Identification of ribavirin-responsive *cis*-elements for *GPAM* suppression in the *GPAM* genome

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Abbreviations: C/EBPα, CCAAT/enhancer-binding protein α; FOXA1, forkhead box protein A1; GPAM, glycerol-3-phosphate acyltransferase, mitochondrial; GPAT, glycerol-3-phosphate acyltransferase; IMPDHs, inosine monophosphate dehydrogenases; RBV, ribavirin; SREBP-1c, sterol regulatory element-binding protein-1c; SREBP-RE, SREBP-1 responsive element; TG, triglyceride; TSSs, transcriptional start sites.

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Conflicts of interest: none.

Highlights

- RBV suppresses *GPAM* expression by downregulating not only C/EBPα, but also SREBP-1c.
- *Cis*-elements regulated by C/EBPα work as distal enhancers for *GPAM* expression.
- RBV suppresses hepatocytes- and adipocytes-specific *GPAM* variant expression.

Abstract

Glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) is a rate-limiting enzyme catalyzing triglyceride synthesis. Recently, we demonstrated that the anti-viral drug ribavirin (RBV) reduces *GPAM* expression by downregulating CCAAT/enhancer-binding protein α (C/EBPα). However, the precise mechanisms of *GPAM* suppression have remained unclear. Here, we found that RBV suppressed *GPAM* expression by downregulating not only C/EBPα, but also sterol regulatory element-binding protein-1c (SREBP-1c). We also found that *cis*-elements regulated by C/EBPα and SREBP-1c functioned as distal and proximal enhancers, respectively, to express hepatocyte- and adipocytes-specific *GPAM* variants. These results imply that RBV disrupts formation of

45 the enhancer machineries on the *GPAM* genome by downregulating both transcription
46 factors. Our findings may contribute to the development of treatments for fatty liver
47 diseases caused by aberrant triglyceride synthesis. (120 words)
48
49

1. Introduction

The anti-viral drug ribavirin (RBV) has been used for patients with chronic hepatitis C [1]. RBV has been shown to exhibit various functions against viral infection, such as viral RNA polymerase inhibition, immunomodulation of the Th cell phenotype, and mutagenesis of the viral genome [2]. We have been studying the mechanisms of the RBV action against hepatitis C virus (HCV) and demonstrated that inhibition of inosine monophosphate dehydrogenases (IMPDHs) is the main function by which RBV suppresses HCV RNA replication [3]. IMPDHs are rate-limiting enzymes for the *de novo* synthesis of guanine nucleotides, catalyzing the synthesis of xanthine monophosphate (XMP) from inosine monophosphate (IMP) [4,5]. Our recent study demonstrated that IMPDH inhibition by RBV suppressed lipogenesis through downregulation of genes related to fatty acid synthesis, such as *sterol regulatory element binding protein 1c (SREBP-1c), fatty acid synthase (FASN),* and *stearoyl-coenzyme A desaturase (SCD)* [6]. In addition, we also demonstrated that RBV suppressed *glycerol-3-phosphate acyltransferase, mitochondrial* (*GPAM*) expression through downregulation of CCAAT/enhancer-binding protein α (C/EBPα) via its

proteasomal degradation [7]. GPAM is a rate-limiting enzyme for triglyceride (TG) synthesis, catalyzing the reaction of glycerol-3'-phosphate to monoacylglycerol (MAG) [8]. TG is the main component of neutral lipids in the liver, and over-accumulation of TG causes various fatty diseases such as fatty liver [9].

The catalytic reaction of glycerol-3'-phosphate to MAG is mediated by four glycerol-3-phosphate acyltransferase (GPAT) family proteins, i.e., GPAM (a human homologue of GPAT1), GPAT2, GPAT3 and GPAT4. Among these proteins, GPAM accounts for 30–50% of total GPAT activities in the liver [8]. Therefore, development of GPAM-targeted treatments may provide benefit for patients with fatty liver disease. However, there are currently no GPAM-targeted drugs. Therefore, elucidation of the mechanism by which RBV suppresses *GPAM* may be useful for the treatment of fatty liver diseases. In the present study, we demonstrated that RBV suppressed *GPAM* expression by downregulating not only C/EBPα, but also SREBP-1c. Moreover, we identified C/EBPα-binding elements in the *GPAM* genome and found that C/EBPα regulated the expression of two types of *GPAM* gene variants as a distal enhancer.

2. Materials & Methods

Details of Materials and Methods section are given in Supplementary Material.

2.1. Cell culture

Adenosine kinase (ADK)-expressing HuH-7 cells (HuH-7A) were maintained and treated with RBV as previously described [7].

2.2. Western blot analysis

Protein sample preparation and immunoblotting analysis were performed as described previously [10]. Rat anti-HA was purchased from Roche Diagnostics (Basel, Switzerland). Rabbit anti-FOXA1 was purchased from Abcam (Cambridge, UK). Mouse anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as the control for the amount of protein loaded per lane.

2.3. RT-quantitative PCR (RT-qPCR)

After extraction of total RNA from HuH-7A cells, cDNA was synthesized by using an M-MLV reverse transcriptional kit (Invitrogen, Carlsbad, CA, USA). The PCR reaction was carried out using KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan). For qPCR, we used TBGreen premix Ex Taq (TaKaRa, Shiga, Japan). The primer sets used to amplify the *GPAM* and *ATP synthase, H+ transporting mitochondrial Fo complex*

98 *subunit B1* (*ATP5F1*) genes were previously described [6,7]. The primers used to
99 amplify each *GPAM* variant are shown in Supplementary Table 1. The gene expression
100 levels were normalized to that of *ATP5F1*.

101 **2.4. Measurement of *GPAM* promoter activity**

102 HuH-7A cells were transfected with 400 ng of *GPAM* promoter-containing Luciferase
103 reporter plasmid and 1.5 ng of phRL-SV40 reporter plasmid (Promega) along with 100
104 ng of pCX4pur, pCX4pur/HA-SREBP-1c, or pCXpur/C/EBPα [11] plasmid by using
105 FuGENE® 6 (Promega), and cultured for 72 h. Luciferase activity was measured using a
106 Dual-Luciferase® Reporter Assay System (Promega).

107 **2.5. Statistical analysis**

108 Data are presented as the means±standard deviation from three independent
109 experiments. Determination of the significance of differences among groups was
110 assessed using the Student's *t*-test with a two-sided test. $P < 0.05$ was considered
111 statistically significant.

112

3. Results

3.1. Downregulation of SREBP-1c by RBV partially contributes to *GPAM* reduction.

In our recent study, we demonstrated that RBV suppressed *GPAM* expression by downregulating C/EBPα [7]. Other groups previously showed that SREBP-1 and Forkhead box protein A1 (FOXA1) regulate *GPAM* expression [12,13]. In another previous study, we revealed that RBV suppressed *SREBP-1c* expression [6]. Collectively, these facts led us to speculate that the RBV-induced downregulation of SREBP-1c would lead to *GPAM* suppression. To investigate this assumption, we performed a gain of function analysis by using HA-tagged SREBP-1c-overexpressed HuH-7A cells (designated as HuH-7AS cells). We found that the *GPAM* expression in HuH-7AS cells was significantly increased compared with that in control cells (Fig. 1A). We next examined the effect of RBV on *GPAM* in HuH-7AS cells. In line with our previous finding [7], *GPAM* expression was decreased by RBV in both HuH-7Ctrl and HuH-7AS cells; however, the suppressive effect of RBV on *GPAM* expression was attenuated in HuH-7AS cells (Fig. 1B). We also performed knockdown experiments by

using siRNAs targeting *SREBP-1* and *C/EBPα* (Fig. 1C). Confirming our previous finding [7], knockdown of *C/EBPα* strongly suppressed *GPAM* expression (Fig. 1D). We also observed that knockdown of *SREBP-1* decreased *GPAM* expression and knockdown of both transcription factors additively decreased it. These results suggest that downregulation of SREBP-1c by RBV partially contributes to *GPAM* suppression.

Next, we analyzed the expression of FOXA1, which was previously shown to be a regulator of *GPAM* expression [13]. We found that its expression was not altered by RBV treatment in HuH-7A cells (Supplementary Figure 1). These results suggest that not only C/EBPα downregulation but also SREBP-1c downregulation by RBV is involved in *GPAM* suppression.

3.2. Analysis of RBV-responsive elements in the *GPAM* promoter region

According to the FANTOM5 database [14], the *GPAM* gene has three transcriptional start sites（TSSs）(designated as 1a, 1d and 1e) (Fig. 2A). Since the mainly used-TSS of the *GPAM* gene differs among tissues, we first tried to determine the TSS of the *GPAM* gene in HuH-7A cells using the 5'-RACE method. We analyzed 15 clones obtained from 5'-RACE products and found that the transcripts of all clones started at 1d TSS of

145 the *GPAM* gene, indicating that *GPAM*-1d TSS was mainly used for *GPAM* expression
146 in HuH-7A cells (Fig. 2B). The FANTOM5 database shows that the 1d TSS of *GPAM*
147 is the most active TSS in primary human hepatocytes (FANTOM CAT:
148 https://fantom.gsc.riken.jp/zenbu/gLyphs/index.html#config=Jdb4advGDdUhpyqAEJY
149 rF;loc=hg19::chr10:113851769.114054872-), suggesting that HuH-7A cells would be a
150 useful model for analysis of *GPAM* expression in hepatocytes. We next investigated
151 whether the *GPAM*-1d promoter possesses RBV responsiveness by using a reporter
152 assay system. We cloned 2-kb genomic DNA upstream from the *GPAM*-1d TSS as a
153 *GPAM*-1d promoter and constructed a luciferase reporter plasmid. We found that the
154 *GPAM*-1d promoter activity was suppressed by RBV (Fig. 2C). To determine RBV-
155 responsive elements in the 1d promoter region, we constructed reporter plasmids with
156 serial deletions of the 1d promoter and used them to perform reporter assays. The basal
157 *GPAM* promoter activities of -654, -91, and -60 were significantly lower than those of -
158 1329, -235, and -91, respectively (Fig. 2D). We also observed that the RBV-suppressive
159 effect on the -91 *GPAM* promoter was weaker compared to that on other longer *GPAM*
160 promoters, and the -60 *GPAM* promoter activity was no longer suppressed by RBV

(Fig. 2E), suggesting that RBV-responsive elements were present in the region between -235 and -60.

A previous study showed that the region from -91 to -60 contains the SREBP-1 responsive element (SREBP-RE) [12]. Thus, we investigated whether SREBP-RE in the *GPAM*-1d promoter was an RBV-responsive element. We generated a mutant *GPAM*-1d promoter-reporter plasmid that possessed nucleotide mutations in the SREBP-RE (Fig. 2F). We observed that the *GPAM*-1d promoter activity and RBV responsiveness were decreased by introducing mutations into the SREBP-RE (Fig. 2G). These results suggest that SREBP-RE in the *GPAM*-1d promoter is an RBV-responsive element.

3.3. C/EBPα activates *GPAM* expression through binding to the distal enhancers

C/EBPα is known to be a key transcriptional factor for lipogenesis [15]. The putative C/EBPα binding site in the mouse *Gpam* (called *Gpat1*) promoter was shown previously [16], but the binding site in the human *GPAM* promoter has not been determined. Therefore, we first explored C/EBPα binding sites in the *GPAM* genomic region using public ChIP-seq data [17]. Because there were no ChIP-seq data for C/EBPα in hepatocytes, we analyzed the data in adipocytes [18], which use the same

177 TSS for *GPAM* expression as hepatocytes. We explored the regions having intense
178 DNase-sensitivity, H3K27ac-positivity and C/EBPα-positivity on the genomic region
179 around the *GPAM* gene as putative C/EBPα binding regions. We found two putative
180 regions; one was located around the 1a TSS (designated as the E1 region) and the other
181 was located approximately 34 kb-upstream from the 1a TSS (designated as the E2
182 region) (Fig. 3A). We cloned 2-kb genomic DNA upstream from *GPAM*-1a TSS as the
183 *GPAM*-1a promoter and investigated whether the *GPAM*-1a promoter had C/EBPα
184 responsiveness. By overexpression of C/EBPα, the *GPAM*-1a promoter was strongly
185 activated, but the *GPAM*-1d promoter was not (Fig. 3B). We searched for C/EBPα
186 binding sequences in the E1 and E2 regions and found sequences similar to the C/EBPα
187 consensus sequence (Fig. 3C). To examine whether C/EBPα bound to these sequences,
188 we performed EMSA using nuclear extracts prepared from C/EBPα-overexpressing
189 cells. By using an E1, E2-a, or E2-b DNA probe containing the putative C/EBPα
190 binding sequence shown in Fig. 3C, shifted bands were detected in all of these probes
191 (arrows in Fig. 3D). When a C/EBPα-specific antibody was added to the reactions, the
192 shifted bands disappeared, and supershifted bands appeared in all of the probes

193 (arrowheads in Fig. 3D). In addition, when each of the putative C/EBPα binding
194 sequences, GTTGCTCAA (E1), ATTGCACCAT (E2-a) or ATTGCTCAA (E2-b), was
195 respectively replaced with GTTCACCCC to disrupt C/EBPα binding, no bands were
196 observed (Fig. 3D). These results suggest that C/EBPα has the ability to bind to these
197 genomic regions. As shown in Fig. 2B, *GPAM* transcription occurs mainly at the 1d
198 TSS, but the identified C/EBPα binding sites were located upstream of the 1a TSS.
199 Thus, we hypothesized that C/EBPα could activate both the promoter of *GPAM*-1a and
200 that of *GPAM*-1d. To assess this possibility, we examined the effect of C/EBPα
201 knockdown on the transcriptional levels of each *GPAM* mRNA variant (the 1a, 1d or 1e
202 variant starting at each TSS) by performing RT-PCR with primers specific for each
203 variant. A previous study demonstrated that mRNAs transcribed from *GPAM*-1a TSS
204 are classifiable into three splicing variants (V1-3) [12], as observed in Fig. 3E. We
205 found that knockdown of C/EBPα reduced the expression of the *GPAM*-1d and *GPAM*-
206 1a-V2 variants, but not the *GPAM*-1e variant (Fig. 3E). In addition, only the *GPAM*-1d
207 and *GPAM*-1a-V2 variants were reduced by RBV treatment (Fig. 3F). These results
208 suggest that C/EBPα works on the expression of 1a and 1d variants of *GPAM*.

209 Interestingly, knockdown of C/EBPα or RBV treatment reduced only *GPAM*-1a-V2
210 variant expression, but not V1 or V3, suggesting that C/EBPα affects the splicing
211 pattern of *GPAM*-1a variants.

212 Next, we considered how C/EBPα activated the *GPAM*-1d promoter. It is known
213 that C/EBPα activates gene expression by altering the chromatin architecture via its
214 interaction with p300, a histone acetyltransferase [19]. Thus, we examined whether
215 C/EBPα affected the chromatin structure of the *GPAM* genomic region using FAIRE-
216 qPCR, a method used to detect open chromatin regions [20]. Knockdown of C/EBPα
217 reduced the chromatin accessibility of both the E1 and E2 regions but not the *GPAM*-1d
218 TSS region (Fig. 3G), suggesting that C/EBPα is associated with the E1 and E2
219 genomic regions and maintains an open chromatin structure at these genomic loci. From
220 these data, we hypothesized that the E1 and E2 regions functioned as distal enhancers
221 for *GPAM*-1d promoter activation. To test this possibility, we constructed reporter
222 plasmids containing the *GPAM*-1d promoter (-91) fused with the E1 and/or E2
223 sequences and examined their responsiveness to C/EBPα. When the *GPAM*-1d
224 promoter (-91) was fused with the E1 or E2 region, it acquired C/EBPα responsiveness

225 (Fig. 3 H). In addition, we observed that the C/EBPα responsiveness of the *GPAM*-1d
226 promoter (-91) fused with the E1 or E2 region was abolished when C/EBPα consensus
227 binding sites were deleted from each region (Fig. 3H). Moreover, we found that the
228 *GPAM*-1d promoter acquired more C/EBPα responsiveness by fusion with both the E1
229 and E2 regions (Fig. 3H). These results suggest that C/EBPα binding sites are located at
230 the regions 13-kb and 47-kb upstream from *GPAM*-1d TSS and regulate the expression
231 of the main transcript of *GPAM*, *GPAM*-1d, in hepatocytes as distal enhancers.
232

4. Discussion

In this study, we identified the TSS of *GPAM* mRNA in HuH-7A cells and the functional binding sites of SREBP-1 and C/EBPα in the *GPAM* genome, and revealed the underlying mechanism of *GPAM* suppression by RBV via downregulation of these transcription factors. Consistent with another group's study using HepG2 cells [12], we found that the SREBP-RE in the *GPAM*-1d promoter region was functional as a *cis* element for *GPAM* expression in HuH-7A cells. Moreover, we found that this site was responsive to RBV for *GPAM* suppression. Previous authors identified a *Gpat1* promoter region in rodents corresponding to human *GPAM*-1d, and showed that the proximal promoter sequences were highly conserved between the species, with both sequences including the SREBP-RE and CCAAT-box [21]. These data suggest that the regulatory mechanism of *GPAM* expression by SREBP-1 is conserved among species.

By assessing the *GPAM*-1d promoter activity with serial deletions, we found an RBV-responsive region between -235 and -91 of the *GPAM*-1d promoter, which lies outside of the SREBP-1 binding region (between -91 and -60). In addition, we found that the basal promoter activity of *GPAM*-1d (-91) was drastically reduced compared to

249 that of *GPAM*-1d (-235), suggesting that some factor(s) binding to the region between -
250 235 and -91 activate *GPAM* expression, and the activity of this factor(s) is suppressed
251 by RBV. Currently, we are trying to identify this unknown factor(s).

252 The mouse *Gpat1* gene has two transcriptional variants, *Gpat1*a and *Gpat1*b,
253 which are equivalent to 1d and 1e of human *GPAM*, respectively, and C/EBPα binding
254 sites have been identified in the *Gpat1a*-promoter region [16]. The human *GPAM*-1d
255 promoter has a sequence highly homologous to the C/EBPα binding region of the
256 mouse *Gpat1a* promoter. However, our promoter assay did not show any effect of
257 C/EBPα on the activation of the authentic *GPAM*-1d promoter (Fig. 3B), suggesting
258 that the regulatory mechanism of *GPAM* expression by C/EBPα differs among species.
259 Our knockdown experiments showed that C/EBPα contributes to expression of the
260 *GPAM*-1a and -1d variants and chromatin accessibility at the E1 and E2 regions. The
261 ChIP-seq data of adipocytes show that binding signals of p300, mediator of RNA
262 polymerase II transcription subunit 1 (MED1), and bromodomain-containing protein 4
263 (BRD4) are present in the E1 and E2 regions (data not shown). Since these factors are

264 involved in formation of a super enhancer[22], C/EBPα may play roles in the formation
265 of a super enhancer to activate the expression of *GPAM*-1a and -1d variants.

266 GPAM acts as a rate-limiting enzyme of TG synthesis. Since GPAM accounts for
267 30–50% of the total GPAT family protein activity in hepatocytes, it has been suggested
268 that GPAM is associated with hepatic steatosis caused by accumulation of TG [23]. In
269 mouse experiments, previous reports have shown that knockout of the *Gpat1* gene
270 reduced liver TG content and plasma cholesterol [24], implying that GPAM-targeted
271 therapy may be useful for the treatment of fatty diseases. On the other hand, another
272 report showed that *GPAM* expression is important for the anti-viral response of T cells
273 [25]. These reports suggest that the suppression of *GPAM* expression in a wide range of
274 tissues is paradoxically both beneficial to health and, in some cases, harmful. As we
275 have shown in this study, RBV suppressed only the *GPAM*-1a and -1d variants, but not
276 the -1e variant, in HuH-7A cells. According to the FANTOM5 database [14], the
277 *GPAM*-1a and -1d variants are mainly expressed only in hepatocytes and adipocytes,
278 and the *GPAM*-1e variant is mainly expressed in other types of cells. These data suggest
279 that RBV does not affect *GPAM* expression in cells other than hepatocytes and

adipocytes, implying its usefulness for liver- or adipose tissue-targeting treatments of fatty disease. However, since RBV is known to cause hemolytic anemia [26], the discovery and exploration of drugs with fewer side effects could lead to the development of more useful treatments.

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372 **Figure legends**

373 **Fig. 1 The effect of SREBP-1c on *GPAM* expression.**

374 (A) *GPAM* mRNA expression level (upper panel) and HA-tagged SREBP-1c protein

375 level (lower panel) in HA-tagged SREBP-1c-expressing HuH-7A cells and control cells.

376 $^{**}P < 0.01$ versus control cells. (B) Relative *GPAM* expression level when the cells

377 were treated with RBV for 48 h. The expression level in the cells not treated with RBV

378 was assigned a value of 1. $^{*}P < 0.05$ versus control cells. (C, D) mRNA expression

379 levels in HuH-7A cells transfected with the corresponding siRNAs. siRNA-transfected

380 HuH-7A cells were cultured for 96 h. $^{*}P < 0.05$, $^{**}P < 0.01$ versus siCtrl.

381

382 **Fig. 2 Determination of *GPAM* TSS in HuH-7A cells and analysis of *GPAM***

383 **promoter activity.**

384 (A) The genomic structure of three GPAM variants. The black boxes show exons. (B)

385 The results of 5'-RACE analysis. The upper portion shows the locus of GPAM and the

386 lower one shows the cloned transcripts in HuH-7A cells. (C) The RBV responsiveness

387 of *GPAM*-1d promoter (-2022) activity. At 24 h after transfection of each reporter

388 plasmid into HuH-7A cells, the cells were treated with RBV for 48 h and then luciferase
389 activity was measured. $^{**}P < 0.01$ versus RBV 0 μM. (D) Basal activity of the *GPAM*-
390 1d promoter with various lengths of genomic DNA in HuH-7A cells. At 72 h after
391 transfection of each reporter plasmid into HuH-7A cells, luciferase activity was
392 measured. $^{*}P < 0.05$, $^{**}P < 0.01$. (E) The RBV-responsiveness of *GPAM*-1d promoters.
393 At 24 h after transfection of each reporter plasmid into HuH-7A cells, cells were treated
394 with RBV for 48 h and then luciferase activity was measured. $^{*}P < 0.05$, $^{**}P < 0.01$
395 versus RBV 0 μM. (F) The schematics of *GPAM*-1d promoter construction. The gray
396 box shows SREBP-RE. In mutagenesis experiments, SREBP-RE TCAGCCTAGC was
397 replaced with aaAaCCTAGC. The lower case letters show mutations in SREBP-RE.
398 (G) Promoter activity of the wild type *GPAM*-1d promoter (-2022) or mutated promoter
399 (-2022)-SREBPmt. $^{**}P < 0.01$ (left panel). RBV-responsiveness of the wild type and
400 mutant *GPAM*-1d promoters. At 24 h after transfection of each reporter plasmid into
401 HuH-7A cells, the cells were treated with RBV for 48 h and then luciferase activity was
402 measured. $^{*}P < 0.05$, $^{**}P < 0.01$ versus RBV 0 μM (right panel).
403

Fig. 3 Determination of C/EBPα binding sites and their involvement in *GPAM* expression.

(A) The Dnase-seq and ChIP-seq data at the locus of *GPAM*. The Dnase-seq data are derived from HuH-7 cells. The ChIP-seq data for C/EBPα are derived from adipocytes, and the data for H3K27ac are derived from HuH-7 cells. The square areas show putative C/EBPα binding sites (designated as the E1 and E2 regions). The bars on the data panels show the distances between each region. (B) *GPAM*-1a (-1979) and *GPAM*-1d (-2022) promoter activities by overexpression of C/EBPα. HuH-7A cells co-transfected with reporter plasmids and C/EBPα overexpression plasmid were cultured for 72 h and then luciferase activity was measured. $^{**}P < 0.01$ versus Ctrl. (C) The putative C/EBPα binding sequences at the E1 and E2 regions. The C/EBPα primary binding motif is shown under the putative C/EBPα binding sequence in the E1 region. (D) The EMSA showing C/EBPα binding to E1, E2-a, and E2-b. The arrows show shifted bands. The competition experiment included the addition of a 10-fold excess of non-labeled probe. The arrowheads show supershifted bands. (E, F) The expression pattern of *GPAM* gene variants. HuH-7A cells were transfected with siRNAs (E) or treated with RBV (F) for

48 h. (G) The changes of chromatin accessibility at the 1d-TSS, E1 and E2 regions by C/EBPα knockdown. HuH-7A cells transfected with siRNAs were cultured for 48 h and then the cells were crosslinked. The DNA recovery ratios were normalized to that of β-actin promoter (ACTB-p). $^{**}P < 0.01$ versus siCtrl. (H) The promoter activity of the *GPAM*-1d promoter (-91) fused with the E1 and/or E2 region. HuH-7A cells co-transfected with reporter plasmids and the C/EBPα over-expression plasmid were cultured for 72 h and then luciferase activity was measured. The red box shows the putative C/EBPα binding sequence and the mark (indicated by an X) shows the deletion site. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Ctrl.