TFE3 Controls Lipid Metabolism in Adipose Tissue of Male Mice by Suppressing Lipolysis and Thermogenesis


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Transcription factor E3 (TFE3) is a transcription factor that binds to E-box motifs and promotes energy metabolism-related genes. We previously reported that TFE3 directly binds to the insulin receptor substrate-2 promoter in the liver, resulting in increased insulin response. However, the role of TFE3 in other tissues remains unclear. In this study, we generated adipose-specific TFE3 transgenic (aP2-TFE3 Tg) mice. These mice had a higher weight of white adipose tissue (WAT) and brown adipose tissue than wild-type (WT) mice under fasting conditions. Lipase activity in the WAT in these mice was lower than that in the WT mice. The mRNA level of adipose triglyceride lipase (ATGL), the rate-limiting enzyme for adipocyte lipolysis, was significantly decreased in aP2-TFE3 Tg mice. The expression of Foxo1, which directly activates ATGL expression, was also suppressed in transgenic mice. Promoter analysis confirmed that TFE3 suppressed promoter activities of the ATGL gene. In contrast, G0S2 and Perilipin1, which attenuate ATGL activity, were higher in transgenic mice than in WT mice. These results indicated that the decrease in lipase activity in adipose tissues was due to a decrease in ATGL expression and suppression of ATGL activity. We also showed that thermogenesis was suppressed in aP2-TFE3 Tg mice. The decrease in lipolysis in WAT of aP2-TFE3 Tg mice inhibited the supply of fatty acids to brown adipose tissue, resulting in the inhibition of the expression of thermogenesis-related genes such as UCP1. Our data provide new evidence that TFE3 regulates lipid metabolism by controlling the gene expression related to lipolysis and thermogenesis in adipose tissue. (Endocrinology 154: 3577–3588, 2013)

Adipose tissue plays a critical role in regulating energy balance. In addition to being a reserve of fuel in mammals, white adipose tissue (WAT) plays an important role in lipid metabolism, including the storage of triacylglycerol (TAG) for lipolysis to provide fatty acids (FAs) as an energy source for other organs. Brown adipose tissue (BAT) functions in thermogenesis using FAs produced from lipolysis in WAT to activate uncoupling protein (UCP) 1 and mitochondrial β-oxidation (1).

Three enzymes are involved in the complete hydrolysis of TAG molecules in cellular lipid stores. The first and rate-limiting step hydrolyzes TAGs to generate diacylglycerols and nonesterified fatty acids, which are then cat-

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Abbreviations: ADRP/ADFP, adipose differentiation-related protein; aP2, fatty acid-binding protein 4; aP2-TFE3 Tg, adipose-specific TFE3 transgenic; ATG, adipose triglyceride lipase; ATGL KO, ATGL deficient; BAT, brown adipose tissue; C/EBP, CCAAT/enhancer-binding protein; CGI-58, comparative gene identification-58; FA, fatty acid; FAS, fatty acid synthase; FBS, fetal bovine serum; FFA, free fatty acid; Foxo, Forkhead box; HFHS, high-fat, high-sucrose; HG, hormone-sensitive lipase; IRS, insulin receptor substrate; IRS, insulin receptor substrate; PGC-1α, PPAR, peroxisome proliferative-activated receptor, gamma, coactivator 1 alpha; Q-PCR, quantitative PCR; RNAi, RNA interference; RT, room temperature; TAG, triacylglycerol; TFE3, transcription factor E3; UCP, uncoupling protein; WAT, white adipose tissue; WT, wild type.
analyzed by adipose triglyceride lipase (ATGL). The next step is catalyzed by hormone-sensitive lipase (HSL), a multifunctional enzyme that hydrolyzes various acyl esters, including TAG, diacylglycerols, and monoacylglycerol. The last step involves monoglyceride lipase, which cleaves monoacylglycerol into glycerol and nonesterified fatty acids (2).

The physiological functions of ATGL have been investigated in whole-body and adipose-specific knockout (KO) mouse models (3–5). The whole-body ATGL-deficient (ATGL KO) mouse has increased adipose mass and develops TAG deposition in multiple tissues. The mice also accumulate large amounts of lipid in the heart, which causes cardiac dysfunction and premature death (5). The decreased availability of ATGL-derived free fatty acids (FFAs) leads to increases in glucose use and tolerance and increased insulin sensitivity (5). It was recently reported that adipose-specific ATGL-deficient mice possessed adipose lipolysis that was crucial for fasting energy homeostasis (4) and had markedly deficient lipolysis in the fasted state. These mice also had a mild increase in the body fat content, insulin sensitivity, and light-phase hyperphagia. A supply of FFAs from lipolysis is critical for β-oxidation and UCP1 activation for thermogenesis. ATGL KO mice therefore have impaired thermogenesis because of decreased UCP1 expression with lower peroxisome proliferator-activated receptor (PPAR)-α binding to its promoter and subsequent activation (3).

The leucine zipper-containing basic helix loop helix protein transcription factor E3 (TFE3) was originally identified as a transcription factor involved in regulation of the expression of the gene encoding the immunoglobulin heavy chain by binding to the intron enhancer (6). The TFE gene family has also been shown to be involved in the biology of melanocytes and development of their malignancies. In addition to functioning as homodimers, TFEs work as partners of other transcriptional regulators such as E2F, PU.1, Max, and Smads in the regulation of various genes (7–9). Moreover, at least 5 different translocations involving the Xp11.2 chromosomal region reportedly fuse with TFE3 and are predicted to produce ASPL-TFE3, PRCC-TFE3, NonO-TFE3, CLTC-TFE3, and protein-associated splicing factor-TFE3 fusion proteins (10). The involvement of TFE3 in these 5 different gene fusions in renal cell carcinomas is consistent with a central role for TFE3-related transcriptional deregulation in these tumors. TFE3 acts as a rheostat for controlled disequilibration of the regulatory network that establishes ground-state pluripotency in the embryo, thereby sustaining embryonic stem cells (11).

In the immune system, TFE3 plays an important regulatory role in B and T cells. TFE3 and TFEB, one of the TFE3 family molecules, are the direct activators of CD40 ligand expression in activated CD4+ T cells critical for T cell-dependent antibody responses (12). Recently we showed that this transcriptional factor is involved in hepatic glucose metabolism (13). In primary hepatocytes and the mouse liver, in which TFE3 was overexpressed, we observed a significant up-regulation in gene expression of insulin receptor substrate (IRS)-2, Akt/protein kinase B, and hexokinase 2, all of which are involved in insulin signaling or its action. In addition, increased levels of the IRS-2 protein were associated with enhanced phosphorylation of Akt and glycogen synthase kinase-3 and concomitant activation of glycogen synthesis. These changes decreased hyperglycemia in both normal and diabetic mice by enhancing insulin signaling (13). In muscle-specific TFE3 transgenic mice, muscle glycogen stores increased in accordance with regulation of the expression of genes involved in glucose metabolism. Consequently, these mice have high exercise endurance capacity, and after exercise training, they have a greater insulin response than wild-type (WT) mice (14).

However, the functions of TFE3 in adipose tissue remain unclear. In this study, we generated adipose-specific TFE3 transgenic (aP2-TFE3 Tg) mice to investigate the effects of TFE3 on lipid metabolism in adipose tissues.

**Materials and Methods**

**Generation of transgenic mice**

A chimeric gene was constructed that included 5.4 kb of the mouse fatty acid-binding protein 4 (aP2) gene promoter linked to 1.7 kb of mouse TFE3 cDNA (NM_172472) with an hemagglutinin (HA) tag in the N terminus, followed by a polyadenylation signal from the human GH gene. The cDNA of mouse TFE3 was obtained by PCR, as described previously. Constructed transgene fragments were inserted by microinjection into C57BL/6J mouse eggs at the Laboratory Animal Resource Center (University of Tsukuba). We used male mice in all experiments. The mice were maintained on a fixed light/dark cycle and fed a regular chow diet (MF; Oriental Yeast, Tokyo, Japan). All the experiments were performed according to the Guide for the Care and Use of Laboratory Animals of the University of Tsukuba and were approved by the institutional review board.

**Western blotting**

Total cell lysates were prepared from tissues as described previously (13). Anti-TFE3 antibody was purchased from BD Biosciences (Franklin Lakes, New Jersey), anti-α-tubulin antibody from Calbiochem (La Jolla, California), and antiphospho-Akt (Ser473) and Akt antibodies from Cell Signaling Technology (Beverly, Massachusetts). The blots were visualized by ChemiDoc XRS+ (Bio-Rad Laboratories, Hercules California) and quantified by Image Lab Software (Bio-Rad Laboratories).
Metabolic measurements

The plasma levels of glucose, insulin, triglycerides, FFAs, and cholesterol were measured as described previously (13).

Histological analysis

WAT was fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Adipocyte cell size was determined by Image J software (National Institutes of Health, Bethesda, Maryland), with the measurement of at least 300 cells in each sample.

Primary adipocyte cell isolation and culture

Mouse primary adipocytes from WAT were isolated using a modification of the method of Ahmadian et al (3). In brief, WAT was digested for 1 hour at 37°C with collagenase (type II; Sigma, St Louis, Missouri) in Krebs-Ringer buffer (12 mM HEPES, 121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO4, and 0.33 mM CaCl2) supplemented with 0.1% glucose and 1% FA-free BSA, filtered through nylon mesh, and centrifuged, after which adipocytes were collected from the upper phase. The pellet containing the stromal vascular fraction was filtered through a 40-μm cell strainer and centrifuged. Primary adipocytes were cultured in MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. The mouse primary adipocytes were then induced to differentiate into mature adipocytes (15), and after 8 days the cells were stained with Oil Red-O, followed by extraction of the absorbed stain using 100% isopropanol and measurement at 490 nm, as described previously (16).

Analysis of gene expression

Total RNA from the cells and tissues was prepared using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). For real-time PCR analysis, total RNA was used for cDNA synthesis (Invitrogen, Carlsbad, California). Real-time PCR was performed using the ABI Prism 7300 System (Applied Biosystems Inc, Foster City, California) with SYBR Green master mix (Roche, Stock-holm, Sweden). The primer sequences are shown in Supplemental Table 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org. Gene expression was normalized to cyclophilin expression, with the data analyzed using the comparative cycle threshold method.

Adipocyte isolation and in vitro lipolysis

Adipocytes were isolated from the WAT of 8-week-old male WT mice and aP2-TFE3 Tg mice as described by Yang et al (17). Lipolysis was measured in the presence of 10 μM isoproterenol for the times indicated.

In vivo lipolysis assay

Eight-week-old male mice were fasted for 24 hours and then ip injected with isoproterenol (10 mg/kg body weight). Blood was collected from the mice before and 2 hours after the injection. The levels of glycerol and FFAs in plasma were determined as described previously (17).

Plasmid constructs

The expression plasmids TFE3 and Forkhead box O (Foxo)-1 3A (dominant active form of Foxo1; (NM_019739) were con-structed as described previously (13, 18). The ATGL promoter (base pairs −3979 to +81) was subcloned into the pGL4.10 luciferase vector (Promega, Madison, Wisconsin) as described previously (19).

Luciferase analysis

Human embryonic kidney (HEK)-293 cells were grown at 37°C in an atmosphere of 5% CO2 in DMEM (Invitrogen) supplemented with 10% FBS and penicillin-streptomycin. Before transfection, the HEK293 cells were seeded in 24-well plates at a density of 2.5 × 104 cells/well. After adhesion, the cells were transfected with FuGENE 6 (Roche) according to the manufacturer’s protocol. The total amount of DNA was adjusted to 0.3 μg/well in 24-well plates with empty vector DNA. After 24 hours of transfection, the cells were washed with PBS and harvested. Luciferase assays were performed according to the manufacturer’s protocol using the dual-luciferase assay kit (Promega), with luciferase activity being quantified by a Wallac 1420 multilabel counter (PerkinElmer Life Sciences, Boston, Massachusetts). The internal standard, Simian virus 40 Renilla luciferase control vec-tor, was also cotransfected to normalize for transfection efficiency.

3T3-L1 cell culture, adipocyte differentiation, and adenovirus infection

3T3-L1 cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS and penicillin-streptomycin. The cells were cultured to confluence. 3T3-L1 adipocytes were infected with adenoviruses (1000 OPU/cell) encoding LacZ RNA interference [(RNAi); LacZi] or TFE3 RNAi (TFE3i), followed by incubation for 2 days.

Statistical analyses

Statistical significance was calculated by unpaired Student’s t tests, with P < .05 considered significant. All data were expressed as the mean ± SEM.

Results

The expression pattern of TFE3 mRNA in adipose tissues

We reported previously that the expression of TFE3 in mice was high in tissues involved in energy metabolism such as the liver and WAT (13). In this study, we focused on the roles of TFE3 in adipose tissues. First, we checked whether the expression of TFE3 mRNA was nutritionally regulated using quantitative PCR (Q-PCR) analysis to evaluate the level of expression in different nutritional states. The expression levels of TFE3 mRNA in WAT of WT mice were higher in the refed condition than in the fasted condition (Figure 1A). The levels of TFE3 mRNA from ob/ob (leptin deficient) mice were significantly higher than those in the WT mice in both the fasted and refed conditions (Figure 1A). In addition, the expression levels of TFE3 mRNA in WAT of mice fed a high-fat,
high-sucrose (HFHS) diet were up-regulated compared with those in WT mice (Figure 1B). However, there were no apparent differences in the expression levels in the BAT between the fasted and refed conditions (Figure 1C). Levels of TFE3 mRNA in the BAT of ob/ob mice and HFHS-fed mice tended to be increased compared with those in WT mice (Figure 1, D and E). These results suggested that TFE3 mRNA is nutritionally regulated in WAT and that TFE3 may play an important role in lipid metabolism and the development of obesity.

**Generation of aP2-TFE3 Tg mice**

To investigate the roles of TFE3 in adipose tissues, we generated aP2-TFE3 Tg mice using an HA-tagged TFE3 cDNA and an adipose tissue specific promoter/enhancer region of the mouse aP2 gene, which is also called fatty acid-binding protein 4 (Figure 2A). Although aP2 was originally identified as an adipocyte-specific protein, recent studies have shown that aP2 is also expressed in other tissues, including the brain, heart, kidney, spleen, skeletal muscle, and skin (Figure 2C). The increase in TFE3 mRNA was observed in both adipocyte and stromal vascular fraction isolated from WAT (Figure 2C). We also confirmed the TFE3 protein from the transgene in WAT and BAT by Western blotting. The ectopic TFE3 protein was detected at higher levels in the BAT than in WAT, a finding consistent with the levels of TFE3 transgene (Figure 2D). Although Figure 2D shows 2 different sizes of TFE3 proteins, these proteins had similar transcriptional activity as reported previously (13). Histological analysis and cell size measurement revealed that the adipocytes in epididymal WAT from the aP2-TFE3 Tg mice under fasting conditions tended to be slightly larger than those from the WT mice (Figure 2, E and F). However, these differences were not significantly different.

**The phenotypes of the aP2-TFE3 Tg mice**

We investigated the differences between the WT and aP2-TFE3 Tg mice under fasting conditions. At 8 weeks of age, body weight and liver weight were not different between the 2 groups of mice, whereas the weight of WAT and BAT were significant higher in the aP2-TFE3 Tg mice than in the WT mice. Plasma triglyceride contents in the aP2-TFE3 Tg mice during fasting were significantly higher than those in the WT mice, whereas plasma glucose, insulin, and cholesterol levels were similar (Table 1). The levels of plasma FFAs in the aP2-TFE3 Tg mice were marginally higher under fasting conditions, although this difference was not statistically significant (Table 1). The levels of metabolic parameters were not significantly different between the 2 groups of mice under ad libitum conditions (Table 1). These findings indicate that there are differences in lipid metabolism between the WT and aP2-TFE3 Tg mice under fasting conditions.

**Dysregulation of lipolysis in WAT in aP2-TFE3 Tg mice**

Adipose tissue triglyceride metabolism was investigated in the aP2-TFE3 Tg mice based on the difference in plasma triglyceride levels. To study the direct effects of
TFE3 on lipid metabolism, we measured lipolysis activity in WAT by isolating adipocytes from the WT and aP2-TFE3 Tg mice and measuring the release of glycerol and FFAs from these cells for 2 hours after treatment with isoproterenol, which stimulates lipolysis by increasing cellular cAMP levels. Under basal conditions, the rates of glycerol and FFA release were similar in isolated adipocytes from both the WT and aP2-TFE3 Tg mice. Under isoproterenol-stimulated (stimulated) conditions, lipolysis was induced in adipocytes from both mice. However, compared with the WT mice, glycerol and FFA release were significantly lower in isolated adipocytes from the aP2-TFE3 Tg mice (Figure 3A). To further investigate lipolysis activity, the mice were iv injected with isoproterenol in the fasted condition, and plasma glycerol and FFA concentrations measured before (pre) and 2 hours after (post) the injection. Under basal conditions (pre), the amount of glycerol and FFAs released was not different in the WT and aP2-TFE3 Tg mice. However, the levels of plasma FFAs in the aP2-TFE3 Tg mice after the injection of isoproterenol were lower than those in the WT mice, whereas the levels of plasma glycerol remained unchanged (Figure 3B). These results suggested that lipolysis activity of WAT was suppressed in the aP2-TFE3 Tg mice.

**Gene expression in WAT of aP2-TFE3 Tg mice**

Because the aP2-TFE3 Tg mice had defects in lipolysis in WAT, we hypothesized that TFE3 may suppress the expression of lipases, including ATGL and HSL, in these mice. We determined gene expression related to lipolysis in WAT of the WT and aP2-TFE3 Tg mice using Q-PCR. In the fasted state, ATGL mRNA in WAT in the aP2-TFE3 Tg mice was significantly down-regulated compared with that in the WT mice (Figure 4A). Previous studies (3, 28) showed ATGL expression was regulated by Foxo1 and interferon regulatory factor 4 (IRF4). Foxo1 mRNA in WAT from the aP2-TFE3 Tg mice was down-regulated significantly compared with that in WAT from the WT mice. The expression of IRF4 mRNA also tended to be reduced in the aP2-TFE3 Tg mice compared with that in the WT mice, although this difference was not statistically significant.

In addition, mRNA levels of Perilipin1 and G0/G1 switch 2 (G0S2), which inactivate ATGL and HSL, were up-regulated in the aP2-TFE3 Tg mice. The expression of comparative gene identification-58 (CGI-58), which activates lipolysis, did not differ in the 2 strains of mice. Taken together, these results suggest that the suppression of lipolysis in the aP2-TFE3 Tg mice depends on the suppression of both ATGL expression and its enzymatic activation. As shown in Figure 4B, thermogenesis genes such as UCP1 and UCP2 were reduced significantly in the aP2-TFE3 Tg mice, whereas UCP3 and PPARa remained unchanged in both strains of mice. Peroxisome proliferative-activated receptor gamma coactivator 1 alpha (PGC-1α) mRNA tended to decrease in the aP2-TFE3 Tg mice, although this change was not statistically signif-
significant. We next checked the expression of adipogenic and lipogenic genes in WAT in the fasted state, including PPARγ, fatty acid synthase (FAS), CCAAT/enhancer-binding protein-α (C/EBP)-α, C/EBPβ, diacylglycerol O-acyltransferase-1, and adipose differentiation-related protein (ADFP; also known as ADRP). There were no significant differences in the expression levels of FAS, C/EBPβ, and diacylglycerol O-acyltransferase-1 between the WT and aP2-TFE3 Tg mice (Figure 4C). However, the levels of PPARγ and C/EBPα mRNA tended to increase in the aP2-TFE3 Tg mice, whereas those of ADFP tended to decrease, although not significantly (Figure 4C).

We also investigated whether there were differences in adipocyte differentiation using primary adipocytes isolated from the mice. After undergoing differentiation into mature adipocytes, we stained the cells with Oil Red-O to confirm the accumulation of triglyceride and adipose differentiation. We observed no apparent morphological differences in adipocyte differentiation between the WT and aP2-TFE3 Tg mice (Figure 4D, left panel). Quantification of Oil Red-O staining in those cells revealed that the accumulation of triglycerides was not different between the WT and aP2-TFE3 Tg mice (Figure 4D, right panel). However, expression of the PPARγ and ADFP genes was significantly increased in isolated adipocytes from the aP2-TFE3 Tg mice, whereas the expression of the other genes was not different (Figure 4E). These results

### Table 1. Metabolic Parameters in 8-Week-Old Male Mice in 24 Hour-Fasted and Fed ad Libitum Conditions

<table>
<thead>
<tr>
<th></th>
<th>Fasted</th>
<th>Tg</th>
<th>Fed</th>
<th>Tg</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>22.4 ± 0.4</td>
<td>21.2 ± 0.36</td>
<td>ns</td>
<td>23.3 ± 0.37</td>
<td>22.51 ± 0.37</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>0.828 ± 0.02</td>
<td>0.818 ± 0.03</td>
<td>ns</td>
<td>1.059 ± 0.02</td>
<td>1.047 ± 0.004</td>
</tr>
<tr>
<td>WAT weight, g</td>
<td>0.234 ± 0.03</td>
<td>0.29 ± 0.02</td>
<td>P &lt; 0.05</td>
<td>0.298 ± 0.012</td>
<td>0.336 ± 0.023</td>
</tr>
<tr>
<td>BAT weight, g</td>
<td>0.047 ± 0.006</td>
<td>0.066 ± 0.004</td>
<td>P &lt; 0.01</td>
<td>0.069 ± 0.004</td>
<td>0.07 ± 0.004</td>
</tr>
<tr>
<td>Plasma glucose, mg/dL</td>
<td>93.2 ± 3.2</td>
<td>102.6 ± 5.3</td>
<td>ns</td>
<td>179.9 ± 6.4</td>
<td>186.5 ± 10.2</td>
</tr>
<tr>
<td>Plasma insulin, pg/mL</td>
<td>481.2 ± 128.2</td>
<td>373.7 ± 206.1</td>
<td>ns</td>
<td>862.1 ± 131.5</td>
<td>864.7 ± 55.5</td>
</tr>
<tr>
<td>Plasma TG, mg/dL</td>
<td>99.4 ± 12.2</td>
<td>149.9 ± 14.6</td>
<td>P &lt; 0.05</td>
<td>85.2 ± 8.6</td>
<td>80.8 ± 4.8</td>
</tr>
<tr>
<td>Plasma TC, mg/dL</td>
<td>73.4 ± 4.2</td>
<td>76.4 ± 6.1</td>
<td>ns</td>
<td>67.2 ± 3.8</td>
<td>75 ± 5.6</td>
</tr>
<tr>
<td>Plasma FFAs, mEq/L</td>
<td>0.73 ± 0.05</td>
<td>0.81 ± 0.1</td>
<td>ns</td>
<td>0.45 ± 0.02</td>
<td>0.429 ± 0.04</td>
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</table>

Abbreviations: ns, not significant; TC, total cholesterol; TG, triglyceride; Tg, aP2-TFE3 transgenic mice. Data represent means ± SEM: n = 4–11 in each group.
indicated that TFE3 suppressed lipolysis, whereas the effects of TFE3 on adipogenesis and lipogenesis in WAT were restrictive.

**TFE3 has no effect of insulin signaling in WAT in the fasted condition**

Insulin inhibits the expression of ATGL in adipocytes by restraining the nuclear localization of Foxo1; therefore, insulin signaling is important for regulating the expression of ATGL (29). We previously reported that TFE3 up-regulated the expression of IRS-2 and activated insulin signaling in the liver from mice overexpressing TFE3 (13). We determined the expression of IRS-2 and phosphorylated Akt level in WAT from the aP2-TFE3 Tg mice. The expression of IRS-2 tended to be lower in the aP2-TFE3 Tg mice than in the WT mice, although this difference was not significant (Figure 5A). Akt is the downstream protein of IRS-2 and is phosphorylated in response to insulin. The levels of phosphorylated Akt protein were similar in WAT from both the WT and aP2-TFE3 Tg mice (Figure 5B). These findings indicated that TFE3 did not affect insulin signaling in WAT under fasting conditions.

**TFE3 suppresses ATGL promoter activity**

As shown in Figure 4A, the gene expression of ATGL was significantly lower in the aP2-TFE3 Tg mice than in the WT mice. To investigate the role of TFE3 as a regulator of ATGL promoter activity, we tested whether TFE3 affected the ATGL promoter in HEK293 cells using a luciferase reporter gene linked to the mouse ATGL promoter. The transfection of TFE3 suppressed basal ATGL promoter activity, indicating that TFE3 represses ATGL at the transcriptional level (Figure 6A). A previous report showed that Foxo1 directly bound to the promoter region of ATGL via the insulin response element sequence (29). Using the active mutant of Foxo1, Foxo1 3A, we con-
firmed there was a relationship between TFE3 and Foxo1. Foxo1 3A has 3 Akt-mediated serine-phosphorylation sites, which substitute for alanine, and it is resistant to nuclear exclusion by Akt and is therefore constitutively active (18). Foxo1-mediated activation of the ATGL promoter was enhanced by this mutation but was still completely suppressed by the coexpression of TFE3 (Figure 6A). As shown in Figure 6B, ATGL promoter activity induced by Foxo1 3A was repressed by TFE3 in a dose-dependent manner. To confirm the results of luciferase analysis, we next performed knockdown analysis for TFE3 in 3T3-L1 preadipocytes. When 3T3-L1 adipocytes were infected with adenoviruses encoding short hairpin RNA against TFE3 (TFE3i), TFE3i significantly activated ATGL expression compared with LacZi (Figure 6C). These findings raised the possibility that the inhibitory action of TFE3 on ATGL expression was the result of transcriptional repression mediated by interference with Foxo1 activation.

**TFE3 suppresses thermogenesis in BAT**

Cold exposure induces thermogenesis in BAT, thereby maintaining body temperature. We examined cold-induced thermogenesis in the WT and aP2-TFE3 mice by measuring the body temperature before and after cold exposure (4°C for 4 hours). At room temperature (RT), both strains of mice had similar body temperatures (data not shown), whereas exposure to 4°C for 4 hours caused a lower body temperature in the aP2-TFE3 Tg mice compared with that in the WT mice. This indicated that the rate of decrease in temperature was greater in the aP2-TFE3 Tg mice (Figure 7A). ATGL and Foxo1 expression in BAT was significantly lower in the aP2-TFE3 Tg mice than in the WT mice, similar to the gene expression pattern observed in WAT (Figure 7B). We next investigated the expression of genes related to thermogenesis in BAT of the WT and aP2-TFE3 Tg mice. At RT in the fasted state, the expression of UCP1 and UCP2, the genes responsible for thermogenesis, was significantly decreased in BAT from the aP2-TFE3 Tg mice (Figure 7C). The activator for these genes, PPARα, was apparently increased. Consistent with the finding at RT, the aP2-TFE3 Tg mice showed a decrease in expression of the thermogenesis genes, UCP1, UCP2, and UCP3, and increased levels of the activators of this expression, PPARα and PGC-1α (Figure 7D). These
results indicated that the aP2-TFE3 Tg mice have impaired adaptation to cold exposure because of down-regulation of the expression of thermogenesis-related genes such as UCP1 and UCP2.

Discussion

Although TFE3 is ubiquitously expressed in tissue, its roles in regulating genes involved in metabolism are largely unknown. To date, we had proposed that TFE3 has important roles in energy homeostasis in the liver and skeletal muscle (13, 14). The expression of TFE3 is known to change in response to nutrient intake. However, the function of TFE3 in adipose tissues remains unclear. In this study, we revealed that TFE3 inhibited lipolysis in WAT by decreasing the gene expression of ATGL, the rate-limiting enzyme for lipolysis. We also showed TFE3 was involved in cold intolerance by decreasing the expression of the thermogenic genes UCP1 and UCP2 in BAT. Taken together these results indicate that TFE3 plays a crucial role in lipid metabolism in adipose tissues.

To examine the function of TFE3 to modulate lipid metabolism and energy expenditure in adipose tissues, including WAT and BAT of mice, we generated an adipose-specific TFE3 transgenic mouse. The weight of WAT and BAT in the aP2-TFE3 Tg mice in the fasted state was slightly but significantly increased compared with that in WT mice. Overexpression of TFE3 in the transgenic mice also increased the fasting plasma triglyceride concentration compared with that in the WT mice. These results indicated that TFE3 affected lipid metabolism in adipose tissues. Adipocyte lipolysis produces FAs as an energy source during fasting, leading us to hypothesize that TFE3 may affect lipolysis in WAT. Lipolysis activity was markedly lower in the aP2-TFE3 Tg mice than in the WT mice in both in vitro and in vivo.

β-Adrenergic signaling-stimulated lipolysis was also significantly decreased in adipocytes from the aP2-TFE3 Tg mice. Lipolysis is catalyzed by 2 lipases, including ATGL and HSL. HSL knockout mice are not obese (30, 31). Unlike HSL knockout mice, ATGL KO mice have increased deposition of TG and exhibit mild obesity (5). The whole-body and adipose-specific ATGL KO mice had markedly decreased lipolysis, indicating that ATGL plays an important role in lipolysis in WAT (4, 5). Overexpression of TFE3 in WAT also impaired the gene expression of ATGL and lipolysis under fasting conditions. There is also evidence that Foxo1 and PPARγ increase ATGL mRNA at the transcription level (29, 32), and it was recently demonstrated that IRF4 directly activates the ATGL promoter by up-regulating its expression (28). We showed that this expression of Foxo1 and IRF4 was suppressed in WAT of aP2-TFE3 Tg mice. These findings explain the decrease in ATGL mRNA in WAT of the aP2-TFE3 Tg mice. We then verified the direct effects of TFE3 on ATGL expression. We have previously reported that TFE3 activates IRS-2 expression, resulting in the activation of insulin signaling in the liver (13). However, in contrast to these findings, TFE3 did not affect these activations in WAT (Figure 5). Therefore, TFE3 could not affect the transcriptional activity of Foxo1 regulated by insulin signaling in WAT. In luciferase analysis, TFE3 caused a significant suppression of both basal and Foxo1-induced ATGL promoter activity (Figure 6). In addition, TFE3 also down-regulated Foxo1 promoter activity in luciferase analysis (Supplemental Figure 1). These results indicated that TFE3 has a direct effect on ATGL promoter activity.

Figure 7. aP2-TFE3 Tg mice were intolerant to cold exposure. A, Eight-week-old male WT and aP2-TFE3 Tg mice were exposed to a cold temperature (4°C) for 4 hours after 17 hours of fasting. Changes in the body temperature were compared before and after cold exposure in the 2 strains of mice (n = 4). B–D, The expression of lipolysis- (B) and thermogenic (C)-related genes in BAT of 8-week-old male WT and aP2-TFE3 Tg mice after 17 hours of fasting (n = 4) (B and C), followed by cold exposure for 4 hours (n = 4) (D). The results are expressed as means ± SEM. *, P < .05 vs WT mice.
and an indirect effect via a decrease in Foxo1 expression. However, there was no evidence that there are cis elements of TFE3 in the promoter regions of both the ATGL and Foxo1 genes.

Several other factors have also been shown to regulate lipolysis, including CGI-58 (33), Perilipin1 (34), and G0S2 (17). Overexpression of Perilipin1 was shown to suppress lipolysis activity of ATGL (34), whereas G0S2 was reported to bind directly to ATGL, thereby inhibiting ATGL-mediated lipolysis by suppressing its TAG hydro-lase activity (17). Although CGI-58 also binds to ATGL and activates lipolysis, it has been shown that G0S2 inhibits ATGL activity, even in the presence of CGI-58 (17). This indicates that G0S2 is more important than CGI-58 for regulating ATGL activity. The combined marked increase in gene expression of G0S2 and Perilipin1 that we observed in the WAT of the aP2-TFE3 Tg mice resulted in the down-regulation of ATGL enzymatic activity. These findings suggested that the decrease in lipolysis activity in WAT of aP2-TFE3 Tg mice was caused by a decrease in both ATGL gene expression and enzymatic activity.

Despite the decrease in lipolysis in the WAT of aP2-TFE3 Tg mice, plasma triglyceride and FFA levels were not decreased. Plasma FA levels represent a balance between liberation from adipose tissue and uptake by peripheral tissues. Therefore, some factors play a role in regulating the plasma lipid concentration. In fact, some studies have reported that the relationship between lipolysis and plasma lipid contents shows discrepancies in some genetically modified mice. For example, in the transcriptional regulator interacting with the PHD-bromodomain 2 (TRIP-Br2) KO mice, the plasma FFA concentration did not change despite elevated lipolysis activity in WAT (35).

In addition, the aP2-ATGL Tg mice showed the same phenotypes as the TRIP-Br2 KO mice (36). Taken together, we hypothesized that the transgene is expressed in the WAT and other tissues in the aP2-TFE3 Tg mice; therefore, the transgene affected lipid metabolism including lipolysis not only in WAT but also in nonadipose tissues, leading to the unexpected results in plasma lipid concentrations observed in this study.

In addition to focusing on the functions of TFE3 in WAT, we also examined its effect in BAT. The aP2-TFE3 Tg mice were found to express the transgene in BAT as well as WAT. These mice could not maintain the body temperature during cold exposure in the fasted state (Figure 7A). Lipolysis by ATGL generates essential mediators involved in the generation of lipid ligands for PPARα activation (37). We hypothesized that cold intolerance in the aP2-TFE3 Tg mice was due to the decreased availability of FAs from WAT stores that prevented BAT function by diminishing FAs as a substrate for thermogenesis and PPARα ligands. PPARα directly regulates UCP1 gene expression, whereas PPARα/PGC-1α coordinately regulates the control of thermogenic and lipid oxidation pathways in BAT (38). ATGL KO mice reportedly have significant decreases in UCP1 gene expression in the liver and muscle, with the pattern of PGC-1α expression being different in these tissues (37, 39). Despite the levels of PGC-1α mRNA in BAT being higher in our aP2-TFE3 Tg mice than in WT mice in response to cold exposure, the levels of UCP1 were shown to be down-regulated. Consistent with the gene expression in WAT, ATGL and Foxo1 were decreased in BAT of aP2-TFE3 Tg mice (Figures 4A and 7B).

A previous report also showed that adipose-specific ATGL KO mice have a similar thermogenic phenotype to PPARα null mice. PPARα null mice are unable to maintain their body temperature during cold exposure (3). The supply of FAs by ATGL-catalyzed lipolysis activates PPARα to promote transcriptional activity and thermogenesis (3). The supply of FAs from both WAT to BAT and inside BAT itself was therefore suppressed in the aP2-TFE3 Tg mice, resulting in the inactivation of thermogenesis in BAT. Moreover, in the aP2-TFE3 Tg mice, the expression of thermogenic genes such as UCP1 and UCP2 was suppressed significantly in WAT and BAT at both RT and during cold exposure (Figures 4B, 7C, and 7D). Down-regulation of these genes may have TFE3-specific effects in BAT. These results therefore explain, in part, the mechanism underlying the reduction in body temperature observed in the aP2-TFE3 Tg mice. However, there is no evidence to date that the promoter regions of thermogenesis-related genes have cis elements, which may bind to TFE3. It is therefore necessary to determine the mechanism by which TFE3 regulates gene expression by acting as a transcription factor.

A recent clinical study showed that increased ATGL activity may contribute to elevated lipolysis and circulating FFA levels, leading to metabolic dysregulation in patients with type 2 diabetes (40). There is also evidence of decreases in mRNA and protein levels of Perilipin1 and G0S2 and increases in the ATGL protein in adipose tissues from patients with poorly controlled type 2 diabetes (40). TFE3 inhibits ATGL activity in adipose tissues by suppressing its expression and regulating the expression of regulatory molecules, including Perilipin1 and G0S2. Therefore, we consider that TFE3 has the potential to improve the metabolic syndrome by restoring insulin sensitivity as a consequence of inhibiting the synthesis of excess FAs in WAT.

Acknowledgments

We thank Enago for the English language review.

Author contributions are as follows: Y.N. and H.Sh. wrote
the manuscript; Y.F., A.Sa., K.O., A.Sh., A.N., S.Yo., and T.I. performed the experiments; Y.F., Y.N. and T.M. analyzed the data; H.I., K.K., N.Yah., M.S., S.Ya., H.Su., H.So., O.U., and N.Yam. interpreted the results of the experiments; and Y.F., Y.N., and K.O. prepared the figures.

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This work was supported by Grants-in-Aid from the Japanese Ministry of Science, Education, Culture, and Technology (to Y.N., H.Sh.), a grant from Takeda Science Foundation from (to Y.N.), a grant from the Novartis Foundation (Japan) for the Promotion of Science (to Y.N.), a grant from the Kowa Life Science Foundation (to Y.N.), a grant from Mochida Memorial Foundation for Medical and Pharmaceutical Research (to Y.N.), a grant from The Ichiro Kanehara Foundation for the Promotion of Science Foundation (to Y.N.), a grant from The Sumitomo Foundation (to Y.N.), and a grant from the Novartis Foundation (Japan) for the Promotion of Science (to Y.N.).

This work was supported by Grants-in-Aid for Scientific Research (C) (29590087) (to Y.N.), a grant from The Ichiro Kanehara Foundation for the Promotion of Science (to Y.N.), a grant from The Sumitomo Foundation (to Y.N.), and a grant from The Takeda Science Foundation (to Y.N.).

This work was supported by Grants-in-Aid from the Japanese Ministry of Science, Education, Culture, and Technology (to Y.N., H.Sh.), a grant from Takeda Science Foundation from (to Y.N.), a grant from the Novartis Foundation (Japan) for the Promotion of Science (to Y.N.), a grant from the Kowa Life Science Foundation (to Y.N.), a grant from Mochida Memorial Foundation for Medical and Pharmaceutical Research (to Y.N.), a grant from The Ichiro Kanehara Foundation for the Promotion of Medical Sciences and Medical Care (to Y.N.), a grant from the Astellas Foundation for Research on Metabolic Disorders (to Y.N.), and a grant from The Sumitomo Foundation (to Y.N.).

Disclosure Summary: The authors have nothing to disclose.

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