

1 **Title.**

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

4

5 **Author names.**

6 Takeo Iwata^{a*}, Kyoko Kuribayashi^b, Masahiko Nakasono^c, Noriko Saito-Tarashima^d, Noriaki
7 Minakawa^d, Noriko Mizusawa^a, Rie Kido^c, Katsuhiko Yoshimoto^a

8

9 **Affiliations.**

10 ^aDepartment of Medical Pharmacology, Graduate School of Biomedical Sciences, Tokushima
11 University, Tokushima, Japan

12 ^bDepartment of Oral and Maxillofacial Surgery, Dokkyo Medical University School of
13 Medicine, Mibu, Japan

14 ^cDepartment of Internal Medicine, Handa Hospital, Tsurugi, Japan.

15 ^dGraduate School of Pharmaceutical Science, Tokushima University, Tokushima, Japan

16 ^eDepartment of Periodontology and Endodontology, Graduate School of Biomedical Sciences,
17 Tokushima University, Tokushima, Japan.

18

19 ***Corresponding author:**

20 T. Iwata

21 Department of Medical Pharmacology, Graduate School of Biomedical Sciences, Tokushima
22 University, 3-18-15, Kuramoto-cho, Tokushima-City, Tokushima 770-8504, Japan.

23 Tel/Fax: 81-88-633-9137 / 81-88-633-7331

24 E-mail: iwatakeo@tokushima-u.ac.jp

25

26 **Abstract**

27 In adipose tissue, D-dopachrome tautomerase (DDT), a cytokine with structural similarity to
28 macrophage migration inhibitory factor, is mainly expressed in adipocytes rather than
29 preadipocytes and acts as an anti-obesity adipokine in an autocrine manner. However, its
30 transcriptional regulation is largely unknown. In order to explore molecules affecting *DDT*
31 transcription, a chemical library screening using HEK293 cells stably expressing a *DDT*
32 promoter-reporter construct was performed. Several derivatives of
33 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), an AMP-activated protein
34 kinase (AMPK) activator, were identified as transcriptional activators of the *DDT* gene.
35 Furthermore, *DDT* mRNA levels were reduced in SGBS adipocytes treated with compound C,
36 an AMPK inhibitor, suggesting involvement of AMPK in *DDT* transcription. Overexpression
37 of the FOXO1 constitutive active form reduced transcriptional activity of the *DDT* gene in
38 SGBS cells, but increased it in HEK293 cells. Cell-type specific effects were also observed in
39 the *DDT* gene expression of cells treated with AS1842856, a FOXO1 inhibitor. Finally,
40 involvement of the mammalian target of rapamycin (mTOR) signaling in *DDT* transcription in
41 SGBS adipocytes was investigated. Rapamycin, an inhibitor of mTOR, increased *DDT* mRNA
42 levels and attenuated the inhibitory effects of compound C on *DDT* mRNA levels in SGBS
43 adipocytes. In conclusion, *DDT* transcription may be regulated in a cell-dependent manner,
44 and were enhanced by AMPK activation in SGBS adipocytes through inhibiting the mTOR
45 signaling.

46
47 **Keywords**

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

49
50 **Abbreviations**

51 DDT, D-dopachrome tautomerase; MIF, macrophage migration inhibitory factor; AICAR,
52 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside; AMPK, AMP-activated protein kinase;
53 S6K, S6 kinase; FOXO1, forkhead transcription factor O1; DMSO, dimethyl sulfoxide;
54 qRT-PCR, quantitative real-time reverse transcription PCR; FOXO1(CA), constitutive active
55 form of FOXO1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA binding
56 protein; SDS, sodium dodecyl sulfate; CHIP, Chromatin immunoprecipitation; DEPP, decidual
57 protein induced by progesterone; mTOR, the mammalian target of rapamycin

58 **1. Introduction**

59

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

67 DDT has been originally identified as an enzyme that converts D-dopachrome into
68 5,6-dihydroxyindole, but its physiological significance is unknown [4]. Recently, DDT has
69 come to be regarded as a family member of macrophage migration inhibitory factor (MIF), a
70 cytokine involved in the amplification of inflammatory and immune responses, due to
71 similarities in structure and function [5-8]. DDT acts co-operatively with MIF through CD74,
72 a MIF receptor, to activate the proinflammatory pathway [8] and to promote tumor growth [7, 9,
73 10]. However, DDT has been reported to have distinct functions from MIF in adipose tissue.
74 MIF knockout mice exhibit improvement of insulin sensitivity in adipose tissue by an increase
75 of the glucose uptake [11] and reduction of the macrophage infiltration [12], suggesting the
76 unfavorable function of MIF in adipose tissue. On the other hand, administration of
77 recombinant DDT ameliorates the glucose intolerance of obese mice [3] and inhibits
78 adipogenesis in SGBS cells, a human preadipocyte cell line [13]. More recently, DDT was
79 demonstrated to accelerate wound healing in adipose tissue in the presence of neutralizing
80 anti-MIF antibody and the ability of DDT to recruit macrophages into the inflamed adipose
81 tissue was lower than that of MIF [14]. These suggest that DDT has more beneficial roles than
82 MIF in adipose tissue.

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

91 underlying transcriptional mechanism is largely unexplored.

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

98 **2. Materials and Methods**

99

100 *2.1. Materials*

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

111

112 *2.2. A Chemical library*

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

117

118 *2.3. Cell culture*

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

128

129 *2.4. Construction of reporter and expression vectors*

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

131 and the deletion mutants were amplified from genomic DNA extracted from HEK293 cells.
132 PCR products were inserted into the pGL4.17 luciferase vector (Promega, Madison, WI, USA)
133 or pGL4.17 without the putative FOXO1 binding motifs (nucleotides at position from 5,526 to
134 5,547 in pGL4.17), which was made using site-directed mutagenesis. Then, construction of
135 each mutated luciferase reporter plasmid with lacking putative FOXO1 binding motif(s) (-1543
136 to -1536 and/or -172 to -166) in the *DDT* promoter was carried out by standard PCR-based
137 site-directed mutagenesis. The cDNA encoding human FOXO1 was amplified from total RNA
138 extracted from SGBS cells by RT-PCR using each specific primer set and was inserted into the
139 expression vector, pcDNA3.1+. An expression plasmid for a constitutive active form of
140 FOXO1 (FOXO1(CA)), a triple alanine mutant of FOXO1 at T24, S256, and S319 sites, was
141 made using site-directed mutagenesis.

142

143 *2.5. Luciferase assay*

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

149

150 *2.6. Screening from the chemical library*

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

158

159 *2.7. qRT-PCR*

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

164 qPCR Mix (Toyobo, Osaka, Japan). The expression of each gene was normalized to that of the
165 genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or TATA binding protein
166 (TBP). The following pairs of primers were used: *DDT* forward: 5'-CTT GGA GTC CTG
167 GCA GAT TG-3', reverse: 5'-AAT GTT GCA TGC GGG ATA AT-3'; *GAPDH* forward:
168 5'-GAA GGT GAA GGT CCG AGT C-3', reverse: 5'-GAA GAT GGT GAT GGG ATT TC-3';
169 *TBP* forward: 5'-CAGCGTGACTGTGAGTTGCT-3', reverse: 5'-TGG TTC ATG GGG AAA
170 AAC AT-3'.

171

172 2.8. Chromatin immunoprecipitation (ChIP) assay

173 SGBS cells overexpressing FOXO1(CA) were fixed with 1% formaldehyde for 10 min and
174 the cross-linking was stopped by addition of 0.15 M glycine. Then, the cells were lysed in
175 SDS buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS)) and
176 sonicated to yield chromatin fragments of approximately 500 bp in length with the
177 Bioruptor-UCW310 (Diagenode, Liège, Belgium). The lysates were incubated with
178 anti-FOXO1 antibody or normal rabbit IgG for 24 h, followed by incubation with Protein
179 G-sepharose 4 Fast Flow (GE Healthcare, Buckinghamshire, UK) for 2 h. The beads were
180 extensively washed and subjected to heat denaturation and digestion by proteinase K. DNA
181 was further purified by phenol/chloroform extraction and ethanol precipitation. PCR was
182 performed with Ex Taq Polymerase (Takara) using each primer set. As a positive control for
183 ChIP using anti-FOXO1 antibody, FOXO1 binding region of the decidual protein induced by
184 progesterone (*DEPP*) gene was amplified [18]. Normal human genomic DNA (Promega) was
185 used as a positive control for PCR amplification. The following pairs of primers were used:
186 F1 forward: 5'-GAG ACA GGG TGG GTC CAC TA-3', reverse: 5'-ACA GCA ACC TGG CTT
187 CTC AT-3'; F2 forward: 5'-CTC TCC CAT GCC TCC TCA TA-3', reverse: 5'-CAC TGA AAG
188 GCC GAC AGA GT-3'; NC1 forward: 5'-GTA GAG ACG GGG TTT CGT CA-3', reverse:
189 5'-TGC CTG TGT AGC CTC TTG TG-3'; NC2 forward: 5'-AGC TCT GAC TTT CCG TGC
190 TC-3', reverse: 5'-TGA AAA GTT TTG CCC GAA GT-3'; PC1 forward: 5'-CCT GTG CTG
191 CTG ATT TTT CA-3', reverse: 5'-TCG GAA GGC TGT CTT AGG AA-3'.

192

193 2.9. Western blotting

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

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

200

201 *2.10. Statistical analysis*

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

205 3. Results

206

207 3.1. Identification of proximal promoter region of the human *DDT* gene

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

220

221 3.2. AICAR and its derivatives enhanced *DDT* transcription in HEK293 and SGBS cells

222 In order to find a clue for the molecular mechanisms of *DDT* transcription, we attempted to
223 explore the molecules that affect transcriptional activity of the *DDT* gene from a chemical
224 library. We used HEK293 cells that stably express the *DDT* promoter (-2,903/+135)-reporter
225 for high-throughput screening because SGBS cells were not suitable for establishment of stable
226 clones due to difficulty in maintaining a long-term culture after transfection. *DDT* mRNA
227 levels in HEK293 cells were comparable to those in SGBS cells (data not shown). We
228 obtained 28 compounds (10 activators and 18 inhibitors) with more than a 2-fold change in the
229 luciferase activity compared with the DMSO treatment as a control. Of note, 7 of the 10
230 activator candidates had similar structures with AICAR, a selective activator of AMPK (Figs.
231 2A and B). Next, whether AICAR and the 3 derivatives of SY142, SY145, and SY148 [19],
232 whose luciferase activities were relatively high, affect *DDT* mRNA levels in both SGBS cells
233 and SGBS adipocytes was investigated. AICAR and SY148 increased *DDT* mRNA levels in
234 both SGBS cells and the differentiated adipocytes (Figs. 2C and D). SY148 as well as AICAR
235 were confirmed to have the potential to activate AMPK in SGBS adipocytes (Fig. 2E).
236 Furthermore, compound C, an AMPK inhibitor, decreased *DDT* mRNA levels in SGBS

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

239

240 3.3. *FOXO1 binds to the promoter region of the DDT gene.*

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

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

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

268

269 3.4. *Inhibition of mTOR signaling enhanced DDT mRNA expression*

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

277 4. Discussion

278

279 In this study, we found that AICAR and its derivatives induced transcription of the *DDT*
280 gene in SGBS adipocytes by screening a chemical library. AICAR is transformed into the
281 corresponding 5'-monophosphate in cells and activates AMPK in several cell-types including
282 adipocytes [21-25]. AMPK, a serine/threonine kinase protein complex, plays a central role to
283 regulate cellular energy homeostasis. Activation of AMPK in response to different cellular
284 stresses that cause cellular ATP depletion, such as low glucose, hypoxia, ischemia, and heat
285 shock, positively regulates signaling pathways for energy production and negatively regulates
286 ATP-consuming biosynthetic processes [26]. AMPK phosphorylates a number of enzymes
287 involved in these processes and transcription factors, including co-activators and co-repressors,
288 to control the transcription [27]. In adipose tissue, activated AMPK inhibits both lipogenesis
289 and lipolysis [21], resulting in restriction of efflux of fatty acids, molecules that induce insulin
290 resistance. Furthermore, AMPK has been reported to regulate the expression and secretion of
291 adipokines in adipocytes. In human adipose tissue, AICAR stimulates the expression of
292 adiponectin that enhances insulin sensitivity and inhibits secretion of inflammatory cytokines
293 including TNF- α , IL-6, and MIP-1 α/β [24, 28]. Given that DDT is an adipokine that improves
294 insulin resistance in obesity [3], our data suggest that activation of AMPK in adipose tissue may
295 be beneficial in insulin-resistant states. On the other hand, metformin, another AMPK
296 activator, has been reported to reduce adiponectin expression in adipocytes differentiated from
297 3T3-L1 cells, a mouse preadipocyte cell line [29]. Of note, we could not observe any
298 significant effects of AICAR or compound C on mouse *Ddt* mRNA levels in 3T3-L1 cells (data
299 not shown). To uncover whether the involvement of AMPK in transcriptional regulation of
300 the *DDT* gene is species-specific, further investigations are necessary.

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

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

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

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

332 Throughout this study, alterations in *DDT* mRNA levels in SGBS cells or SGBS adipocytes
333 were modest, even if statistically significant, compared with those in the transcriptional activity.
334 This suggests that regions except for the promoter used in this study also participated in
335 transcriptional regulation of the *DDT* gene and the regions may alleviate the positive effects on
336 the mRNA expression. Indeed, HIF1 α and HIF2 α have been reported to bind to the
337 neighborhood of +365 bp downstream of the transcription start site of the *DDT* gene [10];
338 however, the DNA sequences were not included in our experiment. Thus, our approach could
339 not cover the entire aspect of *DDT* transcriptional regulation; however, this study demonstrated
340 at least a partial involvement of AMPK/mTOR signaling in its transcription in SGBS
341 (pre)adipocytes.

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

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

346

347 **Declaration of interest**

348 The authors have no conflicts of interest to declare.

349

350 **Acknowledgements**

351 We thank Dr. Martin Wabitsch (Division of Pediatric Endocrinology, Department of
352 Pediatrics and Adolescent Medicine, University of Ulm, Ulm, Germany) for providing the
353 SGBS cells. This research was supported by MEXT KAKENHI, Grant number JP15K11040.

354 **References**

355

356 [1] Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose
357 homeostasis. *Nature* 2006; 444: 847-53. doi: 10.1038/nature05483

358

359 [2] Fasshauer M, Blüher M. Adipokines in health and disease. *Trends Pharmacol Sci* 2015; 36:
360 461-70. doi: 10.1016/j.tips.2015.04.014

361

362 [3] Iwata T, Taniguchi H, Kuwajima M, Taniguchi T, Okuda Y, Sukeno A, *et al.* The action of
363 D-dopachrome tautomerase as an adipokine in adipocyte lipid metabolism. *PLoS One* 2012;
364 7: e33402. doi: 10.1371/journal.pone.0033402

365

366 [4] Odh G, Hindemith A, Rosengren AM, Rosengren E, Rorsman H. Isolation of a new
367 tautomerase monitored by the conversion of D-dopachrome to 5,6-dihydroxyindole. *Biochem*
368 *Biophys Res Commun* 1993; 197:619-24. doi:10.1006/bbrc.1993.2524

369

370 [5] Nishihira J, Fujinaga M, Kuriyama T, Suzuki M, Sugimoto H, Nakagawa A *et al.* Molecular
371 cloning of human D-dopachrome tautomerase cDNA: N-terminal proline is essential for
372 enzyme activation. *Biochem Biophys Res Commun* 1998; 243: 538-44. doi:
373 10.1006/bbrc.1998.8123

374

375 [6] Sugimoto H, Taniguchi M, Nakagawa A, Tanaka I, Suzuki M, Nishihira J. Crystal structure
376 of human D-dopachrome tautomerase, a homologue of macrophage migration inhibitory
377 factor, at 1.54 Å resolution. *Biochemistry* 1999; 38: 3268–79. doi: 10.1021/bi982184o

378

379 [7] Coleman AM, Rendon BE, Zhao M, Qian MW, Bucala R, Xin D, Mitchell RA. Cooperative
380 regulation of non-small cell lung carcinoma angiogenic potential by macrophage migration
381 inhibitory factor and its homolog, D-dopachrome tautomerase. *J immunol* 2008; 181: 2330-7.
382 doi: 10.4049/jimmunol.181.4.2330

383

384 [8] Merk M, Zierow S, Leng L, Das R, Du X, Schulte W, *et al.* The D-dopachrome tautomerase
385 (DDT) gene product is a cytokine and functional homolog of macrophage migration inhibitory
386 factor (MIF). *Proc Natl Acad Sci USA* 2011; 108: E577-85. doi: 10.1073/pnas.1102941108

387

388 [9] Guo D, Guo J, Yao J, Jiang K, Hu J, Wang B, *et al.* D-dopachrome tautomerase is over-
389 expressed in pancreatic ductal adenocarcinoma and acts cooperatively with macrophage
390 migration inhibitory factor to promote cancer growth. *Int J Cancer* 2016; 139:2056-67. *doi:*
391 *10.1002/ijc.30278*

392

393 [10] Pasupuleti V, Du W, Gupta Y, Yeh IJ, Montano M, Magi-Galuzzi C, Welford SM.
394 D-dopachrome tautomerase, a hypoxia-inducible factor-dependent gene, cooperates with
395 macrophage, migration inhibitory factor in renal tumorigenesis. *J Biol Chem* 2014; 289:
396 3713-23. *doi: 10.1074/jbc.M113.500694*

397

398 [11] Atsumi T, Cho YR, Leng L, McDonald C, Yu T, Danton C, *et al.* The proinflammatory
399 cytokine macrophage migration inhibitory factor regulates glucose metabolism during
400 systemic inflammation. *J Immunol* 2007; 179:5399-406. *doi: 10.4049/jimmunol.179.8.5399*

401

402 [12] Verschuren L, Kooistra T, Bernhagen J, Voshol PJ, Ouwens DM, van Erk M, *et al.* MIF
403 deficiency reduces chronic inflammation in white adipose tissue and impairs the development
404 of insulin resistance, glucose intolerance, and associated atherosclerotic disease. *Circ Res*
405 2009; 105:99-107. *doi: 10.1161/CIRCRESAHA.109.199166*

406

407 [13] Ishimoto K, Iwata T, Taniguchi H, Mizusawa N, Tanaka E, Yoshimoto K. D-dopachrome
408 tautomerase promotes IL-6 expression and inhibits adipogenesis in preadipocytes. *Cytokine*
409 2012; 60:772-7. *doi: 10.1016/j.cyto.2012.07.037*

410

411 [14] Kim BS, Tilstam PV, Hwang SS, Simons D, Schulte W, Leng L, *et al.* D-dopachrome
412 tautomerase in adipose tissue inflammation and wound repair. *J Cell Mol Med*; Published
413 Online First: 2016. *doi:10.1111/jcmm.12936*

414

415 [15] Skurk T, Herder C, Kräfft I, Müller-Scholze S, Hauner H, Kolb H. Production and release of
416 macrophage migration inhibitory factor from human adipocytes. *Endocrinology* 2004;
417 146:1006-11. *doi: 10.1210/en.2004-0924*

418

- 419 [16] Koska J, Stefan N, Dubois S, Trinidad C, Considine RV, Funahashi T, *et al.* mRNA
420 concentrations of MIF in subcutaneous abdominal adipose cells are associated with adipocyte
421 size and insulin action. *Int J Obes* 2009; 33:842-50. *doi: 10.1038/ijo.2009.106*
422
- 423 [17] Wabitsch M, Brenner RE, Melzner I, Braun M, Möller P, Heinze E, *et al.* Characterization
424 of a human preadipocyte cell strain with high capacity for adipose differentiation. *Int J Obes*
425 *Relat Metab Disord* 2001; 25:8-15. *doi: 10.1038/sj.ijo.0801520.*
426
- 427 [18] Chen S, Gai J, Wang Y, Li H. Foxo regulates expression of decidual protein induced by
428 progesterone (DEPP) in human endothelial cells. *FEBS Lett* 2011; 585:1796-800. *doi:*
429 *10.1016/j.febslet.2011.04.024*
430
- 431 [19] Minakawa N, Takeda T, Sasaki T, Matsuda A, Ueda T. Nucleosides and nucleotides. 96.
432 Synthesis and antitumor activity of 5-ethynyl-1- β -D-ribofuranosylimidazole-4-carboxamide
433 (EICAR) and its derivatives. *J Med Chem* 1991; 34:778-86. *doi: 10.1021/jm00106a045*
434
- 435 [20] Cantó C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, *et al.* AMPK
436 regulates energy expenditure by modulating NAD⁺ metabolism and SIRT1 activity. *Nature*
437 2009; 458:1056-60. *doi: 10.1038/nature07813*
438
- 439 [21] Sullivan JE, Brocklehurst KJ, Marley AE, Carey F, Carling D, Beri RK. Inhibition of
440 lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of
441 AMP-activated protein kinase. *FEBS Lett* 1994; 353:33-6. *doi:*
442 *10.1016/0014-5793(94)01006-4*
443
- 444 [22] Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-aminoimidazole-4-carboxamide
445 ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells?
446 *Eur J Biochem* 1995; 229:558-65. *doi: 10.1111/j.1432-1033.1995.0558k.x*
447
- 448 [23] Salt IP, Connell JM, Gould GW. 5-aminoimidazole-4-carboxamide ribonucleoside
449 (AICAR) inhibits insulin-stimulated glucose transport in 3T3-L1 adipocytes. *Diabetes* 2000;
450 49, 1649-56. *doi: 10.2337/diabetes.49.10.1649*
451

- 452 [24] Lihn AS, Jessen N, Pedersen SB, Lund S, Richelsen B. AICAR stimulates adiponectin and
453 inhibits cytokines in adipose tissue. *Biochem Biophys Res Commun* 2004; 316:853-8. *doi:*
454 *10.1016/j.bbrc.2004.02.139*
- 455
- 456 [25] Daval M, Diot-Dupuy F, Bazin R, Hainault I, Viollet B, Vaulont S, *et al.* Anti-lipolytic
457 action of AMP-activated protein kinase in rodent adipocytes. *J Biol Chem* 2005; 280:25250-7.
458 *doi: 10.1074/jbc.M414222200*
- 459
- 460 [26] Steinberg GR, Kemp BE. AMPK in health and disease. *Physiol Rev* 2009; 89:1025-78
461 *doi: 10.1152/physrev.00011.2008.*
- 462
- 463 [27] Cantó C, Auwerx J. AMP-activated protein kinase and its downstream transcriptional
464 pathways. *Cell Mol Life Sci* 2010; 67:3407-23. *doi: 10.1007/s00018-010-0454-z*
- 465
- 466 [28] Sell H, Dietze-Schroeder D, Eckardt K, Eckel J. Cytokine secretion by human adipocytes is
467 differentially regulated by adiponectin, AICAR, and troglitazone. *Biochem Biophys Res*
468 *Commun* 2006; 343:700-6. *doi: 10.1016/j.bbrc.2006.03.010*
- 469
- 470 [29] Huypens P, Quartier E, Pipeleers D, Van de Casteele M. Metformin reduces adiponectin
471 protein expression and release in 3T3-L1 adipocytes involving activation of AMP activated
472 protein kinase. *Eur J Pharmacol* 2005; 518:90-5. *doi: 10.1016/j.ejphar.2005.06.016*
- 473
- 474 [30] Bijland S, Mancini SJ, Salt IP. Role of AMP-activated protein kinase in adipose tissue
475 metabolism and inflammation. *Clin Sci* 2013; 124:491-507. *doi: 10.1042/CS20120536.*
- 476
- 477 [31] Wu X, Motoshima H, Mahadev K, Stalker TJ, Scalia R, Goldstein BJ. Involvement of
478 AMP-activated protein kinase in glucose uptake stimulated by the globular domain of
479 adiponectin in primary rat adipocytes. *Diabetes* 2003; 52:1355-63. *doi:*
480 *10.2337/diabetes.52.6.1355*
- 481
- 482 [32] Wang MY, Orci L, Ravazzola M, Unger RH. Fat storage in adipocytes requires inactivation
483 of leptin's paracrine activity: Implications for treatment of human obesity. *Proc Natl Acad Sci*
484 *USA* 2005; 102:18011-6. *doi: 10.1073/pnas.0509001102*

485

486 [33] Giri S, Rattan R, Haq E, Khan M, Yasmin R, Won JS, *et al.* AICAR inhibits adipocyte
487 differentiation in 3T3L1 and restores metabolic alterations in diet-induced obesity mice model.
488 Nutr Metab (Lond) 2006; 3:1-20. doi: 10.1186/1743-7075-3-31

489

490 [34] Kousteni S. FoxO1, the transcriptional chief of staff of energy metabolism. Bone 2012;
491 50:437-43. doi: 10.1016/j.bone.2011.06.034

492

493 [35] van der Vos KE, Coffey PJ. The extending network of FOXO transcriptional target genes.
494 Antioxid Redox Signal 2011; 14:579-92. doi: 10.1089/ars.2010.3419

495

496 [36] Qiao L, Shao J. SIRT1 Regulates Adiponectin Gene Expression through
497 Foxo1-C/Enhancer-binding Protein α Transcriptional Complex. J Biol Chem 2006;
498 281:39915-24. doi: 10.1074/jbc.M607215200

499

500 [37] Laplante M, Sabatini DM. An emerging role of mTOR in lipid biosynthesis. Curr Biol
501 2009; 19:R1046-52. doi: 10.1016/j.cub.2009.09.058

502

503 [38] Cai H, Dong LQ, Liu F. Recent advances in adipose mTOR signaling and function:
504 therapeutic prospects. Trends Pharmacol Sci 2016; 37:303-17. doi:
505 10.1016/j.tips.2015.11.011

506 **Figure legends**

507

508 **Figure 1**

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

522

523 **Figure 2**

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

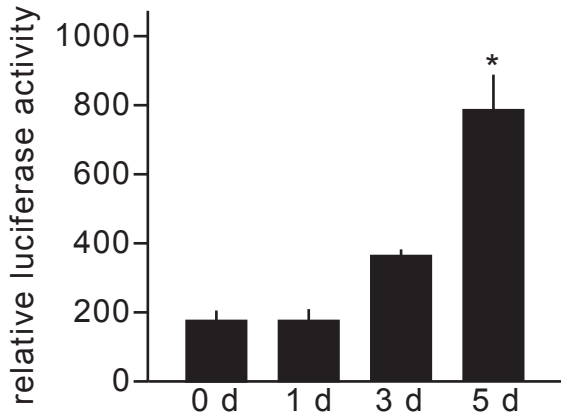
542 Involvement of FOXO1 on transcription and mRNA expression of the *DDT* gene. (A) A
543 diagram of the *DDT* promoter region and the position of each primer set used for the ChIP assay.
544 Two putative FOXO1 binding sites (TTGTTT and AAACAA) were identified in the *DDT*
545 promoter region. F1 and 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 μ M 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

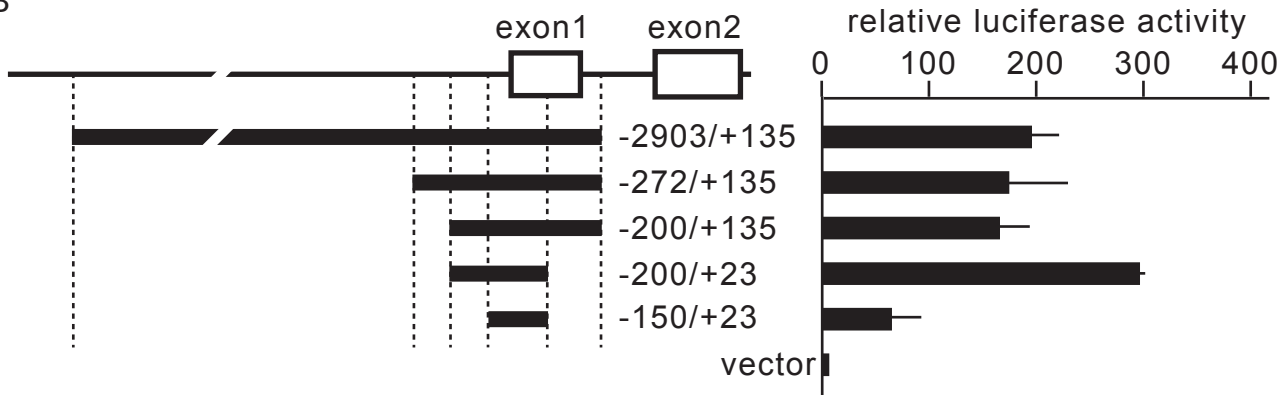
572 Figure 4

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 μ M 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 μ M 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 μ M rapamycin (black column) or DMSO (gray column) with 10 μ M 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



B



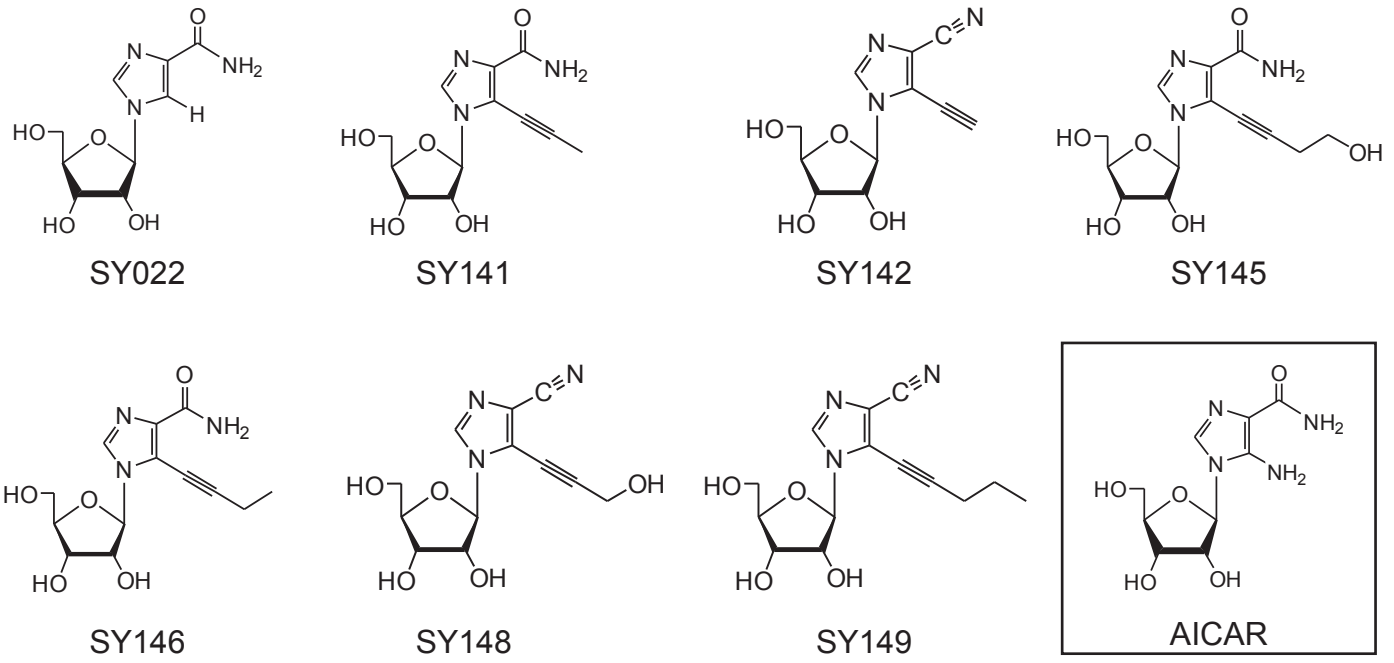
C

```

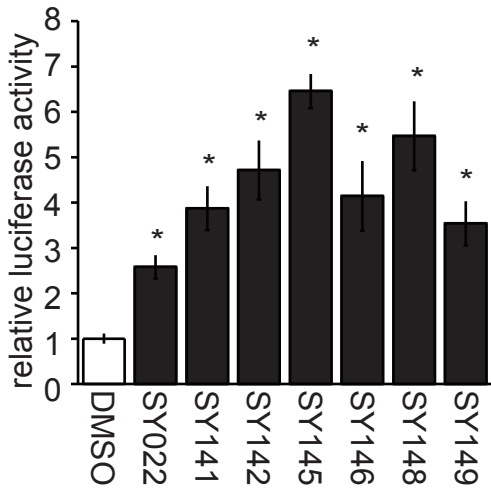
-200  ggggagacaggggtgggtccactaccgggttaaagacctgtagtgggtggggctacacgta  -141
-140  gggcggagacgatgggacttccggaaatcagccggcacacgtgacttttgtttgcagaag  -81
-80   cgggaggtaccctaggcagccaatcggggagcgccgagtcctctgtccagccaatgagaag  -21
-20   ccaggttgctgtgggcgcctcgccctcctccttggtccgcgagc  +23
  
```

Fig. 1

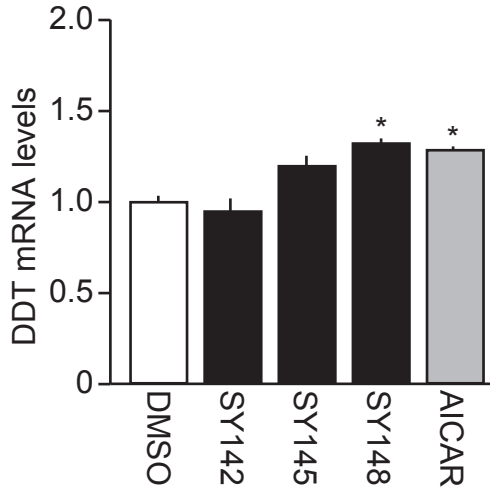
A



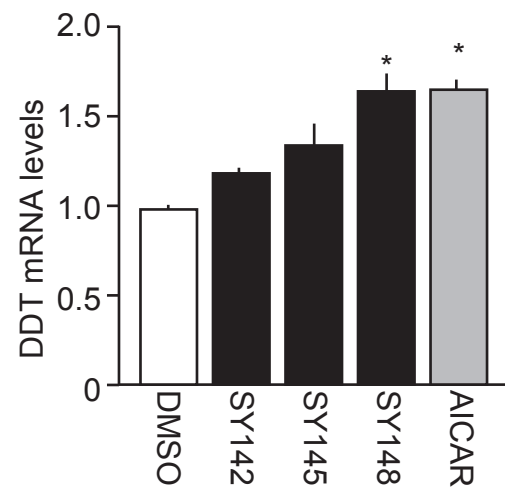
B



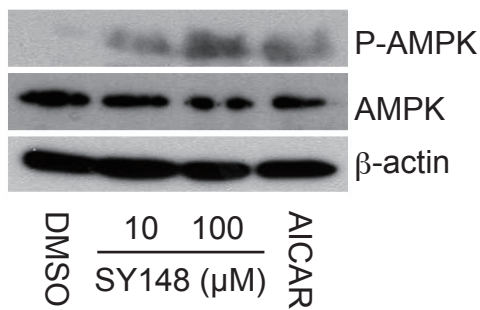
C



D



E



F

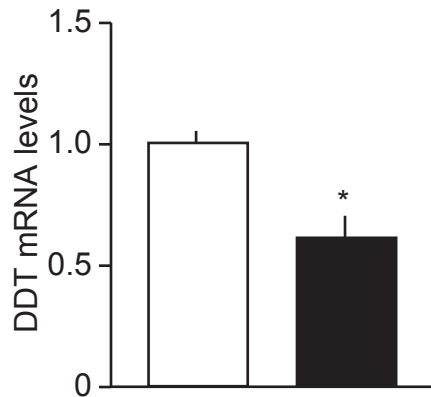


Fig. 2

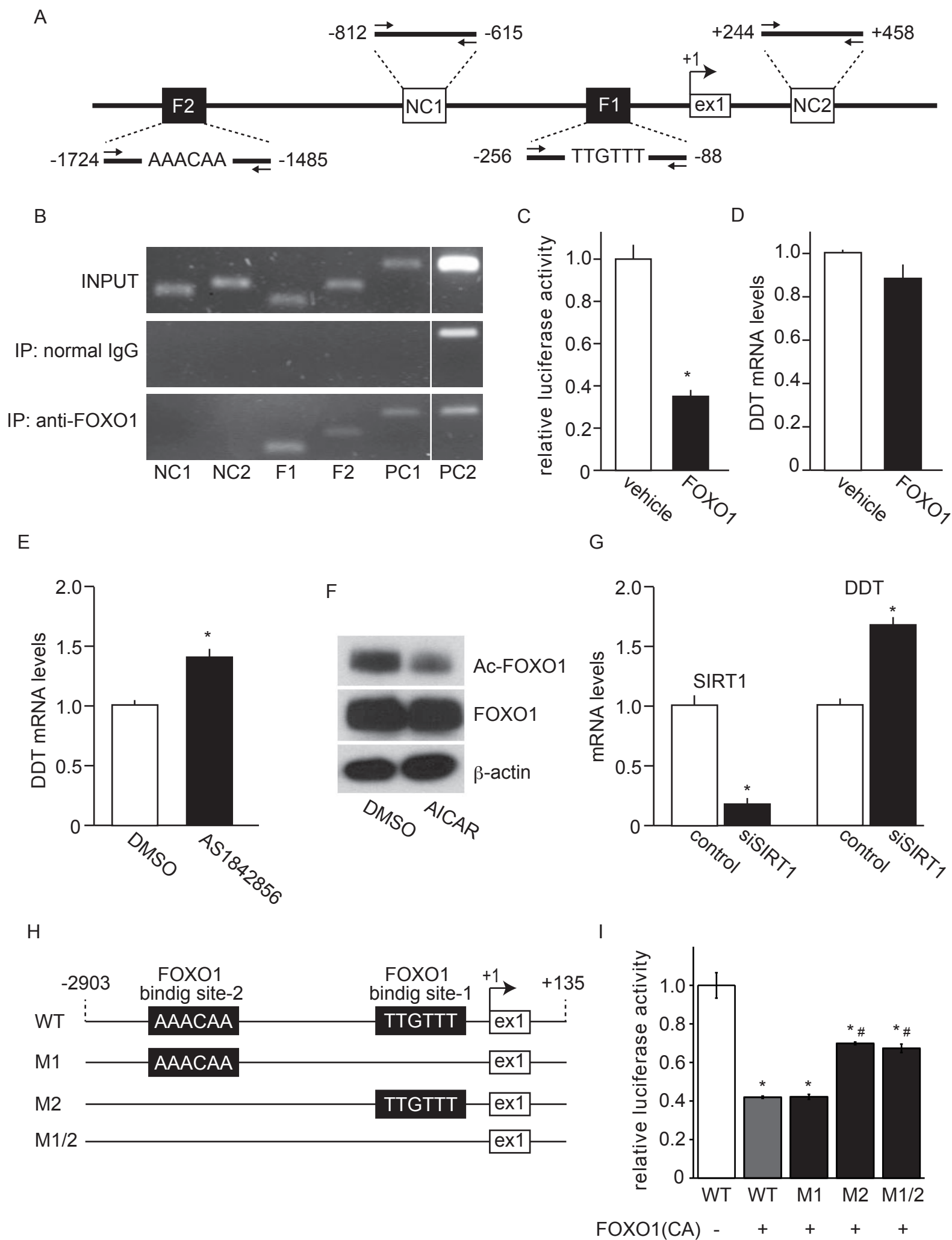
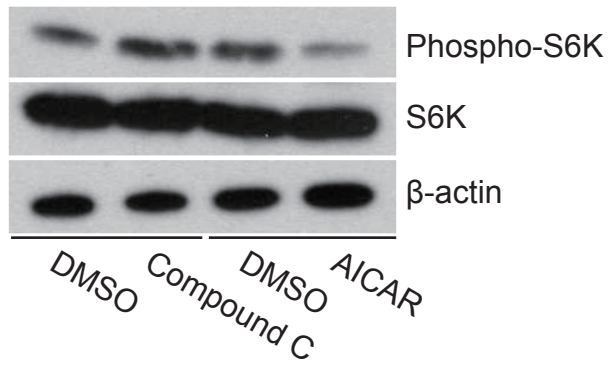
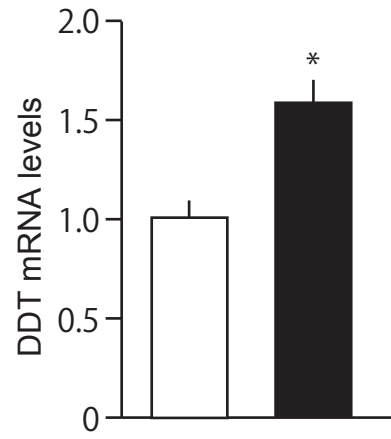


Fig. 3

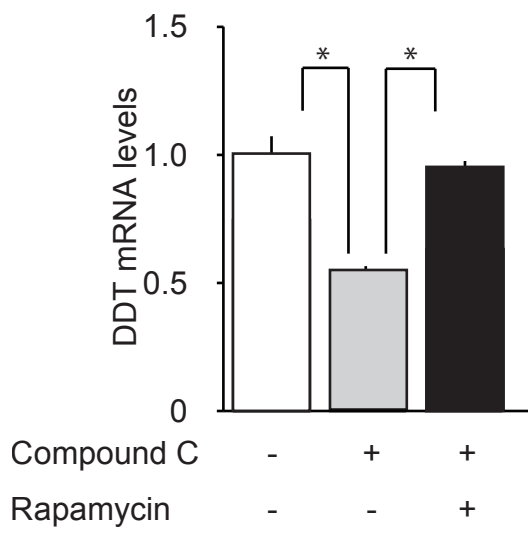
A



B



C



D

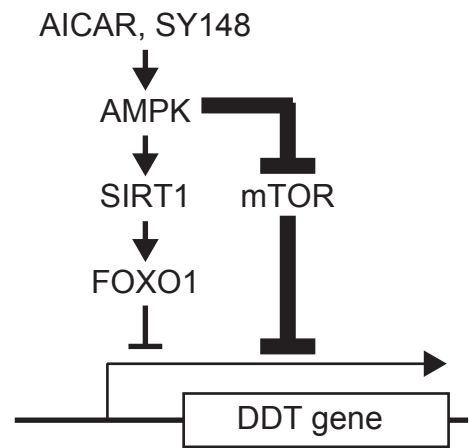
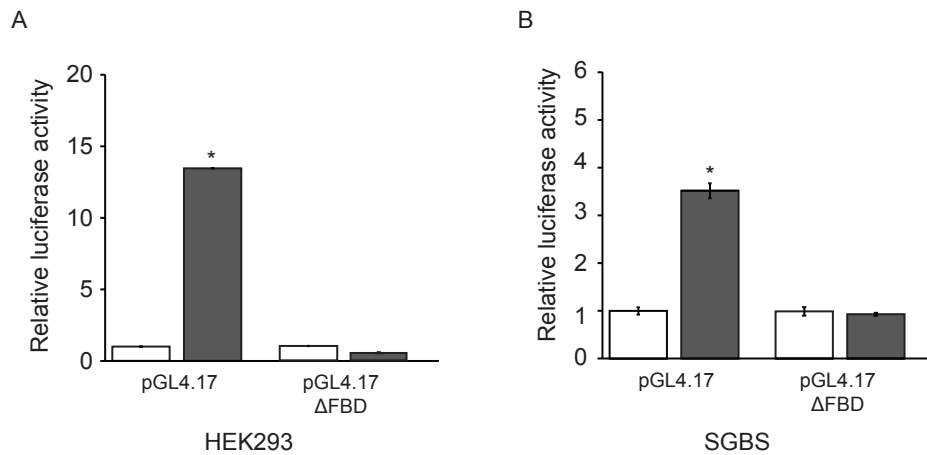


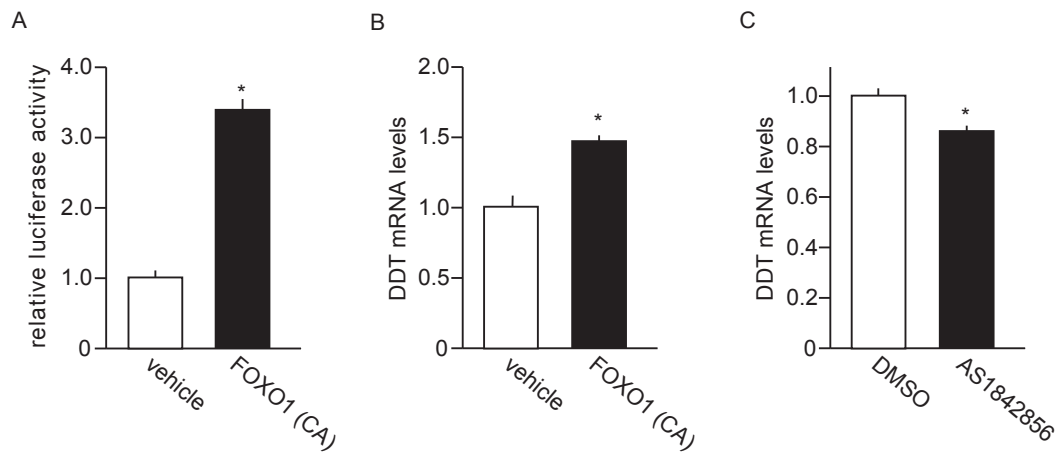
Fig. 4

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 μ M AS1842856 or DMSO for 24 h. Data are shown relative to empty vector-transfected samples or DMSO treatment. * $P < 0.05$ (n=3).