

NFATc4 and ATF3 Negatively Regulate Adiponectin Gene Expression in 3T3-L1 Adipocytes

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Expression of adiponectin decreases with obesity and insulin resistance. At present, the mechanisms responsible for negatively regulating adiponectin expression in adipocytes are poorly understood. In this investigation, we analyzed the effects of 5' serial deletion constructs on the murine adiponectin promoter. Here, we identified the repressor region located between -472 and -313 bp of the promoter. Removal of the putative nuclear factor of activated T-cells (NFATs) binding site increased the promoter activity, and overexpression of NFATc4 reduced the promoter activity. Treatment with the calcium ionophore A23187, an activator of NFAT, reduced mRNA as well as promoter activity. The binding of NFATc4 to the promoter was associated with increased recruitment of histone deacetylase 1 and reduced acetylation of histone H3 at the promoter site. In addition, binding of activating transcription factor 3 (ATF3) to the putative activator protein-1 site located adjacent to the NFAT binding site also repressed the promoter activity. Treatment with thapsigargin, an inducer of ATF3, reduced both mRNA and promoter activity. Importantly, the binding activities of NFATc4 and ATF3, increased significantly in white adipose tissues of *ob/ob* and *db/db* mice compared with controls. Taken together, this study demonstrates for the first time that NFATc4 and ATF3 function as negative regulators of adiponectin gene expression, which may play critical roles in downregulating adiponectin expression in obesity and type 2 diabetes. *Diabetes* 55:1342–1352, 2006

Adipose tissues are known to store triglycerides and release free fatty acid/glycerol in response to changing energy demands (1). Additionally however, adipose tissues also regulate energy homeostasis by secreting biologically active adipocytokines, such as adiponectin, adiponectin, leptin, plasminogen activator inhibitor-1, resistin, and tumor necrosis factor

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AP-1, activator protein-1; ATF3, activating transcription factor 3; C/EBP, CCAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; CsA, cyclosporin A; EMSA, electrophoretic mobility shift assay; HDAC, histone deacetylase; HEK, human embryonic kidney; IL, interleukin; NFAT, nuclear factor of activated T-cell; TNF, tumor necrosis factor; TSA, trichostatin A.

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(TNF)- α (2). Adiponectin in particular possesses insulin-sensitizing, antiatherogenic, anti-inflammatory, and antiangiogenic properties (3,4). In humans and animals with insulin resistance, obesity, or type 2 diabetes, serum levels of adiponectin are reduced (5–8). Although the physiological effects of adiponectin have been investigated intensively, regulatory mechanisms for adiponectin gene expression are poorly understood. Several insulin resistance-inducing factors such as TNF- α (9), interleukin (IL)-6 (10), and β -adrenergic agonists (11) have been shown to reduce adiponectin expression. Conversely, increases in adiponectin expression have been reported during adipocyte differentiation and transcriptional factors implicated in adipogenesis, including peroxisome proliferator-activated receptor- γ (12), CCAAT/enhancer-binding protein (C/EBP) α (13,14), and sterol regulatory element-binding protein-1c (15), have been shown to upregulate adiponectin gene expression. However, little is known about negative transcriptional control of adiponectin gene expression.

Nuclear factor of activated T-cell (NFAT) is a family of transcription factors originally identified as important mediators in cytokine gene expression during the immune response (16). However, recent evidence has demonstrated that NFAT is expressed in different cell types and regulates diverse cellular functions such as adipocyte differentiation (17), cardiac hypertrophy (18), neuronal development (19), and angiogenesis (20). Four members of the NFAT family share significant sequence and functional similarity. Calcium-sensitive NFATc1, -c2, and -c3 are tightly restricted to the immune system, whereas NFATc4 is fairly ubiquitous and regulates cardiac hypertrophy and hippocampal neuronal signaling (21). NFAT proteins are primarily phosphorylated and found in the cytoplasm of resting cells. Increased intracellular calcium levels activate calcineurin, a serine-threonine phosphatase that dephosphorylates NFAT, which then translocates into the nucleus. Inside the nucleus, NFAT bind to a purine-rich core motif, (A/T)GGAAA, and regulate transcription of NFAT-dependent genes (21). Cyclosporin A (CsA) and FK506 block calcineurin phosphatase activity and inhibit NFAT activation by preventing nuclear NFAT translocation (22).

Activating transcription factor 3 (ATF3) is a member of the ATF/cAMP responsive element-binding family of proteins that acts as a stress-inducible transcriptional repressor (23). ATF3 has been shown to be induced in cells exposed to a variety of physiological and pathological stimuli, including carbon tetrachloride exposure (24), anti-cancer drugs (25), proteasome inhibitors (26), genotoxic agents (27), homocysteine (28) and ischemia-reperfusion

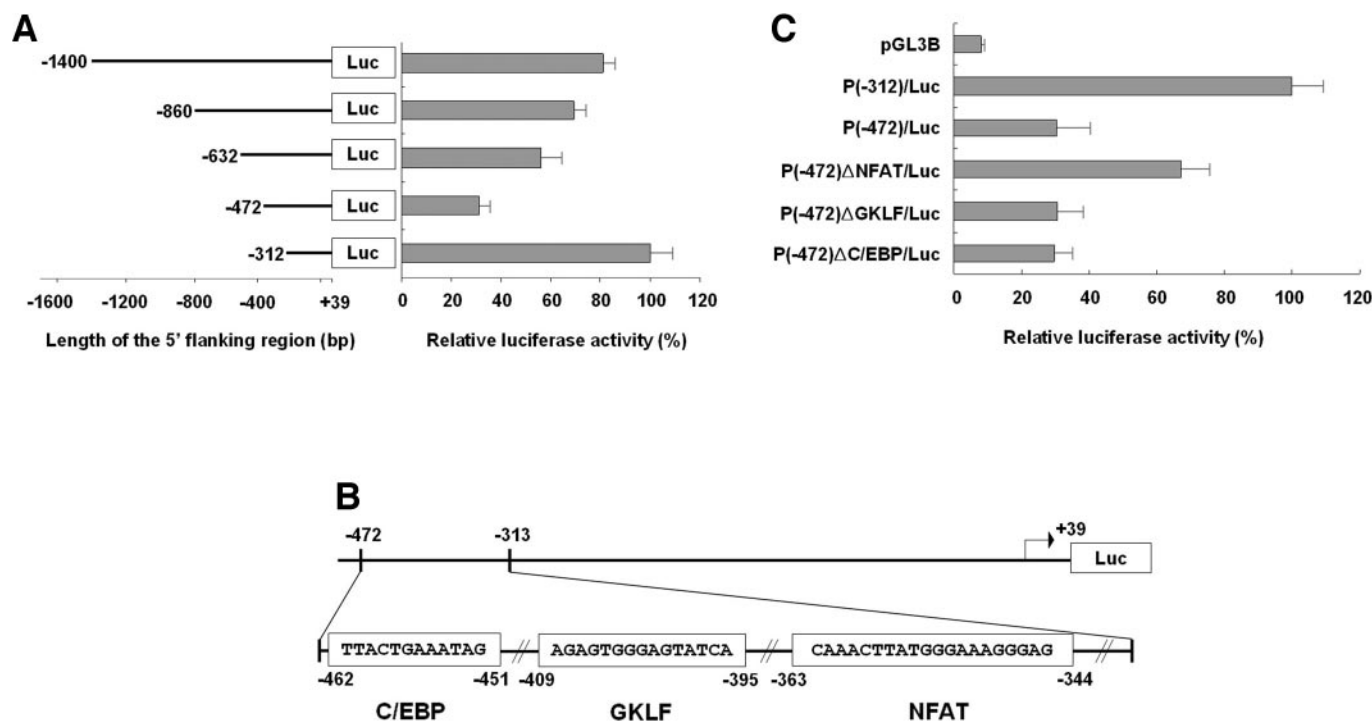


FIG. 1. Location of repressor element between -472 and -313 bp of the mouse adiponectin promoter. **A:** Constructs containing various lengths of serial deletions of the 5' flanking region of the mouse adiponectin gene were generated in pGL3 basic reporter plasmid. Luciferase activity is presented relative to the observable highest activity from the p(-312)/Luc reporter. **B:** Schematic representation of mouse adiponectin promoter (-472 – -313). The putative binding sites for NFAT, GKLf, and C/EBP are boxed. **C:** Deletion of putative NFAT site increases adiponectin promoter activity. The data are presented as means \pm SD of five independent experiments.

(29). ATF3 is also induced in response to endoplasmic reticulum stress or amino acid deprivation (30). Activated ATF3 can either homodimerize and repress transcription of various promoters with ATF sites (31) or heterodimerize with bZip proteins, c-jun, Jun B, ATF2, or gadd153/CHOP10 (C/EBP homologous protein) and activate transcription of target genes (32).

To understand mechanisms involved in regulating adiponectin gene expression, we isolated the mouse adiponectin promoter and analyzed the activities of promoters with 5' serial deletions. Here, we demonstrate that NFATc4 and ATF3 negatively regulate adiponectin gene expression. The binding activities of both NFATc4 and ATF3 from the nuclear extracts of white adipose tissue from *ob/ob* and *db/db* mice increase, inconsistent with reductions in adiponectin mRNA. These results suggest that NFATc4 and ATF3 play crucial roles in repression of adiponectin expression in obesity and type 2 diabetes.

RESEARCH DESIGN AND METHODS

Cell culture and treatments. 3T3-L1 preadipocytes and human embryonic kidney (HEK) 293 cell lines (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium with high glucose (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) FCS (GibcoBRL, Gaithersburg, MD.). 3T3-L1 preadipocytes were differentiated as described previously (15). To investigate the effects of stimulation on the adiponectin promoter, transfected 3T3-L1 adipocytes were treated with 2 μ mol/l of A23187 (Calbiochem, San Diego, CA), 0.3 μ mol/l of trichostatin A (TSA) (Biomol, Plymouth Meeting, PA), or 0.3 μ mol/l of thapsigargin (Sigma, St Louis, MO) alone or in combination and 10 ng/ml of TNF- α (Calbiochem). For the determination of adiponectin mRNA levels and assay of the chromatin immunoprecipitation (ChIP), the compounds were added to fully differentiated 3T3-L1 cells for the indicated times. **Plasmid constructs.** The mouse adiponectin promoter region spanning $-1,500$ to $+50$ bp was amplified by PCR with mouse genomic DNA and inserted into KpnI/BglII restriction sites of the pGL3 basic luciferase reporter

(Promega, Madison, WI). Deletions in the 5' flanking regions of the adiponectin promoter were constructed by PCR using a pairwise combination of the following sense primers: 5'-GGT ACC GAG GAT AAT TTT CAT TGC AC-3' [for P(-312)/Luc], 5'-GGT ACC CTC TTC ATT CTT ACT GAA AT-3' [for P(-472)/Luc], 5'-GGT ACC TTT GGC TGC ATG CAT ATT TG-3' [for P(-632)/Luc], 5'-GGT ACC ATG GTT CTC CAA TGT CAA GG-3' [for P(-860)/Luc], and 5'-GGT ACC CCT AAC GTG ATT TCT CTA GA-3' [for P($-1,400$)/Luc], with the antisense primer 5'-AGA TCT CTT TTG GTG TCG TCA GAT CC-3'.

Deletion-mutant plasmids lacking putative transcription factor binding sites and site-mutated plasmids were constructed in the pGL3 adiponectin luciferase plasmid by the two-step PCR method. Expression plasmids encoding mouse NFATc1, c2, c3, or c4 were generous gifts from Dr. G. R. Crabtree (Department of Molecular Pharmacology, Albert Einstein College of Medicine). The ATF3 expression vector was kindly provided from Dr. T. Hai (Department of Molecular and Cellular Biochemistry, Ohio State University, Columbus, OH).

Transient transfection and luciferase activity. Fully differentiated 3T3-L1 adipocytes were grown in six-well plates and subjected to transient transfection using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. After transfection for 24 h, cells were lysed in lysis buffer (Promega, Madison, WI), and luciferase activity was measured using the Luciferase Assay System (Promega).

Electrophoretic mobility shift assay. Probes corresponding to each promoter binding region were generated, and electrophoretic mobility shift assays (EMSA) were performed as previously described (13).

RT-PCR. Total RNA was extracted using TRizol reagent (Invitrogen) and was subjected to reverse transcription using reverse transcriptase (Promega) at 42°C for 1 h, and the resulting cDNA was amplified by PCR using gene-specific primers.

Preparation of nuclear extracts. Nuclear extracts from 3T3-L1 adipocytes were prepared as described by Crabtree (16). Nuclear extracts from mouse epididymal adipose tissues were prepared as previously described (13).

Western blot analysis. Total proteins were extracted by using PRO-PREP reagent (Intron Biotechnology, Sungnam, Korea), and the immune complexes were identified using the enhanced chemiluminescence detection system (Amersham Biosciences, Uppsala, Sweden).

ChIP. The ChIP assays were performed as described previously (33). Fully differentiated 3T3-L1 cells were stimulated with the indicated compounds or left unstimulated. Immunoprecipitated DNA was amplified by PCR using

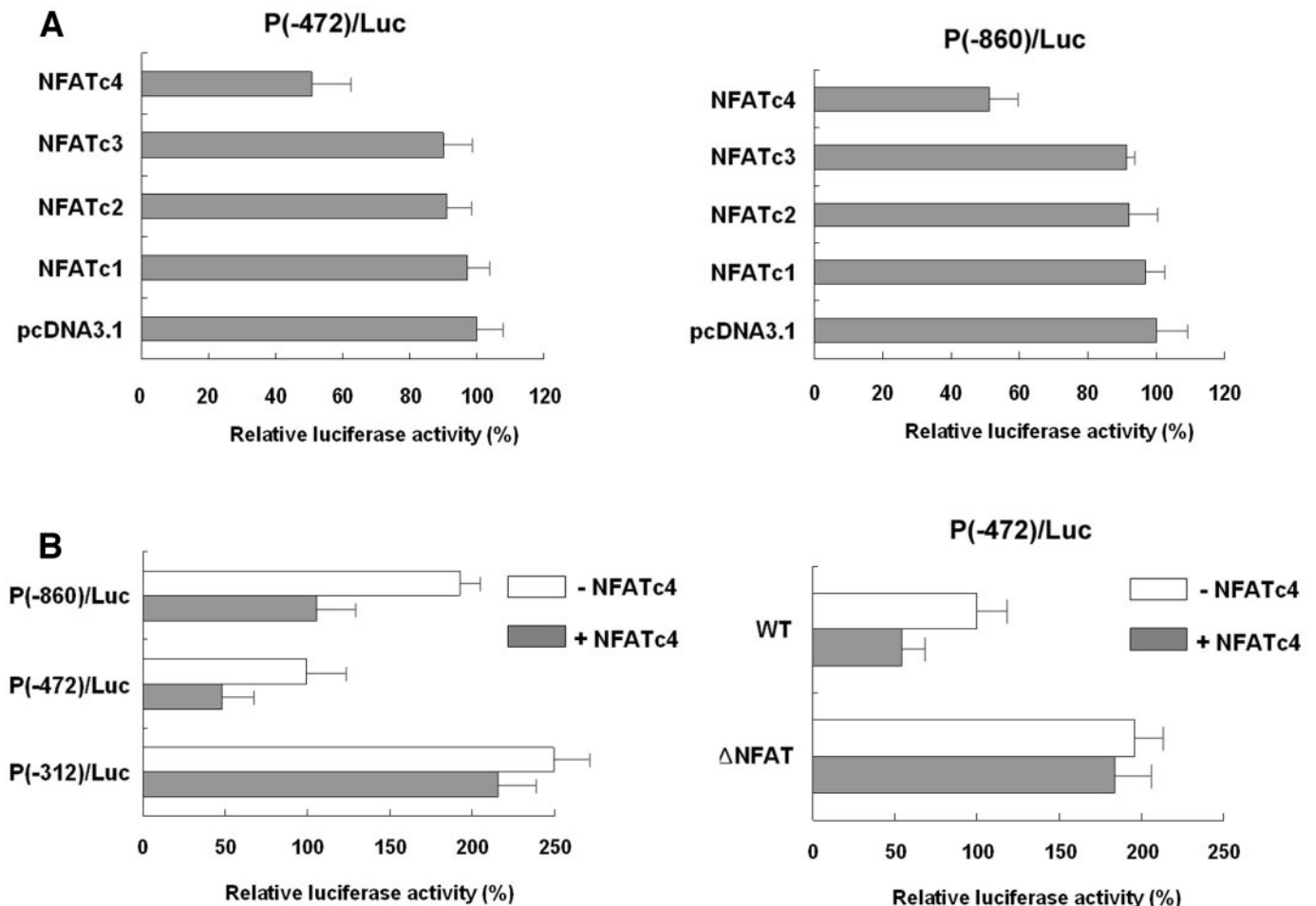


FIG. 2. NFATc4 downregulates transcription of adiponectin promoter dependent on putative NFAT binding site. **A:** NFATc4 represses adiponectin promoter activity in 3T3-L1 cells. The p(-472)/Luc or p(-860)/Luc reporter plasmids were transfected into 3T3-L1 adipocytes with the indicated NFAT expression vectors. **B:** NFATc4-mediated repression is dependent on putative NFAT binding site. The p(-860)/Luc, p(-472)/Luc, p(-312)/Luc reporters (*left panel*) and the mutant reporter lacking the NFAT binding site (*right panel*) were transfected into 3T3-L1 adipocytes along with the NFATc4 expression vector. The data are presented as means \pm SD of five independent experiments.

primers specific for the adiponectin promoter, sense (-432 to -413), 5'-GCT TCA CAT TTA ACA AA-3' and antisense (-292 to -273), 5'-ATA ATT CAG CAT GTT TCT GA-3' or for exon 3 region, sense, 5'-GTCTCCACGACTCT TACATG-3' and antisense, 5'-ACATTCATACACTCAGCCTG-3'.

Experimental animals. Obese (*ob/ob*) and diabetic (*db/db*) mice and their age-matched control lean mice (C57BL/6J, 10 weeks) were purchased from The Jackson Laboratory (Bar Harbor, ME). After overnight fasting, the mice were killed and the epididymal fat tissues collected and stored at -80°C for mRNA and nuclear protein isolation.

RESULTS

Location of repressor element between -472 to -313 bp of the mouse adiponectin promoter. To dissect the regulatory DNA region involved in regulating adiponectin gene expression, mouse adiponectin promoters with 5' serial deletions were inserted into the pGL3 basic luciferase plasmid and transiently transfected into 3T3-L1 adipocytes. Analysis of the luciferase activities of transfected cells revealed that the proximal -312-bp region (-312 to +39) of the adiponectin promoter conferred maximal transcriptional activity in 3T3-L1 cells (Fig. 1A). However, promoter activity was reduced by $>70\%$ with plasmid containing the -472-bp (-472 to +39) region of the adiponectin promoter (Fig. 1A). This suggests that the presence of a repressor binding site was situated between -472 and -313 bp of the 5' flanking region of the

adiponectin promoter. A search of the TRANSFAC transcription factor binding database for a 160-bp consensus sequence revealed several potential binding sites for NFAT (-363 to -344), gut-enriched kruppel-like factor (-409 to -395), and C/EBP (-462 to -451) transcription factors, all located within the -472 and -313 region (Fig. 1B). To evaluate the functional significance of the putative repressor binding sites, deletion-mutant constructs lacking binding sites for the transcription factors were introduced into the p(-472)/Luc reporter. As shown in Fig. 1C, the NFAT site-deleted mutant increased promoter activity compared with the wild type, whereas the gut-enriched kruppel-like factor and C/EBP-deleted mutants produced minimal effects on the promoter activity, indicating that NFAT may be involved in repressing adiponectin promoter activity.

NFATc4 represses adiponectin promoter activity dependent on the putative NFAT binding site. To examine whether NFAT is directly involved in repressing adiponectin expression, NFAT expression vectors were transfected along with p(-472)/Luc or p(-860)/Luc reporters. As shown in Fig. 2A, exogenous overexpression of NFATc4 significantly reduced the promoter activity of both p(-472)/Luc and p(-860)/Luc by 50% compared with the control vector. In contrast, the other NFAT family members, NFATc1, c2, and c3 all failed to reduce pro-

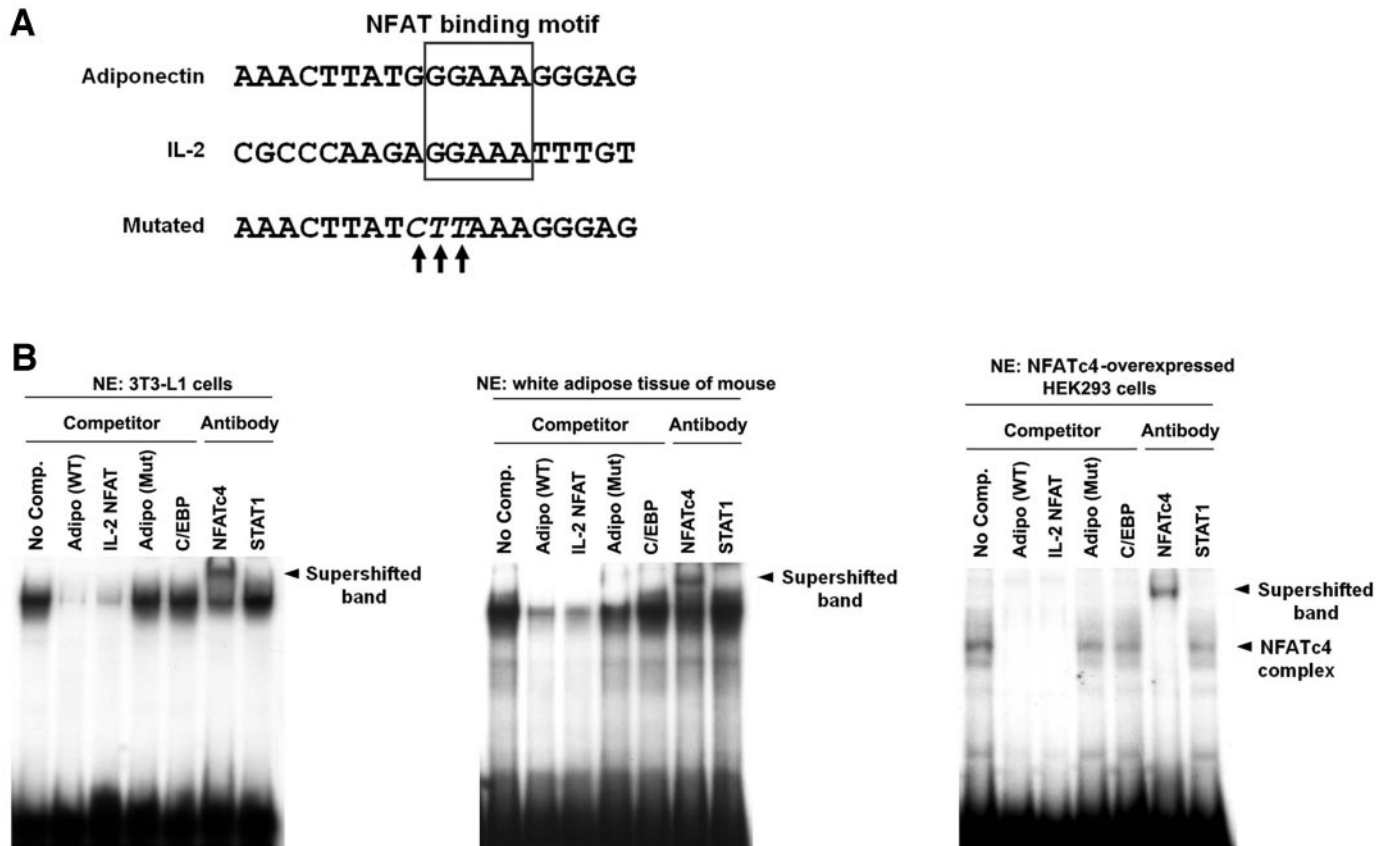


FIG. 3. NFATc4 binds to the adiponectin promoter *in vitro*. **A:** Sequence comparison of putative NFAT binding site in the adiponectin promoter with the canonical NFAT binding site located on the IL-2 gene. The NFAT core binding site is boxed. Arrow indicates mutated site. **B:** EMSAs were performed on the NFAT site (–363 to –344 bp) using nuclear extracts from 3T3-L1 adipocytes (*left panel*), murine white adipose tissue (*middle panel*), and NFATc4-overexpressing HEK293 cells (*right panel*). For oligonucleotide competition experiments, a 100-fold excess of oligonucleotides were used (*lane 1*: no competition; *lane 2*: adiponectin wild NFAT oligonucleotides; *lane 3*: IL-2 NFAT oligonucleotides; *lane 4*: adiponectin mutant NFAT oligonucleotides; *lane 5*: C/EBP oligonucleotides). The protein-DNA complex was supershifted by NFATc4 antibody (*lane 6*) but not by the nonspecific antibody (*lane 7*).

motor activity, demonstrating that NFATc4 is likely responsible for repression of adiponectin expression in the natural promoter context. Next, we determined whether NFATc4-dependent repression is mediated through the repressor region containing the putative NFAT binding site. As shown in Fig. 2B (*left panel*), the promoter activities of both p(–860)/Luc and p(–472)/Luc reporters were reduced by NFATc4, while the p(–312)/Luc reporter, which does not contain the repressor region, was unaffected. To further confirm the role of the putative NFAT binding site, an NFAT binding site–deleted reporter was constructed in p(–472)/Luc. As shown in Fig. 2B (*right panel*), NFATc4 repressed the promoter activity of wild-type p(–472)/Luc but did not affect the NFAT site–deleted reporter. Taken together, these results demonstrate that NFATc4 represses the promoter activity of adiponectin genes, dependent upon the putative NFAT binding site (–363 to –344) of the mouse adiponectin promoter.

NFATc4 binds to putative NFAT binding site on the adiponectin promoter. To determine whether NFATc4 interacts with the NFAT-binding site, EMSAs were performed using nuclear extracts from fully differentiated 3T3-L1 cells and mouse white adipose tissue. For this experiment, a 20-bp wild-type oligonucleotide (–363 to –344) was designed that cover the putative NFAT binding site containing the core sequence of the NFAT response element (GGAAA) (Fig. 3A). As shown in Fig. 3B, using nuclear extracts from both 3T3-L1 cells (Fig. 3B, *left*

panel) and mouse white adipose tissue (Fig. 3B, *middle panel*), binding complexes were observed with the labeled 20-bp oligonucleotides. A 100-fold molar excess of unlabeled oligonucleotides for homologous adiponectin and IL-2 NFAT nearly abolished the bands, whereas unlabeled mutant oligonucleotides bore no significant effect on binding (Fig. 3B). To ascertain whether the complex consisted of bound NFATc4, a supershift assay was conducted with the antibody against NFATc4. Results show that anti-NFATc4 shifted the binding complex. In contrast, no supershifted complex was detected with anti-STAT-1 (Fig. 3B). Nuclear extracts from HEK293 cells overexpressing NFATc4 were also used to examine specific binding of NFATc4 to the binding site. The overexpression of NFATc4 was confirmed by Western blot (data not shown). As shown in Fig. 3B (*right panel*), overexpressing NFATc4 formed complexes with the labeled oligonucleotides and competed against oligonucleotides for homologous adiponectin and IL-2 NFAT. Moreover, the NFATc4 antibody successfully supershifted the complex. All together, these data demonstrate that NFATc4 directly binds to the NFAT binding site located between –363 and –344 of the adiponectin promoter.

NFATc4 activation reduces mRNA expression and promoter activity of adiponectin. To address whether NFATc4 activation downregulates adiponectin expression, fully differentiated 3T3-L1 cells were treated with A23187 that activates NFAT, and adiponectin mRNA levels were

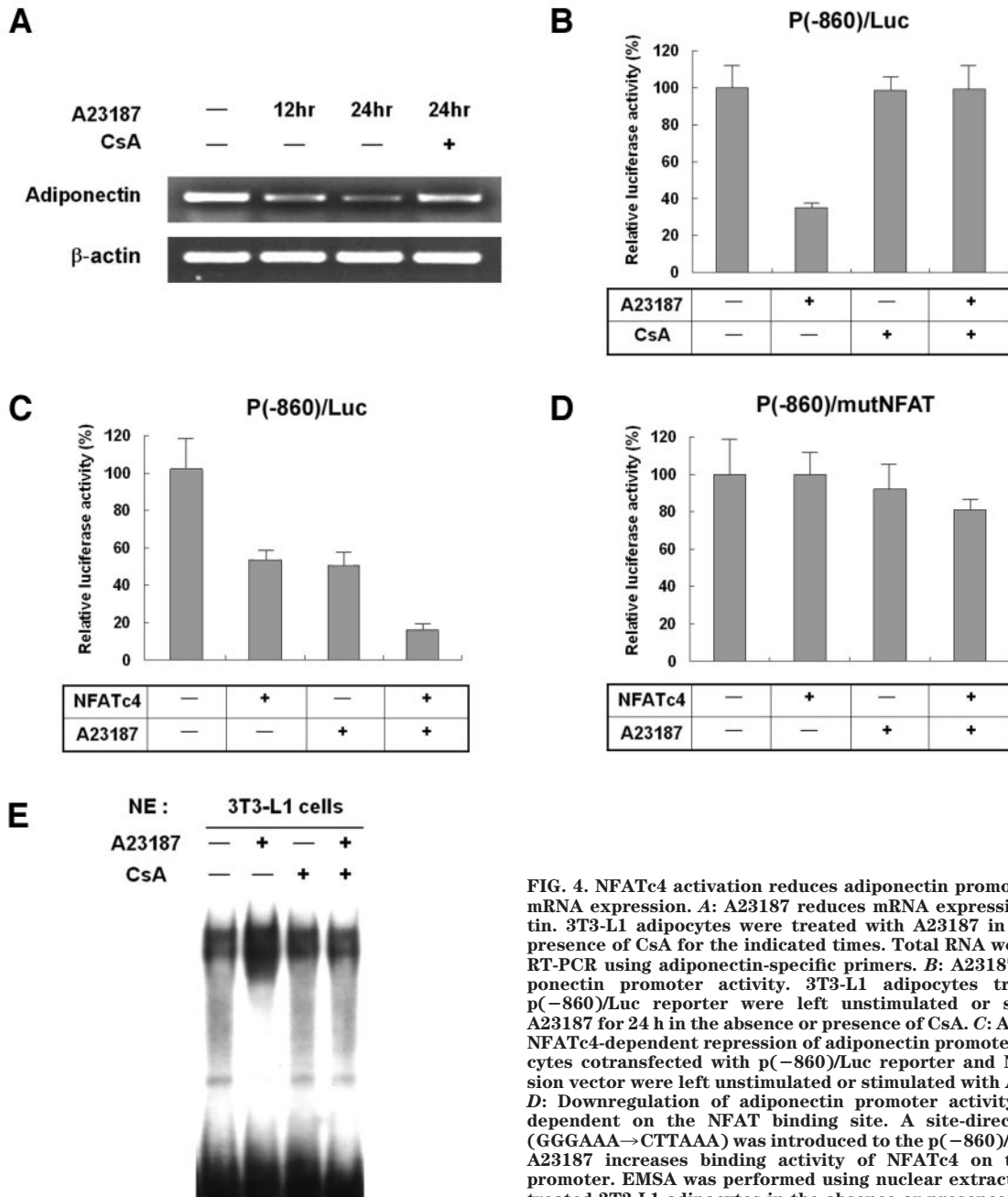


FIG. 4. NFATc4 activation reduces adiponectin promoter activity and mRNA expression. **A:** A23187 reduces mRNA expression of adiponectin. 3T3-L1 adipocytes were treated with A23187 in the absence or presence of CsA for the indicated times. Total RNA were subjected to RT-PCR using adiponectin-specific primers. **B:** A23187 represses adiponectin promoter activity. 3T3-L1 adipocytes transfected with p(-860)/Luc reporter were left unstimulated or stimulated with A23187 for 24 h in the absence or presence of CsA. **C:** A23187 increases NFATc4-dependent repression of adiponectin promoter. 3T3-L1 adipocytes cotransfected with p(-860)/Luc reporter and NFATc4 expression vector were left unstimulated or stimulated with A23187 for 24 h. **D:** Downregulation of adiponectin promoter activity by A23187 is dependent on the NFAT binding site. A site-directed mutation (GGGAAA→CTTAAA) was introduced to the p(-860)/Luc reporter. **E:** A23187 increases binding activity of NFATc4 on the adiponectin promoter. EMSA was performed using nuclear extracts from A23187-treated 3T3-L1 adipocytes in the absence or presence of CsA.

measured by RT-PCR. As shown in Fig. 4A, A23187 significantly reduced mRNA. However, treatment with CsA, an inhibitor of NFAT, reversed the repressive effect of A23187. We next determined whether reductions in adiponectin mRNA by A23187 is correlated with decreased transcriptional activity mediated by the adiponectin promoter. Consistent with decreased mRNA, A23187 also repressed promoter activity of p(-860)/Luc, which was reversed by CsA treatment (Fig. 4B). Moreover, combining overexpression of NFATc4 and A23187 treatment further reduced the promoter activity of p(-860)/Luc (Fig. 4C). However, constructs containing the mutant NFAT binding site diminished the transcriptional repression of the adiponectin promoter from A23187 stimulation (Fig. 4D). These data suggest that NFATc4 activation represses promoter activity dependent on the NFAT binding site. To identify whether A23187-mediated repression of the adi-

ponectin gene is mediated through increased NFATc4 binding to the adiponectin promoter, EMSA was performed using nuclear extracts from A23187-treated 3T3-L1 adipocytes. As shown in Fig. 4E, the NFATc4 binding complex was strongly induced by A23187 treatment and pretreatment with CsA abolished induction of the binding complexes.

NFATc4 interacts with adiponectin promoter in vivo, and NFATc4-mediated repression is associated with recruitment of histone deacetylase activity. To examine binding of NFAT to the adiponectin promoter in vivo, ChIP was performed in 3T3-L1 adipocytes stimulated with A23187 and unstimulated cells. The immunoprecipitated chromatin was amplified by PCR using adiponectin promoter-specific primers (Fig. 5A). As shown in Fig. 5B, the 160-bp PCR product was compared with the NFATc4 antibody, whereas no bands were detected with primers in

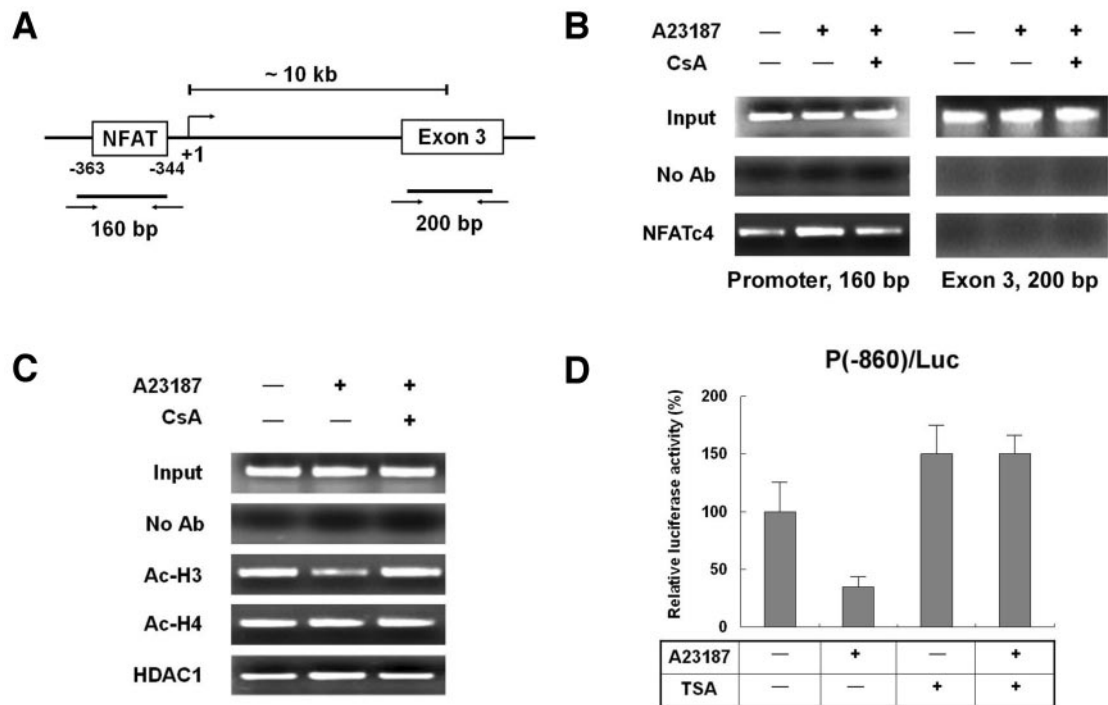


FIG. 5. NFATc4 binds to the adiponectin promoter in vivo, whose binding reduces histone H3 specific-acetylation. **A:** Diagram showing the oligonucleotides used to amplify the adiponectin promoter in the ChIP assay. **B:** ChIP assay was performed with or without antibody against NFATc4. 3T3-L1 adipocytes were incubated with A23187 in the absence or presence of CsA. **C:** A23187 decreases acetylation of histone H3. 3T3-L1 adipocytes were treated with A23187 in the absence or presence of CsA. ChIP assay was performed with or without antibody against acetyl H3, acetyl H4, and HDAC1. **D:** TSA reverses A23187-dependent repression of adiponectin promoter. 3T3-L1 adipocytes transfected with p(-860)/Luc reporter were left unstimulated or stimulated with A23187 for 24 h in the absence or presence of TSA. Luciferase activity is presented relative to unstimulation. The data are represented as means \pm SD of five independent experiments.

exon3, which is located 10 kb downstream of NFAT binding region, indicating that NFATc4 formed a specific complex with the adiponectin promoter in vivo. The intensity of the PCR product was increased with stimulation of A23187 compared with nonstimulation. Moreover, treatment with CsA reversed the stimulated intensity (Fig. 5B), indicating that NFAT activation increased binding of NFATc4 to the promoter in vivo.

Previously, a published report found that NFATc2 repressed CDK4 promoter activity by reducing acetylation of histone H3 through recruitment of a histone deacetylase (HDAC) family member to the CDK promoter (34). Therefore, we investigated whether NFATc4-dependent repression of adiponectin promoter is also regulated by HDAC activity. To this end, we utilized ChIP analysis to examine the status of histone H3 and H4 acetylation of the adiponectin promoter when treated with A23187. As shown in Fig. 5C, A23187 decreased histone H3 acetylation, but left acetylation of histone H4 unchanged. However, the addition of CsA reversed the histone H3 acetylation to levels comparable to unstimulated cells. To characterize the involvement of HDAC in NFATc4-mediated decrease in histone H3 acetylation, recruitment of HDAC1 to the adiponectin promoter was investigated in A23187-stimulated cells by ChIP analysis. As shown in Fig. 5C, A23187 treatment increased recruitment of HDAC1, which was prevented by treatment with CsA. Moreover, to further confirm these findings, the effect of TSA, an irreversible HDAC inhibitor, on A23187-mediated repression of promoter activity was examined. As shown in Fig. 5D, treatment with TSA potentially reversed repression of the promoter by A23187. These results suggest that NFATc4

negatively regulates adiponectin promoter through histone H3-specific deacetylation.

ATF3 also represses transcriptional activity of the adiponectin gene. It has been demonstrated that activated NFAT interacts with other transcription factors such as MEF2, GATA2, GATA4, or, more frequently, with activator protein-1 (AP-1) at composite DNA elements to regulate gene expression (18,35,36). In the adiponectin promoter, a putative AP-1 binding site (TGACTCTC, -376/-369) was found 15 bp apart from the core NFAT binding sequence. To test the functional role of this site, a deletion mutant was generated using the p(-472)/Luc reporter. As shown in Fig. 6A, removal of the putative AP-1 binding site increased promoter activity equivalent to that of the NFAT binding site-deleted mutant, suggesting that the putative AP-1 binding site may also be involved in repressing the adiponectin promoter. Next, we investigated whether AP-1 could directly regulate the adiponectin promoter. When the p(-860)/Luc reporter was cotransfected with the c-jun expression vector into 3T3-L1 adipocyte cells, it did not affect promoter activity (data not shown). Since it had been reported that ATF3, a stress-responsive transcription repressor, can effectively bind to the AP-1 site and repress transcriptional activity (30), we examined whether ATF3 represses adiponectin gene expression. As shown in Fig. 6B, expression of ATF3 repressed the promoter activity of both the p(-860)/Luc and p(-472)/Luc reporters, whereas it did not repress the promoter activity of the p(-312)/Luc reporter missing the AP-1 binding site. These findings demonstrate that ATF3 downregulates the adiponectin promoter. Thapsigargin is a known activator of ATF3 and inducer of endoplasmic reticulum stress. Therefore, we

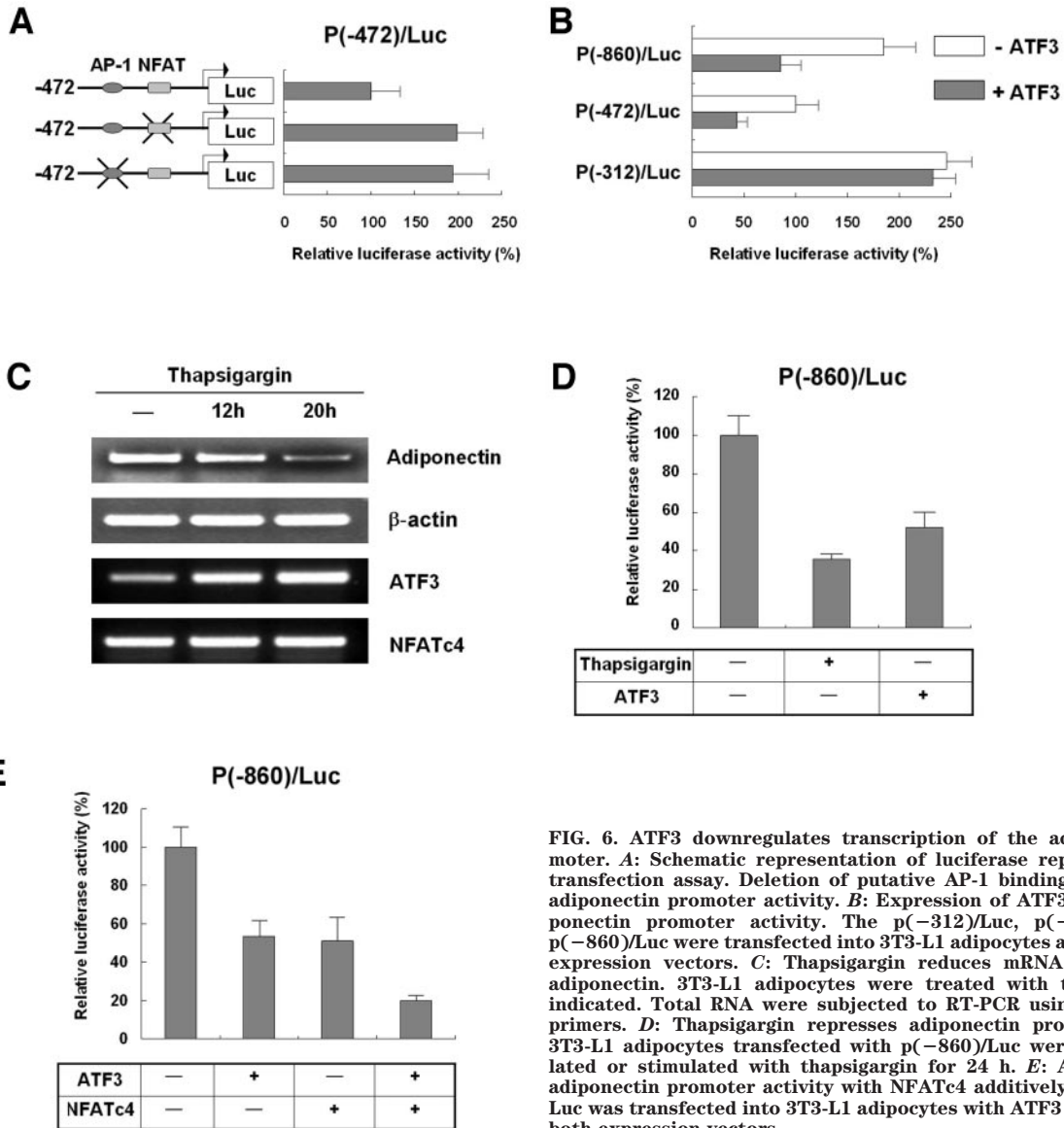


FIG. 6. ATF3 downregulates transcription of the adiponectin promoter. **A:** Schematic representation of luciferase reporters used in transfection assay. Deletion of putative AP-1 binding site increases adiponectin promoter activity. **B:** Expression of ATF3 represses adiponectin promoter activity. The p(-312)/Luc, p(-472)/Luc, and p(-860)/Luc were transfected into 3T3-L1 adipocytes along with ATF3 expression vectors. **C:** Thapsigargin reduces mRNA expression of adiponectin. 3T3-L1 adipocytes were treated with thapsigargin as indicated. Total RNA were subjected to RT-PCR using gene-specific primers. **D:** Thapsigargin represses adiponectin promoter activity. 3T3-L1 adipocytes transfected with p(-860)/Luc were left unstimulated or stimulated with thapsigargin for 24 h. **E:** ATF3 represses adiponectin promoter activity with NFATc4 additively. The p(-860)/Luc was transfected into 3T3-L1 adipocytes with ATF3 or NFATc4 and both expression vectors.

used thapsigargin to investigate whether ATF3 activation represses transcription of the adiponectin gene. As shown in Fig. 6C, treatment of fully differentiated 3T3-L1 cells with thapsigargin significantly reduced adiponectin mRNA with a concomitant increase in ATF3 levels, as well as efficiently repressing the promoter activity of the p(-860)/Luc reporter (Fig. 6D). In combination, NFATc4 and ATF3 additively repressed promoter activity by >80% (Fig. 6E) compared with ATF3 or NFATc4 expression alone.

ATF3 binds to the adiponectin promoter in vitro and in vivo. To demonstrate whether ATF3 binds to the putative AP-1 site of the adiponectin promoter, we performed EMSAs using nuclear extracts from fully differentiated 3T3-L1 cells, mouse white adipose tissue, and ATF3-overexpressing HEK293 cells. Using the labeled oligonucleotide covering the -383/-364-bp region (Fig. 7A), binding complexes were observed in the nuclear extracts (Fig. 7B). Unlabeled ATF/cAMP response element consensus oligonucleotides, as well as homologous oligonucleotides, nearly eliminated the retarded bands, but the unlabeled mutant oligonucleotides exerted no significant effect on binding. To further confirm ATF3 binding, the antibody supershift assay was conducted with the ATF3

antibody. As shown in Fig. 7B, anti-ATF3 inhibited binding of complexes formed in the nuclear extracts, whereas no inhibition was detected with anti-STAT1 (Fig. 7B), indicating that ATF3 specifically binds to the putative AP-1 binding site of adiponectin promoter. To examine binding of ATF3 to the adiponectin promoter in vivo, ChIP was performed. The 160-bp PCR product was produced from the immunoprecipitated chromatin (Fig. 7C, left panel), and the intensity of the PCR product was increased with thapsigargin treatment compared with unstimulated cells (Fig. 7C, left panel). In agreement with this result, thapsigargin treatment increased ATF3 binding activity on adiponectin promoter (Fig. 7C, right panel). Therefore, these results suggest that reduced adiponectin expression by thapsigargin may be mediated through increased ATF3 binding to the adiponectin promoter. Furthermore, since recent studies have reported that TNF- α , whose expression increased in obesity animal models, induces ATF3 in vascular endothelial cells (37), we investigated whether TNF- α also induces ATF3 expression in 3T3-L1 adipocytes. As shown in Fig. 7D, treatment of TNF- α significantly increased ATF3 expression (left panel) and the binding activity on adiponectin promoter (right panel), strongly

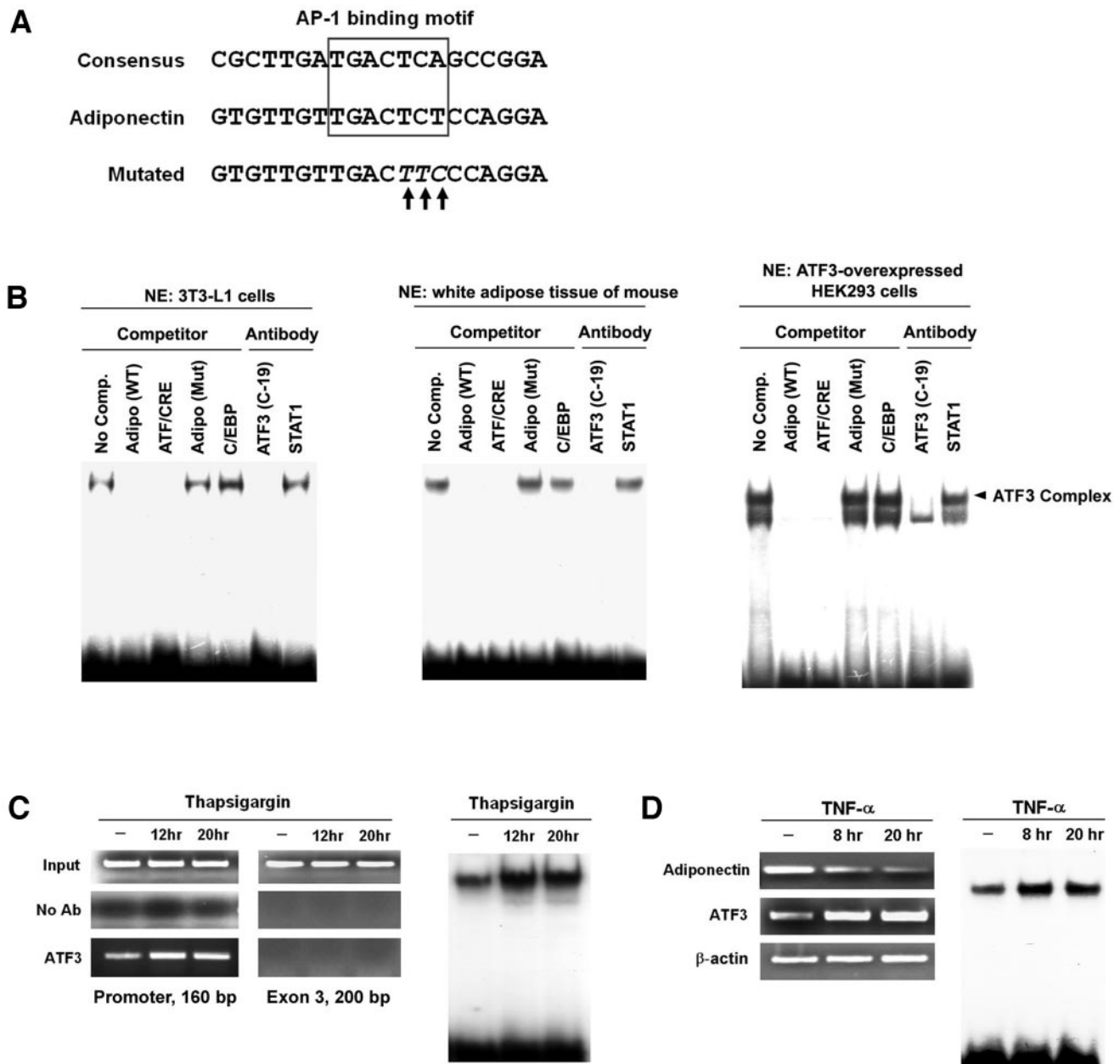


FIG. 7. ATF3 binds to the adiponectin promoter in vitro and in vivo. **A:** Sequence comparison of the putative AP-1 site in the adiponectin promoter with the canonical AP-1 binding site. The AP-1 consensus sequence is boxed. Arrow indicates mutated site. **B:** EMSAs were performed on the putative AP-1 site using nuclear extracts from 3T3-L1 adipocytes (*left panel*), murine white adipose tissue (*middle panel*), and ATF3-overexpressing HEK293 cells (*right panel*). For oligonucleotide competition experiments, a 100-fold excess was used (*lane 1*: no competition; *lane 2*: adiponectin wild AP-1 oligonucleotides; *lane 3*: consensus ATF/cAMP response element oligonucleotides; *lane 4*: adiponectin mutant AP-1 oligonucleotides; *lane 5*: C/EBP oligonucleotides). The protein-DNA complex was diminished by anti ATF-3 (*lane 6*), but not by the nonspecific antibody (*lane 7*). **C:** ChIP assay was performed with or without antibody against ATF3 (*left panel*). The immunoprecipitated DNA fragments from thapsigargin-treated 3T3-L1 adipocytes were amplified by PCR with primers used in Fig. 5. Thapsigargin increases the binding activity of ATF3 on adiponectin promoter (*right panel*). **D:** TNF- α increases the expression of ATF3 (*left panel*) and the binding activity (*right panel*).

suggesting that suppression of adiponectin in obesity by TNF- α may be partly mediated through ATF3.

Increase in binding activities of both NFATc4 and ATF3 to the adiponectin promoter from the nuclear extracts of the white adipocytes of *ob/ob* and *db/db* mice. Since adiponectin expression is significantly reduced in the white adipose tissue of obese and diabetic animal models, we examined the involvement of NFATc4 and ATF3 in the repression of adiponectin expression due to obesity and type 2 diabetes. To this end, we examined the binding activities of NFATc4 and ATF3 in nuclear extracts from the white adipose tissue of *ob/ob* and *db/db*

mice by EMSAs. As shown in Fig. 8A, the binding activities of both NFATc4 and ATF-3 increased significantly in *ob/ob* and *db/db* mice compared with control mice, whereas Sp1 used as a control and binding activity was unchanged in nuclear extracts, suggesting that both NFATc4 and ATF3 may play critical roles in repression of adiponectin expression in obese and diabetic subjects. Moreover, when expression of NFATc4 or ATF3 was examined from white adipose tissue of *ob/ob* or *db/db* mice, ATF3 was drastically upregulated in both types of mice consistent with reductions in adiponectin mRNA, whereas NFATc4 was not significantly changed (Fig. 8B). Therefore, increased ATF3

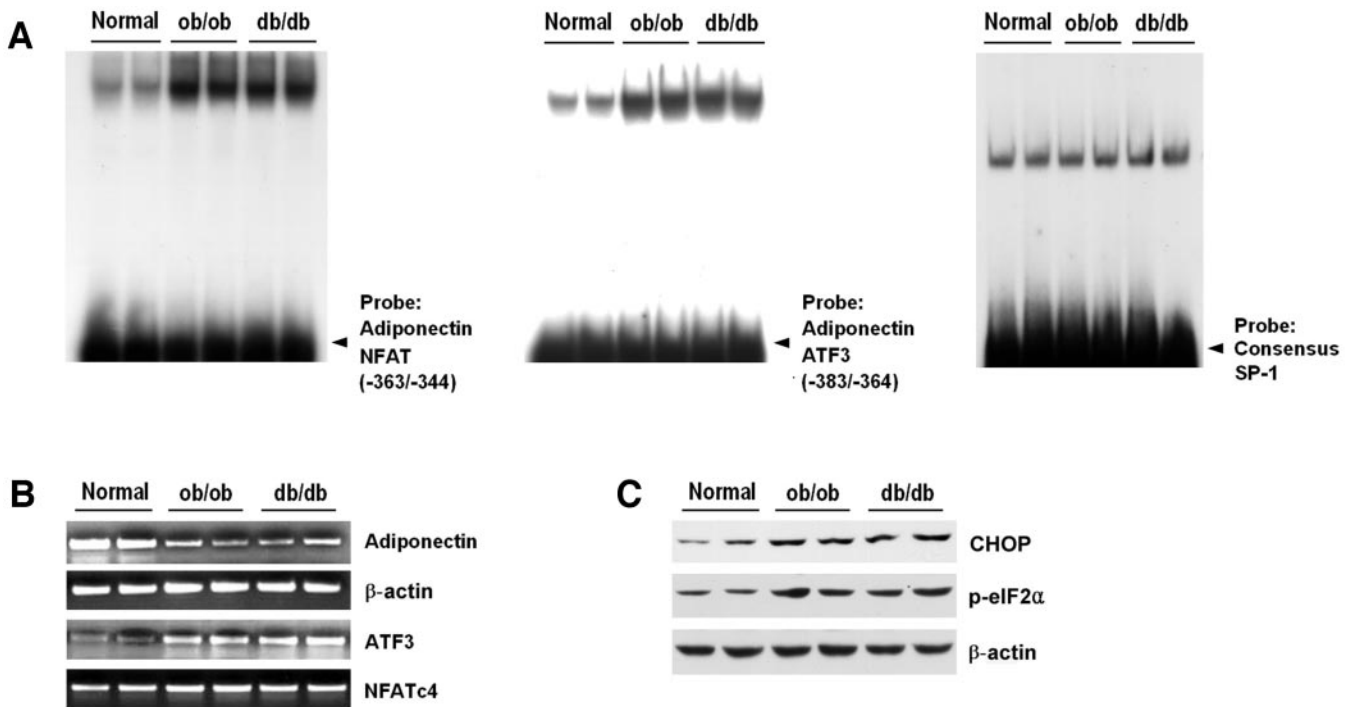


FIG. 8. The binding activities of NFATc4 and ATF3 increase in nuclear extracts from the white adipose tissue of *ob/ob* and *db/db* mice. **A:** Nuclear extracts were prepared from the white adipose tissues of *ob/ob* and *db/db* mice and analyzed by EMSA using probes for NFATc4 (left panel), ATF3 (middle panel), and SP-1 as a control (right panel). **B:** The mRNA expression of ATF3 increases in white adipose tissue of *ob/ob* and *db/db* mice, concomitant with an increase in endoplasmic reticulum stress. Total RNA were isolated from the white adipose tissues of *ob/ob* and *db/db* mice and subjected to RT-PCR using primers for ATF3, NFATc4, and adiponectin. **C:** Endoplasmic reticulum stress increases in white adipose tissue of *ob/ob* and *db/db* mice. Western blotting was performed for the detection of CHOP and eIF2 α phosphorylation.

binding activity in *ob/ob* and *db/db* mice may result from elevated ATF3 expression, whereas NFATc4 may be activated by calcineurin signaling in obesity and type 2 diabetes. Since endoplasmic reticulum stress-inducible ATF3 was upregulated in *ob/ob* and *db/db* mice, we then examined the expression of molecular indicators of endoplasmic reticulum stress in the white adipose tissue of *ob/ob* and *db/db* mice. As shown in Fig. 8C, expression of CHOP and eIF2 α phosphorylation increased in *ob/ob* and *db/db* mice compared with controls, suggesting that induction of endoplasmic reticulum stress in obesity and type 2 diabetes may involve in downregulation of adiponectin expression via ATF3.

DISCUSSION

Even though several positive transcription factors including peroxisome proliferator-activated receptor- γ (12), C/EBP α (13,14), and ADD1/sterol regulatory element-binding protein-1c (15), have been identified as regulators of adiponectin gene expression, there is little information about negative transcription factors, particularly under conditions of obesity, insulin resistance, or type 2 diabetes. Here, we provide evidence that both NFATc4 and ATF3 negatively regulate adiponectin gene expression in 3T3-L1 adipocytes, which may contribute to repression of adiponectin expression in obese and diabetic subjects.

Several lines of evidence indicate that NFAT is involved in stimulating transcription of inducible genes in immune and nonimmune cells, but very recently, a regulatory role for NFAT has been reported where NFATc2 suppresses CDK4 gene expression through binding to a site immediately downstream of the transcriptional start site (34) and expression of cartilage markers, type II, and type X

collagen (38). In this study, we demonstrate that NFATc4 suppresses adiponectin gene expression in 3T3-L1 adipocytes dependent on an NFAT binding site (-363 to -344) in the mouse adiponectin promoter. To understand the mechanism involved in negative regulation of adiponectin expression by NFATc4, we investigated chromatin remodeling-associated factors since chromatin structure remodeling such as acetylation and deacetylation of histone plays a critical role in regulating gene expression. Both histone H3 and H4 acetylation allows transacting factors to associate with cognate DNA binding sites and transcription to occur (39,40). Conversely, HDAC reverses histone acetylation, resulting in a more compact chromatin that is transcriptionally repressive. Our study results show that under conditions that maximize promoter repression by treatment with A23187, reductions in histone H3 acetylation within the adiponectin promoter region were observed together with recruitment of HDAC1. This suggests that increased NFAT binding to the adiponectin promoter reduces H3 acetylation through recruitment of HDAC1. These events are inhibited by the addition of the NFAT inhibitor, CsA. Even though we did not investigate a direct association between HDAC1 and NFATc4, repression of the adiponectin gene expression by NFATc4 may be partly mediated by reduced histone acetylation associated with binding of NFATc4 in mature adipocytes.

Here, we also identified that ATF3 additionally functions as a transcriptional repressor by binding to a putative AP-1 site adjacent to the NFAT binding site of the adiponectin promoter. It has been demonstrated that ATF3 homodimers recognize both the ATF/cAMP response element site and the AP-1 site and plays a transcriptional repressor role. Therefore, ATF3 can repress expression of

itself (41), its inhibitor (gadd153/Chop10) (42), PEPCK (43), and the TNF- α -induced E-selectin gene (44). In the present study, we verified that ATF3 binds to a putative AP-1 site on the adiponectin promoter and that expression of ATF3 significantly reduces promoter activity. Importantly, treatment with thapsigargin, an endoplasmic reticulum stress inducer, reduces adiponectin gene expression together with an increase in ATF3. Therefore, it is suggested that endoplasmic reticulum stress is implicated in downregulating adiponectin expression through induction of ATF3. Studying the cooperation of NFATc4 and ATF3 in regulating adiponectin promoter, both NFATc4 and ATF3 appear to negatively regulate adiponectin gene expression independently. The coexistence of both NFATc4 and ATF3 results in additive repression of adiponectin expression.

The mechanism responsible for the negative regulation of adiponectin in obesity and insulin resistance have not been determined in details so far. As described earlier, increased TNF- α and IL-6 may partially be responsible for decreased adiponectin production in obese subjects (9,10). Based on our present findings that the binding activities of both NFATc4 and ATF3 increases in the nuclear extracts of white adipose tissue of *ob/ob* and *db/db* mice and ATF3 expression also increases in white adipose tissue of *ob/ob* and *db/db* mice, we suggest that NFATc4 and ATF3 play crucial roles in reducing adiponectin gene expression in obesity and type 2 diabetes. Moreover, TNF- α significantly increases ATF3 expression in 3T3-L1 cells with reduction of adiponectin gene expression. Therefore, we also suggest that ATF3 may involve in TNF- α -mediated downregulation of adiponectin gene in the adipocyte under obesity states. Obesity causes endoplasmic reticulum stress, which in turn leads to impaired insulin resistance and type 2 diabetes through suppression of insulin receptor signaling by the hyperactivation of c-Jun NH₂-terminal kinase and subsequent serine phosphorylation of insulin receptor substrate-1 in the liver and adipocytes (45). Since our present study shows that ATF3 induced by endoplasmic reticulum stress represses adiponectin expression, endoplasmic reticulum stress may be responsible for repression of adiponectin gene expression due to obesity and type 2 diabetes via ATF3. Furthermore, this finding strongly supports another role of endoplasmic reticulum stress in causing insulin resistance and type 2 diabetes through downregulation of adiponectin expression in adipocyte.

In summary, this is the first report showing that NFATc4 and ATF3 act as transcriptional repressors in regulating adiponectin gene expression, providing a potential mechanism for downregulating adiponectin expression in obesity and type 2 diabetes, and implicating an additional role of endoplasmic reticulum stress in causing insulin resistance and type 2 diabetes.

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