

Scaffold attachment factor B1 directly interacts with nuclear receptors in living cells and represses transcriptional activity

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Abstract

Transcriptional activity relies on coregulators that modify the chromatin structure and serve as bridging factors between transcription factors and the basal transcription machinery. Using the DE domain of human peroxisome proliferator-activated receptor gamma (PPAR γ) as bait in a yeast two-hybrid screen of a human adipose tissue library, we isolated the scaffold attachment factor B1 (SAFB1/HET/HAP), which was previously shown to be a corepressor of estrogen receptor α . We show here that SAFB1 has a very broad tissue expression profile in human and is also expressed all along mouse embryogenesis. SAFB1 interacts in pull-down assays not only with PPAR γ but also with all nuclear receptors tested so far, albeit with different affinities. The association of SAFB1 and PPAR γ *in vivo* is further demonstrated by fluorescence resonance energy transfer (FRET) experiments in living cells. We finally show that SAFB1 is a rather general corepressor for nuclear receptors. Its change in expression during the early phases of adipocyte and enterocyte differentiation suggests that SAFB1 potentially influences cell proliferation and differentiation decisions.

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Introduction

In eukaryotic cells, the structure of chromatin has been shown to repress mainly gene activation, and both remodeling and repositioning of nucleosomes are necessary to allow binding of transcription factors and the formation of the transcriptional preinitiation complex (Horn & Peterson 2002). Chromatin-modifying complexes have been classified into two main groups. The first one comprises factors which covalently modify histone tails (Chen *et al.* 1999, Roth *et al.* 2001, Wang *et al.* 2001, Bauer *et al.* 2002). The second group of chromatin modifiers comprises complexes that change the location or conformation of nucleosomes and remodel chromatin by using energy from ATP hydrolysis (Sudarsanam & Winston 2000, Becker & Horz 2002, Neely & Workman 2002).

The peroxisome proliferator-activated receptor gamma (PPAR γ or NR1C3) is one of the three PPARs, which together constitute a distinct subfamily of the nuclear receptors. PPAR γ has mostly been studied because of its key role in adipocyte differentiation, where it acts as a master controller of the ‘thrifty gene response’ (Auwerx 1999, Rosen & Spiegelman 2001), but it has many additional functions (Debril *et al.* 2001). PPAR γ heterodimerizes with the retinoid X receptors (RXRs) and is activated by naturally occurring fatty acids or

fatty acid derivatives (Schoonjans *et al.* 1997). In addition to these natural PPAR γ ligands, several classes of synthetic PPAR γ agonists have been described, including the thiazolidinediones, which are potent insulin sensitizers used in the treatment of type 2 diabetes mellitus (Schoonjans & Auwerx 2000, Willson *et al.* 2001). In order to modulate gene transcription, PPARs, like most transcription factors, rely on coregulators that modify chromatin (Robyr *et al.* 2000, Urnov & Wolffe 2001). Coregulators partially determine the specificity of action of nuclear receptors and integrate their different activities to orchestrate a specific cellular response.

The aim of the study was to identify novel PPAR γ -interacting proteins that could influence the activity of this nuclear receptor. We characterized here a new coregulator isolated from human adipose tissue with a yeast two-hybrid screen, with the DE domain of PPAR γ as bait (including the ligand-binding domain). This regulator is known as SAFB1/HET/HAP (scaffold attachment factor B1/heat-shock protein 27 estrogen response element TATA (hsp27 ERE TATA)-binding protein/heterogeneous nuclear ribonucleoprotein (hnRNP) A1-associated protein). The nuclear protein SAFB1 was initially identified independently by three groups as a scaffold/matrix attachment region (S/MAR)-binding protein (Renz & Fackelmayer 1996), binding to an ERE flanking a TATA box in the

promoter of the hsp27 (Oesterreich *et al.* 1997), and as a partner of hnRNP A1 (Weighardt *et al.* 1999). Additionally, it was shown to bind to the C-terminal domain of RNA polymerase II (RNA Pol II) and to certain serine/arginine-rich RNA-processing factors (SR proteins) and hence to have a role in mRNA splicing (Nayler *et al.* 1998). This protein functions as an ER α corepressor (Oesterreich *et al.* 2000), and represses cell proliferation (Townson *et al.* 2000). Hence, a role for SAFB1 in tumor development has been suggested (reviewed in Oesterreich 2003). Recently, a second SAFB gene, SAFB2, has been described (Townson *et al.* 2003). Both genes are arranged in head-to-head orientation on the same chromosome. They are separated by a region that functions as a bidirectional promoter (Townson *et al.* 2003). We show here that SAFB1 binds in fact to multiple nuclear receptors, mostly in a ligand-independent manner, and inhibits their transcriptional activity. This suggests that SAFB1 is a general corepressor for nuclear receptors. Furthermore, SAFB1 expression level is modified during adipocyte differentiation of 3T3-L1 preadipocytes and enterocyte differentiation of Caco-2 cells. SAFB1 might hence be involved in cell proliferation and differentiation choices.

Materials and methods

Materials

Rosiglitazone and GW4064 were gifts of R Heyman (X-ceptor, San Diego, CA, USA). Wy14 643 compound was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA), and the L-165041 compound was synthesized in the laboratory by Marco Alves. All chemicals, unless stated otherwise, were purchased from Sigma. Suberoylanilide hydroxamic acid (SAHA) was purchased from Biomol (Buttler Pike, PA, USA), and MS-275 was from Calbiochem (San Diego, CA, USA).

Plasmids

hSAFB1 full-length cDNA was obtained by a two-step PCR amplification. First, SAFB1 N-terminus was amplified from IMAGE clone 4999249 (UK HGMP Resource Center, Cambridge, UK) with 5'-gTC gAg ggA CCg AAC ggA CTg Tag-3' and 5'-CCg gAA TTC Cgg TCA gTA gCg gCg AgT gAA gCg-3' and from SAFB1 C-terminus (our clone no. 66, isolated by yeast two-hybrid screening) with 5'-CgC ggA TCC gCg gTC CCT ggA ATg gCg gAg ACT CTg-3' and 5'-CTA CAg TCC gTT Cgg TCC CTC gAC-3'. The full-length cDNA was then amplified from these two fragments with the external primers and inserted into pBS(SK+) (Stratagene, La Jolla, CA, USA). This cDNA was then introduced into the expression vector pSG5 (Stratagene). pSG5-SAFB-HA, containing the hSAFB1 C-terminus (aa 532–915) fused to HA tag, was a kind gift of S Kato

(Arao *et al.* 2000). hSAFB1 and mPPAR γ 1 were cloned after PCR amplification in pEYFP-C1 and pECFP-C1 using Sal I/BamHI and XhoI/KpnI restriction sites respectively. pSG5-mFXR α was obtained by PCR amplification of mFXR from liver cDNA, using the primers 5'-A CgC ggA TCC Agg ATg gTg ATg CAg TTT CAg gg-3' and 5'-CgC gCT AgC CCA CTg gTg TCC ATC ACT gC-3'. The primers introduce a BamHI site (underlined). The PCR product was cloned into pSG5, using the BamHI site and a blunted BglII site. The luciferase reporter construct for FXR α contains the -496/+40 part of mouse intestinal bile acid-binding protein promoter and is a kind gift of D Mangelsdorf. pCMX-hROR α 1, pSG5-hPPAR γ 2, pCMX-hLRH-1, pSG5-mSF1, pSG5-hcJun (a gift of P Sassone Corsi) and pSG5-hVDR (a gift from S Kato) are expression vectors used for *in vitro* translation and/or transfection. pGL3-J3-TK-Luc (luciferase construct with a PPAR response element), pcDNA3-BDGal4-hPPAR γ DE (amino acids (aa) 181–507) and pGL3-(GAL5)-TK-Luc reporter plasmids were already described (Debril *et al.* 2004). pGL3-RORE(3)-TKLuc was a gift from V Giguere. pCMV- β Gal was used as a control of transfection efficiency. hSAFB1 cDNA was subcloned into pLPCX (Clontech) for retroviral experiments. The mouse PPAR α and mouse PPAR β expression vectors were kind gifts of Drs Green and Grimaldi respectively.

Construction of the yeast two-hybrid library and screening

Adipose tissue was obtained from a female nonobese adult subject undergoing endoscopic cholecystectomy after informed consent was obtained. The local ethics committee of the Centre Hospitalier Universitaire in Lille, France, approved the project. cDNA library preparation, library screening and prey construct purification, were done according to the manufacturer (Stratagene). To create the bait vector, the DE domains of PPAR γ 2 (residues 179–505) were cloned downstream of the DNA-binding domain of Gal4. YRG-2 yeast (Stratagene) was sequentially transformed, with the bait construct and with the library, and grown on the appropriate selective medium in the presence of the PPAR γ ligand rosiglitazone (1 μ M). About 5·10⁶ cotransformants were obtained. The first 100 clones which grew on the selective medium were selected for prey construct purification and sequencing.

RNA isolation, RT-PCR and quantitative PCR, oligonucleotides

RNA was isolated from cell lines and embryos by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski & Sacchi 1987). RNAs from human tissues were purchased at Clontech (human total

RNA master panel II). Reverse transcription (RT) of RNA was performed as described (Fayard *et al.* 2003). Quantitative real-time PCRs (Q-PCRs) were performed with a LightCycler and the DNA double-strand specific SYBR Green I dye for detection (Roche). The oligonucleotides used are as follows: 5'-CTG TGG TAG GAA TTT CTG GG-3' and 5'-AA TCT CCA GCC GCT CTC GCT-3' for SAFB1, and 5'-ATG GGT GAA ACT CTG GGA GAT TCT-3' and 5'-CTT GGA GCT TCA GGT CAT ATT TGT A -3' for PPAR γ 2.

Cell culture, transfections, retroviral overexpression

Cell lines were maintained at 37 °C, 5% CO₂ according to the supplier's instructions (ATCC, Manassas, VA, USA). Cells were transfected with lipofectamine (Life Technologies, Burlington, Canada) in 48-well plates. Empty expression vectors were used to maintain equivalent amounts of DNA in the transfections. Luciferase and β -galactosidase activities were measured as described (Gelman *et al.* 1999). Histograms on figures 5 and 6 represent the mean of three independent experiments, in which each point was performed in triplicate. Retroviral overexpression was performed as previously described (Debril *et al.* 2004).

Adipocyte and enterocyte differentiation

Differentiation of 3T3-L1 cells into adipocytes was performed as described (Debril *et al.* 2004). Briefly, proliferating 3T3-L1 cells (day 2) are grown to confluence (day 0). Cells are then treated with the differentiation mix for 2 days. Medium is then changed every other day with a medium containing insulin. The differentiation starts with a clonal expansion phase, during which cells re-enter cell cycle, followed by an accumulation of lipids during terminal adipocyte differentiation. For enterocyte differentiation, proliferating Caco-2 cells (day 1) are grown to confluence and maintained for 21 days after confluence. Culture medium was changed every other day.

Protein production, pull-down assays, immunoblotting

For glutathione S-transferase (GST) pull-down assays, SAFB1 full-length protein and deletion mutants of PPAR γ (PPAR γ AB, aa 31–129; PPAR γ bABC, aa 1–211; and PPAR γ DE, aa 204–507, a kind gift from G Zhou) and p300 (p300Nt: aa 2–516) were subcloned downstream of the GST cDNA in the pGex-4T1 vector (Pharmacia, Orsay, France). The GST fusion proteins were prepared as described (Gelman *et al.* 1999). Pull-down assays were performed with *in vitro* ³⁵S-labeled translated proteins (TNT Quick Rabbit Reticulocyte; Promega) as described (Gelman *et al.* 1999). Ligand was added when indicated. Immunoblotting was performed as described (Gelman

et al. 1999). Antibodies directed against SAFB1 (Upstate, Lake Placid, NY, USA), PPAR γ (Santa Cruz, Heidelberg, Germany) and actin (Santa Cruz) were used at the following dilutions: 1:500, 1:1000 and 1:5000 respectively.

Confocal imaging

Live cells on LabTek chambered cover glasses were washed once with phenol red free Optimum medium (Gibco) containing ligands or their vehicles and observed in the same medium. Observations were performed at 37 °C on a TCS SP2 AOBS confocal microscope (Leica, Wetzlar, Germany) equipped with a whole-microscope incubator (Life Imaging Service, Reinach, Switzerland). Acquisitions were performed with a 63/NA 1.2 water immersion objective. Observation of EYFP fusion proteins was done by exciting at 514 nm and detecting emission at 525–575 nm.

Fluorescence resonance energy transfer (FRET) experiments

Transfections were performed as described above, but expression levels of donor and acceptor proteins were adjusted to similar levels by western blot. Fluorescence was recorded in three different settings: CFPex: 405 nm/CFPem: 465–485 nm; YFPex: 514 nm/YFPem: 525–545 nm; and FRETex: 405 nm/FRETem: 525–545 nm. Laser power and detector gain were adjusted in the different channels in order to observe equimolar concentrations of CFP and YFP at equal intensities (equimolar concentrations of CFP and YFP were obtained by expressing a fusion protein of CFP and YFP spaced by 475 residues). Settings were kept unchanged for analysis of all samples, and background-corrected 8-bit images were quantified by measuring the average intensity of a region of interest, using the Leica Confocal Software (LCS), Version 2.4. CFP and YFP spectral bleed-throughs (BT) in the FRET setting were determined on cells expressing CFP or YFP alone by fitting the intensity ratios IFRET/ICFP and IFRET/IYFP to an exponential model (Feige *et al.* 2005). FRET measured in coexpressing cells was then corrected for spectral bleed-throughs and normalized (NFRET) for expression levels according to the following formula (Xia & Liu 2001):

$$NFRET = \frac{I_{FRET} - I_{CFP} * BT_{CFP} - I_{YFP} * BT_{YFP}}{\sqrt{I_{CFP} * I_{YFP}}}$$

Results

Isolation of SAFB1, a ubiquitously expressed protein

PPAR γ is highly expressed in adipose tissue, where its function has been well characterized (Cock *et al.* 2004).

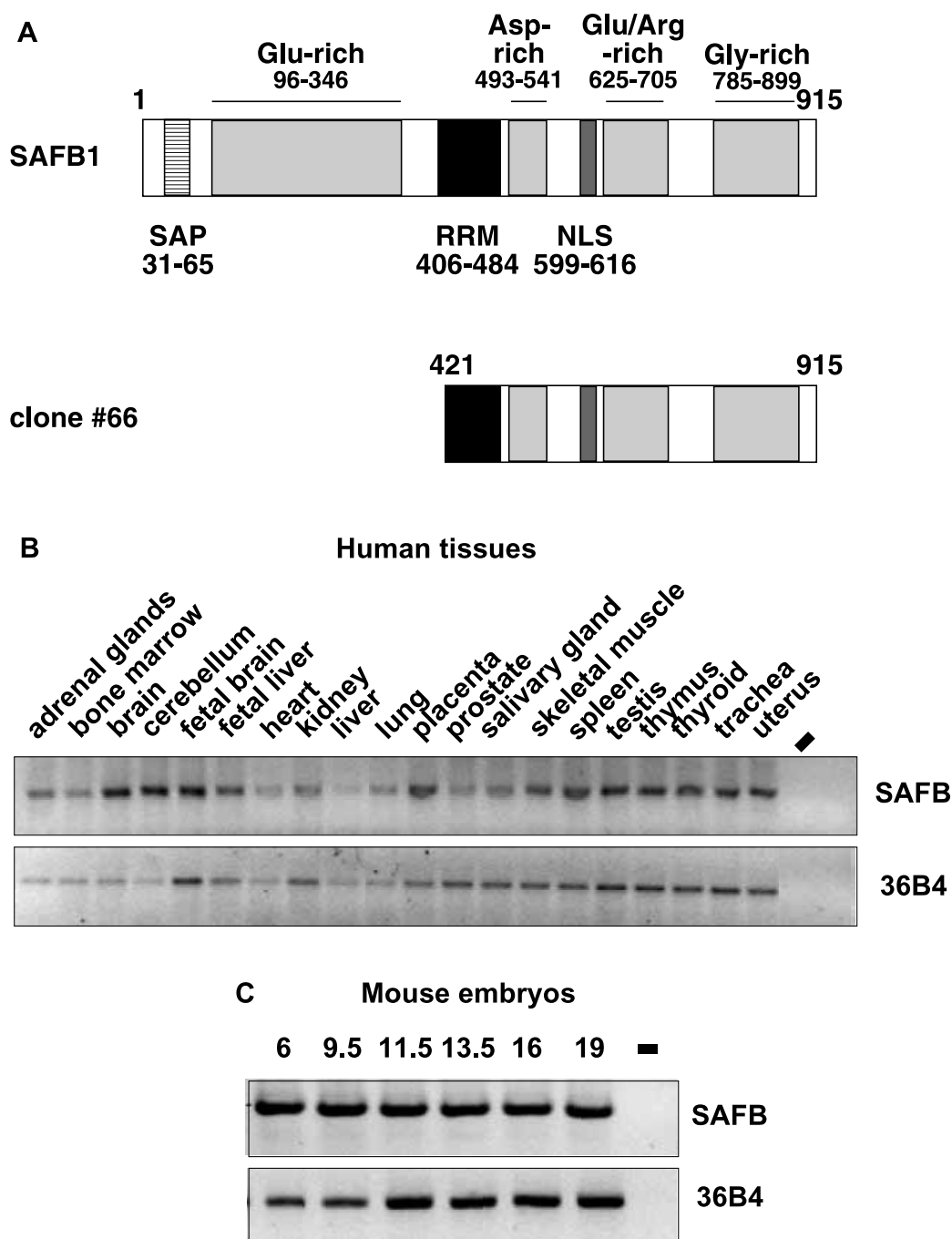
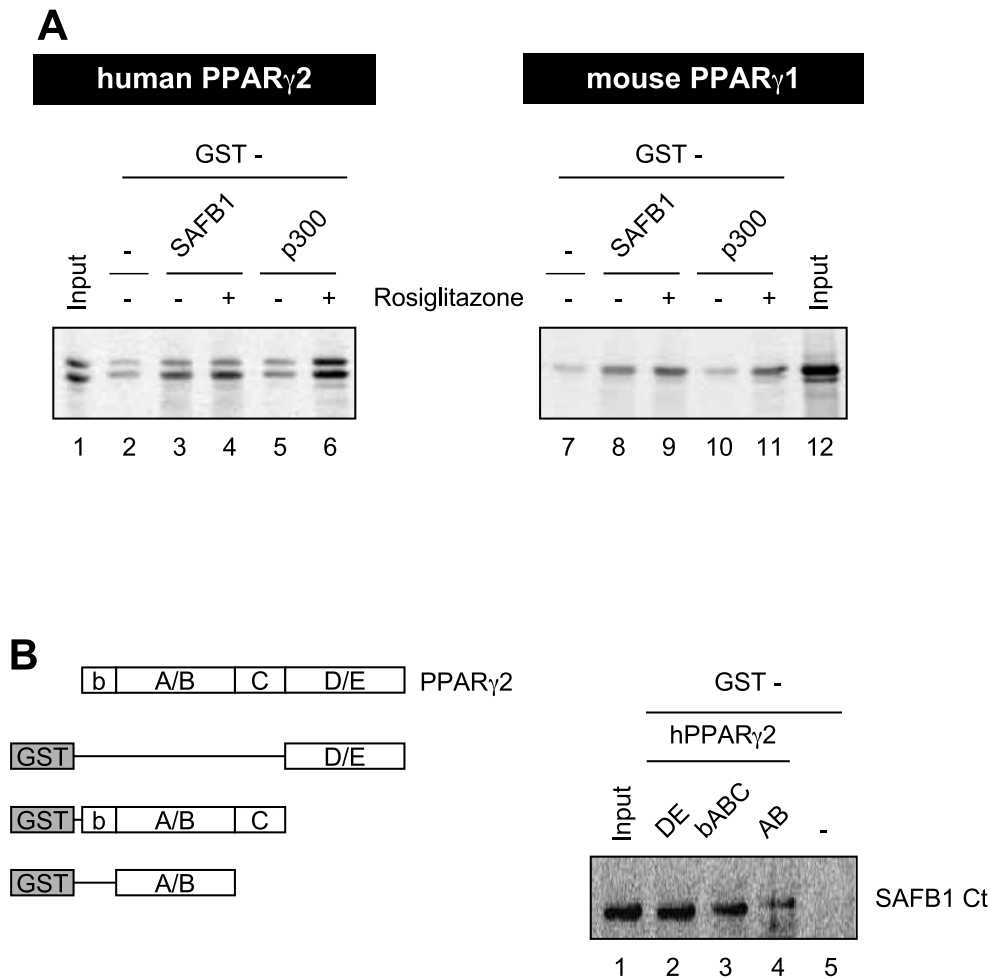


Figure 1 SAFB1 structure and mRNA expression. (A) SAFB1 and clone no. 66 secondary structures. SAFB1 is a large protein of 915 amino acids. It possesses an N-terminal SAF box also called SAP domain (hatched box), a central RNA recognition motif (RRM, black box) and a nuclear localization signal (NLS, dark gray). SAFB1 also contains some regions rich in the indicated amino acids (light gray boxes), which might be important for protein–protein interactions. Clone no. 66 was isolated by yeast two-hybrid screening. It corresponds to the C-terminal part of SAFB1 (amino acids 421–915). Amino-acid numbering was added on the figure to define the described domains. (B and C) SAFB1 mRNA expression was analyzed by RT–PCR. 36B4 is used as the internal control. SAFB1 mRNA is ubiquitously expressed in various human tissues. Negative control of PCR is indicated by (–) (B). SAFB1 mRNA is expressed early during mouse embryo development (C). Numbers in C indicate the number of days since fecondation.



GST -

hPPAR γ 2

Input	DE	bABC	AB	-
	-	-	-	-

1 2 3 4 5

SAFB1 Ct

Figure 2 SAFB1 interacts with some nuclear receptors PPAR γ *in vitro*. (A) *In vitro* translated PPAR γ was incubated with GST-SAFB1, GST-p300 or GST alone, in the presence or absence of ligand (rosiglitazone, 10^{-5} M). PPAR γ was detected by autoradiography. (B) Various domains of hPPAR γ 2 were incubated with the SAFB1 C-terminus fused to HA. A scheme of the various GST-PPAR γ proteins used is provided on top left of the panel. The SAFB1-HA bound protein was detected with an anti-HA antibody.

To isolate new PPAR γ coregulators, we performed a yeast two-hybrid screen of a human adipose tissue cDNA library with the DE domains of PPAR γ 2 fused to the DNA-binding domain of the yeast Gal4 activator as bait. The C-terminal DE domain of PPAR γ encompasses the ligand-binding domain and the ligand-dependent activation function AF-2. One of the constructs isolated in our yeast two-hybrid screen (clone no. 66) was a cDNA containing an open reading frame of 1488 bp. This region corresponds to the C-terminal part of hSAFB1 protein (nt 1314–2801 of NM_002967) (Fig. 1A). We then cloned the full-length hSAFB1 cDNA. SAFB1 contains a central RNA recognition motif (RRM) and an N-terminal SAF box (also called SAP motif (for SAFA/B, acinus and protein inhibitor of activated signal transducer and activator

of transcription), which is a putative DNA-binding domain found in diverse nuclear proteins involved in chromosomal organization (Aravind & Koonin 2000, Kipp *et al.* 2000). This protein also contains some Glu/Arg- and Gly-rich regions that may be involved in protein–protein interactions. SAFB1 does not contain any coactivator motif (such as a LXXLL motif, where L is a leucine and X any amino acid) or corepressor box.

We then tested the mRNA expression level of SAFB1 in various tissues or cells. RT–PCR analysis shows that SAFB1 is ubiquitously expressed in human tissue (Fig. 1B), and in cell lines such as Caco-2 and 3T3-L1 (data not shown). Additionally, SAFB1 mRNA is already expressed in mouse ES cells (data not shown) and embryos from day 6 postcoitum onward (Fig. 1C).

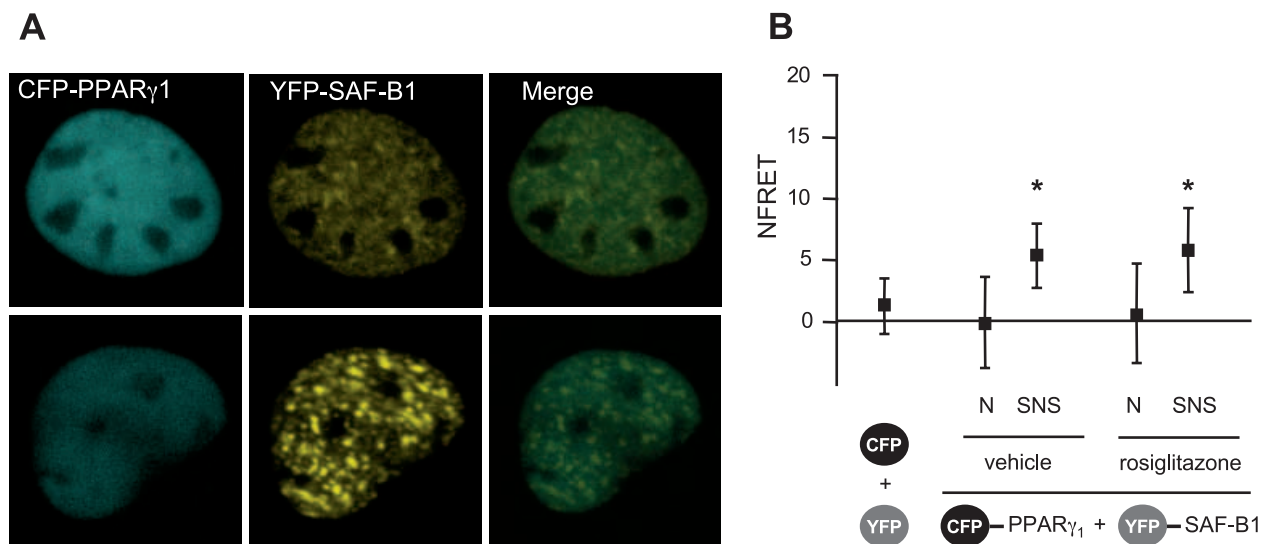


Figure 3 SAFB1 and PPAR γ interact in cells. (A) EYFP-SAFB1 and ECFP-mPPAR γ 1 were expressed in living COS-7 cells and imaged by confocal microscopy. (B) SAFB1 interacts with PPAR γ in living cells: EYFP-SAFB1 and ECFP-mPPAR γ 1 were coexpressed in living COS-7 cells, and their interaction was assessed by FRET either in the nucleoplasm (N) or in subnuclear structures (SNS). As a negative control, FRET was measured in cells expressing ECFP and EYFP alone. FRET was corrected for spectral bleed-throughs and normalized as described in the Materials and methods section. Average normalized FRET (NFRET) values over at least 15 cells are represented. The asterisk denotes *P* value smaller than 0.01 compared with the negative control, using Student's *t*-test.

Hence, these data extend the previously reported results on SAFB1 expression (Renz & Fackelmayer 1996).

SAFB1 interacts with PPAR γ and other nuclear receptors

To confirm the interaction between SAFB1 and PPAR γ detected in the yeast two-hybrid screen, we produced GST-fusion proteins comprising full-length coding sequence of SAFB1 downstream of the GST protein. *In vitro* interaction between GST-SAFB1 and *in vitro* translated PPAR γ was assayed in pull-down experiments. p300 is a known partner of PPAR γ and was used as a positive control. When fused to GST, p300 interacts with human PPAR γ 2 (hPPAR γ 2) and mouse PPAR γ 1 (mPPAR γ 1) in a ligand-dependent manner, as previously described (Gelman *et al.* 1999) (Fig. 2A, lanes 5 and 6; 11 and 12). When fused to GST, SAFB1 interacted also with hPPAR γ 2 and mPPAR γ 1 (Fig. 2A, lanes 4 and 5; 8 and 9). No interaction was observed between GST alone (negative control) and the two PPARs (Fig. 2A, lanes 2 and 7).

In our yeast two-hybrid screen, we isolated the C-terminal part of SAFB1 as an interacting partner of PPAR γ DE domain (C-terminal part). We next mapped the interacting domains between both proteins. Various domains of PPAR γ (AB, bABC and DE) were fused to GST and incubated with the SAFB1 C-terminus fused to

a HA tag. In a GST pull-down assay, both the bABC and DE domains of PPAR γ interacted with the SAFB1 construct (Fig. 2B). From this, we can conclude that both the A/B and D/E domains of PPAR γ interact with SAFB1 (specifically, here, its C-terminal region), as previously shown for the p300 cofactor (Gelman *et al.* 1999).

We subsequently analyzed the interaction between both proteins *in vivo*, in living cells, using FRET. First of all, we characterized the cellular localization of SAFB1, using an EYFP-SAFB1 fusion protein expressed alone in COS-7 cells. SAFB1 was exclusively nuclear, was excluded from the nucleolus and formed subnuclear speckles in some but not all transfected cells. Cells expressing EYFP-SAFB1 displayed two different nuclear patterns, one diffuse and one with speckles (Fig. 3A). Importantly, these subnuclear structures did not result from overexpression, as only cells with low expression levels were analyzed and similar structures were also observed with other nuclear receptor cofactors such as CBP/p300, GRIP1 and SRC-1 (our unpublished data; Doucas *et al.* 1999, Stenoien *et al.* 2000, Baumann *et al.* 2001, McManus & Hendzel 2003, Black *et al.* 2004). Then, EYFP-SAFB1 was coexpressed with ECFP-mPPAR γ 1, which localized to the nucleus as well, with no sign of enrichment in the subnuclear structures formed by EYFP-SAFB1. When FRET was quantified in the nucleoplasm of cells not harboring subnuclear speckles, the average FRET value was comparable to that of the negative control expressing ECFP and EYFP

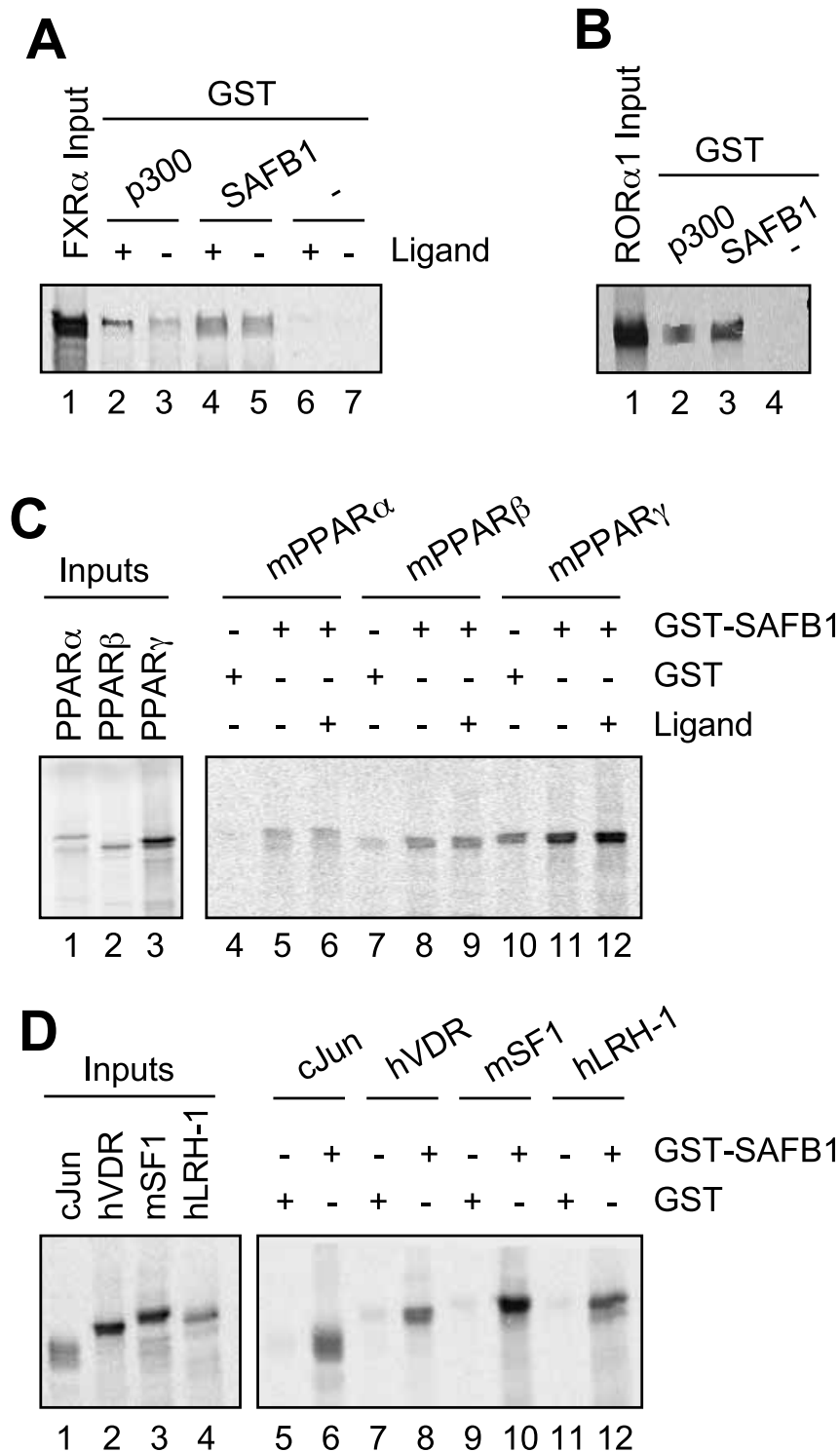
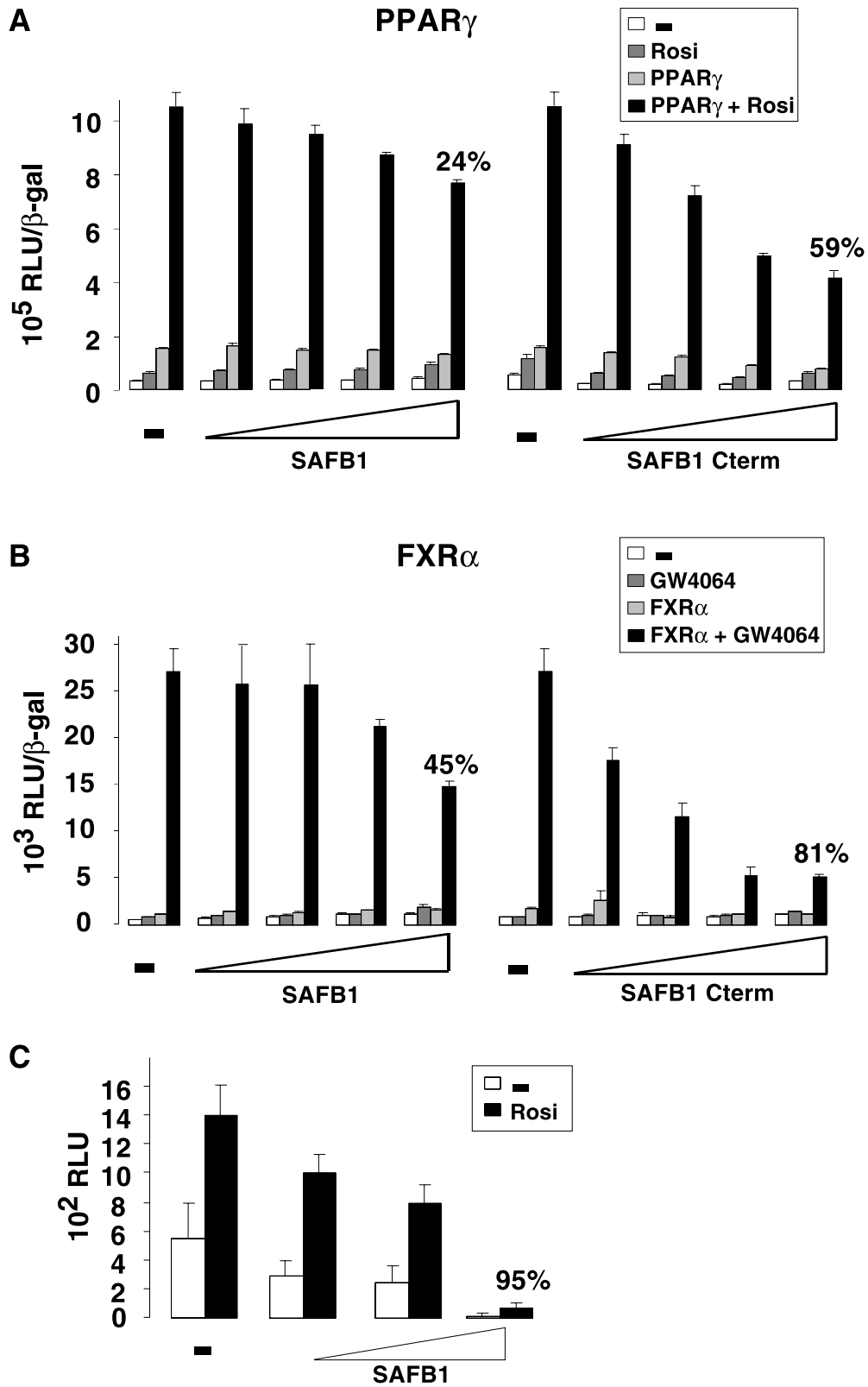


Figure 4 SAFB1 interacts with numerous transcription factors. GST-fusion proteins were incubated with *in vitro* translated nuclear receptors as well as cJun, eventually in the presence or absence of their respective ligands. (A) FXR α , with or without GW4064; (B) hROR α 1; (C) mPPAR α , mPPAR β and mPPAR γ , with or without Wy14643, L-165041 or rosiglitazone respectively; (D) hcJun, hVDR, mSF1 and hLRH-1.



alone (Fig. 3B). However, FRET quantification in the EYFP-SAFB1 speckles revealed a significant interaction between the two fusion proteins, which occurred at similar levels in both the presence and absence of ligand.

The fact that SAFB1 interacts with both ER and PPAR γ prompted us to investigate the specificity of the interaction with other nuclear receptors, as well as the nonrelated cJun transcription factor. We first performed GST pull-down assays by incubating GST-SAFB1 or GST-p300 with *in vitro* translated Farnesoid X receptor (FXR) α . We observed a clear interaction between FXR α and GST-SAFB1 that was ligand-independent (Fig. 4A, lanes 4 and 5), whereas the interaction between FXR α and p300 was ligand-dependent (Fig. 4A, compare lanes 2 and 3), mimicking the mode of interaction of PPAR γ with these two cofactors (Fig. 2A). No interaction was observed with GST alone (Fig. 4A, lanes 6 and 7). Furthermore, GST-SAFB1 also interacted with human ROR α 1 (hROR α 1, Fig. 4B), mouse (m) PPAR α , β and γ (Fig. 4C), the human vitamin D receptor (hVDR), the mouse steroidogenic factor 1 (mSF1) and the human liver receptor homolog 1 (hLRH-1) (Fig. 4D). Again, none of these receptors interacted with GST alone. Interestingly, SAFB1 also interacted with the human cJun (Fig. 4D). We hence conclude that SAFB1 interacts promiscuously with various nuclear receptors, although the strength of interaction varies with the receptor tested. Indeed, SF1, LRH1 and cJun interact on average four times better than PPAR α , PPAR β , PPAR γ and VDR.

SAFB1 inhibits nuclear receptor transcriptional activity

SAFB1 has been described as a protein repressing ER α transactivation (Oesterreich *et al.* 2000), and hence we tested the possibility that SAFB1 could also act as a corepressor for other nuclear receptors. COS-1 cells were cotransfected with reporter constructs under the control of PPAR or FXR responsive elements (RE), together with expression vectors for PPAR γ or FXR α and increasing amounts of an expression vector for

SAFB1 or the corresponding empty vector, in the presence or absence of ligand. PPAR γ transcriptional activity is stimulated in the presence of rosiglitazone, and increasing concentrations of SAFB1 inhibit PPAR γ activation by up to 24% (Fig. 5A, left panel). When a similar experiment was performed with the C-terminal part of SAFB1, the inhibition of PPAR γ activity reached 59% (Fig. 5A, right panel). SAFB1 repressed the transcriptional activity of FXR α as well in a dose-dependent manner (Fig. 5B). This inhibition was more pronounced than for PPAR γ (45% vs 24%). Again, the SAFB1 C-terminal domain was a better repressor (up to 81% of inhibition) than the full-length SAFB1 protein. It is noteworthy that SAFB1 did not affect the basal activity of the TK-Luc reporter backbone (data not shown), indicating that SAFB1-mediated modulation of nuclear receptor activity is specific for the nuclear receptors tested and does not correspond to a general repressive effect on transcription.

The repressive action of SAFB1 on PPAR γ transcriptional activity was then tested in another promoter context. HEK293 cells were transfected with an expression vector for a fusion protein comprising the DNA-binding domain of Gal4 and the D/E domain of PPAR γ , together with a reporter construct comprising five Gal4 responsive elements cloned upstream of a TK promoter and driving the expression of the luciferase cDNA (Fig. 5C). Cotransfection of increasing amounts of an expression vector for SAFB1 inhibited Gal4-PPAR γ D/E transcriptional activity by up to 95%.

These data show that SAFB1 decreases PPAR γ , FXR α and Gal4-PPAR γ D/E transcriptional activity and that the extent of inhibition varies according to the receptor and the promoter tested, reflecting most probably a specific action rather than a common effect of the basic transcription machinery.

Transcriptional repression often involves the presence of proteins called histone deacetylases (HDACs). We thus assessed the action of HDAC inhibitors on SAFB1-mediated transcriptional repression. COS-1 cells were cotransfected as previously, in the presence or absence of various HDAC inhibitors. Each of these

Figure 5 SAFB1 represses PPAR γ 2 and FXR α transcriptional activities. (A and B) SAFB1 inhibits the ligand-dependent and -independent transcriptional activities of PPAR γ 2 (A) and FXR α (B). COS-1 cells were transfected with a PPAR (A) or a FXR (B) reporter construct (PPRE-TK-Luc or FXR-TK-Luc respectively), an expression vector for PPAR γ (18 ng) or for FXR α (20 ng), an expression vector for FXR α (18 ng with PPAR γ or 2 ng with FXR α respectively), and increasing amounts of an expression vector for full-length SAFB1, for the truncated SAFB1 C-terminal protein (10, 20, 50 or 100 ng/well) or with the corresponding empty vectors (-). The presence of agonist (10^{-7} M rosiglitazone for PPAR or 10^{-7} M GW4064 for FXR) or vehicle (dimethyl sulfoxide (-)) is indicated. The numbers above the shaded bars indicate the percentage of reduction of normalized luciferase activity compared with the same condition in the absence of SAFB1. RLU: relative luciferase units; β -gal: β -galactosidase activity. (C) SAFB1 inhibits the transcriptional activity of a Gal4 DBD-PPAR γ 2 chimera. The same experiments were conducted as in A and B but with an expression vector for the DNA-binding domain of Gal4 fused to the ligand-binding domain of PPAR γ (pcDNA3-BDGal4-PPAR γ DE, 18 ng/well) together with a reporter construct where the transcription of the luciferase gene is driven by a TK promoter and five Gal4-binding elements (pGL3-(GAL)5-TK-Luc, 90 ng/well) and with increasing amounts of an expression vector for full-length SAFB1 (18, 72 or 216 ng/well) or with the corresponding empty vector (-). Cells were grown for 24 h in the presence or absence of 10^{-6} M rosiglitazone.

inhibitors affected SAFB1 action very differently, depending also on the receptor tested (Fig. 6). Neither trichostatin A (TSA), nicotinamide nor suberoylanilide hydroxamic acid (SAHA) had any effect on the repression of PPAR γ activity by SAFB1, whereas MS-275 relieved the action of SAFB1, but not that of its C-terminus. TSA reversed very slightly the action of SAFB1 on FXR, whereas SAHA, nicotinamide and MS-275 exacerbated it. We did not find any synergistic effect when inhibitors of class I/II (TSA, MS-275 or SAHA) and of class III (nicotinamide) were added together (data not shown). It is also noteworthy that these inhibitors had no effect on the activity of the CMV- β Gal control (data not shown).

SAFB1 expression is decreased during 3T3-L1 differentiation into adipocytes, and Caco-2 differentiation into enterocytes

SAFB1 was cloned from a human adipose tissue library on the basis of its interaction with PPAR γ in a yeast two-hybrid system, an interaction further confirmed by pull-down and FRET experiments. We therefore hypothesized that SAFB1 may play a role in adipocyte differentiation. We first monitored the expression level of SAFB1 during 3T3-L1 adipocyte differentiation. The preadipocyte 3T3-L1 cell line can differentiate into adipocytes when treated with an adipocyte differentiation mix for 2 days and then with insulin alone. After 12 days, such 3T3-L1 cells display a differentiated adipocyte-like phenotype, as evidenced by the accumulation of lipid droplets in their cytoplasm and revealed by Oil red O staining (data not shown). Quantitative real-time PCR experiments showed that the highest level of SAFB1 mRNA expression is obtained immediately after the induction of the differentiation (days 0–1 of the adipocyte differentiation process) (Fig. 7A, upper panel). SAFB1 mRNA expression level then returns to basal levels. At the protein level, a rapid decrease of SAFB1 expression was observed during the first 2 days of differentiation (Fig. 7B, upper panel). As previously reported, PPAR γ 2 expression level increased later during 3T3-L1 differentiation (mRNA level, Fig. 7A, upper panel); protein level (Fig. 7B, middle panel). Hence, SAFB1 and PPAR γ protein expression followed an opposite expression pattern during 3T3-L1 differentiation (Fig. 7B).

SAFB1 has been shown to have antiproliferative properties (Townson *et al.* 2000). It is hence tempting to speculate that the high expression of SAFB1 at the beginning of adipocyte differentiation contributes to the onset of differentiation. To validate experimentally such an antiproliferative role of SAFB1, we set out to change the basal expression level of SAFB1 in 3T3-L1 cells. For stable overexpression of SAFB1, we infected cells with a

retrovirus encoding SAFB1 or with an empty control retrovirus. However, we were unable to obtain a stable cell line overexpressing SAFB1, whereas clones were obtained for the cell line infected with the control retrovirus. This strongly suggests that SAFB1 inhibits 3T3-L1 cell proliferation, as was demonstrated for other cell lines. Moreover, our attempt to decrease SAFB1 expression in cells by the RNA interference technique led to only very modest reductions of SAFB1 levels, not sufficient to carry out proliferation and differentiation analyses in this context.

Since we suspected SAFB1 to have a general action in cell proliferation and differentiation, we also monitored SAFB1 protein levels in differentiating Caco-2 cells. When grown for 21 days after confluence, these cells acquire an enterocyte phenotype). Caco-2 cells express the highest level of SAFB1 protein early during the differentiation process at day 7 (Fig. 7C), these levels decreasing thereafter. Together with the data obtained in adipocytes, these results suggest that SAFB1 might be involved in cell decisions leading to differentiation.

Discussion

In an attempt to isolate new coregulators of PPAR γ activity, we identified, cloned and characterized SAFB1, a protein also known as HET or HAP. PPAR γ and SAFB1 interact both *in vitro* and *in vivo*. Other nuclear receptors, such as FXR α , ROR α 1, PPAR α , PPAR β , VDR, SF1 and LRH-1 are also shown here to interact with SAFB1. This interaction between the nuclear receptors and SAFB1 was ligand-independent, as previously reported for the interaction between ER α and SAFB1 (Oesterreich *et al.* 2000). The promiscuous interaction of SAFB1 with all nuclear receptors tested in this study and with ER, as well as with the unrelated cJun transcription factor, raises the question of the specificity of the interaction. Such a promiscuous mode of action has been already reported for the p300/CBP and SRC-1 coactivators. SAFB1 is a big protein of 915 residues that may offer numerous docking sites for other proteins. It will be interesting in the future to map precisely the domains within SAFB1 that are involved in the recruitment of these different transcription factors. It is noteworthy that we found a very broad expression pattern for SAFB1, supporting the idea that SAFB1 should be able to interact with numerous cellular components in different cellular contexts. As reported for ER α , we demonstrated here that SAFB1 inhibited the transcriptional activity of PPAR γ and FXR α , suggesting that SAFB1 is a genuine nuclear receptor corepressor. We demonstrated that the SAFB1 C-terminus is a much more potent repressor of transcriptional activity than the full-length protein. This is consistent with recent data showing that the repression

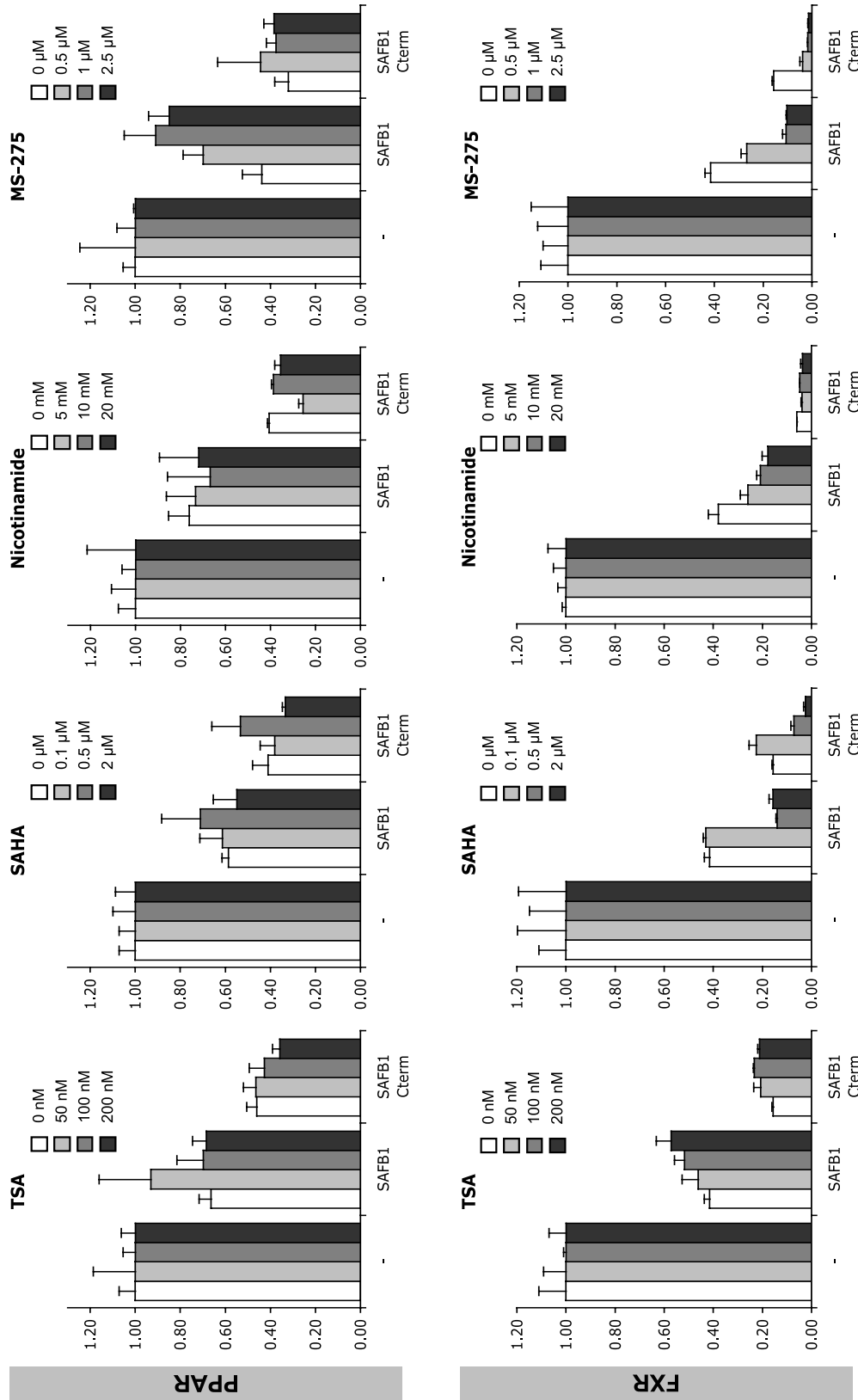


Figure 6 HDAC inhibitors influence SAFB1 repression of FXR α nuclear receptor. COS-1 cells were transfected as described for Fig. 5, in the presence or absence of HDAC inhibitors. Increasing concentrations of trichostatin A (TSA) (0, 50, 100 or 200 nM, from white to black respectively) (B) SAHA (0, 0.1, 0.5 or 2 μ M) (C) nicotinamide (0, 5, 10 or 20 mM) (D) or MS-275 (0, 0.5, 1 or 2.5 μ M) were added. Data are presented as relative values compared with the values in the absence of SAFB1 protein (-) since it was the most appropriate way to analyze the influence of HDAC inhibitors on SAFB1-mediated transcriptional repression. The top panels represent transfections with PPAR γ and the bottom panels those with FXR α .

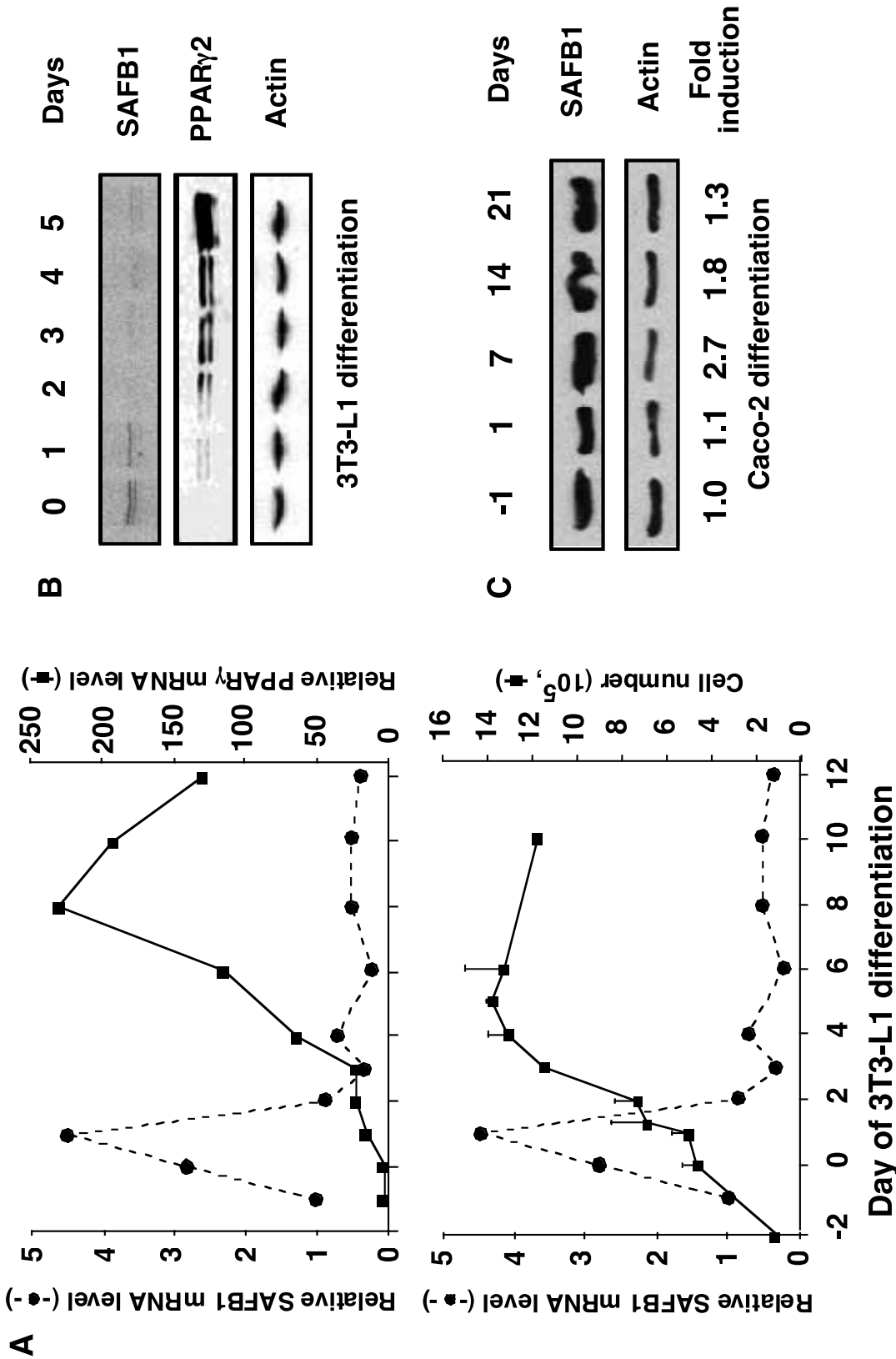


Figure 7 SAFB1 expression changes during adipocyte and enterocyte differentiation. (A) Upper Panel, SAFB1 and PPAR γ mRNA expression during 3T3-L1 differentiation, as observed by quantitative PCR. (A) Lower Panel, SAFB1 mRNA expression and cell numbers along 3T3-L1 differentiation. (B) SAFB1 and PPAR γ protein expression during 3T3-L1 differentiation. Actin was used as a loading control (lower panel). (C) SAFB1 protein levels during Caco-2 differentiation, observed by Western blot before (day 1) and after the induction of enterocyte differentiation. Actin was used as a loading control. Numbers below the blots correspond to the fold induction of SAFB1 protein level, compared with day 1, and normalized to actin levels.

domain of SAFB1 resides in the C-terminal part of the protein (Townson *et al.* 2004). Corepressors of nuclear receptors often possess HDAC activity or interact with HDACs. We show here that SAFB1 action is specifically affected by four different HDAC inhibitors, depending on the receptor tested (FXR or PPAR). Although these results may appear confusing at first sight, they most probably reflect the recruitment of different types of HDACs by each receptor, as well as some specific interplay between HDACs on the one hand and promoters and ligands on the other hand, as recently exemplified with the estrogen receptor (ER), whose action is differently modulated by HDAC inhibitors, depending upon the cell line and the ligand tested (Margueron *et al.* 2004). It is also possible that these compounds have some side effects and modulate other cellular components which indirectly affect the transcription of the reporter construct. However, these inhibitors had no effect on the activity of the CMV- β Gal control, ruling out some general and unspecific effects on basal transcription.

PPAR γ activity in adipocytes is modulated by a number of coregulators. More particularly, the CREB-binding protein (Yamauchi *et al.* 2002), the steroid receptor coactivator-1/transcriptional intermediary factor 2 (Picard *et al.* 2002), the PPAR γ coactivator-1 (Puigserver *et al.* 1998), the receptor interacting protein 140 (Leonardsson *et al.* 2004), and the thyroid hormone receptor-associated protein 220 (Ge *et al.* 2002) have all been shown to have an impact on adipocyte differentiation. Hence, we hypothesized that SAFB1, which was isolated from an adipose tissue cDNA library, could also affect adipocyte differentiation. Consistent with such a role, we found that SAFB1 expression level in 3T3-L1 cells changed during the initial phases of differentiation into adipocytes. SAFB1 expression levels are high in preadipocytes and decrease rapidly upon differentiation. This is consistent with the hypothesis that high levels of SAFB1 prevent 3T3-L1 cell proliferation. An inhibitory effect of SAFB1 on cell proliferation was also reported for NIH3T3 and HEK293 cells as well as some breast cancer cell lines (Townson *et al.* 2000).

In view of the potential of SAFB1 to repress cell proliferation and of our observations of regulated expression of SAFB1 during adipogenesis, one hypothesis merits particular attention. The level of expression of SAFB1 during cell cycle has been correlated to a role of this protein in transcription control (Townson *et al.* 2000). Indeed, when cells are in the G2/M phase of the cell cycle, SAFB1 might be involved in the packaging of chromatin for mitosis, hence inhibiting transcription during the clonal expansion phase. SAFB1 expression could, however, in view of its effects on cell proliferation, subsequently indicate the end of the clonal expansion phase and limit the length of this phase. SAFB1 might thus be a factor both facilitating the entry and triggering

the subsequent exit from the clonal expansion phase and consequently permitting progression of cells through the initial phases of adipogenesis. Through inhibition of cell proliferation, SAFB1 would thus set the scene for appropriate differentiation.

SAFB1 is a S/MAR-binding protein (Renz & Fackelmayer 1996), and, as such, connects chromatin to the nuclear matrix. SAFB1 binds to the C-terminal domain of RNA Pol II and to certain splicing factors as well (Nayler *et al.* 1998) and could thereby affect the subcellular distribution of factors involved in transcription and RNA processing. SAFB1 might therefore act as an architectural factor that attracts nuclear receptors to the nuclear matrix and influences their effect on transcription and RNA processing.

In summary, we report here that SAFB1 interacts promiscuously with nuclear hormone receptors, and inhibits their transcriptional activities. SAFB1 expression decreases at the beginning of adipocyte differentiation and is modulated along enterocyte differentiation, suggesting a role for this factor in cell proliferation and differentiation. Finally, we may speculate that SAFB1 may influence many other biologic processes, such as metabolic homeostasis, whose coordination depends on several nuclear receptor activities.

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