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(54) Titre : INHIBITEURS DE CaMKII, IP3R, CALCINEURINE, P38 ET MK2/3 POUR TRAITER DES PERTURBATIONS METABOLIQUES DE L'OBESITE

(54) Title: CaMKII, IP3R, CALCINEURIN, P38 AND MK2/3 INHIBITORS TO TREAT METABOLIC DISTURBANCES OF OBESITY

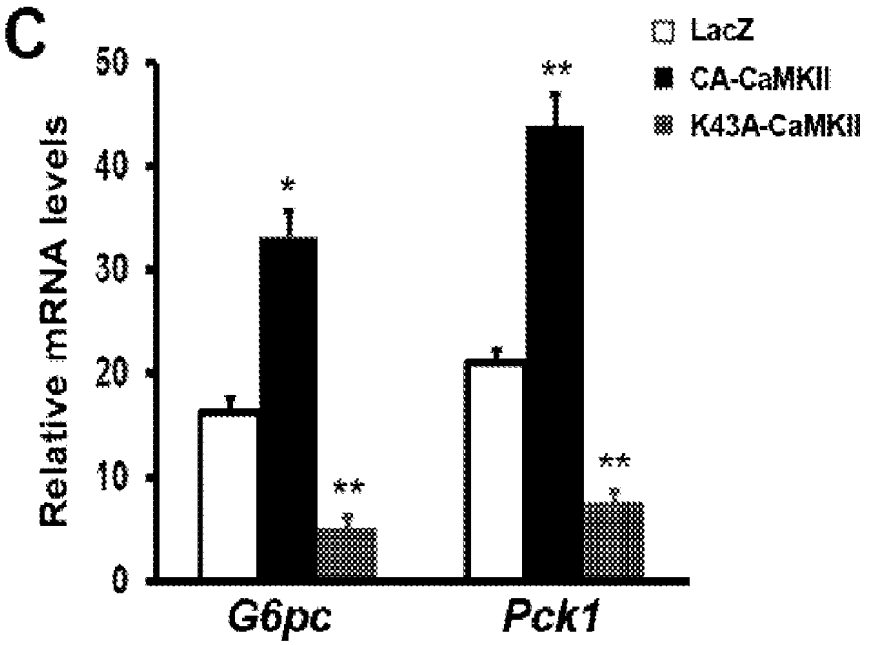

Figure 2C

(57) Abrégé/Abstract:
The present invention relates to methods of treating or preventing a metabolic disorder in a subject, methods of treating or preventing coronorary artery disease in a subject with a metabolic disorder, as well as methods of reducing hepatic glucose production in a subject. Such methods include, but are not limited to, the administration to the subject of inhibitors of CaMKII, IP3Rs, calcineurin, p38, MK2/3, or any combination thereof. The invention also provides methods of identifying a compound that inhibits the activity of CaMKII, IP3Rs, calcineurin, p38, or MK2/3, or reduces the activity and/or activation of CaMKII, IP3Rs, calcineurin, p38, or MK2/3.

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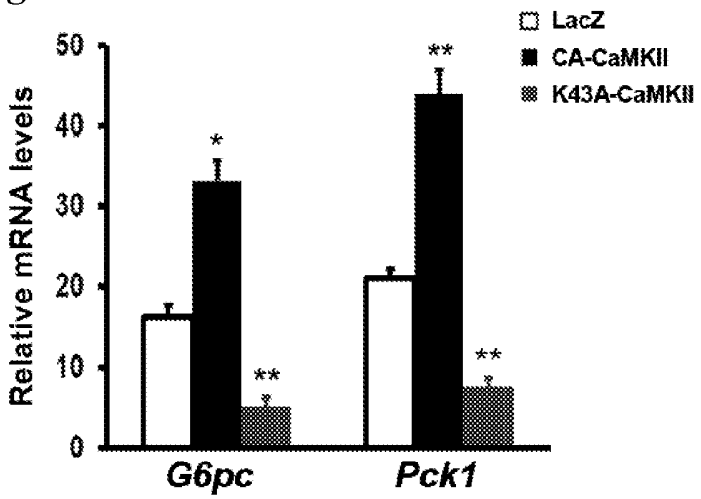
(54) Title: CaMKII, IP3R, CALCINEURIN, P38 AND MK2/3 INHIBITORS TO TREAT METABOLIC DISTURBANCES OF OBESITY

Figure 2C



(57) Abstract: The present invention relates to methods of treating or preventing a metabolic disorder in a subject, methods of treating or preventing coronorary artery disease in a subject with a metabolic disorder, as well as methods of reducing hepatic glucose production in a subject. Such methods include, but are not limited to, the administration to the subject of inhibitors of CaMKII, IP3Rs, calcineurin, p38, MK2/3, or any combination thereof. The invention also provides methods of identifying a compound that inhibits the activity of CaMKII, IP3Rs, calcineurin, p38, or MK2/3, or reduces the activity and/or activation of CaMKII, IP3Rs, calcineurin, p38, or MK2/3.

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CaMKII, IP3R, CALCINEURIN, P38 AND MK2/3 INHIBITORS TO TREAT METABOLIC DISTURBANCES OF OBESITY

[0001] This application claims priority to United States Provisional Application No. 61/530,851, filed September 2, 2011, United States Provisional Application No. 61/618,551, filed March 30, 2012, United States Provisional Application No. 61/621,407, filed April 6, 2012, United States Provisional Application No. 61/676,091, filed July 26, 2012, and United States Provisional Application No. 61/676,152, filed July 26, 2012, all of which are herein incorporated by reference in their entireties.

[0002] This patent disclosure contains material that is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or the patent disclosure as it appears in the U.S. Patent and Trademark Office patent file or records, but otherwise reserves any and all copyright rights.

[0003] All patents, patent applications and publications cited herein are hereby incorporated by reference in their entirety. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described herein.

GOVERNMENT SUPPORT

[0004] This invention was made with government support under Grant No. P01 HL087123 awarded by the National Institute of Health and the National Heart, Lung and Blood Institute. Thus, the United States Government has certain rights in the present invention.

BACKGROUND OF THE INVENTION

[0005] Obesity-induced insulin resistance and disturbances in liver glucose and fat metabolism increase the risk for heart disease, cancer, and other widespread and devastating diseases. Current treatments options are severely limited, leading to a critical unmet clinical need affecting hundreds of millions of overweight people in the current obesity epidemic. There is a need for methods to treat and diagnose obesity-induced insulin resistance and disturbances in liver glucose and fat metabolism. This invention addresses these needs.

SUMMARY OF THE INVENTION

[0006] The present disclosure provides methods for the treatment and/or prevention of a metabolic disorder. The present disclosure also provides methods for identifying a compound or a combination of compounds for the treatment and/or prevention of a metabolic disorder in a subject.

[0007] The present disclosure provides methods for the treatment and/or prevention of a metabolic disorder. In one aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of CaMKII, thereby treating or preventing the disorder. In one embodiment, the disorder is selected from the group consisting of Type 1 diabetes, Type 2 diabetes, insulin resistance and metabolic syndrome. In another embodiment, the treating or preventing affects glycogenolysis or gluconeogenesis in the subject. In one embodiment, the treating or preventing reduces hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject. In another embodiment, the treating or preventing reduces phosphorylation and/or activation of CaMKII. In one embodiment, the activity of CaMKII is a glucagon-induced activity. In one embodiment, the treating or preventing reduces expression of G6pc and/or Pck1 in a cell of the subject. In another embodiment, the treating or preventing reduces the amount of FoxO1 protein level in the nucleus of a cell of the subject. In another embodiment, the treating or preventing reduces phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein.

[0008] In another aspect, the present disclosure provides a method of treating or preventing coronary artery disease in a subject with a metabolic disorder, the method comprising reducing the activity of CaMKII in the subject, thereby treating or preventing the coronary artery disease. In one embodiment, the disorder is selected from the group consisting of Type 1 diabetes, Type 2 diabetes, insulin resistance and metabolic syndrome. In another embodiment, the treating or preventing affects glycogenolysis or gluconeogenesis in the subject. In one embodiment, the treating or preventing reduces hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject. In another embodiment, the treating or preventing reduces phosphorylation and/or activation of CaMKII. In one embodiment, the

activity of CaMKII is a glucagon-induced activity. In one embodiment, the treating or preventing reduces expression of G6pc and/or Pck1 in a cell of the subject. In another embodiment, the treating or preventing reduces the amount of FoxO1 protein level in the nucleus of a cell of the subject. In another embodiment, the treating or preventing reduces phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein. In one embodiment, the treating or preventing comprises reducing CaMKII activity in a macrophage of the subject.

[0009] In one embodiment, the coronary artery disease is associated with atherogenesis and/or atherosclerosis. In another embodiment, the method further comprises treating or preventing heart failure, hypertension and/or renal disease. In another embodiment, the disorder is associated with advanced lesional macrophage apoptosis. In another embodiment, the disorder is associated with plaque necrosis. In another embodiment, the treating or preventing coronary artery disease in a subject with a metabolic disorder results in lowering hyperinsulinemia and/or dyslipidemia. In another embodiment, the treating or preventing coronary artery disease in a subject with a metabolic disorder results in lowering atherogenesis and/or atherosclerosis.

[0010] In one embodiment, the treating or preventing comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the CaMKII protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the CaMKII protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a small molecule. In one embodiment, the small molecule is a CaMKII inhibitor selected from the group consisting of KN-93, lavendustin C, CK59, Ant-CaMKIINtide, KN62, DY9760e, K-252a *Nocardiopsis sp.*, H89 dihydrochloride, PP1 Analog II, 1NM-PP1, eEF-2 kinase inhibitor NH125, and STO-609.

[0011] In another aspect, the present disclosure provides a method of reducing hepatic glucose production in a subject, the method comprising reducing the activity of CaMKII, thereby resulting in the reduction of hepatic glucose in the subject. In one embodiment, the reducing hepatic glucose production reduces phosphorylation and/or activation of CaMKII. In another embodiment, the activity of CaMKII is a glucagon-induced activity. In another embodiment, the reducing hepatic glucose production reduces expression of G6pc and/or Pck1 in a cell of the subject. In one embodiment, the reducing hepatic glucose production reduces hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject. In another embodiment, the reducing hepatic glucose production reduces the amount of FoxO1 protein level in the nucleus of

a cell of the subject. In another embodiment, the reducing hepatic glucose production reduces phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein.

[0012] In one embodiment, the reducing hepatic glucose production comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the CaMKII protein. In another embodiment, the reducing hepatic glucose production comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the CaMKII protein. In another embodiment, the reducing hepatic glucose production comprises a step of administering to the subject a small molecule. In one embodiment, the small molecule is a CaMKII inhibitor selected from the group consisting of KN-93, lavendustin C, CK59, Ant-CaMKIINtide, KN62, DY9760e, K-252a *Nocardiopsis sp.*, H89 dihydrochloride, PP1 Analog II, 1NM-PP1, eEF-2 kinase inhibitor NH125, and STO-609.

[0013] In another aspect, the present disclosure provides a method for identifying a compound that inhibits the activity of CaMKII, the method comprising a) contacting a cell with a CaMKII fusion protein, wherein the CaMKII fusion protein comprises an acceptor fluorophore protein at one terminus, and a donor fluorophore protein at the other terminus; and b) measuring FRET efficiency in the absence and in the presence of a test compound, wherein a greater FRET efficiency in the presence of the test compound compared to the FRET efficiency in the absence of the test compound indicates that the test compound inhibits the activity of CaMKII.

[0014] In one embodiment the acceptor fluorophore protein is selected from the group consisting of mOrange, mStrawberry, Venus, yellow fluorescent protein, cyan fluorescent protein, red fluorescent protein and green fluorescent protein.

[0015] In another embodiment, the cell is a HEK293T cell, a hepatocyte, a U2OS cell, a HeLa cell, a murine embryonic fibroblast (MEF) or a macrophage. In another embodiment, the cell is from a *Insr-/-* mouse, a *Camk2g-/-* mouse, a *Foxo1 -/-* mouse, a *db/db* mouse, a *ob/ob* mouse, a *p38 -/-* mouse, a Non Obese Diabetes (NOD) mouse, a mouse fed a high fat diet, or a streptozotocin-treated mouse. In another embodiment, the cell is from a mouse expressing a mutant FoxO1 protein. In one embodiment, the mutant FoxO1 protein comprises alanine substitutions at S284, S295, S326, S467, S475, S246, S253, S413, or S415, or aspartic acid substitutions at S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof.

[0016] In one embodiment, the cell is subjected to ER stress. In another embodiment, the cell is treated with glucagon, 8-bromo cAMP, H89 dihydrochloride, Xestospongin C, forskolin, saturated fatty acids, or any combination thereof.

[0017] In one aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of IP3R1, IP3R2 or IP3R3, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of calcineurin, thereby treating or preventing the disorder.

[0018] In one embodiment, the disorder is selected from the group consisting of Type 1 diabetes, Type 2 diabetes, insulin resistance, obesity, and metabolic syndrome.

[0019] In another embodiment, the treating or preventing affects glycogenolysis or gluconeogenesis in the subject.

[0020] In one embodiment, the treating or preventing reduces hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject.

[0021] In one embodiment, the treating or preventing comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the IP3R1 protein, IP3R2 protein or IP3R3 protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the IP3R1 protein, IP3R2 protein or IP3R3 protein.

[0022] In one embodiment, the treating or preventing comprises a step of administering to the subject a small molecule. In one embodiment, the small molecule is a IP3R1 protein inhibitor, IP3R2 protein inhibitor or IP3R3 protein inhibitor.

[0023] In one embodiment, the treating or preventing comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the calcineurin protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the calcineurin protein.

[0024] In one embodiment, the treating or preventing comprises a step of administering to the subject a small molecule. In another embodiment, the small molecule is a calcineurin inhibitor.

[0025] In another aspect, the present disclosure provides a method for identifying a compound that inhibits the activity of IP3R1, IP3R2 or IP3R3, the method comprising a) contacting a cell with a test compound; and b) measuring IP3R1, IP3R2 or IP3R3 activity, wherein a reduction of the activity of IP3R1, IP3R2 or IP3R3 in the presence of the compound compared to the activity of IP3R1, IP3R2 or IP3R3 in the absence of the compound indicates that the compound is an inhibitor of IP3R1, IP3R2 or IP3R3, respectively.

[0026] In one embodiment, the activity is measured by calcium release into the cytosol of the cell after stimulation with an inducer of IP3. In one embodiment, calcium release is measured by an increase in the fluorescence of a cytosolic calcium dye. In one embodiment, the cytosolic calcium dye is Fluo-3.

[0027] In another aspect, the present disclosure provides a method for identifying a compound that inhibits the activity of calcineurin, the method comprising a) contacting a cell with a test compound; and b) measuring calcineurin activity, wherein a reduction of the activity of calcineurin in the presence of the compound compared to the activity of calcineurin in the absence of the compound indicates that the compound is an inhibitor of calcineurin.

[0028] In one embodiment, the activity of calcineurin is measured through detection of phosphatase activity using a calcineurin substrate peptide. In one embodiment, the cell is a HEK293T cell, a hepatocyte, a U2OS cell, a HeLa cell, or a murine embryonic fibroblast (MEF). In another embodiment, the cell is from a db/db mouse, a ob/ob mouse, a Non Obese Diabetes (NOD) mouse, a mouse fed a high fat diet, or a streptozotocin-treated mouse.

[0029] In one embodiment, the cell is treated with glucagon, H89 dihydrochloride, insulin, forskolin, an inducer of ER stress, tunicamycin, saturated fatty acids, or any combination thereof.

[0030] In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of p38, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of MK2/3, thereby treating or preventing the disorder.

[0031] In one embodiment, the disorder is selected from the group consisting of Type 1 diabetes, Type 2 diabetes, insulin resistance, obesity and metabolic syndrome.

[0032] In one embodiment, the treating or preventing affects glycogenolysis or gluconeogenesis in the subject. In another embodiment, the treating or preventing reduces hepatic glucose

production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject.

[0033] In one embodiment, the treating or preventing comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the p38 protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the p38 protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a small molecule. In one embodiment, the small molecule is a p38 inhibitor.

[0034] In one embodiment, the treating or preventing comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the MK2/3 protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the MK2/3 protein. In one embodiment, the treating or preventing comprises a step of administering to the subject a small molecule. In one embodiment, the small molecule is a MK2/3 inhibitor.

[0035] In another aspect, the present disclosure provides a method for identifying a compound that inhibits the activity of p38, the method comprising a) contacting a cell with a test compound; and b) measuring p38 kinase activity, wherein a reduction of the kinase activity of p38 in the presence of the compound compared to the kinase activity of p38 in the absence of the compound indicates that the compound is an inhibitor of p38. In another aspect, the present disclosure provides a method for identifying a compound that inhibits the activity of MK2/3, the method comprising a) contacting a cell with a test compound; and b) measuring MK2/3 kinase activity, wherein a reduction of the kinase activity of MK2/3 in the presence of the compound compared to the kinase activity of MK2/3 in the absence of the compound indicates that the compound is an inhibitor of MK2/3.

[0036] In one embodiment, p38 kinase activity is measured using a p38-specific peptide. In one embodiment, MK2/3 kinase activity is measured using a MK2/3-specific peptide.

[0037] In one embodiment, the cell is a HEK293T cell, a hepatocyte, a U2OS cell, a HeLa cell, a macrophage or a murine embryonic fibroblast (MEF). In another embodiment, the cell is from a Insr-/- mouse, a db/db mouse, a ob/ob mouse, a Non Obese Diabetes (NOD) mouse, a mouse fed a high fat diet, or a streptozotocin-treated mouse. In another embodiment, the cell is treated with glucagon, 8-bromo cAMP, H89 dihydrochloride, forskolin, saturated fatty acids, or any combination thereof.

[0038] These, and other, embodiments of the invention will be better appreciated and understood when considered in conjunction with the following description and the accompanying drawings. It should be understood, however, that the following description, while indicating various embodiments of the invention and numerous specific details thereof, is given by way of illustration and not of limitation. Many substitutions, modifications, additions and/or rearrangements may be made within the scope of the invention without departing from the spirit thereof, and the invention includes all such substitutions, modifications, additions and/or rearrangements.

BRIEF DESCRIPTION OF THE FIGURES

[0039] Figs. 1A-J. Glucagon and fasting activates hepatic CaMKII. Fig. 1A. CaMKII enzyme activity was assayed in triplicate wells of primary mouse HCs stimulated with 100 nM glucagon (Gluc) or vehicle control (Veh) for the indicated times (*P < 0.05 and **P < 0.01 vs. Veh; mean ± S.E.M.). **Figs. 1B-1J**. Extracts of HCs or liver were probed for phospho-CaMKII, total CaMKII, and β-actin by immunoblot assay. **Fig. 1B**. HCs were incubated with 00 nM glucagon for the indicated times; **Fig. 1C**. Glucagon was added to HCs that were pre-treated for 1 h with vehicle control (Veh) or 5 μM BAPTA-AM; **Fig. 1D**. Glucagon was added to HCs that were pre-treated with 0.5 μM xestospongin (XesC) or to HCs from Ip3r1fl/fl mice transduced with adeno-LacZ control or adeno-Cre (bar graph = Ip3r1 mRNA levels); **Fig. 1E**. Glucagon was added to HCs that were pre-treated for 1 h with vehicle control (Veh) or 10 μM H89. **Fig. 1F**. HCs were incubated with 100 μM 8-bromo-cAMP for the indicated times. **Figs. 1G-1J**. In vivo experiments. In **Fig. 1G**, mice were treated for 30 min with 200 μg kg-1 body weight of glucagon i.p., and in **Fig. 1H**, mice were pre-treated with 10 pmol g-1 xestospongin C or vehicle control i.p. 4 days prior to glucagon treatment. In **Figs. 1I-1J**, mice were fed ad libitum or fasted for 12 h, or fasted for 12 h and then re-fed for 4 h.

[0040] Figs. 2A-E. CaMKII regulates glucose production and hepatic G6Pc and Pck1 expression in primary hepatocytes. **Fig. 2A** Primary mouse hepatocytes were transduced with adenoviral vectors expressing LacZ, CA-CaMKII, or K43A-CaMKII at an MOI of 20. Cells were serum depleted overnight and then incubated with forskolin (10 μm) for 14 h in serum- and glucose-free media. Glucose content was measured as described. **Fig. 2B** As in **Fig. 2A** except that WT and Camk2g-/- hepatocytes were used. **Fig. 2C** Primary mouse hepatocytes were infected with adenoviral vectors expressing LacZ, CA-CaMKII, or K43A-CaMKII at an MOI of 20. Cells were serum depleted overnight and were then incubated with forskolin (10 μm) for 5 h

in serum-free media. RNA was isolated and gene expression was analyzed by real-time Q-PCR. (*, P < 0.05; **, P < 0.01) **Fig. 2D** Primary hepatocytes from WT and Camk2g-/- mice were serum depleted overnight and then incubated with forskolin (10 μm) for 5 h in serum-free media. RNA was assayed for G6pc and Pck1 mRNA by real-time Q–PCR. (**, P < 0.01; ****, P < 0.0005). **Fig. 2E**. As in **Fig. 2D**, except that glucagon (100 nm) was used to stimulate gluconeogenesis. (*, P < 0.05; **, P < 0.01)

[0041] Figs. 3A-C. CaMKII regulates hepatic FoxO1 subcellular localization in primary hepatocytes. **Fig. 3A** Primary hepatocytes from WT and Camk2g-/- mice were transduced with an adenovirus expressing murine GFP-FoxO1 at an MOI of 2. Cells were serum depleted overnight and then incubated for 5 h in serum-free media. FoxO1 subcellular localization was assessed by indirect immunofluorescence. Bar, 10 μm. Data are quantified in the right panel. (****, P < 0.0005). **Fig. 3B** Primary mouse hepatocytes were transduced with adenoviral vectors expressing LacZ, CA-CaMKII, or K43A-CaMKII at an MOI of 20. 4 h later cells were infected with murine GFP-FoxO1 adenovirus at an MOI of 5. Cells were serum depleted overnight and then incubated for 5 h in serum-free media. FoxO1 subcellular localization was subsequently assessed by indirect immunofluorescence. Bar, 5 μm. Data are quantified in the lower panel. (****, P < 0.0005; #### P < 0.0005). **Fig. 3C** Primary mouse hepatocytes were infected with adenoviral vectors expressing LacZ or CA-CaMKII. Nuclear extracts were assayed by immunoblot for FoxO1 and nucleophosmin (nuclear loading control).

[0042] Figs. 4A-D. CaMKIIγ deficiency decreases blood glucose, G6pc and Pck1 expression, and nuclear FoxO1. **Fig. 4A** Blood glucose concentrations in 8 week-old WT and Camk2g-/- mice fasted for 12 h. (*, P < 0.05) **Fig. 4B** 8-10 week-old WT and Camk2g-/- mice were fasted for 18 h and then challenged with pyruvate (2 mg kg-1). (*, P < 0.05; **, P < 0.01; ***, P < 0.005) **Fig. 4C** Liver G6pc and Pck1 gene expression levels in WT and Camk2g-/- mice fasted for 12 h. (**, P < 0.01; ***, P < 0.001) **Fig. 4D** As in **Fig. 4C**, except that liver nuclear extracts were assayed for FoxO1 and nucleophosmin by immunoblot. Densitometric quantification of these data are shown in the lower panel. (***, P < 0.001)

[0043] Figs. 5A-F. Acute inhibition of CaMKII decreases blood glucose, G6pc and Pck1 expression, and nuclear FoxO1. 9 wk-old WT mice were injected through the tail vein with 1.5 x 109 pfu of adenovirus containing either control LacZ (n = 5) or K43A-CaMKII (n = 5). **Fig. 5A** Blood glucose levels at day 5 after a 12-h fast. (***, P < 0.001) **Fig. 5B** Liver gluconeogenic gene expression in mice fasted for 12 h. (**, P < 0.01; ***, P < 0.001). **Fig. 5C**. As in **Fig. 5B**, except that liver nuclear extracts were assayed for FoxO1 and nucleophosmin by immunoblot.

Fig. 5D Liver glycogen content. **Fig. 5E-5F** LacZ or K43A-CaMKII treated mice were injected with 200 μg kg-1 body weight of glucagon intraperitoneally and sacrificed 30 min later. Liver G6pc mRNA was assayed, and liver sections were examined for glycogen content by PAS staining, which was quantified as percent of PAS-positive hepatocytes (n = 4 mice). Bar, 20 μm.

[0044] Figs. 6A-D. Constitutively active CaMKII increases blood glucose, *G6pc* and *Pck1* expression, and nuclear FoxO1. 9 week-old WT mice were injected with 1.5 x 109 pfu of adenoviruses containing either control LacZ (n = 5) or CA-CaMKII (n = 5). **Fig. 6A** Blood glucose was assayed at day 6 after a 12-h fast. (*, P < 0.05) **Fig. 6B** Pyruvate challenge test was conducted in mice fasted for 17 h. (*, P < 0.05; **, P < 0.01; ***, P < 0.005) **Fig. 6C** *G6pc* and *Pck1* mRNA levels were assayed in mice fasted for 12 h. (*, P < 0.05) or fed ad libitum (*, P < 0.05; ***, P < 0.005). **Fig. 6D** Liver nuclear extracts were assayed for FoxO1 and nucleophosmin by immunoblot in fed mice injected with either control LacZ (n = 5) or CA-CaMKII (n = 5).

[0045] Figs. 7A-D. Glucose metabolism impairment in *Camk2g-/-* hepatocytes and *in vivo* is rescued by transduction with constitutively nuclear FoxO1-ADA. **Fig. 7A** Primary hepatocytes from WT and *Camk2g-/-* mice were transduced with an adenovirus expressing FoxO1-ADA at an MOI of 0.2. Cells were serum depleted overnight and then incubated with forskolin (10 μm) for 5 h in serum-free media. RNA was isolated and gene expression was analyzed by real-time Q-PCR. (**, P < 0.01; #, P < 0.05; ##, P < 0.01) **Fig. 7B** 8 wk-old WT mice were injected with 1.5 x 10^9 pfu of adenovirus containing either control LacZ (n = 5) or K43A-CaMKII (n = 10). One day later, half of the adeno-K43A-CaMKII injected mice received 0.1 x 10^9 pfu of adenovirus containing FoxO1-ADA, while rest of the mice received the LacZ control. Blood glucose levels were assayed at day 5 after a 12-h fast. (*, P < 0.05; #, P < 0.05) **Fig. 7C** Liver nuclear extracts were assayed for FoxO1 and nucleophosmin by immunoblot **Fig. 7D** *G6pc*, *Pck1* and *Igfbp1* mRNA levels were assayed from livers of mice fasted for 12 h. (*, P < 0.05; **, P < 0.01; #, P < 0.05)

[0046] Figs. 8A-B. P-CaMKIIγ in obese liver. Immunoblots of liverextracts from **Fig. 8**A 10-wk/oWT or ob/ob mice and mice fed chow or high-calorie (DIO) diet for 20 wks; or **Fig. 8B** lean or morbidly obese humans. Densitometry quantification is shown for the mouse data in **Fig. 8A**; P<0.001.

[0047] Figs. 9A-I. CaMKIIγdeficiency reduces hyperinsulinemia and improves insulin and glucose metabolism in obese mice. **Fig. 9A-9B**, Plasma insulin in fed and fasted WT(n=10) and Camk2g-/-(n=7) DIO-obese male mice. **Fig. 9C**, Insulin tolerance test (ITT) in WT and

Camk2g-/-mice (n=5). **Fig. 9D**, Body weight. **Figs. 9E-G**, Plasma insulin, glucose tolerance test (GTT), and liver mRNAs for FoxO1 targets in DIO mice before or after treatment with adeno-LacZ control or adeno-K43ACaMKII (dominant-negative)(n = 6). In **Fig. 9E**, *, P<0.01 vs. pre-adeno and <0.005 vs. day 7 LacZ. In **Fig. 9G**, *, P<0.01. **Fig. 9H-I**, ob/ob obese mice were treated with the CaMKII inhibitor KN93 or its inactive homologue KN92. Blood glucose and expression of G6pcand Pck1in the liver were then assayed (duplicate values that varied by10%).

[0048] Figs. 10A-D. CaMKIIγdeficiency improves steatosis, dyslipidemia, and inflammation in obese mice. **Figs. 10A-B, 10D**, Liver TG content, plasma lipids, and liver TnfamRNA were assayed in WT (n = 10) and Camk2g-/-(n = 7) mice after DIO diet for 20 wks (G, *, P<0.005; H, *, P<0.05 **, P<0.01; I, *,P<0.05). **Fig. 10C**, Immunoblots of p-and total S6Kand Sortilin-1in livers from DIO mice before or after treatment with adeno-LacZ control or adeno-K43ACaMKII (dominant-negative).

[0049] Fig. 11. CaMKIIγdeficiency mediated suppression of atherosclerosis by complementary mechanisms.

[0050] Fig. 12. Role of CaMKIIγin UPR-induced Mφapoptosis

[0051] Fig. 13. CaMKIIγ haploinsufficency protects Mφs from ER stress-induced apoptosis.Mφs from WT, *Camk2g+/-*,or *Camk2g-/-*mice were treated under control or FC-loading (Chol) conditions and then assayed for apoptosis. *,#, @= P<0.01 *vs*. each other.

[0052] Fig. 14. Overview of key role of CaMKIIγin ER stress-induced Mφapoptosis in the setting of insulin resistance.

[0053] Figs. 15A-B. Mφinsulin resistance and ERK1/2 inhibition increase p-CaMKII. Immunoblots of pThr287-CaMKII in **Fig. 15A** WT, *Insr-/-*, and *ob/ob*Mφs ±FC loading or oxLDL and **Fig. 15B** WT Mφs ±4 h treatment with 5 μM U0126 ERK inhibitor.

[0054] Fig. 16. CaMKIIγ deficiency suppresses the UPR. Mφs from WT or *Camk2g-/-*mice were incubated with thapsigargin (Thaps) or under FC-loading conditions (Chol) and then immuno-blotted for UPR proteins, CaMKIIγ, and β-actin.

[0055] Fig. 17. p-CaMKI in marcrophages in advanced human coronary artery plaque. The area examined is depicted by the box in the Movat Pentachrome stained plaque specimen; NC, necrotic core; Bar, 2mm. The antibody reaction yields a reddish-brown product if positive and is light blue if negative. Bar, 100μm for enlarged images.

[0056] Fig. 18. Hepatocyte CaMKIIγ deficiency lessens hyperinsulinemia and subsequent dyslipidemia by decreasing gluconeogenesis (GNG) and hepatic glucose production (HGP) via a mechanism involving nuclear exclusion of FoxO1.

[0057] Figs. 19A-H. Fasting activates hepatic CaMKII, and CaMKII deficiency or inhibition suppresses GNG and nuclear FoxO1. **Fig. 19A**, WT mice were fed ad libitum or fasted for 12 h (upper); or fasted for 12 h and then re-fed for 4 h (lower). Liver extracts were immunoblotted for p-CaMKII, total CaMKII, and β-actin. **Fig. 19B**, Blood glucose in 12-h-fasted WT and Camk2g-/-mice, or in WT mice treated with adeno-LacZ or adeno-K43A-CaMKII(*, P< 0.001). **Fig. 19C**, Blood glucose in 18-h-fasted WT and Camk2g-/-mice challenged with 2 mg/kg pyruvate. **Fig. 19D**, Hepatic G6pc and Pck1mRNA in mice treated with adeno-LacZ or adeno-K43A-CaMKII (*, P< 0.01). **Fig. 19E**, Nuclear extracts from the livers from the experiment in B (left)were immunoblotted for FoxO1 and nucleophosmin (Np; nuclear loading control) and quantified by densitometry (*, P< 0.001). **Fig. 19F**, Primary HCs from WT or Camk2g-/-mice (left)—or WT HCs transduced with adeno-LacZ, constitutive-active CaMKII (T287D),or K43A-CaMKII (right)—were (co)-transducedwith adeno-GFP-FoxO1. FoxO1 subcellular localization in serum-starved cells was quantified by image analysis of immunofluorescence confocal microscopy data (*,#, P < 0.005). **Fig. 19G**, HCs from WT or Camk2g-/-mice were transduced with adeno-LacZ or FoxO1-ADA, then serum-starved, treated with forskolin, and assayed for G6pc mRNA (*, P< 0.01). **Fig. 19H**, Ser residues that were phosphorylated in serum starved WT HCs but not in CaMKII-deficient HCs; DBD = DNA-binding domain.

[0058] Figs. 20A-B. CaMKIIgamma is phosphorylated in the livers of WD-fed Ldlr-/- mice. Ldlr -/- mice on a chow or Western diet for 11 wks were assayed for blood glucose **Fig. 20A**; P<0.001) and hepatic p-CaMKII, total CaMKII, and beta-actin by immunoblot (**Fig. 20B)**.

[0059] Figs. 21A-21AV show a molecular link in glucagon- induced hepatic glucose production in fasting and obesity. **Fig. 21A** shows direct signaling and transcriptional regulation of glycogenolysis and gluconeogenesis during hepatic glucose production. **Fig. 21B** shows transcriptional control of hepatic glucose production (HGP). **Fig. 21C** shows a link between hepatic glucose production, fasting/glucagon, insulin resistance/DM2 and calcium and obesity. The link between an increase in calcium and HGP will be studied. **Figs. 21D-F** show a brief historical perspective of the link between calcium, hepatic glucose production and apoptosis. **Fig. 21D** is a diagram showing macrophage death in advanced atherosclerosis. **Fig. 21E** is a diagram that shows the calcium-sensing signaling molecule calcium/calmodulin-dependent

protein kinase II (CaMKII). **Fig. 21F** is a diagram that shows a mechanism of macrophage death in atherosclerosis. **FIG. 21G** is a diagram showing that, without being bound by theory, CaMKII-γ can regulate HGP. **Figs. 21H-L** show that glucagon activates CaMKII in cultured primary murine hepatocytes. **Fig. 21H** shows that glucagon activates CaMKII in primary murine hepatocytes. **Fig. 21I** shows the link between glucagon and activation of CaMKII. **Fig. 21J** shows the link between glucagon and an increase in calcium. **Fig. 21K** shows that glucagon, via IP3R, activates hepatic CaMKII *in vivo*. **Fig. 21L** shows that fasting activates and re-feeding suppresses hepatic CaMKII *in vivo*. **Fig. 21M** shows that CaMKII plays an important role in PKA-induced glucose production by primary hepatocytes. **Fig. 21N** shows that CamKII plays an important role in PKA and glucagon-mediated *Gcpc* and *Pck1* induction in primary hepatocytes. **Fig. 21O** is a diagram showing the link between fasting and glucagon, CaMKII and glucose production through a mechanism of gene induction. **Fig. 21P** is a diagram showing the transcriptional control of HGP. **Fig. 21Q** shows that FoxO1 is nuclear in serum-starved primary hepatocytes. **Fig. 21R** shows that CamKII plays an important role in FoxO1 nuclear localization in serum-starved primary hepatocytes. **Fig. 21S** shows that CaMKII plays an important role in FoxO1 nuclear localization in serum-starved primary hepatocytes. **Fig. 21T** shows a Western blot assay of nuclear extracts that shows that CaMKII plays an important role in FoxO1 nuclear localization in serum-starved primary hepatocytes. **Fig. 21U** is a diagram showing the transcriptional control of HGP, including the CRTC2-PGC1α pathway. **Fig. 21V** shows that CaMKII does not play a role in CRTC2 nuclear localization of *Pgc1* induction in primary hepatocytes. **Fig. 21W** is a diagram that shows the link between fasting and glucagon production, CaMKII, FoxO1 and glucose production. **Fig. 21X** presents *in vivo* evidence in fasting CaMKII$\gamma^{-/-}$ mice of a blood glucose and pyruvate challenge test. **Fig. 21Y** presents *in vivo* evidence in fasting CaMKII$\gamma^{-/-}$ mice of hepatic *Gcpc/Pck1* mRNA and nuclear FoxO1. **Fig. 21Z** presents *in vivo* evidence in fasting adeno-K43A CaMKII-transduced WT mice of blood glucose and glycogen stores. **Fig. 21AA** presents *in vivo* evidence in fasting adeno-K43A CaMKII-transduced WT mice of hepatic *Gcpc/Pck1* mRNA and nuclear FoxO1. **Fig. 21AB** presents *in vivo* evidence in glucagon-treated adeno-K43A CaMKII-transduced WT mice of hepatic *Gcpc* mRNA and glycogen stores. **Fig. 21AC** presents *in vivo* evidence in fasting adeno-CA CaMKII transduced WT mice of a blood glucose and pyruvate challenge test. **Fig. 21AD** presents *in vivo* evidence in fasting and fed adeno-CA CaMKII transduced WT mice of hepatic *Gcpc/Pck1* mRNA. **Fig. 21AE** presents *in vivo* evidence in fasting and fed adeno-CA CaMKII transduced WT mice of nuclear FoxO1. **Figs. 21AF-AH** show causative evidence that the suppressive effects of CaMKII deficiency on HGP are mediated through nuclear exclusion of

FoxO1. **Fig. 21AF** is a diagram that indicates that, without being bound by theory, the effects of CaMKII deficiency can be prevented by transduction with constitutively nuclear FoxO-ADA. **Fig. 21AG** shows G6pc/Pck1 in forskolin-treated primary hepatocytes. **Fig. 21AH** shows blood glucose levels and relative mRNA levels in blood glucose and FoxO1 genes in fasting adeno-K43A CaMKII-transduced mice. **Fig. 21AI** is a diagram that shows the relevance of CaMKII-mediated regulation of glucose production through FoxO1, to obesity and insulin resistance. **Fig. 21AJ** shows that CaMKIIγ is activated in the liver of obese mice. **Fig. 21AK** shows that CaMKIIγ is activated in the liver of obese humans. **Fig. 21AL** shows that CaMKIIγ deficiency reduces plasma insulin and improves insulin sensitivity in obese mice. **Fig. 21AM** shows that CaMKIIγ deficiency improves hepatic response to acute insulin in obese mice. **Figs. 21AN-AO** show that acute adenovirus-mediated inhibition of CaMKIIγ improves metabolic parameters in *ob/ob* mice. **Fig. 21AP** shows that acute adenovirus-mediated inhibition of CaMKIIγ decreases expression of *G6pc* and *Pck1* in livers of *ob/ob* mice. **Fig. 21AQ** shows that acute adenovirus-mediated inhibition of CaMKIIγ decreases nuclear FoxO1 in livers of *ob/ob* mice. **Fig. 21AR** shows that CaMKIIγ deficiency reduces fatty liver in obese mice. **Fig. 21AS** shows that CaMKII inhibition decreases hepatic lipogenic gene expression in *ob/ob* mice. **Fig. 21AT** shows that CaMKIIγ deficiency partially reduces plasma cholesterol and triglycerides in obese mice. **Fig. 21AU** shows that CaMKIIγ deficiency reduces heatic TNFα in obese mice. **Fig. 21AV** shows a working model of the regulation of glucagon-induced glucose production. Future studies include, but are not limited to, a direct assay of HGP and glucose utilization (clamp study), the mechanism of CaMKII regulation of nuclear FoxO1, how CaMKII deficiency can affect hepatic ER stress and *vice-versa*, what effect does CaMKII deficiency have on insulin sensitivity, whether CaMKII in liver macrophages plays a role in obesity, the effect of CaMKII on atherosclerosis and the therapeutic potential of CaMKII.

[0060] Figs. 22A-E. CaMKII regulates glucose production and hepatic *G6Pc* and *Pck1* expression in primary HCs. Fig. 22A RNA from HCs from 3 WT and 3 *Camk2g*$^{-/-}$ mice and mouse brain from a WT mouse were probed for the indicated *Camk2* isoform mRNAs by RT-PCR. **Fig. 22B** HCs from WT and *Camk2g*$^{-/-}$ mice were serum-depleted overnight and then incubated with forskolin (10 μm) for 14 h in serum- and glucose-free media, and then glucose in the medium was assayed (**P < 0.01 vs. WT in each group; mean ± S.E.M.). **Fig. 22C** HCs from WT mice were transduced with adenoviral vectors expressing LacZ, CA-CaMKII, or KD-CaMKII at an MOI of 20 and then assayed for glucose production as in **Fig. 22B** (*P < 0.05 and **P < 0.01 vs. LacZ in each group; mean ± S.E.M.). **Fig. 22D-22E** HCs similar to those in **Fig.**

22B and **Fig. 22C** were serum-depleted overnight and then incubated for 5 h with 10 μM forskolin or 100 nM glucagon in serum-free media, as indicated. RNA was assayed for *G6pc* and *Pck1* mRNA by RT-qPCR (*P < 0.05 and **P < 0.01 vs. LacZ or WT in each group; mean ± S.E.M.).

[0061] Figs. 23A-G. CaMKIIγ deficiency or acute inhibition *in vivo* decreases blood glucose and hepatic *G6pc* and *Pck1*. Fig. 23A Blood glucose of 12-h-fasted 8-wk/o WT and *Camk2g*$^{-/-}$ mice (*P < 0.05). **Fig. 23B** As in **Fig. 23**A, but the mice were fasted for 18 h and then challenged with 2 mg kg^{-1} pyruvate (**Fig. 23B**). (*P < 0.05; **P < 0.01; ***P < 0.005; mean ± S.E.M.). **Fig. 23C** Liver *G6pc* and *Pck1* mRNA in 12-h-fasted WT and *Camk2g*$^{-/-}$ mice (**P < 0.01; ***P < 0.001; mean ± S.E.M.). **Fig. 23D** WT and *Camk2g*$^{-/-}$ mice were injected i.p. with glucagon (200 μg kg^{-1}) and sacrificed 30 min later. Liver *G6pc* mRNA was assayed (*P < 0.05; mean ± S.E.M.). **Figs. 23E-G** 9-wk/o WT mice were administered 1.5 x 10^{9} pfu of adeno-LacZ or KD-CaMKII, and 5 days later the following parameters were assayed in 12-h-fasted mice: **Fig. 23E**, blood glucose (***P < 0.001; mean ± S.E.M.); **Fig. 23F**, liver *G6pc* and *Pck1* mRNA (**P < 0.01; ***P < 0.001; mean ± S.E.M.); and **Fig. 23G**, liver glycogen content and PAS-positive cells (**P < 0.01; mean ± S.E.M.). Panel G also shows liver glycogen content in fasted WT and *Camk2g*$^{-/-}$ mice (*P < 0.05; mean ± S.E.M.). For all panels, n = 5/group except panel D, where n = 4/group.

[0062] Figs. 24A-D. CaMKII regulates hepatic FoxO1 subcellular localization. Fig. 24A HCs from WT and *Camk2g*$^{-/-}$ mice were transduced with an adenovirus expressing murine GFP-FoxO1 at an MOI of 2. Cells were serum-depleted overnight and then incubated for 5 h in serum-free media. FoxO1 subcellular localization was assessed by indirect immunofluorescence. Bar, 10 μm. Data are quantified in the right panel. ($^{\#}$P < 0.0005; mean ± S.E.M.). **Fig. 24B** HCs were transduced with adenoviral vectors expressing LacZ, CA-CaMKII, or KD-CaMKII at an MOI of 20 and then transduced 4 h later with adeno-GFP-FoxO1, followed by fluorescence microscopy and quantification as in **Fig. 24A** ($^{\#}$P < 0.005 vs. LacZ; mean ± S.E.M.). Bar, 5 μm. **Fig. 24C** HCs were transduced with adeno-LacZ or CA-CaMKII and then adeno-GFP-FoxO1 as in **Fig. 24B**. After incubation in serum-depleted medium o.n. and then serum-free medium for 5 h, the cells were treated with 100 nM insulin for the indicated times. FoxO1 subcellular localization was quantified as in **Fig. 24B** (*P < 0.005 vs. LacZ in each group; mean ± S.E.M.). **Fig. 24D** Nuclear FoxO1 and nucleophosmin were probed by immunoblot in livers from fasted WT mice, *Camk2g*$^{-/-}$ mice, or WT mice treated with adeno-LacZ or KD-CaMKII; from fed WT mice treated with adeno-LacZ or CA-CaMKII; or from fed

WT mice treated for 30 min with 200 μg kg-1 body weight of glucagon i.p. For the glucagon experiment, the average FoxO:Np densitometric ratio values are in the graph (*P = 0.029; blemishes in lanes 6 and 8 were excluded from the densitometry analysis).

[0063] Figs. 25A-E. Impairment of glucose metabolism by CaMKII inhibition is rescued by transduction with constitutively nuclear FoxO1-ADA. **Fig. 25A** HCs from wild-type or L-FoxO1 knockout mice were transduced with adeno-LacZ or CA-CaMKII. The cells were serum-depleted overnight and then incubated for 5 h in the absence or presence of forskolin (10 μm) in serum-free media. RNA was assayed for *G6pc* mRNA (*P < 0.001; mean ± S.E.M.). **Fig. 25B** HCs from WT and *Camk2g*$^{-/-}$ mice were administered adeno-LacZ or FoxO1-ADA at an MOI of 0.2. Cells were serum-depleted overnight and then incubated for 5 h with 10 μM forskolin in serum-free media. RNA was assayed for *G6pc* and *Pck1* mRNA (**P < 0.01 vs. WT groups; $^{\#}$P < 0.05 and $^{\#\#}$P < 0.01 vs. *Camk2g*$^{-/-}$/LacZ group; mean ± S.E.M.). Inset, the nuclei from a parallel set of cells were probed for FoxO1 and nucleophosmin by immunoblot; the average densitometric ratio appears below each pair of lanes. **Fig. 25C-E** 8-wk/o WT mice were administered adeno-LacZ or KD-CaMKII, and then one day later, half of the adeno-KD-CaMKII mice received adeno-FoxO1-ADA, while the other half received adeno-LacZ control. Blood glucose levels were assayed at day 5 after a 12-h fast (*P < 0.05 vs. LacZ/LacZ; $^{\#}$P < 0.05 vs. KD/LacZ; n = 5/group; mean ± S.E.M.), and liver was assayed for nuclear FoxO1 protein (**P < 0.01 vs. KD/LacZ; mean ± S.E.M.); *G6pc*, *Pck1* and *Igfbp1* mRNA (*P < 0.05 and **P < 0.01 vs. LacZ/LacZ; $^{\#}$P < 0.05 vs. KD/LacZ; n = 3/group; mean ± S.E.M.). The inset to panel E shows the level of hemagglutinin (HA)-tagged KD-CaMKII protein (anti-HA immunoblot).

[0064] Figs. 26A-C. The role of non-AKT-phospho-sites of FoxO1 in CaMKII-mediated FoxO1 nuclear localization. Fig. 26A HCs from WT and *Camk2g*$^{-/-}$ mice were transduced with adeno-FLAG-FoxO1 at an MOI of 2. Cells were serum-depleted overnight and then incubated for 5 h in serum-free media. FoxO1 was immunopurified using anti-FLAG, followed by reduction, alkylation, and proteolytic digestion. Phosphorylated peptides were enriched by TiO_2 chromatography and then analyzed by LC-MS/MS as described in Experimental Procedures. The table shows spectral count number, Debunker score, and Ascore of phosphorylated peptides in KO and WT samples; Δ in the peptide sequence indicates the phosphorylation site. The cut-off values for spectral count #, Debunker score, and Ascore are set at 5, 0.5 and 12 respectively. The spectra of peptides with scores that are below these values (italics) were checked manually to eliminate uncertain phosphorylation sites (**Figs. 31A-B** for WT peptides 4 and 5; and http://fields.scripps.edu/published/foxo1_Tabas_2012/, incorporated by reference in their

entireties, for KO peptides 7, 10, and 11). The KO/WT ratio of spectral counts was calculated only for peptides with a combined spectral count in KO and WT > 10. **Fig. 26B** HCs from L-FoxO1 mice were transfected with expression plasmids encoding murine Flag-FoxO1 or Flag-7A-FoxO1 mutant. After 48 h, the cells were serum-depleted overnight and then incubated with glucagon (100 nm) for 4 h in serum-free media. Nuclear extracts were assayed by immunoblot for Flag and nucleophosmin (nuclear loading control), and RNA from a parallel set of cells was probed for Foxo1 mRNA by RT-qPCR. Densitometric quantification of the mRNA and immunoblot data is shown in the graph (*P < 0.005; mean ± S.E.M.) **Fig. 26C** Similar to **Fig. 26B**, except the HCs were transduced with adeno-LacZ or CA-CaMKII one day after the transfection with the WT or mutant FoxO1 plasmids (*P = 0.003; mean ± S.E.M.).

[0065] Figs. 27A-D. The role of CaMKII in hepatic glucose metabolism in obesity Fig. 27A Liver extracts from 10-wk/o WT or *ob/ob* mice, or WT mice fed a chow or high-calorie diet for 20 wks (diet-induced obesity; DIO), were probed for p- and total CaMKIIγ and β-actin by immunoblot. Densitometric quantification is shown in the bar graph (***P < 0.001; mean ± S.E.M.). Antibody specificity is shown by the absence of p- and total CaMKIIγ bands in liver extracts from DIO *Camk2g*$^{-/-}$ mice. **Figs. 27B-D** Fasting blood glucose, blood glucose after pyruvate challenge, and liver *G6pc*, *Pck1* and *Igfbp1* mRNA in *ob/ob* mice before or after treatment with adeno-LacZ or KD-CaMKII (n = 5/group; *P < 0.05, **P < 0.01, ***P < 0.005, and ****P < 0.001 vs. LacZ; mean ± S.E.M.).

[0066] Figs. 28A-G. (Related to Figs. 1 and 22). Fig. 28A Mice were injected i.p. with the indicated doses of glucagon and sacrificed 15 min later. The livers were probed for phospho- and total CaMKII and β-actin by immunoblot. **Fig. 28B** Mice (n= 5/group) were fed ad libitum or fasted for 12 h and then plasma was assayed for glucagon (*P = 0.007; mean ± S.E.M.). **Fig. 28C** Mice were injected i.p. with 10 pmol g^{-1} xestospongin C (XesC) or vehicle control each day for 4 days and then fed ad libitum or fasted for 12 h. Liver phospho-CaMKII, total CaMKII, and β-actin levels were assayed by immunoblot. **Fig. 28D** Immunoblot of CaMKII in HCs transduced with adeno-LacZ vs. CA-CaMKII. **Fig. 28E** HCs were transduced with adeno-LacZ or KD-CaMKII. Cells were serum-depleted overnight and then assayed for CaMKII activity (*P < 0.005; mean ± S.E.M.). **Fig. 28F** *G6pc* and *Pck1* mRNA levels in untreated HCs from WT vs. *Camk2g*$^{-/-}$ mice (*P 0.05). In comparison to the mRNA levels in forskolin-treated WT HCs in **Fig. 22D**, the levels of *G6pc* and *Pck1* in untreated WT HCs was ~165-fold and ~2500-fold lower, respectively. **Fig. 28G** HCs from *Camk2g*$^{-/-}$ mice were transduced with adeno-LacZ or KD-CaMKII as in **Fig. 28D**, incubated for 14 h in media ± forskolin, and then assayed for *G6pc*

mRNA (*P < 0.05 vs. the other 2 groups; mean ± S.E.M.). The black circle represents the *G6pc* mRNA value for forskolin-treated, adeno-LacZ WT HCs (1.99 ± 0.3; *P < 0.001 vs. *Camk2g*$^{-/-}$ value; mean ± S.E.M.).

[0067] Figs. 29A-E. Related to Fig. 23. Fig. 29A Fasting glucagon and insulin levels were assayed in the plasma of WT and *Camk2g*$^{-/-}$ mice and in WT mice treated with adeno-LacZ or KD-CaMKII (n = 5/group); n.s., non-significant. **Fig. 29B** CaMKII was immunoprecipitated (IP) from the livers of mice treated with adeno-LacZ or KD-CaMKII and then assayed for CaMKII activity. The data in the **inset** validates the CaMKII assay by showing that kinase activity was negligible in the livers of *Camk2g*$^{-/-}$ mice and when non-immune IgG was used in the CaMKII IP part of the protocol (*P < 0.006; mean ± S.E.M.). **Fig. 29C-D** WT mice were treated with adeno-LacZ or CA-CaMKII (n = 5/group). After 5 days, the mice were assayed for blood glucose before or after pyruvate challenge, and then, after a period of ad libitum feeding, the mice were sacrificed, and the livers were assayed for *G6pc* and *Pck1* mRNA (*P < 0.05; **P < 0.01; ***, P < 0.005; mean ± S.E.M.). **Fig. 29E** Mice were treated with adeno-LacZ or CA-CaMKII (n = 5/group). After 5 days, the mice were fasted overnight and then the livers were assayed for glycogen content (*P < 0.05; mean ± S.E.M.).

[0068] Figs. 30A-D. Related to Figs. 24-25. Fig. 30A HCs were transduced with adeno-LacZ or CA-CaMKII at an MOI of 1 and transduced 4h later with adeno-GFP-FoxO1. After incubation in serum-depleted medium o.n. and then serum-free medium for 5 h, the cells were treated with 100 nM insulin for 15 min. FoxO1 subcellular localization was quantified. (*P < 0.005; mean ± S.E.M.). *Inset*, the lysate from a parallel set of cells were probed for CaMKII and β-actin by immunoblot. **Fig. 30B** FAO hepatocytes were transfected with luciferase fusion constructs encoding nucleotides -1227 to +57 of the G6PC promoter containing either intact (-1227 WT) or mutated FoxO binding sites (-1227 Mut). Relative luciferase activity was measured following a 16-h incubation in the absence or presence of 0.1 mM cAMP and 1 μM dexamethasone. *P < 0.001 vs. all other groups; **P = 0.009 vs. untreated Mut (mean ± S.E.M.). **Fig. 30C** Nuclei from the livers of the adeno-LacZ and KD-CaMKII-treated mice were probed by immunoblot for CREB or Crtc2 and nucleophosmin (Np), and cell extracts of glucagon-treated HCs from WT or *Camk2g*$^{-/-}$ mice were probed for p-CREB and β-actin. **Fig. 30D** Nuclei from glucagon-treated HCs from WT or *Camk2g*$^{-/-}$ mice were probed for Crtc2 and nucleophosmin (Np).

[0069] Figs. 31A-F. Related to Fig. 26—FoxO1 phosphorylation. Fig. 31A Extracts of liver from fasted WT and *Camk2g*$^{-/-}$ mice were immunoblotted for phospho-T24-, 253-, and 316-

FoxO1 and β-actin. **Fig. 31B** Extracts of liver from fasted adeno-FoxO1-treated WT and *Camk2g*$^{-/-}$ mice immunoblotted for acetylated FoxO1 (Ac-FoxO1) and β-actin. Average Ac-FoxO:β-actin densitometric ratio values appear below the blot; n.s., non-significant. **Fig. 31C** Murine FoxO1 sequence, with identified phospho-sites in bold font; summary of data for key residues relevant to **Figs. 26, 31F, and 32** are shown below in the bullet list below the sequence. **Fig. 31D** MS/MS spectra and b, y ion table of phosphorylated peptide #4 from *Camk2g*$^{-/-}$ HCs. **Fig. 31E** MS/MS spectra and b, y ion table of phosphorylated peptide #5 from *Camk2g*$^{-/-}$ HCs. **Fig. 31F** Similar to the experiment in **Fig. 26B**, except the L-Foxo1 HCs were transfected with FLAG-9A-FoxO1 mutant instead of FLAG-7A-Foxo1 (*P = 0.006; **P = 0.02; mean ±S.E.M.).

[0070] Fig. 32A-E. Related to Fig. 26—p38. Fig. 32A Mice were fed ad libitum or fasted for 12 h. Liver phospho-p38, total p38, p-MK2, total MK-2, and β-actin were assayed by immunoblot. **Fig. 32B** WT HCs were serum-depleted overnight and then incubated for 5 h with 100 nm glucagon in serum-free media with or without SB202190; or glucagon was added to HCs from *P38a*$^{fl/fl}$ mice treated with adeno-LacZ (control) or adeno-Cre. RNA was assayed for *G6pc* and *Pck1* mRNA (*P < 0.05; mean ± S.E.M.). **Fig. 32C** HCs from fasting WT and *Camk2g*$^{-/-}$ mice were assayed for p-p38, total p38, and β-actin. **Fig. 32D** WT mice were injected i.p. with 12.5 mg kg^{-1} body weight of p38 inhibitor (SB202190) or vehicle control. 12 h later, the mice were injected with an additional dose of SB202190 and fasted overnight. The livers were assayed for nuclear FoxO1, nucleophosmin, p-MK2, total MK-2, and β-actin. The correlation between nuclear FoxO1 and p-MK2 band intensities is shown in the graph. **Fig. 32E** Adeno-GFP-FoxO1-transduced HCs were serum-depleted overnight, and then incubated for 5 h in serum-free media with SB202190 or vehicle (Veh) control. FoxO1 localization was assessed by indirect immunofluorescence (Bar, 10 μm; *P < 0.05; mean ± S.E.M.).

[0071] Figs. 33A-D. Calcineurin promotes CRTC2 activation during fasting. Fig. 33A. Effect of Ser/Thr phosphatase inhibitors (okadaic acid (OA), cyclosporin A (CsA) on CRTC2 dephosphorylation and CRE-luciferase (luc) reporter activation (*P < 0.001; n=3). **Fig. 33B.** Effect of glucagon (Gcg) on dephosphorylation (left) and activity (right) of wild-type (WT) and calcineurin-defective (ΔCalna) CRTC2 in hepatocytes (*P < 0.001; n=3). **Fig. 33C.** Effect of calcineurin A over-expression (left) or knockdown (right) on CRTC2 dephosphorylation (top), CRE-luc reporter activity (middle, *P < 0.001; n=3), and glucose output (bottom, *P < 0.001; n=3) from hepatocytes. **Fig. 33D.** Effect of hepatic calcineurin over-expression (left) or knockdown (right) on CRE-luc activity, gluconeogenic gene (Pck1, G6pc) expression, and blood

glucose concentrations in 6-8 hour fasted mice (*P < 0.01; n=5). For this and other figures, data are shown as mean ± s.e.m.

[0072] Figs. 34A-D. Glucagon stimulates CRTC2 dephosphorylation via activation of InsP3 receptors. **Fig. 34A**. Effect of glucagon (Gcg) on calcium mobilization in hepatocytes by fluorescence imaging. Calcium mobilization and calcineurin activation following knockdown of all three InsP3R family members shown (*P < 0.001; n=3). **Fig. 34B**. Effect of calcium chelator (BAPTA) on CRTC2 dephosphorylation and CRE-luc activation (*P < 0.001; n=3). **Fig. 34C**. Effect of InsP3R depletion on CRTC2 dephosphorylation, CRE-luc activity, and glucose output from hepatocytes (*P < 0.001; n=3). **Fig. 34D**. Effect of hepatic InsP3R knockdown on CRE-luc activity, blood glucose, and gluconeogenic gene expression (*P < 0.01; n=5).

[0073] Figs. 35A-F. Glucagon stimulates CRTC2 activity via PKA-dependent phosphorylation of InsP3Rs. **Figs. 35A. and B**. Immunoblots of InsP3R1 immunoprecipitates using phospho-PKA substrate antiserum to show effect of H89 (A) and Ala mutations (B) at one or both (DM) PKA consensus sites (Ser1589, Ser1756) on InsP3R1 phosphorylation in hepatocytes exposed to glucagon (Gcg). Effect of wild-type and PKA-mutant InsP3R1 on calcium mobilization in response to Gcg (B) shown (*P < 0.001; n=3). **Figs. 35C. and D**. Effect of wild-type or PKA-defective InsP3R1 (DM) on calcineurin (Calna) activation (C) and CRTC2 dephosphorylation (C), as well as CRE-luc activation (D) and glucose output (D) from hepatocytes (*P < 0.001; n=3). **Fig. 35E**. Effect of wild-type and PKA-defective InsP3R1 on hepatic CRE-luc activity, fasting blood glucose, and gluconeogenic gene expression (*G6pc, Pck1*) (*P < 0.01 versus wild-type; n=5). **Fig. 35F**. Co-immunoprecipitation of CRTC2 with InsP3R1 in primary hepatocytes. Exposure to glucagon (100 nM; 15 minutes) indicated. Input levels of CRTC2 and InsP3R1 in nuclear (Nu) and post-nuclear (p/Nu) supernatant fractions shown.

[0074] Figs. 36A-C. InsP3R activity is upregulated in diabetes. **Fig. 36A**. Hepatic CRE-luc and calcineurin activity in lean and *db/db* mice (*P < 0.001; n=5). **Fig. 36B**. Immunoblots showing relative amounts and phosphorylation of InsP3R family members in livers of ad libitum fed lean, *db/db*, or *ob/ob* mice. InsP3R phosphorylation at PKA or AKT sites indicated. **Fig. 36C**. Effect of RNAi-mediated depletion of InsP3Rs or calcineurin A on CRE-luc activity, gluconeogenic gene expression, and hepatic glucose production in *db/db* mice, determined by pyruvate tolerance testing (*P < 0.01; n=5).

[0075] Figs. 37A-E. Glucagon stimulates CRTC2 activity in hepatocytes via a calcineurin-dependent mechanism. **Fig. 37A**. Effect of Ser/Thr phosphatase inhibitors (okadaic acid (OA), cyclosporin A (CsA)) on CRTC2 localization in primary mouse hepatocytes exposed to glucagon (Gcg). Scale bar, 5 μm. **Fig. 37B**. Effect of calcineurin inhibitors (calcineurin autoinhibitory peptide (CAP), CN585) and PP1/ PP2A inhibitor (calyculin A) on CRE-luciferase reporter activity in primary hepatocytes (*$P < 0.001$; n=3). **Figs. 37C. and D**. Effect of wild-type and phosphorylation-defective (S171A, S275A) active CRTC2 mutants on CRE-luc activity (C) (*$P < 0.001$; n=3) and CRTC2 cellular localization **Fig. 37D**. Glucagon treatment indicated. Scale bar, 5 μm. **Fig. 37E**. Effect of CsA on CRE-luc activity in cells expressing wild-type or constitutively active (S171A/S275A) mutant CRTC2 (*$P < 0.001$; n=3). *NS,* no statistical difference.

[0076] Figs. 38A-D. Calcineurin modulates CRTC2 activity in hepatocytes. Fig. 38A. Analysis of calcineurin binding sites in CRTC2. Different CRTC2 polypeptides and amino acid substitutions/deletions indicated in schematic. Immunoblot of flag-tagged CRTC2 recovered from IPs of HA-tagged calcineurin (Calna) in HEK293T cells shown. **Fig. 38B**. Co-immunoprecipitation assay showing relative binding of wild-type and mutant CRTC2 polypeptides to calcineurin in cultures of primary hepatocytes. Cells infected with adenovirus encoding wild-type or calcineurin-defective (ΔCalna) Flag-tagged CRTC2 plus HA-tagged calcineurin A (Calna) indicated. Recovery of CRTC2 polypeptides from HA-Calcineurin IPs shown. **Fig. 38C**. Nuclear shuttling of wild-type (WT) and calcineurin-defective (ΔCalna) CRTC2 in hepatocytes exposed to glucagon. Scale bar, 5 μm. **Fig. 38D**. Effect of Calna over-expression (O.E.) or depletion by RNAi-mediated knockdown on downstream PKA signaling in hepatocytes using phospho-PKA substrate antibody (anti-RRXpS/T).

[0077] Figs. 39A-B. Calcineurin regulates CRTC2 activity in liver. Effect of calcineurin (Calna) over-expression (A) or RNAi-mediated depletion (B) on mRNA amounts for fasting-inducible CREB target genes (*Nr4a1, Nr4a2, Pgc1α)* in livers of fasted mice (*$P < 0.01$; n=5). Immunoblot showing relative calcineurin protein amounts in livers from calcineurin over-expressing or knockdown mice compared to controls.

[0078] Figs. 40A-C. Calcineurin and InsP3Rs regulate gluconeogenesis by modulating CRTC2 activity. **Figs. 40A-B**. Effect of adenovirally encoded *Crtc2* RNAi on blood glucose concentrations (A) and hepatic gene expression (B), in fasted mice expressing adenoviraly encoded calcineurin or InsP3R1, as indicated. n=4. *NS,* no statistical difference. **Fig. 40C**.

Immunoblot showing hepatic protein amounts for CRTC2, Calcineurin (Calna), and InsP3R1 in mice characterized in panels A and B.

[0079] Figs. 41A-C. Glucagon stimulates calcium mobilization in hepatocytes. Figs. 41A. and B. Effect of glucagon (Gcg) and PKA inhibitor H89 on calcium mobilization in primary hepatocytes by fluorescence imaging (*$P < 0.001$; n=3). **Fig. 41C**. InsP3R1 peptides identified by MS analysis of immunoprecipitates prepared with phospho-PKA substrate antiserum on lysates from primary hepatocytes exposed to glucagon (Gcg).

[0080] Figs. 42A-E. InsP3 Receptors are required for CRTC2 activation in response to cAMP agonists. Fig. 42A, B. Effect of Xestospongin C (Xc) on calcium mobilization and CRTC2 dephosphorylation (A) and on mRNA amounts for glucose-6-phosphatase (*G6pc*) and PEPCK (*Pck1*) (B) in primary hepatocytes exposed to Forskolin (FSK) (*$P < 0.001$; n=3). **Fig. 42C-E**. Effects of glucagon on calcium mobilization and calcineurin activation (C) (*$P < 0.001$; n=4) as well as CRE-luc reporter activation and glucose secretion (D) (*$P < 0.001$; n=4) and CRTC2 dephosphorylation (E) in primary hepatocytes from InsP3R2 knockout mice and control littermates. **Fig. 42E**. Bar graph on right shows relative dephosphorylation of CRTC2 in wild-type and InsP3R2 knockout hepatocytes exposed to glucagon, determined by densitometry (*$P < 0.01$; n=3).

[0081] Figs. 43A-E. InsP3Rs modulate hepatic gluconeogenesis. Fig. 43A. Effect of RNAi-mediated depletion of all three InsP3R family members on calcineurin activation in fasted livers (*$P < 0.001$; n=5). **Fig. 43B and C.** Effect of hepatic InsP3R knockdown on mRNA amounts for fasting inducible CREB target genes (B) and on InsP3R protein amounts (C) in livers of mice (*$P < 0.01$; n=5). **Fig. 43D. and E**. Hepatic gluconeogenesis, measured by pyruvate tolerance testing (D) (*$P < 0.02$; **$P < 0.01$; n=4) and gluconeogenic gene expression (E) (*$P < 0.01$; n=4) in wild-type and InsP3R2 knockout mice. Immunoblot shows InsP3R2 protein amounts in hepatic extracts from control and InsP3R2 knockout mice.

[0082] Figs. 44A-D. Glucagon modulates InsP3R activity via PKA-mediated phosphorylation. Fig. 44A. Immunoblots showing effect of glucagon (Gcg) on InsP3R phosphorylation, using phospho-PKA substrate antiserum on immunoprecipitates of InsP3Rs prepared from primary hepatocytes. **Fig. 44B**. Immunoblots showing phosphorylation of InsP3R at PKA or AKT sites in livers of 6-8 hour fasted or 2 hour-refed mouse livers. **Figs. 44C. and D**. Effect of wild-type and PKA-defective (DM) InsP3R1 on calcineurin (Calna) activation (C, *$P < 0.001$, n=5) and CREB target genes (D, *$P < 0.01$, n=5) in fasted livers. Right, immunoblot

showing relative InsP3R protein amounts in livers of mice injected with adenovirus encoding wild-type or PKA-defective (DM) InsP3R1.

[0083] Figs. 45A-I. Association of InsP3Rs with CRTC2 in hepatocytes. Fig. 45A. InsP3R1 peptides recovered from anti-CRTC2 immunoprecipitates by MS analysis. **Fig. 45B**. Co-immunoprecipitation assay showing amounts of Flag-tagged CRTC2 recovered from IPs of HA-tagged InsP3R1 in HEK293T cells. **Figs. 45C. and D**. Deletion analysis of regions in CRTC2 (C) and InsP3R1 (D) required for the CRTC2:InsP3R1 interaction. Interactioncompetent CRTC2 and InsP3R1 polypeptides indicated in each schematic (+). **Fig. 45E**. Effect of InsP3R depletion on CRTC2 protein amounts associated with ER enriched high density microsomes (HDM) as well as cytosolic (Cyto), and nuclear (Nucl) fractions in hepatocytes exposed to glucagon (Gcg). Relative amounts of ER-localized (GRP78), cytosolic (Tubulin), and nuclear (CREB) proteins in each fraction indicated. **Fig. 45F**. Effect of InsP3R depletion on CRTC2 localization in hepatocytes. Scale bar, 5 μm. **Figs. 45G.- I**. Cellular localization (G), phosphorylation state (H) and activity (I) of wild-type, InsP3R-defective (ΔCBD, 51-692 aa), and myristoylated CRTC2 mutant polypeptides (*$P < 0.001$; n=3). Scale bar, 5 μm.

[0084] Figs. 46A-D. Insulin down-regulates CRTC2 activity via the AKT-mediated phosphorylation of InsP3Rs. **Fig. 46A**. Left, immunoblots showing effect of insulin (INS) on InsP3R phosphorylation, using phospho-AKT substrate antiserum on immunoprecipitates of InsP3Rs prepared from primary hepatocytes. Bottom right, effect of insulin (INS) on phosphorylation of wild-type and mutant InsP3R1 containing an alanine substitution at the consensus AKT phosphorylation site (Ser2682). **Fig. 46B**. Effect of insulin on glucagon (Gcg)-induced calcium mobilization and calcineurin (Calna) activation in primary hepatocytes expressing wild-type or AKT-defective (S2682A) InsP3R1 (*$P < 0.001$; n=3). **Fig. 46C**. Effect of wild-type and AKT-defective (S2862A) InsP3R1 on CRTC2 dephosphorylation (top), CRE-luc reporter activation (bottom left), and glucose output (bottom right) from hepatocytes (*$P < 0.001$; n=3). **Fig. 46D**. Effect of wildtype and AKT-defective (S2682A) InsP3R1 on CRE-luc activity, blood glucose, and CREB target gene expression (*G6pc, Pck1, Nr4a1, Nr4a2, Pgc1α*) in fasting or refed mice (*$P < 0.01$; n=5). Bottom right, immunoblot showing relative InsP3R protein amounts in livers of mice injected with adenovirus encoding wild-type or AKT defective (S2682A) InsP3R1 (*$P < 0.01$; n=5).

[0085] Figs. 47A-C. Hepatic cAMP signaling and calcineurin activity are increased in obesity. Fig. 47A. and B. Hepatic CRE-luc reporter activity and calcineurin activity (A) as well as cAMP content (B) in lean, *db/db,* and *ob/ob* mice (*$P < 0.001$; n=5). **Fig. 47C**. Immunoblot

of InsP3R and calcineurin (Calna) proteins amounts in livers of mice injected with *Insp3r* or *Calna* RNAi adenovirus.

[0086] Fig. 48. Fasting and feeding pathways regulate CRTC2-dependent gluconeogenesis through antagonistic effects on InsP3R activity. Fasting signaling activates InsP3Rs via PKA-dependent phosphorylation, leading to increases in calcineurin (Calna) activity and in the subsequent dephosphorylation of CRTC2. By contrast, feeding inhibits InsP3R activity via AKT-dependent phosphorylation, thereby blocking the calcineurin-dependent dephosphorylation of CRTC2.

[0087] Figs. 49A-S. **The role of IP3R in forskolin/glucagon-induced G6Pase/PEPCK in cultured hepatocytes and in fasting blood glucose in vivo**. **Fig. 49 A,B**. FSK-Induced G6Pase and PEPCK is Calcium-Dependent. **Fig. 49C-D.** IP3R Activity is Required for Forskolin- and Glucagon-Induced G6Pase and PEPCK (FAO rat hepatocyte cell line). **Fig. 49E-F.** IP3R Activity is Required for Forskolin-Induced G6Pase and PEPCK (primary murine hepatocytes). **Fig. 49G-H.** IP3R1 Anti-Sense Oligonucleotide Suppresses Forskolin-Induced G6Pase and PEPCK (primary murine hepatocytes). **Fig. 49I**. Forskolin Activates IP3R Activity in FAO Hepatocytes (Inositol/ATP-Induced Calcium Release Assay). **Fig. 49J**. The PKA Inhibitor H89 Suppresses Forskolin-Induced IP3R Activation. **Fig. 49K-L**. IP3R Activity is Required for Forskolin-Induced PGC1α. **Fig. 49M-N**. Calcineurin Inhibitory Peptide Suppresses Forskolin-Induced G6Pase and PEPCK. **Fig. 49O.** IP3R Activity is Required for Forskolin-Induced Calcineurin Activation. **Fig. 49P-Q.** IP3R Activity is Required for Forskolin-Induced TORC2 Dephosphorylation and Nuclear Localization. **Fig. 49R.** TORC2 Transduction Partially Restores Forskolin-Induced G6Pase in IP3R-Inhibited Hepatocytes. **Fig. 49S**. Xestospongin C Treatment Reduces Fasting Glucose Levels in C57BL/6J Mice.

[0088] Figs. 50A-M. The role of IP3R in obesity/insulin resistance induced hyperglycemia and gluconeogenesis. **Fig. 50A**. Evidence that Xestospongin C Treatment of *ob/ob* Mice Inhibits IP3R Assay of IICR in Freshly Isolated Peritoneal Cavity Macrophages. Fig. **50B-E**. Inhibition of IP3R Reduces Fasting Blood Glucose Levels in *ob/ob Mice*. **Fig. 50F.** Inhibition of IP3R Reduces Hepatic Expression of PEPCK, G6Pase, and PGC1α in *ob/ob*. **Fig. 50G-I**. Inhibition of IP3R Reduces Expression of PEPCK, G6Pase, and PGC1α in Palmitate-Treated Hepatocytes as a Model of ER Stress and Insulin Resistance. **Fig. 50J-K.** Inhibition of IP3R Reduces Expression of G6Pase and PEPCK in Akt-Inhibited Hepatocytes as a Model of Insulin Resistance. **Fig. 50L.** IP3R Activity is Required for Forskolin-Induced TORC2 Dephosphorylation. **Fig. 50M.** IP3R Activation is Required for TORC2 Nuclear Retention.

[0089] Fig. 51. Diagram showing the IP3R pathway.

[0090] Fig. 52. CA-CaMKII does not increase nuclear FoxO1 in p38 deficient hepatocytes. Primary hepatocytes (HCs) from p38 $^{fl/fl}$ mice were transduced with adeno-LacZ or adeno-Cre. After 12h, half of the cells were transduced with adeno-CA-CaMKII while the other half was transduced with adeno-LacZ. Cells were incubated in serum-depleted medium o.n. and then serum-free medium for 5 h. Nuclear extracts were assayed by immunoblot for FoxO1 and nucleophosmin (Np).

[0091] Figs. 53A-B. MK-2 regulates hepatic FoxO1 subcellular localization and hepatic *G6Pc* and *Pck1*. Fig. 53A. Adeno-GFP-FoxO1-transduced HCs were serum-depleted overnight, and then incubated for 30 min in serum-free media with 2 uM MK-2 inhibitor or vehicle (Veh) control. FoxO1 localization was assessed by indirect immunofluorescence (Bar, 10 μm; *P < 0.005; mean ± S.E.M.). **Fig. 53B** WT HCs were serum-depleted overnight and then incubated for 5 h with 100 nm glucagon in serum-free media with or without MK-2 inhibitor. RNA was assayed for *G6pc* and *Pck1* mRNA (*P < 0.005; mean ± S.E.M.). MK-2 Inhibitor is from Calbiochem (cat#475864): 2-(2-Quinolin-3-ylpyridin-4-yl)-1,5,6,7-tetrahydro-4H-pyrrolo-[3,2-c]pyridin-4-one.

[0092] Fig. 54. **p38 deficiency suppresses tunicamycin induced UPR activation**. Tunicamycin was added to HCs from p38$^{fl/fl}$ mice transduced with adeno-LacZ control or adeno-Cre. Lysates were immunoblotted for p-PERK, PERK, CHOP, p58IPK and actin.

[0093] Fig. 55. p38 deficiency suppresses palmitate induced UPR activation. Palmitate was added to HCs from p38$^{fl/fl}$ mice transduced with adeno-LacZ control or adeno-Cre. Lysates were immunoblotted for p-PERK, CHOP and actin.

[0094] Fig. 56. **Acute insulin-induced p-AKT in HCs is enhanced by p38 deficiency**. As in **Fig. 55**, except the HCs were treated with 100 nM insulin for 5 min. Lysates were immunoblotted for p-Akt, Akt and actin.

[0095] Figs. 57A-E. Fig. 57A. Mice were fed ad libitum or fasted for 12 h. Liver phospho-p38, total p38, p-MK2, total MK-2, and β-actin were assayed by immunoblot. **Fig. 57B** WT HCs were serum-depleted overnight and then incubated for 5 h with 100 nm glucagon in serum-free media with or without SB202190; or glucagon was added to HCs from *P38a*fl/fl mice treated with adeno-LacZ (control) or adeno-Cre. RNA was assayed for *G6pc* and *Pck1* mRNA (*P < 0.05; mean ± S.E.M.). **Fig. 57C**. HCs from fasting WT and *Camk2g*-/- mice were assayed for p-p38, total p38, and β-actin. **Fig. 57D**. WT mice were injected i.p. with 12.5 mg kg-1 body

weight of p38 inhibitor (SB202190) or vehicle control. 12 h later, the mice were injected with an additional dose of SB202190 and fasted overnight. The livers were assayed for nuclear FoxO1, nucleophosmin, p-MK2, total MK-2, and β-actin. The correlation between nuclear FoxO1 and p-MK2 band intensities is shown in the graph. **Fig. 57E.** Adeno-GFP-FoxO1-transduced HCs were serum-depleted overnight, and then incubated for 5 h in serum-free media with SB202190 or vehicle (Veh) control. FoxO1 localization was assessed by indirect immunofluorescence (Bar, 10 μm; *P < 0.05; mean ± S.E.M.).

[0096] Fig. 58. p38 deficiency decreases tunicamycin-induced UPR activation. Primary hepatocytes from $p38\alpha^{flox/flox}$ mice were transduced at an MOI of 5 with control adeno-LacZ (Ad-LacZ) or adeno-Cre (Ad-Cre) to delete p38. 36 h later, the cells were treated with 0.5 ug/ml tunicamycin for 4 hours. Lysates were then immunoblotted for p-Perk, CHOP, Trb3, p58IPK and actin.

[0097] Fig. 59. p38 deficiency blunts palmitate-induced insulin resistance. Primary hepatocytes from $p38^{fl/fl}$ mice were transduced with adeno-LacZ (Ad-LacZ) or adeno-Cre (Ad-Cre) at an MOI of 5. 24 h later, cells were treated o/n with palmitate. After incubation in serum-free medium for 5 h with palmitate, the cells were treated with 100 nM insulin for 5 min. Lysates were then immunoblotted for p-Akt, Akt, actin, p-perk, CHOP and Trb3.

[0098] Fig. 60. CA-CaMKII does not increase nuclear FoxO1 in p38-deficient hepatocytes: Evidence that CaMKII and p38 are in the same signaling pathway. Primary hepatocytes from $p38^{fl/fl}$ mice were transduced o/n with adeno-LacZ (Ad-LacZ) or adeno-Cre (Ad-Cre) at an MOI of 5, followed by transduction with with adeno-LacZ or adeno-CA-CaMKII (CA) at an MOI of 1. After 12 h, the cells were serum-depleted overnight and then incubated for 4 h in serum-free media. Nuclear extracts were assayed by immunoblot for FoxO1 and nucleophosmin (nuclear loading control).

[0099] Fig. 61. Dominant negative (DN)-MK2 suppresses forskolin-induced *Pck1* and *G6Pc* induction. Primary hepatocytes from WT mice were transduced with adenoviral vectors expressing either LacZ or DN-MK2. Cells were then serum-depleted overnight and then incubated for 5 h with 2 μM forskolin in serum-free media. RNA was assayed for *G6p*c and *Pck1* mRNA by RT-qPCR. *, $P < 0.0001$

[00100] Fig. 62. DN-MK2 decreases nuclear FoxO1 levels. Primary hepatocytes from WT mice were transduced with adeno-LacZ (LacZ) or adeno-DN-MK2 (DN-MK2) at an MOI of 1. After 24 h, the cells were serum-depleted overnight and then incubated for 4 h in serum-free

media. Nuclear extracts were assayed by immunoblot for FoxO1 and nucleophosmin (nuclear loading control).

[00101] Fig. 63. Constitutively active (CA)-CaMKII does not increase nuclear FoxO1 in MK-2 inhibited hepatocytes: Evidence that CaMKII and MK2 are in the same signaling pathway. Primary hepatocytes from WT mice were transduced o/n with adeno-LacZ (LacZ) or adeno-DN-MK2 (DN-MK2) at an MOI of 1, followed by transduction with with adeno-LacZ or adeno-CA-CaMKII (CA-CaMKII) at an MOI of 1. After 12 h, the cells were serum-depleted overnight and then incubated for 4 h in serum-free media. Nuclear extracts were assayed by immunoblot for FoxO1 and nucleophosmin (nuclear loading control).

[00102] Fig. 64. Hepatic MK-2 inhibition does not change body weight or food intake. 10 week-old *ob/ob* mice were injected with 1 x 10^9 pfu of adenovirus containing either control LacZ (n=5) or DN-MK-2 (n=5)

[00103] Fig. 65. Hepatic MK-2 inhibition improves hyperglycemia in obese mice. 10 week-old *ob/ob* mice were injected with 1 x 10^9 pfu of adenovirus containing either control LacZ (n=5) or DN-MK-2 (n=5). Blood glucose levels on day 3 after 6 h fasting ; ** = $p<0.05$

[00104] Fig. 66. Hepatic MK-2 inhibition improves hyperglycemia in obese mice (absolute values). Glucose tolerance test (0.5 g/kg i.p.) was performed on day 5 (n=5 for each group) * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$

[00105] Fig. 67. Hepatic MK-2 inhibition improves hyperglycemia in obese mice (relative to time = 0). Glucose tolerance test (0.5 g/kg i.p.) was performed on day 5 (n=5 for each group) * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$

[00106] Fig. 68. Hepatic MK-2 inhibition improves hyperinsulinemia in obese mice. 10 week-old *ob/ob* mice were injected with 1 x 10^9 pfu of adenovirus containing either control LacZ (n=5) or DN-MK-2 (n=5). Serum insulin levels on day 8 after 6 h fasting ; ** = $p<0.05$

[00107] Fig. 69. MK-2 inhibitor suppresses forskolin-induced *Pck1* and *G6Pc* induction in primary hepatocytes. Primary hepatocytes from WT mice were serum-depleted overnight and then pre-treated with 300 nM MK-2 inhibitor (compound 28) or vehicle for 1 h. Cells were then treated with 2 μM forskolin in serum-free media with or without the inhibitor for 4 h. RNA was assayed for *G6pc* and *Pck1* mRNA by RT-qPCR. *, $P < 0.0001$.

[00108] Figs. 70-76 show MK-2 inhibitor studies in obese mice (Compound 28 from Huang et al.*). - MK-2 inhibitor (10 μg/mouse; 0.2 mg/kg) or vehicle control was administered to *ob/ob* mice once daily by intraperitoneal injections (in 150 ul total volume). *See* Huang X,

Zhu X, Chen X, Zhou W, Xiao D, Degrado S, Aslanian R, Fossetta J, Lundell D, Tian F, Trivedi P, Palani A. *Bioorg Med Chem Lett.* 2012 Jan 1;22(1):65-70, "*A three-step protocol for lead optimization: quick identification of key conformational features and functional groups in the SAR studies of non-ATP competitive MK2 (MAPKAPK2) inhibitors*". Department of Medicinal Chemistry, Merck Research Laboratories, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA.

[00109] Fig. 70 shows that MK-2 inhibitor does not change body weight. 10 week-old *ob/ob* mice were administered with MK-2 inhibitor once daily by intraperitoneal injections (150 ul total volume).

[00110] Fig. 71 shows that MK-2 inhibitor does not change food intake. 10 week-old *ob/ob* mice were administered with MK-2 inhibitor once daily by intraperitoneal injections (150 ul total volume).

[00111] Fig. 72 shows that MK-2 inhibitor decreases fasting blood glucose. 10 week-old *ob/ob* mice were administered with MK-2 inhibitor once daily by intraperitoneal injections (150 ul total volume). 6h fasting blood glucose levels on day 5. *, $p < 0.05$

[00112] Fig. 73 shows that MK-2 inhibitor improves hyperglycemia in obese mice. 10 week-old *ob/ob* mice were administered with MK-2 inhibitor once daily by intraperitoneal injections (150 ul total volume). Glucose tolerance test (0.5 g/kg i.p.) was performed on day 7. *, $p < 0.05$

[00113] Fig. 74 shows that MK-2 inhibitor improves insulin resistance in obese mice. 10 week-old *ob/ob* mice were administered with MK-2 inhibitor once daily by intraperitoneal injections (150 ul total volume). Insulin tolerance test (1.5 IU/kg) was performed on day 12. *, $p < 0.05$

[00114] Fig. 75 shows that MK-2 inhibitor decreases fasting insulin levels. 10 week-old *ob/ob* mice were administered with MK-2 inhibitor once daily by intraperitoneal injections (150 ul total volume). 6h fasting insulin levels on day 10. *, $p < 0.05$

[00115] Fig. 76 shows that MK-2 inhibitor does not change total WBC. 10 week-old *ob/ob* mice were administered with MK-2 inhibitor once daily by intraperitoneal injections (150 ul total volume). Total WBC count levels on day 18.

[00116] Fig. 77. *In vitro* phosphorylation of FoxO1 by p38. 1 ug WT GST-FoxO1 or GST control were incubated with 25 ug activated p38 (Upstate Biotechnology) at 30 °C for 30 minutes in 15 ul kinase buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM beta-

glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na3VO4, 10 mM MgCl2 and 2.5 μCi [γ–32P] ATP. Samples were separated by SDS-PAGE and visualized by autoradiography followed by immunoblot analysis.

[00117] Fig. 78. *In vitro* phosphorylation of FoxO1 by MK-2. 1 ug WT GST-Foxo1, GST (control) or GST-Hsp25 were incubated with or without 25 ug activated MK-2 (Upstate Biotechnology) at 30 °C for 30 minutes in 15 ul kinase buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na3VO4, 10 mM MgCl2 and 2.5 μCi [γ–32P] ATP. Samples were separated by SDS-PAGE and visualized by autoradiography followed by immunoblot analysis.

[00118] Fig. 79. Dominant-Negative MK2 Lowers Hepatic *G6pc* in *ob/ob* Mice. 9 week-old *ob/ob* mice were injected with 1 x 10^9 pfu of adenoviruses containing either control LacZ (n = 5) or DN-MK2 (n = 5). Liver *G6pc* mRNA was analyzed 8 days after the injection. (*p <0.05).

[00119] Fig. 80. MK-2 Inhibitor Lowers FoxO1 Target Genes in the Livers of *ob/ob* Mice. 10 week-old *ob/ob* mice were treated with MK-2 inhibitor (0.2 mg/kg) or vehicle control once daily by intraperitoneal injections for 20 days. Liver *G6pc*, *Pck1* and *Igfbp-1* mRNAs were analyzed by qRT–PCR. (*p <0.05, ** p <0.05). The MK-2 inhibitor used in **Figs. 80-85** is Compound 28 described in Huang X, Zhu X, Chen X, Zhou W, Xiao D, Degrado S, Aslanian R, Fossetta J, Lundell D, Tian F, Trivedi P, Palani A. *Bioorg Med Chem Lett.* 2012 Jan 1;22(1):65-70, "*A three-step protocol for lead optimization: quick identification of key conformational features and functional groups in the SAR studies of non-ATP competitive MK2 (MAPKAPK2) inhibitors*". Department of Medicinal Chemistry, Merck Research Laboratories, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA.

[00120] Fig. 81: MK-2 Inhibitor Improves Hepatic Insulin Signaling in *ob/ob* Mice. 10 week-old *ob/ob* mice were treated with MK-2 inhibitor (0.2 mg/kg) or vehicle control once daily by intraperitoneal injections for 20 days. Following 6 hours of fasting, insulin (2IU/kg) was injected into the mice through portal vein for 3 minutes. Liver phospho-Akt (serine 473) and total Akt levels were analyzed via western blotting. Densitometric quantification of the immunoblot data is shown in the graph. (*p <0.05).

[00121] Fig. 82. MK-2 Inhibitor Lowers Chop and Trib3 mRNAs in the Livers of *ob/ob* Mice. As in Figure 80, except that liver *Chop* and *Trb3* mRNA levels were analyzed. (*p <0.05).

[00122] Fig. 83. MK-2 Inhibitor Lowers FA Synthesis mRNAs and *Tnfa* mRNA in the Livers of *ob/ob* Mice. As in Figure 80, except that liver *Fas*, *Scd1*, *Srebp1*, *Tnfa* mRNA levels were analyzed. (*p <0.05).

[00123] Fig. 84. MK-2 Inhibitor-Mediated Decrease in FBG is Additive with Metformin. 10 week-old *ob/ob* mice were treated with once daily injections of vehicle control, MK-2 inhibitor (0.2 mg/kg) alone, metformin (250 mg/kg) alone or MK-2 inhibitor and metformin together for 5 days. 6-hour fasting blood glucose levels are shown. (*p <0.05).

[00124] Fig. 85. MK-2 Inhibitor-Mediated Decrease in Hepatic *G6pc* is Additive with Metformin. As in Figure 84, except that liver *G6Pc* mRNA was analyzed by qRT–PCR. (*p <0.05).

[00125] Fig. 86. p38 deficiency suppresses tunicamycin induced UPR activation and Trb3 upregulation. Primary mouse hepatocytes from p38 floxed mice were transduced with adenoviral vectors expressing LacZ or Cre at an MOI of 10. 36 hours after transfection, cells were treated with tunicamycin (1 μg/ml) for 4 hours. Cell lysates were assayed by immunoblot for phospho-Perk, Perk, Chop, β-actin and Trb3.

[00126] Fig. 87. p38 deficiency suppresses *Trb3* mRNA levels. Primary mouse hepatocytes from p38 floxed mice were transduced with adenoviral vectors expressing LacZ or Cre at an MOI of 10. 24 hours after transfection, cells were treated with palmitate (300 μm) for 18 hours. Trb3 mRNA was assayed by qRT–PCR. (†, $P<0.0005$; *, $P<0.0001$).

[00127] Fig. 88. Acute insulin-induced p-AKT enhancement in *p38-/-* hepatocytes is rescued by transduction with Ad-Trb3. Primary mouse hepatocytes from p38 floxed mice were transduced with adenoviral vectors expressing LacZ or Cre at an MOI of 10. After overnight incubation, half of the adeno-Cre treated cells were transduced with adeno-Trb3 while rest of the cells received adeno-LacZ. After 12 hours, cells were treated with palmitate (300 μm) for 14 hours, followed by treatment in serum-free media for 5 hours. After 5-hour incubation in serum-free media, cells were stimulated with insulin (100nm) for 5 minutes. Cell lysates were then assayed by immunoblot for phospho-Akt (serine 473), Akt, Trb3 and β-actin.

DETAILED DESCRIPTION OF THE INVENTION

[00128] The patent and scientific literature referred to herein establishes knowledge that is available to those skilled in the art. The issued patents, applications, and other publications that

are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference.

[00129] As would be apparent to one of ordinary skill in the art, any method or composition described herein can be implemented with respect to any other method or composition described herein.

Definitions and Abbreviations

[00130] The singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

[00131] The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.”

[00132] The term "about" is used herein to mean approximately, in the region of, roughly, or around. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20%.

[00133] As used herein, the abbreviation “CaMKII” refers to the enzyme calcium calmodulin-dependent kinase II, including any of its isoforms and their splice variants. The nucleic acid sequences of the genes encoding the different isoforms of CaMKII, including, but not limited to, the nucleic acid sequences of the open reading frames of the genes, are known in the art. The nucleic acid sequences of the genes encoding the different isoforms of human CaMKII, including, but not limited to, the nucleic acid sequences of the open reading frames of the genes, are known in the art. The amino acid sequences of the CaMKII polypeptides and proteins, including, but not limited to, the amino acid sequences of the human CaMKII polypeptides and proteins, are known in the art. Sequences of transcript variants and splice variants are also known in the art (*see e.g.* Couchonnal and Anderson, 2008, which is incorporated by reference in its entirety). The accession number of the nucleic acid sequence of *mus musculus* CaMKII-γ is NM_178597 and the accession number of the nucleic acid sequence of human CaMKII- γ is NM_172171.2. The accession number of the amino acid sequence of *mus musculus* CaMKII-γ is NP_848712.2 and the accession number of the amino acid sequence of human CaMKII- γ is NP_751911.1.

[00134] As used herein, the abbreviation "IP3R" refers to the inositol 1,4,5-triphosphate receptor, including any of its isoforms and their splice variants. Within the IP3R family, several isoforms have been identified and characterized. The abbreviation "IP3R1" refers to isoform IP3R type I, the abbreviation "IP3R2" refers to isoform IP3R type II, and the abbreviation "IP3R3" refers to isoform IP3R type III. The nucleic acid sequences of the genes encoding the different isoforms of IP3R, including, but not limited to, the nucleic acid sequences of the open reading frames of the genes, are known in the art. The nucleic acid sequences of the genes encoding the different isoforms of human IP3R, including, but not limited to, the nucleic acid sequences of the open reading frames of the genes, are known in the art. The amino acid sequences of the IP3R polypeptides and proteins, including, but not limited to, the amino acid sequences of the human IP3R polypeptides and proteins of the different IP3R isoforms, are known in the art. The accession number of the nucleic acid sequence of *mus musculus* IP3R1 is NM_010585.5 and the accession number of the nucleic acid sequence of human IP3R1 is NM_001099952.2. The accession number of the amino acid sequence of *mus musculus* IP3R1 is NP_034715.3 and the accession number of the amino acid sequence of human IP3R1 is NP_001093422.2. For additional information on IP3R, *see* Wehrens *et al.*, 2005, Annu Rev Physiol., 67:69-98. "Intracellular calcium release and cardiac disease"; *see also* Patterson *et al.*, 2004, Annu Rev Biochem. 73:437-65. "Inositol 1,4,5-trisphosphate receptors as signal integrators." and Volpe *et al.*, 1990, Am J Physiol.;258(6 Pt 1):C1086-91. "Regulation of inositol 1,4,5-trisphosphate-induced Ca2+ release. II. Effect of cAMP-dependent protein kinase.", all of which are incorporated by reference herein.

[00135] As used herein, the abbreviation "p38" refers to any of the p38 mitogen-activated protein (MAP) kinases. Several p38 MAP kinases have been identified, including p38-α (also known as MAPK14), p38- β (also known as MAPK11), p38-γ (also known as MAPK12/ERK6) and p38-δ (also known as MAPK13 / SAPK4). The nucleic acid sequences of the genes encoding p38, including, but not limited to, the nucleic acid sequences of the open reading frames of the genes, are known in the art. The nucleic acid sequences of the genes encoding the human p38, including, but not limited to, the nucleic acid sequences of the open reading frames of the genes, are known in the art. The amino acid sequences of p38 polypeptides and proteins, including, but not limited to, the amino acid sequences of the human p38 polypeptides and proteins, are known in the art. The accession number of the nucleic acid sequence of *mus musculus* p38-α (MAPK14) is NM_011951.3 and the accession number of the nucleic acid sequence of human p38-α (MAPK14) is NM_001315.2. The accession number of the amino

acid sequence of *mus musculus* p38-α (MAPK14) is NP_036081.1 and the accession number of the amino acid sequence of human p38-α (MAPK14) is NP_001306.1. For additional information on p38, see Marber *et al.*, 2011, *J Mol Cell Cardiol.*;51(4):485-90 "The p38 mitogen-activated protein kinase pathway--a potential target for intervention in infarction, hypertrophy, and heart failure."; *see also* Kostenko *et al.*, 2011, *World J Biol Chem.* 26;2(5):73-89. "Physiological roles of mitogen-activated-protein-kinase-activated p38-regulated/activated protein kinase." and Cuadrado *et al.*, 2010, *Biochem J.*;429(3):403-17. "Mechanisms and functions of p38 MAPK signalling.", all of which are incorporated by reference herein.

[00136] As used herein, the abbreviation "MK2" refers to the p38-activated kinase MK2, also known as MAP kinase activated protein kinase 2 (or MAPKAPK2). The nucleic acid sequence of the gene encoding MK2, including, but not limited to, the nucleic acid sequence of the open reading frame of the gene, is known in the art. The nucleic acid sequence of the gene encoding the human MK2, including, but not limited to, the nucleic acid sequence of the open reading frame of the gene, is known in the art. The amino acid sequence of MK2 polypeptides and proteins, including, but not limited to, the amino acid sequences of the human MK2 polypeptides and proteins, are known in the art. The accession number of the nucleic acid sequence of *mus musculus* MK2 is NM_008551 and the accession number of the nucleic acid sequence of human MK2 is NM_004759.4. The accession number of the amino acid sequence of *mus musculus* MK2 is NP_032577.1 and the accession number of the amino acid sequence of human MK2 is NP_004750.1.

[00137] As used herein, the abbreviation "MK3" refers to the p38-activated kinase MK3, also known as MAP kinase activated protein kinase 3 (or MAPKAPK3). The nucleic acid sequence of the gene encoding MK3, including, but not limited to, the nucleic acid sequence of the open reading frame of the gene, is known in the art. The nucleic acid sequence of the gene encoding the human MK3, including, but not limited to, the nucleic acid sequence of the open reading frame of the gene, is known in the art. The amino acid sequence of MK3 polypeptides and proteins, including, but not limited to, the amino acid sequences of the human MK3 polypeptides and proteins, are known in the art. The abbreviation "MK2/3" refers to either MK2, or MK3 or both MK2 and MK3. The accession number of the nucleic acid sequence of *mus musculus* MK3 is NM_178907.3 and the accession number of the nucleic acid sequence of human MK3 is NM_001243926.1. The accession number of the amino acid sequence of *mus musculus* MK3 is NP_849238 and the accession number of the amino acid sequence of human MK3 is NP_004626.1. For additional information on MK2 and MK3, see Gaestel *et al.*, 2006, *Nat Rev*

Mol Cell Biol. 7(2):120-30. "MAPKAP kinases - MKs - two's company, three's a crowd."; see also Shiryaev et al., 2010, *Cell Signal.*;22(8):1185-92. "Mitogen-activated protein kinase p38 and MK2, MK3 and MK5: ménage à trois or ménage à quatre?" and Kotlyarov et al., 2002, *Biochem Soc Trans.*;30(Pt 6):959-63. "Is MK2 (mitogen-activated protein kinase-activated protein kinase 2) the key for understanding post-transcriptional regulation of gene expression?", all of which are incorporated by reference herein.

[00138] As used herein, the term "calcineurin" refers to either the catalytic subunit of the protein phosphatase calcineurin, or the regulatory subunit of the protein phosphatase calcineurin, or both, including any of their isoforms and their splice variants. The nucleic acid sequences of the genes encoding the different isoforms of the subunits of calcineurin, including, but not limited to, the nucleic acid sequences of the open reading frames of the genes, are known in the art. The nucleic acid sequences of the genes encoding the different isoforms of the subunits of human calcineurin, including, but not limited to, the nucleic acid sequences of the open reading frames of the genes, are known in the art. The amino acid sequences of the calcineurin polypeptides and proteins, including, but not limited to, the amino acid sequences of the human calcineurin polypeptides and proteins, are known in the art. The accession number of the nucleic acid sequence of *mus musculus* calcineurin subunit B is NM_024459.2 and the accession number of the nucleic acid sequence of human calcineurin catalytic subunit is NM_000944.4. The accession number of the amino acid sequence of *mus musculus* calcineurin subunit B is NP_077779.2 and the accession number of the amino acid sequence of human calcineurin catalytic subunit is NP_000935.1. For additional information on calcineurin, see Wilkins *et al.*, 2004, *Biochem Biophys Res Commun.* 1;322(4):1178-91. "Calcium-calcineurin signaling in the regulation of cardiac hypertrophy."; *see also* Periasamy, 2002, *J Mol Cell Cardiol.* 34(3):259-62. "Calcineurin and the heartbeat, an evolving story." and Buchholz *et al.*, 2007, *Cell Cycle.* 6(1):16-9. "An emerging role for Ca2+/calcineurin/NFAT signaling in cancerogenesis.", all of which are incorporated by reference herein.

[00139] As used herein, the term "metabolic syndrome" is used to describe a combination of medical disorders that, when occurring together, increase the risk for cardiovascular disease, stroke and type 2 diabetes. These disorders include, but are not limited to, central obesity (extra weight around the middle and upper parts of the body); insulin resistance; aging; stress; hormonal changes; excess blood clotting; dyslipidemia, which includes low HDL; a type of LDL that promotes heart disease; and elevated apolipoprotein B100.

Methods for Treatment and Prevention

[00140] The present invention relates to the discovery that a calcium-sensing enzyme, CaMKII, is activated in a calcium- and IP3R-dependent manner by cAMP and glucagon in primary HCs and by glucagon and fasting *in vivo*. Genetic deficiency or inhibition of CaMKII blocks nuclear translocation of FoxO1 by affecting its phosphorylation, impairs fasting- and glucagon/cAMP-induced glycogenolysis and gluconeogenesis, and lowers blood glucose levels, while constitutively active CaMKII has the opposite effects. The suppressive effect of CaMKII deficiency on glucose metabolism is abrogated by transduction with constitutively nuclear FoxO1, indicating that the effect of CaMKII deficiency requires nuclear exclusion of FoxO1. This same pathway is also involved in excessive HGP in the setting of obesity. These results reveal a calcium-mediated signaling pathway involved in FoxO1 nuclear localization and hepatic glucose homeostasis.

[00141] The present invention also relates to discoveries, including validation in models of obesity, that indicate that inhibitors of two drug targets—a liver calcium trasporter called IP3 receptor and a liver phosphatase enzyme called calcineurin—can be invaluable in this niche. A new role for IP3 receptors and calcineurin in hepatic glucose production (HGP), which is excessively activated in obesity and type 2 diabetes, was recently discovered. Glucagon in fasting or obesity activates IP3 receptors and IP3R-induced calcium release from the endoplasmic reticulum to the cytosol. The released calcium then activates 2 calcium-sensitive enzymes. One of the enzymes is CaMKII. Activation of CaMKII is essential, because it facilitates the entry of FoxO1 into the nucleus, which then induces genes for HGP and is partially responsible for fasting hyperglycemia and metabolic disturbances in a mouse model of obesity. Calcineurin is the other enzyme that is activated by the released calcium. Calcineurin is a phosphatase that dephosphorylates another transcription factor called Crtc2, thus facilitating its entry into the nucleus. Crtc2 works together with FoxO1 to induce genes for HGP in obesity. Thus inhibition of Crtc2, like that of CaMKII, suppresses fasting hyperglycemia in a mouse model of obesity. Thus, this invention provides for the development and testing of IP3 receptor and calcineurin inhibitors in a pre-clinical model of obesity and insulin resistance.

[00142] Finally, this invention relates to the discovery that p38 and MK2/3 inhibitors can also be invaluable in this niche.

[00143] The invention provides for methods of treating or preventing a metabolic disorder in a subject, methods of treating or preventing coronorary artery disease in a subject with a metabolic disorder, as well as methods of reducing hepatic glucose production in a subject. The

invention provides for the treatment and/or prevention of a metabolic disorder in a subject, the treatment and/or prevention of a coronorary artery disease in a subject with a metabolic disorder, as well as methods of reducing hepatic glucose production in a subject, by administering to the subject compound(s) that inhibit or reduce the activity of CaMKII, IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, calcineurin, p38, MK2/3, or any combination thereof.

[00144] The invention also provides methods of identifying a compound that inhibits the activity of CaMKII or reduces the activity and/or activation of CaMKII. The present invention thus also provides for the screening, development, and testing of inhibitors of CaMKII to prevent the metabolic disturbances of obesity, type 1 diabetes and type 2 diabetes. The invention further provides for the screening, development, and testing of inhibitors of CaMKII to prevent the metabolic disturbances of obesity, type 1 diabetes and type 2 diabetes in combination with other inhibitors, such as, but not limited to, inhibitors of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, calcineurin, p38 and/or MK2/3.

[00145] The present invention also provides for the screening, development, and testing of inhibitors of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, and inhibitors of calcineurin, to prevent the metabolic disturbances of obesity, type 1 diabetes and type 2 diabetes. The invention further provides for the screening, development, and testing of inhibitors of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3 and/or inhibitors of calcineurin, to prevent the metabolic disturbances of obesity, type 1 diabetes and type 2 diabetes in combination with other inhibitors, such as, but not limited to, CaMKII, calcineurin, p38 and/or MK2/3 inhibitors.

[00146] The present invention also provides for the screening, development, and testing of inhibitors of p38 and MK2/3 to prevent the metabolic disturbances of obesity, type 1 diabetes and type 2 diabetes. The invention further provides for the screening, development, and testing of inhibitors of p38 and MK2/3 to prevent the metabolic disturbances of obesity type 1 diabetes, and type 2 diabetes in combination with other inhibitors, such as, but not limited to, CaMKII, IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3 and/or calcineurin inhibitors.

[00147] In certain aspects, the invention described herein is based on the finding that inhibitors of CaMKII can treat obesity-induced insulin resistance and disturbances in liver glucose and fat metabolism.

[00148] In certain aspects, the invention described herein provides CaMKII inhibitors for the purpose of drug development to improve the metabolic disturbances and their consequences in obesity, metabolic syndrome, type 1 diabetes and and type 2 diabetes.

[00149] In certain aspects, the invention described herein provides methods for treating and diagnosing metabolic disturbances including heart disease, of insulin resistant states such as obesity, metabolic syndrome, and type 2 diabetes.

[00150] In certain aspects, the invention relates to findings related to the role of hepatic CaMKII in glucagon-mediated hepatic glucose production (HGP).

[00151] As described herein, CaMKII is activated by glucagon in primary hepatocytes and by glucagon and fasting in vivo. Genetic deficiency or inhibition of hepatic CaMKII lowered blood glucose levels, suppresses the HGP genes G6pc and Pck1, decreases glycogen depletion, and blocks nuclear translocation of the HGP transcription factor FoxO1. Conversely, constitutively active CaMKII induces G6pc and Pck1, stimulates glucose production, and raises blood glucose levels. The suppressive effect of CaMKII deficiency on glucose metabolism is abrogated by constitutively nuclear FoxO1, indicating that the effect of CaMKII deficiency can require nuclear exclusion of FoxO1.

[00152] The results described herein identify a molecular pathway regulated by CaMKII in the control of HGP.

[00153] In certain aspects, the invention is related to the finding that fasting and glucagon activate hepatic CaMKII. In certain aspects, the invention is related to the finding that CaMKII stimulates hepatic glucose production. In certain aspects, the invention is related to the finding that CaMKII promotes nuclear localization and activation of FoxO1. In certain aspects, the invention is related to the finding that impaired glucose production in CaMKII deficiency requires nuclear exclusion of FoxO1. In certain aspects, the methods described herein are useful for treating and diagnosing heart failure since overactive CaMKII has been implicated in heart failure.

[00154] The present disclosure provides methods for the treatment and/or prevention of a metabolic disorder. In one aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an inhibitor of CaMKII, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an inhibitor of IP3R1, IP3R2, and/or IP3R3, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject in need thereof, the method comprising administering to the subject a therapeutically effective

amount of an inhibitor of calcineurin, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an inhibitor of p38, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an inhibitor of MK2/3, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an inhibitor of CaMKII, IP3R1, IP3R2, IP3R3, calcineurin, p38, MK2/3, or any combination thereof, thereby treating or preventing the disorder.

[00155] In one aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, the method comprising reducing the activity of CaMKII, thereby treating or preventing the disorder. In one aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of CaMKII, thereby treating or preventing the disorder. In one aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is not induced by obesity, the method comprising reducing the activity of CaMKII, thereby treating or preventing the disorder.

[00156] In one embodiment, the disorder is selected from the group consisting of Type 1 diabetes, Type 2 diabetes, insulin resistance and metabolic syndrome.

[00157] In one embodiment, the treating or preventing affects glycogenolysis or gluconeogenesis in the subject. In another embodiment, the treating or preventing does not affect glycogenolysis or gluconeogenesis in the subject.

[00158] In one embodiment, the treating or preventing reduces hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject. In another embodiment, the treating or preventing increases hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject. In another embodiment, the treating or preventing has no effect on hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject.

[00159] In one embodiment, the treating or preventing reduces phosphorylation and/or activation of CaMKII. In another embodiment, the treating or preventing inhibits phosphorylation of CaMKII. In another embodiment, the treating or preventing inhibits the activity and/or activation of CaMKII. In one embodiment, the treating or preventing increases phosphorylation and/or activation of CaMKII. In another embodiment, the treating or preventing increases the activity and/or activation of CaMKII.

[00160] In one embodiment, the activity of CaMKII is a glucagon-induced activity.

[00161] In one embodiment, the treating or preventing reduces or inhibits expression of G6pc and/or Pck1 in a cell of the subject. In one embodiment, the treating or preventing increases expression of G6pc and/or Pck1 in a cell of the subject

[00162] In another embodiment, the treating or preventing reduces the amount of FoxO1 protein level in the nucleus of a cell of the subject. In another embodiment, the treating or preventing increases the amount of FoxO1 protein level in the nucleus of a cell of the subject.

[00163] In another embodiment, the treating or preventing reduces or inhibits phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein. In another embodiment, the treating or preventing increases phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein.

[00164] In another aspect, the present disclosure provides a method of treating or preventing coronary artery disease in a subject, the method comprising reducing the activity of CaMKII in the subject, thereby treating or preventing the coronary artery disease. The present disclosure provides a method of treating or preventing coronary artery disease in a subject with a metabolic disorder, the method comprising increasing the activity of CaMKII in the subject, thereby treating or preventing the coronary artery disease.

[00165] In one embodiment, the disorder is selected from the group consisting of Type 1 diabetes, Type 2 diabetes, insulin resistance and metabolic syndrome.

[00166] In another embodiment, the treating or preventing affects glycogenolysis or gluconeogenesis in the subject. In another embodiment, the treating or preventing does not affect glycogenolysis or gluconeogenesis in the subject.

[00167] In one embodiment, the treating or preventing reduces hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject. In another

embodiment, the treating or preventing increases hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject.

[00168] In one embodiment, the treating or preventing reduces phosphorylation and/or activation of CaMKII. In another embodiment, the treating or preventing increases phosphorylation and/or activation of CaMKII.

[00169] In one embodiment, the activity of CaMKII is a glucagon-induced activity. In another embodiment, the activity of CaMKII is not a glucagon-induced activity.

[00170] In one embodiment, the treating or preventing reduces expression of G6pc and/or Pck1 in a cell of the subject. In another embodiment, the treating or preventing increases expression of G6pc and/or Pck1 in a cell of the subject.

[00171] In one embodiment, the treating or preventing reduces the amount of FoxO1 protein level in the nucleus of a cell of the subject. In another embodiment, the treating or preventing increases the amount of FoxO1 protein level in the nucleus of a cell of the subject.

[00172] In another embodiment, the treating or preventing reduces phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein. In another embodiment, the treating or preventing increases phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein.

[00173] In one embodiment, the treating or preventing comprises reducing CaMKII activity in a macrophage of the subject. In another embodiment, the treating or preventing comprises increasing CaMKII activity in a macrophage of the subject.

[00174] In one embodiment, the coronary artery disease is associated with atherogenesis and/or atherosclerosis. In another embodiment, the method further comprises treating or preventing heart failure, hypertension and/or renal disease. In another embodiment, the disorder is associated with advanced lesional macrophage apoptosis. In another embodiment, the disorder is associated with plaque necrosis.

[00175] In one embodiment, the treating or preventing coronary artery disease in a subject with a metabolic disorder results in lowering hyperinsulinemia and/or dyslipidemia. In another embodiment, the treating or preventing coronary artery disease in a subject with a metabolic disorder results in increasing hyperinsulinemia and/or dyslipidemia.

[00176] In one embodiment, the treating or preventing coronary artery disease in a subject with a metabolic disorder results in lowering atherogenesis and/or atherosclerosis. In another embodiment, the treating or preventing coronary artery disease in a subject with a metabolic disorder results in increasing atherogenesis and/or atherosclerosis.

[00177] In one embodiment, the treating or preventing comprises a step of administering to the subject an inhibitor of the CaMKII protein. In one embodiment, the treating or preventing comprises a step of administering to the subject an antisense RNA, or a siRNA, that reduces expression of the gene encoding the CaMKII protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the CaMKII protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a small molecule. In one embodiment, the small molecule is a CaMKII inhibitor selected from the group consisting of KN-93, lavendustin C, CK59, Ant-CaMKIINtide, KN62, DY9760e, K-252a *Nocardiopsis sp.*, H89 dihydrochloride, PP1 Analog II, 1NM-PP1, eEF-2 kinase inhibitor NH125, and STO-609.

[00178] In another aspect, the present disclosure provides a method of reducing hepatic glucose production in a subject, the method comprising reducing the activity of CaMKII, thereby resulting in the reduction of hepatic glucose in the subject. In another aspect, the present disclosure provides a method of reducing hepatic glucose production in a subject, the method comprising increasing the activity of CaMKII, thereby resulting in the reduction of hepatic glucose in the subject.

[00179] In another aspect, the present disclosure provides a method of increasing hepatic glucose production in a subject, the method comprising increasing the activity of CaMKII, thereby resulting in the increase of hepatic glucose in the subject. In another aspect, the present disclosure provides a method of increasing hepatic glucose production in a subject, the method comprising reducing the activity of CaMKII, thereby resulting in the increase of hepatic glucose in the subject.

[00180] In one embodiment, the reducing hepatic glucose production reduces phosphorylation and/or activation of CaMKII. In another embodiment, the reducing hepatic glucose production increases phosphorylation and/or activation of CaMKII.

[00181] In one embodiment, the increasing hepatic glucose production reduces phosphorylation and/or activation of CaMKII. In another embodiment, the increasing hepatic glucose production increases phosphorylation and/or activation of CaMKII.

[00182] In one embodiment, the activity of CaMKII is a glucagon-induced activity.

[00183] In one embodiment, the reducing hepatic glucose production reduces expression of G6pc and/or Pck1 in a cell of the subject. In another embodiment, the reducing hepatic glucose production increases expression of G6pc and/or Pck1 in a cell of the subject. In one embodiment, the increasing hepatic glucose production reduces expression of G6pc and/or Pck1 in a cell of the subject. In another embodiment, the increasing hepatic glucose production increases expression of G6pc and/or Pck1 in a cell of the subject.

[00184] In one embodiment, the reducing hepatic glucose production reduces hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject. In another embodiment, the reducing hepatic glucose production increases hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject. In one embodiment, the increasing hepatic glucose production reduces hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject. In another embodiment, the increasing hepatic glucose production increases hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject.

[00185] In another embodiment, the reducing hepatic glucose production reduces the amount of FoxO1 protein level in the nucleus of a cell of the subject. In another embodiment, the reducing hepatic glucose production reduces phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein. In another embodiment, the reducing hepatic glucose production increases the amount of FoxO1 protein level in the nucleus of a cell of the subject. In another embodiment, the reducing hepatic glucose production increases phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein.

[00186] In another embodiment, the increasing hepatic glucose production reduces the amount of FoxO1 protein level in the nucleus of a cell of the subject. In another embodiment, the increasing hepatic glucose production reduces phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein. In another embodiment, the increasing hepatic glucose production increases the amount of FoxO1 protein level in the nucleus of a cell of the subject. In another embodiment, the increasing hepatic glucose production increases phosphorylation of amino acid S284, S295,

S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein.

[00187] In one embodiment, the reducing hepatic glucose production comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the CaMKII protein. In one embodiment, the increasing hepatic glucose production comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the CaMKII protein.

[00188] In another embodiment, the reducing hepatic glucose production comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the CaMKII protein. In another embodiment, the increasing hepatic glucose production comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the CaMKII protein.

[00189] In another embodiment, the reducing hepatic glucose production comprises a step of administering to the subject a small molecule. In another embodiment, the increasing hepatic glucose production comprises a step of administering to the subject a small molecule.

[00190] In one embodiment, the small molecule is a CaMKII inhibitor selected from the group consisting of KN-93, lavendustin C, CK59, Ant-CaMKIINtide, KN62, DY9760e, K-252a *Nocardiopsis sp.*, H89 dihydrochloride, PP1 Analog II, 1NM-PP1, eEF-2 kinase inhibitor NH125, and STO-609.

[00191] In one aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, the method comprising reducing the activity of IP3R1, IP3R2 or IP3R3, thereby treating or preventing the disorder. The present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of IP3R1, IP3R2 or IP3R3, thereby treating or preventing the disorder. The present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is not induced by obesity, the method comprising reducing the activity of IP3R1, IP3R2 or IP3R3, thereby treating or preventing the disorder.

[00192] In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, the method comprising reducing the activity of calcineurin, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the

disorder is induced by obesity, the method comprising reducing the activity of calcineurin, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is not induced by obesity, the method comprising reducing the activity of calcineurin, thereby treating or preventing the disorder.

[00193] In one embodiment, the disorder is selected from the group consisting of Type 1 diabetes, Type 2 diabetes, insulin resistance, obesity, and metabolic syndrome.

[00194] In one embodiment, the treating or preventing affects glycogenolysis or gluconeogenesis in the subject. In another embodiment, the treating or preventing does not affect glycogenolysis or gluconeogenesis in the subject.

[00195] In one embodiment, the treating or preventing reduces hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject. In another embodiment, the treating or preventing increases hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject.

[00196] In one embodiment, the treating or preventing comprises a step of administering to the subject an inhibitor of the IP3R1 protein, IP3R2 protein, IP3R3 protein, or any combination thereof. In one embodiment, the inhibitor is Xestospongin C. In another embodiment, the inhibitor is 2-APB. In another embodiment, the inhibitor is caffeine.

[00197] In one embodiment, the treating or preventing comprises a step of administering to the subject an antisense RNA, or a siRNA, that reduces expression of the gene encoding the IP3R1 protein, IP3R2 protein or IP3R3 protein, or any combination thereof. In another embodiment, the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the IP3R1 protein, IP3R2 protein or IP3R3 protein, or any combination thereof.

[00198] In one embodiment, the treating or preventing comprises a step of administering to the subject a small molecule. In one embodiment, the small molecule is a IP3R1 protein inhibitor, IP3R2 protein inhibitor or IP3R3 protein inhibitor. In one embodiment, the small molecule is Xestospongin C. In another embodiment, the small molecule is 2-APB. In another embodiment, the small molecule is caffeine.

[00199] In one embodiment, the treating or preventing comprises a step of administering to the subject an inhibitor of calcineurin. In one embodiment, the inhibitor of calcineurin is cyclosporin A. In another embodiment, the inhibitor of calcineurin is pimecrolimus. In another embodiment, the inhibitor of calcineurin is tacrolimus.

[00200] In one embodiment, the treating or preventing comprises a step of administering to the subject an antisense RNA, or a siRNA, that reduces expression of the gene encoding the calcineurin protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a peptide or a oligopeptide or a polypeptide that specifically binds to the calcineurin protein. In one embodiment, the oligopeptide is cyclosporin A.

[00201] In one embodiment, the treating or preventing comprises a step of administering to the subject a small molecule. In another embodiment, the small molecule is a calcineurin inhibitor. In one embodiment, the small molecule is pimecrolimus. In another embodiment, the small molecule is tacrolimus.

[00202] In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, the method comprising reducing the activity of p38, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of p38, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is not induced by obesity, the method comprising reducing the activity of p38, thereby treating or preventing the disorder.

[00203] In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, the method comprising reducing the activity of MK2/3, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of MK2/3, thereby treating or preventing the disorder. In another aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject, wherein the disorder is not induced by obesity, the method comprising reducing the activity of MK2/3, thereby treating or preventing the disorder.

[00204] In one embodiment, the disorder is selected from the group consisting of Type 1 diabetes, Type 2 diabetes, insulin resistance, obesity and metabolic syndrome.

[00205] In one embodiment, the treating or preventing affects glycogenolysis or gluconeogenesis in the subject. In another embodiment, the treating or preventing does not affect glycogenolysis or gluconeogenesis in the subject.

[00206] In one embodiment, the treating or preventing reduces hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject. In another embodiment, the treating or preventing increases hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject.

[00207] In one embodiment, the treating or preventing comprises a step of administering to the subject an inhibitor of p38. In one embodiment, the inhibitor of p38 is SB203580. In another embodiment, the inhibitor of p38 is SB 202190. In another embodiment, the inhibitor of p38 is SB 239063.

[00208] In one embodiment, the treating or preventing comprises a step of administering to the subject an antisense RNA, or a siRNA, that reduces expression of the gene encoding the p38 protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the p38 protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a small molecule. In one embodiment, the small molecule is a p38 inhibitor. In one embodiment, the small molecule is SB203580. In another embodiment, the small molecule is SB 202190. In another embodiment, the small molecule is SB 239063.

[00209] In one embodiment, the treating or preventing comprises a step of administering to the subject an inhibitor of MK2/3. In one embodiment, the inhibitor of MK2/3 is Hsp25 kinase inhibitor.

[00210] In one embodiment, the treating or preventing comprises a step of administering to the subject an antisense RNA, or siRNA, that reduces expression of the gene encoding the MK2/3 protein. In another embodiment, the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the MK2/3 protein. In one embodiment, the peptide is Hsp25 Kinase Inhibitor. In one embodiment, the treating or preventing comprises a step of administering to the subject a small molecule. In one embodiment, the small molecule is a MK2/3 inhibitor.

Methods for screening

[00211] The disclosure provides methods for identifying a compound or combination of compounds that treat or prevent a metabolic disorder in a subject. The disclosure also provides methods for identifying a compound or combination of compounds that treat or prevent coronary artery disease in a subject with a metabolic disorder. In one embodiment, the disorder is induced by obesity. In another embodiment, the disorder is not induced by obesity. The disclosure also provides methods for identifying a compound or combination of compounds that reduce hepatic glucose production in a subject.

[00212] The disclosure also provides methods for the identification of a compound or a combination of compounds that inhibit, or reduce, the activity and/or activation of CaMKII. The disclosure also provides methods for the identification of a compound or a combination of compounds that inhibit, or reduce, the activity and/or activation of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3. The disclosure also provides methods for the identification of a compound or a combination of compounds that inhibit, or reduce, the activity and/or activation of calcineurin. The disclosure also provides methods for the identification of a compound or a combination of compounds that inhibit, or reduce, the activity and/or activation of p38. The disclosure also provides methods for the identification of a compound or a combination of compounds that inhibit, or reduce, the activity and/or activation of MK2/3.

[00213] The disclosure also provides methods for the identification of a compound or a combination of compounds that inhibit the activity and/or activation of CaMKII, IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, calcineurin, p38, MK2/3, or any combination thereof. The disclosure also provides methods for the identification of a compound or a combination of compounds that reduce the activity of CaMKII, IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, calcineurin, p38, MK2/3, or any combination thereof.

[00214] The present disclosure provides a method for identifying a compound that inhibits the activity of CaMKII, the method comprising a) contacting a cell with a CaMKII fusion protein, wherein the CaMKII fusion protein comprises an acceptor fluorophore protein at one terminus, and a donor fluorophore protein at the other terminus; and b) measuring FRET efficiency in the absence and in the presence of a test compound, wherein a greater FRET efficiency in the presence of the test compound compared to the FRET efficiency in the absence of the test compound indicates that the test compound inhibits the activity of CaMKII (*see* Takao *et al.*, 2005; and Kwok *et al.*, 2008, which are incorporated by reference in their entireties).

[00215] The present disclosure provides a method for identifying a compound that inhibits the activity of CaMKII, the method comprising a) contacting a cell with a CaMKII fusion protein, wherein the CaMKII fusion protein comprises an acceptor fluorophore protein at one terminus, and a donor fluorophore protein at the other terminus; and b) measuring the ratio of the donor protein to the acceptor protein in the absence and in the presence of a test compound, wherein a decrease of the ratio in the presence of the test compound compared to the ratio in the absence of the test compound indicates that the test compound inhibits the activity of CaMKII (*see* Takao *et al.*, 2005; and Kwok *et al.*, 2008, which are incorporated by reference in their entireties).

[00216] In one embodiment the acceptor fluorophore protein is selected from the group consisting of mOrange, mStrawberry, Venus, yellow fluorescent protein, cyan fluorescent protein, red fluorescent protein and green fluorescent protein. In another embodiment, the donor protein is selected from the group consisting of mOrange, mStrawberry, Venus, yellow fluorescent protein, cyan fluorescent protein, red fluorescent protein and green fluorescent protein (*see* Takao *et al.*, 2005; and Kwok *et al.*, 2008, which are incorporated by reference in their entireties).

[00217] In one embodiment, the cell is a HEK293T cell, a hepatocyte, a U2OS cell, a HeLa cell, a murine embryonic fibroblast (MEF) or a macrophage. In another embodiment, the cell is from a *Insr-/-* mouse, a *Camk2g-/-* mouse, a *Foxo1 -/-* mouse, a *db/db* mouse, a *ob/ob* mouse, a *p38 -/-* mouse, a Non Obese Diabetes (NOD) mouse, a mouse fed a high fat diet, or a streptozotocin-treated mouse. In another embodiment, the cell is from a mouse expressing a mutant FoxO1 protein. In one embodiment, the mutant FoxO1 protein comprises alanine substitutions at S284, S295, S326, S467, S475, S246, S253, S413, or S415, or aspartic acid substitutions at S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof.

[00218] In one embodiment, the cell is subjected to ER stress. In another embodiment, the cell is treated with glucagon, 8-bromo cAMP, H89 dihydrochloride, Xestospongin C, forskolin, saturated fatty acids, or any combination thereof.

[00219] In another aspect, the present disclosure provides a method for identifying a compound that inhibits the activity of IP3R1, IP3R2 or IP3R3, the method comprising a) contacting a cell with a test compound; and b) measuring IP3R1, IP3R2 or IP3R3 activity, wherein a reduction of the activity of IP3R1, IP3R2 or IP3R3 in the presence of the compound compared to the activity of IP3R1, IP3R2 or IP3R3 in the absence of the compound indicates that the compound is an inhibitor of IP3R1, IP3R2 or IP3R3, respectively.

[00220] In one embodiment, the activity is measured by calcium release into the cytosol of the cell after stimulation with an inducer of IP3. In one embodiment, calcium release is measured by an increase in the fluorescence of a cytosolic calcium dye. In one embodiment, the cytosolic calcium dye is Fluo-3.

[00221] In another aspect, the present disclosure provides a method for identifying a compound that inhibits the activity of calcineurin, the method comprising a) contacting a cell with a test compound; and b) measuring calcineurin activity, wherein a reduction of the activity of calcineurin in the presence of the compound compared to the activity of calcineurin in the absence of the compound indicates that the compound is an inhibitor of calcineurin.

[00222] In one embodiment, the activity of calcineurin is measured through detection of phosphatase activity using a calcineurin substrate peptide. In one embodiment, the cell is a HEK293T cell, a hepatocyte, a U2OS cell, a HeLa cell, or a murine embryonic fibroblast (MEF). In another embodiment, the cell is from a db/db mouse, a ob/ob mouse, a Non Obese Diabetes (NOD) mouse, a mouse fed a high fat diet, or a streptozotocin-treated mouse.

[00223] In one embodiment, the cell is treated with glucagon, H89 dihydrochloride, insulin, forskolin, an inducer of ER stress, tunicamycin, saturated fatty acids, or any combination thereof.

[00224] In another aspect, the present disclosure provides a method for identifying a compound that inhibits the activity of p38, the method comprising a) contacting a cell with a test compound; and b) measuring p38 kinase activity, wherein a reduction of the kinase activity of p38 in the presence of the compound compared to the kinase activity of p38 in the absence of the compound indicates that the compound is an inhibitor of p38. In another aspect, the present disclosure provides a method for identifying a compound that inhibits the activity of MK2/3, the method comprising a) contacting a cell with a test compound; and b) measuring MK2/3 kinase activity, wherein a reduction of the kinase activity of MK2/3 in the presence of the compound compared to the kinase activity of MK2/3 in the absence of the compound indicates that the compound is an inhibitor of MK2/3.

[00225] In one embodiment, p38 kinase activity is measured using a p38-specific peptide. In one embodiment, MK2/3 kinase activity is measured using a MK2/3-specific peptide.

[00226] In one embodiment, the cell is a HEK293T cell, a hepatocyte, a U2OS cell, a HeLa cell, a macrophage or a murine embryonic fibroblast (MEF). In another embodiment, the cell is from a Insr-/- mouse, a db/db mouse, a ob/ob mouse, a Non Obese Diabetes (NOD)

mouse, a mouse fed a high fat diet, or a streptozotocin-treated mouse. In another embodiment, the cell is treated with glucagon, 8-bromo cAMP, H89 dihydrochloride, forskolin, saturated fatty acids, or any combination thereof.

[00227] In one aspect, the method comprises administering a test compound or a combination of test compounds to an animal that is a model of a metabolic disorder, or cardiovascular disease, such as coronary artery disease, and determining whether the compound or combination of compounds improves metabolic function, and/or cardiovascular function in the animal, compared to an animal not so treated.

[00228] The invention provides methods for identifying compounds which can be used for treating or preventing a metabolic disorder in a subject, treating or preventing coronary artery disease in a subject with a metabolic disorder and/or reducing hepatic glucose production in a subject. The invention provides methods for identifying compounds that inhibit the activity of CaMKII or reduce the phosphorylation and/or activation of CaMKII. The invention provides methods for identifying compounds that inhibit the activity of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, or reduce the phosphorylation and/or activation of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3. The invention provides methods for identifying compounds that inhibit the activity of calcineurin, or reduce the phosphorylation and/or activation of calcineurin. The invention provides methods for identifying compounds that inhibit the activity of p38, or reduce the phosphorylation and/or activation of p38. The invention provides methods for identifying compounds that inhibit the activity of MK2/3, or reduce the phosphorylation and/or activation of MK2/3.

[00229] The methods can comprise the identification of test compounds or agents (e.g., peptides (such as antibodies or fragments thereof), small molecules, or nucleic acids (such as siRNA or antisense RNA), or other agents).

[00230] In one embodiment, a compound can be a peptide fragment. Fragments include all possible amino acid lengths between and including about 8 and about 100 amino acids, for example, lengths between about 10 and about 100 amino acids, between about 15 and about 100 amino acids, between about 20 and about 100 amino acids, between about 35 and about 100 amino acids, between about 40 and about 100 amino acids, between about 50 and about 100 amino acids, between about 70 and about 100 amino acids, between about 75 and about 100 amino acids, or between about 80 and about 100 amino acids. These peptide fragments can be obtained commercially or synthesized via liquid phase or solid phase synthesis methods (Atherton *et al.*, (1989) Solid Phase Peptide Synthesis: a Practical Approach. IRL Press, Oxford,

England). The peptide fragments can be isolated from a natural source, genetically engineered, or chemically prepared. These methods are well known in the art.

[00231] A compound can be a protein, such as an antibody (monoclonal, polyclonal, humanized, chimeric, or fully human), or a binding fragment thereof. An antibody fragment can be a form of an antibody other than the full-length form and includes portions or components that exist within full-length antibodies, in addition to antibody fragments that have been engineered. Antibody fragments can include, but are not limited to, single chain Fv (scFv), diabodies, Fv, and $(Fab')_2$, triabodies, Fc, Fab, CDR1, CDR2, CDR3, combinations of CDR's, variable regions, tetrabodies, bifunctional hybrid antibodies, framework regions, constant regions, and the like (*see,* Maynard *et al.*, (2000) *Ann. Rev. Biomed. Eng.* 2:339-76; Hudson (1998) *Curr. Opin. Biotechnol.* 9:395-402). Antibodies can be obtained commercially, custom generated, or synthesized against an antigen of interest according to methods established in the art (Janeway *et al.*, (2001) Immunobiology, 5th ed., Garland Publishing).

[00232] A compound can be selected from the group comprising: siRNA; interfering RNA or RNAi; dsRNA; RNA Polymerase III transcribed DNAs; ribozymes; and antisense nucleic acids, which can be RNA, DNA, or an artificial nucleic acid. Antisense oligonucleotides, including antisense DNA, RNA, and DNA/RNA molecules, act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. Antisense oligonucleotides of at least about 15 bases can be synthesized, e.g., by conventional phosphodiester techniques (Dallas *et al.*, (2006) *Med. Sci. Monit.*12(4):RA67-74; Kalota *et al.*, (2006) *Handb. Exp. Pharmacol.* 173:173-96; Lutzelburger *et al.*, (2006) *Handb. Exp. Pharmacol.* 173:243-59). Antisense nucleotide sequences include, but are not limited to: morpholinos, 2'-O-methyl polynucleotides, DNA, RNA and the like.

[00233] siRNA comprises a double stranded structure containing from about 15 to about 50 base pairs, for example from about 21 to about 25 base pairs, and having a nucleotide sequence identical or nearly identical to an expressed target gene or RNA within the cell. The siRNA comprise a sense RNA strand and a complementary antisense RNA strand annealed together by standard Watson-Crick base-pairing interactions. The sense strand comprises a nucleic acid sequence which is substantially identical to a nucleic acid sequence contained within the target miRNA molecule. "Substantially identical" to a target sequence contained within the target mRNA refers to a nucleic acid sequence that differs from the target sequence by about 3% or less. The sense and antisense strands of the siRNA can comprise two complementary, single-stranded RNA molecules, or can comprise a single molecule in which two complementary

portions are base-paired and are covalently linked by a single-stranded "hairpin" area. See also, McMnaus and Sharp (2002) *Nat Rev Genetics*, 3:737-47, and Sen and Blau (2006) *FASEB J.*, 20:1293-99, the entire disclosures of which are herein incorporated by reference.

[00234] The siRNA can be altered RNA that differs from naturally-occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siRNA or to one or more internal nucleotides of the siRNA, or modifications that make the siRNA resistant to nuclease digestion, or the substitution of one or more nucleotides in the siRNA with deoxyribonucleotides. One or both strands of the siRNA can also comprise a 3' overhang. As used herein, a 3' overhang refers to at least one unpaired nucleotide extending from the 3'-end of a duplexed RNA strand. For example, the siRNA can comprise at least one 3' overhang of from 1 to about 6 nucleotides (which includes ribonucleotides or deoxyribonucleotides) in length, or from 1 to about 5 nucleotides in length, or from 1 to about 4 nucleotides in length, or from about 2 to about 4 nucleotides in length. For example, each strand of the siRNA can comprise 3' overhangs of dithymidylic acid ("TT") or diuridylic acid ("uu").

[00235] siRNA can be produced chemically or biologically, or can be expressed from a recombinant plasmid or viral vector (for example, see U.S. Patent No. 7,294,504 and U.S. Patent No. 7,422,896, the entire disclosures of which are herein incorporated by reference). Exemplary methods for producing and testing dsRNA or siRNA molecules are described in U.S. Patent Application Publication No. 2002/0173478 to Gewirtz, U.S. Patent Application Publication No. 2007/0072204 to Hannon *et al.*, and in U.S. Patent Application Publication No.2004/0018176 to Reich *et al.*, the entire disclosures of which are herein incorporated by reference.

[00236] RNA polymerase III transcribed DNAs contain promoters, such as the U6 promoter. These DNAs can be transcribed to produce small hairpin RNAs in the cell that can function as siRNA or linear RNAs that can function as antisense RNA. A compound can contain ribonucleotides, deoxyribonucleotides, synthetic nucleotides, or any suitable combination such that the target RNA and/or gene is inhibited. In addition, these forms of nucleic acid can be single, double, triple, or quadruple stranded. (see for example Bass (2001) *Nature*, 411, 428 429; Elbashir *et al.*, (2001) *Nature*, 411, 494 498; and PCT Publication Nos. WO 00/44895, WO 01/36646, WO 99/32619, WO 00/01846, WO 01/29058, WO 99/07409, WO 00/44914).

[00237] A compound can be a small molecule that binds to a protein and disrupts its function, or conversely, enhances its function. Small molecules are a diverse group of synthetic and natural substances generally having low molecular weights. They can be isolated from natural

sources (for example, plants, fungi, microbes and the like), are obtained commercially and/or available as libraries or collections, or synthesized. Candidate small molecules can be identified via *in silico* screening or high-through-put (HTP) screening of combinatorial libraries. Most conventional pharmaceuticals, such as aspirin, penicillin, and many chemotherapeutics, are small molecules, can be obtained commercially, can be chemically synthesized, or can be obtained from random or combinatorial libraries as described below (Werner *et al.*, (2006) *Brief Funct. Genomic Proteomic* 5(1):32-6).

[00238] Knowledge of the primary sequence of a molecule of interest, and the similarity of that sequence with proteins of known function, can provide information as to the inhibitors or antagonists of the protein of interest in addition to agonists. Identification and screening of agonists and antagonists is further facilitated by determining structural features of the protein, e.g., using X-ray crystallography, neutron diffraction, nuclear magnetic resonance spectrometry, and other techniques for structure determination. These techniques provide for the rational design or identification of agonists and antagonists.

[00239] Test compounds can be screened from large libraries of synthetic or natural compounds (see Wang *et al.*, (2007) *Curr Med Chem*, 14(2):133-55; Mannhold (2006) *Curr Top Med Chem*, 6 (10):1031-47; and Hensen (2006) *Curr Med Chem* 13(4):361-76). Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based compounds. Synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), AMRI (Albany, NY), ChemBridge (San Diego, CA), and MicroSource (Gaylordsville, CT). A rare chemical library is available from Aldrich (Milwaukee, Wis.). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from e.g. Pan Laboratories (Bothell, Wash.) or MycoSearch (N.C.), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle *et al.*, (1996) *Tib Tech* 14:60).

[00240] Methods for preparing libraries of molecules are well known in the art and many libraries are commercially available. Libraries of interest in the invention include peptide libraries, randomized oligonucleotide libraries, synthetic organic combinatorial libraries, and the like. Degenerate peptide libraries can be readily prepared in solution, in immobilized form as bacterial flagella peptide display libraries or as phage display libraries. Peptide ligands can be selected from combinatorial libraries of peptides containing at least one amino acid. Libraries can be synthesized of peptoids and non-peptide synthetic moieties. Such libraries can further be

synthesized which contain non-peptide synthetic moieties, which are less subject to enzymatic degradation compared to their naturally-occurring counterparts. For example, libraries can also include, but are not limited to, peptide-on-plasmid libraries, synthetic small molecule libraries, aptamer libraries, in vitro translation-based libraries, polysome libraries, synthetic peptide libraries, neurotransmitter libraries, and chemical libraries.

[00241] Examples of chemically synthesized libraries are described in Fodor *et al.*, (1991) *Science* 251:767-773; Houghten *et al.*, (1991) *Nature* 354:84-86; Lam *et al.*, (1991) *Nature* 354:82-84; Medynski, (1994) *BioTechnology* 12:709-710; Gallop *et al.*, (1994) *J. Medicinal Chemistry* 37(9):1233-1251; Ohlmeyer *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90:10922-10926; Erb *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:11422-11426; Houghten *et al.*, (1992) *Biotechniques* 13:412; Jayawickreme *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:1614-1618; Salmon *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90:11708-11712; PCT Publication No. WO 93/20242, dated Oct. 14, 1993; and Brenner *et al.*, (1992) *Proc. Natl. Acad. Sci. USA* 89:5381-5383. Examples of phage display libraries are described in Scott *et al.*, (1990) *Science* 249:386-390; Devlin *et al.*, (1990) *Science*, 249:404-406; Christian, *et al.*, (1992) *J. Mol. Biol.* 227:711-718; Lenstra, (1992) *J. Immunol. Meth.* 152:149-157; Kay *et al.*, (1993) *Gene* 128:59-65; and PCT Publication No. WO 94/18318. *In vitro* translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058; and Mattheakis *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:9022-9026.

[00242] Screening the libraries can be accomplished by any variety of commonly known methods. See, for example, the following references, which disclose screening of peptide libraries: Parmley and Smith, (1989) *Adv. Exp. Med. Biol.* 251:215-218; Scott and Smith, (1990) *Science* 249:386-390; Fowlkes *et al.*, (1992) *BioTechniques* 13:422-427; Oldenburg *et al.*, (1992) *Proc. Natl. Acad. Sci. USA* 89:5393-5397; Yu *et al.*, (1994) *Cell* 76:933-945; Staudt *et al.*, (1988) *Science* 241:577-580; Bock *et al.*, (1992) *Nature* 355:564-566; Tuerk *et al.*, (1992) *Proc. Natl. Acad. Sci. USA* 89:6988-6992; Ellington *et al.*, (1992) *Nature* 355:850-852; U.S. Patent Nos. 5,096,815; 5,223,409; and 5,198,346, all to Ladner *et al.*; Rebar *et al.*, (1993) *Science* 263:671-673; and PCT Pub. WO 94/18318.

[00243] Small molecule combinatorial libraries can also be generated and screened. A combinatorial library of small organic compounds is a collection of closely related analogs that differ from each other in one or more points of diversity and are synthesized by organic techniques using multi-step processes. Combinatorial libraries include a vast number of small organic compounds. One type of combinatorial library is prepared by means of parallel

synthesis methods to produce a compound array. A compound array can be a collection of compounds identifiable by their spatial addresses in Cartesian coordinates and arranged such that each compound has a common molecular core and one or more variable structural diversity elements. The compounds in such a compound array are produced in parallel in separate reaction vessels, with each compound identified and tracked by its spatial address. Examples of parallel synthesis mixtures and parallel synthesis methods are provided in U.S. Ser. No. 08/177,497, filed Jan. 5, 1994 and its corresponding PCT published patent application W095/18972, published Jul. 13, 1995 and U.S. Pat. No. 5,712,171 granted Jan. 27, 1998 and its corresponding PCT published patent application W096/22529, which are hereby incorporated by reference.

[00244] In one non-limiting example, non-peptide libraries, such as a benzodiazepine library (see e.g., Bunin *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:4708-4712), can be screened. Peptoid libraries, such as that described by Simon *et al.*, (1992) *Proc. Natl. Acad. Sci. USA* 89:9367-9371, can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh *et al.* (1994), *Proc. Natl. Acad. Sci. USA* 91:11138-11142.

[00245] Computer modeling and searching technologies permit the identification of compounds, or the improvement of already identified compounds, that can treat or prevent a metabolic disorder in a subject, treat or prevent coronary artery disease in a subject with a metabolic disorder, reduce hepatic glucose production in a subject, and/or inhibit or reduce the activity and/or activation of CaMKII, IP3R, including but not limited to, IP3R1, IP3R2, IP3R3, calcineurin, p38, MK2/3, or any combination thereof. Other methods for preparing or identifying peptides that bind to a target are known in the art. Molecular imprinting, for instance, can be used for the de novo construction of macromolecular structures such as peptides that bind to a molecule. See, for example, Kenneth J. Shea, Molecular Imprinting of Synthetic Network Polymers: The De Novo synthesis of Macromolecular Binding and Catalytic Sites, TRIP Vol. 2, No. 5, May 1994; Mosbach, (1994) *Trends in Biochem. Sci.*, 19(9); and Wulff, G., in Polymeric Reagents and Catalysts (Ford, W. T., Ed.) ACS Symposium Series No. 308, pp 186-230, American Chemical Society (1986). One method for preparing such structures involves the steps of: (i) polymerization of functional monomers around a known substrate (the template) that exhibits a desired activity; (ii) removal of the template molecule; and then (iii) polymerization of a second class of monomers in, the void left by the template, to provide a new

molecule which exhibits one or more desired properties which are similar to that of the template. In addition to preparing peptides in this manner other binding molecules such as polysaccharides, nucleosides, drugs, nucleoproteins, lipoproteins, carbohydrates, glycoproteins, steroids, lipids, and other biologically active materials can also be prepared. This method is useful for designing a wide variety of biological mimics that are more stable than their natural counterparts, because they are prepared by the free radical polymerization of functional monomers, resulting in a compound with a nonbiodegradable backbone. Other methods for designing such molecules include for example drug design based on structure activity relationships, which require the synthesis and evaluation of a number of compounds and molecular modeling.

[00246] Screening Assays. Test compounds or agents can be identified by two types of assays: (a) cell-based assays; or (b) cell-free assays. The assay can be a binding assay comprising direct or indirect measurement of the binding of a test compound. The assay can also be an activity assay comprising direct or indirect measurement of the activity of a compound. The assay can also be an expression assay comprising direct or indirect measurement of the expression of mRNA nucleic acid sequences or a protein encoded by a gene of interest. The various screening assays can be combined with an *in vivo* assay comprising measuring the effect of the test compound on the symptoms of a metabolic disorder or coronary artery disease or elevated hepatic glucose. An *in vivo* assay can also comprise assessing the effect of a test compound on a metabolic disorder or coronary artery disease or elevated hepatic glucose in known mammalian models.

[00247] Assays for screening test compounds that bind to or modulate the activity of a protein of interest can also be carried out. The test compound can be obtained by any suitable means, such as from conventional compound libraries. Determining the ability of the test compound to bind to a membrane-bound form of the protein can be accomplished via coupling the test compound with a radioisotope or enzymatic label such that binding of the test compound to the cell expressing a protein of interest can be measured by detecting the labeled compound in a complex. For example, the test compound can be labeled with ^{3}H, ^{14}C, ^{35}S, or ^{125}I, either directly or indirectly, and the radioisotope can be subsequently detected by direct counting of radioemmission or by scintillation counting. Alternatively, the test compound can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

[00248] A protein of interest or the target of a protein of interest can be immobilized to facilitate the separation of complexed from uncomplexed forms of one or both of the proteins. Binding of a test compound to a protein of interest, such as CaMKII, IP3R, including but not limited to, IP3R1, IP3R2, IP3R3, calcineurin, p38, or MK2/3, or a variant thereof, or interaction of a protein of interest with a target molecule in the presence and absence of a test compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix (for example, glutathione-S-transferase (GST) fusion proteins or glutathione-S-transferase fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical; St. Louis, Mo.) or glutathione derivatized microtiter plates).

[00249] A protein of interest, or a variant thereof, can also be immobilized via being bound to a solid support. Non-limiting examples of suitable solid supports include glass or plastic slides, tissue culture plates, microtiter wells, tubes, silicon chips, or particles such as beads (including, but not limited to, latex, polystyrene, or glass beads). Any method known in the art can be used to attach a polypeptide (or polynucleotide) or a variant thereof, or test compound to a solid support, including use of covalent and non-covalent linkages, or passive absorption.

[00250] The screening methods of the invention can also involve monitoring the expression of a protein of interest, such as CaMKII, IP3R, including but not limited to, IP3R1, IP3R2, IP3R3, calcineurin, p38, or MK2/3, or a variant thereof. For example, regulators of the expression of a protein of interest can be identified via contacting a cell with a test compound and determining the expression of a protein of interest in the cell. The expression level of a protein of interest in the cell in the presence of the test compound is compared to the expression level of a protein of interest in the absence of the test compound. The test compound can then be identified as a regulator of the expression of a protein of interest based on this comparison. For example, when expression of a protein of interest in the cell is statistically or significantly greater in the presence of the test compound than in its absence, the test compound is identified as a stimulator/enhancer of expression of a protein of interest in the cell. Alternatively, when expression of a protein of interest in the cell is statistically or significantly less in the presence of the test compound than in its absence, the compound is identified as an inhibitor of the expression of a protein of interest in the cell. The test compound can also be said to be an antagonist. The methods to determine the expression level of a protein encoded by a gene or mRNA of interest in the cell are well known in the art.

[00251] For binding assays, the test compound can be a small molecule which binds to and occupies the binding site of a polypeptide encoded by a gene of interest, or a variant thereof. This can make the ligand binding site inaccessible to substrate such that normal biological activity is prevented. Examples of such small molecules include, but are not limited to, small peptides or peptide-like molecules. In binding assays, either the test compound or a polypeptide encoded by a gene of interest can comprise a detectable label, such as a fluorescent, radioisotopic, chemiluminescent, or enzymatic label (for example, alkaline phosphatase, horseradish peroxidase, or luciferase). Detection of a test compound which is bound to a polypeptide encoded by a gene of interest can then be determined via direct counting of radioemmission, by scintillation counting, or by determining conversion of an appropriate substrate to a detectable product.

[00252] Determining the ability of a test compound to bind to a protein of interest also can be accomplished using real-time Biamolecular Interaction Analysis (BIA) [McConnell *et al.*, 1992 , *Science* 257, 1906-1912; Sjolander, Urbaniczky, 1991 , *Anal. Chem.* 63, 2338-2345]. BIA is a technology for studying biospecific interactions in real time, without labeling any of the interactants (for example, BIA-core™). Changes in the optical phenomenon surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

[00253] To identify other proteins which bind to or interact with a protein of interest and modulate its activity, a polypeptide encoded by a gene of interest can be used as a bait protein in a two-hybrid assay or three-hybrid assay (Szabo *et al.*, 1995 , *Curr. Opin. Struct. Biol.* 5, 699-705; U.S. Pat. No. 5,283,317), according to methods practiced in the art. The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains.

[00254] Functional Assays. Compounds can be tested for the ability to increase or decrease the activity of a protein of interest, such as CaMKII, IP3R, including but not limited to, IP3R1, IP3R2, IP3R3, calcineurin, p38, or MK2/3, or a variant thereof. Activity can be measured after contacting a purified protein of interest, a cell membrane preparation, or an intact cell with a test compound. A test compound that decreases the activity of a protein of interest by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, at least about 95% or 100% is identified as a potential agent for decreasing the activity of a protein of interest, for example an antagonist. A test compound that increases the activity of a protein of

interest by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, at least about 95% or 100% is identified as a potential agent for increasing the activity of a protein of interest, for example an agonist.

Compounds

[00255] The present disclosure provides methods for the treatment and/or prevention of a metabolic disorder in a subject. The disclosure also provides methods for the treatment and/or prevention of coronary artery disease in a subject with a metabolic disorder. In one embodiment, the disorder is induced by obesity. In another embodiment, the disorder is not induced by obesity. The disclosure also provides methods for reducing hepatic glucose production in a subject. The disclosure also provides methods for reducing the activity or inhibiting the activity of CaMKII, IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, calcineurin, p38 or MK2/3. The disclosure also provides methods for reducing the phosphorylation and/or activation of CaMKII, IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, calcineurin, p38 or MK2/3.

[00256] In one aspect, the present disclosure provides a method of treating or preventing a metabolic disorder in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a compound, thereby treating or preventing the disorder.

[00257] In one embodiment, the compound is an inhibitor of CamKII. In another embodiment, the compound is an inhibitor of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3. In one embodiment, the compound is an inhibitor of calcineurin. In one embodiment, the compound is an inhibitor of p38. In another embodiment, the compound is an inhibitor of MK2/3. In another embodiment, the compound is an inhibitor of CamKII, IP3R, including, but not limited to, IP3R1, IP3R2, and/or IP3R3, calcineurin, p38, MK2/3, or any combination thereof.

[00258] In one embodiment, the compound is an activator of CaMKII. In another embodiment, the compound is an activator of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3. In one embodiment, the compound is an activator of calcineurin. In one embodiment, the compound is an activator of p38. In another embodiment, the compound is an activator of MK2/3. In another embodiment, the compound is an activator of CamKII, IP3R,

including, but not limited to, IP3R1, IP3R2, and/or IP3R3, calcineurin, p38, MK2/3, or any combination thereof.

[00259] Any suitable compound, either an inhibitor or activator of the CaMKII protein, IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, calcineurin, p38, and/or MK2/3, may be used in the methods of the invention. Such compounds may be, for example, small molecule drugs, peptide agents, peptidomimetic agents, antibodies (including, but not limited to monoclonal, poycloncal, humanized, and fully human antibodies, as well as antibody fragments), inhibitory RNA molecules (such as siRNA) and the like. One of skill in the art will understand that these and other types of agents may be used to inhibit or reduce or increase the activity of CaMKII, IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, calcineurin, p38, MK2/3, or any combination thereof, or reduce or increase the phosphorylation and/or activation of CaMKII, IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, calcineurin, p38, MK2/3, or any combination thereof.

[00260] In one embodiment, a compound of the invention is a small molecule inhibitor of CaMKII. Such inhibitors include, but are not limited to, KN-93 (*N*-[2-[[[3-(4-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-*N*-(2-hydroxyethyl)-4-methoxybenzenesulphonamide); lavendustin C (5-((2,5-Dihydroxybenzyl)amino)-2-hydroxybenzoic acid); CK59 (2-(2-Hydroxyethylamino)-6-aminohexylcarbamic acid tert-butyl ester-9-isopropylpurine); KN62 (*see* Clyne *et al.*, 1995); DY9760e (*see* Sugimura *et al.*, 1997); K-252a *Nocardiopsis sp.* (*see* Kase *et al.*, 1987); H89 dihydrochloride (N-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride); PP1 Analog II, 1NM-PP1 (Mutant Kinases Inhibitor II, 4-Amino-1-tert-butyl-3-(1′-naphthylmethyl)pyrazolo[3,4-d]pyrimidine, NM); eEF-2 kinase inhibitor NH125 (1-Benzyl-3-cetyl-2-methylimidazolium iodide, 1-Cetyl-3-benzyl-2-methylimidazolium iodide, CaM-Dependent Kinase III Inhibitor, NH125), and STO-609 (*see* Tokumitsu *et al.*, 2002).

[00261] In another embodiment, a compound of the invention is a peptide or peptidomimetic inhibitor or activator of CaMKII. Such peptides or peptidomimetic inhibitors or activators include, but are not limited to, Ant-CaMKIINtide, [Ala^{286}]-Ca^{2+}/Calmodulin Kinase II inhibitor 281-301, [Ala^{286}]-Ca^{2+}/Calmodulin Kinase II inhibitor 281-309 and CaM Kinase II (290-309) calmodulin antagonist.

[00262] In another embodiment, a compound of the invention is a protein or polypeptide inhibitor or activator of CaMKII. Such a protein or polypeptide inhibitor may be a recombinant protein or polypeptide, such as, but not limited to, oncomodulin/MDP14.

[00263] In another embodiment, a compound of the invention is an antibody inhibitor or activator of a component of the CaMKII protein. In yet another embodiment, a compound of the invention is a nucleotide-based inhibitor or activator of CaMKII. Such inhibitors include, but are not limited to siRNAs, shRNAs, dsRNAs, microRNAs, antisense RNA molecules, and ribozymes, that inhibit the expression or activity of CaMKII, or a variant thereof. Such nucleotide-based inhibitors may comprise ribonucleotides, deoxyribonucleotides, or various artificial nucleotide derivatives.

[00264] Other compounds that may inhibit the activity of CaMKII or reduce the activation of CaMKII are further described in U.S. Patent No. 7,205,298 (Kuo et al.,), U.S. Publication No. 2004/0086973 (Duecker K.), U.S. Publication No. 2010-0056494 (Winzeler et al.), U.S. Patent No. 5,386,019 (Danishefsky et al.) and U.S. Patent No. 6,828,327 (Kuo et al.), which are incorporated by reference in their entireties.

[00265] In one embodiment, a compound of the invention is a small molecule inhibitor of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3. Such inhibitors include, but are not limited to, Xestospongin C from Calbiochem (EMD Millipore) cat# 682160. Xestospongin C is an oxaquinolizidine alkaloid isolated from a marine sponge and a very potent, reversible, and membrane-permeable blocker of IP_3-mediated Ca^{2+} release (IC_{50} = 358 nM) that does not interact with the IP_3-binding site. It displays high selectivity over the skeletal isoform of the ryanodine receptor type 1 (RyR-1). It also blocks bradykinin- and carbamylcholine-induced Ca^{2+} efflux from the endoplasmic reticulum stores in a reversible manner. Other small molecule inhibitors of IP3R include aminoethoxydiphenyl borate (also called 2-APB), from Tocris, Bristol, UK (*see* Sugawara *et al.*, 1997, EMBO J., 16:3078-88, herein incorporated by reference in its entirety), and caffeine. In another embodiment, a compound of the invention is a small molecule activator of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3.

[00266] In another embodiment, a compound of the invention is a peptide or peptidomimetic inhibitor or activator of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3. In another embodiment, a compound of the invention is a protein or polypeptide inhibitor or activator of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3. Such a protein or polypeptide inhibitor may be a recombinant protein or polypeptide.

[00267] In another embodiment, a compound of the invention is an antibody inhibitor or activator of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3. In yet another embodiment, a compound of the invention is a nucleotide-based inhibitor or activator of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3. Such inhibitors include, but are not

limited to siRNAs, shRNAs, dsRNAs, microRNAs, antisense RNA molecules, and ribozymes, that inhibit the expression or activity of IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, or a variant thereof. Such nucleotide-based inhibitors may comprise ribonucleotides, deoxyribonucleotides, or various artificial nucleotide derivatives.

[00268] In one embodiment, a compound of the invention is a small molecule inhibitor of calcineurin. Such inhibitors include, but are not limited to, pimecrolimus and tacrolimus. In another embodiment, a compound of the invention is a small molecule activator of calcineurin.

[00269] In another embodiment, a compound of the invention is a peptide or peptidomimetic inhibitor or activator of calcineurin. Such a peptide or peptidomimmetic inhibitor includes the calcineurin (PP2B) inhibitor cyclosporin A from Calbiochem (EMD Millipore) cat#239835. This compound is a cyclic oligopeptide with immunosuppressant properties that induces apoptosis in rat thymocytes and in the murine B cell lymphoma cell line, WEH1-231. It prevents anti-IgM and ionomycin-induced apoptosis in BLB cell lines. The complex of cyclosporin A with cyclophilin inhibits protein phosphatase 2B with nanomolar affinity and inhibits nitric oxide synthesis induced by interleukin-1α, lipopolys*a*cch*a*rides, *a*nd TNF-α. It also induces c*a*rdiomyocytes from embryonic stem cells.

[00270] In another embodiment, a compound of the invention is a protein or polypeptide inhibitor or activator of calcineurin. Such a protein or polypeptide inhibitor may be a recombinant protein or polypeptide.

[00271] In another embodiment, a compound of the invention is an antibody inhibitor or activator of calcineurin. In yet another embodiment, a compound of the invention is a nucleotide-based inhibitor or activator of calcineurin. Such inhibitors include, but are not limited to siRNAs, shRNAs, dsRNAs, microRNAs, antisense RNA molecules, and ribozymes, that inhibit the expression or activity of calcineurin, or a variant thereof. Such nucleotide-based inhibitors may comprise ribonucleotides, deoxyribonucleotides, or various artificial nucleotide derivatives.

[00272] In one embodiment, a compound of the invention is a small molecule inhibitor of p38. Such inhibitors include, but are not limited to, SB 202190, SB203580 and SB 239063. SB203580 is a compound available from Calbiochem (EMD Millipore) cat#559389. It is a highly specific, potent, cell-permeable, selective, reversible, and ATP-competitive inhibitor of p38 MAP kinase (IC50 = 34 nM in vitro, 600 nM in cells). It does not significantly inhibit the JNK and p42 MAP kinase at 100 μM. It reduces epirubicin-induced cell injury and caspase-3/7 activity and inhibits IL-1 and TNF-α production from LPS-stimulated human monocytes and the

human monocyte cell line THP-1 (IC50 = 50-100 nM). It inhibits bone morphogenetic protein-2-induced neurite outgrowth in PC12 cells. In another embodiment, a compound of the invention is a small molecule activator of p38.

[00273] In another embodiment, a compound of the invention is a peptide or peptidomimetic inhibitor or activator of p38.

[00274] In another embodiment, a compound of the invention is a protein or polypeptide inhibitor or activator of p38. Such a protein or polypeptide inhibitor may be a recombinant protein or polypeptide.

[00275] In another embodiment, a compound of the invention is an antibody inhibitor or activator of p38. In yet another embodiment, a compound of the invention is a nucleotide-based inhibitor or activator of p38. Such inhibitors include, but are not limited to siRNAs, shRNAs, dsRNAs, microRNAs, antisense RNA molecules, and ribozymes, that inhibit the expression or activity of p38, or a variant thereof. Such nucleotide-based inhibitors may comprise ribonucleotides, deoxyribonucleotides, or various artificial nucleotide derivatives.

[00276] In one embodiment, a compound of the invention is a small molecule inhibitor of MK2/3. In another embodiment, a compound of the invention is a small molecule activator of MK2/3. Such small molecule inhibitors of MK2/3 include, but are not limited, aminocyoanopyridine compounds, pyrrolopyridines, and carboline-based MK2 inhibitors (*see* Fyhrquist et al., 2010, *J. Investig, Dermatol.*, 130: 342-344, which is incorporated by reference herein in its entirety).

[00277] In another embodiment, a compound of the invention is a peptide or peptidomimetic inhibitor or activator of MK2/3. Such peptide inhibitors include, but are not limited to, Hsp25 Kinase Inhibitor, available from Calbiochem (EMD Millipore) cat# 385880. The inhibitor is a 13-residue, cell-permeable peptide that acts as a potent and selective inhibitor of mammalian heat-shock protein (Hsp25) kinase [also called mitogen-activated protein kinase-activated protein kinase-2 (MAPKAP kinase-2)]. Inhibition is competitive with respect to the substrate peptide (Ki = 8.1 μM) and non-competitive with respect to ATP (Ki = 134 μM).

[00278] In another embodiment, a compound of the invention is a protein or polypeptide inhibitor or activator of MK2/3. Such a protein or polypeptide inhibitor may be a recombinant protein or polypeptide.

[00279] In another embodiment, a compound of the invention is an antibody inhibitor or activator of MK2/3. In yet another embodiment, a compound of the invention is a nucleotide-

based inhibitor or activator of MK2/3. Such inhibitors include, but are not limited to siRNAs, shRNAs, dsRNAs, microRNAs, antisense RNA molecules, and ribozymes, that inhibit the expression or activity of MK2/3, or a variant thereof. Such nucleotide-based inhibitors may comprise ribonucleotides, deoxyribonucleotides, or various artificial nucleotide derivatives.

[00280] One of skill in the art will understand that other agents may be useful as inhibitors or activators of CaMKII, IP3R, including, but not limited to, IP3R1, IP3R2, and IP3R3, calcineurin, p38, and/or MK2/3, and may be used in conjunction with the methods of the invention.

Pharmaceutical Compositions and Administration for Therapy

[00281] Compounds of the invention can be administered to the subject once (e.g., as a single injection or deposition). Alternatively, compounds of the invention can be administered once or twice daily to a subject in need thereof for a period of from about two to about twenty-eight days, or from about seven to about ten days. Compounds of the invention can also be administered once or twice daily to a subject for a period of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 times per year, or a combination thereof. Furthermore, compounds of the invention can be co-administrated with another therapeutic. Where a dosage regimen comprises multiple administrations, the effective amount of the compound(s) administered to the subject can comprise the total amount of the compound(s) administered over the entire dosage regimen.

[00282] Compounds can be administered to a subject by any means suitable for delivering the compounds to cells of the subject. For example, compounds can be administered by methods suitable to transfect cells. Transfection methods for eukaryotic cells are well known in the art, and include direct injection of a nucleic acid into the nucleus or pronucleus of a cell; electroporation; liposome transfer or transfer mediated by lipophilic materials; receptor mediated nucleic acid delivery, bioballistic or particle acceleration; calcium phosphate precipitation, and transfection mediated by viral vectors.

[00283] The compositions of this invention can be formulated and administered to reduce the symptoms associated with a metabolic disorder, or coronary artery disease, or elevated hepatic glucose production, by any means that produces contact of the active ingredient with the agent's site of action in the body of a subject, such as a human or animal (e.g., a dog, cat, or horse). They can be administered by any conventional means available for use in conjunction with pharmaceuticals, either as individual therapeutic active ingredients or in a combination of therapeutic active ingredients. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

[00284] The compounds of the invention may be administered to a subject in an amount effective to treat or prevent a metabolic disorder, or coronary artery disease, or to reduce hepatic glucose production. One of skill in the art can readily determine what will be an effective amount of the compounds of the invention to be administered to a subject, taking into account whether the compound is being used prophylactically or therapeutically, and taking into account other factors such as the age, weight and sex of the subject, any other drugs that the subject may be taking, any allergies or contraindications that the subject may have, and the like. For example, an effective amount can be determined by the skilled artisan using known procedures, including analysis of titration curves established *in vitro* or *in vivo*. Also, one of skill in the art can determine the effective dose from performing pilot experiments in suitable animal model species and scaling the doses up or down depending on the subjects weight etc. Effective amounts can also be determined by performing clinical trials in individuals of the same species as the subject, for example starting at a low dose and gradually increasing the dose and monitoring the effects on a metabolic disorder, or coronary artery disease. Appropriate dosing regimens can also be determined by one of skill in the art without undue experimentation, in order to determine, for example, whether to administer the agent in one single dose or in multiple doses, and in the case of multiple doses, to determine an effective interval between doses.

[00285] A therapeutically effective dose of a compound that treats or prevents a metabolic disorder or coronary artery disease, or reduces hepatic glucose production, can depend upon a number of factors known to those of ordinary skill in the art. The dose(s) of the compounds can vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the compound to have upon the target of interest. These amounts can be readily determined by a skilled artisan. These amounts include, for example, mg or microgram (μg) amounts per kilogram (kg) of subject weight, such as about 0.25 mg/kg, 0.5 mg/kg, about 1 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 6 mg/kg, about 7 mg/kg, about 8 mg/kg, about 9 mg/kg or about 10 mg/kg, or between about 0.25 mg/kg to about 0.5 mg/kg, 0.5 mg/kg to 1 mg/kg, 1 mg/kg to 2 mg/kg, 2 mg/kg to 3 mg/kg, 3 mg/kg to 4 mg/kg, 4 mg/kg to 5 mg/kg, 5 mg/kg to 6 mg/kg, 6 mg/kg to 7 mg/kg, 7 mg/kg to 8 mg/kg, 8 mg/kg to 9 mg/kg, or 9 mg/kg to 10 mg/kg, or any range in between. These amounts also include a unit dose of a compound, for example, at least about 0.5 mg, 1 mg, 2 mg, 3 mg, 5 mg, 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60 mg, 70 mg, 80 mg, 90 mg, 100 mg, 110 mg, 120 mg, 130 mg, 140 mg, 150 mg, 160 mg, 170 mg, 180 mg, 190 mg, 200 mg, 225 mg, 250 mg, 275 mg, 300 mg, 325 mg, 350 mg, 375 mg, 400 mg, 425 mg, 450 mg, 475 mg,

500 mg, 525 mg, 550 mg, 575 mg, 600 mg, 625 mg, 650 mg, 675 mg, 700 mg, 750 mg, 800 mg, 850 mg, 900 mg, or more. Any of the therapeutic applications described herein can be applied to any subject in need of such therapy, including, for example, a mammal such as a dog, a cat, a cow, a horse, a rabbit, a monkey, a pig, a sheep, a goat, or a human.

[00286] Pharmaceutical compositions for use in accordance with the invention can be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. The therapeutic compositions of the invention can be formulated for a variety of routes of administration, including systemic and topical or localized administration. Techniques and formulations generally can be found in Remmington's Pharmaceutical Sciences, Meade Publishing Co., Easton, Pa (20th Ed., 2000), the entire disclosure of which is herein incorporated by reference. For systemic administration, an injection is useful, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the therapeutic compositions of the invention can be formulated in liquid solutions, for example in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the therapeutic compositions can be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included. Pharmaceutical compositions of the present invention are characterized as being at least sterile and pyrogen-free. These pharmaceutical formulations include formulations for human and veterinary use.

[00287] According to the invention, a pharmaceutically acceptable carrier can comprise any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Any conventional media or agent that is compatible with the active compound can be used. Supplementary active compounds can also be incorporated into the compositions.

[00288] The invention also provides for a kit that comprises a pharmaceutically acceptable carrier and a compound identified using the screening assays of the invention packaged with instructions for use.

[00289] A pharmaceutical composition containing a compound of the invention can be administered in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed herein. Such pharmaceutical compositions can comprise, for example antibodies directed to polypeptides encoded by genes of interest or variants thereof, or agonists and antagonists of a polypeptide encoded by a gene of interest. The compositions can be administered alone or in combination with at least one other agent, such as a stabilizing

compound, which can be administered in any sterile, biocompatible pharmaceutical carrier including, but not limited to, saline, buffered saline, dextrose, and water. The compositions can be administered to a patient alone, or in combination with other agents, drugs or hormones.

[00290] A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[00291] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EM™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, a pharmaceutically acceptable polyol like glycerol, propylene glycol, liquid polyetheylene glycol, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it can be useful to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of injectable compositions can be brought about by incorporating an agent which delays absorption, for example, aluminum monostearate and gelatin.

[00292] Sterile injectable solutions can be prepared by incorporating the compound (e.g., a small molecule, peptide or antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated herein. In the case of sterile powders for the preparation of sterile injectable solutions, examples of useful preparation methods are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[00293] Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed.

[00294] Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[00295] Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art. In some embodiments, the compound can be applied via transdermal delivery systems, which slowly releases the active compound for percutaneous absorption. Permeation enhancers can be used to facilitate transdermal penetration of the active factors in the conditioned media. Transdermal patches are described in for example, U.S. Pat. No. 5,407,713; U.S. Pat. No. 5,352,456; U.S. Pat. No.

5,332,213; U.S. Pat. No. 5,336,168; U.S. Pat. No. 5,290,561; U.S. Pat. No. 5,254,346; U.S. Pat. No. 5,164,189; U.S. Pat. No. 5,163,899; U.S. Pat. No. 5,088,977; U.S. Pat. No. 5,087,240; U.S. Pat. No. 5,008,110; and U.S. Pat. No. 4,921,475.

[00296] Administration of the compound is not restricted to a single route, but may encompass administration by multiple routes. For instance, exemplary administrations by multiple routes include, among others, a combination of intradermal and intramuscular administration, or intradermal and subcutaneous administration. Multiple administrations may be sequential or concurrent. Other modes of application by multiple routes will be apparent to the skilled artisan.

[00297] The compounds of the invention may be formulated into compositions for administration to subjects for the treatment and/or prevention of a metabolic disorder or coronary artery disease, or the reduction of hepatic glucose production. Such compositions may comprise the compounds of the invention in admixture with one or more pharmaceutically acceptable diluents and/or carriers and optionally one or more other pharmaceutically acceptable additives. The pharmaceutically-acceptable diluents and/or carriers and any other additives must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the subject to whom the composition will be administered. One of skill in the art can readily formulate the compounds of the invention into compositions suitable for administration to subjects, such as human subjects, for example using the teaching a standard text such as Remington's Pharmaceutical Sciences, 18th ed, (Mack Publishing Company: Easton, Pa., 1990), pp. 1635-36), and by taking into account the selected route of delivery.

[00298] Examples of diluents and/or carriers and/or other additives that may be used include, but are not limited to, water, glycols, oils, alcohols, aqueous solvents, organic solvents, DMSO, saline solutions, physiological buffer solutions, peptide carriers, starches, sugars, preservatives, antioxidants, coloring agents, pH buffering agents, granulating agents, lubricants, binders, disintegrating agents, emulsifiers, binders, excipients, extenders, glidants, solubilizers, stabilizers, surface active agents, suspending agents, tonicity agents, viscosity-altering agents, carboxymethyl cellulose, crystalline cellulose, glycerin, gum arabic, lactose, magnesium stearate, methyl cellulose, powders, saline, sodium alginate. The combination of diluents and/or carriers and/or other additives used can be varied taking into account the nature of the active agents used (for example the solubility and stability of the active agents), the route of delivery (e.g. oral, parenteral, etc.), whether the agents are to be delivered over an extended period (such as from a controlled-release capsule), whether the agents are to be co-administered with other

agents, and various other factors. One of skill in the art will readily be able to formulate the compounds for the desired use without undue experimentation.

[00299] The compounds of the invention may be administered to a subject by any suitable method that allows the agent to exert its effect on the subject *in vivo*. For example, the compositions may be administered to the subject by known procedures including, but not limitated to, by oral administration, sublingual or buccal administration, parenteral administration, transdermal administration, via inhalation, via nasal delivery, vaginally, rectally, and intramuscularly. The compounds of the invention may be administered parenterally, or by epifascial, intracapsular, intracutaneous, subcutaneous, intradermal, intrathecal, intramuscular, intraperitoneal, intrasternal, intravascular, intravenous, parenchymatous, or sublingual delivery. Delivery may be by injection, infusion, catheter delivery, or some other means, such as by tablet or spray. In one embodiment, the compounds of the invention are administered to the subject by way of delivery directly to the heart tissue, such as by way of a catheter inserted into, or in the proximity of the subject's heart, or by using delivery vehicles capable of targeting the drug to the heart. For example, the compounds of the invention may be conjugated to or administered in conjunction with an agent that is targeted to the heart, such as an antibody or antibody fragment. In one embodiment, the compounds of the invention are administered to the subject by way of delivery directly to the muscle tissue of interest, such as by way of a catheter inserted into, or in the proximity of the subject's muscle of interest, or by using delivery vehicles capable of targeting the drug to the muscle, such as an antibody or antibody fragment.

[00300] For oral administration, a formulation of the compounds of the invention may be presented as capsules, tablets, powders, granules, or as a suspension or solution. The formulation may contain conventional additives, such as lactose, mannitol, cornstarch or potato starch, binders, crystalline cellulose, cellulose derivatives, acacia, cornstarch, gelatins, disintegrators, potato starch, sodium carboxymethylcellulose, dibasic calcium phosphate, anhydrous or sodium starch glycolate, lubricants, and/or or magnesium stearate.

[00301] For parenteral administration (*i.e.*, administration by through a route other than the alimentary canal), the compounds of the invention may be combined with a sterile aqueous solution that is isotonic with the blood of the subject. Such a formulation may be prepared by dissolving the active ingredient in water containing physiologically-compatible substances, such as sodium chloride, glycine and the like, and having a buffered pH compatible with physiological conditions, so as to produce an aqueous solution, then rendering the solution sterile. The formulation may be presented in unit or multi-dose containers, such as sealed

ampoules or vials. The formulation may be delivered by injection, infusion, or other means known in the art.

[00302] For transdermal administration, the compounds of the invention may be combined with skin penetration enhancers, such as propylene glycol, polyethylene glycol, isopropanol, ethanol, oleic acid, *N*-methylpyrrolidone and the like, which increase the permeability of the skin to the compounds of the invention and permit the compounds to penetrate through the skin and into the bloodstream. The compounds of the invention also may be further combined with a polymeric substance, such as ethylcellulose, hydroxypropyl cellulose, ethylene/vinylacetate, polyvinyl pyrrolidone, and the like, to provide the composition in gel form, which are dissolved in a solvent, such as methylene chloride, evaporated to the desired viscosity and then applied to backing material to provide a patch.

[00303] In some embodiments, the compounds of the invention are provided in unit dose form such as a tablet, capsule or single-dose injection or infusion vial.

Combination Therapy

[00304] According to the methods of the invention, a compound of the invention can be administered to a subject either as a single agent, or in combination with one or more other agents. In one embodiment, a compound of the invention is administered to a subject as a single agent. In one embodiment, a compound of the invention is administered to a subject alone. In one embodiment, a compound of the invention is administered to a subject in combination with one or more other agents.

[00305] In certain embodiments, a compound of the invention may be used in combination with other agents that are used for the treatment or prevention of a metabolic disorder or coronary artery disease, or the reduction of hepatic glucose in a subject. In certain embodiments, a compound of the invention may be used in combination with other agents that are not used for the treatment or prevention of a metabolic disorder or coronary artery disease, or the reduction of hepatic glucose in a subject. In one embodiment, a compound of the invention may be delivered to a subject as part of the same pharmaceutical composition or formulation containing one or more additional active agents. In another embodiment, a compound of the invention may be delivered to a subject in a composition or formulation containing only that active agent, while one or more other agents are administered to the subject in one or more separate compositions or formulations. In one embodiment, the other agents are not used for the treatment or prevention of a metabolic disorder or coronary artery disease, or the reduction of hepatic glucose in a

subject. In another embodiment, the other agents are used for the treatment or prevention of a metabolic disorder or coronary artery disease, or the reduction of hepatic glucose in a subject.

[00306] A compound of the invention and the other agents that are used for the treatment or prevention of a metabolic disorder or coronary artery disease, or the reduction of hepatic glucose in a subject, may be administered to the subject at the same time, or at different times. A compound of the invention and the other agents that are not used for the treatment or prevention of a metabolic disorder or coronary artery disease, or the reduction of hepatic glucose, may be administered to the subject at the same time, or at different times. For example, a compound of the invention and the other agents may be administered within minutes, hours, days, weeks, or months of each other, for example as part of the overall treatment regimen of a subject. In some embodiments, a compound of the invention may be administered prior to the administration of other agents. In other embodiments, a compound of the invention may be administered subsequent to the administration of other agents.

[00307] Compounds of the invention, as described above, including, but not limited to, inhibitors of CaMKII, IP3R, including, but not limited to, IP3R1, IP3R2, IP3R3, calcineurin, p38 and MK2/3, may be used in combination with each other for the treatment or prevention of a metabolic disorder or coronary artery disease, or the reduction of hepatic glucose in a subject.

[00308] In some embodiments, the administration of a compound of the invention in combination with one or more other agents has an additive effect, in comparison with administration of the compound of the invention alone, or administration of the one or more other agents alone. In other embodiments, the administration of a compound of the invention in combination with one or more other agents has a synergistic effect, in comparison with administration of the compound of the invention alone, or administration of the one or more other agents alone. In some embodiments, the administration of a compound of the invention in combination with one or more other agents can help reduce side effects, in comparison with administration of the compound of the invention alone, or administration of the one or more other agents alone.

[00309] In some embodiments, the compound of the invention is used as an adjuvant therapy. In other embodiments, the compound of the invention is used in combination with an adjuvant therapy.

Subjects

[00310] According to the methods of the invention, the subject or patient can be any animal that has or is diagnosed with a metabolic disorder or coronary artery disease, or that has elevated hepatic glucose. According to the methods of the invention, the subject or patient can be any animal that is predisposed to or is at risk of developing a metabolic disorder or coronary artery disease, or elevated hepatic glucose. In preferred embodiments, the subject is a human subject. In some embodiments, the subject is a rodent, such as a mouse. In some embodiments, the subject is a cow, pig, sheep, goat, cat, horse, dog, and/or any other species of animal used as livestock or kept as pets.

[00311] In some embodiments, the subject is already suspected to have a metabolic disorder, coronary artery disease or elevated hepatic glucose. In other embodiments, the subject is being treated for a metabolic disorder, coronary artery disease or elevated hepatic glucose, before being treated according to the methods of the invention. In other embodiments, the subject is not being treated for a metabolic disorder, coronary artery disease or elevated hepatic glucose, before being treated according to the methods of the invention.

EXAMPLES

[00312] The following examples illustrate the present invention, and are set forth to aid in the understanding of the invention, and should not be construed to limit in any way the scope of the invention as defined in the claims which follow thereafter.

Example 1: Regulation of hepatocyte glucose production by a calcium sensing enzyme, CaMKII

[00313] Hepatic glucose production is crucial for glucose homeostasis. Several transcription factors and co-activators have been shown to regulate this process, however, the underlying mechanisms have not been fully elucidated. As described herein, a calcium sensing enzyme, CaMKII, is activated in a calcium- and IP3R-dependent manner by cAMP and glucagon in primary hepatocytes and by glucagon and fasting in vivo. Genetic deficiency or inhibition of CaMKII blocks nuclear translocation of FoxO1, impairs fasting- and glucagon/cAMP-induced glycogenolysis and gluconeogenesis, and lowers blood glucose levels. Conversely, adenoviral expression of constitutively active CaMKII induces genes involved in gluconeogenesis and glycogenolysis, stimulates glucose production both in vitro and in vivo, and raises blood glucose levels. The suppressive effect of CaMKII deficiency on glucose metabolism is abrogated by transduction with constitutively nuclear FoxO1, indicating that the effect of CaMKII deficiency requires nuclear exclusion of FoxO1. These results reveal a new, calcium-sensing molecular pathway in the control of hepatic glucose homeostasis by glucagon and fasting.

Example 2: Regulation of hepatocyte glucose production by a calcium sensing enzyme, CaMKII

[00314] Hepatic glucose production is crucial for glucose homeostasis. Several transcription factors and co-activators have been shown to regulate this process, however, the underlying mechanisms have not been fully elucidated. As described herein, a calcium sensing enzyme, CaMKII, is activated in a calcium- and IP3R-dependent manner by cAMP and glucagon in primary hepatocytes and by glucagon and fasting in vivo. Genetic deficiency or inhibition of CaMKII blocks nuclear translocation of FoxO1, impairs fasting- and glucagon/cAMP-induced glycogenolysis and gluconeogenesis, and lowers blood glucose levels. Conversely, adenoviral expression of constitutively active CaMKII induces genes involved in gluconeogenesis and glycogenolysis, stimulates glucose production both in vitro and in vivo, and raises blood glucose

levels. Importantly, the suppressive effect of CaMKII deficiency on glucose metabolism is abrogated by transduction with constitutively nuclear FoxO1, indicating that the effect of CaMKII deficiency requires nuclear exclusion of FoxO1. The results described herein show a calcium-sensing molecular pathway in the control of hepatic glucose homeostasis by glucagon and fasting.

Example 3: Regulation of hepatocyte glucose production by a calcium sensing enzyme, CaMKII

[00315] Liver is the main organ responsible for maintaining euglycemia under conditions of nutrient deprivation. During the early stages of fasting, liver uses glycogen stores to mobilize glucose (Radziuk and Pye, 2001). As fasting progresses, de novo synthesis of glucose from non-carbohydrate precursors, gluconeogenesis, becomes the main contributor to hepatic glucose production (Klover and Mooney, 2004). Glucose production is also regulated by substrate flux through glycolysis, glycogen synthesis, and glycogenolysis. These changes occur rapidly in response to direct hormonal signaling. In addition, both insulin and glucagon affect transcription of glycogenolytic and gluconeogenic enzymes, glucose-6-phosphatase (G6pc) and phosphoenolpyruvate carboxykinase (Pck1), respectively (Pilkis and Granner, 1992). During fasting, glucagon and its downstream effector, cAMP, induce changes in the subcellular localization of "glucogenic" transcription factors, such as FoxO (1, 3, and 4) and Crct2, which activate expression of these genes (Lin and Accili, 2011). In addition, different co-activators, such as peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and CBP, are thought to interact with different components of the cAMP response, including CREB, hepatic nuclear factor 4α (HNF4α), Sirt1, and Clock genes, leading to an increase in transcription of gluconeogenic genes (Hall et al., 1995; Matsumoto et al., 2007; Puigserver et al., 2003; Rhee et al., 2003).

[00316] Calcium (Ca+2) has been linked to the regulation of gluconeogenesis, however the underlying mechanisms have not been fully elucidated (Friedmann and Rasmussen, 1970; Kraus-Friedmann and Feng, 1996; Marques-da-Silva et al., 1997). Evidence indicates that glucagon and cAMP alter Ca+2 fluxes in the liver. Stimulation by glucagon leads to calcium influx which is then followed by the release of Ca+2 from intracellular stores, and as a result of these changes, cytosolic Ca+2 concentration increases (Bygrave and Benedetti, 1993; Staddon and Hansford, 1989). Noteworthy, intracellular Ca+2 chelation has been shown to reduce glucagon induced glucose production (Mine et al., 1993). However the mechanism of how Ca+2 regulates this phenomenon is not known. Based on these previous studies showing the importance of

intracellular Ca+2, the results described herein show that CaMKII, the activity of which is increased by Ca+2, can play a role in glucagon-induced hepatic glucose production.

[00317] Calcium calmodulin-dependent kinase II (CaMKII) is a serine–threonine kinase that is an important mediator of cellular Ca+2 signaling in cells. There are four genes for different CaMKII isoforms: α, β, γ and δ. The α and β isoforms are mostly neuronal, while CaMKIIγ and δ are expressed in a wide variety of tissues. After binding Ca+2/calmodulin complex, autophosphorylation on Thr287 results in Ca+2/calmodulin independent activity (Couchonnal and Anderson, 2008). Most studies on CaMKII have been carried out in neurons and cardiomyocytes and there is only a limited understanding of CaMKII in other tissues, and the specific role of CaMKII in metabolism remains unknown. The results described herein show that CaMKII activity is increased by cAMP and glucagon and also in response to fasting in vivo. The results described herein demonstrate that CaMKII plays a role in the regulation of glycogenolysis and gluconeogenesis. In particular, these results show that CaMKII has a profound effect on FoxO1 nuclear localization in a manner that regulates the expression of two key enzymes, G6pc and Pck1 in vitro and in vivo.

Results

[00318] Fed-to-fasting metabolic switch leads to activation of hepatic CaMKII

[00319] Fasting leads to an increase in circulating levels of glucagon, which has been shown to increase intracellular calcium (Staddon and Hansford, 1989). The results described herein show that CaMKII, the activity of which is increased by a rise in intracellular Ca+2, might be activated by fasting. To test this idea, a CaMKII activity assay from primary mouse hepatocytes challenged with glucagon for various times showed that CaMKII activity increased steadily as a function of time (**Fig. 1A**). The activation state of CaMKII by immunoblotting with anti-pThr287-CaMKII antibody. Consistent with the kinase assay results, phosphorylation at Thr287 was induced by glucagon treatment (**Fig. 1B**).

[00320] To determine the role of cytosolic Ca+2 on CaMKII activation, the effect of cytosolic Ca+2 chelator,1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid tetrakis [acetoxymethyl ester] (BAPTA-AM), which markedly decreased glucagon-induced CaMKII phosphorylation, was tested. (**Fig. 1C**). Inositol 1,4,5-trisphosphate receptor (IP3R) channels, located in the endoplasmic reticulum (ER), release Ca2+ in response to binding of the second messenger, IP3, and play a major role in intracellular Ca+2 homeostasis. Glucagon-induced PKA phosphorylates and activates IP3R activity and that this event is important in hepatic glucose production. To investigate the contribution of IP3Rs in glucagon-induced CaMKII activation,

pretreatment with the IP3R inhibitor, xestospongin C. Xestospongin C led to a significant decrease in glucagon-induced CaMKII phosphorylation, demonstrating the critical role of IP3Rs in this process (**Fig. 1D**).

[00321] The increase in cAMP levels in response to glucagon increases the activity of PKA, a key enzyme involved in gluconeogenesis. In this context, treatment of hepatocytes with a membrane permeant analog of cAMP, 8-bromo-cAM, mimicked the effect of glucagon and led to a marked increase in phospho-CaMKII (**Fig. 1E**). To investigate whether CaMKII phosphorylation is causally related to PKA activation, hepatocytes were treated with a PKA inhibitor, H89, prior to the addition of glucagon. Inhibition of PKA markedly inhibited the glucagon-mediated increase in phospho-CaMKII (**Fig. 1F**). These data support the existence of a pathway in which glucagon promotes phosphorylation/activation of CaMKII through its effects on IP3R-mediated intracellular Ca+2 release in a cAMP-PKA-dependent manner.

[00322] To examine whether CaMKII is regulated by glucagon in vivo, mice were challenged with a bolus of intraperitoneal (i.p.) glucagon. Consistent with the effects observed in cultured hepatocytes, hepatic CaMKII phosphorylation was induced by glucagon treatment (**Fig. 1G**). A glucagon dose as low as 1 μg kg-1 was capable of phosphorylating CaMKII in the liver. To gain in vivo evidence that IP3Rs are important in the regulation of glucagon-mediated CaMKII phosphorylation, mice were treated with i.p. xestospongin C for 4 days. The mice were then challenged with glucagon, and liver extracts were assayed for p-CaMKII. As shown in **Fig. 1H**, xestospongin C treatment reduced glucagon-induced CaMKII phosphorylation. Next, hepatic CaMKII phosphorylation during the transition from a fed to fasting state was compared. The hepatic CaMKII phosphorylation was significantly increased upon fasting, whereas the total amount of CaMKII appeared to be unaffected by nutrient status (**Fig. 1I**). Moreover, upon re-feeding, the level of p-CaMKII in liver diminished (**Fig. 1J**). These data show that activity of hepatic CaMKII is regulated by nutrient status in a manner that is consistent with its role in fasting-induced hepatic glucose production

[00323] <u>CaMKII promotes hepatic glucose production, the expression of G6pc and Pck1, and FoxO1 nuclear localization in primary hepatocytes</u>

[00324] The regulation of hepatic CaMKII activity in response to fasting/re-feeding in vivo lead to directly testing its role in glucose production by hepatocytes. Glucose production was examined from pyruvate and lactate in primary hepatocytes transduced with adenoviruses expressing constitutively active CaMKII (adeno-CA-CaMKII), a kinase-inactive, dominant negative form of CaMKII (adeno-K43A-CaMKII), or control adeno-LacZ. The CA-CaMKII

construct possesses an amino acid substitution at T287D, which mimics autophosphorylation at that site and results in autonomous activity in the absence of bound Ca+2/calmodulin (Pfleiderer et al., 2004). The cells were examined under basal conditions and after stimulation with forskolin, a glucagon mimetic and a potent adenylate cyclase activator. An increase in both basal and forskolin-induced glucose release was observed in cells transduced with adeno-CA-CaMKII (**Fig. 2A**). Conversely, infection of cells with adeno-K43A-CaMKII decreased both basal and forskolin-induced glucose production (**Fig. 2A**). To further substantiate these results, glucose production in hepatocytes was assayed from WT mice and from mice lacking CaMKIIγ, the major isoform of the enzyme expressed in hepatocytes. Glucose production was suppressed in CaMKIIγ-deficient hepatocytes (**Fig. 2B**). Thus, CaMKII plays an important role in the hormonal regulation of glucose production in hepatocytes.

[00325] The role of CaMKII on hepatic glucose production prompted an investigation of transcriptional effects on genes encoding the rate-limiting enzymes in glycogenolysis and gluconeogenesis. To this end, primary hepatocytes were transduced with adeno-LacZ, CA-CaMKII, or K43A-CaMKII and measured forskolin-induced G6pc and Pck1 gene expression. Both G6pc and Pck1 mRNA levels were significantly higher in hepatocytes transduced with adeno-CA-CaMKII compared with adeno-LacZ, while transduction with adeno-K43A-CaMKII decreased the mRNA levels for these two genes (**Fig. 2C**). The effect of CaMKII on gluconeogenesis is further supported by the observation that forskolin-induced G6pc and Pck1 mRNA levels were significantly lower in Camk2g-/- versus WT hepatocytes (**Fig. 2D**). Similar results were obtained with glucagon treatment (**Fig. 2E**). Thus, CaMKII is necessary for the expression of G6Pc and Pck1.

[00326] A major transcription factor involved in the induction of G6pc and Pck1 is FoxO1, whose activity is primarily regulated by changes in its cytoplasmic vs. nuclear localization (Greer and Brunet, 2005). The distribution of GFP-tagged FoxO1 was assayed in hepatocytes from WT vs. Camk2g-/- mice. Under serum-starved conditions in WT hepatocytes, the majority of GFP-FoxO1 was in the nucleus (**Fig. 3A**). In contrast, Camk2g-/- hepatocytes displayed primarily cytosolic localization of GFP-FoxO1. Moreover, when hepatocytes were transduced with constitutively active CaMKII, FoxO1 became predominantly nuclear (**Fig. 3B-C**). Consistent with the Camk2g -/- data, FoxO1 was located mostly in cytoplasm WT hepatocytes transduced with dominant-negative adeno-K43A-CaMKII (**Fig. 3B**). These data indicate that CaMKII facilitates FoxO1 subcellular localization in serum-starved hepatocytes.

[00327] CaMKIIγ deficiency impairs and constitutively active hepatic CaMKII stimulates hepatic glucose production in vivo

[00328] To assess the functional role of CaMKII in hepatic glucose metabolism in vivo, fasting blood glucose levels in WT and Camk2g-/- mice were examined. Consistent with in vitro data, a modest but statistically significant decrease in blood glucose levels in fasted Camk2g-/- vs. WT mice was observed (**Fig. 4A**). The difference in fasting glucose concentration was not associated with an increase in circulating insulin concentration in knockout vs. WT mice. The mutant mice also showed lower plasma glucose in response to a pyruvate challenge test (**Fig. 4B**). Consistent with the primary hepatocyte data, there was a decrease in G6pc and Pck1 mRNA levels and nuclear FoxO1 in the livers of Camk2g-/- mice (**Figs. 4C-D**).

[00329] To further substantiate this important result, an adenoviral approach to inhibit hepatic CaMKII in vivo was used. Treatment of C57BL/6 mice with adeno-K43A-CaMKII led to a decrease in fasting blood glucose levels compared with mice treated with adeno-LacZ (**Fig. 5A**). In line with this finding, hepatic expression of G6pc and Pck1 mRNA and nuclear FoxO1 levels were lower in mice injected with K43A-CaMKII (**Fig. 5B-C**). Because FoxO1 ablation impairs both fasting gluconeogenesis and glycogenolysis, and CaMKII inhibition has a profound effect on the key glycogenolytic enzyme G6pc, the effect of acute CaMKII inhibition on hepatic glycogen content was examined. The data show a 60% increase in hepatic glycogen content in the mice treated with adeno-K43A-CaMKII (**Fig. 5D**). To directly link these findings to glucagon, mice treated with adeno-LacZ or adeno-K43A-CaMKII were injected i.p. with a bolus of glucagon and then the livers were analyzed 30 min later. G6pc was induced markedly in the livers of LacZ treated mice after glucagon injection but much less so in the livers of K43A-CaMKII-treated mice (**Fig. 5E**). Moreover, the K43A-CaMKII-treated mice had higher levels of hepatic glycogen as visualized by Periodic acid-Schiff (PAS) stain (**Fig. 5F**). These results further substantiate the role of CaMKII in hepatic glucose production.

[00330] The effect of constitutively active hepatic CaMKII in mice by treating mice with adeno-CA-CAMKII was examined. This treatment led to an increase in fasting glucose levels (**Fig. 6A**). There was also a slight increase in fed blood glucose levels, but it did not reach statistically significance. Furthermore, mice overexpressing CA-CaMKII showed a significant increase in blood glucose levels in response to pyruvate administration (**Fig. 6B**). Consistent with these observations, there was an increase in hepatic mRNA levels of G6pc and Pck1 and in nuclear FoxO1 in mice treated with adeno-CA-CAMKII (**Fig. 6C-D**). These combined in vivo

data show that CaMKII affects plasma glucose levels, pyruvate conversion into glucose, nuclear FoxO1, and the expression of hepatic glucose metabolism genes.

[00331] Impairment of glucose metabolism in Camk2g-/- hepatocytes and CaMKII-inhibited mice is rescued by transduction with constitutively nuclear FoxO1

[00332] To validate the importance of FoxO1 in the control of hepatic glucose metabolism by CaMKII, hepatocytes were transduced from Camk2g-/- mice with adenovirus containing a phosphorylation-defective, constitutively nuclear FoxO1 mutant (FoxO1-ADA) (Nakae et al., 2001) such that the level of nuclear FoxO1 was similar to that in WT hepatocytes. The suppressive effect of CaMKIIγ deficiency on G6pc and Pck1 mRNAs was abrogated by transduction with adeno-FoxO1-ADA (**Fig. 7A**). Treatment of mice with adeno-FoxO1-ADA adenovirus rescued the impairment in glucose homeostasis in adeno-K43A-CaMKII-treated mice (**Fig. 7B-D**). Taken together, these results are consistent with a model in which CaMKII contributes to hepatic glucose production through promoting nuclear localization of FoxO1.

Discussion

[00333] Glucose metabolism in the liver is tightly regulated by the opposing actions of insulin and glucagon. A number of signaling molecules and transcription factors have been implicated in the control of glycogenolysis and gluconeogenesis during periods of food deprivation. The in vitro and in vivo data herein add CaMKII to this list and, by doing so, provide a molecular link to the role of intracellular calcium in hepatic glucose metabolism. In particular, the data herein show that hepatic CaMKII is activated in response to fasting, leading to nuclear translocation of FoxO1 and the induction of glycogenolytic and gluconeogenic genes. The role of FoxO1 in CaMKII action is supported by the finding that the suppressive effect of CaMKII deficiency on glucose production is abrogated when constitutively nuclear FoxO1 is introduced into CaMKIIγ-deficient hepatocytes.

[00334] The glucagon-cAMP-PKA pathway not only leads to CaMKII activation, as shown here, but also directly phosphorylates cAMP response element binding (CREB) protein on Ser133. Phosphorylated CREB transcriptionally induces PGC1α, which acts with FoxO1 to promote the transcription of G6pc1 and Pck1 (Herzig et al., 2001). Previous work has shown that CREB from brain tissue can also be phosphorylated on Ser133 by CaMKII in vitro (Dash et al., 1991; Sheng et al., 1991), and a drug inhibitor of CaMKII blocked CREB transcriptional activity in a cell culture model of osteoclastogenesis (Ang et al., 2007). A difference in either nuclear CREB or phospho-CREB in WT vs. CaMKII-deficient hepatocytes was not obsevered,

excluding the possibility that CaMKII exerts its actions on hepatic glucose metabolism through modifying CREB.

[00335] FoxO1 activity is primarily regulated by post-translational modifications including phosphorylation and acetylation (van der Horst and Burgering, 2007). It is well documented that FoxO1 is phosphorylated at Thr24, Ser256, and Ser319 by growth factors via Akt to promote its nuclear exclusion. CaMKII promotes FoxO1 nuclear localization. Indeed, CaMKIIγ deficiency did not affect the phosphorylation of these three residues. There is evidence that FoxO phosphorylation on non-Akt sites by other kinases, such as JNK and AMPK, might actually promote its nuclear retention. Thus, the balance of FoxO activity can result from a combination of stimulatory and inhibitory phosphorylation events, and, without being bound by theory, CaMKII can affect this balance either through direct kinase action or through affecting a FoxO phosphatase activity. FoxO1 de-acetylation can also promote its nuclear localization (Frescas et al., 2005), but FoxO1 acetylation was also not affected by CaMKII deficiency. Alternatively, CaMKIIγ might somehow affect the import or export machinery involved in FoxO1 translocation or the expression or activity of FoxO1-interacting molecules in the cytoplasm or nucleus that might affect this process. Future studies will be directed toward the elucidation of this mechanism.

[00336] The discovery of a new molecule involved in hepatic glucose production not only provides insight into the physiologic defense against fasting hypoglycemia but may also reveal new therapeutic targets for the disturbed glucose metabolism that occurs in the setting of insulin resistance. Indeed, in type 2 diabetes, disproportionate hepatic glucose output and an imbalance of glucagon vs. insulin signaling contributes to fasting hyperglycemia (Saltiel, 2001). In this context, future studies will address whether inhibition of hepatic CaMKII ameliorates the metabolic abnormalities of obesity and insulin resistance.

Example 4: Experimental Procedures Suitable for Use with the Methods Described Herein

[00337] Reagents and Antibodies

[00338] Glucagon, pyruvate, forskolin, H89, and 8-bromo-cAMP were from Sigma. BAPTA-AM, and anti-nucleophosmin (Np) antibody were from Invitrogen. Xestospongin C was from EMD Chemicals. Anti–phospho-Thr287 CaMKII antibody was from Imgenex and Novus; anti–total CaMKII and anti-FoxO1 antibodies were from Santa Cruz Biotechnology Inc, anti-β-actin antibody was from Abcam.

[00339] Cell Culture

[00340] Primary mouse hepatocytes were isolated from 8- to 12-week-old mice as described previously (Matsumoto et al., 2002). Cells were serum depleted overnight and were then incubated with forskolin (10 μm) for 5 h in serum-free media.

[00341] Measurement of CaMKII Activity

[00342] CaMKII activity was assayed using a CaMKII assay kit from Promega according to the manufacturer's instructions. After the hepatocytes were treated as indicated in the figure legends, they were lysed by a 5-min exposure to 1% Triton-X in 50 mM HEPES, 150 mM NaCl, 10 mM Na pyrophosphate, 10 mM EDTA, 10 mM EGTA, 1 mM Na3VO4, 50 mM NaF, 1 mM PMSF, and 5 μg/ml leupeptin. Next, [γ-32P]ATP and CaMKII biotinylated peptide substrate were added to the lysate and after incubation for 10 minutes at 30°C, the [32P]-phosphorylated substrate was separated from the residual [32P]ATP using SAM biotin capture membrane and quantitated using a scintillation counter.

[00343] Adenovirus Infection

[00344] Adenoviruses encoding LacZ, CA-CaMKII, K43A-CaMKII and GFP-FoxO1 were described previously (Pfleiderer et al., 2004; Tanaka et al., 2009) and amplified by Viraquest, Inc. (North Liberty, IA). Primary hepatocytes were transduced 12 h after plating. RNA and protein isolation and glucose production were carried out 24 h after transduction.

[00345] Glucose Production in Primary Hepatocytes

[00346] Glucose production assays were carried out as described (Backs et al., 2010; Yoon et al., 2001). Briefly, after primary mouse hepatocytes were harvested and cultured as described above, cell culture medium was switched to glucose- and phenol-free DMEM (pH 7.4) supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate. After 16 h of culture, 500 μl medium was collected, and the glucose content was measured using a colorimetric glucose assay kit (Abcam). The readings were then normalized to the total protein amount in the whole-cell lysates.

[00347] Mouse Experiments

[00348] Camk2g-/- mice were generated as described previously (Backs et al., 2010) and crossed onto the C57BL6/J background. Mice were fed a standard chow diet and maintained on a 12-h light-dark cycle. Recombinant adenovirus (1.5 × 109 plaque-forming unit/mice) was delivered by tail vein injection. Fasting blood glucose was measured in mice that were fasted for 12-14 h, with free access to water, using a glucose meter (One Touch Ultra, Lifescan). Pyruvate-tolerance tests were carried out with an intraperitoneal injection of 2 g kg-1 body weight

pyruvate after 17 h of fasting. Blood glucose levels were measured over the following 2 h. Xestospongin C was administered by daily i.p. injections to mice at a dose of 10 pmol g-1 for 4 days.

[00349] Hepatic Glycogen Measurement

[00350] 50 -100 mg of frozen livers were homogenized in 1mL of H2O with protease and phosphatase inhibitors. Samples were then mixed with KOH (1:2), boiled for 25 minutes and washed with 70% ethanol. The pellet was dried and dissolved in 100 µl H2O and the glycogen content was assessed using Glycogen Assay Kit (Abcam) according to manufacturer's instructions. Data represent the mean ± SEM.

[00351] PAS Staining of Mouse Liver Sections

[00352] Liver samples were fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin. Sections (5 micron) were stained for glycogen using Periodic acid-Schiff (PAS) stain (Sigma) according to manufacturer's instructions. The sections were then counterstained with hematoxylin and examined by light microscopy. For the quantification of PAS staining, 5 fields from 4 different sections were chosen randomly and the number of PAS-positive cells was counted and expressed as a percentage of the total number of cells, as described before (Raza Asim et al., 2010). Two independent investigators, blinded to the identity of the samples, performed the analysis.

[00353] Immunoblotting and Quantitative RT-PCR

[00354] Total RNA was extracted from hepatocytes using the RNeasy kit (Qiagen). cDNA was synthesized from 2 µg total RNA using oligo (dT) and Superscript II (Invitrogen). Real-time qPCR analysis and western blotting were performed as previously described (Timmins et al., 2009). Nuclear extraction from liver was performed using the Nuclear Extraction Kit from Panomics according to the manufacturer's instructions.

[00355] Statistical Analysis

[00356] All results are presented as mean ± SEM. P values were calculated using the student's t-test.

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Example 5: The role of hepatic CaMKII in glucagon-mediated hepatic glucose production (HGP)

[00357] Obesity-induced insulin resistance and disturbances in liver glucose and fat metabolism increase the risk for heart disease, cancer, and other widespread and devastating diseases. Current treatments options are severely limited, leading to a critical unmet clinical need affecting hundreds of millions of overweight people in the current obesity epidemic. Over the last 2 years, several discoveries, including validation in models of obesity, indicate that

inhibitors of a unique drug target—a liver enzyme called CaMKII—can be invaluable in this niche. Thus, the overall goal of this invention is to develop and test CaMKII inhibitors for the purpose of drug development to improve the metabolic disturbances and their consequences in obesity, metabolic syndrome, and type 2 diabetes.

[00358] It was shown that CaMKII is activated by glucagon in primary hepatocytes and by glucagon and fasting in vivo (**Fig. 1**). Genetic deficiency or inhibition of hepatic CaMKII lowered blood glucose levels, suppressed the HGP genes G6pc and Pck1, decreased glycogen depletion, and blocked nuclear translocation of the HGP transcription factor FoxO1 (data with dominant-negative CaMKII shown in **Fig. 5**). Conversely, constitutively active CaMKII induced G6pc and Pck1, stimulated glucose production, and raised blood glucose levels. Importantly, the suppressive effect of CaMKII deficiency on glucose metabolism was abrogated by constitutively nuclear FoxO1, indicating that the effect of CaMKII deficiency requires nuclear exclusion of FoxO1 (**Fig. 7**). These results reveal a new molecular pathway regulated by CaMKII in the control of HGP.

[00359] Obesity was examined because an imbalance of glucagon over insulin signaling contributes to hyperglycemia and insulin resistance in obesity. CaMKII is activated in the livers of leptin-deficient (ob/ob) and diet-induced obese (DIO) mice but not in the livers of lean mice (**Fig. 8A**). CaMKII is also activated in the livers of obese but not lean humans (**Fig. 8B**). Most important, genetic or pharmacologic inhibition of hepatic CaMKII in obese mice lowered fasting blood glucose; improved insulin resistance; decreased the expression of key genes involved in HGP; decreased fatty liver and inflammation, and improved dyslipidemia (**Figs. 9-10**). These data support the concept that therapeutic targeting of CaMKII will improve hyperglycemia and insulin resistance in obese subjects.

[00360] <u>CaMKIIγ anti-sense oligonucleotides (ASO's) will be tested in a mouse model of obesity</u>

[00361] ASOs directed against Camk2g will be tested in a mouse model of obesity. Isis has a control ASO and 4 different Camk2g ASO's, which will be used to treat DIO obese mice (i.p. injection once a week for 6 weeks). The level of Camk2g mRNA in the liver and other tissue will be measured and overall tolerance including liver weight, liver function tests, and tolerance to fasting will be assesed. Efficacy in improving the following parameters will be tested: fasting and fed plasma glucose and insulin; HGP gene expression and FoxO1 nuclear localization; HGP and peripheral insulin sensitivity via a hyperinsulinemic euglycemic clamp study; fatty liver; and plasma triglycerides and fatty acids.

[00362] Screen for and test novel chemical inhibitors of CaMKII:

[00363] "Druggable" chemical libraries will be screened using a high-throughput fluorescence-based assay of CaMKII activity that relies on changes in fluorescence emission ratio when the enzyme is inactivated. Structure-activity relationships will be defined and potency and specificity of CaMKII inhibitors that already exist and are commercially available for in vitro and pre-clinical studies will be optimized. The most promising hits will then be secondarily screened in a model of insulin-resistant primary hepatocytes, which involves treating the cells with saturated fatty acids in a manner that mimics the increase in glucose production and insulin resistance seen in liver in obese mice. Using this model, the most potent inhibitors of CaMKII, glucose production, and insulin resistance will be examined. The most promising drugs, prepared in gram quantities, will be tested in vivo according to the strategy outlined above for the ASO's, with vigilance on monitoring the activity of CaMKII and consequences of inhibition in non-liver tissues. Orally available drugs will be assessed for ease of delivery and delivery to the liver, i.e., via the portal circulation. Drugs can be altered to achieve oral availability if that becomes necessary.

Example 6

[00364] Two major factors contributing to the increased risk of coronary artery disease (CAD) in obesity/insulin resistance are pro-atherogenic processes in the plaques themselves and an increase in systemic risk factors, notably hepatic-derived dyslipidemia. Human diabetic plaques are characterized by especially large necrotic areas, which promote inflammation, plaque disruption, and acute CAD. Plaque necrosis develops as a consequence of macrophage (Mφ) apoptosis in the setting of defective clearance of the dead cells. The mechanisms by which prolonged endoplasmic reticulum (ER) stress in advanced plaques triggers Mφ death and plaque necrosis will be examined. These processes are amplified in Mφs with defective insulin signaling. Prolonged ER stress promotes elevated cytoplasmic calcium (Ca2+), which triggers apoptosis through activation of Ca2+/calmodulin-dependent protein kinase II-γ (CaMKIIγ). Deletion or inhibition of CaMKIIγ in obese mice was protective against hyperinsulinemia, gluconeogenesis, dyslipidemia, fatty liver, and ER stress. These new findings indicate that one enzyme may have critical effects on two complementary processes that promote CAD in insulin-resistant subjects (**Fig. 11**). Thus, Mφ CaMKIIγ deficiency can lessen advanced lesional Mφ apoptosis and plaque necrosis in a murine model of Mφ insulin resistance and that hepatic CaMKIIγ deficiency will improve atherogenic metabolic idsturbances in obese mice.

[00365] Mφ CaMKIIγ deficiency will lessen advanced lesional Mφ apoptosis and plaque necrosis in insulin-resistant mice

[00366] In vitro data shows that CaMKIIγ orchestrates a number of critical apoptotic pathways in ER-stressed Mφs. PPG data shows perturbed Ca2+ metabolism and activated CaMKIIγ in insulin-resistant Mφs. Thus, CaMKIIγ can play a particularly important role in apoptosis of ER-stressed insulin-resistant Mφs. It will be first determined whether siRNA-mediated silencing of Camk2g in Mφs isolated from Insr-/- and ob/ob, two proof-of-concept models of defective Mφ insulin signaling, suppresses the very high level of ER stress-induced apoptosis in these cells and then explore the molecular/cellular mechanisms of protection. p-CaMKII, a measure of CaMKII activation, will be measured to determine if it is higher in lesional Mφs in advanced vs. earlier stage atherosclerotic lesions in humans and mice. To test causation, bone marrow cells from Camg2gfl/flLysmcre+/-Insr-/- mice, as well as from WT mice and from mice lacking Mφ CaMKIIγ or insulin receptors will be transplanted separately, into Ldlr-/- mice, and then place on the Western diet. The high level of advanced lesional Mφ apoptosis and plaque necrosis in Insr-/- → Ldlr-/- mice will be markedly ameliorated by Mφ CaMKII deficiency.

[00367] Mechanisms whereby liver CaMKIIγ deficiency improves the metabolic disturbances of obesity and liver-specific CaMKIIγ deficiency will suppress atherosclerosis in obese mice.

[00368] Camk2gfl/fl X α1-antitrypsin-Cre mice will be used to test whether hepatic-specific CaMKIIγ deficiency improves glucose, lipid, and lipoprotein metabolism in obesity. The idea that obesity activates hepatic CaMKIIγ through a mechanism involving the ER stress effector CHOP will be tesed. One mechanism underlying the beneficial effects of hepatic CaMKIIγ deficiency on glucose and lipid metabolism will be explored, namely, nuclear exclusion of FoxO1 and suppression of hepatic glucose production/ gluconeogenesis. CaMKII activation and related mechanisms in liver specimens from obese vs. lean human subjects will be probed. The LysMCre model will be used to investigate whether CaMKIIγ deficiency in liver Mφs, by suppressing ER stress and/or inflammation, and whether it can also contribute to the suppression of liver-mediated metabolic disturbances in obesity. The following will be tested: (a) whether liver CaMKIIγ deficiency in Western diet-fed Ldlr-/- mice will suppress atherogenesis; and (b) whether combined hepatic and Mφ CaMKIIγ deficiency will have marked beneficial effect on all stages of atherosclerosis.

Example 7: Plaque necrosis

[00369] The 2-3% of lesions that cause acute CAD are distinguished not by their larger size but by the presence of plaque necrosis1,2, which promotes plaque disruption, acute lumenal

thrombosis, and tissue infarction (3). Plaque necrosis is caused by the combination of Mφ apoptosis and defective clearance, or "efferocytosis," of the dead Mφs, resulting in post-apoptotic necrosis (4-6). This concept is particularly important in considering how diabetes and insulin resistance promotes CAD, because advanced atherosclerotic lesions in diabetic subjects are characterized by particularly large necrotic cores when compared with similarly sized lesions from non-diabetic individuals (7-12). A prospective study of subjects with CAD found that only diabetes and age were associated positively with necrotic core size (12). These data raise the issue as to whether advanced lesional Mφ apoptosis is enhanced in the setting of diabetes.

[00370] Prolonged ER stress as a mechanism of advanced lesional Mφ apoptosis

[00371] Mechanistic and *in vivo* data support a role for prolonged ER stress in advanced lesional Mφ apoptosis and plaque necrosis{4997, 5081}. ER stress-induced Mφ apoptosis is largely dependent on the CHOP branch of the so-called the Unfolded Protein Response (UPR) (15,16). In human coronary arteries, there is a very strong correlation among CHOP expression, apoptosis, and advanced plaque stage (17). CHOP-deficient atherosclerosis-susceptible mice have suppressed advanced plaque progression (16), which was then confirmed by another group who used bone marrow transfer to implicate the role of Mφ CHOP (18). A third study showed an atheroprotective effect of a "chemical chaperone" that is thought to relieve ER stress (19). CHOP-induced apoptosis can be modeled in cultured Mφs by: (a) potent inducers of ER stress, such as 7-ketocholesterol (16,20), which is the most abundant oxysterol in advanced atherosclerotic lesions (17); or (b) the combination of more subtle ER stress plus an athero-relevant "2nd hit," notably, pattern recognition receptor (PRR) activation by modified lipoproteins or saturated fatty acids, which amplifies pro-apoptotic signaling and suppresses cell-survival signaling (21,22). The 2-hit concept also applies to another model of advanced lesional Mφs, namely, Mφs loaded with lipoprotein-derived free cholesterol (FC) (23-26), because excess FC in the ER membrane activates the UPR, while modified lipoproteins activate PRRs. Most importantly for this proposal, the PPG has shown that ER stress-induced apoptosis is markedly enhanced in Mφs with defective insulin signaling.

[00372] Pro-atherogenic roles of the liver in obesity and insulin resistance. The liver plays a major role in the increased CAD risk in insulin resistance (27,28). A major link is through increased VLDL apoB and triglyceride (TG) secretion, which is triggered by *de novo* lipogenesis coupled with increased hepatic delivery of adipose-derived free fatty acids; suppression of apoB degradation; and enhancement of apoB translation28. SREBP1c-mediated lipogenesis and VLDL secretion are stimulated by the activation of "residual" insulin receptor signaling

pathways by hyperinsulinemia (29-33). An insulin receptor-mTORC1 pathway suppresses sortilin-1-mediated apoB degradation. Two other potential links among obesity, insulin resistance, and metabolic disturbances are hepatic ER stress and inflammation. Hepatic ER stress in obesity can suppress insulin signaling by IRE1-JNK-mediated phosphorylation of IRS-1 on Ser30734 or by activation of GSK-3β, which inhibits rictor-mediated AKT activation (35,36). Manipulations that relieve hepatic ER stress in animal models improve insulin resistance (37,38), and studies in humans have shown that weight loss decreases hepatic ER stress (39). Hepatic ER stress promotes SREBP-1c activation and steatosis (34,38,40), but the effects on VLDL secretion *per se* remain to be fully explored (41-43). In terms of inflammation, cytokines can enter the liver from the blood or through secretion by resident Kupffer cells or newly recruited macrophages, which have been implicated in obesity-induced insulin resistance (28,44,45). Inflammatory cytokines activate JNK, which has been linked to insulin resistance by disrupting insulin receptor signaling (above) and through activation of the kinase PKR46.

[00373] CaMKII.

[00374] CaMKII is a Ca2+-activated Ser/Thr kinase whose active form is a homo-multimer of 12-14 subunits encoded by one of 4 genes, α, β, γ, or δ47. (*NB*: CaMKII is distinct from CaMKK, which activates AMPK). The α and β isoforms are neuronal, while CaMKIIγ and δ are expressed in a wide variety of tissues. Mφs and HCs express the γ form48. In the basal state, a regulatory domain interacts with and inhibits the catalytic domain. When cytosolic Ca2+ is increased, Ca2+-calmodulin complex disrupts this interaction, thus relieving auto-inhibition and promoting auto-phosphorylation of Thr287. This process increases the affinity of Ca2+-CaM binding and results in Ca2+-independent kinase activation. Most studies on CaMKII have been carried out in neurons and cardiomyocytes. In Mφs, *in vitro* inhibitor studies have reported roles in phagolysosomal killing of bacteria; LPS-mediated adenylyl cyclase activation and HIF-1α induction; PKR-mediated p38 activation; auto-immune induced apoptosis; and de-repression of inflammatory genes (49-54). Published data with CaMKII in hepatocytes is very limited. As explained in the following sections, genetic targeting of *Camk2g* was used to show new roles in ER stress-induced Mφ apoptosis (48) and in liver-driven metabolic disturbances in the setting of obesity.

[00375] The role of Mφ CaMKIIγ in atherosclerosis has never before been explored, and its importance in the setting of insulin resistance because of altered calcium dynamics in insulin-resistant Mφs is a new concept. A new cre-lox model will be used to test these ideas . The role of hepatocyte CaMKIIγ in the metabolic disturbances of obesity can reveal additional new

principles in this critical area. The cre-lox model will also be ideal to test this idea. Finally, the proposed integrated role of this single molecule in mediating atherosclerosis in the setting of obesity and insulin resistance, through effects on lesional Mφs, hepatocytes, and possibly hepatic Mφs, represents a novel concept that can have important therapeutic implications.

[00376] CAD risk factors associated with the epidemic of obesity and insulin resistance will be the major drivers of CAD over the next decades (55). This finding addresses new mechanisms and *in vivo* consequences of two critical processes in this area, one centered on plaque Mφ apoptosis and the other on metabolic processes in the liver that promote CAD risk. The Mφ studies are focused on advanced plaque morphology, notably necrosis, which is the most important feature of the small minority of human plaques that actually cause acute CAD (56). Moreover, the finding includes future studies using human plaque specimens at various stages of progression and liver specimens from obese *vs.* lean human subjects. Upon completion of the proposed studies, new therapeutic strategies for obese/insulin-resistant subjects that target common pro-atherogenic signaling pathways in the arterial wall and liver will be obtained. Of note, CaMKII inhibitors have been tested successfully in animal models in other scenarios in which CaMKII is thought to contribute to disease progression (57,58). As described herein, *partial* inhibition of the enzyme has a significant effect on protecting Mφs and HCs, which further increases the feasibility of a therapeutic approach. Specificity can be obtained through selective inhibition of the gamma isoform and by using strategies that target drugs to atherosclerotic lesions and the liver (59,60). However, this specificity may not be needed given the benefit of CaMKII inhibition in other aspects of diabetes, such as heart failure (61), hypertension and renal disease (62), and retinal disease (63).

[00377] <u>Testing whether Mφ CaMKIIγ deficiency will lessen advanced lesional Mφ apoptosis and plaque necrosis in insulin-sensitive and especially Mφ-insulin-resistant mice.</u>

[00378] There is *in vitro* and *in vivo* evidence for the aforementioned "2-hit" mechanism of Mφ apoptosis involving prolonged ER stress in combination with PRR signaling (22,48,64-72). A key ER stress pathway involves CHOP-mediated induction of the ER oxidase ERO1α, which then activates the ER Ca2+ release channel IP3R. The released Ca2+ activates CaMKIIγ, which in turn triggers downstream apoptosis pathways, including JNK-mediated induction of the Fas death receptor; permeabilization of the outer mitochondrial membrane and release of cytochrome c; and induction of NADPH oxidase-mediated ROS, which also amplifies CHOP through an upstream kinase called PKR (**Fig. 12**). With regard to the therapeutic potential mentioned above, partial inhibition of CaMKIIγ is adequate to protect Mφs (**Fig. 13**). Importantly, ER-stressed

CaMKIIγ-deficient mice are protected from apoptosis in peritoneal and splenic Mφs and renal epithelial cells *in vivo*. However, the role of Mφ-CaMKIIγ in advanced atherosclerotic lesional Mφ apoptosis and plaque necrosis is not known.

[00379] Application of these concepts to Mφs in the setting of insulin resistance has been a major goal of the PPG. Mφs enhances ER-stress-induced apoptosis by at least 3 mechanisms: (a) up-regulation of scavenger receptors (74); (b) suppression of the AKT and NF-κB cell-survival pathways (66); and (**c**) down-regulation of the ER Ca2+ pump SERCA75. Most importantly, advanced lesional Mφ apoptosis and plaque necrosis are increased in lesions in which Mφs have defective insulin signaling76, which is consistent with the very large necrotic cores of human diabetic lesions. Another study showed that mixed-genetic-background Apoe-/- mice fed a very-high-cholesterol/cholate ("Paigen") diet had a modest decrease in lesion area when Mφs were Insr -/- or Irs2 -/-77. This can be related to anti-inflammatory effect in the setting of the pro-inflammatory Paigen diet (77-79), consistent with the above NF-κB study (66). Most importantly, the critical endpoints of advanced lesional Mφ apoptosis and plaque necrosis, which are the focus of the PPG, were not included.

[00380] Without being bound by theory, links between these 3 mechanisms and the ER stress/PRR 2-hit model are readily apparent based upon these mechanisms and new data (**Fig. 14**): (a) in insulin-resistant Mφs, an ERK1/2-SERCA pathway is inhibited, which increases release of ER Ca2+ into the cytosol (75). The results described herein show increased p-CaMKII, a marker of its activation, in *Insr* -/- and *ob/ob* Mφs and, consistent with the above mechanism (75), in ERK-inhibited WT Mφs (**Fig. 15**); (b) inhibition of SERCA can prolong ER stress (80), and CaMKII activation amplifies ER stress through a feed-forward cycle (**Fig. 16**)—all consistent with the finding of increased CHOP in Mφs freshly isolated from *ob/ob* mice (68).

[00381] Mechanistic studies.

[00382] CaMKII activity will be assayed in control *vs.* insulin-resistant ER-stressed Mφs. To broaden significance, different sources of Mφs, inducers of insulin resistance, activators of ER stress, and methods for silencing CaMKIIγ will be used. Peritoneal Mφs from *Insr* -/- mice (76), which is based on the marked down-regulation of insulin receptors by hyperinsulinemia (81-84), including on monocytes from insulin-resistant humans (85,86) will be used. These Mφs *v*WT Mφs will be challenged with athero-relevant ER stress activators (13): FC loading; 7-ketocholesterol; or low-dose ER stress plus a PRR activator, *e.g.*, the peroxynitrite donor SIN-1 plus oxidized phospholipids (oxPLs)72. Lipoprotein Lp(a)which is a risk factor for CAD (87-91), a carrier of oxPLs in humans (92), and a potent stimulator oapoptosis in ER-stressed Mφs

(72) will be tested. CaMKII activation will be assayed by pThr287 immunoblot (**Fig. 15**) or by direct CaMKII kinase assay (48).

[00383] The level of apoptosis in ER-stressed insulin-resistant Mφs consists of two components: "basal" (*i.e.*, that seen in ER-stressed WT Mφs) plus the increment afforded by insulin resistance, with each contributing ~50% to the final apoptotic response (76). In *ob/ob* Mφs, partial CaMKII inhibition resulted in a ~40% decrease in the basal component of ER stress-induced apoptosis and a ~65% inhibition in the *ob/ob*-induced incremental portion, *i.e.*, total apoptosis was decreased bthe inhibitor to a greater extent in *ob/ob* than in WT ER-stressed Mφs (5.0 ± 0.4 → 3.1 ± 0.2% in WT *vs.* 11.9 ± 1.1 → 5.6 ± 0.5% in *ob/ob*). These experiments will be repated using (a) *Camk2g* siRNA in *ob/o*Mφs; (b) *ob/ob vs. ob/obCamk2g* -/- Mφs; and (c) Mφs from the atherosclerosis study below, *i.e.*, *Ldlr*-/- mice transplanted with marrowWT, *Camk2g* KO, *Insr* KO, or *Camk2gInsr* DKO mice. Mechanism will be probed by determining which of the effectors of CAMKIIgamma-induced pro-apoptotic signaling are increased in insulin-resistant Mφs and decreased by CaMKIIγ deficiency: Fas death receptor induction; STAT1 activation; mitochondrial Ca2+ uptake and cytochrome c release; Nox2 induction/ROS48; UPR amplification; and PKR activation. Four groups of Mφs—con-WT; insulin-resistant-WT; con-Camk2g -/-; and insulin-resistant-Camk2g -/-—will be assayed as follows (48,68,93,94): RT-QPCR for Fas mRNA and FACS analysis for Fas cell-surface expression; immunoblot for p-STAT1; rhodamine-2 staining and mt-pericam fluorescence (95) for mitochondrial Ca2+; immunoblot for mitochondrial and cytosolic cytochrome c and caspase-9 activity assay; RT-QPCR for Nox2 mRNA and DCF staining for cellular peroxide accumulation; immunoblots for p-PERK and CHOP; and immunoblot for p-PKR. These effector endpoints will be increased in insulin-resistant vs. -sensitive ER-stressed Mφs and that both the basal ER stress-induced component and the further increment in insulin-resistant Mφs will be decreased in the absence of CaMKIIγ, leading to an overall dramatic decrease the Camk2g -/- Mφs. However, insulin resistance causes a selective increase in specific endpoints or if the data indicate that factors other than CaMKIIγ can be involved in the insulin resistance-induced increment, mechanistic experiments driven by the data will be conceived and executed.

[00384] Without being bound by theory, insulin resistance (IR) enhances the pro-apoptotic effects of CaMKIIγ in ER-stressed Mφs (IR → CaMKII). Positive-feedback cycles are often involved in ER stress-induced apoptotic signaling pathways (93) (**Fig. 16**). Thus, whether CaMKIIγ can feedback-enhance insulin resistance (IR ↔ CaMKII) will be explored. If so, CaMKIIγ-deficiency can have the following protective effects in insulin-resistant Mφs based on

previous studies (66,72,74,75,75): (a) ↓ SRA and CD36, ↑ p-Akt, ↑ p-ERK, ↑ SERCA2b, and ↑ p-FoxO1 (immunoblot); (b) ↑ *Serca2b,* ↓ *Ikbe*, and ↑ *Bcl2*, an NF-κB target decreased in insulin-resistant Mφs66 (RT-QPCR); (c) ↓ ER Ca2+ (Fluo-3-thapsigargin fluorescence microscopy assay); and (d) ↓ nuclear FoxO (immunofluorescence in adeno-GFP-FoxO1-transduced Mφs). The latter assay is of particular interest given that CaMKIIγ is necessary for FoxO1 nuclear localization in fasting and insulin-resistant hepatocytes. Together, these studies will provide a comprehensive view of the roles of CaMKIIγ in the pro-apoptotic effects of insulin resistance in ER-stressed Mφs.

[00385] Human atherosclerosis.

[00386] Whereas causation studies will be conducted in mice (next section), whether activation of CaMKIIγ in plaque Mφs increases will be tested (a) as a function of advanced plaque stage; and (b) in the setting of defective Mφ insulin signaling. Freshly isolated/flash-frozen carotid plaque specimens will be divided into 3 small, adjacent sections in the middle of the plaque. The middle section will be used for anti-p-CaMKII immunoblot analysis and quantified by densitometric ratio with total CaMKIIγ and β-actin loading controls. The immediate flanking regions will be microtome-sectioned and then (a) stained with Movat pentachrome for necrotic area measurement (quantified as percent of total area); and (b) immunostained for Mφs, p-CaMKII (**Fig. 17**), and TUNEL for apoptosis (quantified as percent of Mφs that are positive). The goal is to show that the correlation between p-CaMKII and necrotic area (and Mφ TUNEL) is significant at P=0.05. In addition, these specimens will be classified in a blinded manner into 5 stages using the Virmani-AHA scheme (96): 1=diffuse intimal thickening; 2=fibrous plaques; 3=thick-cap atheroma; 4=thin-cap atheroma; and 5=ruptured plaques. The goal is to test whether if p-CaMKII is enriched in stages 4+5 *vs.* 1+2. n=15 will be sufficient with 80% power to show at least a 2-fold difference in p-CaMKII at P=0.05 between 4+5 lesions and 1+2 lesions. Finally, all data will be analyzed for correlations with the presence of type 2 diabetes, corrected for lesion stage, and positive correlations between p-CaMKII and both lesion stage and the presence of type 2 diabetes.

[00387] Murine atherosclerosis study.

[00388] The two objectives are: (a) to test the whether CaMKIIγ activation and its pro-apoptotic effects are increased as a function of advanced plaque stage and in the setting of Mφ insulin resistance; and (b) as a causation study, to test the effect and mechanisms of CaMKIIγ deficiency on advanced lesional Mφ apoptosis and plaque necrosis. Using the same overall strategy as previously described (76), 4 groups of mice will be used as bone marrow

transplantation (BMT) donors into male *Ldlr*-/- mice: *Camk2g*fl/fl*Insr*+/+ (WT→*Ldlr*-/-); *Camk2g*fl/fl*Lysmcre*+/-*Insr*+/+ (Mφ-CK KO → *Ldlr*-/-); *Camk2g*fl/fl*Insr*-/- (Mφ-InsR KO → *Ldlr*-/-); and *Camk2g*fl/fl*Lysmcre*+/-*Insr*-/- (Mφ-CK/InsR DKO → *Ldlr*-/-). All genotypes have been backcrossed onto the C57BL6/J background for >8 generations., and all experiments will use littermate controls. Previous studies have shown that there is almost complete deletion of floxed genes in lesional Mφs on the *Lysmcre*+/- background (67,97). Six wks after BMT, the mice will be fed the Western diet (WD) for 8, 12, 16, and 20 weeks, which will enable an analysis of early, mid-stage, and advanced/necrotic lesions (16,65,67,71,97-102). Previous atherosclerosis studies with similar strategies and goals (16,65,71,97,101,102), together with the statistics from the previous *in vivo* study (76), allow an estimate that n=30 male mice per group will give us an 80% chance of detecting a 33% difference in these endpoints among the four groups of mice.

[00389] The overall protocols for the mouse atherosclerosis studies, including Mφ apoptosis and necrotic core quantification, have been detailed in publications in this area (16,65,67,71,72,97,101-103). Briefly, plasma is obtained for lipid/lipoprotein analysis, fasting glucose, and insulin. The aortic root and brachiocephalic artery are analyzed for lesion and necrotic area; apoptosis (TUNEL and activated caspase-3); and in-situ efferocytosis. *In situ* efferocytosis in lesions will be assayed (103). Necrotic areas are defined as acellular areas with Mφ debris (Mφ-specific antigens in the absence of cells) and by the absence of collagen staining. Mφs, SMCs, ECs, and T cells will be identified by IHC, and fibrous caps are quantified for thickness using Verhoeff's and Masson's trichrome stains for elastin and collagen (97). The latter assays will be complemented by probing the 20-wk lesions for complete degradation of medial elastic fibers ("medial erosion") in Verhoeff-stained samples, as described (104), because suppression of Mφ secondary necrosis may lead to less leakage of MMPs, which can be causative for this endpoint (105). The data will be quantified as number of mice in each group whose lesions show medial erosions, as described (104). Positive results would be followed by assaying lesion sections for active MMPs by zymography and near-infrared fluorescence using MMP gelatinase (MMP2/9) substrates, as described (106). The advanced plaque property of intraplaque hemorrhage (107,108) will be assayed using Perl's iron stain (109) and inspection of H&E section and 560-nm fluorescence for RBCs (107,110). IHC using anti-F4/80 (Mφs) and anti-p-CaMKII to will be used evaluate whether p-CaMKII is increased as a function of advanced plaque stage and in the setting of defective Mφ insulin signaling. The anti-p-CaMKII antibody gives no signal above background in Mφs from *Camk2g*-/- mice. As with human lesions (**Fig. 17**), a staining pattern that is consistent with the presence of p-CaMKII has been

obtained in advanced lesional Mφs, and this signal will be validated using the Mφ-CK KO groups. The IHC data will be quantified as area of p-CaMKII staining per lesion area; percent of CaMKIIγ-stained cells that are Mφs; and percent of Mφs that stain with p-CaMKII. Immunoblots will be conducted on aortic root and arch segments from which the adventitia has been removed so that p-CaMKII (and total CaMKII) can be precisely identified. This analysis does not exclude the contribution of other arterial-wall cells, but IHC studies should allow us to assess whether or not these other cells even express the gamma isoform of the enzyme. Pro-inflammatory (TNFα and IL-6) and anti-inflammatory (TGFβ and IL-10) cytokine mRNA expression laser-capture microdissection (LCM)-RT-QPCR will be conducted, because a decrease in Mφ apoptosis and ER stress would likely decrease plaque inflammation (70,111), and absence of CaMKIIγ may decrease the transcription of inflammatory genes by stabilizing NCoR corepressor function (53,54). The captured RNA will also be used to explore *in vivo* mechanisms, as guided by cell culture studies. Pro-apoptotic transcriptional targets of activated CaMKIIγ, notably *Fas*, *Nox2*, and *Chop* (*Ddit3*) mRNA48 will be assessed. mRNA for *Serca2b*, *Ikbe*, and *Bcl2* (an NF-κB target decreased in insulin-resistant Mφs66) will also be assayed. Mφ *Tlr4* and *Tlr2* mRNA expression, which are FoxO1 targets (112) will be assayed. With regard to Nox2 and oxidative stress (93), lesional ROS will be assayed using DHE. These results will show that (a) advanced lesional Mφ apoptosis and plaque necrosis, and markers of the CaMKII-apoptosis pathway, will be lower in the CKO *vs*. WT group, and particularly in the CK/InsR DKO *vs*. WT group; (b) Mφ *Fas*, *Nox2*, and *Chop* mRNA and ROS will be lower in the CKO *vs*. WT group, and particularly the CK/InsR DKO *vs*. WT group; and (c) *Serca2b* and *Bcl2* will be lower, and *Ikbe* higher, in the insulin-resistant *vs*. WT group, and it will be interesting to see whether these trends are reversed in the CK/InsR DKO group.

Model	Mφ apoptosis	Plaque necrosis	Mφ-p-CaMKII	Pro-apoptotic effects of activated CaMKII (*Fas*, *Nox2*, *Chop*, ROS)	*Serca2b* *Bcl2*	*Ikbe*
WT → *Ldlr*$^{-/-}$	++	++	++	++	++	+
Mφ-CK KO → *Ldlr*$^{-/-}$	+	+	+	+	?	?
Mφ-insR KO → *Ldlr*$^{-/-}$	++++	++++	++++	++++	+	++
Mφ-CK/insR DKO → *Ldlr*$^{-/-}$	+	+	+	+	?++	?+

Table 1: Summary of mouse models and predictions for endpoints

[00390] Necrosis, and markers of the CaMKII-apoptosis pathway, will be lower in the CKO *vs*. WT group, and particularly in the CK/InsR DKO *vs*. WT group; (b) Mφ *Fas*, *Nox2*, and *Chop* mRNA and ROS will be lower in the CKO *vs*. WT group, and particularly the CK/InsR DKO *vs*. WT group; and (c) *Serca2b* and *Bcl2* will be lower, and *Ikbe* higher, in the insulin-resistant *vs*.

WT group, and it will be interesting to see whether these trends are reversed in the CK/InsR DKO group.

[00391] Characterization of the mechanisms whereby liver CaMKIIγ deficiency improves the metabolic disturbances of obesity and assaying whether liver-specific CaMKIIγ deficiency will suppress atherosclerosis in obese mice.

[00392] Deficiency of HC CaMKIIγ—the same enzyme that plays a critical role in ER stress-induced Mφ apoptosis— improves atherogenic metabolic disturbances in obesity. CaMKIIγ was the major isoform of CaMKII in mouse and human liver and that p-CaMKIIγ, a measure of its activation state, was increased in the livers of *ob/ob* mice, diet-induced (DIO) mice (5.24 kcal/g, 60% cals from fat, x 20 wks), and morbidly obese humans compared with lean controls (**Figs. 8A-B**). Consistent with these data and the fact that obesity leads to hepatic ER stress (34), ER stress activation by azetidine or palmitate increased p-CaMKIIγ in HCs, and CaMKIIγ deficiency in DIO mice suppressed UPR activation, suggesting that CaMKIIγ participates in a UPR positive feedback cycle in HCslike it does inMφs (see **Fig. 16**). The functional importance CaMKIIγ in obesity was shown byfinding that CaMKIIγ-deficient DIO michave markedly lower plasma insulin and armore responsive to insulin-mediated glucose lowering than DIO WT mice despite no difference in weight (**Fig9A-D**). The mice also had an improvement in blood glucose during a glucose tolerance test. Similar results were found using acute inhibition of liver CaMKII through the use of adeno-K43A-CaMKII (**Fig. 9E-F**), which is a kinase-inactive, dominant-negative form of CaMKII123 that were verified inhibits hepatic CaMKII. The mRNAs for the gluconeogenic (GNG)genes G6Pase and PEPCK were decreased in the livers of adeno-K43A-CaMKII-treated *ob/ob* mice (**Fig. 9G).**

[00393] Both genes are targets of FoxO1, as is *Igfbp1*, which was also decreased in CaMKII-inhibited livers. These data are consistent with CaMKII inhibition suppressing FoxO1 nuclear localization (see below). CaMKIIγ-deficient DIO mice also had decreased (**a**) liver triglyceride (TG) content (**Fig. 10A**); (**b**) SERBP-1c-induced lipogenic mRNAs but not mRNAs associated with fatty acid oxidation; and (**c**) plasma cholesterol and TG (**Fig. 10B**). High fat diet-induced dyslipidemia involves stimulation of an mTORC1-pS6K pathway by hyperinsulinemia acting on residual hinsulin receptors, leading to suppression of Sortilin 1 (Sort). Decreased Sort has been linked genetically to human dyslipidemia, and the mechanism appears to be decreased Sort - mediated degradation of LDL apoB-100, perhaps through a Sort chaperone-mediated lysosomal degradation pathway124. This pathway is partially prevented by CaMKII inhibition (**Fig10C**). Finally, hepatic *Tnfa* mRNA was decreased ~50% in *Camkg2g-/- vs*. WT DIO liver (**Fig. 10D**).

[00394] Hepatocyte CaMKIIγ deficiency lessens hyperinsulinemia and subsequent dyslipidemia by decreasing gluconeogenesis (GNG) and hepatic glucose production (HGP) via a mechanism involving nuclear exclusion of FoxO1 (**Fig. 18**).

[00395] This analysis is based on (a) the role of elevated HGP in promoting hyperinsulinemia in obesity and the importance of hyperinsulinemia in driving dyslipidemia; (b) the finding that CaMKII inhibition in obese mice decreases FoxO1-induced GNG genes and the FoxO1 target, *Igfbp1* (**Fig. 8G**); and (c) data on links among hepatic CaMKIIγ, FoxO1, and GNG in fasting lean mice.

[00396] The *Camk2g* mice described herein, which are now being crossed with α1-antitrypsin Cre (*A1atcre+/-*) mice to delete CaMKIIγ in HCs125 will be used. Four groups of male mice will be fed the DIO diet for 20 wks starting at 4 wks of age: DIO/*Camk2g*fl/fl (DIO/WT) and chow-fed control (lean/WT); and DIO/*Camk2g*fl/fl*A1atcre+/-* (DIO/Li-CK KO); and chow-fed control (lean/Li-CK KO). To rule out compensatory effects of germline knockout, a cohort of DIO/Li-CK KO mice will be "restored" with adeno-T287D-CaMKII (*vs.* adeno-LacZ control), which is a constitutively active form of the kinase (123) that should reverse the predicted beneficial metabolic effects in the DIO/Li-CK KO mice. Food intake and body weight will be monitored, and fasting plasma glucose and insulin will be obtained every 4 wks. At the end of the feeding period, the mice will be fasted for 6 h, weighed, and sacrificed. In view of links between circadian rhythm and metabolism (126-128), there will be strict adherence to a 12-h light/dark cycle, and all mice will be sacrificed at the same time of day. Although *Camk2g-/-* mice develop to adulthood without obvious morphological or functional abnormalities, including those related to the liver48, plasma will be obtained for the metabolic and lipid parameters described below and for alanine transaminase (ALT) and aspartate transaminase (AST) as a measure of HC dysfunction. The liver will then be removed immediately, snap-frozen in liquid nitrogen, and stored at -80oC. Deletion of CaMKIIγ will be confirmed by RT-PCR and immunoblot, and specificity for liver deletion will be determined by similar analysis on snap-frozen samples of brain, heart, kidney, adipose tissue, skeletal muscle, and intestine. The liver will also be analyzed for p-CaMKII, p-JNK134, and UPR markers (34) by immunoblot, and, in the context of liver inflammation, for *Tnfa* by QPCR. Without being bound by theory, these endpoints will be elevated in the DIO/WT group *vs.* lean-WT but suppressed in the DIO/Li-CK KO group.

[00397] HC CaMKIIγ deficiency improvement of glucose and lipid metabolism

[00398] 8 mice in each groups will undergo a complete hyperinsulinemic-euglycemic clamp study, which will further assess insulin sensitivity and quantify basal and insulin-stimulated hepatic glucose production (HGP) and insulin-stimulated glucose uptake, glycolysis, and glycogen synthesis in peripheral tissues (*e.g.*, skeletal muscle, adipose tissue) (129). The DIO/WT group *vs*. lean-WT group should show decreased glucose infusion, increased HGP, and decreased peripheral tissue glucose disposal, reflective of insulin resistance in these mice. These parameters will be improved (*i.e.*, increased glucose infusion and reduced HGP) in DIO/Li-CK KO *vs*. DIO/WT mice. 8 mice per group may have 80% power to show statistically significant differences between the DIO/Li-CK KO and DIO/WT groups ($P<0.05$).

[00399] DIO/Li-CK KO mice will have a less atherogenic lipid profile compared with DIO/WT mice, which will be tested by assaying plasma TG, total cholesterol, HDL-cholesterol, FPLC lipoprotein profile, and atherogenic and are elevated in insulin-resistant states (130). To complement the plasma studies, liver samples from the mice will be analyzed for TG content, LDL receptor protein and mRNA, and mRNA for *Srebp1c* and its transcriptional targets. The predicted decrease in plasma TG-rich lipoproteins in the DIO/Li-CK KO *vs*. DIO/mice mice can be due to decreased hepatic secretion or increased hepatic clearance. To assay TG secretion, a Triton WR-1339 study will be conducted (131). Briefly, fasted mice will be injected with [35S]-methionine to metabolically label apoB and with Triton WR-1339 to block lipoprotein clearance. Plasma will be collected throughout a 2-h post-injection period and analyzed for TG content and VLDL, and apoB100 and apoB48 will be assayed by SDS-PAGE followed by autoradiography. The rate of rise of labeled apoB-labeled lipoproteins during the 2-h period is a measure of secretion rate. CaMKIIγ deficiency increases (de-represses) hepatic Sort expression in the setting of insulin resistance (**Fig. 10C**). The goal is to determine if silencing hepatic *Sort* in DIO/Li-CK KO mice increases VLDL secretion. Fatty liver of DIO mice is markedly improved by CaMKIIγ deficiency (**Fig. 10A**). TG content will be measured and stained for Oil Red O-positive lipid droplets in liver specimens of the 4 groups of mice. If DIO/Li-CK KO mice show decreased liver TG accumulation, mechanism will be probed by assaying the expression of lipogenic genes, including *Srebf1*, *Fasn*, *Acaca*, *Scd1*; genes involved in fatty acid oxidation, including *Ppara*, and *Acox1*; and *Nampt* in view of its role in steatosis in liver-FoxO KO mice (132). Follow-up studies will explore the molecular mechanisms linking CaMKII to the positive findings, as guided by the results of the above experiments. For example, preliminary data support a link between the UPR effector ATF3 and the mTORC1-SORT suppression pathway, and thus, given the ability of CaMKIIγ to amplify the UPR.

[00400] Obesity is promoted by CaMKIIγ phosphorylation

[00401] In Mφs, ER stress activates CaMKIIγ through a pathway involving CHOP-induced release of ER Ca2+ stores (48,68). In view of the induction of hepatic ER stress and CHOP in obesity (34) (above) and the causative role for CHOP in ER stress-induced hepatic steatosis (94), whether CHOP is necessary for CaMKIIγ activation in obesity will be tested. *Chop*fl/fl mice on a pure C57BL6/J background have been made. CHOP deletion occurs when the mice were crossed with a deletor-Cre mouse. These mice will be crossed with *A1atcre+/-* mice to test whether these mice, compared with control *Chop*fl/fl mice, have less hepatic p-CaMKIIγ on the DIO diet (see **Fig. 8**). If so, and if glucose and lipid metabolism are improved in these mice using the above assays, they will be transduced with constitutively active adeno-T287D-CaMKII to show that the beneficial effects of hepatic CHOP deficiency are reversed. Follow-up metabolic and mechanistic experiments will follow pending the results.

[00402] GNG is promoted by CaMKII.

[00403] Fasting promoted phosphorylation of CaMKIIγ and that this was decreased by re-feeding (**Fig. 19A**). Consistent with these data, glucagon (and forskolin) promoted CaMKIIγ phosphorylation in primary HCs. Functional importance *in vivo* was shown by decreases in fasting and pyruvate-stimulated blood glucose and in liver *G6pc* and *Pck1* mRNA in fasting *Camk2g-/-* mice or in WT mice treated with adeno-K43A-CaMKII to more acutely inhibit liver CaMKII (**Fig. 19B-D**). CaMKIIγ deficiency or inhibition in fasting liver or in serum-starved HCs also led to a dramatic decrease in nuclear FoxO1 (**Fig 19E-F**), and constitutively active T287D CaMKII increased nuclear FoxO1 (**Fig. 19F**), plasma glucose and insulin, and hepatic *G6pc* and *Pck1* mRNA. The suppressive effect of CaMKIIγ deficiency on *G6pc* mRNA was abrogated by transduction with constitutively nuclear FoxO1-ADA (**Fig. 19G**), consistent with the idea that the effect of CaMKII deficiency requires nuclear exclusion of FoxO1. It may seem inconsistent that a kinase can promote nuclear FoxO1 given that Akt-induced FoxO1 phosphorylation at T24, S316, and S253 does the opposite (133). Indeed, CaMKIIγ deficiency did not promote phosphorylation at these sites. However, LC-MS phospho-peptide mapping studies (134) showed that two other sites in FoxO1—Ser284 and 295—were phosphorylated in serum-starved HCs from WT mice but were completely unphosphorylated in HCs from *Camk2g-/-* mice (**Fig. 19H**). Thus, without being bound by theory, the absence of pSer284/295 in CaMKII-deficient HCs promotes FoxO1 nuclear exclusion and thus suppresses GNG.

[00404] *G6pc* and *Pck1* mRNA and nuclear FoxO1 in the 4 groups of mice will be assayed. Both will be suppressed in DIO/Li-CK KO *vs.* DIO/WT mice. FoxO1 from the mice will be

immunoprecipitated for phospho-peptide mapping. CaMKII deficiency will be associated with absent pSer284/295-FoxO1. S284/295A-FoxO1 can mimic the effect of CaMKIIγ inhibition (↓ nuclear FoxO1 and *G6pc* and *Pck1* mRNA), while S284/295D FoxO1 should be resistant to the effects of CaMKIIγ inhibition. HCs of liver-specific FoxO1 KO mice, which have decreased GNG125, will be transduced with GFP-tagged WT or mutant FoxO1 constructs, ± adeno-K43A-CaMKII to inhibit CaMKII, and then FoxO1 localization and *G6pc* & *Pck1* mRNA will be assayed. To complement the use of K43A-CaMKII to inhibit CaMKII, HCs from Li-CK KO will be transduced with adeno-WT-FoxO1 *vs*. adeno-S284/295D-FoxO1. Without being bound by theory, this mutant will be constitutively nuclear and have a dominant negative effect on the ability of CaMKIIγ deficiency to suppress GNG. To summarize:

Table 2

HCs lacking FoxO1		Predictions		
adeno-CaMKII	adeno-FoxO1	nucl FoxO1	*G6pc*, *Pck1*	Explanation of Predictions
control	control	N/A	↓	GNG requires FoxO1[125]
control	WT	nucl	↑	FoxO1 should restore GNG in L1-FoxO1 HCs[125]
K43A	WT	cytopl	↓	CaMKII inhibition should suppress GNG—see Fig. 8
control	$S^{284/295}$A	cytopl	↓	$S^{284/295}$A-FoxO1 should **mimic** CaMKIIγ deficiency if pSer284/295 is important
control	$S^{284/295}$D	nucl	↑	$S^{284/295}$D-FoxO1 may be constitutively nuclear
K43A	$S^{284/295}$D	nucl	↑	$S^{284/295}$D-FoxO1 should **block** the GNG-suppressive effect dom-neg CaMKII

ad, adenovirus; control, ad-LacZ; K43A, dom. neg. CaMKII; S→A FoxO1, constitutively de-phos model; S→D FoxO1, constitutively "phos" model

[00405] These predictions will be translated to DIO-fed L-FoxO1 mice transduced with the above adenoviral constructs. The endpoints will be hepatic *G6pc* and *Pck1* mRNA; nuclear FoxO1 where applicable; plasma glucose (including post-pyruvate challenge) and insulin; and the lipid and lipoprotein parameters described above. The predictions are similar to those in the table, where decreases in the GNG genes will be accompanied by improvements in glucose and lipid/lipoprotein metabolism. In the unlikely situation that HGP is affected but *G6pc* and *Pck1* are not, the GNG genes *Pdk4*, *Fbp1*, and *Gck* will be assayed.

[00406] Activation of CaMKIIγ in the liver of obese humans.

[00407] Flash-frozen fresh liver biopsy specimens from obese subjects undergoing bariatric surgery and from lean subjects undergoing general surgery will be examined. There will be no patient identifiers, but there will be a coded list for each subject that includes weight, waist:hip ratio, age, and sex; diabetic/metabolic medicines; fasting plasma glucose, insulin, HbA1c, and free fatty acids; and based on histological analysis, degree of steatosis and steatohepatitis (graded on a scale from 1 to 5). The specimens will be immunoblotted for p- and total CaMKIIγ and for ER stress markers, including CHOP and Sort. The data will be quantified by densitometric

analysis relative to loading controls. The primary goal is to determine whether p-CaMKII is higher in liver from obese *vs*. lean subjects and whether within the obese group there is an increase of p-CaMKII as a function of worsening insulin resistance. A secondary goal is to determine whether high levels of p-CaMKII correlate with high levels of CHOP and a low level or Sort. The results described hereib (**Fig. 8B**) show that every lean liver sample has a lower p-CaMKII level than of every obese sample, and so the planned sample size of n = 20 for obese and lean livers should have a >95% power to show statistically significant differences in level of p-CaMKII between lean- and obese-derived specimens ($P<0.05$). For the associations within obese livers between p-CaMKII and insulin resistance and between p-CaMKII and CHOP and Sort expression, the 20 obese liver samples will provide 80% power to find a correlation coefficient of 0.6 to be statistically significant. Further studies will be guided by mechanistic studies, *e.g.*, those related to FoxO in the previous section.

[00408] CaMKIIγ in *liver Mφs* plays a role in insulin resistance and glucose and lipid metabolism in the setting of obesity.

[00409] HCs in obese mice express the chemokine CCL2 (MCP-1), which attracts new myeloid cells that subsequently play an important role in hepatosteatosis by promoting the transcription of genes involved in fatty acid esterification and TG accumulation (45). In addition, activated liver Mφs, including Kupffer cells, may suppress HC fatty acid oxidation through a paracrine mechanism (44). While HC CaMKIIγ can be instrumental in attracting new Mφs, without being bound by theory, Mφ CaMKIIγ can play an important role in Mφ-mediated effects on HC lipid metabolism. In particular, saturated fatty acids, which Mφs can be exposed to in obese liver, activate the UPR in Mφs and that UPR activation is linked to Mφ inflammation (72,111). If so, and given the role of CaMKIIγ in mediating and amplifying ER stress processes in Mφs (**Fig. 16**), liver Mφ CaMKIIγ can have important effects on HC lipid metabolism and insulin resistance in the setting of obesity. As described herein, TNFα is decreased in obese liver with inhibited CaMKII (**Fig. 10D**), and CaMKIIγ deficiency is linked to decreased Mφ inflammation (53,54). A DIO study similar will be performed wherein LysMCre mice will be used instead of A1at-Cre mice. If Mφ CaMKIIγ deficiency improves metabolic parameters, including steatosis, in the setting of obesity, mechanism will be examined. The primary concept is that ER stress-induced inflammation of Mφs can be lower, and so liver Mφ UPR and cytokine mRNAs by LCM-RT-QPCR will be assayed. In theory, there can also be a decrease in the *number* of liver Mφs if, for example, CaMKIIγ deficiency deceased monocyte chemokine receptor expression. Thus, liver Mφ content will be quantified using Mφ IHC. Further

mechanistic studies will be driven by the data. For example, there is evidence that PPARδ activation can be an ameliorating process in liver Mφs in the setting of obesity (44), which will prompt experiments investigating links among CaMKII, ER stress, inflammation, and PPARδ pathways in liver Mφs from the different groups of mice.

[00410] Atherosclerosis studies related to liver CaMKIIγ.

[00411] Hepatic CaMKIIγ deficiency, by improving dyslipidemia and possibly other parameters, *e.g.*, inflammation, circulating FFAs, arterial wall effects of hyperinsulinemia, and hyperglycemia, will lessen atherosclerosis in the setting of insulin resistance. Western diet (WD) feeding of *Ldlr*-/- mice is required for the atherogenic lipoprotein phenotype. On the C57BL/6 background, feeding the WD, whose fat content is only modestly lower than the DIO diet, leads to insulin resistance135 and, as shown in **Fig. 20**, phosphorylation of hepatic CaMKIIγ. To evaluate this model, mice will be bred to obtain the following 2 groups: *Camk2g*fl/fl*Ldlr*-/- (LDLR KO) and *Camk2g*fl/fl*A1atcre*+/-*Ldlr*-/- (Li-CK LDLR DKO). After 8, 12, 16 and 20 wks on WD, metabolic parameters, plasma and inflammatory cytokines and FFAs will be assayed. 8 wks on WD is sufficient time to develop insulin resistance in this model (135). There will be a focus on lesion endpoints that may be exacerbated by these factors. For lesion initiation, endothelial activation (*e.g.*, acetylchdependent vasodilation, VCAM-1 expression, p-eNOS, ROS, and monocyte adhesion to endothelium) and Mφ accumulation will be assayed. These are processes that have been linked to dyslipidemia (136), hyperglycemia (137), and the effect of increased FFAs and insulin resistance on the endothelium (130,138,139). For plaque progression, inflammatory cytokine expression will be assayed in plaque cells, especially Mφs28; Mφ apoptosis, which can be triggered by saturated FAs and insulin resistance (72,73); defective efferocytosis, which can be worsened by saturated FAs in obesity (140); and intraplaque hemorrhage in advanced plaques, which has been linked to the dyslipidemia of diabetes (141).

[00412] Absence of CaMKIIγ in liver *Mφs* can improve insulin sensitivity and glucose and lipid metabolism. In that case, the LysMCre model can have two mechanisms for improvement in atherosclerosis. Thus, whether deficiency of both Mφ *and* HC CaMKIIγ will have marked protective effects on all stages of atherosclerosis will be tested. For this purpose, 2 additional groups will be added to the study described herein in which the Li-CK LDLR DKO mice are transplanted with bone marrow from WT *vs*. *Camk2g*fl/fl*Lysmcre*+/- mice. This will be evaluated by testing all aspects of atherosclerosis and metabolism.

Table 3

Summary of mouse models and predictions for endpoints:

Model	Plasma glucose	Plasma insulin	Insulin resistance (clamp)	Dyslipid-emia	Fatty liver	Model	Athero-sclerosis
lean/WT	normal	normal	normal	normal	normal	LDLR KO	+++
DIO/WT	↑	↑	↑	↑	↑	Li-CK LDLR DKO	++
lean/Li-CK KO	normal	normal	normal	normal	normal	Mφ CK → Li-CK LDLR DKO	+
DIO/Li-CK KO	normal	normal	normal	normal	normal		
ad-T287D → DIO/Li-CK KO	↑	↑	↑	↑	↑		

[00413] With regard to the KO model, controls will be added through inclusion of the adeno-T287D-CaMKII → DIO/Li-CK KO group. If phosphorylation of FoxO1 Ser284/295 is not involved in mediating the metabolic effects of CaMKII, parameters will be explored including those that affect the nuclear import/export machinery involved in FoxO1 localization, such as interaction with 14-3-3 sites (142), and those related to possible effects of CaMKIIγ on the hepatic insulin receptor signaling pathway. If Ser284/295 phosphorylation is important, future studies will investigate (a) whether CaMKIIγ is directly involved or whether it induces another kinase for this purpose (143); and (b) how defective phosphorylation of these sites can promote nuclear export of FoxO1. A FoxO1-S284/293A mouse will be created to study the role of these p-Ser residues in liver glucose and lipid metabolism. Relief of ER stress in the setting of CaMKIIγ deficiency may improve the response of HCs to acute insulin. For example, suppression of ER stress can decrease Ser207 phosphorylation of IRS1, which improves insulin signaling (34).

Example 8: Calcium signaling through CaMKII regulates hepatic glucose production in fasting and obesity

[00414] Hepatic glucose production (HGP) is crucial for glucose homeostasis, but the underlying mechanisms have not been fully elucidated. Here it is shown that a calcium-sensing enzyme, CaMKII, is activated in a calcium- and IP3R-dependent manner by cAMP and glucagon in primary HCs and by glucagon and fasting in vivo. Genetic deficiency or inhibition of CaMKII blocks nuclear translocation of FoxO1 by affecting its phosphorylation, impairs fasting- and glucagon/cAMP-induced glycogenolysis and gluconeogenesis, and lowers blood glucose levels, while constitutively active CaMKII has the opposite effects. Importantly, the suppressive effect of CaMKII deficiency on glucose metabolism is abrogated by transduction with constitutively nuclear FoxO1, indicating that the effect of CaMKII deficiency requires nuclear exclusion of FoxO1. This same pathway is also involved in excessive HGP in the setting of obesity. These results reveal a calcium-mediated signaling pathway involved in FoxO1 nuclear localization and hepatic glucose homeostasis.

[00415] Highlights include:

- Fasting and glucagon activate hepatic CaMKII in a PKA-IP3R1-Ca2+i-dependent manner
- CaMKII promotes fasting/glucagon-induced hepatic glucose production (HGP)
- CaMKII promotes nuclear FoxO1, which is required for CaMKII-mediated stimulation of HGP.
- The CaMKII-FoxO1 pathway is also involved in excessive HGP in the setting of obesity.

[00416] Liver is the main organ responsible for maintaining euglycemia under conditions of nutrient deprivation. During the early stages of fasting, liver uses glycogen stores to mobilize glucose (Radziuk and Pye, 2001). As fasting progresses, de novo synthesis of glucose from non-carbohydrate precursors, gluconeogenesis, becomes the main contributor to hepatic glucose production (HGP) (Lin and Accili, 2011). These changes occur rapidly in response to direct hormonal signaling. In addition, both insulin and glucagon affect transcription of glucose-6-phosphatase (G6pc), which is involved in both gluconeogenesis and glycogenolysis, and phosphoenolpyruvate carboxykinase (Pck1), which also regulates HGP (Pilkis and Granner, 1992; Burgess et al., 2007). During fasting, changes in the subcellular localization of "glucogenic" transcription factors, such as FoxO (1, 3, and 4) and Crct2, activate expression of these genes (Lin and Accili, 2011). In addition, different co-activators, such as peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and CBP, are thought to interact with components of the cAMP response, including CREB, hepatic nuclear factor 4α (HNF4α), Sirt1, and Clock genes, leading to an increase in transcription of gluconeogenic genes (Hall et al., 1995; Matsumoto et al., 2007; Puigserver et al., 2003; Rhee et al., 2003). In addition to its role in stimulating HGP during fasting, excessive glucagon signaling is thought to play an important role in hyperglycemia in type 2 diabetes (Sorensen et al., 2006; Unger and Cherrington, 2012; Saltiel, 2001).

[00417] The intracellular signal transduction pathways through which glucagon stimulates the nuclear translocation of HGP transcription factors in general, and FoxO1 in particular, to stimulate HGP is not well understood. In this context, previous reports that linked intracellular calcium ($Ca^{2+}{}_i$) to the regulation of gluconeogenesis were of interest (Friedmann and Rasmussen, 1970; Kraus-Friedmann and Feng, 1996; Marques-da-Silva et al., 1997). For example, glucagon and cAMP can increase $Ca^{2+}{}_i$, and $Ca^{2+}{}_i$ chelation has been shown to reduce glucagon induced HGP gene expression and glucose production (Bygrave and Benedetti, 1993; Staddon and Hansford, 1989; Mine et al., 1993). Based on these previous studies, which did not offer a

molecular mechanism linking Ca^{2+}_i to hepatic glucose metabolism, without being bound by theory, a role for the Ca^{2+}_i -sensing enzyme CaMKII is implicated.

[00418] Calcium calmodulin-dependent kinase II (CaMKII) is a serine–threonine kinase that is an important mediator of Ca^{2+}_i signaling in cells (Couchonnal and Anderson, 2008; Singer, 2011). There are four CaMKII isoforms—α , β, γ and δ—each encoded by a separate gene. The α and β isoforms are mostly neuronal, while CaMKIIγ and δ are expressed in a wide variety of tissues. After binding calcium/calmodulin complex, autophosphorylation on Thr287 results in calcium/calmodulin independent activity (Couchonnal and Anderson, 2008; Singer, 2011). Most studies on CaMKII have been carried out in neurons and cardiomyocytes, and there is only a limited understanding of CaMKII in other tissues, with none to date related to glucose metabolism. In the present study, it is shown that CaMKII activity is increased by cAMP and glucagon and also in response to fasting *in vivo*. It is further demonstrated that CaMKII plays an essential role in the regulation of glycogenolysis and gluconeogenesis. In particular, evidence is provided that CaMKII has a profound effect on FoxO1 nuclear localization in a manner that regulates the expression of two key enzymes, *G6pc* and *Pck1*, *in vitro* and *in vivo*. Finally, evidence indicating that this same pathway is involved in excessive HGP in the setting of obesity is presented.

Results

[00419] Glucagon and fasting activate hepatic CaMKII in a IP3R- and Ca^{2+}_i-dependent manner

[00420] Glucagon has been shown to increase intracellular calcium (Ca^{2+}_i) in hepatocytes (HCs) (Staddon and Hansford, 1989), which was recently verified (Y. Wang, G. Li, J. Goode, J. C. Paz, R. Screaton, W. H. Fischer, I. Tabas, and M. Montminy, manuscript submitted for publication). To determine whether glucagon activates the Ca^{2+}_i-sensing enzyme, CaMKII, primary murine HCs were treated with glucagon for various periods of time and then assayed CaMKII enzymatic activity and CaMKII phosphorylation at Thr287, which is a measure of its activation state (Couchonnal and Anderson, 2008; Singer, 2011). The results of both assays show that CaMKII activity increases as a function of time of glucagon treatment (**Fig. 1A-B**). The cytosolic calcium chelator,1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid tetrakis [acetoxymethyl ester] (BAPTA-AM), was used to determine the role of Ca^{2+}_i on CaMKII activation and it was found that BAPTA-AM markedly decreased glucagon-induced CaMKII phosphorylation (**Fig. 1C**).

[00421] Inositol 1,4,5-trisphosphate receptor (IP_3R) channels, located in the endoplasmic reticulum (ER), release Ca^{2+} in response to IP_3 binding and play a major role in intracellular $Ca^{2+}{}_i$ homeostasis. Additional studies have revealed that glucagon-induced PKA phosphorylates and increases IP3R activity, leading to an increase in $Ca^{2+}{}_i$ (Y. Wang, G. Li, J. Goode, J. C. Paz, R. Screaton, W. H. Fischer, I. Tabas, and M. Montminy, manuscript submitted for publication). Glucagon has also been shown to induce phospholipase C-mediated IP3 release (Hansen et al., 1998). To investigate the contribution of IP3Rs in glucagon-induced CaMKII activation, the IP3R inhibitor xestospongin C and, as a complementary approach, adeno-Cre-treated HCs from *Ip3r1*$^{fl/fl}$ mice, were used. Both xestospongin C treatment and Cre-mediated deletion of IP3R1 led to a significant decrease in glucagon-induced CaMKII phosphorylation, demonstrating the critical role of IP3Rs in this process **(Fig. 1D)**.

[00422] Glucagon receptor signaling, including that involved in the increase in $Ca^{2+}{}_i$ (Staddon and Hansford, 1989), is mediated by activation of adenylate cyclase to produce cAMP, followed by activation of protein kinase A (PKA), a key enzyme involved in HGP. In this context, it was found that treatment of HCs with 8-bromo-cAMP mimicked the effect of glucagon and led to a marked increase in phospho-CaMKII **(Fig. 1E)**. Moreover, when HCs were treated with the PKA inhibitor H89 prior to the addition of glucagon, glucagon-mediated increase in phospho-CaMKII was markedly inhibited **(Fig. 1F)**. These data support the existence of a pathway in which glucagon-cAMP-PKA signaling promotes phosphorylation/activation of CaMKII through its effects on IP3R-mediated intracellular Ca^{2+} release.

[00423] To examine whether CaMKII is regulated by glucagon *in vivo*, mice were challenged with a bolus of intraperitoneal (i.p.) glucagon. Consistent with the effects observed in cultured HCs, hepatic CaMKII phosphorylation was induced by glucagon treatment **(Fig. 1G)**. It was found that a glucagon dose as low as 1 $\mu g\ kg^{-1}$ was capable of phosphorylating CaMKII in the liver (**Fig. 28A**). To gain *in vivo* evidence that IP3Rs are important in the regulation of glucagon-mediated CaMKII phosphorylation, mice were treated with i.p. xestospongin C for 4 days. The mice were then challenged with glucagon, and liver extracts were assayed for p-CaMKII. As shown in **Fig. 1H**, xestospongin C treatment markedly reduced glucagon-induced CaMKII phosphorylation. Next, hepatic CaMKII phosphorylation was compared during the transition from a fed to fasting state, which is known to elevate plasma glucagon (Lin and Accili, 2011) (**Fig. 28B**). The data show that hepatic CaMKII phosphorylation was significantly increased upon fasting, whereas the total amount of CaMKII appeared to be unaffected by nutrient status **(Fig. 1I)**. Moreover, upon re-feeding, the level of p-CaMKII in liver diminished

(Fig. 1J). As with glucagon treatment, fasting-induced phosphorylation of CaMKII was suppressed by xestospongin C treatment of the mice (**Fig. 28C**). These data show that activity of hepatic CaMKII is regulated by nutrient status in a manner that is consistent with a potential role in fasting-induced HGP.

[00424] CaMKII promotes glucose production in primary HCs

[00425] CaMKIIγ is the major CaMKII isoform in HCs, and the other isoforms are not induced in HCs lacking the γ isoform (**Fig. 22A**). In view of the regulation of hepatic CaMKII activity by glucagon and fasting *in vivo*, glucose production was assayed in HCs from WT and *Camk2g*$^{-/-}$ mice. The cells were examined under basal conditions and after stimulation with forskolin, a glucagon mimetic and a potent adenylate cyclase activator (Harano et al., 1985). The data show that both basal and forskolin-induced glucose production was suppressed in CaMKIIγ-deficient HCs **(Fig. 22B)**. Glucose production was next examined in WT HCs transduced with adenoviruses expressing constitutively active CaMKII (adeno-CA-CaMKII), "kinase-dead" dominant-negative CaMKII (adeno-KD-CaMKII) (Pfleiderer et al., 2004), or LacZ control. CA-CaMKII possesses an amino acid substitution, T287D, which mimics autophosphorylation at T287 and results in autonomous activity in the absence of bound calcium/calmodulin, while KD-CaMKII has a disabling mutation in the kinase domain (Pfleiderer et al., 2004). An increase in both basal and forskolin-induced glucose release was observed in cells transduced with adeno-CA-CaMKII (**Fig. 22C and 28D**). HCs transduced with adeno-KD-CaMKII, which resulted in ~40% decrease in CaMKII activity (**Fig. 28E**), showed decreased forskolin-induced glucose production **(Fig. 22C)**.

[00426] The role of CaMKII on HGP prompted us to investigate transcriptional effects on two genes encoding enzymes that regulate HGP, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase. To this end, *G6pc* and *Pck1* mRNA levels were assayed in the models described above (**Fig. 22D-E**). In all cases, knockout or KD-CaMKII-mediated inhibition of CaMKII lowered forskolin- or glucagon-induced gene expression, whereas CA-CaMKII increased gene expression. In the absence of forskolin or glucagon, expression levels of *G6pc* and *Pck1* mRNA in WT HCs were much lower than those in hormone-treated WT HCs, but even under these conditions CaMKIIγ deficiency led to a lowering of gene expression (**Fig. 28F**). Moreover, adeno-KD-CaMKII did not decrease the low but detectable level of forskolin-induced *G6pc* mRNA in HCs lacking CaMKIIγ (**Fig. 28G**), consistent with the premise that the suppressive effect of KD-CaMKII on *G6pc* in **Fig. 22E** is due to CaMKII inhibition.

[00427] In summary, the CaMKII deficiency and inhibition data show the importance of endogenous CaMKII in glucose production and *Pck1/G6pc* gene expression, while the data with CA-CaMKII show that when the enzyme is expressed at a high level, it can force these processes in the absence of hormones or increase them in the presence of hormones.

[00428] Hepatic glucose production *in vivo* is impaired by CaMKIIγ deficiency and stimulated by constitutively active CaMKII

[00429] To assess the functional role of CaMKII in hepatic glucose metabolism *in vivo*, fasting blood glucose levels were examined in WT and *Camk2g*$^{-/-}$ mice. Consistent with the *in vitro* data, a modest but statistically significant decrease in blood glucose levels was observed in fasted *Camk2g*$^{-/-}$ *vs*. WT mice **(Fig. 23A)**. The difference in fasting glucose concentration was not associated with an increase in circulating insulin or a decrease in glucagon concentrations in knockout vs. WT mice (**Fig. 29A, left**). The mutant mice also showed lower plasma glucose in response to a pyruvate challenge test **(Fig. 23B)**. Consistent with the primary HC data, there was a decrease in *G6pc* and *Pck1* mRNA levels in the livers of fasting *Camk2g*$^{-/-}$ mice **(Fig. 23C)**. Similar data were obtained in mice treated with glucagon (**Fig. 23D**).

[00430] Consistent with these data, treatment of C57BL/6 mice with adeno-KD-CaMKII, which inhibited liver CaMKII activity by ~45% (**Fig. 29B**), decreased fasting blood glucose **(Fig. 23E)**. As above, plasma glucagon and insulin were not different between the control and experimental groups (**Fig. 29A, right**). In line with blood glucose data, hepatic expression of *G6pc* and *Pck1* mRNA was lower in mice injected with KD-CaMKII **(Fig. 23F)**. Because CaMKII inhibition lowers the level the mRNA for the key glycogenolytic enzyme glucose-6-phosphatase, the effect of acute and chronic CaMKII inhibition on liver glycogen content was examined and, as another indicator of glycogen, the percent of periodic acid-Schiff (PAS) - positive cells was also examined. The data show that adeno-KD-CaMKII or CaMKII gene targeting increases hepatic glycogen in fasting mice **(Fig. 23G)**.

[00431] The effect of constitutively active hepatic CaMKII was next examined in mice by treating mice with adeno-CA-CAMKII. The CA-CaMKII group had elevated blood glucose levels after pyruvate challenge, increased liver *G6pc* and *Pck1* mRNA levels, and increased liver glycogen content (**Fig. 29C-E**). CA-CaMKII administration did not alter plasma glucagon or insulin. These combined *in vivo* data show that CaMKII affects plasma glucose levels, pyruvate conversion into glucose, and the expression of hepatic glucose metabolism genes.

[00432] CaMKII promotes nuclear localization of FoxO1

[00433] A major transcription factor involved in HGP is FoxO1, which is regulated primarily by changes in its localization between the cytoplasm and nucleus (Accili and Arden, 2004). The distribution of GFP-tagged FoxO1 that was transduced into HCs isolated from WT vs. *Camk2g*$^{-/-}$ mice was assayed. Under serum-starved conditions, the majority of GFP-FoxO1 was in the nucleus in WT HCs, whereas *Camk2g*$^{-/-}$ HCs displayed primarily cytosolic localization of GFP-FoxO1 (**Fig. 24A**). Moreover, when HCs were transduced with adeno-CA-CaMKII, FoxO1 became predominantly nuclear, while transduction with adeno-KD-CaMKII caused mostly cytoplasmic FoxO1 (**Fig. 24B**). It was noted that nuclear FoxO1 was substantial in cultured HCs under the "basal" cell culture conditions used here, and so the fold increase with CA-CaMKII was limited. Basal nuclear FoxO1 was therefore lowered using short incubations with insulin, which then revealed a marked increase in nuclear FoxO1 with CA-CaMKII (**Fig. 24C**). Although this experiment was done using a high level of total CaMKII protein expression (see **Fig. 28D**), a similar experiment using a lower MOI of CA-CaMKII showed an increase in nuclear FoxO1 at a level of total CaMKII protein that was similar to endogenous CaMKII (**Fig. 30A**). To show relevance *in vivo*, the effect of CaMKIIγ deficiency or inhibition was tested in fasting mice. Nuclear FoxO1 in liver was prominent under fasting conditions (**Fig. 24D, top blot**), and it was markedly diminished in *Camk2g*$^{-/-}$ mice or mice transduced with adeno-KD-CaMKII (**Fig. 24D, middle 2 blots**). Feeding diminished nuclear FoxO1, and nuclear FoxO1 was increased under these conditions by both CA-CaMKII and glucagon (**Fig. 24D, bottom blots)**.

[00434] cAMP-mediated induction of *G6pc* mRNA in primary hepatocytes is suppressed >50% by *Foxo1* shRNA, indicating an important role for FoxO1 in the endogenous setting (Matsumoto et al., 2007). Consistent with these data, it was found that induction of luciferase downstream of the human *G6PC* promoter was blunted when three consensus FoxO-binding sites were mutated (Ayala et al., 1999; von Groote-Bidlingmaier et al., 2003) (**Fig. 30B**). However, this hepatoma cell line—reporter construct experiment does not distinguish between cAMP and dexamethasone effects and cannot accurately reflect the endogenous situation. For example, reporter induction here was much less robust than actual *G6pc* mRNA induction in primary hepatocytes (Matsumoto et al., 2007), and the identified promoter element may not be the only site required for regulation. Therefore, rather than pursue this model further as a way to assess the functional importance of FoxO1 in CaMKII-mediated *G6pc* expression, the induction of endogenous *G6pc* in primary hepatocytes and, most importantly, *in vivo*, was studied. To begin, the ability of CA-CaMKII to induce *G6pc* in HCs from WT vs. L-Foxo1 KO mice, which lack FoxO1 in liver, was compared (Matsumoto et al., 2007). Consistent with previous studies (Puigserver et al., 2003;

Matsumoto et al., 2007), forskolin-induced expression of *G6pc* was suppressed in the absence of FoxO1 (**Fig. 25A, right graph**). Most importantly, the increase in *G6pc* mRNA expression by CA-CaMKII was markedly blunted by FoxO1 deficiency. In the absence of forskolin, gene expression was much lower as expected (**Fig. 25A, left graph**; note Y-axis scale), but even here CA-CaMKII-induced gene expression was almost completely dependent on FoxO1. Finally, the fact that the nuclear localization of two other transcription factors involved in HGP, CREB and Crtc2, were not decreased by CaMKII inhibition of deficiency, was documented (**Fig. 30C-D**). These combined data are consistent with a model in which CaMKII promotes FoxO1 nuclear localization, which then leads to induction of *G6pc*.

[00435] To further validate the importance of FoxO1 in the impairment of HGP by CaMKII deficiency or inhibition, HCs were transduced from *Camk2g*$^{-/-}$ mice with adenovirus containing a phosphorylation-defective, constitutively nuclear FoxO1 mutant (FoxO1-ADA) (Nakae et al., 2001). It was observed that the suppressive effect of CaMKIIγ deficiency on forskolin-induced *G6pc* and *Pck1* mRNA expression was abrogated by transduction with adeno-FoxO1-ADA **(Fig. 25B)**. Note that the level of FoxO1-ADA used here was low enough so as not to increase *G6pc* or *Pck1* in forskolin-treated WT HCs, and the level of nuclear FoxO1 in the *Camk2g*$^{-/-}$ + ADA group was similar to the endogenous level in the WT + LacZ group (**inset, Fig. 25B**). The *Pck1* results in **Fig. 25B**, as well as those in **Figs. 22-23**, are interesting in view of the finding that germline knockdown of FoxO1 does not affect *Pck1* induction (Nakae et al., 2002; Barthel et al., 2001). However, more acute silencing of FoxO1 does suppress *Pck1* (Matsumoto et al., 2007), and so manipulations of CaMKII may be more parallel to that setting. Consistent with the effect of FoxO1-ADA on HGP gene expression, treatment of mice with adeno-FoxO1-ADA adenovirus rescued the impairment of glucose homeostasis in adeno-KD-CaMKII-treated mice **(Fig. 25C-E)**. Note that this result cannot be explained by defective transduction with adeno-KD in the ADA group (**inset, Fig. 25E**). Taken together, these results are consistent with a model in which CaMKII contributes to HGP through promoting nuclear localization of FoxO1.

[00436] <u>The role of non-AKT-phospho-FoxO1 sites and p38 MAP kinase in CaMKIIγ-mediated FoxO1 nuclear localization</u>

[00437] Insulin/Akt promotes FoxO1 nuclear exclusion through phosphorylation of T24, S253, and S316 (murine residues) (Brunet et al., 1999). Although CaMKII is a kinase, it can activate a phosphatase and thereby promote nuclear localization of FoxO1 by indirectly decreasing the phosphorylation at these sites. However, it was found that phosphorylation at these three sites was not altered in liver from *Camk2g*$^{-/-}$ mice (**Fig. 31A**). FoxO1 acetylation, which can also

affect FoxO1 localization and activity in HCs (Accili and Arden, 2004), was also not affected by CaMKII deficiency (**Fig. 31B**).

[00438] FoxO1 can also be phosphorylated at other Ser/Thr residues by other kinases, such as p38 MAP kinase (Asada et al., 2007), and these phosphorylation events might promote FoxO1 nuclear localization, not exclusion. To assess the possible role of CaMKII in the phosphorylation of non-Akt sites, the model displayed in **Fig. 24A** was used, i.e., serum-starved WT and *Camk2g*$^{-/-}$ (KO) HCs transduced with GFP-FoxO1, which also carries a FLAG tag. FoxO1 was immunopurified using anti-FLAG, followed by reduction, alkylation, and proteolytic digestion using a triple protease protocol developed by MacCoss *et al.* (MacCoss et al., 2002). Phosphorylated peptides were enriched by TiO_2 chromatography and then analyzed by LC-MS/MS (Cantin et al., 2007) using MS-based shotgun proteomic methods and label-free quantitation by spectral counting (Cantin et al., 2007). The FoxO1 protein was identified with ~70% sequence coverage, and a total of 57 phosphopeptides for WT and 63 phosphopeptides for KO samples were identified (**Tables S1 and S2** at http://fields.scripps.edu/published/foxo1_Tabas_2012/, which are incorporated by reference in their entireties). The peptide false discovery rate (FDR) was less than one percent. Stringent selection criteria were used so that all identified phosphopeptides would have high confidence.

[00439] These criteria, with further validation using the phosphopeptide analysis tools Debunker and Ascore (Lu et al., 2007; Beausoleil et al., 2006), enabled the identification of 11 phosphorylation sites: S284, S295, S326, S467, S475, T24, S246, S253, S413, S415 and T553 (see **Fig. 31C** for murine FoxO1 sequence). **Fig. 26A** shows the spectral counts, the Debunker score, and Ascore of each of these 11 FoxO1 phosphopeptides from the KO and WT samples. Most phosphorylation sites are well above the high confidence cut-off values of 0.5 and 12 for Debunker and Ascore, respectively. Five sites with slightly lower values of Debunker or A scores were subjected to manual verification, and their annotated tandem mass spectra are shown in **Fig. 32D-E** for KO peptides 4 and 5 and the aforementioned website for WT peptides 7, 10, and 11. The characteristic b- and/or y-ions for the phospho-sites are all identified. The lower scores are most likely due to the low-abundance fragment ions or lack of neutral losses of phosphoric acid because of the nature of the amino acid sequence for these peptides.

[00440] The ratio of spectral KO:WT counts, which was calculated only for peptides with a combined spectral count in KO and WT samples above 10, was used to obtain a measure of the relative expression of identified phosphorylated peptides. By this analysis, only phosphorylation of S295, S467, S475 (peptides 2, 4, and 5) were significantly lower in the KO based on a cut-off

value of < 0.5, with the ratio of spectral counts in KO vs. WT of 0.45, 0.38 and 0.5, respectively. Although the peptide containing p-S246 had a combined spectral count of 7, and thus did not reach the pre-specified criterion of >10, it showed a lower trend in the KO vs. WT (2 vs. 5, 0.4). In contrast, S326 (peptide 3) had a ratio of 1.65, indicating upregulation in KO vs. WT.

[00441] As an initial test of function for the some of the sites lower in the KO, an available plasmid encoding FoxO1 with S-A mutations at seven Ser residues (7A-FoxO1), including Ser295 and 475, as well as Ser246, was used (Asada et al., 2007). When transfected to a similar levels in *Foxo1*$^{-/-}$ HCs, 7A-FoxO1 showed strikingly less nuclear localization than WT FoxO1 in response to glucagon, while cytoplasmic FoxO1 was higher in the cells transfected with the mutant FoxO1 (**Fig. 26B**). A construct that had the same seven S-A mutations as in 7A plus 2 additional S-A mutations in S326 and S467 (mutant 9A) was also tested (Asada et al., 2007). This mutant showed similarly defective nuclear localization (**Fig. 31F**). Moreover, whereas adeno-CA-CaMKII transduction increased nuclear WT-FoxO1, consistent with the data in **Fig. 24D**, CA-CaMKII did not increase nuclear 7A-FoxO1 (**Fig. 26C**). These combined data are consistent with a model in which CaMKII directly or indirectly alters the phosphorylation of certain Ser residues in FoxO1 in a manner that promotes its nuclear localization.

[00442] Asada et al. (Asada et al., 2007) found evidence of FoxO1 phosphorylation at several sites, including Ser284, 295, 467, and 475, in HEK293T cells transfected with the upstream p38 kinase MKK6. Because p38 has been implicated in the stimulation of HGP (Cao et al., 2005), and CaMKII can activate p38 when studied in neurons (Blanquet, 2000), a CaMKII → p38 → FoxO1 phosphorylation/nuclear localization pathway involved in HGP was considered. It was first confirmed that p38 was phosphorylated, which is a measure of its activation, in the livers of fasting mice (**Fig. 32A**) and that inhibition of p38 by the high-affinity competitive inhibitor SB202190 blocked the expression of *G6pc* and *Pck1* in glucagon-stimulated HC (**Fig. 32B, left**). The inhibitor data were confirmed using adeno-Cre-transduced HCs isolated from the livers of from *P38a*$^{fl/fl}$ mice (**Fig. 32B, right**). To test a potential link between CaMKII and p38, serum-starved HCs from WT and *Camk2g*$^{-/-}$ mice were compared and a striking decrease in phospho-p38 in the CaMKIIγ-deficient HCs was found (**Fig. 32C**). It was then determined whether p38 was involved in hepatic FoxO1 nuclear localization *in vivo* by comparing fasting mice treated with SB202190 vs. vehicle control. Among the 7 mice in each group, there was a certain degree of variability in both the basal level of p-MK2, a p38 kinase target that reflects p38 activity, and in the level of inhibition of MK2 phosphorylation by SB202190. Nuclear FoxO1 for all 14 mice was therefore plotted as a function of p-MK2. The data show a clear decrease in nuclear FoxO1

in mice as a function of p38 inhibition, *i.e.*, as indicated by lower p-MK2 (**Fig. 32D**). Moreover, the p38 inhibitor decreased nuclear GFP-FoxO1 in HCs treated with glucagon (**Fig. 32E**). These combined data are consistent with a model in which CaMKII promotes FoxO1 nuclear localization through p38 activation. Whether p38 functions in CaMKII-induced FoxO1 nuclear localization by directly phosphorylating the aforementioned Ser residues in FoxO1 (Asada et al., 2007) remains to be determined.

[00443] The role of CaMKII in hepatic glucose metabolism in obesity

[00444] Elevated HGP, in part due to an imbalance of glucagon-to-insulin signaling, contributes to fasting hyperglycemia in obesity and other insulin-resistant states (Sorensen et al., 2006; Unger and Cherrington, 2012; Saltiel, 2001). To test the role of CaMKIIγ in hepatic glucose metabolism in the setting of obesity, evidence of hepatic CaMKIIγ activation was sought in two mouse models of obesity. It was found that the level of p-CaMKII, but not total CaMKII, was markedly higher in the livers of both *ob/ob* mice and WT mice placed on a high-fat, high-calorie diet for 20 wks (diet-induced obesity; DIO) (**Fig. 27A**). Antibody specificity for both anti-p-CaMKII and anti-CaMKII in obese liver is shown by the absence of the immunoblot bands in obese *Camk2g*$^{-/-}$ mice. The functional importance was next tested by comparing fasting plasma glucose and hepatic FoxO1-target gene expression in *ob/ob* mice transduced with adeno-KD-CaMKII vs. adeno-LacZ control. The mice treated with adeno-KD-CaMKII had lower fasting glucose, lower blood glucose after pyruvate challenge, and lower expression of three FoxO1-target genes, including *G6pc* and *Pck1* (**Fig. 27B-D**) These changes were not associated with either higher plasma insulin or lower weight in the adeno-KD-CaMKII-treated mice. Thus, hepatic CaMKII is activated in the livers of obese mice and regulates hepatic glucose metabolism and FoxO1-target gene expression.

Discussion

[00445] The data in this report provide evidence for calcium-mediated regulation of HGP as part of a pathway that can be summarized as follows: glucagon/fasting → cAMP/PKA → IP3R1 → Ca^{2+}_{I} → **CaMKII** → nuclear FoxO1 → HGP. CaMKII also mediates elevated HGP in obese mice (**Fig. 27**), and although more work is needed in this area, it is possible that driving force here is also glucagon (Sorensen et al., 2006; Unger and Cherrington, 2012; Saltiel, 2001). As such, the present findings have implications for three fundamental areas related to HGP: the molecular mechanisms whereby glucagon and fasting, as well as obesity/insulin resistance, stimulate HGP; the molecular links between intracellular calcium and HGP; and the regulation of FoxO1 nuclear transport. The latter issue is of particular interest, because while there have

been many reports on how insulin/AKT-mediated phosphorylation of FoxO1, as well as FoxO1 acetylation, promote nuclear exclusion of FoxO1 (Lin and Accili, 2011; van der Horst and Burgering, 2007), there has been little emphasis on the regulation of FoxO1 nuclear *entry* that occurs in the absence of insulin or in the setting of insulin resistance.

[00446] The CaMKII pathway is downstream of cAMP/PKA, and so it would naturally complement other glucagon-PKA pathways that stimulate HGP. Thus far, the data herein indicate that these other pathways occur in parallel with the CaMKII pathway rather than also being downstream of CaMKII. For example, glucagon-PKA directly phosphorylates cAMP response element binding (CREB) protein, which transcriptionally induces the FoxO1 transcriptional co-factor PGC1α (Herzig et al., 2001), but there was no difference in nuclear CREB in livers from adeno-LacZ vs. KD-CaMKII mice (**Fig. 30B**). This was an important finding, because there are *in vitro* data in neurons and in RANKL-treated RAW264.7 cells that CaMKII can activate/phosphorylate CREB in certain settings (Dash et al., 1991; Sheng et al., 1991; Ang et al., 2007). It was also found that CaMKII deficiency did not affect nuclear Crtc2 (**Fig. 30C**), which is another transcriptional activator involved in HGP. These data indicate that CaMKII in liver works in parallel with these other pathways, which together effect the nuclear localization of the proper array of transcriptional factors to mediate HGP. The case with Crtc2 is particularly interesting, because glucagon/PKA-mediated IP3R activation and ER calcium release promotes Crtc2 nuclear localization through another calcium-sensing enzyme, calcineurin. Indeed, it was found that inhibition of CaMKII and calcineurin are additive in terms of suppressing forskolin-induced *G6pc* mRNA. Thus, a common proximal signaling pathway leads to the coordinated nuclear entry of two key HGP transcription factors, Crtc2 and FoxO1, by different distal mechanisms. In this regard, it is interesting to note a previous study showing that drugs that promote calcium entry through the plasma membrane actually decrease *Pck1* mRNA in HCs (Valera et al., 1993), which can indicate that the route of calcium entry into the cytoplasm is a factor in determining downstream events.

[00447] FoxO1 is phosphorylated at Thr24, Ser253, and Ser316 (murine sequence numbers) by insulin/growth factors via Akt to promote its nuclear exclusion. CaMKII promotes FoxO1 nuclear localization, but, without being bound by theory, CaMKII can activate a phosphatase that de-phosphorylates these sites. However, CaMKIIγ deficiency did not affect the phosphorylation of these three residues, and it also did not affect FoxO1 acetylation (**Fig. 31A-B**). Instead, evidence that CaMKII mediates the phosphorylation of other Ser residues on FoxO1 was found,

and the Ser-Ala FoxO1 mutant experiments indicate that this action plays a role in CaMKII-mediated FoxO1 nuclear localization.

[00448] The link between CaMKII and FoxO1 phosphorylation can be direct or indirect. An indirect mechanism, *i.e.*, whereby CaMKII activates another kinase, can be linked to previous findings that other kinases can phosphorylate FoxO on non-Akt sites in a manner that promotes their nuclear retention (Essers et al., 2004; Chiacchiera and Simone, 2010). Based on the p38 inhibitor and gene-targeting data herein and the study of Asada *et al.* (Asada et al., 2007), without being bound by theory, p38 MAPK can also be able to carry out this function and, indeed, can be the mediator of CaMKII-induced FoxO1 nuclear localization. In support of this are reports of links between CaMKII and p38 and between p38 and HGP (Cao et al., 2005; Blanquet, 2000). While there is no direct evidence yet that p38 phosphorylates and thereby activates FoxO1, the ability of glucocorticoids to promote FoxO1 nuclear localization in rat cardiomyocytes correlated with activation/phosphorylation of nuclear p38, and immunofluorescence microscopy and IP/immunoblot data indicated that p-P38 and FoxO1 can interact with each other (Puthanveetil et al., 2010). Interestingly, there is evidence that FoxO1 can be able to activate p38 in HCs (Naimi et al., 2007), and so it is possible that a FoxO1-p38 feed-forward pathway can amplify the effect the CaMKII-p38 pathway indicated here on FoxO1 nuclear localization. However, more work is needed to establish the role of p38 and to further elucidate the mechanisms whereby CaMKII promotes FoxO1 nuclear localization.

[00449] The discovery of the role of calcium-CaMKII in HGP not only provides insight into the physiologic defense against fasting hypoglycemia but can also reveal therapeutic targets for the disturbed glucose metabolism that occurs in the setting of insulin resistance, as indicated by the data in **Fig. 27**. Indeed, in type 2 diabetes, disproportionate HGP and an imbalance of glucagon vs. insulin signaling contributes to fasting hyperglycemia (Sorensen et al., 2006; Saltiel, 2001). Moreover, glucagon signaling has also been implicated in type 1 diabetes (Unger and Cherrington, 2012). In this context, future studies will further address the pathophysiologic role(s) and mechanisms of hepatic CaMKIIγ in obesity, insulin resistance, and diabetes and thereby evaluate its potential as a therapeutic target in these disorders.

[00450] Experimental Procedures

[00451] Measurement of CaMKII Activity

[00452] CaMKII activity was assayed using a CaMKII assay kit from Promega according to the manufacturer's instructions. After the HCs were treated as indicated in the figure legends,

they were lysed by a 5-min exposure to 1% Triton-X100 in 50 mM HEPES, 150 mM NaCl, 10 mM Na pyrophosphate, 10 mM EDTA, 10 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, and 5 μg ml^{-1} leupeptin. Next, [γ-^{32}P]ATP and biotinylated CaMKII peptide substrate were added to the lysate or to the immunoprecipitated complexes (see below). After incubation for 10 min at 30°C, the [^{32}P]-phosphorylated substrate was separated from the residual [^{32}P]ATP using SAM biotin-capture membrane and then quantitated using a scintillation counter. Assays were conducted ± calmodulin, and the activity value in the absence of calmodulin was subtracted from those obtained in the presence of calmodulin.

[00453] Glucose Production in Primary HCs

[00454] Glucose production assays were carried out as described (Yoon et al., 2001). Briefly, after primary mouse HCs were harvested and cultured as described above, the cell culture medium was switched to glucose- and phenol-free DMEM (pH 7.4) supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate. After 16 h of culture, 500 μl medium was collected, and the glucose content was measured using a colorimetric glucose assay kit (Abcam). The readings were then normalized to the total protein amount in the whole-cell lysates.

[00455] Mouse Experiments

[00456] *Camk2g*$^{-/-}$ mice were generated as described previously (Backs et al., 2010) and crossed onto the C57BL6/J background. *ob/ob* mice were obtained from Jackson Labs. Mice were fed a standard chow diet, or a high-fat diet with 60% kcal from fat for the experiments in **Fig. 27**, and maintained on a 12-h light-dark cycle. Recombinant adenovirus (1.5×10^9 plaque-forming unit/mice) was delivered by tail vein injection, and experiments were commenced after 5 days. Fasting blood glucose was measured in mice that were fasted for 12-14 h, with free access to water, using a glucose meter (One Touch Ultra, Lifescan). Pyruvate-tolerance tests were carried out with an intraperitoneal injection of 2 g kg^{-1} body weight pyruvate after 17 h of fasting. Blood glucose levels were measured over the following 2 h. Xestospongin C was administered by daily i.p. injections to mice at a dose of 10 pmol g^{-1} for 4 days. *P38a*$^{fl/fl}$ mice were generated as described previously (Engel et al., 2005).

[00457] Hepatic Glycogen Measurement

[00458] 50 -100 mg of frozen livers were homogenized in 1 ml of H_2O with protease and phosphatase inhibitors. Samples were then mixed with KOH (1:2), boiled for 25 min and washed with 70% ethanol. The pellet was dried and dissolved in 100 μl H_2O, and the glycogen

content was assessed using the Glycogen Assay Kit (Abcam) according to the manufacturer's instructions. Data represent the mean ± SEM.

[00459] PAS Staining of Mouse Liver Sections

[00460] Liver samples were fixed in 10% neutral-buffered formalin for 24 h and embedded in paraffin. Sections (5 micron) were stained for glycogen using Periodic acid-Schiff (PAS) stain (Sigma) according to manufacturer's instructions. The sections were then counterstained with hematoxylin and examined by light microscopy. For the quantification of PAS staining, 5 fields from 4 different sections were chosen randomly, and the number of PAS-positive cells was counted and expressed as the percentage of the total number of cells (Hammad et al., 1982). Two independent investigators, blinded to the identity of the samples, performed the analysis.

[00461] Analysis of Mass Spectrometric Data

[00462] Protein and phosphopeptide identification, quantification, and phospho analysis were performed with Integrated Proteomics Pipeline - IP2 (Integrated Proteomics Applications, Inc., San Diego, CA. http://www.integratedproteomics.com/) using ProLuCID, DTASelect2, Census, DeBunker and Ascore. Spectrum raw files were extracted into ms1 and ms2 files (McDonald et al., 2004) from raw files using RawExtract 1.9.9 (http://fields.scripps.edu/downloads.php), and the tandem mass spectra were searched against EBI IPI mouse protein database (http://www.ebi.ac.uk/IPI/IPImouse.html, released on March 24, 2010). In order to accurately estimate peptide probabilities and false discovery rates, a decoy database containing the reversed sequences of all the proteins appended to the target database was used (Peng et al., 2003). Tandem mass spectra were matched to sequences using the ProLuCID (Xu et al., 2006) algorithm with 50 ppm peptide mass tolerance. ProLuCID searches were done on an Intel Xeon cluster running under the Linux operating system. The search space included all fully- and half-tryptic peptide candidates that fell within the mass tolerance window. Carbamidomethylation (+57.02146 Da) of cysteine was considered as a static modification, while phosphorylation (+79.9663) on serine, threonine, and tyrosine were considered as variable modifications.

[00463] The validity of peptide/spectrum matches (PSMs) was assessed in DTASelect (Tabb et al., 2002; Cociorva et al., 2007) using two SEQUEST (Eng et al., 1994) defined parameters, the cross-correlation score (XCorr), and normalized difference in cross-correlation scores (DeltaCN). The search results were grouped by charge state (+1, +2, +3, and greater than +3) and tryptic status (fully tryptic, half-tryptic, and non-tryptic), resulting in 12 distinct sub-groups. In each one of these sub-groups, the distribution of Xcorr, DeltaCN, and DeltaMass values for (a) direct and (b) decoy database PSMs was obtained, and then the direct and decoy subsets were

separated by discriminant analysis. Full separation of the direct and decoy PSM subsets is not generally possible; therefore, peptide match probabilities were calculated based on a nonparametric fit of the direct and decoy score distributions. A peptide confidence of 99.5% was set as the minimum threshold, and only phosphopeptides with delta mass less than 10 ppm were accepted. The false discovery rate was calculated as the percentage of reverse decoy PSMs among all the PSMs that passed the 99.5% confidence threshold. After this last filtering step, it is estimated that both the protein and peptide false discovery rates were both below 0.1%. After database searching and DTASelect2 filtering, phosphopeptides were analyzed with IP2 phospho analysis tool that uses Ascore (Beausoleil et al., 2006) and Debunker (Lu et al., 2007). Peptides and phosphopeptides were quantified using the Spectral Count method (Liu et al., 2004).

[00464] Statistical Analysis

[00465] All results are presented as mean ± SEM. P values were calculated using the student's t-test for normally distributed data and the Mann-Whitney rank sum test for non-normally distributed data.

[00466] Supplemental Experimental Procedures

[00467] Reagents and Antibodies

[00468] Glucagon, pyruvate, forskolin, H89, 8-bromo-cAMP and SB202190 were from Sigma. BAPTA-AM and anti-nucleophosmin (Np) antibody were from Invitrogen. Xestospongin C was from EMD Chemicals. Anti–phospho-Thr287 CaMKII antibody was from Imgenex and Novus; anti–total CaMKII, anti-FoxO1 and anti-Ac-FoxO1 antibodies were from Santa Cruz Biotechnology Inc. Anti-β-actin and anti-phospho-S316 FoxO1 antibodies were from Abcam. Anti-phosho-p38, anti-phospho-MK2, anti-phospho-CREB, anti-p38, anti-MK2, anti-CREB, anti-phospho-T24 FoxO1, anti-phospho-S253 FoxO1, anti-HA and anti-FLAG antibodies were from Cell Signaling. Anti-CRTC2 antibody was a gift from Dr. Marc Montminy. Adenoviruses encoding LacZ, CA-CaMKII, KD-CaMKII, GFP-FoxO1, and Cre were described previously (Pfleiderer et al., 2004; Tanaka et al., 2009; Akagi et al., 1997) and amplified by Viraquest, Inc. (North Liberty, IA). Plasmids encoding FoxO1 mutants 7A and 9A were constructed as described (Asada et al., 2007).

[00469] Primary Hepatocytes

[00470] Primary mouse HCs were isolated from 8- to 12-week-old mice as described previously (Matsumoto et al., 2002). For most experiments, the HCs were serum-depleted overnight by incubation in medium containing 0.5% fetal calf serum and were then incubated for

5 h in serum-free media, with individual treatments noted in the figure legends. HCs were transduced with adenoviral constructs 12 h after plating, and experiments were conducted 24 h after transduction. Transfections with WT, 7A- and 9A-*Foxo1* were carried out using jetPEI™-hepatocyte DNA transfection reagent (Polyplus-transfection, Inc.) according to manufacturer's instructions.

[00471] Immunoprecipitation

[00472] Cells were lysed by a 5-min exposure to 1% Triton-X in 50 mM HEPES, 150 mM NaCl, 10 mM Na pyrophosphate, 10 mM EDTA, 10 mM EGTA, 1 mM Na3VO4, 50 mM NaF, 1 mM PMSF, and 5 μg/ml leupeptin. The lysate (500 μg of protein) was brought to a total volume of 1 ml with lysis buffer containing 0.3–0.6 μg antibody and 80 μl Sepharose beads. The mixture was rotated in a 1.5-ml microfuge tube at 4°C for 14 h. Immune complexes were collected by centrifugation at 16,000 g and washed 3 times with chilled lysis buffer.

[00473] Generation of $Ip3r1^{flox}$ Mice

[00474] The $Ip3r1^{flox}$ mouse line was created as follows: a targeting construct was generated by recombineering using the BAC clone RP24-245H16 (CHORI), containing a fragment of chromosome 6, encompassing exons 1 through 9 of IP_3R1 from C57Bl/6J mice (Liu et al., 2003; Warming et al., 2005). Plasmids pL451, pL452 & pL253 were kindly provided by Dr. Neal Copeland (NCI, NIH). Briefly, a Frt-Neo-Frt-loxP cassette (from plasmid pL451) was inserted upstream of exon 4 (second coding exon), then the Neo cassette was removed by agarose-induced flp recombination. The second and third loxP sites were introduced downstream of exon 4 by inserting loxP-Neo-loxP cassette (from vector pL452). Finally, a DTA cassette (from plasmid pL253), containing thymidine kinase, was inserted further downstream of the third loxP site. All intermediate BAC constructs and the final construct were screened by PCR, and the final construct was "fingerprinted" by Acc65I digestion and tested for loxP functionality. Crucial junction sites were confirmed by sequencing. The resulting modified BAC was electroporated into chimeric ES cells (CSL3 cell line, derived from 129S6/SvEvTac mouse line). The correct recombinant ES cells were injected into C57BL/6 blastocysts-stage mouse embryos. Chimeric male mice were bred to C57BL/6 female mice to establish a hybrid line. Germ-line transmission generated $Ip3r1^{3xflox}$ mice, and females were crossed with EIIa-cre males to create $Ip3r1^{flox}$ mice in which the floxed Neo cassette was eliminated. These mice were subsequently crossed with C57BL/6J mice to breed out the EIIa-cre allele. The final $Ip3r1^{fl/fl}$ line was derived through heterozygous breeding.

[00475] Immunoblotting and RT-qPCR

[00476] Immunoblot and RT-qPCR assays were performed as previously described (Timmins et al., 2009). Total RNA was extracted from HCs using the RNeasy kit (Qiagen). cDNA was synthesized from 2 μg total RNA using oligo (dT) and Superscript II (Invitrogen). Nuclear extraction from liver was performed using the Nuclear Extraction Kit from Panomics according to the manufacturer's instructions. With regard to anti-Thr287-p and total CaMKII immunoblots, 2 bands and occasionally 3 bands, which were absent in CaMKIIγ-deficient HCs, were routinely seen. Whether their origin is alternative splicing or post-translational modification remains to be determined.

[00477] Mass Spectrometry of FoxO1 Phosphopeptides

[00478] HCs from WT and *Camk2g*$^{-/-}$ mice were transduced with adeno-FLAG-FoxO1 at an MOI of 2. Cells were serum-depleted overnight and then incubated for 5 h in serum-free media. FoxO1 was immunopurified using anti-FLAG. FLAG-FoxO1 in ice-cold Tris-buffered saline was precipitated by mixing 1 volume of the sample solution (cold) with 1/3 volume of 100% (w/v) TCA (6.1 N, Sigma). After 3 h on ice, the samples were centrifuged for 30 min at 4°C, and the supernate was aspirated leaving ~5-10 μl in the tube so as to not disturb the pellet. The pellet was washed twice with ice-cold acetone (500 μl each). After each wash, the solution was centrifuged for 10 min. The final pellet was then dried on a Speed-vac for 1-2 min.

[00479] Peptides were generated by proteolysis as described (Delahunty and Yates, III, 2005; MacCoss et al., 2002). The TCA pellets were solubilized in 60 μl of 100 mM Tris-HCl, pH 8.5, containing 8 M urea, and then the proteins were reduced by the addition of 500 mM Tris (2-carboxyethyl)phosphine (TCEP) to a final concentration of 5 mM. After a 20-min incubation at room temperature, cysteine residues were carboxymethylated by the addition of 500 mM iodoacetamide to achieve a final concentration of 10 mM. The solution was incubated for 30 min at room temperature in the dark and then split equally into three tubes. In one of the tubes, the concentration of urea was then diluted 2-fold (to 4 M) by the addition of an equal volume of 100 mM Tris-HCl, pH 8.5, and then subtilisin (Promega) was added at ~1:100 enzyme:substrate ratio (wt:wt) and incubated at 37°C for 4 h in the dark. The other two samples were diluted 4-fold (to 2 M), and elastase and trypsin (Promega) were added at ~1:100 enzyme:substrate ratio (wt:wt), and then both samples were incubated at 37°C overnight in the dark. The resulting peptides from the three digests were combined into one tube and dissolved in 90% formic acid to a final concentration of 2% in 10% acetonitrile. The samples were stored at -20°C prior to TiO2 enrichment and LC-MS/MS analysis.

[00480] TiO2 enrichment for phosphopeptides was done as described by Cantin *et al.* (Cantin et al., 2007). A TiO2 column was made by pressure-slurry packing TiO2 (5-μ partisphere, Whatman, Clifton, NJ) into fused-silica capillary (250-μm i.d.) to a length of 5 cm, and the peptide mixtures were pressure-loaded onto the column. The column was washed with buffer A and B (see the following section for buffer compositions) in succession, and then phosphopeptides were eluted using 250 mM ammonium bicarbonate (pH 9) directly into 100-μm-i.d. Kasil-fritted end packed column with 5 cm of 5-μm reversed phase (Gemini C18, Phenomenex, Torrance, CA), which was linked to a pulled-tip analytical column with a bed volume of 15 cm of the same reversed phase. This second column was in-line with an Agilent 1200 quaternary HPLC pump (Palo Alto, CA) for mass spectrometry analysis.

[00481] The HPLC buffer solutions used were water/acetonitrile/formic acid (95:5:0.1, v/v/v) as buffer A and water/acetonitrile/ formic acid (20:80:0.1, v/v/v) as buffer B. The elution gradient was as follows: 10 min of 100% buffer A, a 5-min gradient from 0 to 15% buffer B, a 65-min gradient from 15 to 45% buffer B, a 15-min gradient from 45 to 100% buffer B, and 5 min of 100% buffer B. Data-dependent tandem mass spectrometry (MS/MS) analysis was performed with a LTQ-Velos-Orbitrap mass spectrometer (ThermoFisher, San Jose, CA). Peptides eluted from the LC column were directly electrosprayed into the mass spectrometer with the application of a distal 2.5-kV spray voltage. A cycle of one full-scan MS spectrum (m/z 300-1800) was acquired followed by twenty MS/MS events, sequentially generated on the first to the twentieth most intense ions selected from the full MS spectrum at a 35% normalized collision energy. The number of microscans was one for both MS and MS/MS scans, and the maximum ion injection time was 25 and 50 ms respectively. The dynamic exclusion settings used were as follows: repeat count, 1; repeat duration, 30 second; exclusion list size, 500; and exclusion duration, 120 second. MS scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (ThermoFisher).

[00482] <u>*G6PC* Promoter-Luciferase Assay</u>

[00483] FAO hepatocytes were transfected with a construct encoding nucleotides -1227 to +57 of the human *G6PC* promoter fused to luciferase (-1227 WT) or the same construct with three consensus FoxO binding sites mutated (-1227 Mut): -187 to -183 (GTTT → CGAG); -171 (G → C); and -164 (A → C), disrupting the consensus IRS sequences T(G/A)TTT in the *G6PC* promoter (Ayala et al., 1999; von Groote-Bidlingmaier et al., 2003). The final mutation at position -164 is upstream of the cAMP-response element (CRE; -161 to -152), leaving the CRE unaffected (Barthel et al., 2001). Hepatocytes were treated for 16 h with 0.1 mM cAMP and 1

μM dexamethasone in serum-free medium with 1% BSA prior to lysis and analysis of luciferase activity. The luciferase units (RLU) were normalized to the untreated cells in each group.

Example 9: InsP3 Receptor Regulates Hepatic Gluconeogenesis in Fasting and Diabetes

[00484] In the fasted state, increases in circulating glucagon promote hepatic glucose production through induction of the gluconeogenic program. Triggering of the cAMP pathway increases gluconeogenic gene expression via the dephosphorylation of the CREB coactivator CRTC2 (1). Glucagon promotes CRTC2 dephosphorylation in part through the PKA-mediated inhibition of the CRTC2 kinase SIK2. A number of Ser/Thr phosphatases appear capable of dephosphorylating CRTC2 (2,3), but the mechanisms by which hormonal cues regulate these enzymes remain unclear. Here it is shown that glucagon stimulates CRTC2 dephosphorylation in hepatocytes by mobilizing intracellular calcium stores and activating the calcium/calmodulin dependent Ser/Thr phosphatase calcineurin/PP2B. Glucagon increased cytosolic calcium through the PKAmediated phosphorylation of inositol 1,4,5-trisphosphate receptors (InsP3Rs),which is shown here to associate with CRTC2. Following their activation, InsP3Rs enhanced gluconeogenic gene expression by promoting the calcineurin-mediated dephosphorylation of CRTC2. During feeding, increases in insulin signaling reduced CRTC2 activity via the AKT-mediated inactivation of InsP3Rs. InsP3R activity was increased in diabetes, leading to upregulation of the gluconeogenic program. As hepatic down-regulation of InsP3Rs and calcineurin improved circulating glucose levels in insulin resistance, these results demonstrate how cross-talk between cAMP and calcium pathways at the level of the InsP3 receptor modulates hepatic glucose production under fasting conditions and in diabetes.

[00485] Results and Discussion

[00486] A series of Ser/Thr protein phosphatase (PP) inhibitors were tested for their ability to block CRTC2 activation in response to glucagon. Exposure to the PP2B/calcineurin inhibitor cyclosporine A (CsA) disrupted the glucagon-induced dephosphorylation and nuclear translocation of CRTC2, but okadaic acid (OA), an inhibitor of PP1, PP2A, and PP4 did not (**Fig. 33A, Fig. 37A**). CsA and other calcineurin inhibitors also reduced CRE-luciferase (luc) reporter activity (**Fig. 33A, Fig. 37B**), but they had no effect in cells expressing phosphorylation-defective (Ser171, 275 Ala) and therefore active forms of CRTC2 (**Figs. 37C-E**).

[00487] Based on the ability for CsA to interfere with CRTC2 activation, it was considered whether calcineurin can promote the dephosphorylation of CRTC2 in response to glucagon.

Supporting this idea, CRTC2 contains two consensus (PXIXIT) motifs that mediate an association with calcineurin (3, 4) (**Fig. 38A, B**). Moreover, mutation of both motifs disrupted the glucagon-dependent dephosphorylation of CRTC2 (**Fig. 33B**) and prevented its nuclear translocation (**Fig. 38C**), thereby down-regulating CRE-luc activation (**Fig. 33B**).

[00488] Based on these results, whether calcineurin modulates expression of the gluconeogenic program was tested. Adenoviral over-expression of the calcineurin catalytic subunit in hepatocytes augmented CRTC2 dephosphorylation, CRE-luc activity, and glucose secretion in response to glucagon, whereas calcineurin knockdown had the opposite effect (**Fig. 33C**). Although calcineurin can, in principle, modulate CRTC2 activity indirectly through effects on cAMP signaling, calcineurin over-expression or knockdown did not alter the phosphorylation of cellular PKA substrates in cells exposed to glucagon (**Fig. 38D**).

[00489] Whether calcineurin modulates hepatic gluconeogenesis *in vivo* was next examined. Modest (2-fold) over-expression of calcineurin in liver increased gluconeogenic gene expression, hepatic CRE-luc activity, and fasting blood glucose concentrations (**Fig. 33D, Fig. 39A**). Conversely, knockdown of hepatic calcineurin reduced expression of the gluconeogenic program and lowered circulating glucose levels (**Fig. 33D, Fig. 39B**), demonstrating that this phosphatase contributes to the fasting adaptation in liver. Calcineurin appeared to stimulate gluconeogenesis via the CREB pathway; depletion of CRTC2 blocked effects of calcineurin over-expression in this setting (**Fig. 40**).

[00490] Realizing that calcineurin activity is dependent on increases in intracellular calcium, whether the cAMP pathway stimulates calcium mobilization was tested. Exposure of primary hepatocytes to glucagon triggered a rapid increase in cellular free calcium (**Fig. 34A, Fig. 41A**); these effects were partially reversed by co-treatment with the PKA inhibitor H89 (**Fig. 41B**). The rise in intracellular calcium appears critical for CRTC2 activation because co-incubation with the calcium chelator BAPTA disrupted CRTC2 dephosphorylation and CRE-luc activation in response to glucagon (**Fig. 34B**). Arguing against an effect of calcium on cAMP signaling, exposure to BAPTA did not block the PKA-mediated phosphorylation of CREB in response to glucagon.

[00491] Without being bound by theory, cAMP can increase calcium mobilization through the PKA-dependent phosphorylation of an intracellular calcium channel. In mass spectrometry studies to identify proteins that undergo phosphorylation by PKA in response to glucagon, the inositol 1,4,5-trisphosphate receptor 1 (InsP3R1) was recovered from immunoprecipitates of phospho-PKA substrate antiserum (**Fig. 41C**). InsP3R1 and its related family members

(InsP3R2, InsP3R3) are calcium release channels that promote the mobilization of endoplasmic reticulum (ER) calcium stores following their activation in response to extracellular signals (5-9). Moreover, cAMP agonists have also been shown to enhance InsP3R receptor activity through PKA-mediated phosphorylation.

[00492] Inhibiting InsP3Rs, either by exposure of hepatocytes to Xestospongin C (Xc) or by knockdown of all three InsP3Rs, disrupted cytosolic calcium mobilization and calcineurin activation in response to glucagon and forskolin (**Fig. 34A, Fig. 42A**). Moreover, Xc treatment and InsP3R knockdown also blocked glucagon effects on CRTC2 dephosphorylation, CRE-luc activation, and induction of the gluconeogenic program (**Fig. 34C, Fig. 42A, B**). Effects of InsP3R depletion using hepatocytes from mice with a knockout of InsP3R2 (10), the predominant InsP3R isoform in these cells, were confirmed (**Figs. 42C-E**).

[00493] Based on these results, without being bound by theory, InsP3Rs can also modulate fasting glucose production *in vivo*. Decreasing hepatic InsP3R expression, either by knockdown of all three Insp3Rs in liver or by targeted disruption of the InsP3R2 gene, reduced fasting CRE-luc activity, gluconeogenic gene expression, and circulating glucose concentrations, demonstrating the importance of these receptors in glucose homeostasis (**Fig. 34D, Fig. 43**).

[00494] It was tested whether glucagon modulates InsP3R activity through PKA-mediated phosphorylation. Exposure of hepatocytes to glucagon increased the phosphorylation of InsP3R1 as well as InsP3R2 and InsP3R3 by immunoblot assay with phospho-PKA substrate antiserum; these effects were blocked by the PKA inhibitor H89 (**Fig. 35A, Fig. 44A**). Moreover, mutation of serine residues at consensus PKA sites in InsP3R1 (Ser1589, Ser1756) to alanine completely disrupted InsP3R1 phosphorylation in response to glucagon (**Fig. 35B**). As a result, over-expression of PKA-defective (S1589,1756A) InsP3R1 interfered with calcium mobilization and calcineurin activation, and it reduced CRE-luc activation and glucose secretion from hepatocytes exposed to glucagon (**Figs. 35B-D**).

[00495] Similar to glucagon, fasting also stimulated hepatic InsP3R1 phosphorylation at Ser1589 and Ser1756 (**Fig. 44B**). And over-expression of PKA-defective InsP3R1 reduced fasting CRE-luc induction, calcineurin activation, and gluconeogenic gene expression, leading to lower circulating glucose concentrations (**Fig. 35E, Fig. 44C, D**). Taken together, these results support an important role for the PKA-mediated phosphorylation of InsP3R in hepatic gluconeogenesis.

[00496] Without being bound by theory, the proximity of CRTC2 to the calcium signaling machinery can be important for its activation. Supporting this notion, CRTC2 was found to

associate with InsP3R1 via its N-terminal CREB binding domain (CBD) in co-immunoprecipitation assays (**Fig. 35F, Figs. 45A-D**). Moreover, CRTC2 was enriched in ER-containing high density microsomal (HDM) fractions, which also contain the InsP3Rs (**Fig. 45E**). The InsP3R:CRTC2 association appears critical for CRTC2 localization in the perinuclear space because RNA-mediated knockdown of the InsP3Rs led to redistribution of CRTC2 in the cytoplasm (**Fig. 45F**). Disrupting the CRTC2:InsP3R interaction, by deletion of the CBD in CRTC2 or by addition of an N-terminal myristoylation signal that targets CRTC2 to the plasma membrane, blocked CRTC2 dephosphorylation and CRE-reporter activation in response to glucagon (**Figs. 45G-I**). Taken together, these results indicate that the association of CRTC2 with InsP3Rs enhances its sensitivity to fasting signals.

[00497] Under feeding conditions, insulin inhibits gluconeogenesis in part by increasing CRTC2 phosphorylation. Whether insulin interferes with InsP3R effects on CRTC2 activitation was examined. Supporting this idea, AKT has been shown to block calcium mobilization by phosphorylating InsP3Rs at Ser 2682 (in InsP3R1) (11). Indeed, exposure of hepatocytes to insulin increased InsP3R phosphorylation by immunoblot analysis with phospho-AKT substrate antiserum (**Fig. 46A**); mutation of Ser 2682 (in InsP3R1) to alanine blocked these effects. Insulin treatment also reduced glucagon-dependent increases in calcium mobilization and calcineurin activation in cells expressing wild-type InsP3R1, but it had no effect in cells expressing AKT-defective (S2682A) InsP3R1 (**Fig.46B**). As a result, CRTC2 dephosphorylation, CRE-luc activity, and glucose output were elevated in hepatocytes expressing (S2682A)-InsP3R compared to wild-type (**Fig. 46C**).

[00498] Whether InsP3R1 phosphorylation by AKT is important in regulating hepatic glucose production *in vivo* was next examined. Feeding increased hepatic InsP3R1 phosphorylation at Ser2682 (**Fig. 44B**). Moreover, over-expression of AKT-defective InsP3R1 partially suppressed feeding-induced decreases in CRE-luc activity and gluconeogenic gene expression, leading to elevations in circulating glucose concentrations (**Fig. 46D**). Taken together, these results indicate that the AKT-mediated phosphorylation of InsP3Rs during feeding inhibits hepatic gluconeogenesis by blocking the calcineurin-dependent dephosphorylation of CRTC2.

[00499] Whether hepatic InsP3R signaling contributes to increases in gluconeogenesis in the setting of insulin resistance was examined. Hepatic calcineurin activity was enhanced in both *ob/ob* and *db/db* diabetic animals, leading to increases in CRE-luc activity (**Fig. 36A, Fig. 47A,B**). Pointing to a role for InsP3R, hepatic amounts of PKA-phosphorylated, active InsP3Rs were increased in these diabetic mice, while amounts of AKT-phosphorylated, inactive InsP3R

were reduced (**Fig. 36B**). Correspondingly, knockdown of either calcineurin or InsP3Rs in *db/db* mice reduced CRE-luc activity, gluconeogenic gene expression, and hepatic gluconeogenesis (**Fig. 36C, Fig. 47C**).

[00500] Collectively, the results herein demonstrate that glucagon promotes CRTC2 dephosphorylation during fasting by triggering increases in cytoplasmic calcium that lead to calcineurin activation (**Fig. 48**). The ability for glucagon to increase calcium signaling via the PKA-mediated phosphorylation of InsP3Rs demonstrates an important regulatory node for cross-talk between cAMP and calcium signaling pathways in liver and perhaps other insulin sensitive tissues. The partial inhibition of calcium entry by the PKA inhibitor H89 also points to additional regulatory inputs (12,13) that may function with PKA to increase InsP3R activity in response to glucagon. CRTC2 has also been found to stimulate metabolic gene expression by upregulating the nuclear hormone receptor coactivator PGC1α in liver (14,15) and muscle (16). Based on the well-recognized role of calcium signaling in PGC1α dependent transcription, InsP3Rs can also function importantly in this setting.

[00501] Methods

[00502] Adenoviruses were delivered by tail vein injection (17). Hepatic CRE-luc activity was visualized using an IVIS Imaging system. Mice were imaged 3-5 days after injection of CRE-luc adenovirus. Pyruvate tolerance testing was performed on mice fasted overnight and injected intraperitoneally with pyruvate (2g kg-1). *Insp3r2* knockout mice have been described (10). Cultured primary mouse hepatocytes were prepared as reported (18). Cellular fractionation studies were conducted using primary mouse hepatocytes (18). Calcium imaging experiments were performed using a CCD camera on primary hepatocytes loaded with fura-2 dye. Mass spectrometry studies were performed on CRTC2 immunoprecipitates prepared from HEK293T cells and on immunoprecipitates of phospho-PKA substrate antiserum prepared from primary hepatocytes exposed to glucagon. Anti-InsP3R1 (A302-158A) and InsP3R3 (A302-160A) antibodies were purchased from Bethyl Laboratories, anti-InsP3R2 (ab77838) antiserum was from Abcam, anti-Calcineurin (610260) from BD Biosciences, anti-GRP78 (ADISPA-826-F) from Enzo Life Sciences, anti-phospho-PKA substrate (RRXS/T, 9624), anti-phospho-AKT substrate (RXXS/T, 9614) and CRTC2 (pS171, 2892) from Cell Signaling. Phospho (Ser275) CRTC2 antibody was used as described (19). Details are included below.

[00503] Mouse strains and adenovirus

[00504] Adenoviruses (1 X10^8 plaque forming units (pfu) GFP, Calcineurin, InsP3R1, InsP3R1 DM (S1589A/S1756A), unspecific (US) RNAi, *Calcineurin* RNAi, *Insp3r1* RNAi, *Insp3r2* RNAi, *Insp3r3* RNAi, *Crtc2* RNAi, 1 X10^9 pfu CRE-luc reporter, 5X107 pfu RSV β-gal) were delivered to 8-10 week old male C57BL/6J, B6.V-*lep*<ob>/J, B6.Cg-*m*+/+*Lepr*<*db*>/J by tail vein injection (17). *Insp3r2* knockout mice were described previously (10). All mice were adapted to their environment for 1 week before study and were housed in colony cages with 12h light/dark cycle in a temperature-controlled environment. For *in vivo* imaging experiments, mice were imaged on day 3-5 after adenovirus delivery. Wild-type CRTC2, CRTC2 (S171A), GFP, unspecific RNAi, *Crtc2* RNAi, CRE-luc, and RSV β-gal adenoviruses have been described previously (17,20). The adenoviruses containing rat InsP3R1, InsP3R1 DM and InsP3R1 (S2682A) were generated from the InsP3R1 plasmid. Calcineurin adenovirus was constructed using a mouse Calcineurin plasmid (Addgene). CRTC2 ΔCBD (51-692aa), S275A and S171A/S275A adenoviruses were made from mouse CRTC2. Myristoylated-CRTC2 (Myr-CRTC2) adenovirus was generated with mouse CRTC2 fused to an N-terminal myristoylation tag (MGSSKSKPKDPSQR) from Src. *Calcineurin* RNAi, *Insp3r1* RNAi, *Insp3r2* RNAi, *Insp3r3* RNAi adenoviruses were constructed using the sequence 5'-GGGTACCGCATGTACAGGAAAA-3', 5'-GGGTACTGGAATAGCCTCTTCC-3', 5'-GGGTAACAAGCACCACCATCCC-3' and 5'-GGGCAAGCTGCAGGTGTTCCTG-3', respectively. All expressed constructs used in this study were confirmed by sequencing.

[00505] <u>*In vivo* analysis</u>

[00506] For *in vivo* imaging, mice were imaged as described (17,20) under ad libitum feeding conditions or after fasting for 6 hours. For pyruvate challenge experiments, mice were fasted overnight and injected intraperitoneally with pyruvate (2g kg^{-1}). Blood glucose values were determined using a LifeScan automatic glucometer. For immunoblot, mouse tissues were sonicated, centrifuged and supernatants were reserved for protein determinations, and SDS–PAGE analysis.

[00507] <u>Cell culture, cellular fractionation, luciferase assay, calcineurin activity and cAMP measurement</u>

[00508] HEK293T (ATCC) cells were cultured in DMEM containing 10% FBS (HyClone), 100 mg ml^{-1} penicillin-streptomycin. Mouse primary hepatocytes were isolated and cultured as previously described (18). Cellular fractionation studies were conducted as previously reported (18). For reporter studies, Ad-CRE-luc infected hepatocytes (1 pfu per cell) were exposed to glucagon (Gcg, 100 nM) for 2~4h. For cyclosporine A (CsA, 10 μM) or okadaic acid (OA, 100

nM) or cell permeable calcineurin autoinhibitory peptide (10 μM) or CN585 (100 μM) or Calyculin A (10 nM) or Xestospongin C (Xc, 2 μM) or H89 (30 μM) or BAPTA (50 μM) inhibition, hepatocytes were pre-treated with the inhibitors for one hour. Luciferase activities were normalized to β-galactosidase activity from adenoviral-encoded RSV β-gal. Calcineurin activity (test kit from Enzo Life Sciences) and cellular cAMP levels (test kit from Cayman Chemical Company) were measured according to manufacturer's instructions.

[00509] Calcium imaging

[00510] Mouse primary hepatocytes were plated on glass coverslips and loaded with 5 μM Fura-2 acetoxymethyl ester (Molecular Probes) in the presence of 0.025% (w/v) pluronic F127 (Sigma-Aldrich) in Media 199 (Mediatech) for 30 minutes. Coverslips were mounted on a laminar flow perfusion chamber (Warner Instruments Corp.) and perfused with Media 199 or a solution of 100 nM glucagon in Media 199. Images of Fura-2 loaded cells were collected with a cooled CCD camera while the excitation wavelength was alternated between 340 nm and 380 nm. The ratio of fluorescence intensity at the two excitation wavelengths was calculated after subtracting background fluorescence. [Ca2+]I (cytosolic free calcium concentration) was calculated using a Fura-2 calcium imaging calibration kit (Invitrogen). Images were collected and analyzed using the MetaFluor software package (Universal Imaging Corp.). Graphs represent average responses from groups of 30-40 individual cells from representative single experiments. Bar graphs represent average responses (fold over average baseline) from 150-200 cells per condition. All experiments were repeated at least three times with similar results.

[00511] Immunoblot, immunoprecipitation, immunostaining

[00512] Immunoblot, immunoprecipitation, and immunostaining assays were performed as described (18). CRTC2, pCREB (Ser133), CREB, pAKT (Thr308), AKT, tubulin, HA, and FLAG antibodies were previously described (18). The antibodies anti-InsP3R1 (A302-158A) and InsP3R3 (A302-160A) were purchased from Bethyl Laboratories, anti-InsP3R2 (ab77838) from Abcam, anti-Calcineurin (610260) from BD Biosciences, anti-GRP78 (ADI-SPA-826-F) from Enzo Life Sciences, anti-phospho-PKA substrate (RRXS/T, 9624), anti-phospho-AKT substrate (RXXS/T, 9614) and CRTC2 (pS171, 2892) from Cell Signaling. CRTC2 (pS275) antibody was employed as described (19).

[00513] Quantitative PCR

[00514] Total cellular RNAs from whole liver or from primary hepatocytes were extracted using the RNeasy kit (Qiagen) and used to generate cDNA with SuperScript II enzyme (Invitrogen). cDNA were analyzed by quantitative PCR as described (18).

[00515] Mass spectrometry

[00516] Immunoprecipitates of endogenous CRTC2 from HEK293T cells and of phospho-PKA substrate antiserum from glucagon stimulated hepatocytes were prepared for mass spectrometric studies as previously reported (21), and analyzed by electrospray ionization tandem mass spectrometry on a Thermo LTQ Orbitrap instrument.

[00517] Statistical analyses

[00518] All studies were performed on at least three independent occasions. Results are reported as mean ± s.e.m. The comparison of different groups was carried out using two-tailed unpaired Student's *t*-test. Differences were considered statistically significant at $P<0.05$.

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Example 10: p38 and MK2/3 inhibitors for treatment and prevention of metabolic diseases

[00519] There are two kinases downstream of CaMKII that can mediate the adverse effects of CaMKII—p38 and MK2 (see **Fig. 51**). While drugs blocking the more upstream targets like IP3 receptor and CaMKII can cover more pathways, they also can be more toxic. In contrast, targeting the more downstream mediators can lead to less off-target effects. MK2 and MK3 are

very similar, and reagents that block MK2 (DN-MK2 and MK2 inhibitor) block both MK2 and MK3. If one blocked only one of them, the other would compensate.

[00520] Thus, p38 inhibitors and MK2/3 inhibitors can also be used to treat metabolic diseases induced by obesity, such as Type 1 diabetes, Type 2 diabetes, insulin resistance and metabolic syndrome.

[00521] The studies described in **Figs. 52-56** use primary murine hepatocytes (HCs). For p38 experiments, p38 is deleted in samples labeled as "Cre" or "adeno-Cre" or "ad-Cre" and the control for this is adeno-LacZ (sometimes labeled ad-LacZ or LacZ). (The HCs have a form of the p38 gene that is deleted when exposed to Cre recombinase, which is delivered using an adenoviral vector. The control is an adenoviral vector encoding an irrelevant protein called LacZ.)

[00522] **Fig. 52** directly relates p38 to the key distal consequence of CaMKII, namely, FoxO1 nuclear localization: in normal HCs with p38 present (LacZ), constitutively active (CA) CaMKII pushes FoxO1 into the nucleus, but it cannot do this HCs lacking p38 (Cre).

[00523] **Fig. 53** uses an MK2 inhibitor to show the importance of MK2 in both nuclear FoxO1 and in induction of genes involved in hepatic glucose production. MK-2 Inhibitor is from Calbiochem (cat#475864): 2-(2-Quinolin-3-ylpyridin-4-yl)-1,5,6,7-tetrahydro-4H-pyrrolo-[3,2-c]pyridin-4-one.

[00524] **Fig. 54** uses an experimental model (an inducer of protein misfolding called tunicamycin) that mimics a process in obesity and type 2 diabetes in which liver cells are subjected to endoplasmic reticulum (ER) stress. ER stress is represented by 'PERK/PKR' and 'ATF4/CHOP' (lower right branch) (see **Fig. 51**). What is shown in **Fig. 54** is that p38 is needed for the tunicamycin (ER stress)-induced increase in p-PERK (its active form) and CHOP. One will also see that p58IPK is slightly but consistently elevated in the HCs lacking p38 (Cre)—this also fits the lower right branch of **Fig. 51**.

[00525] **Fig. 55** uses another experimental model (a fatty acid called palmitate) that mimics liver ER stress in obesity and diabetes. As in **Fig. 54**, p38 is needed for the increase in CHOP and p-PERK.

[00526] **Fig. 56** If one looks at the bottom of the right branch of **Fig. 51**, one will see that the readout is p-Akt response to insulin. All the HCs in **Fig. 56** received an acute dose of insulin to test insulin's ability to activate (phosphorylate) Akt. The model used here to mimic obesity/diabetes is the palmitate model used in **Fig. 55**. One can see that in HCs with normal

p38 (Ad-LacZ), insulin gave a robust p-Akt signal (first 2 lanes) but this was lowered by palmitate ("palm"). This "insulin resistance" in the face of palmitate mimics what happens in obesity and type 2 diabetes. If one then looks at HCs without p38 (Ad-Cre), the ability of insulin to activate Akt (p-Akt) is improved. This is consistent with the p38-p-Akt link shown in **Fig. 51**.

[00527] **Figs. 86, 87 and 88** relate to how inhibition of CaMKII, p38, or MK2/3 causes an improvement in insulin signaling, i.e., how these inhibitors prevent insulin resistance. The data therein support the following mechanism: Inhibitors of CaMKII, p38, or MK2/3 lower Tribble3 (Trb3), which then improves the ability of insulin to phosphorylate AKT (Trb3 is an endogenous inhibitor of AKT phosphorylation).

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CLAIMS

In the claims:

1. A method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of CaMKII, thereby treating or preventing the disorder.

2. A method of treating or preventing coronary artery disease in a subject with a metabolic disorder, the method comprising reducing the activity of CaMKII in the subject, thereby treating or preventing the coronary artery disease.

3. A method of reducing hepatic glucose production in a subject, the method comprising reducing the activity of CaMKII, thereby resulting in the reduction of hepatic glucose in the subject.

4. The method of claim 2, wherein the treating or preventing comprises reducing CaMKII activity in a macrophage of the subject.

5. The method of claim 1 or 2, wherein the disorder is selected from the group consisting of Type 1 diabetes, Type 2 diabetes, insulin resistance and metabolic syndrome.

6. The method of claim 1 or 2, wherein the treating or preventing affects glycogenolysis or gluconeogenesis in the subject.

7. The method of claim 1 or 2, wherein the treating or preventing reduces hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject.

8. The method of claim 1 or 2, wherein the treating or preventing reduces phosphorylation and/or activation of CaMKII.

9. The method of claim 1 or 2, wherein the activity of CaMKII is a glucagon-induced activity.

10. The method of claim 1 or 2, wherein the treating or preventing reduces expression of G6pc and/or Pck1 in a cell of the subject.

11. The method of claim 1 or 2, wherein the treating or preventing reduces the amount of FoxO1 protein level in the nucleus of a cell of the subject.

12. The method of claim 1 or 2, wherein the treating or preventing reduces phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein.

13. The method of claim 1 or 2, wherein the treating or preventing comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the CaMKII protein.

14. The method of claim 1 or 2, wherein the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the CaMKII protein.

15. The method of claim 1 or 2, wherein the treating or preventing comprises a step of administering to the subject a small molecule.

16. The method of claim 15, wherein the small molecule is a CaMKII inhibitor selected from the group consisting of KN-93, lavendustin C, CK59, Ant-CaMKIINtide, KN62, DY9760e, K-252a Nocardiopsis sp., H89 dihydrochloride, PP1 Analog II, 1NM-PP1, eEF-2 kinase inhibitor NH125, and STO-609.

17. The method of claim 2, wherein the coronary artery disease is associated with atherogenesis and/or atherosclerosis.

18. The method of claim 2, which further comprises treating or preventing heart failure, hypertension and/or renal disease.

19. The method of claim 2, wherein the disorder is associated with advanced lesional macrophage apoptosis.

20. The method of claim 2, wherein the disorder is associated with plaque necrosis.

21. The method of claim 2, wherein the treating or preventing coronary artery disease in a subject with a metabolic disorder results in lowering hyperinsulinemia and/or dyslipidemia.

22. The method of claim 2, wherein the treating or preventing coronary artery disease in a subject with a metabolic disorder results in lowering atherogenesis and/or atherosclerosis.

23. The method of claim 3, wherein the reducing hepatic glucose production reduces phosphorylation and/or activation of CaMKII.

24. The method of claim 3, wherein the activity of CaMKII is a glucagon-induced activity.

25. The method of claim 3, wherein the reducing hepatic glucose production reduces expression of G6pc and/or Pck1 in a cell of the subject.

26. The method of claim 3, wherein the reducing hepatic glucose production reduces hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject.

27. The method of claim 3, wherein the reducing hepatic glucose production reduces the amount of FoxO1 protein level in the nucleus of a cell of the subject.

28. The method of claim 3, wherein the reducing hepatic glucose production reduces phosphorylation of amino acid S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof, of the FoxO1 protein.

29. The method of claim 3, wherein the reducing hepatic glucose production comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the CaMKII protein.

30. The method of claim 3, wherein the reducing hepatic glucose production comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the CaMKII protein.

31. The method of claim 3, wherein the reducing hepatic glucose production comprises a step of administering to the subject a small molecule.

32. The method of claim 31, wherein the small molecule is a CaMKII inhibitor selected from the group consisting of KN-93, lavendustin C, CK59, Ant-CaMKIINtide, KN62, DY9760e, K-252a Nocardiopsis sp., H89 dihydrochloride, PP1 Analog II, 1NM-PP1, eEF-2 kinase inhibitor NH125, and STO-609.

33. A method for identifying a compound that inhibits the activity of CaMKII, the method comprising:

a) contacting a cell with a CaMKII fusion protein, wherein the CaMKII fusion protein comprises an acceptor fluorophore protein at one terminus, and a donor fluorophore protein at the other terminus; and

b) measuring FRET efficiency in the absence and in the presence of a test compound, wherein a greater FRET efficiency in the presence of the test compound compared to the FRET efficiency in the absence of the test compound indicates that the test compound inhibits the activity of CaMKII.

34. The method of claim 33, wherein the acceptor fluorophore protein is selected from the group consisting of mOrange, mStrawberry, Venus, yellow fluorescent protein, cyan fluorescent protein, red fluorescent protein and green fluorescent protein.

35. The method of claim 33 or 34, wherein the cell is a HEK293T cell, a hepatocyte, a U2OS cell, a HeLa cell, a murine embryonic fibroblast (MEF) or a macrophage.

36. The method of any one of claims 33 to 35, wherein the cell is from a Insr-/- mouse, a Camk2g-/- mouse, a Foxo1 -/- mouse, a db/db mouse, a ob/ob mouse, a p38 -/- mouse, a Non Obese Diabetes (NOD) mouse, a mouse fed a high fat diet, or a streptozotocin-treated mouse.

37. The method of any one of claims 33 to 35, wherein the cell is from a mouse expressing a mutant FoxO1 protein.

38. The method of claim 37, wherein the mutant FoxO1 protein comprises alanine substitutions at S284, S295, S326, S467, S475, S246, S253, S413, or S415, or aspartic acid substitutions at S284, S295, S326, S467, S475, S246, S253, S413, or S415, or any combination thereof.

39. The method of any one of claims 33 to 38, wherein the cell is subjected to ER stress.

40. The method of any one of claims 33 to 39, wherein the cell is treated with glucagon, 8-bromo cAMP, H89 dihydrochloride, Xestospongin C, forskolin, saturated fatty acids, or any combination thereof.

41. A method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of IP3R1, IP3R2 or IP3R3, thereby treating or preventing the disorder.

42. A method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of calcineurin, thereby treating or preventing the disorder.

43. The method of claim 41 or 42, wherein the disorder is selected from the group consisting of Type 1 diabetes, Type 2 diabetes, insulin resistance, obesity, and metabolic syndrome.

44. The method of claim 41 or 42, wherein the treating or preventing affects glycogenolysis or gluconeogenesis in the subject.

45. The method of claim 41 or 42, wherein the treating or preventing reduces hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated

inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject.

46. The method of claim 41, wherein the treating or preventing comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the IP3R1 protein, IP3R2 protein or IP3R3 protein.

47. The method of claim 41, wherein the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the IP3R1 protein, IP3R2 protein or IP3R3 protein.

48. The method of claim 41, wherein the treating or preventing comprises a step of administering to the subject a small molecule.

49. The method of claim 48, wherein the small molecule is a IP3R1 protein inhibitor, IP3R2 protein inhibitor or IP3R3 protein inhibitor.

50. The method of claim 42, wherein the treating or preventing comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the calcineurin protein.

51. The method of claim 42, wherein the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the calcineurin protein.

52. The method of claim 42, wherein the treating or preventing comprises a step of administering to the subject a small molecule.

53. The method of claim 52, wherein the small molecule is a calcineurin inhibitor.

54. A method for identifying a compound that inhibits the activity of IP3R1, IP3R2 or IP3R3, the method comprising:

a) contacting a cell with a test compound; and

b) measuring IP3R1, IP3R2 or IP3R3 activity, wherein a reduction of the activity of IP3R1, IP3R2 or IP3R3 in the presence of the compound compared to the activity of IP3R1, IP3R2 or IP3R3 in the absence of the compound indicates that the compound is an inhibitor of IP3R1, IP3R2 or IP3R3, respectively.

55. The method of claim 54, wherein the activity is measured by calcium release into the cytosol of the cell after stimulation with an inducer of IP3.

56. The method of claim 55, wherein calcium release is measured by an increase in the fluorescence of a cytosolic calcium dye.

57. The method of claim 56, wherein the cytosolic calcium dye is Fluo-3.

58. A method for identifying a compound that inhibits the activity of calcineurin, the method comprising:

a) contacting a cell with a test compound; and

b) measuring calcineurin activity, wherein a reduction of the activity of calcineurin in the presence of the compound compared to the activity of calcineurin in the absence of the compound indicates that the compound is an inhibitor of calcineurin.

59. The method of claim 58, wherein the activity of calcineurin is measured through detection of phosphatase activity using a calcineurin substrate peptide.

60. The method of any one of claims 54 to 59, wherein the cell is a HEK293T cell, a hepatocyte, a U2OS cell, a HeLa cell, or a murine embryonic fibroblast (MEF).

61. The method of any one of claims 54 to 60, wherein the cell is from a db/db mouse, a ob/ob mouse, a Non Obese Diabetes (NOD) mouse, a mouse fed a high fat diet, or a streptozotocin-treated mouse.

62. The method of any one of claims 54 to 61, wherein the cell is treated with glucagon, H89 dihydrochloride, insulin, forskolin, an inducer of ER stress, tunicamycin, saturated fatty acids, or any combination thereof.

63. A method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of p38, thereby treating or preventing the disorder.

64. A method of treating or preventing a metabolic disorder in a subject, wherein the disorder is induced by obesity, the method comprising reducing the activity of MK2/3, thereby treating or preventing the disorder.

65. The method of claim 63 or 64, wherein the disorder is selected from the group consisting of Type 1 diabetes, Type 2 diabetes, insulin resistance, obesity and metabolic syndrome.

66. The method of claim 63 or 64, wherein the treating or preventing affects glycogenolysis or gluconeogenesis in the subject.

67. The method of claim 63 or 64, wherein the treating or preventing reduces hepatic glucose production, hyperglycemia, fatty liver, insulin resistance, insulin resistance-associated inflammation, insulin resistance-associated dyslipidemia, or any combination therof, in the subject.

68. The method of claim 63, wherein the treating or preventing comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the p38 protein.

69. The method of claim 63, wherein the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the p38 protein.

70. The method of claim 63, wherein the treating or preventing comprises a step of administering to the subject a small molecule.

71. The method of claim 70, wherein the small molecule is a p38 inhibitor.

72. The method of claim 64, wherein the treating or preventing comprises a step of administering to the subject an antisense RNA that reduces expression of the gene encoding the MK2/3 protein.

73. The method of claim 64, wherein the treating or preventing comprises a step of administering to the subject a peptide or a polypeptide that specifically binds to the MK2/3 protein.

74. The method of claim 64, wherein the treating or preventing comprises a step of administering to the subject a small molecule.

75. The method of claim 74, wherein the small molecule is a MK2/3 inhibitor.

76. A method for identifying a compound that inhibits the activity of p38, the method comprising:

a) contacting a cell with a test compound; and

b) measuring p38 kinase activity, wherein a reduction of the kinase activity of p38 in the presence of the compound compared to the kinase activity of p38 in the absence of the compound indicates that the compound is an inhibitor of p38.

77. A method for identifying a compound that inhibits the activity of MK2/3, the method comprising:

a) contacting a cell with a test compound; and

b) measuring MK2/3 kinase activity, wherein a reduction of the kinase activity of MK2/3 in the presence of the compound compared to the kinase activity of MK2/3 in the absence of the compound indicates that the compound is an inhibitor of MK2/3.

78. The method of claim 76, wherein p38 kinase activity is measured using a p38-specific peptide.

79. The method of claim 77, wherein MK2/3 kinase activity is measured using a MK2/3-specific peptide.

80. The method of any one of claims 76 to 79, wherein the cell is a HEK293T cell, a hepatocyte, a U2OS cell, a HeLa cell, a macrophage or a murine embryonic fibroblast (MEF).

81. The method of any one of claims 76 to 80, wherein the cell is from a Insr-/- mouse, a db/db mouse, a ob/ob mouse, a Non Obese Diabetes (NOD) mouse, a mouse fed a high fat diet, or a streptozotocin-treated mouse.

82. The method of any one of claims 76 to 81, wherein the cell is treated with glucagon, 8-bromo cAMP, H89 dihydrochloride, forskolin, saturated fatty acids, or any combination thereof.

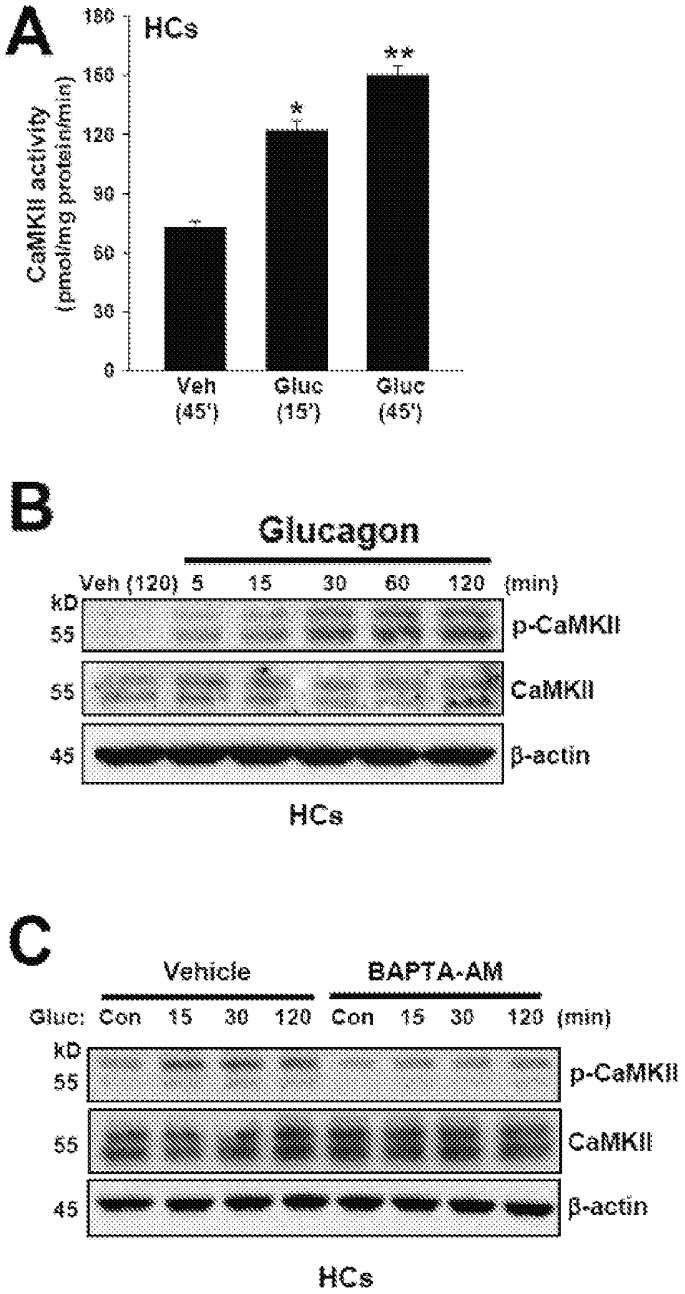

A
HCs
CaMKII activity
(pmol/mg protein/min)
180
150
120
90
60
30
0
*
**
Veh
(45')
Gluc
(15')
Gluc
(45')
B
Glucagon
Veh (120) 5 15 30 60 120 (min)
kD
55
p-CaMKII
55
CaMKII
45
β-actin
HCs
C
Vehicle
BAPTA-AM
Gluc: Con 15 30 120 Con 15 30 120 (min)
kD
55
p-CaMKII
55
CaMKII
45
β-actin
HCs

Figure 1A-1C

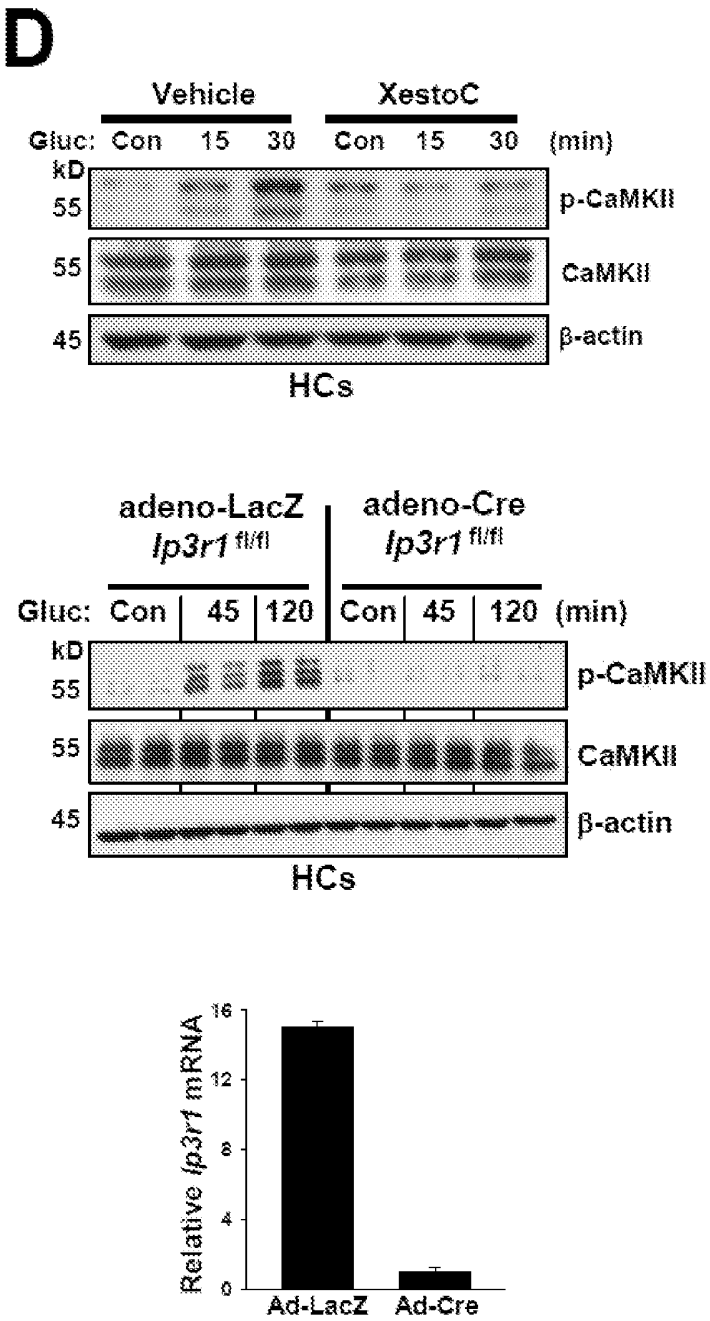

D
Vehicle
XestoC
Gluc: Con 15 30 Con 15 30 (min)
kD
55
p-CaMKII
55
CaMKII
45
β-actin
HCs
adeno-LacZ
Ip3r1 fl/fl
adeno-Cre
Ip3r1 fl/fl
Gluc: Con 45 120 Con 45 120 (min)
kD
55
p-CaMKII
55
CaMKII
45
β-actin
HCs
Relative Ip3r1 mRNA
16
12
8
4
0
Ad-LacZ
Ad-Cre

Figure 1D

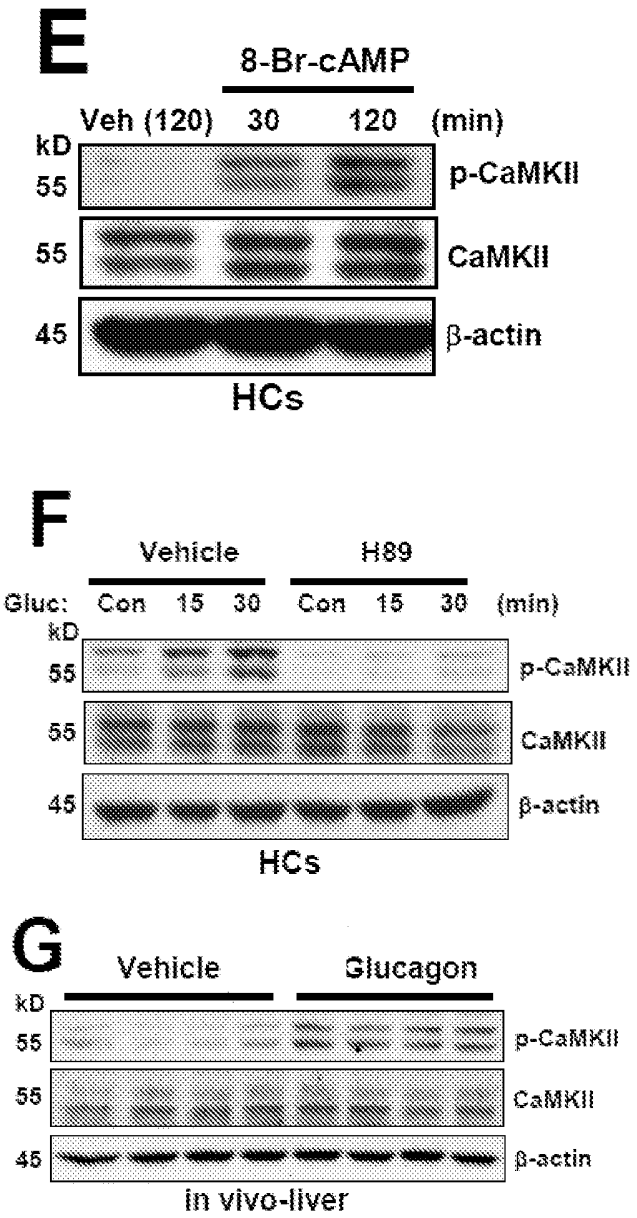

E
8-Br-cAMP
Veh (120) 30 120 (min)
kD
55
p-CaMKII
55
CaMKII
45
β-actin
HCs
F
Vehicle
H89
Gluc: Con 15 30 Con 15 30 (min)
kD
55
p-CaMKII
55
CaMKII
45
β-actin
HCs
G
Vehicle
Glucagon
kD
55
p-CaMKII
55
CaMKII
45
β-actin
in vivo-liver

Figure 1E-1G

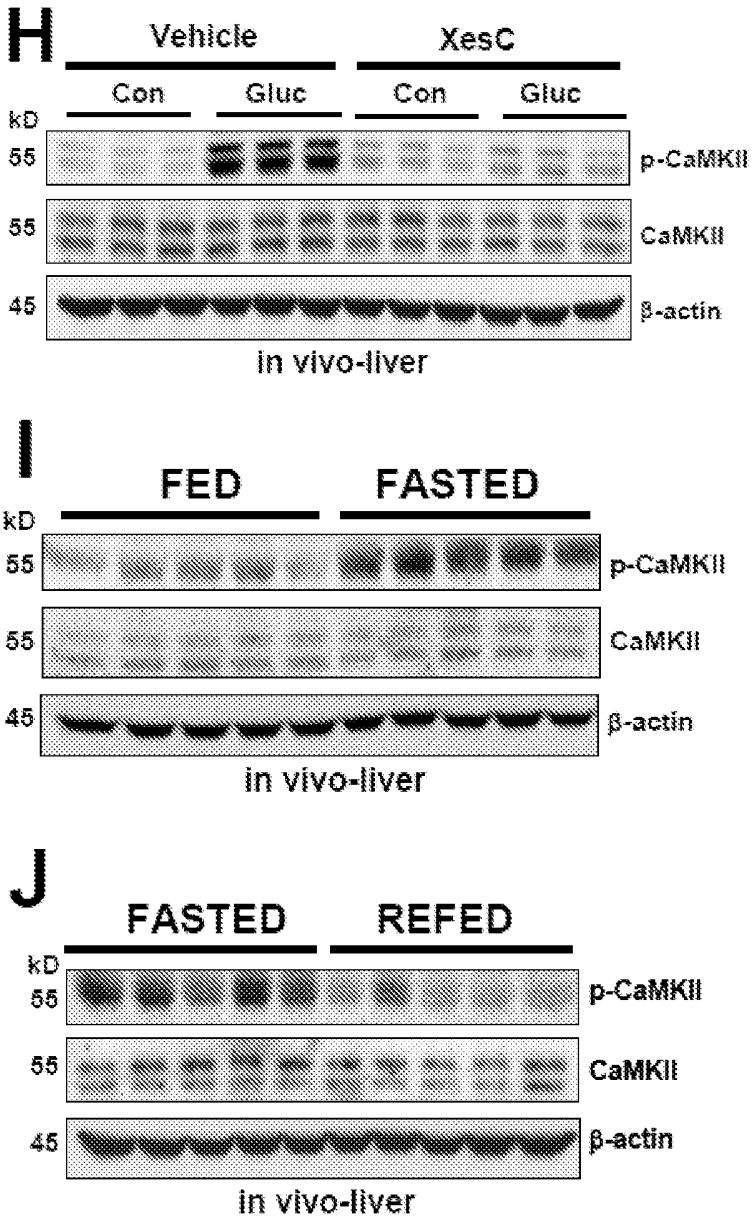
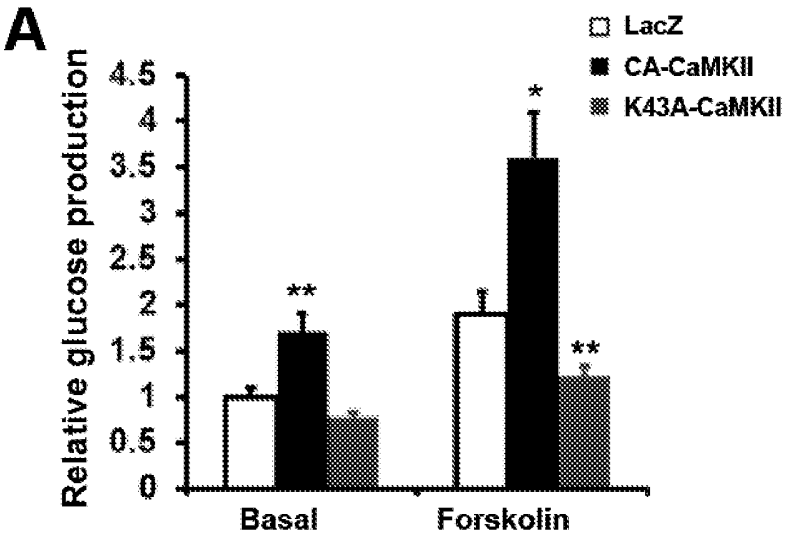

H
Vehicle
XesC
Con
Gluc
Con
Gluc
kD
55
p-CaMKII
55
CaMKII
45
β-actin
in vivo-liver
I
FED
FASTED
kD
55
p-CaMKII
55
CaMKII
45
β-actin
in vivo-liver
J
FASTED
REFED
kD
55
p-CaMKII
55
CaMKII
45
β-actin
in vivo-liver

Figure 1H-1J


A
LacZ
CA-CaMKII
K43A-CaMKII
Relative glucose production
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
*
**
Basal
Forskolin

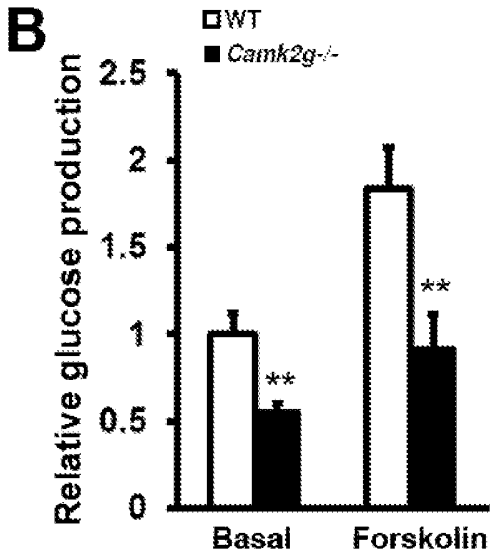

B
WT
Camk2g-/-
Relative glucose production
2.5
2
1.5
1
0.5
0
**
**
Basal
Forskolin

Figure 2A-2B

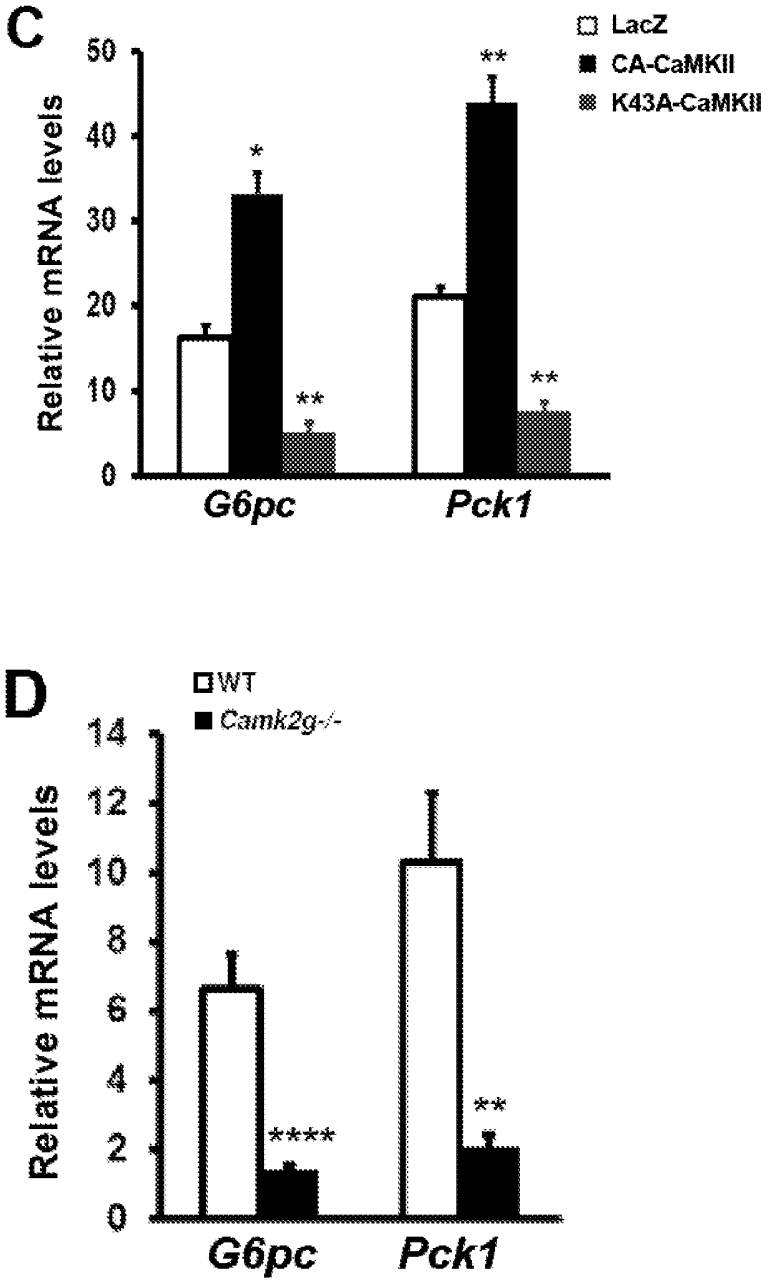

C
LacZ
CA-CaMKII
K43A-CaMKII
Relative mRNA levels
50
40
30
20
10
0
*
**
**
**
G6pc
Pck1
D
WT
Camk2g-/-
Relative mRNA levels
14
12
10
8
6
4
2
0
****
**
G6pc
Pck1

Figure 2C-2D

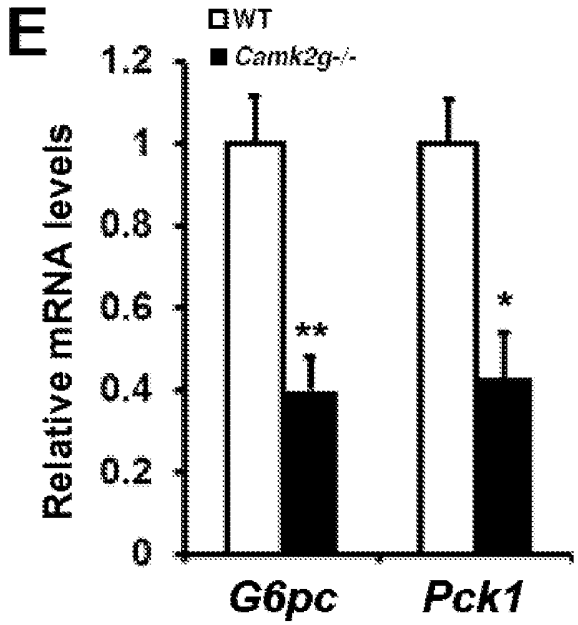


Figure 2E

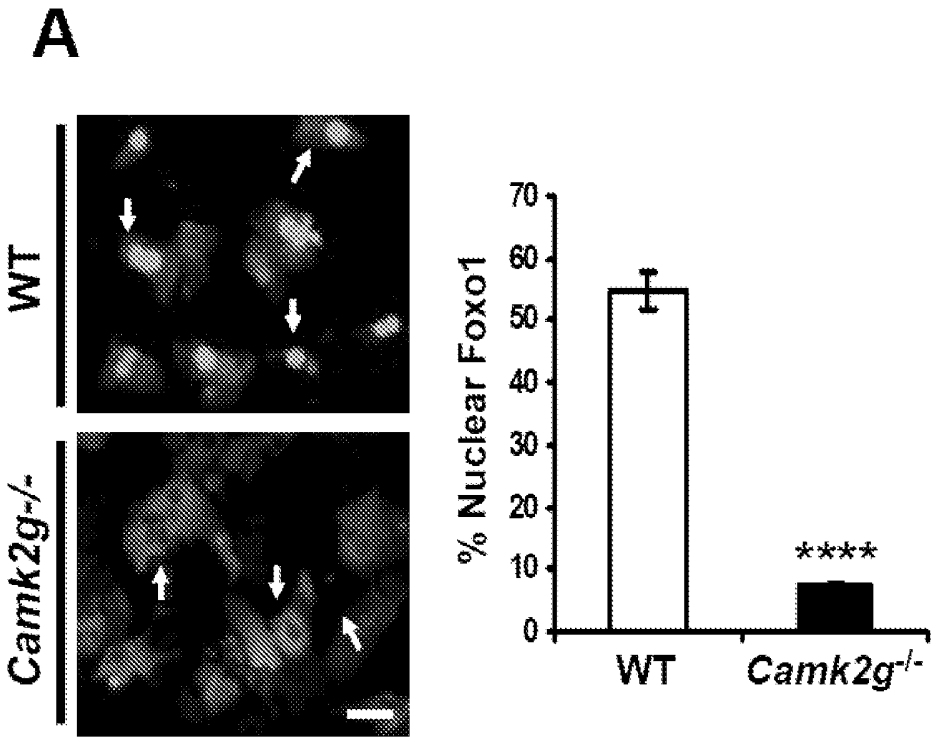


Figure 3A

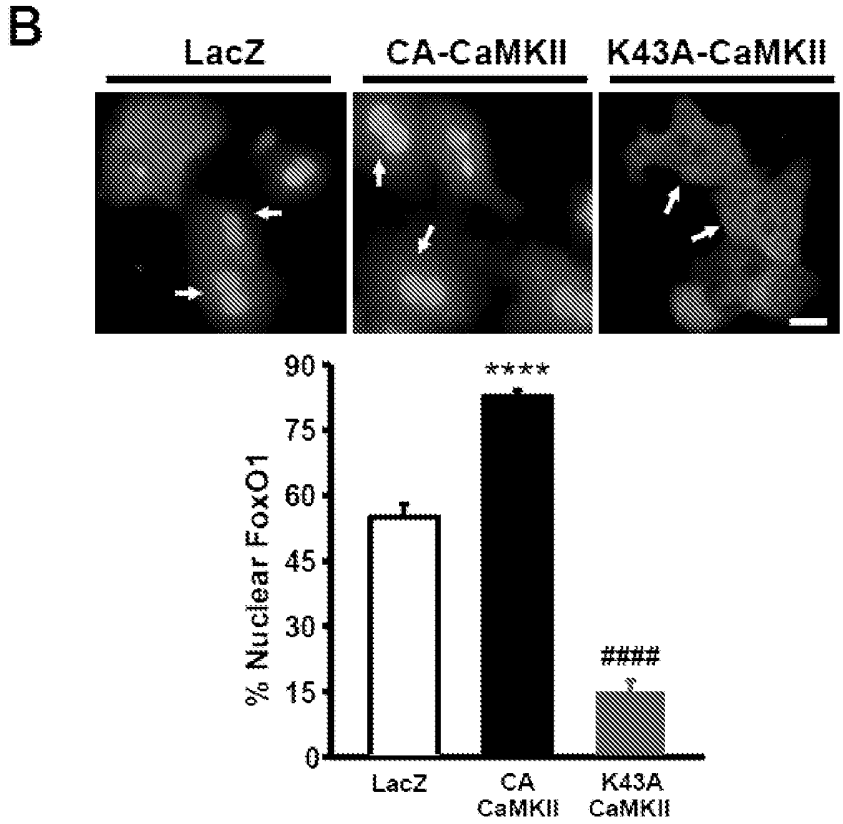

B
LacZ
CA-CaMKII
K43A-CaMKII
% Nuclear FoxO1
90
75
60
45
30
15
0
****
####
LacZ
CA CaMKII
K43A CaMKII

Figure 3B

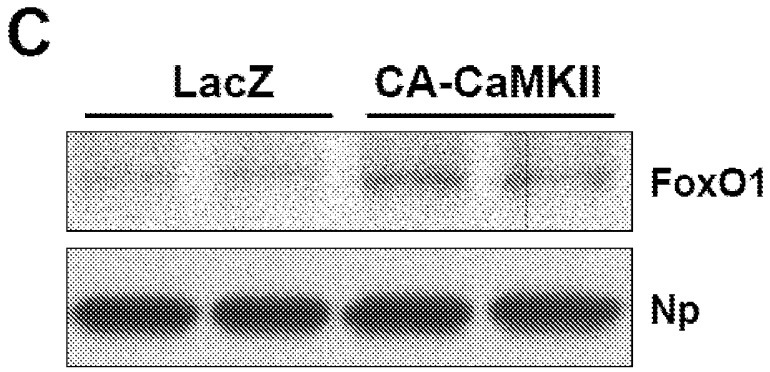
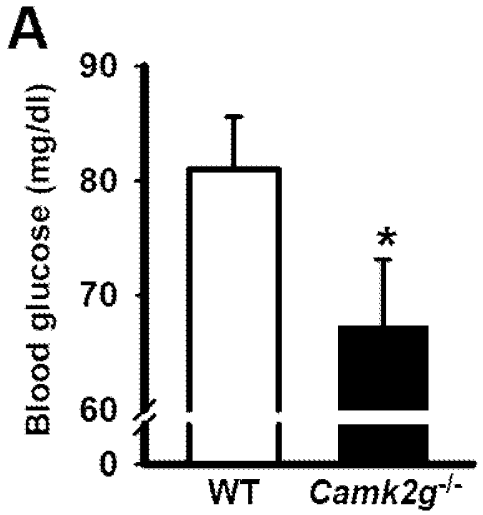


Figure 3C


A
Blood glucose (mg/dl)
90
80
70
60
0
*
WT
Camk2g-/-

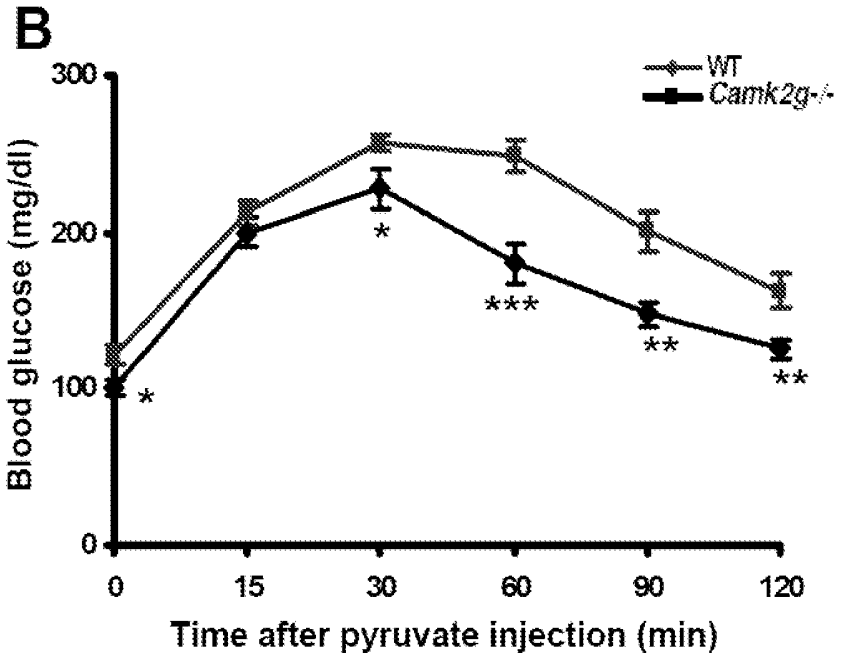

B
WT
Camk2g-/-
Blood glucose (mg/dl)
300
200
100
0
*
*
***
**
**
0
15
30
60
90
120
Time after pyruvate injection (min)

Figure 4A-4B

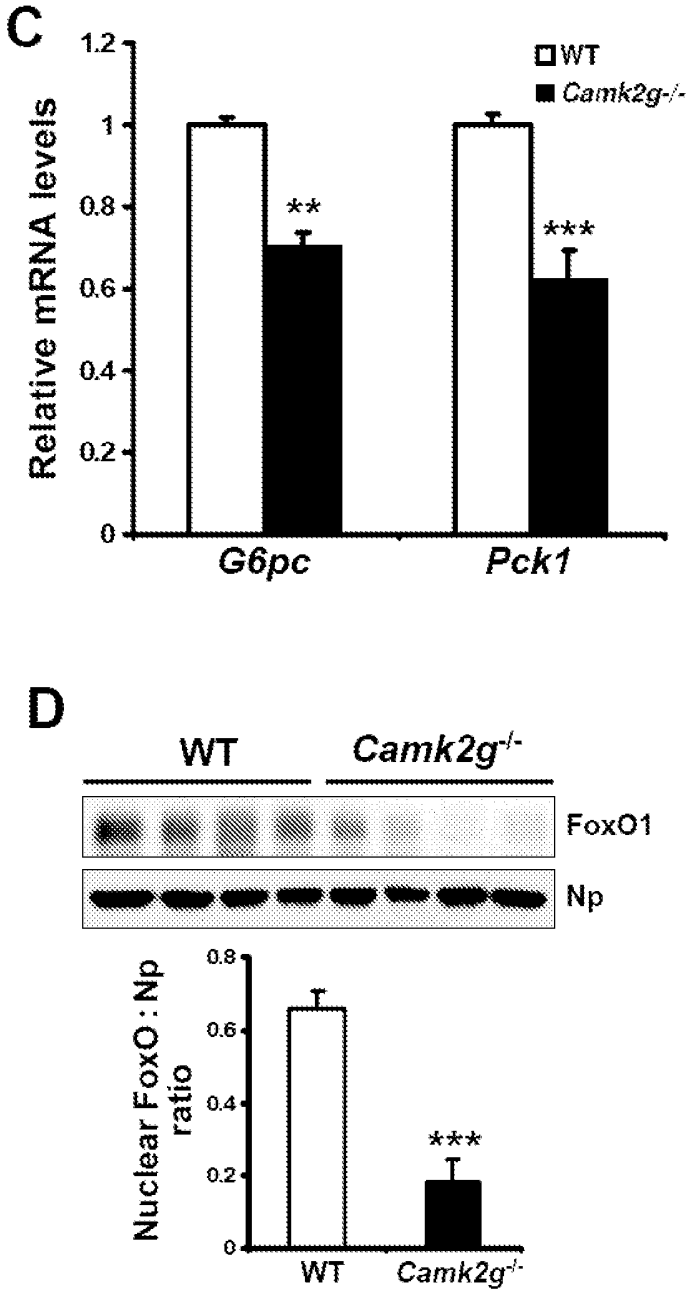

C
Relative mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
WT
Camk2g-/-
**
***
G6pc
Pck1
D
WT
Camk2g-/-
FoxO1
Np
Nuclear FoxO : Np ratio
0.8
0.6
0.4
0.2
0
***
WT
Camk2g-/-

Figure 4C-4D

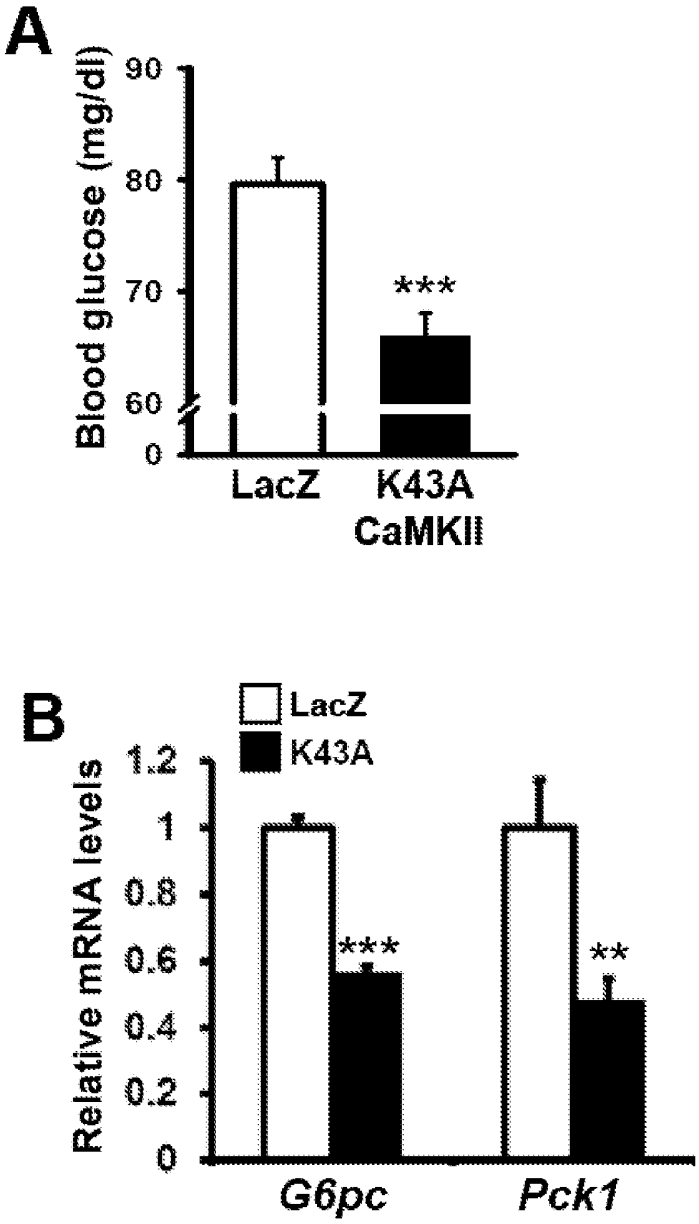
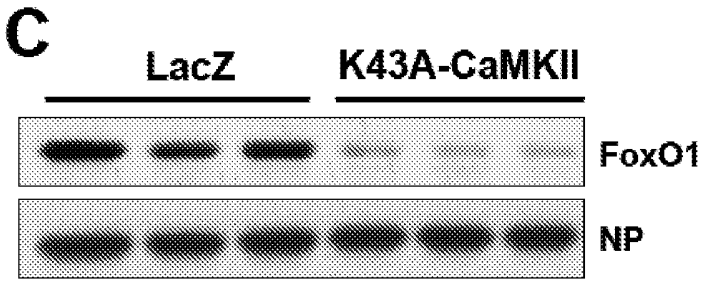

A
Blood glucose (mg/dl)
90
80
70
60
0
***
LacZ
K43A
CaMKII
B
LacZ
K43A
Relative mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
***
**
G6pc
Pck1

Figure 5A-5B


C
LacZ
K43A-CaMKII
FoxO1
NP

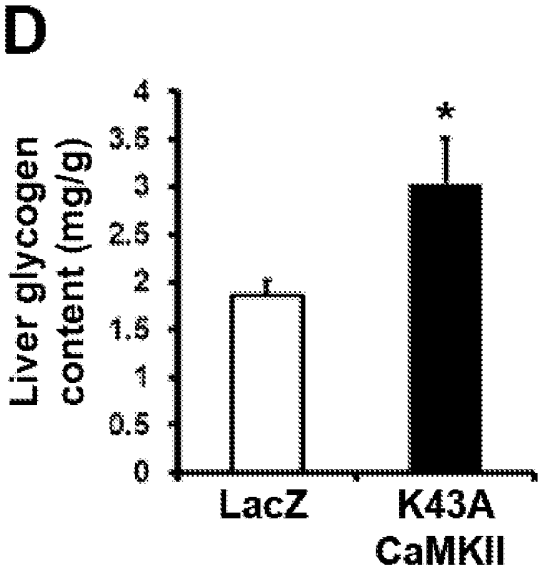
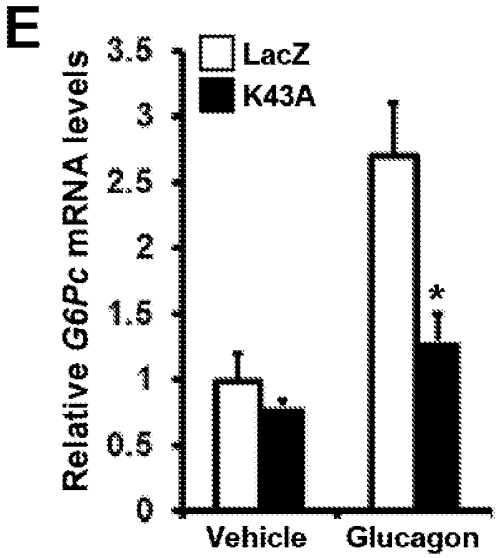

D
Liver glycogen content (mg/g)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
LacZ
K43A CaMKII

Figure 5C-5D


E
LacZ
K43A
Relative G6Pc mRNA levels
3.5
3
2.5
2
1.5
1
0.5
0
*
*
Vehicle
Glucagon

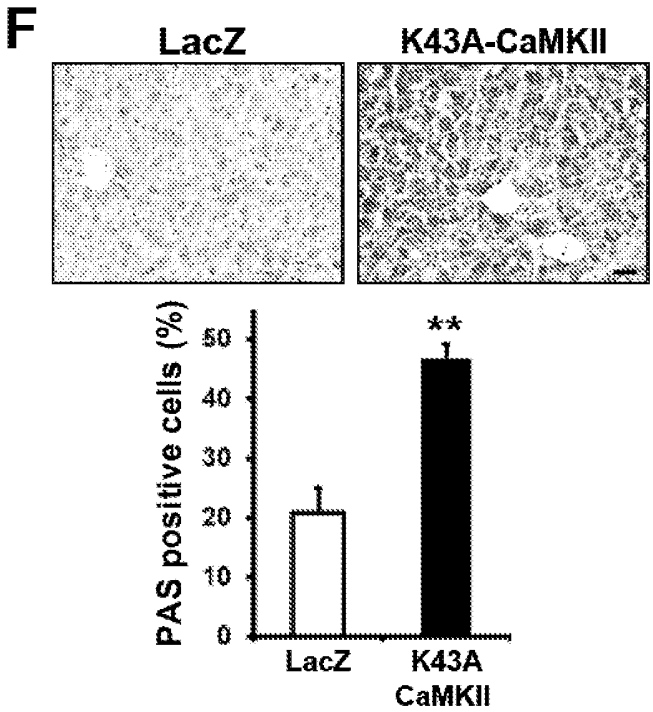

F
LacZ
K43A-CaMKII
PAS positive cells (%)
50
40
30
20
10
0
**
LacZ
K43A
CaMKII

Figure 5E-5F

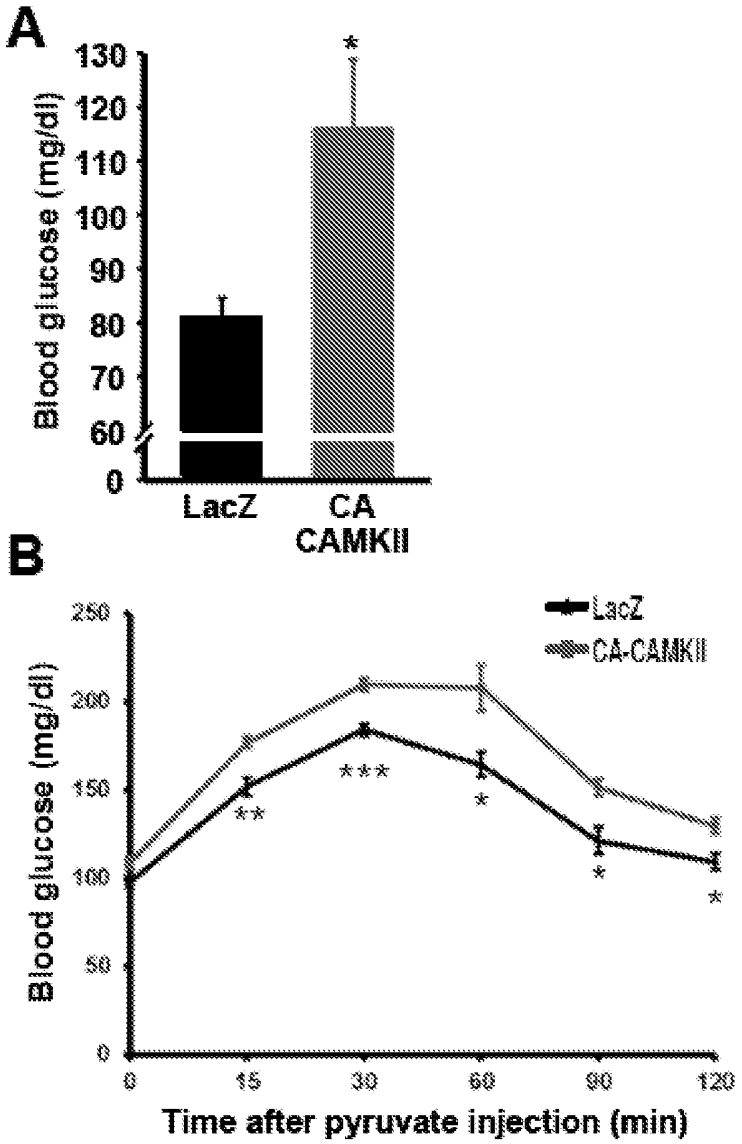

A
Blood glucose (mg/dl)
130
120
110
100
90
80
70
60
0
*
LacZ
CA CAMKII
B
Blood glucose (mg/dl)
250
200
150
100
50
0
LacZ
CA-CAMKII
**
***
*
*
*
0
15
30
60
90
120
Time after pyruvate injection (min)

Figure 6A-6B

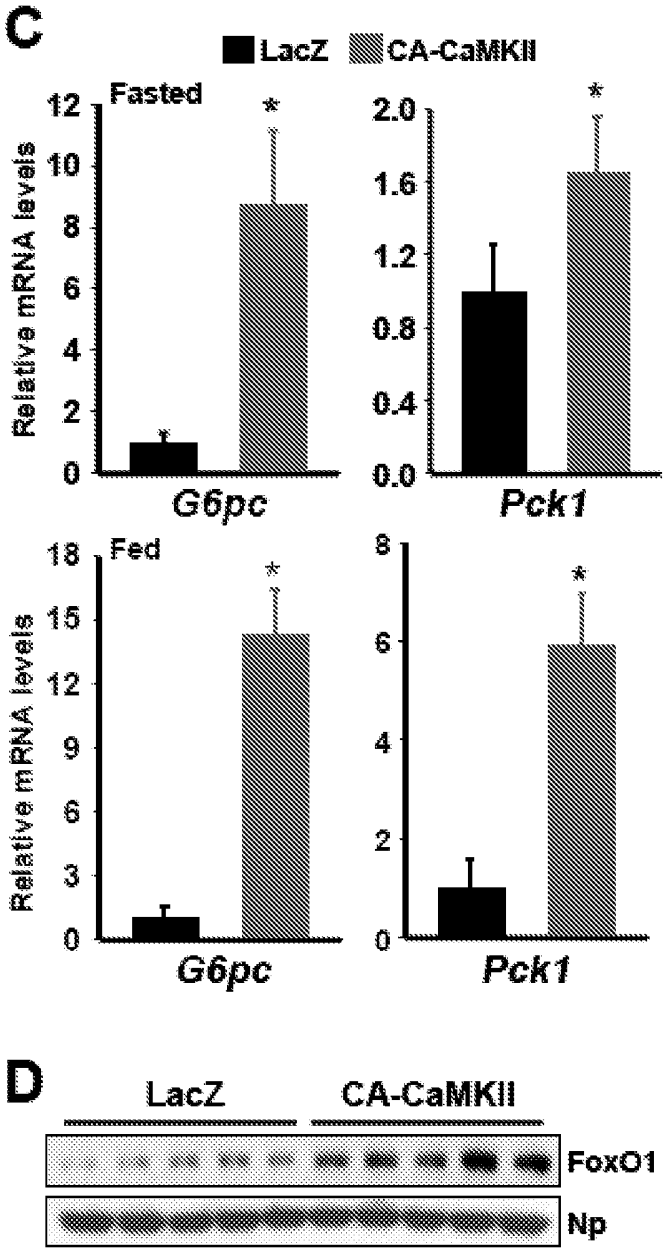

C
LacZ
CA-CaMKII
Fasted
Relative mRNA levels
12
10
8
6
4
2
0
*
G6pc
2.0
1.6
1.2
0.8
0.4
0.0
Pck1
Fed
18
15
12
9
6
3
0
8
6
4
2
0
D
LacZ
CA-CaMKII
FoxO1
Np

Figure 6C-6D

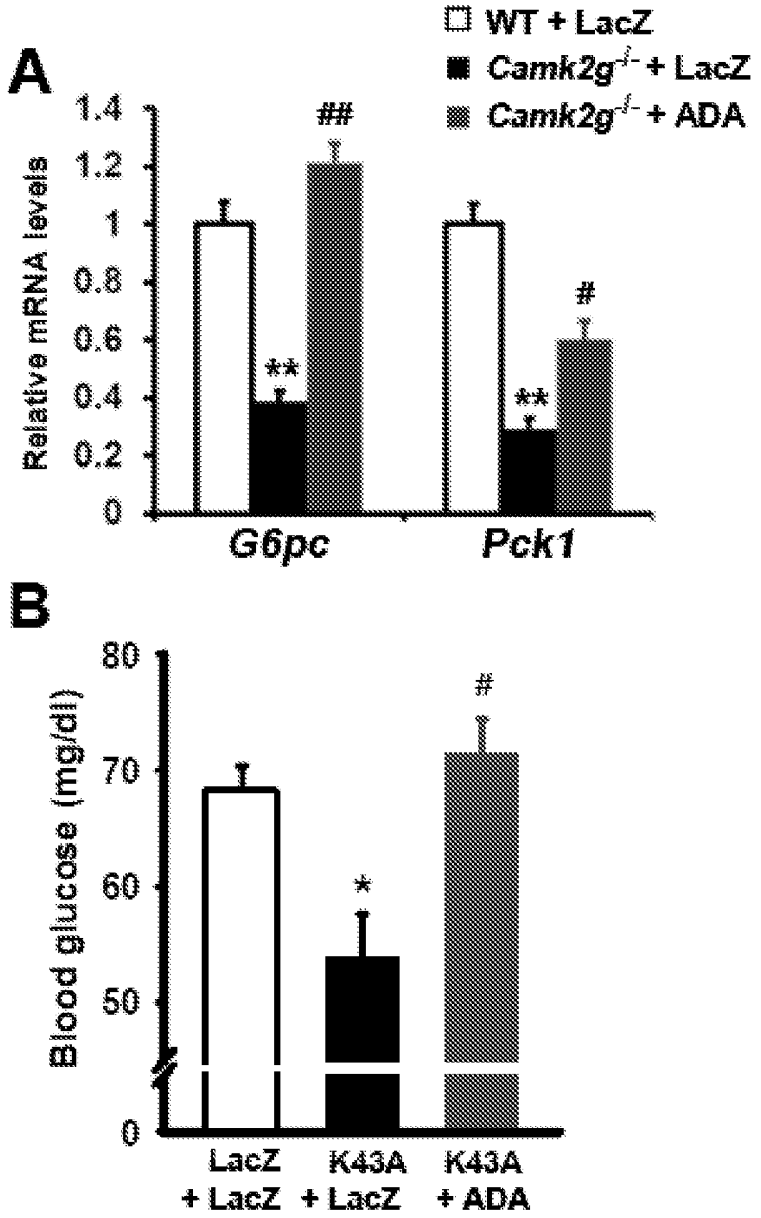

A
WT + LacZ
Camk2g-/- + LacZ
Camk2g-/- + ADA
Relative mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
##
**
**
#
G6pc
Pck1
B
Blood glucose (mg/dl)
80
70
60
50
0
#
*
LacZ + LacZ
K43A + LacZ
K43A + ADA

Figure 7A-7B

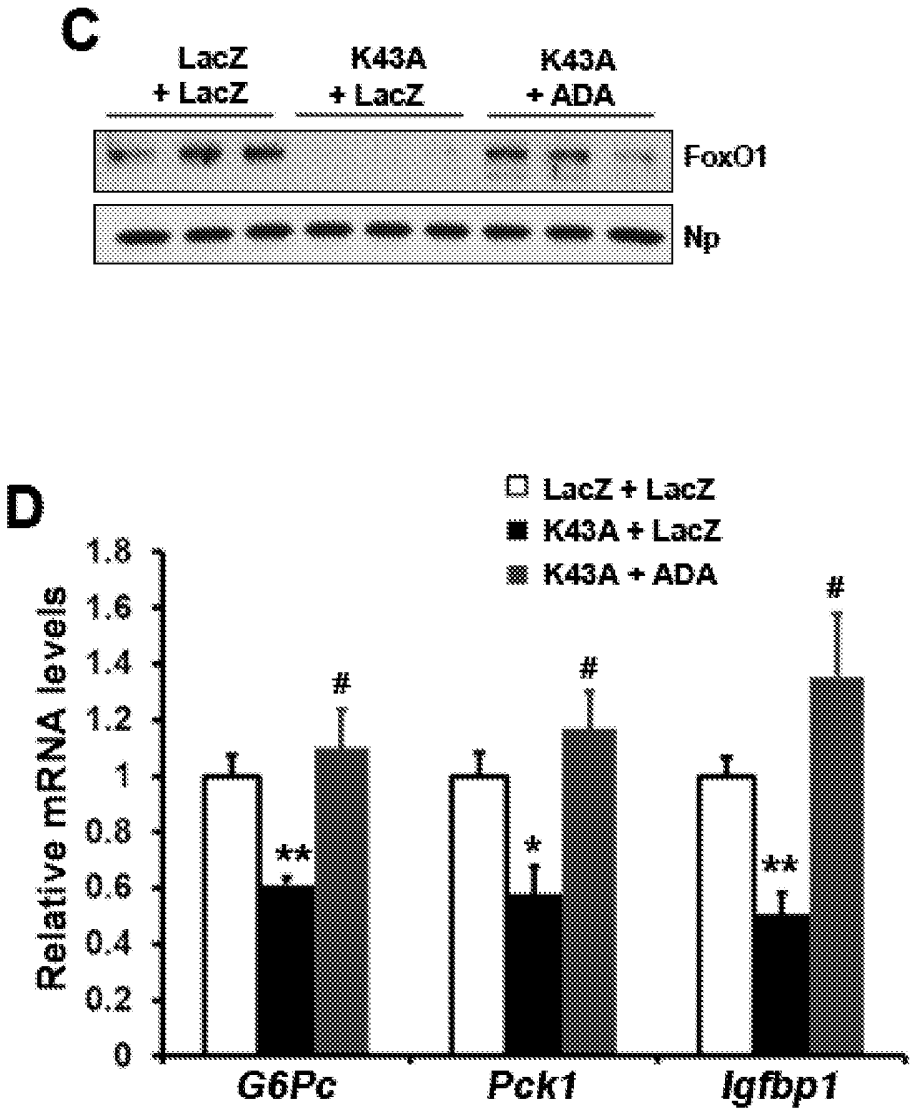

C
LacZ + LacZ
K43A + LacZ
K43A + ADA
FoxO1
Np
D
LacZ + LacZ
K43A + LacZ
K43A + ADA
Relative mRNA levels
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
**
*
G6Pc
Pck1
Igfbp1

Figure 7C-7D

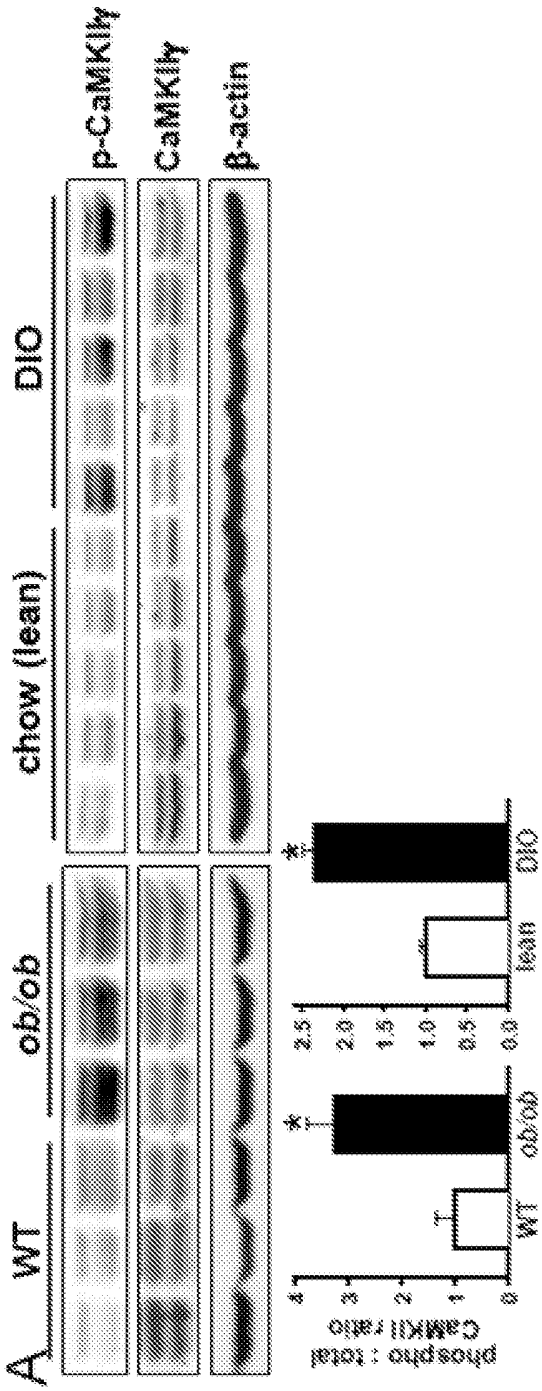

A
WT
ob/ob
chow (lean)
DIO
p-CaMKIIγ
CaMKIIγ
β-actin
phospho : total CaMKII ratio
4
3
2
1
0
WT
ob/ob
2.5
2.0
1.5
1.0
0.5
0.0
lean
DIO

Figure 8A

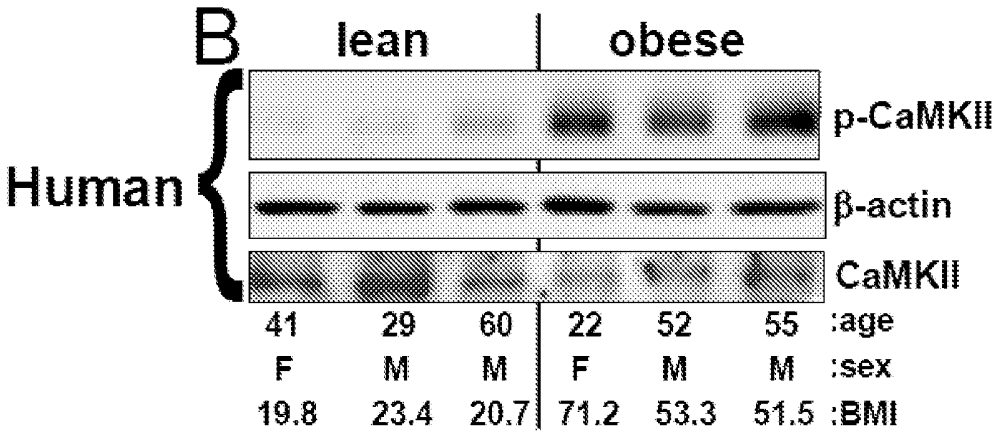

B
lean
obese
Human
p-CaMKII
β-actin
CaMKII
41 29 60 22 52 55 :age
F M M F M M :sex
19.8 23.4 20.7 71.2 53.3 51.5 :BMI

Figure 8B

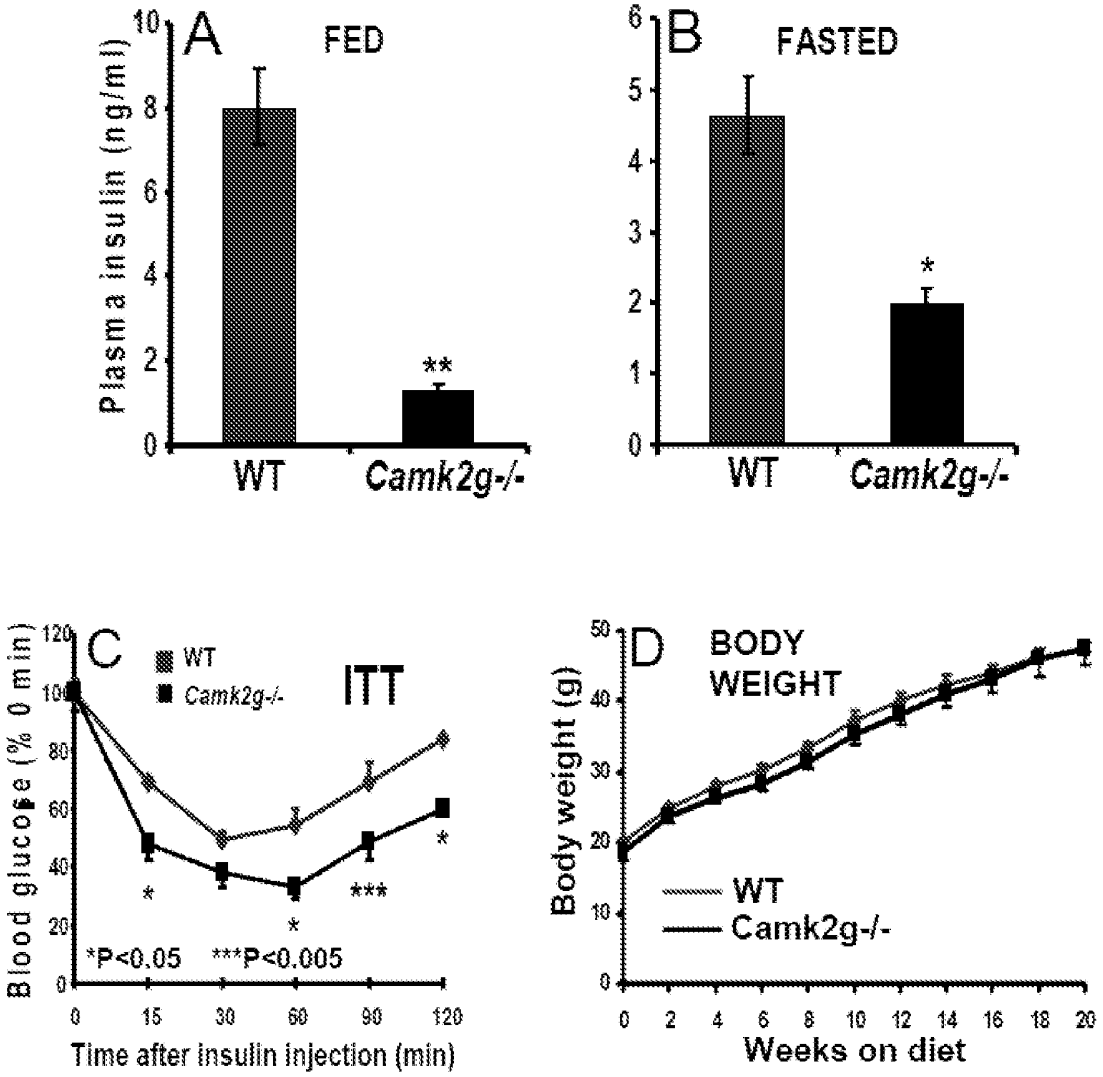
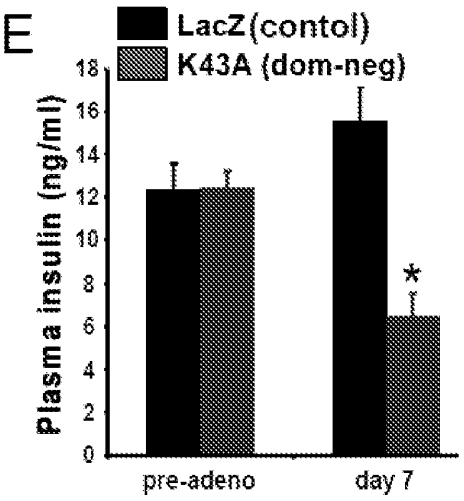

A
FED
Plasma insulin (ng/ml)
10
8
6
4
2
0
**
WT
Camk2g-/-
B
FASTED
6
5
4
3
2
1
0
*
WT
Camk2g-/-
C
WT
Camk2g-/-
ITT
Blood glucose (% 0 min)
120
100
80
60
40
20
0
*
*
***
*
*P<0.05 ***P<0.005
0 15 30 60 90 120
Time after insulin injection (min)
D
BODY WEIGHT
Body weight (g)
50
40
30
20
10
0
WT
Camk2g-/-
0 2 4 6 8 10 12 14 16 18 20
Weeks on diet

Figure 9A-9D


E
LacZ(contol)
K43A (dom-neg)
Plasma insulin (ng/ml)
18
16
14
12
10
8
6
4
2
0
*
pre-adeno
day 7

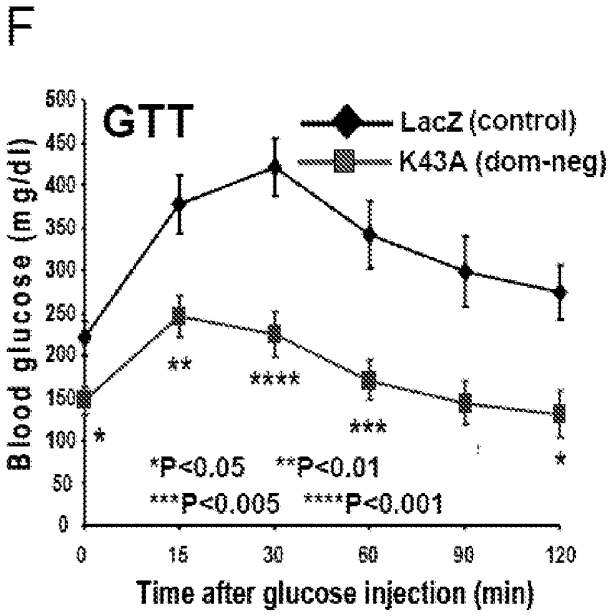
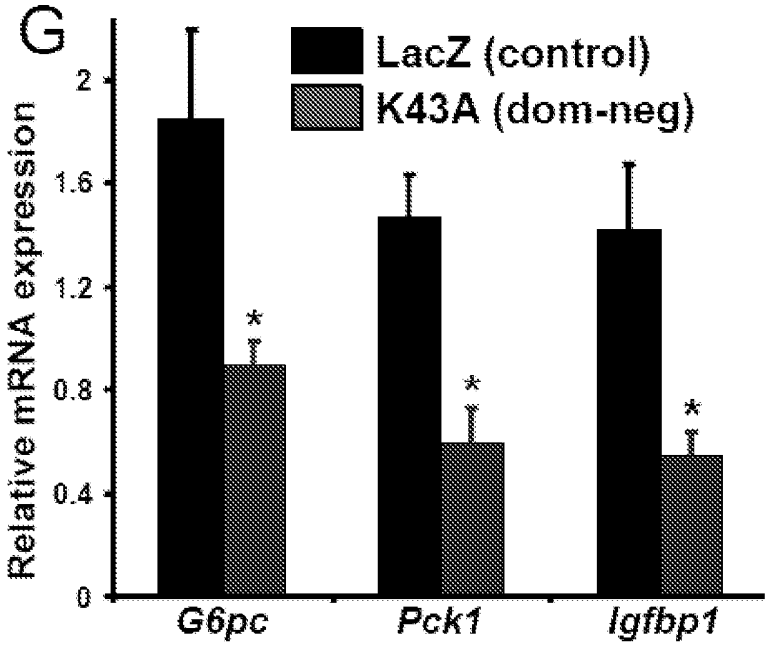

F
GTT
LacZ (control)
K43A (dom-neg)
Blood glucose (mg/dl)
500
450
400
350
300
250
200
150
100
50
0
*
**
****
***
*
*P<0.05 **P<0.01
***P<0.005 ****P<0.001
0
15
30
60
90
120
Time after glucose injection (min)

Figure 9E-9F


G
LacZ (control)
K43A (dom-neg)
Relative mRNA expression
2
1.6
1.2
0.8
0.4
0
*
*
*
G6pc
Pck1
Igfbp1

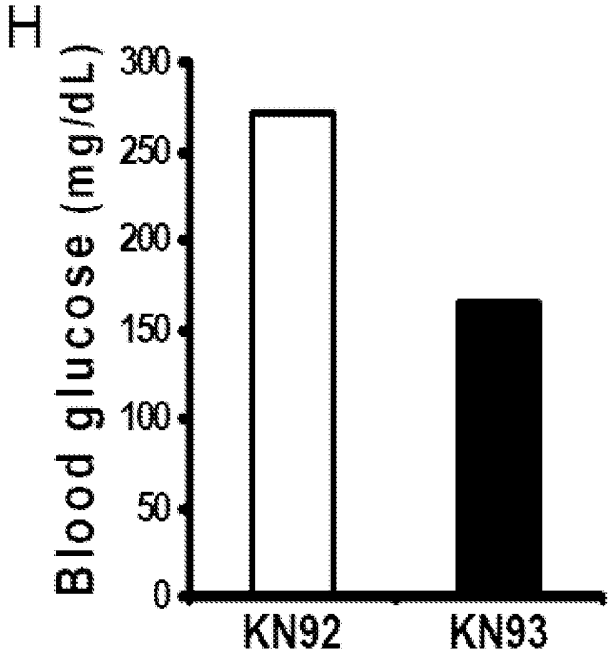

H
Blood glucose (mg/dL)
300
250
200
150
100
50
0
KN92
KN93

Figure 9G-9H

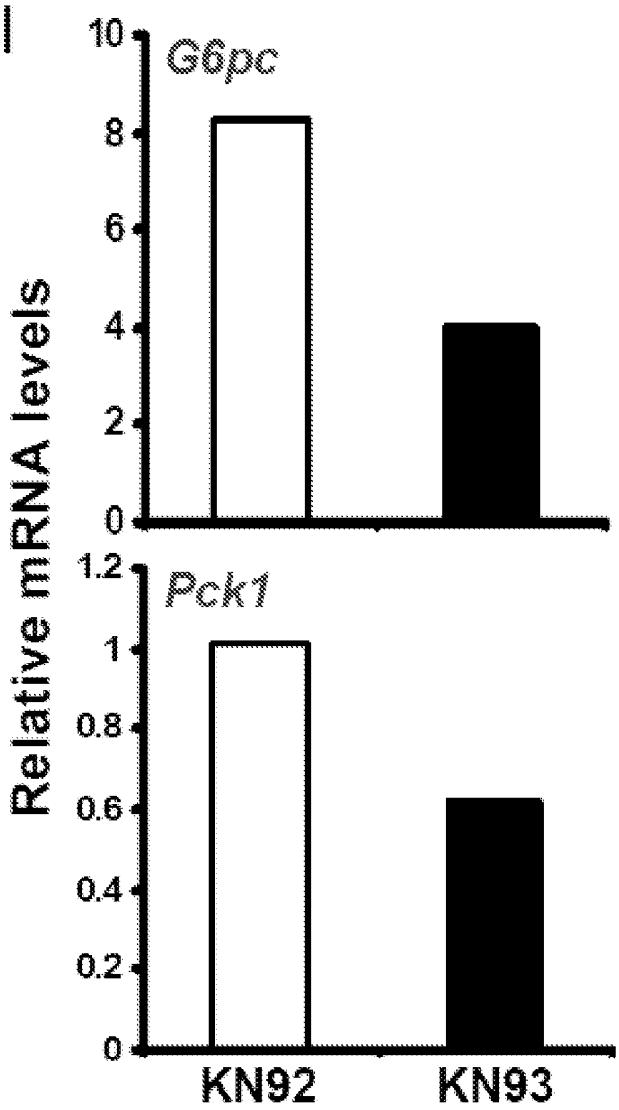

I
G6pc
Pck1
Relative mRNA levels
10
8
6
4
2
0
1.2
1
0.8
0.6
0.4
0.2
0
KN92
KN93

Figure 9I

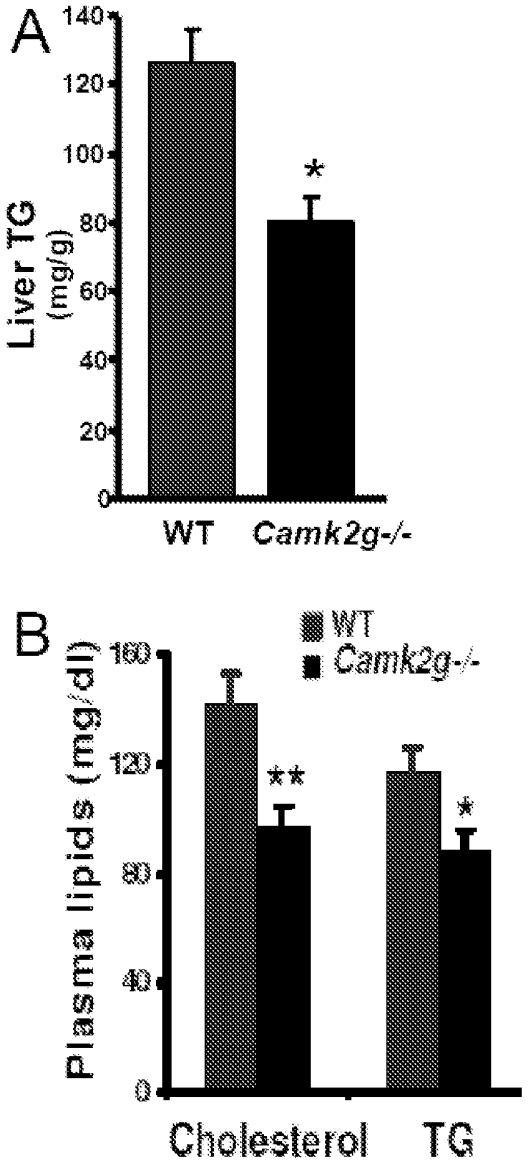


Figure 10A-10B

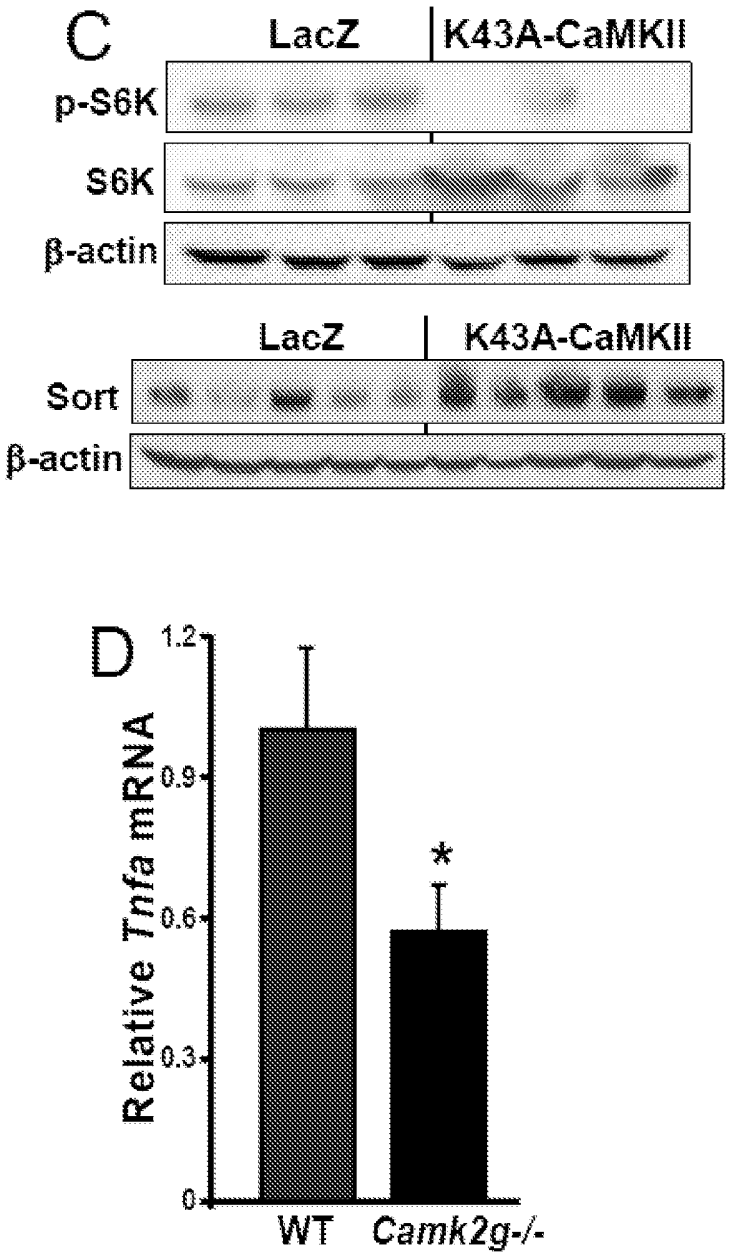


Figure 10C-10D

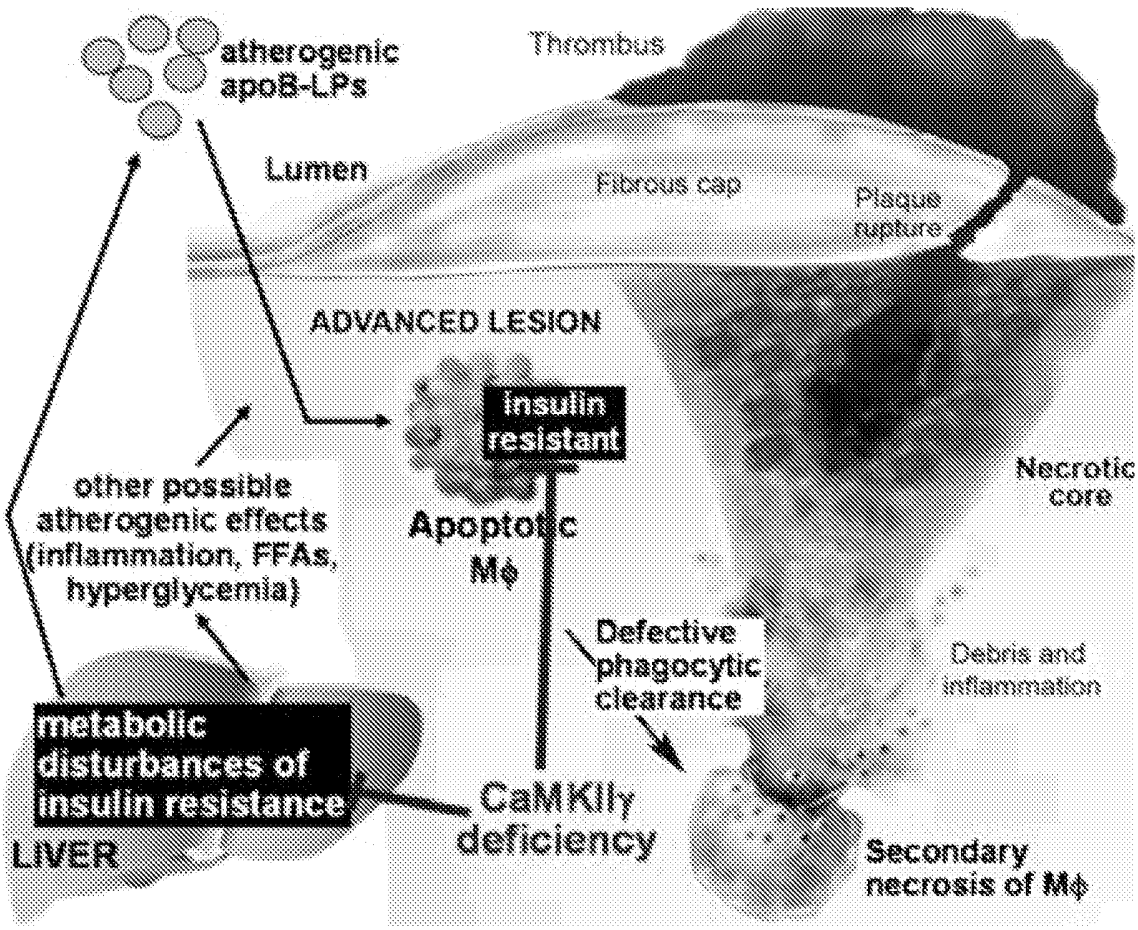

atherogenic
apoB-LPs
Thrombus
Lumen
Fibrous cap
Plaque
rupture
ADVANCED LESION
insulin
resistant
Apoptotic
Mϕ
other possible
atherogenic effects
(inflammation, FFAs,
hyperglycemia)
Necrotic
core
Defective
phagocytic
clearance
Debris and
inflammation
metabolic
disturbances of
insulin resistance
CaMKIIγ
deficiency
LIVER
Secondary
necrosis of Mϕ

Figure 11

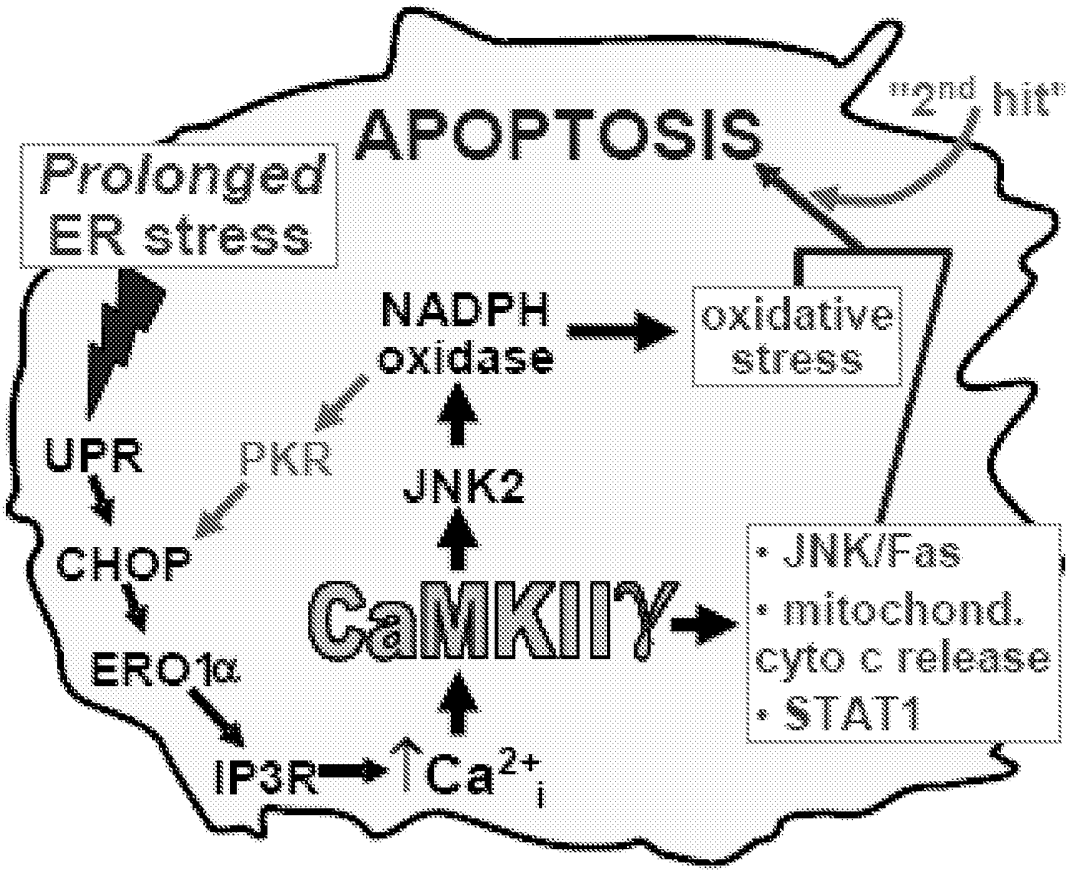

APOPTOSIS
"2nd hit"
Prolonged ER stress
NADPH oxidase
oxidative stress
UPR
PKR
JNK2
CHOP
CaMKIIγ
• JNK/Fas
• mitochond. cyto c release
• STAT1
ERO1α
IP3R
↑Ca2+i

Figure 12

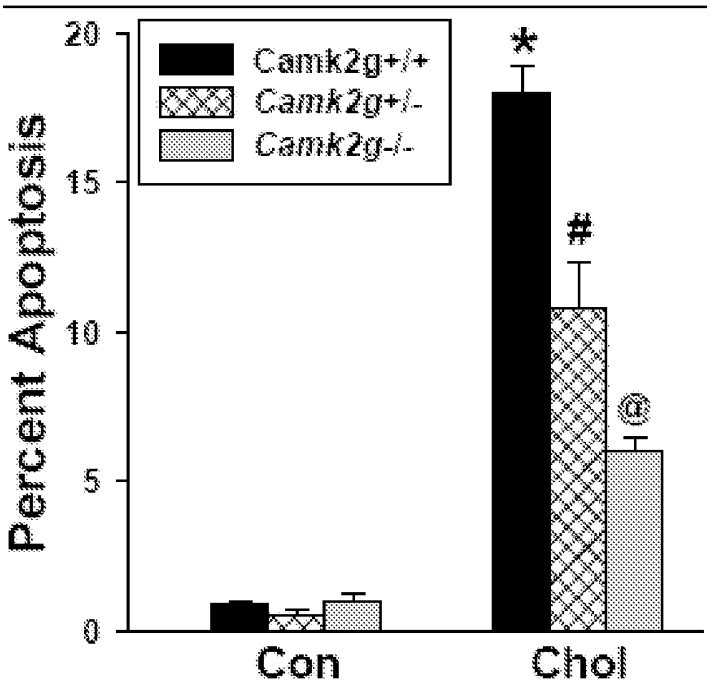


Figure 13

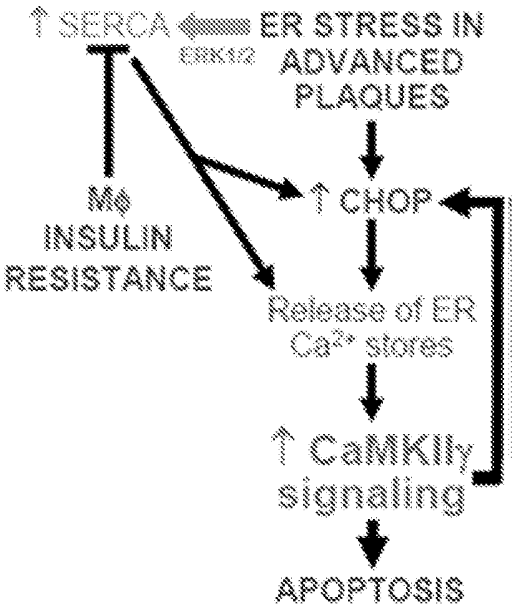

↑ SERCA
ERK1/2
ER STRESS IN ADVANCED PLAQUES
↑ CHOP
Mϕ INSULIN RESISTANCE
Release of ER Ca2+ stores
↑ CaMKIIγ signaling
APOPTOSIS

Figure 14

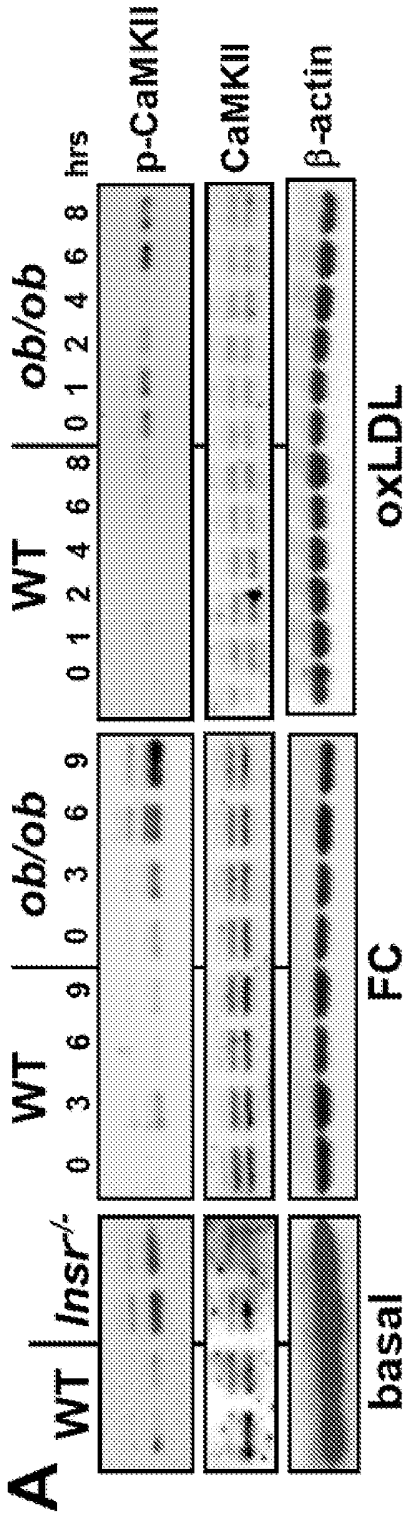

A
WT
Insr-/-
WT
0 3 6 9
ob/ob
0 3 6 9
WT
0 1 2 4 6 8
ob/ob
0 1 2 4 6 8
hrs
p-CaMKII
CaMKII
β-actin
basal
FC
oxLDL

Figure 15A

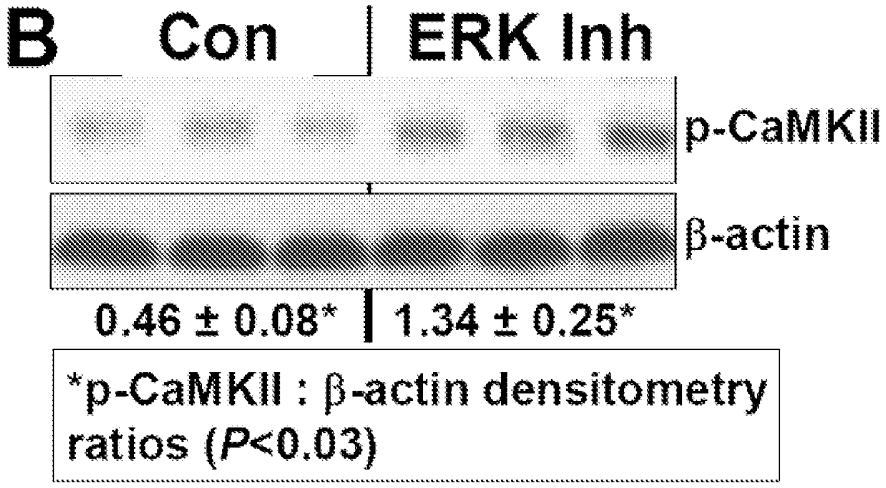

B
Con
ERK Inh
p-CaMKII
β-actin
0.46 ± 0.08*
1.34 ± 0.25*
*p-CaMKII : β-actin densitometry ratios (P<0.03)

Figure 15B

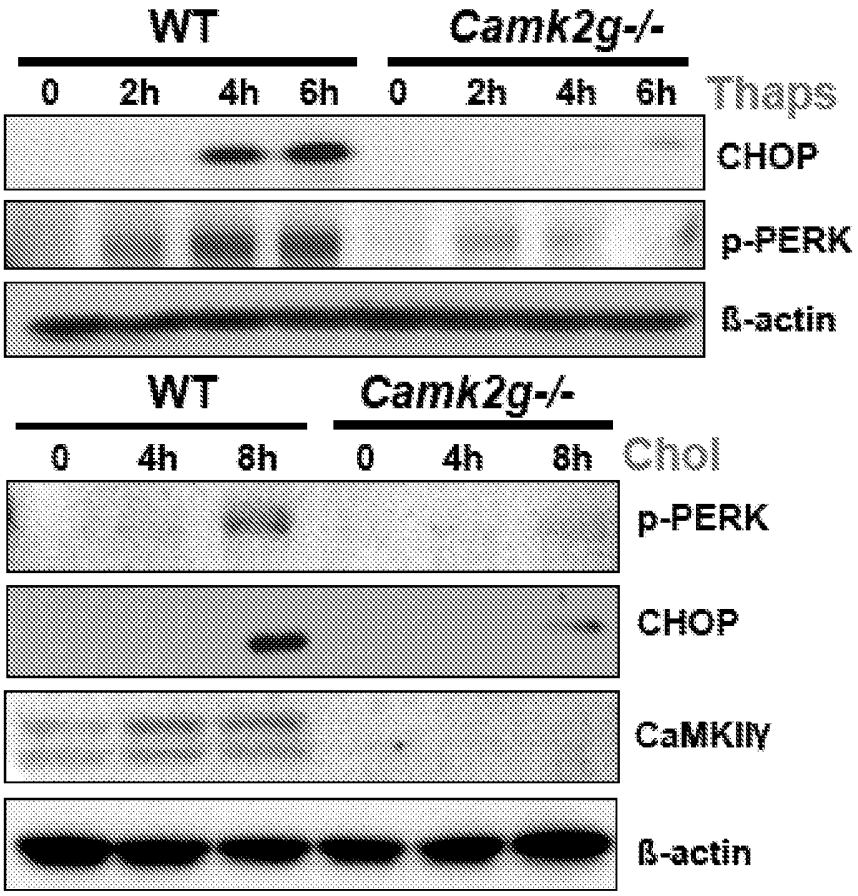

WT
Camk2g-/-
0
2h
4h
6h
0
2h
4h
6h
Thaps
CHOP
p-PERK
ß-actin
WT
Camk2g-/-
0
4h
8h
0
4h
8h
Chol
p-PERK
CHOP
CaMKIIγ
ß-actin

Figure 16

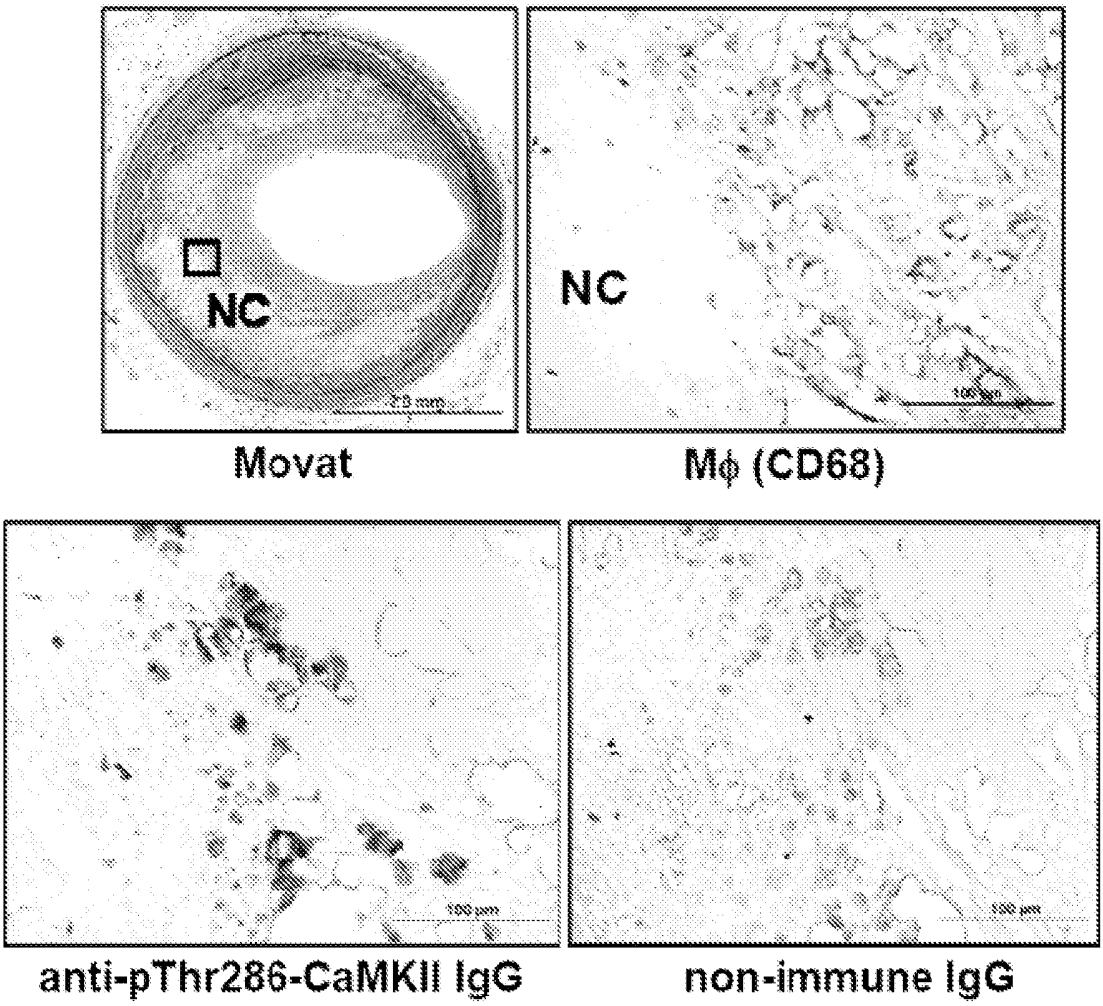


Figure 17

Hepatic CaMKIIγ deficiency → ↓ nuclear FoxO1 → ↓ GNG/HGP → ↓ hyperinsulinemia → ↓ mTORC1 → ↓ dyslipidemia

Figure 18

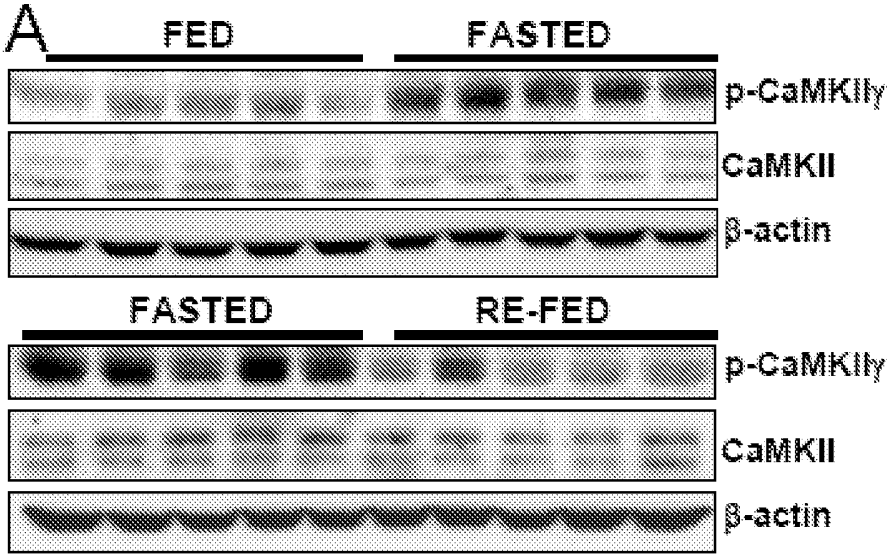

A
FED
FASTED
p-CaMKIIγ
CaMKII
β-actin
FASTED
RE-FED
p-CaMKIIγ
CaMKII
β-actin

Figure 19A

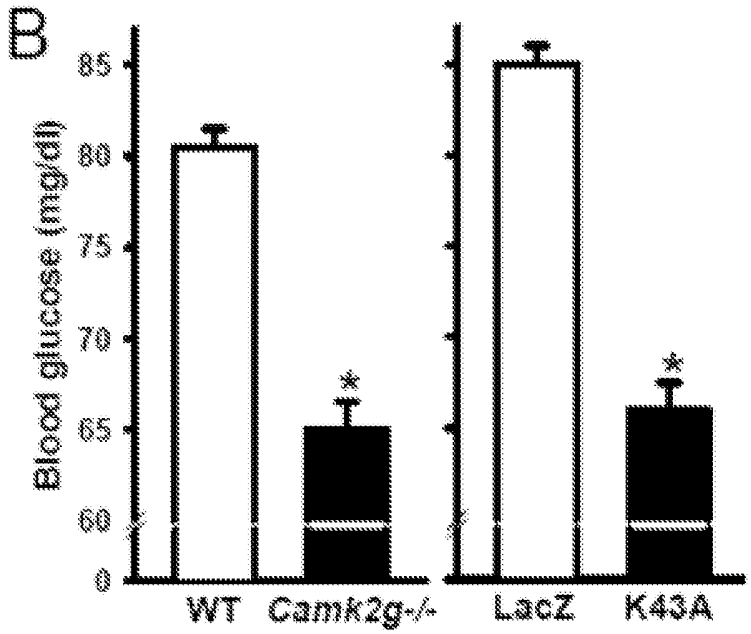


Figure 19B

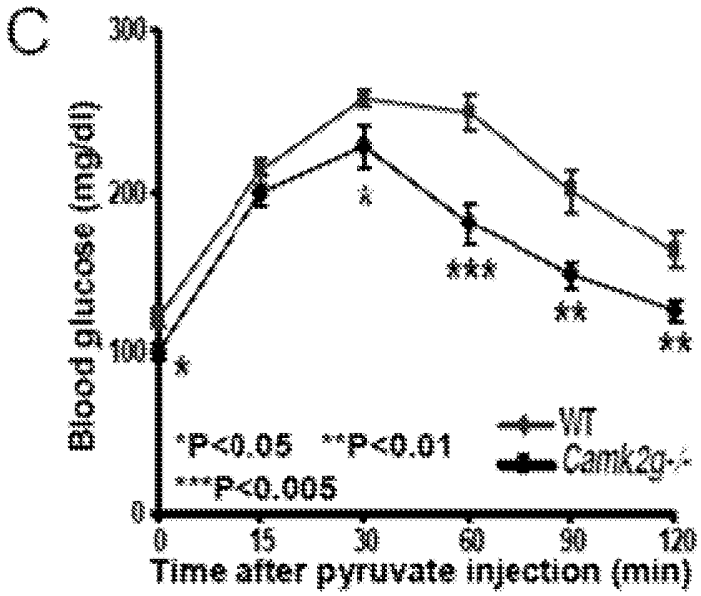

C
300
200
100
0
Blood glucose (mg/dl)
*
*
***
**
**
*P<0.05 **P<0.01
***P<0.005
WT
Camk2g-/-
0
15
30
60
90
120
Time after pyruvate injection (min)

Figure 19C

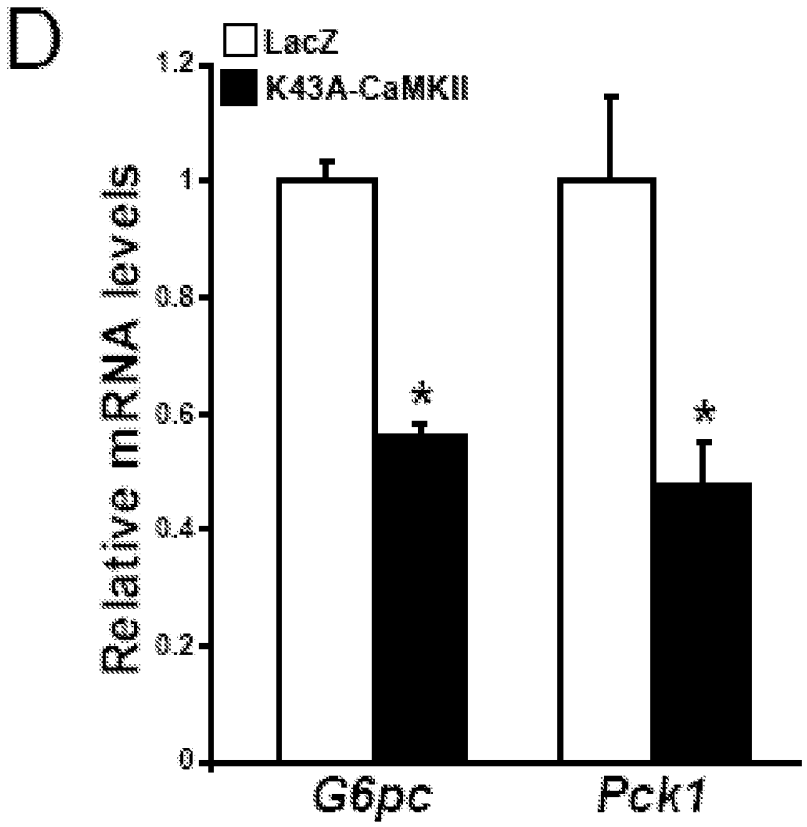

D
LacZ
K43A-CaMKII
Relative mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
*
*
G6pc
Pck1

Figure 19D

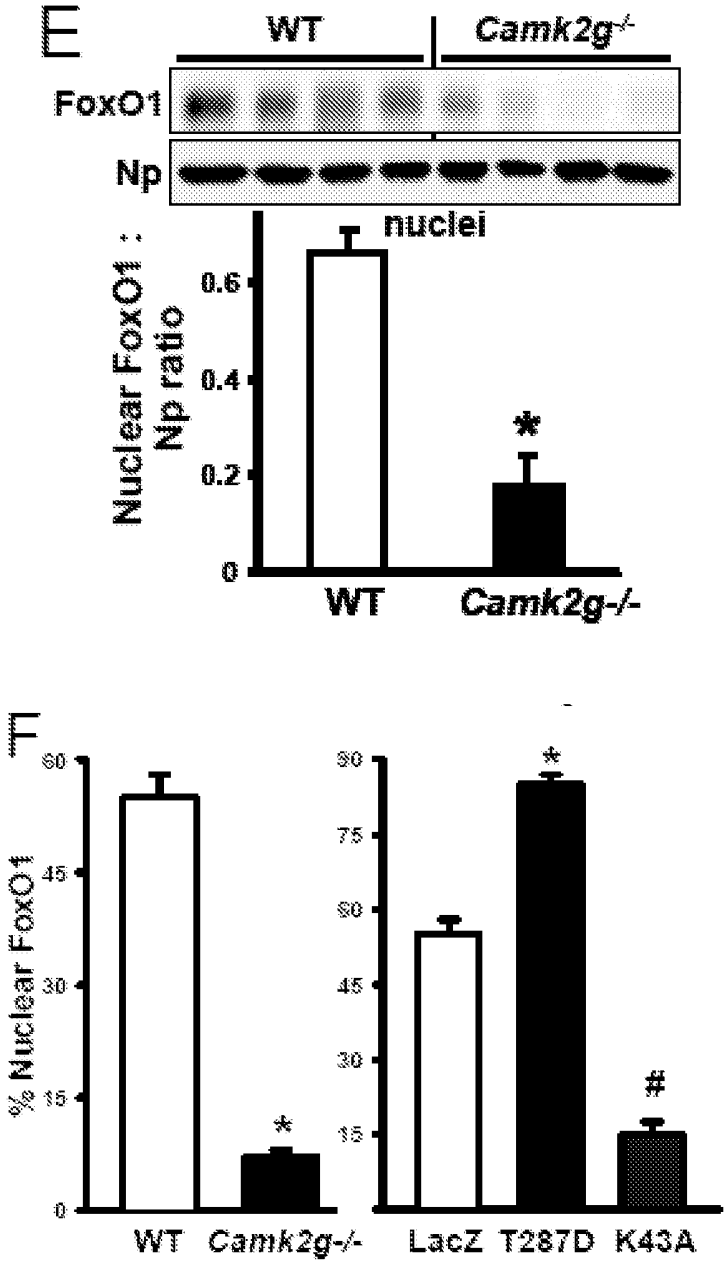


Figure 19E-19F

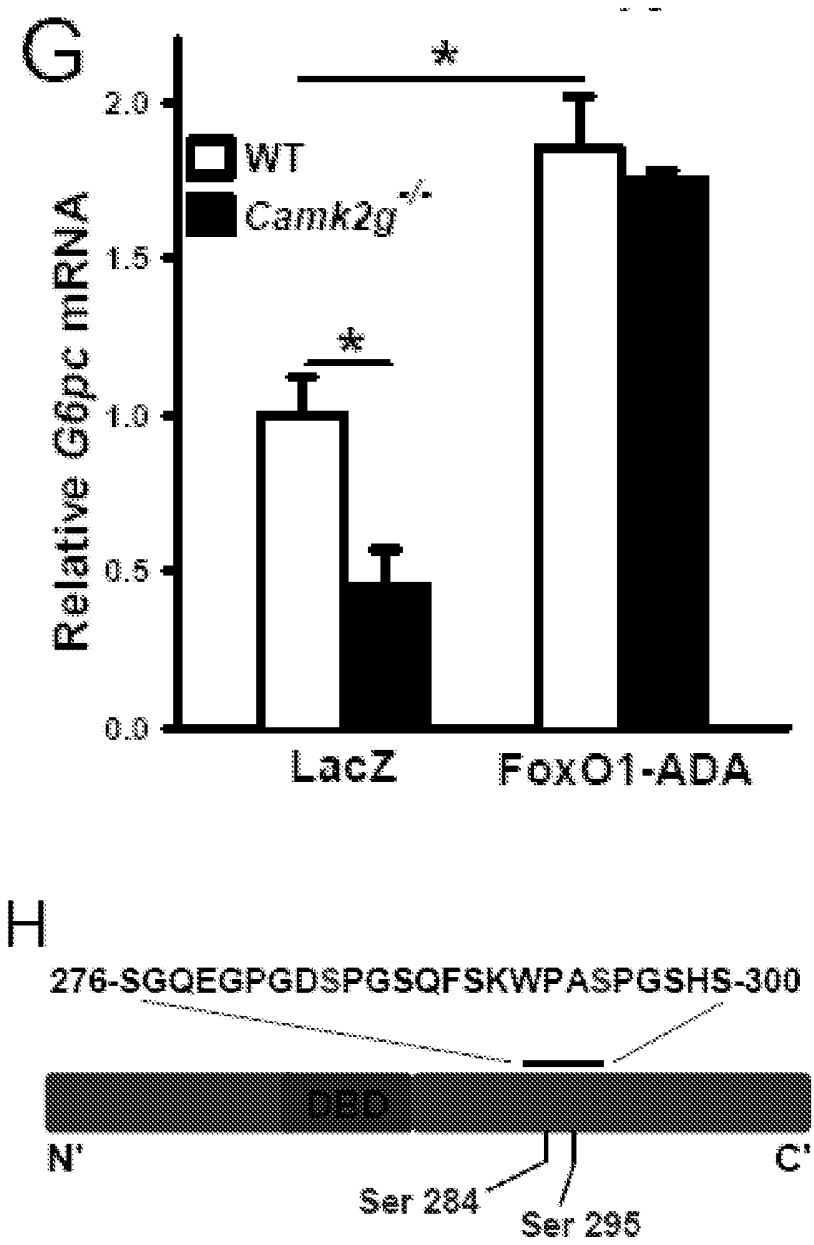
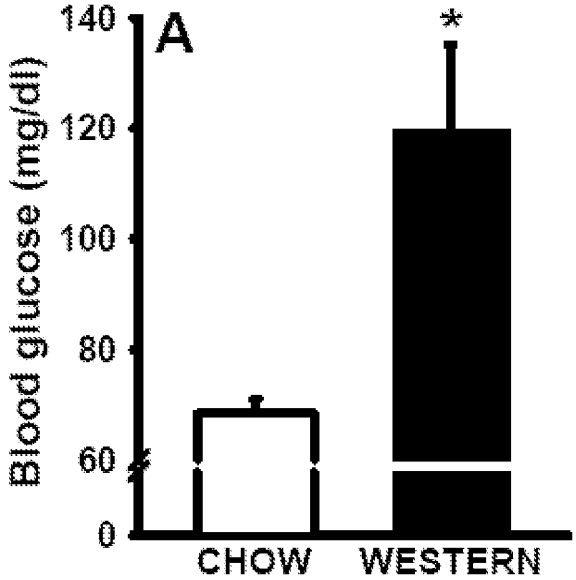


Figure 19G-19H



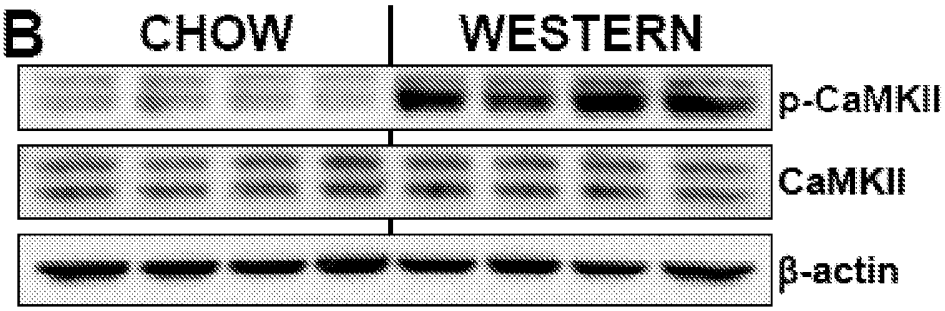


Figure 20A-20B

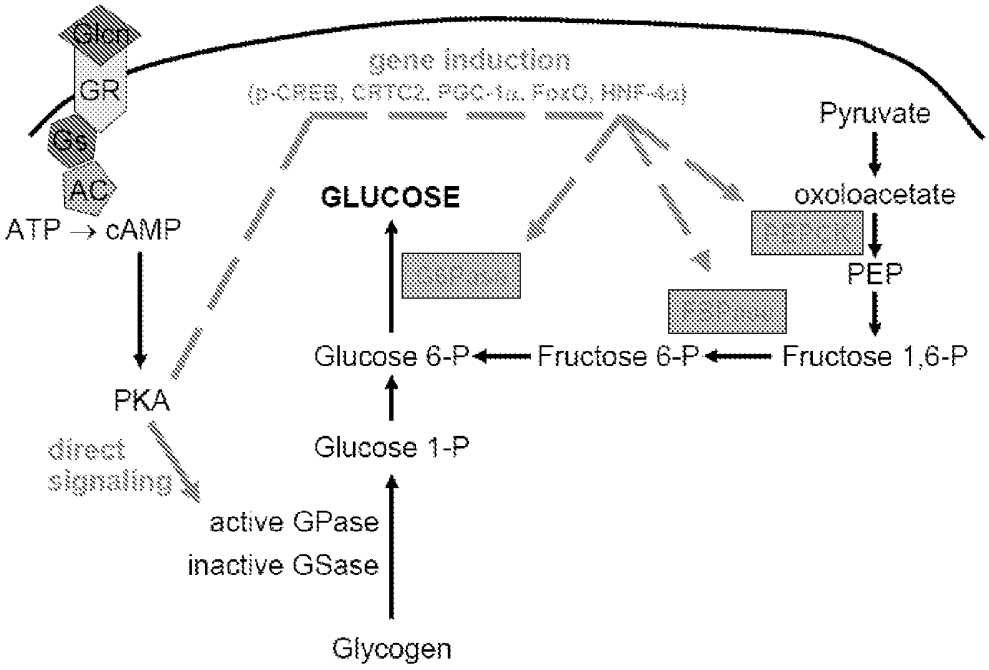

Glcn
GR
Gs
AC
ATP → cAMP
PKA
direct signaling
gene induction
(p-CREB, CRTC2, PGC-1α, FoxO, HNF-4α)
GLUCOSE
Pyruvate
oxoloacetate
PEP
Fructose 1,6-P
Fructose 6-P
Glucose 6-P
Glucose 1-P
active GPase
inactive GSase
Glycogen

Figure 21A

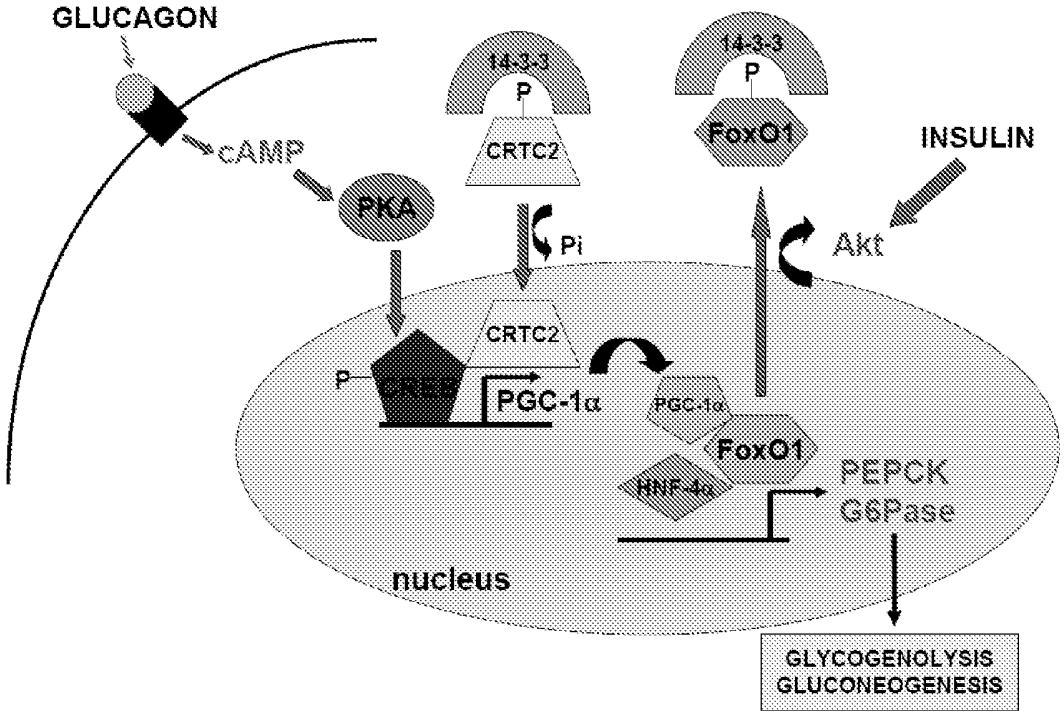

GLUCAGON
cAMP
PKA
14-3-3
P
CRTC2
Pi
14-3-3
P
FoxO1
INSULIN
Akt
CRTC2
P
CREB
PGC-1α
PGC-1α
FoxO1
HNF-4α
PEPCK
G6Pase
nucleus
GLYCOGENOLYSIS
GLUCONEOGENESIS

Figure 21B

- Fasting glucagon → HGP: glycogenolysis, then gluconeogenesis (GNG)
- Regulation: direct hormonal signaling, then transcriptional
- Insulin resistance/DM2: ↑ glucagon/insulin balance → ↑ HGP
- Calcium ion: glucagon → ↑Ca^{2+}_i and Ca-chelation reported to ↓ HGP, but mechanism completely unknown
- Obesity: ↑Ca^{2+}_i (↓ SERCA) → ER stress → insulin resistance

Figure 21C

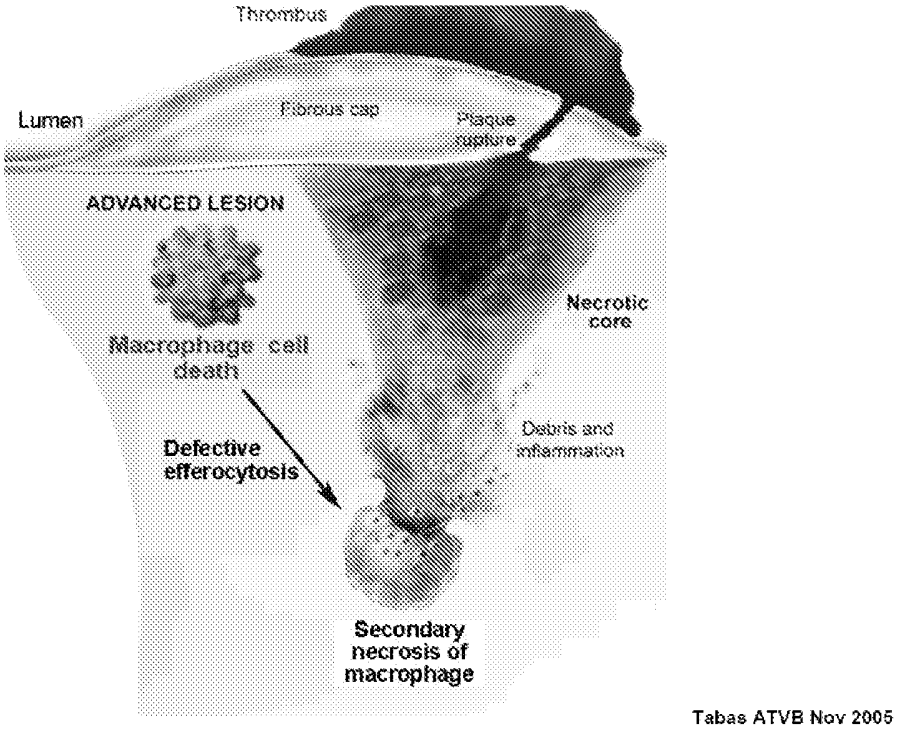

Thrombus
Lumen
Fibrous cap
Plaque rupture
ADVANCED LESION
Macrophage cell death
Defective efferocytosis
Necrotic core
Debris and inflammation
Secondary necrosis of macrophage
Tabas ATVB Nov 2005

Figure 21D

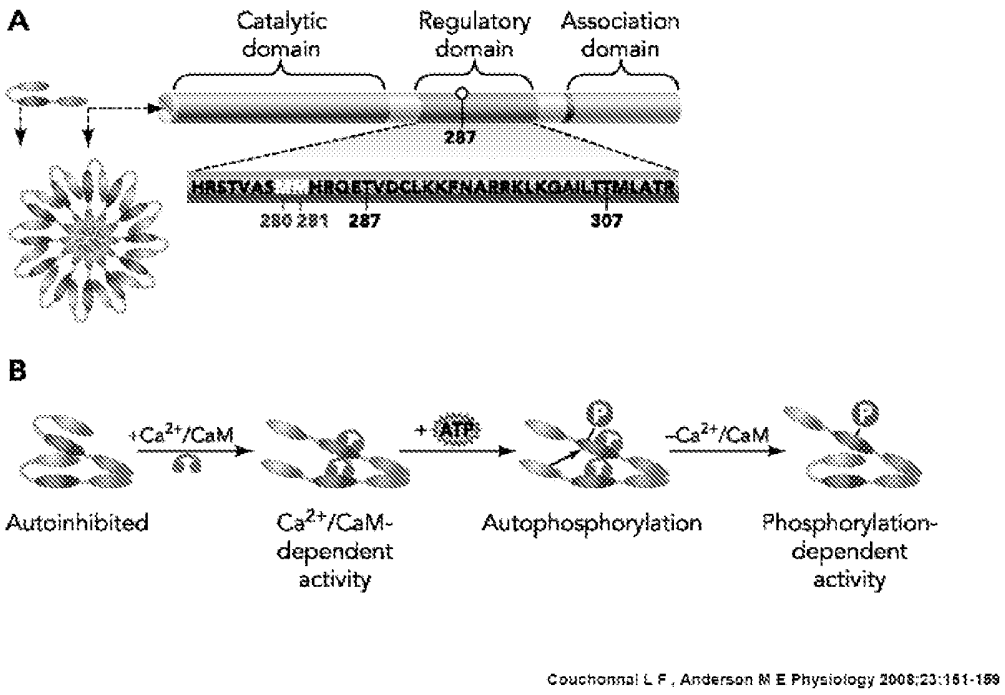
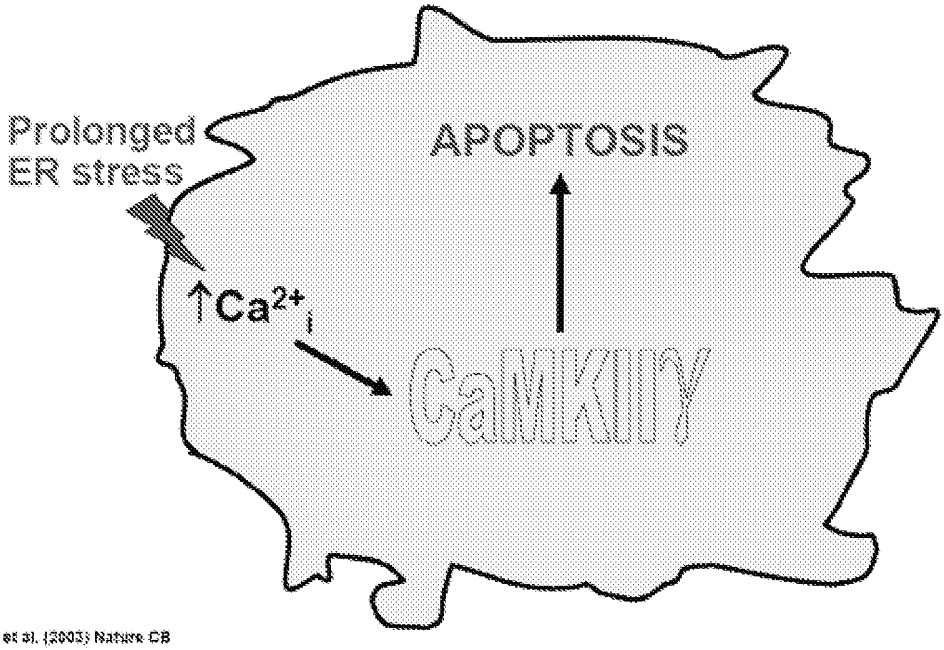

A
Catalytic domain
Regulatory domain
Association domain
287
HRSTVAS ... NRQETVDCLKKFNARRKLKGAILTTMLATR
280 281 287
307
B
+Ca2+/CaM
+ ATP
-Ca2+/CaM
Autoinhibited
Ca2+/CaM-dependent activity
Autophosphorylation
Phosphorylation-dependent activity
Couchonnal L F , Anderson M E Physiology 2008;23:151-159

Figure 21E



Feng et al. (2003) Nature CB
Feng et al. (2003) PNAS
Li et al. (2005) JBC
Selmon et al. (2005) J Cell Biol
Selmon et al. (2006) PNAS
Thorp et al. (2008) Cell Metab.
Li et al. (2009) J. Cell Biol.
Timmins et al. (2010) J. Clin. Invest.
Selmon et al. (2010) Cell Metab.
Li et al. (2010) J. Cell Biol.

Figure 21F

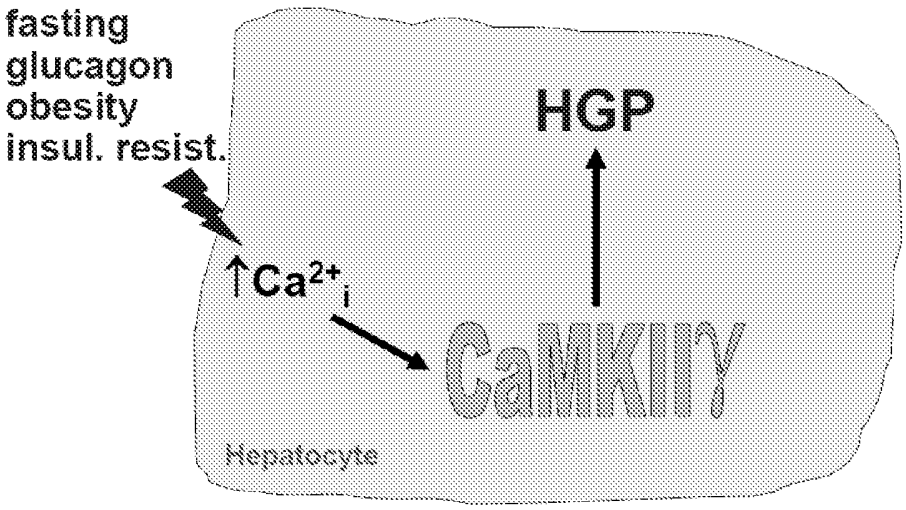

fasting
glucagon
obesity
insul. resist.
HGP
↑Ca2+i
CaMKIIγ
Hepatocyte

Figure 21G

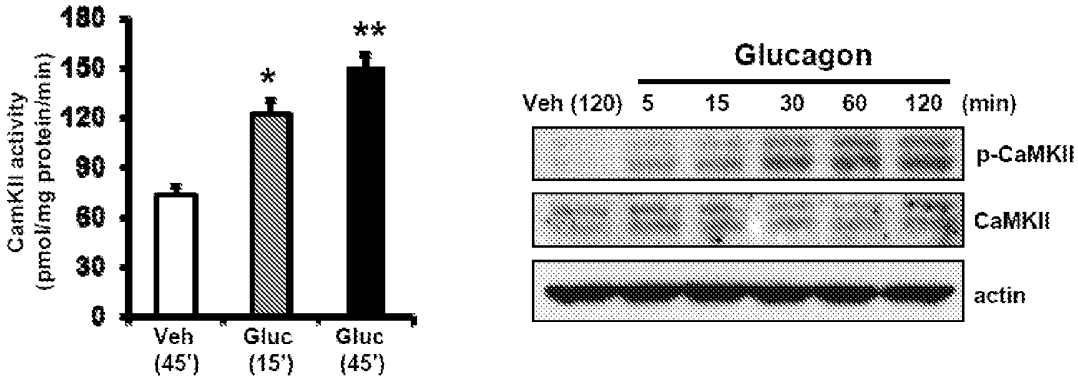

CamKII activity
(pmol/mg protein/min)
180
150
120
90
60
30
0
*
**
Veh
(45')
Gluc
(15')
Gluc
(45')
Glucagon
Veh (120)
5
15
30
60
120
(min)
p-CaMKII
CaMKII
actin

Figure 21H

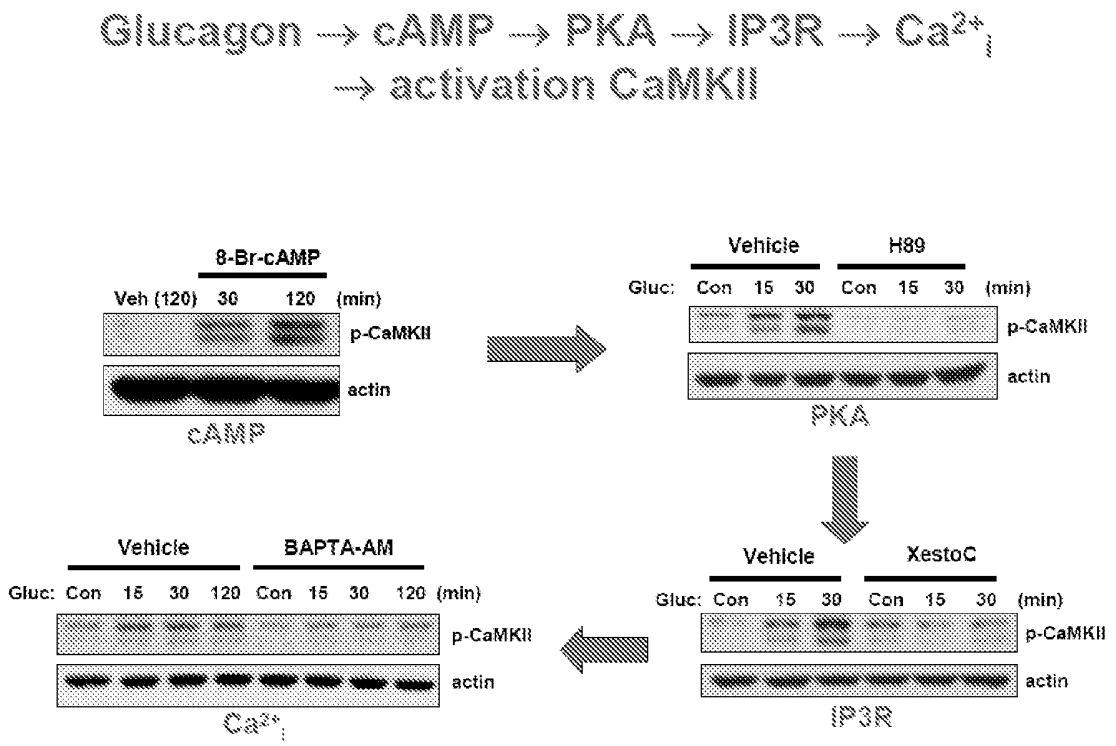
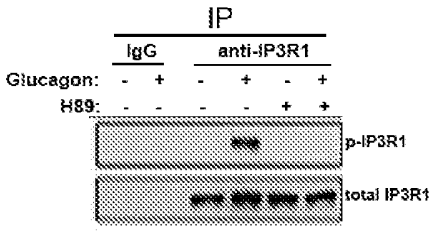
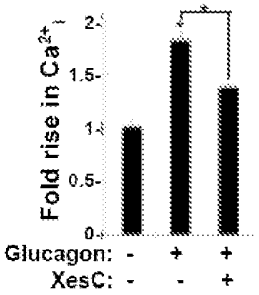

Glucagon → cAMP → PKA → IP3R → Ca2+i
→ activation CaMKII
8-Br-cAMP
Veh (120) 30 120 (min)
p-CaMKII
actin
cAMP
Vehicle
H89
Gluc: Con 15 30 Con 15 30 (min)
p-CaMKII
actin
PKA
Vehicle
XestoC
Gluc: Con 15 30 Con 15 30 (min)
p-CaMKII
actin
IP3R
Vehicle
BAPTA-AM
Gluc: Con 15 30 120 Con 15 30 120 (min)
p-CaMKII
actin
Ca2+i

Figure 21I

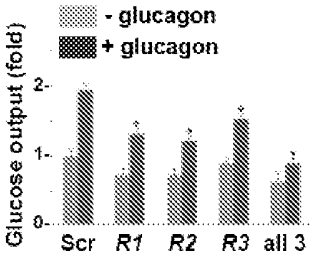
Glucagon → cAMP → PKA → IP3R → ↑Ca^{2+}_i



Glucagon → PKA → IP3R



Glucagon → IP3R → ↑Ca^{2+}_i



Ip3r shRNA complements XesC-HGP data

Figure 21J

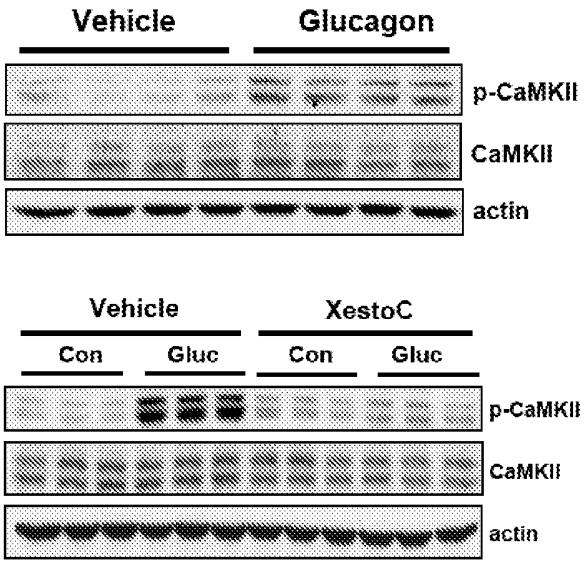

Vehicle
Glucagon
p-CaMKII
CaMKII
actin
Vehicle
XestoC
Con
Gluc
Con
Gluc
p-CaMKII
CaMKII
actin

Figure 21K

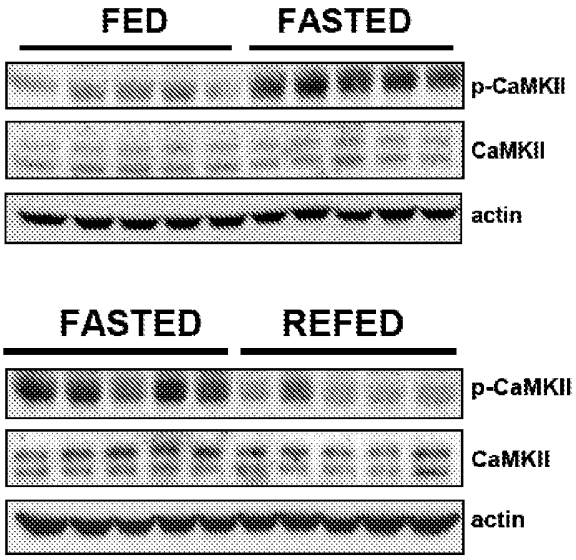

FED
FASTED
p-CaMKII
CaMKII
actin
FASTED
REFED
p-CaMKII
CaMKII
actin

Figure 21L

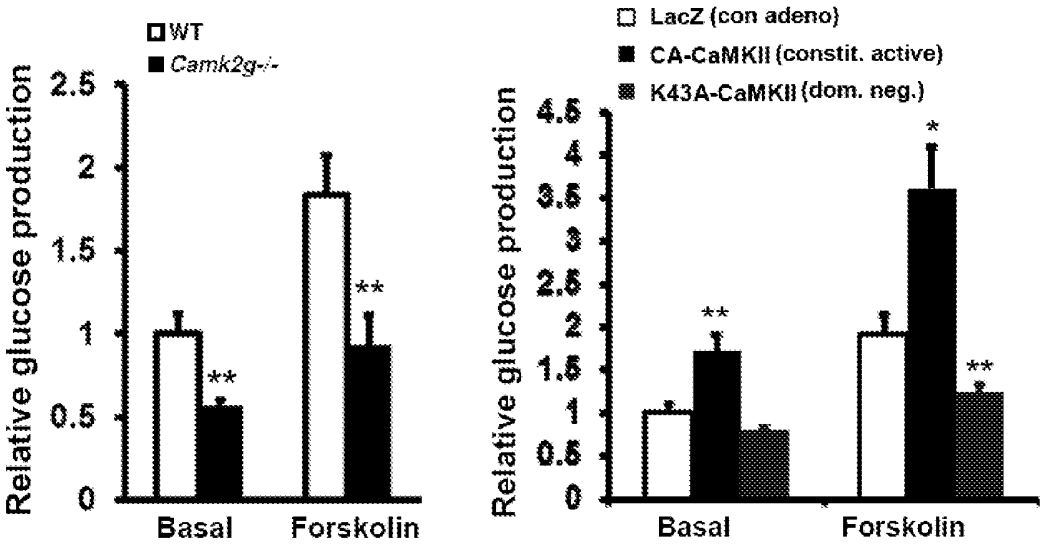

WT
Camk2g-/-
Relative glucose production
2.5
2
1.5
1
0.5
0
**
**
Basal
Forskolin
LacZ (con adeno)
CA-CaMKII (constit. active)
K43A-CaMKII (dom. neg.)
Relative glucose production
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
*
**
Basal
Forskolin

Figure 21M

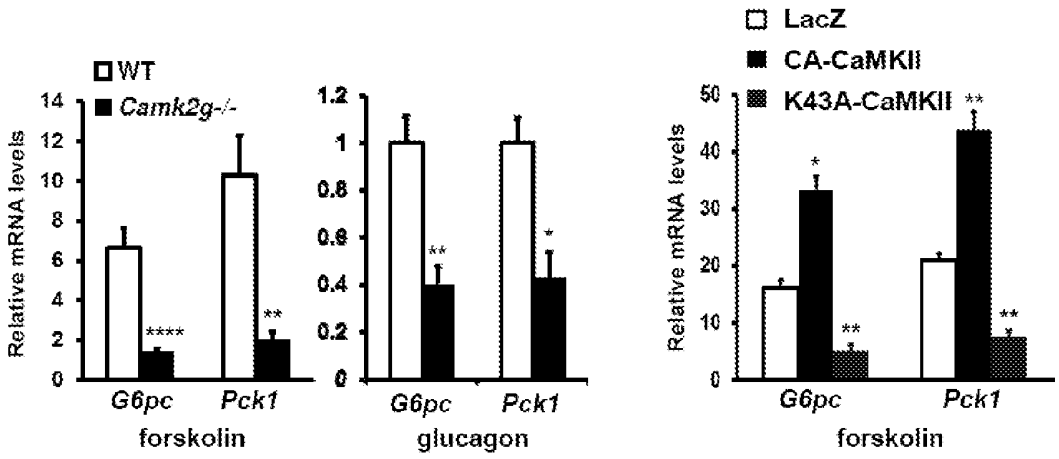

WT
Camk2g-/-
Relative mRNA levels
14
12
10
8
6
4
2
0
****
**
G6pc
Pck1
forskolin
1.2
1
0.8
0.6
0.4
0.2
0
**
*
G6pc
Pck1
glucagon
LacZ
CA-CaMKII
K43A-CaMKII
Relative mRNA levels
50
40
30
20
10
0
*
**
**
**
G6pc
Pck1
forskolin

Figure 21N

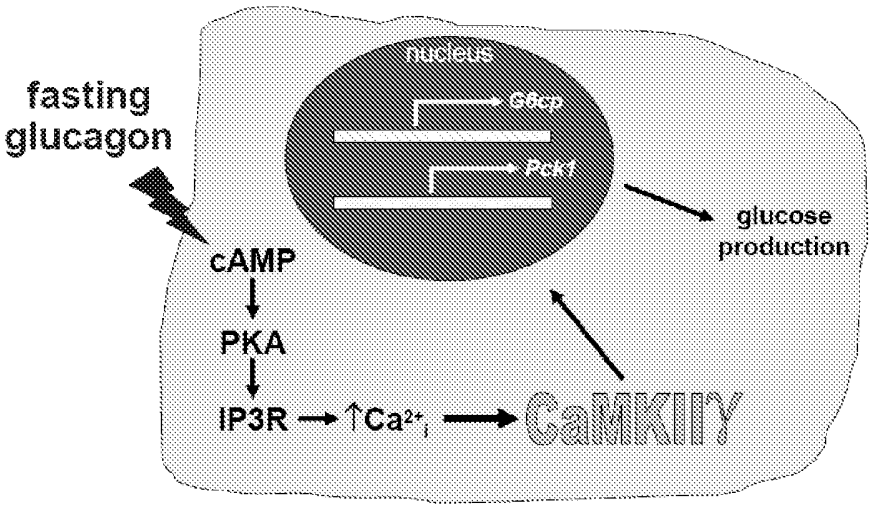

fasting
glucagon
nucleus
G6cp
Pck1
glucose
production
cAMP
PKA
IP3R
↑Ca²⁺i
CaMKIIγ

Figure 21O

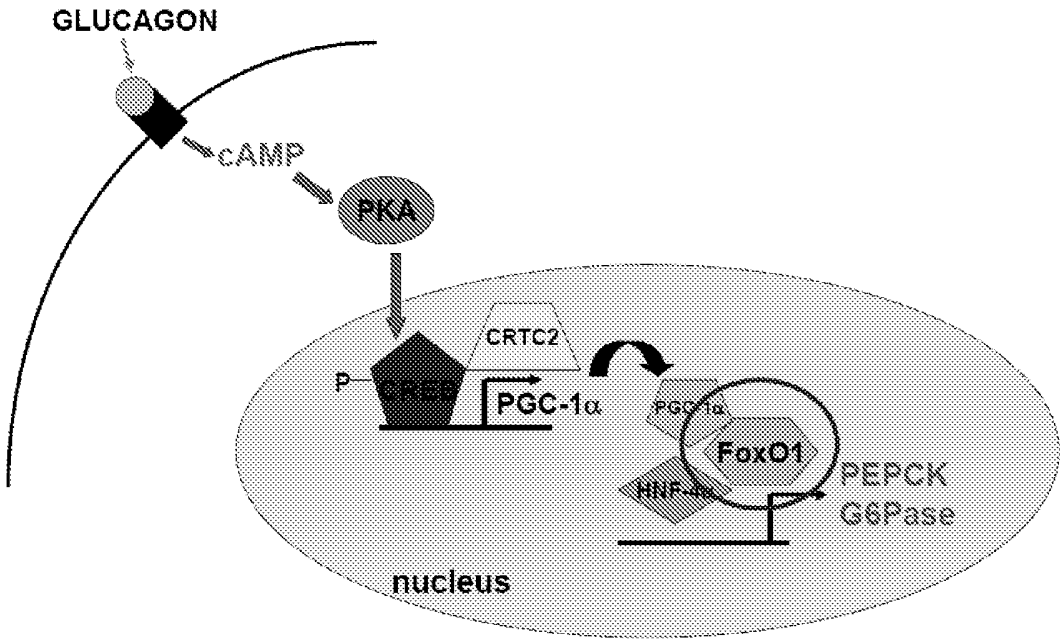
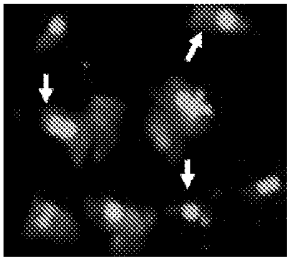

GLUCAGON
cAMP
PKA
CRTC2
P
CREB
PGC-1α
PGC-1α
FoxO1
HNF-4α
PEPCK
G6Pase
nucleus

Figure 21P

GFP-FoxO1-transduced 1° HCs



no insulin → no Akt → no phosphorylation of FoxO1

↓ ? CaMKII

nuclear localization of FoxO1

Figure 21Q

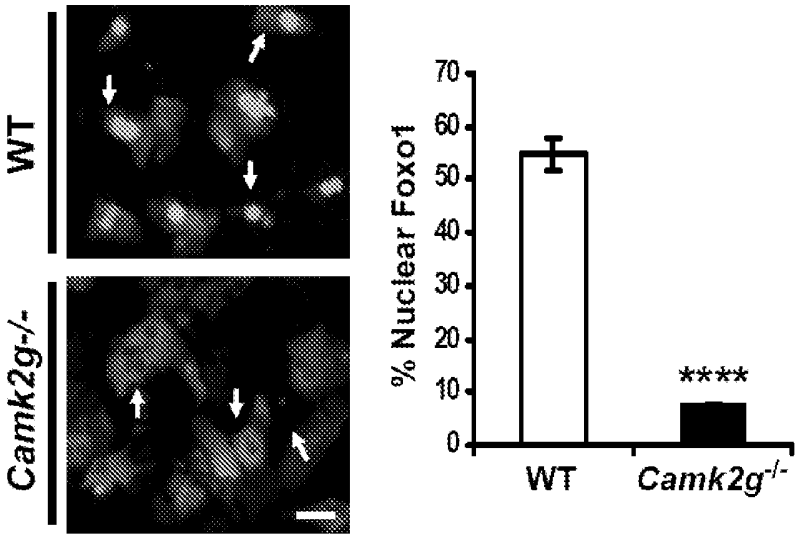


Figure 21R

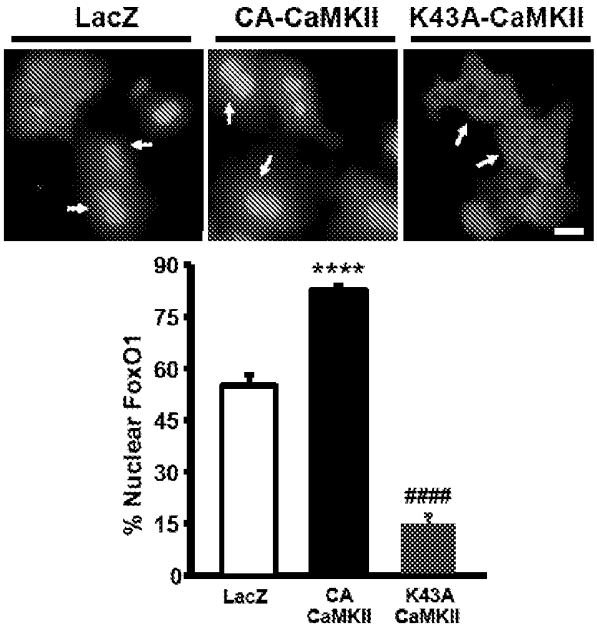

LacZ
CA-CaMKII
K43A-CaMKII
% Nuclear FoxO1
90
75
60
45
30
15
0
****
####
*
LacZ
CA CaMKII
K43A CaMKII

Figure 21S

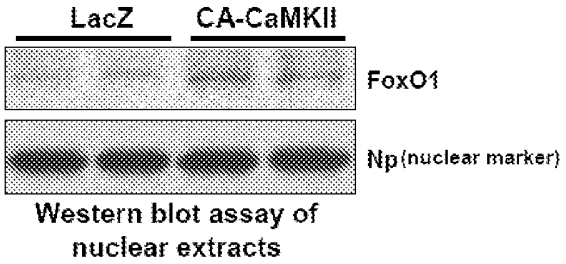

LacZ
CA-CaMKII
FoxO1
Np (nuclear marker)
Western blot assay of
nuclear extracts

Figure 21T

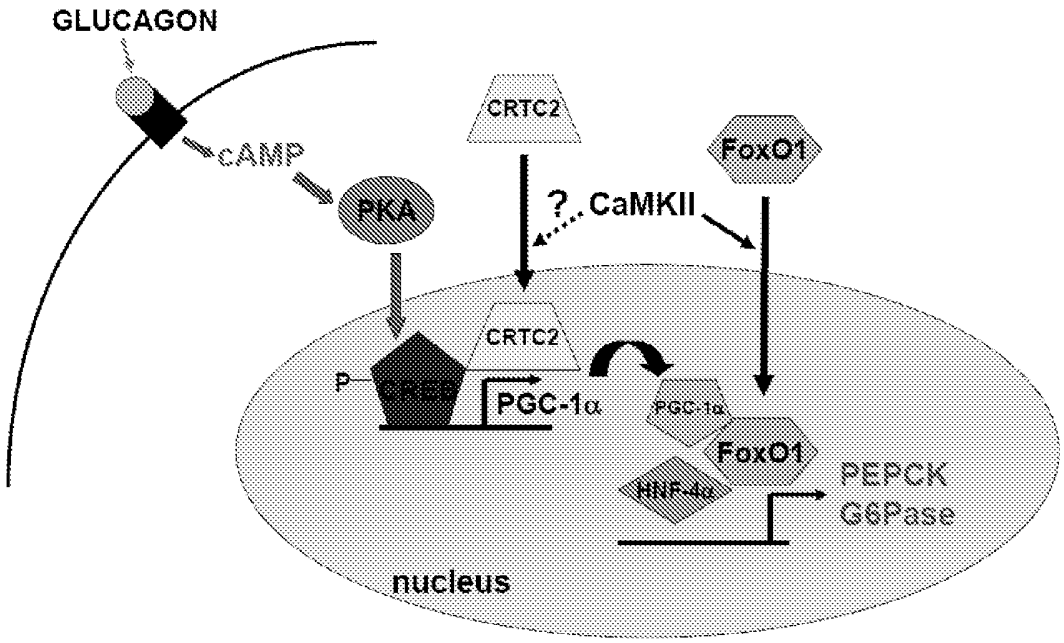
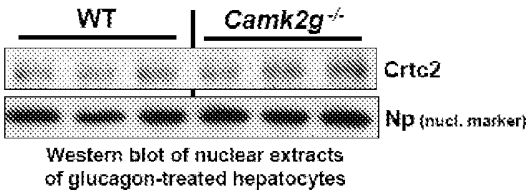

GLUCAGON
cAMP
PKA
CRTC2
? CaMKII
FoxO1
CRTC2
P
CREB
PGC-1α
PGC-1α
FoxO1
HNF-4α
PEPCK
G6Pase
nucleus

Figure 21U


WT
Camk2g-/-
Crtc2
Np (nucl. marker)
Western blot of nuclear extracts
of glucagon-treated hepatocytes

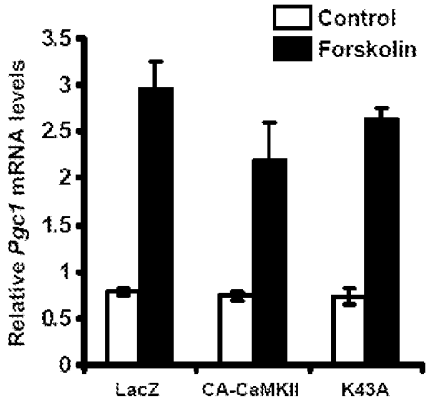

Control
Forskolin
Relative Pgc1 mRNA levels
3.5
3
2.5
2
1.5
1
0.5
0
LacZ
CA-CaMKII
K43A

Figure 21V

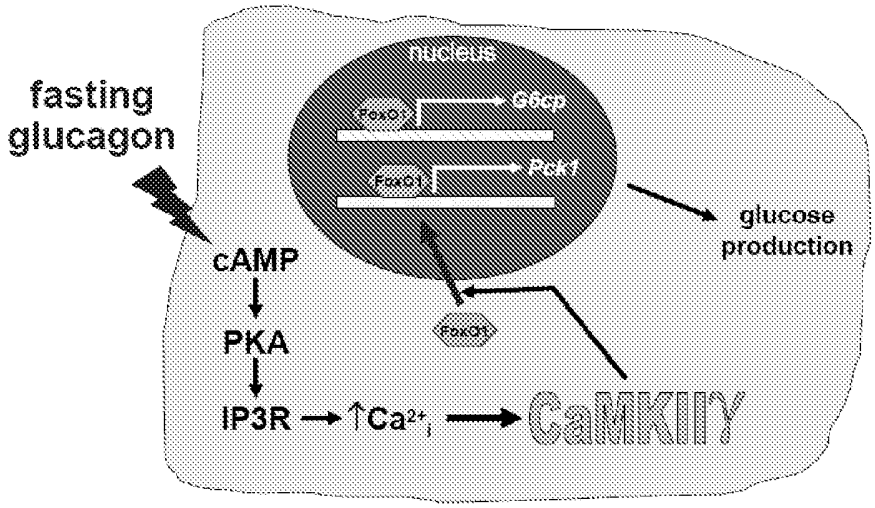

fasting
glucagon
nucleus
FoxO1
G6cp
FoxO1
Pck1
glucose
production
cAMP
PKA
IP3R
↑Ca2+i
CaMKIIγ
FoxO1

Figure 21W

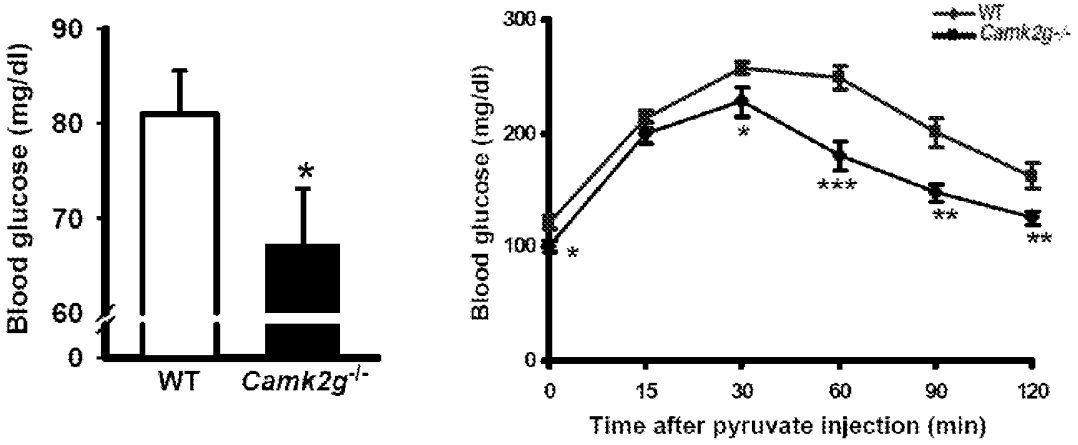

Blood glucose (mg/dl)
90
80
70
60
0
*
WT
Camk2g-/-
Blood glucose (mg/dl)
300
200
100
0
WT
Camk2g-/-
*
*
***
**
**
0
15
30
60
90
120
Time after pyruvate injection (min)

Figure 21X

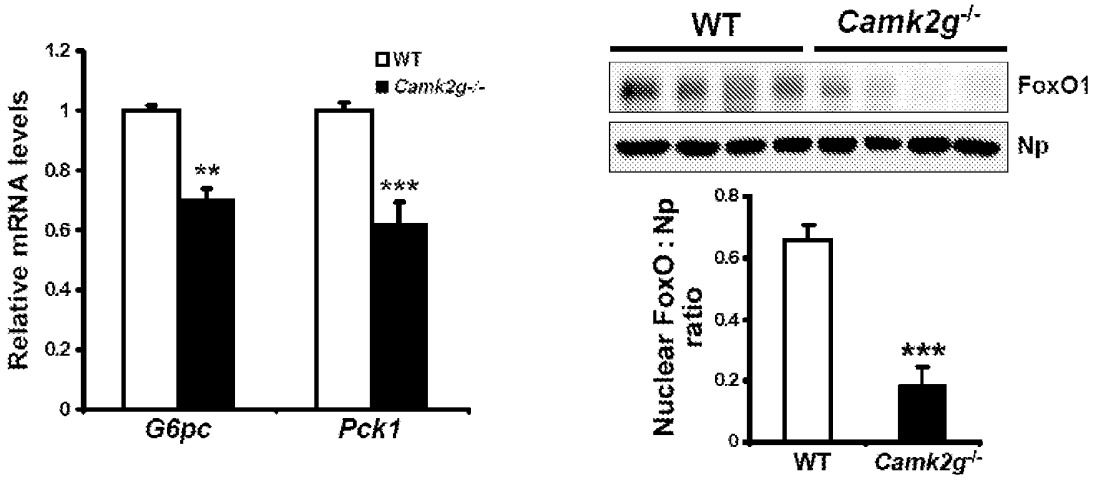

Relative mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
WT
Camk2g-/-
**
***
G6pc
Pck1
WT
Camk2g-/-
FoxO1
Np
Nuclear FoxO : Np ratio
0.8
0.6
0.4
0.2
0
***
WT
Camk2g-/-

Figure 21Y

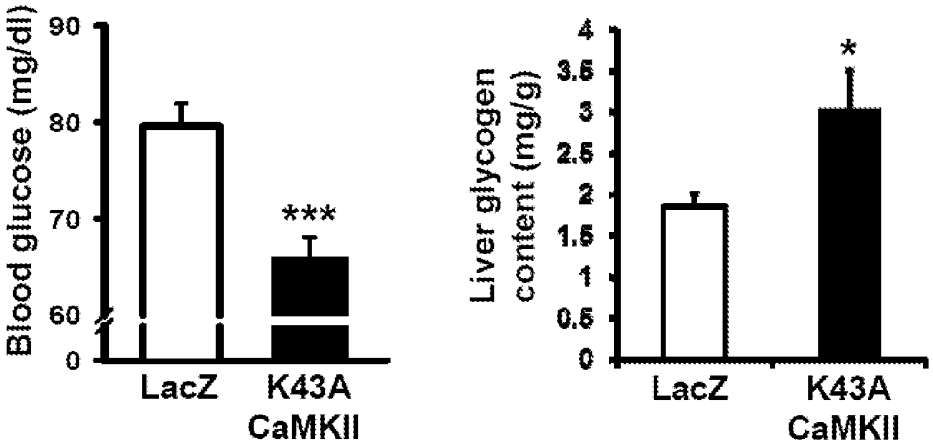

Blood glucose (mg/dl)
90
80
70
60
0
***
LacZ
K43A CaMKII
Liver glycogen content (mg/g)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
LacZ
K43A CaMKII

Figure 21Z

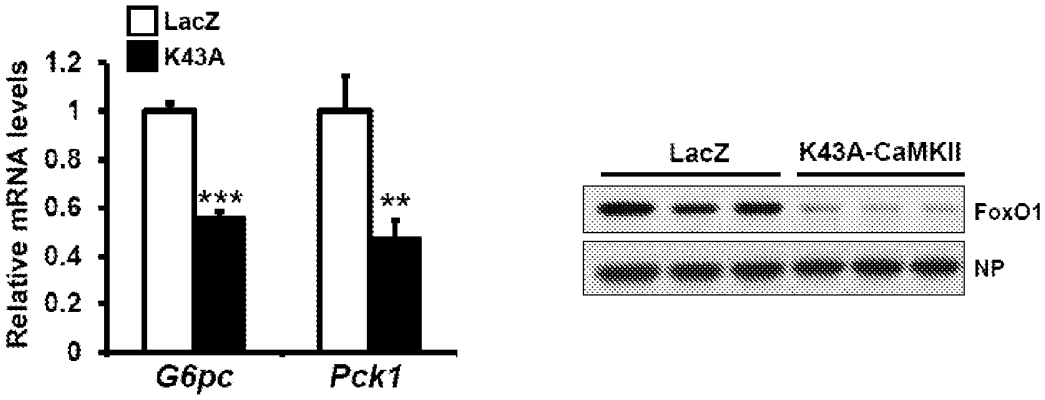

LacZ
K43A
Relative mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
***
**
G6pc
Pck1
LacZ
K43A-CaMKII
FoxO1
NP

Figure 21AA

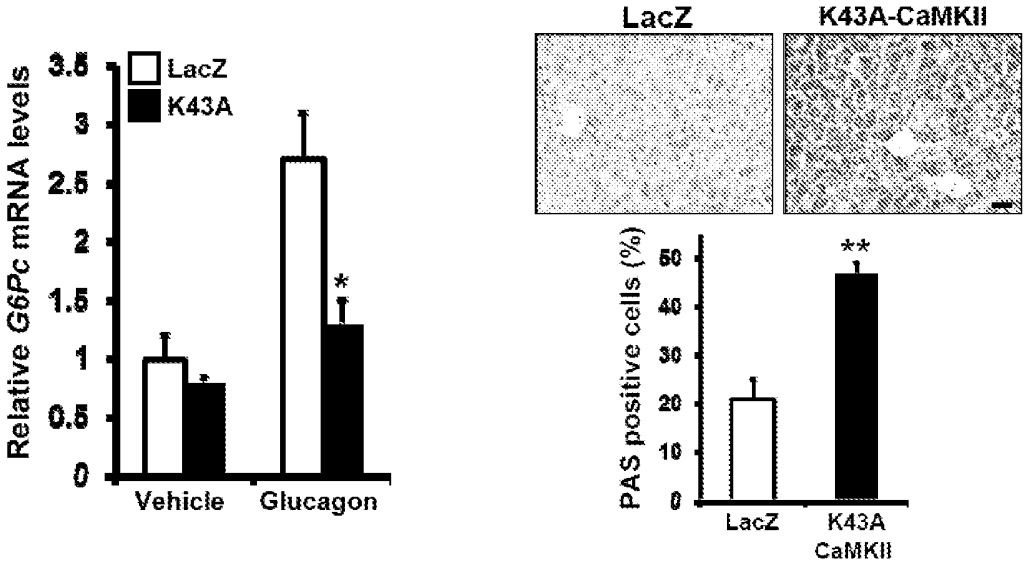
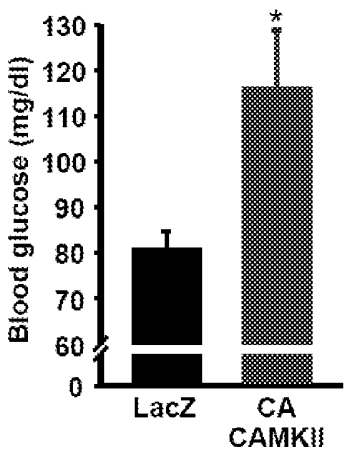

LacZ
K43A
Relative G6Pc mRNA levels
3.5
3
2.5
2
1.5
1
0.5
0
*
Vehicle
Glucagon
LacZ
K43A-CaMKII
PAS positive cells (%)
50
40
30
20
10
0
**
LacZ
K43A
CaMKII

Figure 21AB


Blood glucose (mg/dl)
130
120
110
100
90
80
70
60
0
*
LacZ
CA CAMKII

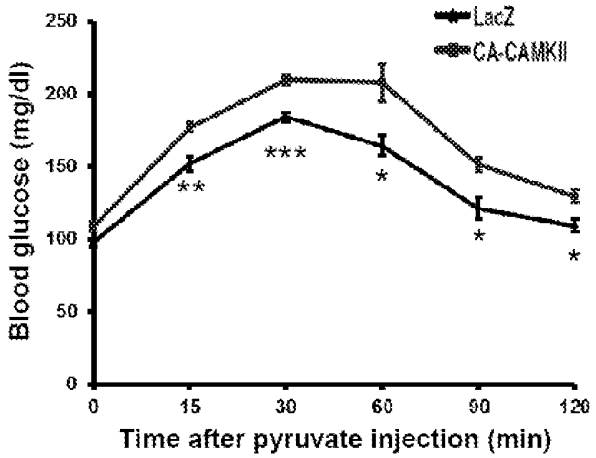

LacZ
CA-CAMKII
Blood glucose (mg/dl)
250
200
150
100
50
0
**
***
*
*
*
0
15
30
60
90
120
Time after pyruvate injection (min)

Figure 21AC

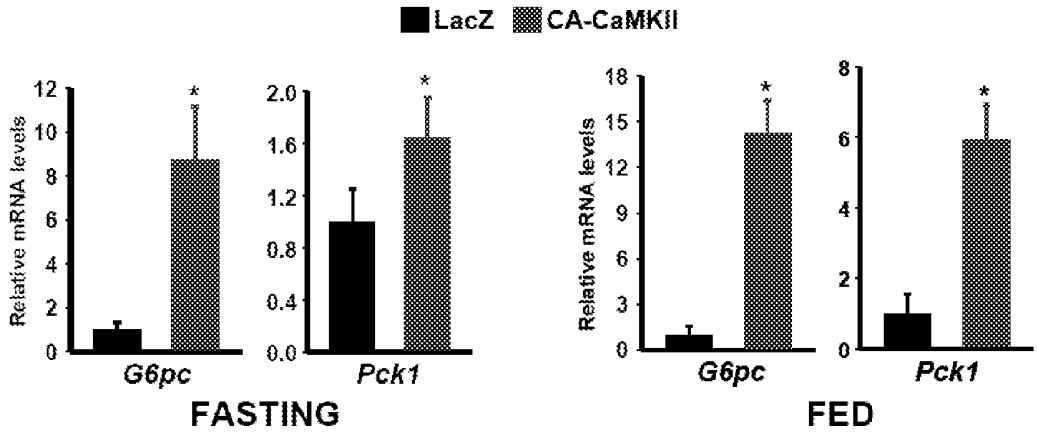

LacZ
CA-CaMKII
Relative mRNA levels
12
10
8
6
4
2
0
*
G6pc
2.0
1.6
1.2
0.8
0.4
0.0
*
Pck1
FASTING
Relative mRNA levels
18
15
12
9
6
3
0
*
G6pc
8
6
4
2
0
*
Pck1
FED

Figure 21AD

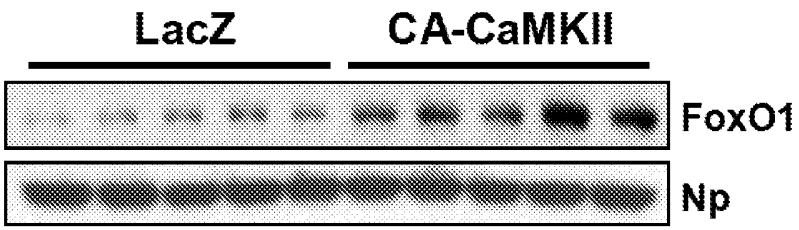


Figure 21AE

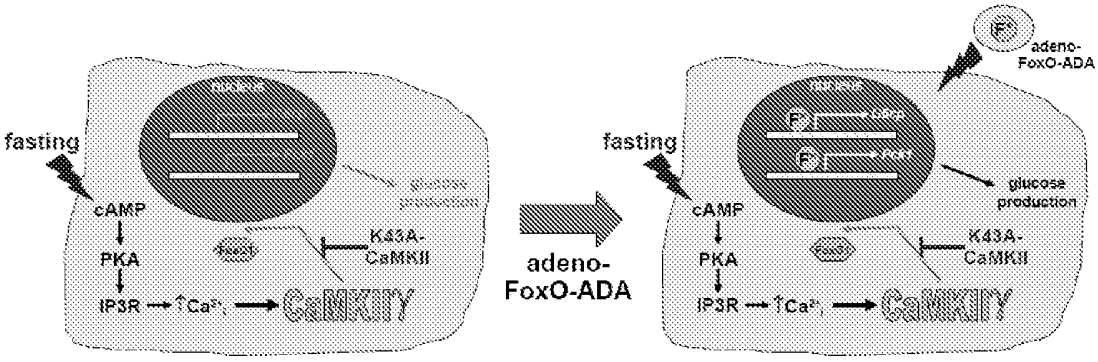

fasting
cAMP
PKA
IP3R
K43A-
CaMKII
adeno-
FoxO-ADA
fasting
adeno-
FoxO-ADA
cAMP
PKA
IP3R
K43A-
CaMKII
glucose
production

Figure 21AF

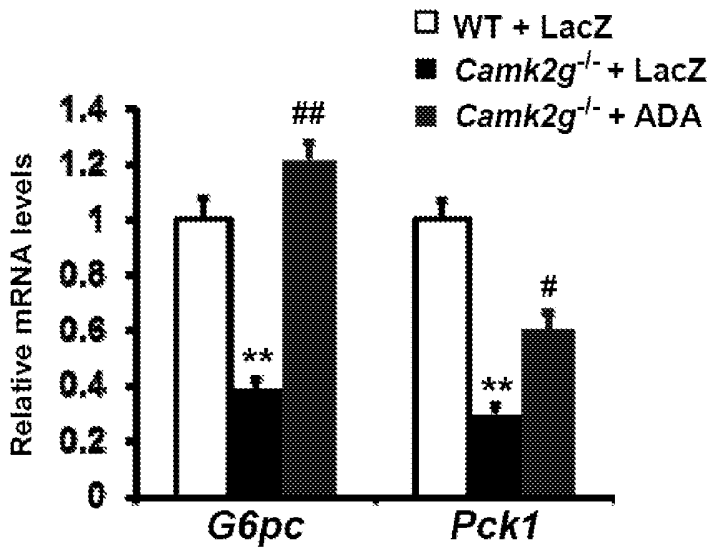

WT + LacZ
Camk2g-/- + LacZ
Camk2g-/- + ADA
Relative mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
##
**
#
**
G6pc
Pck1

Figure 21AG

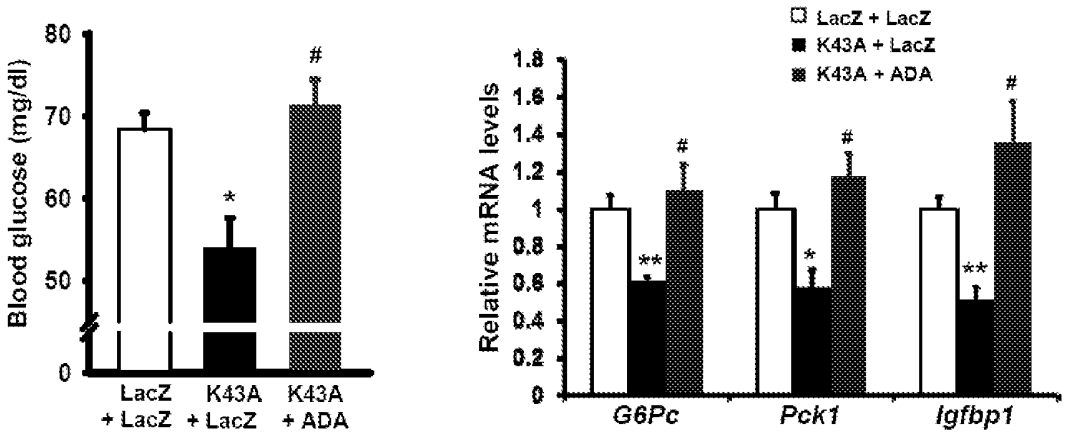

Blood glucose (mg/dl)
80
70
60
50
0
LacZ + LacZ
K43A + LacZ
K43A + ADA
*
#
LacZ + LacZ
K43A + LacZ
K43A + ADA
Relative mRNA levels
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
*
**
#
#
#
G6Pc
Pck1
Igfbp1

Figure 21AH

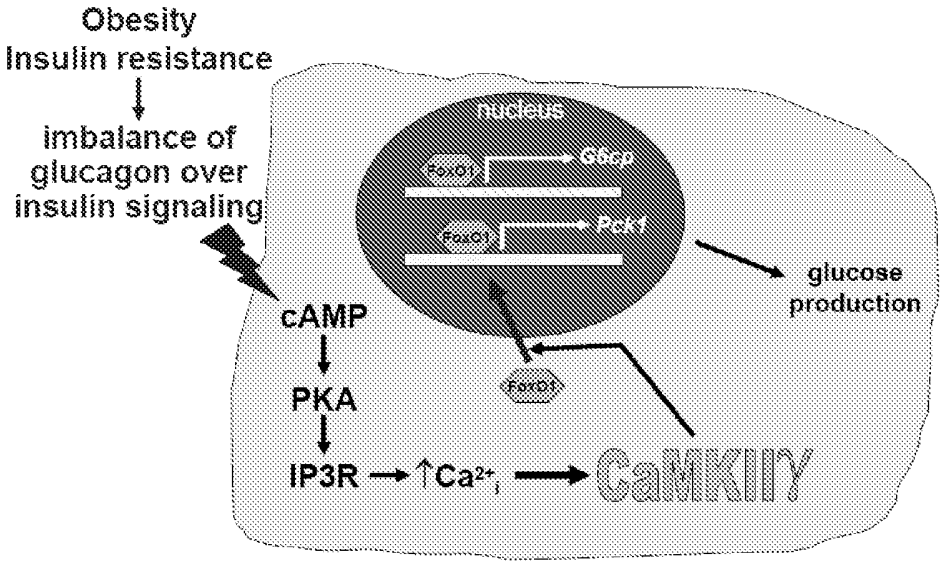

Obesity
Insulin resistance
imbalance of
glucagon over
insulin signaling
nucleus
FoxO1
G6Pase
FoxO1
PEPCK
glucose
production
cAMP
PKA
FoxO1
IP3R
↑Ca2+i
CaMKIIγ

Figure 21AI

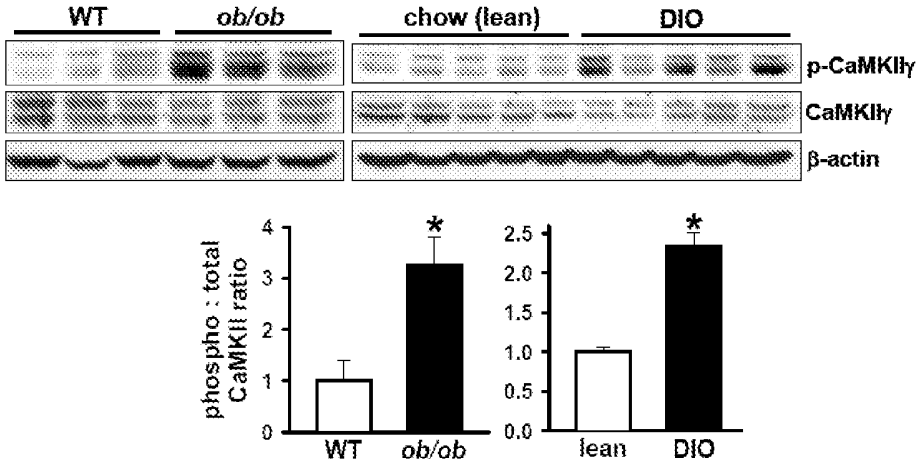

WT
ob/ob
chow (lean)
DIO
p-CaMKIIγ
CaMKIIγ
β-actin
phospho : total CaMKII ratio
4
3
2
1
0
*
WT
ob/ob
2.5
2.0
1.5
1.0
0.5
0.0
*
lean
DIO

Figure 21AJ

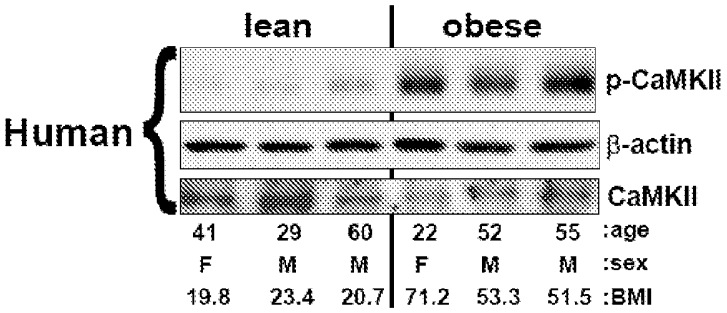

lean
obese
Human
p-CaMKII
β-actin
CaMKII
41 29 60 22 52 55 :age
F M M F M M :sex
19.8 23.4 20.7 71.2 53.3 51.5 :BMI

Figure 21AK

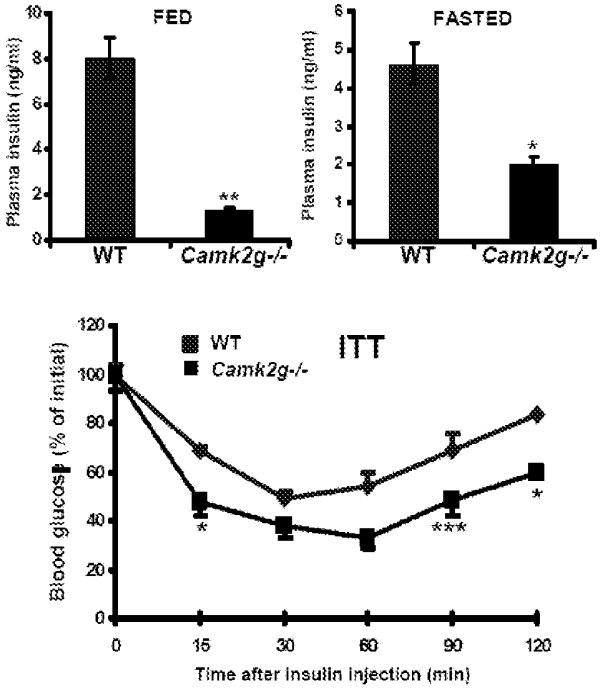

FED
Plasma insulin (ng/ml)
10
8
6
4
2
0
**
WT
Camk2g-/-
FASTED
Plasma insulin (ng/ml)
6
5
4
3
2
1
0
*
WT
Camk2g-/-
ITT
WT
Camk2g-/-
Blood glucose (% of initial)
120
100
80
60
40
20
0
*
***
*
0
15
30
60
90
120
Time after insulin injection (min)

Figure 21AL

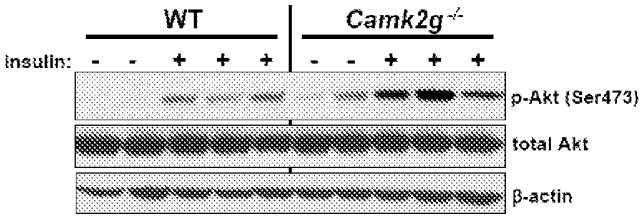


Figure 21AM

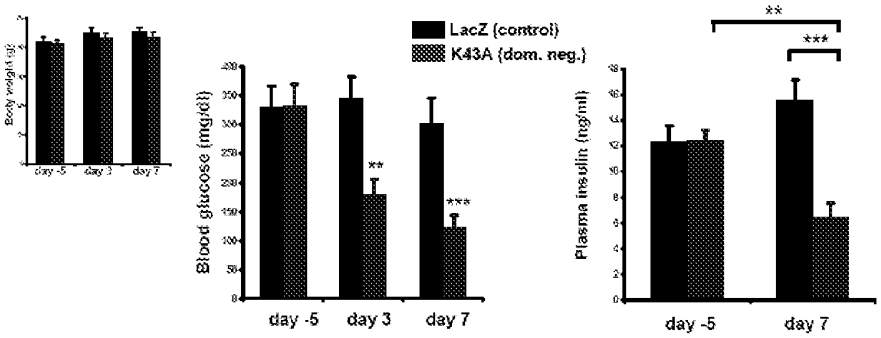

LacZ (control)
K43A (dom. neg.)
Blood glucose (mg/dl)
day -5
day 3
day 7
**
***
Plasma insulin (ng/ml)
day -5
day 7
**
***

Figure 21AN

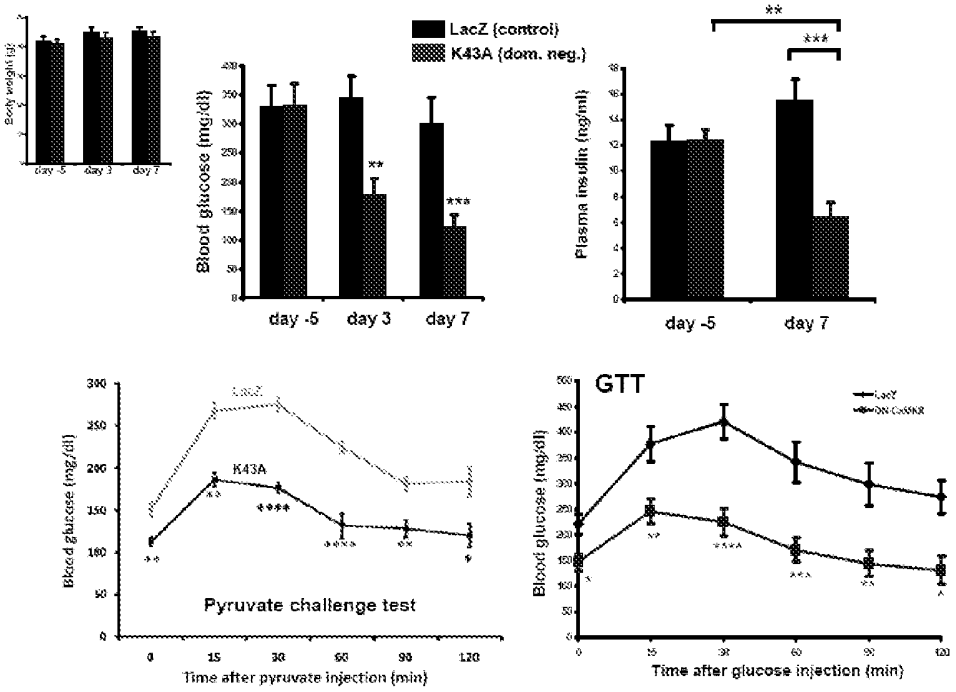

LacZ (control)
K43A (dom. neg.)
Blood glucose (mg/dl)
day -5
day 3
day 7
Plasma insulin (ng/ml)
Pyruvate challenge test
Time after pyruvate injection (min)
GTT
Time after glucose injection (min)

Figure 21AO

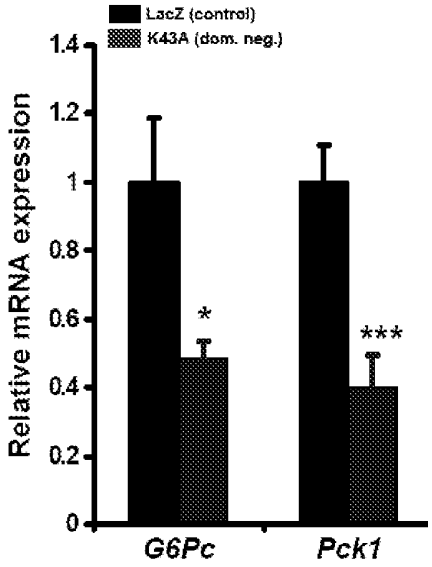

LacZ (control)
K43A (dom. neg.)
Relative mRNA expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
***
G6Pc
Pck1

Figure 21AP

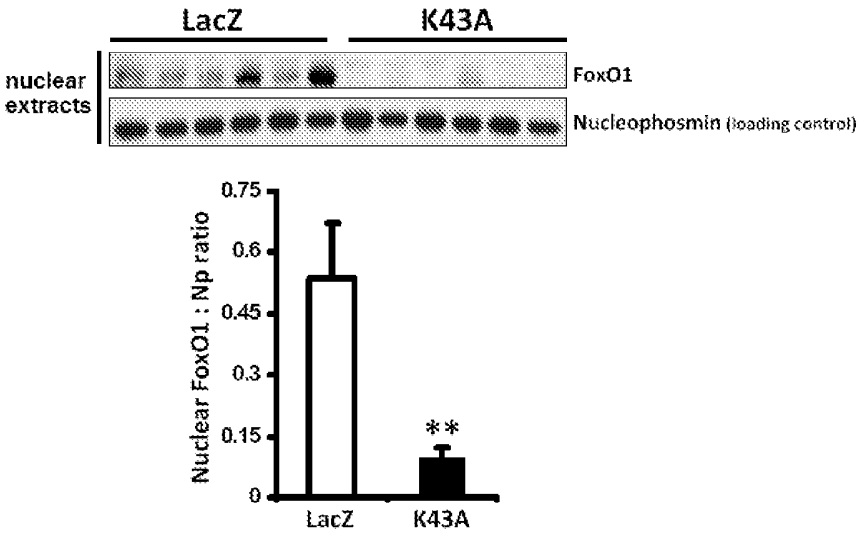

LacZ
K43A
nuclear extracts
FoxO1
Nucleophosmin (loading control)
Nuclear FoxO1 : Np ratio
0.75
0.6
0.45
0.3
0.15
0
**
LacZ
K43A

Figure 21AQ

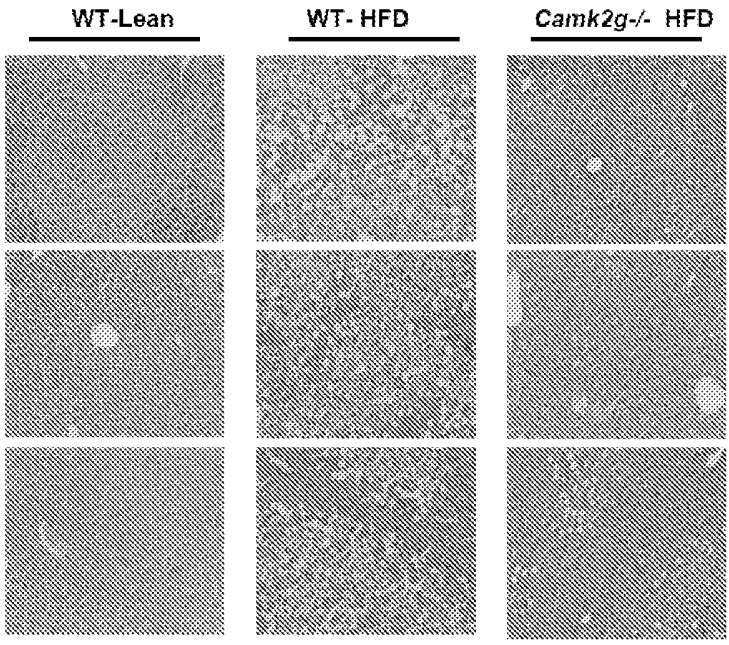


Figure 21AR

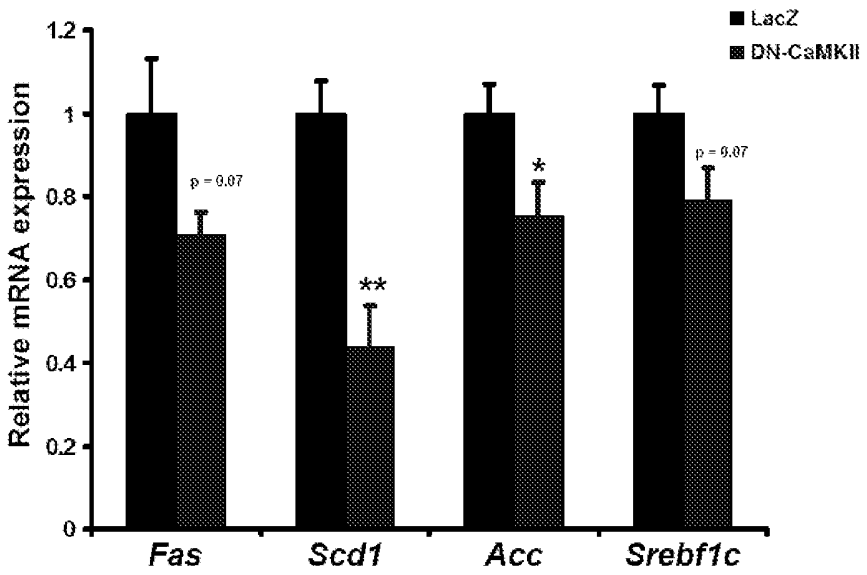

LacZ
DN-CaMKII
Relative mRNA expression
1.2
1
0.8
0.6
0.4
0.2
0
p = 0.07
**
*
p = 0.07
Fas
Scd1
Acc
Srebf1c

Figure 21AS

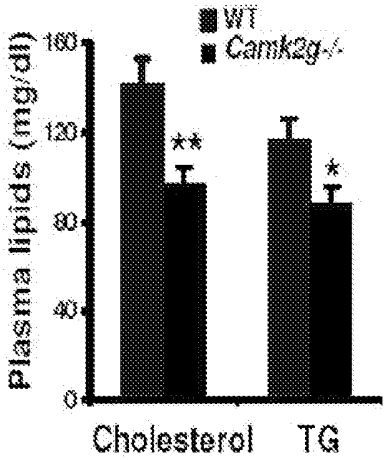


Figure 21AT

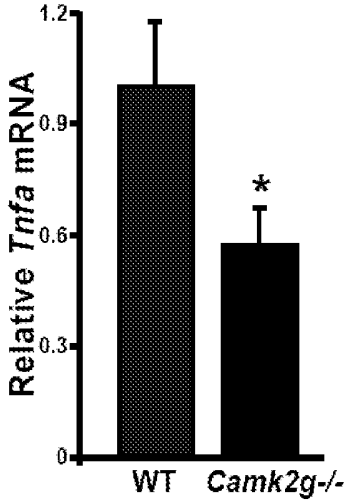


Figure 21AU

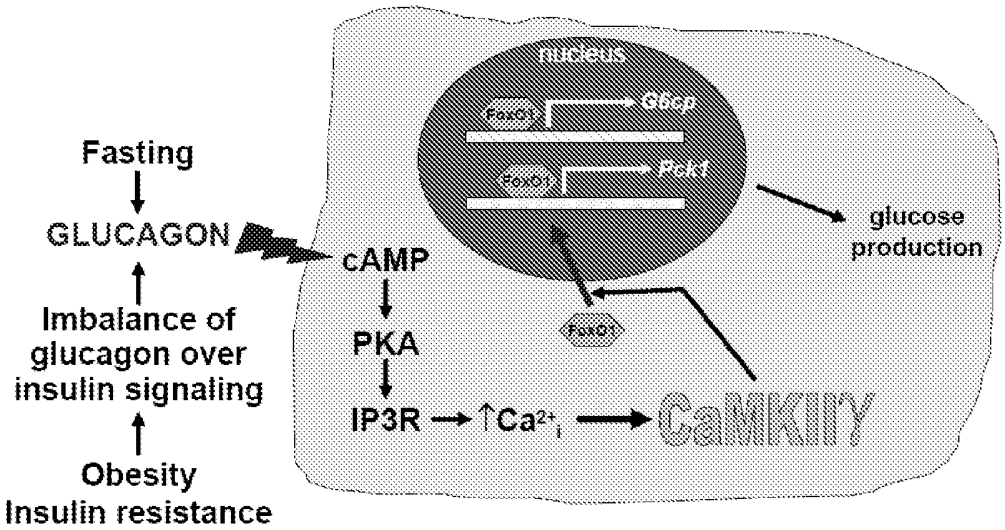

Fasting
GLUCAGON
Imbalance of glucagon over insulin signaling
Obesity
Insulin resistance
cAMP
PKA
IP3R
↑Ca2+i
CaMKIIγ
nucleus
FoxO1
G6cp
FoxO1
Pck1
FoxO1
glucose production

Figure 21AV

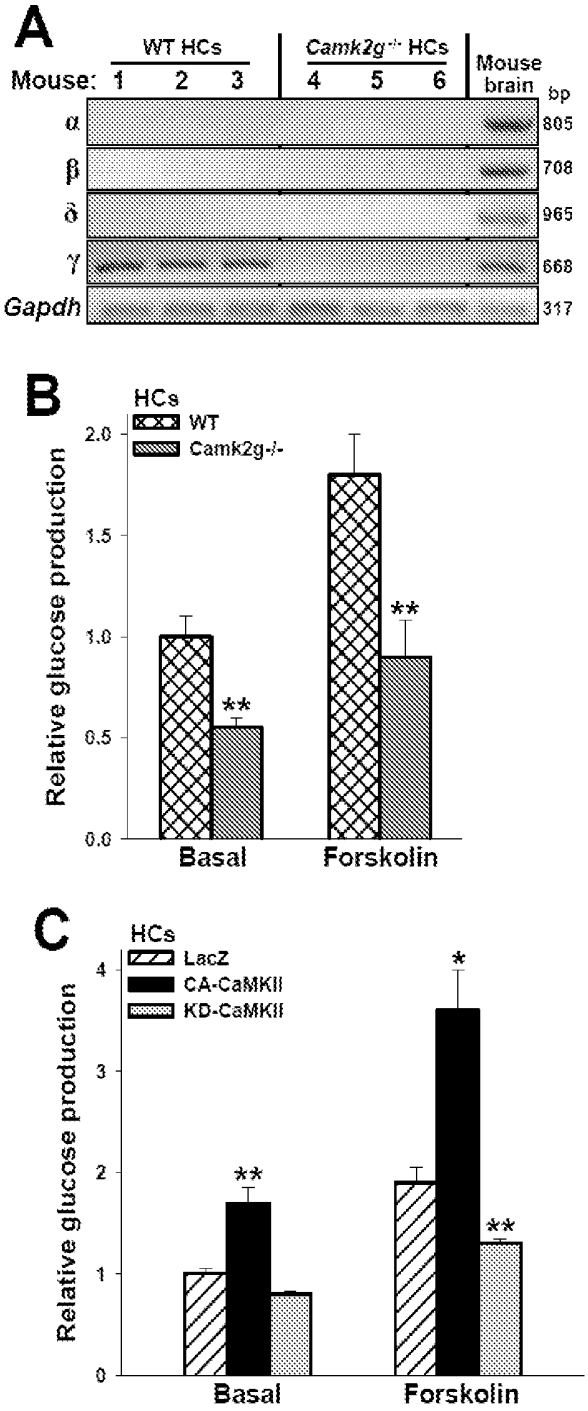
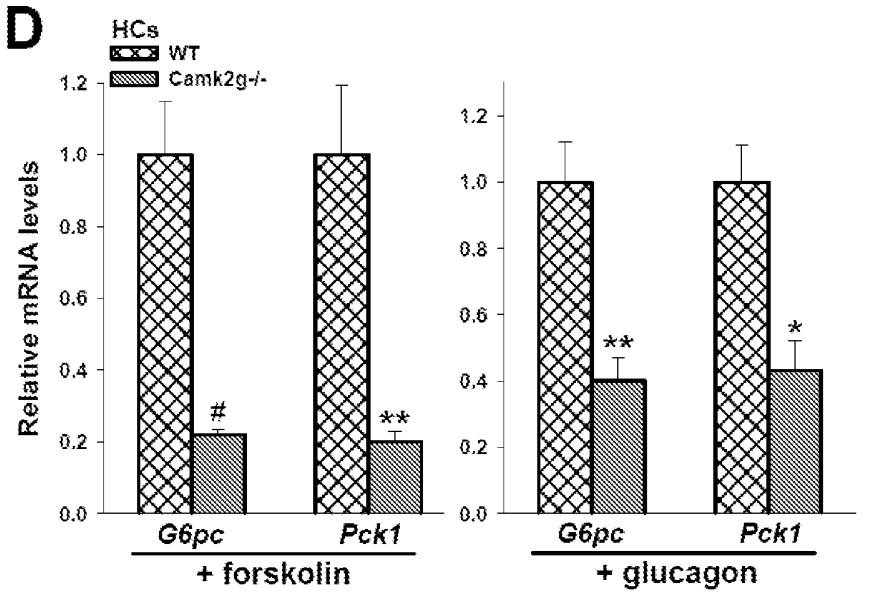


Figure 22A-22C



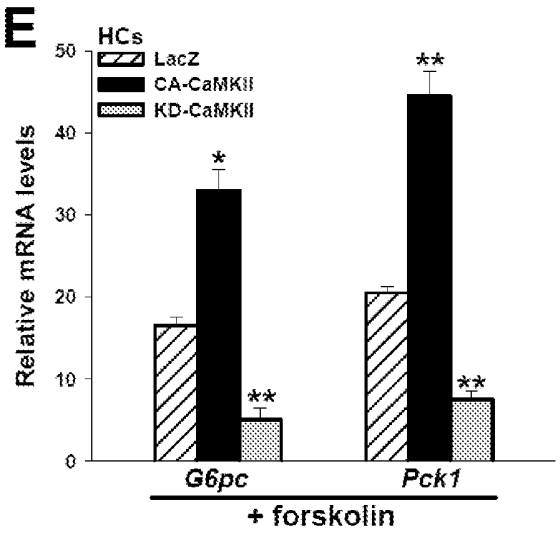


Figure 22D-22E

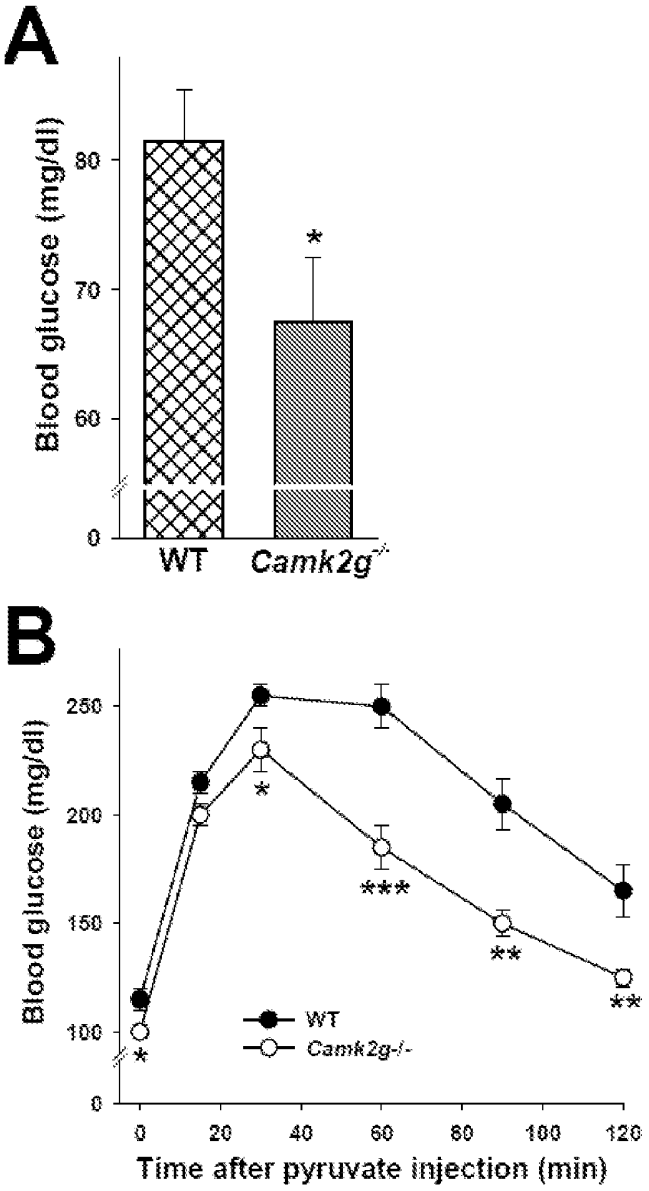


Figure 23A-23B

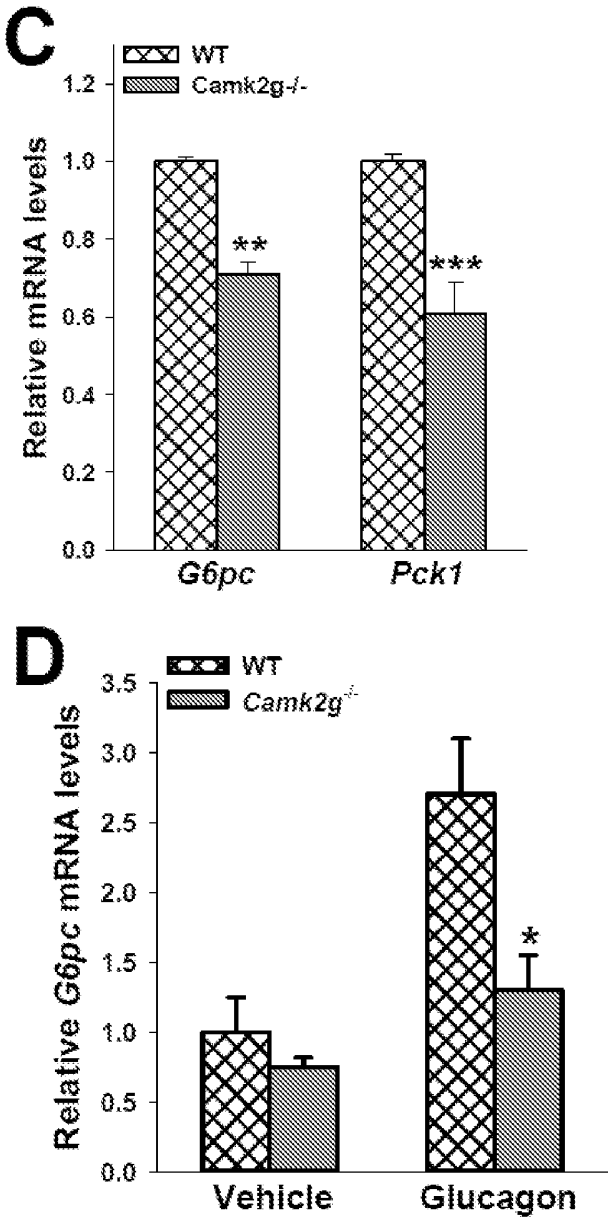


Figure 23C-23D

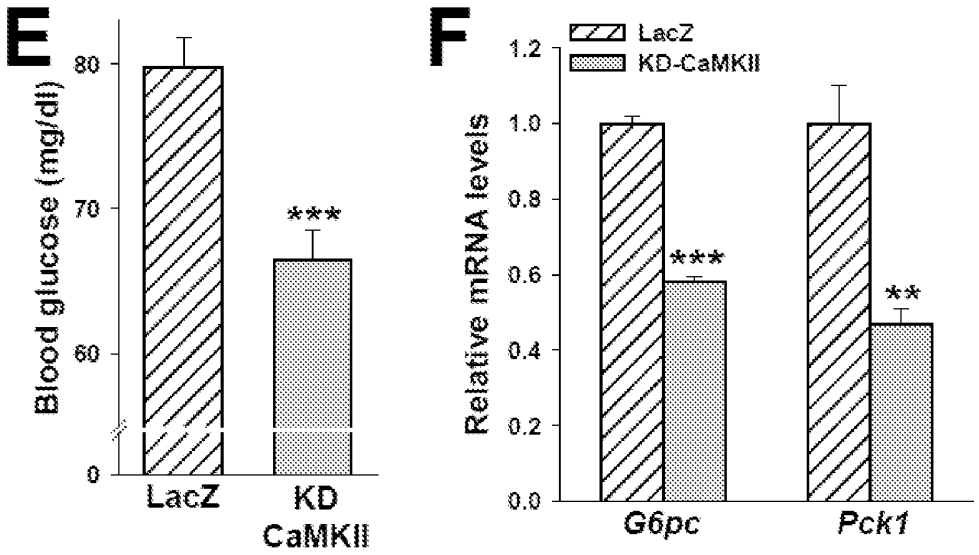


Figure 23E-23F

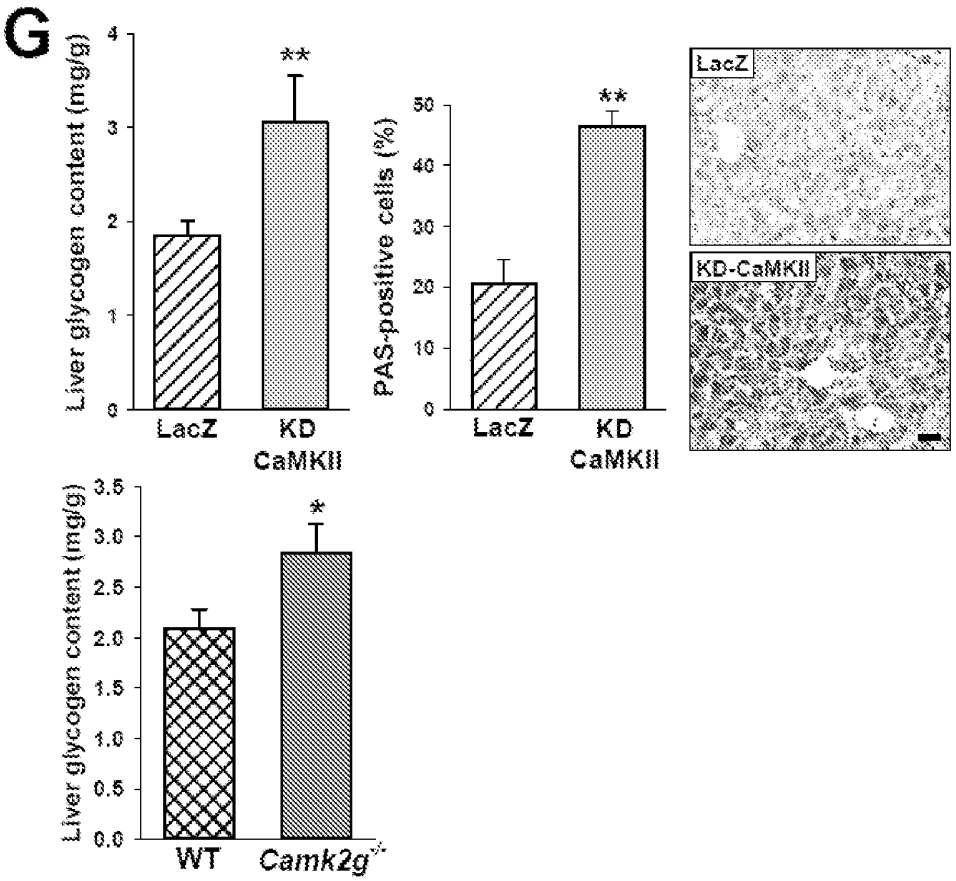
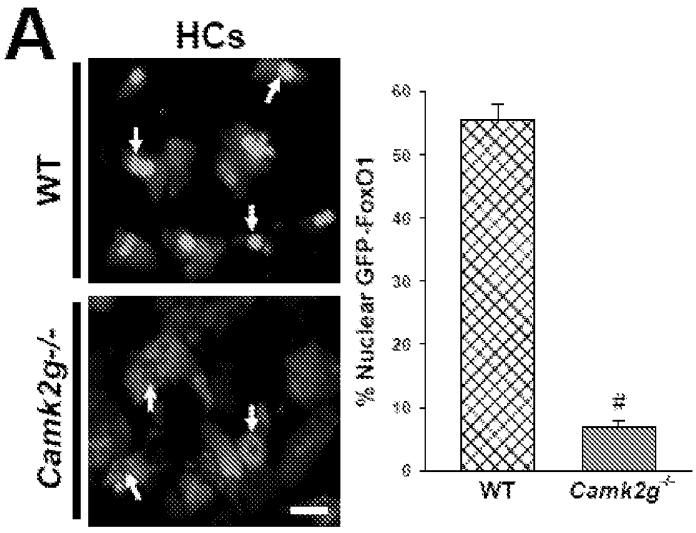

G
Liver glycogen content (mg/g)
4
3
2
1
0
**
LacZ
KD CaMKII
PAS-positive cells (%)
50
40
30
20
10
0
**
LacZ
KD CaMKII
LacZ
KD-CaMKII
Liver glycogen content (mg/g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
WT
Camk2g-/-

Figure 23G



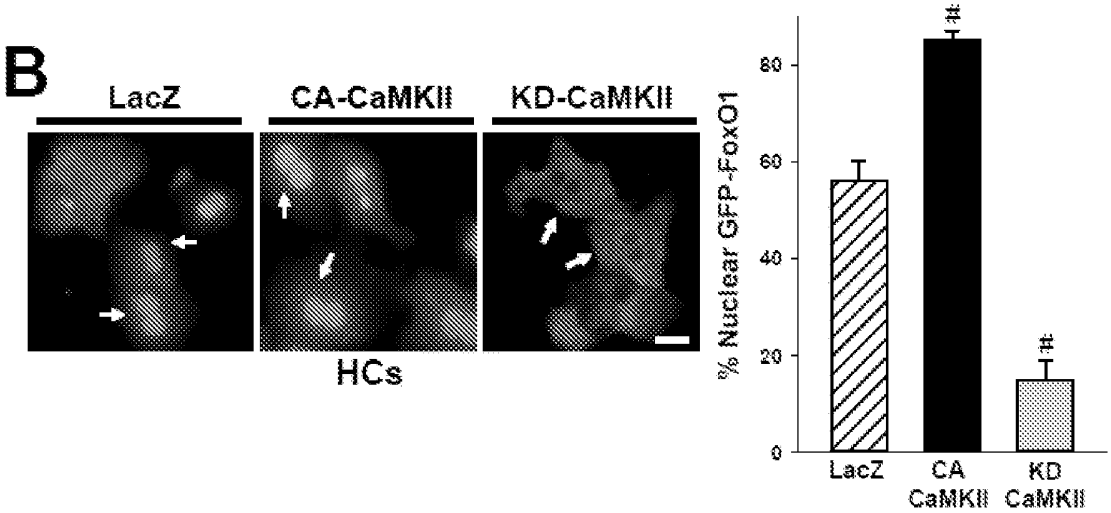


Figure 24A-24B

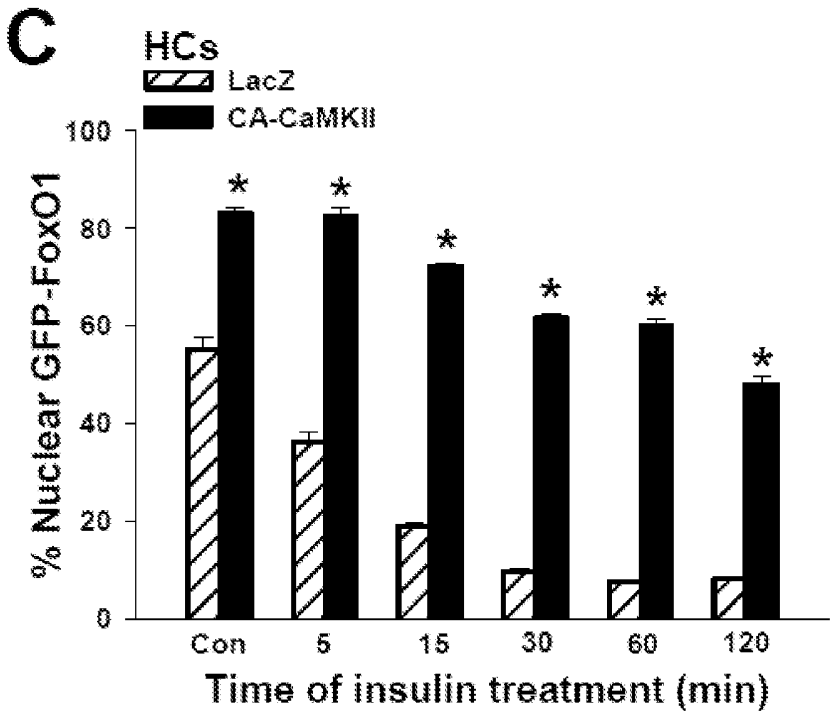

C
HCs
LacZ
CA-CaMKII
% Nuclear GFP-FoxO1
100
80
60
40
20
0
Con
5
15
30
60
120
Time of insulin treatment (min)

Figure 24C

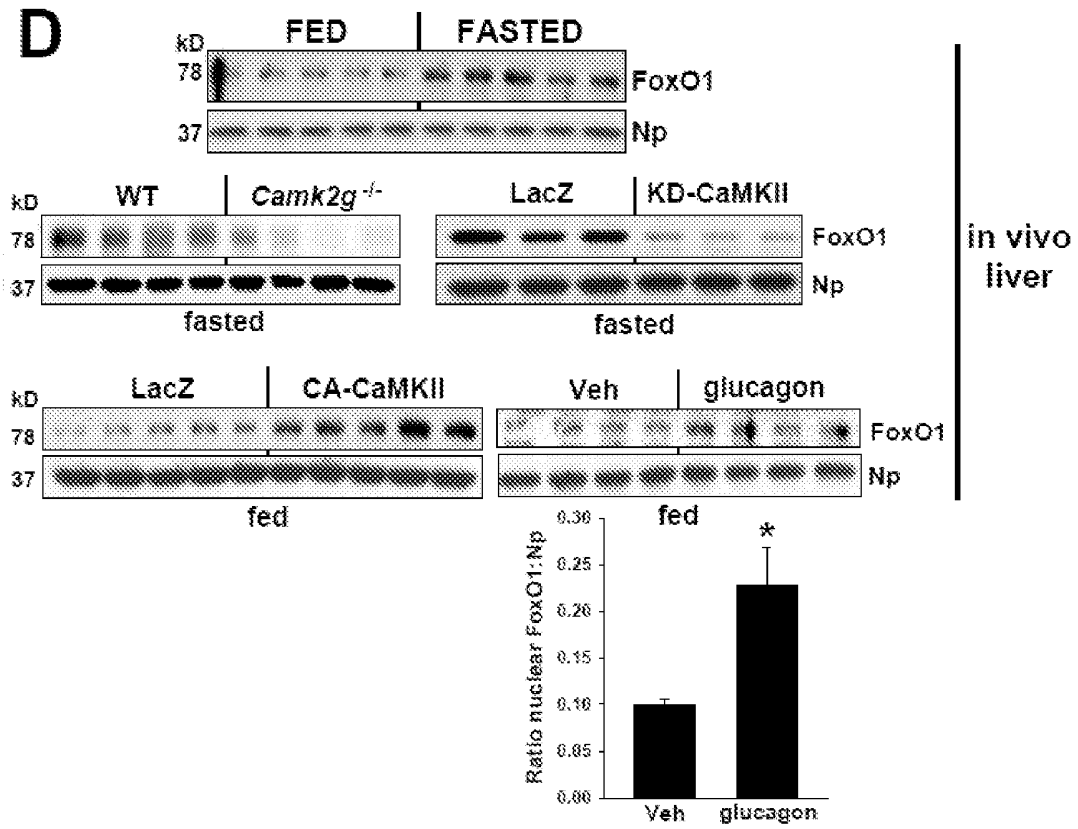

D
FED
FASTED
kD
78
37
FoxO1
Np
WT
Camk2g -/-
LacZ
KD-CaMKII
fasted
fasted
LacZ
CA-CaMKII
Veh
glucagon
fed
fed
in vivo
liver
Ratio nuclear FoxO1:Np
Veh
glucagon

Figure 24D

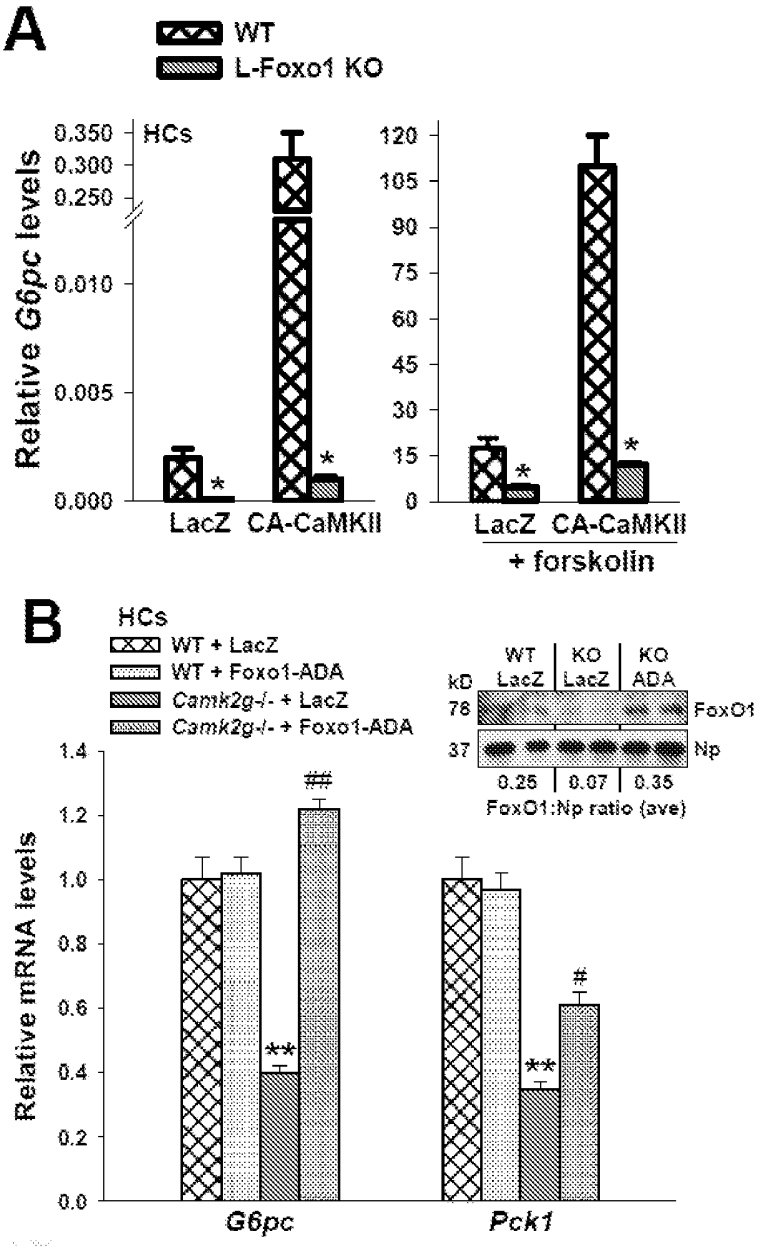


Figure 25A-25B

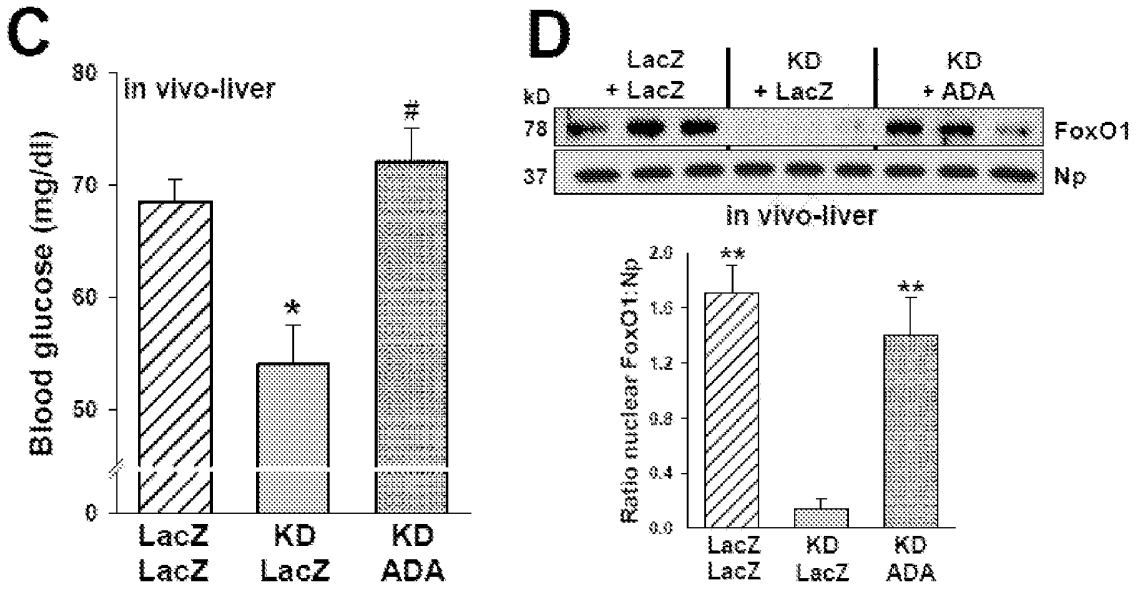


Figure 25C-25D

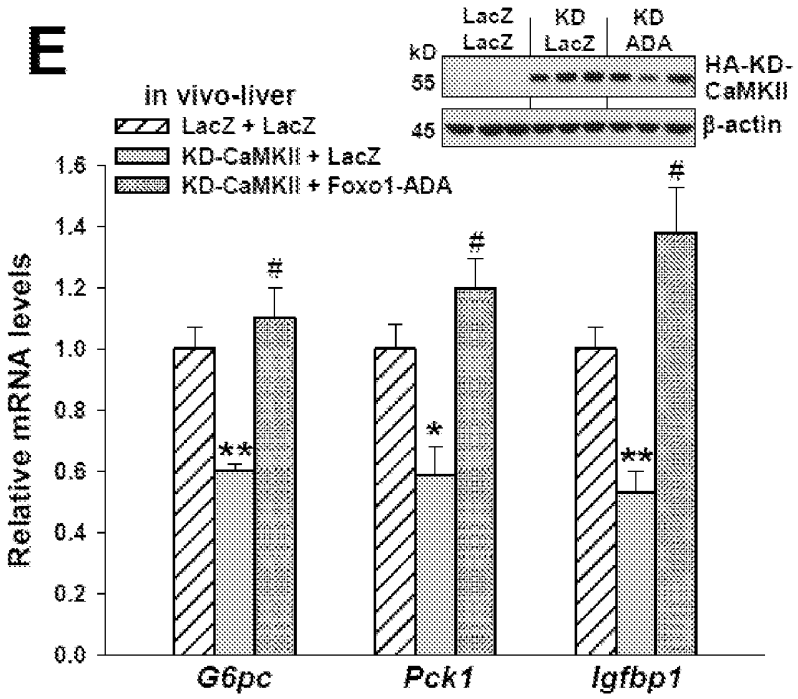

E
in vivo-liver
LacZ + LacZ
KD-CaMKII + LacZ
KD-CaMKII + Foxo1-ADA
LacZ LacZ
KD LacZ
KD ADA
kD
55
45
HA-KD-CaMKII
β-actin
Relative mRNA levels
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
**
#
#
#
G6pc
Pck1
Igfbp1

Figure 25E

A

Peptide*	Site	Spectral Count # (KO)	Debunker Score (KO)	A Score (KO)	Spectral Count # (WT)	Debunker Score (WT)	A Score (WT)	Spectral Count # Ratio (KO/WT)
1: K.KASLQSGQEGPGDS$^{\Delta}$PGSQFSK.W	S284	4	0.99	30.66	6	0.99	57.5	-
2: K.WPAS$^{\Delta}$PGSHSNDDFDNWSTFRPR.T	S295	17	0.99	29.82	37	0.99	40.35	0.45
3: R.TSSNASTISGRLS$^{\Delta}$PIMTEQDDLGDGDVHSLVYPPSAAK.M	S326	33	0.90	30.65	20	0.60	24.89	1.65
4: K.ELLTSDS$^{\Delta}$PPHNDIMSPVDPGVAQPNSR.V	S467	5	0.34	16.22	13	0.59	12.09	0.38
5: K.ELLTSDSPPHNDIMS$^{\Delta}$PVDPGVAQPNSR.V	S475	5	0.34	32.44	10	0.72	44.72	0.5
6: R.SCT$^{\Delta}$WPLPR.P	T24	0	0	0	1	0.80	42.68	-
7: K.SSWWMLNPEGGKSGKS$^{\Delta}$PR.R	S246	2	0.99	16.33	5	0.99	10.49	-
8: R.AAS$^{\Delta}$MDNNSKFAKSR.G	S253	3	0.99	45.45	1	0.99	31.85	-
9: C.YSFAPPNTSLNS$^{\Delta}$PSPNYSK.Y	S413	0	0	0	1	0.31	14.42	-
10: C.YSFAPPNTSLNSPS$^{\Delta}$PNYSK.Y	S415	0	0	0	1	0.82	11.56	-
11: R.TLPHVVNTMPHTSAMNRLT$^{\Delta}$PVK.T	T553	2	0.99	37.41	1	0.36	12.03	-

Figure 26A

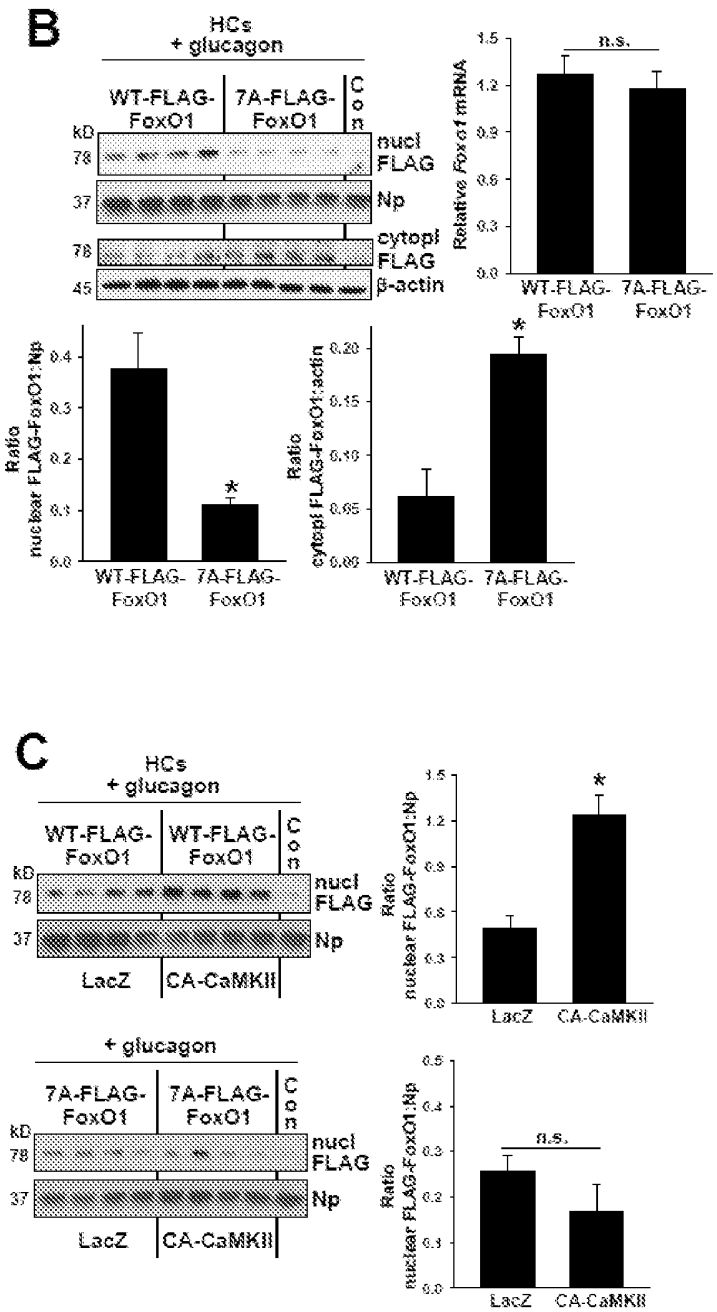


Figure 26B-26C

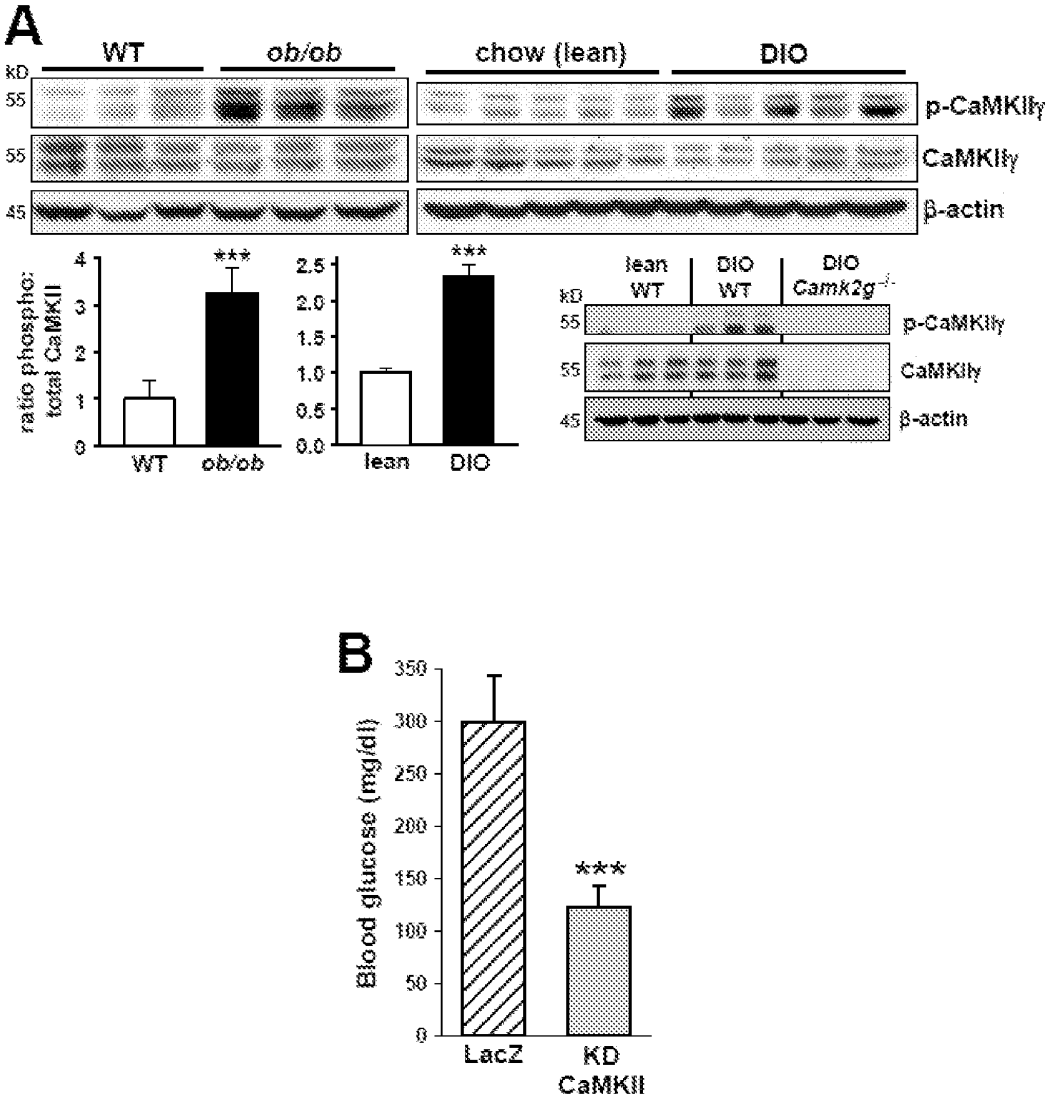
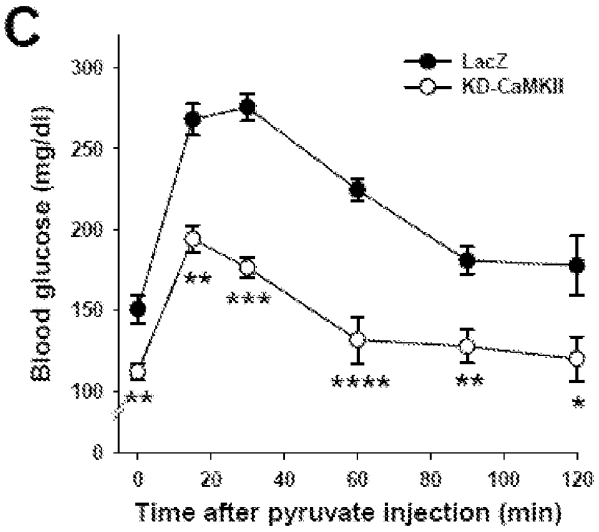


Figure 27A-27B



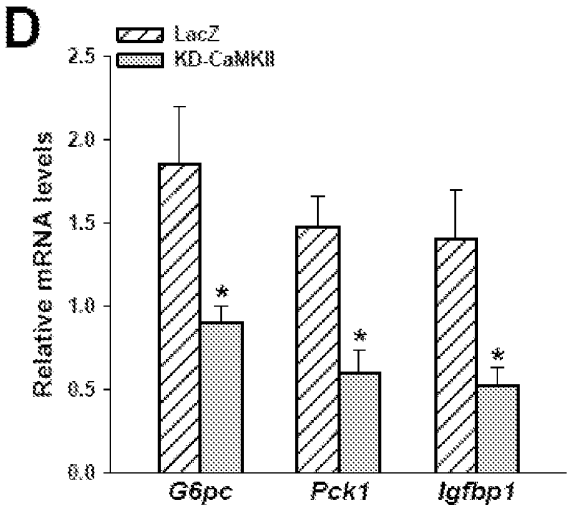


Figure 27C-27D

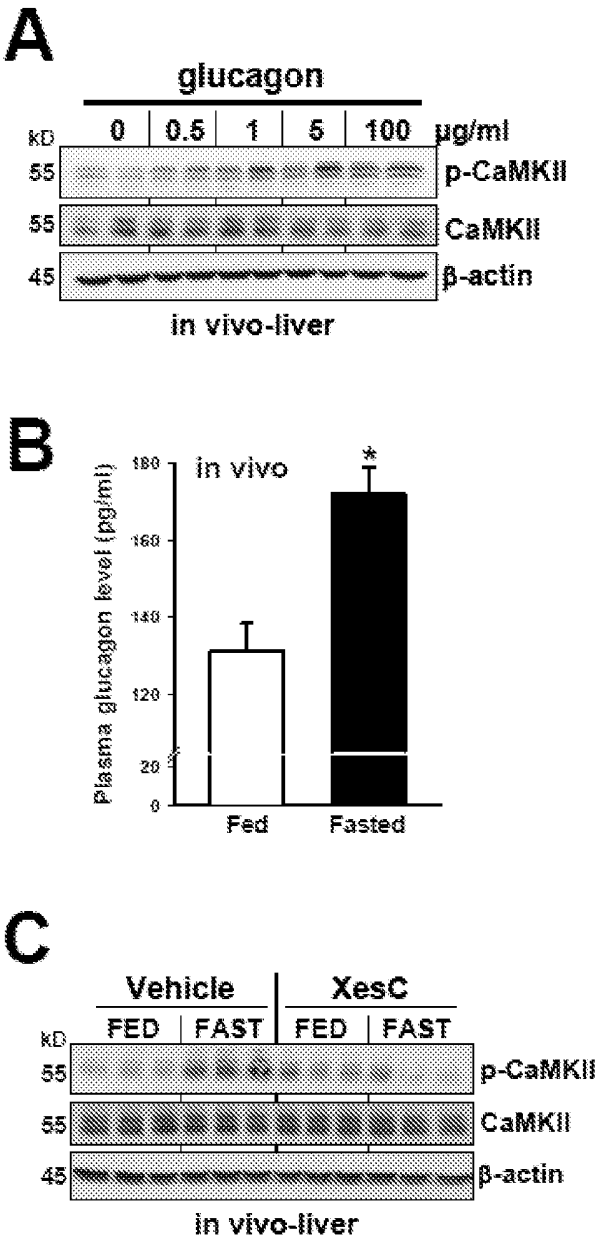


Figure 28A-28C

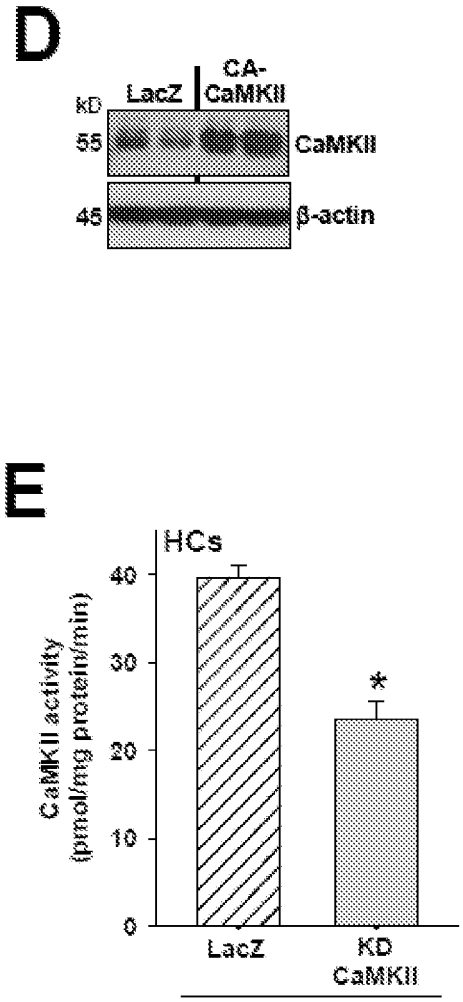


Figure 28D-28E

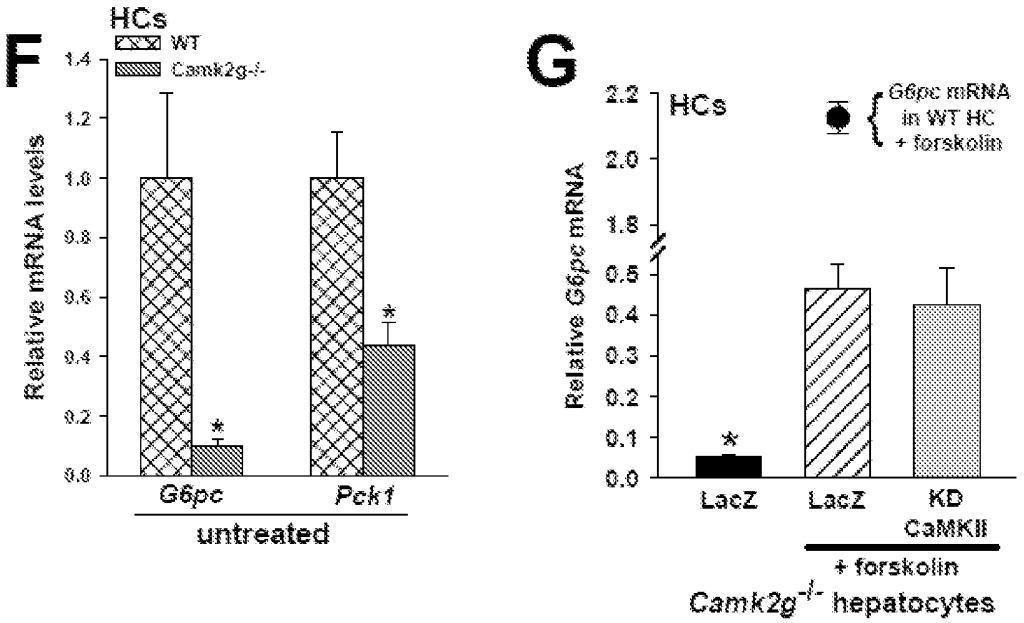


Figure 28F-28G

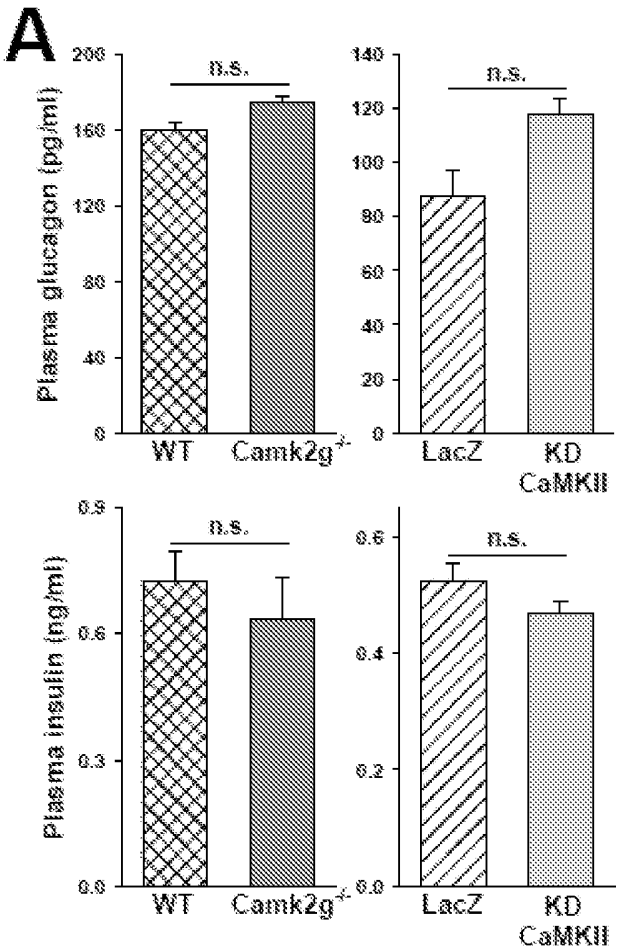
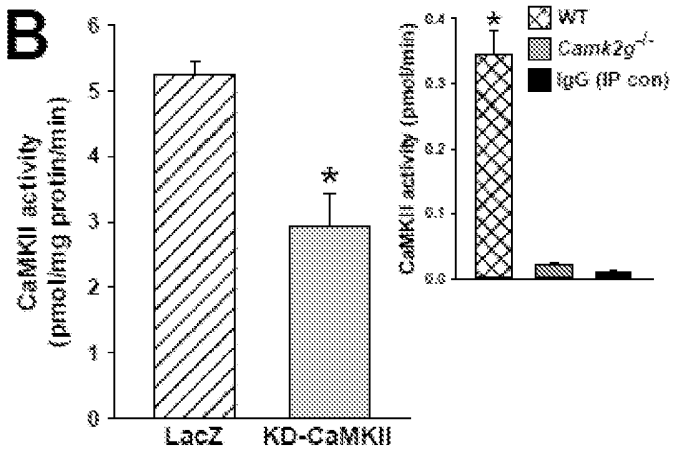

A
Plasma glucagon (pg/ml)
200
160
120
80
40
0
n.s.
WT
Camk2g-/-
140
120
100
80
60
40
20
0
n.s.
LacZ
KD CaMKII
Plasma insulin (ng/ml)
0.9
0.6
0.3
0.0
n.s.
WT
Camk2g-/-
0.6
0.4
0.2
0.0
n.s.
LacZ
KD CaMKII

Figure 29A



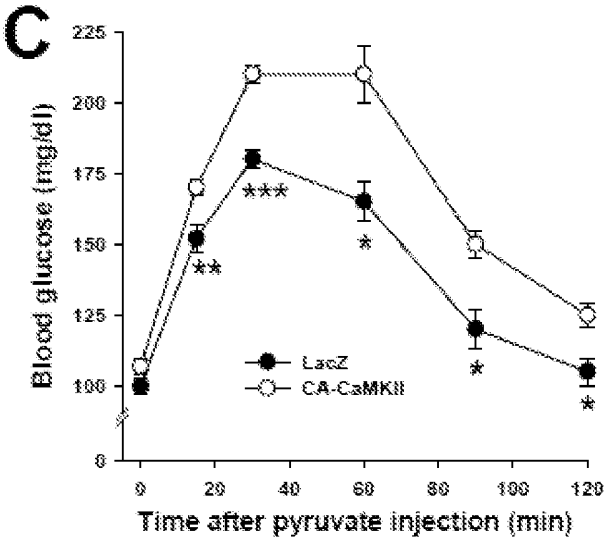


Figure 29B-29C

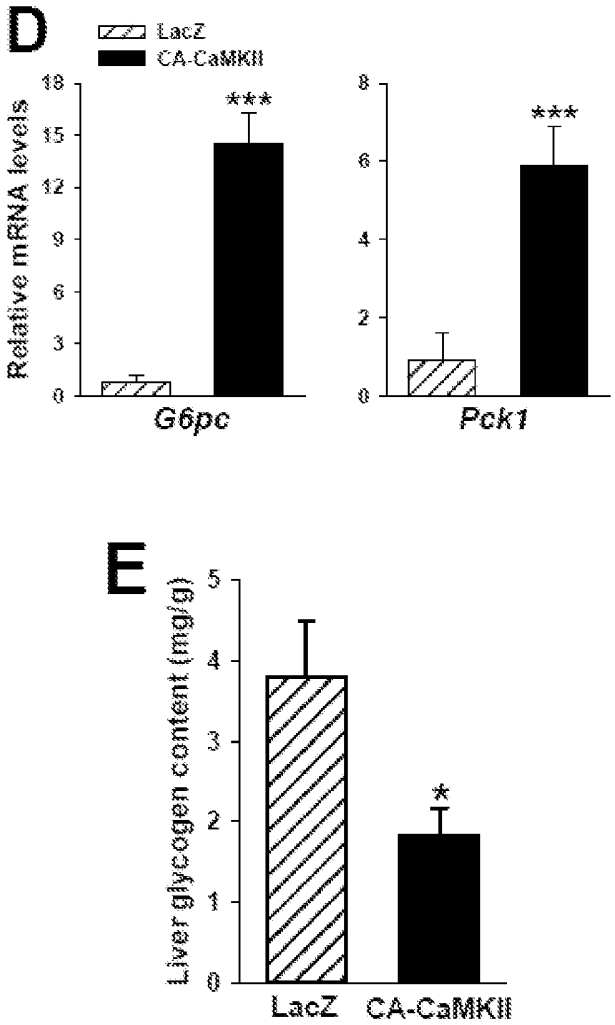
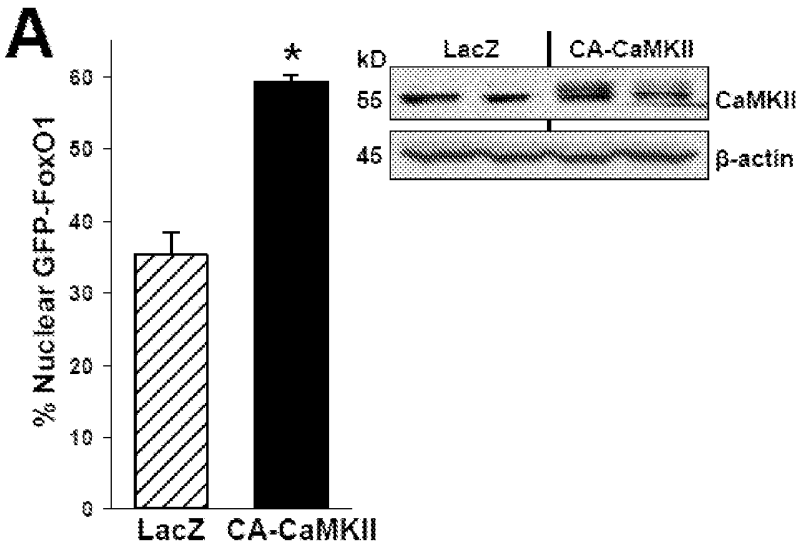


Figure 29D-29E



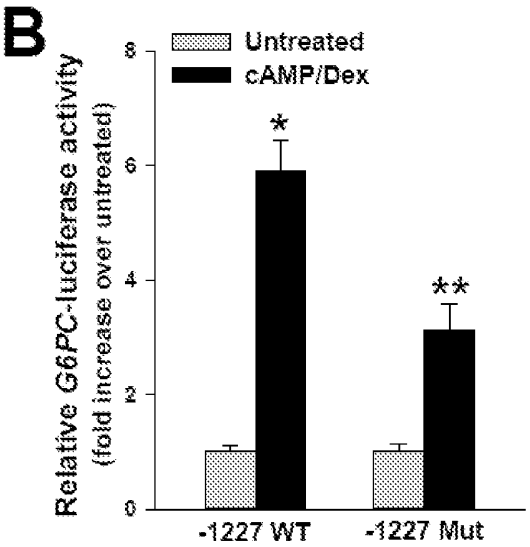


Figure 30A-30B

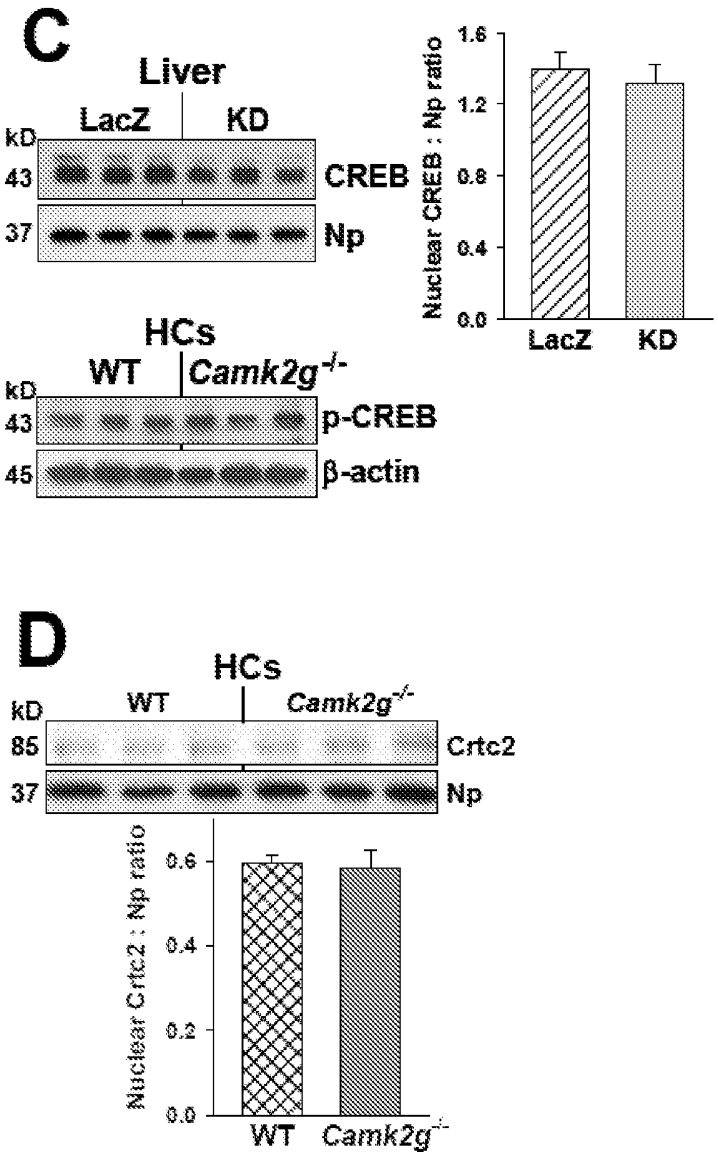


Figure 30C-30D

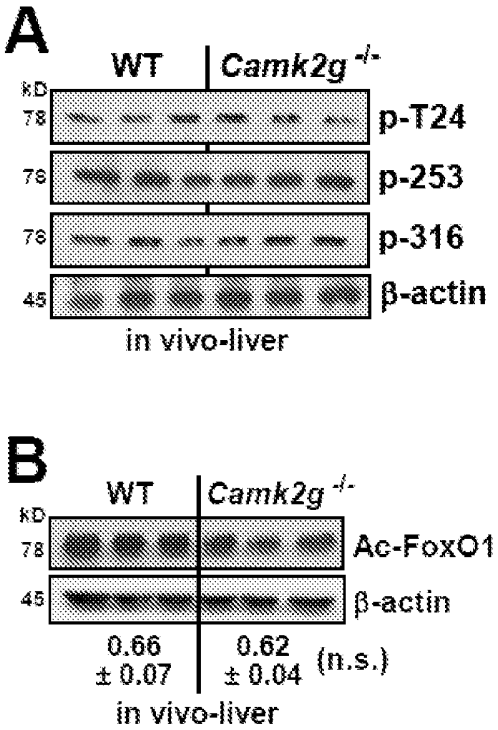


Figure 31A-31B

C

1 maeapqvvet dpdfeplprq rsc**T**[24]wplprp efnqsnstts
41 spapsggaaa npdaaaslas asavstdfms nlslleesed
81 farapgcvav aaaaaasrgl cgdfqgpeag cvhpappqpp
121 ptgplsqppp vppsaaaaag plagqprkts ssrrnawgnl
161 syadlitkai essaekrltl sqiyewmvks vpyfkdkgds
201 nssagwknsi rhnlslhskf irvqnegtgk sswwmlnpeg
241 gksgk**S**[246]prr aa**S**[253]mdmskf aksrgraakk kaslqsgqeg
281 pgd**S**[284]pgsqfs kwpa**S**[295]pgshs nddfdnwstf rprtssnast
321 isgrl**S**[326]pimt eqddlgdgdv hslvyppsaa kmastlpsls
361 eisnpenmen lldnlnllss ptsltvstqs spgsmmqqtp cysfappnts
411 ln**S**[413]p**S**[415]pnysk ytygqssmsp lpqmpmqtlq dsksyggln
451 qyncapgllk ellts**S**[467]pph ndim**S**[475]pvdpg vaqpnsrvlg
491 qnvmmgpnsv mpaygsqash nkmmmpsshl hpghaqqtas
531 vngrtlphvv ntmphtsamm rl**T**[553]pvktplq vplshpmqms
571 algsyssvss cngygrmgvl hqeklpsdld gmfierldcd
611 mesiimdlm dgdtldfnfd nvlpnqsfph svkttthswv sg

(amino acids whose phospho sites were identified by MS/MS using stringent selection criteria are in bold font—see Fig. 6A)

- Lower phospho in KO: **S295, 467, 475** (S246 showed lower trend in KO but spectral count not >10)
- Higher phospho in KO: **S326**
- Possible sites phosphorylated by p38 according to Asada *et al.*, Cellular Signalling, 19:519 (2007): **Ser284, 295, 467, and 475**
- S-A residues in 7A-FoxO1: **S246, 284, 295, 413, 415, 429, 475**
- Additional S-A residues in 9A-FoxO1: **S326, 467**

Figure 31C

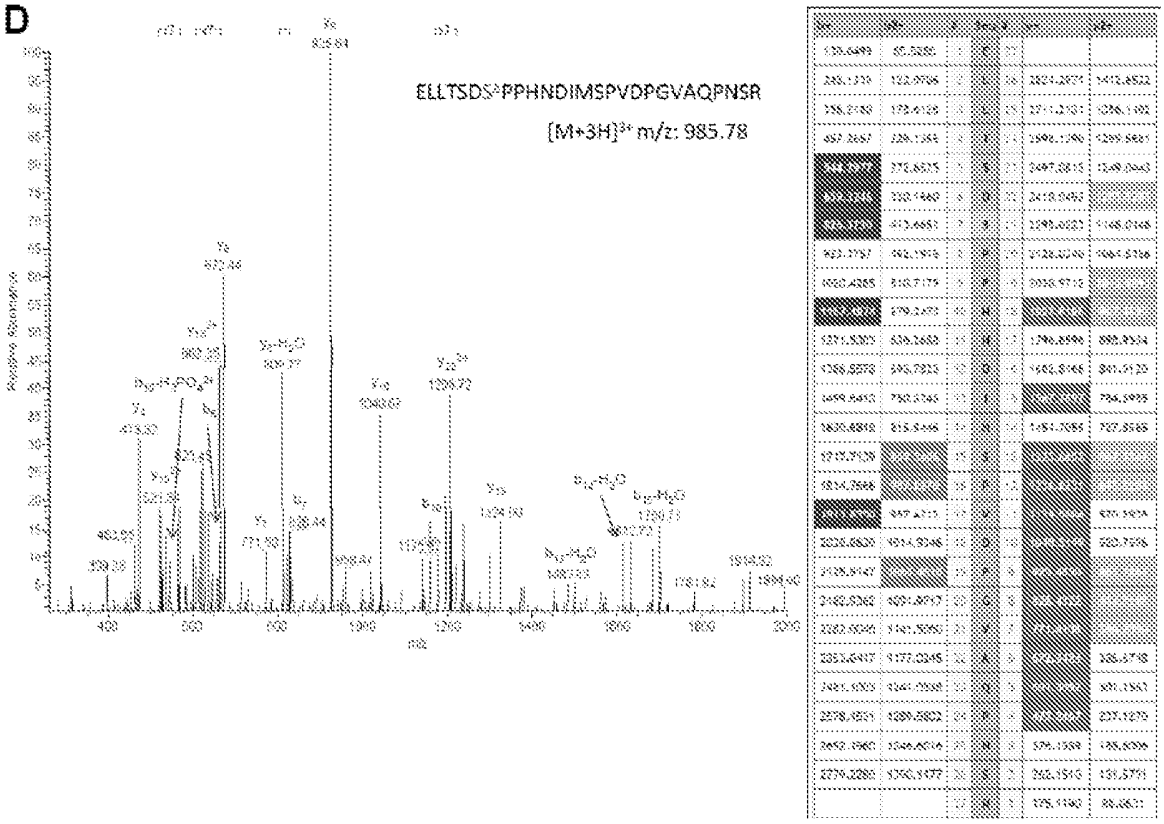


Figure 31D

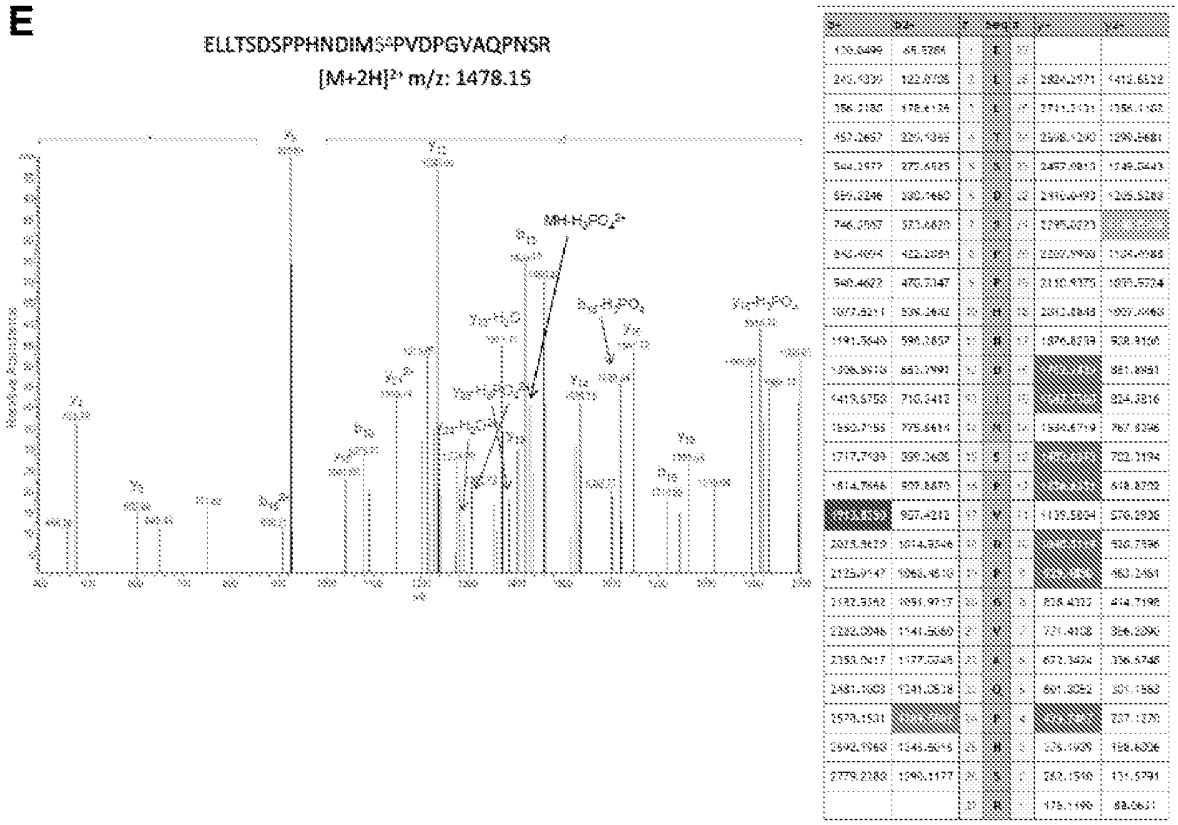


Figure 31E

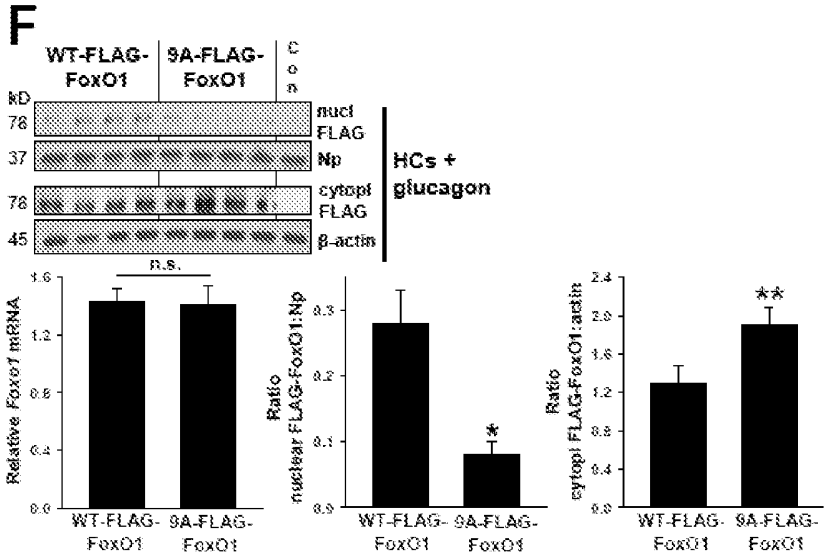

F
WT-FLAG-FoxO1
9A-FLAG-FoxO1
Con
kD
78
37
78
45
nucl FLAG
Np
cytopl FLAG
β-actin
HCs + glucagon
n.s.
Relative Foxo1 mRNA
Ratio nuclear FLAG-FoxO1:Np
Ratio cytopl FLAG-FoxO1:actin
*
**
WT-FLAG-FoxO1
9A-FLAG-FoxO1

Figure 31F

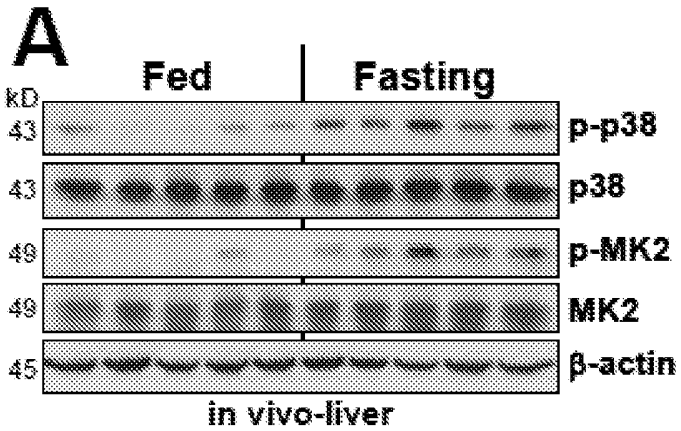

A
Fed
Fasting
kD
43
43
49
49
45
p-p38
p38
p-MK2
MK2
β-actin
in vivo-liver

Figure 32A

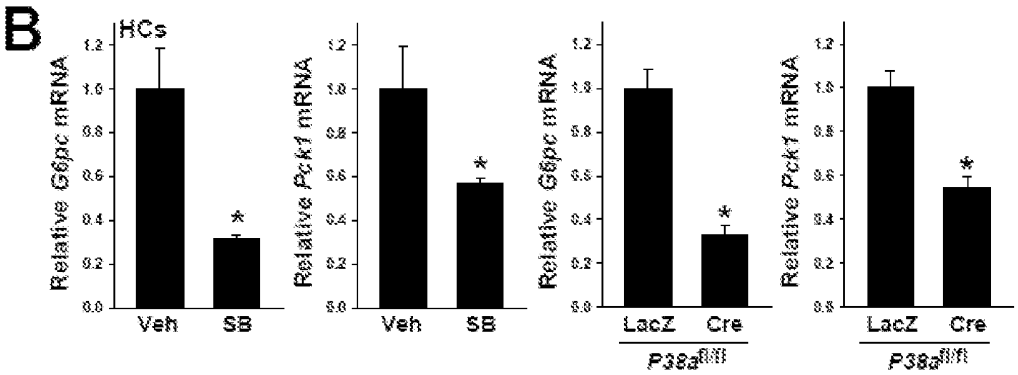

B
HCs
Relative G6pc mRNA
Relative Pck1 mRNA
Relative G6pc mRNA
Relative Pck1 mRNA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Veh
SB
LacZ
Cre
P38a fl/fl
*

Figure 32B

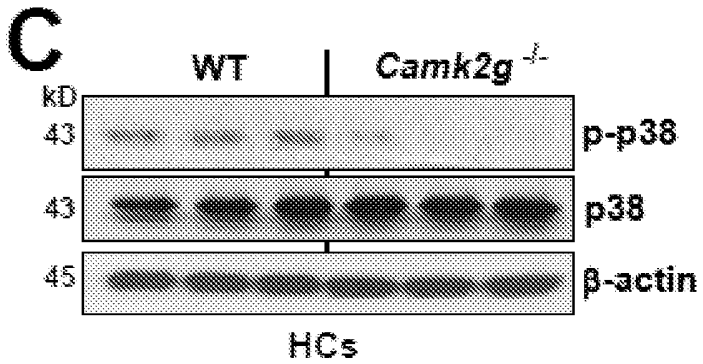


Figure 32C

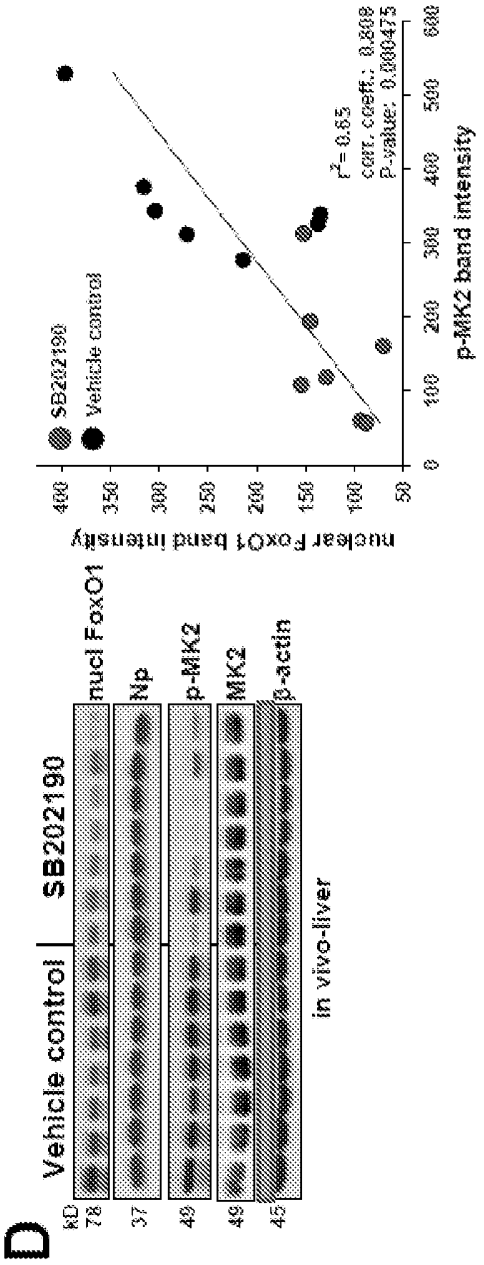

D
Vehicle control
SB202190
kD
78
37
49
49
45
nucl FoxO1
Np
p-MK2
MK2
β-actin
in vivo-liver
nuclear FoxO1 band intensity
400
350
300
250
200
150
100
50
0
100
200
300
400
500
600
p-MK2 band intensity
SB202190
Vehicle control
r² = 0.85
corr. coeff.: 0.808
p-value: 0.000475

Figure 32D

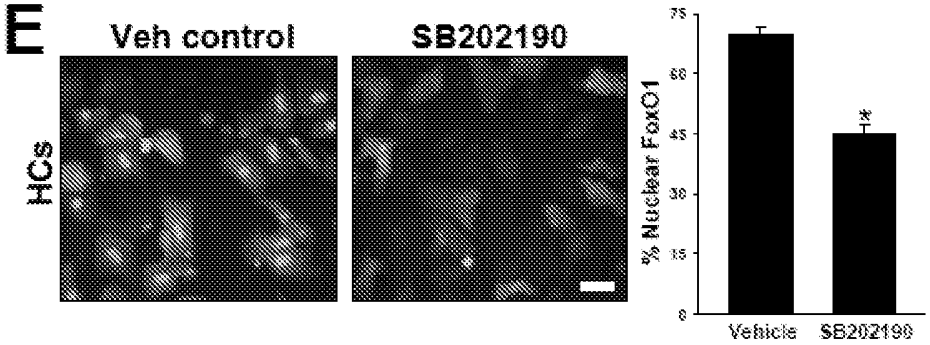

E
Veh control
SB202190
HCs
% Nuclear FoxO1
75
60
45
30
15
0
*
Vehicle
SB202190

Figure 32E

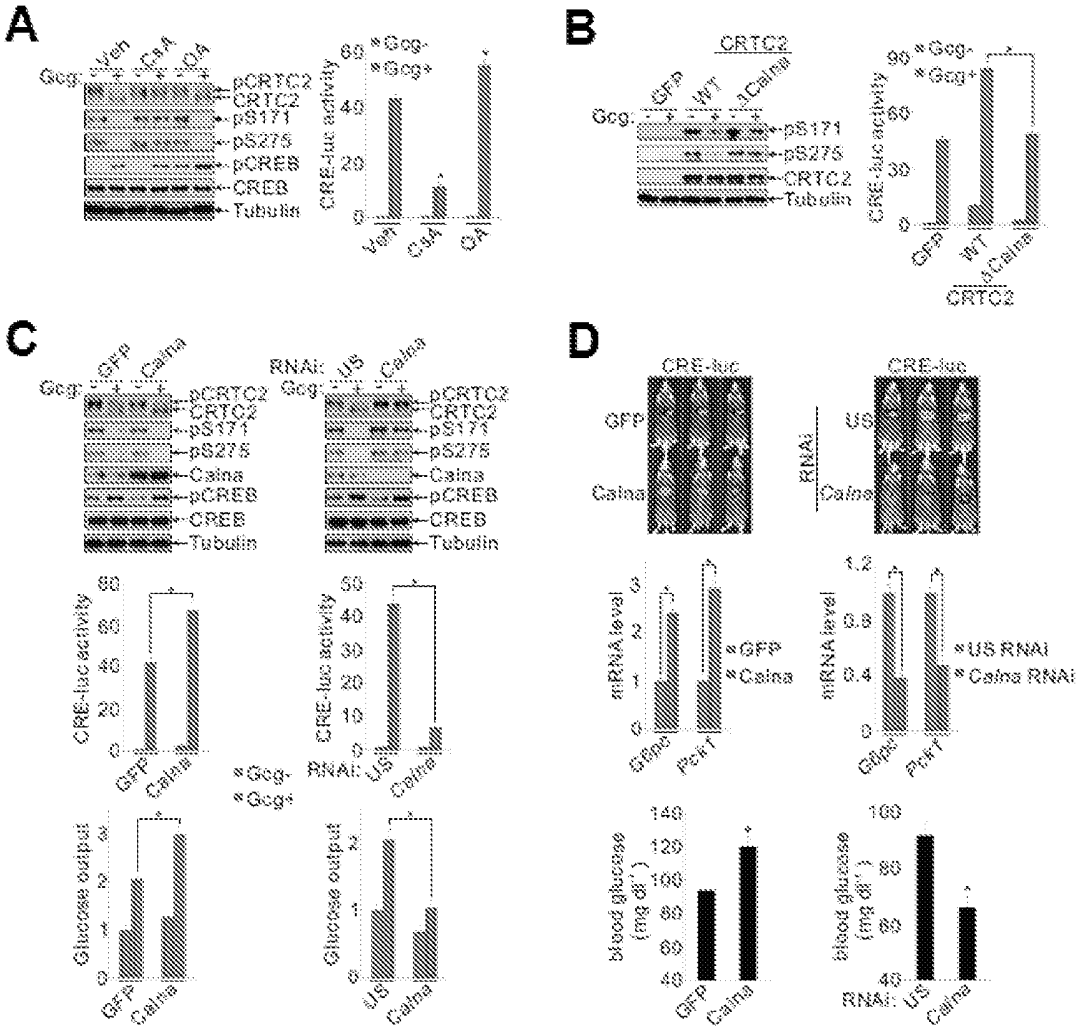


Figure 33A-33D

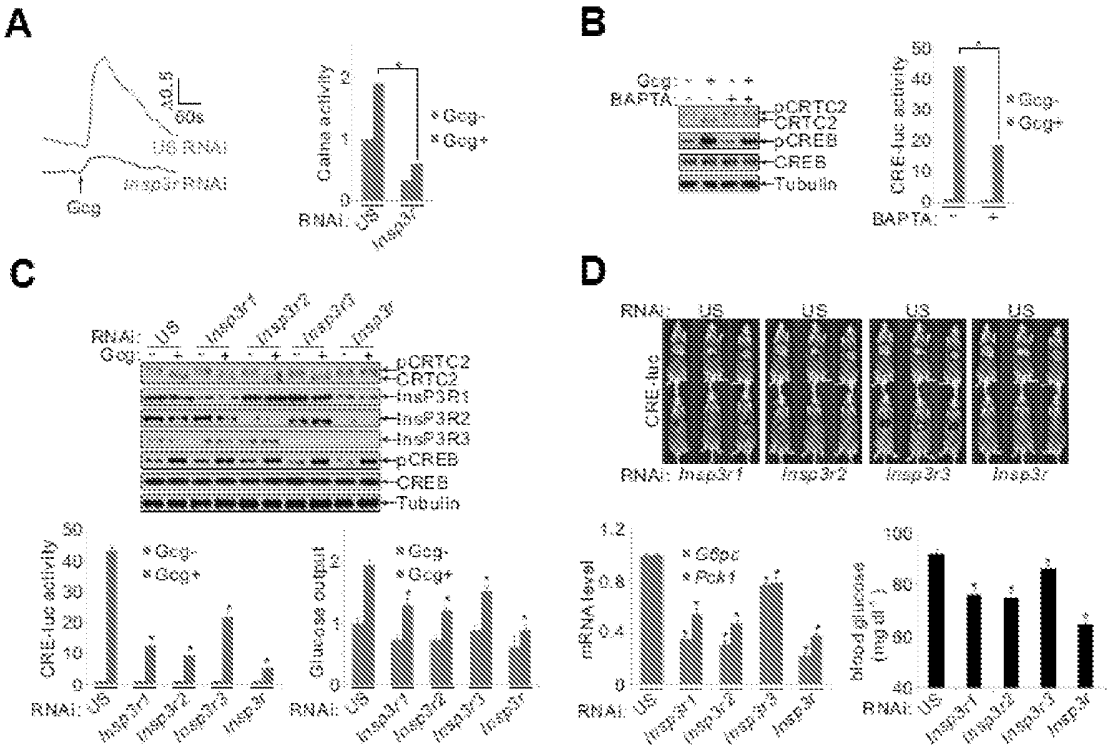


Figure 34A-34D

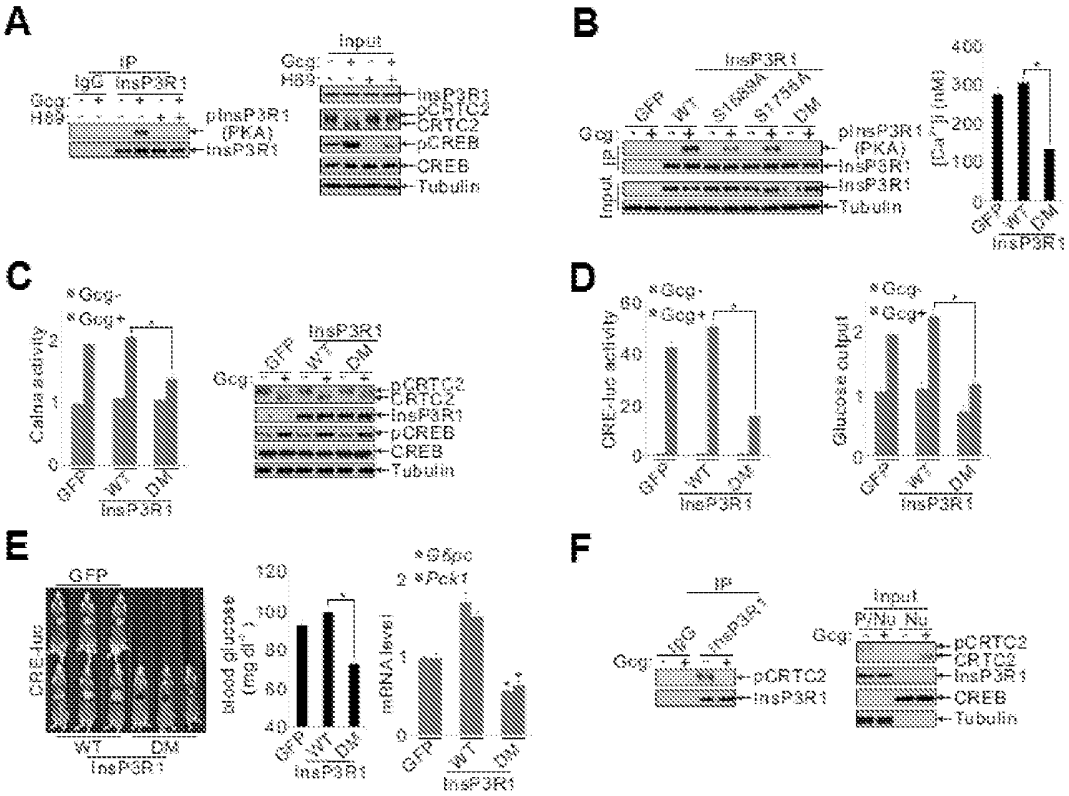


Figure 35A-35F

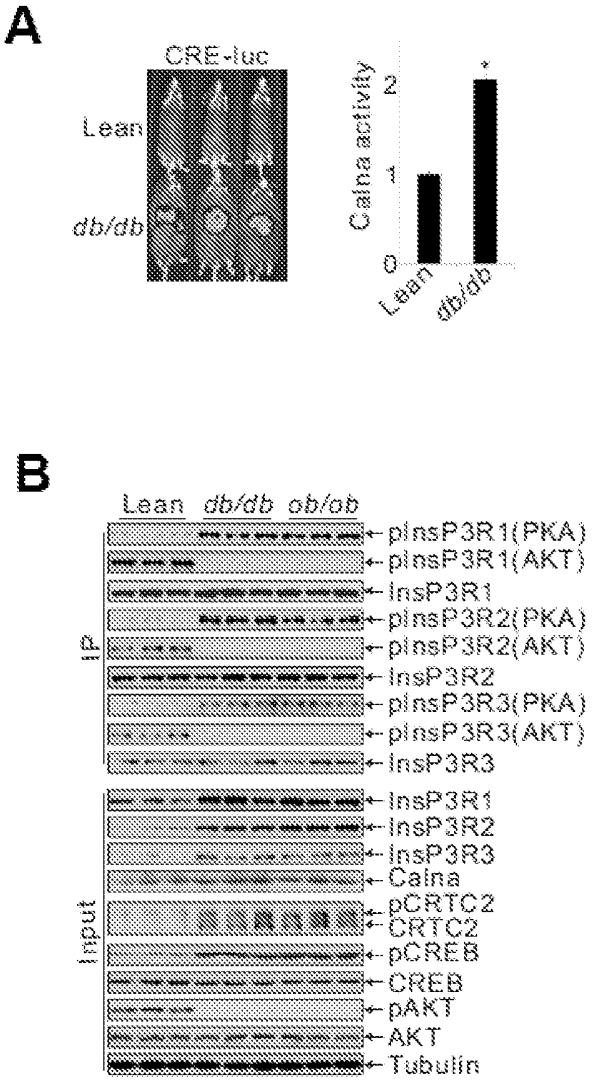


Figure 36A-36B

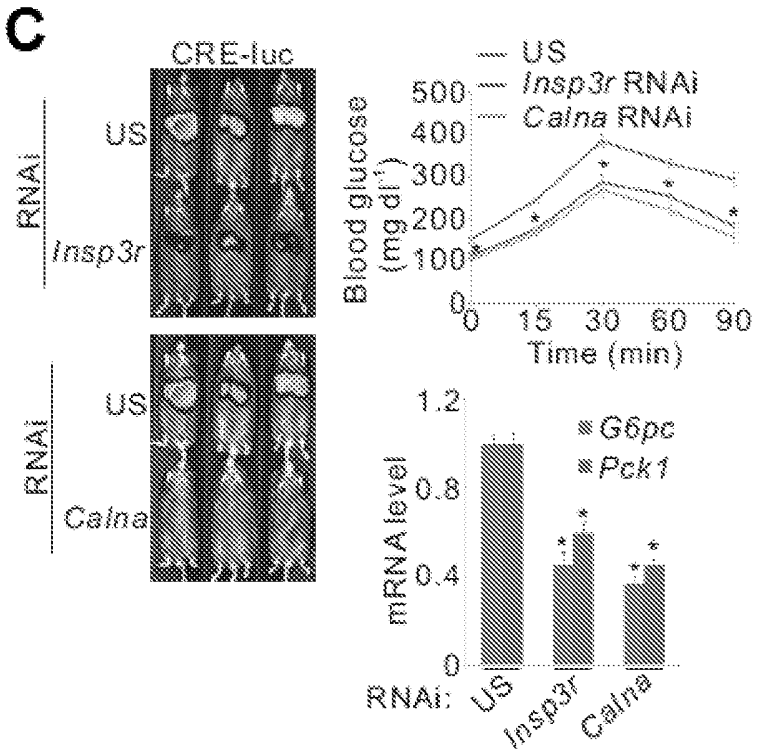

C
CRE-luc
RNAi
US
Insp3r
RNAi
US
Calna
US
Insp3r RNAi
Calna RNAi
Blood glucose (mg dl⁻¹)
500
400
300
200
100
0
0 15 30 60 90
Time (min)
1.2
0.8
0.4
0
mRNA level
G6pc
Pck1
RNAi: US Insp3r Calna

Figure 36C

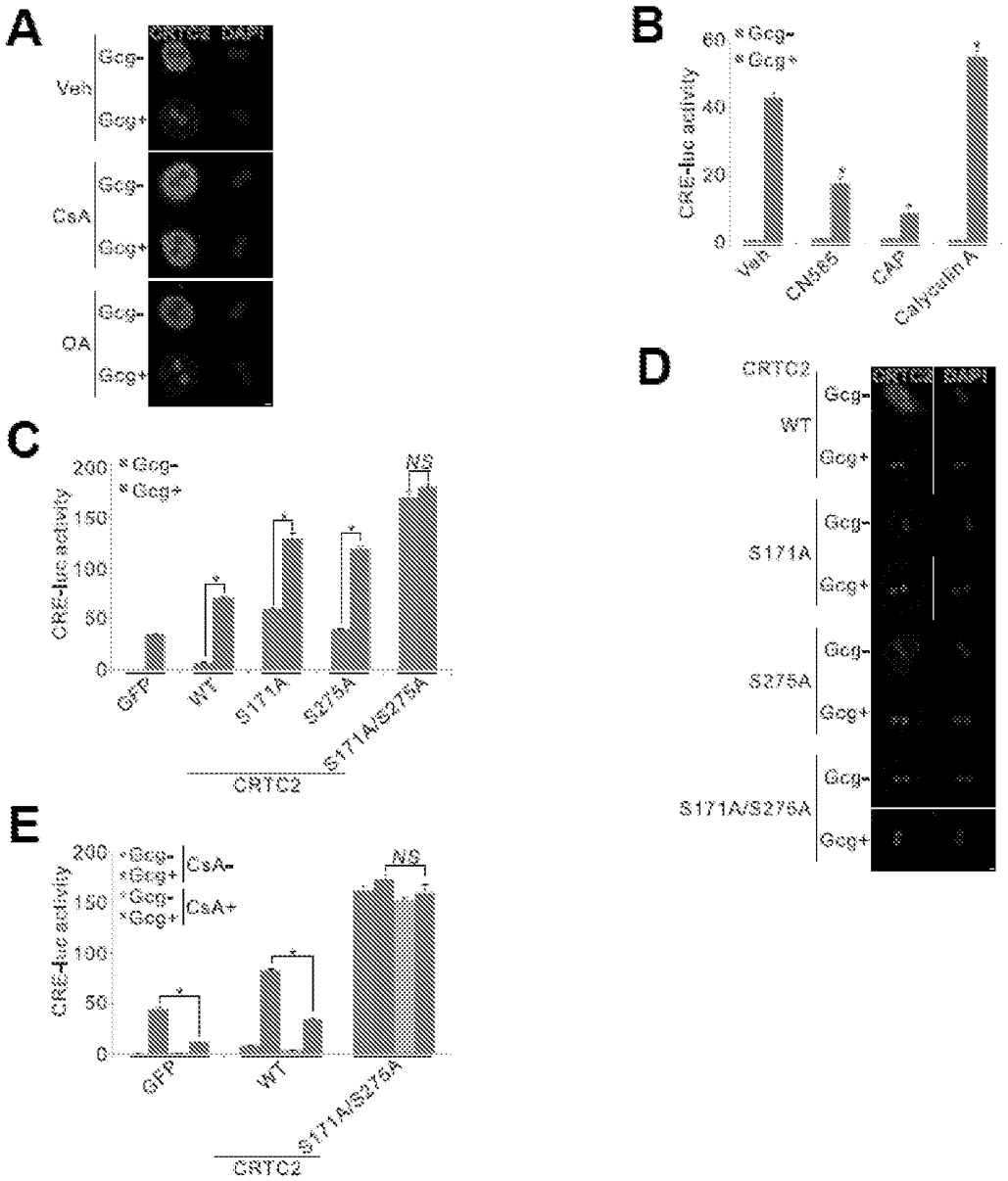


Figure 37A-37E

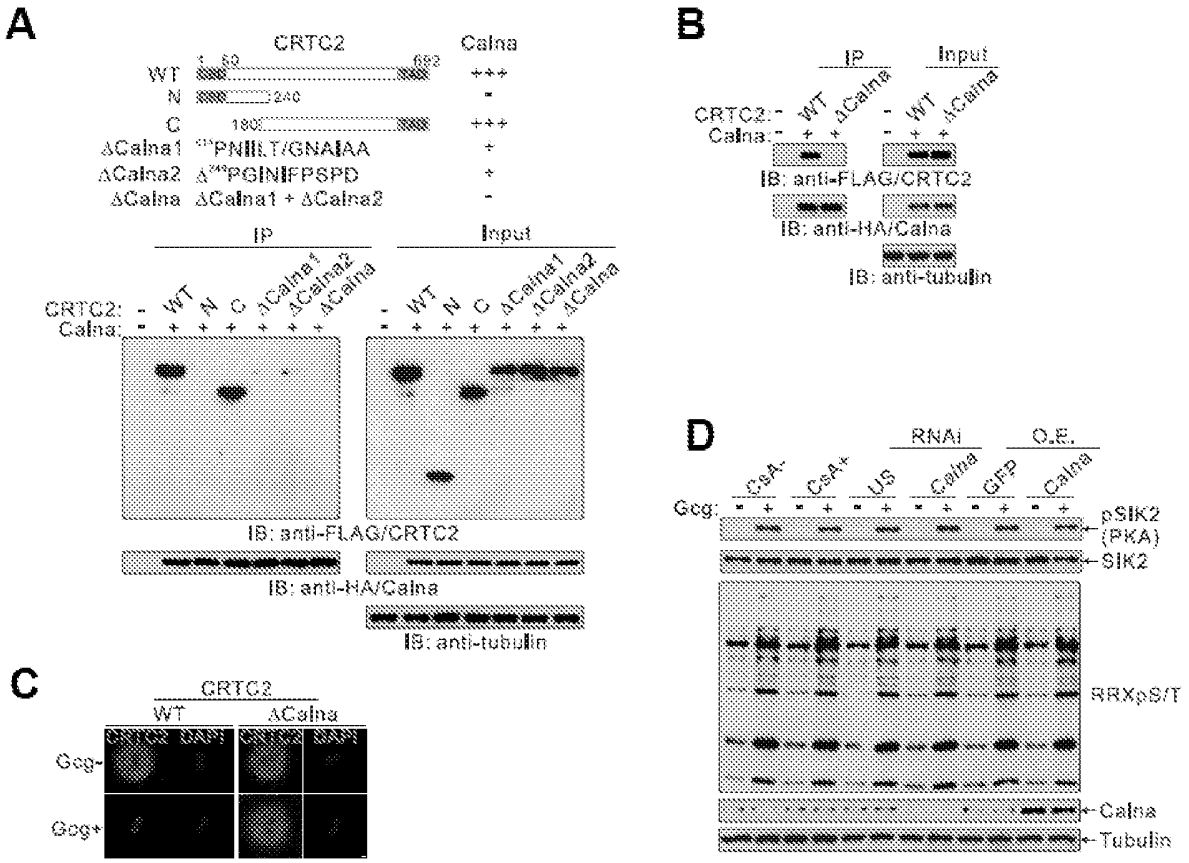


Figure 38A-38D

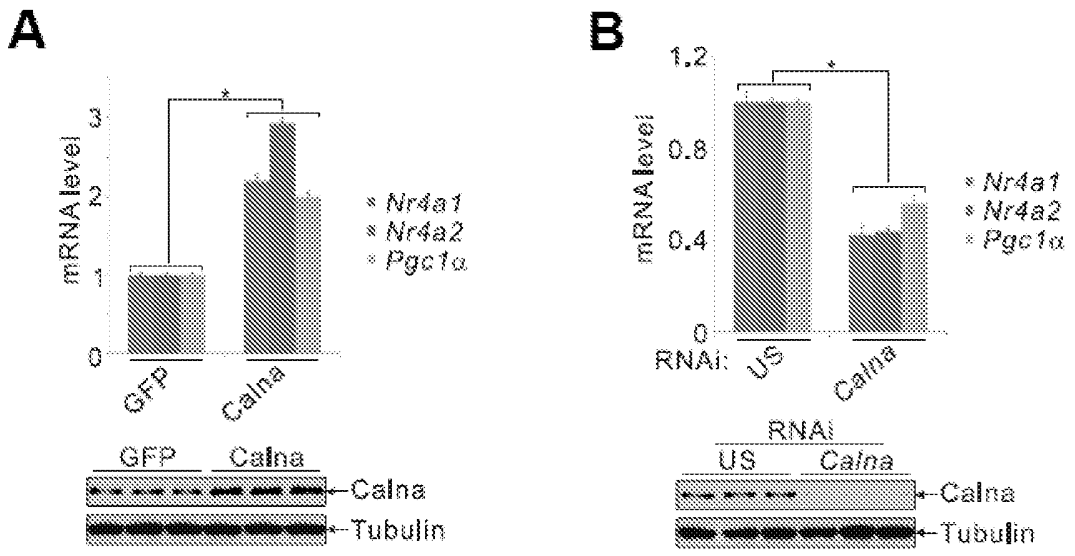


Figure 39A-39B

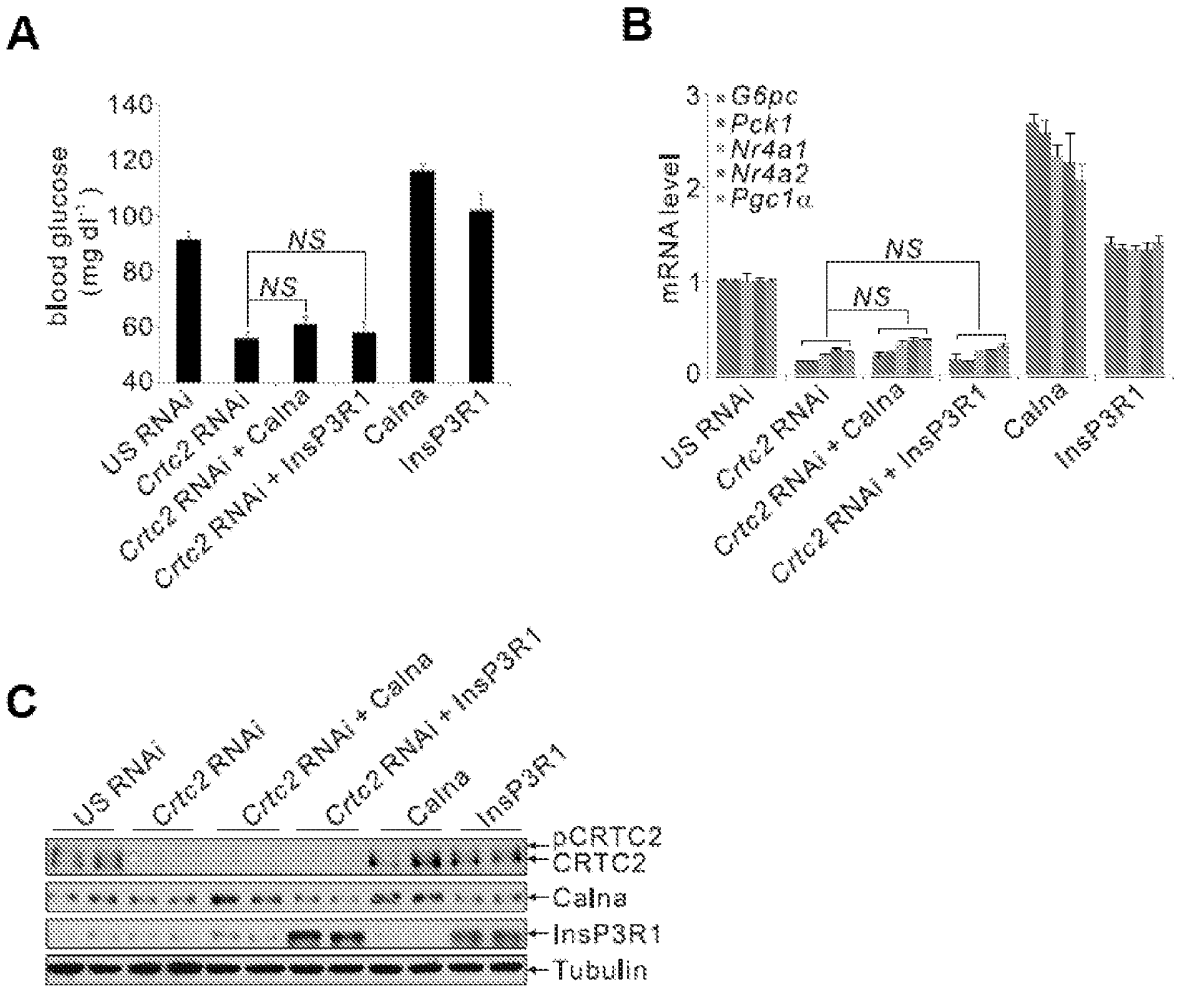


Figure 40A-40C

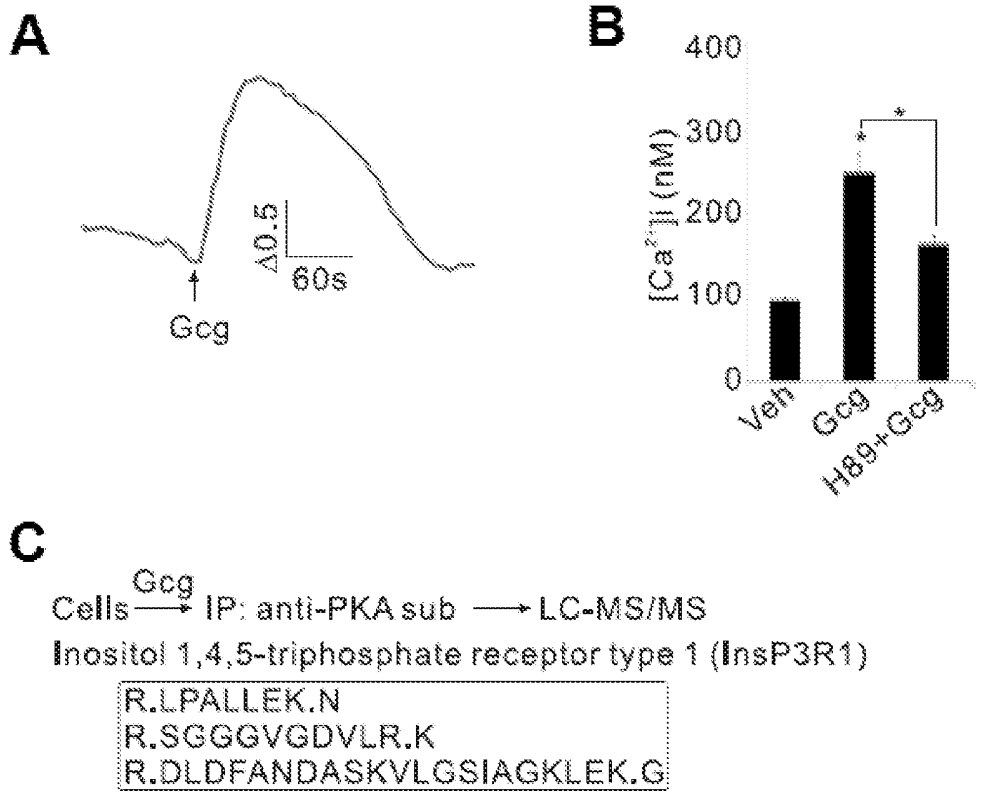


Figure 41A-41C

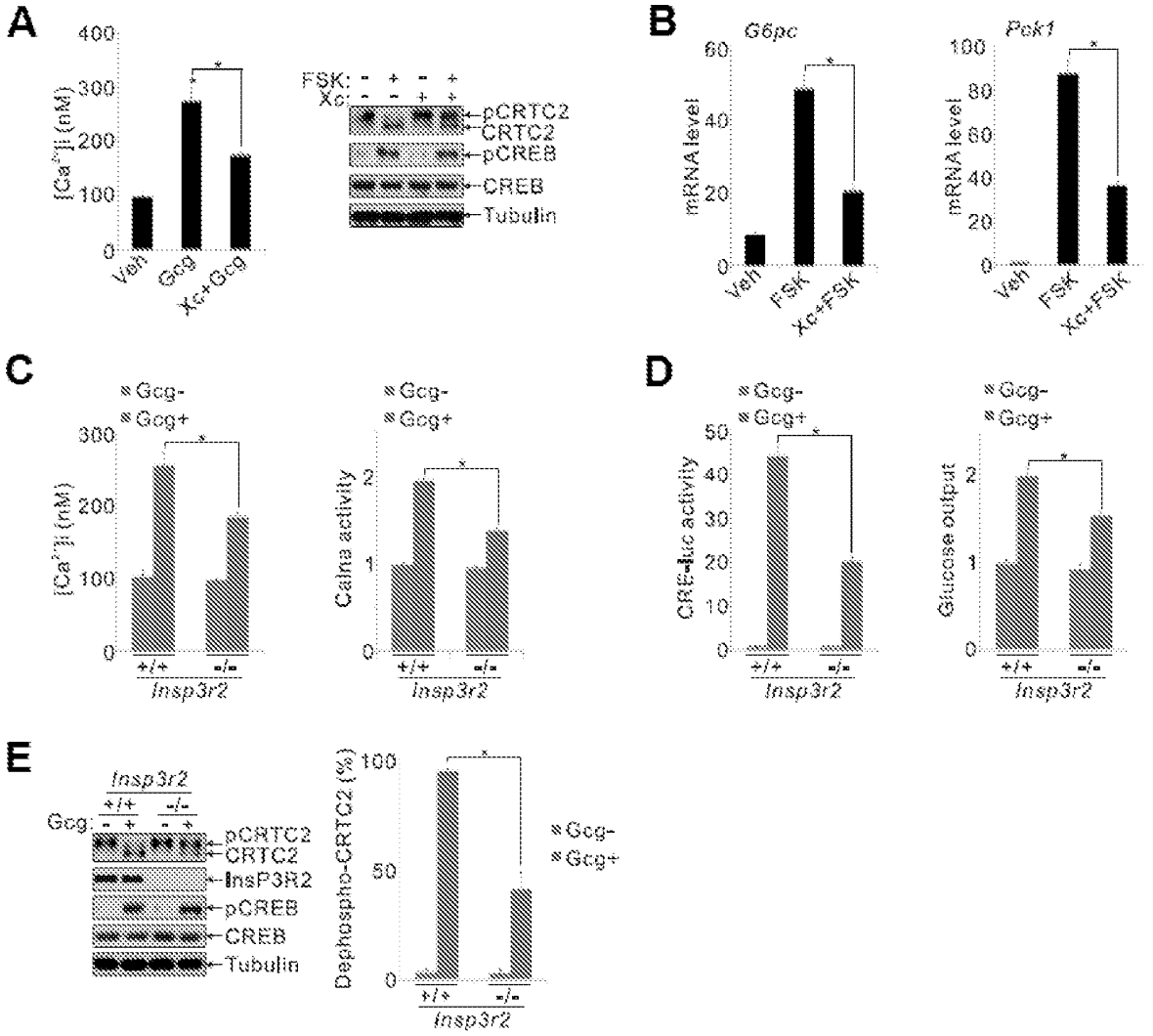


Figure 42A-42E

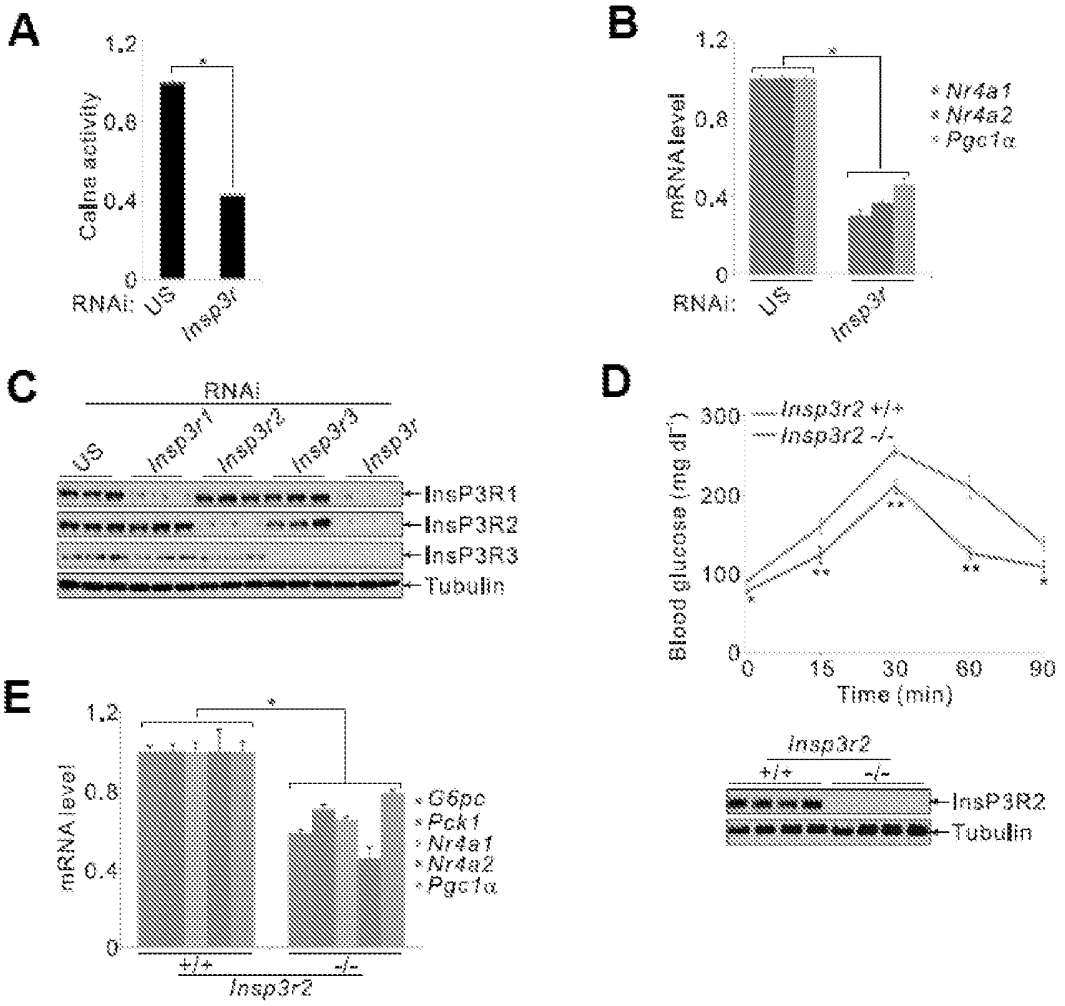


Figure 43A-43E

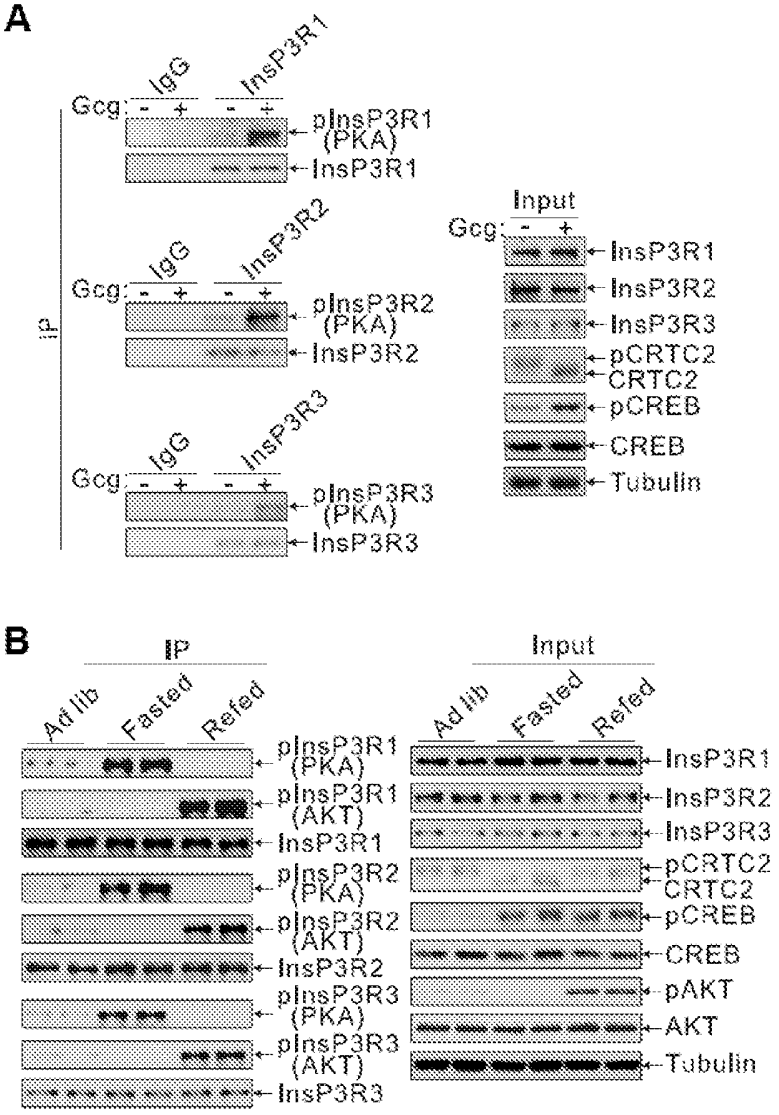


Figure 44A-44B

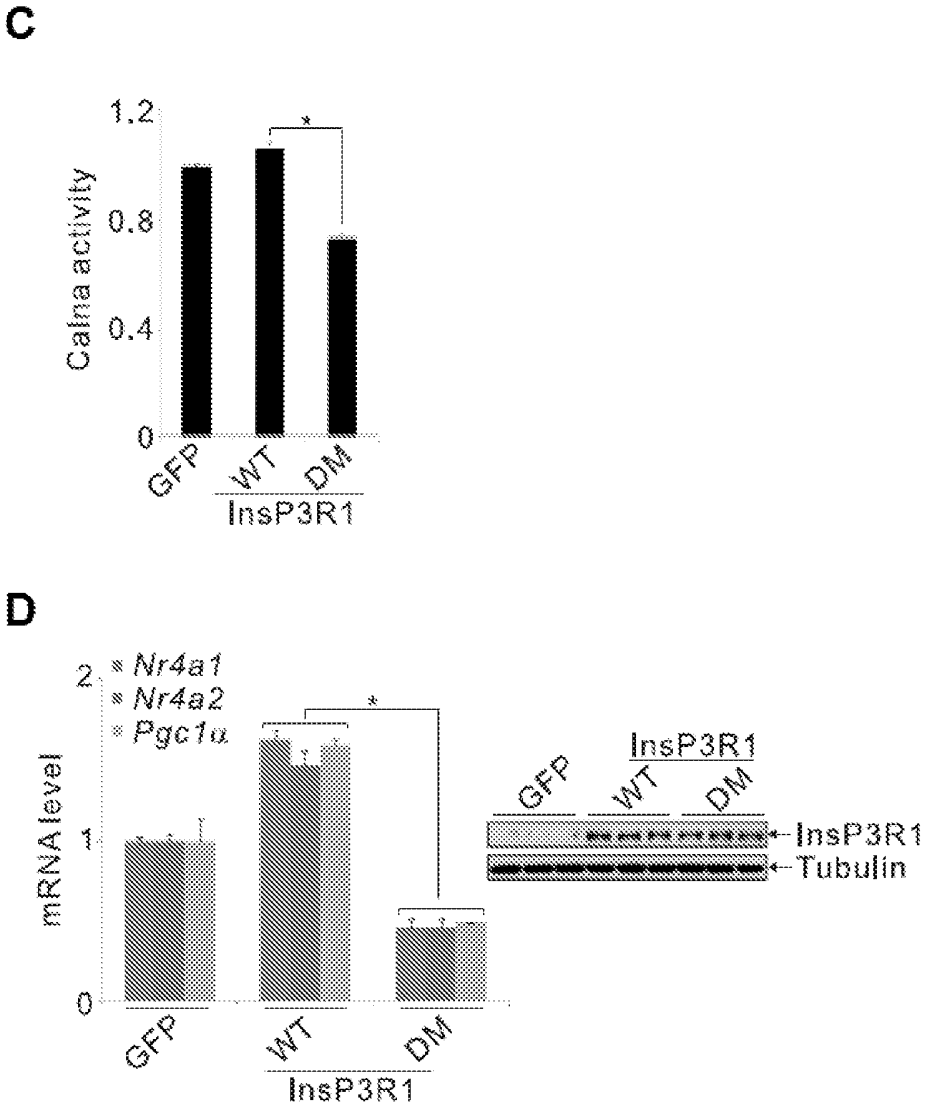


Figure 44C-44D

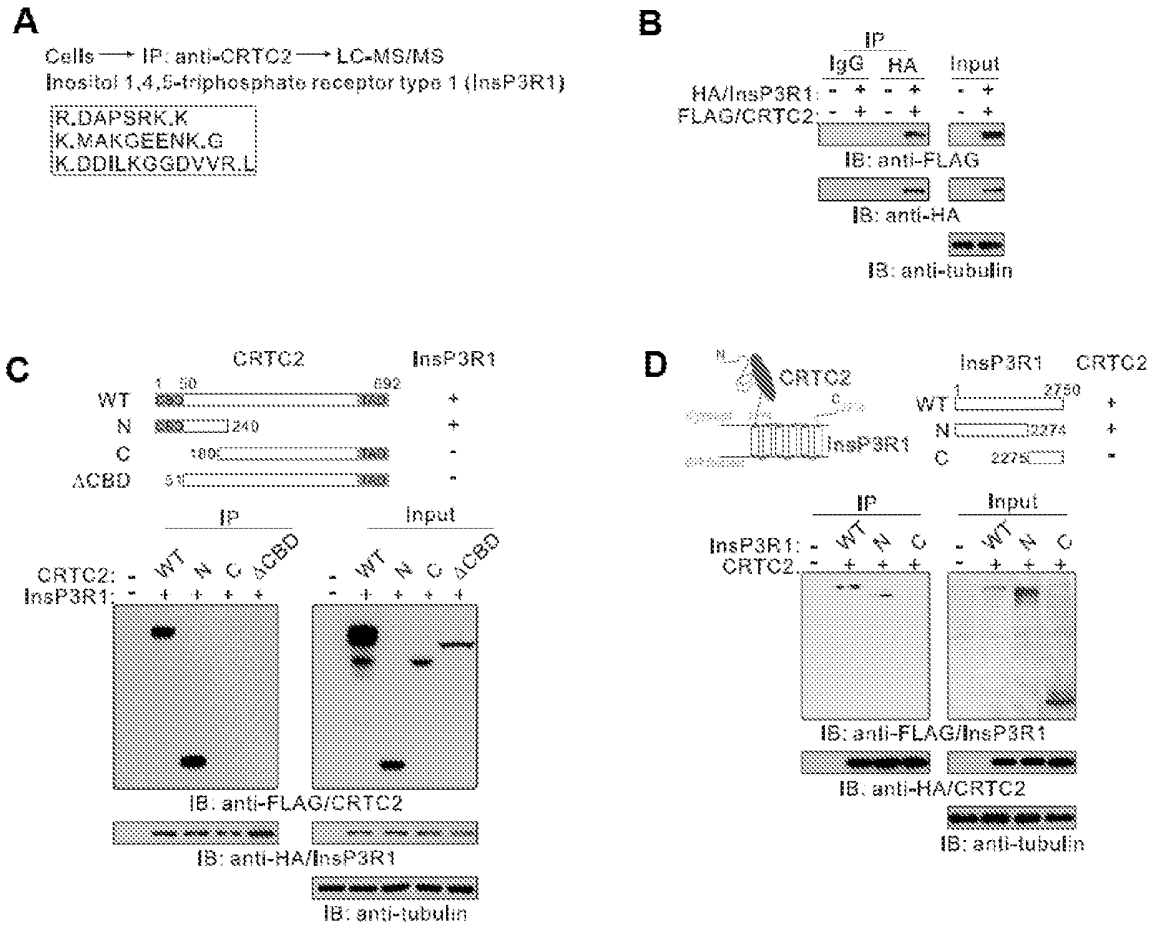


Figure 45A-45D

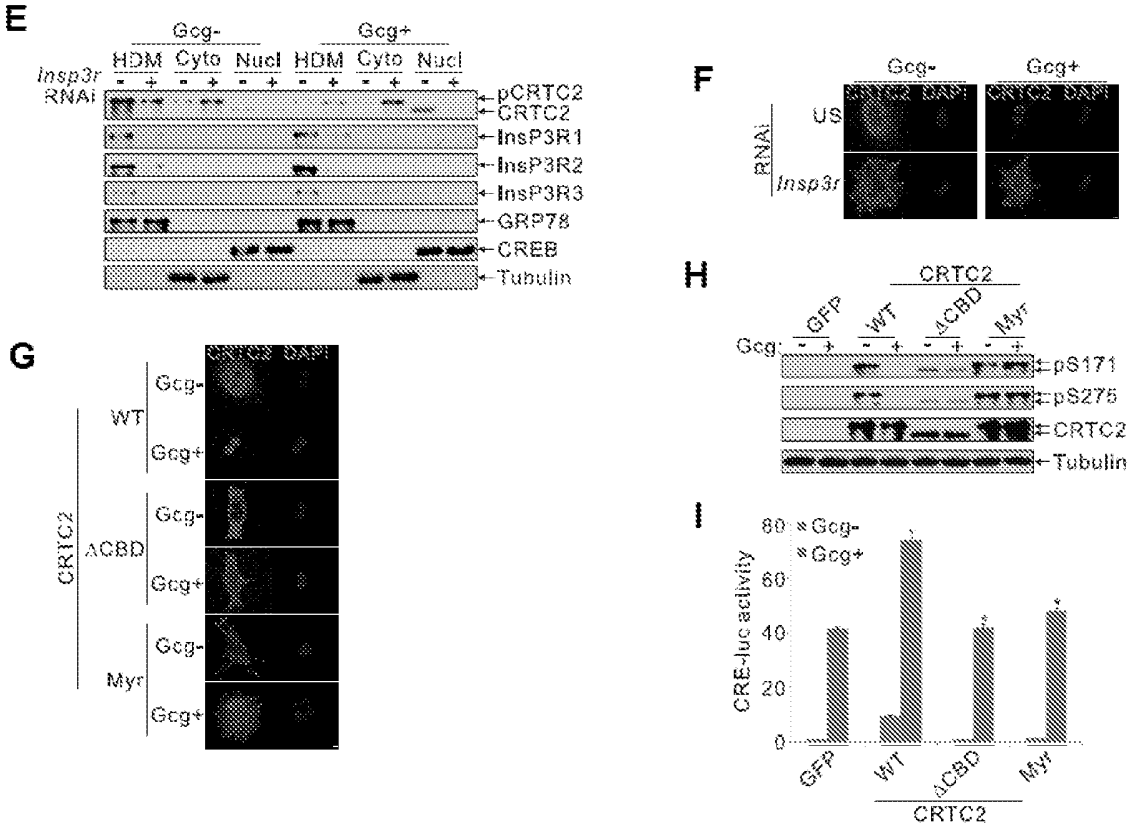


Figure 45E-45I

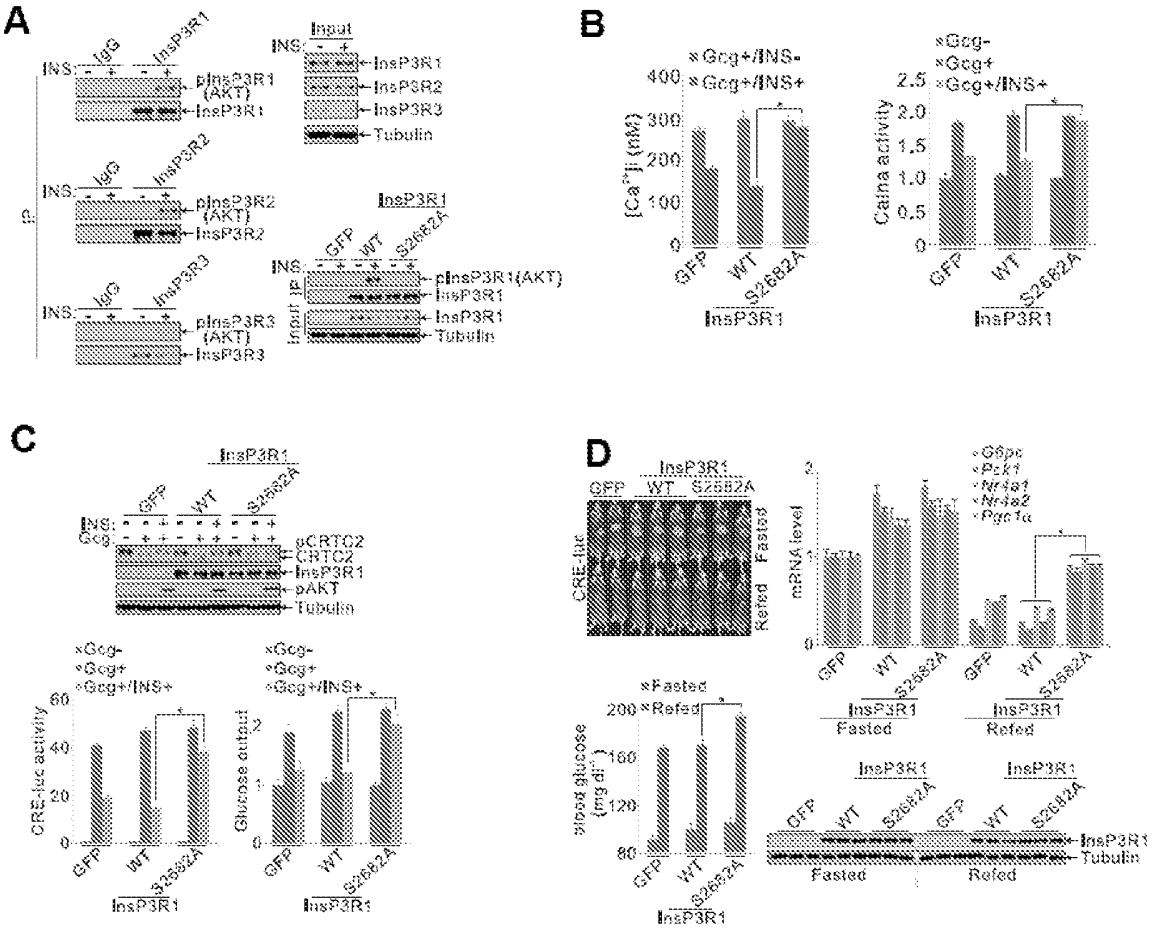


Figure 46A-46D

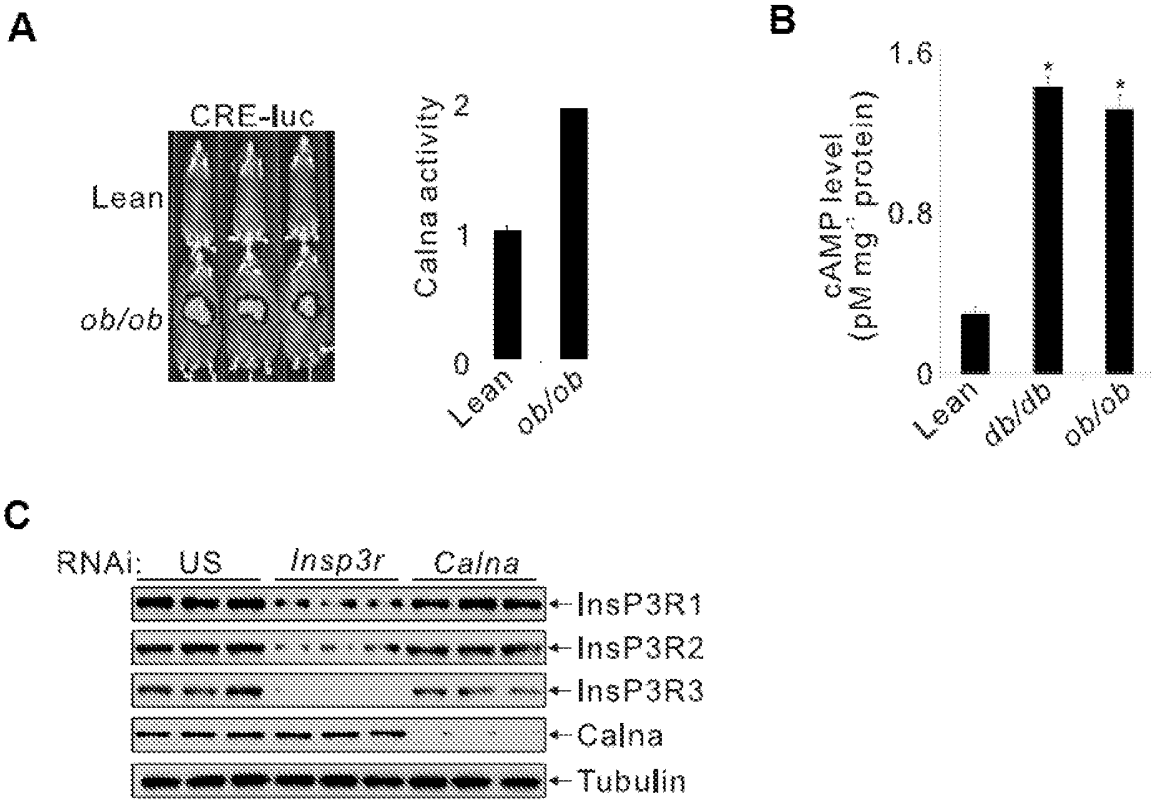


Figure 47A-47C

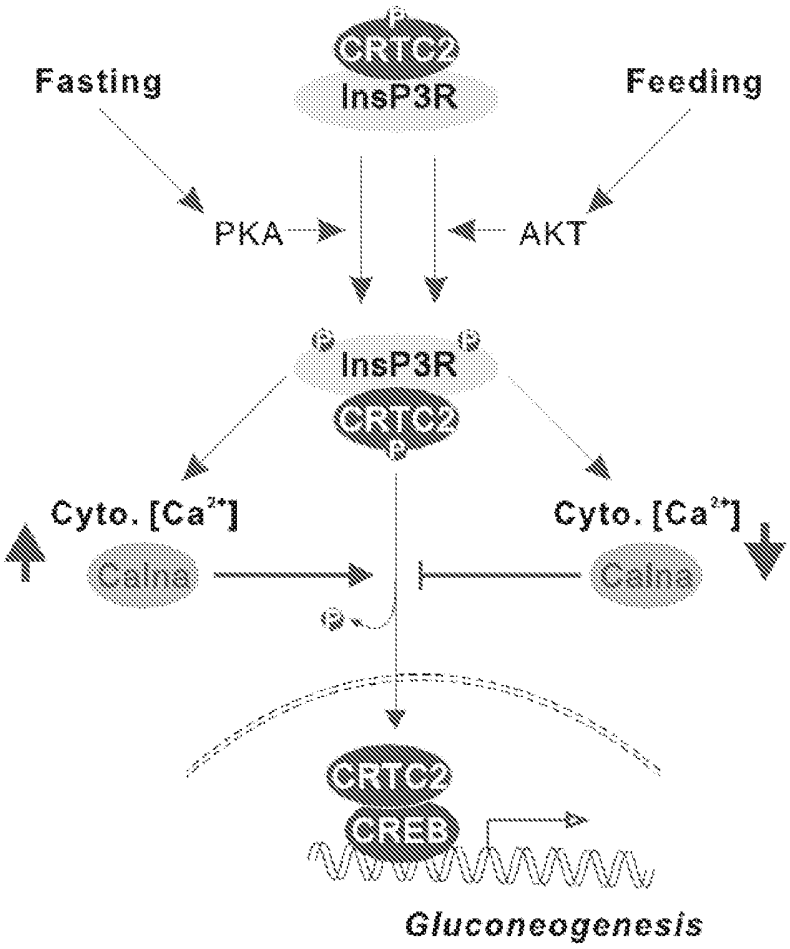
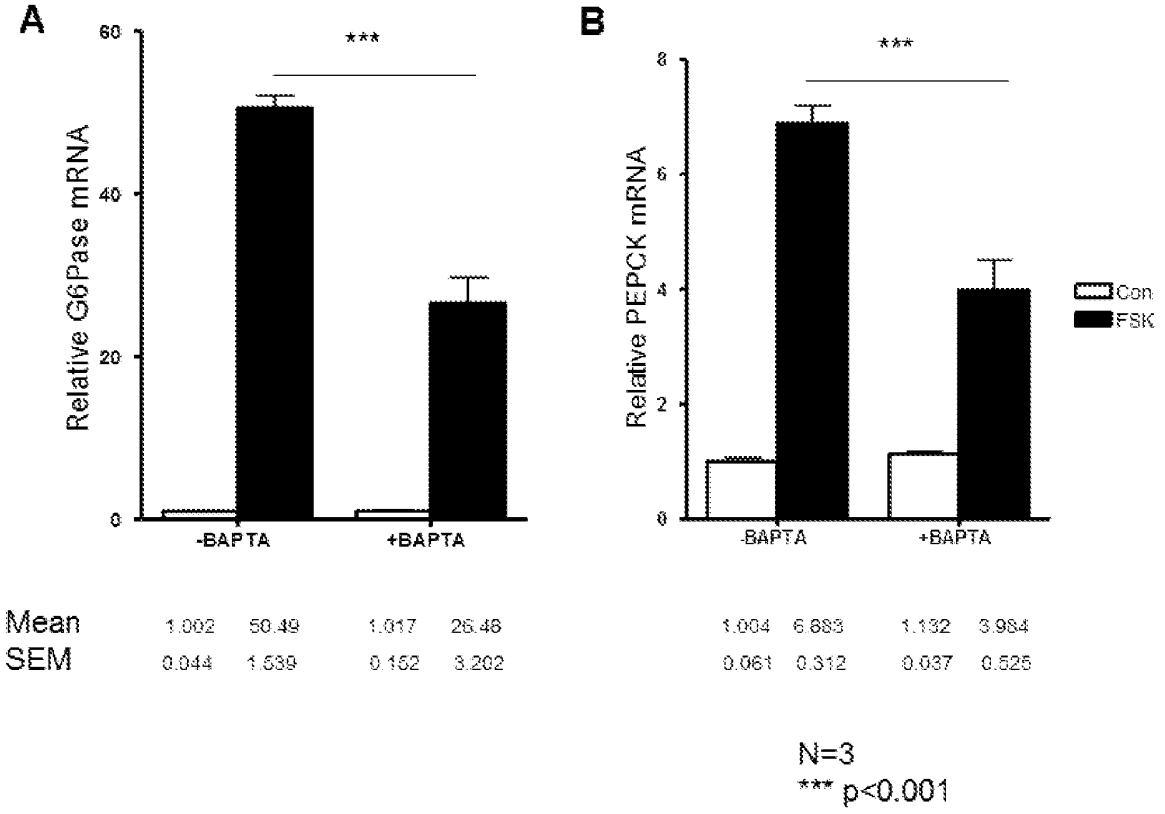
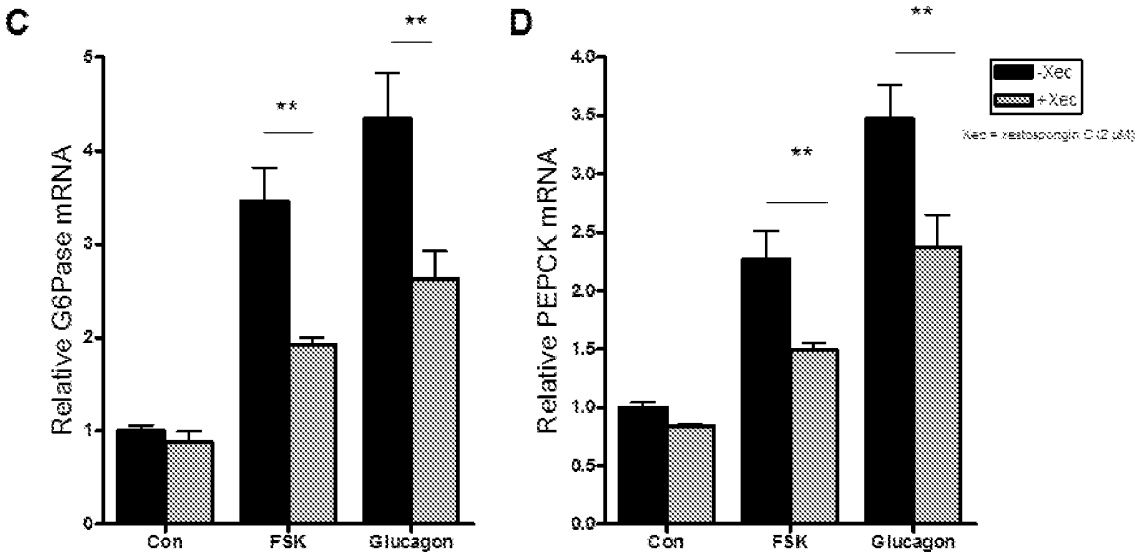

CRTC2
InsP3R
Fasting
Feeding
PKA
AKT
InsP3R
CRTC2
Cyto. [Ca2+]
Cyto. [Ca2+]
Calmo
Calmo
CRTC2
CREB
Gluconeogenesis

Figure 48



Figures 49A-49B



Mean±SEM	Con-G6P	FSK-G6P	Glucagon-G6P	Con-PEPCK	FSK-PEPCK	Glucagon-PEPCK
-Xec	1.004±0.06	3.462±0.357	4.34±0.486	1.002±0.042	2.273±0.238	3.476±0.287
+Xec	0.88±0.113	1.93±0.066	2.634±0.29	0.843±0.011	1.491±0.068	2.37±0.283

** p<0.01

Figure 49C-49D

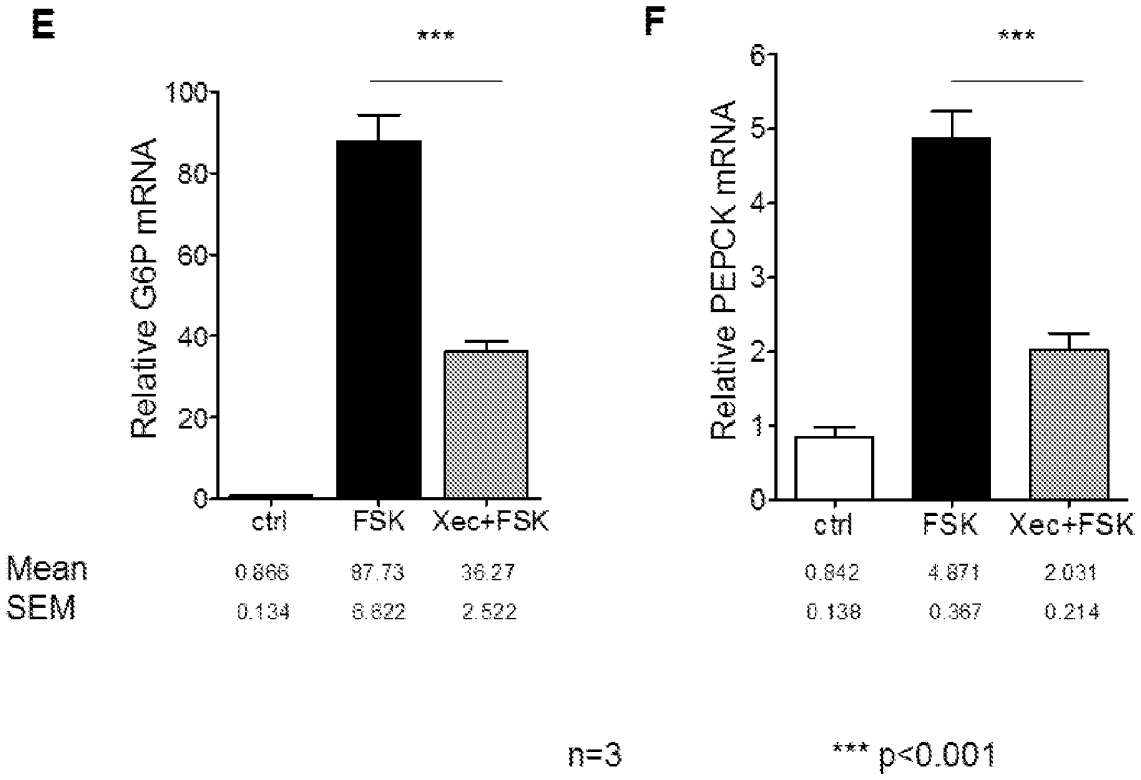


Figure 49E-49F

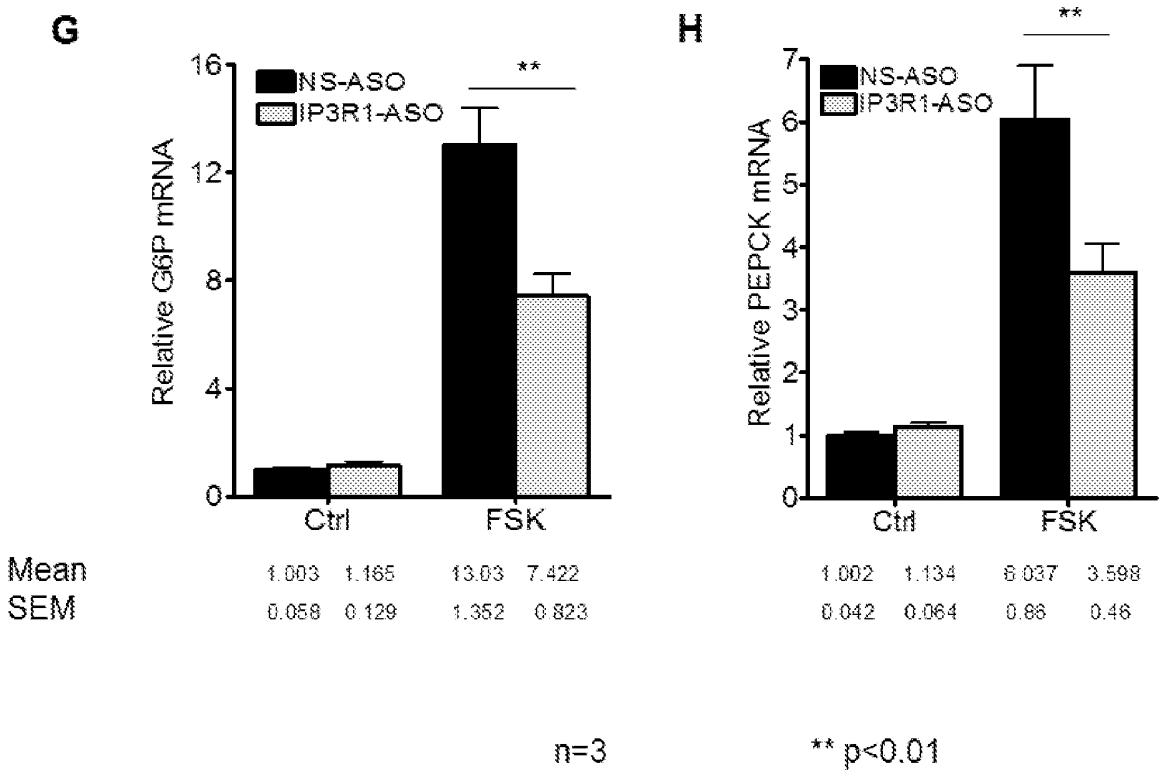


Figure 49G-49H

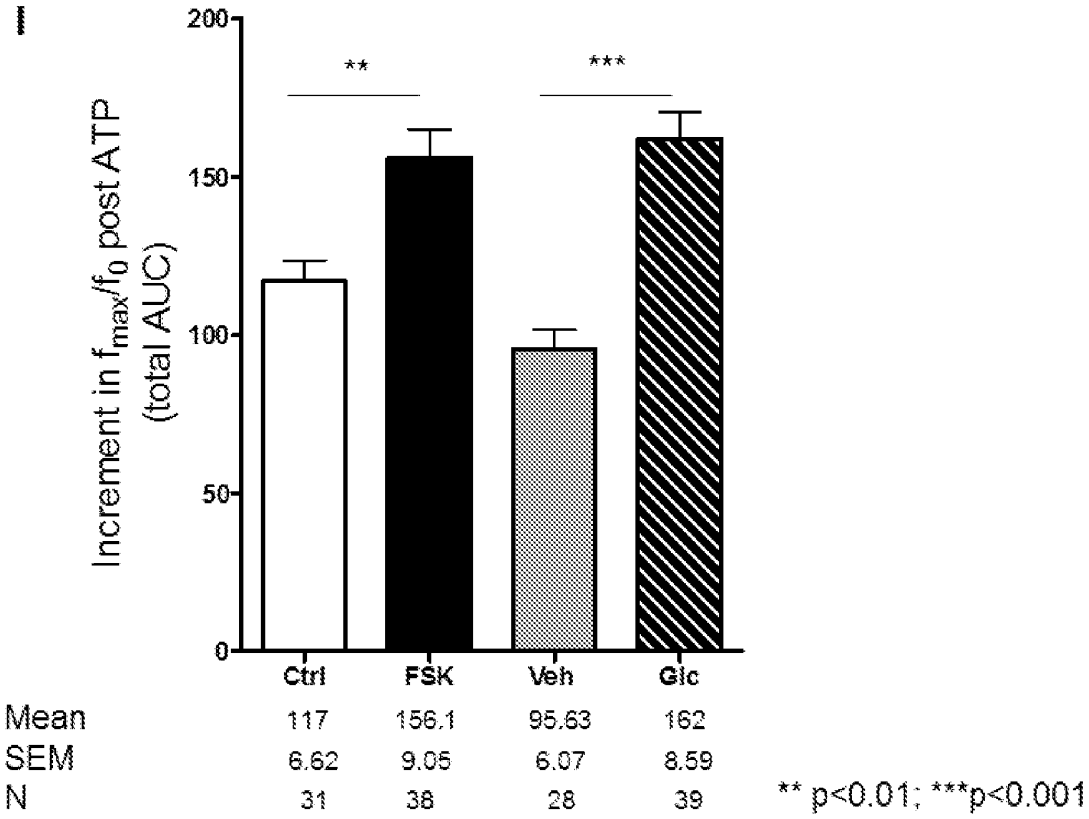

Increment in f_{max}/f_0 post ATP (total AUC)
200
150
100
50
0
**
***
Ctrl
FSK
Veh
Glc
Mean 117 156.1 95.63 162
SEM 6.62 9.05 6.07 8.59
N 31 38 28 39
** p<0.01; ***p<0.001

Figure 49I

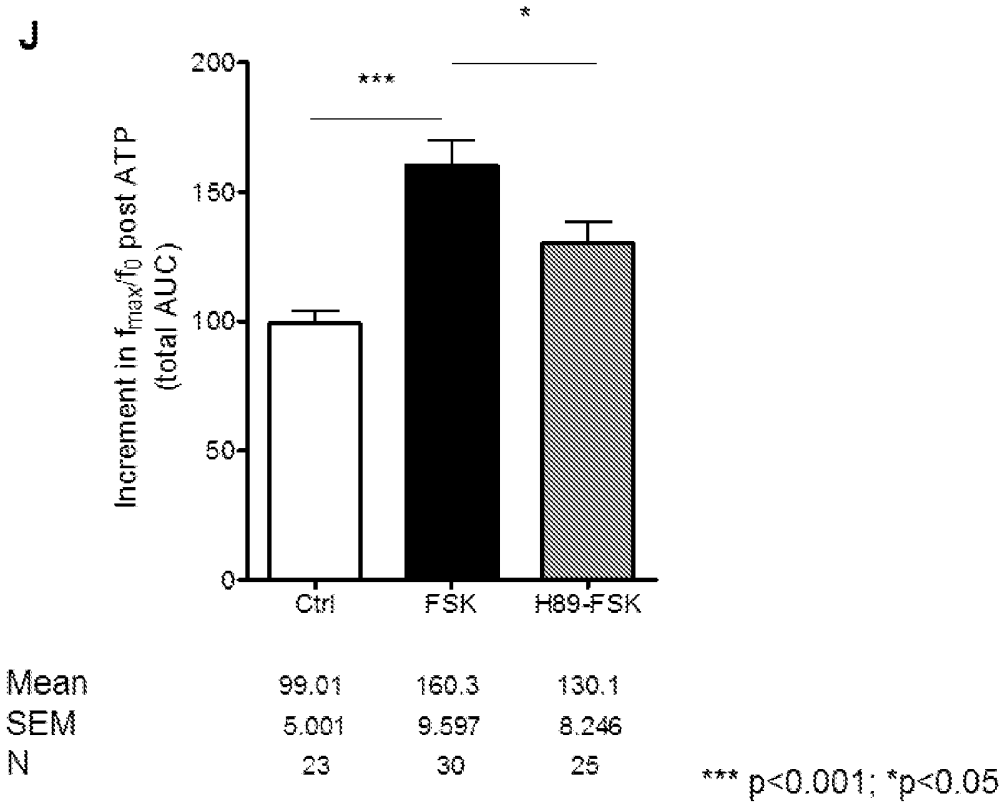
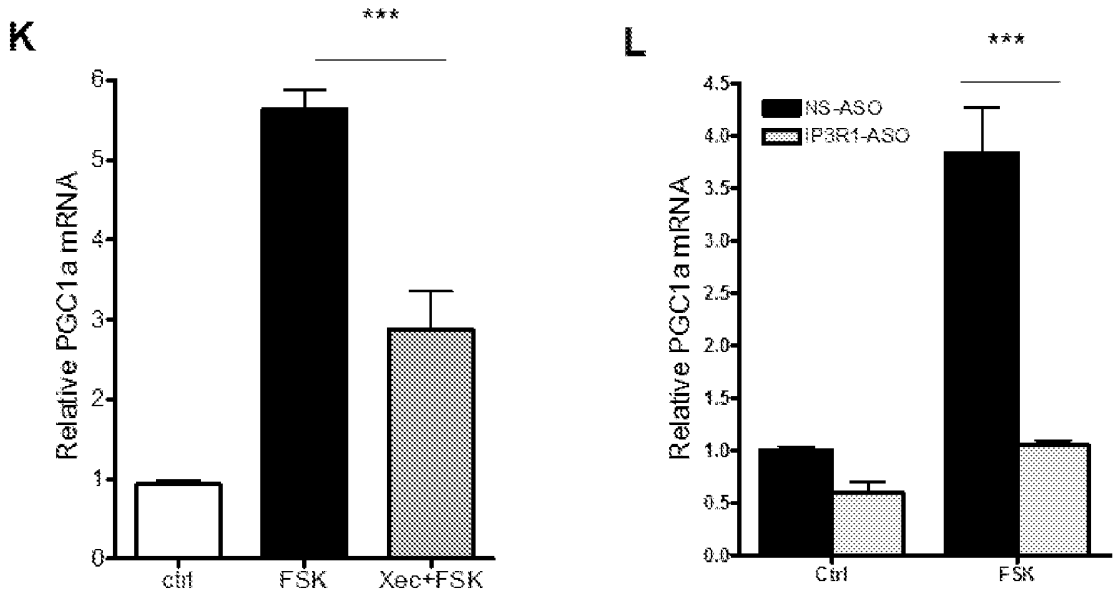

J
Increment in f_{max}/f_0 post ATP (total AUC)
200
150
100
50
0
***
*
Ctrl
FSK
H89-FSK
Mean 99.01 160.3 130.1
SEM 5.001 9.597 8.246
N 23 30 25
*** p<0.001; *p<0.05

Figure 49J



Mean	0.938	5.641	2.874		1.001	0.593	3.841	1.049
SEM	0.046	0.237	0.479		0.038	0.104	0.434	0.044

n=3 *** p<0.001

Figure 49K-49L

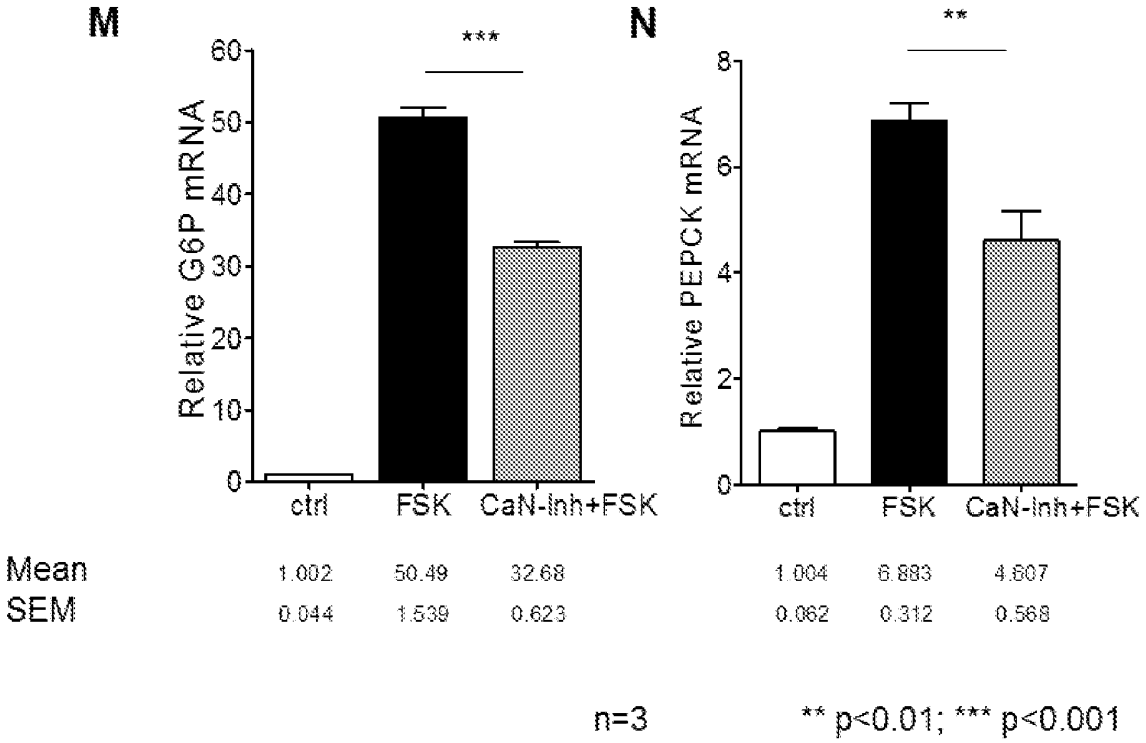
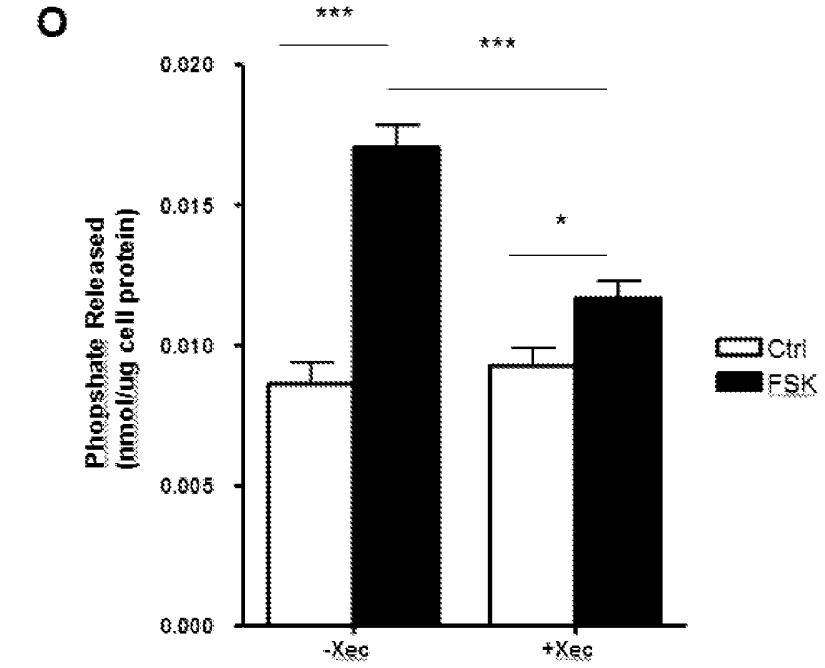


Figure 49M-49N



Mean	0.0086	0.017	0.0093	0.0117
SEM	0.00077	0.00081	0.00083	0.00082

n=3 * p<0.05; ** p<0.01; *** p<0.001

Figure 49O

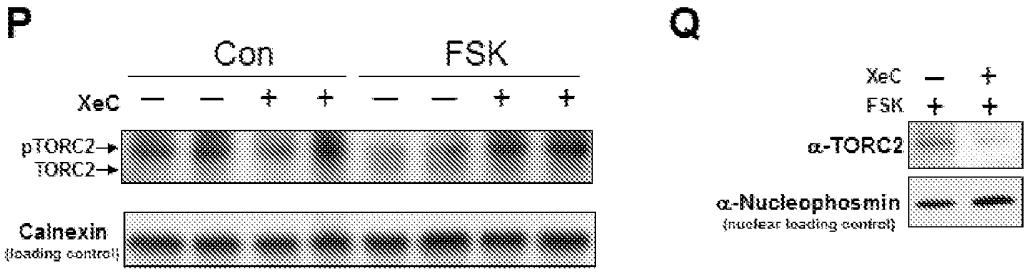
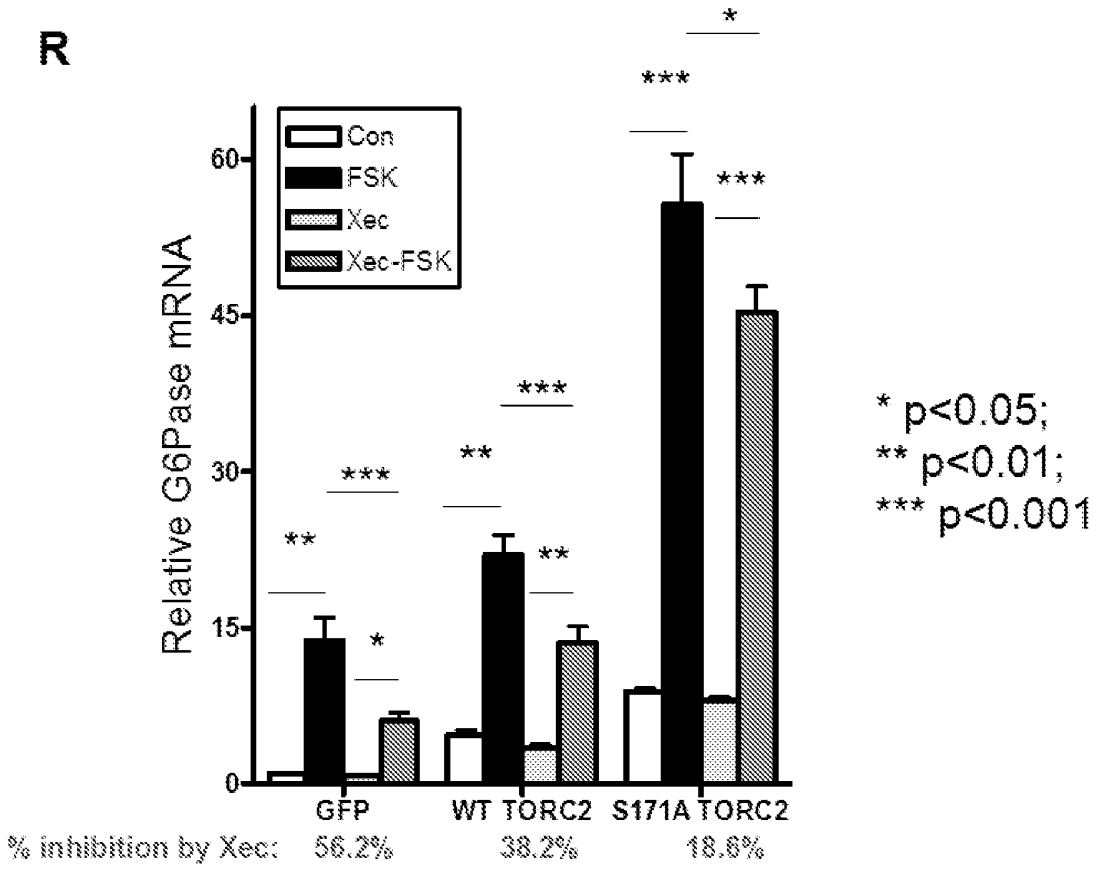
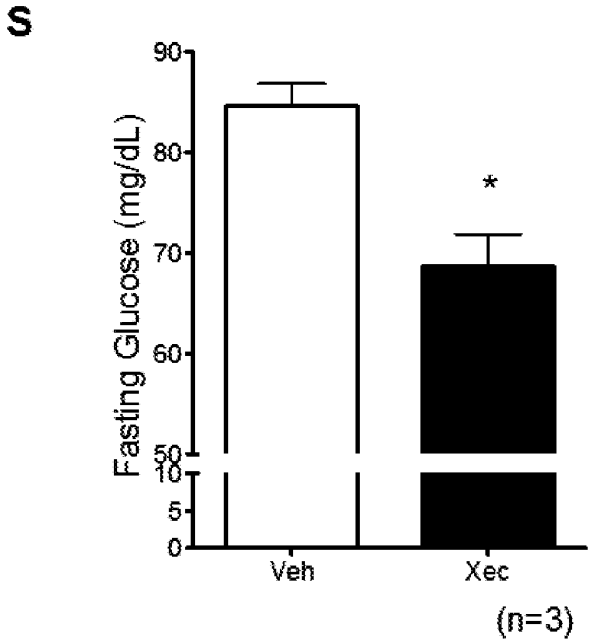


Figure 49P -49Q



Mean±SEM	Con	FSK	Xec	Xec-FSK
GFP	1.001±0.039	13.76±2.206	0.844±0.075	6.031±0.817
WT TORC2	4.755±0.436	21.98±1.913	3.472±0.278	13.59±1.535
S171A TORC2	8.925±0.26	55.64±4.85	7.96±0.352	45.29±2.5

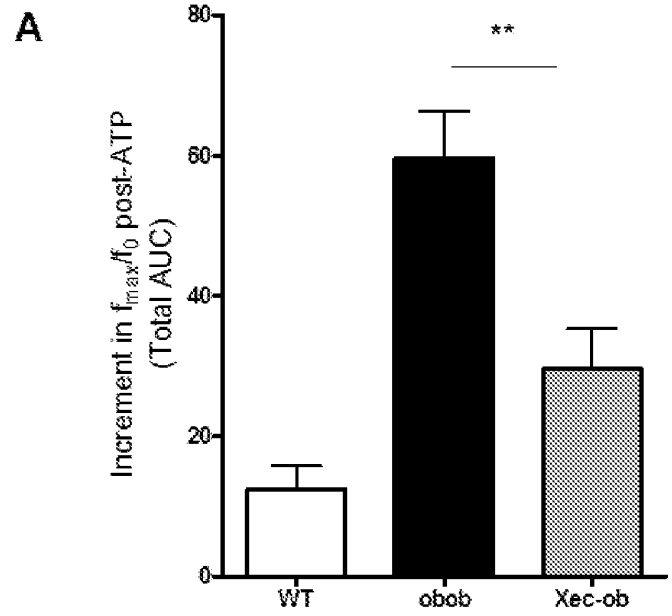
Figure 49R



Mean	84.67	68.67
SEM	2.186	3.18

* $p<0.05$

Figure 49S



	WT	obob	Xec-ob
Mean	12.43	59.47	29.55
SEM	3.31	6.883	5.703
N	12	20	15

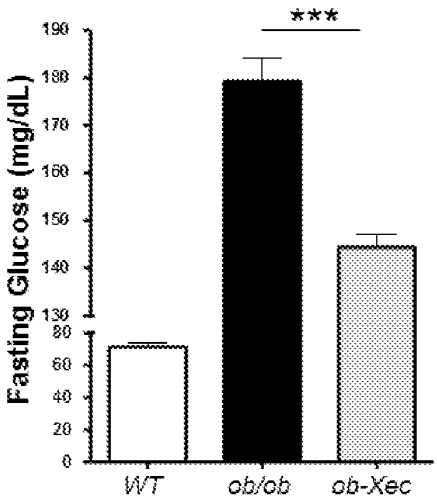
Peritoneal macrophages

** $p<0.01$

Figure 50A

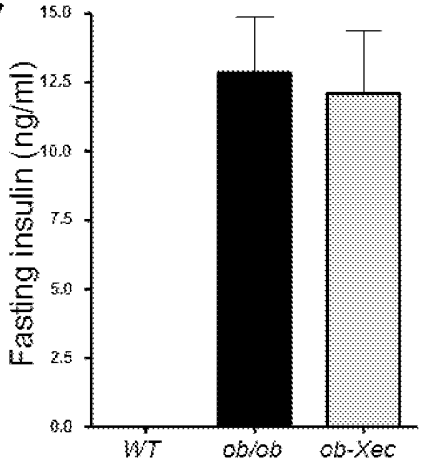
B

	Mean	SEM
WT	71.2	1.985
Ob/ ob	179.2	4.934
Xec -ob	144.4	2.638



(n=5) *** p<0.001

C

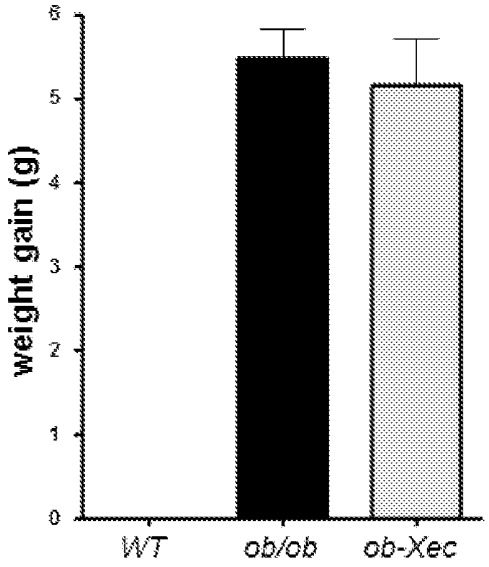


	Mean (ng/ml)	SEM
WT		
Ob/ ob	12.85	2.012
Xec -ob	12.09	2.274

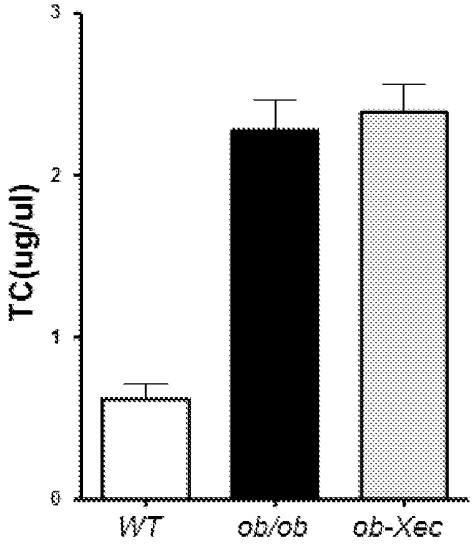
Figure 50B-50C

D

	Mean	SEM
WT		
Ob/ ob	5.48	0.35
Xec -ob	5.16	0.552



E



	Mean	SEM
WT	0.619	0.094
Ob/ ob	2.278	0.184
Xec -ob	2.395	0.171

Figure 50D-50E

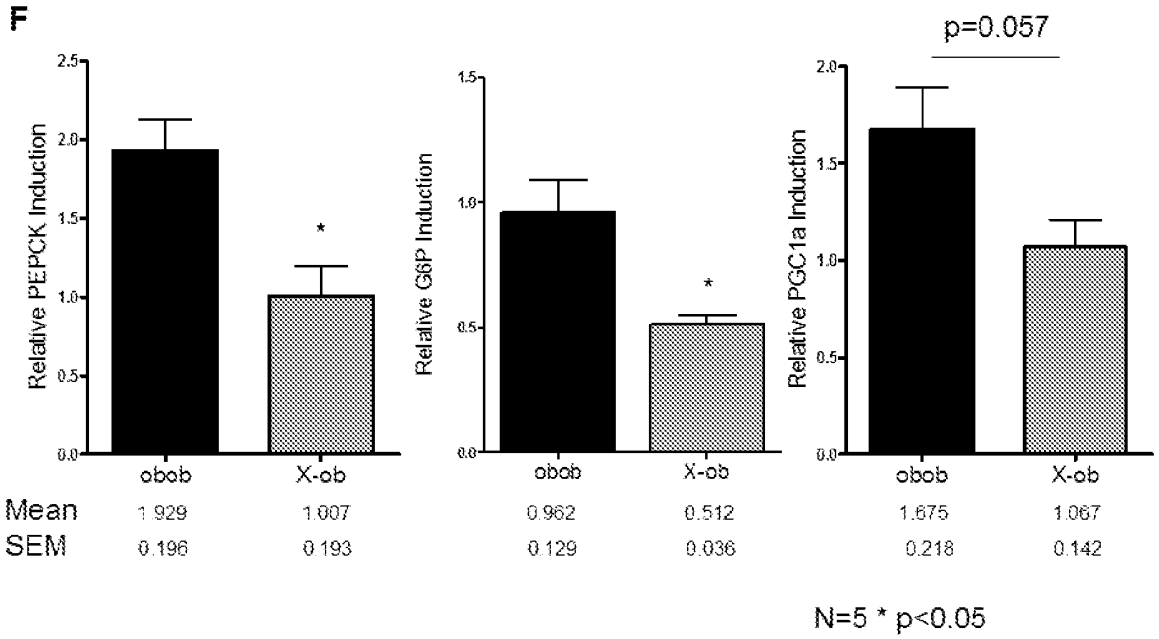
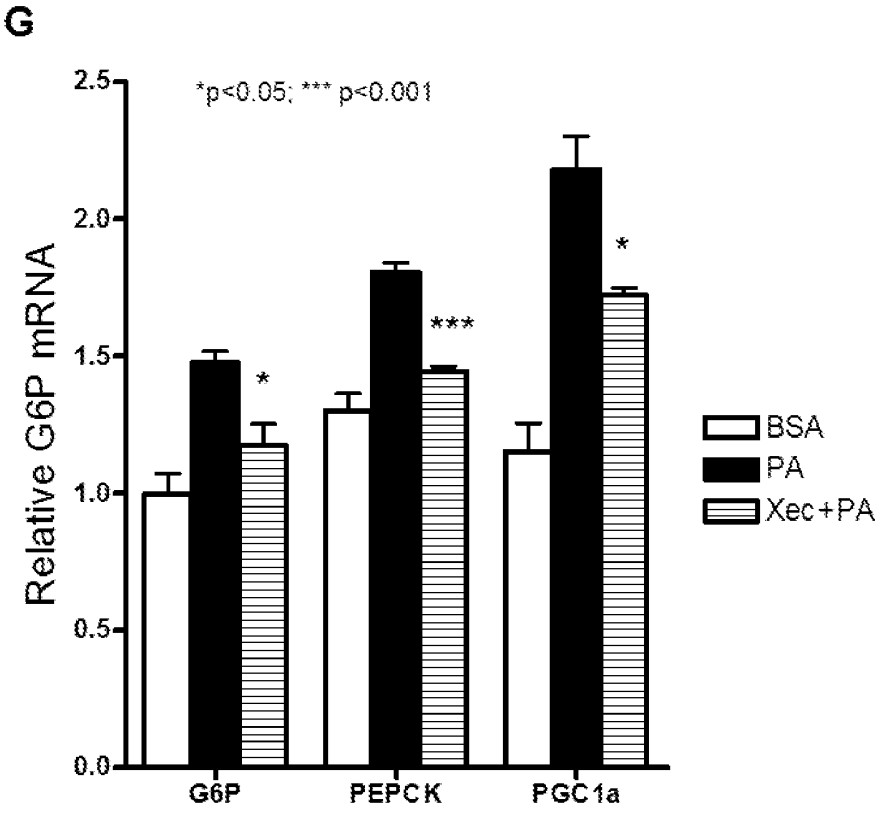

F
Relative PEPCK Induction
2.5
2.0
1.5
1.0
0.5
0.0
*
obob
X-ob
Relative G6P Induction
1.5
1.0
0.5
0.0
*
obob
X-ob
p=0.057
Relative PGC1a Induction
2.0
1.5
1.0
0.5
0.0
obob
X-ob
Mean 1.929 1.007 0.962 0.512 1.675 1.067
SEM 0.196 0.193 0.129 0.036 0.218 0.142
N=5 * p<0.05

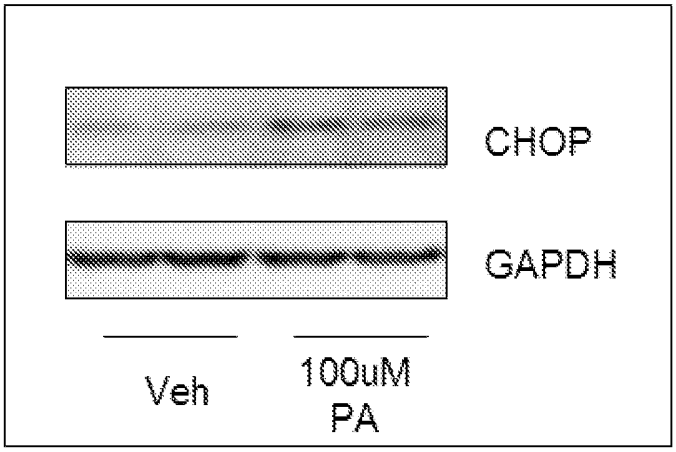
Figure 50F



Mean±SEM	BSA	PA	Xec-PA
G6P	0.999±0.073	1.475±0.042	1.175±0.077
PEPCK	1.301±0.063	1.807±0.033	1.444±0.018
PGC1a	1.153±0.103	2.058±0.232	1.456±0.049

Figure 50G

H



I

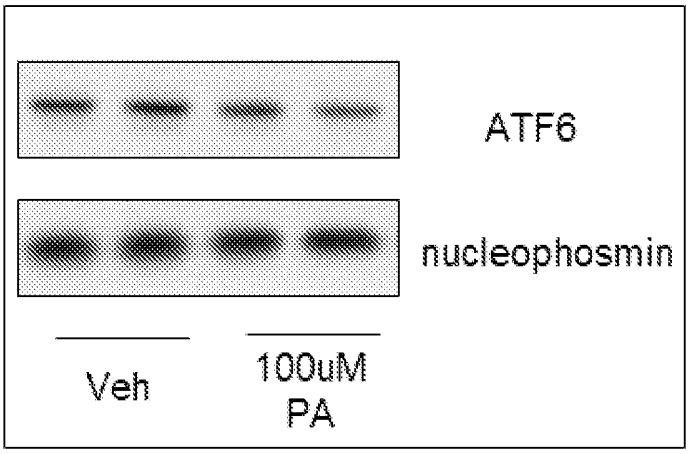
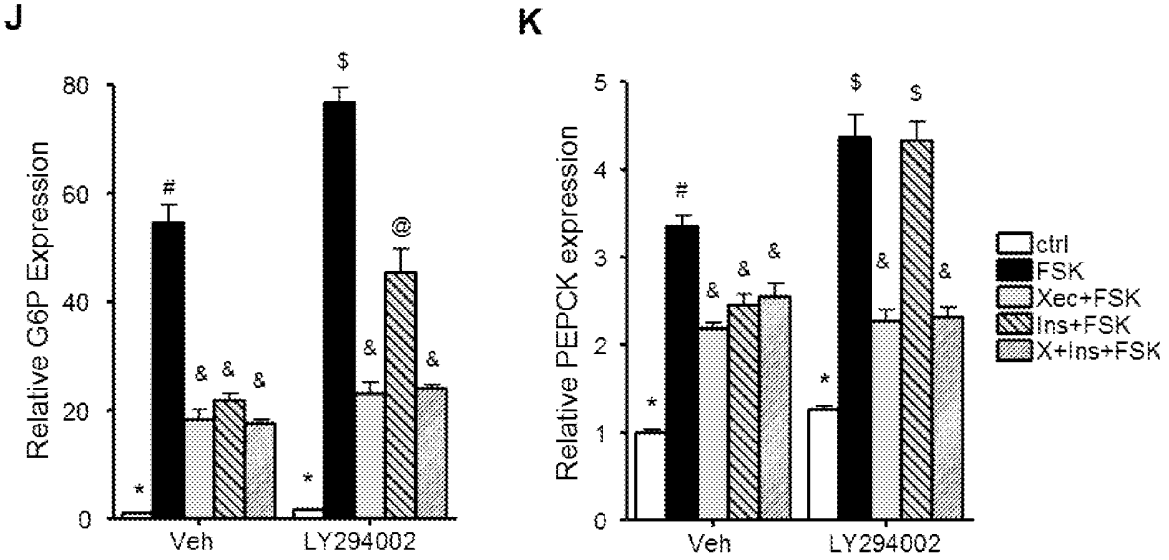


Figure 50H-50I



G6Pase	Ctrl	FSK	Xec+FSK	Ins+FSK	X-Ins+FSK
Veh	1.001± 0.0365	54.53 ±3.366	18.24 ±1.868	21.8 ±1.288	17.54 ±0.741
LY	1.666±0.0168	70.74 ±2.778	23.05 ±2.142	45.36 ±4.423	23.94 ±0.79

PEPCK	Ctrl	FSK	Xec+FSK	Ins+FSK	X-Ins+FSK
Veh	1.001± 0.0338	3.353 ±0.122	2.186 ±0.066	2.452 ±0.127	2.547 ± 0.154
LY	1.261±0.043	4.362 ±0.263	2.269 ±0.133	4.328 ±0.22	2.314 ±0.114

Figure 50J-50K

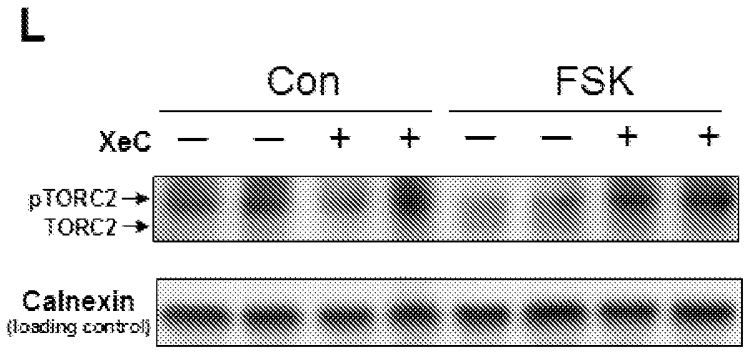
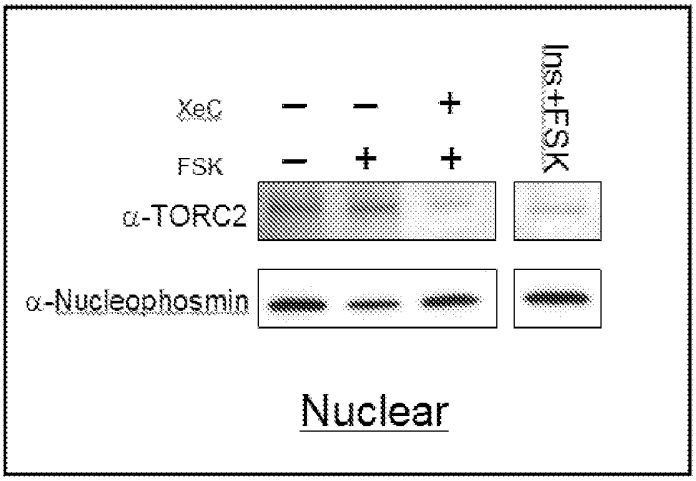


Figure 50L

M



Primary HCs

Figure 50M

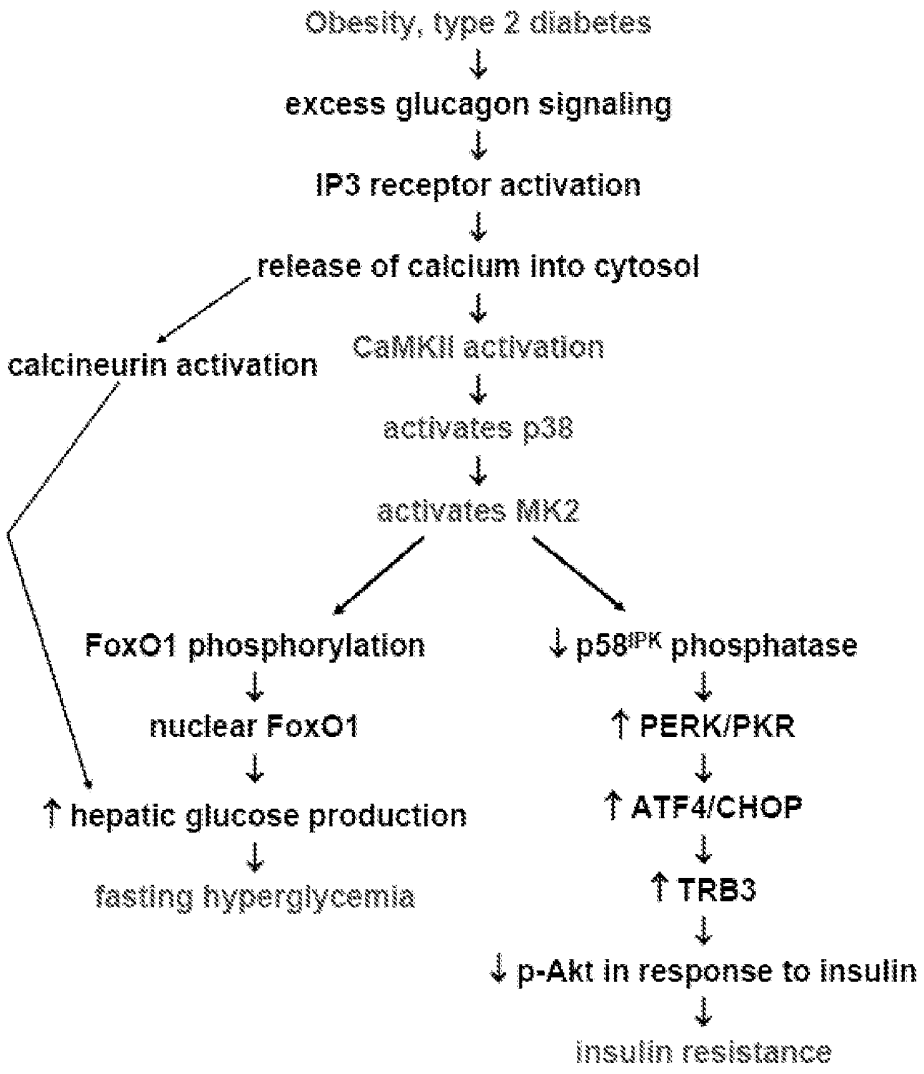
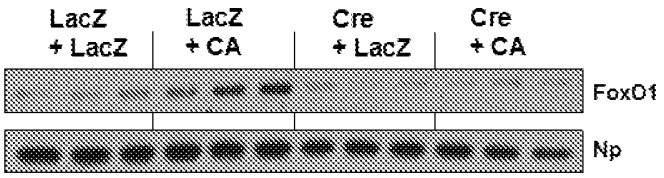

Obesity, type 2 diabetes
excess glucagon signaling
IP3 receptor activation
release of calcium into cytosol
calcineurin activation
CaMKII activation
activates p38
activates MK2
FoxO1 phosphorylation
nuclear FoxO1
↑ hepatic glucose production
fasting hyperglycemia
↓ p58IPK phosphatase
↑ PERK/PKR
↑ ATF4/CHOP
↑ TRB3
↓ p-Akt in response to insulin
insulin resistance

Figure 51



p38$^{flx/}$ primary hepatocytes

Figure 52

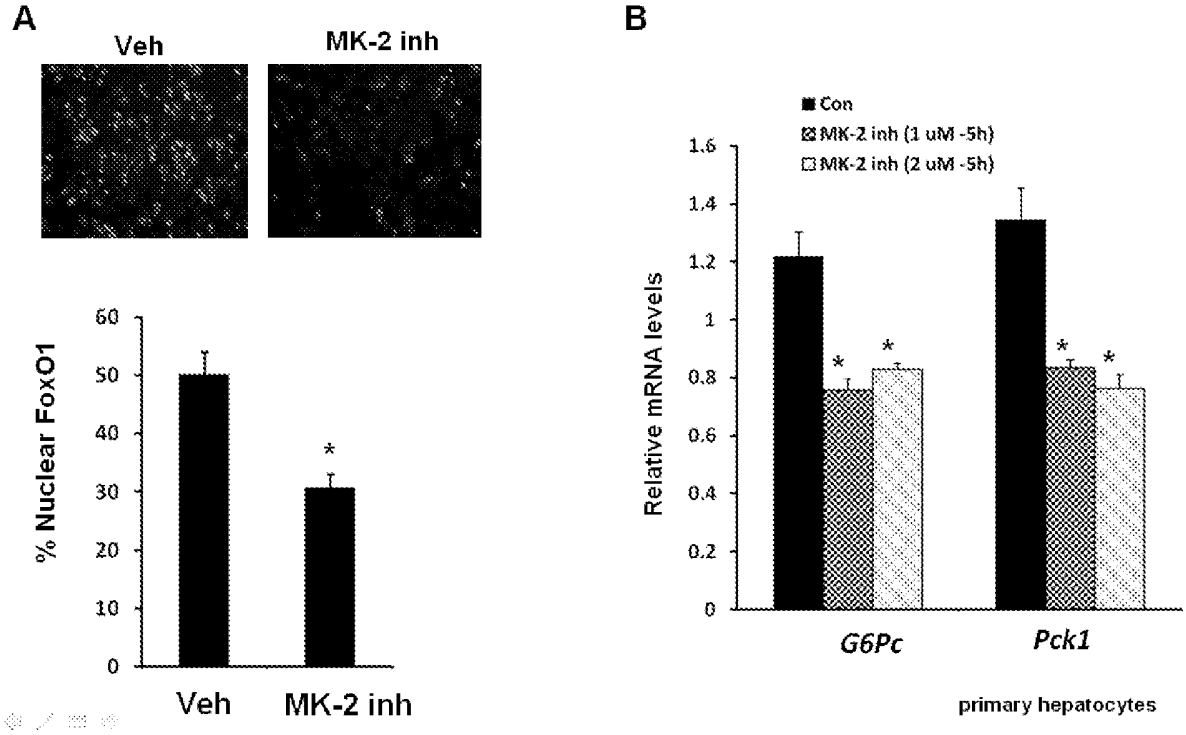


Figure 53A-53B

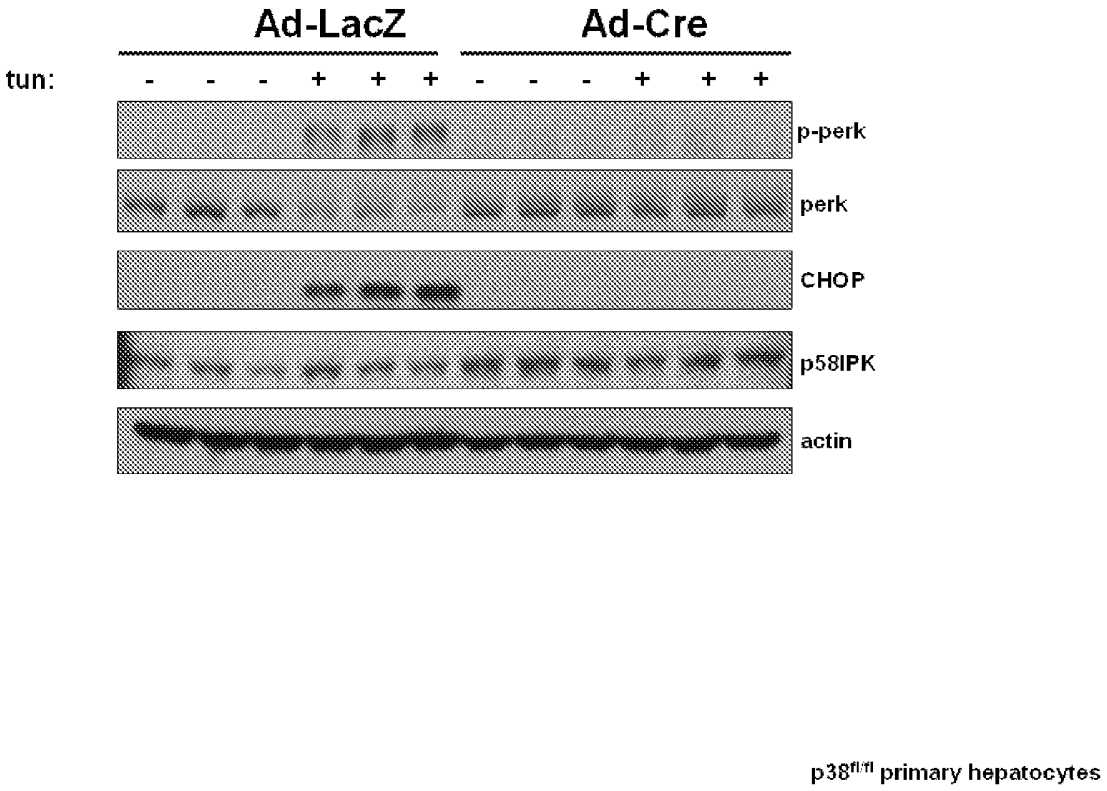

Ad-LacZ
Ad-Cre
tun:
- - - + + + - - - + + +
p-perk
perk
CHOP
p58IPK
actin
p38fl/fl primary hepatocytes

Figure 54

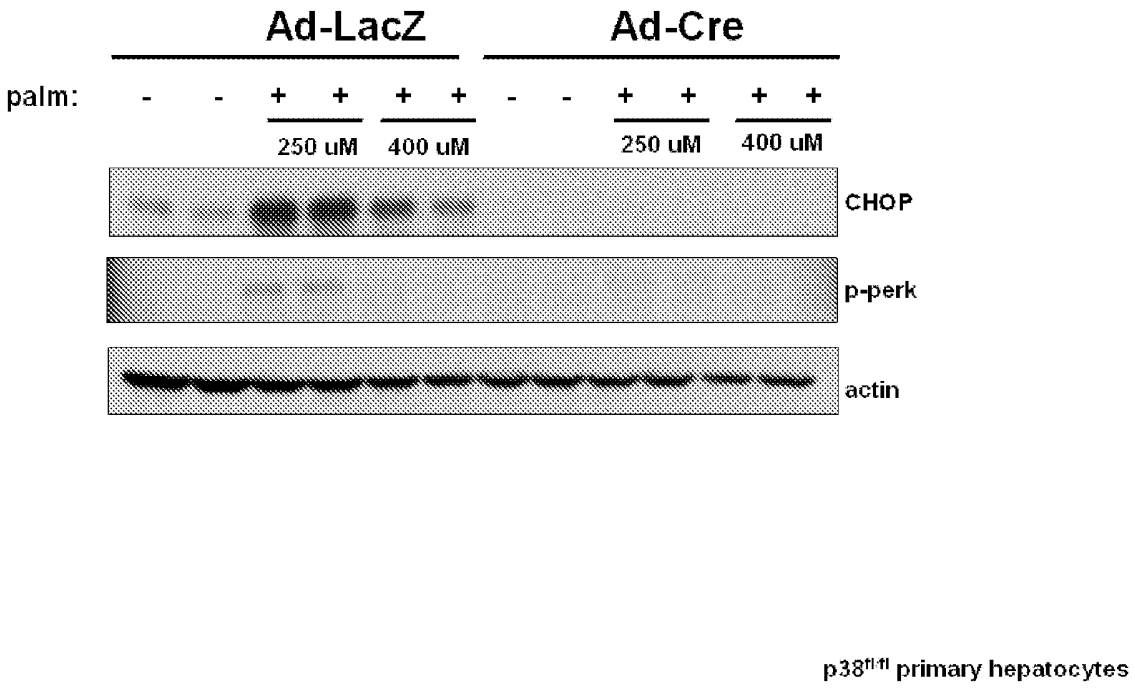

Ad-LacZ
Ad-Cre
palm:
- - + + + + - - + + + +
250 uM
400 uM
250 uM
400 uM
CHOP
p-perk
actin
p38fl/fl primary hepatocytes

Figure 55

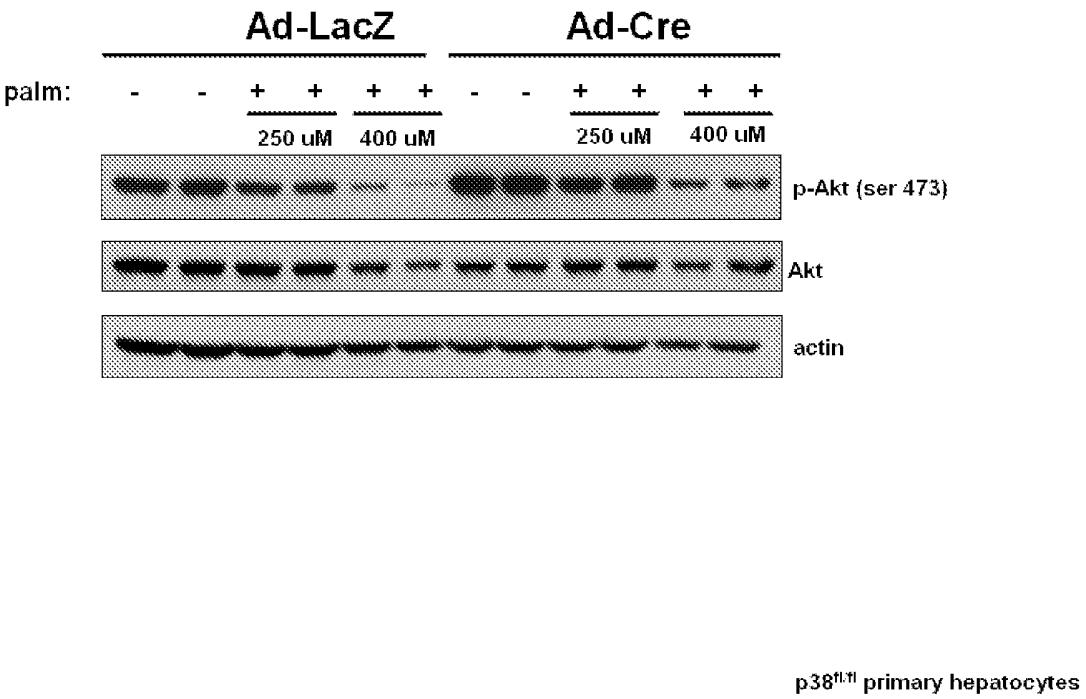

Ad-LacZ
Ad-Cre
palm:
- - + + + + - - + + + +
250 uM 400 uM
250 uM 400 uM
p-Akt (ser 473)
Akt
actin
p38fl/fl primary hepatocytes

Figure 56

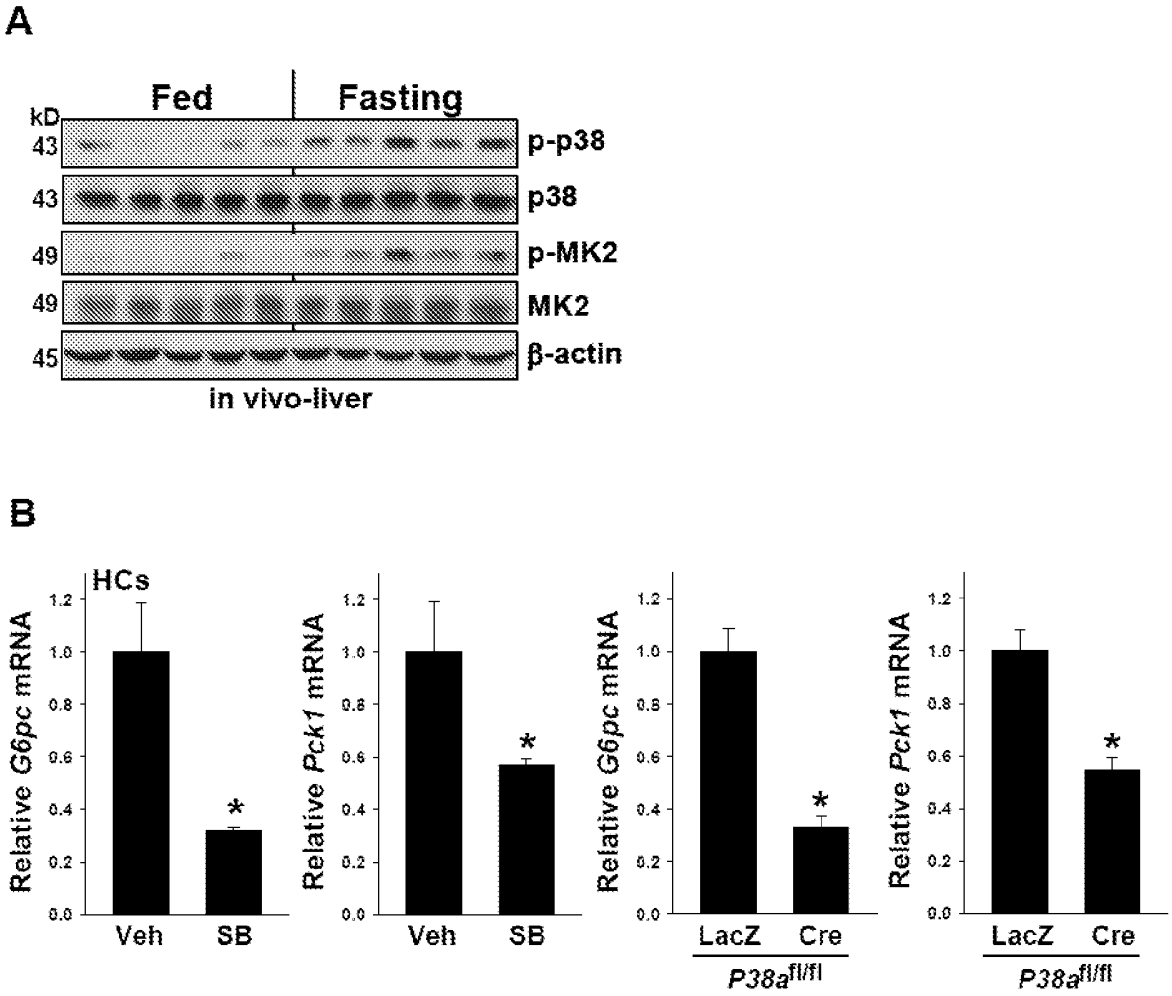
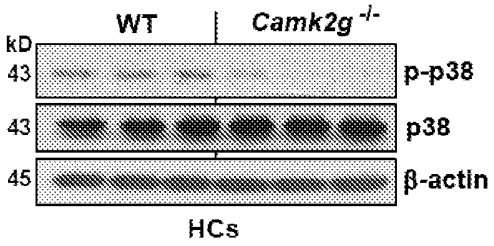


Figure 57A-57B

C



D

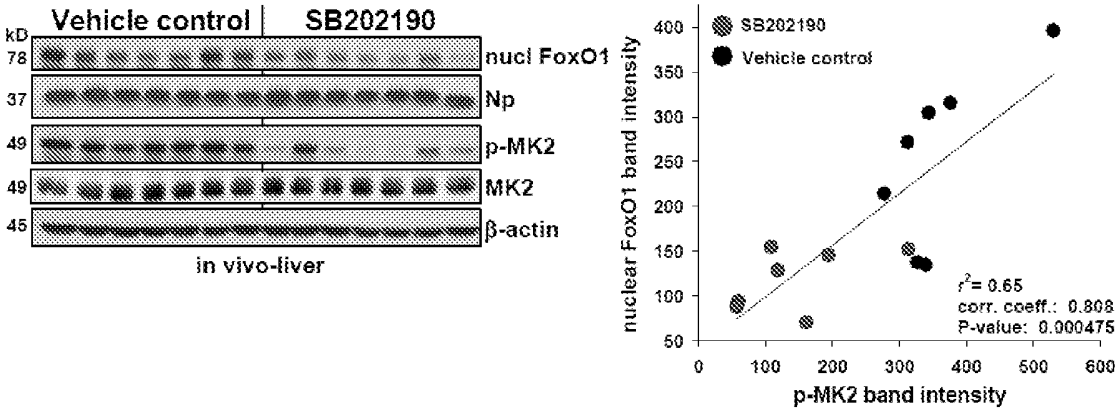


Figure 57C-57D

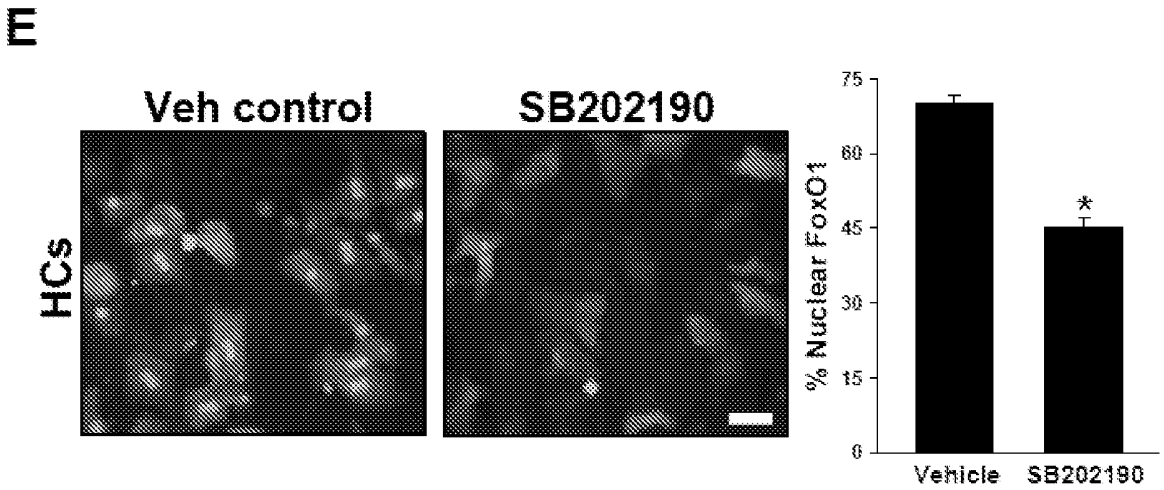

E
Veh control
SB202190
HCs
% Nuclear FoxO1
75
60
45
30
15
0
*
Vehicle
SB202190

Figure 57E

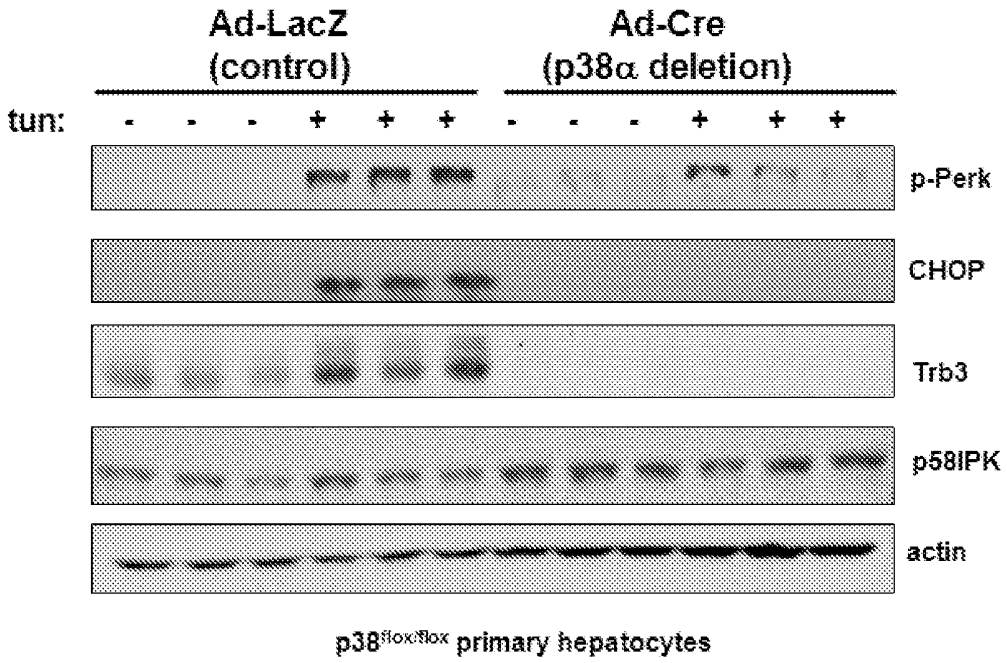

Ad-LacZ
(control)
Ad-Cre
(p38α deletion)
tun:
- - - + + + - - - + + +
p-Perk
CHOP
Trb3
p58IPK
actin
p38flox/flox primary hepatocytes

Figure 58

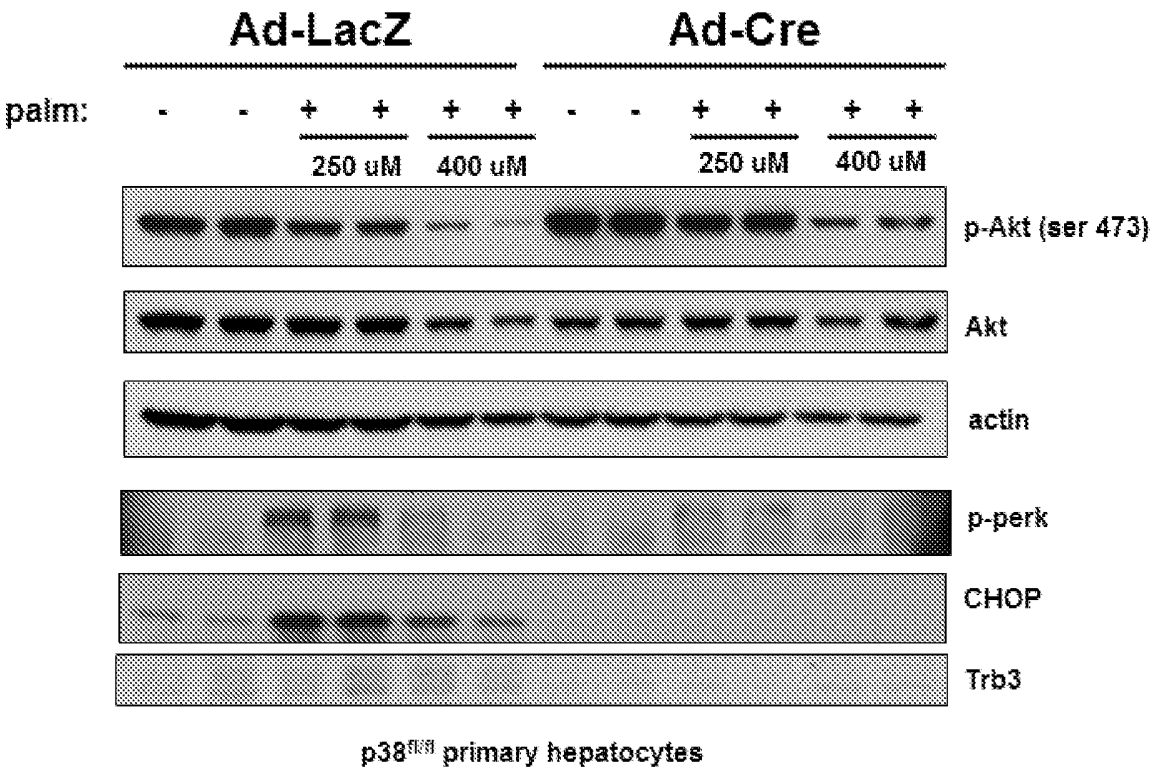

Ad-LacZ
Ad-Cre
palm:
- - + + + + - - + + + +
250 uM
400 uM
250 uM
400 uM
p-Akt (ser 473)
Akt
actin
p-perk
CHOP
Trb3
p38fl/fl primary hepatocytes

Figure 59

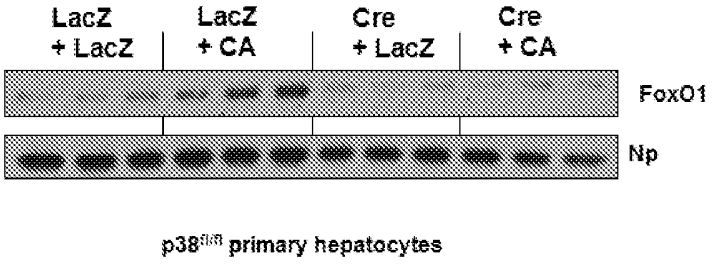

LacZ
+ LacZ
LacZ
+ CA
Cre
+ LacZ
Cre
+ CA
FoxO1
Np
p38fl/fl primary hepatocytes

Figure 60

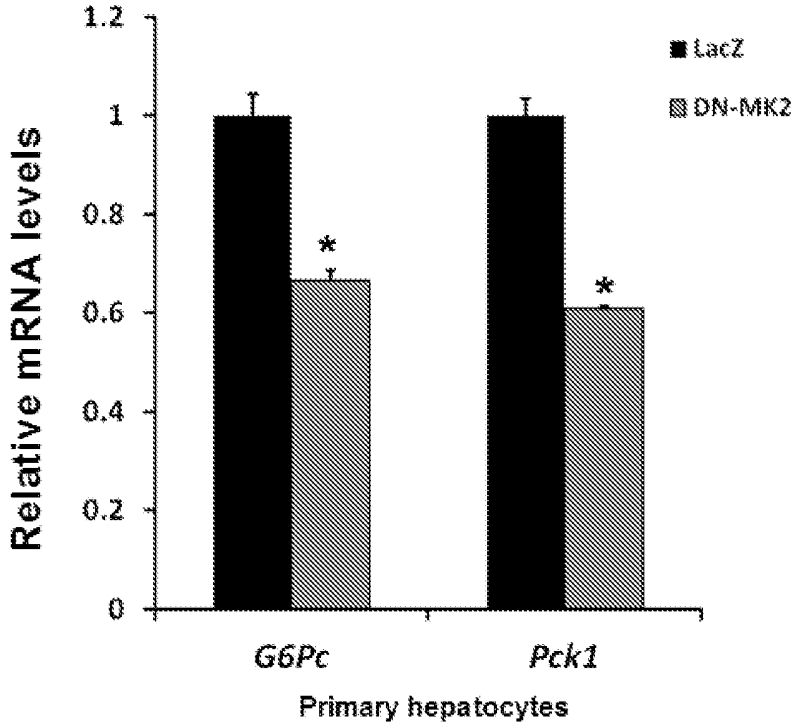

1.2
1
0.8
0.6
0.4
0.2
0
Relative mRNA levels
LacZ
DN-MK2
*
*
G6Pc
Pck1
Primary hepatocytes

Figure 61

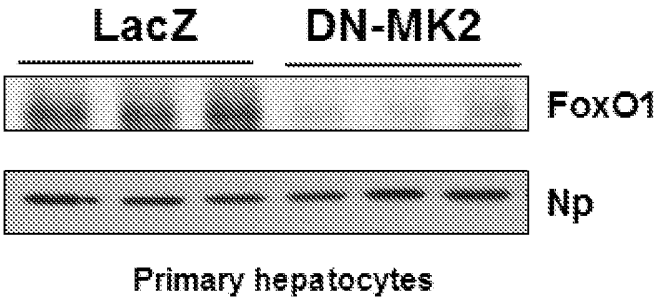


Figure 62

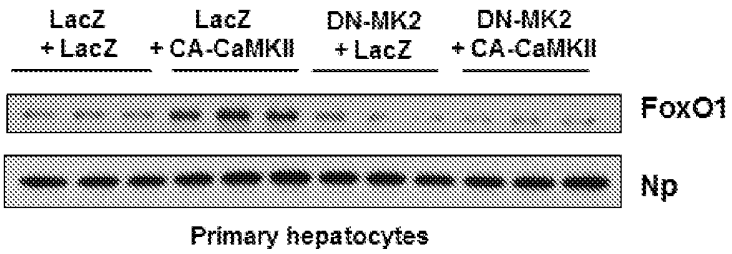

LacZ
+ LacZ
LacZ
+ CA-CaMKII
DN-MK2
+ LacZ
DN-MK2
+ CA-CaMKII
FoxO1
Np
Primary hepatocytes

Figure 63

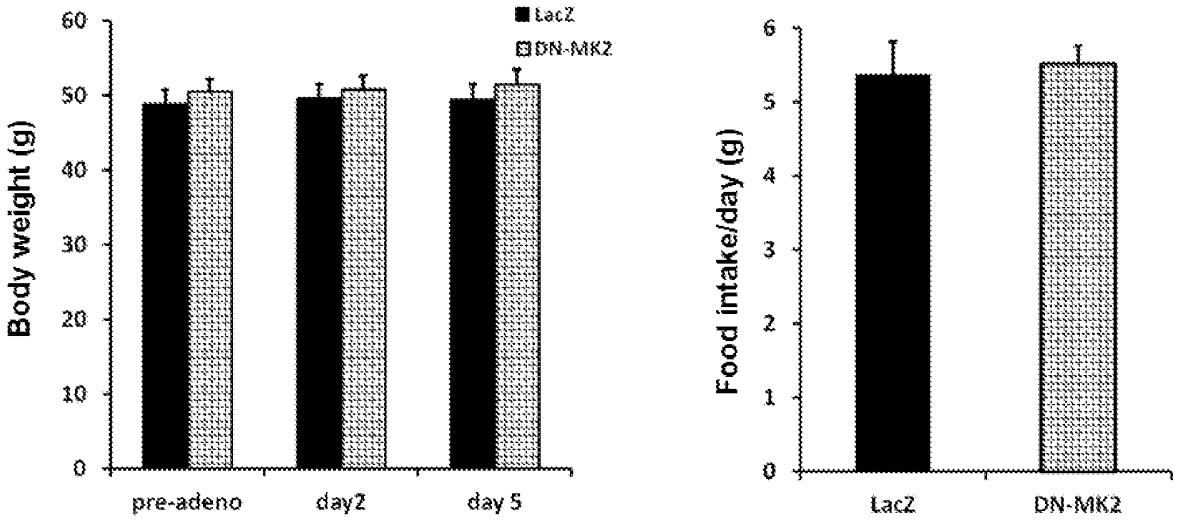

LacZ
DN-MK2
Body weight (g)
60
50
40
30
20
10
0
pre-adeno
day2
day 5
Food intake/day (g)
6
5
4
3
2
1
0
LacZ
DN-MK2

Figure 64

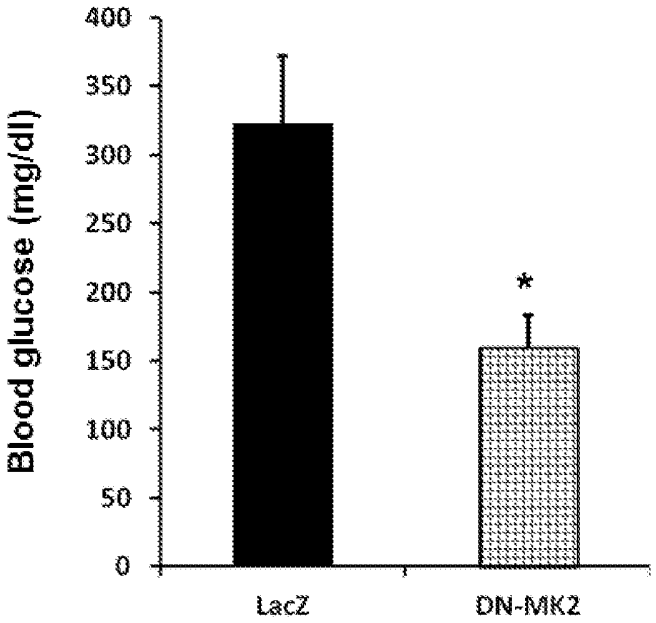


Figure 65

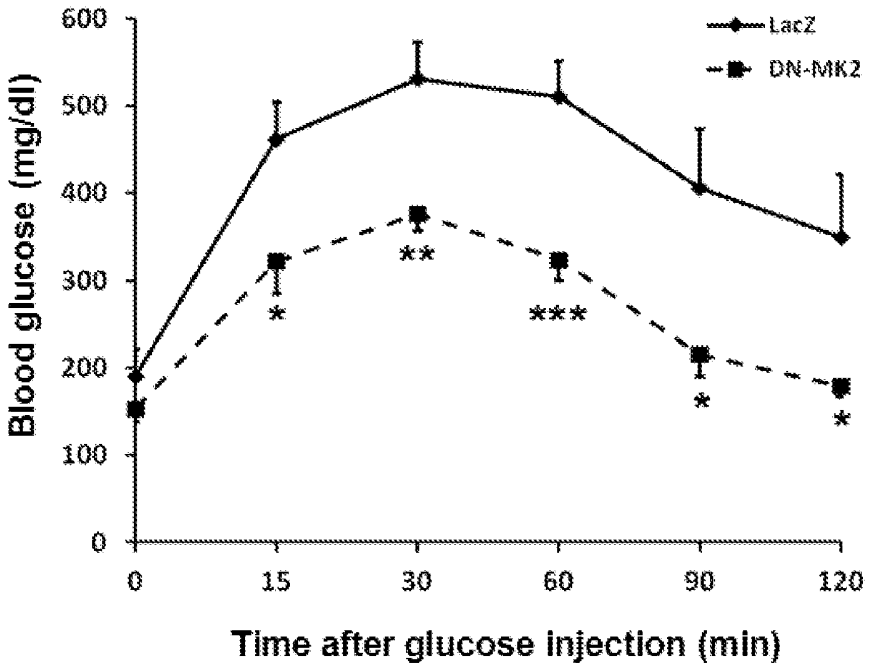

LacZ
DN-MK2
Blood glucose (mg/dl)
600
500
400
300
200
100
0
*
**
***
*
*
0
15
30
60
90
120
Time after glucose injection (min)

Figure 66

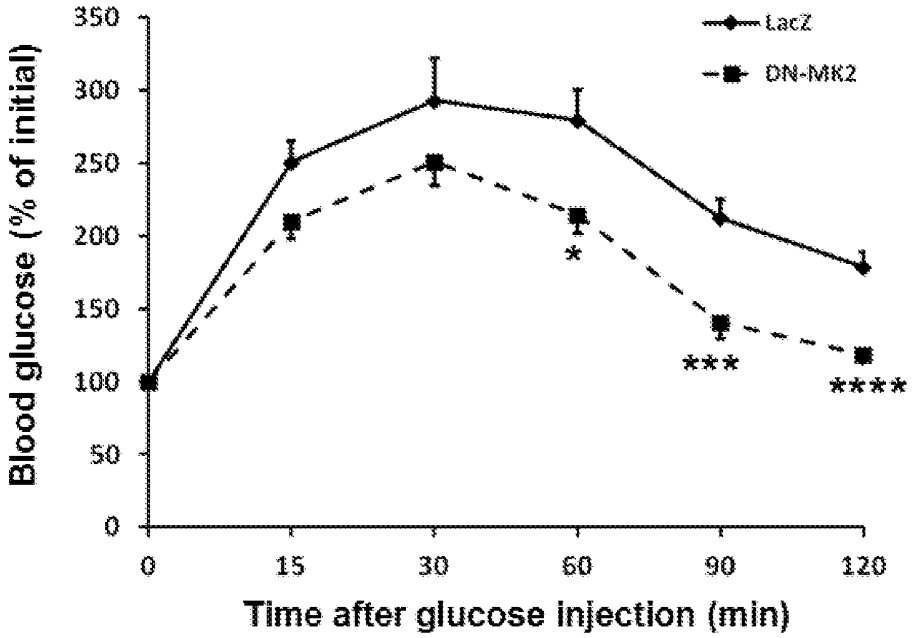

LacZ
DN-MK2
Blood glucose (% of initial)
350
300
250
200
150
100
50
0
0
15
30
60
90
120
Time after glucose injection (min)
*
***
****

Figure 67

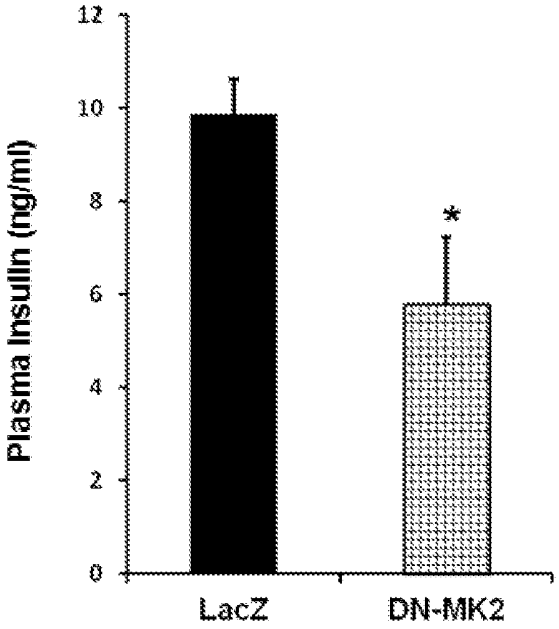


Figure 68

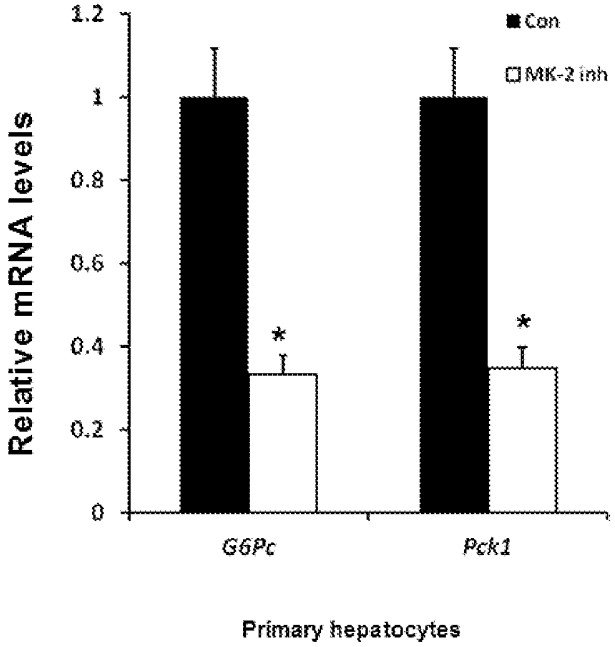

1.2
1
0.8
0.6
0.4
0.2
0
Relative mRNA levels
Con
MK-2 inh
*
*
G6Pc
Pck1
Primary hepatocytes

Figure 69

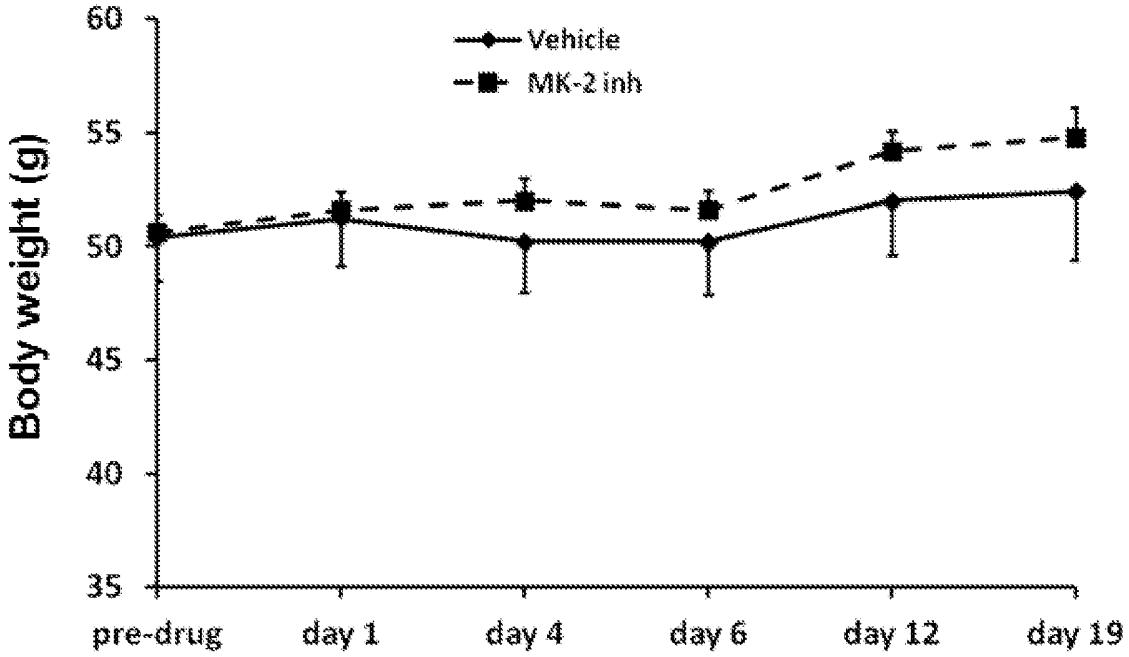

Vehicle
MK-2 inh
Body weight (g)
60
55
50
45
40
35
pre-drug
day 1
day 4
day 6
day 12
day 19

Figure 70

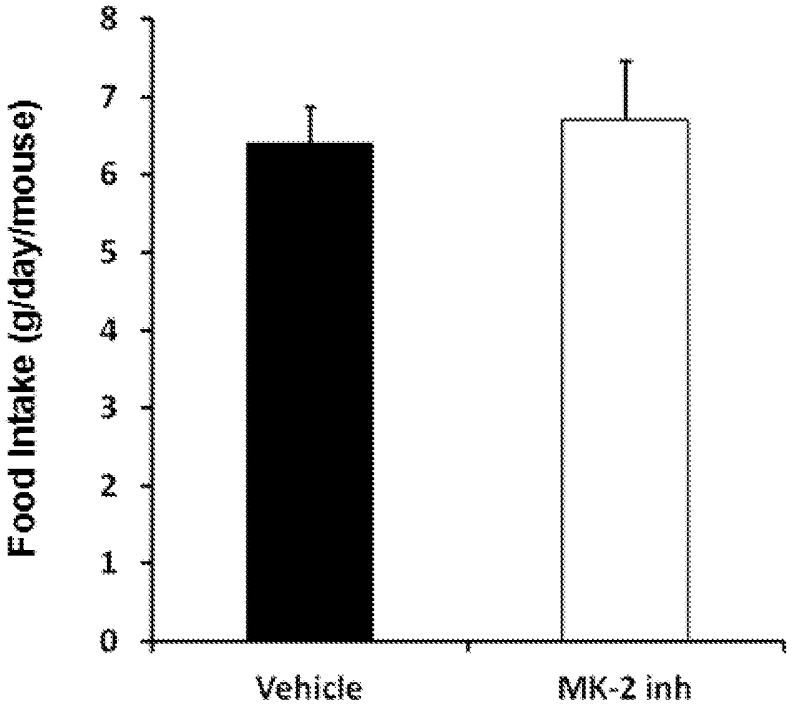


Figure 71

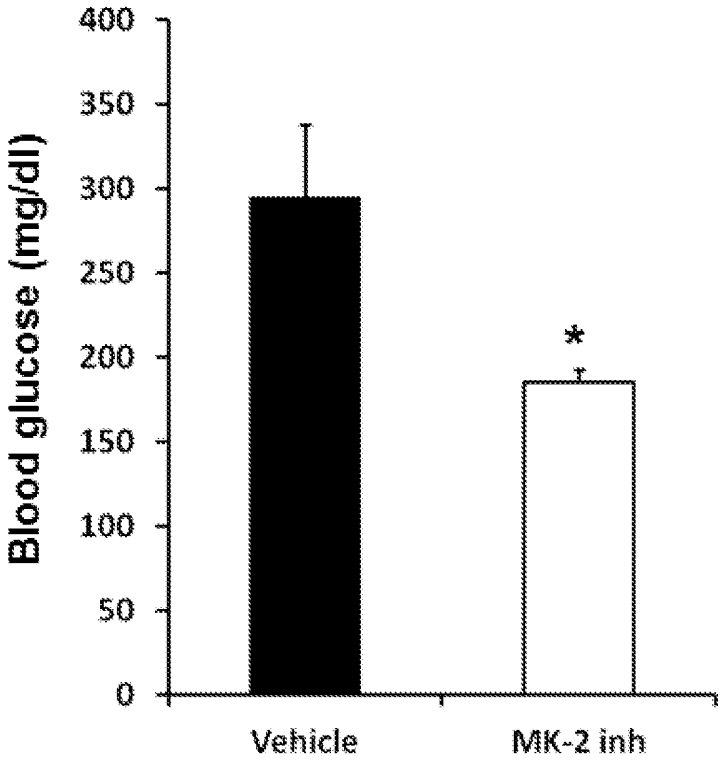

Blood glucose (mg/dl)
400
350
300
250
200
150
100
50
0
*
Vehicle
MK-2 inh

Figure 72

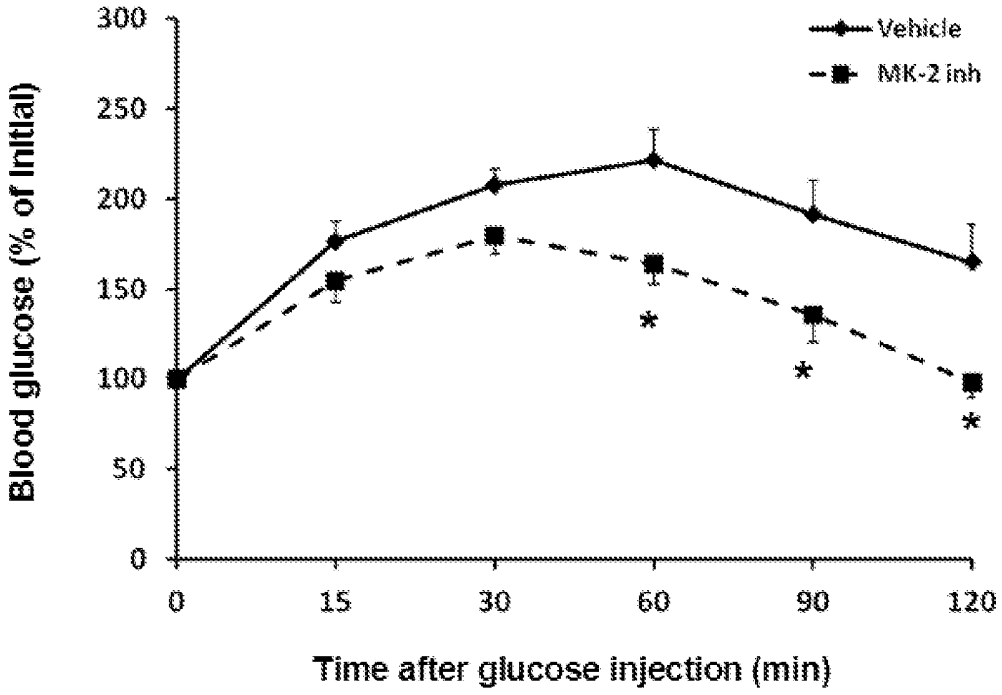

Vehicle
MK-2 inh
Blood glucose (% of Initial)
300
250
200
150
100
50
0
0
15
30
60
90
120
Time after glucose injection (min)
*
*
*

Figure 73

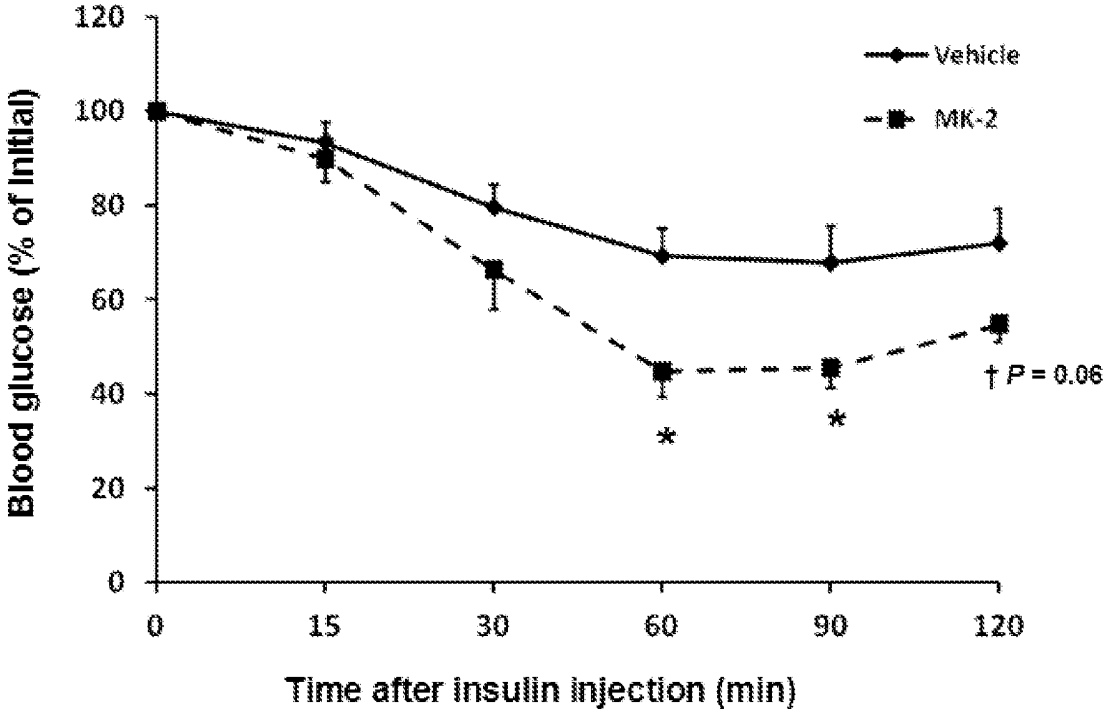

120
100
80
60
40
20
0
Blood glucose (% of initial)
Vehicle
MK-2
† P = 0.06
*
*
0
15
30
60
90
120
Time after insulin injection (min)

Figure 74

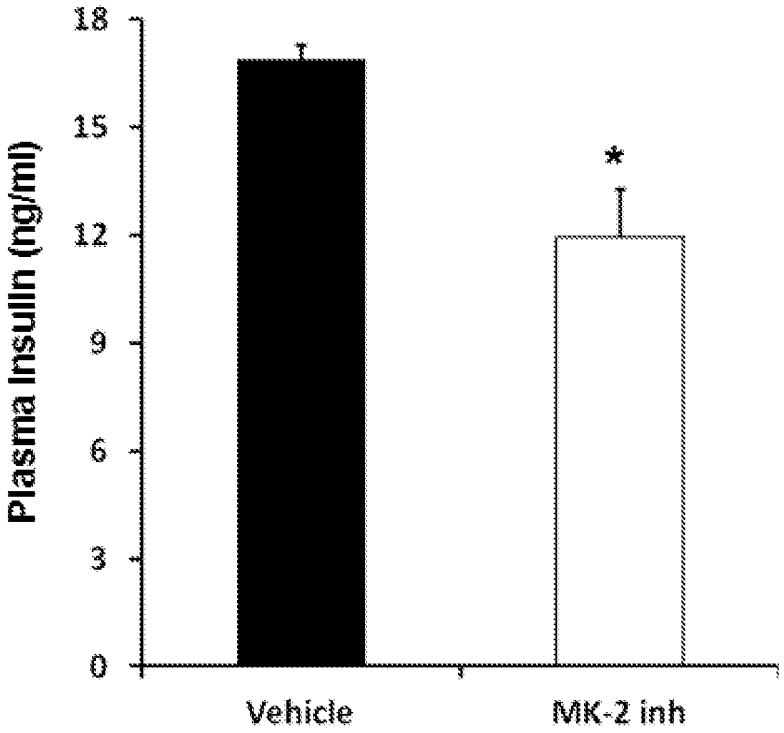


Figure 75

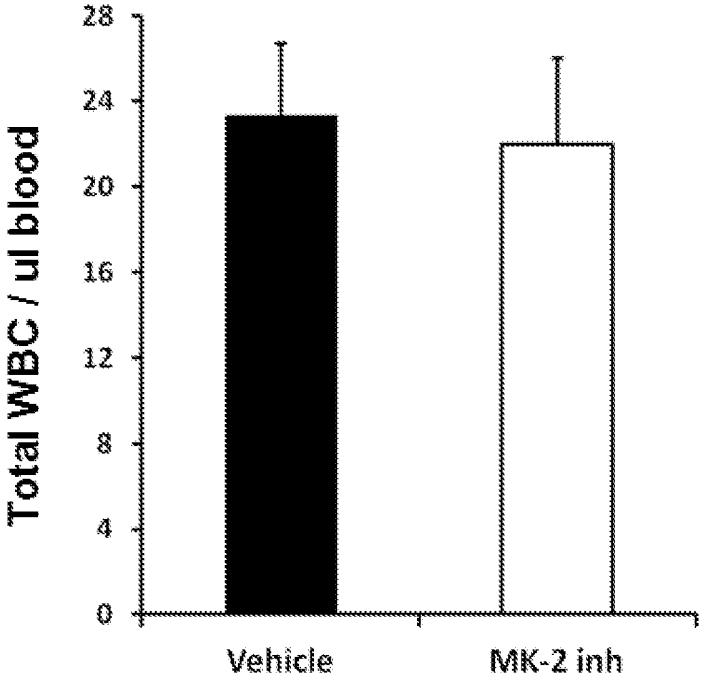


Figure 76

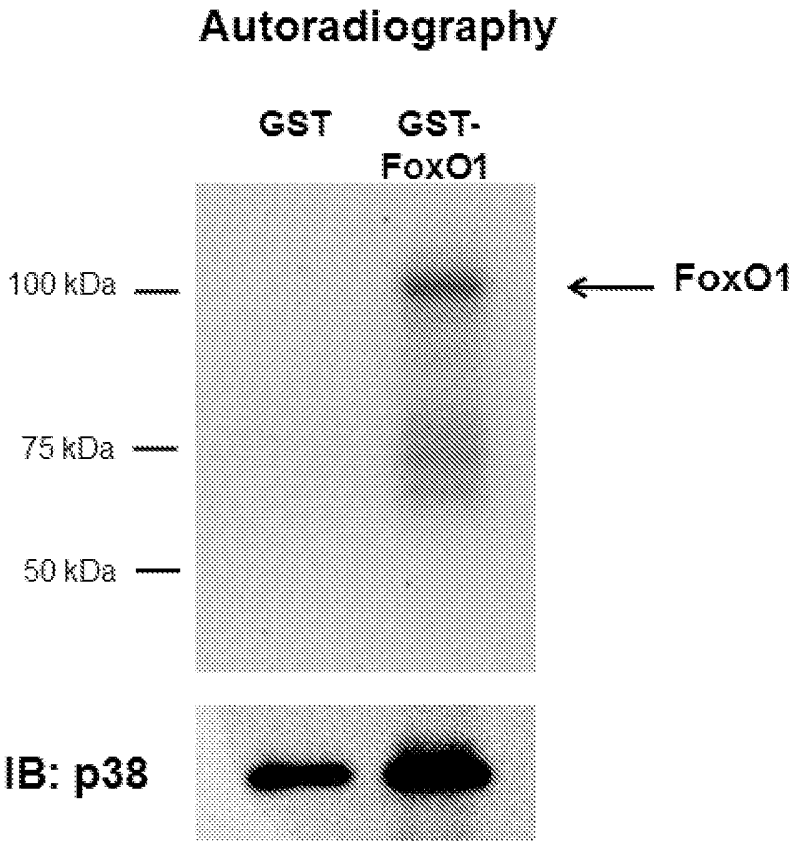

Autoradiography
GST
GST-FoxO1
100 kDa
75 kDa
50 kDa
FoxO1
IB: p38

Figure 77

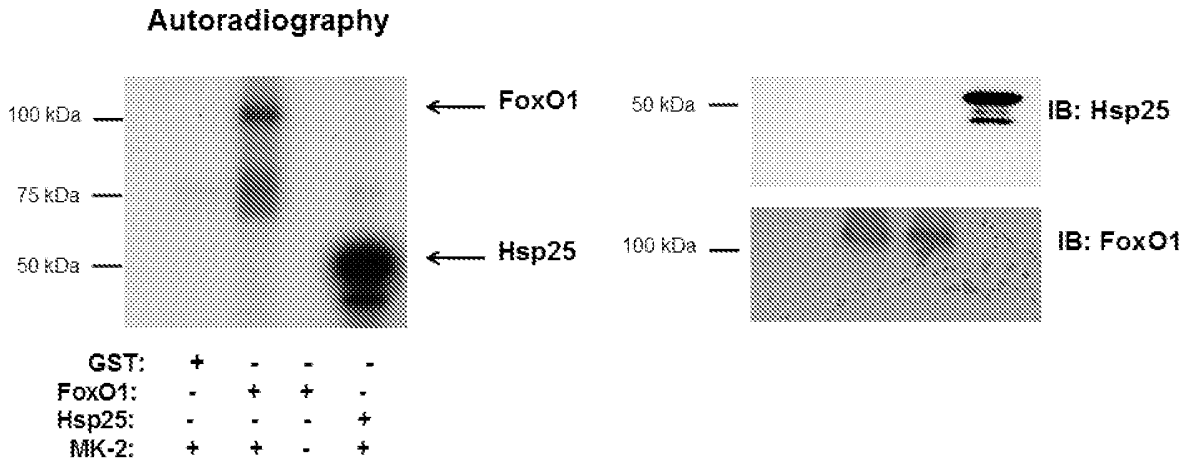

Autoradiography
100 kDa
75 kDa
50 kDa
FoxO1
Hsp25
GST: + - - -
FoxO1: - + + -
Hsp25: - - - +
MK-2: + + - +
50 kDa
IB: Hsp25
100 kDa
IB: FoxO1

Figure 78

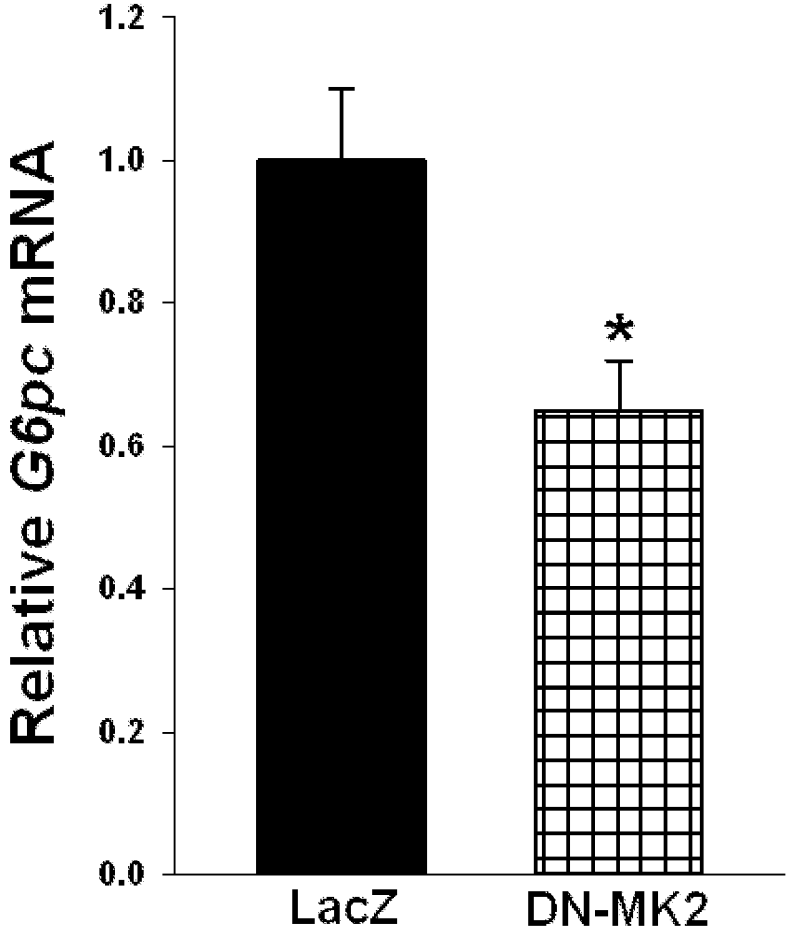
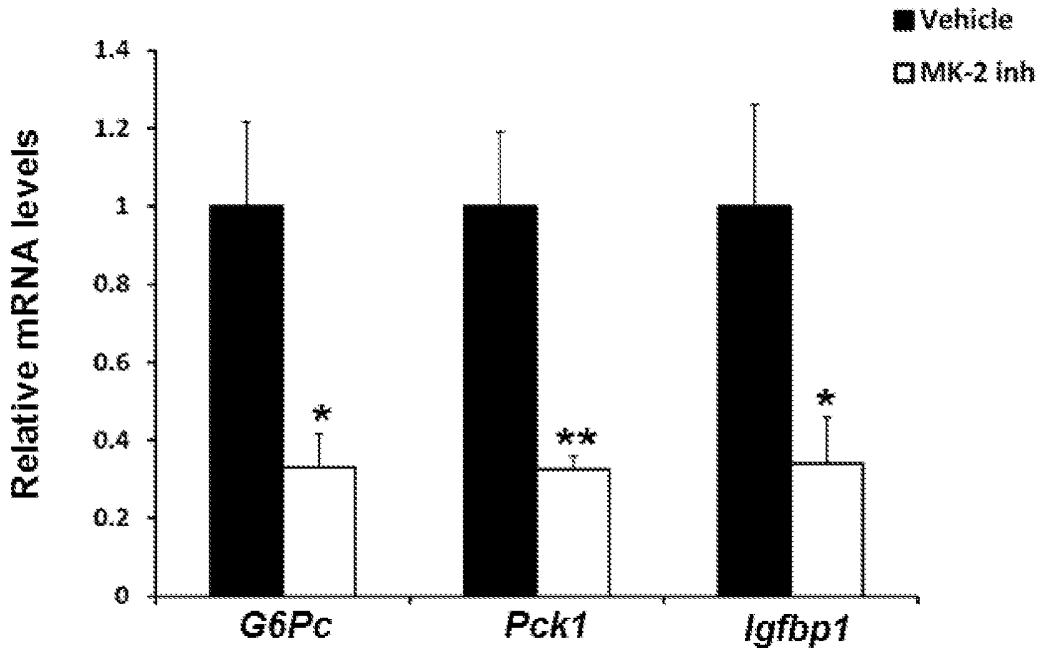
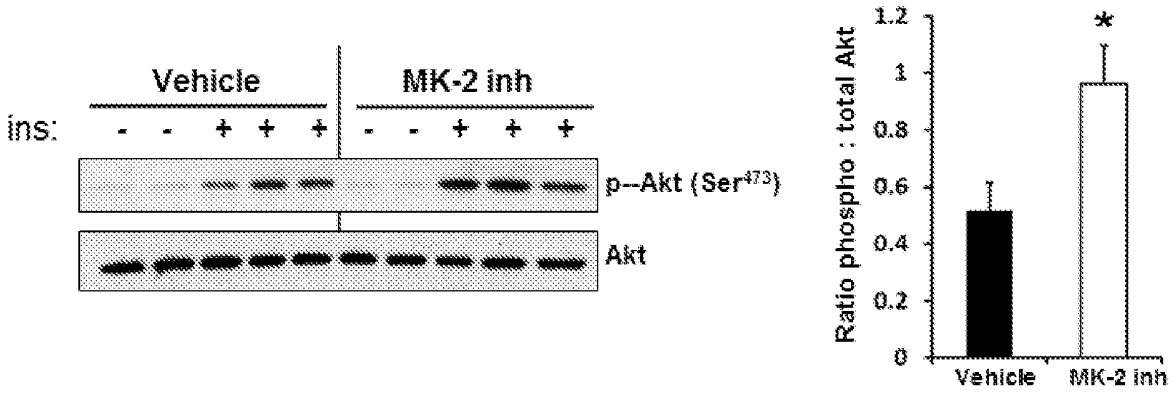


Figure 79



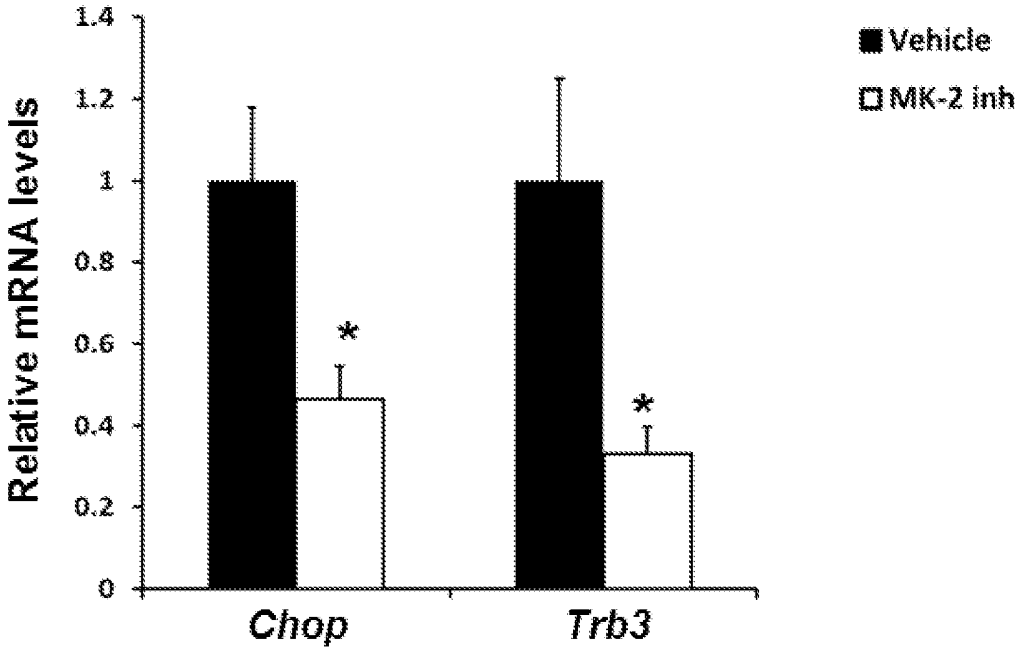
* $p < 0.05$, ** $p < 0.01$

Figure 80



* $p = 0.05$

Figure 81



* $p < 0.05$

Figure 82

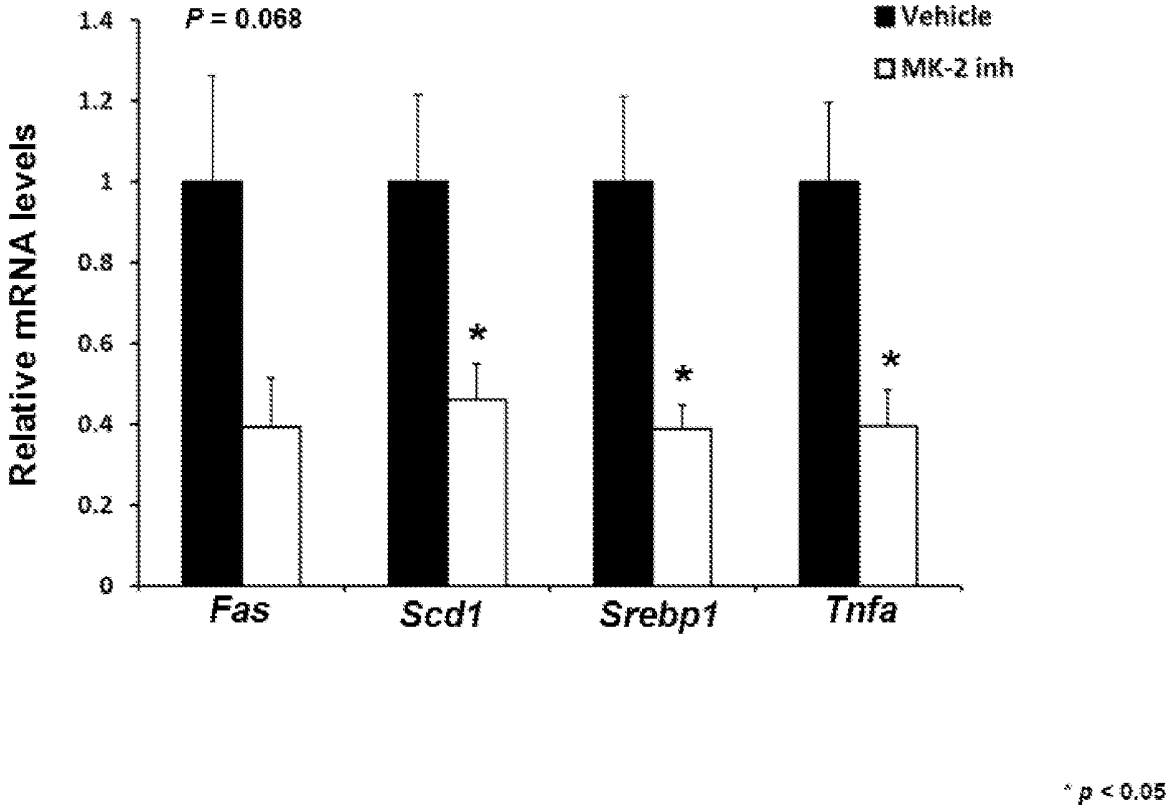
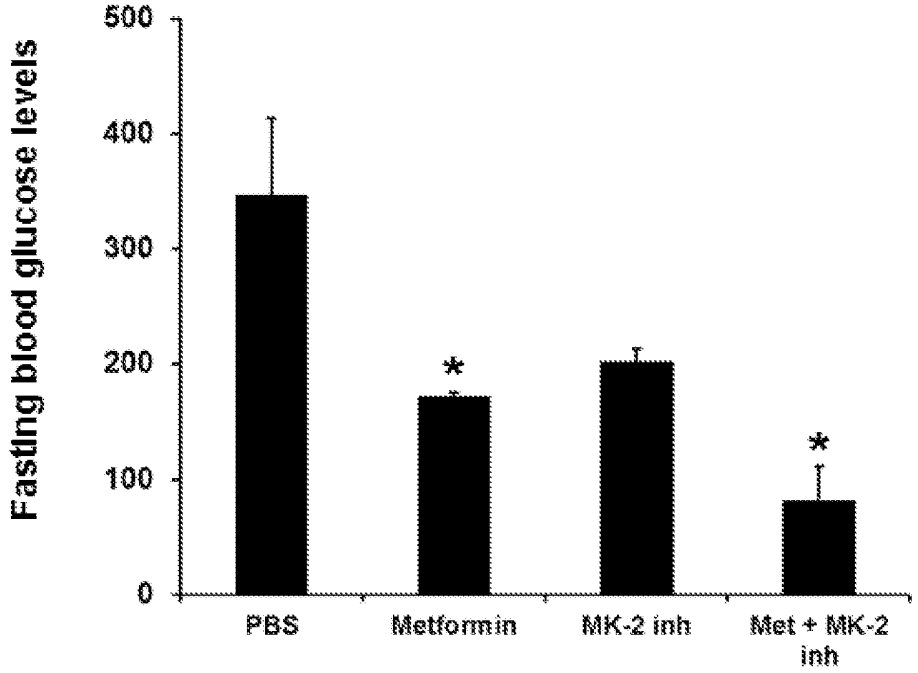
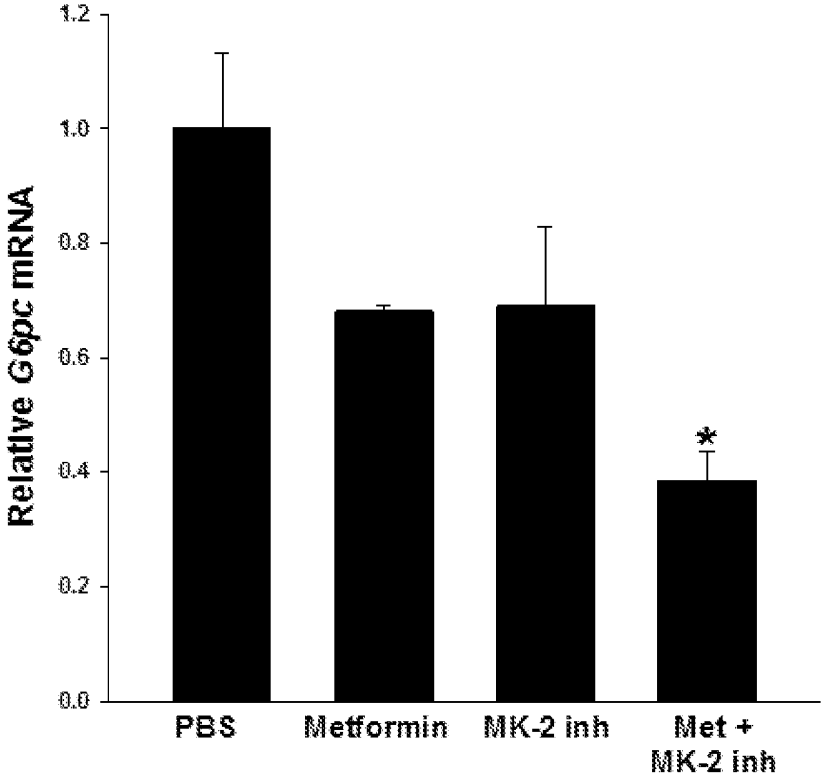

P = 0.068
Vehicle
MK-2 inh
Relative mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
Fas
Scd1
Srebp1
Tnfa
* p < 0.05

Figure 83



* $p < 0.05$

Figure 84



* $p < 0.005$

Figure 85

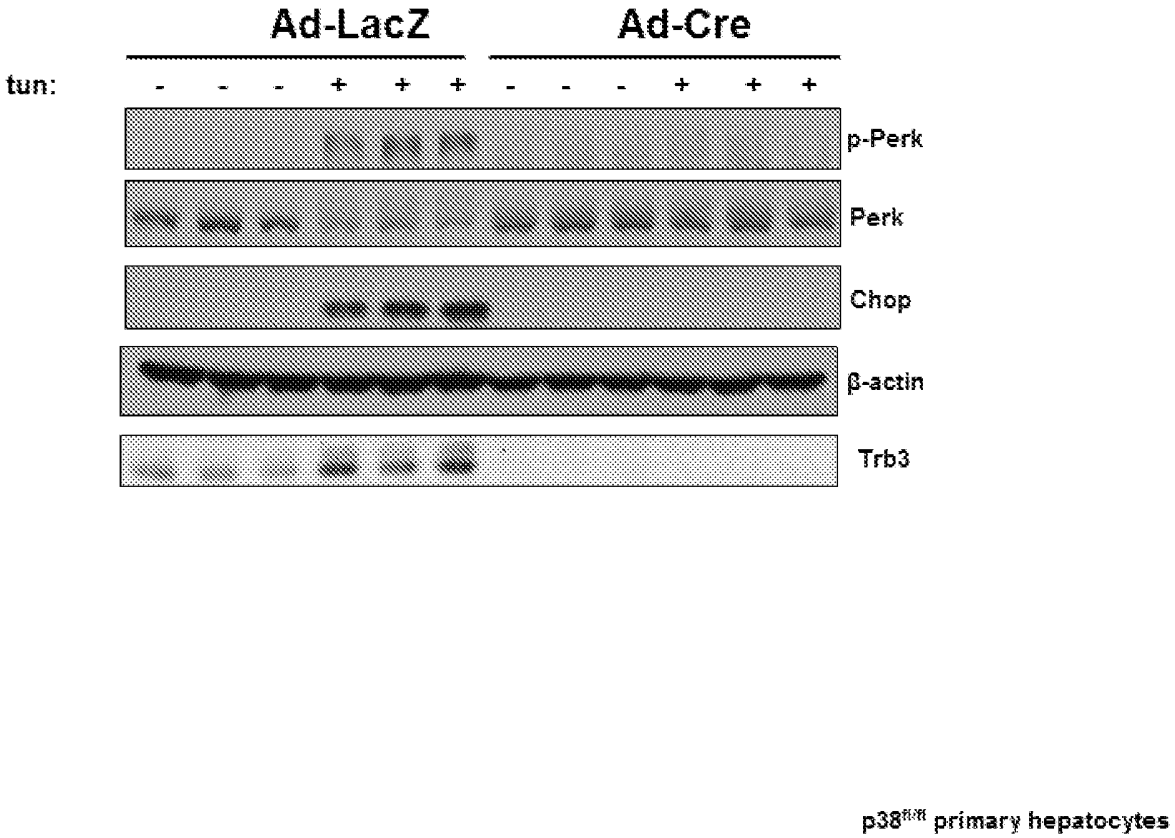
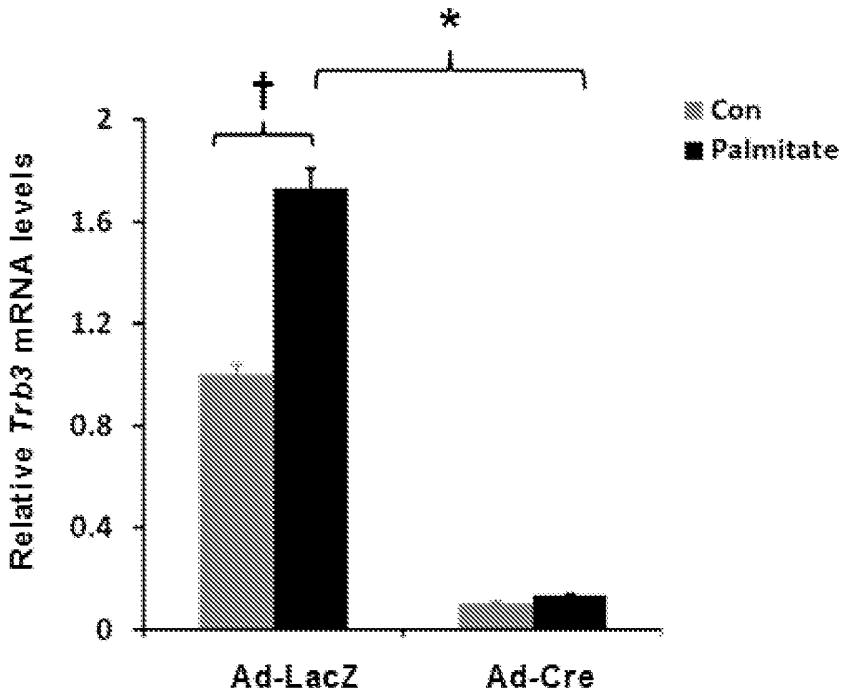
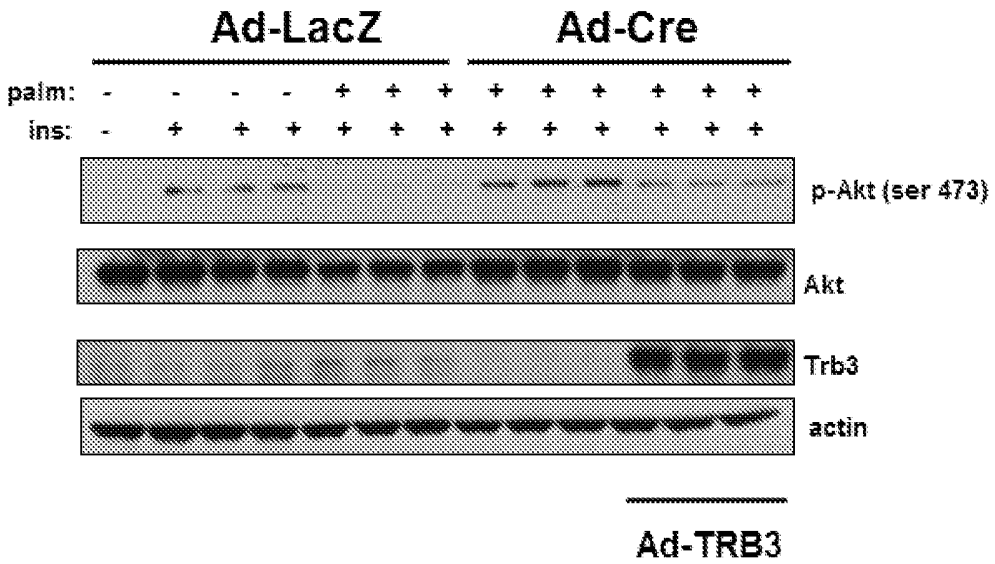

Ad-LacZ
Ad-Cre
tun:
- - - + + + - - - + + +
p-Perk
Perk
Chop
β-actin
Trb3
p38fl/fl primary hepatocytes

Figure 86



p38$^{fl/fl}$ primary hepatocytes

Figure 87



p38$^{fl/fl}$ primary hepatocytes

Figure 88

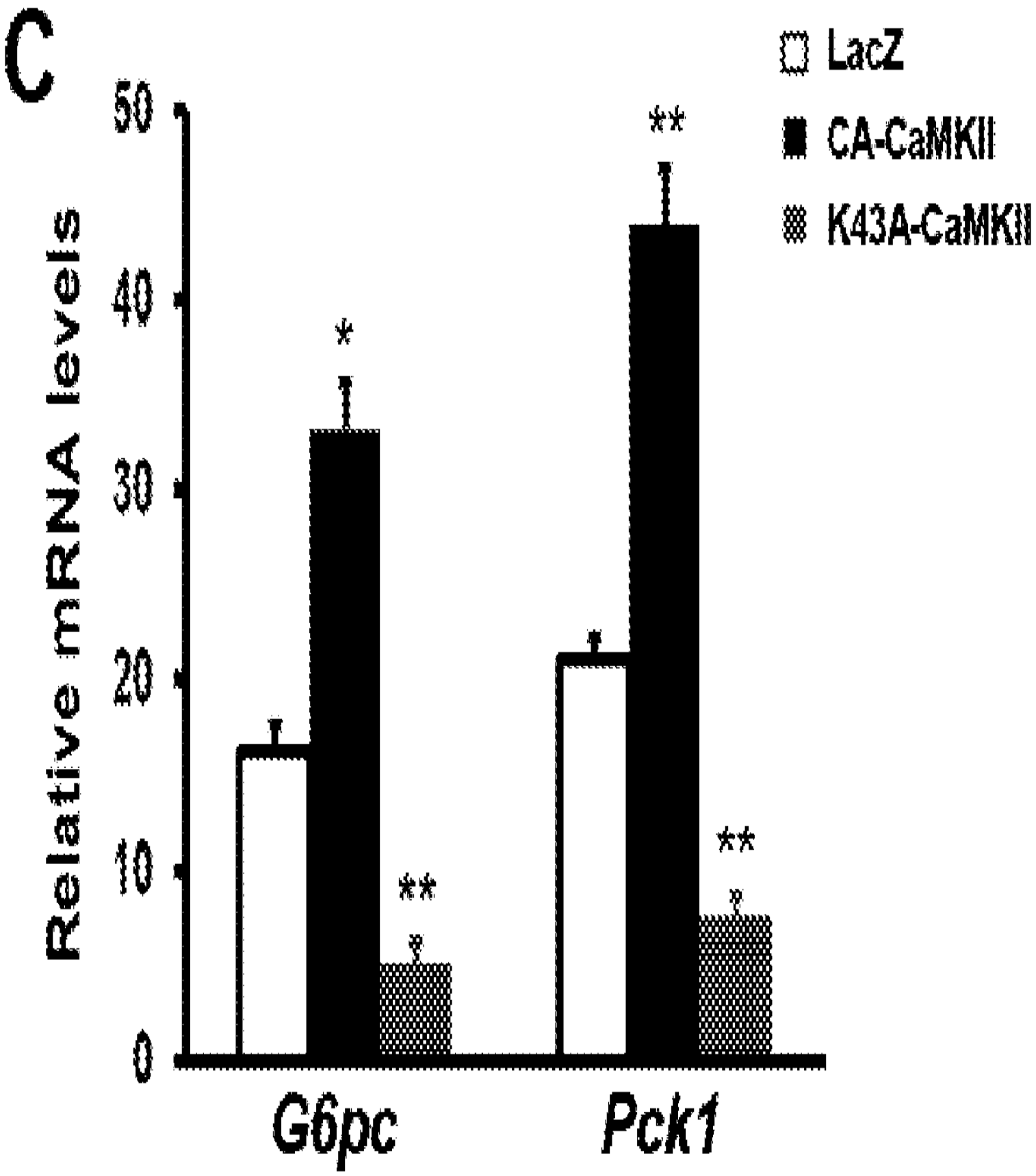

C
LacZ
CA-CaMKII
K43A-CaMKII
Relative mRNA levels
50
40
30
20
10
0
*
**
**
**
G6pc
Pck1

Figure 2C