ARTICLES

The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism

Seung-Hoi Koo[1]*, Lawrence Flechner[1]*, Ling Qi[1], Xinmin Zhang[1], Robert A. Screaton[1], Shawn Jeffries[1], Susan Hedrick[1], Wu Xu[2], Fayçal Boussouar[2], Paul Brindle[2], Hiroshi Takemori[3] & Marc Montminy[1]

Glucose homeostasis is regulated systemically by hormones such as insulin and glucagon, and at the cellular level by energy status. Glucagon enhances glucose output from the liver during fasting by stimulating the transcription of gluconeogenic genes via the cyclic AMP-inducible factor CREB (CRE binding protein). When cellular ATP levels are low, however, the energy-sensing kinase AMPK inhibits hepatic gluconeogenesis through an unknown mechanism. Here we show that hormonal and energy-sensing pathways converge on the coactivator TORC2 (transducer of regulated CREB activity 2) to modulate glucose output. Sequestered in the cytoplasm under feeding conditions, TORC2 is dephosphorylated and transported to the nucleus where it enhances CREB-dependent transcription in response to fasting stimuli. Conversely, signals that activate AMPK attenuate the gluconeogenic programme by promoting TORC2 phosphorylation and blocking its nuclear accumulation. Individuals with type 2 diabetes often exhibit fasting hyperglycaemia due to elevated gluconeogenesis; compounds that enhance TORC2 phosphorylation may offer therapeutic benefits in this setting.

Type 2 diabetes affects nearly 20 million individuals in the United States alone[1,2]. Diabetes complications such as renal failure, microvascular disease and peripheral neuropathy account for an increasing proportion of annual health care costs. Tight glycaemic control has been associated with a reduced incidence of diabetes complications, underscoring efforts to characterize regulatory pathways that function importantly in glucose metabolism[3].

Circulating glucose levels reflect a balance between glucose production by the liver and glucose utilization by skeletal muscle. Under fasting conditions, this balance is largely maintained by decreasing glucose uptake into muscle and increasing glucose output from the liver via gluconeogenesis. Pancreatic glucagon normally triggers the activation of catabolic programmes in the liver in part via the cAMP responsive factor CREB[4–6]. CREB in turn stimulates hepatic gluconeogenesis as well as fatty acid oxidation by inducing expression of the nuclear hormone receptor coactivator PGC-1α (peroxisome-proliferation-activated receptor-γ coativator-1)[7–10]. Mice deficient in PGC-1α, due either to acute RNA interference (RNAi)-mediated knockdown or to targeted gene disruption of the *PGC-1a* gene, display fasting hypoglycaemia with reduced expression of gluconeogenic genes[9–11].

Under feeding conditions, insulin silences hepatic PGC-1α expression and transcriptional activity via the Akt-mediated phosphorylation and nuclear export of the forkhead family activator FOXO1 (refs 6, 12). Indeed, loss of insulin signalling in diabetes leads to elevated hepatic PGC-1α expression and excessive hepatic glucose production[8]. Within this regulatory framework the gluconeogenic programme is further modified by adipocyte-derived hormones called adipokines[13,14]. Among these, resistin and adiponectin have been shown to modulate gluconeogenesis via the energy-sensing kinase AMPK, although the underlying mechanisms have not been determined.

Role of CREB coactivators during fasting

Glucagon is thought to enhance hepatic gene expression by stimulating the protein kinase A (PKA)-mediated phosphorylation of CREB at Ser 133 and by promoting the subsequent recruitment of the coactivator CBP (CREB-binding protein) to the promoter[15,16]. To test this notion, we injected mice intraperitoneally with glucagon or insulin, and monitored CREB activation in liver. Intraperitoneal injection of glucagon stimulated hepatic CREB phosphorylation (p-CREB) within 10 min, as detected by histochemical and western blot analysis (Fig. 1a, b). Unexpectedly, intraperitoneal insulin administration had comparable effects on p-CREB levels (Fig. 1a, b). To rule out potential counter-regulatory effects of hypoglycaemia after insulin treatment, we co-injected glucose (0.5 g per kg body weight) with insulin and observed comparable CREB phosphorylation relative to insulin alone (Supplementary Fig. S1). Taken together, these results indicate that the CREB–CBP pathway is unlikely to discriminate between fasting and feeding signals in liver.

We next tested the potential involvement of other CREB coactivators in this setting. In recent studies, cAMP has been found to stimulate cellular gene expression by triggering the dephosphorylation and nuclear entry of TORCs, a family of latent cytoplasmic coactivators that enhances cellular gene expression via an interaction with the CREB basic region/leucine zipper (bZIP) DNA-binding domain[17–20]. We examined TORC2 activity because this family member was expressed at highest levels relative to TORC1 and TORC3 in liver (data not shown).

Adenovirus-expressed TORC2 (Ad-TORC2) was localized primarily in the cytoplasm under *ad libitum* feeding conditions, translocating to the nucleus within 10 min after intraperitoneal glucagon administration (Fig. 1a). By contrast with its effect on CREB phosphorylation, insulin did not promote Ad-TORC2 nuclear entry, demonstrating the capacity of TORC2 to distinguish between

[1]Peptide Biology Laboratories, Salk Institute for Biological Studies, 10010 N Torrey Pines Rd, La Jolla, California 92037-1002, USA. [2]Department of Biochemistry, St Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, Tennessee 38105, USA. [3]Department of Biochemistry and Molecular Biology, Graduate School of Medicine (H-1), Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.
*These authors contributed equally to this work.

fasting and feeding cues. Consistent with this activation profile, both Ad-TORC2 and endogenous TORC2 were highly phosphorylated at Ser 171 under *ad libitum* feeding or insulin-stimulated conditions, and were dephosphorylated only in response to glucagon or fasting (Fig. 1b; see also Supplementary Figs S1 and S2). Arguing against an inhibitory role for insulin itself in modulating TORC2 localization directly, endogenous hepatic TORC2 was comparably dephosphorylated in mice injected with glucagon or glucagon plus insulin (Supplementary Fig. S1).

After nuclear entry, TORC2 is thought to stimulate cellular gene expression via an association with CREB on relevant promoters[19]. In chromatin immunoprecipitation assays of liver tissue samples, glucagon but not insulin promoted recruitment of TORC2 to gluconeogenic genes (Fig. 1c). Similarly, addition of the cAMP agonist forskolin (FSK) to primary cultures of rat hepatocytes induced TORC2 occupancy over *PEPCK* (phospho-enol pyruvate carboxykinase), *G6Pase* (glucose-6-phosphatase) and *PGC-1a* promoters (Fig. 1d). FSK also stimulated CREB phosphorylation over gluconeogenic genes, indicating that both CREB–TORC and CREB–CBP pathways are probably activated in this setting (Supplementary Fig. S3). Consistent with this notion, we observed CBP recruitment to *PEPCK* and *G6Pase* promoters in response to FSK (Supplementary Fig. S3).

Although CREB activity is regulated by both CBP and P300, CBP has been proposed to exert a key role in modulating hepatic gluconeogenesis; hepatic glucose output during feeding can be blocked by insulin-dependent phosphorylation of CBP at a site (Ser 436) that is absent in P300 (ref. 21). To test the importance of

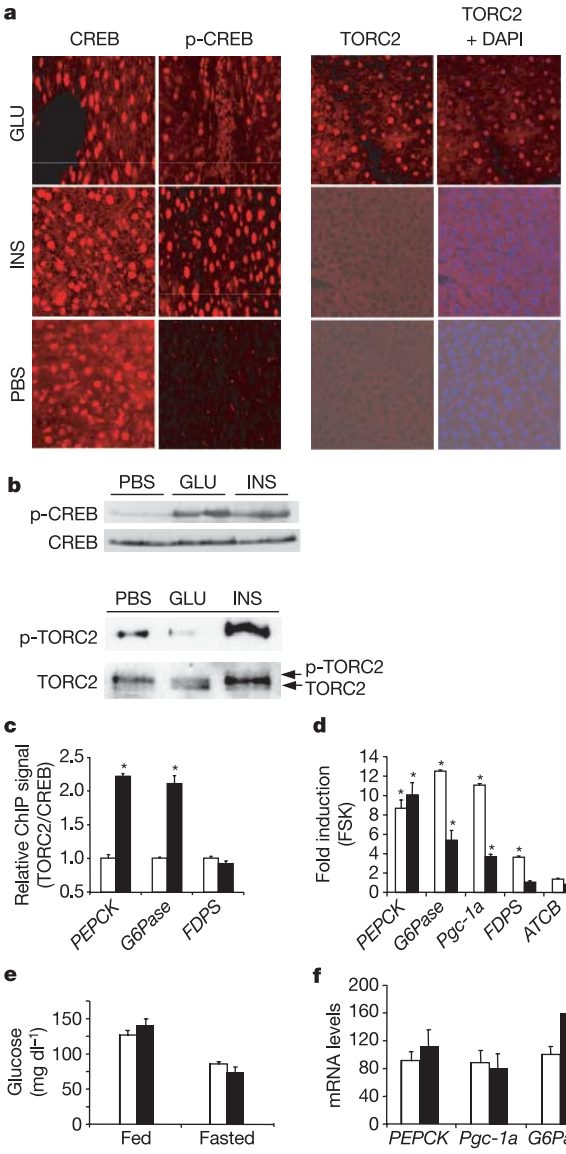

Figure 1 | Fasting hormones activate TORC2. **a**, **b**, Immunohistochemical (**a**) and western blot (**b**) assays showing effect of insulin (INS), glucagon (GLU) or saline (PBS) injection (10 min, intraperitoneal injection) on CREB, phospho-CREB (p-CREB), TORC2 and phospho-TORC2 (p-TORC2) localization and levels in liver. **c**, Relative effect of insulin (open columns) and glucagon (filled columns) on TORC2 occupancy over PEPCK and G6Pase genes relative to CREB by chromatin immunoprecipitation (ChIP) assay (TORC2/CREB; asterisk, $P < 0.05$, *t*-test). **d**, Effect of FSK on p-CREB (open columns) and TORC2 (filled columns) occupancy over gluconeogenic and control (FDPS and ATCB) promoters in primary hepatocytes (asterisk, $P < 0.05$, *t*-test). **e**, **f**, CREB–CBP complexes are not required for glucose homeostasis. **e**, Blood glucose levels for $CBP^{+/+}$ (open columns; $n = 10$) and $CBP^{kix/kix}$ (filled columns; $n = 7$) mice under fed ($P = 0.19$) and fasted (18 h, $P = 0.19$) conditions (*t*-test). **f**, Quantitative PCR analysis of *PEPCK* ($P = 0.43$), *Pgc-1a* ($P = 0.83$) and *G6Pase* ($P = 0.11$) mRNA levels in livers from fasted $CBP^{+/+}$ (open columns; $n = 7$) and $CBP^{kix/kix}$ (filled columns; $n = 7$) mice. mRNA levels in this and other figures are shown as relative values. Data in **c**–**f** represent mean ± s.e.m.

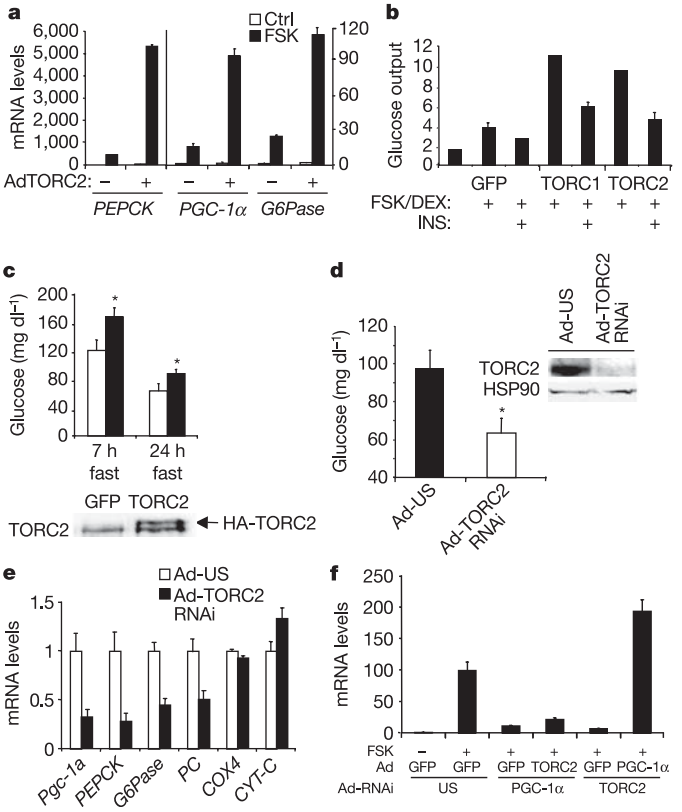

Figure 2 | TORC2 is required for fasting hepatic gluconeogenesis. **a**, **b**, Effect of Ad-TORC2 on gluconeogenic gene expression (**a**) and glucose output (**b**) in primary hepatocytes exposed to FSK, FSK plus dexamethasone (FSK/DEX) and insulin, as indicated. **c**, Blood glucose levels in Ad-GFP (open columns) and Ad-TORC2 (filled columns) mice after 7-h and 24-h fasts (asterisk, $P < 0.01$, *t*-test) ($n = 3$). Bottom: western blot showing levels of Ad-TORC2 (HA-TORC2) relative to endogenous TORC2 in livers from mice above. **d**, **e**, Effect of Ad-TORC2 RNAi or control (Ad-US) RNAi on fasting glucose levels ($n = 3$) (asterisk, $P < 0.01$, *t*-test) (**d**) and gluconeogenic gene expression (**e**). Inset in **d** shows a western blot of hepatic TORC2 levels in Ad-US and Ad-TORC2 RNAi mice. **f**, Effect of Ad-PGC-1α and Ad-TORC2 on *PEPCK* mRNA levels in primary hepatocytes co-expressing Ad-TORC2 RNAi, Ad-PGC-1α RNAi, or Ad-US. Data in **a**–**f** represent mean ± s.e.m.

the CREB–CBP interaction in this process we used $CBP^{kix/kix}$ mice (*CBP* is also known as *Crebbp*), which contain knockin mutations in the CREB-binding domain (KIX; amino acids 586–672) that specifically block CREB–CBP complex formation[22]. If insulin disrupts gluconeogenesis by blocking the CREB–CBP interaction, then $CBP^{kix/kix}$ mice would be expected to display fasting hypoglycaemia and reduced gluconeogenic gene expression. Remarkably, blood glucose levels were very similar between $CBP^{kix/kix}$ mice and control littermates under either feeding or fasting conditions (Fig. 1e). Moreover, we observed no discernable difference in fasting levels of *PEPCK* (also known as *Pck2*) *G6Pase* (also known as *G6pc*) or *Pgc-1a* (also known as *Ppargc1a*, the product of which is PGC-1α) messenger RNAs between $CBP^{kix/kix}$ and wild-type mice (Fig. 1f), arguing against the notion that recruitment of CBP to CREB target genes *per se* is necessary for induction of the gluconeogenic programme.

Role of TORC2 in glucose metabolism

On the basis of the ability of TORC2 to enter the nucleus and to associate with CREB target genes in response to fasting signals, we explored the role of this coactivator in directing the gluconeogenic programme. Ad-TORC2 had marginal effects on gluconeogenic genes under basal conditions, but strongly potentiated their expression after exposure to FSK (Fig. 2a). The stimulatory effects of TORC2 were CREB-dependent; expression of a dominant negative CREB polypeptide (A-CREB), which specifically inhibits binding of CREB but not other bZIP family members to DNA[23], disrupted TORC2 activity on *PECPK*, *G6Pase* and *PGC-1a* genes (Supplementary Fig. S4 and data not shown).

The ability of TORC2 to stimulate the gluconeogenic programme in hepatocytes suggests that it may promote glucose output in response to fasting signals. Ad-TORC2 enhanced glucose production in primary rat hepatocytes after exposure to FSK plus dexamethasone; these effects were inhibited by co-treatment with insulin (Fig. 2b; see also Supplementary Fig. S5). When expressed at levels comparable to endogenous TORC2 in liver, Ad-TORC2 also promoted fasting hyperglycaemia *in vivo* (Fig. 2c). Indeed, levels of circulating insulin were elevated in Ad-TORC2 mice, indicating that the enhanced hepatic glucose output in this setting is sufficient to trigger a counter-regulatory response (Supplementary Fig. S6).

On the basis of its ability to stimulate the gluconeogenic programme and to promote hyperglycaemia when overexpressed in mice, endogenous TORC2 may be required for the metabolic response to fasting signals in liver. To test this idea, we used an Ad-TORC2 RNAi construct to reduce hepatic TORC2 expression (80% by western blot assay; Fig. 2d). Mice made acutely deficient in TORC2 exhibited fasting hypoglycaemia (65 mg dl^{-1} versus 100 mg dl^{-1}); hepatic mRNA levels for gluconeogenic genes were reduced 2–4-fold relative to control littermates (Fig. 2e). Taken together, these results indicate that TORC2 exerts significant regulatory control over hepatic gluconeogenesis in the fasted state.

In previous studies, the nuclear hormone receptor coactivator PGC-1α was found to direct expression of the gluconeogenic programme in liver during fasting[7–10]. Although the ability of TORC2 to stimulate PGC-1α expression and to associate with its promoter suggests that TORC2 lies upstream of PGC-1α, our results did not exclude a scenario in which TORC2 and PGC-1α individually regulate fasting metabolism in liver via parallel pathways. In that case, overexpression of one coactivator (TORC2 or PGC-1α) might be expected to compensate for deficiencies in the other. Knockdown of either PGC-1α or TORC2 with Ad-RNAi constructs disrupted *PEPCK* gene expression in primary hepatocytes exposed to FSK (Fig. 2f). Ad-PGC-1α rescued expression of the *PEPCK* gene in TORC2-deficient cells, but Ad-TORC2 had no effect on *PEPCK* expression in PGC-1α-deficient cells (Fig. 2f), demonstrating that PGC-1α probably acts downstream of TORC2 in the gluconeogenic pathway.

Attenuation of TORC2 by a feedback loop

Similar to other second messengers, cAMP stimulates cellular gene expression with burst-attenuation kinetics[24]. After exposure of primary hepatocytes to glucagon, *PEPCK* mRNA levels became maximal after 2 h and returned to near baseline after 4 h (Fig. 3a). Consistent with this profile, TORC2 was re-phosphorylated at Ser 171, and hence inactivated, 3 h after glucagon stimulation in primary rat hepatocytes (Fig. 3b). Pre-treatment with the protein synthesis inhibitor cycloheximide (CHX) blocked the phosphorylation of TORC2 at Ser 171 by glucagon, suggesting that fasting signals promote the synthesis of an inhibitor, which in turn feeds back to shut down the CREB–TORC pathway (Fig. 3b). Supporting this notion, CHX pre-treatment enhanced gluconeogenic gene expression 2–3-fold in primary rat hepatocytes exposed to glucagon (Fig. 3c).

On the basis of the ability of CHX to disrupt TORC2 Ser 171 phosphorylation during the attenuation period, we reasoned that glucagon may induce the expression of an inhibitory kinase. In previous studies, the SIK (salt-inducible kinase; also known as SNF1-like kinase) family of AMP kinases was found to associate with and to phosphorylate TORC2 at Ser 171 (ref. 19), an optimal consensus site for phosphorylation by AMPK family members (LNRTSSDSAL; Ser 171 is underlined). Indeed, fasting increased *SIK1* mRNA and protein levels in liver fourfold relative to feeding conditions, leaving other SIK family members (SIK2 and SIK3) relatively unaffected (Fig. 3d). Similarly, exposure of primary rat hepatocytes to glucagon or FSK stimulated *SIK1* mRNA and protein accumulation via a CREB-dependent mechanism; Ad-TORC2 potentiated *SIK1* gene expression in cells exposed to FSK (Fig. 3e; see also Supplementary Fig. S7). Notably, the accumulation of SIK1 protein in cells exposed

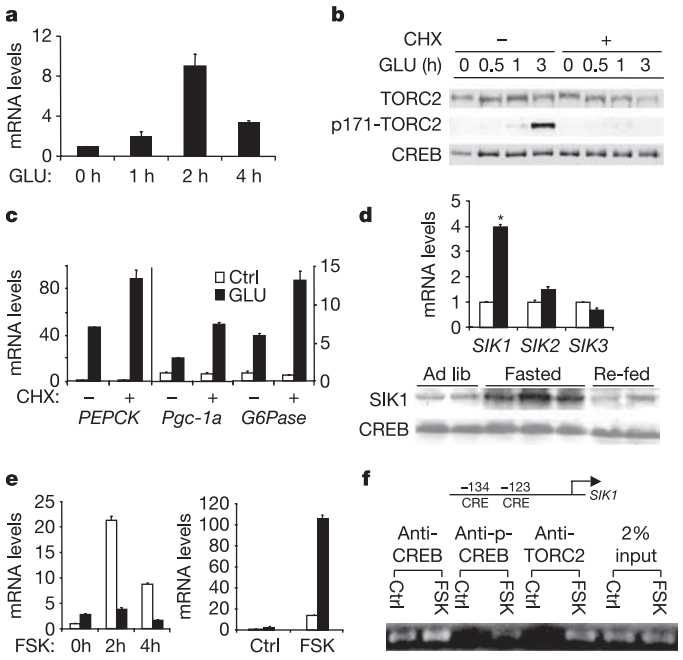

Figure 3 | Induction of SIK1 during fasting attenuates the gluconeogenic programme in primary hepatocytes. **a**, Time course of PEPCK gene expression after exposure to glucagon. **b**, **c**, Effect of glucagon and CHX on TORC2 or p-TORC2 protein levels (**b**), and on mRNA levels for gluconeogenic genes (**c**). **d**, Effect of feeding, fasting, or re-feeding on *SIK1*, *SIK2* and *SIK3* mRNA levels (top, $n = 3$) (asterisk, $P < 0.01$, *t*-test) and on SIK1 protein levels in liver (bottom). Control, open columns; fasted, filled columns. **e**, Effect of Ad-GFP (open columns) or Ad-A-CREB (filled columns) (left) and Ad-GFP (open columns) or Ad-TORC2 (filled columns) (right) on *SIK1* mRNA levels in primary hepatocytes exposed to FSK. **f**, Top: CREs on SIK1 promoter. Bottom: chromatin immunoprecipitation assay of CREB, p-CREB and TORC2 on SIK1 promoter from control or FSK-treated (10 μM, 2 h) cells. Data in **a**, **c**–**e** represent means ± s.e.m.

to glucagon was blocked by CHX, implicating SIK1 as an inducible silencer of gluconeogenic genes (Supplementary Fig. S7).

In studies to determine how fasting signals regulate SIK1 expression, we noticed two consensus cAMP-responsive promoter elements (CREs) in rat, mouse and human orthologues of the SIK1 promoter (Fig. 3f). In transient assays of human HepG2 hepatocytes, PKA stimulated *SIK1* promoter activity about 20-fold; these effects were disrupted by co-expression of A-CREB (Supplementary Fig. S8). Indeed, CREB was found to occupy the *SIK1* promoter in chromatin immunoprecipitation assays of primary rat hepatocytes; TORC2 was recruited to this promoter in response to FSK treatment, indicating that fasting signals regulate SIK1 gene expression via the CREB–TORC pathway (Fig. 3f).

SIK1 inhibits hepatic gluconeogenesis

If SIK1 functions as part of a negative feedback loop for the gluconeogenic programme, then reducing cellular SIK1 levels would be expected to enhance the expression of these genes by increasing TORC2 activity. Levels of unphosphorylated—and therefore activated—TORC2 were twofold higher in Ad-SIK1 RNAi-expressing hepatocytes compared to control cells after exposure to glucagon (Fig. 4a; see also Supplementary Fig. S9); we observed a corresponding increase in mRNA levels for *PEPCK*, *Pgc-1a* and *G6Pase* in SIK1-deficient cells, confirming the inhibitory role of this kinase on the gluconeogenic programme (Fig. 4b). Indeed, knockdown of SIK1 in mice promoted both fasting hyperglycaemia and gluconeogenic gene expression, demonstrating a key role for this kinase *in vivo* (Fig. 4c, d).

On the basis of the ability of Ad-SIK1 RNAi to enhance gluconeogenesis, we suspected that, conversely, SIK1 overexpression would block induction of the gluconeogenic programme. When expressed in hepatocytes, SIK1 promoted Ser 171 phosphorylation and hence disruption of TORC2 activity on the *PEPCK* promoter in cells exposed to FSK (Supplementary Figs S10 and S11, left panel). By contrast, SIK1 had no effect on the ability of PGC-1α to stimulate transcription from a PPAR-α target gene (acyl CoA oxidase (*AOX*)), arguing against an inhibitory effect of this kinase on gluconeogenic regulators downstream of TORC2 (Supplementary Fig. S11, right panel).

Having seen that SIK1 inhibits TORC2 activity on gluconeogenic promoters, we evaluated its role in fasting glucose metabolism. Relative to control Ad-GFP littermates, Ad-SIK1 as well as Ad-SIK2 mice exhibited fasting hypoglycaemia and reduced gluconeogenic gene expression (Fig. 4e, f). Although the ability of Ad-SIK1 to block hepatic glucose output in this setting could reflect an unanticipated induction of the insulin signalling pathway, fasting insulin levels were actually lower in Ad-SIK1 and Ad-SIK2 compared to

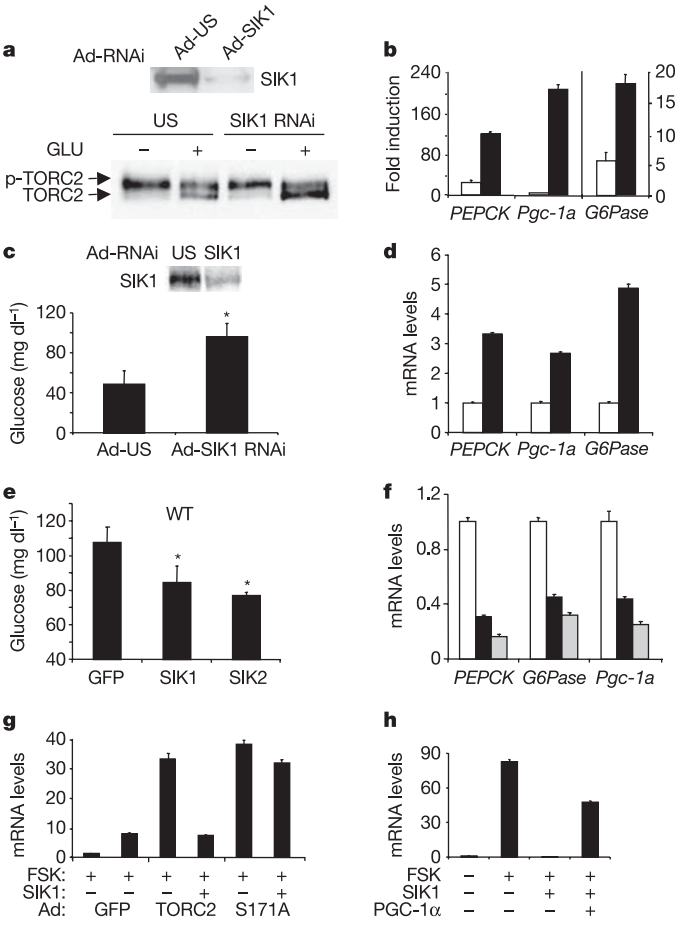

Figure 4 | SIKs inhibit hepatic gluconeogenesis via Ser 171 phosphorylation of TORC2. **a**, **b**, Effect of Ad-US (open columns) and Ad-SIK1 RNAi (filled columns) on SIK1, TORC2 and p-TORC2 protein levels (**a**) and on mRNA levels for gluconeogenic genes (**b**) in primary hepatocytes exposed to glucagon. **c**, **d**, Fasting glucose (**c**) and gluconeogenic mRNA levels (**d**) in Ad-US (open columns in **d**) or Ad-SIK1 RNAi (filled columns in **d**) mice fasted for 4 h (asterisk, $P < 0.01$, *t*-test). Inset in **c** shows a western blot of hepatic SIK1 levels in Ad-US and Ad-SIK1 RNAi mice. **e**, **f**, Effect of Ad-SIK1 (black columns in **f**), Ad-SIK2 (grey columns in **f**) or control Ad-GFP (white columns in **f**) on fasting glucose levels (**e**) (asterisk, $P < 0.01$, *t*-test) ($n = 4$) and on mRNA levels for gluconeogenic genes (**f**). **g**, **h**, Effect of Ad-SIK1 on *PEPCK* mRNA levels in primary hepatocytes co-expressing Ad-TORC2 and Ad-TORC2(Ser171Ala) (**g**) or Ad-PGC-1α (**h**). Treatment with FSK is as indicated. Data in **b**–**h** represent mean ± s.e.m.

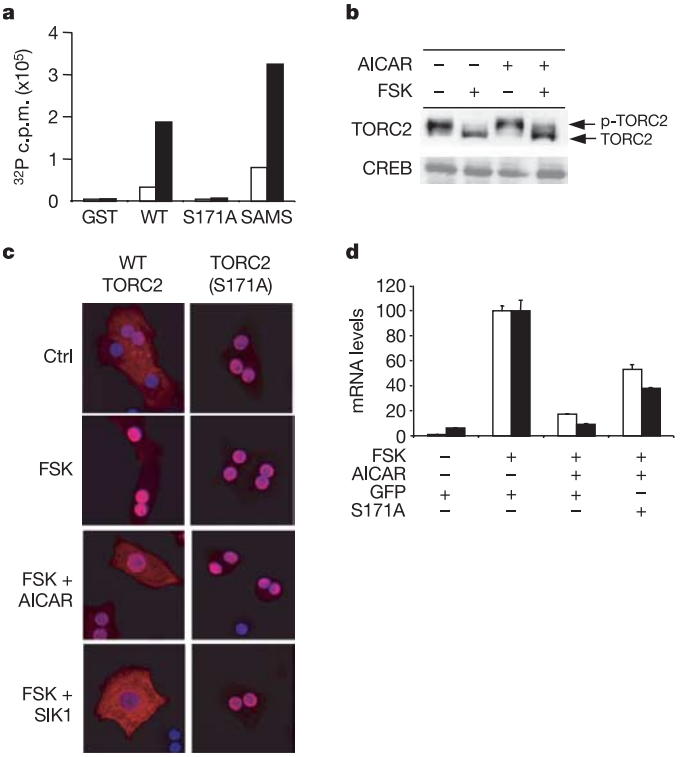

Figure 5 | AMPK inhibits activation of TORC2 by fasting signals. **a**, *In vitro* phosphorylation of wild-type, GST–TORC2(Ser171Ala) (161–181), GST, or optimal substrate (SAMS) by activated AMPK with (filled columns) or without (open columns) AMP addition to reactions. c.p.m., counts per min. **b**, Effect of AMPK inducer AICAR on p-TORC2 levels in primary hepatocytes. Treatment with FSK is indicated. **c**, Immunocytochemical analysis showing effects of AICAR and Ad-SIK1 on Ad-TORC2 and Ad-TORC2(Ser171Ala) localization in primary hepatocytes. **d**, Effect of AICAR on expression of gluconeogenic genes in primary hepatocytes. *PEPCK*, open columns; *Pgc-1a*, filled columns. Rescue with Ad-TORC2(Ser171Ala) is indicated. Data represent mean ± s.e.m.

control mice (Supplementary Fig. S12). Ad-SIK1 and Ad-SIK2 were also effective in reducing blood glucose levels in fasted *db*/*db* diabetic mice, which exhibit hepatic insulin resistance (Supplementary Fig. S13). Indeed, SIK expression did not improve insulin signalling in *db*/*db* animals, as measured by western blot analysis of phosphorylated (Ser 473) Akt levels in liver extracts from mice after acute intraperitoneal insulin injection (10 min; Supplementary Fig. S14). Taken together these results suggest that SIKs disrupt hepatic gluconeogenesis via an insulin-independent mechanism.

To evaluate whether SIK1 inhibits the gluconeogenic programme via TORC2 phosphorylation, we prepared an adenovirus expressing phosphorylation-defective TORC2 (TORC2(Ser171Ala)). Wild-type and mutant Ad-TORC2(Ser171Ala) were expressed at comparable levels and had similar effects on *PEPCK* and *PGC-1a* gene expression in primary hepatocytes exposed to FSK (Fig. 4g; see also Supplementary Fig. S15). Ad-SIK1 blocked wild-type TORC2 activity almost completely but had little effect on TORC2(Ser171Ala), demonstrating the importance of Ser 171 for inhibition of the gluconeogenic programme by this kinase (Fig. 4g). Indeed, Ad-PGC-1α was competent to rescue *PEPCK* expression in Ad-SIK1 infected cells, confirming the notion that SIK1 inhibits hepatic gene expression specifically at the level of TORC2 phosphorylation (Fig. 4h).

AMPK blocks TORC2 activation

Activation of the AMPK pathway in response to energy stress has been shown to block expression of the gluconeogenic programme, although the relevant targets for inhibition have not been identified[14,25]. Changes in cellular ATP levels seem to be uniquely sensed by AMPK, the founding member of this family[26–28]. The presence of a consensus AMPK phosphorylation site (Ser 171) that modulates TORC2 activity in liver prompted us to consider whether, in a manner analogous to SIK1, AMPK triggers TORC2 phosphorylation in response to ATP depletion and thereby inhibits hepatic gluconeogenesis. Activated AMPK was found to phosphorylate a wild-type but not Ser171Ala mutant GST–TORC2 (161–181) peptide *in vitro* at levels comparable to an optimal AMPK peptide substrate (SAMS)[27] (Fig. 5a). Addition of AMP to reactions further stimulated Ser 171 phosphorylation by AMPK on both GST–TORC2(Ser171Ala) (161–181) and GST–TORC2 (1–240) polypeptides (Supplementary Fig. S16). Consistent with this result, selective activation of cellular AMPK by exposure of primary hepatocytes to the AMP analogue 5-aminoimidazole-4-carboxamide riboside (AICAR) triggered phosphorylation of endogenous TORC2 twofold even in the presence of FSK (Fig. 5b; see also Supplementary Figs S17 and S18).

Given that it lies on a different signalling axis from the cAMP–PKA pathway, AMPK would be predicted to inhibit nuclear entry of TORC2 in cells exposed to FSK. Using primary rat hepatocytes infected with Ad-TORC2 constructs, we found that FSK triggered translocation of wild-type TORC2 (Fig. 5c). Confirming the importance of Ser 171 in this regard, mutant TORC2(Ser171Ala) protein remained constitutively nuclear under both conditions. Treatment with AICAR inhibited nuclear entry of wild-type but not TORC2(Ser171Ala) in cells exposed to FSK; Ad-SIK1 similarly blocked TORC2 nuclear entry in a Ser 171-dependent manner (Fig. 5c).

The ability of AICAR to limit TORC2 nuclear entry in response to fasting signals suggests that AMPK disrupts gluconeogenesis via Ser 171 phosphorylation. Indeed, exposure of primary hepatocytes to AICAR blocked induction of *PEPCK* and *PGC-1a* genes in response to FSK (Fig. 5d). However, phosphorylation-defective Ad-TORC2(Ser171Ala) restored expression of *G6Pase*, *PEPCK* and *PGC-1a* in the presence of AICAR inhibitor, demonstrating the importance of Ser 171 in mediating inhibitory effects of AMPK on gluconeogenic genes (Fig. 5d; see also Supplementary Fig. S19).

Discussion

Our studies suggest that TORC2 is a critical switch that modulates the gluconeogenic programme in response to both hormonal and intracellular signals. Although the CREB–CBP interaction itself did not seem to be critical for hepatic gene activation during fasting, we imagine that CBP and P300 together provide necessary histone acetylase activities, which further enhance TORC2 function in this setting.

TORC2 activity is tightly regulated during fasting by SIK1, which forms part of an auto-regulatory loop that attenuates the gluconeogenic programme by phosphorylating TORC2 at Ser 171 and thus promoting its export to the cytoplasm. Other SIK family members (SIK2 and SIK3) probably inhibit TORC2 activity as well, particularly under feeding conditions when SIK1 levels are low.

Superimposed on this regulatory circuit, TORC2 activity is additionally modulated at the cellular level by AMPK, the induction of which disrupts hepatic glucose production in response to stressors. Indeed, the ability of AMPK to catalyse Ser 171 phosphorylation even in the presence of cAMP agonist suggests that this pathway may override the inductive effects of hormones such as glucagon on fasting metabolism. A number of adipokines such as adiponectin[14] and resistin[13] have been shown to modulate hepatic gluconeogenesis via an AMPK-dependent mechanism; our results provide a regulatory framework with which to test these regulatory inputs. Metformin, a compound that also activates AMPK[29], has been widely used for treatment of type 2 diabetes owing to its effects in blocking hepatic gluconeogenesis and in stimulating glucose uptake in muscle[30]. Other compounds that enhance TORC2 phosphorylation in liver may provide similar therapeutic benefit for individuals with insulin resistance.

METHODS

Primary cultures. Primary rat hepatocytes were prepared from Sprague–Dawley rats as described[9]. After 16-h adenoviral infection, cells were maintained in serum-free medium 199 (Invitrogen) for 24 (cDNA expression adenoviruses) or 48 h (RNAi adenoviruses); cells were exposed to 10 μM forskolin or 100 nM glucagon for 2 h unless noted otherwise. Cells were treated with AICAR (1 mM) for either 30 min (western blot, immunostaining) or 2 h (quantitative PCR analysis). CHX (10 μg ml^{-1}) was added 30 min before addition of 100 nM glucagon. Glucose output assays were performed as reported using a glucose Trinder kit (Sigma)[31]. Cells infected with TORC1, TORC2 or GFP adenovirus were treated with FSK plus dexamethasone for 4 h or with insulin (100 nM) for 16 h.

Adenoviruses. Control GFP and unspecific RNAi adenoviruses were as described[9]. Adenoviruses for SIK1, SIK2 as well as wild-type and TORC2(Ser171Ala) expression were generated as described[9].

Animals. Male 7-week-old C57BL6 mice (Harlan) or diabetic *db*/*db* mice (Jackson laboratory) were used. Adenovirus injections were performed as reported[9]. Sixteen-hour fasting blood glucose levels were measured either 5 days (Ad-TORC2 RNAi, Ad-SIK1/2) or 9 days (Ad-TORC2) after injection. Four-hour fasting blood glucose levels were measured for Ad-SIK1 RNAi mice 2 days after injection. Ad-TORC2 mice (Fig. 1) were injected intraperitoneally with glucagon (5 μg per kg body weight), insulin (1 unit per kg), or PBS, and livers were collected for immunostaining or western blot analysis after 10 min. Absence of adenovirus-induced hepatitis was confirmed by measuring circulating ALT/AST levels.

Metabolites. Blood glucose levels were monitored from tail vein blood using an automatic glucose monitor (One Touch, Lifescan). Plasma insulin levels were determined using commercial insulin assay kit (ALPCO Diagnostics).

Quantitative PCR. Total RNA was prepared and mRNA levels were measured as previously described[9].

Western blot. Whole-cell extracts were prepared using SDS-urea-lysis buffer. Western blot assays on 50–100 μg of protein were performed as described[19,31,32]. For Fig. 1b, western blot assays were performed on haemagglutinin (HA)-tagged TORC2 immunoprecipitates using phospho (Ser 171) specific and non-discriminating TORC2 antisera. Immunoprecipitation experiments were performed as described[19].

Immunostaining. Formalin-fixed, paraffin-embedded liver sections (5 μm) were deparaffinized in two changes of xylenes and hydrated by extensive washes with ethanol and distilled H_2O. After antibody incubation (anti-TORC2 (1:1,600), anti-p-CREB (5322, 1:500)), slides were incubated with donkey anti-rabbit Cy3 (1:600; 45 min, room temperature). For primary hepatocytes, cells were fixed in 4% paraformaldehyde for 10 min; anti-HA antibody (1:1,000, Santa Cruz) was used for HA-TORC2 detection. Slides were washed and

mounted with coverslips using Vectashield mounting media containing 4,6-diamidino-2-phenylindole (DAPI).

Chromatin immunoprecipitation. Mice were injected intraperitoneally with either glucagon or insulin for 10 min. Nuclear isolation, cross-linking and chromatin immunoprecipitation assays on liver samples were performed as described[19]. Precipitated DNA fragments were analysed by quantitative PCR using primers against relevant mouse promoters.

***In vitro* kinase assays.** Recombinant GST–TORC2 (1–240) and GST–TORC2 (161–181) proteins (wild-type and Ser171Ala) were phosphorylated *in vitro* with 100 mU purified AMPK (Upstate Biotech) with or without 300 μM AMP according to the manufacturer's instructions. After 15 min incubation with [γ-^{32}P]ATP, ^{32}P incorporation was measured by scintillation.

Received 14 April; accepted 27 June 2005.
Published online 7 September 2005.

1. Saltiel, A. & Kahn, C. R. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414,** 799–806 (2001).
2. Saltiel, A. R. New perspectives into the molecular pathogenesis and treatment of type 2 diabetes. *Cell* **104,** 517–529 (2001).
3. Phillips, C. A. & Molitch, M. E. The relationship between glucose control and the development and progression of diabetic nephropathy. *Curr. Diab. Rep.* **2,** 523–529 (2002).
4. Hanson, R. W. & Reshef, L. Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu. Rev. Biochem.* **66,** 581–611 (1997).
5. Hall, R. K. & Granner, D. K. Insulin regulates expression of metabolic genes through divergent signalling pathways. *J. Basic Clin. Physiol. Pharmacol.* **10,** 119–133 (1999).
6. Herzig, S. *et al.* CREB regulates hepatic gluconeogenesis via the co-activator PGC-1. *Nature* **413,** 179–183 (2001).
7. Herzig, S. *et al.* CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-γ. *Nature* **426,** 190–193 (2003).
8. Yoon, J. *et al.* Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* **413,** 131–138 (2001).
9. Koo, S. H. *et al.* PGC-1 promotes insulin resistance in liver through PPAR-α-dependent induction of TRB-3. *Nature Med.* **10,** 530–534 (2004).
10. Lin, J. *et al.* Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1α null mice. *Cell* **119,** 121–135 (2004).
11. Leone, T. C. *et al.* PGC-1α deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. *PLoS Biol.* **3,** e101 (2005).
12. Puigserver, P. *et al.* Insulin-regulated hepatic gluconeogenesis through FOXO1–PGC-1α interaction. *Nature* **423,** 550–555 (2003).
13. Banerjee, R. R. *et al.* Regulation of fasted blood glucose by resistin. *Science* **303,** 1195–1198 (2004).
14. Yamauchi, T. *et al.* Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature Med.* **8,** 1288–1295 (2002).
15. Chrivia, J. C. *et al.* Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365,** 855–859 (1993).
16. Arias, J. *et al.* Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature* **370,** 226–228 (1994).
17. Conkright, M. D. *et al.* TORCs: transducers of regulated CREB activity. *Mol. Cell* **12,** 413–423 (2003).
18. Iourgenko, V. *et al.* Identification of a family of cAMP response element-binding protein coactivators by genome-scale functional analysis in mammalian cells. *Proc. Natl Acad. Sci. USA* **100,** 12147–12152 (2003).
19. Screaton, R. A. *et al.* The CREB coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector. *Cell* **119,** 61–74 (2004).
20. Bittinger, M. A. *et al.* Activation of cAMP response element-mediated gene expression by regulated nuclear transport of TORC proteins. *Curr. Biol.* **14,** 2156–2161 (2004).
21. Zhou, X. Y. *et al.* Insulin regulation of hepatic gluconeogenesis through phosphorylation of CREB-binding protein. *Nature Med.* **10,** 633–637 (2004).
22. Kasper, L. H. *et al.* A transcription-factor-binding surface of coactivator p300 is required for haematopoiesis. *Nature* **419,** 738–743 (2002).
23. Ahn, S. *et al.* A dominant negative inhibitor of CREB reveals that it is a general mediator stimulus-dependent transcription of c-fos. *Mol. Cell. Biol.* **18,** 967–977 (1998).
24. Sasaki, K. *et al.* Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. *J. Biol. Chem.* **259,** 15242–15251 (1984).
25. Lochhead, P. A., Coghlan, M., Rice, S. Q. & Sutherland, C. Inhibition of GSK-3 selectively reduces glucose-6-phosphatase and phosphatase and phosphoenolypyruvate carboxykinase gene expression. *Diabetes* **50,** 937–946 (2001).
26. Kahn, B. B., Alquier, T., Carling, D. & Hardie, D. G. AMP-activated protein kinase: Ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* **1,** 15–25 (2005).
27. Lizcano, J. M. *et al.* LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J.* **23,** 833–843 (2004).
28. Sakamoto, K., Goransson, O., Hardie, D. G. & Alessi, D. R. Activity of LKB1 and AMPK-related kinases in skeletal muscle: effects of contraction, phenformin, and AICAR. *Am. J. Physiol. Endocrinol. Metab.* **287,** E310–E317 (2004).
29. Radziuk, J., Bailey, C. J., Wiernsperger, N. F. & Yudkin, J. S. Metformin and its liver targets in the treatment of type 2 diabetes. *Curr. Drug Targets Immune Endocr. Metab. Disord.* **3,** 151–169 (2003).
30. Bergeron, R. *et al.* Effect of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside infusion on *in vivo* glucose and lipid metabolism in lean and obese Zucker rats. *Diabetes* **50,** 1076–1082 (2001).
31. Du, K., Herzig, S., Kulkarni, R. N. & Montminy, M. TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. *Science* **300,** 1574–1577 (2003).
32. Katoh, Y. *et al.* Salt-inducible kinase-1 represses cAMP response element-binding protein activity both in the nucleus and in the cytoplasm. *Eur. J. Biochem.* **271,** 4307–4319 (2004).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements This work was supported by grants from the NIH, the Keickhefer Foundation, the Hillblom Foundation, the American Diabetes Association, the Juvenile Diabetes Foundation and the Leukemia and Lymphoma Society. We thank L. Vera for mouse injections and ProteinExpress Co. Ltd for the gift of p-TORC2 antiserum.

Author Information Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to M.M. (montminy@salk.edu).