E4BP4 is an insulin-induced stabilizer of nuclear SREBP-1c and promotes SREBP-1c-mediated lipogenesis.

Xin Tong,¹,*, Pei Li,¹,† Deqiang Zhang,* Kyle VanDommelen,* Neil Gupta,* Liangyou Rui,* M. Bishr Omary,* and Lei Yin¹,†

Department of Molecular and Integrative Physiology,* University of Michigan Medical School, Ann Arbor, MI 48019; and Xiangya School of Medicine,† Central South University, Changsha 410013, People’s Republic of China.

Abstract Upon food intake, insulin stimulates de novo lipogenesis (DNL) in hepatocytes via the AKT-mTORC1-sterol regulatory element-binding protein (SREBP)-1c pathway. How insulin maintains the maximal SREBP-1c activities during the entire feeding state remains elusive. We previously reported that insulin induced b-ZIP transcription factor, E4-binding protein 4 (E4BP4), in hepatocytes. In the current study, we show that insulin injection increases hepatic E4bp4 expression by activating the AKT-mTORC1-SREBP-1c pathway in hepatocytes. E4bp4-deficient hepatocytes not only fail to maintain robust DNL but also become resistant to SREBP-1c-induced lipogenesis. In vivo, acute depletion of E4bp4 in the liver by adenoviral shRNA reduces the expression of lipogenic enzymes and results in reduced levels of serum triglycerides and cholesterol during the postprandial phase. In hepatocytes, E4BP4 interacts with nuclear SREBP-1c to preserve its acetylation, and subsequently protects it from ubiquitination-dependent degradation. In conclusion, the current studies uncover a novel positive feedback pathway mediated by E4BP4 to augment SREBP-1c-mediated DNL in the liver during the fed state.—Tong, X., P. Li, D. Zhang, K. VanDommelen, N. Gupta, L. Rui, M. B. Omary, and L. Yin. E4BP4 is an insulin-induced stabilizer of nuclear SREBP-1c and promotes SREBP-1c-mediated lipogenesis. J. Lipid Res. 2016. 57: 1219–1230.

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During the feeding cycle, insulin activates the insulin receptor-AKT-mTORC1-sterol regulatory element-binding protein (SREBP)-1c pathway to promote de novo lipogenesis (DNL) in the liver (1–3). In Irs (4), Akt2 (5), or mTorc1-deficient mice (6, 7), DNL is significantly impaired following food intake. In turn, DNL is suppressed during fasting when the rate of fatty acid oxidation is high (8, 9). The energy sensors, such as AMPK and SIRT1, are potent repressors of DNL via protein posttranslational modifications (10, 11). In the case of obesity and insulin resistance, dysregulated DNL has been shown to contribute to liver steatosis (1, 5, 12, 13). In a human study, 26% of radiolabeled triglycerides in the liver were derived from elevated DNL in human patients with nonalcoholic fatty liver disease (14). Thus, an in-depth understanding of how DNL is regulated during physiological and pathological conditions will shed light on therapeutic targets for the treatment of liver steatosis.

Insulin stimulates the expression of DNL genes mainly via the activation of SREBP-1c, a member of the basic-helix-loop-helix leucine zipper transcription factors (15, 16). SREBP-1c recognizes sterol-regulatory elements on the target promoters and activates the expression of lipogenic enzymes such as Fasn, Acc1, LDL receptor (Ldlr), and Hmgcs (16, 17). Persistent activation of SREBP-1c has been implicated in the pathogenesis of fatty liver disease (18–20). Transgenic mice overexpressing the nuclear form of SREBP-1a or SREBP-1c (21, 22) in hepatocytes develop hepatic steatosis along with elevated lipogenic gene expression, such as Fasn and Scd1. Conversely, Sreb1 deficiency in mice leads to significant reduction in hepatic triglycerides and lipogenic gene expression after refeeding and during obesity (23, 24). Therefore, SREBP-1c activity serves as a master regulator to control DNL during the

Abbreviations: CBP, calmodulin-binding protein; ChIP, chromatin immunoprecipitation; DNL, de novo lipogenesis; E4BP4, E4-binding protein 4; E4bp4-LKO, E4bp4 liver-specific knockout; Ldlr, Low density lipid receptor; PMH, primary mouse hepatocyte; qPCR, quantitative PCR; SBP, streptavidin-binding protein; SREBP, sterol regulatory element-binding protein; UTR, untranslated region.

¹X. Tong and P. Li contributed equally to this work.
†To whom correspondence should be addressed.
e-mail: leiyin@umich.edu
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cycle of fasting and feeding. However, how SREBP-1c achieves the maximal efficiency of DNL during the postprandial phase remains largely unknown.

Insulin signaling has been shown to regulate the biogenesis of a functional SREBP-1c (13, 16). The precursor form of SREBP-1c protein is tethered within the ER membrane in a complex with SCAP and INSIG. Upon insulin stimulation, the dissociation of INSIG from the SCAP/SREBP-1c complex triggers the release of the full-length SREBP-1c from the ER membrane and proteolysis at the Golgi (25–27). The cleaved SREBP-1c translocates into the nucleus to activate its targets. Nuclear SREBP-1c is very unstable and quickly degrades through the ubiquitination-pathway (28). Aside from ubiquitination, other posttranslational modifications, such as phosphorylation, sumoylation, and acetylation, have been identified to modify nuclear SREBP-1c and modulate its protein stability, DNA binding, and transcriptional activity (28–33). Specifically, acetylation of SREBP-1c is dynamically regulated by histone acetyl transferase p300/calmodulin-binding protein (CBP) and NAD-dependent deacetylase SIRT1 (30, 32, 33). It has been shown that acetylation of SREBP-1c negatively correlates with its ubiquitination and tends to stabilize SREBP-1c (32). Furthermore, insulin promotes acetylation of SREBP-1c in hepatocytes, suggesting that insulin can influence its protein stability in addition to the induction of Srebp-1c mRNA expression and protein cleavage. Until now, it has remained unclear how insulin promotes the SREBP-1c stability by retaining its acetylation during the postprandial phase.

The b-ZIP transcription repressor, E4-binding protein 4 (E4BP4), also called nuclear factor interleukin-3-regulated (NFIL3), plays an essential role in innate immunity (34, 35). The mRNA oscillations of E4bp4 were detected in the suprachiasmatic nuclei, liver, adipose tissues, aorta, skeletal muscles, and adrenal glands (36, 37). In addition to its regulation by the circadian clock, E4bp4 mRNA can be induced by glucocorticoids (38), insulin (39), and cAMP (40) in various cell types and tissues, indicative of tissue-specific regulation and functions of E4BP4. Previously, we reported that levels of E4bp4 mRNA and protein are elevated in hepatocytes from refed mice and induction of E4BP4 is required for the suppression of the fasting hormone, Fgf21, during the refed state (39). In cultured hepatocytes, we demonstrated that insulin induces E4bp4 mRNA in a PI3K-dependent manner. All of these observations suggest that E4BP4 could participate in insulin-dependent hepatic anabolism.

Here we present evidence, for the first time, that E4BP4 is an insulin-induced enhancer of hepatic lipogenesis. Upon insulin stimulation, newly synthesized E4BP4 promotes the stability of nuclear SREBP-1c and SREBP-1c-dependent DNL. In the case of E4bp4 deletion, primary mouse hepatocytes (PMHs) display reduced DNL and fail to activate DNL in response to SREBP-1c. Our data support that E4BP4 interacts with nuclear SREBP-1c and protects it from ubiquitination and proteasome degradation. Furthermore, interaction of E4BP4 with the nuclear SREBP-1c preserves SREBP-1c acetylation. These findings, for the first time, highlight a novel role of insulin-induced E4BP4 in promoting SREBP-1c-mediated DNL via a feed-forward loop.

**MATERIALS AND METHODS**

**Animals**

All animal care and use were in accordance with guidelines of the University of Michigan Institutional Animal Care and Use Committee. WT C57/BL6 male mice (8–10 weeks) were directly purchased from the Jackson Laboratory and maintained on a 12:12 h light-dark cycle with free access to standard diet and water. For acute insulin treatment, mice were fasted for 6 h before injection with insulin at 0.5 U/kg via an intra-peritoneal route.

**Generation of liver-specific E4bp4 knockout mice**

The E4bp4 targeting construct was created by flanking its exon 2 with Lox-P site and homology sequence on both arms. After injection into C57BL/6 ES cells and selection with neomycin, positive ES cells were microinjected into blastocysts and implanted into pseudopregnant females, which gave birth to the F0 chimeric pups. Germ-line transmission of the transgene was confirmed in the F1 generation after crossing chimeric males with WT C57BL/6 females. All the above procedures were performed at Cyagen (Sunnyvale, CA). The conditional mice were generated by crossing the positive F1 mice with FLPeR mice from the Jackson Laboratory. Both the deletion of the Frt-flanked Neo-cassette by FLPeR and the locations of Lox-P sites were confirmed by sequencing PCR products amplified by site-specific primers. The WT allele was determined by a 236 bp PCR band, whereas the conditional allele was defined by a 351 bp PCR band encompassing both Frt and Lox-P sites. E4bp4 liver-specific knockout (E4bp4-LKO) mice were generated by crossing E4bp4flox/flox mice with Alb-Cre transgenic mice (Jackson Laboratory) and confirmed by genotyping PCR reactions.

**Cell culture and reagents**

The Hepa1c1c7 mouse hepatoma cell line and 293T human embryonic kidney cell line were grown in complete MEM supplemented with 5% fetal calf serum, 1% penicillin-streptomycin mixture, and L-glutamine. MG132 was purchased from Biomol. Rapamycin and MK2206 were from Sigma. All the commercial antibodies used in this work were: anti-E4BP4 (sc-28203 and sc-74414), SREBP-1c (sc-13551) or knockdown of mouse E4BP4 has been described previously (39). Ad-shSrebp-1c or knockdown of mouse E4BP4 has been described previously (39). Ad-shSrebp-1c plasmid was generated through Gateway LR recombination between pEntry/U6-shSrebp-1c targeting GTCTTCTATCAATGACAAAG and pAdBlock-I vector (Invitrogen). To make pAdCMV-2xFlag-Srebp-1c vector, the 2x Flag-tagged nuclear form of human Srebp-1c cDNA was first cloned into pDONR/Zeo (Invitrogen) and then in vitro recombined with pAdCMV-Dest vector (Invitrogen) with LR recombinase I (Invitrogen). All the adenoviruses were produced in 293AD packaging
cells (Agilent) after Lipofectamine-mediated transfection and concentrated after ultracentrifugation in cesium chloride gradient solutions.

**Tail vein injection of adenovirus or adenoviral-associated virus**

For adenoviral injections, 1 × 10^12 plaque-forming units (pfu) per adenovirus were administered via tail-vein injection. For each virus, a group of four to five mice were injected with the same dose treatment. Ten days after injection, mice were euthanized around ZT23 (~6 AM at the end of dark cycle) and both serum and liver tissues were harvested for analysis. For acute hepatic dose treatment. Ten days after injection, mice were euthanized with the endogenous nuclear SREBP-1c, PMH lysates were incubated with 10 μl of anti-FLAG-M2 agarose beads for 16 h at 4°C. The beads were washed five times in lysis buffer and eluted in 30 μl of 2× SDS loading buffer. Western blotting was performed to detect the presence of targeted proteins with specific antibodies.

**Protein extraction, immunoprecipitation, and immunoblotting**

The general protocol for protein extraction from liver tissue and cells was described previously (41). The modified RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 0.05% SDS, 0.25% sodium deoxycholate, and protease inhibitor cocktail] was used to lyse cells and extract protein. Cleared lysates (about 20–50 μg total protein) were resolved by SDS-PAGE, transferred to PVDF membrane, and incubated with primary antibodies; immunoreactive bands were detected using homemade ECL reagents [1.25 mM Luminol, 0.2 mM p-coumaric acid, 0.1 M Tris-HCl (pH 8.5)]. For detecting the interaction of CBP-streptavidin-binding protein (SBP)-E4BP4 and nuclear SREBP-1c in overexpressed condition, precleared protein lysates (1 mg of protein) were incubated with 20 μl of Streptavidin magnetic beads (GE Healthcare) for 16 h. The beads were then washed with lysis buffer five times and eluted in 20 μl of 2× SDS loading buffer. For detecting FLAG-E4BP4 interaction with the endogenous nuclear SREBP-1c, PMH lysates were incubated with 10 μl of anti-FLAG-M2 agarose beads for 16 h at 4°C. The beads were washed five times in lysis buffer and eluted in 30 μl of 2× SDS loading buffer. Western blotting was performed to detect the presence of targeted proteins with specific antibodies.

**Gene expression analysis**

Total RNA extraction, cDNA synthesis, and quantitative (q)PCR analysis were described previously (42). The value of each cDNA was calculated using the ΔΔCt method and normalized to the value of the housekeeping gene control (18S RNA). Data were plotted as fold change with the value of the control group set as 1. PCR primers for lipogenic genes were reported previously (43). PMHs were isolated in DMEM supplemented with 5% FBS and subjected to adenoviral transduction prior to RT-qPCR, Western blot, or lipogenesis assay.

**PMH isolation and treatment**

PMHs were isolated from the livers of 8- to 10-week-old WT C57/BL6 mice after a two-step perfusion with EGTA and collage nase using a protocol described previously (43). PMHs were cultured in DMEM supplemented with 5% FBS and subjected to adenoviral transduction prior to RT-qPCR, Western blot, or lipogenesis assay.

**DNL assay**

The assay protocol was described previously (44). In brief, PMHs were seeded into 12-well plates (2 × 10^4 cells per well) and cultured overnight. In some experiments, primary hepatocytes were transduced with recombinant adenovirus and assayed for the rate of lipogenesis 48 h later. After rinsing with 1× PBS, hepatocytes were incubated with culture medium containing both cold acetate and 1 μCi 3H-labeled acetate (Moravek Biochemicals) for 4 h. Lipids were then extracted by adding 800 μl of chloroform/methanol (2:1, v/v). The organic fraction was transferred to a fresh tube and left to air dry at room temperature overnight. The pellet was dissolved in hexane and 5% H_2SO_4 in methanol and heated at 100°C for 30 min. The final radiolabeled lipids were extracted with 500 μl of petroleum and the 3H radioactivity was measured on a Beckman scintillation counter. DNL rate represents nanomoles per hour of incorporated 3H-labeled acetate after normalization by the protein amount in milligrams.

**Liver and plasma lipid measurements**

To extract total lipid from mouse liver tissues, about 60–80 mg liver tissues were homogenized in 1% acetic acid solution and mixed with 2× SDS loading buffer. After centrifugation, 3H-labeled acetate content was normalized by protein amount for each sample. Serum triglycerides and cholesterol were measured using an assay kit (Point Scientific) according to the manufacturer’s instructions.

**Promoter activity assay**

A 1.2 kb mouse E4bp4 promoter was subcloned from the mouse genomic DNA into the pGL4-luc reporter with T4 ligase and confirmed by sequencing. The cloning primer sequences were 5′-AGAAGGCTTTGCTGCTGACGACACAA-3′ and 5′-CGCACGCGGCAACACACACAG-3′. The luciferase activity driven by E4bp4 promoter was assayed in Hepa1 cells that were plated in a 24-well plate and transfected with E4bp4-luc alongside the nuclear Srebplc expression vector. Thirty-six hours post transfection, cells were lysed for luciferase activity measurement on a BioTek Synergy 2 microplate reader. A β-galactosidase construct was cotransfected in each well for normalizing luciferase activity.

**Chromatin immunoprecipitation (ChIP)**

Chromatin immunoprecipitation (ChIP) assay in cultured cells was performed as described before (43). PMHs were transduced with either Ad-GFP or Ad-Flag-Srebplc and harvested 48 h later for ChIP assay. Hepatocytes were cross-linked in 1% formaldehyde-PBS buffer. Nuclei pellets were isolated and sonicated to generate soluble chromatin materials with sizes between 1 and 2 kb. The chromatin was immunoprecipitated with anti-FLAG M2 beads (Sigma) or Ad-Flag-Srebplc and half of 36 h later for ChIP assay. Hepatocytes were cross-linked in 1% formaldehyde-PBS buffer. Nuclei pellets were isolated and sonicated to generate soluble chromatin materials with sizes between 1 and 2 kb. The chromatin was immunoprecipitated with anti-FLAG M2 beads (Sigma) or Ad-Flag-Srebplc and half of the purified DNA fragments were subjected to PCR amplification using PCR primers designed to encompass an E-box element in the mouse E4bp4 5′-promoter region. The E-box upstream of the transcription start site. Primers targeting a 3′-untranslated region (UTR) of E4bp4 were included as negative control, whereas the mouse Ldrb promoter region was amplified as positive control for SREBP-1c binding. The sequences of the PCR primers are as follows: mE4bp4 5′-promoter forward, 5′-CTTGCCTCAATTGTGTCCGC-3′; mE4bp4 5′-promoter reverse, 5′-CTTGTACCAATAGAACCAGC-3′; mLdhb 5′-promoter forward, 5′-GAATGCTAGCAGGGAAGGAA-3′; mLdhb 5′-promoter reverse, 5′-GAATGCTAGCAGGGAAGGAA-3′; mLdhb 5′-UTR forward, 5′-GAATGCTAGCAGGGAAGGAA-3′; mLdhb reverse, 5′-GAATGCTAGCAGGGAAGGAA-3′; mLdhb reverse, 5′-CAAGCCACATTCACCTC-3′; 18S RNA forward, 5′-TTGAGGGAAGGCCACCCACG-3′; 18S RNA reverse, 5′-CGACCCACCCACGGAATCT-3′.

E4BP4 promotes lipogenesis by stabilizing SREBP-1c
Cell-based nuclear SREBP-1c acetylation and ubiquitination assay

PMHs were transduced with Ad-F-Srebp-1c along with either Ad-shLacZ or Ad-shE4bp4. Cells were then treated with MG132 for 8 h before harvest for measuring ubiquitination or acetylation levels of the FLAG-tagged nuclear SREBP-1c. A denaturing immunoprecipitation protocol (41) was followed to pull down FLAG-tagged nuclear SREBP-1c with anti-FLAG M2 antibody. The lysis buffer was supplemented with histone deacetylase inhibitors (500 nM TSA, 10 μM EK547, and 100 mM nicotinamide) to preserve the acetylated SREBP-1c. The polyubiquitin-SREBP-1c conjugates were detected by anti-ubiquitin (Sigma). The acetylated SREBP-1c was detected by anti-acetyl-lysine (Abcam).

Statistical analysis

Differences between two groups were assessed by two-tailed Student’s t-test. Differences between more than two groups were assessed by ANOVA followed by either Dunnett’s test or Tukey’s test for all possible comparisons of means. All data are reported as mean ± SD. P < 0.05 was deemed as statistically different.

RESULTS

Insulin stimulates hepatic E4BP4 in an AKT-mTORC1-dependent manner

We previously reported that insulin potently induces the transcription repressor, E4BP4, in PMHs (39). However, the mechanism of hepatic E4BP4 induction by insulin remains largely unclear. We sought to determine whether the classical AKT-mTORC1 activity contributes to this induction of E4bp4 in hepatocytes. We first confirmed that insulin treatment induces the mRNA levels of E4bp4 in both mouse liver and PMHs. In WT mice injected with insulin, induction of E4bp4 in the liver peaked at 2 h and then declined after 6 h (Fig. 1A). As a positive target of insulin in the liver (45, 46), Sreb1c paralleled the expression pattern of E4bp4 in those mouse livers (supplementary Fig. 1A). Similarly, insulin induced both E4bp4 and Sreb1c in PMHs (Fig. 1B, supplementary Fig. 1B), suggesting that insulin may trigger the same signaling pathway to transactivate E4bp4 and Sreb1c. It has been established that the AKT-mTORC1 pathway is required for the induction of Sreb1c by insulin (47, 48). To test whether this pathway is also required for the induction of E4bp4 by insulin, PMHs were pretreated with the AKT inhibitor, MK2206 (49, 50), or the mTORC1 inhibitor, rapamycin (47, 48), for 1 h before insulin stimulation for 6 h. The inhibition of the AKT-mTORC1 signaling was confirmed by the reduced phosphorylation of AKT (AKT-P$\text{S}^{473}$) and S6 (S6-P) (Fig. 1C). In both conditions, insulin induction of E4bp4 mRNA was completely abrogated (Fig. 1D). Taken together, these results indicate that the AKT-mTORC1 signaling is a critical effector downstream of insulin required for the induction of E4bp4 in hepatocytes.

The lipogenic transcription factor, SREBP-1c, induces E4BP4 via direct binding to an E-box element in its promoter

An interesting observation from the chicken pineal gland demonstrated that the avian SREBP-1 binds to the promoter of E4bp4 in response to light (51), suggesting a possible conserved regulation between SREBP-1 and E4BP4. Due to the pivotal actions of SREBP-1c in DNL in response to feeding, we asked whether SREBP-1c is involved in the transcriptional induction of E4bp4 by insulin.

To efficiently manipulate SREBP-1c expression in hepatocytes, we generated adenoviruses for overexpression or knockdown of Sreb1c in PMHs. In the case of Sreb1c knockdown by adenoviral shRNA, insulin failed to induce not only Fasn (Fig. 2A, left panel), a known downstream target of SREBP-1c (45), but also E4bp4 mRNA in PMHs (Fig. 2A, right panel). In the meantime, E4BP4 protein was almost obliterated in those Ad-shSreb1c-transduced cells (Fig. 2B). In contrast, adenovirus-mediated overexpression of FLAG-tagged nuclear SREBP-1c significantly induced both mRNA and protein levels of E4bp4 in PMHs (Fig. 2C, D). Hence, our data indicate that SREBP-1c is both necessary and sufficient for E4bp4 induction in PMHs upon insulin stimulation.

SREBP-1c functions by binding to sterol regulatory elements (5′-AGCAGATTGTG-3′) or, in some cases, E-box-like elements (5′-CACGTG-3′) within the target promoters (17, 52). We sought to address whether SREBP-1c activates E4bp4 transcription by direct binding to the promoter of E4bp4. We first generated an E4bp4-luciferase reporter construct driven by a 1.2 kb promoter sequence containing one putative E-box binding site (Fig. 2E). In a co-transfection assay, overexpression of nuclear SREBP-1c activated both E4bp4-luc and Fasn-luc, a known target of nuclear SREBP-1c (supplementary Fig. 2). Compared with E4bp4-luc WT, deletion of the putative E-box blocked the SREBP-1c-induced E4bp4-luc activity in Hepa1 cells, suggesting that the E-box element is critical for the transactivation of E4bp4 by SREBP-1c (Fig. 2E). To test whether SREBP-1c directly binds to the E4bp4 promoter, we performed a ChIP assay after pulling down the chromatin-bound FLAG-SREBP-1c with anti-FLAG antibody from Ad-Flag-Srebp-1c (encoding nuclear SREBP-1c protein)-transduced PMHs. As a positive control, we detected about a 5-fold increase of SREBP-1c binding on the Ldlr promoter, a known target of SREBP-1c (17) (Fig. 2F, left panel). With the same set of samples, we observed about a 6-fold enrichment of FLAG-SREBP-1c on the E4bp4 E-box-containing 5′-promoter. However, there was no difference between Ad-GFP and Ad-SREBP-1c when the 5′-UTR of E4bp4 was examined (Fig. 2F, right panel). Taken together, these findings support that SREBP-1c is both necessary and sufficient for the induction of E4bp4 in hepatocytes via direct transcriptional activation.

Liver-specific knockdown of E4bp4 impairs lipogenesis program during the postprandial phase

Insulin signaling and downstream actions are essential for liver lipid biosynthesis during the postprandial state (1). To determine whether E4BP4 is involved in insulin-induced lipid metabolism, we employed adenoviral shRNA to deplete E4bp4 in the mouse liver and then measured systemic and liver lipid profiles after feeding. Compared with Ad-shLacZ-injected mice, Ad-shE4bp4 mice showed a reduced
E4BP4 promotes lipogenesis by stabilizing SREBP-1c

So far, our data have demonstrated that one of the physiological activities of E4BP4 is to promote hepatic lipogenesis during the feeding phase. To our knowledge, this is the first report signifying a role of E4BP4 in hepatic lipogenesis. To test whether E4BP4 regulates this process in a cell-autonomous manner, we took advantage of the E4bp4flox/flox mice recently generated in our laboratory (supplementary Fig. 3). Acute E4bp4 deletion was achieved by transducing E4bp4flox/flox PMHs with Ad-Cre (Fig. 4A). We then asked whether acute E4bp4 deletion could impact DNL in hepatocytes. To maximize the rate of DNL (43), we treated PMHs with insulin at 50 nM plus glucose at 25 mM. Compared with Ad-GFP control, Ad-Cre-transduced cells showed a significantly reduced rate of DNL (Fig. 4B). Such impairment in DNL in the absence of E4BP4 was consistent with a suppression of DNL genes, including Fasn and Scd1, at both the mRNA and protein levels (Fig. 4C, D). In the meantime, acute E4bp4 deletion in PMHs resulted in about a 15-fold increase in the Fgf21 mRNA (supplementary Fig. 4), consistent with our previous finding (39). Interestingly, we noticed a marked suppression of Ldlr in E4bp4-deficient hepatocytes, suggesting that E4BP4 might also play a positive role in cholesterol metabolism. Altogether, our data strongly support that E4BP4 is required to maintain a robust lipogenic program in insulin- and high glucose-stimulated hepatocytes.

E4bp4 deficiency represses DNL in PMHs

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E4bp4 promotes lipogenesis by stabilizing SREBP-1c

hepatic E4bp4 level by 90% (Fig. 3A). No significant changes in body weight and blood glucose levels were detected upon euthanization (Fig. 3B, C). However, significantly lower levels of serum triglycerides and cholesterol were observed in the Ad-shE4bp4 group (Fig. 3D, E). The liver triglyceride content was about 20% lower without statistical significance in Ad-shE4bp4-injected mice (Fig. 3F). These metabolic changes indicate the E4BP4 actively participates in hepatic lipid metabolism during the postprandial phase. To gain further insight into the molecular pathways that are regulated by E4BP4, we measured the mRNA levels of lipid metabolic genes in both groups of mice. Acute depletion of E4bp4 expression greatly reduced the expression of key lipogenic genes, including Fasn and Scd1 (Fig. 3G, left panel), as well as the protein levels of FASN, ACC1, and SCD1 in the liver (Fig. 3H). Notably, the ACC1 protein level was very low in the liver of Ad-shE4bp4-injected mice even though its mRNA was only partially reduced, indicating that E4BP4 might regulate ACC1 via posttranslational modifications in addition to transcriptional suppression. In the meantime, E4bp4 depletion did not affect genes of lipid uptake or secretion, CD36 and Mttp1 (3) (Fig. 3G, middle panel). E4bp4 depletion also showed a modest effect on a subset of genes of fatty acid oxidation (Acox1 and Cpt1a) (Fig. 3G, right panel) (53). In summary, our data illustrate a crucial and selective role of E4BP4 in activating the hepatic lipogenesis program during the postprandial phase.

Fig. 1. Insulin stimulates hepatic E4bp4 gene expression in an AKT-mTORC1-dependent manner. A: Insulin injection (0.5 U/kg) induces hepatic E4bp4 mRNA in a time-dependent manner in WT mice (n = 3–5 per group). Data represent mean ± SD, *P < 0.05 versus control by ANOVA with Dunnett’s test. B: Insulin (100 nM) induces E4bp4 mRNA in PMHs (n = 3–4) isolated from C57/BL6 WT mice. The experiments were repeated five times with similar results. C: Pharmacological inhibition of the insulin-AKT-mTORC1 pathway impairs insulin-induced E4bp4 mRNA in PMHs (n = 3) treated with AKT inhibitor, MK2206 (50 nM), or mTORC1 inhibitor, rapamycin (50 nM), for 1 h prior to insulin (100 nM) stimulation. The effects of MK2206 and rapamycin were confirmed by the levels of AKT-PSer473 and S6-P (left panel). The experiments were repeated twice with similar results. Data represent mean ± SD, *P < 0.05 versus control by ANOVA with Dunnett’s test.
E4BP4 overexpression promotes lipogenic gene expression in PMHs

So far, all our findings point to the essential role of E4BP4 in promoting lipogenesis in hepatocytes and liver. To determine whether overexpression of E4BP4 is sufficient to drive lipogenesis, we overexpressed E4BP4 by transducing PMHs with Ad-Flag-E4bp4 versus Ad-GFP control (supplementary Fig. 5A). Next, we measured the mRNA expression of two lipogenic enzymes in transduced hepatocytes and found that overexpression of E4BP4 induced a 7-fold increase of Fasn mRNA and a 5-fold increase of Scd1 mRNA (supplementary Fig. 5B). In parallel with the mRNA changes, the protein abundance of FASN and SCD1 was also increased as well as decreased total triglyceride content (Fig. 5E).

Deletion of E4bp4 impairs the SREBP-1c-driven lipogenesis in PMHs

One of the main metabolic functions of the insulin signaling pathway in the liver during refeeding is to activate the transcription of lipogenic enzymes through SREBP-1c (1, 47). In E4bp4-deficient hepatocytes, the transcriptional targets of SREBP-1c, Fasn and Ldlr, were greatly reduced (Fig. 4C); raising the possibility that E4BP4 is required for the full activity of SREBP-1c. To test a potential cross-talk between E4BP4 and SREBP-1c, we generated Hepa1-stable cell lines stably expressing shE4bp4. In two independent clones, reduction of E4BP4 protein was confirmed by immunoblotting (supplementary Fig. 6A). Compared with shLacZ Hepa1 cells, both shE4bp4 cell lines showed an abrogation of SREBP-1c-induced activation of the Fasn-luc promoter activity (supplementary Fig. 6B). Meanwhile, the nuclear SREBP-1c was greatly reduced in the shE4bp4 Hepa1 cells (supplementary Fig. 6C). These results demonstrated that E4BP4 is required for the full activation of lipogenic genes by SREBP-1c, as well as for maintaining the stability of SREBP-1c.

To confirm the role of E4BP4 on SREBP-1c-mediated lipogenesis in hepatocytes, we transduced PMHs from both E4bp4flox/flox and E4bp4-LKO (albumin-Cre-driven E4bp4 liver-specific knockout) mice with Ad-Srebp-1c. The absence of E4BP4 protein in E4bp4-LKO hepatocytes was confirmed by immunoblotting (Fig. 5A). SREBP-1c overexpression increased the rate of DNL by 12-fold along with the increased expression of Fasn (8-fold), Scd1 (2.5-fold), and Ldlr (2.3-fold) in E4bp4flox/flox PMHs, whereas its effects on DNL and lipogenic gene expression were lost in E4bp4-LKO PMHs (Fig. 5B, C). E4bp4-LKO hepatocytes also showed reduced levels of FASN and SCD1 (Fig. 5D), as well as decreased total triglyceride content (Fig. 5E).
E4BP4 promotes lipogenesis by stabilizing SREBP-1c

Consistent with the observation in Hepa1 shE4bp4-stable cells (supplementary Fig. 6C), the protein level of FLAG-SREBP-1c by adenoviral transduction was greatly diminished in the absence of E4BP4 (Fig. 5D), indicating that E4BP4 may regulate nuclear SREBP-1c stability. Collectively, these findings support that E4BP4 is required for SREBP-1c-driven lipogenesis and its stabilization in hepatocytes.

E4BP4 regulates nuclear SREBP-1c acetylation, ubiquitination, and stability

So far, we have generated evidence supporting the lipogenic action of E4BP4 in hepatocytes, consistent with E4BP4 being a downstream target of the insulin-AKT-mTORC1-SREBP-1c pathway. Yet how E4BP4, a canonical transcription repressor, enhances the SREBP-1c-mediated lipogenic action remains unclear. We have so far observed that either E4bp4 depletion (supplementary Fig. 6C) or deficiency (Fig. 5D) in vitro leads to a lower level of nuclear SREBP-1c, prompting us to examine how E4bp4 impairment affects nuclear SREBP-1c abundance in the liver. To this end, E4bp4<sup>−/−</sup> mice were injected with AAV-Cre to acutely delete E4bp4 in the liver. AAV-Cre-mediated deletion of E4bp4 resulted in efficient downregulation of the total E4BP4 protein. In the same AAV-Cre-injected liver samples, the expression of nuclear SREBP-1c and FASN was markedly reduced (Fig. 6A). However these changes were independent of AKT activity because the AKT phosphorylation level remained unchanged. Thus, E4BP4 also controls the protein abundance of nuclear SREBP-1c in the liver.

We postulated that E4BP4 might affect nuclear SREBP-1c stability through posttranslational modifications. To test this hypothesis, we first investigated whether E4bp4 deficiency alters ubiquitination or acetylation status of nuclear SREBP-1c because both modifications have been shown to regulate nuclear SREBP-1c stability (28, 30). After acute deletion of E4bp4 in PMHs by Ad-Cre, the polyubiquitinated FLAG-SREBP-1c was increased, whereas its acetylation level was reduced (Fig. 6B). Treatment of Ad-shE4bp4-transduced PMHs with MG132, a nonspecific proteasome inhibitor, restored the protein level of FLAG-SREBP-1c (Fig. 6C), suggesting that depletion of E4bp4 augments the proteasome-dependent degradation of nuclear SREBP-1c. Next, we examined the impact of Ad-Flag-E4bp4 on nuclear SREBP-1c protein stability, acetylation, and ubiquitination in PMHs. Indeed, E4BP4 overexpression increased both the endogenous SREBP-1 and the FLAG-tagged nuclear SREBP-1c abundance (Fig. 6D, E). In an immunoprecipitation experiment with PMHs, overexpression of E4BP4 reduced SREBP-1c ubiquitination, while increasing its acetylation (Fig. 6F). These data support the
truncation mutants and tested their abilities to interact with nuclear SREBP-1c in 293T cells. Both E4BP4 WT and the 1-300aa mutant showed interaction with nuclear SREBP-1c, although the interaction of the E4BP4 1-300aa mutant (E4BP4-S300) with nuclear SREBP-1c was considerably weaker (Fig. 7C). In contrast, the E4BP4 1-200aa mutant (E4BP4-S200) completely lost interaction with nuclear SREBP-1c, indicating that the E4BP4 200aa-300aa region is critical for interaction of E4BP4 with nuclear SREBP-1c. Moreover, E4BP4-S200 failed to enhance the acetylation of SREBP-1c in Hepa1 cells in comparison with E4BP4-WT (Fig. 7D), suggesting that the interaction of E4BP4 with nuclear SREBP-1c is a prerequisite for its ability to enhance nuclear SREBP-1c acetylation. Because acetylation of SREBP-1c is positively associated with its protein stability, we compared the protein half-lives of FLAG-tagged

Fig. 4. Loss of E4BP4 suppresses DNL in PMHs. A: Confirmation of loss of E4BP4 protein in Ad-Cre-transduced E4bp4flox/flox primary hepatocytes. Forty-eight hours post transduction of either Ad-Cre or Ad-GFP viruses, E4bp4flox/flox PMHs were harvested for immunoblotting with anti-E4BP4 antibody. B: Acute deletion of E4bp4 by Ad-Cre suppresses the rate of DNL in E4bp4flox/flox PMHs. Twenty-four hours after Ad-Cre versus Ad-GFP transduction, E4bp4flox/flox PMHs in high glucose and insulin were with 3H-labeled acetate for 4 h. Radiolabeled lipids were extracted in petroleum and the 3H radioactivity was normalized by protein amount for each sample. Data were plotted as mean ± SD (n = 4); *P < 0.05, **P < 0.01. C: The mRNA levels of lipogenic genes in Ad-Cre-transduced E4bp4flox/flox PMHs. The mRNA levels of Fasn, Scd1, and Ldh were measured by RT-qPCR. Data were plotted as mean ± SD (n = 4). D: The protein levels of lipogenesis enzymes in Ad-Cre-transduced E4bp4flox/flox PMHs. Twenty-four hours after Ad-Cre transduction, the protein abundance of FASN, SCD1, and GCK was measured by immunoblotting.

Fig. 5. E4bp4 deficiency impairs SREBP-1c-driven lipogenesis in PMHs. A: Loss of E4BP4 protein in E4bp4 LKO (liver-specific knockout) mouse liver by immunoblotting. B: Impaired induction of DNL by SREBP-1c overexpression in E4bp4 LKO PMHs. Twenty-four hours post transduction with Ad-Srebp-1c versus Ad-GFP, E4bp4flox/flox and E4bp4 LKO PMHs were incubated with 3H-labeled acetate in complete medium for 4 h. Radiolabeled lipids were extracted in petroleum and 3H radioactivity was normalized by protein amount for each sample. Data represent mean ± SD, *P < 0.05, and **P < 0.01 by ANOVA with Tukey’s test. C: Effects of SREBP-1c overexpression on the mRNA levels of DNL genes (Fasn, Scd1, and Ldh) in PMHs of E4bp4flox/flox versus E4bp4 LKO mice. D: Effects of SREBP-1c overexpression on the protein levels of DNL enzymes in PMHs of E4bp4flox/flox versus E4bp4 LKO mice. E: Effects of SREBP-1c overexpression on the cellular TG levels in PMHs of E4bp4flox/flox versus E4bp4 LKO mice. Data were plotted as mean ± SD (n = 4), *P < 0.05 by Student’s t-test.
E4BP4 promotes lipogenesis by stabilizing SREBP-1c. In cells cotransfected with either E4BP4 WT or E4BP4-S200 after cycloheximide treatment. In the case of E4BP4 WT overexpression, nuclear SREBP-1c displayed a half-life longer than 6 h. However, its half-life was shortened to 3 h in the presence of the E4BP4-S200 mutant (Fig. 7E), demonstrating that the E4BP4-S200 mutant is defective in promoting stabilization of nuclear SREBP-1c. In summary, E4BP4 promotes acetylation and stabilization of nuclear SREBP-1c via a protein-protein interaction.
DISCUSSION

There is ample evidence that insulin via AKT-mTORC1-SREBP-1c functions as a major regulator of hepatic lipogenesis (1, 6, 47, 48). However, how this insulin-induced SREBP-1c activity is maintained in hepatocytes remains elusive. Our current work revealed the crucial role of E4BP4 in an insulin-induced feed-forward loop in hepatic lipogenesis. E4BP4 is a novel downstream target of the insulin-AKT-mTORC1-SREBP-1c pathway. In E4bp4-deleted or -depleted mouse hepatocytes, nuclear SREBP-1c protein becomes highly unstable, resulting in reduced DNL. E4BP4 binds to nuclear SREBP-1c and preserves its acetylation status and stability. Thus, E4BP4, as a direct transcriptional target of SREBP-1c, acts in a positive feedback loop to stabilize the matured SREBP-1c protein and maintain its activity in promoting lipogenesis during insulin action.

The b-ZIP transcription factor, E4BP4, is abundantly expressed in immune cells, liver, and bone (35). We previously reported an elevated level of E4bp4 mRNA in the refeed liver and its induction by insulin in hepatocytes (39). However, the signaling pathways that mediate hepatic E4bp4 induction by insulin are not fully understood. In this current study, we discovered that E4BP4 is a bona fide target of the insulin-AKT-SREBP-1c pathway in hepatocytes. Pharmacological inhibition of either AKT or mTORC1 activity abrogates E4bp4 mRNA induced by insulin. Consistent with its primary role in insulin action, we found that nuclear SREBP-1c activates E4bp4 by binding to the E4bp4 5′-promoter sequence containing an E-box element. This finding sheds new light on nutrient-dependent transcriptional regulation of E4BP4, which is traditionally considered as a circadian target gene. This finding also highlights an evolutionarily conserved SREBP-E4BP4 axis in both the chicken pineal gland and mouse hepatocytes. It is also possible that SREBP-1c might control E4bp4 expression in human hepatocytes because an identical E-box sequence is located in the promoter of the human E4bp4 gene. In the obese condition, SREBP-1c activity was shown to be upregulated (18, 19). It will be of great interest to examine whether the level of E4bp4 is altered and whether SREBP-1c regulates its expression during the course of obesity.

E4BP4 is well-known for its critical role in NK cell development, the survival of preB lymphocytes, and IgG switch in intestinal lymphoid cells (34). However, the E4BP4’s functions in other tissues are largely unknown, even though this protein is ubiquitously expressed. We previously reported that feeding-induced E4BP4 suppresses the fasting hormone, Fgf21, and may contribute to feeding-associated inhibition of ketogenesis (39, 55). In the current study, we discovered that E4BP4 also plays an important role in hepatic lipogenesis during the postprandial phase. In WT mice, acute depletion of E4BP4 during refeeding significantly decreases serum triglycerides and cholesterol levels. At the molecular level, the expression of DNL enzymes, including Fasn, Acc1, and Scd1, was significantly reduced in E4bp4-depleted mouse liver. Together with our previous finding that E4BP4 inhibits Fgf21 and ketogenesis, we speculate that E4BP4 is a metabolic node turned on by the insulin-AKT-mTORC1 pathway to reprogram lipid metabolism upon food intake. Interestingly, the metabolic changes in our model share many similarities with other models of impaired DNL, for instance, genetic deletion of Srebp-1c (23, 24) or its proteolytic regulator, Scap (56). In future studies, the lipogenic action of E4BP4 will be investigated in the pathogenesis of nonalcoholic fatty liver disease induced by chronic high-fat diet feeding.

The most unexpected finding in our study is that E4BP4 positively regulates the DNL pathway via protein-protein interaction with nuclear SREBP-1c. We have provided evidence showing that E4BP4 is required for the induction of lipogenic genes by nuclear SREBP-1c. The presence of E4BP4 is necessary to protect nuclear SREBP-1c against ubiquitination-dependent degradation. Given the evidence that E4BP4 is a direct downstream target of SREBP-1c, we argue that E4BP4 forms a feed forward loop to enhance nuclear SREBP-1c protein stability and subsequent transcriptional activity. It has already been shown that SREBP-1c feeds back to upregulate its own transcription (57, 58). Our work points to an additional forward loop initiated by SREBP-1c and executed by E4BP4 during insulin-driven lipogenesis. Of note, the regulation of protein abundance of nuclear SREBP-1c by E4BP4 is independent of AKT activity in hepatocytes, even though E4BP4 induction by insulin clearly requires AKT. So far, whether E4BP4 might directly inhibit the suppressors of lipogenesis, such as AMPK (11, 59), has not been tested.

The transcriptional activity of nuclear SREBP-1c has been shown to be controlled by multiple posttranslational modifications, including phosphorylation, ubiquitination, and acetylation (28, 30–32). In agreement with the literature (28), we observed that nuclear SREBP-1c is quickly turned over with a half-life of less than 3 h (data not shown), suggesting that this mature form of SREBP-1c is consistently undergoing proteolysis in hepatocytes. In fact, nuclear SREBP-1c has been shown to be targeted by several mechanisms for proteasomal degradation. It has been reported that GSK3β phosphorylates SREBP-1c and promotes its degradation via E3 ligase FBW7 (31). During fasting, glucagon signaling promotes ubiquitination-mediated SREBP-1c degradation through PKA signaling and ubiquitin E3 ligase RNF20 (60). Conversely, SREBP-1c stability has been shown to positively correlate with its acetylation status. Insulin is known to promote acetylation of nuclear SREBP-1c (32), though the molecular pathways downstream of insulin signaling have not been identified as yet. In our study, E4BP4 overexpression increased SREBP-1c acetylation, whereas acute depletion of E4bp4 reduced SREBP-1c acetylation. As a result, E4BP4 is both necessary and sufficient to protect the nuclear SREBP-1c protein from degradation. In turn, SREBP-1c could self-preserve its acetylation by inducing E4BP4 in response to insulin. Because modulation of SREBP-1c acetylation is critical for its lipogenic action not only during the cycles of fasting and feeding but also in chronic high-fat diet feeding (32), E4BP4 may also protect nuclear SREBP-1c by promoting its acetylation and stability during the course of obesity.
How does E4BP4 promote the acetylation status of nuclear SREBP-1c following insulin stimulation? It is unlikely that E4BP4 directly acetylates nuclear SREBP-1c because E4BP4 protein itself does not contain any putative acetyl transferase domains. Two independent studies showed that the nutrient sensor, SIRT1, directly binds to SREBP-1c and destabilizes SREBP-1c through deacetylation in both mammals and Drosophila (32, 33). In our current study, we monitored the expression and activity of SIRT1 in hepatocytes treated in the presence or absence of insulin. The overall protein levels of SIRT1 remained constant (data not shown). Also, the acetylation levels of two known SIRT1 targets, p53 and p65, were not affected by insulin treatment (data not shown). In contrast, the acetylation levels of nuclear SREBP-1c are increased by insulin, indicative of an insulin-specific action on SREBP-1c acetylation. Using E4BP4 truncation mutants, we clearly demonstrated that interaction between E4BP4 and nuclear SREBP-1c is necessary for maintaining acetylation and stability of nuclear SREBP-1c. It is plausible that E4BP4 interaction with SREBP-1c blocks the access of SIRT1 or the ubiquitin E3 ligase to SREBP-1c. It is also conceivable that E4BP4 might recruit specific histone acetyl-transferases via protein-protein interaction to modify SREBP-1c. We previously reported that E4BP4 can form a transcriptional repression complex with the histone methyl-transferase, G9a (55), pointing to the possibility of E4BP4 recruiting other histone-modifying enzymes.

Dysregulation of hepatic lipid metabolism serves as an important pathogenic factor during the development of NAFLD. Both human and animal studies have highlighted elevated DNL in fatty liver disease (12, 14). We suspect that E4BP4 is very likely to participate in the pathogenesis of NAFLD, given its obligatory role in stabilizing nuclear SREBP-1c. The liver nuclear SREBP-1c levels were found to be significantly higher in both db/db and Ap2-Srebplc transgenic mouse models of diabetes (18, 21). Short-term inhibition of Srebp-1c using an antisense approach reverses diet-induced liver steatosis, indicative of a therapeutic potential (61). However, how to specifically inhibit nuclear Srebp-1c has not been fully explored. Our work showed a strong protein-protein interaction between E4BP4 and nuclear SREBP-1c and such an interaction might protect nuclear SREBP-1c from degradation by preserving SREBP-1c acetylation. In this regard, inhibitors that block E4BP4 and nuclear SREBP-1c interaction could suppress lipogenesis to treat fatty liver disease. This approach might offer the advantage of specifically targeting nuclear SREBP-1c for degradation without affecting other E4BP4 functions.

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