Title.

The AMPK/mTOR pathway is involved in D-dopachrome tautomerase gene transcription in adipocytes differentiated from SGBS cells, a human preadipocyte cell line

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Abstract

In adipose tissue, D-dopachrome tautomerase (DDT), a cytokine with structural similarity to macrophage migration inhibitory factor, is mainly expressed in adipocytes rather than preadipocytes and acts as an anti-obesity adipokine in an autocrine manner. However, its transcriptional regulation is largely unknown. In order to explore molecules affecting *DDT* transcription, a chemical library screening using HEK293 cells stably expressing a *DDT* promoter-reporter construct was performed. Several derivatives of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an AMP-activated protein kinase (AMPK) activator, were identified as transcriptional activators of the *DDT* gene. Furthermore, *DDT* mRNA levels were reduced in SGBS adipocytes treated with compound C, an AMPK inhibitor, suggesting involvement of AMPK in *DDT* transcription. Overexpression of the FOXO1 constitutive active form reduced transcriptional activity of the *DDT* gene in SGBS cells, but increased it in HEK293 cells. Cell-type specific effects were also observed in the *DDT* gene expression of cells treated with AS1842856, a FOXO1 inhibitor. Finally, involvement of the mammalian target of rapamycin (mTOR) signaling in *DDT* transcription in SGBS adipocytes was investigated. Rapamycin, an inhibitor of mTOR, increased *DDT* mRNA levels and attenuated the inhibitory effects of compound C on *DDT* mRNA levels in SGBS adipocytes. In conclusion, *DDT* transcription may be regulated in a cell-dependent manner, and were enhanced by AMPK activation in SGBS adipocytes through inhibiting the mTOR signaling.

Keywords

D-dopachrome tautomerase; adipose tissue; AMPK; FOXO1; mTOR

Abbreviations

DDT, D-dopachrome tautomerase; MIF, macrophage migration inhibitory factor; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; S6K, S6 kinase; FOXO1, forkhead transcription factor O1; DMSO, dimethyl sulfoxide; qRT-PCR, quantitative real-time reverse transcription PCR; FOXO1(CA), constitutive active form of FOXO1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA binding protein; SDS, sodium dodecyl sulfate; ChIP, Chromatin immunoprecipitation; DEPP, decidual protein induced by progesterone; mTOR, the mammalian target of rapamycin

1. Introduction

Adipose tissue is not only an organ contributing to energy homeostasis by storage and supply of energetic molecules, but is also an endocrine organ secreting biologically active proteins termed “adipokines” [1]. Adipokines have important roles in health maintenance by regulating diverse processes including appetite and satiety, fat distribution, inflammation, blood pressure, hemostasis, and endothelial function [2]. In the previous study, D-dopachrome tautomerase (DDT) was found as an adipokine that improved insulin resistance caused by obesity and regulated lipid metabolism in adipocytes [3].

DDT has been originally identified as an enzyme that converts D-dopachrome into 5,6-dihydroxyindole, but its physiological significance is unknown [4]. Recently, DDT has come to be regarded as a family member of macrophage migration inhibitory factor (MIF), a cytokine involved in the amplification of inflammatory and immune responses, due to similarities in structure and function [5-8]. DDT acts co-operatively with MIF through CD74, a MIF receptor, to activate the proinflammatory pathway [8] and to promote tumor growth [7, 9, 10]. However, DDT has been reported to have distinct functions from MIF in adipose tissue. MIF knockout mice exhibit improvement of insulin sensitivity in adipose tissue by an increase of the glucose uptake [11] and reduction of the macrophage infiltration [12], suggesting the unfavorable function of MIF in adipose tissue. On the other hand, administration of recombinant DDT ameliorates the glucose intolerance of obese mice [3] and inhibits adipogenesis in SGBS cells, a human preadipocyte cell line [13]. More recently, DDT was demonstrated to accelerate wound healing in adipose tissue in the presence of neutralizing anti-MIF antibody and the ability of DDT to recruit macrophages into the inflamed adipose tissue was lower than that of MIF [14]. These suggest that DDT has more beneficial roles than MIF in adipose tissue.

Differences in gene expression between DDT and MIF in adipose tissue have been reported. MIF is expressed in both adipocytes and preadipocytes, and its mRNA levels were not elevated during adipogenesis [15]. *MIF* mRNA levels in subcutaneous abdominal adipocytes are positively associated with adipocyte size and insulin resistance [16]. On the other hand, *DDT* mRNA levels are increased in an adipocyte differentiation-dependent manner and the mRNA levels in human adipocytes are negatively correlated with obesity-related clinical parameters such as body mass index, and visceral and subcutaneous fat areas [3]. Thus, transcriptional regulation of the *DDT* gene may be different from that of *MIF* in adipose tissue; however, the

underlying transcriptional mechanism is largely unexplored.

In order to uncover the molecular mechanisms of transcriptional regulation of the *DDT* gene in adipocytes, we first explored the molecules that influence transcription from a chemical library using HEK293 cells stably expressing a *DDT* promoter-reporter construct, and then validated the effects using SGBS cells and the differentiated adipocytes (SGBS adipocytes). Consequently, we found that 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and its derivatives enhance *DDT* transcription; therefore, we examined the signaling pathway.

2. Materials and Methods

2.1. Materials

AICAR, compound C, three kinds of siRNAs against human *SIRT1* (Mission siRNA: Hs_SIRT1_3666, 3669, 3671) and the control siRNAs, and mouse anti-β-actin antibody were purchased from Sigma (St Louis, MO, USA). AS1842856 was purchased from Millipore (Billerica, MA, USA). Rapamycin was purchased from Tokyo Chemical Industry (Tokyo, Japan). Rabbit antibodies against AMP-activated protein kinase (AMPK)-α, phosphorylated AMPKα (Thr-172), S6 kinase (S6K), and phosphorylated S6K were purchased from Cell signaling (Danvers, MA, USA). Rabbit anti-forkhead transcription factor O1 (FOXO1) antibody was purchased from Abcam (Cambridge, UK). Rabbit anti-acetylated FOXO1 (Ac-FKHR (D-19)) antibody was purchased from SantaCruz Biotechnologies (Santa Cruz, CA, USA).

2.2. A Chemical library

A chemical library, including natural products and their synthetic intermediates, heterocyclic compounds, peptides, and nucleosides (1,599 compounds) from the Graduate School of Pharmaceutical Science, Tokushima University was prepared as an approximately 10 mM stock solution in dimethyl sulfoxide (DMSO).

2.3. Cell culture

SGBS cells were maintained and made to differentiate into adipocytes as described by Wabitsch *et al.* [17]. The differentiated adipocytes were used in experiments on day 7 after adipogenic induction. HEK293 cells were cultured with Dulbecco's modified eagle medium containing 10% fetal bovine serum. These cells were treated with each chemical compound for 24 h or indicated time, and then used for quantitative real-time reverse transcription PCR (qRT-PCR) and western blotting. Cells treated with equal amounts of DMSO were used as controls. Transfection to SGBS cells and HEK293 cells was performed using the Neon Transfection System (Thermo Fisher Scientific, Waltham, MA, USA) and Effectene Transfection Reagent (Qiagen, Valencia, CA, USA), respectively.

2.4. Construction of reporter and expression vectors

A putative promoter region (-2,903/+135 from the transcription start site of the *DDT* gene)

and the deletion mutants were amplified from genomic DNA extracted from HEK293 cells. PCR products were inserted into the pGL4.17 luciferase vector (Promega, Madison, WI, USA) or pGL4.17 without the putative FOXO1 binding motifs (nucleotides at position from 5,526 to 5,547 in pGL4.17), which was made using site-directed mutagenesis. Then, construction of each mutated luciferase reporter plasmid with lacking putative FOXO1 binding motif(s) (-1543 to -1536 and/or -172 to -166) in the *DDT* promoter was carried out by standard PCR-based site-directed mutagenesis. The cDNA encoding human FOXO1 was amplified from total RNA extracted from SGBS cells by RT-PCR using each specific primer set and was inserted into the expression vector, pcDNA3.1+. An expression plasmid for a constitutive active form of FOXO1 (FOXO1(CA)), a triple alanine mutant of FOXO1 at T24, S256, and S319 sites, was made using site-directed mutagenesis.

2.5. Luciferase assay

Firefly and *Renilla* luciferase activities were measured using the Dual Luciferase® Reporter Assay System (Promega) on the TriStar LB 941 Multi-label plate reader (Berthold Technologies, Pforzheim, Germany) 24 h after co-transfection of reporter vectors and *Renilla* luciferase vector (pGL4.74; Promega). The firefly luciferase activity was normalized by the *Renilla* luciferase activity.

2.6. Screening from the chemical library

HEK293 cells stably expressing the *DDT* promoter (-2,903/+135) reporter vector were selected in medium containing G418 (Sigma) for 2 weeks after transfection. The cells were seeded at 10,000 cells/well in white 96-well plates and incubated for 2 days. Then, the cells were treated with each 10 μM compound from the chemical library or equal amount of DMSO for 24 h and the luciferase activity was measured using a Luciferase Assay System (Promega). The activity was normalized with that of cells stably expressing the control reporter plasmid, pGL4.24 (Promega), which were treated in the same way.

2.7. qRT-PCR

Total RNA from the cells was extracted with ISOGEN (Nippongene, Toyama, Japan). Each cDNA was synthesized from total RNA using the Primescript™ RT Reagent Kit (Takara, Shiga, Japan). qRT-PCR was performed on an Applied Biosystems Prism 7300 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) using THUNDERBIRD™SYBR®

qPCR Mix (Toyobo, Osaka, Japan). The expression of each gene was normalized to that of the genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or TATA binding protein (TBP). The following pairs of primers were used: *DDT* forward: 5'-CTT GGA GTC CTG GCA GAT TG-3', reverse: 5'-AAT GTT GCA TGC GGG ATA AT-3'; *GAPDH* forward: 5'-GAA GGT GAA GGT CGG AGT C-3', reverse: 5'-GAA GAT GGT GAT GGG ATT TC-3'; *TBP* forward: 5'-CAGCGTGACTGTGAGTTGCT-3', reverse: 5'-TGG TTC ATG GGG AAA AAC AT-3'.

2.8. Chromatin immunoprecipitation (ChIP) assay

SGBS cells overexpressing FOXO1(CA) were fixed with 1% formaldehyde for 10 min and the cross-linking was stopped by addition of 0.15 M glycine. Then, the cells were lysed in SDS buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS)) and sonicated to yield chromatin fragments of approximately 500 bp in length with the Bioruptor-UCW310 (Diagenode, Liége, Belgium). The lysates were incubated with anti-FOXO1 antibody or normal rabbit IgG for 24 h, followed by incubation with Protein G-sepharose 4 Fast Flow (GE Healthcare, Buckinghamshire, UK) for 2 h. The beads were extensively washed and subjected to heat denaturation and digestion by proteinase K. DNA was further purified by phenol/chloroform extraction and ethanol precipitation. PCR was performed with Ex Taq Polymerase (Takara) using each primer set. As a positive control for ChIP using anti-FOXO1 antibody, FOXO1 binding region of the decidual protein induced by progesterone (*DEPP)* gene was amplified [18]. Normal human genomic DNA (Promega) was used as a positive control for PCR amplification. The following pairs of primers were used: F1 forward: 5'-GAG ACA GGG TGG GTC CAC TA-3', reverse: 5'-ACA GCA ACC TGG CTT CTC AT-3'; F2 forward: 5'-CTC TCC CAT GCC TCC TCA TA-3', reverse: 5'-CAC TGA AAG GCC GAC AGA GT-3'; NC1 forward: 5'-GTA GAG ACG GGG TTT CGT CA-3', reverse: 5'-TGC CTG TGT AGC CTC TTG TG-3'; NC2 forward: 5'-AGC TCT GAC TTT CCG TGC TC-3', reverse: 5'-TGA AAA GTT TTG CCC GAA GT-3'; PC1 forward: 5'-CCT GTG CTG CTG ATT TTT CA-3', reverse: 5'-TCG GAA GGC TGT CTT AGG AA-3'.

2.9. Western blotting

The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and blotted to PVDF membranes (Immobilon Transfer Membranes; Millipore). After incubation in blocking solution (Blocking One; Nakalai tesque, Kyoto, Japan), the membranes were incubated with

each primary antibody. Then, the membranes were incubated with an anti-rabbit or -mouse IgG-horseradish peroxidase-conjugated secondary antibody (GE Healthcare). Signal was detected using Immobilon Western Detection Reagent (Millipore) and exposed to X-ray film.

2.10. Statistical analysis

Each experiment was repeated at least three times. Data were expressed as the mean ± SEM. Statistical analyses were performed using a Student's *t*-test. Differences were considered to be significant when the *P*-value was less than 0.05.

3. Results

3.1. Identification of proximal promoter region of the human DDT gene

To identify the promoter region of the human *DDT* gene, we first constructed a reporter plasmid that has a fragment containing the sequence between -2,903 and +135 bp relative to the transcription start site of the *DDT* gene upstream of the firefly luciferase gene, and assessed the luciferase activity of SGBS cells transfected with the reporter plasmid during adipogenic differentiation. The promoter activity was approximately 190-fold higher than cells transfected with the empty reporter plasmid even if under basal conditions without adipogenic induction (day 0), and the activity was further increased in a differentiation-dependent manner (Fig. 1A) in accordance with the mRNA expression pattern as previously reported [3]. To narrow the promoter region, a series of deletion mutants of the promoter was investigated using SGBS cells. Consequently, the reporter construct containing regions from -200 to +23 of the *DDT* promoter exhibited the highest activity among the constructs (Fig. 1B). DNA sequences in this region lacked a TATA box but contained two CAAT boxes and one GC box (Fig. 1C).

3.2. AICAR and its derivatives enhanced DDT transcription in HEK293 and SGBS cells

In order to find a clue for the molecular mechanisms of *DDT* transcription, we attempted to explore the molecules that affect transcriptional activity of the *DDT* gene from a chemical library. We used HEK293 cells that stably express the *DDT* promoter (-2,903/+135)-reporter for high-throughput screening because SGBS cells were not suitable for establishment of stable clones due to difficulty in maintaining a long-term culture after transfection. *DDT* mRNA levels in HEK293 cells were comparable to those in SGBS cells (data not shown). We obtained 28 compounds (10 activators and 18 inhibitors) with more than a 2-fold change in the luciferase activity compared with the DMSO treatment as a control. Of note, 7 of the 10 activator candidates had similar structures with AICAR, a selective activator of AMPK (Figs. 2A and B). Next, whether AICAR and the 3 derivatives of SY142, SY145, and SY148 [19], whose luciferase activities were relatively high, affect *DDT* mRNA levels in both SGBS cells and SGBS adipocytes was investigated. AICAR and SY148 increased *DDT* mRNA levels in both SGBS cells and the differentiated adipocytes (Figs. 2C and D). SY148 as well as AICAR were confirmed to have the potential to activate AMPK in SGBS adipocytes (Fig. 2E). Furthermore, compound C, an AMPK inhibitor, decreased *DDT* mRNA levels in SGBS

adipocytes (Fig. 2F). These results suggested that AMPK activation enhanced *DDT* gene expression in SGBS adipocytes.

3.3. FOXO1 binds to the promoter region of the DDT gene.

Next, we sought transcriptional regulation factors downstream of AMPK signaling and therefore focused on FOXO1, a transcription factor known to be activated through AMPK/SIRT1 pathway [20], because there are two putative FOXO1 binding consensus sequences (AAACAA/TTGTTT) in the *DDT* promoter region (Fig. 3A). A ChIP assay revealed that FOXO1 bound to these two sites (Fig. 3B). Next, the effects of FOXO1(CA) overexpression on transcriptional activity of the *DDT* gene in SGBS cells were investigated. As FOXO1(CA) markedly induced luciferase activity in control cells with an empty reporter plasmid, presumably due to the putative FOXO1 binding sequences in the vicinity of the multiple cloning site in the plasmid, we reconstructed the *DDT* promoter (-2,903/+135)-reporter and control empty plasmids lacking the sequences, and confirmed that influence of FOXO1(CA) on the basal luciferase activity of the newly constructed plasmids was negligible (supplementary Fig. 1). Unexpectedly, FOXO1(CA) significantly inhibited transcriptional activity of the *DDT* gene and tended to decrease *DDT* mRNA levels in SGBS cells (Figs. 3C and D) and treatment of AS1842856, a FOXO1 inhibitor, in SGBS adipocytes increased *DDT* mRNA levels (Fig. 3E). Furthermore, AICAR-induced deacetylation of FOXO1 in SGBS adipocytes (Fig. 3F) and increased DDT mRNA levels in SIRT1-knockdown SGBS cells were observed (Fig. 3G). Thus, AMPK/SIRT1/FOXO1 pathway seems to rather inhibit the *DDT* gene expression in the SGBS adipocytes.

In contradiction to SGBS cells, overexpression of FOXO1(CA) in HEK293 cells demonstrated enhancement both of transcriptional activity and the mRNA levels of the *DDT* gene and AS1842856 also had opposite effects on *DDT* mRNA levels (supplementary Fig. 2). These results suggested that FOXO1 regulated *DDT* transcription in a cell-type specific manner.

Next, we performed luciferase assay using reporter constructs containing the mutated *DDT* promoter that lacks either or both of two putative FOXO1 binding sites (Fig. 3H). Inhibition of luciferase activity by FOXO1(CA) overexpression was attenuated in SGBS cells transfected with reporter plasmids lacking FOXO1 binding site-2 (Fig. 3I), indicating that the binding site-2 is involved in FOXO1-inhibited transcription of the *DDT* gene in SGBS cells.

3.4. Inhibition of mTOR signaling enhanced DDT mRNA expression

Finally, we investigated involvement of the mammalian target of rapamycin (mTOR) signaling in transcriptional regulation of the *DDT* gene. AICAR and compound C decreased and increased phosphorylated levels of S6K, a target of mTOR, in SGBS adipocytes (Fig. 4A), respectively, suggesting AMPK/mTOR pathway in adipocytes. Rapamycin, an inhibitor of mTOR, increased *DDT* mRNA levels (Fig. 4B) and abolished the inhibitory effects of compound C on *DDT* mRNA levels in SGBS adipocytes (Fig. 4C), suggesting that AMPK/mTOR signaling was one of the key pathways of DDT transcription in adipocytes.

4. Discussion

In this study, we found that AICAR and its derivatives induced transcription of the *DDT* gene in SGBS adipocytes by screening a chemical library. AICAR is transformed into the corresponding 5'-monophosphate in cells and activates AMPK in several cell-types including adipocytes [21-25]. AMPK, a serine/threonine kinase protein complex, plays a central role to regulate cellular energy homeostasis. Activation of AMPK in response to different cellular stresses that cause cellular ATP depletion, such as low glucose, hypoxia, ischemia, and heat shock, positively regulates signaling pathways for energy production and negatively regulates ATP-consuming biosynthetic processes [26]. AMPK phosphorylates a number of enzymes involved in these processes and transcription factors, including co-activators and co-repressors, to control the transcription [27]. In adipose tissue, activated AMPK inhibits both lipogenesis and lipolysis [21], resulting in restriction of efflux of fatty acids, molecules that induce insulin resistance. Furthermore, AMPK has been reported to regulate the expression and secretion of adipokines in adipocytes. In human adipose tissue, AICAR stimulates the expression of adiponectin that enhances insulin sensitivity and inhibits secretion of inflammatory cytokines including TNF-α, IL-6, and MIP-1α/β [24, 28]. Given that DDT is an adipokine that improves insulin resistance in obesity [3], our data suggest that activation of AMPK in adipose tissue may be beneficial in insulin-resistant states. On the other hand, metformin, another AMPK activator, has been reported to reduce adiponectin expression in adipocytes differentiated from 3T3-L1 cells, a mouse preadipocyte cell line [29]. Of note, we could not observe any significant effects of AICAR or compound C on mouse *Ddt* mRNA levels in 3T3-L1 cells (data not shown). To uncover whether the involvement of AMPK in transcriptional regulation of the *DDT* gene is species-specific, further investigations are necessary.

We found that transcriptional activity of the *DDT* gene increased at the late stage of adipogenesis in SGBS cells. Although AMPK activation has been reported to inhibit preadipocyte differentiation [30], the activity and physiological role of AMPK during adipocyte differentiation is not clear. Based on reports that adiponectin and leptin secreted from differentiated adipocytes activate adipocyte AMPK in an autocrine manner [31, 32], AMPK activity should be increased in the late stage of adipogenesis as secretion of these adipokines increases. Indeed, Thr172-phosphorylated levels of AMPK are reported to be increased in a differentiation-dependent manner in 3T3-L1 cells [33]. Thus, increase of transcriptional activity of the *DDT* gene at the late stage of adipogenesis in SGBS cells may depend on AMPK

activation. Furthermore, DDT can activate AMPK in SGBS cells [3], suggesting that AMPK activation and DDT expression may form a positive loop to promote transcriptional activity of the *DDT* gene.

AMPK enhances SIRT1 activity by increasing cellular NAD^+ levels, resulting in activation of FOXO1 [20]. Among all FOXO members, FOXO1 is highly expressed in insulin-responsive tissues including the pancreas, liver, skeletal muscle, and adipose tissue, and regulates gene transcription involved in glucose metabolism [34]. In the present study, FOXO1 exhibited opposite effects on transcriptional activity and mRNA levels of the *DDT* gene between SGBS cells and HEK293 cells, suggesting cell-type specific transcriptional regulation. FOXO associates with a variety of unrelated transcription factors to regulate activation or repression of the target genes, and the complement of transcription factors is critical in determining the functions of FOXO in each tissue [35]. For example, CCAAT/enhancer binding protein α acts as a co-activator of FOXO1 to up-regulate mouse adiponectin gene expression in 3T3L1 cells [36]. Identification of the co-factors of FOXO1 involved in *DDT* transcription in human adipocytes requires further consideration.

DDT mRNA levels down-regulated by the SIRT1/FOXO1 signaling pathway in SGBS cells implied another pathway downstream of AMPK in the *DDT* transcription and as a result, we found involvement of mTOR signaling (Fig. 4D). AMPK suppresses mTOR complex 1 which controls the cellular metabolism in response to growth factors, hormones, nutrients, and energy levels, and stress signals [37]. Synthesis or secretion of adipokines appears to be under the control of mTOR signaling, but how mTOR signaling controls the expression and secretion of adipokines remains unknown [38].

Throughout this study, alterations in *DDT* mRNA levels in SGBS cells or SGBS adipocytes were modest, even if statistically significant, compared with those in the transcriptional activity. This suggests that regions except for the promoter used in this study also participated in transcriptional regulation of the *DDT* gene and the regions may alleviate the positive effects on the mRNA expression. Indeed, HIF1α and HIF2α have been reported to bind to the neighborhood of +365 bp downstream of the transcription start site of the *DDT* gene [10]; however, the DNA sequences were not included in our experiment. Thus, our approach could not cover the entire aspect of *DDT* transcriptional regulation; however, this study demonstrated at least a partial involvement of AMPK/mTOR signaling in its transcription in SGBS (pre)adipocytes.

In conclusion, the present study revealed that the activation of AMPK enhanced *DDT*

transcription in SGBS cells by inhibiting the mTOR pathway. Our results provided a clue for further understanding of *DDT* transcriptional regulation and for developing pharmaceutical drugs targeting transcription of *DDT* that have anti-obesity properties.

Declaration of interest

The authors have no conflicts of interest to declare.

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Figure legends

Figure 1

Identification of the proximal promoter region of the human *DDT* gene. (A) Time-course of luciferase activity during adipogenesis in SGBS cells. SGBS cells transfected with a reporter construct containing the upstream region (-2,903/+135) of the human *DDT* gene were subjected to adipogenic induction and the luciferase activities were measured at indicated time points. Data are shown relative to those of cells transfected with the control reporter plasmid. **P*<0.05 (n=4). (B) Comparison of luciferase activity in SGBS cells transfected with reporter constructs containing different lengths of 5'-flanking regions of the *DDT* gene. Upstream region of the *DDT* gene and the 5'-flanking regions used are shown in the left diagrams and the activities are shown in the right graph. The activities were measured 24 h after transfection. Data are shown relative to the control reporter plasmid-transfected samples. **P*<0.05 (n=4). (C) Nucleotide sequence of proximal promoter region of the human *DDT* gene. Sequence numbering is relative to the transcription start site. Putative GC box and CAAT boxes are underlined and boxed, respectively.

Figure 2

Involvement of AMPK in transcription and mRNA expression of the *DDT* gene. (A) Chemical structures of AICAR (boxed) and its derivatives that were screened from a chemical library as transcriptional activators of *DDT*. (B) Effects of derivatives of AICAR on luciferase activity in HEK293 cells stably expressing a *DDT* promoter (-2,903/+135)-reporter construct. The cells were treated with each 10 μM derivative for 24 h. The activity was normalized to that in HEK293 cells stably expressing a control reporter construct, which were treated with the same compounds. Data are shown relative to DMSO treatment. **P*<0.05 (n=4). (C, D) Effects of AICAR and 3 derivatives on *DDT* mRNA expression in SGBS cells (C) and SGBS adipocytes (D). The cells were treated with 1 mM AICAR and each 10 μM derivative for 24 h, and then *DDT* mRNA levels were quantified by qRT-PCR. Data are shown relative to DMSO treatment. **P*<0.05 (n=3). (E) AMPK activation by AICAR and SY148 in SGBS adipocytes. SGBS adipocytes were treated with 1 mM AICAR and indicated concentration of SY148 for 24 h. As an internal control, β-actin levels are shown. (F) Effects of compound C on *DDT* mRNA levels in SGBS adipocytes. *DDT* mRNA levels were measured by qRT-PCR in SGBS adipocytes treated with 10 μM compound C (black column) or DMSO (white column) for 24 h.

539 Data are shown relative to DMSO treatment. *P<0.05 (n=3).

540

541 Figure 3

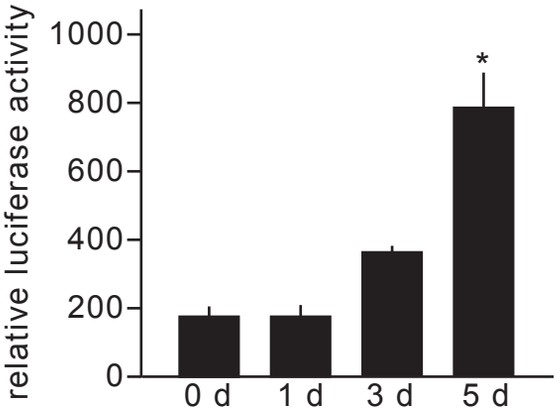
542 Involvement of FOXO1 on transcription and mRNA expression of the *DDT* gene. (A) A
543 diagram of the *DDT* promoter region and the position of each primer set used for the ChIP assay.
544 Two putative FOXO1 binding sites (TTGTTT and AAACAA) were identified in the *DDT*
545 promoter region. F1 and F2 primer sets were designed to amplify regions including each motif.
546 (B) Representative images of the ChIP assay in SGBS cells transiently overexpressing
547 FOXO1(CA). PCR using each primer set of F1 (178 bp), F2 (239 bp), and negative controls
548 (NC1 (197 bp) and NC2 (214 bp)) was performed. As a positive control for the ChIP assay, a
549 primer set including the FOXO1 binding region of *DEPP* was used (PC1: 305 bp). As a
550 positive control for PCR, the FOXO1 binding region of *DEPP* amplified from normal human
551 genomic DNA was applied to lane PC2. (C) Effects of overexpressed FOXO1(CA) on
552 transcriptional activity of the *DDT* gene in SGBS cells. The cells were co-transfected with the
553 *DDT* promoter (-2,903/+135)-reporter construct and FOXO1(CA) expression vector or the
554 empty vector and the luciferase activities were measured 24 h after transfection. (D) Effects of
555 FOXO1 (CA) overexpression on *DDT* mRNA levels in SGBS cells. The cells were transfected
556 with the FOXO1(CA) expression vector or the empty vector and *DDT* mRNA levels were
557 measured by qRT-PCR 24 h after transfection. (E) Effects of AS1842856, a FOXO1 inhibitor,
558 on *DDT* mRNA expression in SGBS adipocytes. *DDT* mRNA levels were measured by
559 qRT-PCR in the cells treated with 1 μM AS1842856 or DMSO for 24 h. (F) Representative
560 images of western blotting using anti-acetylated FOXO1 antibody (Ac-FOXO1), anti-FOXO1
561 antibody, and β-actin in SGBS adipocytes treated with DMSO or 1 mM AICAR for 12 h. (G)
562 Effects of SIRT1 knockdown on *DDT* gene expression in SGBS cells. SGBS cells were
563 transfected with siRNA against SIRT1 (siSIRT1) or control siRNA (control), and *DDT* (right)
564 and *SIRT1* (left) mRNA levels were measured by qRT-PCR 24 h after transfection. (H)
565 Diagrams of wild type *DDT* promoter (WT) and each mutant that lacks either of FOXO1
566 binding site-1 (M1) or FOXO binding site-2 (M2), or both (M1/2). (I) Effect of overexpressed
567 FOXO1(CA) on luciferase activity in SGBS cells transfected with each reporter construct. #P
568 < 0.05 vs. data of cells co-transfected with WT reporter construct and FOXO1(CA) (gray
569 column) (n=3). Data are shown relative to each control (white column). *P < 0.05 vs. each
570 control (white columns) (n=3).

571

572 Figure 4

573 Involvement of the mTOR pathway in transcription and mRNA expression of the *DDT* gene in
574 SGBS adipocytes. (A) Inhibitory effects of AICAR on the mTOR pathway. Phosphorylated
575 S6K protein levels were assessed by Western blot analysis of SGBS adipocytes treated with 1
576 mM AICAR, 1 μM compound C, or DMSO for 12 h. Representative images are shown. (B)
577 Effects of rapamycin, an mTOR inhibitor, on *DDT* mRNA levels in SGBS adipocytes. *DDT*
578 mRNA levels were measured by qRT-PCR of SGBS adipocytes treated with 0.1 μM rapamycin
579 (black column) or DMSO (white column) for 24 h. Data are shown relative to DMSO
580 treatment. (C) Effects of rapamycin on *DDT* mRNA levels in SGBS adipocytes in the
581 presence of compound C. The levels were measured by qRT-PCR of SGBS adipocytes treated
582 with 0.1 μM rapamycin (black column) or DMSO (gray column) with 10 μM compound C for
583 24 h. Data are shown relative to samples treated with DMSO only (white column). **P*<0.05
584 (n=3). (D) Schematic model of transcriptional regulation of the *DDT* gene in SGBS
585 adipocytes.

A



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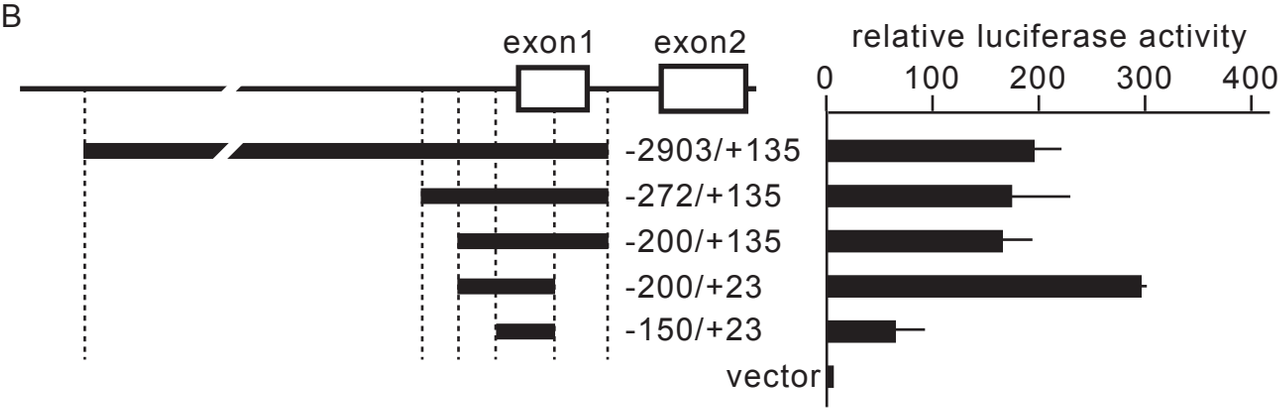

C

```
-200  ggggagacagggtgggtccactaccgggttaaagacctgtagtgggtggggctacacgta  -141
-140  gggcggagacgatgggacttccggaaatcagccggcacacgtgacttttgtttgcagaag  -81
 -80  cgggaggtaccctaggcagccaatcggggagcgccgagtctctgtccagccaatgagaag  -21
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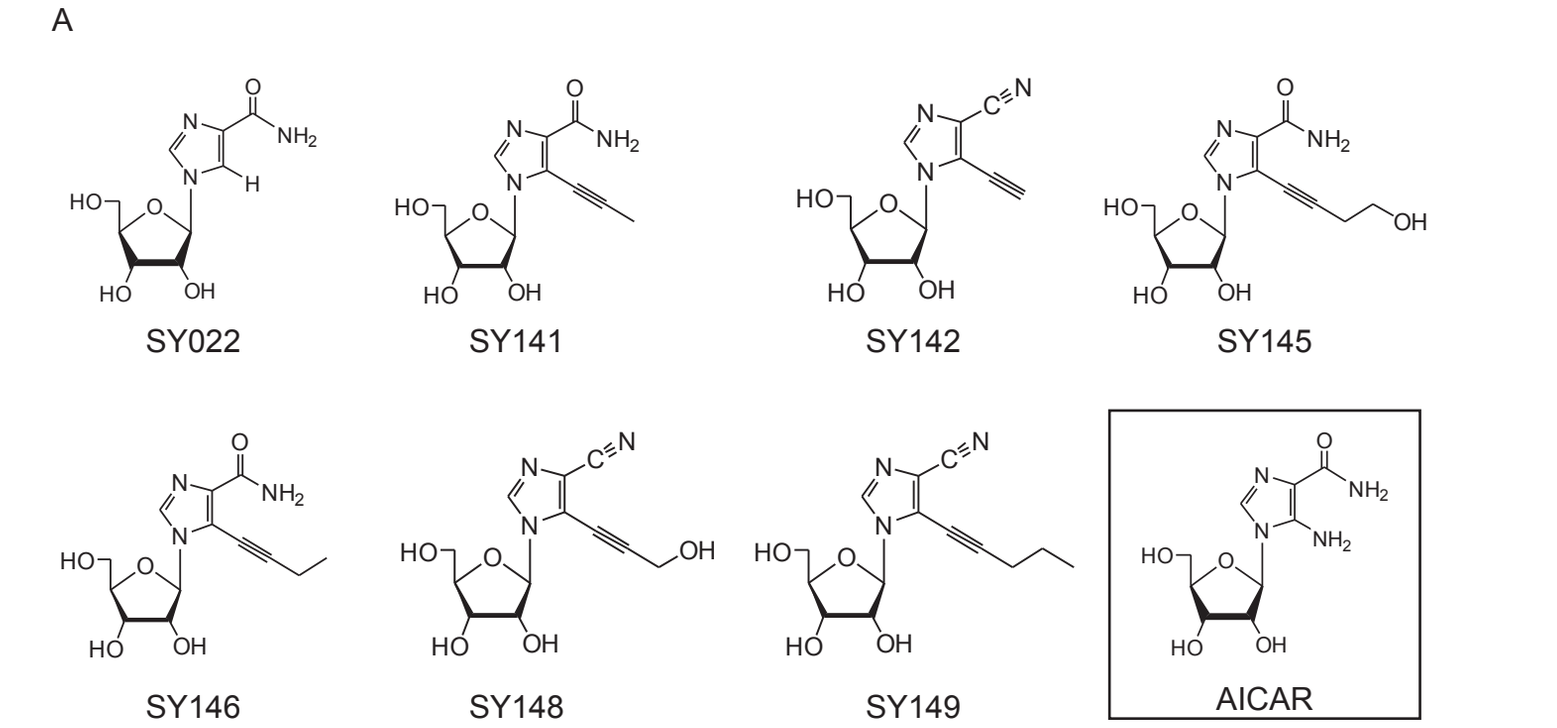
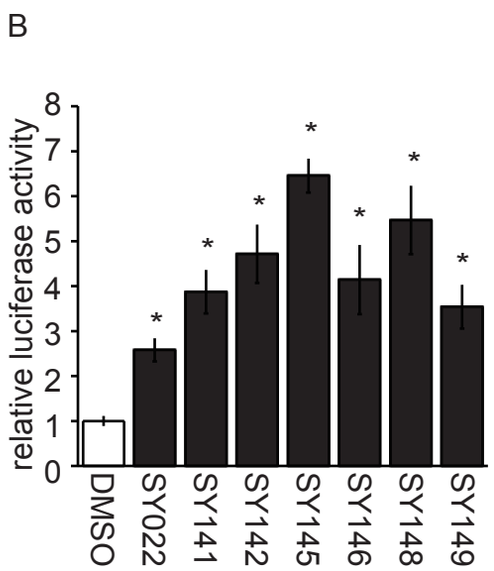
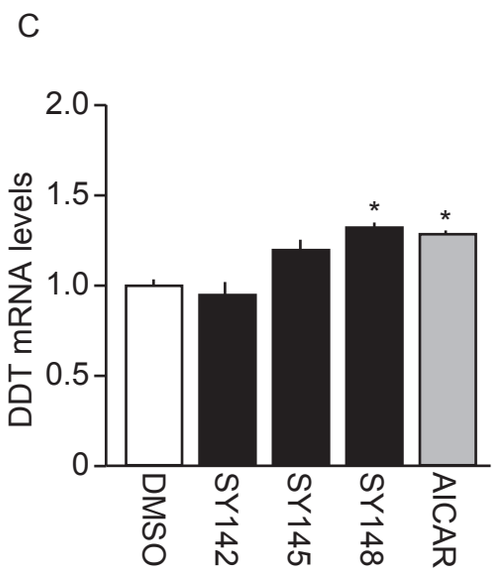
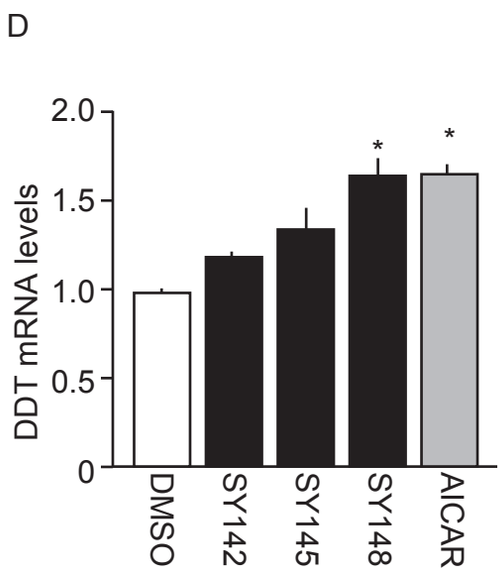
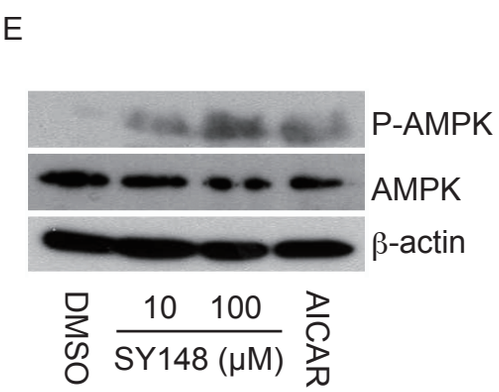
Fig. 1


A
SY022
SY141
SY142
SY145
SY146
SY148
SY149
AICAR


B
relative luciferase activity
DMSO
SY022
SY141
SY142
SY145
SY146
SY148
SY149


C
DDT mRNA levels
DMSO
SY142
SY145
SY148
AICAR


D
DDT mRNA levels
DMSO
SY142
SY145
SY148
AICAR


E
P-AMPK
AMPK
β-actin
DMSO
10
100
SY148 (μM)
AICAR

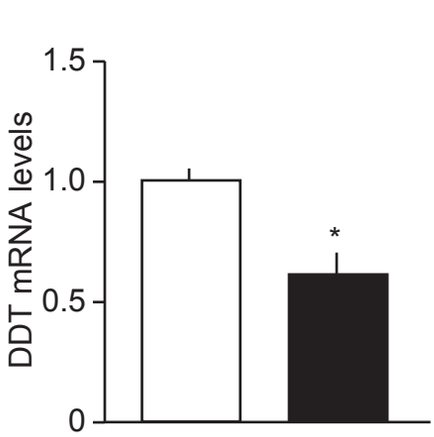

F
DDT mRNA levels

Fig. 2

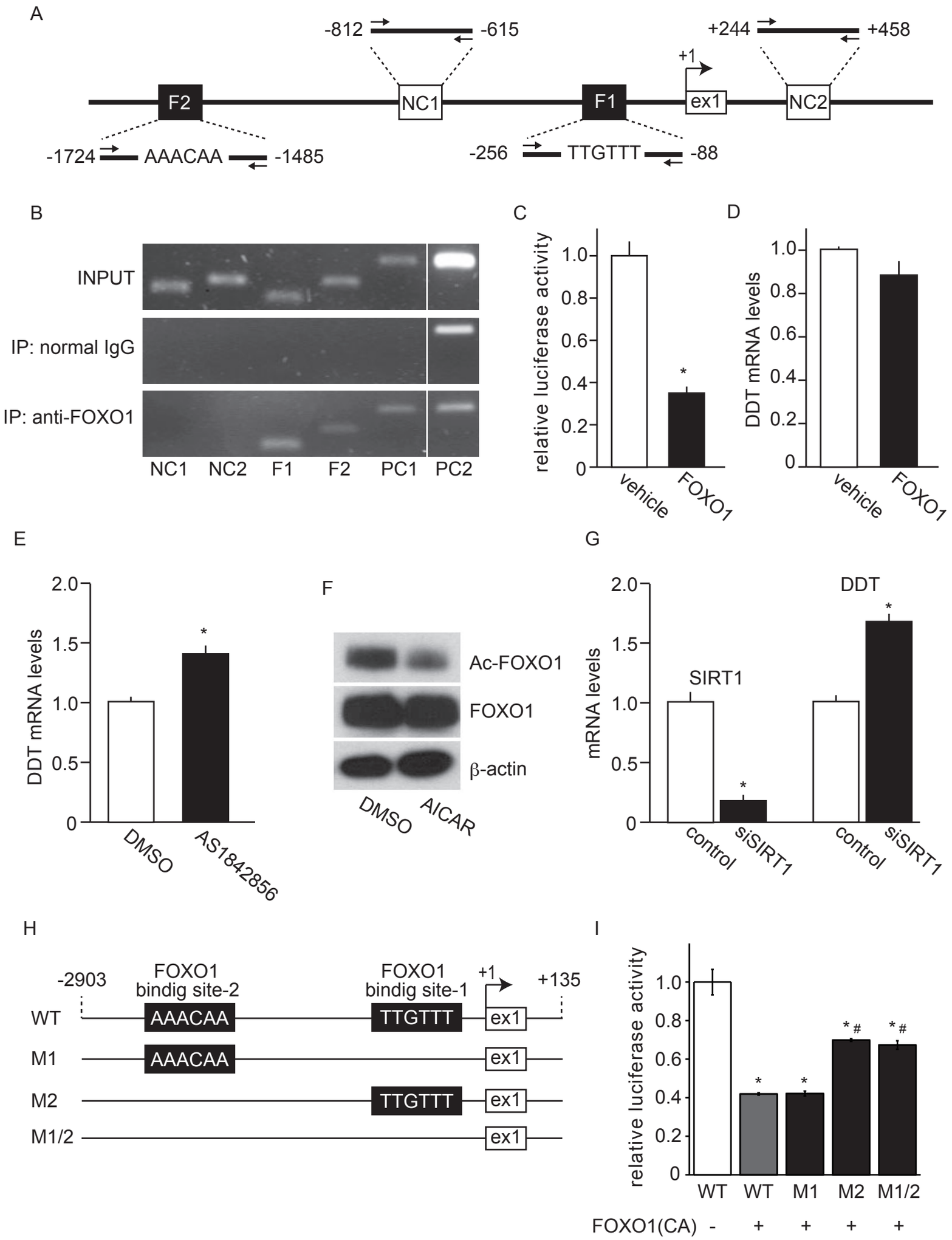

A
-812
-615
+244
+458
+1
F2
NC1
F1
ex1
NC2
-1724
AAACAA
-1485
-256
TTGTTT
-88
B
INPUT
IP: normal IgG
IP: anti-FOXO1
NC1
NC2
F1
F2
PC1
PC2
C
relative luciferase activity
vehicle
FOXO1
D
DDT mRNA levels
vehicle
FOXO1
E
DDT mRNA levels
DMSO
AS1842856
F
Ac-FOXO1
FOXO1
β-actin
DMSO
AICAR
G
mRNA levels
SIRT1
DDT
control
siSIRT1
control
siSIRT1
H
-2903
FOXO1 bindig site-2
FOXO1 bindig site-1
+1
+135
WT
AAACAA
TTGTTT
ex1
M1
AAACAA
ex1
M2
TTGTTT
ex1
M1/2
ex1
I
relative luciferase activity
WT
WT
M1
M2
M1/2
FOXO1(CA) -
+
+
+
+

Fig. 3

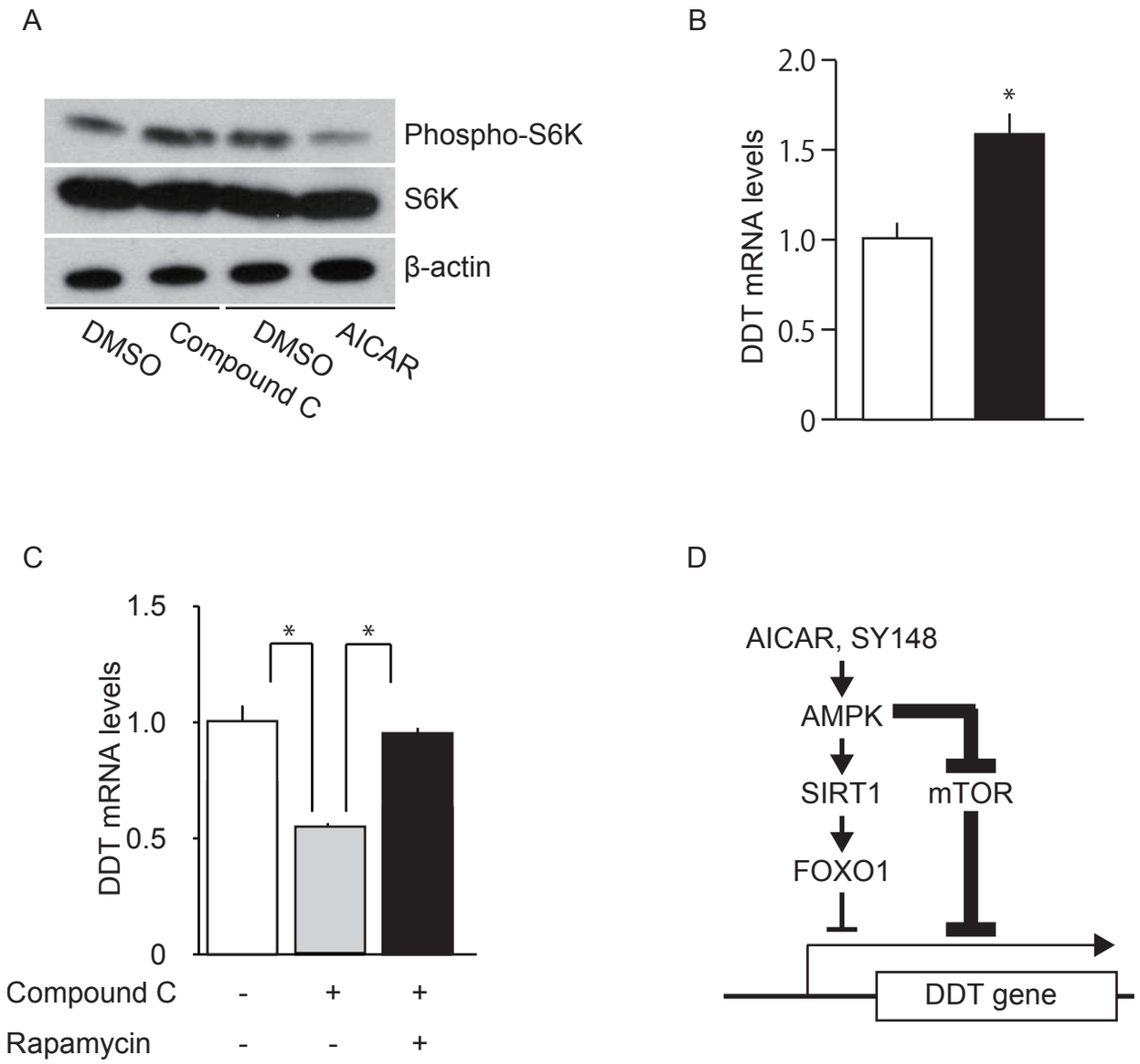
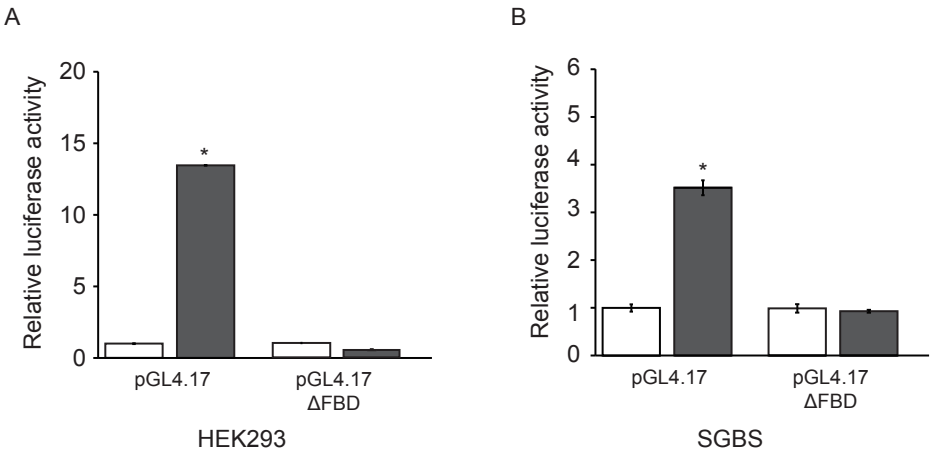

A
Phospho-S6K
S6K
β-actin
DMSO
Compound C
DMSO
AICAR
B
DDT mRNA levels
2.0
1.5
1.0
0.5
0
*
C
DDT mRNA levels
1.5
1.0
0.5
0
*
*
Compound C - + +
Rapamycin - - +
D
AICAR, SY148
AMPK
SIRT1
mTOR
FOXO1
DDT gene

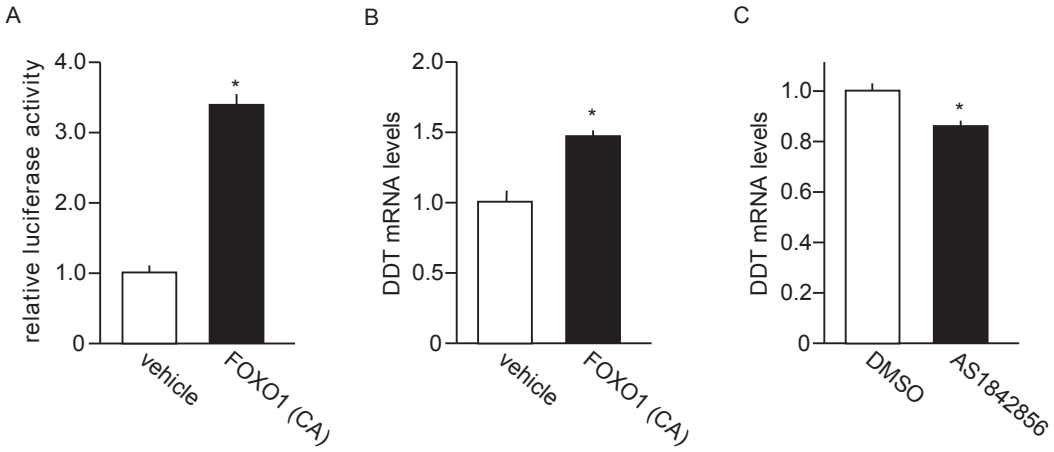
Fig. 4

Supplementary Figure 1.



Luciferase activities of pGL4.17 in HEK293 (A) and SGBS cells (B) transfected with pcDNA3.1+ (open columns) or FOXO1(CA) (gray columns). Luciferase activities of pGL4.17 increased in response to FOXO1(CA) in both cell-types despite lack of promoter sequences. The pGL4.17 lacking sequences (at the position from 5,526 to 5,547), including the putative FOXO1 binding motifs (pGL4.17 ΔFBD), exhibited no FOXO1(CA) effects on the basal luciferase activity.

Supplementary Figure 2.



Involvement of FOXO1 on transcription and mRNA levels of the *DDT* gene in HEK293 cells. (A) Effects of overexpressed FOXO1(CA) on transcriptional activity of the *DDT* gene in HEK293 cells. The cells were co-transfected with the *DDT* promoter (-2,903/+135)-reporter construct and FOXO1(CA) expression vector or the empty vector and the luciferase activities were measured 24 h after transfection. (B) Effects of FOXO1 (CA) overexpression on *DDT* mRNA levels in HEK293 cells. The cells were transfected with the FOXO1(CA) expression vector or the empty vector and *DDT* mRNA levels were measured by qRT-PCR 24 h after transfection. (C) Effects of AS1842856, a FOXO1 inhibitor, on *DDT* mRNA expression in HEK293. *DDT* mRNA levels were measured by qRT-PCR in the cells treated with 1 μM AS1842856 or DMSO for 24 h. Data are shown relative to empty vector-transfected samples or DMSO treatment. **P*<0.05 (n=3).