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Supplemental Information

Neonatal β Cell Development in Mice and Humans Is Regulated by Calcineurin/NFAT

William R. Goodyer, Xueying Gu, Yinghua Liu, Rita Bottino, Gerald R. Crabtree, and Seung K. Kim

Inventory of Supplementary Information

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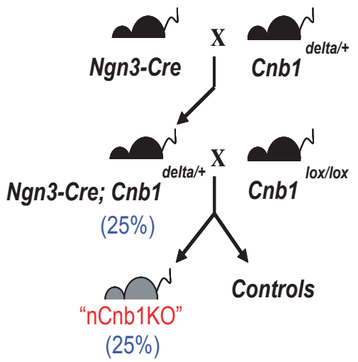

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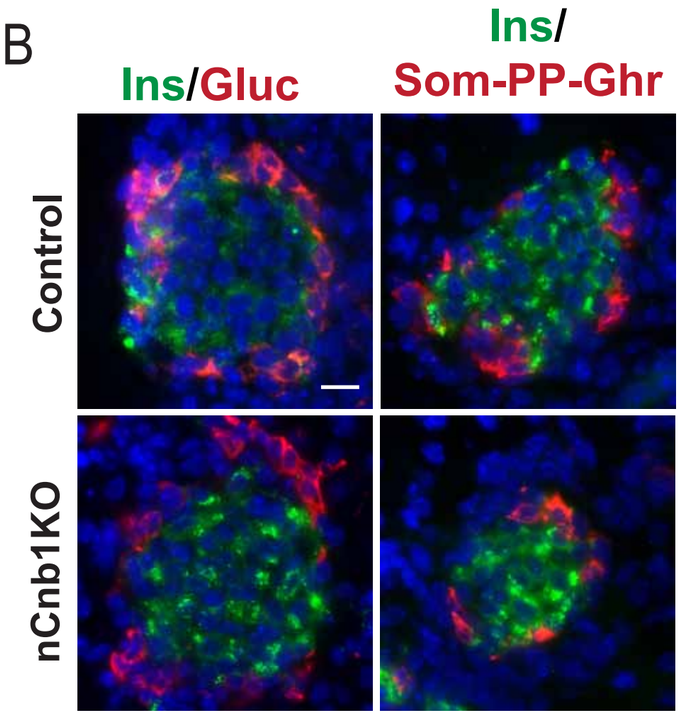

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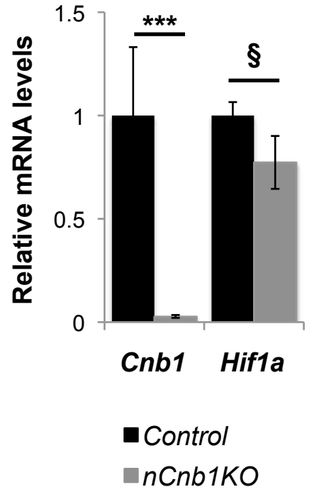

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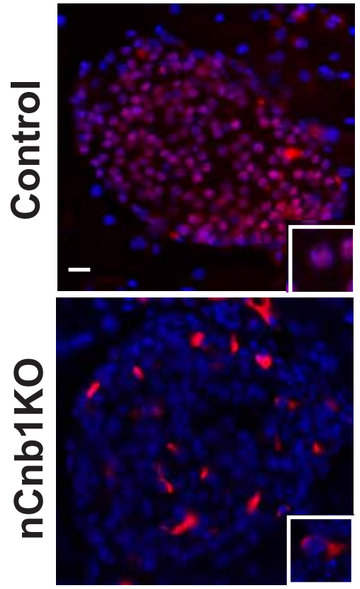

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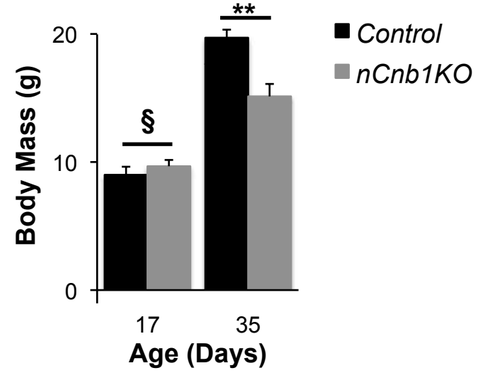

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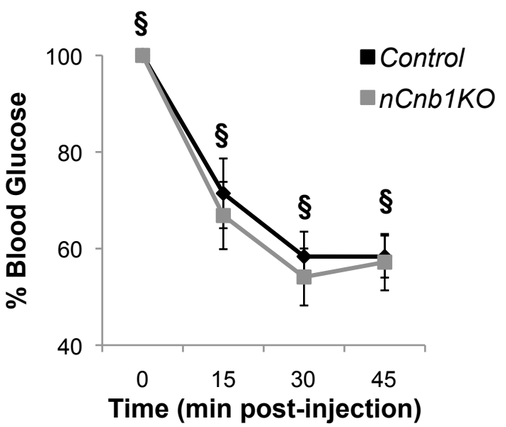

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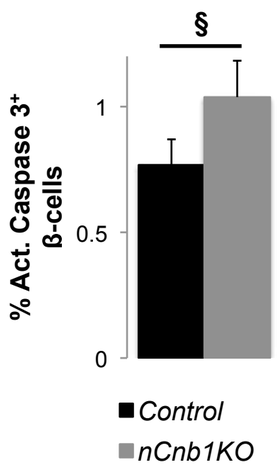

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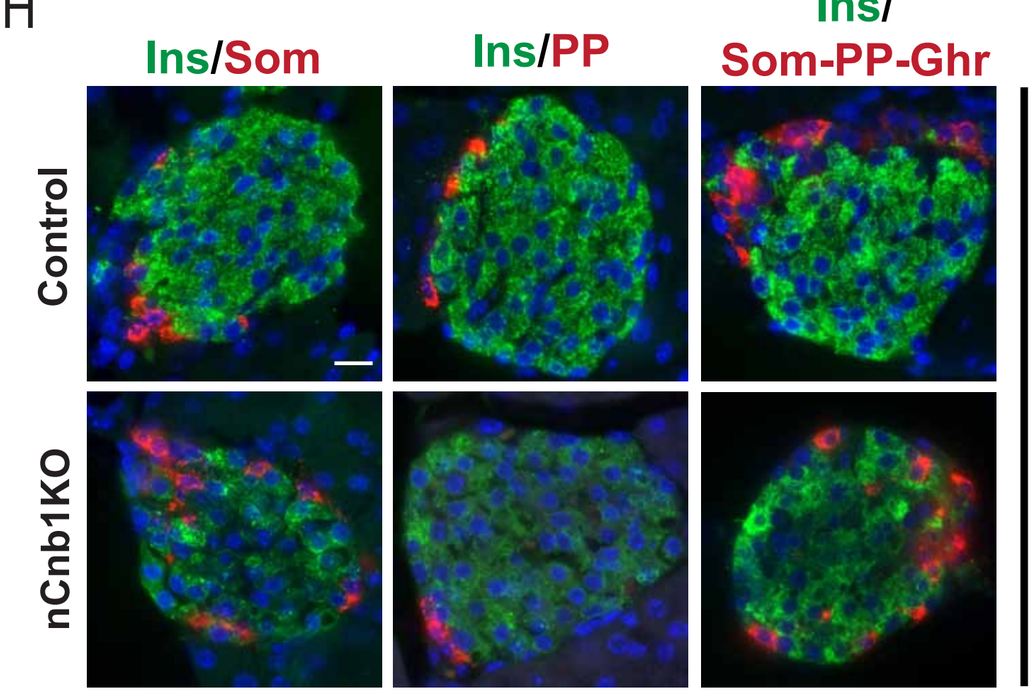
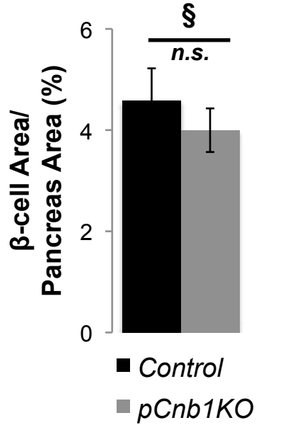


P26

P1

I



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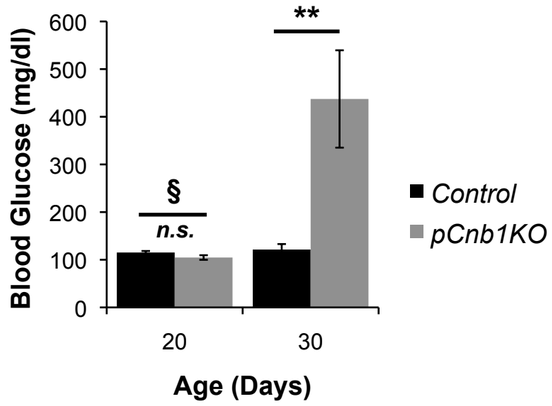

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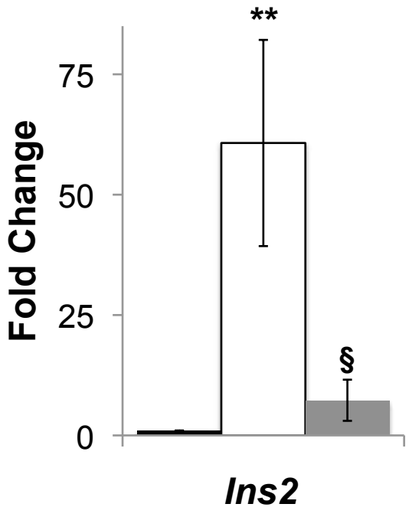
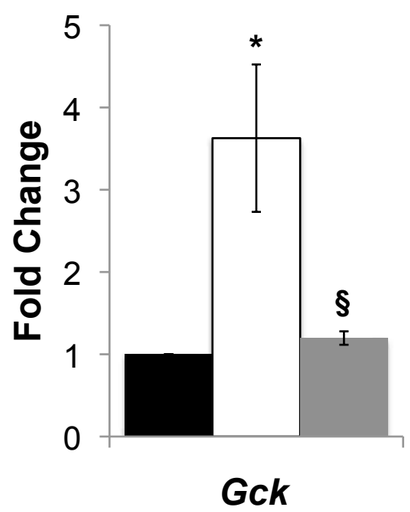
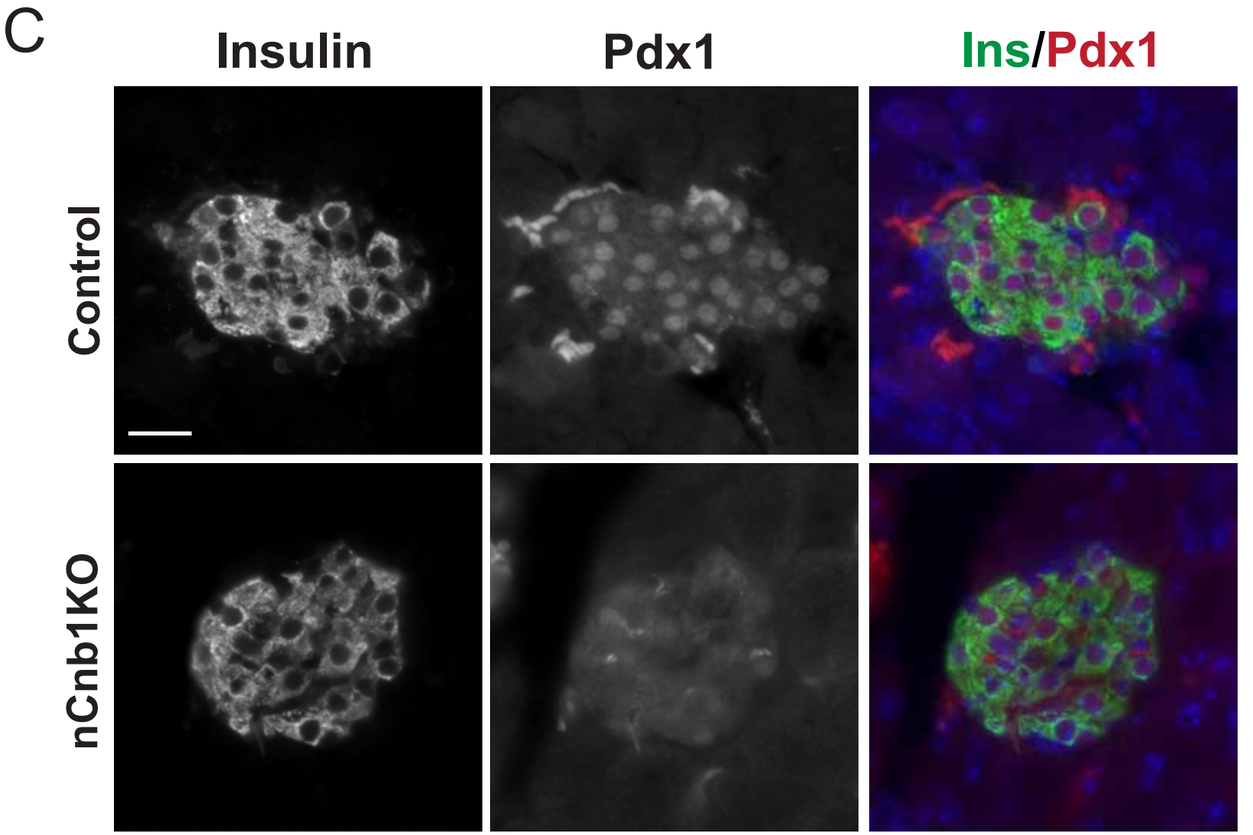

**
75
50
25
0
Fold Change
§
Ins2

B

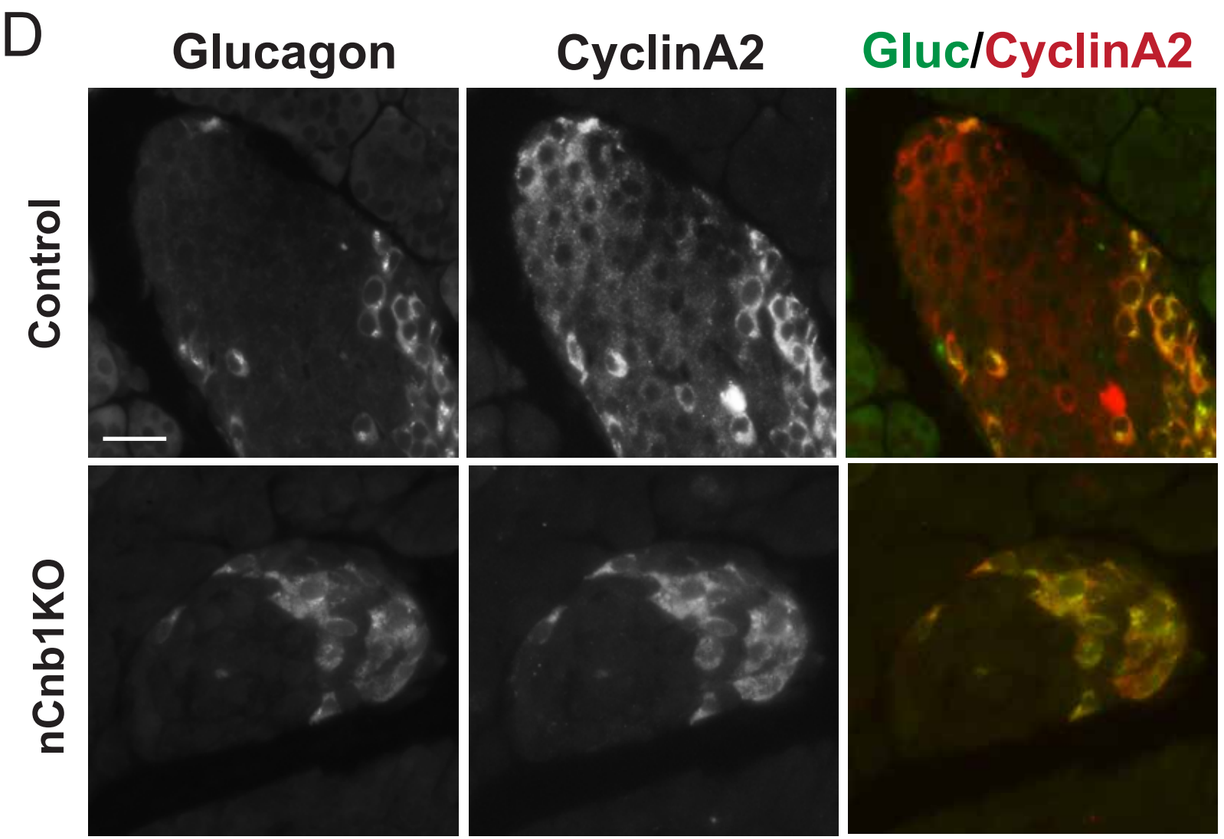

5
4
3
2
1
0
Fold Change
*
§
Gck

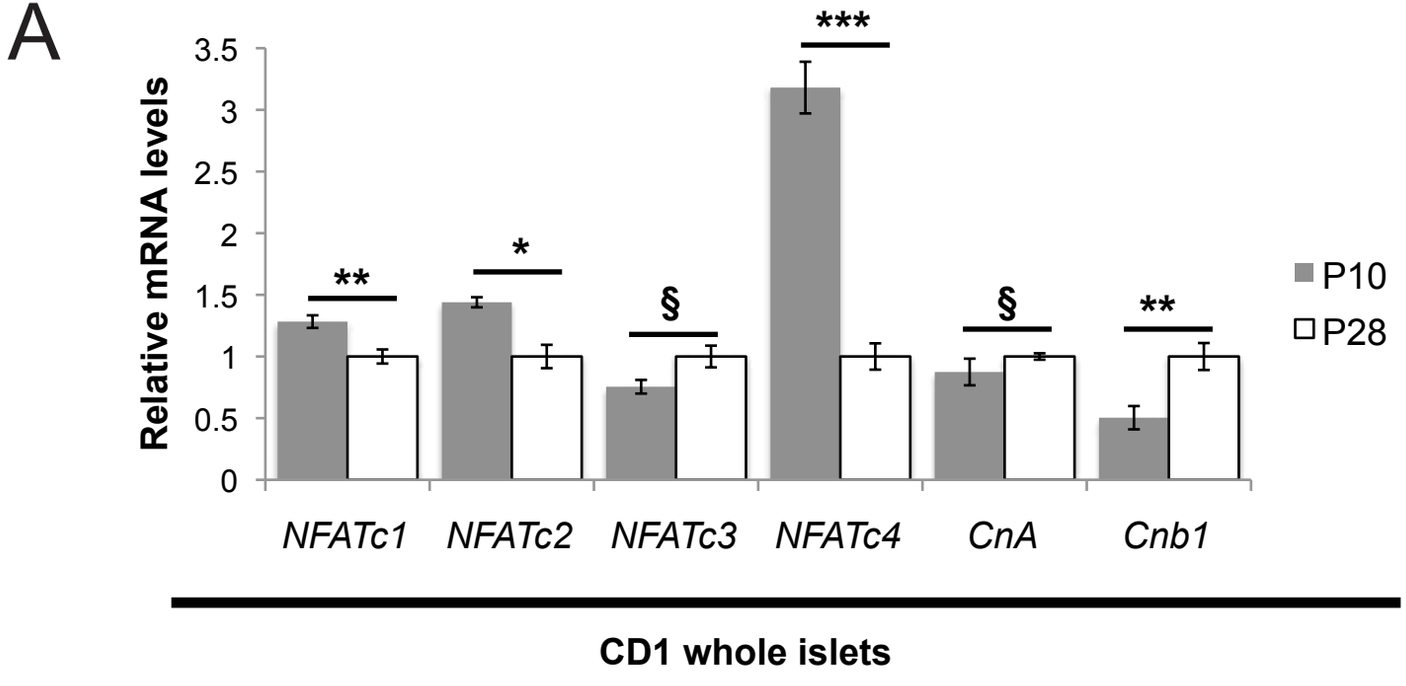

IgG
NFATc1
NFATc1+FK
* p<0.05
** p<0.025
§ p>0.05

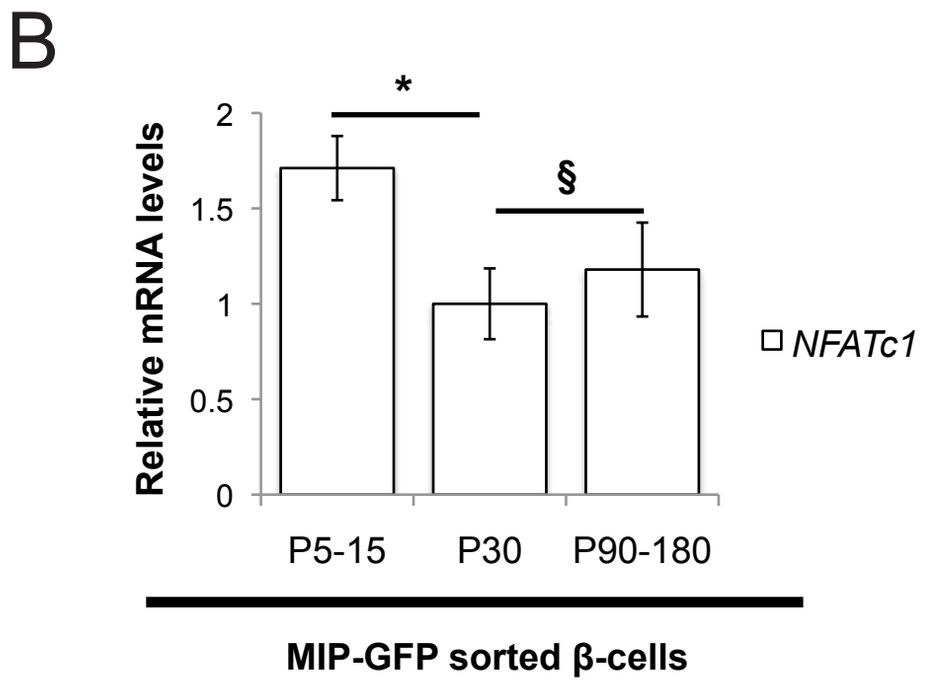
C


Insulin
Pdx1
Ins/Pdx1
Control
nCnb1KO

D


Glucagon
CyclinA2
Gluc/CyclinA2
Control
nCnb1KO


A
Relative mRNA levels
3.5
3
2.5
2
1.5
1
0.5
0
**
*
§
***
§
**
NFATc1
NFATc2
NFATc3
NFATc4
CnA
Cnb1
P10
P28
CD1 whole islets


B
Relative mRNA levels
2
1.5
1
0.5
0
*
§
P5-15
P30
P90-180
NFATc1
MIP-GFP sorted β-cells

Supplemental Figure Legends

Figure S1. Additional physiologic and cellular analyses of nCnb1KO mice.

A. Mating scheme for generating *Ngn3-Cre; Cnb1*$^{\Delta/f}$ (nCnb1KO) mice.

B. Representative immunofluorescent stains demonstrating normal islet cell composition in postnatal day 1 (P1) nCnb1KO islets and littermate controls.

C. QPCR of *calcineurin b1* (*Cnb1*) and *hypoxia-inducible factor 1* (*Hif1a*) transcript levels of islets isolated from P20 control and nCnb1KO mice.

D. Immunohistology of NFATc1 within P20 control and KO pancreatic islets. Insets are of single β-cells illustrating nuclear localization of NFATc1 in controls and cytoplasmic localization in nCnb1KOs.

E. Body mass (in g) of littermate mice (n=8 min. per genotype).

F. Insulin tolerance test (ITT) in P20 mice (mixed sex; Control, n=6; nCnb1KO, n=4; Insulin [1U/kg]). Blood glucose levels were measured at times 0, 15, 30 and 45 min.

G. Quantification of the percentage of β-cells positive for activated caspase 3 staining in either control (black bar) or nCnb1KO (grey bar).

H. Representative immunofluorescent stains on pancreatic islets from diabetic, P26 nCnb1KO mice and controls.

I. Quantification of total β-cell Area/Pancreatic Area (in percentage) in P1 control (black bar) and *Pdx1-Cre; Cnb1*$^{\Delta/f}$ (pCnb1KO) (grey bar) pancreas (n=3 per genotype).

J. Random fed blood glucose levels of postnatal nCnb1KO mice (grey bars) and littermate controls (black bars) fed ad libitum (n=3 per genotype min. per timepoint).

DAPI in blue; Ins (Insulin) in green; Som (Somatostatin), PP (Pancreatic polypeptide), Ghr (Ghrelin) and NFATc1 in red. Scale bar = 10 μM. All data presented as means ± s.e.m. **, P<0.025. ***, P<0.002. §, not significant (n.s.).

Figure S2. NFATc1 binding of mouse *Ins2* and *Gck* promoter regions *in vivo* by ChIP and decreased Pdx1 and CcnA2 protein expression in nCnb1KO β-cells.

A-B. Chromatin immunoprecipitation of NFATc1 on islets isolated and fixed from postnatal day 20 (P20) C57BL/6 mice. Islets were treated for 24hrs with either vehicle (EtOH) or FK506 (10 μM) (n=4 per condition). Putative NFAT consensus sites were assessed in the upstream (within 2kb from start site) promoter regions of **A.** *Insulin 2* (*Ins2*) and **B.** *Glucokinase* (*Gck*). ChIP data is presented as Fold Change of signal relative to IgG background signal. All data presented as means ± s.e.m. *, P<0.05. **, P<0.025. §, not significant (n.s.).

C-D. Representative immunohistology of insulin or glucagon (green in merge) and **C.** Pdx1 or **D.** CyclinA2 (red in merge) in pancreatic islets from P26 control and nCnb1KO mice. Scale bar is 10 μM.

Figure S3. NFATc1, NFATc2 and NFATc4 mRNA transcript levels are enriched in postnatal islets.

A. Relative mRNA transcript levels of Cn/NFAT signaling components by QPCR in islets isolated from early postnatal day 10 (P10) (grey bars) vs. mature (P28) (white bars) CD1 mice.

B. Relative mRNA levels of NFATc1 in FAC-sorted β-cells from MIP-GFP mice of indicated ages (in days) on x-axis. *Calcineurin A (CnA), Calcineurin b1 (Cnb1)*. All data are from male mice and are represented as means ± s.e.m. *, P<0.05. **, P<0.025. ***, P<0.002. §, not significant.

Supplemental Experimental Procedures

Animals and genotyping

Mice harboring the *Cnb1*^{f} or *Cnb1*$^{\Delta}$ alleles, have exons 3 to 5 flanked by loxP sites or are excised, respectively (Winslow et al., 2006). Transgenic *Ngn3-Cre* and *Pdx1-Cre* mice were provided by the Leiter and Melton laboratories, respectively and previously described (Gu et al., 2002; Schonhoff et al., 2004). These strains were crossed to generate *Ngn3-Cre; Cnb1*$^{\Delta/f}$ (***nCnb1KO***) mice or *Pdx1-Cre; Cnb1*$^{\Delta/f}$ (***pCnb1KO***) and their littermate controls on a mixed 129/Sv and C57/BL6 genetic background. Mice were genotyped routinely by PCR from tail genomic DNA for the deleted, floxed and WT alleles of *Cnb1* as well as the *Cre* transgenes as previously described. Inbred C57/BL6 mice for ChIP analyses were purchased from Charles River. MIP-EGFP mice were obtained from M. Hara and G. Bell (University of Chicago, Chicago, IL). All animals were maintained on a 12-hour light/dark cycle with ad libitum access to water and chow. All handling, experimentation and methods were in accordance with the Institutional Animal Care and Use Committee (IACUC) of Stanford University.

Quantitative Real-time RT–PCR

Mouse islets were isolated by standard collagenase pancreatic perfusion as previously described (Heit et al., 2006a). Total islet mRNA was then isolated using the RNeasy Microkit (QIAGEN) according to the manufacturer's instructions and RNA amount and purity were assessed by Nanodrop spectrophotometry. Any remaining contaminant DNA was removed by treating samples with 1 unit of RNAse-free DNAse (Fermentas). Next, cDNA was prepared from 750 ng of total islet RNA using the RETROscript kit (Ambion) and analyzed by quantitative real-time PCR (QPCR) using TaqMan Universal PCR Master Mix (ABI) and the ABI Prism 7500 detection system.

For culture studies, islets harvested from mice of the indicated genotype were placed in standard media (RPMI 1640 with 4.5 mM glucose containing 10% FBS and 1% pen/strep) at 37°C and 5% CO_2. For some experiments islets were maintained in media containing vehicle (DMSO), glucokinase activator (GKA) R0-28-1675 (10 µM; Axon Ligands) and/or FK506 (10 µM; LC Laboratories) for 72 hours, with media changes every 24 hours. Additionally, β-cells from MIP-EGFP islets were isolated using fluorescence-activated cell sorting as previously described (Sugiyama et al., 2007) and processed as above for analysis by QPCR. See Supplementary Table 1 for TaqMan probes (ABI) used. Each quantitative analysis was performed in triplicate and islets from 3 to 6 mice of each genotype were independently tested. Data are normalized to β-actin and results are expressed as the mean ± S.E.M.

Physiological studies

Random fed glucose levels were measured in ad libitum fed mice from tail vein blood using the Ascensia Contour glucometer. Glucose tolerance tests were performed following a 16-hour fast and blood glucose levels measured immediately before (0) and 15, 30, 45, 60, and 75 min after intraperitoneal (IP) injection of D-glucose (1 g/Kg body weight). For insulin sensitivity studies, random-fed mice were IP injected with insulin (Sigma) at 1U/kg body weight. Blood glucose levels were taken at indicated times and expressed as a percentage of the initial blood glucose concentration. Both serum insulin and glucagon levels were assessed in P26 mice that were fasted for 4 hours. Following euthanasia, blood was collected by cardiac puncture and serum was isolated after centrifugation. Serum insulin and glucagon levels were measured using the Mouse Insulin Ultrasensitive EIA kit (Alpco) and Glucagon ELISA kit (Alpco), respectively.

Histology, immunofluorescence, and immunohistochemistry

Pancreata were isolated, fixed in 4% paraformaldehyde at 4°C for 2 hours and washed 3 times in PBS. For paraffin sections, samples were then serially dehydrated in increasing concentrations of ethanol (25, 50, 75, 90, 100%) for 2 minutes at each concentration and then placed in xylenes for 2 hours. The samples were then embedded in paraffin wax blocks and sectioned at a thickness of 6 μm. For cryo sections, samples were cryoprotected in 30% sucrose overnight, embedded in O.C.T. (TissueTek) and 8 μm sections were obtained by cryosection (Leica). All immunohistochemistry was performed on paraffin sections while β-cell mass morphometry was done on cryosections.

The following primary antibodies and dilutions were used: chicken anti-insulin (1:200; Abcam, ab14042), guinea pig (GP) anti-glucagon (1:200; Linco, 4031-01), goat anti-glut2 (1:200; Santa Cruz, sc-7580), rabbit anti-pdx1 (1:100; Chemicon, AB3503), rabbit anti-cyclin A2 (1:100; Thermo Scientific, RB-1548P0), rabbit anti-cyclin D2 (1:100; Santa Cruz, sc-593), rabbit anti-FoxM1 (1:100; Santa Cruz, sc-500), rabbit anti-Cdk4 (1:100; Santa Cruz, sc-260), rabbit anti-chromogranin A (1:100; Immunostar, 20085), rabbit anti-chromogranin B (1:100; Abcam, ab12242), mouse anti-IAPP (1:50; Serotec, MCA1126T), mouse anti-IA2 (1:50; Santa Cruz, sc-130570), rabbit anti-Ki67 (1:100; NovoCastra, NCL-Ki67p), rabbit polyclonal anti-cleaved caspase-3 (Asp175) (1:500; Cell Signaling, 9661), mouse monoclonal anti-BRDU (1:100; Sigma). Antigen retrieval was performed for certain markers using either antigen unmasking solution (Vector Laboratories, H-3300) [anti-CcnA2, anti-CcnD2, anti-Cdk4, anti-IAPP, anti-Glut2] or Retrievit-8 target retrieval solution (Biogenex) [anti-activated caspase 3, anti-ChgB, anti-FoxM1, and anti-IA2]. We detected immune complexes with secondary antibodies

conjugated with either Alexa 488, Alexa 555 (Molecular Probes) or horseradish peroxidase (Vector Laboratories). All images were collected using the AxioCam microscope equipped with a CCD digital camera (Carl Zeiss) and represesentative of over 50 islets of a minimum of 3 different mice per genotype.

For measurement of β-cell mass, a minimum of 30 pancreas sections (spanning the entire pancreas) were assessed for at least 3 different mice per genotype. Cross-sectional area of insulin$^+$ cells were measured and normalized to total pancreatic area using Image-Pro Plus analysis software (Media Cybernetics). β-cell mass is expressed in mg, normalized to total pancreas mass.

β-cell proliferation and apoptosis levels were assessed by scoring the number of Ki67$^+$ or activated caspase-3$^+$ β-cells and expressed as a percentage of the total number of β-cells counted. For each experiment, a minimum of 30 islets/mouse for at least 3 mice/genotype were scored.

Transmission Electron Microscopy (TEM)

For each experiment, roughly 50 size-matched islets were isolated by collagenase perfusion from 3 pre-diabetic P20 nCnb1KO mice and 3 littermate controls as described above. Islets were fixed in Karnovsky's fixative (2% glutaraldehyde [EMS] and 4% paraformaldehyde [EMS] in 0.1M sodium cacodylate [EMS] pH 7.4) for 1 hour at room temperature (RT). The samples were then cut, post-fixed in 1% osmium tetroxide (EMS) for 1 hour at RT, washed 3x with ultrafiltered water and en bloc stained for 2 hrs at RT or left at 4^{o}C overnight. The samples were then dehydrated in a series of ethanol washes

(50%, 70%, 95%) for 15 minutes each at 4^{o}C, where the samples were then allowed to rise to RT. They were then moved to 100% ethanol 2x, followed by Acetonitrile for 15 min. Samples were infiltrated with EMbed-812 resin (EMS) mixed 1:1 with Acetonitrile for 2 hrs followed by 2 parts EMbed-812 to 1 part Acetonitrile for 2 hours. Finally, they were placed in EMbed-812 for 2 hours, moved into molds and resin filled gelatin capsules and placed into a 65^{o}C oven overnight.

Sections were taken between 75 and 90nm, picked up on formvar/carbon coated slot grids (EMS) or 100 mesh Cu grids (EMS). Grids were contrast stained for 15 minutes in 1:1 saturated UrAcetate (~7.7%) to 100% ethanol followed by staining in 0.2% Lead Citrate for 3 to 4 minutes. Samples were observed in the JEOL 1230 TEM at 80kV and final images were taken using a Gatan Orius digital camera. A total of three experiments were performed, with a minimum of 30 β-cells scored (blinded to genotype) per genotype per experiment.

Islet insulin secretion and islet insulin content measurement

Islets were isolated and cultured overnight in islet medium (as above) and passed three times through 10 cm petri dishes containing 3mM glucose islet media and allowed to equilibrate at 37°C for 1 hour. Five islets were then transferred into each well of an untreated, 24-well plate containing 1 ml of media for each condition (3mM or 20 mM glucose; 3mM or 20 mM arginine) and incubated at 37°C for one hour. Each condition was performed in quadruplicate using islets from at least 3 mice per genotype.

Islet DNA content was assessed by nanodrop for each replicate of all conditions and genotypes and media for each condition was removed and levels of secreted insulin were determined using the Mouse Insulin Ultrasensitive EIA kit (Alpco). Values of insulin were then normalized to islet DNA content. For whole islet insulin content, isolated islets were sonicated in 150 μl sonication buffer (150 ml 10 mmol/l Tris HCl, 1 mmol/l EDTA,

and 1 mg/ml radioimmunoassay grade BSA (pH 7.0) for 30 s. Fifty μl were used to extract islet insulin with 100 μl acid ethanol (75 ethanol:2 concentrated HCl:23 H2O, vol:vol:vol) at 4°C, overnight. The remainder of the sonicate (100 μl) was digested with an equal volume of lysis buffer at 55°C for 2 hours and used for islet DNA quantification for normalization.

Pancreatic islet chromatin immunoprecipitation (ChIP)

Islets from P20 C57/BL6 mice were isolated (as above) and fixed with 1% formaldehyde for 10 minutes at room temperature. Cross-linking was quenched by the addition of 0.125 M glycine and islets were washed in DPBS. ChIPs were then performed using the EZ-Magna ChIP™ G Chromatin Immunoprecipitation Kit (Millipore), following the manufacturer's protocol. Briefly, following cell and nuclear lysis, islets were sonicated to shear chromatin using a Bioruptor Sonicator (Diagenode) at maximum power; set for 15 seconds ON followed by 45 seconds OFF for a total time of 10 minutes. Precleared chromatin from 200-300 islets was used for each ChIP sample with incubation of 1 to 10 ug of the appropriate antibodies overnight at 4°C. Before IP, 1/10 of the extract was saved for use as the input. Antibodies used for IP were mouse monoclonal anti-RNA Polymerase (1 ug, Millipore, 05-623B), rabbit anti-IgG (1 ug, Santa Cruz, sc-2027), and rabbit anti-NFATc1 (5 ug, Imgenex, #IMG-5101A). Resulting chromatin was then amplified using the GenomePlex WGA4 Whole Genome Amplification Kit (Sigma) and quantified using nanodrop. Equal amounts of chromatin DNA were then analyzed by quantitative PCR in the ABI Prism 7500 detection system (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). All mouse and human ChIP primer sequences used are listed in the Supplementary Table 2 and flank putative NFAT

concensus binding sites (T/AGGAAAA/N) within the first 2 kb upstream of the transcriptional start site of each gene.

Human islet studies

Human islet samples were obtained from healthy, non-diabetic organ donors deceased due to acute traumatic or anoxic death and offered by NDRI (National Diseases Resource Interchange). Islets were isolated by Bottino, R. at the University of Pittsburg, or by Bryant, S. and Thompson A. at the University of Alabama, Birmingham. Seven independent human islet batches from juvenile donors at the ages of 13 mo (months old), 19 mo, 23 mo, 4 yo (years old), 5 yo, 19 yo and 20 yo, as well as five adult batches from donors of 28, 29, 49, 55, and 56 years of age were used in this study. After isolation, islets were shipped directly to our laboratory and were transferred to fresh islet culture medium (RPMI 1640 with 4.5 mM glucose containing 10% FBS and 1% pen/strep). As with mouse islets, islet samples were split and treated with either vehicle (DMSO) or FK506 (10 mM, LC laboratories) for 72 hours, with medium changes every 24 hours. Islets were then handpicked by dithizone staining and aliquots of 1000 IEQ were spun down and snap frozen for mRNA isolation or crosslinked as described above for ChIP studies. BrdU analysis was performed on the 4 yo donor batch by treating islets with 50 uM BrdU and chasing for 24 hrs. Afterwards, islets were placed in 2% agarose and processed within paraffin blocks as described above. Islets were immunostained for insulin and BrdU and β-cell proliferation rate was determined by quantifying the percentage of insulin$^+$ and BrdU$^+$ cells. A minimum of 50 islets and over 2000 β-cells were scored per condition.

***In vitro* MIN6 cell culture experiments**

For cell culture experiments, MIN6 murine insulinoma cells (passage 26) were transfected using lipofectamine 2000 (Invitrogen) with 2 ug of either the expression vector alone (pcDNA) or containing human NFATc1 cDNA (courtesy of Dr. Gerald Crabtree) as previously described (Beals et al., 1997). Following transfection, cells were grown for 48 hours and then treated with vehicle (DMSO) or a combination of ionomycin (1 uM) and phorbol 12-myristate 13-acetate (PMA) (25 uM) in order to activate calcineurin/NFAT. Eight hours after induction, cells were harvested for mRNA isolation.

Statistical analysis

Results were expressed as the mean ± S.E.M. Statistical analysis was performed using the two-tailed or one-tailed, unpaired Student's t-test. Differences were considered to be significant at $P < 0.05$.

Table S1. Lists of QPCR TaqMan probes used for mouse and human islet mRNA expression assays (purchased from Applied Biosystems).

MOUSE	
Insulin 1	Mm01259683_g1
Insulin 2	Mm00731595_gH
Glucokinase	Mm00439129_m1
Glut2	Mm00446224_m1
Hnf4a	Mm00433964_m1
Chromogranin A	Mm00514341_m1
Chromogranin B	Mm00483287_m1
IAPP	Mm00439403_m1
IA2	Mm00436138_m1
Cdk4	Mm00726334_s1
CyclinA2	Mm00438064_m1
CyclinD1	Mm00432360_m1
CyclinD2	Mm00438072_m1
FoxM1	Mm00514924_m1
HUMAN	
INSULIN	Hs00355773_m1
HNF4a	Hs00230853_m1
CHROMOGRANIN A	Hs00900373_m1
CHROMOGRANIN B	Hs01084631_m1
IAPP	Hs00169095_m1
IA2	Hs00160947_m1
CYCLINA2	Hs00996788_m1
CYCLIND2	Hs00153380_m1
FOXM1	Hs01073586_m1

Table S2. PCR Primers in ChIP analysis of mouse and human gene promoters.

Gene Promoter	Forward	Reverse	Targeted NFAT site
MOUSE			-BP upstream of start
CyclinA2	5'-GCC TTG CAC TCA AGA GAT CC	5'-TGA AGT TCC ACT GAC CCA AA	-979 **GGAAAA** -971
CyclinD2	5'-AGA GGG CCT CGG AGA AGT AG	5'-CAA GCT GGA AGG GCA GTT AG	-65 **AGGAAA** -58
FoxM1	5'-TCA AAG CAG CTC TCC CTT CT	5'-CGC AGC CTC CTG TGA TAA CT	-793 **GGAAA** -787
Chromogranin A	5'-AGT TTC AGC TGT GCC ACC TT	5'-CAA TGC TAT GCC GGC TTT TA	-311 **AGGAAAAC** -302
Chromogranin B	5'-GAG AAA GAG GGG GAG AGG AA	5'-AAA TCA AAC AGG CCA AAG GA	-329 **AGGAAA** -322
IA2	5'-TCC AAG ACA TCC AGG GCT AC	5'-TGA CAT TTG GGG TGT GTT TG	-1589 **TGGAAATA** -1580
Insulin	5'-AAC TGG TTC ATC AGG CCA TC	5'-ACT GGG TCC CCA CTA CCT TT	-318 **TGGAAAA** -310
Glucokinase	5'-GAA GGA GAA GGG GAA GGA GA	5'-ATG TTC AGG GCT TGT TCA GG	-1731 **GGAAA** -1725
HUMAN			
CYCLINA2	5'-AAT TTT TGG CAA GTG GCT GT	5'-TTT GAA GCC TAT AAA GCG GTC T	-1636 **TGGAAAAT** -1627
CYCLIND2	5'-TTG GCG TGC TAC ACC TAC AG	5'-CCC CTC CTC CTT TCA ATC TC	-113 **GGAAA** -107
FOXM1	5'-AGG GGC AAA AGA CAG GTT TC	5'-TCA AAG CTC GGC TTT AGT TGA	-394 **AGGAAATC** -385
CHROMOGRANIN A	5'-GTC AGG TGG CAA AGA GCT TC	5'-CCT TGC AAC ACC TAC CCA TT	-902 **AGGAAACT** -893
CHROMOGRANIN B	5'-TGA CTG AAA GAG GAA TTG AGG A	5'-AAG TGC AGC CGG AGA ATA TG	-523 **TGGAAATA** -514
IAPP	5'-GGC GGT TTT GCA GTC ATA TT	5'-CTA AAA CAG GGC CAA TGG AA	-1701 **TGGAAA** -1695
IA2	5'-TCA TTA TGC ATT TCT GTC CTT TTT	5'-GCT CTT TCA CCA CGA CCA CT	-1212 **TGGAAAGC** -1203
Negative Sites			
CYCLINA2 #2	5'-GGA GCT ATT CAG CGT GCT TC	5'-TTC GTG AGT CTG CCC TTC TT	-977 **TGGAAAAT** -968
CYCLIND2 #2	5'-TCA AGC ATG CGT TAG AGC AC	5'-GGC GAG TGA GGG ATT AGG TC	-987 **GGAAA** -981
FOXM1 #2	5'-TCG TGA CCT CAA GTG ATC CA	5'-CGC TAG GCC CTG AAG ATA CA	-696 **AGGAAAGA** -687
CHROMOGRANIN A #2	5'-TCT GCC CAA ACT CTG TAC CC	5'-CTT GAA CCC AAG AGG TGG AG	-1801 **TGGAAACC** -1792
CHROMOGRANIN B #2	5'-GAT TAC AGG CGT GAG CTT CC	5'-AAG ACC ACA GCC ACA GAA CA	-1328 **AGGAAATC** -1319
IAPP #2	5'-CTG GGC AAC AAA GAG TGA AA	5'TGGATGACCTTTTCCATTCA	-611 **GGAAA** -605
IA2 #2	5'-GGA GGG GAG AGA GGA TAT GG	5'-TCT CGA TCT CCT GAC CTC GT	-1829 **GGAAACA** -1221