

Pancreatic and Duodenal Homeobox Protein 1 (Pdx-1) Maintains Endoplasmic Reticulum Calcium Levels through Transcriptional Regulation of Sarco-endoplasmic Reticulum Calcium ATPase 2b (SERCA2b) in the Islet β Cell*

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Background: Altered sarco-endoplasmic reticulum Ca^{2+} ATPase 2b (SERCA2b) expression and activity contributes to β cell dysfunction in diabetes.

Results: SERCA2b deficiency occurs secondary to loss of pancreatic and duodenal homeobox 1 (Pdx-1)-mediated transcriptional regulation.

Conclusion: Pdx-1 maintains SERCA2b expression and endoplasmic reticulum (ER) calcium levels in the β cell.

Significance: These findings elucidate a novel pathway that contributes to β cell ER stress.

Although the pancreatic duodenal homeobox 1 (Pdx-1) transcription factor is known to play an indispensable role in β cell development and secretory function, recent data also implicate Pdx-1 in the maintenance of endoplasmic reticulum (ER) health. The sarco-endoplasmic reticulum Ca^{2+} ATPase 2b (SERCA2b) pump maintains a steep Ca^{2+} gradient between the cytosol and ER lumen. In models of diabetes, our data demonstrated loss of β cell Pdx-1 that occurs in parallel with altered SERCA2b expression, whereas *in silico* analysis of the SERCA2b promoter revealed multiple putative Pdx-1 binding sites. We hypothesized that Pdx-1 loss under inflammatory and diabetic conditions leads to decreased SERCA2b levels and activity with concomitant alterations in ER health. To test this, siRNA-mediated knockdown of Pdx-1 was performed in INS-1 cells. The results revealed reduced SERCA2b expression and decreased ER Ca^{2+} , which was measured using fluorescence lifetime imaging microscopy. Cotransfection of human Pdx-1 with a reporter fused to the human SERCA2 promoter increased luciferase activity 3- to 4-fold relative to an empty vector control, and

direct binding of Pdx-1 to the proximal SERCA2 promoter was confirmed by chromatin immunoprecipitation. To determine whether restoration of SERCA2b could rescue ER stress induced by Pdx-1 loss, Pdx1^{+/-} mice were fed a high-fat diet. Isolated islets demonstrated an increased spliced-to-total Xbp1 ratio, whereas SERCA2b overexpression reduced the Xbp1 ratio to that of wild-type controls. Together, these results identify SERCA2b as a novel transcriptional target of Pdx-1 and define a role for altered ER Ca^{2+} regulation in Pdx-1-deficient states.

Diabetes mellitus is a worldwide epidemic affecting an estimated 285 million people, and the prevalence of this disorder is expected to rise to 439 million by the year 2030 (1). Altered pancreatic β cell function and survival are central components of the pathophysiology of type 1 (T1D)⁴ and type 2 diabetes (T2D). As secretory cells, β cells are dependent upon a highly developed endoplasmic reticulum (ER) to ensure that proteins are produced robustly and folded efficiently. Under conditions of metabolic stress, proteins may fail to fold correctly within the β cell ER lumen, initiating an unfolded protein response (UPR). The UPR limits the delivery of new proteins to the ER and, ultimately, increases protein folding capacity through increased transcription and activity of protein chaperones and foldases. However, if the inciting stress is unresolved, continual stimulation of the UPR leads to the activation of apoptotic path-

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⁴ The abbreviations used are: T1D, type 1 diabetes; T2D, type 2 diabetes; ER, endoplasmic reticulum; UPR, unfolded protein response; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase; FLIM, fluorescence lifetime imaging microscopy; HG, high glucose; HFD, high-fat diet.

ways and β cell death, a transition referred to as ER stress (2–4). Data from rodent and human studies illustrate a pivotal role for β cell ER stress during the progression of both major forms of diabetes mellitus (5–9).

β Cell ER stress is induced in response to a number of insults, including hyperglycemia and downstream oxidative stress, increased saturated free fatty acids, and proinflammatory cytokines. One unifying consequence of these insults and accumulation of unfolded proteins is thought to be altered ER calcium (Ca^{2+}) regulation (10). Under normal conditions, ER Ca^{2+} is estimated to be at least three orders of magnitude higher than cytosolic Ca^{2+} , and Ca^{2+} within the ER lumen serves as a required cofactor for a number of steps involved in protein processing and maturation (11–14). The integrity of this gradient is actively maintained by the sarco-endoplasmic reticulum calcium ATPase (SERCA) pump, which transports two Ca^{2+} ions into the ER at the expense of one ATP molecule (15). At least 14 different SERCA isoforms have been identified to date (16, 17), and we have previously shown SERCA2b to be the most prevalent isoform expressed in the mouse pancreatic islet. We and others have also shown that β cell SERCA2b levels are diminished in human and rodent models of T1D and T2D (18–21), resulting in Ca^{2+} dysregulation, impaired insulin secretion, and ER stress (19). However, the specific transcriptional pathways that regulate SERCA2b expression in the β cell remain largely uncharacterized.

The pancreatic duodenal homeobox 1 (Pdx-1) transcription factor is known to play an indispensable role in β cell development and secretory function, and recent data also implicate Pdx-1 in the maintenance of ER health (22–27). Islets isolated from Pdx-1 haploinsufficient mice demonstrate ER stress when animals are exposed to a high-fat diet, and Pdx-1 has been shown to enhance the transcription of a number of proteins involved in UPR signaling and ER function, including activating transcription factor 4 (Atf4) and Wolframin (Wfs1) (9, 28). Furthermore, reductions in total Pdx-1 levels as well as impaired nuclear localization of Pdx-1 have been described under hyperglycemic and diabetic conditions (29–31). Interestingly, our *in silico* analysis of the human SERCA2b promoter revealed multiple putative binding sites for Pdx-1. Given an emerging role for Pdx-1 in the maintenance of an ER-specific subgenome, we hypothesized that Pdx-1 may serve as a transcriptional regulator of the SERCA2 gene and that loss of Pdx-1 may underlie decreased SERCA2b expression, altered Ca^{2+} regulation, and activation of β cell ER stress signaling pathways in diabetes.

EXPERIMENTAL PROCEDURES

Animals and Islet Preparations—Animals were maintained under protocols approved by the Indiana University Institutional Animal Care and Use Committee, the United States Department of Agriculture Animal Welfare Act (9 CFR, Parts 1, 2, and 3), and the Guide for the Care and Use of Laboratory Animals (32). C57BLKs/J-db/db mice and heterozygote littermate controls were obtained from The Jackson Laboratory (Bar Harbor, ME) at 12 weeks of age. Pdx-1 haploinsufficient mice on a mixed background were a gift from Chris Wright (Vanderbilt University School of Medicine, Nashville, TN). Male Pdx-1 haploinsufficient and wild-type littermates were weaned at 4

weeks of age and the following week, begun on either a normal chow diet (17% kcal from fat) or a high-fat diet (45% kcal from fat) for 8 weeks. Mouse cages were kept in a standard light-dark cycle with *ad libitum* access to food and water. Intraperitoneal glucose tolerance tests were performed at 11 weeks of age using a protocol described previously (33, 34). Blood glucose was measured using an AlphaTRAK glucometer from Abbott Laboratories (Abbott Park, IL). After 8 weeks of diet treatment, mouse pancreatic islets were isolated by collagenase digestion as described previously (35). Human islets isolated from cadaveric non-diabetic and diabetic donors were obtained from the National Disease Research Interchange or the Integrated Islet Distribution Program. On arrival, islets were immediately placed in DMEM containing 5.5 mM glucose, 10% fetal bovine serum, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen) and incubated overnight at 37 °C with 5% CO_2 . Our analysis included islets from five non-diabetic donors and three donors with an established diagnosis of T2D. The average age of non-diabetic donors was 45.2 ± 6.1 years (S.E.), and the average body mass index was 27.6 ± 2.8 kg/m^2 . The average age of the donors with T2D was 53.0 ± 4.4 years, and the average body mass index was 22.7 ± 2.7 kg/m^2 .

Cell Culture and *in Vitro* Islet Treatment—INS-1 832/13 rat insulinoma cells were cultured as described previously (36, 37). NIH-3T3 immortalized mouse fibroblast cells were cultured at 37 °C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin (38). For *in vitro* viral overexpression and knockdown experiments, viruses were replicated and purified using HEK-293T cells. For adenoviral transduction in cell lines, culture medium was replaced with fresh medium containing adenovirus-expressing mouse Pdx-1 (39), mouse SERCA2b (40), Pdx-1 siRNA (41), random siRNA (42), or a LacZ control virus, followed by overnight incubation. The anti-Pdx-1 siRNA sequence used was 5'-GAAAGAG-GAAGATAAGAAA-3', and the random siRNA sequence was 5'-GAGACCCTATCCGTGATTA-3'. After 24–48 h, RNA or protein was isolated for the indicated analyses, or cells were used for Ca^{2+} imaging. For viral transduction of isolated mouse islets, groups of 50–100 islets were hand-picked within 1 h of isolation, incubated with adenovirus for 24 h, and washed with PBS followed by Ca^{2+} imaging or RNA or protein isolation. To simulate hyperglycemic and proinflammatory conditions *in vitro*, INS-1 cells were treated with 25 mM glucose and 5 ng/ml IL-1 β for 16–24 h using a protocol established previously (19).

Immunoblot Analysis—Isolated islets or cultured cells were washed with PBS and lysed with buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 0.05% desoxycholate, 0.1% IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO), 0.1% SDS, 0.2% sarcosyl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 10 mM NaF, EDTA-free protease inhibitors (Roche Applied Science), phosphatase inhibitors (Roche Applied Science), 2 mM MgCl_2 , and 0.05% v/v benzonase nuclease (Sigma-Aldrich). Lysate from isolated islets was further disrupted by mechanical shearing using a 20-gauge needle and syringe. Protein concentration was measured using the Bio-Rad DC protein assay (Bio-Rad) and a SpectraMax M5 multiwell plate reader (Molecular Devices, Sunnyvale, CA). Equal concentrations of proteins were suspended in 10% SDS solution prior to electrophoresis using a 4–20% Mini-Protean

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TGX gel in a Mini-Protean Tetra apparatus (Bio-Rad). Proteins were transferred to a PVDF membrane and blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) prior to incubation with primary antibodies. The primary antibodies used included the following: Pdx-1 (catalog no. 07-696 Millipore, Billerica, MA), SERCA2 (catalog no. sc-8095, Santa Cruz Biotechnology, Dallas, TX), Actin (catalog no. 691002, MP Biomed, Santa Ana, CA), and GAPDH (catalog no. ab9484, Abcam, Cambridge, UK). Of note is that the SERCA2 antibody does not distinguish between the SERCA2a and SERCA2b isoforms. However, we have shown previously that SERCA2a is expressed at very low levels in mouse islets (19). Immunoblots were scanned using a LI-COR Odyssey 1828 scanner and analyzed with LI-COR Image Studio software, with the densitometry of the scanned images calculated by ImageJ software (National Institutes of Health, Bethesda, MD).

Real-time quantitative RT-PCR—Cultured cells or isolated islets were processed for total RNA using RNeasy Mini plus or Micro plus kits (Qiagen, Valencia, CA), according to the instructions of the manufacturer and as described previously (43, 44). Total RNA was reverse-transcribed at 37 °C for 1 h using random hexamers, 0.5 mM dNTPs, 5 \times first-strand buffer, 0.01 mM DTT, and Moloney murine leukemia virus reverse transcriptase (all from Invitrogen). Quantitative RT-PCR was performed using either TaqMan proprietary primers (Invitrogen) or SYBR Green I dye and primer sequences and methods published previously (37).

Fura-2/AM Cytoplasmic Calcium Imaging—Intracellular cytosolic Ca²⁺ was measured using the ratiometric calcium indicator Fura-2/AM from Invitrogen and a modification of methods published previously (37). INS-1 832/13 cells were seeded in a glass bottom 50-mm plate. After 24 h, cells were transduced with siPdx-1 or random siRNA adenovirus, and isolated mouse islets were transduced with SERCA2 or LacZ adenovirus as described. Prior to imaging, INS-1 cells and islets were incubated at 37 °C and 5% CO₂ in 4 μ M Fura-2/AM and 0.02% pluronic F127 (Invitrogen) for 1 h and then washed and incubated with Hanks' balanced salt solution (Invitrogen) supplemented with 0.1% BSA.

Islets were next transferred to a small volume chamber (Warner Instruments, Hamden, CT) mounted on the microscope stage. INS-1 cells and islets were perfused with a gradient pump (Bio-Rad) and maintained at 37 °C. For INS-1 cells, 10 mM caffeine or 1 μ M thapsigargin were used to activate ryanodine receptors or inhibit SERCA activity, respectively. Fura-2/AM fluorescence was measured with excitation at 340 and 380 nm and emission at 510 nm. Images were captured using a Zeiss Z1 microscope with a \times 10 or \times 20 objective, and results were analyzed with Zen Blue software (Zeiss, Oberkochen, Germany).

Fluorescence Lifetime Imaging Microscopy (FLIM)—A D4ER adenovirus described previously was used for direct analysis of ER Ca²⁺ levels (45, 46). The probe was created by replacing the Ca²⁺ binding domain of the D1ER construct with D4 to provide lower Ca²⁺ affinity, with the new construct placed downstream of the rat insulin promoter. Briefly, INS-1 cells were cultured for 18 h with the D4ER adenovirus and siPdx-1 adenovirus or

random siRNA adenovirus. Cells were then allowed to recover for 6 h. FLIM was carried out in accordance with a protocol published previously (47). In brief, the Alba FastFLIM system (ISS Inc., Champaign, IL) was coupled to an Olympus IX71 microscope using a \times 60 water-immersion lens (Olympus, Tokyo, Japan). Confocal scanning was controlled by Build 143 VistaVision software (ISS Inc.) at 530/43 nm acceptor and 480/40 nm receptor wavelengths. Regions of interest were selected with >75 count averages. The lifetime determination was obtained by analyzing the first 11–12 modulation frequencies (10–120 MHz). Efficiency of FRET was estimated next using the following equation: $E_{\text{FRET}} = 1 - (\tau_{\text{DA}} / \tau_{\text{D}})$, where τ_{D} and τ_{DA} are the donor fluorescence lifetime obtained in the absence and presence of the acceptor, respectively.

Luciferase Reporter Assays—Our previous publication utilized luciferase constructs incorporating different lengths of the human SERCA2 promoter (19), and the same constructs were used for this series of experiments. Approximately 2.0×10^4 NIH-3T3 mouse fibroblast cells were seeded in 12-well plates 24 h before transfection. Next, 100 μ g of plasmid was transfected into cells using Metafectene Pro transfection reagent (Biotex, Munich, Germany) according to the instructions of the manufacturer. Total luminescence was measured using a luciferase assay system kit (Promega, Madison, WI) using a SpectraMax M5 plate reader (Molecular Devices). Luminosity results were normalized to total protein content as measured with the same BCA assay technique described for immunoblotting.

A luciferase construct with a deleted proximal Pdx-1 binding region was created by mutagenesis using the Stratagene QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the instructions of the manufacturer, and the site-specific deletion was confirmed by automated DNA sequencing (Genewiz, South Plainfield, NJ).

ChIP—Approximately 3.25×10^6 INS-1 cells were fixed in 1% formaldehyde for 10 min, sonicated to shear DNA fragments into the size range of 800–2000 bp and then subjected to ChIP as detailed previously (19, 48). Cross-linked protein and promoters were incubated for \sim 35 h under nutation at 4 °C using anti-Pdx-1 antibody (Santa Cruz Biotechnology) with normal rabbit immunoglobulin G (Santa Cruz Biotechnology) as a control. Samples were quantitated in triplicate by SYBR Green I-based quantitative real-time PCR as described previously (44) using forward and reverse primer sequences for the rat SERCA2 promoter with the sequences 5'-CGCTTTTGGC-TGTGTGGGAAG-3' (forward) and 5'-TGGTGTCTTGGC-TTGCCTC-3' (reverse) and the rat insulin 1 (INS1) promoter with the sequences 5'-TCAGCCAAAGATGAAGAAGGTCTC-3' (forward) and 5'-GCATTTTCCACATCATTC-CCC-3' (reverse).

Statistical Analysis—Differences between groups were analyzed for significance using unpaired Student's *t* test, one-way analysis of variance with multiple comparisons and Tukey-Kramer post test, or multiple *t* tests with Sidak-Bonferroni correction (49), as calculated by GraphPad Prism 6.01 statistics software. *p* < 0.05 was considered to indicate a significant difference between groups.

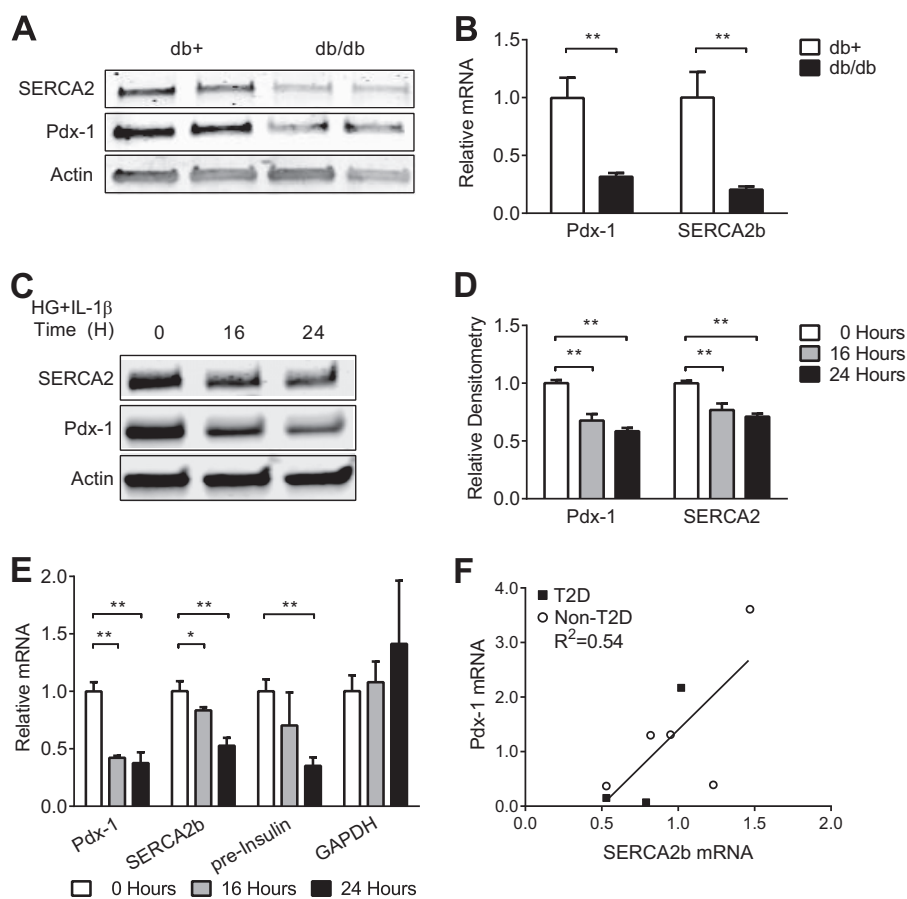


FIGURE 1. *Pdx-1* and *SERCA2b* levels are decreased in parallel in the β cell under diabetic stress conditions. *A* and *B*, protein and RNA were isolated from 12-week-old C57BLKs/J-*db/db* (*db/db*) and littermate heterozygous control (*db/+*) mouse islets. Immunoblot analysis was performed using antibodies against *SERCA2*, *Pdx-1*, and actin, and reverse-transcribed RNA was subjected to real-time quantitative RT-PCR to measure *SERCA2b* and *Pdx-1* transcript levels. *C–E*, INS-1 832/13 rat insulinoma cells were treated with 25 mM glucose and 5 ng/ml IL-1 β (HG + IL-1 β) for 16 and 24 h. *C*, immunoblot analysis was performed using antibodies against *SERCA2*, *Pdx-1*, and actin. *D*, quantitative protein levels are shown graphically. *E*, reverse-transcribed RNA was subjected to real-time PCR for quantification of *SERCA2b*, *Pdx-1*, preinsulin, and GAPDH transcript levels. *F*, *Pdx-1* and *SERCA2b* mRNA levels in cadaveric human islets were graphed using a best fit line. The indicated comparisons are significantly different. *, $p < 0.05$; **, $p < 0.01$ ($n =$ at least 6, except in *A*, where $n = 2$ samples of 100 islets from three biological replicates). Results are displayed as mean \pm S.E.

RESULTS

Pdx-1 and *SERCA2b* Levels Are Decreased in Parallel in the β Cell under Diabetic Stress Conditions—Our previous work has shown significantly decreased expression of *SERCA2b* mRNA and protein in islets isolated from C57BLKs/J-*db/db* (*db/db*) mice that worsened with advancing age and increasing hyperglycemia (19, 37). To define the relationship between both *Pdx-1* and *SERCA2b* expression in this model, islets were isolated from 12-week-old *db/db* mice and heterozygote littermate controls, and *Pdx-1* and *SERCA2b* protein and mRNA were found to be decreased in parallel (Fig. 1, *A* and *B*). Previous work has also demonstrated altered *SERCA2b* expression in INS-1 832/13 rat insulinoma cells treated with 25 mM glucose (high glucose, or HG) combined with 5 ng/ml of IL-1 β , a model that is intended to mimic the proinflammatory milieu of diabetes (19).

Using this model, time course experiments were performed. At both 16 and 24 h post-HG + IL-1 β treatment, *Pdx-1* and *SERCA2b* protein levels were decreased coordinately (Fig. 1, *C* and *D*), whereas reductions in *Pdx-1* mRNA levels preceded the loss of *SERCA2b* mRNA (Fig. 1*E*). Because the insulin gene is a known transcriptional target of *Pdx-1* (50), measurement of preinsulin mRNA was measured as a positive control to confirm the tran-

scriptional effects of *Pdx-1* loss. As anticipated, decreased preinsulin mRNA expression was observed with HG + IL-1 β treatment. In contrast, GAPDH has not been described to be a *Pdx-1* target (51). Therefore, GAPDH mRNA was measured as a negative control to rule out nonspecific changes in gene expression under inflammatory conditions. No change in GAPDH expression was observed with HG + IL-1 β treatment (Fig. 1*E*).

Levels of *SERCA2b* and *Pdx-1* mRNA were next quantitated in cadaveric human islets from a cohort that included donors with no previous diagnosis of T2D ($n = 5$) and three donors with established T2D. Levels of *SERCA2b* and *Pdx-1* mRNA were graphed as a line, with the x axis corresponding to *SERCA2b* and the y axis corresponding to *Pdx-1* levels, respectively. A significant linear relationship ($p = 0.038$) was observed with a slope of 2.760 ± 1.044 and a coefficient of determination (R^2) value of 0.5380, suggesting a positive correlation between *Pdx-1* and *SERCA2b* levels of expression in cadaveric human islets from diabetic and non-diabetic donors (Fig. 1*F*).

SERCA2b Is Decreased in Islets Isolated from *Pdx-1* Haploinsufficient Mice—Homozygous deletion of *Pdx-1* leads to pancreatic agenesis and perinatal death (25). As such, *Pdx-1* haploinsufficient mice were used to study the *in vivo* relationship

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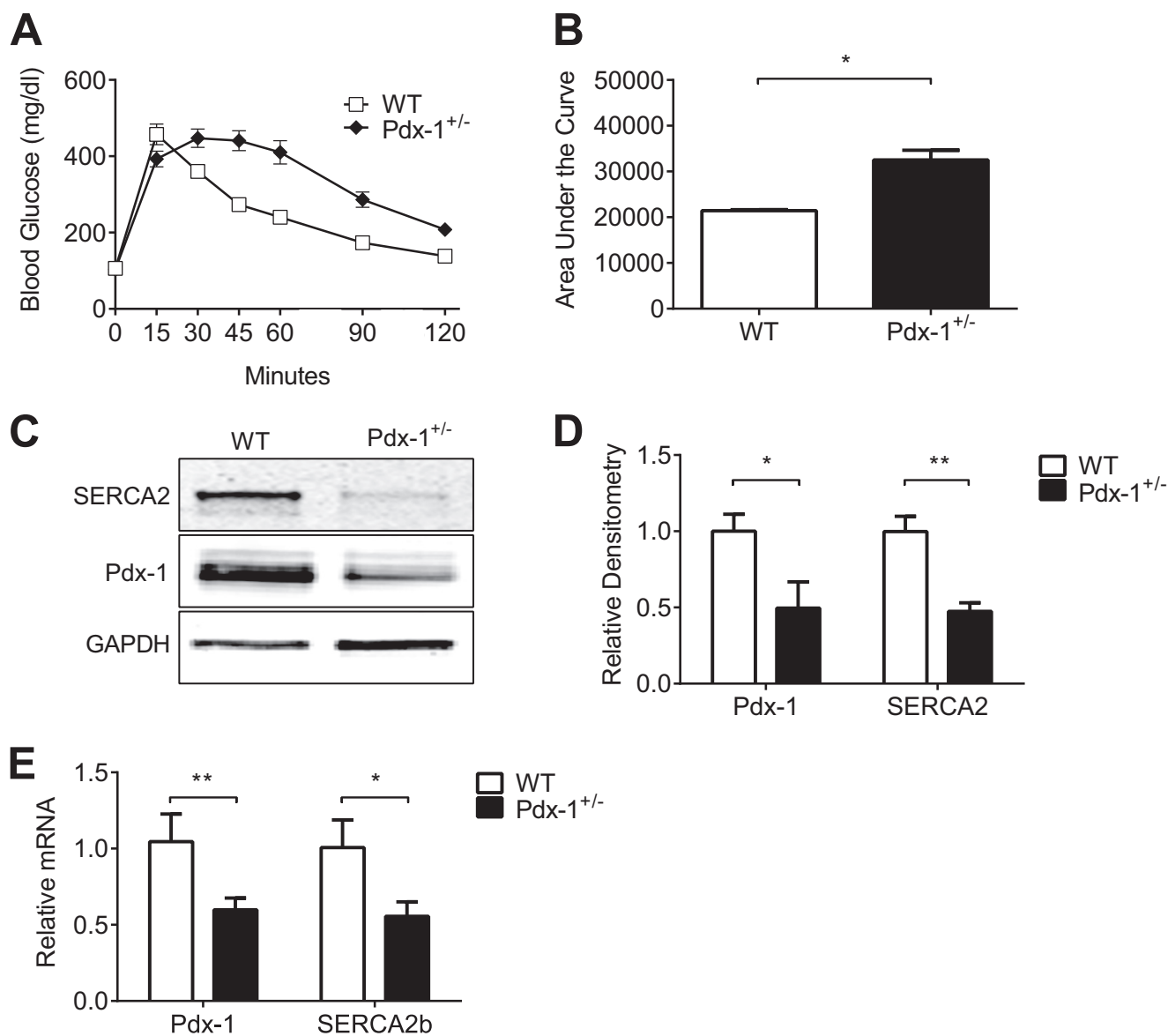


FIGURE 2. SERCA2b is decreased in islets isolated from Pdx-1 haploinsufficient mice. Pdx-1 haploinsufficient mice ($Pdx-1^{+/-}$) and wild-type littermates (WT) were fed a normal chow diet containing 17% of kilocalories from fat. *A* and *B*, intraperitoneal glucose tolerance tests (IPGTTs) were performed at 11 weeks of age in $Pdx-1^{+/-}$ and WT mice, and the area under the curve analysis is shown graphically. *C–E*, protein and RNA were isolated from 13-week-old Pdx-1 haploinsufficient and WT littermate control mouse islets. *C*, immunoblot analysis was performed using antibodies against SERCA2, Pdx-1, and GAPDH. *D*, quantitative protein levels are shown graphically. *E*, reverse-transcribed islet RNA was subjected to real-time quantitative RT-PCR for quantification of SERCA2b and Pdx-1. The indicated comparisons are significantly different. *, $p < 0.05$; **, $p < 0.01$ ($n = 3–10$ for the intraperitoneal glucose tolerance test and $n =$ at least 4 for immunoblot and quantitative RT-PCR). Results are displayed as mean \pm S.E.

between Pdx-1 and SERCA2b. Pdx-1 haploinsufficient mice and wild-type littermates were fed a normal chow diet containing 17% of calories from fat. At 11 weeks of age, intraperitoneal glucose tolerance tests were performed. As observed previously by other groups, Pdx-1 haploinsufficient mice were glucose-intolerant compared with wild-type littermates (Fig. 2, *A* and *B*) (28, 52–54). Similar to results obtained in db/db islets and HG + IL-1 β -treated INS-1 cells, SERCA2b protein and mRNA were decreased significantly in islets isolated from Pdx-1 haploinsufficient mice compared with controls (Fig. 2, *C–E*).

Pdx-1 Overexpression Increases SERCA2 Expression, whereas Pdx-1 Knockdown Decreases SERCA2b Levels in Vitro—NIH-3T3 mouse fibroblast cells express SERCA2b but do not natively express Pdx-1. To test the relationship between Pdx-1

and SERCA2b expression directly, NIH-3T3 fibroblasts were transduced with Pdx-1 adenovirus, and the change in SERCA2 expression was assessed. Pdx-1 overexpression in NIH-3T3 cells resulted in a nearly 2-fold increase in SERCA2 protein levels (Fig. 3, *A* and *B*). Next, adenoviral siRNA-mediated knockdown of Pdx-1 was performed in INS-1 832/13 cells that express Pdx-1 at high levels under normal conditions (55, 56). In cells treated with siRNA, Pdx-1 levels were reduced by ~90%, resulting in a 50% decrease in SERCA2b protein and mRNA expression (Fig. 3, *C–E*). Taken together, these results demonstrate that islet Pdx-1 and SERCA2b levels are altered in parallel in rodent models of diabetes, whereas direct modulation of Pdx-1 levels using overexpression and knockdown strategies results in reciprocal changes in SERCA2b expression.

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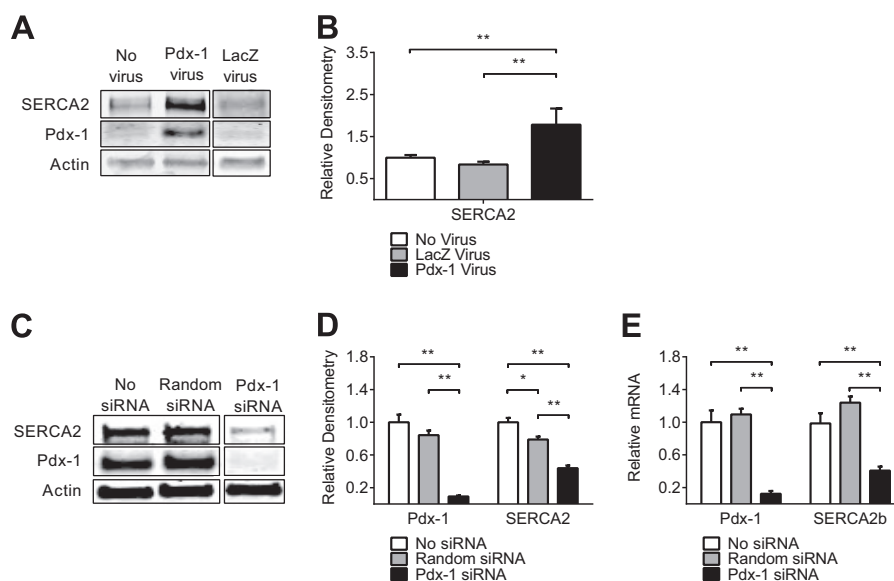


FIGURE 3. Pdx-1 overexpression increased SERCA2 expression, whereas Pdx-1 knockdown decreased SERCA2b levels *in vitro*. *A* and *B*, Pdx-1 was overexpressed via adenoviral transduction in NIH-3T3 mouse fibroblast cells that lack native expression of Pdx-1, and immunoblot analysis was performed using antibodies against SERCA2, Pdx-1, and actin. *C–E*, INS-1 cells were transduced with an adenovirus that expressed siRNA against Pdx-1. *C*, immunoblot analysis was performed using antibodies against SERCA2, Pdx-1, and actin. *D*, quantitative protein levels are shown graphically. *E*, RNA was subjected to real-time quantitative RT-PCR for quantification of SERCA2b and Pdx-1 transcript levels. The indicated comparisons are significantly different. *, $p < 0.05$; **, $p < 0.01$ ($n =$ at least 4). Results are displayed as mean \pm S.E.

Pdx-1 Knockdown Alters β Cell Calcium Homeostasis—The anticipated effect of diminished SERCA2b expression or activity is a reduction in ER Ca^{2+} . To directly assess the impact of Pdx-1 loss on ER Ca^{2+} levels, INS-1 cells were transduced with an ER-targeted D4ER adenovirus in combination with an siPdx-1 or random siRNA adenovirus. FLIM experiments were then performed as outlined under “Experimental Procedures.” Using this strategy, an increase in the lifetime of the enhanced cyan fluorescent protein (ECFP) donor indicates less FRET efficiency and, therefore, lower ER Ca^{2+} .

Representative micrographs of each treatment group are shown in Fig. 4A. ECFP donor lifetime increased significantly from 1.72 ± 0.02 ns under control conditions to 1.83 ± 0.02 ns with Pdx-1 knockdown, consistent with reduced ER Ca^{2+} in the setting of Pdx-1 deficiency (Fig. 4, *A* and *B*). In separate experiments, random siRNA-treated INS-1 cells were treated with the SERCA inhibitor thapsigargin. As expected, within 2 min, thapsigargin treatment resulted in a significantly increased donor life time, and changes were comparable to those observed with Pdx-1 knockdown (Fig. 4C).

Next, INS-1 cells that had been transduced with siPdx-1 adenovirus or control siRNA were incubated with Fura-2/AM Ca^{2+} dye to measure cytosolic Ca^{2+} levels. Fura-2/AM imaging revealed that Pdx-1 knockdown significantly increased basal Ca^{2+} levels within the cytosolic compartment (Fig. 5A). To assess changes in Ca^{2+} transit following ER Ca^{2+} depletion, cells were next treated with either caffeine or thapsigargin. Changes in cytosolic Ca^{2+} were analyzed by calculating the change in the Fura-2/AM ratio between the peak and basal measurements (ΔF) and dividing by the average of the basal ratio measured over 30 s (F_0) according to the formula $\Delta F/F_0$, as indicated in Fig. 5B. Indeed, rapid Ca^{2+} release following caffeine and thapsigargin treatment was detected (Fig. 5, *C* and *E*). Notably, the $\Delta F/F_0$ was significantly lower in Pdx-1

siRNA-transduced cells (Fig. 5, *D* and *F*). Together, FLIM and Fura-2/AM imaging experiments suggest that loss of Pdx-1 alters β cell Ca^{2+} compartmentalization, leading to decreased ER Ca^{2+} , increased basal cytosolic Ca^{2+} levels, and decreased Ca^{2+} transit following ER store depletion.

Pdx-1 Enhances SERCA2 Promoter Activity—Pdx-1 is known to bind to TA-rich sequences, including TAAT, ATTA, and TAAAT, in the promoters of target genes (41, 57). *In silico* analysis demonstrated five putative Pdx-1 binding regions in the SERCA2 promoter (Fig. 6A). To determine whether Pdx-1 is a transcriptional regulator of the SERCA2 gene, reporter assays were undertaken using different lengths of the human promoter fused to the luciferase coding region. NIH-3T3 cells were cotransfected with SERCA2 promoter constructs and a human Pdx-1 plasmid. Luciferase activity was measured 24 h after transfection and normalized to total protein content. Cotransfection of Pdx-1 increased luciferase expression 3- to 4-fold over the empty vector control in all constructs tested (Fig. 6B), suggesting that the binding region closest to the transcriptional start site might serve as a key regulatory region for Pdx-1-mediated transcriptional regulation of the SERCA2 gene. This region of the SERCA2 promoter maintains close homology between several species of mammals, including human, mouse, and rat (Fig. 6C). To confirm these findings, luciferase assays were performed following site-directed mutagenesis of this most proximal binding region. Eight base pairs were deleted, as depicted in the schematic in Fig. 6C. No increase in luciferase activity was observed using this mutant construct, suggesting that deletion of this element in the proximal promoter was sufficient to block Pdx-1 mediated transcriptional activation of the SERCA2 promoter (Fig. 6D).

Pdx-1 Directly Binds the Proximal SERCA2 Promoter—For *in vivo* confirmation, ChIP experiments were performed using whole cell extract isolated from INS-1 cells. Results showed a

Pdx-1 Enhances Transcription of SERCA2b in the Islet β Cell

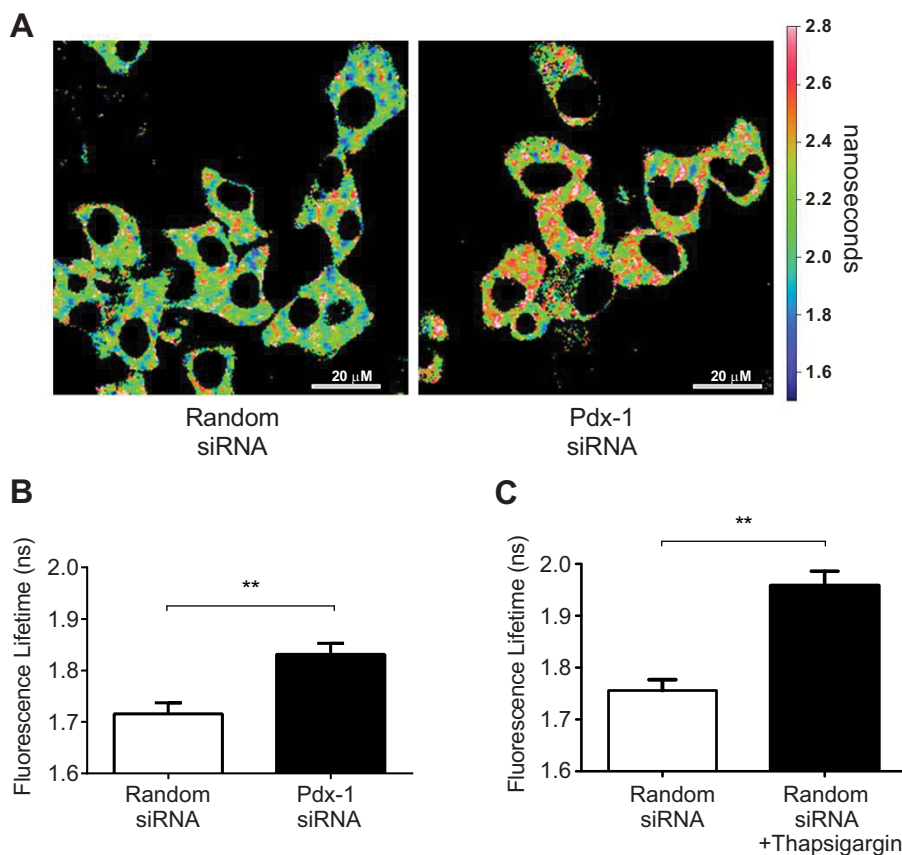


FIGURE 4. **Pdx-1 knockdown reduced β cell ER calcium levels.** INS-1 cells were transduced with a D4ER Ca^{2+} reporter adenovirus in combination with adenovirus-expressing siPdx-1 or random siRNA. FLIM was used to measure endoplasmic reticulum Ca^{2+} . *A*, representative micrograph of the average donor lifetime in random siRNA and Pdx-1 siRNA-treated cells. *B*, amplitude-weighted donor fluorescence lifetime in random siRNA and siPdx-1-treated INS-1 cells was quantified as described under "Experimental Procedures." *C*, amplitude-weighted donor fluorescence lifetime in random siRNA-treated INS-1 cells was quantitated at baseline and after 2 min of treatment with thapsigargin ($n =$ at least 26 regions of interest quantitated per treatment in three independent experiments for *B* and *C*). Results are displayed as mean \pm S.E. The indicated comparisons are significantly different. **, $p < 0.01$).

2-fold increase in recovery of the proximal *SERCA2* promoter following immunoprecipitation with anti-Pdx-1 antibody compared with immunoprecipitation with rabbit IgG (Fig. 7A). Because the *INS1* gene is known to be a transcriptional target of Pdx-1 (50), recovery of the *INS1* promoter was quantitated to confirm successful pulldown, and a 3-fold increase in *INS1* promoter recovery was observed. In aggregate, results from luciferase and ChIP experiments identify *SERCA2* as a novel transcriptional target of Pdx-1 in the β cell.

Reconstitution of SERCA2 Expression Ameliorates ER Stress in Islets Isolated from Pdx-1 Haploinsufficient Mice Fed a High-fat Diet—Results from Fig. 2 demonstrated decreased *SERCA2b* levels in islets isolated from Pdx-1^{+/-} mice fed a normal diet (Fig. 2, B–D). A previous publication by Sachdeva *et al.* (28) revealed the presence of ER stress in islets isolated from Pdx-1 haploinsufficient mice challenged with a high-fat diet.

SERCA2b and Pdx-1 mRNA levels were measured in islets isolated from wild-type mice fed a normal chow or diet containing 45% of calories from fat (HFD) for 8 weeks. No significant difference in *SERCA2b* or Pdx-1 gene expression was observed between wild-type mice fed either diet (Fig. 8A). Next, Pdx-1 haploinsufficient mice and wild-type mice were fed an HFD for 8 weeks. *SERCA2b* levels were again found to be significantly lower in Pdx-1^{+/-} mice (Fig. 8, B–D). Interestingly, compared with Pdx-1^{+/-} mice fed normal chow (Fig. 2, C and D), a further

reduction in *SERCA2b* and Pdx-1 mRNA levels was observed (Figs. 8, B–D, and 2, C and D). Consistent with the activation of ER stress signaling, the spliced-to-total X-box binding protein 1 (Xbp1) ratio was increased in islets from Pdx-1^{+/-} mice fed an HFD compared with WT littermate controls (Fig. 8E).

To determine whether reconstitution of islet *SERCA2b* in this model was sufficient to rescue components of ER stress or Ca^{2+} signaling, isolated islets from both HFD-fed Pdx-1^{+/-} mice and HFD-fed WT controls were transduced with *SERCA2b* adenovirus or a LacZ control adenovirus. Successful transduction was verified by immunoblot analysis (Fig. 8F). Notably, *SERCA2b* reconstitution in HFD-Pdx-1^{+/-} islets significantly decreased the spliced Xbp1 ratio (Fig. 8G).

Finally, to assess overall glucose-stimulated Ca^{2+} responses, Fura-2/AM imaging was performed in HFD-Pdx-1^{+/-} islets that had been transduced with either *SERCA2b* or a LacZ control adenovirus. Interestingly, in response to 20 mM glucose, the $\Delta F/F_0$ ratio was increased significantly in HFD-Pdx-1^{+/-} islets transduced with *SERCA2b*, compared with LacZ control HFD-Pdx-1^{+/-} islets (Fig. 9, A and B).

DISCUSSION

A single β cell synthesizes approximately 1 million insulin molecules/min, an arduous task that requires an extremely well developed and highly functional ER (58). ER stress in the pan-

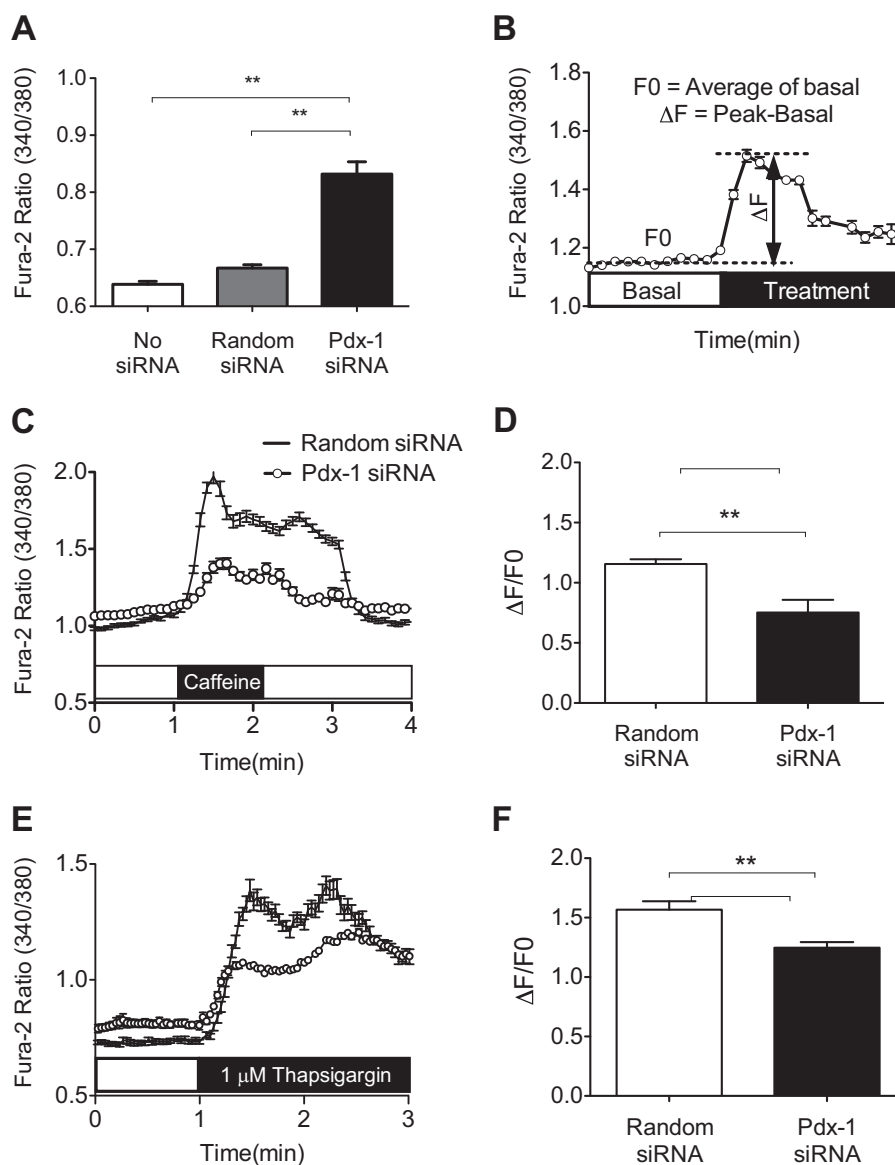


FIGURE 5. **Pdx-1 knockdown altered β cell calcium homeostasis.** *A*, to assess basal cytosolic Ca^{2+} levels, the Fura-2/AM fluorescence ratio was measured as described under "Experimental Procedures" in untreated INS-1 cells and INS-1 cells transduced with adenovirus-expressing siPdx-1 or random siRNA. *B–F*, siPdx-1 or random siRNA-transduced INS-1 cells were treated with 10 mM caffeine or 1 μ M thapsigargin to determine the effect of Pdx-1 knockdown on ER Ca^{2+} storage and cytosolic Ca^{2+} transit following ER Ca^{2+} depletion. *B*, data are described as a normalized ratio according to the formula $\Delta F/F_0$. *C* and *E*, representative Fura-2/AM Ca^{2+} recording with caffeine (*C*) and thapsigargin (*E*) treatment in siPdx-1- or random siRNA-transduced INS-1 cells. The calculated $\Delta F/F_0$ for each group is shown graphically in *D* and *F*. The Fura-2/AM ratio was collected from at least 120 regions of interest over three independent experiments. Results are displayed as mean \pm S.E., and the indicated comparisons are significantly different. **, $p < 0.01$.

cretic β cell is well appreciated in the context of obesity and type 2 diabetes (59, 60), whereas more recent and emerging data suggest an expanding role for ER stress in the development and progression of T1D (5, 8, 9). A key determinant of ER homeostasis is the maintenance of a robust intraluminal Ca^{2+} pool (13, 14), and this pool serves as an important store for Ca^{2+} release, leading to activation of a variety of signaling pathways, including incretin-induced insulin secretion (61, 62). Moreover, ER Ca^{2+} plays a central role in protein processing and maturation through the regulation of protein chaperone activity and the formation of chaperone complexes (13), whereas depletion of ER Ca^{2+} inhibits protein synthesis and facilitates protein degradation (63, 64). Studies also illustrate a strict requirement for ER-derived Ca^{2+} in proinsulin processing in

secretory granules, where Ca^{2+} is needed for activity of the endopeptidases prohormone convertases 1 and 2 (65, 66).

The SERCA family of ion pumps serves as a primary gatekeeper of this ER calcium gradient. At least three SERCA isoforms are known to be expressed in the pancreatic β cell: SERCA2a, SERCA2b, and SERCA3, and expression of SERCA2 and SERCA3 isoforms has been shown to be regulated independently in the β cell (67). Although we previously observed decreased expression of all three isoforms in islets isolated from diabetic rodents (37), this series of experiments was focused on SERCA2b because it is the most highly expressed isoform in mouse islets (19). Furthermore, SERCA2b is structurally unique because it possesses an extra transmembrane domain providing this isoform with the highest relative calcium affinity

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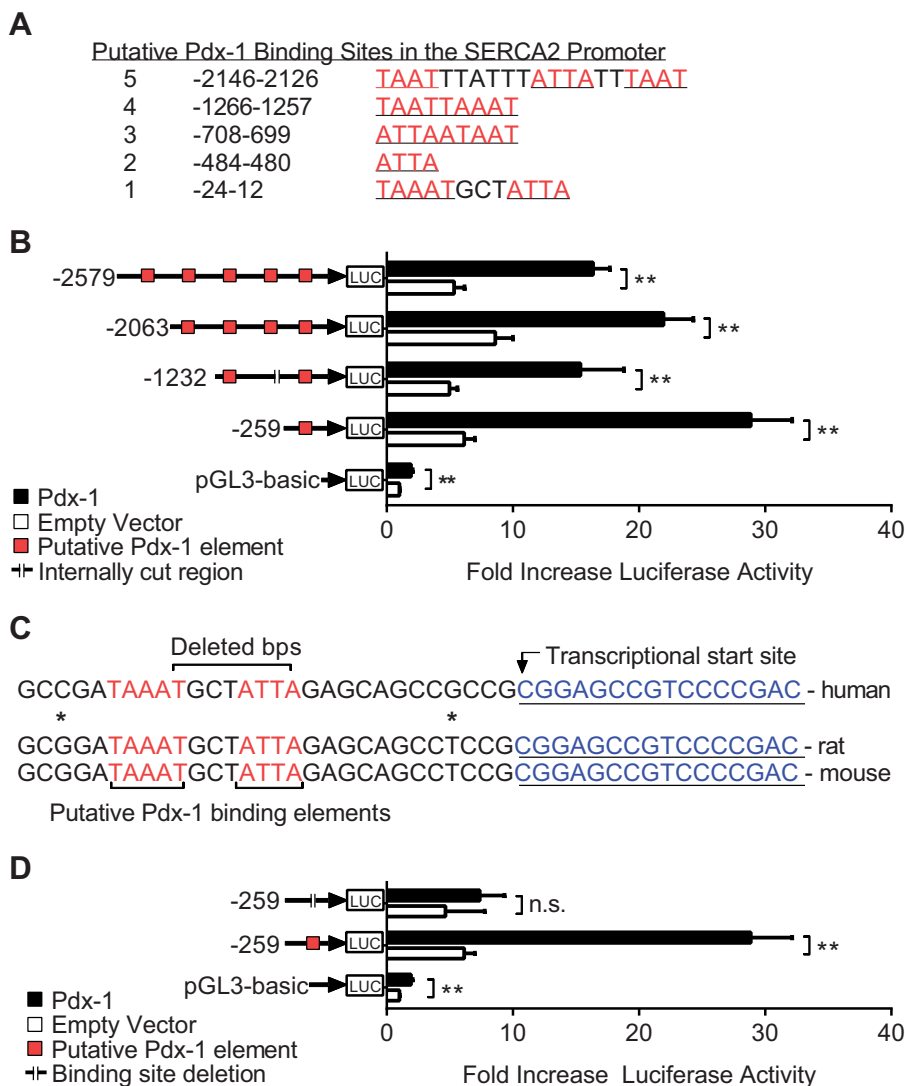


FIGURE 6. Pdx-1 enhances SERCA2 promoter activity. *A*, five putative Pdx-1 binding regions were identified in the human SERCA2 promoter. *B*, NIH-3T3 mouse fibroblast cells were cotransfected with deletion constructs of the human SERCA2 promoter placed upstream of a luciferase (*Luc*) coding region and also transfected with human Pdx-1 or empty vector control plasmids. Reporter assays were performed as outlined under "Experimental Procedures." *C*, depiction of the mutation strategy undertaken to delete the most proximal Pdx-1 binding element. Homology between human, rat, and mouse promoters is indicated. *D*, NIH-3T3 cells were cotransfected with wild-type or mutated constructs combined with the Pdx-1 plasmid or the empty vector control, and reporter assays were performed ($n =$ at least 5 independent experiments with samples analyzed in triplicate). Results are displayed as mean \pm S.E. The indicated comparisons are significantly different. **, $p < 0.01$; *n.s.*, not significant.

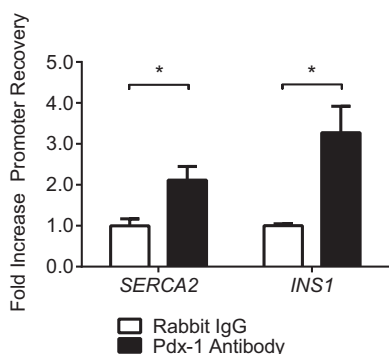


FIGURE 7. Pdx-1 directly binds the proximal SERCA2 promoter. INS-1 cells were harvested and subjected to ChIP analysis. Quantitative PCR was used to measure recovery of the SERCA2 or INS1 promoters following immunoprecipitation with anti-Pdx-1 antibody or normal rabbit IgG. Results are expressed as fold increase in percent recovery of the target gene compared with rabbit IgG and represent the mean \pm S.E. for three independent experiments. The indicated comparisons are significantly different. *, $p < 0.05$.

(68). Our previous work has shown that altered SERCA2b expression leads to altered insulin secretion, activation of ER stress signaling pathways, and decreased β cell survival (19). The goal of this work was to identify additional transcriptional pathways that underlie dysregulated SERCA2b expression under inflammatory and diabetic conditions.

The homeobox protein Pdx-1 plays an essential role in pancreatic and β cell development and the maintenance of postnatal β cell function. Importantly, Pdx-1 is a transcriptional regulator of the insulin gene as well as other key genes involved in stimulus-secretion coupling (25, 54, 69). Pdx-1 is also recognized to play a role in β cell adaptation to metabolic stress, and Pdx-1 haploinsufficiency superimposed on a background of severe insulin resistance leads to impaired compensatory β cell mass expansion, diabetes, and premature mortality (54). Sachdeva *et al.* (28) recently examined the effects of diet-induced obesity in Pdx-1^{+/-} mice and similarly found that Pdx-1 defi-

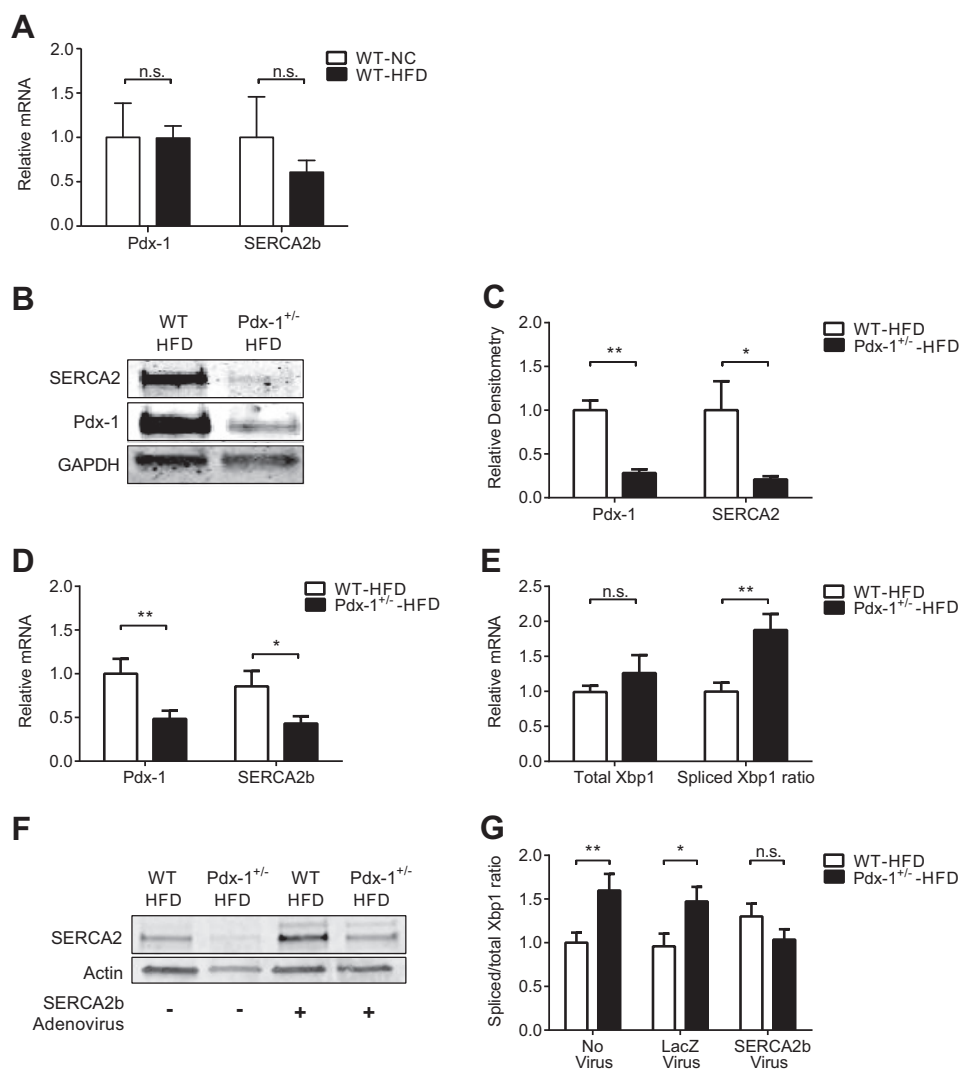


FIGURE 8. Reconstitution of *SERCA2* expression ameliorates ER stress in islets isolated from *Pdx-1* haploinsufficient mice fed a high-fat diet. *Pdx-1* haploinsufficient (*Pdx-1*^{+/-}) mice or wild-type littermates were fed normal chow (NC) or an HFD containing 45% kilocalories from fat for 8 weeks prior to islet isolation. *A*, real-time RT-PCR was used to quantitate *Pdx-1* and *SERCA2b* transcript levels in islets isolated from WT littermate control mice fed NC or an HFD. *B–E*, protein and RNA were isolated from 13-week-old *Pdx-1*^{+/-} and WT littermate control islets. *B*, immunoblot analysis was performed using antibodies against *SERCA2*, *Pdx-1*, and *GAPDH*. *C*, quantitative protein levels are shown graphically. *D–E*, reverse-transcribed RNA was subjected to real-time PCR for quantification of *Pdx-1*, *SERCA2b*, and total and spliced Xbp1 transcript levels. The ratio of spliced-to-total Xbp1 is indicated in *D*. *E*, immunoblot analysis demonstrating successful adenoviral overexpression of *SERCA2b* in islets isolated from *Pdx-1*^{+/-} mice and WT littermate controls fed an HFD. *G*, quantification of the ratio of spliced to unspliced Xbp1 mRNA in isolated islets from *Pdx-1*^{+/-} and WT mice fed an HFD and treated *ex vivo* with no virus or transduced with *SERCA2b* adenovirus or LacZ-expressing control virus ($n = 12–18$). The results are displayed as mean \pm S.E. The indicated comparisons are significantly different. *, $p < 0.05$; **, $p < 0.01$; n.s., not significant.

ciency significantly limits β cell mass expansion under HFD conditions. Interestingly, these effects were not secondary to decreased proliferation but, rather, due to impaired β cell survival. Similarly, increased β cell apoptosis in *Pdx-1*^{+/-} islets has also been observed by Johnson *et al.* (53). Interestingly, islets from HFD-fed *Pdx-1*^{+/-} mice demonstrated activation of specific components of ER stress signaling, whereas similar results were seen in *Pdx-1*-deficient MIN6 cells. Microarray studies performed in MIN6 cells transduced with an shRNA against *Pdx-1* further revealed alterations in a large subset of genes with well defined roles in the maintenance of ER function and UPR signaling, including *Atf4*, *Wfs1*, ER oxidoreductin 1b, heat shock 70-kDa protein 5/Bip/GRP78, and neuronatin. *Pdx-1* was confirmed to directly bind the promoters of *Atf4* and *Wfs1*,

further confirming its role in maintenance of a specific β cell ER subgenome (28).

In addition to key ER-related genes identified previously, our results suggest that altered *SERCA2b* expression with concomitant alterations in ER Ca^{2+} also contributes to ER stress observed in *Pdx-1*-deficient states. Here we show that *Pdx-1* and *SERCA2b* expression are altered in parallel in db/db islets and in an *in vitro* model of inflammatory diabetes. Moreover, we demonstrate a significant and positive correlation between *Pdx-1* and *SERCA2b* mRNA levels in human islets from subjects with and without T2D. To test further whether *Pdx-1* directly regulates *SERCA2b* expression, overexpression and knockdown strategies were employed in NIH-3T3 and INS-1 cells, respectively. The results showed that *Pdx-1* overexpres-

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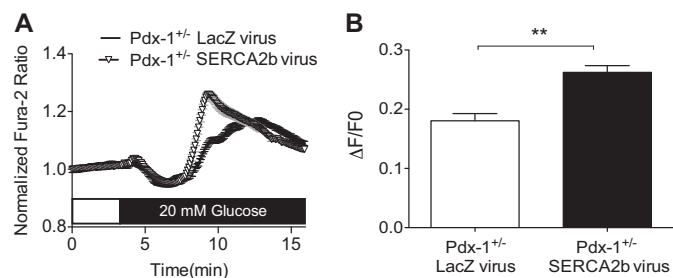


FIGURE 9. SERCA2b overexpression leads to an improved glucose-stimulated Ca^{2+} response in islets from Pdx-1 haploinsufficient mice fed a high-fat diet. Islets isolated from Pdx-1^{+/+} mice fed a high fat diet for 8 weeks were transduced *ex vivo* with either SERCA2b or LacZ-expressing adenovirus. **A**, representative Fura-2/AM Ca^{2+} imaging recording performed at 2.5 mM glucose and following stimulation with 20 mM glucose. **B**, data were analyzed according to the formula $\Delta\text{F}/\text{F}_0$, and results are shown graphically. The Fura-2/AM ratio was measured from at least two regions of interest per islet, and islets were isolated from at least three biological replicates. The results are displayed as the means \pm S.E. The indicated comparisons are significantly different. **, $p < 0.01$.

sion increases SERCA2b levels, whereas siRNA-mediated knockdown of Pdx-1 leads to a reciprocal reduction in SERCA2b expression. Luciferase assays and ChIP assays confirm that this relationship is direct and show that Pdx-1 binds a proximal region of the human SERCA2 promoter. Importantly, we recapitulated the stress paradigm employed by Sachdeva *et al.* (28) by treating Pdx-1^{+/+} mice with an HFD with the goal of testing whether reconstitution of SERCA2b in islets *ex vivo* is sufficient to limit ER stress. Indeed, using the spliced-to-total Xbp1 ratio as a readout, we show that restoration of SERCA2b expression is capable of mitigating against ER stress in this model. Furthermore, we also show that restoration of SERCA2b improved the overall glucose-stimulated Ca^{2+} response in Pdx-1^{+/+} islets from HFD-fed mice.

A potential limitation of our study is that, aside from changes in the spliced Xbp1 ratio following SERCA2b reconstitution in islets from HFD-fed Pdx-1^{+/+} mice, we did not observe significant changes in mRNA expression of other markers of ER stress (data not shown). Although future studies will be needed to fully investigate the specific signaling arms regulated by this pathway, a novel aspect of our study is that we link modulation of Pdx-1 expression with alterations in β cell Ca^{2+} homeostasis. Specifically, using a genetically encoded and ER-localizedameleon reporter (45), our results show that ER Ca^{2+} is decreased and cytosolic Ca^{2+} is increased in a Pdx-1-deficient state. Although our proposed model would suggest that these changes are the result of alterations in SERCA2b expression and activity, previous literature has shown that Pdx-1 also enhances transcription of the *Wfs1* gene. WFS1 is a transmembrane protein localized to the β cell ER, plasma membrane, and secretory granules (70, 71), and mutations in this gene lead to Wolfram syndrome, a disorder characterized by childhood onset of diabetes mellitus, hypoinsulinemia, diabetes insipidus, optic atrophy, and deafness in humans (72, 73). Furthermore, mouse models of diminished WFS1 also exhibit β cell apoptosis because of ER stress (74, 75). Although the precise function of WFS1 in the β cell has remained somewhat enigmatic, the function of this protein has been linked to the regulation of ER Ca^{2+} , raising the possibility that a component of our ER Ca^{2+} phenotype could be secondary to WFS1 deficiency (76). In addition,

we observed an increase in basal cytosolic Ca^{2+} levels with Pdx-1 knockdown, which could reflect a reciprocal increase in cytosolic Ca^{2+} resulting directly from a ER Ca^{2+} leak. Although not directly interrogated in our study, another possibility is that the activation of store-operated Ca^{2+} entry from the extracellular space also contributed to this finding (77).

Despite these caveats, our results identify SERCA2b as a new transcriptional target of Pdx-1 and identify an additional pathway through which SERCA2b expression is altered in the β cell under diabetic conditions. Moreover, we define a novel role for altered ER Ca^{2+} regulation in Pdx-1-deficient states.

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