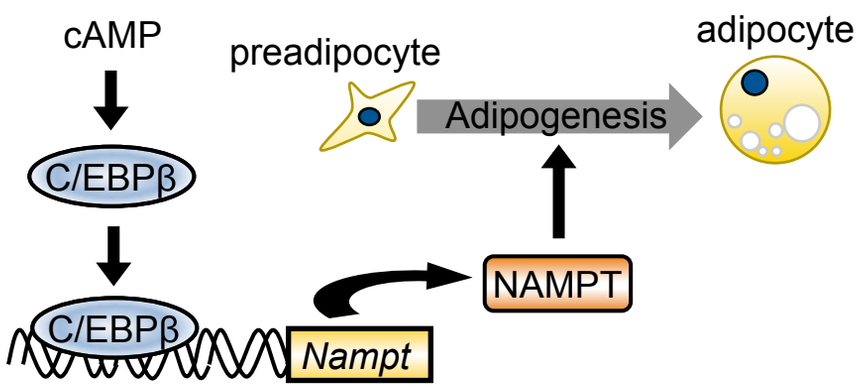
Highlights

- Intracellular cAMP activates *Nampt* promoter activity in 3T3-L1 cells.
- cAMP-induced C/EBPβ expression stimulates *Nampt* promoter activity.
- C/EBPβ bound to promoter region of *Nampt*.
- NAMPT-mediated NAD synthesis is involved in adipogenesis of 3T3-L1 cells.

Graphical Abstract



Intracellular cAMP contents regulate NAMPT expression via induction of C/EBPβ in adipocytes

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Running title: cAMP induces NAMPT expression

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Abstract

A decline in intracellular nicotinamide adenine mononucleotide (NAD^+) causes adipose tissue dysfunction. Nicotinamide phosphoribosyltransferase (NAMPT) the rate-limiting step in the NAD^+ biosynthesis pathway. However, the molecular mechanism that mediates regulation of NAMPT expression in adipocytes is yet to be elucidated. This study found that intracellular cAMP regulates NAMPT expression and promoter activity in 3T3-L1 adipocytes. cAMP-mediated *Nampt* promoter activity was suppressed by protein kinase A inhibitor H89, whereas AMP-activated protein kinase inhibitor compound C did not affect cAMP-mediated *Nampt* promoter activity. Intracellular cAMP induced CCAAT/enhancer-binding protein β (C/EBPβ) expression. Knockdown of C/EBPβ suppressed NAMPT expression and promoter activity. Furthermore, the *Nampt* promoter was activated by C/EBPβ, while LIP activated the dominant-negative form of C/EBPβ. Promoter sequence analysis revealed that the region from −96 to −76 on *Nampt* was required for C/EBPβ-mediated promoter activity. Additionally, chromatin immunoprecipitation assay demonstrated that C/EBPβ was bound to the promoter sequences of *Nampt*. Finally, NAMPT inhibitor FK866 suppressed adipogenesis in 3T3-L1 cells, and this suppressive effect was restored by

nicotinamide mononucleotide treatment. These findings showed that intracellular cAMP increased NAMPT levels by induction of C/EBPβ expression and indicated that the induction of NAMPT expression was important for adipogenesis.

Keywords; adipogenesis; cAMP; CCAAT-enhancer-binding protein β; nicotinamide adenine dinucleotide; nicotinamide phosphoribosyltransferase

Abbreviations: CCAAT/enhancer-binding protein, C/EBP; 3-isobutyl-1-methylxanthine, IBMX; nicotinamide adenine dinucleotide, NAD^+; nicotinamide phosphoribosyltransferase, NAMPT; peroxisome proliferator-activated receptor, PPAR.

1. Introduction

Adipocytes store triglycerides as energy and are involved in secretory functions via cytokines, known as adipokines. Secretory adipokines, such as leptin, adiponectin, and interleukin-6, are carried to distant organs via the circulatory system; they maintain metabolic functions, including appetite, body weight, insulin sensitivity, and inflammation. Therefore, adipocyte dysfunction causes metabolic diseases, such as type 2 diabetes mellitus, cardiovascular disease, and hypertension [1,2].

Adipogenesis is a unique process of adipocyte differentiation, which is regulated by transcriptional factors such as CCAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptor γ (PPARγ), and sterol regulatory element-binding protein 1 (SREBP-1) [3,4]. The second messenger molecule cAMP increases in the early stage of adipogenesis. Intracellular cAMP stimulates two signal pathways, namely protein kinase A (PKA) and AMP-activated protein kinase (AMPK) pathways [5,6]. PKA signaling mediates the induction of C/EBPβ expression [5]. Subsequently, C/EBPβ induces the expression of PPARγ and C/EBPα to promote adipogenesis. C/EBPβ mRNA is translated into three different isoforms, full-length C/EBPβ, LAP, and LIP, by alternative translation of the start codon [7]. LAP provides

transactivation capacity and is associated with adipogenesis, whereas LIP represents a strongly shortened isoform, which is transcriptionally inactive and supports proliferation [8].

Nicotinamide adenine dinucleotide (NAD^+) plays a vital role in cellular energy metabolism and homeostasis pathways in multiple organs. In addition, NAD^+ acts as a multifunctional coenzyme by modulating the key NAD^+-dependent enzymes, such as sirtuins and poly (ADP-ribose) polymerases [9]. Through these activities, NAD^+ is involved in cellular processes that regulate signaling and transcriptional events, such as survival, stress-response, circadian rhythm, and aging. Thus, a decline in NAD^+ content causes cellular dysfunction and is particularly problematic for adipocytes. NAD^+ is synthesized in the *de novo* and salvage pathways. In the salvage pathway, nicotinamide (an NAD^+ precursor) is metabolized into nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT), which is the rate-limiting enzyme in the NAD^+ salvage pathway. Subsequently, NMN is rapidly converted into NAD^+ by nicotinamide mononucleotide adenylyltransferase [9-11]. NAMPT is also known as visfatin, and its expression is gradually increased in adipogenesis [12]. NAMPT expression in adipocytes is regulated by environmental and nutritional conditions. For

example, diet-induced obesity decreases NAMPT expression levels and NAD^+ content, resulting in increased metabolic dysfunction [13,14]. In contrast, calorie restriction increases NAMPT expression and NAD^+ content [15]. However, the molecular mechanism by which NAMPT expression is regulated in the adipocytes has yet to be elucidated. In this study, we determined the role of cAMP in the induction of NAMPT expression in the 3T3-L1 adipocytes.

2. Material and methods

2.1. Cell culture

Murine 3T3-L1 preadipocytes were obtained from the Japanese Collection of Research Bioresources (IFO050416), and the culture and adipocyte differentiation methods were described previously [16]. Briefly, for induction of adipocyte differentiation, confluent cells were treated with a DMI cocktail [1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 10 μg/mL insulin] in Dulbecco's modified Eagle's medium with high glucose (4.5 g/L glucose), supplemented with 10% fetal bovine serum for the first 2 days. Then the cells were cultured in the same medium with insulin for another 5 days. The medium was changed every 2 days.

2.2. siRNA oligonucleotides

Double-stranded siRNA for mouse C/EBPβ (siC/EBPβ) was chemically synthesized by Sigma-Aldrich, and the sequences for the siRNA duplexes were as follows: siC/EBPβ 5′-GAGCGACGAGTACAAGAT-3′. The control siRNA (siCTL) was purchased from Sigma-Aldrich (MISSION siRNA Universal Negative Control#1). The duplexes (20 nM) were transfected into the 3T3-L1 cells using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) for 24 h, according to the manufacture's protocol.

2.3. Plasmids

Myc tag was inserted into a pLVSIN-CMV Pur vector (Takara Bio., Shiga, Japan), creating pLVSIN-Myc. Then mouse full-length C/EBPβ (1-296 amino acid), splicing variant C/EBPβ (LIP; 152-296 amino acid) cDNAs were amplified by PCR and subcloned into the pLVSIN-Myc vector, yielding a C/EBPβ expression vector with N-terminal Myc tag (pLVSIN-Myc-C/EBPβ and pLVSIN-Myc-LIP). Mouse PPARγ cDNAs were amplified by PCR and subcloned into a pLVSIN-Myc vector, yielding a

Myc-tag fused PPARγ expression vector (pLVSIN-Myc- PPARγ). The promoter region of the mouse *Nampt* gene (−2000/+72) was amplified by PCR using mouse genomic DNA from adipose tissues as a template. The amplified DNA was subcloned into the pGL4.14 plasmid (Promega), and termed pGL4-*Nampt* (−2000/+72). We then generated other reporter vectors, which were inserted at various lengths into the *Nampt* promoter (−1500/+72, −1000/+72, −500/+72, −50/+72, −2000/−501, −2000/−51 and −500/−51), which was constructed using pGL4-*Nampt* (−2000/+72) as a template. The introduction of mutations from −91 to −83 into the promoter region of *Nampt* was performed by two-step PCR, and the resultant DNAs were subcloned into pGL4.14 and termed pGL4-*Nampt* (−500/+72 Mut). The nucleotide sequence of the *Nampt* promoter changed from 5′-TTAAGCAA-3′ to 5′-AATTCGTT-3′. The pGL4-3×PPRE-Luc plasmid was constructed by the insertion of three tandem repeats of PPARγ responsive elements (PPREs) in pGL4.14. The resultant sequence of the PPREs oligonucleotide is as follows:

5′-AGGTCAAAGGTCAGACAGGTCAAAGGTCAGACAGGTCAAAGGTCA-3′.

2.4. Measurement of intracellular lipid and culture adiponectin contents, and cell

survival

Adipocyte differentiation was induced by treatment with a DMI cocktail for 7 days. The cells were fixed with 4% paraformaldehyde in PBS and incubated with Oil red O solution (0.5% w/v) for 10 min at 23°C. Following staining, cells were washed, and the lipid contents were extracted using isopropanol containing 4% (v/v) TritonX-100. The extracted dye was measured at an absorbance of 492 nm. The culture adiponectin contents were measured using a commercial ELISA kit (Shibayagi, Gunma, Japan). Cell survival was determined by crystal violet staining assay, as described previously [16]. Briefly, the cells were incubated with the indicated concentrations of FK866 for 72 h. The cells were fixed and stained. Then, the dye was extracted, and the absorbance was measured at 595 nm with a reference wavelength at 630 nm.

2.5. Luciferase reporter assay

The cells were transiently transfected with various reporter vectors [*Nampt* promoter-inserted pGL4.14, pGL4-3×PPRE-Luc, and pRL-SV40 (control reporter vector; Promega)] using Lipofectamine 3000 (Thermo Fisher Scientific). Following 24 h transfection, the medium was changed to fresh medium, and the cells were incubated

with or without compounds for 24 h. Luciferase activities were measured using the dual-luciferase reporter assay kit and GloMax 20/20 Luminometer (Promega).

2.6. Western blotting

Western blotting was performed as described previously [16]. Briefly, the cell lysates were subjected to SDS-PAGE and analyzed by western blotting using the following mouse monoclonal antibodies: anti-β-actin (1/10000, clone; C4), anti-PPARγ (1/5000, clone; E-8, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Myc (1/3000, clone; MC045, Nacalai Tesque, Kyoto, Japan); other antibodies used were rabbit polyclonal anti-NAMPT (1/5000, GeneTex, Irvine, CA) and C/EBPβ (1/5000, Bethyl Laboratories, Edison, NJ) antibodies. The immunoreactive proteins were visualized using LAS500 (GE healthcare). The intensity of each band was quantified using ImageJ (version 1.44o; NIH, Bethesda, MD), and the ratio of each protein level was normalized to that of the β-actin (loading control) level.

2.7. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously [16]. Briefly, cells were incubated with

IBMX for 24 h, followed by fixation and lysis. The cell lysates were incubated with rabbit polyclonal anti-C/EBPβ IgG or control rabbit IgG overnight at 4ºC. Then protein-DNA complexes were washed and eluted with the fragments. The promoter regions of *Nampt* were amplified by PCR using the following primer sets; P1 (5′-CAAAGGCCTTGAGAACCAGAGC-3′ and 5′-CTTGTGAGACTATGCCGGGG-3′); P2 (5′-CGCGCTCCGTTCCCTGCTCT-3′ and 5′-GCGGCTGCGAGCAAGGAGAAAA-3′).

2.8. Statistical analysis

Data were analyzed by one- or two-way analysis of variance via Tukey's post hoc or Dunnett post hoc testing. Statistical analysis was performed with JMP statistical software version 11.2.0 (SAS Institute. Cary, NC). Data are expressed as means ± SD, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. cAMP stimulates Nampt promoter activity in 3T3-L1 cells

We examined the effect of differentiation inducers on the promoter activity of *Nampt*

in 3T3-L1 cells using luciferase reporter assay. Although insulin and dexamethasone did not stimulate *Nampt* promoter activity, IBMX increased its promoter activity in a dose-dependent manner (Fig. 1A and 1B). Adipocyte differentiation increased NAMPT protein levels, and IBMX enhanced NAMPT protein levels in the differentiated 3T3-L1 adipocytes (Fig. 1C), while IBMX did not affect the protein levels of PPARγ. Phosphodiesterase (PDE) is an enzyme that breaks cAMP into AMP, and IBMX increases intracellular cAMP contents by inhibiting PDE function. Thus, to determine whether increases in cAMP contents could activate *Nampt* promoter, 3T3-L1 cells were incubated in the presence of other cAMP inducers. Forskolin increased intracellular cAMP contents by stimulation of adenylate cyclase. Forskolin and cAMP analog bucladesine increase the *Nampt* promoter activity and NAMPT protein levels (Fig. 1D and 1E). IBMX-mediated promoter activity suppresses H89, an inhibitor of PKA signaling, but did not suppress the AMPK inhibitor compound C (Fig. 1F). These results indicated that the *Nampt* promoter activity was enhanced by cAMP-PKA signaling in the 3T3-L1 adipocytes.

3.2. C/EBPβ is involved in Nampt promoter activity in the adipocytes

IBMX induced C/EBPβ expression in the 3T3-L1 cells [17]. We determined whether the involvement of the C/EBPβ protein-induced NAMPT expression using C/EBPβ-specific siRNA. NAMPT protein levels increased significantly in the control siRNA-treated 3T3-L1 cells at 5-7 days following the start of adipocyte differentiation (Fig. 2A). While C/EBPβ knockdown was suppressed, an increase in adipocyte differentiation-induced NAMPT protein levels. The knockdown of C/EBPβ also suppresses IBMX-stimulated *Nampt* promoter activity (Fig. 2B). Furthermore, when the C/EBPβ expression vector is transfected into 3T3-L1 cells, *Nampt* promoter activity is induced by exogenous C/EBPβ (Fig. 2C). However, exogenous C/EBPβ did not influence the empty luciferase vector. *Cebpb*-encoded protein isoforms, LIP (152-296) were examined for their effect on the *Nampt* promoter activity. The results showed that the C/EBPβ full length and LIP isoforms increased promoter activity, and LIP was more potent than C/EBP full length in the upregulation of the *Nampt* promoter activity (Fig. 2D). We determined whether C/EBPβ-induced PPARγ expression was involved in *Nampt* promoter activity. Rosiglitazone, a PPARγ ligand, stimulated the transcriptional activity of the 3×PPRE-Luc but did not influence the *Nampt* promoter activity (Fig. 2E). These results indicated that the *Nampt* promoter activity was regulated by the C/EBPβ

protein, but not by PPARγ.

3.3. Located from −96 to −76 bp is required for the C/EBPβ-induced Nampt promoter activity

To identify the C/EBPβ responsive region of the *Nampt* promoter, we generated luciferase reporter constructs, which contained six series of deletion constructs of the *Nampt* promoter (Fig. 3A, *left panels*). The promoter activity was driven by −2000/+72, −1500/+72, −500/+72, and −2000/−51 *Nampt* promoters (Fig. 3A, *right panels*), induced by IBMX, while deletion of the sequence from −500 to −51 bp abolished IBMX responsiveness. Multi-genome analysis using the database showed that the regions located at −1934/–1925 and −96/–76 in the *Nampt* promoter region contained the C/EBPβ-binding element candidate, and the region from −96 to –76 bp was highly conserved among mammalians, such as mice, humans, chimpanzee, rats, cattle, and pigs (Fig. 3B). Therefore, we generated mutant constructs located from −96 to –76 bp in the *Nampt* promoter region. In the mutant construct, IBMX, and exogenous C/EBPβ did not influence the *Nampt* promoter activity (Fig. 3C). Furthermore, we determined whether C/EBPβ could bind to the promoter region of *Nampt* using a ChIP assay with

specific-primer sets, as depicted in Fig. 3D (*upper panel*s). IBMX treatment increased the levels of C/EBPβ on the DNA, which were located at −138/–41 of the *Nampt* promoter region. While IBMX treatment did not affect the interaction between C/EBPβ and the DNA in the *Nampt* promoter region from −2144 to –1828 (Fig. 3D, *bottom panels*). These results indicated that C/EBPβ induced NAMPT expression via binding to the promoter region (−97/−76) on *Nampt* in 3T3-L1 cells.

3.4. NAMPT promotes adipogenesis in 3T3-L1 cells.

High-fat diet-induced adipose expansion was suppressed in the NAMPT knockout mice [18]. We observed that NAMPT inhibitor FK866 decreased the intracellular lipid contents in 3T3-L1 cells (Fig. 4A). In addition, the treatment of NMN restored the FK866-decreased lipid contents. By contrast, FK866 did not influence cell survival in the 3T3-L1 cells (Fig. 4B). Interestingly, NAMPT inhibition decreased the production of adiponectin that was an insulin-sensitizing adipokine, and this effect was canceled by the NMN treatment (Fig. 4C). These results indicated that NAMPT-mediated NAD^+ synthesis was involved in adipogenesis of 3T3-L1 cells.

4. Discussion

NAMPT is a rate-limiting enzyme in the NAD^+ biosynthesis pathway, and a decline in NAMPT could cause a decrease in intracellular NAD^+. NAMPT expression and NAD^+ were decreased in the diet-induced obesity and aging mice and resulted in the development of type 2 diabetes mellitus [19,13]. Therefore, supplementation with NAD^+ or increasing the expression of NAMPT could lead to the prevention of obesity- or aging-associated diseases. A previous report has indicated that NAMPT expression was induced during adipogenesis in the adipocytes [12]. However, it was unclear how NAMPT affected adipogenesis in the adipocytes, as the regulatory mechanism of NAMPT expression has not yet been elucidated. In this study, we revealed that NAMPT was involved in the adipogenesis of the 3T3-L1 cells. In addition, our report was the first to show that cAMP-induced C/EBPβ was the primary regulator of *Nampt* expression during adipogenesis.

NAMPT inhibition suppressed adipogenesis and adiponectin production in the 3T3-L1 cells. In the fat-specific NAMPT knockout mice, the high-fat diet interfered with the healthy expansion of the adipose tissue mass, and adipose deposits resulted in adipose tissue fibrosis [18]. Adipocyte hypertrophy induced insulin resistance via the

secretion of pro-inflammatory adipokines from the adipocytes [9]. By contrast, healthy adipocyte differentiation is associated with improved insulin resistance through the secretion of insulin-sensitizing adipokine, adiponectin in the adipocytes [20]. The knockout of NAMPT reduced adiponectin expression in the adipose tissue, and adipose tissue expression was restored by NMN administration [19]. Thus, these results indicated that NAMPT contributed to the healthy functions of the adipocyte as well as adipogenesis.

Nampt expression was stimulated by the full-length C/EBPβ and LIP. C/EBPβ plays a pivotal role in adipogenesis and induces the expression of PPARγ [21]. The LIP isoform is regarded as a dominant-negative inhibitor for the full-length C/EBPβ, and overexpression of LIP resulted in anti-adipogenic activity [8]. However, Bégay et al. (2018) reported that the LIP isoform was sufficient to function in the development of adipose in the *Cebpb* knockout mice, and proposed that the LIP isoform likely had more physiological functions than its known role as a dominant-negative inhibitor [22]. These results indicated that C/EBPβ promoted adipogenesis through two independent pathways, such as the C/EBPβ-mediated PPARγ or NAMPT induction pathways.

There are multiple cAMP-binding proteins in all cells. cAMP-bound Epac1 enhanced

phosphorylation of AMPK, an intracellular energy sensor, as it increased the intracellular calcium concentration [23]. In the skeletal muscle, exercise-mediated AMPK activation increased the *Nampt* expression [24]. However, our data showed that the AMPK inhibitor compound C did not influence the IBMX-induced *Nampt* promoter activity (Fig. 1F). cAMP-PKA signaling-activated CREB positively controls the C/EBPβ expression [25]. In addition, CREB improved the protein stability of C/EBPβ as it induced SUMO-specific protease [26]. Therefore, our results indicated that cAMP signaling contributed to *Nampt* promoter activity through increased C/EBPβ expression levels and protein stability in the adipocytes.

C/EBPβ was bound to the −138/−42 in the *Nampt* promoter region of the 3T3-L1 cells. There were two candidate C/EBPs-binding sequences in the −2000/+72 *Nampt* promoter region. Specifically, the −96/–76 region showed high homology between a mouse and human, which indicated that the regulation of *Nampt* expression in the human adipocytes was by the same mechanism characterized in this study. Furthermore, another research group has found a transcription factor that binds to DNA near the transcription start site of *Nampt*, which was similar to C/EBPβ [27]. Yoon et al. (2017) found that SREBP-1c stimulated *Nampt* promoter activity in the murine pancreatic

islets, and the region at −450/−455 of *Nampt* was important for SREBP-1c as it stimulated promoter activity [27]. SREBP-1c expression increased during differentiation in the adipocytes [28]. These results suggested that *Nampt* expression was additively or synergistically regulated by transcriptional factors, which could bind near the transcription start site, such as C/EBPβ and SREBP-1c during adipogenesis.

In summary, we showed that cAMP contents regulated *Nampt* expression in adipocytes and NAMPT contributed to adipogenesis. Selective PDE inhibitors have been identified as therapeutic agents for hypertension and coronary heart disease to increase the intracellular cAMP contents [29]. Thus, these selective PDE inhibitors might contribute to healthy adipogenesis and the maintenance of adipocyte function through the regulation of NAMPT levels.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1. Effect of cAMP contents on the promoter activity of *Nampt* in 3T3-L1 cells. (A)

Nampt promoter activity in 3T3-L1 cells transiently transfected with pGL4-*Nampt* (−2000/+72) vector, then incubated with insulin (10 μg/mL), DEX (1 μM), or IBMX (0.5 mM) for 24 h. (B) Luciferase reporter assay in 3T3-L1 cells is transiently transfected with pGL4-*Nampt* (−2000) vector, then incubated with various concentrations of IBMX for 24 h. (C) NAMPT and PPARγ protein levels in 3T3-L1 cells are treated with IBMX (0.5 mM) for 24 h. (D) *Nampt* promoter activity in 3T3-L1 cells treated with cAMP inducers, such as bucladesine (cAMP analogue; 100 μM) and forskolin (adenylyl cyclase activator; 10 μM). (E) NAMPT protein levels in 3T3-L1 cells treated with bucladesine (100 μM) and forskolin (10 μM). *$p < 0.05$ versus vehicle groups. (F) *Nampt* promoter activity in 3T3-L1 cells treated with H89 (1 μM) or compound C (5 μM) in the presence of IBMX (0.5 mM) for 24 h. Error bars represent the mean ± SD (n = 4). In (C) and (E), the band intensities of NAMPT or PPARγ are normalized to those of β-actin, and the relative values for the vehicle are indicated under the lower panels. Significant differences ($p < 0.05$) are indicated by the corresponding letters. All data shown are representative of triplicate independent experiments.

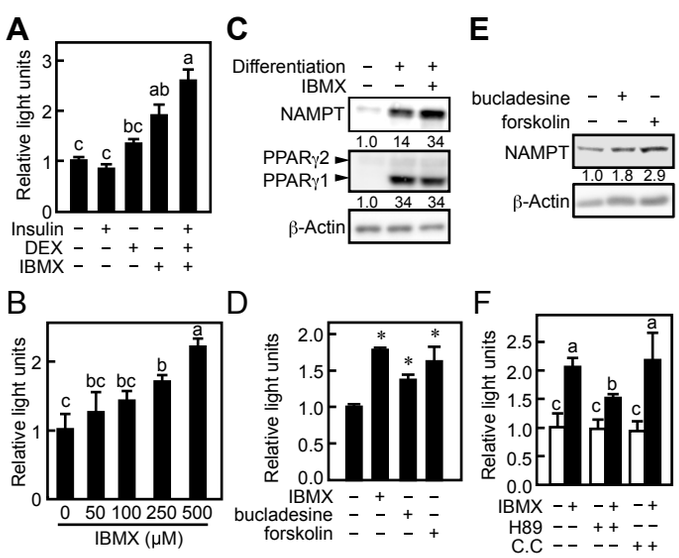
Fig. 2. Involvement of C/EBPβ in *Nampt* promoter activity. (A) NAMPT protein levels in 3T3-L1 cells transfected with the control siRNA (siCTL) or C/EBPβ-specific siRNA (siC/EBPβ). Following siRNA transfection, adipogenesis is induced for 7 days. The band intensities of NAMPT and C/EBPβ are normalized to those of β-actin, and the relative values to Day 0 are indicated under the lower panels. n.d. (not detected) (B) *Nampt* promoter activity in 3T3-L1 cells treated with siRNA. Following transfection with siCTL or siC/EBPβ, the cells are transiently transfected with pGL4-*Nampt* (−2000/+72) vector, followed by incubation with or without IBMX (0.5 mM) for 24 h. (C) *Nampt* promoter activity (upper panel) and Myc-C/EBPβ protein levels (bottom panel) in the 3T3-L1 cells. The cells are transiently transfected with luciferase reporter vectors [pGL4.14 (open bars) or pGL4-*Nampt* (−2000/+72) (closed bars)] and Myc-C/EBPβ expression vector, and incubated for 24 h. (D) *Nampt* promoter activity in full-length C/EBPβ (FL) or LIP expressed 3T3-L1 cells. (E) The cells are transiently transfected with luciferase reporter vectors and Myc-PPARγ expression vector, followed by incubation with or without rosiglitazone (Rosi; 10 μM) for 24 h. *$p < 0.05$ versus Rosi non-treated group. Error bars represent the mean ± SD (n = 4). Significant differences ($p < 0.05$) are indicated by the corresponding letters. All data shown are

representative of triplicate independent experiments.

Fig. 3. Identification of C/EBPβ-interaction with the promoter region in *Nampt*. (A) Schematic representation of the reporter plasmids (*left panels*). *Nampt* promoter activity in 3T3-L1 cells transiently transfected with reporter vectors, followed by incubation with IBMX for 24 h (*right panels*). (B) DNA sequences of various mammalian *Nampt* promoters from −98 to −76. Conserved DNA sequences are shown in the gray sheet. Underline shows a candidate sequence for the C/EBP-binding element. (C) Comparison of *Nampt* promoter activity in 3T3-L1 cells transfected with pGL4-*Nampt* (−500/−51) or pGL4-*Nampt* (−500/−51 Mut), followed by incubation with IBMX (0.5 mM) for 24 h. (D) Schematic representation in the promoter regions of *Nampt*. The open-box indicates putative C/EBP-binding elements. Double-headed arrows indicate the DNA regions amplified using PCR primer sets designated as P1 and P2 (*upper panel*). ChIP assay in 3T3-L1 cells incubated with IBMX (0.5 mM) for 24 h. Co-immunoprecipitated protein-DNA complex is analyzed by PCR (*bottom panels*). Error bars represent the mean ± SD (n = 4). All data shown are representative of triplicate independent experiments.

Fig. 4. Effect of NAMPT on adipogenesis in 3T3-L1 cells. (A) Lipid accumulation in 3T3-L1 cells after adipocyte differentiation in the presence or absence of FK866 (100 nM, NAMPT inhibitor) and NMN (100 μM) for 7 days. (B) Cell survival of 3T3-L1 cells treated with the indicated concentrations of FK866 for 72 h. (C) Measurement of secretory adiponectin from 3T3-L1 cells following adipocyte differentiation with FK866 (100 nM) or NMN (100 μM) for 7 days. Error bars represent the mean ± SD (n = 4). Significant differences ($p < 0.05$) are indicated by the corresponding letters. All data shown are representative of triplicate independent experiments.

Figure 1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Relative light units
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2
1
0
c
c
bc
ab
a
Insulin − + − − +
DEX − − + − +
IBMX − − − + +
B
Relative light units
2
1
0
c
bc
bc
b
a
0 50 100 250 500
IBMX (μM)
C
Differentiation − + +
IBMX − − +
NAMPT
1.0 14 34
PPARγ2
PPARγ1
1.0 34 34
β-Actin
D
Relative light units
2.0
1.5
1.0
0.5
0.0
*
*
*
IBMX − + − −
bucladesine − − + −
forskolin − − − +
E
bucladesine − + −
forskolin − − +
NAMPT
1.0 1.8 2.9
β-Actin
F
Relative light units
2.5
2.0
1.5
1.0
0.5
0.0
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a
c
b
c
a
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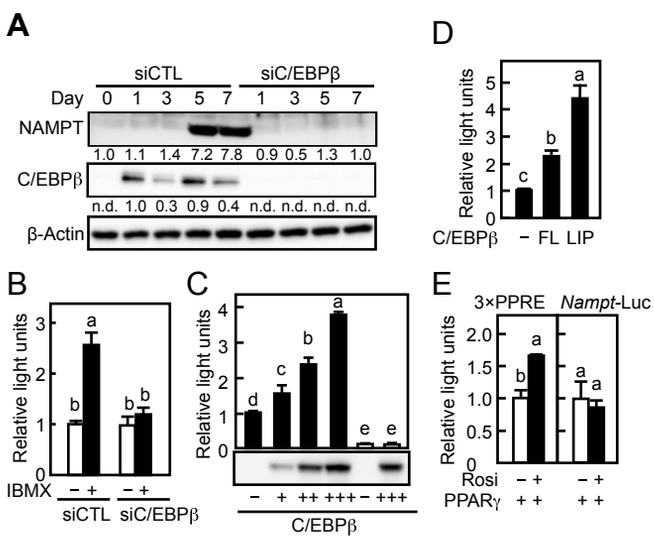
H89 − − + + − −
C.C − − − − + +

Figure 2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siCTL
siC/EBPβ
Day 0 1 3 5 7 1 3 5 7
NAMPT
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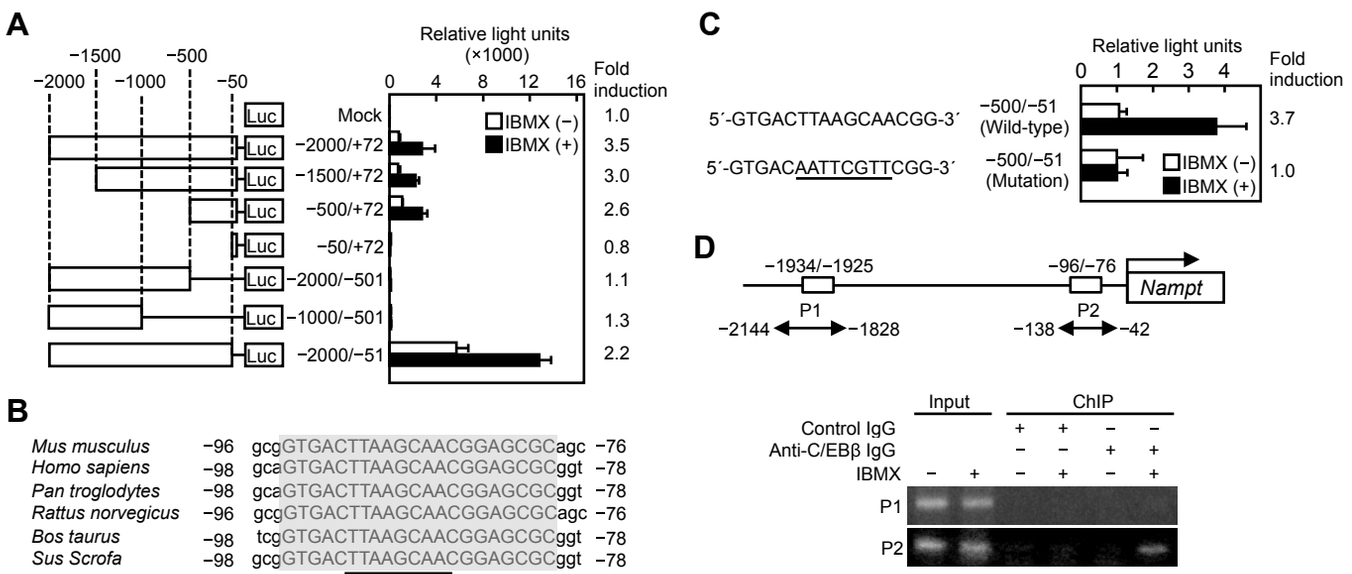
C/EBPβ
n.d. 1.0 0.3 0.9 0.4 n.d. n.d. n.d. n.d.
β-Actin
B
Relative light units
IBMX – + – +
siCTL siC/EBPβ
C
Relative light units
– + ++ +++ – +++
C/EBPβ
D
Relative light units
C/EBPβ – FL LIP
E
3×PPRE Nampt-Luc
Relative light units
Rosi – + – +
PPARγ + + + +

Figure 3


A
−1500
−500
−2000
−1000
−50
Relative light units
(×1000)
0
4
8
12
16
Fold induction
Luc
Mock
1.0
IBMX (−)
IBMX (+)
−2000/+72
3.5
−1500/+72
3.0
−500/+72
2.6
−50/+72
0.8
−2000/−501
1.1
−1000/−501
1.3
−2000/−51
2.2
B
Mus musculus −96 gcgGTGACTTAAGCAACGGAGCGCagc −76
Homo sapiens −98 gcaGTGACTTAAGCAACGGAGCGCggt −78
Pan troglodytes −98 gcaGTGACTTAAGCAACGGAGCGCggt −78
Rattus norvegicus −96 gcgGTGACTTAAGCAACGGAGCGCagc −76
Bos taurus −98 tcgGTGACTTAAGCAACGGAGCGCggt −78
Sus Scrofa −98 gcgGTGACTTAAGCAACGGAGCGCggt −78
C
Relative light units
0
1
2
3
4
Fold induction
5′-GTGACTTAAGCAACGG-3′
−500/−51
(Wild-type)
3.7
5′-GTGACAATTCGTTCGG-3′
−500/−51
(Mutation)
IBMX (−)
IBMX (+)
1.0
D
−1934/−1925
−96/−76
Nampt
P1
P2
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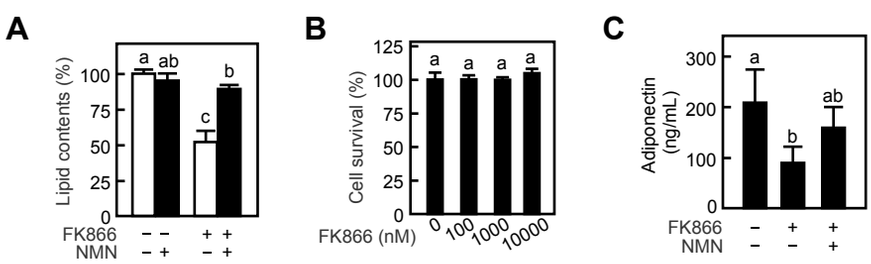
−1828
−138
−42
Input
ChIP
Control IgG
Anti-C/EBβ IgG
IBMX
P1
P2

Figure 4


A
Lipid contents (%)
100
75
50
25
0
a
ab
c
b
FK866 – – + +
NMN – + – +
B
Cell survival (%)
125
100
75
50
25
0
a
a
a
a
FK866 (nM) 0 100 1000 10000
C
Adiponectin (ng/mL)
300
200
100
0
a
b
ab
FK866 – + +
NMN – – +