LETTER

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# Inositol-1,4,5-trisphosphate receptor regulates hepatic gluconeogenesis in fasting and diabetes

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**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 ($InsP_3Rs$), which associate with CRTC2. After their activation, $InsP_3Rs$ 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 $InsP_3Rs$. $InsP_3R$ activity was increased in diabetes, leading to upregulation of the gluconeogenic program. As hepatic downregulation of $InsP_3Rs$ and calcineurin improved circulating glucose levels in insulin resistance, these results demonstrate how interactions between cAMP and calcium pathways at the level of the $InsP_3R$ modulate hepatic glucose production under fasting conditions and in diabetes.**

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[3,4] (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 modulates 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 CREB 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 ($InsP_3R1$) from immunoprecipitates of phospho-PKA substrate antiserum (Supplementary Fig. 5c). $InsP_3R1$ and its related family members ($InsP_3R2$, $InsP_3R3$) are calcium release channels that promote the mobilization of endoplasmic reticulum calcium stores following their activation in response to extracellular signals[5–9]. Moreover, cAMP agonists have also been shown to enhance $InsP_3R$ receptor activity through PKA-mediated phosphorylation.

Inhibiting $InsP_3Rs$, either by exposure of hepatocytes to xestospongin C or by knockdown of all three $InsP_3Rs$, disrupted cytosolic calcium mobilization and calcineurin activation in response to glucagon and forskolin (Fig. 2a and Supplementary Fig. 6a). Moreover, xestospongin C treatment and $InsP_3R$ 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

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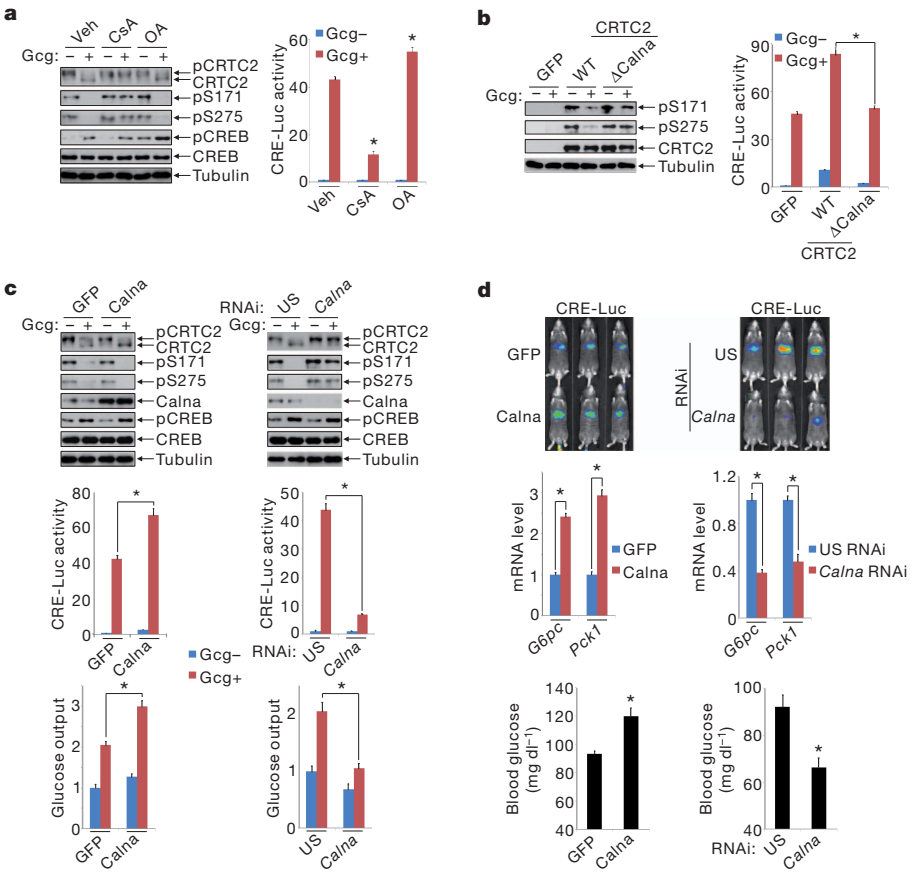


**Figure 1 | Calcineurin promotes CRTC2 activation during fasting.** **a**, Effect of Ser/Thr phosphatase inhibitors (okadaic acid (OA), CsA) on CRTC2 dephosphorylation and CRE-Luc reporter activation (*$P < 0.001$; $n = 3$). Gcg, glucagon; Veh, vehicle. **b**, Effect of glucagon on dephosphorylation (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 calcineurin overexpression (left) or knockdown (right) on CRE-Luc activity, gluconeogenic gene (*Pck1*, *G6pc*) expression, and blood glucose concentrations in 6–8 h fasted mice (*$P < 0.01$; $n = 5$). Data are shown as mean ± s.e.m.

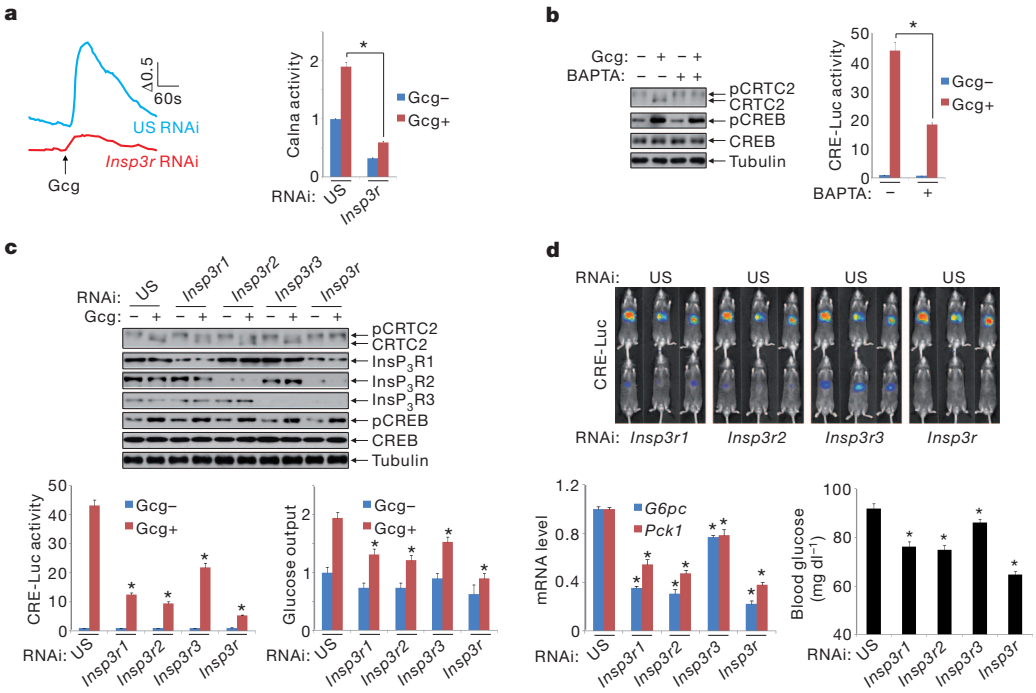


**Figure 2 | Glucagon stimulates CRTC2 dephosphorylation via activation of $InsP_3Rs$.** **a**, Effect of glucagon (Gcg) on calcium mobilization in hepatocytes by fluorescence imaging. Calcium mobilization and calcineurin activation following knockdown of all three $InsP_3R$ family members shown (*$P < 0.001$; $n = 3$). **b**, Effect of calcium chelator (BAPTA) on CRTC2 dephosphorylation and CRE-Luc activation (*$P < 0.001$; $n = 3$). **c**, Effect of $InsP_3R$ depletion on CRTC2 dephosphorylation, CRE-Luc activity, and glucose output from hepatocytes (*$P < 0.001$; $n = 3$). **d**, Effect of hepatic $InsP_3R$ 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 $InsP_3R$ depletion using hepatocytes from mice with a knockout of the $InsP_3R2$ (ref. 10), the predominant $InsP_3R$ isoform in these cells (Supplementary Fig. 6c–e).

On the basis of these results, $InsP_3Rs$ would also be expected to modulate fasting glucose production *in vivo*. Decreasing hepatic $InsP_3R$ expression, either by knockdown of all three $InsP_3Rs$ 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 $InsP_3R$ activity through PKA-mediated phosphorylation. Exposure of hepatocytes to glucagon increased the phosphorylation of $InsP_3R1$ as well as $InsP_3R2$ and $InsP_3R3$ 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 $InsP_3R1$ (Ser 1589, Ser 1756) to alanine completely disrupted $InsP_3R1$ phosphorylation in response to glucagon (Fig. 3b). As a result, overexpression of PKA-defective (S1589,1756A) $InsP_3R1$ 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 $InsP_3R1$ phosphorylation at Ser 1589 and Ser 1756 (Supplementary Fig. 8b). And overexpression of PKA-defective $InsP_3R1$ 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 $InsP_3R$ 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 $InsP_3R1$ 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 $InsP_3Rs$ (Supplementary Fig. 9e). The $InsP_3R$–CRTC2 association seems to be critical for CRTC2 localization in the perinuclear space, because RNA interference (RNAi)-mediated knockdown of the $InsP_3Rs$ led to redistribution of CRTC2 in the cytoplasm (Supplementary Fig. 9f). Disrupting the CRTC2–$InsP_3R$ 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 $InsP_3Rs$ enhances its sensitivity to fasting signals.

Under feeding conditions, insulin inhibits gluconeogenesis in part by increasing CRTC2 phosphorylation. We wondered whether insulin interferes with $InsP_3R$ effects on CRTC2 activitation. Supporting this idea, AKT has been shown to block calcium mobilization by phosphorylating $InsP_3Rs$ at Ser 2682 (in $InsP_3R1$)[11]. Indeed, exposure of hepatocytes to insulin increased $InsP_3R$ phosphorylation by immunoblot analysis with phospho-AKT substrate antiserum (Supplementary Fig. 10a); mutation of Ser 2682 (in $InsP_3R1$) to alanine blocked these effects. Insulin treatment also reduced glucagon-dependent increases in calcium mobilization and calcineurin activation in cells expressing wild-type $InsP_3R1$, but it had no effect in cells expressing AKT-defective (S2682A) $InsP_3R1$ (Supplementary Fig. 10b). As a result, CRTC2 dephosphorylation, CRE-Luc activity, and glucose output were elevated in hepatocytes expressing $InsP_3R$(S2682A) compared to wild type (Supplementary Fig. 10c).

We examined whether $InsP_3R1$ phosphorylation by AKT is important in regulating hepatic glucose production *in vivo*. In line with this

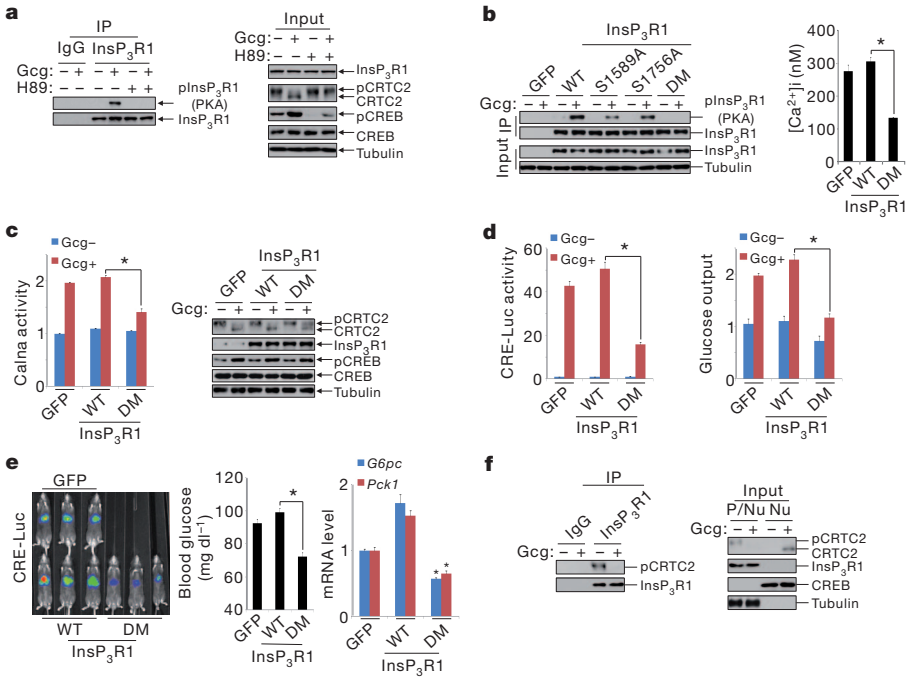


**Figure 3 | Glucagon stimulates CRTC2 activity via PKA-dependent phosphorylation of $InsP_3Rs$.** **a**, **b**, Immunoblots of $InsP_3R1$ immunoprecipitates (IP) using phospho-PKA substrate antiserum to show effect of H89 (**a**) and Ala mutations (**b**) at one or both (DM) PKA consensus sites (Ser 1589, Ser 1756) on $InsP_3R1$ phosphorylation in hepatocytes exposed to glucagon (Gcg). Effect of wild-type and PKA-mutant $InsP_3R1$ on calcium mobilization in response to Gcg (**b**) shown ($^*P < 0.001$; $n = 3$). **c**, **d**, Effect of wild-type or PKA-defective $InsP_3R1$ (DM) on calcineurin (Calna) 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 $InsP_3R1$ 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 $InsP_3R1$ in primary hepatocytes. Exposure to glucagon (100 nM, 15 min) indicated. Input levels of CRTC2 and $InsP_3R1$ in nuclear (Nu) and post-nuclear (p/Nu) supernatant fractions shown. Data are shown as mean ± s.e.m.

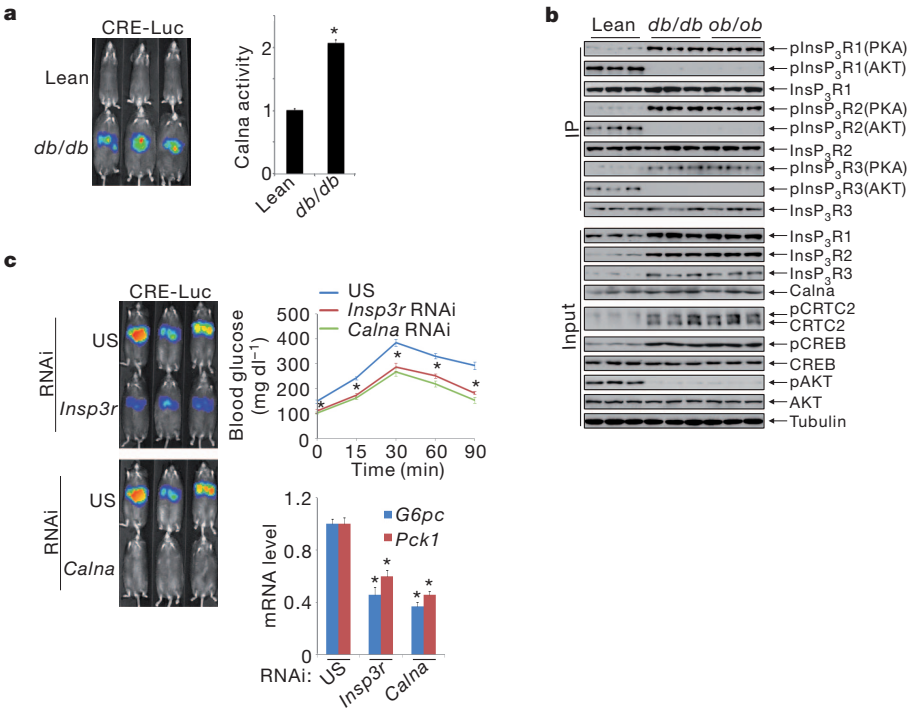


**Figure 4 | $InsP_3R$ activity is upregulated in diabetes.** **a**, Hepatic CRE-Luc and calcineurin activity in lean and *db/db* mice ($*P < 0.001$; $n = 5$). **b**, Immunoblots showing relative amounts and phosphorylation of $InsP_3R$ family members in livers of ad libitum fed lean, *db/db*, or *ob/ob* mice. $InsP_3R$ phosphorylation at PKA or AKT sites indicated. **c**, Effect of RNAi-mediated depletion of $InsP_3Rs$ 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.

notion, feeding increased hepatic $InsP_3R1$ phosphorylation at Ser 2682 (Supplementary Fig. 8b). Moreover, overexpression of AKT-defective $InsP_3R1$ partially suppressed feeding-induced decreases in CRE-Luc activity and gluconeogenic gene expression, leading to elevations in circulating glucose concentrations (Supplementary Fig. 10d). Taken together, these results suggest that the AKT-mediated phosphorylation of $InsP_3Rs$ during feeding inhibits hepatic gluconeogenesis by blocking the calcineurin-dependent dephosphorylation of CRTC2.

We wondered whether hepatic $InsP_3R$ signalling contributes to increases in gluconeogenesis in the setting of insulin resistance. Supporting this notion, hepatic calcineurin activity was enhanced in both *ob/ob* and *db/db* diabetic animals, leading to increases in CRE-Luc activity (Fig. 4a and Supplementary Fig. 11a, b). Pointing to a role for $InsP_3R$, hepatic amounts of PKA-phosphorylated, active $InsP_3Rs$ were increased in these diabetic mice, whereas amounts of AKT-phosphorylated, inactive $InsP_3Rs$ were reduced (Fig. 4b). Correspondingly, knockdown of either calcineurin or $InsP_3Rs$ in *db/db* mice reduced CRE-Luc activity, gluconeogenic gene expression, and hepatic gluconeogenesis (Fig. 4c and Supplementary Fig. 11c).

Collectively, our results demonstrate that glucagon promotes CRTC2 dephosphorylation during fasting by triggering increases in cytoplasmic calcium that lead to calcineurin activation (Supplementary Fig. 12). The ability for glucagon to increase calcium signalling via the PKA-mediated phosphorylation of $InsP_3Rs$ demonstrates an important regulatory node for cross-talk between cAMP and calcium signalling pathways in liver and perhaps other insulin sensitive tissues. The partial inhibition of calcium entry by the PKA inhibitor H89 also points to additional regulatory inputs[12,13] that may function with PKA to increase $InsP_3R$ activity in response to glucagon. CRTC2 has also been found to stimulate metabolic gene expression by upregulating the nuclear hormone receptor co-activator PGC-1α in liver[14,15] and muscle[16]. On the basis of the well-recognized role of calcium signalling in PGC-1α-dependent transcription, $InsP_3Rs$ may also have an important function in this setting.

## 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 $kg^{-1}$). *Insp3r2* knockout mice have been described[10]. Cultured primary mouse hepatocytes were prepared as reported[18]. Cellular fractionation studies were conducted using primary mouse hepatocytes[18]. Calcium imaging experiments were performed using a CCD camera on primary hepatocytes loaded with fura-2 dye. Mass spectrometry studies were performed on CRTC2 immunoprecipitates prepared from HEK293T cells and on immunoprecipitates of phospho-PKA substrate antiserum prepared from primary hepatocytes exposed to glucagon. Anti-$InsP_3R1$ (A302–158A) and $InsP_3R3$ (A302–160A) antibodies were purchased from Bethyl Laboratories, anti-$InsP_3R2$ (ab77838) antiserum was 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. Phospho (Ser 275) CRTC2 antibody was used as described[19]. For more details, see Supplementary Methods.

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

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

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**Author Contributions** Y.W., I.T. and M.M. designed and interpreted the experiments. Y.W., G.L., J.C.P., R.S. and J.G. carried out the experimental work. Y.W., K.O. and J.C. characterized glucose metabolism in *Insp3r2* knockout mice. W.H.F. performed proteomic studies, and Y.W. and M.M. wrote the paper.

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## METHODS

**Mouse strains and adenovirus.** Adenoviruses ($1 \times 10^8$ plaque forming units (p.f.u.) GFP, calcineurin, $InsP_3R1$, $InsP_3R1$ DM (S1589A/S1756A), unspecific RNAi, calcineurin RNAi, *Insp3r1* RNAi, *Insp3r2* RNAi, *Insp3r3* RNAi, *Crtc2* RNAi, $1 \times 10^9$ p.f.u. CRE-Luc reporter, $5 \times 10^7$ p.f.u. RSV β-galactosidase) were delivered to 8–10-week-old male C57BL/6J, B6.V-*lep* < ob>/J, B6.Cg-*m*+/+*Lepr* < *db*>/J mice by tail vein injection[17]. *Insp3r2* knockout mice were described previously[10]. 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[17,20]. The adenoviruses containing rat $InsP_3R1$, $InsP_3R1$ DM and $InsP_3R1$(S2682A) were generated from the $InsP_3R1$ plasmid, provided by I. Bezprozvanny (UT Southwestern Medical Center at Dallas). Calcineurin adenovirus was constructed using a mouse calcineurin plasmid (Addgene). CRTC2 ΔCBD (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′-GGGTACCGCATGTACAGGAAAA-3′, 5′-GGGTACTGGAATAGCCTCT TCC-3′, 5′-GGGTAACAAGCACCACCATCCC-3′ and 5′-GGGCAAGCTGCA GGTGTTCCTG-3′, respectively. All expressed constucts used in this study were confirmed by sequencing.

***In vivo* analysis.** For *in vivo* imaging, mice were imaged as described[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[18]. Cellular fractionation studies were conducted as previously reported[18]. For reporter studies, Ad-CRE-Luc-infected hepatocytes (1 p.f.u. per cell) were exposed to glucagon (100 nM) for 2 to ~4 h. For CsA (10 μM), okadaic acid (100 nM), cell permeable calcineurin autoinhibitory peptide (10 μM), CN585 (100 μM), calyculin A (10 nM), xestospongin C (2 μM), H89 (30 μM) or BAPTA (50 μM) inhibition, hepatocytes were pre-treated with the inhibitors for 1 h. Luciferase activities were normalized to β-galactosidase activity from adenoviral-encoded RSV β-galactosidase. Calcineurin activity (test kit from Enzo Life Sciences) and cellular cAMP levels (test kit from Cayman Chemical Company) were measured according to the manufacturer's instructions.

**Calcium imaging.** Mouse primary hepatocytes were plated on glass coverslips and loaded with 5 μM Fura-2 acetoxymethyl ester (Molecular Probes) in the presence of 0.025% (w/v) pluronic F127 (Sigma-Aldrich) in Media 199 (Mediatech) for 30 min. Coverslips were mounted on a laminar flow perfusion chamber (Warner Instruments) and perfused with Media 199 or a solution of 100 nM glucagon in Media 199. Images of Fura-2 loaded cells were collected with a cooled CCD camera while the excitation wavelength was alternated between 340 nm and 380 nm. The ratio of fluorescence intensity at the two excitation wavelengths was calculated after subtracting background fluorescence. $[Ca^{2+}]i$ (cytosolic free calcium concentration) was calculated using a Fura-2 calcium imaging calibration kit (Invitrogen). Images were collected and analysed using the MetaFluor software package (Universal Imaging Corp.). Graphs represent average responses from groups of 30–40 individual cells from representative single experiments. Bar graphs represent average responses (fold over average baseline) from 150–200 cells per condition. All experiments were repeated at least three times with similar results.

**Immunoblot, immunoprecipitation and immunostaining.** Immunoblot, immunoprecipitation and immunostaining assays were performed as described[18]. CRTC2, pCREB (Ser 133), CREB, pAKT (Thr 308), AKT, tubulin, HA and Flag antibodies were previously described[18]. The antibodies anti-$InsP_3R1$ (A302–158A) and $InsP_3R3$ (A302–160A) were purchased from Bethyl Laboratories, anti-$InsP_3R2$ (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[19].

**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[18].

**Mass spectrometry.** Immunoprecipitates of endogenous CRTC2 from HEK293T cells and of phospho-PKA substrate antiserum from glucagon-stimulated hepatocytes were prepared for mass spectrometric studies as previously reported[21], 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 $P < 0.05$.