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SREBPs suppress IRS-2-mediated insulin signalling in the liver

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Insulin receptor substrate 2 (IRS-2) is the main mediator of insulin signalling in the liver, controlling insulin sensitivity. Sterol regulatory element binding proteins (SREBPs) have been established as transcriptional regulators of lipid synthesis. Here, we show that SREBPs directly repress transcription of IRS-2 and inhibit hepatic insulin signalling. The IRS-2 promoter is activated by forkhead proteins through an insulin response element (IRE). Nuclear SREBPs effectively replace and interfere in the binding of these transactivators, resulting in inhibition of the downstream PI(3)K/Akt pathway, followed by decreased glycogen synthesis. These data suggest a molecular mechanism for the physiological switching from glycogen synthesis to lipogenesis and hepatic insulin resistance that is associated with hepatosteatosis.

Insulin has long been known to have a major role in the anabolic effects associated with carbohydrate and lipid metabolism. IRS proteins link insulin receptor binding to its final biological actions through a series of intermediate effectors in which the phosphatidylinositol-3-OH kinase (PI(3)K)/Akt cascade is the main pathway (reviewed in ref. 1). The roles of insulin signalling in the liver include activation of glycogen synthesis for energy storage, and suppression of hepatic glucose output by inhibiting phosphoenol pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Gene targeting experiments have demonstrated that hepatic insulin signalling for these effects is mediated mainly through IRS-2, rather than IRS-1 (refs 2, 3). After these acute actions of insulin on glucose metabolism, the liver initiates lipogenesis through induction of lipogenic enzymes. SREBPs are membrane-bound transcription factors that regulate genes involved in lipid synthesis. After sterol-regulated cleavage, the amino-terminal basic helix-loop-helix leucine zipper domains (nuclear SREBPs) are translocated to the nucleus, where they activate their target-gene promoters containing SREs (reviewed in ref. 4). Whereas SREBP-2 is crucial for the regulation of cholesterol synthesis genes, SREBP-1c controls gene expression of lipogenic enzymes (reviewed in refs 5, 6). Insulin and glucose are well known to stimulate lipogenesis, and SREBP-1c expression is nutritionally regulated in the liver and adipose tissue. Thus, SREBP-1c has been thought of as a mediator for insulin action on gene transcription.

Hepatic insulin resistance is an important pathophysiological feature of type-2 diabetes mellitus and metabolic syndrome. Decreased IRS-2 expression (and the resultant impairment of PI(3)K/Akt signalling) has been reported in the livers of animal models for insulin resistance, such as *ob/ob* mice and lipodystrophic mice[7,8]. These studies, in conjunction with the hepatic insulin resistance observed in IRS-2 knockout mice, led to the hypothesis that hepatic insulin resistance is mediated through inhibition of IRS-2. In these animals, SREBP-1c is highly expressed and is important for the formation of fatty liver[9].

In this study, hepatic SREBP-1 and IRS-2 gene expression were compared under various nutritional conditions. First, refeeding of normal mice with a high-sucrose/fat-free diet after fasting (a potent insulin-stimulating diet formula) markedly induced hepatic *SREBP-1c* expression from almost undetectable levels, as estimated by northern blotting (Fig. 1a). In contrast, *IRS-2* mRNA levels were very high during fasting and markedly reduced by refeeding, whereas *IRS-1* levels did not show a significant change. The suppression of hepatic *IRS-2* gene expression in a refed state was accompanied by a concomitant decrease in IRS-2 protein levels (Fig. 1b). Second, the chronic effects of forced SREBP expression on *IRS-2* gene expression were estimated in transgenic mice. Overproduction of nuclear-active SREBP-1a and -1c caused a marked reduction in *IRS-2* mRNA levels, whereas *IRS-1* mRNA levels were not much affected (Fig. 1c). Next, *IRS-2* mRNA levels were examined in the livers of SREBP-1-deficient mice, which have been reported to show impaired nutritional induction of lipogenic genes[10]. *IRS-2* mRNA was elevated in the livers of SREBP-1-deficient mice, compared with those of wild-type mice both at fasting and at refeeding (Fig. 1d). Increased *IRS-2* RNA levels were also detected in SREBP-1c-knockout mice[11]. Together, these data consistently show an inverse correlation between hepatic *SREBP-1* and *IRS-2* expression in different nutritional states, and raise the possibility that SREBP-1 might regulate *IRS-2* gene expression.

To test this hypothesis in a more acute and transient manner, the effects of SREBPs on the *IRS-2* gene were examined by infection of rat primary hepatocytes with adenoviral vectors expressing either

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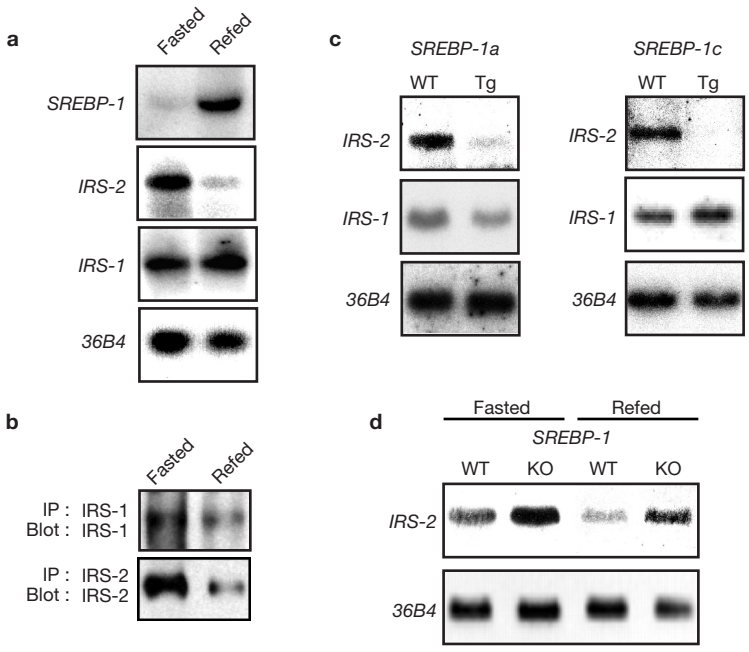

Figure 1 An inverse correlation between hepatic *IRS-2* and *SREBP-1* gene expression. Amounts of hepatic *IRS-2* and *SREBP-1* mRNA (**a**, **c**, **d**) and protein (**b**) were measured by northern blotting and by immunoblot analysis after immunoprecipitation, respectively, using the indicated animals. **a**, **b**, fasted or refed C57BL6 mice; **c**, fasted wild-type (WT) mice, and *SREBP-1a* or *SREBP*-1c transgenic (Tg) mice. **d**, Wild-type mice and *SREBP-1*-knockout (KO) mice in fasted and refed states.

SREBP-1a, -1c or -2. Transduction of cultured hepatocytes with increasing titres of the SREBP adenovirus resulted in dose-dependent expression of each SREBP isoform (Fig. 2a). The accumulation of nuclear SREBP-1c or SREBP-1a induced expression of *FAS* mRNA, a target gene for SREBP-1, while decreasing *IRS-2* mRNA levels in a dose-dependent manner. SREBP-2 also had a similar inhibitory action on *IRS-2* expression. The suppression of *IRS-2* mRNA levels through overproduction of SREBP-1c, -1a and -2 was accompanied by a reduction in IRS-2 protein levels (Fig. 2b). Interestingly, the level of IRS-1 protein was also reduced. To determine whether the SREBP-induced reduction of IRS-2 protein levels affects insulin signalling, insulin-mediated stimulation of IRS-2 tyrosine phosphorylation, Akt-Ser 473 phosphorylation and glycogen synthesis were measured. As estimated by immunoblotting after immunoprecipitation in insulin-stimulated hepatocytes, tyrosine phosphorylation of IRS-2 and Ser 473 phosphorylation of Akt were reduced by expression of SREBPs in a dose-dependent manner (corresponding to reduced IRS-2 protein levels), whereas Akt protein levels were not affected (Fig. 2c). This inhibition caused a resultant suppression of glycogen synthesis under both basal and insulin-induced conditions (Fig. 2d). In contrast, but consistent with a well-established theory, SREBPs activated fatty acid synthesis, which is a late metabolic effect of insulin (Fig. 2e). Similar results were observed in SREBP-1a-expressing hepatocytes, but with a slightly more potent inhibition. These data indicate that activation of SREBPs markedly suppresses hepatic insulin signalling through repression of *IRS-2*. The diminished level of IRS-1 protein could also contribute to impaired insulin signalling caused by adenovirus-mediated overproduction of SREBPs in primary hepatocytes, although the mechanism is currently unknown.

To investigate mechanisms for SREBP-mediated suppression of *IRS-2* expression, promoter analysis of the 5′-flanking region of the human *IRS-2* gene was performed in transfection studies in rat primary hepatocytes. The *IRS-2* gene promoter contains an IRE through which chronic insulin treatment down-regulates *IRS-2* expression[12]. Intriguingly, a highly probable SREBP-binding site was identified in a region that overlaps with the IRE[13] (Fig. 3a). In luciferase reporter assays, IRS-2 promoter activity was consistently suppressed by insulin and increased by dexamethasone, as previously described[14] (Fig. 3b). Transfection of nuclear SREBP-1a and -1c suppressed basal IRS-2 promoter activity, indicating that SREBPs repress IRS-2 at the transcriptional level (Fig. 3c). Similar consensus IRE sequences in the promoters of genes involved in hepatic glucose output, such as *PEPCK* and *G6Pase* genes, have been reported[14]. Forkhead (FKH) proteins such as FKHR and FKHRL1 (also known as FOXO1 and FOXO3a, respectively) have been shown to be crucial for the regulation of PEPCK and G6Pase through their IREs[15,16]. Insulin-mediated repression of these genes occurs through PI(3)K/Akt-mediated phosphorylation of the FKHR proteins, resulting in their translocation out of nucleus[17,18]. Therefore, it is highly conceivable that the FKH proteins could activate *IRS-2* gene expression through its IRE in a similar manner. Supporting this prediction, overexpression of FKHRL1 increased IRS-2 promoter activity (Fig. 3c). Even in this FKHRL1-induced condition, IRS-2 promoter activity was efficiently suppressed by co-transfection of SREBP-1a and -1c. Overproduction of SREBP did not affect FKHRL1 expression (Fig. 3c, inset). Similar results were obtained in HepG2 cells, where FKHRL1 and FKHR were shown to activate, and SREBPs were shown to inhibit, the IRS-2 promoter in a competitive manner (Fig. 3d, e). Competition between SREBP-1 and FKH was further confirmed with mutants of FKHR (Fig. 3f, g). FKHR3A, in which three Akt-mediated serine-phosphorylation sites were substituted for alanine, is resistant to nuclear exclusion by Akt and is therefore constitutively active[19]. The FKHR-mediated activation of the IRS-2 promoter was enhanced by this mutation, but still robustly suppressed by co-expression of SREBP-1. A mutation that disrupts binding to IRE ($FKHR^{H212R}$; ref. 20) abolished activation of IRS-2 by FKH and

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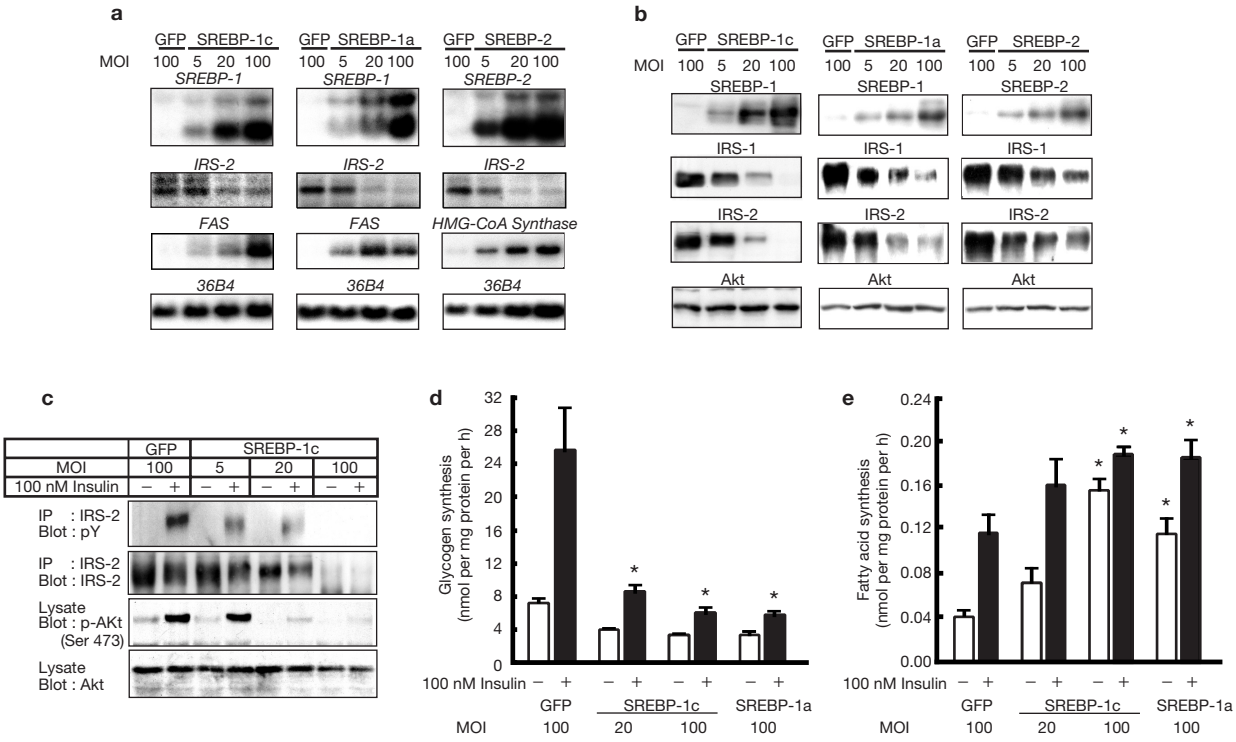

Figure 2 Nuclear SREBPs reduce *IRS-2* expression and insulin signalling in primary hepatocytes. (**a**) Expression of *IRS-2*, *FAS* and *HMG-CoA synthase* in adenovirus-mediated SREBP-overexpressing hepatocytes, as estimated by northern blotting. Hepatocytes were infected with green fluorescent protein (GFP), SREBP-1c, SREBP-1a and SREBP-2 adenovirus at the indicated multiplicity of infection (MOI). (**b**) Protein levels of IRS-1, IRS-2 and Akt were examined by immunoblotting of hepatocytes expressing SREBP-1c, -1a, and –2. (**c**) Insulin-induced tyrosine phosphorylation of IRS-2 and serine (Ser) phosphorylation of Akt in SREBP-1c-expressing hepatocytes. (**d**, **e**) Nuclear SREBPs suppress the hepatic insulin effect, as measured by glycogen synthesis. Insulin-induced glycogen (**d**) and fatty acid (**e**) synthesis from U-^{14}C-glucose were measured in SREBP-1c- and SREBP-1a-expressing hepatocytes. Error bars represent standard errors from three independent experiments. *$P < 0.05$, compared with GFP-treated cells.

FKH3A (Fig. 3f). PGC-1 (peroxisome proliferative activated receptor-γ co-activator 1) was recently established as a co-activator for FKH-mediated activation of gluconeogenic genes[21]. As shown in Fig. 3h, cotransfection of PGC-1 enhanced FKHRL1-mediated activation of the IRS-2 promoter, suggesting that PGC-1 is involved in transactivation of IRS-2 by FKHs. SREBP-1 suppressed this PGC-1-mediated activation.

On the basis of these results from luciferase assays, direct binding of SREBP to the IRS-2 promoter was examined. Using different probes covering the region neighbouring SRE/IRE (see Supplementary Information, Fig. S1a), the binding site was determined and tentatively designated as IRS-2-SRE (see Fig. 3a and Supplementary Information, Fig. S1b). Gel-shift assays demonstrated that *in-vitro*-translated SREBP-1a, -1c and -2 proteins bound specifically to this region with a higher affinity than they do to an authentic low-density lipoprotein receptor (LDLR)-SRE (see Supplementary Information, Fig. S1c). Both FKH proteins were shown to bind specifically to the IRE-containing region (see Supplementary Information, Fig. S1d, e), as well as FKHR (data not shown). Although IRS-2-SRE and IRS-2-IRE partially overlap, SREBPs and FKHs bind to this region in a different way, because the mutation that abolishes FKH binding did not affect SREBP binding (see Supplementary Information, Fig. S1e). Fig. 3i shows luciferase assays with the IRS-2 promoter construct in which this newly identified IRS-2-SRE was disrupted, but in which the IRS-2-IRE remained. The construct retained the activation by FKHRL1, but lost the suppression by SREBP-1c on the IRS-2 promoter activity. These data demonstrate that IRS-2-SRE is responsible for the repression of IRS-2 by SREBP-1.

When both FKHRL1 and SREBP-1c proteins were added competitively to the IRS-2-SRE/IRE region for gel-shift assays, SREBP binding replaced FKHRL1 binding in a dose-dependent manner (Fig. 4a). This indicates that SREBP can prevent FKHRL1 from binding to the IRS-2 promoter. In a more physiologically relevant experiment, nuclear extracts from mouse livers were used to estimate binding to the IRS-2-SRE/IRE region (Fig. 4b, c). By the time of refeeding, nuclear SREBP-1c protein accumulated and FKHR proteins declined, as shown by immunoblotting (Fig. 4b). Nuclear extracts from refed mouse livers shifted the IRS-2-SRE probe more prominently than those from fasted mice (Fig. 4c). The strong signal was almost exclusively specific to SREBP-1c, as shown with the anti-SREBP antibody. Finally, to confirm direct binding of SREBP to the IRS-2 promoter *in vivo*, chromatin immunoprecipitation (ChIP) assays were performed on hepatic nuclei from fasted and refed mice (Fig. 4d). These results support the idea that SREBP-1 binds to the IRS-2-SRE in liver nuclei. Reflecting nutritional induction of nuclear SREBP-1c, the signal was highly enhanced by refeeding. In contrast, binding of FKH to the IRS-2-IRE exhibited a completely reciprocal change, suggesting that SREBP-1c prevents binding of FKH to the IRS-2 promoter. The ChIP assay with the PGC-1 antibody also detected the IRS-2 promoter, supporting our observations that this co-activator could be involved in the transcription of IRS-2 (Fig. 4d).

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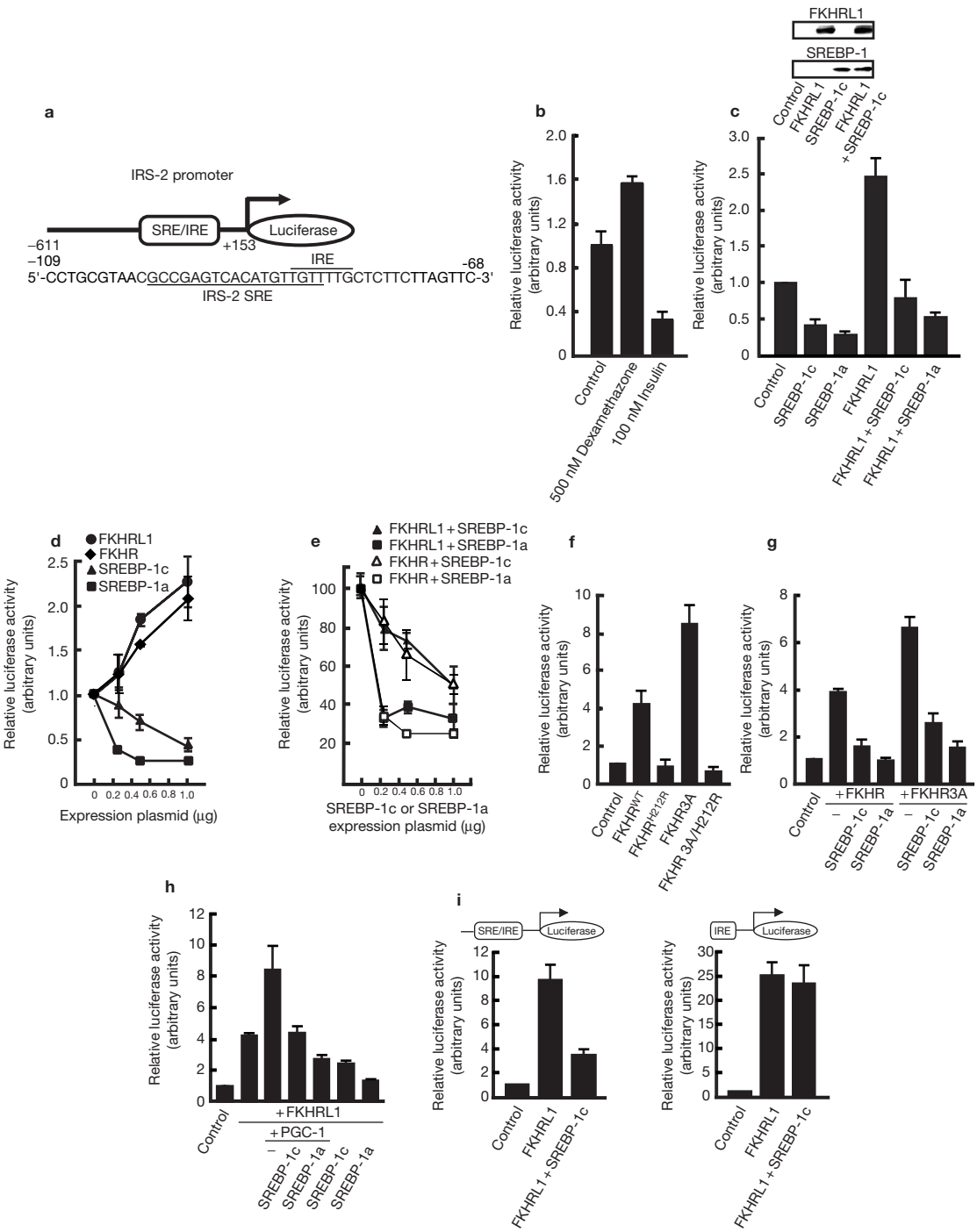

Figure 3 Nuclear SREBP-1c and SREBP-1a inhibit IRS-2 promoter activity competing with FKHs. (**a**) The human IRS-2 promoter-luciferase reporter plasmid was used for transfections. The SRE/IRE region contains an SREBP-binding site (SRE) and insulin response element (IRE). (**b**) Dexamethasone-mediated induction and insulin-mediated inhibition of IRS-2 promoter activity in rat primary hepatocytes. (**c**) Effects of SREBP-1c, SREBP-1a and FKHRL1 on IRS-2 promoter activity in primary rat hepatocytes. Immunoblotting of the cotransfected SREBP-1c and FKHRL1 proteins is also shown (inset). (**d**) Dose-dependent effects of SREBP-1c, SREBP-1a, FKHRL1 and FKHR on IRS-2 promoter activity in HepG2 cells. (**e**) Inhibition of FKHRL1- or FKHR-induced IRS-2 promoter activity by SREBP-1c and SREBP-1a in HepG2 cells. (**f**) Effects of wild-type and mutant FKHR. $FKHR^{H212R}$ (ref. 20), DNA-binding-defective mutant; FKHR3A (ref. 19), phosphorylation-deficient, and thus constitutively active, mutant. (**g**) Effects of FKHR3A mutants on IRS-2 promoter activity and SREBP inhibition. (**h**) Effects of PGC-1 on FKHRL1-induced IRS-2 promoter activity and SREBP-1 inhibition. (**i**) IRS-2-SRE is responsible for repression of SREBP. HepG2 cells were transfected with the indicated expression plasmids, IRS-2 promoter luciferase constructs and a renilla luciferase plasmid as a reference, before a 24-h incubation in the presence or absence of the indicated hormones. Luciferase assays were then performed. The firefly luciferase activity of transfectants was normalized to renilla luciferase activity as an internal reference. Error bars represent standard errors for three independent experiments.

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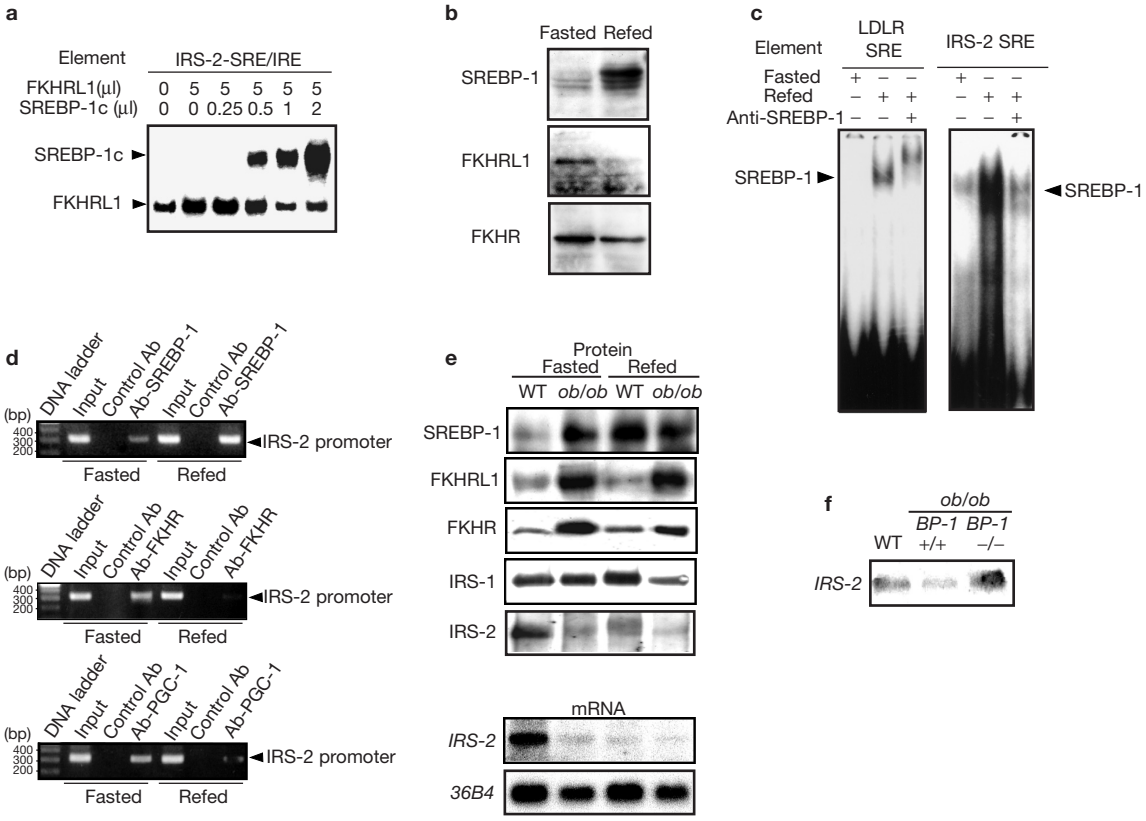

Figure 4 SREBP-1c in hepatic nuclear extracts dominates binding to the IRS-2-SRE. (**a**) Competition between SREBP-1c and FKHRL1 in binding to IRS-2-SRE/IRE. Both *in vitro*-translated proteins were used in the indicated amounts for an EMSA assay with the IRS-2-SRE/IRE probe. (**b**) The amounts of SREBP-1c and forkhead proteins in hepatic nuclei from fasted or refed normal mice. Liver nuclear extracts were subjected to immunoblotting with the indicated antibodies. (**c**) Endogenous SREBP-1c from hepatic nuclear extracts binds to IRS-2-SRE. Binding reactions were performed by incubation with hepatic nuclear extracts from fasted or refed mice using IRS-2-SRE (probe G in Supplementary Information, Fig. S2a) or LDLR-SRE as probes. An anti-SREBP-antibody was used to confirm the specificity of the binding. (**d**) SREBP-1c and FKH proteins bind to the IRS-2 gene promoter *in vivo*. ChIP assays were performed for livers from mice in fasted and refed states using anti-SREBP-1 (top), anti-FKHR antibody (middle), anti-PGC-1 antibodies (bottom), or IgG as a negative control. PCR reactions were conducted with primers for the IRS-2 promoter region containing IRS-2-SRE/IRE. (**e**) SREBP-1, FKH and IRS protein levels in hepatic nuclei and *IRS-2* mRNA level from wild-type (WT) and leptin-deficient *ob/ob* mice in fasted and refed states. (**f**) Hepatic *IRS-2* mRNA level of wild-type, *ob/ob* and *ob/ob-SREBP-1* double-knockout mice.

In this study, we demonstrate that the competition between SREBP-1c and FKH proteins is observed *in vivo* in pathophysiological livers. In leptin-deficient *ob/ob* mice, extreme insulin resistance results in dysregulation of both SREBP-1c and FKH proteins in the liver. Hepatic nuclear SREBP-1c is unsuppressed at fasting, whereas nuclear FKH proteins are resistant to nuclear exclusion by insulin treatment and remain in the nucleus even in a fed state (Fig. 4e). Therefore, hepatic nuclear extracts from these insulin-resistant mice contained an abundance of both FKH and SREBP-1c proteins in either fasted or refed states. Nevertheless, hepatic *IRS-2* mRNA and protein were markedly decreased, supporting the observation that SREBP-1c dominates binding to the IRS-2 promoter. Furthermore, the low level of hepatic *IRS-2* mRNA in insulin-resistant *ob/ob* mice was restored by deletion of SREBP-1, as shown in *ob/ob SREBP-1* double-knockout mice[9] (Fig. 4f). In conjunction with the results from the luciferase assays, these data illustrate that SREBPs bind strongly to the IRS-2-SRE and efficiently inhibit binding of trans-activators (FKHs) to the IRS-2-IRE to repress *IRS-2* expression and sustain insulin resistance.

Here, we clearly demonstrate that SREBPs — traditionally characterized as transcriptional activators for lipid synthesis — also regulate expression of *IRS-2*, the major hepatic insulin signal mediator. An abundance of nuclear SREBPs, as observed by a high enrichment of SREBP-1c in the liver nuclei of refed or insulin-resistant mice, causes efficient repression of *IRS-2* expression, resulting in impaired PI(3)K/Akt-mediated insulin signalling. The molecular mechanism for SREBP-mediated inhibition of IRS-2 is transcriptional repression through direct binding to the IRS-2-SRE in competition with putative trans-activators, including FKHRL1, FKHR and HNF3 (data not shown). Although it is possible that other factors could be involved in *IRS-2* expression through the IRS-2-IRE, the high affinity of SREBPs for the IRS-2-SRE seems to function in a dominant manner to remove their activators from the IRE. Our data also suggest that PGC-1 could be involved in transcription of IRS-2 by interacting with FKHs on the IRS-2-IRE, and that SREBP represses IRS-2 expression through inhibition of PGC-1 recruitment because SREBP-1 does not interact with PGC-1 (ref. 22).

Reciprocal effects of FKH and SREBP-1c on IRS-2 promoter activity could provide a mechanism for nutritional regulation of hepatic insulin sensitivity and expression of metabolic genes by these factors (see Supplementary Information, Fig. S2). In insulin-depleted or fasted states, high nuclear FKH and low nuclear SREBP-1c assure high expression of *IRS-2*, presumably to sensitize hepatocytes for

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subsequent calorie intake and insulin influx (see Supplementary Information, Fig. S2a). After food intake, glucose consumption and glycogen synthesis proceed according to insulin signalling pathways. As feeding proceeds, insulin signalling stimulates phosphorylation of FKHs through PI(3)K/Akt activation and excludes them from the nucleus, reducing *PEPCK* and *G6Pase* gene expression (see Supplementary Information, Fig. S2b). Meanwhile, nuclear SREBP-1c is accumulated and lipogenesis is initiated. High SREBP-1c and low FKHs in the liver nuclei are prominent in the fully fed state, resulting in a further reduction of *IRS-2* expression and insulin sensitivity (see Supplementary Information, Fig. S2c). Thus, the physiological consequence of IRS-2 suppression by SREBP-1c is a transitional switch from glycogen synthesis to lipogenesis, and could be considered as a feedback response to prolonged insulin/glucose action. It has been reported that chronic exposure of hepatocytes to insulin represses IRS-2 promoter activity through the IRE, implying that prolonged hyperinsulinemia causes secondary insulin resistance[12]. Suppression of IRS-2 in the refed state mimics this situation *in vivo*.

This study also suggests that SREBP-1c may be involved in the pathogenesis of hepatic insulin resistance. High amounts of hepatic SREBP-1c were observed in numerous insulin-resistant animals[8,23]. Excess carbohydrate intake, an exacerbating factor for insulin resistance, also increases hepatic SREBP-1c expression. Our studies demonstrate that a high level of SREBP-1c results in further hepatic insulin resistance through suppression of *IRS-2* expression, as well as formation of fatty liver and production of triglyceride-rich VLDL that could be converted to atherogenic remnant particles. These are the main features of insulin-resistant animals, and clinical components of the metabolic syndrome. Concomitantly, nuclear FKHs sustain gluconeogenesis and could cause the onset of diabetes (Fig. 4e; also see Supplementary Information, Fig. S2d). Resultant hyperglycemia would also enhance further glucose-mediated induction of SREBP-1c, forming an unfavourable positive feedback loop of insulin resistance[24]. The current studies suggest that SREBP-1c could be central to the pathogenesis of hepatic insulin resistance and represent a potential therapeutic target. □

METHODS

Materials. Anti-IRS-1 and anti-IRS-2 antibodies were purchased from Upstate Biotechnology Institute (Lake Placid, NY), anti-FKHRL1 and anti-FKHR antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Affinity purified antibodies against Akt and phosphorylated Akt (Ser 473) were obtained from Cell Signaling Technology Inc. (Beverly, MA). The anti-phosphotyrosine monoclonal antibody (4G10) was a kind gift from T. Asano. The adenovirus expression vector kit (AdEasy system) was from B. Vogelstein and T.C. He.

Animals. Male mice (C57BL/6J) were obtained from CLEA Japan (Tokyo, Japan). For fasting and refeeding, mice were fasted for 24 h and then fed a high-sucrose/fat-free diet for 12 h, as previously described[10]. *SREBP-1* knockout mice and *ob/ob SREBP-1* double-knockout mice are previously described[9,10]. *SREBP-1a* and *SREBP-1c* transgenic mice, and the wild-type littermates, were put on a high-protein/low-carbohydrate diet for two weeks to induce transgene expression and then fasted for 12 h before sacrifice[25,26]. *ob/+* mice on a C57BL/6 background were purchased from Jackson Laboratories (West Grove, PA) and intercrossed to obtain leptin-deficient *ob/ob* mice and wild-type mice.

Isolation and culture of hepatocytes. Primary hepatocytes were isolated from male Sprague-Dawley rats (200–300 g, Japan Clea, Tokyo, Japan). Cells were resuspended in DMEM containing penicillin and streptomycin supplemented with 10% foetal bovine serum (FBS) and 30 nM dexamethasone before being seeded on 100-mm collagen-coated dishes at a final density of 4×10^4 cells cm^{-2}. After incubation for 4 h to allow attachment, the medium was replaced with serum-free DMEM containing adenovirus.

Preparation of recombinant adenovirus. cDNAs encoding the active amino-terminal fragment of human SREBP-1c (amino acids 1–436), SREBP-1a (amino acids 1–460) and SREBP-2 (amino acids 1–460) were integrated into the adenovirus vector[27]. SREBP adenoviral vectors were propagated in 293 cells and purified by caesium chloride density centrifugation.

Glycogen and fatty acid synthesis. After adenoviruses infection, rat primary hepatocytes were incubated for 24 h in DMEM containing 10% FBS and 30 nM dexamethasone before a 4-h incubation in serum-free DMEM. Cells were then incubated for 3 h in DMEM containing 5.6 mM glucose and 2.5 μCi ml^{-1} U-^{14}C-glucose in the presence or absence of 100 nM insulin. Glycogen and fatty acid synthesis were determined by measuring the incorporation of U-^{14}C-glucose into glycogen and fatty acid, as described previously[28].

Plasmids. Human SREBP-1c, SREBP-1a, SREBP-2, FKHRL1 and FKHR cDNAs were cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) containing a CMV promoter. $FKHR^{H212R}$, FKHR3A and FKHR3A-H212R expression plasmids were previously described[19,20]. The human IRS-2 promoter (base pairs −611 to +153, relative to the transcriptional start site) was amplified by PCR[12,29] and sub-cloned into the pGL3-basic luciferase vector (Promega, Madison, WI).

Northern blotting. After adenovirus infection, rat primary hepatocytes were incubated for 24 h. The cDNA probes for SREBP-1, SREBP-2, FAS, HMG-CoA synthase and acidic ribosomal phosphoprotein PO (36B4) were prepared as previously described[10]. The cDNA probes for mouse IRS-1 and IRS-2 were prepared by RT-PCR using mouse liver total RNA as a template. The 5′- and 3′-PCR primers used to generate these probes were as follows: IRS-1, 5′-CGCCTGGAGTATTATGAGAACGAG-3′ and 5′-TGATGGGAAATGGTAGGAGATGTG-3′ (GenBank accession number: X69722); IRS-2, 5′- CCTTAGGAGTGGTGGTCCCAATAG-3′ and 5′-TGACGGTGGTGGTAGAGGAAAAG-3′ (GenBank accession number: AF090738).

Transfections and luciferase assays. HepG2 cells were grown in serum-free DMEM supplemented with 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37 °C in 12-well plates for 6 h before transfection. Cells were transfected with 50 ng each of a pIRS-2 luciferase plasmid and a pRL-SV40 plasmid (Promega) using the SuperFect reagent (Qiagen, Valencia, CA). Rat primary hepatocytes were placed in serum-free DMEM and transfected with 1.5 μg of pIRS-2-Luciferase and 0.5 μg of pRL-SV40 using the Lipofectin reagent (Invitrogen). An SREBP-1c or SREBP-1a (2 μg) expression plasmid was cotransfected with or without an FKHRL1 or FKHR expression plasmid (2 μg). After a 24-h incubation, the amount of firefly luciferase activity in transfectants was measured and normalized to the amount of renilla luciferase activity.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from livers as previously described[10]. Nuclear SREBP-1c, SREBP-1a, SREBP-2 and FKHRL1 proteins were generated from expression vectors using a coupled *in vitro* reticulocyte transcription-translation system (Promega). Double-stranded oligonucleotides used in gel mobility shift assays were directed against the SRE of the LDLR promoter, 5′-TTTGAAAATCACCCCACTGCA-3′ and IRS-2 SRE/IRE promoter, 5′-CCTGCGTAACGCCGAGTCACATGTTGTTTTGCTCTTC-3′ (G probe in Supplementary Information, Fig. S1) or 5′-GCCGAGTCACATGTTGTTTTGCTCTTCTTAGTTC-3′ (C probe in Supplementary Information, Fig. S1). *In vitro* synthetic protein lysate (0.25–5-μl) or nuclear extracts (1.0 μg) were incubated in a reaction mixture as previously described. DNA–protein complexes were resolved on a 4.0% polyacrylamide gel.

Immunoblotting. Proteins were extracted from rat primary hepatocytes or immunoprecipitated from livers of fasted or refed mice as described previously[10].

ChIP assay. Hepatic nuclei were prepared from livers as previously described[10]. ChIP assays were performed as described by the manufacturer (Upstate Biotechnology, Lake Placid, NY) with some modifications. Cross-linking between transcription factors and chromatin was achieved by adding formaldehyde (final concentration 1%) to hepatic nuclei for 15 min for 37 °C. Chromatin solutions were sonicated and incubated with 15 μg of anti-PGC-1 antibody (H-300; Santa Cruz), anti-SREBP-1 antibody (H-160, Santa Cruz),

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anti-FKHR antibody[30], or with control IgG, and rotated overnight at 4 °C. Immune complexes were collected with salmon-sperm DNA/protein G–sepharose slurry for 4 h with rotation, washed and then incubated at 65 °C for 6 h for reverse crosslinking. Chromatin DNA was extracted with phenol-chloroform, precipitated with ethanol, resuspended in water and subjected to PCR analysis. To amplify the IRS-2 promoter regions containing SRE, the following primer sets were used: 5′-TCCAGCAAACAGATGCTGAC-3′ and 5′-CATTTAACTTGACTCTCCAGTGAA CT-3′. After amplification, PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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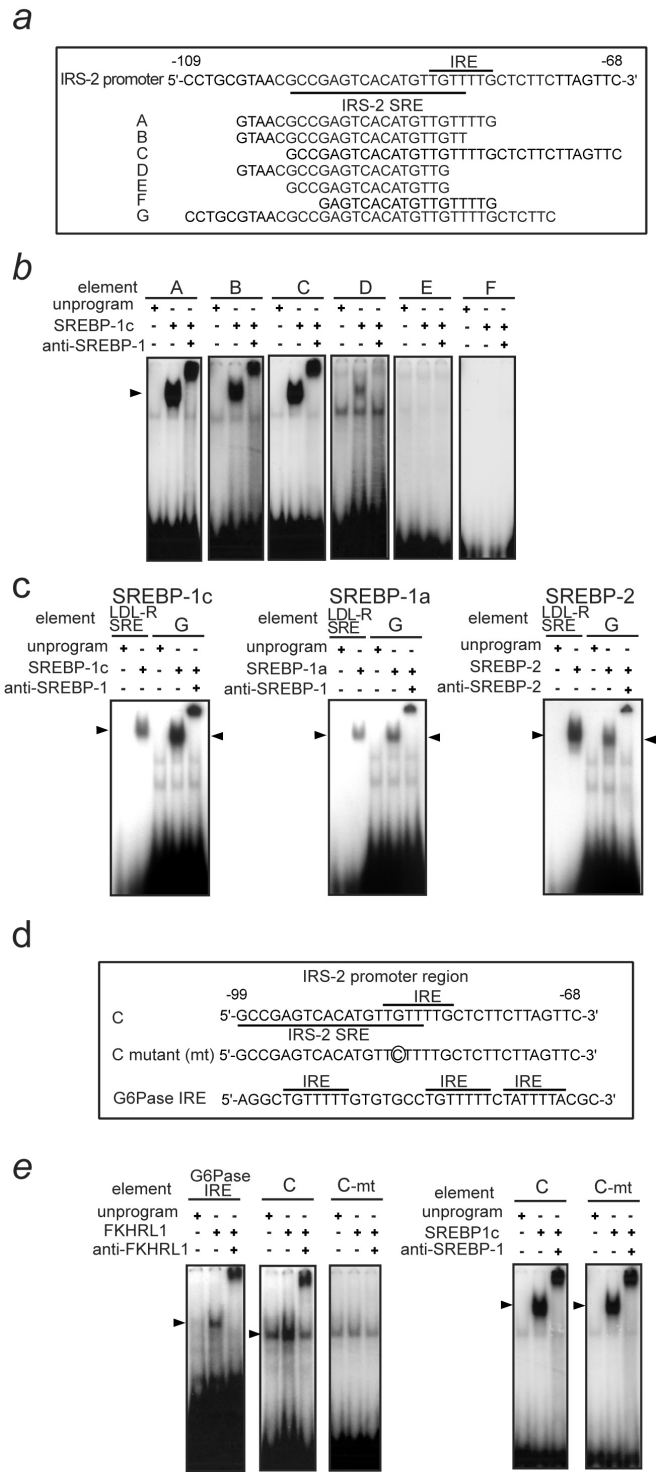

Figure S1. Identification of SREBP binding site (IRS-2-SRE) and FKH binding to IRS-2-IRE in the IRS-2 promoter. ***a,*** Neighboring DNA sequences around a newly identified SREBP binding site (IRS-2-SRE) and insulin response element (IRE) in human IRS-2 promoter and various oligonucleotides used for gel shift assays to determine the IRS-2-SRE (A-F). ***b,*** *Identification of IRS-2-SRE* ***c***, Binding of each SREBP isoform (-1c, -1a, and -2) to IRS-2-SRE (G) and LDLR-SRE. ***d***, A mutation in IRS-2-SRE/IRE (TGTTTTG→ T***C***TTTG) that abolishes FKH binding was introduced into C probe (indicated by an oval in C mt). *e*, DNA binding assays performed with FKHRL1 and SREBP1c by using probes as IRS-2-SRE/IRE (C), IRS-2-SRE/IRE-mutation (C-mt), or G6Pase IRE. ***e,*** IRS-2-SRE as the element responsible for SREBP repression. The indicated IRS-2 promoter constructs with or without IRS-2-SRE were compared in luciferase assays in HepG2 cells. In EMSA assays, each SREBP and FKHRL1 proteins were in vitro-translated. The specific binding of the protein/DNA complex (arrowhead) was confirmed by super-shift induced by the indicated antibodies.

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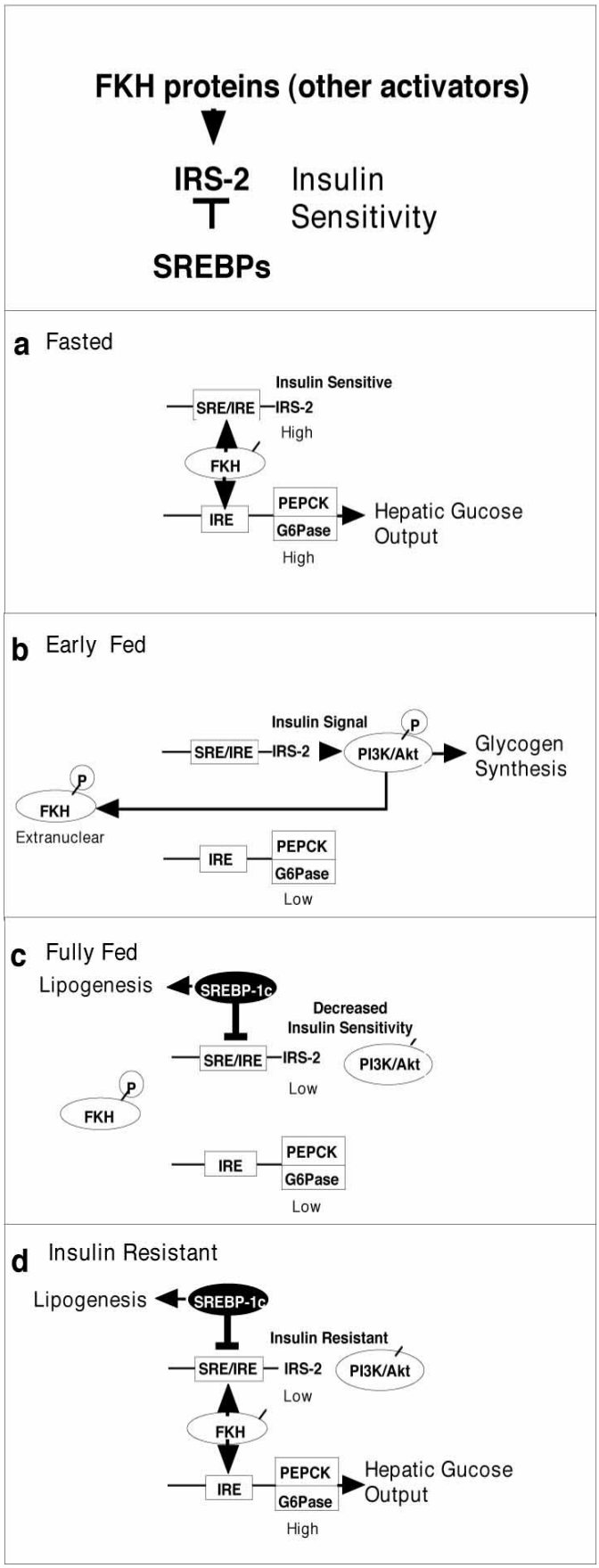

Figure S2. Reciprocal regulation of IRS-2 expression by SREBP-1c and FKHs for hepatic insulin signaling in feeding cycles (a-c) and insulin resistant state (d) Activation by FKHs and inhibition by SREBP-1c of IRS-2 expression could illustrate physiological and pathophysiological regulation of insulin sensitivity, gluconeogenic gene expression, glycogen synthesis, and lipogenic gene expression in fasted (a, insulin-depletion) , early fed (b, insulin action), fully fed (c, chronic insulin action), and insulin resistant (d) states

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