In the fasted state, increases in circulating glucagon promote hepatic glucose production through induction of the gluconeogenic program. Triggering of the cyclic AMP pathway increases gluconeogenic gene expression via the de-phosphorylation of the CREB co-activator CRTC2 (ref. 1). Glucagon promotes CRTC2 dephosphorylation in part through the protein kinase A (PKA)-mediated inhibition of the CRTC2 kinase SIK2. A number of Ser/Thr phosphatases seem to be capable of dephosphorylating CRTC2 (refs 2, 3), but the mechanisms by which hormonal cues regulate these enzymes remain unclear. Here we show in mice that glucagon stimulates CRTC2 dephosphorylation in hepatocytes by mobilizing intracellular calcium stores and activating the calcium/calmodulin-dependent Ser/Thr-phosphatase calcineurin (also known as PP3CA). Glucagon increased cytosolic calcium concentration through the PKA-mediated phosphorylation of inositol-1,4,5-trisphosphate receptors (InsP3Rs), which associate with CRTC2. After their activation, InsP3Rs enhanced gluconeogenic gene expression by promoting the calcineurin-mediated dephosphorylation of CRTC2. During feeding, increases in insulin signalling reduced CRTC2 activity via the AKT-mediated inactivation of InsP3Rs. InsP3R activity was increased in diabetes, leading to upregulation of the gluconeogenic program. As hepatic downregulation of InsP3Rs and calcineurin improved circulating glucose levels in insulin resistance, these results demonstrate how interactions between cAMP and calcium pathways at the level of the InsP3R
capable of dephosphorylating CRTC2 (refs 2, 3), but the mechanisms that undergo phosphorylation by PKA in response to glucagon, we

We tested a series of Ser/Thr protein phosphatase inhibitors for their ability to block CRTC2 activation in response to glucagon. Exposure to the calcineurin inhibitor cyclosporine A (CsA) disrupted the glucagon-induced dephosphorylation and nuclear translocation of CRTC2, but okadaic acid, an inhibitor of PP1, PP2A and PP4 did not (Fig. 1a and Supplementary Fig. 1a). CsA and other calcineurin inhibitors also reduced cAMP response element (CRE)-luciferase (Luc) reporter activity (Fig. 1a and Supplementary Fig. 1b), but they had no effect in cells expressing phosphorylation-defective (Ser 171, 275 Ala) and therefore active forms of CRTC2 (Supplementary Fig. 1c–e).

On the basis of the ability of CsA to interfere with CRTC2 activation, we considered that calcineurin may promote the dephosphorylation of CRTC2 in response to glucagon. Supporting this idea, CRTC2 contains two consensus (PXIXIT) motifs that mediate an association with calcineurin (Supplementary Fig. 2a, b). Moreover, mutation of both motifs disrupted the glucagon-dependent dephosphorylation of CRTC2 (Fig. 1b) and prevented its nuclear translocation (Supplementary Fig. 2c), thereby down-regulating CRE-Luc activation (Fig. 1b).

On the basis of these results, we tested whether calcineurin regulates expression of the gluconeogenic program. Adenoviral overexpression of the calcineurin catalytic subunit in hepatocytes augmented CRTC2 dephosphorylation, CRE-Luc activity, and glucose secretion in response to glucagon, whereas calcineurin knockdown had the opposite effect (Fig. 1c). Although calcineurin could, in principle, modulate CRTC2 activity indirectly through effects on cAMP signalling, calcineurin overexpression or knockdown did not alter the phosphorylation of cellular PKA substrates in cells exposed to glucagon (Supplementary Fig. 2d).

We examined next whether calcineurin modulates hepatic gluconeogenesis in vivo. Modest (twofold) overexpression of calcineurin in liver increased gluconeogenic gene expression, hepatic CRE-Luc activity, and fasting blood glucose concentrations (Fig. 1d and Supplementary Fig. 3a). Conversely, knockdown of hepatic calcineurin reduced expression of the gluconeogenic program and lowered circulating glucose levels (Fig. 1d and Supplementary Fig. 3b), demonstrating that this phosphatase contributes to fasting adaptation in the liver. Calcineurin seemed to stimulate gluconeogenesis via the CREB pathway; depletion of CRTC2 blocked the effects of calcineurin overexpression (Supplementary Fig. 4).

Realizing that calcineurin activity is dependent on increases in intracellular calcium, we tested whether the cAMP pathway stimulates calcium mobilization. Exposure of primary hepatocytes to glucagon triggered a rapid increase in cellular free calcium (Fig. 2a and Supplementary Fig. 5a); these effects were partially reversed by co-treatment with the PKA inhibitor H89 (Supplementary Fig. 5b). The rise in intracellular calcium seems to be critical for CRTC2 activation, because co-incubation with the calcium chelator BAPTA disrupted CRTC2 dephosphorylation and CRE-Luc activation in response to glucagon (Fig. 2b). Arguing against an effect of calcium on cAMP signalling, exposure to BAPTA did not block the PKA-mediated phosphorylation of CRET in response to glucagon.

We imagined that cAMP may increase calcium mobilization through the PKA-dependent phosphorylation of an intracellular calcium channel. In mass spectrometry studies to identify proteins that undergo phosphorylation by PKA in response to glucagon, we recovered the inositol 1,4,5-trisphosphate receptor 1 (InsP3R1) from immunoprecipitates of phospho-PKA substrate antisera (Supplementary Fig. 5c). InsP3R1 and its related family members (InsP3R2, InsP3R3) are calcium release channels that promote the mobilization of endoplasmic reticulum calcium stores following their activation in response to extracellular signals. Moreover, cAMP agonists have also been shown to enhance InsP3R receptor activity through PKA-mediated phosphorylation.

Inhibiting InsP3Rs, either by exposure of hepatocytes to xestospongin C or by knockdown of all three InsP3Rs, disrupted cytosolic calcium mobilization and calcineurin activation in response to glucagon and forskolin (Fig. 2a and Supplementary Fig. 6a). Moreover, xestospongin C treatment and InsP3R1 knockdown also blocked the effects of glucagon on CRTC2 dephosphorylation, CRE-Luc activation, and induction of the gluconeogenic program (Fig. 2c and Supplementary Fig. 6a, b). We
Figure 1 | Calcineurin promotes CRTC2 activation during fasting. 

- **a**: Effect of Ser/Thr phosphatase inhibitors (okadaic acid (OA), CsA) on CRTC2 dephosphorylation and CRE-Luc reporter activity (*P* < 0.001; *n* = 3). Gcg = glucagon; Veh = vehicle. 
- **b**: Effect of glucagon on dephosphorylation (left) and activity (right) of wild-type (WT) and calcineurin-defective (ΔCalna) CRTC2 in hepatocytes (*P* < 0.001; *n* = 3). 
- **c**: Effect of calcineurin A overexpression (left) or knockdown (right) on CRTC2 dephosphorylation (top), CRE-Luc reporter activity (middle, *P* < 0.001; *n* = 3), and glucose output (bottom, *P* < 0.001; *n* = 3) from hepatocytes. US = unspecific. 
- **d**: Effect of hepatic InsP3R overexpression on CRTC2 dephosphorylation, CRE-Luc activity, and glucose output from 6–8 h fasted mice (*P* < 0.01; *n* = 5). Data are shown as mean ± s.e.m.

Figure 2 | Glucagon stimulates CRTC2 dephosphorylation via activation of InsP3Rs. 

- **a**: Effect of glucagon (Gcg) on calcium mobilization in hepatocytes by fluorescence imaging. Calcium mobilization and calcineurin activation following knockdown of all three InsP3R family members shown (*P* < 0.001; *n* = 3). 
- **b**: Effect of calcium chelator (BAPTA) on CRTC2 dephosphorylation and CRE-Luc activation (*P* < 0.001; *n* = 3). 
- **c**: Effect of InsP3R depletion on CRTC2 dephosphorylation, CRE-Luc activity, and glucose output from hepatocytes (*P* < 0.001; *n* = 3). 
- **d**: Effect of hepatic InsP3R knockdown on CRE-Luc activity, blood glucose, and gluconeogenic gene expression (*P* < 0.01; *n* = 5). Data are shown as mean ± s.e.m.
confirmed the effects of InsP₃R depletion using hepatocytes from mice with a knockout of the InsP₃R2 (ref. 10), the predominant InsP₃R isoform in these cells (Supplementary Fig. 6c–e).

On the basis of these results, InsP₃R would also be expected to modulate fasting glucose production in vivo. Decreasing hepatic InsP₃R expression, either by knockdown of all three InsP₃Rs in liver or by targeted disruption of the InsP₃R2 gene, reduced fasting CRE-Luc activity, gluconeogenic gene expression, and circulating glucose concentrations, demonstrating the importance of these receptors in glucose homeostasis (Fig. 2d and Supplementary Fig. 7).

We tested whether glucagon modulates InsP₃R activity through PKA-mediated phosphorylation. Exposure of hepatocytes to glucagon increased the phosphorylation of InsP₃R₁ as well as InsP₃R₂ and InsP₃R₃ by immunoblot assay with phospho-PKA substrate antiserum; these effects were blocked by the PKA inhibitor H89 (Fig. 3a and Supplementary Fig. 8a). Moreover, mutation of serine residues at consensus PKA sites in InsP₃R₁ (Ser 1589, Ser 1756) to alanine completely disrupted InsP₃R₁ phosphorylation in response to glucagon (Fig. 3b). As a result, overexpression of PKA-defective (S1589,1756A) InsP₃R₁ interfered with calcium mobilization and calcineurin activation, and it reduced CRE-Luc activation and glucose secretion from hepatocytes exposed to glucagon (Fig. 3b–d).

Similar to glucagon, fasting also stimulated hepatic InsP₃R₁ phosphorylation at Ser 1589 and Ser 1756 (Supplementary Fig. 8b). And overexpression of PKA-defective InsP₃R₁ reduced fasting CRE-Luc induction, calcineurin activation, and gluconeogenic gene expression, leading to lower circulating glucose concentrations (Fig. 3e and Supplementary Fig. 8c, d). Taken together, these results support an important role for the PKA-mediated phosphorylation of InsP₃R in hepatic gluconeogenesis.

We considered that the proximity of CRTC2 to the calcium signalling machinery may be important for its activation. Supporting this notion, CRTC2 was found to associate with InsP₃R₁ via its amino-terminal CREB binding domain (CBD) in co-immunoprecipitation assays (Fig. 3f and Supplementary Fig. 9a–d). Moreover, CRTC2 was enriched in endoplasmic reticulum containing high density microsomal fractions, which also contain the InsP₃Rs (Supplementary Fig. 9e). The InsP₃R-CRTC2 association seems to be critical for CRTC2 localization in the perinuclear space, because RNA interference (RNAi)-mediated knockdown of the InsP₃Rs led to redistribution of CRTC2 in the cytoplasm (Supplementary Fig. 9f). Disrupting the CRTC2-InsP₃R interaction, by deletion of the CBD in CRTC2 or by addition of an N-terminal myristoylation signal that targets CRTC2 to the plasma membrane, blocked CRTC2 dephosphorylation and CRE-reporter activation in response to glucagon (Supplementary Fig. 9g–i). Taken together, these results suggest that the association of CRTC2 with InsP₃Rs enhances its sensitivity to fasting signals.

Under feeding conditions, insulin inhibits gluconeogenesis in part by increasing CRTC2 phosphorylation. We wondered whether insulin interferes with InsP₃R effects on CRTC2 activation. Supporting this idea, AKT has been shown to block calcium mobilization by phosphorylating InsP₃Rs at Ser 2682 (in InsP₃R₁)11. Indeed, exposure of hepatocytes to insulin increased InsP₃R phosphorylation by immunoblot analysis with phospho-AKT substrate antiserum (Supplementary Fig. 10a). Moreover, Ser 2682 (in InsP₃R₁) to alanine blocked these effects. Insulin treatment also reduced glucagon-dependent increases in calcium mobilization and calcineurin activation in cells expressing wild-type InsP₃R₁, but it had no effect in cells expressing AKT-defective (S2682A) InsP₃R₁ (Supplementary Fig. 10b). As a result, CRTC2 dephosphorylation, CRE-Luc activity, and glucose output were elevated in hepatocytes expressing InsP₃R₁(S2682A) compared to wild type (Supplementary Fig. 10c).

We examined whether InsP₃R₁ phosphorylation by AKT is important in regulating hepatic glucose production in vivo. In line with this

Figure 3 | Glucagon stimulates CRTC2 activity via PKA-dependent phosphorylation of InsP₃Rs. a, b, Immunoblots of InsP₃R₁ immunoprecipitates (IP) using phospho-PKA substrate antiserum to show effect of H89 (a) and Ala mutations (b) at one or both (DM) PKA consensus sites (Ser 1589, Ser 1756) on InsP₃R₁ phosphorylation in hepatocytes exposed to glucagon (Gcg). Effect of wild-type and PKA-mutant InsP₃R₁ on calcium mobilization in response to Gcg (b) shown (⁎P < 0.001; n = 3). c, d, Effect of wild-type or PKA-defective InsP₃R₁ (DM) on calcineurin (Caln) activation (c) and CRTC2 dephosphorylation (c), as well as CRE-Luc activation (d) and glucose output (d) from hepatocytes (⁎P < 0.001; n = 3). e, Effect of wild-type and PKA-defective InsP₃R₁ on hepatic CRE-Luc activity, fasting blood glucose, and gluconeogenic gene expression (G6pc, Pck1) (⁎P < 0.01 versus wild type; n = 5). f, Co-immunoprecipitation of CRTC2 with InsP₃R₁ in primary hepatocytes. Exposure to glucagon (100 nM, 15 min) indicated. Input levels of CRTC2 and InsP₃R₁ in nuclear (Nu) and post-nuclear (p/Nu) supernatant fractions shown. Data are shown as mean ± s.e.m.
phosphorylation at PKA or AKT sites indicated. c. Effect of RNAi-mediated depletion of InsP3Rs or calcineurin A on CRE-Luc activity, gluconeogenic gene expression, and hepatic glucose production in db/db mice, determined by pyruvate tolerance testing (*P < 0.01; n = 5). Data are shown as mean ± s.e.m.

**METHODS SUMMARY**

Adenoviruses were delivered by tail vein injection[17]. Hepatic CRE-Luc activity was visualized using an IVIS Imaging system. Mice were imaged 3–5 days after injection of CRE-Luc adenovirus. Pyruvate tolerance testing was performed on mice fasted overnight and injected intraperitoneally with pyruvate (2 g kg⁻¹). Ins3p2 knockout mice have been described[13]. Cultured primary mouse hepatocytes were prepared as described[14]. Cellular fractionation studies were conducted using primary mouse hepatocytes[16]. Calcium imaging experiments were performed using a CCA camera on primary hepatocytes loaded with fura-2 dye. Mass spectrometry studies were performed on CRTC2 immunoprecipitates prepared from Heko293T cells and on immunoprecipitates of phosho-PKA substrate antiserum prepared from primary hepatocytes exposed to glucagon. Anti-InsP3R1 (A302–158A) and InsP3R3 (A302–1096) monoclonal antibodies were purchased from Abcam, anti-calcineurin (610260) from BD Biosciences, anti-phospho-AKT substrate (RXXS/T, 9614) and CRTC2 (pS171, RXXS/T, 9624) polyclonal antibodies were purchased from Cell Signaling Technology. Cyclic AMP-sensitive coincidence detector

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Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

Mouse strains and adenovirus. Adenoviruses (1 × 10^6 plaque forming units (p.f.u.)) GFP, calcineurin, Inp3r1, Inp3r1 DM (S1589A/S1756A), unspecific RNAi, calcineurin RNAi, Insp3r1 RNAi, Insp3r2 RNAi, Insp3r3 RNAi, Crtc2 RNAi, 1 × 10^7 p.f.u. CRE-Luc reporter, 5 × 10^7 p.f.u. RSV β-galactosidase) were delivered to 8- to 10-week-old male C57BL/6J, B6.V-lep (ob/) B6, C57BL/6J-m/+ Lepr < db/>J mice by tail vein injection\(^\text{17}\). Insp3r2 knockout mice were described previously\(^\text{20}\). All mice were adapted to their environment for 1 week before study and were housed in colony cages with a 12 h light/dark cycle in a temperature-controlled environment. For in vivo imaging experiments, mice were imaged on day 3–5 after adenovirus delivery. Wild-type CRTC2, CRTC2(S171A), GFP, unspecific RNAi, Crtc2 RNAi, CRE-Luc and RSV β-gal adenoviruses have been described previously\(^\text{17,20}\). The adenoviruses containing rat Inp3r1, Inp3r1 DM and Inp3r1(RS268A) were generated from the Inp3r1 plasmid, provided by I. Bezprozvanny (UT Southwestern Medical Center at Dallas). Calcineurin adenovirus was constructed using a mouse calcineurin plasmid (Addgene). CRTC2ΔCBD (51–692 amino acids), S275A and S171A/S275A adenoviruses were made from mouse CRTC2. Myristoylated CRTC2 (Myr-CRTC2) adenovirus was generated with mouse CRTC2 fused to an N-terminal myristoylation tag (MGSSKSKPKDPSQR) from Src. Calcineurin RNAi, Insp3r1 RNAi, Insp3r2 RNAi, Insp3r3 RNAi adenoviruses were constructed using the sequence 5’-GGGTACCGCATGTAAGAGAAA-3’, 5’-GGGTACTGGAATAGCCTCTG-3’ (TOC-3), 5’-GGTAAACAAGCCACACCATCC-3’ and 5’-GGCAAGGTGCGGTGTTCTG-3’. All expressed constructs used in this study were confirmed by sequencing.

In vitro analysis. For in vitro imaging, mice were imaged as described\(^\text{17,20}\) under ad libitum feeding conditions or after fasting for 6 h. For pyruvate challenge experiments, mice were fasted overnight and injected intraperitoneally with pyruvate (2 g kg\(^{-1}\)). Blood glucose values were determined using a LifeScan automatic glucometer. For immunoblot, mouse tissues were sonicated, centrifuged and supernatants were reserved for protein determinations, and SDS–PAGE analysis.

In vitro analysis. HEK293T (ATCC) cells were cultured in DMEM containing 10% FBS (HyClone), 100 mg ml\(^{-1}\) penicillin-streptomycin. Mouse primary hepatocytes were isolated and cultured as previously described\(^\text{18}\). Cellular fractionation supernatants were reserved for protein determinations, and SDS–PAGE analysis.

Immunoblot, immunoprecipitation and immunostaining. Immunoblot, immunoprecipitation and immunostaining assays were performed as described. CRTC2, pCREB (Ser 133), CREB, pAKT (Thr 308), AKT, tubulin, HA and Flag antibodies were previously described\(^\text{18}\). The antibodies anti-Inp3r1, Anti-(1302–158A) and Inp3p, R3 (1302–160A) were purchased from Bethyl Laboratories, anti-Inp3p, R2 (ab77838) from Abcam, anti-calcineurin (610260) from BD Biosciences, anti-GRP78 (ADI-SPA-826-F) from Enzo Life Sciences, anti-phospho-PKA substrate (RRXS/T, 9624), anti-phospho-AKT substrate (RXXS/T, 9614) and CRTC2 (pS171, 2892) from Cell Signaling. CRTC2 (pS275) antibody was used as described\(^\text{18}\).

Quantitative PCR. Total cellular RNAs from whole liver or from primary hepatocytes were extracted using the RNeasy kit (Qiagen) and used to generate cDNA with SuperScript II enzyme (Invitrogen). cDNA were analysed by quantitative PCR as described\(^\text{18}\).

Mass spectrometry. Immunoprecipitates of endogenous CRTC2 from HEK293T cells and of phospho-PKA substrate antisera from glucagon-stimulated hepatocytes were prepared for mass spectrometric studies as previously reported\(^\text{18}\), and analysed by electrospray ionization tandem mass spectrometry on a Thermo LTQ Orbitrap instrument.

Statistical analyses. All studies were performed on at least three independent occasions. Results are reported as mean ± s.e.m. The comparison of different groups was carried out using two-tailed unpaired Student’s t-test. Differences were considered statistically significant at \(P < 0.05\).