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RESEARCH ARTICLE

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The transcriptional regulator Zfat is essential for maintenance and differentiation of the adipocytes

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Abstract

Adipocytes play crucial roles in the control of whole-body energy homeostasis. Differentiation and functions of the adipocytes are regulated by various transcription factors. Zfat (zinc-finger protein with AT-hook) is a transcriptional regulator that controls messenger RNA expression of specific genes through binding to their transcription start sites. Here we report important roles of Zfat in the adipocytes. We establish inducible *Zfat*-knockout (Zfat iKO) mice where treatment with tamoxifen causes a marked reduction in Zfat expression in various tissues. Tamoxifen treatment of Zfat iKO mice reduces the white adipose tissues (WATs) mass, accompanied by the decreased triglyceride levels. Zfat is expressed in both the adipose-derived stem cells (ADSCs) and mature adipocytes in the WATs. In ex vivo assays of the mature adipocytes differentiated from the Zfat iKO ADSCs, loss of Zfat in the mature adipocytes reduces the triglyceride levels, suggesting cell autonomous roles of Zfat in the maintenance of the mature adipocytes. Furthermore, we identify the *Atg13, Brf1, Psmc3*, and *Timm22* genes as Zfat-target genes in the mature adipocytes. In contrast, loss of Zfat in the ADSCs impairs adipocyte differentiation with the decreased expression of C/EBPα and adiponectin. Thus, we propose that Zfat plays crucial roles in maintenance and differentiation of the adipocytes.

KEYWORDS

adipocyte, CCAAT-enhancer-binding protein (C/EBP), lipid metabolism, transcription regulation, transgenic mice, Zfat

1 | INTRODUCTION

Adipocytes serve the primary function of lipid storage in the form of triglyceride (TG) in the fed state, with a release of fatty acids from the breakdown of TG into the circulation in the fasting state. In addition to the lipid metabolism, the adipocytes are important to maintain whole-body energy homeostasis through the secretion of various adipokines. The increases in size and number of the adipocytes are major contributors to the development of obesity and obesity-related complications, such as type 2 diabetes mellitus (T2DM), hypertension, hyperlipidemia,

and arteriosclerosis. Therefore, differentiation and functions of the adipocytes are tightly regulated by various transcription factors, including the CCAAT/enhancer-binding protein (C/EBP) family members (C/EBPα, C/EBPβ, and C/EBPδ) and the peroxisome proliferator-activated receptor γ (PPARγ).[1]

Zinc-finger protein with AT-hook (Zfat) is a nuclear protein harboring one AT-hook motif and 18 zinc-finger domains.[2,3] The amino acid sequences of Zfat are evolutionally highly conserved from fish to humans, suggesting crucial roles of Zfat in vertebrates.[4] Zfat controls the messenger RNA (mRNA) transcription of specific genes through binding to the proximal region of their transcription start sites.[5] Furthermore, Zfat also regulates noncoding RNA transcription at the centromeres through histone acetylation.[6] We have established several transgenic mice of the *Zfat* gene. Homozygous *Zfat*-knockout mice exhibit embryonic lethality by embryonic day 8.5.[7] Furthermore, T-cell specific deletion of the *Zfat* gene in mice results in a marked reduction in the number of T cells.[8-10] Therefore, Zfat has been thought to be an essential molecule for embryonic development and T-cell homeostasis.

On the other hand, genetic variants of *Zfat* have been reported to be associated with various human diseases, including autoimmune thyroid diseases,[2,11] cerebral aneurysms,[12] hypertension,[13] and cancer.[14] Furthermore, a recent genome-wide association study showed that genetic variations of the *Zfat* gene were associated with the onset of T2DM.[15] Indeed, the *Zfat* gene locus showed 10^3-fold higher *p* value in the association with T2DM than the *TCF7L2* gene that is known to be the strongest susceptibility gene of T2DM, suggesting the possibility that Zfat may play an important role in the onset of T2DM.[15] We recently reported that Zfat is widely expressed in various cells and tissues using the *ZsGreen* reporter gene knock-in mice.[16] However, roles of Zfat in these tissues and cells remain unexplored. Here we report creation of inducible *Zfat*-knockout mice and important roles of Zfat in the adipocytes.

2 | MATERIALS AND METHODS

2.1 | Mice

Zfat$^{flox/flox}$ mice, described in Reference [10], were crossed with *ROSA26-CreERT2* mice (Taconic) to generate *CreERT2-Zfat*$^{flox/flox}$ mice. *CreERT2-Zfat*$^{flox/flox}$ mice were further crossed with *Zfat*$^{WT/ZsGreen}$ mice, described in Reference [16], to generate *CreERT2-Zfat*$^{flox/WT}$ (Control) and *CreERT2-Zfat*$^{flox/ZsGreen}$ (Zfat iKO) mice. To delete the *Zfat* gene in these mouse lines, 6–7-week-old mice were intraperitoneally injected daily with tamoxifen (0.1 mg/g of body weight) dissolved in sunflower oil for 7 consecutive days, and killed at 48 h after the final administration.

Mice were housed on a 12 h light/dark cycle at 23°C with free access to water and food. To determine food intake, mice were housed individually. Food intake was measured manually by weighing of preweighed food pellets every 24 h. Core body temperature was measured with a microcomputer thermometer (BAT-7001H, Physitemp). To measure organ weight, age-matched Control and Zfat iKO mice were killed, and tissues were dissected and weighed.

All animal experiments were performed under the Institutional Animal Care and Use Committee of Fukuoka University-approved guidelines in accordance with approved protocols.

2.2 | Lysate preparation from tissues and cells, and immunoblotting analysis

The lymph nodes were removed from the inguinal white adipose tissues (WATs) before lysate preparation. Tissues minced with scissors and cells were sonicated using a Bioruptor (Cosmo Bio) for two cycles of 1 min with 30 s on/off in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1% Triton X-100) supplemented with complete ethylenediaminetetraacetic acid-free protease inhibitors (1183617001, Roche), and then incubated for 30 min at 4°C. Cell pellets were removed by centrifugation and then the supernatants were mixed with Laemmli sample buffer. Equal amounts of protein were resolved via sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (GE Healthcare). Immunoblotting was performed as described previously,[17,18] with antibodies against, β-actin (A2066, Sigma), Armc8 (ab108158, Abcam), Atg13 (#13273, Cell Signaling Technologies), Brf1 (#2119, Cell Signaling Technologies), Brpf1 (Invitrogen, PA5-27783), C/EBPα (SC-61, SantaCruz), Harbi1 (SAB1306206, Sigma), Ppp1r37 (ab107843, Abcam), Psmc3 (13923, Cell Signaling Technologies), Rad51ap1 (11255-1-AP, Proteintech), Timm22 (ab167423, Abcam), and Zfat.[4] The primary antibodies were dissolved in Can Get Signal Solution 1 (NKB-201, Toyobo).

2.3 | Histological analysis

The inguinal WATs were fixed with 10% formalin for 3 days, and then embedded in paraffin. The embedded

samples were cut into 5 μm sections, and stained with hematoxylin and eosin for morphological analysis. Pictures were taken with a BZ-9000 microscope (KEYENCE). The area of lipid droplets in the pictures was measured by counting 150 droplets per section using the BZ-9000 Analysis Software (KEYENCE).

To detect fluorescent ZsGreen signals in the inguinal WATs, the sections were analyzed using a TCS SP5 laser-scanning confocal microscope (Leica Microsystems) as described previously.[16]

2.4 | TG levels in tissues and cells

Lipids were extracted from the inguinal WATs and adipocytes using Lipid Extraction Kit (Chloroform Free) (ab211044, Abcam), and then TG was measured with LabAssay Triglyceride Kit (#290-63701, Wako).

2.5 | Serum measurements

Blood samples were collected from the heart. Serum levels of free fatty acid and glycerol were measured with LabAssay NEFA Kit (294-63601, Wako) and Free Glycerol Assay Kit (ab65337, Abcam), respectively, according to the manufacture's protocols.

2.6 | Isolation of stromal vascular fraction from the inguinal WATs

The inguinal WATs, where the lymph nodes were removed, were minced with scissors and incubated in digestion medium (1 ml/g adipose tissue, PBS containing 1.5 unit/ml Collagenase D [#11088866001, Sigma-Aldrich], 2.4 unit/ml Dispase II [#4942078001, Sigma-Aldrich] and 10 mM $CaCl_2$) at 37°C with rotating (150 rpm) for 45 min. The digested WATs were mixed with 5 ml of Dulbecco's modified Eagle medium (DMEM; 043-30085, Wako) containing 10% fetal bovine serum (FBS; DMEM/FBS), and centrifuged for 10 min at 700*g*. The resulting cell pellet was suspended in DMEM/FBS, filtered through 100 μm cell strainer, and then recentrifuged for 10 min at 700*g*. The pellets were resuspended in DMEM/FBS.

2.7 | Flow cytometry

The stromal vascular fraction from inguinal WATs was prepared as described above. Cells were stained with fluorophore-conjugated antibodies in the presence of Mouse BD Fc Block (#553141, BD Biosciences). Data were collected with a flow cytometer (FACSAria II, BD Biosciences) and analyzed with FlowJo software (Tomy Digital Biology) as described previously.[8] The fluorophore-conjugated antibodies used for flow cytometry analysis were as follows: CD11b (#561098, BD Pharmingen), F4/80 (#123115, Biolegend), CD45 (#109823, Biolegend), CD31 (#102509, Biolegend), and Sca-1 (#561076, BD Pharmingen).

2.8 | Ex vivo differentiation of adipose-derived stem cells into mature adipocytes

The stromal vascular fraction in inguinal WATs was stained with fluorophore-conjugated antibodies as described above, and the adipose-derived stem cells (ADSCs, $CD45^{-}CD31^{-}Sca1^{+}$ cells) were sorted by the FACSAria II and cultured in adipocyte maintenance medium (DMEM supplemented with 10% FBS, 1% Pen Strep (10378-016, Life Technologies), 20 nM insulin (I4011, Sigma-Aldrich)). For adipocyte differentiation, confluent cells were incubated in adipocyte differentiation medium (DMEM supplemented with 10% FBS, 1% Pen Strep, 20 nM insulin, 0.5 mM 3-isobutyl-1-methylxanthine [I5879, Sigma-Aldrich], 0.5 μM dexamethasone [D1756, Sigma-Aldrich] and 125 μM indomethacin [I7378, Sigma-Aldrich]). After 48 h of exposure to the adipocyte differentiation medium, cells were maintained in the adipocyte maintenance medium with changing the medium every 2 days until analysis. To delete *Zfat* gene ex vivo, cells were treated with 1 μM 4-hydroxytamoxifen (H7904, Sigma-Aldrich) in the adipocyte maintenance medium for 4 days.

2.9 | Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted by ISOGEN (319-90211, Nippon Gene), and complementary DNA (cDNA) was synthesized using superscript VILO cDNA synthesis kit (11754-250, Life Technologies), according to the manufacturer's protocols. Quantitative-PCR was performed by using Thunderbird SYBR qPCR Mix (QPS-201, Toyobo) with ABI PRISM 7900HT (Applied Biosystems) as describe previously.[19] The sequences of primers used were as follows:

Arl1 Fw; cagttgtgaccgagatcgaa
Arl1 Rv; gccatttctgagggtgtcat
Armc8 F1; gagctgagaccctcgcctat
Armc8 R1; catgatcaagccttttaatgtca
Atg13 F; tgcttatagaattaacttggcattca
Atg13 R; gcgtcctcagcagttctgta

Brf1 F; ggaagacgttcctgctcttg
Brf1 R; ggttcttctccccaaactcc
Brpf1 F; caccacactgaagatgtagagga
Brpf1 R; atttttgcacgccgagag
Commd7 F; tccaaatggtgcactgaaga
Commd7 R; gcgttctgcttccacttttc
Dclre1a F; ctgcatcggaaaggagaaag
Dclre1a R; agagtcgcacatgtctgtgg
Ggnbp2 F; agttcgcaggagggtatgag
Ggnbp2 R; atctgccacgtctgctcttc
Harbi1 F; ctggcttcttggtgacagttc
Harbi1 R; ctacagcagagggtctgcaa
Nhlrc2 F; accttcaccattgctgttcc
Nhlrc2 R; ccttccagggttaggtcctc
Pde12 F; agctggctttgaacccatta
Pde12 R; ccttttgggtgccagtagag
Ppp1r37 F; cacagccctgaagatgaaca
Ppp1r37 R; tgtaccccagacctgagtcc
Psmc3 F; agacctggtgggtgtgaac
Psmc3 R; agctcctggatctgcttgtc
Rad51ap1 F; ccactggtgaaggttcagaaa
Rad51ap1 R; gatgcctcacatttgggttt
Setd1a F; tggcgctgactggcttaatg
Setd1a R; cgtgcagagcctgtctgatg
Timm22 F; gtcttaccggggaaagtcg
Timm22 R; ccgtaggtaataatcgattgcag
Gapdh F; accacagtccatgccatcact
Gapdh R; gtccaccaccctgttgctgta

2.10 | Statistical analysis

The data were expressed as the mean ± *SD*. The statistical analyses were performed using an unpaired two-tailed Student's *t*-test. Differences at $p < .05$ were considered to be statistically significant.

3 | RESULTS

3.1 | Creation of tamoxifen-induced Zfat knockout mouse

To elucidate roles of Zfat in vivo after the birth, we generated tamoxifen-induced *Zfat*-knockout mice (*CreERT2-Zfat*$^{flox/flox}$ mice) by crossing *Zfat*$^{flox/flox}$ mice with *CreERT2* mice.[10] To induce Cre-mediated recombination of the *Zfat* gene, *CreERT2-Zfat*$^{flox/flox}$, and *CreERT2-Zfat*$^{flox/WT}$ (Control) mice were intraperitoneally administrated with tamoxifen for 7 consecutive days (Figure 1A). Then the expression levels of Zfat were examined at 48 h after the final administration through immunoblotting analysis using an anti-Zfat antibody. However, tamoxifen treatment did not cause a significant decrease in the expression levels of Zfat in the thymus and spleen from *CreERT2-Zfat*$^{flox/flox}$ mice, compared with those from Control mice (Figure 1B).

To improve the efficiency of the *Zfat* gene knockout, we, next, generated *CreERT2-Zfat*$^{flox/ZsGreen}$ (inducible *Zfat*-knockout, Zfat iKO) mice by crossing *CreERT2-Zfat*$^{flox/flox}$ mice with *Zfat*$^{WT/ZsGreen}$ mice, which have the knock-in allele containing the *ZsGreen* gene inserted in-frame with the ATG translation initiation site of the *Zfat* gene.[16] Before tamoxifen treatment, the expression levels of Zfat in various tissues were similar between Zfat iKO and Control mice (Figure 1C). Treatment of Zfat iKO mice with tamoxifen resulted in a marked reduction in the Zfat expression levels in various tissues, including the thymus, spleen, testis, and WAT, compared with those in Control mice (Figure 1C). These results indicate the usefulness of Zfat iKO mice in the elucidation of Zfat roles in vivo.

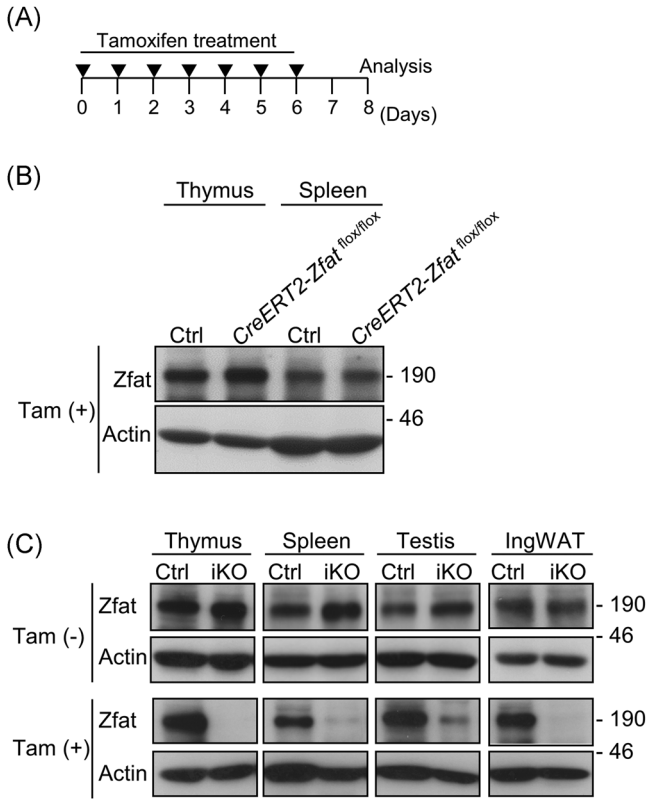

FIGURE 1 Creation of Tam-induced Zfat knockout mouse. (A) Scheme of time course for Tam treatment of mice. (B, C) Immunoblotting analysis of Zfat in tissues from the indicated genotype mice. Actin was used as a loading control. Molecular weights in kDa are indicated on the right. Mice were treated with Tam (Tam (+)) or vehicle (Tam (−)) for 7 consecutive days, and analyzed at day 8, as described in (A). Data are representative of three independent experiments. Ctrl, control mice; iKO, inducible Zfat-knockout mice; Tam, tamoxifen; Zfat, zinc-finger protein with AT-hook

3.2 | Tamoxifen-induced loss of Zfat expression causes significant reductions in body weight and WAT mass

We compared body weights between Control and Zfat iKO mice during and after tamoxifen treatment, and found that the tamoxifen treatment caused a significant decrease in the body weights of Zfat iKO mice, compared with those of Control mice (Figure 2A). In contrast, daily food intake and body temperature were similar between Control and Zfat iKO mice, suggesting that the reduction in body weights of tamoxifen-treated Zfat iKO mice is not due to hypophagia or hyperthermia (Figures 2B,C). Next, we compared the relative weights of various tissues against body weights between Control and Zfat iKO mice treated with tamoxifen. Intriguingly, tamoxifen-treated Zfat iKO mice showed significant decreases in the relative weights and size of inguinal and epididymal WATs, compared with those of Control mice (Figures 2D,E). In contrast, the relative weights of other tissues examined in this study, including the thymus, spleen, heart, lung, kidney, liver, and brown adipose tissue (BAT) were unaffected by loss of Zfat expression (Figure 2D). These results suggest that loss of Zfat expression in vivo causes significant reductions in body weight and WAT mass.

3.3 | Loss of Zfat expression results in the decreased TG levels in the WAT

To elucidate mechanisms by which loss of Zfat expression causes a significant decrease in WAT mass, we performed histological analysis of the inguinal WATs by staining with hematoxylin and eosin. As shown in Figure 3A, the adipocytes in the tamoxifen-treated Zfat iKO inguinal WATs were apparently smaller than those in the Control inguinal WATs. The histological differences were confirmed by quantification analysis of the area of lipid droplets. The average of lipid droplet area in

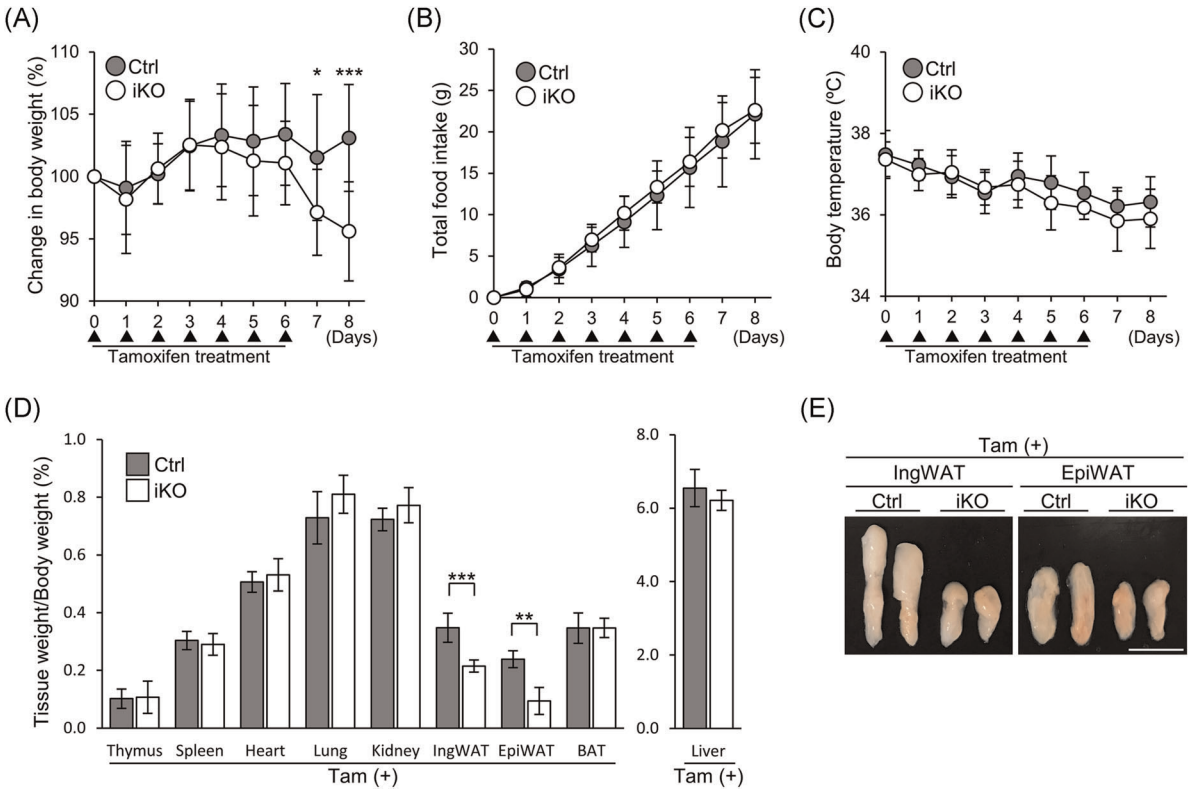

FIGURE 2 Tam-induced loss of Zfat expression causes significant reductions in body weight and WAT mass. (A–C) Time courses of changes in body weight (A), total food intake (B), and body temperature (C) for Ctrl and iKO mice during and after Tam treatment. $n = 11$/genotype. (D) The ratio of tissue weight against body weight for Ctrl and iKO mice treated with tam. $n = 4$–6/genotype. (E) Representative images of the IngWAT and EpiWAT WATs from Ctrl and iKO mice treated with Tam (scale bar = 1 cm). (A–E) Mice were treated with Tam (Tam (+)) or vehicle (Tam (−)) for 7 consecutive days, and analyzed during tamoxifen treatment or at day 8, as described in Figure 1A. (A–D) Data represent the mean ± *SD*. *, $p < .05$. **, $p < .005$. ***, $p < .001$. (E) Data are representative of three independent experiments. Ctrl, Control; EpiWAT, epididymal WAT; iKO, inducible Zfat-knockout; IngWAT, inguinal WAT; Tam, tamoxifen; Zfat, zinc-finger protein with AT-hook; WAT, white adipose tissue

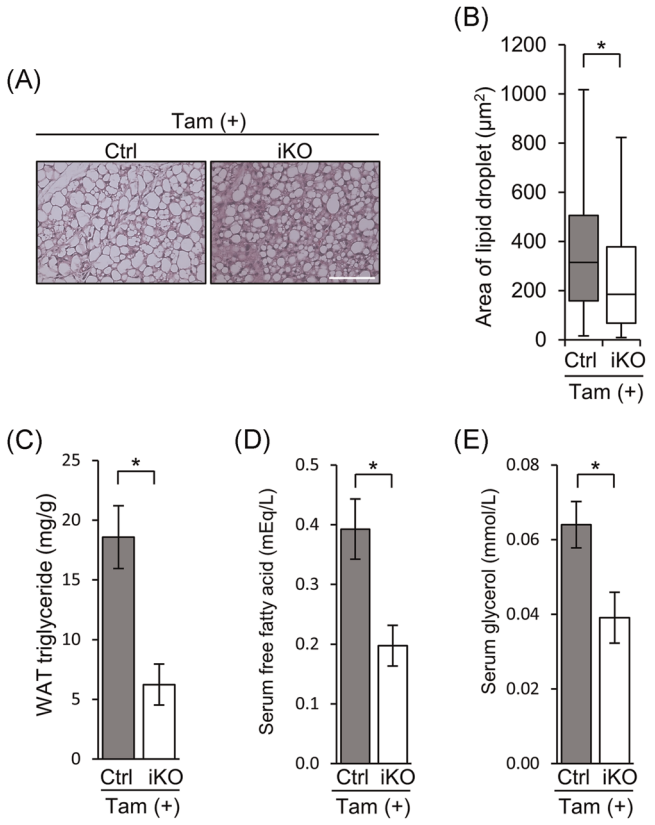

FIGURE 3 Loss of Zfat expression results in the decreased TG levels in the WAT. (A) Representative images of the Ctrl and iKO inguinal WATs stained with hematoxylin and eosin (scale bar = 100 μm). (B, C) Quantification of the lipid droplet area (B, $n = 4$/genotype) and the TG levels (C, $n = 4$–5/genotype) in the inguinal WATs from Ctrl and iKO mice treated with Tam. (B) Area of lipid droplets are shown by Box and Whiskers plot. Whiskers indicate minimum and maximum values. (D, E) Serum levels of free fatty acid (D) and glycerol (E) in Ctrl and iKO mice treated with Tam. $n = 8$/genotype. (A–E) Mice were treated with Tam for 7 consecutive days, and analyzed at day 8, as described in Figure 1A. (A) Data are representative of three independent experiments. (B–E) Data represent the mean ± *SD*. *$p < .001$. Ctrl, Control; iKO, inducible Zfat-knockout; Tam, tamoxifen; TG, triglyceride; WAT, white adipose tissue; Zfat, zinc-finger protein with AT-hook

the tamoxifen-treated Zfat iKO inguinal WATs was significantly smaller than that in the Control inguinal WATs (Figure 3B). Consistent with the morphological changes, the TG levels in the inguinal WATs were significantly decreased in tamoxifen-treated Zfat iKO mice, compared with those in Control mice (Figure 3C). It has been known that TG in the adipocytes is hydrolyzed into free fatty acid and glycerol that are released into the blood. The serum levels of free fatty acid and glycerol were significantly decreased in tamoxifen-treated Zfat iKO mice, compared with those in Control mice (Figures 3D,E), suggesting that the decreased TG levels in the WATs caused by Zfat loss are not due to excessive hydrolysis of TG. These results suggest that loss of Zfat expression in mice results in the decreased TG levels in the WATs.

3.4 | Zfat is expressed in the mature adipocytes and adipose-derived stem cells in the WAT

The WAT contains various types of cells, including the mature adipocytes, adipose-derived stem cells (ADSCs), endothelial cells and macrophages. To identify Zfat-expressing cells in the WAT, we examined ZsGreen signals in the inguinal WAT of *Zfat*$^{WT/ZsGreen}$ mice in which a green fluorescence signal from ZsGreen in tissues and cells reflects endogenous Zfat expression.[16] Histological analysis showed that ZsGreen signals were detected in the mature adipocytes in the *Zfat*$^{WT/ZsGreen}$ inguinal WATs, but not in those in the wild-type (WT) inguinal WATs (Figure 4A), indicating that Zfat is expressed in the mature adipocytes. Next, stromal vascular fractions were isolated from the inguinal WATs, stained with anti-Sca1, anti-CD45, anti-CD31, anti-CD11b and anti-F4/80 antibodies, and classified into the ADSCs ($CD45^-CD31^-Sca1^+$ cells), endothelial cells ($CD45^-CD31^+Sca1^+$ cells), macrophages ($CD45^+CD11b^+F4/80^+$ cells) and other cells ($CD45^-CD31^-Sca1^-$ cells). ZsGreen signals in each cell population were examined through flow cytometry analysis (Figure 4B). The majority of the ADSCs from the *Zfat*$^{WT/ZsGreen}$ inguinal WATs exhibited ZsGreen signals. Furthermore, a part of the endothelial cells and macrophages from the *Zfat*$^{WT/ZsGreen}$ inguinal WATs contained $ZsGreen^+$ cells. In contrast, ZsGreen signals were hardly detected in the population of other cells from the *Zfat*$^{WT/ZsGreen}$ inguinal WATs. These results indicate that Zfat is mainly expressed in adipocyte lineage cells, including the mature adipocytes and ADSCs, with moderate expression in the endothelial cells and macrophages.

Then, we assessed the effects of tamoxifen-induced loss of Zfat expression on the ADSCs, endothelial cells and macrophages. Tamoxifen treatment did not affect the number of these cells from the Zfat iKO inguinal WATs, compared with those from the Control inguinal WATs (Figure 4C), suggesting that Zfat is not essential for survival of the ADSCs, endothelial cells or macrophages in the WAT.

3.5 | Loss of Zfat expression in the mature adipocytes results in the decreased TG levels

We examined cell autonomous roles of Zfat in the mature adipocytes through ex vivo assay of the mature

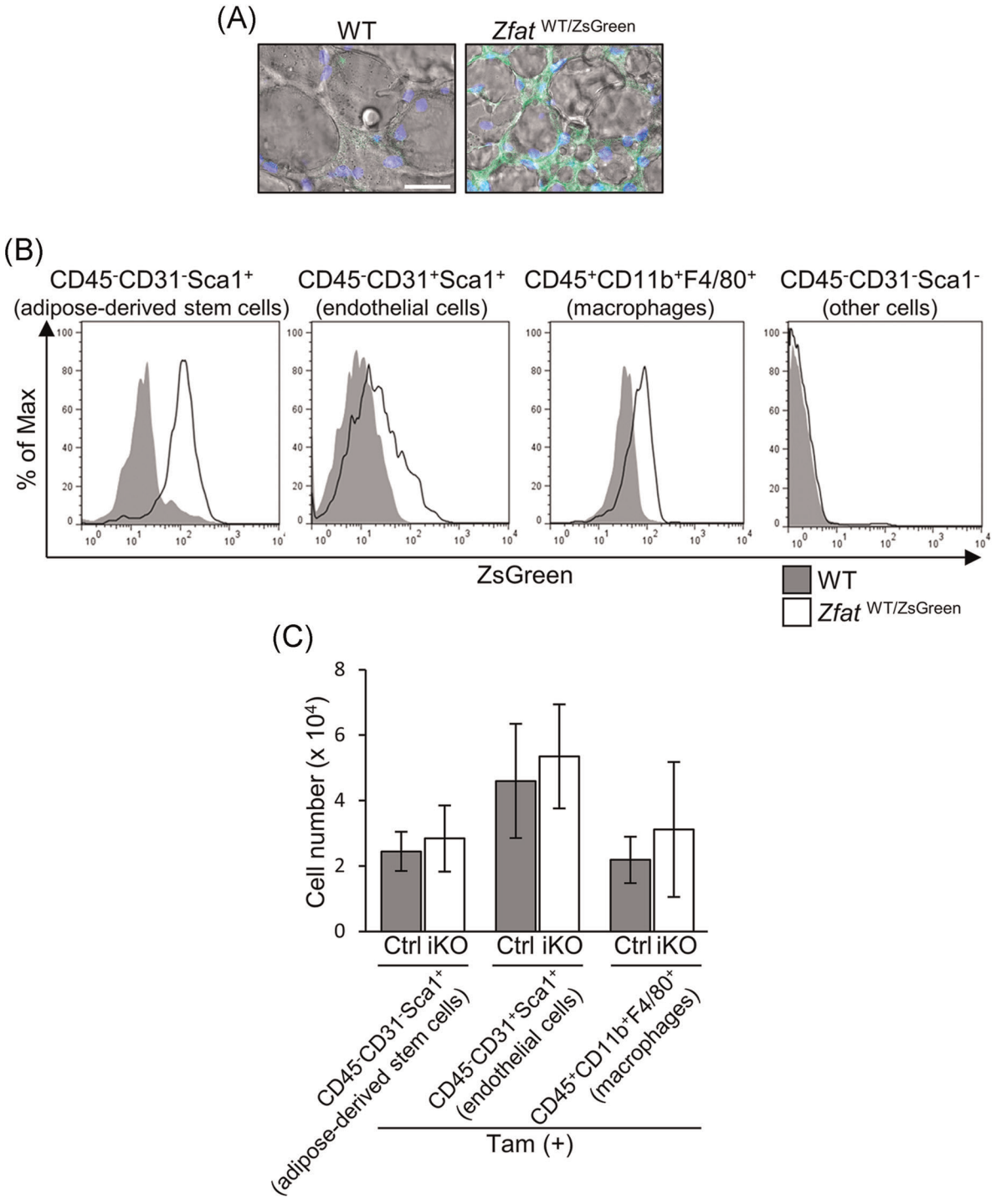

FIGURE 4 Zfat is expressed in the mature adipocytes and adipose-derived stem cells in the WAT. (A) Representative merged images of ZsGreen (green), DAPI (blue) and bright field in the inguinal WAT cryosections from WT and *Zfat*$^{WT/ZsGreen}$ mice. (B) ZsGreen signals in $CD45^{-}CD31^{-}Sca1^{+}$ (ADSCs), $CD45^{-}CD31^{+}Sca1^{+}$ (endothelial cells), $CD45^{+}CD11b^{+}F4/80^{+}$ (macrophages) and $CD45^{-}CD31^{-}Sca1^{-}$ (other cells) cells in stromal vascular fractions isolated from the WT and *Zfat*$^{WT/ZsGreen}$ inguinal WATs. (C) The number of ADSCs, endothelial cells and macrophages in stromal vascular fractions isolated from the Ctrl and iKO inguinal WATs. $n = 3$/genotype. Mice were treated with tamoxifen for 7 consecutive days, and analyzed at day 8, as described in Figure 1A. Data represent the mean ± *SD*. (A, B) Data are representative of three independent experiments. ADSC, adipose-derived stem cells; Ctrl, Control; DAPI, 4′, 6-diamidino-2-phenylindole; iKO, inducible Zfat-knockout; Tam, tamoxifen; TG, triglyceride; WAT, white adipose tissue; WT, wild-type; Zfat, zinc-finger protein with AT-hook

adipocytes differentiated from the ADSCs. The primary ADSCs isolated from the inguinal WATs of Zfat iKO and Control mice were cultured *ex vivo*, differentiated into the mature adipocytes and then treated with 4-hydroxytamoxifen (4OHT), a tamoxifen analogue, to induce Cre-mediated recombination of the *Zfat* gene (Figure 5A). After the 4OHT treatment, the mature adipocytes were cultured for 14 days. Immunoblotting analysis confirmed that 4OHT treatment resulted in a marked decrease in Zfat expression in the mature adipocytes differentiated from the Zfat iKO ADSCs (Figure 5B). Treatment with 4OHT caused a decrease in the size of lipid droplets in the Zfat iKO adipocytes, compared with those in the Control adipocytes (Figure 5C). Consistent with the size of lipid droplets, the TG levels in the 4OHT-treated Zfat iKO adipocytes were significantly decreased, compared with those in the Control adipocytes (Figure 5D), indicating that loss of Zfat expression in the mature adipocytes results in the decreased TG levels. These results suggest that Zfat plays cell autonomous roles in maintenance of the mature adipocytes.

3.6 | Zfat functions as a transcriptional regulator in the mature adipocytes

We previously reported that Zfat controls the mRNA transcription of specific genes in the thymocytes.[5] To identify Zfat-target genes in the mature adipocytes, we examined the effects of Zfat loss on the mRNA levels of genes of which Zfat-binding was observed at the promoter regions in the thymocytes. RNA was extracted from the 4OHT-treated mature adipocytes differentiated from the ADSCs of Zfat iKO and Control WATs, and the mRNA levels were determined through qRT-PCR (qRT-PCR) analysis. 4OHT treatment of the Zfat iKO adipocytes caused a significant decrease in the mRNA expression levels of eight genes in the 16 genes examined in this study, compared with those in the Control adipocytes, whereas only *Rad51ap1* mRNA levels were upregulated by loss of Zfat expression (Figure 6A), suggesting that Zfat mainly positively regulates the expression of specific genes in the mature adipocytes, similar to the thymocytes. We previously reported *Arl1, Brpf1, Commd7*, and *Nhlrc2* as Zfat-target genes in the thymocytes.[5] However, loss of Zfat in the mature adipocytes did not cause a significant decrease in

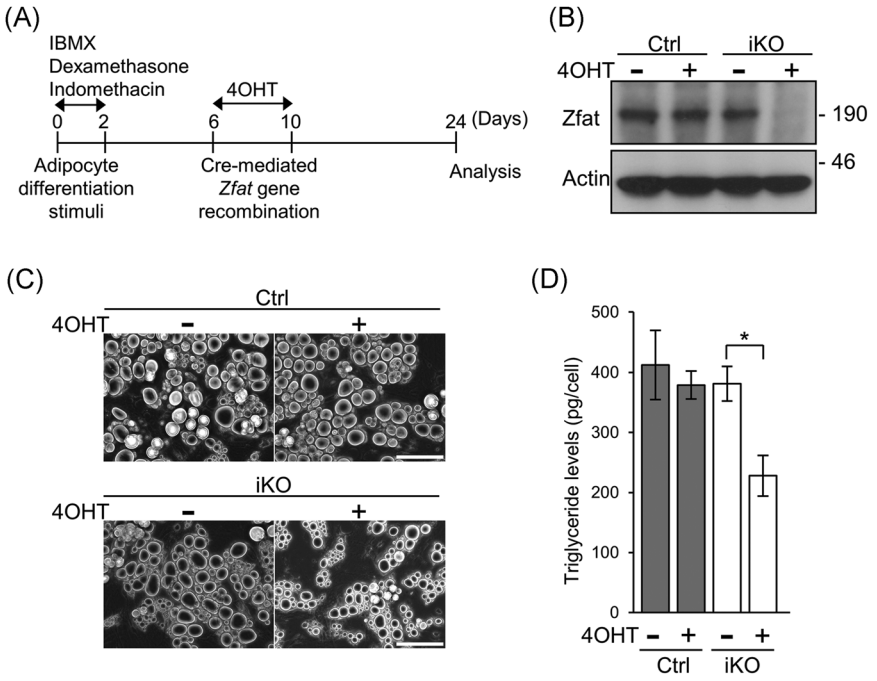

FIGURE 5 Loss of Zfat expression in the mature adipocytes results in the decreased TG levels. (A) Scheme of time courses for ex vivo assay of the mature adipocytes differentiated from the ADSCs isolated from the Ctrl and iKO inguinal WATs. (B) Immunoblotting analysis of Zfat in the mature adipocytes at day 24. Actin was used as a loading control. Molecular weights in kDa are indicated on the right. (C, D) Representative bright field images (C) and the TG levels (D, $n = 6$/genotype) of the mature adipocytes at day 24. (B–D) ADSCs were differentiated into the mature adipocytes and then treated with 4OHT (+) or nontreated (−), as described in (A). (B, C) Data are representative of three independent experiments. (C) Scale bar = 100 μm. (D) Data represent the mean ± *SD*. *$p < .05$. 4OHT, 4-hydroxytamoxifen; ADSCs, adipose-derived stem cells; Ctrl, Control; iKO, inducible Zfat-knockout; TG, triglyceride; WAT, white adipose tissue; WT, wild-type; Zfat, zinc-finger protein with AT-hook

the mRNA levels of *Arl1, Commd7*, and *Nhlrc2*, with moderate reduction in the *Brpf1* mRNA levels. These results suggest that Zfat regulates the transcription of particular genes through cell-type specific manner.

Next, we assessed the protein levels of genes of which the mRNA levels were decreased in the Zfat iKO adipocytes, through immunoblotting analysis. 4OHT treatment caused a significant decrease in the protein levels of Atg13, Brf1, Psmc3, and Timm22 in the Zfat iKO adipocytes, whereas the Armc8 and Ppp1r37 protein levels were unaffected by loss of Zfat expression (Figure 6B). On the other hand, Brpf1, Harbi1, and Rad51ap1 proteins were not detected in the mature adipocytes through immunoblotting analysis (data not shown). These results suggest that *Atg13, Brf1, Psmc3*, and *Timm22* are Zfat-target genes in the mature adipocytes.

3.7 | Loss of Zfat expression in the ADSCs impairs adipocyte differentiation with the decreased expression of C/EBPα and adiponectin

Since Zfat was also expressed in the ADSCs as shown in Figure 4B, we examined Zfat functions in adipocyte differentiation through *ex vivo* assays. To this end, 4OHT was added into the ADSCs before initiation of differentiation (Figure 7A). Treatment of the Zfat iKO ADSCs with 4OHT resulted in a significant decrease in Zfat expression at the beginning of differentiation (Figure 7B). After differentiation into the mature adipocytes, we examined the mRNA expression levels of several adipocyte differentiation markers by qRT-PCR analysis. Interestingly, the expression levels of *C/EBPα* mRNA were significantly decreased in the 4OHT-

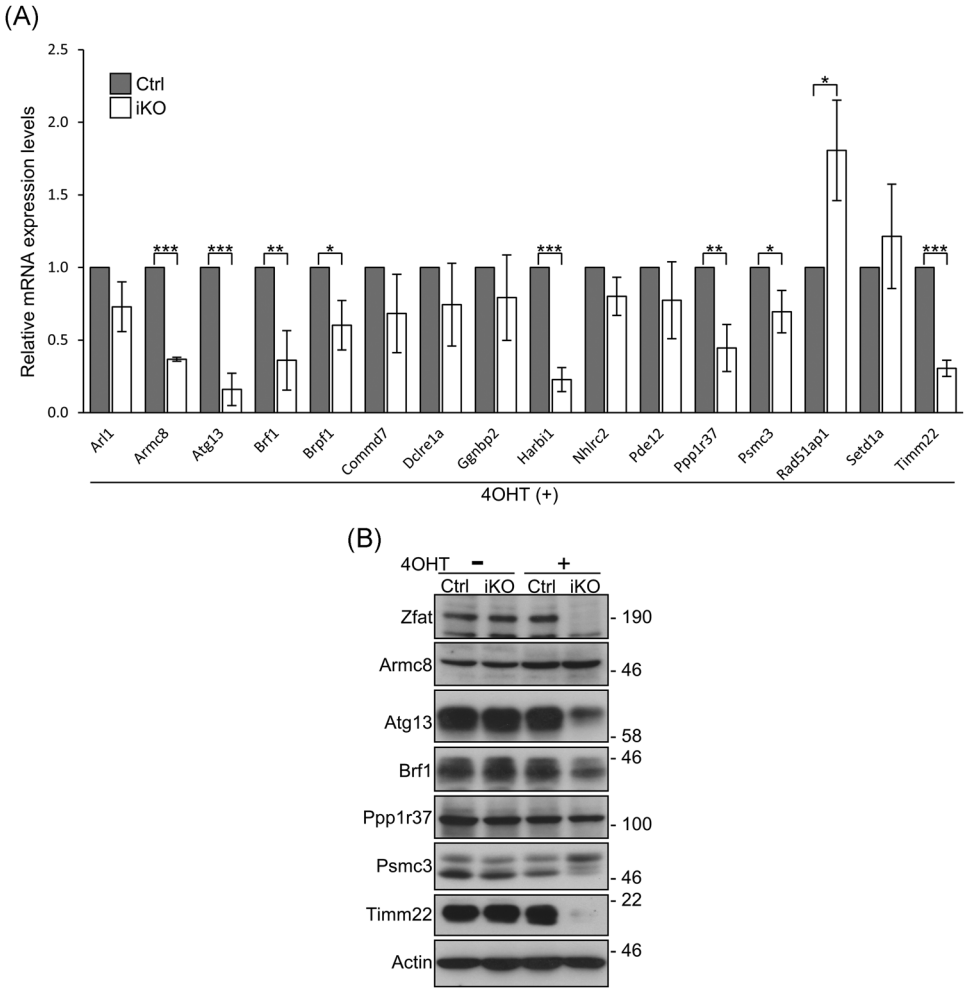

FIGURE 6 Zfat functions as a transcriptional regulator in the mature adipocytes. (A,B) qRT-PCR (A, $n = 3$/genotype) and immunoblotting (B) analyses of candidate molecules for Zfat-target gene in the mature adipocytes. ADSCs were differentiated into the mature adipocytes, treated with 4OHT (+) or vehicle (−), and analyzed at day 24, as described in Figure 5A. (A) Data represent the mean ± *SD*. *$p < .05$. **$p < .01$. ***$p < .001$. (B) Actin was used as a loading control. Molecular weights in kDa are indicated on the right. Data are representative of three independent experiments. 4OHT, 4-hydroxytamoxifen; ADSC, adipose-derived stem cell; qRT-PCR, quantitative reverse transcription polymerase chain reaction; Zfat, zinc-finger protein with AT-hook

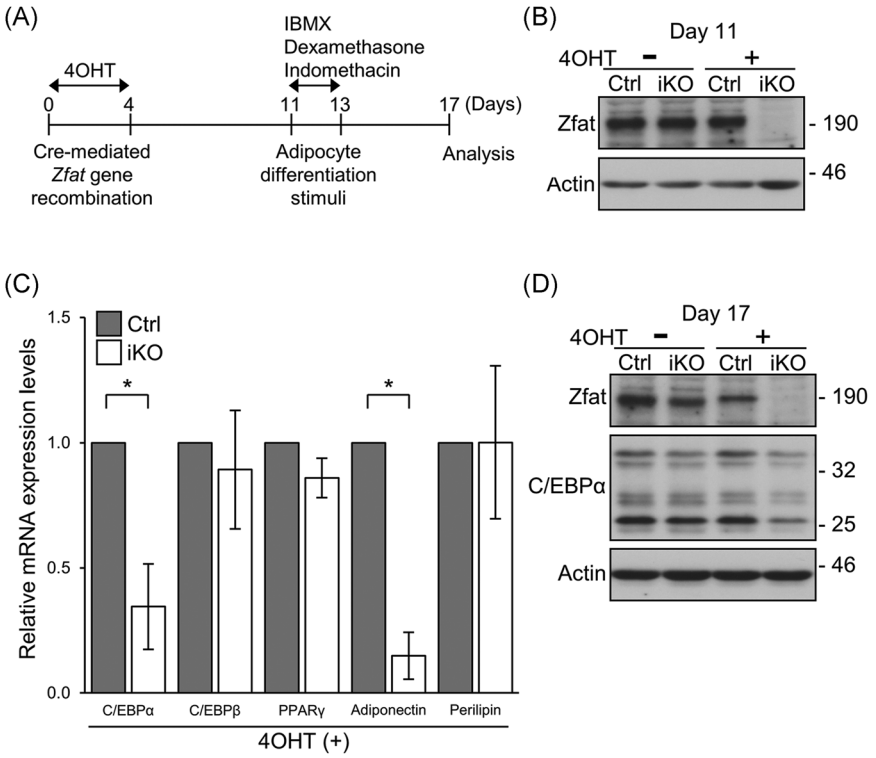

FIGURE 7 Loss of Zfat expression in the ADSCs impairs adipocyte differentiation with the decreased expression of C/EBPα and adiponectin. (A) Scheme of time courses for ex vivo assay of adipocyte differentiation of the ADSCs from the Ctrl and iKO inguinal WATs. (B) Immunoblotting analysis of Zfat in the ADSCs. ADSCs were treated with 4OHT (+) or nontreated (−), and analyzed at day 11, as described in (A). (C, D) qRT-PCR (C, $n = 3$/genotype) and immunoblotting (D) analyses of adipocyte differentiation markers in the adipocytes at day 17. ADSCs were treated with 4OHT (+) or nontreated (−) and then differentiated into the adipocytes, as described in (A). (B, D) Actin was used as a loading control. Molecular weights in kDa are indicated on the right. Data are representative of three independent experiments. (C) Data represent the mean ± *SD*. *$p < .05$. 4OHT, 4-hydroxytamoxifen; ADSC, adipose-derived stem cell; Crtl, Control; iKO, inducible Zfat-knockout; qRT-PCR, quantitative reverse transcription polymerase chain reaction; WAT, white adipose tissue; Zfat, zinc-finger protein with AT-hook

treated Zfat iKO adipocytes, compared with those in the Control adipocytes (Figure 7C). There are several splicing isoforms of the *C/EBPα* gene. Immunoblotting analysis using an anti-C/EBPα antibody showed that 4OHT treatment of Zfat iKO adipocytes decreased the expression levels of C/EBPα proteins, including all splicing isoforms (Figure 5D). It has been reported that C/EBPα controls the mRNA transcription of the *adiponectin* gene.[20,21] Indeed, the mRNA levels of adiponectin were significantly decreased in the 4OHT-treated Zfat iKO adipocytes. On the other hand, the mRNA expression levels of *C/EBPβ, PPARγ* and *perilipin* were unaffected by loss of Zfat. These results suggest that Zfat is involved in adipocyte differentiation.

3.8 | Roles of Zfat in lipid metabolism and gene expression in the BAT and liver

We examined the roles of Zfat in other metabolic tissues, including the BAT and liver. Immunoblotting analysis using an anti-Zfat antibody revealed that Zfat protein was hardly detected in the BAT and liver (Figure 8A). Indeed, the TG levels in the BAT and liver were comparable between tamoxifen-treated Zfat iKO and Control mice (Figure 8B). Furthermore, the protein levels of Timm22 were unaffected in the Zfat iKO BAT and liver by tamoxifen treatment (Figure 8C). These results suggest that Zfat is not involved in lipid metabolism or gene expression in the BAT and liver, and plays important roles, specifically in the WATs.

4 | DISCUSSION

We have previously shown that Zfat is an essential molecule for embryonic development and T-cell homeostasis in mice. However, roles of Zfat in vivo after the birth remained unknown, except for roles in the T-cells, because homozygous Zfat knockout mice exhibit embryonic lethality. Here we show that Zfat functions as an important transcriptional regulator in the adipocytes by generating inducible *Zfat*-knockout mice. Adipocytes

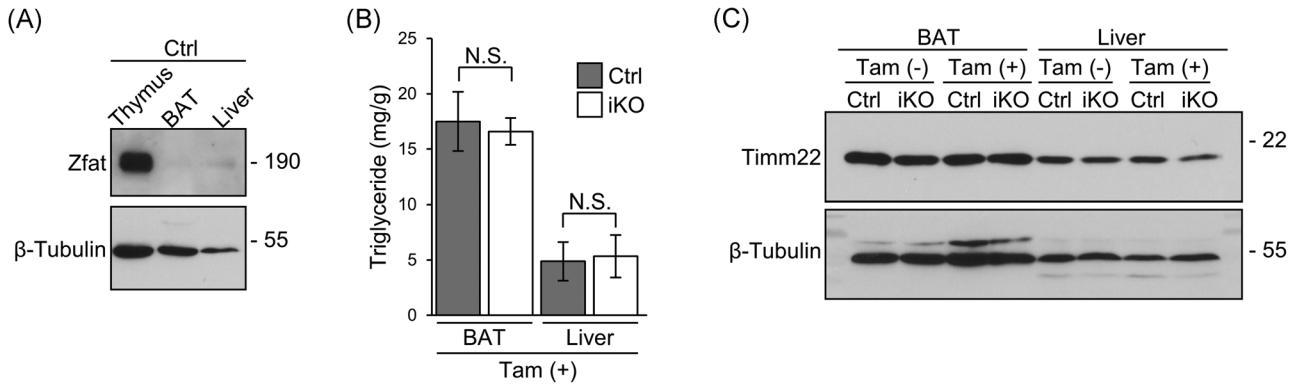

FIGURE 8 Roles of Zfat in lipid metabolism and gene expression in the BAT and liver. (A) Immunoblotting analysis of Zfat in the BAT and liver from Ctrl mice. (B) Quantification of the TG levels in the BAT and liver from Ctrl and iKO mice treated with Tam. (C) Immunoblotting analysis of Timm22 in the BAT and liver from Tam-treated Ctrl and iKO mice. BAT, brown adipose tissue; Crtl, Control; iKO, inducible Zfat-knockout; TAM, tamoxifen; TG, triglyceride; Zfat, zinc-finger protein with AT-hook

play crucial roles in whole-body energy homeostasis, and their dysregulation leads to obesity and obesity-related complications, including T2DM. Differentiation and functions of the adipocytes are tightly regulated by various transcription factors. However, transcriptional program in the adipocytes has not been completely explained at the molecular levels. In this study, loss of Zfat in vivo causes the reduction in the WAT mass, accompanied by the decreased TG levels. In ex vivo assays of the mature adipocytes, Zfat loss reduces the TG levels, suggesting cell autonomous roles of Zfat in maintenance of the mature adipocytes. Furthermore, we show that Zfat is required for the mRNA expression of specific genes in the mature adipocytes. On the other hand, loss of Zfat in the ADSCs impairs adipocyte differentiation with the decreased expression of both C/EBPα and adiponectin. These results demonstrate that Zfat plays important roles in the transcriptional program for maintenance and differentiation of the adipocytes.

We identify *Atg13, Brf1, Psmc3*, and *Timm22* as Zfat-target genes in the mature adipocytes. Decreased expression of some or all of these genes may cause the reduced C/EBPα expression and TG levels observed in the Zfat-depleted adipocytes. Among these Zfat-target genes, Atg13 is a component of the protein complex essential for autophagy initiation.[22] Loss of Atg13 inhibits autophagy by suppressing autophagosome formation.[23,24] Autophagy has been reported to play important roles in the adipocyte differentiation. Loss of Atg5 or Atg7 in cultured cells, both of which are autophagy-related proteins, impairs the adipocyte differentiation.[25,26] Interestingly, Atg7 knockout mice exhibit reduced body weight and WAT mass, similar to tamoxifen-treated Zfat iKO mice.[26] Therefore, loss of Atg13 may be one of the causes for the reduced C/EBPα expression and TG levels observed in Zfat-depleted adipocytes.

On the other hand, Timm22 modulates the membrane insertion of mitochondrial carrier proteins, which mediate the exchange of metabolites across the inner membrane.[27,28] Mitochondria have been known to play important roles in lipid metabolism in the adipocytes.[29-31] Especially, mitochondria regulate lipogenesis by providing key intermediates for the synthesis of TG, such as glycerol 3-phosphate and acetyl-CoA. Therefore, loss of Timm22 would cause the impairment of lipogenesis through dysfunction of mitochondrial carrier proteins, leading to the decreased TG levels observed in Zfat-depleted adipocytes.

Furthermore, Brf1 is a subunit of the RNA polymerase III transcription factor complex,[32] and Psmc3, also known as Rpt5, is a subunit of the proteasome.[33] The roles of Brf1 and Psmc3 in lipid metabolism have not been reported. Future studies will be required to elucidate their contribution to the decreased TG levels caused by Zfat loss.

Here, we show that loss of Zfat in the ADSCs decreases the mRNA levels of the *C/EBPα* gene. On the other hand, a 8-bp consensus DNA sequence (GAA(T/A)(C/G)TGC) for Zfat-binding, which we previously reported,[5,6] is not located at the promoter region of *C/EBPα* gene. Indeed, in the Zfat ChIP-seq analysis, Zfat-binding was not observed in the promoter region of *C/EBPα* gene.[5] These results suggest that Zfat does not directly regulate the transcription of *C/EBPα*. Elucidating the mechanism by which Zfat loss causes the decrease in the C/EBPα expression levels will be addressed in future studies.

Here, we report important roles of Zfat as a transcription regulator in the adipocytes. Furthermore, genetic variations of the *Zfat* gene are associated with the onset of T2DM although the mechanism by which dysregulation of Zfat leads to T2DM is unknown.[15] Interestingly, in our preliminary experiments, even under conditions of obesity

induced by a high-fat diet, the TG levels in the WATs decreased by Zfat loss (unpublished data). Thus, Zfat may also play important roles in the adipocytes in obese mice. Further elucidation of Zfat roles in maintenance and differentiation of the adipocytes will contribute to understanding of the onset mechanism of T2DM at the molecular levels. Performing both RNA-seq and Zfat ChIP-seq analyses in the adipocytes would lead to identify the novel Zfat-target genes in the adipocytes.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Shuhei Ishikura, Masayoshi Nagai, Toshiyuki Tsunoda, Kensuke Nishi, Yoko Tanaka, and Midori Koyanagi performed experiments. Shuhei Ishikura and Senji Shirasawa wrote the paper. Senji Shirasawa supervised the project.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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