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TFE3 transcriptionally activates hepatic IRS-2, participates in insulin signaling and ameliorates diabetes

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Using an expression cloning strategy, we have identified TFE3, a basic helix-loop-helix protein, as a transactivator of metabolic genes that are regulated through an E-box in their promoters. Adenovirus-mediated expression of TFE3 in hepatocytes in culture and *in vivo* strongly activated expression of IRS-2 and Akt and enhanced phosphorylation of insulin-signaling kinases such as Akt, glycogen synthase kinase 3β and p70S6 kinase. TFE3 also induced hexokinase II (*HK2*) and insulin-induced gene 1 (*INSIG1*). These changes led to metabolic consequences, such as activation of glycogen and protein synthesis, but not lipogenesis, in liver. Collectively, plasma glucose levels were markedly reduced both in normal mice and in different mouse models of diabetes, including streptozotocin-treated, *db/db* and KK mice. Promoter analyses showed that *IRS2*, *HK2* and *INSIG1* are direct targets of TFE3. Activation of insulin signals in both insulin depletion and resistance suggests that TFE3 could be a therapeutic target for diabetes.

Insulin has long been known to have a major role in the anabolic effects associated with carbohydrate and lipid metabolism. Insulin receptor substrate proteins (IRS-1 and IRS-2) link insulin receptor binding to its final biological actions through a series of intermediate effectors including the phosphatidylinositol 3-kinase (PI3K)-Akt cascade, which is the primary physiological pathway used (reviewed in refs. 1,2). Through gene-targeting experiments, it has been shown that hepatic insulin signaling is mediated mainly through IRS-2 rather than IRS-1 (refs. 3–6). After these acute insulin actions on glucose metabolism, the liver initiates lipogenesis through the induction of lipogenic enzymes.

Sterol regulatory element–binding proteins (SREBPs) are membrane-bound transcription factors that regulate genes involved in lipid synthesis. Upon sterol-regulated cleavage leading to nuclear translocation, the amino-terminal basic helix-loop-helix (bHLH) leucine zipper domains (nuclear SREBPs) transactivate their target gene promoters containing SREBP-response elements (SREs). The rough endoplasmic reticulum membrane proteins SREBP cleavage–activating protein (SCAP) and insulin-induced gene have a central role in sterol regulation: SCAP activates and Insig inhibits cleavage of SREBPs as a sensor and regulator of sterols[7]. The alternate SREBP isoforms have different roles in lipid synthesis. Whereas SREBP-2 is crucial for regulation of genes involved in cholesterol synthesis, SREBP-1c controls the expression of genes encoding lipogenic enzymes[8,9]. Insulin and glucose are well known to stimulate lipogenesis and activate hepatic expression of SREBP-1c[10,11]. Thus, SREBP-1c is thought to mediate insulin action on lipid gene transcription[10].

Hepatic insulin resistance is an important pathophysiological feature of type 2 diabetes mellitus and the metabolic syndrome. Decreased *Irs2* expression and the resultant impairment of PI3K-Akt signaling has been reported in the livers of several animal models of insulin resistance[12,13], suggesting that hepatic insulin resistance is mediated through inhibition of *Irs2*. In these animals, SREBP-1c is highly expressed and is crucial for the formation of fatty liver[14]. We recently reported that SREBPs directly bind to the promoter of *Irs2* and suppress *Irs2* expression, leading to hepatic insulin resistance[15]. Conversely, activation of IRS-2 should ameliorate insulin resistance in the liver. As IRS-2 is regulated at the transcriptional level, identification of transactivators of IRS-2 will be important for development of new treatments for type 2 diabetes. We found that the *Irs2* promoter was activated by Foxos, but their activation was not very potent[15]. There could be some additional transcriptional activators for *Irs2* presumably mediating transcription through its SRE, which also contains an E-box.

E-boxes are consensus *cis*-elements for bHLH proteins, a large superfamily of transcription factors. E-boxes are found in many genes involved in metabolism, including those related to glycolysis, lipogenesis and insulin signaling, suggesting that the responsible transcription factors could be involved in both carbohydrate and lipid metabolism. Although some bHLH proteins such as upstream stimulatory factors (USFs)[16,17], c-myc[18], SREBP-1 (ref. 19) and carbohydrate response element binding protein (ChREBP)[20] have been well characterized and have been shown to regulate genes involved in metabolism, the low stringency of E-boxes prompted us

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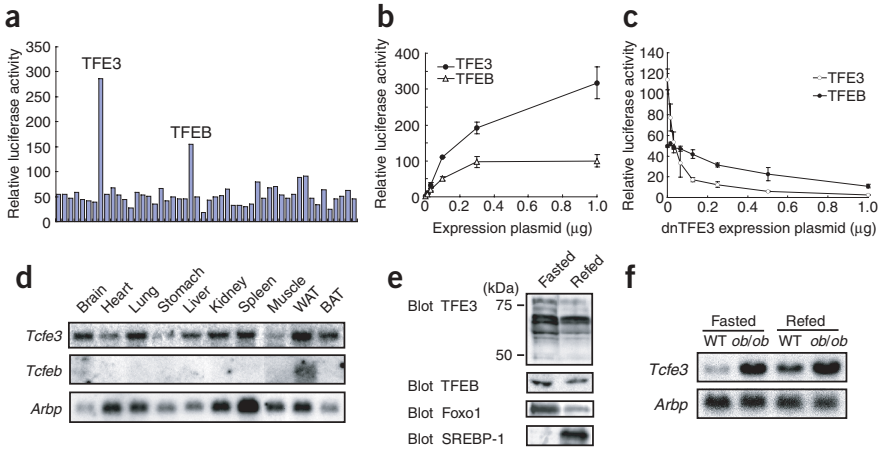

Figure 1 Identification of TFE3 as a transactivator of E-box–containing promoters of genes involved in metabolism. (**a**) Results from initial screening of the expression library to identify cDNA clones that activate the E-box–containing enhancer luciferase reporter (pE-box-Luc). (**b**) Dose-dependent activation of pE-box-Luc by TFE3 and TFEB. (**c**) Dose-dependent inhibition of pE-box-Luc by a dominant negative form of TFE3 (dnTFE3). (**d**) Tissue distribution of *Tcfe3* and *Tcfeb* mRNA as estimated by northern blotting. *Arbp* (which encodes acidic ribosomal protein P0) was used as an internal control. WAT, white adipose tissue; BAT, brown adipose tissue. (**e**) Amounts of hepatic TFE3, TFEB and SREBP-1 as measured by immunoblotting in fasted and refed C57BL6 mice. (**f**) Amounts of *Tcfe3* mRNA in white adipose tissue from wild-type mice and *ob*/*ob* mice in fasted and refed states. Values in graphs represent results from three independent experiments (mean ± s.e.m.).

to speculate that a yet-unidentified factor regulates genes related to insulin signal transduction.

The leucine zipper–containing bHLH protein TFE3 (encoded by *Tcfe3*) was originally identified as a transcription factor involved in regulating the expression of the gene encoding the immunoglobulin heavy chain by binding to the intron enhancer[21]. The TFE gene family has been also shown to be involved in the biology of melanocytes and their malignancies. In addition to functioning as homodimers, TFEs also work as partners of other transcriptional regulators such as E2F, PU-1, Max and Smads in the regulation of a variety of genes[22–24]. Although TFEs have ubiquitous tissue expression, their roles in regulating genes involved in metabolism are largely unknown.

RESULTS

TFE3 transactivates genes involved in metabolism

Many genes involved in glucose and lipid metabolism have E-boxes, a consensus DNA cognate sequence for bHLH proteins, in their promoter regions. In an attempt to identify new transcription factors that could have a role in glucose and insulin metabolism, we used an expression cloning strategy. We used a luciferase reporter containing a representative E-box in a carbohydrate responsive element from the *Thrsp* gene (encoding S14), which is known to be highly upregulated by both insulin and glucose. We screened 210,000 clones from an expression library prepared from SREBP-1 knockout mice and identified two clones, TFE3 and TFEB, that robustly activated the E-box reporter (**Fig. 1a**). Consistent with the rationale for this cloning, TFE3 activated the E-box enhancer activity in a dose-dependent manner (**Fig. 1b**), and TFEB had a weaker activity. TFEC is another TFE family member that is an endogenous negative regulator owing to a lack of a transactivation domain[25]. We constructed a dominant negative form of TFE3 (dnTFE3) based upon the structure of TFEC. Cotransfection of TFE3 with dnTFE3 competitively suppressed the TFE3-induced activity (**Fig. 1c**). *Tcfe3* was highly expressed in organs involved in energy metabolism such as liver and white adipose tissue of mice (**Fig. 1d**). Multiple immunoreactive bands of TFE3 were detected in hepatic nuclear extracts and were presumably derived from multiple ATG start sites (**Fig. 1e** and **Supplementary Fig. 1** online). These TFE3 proteins of different sizes have similar transcriptional activity, and their physiological relevance is currently unknown (**Supplementary Fig. 1** online). Nutritional manipulations such as fasting and refeeding are known to highly regulate hepatic nuclear SREBP-1 and nuclear Foxo1, but not TFE3 (**Fig. 1e**). But expression of *Tcfe3* in white adipose tissue from leptin-deficient *ob*/*ob* mice was increased (**Fig. 1f**), suggesting a potential involvement of TFE3 in some pathophysiological states of fat energy metabolism.

TFE3 activates insulin signaling in primary hepatocytes

To obtain a blueprint of potential metabolic functions of TFE3, we used DNA microarray technology with mouse livers infected with adenoviral vector encoding mouse TFE3. *Irs2*, *Akt*, hexokinase 2 (*Hk2*) peroxisome proliferator–activated receptor γ (*Pparg*) and *Insig1* were highly upregulated by TFE3 overexpression (data not shown). These data were confirmed by northern blot analysis of rat primary hepatocytes infected by adenoviral TFE3 (**Fig. 2a**). These genes are either regulated by insulin, are involved in insulin signaling, or both, indicating the involvement of TFE3 in insulin action.

In support of this, insulin signaling in hepatocytes was highly activated by overexpression of TFE3, as estimated by levels of total

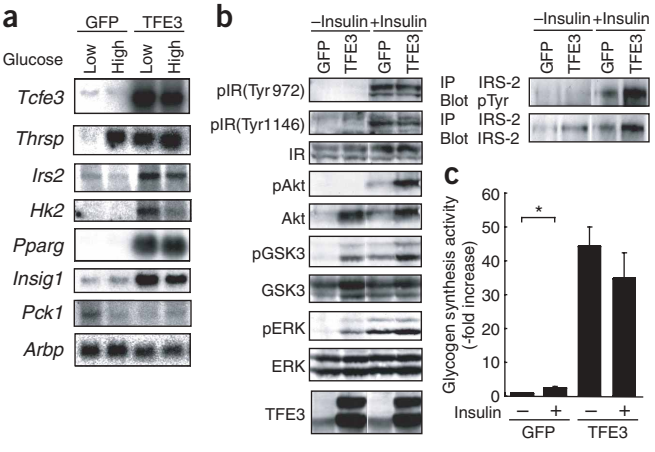

Figure 2 Effects of TFE3 on insulin signaling and glycogen synthesis in primary hepatocytes. (**a**) Gene expression in hepatocytes infected with adenovirus expressing either green fluorescent protein (GFP) or TFE3, as estimated by northern blotting. (**b**) Overexpression of TFE3 strongly stimulates hepatic glycogen synthesis in an insulin-dependent manner. 'p' indicates phosphorylated. (**c**) TFE3 activates the hepatic insulin effects, as measured by glycogen synthesis in TFE3-expressing hepatocytes. Error bars represent s.e.m. from three independent experiments. * $P < 0.01$, compared with GFP-overexpressing cells.

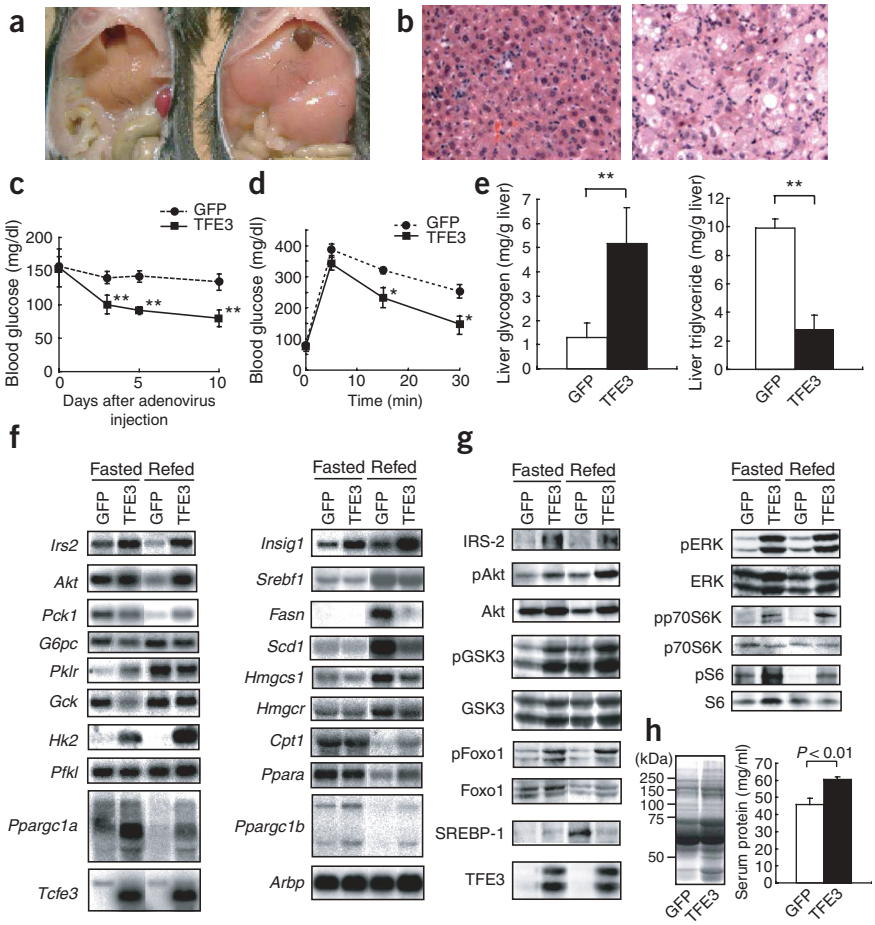

Figure 3 *In vivo* effects of TFE3 on livers of normal mice. (**a**) Livers of C57BL6 male mice overexpressing adenovirus-mediated GFP (left) and wild-type TFE3 (TFE3, right) in a fasted state. (**b**) Histological analysis of livers of C57BL/6 mice injected with adenovirus encoding GFP (left) and TFE3 (right) in a fasted state. (**c**) Blood glucose concentration of wild-type mice injected with adenovirus encoding GFP ($n = 4$) and TFE3 ($n = 4$) in a fed state at indicated days after adenovirus injection. (**d**) Blood glucose concentration during intravenous glucose tolerance test of wild-type mice overexpressing GFP ($n = 4$) and TFE3 ($n = 6$). (**e**) Hepatic concentrations of glycogen and triglycerides from mice infected with adenovirus encoding GFP ($n = 4$) and TFE3 ($n = 4$) at day 6 after the injection in a 24-h fasted state. (**f**) Gene expression in livers of TFE3-overexpressing wild-type mice, as estimated by northern blotting, in fasted and refed states. (**g**) Levels of insulin signaling molecules were examined by immunoblotting of livers expressing GFP and TFE3. (**h**) Serum protein levels in fed state at day 5 after adenovirus injection were determined by SDS-PAGE and bicinchoninic acid (BCA) assay. $^*P < 0.05$, $^{**}P < 0.01$, compared with mice overexpressing GFP.

protein and phosphorylation of various insulin signaling molecules (**Fig. 2b**). TFE3 did not change either the amount of insulin receptor protein or its autophosphorylation by insulin. When *Irs2* mRNA was upregulated, however, both IRS-2 and phosphorylated IRS-2 were highly increased by overexpression of TFE3 in hepatocytes. Reciprocal repression by the dominant negative form of TFE3 confirmed its specific effect on *Irs2* expression (**Supplementary Fig. 2** online). Akt protein was also elevated, with a marked enhancement in phosphorylated Akt only in the presence of insulin. Facilitating this major insulin metabolic signaling, TFE3 also strongly elevated phosphorylation of GSK3β, a key regulator for glycogen synthesis, without changing its level of expression. In contrast to Akt, phosphorylation of GSK3β by TFE3 did not require insulin, suggesting that this effect of TFE3 was not mediated solely through the IRS-2–Akt signal. In response to these changes, glycogen synthesis was markedly induced by TFE3 in the presence or absence of insulin (**Fig. 2c**). Other insulin signaling pathways, including the extracellular signal–regulated kinase (ERK) pathway, were also activated. The intensity of TFE3 activation of entire pathways for insulin signaling exceeded authentic insulin supplementation.

In vivo effects of TFE3 on livers of normal mice

Next, we intravenously infected C57BL/6 mice with adenoviral TFE3 to determine metabolic effects *in vivo*. TFE3 did not affect body weight unless mice were fasted (**Supplementary Table 1** online). Liver weights of mice infected with adenoviral TFE3 were substantially increased (**Supplementary Table 1** online). Macroscopically, livers overexpressing TFE3 were enlarged (**Fig. 3a**). Microscopically, TFE3-infected livers had increased cellular size, but not increased cellular or nuclear numbers, showing hypertrophic, rather than hyperplastic, changes (**Fig. 3b**). The mice infected with TFE3 adenovirus showed a steady and marked reduction in plasma glucose levels that was sustained after the injection (**Fig. 3c**). Plasma insulin, triglycerides and free fatty acids were substantially reduced by adenoviral TFE3 (**Supplementary Table 1** online). Intravenous glucose tolerance tests showed that hepatic TFE3 expression led to improved glucose tolerance, with lower glucose levels at all time points after glucose loading (**Fig. 3d**). TFE3 overexpression caused a marked increase in hepatic glycogen content, whereas hepatic triglyceride and cholesterol contents were reduced (**Fig. 3e**). Food intake was not affected, and liver enzymes such as serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were elevated but not significantly changed by hepatic overexpression of TFE3 as compared to overexpression of GFP, indicating that the late body weight loss induced by TFE3 was not the result of hepatotoxicity (**Supplementary Table 1** online).

In vivo regulation of hepatic genes and signals by TFE3

We investigated hepatic gene expression and insulin signaling proteins (**Fig. 3f,g**). Northern blot analysis confirmed the *in vivo* gene induction of newly identified TFE3-activated genes (**Fig. 3f**). *Irs2* expression was induced by TFE3, with a more prominent elevation in a refed state than in a fasted state, in which endogenous *Irs2* expression was upregulated. Expression of glycolytic enzymes such as glucokinase (encoded by *Gck*), pyruvate kinase (encoded by *Pklr*) and

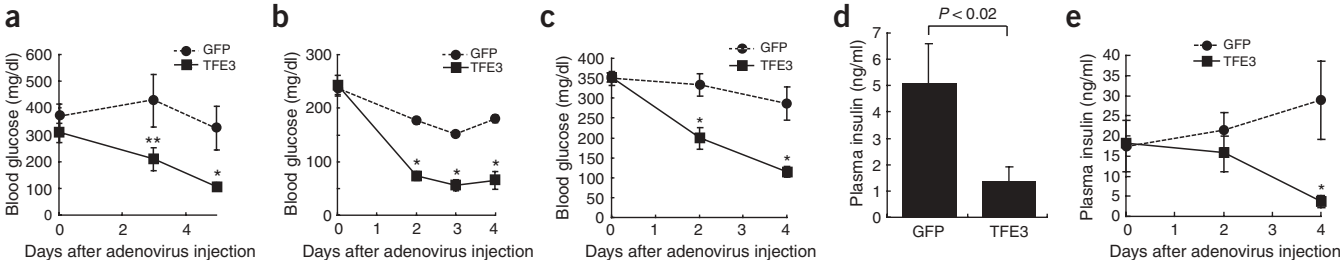

Figure 4 Changes in blood glucose and plasma insulin levels by TFE3 in diabetic mice. Blood glucose concentration of streptozotocin-treated (**a**), KK (**b**), and *Lepr*$^{-/-}$ (**c**) male mice injected with adenovirus encoding GFP ($n = 4$) or TFE3 ($n = 6$) at indicated days in a fed state after adenovirus injection. Plasma insulin concentration of KK at 4 d (**d**) and *Lepr*$^{-/-}$ male (**e**) mice in a fed state at indicated days after adenovirus injection. Data represent mean ± s.e.m. $^{**}P < 0.05$, $^{*}P < 0.01$.

phosphofructokinase (encoded by *Pfkl*) were not markedly changed by TFE3. In contrast, *Hk2*, which encodes an enzyme that normally has a key role in glycolysis in muscle and is regulated by insulin[26], was highly upregulated. These effects by TFE3 were dose dependent (**Supplementary Fig. 3** online). Consistent with the data from primary hepatocytes, the phosphorylation of molecules involved in insulin signaling such as Akt, GSK3β, Foxo1, ERK, p70S6 kinase (p70S6K) and S6 was strongly enhanced by TFE3 in both fasted and fed states (**Fig. 3g**). Genes encoding hepatic gluconeogenic enzymes phosphoenolpyruvate carboxykinase 1 (*Pck1*) and glucose-6-phosphatase (*G6pc*) were not consistently changed by TFE3, although PPARγ coactivators 1α and 1β (*Ppargc1a* and *Ppargc1b*), recently recognized as important coactivators for these gluconeogenic genes[27,28], were highly induced. Genes involved in hepatic fatty acid oxidation, such as *Ppara* (which encodes peroxisome proliferator–activated receptor α) and *Cpt1* (which encodes carnitine palmitoyltransferase 1a), were highly regulated by nutrition, but not by TFE3.

Downregulation of lipogenic genes by TFE3

Along with glucose metabolism, lipid synthesis is another anabolic pathway enhanced by insulin. Genes involved in lipogenesis are controlled at the transcriptional level by SREBPs. Insig-1 suppresses activities of these transcription factors by inhibiting cleavage and expression of SREBPs. TFE3 markedly activated Insig-1 expression (**Fig. 3f**). Owing to activation of this SREBP inhibitor, the nuclear form of SREBP-1, highly abundant in liver nuclei of normal refed mice, was completely abrogated by TFE3 (**Fig. 3g**). Subsequently, SREBP-1 target lipogenic genes *Fasn* (which encodes fatty acid synthase) and *Scd1* (which encodes stearoyl–coenzyme A desaturase 1) were markedly decreased even in the refed condition. Similarly, genes involved in cholesterol synthesis and controlled by SREBP-2, such as *Hmgcr* (which encodes 3-hydroxy-3-methylglutaryl-coenzyme A reductase) and *Hmgcs1* (which encodes 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1), were also suppressed.

Enhancement of protein synthesis by TFE3

Consistent with the enlarged and hypertrophic change of hepatocytes in adenoviral TFE3–infected mice, protein synthesis, another insulin anabolic effect, was markedly enhanced by TFE3 overexpression, as judged by phosphorylation of p70S6 kinase and S6, at least partially as a result of activation of the PI3K-Akt pathway (**Fig. 3g**). Accordingly, plasma proteins such as albumin were markedly increased (**Fig. 3h**).

Lowering of plasma glucose by TFE3 in diabetes

Based on the ability of TFE3 to lower plasma glucose by enhancing insulin signaling in normal mice, we explored the therapeutic

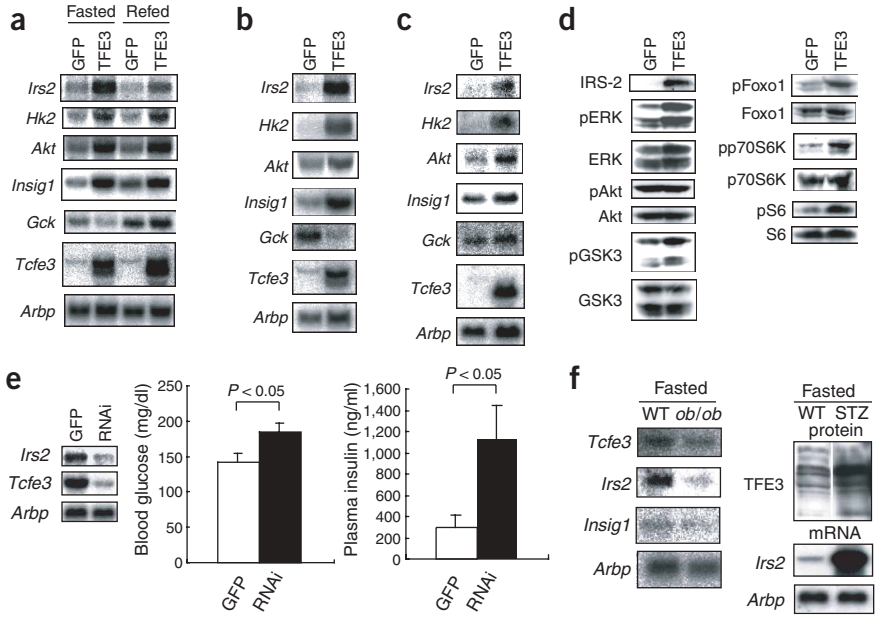

Figure 5 Effects of TFE3 on gene expression and insulin signaling in livers of diabetic mice. Gene expression in livers of (**a**) STZ-treated mice, (**b**) KK mice in a fed state and (**c**) *Lepr*$^{-/-}$ mice in a fed state, as estimated by northern blotting. (**d**) Protein levels of insulin-signaling molecules were examined by immunoblotting of livers of *Lepr*$^{-/-}$ mice infected with adenovirus encoding GFP or TFE3 in a fed state. (**e**) Gene expression in liver, blood glucose and plasma insulin concentrations of normal C57BL/6 mice in a fed state at 6 d after injection of adenovirus encoding *Tcfe3*-specific RNA for RNAi ($n = 4$). (**f**) Regulation of TFE3 and IRS-2 in *ob*/*ob* and STZ-treated mice. Shown are amounts of *Tcfe3* and TFE3 target gene mRNA in liver from wild-type mice and *ob*/*ob* mice in a fasted state, and the amount of nuclear TFE3 protein and *Irs2* mRNA in livers of wild-type and mice STZ-treated mice in a fasted state. WT, wild-type.

potential of this transcription factor for diabetic mice. We administered TFE3 adenovirus intravenously to three different diabetic mouse models: streptozotocin (STZ)-treated, KK and *db*/*db* (also known as *Lepr*$^{-/-}$) mice (**Fig. 4**). The elevated blood glucose levels were normalized in a time-dependent manner in each of the diabetic models that received the TFE3 adenovirus, whereas control diabetic mice infected with adenovirus expressing green fluorescent protein (GFP) had no significant changes in blood glucose levels (**Fig. 4a–c**). These findings suggest that TFE3 has a potent hypoglycemic effect on diabetic mice irrespective of their pathology (that is, insulin depletion or insulin resistance). In KK and *Lepr*$^{-/-}$ mice, high plasma insulin levels caused by insulin resistance were markedly lowered by administration of TFE3 adenovirus (**Fig. 4d**,**e**). The marked improvement of diabetes was supported by changes in hepatic gene expression patterns: expression of *Irs2*, *Akt*, *Hk2* and *Insig1* was very low in these diabetic mice but was restored by TFE3 (**Fig. 5a–c**). Subsequently, in *Lepr*$^{-/-}$ mice, suppressed phosphorylation of molecules involved in insulin signaling was consistently enhanced, indicating amelioration of insulin resistance by TFE3 (**Fig. 5d**).

Finally, to evaluate the physiological function of TFE3 in hepatic insulin signaling, we knocked down hepatic expression of *Tcfe3* using adenoviral overexpression of *Tcfe3*-specific small interfering RNA (siRNA) in C57BL/6 mice. Hepatic TFE3 expression was reduced by 90% (**Fig. 5e**). Subsequently, expression of *Irs2* was markedly suppressed, and both plasma insulin and glucose levels were elevated, suggesting the emergence of insulin resistance (**Fig. 5e**). In accordance with this, we observed coordinated regulation of hepatic *Tcfe3* and *Irs2* in pathophysiological livers from leptin-deficient *ob*/*ob* and STZ-treated mice (**Fig. 5f**). Together, these data support a physiological and pathophysiological role for TFE3 in hepatic *Irs2* expression and insulin signaling.

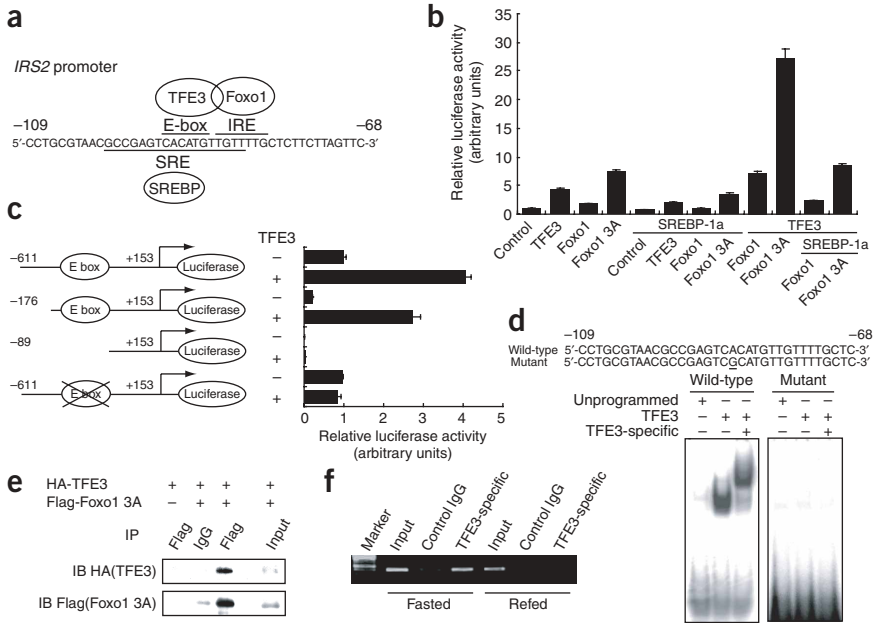

Figure 6 *IRS2* promoter is a direct target of TFE3. (**a**) The human *IRS2* promoter contains an E-box, IRE and SREBP binding site (SRE). (**b**) Effects of TFE3, Foxo1 3A and SREBP1a on *IRS2* promoter activity in HepG2 cells, as estimated by luciferase reporter assay. (**c**) The IRS-2 E-box is responsible for induction of TFE3 in luciferase reporter assay. (**d**) The *IRS2* E-box is the TFE3 binding site. The E-box mutation that abolished the transactivation by TFE3 (**c**) is indicated as mutant probe in comparison with wild-type probe for the EMSA (blot shown in lower right panel). (**e**) Physical interaction between TFE3 and Foxo1. COS7 cell lysates expressing hemagglutinin (HA)-tagged TFE3 and the Flag-tagged constitutively active form of Foxo1 (Foxo1 3A) were immunoprecipitated with Flag-specific or control IgG antibodies, followed by immunoblotting with HA-specific antibody. (**f**) TFE3 binds directly to the *Irs2* promoter *in vivo*, as shown by ChIP assay. Data represent mean ± s.e.m. from three independent experiments.

Irs2 promoter is a direct target of TFE3

As the insulin-related genes *Irs2*, *Hk2* and *Insig1* were highly and consistently regulated by TFE3, we analyzed their promoters for TFE3 target binding sites. As previously described, the human *IRS2* gene has an SRE (IRS-2 SRE) at which SREBPs repress *IRS2* expression in competition with putative activators, Foxos, that bind to insulin response elements (IREs) neighboring the SRE[15]. The IRS-2 SRE also contains an E-box (**Fig. 6a**). We conducted luciferase reporter assays, which showed that TFE3 strongly activates the *IRS2* promoter (**Fig. 6b**). We performed mutational studies using electrophoretic mobility shift assays (EMSAs) and luciferase assays, which confirmed that the *IRS2* E-box is a site for TFE3 binding and activation (**Fig. 6c**,**d**). Notably, cotransfection of TFE3 with a constitutively active mutant of Foxo1 (Foxo1 3A)[29] synergistically activated the *IRS2* promoter. SREBP continued to compete with TFE3 and Foxo1 3A on a region overlapping both the E-box and SRE. Supporting this synergistic activation, TFE3 and Foxo1 physically interact in immunoprecipitation analysis (**Fig. 6e**). We performed a chromatin immunoprecipitation (ChIP) assay, which suggested that TFE3 directly binds to the *Irs2* promoter *in vivo* (**Fig. 6f**). Consistent with high expression of *Irs2*, the signal was prominent only in a fasted state. Taken together with the previous report of direct binding of Foxo1 to this region[15], our current data indicate synergistic activation of *Irs2* by TFE3 and Foxo1, as compared with inhibition by SREBPS (**Fig. 6a**). In addition to the *IRS2* promoter, *HK2* and *INSIG1* promoters were also confirmed to be new TFE3 targets (**Supplementary Fig. 4** online).

DISCUSSION

Our studies show that TFE3 has the potential to enhance insulin signal transduction downstream of the insulin receptor. At least in part, this effect is the result of direct transactivation of IRS-2 and Akt, leading to activation of the IRS-2–PI3K–Akt cascade, the primary hepatic insulin signaling pathway. When TFE3 is overexpressed *in vivo*, these kinase cascades are markedly enhanced in normal mice and restored in different insulin-resistant mice, improving insulin sensitivity and, notably, reducing both plasma insulin and glucose levels of all mice tested. TFE3 activation of insulin signal transduction also includes GSK3β phosphorylation, which is crucial for glycogen synthesis. Notably, GSK3β is fully activated by TFE3 in the absence of insulin in cell-culture experiments and in insulin-depleted fasted mice, whereas activation of Akt phosphorylation by increasing amounts of IRS-2 and Akt proteins requires insulin. The precise mechanism for this activation of insulin signaling without insulin is currently unknown, but TFE3 may also be involved in a yet-unidentified Akt-independent mechanism for GSK3β phosphorylation.

In concert with activation of insulin signaling, TFE3 induces *Hk2* expression. In normal liver, glucokinase is important in conversion of glucose to glucose-6-phosphate, the first key step in glycolysis and glycogen synthesis, and is suppressed in many diabetic models. TFE3 does not elevate expression of genes encoding hepatic enzymes including *Gck*, *Pfkl* and *Pkl*. Instead, TFE3 causes a marked induction of *Hk2*. Induction of *Hk2*, glycogen synthesis through activation of GSK3β and protein synthesis through activation of p70S6K and S6 are all likely to consume glucose, facilitate glucose uptake in hepatocytes and contribute to a marked glucose-lowering effect of TFE3 in both normal and diabetic mice. TFE3 might be an efficient therapeutic target for diabetes because it could improve glucose metabolism both in insulin resistance and deficiency.

In contrast to a marked enhancement of insulin-mediated glucose metabolism, TFE3 does not induce lipid synthesis, although lipogenesis is one of the major anabolic effects of insulin in the liver. SREBPs control lipid synthesis. Insig-1 is a suppressor of SREBP activity by trapping the SCAP-SREBP complex in the rough endoplasmic reticulum. TFE3 directly and markedly induces Insig-1 and inhibits SREBP activation. Thus, despite enhancement of insulin signaling, lipid synthesis is not augmented by TFE3.

Nutritional regulation of IRS-2 is crucial for insulin signaling in the liver. We have shown previously that SREBP-1 represses *Irs2* expression by competing with Foxo1 and Foxo3 in binding to the SRE and IRE and inhibiting recruitment of PGC-1α, a crucial coactivator[15]. Our studies suggest that TFE3 might also be important in nutritional regulation of hepatic *Irs2* expression; the synergism with Foxo1 at fasting and antagonism by SREBP-1c at feeding is schematized in **Supplementary Figure 5** online.

It is notable that one transcription factor has potent effects on such a wide range of insulin-related genes in a coordinated fashion (**Supplementary Fig. 5** online). The presence of E-boxes in a wide variety of genes suggests that there may be systems discovered in the future in which any bHLH superfamily has the potential to activate some sets of E-box–containing genes and exert physiological functions. It has been reported that overexpression of c-myc, member of the bHLH superfamily, counteracts diabetic alterations mainly by altering *Pck* and *G6pc* genes[30]. Here, we find that another bHLH family member has profound effects on regulation of genes involved in insulin actions. Hepatic overexpression of TFE3 causes activation of the major members of the insulin signaling pathway, with differential consequences on the various branches of insulin-mediated metabolic effects: glycogen and protein synthesis are enhanced, whereas lipogenesis is not. Hypertrophic changes without steatosis in hepatocytes expressing TFE3 reflects this mode of action by TFE3. Excess accumulation of triglycerides has been thought to be associated with insulin resistance and type 2 diabetes. Thus, this unique mode of insulin signal enhancement by TFE3 has favorable therapeutic implications for the treatment of type 1 and type 2 diabetes. Further studies are needed to clarify the precise molecular mechanisms of activation of different insulin signals by TFE3 and to address the physiological and pathological roles of TFE3 in other metabolic organs, especially in adipose tissue.

METHODS

Materials. We purchased antibodies to IRβ, Tyr972-phosphorylated IRβ and IRS-2 from Upstate Biotechnology, and antibodies to GSK3β and SREBP-1 (2A4) from Santa Cruz Biotechnology. We obtained antibodies to Akt, Ser473-phosphorylated Akt, ERK1/2, phosphorylated ERK1/2 (Thr202, Tyr204), phosphorylated GSK3α/β (Ser21, Ser9), p70S6K, Thr389-phosphorylated p70S6K, S6, phosphorylated S6 (Ser235, Ser236), Foxo1, Ser256-phosphorylated Foxo1 and Tyr1146-phosphorylated IRβ from Cell Signaling Technology. The adenovirus expression vector kit (AdEasy system) and adenovirus encoding green fluorescent protein (GFP) were from B. Vogelstein (Johns Hopkins Oncology Center) and T.C. He (University of Chicago Medical Center).

Animals. We obtained 7-week-old male and 16-week-old male (the control for leptin-deficient *ob/ob* mice) C57BL/6 and KK mice from CLEA Japan. We obtained 16-week-old male leptin-deficient *ob/ob* and 7-week-old male *Lepr*$^{-/-}$ (*db/db*) mice from Charles River. The fasting and refeeding protocol was as described previously[15]. To induce diabetes, we injected mice with STZ (100 mg/kg body weight) twice, with a 1-d interval between injections. We intravenously injected mice with adenovirus encoding GFP or TFE3 at a dose of 1×10^9 plaque-forming units (p.f.u.). All the animal husbandry and animal experiments were consistent with the University of Tsukuba's Regulation of Animal Experiment and were approved by the Animal Experiment Committee, University of Tsukuba.

Metabolic measurements. We measured levels of glucose, insulin, triglycerides, free fatty acids, cholesterol, protein, AST and ALT in plasma and of glycogen, triglycerides and cholesterol in liver as previously described[14].

Glucose tolerance test. Male mice treated with the adenovirus were fasted for 12 h and intravenously injected with glucose (1 g/kg body weight) for measurement of plasma glucose and insulin levels at the indicated times.

Cloning of TFE3. The expression cloning strategy to identify transactivators of E-box–containing promoters was previously described for identification of liver X receptors (LXRs) as the activators of the promoter of the gene encoding mouse SREBP-1c[31]. We prepared an expression library from adipose tissues of SREBP-1 knockout mice[19,31]. As a probe to screen the expression library, we used pE-box-Luc, the E-box enhancer luciferase reporter gene containing six copies of carbohydrate responsive element at position –1448 to –1422 of the rat S14 gene (5′-GCCAGTTCTCACGTGGTGGCCCTGTGC-3′) fused to SV40 early promoter in the pGL2-Promoter Vector (Promega)[32]. pE-box-Luc and each subpool containing approximately 1,500 cDNA clones were cotransfected into HEK293 cells with pSV-β-gal (Promega) as a reference plasmid. We measured luciferase activity and normalized it using β-galactosidase activity.

Isolation and culture of primary hepatocytes and glycogen synthesis. We prepared primary cultures of rat hepatocytes and subjected them to adenovirus infection as previously described[15]. Using rat hepatocytes, studies of glycogen synthesis, gene expression and immunoblotting were as described previously[33]. For RNA analysis, we incubated cells at 37 °C for 24 h after adenovirus infection at a multiplicity of infection of 100. For protein analysis, we stimulated adenovirus-infected cells with 100 nM insulin for 10 min after a 24-h incubation.

Preparation of recombinant adenovirus. We subcloned mouse *Tcfe3* (TFE3) and the dominant negative form of *Tcfe3* (dnTFE3) coding cDNAs into the pShuttle-CMV vector, and we generated the recombinant adenoviral plasmid by homologous recombination with the pAdEasy-1 plasmid. We subcloned *Tcfe3*-specific RNA interference (RNAi) constructs using the *Tcfe3* coding sequences 5′-GCGATTCAACATTAACGATAAGA-3′ into U6 entry vector (Invitrogen), and generated the recombinant adenoviral plasmid by homologous recombination with pAd promoterless vector (Invitrogen). We produced recombinant adenoviruses in HEK293 cells and purified them by CsCl gradient centrifugation as described[34].

Plasmids. We cloned hemagglutinin-tagged mouse *Tcfe3* cDNA into pcDNA3 (Invitrogen). The expression vector containing Flag-tagged constitutively active form of Foxo1 (Foxo1 3A)[29] was from A. Fukamizu (University of Tsukuba). The human *IRS2* promoters have been previously described[15]. We amplified the mouse *Hk2* promoter (base pairs –671 to +88, relative to transcriptional start site)[35] and the human *INSIG1* promoter (base pairs –1273 to +33) by PCR[36] and subcloned them into pGL3-basic luciferase vector (Promega).

Northern blot analysis. We prepared total RNA from cells and tissues using Trizol reagent (Invitrogen) unless otherwise indicated. Blot hybridization with cDNA probes were performed as previously described[19].

Transfection and luciferase assays. We transfected HepG2 cells with 250 ng each of the indicated luciferase reporter and expression plasmids and a

pRL-SV40 plasmid as a reference (Promega) using FuGENE6 (Roche). After a 24-h incubation at 37 °C, we measured the amount of firefly luciferase activity and normalized it to the amount of renilla luciferase activity.

Electrophoretic mobility shift assay (EMSA). We generated TFE3 from an expression vector using a coupled *in vitro* reticulocyte transcription-translation system (Promega). We used the following double-stranded oligonucleotides in EMSAs: 5′-CCTGCGTAACGCCGAGTCACATGTTGTTTTGCTC-3′ for the *IRS2* promoter, 5′-GCAGCCCCTCGCATGATCCCC-3′ for the *Hk2* promoter, and 5′-GGGCAGGCTCACGTGATGCGGGCC-3′ for the *INSIG1* promoter. We incubated *in vitro* synthetic protein lysate in a reaction mixture as previously described[15]. We resolved DNA-protein complexes on a 4.0% polyacrylamide gel.

Immunoblotting. We performed immunoblot analysis of nuclear extracts, cell and liver lysates using indicated antibodies as described[15,19].

Immunoprecipitation. We performed immunoprecipitation analysis as previously described[15].

Determination of serum protein levels. We determined the serum protein levels from fasted C57BL/6 mice 5 d after adenovirus injection by SDS-PAGE and bicinchoninic acid assay.

Chromatin immunoprecipitation assay. We performed the ChIP assay as previously described[15]. Briefly, we immunoprecipitated complexes of genomic DNA and TFE3 with antibodies to TFE3 (Santa Cruz). We subjected extracted genomic DNA to PCR amplification. To amplify the *Irs2* promoter region containing an E-box, we used the following primer set: 5′-TGCTCTCAGTGTC CTCTTAGGGTC-3′ and 5′-TTGGGTTCGTAGTTCAAGGGC-3′.

Statistical analysis. Data are expressed as the mean ± s.e.m. Statistical significance was tested with an unpaired two-tailed Student *t*-test. The differences were considered to be significant if $P < 0.05$.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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