Supporting Information

Sachdeva et al. 10.1073/pnas.0904849106

SI Text

Animals. Pdx1 heterozygous mice were generated as described previously, maintained on a C57BL/6 background, and housed on a standard 12-h light/12-h dark cycle with ad libitum access to food and water (29). Male mice (*Pdx1*$^{+/-}$ and their wild-type littermates) were randomized to receive either standard chow (11.9% kcal fat; LabDiet, 5053 PicoLab Rodent Diet 20) or a high-fat diet containing 45% kcal fat (Research Diets, D12451) at 4 weeks of age. Body weights and glucose were measured at baseline and followed weekly. All animal procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

For in vivo tunicamycin adminstration, 20-week-old male Pdx1$^{+/-}$ mice ($n = 3$) and their Pdx1$^{+/+}$ littermates ($n = 4$) were each given a single 1 μg/g body weight i.p. injection of a 0.05 mg/mL suspension of tunicamycin in 150 mM dextrose as described (30). After 96 h, pancreata were harvested and TUNEL/insulin co-staining and quantification performed as described below.

Characterization of Glucose Homeostasis. For glucose tolerance tests, mice were fasted overnight and then injected i.p. (IP) with 2g per kg body weight of D-glucose. Glucose measurements were taken at 0, 5, 15, 30, 60, and 120 min post-injection using a Freestyle Freedom glucometer (Abbott Labs). For readings higher than 500 mg/dL, the glucose was measured again with a OneTouch Ultra glucometer (Lifescan, Inc.), which can obtain a value of up to 600 mg/dL. Blood was collected from the tail vein at each time point during the glucose tolerance test and plasma insulin levels determined by ELISA (Crystal Chem). For insulin tolerance tests, mice were fasted for 6 h and then injected IP with 0.75 U/kg body weight of regular human insulin (NovolinR, Novo Nordisk) dissolved in normal saline. Glucose measurements were taken as above at 0, 15, 30, 45, 60, 90, and 120 min post-injection.

Morphological Analyses. To assess rates of β cell proliferation, 1 mg/mL BrdU (Sigma) was added to the drinking water of all mice 1 week before tissue harvest. Pancreata were dissected from mice 5 months on the diets, then fixed overnight in 4% paraformaldehyde (PFA) and embedded in paraffin for sectioning. All morphometric analyses were done on four to eight animals per group.

For measurement of β cell mass, insulin immunohistochemistry was performed on sections representing the maximal pancreatic footprint. After an antigen retrieval step of microwaving in 10 mM citric acid buffer (pH 6.0) and quenching of endogenous peroxidase activity, slides were blocked with AvidinD and Biotin (Vector Laboratories), then incubated in guinea pig anti-insulin (Linco; 1:5,000 dilution) overnight at 4 °C. Following addition of a biotinylated anti-guinea pig secondary antibody, HRP-conjugated avidin-biotin complex reagent was used following the manufacturer's protocol (Vector) and the signal developed using DAB. Images were captured and β cell area as a percent of total pancreatic area quantified using IPLab software (Scanalytics, Inc.). β cell mass was calculated for each animal by multiplying the percentage β cell area by total pancreatic mass.

For BrdU/insulin and double immunofluorescence, the following primary antibodies were used at the following dilutions: sheep anti-BrdU (U.S. Biologicals; 1:750), guinea pig anti-insulin (Linco; 1:400). After incubation in Cy2- and C3-conjugated secondary antibodies, slides were quickly stained with DAPI to visualize nuclei. Using IPlab software, BrdU+/insulin+ cells were counted and divided by the number of insulin+ cells. For BrdU quantification, more than 1,000 β cells were counted per animal.

Apoptosis was assessed by TUNEL assay using the Apoptag Peroxidase In Situ Apoptosis Detection Kit (Chemicon) essentially according to the manufacturer's instructions. To enable double TUNEL/insulin staining, immunofluorescence was performed using the anti-insulin primary antibody (as above) and the following secondary antibodies: Cy2 anti-guinea pig and Cy3 anti-digoxigenin. The number of TUNEL+ β cell nuclei were counted and normalized to total β cell number.

Mean β cell size was calculated by measuring islet area and number of β cells per islet for at least 10 islets per animal using IPlab software. For Bip immunohistochemistry, an antigen retrieval step was not required and the primary antibody (Stressgen SPA-826) was used at a dilution of 1:1,000.

Electron Microscopy. Pancreata were harvested from *Pdx1*$^{+/+}$ and *Pdx1*$^{+/-}$ littermates fed HFD or NC for 16 weeks (i.e., at 20 weeks of age), minced into small pieces, and fixed with 2.5% glutaraldehyde and 2.0% PFA in 0.1M sodium cacodylate buffer, pH 7.4, overnight at 4 °C. After washing with the same buffer, they were postfixed with 2.0% osmium tetroxide for 1 h at room temperature. After several dH_2O washes, samples were *en bloc* stained with 2.0% uranyl acetate for 30 min before dehydration in a graded ethanol series and embedding in PolyBed 812 (Polysciences Inc.) Sections were stained with uranyl acetate and bismuth subnitrite and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu CCD camera and using AMT Advantage image capture software. We examined approximately 50–100 β cells per mouse of eight mice in total (two per experimental group).

Islet Isolation. Islet isolations from mouse pancreata were performed using collagenase digestion similarly to previously-described protocols (1). After anesthetizing each mouse, the pancreas was inflated with cold 1× Hanks buffer, dissected from the abdomen, and minced for 2 min. Islets were released from acinar tissue by digestion with collagenase (Crescent Chemical Co., Inc.) at 37 °C for 10–15 min. After washing with cold Hanks buffer, individual islets underwent three rounds of hand-picking under a light microscope.

RNA Isolation. After addition of TRIzol (Invitrogen), islet RNA was isolated using the RNeasy Mini kit (Qiagen), treated with DNAseI (Ambion), analyzed for integrity with an Agilent 2100 Bioanalyzer, and reverse transcribed with SuperScript II (Invitrogen) using oligo(dT) for priming. When isolating islet RNA for microarray analysis, pancreata from two mice were processed at the same time. Following collagenase digestion, islets were purified through a Ficoll gradient and then subjected to two rounds of hand-picking. RNA was isolated, analyzed, and reverse-transcribed as above. For Min6 cell experiments, cells were harvested and processed for RNA isolation using TRIzol. RNA samples were DNAseI-treated, analyzed, and reverse-transcribed as above.

Pdx1 Gene Silencing and ER Stress Induction in Min6 Cells. Min6 cells were infected with recombinant adenovirus containing an shRNA sequence designed to target either Pdx1 or luciferase as described (2, 3). Briefly, 5×10^5 cells were infected at a multiplicity of infection of 2,500 pfu/cell for 6.5 h and then harvested 72 h later.

To knock down Pdx1 in Min6 cells using siRNA duplexes, we introduced 200 pmol of either siPdx1 (L-040402, Dharmacon ON-TARGETplus SMARTPool) or a pool of non-targeting control duplexes (D-001810, Dharmacon ON-TARGETplus si-

CONTROL) into approximately 5×10^6 cells using nucleofection (Amaxa Biosystems). Cells were harvested 96 h after nucleofection for RNA isolation or preparation of nuclear extracts. Nuclear protein was isolated using a protocol based on published reports (4). Briefly, cells were pelleted and lysed with three volumes of Buffer A (10 mM HEPES, pH7.9, 10 mM KCl, 0.1 mM EDTA, and 0.1 mM EGTA) including protease and phosphatase inhibitors (Calbiochem) by vortexing and incubating on ice for 15 min. Then, after centrifugation and removal of the supernatant (cytoplasmic fraction), the nuclear pellet was lysed for 15 min at 4 °C with constant shaking in Buffer C (20 mM HEPES, pH7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 10% glycerol) plus protease and phosphatase inhibitors. Protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Scientific) and, for Western blotting, 10–20 μg of nuclear extract was resolved on a 4–12% SDS/PAGE gel (Nupage, Invitrogen). The following primary antibodies were used: rabbit anti-Pdx1 antiserum (5) at 1:10,000 dilution, rabbit anti-CREB2 (Atf4) (sc-200, Santa Cruz Biotechnology) at 1:500 dilution, rabbit anti histone H3 (ab1791, Abcam) at 1:5,000 dilution, rat anti-caspase 12 (C 7611, Sigma) at 1:1,000 dilution, and mouse anti ran (610340 BD Biosciences) at 1:5,000 dilution.

For induction of ER stress, Min6 cells were treated with either 1 μM thapsigargin (Sigma, T9033), 10 μg/mL tunicamycin (Sigma, T7765), or DMSO (vehicle) unless specified otherwise in the figure legends. Cells were then harvested at indicated time points after treatment for RNA extraction, analysis, and reverse transcription as above. For measuring ER stress-induced apoptosis in Pdx1-deficient Min6 cells, cells were treated with thapsigargin or tunicamycin 72 h after siRNA-mediated Pdx1 silencing and harvested 24 h later for either Western blot or flow cytometry. The following antibodies were used for Western blot: rat monoclonal anti-caspase 12 (C7611, Sigma) and mouse monoclonal anti-tubulin (Sigma). Floating and adherent cells were collected and stained with annexin V and propidium iodide for flow cytometry.

cDNA Microarrays. Global gene expression profiling of islets was performed using three pairs of RNA samples from islets isolated from 8-week-old *Pdx1*$^{+/-}$ mice or their *Pdx1*$^{+/+}$ littermates. These samples were matched for islet purity by measuring insulin and amylase expression using quantitative RT-PCR. For microarray analysis of Min6 cells, RNA from four independent wells of Min6 cells infected with AdshPdx1 was compared with two sets of controls (both uninfected and AdshLuc-infected cells) on separate chips, each also in quadruplicate.

For all microarray experiments, approximately 100–500 ng total RNA was amplified using the MessageAmpTM II aRNA Amplification Kit (Ambion). After amplification 2.5 μg aRNA was indirectly labeled using amino-allyl dUTP and anchored oligo(d)T prime reverse transcription. The cDNA was purified using the MinElute PCR Purification Kit (Qiagen), eluted in coupling buffer (0.1 M sodium bicarbonate, pH 9) and coupled with the appropriate Cy3 or Cy5 fluorescent label (CyTMDye, Amersham Pharmacia Biotech Ltd.), combined, and purified. Amplified and labeled RNA was hybridized to the Mouse PancChip 6.1 cDNA microarrays. Slides were scanned, images captured, and data analyzed as described (6). The expression microarray data have been deposited into the publicly available database, Array Express (accession numbers E-MTAB-132 and E-MTAB-127).

Quantitative RT-PCR. Real-time PCR was performed in triplicate with a Bio-Rad iCycler by cycling 40 times using the following conditions: 95 °C for 10 s, 61 °C for 45 s. Primers for mouse Pdx1, spliced XBP1, total XBP1, Bip, Ero1l, Wfs1, and Asns have been described previously (3, 7–9). Primers for all other genes were designed using Primer3 as above and tested for linear amplification using serial dilutions of cDNA before use on experimental samples. Sequence information is provided in Table S5.

Quantitative Chromatin Immunoprecipitation. Min6 ChIP assays were performed essentially as described previously, using approximately 10^7 cells per IP (3). Rabbit anti-Pdx1 antiserum (5) and normal rabbit serum (sc-2338, Santa Cruz Biotechnology) were used for the pull-downs.

For each islet ChIP, islets were freshly isolated from 8-week-old CD1 male mice as detailed above, crosslinked with 1% formaldehyde in PBS (PBS) for 10 min at room temperature and quenched with glycine to a final concentration of 0.125 M. Cells were lysed for 15 min at 4 °C with constant shaking in ChIP cell lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40) with added protease and phosphatase inhibitors (Calbiochem). The remainder of the ChIP was carried out using the same protocol used for Min6 ChIPs as above (3). The number of islets isolated for each independent ChIP experiment ranged from 520 to 850, but in each experiment the total chromatin was divided equally between the rabbit anti-Pdx1 antiserum and the control serum.

Data were analyzed quantitatively in duplicate by real-time PCR with SYBR green using a Bio-Rad iCycler. PCR product signals were referenced to a dilution series of the relevant input to account for different efficiencies of primer sets. Forward and reverse primer sequences for the insulin, Pdx1, and albumin promoters have been described elsewhere (10). For detection of the two conserved regions of the mouse Atf4 promoter, primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequences were as follows: Region 1: 5′-tgcacatgcacttgtgacttct-3′ and 5′-cggaaccaaccataaaacacg-3′; Region 2: 5′-cgcagacccctgatcctaga-3′ and 5′-ctgacggtcaaagccgaag-3′.

ChIP on Chip Promoter Array. Five independent Pdx1 ChIPs were performed in Min6 cells as described above. Approximately one-half of each ChIP sample was used for ligation-mediated PCR amplification performed similarly to previously-described methods (11). This technique generated at least 1 μg chromatin per sample. Final chromatin concentration was measured using the Nanodrop ND-1000 spectrophotometer. Amplified material was paired, labeled and hybridized as Test (Cy5) vs. Control (Cy3). Samples were labeled using Ready-To-Go DNA labeling beads (Amersham) per manufacturer's instructions. The Cy3 and Cy5 samples were combined and purified using MinElute columns (Qiagen). After purification, 1 μg Mouse Cot1 DNA (Invitrogen Life Technologies) was added to each sample and denatured at 95 °C for 5 min. The samples were then cooled to 42 °C and an equal volume of 2× hybridization buffer (50% formamide, 10× SSC, and 0.2% SDS) was added, mixed, and applied to the Mouse PromoterChip BCBC-5A.1 microarray slide. Microarray slides were hybridized overnight, then washed and scanned with Agilent G2565BA Microarray Scanner. Images were analyzed with GenePix 5.0 software (Axon Instruments). The ChIP on chip promoter array data have been deposited into the publicly available database, Array Express (accession number E-MTAB-134).

Statistical Analysis. All data represent the mean ± standard error. Glucose tolerance tests and insulin tolerance tests were analyzed with repeated-measures analysis of variance (ANOVA). The IP tunicamycin results were compared using a one-tailed Student's *t* test. For all other comparisons, including qPCR data, ChIP data, and β cell morphometry, we assessed statistical significance using a two-tailed Student's *t* test. Differences were considered significant if $P < 0.05$. For statistical analysis of all microarray data, genes were called differentially expressed using the signif-

icance analysis of microarrays (SAM) one class response package with a false discovery rate (FDR) of 10% (for the AdshPdx1 Min6 cDNA microarray and the Pdx1 ChIP on chip promoter array) or 20% (for the $Pdx1^{+/-}$ islet cDNA array) (12).

1. Scharp DW, et al. (1973) The use of ficoll in the preparation of viable islets of langerhans from the rat pancreas. *Transplantation* 16:686–689.
2. Iype T, et al. (2005) Mechanism of insulin gene regulation by the pancreatic transcription factor Pdx-1: Application of pre-mRNA analysis and chromatin immunoprecipitation to assess formation of functional transcriptional complexes. *J Biol Chem* 280:16798–16807.
3. Deramaudt TB, et al. (2006) The PDX1 homeodomain transcription factor negatively regulates the pancreatic ductal cell-specific keratin 19 promoter. *J Biol Chem* 281:38385–38395.
4. Schreiber E, Matthias P, Muller MM, Schaffner W (1989) Rapid detection of octamer binding proteins with 'mini-extracts,' prepared from a small number of cells. *Nucleic Acids Res* 17:6419.
5. Stoffers DA, Stanojevic V, Habener JF (1998) Insulin promoter factor-1 gene mutation linked to early-onset type 2 diabetes mellitus directs expression of a dominant negative isoprotein. *J Clin Invest* 102:232–241.
6. Gupta RK, et al. (2007) Expansion of adult beta-cell mass in response to increased metabolic demand is dependent on HNF-4alpha. *Genes Dev* 21:756–769.
7. Lipson KL, et al. (2006) Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1. *Cell Metab* 4:245–254.
8. Riggs AC, et al. (2005) Mice conditionally lacking the Wolfram gene in pancreatic islet beta cells exhibit diabetes as a result of enhanced endoplasmic reticulum stress and apoptosis. *Diabetologia* 48:2313–2321.
9. Jousse C, et al. (2007) TRB3 inhibits the transcriptional activation of stress-regulated genes by a negative feedback on the ATF4 pathway. *J Biol Chem* 282:15851–15861.
10. Chakrabarti SK, James JC, Mirmira RG (2002) Quantitative assessment of gene targeting in vitro and in vivo by the pancreatic transcription factor, Pdx1. Importance of chromatin structure in directing promoter binding. *J Biol Chem* 277:13286–13293.
11. Oberley MJ, Farnham PJ (2003) Probing chromatin immunoprecipitates with CpG-island microarrays to identify genomic sites occupied by DNA-binding proteins. *Methods Enzymol* 371:577–596.
12. Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 98:5116–5121.

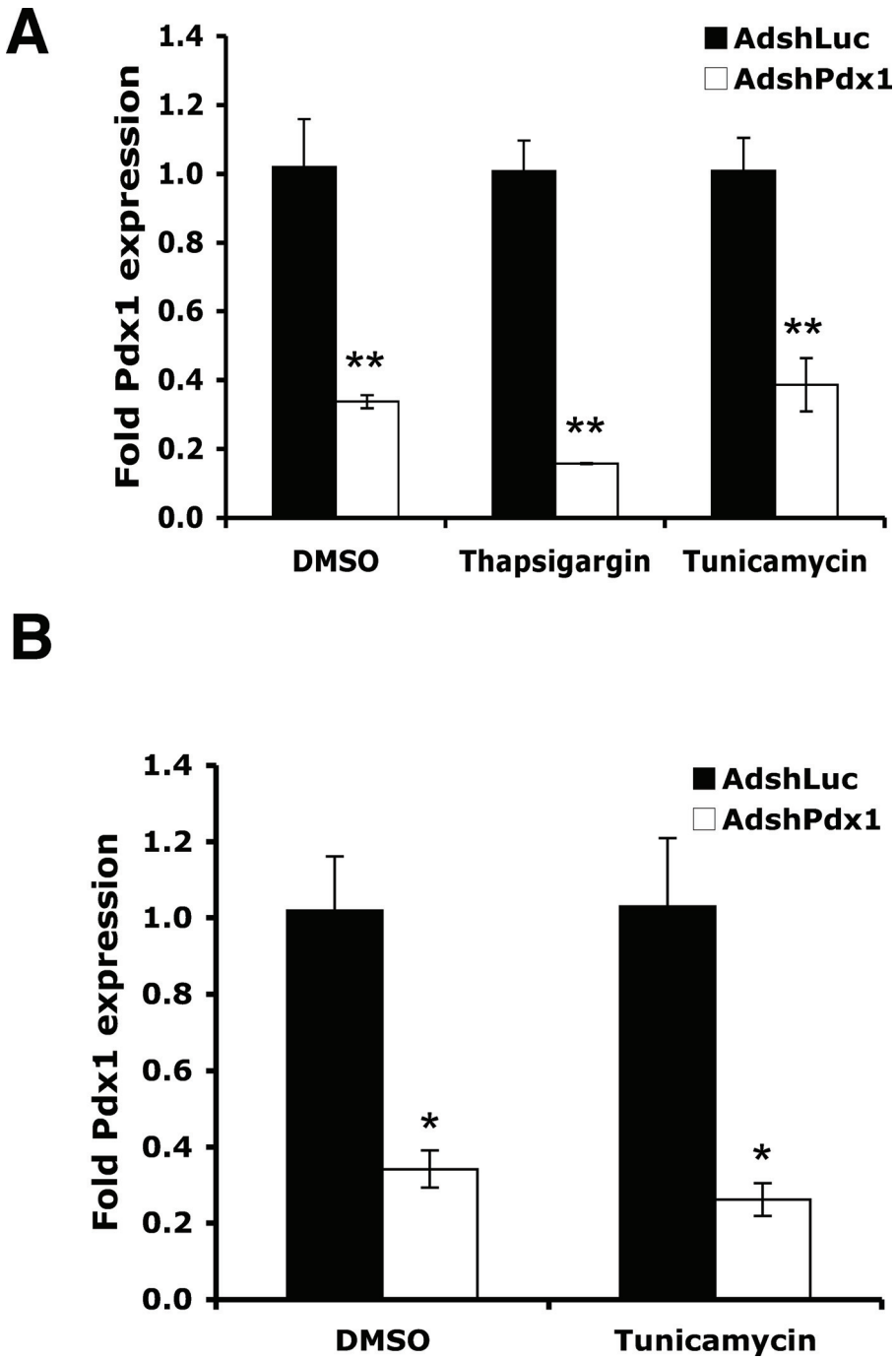

Fig. S1. Pdx1 transcript levels in AdshPdx1-infected Min6 cells treated with 1 μM thapsigargin, 10 μg/mL tunicamycin, or vehicle (DMSO) for 2 h (Fig. S1*A*) or 8 h (Fig. S1*B*). Quantitative RT-PCR data represent the mean of three replicates per condition (*, $P < 0.05$, **, $P < 0.01$ relative to treatment-matched AdshLuc-infected cells). ER stress-mediated induction of spliced XBP1 and Bip transcripts in these samples is depicted in Fig. 5 *H* and *I*.

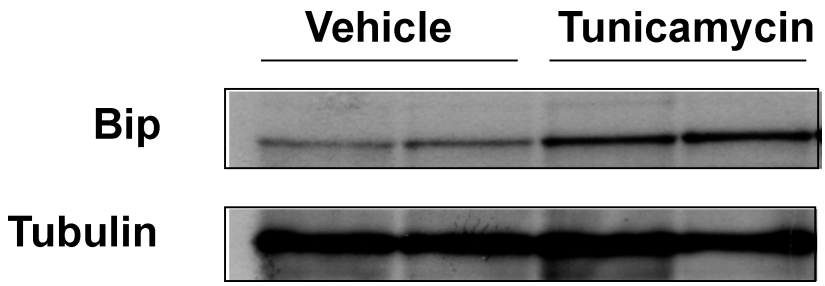

Fig. S2. Tunicamycin induces systemic ER stress. Kidney lysate harvested from mice 96 h after IP injection of TM (1 μg/g body weight) or vehicle (saline). Western blot visualized with anti-BiP and anti-tubulin (loading control).

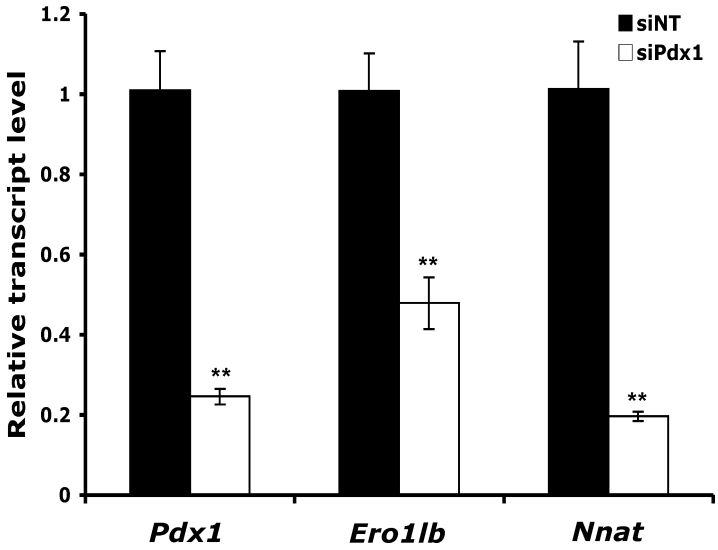

Fig. S3. Confirmation of Pdx1 regulation of Ero1b and Nnat in Min6 cells. Quantitative RT-PCR analysis of Ero1lb and Nnat mRNA levels in Min6 cells 96 h after knockdown of Pdx1 expression using a pool of four siPdx1 duplexes. ($n = 3$ independent replicates per condition; $**P < 0.01$).

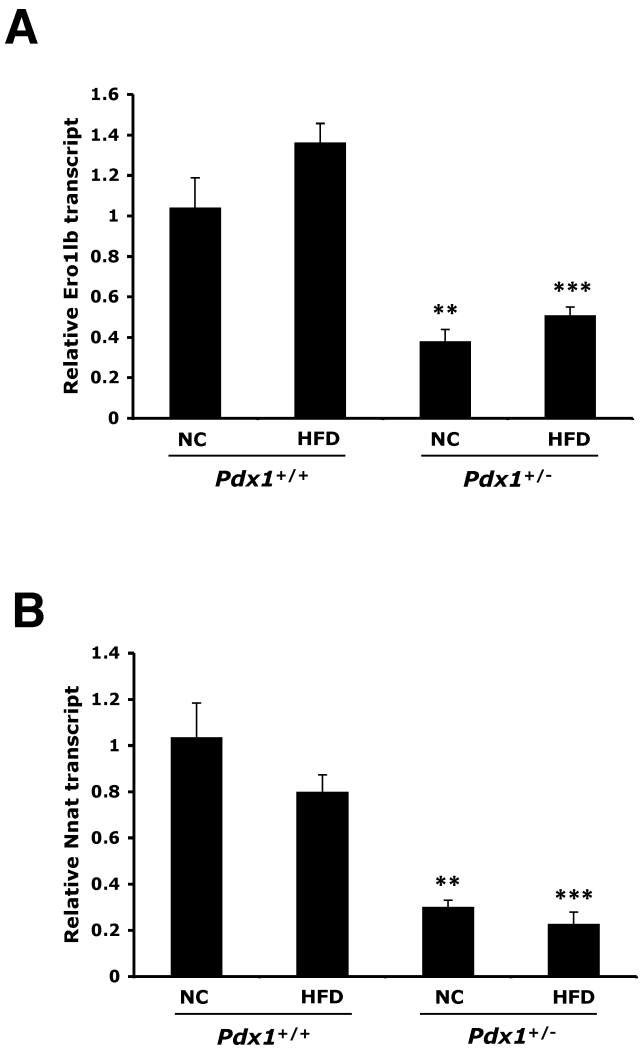

Fig. S4. Pdx1 regulates the ER genes Ero1lb and Nnat in vivo. Ero1lb (*A*) and Nnat (*B*) mRNA levels in islets isolated from individual *Pdx1*$^{+/+}$ and *Pdx1*$^{+/-}$ mice fed either HFD or NC for 8 weeks. $n = 4$ mice per group, **, $P < 0.01$, ***, $P < 0.001$ relative to diet-matched *Pdx1*$^{+/+}$ littermates.

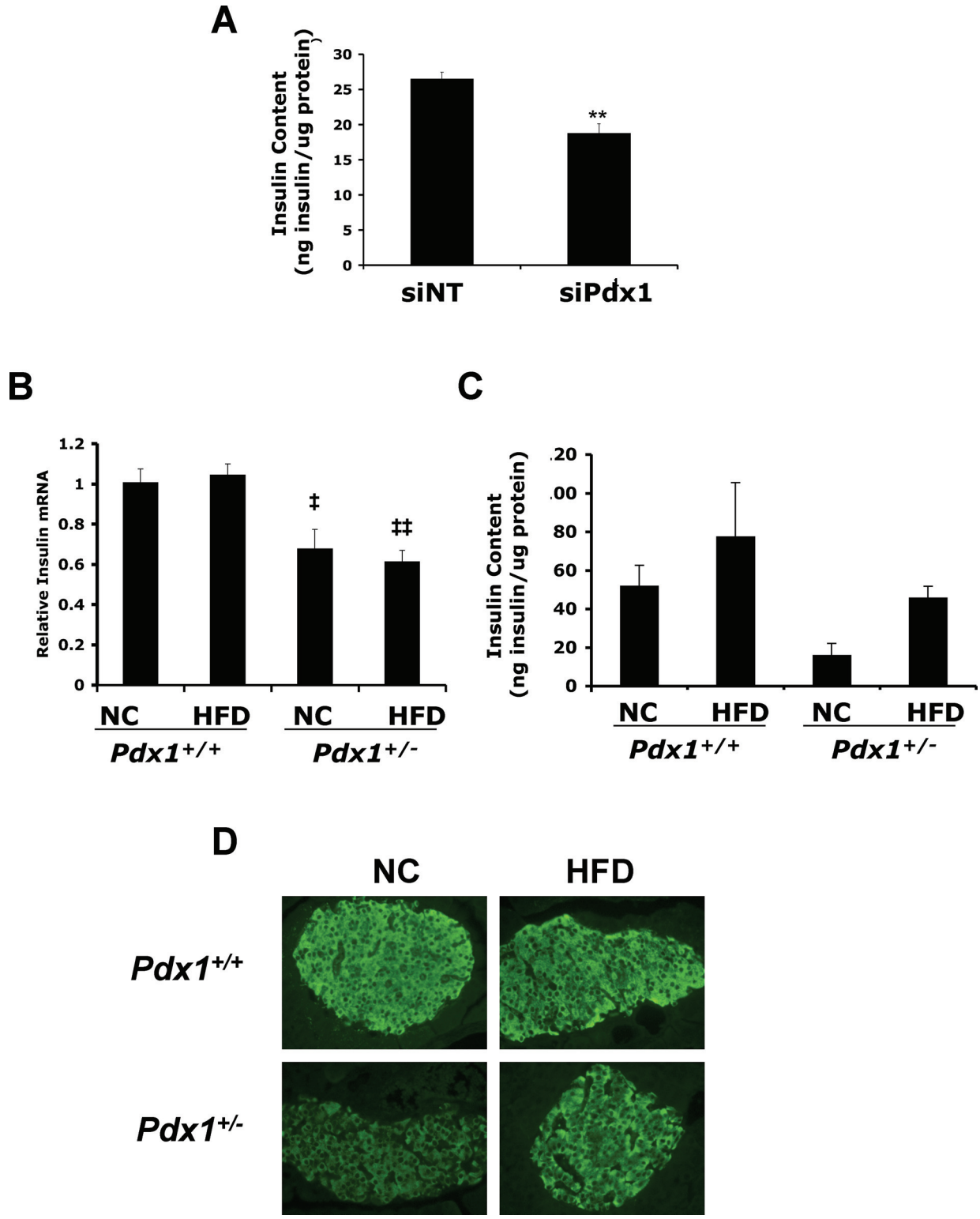

Fig. S5. Pdx1 deficiency reduces β cell insulin content. (*A*) Insulin content measurement in Min6 cells 72 h after silencing of Pdx1 with a pool of four siRNA duplexes or non-targeting siRNA. ($n = 6$, **, $P < 0.001$). (*B*) Quantitative PCR measurement of insulin transcript in mouse islets isolated after 8 weeks on HFD or NC, normalized to HPRT. ($n = 4$ per group, ‡, $P < 0.05$, ‡‡, $P < 0.01$ relative to diet-matched *Pdx1*$^{+/+}$ littermates). (*C*) Insulin content measurement of mouse islets isolated after 8 weeks of NC or HF-feeding ($n = 2$–3 per group). (*D*) Representative images of insulin stained pancreas sections of *Pdx1*$^{+/+}$ *or* *Pdx1*$^{+/-}$ mice after 5 months on NC or HFD.

Other Supporting Information Files

Table S1
Table S2
Table S3
Table S4
Table S5