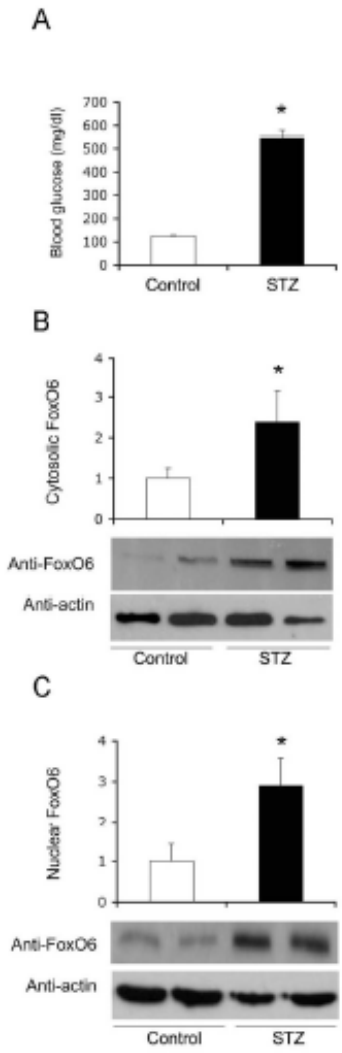
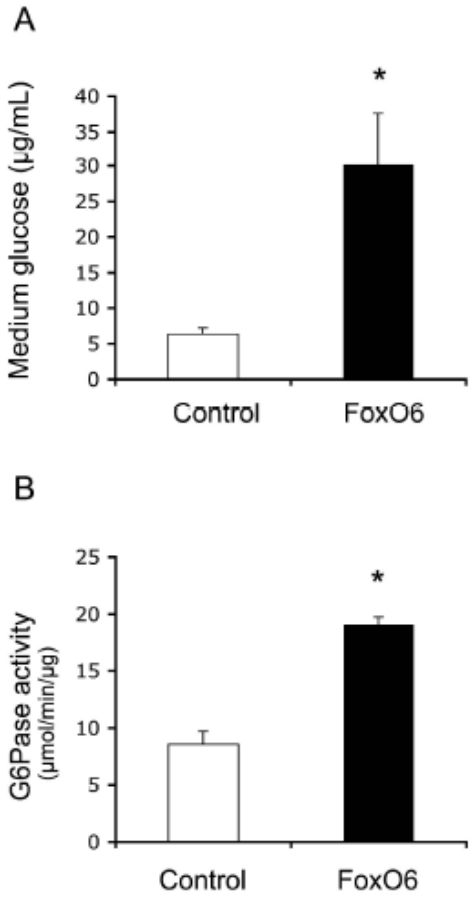
SUPPLEMENTARY DATA

Supplementary Figure 1. Hepatic FoxO6 expression in insulin-deficient diabetic mice. CD1 mice (male, 10 wks, n=8) were intraperitoneally injected with 180 mg/kg STZ. Control mice (n=8) were mock-treated with PBS. One week later, all mice in the streptozotocin group developed severe hyperglycemia (**A**). Mice were sacrificed and liver tissues were subjected to immunoblot analysis for the determination of cytoplasmic (**B**) and nuclear (**C**) FoxO6 protein levels in control and STZ-induced diabetic mice. *P<0.01 vs. control by ANOVA.



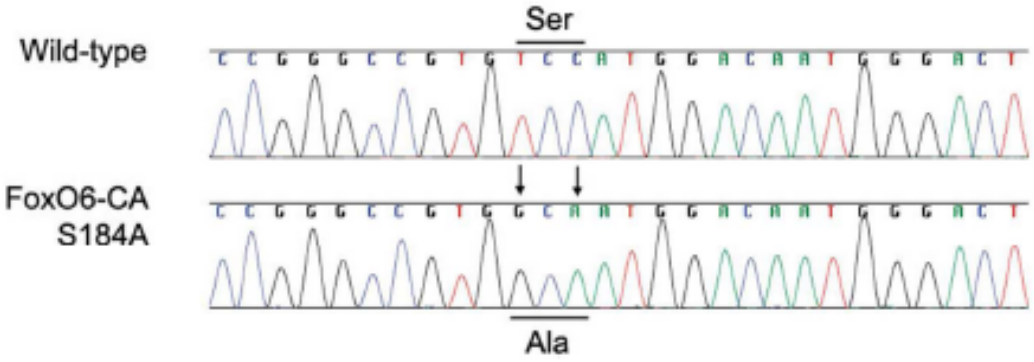
SUPPLEMENTARY DATA

Supplementary Figure 2. FoxO6 induces glucose production in mouse primary hepatocytes. Mouse primary hepatocytes were isolated from CD-1 mice. Hepatocytes were plated in collagen-coated 12-well plates at $2x10^5$ cells/well and transduced with control or FoxO6 vector at the predefined dose of 100 pfu/cell. Each condition was run in 6 replicates. After 24-h incubation, conditioned medium and hepatocytes were harvested for the determination of medium glucose concentrations (**A**) and hepatic G6Pase activity (**B**). The G6Pase activity is defined as the production of Pi (in µmole) per unit time (in minutes) per µg of cellular microsomes in liver. *P<0.001 vs. control by ANOVA.



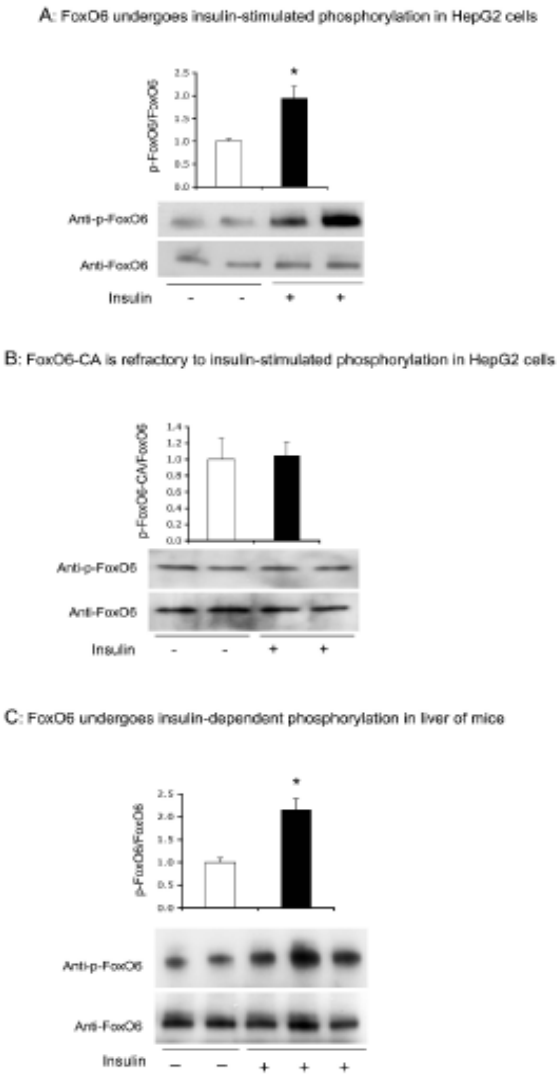
SUPPLEMENTARY DATA

Supplementary Figure 3. DNA sequence profiles of FoxO6 and its constitutive mutant. The FoxO6-CA mutant contains 2 nucleotide substitutions, as indicated by arrows. These alterations converted Ser184 to Ala184 in the FoxO6 polypeptide chain, as confirmed by nucleotide sequencing.



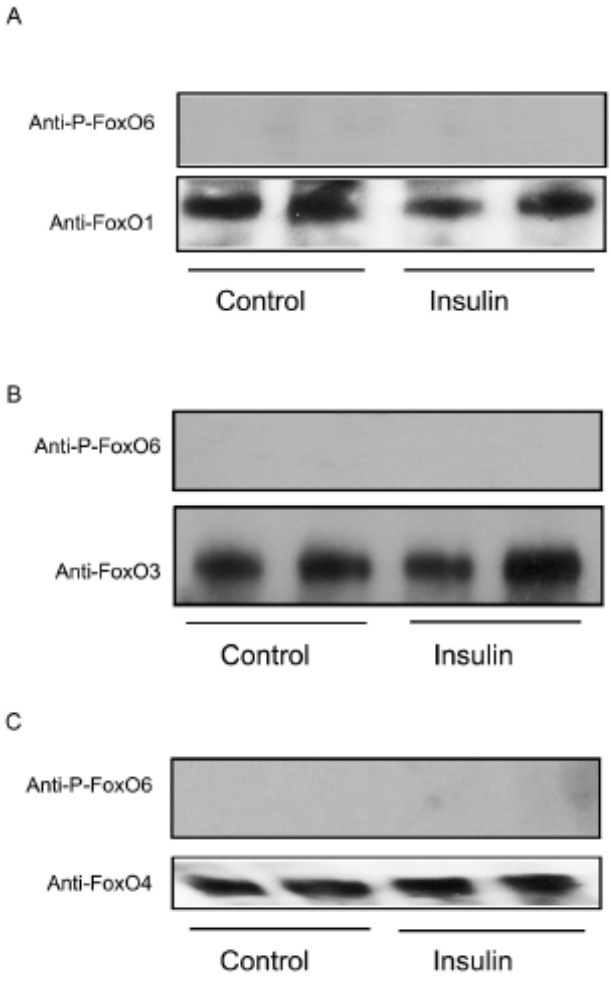
SUPPLEMENTARY DATA

Supplementary Figure 4. Insulin promotes FoxO6 phosphorylation. HepG2 cells pre-transduced with FoxO6 vector were incubated without or with insulin (100 nM) for 30 min, followed by immunoprecipitation using anti-FoxO6 antibody. Immunoprecipitates were analyzed by immunoblot assay for FoxO6 Ser-phosphorylation using anti-phospho-FoxO6 (S184) antibody (**A**). Likewise, HepG2 cells pre-transduced with FoxO6-CA vector encoding the mutant FoxO6-S184A were incubated without or with insulin (100 nM) for 30 min, followed by immunoprecipitation and immunoblot analysis of FoxO6 Ser-phosphorylation for the determination of S184-phosphorylated FoxO6 vs. total FoxO6 proteins (**B**). In addition, CD-1 mice were fasted for 16 h, followed by an intraperitoneal dose of insulin (2 IU/kg, n=5) or 200-µl of saline as control (n=4). Mice were sacrificed 5-min post insulin injection, and liver tissues were subjected to anti-FoxO6 immunoprecipitation and immunoblot analysis of FoxO6 Ser-phosphorylation (**C**). Rabbit anti-phospho-FoxO6 (S184) antibody was derived from AV(S^{184})MDNGAKFLRIK that corresponds to amino acid residues 182-195 of mouse FoxO6 protein, in which the S^{184} residue was phosphorylated. Rabbit anti-FoxO6 antibody was made from the N-terminal epitope (AKLRAHQV DVDPDFA) corresponding to amino acid residues 3-17 of mouse FoxO6 protein. Both antibodies were generated in our lab. *P<0.05 vs. control by ANOVA.



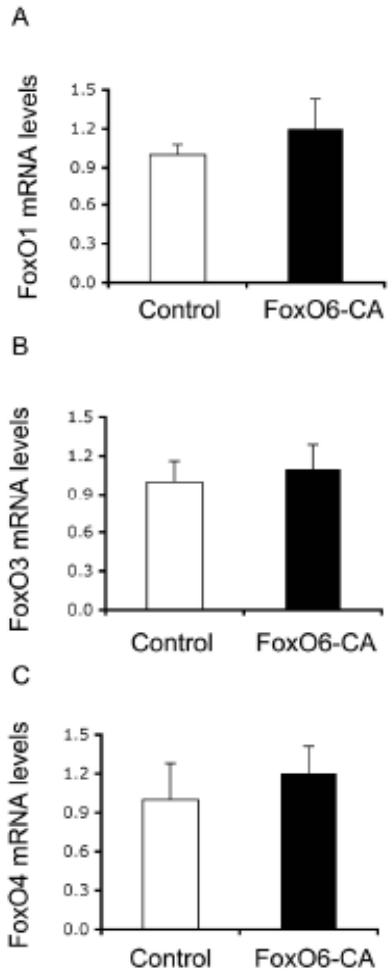
SUPPLEMENTARY DATA

Supplementary Figure 5. Anti-Phospho-FoxO6 antibody does not cross-react with FoxO1, FoxO3 and FoxO4 proteins. CD-1 mice were fasted for 16 h, followed by an intraperitoneal dose of insulin (2 IU/kg) or saline. Mice were sacrificed 5-min post insulin injection, and liver tissues were subjected to immunoprecipitation using anti-FoxO1, anti-FoxO3, and anti-FoxO4 antibodies. Immunoprecipitates were washed thoroughly with PBS buffer plus 0.25% Tween-20, followed by immunoblot analysis using anti-Phospho-FoxO6 antibody. As control, immunoblot analysis was performed for confirming the presence of FoxO1, FoxO3 and FoxO4 proteins in the immunoprecipitates by anti-FoxO1, anti-FoxO3 and anti-FoxO4 antibody, respectively.



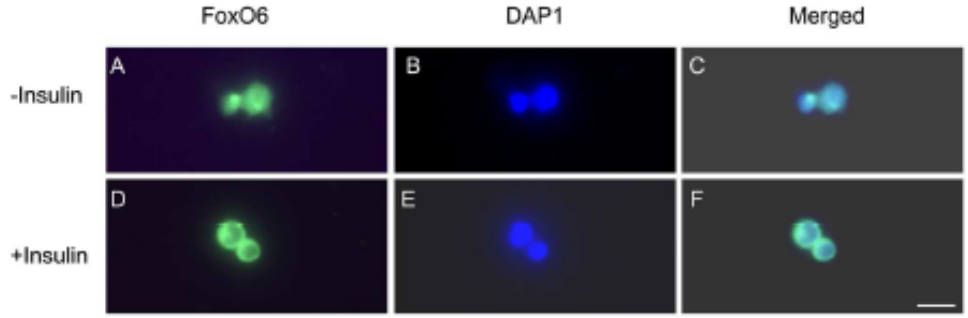
SUPPLEMENTARY DATA

Supplementary Figure 6. Impact of FoxO6 on hepatic FoxO1, FoxO3 and FoxO4 expression. CD1 mice (male, 10 weeks old) were stratified by body weight and randomly assigned to two groups (n=5), which were intravenously injected with Adv-FoxO6-CA or Adv-null vector at $1.5x10^{11}$ pfu/kg body weight. Mice were sacrificed after two weeks of hepatic FoxO6-CA production and liver tissues were subjected to real-time qRT-PCR analysis for the determination of hepatic FoxO1 (**A**), FoxO3 (**B**) and FoxO4 (**C**) mRNA levels. No significant differences were detected in hepatic FoxO1, FoxO3 and FoxO4 expression between control and FoxO6-CA groups.



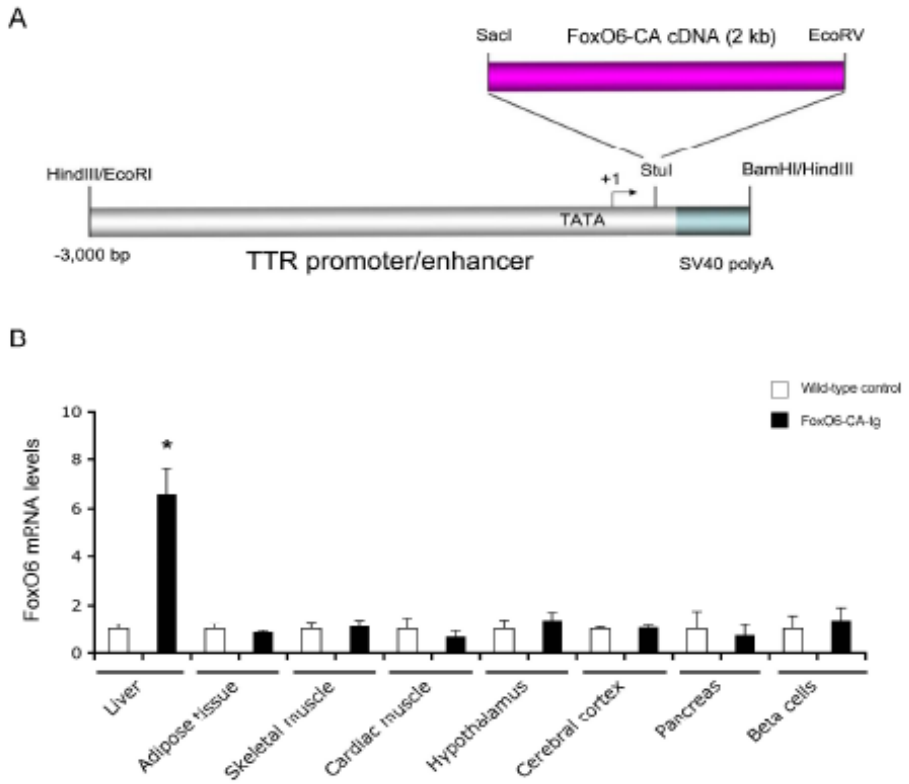
SUPPLEMENTARY DATA

Supplementary Figure 7. Effect of insulin on FoxO6-CA subcellular distribution. HepG2 cells were transduced with FoxO6-CA vector (100 pfu/cell). After 24-h incubation, cells were treated with insulin (100 nM) for 30 min, followed by anti-FoxO6 immunohistochemistry. Like its wild-type counterpart, FoxO6-CA mutant remained in the nucleus regardless of insulin addition in culture medium.



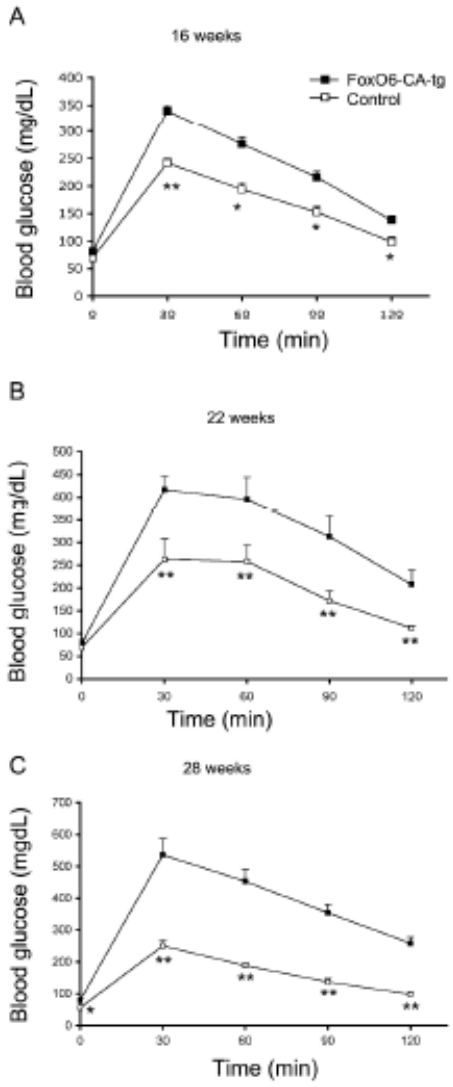
SUPPLEMENTARY DATA

Supplementary Figure 8. Characterization of FoxO6-CA transgenic mice. (**A**) Schematic depiction of the genetic construct used for generating liver-specific FoxO6-CA transgenic mice. The TTR promoter/enhancer (3.5 kb) was used for directing the expression of FoxO6-CA in liver. The mouse FoxO6-CA cDNA was inserted in the first intron of the TTR promoter. (**B**) FoxO6 mRNA profiles in different tissues. Transgenic mice (n=8-10, male, 40 weeks old) and age/sex-matched control littermates (n=5-8, male) in C57BL/6J background were sacrificed for the studies of FoxO6 tissue distribution by real-time qRT-PCR assay. A 6-fold induction of FoxO6 mRNA was detected in liver of FoxO6-CA transgenic mice. In contrast, FoxO6 mRNA expression remained unchanged in other peripheral tissues including brain, cardiac muscle, skeletal muscle, adipose tissue, pancreas and islet β cells. Islets were isolated from male FoxO6-CA transgenic mice (n=6, 12 weeks old) and age/sex-matched control littermates (n=6). Aliquots of 100-150 islets from individual mice were handpicked for the preparation of total islet RNA, which was subjected to real-time qRT-PCR for the determination of FoxO6 mRNA levels in islets. *P<0.005 vs. control by ANOVA.



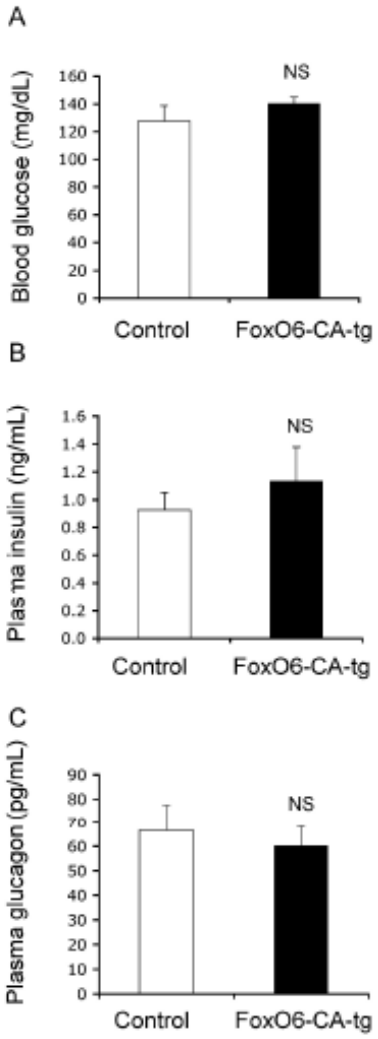
SUPPLEMENTARY DATA

Supplementary Figure 9. Glucose tolerance in FoxO6-CA transgenic mice.
Blood glucose profiles in response to intraperitoneal glucose administration. FoxO6-CA (n=8-10) and control littermates (n=5-8) were subjected to glucose tolerance test at different ages of 16 weeks (A), 22 weeks (B), and 28 weeks old (C). Blood glucose profiles were determined after intraperitoneal glucose infusion (2 g/kg). (□) Control group. (■) FoxO6-CA-tg group. *P<0.05 and **P<0.01 vs. control by ANOVA.



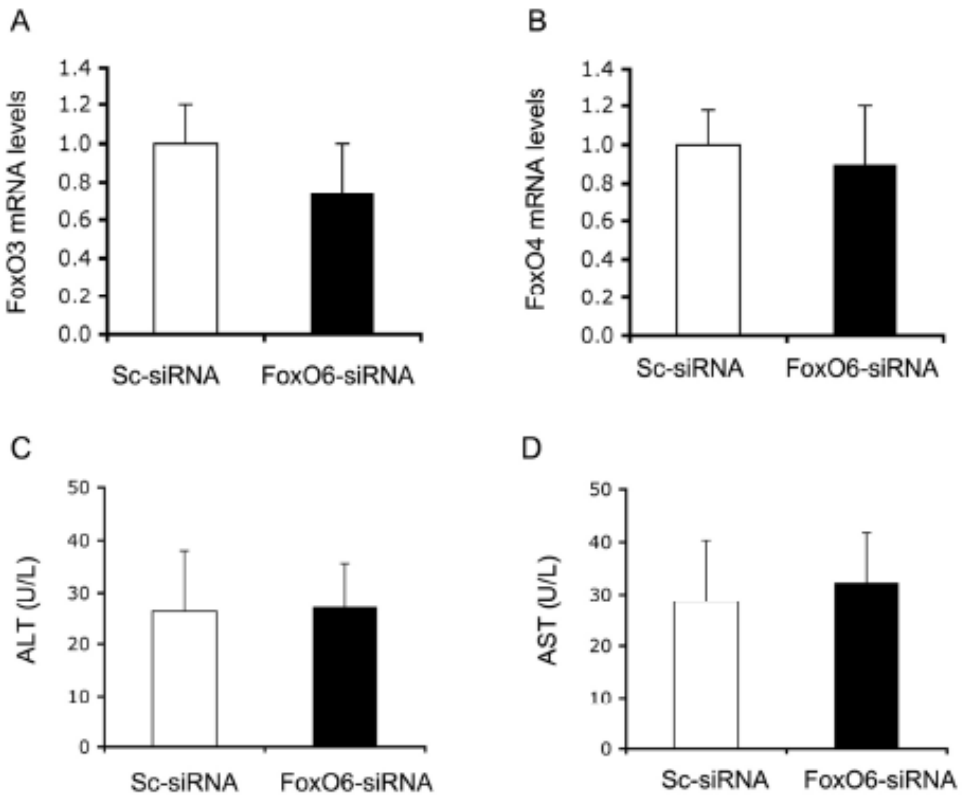
SUPPLEMENTARY DATA

Supplementary Figure 10. Supplemental Fig. 10: Non-fasting blood glucose, plasma insulin and plasma glucagon levels in FoxO6-CA transgenic mice. Under ad libitum conditions, blood glucose (A), and plasma insulin (B) and glucagon (C) levels were determined in FoxO6-CA (n=6, male, 28 weeks old) and sex/age-matched control littermates (n=5). NS, not significant.



SUPPLEMENTARY DATA

Supplementary Figure. 11. Effect of hepatic FoxO6 depletion on FoxO3 and FoxO4 expression and plasma ALT and AST levels. (**A**) Hepatic FoxO3 mRNA levels. (**B**) Hepatic FoxO4 mRNA levels. (**C**) Plasma ALT levels. (**D**) Plasma AST levels. CD1 mice (male, 10 weeks old) were treated with Adv-FoxO6-siRNA (n=10) or control Adv-Sc-siRNA vector (n=10). After two weeks of hepatic FoxO6-siRNA production, liver tissues were subjected to real-time qRT-PCR analysis for the determination of FoxO3 and FoxO4 mRNA levels. Aliquots of blood (25 μl) were used for the determination of plasma ALT and AST levels. No significant differences in hepatic FoxO3 and FoxO4 mRNA levels, as well as plasma ALT and AST levels were detected between FoxO6-siRNA and control groups.



Supplementary Figure 12. Phylogeny of the FoxO family. The phylogenetic tree for the FoxO family was generated using the TreeFam program (http://www.treefam.org). The number in the phylogenic tree denotes the score that is linearly scaled from 1 to 100. The score measures how often the same ortholog (or paralog within-species) pair can be inferred from a resampled tree. The tree branches corresponding to the mammalian FoxO1 and FoxO6 are marked by red box.

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