Research article

**Kbtbd11** contributes to adipocyte homeostasis through the activation of upstream stimulatory factor 1

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**ABSTRACT**

The present study aimed to investigate the transcriptional regulation of Kbtbd11 in adipose tissue. To elucidate the physiological role of Kbtbd11 gene expression, adipose Kbtbd11 mRNA expression levels were estimated under various feeding states in wild-type mice. Kbtbd11 expression increased in a time-dependent manner in the adipose tissue in mice fed on chow diet, whereas the promotion of Kbtbd11 mRNA expression by refeeding was attenuated in mice fed on high-fat (HF) diet, suggesting the suppression of Kbtbd11 mRNA expression under HF diets and that changes in mRNA levels were associated with regulation of the transcription activity of Kbtbd11 by some transcription factors. To investigate the transcriptional regulation of Kbtbd11, the fragment upstream of either mouse Kbd11 or human KBTBD11 promoter was inserted into a luciferase vector. Luciferase reporter assays revealed that both mouse and human KBTBD11 promoter activity was increased by USF1. Direct USF1 binding to the E-box in the Kbtbd11 promoter was confirmed by electrophoretic mobility shift and chromatin immunoprecipitation assays. In addition, the adipocyte differentiation marker levels increased instantly in Kbtbd11-overexpressing Usf1 knockdown cells than in Usf1 knockdown cells. These results imply an association of between Kbtbd11 with Usf1 expression and suggest the involvement of Kbtbd11 in a novel adipogenesis pathway.

1. **Introduction**

Kelch repeat and BTB domain-containing 11 (KBTBD11) is a member of the KBTBD subfamily, which comprised BTB/POZ and Kelch domains. The BTB/POZ domain functions as the protein–protein interaction domain to facilitate dimer formation and interaction with non-BTB domain comprising proteins, involving activities such as transcriptional regulation, cytoskeleton dynamics, ion channel assembly and gating, and protein ubiquitination/degradation [1]. The Kelch domain, which is widely conserved in mammals and insects, usually comprises 2–7 repeats of four-stranded beta-sheet motifs that form the beta-propeller structure [2]. The Kelch β-propellers primarily function as scaffolds for protein–protein interactions. Kelch proteins interact directly with actin, suggesting the regulation of cell–cell interactions, cell–substrate interactions, and cell migration [3].

The putative tumor suppressor gene KBTBD11 is regulated by MYC. A variant allele of KBTBD11—rs11777210—is significantly associated with cell susceptibility to colorectal cancer. KBTBD11 expression is significantly decreased in tumor tissues compared with adjacent paired normal tissues [4]. We have previously reported that Kbd11 is involved in nutritional regulation and is highly expressed in the epididymal white adipose tissue (eWAT) in diet-induced obesity (DIO) mice compared with that in mice fed on chow diet [2]. In addition, the adenovirus-mediated knockdown of Kbd11 in 3T3-L1 cells inhibits mitotic clonal expansion (MCE), which is required during the early stages of 3T3-L1 adipocyte differentiation [2]. In contrast, Kbd11 overexpressing 3T3-L1 promotes MCE, which leads to the expression of adipocyte differentiation markers—C/ebpα and Pparg, and induces lipid accumulation [2], suggesting that Kbd11 expression levels play a major role in MCE and influence triglyceride accumulation and adipocyte differentiation. In this context, the present study aimed to clarify the transcriptional regulation of Kbtbd11 to elucidate the functions underlying the role of Kbtbd11 in adipocyte differentiation.

2. **Materials and methods**

2.1. **Animal experiments**

For mice experiments, we used 8-week-old male C57BL/6 mice from CLEA Japan. The mice were maintained on a normal chow diet.

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fasting–refeeding experiments, C57BL/6 mice were fasted for 24 h and then fed a chow diet or HF diet for 4, 6, 8 and 12 h. Feed ingredient contents were as follows: normal chow diet (CE-2) comprised carbohydrate 50.3%, protein 25.4%, and fat 4.4% and high-fat diet (HFD32) comprised carbohydrate 29.4%, protein 25.5%, and fat 32.0% (CLEA Japan Inc.). Animal experimental protocols were approved by the Animal ethics committee of Jichi Medical University (permit number 17177).

2.2. Cell and adipocyte differentiation

Human kidney 293T (HEK293T) and 3T3-L1 cells were maintained in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 100 units each of penicillin and streptomycin at 37°C in 5% CO2. For adipocyte differentiation experiments, at 2 days after 3T3-L1 cells confluence, the medium was replaced with high-glucose DMEM comprising insulin (5 μg/mL), dexamethasone (1 μM), and 3-isobutyl-1-methylxanthine (0.5 mM). After 2 days of incubation, the medium was replaced with high-glucose DMEM comprising only 5 μg/mL insulin. The medium was replaced every alternate day. For adenovirus infection experiments, 3T3-L1 cells at day –2 after induction of differentiation were infected with adenovirus. 3T3-L1 cells were infected with the adenovirus at a multiplicity of infection (MOI) of 10 plaque-forming units per cell. The adenovirus used in this study demonstrated to be expressed with an efficacy of almost 100% at an MOI of 30 in 3T3-L1 adipocytes, as assessed by GFP (Fig. 1).

2.3. Oil Red O (ORO) stain

Adipocytes were fixed with 10% formalin solution in phosphate-buffered saline (PBS) for 10 min and replaced with 60% isopropanol in PBS for 1 min. The fixed adipocytes were stained with ORO for 20 min at room temperature.

2.4. Transfection and luciferase assays

The Dual-Luciferase Reporter Assay System (Promega) was used to determine promoter expression levels. Mouse Kbtbd11 and human KBTBD11 were generated using PCR with either mouse or human genomic DNA as a template. 3T3-L1 cells were co-transfected with each expression vector, mouse Kbtbd11 and human KBTBD11 promoters that drive firefly luciferase expression (pGL4.10 mKbtbd11-Luc and hKbtbd11-Luc) and Renilla reniformis luciferase vector (pGL4.74) for use as an internal control reporter. The cells were incubated for 24 h post-transfection at 37°C in 5% CO2 and lysed in 100 μL 1x Passive Lysis Buffer (Promega). Lysate was used for the luciferase assay, and luminescence was detected using a Luminometer (Thermo Fisher Scientific).

2.5. Electrophoretic mobility shift assay (EMSA)

The probes shown in Fig. 4A were synthesized by Thermo Scientific and biotin labeled. HEK293T cells were transfected with a USF1 expression vector driven by a CMV promoter. Nuclear protein was extracted 48 h post-transfection. The biotin-labeled probe was mixed with the USF1-overexpressing nuclear protein extract [super-shift lane was generated by adding the USF1 protein and USF1 antibody (sc-229, SantaCruz Biotech)] and allowed to incubate at room temperature for 20 min. For detection of the DNA-protein bands, we used the LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific). The wild-type (wt) and mutant (mut) probes for EMSA were as follows: Kbd11 Ebox (wt), TTTTCTCCACCCAGTAAATG and Kbd11 Ebox (mut), TTTTCTCCACCCaaaATG.

2.6. Chromatin immunoprecipitation (ChIP) assay

For the ChIP assay, we used the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology). Immunoprecipitation was performed using an USF1 tag antibody with mouse IgG as the negative control. After immunoprecipitation, the associated DNA was amplified with a primer pair: Kbtbd11 promoter–832 Fwd 5′-CCGATCCCGTGCTACCCCATTC-3′ and Kbtbd11 promoter–669 Rev 5′-CTCTTGATTGTTCTCCTTTGTC-3′.

2.7. Adenoviral expression vectors

For adenoviruses amplification, we used ViraPower Adenoviral
Expression System (Thermo Fisher Scientific) as described previously [5]. Full-length Kbtbd11-cDNA was subcloned into the pENTR-D-TOPO vector (Thermo Fisher Scientific). The pENTR-Kbtbd11 (C-terminal FLAG-tagged) vector was transferred into the pAd/CMV.V5-DEST vector using the Gateway system (Thermo Fisher Scientific). Sequences corresponding to the shRNAs for Kbtbd11 and lacZ were cloned into pBlock-it (Thermo Fisher Scientific). The sequence of the shRNA for Usf1: 5'–cacc GTACGCTCTCGAAGTGAAG cgtgtgtcgtcgt CTCAGTGGCAAGA GTGAC-3'. The adenoviruses were purified using ViralBind Adenovirus Miniprep Kit (Cell Biolabs) according to the manufacturer's protocol.

2.8. Real-time PCR (RT-PCR)

Total RNA was extracted with acid guanidinium thiocyanate–phenol reagents [6]. CDNA synthesis was performed using 1 μg of total RNA each and the Verso cDNA Kit (Thermo Fisher Scientific) with random hexamer primers. RT-PCR assays were performed using the ViiA7 Real-Time PCR System and Kapa SYBR fast universal qPCR kit (Kapa Biosystems). The following primers were used for this analysis: Kbtbd11 Fwd, 5'-TCACGCTTTTCGCTTACAT-3' and Kbtbd11 Rv, 5'-AACACAAACG AAAGGGTCTGA-3'; C/epbd Fwd, 5'-GCCATGCCGGAGAACTCTA-3' and C/epbd Rv, 5'-GGCTCGAGATGCTGCTT-3'; Ppar Fwd, 5'-TTCCACT ATGGGTTCATTTGTTGTTT-3' and Ppar Rv, 5'-TGGCGCAGTTAGATC ACGTCA-3'; Usf1 Fwd, 5'-ACCTTATTCCGCGAGATCGCA-3' and Usf1 Rv, 5'-CGGGCTTCTCATCTTTGTGTT-3'; Sreb1p Fwd, 5'-CGGCCGG AAAGTGT-3' and Sreb1 Rv, 5'-TCGACTCTCTCTGTGGTGG-3'; Fasn Fwd, 5'-ATCTGGAAAGCAGAACAGATCT-3' and Fasn Rv, 5'-AGAGACGTG TCCTCTGAGCT-3'; αRv Fwd, 5'-TTTCCCTCAACTGACGGGTG-3' and αRv P2 Fwd, 5'-AGGGTTATATGCCTCTCCTTTCT-3'; Tnfα Fwd, 5'-CAGCCG ATGGGTAGTGACTTCT-3' and Tnfα Rv, 5'-GGCTCTTACCCAGGGTTTGA-3'; Il6 Fwd, 5'-GAGGATACCTCCACCAAGACCC-3' and Il6 Rv, 5'-AAGTGC AATCATGGTGTCATTCA-3'; Bax Fwd, 5'-GGCTGAGATGGTTGCT GATTG-3' and Bax Rv, 5'-GATCAGCTCGCCGACATTTAG-3'; Bcl2 Fwd, 5'-CTGGAGTTGCTTCTGGACAC-3' and Bcl2 Rv, 5'-GAGACAGCGCAG AGAATAACAC-3'; Bclx Fwd, 5'-ATGCAGACGATCCCGCATG-3' and Bclx Rv, 5'-TTGCGCATGTTGCTTCA-3'.

2.9. Western blotting

Whole cell lysates were electrophoretically separated on denaturing polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Proteins were detected with USF1 (sc-229, Santa Cruz Biotechnology), and β-actin antibody (G043, abm).

2.10. Statistical analysis

Statistical significance was tested using the unpaired two-tailed Student’s t-test. All data were expressed as mean ± SEM. Statistical significance was set at p < 0.05.

3. Results

3.1. Kbtbd11 expression in epididymal WAT (eWAT) after feeding

We have previously reported that Kbtbd11 expression is upregulated at 12 h after a chow diet feeding and is increased in the adipose tissue in HF DIO mice [2]. To elucidate the nutritional regulation of Kbtbd11 expression in eWAT at an earlier time after feeding, the gene expression levels under various feeding states were estimated in C57BL/6J wild-type mice. Kbtbd11 mRNA expression was increased in a time-dependent manner during chow diet refeeding. The increase in Kbtbd11 expression after feeding was attenuated in mice fed on HF diet compared with mice fed on chow diet (Fig. 2).
3.4. *Usf1* expression in epididymal WAT (eWAT) after feeding

To elucidate the nutritional regulation of *Usf1* expression in eWAT after feeding, the gene expression levels were estimated under various feeding states in C57BL6/J wild-type mice. The *Usf1* mRNA expression was increased in a time-dependent manner during chow diet refeeding. The increase in *Usf1* expression after feeding was attenuated in mice fed on HF diet compared with that in mice fed on chow diet (Fig. 5).

![Fig. 3.](image)

**Fig. 3.** Activation of *Kbtbd11* promoter by USF1. (A) Schematic representation of mouse *Kbtbd11* promoter with the potential binding sites of lipid metabolism-related transcription factors. (B) The mouse *Kbtbd11* promoter region (2303 bp) was fused to a luciferase reporter gene (pGL4.10 m*Kbtbd11*-2030-Luc). 3T3-L1 cells were cotransfected with pGL4.10 *Kbtbd11*-2303-Luc as the reporter gene, *R. reniformis* luciferase vector (pGL4.74) as the internal control reporter, and the indicated expression plasmids or control vector (pcDNA3.1). (C) Ebox of the mouse *Kbtbd11* and human *KBTBD11* promoters. (D) Effects of USF1 on the mouse *Kbtbd11* and human *KBTBD11* promoter activity in 3T3-L1 cells. $n = 3$ per group, **$p < 0.01$ vs. control.

![Fig. 4.](image)

**Fig. 4.** *Kbtbd11* promoter is a direct target of USF1. (A) Ebox of the mouse *Kbtbd11* and mutant *Kbtbd11* promoters. (B) Luciferase assays of the reporter gene pGL4.10 *Kbtbd11*-2303-Luc and its various deletion constructs in the presence or absence of the USF1 expression plasmid in 3T3-L1 cells. $n = 3$ per group, **$p < 0.01$ vs. control. (C) EMSA for USF1 binding to Ebox of *Kbtbd11* promoter. Nuclear extracts of USF1 protein were incubated with biotin-labeled *Kbtbd11* promoter or mutant *Kbtbd11* promoter probe in the presence or absence of unlabeled probes and USF1 antibody. The competition was performed using unlabeled probes as competitors in 1000-fold molar excess. (D) ChIP assay for USF1 binding to Ebox of the *Kbtbd11* promoter using infection of either shLacZ or shUsf1 knockdown adenoviruses in mature adipocytes after 3T3-L1 differentiation at day 8. The cells were harvested at day 2 post-infection. The extracted genomic DNA was subjected to immunoprecipitation, performed using antibody against USF1, with IgG as a negative control. For comparison, amplification derived from unprecipitated chromatin is also shown (input).
group, in various feeding states. The expression of Mice were fasted for 24 h or fasted for 24 h/refed for 4, 6, 8, and 12 h. Wild-type mice were fed on chow diet or HF diet during the refeeding period. \( n = 3 \) per group, \(* p < 0.05, ** p < 0.01\) vs. fasted.

3.5. Plasma insulin and glucose levels after feeding

Because glucose increases USF1 expression \([11]\), Kbtbd11 and Usf1 expression levels might be affected by circulating insulin and/or glucose levels. To examine circulating insulin and glucose levels, plasma insulin and glucose levels were determined under various feeding states in C57BL6/J wild-type mice. Plasma insulin and glucose levels were significantly increased during HF refeeding compared with that during fasting. Plasma insulin levels were significantly increased during HF refeeding compared with that during chow diet refeeding, whereas plasma glucose levels were significantly decreased and plasma insulin levels were increased after HF refeeding (Fig. 6). These results suggested that Kbtbd11 expression was affected by USF1 expression levels via insulin and glucose levels.

3.6. Effects of Kbtbd11 overexpression on mature 3T3-L1 adipocytes

Based on the changes in Kbtbd11 expression levels in the livers of feeding mice, we investigated the potential role of Kbtbd11 in mature 3T3-L1 adipocytes by investigating the effects of Kbtbd11 overexpression on mature 3T3-L1 adipocytes. At day 8 after inducing differentiation, mature 3T3-L1 adipocytes were infected with GFP control or Kbtbd11 overexpression adenovirus. With the GFP control, Kbtbd11 overexpression increased the Kbtbd11 expression levels by 80-folds (Fig. 7B). However, lipid droplet abundance, as observed with ORO staining of mature 3T3-L1 adipocytes, was not different between conditions under the GFP control and Kbtbd11 overexpression (Fig. 7A). Consistent with the observations under ORO staining, the mRNA expression analyses of adipogenic (Pparg and aP2) and inflammation markers (Tnfα and Il6), in addition to lipogenic (Fasn) and proapoptotic genes (Bax and Bcl2), were not altered. We have previously demonstrated that the effects of Kbtbd11 knockdown on mature 3T3-L1 adipocytes do not vary between the control and Kbtbd11 knockdown conditions \([2]\). Taken together, the results suggested that Kbtbd11 plays a role after starvation and/or during lipid accumulation in adipose tissue.

3.7. Inhibition of adipocyte differentiation in Usf1 knockdown 3T3-L1 cells was rescued by Kbtbd11 overexpression

To investigate the role of Kbtbd11 in adipocyte differentiation, we examined the effects of Kbtbd11 in Usf1 knockdown 3T3-L1 cells. The adenooviral coinfection of Usf1 shRNA and GFP (shUsf1 + GFP) constructs reduced Usf1 mRNA expression levels by up to 70% compared with the coinfection of shLacZ and GFP (shLacZ + GFP) control (Fig. 8A). On day 8 after inducing differentiation, shLacZ + GFP control cells showed lipid droplet abundance (observed with ORO staining) (Fig. 8B) and increases in the expressions of C/ebpα, C/ebpβ, and Pparg (Fig. 8C). Consistent with the reduction in Usf1 expression in the coinfected shUsf1 + GFP 3T3-L1 cells, Kbtbd11 expression decreased by 50% compared with that in the shLacZ + GFP control. On day 8 after inducing differentiation, shUsf1 + GFP 3T3-L1 cells exhibited a marked reduction in lipid accumulation (Fig. 8B) and significant decreased in adipocyte differentiation marker levels (C/ebpα, C/ebpβ, and Pparg) (Fig. 8C).

In Kbtbd11 overexpressing Usf1 knockdown 3T3-L1 cells, the Kbtbd11 mRNA expression increased by 24-folds (Fig. 8A). On day 8 after inducing differentiation, Kbtbd11 overexpressing Usf1 knockdown cells exhibited a significant reduction in both lipid accumulation (Fig. 8B) and adipocyte differentiation marker levels (C/ebpα, C/ebpβ, and Pparg) (Fig. 8C) compared with those in the shLacZ + GFP control. However, in Kbtbd11 overexpressing Usf1 knockdown 3T3-L1 cells, C/ebpβ expression increased by 24-folds during the early stage of adipogenesis increased significantly compared with C/ebpβ expression in shUsf1 + GFP 3T3-L1 cells (Fig. 8C). Consistent with that in shUsf1 + GFP 3T3-L1 cells, following rescued C/ebpβ expression in shUsf1 + Kbtbd11 3T3-L1 cells, C/ebpα and Pparg expressions in the late stage of adipogenesis were equally rescued by Kbtbd11 overexpression (Fig. 8C).

3.8. Depression of adipogenic and lipogenic genes in Usf1 knockdown of mature 3T3-L1 adipocytes recovered by Kbtbd11 overexpression

To examine the role of Kbtbd11 in mature adipocytes, we investigated the effects of Kbtbd11 in Usf1 knockdown mature 3T3-L1 adipocytes. On day 8 after inducing differentiation, mature 3T3-L1 adipocytes were coinfected with adenooviral vectors expressing shLacZ and GFP control, shUsf1 and GFP, or shUsf1 and Kbtbd11. Compared with shLacZ and GFP control, coinfection of shUsf1 and GFP decreased Usf1 mRNA expression levels (by up to approximately 80%) (Fig. 9B) as well as USF1 protein levels (Fig. 9C). Consistent with the reduction in Usf1 expression under coinfection of shUsf1 + GFP 3T3-L1 cells, Kbtbd11 expression decreased by 70% compared with Kbtbd11 expression in shLacZ + GFP cells. In Kbtbd11 overexpressing Usf1 knockdown 3T3-L1 cells, Usf1 mRNA expression...
expression decreased by 80% and Kbtbd11 mRNA increased by 36-folds compared with that in shLacZ+GFP cells (Fig. 6B). Endogenous KBTBD11 protein levels could not be detected using a commercial antibody. Because the Kbtbd11-overexpressing adenovirus vector was fused with FLAG tag, only overexpression of KBTBD11 was detected by the anti-FLAG antibody (Fig. 9C). The lipid droplet abundance in mature 3T3-L1 adipocytes did not vary among shLacZ+GFP control, shUsf1+GFP cells, and shUsf1+Kbtbd11 cells (observed using ORO staining) (Fig. 9A). In addition, lipid droplet abundance was not different in mature 3T3-L1 adipocyte cells under shUsf1+GFP and shLacZ+GFP conditions (observed using ORO staining) (Fig. 9A). However, adipogenic (Pparg and aP2) and lipogenic (Srebp1c and Fasn) genes significantly decreased in the shUsf1+GFP mature 3T3-L1 adipocytes (Fig. 9B). PPARg and SREBP1 protein levels were consistent with Pparg and Srebp1c mRNA levels in the shUsf1+GFP cells (Fig. 9C). Similarly, lipid droplet abundance was not different between in mature 3T3-L1 adipocytes under shUsf1+Kbtbd11 and shLacZ+GFP conditions (observed using ORO staining) (Fig. 9A), although adipogenic (Pparg and aP2) and lipogenic (Srebp1c and Fasn) genes increased significantly and nearly recovered in the shLacZ+GFP control (Fig. 9B). PPARg and SREBP1 protein levels were increased in the shUsf1+Kbtbd11 cells compared with those in the shUsf1+GFP cells (Fig. 9C). FASN protein levels were not different between the shUsf1+GFP and shLacZ+GFP cells; however, FASN protein levels were higher in shUsf1+Kbtbd11 cells (Fig. 9C).}

4. Discussion

We have previously reported that Kbtbd11 mRNA expression increases in a time-dependent manner during 3T3-L1 differentiation and increases significantly in eWAT under feeding status and obesity [2]. Moreover, we reported that Kbtbd11 knockdown inhibits mitotic clonal expansion (MCE), and the resulting Kbtbd11 attenuates 3T3-L1 adipocyte differentiation. Unlike Kbtbd11 knockdown in 3T3-L1 cells, Kbtbd11...
Fig. 8. The effects of Kbtbd11 overexpression on USF1 knockdown 3T3-L1 cells. The 3T3-L1 cells were infected with mixture adenoviral vectors (at an MOI of 30) for expressing shLacZ, GFP, shUsf1, and Kbtbd11 at day −2 before inducing differentiation and were then incubated for 8 days. (A) The expressions of Usf1 and Kbtbd11 mRNA in Usf1 knockdown 3T3-L1 cells at day 8. n = 3 per group, *p < 0.05, **p < 0.01 vs. shLacZ+GFP. (B) Triglyceride accumulation in 3T3-L1 cells on day 8 was visualized using Oil Red O staining (scale bar = 100 μm) (left), quantification of Oil Red O stained area (right). (C) The mRNA levels in 3T3-L1 cells expressing each adenovirus mixture (shLacZ+GFP, shUsf1+GFP, and shUsf1 and Kbtbd11) at various time points after inducing differentiation. n = 3 per group, *p < 0.05, **p < 0.01 vs. shLacZ+GFP. Relative mRNA levels for adipogenic markers (C/ebpa, C/ebpb, and Pparg) were estimated after normalization with Rplp0. n = 3 per group, *p < 0.05, **p < 0.01 vs. shLacZ+GFP.
overexpression promotes MCE, thus leading to the expressions of C/ebpα and Pparg. These results suggest that Kbtbd11 expression regulation plays an important role in adipogenesis. Therefore, we hypothesized that the elevated expression of Kbtbd11 in eWAT in obese mice during 3T3-L1 differentiation could be regulated by transcription factors, including adipogenic and lipogenic transcription factors.

To examine the regulation of Kbtbd11 expression in 3T3-L1 cell differentiation, we investigated Kbtbd11 transcriptional control mechanisms. We used LASAGNA-Search 2.0 for analyzing the candidate transcription factor binding sites in Kbtbd11 promoter and identified the potential binding sites of lipogenic and adipogenic transcription factors, SREBP1, LXR, USF1 C/EBPα, PPARγ, and HNF4α. Subsequently, we investigated whether the transcription factors activate the Kbtbd11 promoter and found that USF1 is bound to the Ebox motif in the Kbtbd11 promoter and it enhanced the activation of Kbtbd11 transcription significantly. In fasting–refeeding experiments, Usf1 mRNA expression was increased in a time-dependent manner during chow diet refeeding, which is consistent with the Kbtbd11 mRNA expression. In addition, the attenuation of Usf1 mRNA after HF diet feeding was also consistent with that of Kbtbd11 mRNA (Fig. 5). Furthermore, we evaluated plasma insulin and glucose levels in fasting–refeeding state. The HF diet refeeding after caloric restriction reduced insulin sensitivity and increased circulating plasma insulin levels [12]. Plasma insulin levels were significantly increased during HF refeeding compared with those during chow diet refeeding. Plasma glucose levels were significantly decreased after HF refeeding, accompanied with the increase in plasma insulin levels (Fig. 6). Because glucose increases USF1 expression [11], the reduced plasma glucose levels after HF refeeding might downregulate USF1. Indeed, Usf1 mRNA was decreased after HF refeeding compared with that during chow diet refeeding (Fig. 5), resulting in the reduced expression of Kbtbd11.

USF1 is a basic helix–loop–helix leucine zipper transcription factor, which recognizes the Ebox motif (CACGTG) [13]. USF1 is ubiquitously expressed, and it regulates several proteins involved in lipid metabolism, including C/EBPα [14], fatty acid synthase (FASN) [13], and acetyl-CoA carboxylase-α [15]. In addition, the genetic variants of USF1 are associated with familial combined hyperlipidemia [16, 17], increased risk of cardiovascular diseases [17, 18], risk of type 2 diabetes [19, 20], metabolic syndrome traits [21, 22], and increased levels of obesity [23, 24, 25]. Because Kbtbd11 is a USF1 target gene and is altered by nutritional regulation (Fig. 2) during adipocyte differentiation [2], it plays an important role in lipid metabolism. However, Kbtbd11 upregulation might not play a crucial role in the modulation of mature adipocyte function(s), except after starvation or during energy storage in adipose tissue (Fig. 7).

In addition, we revealed that the effect of Usf1 knockout in 3T3-L1 cell differentiation is rescued through Kbtbd11 overexpression. Usf1 knockout inhibits differentiation into 3T3-L1 adipocytes because USF1, a crucial master regulator of adipocyte differentiation, regulates the transcription of C/ebpα and Fasn by binding to the Ebox region of the promoters [26]. However, on day 4 after inducing differentiation, adipocyte differentiation marker (C/ebpα, Pparg, Fasn, and aP2) levels increased significantly Kbtbd11-overexpressing Usf1 knockout cells compared with those in Usf1 knockout cells (Fig. 8C). These results

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Fig. 9. Effects of Kbtbd11 overexpression on Usf1 knockdown mature 3T3-L1 adipocytes. Mature 3T3-L1 cells were infected with adenoviral vectors (shLacZ, shUsf1, GFP, and Kbtbd11) at an MOI of 30. The differentiated mature 3T3-L1 adipocytes at day 8 after induction were used. (A) Triglyceride accumulation in mature 3T3-L1 adipocytes 48 h after each adenoviral mixture infection (shLacZ + GFP, shUsf1 + GFP, and shUsf1 and Kbtbd11) visualized using Oil Red O staining (scale bar = 100 μm) (left); quantification of Oil Red O stained area (right); (B) Total RNA was harvested 48 h after adenoviral infection, and expression levels of Usf1, Kbtbd11, and adipogenic (Pparg and aP2), and lipogenic (Srebp1 and Fasn) genes were measured using qPCR; n = 3 per group, *p < 0.05, **p < 0.01 vs. shLacZ + GFP. (C) Western blotting showing USF1, KBTBD11, PPARγ, SREBP, FASN, and β-actin protein levels.
suggest that the differentiation of Usf1 knockout 3T3-L1 cells could be partially rescued by Kbtbd11 overexpression. Furthermore, mature 3T3-L1 adipocyte cells under shUsf1 + Kbtbd11 condition showed that lipid droplet abundance was not different when compared with the abundance in the shLacZ + GFP control (observed with ORO staining) (Fig. 9A). However, adipogenic (Pparγ and aP2) and lipogenic (Srebp1c and Fasn) genes significantly increased and nearly recovered in the shLacZ + GFP control (Fig. 9B), suggesting that maintenance of Kbtbd11 expression could be essential during adipocyte differentiation and/or adipocyte tissue homeostasis.

Moreover, Kbtbd11 overexpression did not affect the expressions of adipocyte differentiation markers in 3T3-L1 cells. This result was consistent with Kbtbd11 knockdown in mature 3T3-L1 cells, wherein Kbtbd11 knockdown in mature 3T3-L1 cells was not different from the control adipocytes [2]. These results suggest that Kbtbd11 did not affect the already matured adipocytes and/or adipocytes that have reached the lipogenesis threshold. Conversely, Usf1 knockdown in mature 3T3-L1 cells downregulated adipogenic and lipogenic genes (Pparγ, aP2, Srebp1c, and Fasn) (Fig. 9). In such conditions (wherein the lipogenesis threshold is not reached), Kbtbd11 could play a role in the maintenance of adipocyte homeostasis. However, the molecular mechanism(s) of Kbtbd11 in adipocyte homeostasis and the physiological functions remain to be fully elucidated.

A recent study has reported the methylation and gene expression profiling of B-cell acute lymphoblastic leukemia (ALL) using next-generation sequencing and showed that KBTBD11 differed in methylation and expression levels between two subtypes of patients (TCF3-HLF and TCF3-PBX1) based on the presence of chromosomal translocations [27]. KBTBD11 was overexpressed in TCF3-HLF compared with that in TCF3-PBX1 patients, and the CpG sites in the exons of KBTBD11 were hypomethylated before remission in TCF3-HLF patients. Following remission, KBTBD11 methylation increased and gene expression was downregulated. The results suggested that KBTBD11 hypomethylation, expression levels, and/or transcriptional regulation are associated with the regulation of B-cell proliferation and differentiation in B-cell ALL [28].

In addition, the oncogenic transcription factor MYC bound to a MYC response element in KBTBD11 intron1 and regulated KBTBD11 expression [4, 29]. Furthermore, rs17777210, a variant allele of KBTBD11 in the MYC response element in KBTBD11 intron1, is significantly associated with cell susceptibility to colorectal cancer. KBTBD11 is reported to be downregulated in tumor tissues, and KBTBD11 knockdown promoted cell proliferation and inhibited cell apoptosis [4], suggesting that regulation of KBTBD11 expression and transcript levels of KBTBD11 play important roles in tumorigenesis and function.

Overall, alterations in KBTBD11 expression levels, methylation, and transcriptional regulation using a variant of MYC and a subtype of TCF3-HLF could be an important proliferation and differentiation function in various cell types. Considering that Kbtbd11 is a target USF1 gene that regulates proliferation and differentiation in adipocytes, genetic variation in USF1 and varying Kbtbd11 expression levels could be associated with obesity.

In conclusion, we revealed that USF1, an important regulator of lipid metabolism, is involved in the transcriptional regulation of Kbtbd11 and found that the effect of Usf1 knockdown is rescued through Kbtbd11 overexpression. However, further research is required to clarify Kbtbd11 methylation and expression differences between normal and obese individuals and elucidate the physiological function(s) of KBTBD11. KBTBD11 can be applied to control the progression and differentiation, including the development, of various cell types, such as adipocytes and cancer cells.

Declarations

Author contribution statement

Kazuhisa Watanabe: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kazuha Yokota, Ken Yoshida, Ayumi Matsumoto, Sadahiko Iwamoto: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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