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Lipin 1 Represses NFATc4 Transcriptional Activity in Adipocytes To Inhibit Secretion of Inflammatory Factors▽†

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Lipin 1 is a bifunctional protein that regulates gene transcription and, as a Mg^{2+}-dependent phosphatidic acid phosphatase (PAP), is a key enzyme in the biosynthesis of phospholipids and triacylglycerol. We describe here the functional interaction between lipin 1 and the nuclear factor of activated T cells c4 (NFATc4). Lipin 1 represses NFATc4 transcriptional activity through protein-protein interaction, and lipin 1 is present at the promoters of NFATc4 transcriptional targets *in vivo*. Catalytically active and inactive lipin 1 can suppress NFATc4 transcriptional activity, and this suppression may involve recruitment of histone deacetylases to target promoters. In fat pads from mice deficient for lipin 1 (*fld* mice) and in 3T3-L1 adipocytes depleted of lipin 1 there is increased expression of several NFAT target genes including tumor necrosis factor alpha, resistin, FABP4, and PPARγ. Finally, both lipin 1 protein and total PAP activity are decreased with increasing adiposity in the visceral, but not subcutaneous, fat pads of *ob*/*ob* mice. These observations place lipin 1 as a potentially important link between triacylglycerol synthesis and adipose tissue inflammation.

Lipin 1 was first identified as the protein product of the *Lpin1* gene to which the site of a spontaneous mutation in the fatty liver dystrophy (*fld*) strain of mice had been localized (40). As the name suggests, newborn *fld* mice exhibit fatty liver and hyperlipidemia that resolve at weaning (29). Adult *fld* mice display dramatically reduced adiposity, insulin resistance, and glucose intolerance (47). There are three lipin genes in vertebrates and lipin homologs are found in most species (40).

Although implicated in adipocyte development, the function of lipin 1 was unknown until Han et al. identified the lipin homolog in yeast as a Mg^{2+}-dependent phosphatidic acid phosphatase (PAP) (16). The mammalian enzyme lipin 1 exhibits Mg^{2+}-dependent PAP activity and performs the next-to-last enzymatic reaction in the synthesis of triacylglycerol (16, 46). Lipin 1 contains a haloacid dehalogenase active site in the conserved carboxy-terminal CLIP domain necessary for lipid phosphatase activity. However, the highly conserved amino-terminal NLIP domain is also required for lipin activity (18). Analysis of total PAP activity in tissues from *fld* mice revealed that lipin 1 is responsible for the majority of PAP activity in adipose tissue, skeletal muscle, heart, kidney, and lungs and contributes significantly to total liver PAP activity (9, 18).

Mg^{2+}-dependent PAP activity has long been known to be crucial in the synthesis of phospholipids and triacylglycerol (6). Fatty acids, either synthesized *de novo* or taken up from the circulation, are converted to acyl-coenzymes A that are then used to acylate glycerol-3-phosphate in a stepwise fashion to generate phosphatidic acid. Phosphatidic acid can then be utilized for the synthesis of phospholipids such as phosphatidylinositol and cardiolipin or dephosphorylated by a PAP enzyme such as lipin 1 to form diacylglycerol (DAG). DAG can then be used to form triacylglycerol and phospholipids such as phosphatidylcholine or phosphatidylethanolamine. Lipin 1 expression and PAP activity is induced in 3T3-L1 cells and the liver upon glucocorticoid stimulation, during adipocyte differentiation, and upon fasting in the liver (12, 40, 42, 59). Posttranscriptionally, PAP activity has been hypothesized to be regulated via changes in its intracellular localization (3, 18). Lipin 1 is highly phosphorylated; however, in 3T3-L1 cells the phosphorylation of lipin 1 does not affect its intrinsic enzymatic activity (18, 24). Instead, the localization of lipin 1 correlates with its phosphorylation state. Of note, indirect immunofluorescence studies have demonstrated considerable lipin 1 localization in the nucleus of cells (38–40).

Lipin 1 is a multifunctional protein that has both enzymatic activity and the ability to regulate transcription (reviewed in references 46 and 48). Lipin proteins do not contain DNA-binding domains but instead regulate gene expression indirectly by interacting with transcription factors and regulating their activity. For example, lipin 1 interacts with and activates PPARα transcriptional activity in partnership with the PPAR coactivator PGC-1α (12). The effect of lipin 1 on PPARα transcription does not require lipin 1 PAP activity, demonstrating that the enzymatic activity of lipin 1 can be functionally separated from the ability of lipin 1 to regulate transcription. In yeast, lipin plays a crucial role in nuclear membrane formation (50), at least in part by inhibiting expression of several genes necessary for phospholipid synthesis. Subsequent work

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in yeast cells has shown that the yeast homolog of lipin requires intact PAP activity for function (15).

The ability of lipin 1 to activate PPARα transcription requires a direct interaction between the two proteins, and chromatin immunoprecipitation (ChIP) analysis suggests that this interaction occurs in the nucleus at the promoters of PPARα-regulated target genes (12). A recent study demonstrated that lipin 1 interaction with PPARγ allows lipin 1 to act as a transcriptional coactivator for PPARγ in 3T3-L1 adipocytes and suggested that the ability of lipin 1 to coactivate PPARγ was important for 3T3-L1 adipocyte differentiation (28). Lipin 1 seems to interact with PPARα through a hydrophobic motif similar to domains used by other coactivators to interact with nuclear hormone receptors. This binding motif is found in the CLIP domain of lipin 1 and is adjacent to the PAP catalytic domain (12). Previous work from our laboratory suggested that lipin 1 increases the transcriptional activity of the PPARα/PGC-1α complex via recruitment of transcriptional coactivators with histone-modifying enzymatic activity, such as p300 (12).

We describe nuclear factor of activated T cells c4 (NFATc4) here as a new lipin 1-interacting partner and demonstrate the functional consequence of this interaction. NFAT transcription factors are activated by cytosolic calcium and are recognized to play an important role in the activation of cytokine expression and differentiation of immune cells (20). NFATc2 and NFATc4 have also been implicated in both adipocyte differentiation and expression of adipokines. Mice deficient for both NFATc2 and NFATc4 have decreased production of resistin in adipose tissue and are resistant to diet-induced obesity (55). The role of lipin 1 in triacylglycerol synthesis, when combined with its ability to modulate the activity of a transcription factor that regulates expression of cytokines and adipokines, positions lipin 1 as a potential regulator of the negative consequences of increased triacylglycerol deposition in adipocytes.

MATERIALS AND METHODS

Cell culture and treatments. 3T3-L1 preadipocytes were maintained in Dulbecco modified Eagle medium (DMEM) with 10% newborn bovine serum. Differentiation of 3T3-L1 preadipocytes was initiated with a mix of insulin (0.25 U/ml), 3-isobutyl-1-methylxanthine (0.5 mM), and dexamethasone (0.25 μM) in DMEM plus 10% fetal bovine serum (FBS). On day 4 and every other day thereafter, the differentiation medium was replaced with DMEM containing 10% FBS. BHK cells were maintained in DMEM plus 10% FBS.

Electrophoretic mobility shift assay (EMSA). Double-stranded synthetic oligonucleotides were 5′ end labeled with [γ-^{32}P]dATP. The sequences were as follows: NFAT (sense), 5′-CGCCCAAAGAGGAAAATTTGTTTCATA-3′; and NFAT (antisense), 5′-TATGAAACAAATTTTCCTCTTTGGGCG-3′. The PPARα oligonucleotide was from PPAR binding site in the human apolipoprotein C-III promoter (sc-2587; Santa Cruz Biotechnology). Standard binding reactions were carried out in 10% glycerol (vol/vol), 20 mM HEPES (pH 7.9), 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 1 μg of poly(dI-dC), and 100,000 cpm of DNA probe in a total volume of 20 μl. The reaction mixtures were then incubated for 1 h at room temperature, and protein-DNA was separated on 4% nondenaturing polyacrylamide gels. Electrophoresis was performed at a constant voltage of 160 V for 90 min at 4°C. The gels were then dried under vacuum and autoradiographed at –80°C for several days. For supershift analyses, antibodies against NFAT (FLAG), lipin (HA), and NFATc4 antibody (sc-13036X; Santa Cruz) were used. Incubation with antibodies was on ice for 1 h prior to addition of the ^{32}P-labeled probe.

ChIP. ChIP assays were performed by using a ChIP assay kit (Upstate Biotechnology, Inc.) according to the manufacturer's instructions. Briefly, the differentiated 3T3-L1 cells were cross-linked with 1% (vol/vol) formaldehyde at 37°C for 10 min and harvested. The cells were sonicated for 30 s five times and centrifuged at 13,000 rpm for 10 min at 4°C. The cell supernatant was precleared with a salmon sperm DNA-protein A-agarose slurry for 30 min at 4°C with agitation. The specific DNA-bound transcription factor complexes were precipitated overnight with 4 μg of nonimmune, NFATc4 (Santa Cruz), lipin 1 (LAb1 [23]), or acetylated histone H3 (Millipore) antibody at 4°C before the addition of salmon sperm DNA-protein A-agarose slurry. The antibody-chromatin complexes were eluted from the salmon sperm DNA-protein A-agarose slurry, and cross-links were reversed with 5 M NaCl at 65°C for 4 h. The protein was removed from DNA digestion with 10 mg of proteinase K/ml at 45°C for 1 h, and the DNA was recovered from the solution by phenol-chloroform extraction. PCR was performed with total DNA (input control) and immunoprecipitated DNA using promoter-specific primers (see the supplemental material). The PCR products were subjected to electrophoresis on 1% agarose gels.

Coimmunoprecipitations and electrophoretic analysis. Transfected cells (10-cm plate) were homogenized by glass-Teflon at 1,000 rpm in 1 ml of buffer A (50 mM β-glycerophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, and 10 mM sodium phosphate [pH 7.4] plus 0.1% NP-40 and protease inhibitors). Cellular debris was cleared by centrifugation at 13,000 rpm for 15 min, and the supernatant was retained. The FLAG, myc, and hemagglutinin (HA) antibodies were incubated with protein G-agarose at room temperature for 2 h by gentle inversion and then incubated with aliquots of 400-μl protein lysates at 4°C overnight. After centrifugation at 6,000 rpm for 15 s, the supernatant was removed, and the beads were washed three times with 1 ml of buffer A. The beads were suspended in 80 μl of sodium dodecyl sulfate (SDS) gel loading buffer, boiled for 5 min, and centrifuged at 6,000 rpm for 1 min. The supernatant and cell lysates were analyzed by Western blotting with antibodies to NFATc4 (H-74; Santa Cruz), lipin 1 (22), lipin 2 (14), FLAG (M2), HA (12CA5), and myc (9E10), as previously described (18).

Nuclear extracts. Total nuclear fractions were prepared from 3T3-L1 cells by the following method. 3T3-L1 cells (10-cm plate) were washed twice with ice-cold phosphate-buffered saline (PBS) buffer and incubated at 4°C for 5 min with 1 ml of hypotonic buffer (10 mM Tris [pH 7.6], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, and protease inhibitors). The cells were scraped from the dish and centrifuged at 130 × *g* for 5 min. The supernatants were used as cytosolic fractions, and the pellet was suspended in 500 μl of nuclear extraction buffer (5 mM HEPES [pH 7.9], 26% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.4 M NaCl, 0.5 mM dithiothreitol, and protease inhibitors), incubated at 4°C for 30 min, and then centrifuged at 30,000 × *g* for 20 min.

Northern analysis. For Northern blot analysis, cells were washed with ice-cold PBS and total RNA was extracted with TRIzol RNA isolation reagent (Invitrogen) according to the manufacturer's instructions. A 15-μg portion of total RNA extracted from differentiated 3T3-L1 adipocytes was denatured by heating in denaturing buffer (20 mM morpholinepropanesulfonic acid, 1 mM EDTA, 2.2 M formaldehyde, 50% formamide, bromophenol blue, 5 mM sodium acetate; pH 7.0) at 55°C for 15 min and then electrophoresed on 1.2% agarose-formaldehyde gel. The gel was transferred to Hybond membranes by using 10× SSC (1.5 M NaCl, 150 mm sodium citrate) overnight. The membrane was washed in 2× SSC, cross-linked by UV light (254 nm; UV Stratalinker 1800; Stratagene). The membrane was rinsed in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), and then sequentially probed with [γ-^{32}P]dATP-labeled probes in hybridization buffer (0.5 M $NaPO_4$ [pH 7.2], 7% SDS, 1 mM EDTA, 1% bovine serum albumin) at 61°C overnight. After hybridization, the Northern blot was washed twice with 2× SSC–0.1% SDS for 15 min and once with 1× SSC–0.1% SDS for 15 min and then exposed to X-ray film or phosphorimage screens (Molecular Dynamics) for 24 to 72 h depending on the signal intensity.

RT-PCR. Total RNA from 3T3-L1 cells or white adipose tissue (1 μg) was reverse transcribed in a final incubation volume of 20 μl using an Omniscript RT kit (Qiagen) at 37°C for 1 h. The resulting first-strand cDNA was subjected to real-time PCR using the SYBR green detection system in a final volume of 20 μl. PCR amplifications were performed as follows: 5 min at 94°C for denaturation, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. reverse transcription-PCR (RT-PCR) quantitation was performed according to the method of Pfaffl (41). The data shown are the fold change in expression from the control. The primer sequences are given in the supplemental material.

Enzyme-linked immunosorbent assay. A tumor necrosis factor alpha (TNF-α) enzyme-linked immunoassay kit (mouse TNF-α; eBioscience, San Diego, CA) was used according to the manufacturer's instructions to quantitate TNF-α levels in media from 3T3-L1 cells.

Transfection and luciferase reporter assay. BHK cells were transfected with expression vectors and luciferase reporter vectors with Lipofectamine 2000 reagent (Invitrogen). A β-galactosidase expression vector was also cotransfected to measure the transfection efficiency. Luciferase activity was measured by using a dual-luciferase reporter assay system (Promega) according to the manufacturer's

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instructions, and the luciferase activity was normalized to the β-galactosidase activity.

Measurement of PAP activity. PAP activity was measured as previously described except the cells were homogenized in buffer A plus 10 mM Triton X-100 for a final concentration of 2 mM Triton X-100 and a final concentration of phosphatidic acid (PA) of 0.2 mM (18). The data are expressed as nmol of PA dephosphorylated/min/mg of protein.

Adenovirus constructs and expression vectors. Adenoviral constructs for lipin 1 shRNA and HA-tagged lipin 1b, as well as expression plasmids for HA-tagged lipin 1b the D712E, I-mut, L-mut, ΔN, and ΔC expression plasmids, and the ACO3-tk-luc reporter plasmid have all been previously described (12, 18). The FLAG-tagged NFATc4, constitutively active NFATc4 containing Ser 168 and 170 mutated to Ala (NFAT-CA), and the NFAT-tk-luc reporter plasmid were generously provided by Chi-Wing Chow, Albert Einstein College of Medicine (56). The Ad-EGFP-VIVIT was made by generating an adenoviral expression vector containing enhanced green fluorescent protein (EGFP) fused in frame with two copies of the NFAT inhibitory peptide MAGPHPVIVITGPHEE (1). For infection of 3T3-L1 cells, differentiated adipocytes at 9 to 15 days postdifferentiation were infected with adenovirus for 3 to 4 days before harvest. The infection efficiency as determined by GFP expression was >95%. Myc-tagged histone deacetylase (HDAC) expression vectors have been previously described (32).

Recombinant Lipin 1. The coding sequence of lipin 1b was subcloned downstream of the glutathione *S*-transferase (GST) tag in the pEBG expression vector to generate pEBG-lipin 1b. Ten 15-cm plates of 293T cells were transfected with 20 μg of pEBG-lipin 1b and harvested in buffer A plus 1% Triton X-100. GST-lipin 1b was purified with glutathione-Sepharose 4B beads (GE Healthcare) according to the manufacturer's instructions.

Mouse studies. Breeding colonies of *fld* and *ob*/*ob* mice were established at Washington University School of Medicine from mice originally obtained from the Jackson Laboratory (Bar Harbor, ME). *fld* mice were genotyped according to previously published protocols (40). Adipose tissue was collected from wild-type and *fld*/*fld* mice at 3 to 4 months of age. Adipose tissue was collected from female *ob*/*ob* mice and sex- and age-matched lean control mice at the indicated ages. All animal experiments were approved by the Washington University School of Medicine Animal Studies Committee conformed to criteria outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

RESULTS

Lipin 1 interacts with NFATc4. Using lipin 1 as bait in a two-hybrid screen of a 3T3-L1 expression library, we identified NFATc4 as a potential interacting protein; NFATc4 was present in two independent clones out of nine potential interactors (data not shown). As shown in Fig. 1A, immunoprecipitation of HA-tagged lipin isolated FLAG-tagged NFATc4 and vice versa immunoprecipitations of FLAG-NFATc4 from the same lysates pulled down HA-tagged lipin 1. To examine the functional significance of the lipin 1-NFATc4 interaction, we used a reporter gene that contains the NFAT binding motif from the interleukin-2 (IL-2) promoter (Fig. 1C). NFATc4 expression strongly activated the reporter while coexpression of lipin 1 decreased activity by ca. 50%. The repressive effect of lipin 1 was dependent on the ability to interact with NFATc4. As seen in Fig. 1A, a lipin 1 construct deleted for the entire carboxy-terminal CLIP domain (ΔC) neither interacted with NFATc4 (Fig. 1A) nor repressed NFATc4 transcriptional activity (Fig. 1C). In contrast, lipin 1 with the NLIP domain deleted (ΔN) was capable of both interaction and repression. Because lipin 1 is known to serve as a coactivator of PPARα, we were surprised that lipin 1 exhibited repressive effects on NFAT transcriptional activity. We next tested the ability of lipin 1 to interact with and transcriptionally activate PPARα under identical conditions. Figure 1B shows that similar regions of lipin 1 interacted with PPARα, whereas Fig. 1D shows that lipin 1 activated a PPARα-responsive reporter construct, ACO3-tk-luc, as previously reported (12). As seen with NFAT, the CLIP domain is required for both PPARα interaction and transcriptional activation. These results suggest that lipin 1 can act to repress or activate transcription factors.

We previously reported that lipin 1 requires an LxxIL motif in the carboxy terminus to interact with PPARα (12). There are two pentameric hydrophobic residue-rich motifs in this region of lipin 1 (IIxxI at residues 706 to 711 and LxxIL at residues 719 to 724). When I723 and L724 were mutated to phenylalanine (L-mut), lipin 1 was unable to interact with NFATc4 and had no repressive effect on reporter gene activity. However, lipin 1 that contained I706 and I707 mutated to phenylalanine (I-mut) retained the ability to interact with and repress NFATc4 transcriptional activity (Fig. 1E and G). This was similar to the interaction results previously reported for PPARα (also shown in Fig. 1F and H). In contrast, although mutation of the first aspartic acid residue in the lipin 1 catalytic motif to glutamic acid (D712E) did not affect the interaction of lipin 1 and PPARα (Fig. 1F), it eliminated the ability of lipin 1 to interact with NFATc4 (Fig. 1E). The D712E mutant also failed to repress NFATc4 transcriptional activity (Fig. 1G) but still coactivated PPARα (Fig. 1H). The I-mut, L-mut, and D712E mutants all lack enzymatic activity, but only I-mut mutant can interact with and repress NFATc4 transcription. Therefore, the inability of the D712E mutant to repress NFATc4 transcriptional activity is due to lack of association with lipin 1 rather than a loss of PAP activity. These data also indicate that there are subtle differences in the requirements for NFATc4 and PPARα for binding to lipin 1; the D712E mutant can interact with PPARα but not NFATc4.

PGC-1α is a transcriptional coactivator that interacts with lipin 1 and acts synergistically with lipin 1 to activate the PPARα transcriptional activity. Therefore, we tested whether PGC-1α modulates the repressive effects of lipin 1 on NFATc4-responsive promoters. Figure 2A shows that PGC-1α coimmunoprecipitates PPARα but not NFATc4. Likewise, while PGC-1α increases the transactivation of the PPARα responsive reporter ACO3tk reporter either with or without lipin 1, there was no effect on NFATc4 transcriptional activity (Fig. 2B). Thus, PGC-1α insufficiency is not causing nonspecific squelching of NFATc4 activity, and the ability of lipin 1 to repress NFATc4 transcriptional activity is independent of PGC-1α.

There are four calcium responsive NFAT isoforms and all are expressed in 3T3-L1 adipocytes, with NFATc1 and -c3 constitutively expressed and NFATc2 and -c4 induced during adipocyte differentiation (55). Despite similar expression of the FLAG-tagged NFAT proteins, only NFATc2 and -c4 showed significant levels of interaction with lipin 1 (Fig. 2C). The NFATc4 clone from the two hybrid screen that identified NFATc4 as a potential lipin 1 interactor only contained amino acids 501 to 901. Expression of this portion of NFATc4 produced a truncated NFATc4 protein that retained the ability to interact with lipin 1 by coimmunoprecipitation (data not shown). Therefore, the specific residues required for interaction with lipin 1 are contained within the RHD/DNA-binding domain and carboxy terminus of NFATc4.

Lipin 1 can interact with activated NFAT. NFAT-mediated transcription is activated by intracellular calcium signaling, and in unstimulated cells hyperphosphorylated NFAT is found in the cytoplasm (20). Calcium mobilization activates calcineurin,

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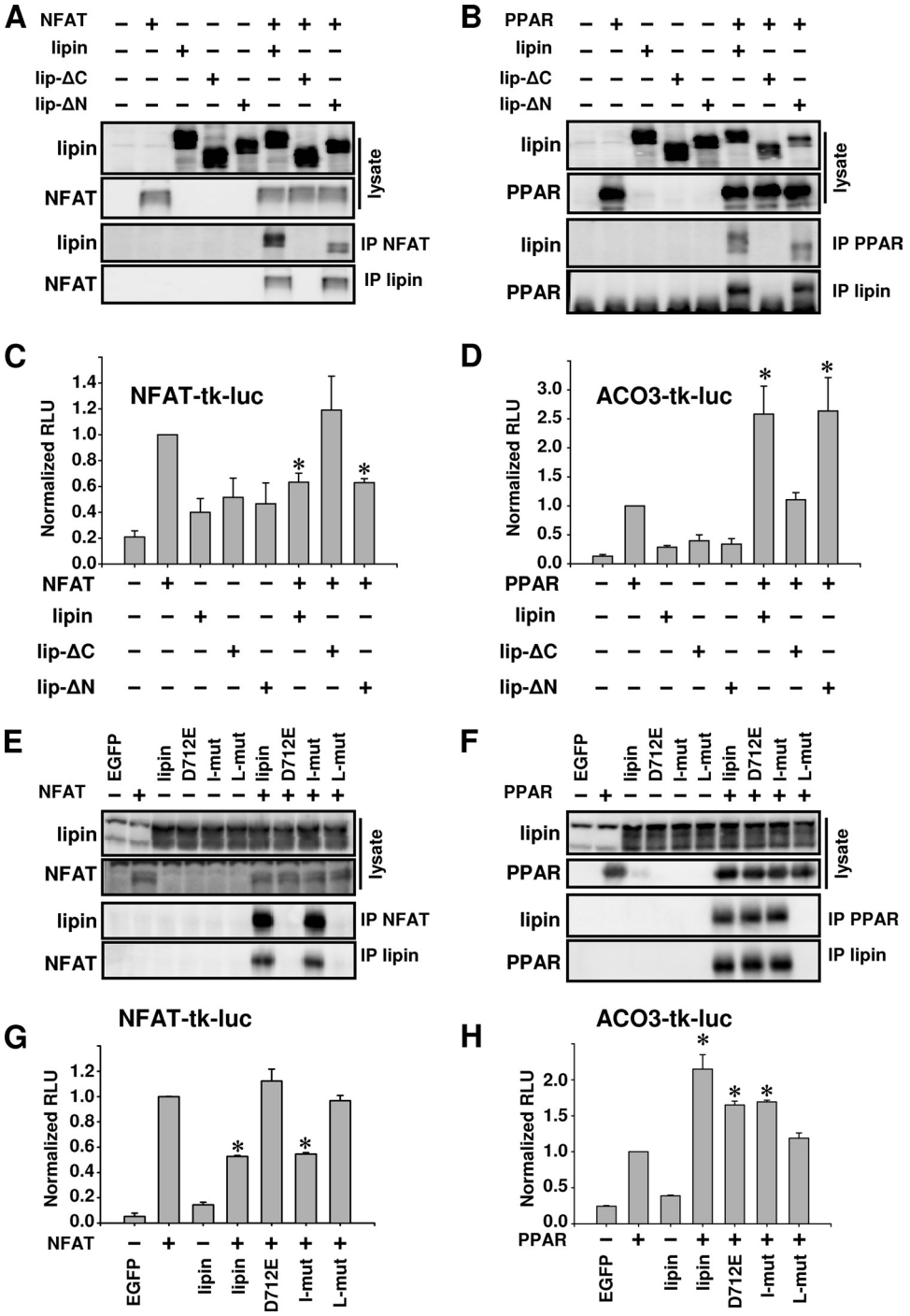

FIG. 1. Lipin 1b interacts with NFATc4 and represses transcriptional activity. (A) BHK cells were transfected with expression vectors for EGFP, FLAG-NFATc4 (NFAT), HA-lipin 1 (lipin), and the lipin 1 deletion mutants lip-ΔC (containing amino acids 1 to 641) and lip-ΔN (containing amino acids 107 to 924). Cell extracts were subjected to immunoprecipitation with either anti-FLAG or anti-HA antibodies. Immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies to the epitope tag of the indicated proteins. (B) BHK cells were transfected as in panel A, but with FLAG-tagged PPARα (PPAR) instead of NFATc4. Immune complexes were isolated and immunoblotted as in panel A. (C) BHK cells were transfected with the indicated combinations of FLAG-NFATc4, HA-lipin 1, lipin 1 deletion mutants, and the NFAT-tk-luc reporter construct. BHK cells transfected with pSV-βgal served as a control for transfection efficiency. The data shown are the relative light units produced by luciferase activity normalized to β-galactosidase expression and are expressed relative to NFATc4 activity alone. Error bars represent standard error ($n = 4$). Student t test indicates significant difference ($P < 0.05$) from NFATc4 alone (∗). (D) Reporter activity of PPARα was performed as in C using the ACO3-tk-luc reporter construct ($n = 4$). The data expressed relative to PPARα alone. (E) BHK cells were transfected with expression vectors for FLAG-NFATc4, HA-lipin 1, and the lipin 1 point mutants D712E, I-mut, and L-mut. Coimmunoprecipitations were performed as in panel A. (F) Transfections and coimmunoprecipitation performed as in panel E, except PPARα was transfected instead of NFATc4. (G) NFATc4 reporter analysis with lipin 1 point mutants was performed as in panel C ($n = 3$). (H) PPARα reporter analysis with lipin 1 point mutants was performed as in panel D ($n = 2$).

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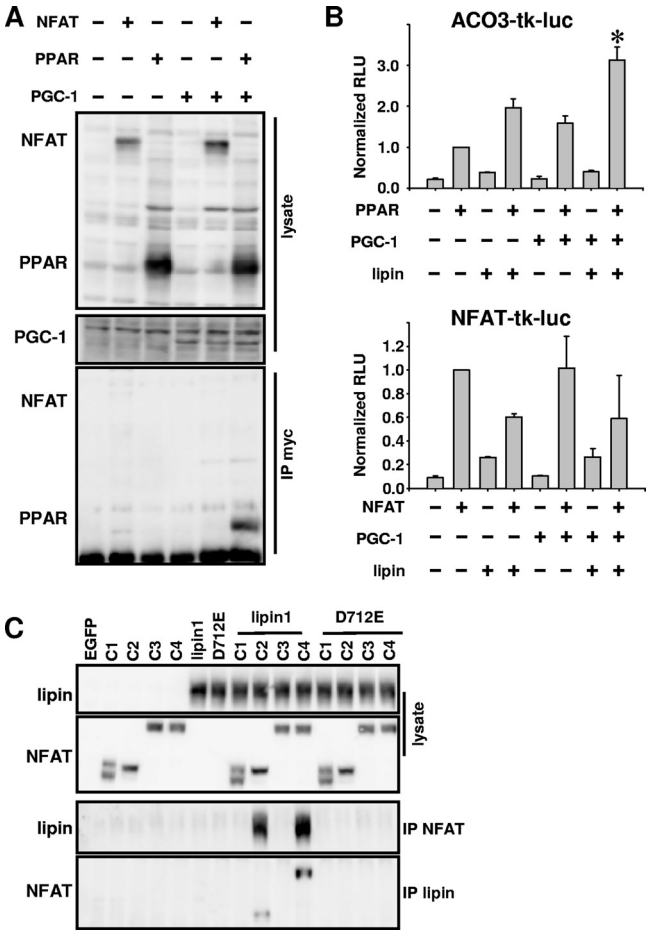

FIG. 2. Lipin 1does not require PGC-1α to repress NFATc4, and lipin 1 can suppress both NFATc2 and NFATc4. (A) Myc-tagged PGC-1α (PGC-1) was transfected with FLAG-tagged NFATc4 (NFAT) or FLAG-tagged PPARα (PPAR). Coimmunoprecipitations were performed with anti-myc antibodies and immunoblotted with anti-FLAG (top and bottom panels) or anti-myc antibodies (middle panel). (B) Reporter activity of PPARα or NFATc4 in the presence of PGC-1α and lipin 1 ($n = 3$ or 2, respectively). *, significant difference (<0.05) from PPARα plus lipin 1 alone. (C) BHK cells were transfected with expression vectors for FLAG-tagged NFATc1 to -c4 and either HA-tagged lipin 1 or the HA-tagged lipin 1 mutant D712E. Coimmunoprecipitations were performed as in Fig. 1A.

also known as PP2B, a calcium-sensitive phosphatase that dephosphorylates NFAT. This leads to NFAT nuclear localization and transcriptional activation (20). Yang et al. demonstrated that mutation of the calcineurin-regulated phosphorylation sites S168 and S170 to alanine creates a constitutively active NFATc4 (NFAT-CA) (Fig. 3B) (56). NFAT-CA interacts with lipin 1 to an extent similar to that of wild-type NFATc4 (Fig. 3A). Despite an overall increase in transcriptional activity the constitutively nuclear NFAT-CA was still effectively repressed by lipin 1 (Fig. 3B). Although treatment of cells with A23187 increased NFAT transcriptional activity >2-fold, this effect was reversed by lipin 1. As shown in Fig. 3C, constitutively active calcineurin (ΔCnA) activated the NFAT reporter and this activation was also largely reversed by lipin 1 overexpression.

We next examined the interaction between endogenous lipin 1 and NFATc4 in 3T3-L1 adipocytes. Cyclosporine (CsA) inhibits calcineurin thus blocking NFAT cytosolic to nuclear transition (Fig. 3D), while A23187 promotes NFAT nuclear accumulation (Fig. 3D). Lipin 1 nuclear accumulation was unaffected by either treatment. Interaction between endogenous nuclear lipin 1 and NFATc4 directly corresponded to the amount of NFATc4 present in the nucleus, since CsA treatment decreased the amount of NFAT in the nucleus and resulted in less NFATc4-lipin 1 interaction, whereas A23187 treatment increased nuclear NFAT as well as the amount of NFATc4 interacting with lipin 1. Together, these results suggest that lipin 1-mediated repression of NFAT transcriptional activity occurs after NFAT mobilization and activation by calcium, perhaps in the nucleus at the level of DNA binding.

It has been previously reported that phosphorylation of NFATc4 by mTORC1, the rapamycin-sensitive mTOR complex, promotes NFATc4 nuclear export (57). Lipin 1 also contains rapamycin-sensitive phosphorylation sites, suggesting that lipin 1 is downstream of mTORC1 as well (18, 24). Rapamycin treatment modestly increases the amount of lipin 1 in the nucleus, suggesting that lipin 1 nuclear localization is at least partially dependent on mTORC1 (data not shown). A recently published study detailing the mechanisms controlling lipin 1 nuclear localization confirms our observation that rapamycin increases lipin 1 nuclear localization (38). Thus, rapamycin treatment opposes NFAT nuclear export and promotes lipin 1 nuclear localization.

NFATc4 provides specificity for lipin 1 repression. Although NFAT transcription factors can bind to DNA as monomers or dimers, the NFATs frequently activate gene expression at composite binding elements through cooperative interactions with other transcription factors (44). The ubiquitous jun/Fos complex, AP-1, was found to bind in a cooperative fashion with NFAT at a composite NFAT binding element in the IL-2 promoter. The reporter gene experiments shown in Fig. 1 utilized the composite NFAT:AP-1 binding element. In 3T3-L1 adipocytes, C/EBPα and NFATc4 have been shown to cooperatively activate the PPARγ2 promoter (54). We sought to determine whether the identity of the NFAT binding partner affects the ability of lipin 1 to repress NFAT transcriptional activity. Cotransfection of C/EBPα did not block the ability of lipin 1 to interact with NFATc4 (Fig. 3E). As previously reported, cotransfection of NFATc4 and C/EBPα caused an additive increase in the transcriptional activation of the PPARγ2 proximal promoter (Fig. 3F) but had no effect on the NFAT-tk-luc promoter, which does not have a C/EBP response element (data not shown). Cotransfection of lipin 1 repressed the PPARγ2 promoter in a manner dependent on NFATc4 expression (Fig. 3F). Therefore, lipin 1 can repress NFATc4 in the context of at least two different composite elements.

Lipin 1 interacts with NFATc4 bound to DNA and is present at the promoters of NFAT target genes. Since lipin 1 expression inhibited nuclear NFAT, we speculated that lipin 1 may either bind directly to NFAT at the promoters of target genes or block NFAT binding to DNA. We sought to determine whether lipin 1 blocks the ability of NFAT to bind to DNA *in vitro* using an EMSA. We found that the coexpression of lipin 1 did not affect NFATc4 binding to DNA (Fig. 4A). In fact, coexpression of lipin 1 and NFATc4 resulted in the formation of a larger DNA-protein complex, seen as a band of retarded mobility. The formation of the higher mobility DNA-binding

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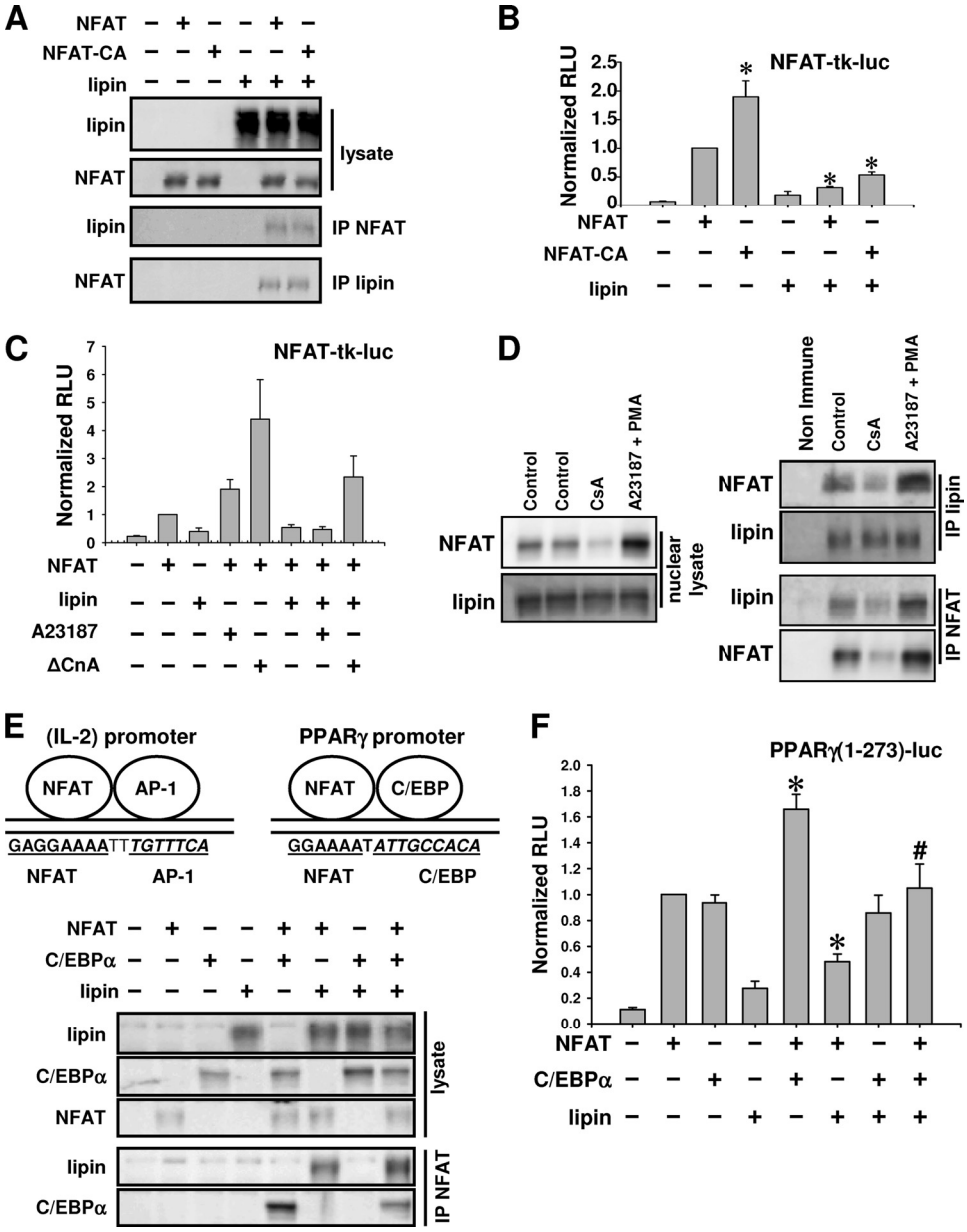

FIG. 3. Lipin 1 represses activated NFATc4 and NFATc4 bound to different composite elements. (A) BHK cells were transfected with the indicated expression vectors for FLAG-NFATc4, constitutively active NFATc4 (FLAG-NFAT-CA), and HA-lipin 1. Coimmunoprecipitations were performed as in Fig. 1A. (B) NFATc4 reporter gene analysis with NFATc4, constitutively active NFATc4, and lipin 1 were performed as described in Fig. 1C ($n = 3$). (C) Reporter gene analysis of BHK cells transfected with expression vectors for FLAG-NFATc4 and HA-Lipin 1 and a constitutively active calcineurin vector (ΔCnA) as indicated. At 24 h after transfection the indicated cells were treated with 1 μM A23187 for 24 h. NFATc4 reporter analysis was performed as in Fig. 1C ($n = 3$). (D) Differentiated 3T3-L1 cells, were either untreated (control), or treated with CsA (5 μg/ml), or A23187 (1 μM) plus phorbol myristate acetate (PMA; 50 nM) for 18 h. After treatment, nuclear extracts were prepared and antibodies to endogenous lipin 1 and NFATc4 were used for coimmunoprecipitation. (E) The top panel is a schematic of NFATc binding to two different composite elements. For the bottom panel, BHK cells were transfected with expression vectors for FLAG-NFATc4, HA-C/EBPα, and HA-lipin 1b. Coimmunoprecipitation of NFATc4 was performed as in Fig. 1A. (F) BHK cells were transfected with the indicated expression vectors along with the NFAT:C/EBPα-responsive reporter from the promoter of PPARγ. NFATc4 reporter analysis was performed as in Fig. 1C ($n = 4$). ∗ and #, significant difference ($P < 0.05$) from NFATc4 alone (∗) or from NFAT plus C/EBPα (#).

complex required NFATc4, since lipin 1 alone showed no complex formation. The addition of unlabeled oligonucleotide containing the NFAT binding site eliminated both bands. In the right hand panel, the identity of the complex is demonstrated by the indicated gel shifts with antibodies to either NFAT (FLAG) or lipin 1 (HA). Overexpression of lipin 1 with PPARα also induced the formation of a larger complex (Fig. 4B). As an additional measure of specificity, recombinant GST-tagged lipin 1 was added to the NFATc4-DNA binding complex generated from cells expressing NFATc4 only. This

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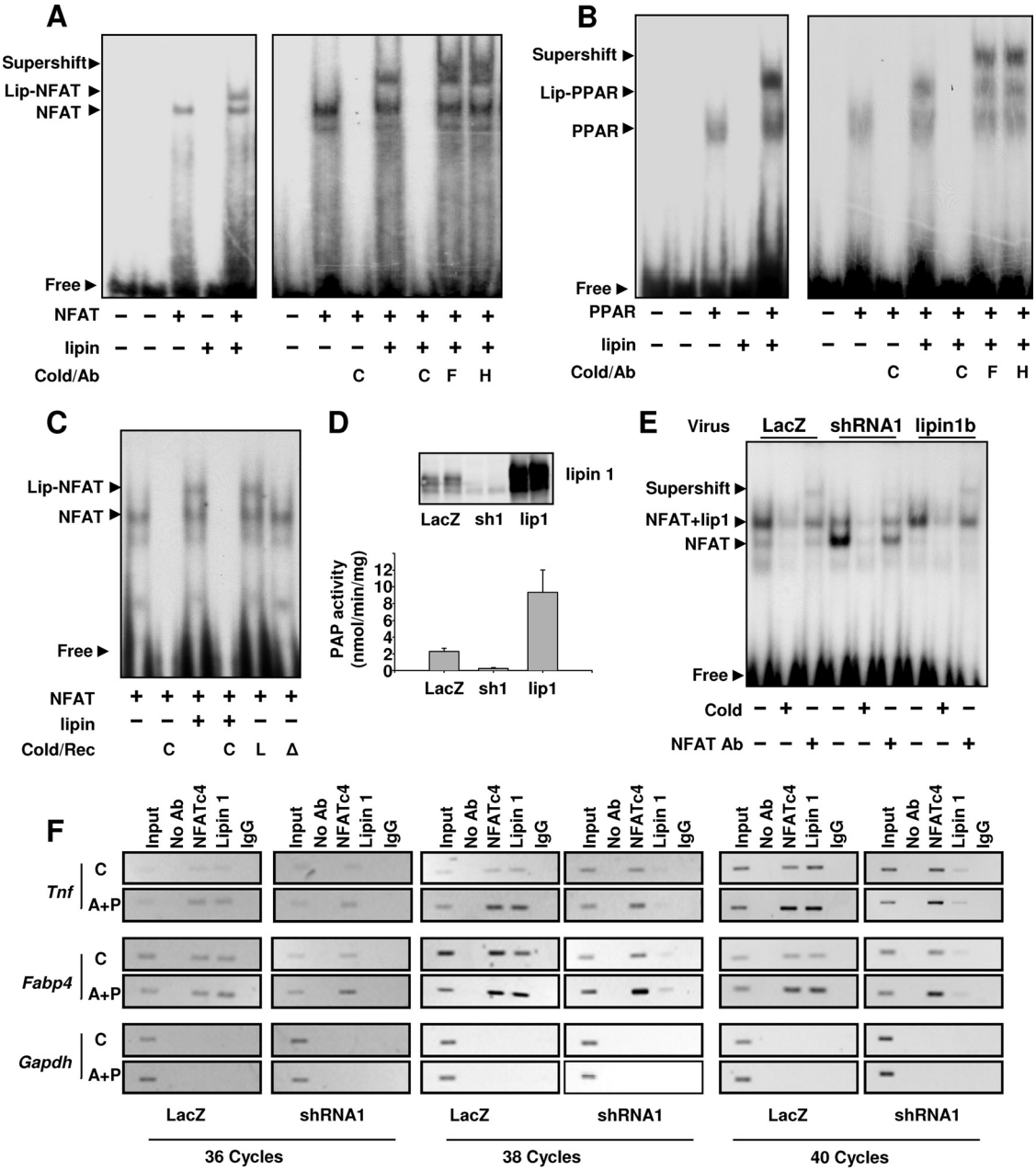

FIG. 4. Lipin 1 forms a complex with both NFATc4 and PPARα on DNA *in vitro* and is present at the promoter of NFAT target genes *in vivo*. (A) BHK cells were transfected with expression vectors for FLAG-tagged NFATc4, HA-tagged lipin 1b, or both. The DNA bound complexes were then analyzed by EMSA. The first lane contains labeled oligonucleotide only, while the second lane is lysates from EGFP-transfected cells. The free probe (free), NFATc4-DNA complex (NFAT), and the lipin 1-NFATc4-DNA complex (Lip-NFAT) and antibody-induced supershift (Supershift) are indicated. The right panel shows the specificity of NFAT binding via competition with 100-fold of excess unlabeled oligonucleotide (lanes C) and verifies the identity of the NFATc4 and lipin 1-NFATc4 bands by preincubation with antibodies specific for the epitope tags of NFATc4 and lipin 1, FLAG (lane F) and HA (lane H), respectively. (B) BHK cells were transfected with expression vectors for FLAG-tagged PPARα and HA-tagged lipin 1b. Gel mobility shift assays performed as described for panel A using an oligonucleotide derived from the PPARα binding site in the apoE III promoter. (C) BHK cells were transfected and binding reactions performed as described above. Where indicated, the DNA-binding reaction was supplemented with recombinant lipin 1 (L) or recombinant lipin 1 that was heat inactivated by boiling for 10 min (Δ). (D) Fully differentiated 3T3-L1 adipocytes were infected with adenovirus expressing LacZ shRNA (LacZ), lipin 1 shRNA (shRNA1), or HA-tagged lipin 1. At 3 to 4 days after infection, adipocytes were harvested for protein analysis. The top panel shows the results of Western blot analysis of lipin 1 expression in LacZ shRNA (LacZ)-, lipin 1 shRNA (shRNA1)-, or HA-tagged lipin 1b-expressing cells. The bottom panel shows results obtained with total Mg^{2+}-dependent PAP in adenovirus-infected cells. (E) 3T3-L1 adipocytes infected with adenovirus expressing LacZ shRNA (LacZ), lipin 1 shRNA (shRNA1), or HA-tagged lipin 1 were used to prepare nuclear extracts to analyze NFATc4 binding activity. Where indicated, cold competitor oligonucleotide or antibodies to endogenous NFATc4 were included in the reactions. (F) Semiquantitative ChIP assays for the *Tnf*, *Fabp4*, and *Gapdh* promoters in 3T3-L1 cells infected with adenovirus expressing shRNA targeting LacZ or lipin 1. Either no antibody, antibodies specific for NFATc4, lipin 1, or nonimmune antibodies were used to immunoprecipitate chromatin-bound proteins from untreated, control cells (rows C) or cells treated with A23187 (1 μM) plus PMA (50 nM) for 18 h (A+P). PCR was performed for the indicated number of cycles using primer pairs specific for the *Tnf*, *Fabp4*, and *Gapdh* promoters. Input is PCR amplification of 1% of the amount of lysates used for the immunoprecipitation. The results in the figure are representative of two independent experiments.

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induced the formation of a retarded mobility band similar to that seen with the overexpression of lipin 1 and NFATc4 (Fig. 4C). Denaturing the recombinant lipin 1 (Δ) by boiling at 95°C for 10 min before addition to the binding reaction eliminated the complex. Thus, lipin 1 can bind to the NFATc4-DNA complex even when supplied from an exogenous source and after the NFATc4-DNA complex is formed.

We next confirmed that lipin 1-NFATc4 complex formed on DNA occurs with endogenous proteins. The NFATs are primarily known for their role in T-cell activation and development but have also been shown to play a role in adipocyte development and expression of several adipocyte-specific genes including FABP4, PPARγ, and resistin (19, 55, 56). We chose 3T3-L1 adipocytes as a model system because they express high levels of lipin 1 and, as previously mentioned, all four isoforms of calcium-regulated NFAT (NFATc1 to -c4) (18, 55). However, both embryonic fibroblasts from *fld* mice and lipin 1-deficient 3T3-L1 cells have a decreased ability to differentiate into adipocytes and show decreased expression of the master regulators of adipogenesis (C/EBPα and PPARγ), indicating that lipin 1 is essential for adipocyte differentiation (28, 39, 42). Precisely how the loss of lipin 1 prevents adipocyte differentiation is not completely clear, but it is likely to have pleiotropic effects on adipocyte differentiation through both transcriptional and lipid biosynthetic pathways. There is evidence that repression of the NFAT genes is essential to allow adipocyte differentiation as constitutive activation of NFAT, either through expression of activated NFAT or activated calcineurin, inhibits 3T3-L1 adipocyte differentiation (36, 37). So, in order to avoid confounding effects on differentiation from the loss of lipin 1, we reduced lipin 1 expression in mature 3T3-L1 adipocytes. At 3 to 4 days after infection with lipin 1 shRNA adenovirus, lipin 1 protein and total PAP activity were both decreased by >95% (Fig. 4D), whereas infection with HA-lipin 1 adenovirus increased lipin 1 protein and PAP activity by ~5-fold. Lysates from LacZ-infected 3T3-L1 adipocytes analyzed for NFAT complex formation (Fig. 4E) demonstrated an upper band and a lower band, a finding similar to what was observed after transient transfection in BHK cells (Fig. 4A). Both bands were eliminated by competition with unlabeled oligonucleotides and were partially supershifted by the addition of NFATc4 antibody to the reaction mix. Ablation of lipin 1 expression with shRNA increased the relative level of the lower band at the expense of the upper band, while forced expression of lipin 1 did the opposite.

Since lipin 1 can bind to NFAT-DNA complexes, ChIP was used to determine whether lipin 1 is present at the promoters of genes that contain NFATc4 response elements. We show that immunoprecipitation of either NFATc4 or lipin 1 results in the isolation of genomic DNA from the promoters of TNF-α or FABP4, but not GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Fig. 4F). Thus, semiquantitative ChIP analyses demonstrate that in 3T3-L1 cells both lipin 1 and NFATc4 are present at the promoters of NFAT target genes, and the presence of both is increased upon NFATc4 activation. When shRNA targeting lipin 1 is used, there is a significant decrease in the amount of lipin 1 found at the promoters of NFAT target genes with little, if any, effect on the amount of NFAT found at these promoters. This observation, and the ability of endogenous lipin 1 to form a complex with NFATc4 and DNA as measured by EMSA analysis, suggests that lipin 1 binds to NFATc4 at the promoters of NFAT target genes in native chromatin.

Lipin 1 recruits a HDAC to repress NFATc4 transcription. Since lipin 1 appears to have no effect on the ability of NFATc4 to bind to DNA, yet represses NFATc4 transcriptional activity, we hypothesized that the repressive effects of lipin 1 on NFAT transcriptional activity might involve active recruitment of a transcriptional repressor. A common mechanism of transcriptional corepression involves the recruitment of HDACs to the promoters of target genes that deacetylate open chromatin, causing it to close, and thereby repressing transcriptional activation (32). Trichostatin A (TSA) inhibits class I and class II HDAC enzymatic activity, thus relieving HDAC-mediated repression. As shown in Fig. 5A, TSA had no effect on the activity of NFATc4 alone, but the repression of NFATc4 by lipin 1 was eliminated. There was no substantial change in the interaction of lipin 1 with NFATc4 (Fig. 5B). To more directly address how lipin 1 might recruit HDACs to NFAT-regulated target genes, we performed coimmunoprecipitation analyses between lipin 1 and HDAC1 to -4. As seen in Fig. 5C, HDAC1 and -3 can both coimmunoprecipitate with lipin 1. As a functional measure of HDAC recruitment to NFAT target genes, we used antibodies targeting acetylated histone H3 to isolate DNA sequences surrounding NFAT binding sites, both before and after calcium mobilization and with or without lipin 1 depletion by shRNA. As shown in Fig. 5D, the loss of lipin 1 results in increased histone H3 acetylation at regions surrounding NFAT binding sites. When combined with the sensitivity of lipin 1-mediated repression of NFAT to TSA and the ability of lipin 1 to interact with HDAC1 and -3, these results suggest that lipin 1 recruits HDAC activity to NFAT-responsive promoters to inhibit transcription via chromatin deacetylation.

Analysis of NFAT-Lipin 1 in 3T3-L1 cells. Lipin 1 depletion in 3T3-L1 adipocytes led to an increase in the expression of several NFAT target genes, including FABP4, PPARγ, resistin, IL-6, and TNF-α (Fig. 6A). This further supports the notion that lipin 1 represses NFAT transcriptional activity. To ensure that the lipin 1 shRNA did not induce nonspecific effects due to off target silencing, a second independent shRNA targeting lipin 1 was used (shRNA2); it generated similar effects on gene expression as shRNA1 (Fig. 6A). RT-PCR analyses were confirmed by Northern blot (Fig. 6B). Importantly, the induction of gene expression caused by lipin 1 knockdown was reversed by the addition of CsA, suggesting that the effects of lipin 1 knockdown by shRNA occur through NFAT transcription. We confirmed that the effects of lipin 1 knockdown by shRNA on mRNA expression occur through NFAT transcriptional activity by coexpression of an EGFP-VIVIT peptide fusion protein, a more specific inhibitor of calcineurin-mediated dephosphorylation of NFAT than CsA (1). Figure 6C shows that EGFP-VIVIT completely reverses the induction of TNF-α message caused by loss of lipin. We also investigated the consequence of lipin 1 overexpression in 3T3-L1 adipocytes. However, despite a substantial increase in lipin 1 protein only TNF-α demonstrated a significant decrease in expression, while other NFAT targets tested showed a nonsignificant trend toward repression (Fig. 6D). Figure 6E shows that, consistent with the interaction studies in Fig. 1E and the reporter gene results in

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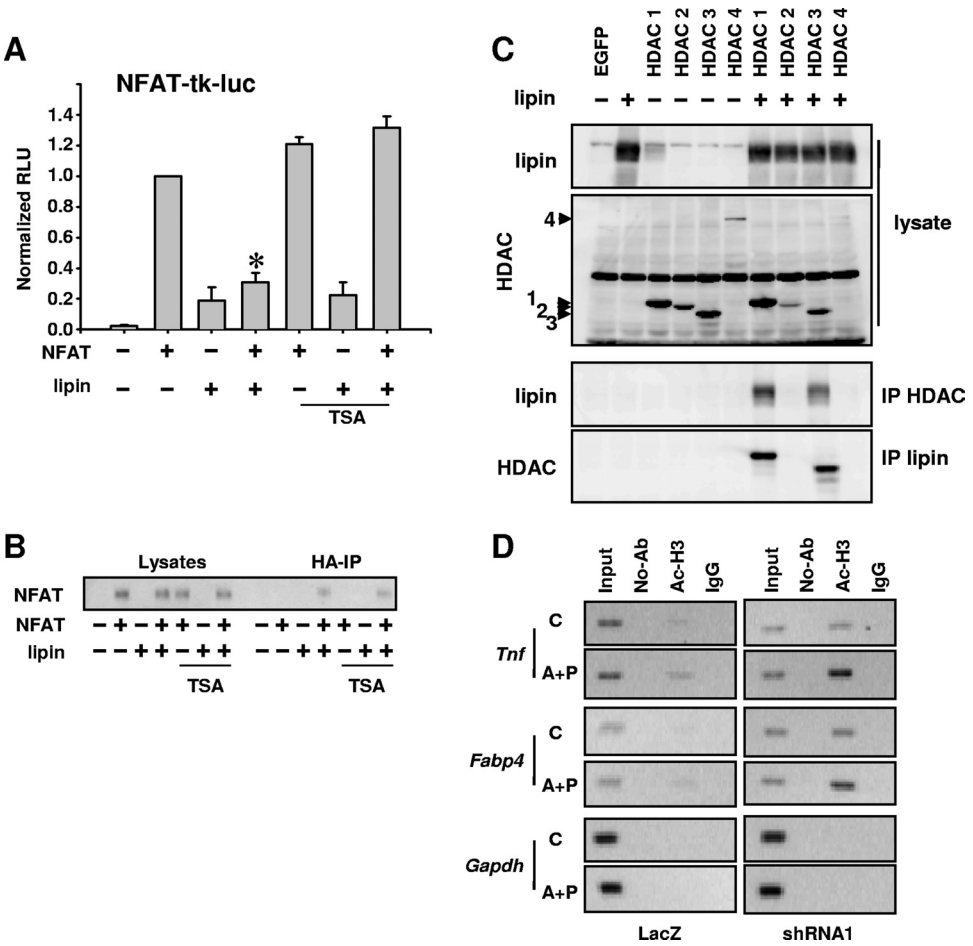

FIG. 5. TSA reverses lipin 1-mediated repression of NFATc4. (A) BHK cells were transfected with expression vectors for FLAG-NFATc4 and HA-lipin 1b and analyzed for transcriptional activation of NFAT-tk-luc in the presence or absence of 500 nM TSA pretreatment for 24 h ($n = 3$). (B) BHK cells were transfected as in panel A and subjected to coimmunoprecipitation as in Fig. 1A. (C) BHK cells were transfected with expression vectors for Myc-tagged HDAC1-4 and HA-tagged lipin 1. Reciprocal immunoprecipitations using Myc or HA antibodies were performed, and immune complexes were analyzed as in Fig. 1A. (D) ChIP assays with acetylated histone H3 antibodies were performed on 3T3-L1 adipocytes infected and treated as described in the legend to Fig. 4F.

Fig. 1G, the I-mut mutant is capable of repressing TNF-α message abundance, whereas the L-mut mutant cannot.

Calcium mobilization acts synergistically with lipin 1 depletion to activate TNF-α message and protein. To further explore the effects of NFAT derepression, we focused on the expression of TNF-α. The depletion of lipin 1 resulted in a 4-fold increase in TNF-α expression (Fig. 7A). However, as early as 2 h after the stimulation of Ca^{2+} signaling by A23187, there was a very robust (~11-fold) increase in TNF-α expression in lipin 1-deficient cells, whereas there was only a modest (33%) increase in TNF-α expression in βgal-infected cells. In contrast, overexpression of lipin 1 decreased basal expression of TNF-α and largely prevented the A23187-mediated increase in TNF-α.

CsA is a nonselective inhibitor of calcineurin activity. The inhibitor of NFAT-calcineurin compounds (INCA) are small organic molecules that are specific for inhibiting the interaction between calcineurin and the NFAT transcription factors (49). These compounds bind to the NFAT-calcineurin docking site and prevent calcineurin-mediated dephosphorylation and activation of NFAT. The addition of 3 μM INCA6, like CsA, also blocks the increase in TNF-α message that occurs with lipin 1 depletion confirming that this increase is mediated through NFAT (Fig. 7B). As in Fig. 7A, A23187 addition to cells lacking lipin 1 expression showed a dramatic activation of TNF-α, an effect that was blocked by the addition of CsA. In order to verify that the increase of TNF-α message levels was functionally relevant, the secretion of TNF-α by the cells into the medium was examined. Figure 7C shows that the increased TNF-α message seen in lipin 1-depleted cells translated into an increase in TNF-α secretion; the combination of lipin 1 shRNA and A23187 leads to a striking further 10-fold increase in TNF-α secretion. In conclusion, depletion of lipin 1 increases NFAT target gene expression. Activation of NFAT in the context of lipin 1 depletion causes such a powerful induction of NFAT target genes to suggest that lipin 1 is a potent repressor of activated NFAT.

Mice lacking lipin 1 exhibit increased expression of NFAT-regulated genes. We examined what effect the loss of lipin 1 expression has on NFATc4 target gene expression *in vivo* using

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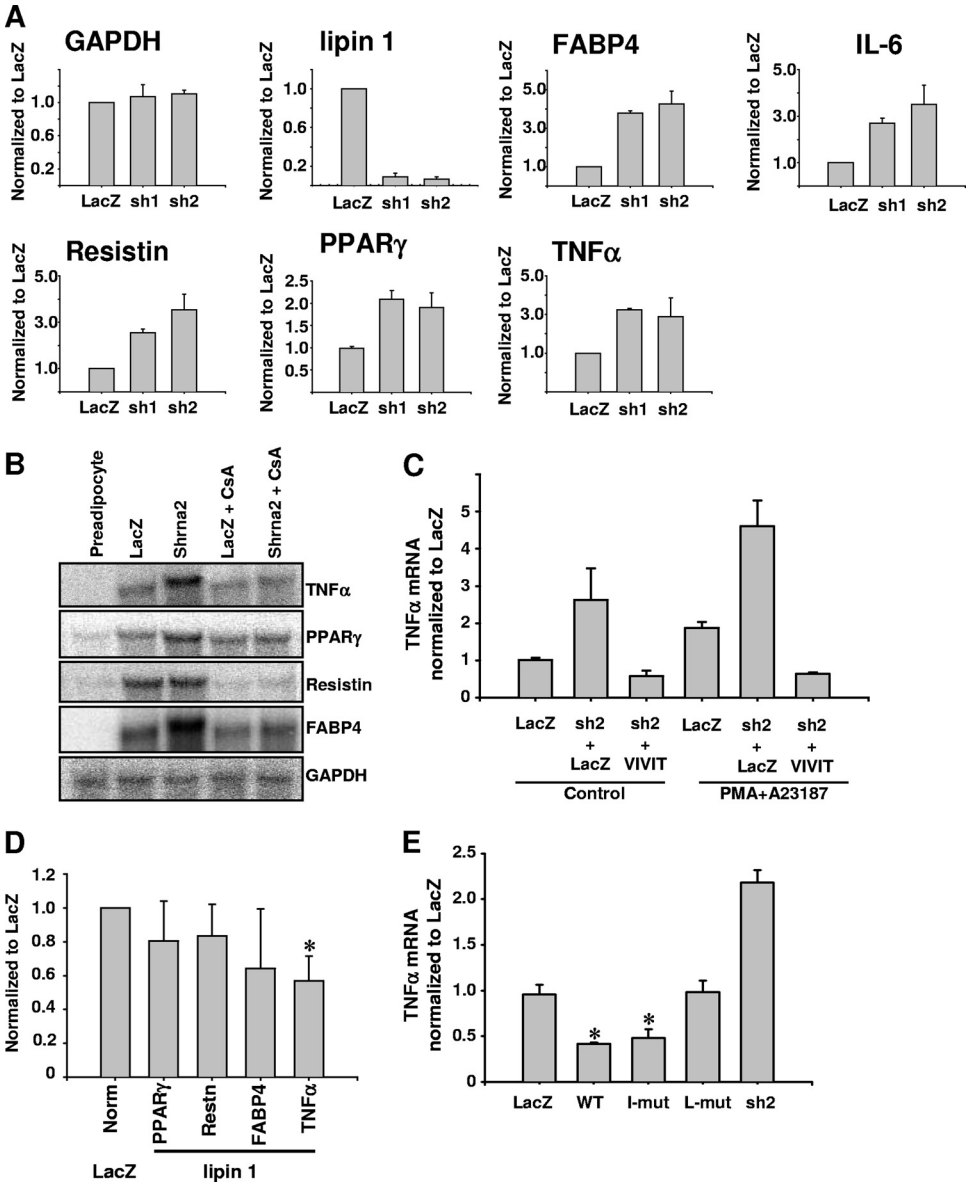

FIG. 6. Depletion of lipin 1 in 3T3-L1 adipocytes induces the expression of NFAT-responsive genes. (A) Real-time PCR analysis of gene expression in 3T3-L1 cells infected with adenovirus expressing LacZ or one of two different lipin 1 shRNAs (sh1 and sh2). In each experiment the results shown are relative to LacZ expression ($n = 3$ to 6). (B) Northern blot analysis of the indicated NFAT target genes in 3T3-L1 cells infected with adenovirus expressing shRNA toward LacZ or lipin 1 (shRNA2). At 18 to 24 h before harvest, CsA (5 μg/ml) was added, as indicated, to inhibit NFAT activity. The results for confluent but undifferentiated 3T3-L1 preadipocytes are shown on the left (preadipocyte). (C) Real-time PCR analysis of gene expression in 3T3-L1 cells infected with adenovirus expressing shRNA targeting LacZ or lipin 1 (sh2), and EGFP-tagged VIVIT peptide. Where indicated, the cells were pretreated with PMA (50 nM) plus A23187 (1 μM) for 18 h. The total amount of virus used for infections was kept consistent using LacZ shRNA virus. (D) Real-time PCR analysis of gene expression in 3T3-L1 cells infected with adenovirus expressing HA-tagged lipin 1b. The results were analyzed as described above for panel A, with the results normalized to control LacZ reference on the left (Norm) ($n = 3$). (E) Real-time PCR analysis of TNF-α expression in 3T3-L1 cells infected with adenovirus expressing HA-tagged lipin 1b, the point mutants I-mut and L-mut, or shRNA2 (sh2). For panels D and E, the Student t test indicated a significant difference ($P < 0.05$) from LacZ alone (*).

visceral white adipose tissue (WAT) isolated from *fld* mice. In agreement with previous observations (47) and consistent with our findings in 3T3-L1 cells, we found increased expression of PPARγ and FABP4. In addition, the expression of resistin, IL-6, and TNF-α was markedly induced in *fld* WAT (Fig. 8). These results are consistent with our observations in the 3T3-L1 model system and provide strong evidence that lipin 1 represses NFATc4 transcriptional activity not only *in vitro* but also *in vivo*.

Decline in lipin 1 expression with increasing obesity in *ob*/*ob* mice. To further evaluate whether lipin 1 could play a role in obesity-induced inflammation, we examined the expression of lipin 1 protein and total PAP activity in visceral and subcutaneous adipose tissue of *ob*/*ob* mice at different ages. At 8 weeks

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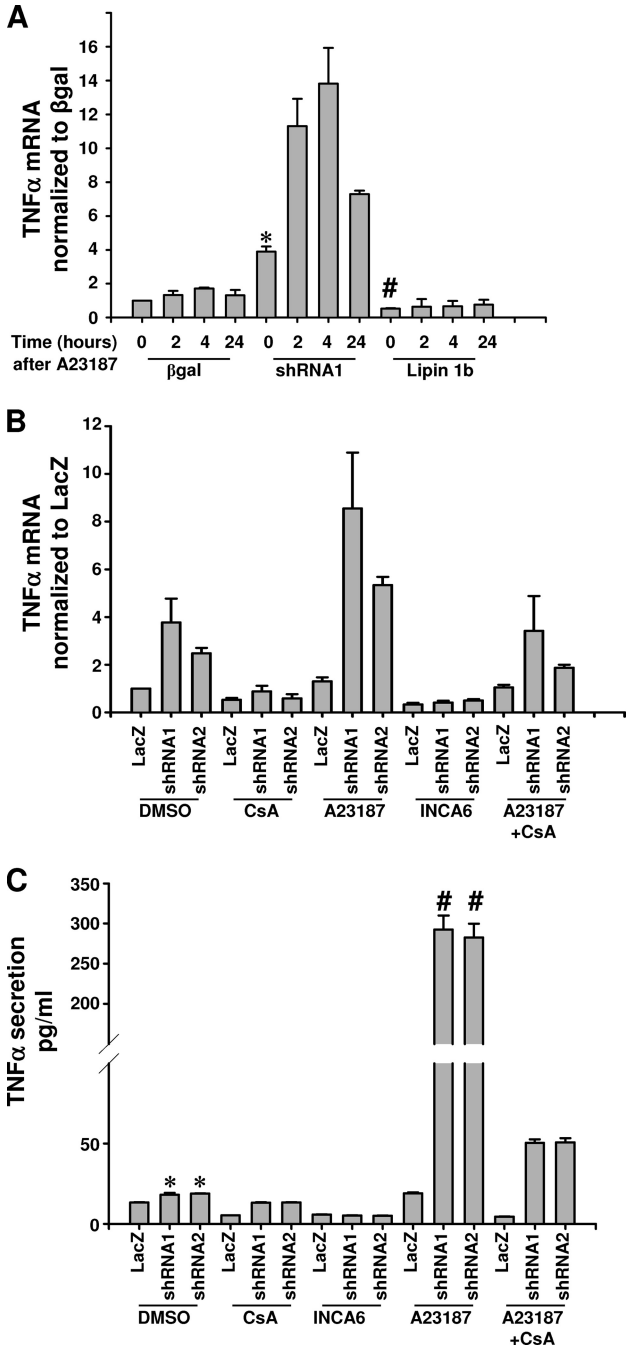

FIG. 7. TNF-α secretion is increased in response to depletion of lipin 1-mediated repression of NFAT. (A) Time course of TNF-α mRNA expression in fully differentiated 3T3-L1 cells infected with adenovirus expressing β-galactosidase (βgal), shRNA construct 1 targeting lipin 1 (shRNA1), and HA-tagged lipin 1 (Lipin 1). At 4 days postinfection, the cells were treated with A23187 (1 μM) for the indicated time. A significant increase (*) or decrease (#) from βgal infected cells ($P < 0.05$) is indicated ($n = 2$). (B) Real-time PCR analysis of TNF-α mRNA expression in differentiated 3T3-L1 cells infected with adenovirus expressing shRNA toward LacZ (LacZ) or lipin 1 (shRNA1 and shRNA2) for 4 days. At 18 to 24 h before harvest, the cells were treated with CsA (5 μg/ml), INCA-6 (3 μM), A23187 (1 μM), or A23187 (1 μM) plus CsA (5 μg/ml) ($n = 3$ to 6). (C) Measurement of TNF-α protein secreted into the media (pg/ml) collected from same cells used for experiments in panel B ($n = 4$; *, $P < 0.001$ from LacZ; #, $P < 0.001$ from LacZ plus A23187).

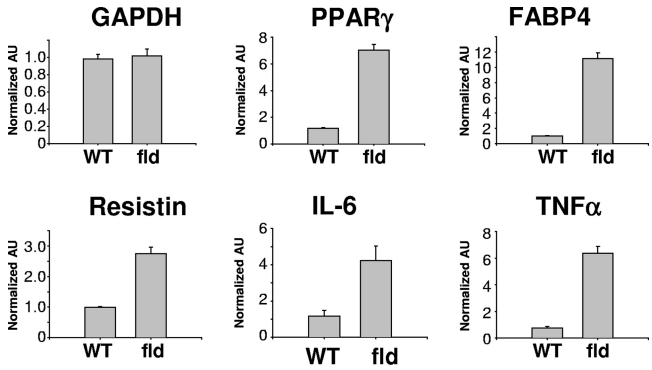

FIG. 8. Expression of NFAT target genes in *fld* mice. RNA was isolated from gonadal fat pads from 42-day-old mice with the indicated genotypes and analyzed by real-time PCR ($n = 4$ to 5 animals for each genotype).

of age there was a clear increase in lipin 1 protein and total PAP activity in both visceral and subcutaneous adipose tissue of *ob*/*ob* mice compared to lean controls (Fig. 9). However, by 12 weeks of age, lipin 1 protein levels in visceral adipose tissue of *ob*/*ob* mice returned to lean control levels, which coincided with a normalization of PAP activity (Fig. 9A and C). The decline in lipin 1 level and PAP activity persisted throughout 19 weeks of age. Between 8 and 12 weeks of age there is a dramatic increase in body weight in the *ob*/*ob* mice in parallel with a significant increase in adipocyte hypertrophy (26, 53). It is thus possible that the loss of lipin 1 expression is related to metabolic complications, increased insulin resistance and glucose intolerance, seen in the 12-week-old mice. Lipin 1 levels decline during the acute phase response in adipocytes, most likely due to the action of proinflammatory cytokines (33). However, lipin 1 levels only declined in the visceral fat pads; both lipin 1 and total PAP activity remained elevated in the *ob*/*ob* subcutaneous fat pads even at 19 weeks of age (Fig. 9B and C). This suggests that elevated levels of insulin, proinflammatory cytokines, and glucose are not necessarily responsible for the decline in lipin 1 levels in *ob*/*ob* visceral fat since the subcutaneous fat is also exposed to the same circulating factors. In addition, we observed an increase in lipin 2 expression in the *ob*/*ob* visceral and subcutaneous fat pads which remained elevated at all times examined (Fig. 9A and B). The data presented here raise the possibility that a decline in lipin 1-mediated repression of NFAT may contribute to the increased visceral fat pad secretion of proinflammatory cytokines observed in *ob*/*ob* mice (17, 21). However, it should be acknowledged that numerous events occur during the development of obesity in *ob*/*ob* mice, not the least of which is the absence of leptin signaling. Thus, decreased lipin 1 expression and activity is unlikely to account for all of the changes in inflammatory cytokine expression observed in these mice.

DISCUSSION

Previously, lipin 1 had been identified as a transcriptional activator of PPARα and PPARγ. Herein, we identify lipin 1 as a repressor of NFATc4 transcriptional activity, a function that does not seem to require lipin 1 lipid phosphatase activity. We show that lipin 1 forms a complex with NFAT and DNA *in vitro*

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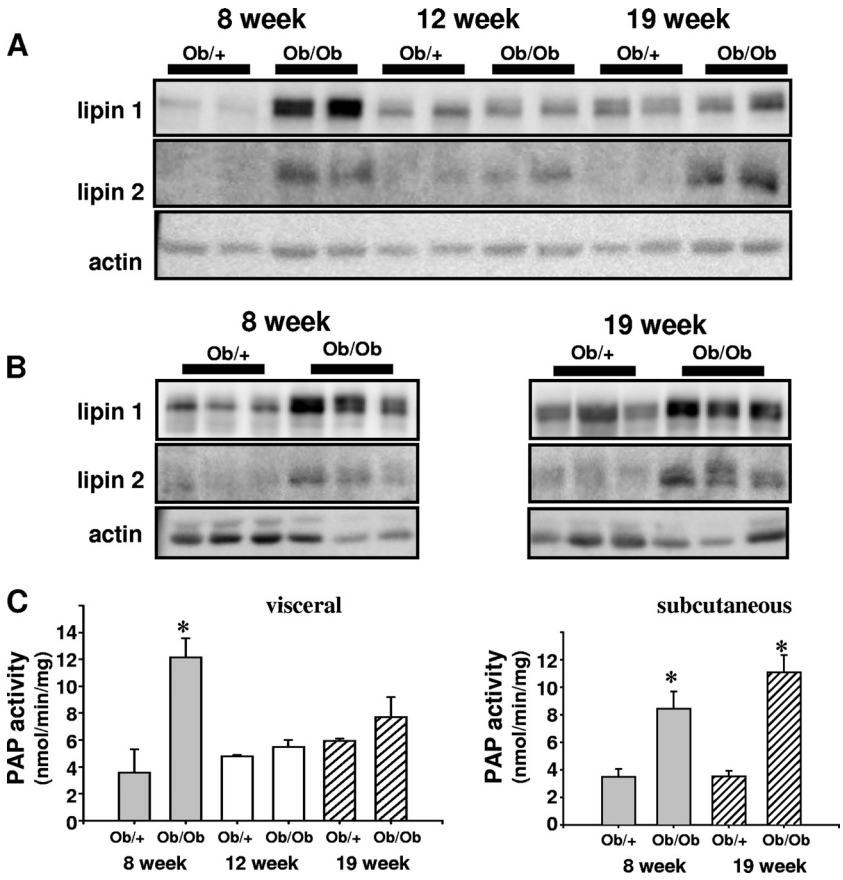

FIG. 9. Age-dependent decline in the expression of lipin 1 in *ob*/*ob* white adipose tissue (WAT). (A) Gonadal fat pads from *ob*/*ob* and *ob*/+ mice were harvested for protein analysis at 8, 12, and 19 weeks of age. Shown are Western blots for lipin 1, lipin 2, and actin. (B) Subcutaneous fat pads from *ob*/*ob* and *ob*/+ mice were harvested for protein analysis at 8 and 19 weeks of age. Shown are Western blots for lipin 1, lipin 2, and actin. (C) Total PAP activity in lysates from visceral and subcutaneous WAT of *ob*/*ob* and *ob*/+ mice ($n = 3$ to 6 for each genotype at each age). A Student *t* test indicated significant differences between *ob*/*ob* and *ob*/+ mice (*; $P < 0.02$ for visceral WAT at 8 weeks, $P < 0.03$ for subcutaneous WAT at 8 weeks, and $P < 0.005$ for subcutaneous WAT at 19 weeks). The data are expressed as nmol of PA dephosphorylated/min/mg of protein.

and is found *in vivo* at the promoters of NFAT target genes. Precisely how lipin 1 functions to modulate transcriptional activity is not entirely clear. Multiple lines of evidence from the present study and others indicate that lipin 1 is physically present at the promoters of regulated genes (12, 28). Our study provides the first evidence for an interaction of the mammalian lipin 1 protein with a non-nuclear receptor transcription factor. With regard to PPARα, lipin 1 coactivates via cooperation with the transcriptional coactivator PGC-1α. However, this does not occur with NFAT; instead, lipin 1 represses NFAT transcriptional activity. The NFATs have been reported to transcriptionally repress certain genes, notably CDK4 and VEGF (2, 4). We found no effect on CDK4 mRNA expression in the absence of lipin 1 (data not shown). However, VEGF mRNA was induced with the loss of lipin 1 expression (data not shown), suggesting that at least in 3T3-L1 cells lipin 1 may play a role in the suppression of VEGF. The ability of TSA to eliminate the repressive effects of lipin 1 on NFATc4 suggests that the lipin 1-NFAT interaction alone is not sufficient for repression but requires the lipin 1-mediated recruitment of an HDAC. It was recently demonstrated that lipin 1 is SUMOylated and that this controls lipin 1 nuclear translocation (31). There is a growing awareness of the importance of SUMOylation of transcriptional repressor complexes in the regulation of transcription (13). Whether SUMOylation of lipin 1 is more directly required for HDAC recruitment and transcriptional repression is under investigation. Although our results suggest that lipin 1 PAP activity is not required for either repression or activation of transcription, it is possible that the overexpressed, catalytically inactive lipin 1 proteins are either directly or indirectly recruiting endogenous, enzymatically active lipin 1 (or lipin 2 or 3), and it is the latter's enzymatic function that contributes to NFAT transcriptional suppression.

In differentiated 3T3-L1 adipocytes the loss of lipin 1 expression leads to a dramatic activation of NFAT transcriptional targets that could be reversed with small molecule inhibitors of NFAT. Calcium mobilization in lipin 1-deficient cells leads to a potent activation of NFAT target genes. These findings suggest that lipin 1 may function as a gatekeeper for NFAT activation in adipocytes. In concert with its role as a lipid phosphatase, lipin may thus provide a novel link between lipid metabolism, particularly synthesis of triacylglycerol, and inflammation. In the obesity of adults, fat mass increases primarily by adipocyte hypertrophy. Fat cell size strongly correlates with adipose tissue inflammation and degree of macrophage infiltration, particularly in abdominal adipose tissue (5).

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It is thought that adipocyte-secreted inflammatory factors, such as TNF-α and MCP1, function to recruit macrophages to the fat tissue and/or convert resident macrophages to proinflammatory, activated macrophages (51). Because NFAT contributes to proinflammatory cytokine expression in adipocytes, the ability of lipin 1 to repress NFAT transcription may be important for regulating macrophage recruitment to the fat tissue. It should also be noted that the lipin 1 expression is increased by the thiazolidinedione (TZD) class of anti-diabetic drugs (11, 58). This is intriguing since the TZDs improve insulin sensitivity and reduce inflammatory gene expression.

Consistent with lipin's role in repressing NFAT target genes that play a role in inflammation associated with obesity, we found lipin expression declining with increasing obesity in *ob*/*ob* mice. Given that triacylglycerol stores expand dramatically in obese adipose tissue and lipin 1 plays a key role in triacylglycerol biosynthesis, this might seem counterintuitive. However, there is abundant evidence that this occurs. Many years before the cloning of lipin 1, it was reported that PAP activity declines with age in rodents (25). Because lipin 1 is responsible for the majority of PAP activity in white adipose tissue, it is likely that this earlier finding could be explained by a decline in lipin 1 levels. In addition, two independent reports have shown that adipose tissue lipin 1 mRNA levels are decreased in obese human subjects (7, 52). We found an age-dependent decline in lipin 1 protein and total PAP activity in visceral, but not subcutaneous, fat depots in *ob*/*ob* mice as they progressively became more obese. Compared to subcutaneous fat, visceral adipose tissue mass in obese subjects is more closely linked to adipose inflammation. It is tempting to speculate that an obesity-driven decline in lipin 1 expression in visceral adipose tissue contributes to depot-specific inflammation. Transgenic mice overexpressing lipin 1 in adipose tissue are insulin sensitive, even when obese on a high fat diet, suggesting decreased fat tissue inflammation (43). Moreover, genetic variations in human *LPIN1* are associated with insulin resistance and the metabolic syndrome (reviewed in reference 45). In addition, several studies have found an inverse correlation between lipin 1 expression in white adipose tissue and inflammatory cytokine levels (30, 35). The data presented herein showing the transcriptional repression of NFATc4 by lipin may provide a molecular basis for this emerging link between lipin 1 and inflammation. We believe the positioning of lipin 1 at an intersection between triacylglycerol synthesis and expression of inflammatory genes is likely to have functional consequences for the regulation of adipose tissue function.

TNF-α is strongly induced by the knockdown of lipin 1. The NFATs have long been known to be involved in the transcription of TNF-α in immune cells, and there are reports of NFATc4 specifically inducing TNF-α expression in lung cells (10, 27, 34). Previous reports have indicated that macrophages are responsible for the synthesis of the majority of TNF-α secreted from adipose tissue (51). However, the inability of macrophages lacking TNF-α to completely protect against high fat diet-induced insulin resistance suggests that adipocyte-secreted TNF-α may play an important role in the initial recruitment of activated macrophages (8).

In conclusion, we have identified a non-nuclear receptor transcription factor, NFATc4, that is regulated by interaction with lipin 1. We have shown that lipin 1 represses the activity of NFATc4 bound to DNA thus expanding the role of lipin 1 in regulating transcription factor activity. We also demonstrated a decline in lipin 1 expression in a mouse model of increased obesity and propose that this may couple obesity with the activation of inflammatory pathways. Determining ways of preventing lipin 1 from declining during obesity, or blocking the loss of lipin 1-mediated repression of NFAT, may lead to treatments that can prevent obesity-induced fat tissue inflammation and subsequent insulin resistance.

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