Coordinated transcriptional control of adipocyte triglyceride lipase (*Atgl*) by transcription factors Sp1 and peroxisome proliferator–activated receptor γ (PPARγ) during adipocyte differentiation

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The breakdown of stored fat deposits into its components is a highly regulated process that maintains plasma levels of free fatty acids to supply energy to cells. Insulin-mediated transcription of *Atgl*, the enzyme that mediates the rate-limiting step in lipolysis, is a key point of this regulation. Under conditions such as obesity or insulin resistance, *Atgl* transcription is often misregulated, which can contribute to overall disease progression. The mechanisms by which *Atgl* is induced during adipogenesis are not fully understood. We utilized computational approaches to identify putative transcriptional regulatory elements in *Atgl* and then tested the effect of these elements and the transcription factors that bind to them in cultured preadipocytes and mature adipocytes. Here we report that *Atgl* is down-regulated by the basal transcription factor Sp1 in preadipocytes and that the magnitude of down-regulation depends on interactions between Sp1 and peroxisome proliferator–activated receptor γ (PPARγ). In mature adipocytes, when PPARγ is abundant, PPARγ abrogated transcriptional repression by Sp1 at the *Atgl* promoter and up-regulated *Atgl* mRNA expression. Targeting the PPARγ–Sp1 interaction could be a potential therapeutic strategy to restore insulin sensitivity by modulating *Atgl* levels in adipocytes.

The breakdown of TGs[4] into glycerol and FAs, called lipolysis, is a catabolic process that serves to maintain FFA homeostasis (1, 2). Under normal physiological conditions, plasma levels of NEFAs are controlled via clearance of circulating FAs and storage as TGs in adipose tissue. After meals, the pool of plasma NEFAs is increased by as much as 50% by lipoprotein lipase–mediated cleavage of newly circulating TGs, whereas basal-level mobilization of NEFAs by lipolysis in adipose tissue is impaired by the release of insulin (1, 3).

Obese individuals display several altered characteristics in this process, including decreased ability of adipose tissue to mobilize stored lipids, loss of insulin-mediated inhibition of NEFA release, and a general increase in plasma NEFA levels. There remains substantial debate regarding the meaning of the correlation between increased hypertrophy of adipocytes and high plasma NEFA levels in obesity (1, 3); however, as circulating lipids exceed the energy requirements of the body, higher plasma NEFA levels do contribute to the accumulation of fat in the liver and skeletal muscle, further aggravating insulin resistance and perpetuating this cycle (4, 5). As FFAs are critical factors in promoting insulin resistance, it is crucial to understand the mechanisms that control the basal breakdown of fat.

Insulin is classically described as an inhibitor of lipolysis (6–9). The initial and rate-limiting step in TG breakdown is mediated by the enzyme ATGL, which yields the metabolites FA and diacylglycerol. The predominant mechanism by which insulin regulates *Atgl* is via transcriptional control (6, 10). Insulin activates the mTORC1 pathway and simultaneously stimulates transcription of the transcription factor Egr1 in a mTORC1-dependent manner. Egr1 suppresses mTORC1-mediated *Atgl* transcription and decreases its expression both in cultured adipocytes and in adipose tissue derived from animals fed a high-fat diet (11). Insulin also controls nucleo-cytoplasmic shuttling of the transcription factor FoxO1, primarily via Akt-mediated phosphorylation and nuclear exclusion of FoxO1, reducing FoxO1-mediated transcription of *Atgl* (12). In addition, the master regulator of adipogenesis PPARγ has consensus binding sites within 3 kb upstream of the *Atgl* transcrip-

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This article contains supplemental Fig. S1 and Table S1.

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[4] The abbreviations used are: TG, triglyceride; FA, fatty acid; NEFA, non-esterified fatty acid; Atgl, adipocyte triglyceride lipase; PPARγ, peroxisome proliferator–activated receptor γ; RXRα, retinoid X receptor α; MEF, mouse embryonic fibroblast; RIPA, radioimmune precipitation assay; RT-qPCR, quantitative RT-PCR.

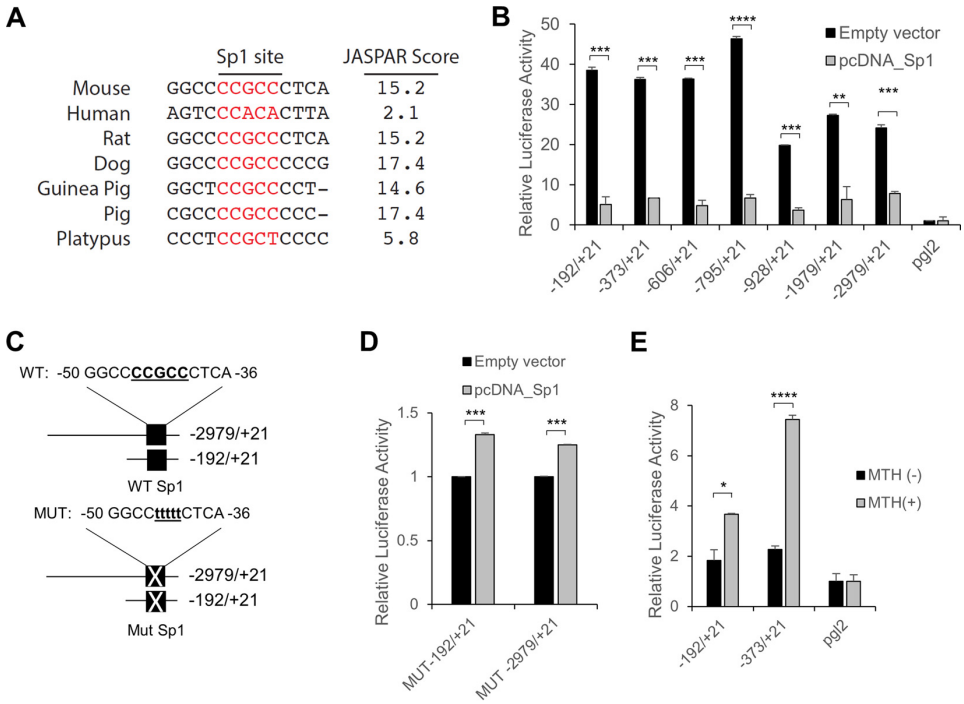

Figure 1. Atgl promoter-driven luciferase expression is inhibited by an Sp1-binding site. *A*, identification of an evolutionarily conserved putative Sp1-binding site in the *Atgl* promoter. All sites shown were identified by JASPAR as putative Sp1-binding sites ($p < 0.05$), with a higher score indicative of a stronger prediction of Sp1 binding. *B*, 3T3-L1 preadipocytes were co-transfected with luciferase reporter constructs of varying 5′-end deletions of the *Atgl* promoter and an Sp1 expression vector (pcDNA_Sp1). *C*, diagram of the putative Sp1-binding site at the *Atgl* minimal promoter (−50 to −36 bp) and the mutation (*MUT*) generated in the *Atgl* promoter constructs to disrupt this binding site. *D*, 3T3-L1 preadipocytes were co-transfected with luciferase reporter constructs of varying 5′-end deletions of the *Atgl* promoter bearing mutations in the predicted Sp1-binding site and an Sp1 expression vector. *E*, 3T3-L1 preadipocytes were co-transfected with variable-length *Atgl* promoter–luciferase reporter constructs and incubated with mithramycin A for 24 h. *pgl2*, empty vector luciferase control. not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

tion start site (13) that respond to insulin treatment to positively regulate *Atgl* transcription (14).

Insulin exhibits control over the expression of other various metabolically important genes via transcriptional regulation as well, including the insulin receptor (15–17). Transcriptional control of a significant cohort of its target genes is mediated by the Sp1 family of transcription factors (18). The Sp1 family binds to GC-rich motifs at promoters and subsequently drives or inhibits transcription (19). Sp1 has been demonstrated to dynamically modify promoter recognition of various genes in response to insulin (17, 18) and plays diverse roles in the control of a wide array of cellular processes, including cell growth (20, 21), apoptosis (21), angiogenesis (22), immune response (23), and differentiation (24). Sp1 has multiple functional domains to regulate gene expression by other mechanisms as well, including via protein–protein interaction with transcription factors such as PPARγ (25), Egr1 (26), Ets1 (27), c-myc (28), c-Jun (29), and Stat1 (30).

Transcription of *Atgl* is regulated by insulin, and insulin-mediated gene expression can be mediated via binding of the transcription factor Sp1 to specific promoters, which is itself transcriptionally regulated by insulin (31). In addition, PPARγ mediates transcription of target genes via a functional interaction with Sp1 (25). This inspired us to investigate the involvement of insulin-responsive Sp1 in the regulation of *Atgl* and to explore the potential of the PPARγ–Sp1 interaction to regulate *Atgl* gene expression during adipocyte differentiation.

Results

Sp1 binding to the Atgl promoter negatively regulates Atgl transcription

Analysis of approximately 3 kb of the DNA sequence immediately upstream of the *Atgl* transcription start site by Genomatix and TRANSFAC matrices revealed a putative Sp1-binding site at the minimal promoter (−50 to −36 bp). This site is conserved across numerous species, including mice and humans, suggesting an important biological function (Fig. 1*A*). Therefore, to test the involvement and mechanism of Sp1 in regulating the expression of *Atgl*, 3T3-L1 preadipocytes were co-transfected with luciferase reporter constructs of varying 5′ end deletions of the *Atgl* promoter and an Sp1 expression vector. Sp1 overexpression significantly decreased luciferase activity across all *Atgl* promoter constructs, indicating that Sp1 binding at the minimal promoter region (−192 to +21) can negatively affect *Atgl* expression (Fig. 1*B*). We examined the predicted Sp1-binding site by selectively mutating five conserved nucleotides (−46 5′-CCGCC-3′ −42), as shown in Fig. 1*C*. These mutations completely prevented the inhibitory effect of Sp1 on the minimal promoter of *Atgl* (Fig. 1*D*), confirming the involvement of this site in the transcriptional inhibition of *Atgl*. When 3T3-L1 preadipocytes were transfected with variable-length *Atgl* promoter-luciferase reporter constructs and incubated with mithramycin A, an Sp1 inhibitor, for 24 h, it also abrogated the inhibitory effect of endogenous Sp1 when transfected with the −192/+21 and −373/+21 constructs contain-

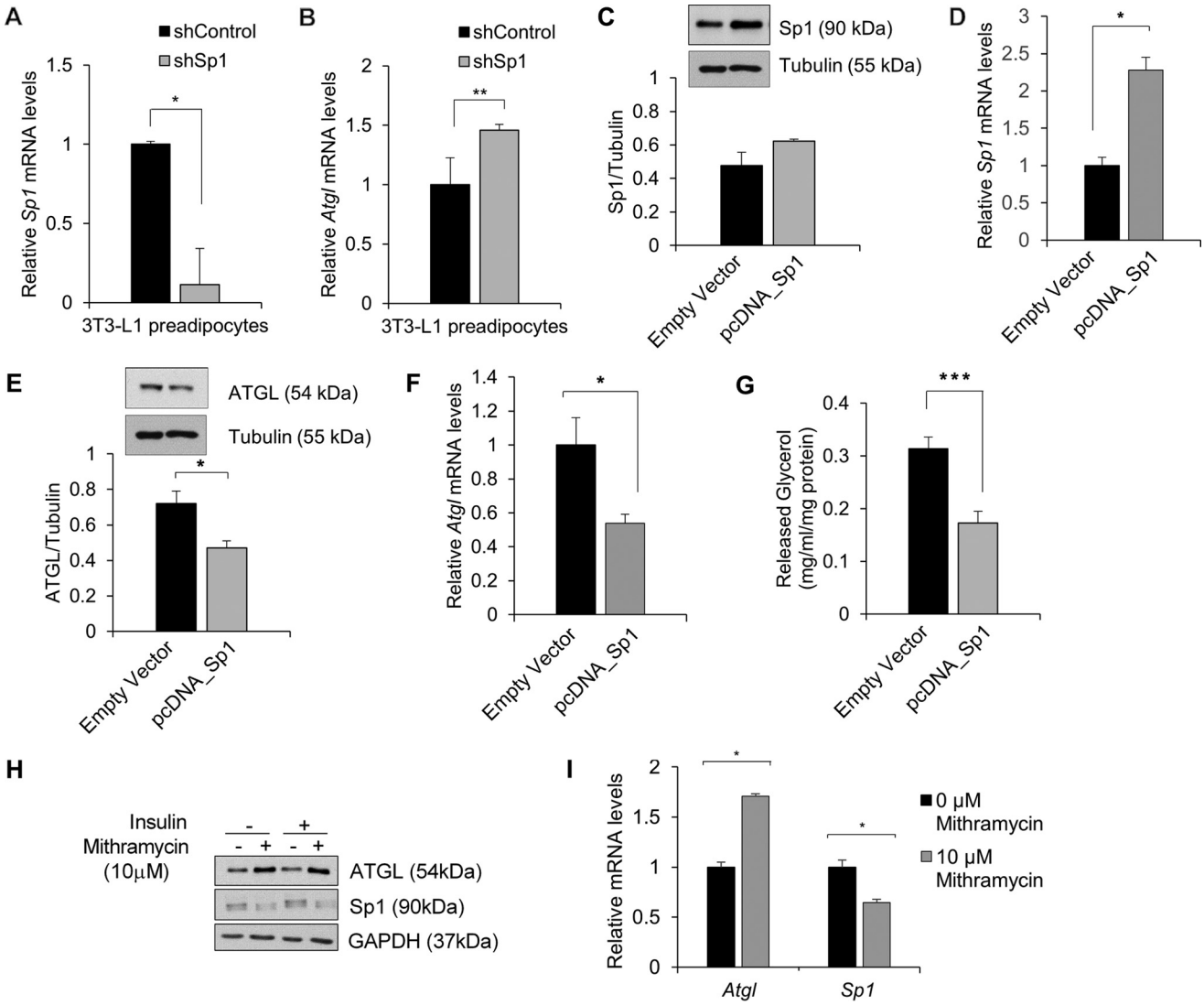

Figure 2. Sp1 mediates the inhibition of *Atgl* expression. *A* and *B*, RT-qPCR analysis of mRNA isolated from 3T3-L1 preadipocytes infected with viral particles expressing shRNA targeting Sp1 or a control shRNA. *C–F*, Western blot and RT-qPCR analyses of differentiated adipocytes transfected with Sp1 overexpression plasmids via electroporation. *G*, glycerol release in cultured adipocytes transfected with an Sp1 overexpression plasmid via electroporation. *H*, Western blot analysis of differentiated adipocytes treated with mithramycin A (10 μM) in the presence or absence of insulin. *I*, RT-qPCR analysis of differentiated adipocytes treated with mithramycin A (10 μM) in the presence of insulin. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ing the Sp1-binding site (Fig. 1*E*). This further demonstrates that Sp1 exerts inhibitory control over *Atgl* gene expression via the Sp1-binding site at −36 to −50.

The significance of Sp1 on endogenous *Atgl* gene expression was further investigated by Sp1 depletion and overexpression studies in 3T3-L1 cells. Knockdown of Sp1 in preadipocytes via a lentiviral vector producing shRNA targeting Sp1 increased the mRNA levels of *Atgl* relative to a control shRNA (Fig. 2, *A* and *B*). Conversely, overexpression of Sp1 in cultured adipocytes by electroporation (Fig. 2, *C* and *D*) resulted in lower expression levels of ATGL mRNA and protein (Fig. 2, *E* and *F*) with a concomitant reduction in basal glycerol release in the medium (Fig. 2*G*). In addition, cultured preadipocytes treated with mithramycin A (10 μM) showed significant increase in ATGL levels both in the presence and absence of insulin (Fig. 2*H*). Also, in the presence of insulin, mithramycin A decreased Sp1 mRNA levels and increased *Atgl* mRNA levels (Fig. 2*G*). Collectively, this demonstrates that Sp1 mediates the negative regulation of *Atgl* in preadipocytes. The data further support that Sp1 reduces glycerol release in adipocytes via mechanisms that involve *Atgl*.

Sp1-mediated negative regulation of Atgl is abrogated by PPARγ in adipogenesis

Levels of PPARγ protein increase dramatically during adipogenesis, correlating with an increase in *Atgl* mRNA and protein levels (supplemental Fig. 1). Bioinformatic analyses identified putative PPARγ-binding sites in the *Atgl* promoter at −2424, −1674, and −1573 bp (14). In the absence of ChIP data in the literature (11–13), we speculated that the profound up-regulation of *Atgl* transcription is due to the binding of the PPARγ/RXRα heterodimer (32–34) at the abovementioned sites.

Of note, PPARγ and Sp1 proteins both functionally and physically interact with each other (25). Thus, we hypothesized that PPARγ may abrogate the Sp1-mediated negative control of *Atgl* transcription. To test this, PPARγ and Sp1 overexpression

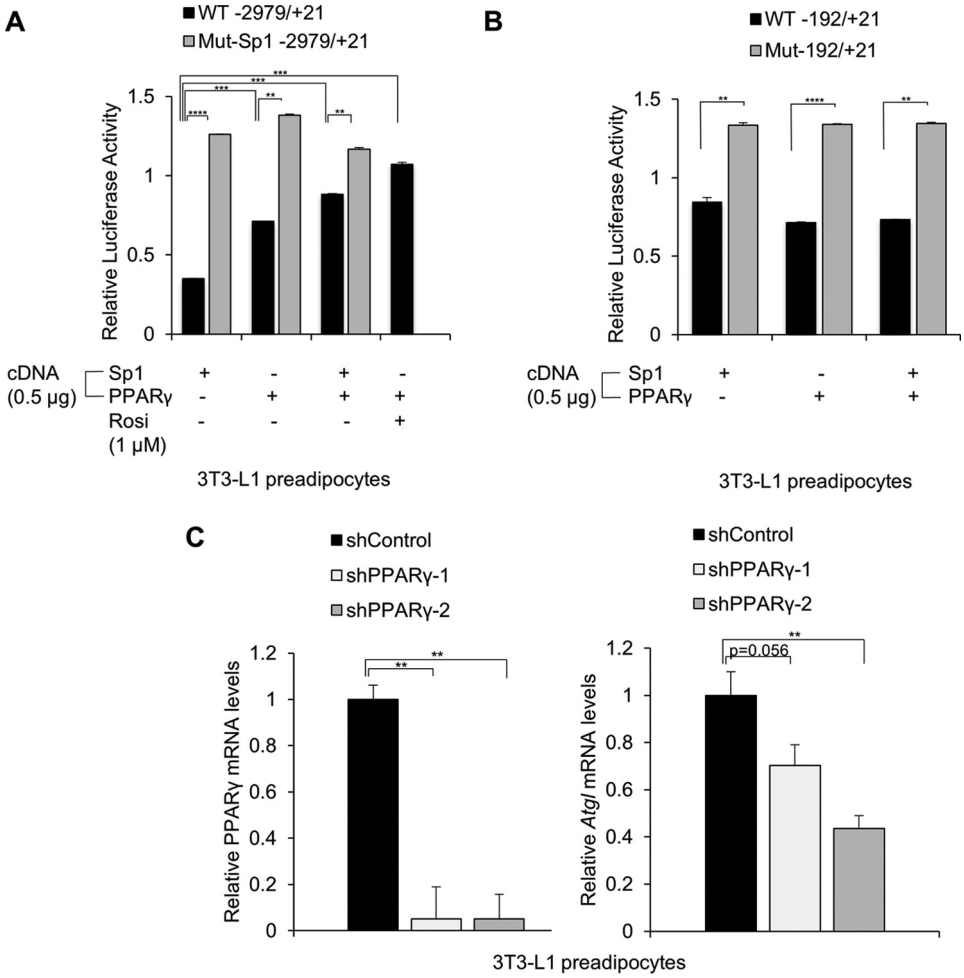

Figure 3. PPARγ reverses Sp1 inhibition of *Atgl* transcription. *A*, luciferase reporter assay of 3T3-L1 preadipocytes co-transfected with PPARγ and Sp1 overexpression plasmids and wild-type or Sp1 site–mutated (*Mut*) full-length *Atgl* promoter luciferase constructs. *B*, luciferase reporter assay of 3T3-L1 preadipocytes co-transfected with PPARγ and Sp1 overexpression plasmids and wild-type or Sp1 site–mutated minimal *Atgl* promoter luciferase constructs. *C*, RT-qPCR analysis of 3T3-L1 preadipocytes infected with viral particles expressing shRNA targeting PPARγ. *Rosi*, rosiglitazone. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

plasmids were co-transfected with wild-type or Sp1 site–mutated −2979/+21 bp *Atgl* promoter constructs in 3T3-L1 preadipocytes. PPARγ overexpression alone or in the presence of Sp1 increased the reporter activity compared with Sp1 overexpression alone in cells transfected with the wild-type −2979/+21 *Atgl* luciferase promoter construct (Fig. 3*A*). Upon mutation of the Sp1-binding site in the full-length *Atgl* promoter, PPARγ overexpression no longer induced Atgl expression (Fig. 3*A*). These data suggest that the Sp1 site (−50 bp to −36 bp) contributes to the regulation of *Atgl* by PPARγ in the context of the −2979 to +21 promoter construct. The Sp1-mediated transcriptional repression of *Atgl* in preadipocytes allows transcriptional activation by PPARγ during adipocyte differentiation. Furthermore, the presence of the Sp1 site has a positive effect on PPARγ-mediated transcriptional activation of the *Atgl* promoter. To further support this hypothesis, PPARγ overexpression alone or with Sp1 did not induce reporter activity of the shorter *Atgl* promoter fragment (−192/+21) in either the presence or absence of the Sp1 mutation (Fig. 3*B*). Taken together, we speculate that induction of PPARγ expression during adipogenesis reverses the negative control of Sp1 over *Atgl* transcription, presumably via direct interaction with Sp1.

The fact that PPARγ increases luciferase activity in the presence of Sp1 in the full-length promoter but not in the minimal promoter fragment shows that PPARγ stimulates *Atgl* transcription via binding sites upstream of −192 and by interacting directly with the Sp1-binding site at −50 bp to −36 bp. We speculate that the Sp1-mediated transcriptional down-regulation that is reversed by PPARγ is a stoichiometric transcriptional phenomenon in which there is a reversal of the role of the transcription complex, depending upon the available transcription factor levels. To further test this hypothesis, we knocked down PPARγ in preadipocytes via shRNA (Fig. 3*C*, *left panel*). PPARγ deficiency decreased *Atgl* mRNA levels (Fig. 3*C*, *right panel*), further supporting the conclusion that PPARγ is a positive transcriptional regulator of *Atgl* in preadipocytes via mechanisms that involve its interaction with Sp1 and inhibition of the Sp1-mediated negative regulation of *Atgl* transcription.

PPARγ-mediated transactivation of Atgl is Sp1-dependent

As the data suggested that Sp1 is a negative regulator of *Atgl* transcription in preadipocytes and that PPARγ overrides this process, we further investigated the importance of the Sp1-binding site on PPARγ-mediated transactivation of *Atgl* tran-

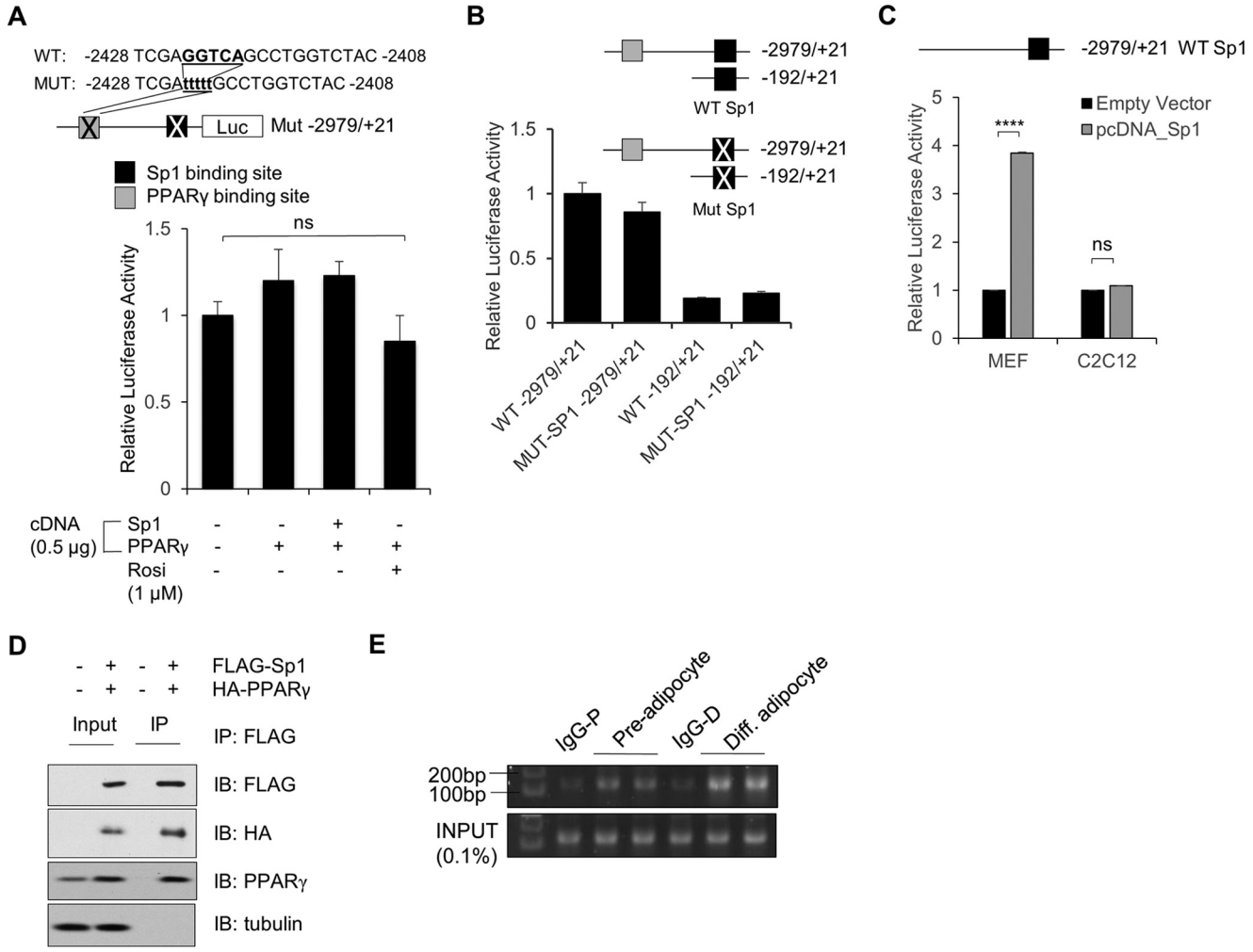

Figure 4. PPARγ-mediated transactivation of *Atgl* is Sp1-dependent. *A*, luciferase reporter assay of 3T3-L1 preadipocytes co-transfected with Sp1 and PPARγ overexpression plasmids and full-length *Atgl* luciferase reporter construct bearing mutations (*MUT*) at the Sp1- and PPARγ-binding site or treated with rosiglitazone (*Rosi*, 1 μM). *B*, luciferase reporter assay of differentiated adipocytes transfected with wild-type or Sp1-binding site mutants of full-length or minimal *Atgl* promoter luciferase constructs via electroporation. *C*, luciferase reporter assay of MEF and C2C12 cells co-transfected with Sp1 overexpression plasmids and a full-length *Atgl* promoter luciferase construct. *D*, Western blot analysis of co-immunoprecipitation in 3T3-L1 preadipocytes. *E*, PCR analysis of ChIP performed with Sp1 antibodies identify binding of Sp1 to the minimal *Atgl* promoter. *IP*, immunoprecipitation; *IB*, immunoblot; *Diff.*, differentiated. *ns*, $p > 0.05$; ****, $p < 0.0001$.

scription. During adipogenesis, there is a decrease in the nuclear levels of Sp1 and an increase in PPARγ levels in 3T3-L1 cells (supplemental Fig. 1). We co-transfected 3T3-L1 preadipocytes with full-length *Atgl* luciferase reporter constructs containing mutations at both the Sp1 (−50 bp to −36 bp) and PPARγ-binding sites (−2428 bp to −2408 bp). We did not observe an increase in reporter activity by overexpression of PPARγ or PPARγ and Sp1 combined (Fig. 4*A*). PPARγ alone did not increase luciferase activity either in the presence or absence of the PPARγ agonist rosiglitazone. This indicates that the effect of the PPARγ-binding site is to positively drive transcription of *Atgl*.

In mature adipocytes, expression from the full-length *Atgl* promoter (−2979/+21) containing the PPARγ- and Sp1-binding sites was significantly greater than that from the minimal promoter (−192/+21) containing only the Sp1-binding site (Fig. 4*B*), suggesting a positive regulatory role of PPARγ. Interestingly, mutation of the Sp1-binding site in the full-length promoter that contains the PPARγ-binding site decreased *Atgl* reporter expression (Fig. 4*B*), suggesting a positive role of Sp1 in *Atgl* transcription in mature adipocytes, where PPARγ is abundant. This effect is lost in the minimal promoter lacking the PPARγ-binding site (Fig. 4*B*) and is in contrast to the data from preadipocytes, in which mutation of the Sp1 site in the full-length promoter increased expression of the *Atgl* reporter construct (Fig. 3*A*). Together, this suggests that PPARγ-mediated transactivation of *Atgl* is partially driven by the Sp1-binding site and that PPARγ binding to the promoter is essential to positively drive transcription of *Atgl* in mature adipocytes. Interestingly, this regulation is cell-type specific, as a luciferase reporter assay performed using Sp1 overexpression vectors and the full-length *Atgl* promoter construct in MEF and C2C12 cells did not result in the reduction in luciferase activity that was detected in preadipocytes (Figs. 1*B* and 4*C*).

To examine the physical interaction between Sp1 and PPARγ proteins, co-immunoprecipitations were performed from 3T3-L1 protein lysates; they demonstrated that Sp1 interacts with PPARγ in this cell type (Fig. 4*E*). In addition, ChIP

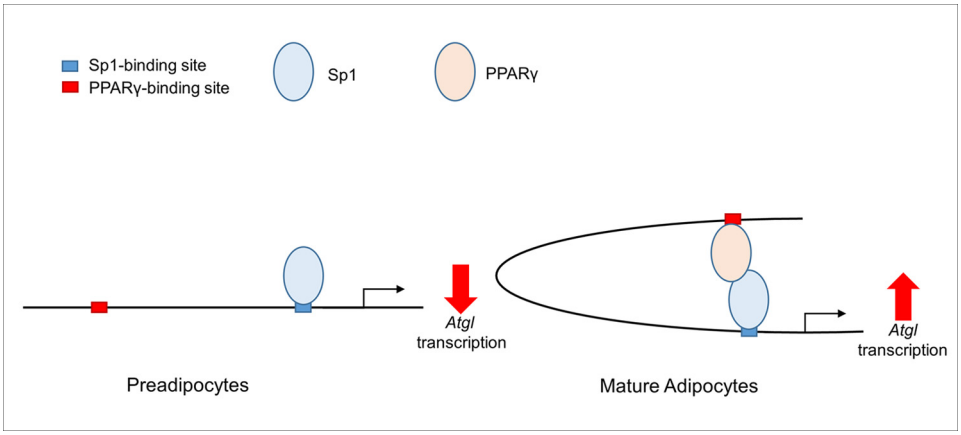

Figure 5. Interaction between PPARγ and Sp1 leads to stage-specific increase in transcription of *Atgl*. We propose a model in which the regulation of *Atgl* transcription is dependent on the relative abundance of and interaction between the transcription factors Sp1 and PPARγ. Although Sp1 negatively regulates *Atgl* expression in preadipocytes, Sp1 coordinates with PPARγ in mature adipocytes to positively regulate *Atgl* transcription.

analysis of Sp1 binding to the minimal *Atgl* promoter identified Sp1 genomic occupancy at the *Atgl1* promoter in both preadipoctyes and mature adipocytes (Fig. 4*F*). Taken together, these data support the hypothesis that Sp1 and PPARγ coordinately directly regulate *Atgl* transcription in a cell type- and stage-dependent manner that is governed by the changes in PPARγ abundance that occur during adipocyte differentiation (Fig. 5).

Discussion

It has been well-documented that active transcriptional complexes can adopt alternative functions when bound to additional co-activators or co-repressors (35–37). For example, Klf1 is a transcription factor that exhibits alternate functions in hematopoietic cells, depending on the stage of development. In erythroid cells, a primitive cell type, Klf1 functions as an activator of β-globin expression. However, in hematopoietic cells, a definitive cell type, Klf1 switches to function as a repressor of β-globin expression (38). As Sp1 is a prominent member of the KLF family of transcription factors, similar mechanisms could explain the transcriptional switch identified in this study.

Our results showed that the shortest fragment of the *Atgl* promoter (−192/+21) contains an evolutionarily conserved Sp1-binding site that is capable of inhibiting *Atgl* transcription when Sp1 is overexpressed exogenously. This negative impact of Sp1 on *Atgl* transcription is modulated by PPARγ. In addition, cell type–specific responses were observed. In MEFs, which produce negligible amounts of PPARγ, a positive regulation of the *Atgl* promoter was observed when Sp1 was expressed exogenously, further supporting the critical nature of the cellular transcription factor milieu in *Atgl* gene regulation. In adipocytes, we found that Sp1 allows PPARγ-mediated transactivation via a protein–protein interaction between Sp1 and PPARγ (Fig. 4*E*). PPARγ-mediated transactivation of the *Atgl* promoter decreased when the conserved Sp1 overlapping binding site at the proximal promoter was mutated (Fig. 4*B*). We propose that, although Sp1 maintains a steady promoter binding at the minimal promoter, as identified by ChIP analysis (Fig. 4*F*), and PPARγ and Sp1 directly interact (Fig. 4*E*), there is a complete reversal of the repressive transcription complex to an activating transcription complex during adipogenesis that governs the fate of *Atgl* transcription (Fig. 5).

PPARγ heterodimerizes with RXRα, binds to peroxisomal proliferator response elements, and exhibits ligand-induced transactivation of gene expression (39). Our results show transcriptional suppression of the *Atgl* promoter reporter by the Sp1-binding site at −192/+21 in the presence of exogenous PPARγ (Fig. 3*A*). As consistent binding of Sp1 at this smaller fragment is observed in both preadipocytes and mature adipocytes (Fig. 4*E*), we propose that physical interaction of Sp1–PPARγ contributes to Sp1-mediated repression and PPARγ-mediated transactivation of the *Atgl* gene in adipocytes. A similar mechanistic role of PPARγ–Sp1 interaction has been shown to regulate thromboxane gene expression (25). The increased abundance of PPARγ in mature adipocytes seems likely to explain this reversal of Sp1 function, warranting further investigation into potential Sp1-binding partners.

Sp1–nuclear hormone receptor interaction is found to enhance Sp1-induced gene transcription (40–42). This study demonstrates the possibility that other interacting partners of Sp1 play a role in mediating this process, in this case PPARγ. However, there are a number of other proteins that interact with Sp1, including Egr1. It has been determined that both Sp1 and Egr1 occupy overlapping binding sites and can compete with each other for DNA binding, with Egr1 displacing Sp1 when the local concentration is more than that of Sp1 (43). Therefore, additional cofactors, such as Egr1, may be involved in the repression of Sp1 target gene expression. Another potential interacting partner is C/ebp β, which, like PPARγ, increases during adipogenesis and has been shown to functionally interact with Sp1 (44, 45). In fact, the presence of additional transcription factors such as C/ebp β in mature adipocytes may contribute to the finding that, in preadipocytes, mutation of the Sp1-binding site in the *Atgl* promoter increased transcription, even in the presence of PPARγ (Fig. 3*A*), whereas mutation of this site in mature adipocytes led to an overall decrease in transcription (Fig. 4*B*).

In conclusion, Sp1 negatively regulates *Atgl* transcription in a PPARγ-dependent fashion. It is interesting to note how, being part of the same transcription complex, the increased abundance of PPARγ during adipogenesis leads to a reversal in the transcriptional action of Sp1, which acts as a repressor in prea-

dipocytes and as an activator in fully differentiated adipocytes (Fig. 5). Understanding the alternating functions of these transcription factors in regulating *Atgl* in adipocytes is relevant to the treatment of insulin resistance and type 2 diabetes. Obesity is a predominant risk factor for insulin resistance, and insulin action is highly impaired in adipocytes of individuals with metabolic syndrome (46–48). In these individuals, there is a reduction in hormone-induced lipolysis and an increase in the basal lipolysis of adipocytes (6, 49), most likely because of the changes in the levels of *Atgl*. Therefore, targeting the specific PPARγ–Sp1 interaction could possibly modulate *Atgl* levels in metabolic syndrome and restore whole-body insulin sensitivity.

Experimental procedures

Cells and reagents

HEK 293T, MEF (C57BL/6), C2C12, and 3T3-L1 cells were grown in DMEM with high glucose (4.5 mM) supplemented with 10% FBS (Gibco), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. 3T3-L1 preadipocytes were induced to differentiation 2 days after reaching 100% confluence via treatment with a differentiation mixture (5 μg/ml insulin (Sigma, 91077C), 1 μM dexamethasone (Sigma, D-2915), and 0.5 mM isobutylmethylxanthine (Sigma, 15879)) for 3 days. Cells were maintained in medium containing 5 μg/ml insulin until harvested 8 days after treatment with the differentiation mixture. Antibodies were used against the following proteins: ATGL (Cell Signaling Technology, 2138S), Sp1 (Santa Cruz Biotechnology, sc-59), and tubulin (Sigma, T9026). The following drugs were used: rosiglitazone (Sigma-Aldrich, R2408) and mithramycin A (Cayman Chemicals, 11434).

RNA isolation and RT-qPCR

Total RNA was isolated from 3T3-L1 cells using TRIzol reagent (Invitrogen). RNA was then reverse-transcribed using SuperScript III First-Strand Synthesis SuperMix (Invitrogen), and levels of cDNA were quantified by RT-qPCR using VeriQuest SYBR Green qPCR Master Mix (Affymetrix) with the StepOnePlus RealTime PCR System (Applied Biosystem). Primers used in the study are listed in supplemental Table S1. Relative mRNA levels were normalized to *Gapdh* using the ΔΔCt method.

Immunoblotting and nuclear localization

For total protein lysates, cells were washed with cold PBS twice and lysed with RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and 0.1% SDS) supplemented with Complete EDTA-free protease inhibitor and PhosSTOP phosphatase inhibitor tablets (Roche, 1 tablet/10 ml of RIPA buffer). Lysates were sonicated and centrifuged at 13,000 rpm for 10 min at 4 °C. Supernatants were collected, and the amount of protein was quantified using the DC Protein Assay Kit (Bio-Rad).

For nuclear fractionation experiments, nuclei were isolated as described previously (50). Briefly, cells were pelleted, washed in PBS, and lysed with a mild detergent buffer (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, and 0.3% Nonidet P-40). Nuclei were collected by centrifugation, washed in this buffer without detergent, and resuspended in a high-salt buffer (20 mM HEPES, (pH 7.9), 0.45 M NaCl, 1 mM EDTA, and 0.5 mM DTT). Nuclei were lysed via sonication, and protein was quantified as described above.

Equal quantities of protein were loaded and separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore) following standard procedures. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST) for 1 h, rinsed in TBST, and incubated with a primary antibody overnight at 4 °C. Membranes were then probed for respective HRP-conjugated secondary antibody for 1 h at room temperature. A Western Lightning ECL substrate kit (PerkinElmer Life Sciences) was used for detection, and bands were quantified using densitometry by Adobe Photoshop CS 5.0.

JASPAR analysis of Sp1-binding site

The Sp1 consensus motif in the promoter region of *Atgl* in mice was aligned to other species using the LastZ track in the Ensembl database (51). Conserved sequences were identified in a number of species. Analysis of these sequences using the JASPAR database (52) identified putative Sp1 motifs in all species shown in Fig. 1*A*.

Transient transfection, electroporation, and luciferase reporter assay

3T3-L1 preadipocytes were transiently transfected with plasmids using X-tremeGENE HP transfection reagent (Roche) according to the instructions of the manufacturer. Briefly, cells were grown to 70–80% confluence and transfected with 500 ng of *Atgl* luciferase constructs (14), 500 ng of a plasmid encoding Sp1, 500 ng of a plasmid encoding PPARγ, and 50 ng of a plasmid encoding *Renilla* luciferase in a 6-well plate format. After 48 h of transfection, cells were lysed using passive lysis buffer (Promega). Luciferase activities were determined by Dual Glo Luciferase Reporter Assay Kit (Promega), with values expressed in relative light units and normalized to *Renilla* luciferase levels.

For electroporation, 3T3-L1 differentiated adipocytes were washed with calcium- and magnesium-free warm PBS and trypsinized. Post-trypsinization, cells were washed twice with PBS (containing Ca^{2+} and Mg^{2+}) and centrifuged at 2000 rpm for 5 min. Cells were resuspended in 500 μl of PBS and electroporated with 50 μg DNA using the Gene Pulser Xcell electroporation system (Bio-Rad) with a pulse setting of 160 V and 950 microfarad. Following electroporation, cells were resuspended in growth medium in 6-well plates. 24 h after electroporation, cells were collected for the luciferase activity assay or immunoblot analysis.

Site-directed mutagenesis

The putative Sp1-binding site at the *Atgl* minimal promoter region was identified using TRANSFAC matrices. Five conserved residues in this putative Sp1-binding site were mutated to thymine residues using the following set of primers: forward, 5′-CGACC AGGCC ttttt CTCAC CCCGC ACTAA AACAC-3′; reverse, 5′-GCAGG GGGCA GGACC TGG-3′. A putative PPARγ-binding site was also located −2408 to −2428 bp upstream of the transcriptional start site, and five conserved

residues were mutated to thymine residues using the following set of primers: forward, 5′-CTGAG TTCGA ttttt GCCTG GTCTA CAATG TGAGT TC-3′; reverse 5′-AAATC CACCT GCCTC TGC-3′. Mutagenesis was carried out using the Q5 site-directed mutagenesis kit (New England Biolabs) following the recommended protocol. Mutations were confirmed by Sanger sequencing.

Lipolysis assay

Differentiated 3T3-L1 adipocytes were incubated with phenol red–free DMEM with 2% fatty acid–free BSA for 6 h. Glycerol release was measured as a function of NADH consumption via absorbance at 340 nm using the Free Glycerol Kit (Megazyme).

Co-immunoprecipitation

3T3-L1 preadipocytes were transfected with human FLAG-Sp1 and human HA-PPARγ. Twenty-four hours post-transfection, cells were harvested in RIPA buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and 1% Triton X-100) with protease inhibitor mixture (Roche). Lysates were incubated with anti-FLAG M2 affinity beads (Sigma, A2220) overnight at 4 °C on an orbital shaker. Beads were washed with RIPA buffer, and proteins were eluted with 30-min incubation in 1 M glycine and analyzed by immunoblot.

ChIP

3T3-L1 preadipocytes and differentiated adipocytes were cross-linked by adding formaldehyde to the culture medium at a final concentration of 1% and incubated for 10 min at 37 °C in a CO_2 incubator. The medium was then aspirated and washed twice with cold PBS (pH 7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). Cells were collected, lysed with buffer containing 0.2% SDS, and then sonicated using a Bioruptor (Diagenode) with eight pulses of 15 s at high setting. Tubes were centrifuged for 10 min, and the supernatant was immunoprecipitated with 2 μg of anti-Sp1 antibody (Cell Signaling Technology) using Dynabeads protein A (Invitrogen). DNA was obtained by reverse cross-linking, purified, and analyzed via RT-qPCR with primers to detect the Sp1-binding site (forward, 5′-CGGCG GAGGC GGAGA CGCT-3′; reverse, 5′-TCCCT GCTTG ATCCA GTTGG AT-3′) using Premix TaqDNA Polymerase, Hot Start (catalog no. R028A, Takara) with 0.1% input.

shRNA-mediated knockdown

HEK-293T cells were grown to 70% confluence in 150-mm-diameter dishes and then transfected with 5 μg of psPAX2 vector (Addgene), 5 μg of PMD2G vector (Addgene), 10 μg of shSp1 (Sigma, TRCN0000017603), 10 μg of shPPARγ (Sigma, TRCN0000001660 (shPPARγ-1) and TRCN0000001657 (shPPARγ-2)) using X-tremeGENE HP reagent (Roche) in serum-free medium. 8 h post-transfection, the medium was supplemented with fresh DMEM containing 10% FBS. 24 h post-transfection, medium containing a high titer of lentiviral particles was harvested and filtered with a 0.45-μm PVDF syringe filter. 3T3-L1 cells were infected with lentiviral particles expressing Sp1-specific shRNA for 48 h.

Statistical analysis

All data are presented as mean ± S.E. To evaluate statistical significance, two tailed Student's *t* test or one-way analysis of variance (followed by post tests, Dunnett's multiple comparison test and Welch correction test) were performed using the GraphPad Prism software package. $p < 0.05$ was considered to be statistically significant and is presented as follows: not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

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