

# The CREB Coactivator TORC2 Functions as a Calcium- and cAMP-Sensitive Coincidence Detector

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## Summary

Elevations in circulating glucose and gut hormones during feeding promote pancreatic islet cell viability in part via the calcium- and cAMP-dependent activation of the transcription factor CREB. Here, we describe a signaling module that mediates the synergistic effects of these pathways on cellular gene expression by stimulating the dephosphorylation and nuclear entry of TORC2, a CREB coactivator. This module consists of the calcium-regulated phosphatase calcineurin and the Ser/Thr kinase SIK2, both of which associate with TORC2. Under resting conditions, TORC2 is sequestered in the cytoplasm via a phosphorylation-dependent interaction with 14-3-3 proteins. Triggering of the calcium and cAMP second messenger pathways by glucose and gut hormones disrupts TORC2:14-3-3 complexes via complementary effects on TORC2 dephosphorylation; calcium influx increases calcineurin activity, whereas cAMP inhibits SIK2 kinase activity. Our results illustrate how a phosphatase/kinase module connects two signaling pathways in response to nutrient and hormonal cues.

## Introduction

Elevations in blood glucose levels during feeding stimulate insulin release and islet cell gene expression in  $\beta$  cells through a specialized pathway that requires mitochondrial glucose oxidation and ATP production (Hinke et al., 2004). The ensuing closure of ATP-sensitive  $K_{ATP}$  channels triggers  $\beta$  cell depolarization and calcium influx through voltage-gated L-type calcium channels.

Feeding also stimulates the release of certain gut hormones such as glucagon-like peptide-1 (GLP-1) into the circulation. These hormones enhance the effects of glucose on insulin secretion and promote  $\beta$  cell viability through a cAMP-dependent mechanism (Hui et al., 2003). The ability of GLP-1 to modulate islet cell gene expression is dependent on glucose elevations, thus insuring against inappropriate release of insulin from the pancreas when glucose levels are low. The mechanism by which glucose and GLP-1 signals synergize in the  $\beta$  cell is unclear, but current studies implicate transcriptional events in this process (Hinke et al., 2004).

The cAMP and calcium-responsive transcription factor CREB is thought to mediate the survival and proliferative effects of GLP-1 and glucose signaling pathways in the pancreas. Transgenic mice expressing a dominant-negative CREB polypeptide in islets develop diabetes with apoptosis of insulin-producing  $\beta$  cells due to reduced expression of IRS2, a direct target of CREB (Jhala et al., 2003). Additionally, transgenic mice expressing the inducible cAMP early repressor (ICER) in islets also develop severe diabetes with reduced islet cell mass, which was attributed to a defect in  $\beta$  cell proliferation (Inada et al., 2004).

cAMP and calcium pathways trigger CREB Ser133 phosphorylation and target gene activation via the Ser/Thr kinases PKA and CaMKIV, respectively (Mayr and Montminy, 2001). Ser 133 phosphorylation, in turn, stimulates target gene expression via recruitment of the coactivator paralogs CBP and p300. CREB and CBP/p300 interact through well-defined domains called KID and KIX, respectively (Radhakrishnan et al., 1997). The ability of cAMP and calcium signals to converge at the level of CREB Ser133 phosphorylation provides a plausible mechanism to explain how CREB mediates the cooperativity between these pathways in electrically excitable cells.

Recent studies showing that calcium signals destabilize the CREB:CBP complex through secondary phosphorylation events on CREB, however, have challenged this model and argued for the involvement of additional CREB coactivators (Kornhauser et al., 2002). Indeed, a potential role for the CREB DNA binding/dimerization domain (bZIP) in mediating transcriptional responses to both calcium influx and cAMP has emerged from previous studies with GAL4-CREB fusion proteins (Sheng et al., 1991). In that case, the bZIP appeared to contribute significantly to induction of CREB activity in response to membrane depolarizing signals, implicating this domain in mediating the association of CREB with a calcium-regulated coactivator.

In a recent screen to identify novel modulators of CREB activity, we and others characterized a family of CREB coactivators called transducers of regulated CREB activity (TORCs) (Conkright et al., 2003a; Iourgenko et al., 2003). The three TORC family members share an N-terminal coiled-coil domain that associates with the CREB bZIP domain. The importance of CREB in mediating cooperativity between cAMP and calcium second messengers in islet cells prompted us to ad-

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dress the regulatory contributions of TORC toward this process. Our studies illustrate a mechanism by which calcium and cAMP pathways converge on TORC in islet cells to regulate cellular gene expression in response to nutrient and hormonal cues.

## Results

### The CREB bZIP Domain Mediates Cooperativity between cAMP and Calcium Signals

We examined the effects of glucose and the gut hormone GLP-1 on CREB target gene expression in MIN6 insulinoma cells. Treatment with high glucose or the GLP-1 analog exendin-4 stimulated expression of the cAMP-responsive NR4A2 gene (Conkright et al., 2003b) 5- and 3-fold individually and 20-fold in combination (Figure 1A). Exposure to nifedipine, a potent L-type calcium channel antagonist, blocked induction of NR4A2 by glucose, demonstrating the importance of calcium influx for these effects (Figure 1B). The calcium-activated Ser/Thr phosphatase calcineurin/PP2B (Cn) appeared to be critical for cooperativity between glucose and exendin-4, as treatment with the Cn inhibitor cyclosporine A (CsA) blocked induction of NR4A2 mRNA in response to both stimuli (Figure 1B).

Based on the ability of glucose to activate L-type calcium channels, we used depolarizing concentrations of KCl (45 mM) to mimic these effects in HIT insulinoma cells. Similarly, we employed forskolin (FSK), an adenyl cyclase agonist, in place of exendin-4 because of its ability to enhance  $\beta$  cell function via the cAMP pathway (Hinke et al., 2004). When added to cultures of HIT cells individually, KCl and FSK stimulated the CREB-dependent EVX1 promoter 5- and 13-fold, respectively. In combination, they induced reporter activity synergistically (80-fold, Figure 1C). Similar to its inhibitory effects on NR4A2 induction by glucose and exendin-4, CsA also blocked cooperativity between KCl and FSK (Figure 1C). Consistent with the involvement of CREB in this process, mutation of the CRE element on the EVX1 promoter disrupted responses to both KCl and FSK (Conkright et al., 2003b) (see Supplemental Figure S1 at <http://www.cell.com/cgi/content/full/119/1/61/DC1/>).

The ability of both calcium influx and cAMP to promote Ser133 phosphorylation of CREB prompted us to test whether activation of the two pathways leads to cooperative changes in Ser133 phosphorylation and recruitment of the coactivator paralogs CBP and p300. When added to HIT cells in combination, KCl and FSK had no greater effect on phospho-CREB levels relative to either stimulus alone (Figure 1D). Moreover, exposure to CsA did not alter CREB Ser133 phosphorylation in response to either stimulus, despite the ability of CsA to block the cooperative effects of KCl and FSK on CREB activity (Schwaninger et al., 1995) (Figure 1D).

Although calcium and cAMP pathways do not synergize at the level of CREB Ser133 phosphorylation, they could exert cooperative effects on the CREB:CBP interaction by modifying CBP (Impey et al., 2002) or a novel component of this pathway. To test this notion, we performed mammalian two-hybrid assays in HIT cells using GAL4-KID and KIX-VP16 expression vectors, which contain the relevant interaction domains for CREB and CBP,

respectively. Exposure to FSK stimulated the CREB:CBP interaction 20-fold by this assay, but KCl had no effect (Figure 1E). Costimulation with both FSK and KCl promoted complex formation at levels comparable to FSK alone, arguing against a role for the CREB:CBP complex in mediating the synergism between cAMP and calcium pathways.

Having observed that cAMP and calcium do not promote CREB:CBP complex formation cooperatively, we tested the role of the CREB bZIP domain, which has been previously implicated in this process (Sheng et al., 1991). In transient assays of HIT cells, KCl and FSK stimulated the activity of a full-length GAL4 CREB (aa 1–341) construct cooperatively, and these effects were blocked by treatment with CsA (Figure 1F). By contrast, a GAL4-CREB  $\Delta$ bZIP (aa 1–283) protein lacking the C-terminal bZIP domain (aa 284–341) was far less responsive to both stimuli as well as to inhibition by CsA, underscoring the involvement of the bZIP domain in this context (Figure 1F).

The importance of the bZIP domain for GAL4-CREB activation by KCl and FSK could reflect either recruitment of a cofactor or dimerization of the bZIP with endogenous CREB proteins. To differentiate between these two possibilities, we performed knockdown studies. In control experiments, CREB siRNAs reduced cellular CREB protein levels to near-undetectable levels and blocked the induction of a transfected EVX1 reporter by FSK (see Supplemental Figure S2 on the *Cell* web site). Despite their effects on endogenous CREB levels and activity, CREB siRNAs had no effect on the induction of the GAL4-bZIP protein by FSK in HEK293T cells (Figure 2A) or on the activation of a full-length RNAi-insensitive GAL4-CREB polypeptide by FSK and KCl in HIT cells (Supplemental Figure S3). These results argue against a significant regulatory contribution of endogenous CREB on transcriptional activation via the bZIP domain.

### Role of TORC2 in cAMP and Calcium Signaling

The recent characterization of TORCs, a family of CREB coactivators that binds to the CREB bZIP domain (Conkright et al., 2003a), led us to hypothesize that these proteins mediate the synergistic effects of cAMP and calcium signals on target gene expression. We performed knockdown studies using siRNAs against TORC2 since this protein is expressed most abundantly in insulinoma and HEK293T cells relative to the other family members (TORC1 and TORC3, data not shown). Indeed, addition of TORC2 siRNAs nearly eliminated the induction of GAL4-bZIP activity by FSK in HEK293T cells (Figure 2A). TORC2 knockdown also reduced the effects of glucose and exendin-4 on EVX1 reporter activity in MIN6 insulinoma cells, demonstrating the importance of TORC2 in these contexts (Figure 2B). The inhibitory effect of TORC2 knockdown on CREB activity appears specific because overexpression of an RNAi-insensitive TORC2 construct rescued EVX1 reporter induction by FSK (Supplemental Figure S4).

Based on the importance of TORC2 for bZIP-dependent activation, we examined whether overexpression of TORC2 was sufficient to potentiate CREB target gene expression in response to cAMP and calcium influx. By contrast with its modest effects in resting HIT cells,

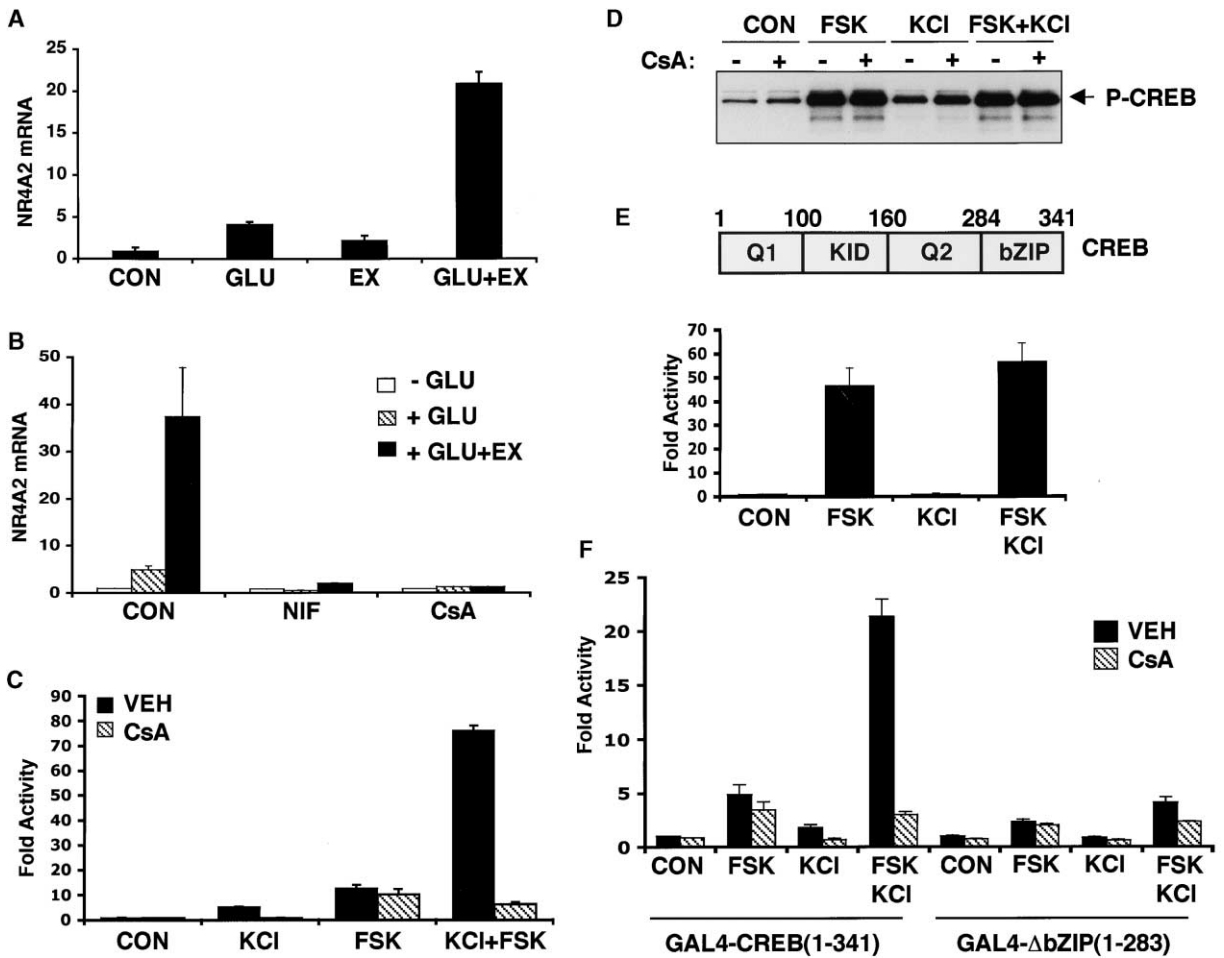


Figure 1. cAMP and Calcium Pathways Stimulate CREB Activity Cooperatively via the CREB bZIP Domain

(A) Q-PCR analysis of NR4A2 mRNA levels in MIN6 cells exposed to glucose (GLU), exendin-4 (EX), or vehicle (CON).  
 (B) Effect of calcium channel antagonist (nifedipine; NIF) and calcineurin antagonist (cyclosporine A; CsA) on NR4A2 mRNA levels in MIN6 cells treated with GLU and EX as indicated.  
 (C) Transient assay of HIT cells transfected with the CREB-dependent EVX1 luciferase reporter. Treatment with KCI and forskolin (FSK) or DMSO (CON) indicated. Effect of CsA or vehicle (VEH) shown.  
 (D) Western blot of phospho (Ser133) CREB levels in HIT cells treated with KCI and/or FSK for 30 min. Effect of CsA shown.  
 (E) Effect of calcium and cAMP agonists on CREB:CBP complex formation. (Top) Schematic of CREB; Q1 and Q2, glutamine rich domains; KID, kinase inducible domain; bZIP, basic region/leucine zipper domain. Amino acid endpoints indicated. (Bottom) Two-hybrid assay of HIT cells transfected with GAL4-KID and KIX-VP16 effector plasmids plus GAL4 luciferase reporter. Treatment with FSK and KCI indicated.  
 (F) Effect of KCI, FSK, and CsA on activity of full-length GAL4-CREB and truncated GAL4-CREB ΔbZIP (aa 1–283) lacking the bZIP domain in HIT cells.

TORC2 stimulated EVX1 reporter activity 60-fold in response to FSK and 6-fold following exposure to KCI (Figure 2C). Cotreatment with FSK and KCI enhanced TORC2 activity more than 300-fold, and this cooperativity was blocked by pretreatment with CsA (Figure 2C). CREB was required for TORC2 potentiation, as siRNA-mediated knockdown of CREB eliminated these effects in transient assays with an EVX1 reporter (Figure 2D).

To determine whether the CREB:TORC complex is necessary for cooperativity between cAMP and calcium signals, we screened for mutations in the bZIP domain that block this interaction. Based on the sensitivity of the CREB:TORC complex to high-salt conditions (data not shown), we tested whether Ala mutations at charged residues within the CREB leucine zipper would disrupt this association. Of five mutants tested, Arg314Ala was

found to be defective in TORC2 binding by GST pull-down and gel mobility shift assays (Figures 2E and 2F). Furthermore, TORC2 was unable to potentiate the activity of Arg314Ala mutant compared to wild-type CREB in transient assays with an EVX1 reporter (Supplemental Figure S6). The Arg314Ala mutation in CREB affected neither dimerization nor DNA binding properties of the CREB bZIP domain, indicating that loss of TORC2 potentiation reflects a direct effect on formation of the TORC:CREB complex and not on CREB occupancy (Supplemental Figure S7). The association of TORC with the bZIP domain also had no effect on binding site selectivity; in gel shift assays using the CREB-related CREM protein, CREM:TORC complexes were formed only on CRE sites as opposed to lower-affinity AP-1 sites (Supplemental Figure S8). These experiments suggest that

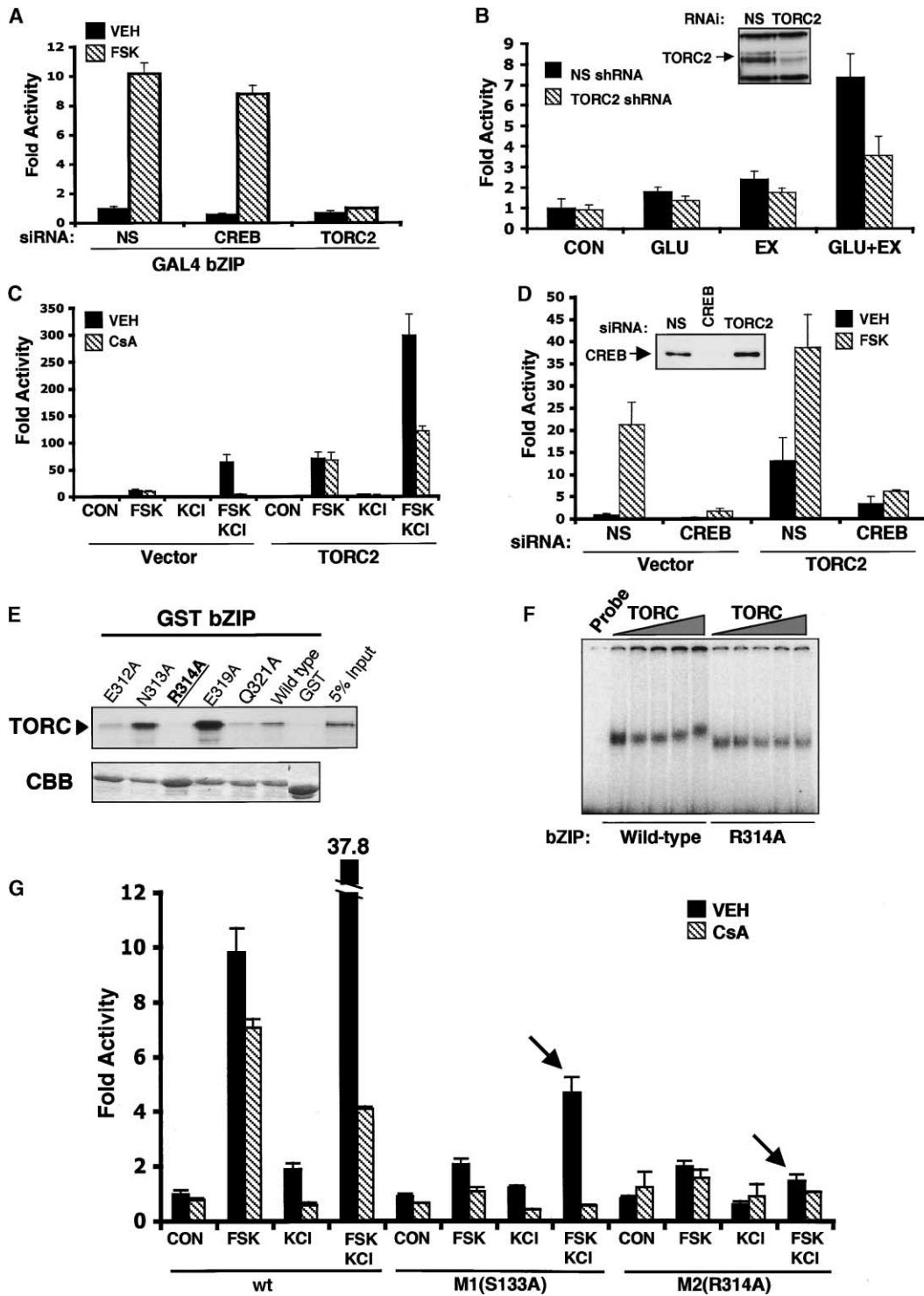


Figure 2. TORC2 Mediates Cooperative Effects of cAMP and Calcium Signals on CREB Activation

(A) Transient assay of HEK293T cells transfected with GAL4 bZIP (aa 271–341) vector plus GAL4 luciferase reporter. Effect of CREB, TORC2, or nonsilencing (NS) siRNAs on GAL4-bZIP activity in cells treated with FSK or vehicle (VEH) shown.

(B) Effect of TORC2 or nonsilencing (NS) shRNA plasmids on induction of cotransfected EVX1 luciferase reporter by glucose and extendin-4 in MIN6 cells. (Inset) Western blot assay showing effect of TORC2 versus nonsilencing (NS) siRNA on TORC2 protein levels by Western blot assay.

(C) Transient assay showing effect of TORC2 or empty vector on EVX1 reporter activity in HIT cells exposed to FSK and/or KCl. Pretreatment with CsA or vehicle (VEH) as indicated.

(D) Transient assay of HEK293T cells transfected with TORC2 or empty vector. Cotransfection with either nonsilencing (NS) or CREB siRNA indicated. Luciferase activity from cotransfected EVX1 reporter shown. Treatment with FSK or vehicle (VEH) indicated. (Inset) Reduction of CREB protein levels in CREB versus NS or TORC2 RNAi treated cells verified by Western blot assay with anti-CREB antiserum.

(E) GST pull-down assay of <sup>35</sup>S-labeled TORC1 with wild-type and mutant GST CREB bZIP (aa 271–341) proteins. Effect of individual bZIP

the effect of TORC on CREB target gene expression reflects its ability to recruit the transcriptional machinery rather than to alter CREB binding to the promoter.

To test the regulatory contributions of TORC and CBP toward CREB activation, we used GAL4-CREB constructs containing point mutations that disrupt either CREB:CBP (Ser133Ala:M1) or CREB:TORC (Arg314Ala:M2) complexes. By contrast with the wild-type GAL4-CREB polypeptide, M1 and M2 mutants were far less responsive to KCl or FSK individually in HIT cells (Figure 2G). The CBP interaction-defective M1 polypeptide was induced 4-fold in response to KCl and FSK, and these effects were blocked by CsA. By contrast, the TORC interaction-defective M2 mutant was completely unresponsive to cotreatment with KCl and FSK, suggesting that TORC2 is primarily responsible for the synergistic effects of calcium and cAMP on cellular gene expression.

Based on the importance of the CREB:TORC2 interaction for transcriptional induction, calcium and cAMP agonists would be predicted to enhance recruitment of TORC2 to CREB-regulated genes. In chromatin immunoprecipitation (ChIP) assays of HIT insulinoma cells, TORC2 was largely absent from the NR4A2 promoter under resting conditions (Figure 3A, left). Although FSK or KCl alone had only modest effects on TORC recruitment (1.0- and 1.7-fold, respectively), cotreatment with FSK and KCl induced TORC2 occupancy 4.6-fold. Moreover, the cooperative recruitment of TORC2 to the NR4A2 promoter was completely blocked by CsA. Indeed, the profile for TORC occupancy paralleled the changes in NR4A2 mRNA levels with each treatment (Figure 3A, right), supporting the notion that TORC2 mediates the cooperative effects of cAMP and calcium on target gene activation.

#### Nuclear Entry of TORC2 Is Regulated by cAMP and Calcium Influx

In the course of experiments to determine TORC cellular localization, we noticed that TORC2 protein was largely confined to the cytoplasm in MIN6 and HIT cells under resting conditions (Figure 3B). Individually, glucose and exendin-4 had no effect on TORC2 localization; in combination, however, they promoted redistribution of TORC2 to the nucleus (Figure 3B, top). Exposure to both KCl and FSK also promoted TORC2 nuclear entry; in all cases, pretreatment with CsA potentially disrupted this process (Figure 3B, top and bottom).

To determine the mechanism by which TORC2 subcellular localization is regulated, we characterized relevant nuclear import and export signals using a human fibroblast line (ATYB1) in which cytoplasmic and nuclear targeting could be readily assessed. In contrast to the electrically excitable MIN6 and HIT cells, FSK treatment of ATYB1 cells was sufficient to trigger nuclear translocation of TORC2 (Figure 3C, left). Using cellular fluorescence assays on truncated TORC polypeptides fused to

green fluorescent protein (GFP), we identified a nuclear localizing sequence (NLS) at aa 56–144 of TORC2 and two nuclear export sequences (NES1 and NES2) within aa 145–320 (Figure 3C, top; Supplemental Figure S10). Both NLS and NES motifs were conserved within the three TORC family members, and mutagenesis of individual leucines in NES1 and NES2 promoted nuclear accumulation of TORCs 1 and 2 (data not shown). Consistent with the presence of both NLS and NES motifs, TORCs appear to cycle in and out of the nucleus under resting conditions; treatment of ATYB1 cells with the exportin inhibitor leptomycin B promoted nuclear accumulation of TORC2 (Figure 3C).

By contrast with TORCs 1 and 2, TORC3 was nuclear localized under basal conditions (Figure 3D) and was far more active in potentiating CREB activity relative to TORC2 (data not shown). An analysis of the relevant export domains between TORC family members revealed that TORC3 contains a Tyr in place of Phe at aa 282 within NES1, which would be predicted to disrupt export activity (Figure 3D, top). Mutagenesis of Tyr282 in TORC3 to Phe, as in TORCs 1 and 2, led to cytoplasmic retention of the protein (Figure 3D, bottom) and repressed basal TORC3 activity (data not shown). Indeed, exposure to FSK triggered nuclear translocation of the Tyr282Phe TORC3 mutant, supporting the involvement of NES1 in signal-dependent shuttling (Figure 3D, bottom).

#### TORC2 Phosphorylation Is Regulated by cAMP and Calcium Influx

The subcellular localization of certain activators, such as NFAT and FOXO family members, is regulated by signal-dependent phosphorylation or dephosphorylation (Brunet et al., 1999; Chow and Davis, 2000). To test whether TORC2 localization is similarly modulated, we compared the mobilities of TORC2 proteins isolated from nuclear and cytoplasmic fractions. Overexpressed Flag-TORC2 migrated as an 85 kDa doublet in cytoplasmic fractions but as a single faster-migrating species in nuclear extracts (Figure 4A, top left). The slower-migrating TORC2 band in cytoplasmic extracts likely represents phosphorylated TORC2, as treatment with calf intestinal alkaline phosphatase (CIP) collapsed this doublet into the single faster-migrating species (Figure 4A, top right).

The accumulation of unphosphorylated TORC2 in nuclear fractions suggests that calcium and cAMP pathways stimulate TORC2 nuclear entry by triggering its dephosphorylation. Indeed, exposure of HIT cells to KCl and FSK in combination promoted extensive dephosphorylation of endogenous TORC2 as determined by Western blot analysis with anti-TORC2 antiserum (Figure 4A, bottom). Moreover, treatment with CsA blocked TORC2 dephosphorylation, implicating the involvement of calcineurin in this process (Figure 4A, bottom).

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mutations on TORC binding shown. CBB, Coomassie gel showing input levels of bZIP proteins.

(F) Gel shift assay of wild-type and Arg314Ala mutant recombinant CREB bZIP (aa 271–341) peptides using <sup>32</sup>P-labeled CRE oligo. Addition of recombinant TORC1 (aa 1–129) to reactions indicated.

(G) Transient assay of HIT cells expressing wild-type (wt) or mutant GAL4-CREB proteins defective in either CBP (M1: Ser133Ala) or TORC (M2: Arg314Ala) binding. Effect of FSK, KCl, FSK+KCl, and CsA or VEH on GAL4 luciferase reporter activity shown.

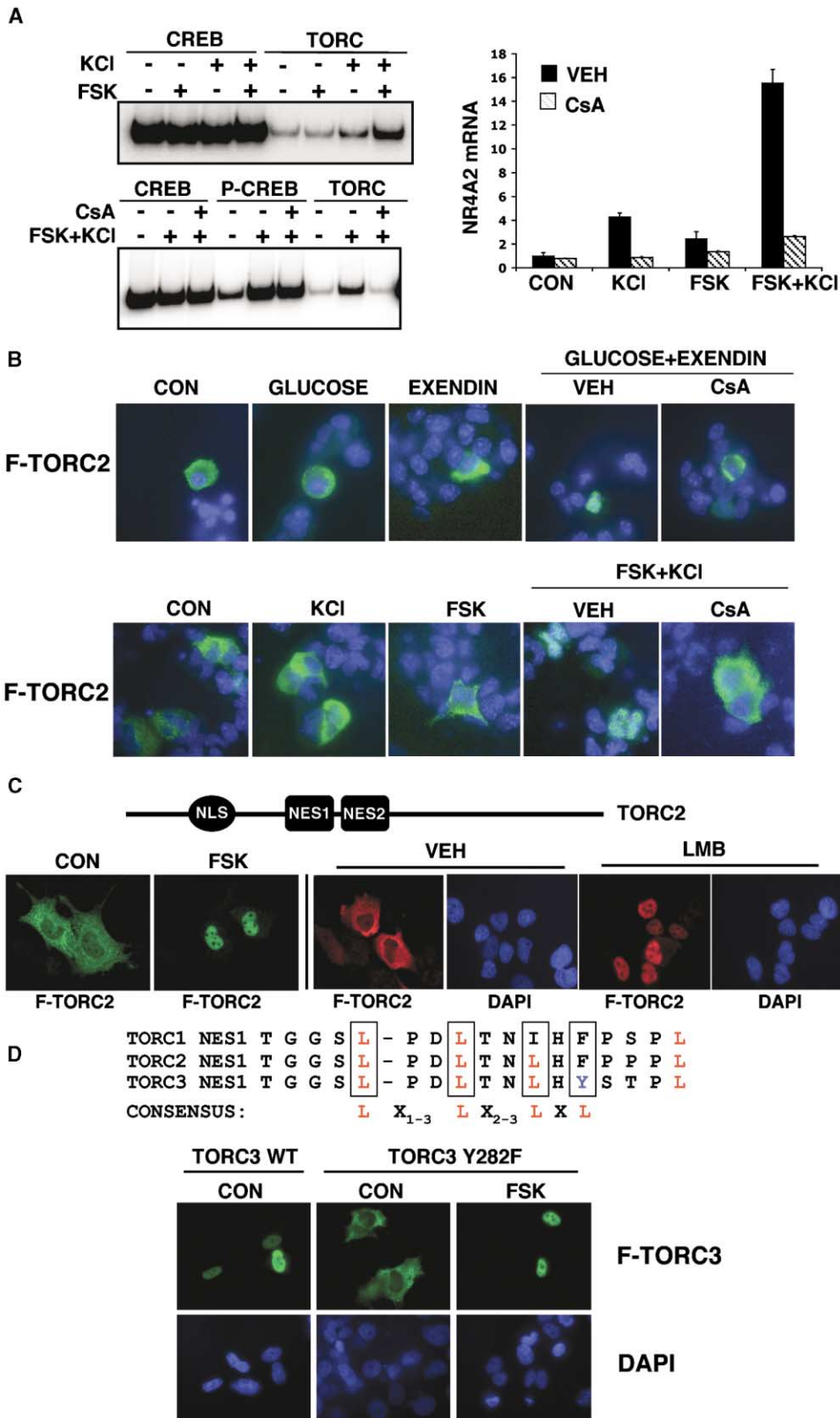


Figure 3. Cooperative Effects of cAMP and Calcium Pathways on TORC Activity

(A) (Top and bottom) ChIP assays of the CREB-regulated NR4A2 gene in HIT cells exposed to KCl, FSK, and CsA as indicated. Levels of NR4A2 promoter recovered from individual IPs (CREB, phospho [Ser133] CREB, and TORC) measured by PCR amplification with <sup>32</sup>P-labeled primers. (Right) Q-PCR analysis of NR4A2 mRNA levels in HIT cells treated with FSK, KCl, and CsA or vehicle (VEH) indicated.

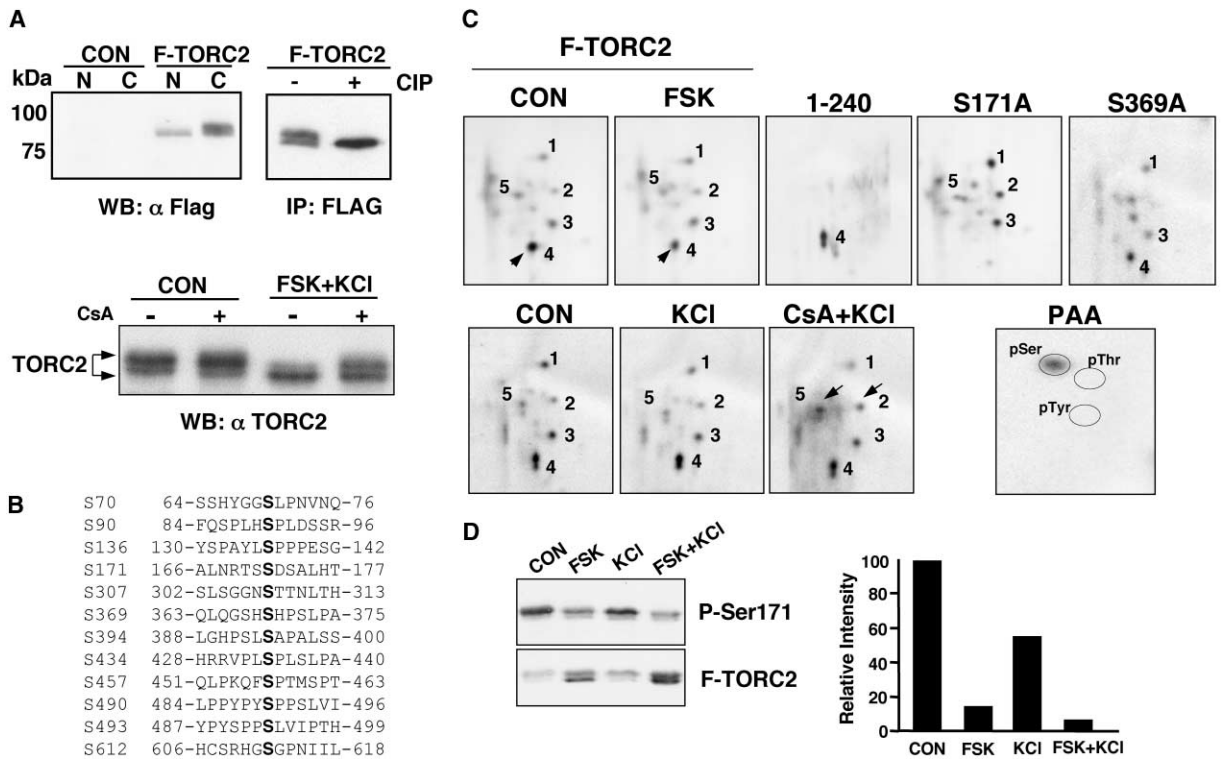


Figure 4. cAMP and Calcium Influx Promote Dephosphorylation of TORC2 at Ser171

(A) (Top left) Western blot (WB) of nuclear (N) and cytoplasmic (C) fractions from HEK293T cells transfected with Flag-TORC2. (Right) Effect of calf intestinal alkaline phosphatase (CIP) treatment on mobility of IPed TORC2. (Bottom) Western blot with  $\alpha$ TORC2 antiserum showing effect of FSK plus KCl treatment on endogenous TORC2 dephosphorylation in HIT cells. Treatment with CsA indicated.

(B) Mass spectrometry (MS) of sites on TORC2 phosphorylated in vivo. Phosphopeptides recovered from Flag-TORC2 IPs identified by MS/MS analysis. Phosphoacceptor Sers in bold.

(C) (Top) 2D phosphotryptic mapping of <sup>32</sup>P-labeled Flag-TORC2 recovered from IPs of HIT cells treated with FSK, KCl, and CsA as indicated. Maps (1000 cpm/map) of wild-type, truncated (aa 1–240), and mutated (S171A, S369A) TORC2 proteins shown. Major tryptic spots labeled 1–5. Arrowheads point to spots regulated by FSK and KCl+CsA. (Bottom right) Phosphoamino acid analysis of <sup>32</sup>P-labeled TORC2 recovered from HIT cells as above. Positions of phosphorylated Ser, Thr, and Tyr indicated.

(D) Western blot of extracts from HIT cells transfected with a Flag-TORC2 vector and treated with DMSO (CON), KCl, FSK, and CsA as indicated and probed with  $\alpha$  phospho (Ser171) TORC2 antiserum. Total amounts of Flag-tagged TORC2 for each condition (F-TORC2) determined by blotting with  $\alpha$ -Flag antiserum. (Right) Graph showing relative levels of phospho (Ser171) TORC2, by densitometry, after normalizing for Flag-TORC2 levels.

We used tandem mass spectrometric (MS) analysis to identify phosphorylation sites on TORC2 that dictate its subcellular localization. Twelve independent phosphorylated serine residues on TORC2 were identified by analysis of Flag-TORC2 immunoprecipitates from HEK293T cells, the majority of these (seven of 12) mapping to a central region (aa 300–500) in TORC2 (Figure 4B).

To identify phosphorylation sites on TORC2 that are regulated by cAMP or calcium influx, we performed two-dimensional (2D) tryptic mapping experiments in HIT cells. Consistent with MS studies, <sup>32</sup>P-labeled TORC2

was phosphorylated exclusively on serine by phosphoamino acid analysis (Figure 4C, bottom right). 2D tryptic maps of <sup>32</sup>P-labeled TORC2 revealed five major spots (1–5, Figure 4C), four of which (1, 2, 3, and 5) were assigned to the C-terminal 350 residues of TORC2; only spot 4 was detected in 2D maps of a truncated TORC2 polypeptide extending from aa 1 to aa 240 (Figure 4C). Indeed, spot 4 corresponds to the primary TORC2 phosphorylation site, accounting for over half of <sup>32</sup>P incorporated by the full-length protein in resting cells.

Following treatment with FSK, the intensity of spot 4 was specifically diminished, suggesting that cAMP

(B) Immunofluorescence (IF) assay showing effect of glucose, exendin-4, and CsA (top) or FSK, KCl, and CsA (bottom) on cellular localization of Flag-TORC2 (F-TORC2) in MIN6 (top) and HIT (bottom) insulinoma cells. Counterstaining with DAPI shown to visualize nuclei.

(C) (Top) Schematic showing relative positions of NLS and NES motifs in TORC2 as determined by IF assay with truncated TORC proteins (Supplemental Figure S10). (Left) IF analysis of Flag-TORC2 in human ATYB1 fibroblasts treated with FSK or vehicle for 30 min. (Right) Effect of exportin inhibitor leptomycin B (LMB) on localization of TORC2 in transfected ATYB1 fibroblasts. DAPI staining shown.

(D) (Top) Amino acid sequence alignment for NES1 motif (aa 271–287 in TORC2) in TORC family members relative to consensus NES motif. Tyr282 in TORC3 highlighted. (Bottom) Effect of Tyr282 to Phe mutagenesis in NES1 on TORC3 localization in control and FSK treated ATYB1 cells. Staining of wild-type TORC3 in untreated cells shown. DAPI staining included.

regulates TORC2 phosphorylation at one principal site (Figure 4C). Although exposure to KCl had minor effects by 2D mapping, treatment with KCl plus CsA specifically enhanced spots 2 and 5. These results support the notion that cAMP and calcium pathways regulate dephosphorylation of TORC2 at distinct sites.

We assigned relevant phosphoacceptor sites that correspond to cAMP- and calcium-regulated spots by performing mutational studies on phosphorylated serines identified by MS. Although a number of substitutions (Ser70Ala and Ser394Ala; data not shown) had no effect on 2D maps of <sup>32</sup>P-labeled TORC2, mutation of Ser171 to Ala eliminated spot 4, and Ser369Ala mutation disrupted spots 2 and 5 (Figure 4C). Spots 2 and 5 appear to correspond to differentially phosphorylated forms of the same 19 kDa tryptic fragment (aa 243–428) of TORC2, which contains 38 potential serine phosphoacceptors.

Having identified Ser171 as a principal phosphorylation site on TORC2 that is dephosphorylated in response to cAMP, we developed a phospho (Ser171)-specific TORC2 antiserum. Consistent with <sup>32</sup>P labeling studies, treatment with FSK reduced levels of phospho (Ser171) TORC2 6-fold within 10 min (Figure 4D). Exposure to KCl alone had a small effect (2-fold) on phospho (Ser171) TORC2 levels, but costimulation with both FSK and KCl reduced amounts of phospho (Ser171) TORC2 cooperatively (12-fold). These results indicate that, although calcium and cAMP pathways promote dephosphorylation at independent sites on TORC2, they converge on Ser171 when activated together.

#### Phosphorylated TORCs Associate with 14-3-3 Proteins

In the course of MS studies on TORC2 phosphorylation, we detected 14-3-3 proteins in immunoprecipitates (IPs) of TORC2. Notably, 14-3-3 proteins have been found to bind and sequester a number of regulatory proteins, including CDC25A, Forkhead, and NFAT family members, in a phosphorylation-dependent manner (Brunet et al., 1999; Chen et al., 2003; Chow and Davis, 2000; Durocher et al., 2000). Confirming the results from MS analysis, endogenous 14-3-3 proteins were recovered from IPs of Flag-TORC2 prepared from HEK293T cells (Figure 5A, left). Given the importance of Ser171 as a primary phosphorylation site on TORC2, we tested whether the Ser171Ala mutant polypeptide was also associated with 14-3-3 in cells. By contrast with the levels of 14-3-3 protein recovered from IPs of wild-type TORC2, 14-3-3 proteins were only weakly detected in IPs of Ser171Ala mutant TORC2. These results support the involvement of Ser171 in the TORC2:14-3-3 interaction.

To further test the importance of TORC2 phosphorylation for complex formation with 14-3-3 protein, we used Far Western blotting assays. A recombinant GST-14-3-3 polypeptide was found to bind phosphorylated TORC2 isolated from HIT cells under basal conditions but not TORC2 dephosphorylated *in vitro* with CIP (Figure 5A, right). Consistent with the importance of Ser171 as a principal phosphorylation site on TORC2, mutation of Ser171 to Ala substantially reduced complex formation with 14-3-3, although not to the same extent as CIP treatment (Figure 5A, right). These results indicate that

14-3-3 proteins bind directly to multiple phosphorylated regions on TORC2.

In view of their ability to promote TORC2 dephosphorylation at Ser171 and other sites, KCl and FSK might be expected to modulate the TORC2:14-3-3 interaction. Indeed, exposure to either stimulus reduced TORC2 binding to 14-3-3 by Far Western blot assay, and co-treatment with CsA reversed these effects (Figure 5B). These results support the idea that cAMP and calcium influx individually disrupt the TORC2:14-3-3 interaction by promoting dephosphorylation of TORC2 at distinct sites.

The ability of CsA to interfere with TORC2 dephosphorylation and liberation from 14-3-3 proteins led us to examine the role of Cn in regulating TORC2 activity. Notably, Cn A and B subunits were recovered from immunoprecipitates of Flag-TORC2 in MS studies, and we confirmed these results in pull-down assays using full-length <sup>35</sup>S-labeled TORC2 with GST-CnA (aa 1–347) (Figure 5C). The Cn:TORC2 interaction also appears direct; in Far Western blotting assays, a recombinant CnA polypeptide was found to bind efficiently to Flag-TORC2 recovered from anti-Flag immunoprecipitates from HIT cells (Figure 5D). By contrast with the TORC2:14-3-3 interaction, however, dephosphorylation of TORC2 with CIP had no effect on CnA binding, indicating that the TORC2:CnA complex is not modulated by phosphorylation (Figure 5D).

TORC2 contains three near-consensus PXIXIT motifs (Cn1, PNVNQIG [aa 72–78]); Cn2, PNIILT [aa 615–620]; and Cn3, PGINIF [aa 248–253]), which have been shown to mediate CnA binding in NFAT and other calcium-regulated proteins (Hogan et al., 2003). Indeed, mutagenesis of either Cn2 or Cn3 motifs in TORC2 reduced binding of CnA to TORC2 by GST pull-down and Far Western blotting assays, but Cn1 mutations had no effect (Figures 5C and 5D). Supporting a functional role for Cn binding in TORC2 activation, the Cn3 mutant TORC2 protein was 2.5-fold less responsive to KCl relative to wild-type TORC2 in HIT cells cotransfected with an EVX1 reporter (Figure 5E).

The importance of Ser171 as a major regulatory site for dephosphorylation in response to FSK and KCl prompted us to examine whether Ser171Ala mutagenesis alters TORC2 activity in response to these stimuli. Compared with the wild-type protein, Ser171Ala mutant TORC2 was 3-fold more active under basal conditions in HIT (Figure 5E) and HEK293T (Figure 5F) cells, and treatment with KCl or FSK had only modest effects on Ser171Ala mutant TORC2 activity. These results support the notion that Ser171 is a key site for cooperative induction of TORC2 activity by cAMP and calcium signaling pathways.

#### TORC2 Associates with the Ser/Thr Kinase SIK2

Based on the extensive phosphorylation of TORC2 in quiescent cells, we speculated that an inhibitory Ser/Thr kinase may interact with and sequester TORC2 in the cytoplasm. Using *in vitro* kinase assays on TORC2 immunoprecipitates, we found that a Ser/Thr kinase activity was associated with TORC2 in cytoplasmic (C) but not nuclear (N) fractions of HEK293T cells (Figure 6A, left). Indeed, the TORC2 kinase activity appeared to



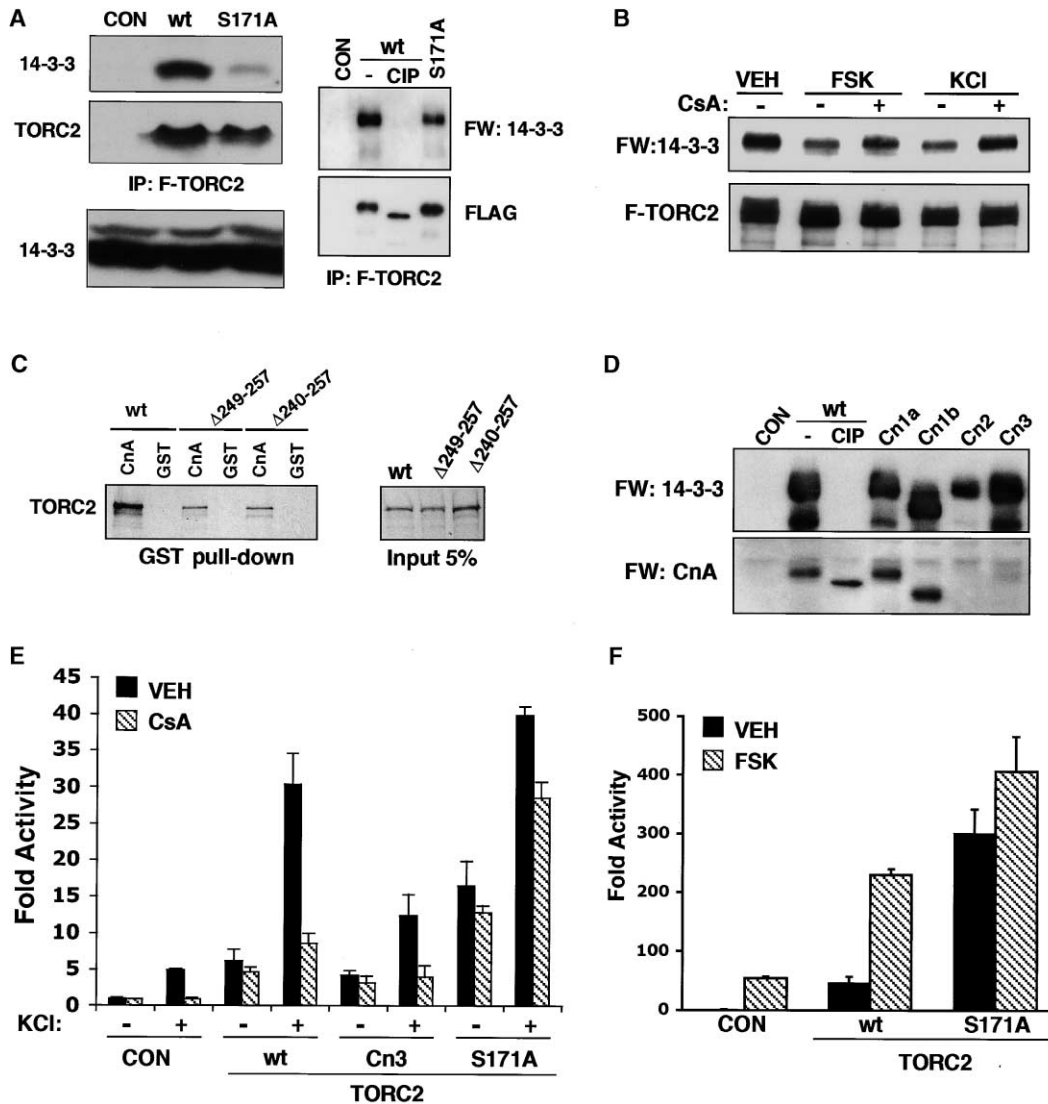


Figure 5. 14-3-3 Proteins Associate with Phosphorylated TORCs

(A) (Left) CoIP of wild-type and Ser171Ala mutant Flag-TORC2 with endogenous 14-3-3 proteins in HEK293T cells. IPed Flag-TORC2 and CoIPed 14-3-3 proteins shown. Input levels of 14-3-3 proteins shown (bottom). (Right) Far Western blot assay of Flag-TORC2 IPs from HIT cells expressing wild-type or Ser171Ala mutant Flag-TORC2. Blots were probed with recombinant GST-14-3-3 protein. Effect of TORC2 dephosphorylation by CIP on 14-3-3 binding shown. (Bottom right) (FLAG) Western blot assay showing recovery of TORC2 proteins from  $\alpha$ -Flag IPs by Western blotting with  $\alpha$ -Flag antiserum.

(B) Effect of FSK, KCl, or vehicle (VEH) on 14-3-3 binding by Far Western blot assay of Flag-TORC2 IPs prepared from HIT cells. Effect of CsA on 14-3-3 binding indicated. (Bottom) Recovery of Flag-TORC2 from each IP by Western blot with  $\alpha$ -Flag antiserum.

(C) Pull-down assay of GST-CnA (aa 1–347) with <sup>35</sup>S-labeled wild-type and mutant TORC2 proteins as indicated. (Right) Input levels of wild-type and mutant TORC2 proteins shown.

(D) Far Western blot assay of Flag IPs from HIT cells transfected with control vector or Flag-TORC2 vector and probed in parallel with either recombinant GST-14-3-3 (top) or GST-CnA (bottom) proteins. Effect of TORC2 dephosphorylation by CIP on CnA and 14-3-3 binding shown. Top bands in each lane represent full-length protein. Wild-type and mutant TORC2 proteins (Cn1a [aa 72–78], PNVNQIG/GNANQAA; Cn1b [deletion of aa 56–144]; Cn2 [aa 615–620], PNIILT/GNAIAA; Cn3 [aa 248–257], PGINIFPSPD [aa 249–257 deleted]) indicated.

(E) Transient assay of HIT cells transfected with wild-type or mutant TORC2 expression vectors defective in either CnA binding (Cn3) or phosphorylation (Ser171A). Luciferase activity from EVX1 reporter plasmid shown. Treatment with KCl and CsA indicated.

(F) Transient assay of HEK293T cells showing effect of wild-type and Ser171Ala mutant TORC2 expression vector or empty vector (CON) on EVX1 reporter activity in cells treated with FSK or vehicle (VEH).

phosphorylate primarily one site corresponding to spot 4 (Ser171) by 2D tryptic mapping (Figure 6A, right). Moreover, exposure to FSK potently inhibited the TORC-associated kinase activity by *in vitro* assay, providing a potential mechanism to explain how cAMP inhibits Ser171 phosphorylation on TORC2.

To determine the identity of the putative cAMP-regulated TORC2 kinase, we performed MS studies on TORC2 immunoprecipitates prepared from HEK293T cytoplasmic lysates. Among other potential kinases identified by this analysis, we detected the salt-inducible kinase 2 (SIK2), a member of the snf1 family of kinases

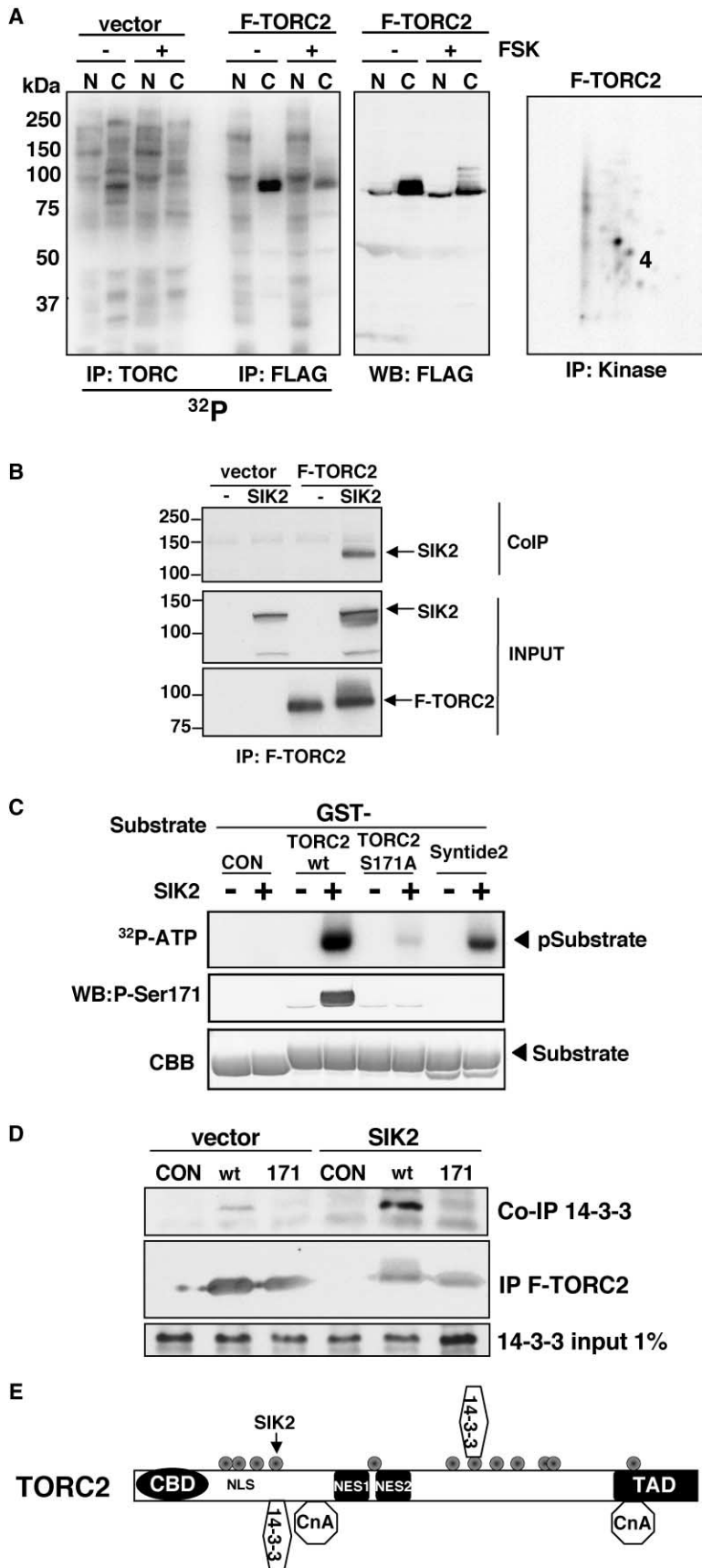


Figure 6. The Ser/Thr Kinase SIK2 Associates with and Phosphorylates TORC2

(A) In vitro kinase assays of endogenous TORC (IP:TORC) and transfected Flag-TORC2 (IP:FLAG) IPs prepared from nuclear (N) or cytoplasmic (C) fractions of control and FSK treated HEK293T cells as shown. <sup>32</sup>P-labeled TORC2 bands (left) and corresponding TORC2 protein levels by Western blot assay with  $\alpha$ -Flag antiserum (right) indicated. (Right) 2D tryptic mapping of Flag-tagged TORC2 IPed from HEK293T cells and phosphorylated with <sup>32</sup>P-ATP by in vitro kinase assay. Position of spot 4 (as shown in Figure 3) indicated.

(B) CoIP assay of HEK293T cells cotransfected with expression vectors for SIK2, Flag-TORC2, or empty vector. Western blot of SIK2 recovered from Flag IPs using anti-SIK2 antiserum shown.

(C) SIK2 phosphorylates TORC2 at Ser171. In vitro kinase assay of wild-type and Ser171-Ala GST TORC2 (aa 162-179) using purified SIK2 as indicated. Effect of SIK2 on GST and GST-syntide 2 containing an optimal SIK2 phosphorylation site shown. (Middle) Western blot assay using phospho (Ser171)-specific TORC2 antiserum. (Bottom) Input levels of individual GST proteins (CBB).

(D) SIK2 stimulates binding of 14-3-3 to TORC2 at Ser171. Flag-TORC2 (aa 1-240) wt or S171A mutant proteins were IPed from HEK293T cells cotransfected with SIK2 plasmid or empty vector control. CoIPed 14-3-3 proteins (top), IPed TORC2 (1-240) proteins (middle), and input levels of 14-3-3 from cell lysates for each condition shown.

(E) Schematic of TORC2 showing position of CREB Binding domain (CBD), nuclear localization and export signals (NLS and NES), and transactivation domain (TAD). In vivo phosphorylation sites, identified by mass spectrometric analysis, shown as circles. Binding sites for 14-3-3 and CnA indicated. Site for SIK2 phosphorylation (Ser171) indicated.

previously found to inhibit transcription of cAMP-responsive genes (Doi et al., 2002). We confirmed the association between SIK2 and TORC2 in coimmunoprecipitation (CoIP) assays of HEK293T cells cotransfected with SIK2 and Flag-TORC2 expression vectors (Figure 6B). Indeed, TORC2 mobility was noticeably reduced in cells coexpressing SIK2, consistent with the idea that this kinase directly phosphorylates TORC2 *in vivo* (Figure 6B).

Based on *in vitro* phosphorylation studies with potential SIK2 substrates, we deduced an optimal consensus motif for SIK2 phosphorylation (LXBS/TXSXXXL, where B is a basic residue; Supplemental Table S1). Remarkably, TORC2 contains a single consensus site for SIK2 phosphorylation corresponding to Ser171 (aa 166–LNR-TSSDSAL). Indeed, SIK2 was found to phosphorylate wild-type but not Ser171Ala mutant GST-TORC2<sub>162–179</sub> *in vitro* (Figure 6C). SIK2 did not appear to phosphorylate other potential sites on TORC2, including Ser70 and Ser394 (data not shown), suggesting that Ser171 is the principal phosphoacceptor site for this kinase.

Based on its ability to phosphorylate Ser171, SIK2 would be expected to promote the association of TORC2 with 14-3-3 proteins. Indeed, overexpression of SIK2 strongly enhanced binding of Flag-TORC2 (aa 1–240) protein to 14-3-3, and this effect was blocked by mutation of Ser171 to Ala (Figure 6D). Demonstrating the direct role of phospho (Ser171) in 14-3-3 binding, SIK2 also stimulated the interaction of 14-3-3 proteins with a wild-type but not Ser171Ala mutant TORC2 peptide (aa 162–179) fused to GST (Supplemental Figure S11). Taken together, these studies indicate that SIK2 and Cn associate with and regulate TORC2 phosphorylation as well as its association with 14-3-3 proteins (Figure 6E).

#### SIK2 Inhibits TORC2 Translocation

cAMP has been found to disrupt SIK2 activity via the PKA-mediated phosphorylation of SIK2 at Ser587 (Okamoto et al., 2004). Supporting this notion, exposure of COS-7 cells to FSK increased phospho (Ser587) SIK2 levels by Western blot assay with a phospho (Ser587)-specific antiserum (Figure 7A). In keeping with its ability to stimulate the TORC:14-3-3 interaction, wild-type SIK2 inhibited induction of an EVX1 reporter by FSK about 3-fold (Figure 7B). SIK2 kinase activity was required for inhibition, as overexpression of a kinase-inactive SIK2 mutant (K49M) had no such effect. Consistent with a role for cAMP in regulating SIK2, the PKA phosphorylation-defective SIK2 (Ser587Ala) mutant was more potent in blocking EVX1 reporter activity in response to FSK (Figure 7B).

Having seen that SIK2 enhances the TORC2:14-3-3 interaction via phosphorylation at Ser171, we reasoned that SIK2 would correspondingly promote cytoplasmic sequestration of TORC2. In immunocytochemical studies of ATYB1 cells, Flag-TORC2 was localized to both nuclear and cytoplasmic compartments under resting conditions (Figure 7C). However, coexpression of SIK2 in these cells resulted in exclusive cytoplasmic localization of TORC2. Consistent with the ability of PKA to disrupt SIK2 activity, treatment with FSK promoted TORC2 nuclear translocation in SIK2-expressing cells. FSK had no such effect in cells expressing the phos-

phorylation-defective (Ser587Ala) mutant SIK2, however, demonstrating the importance of SIK2 phosphorylation by PKA for TORC2 nuclear entry in response to cAMP. The phosphorylation of TORC2 at Ser171 by SIK2 appears pivotal in this regard; TORC Ser171Ala mutant protein was targeted to the nucleus of ATYB1 cells under both basal and cAMP stimulated conditions (Figure 7C, bottom). Taken together, these results indicate that the SIK2-mediated phosphorylation of TORC2 at a single site (Ser171) favors sequestration of TORC2 and inhibition of CREB activity in the basal state (Figure 7D).

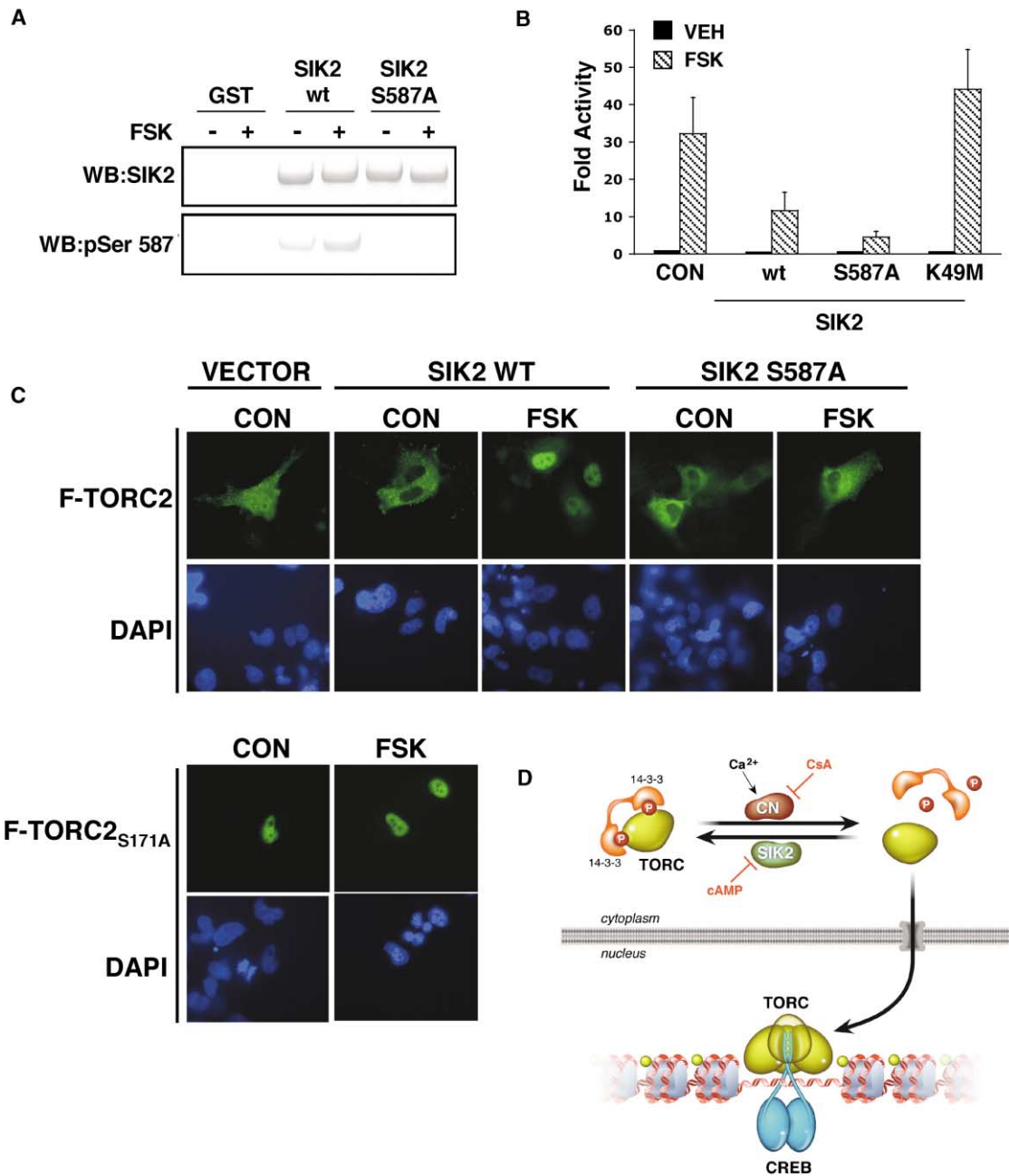
#### Discussion

We have described a mechanism by which two second messenger pathways converge on CREB to regulate  $\beta$  cell gene expression in response to glucose and gut hormones. The synergistic effects of these pathways on cellular gene expression require the CREB bZIP domain, a region which has been found to contribute functionally to cAMP and calcium signaling in excitable cells (Bonni et al., 1995; Sheng et al., 1991). Indeed, the importance of the bZIP domain for target gene activation may explain in part why CREB selectively homodimerizes with related family members (CREB1, ATF1, and CREM) and not with other bZIP proteins (Newman and Keating, 2003). Taken together, these results extend the functional role of bZIP domains to include not only DNA binding and dimerization but also coactivator recruitment.

TORC2 appears to fulfill a number of criteria as a coactivator that mediates cooperativity between cAMP and calcium signals in islet cells. First, TORC2 stimulates CREB activity through a bZIP domain interaction, and point mutants in CREB that disrupt this interaction also block cooperativity between these pathways. Secondly, cAMP and calcium signals act synergistically in promoting TORC2 nuclear translocation and promoter recruitment. Finally, loss of TORC2 expression in insulinoma cells inhibits the transcriptional response to glucose and exendin.

CBP and TORC enhance the expression of cAMP-responsive genes by associating with distinct regions of CREB. Although both interactions are regulated by phosphorylation, the mechanisms are very different. CREB is constitutively targeted to the nucleus, for example, and its phosphorylation at Ser133, along with CREB:CBP complex formation, in response to inductive signals is rate limited by nuclear entry of relevant Ser/Thr kinases via passive diffusion, a comparatively inefficient process. By contrast, TORC2 is cytoplasmic under resting conditions, undergoing rapid dephosphorylation and nuclear translocation within minutes of stimulation. The cytoplasmic localization of TORC in resting cells may thus provide improved coupling with early signaling events at the plasma membrane and insure that short-lived signals are efficiently transmitted to the nucleus.

Under resting conditions, TORC2 is sequestered in the cytoplasm through a phosphorylation-dependent interaction with 14-3-3 proteins. Calcium and cAMP agonists were found to stimulate the dephosphorylation of TORC at distinct sites (Ser171 and Ser369). Although both sites appear to participate in 14-3-3 binding, phospho (Ser171) performs a dominant role. Indeed, binding



**Figure 7. SIK2 Phosphorylates and Sequesters TORC2 in the Cytoplasm under Basal Conditions**

(A) Western blot of total and phospho (Ser587)-SIK2 levels in COS-7 cells transfected with wild-type and PKA phosphorylation defective Ser587Ala mutant GST-SIK2 expression vector. Effect of FSK treatment shown.

(B) Transient assay of HEK293T cells showing effects of wild-type, PKA phosphorylation-defective (S587A), or kinase-inactive (K49M) SIK2 expression vectors relative to empty vector (CON) on activity from cotransfected EVX1 luciferase reporter in HEK293T cells treated with FSK or vehicle (VEH).

(C) Effect of SIK2 on TORC2 subcellular localization. Immunofluorescence assay of AT1YB1 cells transfected with Flag-TORC2 plus wild-type or PKA phosphorylation defective SIK2 (Ser587Ala) constructs as indicated. DAPI staining shown below each panel. (Bottom) Cellular localization of mutant Ser171Ala TORC2 polypeptide shown. Treatment with FSK or vehicle (CON) indicated.

(D) TORC2 mediates synergistic effects of calcium and cAMP pathways on CREB activation. TORC2 is sequestered in the cytoplasm under basal conditions via a phosphorylation-dependent association with dimeric 14-3-3 proteins. Calcium and cAMP pathways trigger release from 14-3-3 proteins by activating a TORC2 phosphatase (Cn) and inhibiting a TORC2 kinase (SIK2), respectively. Dephosphorylated TORC2 migrates into the nucleus and is recruited to the promoter via an interaction with the bZIP domain of CREB.

of ligands to 14-3-3 proteins is generally thought to occur initially at a high-affinity or gatekeeper site and then at a weak secondary site (Yaffe, 2002), resulting

in an altered conformation of the ligand. Based on its proximity to the TORC2 NLS (aa 56–144), phosphorylated Ser171 may promote TORC2 cytoplasmic localiza-

tion by masking this motif from the nuclear import machinery.

In quiescent cells, TORC2 is highly phosphorylated at Ser171 by SIK2, a member of the AMPK family of Ser/Thr kinases. The kinase activity of SIK2 and other AMPK family members requires prior phosphorylation at a conserved Thr in the T loop by the tumor suppressor protein LKB-1 (Carling, 2004). Germline mutations of LKB1 have been described in Peutz-Jehgers syndrome (PJS), an autosomal dominant disorder characterized by intestinal polyposis and increased frequency of certain gastrointestinal malignancies (Carling, 2004; Lizcano et al., 2004; Shaw et al., 2004; Woods et al., 2003). Loss of LKB-1 would be predicted to activate CREB target gene expression by blocking SIK2-mediated phosphorylation of TORC2 at Ser171. It will be of interest to determine to what extent the CREB:TORC pathway contributes to the etiology of PJS.

CsA was found to block CREB target gene activation in islet cells by interfering with the dephosphorylation of TORC2 at Ser171. The use of CsA and FK506, a second Cn inhibitor, for immunosuppressive therapy in organ transplant recipients is frequently associated with development of  $\beta$  cell failure and diabetes (Al-Uzri et al., 2001; Filler et al., 2000). Based on their ability to prevent TORC2 activation in response to glucose and incretin signaling, these inhibitors may promote islet cell death by blocking CREB target gene activation through this pathway. The development of specific SIK antagonists may improve islet cell function and offer useful therapy for insulin-resistant individuals.

The effects of SIK2 and perhaps related AMPK family members on cellular gene regulation by CREB are likely to extend to other electrically excitable tissues. Both SIKs and TORCs are highly expressed in the brain, for example, an area where CREB appears to function in higher-order processes such as learning and memory. Targeted disruption of SIK and TORC genes in these and other tissues will help to elucidate the cellular contexts in which this pathway mediates responses to extracellular signals.

## Experimental Procedures

### Chemicals

LMB and Exendin-4 (Sigma, Saint Louis, MO) were used at 10 ng/ml and 10 nM, respectively. Nifedipine (10  $\mu$ M), cyclosporine A (CsA, 5  $\mu$ M), and okadaic acid (OA, 100 nM) were from Calbiochem (San Diego, CA). Cells were treated with forskolin at 10  $\mu$ M and with KCl at 45 mM.

### Recombinant Proteins

Recombinant GST CREB bZIP (aa 271–341), GST-14-3-3  $\beta$ , GST-Calcineurin A (aa 1–347), and 6XHis-human TORC1 (aa 1–129) proteins were harvested and purified using glutathione-Sepharose (Amersham-Pharmacia) or Ni<sup>2+</sup>-NTA Superflow beads (Qiagen). Gel shift assays were performed as described (Montclare et al., 2001).

### Q-PCR

For glucose experiments, MIN6 cells were starved overnight in DME/10% FBS containing 2.75 mM glucose plus 16 mM mannitol and 10% FBS. The next day, the medium was changed to 20 mM glucose plus or minus exendin and/or inhibitor for the indicated times. Total RNA was used to generate cDNA with SuperScript II enzyme (Invitrogen, Carlsbad, CA). cDNAs were analyzed by quantitative PCR as described (Conkright et al., 2003b).

### Far Western, Western, IP, and GST Pull-Down

Rabbit pan-TORC (aa 1–42 of human TORC1), TORC2 (aa 454–607 of mouse TORC2), and phospho-Ser171 TORC2 (aa 161–181 of mouse TORC2) antisera were generated as described (Conkright et al., 2003a). Chromatin IP, CoIP, and GST pull-downs were performed as described (Asahara et al., 2001; Conkright et al., 2003a). For Far Western blotting assays, blots were probed with 0.1  $\mu$ M GST-14-3-3  $\beta$  or GST-calcineurin A (aa 1–347). TORC:14-3-3 and TORC:CnA complexes were detected by Western blot assay with rabbit anti-GST antiserum.

### Plasmids and RNAi

Mutant TORC and CREB cDNAs were generated using standard cloning protocols. TORC2 shRNAi plasmid has been described (Conkright et al., 2003a). siRNA duplexes against CREB and TORC2 were from Qiagen.

### Transfections

Cells were cotransfected with EVX-LUC or GAL4-LUC reporter plasmid, RSV- $\beta$  gal and Flag-TORC, and/or SIK2 expression plasmids. For RNAi-mediated knockdown, extracts were harvested after 72 hr. Luciferase values were normalized to  $\beta$ -gal activity.

### Phosphorylation Assays

#### *In Vivo*

40 hr posttransfection, HIT cells were incubated with phosphate-free DMEM containing 10% dialyzed serum and 1 mCi of [<sup>32</sup>P]orthophosphate/ml. After 4–12 hr, cells were washed and harvested in boiling SDS or RIPA lysis buffer. Samples were immunoprecipitated using anti-Flag agarose (Sigma) after preclearing with Staph A. Tryptic peptide mapping was performed as described (Hagiwara et al., 1992).

#### *In Vitro*

Flag-TORC2 and endogenous TORC immune complexes were washed with lysis buffer and subjected to kinase assays using 50  $\mu$ Ci  $\gamma$ -ATP.

### Indirect Immunofluorescence

Human ATYB1 fibroblasts, HIT, and MIN6 cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained stepwise with Flag M2 antibody, donkey anti-mouse, or anti-rabbit Alexa 488- or Cy3-conjugated secondary antibody (Molecular Probes, Eugene, OR; Jackson Immunochemicals), and mounted in Vectashield with DAPI counterstain (Vector Labs).

### Mass Spectrometry

TORC2 IPs were prepared (MacCoss et al., 2002), and peptide mixtures were analyzed by MudPIT analysis (Link et al., 1999). Tandem mass spectra were searched against the most recent versions of the predicted rat, mouse, and human proteins, to which common contaminants such as keratin and trypsin were added using a modified version of the PEP\_PROB algorithm. Search results were filtered and grouped using DTASelect (Tabb et al., 2002). For phosphorylation analysis, a subset database was generated containing only TORC2. Phosphorylation sites that were matched by multiple tandem mass spectra representing sequences of different molecular weights (from the nonspecific enzymes used in the digest) were called matches.

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