**ISCI, Volume *23***

## Supplemental Information

# FCoR-Foxo1 Axis Regulates α-Cell Mass through Repression of *Arx* Expression

**Noriko Kodani, Jun Nakae, Masaki Kobayashi, Osamu Kikuchi, Tadahiro Kitamura, and Hiroshi Itoh**

## Transparent Methods

### Antibodies and Cell Cultures

Anti-FCoR antiserum has been described previously (Nakae et al., 2012). We purchased anti-FLAG (M2) and anti-tubulin from Sigma; anti-cMyc (9E10) from Santa Cruz Biotechnology, Inc; anti-FOXO1 (L27) and anti-acetylated lysine polyclonal antibodies from Cell Signaling Technology; anti-insulin from Dako; and anti-glucagon (ab92517) from Abcam. Anti-Arx antibody was a gift from Dr. Ken-ichirou Morohashi and Dr. Kanako Miyabayashi (Kyushu University, Fukuoka, Japan) (Kitamura et al., 2002) (Miyabayashi et al., 2013), and anti-EGFP was a gift from Dr. Naoki Mochizuki (National Cerebral and Cardiovascular Center, Osaka, Japan). Anti-DNMT1 (60B1220.1) was purchased from Novus Biological; anti-DNMT3A (ab13888) from Abcam; anti-Sirt1 (07-131) from Merck Millipore; anti-HA (12CA5) from Santa Cruz Biotechnology, Inc.; anti-MeCP2 (#3456) from Cell Signaling; anti-Histone H3 (tri methyl K4) (ab213224) from Abcam; anti-Histone H3 (tri methyl K9) (ab176916) from Abcam; and anti-Ki67 (RM-9106-S1) from Thermo Fisher Scientific. HEK293, $\alpha$TC1, and MIN6 cells were cultured as described previously (Nakae et al., 1999) (Efrat et al., 1988) (Ishihara et al., 1993).

### Available Mice

RIP-Cre(Herrera, 2000), Glucagon-Cre (Herrera, 2000), FcorKO (Nakae et al., 2012), Foxo1$^{flox/flox}$ (Paik et al., 2007), and CAG-CAT-EGFP (Kawamoto et al., 2000) mice have been previously described elsewhere. Foxo1$^{flox/flox}$ mice were obtained from Dr. Ronald DePinho, University of Texas M.D. Anderson Cancer Center, courtesy of Dr. Domenico Accili, Columbia University.

### Animal Studies, Analytical Procedures, and Intraperitoneal Glucose and Insulin Tolerance Tests

For the following experiments, we used only male mice, which are more susceptible to insulin resistance and diabetes. All experimental protocols using mice were approved by the animal ethics committee of the Keio University School of Medicine. Animals were fed a standard chow diet and water ad libitum, and were housed in sterile cages in a barrier animal facility with a 12-h light/12-h dark cycle. Wild-type littermates were used as controls. At weaning (4 weeks of age), the mice were started on a high-fat diet (HFD). We continued to provide the same diet, and blood glucose and insulin level measurements and glucose and insulin tolerance tests were performed as described previously (Nakae et al., 2008). All assays were performed in duplicate, and each value represents the mean of two independent determinations.

**Generation of Tissue-specific FCoR Transgenic Mice**

We ligated a BspE1-BamHI-BglII adaptor (5'-CCGGAGGATCCA-3') with pAcGFP1-C1 (Clontech) treated with BspE1 and BglII. We amplified a BglII- and EcoRI-treated PCR fragment using 5'-GGGGAGATCTATGGACTACAAAGACGATGAC-3' and 5'-GGGGGAATTCCTAGCACATGCCTTTAGTCCC-3', with pFLAG-CMV2-FCoR (Nakae et al., 2012) as a template, and ligated this fragment with BglII- and EcoRI-treated pAcGFP1-C1 (pAcGFP1-C1-FLAG-FCoR). Next, EcoRI- and SmaI-treated β-globin pA of the pBS II SK(+)-CAG-CAT-EGFP vector (Kawamoto et al., 2000) was ligated with EcoRI- and SmaI-treated pAcGFP1-C1-FLAG-FCoR (pAcGFP1-C1-FLAG-FCoR- β-globin pA). Finally, the BamHI-treated pAcGFP1-C1-FLAG-FCoR- β-globin pA fragment was ligated with the BamHI-treated pBS II SK(+)-CAG-CAT-EGFP vector. The transgene was excised using SalI and SacI, gel-purified, and injected into fertilized eggs from BDF1 x C57BL/6 mice. The resulting embryos were implanted into CD-1 foster mothers, and the offspring were screened for transgene transmission by PCR. Ten independent transgenic lines were obtained, among which seven founders transmitted the transgene through the germ line. To obtain F1 mice, we crossed each founder with C57BL/6 mice. We then crossed the F1 mice with Albumin-Cre transgenic mice (Okamoto et al., 2007). We sacrificed one mouse from each line in which the transgene was transmitted, and examined the transgene expression in the liver. We ultimately obtained two independent *Fcor* transgenic lines. The primers used for genotyping were: 5'- ATG GAC TAC AAA GAC GAT GAC –3' and 5'-CTGCACCGGTCTCATCCTTTC-3'.

**RNA Isolation and Real-time PCR**

We isolated total RNA from tissues and cells using the SV Total RNA Isolation System (Promega) following the manufacturer's protocol. Real-time PCR was performed as previously described(Nakae et al., 2008). The primers used in this study are described in Supplementary Table 1.

**Insulin and Glucagon Secretion Studies**

Pancreatic islets were isolated by collagenase digestion. The isolated pancreatic islets were preincubated for 15 min in KREBS buffer (119 mM NaCl; 2.5mM $CaCl_2$; 1.19mM $KH_2PO_4$; 1.19mM $MgSO_4$; 10mM HEPES, pH 7.4; and 2% bovine serum albumin) with 2.8 mM glucose, and then stimulated for 1 h with glucose or L-arginine at the indicated concentration. We measured the insulin and glucagon released in the incubation buffer using the rat insulin radioimmunoassay kit (RI-13K, Millipore) and glucagon

radioimmunoassay kit (GL-32K, Millipore), respectively. The amounts of insulin secretion were normalized according to the cellular DNA content. For measurement of islet insulin content, islets were solubilized in acid-ethanol solution (74% ethanol, 1.4% HCl) overnight at 4$^{o}$C before insulin measurement. The amounts of insulin content were normalized according to the numbers of islets.

**Bisulfite Sequencing Analysis**

Bisulfite conversion of DNA isolated from αTC1 or MIN6 cells, or from isolated male mice pancreatic islets, was subjected to bisulfite conversion using the EZ DNA Methylation$^{TM}$ Kit (Zymo Research). Bisulfite-treated DNA samples were used for PCR using Arx UR2 primers(Dhawan et al., 2011). PCR products were gel purified using the QIAquick® Gel Extraction Kit (QUIAGEN). Purified products were cloned using the Mighty TA-cloning Kit (Takara), and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher) with the M13 Primer RV (CAGGAAACAGCTATGAC).

**Chromatin Immunoprecipitation (ChIP) Assay**

αTC1 or MIN6 cells were seeded onto 10 cm-culture dishes and transduced with adenovirus encoding constitutively nuclear HA-tagged ADA-Foxo1. At 36-48 h after transduction, the cells were fixed with 1% formaldehyde at 37$^{o}$C for 1 h. The DNA solution for ChIP PCR was prepared according to the protocol for the Chip Assay Kit (Upstate). Immunoprecipitation was performed using anti-HA (12CA5; Santa Cruz Biotechnology, Inc.), anti-MeCP2 (#3456; Cell Signaling), anti-Histone H3 (tri methyl K4) (ab213224, Abcam), anti-Histone H3 (tri methyl K9) (ab176916, Abcam), anti-DNMT1 (60B1220.1, Novus Biological), anti-DNMT3A (ab13888, Abcam), or an equal amount of normal mouse IgG (Santa Cruz Biotechnology, Inc). Samples were subjected to PCR amplification of the UR2 region of the *Arx* promoter(Dhawan et al., 2011).

**Construction of Arx Promoter-directed Luciferase Reporter Vectors**

From mouse genomic DNA, we PCR amplified several DNA fragments containing the mouse *Arx* promoter using the following primers: forward primers, *1813Ccr2* 5'-GGGGAGATCTTCCACCATTTGAGGGTACGGAAAAC-3' and *1432Ccr2* 5'-GGGGAGATCTAGAGAGGAAAACCTGGCGTGGATT-3' (underlining indicates the BglII recognition site); reverse primer, 5'- GGGGAAGCTT GGCTGGTGCTTTTTCCTTGGGCTC -3' (underlining indicates the HindIII recognition site). Their nucleotide sequences were verified by DNA sequencing. Then the *Arx* promoter fragments were treated with BglII- and HindIII-treated luciferase reporter pGL3-

Basic vector (Promega, Madison, WI).

**Site-directed Mutagenesis**

To alter the consensus Foxo1 response element (FRE) in the *Arx* promoter of the PGL3-Basic vectors, we performed site-directed mutagenesis using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The Mutant 1 vector was constructed using the following primers: 5'-ATCCAAGAGTTATGCAGA**T**G**GGGA**TGGCTGGGGGGAAAAATC-3' and 5'-GATTTTTCCCCCCAGCCA**TCCC**C**A**TCTGCATAACTCTTGGAT-3' (mutant bases are indicated by bold font and underlining). The Mutant 2 vector was constructed using the following two primers: 5'-ATCCAAGAGTTATGCAGA**G**G**GGGA**TGGCTGGGGGGAAAAATC-3' and 5'-GATTTTTCCCCCCAGCCA**TCCC**C**C**TCTGCATAACTCTTGGAT-3' (mutant bases are indicated by bold font and underlining). The Mutant 3 vector was constructed using the following two primers: 5'-ATCCAAGAGTTATGCAGA**G**G**GGGG**TGGCTGGGGGGAAAAATC-3' and 5'-GATTTTTCCCCCCAGCCA**CCCC**C**C**TCTGCATAACTCTTGGAT-3' (mutant bases are indicated by bold font and underlining). Mutated nucleotides were confirmed by DNA sequencing.

**Luciferase Assay**

For the mouse *Arx* promoter luciferase assay, MIN6 cells were plated onto 12-well culture dishes. Cells were grown to 70%-80% confluence, and transfections were performed using 1.5μg of pGL3/Basic-*Arx* reporter vector, and/or 0.6μg of pFLAG-CMV-2 empty vector or constitutively nuclear FLAG-CMV-2-CNFoxo1 expression vectors(Nakae et al., 2006). The synthetic renilla luciferase reporter vector (phRL-SV40; Promega) (10ng) was used as an internal control of transfection efficiency. After transfection, cells were cultured in DMEM containing 15% foetal calf serum and 0.0005% β-mercaptoethanol. Following a 36-h incubation, the cells were harvested for the luciferase assay.

**Electrophoretic Mobility Shift Assay (EMSA)**

From HEK293T cells transfected with PCMV5/cMyc-WTFoxo1, we isolated nuclear extracts using the NE-PER extraction reagents (Pierce). The DNA probe was a 31-bp DNA fragment covering the consensus FRE (-1565/-1535 nt) of the mouse *Arx* promoter: (5'-AGAGTTATGCAGATGTTTATGGCTGGGGGGA-3'). The mutant probe contained five bases substitutions within the consensus FRE (underlined letters): 5'-

AGAGTTATGCAGAGGGGGGTGGCTGGGGGGA -3'. The oligonucleotides were labelled with $^{32}$P-ATP using the MEGALABEL™ DNA 5'-End Labeling Kit (Takara Bio, Inc., Shiga, Japan). EMSA was performed as previously described(Nakae et al., 2003). For the super shift assay, we incubated anti-cMyc or normal mouse IgG with nuclear extracts at 4°C for 20 min, added labeled probes, and performed another 20-min incubation at room temperature. Competitor wild-type and mutant probes were incubated in the reaction mixture for 20 min prior to the addition of the radiolabelled probe.

**Construction of Adenoviral Vectors and Adenoviral Transduction**

Previous reports describe the adenoviral vectors encoding cMyc-FCoR (Nakae et al., 2012) or HA-ADA-Foxo1 (Nakae et al., 2001), and the adenovirus vectors for FCoR knockdown(Nakae et al., 2012). To construct an adenoviral vector encoding FCoR, we amplified the FLAG-FCoR cDNA fragment using the pFLAG-CMV2-FCoR expression vector (Nakae et al., 2012) as a template and the following primers: 5' - GG GCTAGC (NheI) ATGGACTACAAAGACGATGACGAC-3' (sense) and 5'-GG GCTAGC (NheI) CTAGCACATGCCTTTAGTCCC-3' (antisense). After treatment with NheI, the PCR fragment was subcloned into a NheI-treated pShuttle2 vector (Clontech). Following sequencing of the vectors to confirm that they had the intended sequences, and confirming protein expression in HEK293 by transient transfection, a fragment treated with I-CeuI- and PI-SceI was subcloned into Adeno-X viral DNA (pAdeno-X-FLAG-FCoR) (Clontech). The adenovirus vector was generated by transfecting HEK293 cells with the pAdeno-X-FLAG-FCoR plasmid. αTC1 or MIN6 cells were grown to 70%-80% confluence, and then transduced every day with adenovirus preparations encoding cMyc-FCoR, FLAG-FCoR, or FCoR-shRNA at different MOI for 7-8 h and removed from the culture dish. The cells were harvested after 48 h.

**Western Blotting**

We homogenized tissues and lysed cells in buffer containing 50mM Tris HCl (pH 8.0), 250mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors (Roche Diagnostics). The lysis solution was centrifuged to remove insoluble material, and the proteins in 30 μg of lysate were separated using 8% or 14 % SDS-PAGE. Western blotting was performed using the indicated antibodies. Immunoprecipitation was performed as previously described (Cao et al., 2006).

**Immunohistochemistry, Immunofluorescence and Histological Analysis**

For histological analysis, dissected pancreases were fixed in 10 % paraformaldehyde

and embedded in paraffin blocks. Tissues were cut in 5μm section by a microtome and used for immunohistochemical staining. 4 pancreatic sections from 3-7 mice from each genotype were sampled 150-200 μm apart. For α- and β-cell, each section was covered systematically by accumulating images from non-overlapping fields with fluorescence microscope (BZ-9000, KEYENCE) using a 10 x objective for whole pancreas sections. All morphometric analyses were performed using ImageJ software. Briefly, individual channels were converted to 8 bits grayscale and measurement scale was converted from pixels to microns. An identical threshold was applied to all images from the same channel to exclude background signals and further converted to binary format before automated analysis of weight. Sections in the lineage tracing study were imaged on Olympus Fluoview V3000 confocal microscope or KEYENCE BZ-X800 fluorescent microscope at 400X. Z-stack images were taken using KEYENCE BZ-X800 and was transformed into 3D images.

**Statistical Analysis**

We calculated descriptive statistics using one-way ANOVA with the Fisher's test. All data are expressed as mean $\pm$ standard error. Significance was set at $p<0.05$.

**SUPPLEMENTAL REFERENCES**

Cao, Y., Kamioka, Y., Yokoi, N., Kobayashi, T., Hino, O., Onodera, M., Mochizuki, N., and Nakae, J. (2006). Interaction of FoxO1 and TSC2 induces insulin resistance through activation of the mammalian target of rapamycin/p70 S6K pathway. J Biol Chem *281*, 40242-40251.

Dhawan, S., Georgia, S., Tschen, S.I., Fan, G., and Bhushan, A. (2011). Pancreatic beta cell identity is maintained by DNA methylation-mediated repression of Arx. Dev Cell *20*, 419-429.

Efrat, S., Linde, S., Kofod, H., Spector, D., Delannoy, M., Grant, S., Hanahan, D., and Baekkeskov, S. (1988). Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. Proc Natl Acad Sci U S A *85*, 9037-9041.

Herrera, P.L. (2000). Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development *127*, 2317-2322.

Ishihara, H., Asano, T., Tsukuda, K., Katagiri, H., Inukai, K., Anai, M., Kikuchi, M., Yazaki, Y., Miyazaki, J.I., and Oka, Y. (1993). Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. Diabetologia *36*, 1139-1145.

Kawamoto, S., Niwa, H., Tashiro, F., Sano, S., Kondoh, G., Takeda, J., Tabayashi, K.,

and Miyazaki, J. (2000). A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. FEBS Lett *470*, 263-268.

Kitamura, K., Yanazawa, M., Sugiyama, N., Miura, H., Iizuka-Kogo, A., Kusaka, M., Omichi, K., Suzuki, R., Kato-Fukui, Y., Kamiirisa, K., et al. (2002). Mutation of ARX causes abnormal development of forebrain and testes in mice and X-linked lissencephaly with abnormal genitalia in humans. Nat Genet *32*, 359-369.

Miyabayashi, K., Katoh-Fukui, Y., Ogawa, H., Baba, T., Shima, Y., Sugiyama, N., Kitamura, K., and Morohashi, K. (2013). Aristaless related homeobox gene, Arx, is implicated in mouse fetal Leydig cell differentiation possibly through expressing in the progenitor cells. PLoS One *8*, e68050.

Nakae, J., Cao, Y., Daitoku, H., Fukamizu, A., Ogawa, W., Yano, Y., and Hayashi, Y. (2006). The LXXLL motif of murine forkhead transcription factor FoxO1 mediates Sirt1-dependent transcriptional activity. J Clin Invest *116*, 2473-2483.

Nakae, J., Cao, Y., Hakuno, F., Takemori, H., Kawano, Y., Sekioka, R., Abe, T., Kiyonari, H., Tanaka, T., Sakai, J., et al. (2012). Novel repressor regulates insulin sensitivity through interaction with Foxo1. EMBO J *31*, 2275-2295.

Nakae, J., Cao, Y., Oki, M., Orba, Y., Sawa, H., Kiyonari, H., Iskandar, K., Suga, K., Lombes, M., and Hayashi, Y. (2008). Forkhead transcription factor FoxO1 in adipose tissue regulates energy storage and expenditure. Diabetes *57*, 563-576.

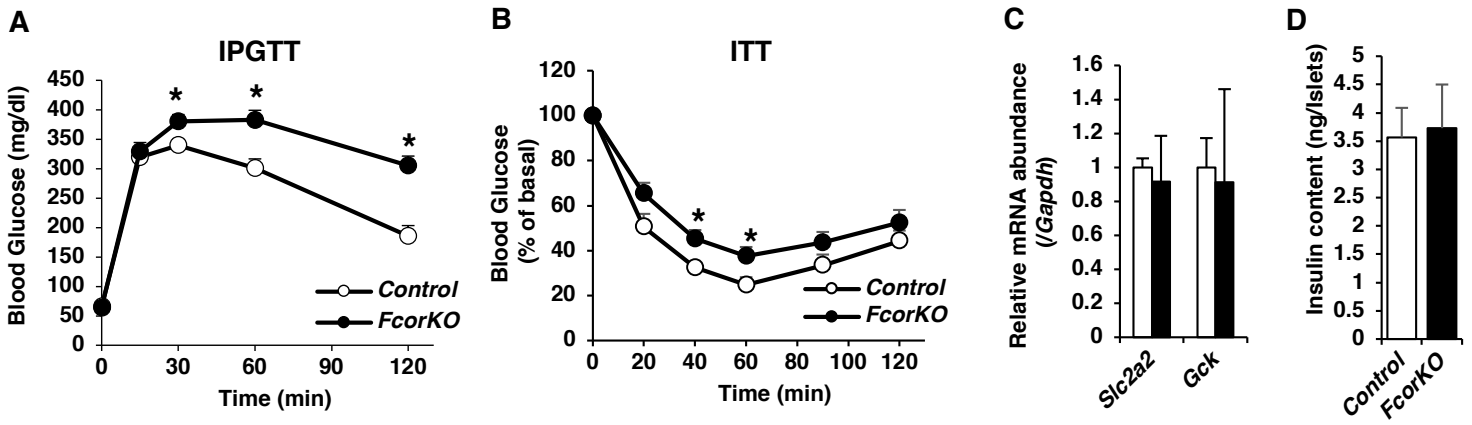
Nakae, J., Kitamura, T., Kitamura, Y., Biggs, W.H., 3rd, Arden, K.C., and Accili, D. (2003). The forkhead transcription factor Foxo1 regulates adipocyte differentiation. Dev Cell *4*, 119-129.

Nakae, J., Kitamura, T., Silver, D.L., and Accili, D. (2001). The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. J Clin Invest *108*, 1359-1367.

Nakae, J., Park, B.C., and Accili, D. (1999). Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. J Biol Chem *274*, 15982-15985.

Okamoto, Y., Ogawa, W., Nishizawa, A., Inoue, H., Teshigawara, K., Kinoshita, S., Matsuki, Y., Watanabe, E., Hiramatsu, R., Sakaue, H., et al. (2007). Restoration of glucokinase expression in the liver normalizes postprandial glucose disposal in mice with hepatic deficiency of PDK1. Diabetes *56*, 1000-1009.

Paik, J.H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R., et al. (2007). FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis. Cell *128*, 309-323.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Blood Glucose (mg/dl)
Time (min)
Control
FcorKO
B
ITT
Blood Glucose (% of basal)
Time (min)
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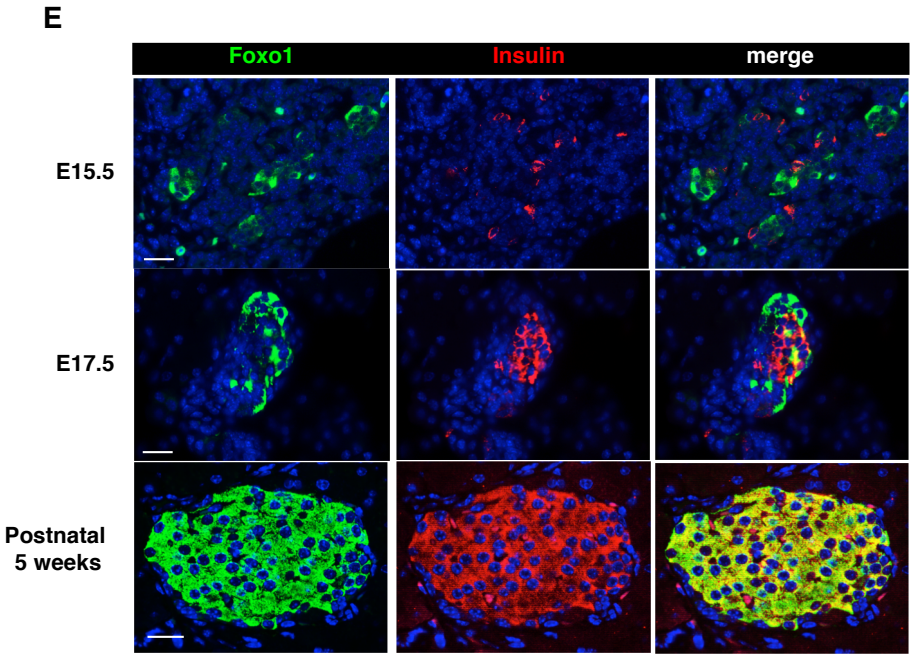
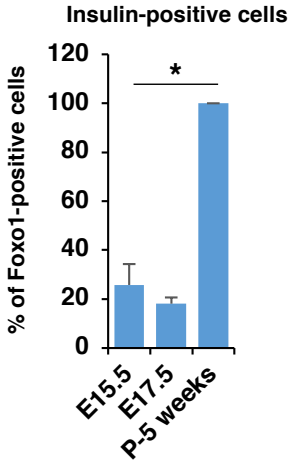
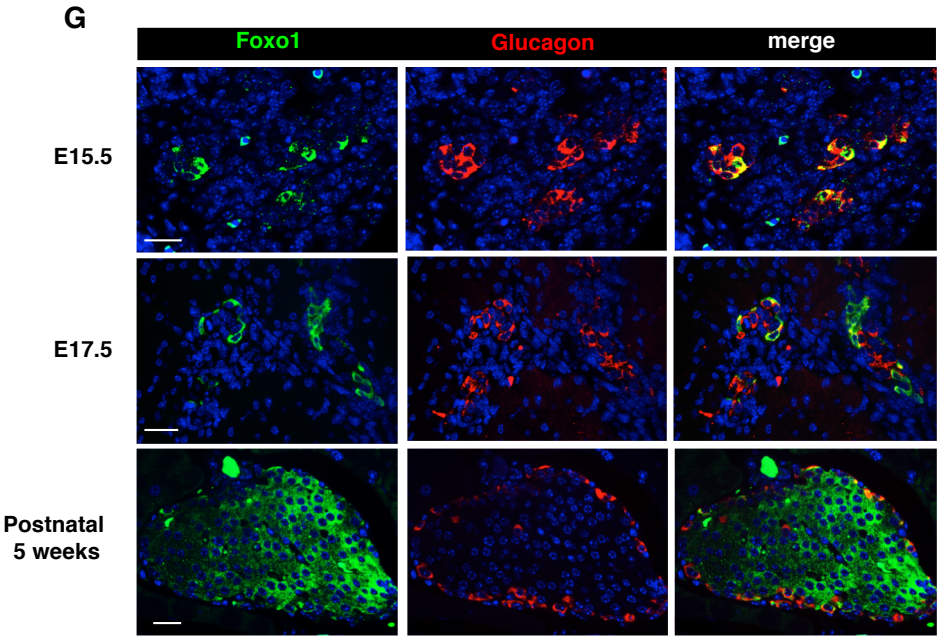
C
Relative mRNA abundance (/Gapdh)
Slc2a2
Gck
D
Insulin content (ng/Islets)
Control
FcorKO


E
Foxo1
Insulin
merge
E15.5
E17.5
Postnatal 5 weeks


F
Insulin-positive cells
% of Foxo1-positive cells
E15.5
E17.5
P-5 weeks


G
Foxo1
Glucagon
merge
E15.5
E17.5
Postnatal 5 weeks

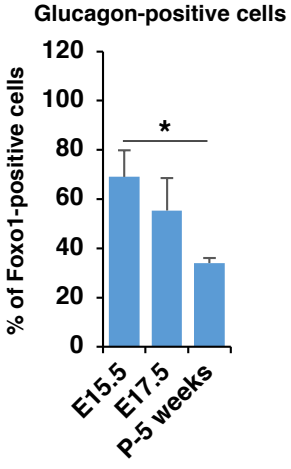
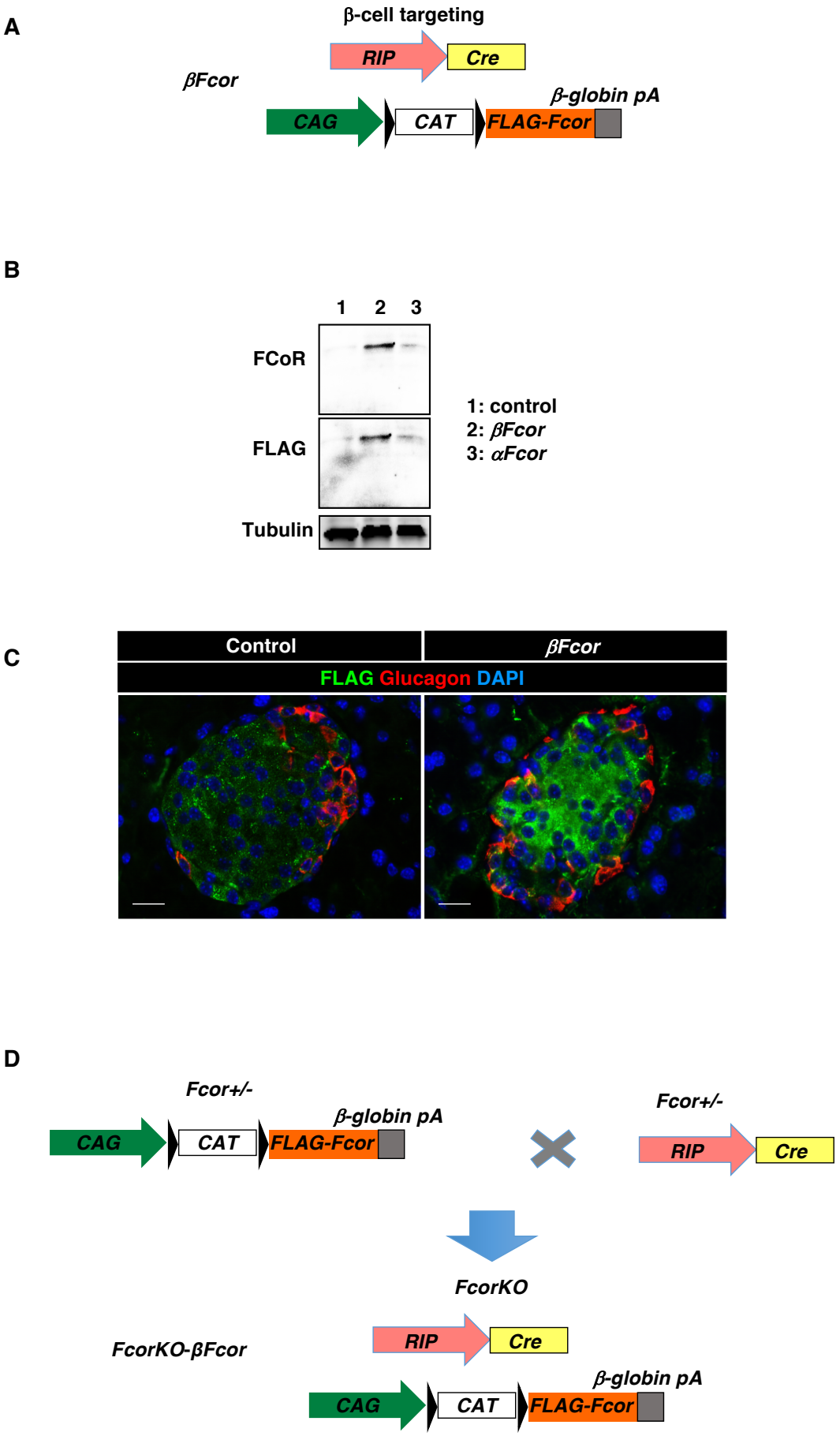

H
Glucagon-positive cells
% of Foxo1-positive cells
E15.5
E17.5
P-5 weeks

**Figure S1**

**Figure S1. Related to Figure 1. Glucose Metabolism and Foxo1 Expression in Fetal Pancreas.**
(A) Intraperitoneal glucose tolerance test (IPGTT) of control (n=11) and *FcorKO* (n=12) at the age of 20 weeks. Data are means ± SEM. *P < 0.05 by two-way ANOVA with Fisher's test.
(B) Insulin tolerance test (ITT) of control (n=11) and *FcorKO* (n=14) at the age of 20 weeks. Data are represented as % of basal glucose level and means ± SEM. *P < 0.05 by two-way ANOVA with Fisher's test.
(C) Expression levels of Glut 2 (*Slc2a2*) and Glucokinase (*Gck*) in islets isolated from control (white bar) (n=4) and *FcorKO* (black bar) (n=4) at the age of 20-24 weeks.
Data represent means ± SEM.
(D) Insulin content of islets isolated from control (n=4) and *FcorKO* (n=4) at the age of 20-24 weeks. Data are represented as insulin content (ng) per islet and means ± SEM.
(E)(F) Representative images of pancreatic islets for Foxo1 and insulin from embryos (n=3) at embryonic days 15.5 (E15.5), 17.5 (E17.5), and postnatal week 5 (E) and the percentages of Foxo1-positive cells among insulin-positive cells (F). Data represent means ± SEM.
* P <0.05 by one-way ANOVA. Scale bar, 20 μm.
(G)(H) Representative images of pancreatic islets for Foxo1 and glucagon from embryos (n=3) at embryonic days 15.5 (E15.5), 17.5 (E17.5), and postnatal week 5 (G) and the percentages of Foxo1-positive cells among glucagon-positive cells (H). Data represent means ± SEM.
* P <0.05 by one-way ANOVA. Scale bar, 20 μm.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Cre
βFcor
β-globin pA
CAG
CAT
FLAG-Fcor
B
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FCoR
FLAG
Tubulin
1: control
2: βFcor
3: αFcor
C
Control
βFcor
FLAG Glucagon DAPI
D
Fcor+/-
β-globin pA
CAG
CAT
FLAG-Fcor
Fcor+/-
RIP
Cre
FcorKO
FcorKO-βFcor
RIP
Cre
β-globin pA
CAG
CAT
FLAG-Fcor

**Figure S2**

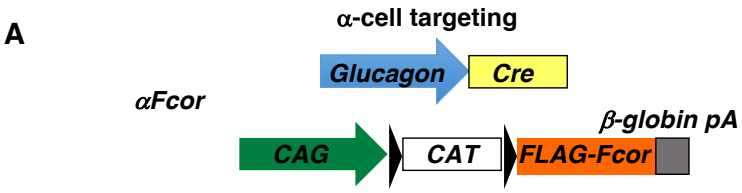
**Figure S2. Related to Figure 2. Generation of β-Cell-Specific *Fcor* Transgenic Mice**

(A) Construction of β-cell-specific *Fcor* transgenic mice (*βFcor*).

(B) Western blotting of islets isolated from control, *βFcor,* and *αFcor* with anti-FCoR, anti-FLAG, and anti-tubulin antibodies.

(C) Representative images of immunofluorescence of islets from control and *βFcor* using anti-FLAG and anti-glucagon antibodies. Scale bar, 20μm.

(D) Generation of *FcorKO-βFcor*.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Glucagon
Cre
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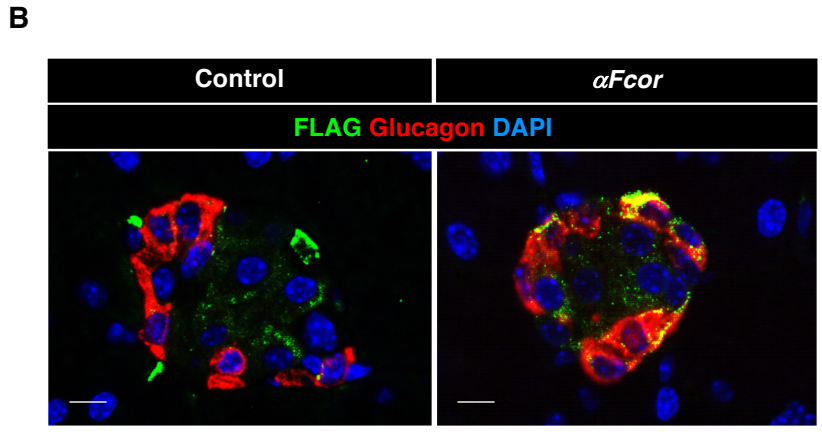
FLAG-Fcor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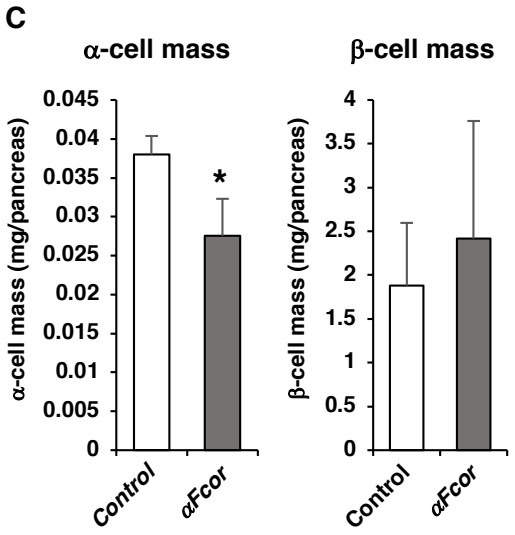
Control
αFcor
FLAG Glucagon DAPI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β-cell mass
α-cell mass (mg/pancreas)
β-cell mass (mg/pancreas)
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
4
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3
2.5
2
1.5
1
0.5
0
*
Control
αFcor

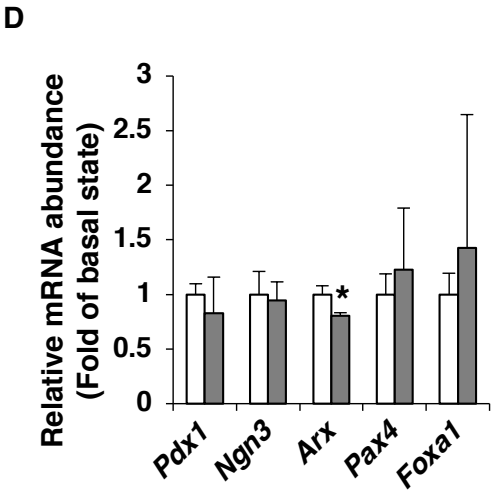

D
Relative mRNA abundance
(Fold of basal state)
3
2.5
2
1.5
1
0.5
0
*
Pdx1
Ngn3
Arx
Pax4
Foxa1

Figure S3

**Figure S3. Related to Figure 2. Generation of α-Cell-Specific *Fcor* Transgenic Mice**

(A) Construction of α-cell-specific *Fcor* transgenic mice (*αFcor*).

(B) Representative images of immunofluorescence of islets from control and *αFcor* using anti-FLAG and anti-glucagon antibodies. Scale bar, 10μm.

(C) Quantification of α-cell and β-cell mass in control (n=7 mice; 174 islets measured) and *αFcor* (n=5 mice; 166 islets measured).

(D) Gene expression in islets isolated from control (white bar) and *αFcor* (gray bar).

Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.

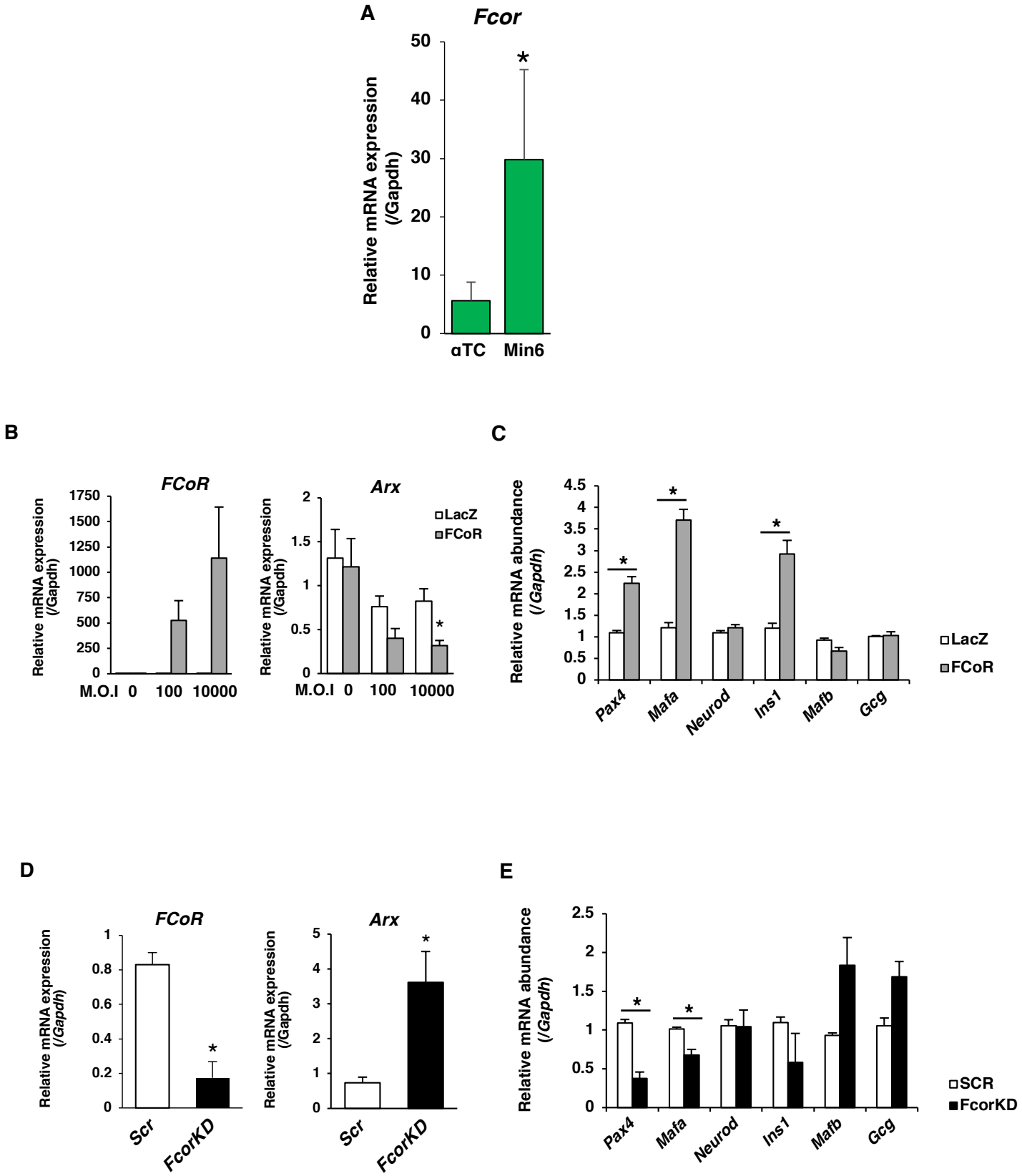

A
Fcor
Relative mRNA expression (/Gapdh)
50
40
30
20
10
0
*
αTC
Min6
B
FCoR
Relative mRNA expression (/Gapdh)
1750
1500
1250
1000
750
500
250
0
M.O.I 0 100 10000
Arx
Relative mRNA expression (/Gapdh)
2
1.5
1
0.5
0
M.O.I 0 100 10000
LacZ
FCoR
C
Relative mRNA abundance (/Gapdh)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Pax4
Mafa
Neurod
Ins1
Mafb
Gcg
LacZ
FCoR
D
FCoR
Relative mRNA expression (/Gapdh)
1
0.8
0.6
0.4
0.2
0
Scr
FcorKD
Arx
Relative mRNA expression (/Gapdh)
5
4
3
2
1
0
Scr
FcorKD
E
Relative mRNA abundance (/Gapdh)
2.5
2
1.5
1
0.5
0
Pax4
Mafa
Neurod
Ins1
Mafb
Gcg
SCR
FcorKD

**Figure S4**

**Figure S4. Related to Figure 2. Endogenous Expression Levels of Islet-specific Genes in Cell Lines.**

(A) *Fcor* Expression in αTC1 and MIN6 Cells. *Fcor* expression in αTC1 and MIN6 cells. Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.

(B) *Fcor* and *Arx* expression in αTC1 cells transduced with adenoviruses encoding LacZ (white bar) and FLAG-FCoR (gray bar). Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.

(C) Expression levels of islet-specific genes in αTC1 cells transduced with adenoviruses encoding LacZ (white bar) and FLAG-FCoR (grey bar). Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.

(D) The effect of FCoR knockdown on *Arx* expression in MIN6 cells. *Fcor* and *Arx* expression in MIN6 cells transduced with adenoviruses encoding scramble-shRNA (*Scr*) (white bar) and *Fcor* knockdown-shRNA (*FcorKD*) (black bar). Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.

(E) Expression levels of islet-specific genes in MIN6 cells transduced with adenoviruses encoding scramble-shRNA (*Scr*) (white bar) and *Fcor* knockdown-shRNA (*FcorKD*) (black bar). Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.

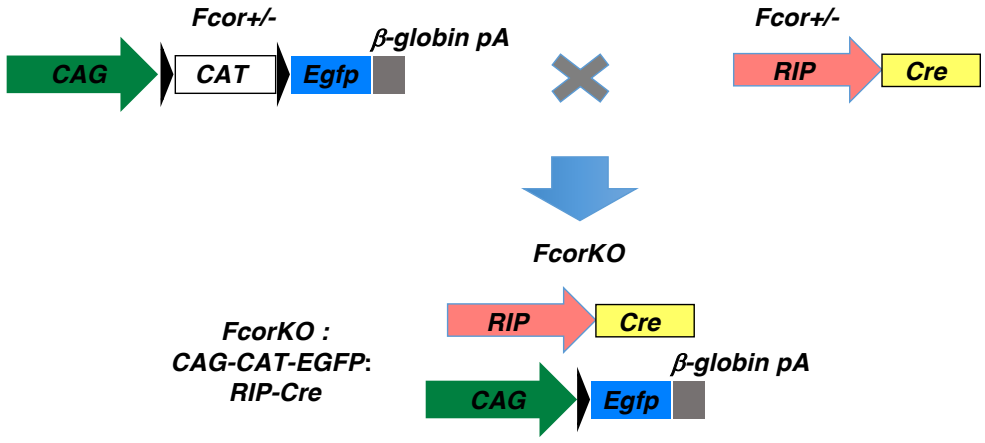

Fcor+/-
β-globin pA
CAG
CAT
Egfp
Fcor+/-
RIP
Cre
FcorKO
FcorKO :
CAG-CAT-EGFP:
RIP-Cre
RIP
Cre
β-globin pA
CAG
Egfp

**Figure S5**

**Figure S5. Related to Figure 3. β-Cell Tracing Analysis of *FcorKO***
Construction of β-cell tracing mice.

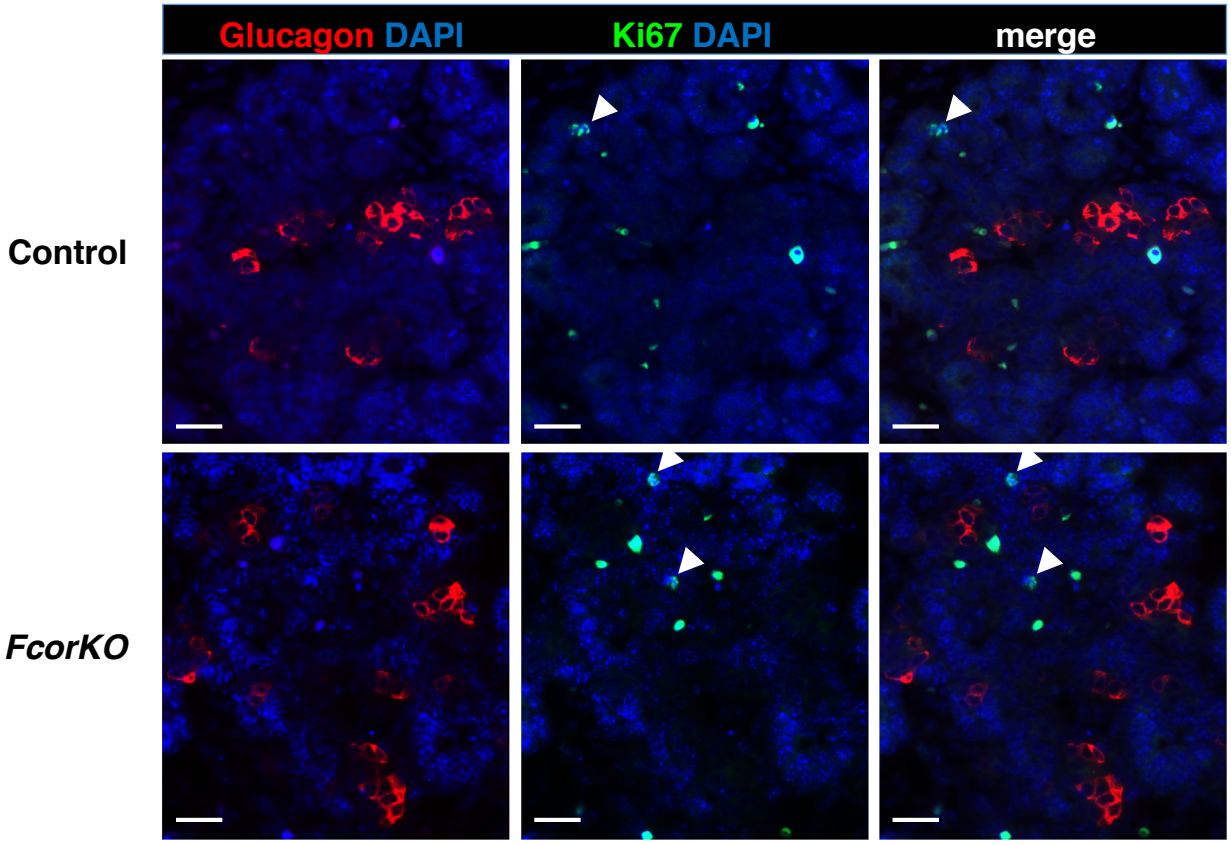



Figure S6

**Figure S6. Related to Figure 3. Glucagon and Ki67 staining of fetal pancreas from control and *FcorKO*.**

Representative images of fetal pancreas for glucagon and Ki67 from control and *FcorKO* at embryonic day 15.5. Scale bar, 20μm.

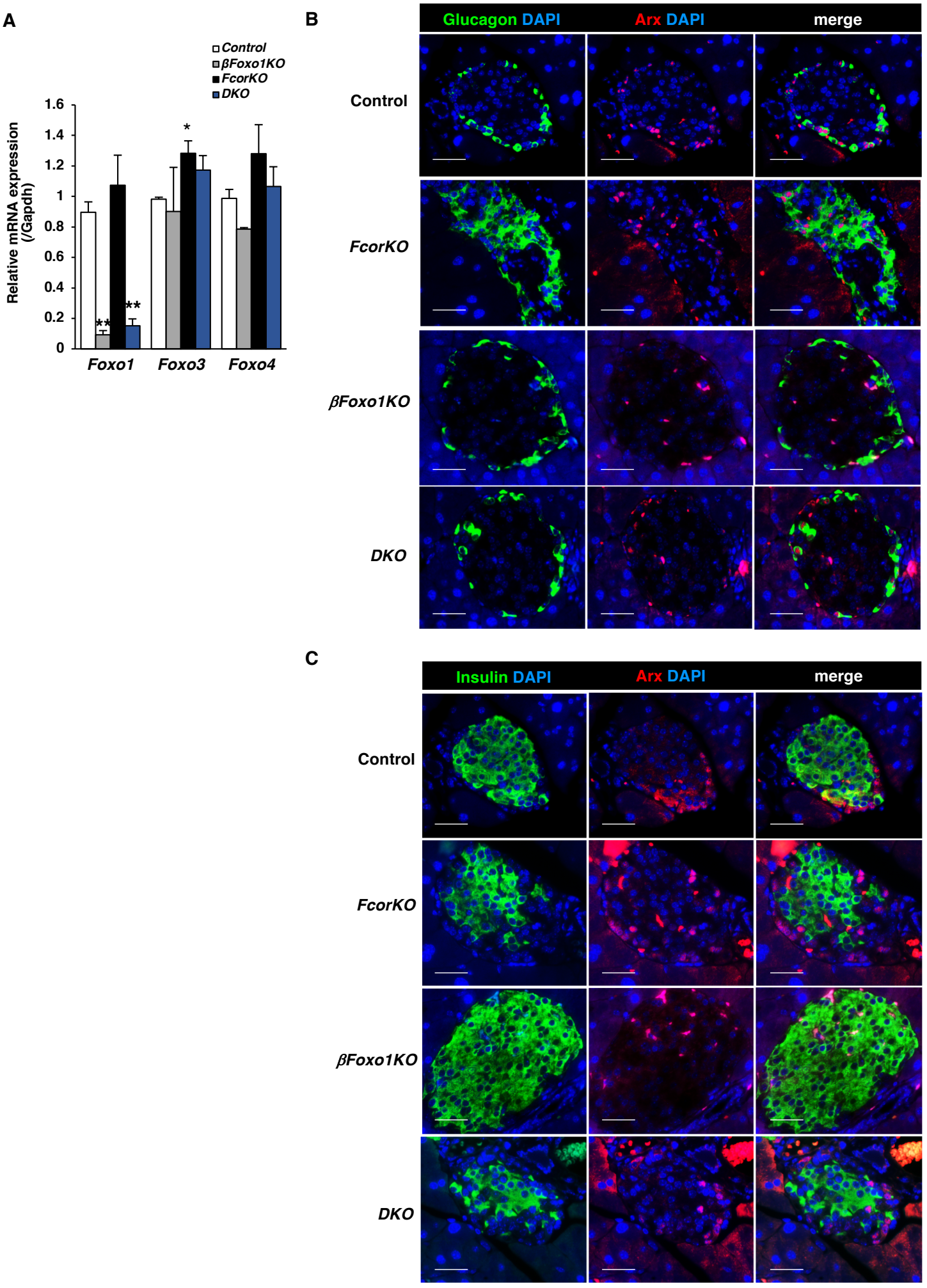
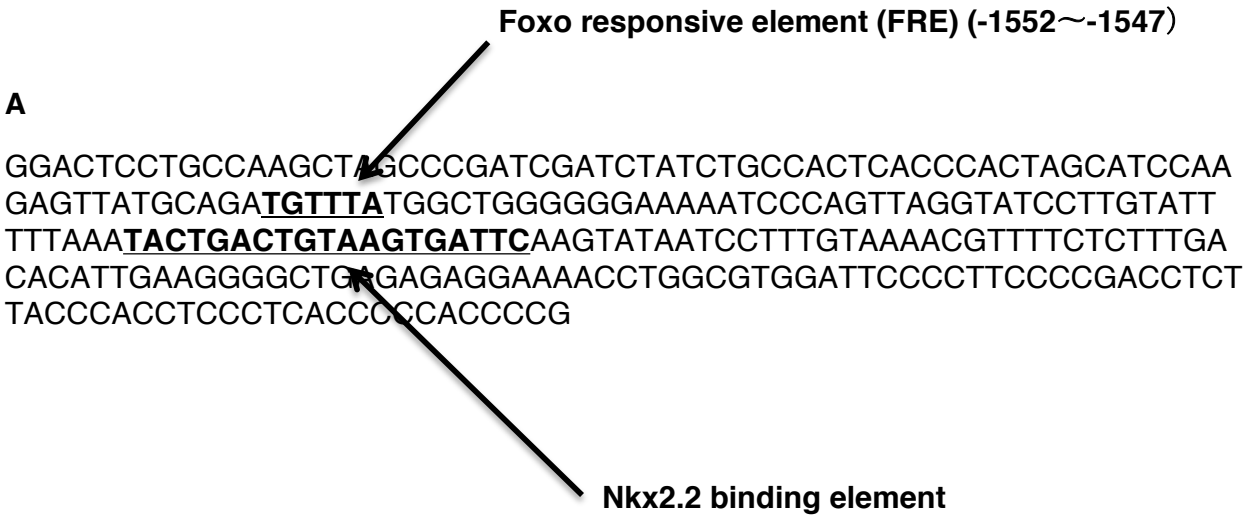

A
Control
βFoxo1KO
FcorKO
DKO
Relative mRNA expression (/Gapdh)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
*
Foxo1
Foxo3
Foxo4
B
Glucagon DAPI
Arx DAPI
merge
Control
FcorKO
βFoxo1KO
DKO
C
Insulin DAPI
Arx DAPI
merge
Control
FcorKO
βFoxo1KO
DKO

Figure S7

**Figure S7. Related to Figure 5. Expression of *Foxo* Family Genes and Arx in Islets.**
(A) Gene Expression Levels of *Foxo* Family Members in Islets. Gene expression levels of *Foxo* family members in islets isolated from 16-week-old controls (n=4), *FcorKO* (n=4), *DKO* (n=4), and *βFoxo1KO* (n=4). Data represent means ± SEM from three independent experiments. *P<0.05, and **P<0.005 by one-way ANOVA.
(B, C) Arx expression in islets from control, *FcorKO*, *βFoxo1KO*, *DKO*.
Representative images of pancreatic islets for Arx and glucagon (B), or Arx and insulin (C) from control, *FcorKO*, *βFoxo1KO*, *DKO* at the age of 24 weeks. Scale bar, 50 μm.




**B**

**FRE(-1576～-1571）**

**Human *ARX*** GAGTTATGCAGATGTTTATGGCTGAAAAAAAAAAATCCCAGTTATGCATCCTTGTAT

**Mouse *Arx*** GAGTTATGCAGATGTTTATGGCTGGGGGGGAAAAATCCCAGTTAGGTATCCTTGTATT

**FRE(-1552～-1547）**

**Figure S8**

**Figure S8. Related to Figure 6. Analysis of Mouse *Arx* Promoter Region**

(A) Sequencing data of the *Arx* promoter region around the Foxo responsive element and NKX2.2 binding element.

(B) Conservation between human and mouse sequences of the *Arx* promoter region near the Foxo responsive element (FRE).

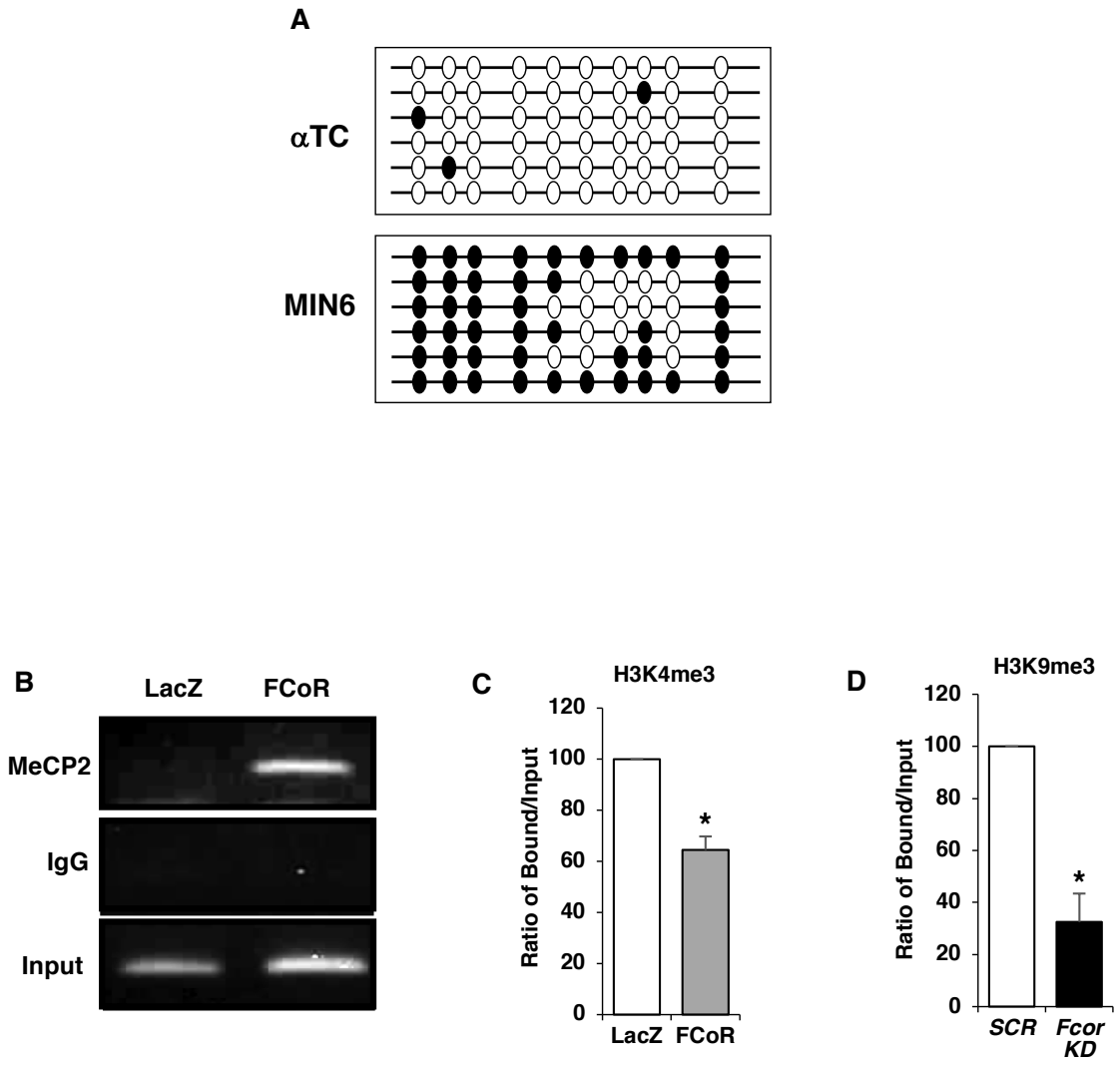

A
αTC
MIN6
B
LacZ
FCoR
MeCP2
IgG
Input
C
H3K4me3
Ratio of Bound/Input
120
100
80
60
40
20
0
*
LacZ
FCoR
D
H3K9me3
Ratio of Bound/Input
120
100
80
60
40
20
0
*
SCR
Fcor KD

**Figure S9**

**Figure S9. Related to Figure 4. DNA Methylation and Histone Modification of *Arx* Promoter Region.**

(A) Methylation Status of the UR2 Region of the *Arx* Promoter in αTC1 and MIN6 Cells. Bisulfite sequencing analysis of the UR2 region of *Arx* promoter in αTC1 and MIN6 cells.

(B) ChIP assay showing the binding of MeCP2 to the UR2 region of *Arx* promoter in αTC1 cells transduced with adenoviruses encoding LacZ or FLAG-FCoR, and harvested 48 h after transduction.

(C)(D) ChIP assay comparing the levels of H3K4 trimethylation (H3K4me3) (C) and H3K9 trimethylation (H3K9me3) (D) at the UR2 region of the Arx promoter in αTC1 cells transduced with adenoviruses encoding LacZ or FCoR (C), and in MIN6 cells transduced with adenoviruses encoding scramble shRNA (SCR) or *Fcor*-knockdown shRNA (*Fcor KD*) (D).

Data represent means ± SEM from three independent experiments. *P<0.05 by one-way ANOVA.

Table S1. Related to Figure 2. Primers used for real-time PCR

| Gene symbol | Sense | Antisense |
|---|---|---|
| *Arx* | GGCCGGAGTGCAAGAGTAAAT | TCGATGCAGTAGGAGGAGAGC |
| *Pax4* | AGGGGGACTCTTTGTGAATGG | ACCTGTGCGGTAGTAGCGT |
| *Pdx1* | TTCCCGAATGGAACCGAGC | GCGTGAGCTTTGGTGGATT |
| *Slc2a2* | TCAGAAGACAAGATCACCGGA | TCAGAAGACAAGATCACCGGA |
| *Mafa* | AGGAGGAGGTCATCCGACTG | CTTCTCGCTCTCCAGAATGTG |
| *Neurod* | ATGACCAAATCATACAGCGAGAG | TCTGCCTCGTGTTCCTCGT |
| *Ucn3* | AAGCCTCTCCCACAAGTTCTA | GAGGTGCGTTTGGTTGTCATC |
| *Ins1* | CACTTCCTACCCCTGCTGG | ACCACAAAGATGCTGTTTGACA |
| *Ins2* | GCTTCTTCTACACACCCATGTC | AGCACTGATCTACAATGCCAC |
| *Neurog3* | AGTGCTCAGTTCCAATTCCAC | CGGCTTCTTCGCTTTTTGCTG |
| *Gcg* | GATCATTCCCAGCTTCCCAG | CTGGTAAAGGTCCCTTCAGC |
| *Irx1* | CTGGGCTACCCGCAATACC | ATACATGCCTAGCACCGAGGT |
| *Irx2* | CCAAGCCCGTAACCTCCTC | GTCCGACGTGGCTATCTCG |
| *Mafb* | TTCGACCTTCTCAAGTTCGACG | GAGATGGGTCTTCGGTTCAGT |
| *Foxa1* | ATGAGAGCAACGACTGGAACA | TCATGGAGTTCATAGAGCCCA |
| *Fcor* | AATGCTTAGGAGGCCCTTCCA | CGGTGCAGTTCCCAGCCCTAC |
| *Dnmt1* | AAGAATGGTGTTGTCTACCGAC | CATCCAGGTTGCTCCCCTTG |
| *Dnmt3a* | GAGGGAACTGAGACCCCAC | CTGGAAGGTGAGTCTTGGCA |
| *Tet1* | ACACAGTGGTGCTAATGCAG | AGCATGAACGGGAGAATCGG |
| *Tet2* | AGAGAAGACAATCGAGAAGTCGG | CCTTCCGTACTCCCAAACTCAT |
| *Foxo1* | GTCCTGGGCCAAAATGTAATG | AGCCTGACACCCAGCTGTGTG |
| *Foxo3a* | CAAACGGCTCACTTTGTCCC | GGTTGATGATCCACCAAGAGCT |
| *Foxo4* | TGACACTCGCCCAGATCTACG | AGGACAGGTTGTGACGGATGG |
| *Gapdh* | CATCTTGGGCTACACTGAGGA | TGGAGGCCATGTAGGCCATGA |