A novel upstream transcription factor 1 target gene \textit{N4bp2l1} that regulates adipogenesis

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\textbf{ABSTRACT}

\textit{N4bp2l1}, which is highly expressed in oral squamous cell carcinoma, is associated with poor prognosis. However, \textit{N4bp2l1}'s role in adipogenesis remains unknown. We aimed to clarify the expression profile and transcriptional regulation of \textit{N4bp2l1} to elucidate the functions underlying the role of \textit{N4bp2l1} in adipocyte differentiation. Our results revealed that \textit{N4bp2l1} mRNA expression increased in 3T3-L1 cells in a differentiation-dependent manner. To investigate the transcriptional regulation of \textit{N4bp2l1}, the 2-kb 5' region upstream of the mouse \textit{N4bp2l1} promoter was cloned into a luciferase vector. Luciferase reporter assays indicated that USF1 induces the \textit{N4bp2l1} promoter activity. Electrophoretic mobility shift and chromatin immunoprecipitation assays confirmed that USF1 directly binds to the Ebox in the \textit{N4bp2l1} promoter. Furthermore, the expressions of adipocyte differentiation markers significantly decreased in \textit{N4bp2l1}-knockdown cells compared with those in control cells. Our results demonstrated that \textit{N4bp2l1} is a novel USF1 target gene that may be involved in adipogenesis regulation.

\textbf{1. Introduction}

Individuals with obesity have an increased risk of developing type 2 diabetes, atherosclerosis, hyperlipidemia, steatosis, and various types of cancer [1–3]. Adipogenesis is a process of progenitor cell differentiation and is controlled by the interplay of many transcription factors and complex molecular regulatory networks. For adipocyte differentiation, several groups of transcription factors regulating the key proteins need to be sequentially activated; this in turn induces gene expression that leads to adipocyte development. The key proteins include CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator- activated receptor gamma (PPAR\gamma) that coordinate the expression of genes that generate and maintain the adipocyte phenotype [4,5]. Over the years, many studies have been performed to identify the transcriptional processes and molecular mechanisms involved in adipocyte differentiation. However, more studies are required to identify novel regulators of adipogenesis and the appropriate targets for anti-obesity drugs for the treatment of obesity pathogenesis. The 3T3-L1 pre-adipocyte is a well-established model for investigating mechanisms underlying adipocyte proliferation, differentiation, and lipid metabolism and is appropriate for the identification of genes that regulate adipocyte physiology, including adipogenesis [6–8].

Upstream transcription factor 1 (USF1), a ubiquitously expressed helix–loop–helix leucine zipper transcription factor, recognizes the Ebox motif CACGTG [9] and regulates several proteins involved in lipid metabolism, including apolipoproteins apoA-II [10], apoA-V [11], apoC-III [12], apoE [13], hepatic lipase [14], ATP-binding cassette transporter-1 [15], fatty acid synthase [9,16], and hormone-sensitive lipase [17]. Moreover, genetic variants of USF1 are associated with familial combined hyperlipidemia [16,17], increased cardiovascular disease risk [17], atherosclerotic lesions [18], and body mass index (BMI) variations [19].

The NEDD4-binding protein 2-like 1 gene (\textit{N4BP2L1}) produces a full-length transcript with 5 exons and encodes a 243-amino-acid protein. This protein possesses several alternatively spliced isoforms that map to the human chromosome 13q13.1. \textit{N4bp2l1} is highly expressed in nasopharyngeal carcinoma and oral squamous cell carcinoma [20], and \textit{N4BP2L1} overexpression is reportedly correlated with nodal metastasis and poor outcomes in oral squamous cell carcinoma [20,21]. \textit{N4bp2l1} has been suggested to be involved in tumor development and progression and to play an important role in cancer pathophysiology. However, the expression profile and functional significance of \textit{N4bp2l1} in adipocyte development remain completely unknown.

In this study, we identified \textit{N4bp2l1} as a novel USF1 target gene and found that its knockdown inhibited adipocyte differentiation in 3T3-L1 cells. This relationship between USF1 and \textit{N4bp2l1} transcription in...
adipocytes could represent a new pathway in the regulation of adipogenesis, thereby providing targets for the therapeutic modulation of obesity.

2. Materials and methods

2.1. Cells and culture conditions

Human embryonic kidney 293T (HEK293T) and 3T3-L1 cells were cultured in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and 100 units of penicillin/streptomycin at 37 °C under 5% CO₂ atmosphere.

2.2. Adipocyte differentiation

3T3-L1 cells were cultured until confluence in high-glucose DMEM. Two days later, fresh high-glucose DMEM containing 5 μg/mL insulin, 1 μM dexamethasone (DEX), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) introduced. After incubation for 48 h, the medium was changed to high-glucose DMEM containing 5 μg/mL insulin. The medium was changed every alternate day [8]. In experiments with adenovirus, 3T3-L1 cells were infected with adenovirus at day 2 before the induction of differentiation.

2.3. Animal experiments

For mice experiments, 8-week-old male C57BL/6J mice from Japan SLC were used. The mice were maintained on a standard chow diet (CE-2) consists of 50.3% carbohydrates, 25.4% protein and 4.4% fat (CLEA Japan). Animal experimental protocols were approved by the animal ethics committee of Jichi Medical University (permit number 17177), and were performed in accordance with the Use and Care of Experimental Animals Guidelines of the Jichi Medical University Guide for Laboratory Animals.

2.4. Western blotting

Total cell lysates were electrophoretically separated on denaturing polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Proteins were detected with N4BP2L1 antibody (HPA038971, Merck) and β-actin antibody (G043, abm) as internal control.

2.5. Transfection and luciferase assays

We used the Dual-Luciferase Reporter Assay System (Promega) to determine promoter expression levels. Mouse N4bp2l1 was generated with PCR using the mouse genomic DNA as a template. 3T3-L1 cells were co-transfected with each expression vector, mouse N4bp2l1 promoter that drives firefly luciferase expression (pGL4.10 mN4bp2l1-Luc), and Renilla reniformis luciferase vector (pGL4.74) for use as an internal control reporter. After transfection, the cells were incubated for 24 h at 37 °C under 5% CO₂ atmosphere and then lysed in 100 μL 1X Passive Lysis Buffer (Promega). Next, 10 μL lysate was used in the luciferase assay, and luminescence was detected using the Fluoroskan Ascent FL Microplate Luminometer (Thermo Fisher Scientific).

2.6. Electrophoretic mobility shift assay (EMSA)

Biotin-labeled probes are mixed with USF1-overexpressing nuclear protein extract (supershift lane is generated by adding the USF1 protein and USF1 antibody [sc-229; SantaCruz Biotech]) and allowed to incubate at room temperature for 20 min. DNA–protein bands were detected using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific). The probes used for EMSA were N4bp2l1 Ebox2 and CAT CTATCACATGGCCGGAG.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology). Immunoprecipitation was performed using an antibody directed against USF1 and mouse IgG as the negative control. The associated DNA was then amplified with a primer pair: N4bp2l1 promoter −86 Fwd 5'-CTCAACGTTAGAGACGC CCCCTC-3' and N4bp2l1 promoter +125 Rev 5'-CAGCCTCCCGAAAGC TTCCAGAAAA-3'.

2.8. Oil Red O stain

The differentiated 3T3-L1 adipocytes were fixed for 10 min at 37 °C using 10% formaldehyde solution and then stained for 20 min at room temperature using freshly prepared 60% Oil Red O solution (Muto Pure Chemicals Co.). The cells were washed with PBS and rinsed with 60% isopropanol.

2.9. Adenoviral expression vectors

Adenoviruses were prepared and amplified with the ViraPower Adenoviral Expression System (Thermo Fisher Scientific), as previously described [22]. The shRNAs of N4bp2l1 and LacZ were cloned into BLOCK-it U6 entry vector (Thermo Fisher Scientific). The shRNA sequence for N4bp2l1 shRNA#1 was 5'-cacc GAATACTAGAAGCTTATC ttcagaga TATAACTCTCATGTATTCT-3' and for N4bp2l1 shRNA#2 was cacc GAAAGAATTTGATGAAAT ttcagaga ATTTCATCATACT TTT. The pENTR vector inserts were transferred into the adenovirus vector pAd/PL-DEST using the Gateway System (Thermo Fisher Scientific). The recombinant adenoviruses were purified using the Adenovirus Purification Miniprep Kit (Cell Biologics) according to the manufacturer's protocol.

2.10. Real-Time PCR (qPCR)

Total RNAs were isolated using acid guanidinium thiocyanate—phenol reagent [23]. cDNA was synthesized using the Verso cDNA Kit (Thermo Fisher Scientific) with random hexamer primers. qPCR assays were performed using the Viia7 Real-Time PCR System and KAPA SYBER FAST ROX Low qPCR Kit (Kapa Biosystems). The relative gene expression levels were quantified with qPCR, followed by normalization to the internal control gene ribosomal protein lateral stalk subunit P0 (Rplp0). The primers used for this analysis were N4bp2l1 Fwd, 5'-TGGAAACAGAAGAGCAAGAAGA-3' and N4bp2l1 Rev, 5'-AGTGTGGGTTCCTCCGGAATATA-3'; C/ebpa Fwd, 5'-GCCATGCGAGTGTAGCTGTC-3'; C/ebpa Rev, 5'-GGGGCTCTGGAGGTGACTGCT-3'; C/ebpa Fwd, 5'-GAGCGCCGACAAAGCCTCAACATTA3'; C/ebpa Rev, 5'-GGCTGTGTCCTCCGCGTCAGC-3'; Pparg Fwd, 5'-TCCACAAATGGAGCTTCTCTT-3'; Pparg Rev, 5'-ATGGCAGCACATTGCTCAGT-3' and Rplp0 Fwd, 5'-TGTCGCTATGGGCTGCTACCT-3' and Rplp0 Rev, 5'-TTGCGGAGATCAGCTGCTACCT-3'.

2.11. Statistical analysis

Experimental studies were performed in triplicates or in greater numbers of sets. Statistical significance was tested using the unpaired two-tailed Student's t-test or one-way ANOVA with Bonferroni test for multiple comparisons. Data were expressed as mean ± SEM. Statistical significance was set at p < 0.05.

3. Results and discussion

3.1. N4bp2l1 expression during 3T3-L1 adipocyte differentiation

To identify novel genes that regulate adipocyte development, we first searched the expression profiles during in vitro adipocyte differentiation. To determine promoter expression levels, mouse N4bp2l1 was generated with PCR using the mouse genomic DNA as a template. 3T3-L1 cells were co-transfected with each expression vector, mouse N4bp2l1 promoter that drives firefly luciferase expression (pGL4.10 mN4bp2l1-Luc), and Renilla reniformis luciferase vector (pGL4.74) for use as an internal control reporter. After transfection, the cells were incubated for 24 h at 37 °C under 5% CO₂ atmosphere and then lysed in 100 μL 1X Passive Lysis Buffer (Promega). Next, 10 μL lysate was used in the luciferase assay, and luminescence was detected using the Fluoroskan Ascent FL Microplate Luminometer (Thermo Fisher Scientific).
differentiation. To follow this approach, we used a publicly available microarray data from Gene Expression Omnibus (GSE20696) [24]; preadipocyte 3T3-L1 cells were compared at day 0 and at day 7 after differentiation. Interestingly, these data indicated that N4bp2l1 was dramatically increased by 23.9-fold at day 7 compared with that at day 0 (GSE20696). Next, to confirm the evidence for N4bp2l1 expression profiling of 3T3-L1 adipogenesis, we analyzed the expression of N4bp2l1 in 3T3-L1 cell differentiation using qPCR. A cocktail mix comprising DEX, IBMX, and insulin was used to induce differentiation in 3T3-L1 cells. Total RNA was extracted from the cells on days 0, 2, 4, 6, and 8 after adipocyte differentiation was induced. N4bp2l1 expression increased by three-fold between days 0 and 4, and during 3T3-L1 cell differentiation, N4bp2l1 expression subsequently increased by six-fold on day 8 compared with that on day 0 (Fig. 1A). N4BP2L1 protein levels markedly increased between days 0 and 4 following adipocyte differentiation (Fig. 1B). These results suggested that N4BP2L1 was constitutively expressed in preadipocyte 3T3-L1 cells at day 0 and remarkably increased after induction of adipocyte differentiation (Fig. 1B). Because the brain and adipose tissue are lipid rich, N4bp2l1 may be related to lipid metabolism. In addition, from the Type 2 Diabetes Knowledge Portal (http://www.type2diabetesgenetics.org/, last accessed on December 18, 2018), we detected nominally significant associations between the variant allele of N4BP2L1 rs497255 and BMI in the GIANT UK Biobank GWAS (p = 1.6 × 10−15). These results suggest that N4bp2l1 expression is associated with the link between lipid accumulation and BMI and plays an important role in lipid-rich organ.

3.2. Regulation of N4bp2l1 promoter by USF1

Fig. 2A shows the structure of mouse N4bp2l1. We used the LASAGNA-Search 2.0 to identify potential binding sites of adipogenesis- and lipid metabolism-related transcription factors in the N4bp2l1 promoter [25]. We recognized putative transcription factor-binding sites for hepatocyte nuclear factor 4 alpha (HNF4a), PPARg, C/EBPa, and USF1 (Fig. 2A). Hepatocyte nuclear factor-4 (HNF4), in addition to inducing numerous genes involved in lipid metabolism, binds to direct
suggests that

3.3. Requirement of Ebox regions for USF1 activation of N4bp2l1 promoter

N4bp2l1 is a novel USF1 target gene.

repeat motifs of the AGGCTA sequence that are separated by one nucleotide (DR1) and regulates gene expression [26]. PPARδ also binds to the DR1 element as a heterodimer with the retinoid X receptor family [27]. C/EBP family members recognize the palindromic DNA sequence CACGTG [9]. To determine whether these transcription factors activate the N4bp2l1 promoter, the 2-kb 5′ region upstream of the mouse N4bp2l1 promoter was cloned into a luciferase vector and subjected to a promoter probe in the presence or absence of unlabeled probe and USF1 antibody. The competition was induced using an unlabeled probe as a competitor at 100- and 500-fold molar excesses. (D) ChIP assay for USF1 binding to Ebox2 of the N4bp2l1 promoter using 3T3-L1 preadipocytes (day 0) or adipocytes after 3T3-L1 differentiation (day 4, 6 and 8). The extracted genomic DNA was subjected to immunoprecipitation performed with an antibody against USF1 and with IgG as the negative control. For comparison, amplification derived from unprecipitated chromatin is also shown (input). n = 3 per group, p < 0.01 vs. day 0.

Fig. 3. N4bp2l1 promoter is a direct target of USF1. (A) A schematic of the potential Ebox element of the mouse N4bp2l1 promoter. (B) Luciferase assays of the reporter gene including the mouse N4bp2l1 promoter region (−468 bp) and its various deletion constructs in the presence or absence of the USF1 expression vector in 3T3-L1 cells. n = 3 per group, p < 0.01 vs. control and *p < 0.01 vs. −12 bp N4bp2l1 promoter with USF1. (C) Upper panel: Ebox2 of the mouse N4bp2l1. Bottom panel: EMSA for USF1 binding to Ebox2 of the N4bp2l1 promoter. Nuclear extract (NE) of USF1 protein was incubated with biotin-labeled Ebox2 of the N4bp2l1 promoter probe in the presence or absence of unlabeled probe and USF1 antibody. The competition was induced using an unlabeled probe as a competitor at 100- and 500-fold molar excesses. (D) ChIP assay for USF1 binding to Ebox2 of the N4bp2l1 promoter using 3T3-L1 preadipocytes (day 0) or adipocytes after 3T3-L1 differentiation (day 4, 6 and 8). The extracted genomic DNA was subjected to immunoprecipitation performed with an antibody against USF1 and with IgG as the negative control. For comparison, amplification derived from unprecipitated chromatin is also shown (input). n = 3 per group, p < 0.01 vs. day 0.

3.3. Requirement of Ebox regions for USF1 activation of N4bp2l1 promoter

USF1 binds to the Ebox motif CACGTG in the promoter of lipid metabolism-related genes, such as apolipoproteins [10–13], hormone-sensitive lipase [17], ATP-binding cassette transporter-1 [15], and fatty acid synthase [9,16]. We identified 2 predicted Ebox sites in the 0.5-kb 5′ region upstream of the mouse N4bp2l1 (Fig. 3A). The region responsible for USF1 activation was located within −468 bp, indicating that the Ebox1 sequence at −403 bp (CAGGTG) and the Ebox2 sequence at +1 (CACATG) could be the USF1-binding sites (Fig. 3A). To confirm whether the Ebox motif of the N4bp2l1 promoter was the correct USF1-binding site, various deletion and mutation constructs (as indicated in Fig. 3B) were prepared and tested for the basal and USF1-induced activities. The N4bp2l1 promoter including Ebox1 and Ebox2 was significantly activated by USF1 coexpression (Fig. 3B). Although there is no Ebox1 site in Ebox1 mutation and Ebox1 deletion (−375 bp) luciferase vectors, the expression was significantly promoted by USF1 coexpression (Fig. 3B). Ebox2 in the −12 bp N4bp2l1 promoter resulted in a significant increase in transcript abundance. Notably, the activation of the −12 bp N4bp2l1 promoter was significantly increased compared with the −468 bp promoter (Fig. 3B), suggesting that other regions (from −468 to −12 bp) of the N4bp2l1 promoter have suppressing domain responsible for N4bp2l1 expression. In addition, activation of the −12 bp mutant N4bp2l1 promoter significantly increased by USF1 compared with that by control. This result indicated that USF1 might slightly bind to the −12bp mutant N4bp2l1 promoter. However, Ebox2 mutation in the −12 bp N4bp2l1 promoter caused a significant decline in USF1 activation compared with the −12 bp N4bp2l1 promoter, which suggests that Ebox2 is a crucial USF1-binding site for N4bp2l1 transcription (Fig. 3B).

EMSA was performed to confirm the direct binding of USF1 to the Ebox2 sequence, which indicated that USF1 binds to the Ebox2 oligonucleotide and USF1 antibody binds to the USF1/Ebox2 oligonucleotide complex, resulting in band shifting (supershift). The protein binding specificity of the Ebox2 probe was demonstrated by competition with 100- and 500-fold molar excesses of the unlabeled Ebox2 probe (Fig. 3C).

To elucidate whether USF1 physically binds to the endogenous mouse N4bp2l1 promoter, we performed the ChIP assay using preadipocytes and mature adipocytes of 3T3-L1. The results of ChIP assay of genomic DNA from before differentiation at day 0 (preadipocytes) or after differentiation at day 4, 6, and 8 (mature adipocytes) of 3T3-L1 confirmed the direct binding of USF1 to Ebox2 in vitro; moreover, signal intensity detected at day 0 was increased at day 8 (Fig. 3D); this finding is consistent with N4bp2l1 expression during 3T3-L1 differentiation (Fig. 1A). Furthermore, during 3T3-L1 cell differentiation, Usf1 expression decreased by 45% between days 0 and 2, and then increased in a time-dependent manner (Supplementary Fig. 2A). N4bp2l1 expression also decreased by 33% between days 0 and 2, and then increased at day 4 following adipocyte differentiation (Fig. 1A). Although Usf1 mRNA expression levels were not consistent with USF1 protein levels, increase expression levels of USF1 protein were consistent with N4BP2L1 protein levels during 3T3-L1 differentiation (Fig. 1B and Supplementary Fig. 2B). These results suggested that increased USF1 following lipid accumulation in adipocytes binds to the N4bp2l1 promoter and upregulates N4bp2l1 transcripts. Our results indicate that USF1 activates N4bp2l1 transcription by specifically binding to the Ebox sequence in the N4bp2l1 promoter and that N4bp2l1 is a novel USF1 target gene involved in lipid metabolism.

Triglyceride accumulation and adipocyte differentiation in 3T3-L1
cells were inhibited by N4bp2l1 knockdown.

To investigate N4bp2l1’s role in adipocyte differentiation, we studied N4bp2l1 knockdown effects on 3T3-L1 differentiation. Adenoviral infection of either of the 2 independent N4bp2l1 shRNA constructs decreased N4bp2l1 mRNA expression levels by approximately 80%–90% compared with that of the shLacZ control (Fig. 4A). On day 8 after differentiation induction, the shLacZ control cells displayed abundant lipid droplets (observed with Oil Red O staining) (Fig. 4B) and significantly increased expression of C/ebpα, C/ebpβ, and Pparg (Fig. 4C). In contrast, the levels of lipid accumulation and adipocyte differentiation markers (C/ebpα, C/ebpβ, and Pparg) significantly decreased in both N4bp2l1–knockdown cells. These results suggest that N4bp2l1 plays an important role in triglyceride accumulation and adipocyte differentiation.

The study results demonstrate that USF1 regulates N4bp2l1 mRNA expression and that they may be associated with lipid metabolism in the early and/or late stage of 3T3-L1 adipocyte differentiation. Elucidation of the physiological function of N4BP2L1 function(s) may contribute to a new therapeutic target for obesity, although further research is needed.

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Appendix A. Supplementary data

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Transparency document

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