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FoxO1-S253 regulates glucose homeostasis

Phosphorylation of forkhead protein FoxO1 at Ser253 regulates glucose homeostasis in mice

Kebin Zhang¹#, Xiaqin Guo²#, Hui Yan¹#, Yuxin Wu¹,³, Quan Pan¹, James Zheng Shen¹, Xiaopeng Li¹, Yunmei Chen¹, Ling Li¹, Yajuan Qi¹, Zhihui Xu¹, Wei Xie², Weiping Zhang¹, David Threadgill¹, Ling He⁴, Daniel Villarreal¹, Yuxiang Sun¹, Morris F. White⁵, Hongting Zheng², Shaodong Guo¹*

¹ Department of Nutrition and Food Science, College of Agriculture and Life Sciences, Texas A&M University, College Station, TX 77843, USA; ² Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China; ³ Queens University Belfast School of Biological Sciences, Belfast, UK; ⁴ Division of Endocrinology, Departments of Medicine, John Hopkins University, Baltimore, ML 04515, USA; ⁵ Division of Endocrinology, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115

ORCiD numbers:

0000-0001-5126-731X

Guo

Shaodong

ORCiD numbers: 0000-0001-5126-731X (S. Guo)

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# equally contributed to this work

The transcription factor forkhead boxO1 (FoxO1) is a key mediator in the insulin signaling pathway and controls multiple physiological functions, including hepatic glucose production (HGP) and pancreatic β-cell function. We previously demonstrated that Ser256 in human FOXO1, equivalent to Ser253 in mouse FoxO1, is a key phosphorylation site mediating the effect of insulin as a target of Akt on suppressing FOXO1 activity and expression of target genes responsible for gluconeogenesis. Here, we investigated the role of FoxO1-Ser253 phosphorylation in control of glucose hemostasis in vivo, by generating global FoxO1-S253A/A knock-in (KI) mice, in which FoxO1-Ser253 alleles were replaced with alanine (A substitution), blocking FoxO1-S253 phosphorylation. FoxO1-S253A/A mice displayed mild increases in feeding blood glucose and insulin levels, but decreases in fasting blood glucose and glucagon concentrations, as well as a reduction in the ratio of pancreatic α-cells/β-cells per islet. FoxO1-S253A/A mice exhibited a slight increase in energy expenditure, but barely altered food intake and glucose uptake among tissues. Further analyses revealed that FoxO1-S253A/A enhances FoxO1 nuclear localization and promotes the effect of glucagon on HGP. We conclude that dephosphorylation of S253 in FoxO1 may reflect a molecular basis of pancreatic plasticity during the development of insulin resistance.

INTRODUCTION

The forkhead protein FoxO1 is an important transcription factor governing a variety of physiological function, including hepatic glucose production (HGP) and pancreatic β-cell
function (1-3). Suppressed by insulin via activation of phosphatidylinositol 3-kinase (PI-3K) and Akt, FoxO1-stimulated HGP by gluconeogenesis is inhibited under feeding conditions, contributing to the maintenance of a steady level of blood glucose (4). Our previous studies demonstrated that the insulin receptor substrate proteins -1 and -2 (Irs-1, -2) are major mediators of insulin signaling cascades in activating endogenous PI3K and Akt and subsequently suppressing FoxO1 in the liver in control of blood glucose homeostasis (5,6). By contrast, upon loss of IRS1, 2 genes, activation of PI-3K and its downstream protein kinase B (Akt) were suppressed and subsequently failed to inhibit FoxO1-stimulated gluconeogenesis in the liver, promoting HGP, hyperinsulinemia, and diabetes mellitus (4-7). Thus, FoxO1 is required for the liver to develop hyperglycemic diabetes.

In hepatocytes, FoxO1 binds to the promoter region of glucose-6-phosphatase (G6pc) or phosphoenolpyruvate carboxykinase (Pck), two key metabolic enzymes, promoting the expression genes for gluconeogenesis and HGP (8). FoxO1 also binds to the insulin responsive element on the promoter region of IRS2, increasing IRS-2 gene transcription and insulin sensitivity (9). In the pancreas, early studies indicated FoxO1 represses FoxA2-dependent Pdx-1 transcription and β-cell apoptosis in IRS2-dependent manners (10). By contrast, it is recently shown that FoxO1 is involved in pancreatic β-cell differentiation and maintenance, while a loss of FoxO1 in pancreatic β-cell lineages can stimulate β-cell dedifferentiation into progenitor-like cells even the α-cells, resulting in hyperglucagonemia (2).

FoxO1 is phosphorylated at Ser256 (human) by Akt, which primes phosphorylation of other two sites at Thr24 and Ser319 (11,12) and enhances FoxO1 interaction with E3 ubiquitin ligase, and promotes FoxO1 nuclear export and/or degradation (13,14), controlling expression of genes responsible for cellular growth, survival, and metabolism. Thus, FoxO1 is a key mediator of insulin and Akt signaling pathway that controls multiple physiological functions (4). In addition to FoxO1-S256 phosphorylation by insulin and/or Akt, FoxO1 subcellular localization, transcriptional activity, and protein stability are modulated by multiple levels of posttranslational modifications, including phosphorylation, ubiquitination, OGlcNacylation, and acetylation (1,4). Given that FoxO1-Ser253 dephosphorylation upon inactivation of Akt, which is observed in tissues of diabetic animals, including the liver of db/db mice or mice lacking both IRS1 and IRS2 genes (6,15), FoxO1 nuclear localization is a hallmark for the progression of type 2 diabetes mellitus (T2D) and dephosphorylation of FoxO1 at Ser253 (mice) is believed to be a potential indicator for FoxO1 activation that is associated with diabetes mellitus (4).

To further explore whether impairing FoxO1 phosphorylation at Ser253 itself is sufficient for impairing blood glucose homeostasis and insulin sensitivity in vivo, we generated FoxO1-S253A/A knock-in (KI) mice, also referred as A/Amice, in which the FoxO1 gene was expressed at its physiological level and the endogenous FoxO1-Ser253 loci were replaced by alanine (A) to prevent FoxO1-Ser253 phosphorylation-mediated functionality. We analyzed the blood glucose and energy metabolism in the KI mice, and the role of FoxO1-Ser253 dephosphorylation in control of systemic glucose homeostasis was determined. In particular, we focus on liver and pancreas in this study to understand the regulation of FoxO1 by its serine 253 in control of energy metabolism at the bodily level.

**MATERIALS AND METHODS**

**Construction of FoxO1-S253A/A gene targeting vector.**

To introduce a point mutation at Ser253 to Alanine (S253A) in the endogenous FoxO1 genomic locus of mice, we first screened PPCI-22 female 129S6/SvEvTac mouse BAC library provided...
by Roswell Park Cancer Institute (Buffalo, New York, www.rosewellpark.org) with a FoxO1 specific probe. A BAC clone of 200kb DNA insert containing FoxO1 genomic DNA was obtained (Fig. 1A). A 4.2kb fragment spanning exon 2 of FoxO1 and a 3.2kb fragment spanning exon 3 of FoxO1 were amplified by PCR. The 4.2kb fragment was amplified by PCR primer 1: 5’-ACCGCTCGAGAAAGCATCGAAATGTGTCTGC-3’ with primer 2: 5’-AGGCGGATCCACCTGTCTTTAATTAGTT-3’, and the 3.2kb fragment was amplified by PCR primer 3: 5’-ACGGGGGTACCTTATGTAACTCATCCCTACC-3’ with primer 4: 5’-TCGGGGTACCTATTTGCTAATTACGTT-3’. These two fragments were then cloned into upstream and downstream regions of loxP-flanked neomycin cassette using Xho1 and Kpn1 linkers, respectively, in gene targeting vector pPN-T-2loxP (Fig. 1B) (5). Two-point mutations at Ser253 site were introduced by changing two nucleotides from GT to AG with primer 5: 5’-CGGAGAAGTGCTGCAGCCATGACACAG-3’, which simultaneously created a Pst1 site without changing any other amino acids. The Pst1 site was used as a selection and genotyping marker for latter gene targeting vector validation, ES cell screening, and mouse offspring genotyping. The pPNT-FoxO1-S253A targeting vector was successfully generated and validated by restriction endonuclease digestions with expected patterns.

**Mouse embryonic stem cell transfection and Southern-blotting.**

The linearized targeting vector pPNTFoxO1-S253A by Not1 was used for ES cell transfection. To determine homologous recombination (HR), we picked 457 ES clones and examined them by performing southern blot experiments. Different from many other negative clones (e.g. clone 68), a positive clone 167 with both 5’ and 3’ homologous recombination near the neo cassette was identified using 32P-dCTP-labeled S5 probe and S3 probe, respectively (Fig. 1C-D). The S5 probe was amplified by PCR primer 6: 5’-CCTTCTCAAGCACACTGGA-3’ with primer 7: 5’-GTGGGTGCTTTATGACAGAAG-3’ and the S3 probe was amplified by PCR primer 8: 5’-TCAGTAGAGTGTGCATTGTGC-3’ with primer 9: 5’-CTCACACTGAGAACCATTG-3’ from the FoxO1 BAC clone. The neo cassette was removed by Cre expression (Fig. 1B). The FoxO1-S253A/+ heterozygous allele in the DNA of 167 ES clone was further confirmed by verification of a 600 bp fragment that was amplified by PCR primer 10: 5’-AGTGCTGCACTTCAACATAC-3’ with primer 11: 5’-GAGAGAAGGTTGAGATTAC-3’. The Pst1 digestion of the 600bp fragment produced a 400 bp and a 200 bp fragment from S253A allele. Using primer 10 as a DNA sequencing primer, we further confirmed that the 600 bp fragment from the positive 167 ES cell clone contained both wild type Ser253 allele (GT) and Ser253A allele (AG), while the 600bp fragment from the negative 68 ES clone only contained two wild type Ser253 alleles (GT).

**Generation of FoxO1-S253A/A mutant mice and genotyping.**

The positive 167 ES clone was used for pronuclear injection in a fertilized mouse egg that was then transferred into two foster female mice. Two chimeric mice were generated and backcrossing the chimeric mice to C57/BL6 mice generated 10 F1 offspring pups. Genotyping of the F1 offspring confirmed the germ-line transmission of the FoxO1- S253A allele. Mating the male with the female heterozygous FoxO1-S253A/+ allele (A/+ mice) generated F2 offspring mice homozygous FoxO1-S253A/A allele (A/A mice) and wild-type FoxO1-S253A/+ control mice (+/+ mice). All mice were with C57/BL6 and 129 Sv mixed background maintained on regular chow (Prolab Isopro 5P76). Male mice around 8-12 weeks old were analyzed, if not specified in the legend. All animal protocols were approved by Texas A&M University Institutional Animal Care and Use.
Chemicals and Antibodies.
Insulin and glucagon were purchased from Sigma. Antibodies against FoxO1 [Cell Signaling Technology; catalog no. 2880 (16)], pFoxO1-Thr24 [Cell Signaling Technology; catalog no. 2599 (17)], pFoxO1-Ser253 [Cell Signaling Technology; catalog no. 9461 (18)], pFoxO1-Ser316 [Cell Signaling Technology; catalog no. 2486 (19)], GADPH [Cell Signaling Technology; catalog no. 2118 (20)], β-actin [Cell Signaling Technology; catalog no. 4970 (21)], and histone H1 antibody [Santa Cruz Biotechnology; catalog no. sc-8030 (22)] were used for Western-blot.

Blood Chemistry and Metabolic Analysis.
Serum samples were analyzed for insulin and glucagon (Crystal Chem.), and albumin (Bioassay System, CA) using commercial protocols and reagents. Blood glucose levels were measured using a glucometer (Bayer). For the glucose tolerance test, mice were fasted overnight and intraperitoneally (i.p.) injected with 2 g D-glucose/kg body weight. For pyruvate tolerance test, the fasting mice were injected i.p. with 2 g pyruvate/kg body weight. In vivo insulin stimulation was carried out in mice that were fasted and deeply anesthetized. Serum insulin and glucagon were measured with commercial kits (Crystal Chem. Inc.). Hepatic glycogen concentration was analyzed as previously described (5,8). For glucagon tolerance tests, the 18 h fasting mice were i.p. injected with glucagon with a dose of 16 µg/kg body weight and blood glucose were measured with a glucometer (Elite XL, Bayer). The dose of glucagon was used as recently described (23).

Hyperinsulinemic-euglycemic clamp of mice.
Catheters were implanted in a carotid artery and a jugular vein of mice for sampling and intravenous infusions 5 days before hyperinsulinemic-euglycemic clamp when mice were at conscious states, as previously described (5,24). Blood glucose was clamped at 150 mg/dL using a variable glucose infusion rate (GIR). Mice received washed erythrocytes from donors to prevent the loss in hematocrit that would otherwise occur. At 120 min, the clamp was sustained and a 1 mCi bolus of 2-[14C]deoxyglucose ([14C]2DG) was administered. Blood was taken at 122–155 min for [14C]2DG determination. Mice were anesthetized after the last sample, and tissues were excised. Plasma and tissue radioactivity of [3-3H]glucose, [14C]2DG, and [14C]2DG-6-phosphate were determined as previously described (24). Glucose appearance (Ra), endogenous glucose appearance (EndoRa), and glucose disappearance (Rd) rates were determined using non steady state equations (24). The glucose metabolic index (Rg) was calculated as described (24).

Indirect calorimetry measurements.
Twelve-week-old mice were acclimated in metabolic cages (TSE Phenomaster) for 2 days of adaptation. After 2 days of housing, mice were recorded for 3 min every hour for two consecutive days with the following measurements: gas exchange (VO2 consumption and VCO2 output), food intake, and physical activities. Energy expenditure (EE) was calculated according to the manufacturer’s manual. Values were normalized by body weight to the power of 0.75. The respiratory exchange ratio (RER) was estimated by calculating the ration of VCO2/VO2 (25).

Pancreas immunohistochemistry, morphometric and chemical analyses.
Pancreases from WT and A/A mice were fixed in 10% formalin overnight, and then processed for paraffin-embedding. Tissues from five mice of each genotype were sectioned to a thickness of 5 µm used for morphometric and immunohistochemical analysis. For morphometric analysis, at least 3 pancreatic sections from 3-5 mice of each genotypes were staining with Hematoxylin-
Eosin (H&E) and scanned on Leica Aperio ScanScope. Islet cross-sectional area and total pancreatic area were measured using Image J software and calculated as ratio of islet to total pancreas. Quantification of the number of pancreatic α- and β-cells was calculated using co-immunofluorescence staining with antibody of insulin or glucagon from non-overlapping field on Leica confocal microscope. The total numbers of glucagon or insulin positive-cells were counted from pancreatic sections immunostained for glucagon and insulin, respectively.

Total insulin and glucagon in pancreas were extracted from the whole pancreas by Acid-Ethanol extraction according to the Animal Models of Diabetic Complication Consortium protocol (https://www.diacomp.org/shared/document.aspx/Protocol). Pancreatic insulin and glucagon levels were determined by Bio-Plex mouse diabetes immunoassay (Bio-Rad Laboratories Inc., Hercules, CA) as described (26).

**Pancreatic islet isolation, islet insulin and glucagon content, and glucose stimulated insulin secretion (GSIS).**

Islets were collected using the collagenase method and cultured overnight in low glucose RPMI medium using our previous protocols (27). Briefly, 3ml of 1mg/ml Collagenase P dissolved in ice cold Hank’s Balanced Salt Solution (HBSS) was injected via the pancreatic duct while the common bile duct near the liver was clamped. Once inflated, the pancreas was excised and placed in a 50 ml tube with 3 ml of Collagenase P solution. Tubes containing digest were placed in 37°C water bath at 100-120 shaking/min for 10-12 minutes. Subsequently, 10% FBS solution was added to digest tubes to stop collagenase P activity. The islets were then centrifuged using a Ficoll gradient Histopaque-1077 (Sigma-Aldrich, Kawasaki, Japan) per manufacturer’s instruction. Islets were then collected from the gradient, washed with HBSS, and handpicked using a 200 µl pipette under a dissection microscope. The handpicked islets were then suspended in the RPMI-1640 medium and incubated overnight in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM Hepes, 2 mM L-glutamine, 1 mM Sodium-pyruvate, 0.05 mM 2-mercaptoethanol, and 5.5 mM glucose. The next day, each islet was transferred to a 12-well plate (about 10 islets per well) and cultured with 1 ml of the HBSS medium, pH 7.2, consisting of 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM HEPES, 2.5 mM CaCl₂, 25.5 mM NaHCO₃, 0.2% bovine serum albumin, and 3.3 mM glucose for 2 h. Following the 3.3mM glucose incubation, islets then were incubated in either 3.3 mM or 22.2 mM glucose for another 2 h. The 10 µl or 50 µl of the HBSS buffer was collected for insulin and glucagon measurement, respectively, using mouse insulin or glucagon ELISA kit (Mercodia, Uppsala, Sweden). The islets were collected for insulin and glucagon measurement using Bio-Plex mouse diabetes immunoassay (Bio-Rad Laboratories Inc., Hercules, CA) as described (26), and normalized to the islet protein content.

**Quantitative Real-Time PCR Analysis.**

RNA was extracted with Trizol reagent (Invitrogen) (28). 1 µg of RNA was used as template for cDNA synthesis utilizing the SuperScript first-strand synthesis system (Bio-Rad). The quantity of cDNA for each transcript was measured using real-time PCR with the SYBER green supermix (Bio-Rad). Relative quantitative analysis was performed by calculating the ratio of mRNA amount for the gene of interest over the amount of internal control cyclophilin with duplicate samples. The PCR primers were described previously (8).

**Primary Hepatocytes Isolation and Culture, and Hepatic Glucose Production Assay.**

Primary hepatocytes were isolated from mice and cultured in DMEM medium, as described as previously (8). For HGP assay, freshly isolated hepatocytes were cultured in DMEM with 2%
FBS. After 3 hours of attachment, cells were cultured in HGP buffer (120 mM NaCl, 5.0 mM KCl, 2.0 mM CaCl₂, 25 mM NaHCO₃, 2.5 mM KH₂PO₄, 2.5 mM MgSO₄, 10 mM HEPES, 0.5% BSA, 10 mM sodium DL-lactate, and 5 mM pyruvate, pH = 7.4) and treated with chemicals. Culture medium was collected to determine the glucose concentration using Amplex Red Glucose Assay kit (Invitrogen, Carlsbad, CA). For glycogenolysis assays, the sodium DL-lactate and pyruvate were removed from the HGP buffer, and then medium glucose contents were determined, and gluconeogenesis is a subtraction of medium glucose of HGP by glycogenolysis.

Western Blot and Immunoprecipitation.
Tissue or cellular protein lysates were prepared, resolved by SDS-PAGE, and transferred to nitrocellulose membrane for immunoblotting analysis using specific antibodies. Signal intensity was measured and analyzed by NIH Image J software as previously described (8,28).

Statistical Analysis.
All results are presented as mean ± SEM determined by two-tailed Student’s t-tests or one-way analysis of variance. Paired comparisons of the means were made, and p<0.05 was defined as statistical significance. The Bonferroni method was used to adjust the observed significance levels for testing of multiple contrasts (5,8).

RESULTS

Generation of FoxO1-S253AA mutant mice
We generated mice with an alanine point mutation at Ser253 (S253A, or A allele) in FoxO1 genetic loci (Fig. 1A-D). We bred the male and female A/+ mice to generate wild-type (WT or +/+), heterozygous (A/+), and homozygous (A/A) mice for the FoxO1-S253 allele, achieving a Mendelian ratio of 1:2:1, respectively. All the mice appeared healthy, ruling out the possibility that homozygous FoxO1-S253AA alleles are embryonic lethal. Initial analysis indicated that body weight of the A/+ and A/A mice was not significantly changed by the age of 16 weeks (Fig. 1E). The serum albumin levels were measured and there were no significant differences among WT and A/A mice (3.4 ± 0.21 g/dl WT versus 3.5 ± 0.26 g/dl A/A) in the feeding state and there were no significant differences among the groups in the fasting state, indicating that the FoxO1-S253A mutation did not cause liver damage.

FoxO1-S253AA mutation enhances FoxO1 stability in vivo
We next examined the levels of total FoxO1 protein in the primary hepatocytes of the mice. Insulin stimulated phosphorylation of FoxO1 at T24, S253, and S316, and reduced total FoxO1 protein level by 60% in WT hepatocytes, analyzed by immunoblotting. However, insulin failed to induce FoxO1 degradation in S253AA primary hepatocytes, and the basal level of total FoxO1 increased in the mutant cells (Fig. 1F). As expected, the FoxO1-S253 phosphorylation by insulin stimulation was diminished in A/A hepatocytes, while other two sites phosphorylation at T24 and S316 were also largely impaired (Fig. 1F). Together, these data confirmed that S253 is a key site in control of FoxO1 stability and phosphorylation at the other two sites in vivo.

Glucose homeostasis of FoxO1-S253AA mice
To evaluate in vivo function of FoxO1-S253 in glucose homeostasis, we measured blood glucose concentrations of WT, A/+ and A/A mice at overnight fasting or random-fed states. Blood glucose under fasting conditions in the A/A mice was significantly decreased by 16-25% compared to that of control mice (Fig. 1G). However, blood glucose under feeding conditions showed a 15-30% increase in the A/A mice compared to that of the control mice at the age of 4
to 16 weeks (P<0.05) (Fig. 1H). There was no significant difference between +/+ and A/+ mice (Fig. 1G-H).

**Insulin sensitivity of FoxO1-S253A/A mice**

We next examined the insulin sensitivity of control and A/A mice by performing euglycemic-hyperinsulinemic clamp analysis and glucose tolerance test. Upon peripheral insulin infusion (2.5 mU kg⁻¹ min⁻¹), the circulating glucose concentrations were maintained at 150 mg/dl in both control WT and A/A mice. The glucose infusion rate (GIR) in KI mice had no significant differences (Fig. 2A). Basal EndoRa for hepatic glucose production in KI mice displayed a lower level without significance compared to WT mice under the hyperinsulinemic condition (Fig. 2B). The Rg for glucose uptake in soleus muscle and hearts were in trend to decrease but insignificantly, and unchanged in the gastro and vastus L muscle, and white adipose tissue (Fig. 2C-D), suggesting that A/A barely affected glucose uptake in muscle and white adipose tissues.

We next performed glucose tolerance test (GTT), KI mice did not display glucose intolerance upon glucose injection (Fig. 2E). However, KI mice were hyperinsulinemic that serum insulin levels increased significantly in A/+ by 2-fold and A/A mice by 3-fold (WT: 0.4 ± 0.07 ng/ml, A/+: 0.78 ± 0.03 ng/ml, versus A/A: 1.2 ± 0.08 ng/ml), in the fasting state (Fig. 2F). A similar effect was also observed in the feeding condition (0.8 ± 0.02 ng/ml WT, 1.1 ± 0.03 ng/ml A/+, versus 1.6 ± 0.02 ng/ml A/A) (Fig. 2F). KI mice also showed high insulin release upon the glucose tolerance test (Fig. 2G). These indicated that the insulin sensitivity was actually reduced in KI mice.

We next performed pyruvate tolerance test (PTT), and both A/+ and A/A mice exhibited pyruvate intolerance (Fig. 2H). We next measured serum glucagon levels in these mice. Compared to WT mice, glucagon concentrations decreased in A/A mice by 50% during the fasting state (118 ± 10 pmol/ml WT vs. 60 ± 5 pmol/ml A/A, P<0.05), as well as in the feeding condition (50 ± 4.3 pg/ml WT vs. 32 ± 3.1 pg/ml A/A) (Fig. 2I). Additionally, KI mice exhibited increases by nearly 1.5-fold in expressions of Pck1 or G6pc in the liver when mice were at either feeding or fasting state (Fig. 2J). These data indicate that A/+ and A/A mutation affect gluconeogenesis, blood insulin, and glucagon levels.

**Oxygen consumption and energy expenditure in FoxO1-S253A/A mice**

We also measured food intake, physical activity, and energy expenditure by analyzing oxygen consumption and carbon dioxide generation in mice in the metabolic cages. WT and A/A mice exhibited no difference in food intake (Fig. 3A), but physical activity was significantly reduced in A/A mutant mice in the dark phase (Fig. 3B). Meanwhile, oxygen consumption and energy expenditure were higher in A/A mice, in particularly during the dark phase (Fig. 3C-D). The respiratory exchange ratio (RER), the ratio between the amount of carbon dioxide production and oxygen consumption, had no significant changes between A/A and control mice (Fig. 3E). The results indicate A/A mutant mice have higher metabolic rates even though the physical activities were reduced.

**Islet morphology of FoxO1-S253A/A mice**

The serum insulin level increases while glucagon level reduces in A/A mice, and we thus suspect that these changes in pancreatic hormones may reflect a remodeling of β-cells and α-cells in the pancreas. To examine this possibility, pancreases were isolated from WT and A/A mice, we measured pancreatic hormone contents and performed immuno-histochemical and morphometric analysis. A/A mice exhibited a 41% higher pancreas weight than WT mice (Fig. 4A). Insulin contents were increased by 50% in the pancreas, and by 52% in the islets of A/A mice, while
glucagon contents were decreased by 38% in the pancreas, and by 40% in the islets of A/A mice, compared with that of WT mice (P<0.05) (Fig. 4B-E). We further investigated the insulin or glucagon releases in the islets isolated from these mice. KI mice showed increased insulin secretion and decreased glucagon secretion (Fig. 4F-G). Immunostaining of pancreas by glucagon and insulin revealed a 36.8% reduction of glucagon-positive α-cells in islets from A/A mice, but insulin-positive β-cells were unchanged in both genotypes (Fig. 4H-J). We also found that WT islets had 2.30% ± 0.29% insulin and glucagon double positive cells, while A/A islets exhibited 4.40% ± 0.06% double positive cells in the pancreas (P<0.01, n=3 mice/group). The ratio of α-cells to β-cells in each islet was reduced by 27.7% in A/A mice compared to WT (P<0.05) (Fig. 4K). Based on quantification of islet area on H&E staining, the percentage of islet area relative to total pancreas area was reduced by 41.9% in A/A mice (Fig. L-M). Considering the 41.4% increase in pancreatic weight (Fig. 4A), the total islet mass did not change in A/A mice compared with WT mice. We further measured gene expression for markers, which are responsible for different stages of pancreatic differentiation (3). The pancreas of A/A mice exhibited a significant increase in the expression of the progenitor cell markers, including Sox9, Sox17 and Hnf1b, and marked decreases in expression of acinar cell markers, such as Amy2a, Cela1, and Ptf1a, suggesting the A mutation of Foxo1-S253 might increase progenitor cells and reduce acinar cells differentiation in the pancreas (Fig. 4N). For β-cell markers, A/A significantly increased Neurod1 and Maf-A expression in the whole pancreas (Fig. 4N), and significantly increased expression of insulin, Neurod1 and Pdx-1 in the islets of pancreas (Fig. 4O). For α-cell markers, A/A significantly decreased expression of Arx, Loxl4, Hmgb3, and Fev mRNA expression in the whole pancreas (Fig. 4N), and significantly decreased expression of glucagon and Fev expression in islets (Fig. 4O). Although the mRNA of acinar cell and progenitor cell markers, such Amy2a, Cela1, Sox17, and Hnf1b were also detected in isolated islets, the expression level of these genes in islet was 10^3 times less than those in whole pancreas (data not shown). Collectively, these data suggest that A/A pancreas have a reduced α-cell number, which may result from reduced progenitor cell differentiation.

Taken together, these data suggest that A/A pancreas may inhibit progenitor cells differentiation into endocrine cells and increase the pancreatic mass. Moreover, it probably impairs differentiation of α-cells and leads to decreased glucagon levels in both serum and pancreas.

**Glycogen metabolism in FoxO1-S253^AA mice**
We next measured liver glycogen contents since both insulin and glucagon control glycogen levels that contribute to blood glucose homeostasis in the fasting and feeding state. In consistent with increased blood insulin and decreased glucagon levels, the glycogen concentrations in the liver of fasted A/A mice were 81.5% higher than that of fasted WT mice, while there were no significant changes in the liver of fed WT and A/A mice (Fig. 5A). These data suggested that glycogenolysis in the liver of fasted A/A mice was inhibited, probably by the elevation of insulin level, while such effect was diminished in the feeding state.

**Hepatic glucose production of FoxO1-S253^AA in response to glucagon**
We further examined whether FoxO1-S253A impairs glucagon-stimulated glucose production in mice. During the feeding state, glucagon enhanced blood glucose by 25% in WT mice and 35% in A/A mice (Fig. 5B). Glucagon tolerance test indicated that A/A mice displayed higher blood glucose concentrations when glucagon injected after 15 min, an effect sustained up to 60 min (Fig. 5C). We further examined whether the effect of A/A on HGP is mediated by gluconeogenesis or glycogenolysis in cells. The primary hepatocytes were isolated from WT and
A/A mice, and HGP analyzed in vitro by measuring glucose release in the cultured medium by hepatocytes. In the basal condition, A/A hepatocytes exhibited a 50% higher HGP and 47% higher gluconeogenesis, compared with WT cells (Fig. 5D-E). Upon glucagon stimulation, A/A hepatocytes also had a 47% higher HGP and 51% higher gluconeogenesis, compared with WT cells (Fig. 5D-E). The fold change of gluconeogenesis by glucagon in WT cells (1.4-fold induction) was not statistically different from that in A/A cells (1.5-fold) (Fig. 5E). These data indicated S253A mutation induced the basal level of HGP and gluconeogenesis and glucagon-stimulated gluconeogenesis was not impaired. The higher response of A/A mice to the exogenous glucagon stimulation in blood glucose may result from a higher concentration of hepatic glycogen and associated glycogenolysis in A/A mice.

Glycogenolysis increased in both WT and A/A hepatocytes in response to glucagon stimulation, similar to the basal level of A/A cells (Fig. 5E). In agreement with the HGP assay, gene expression analyses indicated that glucagon-induced G6pc gene expression significantly increased by nearly 2-fold in A/A cells, similar to the 2-fold increase in control hepatocytes in response to glucagon stimulation (Fig. 5F).

**FoxO1-S253A/A increases glucagon-stimulated FoxO1 nuclear translocation**

Finally, we determined whether glucagon affects FoxO1 nuclear translocation and examined the effect of A/A in the primary hepatocytes. Glucagon induced FoxO1 nuclear translocation by 1.4-fold in WT hepatocytes. However, FoxO1 nuclear translocation markedly increased by 2.2-fold in A/A hepatocytes versus WT cells. In response to glucagon stimulation, nuclear FoxO1 increased by 25% in A/A hepatocytes compared to the non-treatment A/A cells (P<0.05, Fig. 5G).

**DISCUSSION**

In this study, we generated a novel FoxO1-S253A/A mouse model to assess the role of FoxO1-phosphorylation at Ser253 in control of glucose homeostasis and demonstrated that impairment of FoxO1 phosphorylation at Ser253 plays a role in hepatic glucose production and pancreatic function. The homozygous FoxO1-S253A/A mice displayed: 1) normal body weight, slightly increased postprandial blood glucose and decreased fasting blood glucose; 2) elevated blood insulin by 2-fold, reduced blood glucagon by 40%, and significantly decreased the ratio of pancreatic α-cells over the β-cells; and 3) increased FoxO1 stability and HGP. Thus, our data demonstrated that blockage of FoxO1 phosphorylation at Ser253 in vivo resulted in a unique pattern of metabolic dysregulation.

The role of FoxO1 in glucose metabolism in hepatocytes is well-studied (8,29-32), but this study provided novel genetic evidence to decipher the role of FoxO1 phosphorylation at Ser253 in control of HGP, glucose homeostasis, and pancreatic biology in vivo. In the feeding state, blood glucose of FoxO1-S253A/A mice increased by 15% compared to control mice, suggesting that insulin suppression on HGP was blocked by FoxO1-S253A point mutation. The enhanced gluconeogenesis by the point mutation is further supported by the pyruvate tolerance test in mice and in vitro analysis of primary hepatocytes isolated from mice excluding the potential influences of endocrine hormones altered in vivo, including insulin and glucagon.

An increase of HGP and blood glucose during feeding conditions may also contribute to pancreatic β-cell secretion of insulin, which inhibits glycogenolysis and/or stimulate glycogen synthesis compensating for further elevation of blood glucose in A/A mice. However, the elevated level of insulin by 2-fold in KI mice also observed during the fasting state even blood glucose was slightly reduced by 10%, which encouraged us to examine pancreatic α-cells and
glucagon levels. Compared to control mice, the decreased blood glucose in A/A mice during the fasting state can be a resultant from higher levels of insulin that promotes glycogen synthesis and lower levels of glucagon for attenuation of gluconeogenesis (Fig. 6). The key downstream effectors of glucagon include cAMP content and cAMP response element binding protein (CREB) functionality at gene transcriptional levels (33). A potential reduction of cAMP and CREB functionality in the liver of A/A mice, may also play a role in attenuation of hepatic gluconeogenesis gene expression. Importantly, we have recently discovered a novel mechanism by which glucagon and cAMP-dependent protein kinase (PKA) promote FoxO1 nuclear localization and functionality in the liver via FoxO1-Ser273 phosphorylation (34). Another independent study also indicates that glucagon induces Foxo1 nuclear localization and HGP via a Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) on multiple phosphorylation sites of Foxo1 (35). Moreover, HGP is also controlled by the substrate availability through extrahepatic tissues, in addition to hepatic gene transcriptional programming. For example, lipolysis suppression in the peripheral fat tissues, as well as in the liver, by high insulin or low glucagon levels, can result in a reduction of substrates of gluconeogenesis or acetyl CoA content that reduces hepatic pyruvate carboxylase activity and then gluconeogenesis (36,37). This may be the case in the A/A mice that hepatic pyruvate and blood triglycerides were reduced (data not shown), and HGP elevated significantly as we observed in pyruvate tolerance test. The alteration of hepatic substrates availability can be related to a lower glucagon and higher insulin levels in the blood of A/A mice, when compared to control mice.

FoxO1 also plays a role in the pancreatic β-cell differentiation and function, but its role in control of α-cells is unclear even though its expression in adulthood is very low (2,38). In general, FoxO1 inhibits cell proliferation and induces apoptosis in a number of cells (4). Suppression of FoxO1 in the pancreatic β-cells promotes survival and cell mass when IRS2 is absent (10). Conversely, FoxO1 in pancreas also promotes pancreatic progenitor cell differentiation by inducing expression of MafA and neurod1 gene expression (39). Recent study showed that FoxO1 deficiency in pancreatic β-cells promoted dedifferentiation of β-cells into the progenitor-like cells, including Neurogenin-3 and Oct4 gene expression and even the α-cells (2). Our results from the FoxO1-S253\(^{AA}\) mice suggest that FoxO1-S253\(^{AA}\) enhances β-cell function and blood insulin, whereas decreases α-cell numbers and blood glucagon, as indicated by the lower ratio of α-cells/β-cells. The potential mechanism of pancreatic α-cell suppression and β-cell promotion by FoxO1-S253\(^{AA}\) is unclear, but our data indicate that FoxO1-S253\(^{AA}\) mutant pancreas had increased pancreatic mass and expression of marker genes for β-cells and pancreatic progenitor cells, and reduced marker genes for α-cells, even insulin and glucagon gene expression in the pancreas of mutant pancreas are not significantly altered. The reason for the increase in pancreas weight in FoxO1-S253\(^{AA}\) mice is unclear. It may relate to increase in insulin that serves as a paracrine factor promoting the growth in size of pancreatic tissue or differentiation of different of pancreatic cell population. Thus, FoxO1-S253 de-phosphorylation may reflect a molecular modification on FoxO1 as a compensation for pancreatic plasticity during development of insulin resistance where insulin secretion from the β-cells generally suppresses glucagon secretion from the α-cells.

Our data indicate that FoxO1-S253 de-phosphorylation regulates pancreatic plasticity and the clinical relevance of this study is high and here we proposed a model for the role of FoxO1-S253 in the pancreas and liver in control of glucose metabolism during development of insulin resistance. In the pancreas of adulthood, FoxO1-S253 phosphorylated and suppressed by feeding conditions upon insulin secretion; but moderately activated upon fasting conditions by de-
phosphorylation when insulin decreases and glucagon increases. This may be important for β-cell maintenance and function by FoxO1-S253 de-phosphorylation at the fasting condition where FoxO1 is functional for β-cell identity and maintenance when insulin secretion reduced. The β-cells are insulin responsive and FoxO1 deficiency was observed in the islets of individuals of T2D, even though other peripheral tissue such as liver where FoxO1 is activated (40). We speculate that hyperinsulinemia promotes FoxO1 cytoplasmic localization, degradation, or deficiency upon early development of T2D particularly in β-cells, promoting β-cell dedifferentiation into the α-cells in the pancreas. Several lines of evidence over the past decades support the concept by: 1) β-cells are derived from existing β-cells based on genetic tracing studies (41,42); 2) β-cell mass, function and insulin secretion decrease, and hyperglucagonemia develops in late-phase T2D in patients; and 3) a complete FoxO1 deficiency in β-cells promotes β-cells dedifferentiation to progenitor-like or α-cells for glucagon production (43). We thus predict that increase of glucagon, in addition to other metabolic stress, may be resultant from hyperinsulinemia, FoxO1 deficiency in β-cells and FoxO1 activation in other tissues, such as liver in patients with T2D. Indeed, the pancreas mass increased in KI mice, probably by increased progenitor cell populations, as we predicted with limited and little direct evidence, in which elevated insulin secretion may have a role in enhancing the pancreas mass with other mechanisms. The KI mice showed increase in insulin and decrease in glucagon at both fasting and feeding condition, suggesting that A/A mutation may have key roles in maintaining β-cells function/insulin secretion and directing to progenitor cell differentiation increasing pancreas mass, while restraining its dedifferentiation to the α-cells.

Hyper-activation of FoxO1 by multiple mechanisms contributes to development of diabetes from insulin resistance. At the physiological level, phosphorylation of FoxO1-S253 markedly decreases in the liver during the fasting state when blood insulin decreases while glucagon increases, promoting gluconeogenesis. The FoxO1-S253A/A mutation in hepatocytes promotes FoxO1 stability. However, additional activation mechanism is required for enhancing FoxO1 ability at gene transcriptional levels. In this study, we provide an additional activation of FoxO1 by glucagon may play a critical role in FoxO1 enhancing HGP and elevation of blood glucose. In consistent with the previous reports (34,35), we showed that glucagon significantly stimulated FoxO1 nuclear localization and stability. Of note is that the total Foxo1 protein level in A/A pancreas decreased by 40% compared to that of WT pancreas (data not shown), which might relate to the effect of a lower glucagon level in the mutant mice. In diabetic conditions, de-phosphorylation of FoxO1-S253 is present in tissues of subjects with T2D when insulin resistance occurs failing to activate Akt and hyperglucagonemia develops (34,44,45). This study showed that a moderate activation of FoxO1 via de-phosphorylation of S253 alone is not sufficient for induction of diabetes in mice, unexpectedly, as a compensatory benefit to the metabolism for maintaining insulin sensitivity with higher energy expenditure and lower physical activity. The mechanism may relate to increasing gene expression of Irs2 in tissues, such as liver (data not shown).

In the KI mice, the feeding blood glucose and insulin increase, suggesting that insulin dominant role in suppression of gluconeogenesis is attenuated by A/A liver when blood glucagon decreases (Fig. 6A). However, the fasting blood glucose and glucagon decrease in the KI mice, suggesting that glucagon dominant role in stimulation of gluconeogenesis is attenuated, in accompany with an increase of insulin that can stimulate glycogen synthesis or inhibit glycogenolysis (Fig. 6B).
In addition to liver and pancreas, we do not exclude the possibility of other tissues with the FoxO1-S253A/A mutation that can be involved in regulating the blood glucose and energy metabolism. For example, FoxO1 in hypothalamic neurons increases food intake and/or increases energy expenditure (46). FoxO1- S253A/A represent a combination of outcome of contribution from all tissues in the whole body. Taken together, our data provide genetic and cellular evidence for a fundamental mechanism by which impairment of FoxO1 phosphorylation at Ser253 can alter blood glucose, at least via liver and pancreas, involving the control of hormones and pancreatic plasticity. Our novel findings may provide a new strategy in targeting FoxO1 and its interaction with other factors for conquering T2D in the future.

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AUTHOR CONTRIBUTIONS
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* To whom correspondence should be addressed: Shaodong Guo: Department of Nutrition and Food Science, College of Agriculture and Life Sciences, Texas A&M University, College Station, TX 77843; shaodong.guo@tamu.edu; Tel: 979-845-0850; Fax: 979-862-6842.
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The authors have nothing to disclose.

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**FIG. 1.** Generation of FoxO1-S253AA mice and postprandial hyperglycemia. (A): The genomic locus of FoxO1 in mouse genome. (B): Gene targeting strategy for generation of FoxO1-S253A mutation via ES cell and homologous recombination. (C-D): Southern-blot were performed for screening the 5’ and 3’ end homologous recombination in FoxO1 genomic loci. DNA sequencing of endogenous FoxO1 loci of negative and positive ES cells. (E): Body weight curve of mice of control wild type (WT), A/+, and A/A mice at the ages of 4 to 16 weeks. (F): Effect of insulin on FoxO1 protein and phosphorylation in hepatocytes. Primary hepatocytes were isolated from the control and A/A mice and treated with 100 nM insulin for 30 min and 100 µg protein of cell lysates were subjected to Western-blot against antibody of FoxO1, T24, S316 and beta-actin. (G-H): Blood glucose levels were measured in WT, A/+ and A/A mice at the ages 4 to 16 weeks during 16 h fasting (G) and random-fed conditions (H). * p<0.05 vs. WT and A/+ mice, n=10 mice/group.

**FIG. 2.** FoxO1-S253AA mice exhibit impaired insulin and glucagon secretion and glucose homeostasis. (A): GIR of control and A/A mice at the ages of 16 weeks in euglycemic-hyperinsulinemic clamp assays. n=8 mice per group. (B): Hepatic glucose production of mice during the clamp assay. (C-D): Glucose uptake in a variety of tissues of mice during the clamp assay. p<0.05, n=8 mice per group. (E): Glucose tolerance tests were performed in 10-week-
week mice (n=6) under 16 h fasting condition. Blood glucose were plotted against the time after the i.p. 2g D-glucose/kg body weight glucose injection. (F): Serum insulin concentration was measured in mice during 16 h fasting and random-fed states. * p<0.05 vs. WT mice, n=10 mice/group. (G): Serum insulin concentration was measured in mice during the glucose tolerance test from (E). * p<0.05 vs. WT mice, n=10 mice/group. (H): Pyruvate tolerance tests were conducted in mice after 16 h overnight fast. Blood glucose levels (means + SEM, n=6 mice/group) were determined at the indicated time points after i.p. injection of 2g pyruvate/kg body weight. *<0.05 vs. WT. (I): Serum glucagon concentration was measured in mice during 16 h fasting and random-fed states. * p<0.05 vs. WT mice, n=10 mice/group. (J) Expression of Pck1 and G6pc in the liver of WT, A/+, and A/A under a 16 h fasting or random-fed condition. *<0.05 vs. WT, n=6.

**FIG. 3.** Energy expenditure increases in FoxO1-S253A/A mice. The mice at the age of 16 weeks old were placed in metabolic cages for the measurement of food intake, physical activity and oxygen consumption and energy expenditure. Food intake (A), physical activity (B), oxygen consumption (C), RER (D), and energy expenditure (E) were calculated. * p<0.05 vs. WT, n=8 mice per group.

**FIG. 4.** FoxO1-S253A/A mice exhibit impaired alpha-cells, insulin, and glucagon synthesis in the pancreas. (A): Representative pancreas of control (WT) and A/A mice at the age of 12 weeks at random-fed states, * p<0.05 vs. WT, n=6. (B, C): Insulin and glucagon concentration were measured in the pancreas of control and A/A mice at the age of 12 weeks at random-fed states. * p<0.05 vs. WT, n=3. (D, E): Insulin (J) and glucagon (K) concentration were measured in the islets of control and A/A mice and normalized by islet protein. (F): Insulin secretion from the islets isolated from control and A/A mice. * p<0.05 vs. WT, n=3. (G) Glucagon secretion from the islets isolated from control and A/A mice under 3.3mM glucose condition. * p<0.05 vs. WT, n=3. (H): Immunostaining of pancreas from 16-week-old random-fed mice by glucagon and insulin. Representative images are shown. *p<0.05 vs. WT, n=6 mice per group. (I-K): The number of alpha-cells (I) and beta-cells (J) were counted from at least 3 mice per group and the ratio of alpha-cell over beta cell number (K) in each islet were counted and calculated. * p<0.05 versus WT, n=3 mice per group. (L): The representative images of the pancreas and islets of WT and A/A mice. (M): The area of islets in total pancreas was calculated in WT and A/A mice. * p<0.05 vs WT, n=4. (N-O): The expression of marker genes responsible for the pancreatic beta-cell, alpha-cell, acinar-cell and progenitor cell in total pancreas (N) or islets (O) isolated from WT and A/A mice. * p<0.05 vs. WT, n=3.

**FIG. 5.** FoxO1-S253A/A mice exhibit impaired glucagon tolerance test and hepatic gene expression. (A): Glycogen concentration in the liver of mice at 16 h fasting and random-fed states, p<0.05 vs control, n=6 mice per group and N. S. represents no significance. (B): Blood glucose in random-fed mice injected by glucagon. * p<0.05 vs. control, n=8 per group. (C): Glucagon tolerance tests in control and A/A mice at the age of 12 weeks. Blood glucose were measured in 18 h fasting mice by 16 µg/kg body weight of glucagon i.p. injection. * p<0.05 vs. control, n=8 mice per group. (D): HGP assay in primary hepatocytes of control and A/A mice. Cells were stimulated by 100 nM glucagon and glucose concentrations in culture medium were measured. * p<0.05 WT vs. WT + glucagon, # p<0.05 S253A/ vs. S253A/A + glucagon, n=3 experiments. (E): Hepatic glucose production, glycogenolysis, and gluconeogenesis were measured in the primary hepatocytes. * p<0.05 vs. WT, # p<0.05 vs. A/A, n=3 experiments. (F):
Gene expression levels of G6pc were measured in primary hepatocytes by real-time PCR. Data presented as average of triplicate determinations from control and A/A or 100 nM glucagon stimulation for 8 h. * p<0.05 n=3. PCR primer sequences are cyclophilin 5'-ctaaagcatacaggtctgcscatcttg-3', and 5'-tgccatccagcattccagctcttg-3'; G6pc 5'-cattgtggcttcctgggtcc-3' and 5'-ggcagtggtggataagactg-3'. (G): Nuclear and cytoplasmic proteins were extracted from control and A/A primary hepatocytes with or without 100 nM glucagon stimulation for 30 min. 100 µg cytoplasmic proteins and 20 µg nuclear proteins were subjected to SDS-PAGE and Western-blot analysis. Quantification of nuclear FoxO1 was normalized by histone H1 with analyses from image J. * p<0.05 vs. control. n=3 experiments.

**FIG. 6.** Dephosphorylation of FoxO1-S253 in FoxO1-S253A/A mice regulates hepatic gluconeogenesis, glycogen metabolism, and pancreatic plasticity. FoxO1-S253 dephosphorylation controls insulin and glucagon production in pancreas regulating liver glucose metabolism in the feeding state, resulting in an increase of blood glucose (A). At the fasting state (B) where blood glucagon decreases reducing the blood glucose. → stimulation; ↓ inhibition.
Figure 3

A. Accumulative feed intake (g)

B. Physical activity (cm)

C. VO₂ (mL/kg/h)

D. Energy expenditure (kcal/kg/day)

E. P:EE (in 0.1)

WT and A/A groups are compared with respect to their activities in daylight and nighttime.
Figure 4

A. Relative mRNA expression across different cell types in WT and A/A conditions.

B. Pancreatic insulin content (ng/mg).

C. Pancreatic glucagon content (ng/mg).

D. Insulin secretion (ng/mg protein/h) for WT and A/A.

E. Glucagon secretion (pg/mg protein/h) for WT and A/A.

F. Insulin secretion at 3.3 mM and 22.2 mM glucose.

G. Glucagon secretion at 3.3 mM and 22.2 mM glucose.

H. Immunofluorescent staining for Glucagon, Insulin, DAPI, and Merge.

I. Alpha cell counts/islet for WT and A/A.

J. Beta cell counts/islet for WT and A/A.

K. Alpha cell/beta cell ratio for WT and A/A.

L. Islet areas/total pancreatic areas (%).

M. Pancreatic insulin content (ng/mg) for WT and A/A.

N. Pancreatic glucagon content (ng/mg) for WT and A/A.

O. Pancreas weight (mg) for WT and A/A.
**Figure 5**

A. Bar graph showing glycogen levels (mg/g of liver tissue) in WT and S253A/A mice under fasting and feeding conditions. *P<0.05; N.S.

B. Bar graph depicting blood glucose levels (mg/dL) in WT and S253A/A mice under Vehicle and Glucagon conditions. *P<0.05.

C. Line graph illustrating blood glucose levels (mg/dL) in WT and S253A/A mice over time (minutes) after glucagon injection. *P<0.05.

D. Line graph showing HGP (μmol/g protein) in WT, S253A/A, and S253A/A + Glucagon mice. *P<0.05.

E. Bar graph displaying glucose release (μmol/h/g protein) in WT, WT + Glucagon, S253A/A, and S253A/A + Glucagon mice. *P<0.05; #P<0.05.

F. Bar graph showing relative expression of FoxO1 in cortical (C) and nuclear (N) fractions under Vehicle and Glucagon conditions. *P<0.05.

G. Western blot analysis of FoxO1, GAPDH, and Histone-H1 expression in WT and S253A/A mice under Glucagon conditions. *P<0.05.
Figure 6

A

- S253A
- Nuclear FoxO1
- Feeding
- Insulin β-cells
- Gluconeogenesis
- Glycogen synthesis
- HGP

B

- S253A
- Nuclear FoxO1
- Fasting
- Insulin β-cells
- Glucagon α-cells
- Gluconeogenesis
- Glycogen synthesis
- HGP