Sterol regulatory element-binding protein (SREBP) -1-independent regulation of lipogenic gene expression in adipocytes.

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Running Title

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Abbreviations

ABCA1, ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; ACC, acetyl-CoA carboxylase; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; FACE, fatty acyl-CoA elongase; FAS, fatty acid synthase; LXR, liver X receptor; S14, Spot 14; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element-binding protein.

Abstract

Sterol regulatory element-binding protein (SREBP) -1c is now well established as a key transcription factor for the regulation of lipogenic enzyme genes such as fatty acid synthase (FAS) in hepatocytes. Meanwhile, the mechanisms of lipogenic gene regulation in adipocytes remain unclear. Here we demonstrate that those in adipocytes are independent of SREBP-1c. In adipocytes, unlike in hepatocytes, the stimulation of SREBP-1c expression by liver X receptor (LXR) agonist does not accompany lipogenic gene up-regulation, although nuclear SREBP-1c protein is concomitantly elevated, indicating that the activation process of SREBP-1c by cleavage system is intact in adipocytes. Supportively, transcriptional activity of the mature form of SREBP-1c for FAS promoter was negligible when measured by reporter analysis. As an underlying mechanism, accessibility of SREBP-1c to the functional elements was involved, because chromatin immunoprecipitation assays revealed that SREBP-1c does not bind to functional SRE/E-box site on FAS promoter in adipocytes. Moreover, genetic disruption of SREBP-1 did not cause any changes in the lipogenic gene

expression in adipose tissue. In summary, in adipocytes unlike in hepatocytes, increment in nuclear SREBP-1c is not accompanied with transactivation of lipogenic genes, thus, SREBP-1c is not committed to regulation of lipogenesis.

Supplementary key words

Adipocytes, Lipogenesis, Fatty acid synthase

Introduction

The fatty acid biosynthetic pathway, composed of some 25 enzymes, has been elucidated in detail (1). Among these enzymes, fatty acid synthase (FAS), the main synthetic enzyme that catalyzes the condensation of malonyl-CoA to produce the 16-carbon saturated fatty acid palmitate, and acetyl-CoA carboxylase (ACC), which synthesizes malonyl-CoA from acetyl-CoA, are of particular importance. The regulation of these lipogenic enzymes has two remarkable features. First, their overall enzymatic activities largely depend on the amount of expressed protein which is primarily controlled at the transcriptional level, although regulation through phosphorylation is also important for some enzymes such as ACC. Second, their rates of transcription are coordinately regulated (2). Therefore, it has been presumed that these genes share a regulatory sequence in their promoters that interacts with common trans-acting factors. In the liver, the most likely factor conducting this coordinate transcriptional regulation has been revealed to be sterol regulatory

element-binding protein (SREBP) -1 (3,4).

SREBPs are transcription factors that belong to the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family and are considered to be profoundly involved in the transcriptional regulation of cholesterogenic and lipogenic enzymes (5,6). Unlike other members of the bHLH-Zip family, SREBPs are synthesized as precursors bound to the endoplasmic reticulum and nuclear envelope. Upon activation, SREBPs are released from the membrane into the nucleus as mature protein by a sequential two-step cleavage process. To date, three SREBP isoforms, SREBP-1a, -1c and -2, have been identified and characterized. SREBP-1a and -1c are transcribed from the same gene, each by a distinct promoter, and the predominant SREBP-1 isoform in liver and adipose tissue is 1c rather than 1a (7).

The role of SREBP-1c for the regulation of lipogenesis in the liver has been well established by several lines of evidence, especially by those from transgenic and knockout mouse models (4,8). In these models, hepatic mature SREBP-1c protein levels determine mRNA expression levels for a battery of

lipogenic genes in the liver. Moreover, hepatic mature SREBP-1c is physiologically regulated by nutrient availability, *i.e.* it is down-regulated when animals are starved and up-regulated when they are re-fed, thereby adjusting lipogenic gene expression levels to the nutritional conditions (9). Thus, as far as the role in the liver, SREBP-1c is now well established as a key transcription factor for the regulation of lipogenic gene expression and thereby triglyceride storage in liver (10,11).

Despite the extensive knowledge gained in recent years regarding the role of SREBP-1 in lipogenesis in liver, its physiological role in adipocytes remains obscure. Although the mRNA expression of SREBP-1c in adipocytes is also drastically altered by dietary conditions, we have reported that targeted disruption of SREBP-1 gene scarcely affected the dynamic changes of lipogenic gene expression in adipose tissue (4). Conversely, the impact of SREBP-1c over-expression in adipocytes was also evaluated in transgenic mice; however, it disrupted the differentiation processes of adipocytes, thereby resulting in lipodystrophy. Hence the effect of SREBP-1c on lipogenic gene regulation in

adipocytes was not able to be evaluated (12), although ectopic over-expression of SREBP-1a drives fatty acid synthesis in the adipose tissue of transgenic mice (13).

These situations prompted us to investigate the effects of SREBP-1c activation in adipocytes by stimulating the SREBP-1c promoter. We and others have found that Liver X receptors (LXR), nuclear receptor-type transcription factors, activate the transcription of SREBP-1c gene through binding to LXR-elements in the promoter together with retinoid X receptors (RXR) (14,15).

Here we show that the stimulation of SREBP-1c gene with LXR agonist has negligible effects on the expression of lipogenic enzymes in adipocytes, despite concomitant increase in nuclear active SREBP-1c, indicating that transcriptional activity of SREBP-1c against lipogenic genes is almost none in adipocytes in contrast to hepatocytes. In fact, luciferase reporter gene assays demonstrated that recombinant nuclear active form of SREBP-1c had far lower activity for the FAS promoter in 3T3-L1 adipocytes than in HepG2 hepatoma cells. Consistent with these observations, chromatin immunoprecipitation

(ChIP) assays revealed that SREBP-1 is not recruited to the functional cis-element on FAS promoter in LXR-activated adipocytes. Taken together, lipogenic genes are controlled almost independently of SREBP-1c in adipocytes and the triglyceride biosynthetic pathway is differently regulated between liver and adipose tissue.

Materials and Methods

Materials — Synthetic LXR agonist T0901317 was purchased from Cayman Chemical (Ann Arbor, MI). RXR agonist LG100268 was synthesized as described elsewhere (16). Standard laboratory chow (MF, composed of 60% carbohydrate, 13% fat and 27% protein on a caloric basis) was obtained from Oriental Yeast (Tokyo, Japan). Other materials were purchased from Sigma unless otherwise indicated.

Animals — Eight-week-old male Wister rats and 8-week-old C57BL/6J male mice were purchased from CLEA (Tokyo, Japan). All animals were maintained in a temperature-controlled environment with a 12h-light / dark cycle and were given free access to standard chow and water. The dosage of T0901317 (suspended with 0.9% carboxymethylcellulose, 9.95% polyethyleneglycol 400 and 0.05% Tween 80, and orally administered) was 10mg/kg for rats and 50mg/kg for mice. Animals were sacrificed 12h after the administration. The protocol of dietary manipulation was as follows: for the fasting group, animals

were starved 24h for mice or 48h for rats, and for the refeeding group, they were refed 12h or 24h after a 24h (for mice) or 48h (for rats) starvation, respectively, unless otherwise stated. All groups of animals in one experiment were sacrificed at the same time. SREBP-1-null mice (16 weeks of age, female) on the C57BL/6J background have been previously reported (4).

RNA isolation and Northern blotting — Total RNA from liver, epididymal fat pad and cultured cells was isolated with Trizol Reagent (Invitrogen), and 10μg RNA sample equally pooled among each group was run on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane. cDNA probes were cloned as previously described (4,17). The probes were labeled with [α-^{32}P]dCTP using Megaprime DNA Labelling System (Amersham Biosciences). The membranes were hybridized with the radiolabeled probe in Rapid-hyb Buffer (Amersham Biosciences) at 65°C and washed in 0.1xSSC buffer with 0.1% SDS at 65°C. Blots were exposed to both Kodak XAR-5 film and imaging plate for BAS2000 BIO IMAGING ANALYZER (Fuji Photo Film, Tokyo, Japan). The quantification results obtained from BAS2000 system were normalized to the

signal generated from 36B4 (acidic ribosomal phosphoprotein P0) mRNA.

Quantitative real time PCR analysis — 2μg total RNA was reverse transcribed using ThermoScript RT-PCR System (Invitrogen). Quantitative real time PCR was performed using SYBR-Green Dye (Applied Biosystems) in an ABI Prism 7900 PCR instrument (Applied Biosystems). Relative abundance of each transcript was calculated by a standard curve of cycle thresholds for serial dilutions of a cDNA sample and normalized to 36B4. Primer sequences are available upon request.

Nuclear protein extraction from liver and fat — Nuclear extract protein from mouse or rat liver was prepared as previously described (18). Briefly, excised livers (0.5g) were homogenized in a Polytron in 5ml of buffer A (10mM HEPES at pH7.9, 25mM KCl, 1mM EDTA, 2M sucrose, 10% glycerol, 0.15mM spermine, and 2mM spermidine, supplemented with protease inhibitors (6μg/ml N-acetyl-leucyl-leucyl-norleucinal (ALLN, Calbiochem), 2.5μg/ml pepstatin A, 2μg/ml leupeptin, 0.1mM phenylmethylsulfonyl fluoride (PMSF) and 2.5μg/ml aprotinin)). Pooled homogenate was then subjected to one stroke of Teflon pestle in Potter-

Elvejiem homogenizer, followed by filtration through two layers of cheesecloth, and layered over 10ml of buffer A. After centrifugation at 24000 rpm on a Beckman SW28 rotor for 1h at 4°C, the resulting nuclear pellet was resuspended in a buffer containing 10mM HEPES at pH 7.9, 100mM KCl, 2mM $MgCl_2$, 1mM EDTA, 1mM dithiothreitol, and 10% glycerol supplemented with protease inhibitors, after which 0.1 volume of 5M NaCl was added. Each mixture was agitated gently for 30min at 4°C, and then centrifuged at 89000 rpm on a Himac S120AT2 rotor (Hitachi, Tokyo, Japan) for 30min at 4°C. The supernatant was used as nuclear extract.

Nuclear protein from white adipose tissue was prepared as described previously (19). In brief, fresh epididymal fat pads (approximately 3g) were rinsed in ice cold PBS, minced, and homogenized with 10 strokes of Teflon homogenizer in 15ml NDS buffer at 4°C (10mM Tris-HCl at pH 7.5, 10mM NaCl, 60mM KCl, 0.15mM spermine, 0.5mM spermidine, 14mM mercaptoethanol, 0.5mM EGTA, 2mM EDTA, 0.5% NP-40 and 1mM dithiothreitol) supplemented with protease inhibitors (6μg/ml ALLN, 2μg/ml leupeptin, 2.5μg/ml aprotinin,

2.5μg/ml pepstatin A and 0.1mM PMSF). NP-40 concentration was increased to 1% and nuclei were pelleted at 700g for 10min, washed once with 25ml NDS buffer (1% NP-40), filtered through 70μm mesh, pelleted at 500g for 10min, resuspended in 1 volume of 1% citric acid, lysed by the addition of 2.5 volumes of 0.1M Tris-HCl, 2.5% SDS and 0.1M dithiothreitol, sonicated briefly, and heated to 90°C for 5min. Aliquots of nuclear protein (20μg) were subjected to SDS/PAGE.

Primary culture of rat preadipocytes — Fibroblastic preadipocytes were isolated from epididymal fat pads of Wister rats by collagenase digestion as described previously (20). In brief, the epididymal fat pads from male Wister rat were removed and minced in KRBH buffer (130mM NaCl, 5.2mM KCl, 1.3mM KH_2PO_4, 2.7mM $CaCl_2$, 1.3mM $MgSO_4$, 24.8mM $NaHCO_3$ and 10mM HEPES at pH 7.4), supplemented with 3% (wt/vol) BSA, 2mM glucose and 200nM adenosine. After digestion by collagenase (type II, 1.5mg/ml) at 37°C for 1h in shaking water bath, the digest was filtered through sterile 250μm nylon mesh. The adipocytes were allowed to float to the top of the tube and the infranatant

was collected, passed through 25μm stainless steal filter, and centrifuged at 250g for 10min. The pellet was resuspended in MEM Alpha medium (Invitrogen) with 10% FBS supplemented with penicillin and streptomycin (100U/ml and 100μg/ml, respectively, Invitrogen). Red blood cells were lysed by hypotonic shock (21). The cells were plated on 60mm culture dishes at a density of $6x10^5$ cells / well. Medium was changed every 2 days. After the cells reached confluence, differentiation was induced in MEM Alpha medium with 10% FBS by the addition of 0.5mM isobutylmethylxanthine (Wako), 0.25μM dexamethasone, 5μg/ml bovine insulin and 1μM pioglitazone (provided from Takeda pharmaceutical). The differentiation of cells was morphologically confirmed. Differentiated adipocytes were treated with either vehicle (ethanol) or 10μM T0901317 for 12h in MEM Alpha medium containing indicated amount of glucose and insulin.

Primary culture of rat hepatocytes — Hepatocytes were isolated from nonfasted 4-week-old Wister rats by the collagenase perfusion method as described previously (22). Cells were resuspended in DMEM containing 25mM

glucose supplemented with 5% FBS and seeded on collagen-coated 100mm dishes at a final density of $4x10^4$ cells /cm^2. After 4h, attached cells were cultured with Medium 199 containing Earle's salts (Invitrogen) and 5% FBS. Following incubation for 20h, cells were treated with either vehicle (ethanol) or 10μM T0901317 for 12h in similar medium containing indicated amount of glucose and insulin.

Preparation of nuclear extracts from cultured cells — Nuclear proteins from cultured cells were extracted as described previously (23). In brief, 2h prior to collection, ALLN (6μg/ml) was added to the media. After collection, cells were resuspended in buffer A (10mM HEPES at pH7.6, 1mM KCl, 1.5mM $MgCl_2$, 1mM EDTA and 1mM EGTA), and passed through a 26 gauge needle 20 times and then briefly centrifuged. The pellet, containing the nuclei, was resuspended in buffer B (20mM HEPES at pH7.6, 25% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA and 0.5mM dithiothreitol) and rotated at 4°C for 15min and then centrifuged at 15000g for 20min. The supernatant was collected as nuclear extract. Whole-cell lysates were harvested with a buffer (20mM Tris-

HCl at pH7.4) containing detergents (1% Triton X100, 0.1% SDS and 1% sodium deoxycholate) and protease inhibitors according to standard protocol.

Immunoblotting of SREBP Proteins — Membrane fractions from livers and epididymal fat pads were prepared as previously described (18). Aliquots of nuclear extract (20μg) and membrane fraction (50μg) proteins were subjected to SDS/PAGE. Immunoblot analysis was performed using the ECL Western Blotting Detection System (Amersham Biosciences) and exposed to Kodak XAR-5 film. The primary antibodies (rabbit polyclonal; #931 for mouse SREBP-1a and -1c, #772 for SREBP-1c which does not bind to SREBP-1a, and #528 for SREBP-2) were used as previously described (11,17,24). The precursor and mature SREBP-1 bands are around 125kD and 65kD, respectively.

Transfection and luciferase assays — An expression plasmid for human nuclear form of SREBP-1c constructed in pcDNA3.1(+) (Invitrogen) was described previously (25). Luciferase reporter plasmids for SRE promoter (SRE-Luc) and fatty acid synthase gene promoter (FAS-Luc) were prepared as previously described (8,24). Human hepatoma HepG2 cells were cultured in

DMEM containing 25mM glucose, 100U/ml penicillin and 100μg/ml streptomycin sulfate supplemented with 10%FBS. On day 0, cells were plated on a 12 well plate at $4.5x10^4$ cells / well. On day 2, the indicated amount of mature SREBP-1c expression plasmids, mock plasmids (empty pcDNA3.1(+)) to adjust total DNA amount, and luciferase reporter plasmids (FAS-Luc or SRE-Luc, 0.25μg each) mixed with an SV-β-galactosidase reference plasmid (0.1μg, p-SV-β-gal, Promega) were co-transfected into HepG2 cells using SuperFect Transfection Reagent (QIAGEN) according to the manufacturer's protocol. The luciferase activity in transfectants was measured on a luminometer and normalized to β-galactosidase activity measured by standard kits (Promega).

3T3-L1 adipocytes were transfected by electroporation as previously described (26,27). In brief, after cells became confluent (day0), adipose conversion was induced in DMEM containing 25mM glucose, 10% FBS, 0.5mM isobutylmethylxanthine, 5μg/ml bovine insulin, 0.25μM dexamethasone, 1μM pioglitazone, 33μM biotin (Wako), 17μM pantothenate (Wako) and antibiotics. After 48h, the induction medium was removed and replaced by DMEM

containing 25mM glucose, 10% FBS, insulin, pioglitazone, biotin and pantothenate. This medium was changed every 2 days. The adipocytes at day8 of differentiation grown on 60mm dishes were detached from dishes with 0.25% trypsin and 0.5mg/ml of collagenase (Wako) in PBS, washed twice, and were resuspended in PBS. The indicated amount of SREBP-1c expression plasmids, mock plasmids to adjust total DNA amount, luciferase reporter plasmids (FAS-Luc or SRE-Luc, 15μg each) and control plasmids (pSV-β-gal, 10μg) were co-transfected to the cells by a pulse current generated from electroporator (Cell-Porator, Invitrogen) at 160V with 880μF capacitance and low ohm setting. After electroporation, cells were immediately mixed with fresh medium for 10min before reseeded onto 24 well collagen-coated plates and assayed 40h after transfection.

Chromatin immunoprecipitation (ChIP) assays — ChIP assays were performed as described by Farnham et al with minor modifications (28). The supernatant of soluble chromatin derived from $1x10^7$ cells was used. Briefly, rat primary hepatocytes and adipocytes were treated with 1% formaldehyde for

10min. The cross-linking reaction was stopped by addition of glycine to a final concentration of 0.125M for 5min. After wash with cold PBS, the cells were suspended in cell lysis buffer containing 10mM HEPES at pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 0.5% NP40 and protease inhibitors. After incubation for 30min on ice, the cells were passed through a 26-gauge needle 20 times on ice. The nuclei were collected by microcentrifugation at 5000 rpm and resuspended in nuclear lysis buffer (50mM Tris-HCl at pH8.1, 1% SDS, 10mM EDTA and protease inhibitors) on ice for 10min. Samples were sonicated with Branson sonifier at power 2 for six 10-s pulses to an average DNA length of below 2kb and then microcentrifuged at 15000rpm for 10min. The supernatant was diluted 10-fold with buffer containing 0.01% SDS, 16.7mM Tris-HCl at pH 8.1, 1.1% Triton X-100, 1.2mM EDTA, 167mM NaCl and protease inhibitors and precleared for 1.5h with protein G sepharose. The supernatant was incubated with normal rabbit IgG or anti-SREBP-1 antibody over night at 4°C. Samples were subsequently washed four times using wash buffer A (0.1% SDS, 1% Triton X-100, 2mM EDTA, 150mM NaCl and 20mM Tris-HCl at

pH8.1), wash buffer B (0.1% SDS, 1% Triton X-100, 2mM EDTA, 500mM NaCl and 20mM Tris-HCl at pH8.1), wash buffer C (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA and 10mM Tris-HCl at pH 8.1), and wash buffer D (1mM EDTA and 10mM Tris-HCl at pH 8.0), and were eluted by 30min incubation with 100µl of elution buffer (1% SDS, 50mM $NaHCO_3$ and 10mM DTT). Then NaCl was added at a final concentration of 0.3M and RNA was removed by the addition of RNase A, and the samples were incubated at 65°C for 4h to reverse the formaldehyde-induced cross-linking. Protein was digested by proteinase K in 2xPK buffer (20mM Tris-HCl at pH7.5, 10mM EDTA and 1% SDS) at 45°C for 2h. The resulting chromatin DNA was further purified on silica beads (Wizard DNA clean-up system, Promega) and used as a template for PCR. Primers used to amplify FAS promoter regions were as follows: for SRE/E-box site at -65 (-109 to 63 was amplified), 5'-GACGCTCATTGGCCTGG-3' and 5'-CTCTGGAGGCAGACGACAAG-3'; for SRE-1 site at –150 (-94 to -299 was amplified), 5'-AGGACAGAGATGAGGGCGTC-3' and 5'-CCAGGCCAATGAGCGTC-3'; for

non-specific site (-1076 to –904), 5'-AAGCCACTGCCCATAAGGTT-3' and 5'-TTAAAGGGAGGGAGGGTGAG-3'. For SRE/E-box and non-specific sites, PCR was performed using AmpliTaq Gold (Applied Biosystems, Foster City, CA) under the following reaction conditions: after 9min at 95°C, 35 cycles of 1min at 95°C, 30sec at 62°C and 1min at 72°C. For SRE-1 site, PCR was performed using Advantage-GC Genomic Polymerase Mix (Clontech) under the following reaction conditions: after 1min at 95°C, 35cycles of 30sec at 94°C, 30sec at 62°C and 2.5min at 68°C. After amplification, PCR products were electrophoresed in a 3% NuSieve agarose gel and visualized by ethidium bromide staining. These experiments were performed at least three times.

Results

SREBP-1c activation by LXR agonist does not induce lipogenic gene expression in adipose tissue.

In our first series of experiments, we examined the effects of SREBP-1c over-expression on lipogenic gene expression in liver and adipose tissue. As expected, administration of synthetic LXR agonist T0901317 to animals (mice in Fig. 1 and rats in Fig. 2) up-regulated SREBP-1 expression and thereby the downstream lipogenic enzyme genes such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and stearoyl-CoA desaturase 1 (SCD1) in their livers. In contrast, quite unexpectedly, lipogenic genes in their adipose tissues were not influenced by the treatment, although SREBP-1 was markedly up-regulated in the same way as in liver. These results from Northern blotting were confirmed by quantitative real time PCR analyses (Fig 1b). It is notable that SREBP-1 mRNA levels quantified by real time PCR are equivalent in both tissues as shown in Fig.1b, denying the possibility that lack of lipogenic gene induction in

adipose tissue is a quantitative effect. The effect of LXR activation was further verified by the marked elevation of ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1), representatives of LXR activated genes (29,30), which were strongly induced in both tissues. These results were not altered by combined administration of both LXR agonist and RXR agonist LG100268 (30mg/kg), which produced additive effects on SREBP-1 pathway in the liver, but did not influence lipogenic gene expression in adipose tissue (data not shown).

Post-translational activation of SREBP-1 is intact and mature protein is generated in adipose tissue.

SREBP-1 is synthesized as a precursor bound to the endoplasmic reticulum and nuclear envelope, and is released from the membrane into the nucleus as a mature protein by a cleavage process (6). Therefore, it is possible that defective post-translational processing of SREBP-1 in adipocytes might result in this blunted response of lipogenic genes to SREBP-1 over-expression. To test this possibility, we separated nuclei from liver and adipose tissue, and

quantified the amount of mature SREBP-1 by immunoblot analysis. As shown in Fig. 3, nuclear contents of SREBP-1 were similarly elevated by LXR agonist both in liver and adipose tissue, indicating that post-translational processing of SREBP-1 is intact in adipose tissue (Fig. 3a, mice; Fig.3b, rats). It was further confirmed by SREBP-1c-specific antibody that isoform 1c was increased in the nuclei of adipose tissue. These findings demonstrate that the defective response of lipogenic genes to SREBP-1 over-expression in adipose tissue is not due to impairment of the SREBP-1c cleavage process.

To deny the possibility that the expression levels of nuclear form of SREBP-1 protein in adipose tissue stimulated by LXR agonist are too low to elevate lipogenic gene expression, the effect of T0901317 was compared to that of refeeding. As shown in Fig. 3c, mature form of SREBP-1 in the nuclei of LXR-stimulated adipocytes is more abundant than that observed in adipocytes from refed animals. However, this abundant SREBP-1 protein of active form did not induce FAS expression at all (Fig. 3d), demonstrating that the amount of SREBP-1 protein is not the cause of inability of SREBP-1 to up-regulate the FAS

expression in adipocytes.

SREBP-1 and lipogenic gene expression in primary cultured cells.

LXR widely modifies lipid and glucose metabolism, affecting serum levels of insulin and fatty acids (31), which are known to influence the SREBP-1 pathway (32). To eliminate these indirect effects through systemic factors and reinforce the *in vivo* results, we also examined the effects of SREBP-1c activation in primary cultured hepatocytes and adipocytes *in vitro*, whose isoform patterns of SREBP-1 are 1c-predominant as assessed by quantitative real-time PCR assays (data not shown). In these primary models, it was demonstrated that the over-expressions of SREBP-1 mRNA and thereby precursor proteins induced by LXR agonist lead to the elevation of mature SREBP-1 proteins in both hepatocytes and adipocytes (Fig. 4). However, the resulting activation of SREBP-1 produced totally different effects on lipogenic gene expression between hepatocytes and adipocytes; consistent with the *in vivo* results described above, FAS mRNA expression was up-regulated by SREBP-1c in

hepatocytes but was not induced in adipocytes.

Transcriptional activity of SREBP-1c for FAS promoter is negligible in adipocytes.

As we described above, the contribution of SREBP-1 to lipogenic gene regulation was demonstrated to be different between hepatocytes and adipocytes. Because SREBP-1c activation processes are revealed to be equally intact in both cell types, it is presumed that nuclear SREBP-1 would have different transcriptional activity in hepatocytes and adipocytes. To test this hypothesis, we performed luciferase reporter gene assays and compared transcriptional activities of nuclear SREBP-1c between HepG2 hepatoma cells and 3T3-L1 adipocytes. We estimated transcriptional activity of nuclear SREBP-1c against FAS promoter by transfecting cells with FAS-Luc reporter plasmids along with mature SREBP-1c expression plasmids. Transfection efficiency was evaluated using an optimum SRE-Luc as a control. As shown in Fig. 5, in HepG2 cells, FAS-Luc as well as SRE-Luc was vigorously activated by

SREBP-1c. In contrast, activation of FAS-Luc by active form of SREBP-1c was faint in 3T3-L1 adipocytes while SRE-Luc was highly activated. These data demonstrate that the transcriptional activity of SREBP-1c against FAS promoter is far lower in adipocytes compared to hepatocytes.

SREBP-1 does not bind to functional cis-element on FAS promoter in adipocytes.

To further investigate the molecular mechanism against inability of SREBP-1 to activate the transcription from FAS gene in adipocytes, we evaluate the direct binding of SREBP-1 to FAS promoter using ChIP assays. In the FAS promoter, two potential binding sites for SREBP-1 have been identified: one is -65 SRE/E-box which contains two tandem copies of SREs and E-box, and the other is -150 SRE-1 which is not functional because of the lack of co-factor binding sites nearby although the sequence itself is a complete SRE (5'-TCACCCCAC-3') (33,34). In our ChIP assays, SREBP-1 activation augmented its binding to -65 SRE/E-box in rat primary hepatocytes as expected (Fig. 6). In

sharp contrast to this, SREBP-1 activation did not result in its recruitment to -65 SRE/E-box in adipocytes, demonstrating that SREBP-1 does not bind to the functional cis-element on FAS promoter in adipocytes. Meanwhile SREBP-1 activation lead to its binding to -150 SRE-1 in both hepatocytes and adipocytes. These results were also confirmed using mouse primary cells (data not shown).

Genetic disruption of SREBP-1 lowers lipogenic gene expressions as well as their responses to LXR activation in the liver, but not in adipose tissue.

To further validate that SREBP-1 is not involved in the regulation of lipogenic genes in adipocytes, we evaluated FAS expression in SREBP-1 knockout mice with or without LXR stimulation. As shown in Fig. 7, genetic deletion of SREBP-1 impaired transactivation of FAS in response to LXR activation in liver, while in adipose tissue LXR activation did not alter the expression levels of these lipogenic genes in either SREBP-1 knockout or wild type mice. These results demonstrate that SREBP-1 does not substantially contribute to the regulation of FAS expression in adipose tissue while in the liver

SREBP-1 actually regutates the expression level of FAS.

Discussion

The current study has clearly demonstrated that lipogenic gene regulation is primarily independent of SREBP-1c in adipocytes. Hence the involvement of SREBP-1c in fatty acid synthesis differs fundamentally between liver and adipose tissue, the two major lipogenic organs.

Recently, microarray analysis of mouse liver and adipose tissue stimulated by LXR agonist was reported by others (35). In their study, SREBP-1 mRNA expression was induced both in liver and adipose tissue by LXR agonist while lipogenic genes such as fatty acid synthase, malic enzyme and Spot14 were up-regulated only in their liver and not in adipose tissue, supporting our data. We further proceeded to explore the underlying mechanism for this discrepancy, and have revealed that the active form SREBP-1 is definitely generated but does not transactivate lipogenic genes in adipocytes due to the defective binding to functional SRE in promoter regions *in vivo*.

In our first series of experiments, we have demonstrated that both in

adipocytes and hepatocytes the nuclear content of mature SREBP-1 protein parallels the amount of precursor form of SREBP-1 whether its expression is elevated by LXR activation or by refeeding. This observation indicates that the proteolytic activation of SREBP-1 precursor protein is intact in adipocytes and that SREBP-1 is basically activated when its mRNA expression is up-regulated. This feature of SREBP-1 activation provides a sharp contrast to that of SREBP-2, which is tightly regulated through the cleavage processes as a pivotal part of the negative feedback system to maintain cholesterol homeostasis (6). In fact, SREBP-1 and -2 are reported to be regulated distinctly under some specific conditions (18,36). It is intriguing that there is so much difference in the regulation of proteolytic activation of SREBP-1 and -2 while they are both subject to the same cleavage machineries consisting of SREBP cleavage-activating protein (SCAP) and site-1/2 proteases (37).

Nevertheless, in contrast to hepatocytes, elevation of the active form of SREBP-1c protein failed to activate lipogenic genes in adipocytes. This ineffectiveness of SREBP-1c in adipocytes was validated by four lines of

evidence: 1. Over-expression of active SREBP-1c had little effect on mRNA levels of lipogenic genes. 2. Transcriptional activity of SREBP-1c against FAS promoter measured by reporter analysis was faint. 3. Nuclear SREBP-1c failed to bind to the functional cis-element of FAS promoter in adipocytes. 4. Genetic disruption of SREBP-1 hardly affected mRNA levels of lipogenic genes. These results are further supported by the recent report that claims that mRNA levels of SREBP-1c do not coincide with the changes in adipose lipogenic gene expression (38,39).

As to why mature SREBP-1c poorly transactivates lipogenic genes in adipocytes, there can be two potential possibilities: 1. SREBP-1c cannot reach its binding sites on the promoters. 2. SREBP-1c properly binds to its cognate sites, but absence of required coactivator(s) or presence of corepressor(s) specific to adipocytes prevents the genes from being transcribed. Our results form ChIP assays favor the former hypothesis. SREBP-1 was able to access both -65 functional SRE/E-box and -150 non-functional SRE-1 sites in hepatocytes. In contrast, SREBP-1 could not bind to -65 SRE/E-box in

adipocytes although binding to -150 SRE-1 site was intact and similar to that observed in hepatocytes.

Another question our studies bring up is what transcription factors then regulate lipogenic gene expression in adipocytes. Unfortunately, we have few clues on this issue except that this factor should be functionally activated by refeeding. Upstream stimulatory factors-1/2 (USF-1/2) (40-42), carbohydrate response element-binding protein (ChREBP) (43) and CCAAT / enhancer binding protein (C/EBP) α (44) could be the potential candidates, but as yet unknown factor might be the answer. Further studies in adipocytes are imperative.

It has previously been reported by others that fatty acid synthase gene expression is increased by SREBP-1c through adenovirus-mediated gene transduction to 3T3-L1 adipocytes (45). In our experiments, FAS-Luc reporter acitivity was not elevated by SREBP-1c transfection (Fig. 5). The cause of discrepancy is not clear, but it is possible that our condition of transfection by electroporation method resulted in more physiological level of SREBP-1c

elevation compared to adenovirus-mediated over-expression.

The importance of LXR pathway in the regulation of lipogenesis and adipogenesis in adipocytes has been controversial; some reports have concluded that LXR stimulation leads to lipogenic gene activation (46,47), while others have presented the opposing data (35,48). The current data supported no impact of the LXR ligand on gene expression of lipogenic enzymes, but the discrepancy might originate from differences in the detailed experimental conditions such as variable glucose availability that might indirectly influence lipogenesis, considering LXR pathway is reported to upregulate glucose transport (49). Our main conclusion here is that adipocyte lipogenesis is independent of SREBP-1c even if upregulated by LXR activation, consistent with our previous and current data from SREBP-1-null mice.

In summary, we have found that lipogenic genes in adipocytes are primarily regulated by factor(s) other than SREBP-1. Although proteolytic activation of SREBP-1 is intact and the resulting active form of SREBP-1 is up-regulated whenever SREBP-1 gene is stimulated, it does not function as a

transactivator for lipogenic genes in adipocytes.

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Figure legends

Fig. 1 SREBP-1c activation by LXR agonists does not elevate lipogenic gene expression in mouse adipose tissue.

a,**b**, Northern blot (a) and real time PCR (b) analyses of liver and adipose tissue of mice treated with LXR agonist are shown. C57BL/6J mice (8-week-old, male, four animals for each group) were treated with LXR agonist T0901317 (50mg/kg) as indicated, and sacrificed 12h after the treatment in a 24h fasted state. Refed control mice were refed for 12h after 24h starvation. **a**, Total RNA (10μg) pooled equally was subjected to Northern blotting. A cDNA probe for 36B4 (acidic ribosomal phosphoprotein P0) was used to confirm equal loading. **b**, Real time PCR analysis of RNA isolated from liver and adipose tissue. mRNA levels of target genes were normalized to 36B4. Values are mean +- s.e.m. presented as fold changes relative to liver controls. *P<0.05, **P<0.01 vs controls. FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; SCD1, stearoyl-CoA desaturase 1; FACE, fatty acyl-CoA elongase;

ABCA1, ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1.

Fig. 2 SREBP-1c activation by LXR agonist does not induce lipogenic gene expression in rat adipose tissue.

Northern blot analysis of liver and adipose tissue of rats treated with LXR agonist. Wister rats (8-week-old, male, three animals for each group) were administered LXR agonist T0901317 (10mg/kg) or vehicle alone and sacrificed 12h after the treatment in a fasted state. Total RNA (10μg) pooled equally was subjected to Northern blotting. A cDNA probe for 36B4 (acidic ribosomal phosphoprotein P0) was used to confirm equal loading. The quantification results were obtained with BAS2000 system and normalized to the signal generated from 36B4 (acidic ribosomal phosphoprotein P0) mRNA, and the fold-changes against control were displayed above each blot. FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; S14, Spot 14; ABCA1, ATP binding cassette transporter A1.

Fig. 3 Post-translational activation of SREBP-1 is intact and mature protein is generated in adipose tissue.

Immunoblot analysis of SERBPs in nuclear proteins from liver (**a**, **b**) and adipose tissue (**b, c**). **a**, Mature forms of SREBP-1 and -2 in the liver are shown. C57BL/6J mice (8-week-old, male, four mice for each group) were administered LXR agonist T0901317 (50mg/kg) and/or RXR agonist LG100268 (30mg/kg) as indicated in the panel and sacrificed 12h after the treatment in a 24h fasted state. Refed control mice were refed for 12h after 24h starvation. **b**, Mature forms of SREBP-1 and -2 in liver and adipose tissue were quantified from Wister rats (8-week-old, male) administered T0901317 (10mg/kg) or vehicle alone and sacrificed 12h after the treatment in a fasted state. Nuclei were isolated from liver (pooled within groups) and adipose tissue (individually) and aliquots of nuclear proteins (20μg) were subjected to immunoblot analysis. SREBP-1c-specific antibody was also used. **c**, **d**, The effect of administering LXR agonist was compared with that of refeeding rats. **c**, Precursor and mature forms of

SREBP-1 in adipose tissue were visualized by immunoblots. **d**, Northern blotting of SREBP-1 and FAS. Wister rats (8-week-old, male, three animals for each group) were administered T0901317 (10mg/kg) or vehicle alone 12h before sacrifice.

Fig. 4 Northern and Western blot analyses of SREBP-1 in primary hepatocytes and adipocytes.

a, **b**, mRNA quantification of SREBP-1 and lipogenic genes in primary hepatocytes (**a**) and adipocytes (**b**). Hepatocytes isolated from rat livers (**a**) and adipocytes differentiated from rat preadipocytes (**b**) were incubated for 12h with or without T0901317 (10μM) and insulin (100nM). Incubation medium contained either 5mM glucose (LG, low glucose) or 25mM glucose (HG, high glucose). Total RNA (10μg) was subjected to Northern blotting. A cDNA probe for 36B4 (acidic ribosomal phosphoprotein P0) was used to confirm equal loading. The quantification results were obtained with BAS2000 system and normalized to the signal generated from 36B4 (acidic ribosomal phosphoprotein

P0) mRNA, and the fold-changes against control were displayed above each blot. FAS, fatty acid synthase; ABCA1, ATP binding cassette transporter A1. **c**, **d**, Precursor and mature forms of SREBP-1 proteins from primary hepatocytes (**c**) and adipocytes (**d**) are shown. Cells were stimulated in the same way as above and whole-cell lysates (50μg protein) were subjected to immunoblot analysis and the bands corresponding to precursor and mature SREBP-1 proteins are presented as indicated. These experiments were repeated twice, and reproducibility was confirmed.

Fig. 5 Transcriptional activity of SREBP-1c against FAS promoter is negligible in adipocytes.

Transcriptional activities of mature form SREBP-1c in HepG2 hepatoma cells (left panels) and 3T3-L1 adipocytes (right panels) were measured by luciferase reporter gene assay. Both types of cells were transfected with the indicated amount of mature SREBP-1c expression plasmids and mock plasmids (pcDNA3.1(+)) to adjust total DNA amount in combination with FAS-Luc or

SRE-Luc and pSV-β-gal (note that transfection methods and scales are different between HepG2 and 3T3-L1, and that the amounts of transfected DNA cannot be compared between them). Luciferase activities were measured and normalized to β-galactosidase activities. The values are expressed as fold changes against control (*i.e.* no SREBP-1c). These experiments were repeated twice, and reproducibility was confirmed.

Fig.6 SREBP-1 does not bind to functional cis-element on FAS promoter in adipocytes.

a, ChIP assays using antibody to SREBP-1 and amplifying DNA fragments from FAS promoter. Primary hepatocytes and adipocytes differentiated from preadipocytes were incubated for 12h with or without T0901317 (10μM). After cross-linking, the nuclei were isolated and sonicated and chromatin protein-DNA complexes were immunoprecipitated with control rabbit IgG or anti-SREBP-1 antibodies. The resultant DNA was analyzed by PCR with primers amplifying -150 SRE-1, -65 SRE/E-box and non-specific

region (from -1076 to -904) on FAS promoter. The results shown here are representative of three independent experiments.

b, Schematic representations of FAS promoter. The FAS promoter contains two SREBP-1 recognition sites: -150 SRE-1 and -65 SRE/E-box. ChIP assays revealed tissue-dependent recruitment of SREBP-1; SREBP-1 can bind to both functional SRE/E-box and non-functional SRE-1 in hepatocytes while the binding to SRE/E-box is prevented in adipocytes.

Fig. 7 Effects of genetic disruption of SREBP-1.

SREBP-1 disruption lowered lipogenic gene expressions as well as their responses to LXR activation in the liver (left panel), while it does not in adipose tissue (right panel). SREBP-1 knockout mice in C57BL/6J background (16-week-old, female, three mice for each group) and their control littermates were treated with T0901317 (50mg/kg) and analyzed by Northernblot. Mice were sacrificed 12h after the treatment in a 24h fasted state. FAS, fatty acid synthase; ABCA1, ATP binding cassette transporter A1.

Fig. 1

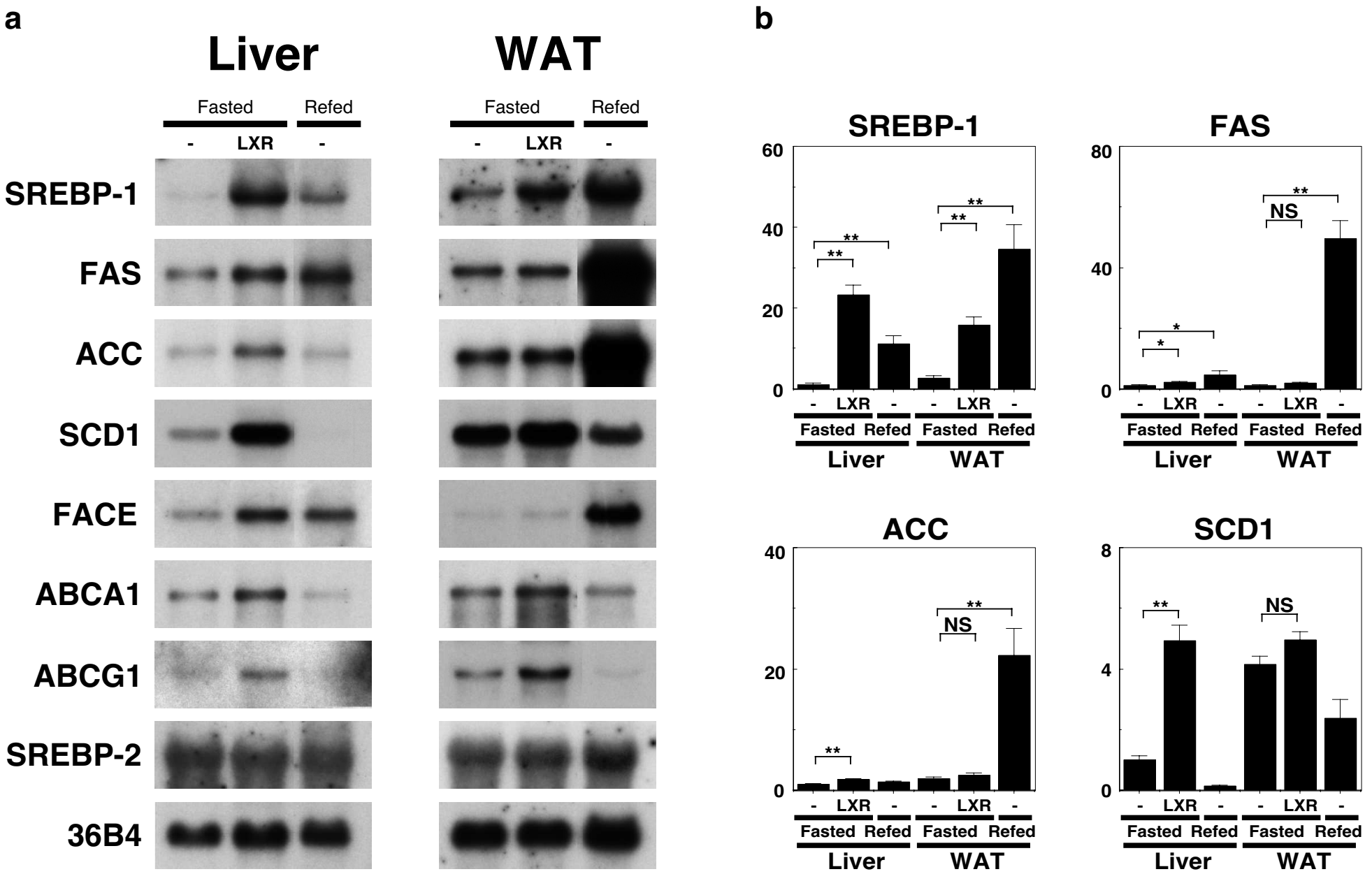


Fig. 2

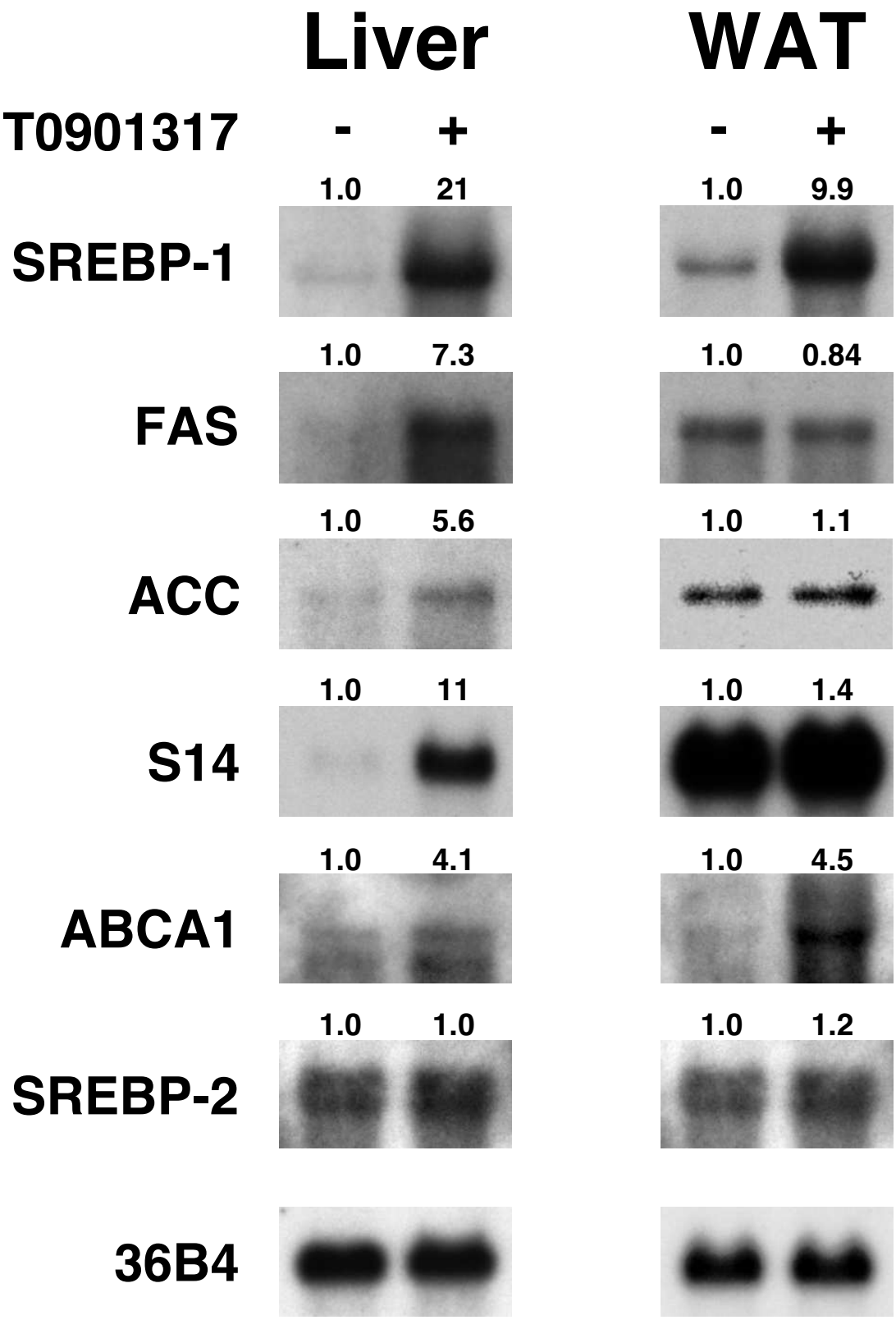


Fig. 3

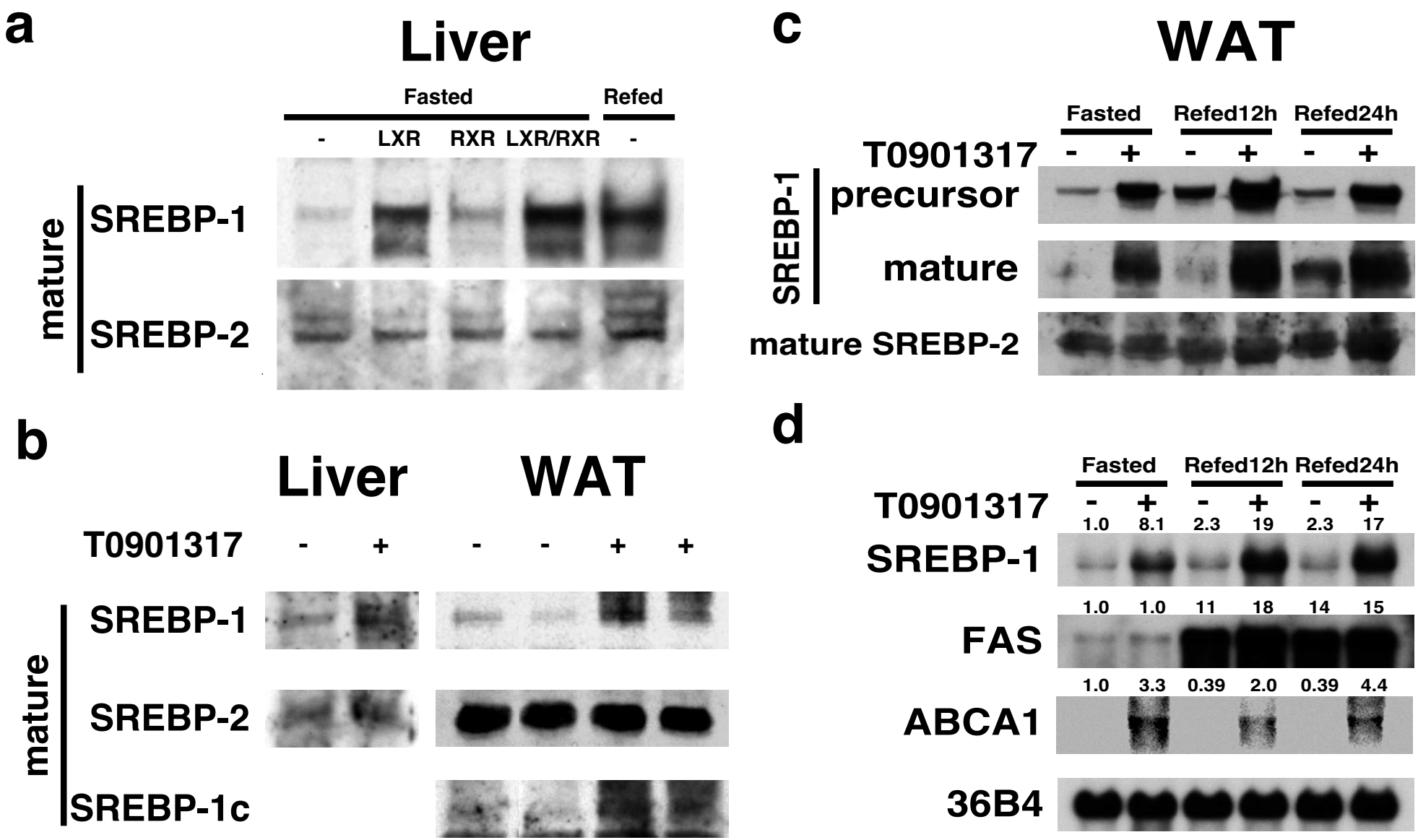


Fig. 4

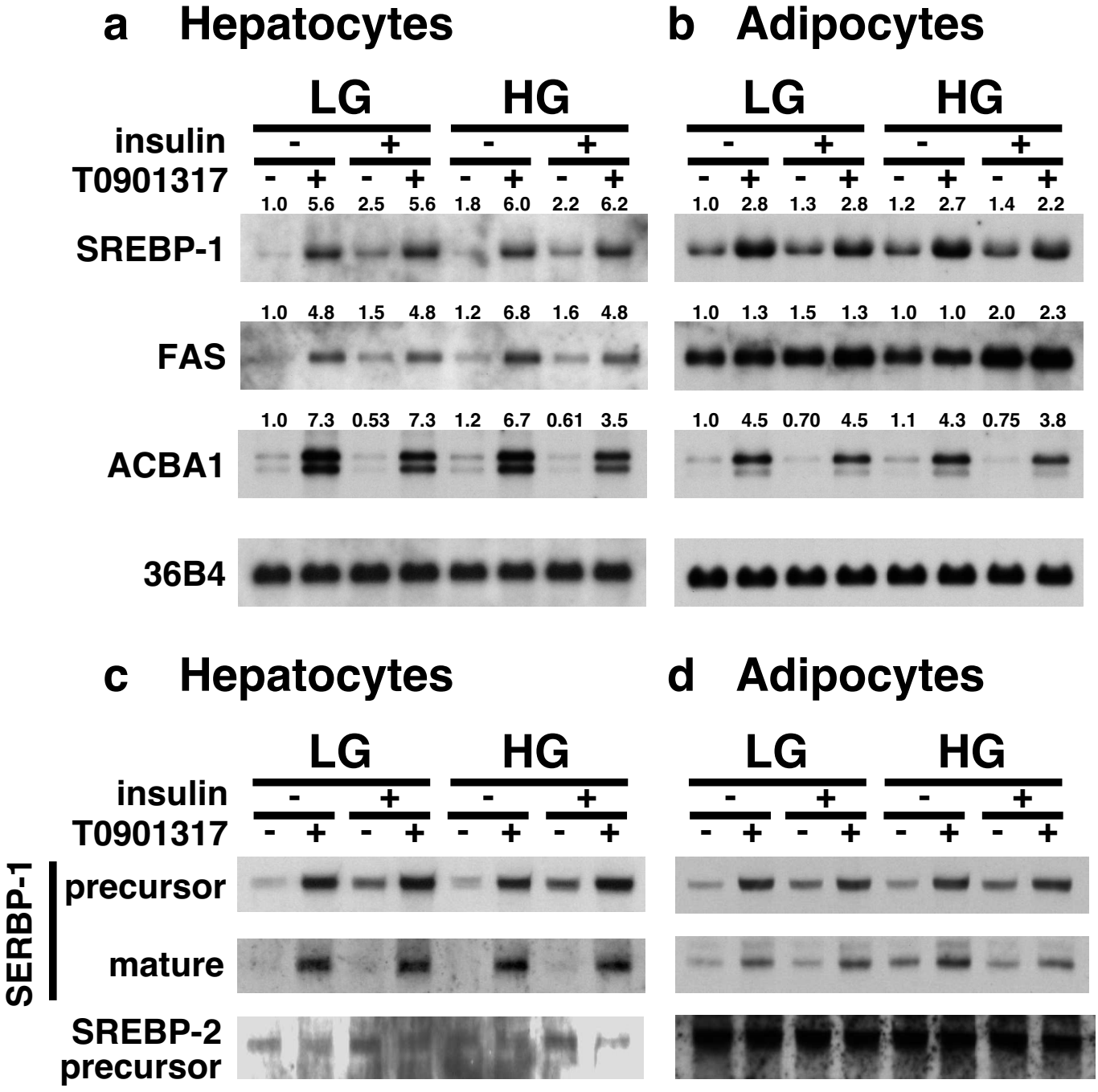


Fig. 5

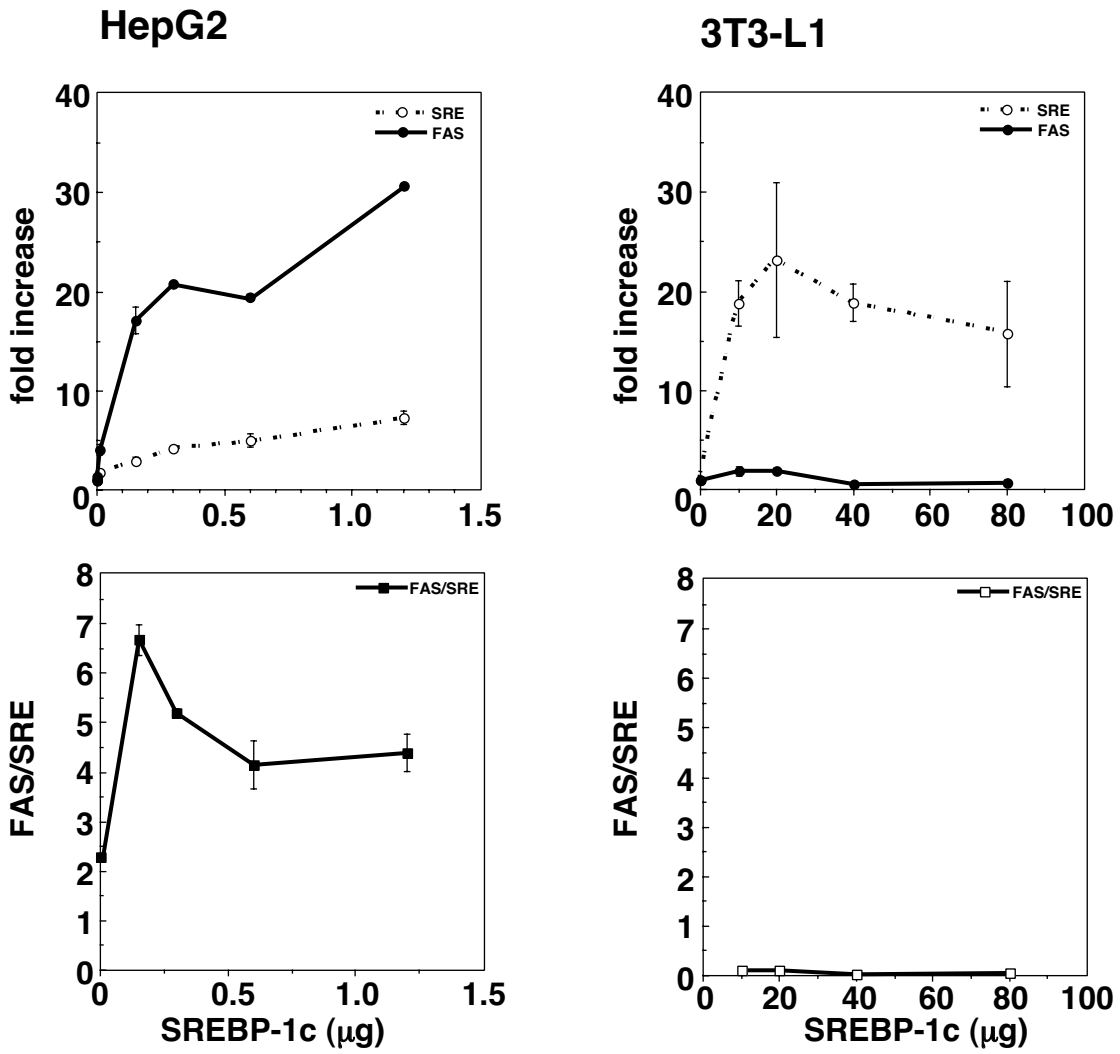
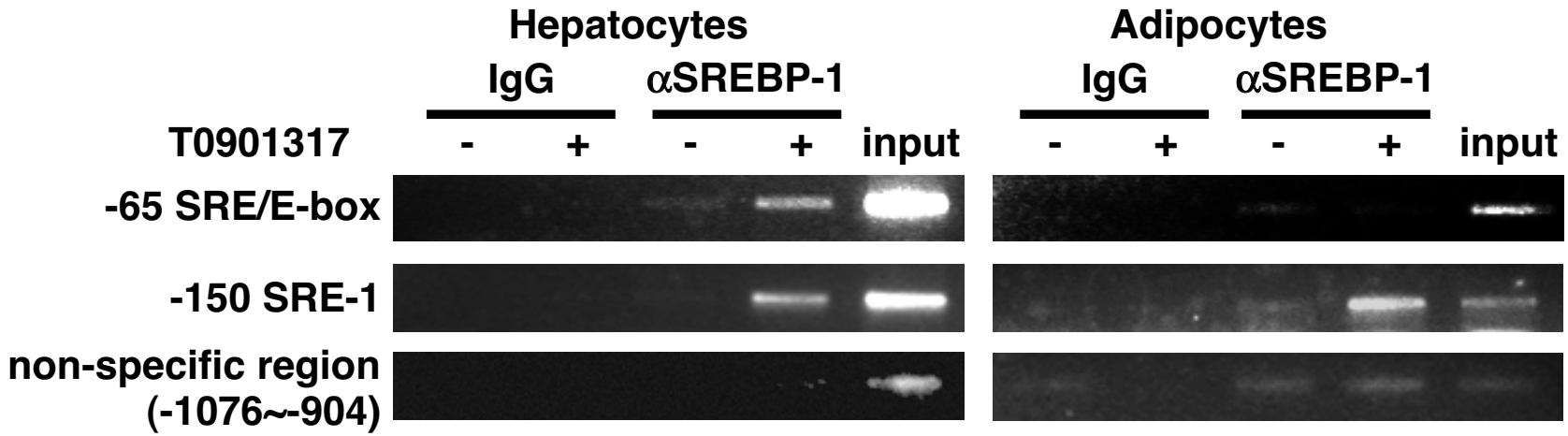


Fig. 6

a



b

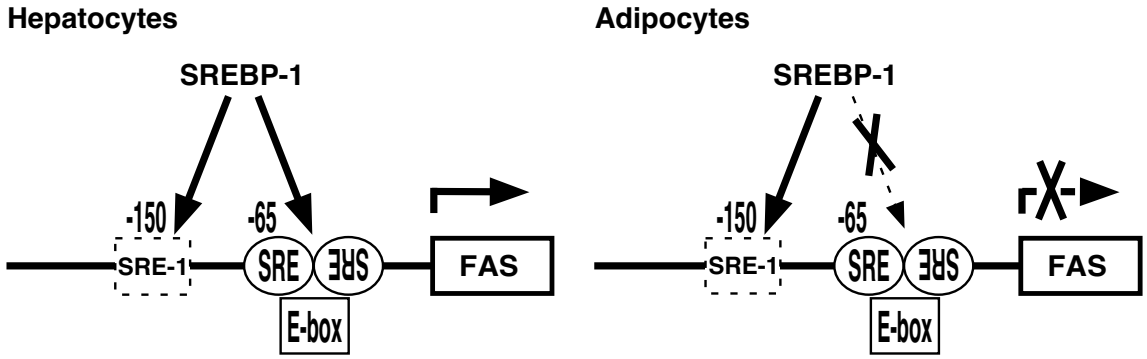


Fig. 7