Title: Pin1 associates with and induces translocation of CRTC2 to the cytosol, thereby suppressing CRE transcriptional activity

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Running title: Pin1 binds to CRTC2 and suppresses CRE activity.

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Pin1 is a unique regulator which catalyzes the conversion of a specific phosphoSer/Thr-Pro containing motif in target proteins. Herein, we identified CRTC2 as a Pin1 binding protein, by over-expressing Pin1 with myc and FLAG tags in mouse livers and subsequent purification of the complex containing Pin1. The association between Pin1 and CRTC2 was observed in not only over-expression experiments but also endogenously in the mouse liver.

Interestingly, Ser136 in NLS of CRTC2 was shown to be involved in the association with Pin1. Pin1 over-expression in HepG2 cells attenuated forskolin-induced nuclear localization of CRTC2 and CRE transcriptional activity, while gene knockdown of Pin1 by siRNA enhanced both. Pin1 also associated with CRTC1, leading to their cytosol localization, essentially similar to the action of CRTC2. Furthermore, it was shown that CRTC2 associated with Pin1 did not bind to CREB. Taken together, these observations indicate the association of Pin1 with CRTC2 to decrease the nuclear CBP-CRTC-CREB complex. Indeed, adenoviral gene transfer of Pin1 into diabetic mice improved hyperglycemia in conjunction with normalizing PEPCK mRNA expression levels, which is regulated by CRE transcriptional activity. In conclusion, Pin1 regulates CRE transcriptional activity, by associating with CRTC1 or CRTC2.

Pin1 was initially cloned as a NIMA kinase interacting protein (1). Since its discovery, numerous proteins have been identified as Pin1 substrates, including p53, cyclin D1 and tau (2-5). Pin1 interacts with a number of target proteins through recognition of pSer/Pro motifs and the proline conformational change induced by Pin1 modifies the structures and functions, such as stabilization, phosphorylation and translocation, of target proteins (4-7). Pin1 possesses the WW and PPIase domains in its N-terminal (a.a 1- 38) and C-terminal (a.a. 39 -163) regions, respectively. To date, many reports have supported an important role for Pin1 in diseases such as cancer and Alzheimer's disease (4, 5). In this study, we demonstrated that Pin1 is also involved in metabolic disease via regulation of CREB Regulated Transcriptional Co-activator 2 (CRTC2, also known as TORC)

The cAMP responsive element (CRE) binding protein (CREB) stimulates transcriptional activity through recruitment of the histone acetylase CBP and through an association with CRTC, leading to formation of the CREB-CBP-CTRC complex on a CRE site (8-16). Thus, multiple molecular mechanisms affect the CREB-CBP-CTRC complex, resulting in the regulation of CRE transcriptional activity. They include the phosphorylations of CREB at Ser133, CBP at Ser436 and CRTC2 at Ser171 (16,17). The phosphorylation of CRTC2 at Ser171 reportedly leads to an association with 14-3-3 protein and thereby to its nuclear exclusion and degradation (16).

The CRTC family consists of three members, CRTC1, CRTC2 and CRTC3 (16, 18). CRTC1 is highly expressed in the brain while the other two are ubiquitously expressed (19). In the liver, insulin induces the phosphorylation of CRTC2 at Ser171, and this phosphorylation leads to the aforementioned association with 14-3-3 protein and the nuclear exclusion and degradation of CRTC2 (16, 20). In contrast, glucagon induces dephosphorylation of CRTC2 and translocation from the cytosol to the nucleus, thereby forming the CREB-CBP-CRTC2 complex and inducing gluconeogenesis (21). Thus, CRTC2 plays important roles in hepatic glucose metabolism.

In this study, we identified CRTC2 as a Pin1 binding protein. Interestingly, the portion of CRTC2 responsible for the association with Pin1 was revealed to be in the Nuclear Localization Signal (NLS) domain. Herein, we demonstrate that Pin1 regulates the functions and subcellular localizations of CRTC family proteins, thereby altering CRE transcriptional activity.

EXPERIMENTAL PROCEDURES

Materials - Anti-Pin1 antibody was generating by immunizing rabbits with the peptide QMQKPFEDASFATRTGEMSGPVFTDSGIHII TRTE (Amino acids 129-163 of human Pin1). Anti-FLAG tag and myc tag antibodies were purchased from Sigma-Aldrich Corporation (St.

Louis, MO). The antibodies against CRTC2, CREB, 14-3-3 protein, GFP and DsRed were purchased from Cell Signaling Technology. Anti-rabbit HRP antibodies conjugated to horseradish peroxidase were obtained from Amersham Pharmacia. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Invitrogen. All other reagents were of analytical grade.

Preparation of adenoviruses expressing MEF-tagged Pin1, CRTC1and CRTC2 - The myc-TEV-FLAG (MEF) tag cassette was generated by DNA synthesis and inserted into cloning sites in the mammalian expression vector pcDNA3 (Invitrogen; termed pcDNA3-MEF), as reported previously (22). To create the N-terminally MEF-tagged Pin1 construct, human Pin1 cDNA was inserted into pcDNA3-MEF. Then, the coding portion of MEF-tagged Pin1 was isolated from pcDNA3-MEF-Pin1 and the recombinant adenoviruses containing the cDNA coding for MEF-tagged Pin1 were constructed as described previously (22). Recombinant adenoviruses expressing human Pin1 with the C-terminal HA tag or N-terminal MEF tag were also constructed and used for adenoviral gene transfer to HepG2 cells and mouse liver. Similarly, adenoviruses expressing GFP-tagged CTRC1, CRTC2 and GFP-tagged CRTC2 were prepared. Adenovirus encoding LacZ served as a control, and the adenoviral gene transfer was performed as reported previously (22).

Purification of MEF-tagged Pin1 from mouse livers - Recombinant adenovirus expressing MEF-tagged Pin1 was generated, purified and concentrated using cesium chloride ultracentrifugation as reported previously (22). Adenovirus encoding LacZ served as a control. Male mice, 9 weeks of age, were obtained from the Nippon Bio-Supp. Center (Tokyo, Japan). They were injected, via the tail vein, with adenovirus at a dose of 2.5×10^7 plaque-forming units/g body wt. Four days after adenovirus injection, the mouse livers were removed and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (w/v) glycerol, 100 mM NaF, 10 mM EGTA, 1 mM Na_3VO_4, 1% (w/v) Triton X-100, 5 µM $ZnCl_2$, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin and 1 µg/ml leupeptin). The lysates were centrifuged at 100,000 x *g* for 20 min at 4°C. The supernatant was passed through a 5-µm filter, incubated with 150µl of Sepharose beads for 60 min at 4°C, and then passed through a 0.65-µm filter. The filtrated supernatant was mixed with 150µl of anti-myc-conjugated Sepharose beads for the first immunoprecipitation. After incubation for 90 min at 4°C, the beads were washed five times with 1.5ml of TNTG buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (w/v) glycerol, 0.1% (w/v) Triton X-100), twice with buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% (w/v) Triton X-100), and finally once with TNT buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (w/v) Triton X-100). The washed beads were incubated with 15 units of TEV protease (Invitrogen) in 150 µl of TNT buffer to release bound materials from the beads. After incubation for 60 min at room temperature, the supernatant was pooled, and the beads were washed twice with 75µl of buffer A. The resulting supernatants were combined and incubated with 25µl of FLAG-Sepharose beads for the second immunoprecipitation. After incubation for 60min at room temperature, the beads were washed three times with 500µl of buffer A, and proteins bound to the FLAG beads were dissociated by incubation with 1mM synthetic FLAG peptides in buffer A for 120min at 4°C. Approximately 3µg of protein (0.01% of starting materials) were routinely recovered by this procedure. The samples were electrophoresed and subjected to SDS-PAGE and immunoblotting.

Cell Culture - Sf9 cells were grown in TC100 (Life Technologies, Inc.) medium containing 10% fetal calf serum at 27°C. HepG2 hepatoma cells were grown in DMEM containing 10% fetal calf serum at 37°C in 5% (v/v) CO_2 in air.

Preparation of baculoviruses expressing Pin1 and CRTC2 constructs - The full-length coding regions of human Pin1, GFP, GFP-tagged Pin1, CRTC2 and DsRed-tagged full-length and various deletion mutant forms of CRTC2 and Ser136Ala CRTC2 were subcloned into pBacPAK9 transfer vector (CLONTECH), and the baculoviruses were prepared according to the manufacturer's instructions. For protein production, Sf9 cells

were infected with these baculoviruses and grown for 48 h.

Preparation of Glutathione S-transferase (GST)-Pin1 Fusion Protein - The cDNAs encoding full-length human Pin1, the WW domain of Pin1 and the PPIase domain of Pin1 were subcloned into a pGEX-5X-1 vector (Pharmacia Biotech Inc.), which was used to transform *E. coli* JM105 (Promega). Transformed cells were grown to an A_{600} of 0.6 in LB medium supplemented with 0.1mg/ml ampicillin and stimulated for 3h with 1.0mM isopropyl- β -D-thiogalactopyranoside. GST fusion proteins were conjugated to Glutathione-Sepharose 4B (Pharmacia), and used for GST-pull down experiments.

GST-pull-down - HepG2 cells expressing MEF-CTRC2 and its mutants were homogenized with 10 vol/wt homogenizing buffer (20mmol/l Tris/HCl (pH 7.4), 1% Triton X-100, 0.25% Sodium deoxycholate, 0.25mol/l NaCl) containing 0.2mmol/l PMSF and 5μg/ml aprotinin, centrifuged at 15,000rpm for 30min at 4°C and the supernatants were then re-centrifuged at 100,000 x g for 1 h. The supernatants (2μg/ml protein concentration) were incubated with 1ml Glutathione Sepharose 4B for 1h at 4°C to remove non-specifically bound proteins, then incubated with purified GST alone, GST-Pin1, and GST-Pin1 deletion mutant proteins for 1h, and finally washed 6 times with homogenizing buffer. Glutathione-Sepharose 4B beads were boiled in Laemmli sample buffer, which was used for the SDS-PAGE and immunoblotting.

Preparation of streptozotocin-treated diabetic mice and gene transfer of Pin1 into mouse livers-Streptozotocin (STZ)-treated diabetic male C57BL/6 mice (8-10 weeks of age) were prepared as reported previously (20). These mice were injected, via the tail vein, with adenovirus at a dose of 2.5×10^7 plaque-forming units/g body weight. Animals were fasted for 14 hours, and then were refed for 4 hr before sacrifice. Blood glucose was measured with a portable blood glucose monitor, "Glutest-Ace" (Sanwa Kagaku Kenkyusho, Nagoya Japan). All animal studies were conducted according to the Japanese guidelines for the care and use of experimental animals.

Immunoprecipitation and immunoblotting- For the immunoprecipitation experiments, whole-cell extracts from HepG2 or Sf-9 cells or mouse liver lysates obtained after an overnight fast were prepared in lysis buffer, as described above. Cell or tissue extracts were incubated for 4 hr at 4°C with the indicated antibody and then for 1hr with 30μl of protein G-sepharose beads. The pellets were washed five times with 1ml of lysis buffer, then resuspended in Laemmli sample buffer, boiled for 3min and analyzed on SDS/ polyacrylamide gels.

Western blot analysis was carried out as described previously (22). In brief, 10μg of protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes in a transfer buffer consisting of 20mM Tris-HCl, 150mM glycine and 20% methanol. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 and incubated with specific antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody interactions were visualized by incubation with ECL chemiluminescence reagent (Amersham).

Immunostaining -HepG2 cells were fixed with 4% paraformaldehyde for 10 min, rinsed with phosphate-buffered saline (PBS), and then exposed to 0.2% Triton X-100 in PBS for 5 min. Cells were subsequently incubated for 1 hr at room temperature with anti rabbit CRTC2 (1:500), and FITC-labeled anti-rabbit IgG (1:750) was used as the secondary antibody. Immunofluorescence was visualized with a laser scanning confocal imaging system.

Luciferase assay- The following plasmids were obtained from commercial sources: pTAL and pTAL-CRE from CLONTECH (Palo Alto, CA); pM from Stratagene (La Jolla, CA); pGL4 and pRL-TK from Promega (Madison, WI).
HepG2 cells in a 24-well collagen-coated plate were co-transfected with pTAL-CRE vector (0.25μg/well) with an internal reporter, pRL-TK (0.25μg). Luciferase activities were determined

using the Dual-Luciferase Reporter Assay System (Promega Corp.).

RNA analysis - RNA extractions were carried out using TRIzol followed by purification over a QIAEASY RNA column. Reverse transcribed- and quantitative-PCR were carried out as already described. The primer sets for hPEPCK (phosphoenolpyruvate carboxykinase) were: GGTTCCCAGGGTGCATGAAA and CACGTAGGGTGAATCCGTCAG (114bp); and for hGAPDH were: ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA (451bp)
Chromatin immunoprecipitation assay with anti-CRTC2, CBP or CREB antibodies-HepG2 cells with or without forskolin stimulation were immunoprecipitated with anti-CRTC2, anti-CBP or anti-CREB antibody, using the Chip-IT^{TM} Express Enzymatic kit (Active Motif Corp.). Then, precipitated DNA was amplified by PCR using primers against the relevant promoters.

Statistical analysis-Results are expressed as means $\pm$ SE, and significance was assessed using one way ANOVA, unless otherwise indicated.

Results

Identification of CRTC2 in the Pin1-containing complex from mouse liver- The adenovirus to MEF-tagged Pin1 was introduced into mice and the Pin1-containing complex was purified. Purified Pin1 in the complex was electrophoresed, and subjected to silver staining, which showed the presence of Pin1bait proteins and many binding proteins (Fig. 1A). The bands of which sizes are around 200kDa and 130kDa were identified to be DNA-directed RNA polymerase II A, DNA-directed RNA polymerase IIB and DNA-directed RNA polymerase I by the analysis using LC/MS, which agree with the previous reports (23). Then, we performed the immunoblotting using many antibodies to detect another protein included in the Pin1-containing complex, since many faint bands were visible with silver staining.

Many transcriptional co-activators are included among the target proteins of Pin1 (4,5). In addition, although one of the regulatory mechanisms of Pin1 is protein stabilization, recent reports have shown that Pin1 is involved in translocation of target proteins, such as Bax (24). These results suggest that CRTC2 is a candidate Pin1 target protein because CRTC2 is a transcriptional co-activator and is translocated between the cytosol and the nucleus. As a result, immunoblotting using anti-CRTC2 antibody indicated the presence of CTRC2 in the Pin1 complexes (Fig. 1B). To confirm the association between CTRC2 and Pin1, CRTC2 and each GFP-Pin1 or GFP were simultaneously over-expressed in HepG2 and Sf-9 cells. As shown in Fig. 1C and supplementary Fig.1, GFP-Pin1, but not GFP alone was detected in the anti CRTC2 immunoprecipitate. Furthermore, CRTC2 was detected in the immunoprecipitate with anti-Pin1 antibody but not that with the control IgG from mouse liver (Fig. 1D). Thus, the association between CRTC2 and Pin1 is physiological.

Pin1 possesses the WW and PPIase domains in its N-terminal (a.a 1- 38) and C-terminal (a.a. 39 -163), respectively. To identify the domain of Pin1 responsible for the association with CRTC2, we prepared GST-Pin1, the GST-Pin1 WW domain, and the GST-Pin1 PPIase domain. These GST-proteins were conjugated to beads followed by incubation with cell lysates from MEF-tagged CRTC2 over-expressing HepG2 cells. GST-Pin1 but not GST alone bound to CRTC2 *in vitro* (Fig. 1E). Using this pull-down system, it was shown that the GST-WW domain, but not the GST-PPIase domain, binds to CRTC2 (Fig. 1F). In addition, okadaic acid treatment significantly increased the association of CRTC2 with Pin1 (Fig. 1G), suggesting the involvement of serine and/or threonine phosphorylation(s) in CRTC2.

Pin1 associates with Ser136-containing motif in the NLS domain of CRTC2- Subsequently, to reveal the domain of CRTC2 responsible for the association with Pin1, six Ds-Red tagged CRTC2 N-terminus deletion mutants (Fig. 2A) and GFP-tagged Pin1 were simultaneously over-expressed in Sf-9 cells. As shown in Fig. 2B, CRTC2 deletion mutant 2 (D-2), containing amino acids 121 to 238, was immunoprecipitated with GFP-tagged Pin1 but not with GFP alone. This portion contains three serine-proline motifs (Fig. 2C). Each of these serine residues was

replaced with alanine, creating a mutant which did not associate with Pin1. As shown in Fig. 2D, CRTC2 with serine 136 replaced by alanine did not bind to Pin1, while CRTC with serine 129 or 131 bound to Pin1 (data not shown). These observations indicated that the association between CRTC2 and Pin1 is mediated via the phospho-serine 136-containing motif in CRTC2 and the WW domain in Pin1. Ser 136 is in the NLS domain, and a high level of Ser136 phosphorylation was demonstrated in a previous report (16)**.**

Pin1 inhibits CRTC2 translocation from the cytosol to the nucleus- To test whether or not the effect of Pin1 on CRE transcriptional activity is mediated via the effect on the subcellular localization of CRTC2, the GFP-tagged CRTC2 was over-expressed and the effects of the Pin1 expression level on the subcellular localization of GFP-tagged CRTC2 were analyzed in the absence or presence of forskolin stimulation (Fig. 3A). In the control LacZ over-expressing or control siRNA treated HepG2 cells, GFP-tagged CRTC2 was translocated from the cytosol to the nucleus, as reported previously (9). Pin1 over-expression markedly inhibited forskolin-induced translocation of CRTC2 into the nucleus. In addition, gene silencing of Pin1 using siRNA markedly enhanced the nuclear translocation of Pin1, in comparison to treatment with control siRNA. While nuclear CRTC2 S136A (unable to bind to Pin1) was required for forskolin stimulation, it had no effect on either Pin1 over-expression or Pin1 siRNA (Fig. 3B).

In addition, we investigated the effect of Pin1 on the distribution of CRTC2 S171A. CRTC2 S171A (unable to bind to 14-3-3) was mainly present in the nucleus regardless of forskolin stimulation (Supplementary Fig. 2). Pin1 over-expression slightly increased CRTC2 S171A in the cytosol, while pin1 siRNA treatment reduced the amount of CRTC2 S171A in the cytosol. This effect of Pin1 was essentially in agreement with the results obtained for wild-type CRTC2.

Similar results were obtained by immunostaining the endogenous CRTC2 in HepG2 cells (Fig. 3C). Pin1 over-expression attenuated the forskolin-induced nuclear translocation of CRTC2 as compared with LacZ over-expression. On the other hand, treatment with Pin1 siRNA increased CRTC2 in the nucleus under forskolin stimulation, as compared with the control siRNA.

Neither the distribution nor the expression of Pin1 was changed by forskolin or insulin stimulation (Supplementary Fig. 3). Thus, a change in Pin1 is not required for regulation of the CRTC2 distribution.

Pin1 associates with CRTC1 and induces its localizationsin the cytosol- The CRTC family consists of three isoforms, CRTC1, CRTC2 and CRTC3. The motif of CRTC2 responsible for the association with Pin1 is present in the NLS and is conserved in CRTC1, but not in CRTC3 (Supplementary Fig. 4A). Thus, the associations of Pin1 with CRTC1 were also investigated using HepG2 cells. As shown in Supplementary Fig.4B, FLAG-tagged CRTC1 were detected in anti-GFP immunoprecipitates from the cells expressing GFP-tagged Pin1 and FLAG-tagged CRTC1. As shown in Supplementary Figure 4C, FLAG-tagged CRTC1, in which serine 155 is replaced with alanine, did not bind to GFP-tagged Pin1, unlike the FLAG-tagged wild-type CRTC1.

Then, the effects of Pin1 on localizations of CRTC1 were examined. When LacZ was overexpressed, GFP-tagged CRTC1 was present in the cytosol and translocated to the nucleus in response to forskolin stimulation (Supplementary Figure 4D). This translocation was markedly inhibited by Pin1over-expression (Supplementary Figure 4D).

CRTC2 associated with Pin1 did not bind to CREB- Formation of the CREB-CBP-CTRC complex, which binds to a CRE site, is critical for CRE transcriptional activation. We investigated whether or not the CREB-CBP-CRTC2-Pin1 complex can form, using the baculovirus and Sf-9 cell over-expression system. When CRTC2 and CREB were both over-expressed in HepG2 or Sf-9 cells, CREB was detected in the CRTC2 immunoprecipitate. Interestingly, the over-expression of Pin1 markedly reduced the association between CREB and CRTC2, in either HepG2 or Sf-9 cells (Fig. 4A and B).

Furthermore, the effect of Pin1 on the association between CRTC2 and 14-3-3 was investigated. In Sf-9 cell lysates over-expressing CREB and CRTC2, both CRTC2 and endogenously expressed 14-3-3 protein were

detected in anti-CREB immunoprecipitates (Fig. 4C). In the case of triple over-expressions of CRTC2, CREB and GFP-tagged Pin1, CRTC2 and 14-3-3 were detectable in the GFP-tagged Pin1 immunoprecipitate (Fig. 4D).

Similar results were obtained in the HepG2 cells. The association between MEF-tagged CRTC2 and endogenously expressed 14-3-3 was not affected by the over-expression of Pin1 (Supplementary Fig. 5A). In addition, Pin1 over-expression did not affect the phosphorylation level of Ser171, responsible for the association with 14-3-3, in either basal or forskolin-stimulated condition (Supplementary Fig. 5B). These results suggest that Pin1-associated CRTC2 is capable of binding to 14-3-3 protein but not to CREB.

Pin1 inhibits CRE transcriptional activity and its downstream PEPCK expression- Subsequently, to elucidate the role of Pin1 in CRE transcriptional activity, the effects of Pin1 over-expression and Pin1 gene silencing using siRNA on the CRE and PEPCK luciferase assay, and PEPCK mRNA level were investigated in HepG2 cells (Fig. 5). The amount of over-expressed Pin1 was approximately 5 times that of endogenous Pin1, in HepG2 cells. Under these conditions, forskolin-induced transcriptional activity and PEPCK mRNA induction were significantly attenuated (Fig. 5A, B and C). On the contrary, gene suppression of Pin1 using siRNA significantly enhanced these events (Fig. 5D, E and F). In addition, suppressions of CRE-luciferase and PEPCK-luciferase activities by Pin1 over-expression were observed in immortalized human hepatocytes (25) (Supplementary Fig. 6), suggesting that this mechanism is independent of the glucose sensitivity of the cell type. An inhibitory effect of Pin1 on CRE luciferase activity was observed when wild type or S171A CRTC2, but not S136A, was over-expressed, consistent with the results showing Pin1 to regulate the translocation of CRTC2 (Supplementary Fig.7).Thus, the Pin1 expression level was revealed to negatively regulate CRE transcriptional activity.

Chromatin immunoprecipitation assay with anti-CRTC2 and CREB antibodies-Because Pin1-associated CRTC2 did not bind CREB, we performed chip assay to investigate whether or not Pin1 affected recruitment of CRTC2 to cAMP responsive (PEPCK, NR4A2, CGA) (Fig. 5G). The PCR product obtained using the anti-CREB immunoprecipitate was unchanged regardless of forskolin stimulation or Pin1 over-expression. In contrast, the PCR product of the anti-CRTC2 immunoprecipitate was markedly increased by forskolin stimulation, and Pin1 over-expression abolished this increase. Forskolin stimulation induced CBP recruitment to the promoter as well as CRTC2, but Pin1 over-expression had no effect. Thus, it was suggested that CRTC2 associated with Pin1 was removed from CREB located in the CRE sequence in the PEPCK, NR4A2 and CGA promoter region.

Hepatic Pin1 over-expression reduces PEPCK expression and decreases hyperglycemia in STZ-induced diabetic mice- CRTC2 is a major transcriptional co-activator for hepatic glucose regulation, via its effects on PEPCK expression. Thus, we considered the possibility of the regulation of PEPCK expression by Pin1 in the liver, and an adenovirus expressing Pin1 was introduced into STZ-induced insulin-deficient diabetic mice. Due to the insulin deficiency, as reported previously, hepatic PEPCK mRNA and serum blood glucose levels were markedly increased in fed and fasted state, as compared with the control mice (Fig. 6). The adenovirus for Pin1 expression was injected intravenously, and 96 hours later, over-expressed Pin1 was detected only in the liver (Fig. 6A) and not in other tissues. With Pin1 over-expression in the liver, the increased hepatic PEPCK mRNA level in STZ-mice was normalized, and blood glucose elevation was also partially but significantly reduced in both the fed and the fasting state (Fig. 9B-E). Pin1 over-expression exerted the same effects on other CRE-dependent transcriptional genes, such as G6Pase, PGC-1a and CPT-1. These findings revealed Pin1 to be a regulator of CRE-dependent transcriptional genes *in vivo*.

Pin1 expression is low in fasting state -Finally, we investigated the changes in Pin1 expressions under different nutrient conditions. Interestingly, we found that the Pin1 expression level is low in the fasted state, but is increased by feeding (Fig.7).

Thus, Pin1 expression appears to be regulated by nutrient conditions.

DISCUSSION

CRE transcriptional activity is enhanced through association of the CREB-CBP-CRTC complex on a CRE site. The co-activator of CREB termed the CRTC family consists of three isoforms, CRTC1, CRTC2 and CRTC3 (18). CRTC2 was reported to be important for the regulation of CRE transcriptional activity and its downstream PEPCK gene expression (20). Depletion of nuclear CRTC2 leads to the suppression of CRE transcriptional activity (20). Thus, both the subcellular localization of CRTC2 and CREB-CBP-CRTC complex formation are critical for CRE transcriptional activity. CRTC2 is reportedly phosphorylated by AMPK and SIK, and phosphorylated CRTC2 binds to 14-3-3 protein and is thereby shifted from the nucleus to the cytoplasm (21). The Montiminy group has identified 12 independent phosphorylated serine residues on CRTC2 using tandem mass spectrometric (MS) analysis (16). They demonstrated that PKA inhibits the activity of SIK and reduces Ser171 phosphorylation leading to binding with 14-3-3 protein and translocation to the cytosol (16). However, the importance of other phosphorylation sites identified in their study such as Ser136 remains unknown.

In this study, it was demonstrated that Pin1 associates with the CRTC family of proteins consisting of CRTC1 and CRTC2. Since the portion of CRTC1 and CRTC2 responsible for the association with Pin1 is in the NLS domain, we considered the possibility that the binding of Pin1 to this portion would interrupt NLS function, resulting in their export from the nucleus. In fact, our observations using GFP-tagged CRTC1 and CRTC2 as well as staining of endogenous CRTC2 supported our hypothesis. On the other hand, gene-silencing of Pin1 using siRNA markedly induced nuclear localization of CRTC2, when stimulated with forskolin. It is likely that altered localization of CTRC2 due to Pin1 takes place independently of the binding of 14-3-3 protein to CRTC2, since Pin1 over-expression affected neither Ser171 phosphorylation level of CRTC2 nor the association with 14-3-3.

A further interesting issue is that CRTC2 associated with Pin1 did not bind to CREB. This phenomenon cannot be attributable to the different subcellular distributions of CREB, CBP and CRTC, since highly over-expressed CREB, CBP and CRTC2 are present in the cytosol of Sf-9 cells. Taken together, these observations indicate the association of Pin1 with CRTC2 to decrease the nuclear CBP-CRTC2-CREB complex via two mechanisms, i.e. the export of CRTC2 and interruption of the association between CRTC2 and CREB. Thus, the Pin1 expression level is a key factor regulating CRE transcriptional activity.

We investigated the effects of various kinase inhibitors on the association between CRTC2 and Pin1, using HepG2 cells, in an effort to identify the kinase which is involved in the phosphorylation of Ser136A on CRTC2. However, we were unable to obtain clear results. Although we did not discover which kinase(s) phosphorylates the Ser136 of CRTC2 responsible for the association with Pin1 in this study, high basal phosphorylation of Ser136 was already demonstrated in a previous report (16).

Prior studies have also shown that Pin1 expression generally correlates with cell proliferative potential in normal tissues (1, 26, 27) and is further up-regulated in many human cancers (28-31). In addition, interestingly, we noticed that the amount of Pin1 was higher in the fed than in the fasting state, in both liver and muscle. However, neither insulin nor forskolin has any effect on the expression of Pin1 in HepG2. Thus, the mechanism(s) involved in the altered expression of Pin1 remains unclear, though this is an important issue which merits further investigation.

In the liver, CRE transcriptional activity plays a critical role in gluconeogenesis (32-34). In addition, in the diabetic state, insufficient suppression of CRE transcriptional activity is regarded as a mechanism underlying hyperglycemia under fasted conditions (35). In the present study, our final experiment examined whether Pin1 over-expression might improve the hyperglycemia in insulin deficient STZ- mice. In these mice, gluconeogenic enzymes such as PEPCK under the control of CRE transcriptional activity are reportedly up-regulated (20, 36, 37), due to insulin deficiency and the relatively increased effect of glucagon. The fact that Pin1 over-expression reduced the high PEPCK

expression and its resultant fasting serum glucose elevation in STZ-mice suggest the Pin1 expression level to be involved in regulating glucose metabolism. Thus, an agent affecting Pin1 expression or activity may represent a novel therapeutic strategy for diabetes.

To date, numerous proteins have been identified as substrates of Pin1 (4, 5, 38). With the proline conformational change induced by Pin1, the structure and function of the target protein are modified, which affects protein stabilization, subcellular localization, phosphorylation, transcriptional activity and so on. In the case of CTRC2, both subcellular localization and the complex-forming function with CREB are affected.

Although we did not investigate the physiological effects occurring via CRTC1 induced by the association with Pin1, we did observe that Pin1 is highly expressed in the brain, while its enzymatic activity is blunted by oxidative stress modification that occurs in the early stages of Alzheimer's disease (39). Although the physiological function of Pin1 in neurons remains largely unknown, numerous reports have implicated CRE transcriptional activity in brain function (40-42). Thus, further important evidence may be obtained from studies of Pin1 and CRTC1, in the brain or other tissues.

In summary, CRTC2 was identified as a new Pin1 binding protein. CBP-CRTC2-CREB complex promotes gluconeogenesis. Pin1 binding to CRTC2 prevents this complex formation, thereby suppressing CRE transcriptional activity (Supplementary Fig. 8). These findings indicate that Pin1 is a regulator of gluconeogenesis and may be a new target for diabetic therapy.

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Figure Legends

Figure 1 Pin1 associates with CRTC2

(A, B) Pin1 with the N-terminal MEF tag was over-expressed in the mouse liver using adenovirus gene transfer, and the Pin1-containing complex was purified. The samples were electrophoresed and subjected to silver staining (A). Analysis using LC/MS revealed the band (1), (2) and (3) to be DNA-directed RNA polymerase II A, suppressor of Ty 6 homolog and DNA-directed RNA polymerase II A, and DNA directed RNA polymerase II polypeptide B and DNA-directed RNA polymerase I, respectively. (B) The samples were subjected to the immunoblotting with anti-CRTC2 antibody. (C) CRTC2 or control LacZ was over-expressed with GFP or GFP-Pin1. Then, the cell lysates were immunoprecipitated with anti- CRTC2 antibody, followed by immunoblotting with anti-GFP antibody. (D) The cell lysates from the mouse liver were immunoprecipitated with control IgG or anti-Pin1 and the immunoprecipitates were then immunoblotted with anti-CRTC2 and anti-Pin-1. (E) HepG2 cell lysates expressing CRTC2 with FLAG tag were incubated with Glutathione beads conjugated with GST or GST-Pin1. After washing the beads, SDS-PAGE was performed followed by immunoblotting with anti-FLAG or anti-GST antibodies. (F) HepG2 cell lysates expressing CRTC2 with FLAG tag were incubated with Glutathione beads conjugated with GST, the GST-WW domain or the GST-PPI domain. After washing the beads, SDS-PAGE was performed followed by immunoblotting with anti-FLAG or anti-GST antibodies. (G) CRTC2 and either GFP or GFP-Pin1 were simultaneously over-expressed in HepG2 cells. With or without okadaic acid treatment for 0.5h, the cell lysates were immunoprecipitated with anti-CRTC2 followed by immunoblotting with anti-GFP antibody. Representative immunoblotting data from three independent experiments are shown.

Figure 2 Pin1 associates with the NLS domain of CRTC2.

(A) The constructs of CRTC2 deletion mutants and baculoviruses expressing these 6 mutants with the C-terminal DsRed tag were prepared. (B) The 6 deletion mutants with C-terminal DsRed tags were over-expressed with GFP or GFP-Pin1 in Sf-9 cells. The cell lysates were immunoprecipitated with anti-GFP antibody followed by immunoblotting with anti-DsRed antibody. The upper panel shows the binding of the Deletion-2 mutant to GFP-Pin1, but not to GFP alone. (C) The orientations of 3 candidate Ser/Pro motifs in the Deletion-2 mutant involved in the association with Pin1. (D) Wild-type CRTC2 or CRTC2 S136A was over-expressed with GFP-Pin1 or GFP in Sf-9 cells. The cell lysates were immunoprecipitated with anti-CRTC2 antibody followed by immunoblotting with anti-GFP. The upper panel shows that CRTC2 S136A does not associate with Pin1, unlike the wild-type CRTC2.

Representative immunoblotting data from three independent experiments are shown.

Figure 3　Effect of Pin1 on subcellular localization of GFP-tagged CRTC2

(A, B)　LacZ or Pin1 was over-expressed, or HepG2 cells were treated with control or Pin1 siRNAs. Then, GFP-tagged CRTC2 was over-expressed in HepG2 cells. These cells were treated with forskolin and the subcellular localization of GFP-tagged wild type or S136A CRTC2 was examined at the indicated periods after initiating forskolin stimulation. Representative data from four independent experiments are shown. (C) LacZ or Pin1 was over-expressed in HepG2 cells, or the cells were treated with control or Pin1 siRNAs. These cells were treated with forskolin and the subcellular localization of endogenous CRTC2 was determined by immunostaining at 10 or 30 min after initiating forskolin stimulation. Nuclei were stained with DAPI. Representative data from five independent experiments are shown.

Figure 4 Binding of Pin1 to CRTC2 inhibits the association between CREB and CRTC2, but not that between 14-3-3 and CRTC2

(A) MEF-tagged CRTC2, CREB and Pin1 were over-expressed in HepG2 cells in the indicated combinations. The cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-CREB antibody. (B) CRTC2, CREB and Pin1 were over-expressed in Sf-9 cells in the indicated combinations. The cell lysates were immunoprecipitated with anti-CREB antibody and immunoblotted with anti-CRTC2 antibody. (C) CREB and CRTC2 were over-expressed in Sf-9 cells. The, cell lysates were immunoprecipitated with anti-CREB antibody and immunoblotted with anti-14-3-3 protein antibody. (D) CREB, CRTC2 and either GFP or GFP-Pin1 were over-expressed in Sf-9 cells. The, cell lysates were immunoprecipitated with anti-GFP antibody and immunoblotted with anti-CRTC2 or anti-14-3-3 protein antibody. Representative data from four independent experiments are shown.

Figure 5 Pin1 suppresses CRE luciferase activity and PEPCK mRNA level in HepG2 cells

(A, B)　LacZ or Pin1 was over-expressed in HepG2 cells transfected with pTAL, and pTAL-CRE or pTAL-PEPCK. (D, E)　These transfected HepG2 cells were treated with control siRNA or Pin1 siRNA. In two experiments, with and without forskolin stimulation for 6 hr, the cell lysates from HepG2 cells were subjected to the luciferase assay. (C, F) PEPCK mRNA levels were also measured. Representative data from four independent experiments are shown. $^{**}p < 0.01$ vs LacZ or Negative siRNA　(G) HepG2 cells over-expressing LacZ or Pin1 were subjected to the CHIP assay using anti-CRTC2, anti-CNP or anti-CREB antibodies and primers corresponding to the PEPCK, NR4A2 and CGA promoter regions. Representative

data from four independent experiments are shown.

Figure 6 Hepatic over-expression of Pin1 restored elevated CRE-dependent transcriptional genes and hyperglycemia in STZ-mice

STZ-treated diabetic C57BL/6 male mice were injected with 2.5×10^7 plaque-forming units/g body weight of adenovirus containing β-galactosidase (LacZ) or FLAG-tagged Pin1 construct via the tail vein. (A) Immunoblotting of hepatic tissue lysates with anti-FLAG or anti-Pin1 antibody. (B, C) Serum glucose concentrations in fed and fasting states (n=6, each group). (D, E) CRE-dependent transcriptional gene mRNA levels in the liver $^{**}p < 0.01$ vs STZ, $^{***}p < 0.001$ vs STZ

Figure 7 Pin1 expression is regulated by nutrient conditions. Mice were fed routinely, starved for 20 hours, or re-fed for 4 hours after a 20-hour fast. Liver (A) and muscle (B) cell lysates were prepared, then immunoblotted with anti-Pin1 antibody. A representative immunoblot is shown in the upper panel

Fig. 1

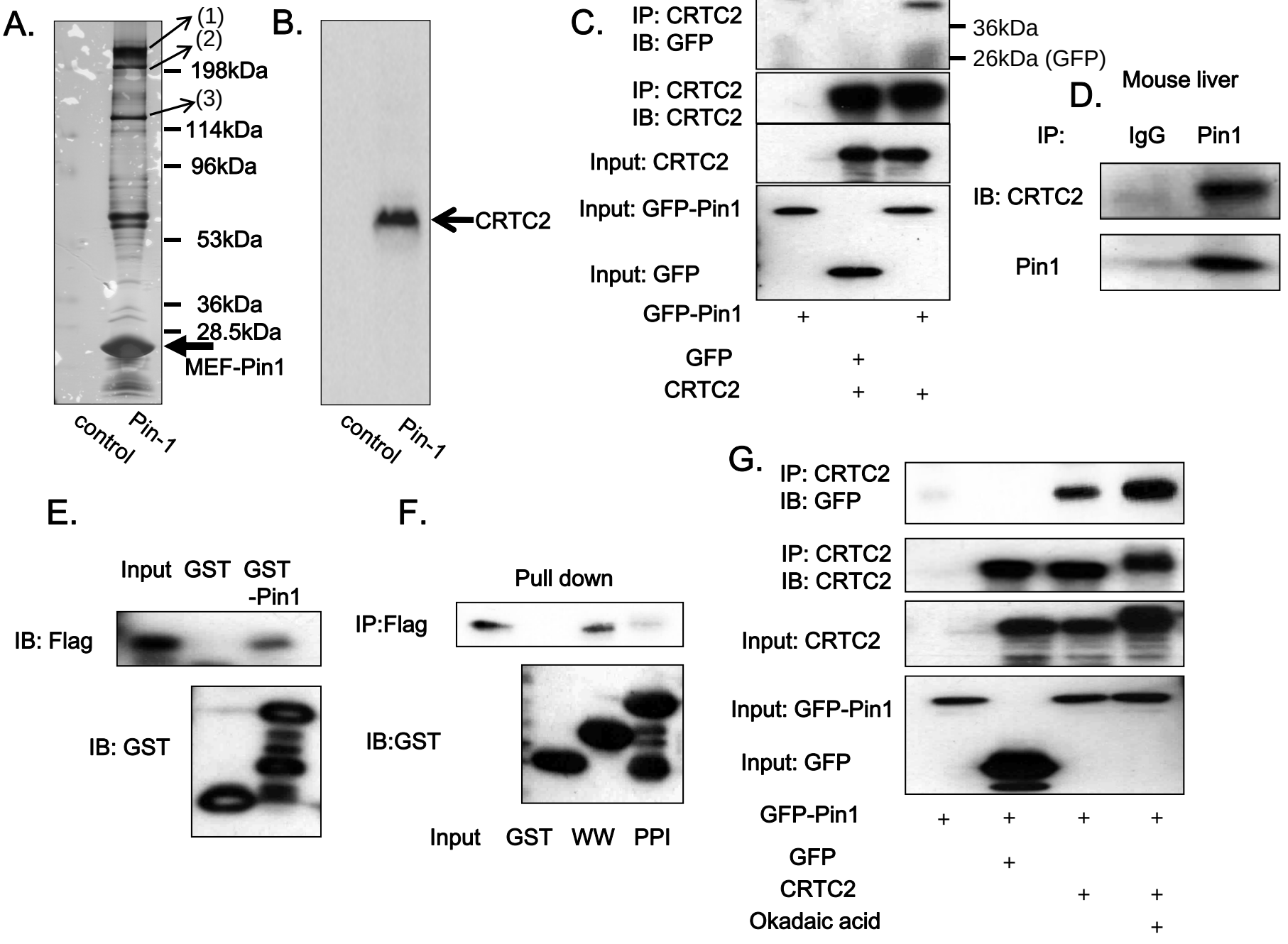
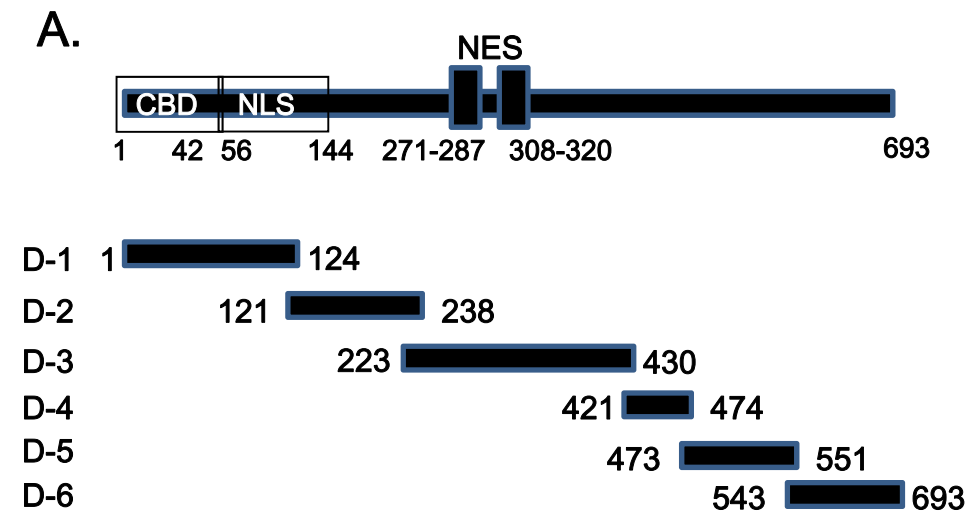
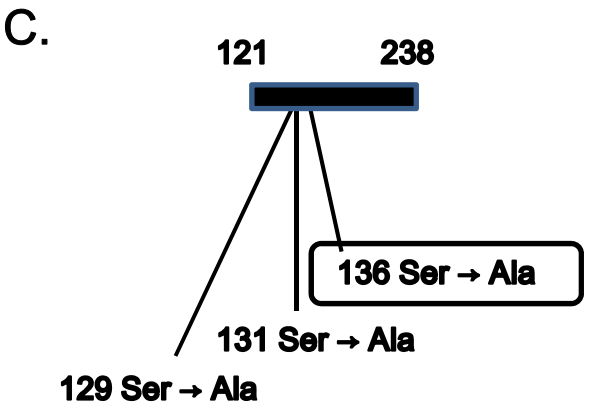
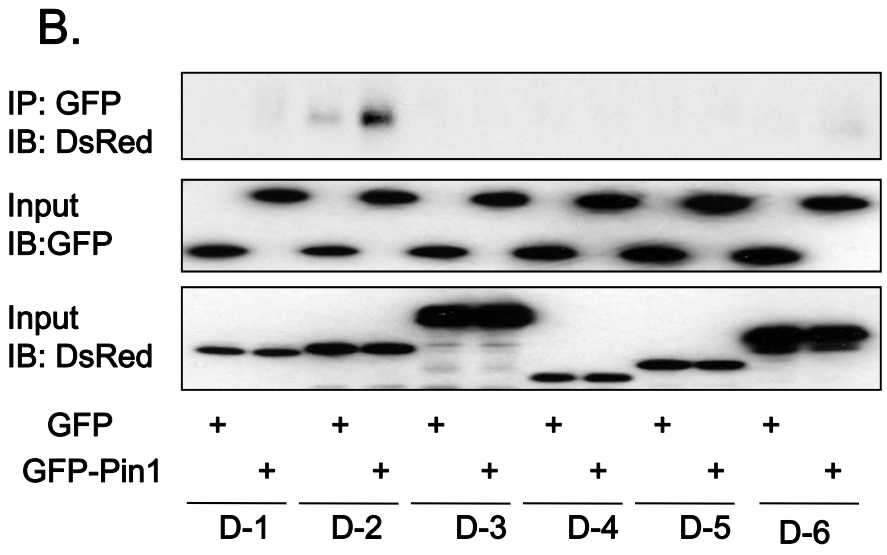

A.
(1)
(2)
198kDa
(3)
114kDa
96kDa
53kDa
36kDa
28.5kDa
MEF-Pin1
control
Pin-1
B.
CRTC2
control
Pin-1
C.
HepG2
49kDa
36kDa
26kDa (GFP)
IP: CRTC2
IB: GFP
IP: CRTC2
IB: CRTC2
Input: CRTC2
Input: GFP-Pin1
Input: GFP
GFP-Pin1 + +
GFP +
CRTC2 + +
D.
Mouse liver
IP: IgG Pin1
IB: CRTC2
Pin1
E.
Input GST GST -Pin1
IB: Flag
IB: GST
F.
Pull down
IP:Flag
IB:GST
Input GST WW PPI
G.
IP: CRTC2
IB: GFP
IP: CRTC2
IB: CRTC2
Input: CRTC2
Input: GFP-Pin1
Input: GFP
GFP-Pin1 + + + +
GFP +
CRTC2 + +
Okadaic acid +

Fig. 2


A.
NES
CBD
NLS
1
42
56
144
271-287
308-320
693
D-1 1 124
D-2 121 238
D-3 223 430
D-4 421 474
D-5 473 551
D-6 543 693


C.
121
238
136 Ser → Ala
131 Ser → Ala
129 Ser → Ala


B.
IP: GFP
IB: DsRed
Input
IB:GFP
Input
IB: DsRed
GFP + + + + + +
GFP-Pin1 + + + + + +
D-1
D-2
D-3
D-4
D-5
D-6

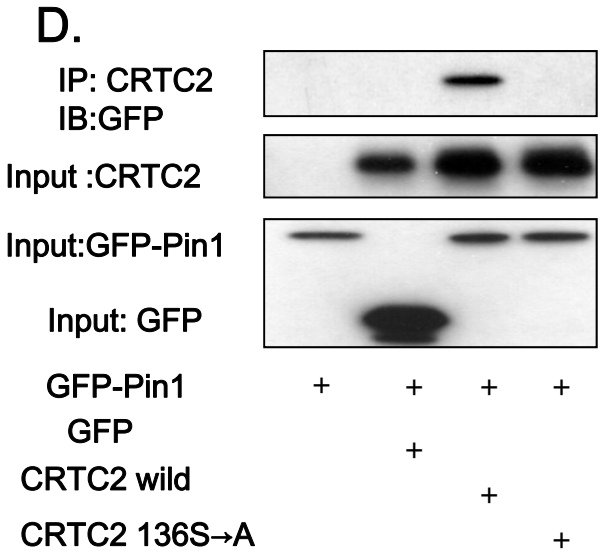
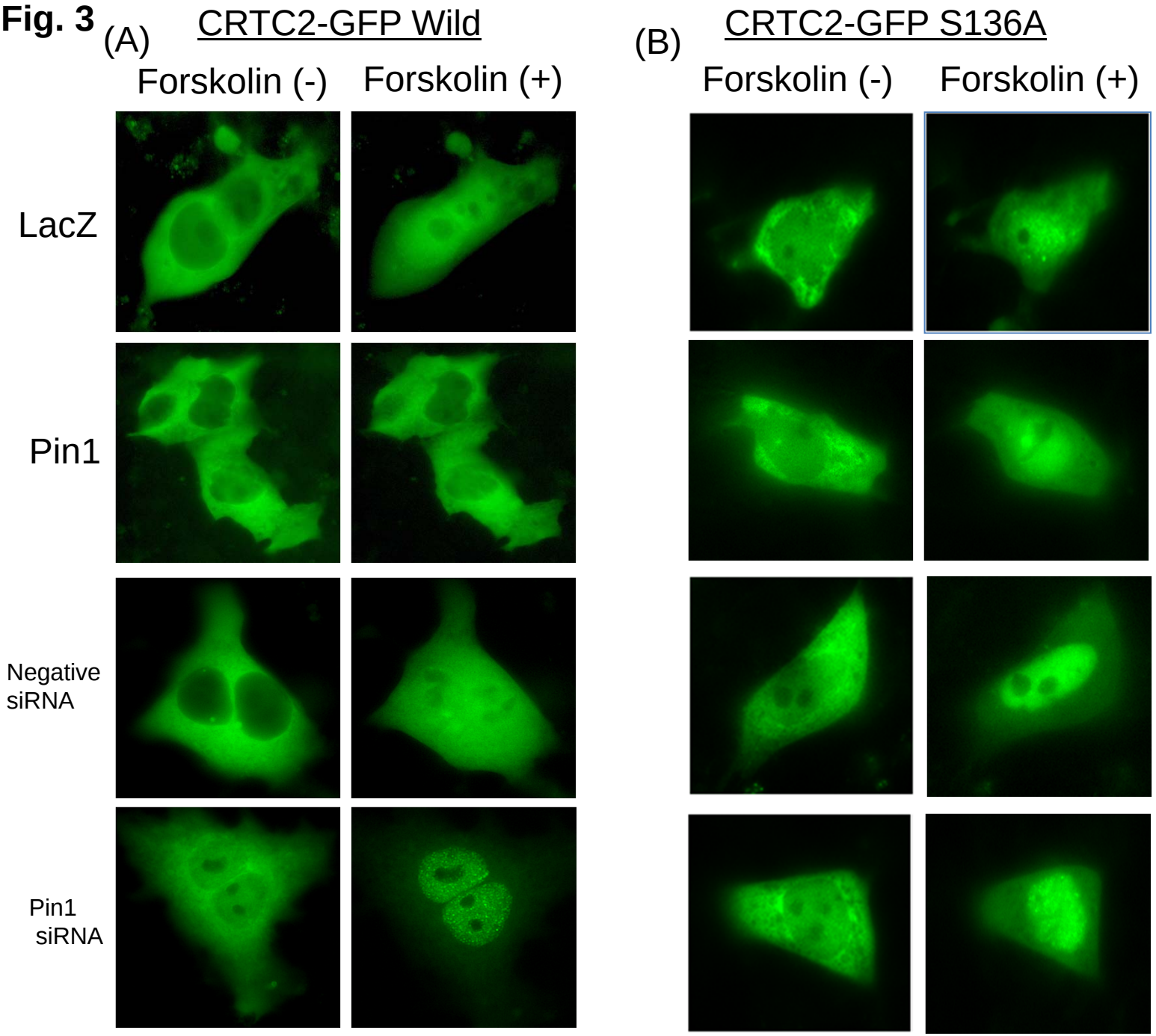

D.
IP: CRTC2
IB:GFP
Input :CRTC2
Input:GFP-Pin1
Input: GFP
GFP-Pin1 + + + +
GFP +
CRTC2 wild +
CRTC2 136S→A +

Fig. 3


(A)
CRTC2-GFP Wild
Forskolin (-)
Forskolin (+)
(B)
CRTC2-GFP S136A
Forskolin (-)
Forskolin (+)
LacZ
Pin1
Negative siRNA
Pin1 siRNA

(C)

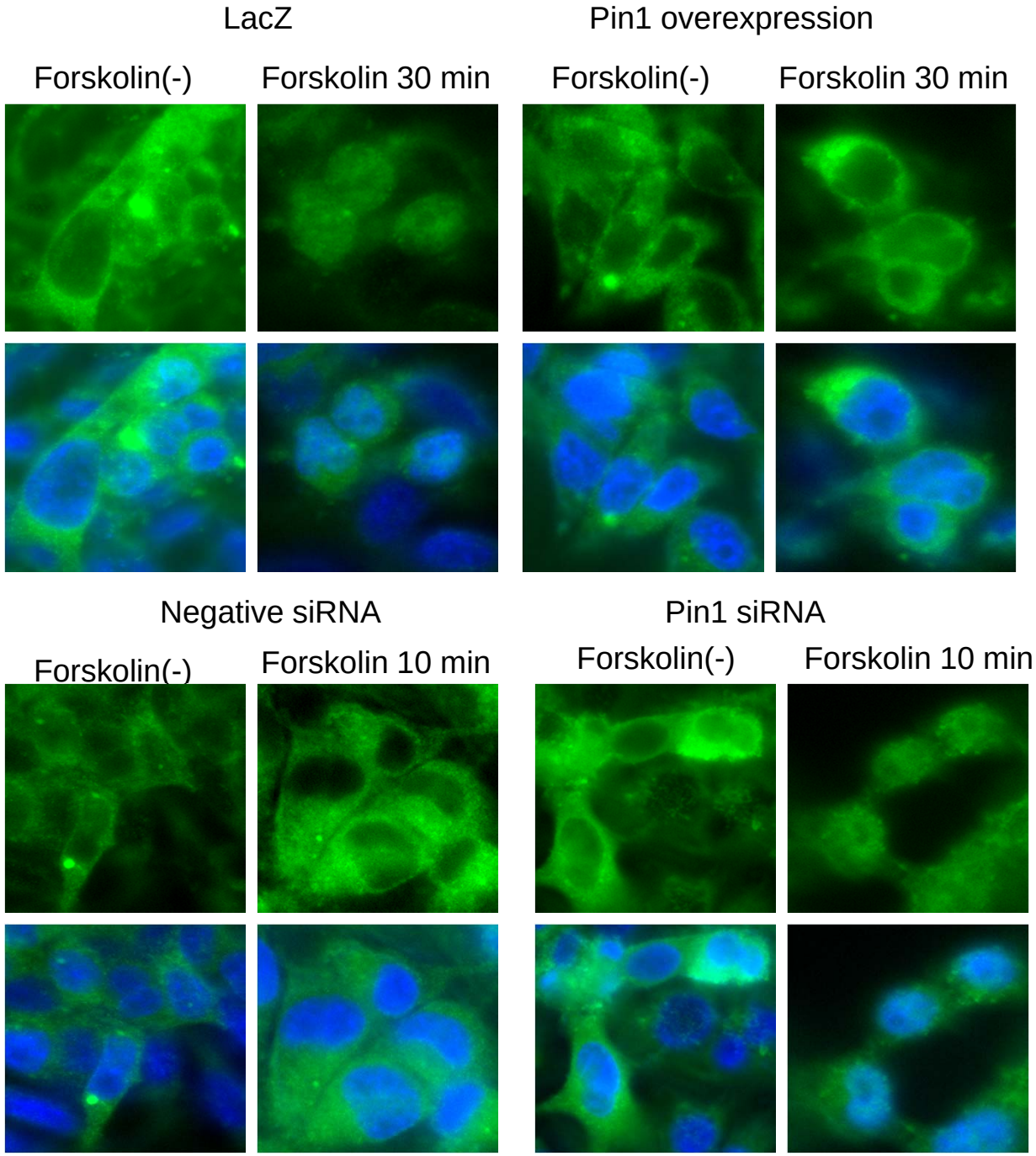
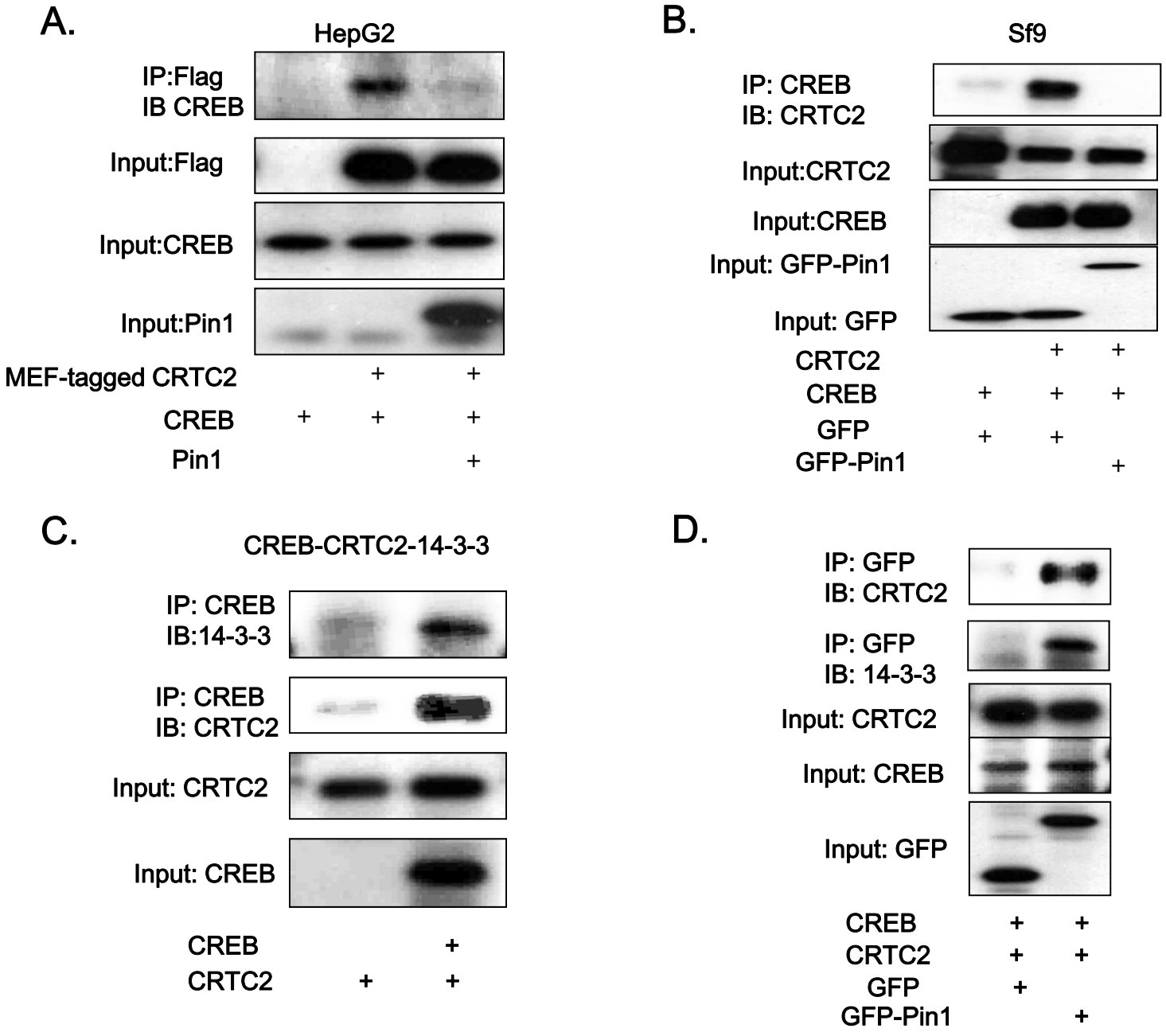

LacZ
Pin1 overexpression
Forskolin(-)
Forskolin 30 min
Forskolin(-)
Forskolin 30 min
Negative siRNA
Pin1 siRNA
Forskolin(-)
Forskolin 10 min
Forskolin(-)
Forskolin 10 min

Fig.4


A.
HepG2
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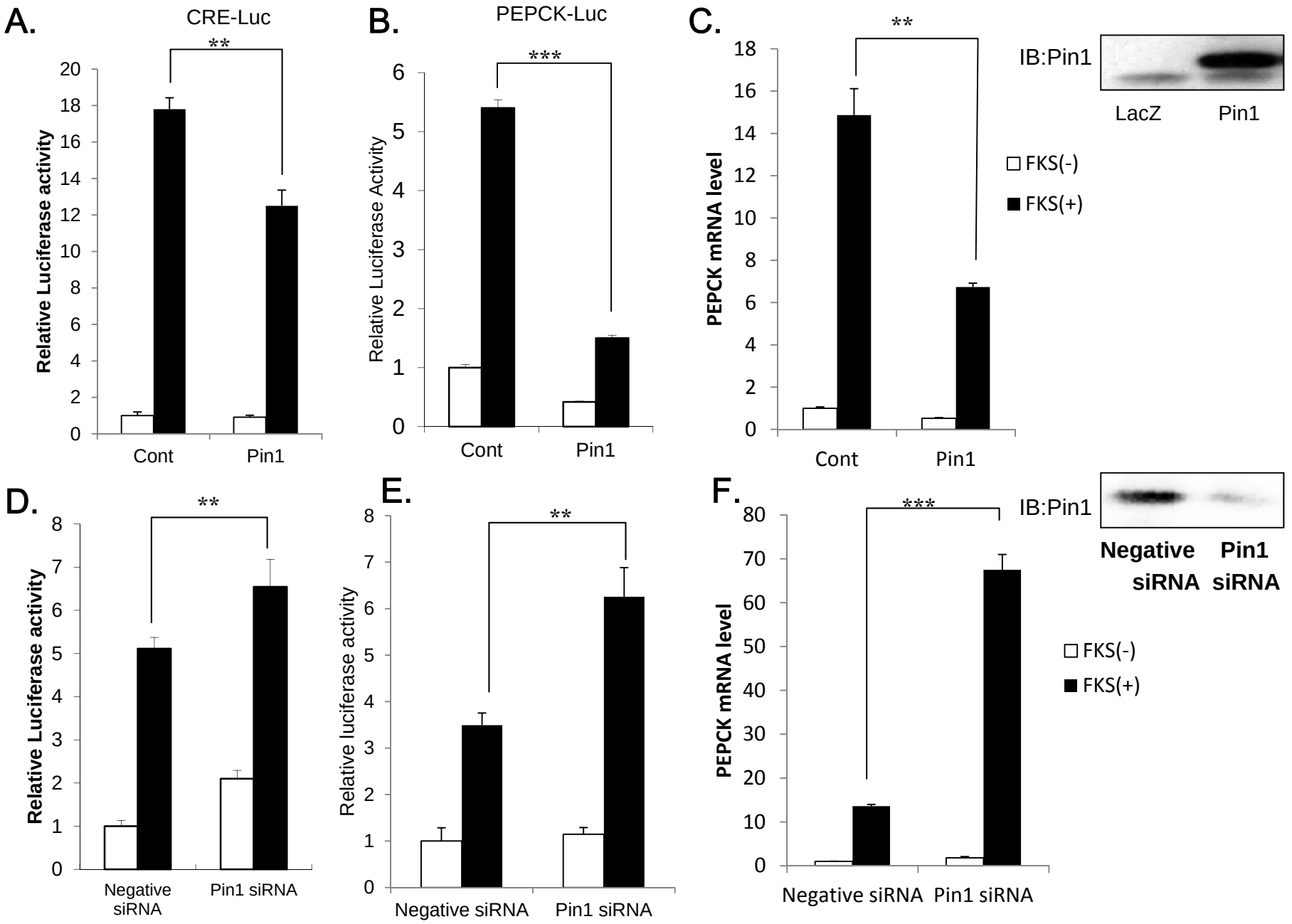
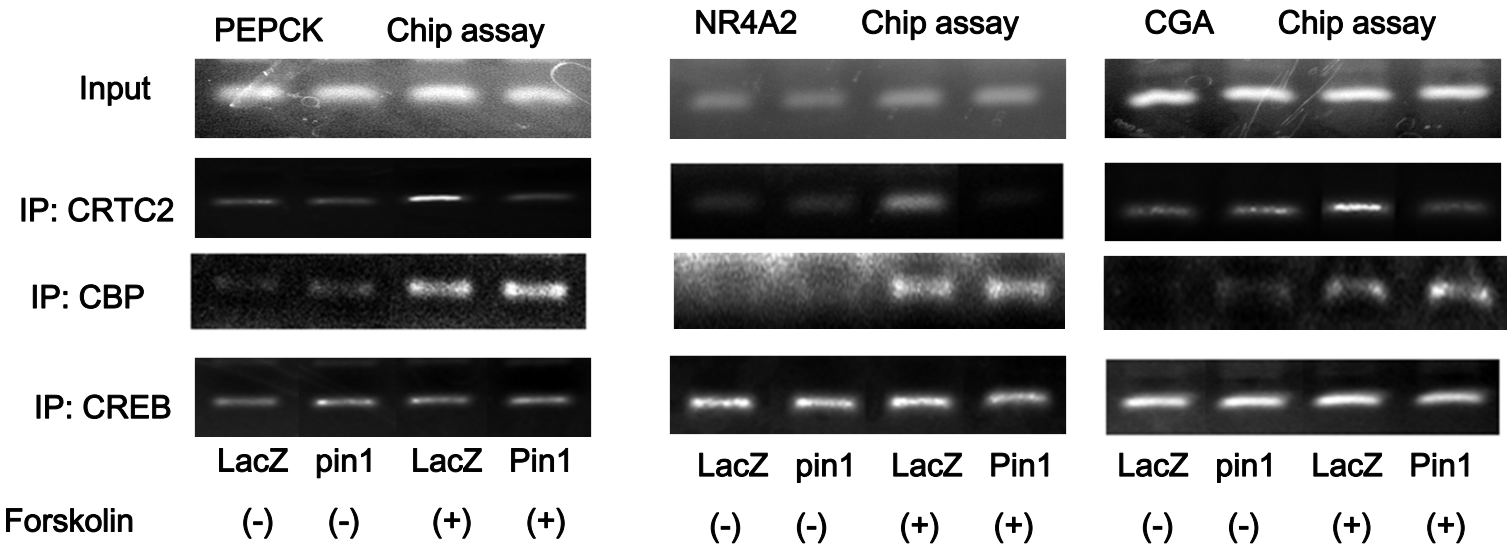
IB CREB
Input:Flag
Input:CREB
Input:Pin1
MEF-tagged CRTC2
CREB
Pin1
B.
Sf9
IP: CREB
IB: CRTC2
Input:CRTC2
Input:CREB
Input: GFP-Pin1
Input: GFP
CRTC2
CREB
GFP
GFP-Pin1
C.
CREB-CRTC2-14-3-3
IP: CREB
IB:14-3-3
IP: CREB
IB: CRTC2
Input: CRTC2
Input: CREB
CREB
CRTC2
D.
IP: GFP
IB: CRTC2
IP: GFP
IB: 14-3-3
Input: CRTC2
Input: CREB
Input: GFP
CREB
CRTC2
GFP
GFP-Pin1

Fig. 5


A.
CRE-Luc
**
Relative Luciferase activity
20
18
16
14
12
10
8
6
4
2
0
Cont
Pin1
B.
PEPCK-Luc
***
Relative Luciferase Activity
6
5
4
3
2
1
0
Cont
Pin1
C.
**
PEPCK mRNA level
18
16
14
12
10
8
6
4
2
0
Cont
Pin1
FKS(-)
FKS(+)
IB:Pin1
LacZ
Pin1
D.
**
Relative Luciferase activity
8
7
6
5
4
3
2
1
0
Negative siRNA
Pin1 siRNA
E.
**
Relative luciferase activity
8
7
6
5
4
3
2
1
0
Negative siRNA
Pin1 siRNA
F.
***
PEPCK mRNA level
80
70
60
50
40
30
20
10
0
Negative siRNA
Pin1 siRNA
FKS(-)
FKS(+)
IB:Pin1
Negative siRNA
Pin1 siRNA

G.


PEPCK
Chip assay
NR4A2
Chip assay
CGA
Chip assay
Input
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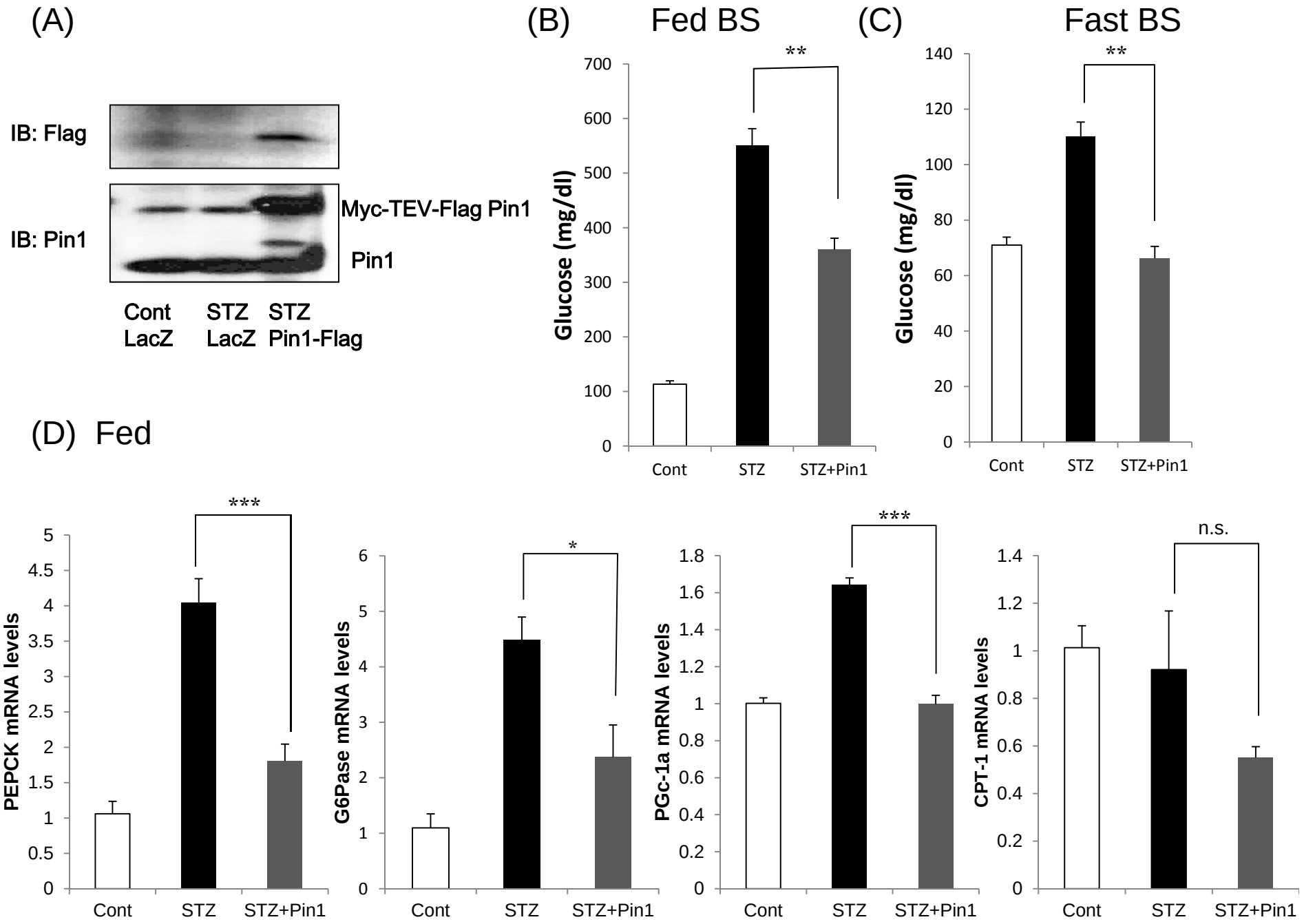
IP: CBP
IP: CREB
LacZ
pin1
LacZ
Pin1
Forskolin
(-)
(-)
(+)
(+)

Fig. 6


(A)
IB: Flag
IB: Pin1
Myc-TEV-Flag Pin1
Pin1
Cont LacZ
STZ LacZ
STZ Pin1-Flag
(B) Fed BS
Glucose (mg/dl)
0
100
200
300
400
500
600
700
**
Cont
STZ
STZ+Pin1
(C) Fast BS
Glucose (mg/dl)
0
20
40
60
80
100
120
140
**
Cont
STZ
STZ+Pin1
(D) Fed
PEPCK mRNA levels
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
***
Cont
STZ
STZ+Pin1
G6Pase mRNA levels
0
1
2
3
4
5
6
*
Cont
STZ
STZ+Pin1
PGc-1a mRNA levels
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
***
Cont
STZ
STZ+Pin1
CPT-1 mRNA levels
0
0.2
0.4
0.6
0.8
1
1.2
1.4
n.s.
Cont
STZ
STZ+Pin1

(E) Fast

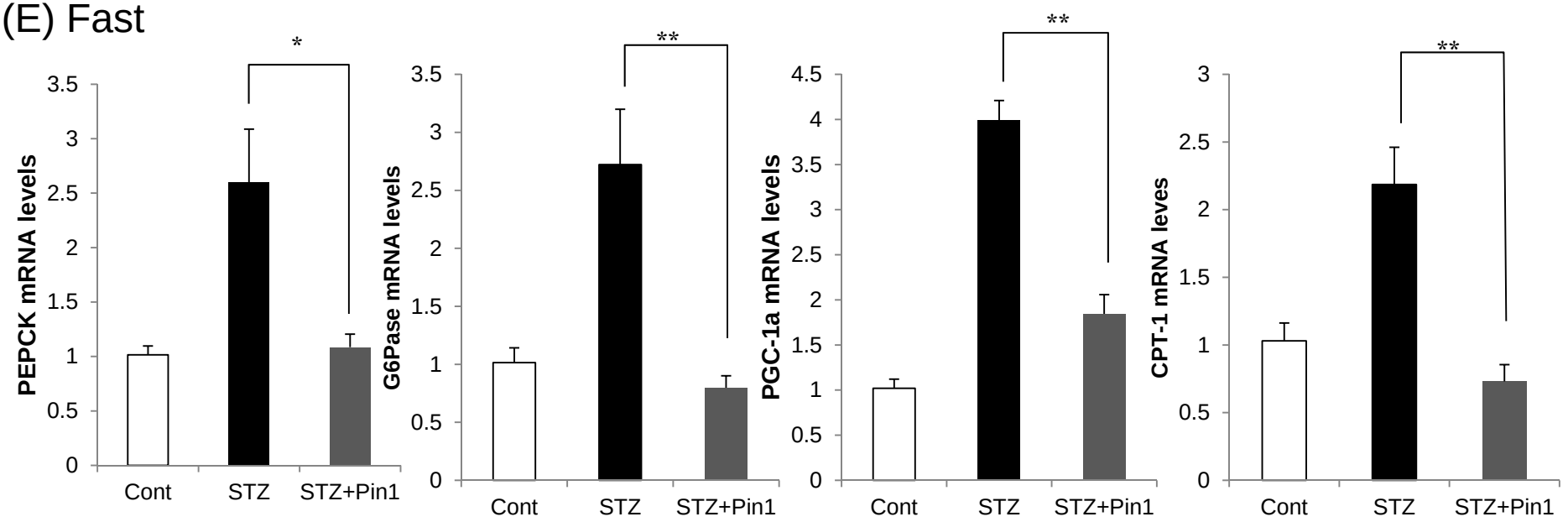
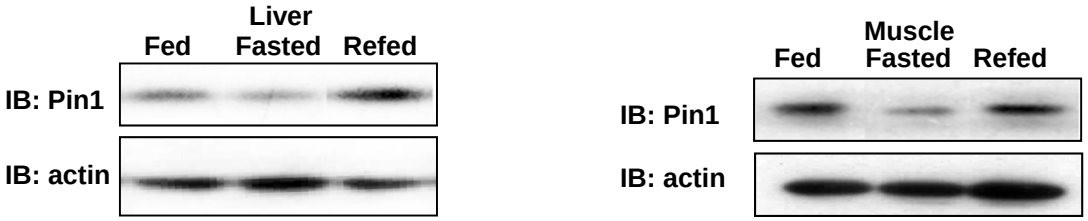

PEPCK mRNA levels
*
3.5
3
2.5
2
1.5
1
0.5
0
Cont
STZ
STZ+Pin1
G6Pase mRNA levels
**
3.5
3
2.5
2
1.5
1
0.5
0
Cont
STZ
STZ+Pin1
PGC-1a mRNA levels
**
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Cont
STZ
STZ+Pin1
CPT-1 mRNA leves
**
3
2.5
2
1.5
1
0.5
0
Cont
STZ
STZ+Pin1

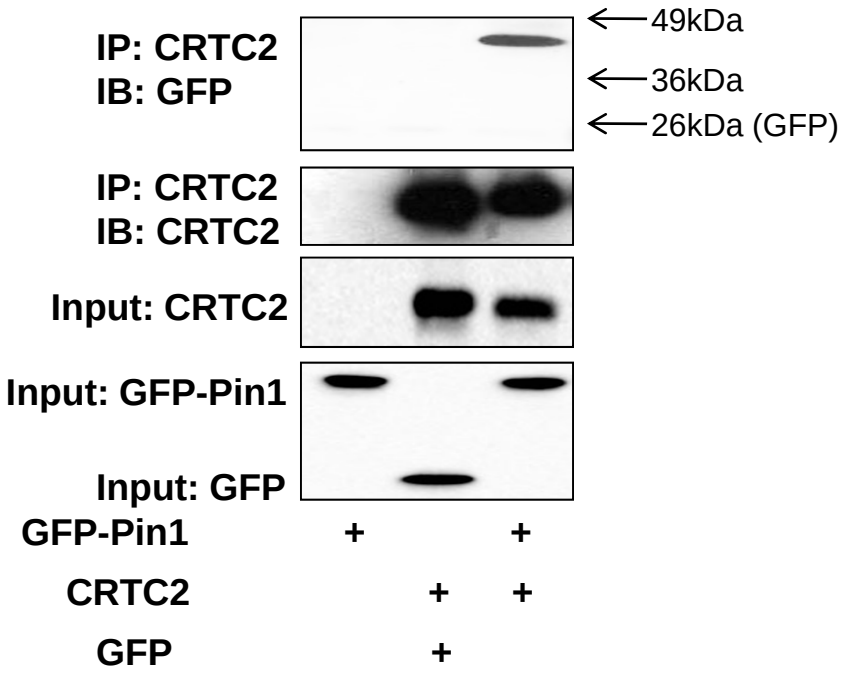
Fig.7


Liver
Fed
Fasted
Refed
IB: Pin1
IB: actin

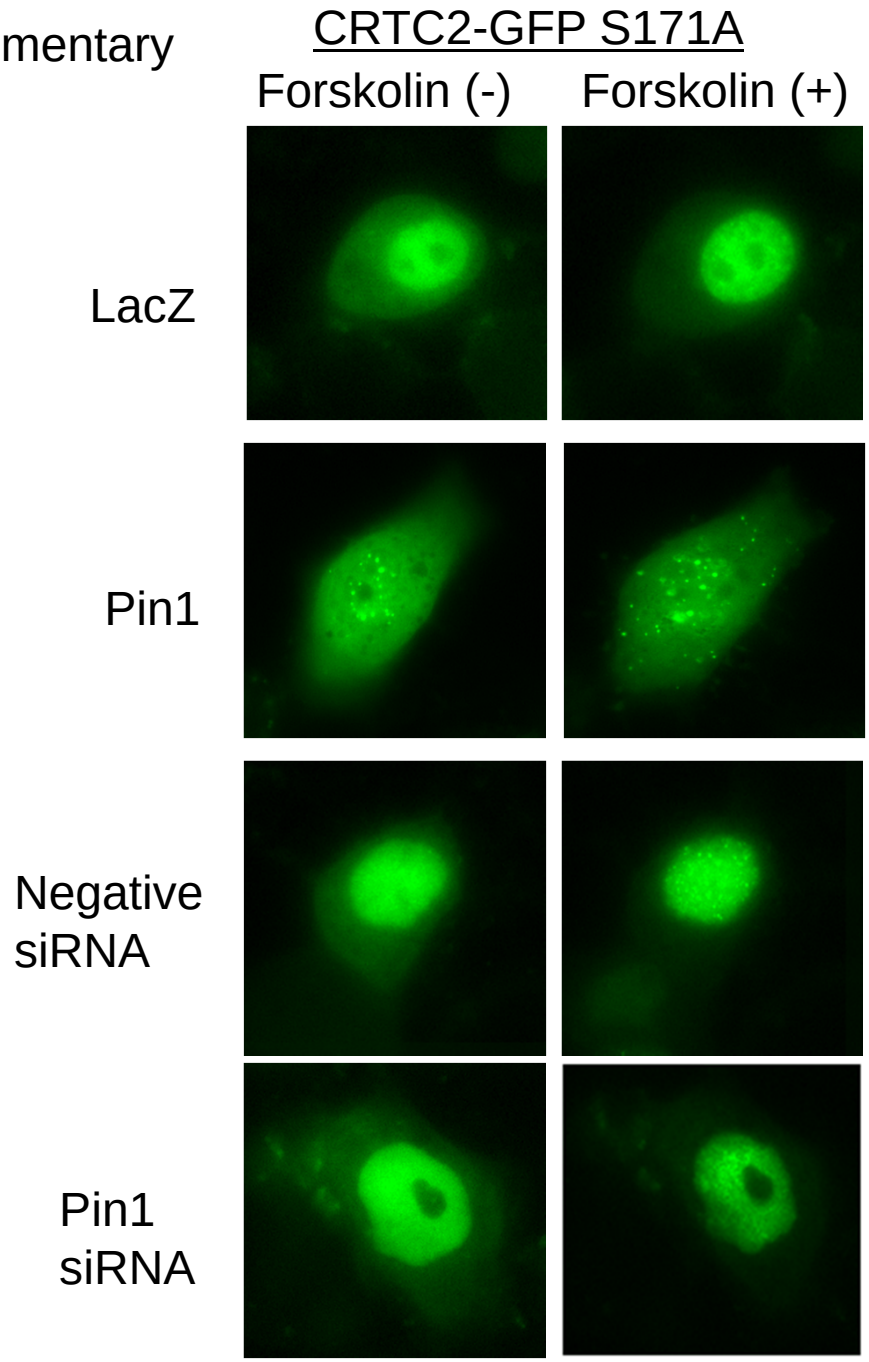
Muscle
Fed
Fasted
Refed
IB: Pin1
IB: actin

Supplementary
Fig.1

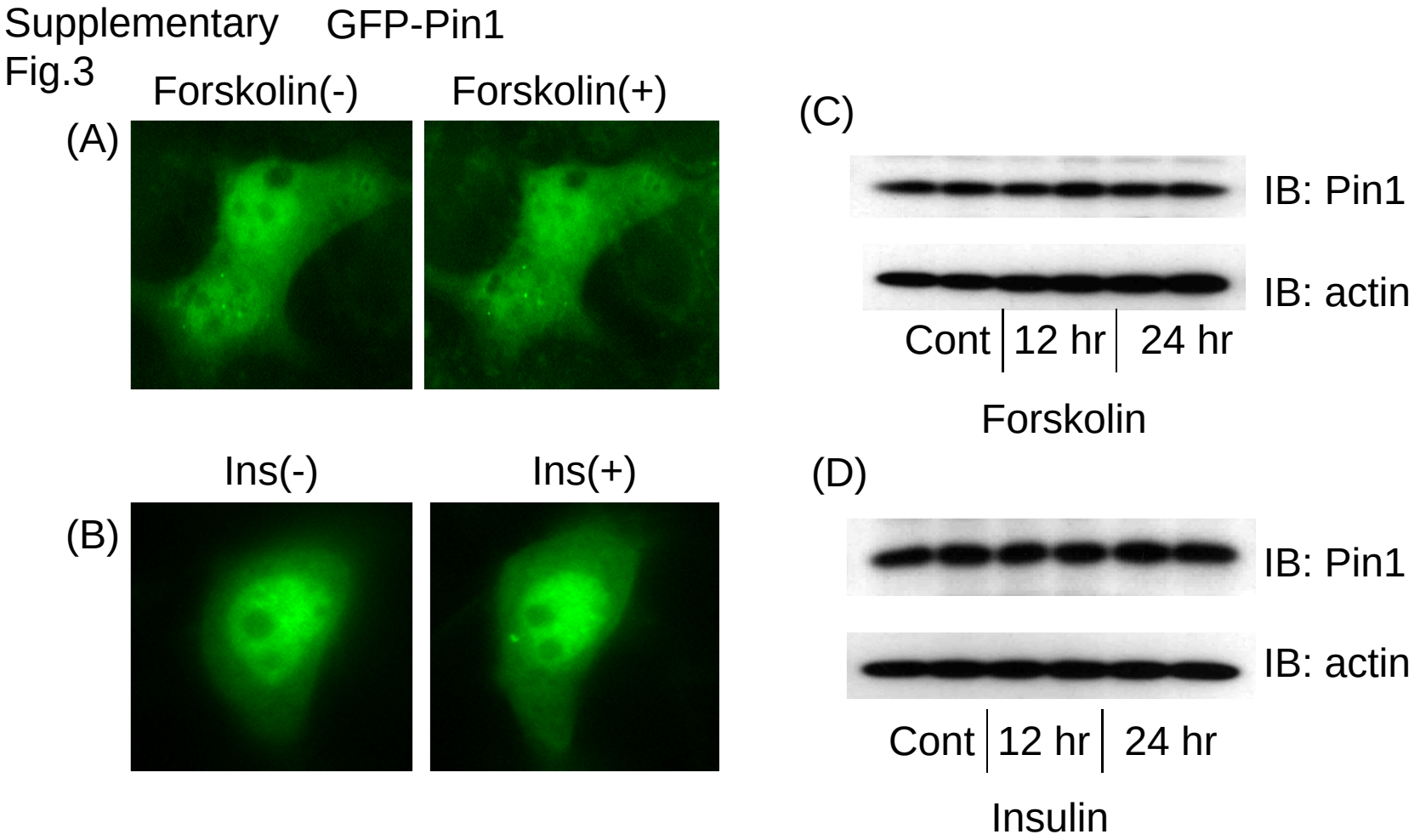
Sf9



Supplementary Fig.2



Supplementary Fig.3

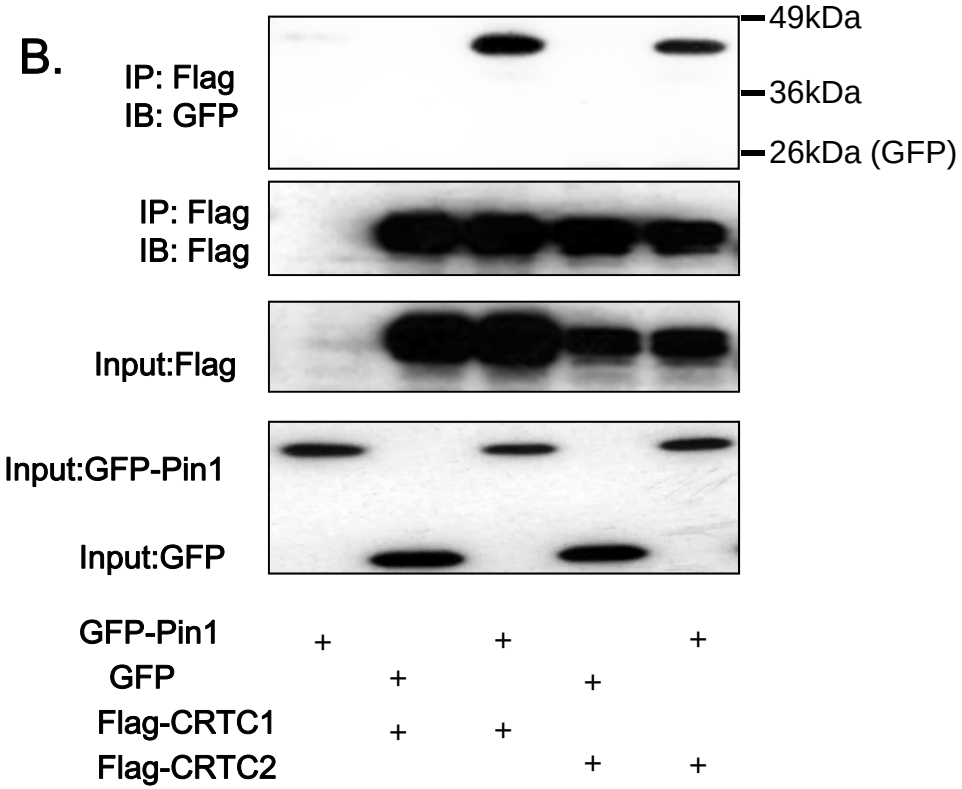


Supplementary Fig. 4

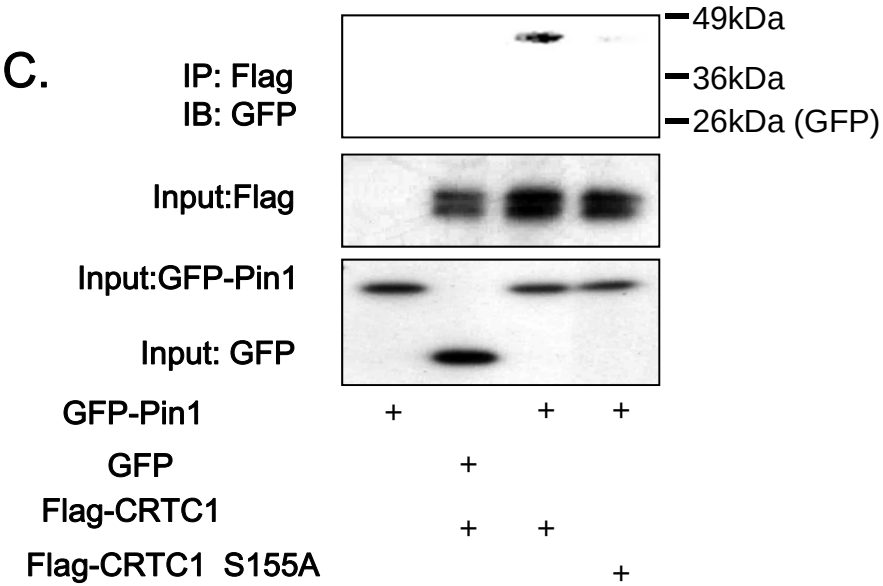
A.

```
hCRTC1: 142 RQADSCPYGTMYLSPPADTSW 162
mCRTC1: 126 RQADSCPYGTVYLSPPADTSW 147
hCRTC2: 123 RHIDSSPYSPAYLSPPPESSW 144
mCRTC2: 123 RHIDSSPYSPAYLSPPPESGW 144
hCRTC3: 116 RQFDGSAFGANYSSQPLDESW 137
mCRTC3: 116 RQFDGNAFAASYSSQHLDESW 137
```

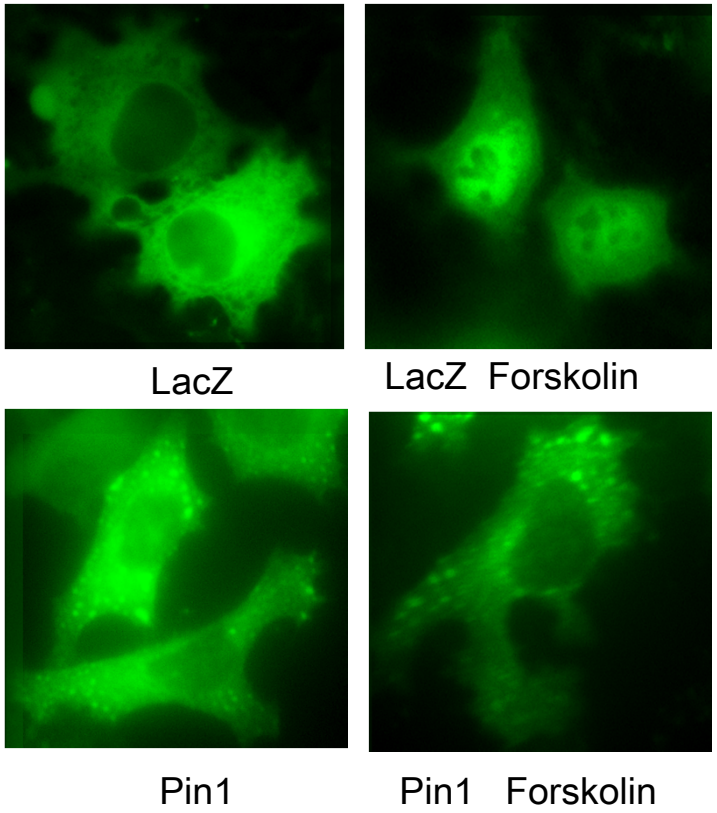
B.



C.

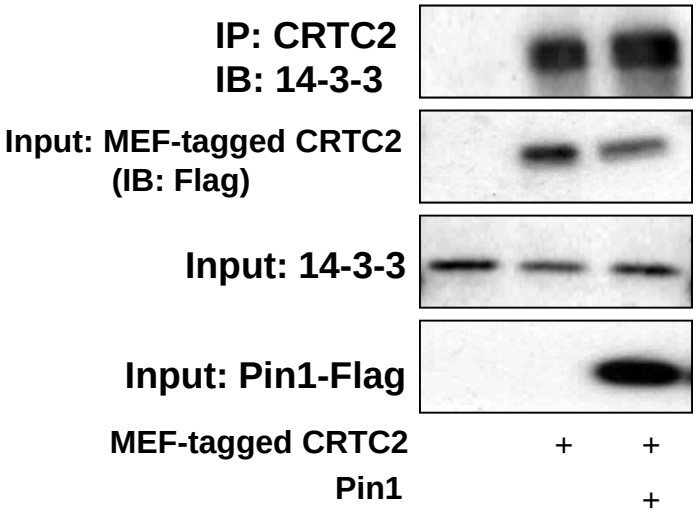


D.

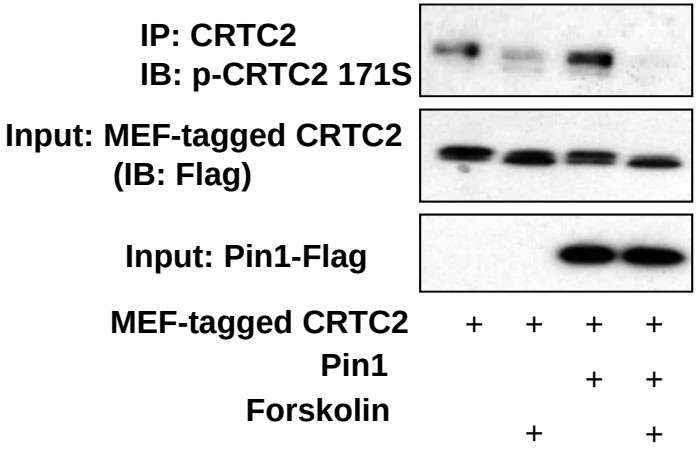


Supplementary
Fig. 5

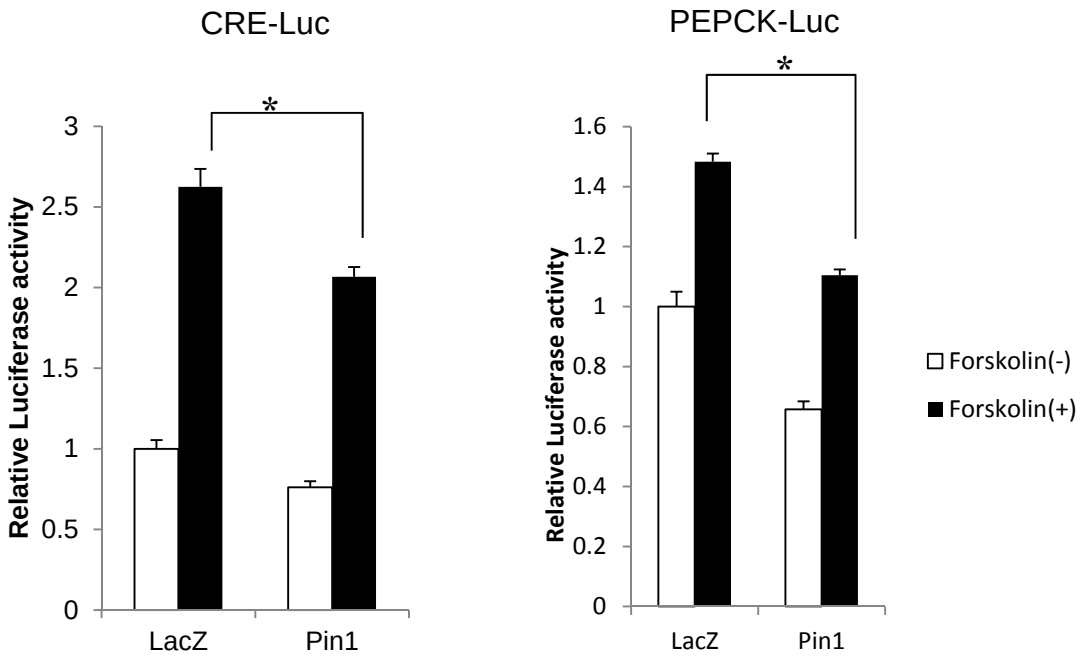
(A)



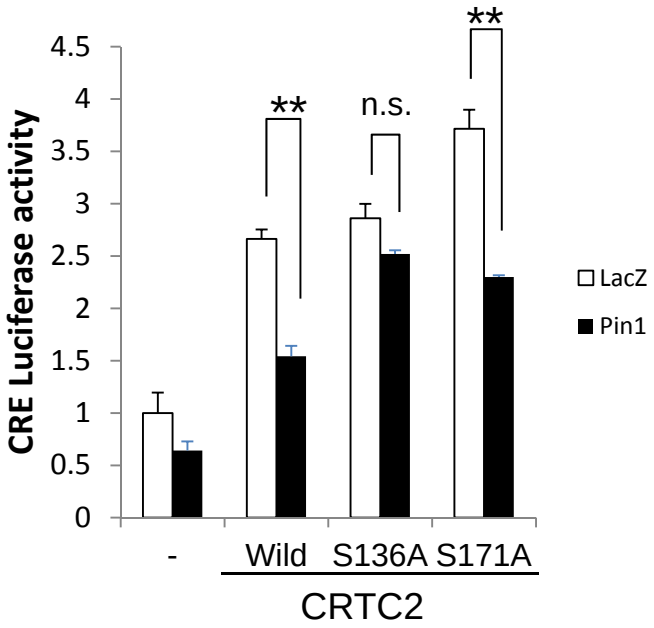
(B)



Supplementary
Fig.6



Supplementary
Fig.7



Supplementary
Fig. 8