FOXO1 Represses Peroxisome Proliferator-activated Receptor-γ1 and -γ2 Gene Promoters in Primary Adipocytes

A NOVEL PARADIGM TO INCREASE INSULIN SENSITIVITY

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FOXO1 and peroxisome proliferator-activated receptor-γ (PPARγ) are crucial transcription factors that regulate glucose metabolism and insulin responsiveness in insulin target tissues. We have shown that, in primary rat adipocytes, both factors regulate transcription of the insulin-responsive GLUT4 gene and that PPARγ2 detachment from the GLUT4 promoter upon thiazolidinedione binding up-regulates GLUT4 gene expression, thus increasing insulin sensitivity (Armoni, M., Kritz, N., Harel, C., Bar-Yoseph, F., Chen, H., Quon, M. J., and Karnieli, E. (2003) J. Biol. Chem. 278, 30614–30623). However, the mechanisms regulating PPARγ gene transcription are largely unknown. We studied the effects of FOXO1 on human PPARγ gene expression in primary rat adipocytes and found that both genes are endogenously expressed. FOXO1 coexpression dose-dependently repressed transcription from either the PPARγ1 or PPARγ2 promoter reporter by 65%, whereas insulin (100 nM, 20–24 h) either partially or completely reversed this effect. Phosphorylation-defective FOXO1 mutants T24A, S256A, S319A, and T24A/S256A/S319A still repressed the PPARγ1 promoter and partially lost their effects on the PPARγ2 promoter in either basal or insulin-stimulated cells. Use of DNA binding-defective FOXO1 (H215R) indicated that this domain is crucial for FOXO1 repression of the PPARγ2 (but not PPARγ1) promoter. Progressive 5′-deletion and gel retardation analyses revealed that this repression involves direct and specific binding of FOXO1 to the PPARγ2 promoter; chromatin immunoprecipitation analysis confirmed that this binding occurs in cellulo. We suggest a novel paradigm to increase insulin sensitivity in adipocytes in which FOXO1 repression of PPARγ, the latter being a repressor of the GLUT4 promoter, consequently leads to GLUT4 derepression/up-regulation, thus enhancing cellular insulin sensitivity. The newly identified FOXO1-binding site on the PPARγ2 promoter may serve as a therapeutic target for type 2 diabetes.

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The peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors and the FOXO (forkhead box class O) family of winged helix/forkhead box factors are two key families of transcription factors that dominate the regulation of glucose metabolism and insulin responsiveness in insulin target tissues. Members of both families are crucial for a multitude of biological processes, including the cell cycle, cell death, differentiation, and metabolism, and have prominent roles in insulin signaling pathways. A convergence of nuclear receptors and forkhead pathways in general and of FOXO1 and PPARγ in particular has been implicated in the pathophysiological states of insulin resistance and diabetes, supporting the importance of these transcription factors (1, 2). However, despite their importance to glucose homeostasis and adipocyte differentiation, the molecular mechanism(s) regulating transcription of the PPARγ gene and the roles of both PPARγ and FOXO1 transcription factors in these processes are not fully known.

The PPAR family of ligand-activated transcription factors includes three PPAR isoforms (α, β/δ, and γ) that differ in their tissue distribution and ligand specificity. PPARβ/δ is expressed ubiquitously in many tissues; PPARα is found predominantly in hepatocytes, cardiomyocytes, and enterocytes; and PPARγ is expressed mainly in insulin-responsive tissues, where it has a pivotal role in adipocyte differentiation and the expression of adipose-specific genes (3). There are two PPARγ isotypes (γ1 and γ2) that arise from the use of different promoters and alternative splicing (4). PPARγ2 is adipose-specific, whereas both are expressed in muscle. We have shown that, in primary adipocytes, both PPARγ1 and PPARγ2 repress GLUT4 transcription via direct and specific binding of the heterodimer PPARγ/retinoid X receptor-α to a GLUT4 promoter region (5). We discovered that rosiglitazone (an important thiazolidinedione ligand of PPARγ that improves insulin sensitivity) exerts its beneficial effect on insulin action by detaching PPARγ from its binding site on the GLUT4 promoter, thus alleviating this transrepression. However, the mechanisms regulating the PPARγ gene promoter itself are largely unknown.

The winged helix/forkhead family of transcription factors is characterized by a 100-amino acid monomeric DNA-binding

2 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PKB, protein kinase B; PRAs, primary rat adipocytes; IRS, insulin response sequence; Luc, luciferase; BSA, bovine serum albumin; HEK, human embryonic kidney; DAPI, 4',6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; DBD, DNA binding domain.
domain called the FOX domain. The DNA-binding domain folds into a variant of the helix-turn-helix motif and is made up of three helices and two characteristic large loops or "wings"; hence, the DNA-binding motif has been named the winged helix DNA-binding domain. Other portions of the forkhead proteins such as the DNA transactivation or DNA transrepression domains are highly divergent (6). The forkhead domain is responsible for DNA binding specificity and binds DNA as a monomer. Following a standardized nomenclature for these proteins (6), all uppercase letters are used for human (e.g. FOXO1), and only the first letter is capitalized for mouse (e.g. Foxo1). The FOXO family of transcription factors stimulates the transcription of target genes involved in many fundamental cell processes, including cell survival, cell cycle progression, DNA repair, and insulin sensitivity (reviewed in Ref. 2). FOXO1 is the most abundant FOXO isoform in insulin-responsive tissues such as hepatic, adipose, and pancreatic cells. Studies have shown that FOXO1 is negatively regulated by human protein kinase B (PKB)/Akt, a serine/threonine kinase that lies downstream of phosphatidylinositol 3-kinase in the insulin signaling cascade, and that this regulation includes a rapid and hierarchic phosphorylation of FOXO1 at three PKB/Akt phosphorylation consensus sites, Thr24, Ser256, and Ser319 (2). Nakae et al. (7) showed that murine Foxo1 is expressed mainly in adipose tissue and is a negative regulator of insulin sensitivity in liver, pancreatic β-cells, and adipocytes. Impaired insulin signaling to Foxo1 provides a unifying mechanism for the metabolic abnormalities of type 2 diabetes. Studying the importance of FOXO1 in both insulin signaling and tumorigenesis, we have shown that FOXO1 (previously FKHR (forkhead homologue rhabdomyosarcoma)) either represses or activates transcription from the GLUT4 gene depending on the cell type, whereas PAX3-FKHR, a chimeric gene product included in mouse FKHR (forkhead homologue rhabdomyosarcoma), enhances GLUT4 promoter activity via direct binding to specific promoter regions (8).

Although both PPARγ and FOXO1 are the main transcription factors in adipose tissue and are involved in adipogenesis and insulin signaling, their interactions have rarely been evaluated in *bona fide* insulin target cells. Furthermore, although PPARγ is involved in multiple regulatory processes, the mechanisms regulating transcription of the PPARγ gene itself are still unknown. Therefore, this study was undertaken to identify the molecular mechanisms by which FOXO1 regulates the expression of the PPARγ gene at the level of PPARγ1 and PPARγ2 transcription in primary rat adipocytes (PRAs).

**EXPERIMENTAL PROCEDURES**

Reverse Transcriptase-PCR—Total cellular RNA was prepared from the various cells using a TriReagent kit (Molecular Research Center, Inc., Cincinnati, OH) and further purified using RNeasy® columns (Qiagen GmbH, Hilden, Germany). Sense and antisense primers specific for β-actin, FOXO1, total PPARγ, and PPARγ2 mRNAs were synthesized based on sequences obtained from the GenBank™ Data Bank. First-strand cDNA synthesis and PCR amplification were performed using a Reverse-iT™ 1st Strand Synthesis kit (ABgene UK, Surrey, UK). Experiments performed in the absence of reverse transcriptase excluded the possibility of amplification from genomic contamination. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining as described previously (8).

Expression Vectors and Luciferase Promoter Reporters—Expression vectors encoding FOXO1 in pcDNA3 were kindly provided by Dr. Eric Tang (University of Michigan Medical School, Ann Arbor, MI) and have been described previously (9). These included a construct containing the full-length open reading frame of wild-type FOXO1; the constitutively active phosphorylation-defective mutants T24A, S256A, S319A, and T24A/S256A/S319A; and the DNA binding-defective mutant H215R. A control synthetic reporter (3×IRS-Luc) containing three repeats of an insulin response element consensus sequence in pGL2-Luc was also provided by Dr. Eric Tang (9). The human full-length PPARγ1 and PPARγ2 promoters in pGL3-Luc (hG1-P3000 and hG2-P587, respectively) were obtained from Dr. Luis Fajas (CNRS-INSERM, Louis Pasteur University, Strasbourg, France) and have been described previously (4). A series of progressively 5′-deleted promoter reporters was generated from the hG2-P587 reporter using the QuikChange® XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). All sequences were confirmed by direct sequencing.

Transient Expression and Promoter Reporter Assays—Isolated adipocytes were prepared from rat epididymal fat pads and transfected according to procedures we described previously (5). Briefly, adipocytes were transfected by electroporation (three pulses at 920 V and 50 microfarads; Gene Pulser II, Bio-Rad) with 2.0 μg of either PPARγ1 or PPARγ2 promoter reporter DNA, 0–5 μg of expression vectors for FOXO1 (wild-type or mutant), and 0.5 μg of pcMV-β-galactosidase. One hour later, an equal volume of incubation medium (supplemented with 7% bovine serum albumin (BSA) and either with or without 100 nM insulin) was added to the DNA-containing medium, and the cells were incubated for additional 20–24 h at 37°C. One set of tubes was transfected with the 3×IRS-Luc promoter reporter to be used as a positive control for FOXO1 transcription activation. In each experiment, the total amount of DNA transfected was held constant by adding the relevant insertless expression vector to account for squelching by the promoter itself. Luciferase activity was assayed at room temperature using a luciferase reporter assay kit (Promega Corp.) and a Lumat LB9501 luminometer (Berthold Systems, Inc., Nashua, NH). Luciferase activity was normalized to β-galactosidase activity as an internal control (10). Within each experiment, values were expressed as a percentage of the induced basal PPARγ promoter activity, i.e. the activity obtained in cells transfected with promoter reporter alone. Cell viability was assessed by trypan blue exclusion. Each experiment was repeated four to six times, with each sample analyzed in triplicates.

Assessment of FOXO1 Proteins by Western Immunoblotting—Endogenous expression of FOXO1 proteins and the levels of the exogenously overexpressed FOXO1 (wild-type and mutant) were assessed by Western immunoblotting in either total cell lysates or subcellular fractions of nuclear extracts and cytosols. Nuclear and cytosolic fractions were prepared as described by us before (11). For preparation of total cell lysates, parallel samples of mock-transfected and FOXO1-transfected PRAs, treated exactly as described for the luciferase reporter assay in 1× Reporter Lysis Buffer® (Promega Corp.), were supplemented with 1% SDS and proteases inhibitors; vortexed; and spun down 1000 × g at 4°C.
The upper fat layer was then removed, and the infranatant, including the total cell lysate fraction, was collected. Cellular protein levels were assessed with the bicinchoninic acid protein assay kit (Pierce). Samples of 10 mg of protein were analyzed by Western immunoblotting using either anti-FLAG monoclonal antibody M2 (Sigma, Rehovot, Israel) for detection of exogenously overexpressed FLAG-tagged FOXO1 or rabbit anti-FOXO1 polyclonal antibody (Cell Signaling Technology, Inc., Beverly, MA) for detection of endogenous FOXO1. Antigen-antibody complexes were detected by ECL with a SuperSignal West Pico chemiluminescent kit (Pierce). Dried blots were exposed to x-ray films, scanned, and quantitatively analyzed.

Immunofluorescence Studies—Human embryonic kidney (HEK) 293 cells were plated on 18-mm glass coverslips in 6-well plates at a density of 25,000 cells/well and transfected as described by us before (5). Cells transfected with expression vectors for FLAG-tagged wild-type or mutant FOXO1 were incubated for 24 h at 37 °C. The next day, cells were washed with magnesium-containing phosphate-buffered saline and transferred to serum-deprived medium supplemented with 2% BSA and either with or without insulin as described above. After 24 h, the cells were fixed in 4% paraformaldehyde and stained for indirect immunofluorescence using an anti-FLAG primary antibody, followed by a Cy3-conjugated goat anti-mouse secondary antibody. FLAG-tagged FOXO1 proteins and 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei were visualized using an Olympus IX81 inverted fluorescence microscope. Combined DAPI/Cy3 images were generated using an Olympus DP70 digital camera with DP Controller and DP Manager software.

In Vitro Translation and Electrophoretic Mobility Shift Assay (EMSA)—In vitro translation of FOXO1 proteins and EMSAs were performed as described (5). The TnT SP6/T7-coupled reticulocyte lysate system (Promega) was used to generate in vitro translated FLAG-tagged FOXO1 proteins from the corresponding cDNA, and the resulting protein lysate was used in EMSA. Protein expression was confirmed by SDS-PAGE, followed by phosphorimaging analysis of proteins translated in the presence of 35S-methionine (cell labeling grade; Amersham Biosciences, Buckinghamshire, UK). In vitro translation reactions generated sufficient protein to use in EMSAs. Sense and antisense PPARγ promoter-derived oligonucleotides corresponding to bp 270–310 of reporter hG2-P587 were synthesized (sense, GACACTGACATGTGGTGCACCGGCGAGACAGTGTGGCAAT); these were annealed and end-labeled with [γ-32P]ATP (6000 Ci/mmole; Amersham Biosciences) in the presence of polymerase kinase. Protein-DNA binding reactions were assembled in a volume of 20 μl, which included 4 μl of the in vitro translated FOXO1 lysate, ~75,000 cpm radiolabeled probe, and 4 μg of poly(dI-dC) in buffer containing 10 mM HEPES (pH 7.9), 1 mM dithiothreitol, 1 mM EDTA, 4% Ficoll, and 50 mM KCl. Competition experiments were performed in the presence of 100- and 200-fold molar excesses of unlabeled probe, which was added 10 min prior to the addition of the radiolabeled probe. For supershift assays, we used either anti-FLAG monoclonal antibody M2 or anti-FOXO1 polyclonal antibodies (FKHR (N-18) and FKHR (H-128), Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein lysates were preincubated with antisera for 10 min prior to the addition of the labeled probe. After incubation for 30 min at 25 °C, protein-DNA complexes were resolved by electrophoresis on 5% nondenaturing polyacrylamide gel at 150 V and 4 °C in 0.5× buffer containing 45 mM Tris (pH 8.3), 45 mm borate, and 1.0 mM EDTA. Gels were fixed in 10% acetic acid for 15 min, dried, and analyzed by phosphorimaging.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed following the method of Shang et al. (12) with modifications. HEK-293 cells were chosen as a source for human PPARγ2 promoter that resides in the context of the
Repression of the PPARγ Promoter by FOXO1

FIGURE 2. Dose-dependent effects of FOXO1 and insulin on PPARγ1 and PPARγ2 promoter activities. Isolated adipocytes were cotransfected with 2 μg of DNA from the human PPARγ1 (A) or PPARγ2 (B) promoter reporter together with 0–5 μg of pcDNA3-FLAG-FOXO1 (wild-type). The empty vector was added to keep the total amount of DNA transfected constant. Cells were incubated in serum-free medium supplemented with 3.5% BSA and either without (○, basal) or with 100 nm insulin (●) and were grown for 20–24 h until harvested. PPARγ promoter activity was determined by measuring luciferase and β-galactosidase activities as described under “Experimental Procedures.” Within each experiment, the results are expressed as a percentage of the basal PPARγ promoter activity, i.e. the activity obtained when each promoter reporter was expressed alone. The data are expressed as the means ± S.E. of four experiments, with each sample analyzed in quadruplicate.

RESULTS

Endogenous Gene Expression—Endogenous gene expression at the mRNA and protein levels was examined by reverse transcription-PCR and Western blot analyses, respectively. As shown in Fig. 1A, isolated PRAs showed endogenous expression of mRNAs for GLUT4, FOXO1, total PPARγ, and PPARγ2. Endogenous expression of GLUT4 was taken as a marker for an insulin-responsive tissue. Western immunoblotting showed endogenous expression of FOXO1 protein in total cell lysates prepared from PRAs; under these basal conditions, FOXO1 was localized to the nuclear fraction and was undetected in the cytosol (Fig. 1B).

We also determined the expression efficiency of FOXO1 in PRAs by Western immunoblotting and found that exogenous FOXO1 was overexpressed to ~20-fold of the endogenous protein (Fig. 1C).

Transcriptional Activities of PPARγ1 and PPARγ2 Are Differentially Regulated by FOXO1 and Insulin—Once establishing the expression patterns of endogenous and exogenous FOXO1 proteins in PRAs, we next studied the effects of FOXO1 on human PPARγ1 and PPARγ2 gene expression at the transcriptional level. PRAs were cotransfected with luciferase-conjugated promoter reporters for either human PPARγ1 or PPARγ2 along with the expression vector for wild-type FOXO1 (Fig. 2). We found that expression of wild-type FOXO1 repressed the transcriptional activities of both coexpressed the PPARγ1 and PPARγ2 promoters in a dose-dependent manner to as much as 65% below basal levels. Incubation of cells with 100 nm insulin resulted in a dose-dependent reversal of the FOXO1 effects on the PPARγ1 promoter, with transcriptional activity reaching 102 ± 3% of basal level at the maximal FOXO1 dose applied. Insulin also interfered with FOXO1 repression of the PPARγ2 promoter, but to a lesser extent. Under similar conditions, FOXO1 activated the insulin response element from the 3×IRS–Luc reporter, which was used as a positive control, by as much as 8.5-fold (data not shown); this excludes the possibility of either cytotoxic or squelching effects in the expression system. These data show that FOXO1 equally represses transcription from the PPARγ1 and PPARγ2 promoters, whereas insulin interferes with this effect in an isoform-specific manner.

Differential Contribution of FOXO1 Domains to PPARγ Promoter Regulation—The differential contribution of the various functional domains of FOXO1 to PPARγ promoter repression was studied under basal as well as insulin-mediated conditions using point mutation constructs as schematically depicted in Fig. 3A. The contribution of each of the three PKB/Akt phosphorylation sites of FOXO1 was studied using non-phosphorylatable mutants of FOXO1 (T24A, S256A, and S319A) and a triple mutant (T24A/S256A/S319A) in which all three phosphorylation sites were mutated to alanine. Cells were cotransfected with PPARγ promoter reporters along with the various FOXO1 mutants and incubated either under basal conditions or with 100 nm insulin for 24 h.
We found that mutations at each of the sites did not affect the basal capacity of FOXO1 to repress the PPARγ1 promoter, but significantly reduced the derepression capacity of insulin (Fig. 3B). However, all these mutants exhibited either partial or complete loss of FOXO1 ability to repress the PPARγ2 promoter (Fig. 3C).

We next used the DNA binding-defective mutant H215R to study the contribution of the FOXO1 DNA-binding domain (DBD). The H215R mutation did not affect the basal capacity of FOXO1 to repress PPARγ1, but slightly reduced promoter derepression in the presence of insulin (Fig. 3B). However, this mutant completely lost its ability to repress the PPARγ2 promoter and showed impaired responsiveness to insulin (Fig. 3C). Western blot analysis performed on total cell lysates prepared from FOXO1-transfected cells showed that all constructs used (wild-type as well as mutant) were successfully expressed as immunoreactive FLAG-tagged FOXO1 proteins to approximately the same extent (Fig. 3D), whereas mock-transfected PRAs showed no FLAG-FOXO1 immunoreactivity (data not shown).

To correlate FOXO1 effects with its cellular localization, we studied the subcellular distribution of wild-type FOXO1 and the various mutants in HEK-293 cells under the same basal and insulin-mediated conditions (Fig. 4). HEK-293 cells were chosen for the immunofluorescence studies because they easily stain to show more clearly the translocated proteins and can serve as a reasonable model to simulate FOXO1 translocation in adipocytes, as they have been shown to express endogenous insulin signaling machinery as well as endogenous FOXO1 (data not shown). In accordance with previous studies (5), we found that, in the basal state, wild-type FOXO1 was distributed in the nucleus and excluded from it upon insulin stimulation. Under both conditions, the DNA binding-defective mutant H215R behaved similarly to the wild-type protein, whereas the T24A/S256A/S319A mutant, which was not responsive to PKB/Akt phosphorylation, was not excluded from the nucleus in response to insulin.

**cis-Elements on the PPARγ2 Promoter Mediate Its Regulation by FOXO1**—Our data indicate that the DBD of FOXO1 is crucial for the repression of PPARγ1, but not of PPARγ2. Therefore, we focused our efforts on identifying cis-elements in the PPARγ2 promoter that may serve as potential FOXO1-binding sites. We performed a progressive 5′-deletion analysis of the full-length PPARγ2 promoter reporter hG2-P587 (Fig. 5). The 5′-deleted promoter reporters are shown in Fig. 5 (left panel). We found that deletion of bp 270–310 in the promoter region led to a major depletion of the ability of FOXO1 to trans-
translated protein-DNA interaction, FLAG-tagged FOXO1 protein was trans-
ferred to serum-free medium supplemented with 3.5% BSA and either without (basal) or with 100 nM insulin and were incubated for 20–24 h before staining. At that time, the cells were fixed, permeabilized, and subjected to indirect immunofluorescence staining using an anti-FLAG primary antibody, followed by a Cy3-conjugated goat anti-mouse secondary antibody. Nuclei were stained with DAPI. Combined images (DAPI/Cy3) were generated using an Olympus IX81 inverted fluorescence microscope and an Olympus DP70 digital camera with DP Controller and DP Manager software. In the Wild Type panels, FOXO1 can be seen all over the cell, mostly in the nuclei under the basal conditions, but was excluded from the nuclei after insulin incubation. This exclusion was prevented in most of the mutants, as can be seen from the clearer blue (DAPI) nuclear staining. AAA, T24A/S256A/S319A.

Repression of the PPARγ Promoter by FOXO1

FIGURE 4. Differential contribution of FOXO1 domains to the subcellular localization of FOXO1 protein. HEK-293 cells were transfected with pcDNA3-FLAG-FOXO1 (wild-type or mutant). After 24 h, cells were transferred to serum-free medium supplemented with 3.5% BSA and either without (basal) or with 100 nM insulin and were incubated for 20–24 h before staining. At that time, the cells were fixed, permeabilized, and subjected to indirect immunofluorescence staining using an anti-FLAG primary antibody, followed by a Cy3-conjugated goat anti-mouse secondary antibody. Nuclei were stained with DAPI. Combined images (DAPI/Cy3) were generated using an Olympus IX81 inverted fluorescence microscope and an Olympus DP70 digital camera with DP Controller and DP Manager software. In the Wild Type panels, FOXO1 can be seen all over the cell, mostly in the nuclei under the basal conditions, but was excluded from the nuclei after insulin incubation. This exclusion was prevented in most of the mutants, as can be seen from the clearer blue (DAPI) nuclear staining. AAA, T24A/S256A/S319A.

or anti-FOXO1 antibody H-128, which is directed against amino acids 471–598 near the C terminus of human FOXO1, but not in the presence of anti-FOXO1 antibody N-18, which is directed against the FOXO1 N terminus. This fact warrants further investigation, as, beyond reflecting the quality of the antibody preparation, it may represent a differential role for the various FOXO1 domains in the interaction with the PPARγ gene promoter.

To determine whether FOXO1 binds to the human PPARγ2 promoter in cellulo, the data obtained in EMSAs were verified by ChIP assays. HEK-293 cells were chosen to study FOXO1 binding to the human PPARγ2 promoter in its native form, i.e. within its native human chromatin context. FOXO1-transfected cells were subjected to either one- or two-step protein-DNA cross-linking. Lysed cells were then sonicated and subjected to immunoprecipitation with either anti-FOXO1 antibody or negative control IgG. DNA cross-linked to immunoprecipitated FOXO1 was subjected to PCR using primers for the human PPARγ2 promoter (bp 81–325) or for the sequence encompassing 3′IRS in pGL2, which was used as a positive control. We found that the conventional ChIP technique using a single formaldehyde cross-linking step did not reproducibly cross-link FOXO1 to DNA. As shown in Fig. 7A, the data obtained using one-step cross-linking yielded no detectable PPARγ2 promoter signal in either anti-FOXO1 or negative control IgG immunoprecipitates, and we could not detect a PPARγ2 promoter signal in anti-FLAG immunoprecipitates (data not shown). Under these conditions, however, FOXO1 complexed with 3′IRS (Fig. 7A, FOXO1–3′IRS), which was used as positive control. Therefore, we have adopted the ChIP assay using a two-step cross-linking procedure as described by Nowak et al. (13). The data obtained using this two-step cross-linking procedure showed that the human PPARγ2 promoter complexed with FOXO1, whereas the PPARγ2 promoter signal was barely detected in negative control IgG immunoprecipitates (Fig. 7B). Similar results were obtained in cells that were transfected with FOXO1 and the 3′IRS reporter (Fig. 7B, FOXO1–3′IRS), which was used as positive control. These data indicate that FOXO1 binds to the native human PPARγ2 promoter in cellulo. However, recapturing the protein-DNA complexes by ChIP assay is possible using a two-step rather than one-step cross-linking procedure.

Suggested Model for FOXO1 Down-regulation of the PPARγ1 and PPARγ2 Promoters—Based on the data we obtained from 5′-deletion, gel retardation, and ChIP analyses on the one hand and from FOXO1 mutation analysis on the other, we suggest the following model for FOXO1 repression of the PPARγ1 and PPARγ2 promoters (Fig. 8). 1) In the basal state, FOXO1 recycles between the nucleus and the cytoplasm, but is localized mostly to the nucleus (see Fig. 4). 2) Upon insulin stimulation, insulin signaling proceeds to PKB/Akt. 3) PKB/Akt activation leads to hierarchic phosphorylation of FOXO1 at three PKB/Akt consensus sites, Thr24, Ser256, and Ser319 (Fig. 8, circled p). 4) PKB/Akt phosphorylation of FOXO1 at any one of these sites leads to its nuclear exclusion, followed by either complete or partial derepression of PPARγ1 or PPARγ2 promoter activity, respectively (see Fig. 2 for insulin effects). 5) When in the nucleus, FOXO1 binds directly to the PPARγ2 promoter via a specific DNA sequence that encompasses bp 270–310, but probably extends beyond this region (as shown by ChIP). This leads to a dose-dependent repression of PPARγ2 transcriptional activity (Fig. 8, straight downward-point-
Repression of the PPARγ Promoter by FOXO1

Mutations at any one of the PKB/Akt phosphorylation sites or in the FOXO1 DBD (which render FOXO1 protein either refractory to PKB/Akt or defective in binding ability, respectively) lead to a partial derepression of the PPARγ2 promoter. 6) FOXO1 also represses transcription from the PPARγ1 promoter. However, this effect probably does not include direct binding to the PPARγ1 promoter, as the H215R mutant (with defective binding capacity) still represses the promoter. Thus, repression of the PPARγ1 promoter by FOXO1 probably involves indirect regulation, maybe via some mediator factor, the nature of which warrants further investigation. One promising candidate for this mediation is the PPARγ coactivator PGC-1, as it has been shown that Foxo1 regulates the activity and expression of PGC-1α, whereas PGC-1 family members interact with and control the activity of PPARγ (14).

**DISCUSSION**

In this work, we have shown for the first time that the human winged helix transcription factor FOXO1 represses transcription from the human PPARγ1 and PPARγ2 gene promoters in *bona fide* insulin target cells and that this regulation is both insulin-dependent and isoform-specific. We have demonstrated that FOXO1 activity is regulated by insulin-mediated phosphorylation of multiple sites that determine nuclear export, nuclear import, and transcriptional activity. We have also shown that regulation of PPARγ2 gene transcription occurs via direct and specific binding of FOXO1 protein to a sequence encompassing bp 270–310 (but probably extends beyond this region, as suggested by ChIP and 5′-deletion analyses in CHO-K1 cells) of the PPARγ2 promoter and that this binding can be demonstrated *in cellulo* within the context of the human nucleosome.

Our data gain support from a large body of evidence showing that the effects of FOXO1 we observed in primary adipocytes indeed reflect *bona fide* features of FOXO1-regulated PPARγ gene expression either *in cellulo* or *in vivo*. First, knock-out studies by Accili and co-workers (7) have shown that Foxo1 haploinsufficiency is associated with a significant enhancement of PPARγ mRNA levels in epididymal adipocytes. Second, arguing that dominant-negative Foxo1 mutants provide a useful reagent to study the effects of Foxo1 knock-out in experimental systems, Accili and co-workers (15) showed that transduction of 3T3-L1 adipocytes with Foxo1-Δ256 leads to earlier induction of adipocyte differentation, which is paralleled by earlier induction of PPARγ gene expression. Third, Kloting et al. (16) have shown that, in the Wistar Ottawa Karlsburg W (WOKW) rat model of human metabolic syndrome, severe insulin resistance is associated with increased Foxo1 levels in epididymal adipocytes, in accordance with decreased PPARγ gene expression. Fourth, in promoter reporter studies, Dowell et al. (17) have shown that Foxo1 and PPARγ functionally interact in a reciprocally antagonistic manner. All these findings support the findings introduced in our present work, that the effects of FOXO1 observed reflect genuine features of PPARγ gene expression. Furthermore, using small interfering RNA techniques in HEK-293 cells, we have recently been able to significantly silence the endogenous expression of the *FOXO1* gene, thus introducing another useful tool for studying *GLUT4* and PPARγ expression in these cells. Indeed, and in accordance with our previous work (8), we have acquired data suggesting that *GLUT4* gene expression directly correlates with FOXO1 levels in *bona fide* insulin target cells. 4

3 M. Armoni, unpublished data.
4 M. Armoni, C. Harel, and E. Karnieli, manuscript in preparation.
Repression of the PPARγ Promoter by FOXO1

A. In Vitro Translation

Control FOXO1

- 67kDa

B. Electromobility Shift Assay (EMSA)

| [32P]-Probe | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|-------------|---|---|---|---|---|---|---|
| TnT FOXO1-FLAG | + | + | + | + | + | + | + |
| Competitor DNA | - | - | x200 | x400 | + | + | + |
| αFOXO1 Ab (N18) | + | + | + | + | + | + | + |
| αFLAG mAb (M2) | + | + | + | + | + | + | + |

FIGURE 6. FOXO1 binding to the human PPARγ2 promoter in vitro: EMSA. A, in vitro translation of FOXO1. The integrity and correct size of the in vitro translated proteins for use in EMSAs were confirmed in parallel reactions performed in the presence of [35S]methionine. The resulting translation products were subjected to 10% SDS-PAGE, followed by phosphorimaging analysis. The size of the band is indicated. B, EMSA and supershift. Binding reactions for EMSA included the [32P]-labeled synthetic oligonucleotide representing region 270–310 on human PPARγ2 and FLAG-tagged FOXO1 protein lysate translated in vitro, as indicated above each lane. An unlabeled oligonucleotide (bp 270–310) was used as a specific DNA competitor, and the -fold molar excess of competitor is indicated above the relevant lanes. The complex was supershifted by the addition of either anti-FLAG monoclonal antibody (αFLAG mAb) or anti-FOXO1 antibody (αFOXO1 Ab), as indicated, 10 min prior to the addition of the probe. Black and white arrows indicate the positions of the bound and free probes, respectively. The checkered arrow indicates the supershift in the presence of the indicated antibodies.

FIGURE 7. FOXO1 binding to the human PPARγ2 promoter in vivo: ChIP. The results represent ChIP assays performed in HEK-293 cells that were transfected with FOXO1 in the pcDNA3-FLAG expression vector and with either pGL2-Luc or pGL2-Luc-3×IRS. Forty-eight hours post-transfection, DNA and protein were subjected to either one-step (A) or two-step (B) cross-linking (13). Cells were lysed and sonicated as described under “Experimental Procedures.” An aliquot of the whole cell lysate was removed for purification of total DNA (T), and immunoprecipitations were conducted using either anti-FOXO1 (αFOXO1) antibody or negative control IgG (αlgG). DNA was extracted from the immunoprecipitates, and PCR (26 cycles) was conducted on total DNA and immunoprecipitated DNA with primers corresponding to promoter region 1–325 of the human PPARγ2 gene (FOXO1-hPPARγ2-P) or to the region encompassing the 3×IRS sequence in pGL2 (FOXO1–3×IRS). PCR products were analyzed on 2% agarose gel and visualized by ethidium bromide staining in the presence of DNA molecular mass markers. Data from one representative assay (of three) are shown.

Our findings underscore the importance of studying the regulation of PPARγ gene expression in the context of genuine insulin target cells, as in this study. Adipogenesis is regulated by the hormonally induced coordinated expression and activation of two main groups of transcription factors, the CCAAT/enhancer-binding protein family and PPARγ (18). Because of its pivotal role in adipocyte differentiation and expression of adipocyte-specific genes, the PPARγ receptor is often called the “master of adipogenesis.” The adipogenic transcription factors then induce the expression of adipocyte-specificgenes, ultimately leading to a morphologically distinct and functional fat cell. Because adipose tissue mass plays a pivotal role in obesity and lipodystrophy (18), in this work, we focused on the regulation of PPARγ1 and PPARγ2 gene expression using bona fide insulin target cells. The pattern of PPARγ gene expression during adipogenesis is now starting to emerge. We studied the endogenous expression of FOXO1 and PPARγ during adipogenesis and found that, whereas the mRNAs for both genes are undetectable in preadipose-like cells (data not shown), they are clearly expressed in fully differentiated adipocytes at both the mRNA and protein levels (Fig. 1). In accordance with this finding, Nakae et al. (15) found that mouse Foxo1 is the most abundant Foxo isoform in murine white and brown adipose tissue and that, being almost undetectable in preadipocytes, its level rises up to 6-fold over basal levels during differentiation.

A role for FOXO1 is emerging as both a transcription activator and repressor of nuclear receptors (2). Looking at the protein structure of FOXO1, it is apparent that, besides a proline-rich and acidic serine/threonine-rich region that serves as a DNA activation domain at the C terminus, it also contains an alanine-rich region at its N terminus, which is believed to serve as a potential transcription repression domain, and an area in its mid-region thought to mediate interactions with nuclear receptors (2). This suggests that, depending on the specific milieu and cellular distribution, FOXO1 can act as either a transrepressor or transactivator of PPARγ gene transcription. Interestingly, we have obtained data showing that it is the adipose-specific isoform (PPARγ2) that is differentially regulated by FOXO1 in preadipocytes versus adipocytes, being immensely activated in the predifferentiated stage and transrepressed in the fully differentiated state. This supports the work of Fajas et al. (19) showing that E2F4 triggers the expression of PPARγ in preadipocytes, resulting in differentiation into adipocytes, but that when cells are terminally differentiated, E2F4 represses PPARγ gene expression through an association with p130/p107. In all, our data point to a
Repression of the PPARγ Promoter by FOXO1

FIGURE 8. Suggested model for FOXO1 regulation of the PPARγ1 and PPARγ2 promoters. Based on the data we obtained from 5′-deletion analysis and EMSAs on the one hand and from FOXO1 mutation analysis on the other, we suggest the following model for FOXO1 repression of the PPARγ1 (PPARγ1-P) and PPARγ2 (PPARγ2-P) promoters. 1) In the basal state, FOXO1 recycles between the nucleus and the cytoplasm and is localized mostly to the nucleus. 2) Upon insulin stimulation, insulin signaling proceeds to PKB/Akt. 3) PKB/Akt activation leads to hierarchical phosphorylation of FOXO1 at three PKB/Akt consensus sites (encircled p, Thr325, Ser256, and Ser319). 4) PKB/Akt phosphorylation of FOXO1 at any one of these sites leads to its nuclear exclusion, followed by either complete or partial derepression of PPARγ1 or PPARγ2 promoter activity, respectively. 5) Once in the nucleus, FOXO1 binds directly to the PPARγ2 promoter via at least one specific DNA sequence encompassing bp 270–310. This leads to a dose-dependent repression of PPARγ2 transcriptional activity (straight downward-pointing arrows). Mutations at any one of the PKB/Akt phosphorylation sites or in the FOXO1 DBD that render FOXO1 protein either refractory to PKB/Akt or defective in binding ability, respectively, lead to partial derepression of the PPARγ2 promoter. 6) FOXO1 also represses transcription from the PPARγ1 promoter; however, this effect probably does not include direct binding to the PPARγ1 promoter, as the H215R mutant, which has defective binding capacity, still represses the promoter. Thus, repression of the PPARγ1 promoter by FOXO1 probably occurs via a different pathway than that of PPARγ2 and involves an indirect regulation via a mediator, the nature of which warrants further investigation.

unique role for FOXO1-regulated PPARγ2 expression during adipogenesis, where FOXO1 greatly enhances PPARγ2 expression in predifferentiated cells, but represses it once PPARγ2 has completed its duties as the master regulator of adipogenesis.

Because FOXO1 was suggested as a downstream mediator of insulin signaling, we examined the effects of insulin on FOXO1-regulated PPARγ transcription. Insulin and other growth factors are known to promote phosphorylation of FOXO1 and PPARγ at phosphoacceptor sites, resulting in changes in the intracellular localization and activity of these transcription factors. The transcriptional activity of FOXO1 is regulated by insulin at the levels of transactivation, DNA binding, and nuclear exclusion. These different regulatory mechanisms allow the precise control of transcription of FOXO1 target genes by insulin. It has been widely accepted that phosphorylation of the three PKB/Akt consensus sites in FOXO1 and other FOXO proteins following incubation with insulin or other serum components inhibits FOXO-stimulated transcription of target genes by inducing the export of FOXO proteins from the nucleus. However, the role of insulin in regulating FOXO1 activity is debatable. Using phosphorylation-defective mutants, we acquired data showing that the effects of insulin on the subcellular distribution of FOXO1 can be segregated from its effects on the transcriptional activity of PPARγ. In accordance with this, Tsai et al. (20) have shown that insulin can inhibit Foxo1-stimulated transcription even when nuclear export of Foxo1 is prevented, indicating that insulin inhibition can occur by direct mechanisms that do not depend on altering the subcellular distribution of the transcription factor. Similarly, Zhang et al. (21) reported that inhibition of FOXO1-stimulated transcription by insulin in HEK-293 cells does not depend solely on nuclear exclusion. Indeed, in this work, we have shown that incubation of FOXO1 with insulin for 24 h affects its regulation of PPARγ in a manner that is both cell type- and isoform-specific, suggesting the potential involvement of different regulatory mechanisms for each isoform, for insulin responsive versus non-responsive cells, and for FOXO1 activity itself.

Several points emerge regarding how this differential regulation is exerted and the contribution of the various functional domains of FOXO1 to PPARγ transcription. First, we found that a mutant with defective DNA binding (H215R) retained its ability to repress PPARγ1, but lost its regulatory effects on the PPARγ2 promoter. These findings indicate that an intact DBD is crucial for FOXO1 regulation of the PPARγ2 (but not PPARγ1) promoter and suggest the involvement of direct binding of FOXO1 to the PPARγ2 promoter. However, as the DBD of FOXO1 is not necessary for transcription repression of the PPARγ1 isoform, FOXO1 likely has an indirect effect on this iso-
Repression of the PPARγ Promoter by FOXO1

form, perhaps via some mediating protein(s). Indeed, Zhao et al. (22) found that FOXO1 can interact with both steroid and non-steroid nuclear receptors in either a ligand-dependent or -independent manner to differentially regulate the transcription mediated by different nuclear receptors. This identifies FOXO1 as a bifunctional transcription factor that functions as both a coactivator and corepressor of the PPARγ promoter via either direct or indirect interactions. Another prominent candidate for mediating FOXO1 effects on PPARγ is the PPARγ coactivator PGC-1. It has been shown that Foxo1 regulates the activity and expression of PGC-1α, whereas PGC-1 family members interact with and control the activity of a large number of nuclear receptors, including PPARγ (see review in Ref. 14).

We next investigated the differential contribution of FOXO1 sites phosphorylated by PKB/Akt. These sites have been mapped to three key regulatory residues that are conserved within the FOXO family. Using mutants of FOXO1 that could not be phosphorylated, we found that the various phosphorylation sites of FOXO1 contribute differently to its basal or insulin-mediated capacity to regulate transcription from each of the PPARγ gene promoters examined. Our data also show clear segregation between the effects of insulin on the subcellular distribution of FOXO1 and on the transcriptional activity of PPARγ.

Looking into this complex pattern of regulation, one should bear in mind that the three PKB/Akt phosphorylation sites are also phosphorylated by SGK1 (serum- and glucocorticoid-induced protein kinase 1). sgk is an immediate-early gene that is activated in response to extracellular stimuli and whose mRNA levels increase dramatically within 30 min of cell exposure to serum or glucocorticoids. It was found that, although Thr24 is robustly phosphorylated by both kinases, Akt preferentially phosphorylates Ser256, and SGK preferentially phosphorylates Ser115 (2). Studies investigating the contribution of Thr24 phosphorylation to FOXO1 localization and subsequent activity have provided inconclusive results. We have shown here that, whereas a T24A mutation in FOXO1 affects neither its basal nor insulin-mediated capacity to repress the PPARγ1 promoter in primary adipocytes, it is associated with a major loss of the ability of FOXO1 to repress the PPARγ2 promoter in the basal state. Immunofluorescence staining indicated that the intracellular localization of the T24A mutant did not differ from that of the wild-type protein (data not shown). Tsai et al. (20) found that, in H4IIE cells, T24A-Foxo1 localizes predominantly to the nucleus after insulin treatment, whereas Scheimann et al. (23) observed intermediate levels of nuclear retention of T24A-Foxo1 in HepG2 cells, but no effect on its export to the cytoplasm in HEK-293 cells. The latter investigators also found that insulin-inhibited IGFBP-1 promoter activity is stimulated to the same extent by wild-type Foxo1 and T24A-Foxo1, but that the insulin inhibition of 3×IRS promoter activity by T24A-Foxo1 decreases by 50%. A similar intermediate reduction of insulin inhibition was seen in HepG2 cells expressing T24A-Foxo1 (16). In contrast, Guo et al. (24) observed no decrease in insulin inhibition. Thus, it is apparent that phosphorylation of FOXO1 in general and of the Thr24 site in particular contributes to FOXO1-mediated transcriptional activity in a manner that is both tissue- and species-specific.

We screened the PPARγ2 promoter (hG2-P587) for the presence of cis-elements that may serve as potential FOXO1-binding sites. Binding site selection studies performed with a variety of forhead proteins have led to the identification of a core recognition motif, T(G/A)TT(G/T)(G/A)(C/T), that is necessary for forhead binding, whereas bases immediately flanking this core contribute to the binding specificity of the different family members; for example, the optimal DNA-binding site for the FOXO members has been determined to be TTTGTTTAC (25). We have found that this core recognition motif is present at bp 431 of reporter hG2-P587. However, as evident from the 5′-deletion and ChIP analyses we performed, this motif lies beyond the region that was found to mediate most of the FOXO1 effects on PPARγ2 and that bound it in a direct and specific manner (Figs. 6 and 7). Thus, we show for the first time that, whereas PPARγ1 may be indirectly regulated by FOXO1, regulation of transcriptional activity from the human PPARγ2 promoter occurs via direct and specific binding of FOXO1 to a novel yet unidentified response element on the PPARγ2 promoter. This FOXO1 response element/binding motif lies in a region that encompasses bp 270–310 of the PPARγ2 promoter and that probably extends to further upstream and downstream regions, as evident from our ChIP and 5′-deletion analyses.

Further screening of the region containing bp 270–310 for known motif sequences revealed that it contains response elements for PPARγ and E2F, two factors that were found to regulate PPARγ transcription. The E2F response element is similar to that found by Fajas et al. (19) on the PPARγ1 promoter and is associated with induction of PPARγ transcription during clonal expansion of 3T3-L1 adipocytes. Interestingly, the PPARγ response element we found is similar to the acyl-coen-
zyme A oxidase PPARγ response element included in the (AOX)₃-Luc reporter. This PPARγ response element may contribute to FOXO1 regulation of the PPARγ promoter via a complex mechanism that involves a FOXO1-PPARγ interaction. Indeed, Dowell et al. (17) have shown that Foxo1 and PPARγ functionally interact in a reciprocally antagonistic manner. Consistent with this, our data support the notion of a convergence of PPARγ and FOXO1 signaling in the action of insulin. A more general convergence of nuclear receptors and forkhead factor pathways may be important for multiple biological processes, and this convergence may be evolutionarily conserved.

In summary, based on the data we obtained, we suggest a model for FOXO1 repression of the PPARγ1 and PPARγ2 promoters that is both tissue- and isoform-specific. According to this model, both promoters are repressed by FOXO1 via a distinct mode of regulation, as only the repression of the PPARγ2 promoter requires an intact DBD of FOXO1, indicating a direct protein-DNA interaction. In accordance with this, we have shown that PPARγ2 promoter repression occurs via specific and direct binding of FOXO1 to defined DNA sequence on the promoter both in vitro and in vivo. This newly identified FOXO1 response element may thus serve as a molecular therapeutic target for the treatment of insulin resistance and type 2 diabetes. As for regulation of PPARγ1, several potential factors can mediate its regulation by FOXO1. Among this, most prominent are members of the PGC-1 family, which have been previously shown to control the activity of PPARγ (see review in Ref. 14).

We introduce a novel paradigm to increase insulin sensitivity in adipocytes (summarized in Fig. 9). According to this paradigm, 1) FOXO1 represses PPARγ gene expression directly (PPARγ2) or indirectly (PPARγ1). 2) As shown by us before (5), both the PPARγ1 and PPARγ2 proteins repress GLUT4 promoter activity. 3) Therefore, repression of PPARγ by FOXO1 leads to GLUT4 up-regulation. 4) This subsequently results in enhanced glucose transport and cellular insulin sensitivity. Considering the prominent roles played by PPARγ and FOXO1 in insulin signaling pathways, our findings may provide a foundation for the better understanding of normal insulin action and its impairment, leading to insulin resistance in type 2 diabetes mellitus.

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REFERENCES
1. Arden, K. C. (2004) Mol. Cell 14, 416–418
2. Tran, H., Brunet, A., Griffith, E. C., and Greenberg, M. E. (2003) Sci. STKE 2003, RE5
3. Gildo, A. J., and Van Bilsen, M. (2003) Acta. Physiol. Scand. 178, 425–434
4. Fajas, L., Auboeuf, D., Raspe, E., Schoonjans, K., Lefebvre, A. M., Saladin, R., Najib, J., Laville, M., Fruchart, J. C., Deeb, S., Vidal-Puig, A., Flier, I., Briggs, M. R., Staels, B., Vidal, H., and Auwerx, J. (1997) J. Biol. Chem. 272, 18779–18789
5. Armoni, M., Kritz, N., Harel, C., Bar-Yoseph, F., Chen, H., Quon, M. J., and Karnieli, E. (2003) J. Biol. Chem. 278, 30614–30623
6. Kaestner, K. H., Nockel, W., and Martinez, D. E. (2000) Genes Dev. 14, 142–146
7. Nakae, J., Biggs, W. H., Ill, Kitamura, T., Cavenee, W. K., Wright, C. V., Arden, K. C., and Accili, D. (2002) Nat. Genet. 32, 245–253
8. Armoni, M., Quon, M. J., Maor, G., Avigad, S., Shapiro, D. N., Harel, C., Esposito, D., Goshen, Y., Yaniv, I., and Karnieli, E. (2002) J. Clin. Endocrinol. Metab. 87, 5312–5324
9. Tang, E. D., Nunez, G., Barr, F. G., and Guan, K. L. (1999) J. Biol. Chem. 274, 16741–16746
10. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
11. Armoni, M., Harel, C., Bar-Yoseph, F., Milo, S., and Karnieli, E. (2005) J. Biol. Chem. 280, 34786–34795
12. Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A., and Brown, M. (2000) Cell 103, 843–852
13. Nowak, D. E., Tian, B., and Brasier, A. R. (2005) BioTechniques 39, 715–729
14. Corton, J. C., and Brown-Borg, H. M. (2005) J. Gerontol. Ser. A Biol. Sci. Med. Sci. 60, 1494–1509
15. Nakae, J., Kitamura, T., Kitamura, Y., Biggs, W. H., Ill, Arden, K. C., and Accili, D. (2003) Dev. Cell 4, 119–129
16. Kloting, N., Bluher, M., and Kloting, I. (2006) Diabetes Metab. Res. Rev. 22, 146–154
17. Dowell, P., Otto, T. C., Adi, S., and Lane, M. D. (2003) J. Biol. Chem. 278, 45485–45491
18. Spiegelman, B. M., and Flier, J. S. (1996) Cell 87, 377–389
19. Fajas, L., Landsberg, R. L., Huss-Garcia, Y., Sardet, C., Lees, J. A., and Auwerx, J. (2002) Dev. Cell 3, 39–49
20. Tsai, W. C., Bhattacharyya, N., Han, L. Y., Hanover, J. A., and Rechler, M. M. (2003) Endocrinology 144, 5615–5622
21. Zhang, X., Gan, L., Pan, H., Guo, S., He, X., Olson, S. T., Mesecar, A., Adam, S., and Unterman, T. G. (2002) J. Biol. Chem. 277, 45276–45284
22. Zhao, H. H., Herrera, R. E., Coronado-Heinsohn, E., Yang, M. C., Ludes-Meyers, J. H., Seybold-Tilson, K. J., Nawaz, Z., Yee, D., Barr, F. G., Diab, S. G., Brown, P. H., Fuqua, S. A., and Osborne, C. K. (2001) J. Biol. Chem. 276, 27907–27912
23. Scheimann, A. O., Durham, S. K., Suwannichkul, A., Snuggs, M. B., and Powell, D. R. (2001) Horm. Metab. Res. 33, 631–638
24. Guo, S., Rena, G., Cichy, S., He, X., Cohen, P., and Unterman, T. (1999) J. Biol. Chem. 274, 17184–17192
25. Burgering, B. M., and Kops, G. J. (2002) Trends Biochem. Sci. 27, 352–360