

ATF6 modulates SREBP2-mediated lipogenesis

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Activating transcription factor 6 (ATF6) and sterol regulatory element-binding proteins (SREBPs) are activated by proteolytic cleavage. The ensuing nuclear translocation of their N-termini (i.e., ATF6(N) and SREBP(N)) activates the respective target genes involved in unfolded protein response and lipogenesis. Here, we report that glucose deprivation activated ATF6 but suppressed the SREBP2-regulated transcription. Overexpression of ATF6(N) had similar inhibitory effects on SREBP2-targeted genes. The blockade of ATF6 cleavage by BiP/grp78 reversed this inhibitory effect. GST pull-down and immunoprecipitation assays revealed that ATF6(N) bound to SREBP2(N). Deletion analysis of the various functional domains of ATF6 indicated that the interaction was through its leucine-zipper domain. Chromatin immunoprecipitation assays revealed that ATF6(N) formed a complex with the SRE-bound SREBP2(N). The attenuated transcriptional activity of SREBP2 was due, in part, to the recruitment of HDAC1 to the ATF6–SREBP2 complex. As a functional consequence, the lipogenic effect of SREBP2(N) in liver cells was suppressed by ATF6(N). Our results provide a novel mechanism by which ATF6 antagonizes SREBP2 to regulate the homeostasis of lipid and glucose.

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Introduction

Secreted proteins, plasma membrane-associated proteins, lipids, and sterols are largely synthesized in the endoplasmic reticulum (ER). In response to unfolded protein accumulation, sterol deficiency, or protein overload, cells elicit several signal transduction pathways from the ER membrane to the nucleus, resulting in the upregulation of target genes (for reviews, see Pahl, 1999; Lee, 2001). Activating transcription

factor 6 (ATF6) is an ER membrane-bound transcription factor. During the quiescent state, the C-terminus of ATF6 resides in the ER lumen, with its N-terminus protruding into the cytoplasm (Haze *et al*, 1999). When unfolded or misfolded proteins accumulate in the ER membrane, the CD1 domain in the C-terminus senses the stress, leading to the translocation of ATF6 from the ER to the Golgi (Chen *et al*, 2002), where ATF6 is cleaved by site 1 protease (S1P) and site 2 protease (S2P) (Ye *et al*, 2000). Such a proteolytic cleavage causes the nuclear translocation of the N-terminal leucine-zipper transcription factor to direct the transcriptional activation of the chaperone molecules and enzymes essential for protein folding (Haze *et al*, 1999; Wang *et al*, 2000; Ye *et al*, 2000; Yoshida *et al*, 2000; Okada *et al*, 2002). Thus, ATF6 is regarded as an ER stress-activated transcription factor. BiP, also known as glucose-regulated protein 78 (grp78), was originally identified as a marker for cellular response to glucose starvation (Munro and Pelham, 1986; Lee, 1987). In cells subjected to glucose starvation, BiP/grp78 is induced to bind the newly synthesized proteins that are hypoglycosylated (Cai *et al*, 1998). Recent studies show that overexpressing ATF6 or treating cells with the ER stress-inducing agent thapsigargin (Tg) activates BiP/grp78 (Haze *et al*, 1999; Li *et al*, 2000; Yoshida *et al*, 2000). Also, BiP/grp78 can bind to ATF6, thus retaining ATF6 in the ER membrane (Shen *et al*, 2002; Sommer and Jarosch, 2002).

Sterol regulatory element-binding proteins (SREBPs), including SREBP1a, SREBP1c, and SREBP2, regulate cellular fatty acid and cholesterol synthesis and metabolism (for a review, see Horton *et al*, 2002). With a hairpin-like structure, SREBPs are also ER membrane-bound transcription factors. In the sterol-loaded cells, the N- and C-termini of SREBPs protrude into the cytoplasm, whereas the central hydrophilic domain spans the ER lumen (Hua *et al*, 1996). Through their C-terminal WD domain, SREBPs form a complex with two other ER membrane-bound proteins, namely, SREBP cleavage-activating protein (SCAP) and insulin-induced gene 1 (insig-1) (Sakai *et al*, 1997; Yang *et al*, 2002). When the ER membrane is deficient in lipids or sterols, the SREBP–SCAP complex dissociates from insig-1. As a consequence, SCAP escorts SREBPs from the ER to the Golgi (Sakai *et al*, 1997). Similar to ATF6, SREBPs are cleaved by S1P and S2P in the Golgi, and the cleaved N-termini translocate into the nucleus to activate genes involved in cholesterol and lipid biosynthesis and metabolism (for a review, see Brown and Goldstein, 1997). SREBP1c-mediated lipogenesis is marked in the liver *in vivo* but is difficult to represent in cultured cells. In contrast, SREBP2 has been shown to regulate both cholesterol and lipogenesis *in vitro* and *in vivo*. SREBP2 upregulation in hepatocytes in culture increases cholesterol uptake and biosynthesis (Hua *et al*, 1993; Sakai *et al*, 1996). The overexpression of SREBP2(N) in transgenic mice resulted in fatty liver, with a large increase in cholesterol ester (Horton *et al*, 1998b).

Under normal conditions, the intracellular glucose concentration is 75–90% of that outside the cell. Depletion

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of intracellular glucose induces ER stress, marked by the upregulated BiP/grp78 (Mote *et al*, 1998). *In vivo*, fasting reduces both extracellular and intracellular glucose levels (Vander *et al*, 1998). Recent studies showed that fasting not only downregulates SREBP1c and its target gene but also induces ER stress in the liver (Horton *et al*, 1998a; Nishihara *et al*, 1998). In addition, ER stress induced by tunicamycin suppresses the SREBP-mediated transcription (Ye *et al*, 2000). Conceptually, the SREBP-mediated lipogenesis would be ablated if the glucose supply is low. But the underlying molecular mechanism is unclear. As ATF6 and SREBPs are all ER membrane-bound transcription factors and their leucine-zipper domains all bind to NF-Y and YY1 for transactivation (Jackson *et al*, 1998; Gauthier *et al*, 1999; Li *et al*, 2000; Yoshida *et al*, 2000), we investigated whether ATF6 interacts with SREBP2 and, if so, whether such interaction affects the SREBP2-regulated lipogenesis.

Results

Glucose deprivation suppresses the SREBP2-mediated but increases the ATF6-regulated transcription

We first tested the effect of glucose deprivation on the expression of genes involved in cholesterol synthesis and metabolism in HepG2 and HEK293 cells. As shown in Figure 1A, the level of mRNA encoding SREBP2 target genes, including 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, HMG-CoA synthase, squalene synthase, and low-density lipoprotein receptor (LDLR), decreased progressively in the glucose-deficient cells within 6 h. In contrast, the marker genes for unfolded protein response (UPR), including BiP/grp78 and growth arrest and DNA damage-inducible 153 (GADD153), were upregulated in these cells. Transient transfection assays were performed to investigate the role of SREBP2 and ATF6 in regulating the increased or decreased level of mRNA in response to glucose deprivation. Compared to the high or low glucose conditions (27.5 and 5 mM, respectively), glucose deprivation reduced luciferase activity by 50% in HepG2 cells transfected with 4 × SRE-Luc, a chimeric construct containing four copies of the SRE consensus sequence fused to the luciferase reporter (Figure 1B). In control experiments, the replacement with 10 mM lactate or treating cells with Tg, an ER inducer, had a similar inhibitory effect. However, sterol depletion using lipoprotein-deficient serum (LDS) increased the reporter activity by four times. A similar trend of modulation was found in cells transfected with LDLR-Luc, the luciferase reporter driven by the LDLR native promoter. As glucose deprivation augmented the UPR marker genes, HepG2 cells were also transfected with 5 × ATF6-Luc, a chimeric construct in which the firefly luciferase reporter is driven by five copies of the ATF6 binding element (Wang *et al*, 2000), or BiP/grp78-Luc, in which the luciferase reporter is driven by a 0.7-kb native promoter of BiP/grp78. The transfected cells were then subjected to the same set of treatments as that for 4 × SRE-Luc and LDLR-Luc. Compared to cells under 27.5 or 5.5 mM glucose, cells under the three conditions suppressing the SREBP2 transcriptional activity, namely, glucose deprivation, lactose replacement, and Tg treatment, all upregulated 5 × ATF6-Luc and BiP/grp78-Luc. In contrast, LDS had no effect on these reporter systems. Thus, glucose deprivation exerts an opposite effect on SREBP2- versus ATF6-mediated

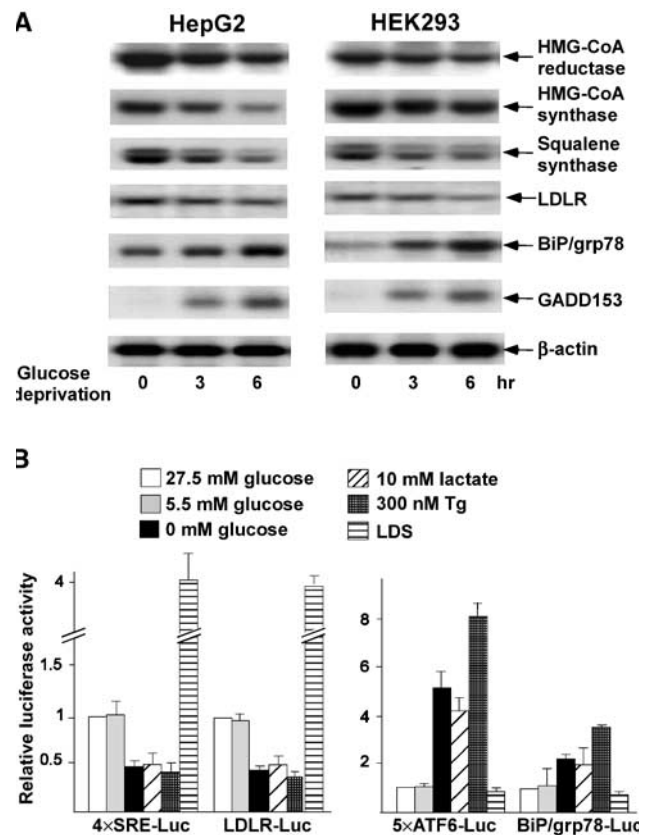


Figure 1 Glucose deprivation suppresses SREBP2-mediated but activates ATF6-mediated transcription. (A) HepG2 and HEK293 cells cultured in high glucose (27.5 mM) DMEM with 10% FBS were subjected to glucose-deficient DMEM with 10% dialyzed FBS for 0, 3, and 6 h. Total RNA was isolated for Northern blotting probed with HMG-CoA reductase, HMG-CoA synthase, squalene synthase, LDLR, BiP/grp78, GADD153, and β-actin cDNA. (B) HepG2 cells in 12-well plates were transiently transfected with 4 × SRE-Luc, LDLR-Luc, 5 × ATF6-Luc, or BiP/grp78-Luc together with renilla-Luc for 24 h. The transfected cells were then cultured in DMEM containing 0, 5.5, 27.5 mM glucose, or 10 mM lactate for 12 h. In parallel control experiments, 300 nM Tg or 10% LDS was included in the DMEM containing 27.5 mM glucose. The cells were then lysed for the measurement of firefly and renilla luciferase activity. The relative luciferase activity is defined as the firefly luciferase activity normalized to that of renilla luciferase and that of cells under 27.5 mM glucose is set as 1.

gene expression. While the ATF6-regulated gene expression increases, the SREBP2-targeted transcription is suppressed.

ATF6 suppresses SREBP2-mediated transactivation

SREBPs and ATF6 regulate the respective target genes through an augmented proteolytic cleavage and nuclear translocation of their N-terminal transactivation domain. The opposite effect of glucose deprivation on the SREBP2- versus ATF6-mediated gene expression could be due to the distinct patterns of SREBP2 and ATF6 proteolytic cleavage. To investigate whether the cleavage of ATF6 and SREBP2 increases and/or decreases as a result of glucose deprivation, we treated HepG2 cells with glucose-deficient media and analyzed the changes in the precursor and mature forms of ATF6 and SREBP2. As shown in Figure 2, glucose deprivation as early as 30 min increased the cleavage of ATF6. The proteolytic product of 50 kDa (i.e., ATF6(N)) increased for

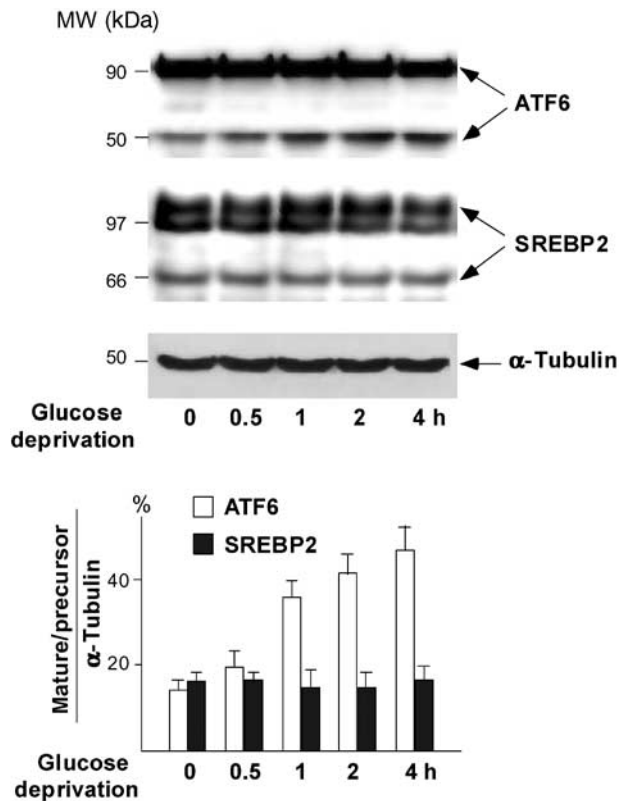


Figure 2 Glucose deprivation causes ATF6 but not SREBP2 cleavage. HepG2 cells were incubated in glucose-deficient DMEM for the times indicated. Cells were lysed and the cell lysates were subjected to immunoblotting with anti-ATF6, anti-SREBP2, and anti- α -tubulin. The intensities of the various protein bands were determined by densitometry and the ratio of the level of mature form to that of precursor (mature/precursor) was normalized to the amount of α -tubulin.

at least 4 h. In contrast, the ratio between the precursor and the mature form of SREBP2 remained the same throughout the deprivation process, which suggests that the decreased transcription mediated by SREBP2 is due to a mechanism independent of SREBP2 cleavage.

As both glucose deprivation and Tg treatment enhanced the ATF6 cleavage but decreased the SREBP-mediated transcription (Haze *et al*, 1999; Figure 2), we hypothesized that the increased ATF6(N) can somewhat inhibit the SREBP2(N)-mediated transcription. To test this hypothesis, HepG2 cells were transfected with pCMV5-HA-SREBP2(N), together with pCI-Flag-ATF6(N) or an empty pCMV5 vector, and the 4 \times SRE-Luc or LDLR-Luc reporters. As shown in Figure 3A, the expression of HA-SREBP2(N) in control cells transfected with pCMV5 greatly increased the luciferase activity of the two reporters. However, this induction was reduced by the expressed Flag-ATF6(N), which was dependent on the dose of pCI-Flag-ATF6(N). To confirm further that the expression of SREBP-targeted genes was suppressed by ATF6(N), we investigated the expression of HMG-CoA reductase and HMG-CoA synthase mRNA in HepG2 cells infected with a recombinant adenovirus encoding ATF6(N). Quantitative real-time PCR revealed that ATF6(N) decreased the levels of HMG-CoA reductase and HMG-CoA synthase mRNA in a dose-dependent manner (Figure 3B). As a control, the expression of the ATF6(N)-targeted BiP/grp78 increased reciprocally. By retain-

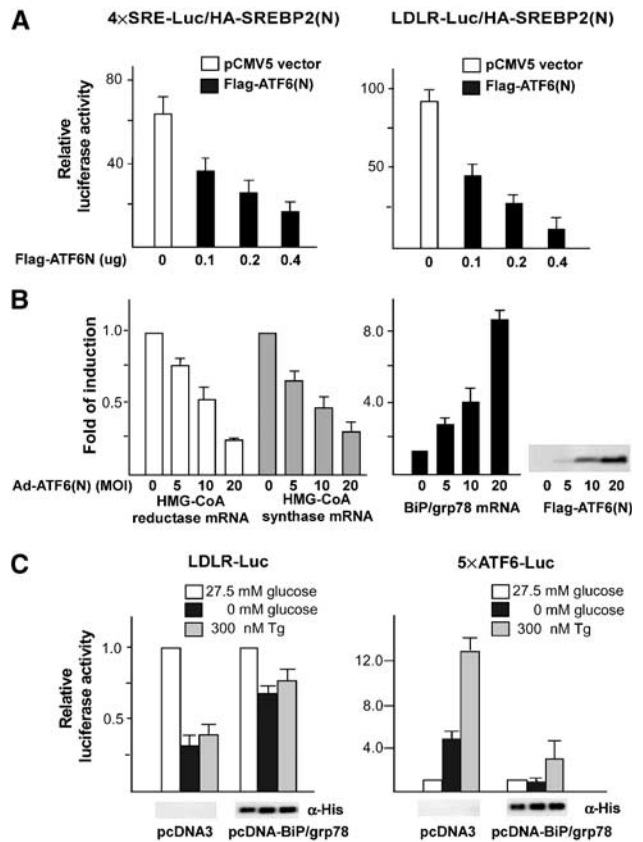


Figure 3 SREBP2(N)-mediated transcription is suppressed by ATF6(N). (A) HEK293 cells were transfected with 4 \times SRE-Luc (0.1 μ g) or LDLR-Luc (0.1 μ g) together with pCMV5-HA-SREBP2(N) (0.1 μ g) in the presence of various amounts of pCI-Flag-ATF6(N) as indicated. pCMV5 empty vector was included as a plasmid control to compensate the plasmid DNA amount. Renilla-Luc (0.01 μ g) was included for transfection control. The cells were then lysed for the measurement of firefly and renilla luciferase activities. The firefly luciferase activity was normalized to that of renilla luciferase, and the relative luciferase activity is the comparison among various samples. (B) HepG2 cells were infected with the indicated amount of Ad-Flag-ATF6(N). Ad- β -gal was used as a control virus and to compensate for the total MOI. At 24 h after virus infection, total cellular RNAs were extracted, and the level of HMG-CoA synthase, HMG-CoA reductase, and BiP/grp78 mRNA was assessed by quantitative real-time RT-PCR. The anti-Flag immunoblotting of the cell lysates is shown on the right, which demonstrates the expression of the exogenous Flag-ATF6(N). (C) HEK293 cells were transiently transfected with LDLR-Luc (0.1 μ g) or 5 \times ATF6-Luc (0.1 μ g) together with pcDNA3 (0.5 μ g) or pcDNA3-BiP/grp78 (0.5 μ g). Renilla-Luc (0.01 μ g) was included as a transfection control. After 12 h, the media of the transfected cells were changed with fresh media containing 27.5 mM glucose in the presence or absence of 300 nM Tg, or media without glucose for another 12 h. The cells were then lysed for the measurement of firefly and renilla luciferase activities. The relative luciferase activity is activity from various samples compared with that from cells with 27.5 mM glucose set as 1. The expression of the exogenous BiP/grp78 was demonstrated by immunoblotting with anti-His shown at the bottom.

ing ATF6 in the ER membrane, BiP/grp78 overexpression prevents the cleavage of ATF6 (Shen *et al*, 2002; Sommer and Jarosch, 2002). We thus examined the expression of LDLR-Luc in cells co-transfected with plasmid encoding BiP/grp78. As shown in Figure 3C, the suppression of LDLR-Luc by glucose deprivation or Tg was largely abolished in cells overexpressing BiP/grp78, whereas the induction of

5 × ATF6-Luc under these conditions was inhibited. These results suggest that ATF6 activation can inhibit the SREBP2-regulated transcription.

ATF6 attenuates SREBP2-mediated lipogenesis

We explored further whether ATF6(N) can inhibit SREBP2-mediated lipogenesis. Adenovirus-mediated gene transfer was used to overexpress SREBP2(N) with or without ATF6(N) (i.e., Ad-SREBP2(N) and Ad-ATF6(N)) in HepG2 cells. As shown in Figure 4, the CMV-driven expression of SREBP2(N) greatly increased the accumulation of lipids in HepG2 cells, as demonstrated by cells showing enhanced Oil-Red-O staining compared with control cells infected with Ad-β-gal. However, the SREBP2(N)-induced lipogenesis was significantly decreased in cells co-infected with Ad-ATF6(N). The levels of exogenous SREBP2(N) were comparable regardless of the expression of ATF6(N), which suggests that the reduced lipogenesis was not due to changes in SREBP2(N) expression.

ATF6 interacts with SREBP2

To explore whether ATF6(N) interacts with SREBP2(N), thereby inhibiting SREBP2-mediated transcription, we transfected HEK293 cells with pCI-Flag-ATF6(N). GST-SREBP2(N) was then used in pull-down assays to precipitate proteins in the cell lysates that interacted with SREBP2. As shown in

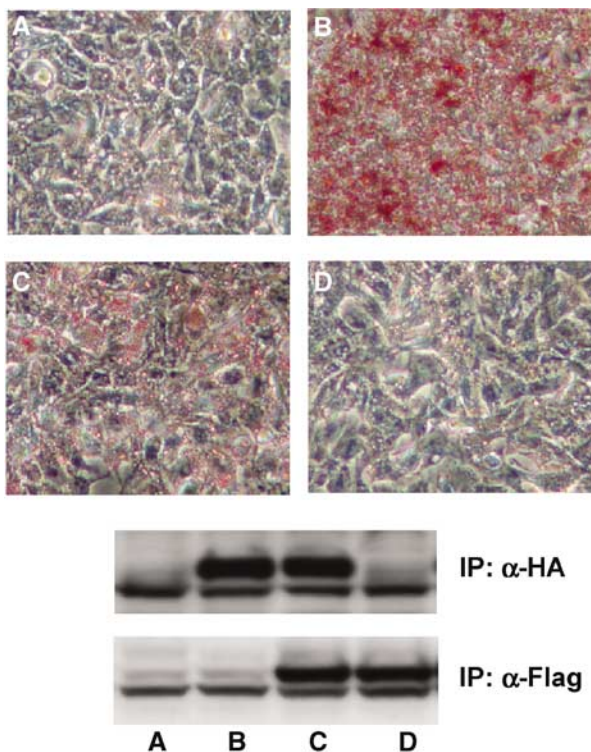


Figure 4 ATF6(N) suppresses SREBP2(N)-mediated lipogenesis. HepG2 cells were infected with (A) Ad-β-gal (20 MOI/ml), (B) Ad-HA-SREBP2(N) (10 MOI/ml), Ad-β-gal (10 MOI/ml), (C) Ad-HA-SREBP2(N) (10 MOI/ml), Ad-Flag-ATF6(N) (10 MOI/ml), and (D) Ad-Flag-ATF6(N) (10 MOI/ml), Ad-β-gal (10 MOI/ml). After 48 h incubation, cells were fixed and stained with Oil-Red-O to detect lipid deposition in various cells. Cells in the parallel set of experiments were lysed, and the expression of HA-SREBP2(N) and Flag-ATF6(N) was examined by immunoblotting with the use of anti-HA or anti-Flag.

Figure 5A, Flag-ATF6(N) was detected in the GST-SREBP2 precipitates, which indicates that ATF6(N) can bind to SREBP2(N) *in vitro*. We also transfected HEK293 cells with pCMV5-HA-SREBP2(N) and pCI-Flag-ATF6(N). The expressed HA-SREBP2 and its associated proteins were immunoprecipitated with anti-HA. As expected, the Flag-tagged ATF6(N) was detected in the precipitates (Figure 5B), indicating that the N-termini of ATF6 and SREBP2 can bind to each other *in vivo*. To investigate further whether ATF6(N) interacts with SREBP2(N) under physiological conditions such as glucose deprivation, we transfected HEK293 cells with pCGN-HA-ATF6 encoding HA-tagged ATF6. The transfected cells were then subjected to glucose deprivation. Indeed, HA-ATF6 increased its binding to the N-termini of the endogenous SREBP2 in response to glucose deprivation (Figure 5C). We also examined whether endogenous ATF6(N) interacts with SREBP2(N) in response to glucose deprivation. ATF6/SREBP2 co-immunoprecipitation shown in Figure 5D indicates that glucose deprivation for 1 h increased the interaction of ATF6(N) with SREBP2(N) in HEK293 cells.

ATF6 interacts with SREBP via its basic zipper domain

To determine the region in ATF6(N) critical for SREBP2 binding, we created ATF6-deletion constructs encoding various regions of ATF6. These constructs were co-transfected with LDLR-Luc for suppression assays. As shown in Figure 6A, pCI-Flag-ATF6(151–366) and pCI-Flag-ATF6(2–366) exerted a similar inhibitory effect on LDLR-Luc expression induced by the co-transfected HA-SREBP2(N). In contrast, pCI-Flag-ATF6(2–330), truncated with the 36-bp leucine-zipper domain, had little inhibition on the transactivity of SREBP2(N). Co-transfection followed by immunoprecipitation assays showed that Flag-ATF6(2–366) and Flag-ATF6(151–366) but not Flag-ATF6(2–330) were able to bind to the co-expressed HA-SREBP2(N) (Figure 6B). These results suggest that the leucine-zipper domain of ATF6 is responsible for its binding to SREBP2(N).

ATF6(N)-SREBP2(N) binds to SRE and recruits HDAC1

The mechanism underlying ATF6(N) inhibition of SREBP2(N) transactivation can be the binding of ATF6(N) to the SRE-bound SREBP2(N), thereby inhibiting the transcriptional activity of SREBP2. Another possible mechanism is that ATF6(N) interaction can inhibit the binding of SREBP2(N) to SRE. We therefore performed chromatin immunoprecipitation (ChIP) assays to investigate whether ATF6(N) and SREBP2(N) can bind to the LDLR promoter concomitantly. As shown in Figure 7A, abundant SREBP2(N), marginal SREBP1(N), and little, if any, ATF6(N) bound to the LDLR promoter in HepG2 cells under high glucose levels (lanes 3–5). Glucose deprivation greatly increased the binding of endogenous ATF6(N) to the LDLR promoter (lane 10 versus lane 5), although the level of binding of SREBP2 remained the same as that under high glucose levels (lane 9 versus lane 4). It is likely that a general co-repressor such as HDAC1 is recruited to SRE, thus inhibiting SRE-mediated transcriptional activation. As shown in Figure 7B, glucose deprivation increased the binding of HDAC1 to the LDLR promoter in HepG2 cells (lane 8 versus lane 4). The increased expression of exogenous ATF6(N) (e.g., Flag-ATF6(N)) augmented the recruitment of HDAC1 to the LDLR promoter even in the presence of high glucose levels (lane 12 versus lane 4), which

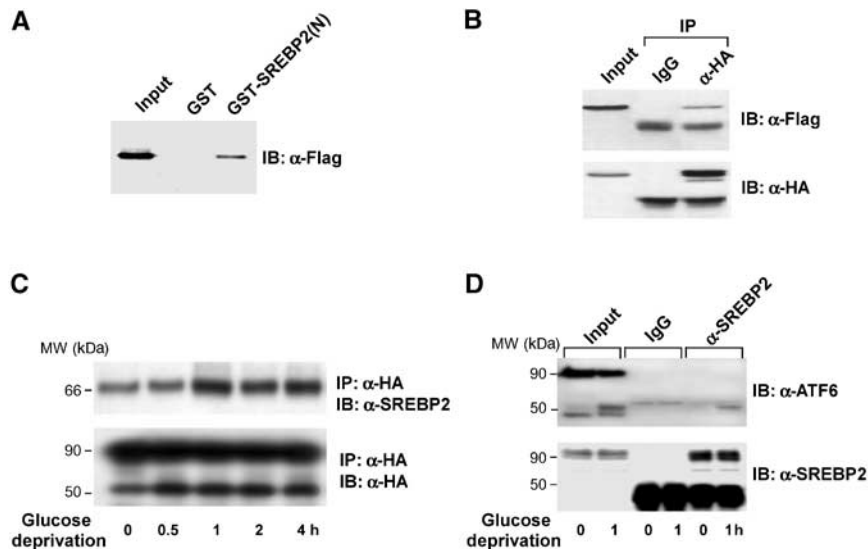


Figure 5 ATF6(N) associates with SREBP2(N). (A) Cell lysates collected from HEK293 cells transfected with pCI-Flag-ATF6(N) were incubated with GST or GST-SREBP2(N) glutathione beads for GST pull-down assays. The precipitates were separated by SDS-PAGE and immunoblotted with anti-Flag. The input control was 10% of the cell lysates. (B) HEK293 cells were transiently transfected with pCMV5-HA-SREBP2(N) and pCI-Flag-ATF6(N). HA-SREBP2(N) was immunoprecipitated from the cell lysates by anti-HA, and the associated ATF6(N) was revealed by immunoblotting with anti-Flag. In control experiments, rabbit IgG was used in immunoprecipitation, and the input was 10% of the cell lysates. Shown at the bottom is the anti-HA immunoblotting demonstrating the expression of HA-SREBP2(N). (C) HepG2 cells were transiently transfected with pCGN-HA-ATF6. At 1 day after transfection, the media were changed to normal growth media or glucose-deprived media for the indicated times. HA-ATF6 was immunoprecipitated from the cell lysates by anti-HA, and the endogenous SREBP2 associated with HA-ATF6 was examined by immunoblotting with anti-SREBP2 pAb. The membrane was stripped and reprobbed with mouse anti-HA antibody to reveal the expression of the ATF6 precursor and its cleavage in response to glucose deprivation (bottom panel). (D) HEK293 cells were subjected to glucose-deficient media containing 10% dialyzed FBS for 1 h or kept in high glucose media for the same period of time. Endogenous SREBP2 was immunoprecipitated from the cell lysates by anti-SREBP2 pAb. The associated ATF6 was detected by immunoblotting with anti-ATF6 pAb. In control experiments, the input was 10% of the cell lysates, and goat IgG was used in an immunoprecipitation control. The membrane was stripped and reprobbed with anti-SREBP2 pAb (bottom panel).

suggests that ATF6(N) is crucial for the recruitment of HDAC1. Data presented in Figure 7 suggest that ATF6(N), SREBP2(N), and HDAC1 bind together to the SREBP2-targeted gene in response to glucose deprivation. Noticeably, trichostatin A (TSA), an HDAC1 inhibitor, abrogated the glucose deprivation-suppressed $4 \times$ SRE-Luc (Figure 7C). Similarly, the inhibitory effect of ATF6(N) on the SREBP2(N)-mediated transcriptional activity was partially reversed by TSA.

Discussion

The homeostasis of catabolic and anabolic processes of sterols, fatty acids, and carbohydrates marks the status of energy expenditure versus storage. Functioning as critical, if not the most important, molecules involved in lipid metabolism, SREBPs would play a central role in mediating the homeostasis of sterols, fatty acids, and glucose. The current study aimed at elucidating the mechanism by which glucose deprivation downregulates SREBP2 transcription. The new findings are as follows: (1) glucose deprivation causes the proteolytic cleavage of the ER stress transducer ATF6; (2) the cleaved ATF6 translocates into the nucleus, binds to SREBP2, and recruits HDAC1; and (3) SREBP2-mediated gene transcription and lipogenesis are downregulated by such a process (summarized in Figure 8).

Changes in carbohydrate content are known to regulate cellular lipid biosynthesis. A high carbohydrate diet increases the expression of lipogenesis-related enzymes (e.g., acetyl-

CoA carboxylase (ACC)) (Granner and Pilkis, 1990; Towle, 2001). Compared to the SREBP1c target genes, those of SREBP2 have been studied less. However, it has been shown that glucose deprivation in rats decreased HMG-CoA reductase activity and cholesterol synthesis in the liver (Easom and Zammit, 1987; Okuyama *et al*, 1995). We show that glucose deprivation suppressed the expression of SREBP2 target genes (e.g., HMG-CoA reductase, HMG-CoA synthase, squalene synthase, LDLR) in HepG2 and HEK293 cells. The decreased expression could be due to the suppressed transcriptional activity of SREBP2, because the expression of the reporter gene driven by SRE was abrogated by glucose deprivation. As the catabolic product of glucose and lactate, acetyl-CoA is the precursor for cholesterol and fatty acid biosynthesis. Glucose deficiency triggers gluconeogenesis, thus depleting the cellular acetyl-CoA pool, leading to the suppression of cholesterologenesis and lipogenesis. SREBP2 transcriptional activity was still suppressed in cells exposed to lactate, which suggests that the decreased SREBP2 transcriptional activity is not due to acetyl-CoA deficiency. Instead, the similar inhibitory effect of tunicamycin, Tg, and glucose deprivation (Ye *et al*, 2000, Figure 1B) demonstrates that ER stress is the main cause of the attenuated transcription activity of SREBP2. Indeed, exhausting intracellular glucose results in hypoglycosylation of newly synthesized proteins and upregulation of chaperone molecules (Mote *et al*, 1998, Figure 1B), which are typical responses to ER stress. Treating HepG2 cells with homocysteine, another ER stress inducer, somehow increased the level of SREBP2 mRNA and

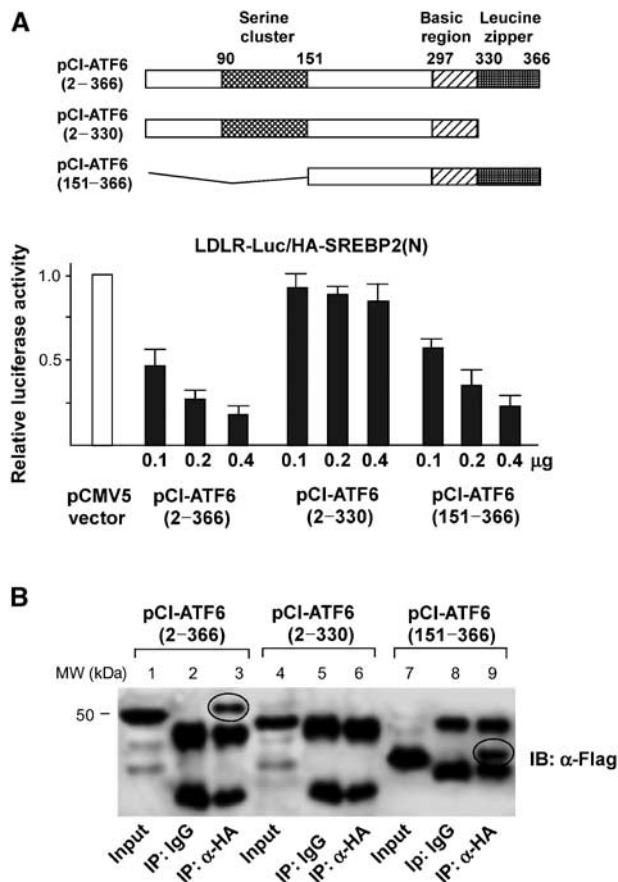


Figure 6 The leucine-zipper domain of ATF6 binds to SREBP2. (A) HEK293 cells were transiently transfected with LDLR-Luc (0.1 μg), HA-SREBP2(N) (0.1 μg), together with different amounts of pCI-Flag-ATF6 expression plasmids as indicated. pCMV5 empty vector was used to compensate the amount of plasmid DNA, and renilla luciferase (0.01 μg) was included as a transfection control. Luciferase activities from various samples were assayed accordingly. The relative luciferase activity is the activity from various samples compared with that from control cells transfected with 0.4 μg pCMV5 set as 1. Shown at the top is the schematic representation of the various Flag-tagged ATF6 constructs. (B) HEK293 cells were transiently transfected with pCMV5-SREBP2(N), together with pCI-Flag-ATF6(2-366), pCI-Flag-ATF6(2-330), or pCI-Flag-ATF6(151-366). HA-SREBP2(N) was immunoprecipitated from the cell lysates using anti-HA. The precipitates were separated by SDS-PAGE and the HA-SREBP2(N)-associated proteins were recognized by immunoblotting with anti-Flag. In control experiments, the input was 10% of the cell lysates, and rabbit IgG was used in an immunoprecipitation control.

the expression of their target genes (Werstuck *et al*, 2001). The discrepancy among glucose deprivation, Tg, tunicamycin, and homocysteine in terms of their effect on SREBP-mediated transcriptional regulation is currently unknown.

We further showed that the suppressed SREBP2 transcriptional activity resulted from the increased ATF6(N) but not the decreased proteolytic cleavage of SREBP2. The evidence is that both glucose deprivation and Tg treatment increased ATF6 cleavage and overexpression of the constitutively active ATF6(N), mimicking the increased ATF6 cleavage, inhibited the HMG-CoA reductase and HMG-CoA synthase mRNAs. Furthermore, the retention of ATF6 in the ER by the overexpressed BiP/grp78 partially reversed the inhibitory effect (Figure 3C). It is reasonable to assume that glucose depriva-

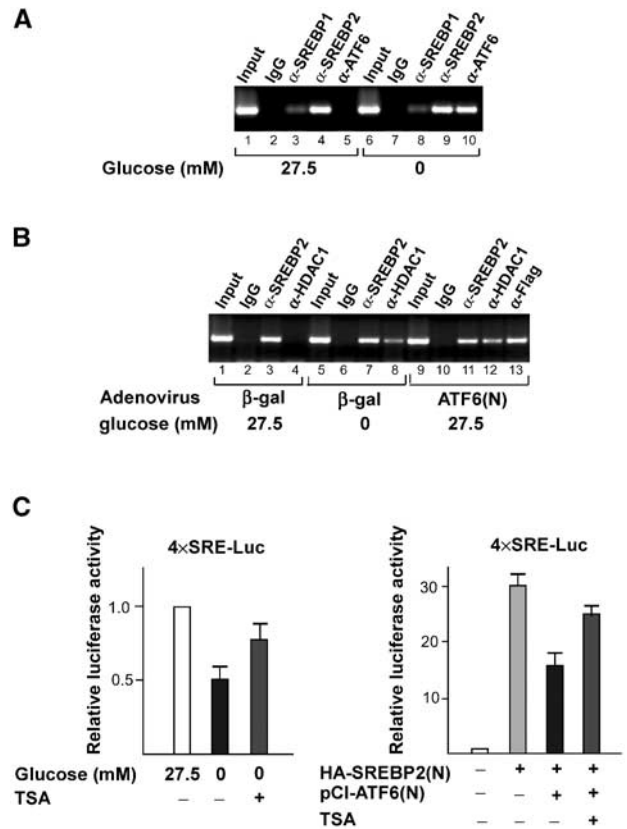


Figure 7 ATF6(N) binds to the LDLR promoter and recruits HDAC1. (A) HepG2 cells cultured under high glucose were changed with media deprived of glucose (lines 6-10). In the parallel controls, cells were changed with fresh media containing 27.5 mM glucose (lanes 1-5). After further incubation for 1 h, cells were lysed, and ChIP assays were performed to detect the binding of SREBP1(N) (lanes 3 and 8), SREBP2(N) (lanes 4 and 9), and ATF6(N) (lanes 5 and 10) to the LDLR promoter. (B) HepG2 cells were infected with Ad-β-gal (lanes 1-8) or Ad-Flag-ATF6(N) (lanes 9-13) at 10 MOI. After 24 h, cells were changed with media containing high glucose (lanes 1-4 and 9-13) or no glucose (lanes 5-8) for 1 h incubation. ChIP assay was performed to detect the binding of endogenous SREBP2 (lanes 3, 7, and 11), exogenous Flag-ATF6(N) (lane 13), and the recruitment of HDAC1 (lanes 4, 8, and 12) to the LDLR promoter in response to glucose deprivation or Flag-ATF6(N) overexpression. Data represent results from three independent repeats. (C) HepG2 cells transiently transfected with 4 × SRE-Luc were subject to glucose deprivation in the presence or absence of TSA (left panel). The 4 × SRE-Luc-transfected cells were also co-transfected with pCMV5-HA-SREBP2(N) and pCI-Flag-ATF6(N) in the presence or absence of TSA (right panel). The luciferase induction was assessed accordingly.

tion induces ER stress but not cholesterol depletion, resulting in the increased cleavage of ATF6 but not SREBP2. ATF6(N) and SREBP2(N) may interact with each other in the ER or Golgi, followed by nuclear translocation of the binary complex, or, alternatively, the two activated factors translocate separately and form a complex in the nucleus. Both ATF6 and SREBPs belong to the basic leucine-zipper protein family. ATF6(N) interacting with SREBP2(N), represents another example of transcription factors in this family that often form a homodimer and/or heterodimer through the leucine-zipper domain (Mitchell and Tjian, 1989; Hai and Hartman, 2001; Haze *et al*, 2001; Nagoshi and Yoneda, 2001). The truncated ATF6(2-330) lacking the basic leucine-zipper domain failed to bind to SREBP2(N), and thus could not inhibit

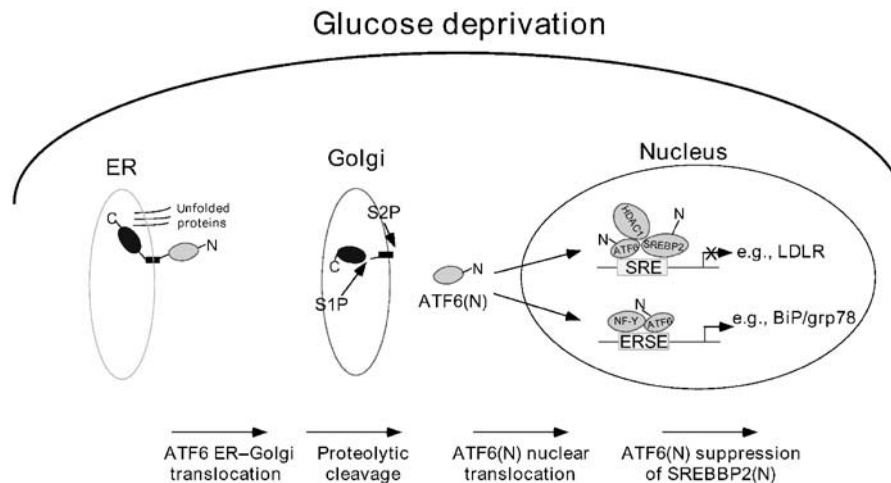


Figure 8 Proposed model for the mechanism by which glucose starvation suppresses the SREBP-mediated lipogenesis. In response to ER stress caused by glucose starvation, ATF6 translocates from the ER to the Golgi, where it is cleaved by S1P and S2P proteases. The cleaved ATF6(N) translocates into the nucleus to bind to SRE-bound SREBPs and recruits HDAC1, thus inhibiting SRE-mediated transcriptional activation. The increased ATF6(N), together with NF-Y, also binds to the ER stress element to activate genes involved in UPR. SRE and ERSE denote sterol regulatory element and ER stress element, respectively.

induction of the $4 \times$ SRE-Luc reporter. This result suggests that the basic leucine-zipper domain of ATF6(N) is responsible for its interaction with SREBP2(N), thus inhibiting SREBP2 transcriptional activity. Under glucose deprivation, SREBP2 target genes as well as SREBP1c ones (e.g., ACC) were suppressed. Furthermore, SREBP1c transactivation was attenuated by overexpressed ATF6(N) (data not shown). Although the current study involves only SREBP2 because of its drastic effect in cultured cells, the suppressive effect of ATF6(N) may be ubiquitous for both SREBP2 and SREBP1c. In addition, SREBP2 may inhibit the ATF6(N)-mediated transcription reciprocally.

SREBPs bind to their targeted DNA sequence in the form of homodimer (Nagoshi and Yoneda, 2001). The results of ChIP assays demonstrate the binding of both ATF6(N) and SREBP2(N) to the LDLR promoter in cells subjected to glucose deprivation. Thus, ATF6(N) did not affect the binding of SREBP2 to SRE, which was also supported by electrophoretic mobility shift assays (data not shown). Under glucose deprivation conditions, mimicked by ATF6(N) overexpression, HDAC1 was also recruited to the LDLR promoter (Figure 7B). The existence of the ATF6(N)-SREBP2(N)-HDAC1 complex is in line with the direct binding of ATF6(N) and HDAC1 *in vitro* (data not shown). By deacetylating histone, HDAC1 would inhibit the transcriptional activation of the SREBP2-targeted genes. This mechanism is revealed by the higher level of induction of $4 \times$ SRE-Luc in cells treated with TSA compared to untreated cells (Figure 7C). It seems that the recruitment of HDAC1 to the DNA-bound ATF6(N)-SREBP(N) exerts a major inhibitory effect on SREBP-regulated transcription. The involvement of HDAC1 in inhibiting lipogenesis is reminiscent of a recent study demonstrating that glucocorticoids potentiate the differentiation of preadipocyte by removing the HDAC1-mSin3A complex from the promoter of CCAAT/enhancer binding protein α (C/EBP α) (Wiper-Bergeron *et al*, 2003). Overexpression of BiP/grp78 has been shown to inhibit SREBP1 mRNA (Werstuck *et al*, 2001). In addition, NF-Y functions as a co-activator for both ATF6 and SREBP

(Ericsson *et al*, 1996; Yoshida *et al*, 2000). Thus, the possibility that ATF6(N) exerts its inhibitory effect on SREBP2 by activating BiP/grp78 and/or competing for NF-Y should not be ruled out. In addition to the ATF6(N)-suppressed SREBP2 transcriptional activity, other mechanism(s) may be involved in the downregulation of SREBP2 in response to glucose deprivation. One possibility is that ER stress can cause PERK phosphorylation of eIF-2, which decreases the translation (Okada *et al*, 2002). Another likelihood is that, being a target gene of itself, SREBP2 level may be decreased, especially after prolonged glucose deprivation.

The physiological and pathophysiological relevance of the current study is that when glucose, an important energy source, is depleted, cells in various tissues in the body abrogate the SREBP2-mediated sterogenesis and lipogenesis through activated ATF6. Such an abrogation would save energy resources to withstand the stress. Consistent with this hypothesis, the overexpression of ATF6(N) can attenuate SREBP2(N)-induced lipid accumulation (Figure 4).

Materials and methods

Cell culture

HepG2 and HEK293 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). For glucose deprivation, HepG2 or HEK293 cells were subjected to glucose-deficient DMEM (Gibco/BRL) supplemented with 10% dialyzed FBS (Omega Scientific, Tarzana, CA) for the time periods indicated. In the TSA treatment experiments, TSA (100 ng/ml) was included in the culture media.

DNA plasmids and transient transfection

pGEX-SREBP2(N) was constructed by subcloning the *EcoRI/BamHI*(blunt) fragment from pCMV5-HA-SREBP2(N) (Lin *et al*, 2003) into the *EcoRI/XhoI*(blunt) site of the pGEX-4T vector. pCI-Flag-ATF6(2-330) and pCI-Flag-ATF6(151-366) were created from pCI-Flag-ATF6(N)(2-366) using a PCR-based mutagenesis kit (Stratagen, La Jolla, CA). For pcDNA-BiP/grp78, six copies of the His-tag and two copies of Ser that serve as spacer were inserted directly after the ER signal peptide of 18 amino acids. The full-length $6 \times$ His-BiP/grp78 cDNA, assembled by multiple subcloning and religation steps, was subcloned into the pKS vector. It was

excised as a *Bam*H1 fragment and subcloned into the *Bam*H1 site of the expression vector pCDNA3 (Invitrogen, Carlsbad, CA). For transient transfection, plasmid DNA was transfected into 293 cells using the lipofectamine method (Invitrogen).

GST pull-down, immunoprecipitation, and immunoblotting

For GST pull-down assay, equal molars of GST-SREBP2(N) or GST together with 20 μ l glutathione Sepharose-4B beads (Amersham Pharmacia Biotech, Arlington Heights, IL) were added to 500 μ g cell lysates isolated from pCI-Flag-ATF6(N)-transfected 293 cells in TNEN buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.1% NP-40) containing 25 μ g/ml ALLN (Calbiochem, San Diego, CA) and a protease inhibitor cocktail (Le Rhoche, Palo Alto, CA). After being incubated on a rotator shaker at 4°C for 4 h and washed with PBS containing 0.2% NP-40, the beads were resuspended in 1 \times SDS loading buffer and subjected to immunoblotting. For immunoprecipitation assays, 2 μ g of rabbit anti-HA and goat anti-SREBP2 (Santa Cruz Biotech, Santa Cruz, CA) and 25 μ l Protein A-Sepharose-4B beads were added to 500 μ g cell lysates in TNEN buffer containing 25 μ g/ml ALLN and the protease inhibitor cocktail. The immunoprecipitates were analyzed by immunoblotting. Cell lysates (50 μ g) were included in the GST pull-down and immunoprecipitation assays as input controls. For immunoblotting assays, the detecting antibodies were rabbit anti-ATF6 (Haze *et al*, 1999), mouse anti-HA, mouse anti-Flag (Sigma, St. Louis, MO), and goat anti-SREBP2. The bound primary antibody was then detected by horseradish peroxidase-conjugated secondary antibody and visualized by an ECL detection kit (Amersham Pharmacia Biotech).

Adenovirus construction and infection

pCMV-shuttle-ATF6(N) was cloned by replacing the *Bgl*II-digested TRE fragment in the pTRE-shuttle2 vector with the *Bgl*II/*Bam*HI-digested pCI-Flag-ATF6(N) fragment containing the CMV promoter, Flag-tagged ATF6(2–366), and the poly A sequence. HA-SREBP2(N) fragments were amplified by PCR from pCMV5-HA-SREBP2(N) and were then used to replace the *Nhe*I/*Sal*I fragment of Flag-ATF6(2–366) to create the pCMV-shuttle-SREBP2(N). Recombinant adenovirus DNA encoding Flag-ATF6(N) or HA-SREBP2(N) (i.e., Ad-ATF6(N) and Ad-SREBP2(N)) were created from respective pCMV-shuttle constructs using Adeno-X Expression Systems (BD Biosciences Clontech, Palo Alto, CA). Recombinant viruses were amplified and the titers were determined in HEK293 cells. For adenovirus infection, HepG2 cells were inoculated in six-well plates at a density of 5×10^5 cells/well. The virus mixture with multiplicity of infection (MOI) indicated was added to the cell culture and incubated for 12 h. The infected cells were then incubated in fresh growth media for 24 h, followed by RNA and protein extraction.

Northern blotting and real-time PCR

Total cellular RNA was extracted using Trizol Reagent method (Invitrogen). For Northern blotting, 10 μ g of isolated RNA for each sample was separated by formaldehyde-denatured gel electrophoresis and then transferred to a nitrocellulose membrane and hybridized with (α -³²P)dCTP-labeled cDNA probes. The hybridized

bands were visualized by autoradiography. For real-time quantitative PCR, the first strand of cDNA was reverse transcribed. PCR was carried out in 384-well plates using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The relative amount of all mRNAs was calculated using the comparative CT method, with the β -actin mRNA used as internal control. The primer set used was HMG-CoA reductase: 5'ggatcgaagggagaggaaag3' and 5'gggcacatgcaatgtagatg3'; HMG-CoA synthase: 5'cttgcgatgactgtggttcag3' and 5'gcactgagtagcactgtatggag3'; BiP/grp78: 5'gagatcatcgccaacgatcag3' and 5'acttgatgtcctgctgcacag3'; β -actin: 5'agaagactcagagctgctc3' and 5'cacacggaactgtgctc3'.

Oil-red-O staining

HepG2 cells were infected with Ad-SREBP2(N) and/or Ad-ATF6(N) for 48 h. The lipid droplets in the infected cells were detected by Oil-Red-O staining (Green and Kehinde, 1975). Briefly, cells were washed with PBS and then fixed in 4% formaldehyde. After being washed in PBS and rinsed in 60% propanol, the cells were stained with filtered Oil-Red-O solution (Sigma) for 10–15 min.

ChIP assay

The ChIP assays were performed as described previously (Boyd and Farnham, 1997) with minor modifications. In brief, HepG2 cells were treated with 1% (v/v) formaldehyde at room temperature for 15 min and then quenched with glycine at room temperature. The medium was removed, and cells were harvested for sonication. The sheared samples were diluted into 1 ml immunoprecipitation buffer containing 25 mM Tris-HCl, pH 7.2, 0.1% NP-40, 150 mM NaCl, 1 mM EDTA, and immunoprecipitation was conducted with mouse anti-SREBP1, anti-HDAC, goat anti-SREBP2, rabbit anti-ATF6, or mouse anti-Flag antibodies, together with single-strand salmon sperm DNA saturated with Protein A-Sepharose-4B beads. Normal IgG was used as a control. Immunoprecipitates were pelleted by centrifugation, and the supernatant of control group was collected as an input control. The immunoprecipitates were eluted from Sepharose-4B beads using 100 μ l elution buffer (50 mM NaHCO₃, 1% SDS). A total of 200 μ l proteinase K solution was added to a total elution volume of 300 μ l and incubated at 60°C for 8 h. DNA was extracted, purified, and then used to amplify target sequences by PCR. The LDLR promoter containing the SRE consensus element was amplified using a primer set of 5'tgaggatcagacttcacg3' and 5'gacctgctgtcctagctg3'.

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