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# Transcriptional regulation of mouse 6-phosphogluconate dehydrogenase by ADD1/SREBP1c

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**Abstract**

6-Phosphogluconate dehydrogenase (6PGDH) constitutes the pentose phosphate pathway and produces NADPH. 6PGDH is also considered as a lipogenic gene since NADPH is a pivotal cofactor for lipogenesis. Thus, it is important to elucidate how 6PGDH is regulated by various signals related to energy homeostasis. Here, we provide several evidences that ADD1/SREBP1c regulates the expression of mouse 6PGDH gene. DNase I footprinting assay and point mutation studies revealed that the E-box (CANNTG) motif in the promoter of mouse 6PGDH is an important *cis*-regulatory element for ADD1/SREBP1c. 6PGDH mRNA is highly expressed in white adipose tissue and tightly modulated by nutritional status. Furthermore, we found that ADD1/SREBP1c mediates insulin-dependent 6PGDH expression and that PI3-kinase is an important linker for its regulation. Taken together, these data suggest that ADD1/SREBP1c is a key transcription factor for 6PGDH gene expression and would coordinate glucose metabolism and lipogenesis for energy homeostasis.


*Keywords:* 6-Phosphogluconate dehydrogenase; NADPH; ADD1/SREBP1c; Insulin; Transcription

6-Phosphogluconate dehydrogenase (6PGDH: EC1.1.1.44) is an enzyme involved in the pentose phosphate pathway (PPP). 6PGDH and glucose 6-phosphate dehydrogenase (G6PDH), another enzyme of the PPP, are the main sources of NADPH in non-photosynthetic cells [1]. NADPH provides the reducing power for biosynthetic processes such as elongation of fatty acids, de novo synthesis of cholesterol [2,3]. Reducing power is also required to maintain the redox potential which is crucial for protection against oxidative stress and regulation of cellular proliferation and survival [4–6]. Since 6PGDH plays an essential role in maintaining cellular NADPH pool, any change of 6PGDH activity would affect energy homeostasis, growth rate, and cellular survival [7]. Both expression level and enzymatic activity of 6PGDH are regulated by diet and several hormones [8,9]. Furthermore, the fact that lipogenesis consumes large amounts of NADPH implies that 6PGDH is required to be activated when lipogenesis is stimulated [10].

ADD1/SREBP1c (adipocyte determination- and differentiation-dependent factor 1/sterol-regulatory element binding protein 1c) is a member of SREBP transcription factors. The SREBPs constitute a family of basic helix–loop–helix (bHLH) transcription factors and three isoforms have been identified [11–13]. A unique feature of SREBPs is dual DNA binding specificity to both classical palindromic E-box (CANNTG) and non-palindromic sterol-regulatory elements (SREs: ATCACCCCAC) [11,14,15]. Among three SREBPs, ADD1/SREBP1c plays a crucial role in fatty acid metabolism and insulin-dependent gene regulation especially in the regulation of lipogenic gene expression in fat and liver [16–19].

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Regulation of gene expression by insulin has been intensely studied for many years. It has been revealed that insulin regulates the expression of several key enzymes in fatty acid synthesis such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) [18,20,21]. Following insulin-induced insulin receptor (IR) autophosphorylation, insulin receptor substrate proteins are phosphorylated and mediate the transmission of insulin signaling pathway. Insulin signaling is also associated with several second messengers including phosphatidylinositol 3-kinase (PI3-kinase) and mitogen-activated protein kinase (MAPK) [22]. Phosphatidylinositol 3,4,5-phosphate regulates the activity or subcellular localization of phosphatidylinositol-dependent kinase and protein kinase B (also known as PKB/Akt) [23,24], and consequently modulates a number of target proteins including glycogen synthetase kinase-3 (GSK3), transcription factors, and coactivators. Recently accumulated evidences suggest that the effects of insulin on the expression of lipogenic genes are mediated by ADD1/SREBP1c [17,18,25]. Although further studies are required, it has been reported that ADD1/SREBP1c is stimulated by the PI3-kinase pathway as well as repressed by GSK3 to link insulin action to its target gene expression [26,27].

Although NADPH produced by 6PGDH is associated with lipogenesis in fat and liver, the transcription factor(s) responsible for 6PGDH expression has not been thoroughly investigated. In this study, we demonstrate that 6PGDH is highly expressed in fat tissue and its expression is enhanced by ADD1/SREBP1c via E-box motif in the proximal promoter region of mouse 6PGDH (m6PGDH) gene. Moreover, we reveal that ADD1/SREBP1c is also involved in insulin-dependent 6PGDH expression. These results suggest that ADD1/SREBP1c is a key transcription factor linking insulin signal and 6PGDH gene expression during lipogenesis.

## Materials and methods

*Animal treatment*. Male C57BL/6J mice were housed (5 mice per cage), and water was given ad libitum, with 12 h light–dark cycle beginning at 07:00 a.m. In experiments, food was withdrawn during the daylight (12 h) before onset of the dark cycle.

*Cell culture*. 3T3-L1 and RatI-IR cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%(v/v) bovine calf serum (BCS, Jeil Biotech, Daegu, South Korea) and 100 U of antibiotic–antimycotic at 10% $CO_2$ and 37 °C. Differentiation into adipocytes was achieved by allowing the cells to reach confluence before the addition of DMEM supplemented with 10% fetal bovine serum (FBS, JBI), 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 5 μg/mL insulin at 5% $CO_2$ and 37 °C. After 2 days and every 2 days thereafter, fresh medium (DMEM plus 10% FBS and 5 μg/mL insulin) was changed. Human embryonic kidney 293 (HEK293) cells were maintained in DMEM supplemented with 10% FBS and 100 U of antibiotic–antimycotic and cultured at 37 °C in a 10% $CO_2$ incubator.

*Cloning of mouse 6PGDH promoter and construction of luciferase reporters.* Mouse genomic DNA was isolated from 3T3-L1 cells using lysis buffer (50 mM Tris, pH 7.5, 50 mM EDTA, 100 mM NaCl, and 2% SDS). The primers used for polymerase chain reaction (PCR) were as follows: −978 to +163 bp fragment—forward, 5′-CGT GGT ACC ACA TGC CTT-3′ and reverse, 5′-CCG GGC CCG ACT ACG CGT GTC GTC ACT CAC TGG GCC ATG-3′; −646 to +163 bp fragment—forward, 5′-TAC AAG CTT AAG GTA CCA CTC ACT TCC AGT CTT GCC-3′; −345 to +163 bp fragment—forward, 5′-GTA CAA GCT TAG GTA CCC ACA GAT AGG ACA GAC-3′. The primers included the sequences for the *Kpn*I and *Mlu*I restriction enzyme sites. The PCR products were digested with *Kpn*I and *Mlu*I, and cloned into the pGL3 basic vector (Promega).

*Electrophoretic mobility shift assay*. Electrophoretic mobility shift assays (EMSAs) were performed in 20 μL volume containing purified recombinant ADD1/SREBP1c protein (20 ng) in the reaction buffer (10 mM Tris, pH 7.6, 50 mM KCl, 2.5 mM $MgCl_2$, 0.05 mM EDTA, 0.1%(v/v) Triton X-100, 8.5%(v/v) glycerol, 1 μg of poly(dI–dC), 1 mM dithiothreitol, and 0.1 mg/mL non-fat dried milk). $^{32}$P-labeled probe (0.1 pmole) was added into the reaction mixture and incubated at RT for 20 min. The samples were resolved in a 4% polyacrylamide gel with 0.25× Tris–borate–EDTA (TBE) buffer, and the gels were processed for autoradiography. For competition assays, unlabeled oligonucleotides (100-fold molar excess) were added into reaction mixture prior to the addition of radioisotope labeled probe. The DNA sequences of the double-stranded oligonucleotides were used as the following (only one strand is shown): ARE7, 5′-GAT CTG TGA ACT CTG ATC CAG TAA G-3′; SRE, 5′-GAT CCT GAT CAC CCC ACT GAG GAG-3′.

*DNase I footprinting assay*. DNA fragments of m6PGDH promoter were labeled in one strand and purified as described in below. m6PGDH promoter fragment was isolated by serial digestion with *Pst*I and *Nhe*I to obtain 5′- and 3′-overhanging ends. Subsequent DNA was labeled with Klenow fragment and [α-$^{32}$P]dCTP, and then purified by PAGE. DNA–protein binding reactions were performed with 50,000 c.p.m. of probe per reaction in the solution containing 10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM EDTA, pH 8.0, 7%(v/v) glycerol, 1 mM dithiothreitol, 2 μg of poly(dI–dC), and indicated amount of recombinant ADD1/SREBP1c protein. After 30 min of incubation on ice, 5 U of DNase I, freshly diluted in a solution containing 10 mM Hepes, pH 7.6, 60 mM KCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$, and 7%(v/v) glycerol, was added to the reaction and then kept at RT for 2 min. Digestion reactions were stopped by the addition of 80 μL of stop solution containing 20 mM Tris–HCl, pH 8.0, 20 mM EDTA, 250 mM NaCl, 0.5% SDS, 4 μg of yeast tRNA, and 10 μg of proteinase K. The samples were incubated for 1 h at 45 °C, extracted with phenol/chloroform, precipitated with ethanol, and resuspended in formamide dye. The samples were resolved in 6%(w/v) polyacrylamide/7 M urea sequencing gel. The protected regions were mapped with reference to the migration of Maxam-Gilbert A + G sequencing products.

## Results

### *6PGDH is predominantly expressed in fat tissue and is induced during adipogenesis*

In order to examine the tissue distribution of mouse 6PGDH mRNA expression, we performed Northern blot analysis. 6PGDH mRNA was highly expressed in white adipose tissue, and low amounts of 6PGDH were detected in several tissues including liver, spleen, kidney, and lung (Fig. 1A). Furthermore, we observed that the

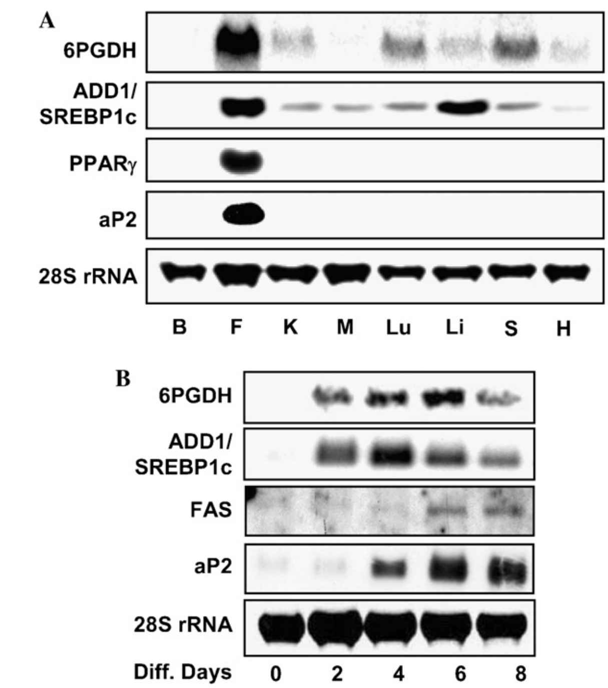


Fig. 1. Expression of 6PGDH in mouse tissue and adipocytes. (A) Tissue distribution of 6PGDH mRNA expression. Total RNA was isolated from several tissues of lean C57BL/6J mice. B, brain; F, white adipose tissue; K, kidney; M, muscle; Lu, lung; Li, liver; S, spleen; H, heart. Northern blot analysis was performed with each radiolabeled cDNA probe. (B) Induction of 6PGDH mRNA during adipogenesis. During 3T3-L1 adipocyte differentiation, total RNA was isolated at the indicated time points and Northern blot analysis was performed.

expression of 6PGDH mRNA increased during adipogenesis (Fig. 1B). The increment of 6PGDH mRNA is comparable to the induction of other adipocyte marker genes. These results implicate that 6PGDH might be regulated by adipogenic transcription factors in fat tissue.

### *ADD1/SREBP1c binds to mouse 6PGDH promoter directly*

To investigate the regulatory mechanisms of 6PGDH gene expression, we decided to characterize the proximal promoter region of the m6PGDH gene. We cloned and analyzed the nucleotide sequence of m6PGDH promoter to search for the transcription factors that might regulate its expression. Within 0.98 kb upstream promoter region of the m6PGDH gene, there are several putative *cis*-elements including a Sp1 binding site and C/EBP binding sites (Fig. 2). In addition, four E-box (CANNTG) motifs were identified at −796, −468, −457, and −8 bp upstream regions. Among E-box binding transcription factors, ADD1/SREBP1c is well known to regulate many lipogenic genes in hepatocytes and adipocytes [16–18].

These findings have prompted us to ask whether ADD1/SREBP1c is involved in the regulation of m6PGDH expression. To answer this question, several luciferase reporter constructs containing m6PGDH promoter regions were generated and cotransfected into HEK293 cells with ADD1/SREBP1c expression vectors. As shown in Fig. 3A, ADD1/SREBP1c efficiently transactivated −345m6PGDH-Luc, suggesting that the proximal region (−345 to +163 bp) of m6PGDH promoter contains enough *cis*-regulatiory elements for ADD1/SREBP1c. In parallel, binding ability of ADD1/SREBP1c to the m6PGDH promoter was examined by electrophoretic mobility shift assay (EMSA). Recombinant ADD1/SREBP1c protein formed a stable DNA–protein complex with the probe containing m6PGDH promoter region from −345 to +163 bp (Fig. 3B). To precisely pinpoint the ADD1/SREBP1c binding site within m6PGDH promoter, we performed DNase I footprinting assay. As shown in Fig. 3C, recombinant ADD1/SREBP1c protein protected the E-box motif at −8 bp of m6PGDH promoter (Fig. 3C). Together, these results indicate that ADD1/SREBP1c is able to bind and transactivate m6PGDH promoter.

### *ADD1/SREBP1c transactivates m6PGDH promoter via the E-box at −8 bp upstream region*

It is well known that ADD1/SREBP1c exhibits unique dual DNA binding specificity to both SRE and E-box motifs [15]. Within −345 bp region of the m6PGDH promoter, there is one E-box, and no conserved SRE motif is found (Fig. 2). Furthermore, DNase I footprinting assays showed that ADD1/SREBP1c specifically bound to the E-box motif (Fig. 3C). To examine whether ADD1/SREBP1c recognizes the E-box motif of m6PGDH promoter, we tested wild-type (WT) and a point mutation form of ADD1/SREBP1c (ADD1 Y → R), which recognizes only E-box motif [15,18]. As shown in Fig. 4A, m6PGDH promoter was effectively transactivated by ADD1 Y → R as well as by ADD1 WT. This result clearly indicates that ADD1/SREBP1c transactivates m6PGDH promoter by recognizing the E-box motif as the major responsive element rather than SRE motif.

To verify the idea that ADD1/SREBP1c stimulates m6PGDH promoter activity via the proximal E-box motif, we examined the promoter activity of −345m6PGDHmutE-Luc reporter construct containing m6PGDH promoter with a mutation in the E-box motif. As shown in Figs. 4A and B, ADD1/SREBP1c did not transactivate the mutated m6PGDH promoter. We also performed EMSA with several DNA oligonucleotides containing the m6PGDH E-box or the point mutated m6PGDH E-box as competitors (Fig. 4C). Excessive cold m6PGDH E-box oligonucleotides abolished the formation of protein–DNA probe complexes (Fig. 4C, lanes 5 and 6). However, oligonucleotides containing mutated E-box failed to compete with the radiolabeled

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-978                                                                  ACATGCCT   TTAATCCCAG

-960   CACTCCGGAG  GCAGAGGCAG  GCGGATTTCT  GAGTTCGAGG  CTAGCCTGGT  CTACTGAGTG

-900   AGTTCCAGGA  CAGGCAGGGC  TACACAGAGA  AAAAATCCCG  CCCCCCGAAA  AAAAAAAAAA

-840   AAAAAAAAAA  AAAAAAGAAA  AGAAAAAAGA  AAGCAGCGCA  CCAACACCTG  AGTCTGAATG
                                                          E-box
-780   GAAAAGTAAA  TTGAAACTAA  AAGTGTCTGA  CCAGCGGGCG  AAACAGCACC  CCAGACCTCC

-720   AGCCCGGAAA  CTGTAGTGGG  GCTGAGGAAA  TGGTAGAGAA  GAGTGCTTGT  GTTCCGCTAC
                         C/EBP
-660   AAGCACAAGG  AAGAACTCAC  TTCCAGTCTT  GCCTCCCCGA  GTATCGGCCA  CGCACGAGGT

-600   ACACGGACAT  ACATGCAGGC  AAACCAATAC  ACAAAAAATA  AATAATTATG  AAACCACCCC

-540   CCCAAAACCC  CTCAAGCCTC  TCCTCCGCTT  CGTTTTCTCT  TTCTGTGCGA  GTGGGCGCAC

-480   GCGCCACTGC  TGCACGTGGA  GGTCAGATGC  CAACTTTGCG  GAGTGGGTTC  CACTTCCACC
                    E-box       E-box
-420   CGCTTGGCTC  TTTACTTGCT  GACCGTCTCG  TCTTAGTTTG  CACCGCTGTT  TCTTGACCTG

-360   CAAAAATGGT  ACACACTGAG  GTTCCCACAG  ATAGGACAGA  CTTGACTCCT  GCCTCAGAGT
                           C/EBP
-300   TGAGGGACAG  TGAAGGGAAG  CAGCAGCCAT  GGATCAAAAA  CGCCAGTGGG  CTTGGTAAGG

-240   GCTGCACCAA  GCCCTTAATG  TCTTCTCTTT  CACGCCGAGC  TGCAGACCCG  AGCTTCCGCA

-180   CGAGCGGGAG  GCATCGTGCA  AGGTCCCGCT  AGAACACTTA  GAAGAAGCCC  TTTAAACGCT
                     C/EBP
-120   CCTAGAACAC  TTAGAAGAAG  CCCTTTAAAC  GCTCCTCTCC  AGCTCGAGGT  GCCATCGACT

 -60   GCTCGGCGCT  CTACAGATCT  GGGCCCCGGG  GGTGGGCGGG  TCCGTTTGCC  CTCAGGTGGT
                                  Sp1 (GC-Box)                   E-box
 + 1   ATAACAGGCG  CCGCAGAGAC  CCAATAGCGT  GGGGCATCGG  CCCACCCCTG  TCTTCTGATT

 + 61  TCTGTAAGCC  TCTCGAGGCG  CCGGGAGCCG  GACTTCACTG  TACTTGCCTC  GGAGCGCTCG

 +121  GTCCTTCGCG  TTTCTTCCTC  CTCGACTCTG  CTTCGTCTGC  CTCCGCC   ATG GCC CAG TGA

                                                                 Met Gln Trp Val
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Fig. 2. The putative *cis*-regulatory elements in the mouse 6PGDH promoter. The nucleotide sequence of m6PGDH promoter from −978 bp to translation initiation site is shown. This sequence is numbered relative to the computer-aided transcription initiation site. Four E-boxes, putative three C/EBP sites, and one Sp1 binding site are marked with arrows.

probe (Fig. 4C, lane 7). Thus, it is very likely that the E-box at −8 bp is important for induction of m6PGDH promoter by ADD1/SREBP1c.

### *6PGDH expression in adipose tissue is influenced by nutritional state*

It has been demonstrated that enzyme activity of 6PGDH changes upon nutritional conditions [28,29]. To investigate how nutritional states affect expression of 6PGDH mRNA, we performed Northern blot analysis with mouse fat tissue isolated under several different nutritional conditions. As shown in Fig. 5A, 6PGDH mRNA dramatically decreased upon fasting. On the contrary, normal-chow refeeding condition recovered 6PGDH mRNA expression close to the control feeding condition. As previously reported, ADD1/SREBP1c and FAS mRNA levels were markedly decreased in the fasting status and significantly induced under refeeding status in white adipose tissue (Figs. 5A and B). These results suggest that 6PGDH expression is also tightly regulated by nutritional status, and ADD1/SREBP1c would regulate the expression of 6PGDH to coordinate energy homeostasis in fat tissue.

### *Insulin stimulates 6PGDH gene expression via ADD1/SREBP1c*

Since ADD1/SREBP1c plays a crucial role in mediating insulin-dependent lipogenic gene expression, it

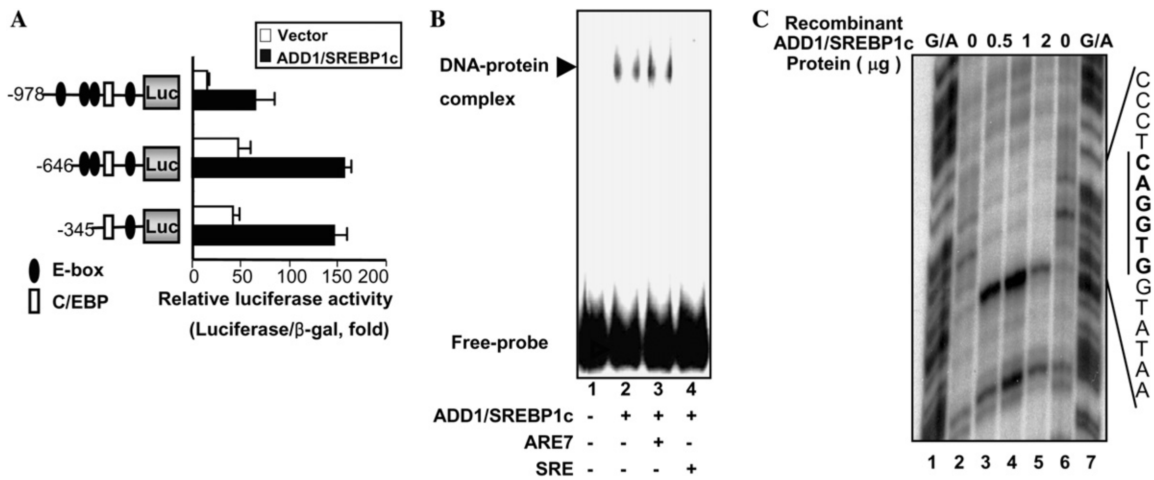


Fig. 3. Binding of ADD1/SREBP1c to the m6PGDH promoter. (A) Diagram of luciferase reporter constructs containing serially deleted m6PGDH promoter region and some putative transcription factor binding sites is shown on the left. Each reporter construct, −967m6PGDH-Luc, −646m6PGDH-Luc, or −345m6PGDH-Luc, was cotransfected with ADD1/SREBP1c expression vector and pCMV-βgal into HEK293 cells. The luciferase activities were normalized by β-galactosidase assays and error bars indicate standard deviations ($n = 2$). (B) Binding of ADD1/SREBP1c to the m6PGDH promoter. DNA fragments corresponding to the m6PGDH promoter (from −345 to +163 bp) were radiolabeled with [$\gamma$-$^{32}$P]ATP and incubated with (lanes 2–4) or without (lane 1) recombinant ADD1/SREBP1c protein. For competition assays, 100-fold molar excess of unlabeled ARE7 (lane 3) or SRE (lane 4) oligonucleotides were used. DNA–ADD1/SREBP1c protein complexes and free probe are indicated by closed and open arrowheads, respectively. (C) DNase I footprinting analysis of the mouse 6PGDH promoter. Lanes 1 and 7, G/A ladder; lanes 2 and 6, DNase I treatment of the mouse 6PGDH promoter in the absence of recombinant ADD1/SREBP1c protein; lanes 3–5, in the presence of increasing concentration of recombinant ADD1/SREBP1c (0.5, 1, and 2 μg).

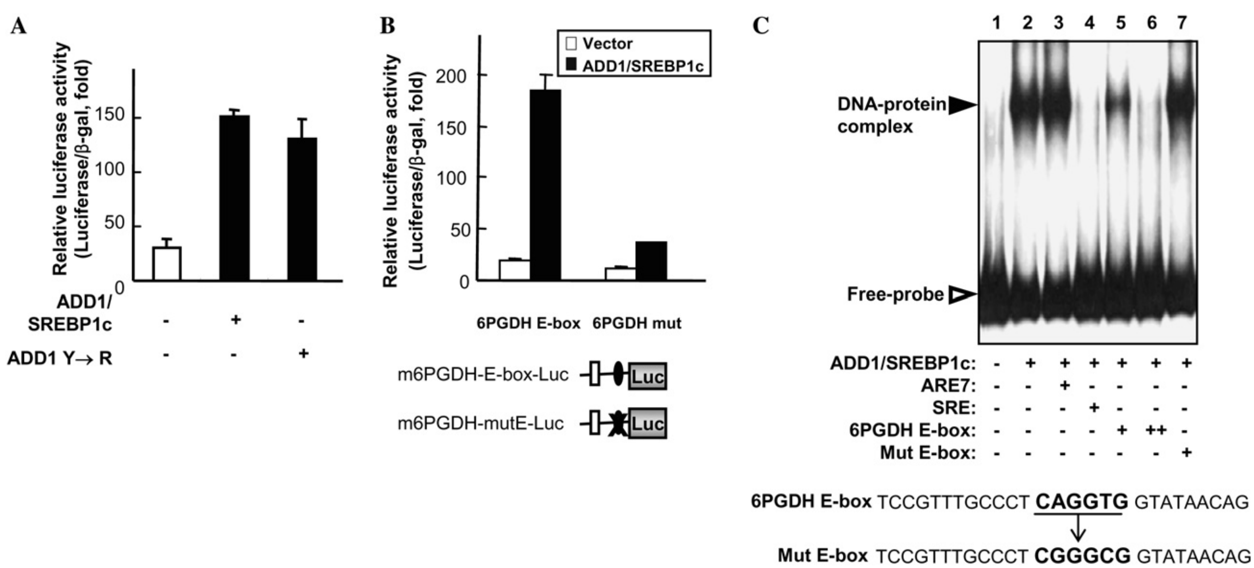


Fig. 4. Transactivation of m6PGDH promoter by ADD1/SREBP1c via E-box motif. (A) Transactivation of m6PGDH promoter by ADD1/SREBP1c via E-box. The −345m6PGDH-Luc was cotransfected with either ADD1 WT (lane 2) or ADD1 Y →R (lane 3) and pCMV-β gal into HEK293 cells. (B) Luciferase reporter constructs, m6PGDH-E-box-Luc and m6PGDH-mutE-Luc, were cotransfected with ADD1/SREBP1c expression vector and pCMV-βgal into HEK293 cells. The luciferase activities were normalized by β-galactosidase assays and error bars indicate standard deviations ($n = 2$). (C) Binding specificity of ADD1/SREBP1c to E-box of m6PGDH promoter. The DNA fragment of m6PGDH promoter (−345 to 163 bp) was radiolabeled with [$\gamma$-$^{32}$P]ATP and incubated with (lanes 2–7) recombinant ADD1/SREBP1c protein or without (lane 1). In competition assays (lanes 3–7), 100-fold molar excess of unlabeled ARE7 (lane 3), SRE (lane 4), oligonucleotides of E-box region of m6PGDH (lane 5, 10-fold; lane 6, 100-fold molar ratio), and mutated E-box region (lane 7, 10-fold molar ratio) were added in the reaction mixture. Nucleotide sequences of E-box region of the m6PGDH promoter and mutated E-box motif are shown.

would be interesting to examine whether ADD1/SREBP1c is involved in insulin-dependent 6PGDH expression in adipocytes. We conducted Northern blot analysis and luciferase reporter assays to elucidate how insulin stimulates 6PGDH gene expression in adipocytes. As shown in Fig. 6A, insulin treatment of 3T3-

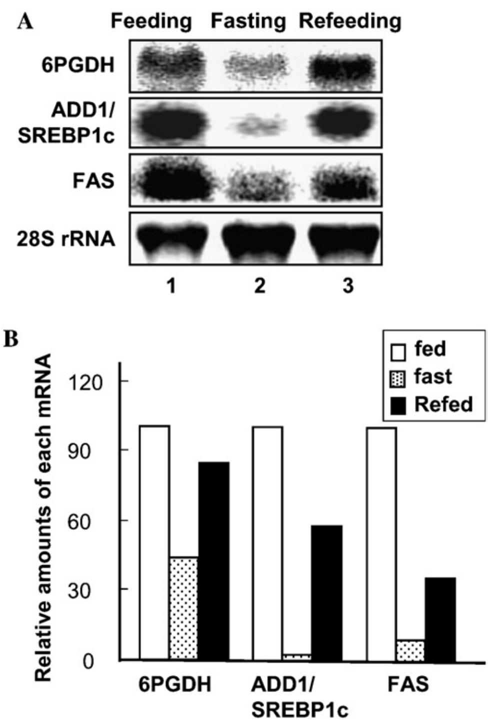


Fig. 5. Regulation of 6PGDH gene expression by nutritional status in mouse white adipose tissue. (A) Nutritional regulation of ADD1/SREBP1c, FAS, and 6PGDH mRNA expression. Each mRNA expression level was analyzed by Northern blot analysis. The feeding controls were allowed free access to food (lane 1). The fasting group was restricted from food access for 16 h (lane 2). The refeeding group was allowed for free access to food for 8 h after 16 h of fasting (lane 3). (B) Relative expression levels of each mRNA for feeding, fasting, and refeeding groups were quantified and normalized to 28S rRNA.

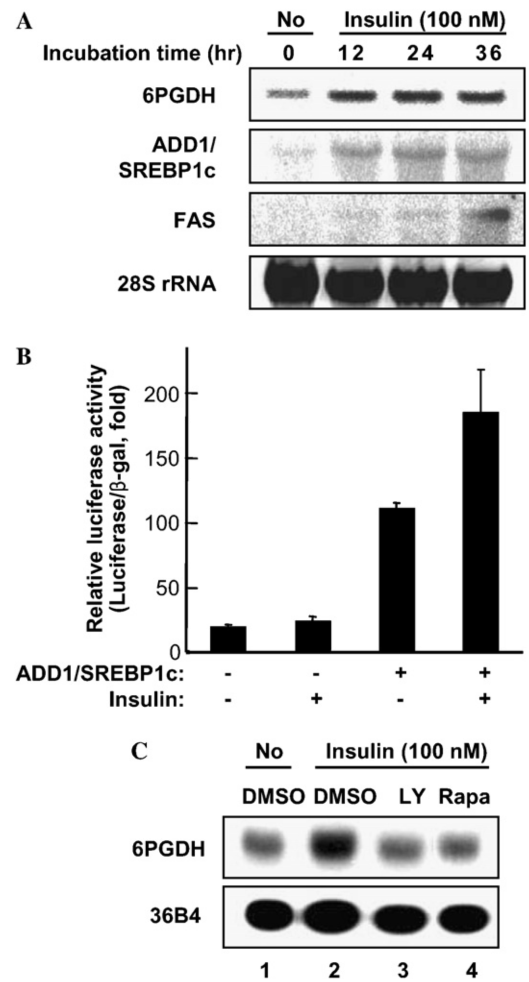


Fig. 6. Insulin-dependent 6PGDH expression through ADD1/SREBP1c. (A) Time-dependent insulin-induced expression of ADD1/SREBP1c, FAS, and 6PGDH in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were incubated overnight in serum-free low glucose DMEM containing 0.5% BSA and treated with insulin (100 nM). mRNA levels were examined by Northern blot analysis. 28S rRNA was used to normalize RNA loading. (B) Transactivation of the mouse 6PGDH promoter by ADD1/SREBP1c. −345m6PGDH-Luc reporter construct was cotransfected with or without ADD1/SREBP1c expression vector into RatI-IR cells. After transfection, cells were incubated overnight in serum-free DMEM with 0.5% BSA and treated with insulin (100 nM) for 24 h. Then the cells were harvested and assayed for luciferase activity and β-galactosidase activity. (C) Inhibitory effect of PI3-kinase blocker on insulin induced expression of 6PGDH in 3T3-L1 adipocytes. Insulin was added after 1 h preincubation of either LY 294002 (50 μM) or rapamycin (100 nM). The cells were harvested and total RNA was isolated after 24 h incubation with insulin.

L1 adipocytes enhanced the expression of 6PGDH mRNA. Next, RatI-IR cells, which stably overexpress IR, were cotransfected with m6PGDH-Luc reporter and ADD1/SREBP1c expression vector in the absence or presence of insulin treatment. Insulin significantly increased the promoter activity of 6PGDH gene in the presence of ADD1/SREBP1c while insulin barely changed basal 6PGDH promoter activity (Fig. 6B). Therefore, these results indicate that 6PGDH mRNA expression is promoted by insulin through ADD1/SREBP1c in adipocytes.

To elucidate how kinase signaling cascades mediate insulin-stimulated 6PGDH expression, we tested the effects of several kinase inhibitors on the insulin-dependent expression of 6PGDH. As shown in Fig. 6C, LY294002, a specific inhibitor of PI3-kinase, and rapamycin, an inhibitor of mTOR, evidently suppressed the insulin-stimulated 6PGDH expression. Thus, it appears that PI3-kinase activity is necessary for the induction of 6PGDH by insulin. This result is also consistent with the previous report that activation of ADD1/SREBP1c by insulin is mediated by PI3-kinase pathway [30]. Taken together, these results suggest that ADD1/SREBP1c is the responsible transcription factor which mediates insulin-induced 6PGDH expression in adipocytes.

## Discussion

In the present study, we demonstrated that m6PGDH gene expression is regulated by ADD1/SREBP1c. There are several lines of evidence to support the above idea. First, the nucleotide sequence analysis revealed that the promoter of m6PGDH gene contains a putative E-box motif. Second, in vitro EMSA showed that recombinant ADD1/SREBP1c protein bound to the m6PGDH promoter in a sequence specific manner. Third, m6PGDH promoter was significantly transactivated by ADD1/SREBP1c. Furthermore, activation of m6PGDH promoter was successfully recapitulated by ADD1 Y $\rightarrow$ R mutant, evidently indicating that activation of m6PGDH promoter by ADD1/SREBP1c is mediated through the E-box motif. Additionally, mutation studies of the 6PGDH promoter and DNase I footprinting analysis confirmed the above observations. Consistent with these results, it has been reported that ADD1/SREBP1c overexpressing transgenic mice exhibit markedly increased 6PGDH mRNA level in liver while ADD1/SREBP1c knockout mice show reduction of 6PGDH mRNA level [19,31]. Together, these data strongly suggest that 6PGDH is a target gene of ADD1/SREBP1c, and the E-box motif at the proximal promoter region of m6PGDH is the responsible *cis*-element.

Interestingly, 6PGDH gene expression is closely correlated with the expression pattern of ADD1/SREBP1c. Like many adipogenic marker genes, 6PGDH mRNA was highly expressed in adipose tissue and increased during adipocyte differentiation (Fig. 1). This result implies that 6PGDH expression may play a role, at least, in lipid metabolism by supplying reducing power, NADPH. ADD1/SREBP1c is also abundantly expressed in adipose tissue and its expression is detected at the early stage of adipocyte differentiation [13,16]. Thus, it appears that ADD1/SREBP1c might be associated with adipogenic induction of 6PGDH gene similar to other ADD1/SREBP1c target genes.

In addition, 6PGDH mRNA expression is modulated by nutritional status; feeding increased 6PGDH mRNA expression whereas fasting reduced it in adipose tissue, which is similar to that of ADD1/SREBP1c (Fig. 5A). In fact, not only ADD1/SREBP1c but also most lipogenic enzymes such as FAS, ACC, and SCD, which are also target genes of ADD1/SREBP1c, are regulated by the nutritional status [18,21,25]. Thus, it is likely that increased expression of 6PGDH by refeeding is stimulated through the activation of ADD1/SREBP1c. Furthermore, expression of 6PGDH mRNA was enhanced by insulin. It has been reported that expression of lipogenic genes including ADD1/SREBP1c and FAS is tightly modulated by insulin [17,20,21], and insulin stimulates expression of ADD1/SREBP1c via PI3-kinase in liver and adipose tissue [30]. Inhibitory effects of LY294002 and rapamycin on insulin-dependent induction of 6PGDH were very similar to those of ADD1/SREBP1c (Fig. 6C) [30,32]. Cumulative evidence suggests that ADD1/SREBP1c is a crucial transcription factor to orchestrate both fatty acid and glucose metabolisms to maintain energy homeostasis in an insulin-dependent manner [17,33]. Insulin has, at least, dual effects on the regulation of ADD1/SREBP1c at the levels of transcription and post-transcription [18,33]. Also, insulin is able to augment transcriptional activity of ADD1/SREBP1c by post-translational modification such as phosphorylation [30].

As insulin sensitive tissues, adipose tissue and liver are crucial to synthesize and store large amounts of energy sources in the form of triglycerides and glycogen, respectively, and are able to utilize these sources to respond to environmental changes. For example, after meals, blood glucose level reaches high enough to block gluconeogenesis and induce lipogenesis [34–36]. Simultaneously, excess supply of glucose is metabolized via glycolysis and glucose shunt pathways. To initiate these physiological changes, ADD1/SREBP1c plays a pivotal role to coordinate both carbohydrate and lipid metabolism in liver and fat [18,33,37]. When pancreatic beta cells secrete more insulin, activated ADD1/SREBP1c suppresses hepatic glucose production and stimulates most lipogenic genes to store surplus energy sources into fat and liver, which needs large amounts of NADPH [17,38]. To satisfy these physiological demands, the expression of 6PGDH might be promoted by ADD1/SREBP1c. Therefore, it is feasible to speculate that ADD1/SREBP1c would regulate 6PGDH gene expression to coordinate both carbohydrate and lipid metabolism in an insulin-dependent manner.

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