phosphate dehydrogenase) as an internal control¹⁶. For experiments with intact mice, 21-day-old female mice were used.

For uterine weight analysis, mice were treated with ligands for 3 days, and the ratio of uterine wet weight to body weight was calculated, followed by *t*-test analysis. Results are given as means $\pm$ s.e.m.

For the BrdU labelling experiment, ovariectomized mice were treated with ligands for 3 days, then injected with BrdU (30 mg kg⁻¹). Paraffin sections from the uteri 8 h after BrdU injection were immunostained with anti-BrdU monoclonal antibody by using the BrdU Labeling and Detection Kit 1 (Roche), and the percentage of BrdU-positive epithelial cells in the sections was calculated.

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1. Bock, K. W. Aryl hydrocarbon or dioxin receptor: biologic and toxic responses. *Rev. Physiol. Biochem. Pharmacol.* **125**, 1–42 (1994).
2. Krishnan, V. *et al.* Molecular mechanism of inhibition of estrogen-induced cathepsin D gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in MCF-7 cells. *Mol. Cell. Biol.* **15**, 6710–6719 (1995).
3. Astroff, B. *et al.* Inhibition of the 17 β -estradiol-induced and constitutive expression of the cellular protooncogene *c-fos* by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the female rat uterus. *Toxicol. Lett.* **56**, 305–315 (1991).
4. Gibbons, A. Dioxin tied to endometriosis. *Science* **262**, 1373 (1993).
5. Mayani, A., Barel, S., Soback, S. & Almogor, M. Dioxin concentrations in women with endometriosis. *Hum. Reprod.* **12**, 373–375 (1997).
6. Rier, S. E., Martin, D. C., Bowman, R. E., Dmowski, W. P. & Becker, J. L. Endometriosis in rhesus monkeys (*Macaca mulatta*) following chronic exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Fundam. Appl. Toxicol.* **21**, 433–441 (1993).
7. Cummings, A. M., Metcalf, J. L. & Birnbaum, L. Promotion of endometriosis by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats and mice: time-dose dependence and species comparison. *Toxicol. Appl. Pharmacol.* **138**, 131–139 (1996).
8. Brown, N. M., Manzillo, P. A., Zhang, J. X., Wang, J. & Lamartiniere, C. A. Prenatal TCDD and predisposition to mammary cancer in the rat. *Carcinogenesis* **19**, 1623–1629 (1998).
9. Davis, B. J., McCurdy, E. A., Miller, B. D., Lucier, G. W. & Tritscher, A. M. Ovarian tumors in rats induced by chronic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin treatment. *Cancer Res.* **60**, 5414–5419 (2000).
10. Sogawa, K. & Fujii-Kuriyama, Y. Ah receptor, a novel ligand-activated transcription factor. *J. Biochem. (Tokyo)* **122**, 1075–1079 (1997).
11. Schmidt, J. V. & Bradfield, C. A. Ah receptor signaling pathways. *Annu. Rev. Cell Dev. Biol.* **12**, 55–89 (1996).
12. Kato, S. *et al.* Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270**, 1491–1494 (1995).
13. McKenna, N. J. & O'Malley, B. W. Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* **108**, 465–474 (2002).
14. Mangelsdorf, D. J. *et al.* The nuclear receptor superfamily: the second decade. *Cell* **83**, 835–839 (1995).
15. Freedman, L. P. Increasing the complexity of coactivation in nuclear receptor signaling. *Cell* **97**, 5–8 (1999).
16. Watanabe, M. *et al.* A subfamily of RNA-binding DEAD-box proteins acts as an estrogen receptor alpha coactivator through the N-terminal activation domain (AF-1) with an RNA coactivator, SRA. *EMBO J.* **20**, 1341–1352 (2001).
17. Yanagisawa, J. *et al.* Nuclear receptor function requires a TIF-2-type histone acetyl transferase complex. *Mol. Cell* **9**, 553–562 (2002).
18. Kamei, Y. *et al.* A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**, 403–414 (1996).
19. Tian, Y., Ke, S., Thomas, T., Meeker, R. J. & Gallo, M. A. Transcriptional suppression of estrogen receptor gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *J. Steroid Biochem. Mol. Biol.* **67**, 17–24 (1998).
20. Tora, L. *et al.* The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* **59**, 477–487 (1989).
21. Kobayashi, Y. *et al.* p300 mediates functional synergism between AF-1 and AF-2 of estrogen receptor alpha and beta by interacting directly with the N-terminal A/B domains. *J. Biol. Chem.* **275**, 15645–15651 (2000).
22. Mimura, J., Ema, M., Sogawa, K. & Fujii-Kuriyama, Y. Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. *Genes Dev.* **13**, 20–25 (1999).
23. Beischlag, T. V. *et al.* Recruitment of the NCoA/SRC-1/p160 family of transcriptional coactivators by the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator complex. *Mol. Cell. Biol.* **22**, 4319–4333 (2002).
24. Daitoku, H. Y. K., Matsuzaki, H., Hatta, M. & Fukamizu, A. Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. *Diabetes* **52**, 642–649 (2003).
25. Weisz, A. & Rosales, R. Identification of an estrogen response element upstream of the human *c-fos* gene that binds the estrogen receptor and the AP-1 transcription factor. *Nucleic Acids Res.* **18**, 5097–5106 (1990).
26. Mueller, M. D. *et al.* Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors alpha and beta. *Proc. Natl Acad. Sci. USA* **97**, 10972–10977 (2000).
27. Puga, A., Nebert, D. W. & Carrier, F. Dioxin induces expression of *c-fos* and *c-jun* proto-oncogenes and a large increase in transcription factor AP-1. *DNA Cell Biol.* **11**, 269–281 (1992).
28. Mimura, J. *et al.* Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. *Genes Cells* **2**, 645–654 (1997).
29. Dupont, S. *et al.* Effect of single and compound knockouts of estrogen receptors α (ER α) and β (ER β) on mouse reproductive phenotypes. *Development* **127**, 4277–4291 (2000).
30. Kuchenhoff, A. *et al.* Arylhydrocarbon receptor expression in the human endometrium. *Fertil. Steril.* **71**, 354–360 (1999).

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Insulin-regulated hepatic gluconeogenesis through FOXO1–PGC-1 α interaction

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Hepatic gluconeogenesis is absolutely required for survival during prolonged fasting or starvation, but is inappropriately activated in diabetes mellitus. Glucocorticoids and glucagon have strong gluconeogenic actions on the liver. In contrast, insulin suppresses hepatic gluconeogenesis^{1–3}. Two components known to have important physiological roles in this process are the forkhead transcription factor FOXO1 (also known as FKHR) and peroxisome proliferative activated receptor- γ co-activator 1 (PGC-1 α ; also known as PPARGC1), a transcriptional co-activator; whether and how these factors collaborate has not been clear. Using wild-type and mutant alleles of FOXO1, here we show that PGC-1 α binds and co-activates FOXO1 in a manner inhibited by Akt-mediated phosphorylation. Furthermore, FOXO1 function is required for the robust activation of gluconeogenic gene expression in hepatic cells and in mouse liver by PGC-1 α . Insulin suppresses gluconeogenesis stimulated by PGC-1 α but co-expression of a mutant allele of FOXO1 insensitive to insulin completely reverses this suppression in hepatocytes or transgenic mice. We conclude that FOXO1 and PGC-1 α interact in the execution of a programme of powerful, insulin-regulated gluconeogenesis.

Two transcriptional components that are targets of insulin signalling, and that can activate the process of gluconeogenesis in liver, are FOXO1 and PGC-1 α . FOXO1 has been shown to bind directly to the promoters of gluconeogenic genes and activate the process of glucose production^{4–6}. FOXO1 is directly phosphorylated by Akt, a key protein kinase downstream of the insulin receptor^{7,8}. This phosphorylation results in exclusion of FOXO1 from the nucleus. A second transcriptional component controlled by insulin and having a role in gluconeogenesis is the co-activator PGC-1 α . PGC-1 α is induced in liver on fasting, and is elevated in several models of diabetes or deficiency in insulin signalling. Notably, expression of PGC-1 α at physiological levels turns on the entire programme of gluconeogenesis^{9,10}.

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Insulin is a dominant suppressor of gluconeogenesis, and several animal models of deficiency in insulin signalling show a rise in hepatic PGC-1 α expression⁹. As insulin may control PGC-1 α expression *in vivo* either through direct action on hepatocytes or through counter-regulatory hormones such as glucagon, we investigated this question using isolated hepatocytes. As shown in Fig. 1a, b, and as expected^{9,11}, PGC-1 α messenger RNA (*Pgc1*) levels were increased by forskolin and/or dexamethasone in both immortalized and in primary mouse hepatocytes. Notably, insulin treatment does not change the level of *Pgc1* mRNA when combined with forskolin alone, or with forskolin and dexamethasone. PGC-1 α protein expression was also induced under the forskolin and dexamethasone treatment, but was unaffected by insulin (Fig. 1a). We also analysed the direct effects of insulin on *Pgc1* mRNA in mice subjected to hyperinsulinaemic, euglycaemic clamps. This procedure elevates insulin levels while maintaining steady glucose concentrations, thus preventing the rise of counter-regulatory hormones such as glucagon. As shown in Fig. 1c, elevated insulin concentrations reduced phosphoenolpyruvate carboxykinase 1 (PEPCK) mRNA (*Pck1*) but did not alter *Pgc1* mRNA levels. Together, these data indicate that insulin does not have a direct effect on PGC-1 α expression in cultured hepatocytes or liver; the effect of this hormone on PGC-1 α expression *in vivo* is probably controlled, at least in part, thorough counter-regulatory hormones, especially glucagon.

To investigate how insulin regulates the function of PGC-1 α , we used adenoviruses expressing this co-activator in Fao hepatoma cells. These cells express very little endogenous PGC-1 α and thus represent an excellent system in which to study the function of this expressed protein. As shown in Fig. 1d, and as expected⁹, expression of PGC-1 α strongly induced the gluconeogenic genes *Pck1* and glucose-6-phosphatase (*G6pc*). The addition of insulin at 10 nM greatly reduced the amount of these mRNAs driven by PGC-1 α . However, mitochondrial genes that are targets of PGC-1 α , such as cytochrome *c* and the β -subunit of ATP synthase, were not regulated by the insulin treatment. These results demonstrate that insulin acts directly on hepatocytes to suppress the regulatory function of PGC-1 α in the expression of a certain subset of genes, especially those encoding the gluconeogenic enzymes.

As the cellular distribution of FOXO1 is regulated by Akt in response to insulin^{8,12}, this factor is considered to be a potential key mediator of the insulin repression of the gluconeogenic genes. To investigate whether PGC-1 α requires FOXO1 to induce gluconeogenic genes, we used adenoviral expression of FOXO1(1–256), a truncated allele of FOXO1 that lacks the transactivation domain and functions as a dominant-negative suppressor of wild-type FOXO1 (ref. 13). Whereas PGC-1 α robustly activated expression of *G6pc* and *Pck1* (Fig. 2a), FOXO1(1–256) had no ability to activate *G6pc*, and had only a relatively weak effect on *Pck1*. However, co-expression of FOXO1(1–256) markedly diminished the ability of

Figure 1 Insulin regulates PGC-1 α action but not PGC-1 α gene expression. **a**, PGC-1 α mRNA and protein amounts are not regulated by insulin in mouse hepatocytes. Immortalized hepatocytes were differentiated as described in Methods. **b**, *Pgc1* mRNA amounts are not changed by insulin in primary hepatocytes. **c**, *Pgc1* mRNA amounts are not changed by insulin in mice. Mice were subjected to glucose clamps as described in

the Methods. Three mice for each group were used. **d**, Insulin markedly decreases PGC-1 α induction of gluconeogenic genes. Fao rat hepatocytes were placed in serum-free medium and infected with adenovirus vectors encoding either GFP or PGC-1 α . Dex, dexamethasone.

A functional FOXO1–PGC-1 α interaction was studied more directly using immortalized hepatocytes transfected with a consensus response sequence for FOXO1 together with a PGC-1 α expression plasmid or a control vector. As shown in Fig. 2b, co-expression of PGC-1 α increased the transcriptional activity of wild-type FOXO1 fourfold. Similar results were obtained when PGC-1 α was co-expressed with a constitutively active mutant of FOXO1 (FOXO1(3A)) in which the three sites for phosphorylation by Akt have been substituted to alanine⁷. The addition of insulin repressed the PGC-1 α activation of wild-type FOXO1 but did not suppress the effect of PGC-1 α on FOXO1(3A). Similar results were obtained using the promoter for glucose-6-phosphatase (Supplementary Fig. S1). Taken together these results show that PGC-1 α augments the tran-

gluconeogenic genes involves the binding of endogenous PGC-1 α to FOXO1 response elements in chromatin.

Whether the functional interaction between PGC-1 α and FOXO1 might be responsible for the ability of insulin to suppress the PGC-1 α -mediated activation of endogenous gluconeogenic genes was investigated using the constitutively active allele of FOXO1. As shown in Fig. 3a, insulin can suppress PGC-1 α -mediated induction of the gluconeogenic genes when this co-activator is expressed alone, or when it is co-expressed with wild-type FOXO1. However, when PGC-1 α is co-expressed with the constitutively active FOXO1 allele, the expression of glucose-6-phosphatase and PEPCK is no longer sensitive to insulin. Adenoviral-mediated expression of FOXO1 proteins without PGC-1 α has little effect on the gluconeogenic genes (data not shown). We next investigated whether these changes in transcriptional activity and induction of gluconeogenic genes were reflected in glucose production *per se*. As shown in Supplementary Fig. S3, adenoviral-mediated PGC-1 α expression increased glucose secretion threefold compared with cells infected with control virus. Consistent with the induction of the gluconeogenic genes shown above, insulin repressed the glucose production by PGC-1 α , but this repression was overcome by co-expression of constitutively active FOXO1.

To investigate whether insulin prevents the physical interaction between PGC-1 α and FOXO1, we analysed this complex in cells by co-immunoprecipitation after transfection of cells. As shown in Fig. 3b, a constitutively active form of Akt, which acts downstream of insulin and phosphorylates FOXO1, clearly interferes with the interaction between PGC-1 α and FOXO1. FOXO1(3A) interacted with PGC-1 α independently of Akt expression. To determine whether phosphorylation of FOXO1 by Akt also disrupts the PGC-1 α -FOXO1 interaction *in vitro*, we performed binding with unmodified and phosphorylated FOXO1 and the glutathione

S-transferase (GST)-PGC-1 α fusion protein. This experiment was done in two ways: using Akt-mediated phosphorylation of FOXO1 before binding to PGC-1 α (Fig. 3c) or after binding to PGC-1 α (Fig. 3d). As shown in Fig. 3c, phosphorylation of FOXO1 by Akt strongly reduces its binding to PGC-1 α ($62 \pm 5\%$); as a control, the binding of FOXO1(3A) was not affected in the presence of Akt and ATP. Markedly similar results were observed when the PGC-1 α -FOXO1 complex was preformed. The PGC-1 α -FOXO1 complex was largely disrupted by Akt ($60 \pm 7\%$), and again the mutant FOXO1(3A) binding was not affected (Fig. 3d). These results strongly indicate that, in addition to causing exclusion from the nucleus, phosphorylation of FOXO1 by Akt specifically disrupts the interaction with PGC-1 α .

We next investigated whether a functional interaction between FOXO1 and PGC-1 α occurs in live animals, by infusing recombinant adenoviruses expressing PGC-1 α or the dominant-negative version of FOXO1 into mice. As expected, PGC-1 α increased the levels of gluconeogenic genes *Pck1* and *G6pc* in infected mice. Notably, co-infection with the dominant-negative allele of FOXO1 reduced the levels of these PGC-1 α -induced gluconeogenic genes, indicating that activation of gluconeogenic genes by PGC-1 α requires FOXO1 function (Fig. 4a). To evaluate whether insulin regulation of PGC-1 α function requires the FOXO1-PGC-1 α interaction *in vivo*, adenoviruses expressing PGC-1 α or control green fluorescent protein (GFP) were infused into wild-type and transgenic mice expressing the constitutively active form of FOXO1 in liver. These transgenic mice are slightly hyperglycaemic and demonstrate a moderate induction of gluconeogenic genes⁶. As expected, PGC-1 α induced an increase of *G6pc* and *Pck1* mRNAs in the liver of wild-type animals⁹, and caused a similar response in transgenic mice (Fig. 4b). However, whereas injection of insulin decreased the PGC-1 α -mediated expression of *Pck1* and *G6pc*

Figure 3 Insulin regulation of PGC-1 α action on gluconeogenesis depends on FOXO1 function in hepatocyte cells. **a**, Insulin regulation of PGC-1 α -mediated gluconeogenic gene expression is abolished by a constitutively active form of FOXO1. Fao rat hepatocytes were treated as described in Fig. 2. **b**, Constitutively activated Akt disrupts PGC-1 α -FOXO1 interaction. BOSC cells were transfected with the indicated plasmids and co-

immunoprecipitation was performed as described in the Methods. **c**, Phosphorylation of FOXO1 by Akt decreases binding to PGC-1 α . Binding assays were performed as described in Supplementary Fig. S2. **d**, Pre-bound PGC-1 α -FOXO1 complex is largely disrupted by Akt-mediated phosphorylation of FOXO1. Similar results were obtained in three different experiments.

Figure 4 Insulin regulation of PGC-1 α action on gluconeogenic genes depends on FOXO1 function in live mice. **a**, Dominant-negative FOXO1 (FOXO1(1–256)) suppresses PGC-1 α induction of gluconeogenic genes. Mice were injected with adenoviruses expressing control LacZ, PGC-1 α and FOXO1(1–256) as described in the Methods. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$. **b**, Insulin does not suppress PGC-1 α -induced

gluconeogenic genes in FOXO1(S253A) transgenic mice. Mice were injected with adenoviruses expressing GFP or PGC-1 α as described in the Methods. Asterisk, $P < 0.01$ compared with wild-type mice; triple asterisk, $P < 0.05$ compared with fasting values in PGC-1 α -expressing wild-type mice. **c**, Model illustrating the influence of the major hormones on the gluconeogenic genes. GR, glucocorticoid receptor.

mRNAs in control mice by 80% and 44%, respectively, a much smaller decrease in these mRNAs in response to insulin was observed in the constitutive FOXO1 transgenic mice (12% and 18%, respectively). Amounts of adenoviral-mediated PGC-1 α and FOXO proteins in mice were equivalent (data not shown). Taken together, these data demonstrate that the ability of PGC-1 α to induce gluconeogenesis requires its interaction with FOXO1, and that the ability of insulin to suppress PGC-1 α -mediated gluconeogenesis, both in cells and in live animals, is dependent on the ability of the insulin pathway to modify FOXO1 activity.

These data, combined with previous studies, allows a model that can readily incorporate all the classical hormonal influences on the process of gluconeogenesis (Fig. 4c). Glucagon and glucocorticoids are elevated in fasting and activate expression of *Pgc1*. In addition, PGC-1 α co-activates the liganded glucocorticoid receptor^{9,15}. Insulin has two distinct modes of action here: the lack of any suppression of PGC-1 α expression by insulin in isolated hepatocytes and in mice under hyperinsulinaemic, euglycaemic clamp conditions suggests that the elevation of PGC-1 α observed in various states of insulin deficiency may occur, at least in part, through its well-known suppression of glucagon release. However, as it is impossible to mimic the exact physiological environment in cell culture, a direct effect of insulin on hepatic PGC-1 α expression cannot be ruled out. Another study has shown a transient effect of insulin on PGC-1 α expression¹⁰, although we have not been able to observe this. What can be readily observed here, however, is a direct suppressive effect of insulin downstream of PGC-1 α . This effect seems to be through modification of FOXO1, in that expression of an allele of FOXO1 that is not modified by insulin/Akt signalling renders the gluconeogenic function of PGC-1 α completely insensitive to insulin. As demonstrated previously⁷, phosphorylation of FOXO1 by Akt causes its sequestration in the cytoplasm. As we show here, this modification of FOXO1 also has another function; that is, it specifically disrupts the FOXO1–PGC-1 α interaction.

Although physical interaction between PGC-1 α and other transcription factors such as the nuclear receptors HNF4- α and glucocorticoid receptor are probably critically important for this

response, FOXO1 is the first transcription factor shown to be required for the gluconeogenic action of PGC-1 α . Hence the PGC-1 α –FOXO1 complex must be considered a potential target for anti-gluconeogenic therapies for diabetes mellitus. The fact that this complex can be disrupted by a small number of phosphorylations raises hope that a small molecule can be developed that also inhibits this interaction. Although the model shown in Fig. 4c can integrate several of the known gluconeogenic hormones and transcription factors as regulators of PGC-1 α , it is worth noting that certain others do not yet fall easily into this scheme. The transcription factors C/EBP- α and - β as well as ADD1/SREBP1 are highly enriched in liver and have been shown to regulate hepatic gluconeogenesis^{16–18}. Whether PGC-1 α also co-activates these transcription factors or is a target of these factors is under investigation. □

Methods

Cell culture and treatments

Primary mouse hepatocytes were isolated and cultured in DMEM with 10% fetal bovine serum (FBS) as described¹⁹. Treatment with insulin (10 nM) and forskolin (1 μ M) was performed in 0.5% bovine serum albumin (BSA) for 14 h. Murine hepatocytes immortalized by SV40 large T antigen¹³ were cultured in α -MEM with 100 nM dexamethasone and 4% fetal calf serum. Fao rat hepatoma cells were cultured in RPMI medium with 10% fetal calf serum.

Transcriptional activation assays

Immortalized mouse hepatocytes were transiently transfected using FuGENE (Roche) or Superfect (Qiagen). After overnight incubation, medium was changed to 0.5% BSA in DMEM medium for 24 h. Insulin was added 2 h before the addition of dexamethasone and forskolin, which were present for the last 6 h. Cells were lysed and aliquots were used to measure β -galactosidase and luciferase activities. pcDNA3-Flag-FOXO1 plasmids were a gift from W. Sellers. PGC-1 α plasmids have been described previously²⁰.

Protein interaction analysis

pGEX2-PGC-1 α and pGEX2-FOXO1 plasmids were generated by cloning the corresponding polymerase chain reaction (PCR) fragments into the *Bam*H1 and *Xho*I cloning sites of these vectors. GST–PGC-1 α and GST–FOXO1 fragments were expressed in bacteria (BL21) by isopropyl- β -D-thiogalactoside induction for 3 h at room temperature. Fusion proteins were purified on Sepharose beads containing glutathione. [³⁵S]-labelled proteins were made with a TNT reticulocyte lysate kit (Promega). Equal amounts of GST fusion proteins (1 μ g) were mixed with 5 μ l of the *in vitro* translated proteins in a binding buffer containing 20 mM HEPES buffer (pH 7.7), 75 mM KCl, 0.1 mM EDTA, 2.5 mM

MgCl₂, 0.05% NP40, 2 mM dithiothreitol and 10% glycerol. Binding reactions were performed as in ref. 20.

For protein interaction experiments involving Akt-mediated phosphorylation, binding was performed as described above, and *in vitro* translated FOXO1 was phosphorylated with activated Akt (Upstate) following the manufacturer's instructions.

Co-immunoprecipitation experiments

Flag-tagged FOXO1 and PGC-1 α proteins were expressed in BOSC23 cells using FuGENE (Roche). Forty-eight hours after transfection, whole-cell extracts were prepared and subjected to an overnight incubation with a monoclonal antibody to Flag (Sigma) linked to agarose beads. The immunoprecipitates were washed four times with lysis buffer, separated by SDS-polyacrylamide gel electrophoresis, and immunoblotted using antibodies directed against the N terminus of PGC-1 α ²⁰.

ChIP assays

Immortalized hepatocytes were fixed with formaldehyde, lysed and then sonicated. Soluble chromatin was co-immunoprecipitated with anti-FOXO1 antiserum, anti-PGC-1 α antiserum or an equal amount of immunoglobulin- γ (IgG). After de-crosslinking of the DNA, samples were subjected to PCR using the following primers: PEPCK (–438 to –326), 5'-GTGGGAGTGACACCTCACAGC-3' and 5'-AGGGCAGGCCTAGCCGAGACG-3'; glucose-6-phosphatase (–251 to –31), 5'-GCCTCTAGCACTCAAGCAGTG-3' and 5'-TGTGCCTTGCCCTGTTTATATG-3'. These regions of amplification contain the FOXO1 binding sites of both the PEPCK promoter (accessory factor 2 site; AF2) and the glucose-6-phosphatase promoter (insulin response unit; IRU). We used standard reaction conditions and 25 cycles of amplification²¹.

Adenoviral infections

Fao hepatocytes and mouse primary hepatocytes were infected with adenoviruses expressing GFP or PGC-1 α at a multiplicity of infection of approximately 50 (ref. 9), and/or FOXO1 wild type, FOXO1(ADA) (where ADA is T24A/S253D/S316A) and FOXO1(1–258) at a multiplicity of infection of approximately 25 (ref. 13). Adenoviral infection was performed in RPMI 1640 medium and 0.5% BSA. After two days cells were treated with 10 nM insulin for 12 h. Cells were collected for RNA isolation using the Trizol reagent (Invitrogen).

Animal experiments

Recombinant adenovirus encoding GFP, PGC-1 α , LacZ and dominant-negative FOXO1 were purified by CsCl gradient centrifugation and concentrated to 1.3×10^{10} plaque-forming units per ml, corresponding to 2.5×10^{12} viral particles (vp) per ml. For the PGC-1 α and dominant-negative FOXO1 experiment (Fig. 4a), ten-week-old CD-1 mice were injected with 0.75×10^{11} vp per mouse. Each animal received the same total of viral particles per body weight and LacZ virus was used as a control. At day 2, blood glucose and insulin was measured and the mice were killed, the livers were removed and analysed for mRNA isolation, and real-time PCR with reverse transcription (RT) was performed. For the PGC-1 α adenoviral infection in FOXO1 transgenic mice (Fig. 4b), six-month-old Ttr/FOXO1(S253A) male transgenic mice and wild-type littermate controls⁶ were injected with purified virus through the tail vein at a dose of 4×10^9 vp g^{–1} in a total volume of 0.1 ml. The total viral load was approximately 1.1×10^{11} per mouse. Blood was taken immediately before and five days after injection to measure plasma glucose and hepatic enzymes ALS (acid-labile subunit) and ALT (alanine-aminotransferase). At the end of the fifth day, mice fed *ad libitum* were injected with insulin (0.75 U per kg body weight) intraperitoneally. After 1 h, plasma glucose levels were measured, animals were killed, and the livers removed and frozen in liquid nitrogen. Total mRNA isolation and real-time RT-PCR was performed⁶. For the glucose clamp experiments, 6–8-week-old mice were subjected to hyperinsulinaemic, euglycaemic clamps. After a 60-min basal period, mice underwent a 90 min euglycaemic clamp period, during which insulin was infused at 18 mU kg^{–1} min^{–1} (ref. 22). At the end of the insulin infusion period, livers were removed and snap-frozen. Liver mRNA was isolated and used for real-time RT-PCR.

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1. Unger, R. H. Glucagon physiology and pathophysiology in the light of new advances. *Diabetologia* **28**, 574–578 (1985).
2. Hanson, R. W. & Reshef, L. Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu. Rev. Biochem.* **66**, 581–611 (1997).
3. Hall, R. K. & Granner, D. K. Insulin regulates expression of metabolic genes through divergent signaling pathways. *J. Basic Clin. Physiol. Pharmacol.* **10**, 119–133 (1999).
4. Hall, R. K. *et al.* Regulation of phosphoenolpyruvate carboxykinase and insulin-like growth factor-binding protein-1 gene expression by insulin. The role of winged helix/forkhead proteins. *J. Biol. Chem.* **275**, 30169–30175 (2000).
5. Schmolli, D. *et al.* Regulation of glucose-6-phosphatase gene expression by protein kinase B α and the forkhead transcription factor FKHR. Evidence for insulin response unit-dependent and -independent effects of insulin on promoter activity. *J. Biol. Chem.* **275**, 36324–36333 (2000).
6. Nakae, J. *et al.* Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nature Genet.* **32**, 245–253 (2002).
7. Brunet, A. *et al.* Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857–868 (1999).
8. Nakae, J., Park, B. C. & Accili, D. Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. *J. Biol. Chem.* **274**, 15982–15985 (1999).
9. Yoon, J. C. *et al.* Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* **413**, 131–138 (2001).

10. Herzig, S. *et al.* CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* **413**, 179–183 (2001).
11. Puigserver, P. *et al.* A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* **92**, 829–839 (1998).
12. Rena, G., Guo, S., Cichy, S. C., Unterman, T. G. & Cohen, P. Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B. *J. Biol. Chem.* **274**, 17179–17183 (1999).
13. Nakae, J., Kitamura, T., Silver, D. L. & Accili, D. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J. Clin. Invest.* **108**, 1359–1367 (2001).
14. Durham, S. K. *et al.* FKHR binds the insulin response element in the insulin-like growth factor binding protein-1 promoter. *Endocrinology* **140**, 3140–3146 (1999).
15. Knutti, D., Kaul, A. & Kralli, A. A tissue-specific coactivator of steroid receptors, identified in a functional genetic screen. *Mol. Cell. Biol.* **20**, 2411–2422 (2000).
16. Darlington, G. J., Wang, N. & Hanson, R. W. C/EBP α : a critical regulator of genes governing integrative metabolic processes. *Curr. Opin. Genet. Dev.* **5**, 565–570 (1995).
17. Arizmendi, C., Liu, S., Croniger, C., Poli, V. & Friedman, J. E. The transcription factor CCAAT/enhancer-binding protein β regulates gluconeogenesis and phosphoenolpyruvate carboxykinase (GTP) gene transcription during diabetes. *J. Biol. Chem.* **274**, 13033–13040 (1999).
18. Becard, D. *et al.* Adenovirus-mediated overexpression of sterol regulatory element binding protein-1c mimics insulin effects on hepatic gene expression and glucose homeostasis in diabetic mice. *Diabetes* **50**, 2425–2430 (2001).
19. Zaher, H. *et al.* The involvement of aryl hydrocarbon receptor in the activation of transforming growth factor- β and apoptosis. *Mol. Pharmacol.* **54**, 313–321 (1998).
20. Puigserver, P. *et al.* Activation of PPAR γ coactivator-1 through transcription factor docking. *Science* **286**, 1368–1371 (1999).
21. Nakae, J. *et al.* The forkhead transcription factor Foxo1 regulates adipocyte differentiation. *Dev. Cell* **4**, 119–129 (2003).
22. Rossetti, L. *et al.* Hepatic overexpression of insulin-like growth factor-II in adulthood increases basal and insulin-stimulated glucose disposal in conscious mice. *J. Biol. Chem.* **271**, 203–208 (1996).

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Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors

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Members of the nuclear receptor (NR) superfamily of transcription factors modulate gene transcription in response to small lipophilic molecules¹. Transcriptional activity is regulated by ligands binding to the carboxy-terminal ligand-binding domains (LBDs) of cognate NRs. A subgroup of NRs referred to as 'orphan receptors' lack identified ligands, however, raising issues about the function of their LBDs². Here we report the crystal structure of the LBD of the orphan receptor Nurr1 at 2.2 Å resolution. The Nurr1 LBD adopts a canonical protein fold resembling that of