SIRT1 Regulates Adiponectin Gene Expression through Foxo1-C/Enhancer-binding Protein α Transcriptional Complex*[^5]

Received for publication, July 31, 2006, and in revised form, October 13, 2006 • Published, JBC Papers in Press, November 6, 2006, DOI 10.1074/jbc.M607215200

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Adiponectin is an adipose-derived hormone that plays an important role in maintaining energy homeostasis. Adiponectin gene expression is diminished in both obesity and type 2 diabetes. However, the mechanism underlying the impaired adiponectin gene expression remains poorly understood. Recent studies have indicated that forkhead transcription factor O1 (Foxo1) and silent information regulator 2 mammalian ortholog SIRT1 are involved in adipogenesis. Here we have shown that Foxo1 up-regulates adiponectin gene transcription through a Foxo1-responsive region in the mouse adiponectin promoter that contains two adjacent Foxo1 binding sites. Foxo1 interacts with CCAAT/enhancer-binding protein α (C/EBPα) to form a transcription complex at the mouse adiponectin promoter and up-regulates adiponectin gene transcription. Our study has revealed that C/EBPα accesses the adiponectin promoter through two Foxo1 binding sites and acts as a co-activator. Further, SIRT1 increases adiponectin transcription in adipocytes by activating Foxo1 and enhancing Foxo1 and C/EBPα interaction. Importantly, both Foxo1 and SIRT1 protein levels were significantly lower in epididymal fat tissues from db/db and high fat diet-induced obese mice compared with normal mice. We propose that low expression of SIRT1 and Foxo1 leads to impaired Foxo1-C/EBPα complex formation, which contributes to the diminished adiponectin expression in obesity and type 2 diabetes.

However, information is limited regarding the underlying mechanisms that impair adiponectin gene expression in obesity and type 2 diabetes.

Foxo1[^2] is a member of the forkhead transcription factor class O family and is involved in adipocyte differentiation (8). Foxo1 gene haploinsufficiency leads to significant reduction of adiponectin gene expression in the adipose tissue (8). SIRT1 is an NAD^+^-dependent protein deacetylase that is also involved in adipogenesis (9). Calorie restriction induces Foxo1 and SIRT1 expression, which mediate the resultant longevity effect in cells from yeast to mammals (10, 11). The expression of Foxo1 and SIRT1 is also up-regulated during adipocyte differentiation (8, 9). SIRT1 regulates Foxo1 transactivation activity by deacetylating three lysine residues within the forkhead DNA binding domain (12). These studies led us to hypothesize that Foxo1 and SIRT1 may regulate adiponectin gene expression.

Here, we report that overexpression of Foxo1 increased adiponectin gene expression in differentiated 3T3-L1 adipocytes. Our study has identified two Foxo1 responding elements at the mouse adiponectin promoter and demonstrated that Foxo1 interacts with C/EBPα and forms a transcriptional complex at the adiponectin promoter. Furthermore, overexpression of SIRT1 or knocking down endogenous SIRT1 increased or decreased adiponectin gene expression in 3T3-L1 adipocytes, respectively. Expression of SIRT1 synergistically increased Foxo1- and C/EBPα-mediated adiponectin promoter activation. In addition, SIRT1 enhanced Foxo1-C/EBPα transcription complex formation. Therefore, we conclude that SIRT1 up-regulates adiponectin gene transcription via a Foxo1-C/EBPα transcription complex. The study also suggests that decreased expression of SIRT1 and Foxo1 in obesity may play a causal role in the diminished adiponectin gene expression.

EXPERIMENTAL PROCEDURES

**Experimental Animals**—Male C57BL/6J and C57BL/6J^+/-^{La}Lepr^db/db (db/db) mice were housed in a pathogen-free animal facility. Obesity of C57BL/6J mice was induced by 17 weeks of high fat diet feeding (60 kcal% as fat, Research Diets, New Brunswick, NJ). Control mice were fed a diet containing 10% fat.

[^5]: This work was supported by grants from the American Diabetes Association (1-04-JF-44 to J. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[^2]: The abbreviations used are: Foxo1, forkhead transcription factor O1; C/EBP, CCAAT/enhancer-binding protein; PPARγ, peroxisome proliferator-activated receptor γ; RXRα, retinoid X receptor α; SIR, silent information regulator; HEK, human embryonic kidney; WT, wild type; siRNA, small interfering RNA; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; IRS, insulin response sequence.
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Cell Culture—3T3-L1 fibroblasts and HEK293 cells were purchased from the American Type Culture Collection (Manassas, VA). 3T3-L1CARΔ1 cells stably express the coxsackie-adenovirus receptor, which improves adenoviral transduction efficiency (13). The cells were maintained at 37 °C, 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA). Adipocyte differentiation was induced by a commonly used protocol (13, 14). For adenovirus transduction studies, purified adenoviruses were used at 100 plaque-forming units/cell.

Plasmid Construction and Adenovirus Vector Preparation—The adenovirus promoter-luciferase gene reporter plasmid and plasmids encoding C/EBPα and PPARγ2 have been described previously (14). Adenovirus vectors and plasmids encoding Foxo1-WT, Foxo1-ADA (constitutive nuclear Foxo1 bearing PKB phosphorylation site mutation) or Foxo1-Δ256 were generously provided by Dr. Domenico Accili (8). The adenovirus vectors encoding siRNA against SIRT1 and control siRNA were kindly provided by Dr. Puigserver (Johns Hopkins University School of Medicine, Baltimore, MD) (15). The plasmids containing mutant Foxo1 cDNA, in which Lys-242, Lys-245, and Lys-262 were replaced by Arg (K3R), Ala (K3A), or Gln (K3Q), were kindly provided by Dr. Fukamizu (University of Tsukuba, Tsukuba, Japan) (16). The plasmids encoding SIRT1 or SIRT1H363Y were gifts from Dr. Gu (Columbia University) (17).

Total Cellular Protein and Nuclear Protein Extraction and Western Blot Analysis—Total cell and nuclear proteins were extracted as described previously (14). Relative protein levels of SIRT1, Foxo1, C/EBPα, and adiponectin were measured by Western blot using specific antibody (Santa Cruz Biotechnology and R & D Systems).

Quantitative Reverse Transcription-PCR Analysis—Total RNA was prepared from 3T3-L1 adipocytes using TRIzol reagent (Invitrogen). Real-time PCR was performed using the Mx3000P Real-time PCR system (Stratagene) using SYBR Green dye (Molecular Probes). The sequences for the primers are: adiponectin (5’-AAAGGAGAGCCTGGAGAGAC-3’ and 5’-TATATGTTTGTGCTGAT-C3’), Perilipin (5’-ACCC-TGCTGGATGGAGAC-3’ and 5’-AGGCAGCTGCAGAAC-TTCT-3’), and IRS2 (5’-AATGCGGCATCTTTATGTC-3’). The levels of PCR product were calculated from standard curves established in mature adipocytes. Eight days after differentiation, the 3T3-L1CARΔ1 adipocytes were transduced with adenovirus vectors encoding wild type (Foxo1-WT), constitutively nuclear (Foxo1-ADA), or dominant negative Foxo1 (Foxo1-Δ256) (21). The results clearly showed that adiponectin protein and RNA levels were significantly increased in adipocytes transduced by adenovirus vector encoding Foxo1-WT or Foxo1-ADA (p < 0.05) (Fig. 1, A and B). However, the magnitude of Foxo1-ADA-induced adiponectin expression was not significantly greater than that induced by Foxo1-WT overexpression. This finding suggests that Foxo1-WT and Foxo1-ADA are equally potent in up-regulating adiponectin gene expression in this cellular model.

Surprisingly, adiponectin protein and RNA levels were also significantly elevated in adipocytes transduced with Foxo1-
FIGURE 1. Foxo1 regulates adiponectin gene expression in mature adipocytes. Fully differentiated 3T3-L1 CAR adipocytes were transduced with adenovirus vectors which encode wild type (WT), constitutively nuclear (ADA), or dominant negative Foxo1 (Δ256). 24 h after transduction, cytoplasm adiponectin was measured by Western blot (A). The mRNA levels of adiponectin and perilipin were measured by real-time reverse transcription-PCR (B). The adiponectin promoter-directed luciferase gene reporter construct was co-transfected with Foxo1-WT-, ADA-, or Δ256-encoding plasmids in 3T3-L1 adipocytes (C) or HEK293 cells (D). Foxo1 protein levels are illustrated in supplemental Fig. S1. The adiponectin promoter-directed luciferase gene reporter construct was co-transfected with Foxo1-, C/EBPα-, PPARγ2-, and RXRα-encoding plasmids in HEK293 cells (E). Luciferase activities were normalized by β-galactosidase activity. Two-way analysis of variance was used for statistical analysis. *, p < 0.05 versus cells transduced with green fluorescent protein vector or pcDNA vector. Data are presented as mean ± S.D. (n = 4 for B; n = 6 for C–E).
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Δ256-encoding adenovirus vectors (Fig. 1, A and B). Foxo1-Δ256 is a truncated protein containing the DNA binding domain but lacking the transactivation domain (8)(supplemental Fig. S1) Theoretically, Foxo1-Δ256 should compete with endogenous Foxo1 for DNA binding and impair Foxo1-mediated gene transcription. Indeed, Foxo1-Δ256 inhibits the transcription of several Foxo1 target genes (8, 22, 23). Our results seem contradictory to the design of the constitutively negative mutation. The following studies demonstrate that the transactivation activity of Foxo1 explains only part of the role of Foxo1 in adiponectin gene transcription. In addition, Foxo1 provides a docking site for C/EBPα at the adiponectin promoter. Nevertheless, these results demonstrate that Foxo1 up-regulates adiponectin gene expression in mature adipocytes.

Foxo1 Activates the Adiponectin Promoter—To study whether and how Foxo1 up-regulates adiponectin gene transcription, a mouse adiponectin promoter-directed luciferase gene reporter construct was co-transfected with Foxo1-encoding plasmids in differentiated 3T3-L1 adipocytes. As expected, expression of Foxo1-WT and Foxo1-ADA dramatically increased adiponectin promoter-directed luciferase expression in 3T3-L1 adipocytes (Fig. 1C). Again, expression of Foxo1-Δ256 increased adiponectin promoter activity significantly (p < 0.05) (Fig. 1C). However, the magnitude of Foxo1-Δ256-stimulated promoter activation is much less than the induction by Foxo1-WT or Foxo1-ADA (Fig. 1C). These results are consistent with the data from adenovirus vector-transduced adipocytes studies (Fig. 1, A and B). Although these studies did not provide any clue for dominant negative Foxo1-Δ256-enhanced adiponectin gene expression, the results indicate that Foxo1 up-regulates adiponectin expression at the transcription level.

To solve the puzzle of Foxo1-Δ256-mediated adiponectin gene expression, we repeated the transient transfection study in HEK293 cells, which have been used as a simplified cellular model for studying transcriptional regulation of adipocyte gene expression (14, 24–26). Similar to the results in 3T3-L1 adipocytes, adiponectin promoter activities were elevated nearly 5-fold by wild type or Foxo1-ADA (p < 0.001) (Fig. 1D). These results further support our conclusion that Foxo1 up-regulates adiponectin gene transcription. In contrast to the result from adipocytes, ectopic expression of Foxo1-Δ256 reduced adiponectin promoter activity in HEK293 cells (p < 0.05) (Fig. 1D), raising the possibility that there is (are) adipocyte-specific protein(s) in adipocytes that prevail over the inhibitory effect of Foxo1-Δ256 and enhance adiponectin gene transcription.

C/EBPα and PPARγ are two master adipogenic transcription factors that regulate most adipose protein expression (14, 27). We used transient transfection to determine the relationship of Foxo1, C/EBPα, and PPARγ in the regulation of adiponectin gene transcription. Consistent with earlier studies (14, 28, 29), ectopic expression of C/EBPα or PPARγ2 and its partner RXRα modestly increased mouse adiponectin promoter-directed luciferase activity (Fig. 1E). The results also showed that expression of C/EBPα and PPARγ2/RXRα increased adiponectin promoter activation additively (Fig. 1E, last lane). Co-expression of Foxo1 and C/EBPα robustly increased the promoter activity nearly 20-fold (p < 0.001) (Fig. 1E), indicating that Foxo1 and C/EBPα synergistically increase adiponectin promoter activity.

Co-expression of Foxo1 did not show any synergistic effect on PPARγ2-induced adiponectin promoter activation (Fig. 1E). Of note, it has been reported that C/EBPα up-regulates adiponectin expression through a C/EBP consensus sequence at the human adiponectin promoter (29). Although a putative C/EBPα binding site in the murine adiponectin promoter can be found by sequence scan, most studies did not demonstrate binding of C/EBPα to this site (14, 26, 30). Our study indicated that C/EBPα and Foxo1 up-regulate adiponectin promoter activity in a synergistic manner. Therefore, we hypothesized that Foxo1 interacts C/EBPα and forms a transcription complex at the adiponectin promoter.

Interaction of Foxo1 and C/EBPα—Initially, a mammalian two-hybrid assay system was used. Foxo1Δ256 was fused with GAL4. Plasmids encoding C/EBPα and GAL4-Foxo1-Δ256 were co-transfected with pFR-luc (Stratagene), which contains five GAL4 binding sites. As expected, GAL4-Foxo1-Δ256 does not exhibit any transactivation activity due to the deletion of the C-terminal region of Foxo1 (Fig. 2A). Because C/EBPα cannot bind at GAL4 responding sites, expression of C/EBPα alone did not alter luciferase activity (Fig. 2A). However, co-expression of GAL4-Foxo1-Δ256 and C/EBPα robustly increased luciferase activity (p < 0.0001) (Fig. 2A). These results suggest that Foxo1 and C/EBPα interact. Co-immunoprecipitation assay further revealed that endogenous C/EBPα interacts with Foxo1 in differentiated adipocytes (Fig. 2B).

To further test the physical interaction of Foxo1 and C/EBPα, GST pulldown was employed. Several recombinant GST–Foxo1 constructs with truncated Foxo1 (Fig. 2, C and D) were also used to define the binding site of Foxo1 with C/EBPα. As shown in Fig. 2D, bottom panel and last lane, there is physical interaction of Foxo1 and C/EBPα. Moreover, the study indicated that the forkhead domain of Foxo1 is the binding site for C/EBPα (Fig. 2D). A positive GST pulldown result has been observed using GST-C/EBPα as bait (supplemental Fig. S2). These results demonstrated that Foxo1 interacts with C/EBPα through the forkhead domain.

Identification of Foxo1-responding Element in the Adiponectin Promoter—The mouse adiponectin promoter was examined to define the Foxo1-responding element. A Foxo1-responding region was located within −760 to −560 bp of the adiponectin promoter by 5′ end deletion (supplemental Fig. S3). Scanning this region revealed three putative insulin response sequences (IRSs) (18, 31). The results of the transient transfection study showed that mutation of IRS1 or IRS3 significantly decreased the basal adiponectin promoter activity (Fig. 3B). In contrast, mutation of IRS2 increased the basal promoter activity significantly (Fig. 3B). Mutation of either IRS1 or IRS3 almost completely abolished Foxo1-induced promoter activation (Fig. 3C).

To determine the binding of Foxo1 to these IRS sites, EMSA and supershift assays were conducted using nuclear proteins from adenovirus vector-transduced HEK293 cells. The results indicated that only IRS1 and IRS3 bind Foxo1 (Fig. 3D). The supershift assay revealed that Foxo1 was present in the protein complex bound to IRS1 and IRS3 probes (Fig. 3E). Taken together, these data demonstrate that IRS1 and IRS3 are Foxo1-
responding elements. Although the mechanism by which IRS2 contributes to basal promoter activity remains to be determined, it appears that the three IRSs regulate adiponectin gene transcription in a functionally distinct manner. IRS1 and IRS3 are co-operative in mediating Foxo1-induced adiponectin promoter activation. A similar observation has been reported in the glucose-6-phosphatase promoter (18, 32).

Interestingly, IRS1 or IRS3 mutation also dampened C/EBPβ-stimulated adiponectin promoter activation. A similar observation has been reported in the glucose-6-phosphatase promoter (18, 32).

FIGURE 2. Foxo1 interacts with C/EBPα. A, GAL4-directed luciferase gene reporter plasmid (pFR-luc) was co-transfected with C/EBPα- and/or GAL4-Foxo1Δ256-encoding plasmids in HEK293 cells. Luciferase activity was measured 24 h after transfection and was normalized by β-galactosidase activity. The values are expressed as relative luciferase activities. The error bar indicates S.E. for four independent experiments. *, p < 0.05 versus pcDNA-transfected cells. Two-way analysis of variance was used for statistical analysis. B, in vivo interaction of Foxo1 and C/EBPα. Nuclear proteins from differentiated 3T3-L1 adipocytes were immunoprecipitated (IP) with C/EBPα antibody or control IgG and probed by Western blot (WB) with the indicated antibodies. The autoradiograph is representative of four independent studies. C, schematics of mouse Foxo1 and truncation mutants for GST fusion proteins. D, GST-Foxo1 fusion proteins were expressed in BL21. The sample proteins were purified using glutathione-Sepharose beads and separated by SDS-PAGE. The gel was stained with Coomassie Blue (top panel). The darkest band in each lane represents the GST fusion protein. After GST pulldown, the eluted proteins were separated by SDS-PAGE and detected by Western blot using C/EBPα antibody (bottom panel).

C/EBPα acts as a co-activator. However, our study does not rule out the possibility that C/EBPα regulates adiponectin gene transcription through its own responding elements at the proximal promoter.

SIRT1 Up-regulates Adiponectin Gene Expression—Foxo1 is a substrate of SIRT1, and both proteins are involved in adipogenesis (8, 9). We therefore investigated the role of SIRT1 in the regulation of adiponectin gene expression. The SIRT1 was overexpressed in fully differentiated 3T3-L1 adipocytes using pcDNA-SIRT1 plasmid introduced by electroporation (14, 17) or knocked down in 3T3-L1CAR1 adipocytes using adenovirus vector encoding SIRT1 siRNA (Fig. 4, A and B). The protein and mRNA levels of adiponectin were significantly elevated in pcDNA-SIRT1-transfected adipocytes (p < 0.05) (Fig. 4A). In contrast, adiponectin expression was significantly reduced in Ad-SIRT1 siRNA-transduced cells (p < 0.05) (Fig. 4B).
larly, incubation of 3T3-L1 adipocytes with SIRT1 activator resveratrol or the inhibitor nicotinamide increased or decreased adiponectin protein levels \((p < 0.05)\) (supplemental Fig. S4). Together, these results demonstrate that SIRT1 regulates adiponectin gene expression in 3T3-L1 adipocytes.

SIRT1 is a nuclear protein that regulates target gene expression via NAD\(^+\)-dependent de-acetylation of histones or transcription factors. We used transient transfection and the HEK293 cellular model to study the effect of SIRT1 on adiponectin promoter activation. The results showed that overexpression of SIRT1 increased adiponectin promoter-directed luciferase expression \((-2\text{-fold}; p < 0.05)\) (Fig. 4C). Co-expression of SIRT1 and Foxo1 robustly increased adiponectin promoter activity \((p < 0.001)\) (Fig. 4C). SIRT1 and Foxo1 appear to synergistically increase adiponectin promoter-directed luciferase expression.

C/EBP\(\alpha\) and PPAR\(\gamma\) are important for adiponectin gene transcription. As shown in Fig. 4C, co-expression of SIRT1 increased C/EBP\(\alpha\)-mediated adiponectin promoter activation in an additive manner \((\text{lanes 6th from the left})\). Overexpression of SIRT1 synergistically increased the adiponectin promoter activities in Foxo1 and C/EBP\(\alpha\) co-transfected cells \((\text{Fig. 4C, 10th lane from the left})\). However, expression of SIRT1 and PPAR\(\gamma2/\text{RXR}\alpha\) only increased the adiponectin promoter-directed luciferase expression additively \((\text{data not shown})\). Together, these data suggest that SIRT1 synergistically up-regulates Foxo1- and C/EBP\(\alpha\)-mediated adiponectin gene transcription. A study has reported that SIRT1 inhibits PPAR\(\gamma\) activity in adipocytes \((9)\). Apparently, our result does not agree with this report in the context of PPAR\(\gamma\)-induced adiponectin promoter activation. We do not have any explanation for this disparity. A most recent study reported that SIRT1 activator resveratrol not only increases SIRT1 expression but also increases PPAR\(\gamma\) activities in Caco-2 cancer cells \((33)\).

To determine whether the deacetylase activity of SIRT1 is required for the transcriptional enhancement of adiponectin gene expression, a deacetylase inactive mutant of SIRT1H363Y was used \((\text{Fig. 4C})\), which contains a base substitution that converts the invariant catalytically active histidine at amino acid residue 363 to a tyrosine \((17)\). Expression of the inactive SIRT1H363Y did not further increase Foxo1- and C/EBP\(\alpha\)-mediated adiponectin promoter activation \((\text{Fig. 4C, 6th and 11th})\).
Regulation of Adiponectin Gene Transcription

To investigate the mechanism by which SIRT1 enhances Foxo1 and C/EBPα-mediated adiponectin gene transcription, we used chromatin immunoprecipitation assays to determine whether SIRT1 alters Foxo1 and C/EBPα binding to the adiponectin promoter. As shown in Fig. 5, both Foxo1 and C/EBPα were detected at the adiponectin promoter. Overexpression of Foxo1 or C/EBPα increased not only binding of the individual proteins to the promoter but also significantly increased the binding of the partner proteins C/EBPα and Foxo1, respectively. Most importantly, SIRT1 knockdown robustly reduced the binding of C/EBPα or Foxo1 to the adiponectin promoter (Fig. 5). However, consistent with our supershift assay (Fig. 3E), we did not detect SIRT1 protein in the complex bound to the adiponectin promoter (data not shown). These results support our hypothesis that Foxo1 and C/EBPα interact and form a complex at the adiponectin promoter, and SIRT1 enhances this process.

We next studied whether SIRT1 alters the Foxo1 and C/EBPα interaction using co-immunoprecipitation of protein samples from transfected HEK293 cells (Fig. 6A). Overexpression of SIRT1 increased the association of Foxo1 and C/EBPα (Fig. 6A). However, overexpression of SIRT1H363Y did not increase Foxo1 and C/EBPα association, suggesting SIRT1 deacetylation activity is required for SIRT1-enhanced Foxo1 and C/EBPα interaction.

SIRT1 deacetylates three lysine residues in the forkhead DNA binding domain of Foxo1 (34, 35), which is the region that interacts with C/EBPα, as we described above. To test the effect of the acetylation status of these three lysine residues on the interaction between Foxo1 and C/EBPα, we performed co-immunoprecipitation. The Lys→Arg substitution (K3R) prevents acetylation but keeps positive charges, thus mimicking the deacetylated form. The Lys→Ala (K3A) and Gln (K3Q) substitutions mimic the constitutively acetylated form through the absence of positive charges (16). Greater amounts of C/EBPα were co-immunoprecipitated with the Foxo1 deacetylation mimic mutant protein than with either wild type or Foxo1 acetylation mimic-mutant proteins (Fig. 6B), which suggest that deacetylation of Foxo1 enhances Foxo1 and C/EBPα interaction.
**Regulation of Adiponectin Gene Transcription**

**A**

IP: flag
IB: Foxo1
IB: C/EBPα

**B**

IP: flag
IB: Foxo1
IB: C/EBPα

**C**

![Graph showing luciferase activity](image)

**FIGURE 6.** SIRT1 enhances adiponectin gene expression by increasing Foxo1 and C/EBPα interaction. A, HEK293 cells were co-transfected with FLAG-Foxo1- and C/EBPα-encoding plasmids. SIRT1 or SIRT1H363Y was over-expressed in the indicated group. B, FLAG-fused wild type or mutant Foxo1 proteins and C/EBPα were expressed in HEK293 cells. Proteins were precipitated using FLAG antibody and probed with Foxo1 or C/EBPα antibodies. The autoradiograph is representative of three independent experiments (A and B). C, plasmids containing the adiponectin promoter (∼1130 to +1 nucleotide(s)) directing luciferase expression and plasmids encoding SIRT1, Foxo1-Δ256, or C/EBPα were transfected into HEK293 cells. Luciferase activities were measured 24 h after transfection and normalized to β-galactosidase activity. The error bar indicates S.E. for four independent experiments. *, p < 0.05 versus pcDNA-transfected cells.

The above studies have demonstrated that SIRT1 up-regulates adiponectin gene transcription by increasing Foxo1 transactivation activity. To determine whether SIRT1 regulates adiponectin transcription through the Foxo1-C/EBPα complex, truncated Foxo1-Δ256 and C/EBPα were co-transfected with adiponectin promoter gene reporter construct into HEK293 cells (Fig. 6C). Expression of SIRT1 did not alter the luciferase activities in Foxo1-Δ256-expressed cells. However, SIRT1 increased the luciferase activity significantly in Foxo1-Δ256- and C/EBPα co-transfected cells. Foxo1-Δ256 does not have transactivation activity but can bind with C/EBPα (Fig. 2, A and D). Taken together with the results presented above, our study indicates that SIRT1 up-regulates adiponectin gene transcription not only by increasing Foxo1 transactivation activity but also by enhancing Foxo1-C/EBPα complex formation at the adiponectin promoter.

Low Level of SIRT1 and Foxo1 Expression in the Adipose Tissues from Obese and db/db Diabetic Mice—The studies described above were performed with cultured cells. Thereafter, we studied the physiological significance of these discoveries. Although Foxo1 gene haploinsufficiency decreased adiponectin expression in mice (8), it is not known whether Foxo1 and SIRT1 contribute to the diminished adiponectin gene expression in obesity and type 2 diabetes. We examined Foxo1 and SIRT1 protein levels in the epididymal fat tissues of high fat diet-induced obese and db/db diabetic mice. Mice were made obese by 17 weeks of high fat diet (60 kcal% as fat). The mice exhibited significantly reduced insulin sensitivity (data not shown). db/db mice have a spontaneous mutation in the leptin receptor gene. They are obese and widely used as a type 2 diabetes mouse model. Consistent with previous studies, serum adiponectin concentrations and adiponectin RNA levels in adipose tissues were severely low in both high fat diet-induced obese and db/db diabetic mice (supplemental Fig. S6, A and B). Parallel with decreased adiponectin expression, Foxo1 and SIRT1 protein levels were also significantly decreased in epididymal fat from high fat diet-induced obese and db/db diabetic mice (supplemental Fig. S6C).

**DISCUSSION**

Adiponectin is an adipose-derived hormone with a variety of beneficial biological functions, such as sensitizing insulin action and protecting against atherogenesis. However, the underlying mechanisms of the diminished adiponectin gene expression in obesity and type 2 diabetes are poorly understood. Here we show that Foxo1 directly up-regulates adiponectin gene transcription but also interacts and recruits C/EBPα to the promoter. SIRT1 enhances the interaction of these two transcription factors and coordinately increases Foxo1-C/EBPα-mediated adiponectin promoter activation. Our data demonstrate that the Foxo1-C/EBPα transcription complex is critical in controlling adiponectin gene expression. Our study also suggests that decreased Foxo1 and SIRT1 expression and impaired Foxo1-C/EBPα transcription complex formation may contribute to the diminished adiponectin gene expression in obesity and type 2 diabetes.

Foxo1 regulates glucose and fat metabolism through its various target genes in the liver, muscle, adipose tissue, and pancreas. Current study is the first to identify its direct regulatory effect on adiponectin gene transcription. Nakae et al. (8) have reported that, with the initiation of adipocyte differentiation, Foxo1 expression is increased up to 6-fold over basal levels, reaching peak level at days 2–4. Overexpression of constitutive nuclear Foxo1 before clonal expansion prevents adipocyte dif-
differentiation by altering the expression of genes involved in cell cycle control and adipogenesis (8). Interestingly, during clonal expansion (days 1 and 2), Foxo1 activity is inhibited through high level phosphorylation and resultant exclusion from the nucleus (8). By day 3 of differentiation, phosphorylation of Foxo1 is substantially decreased, and Foxo1 is located in the nucleus (8). Therefore, despite increased Foxo1 expression, Foxo1 does not suppress adipocyte differentiation physiologically at the early stage, because its transactivation activity is suppressed by protein kinase B-mediated phosphorylation. Both our results3 and the Nakae et al. study (8) demonstrate that overexpression of wild type Foxo1 has no effect on adipocyte differentiation. Therefore, unlike Foxa2 and Foxe2, which suppress adipocyte differentiation by inhibiting PPARγ or increasing preadipocyte factor-1, respectively (36, 37), Foxo1 provides an integrating function for hormone-activated signaling pathways in a transcriptional cascade that promotes adipogenesis (38). Adiponectin gene expression is turned on after clonal expansion (19, 20). During the same time period transactivation of Foxo1 is activated (8). Furthermore, Foxo1 haplo-insufficiency significantly reduces adiponectin expression without significant changes in fat tissue mass (8). Taken together, we conclude that Foxo1 plays an important role in regulating adiponectin gene expression.

There are two main components of obesity: increased adipocyte number (hyperplasia) and cell size (hypertrophy). Adipocyte size is closely related with adiponectin expression, with reduced adiponectin expression in larger adipocytes (20, 39, 40). Despite many factors that influence adipocyte size, ultimately lipid storage plays a determining role. Picard et al. (9) recently reported that SIRT1 increases lipolysis in differentiated adipocytes. They also found that upon calorie restriction, SIRT1 expression is induced and fatty acids are mobilized in white adipose tissues, indicating that SIRT1 regulates lipid metabolism and decreases adipocyte size (9). Our results demonstrate that SIRT1 increases adiponectin gene expression. Thus, our studies provide mechanisms and consolidate the observations of adipocyte size and adiponectin gene expression. In addition, the Picard et al. study suggests that SIRT1 is a nuclear nutrient sensor in mammalian adipocytes (9). A similar function of SIRT1 in sensing metabolic status and regulating hepatic glucose metabolism has been recently reported (15). We speculate that, with nutritional deprivation, SIRT1 expression is induced, which leads to mobilization of fat storage in adipocytes and increased adiponectin gene expression. Increased adiponectin further sensitizes cells to insulin and enhances fatty acid oxidation (2), leading to a new energy homeostasis. In contrast to calorie restriction, long term calorie overload is the main reason for obesity. Here we show that SIRT1 and Foxo1 protein levels are reduced in fat tissues from high fat diet-induced obese and type 2 diabetic mouse models. Furthermore, our study demonstrated that SIRT1 up-regulates adiponectin gene transcription through a Foxo1-C/EBPα complex. Therefore, we propose that decreased expression of SIRT1 and Foxo1 and impaired Foxo1-C/EBPα transcription complex formation contribute to the diminished adiponectin gene expression in obesity. Apparently, more in vivo studies are required to verify this hypothesis.

Acknowledgment—We thank Dr. Jerome Schaack for comments on the manuscript.

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