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 Ca2+ ATPase 2b (SERCA2b) expression and activity contributes to β cell dysfunction in diabetes.

Results: SERCA2b deficiency occurs secondary to loss of pancreatic and duodenal homebox 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 homebox 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 Ca2+ ATPase 2b (SERCA2b) pump maintains a steep Ca2+ 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 Ca2+, which was measured using fluorescence lifetime imaging microscopy. Cotransfection of human Pdx-1 with a reporter fused to the human SERCA2b promoter increased luciferase activity 3- to 4-fold relative to an empty vector control, and direct binding of Pdx-1 to the proximal SERCA2b 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 Ca2+ 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 pat-
ways and \( \beta \) cell death, a transition referred to as ER stress (2–4). Data from rodent and human studies illustrate a pivotal role for \( \beta \) cell ER stress during the progression of both major forms of diabetes mellitus (5–9).

\( \beta \) 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 (\( \text{Ca}^{2+} \)) regulation (10). Under normal conditions, ER \( \text{Ca}^{2+} \) is estimated to be at least three orders of magnitude higher than cytosolic \( \text{Ca}^{2+} \), and \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) 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 \( \beta \) cell SERCA2b levels are diminished in human and rodent models of T1D and T2D (18–21), resulting in \( \text{Ca}^{2+} \) dysregulation, impaired insulin secretion, and ER stress (19). However, the specific transcription pathways that regulate SERCA2b expression in the \( \beta \) cell remain largely uncharacterized.

The pancreatic duodenal homeobox 1 (Pdx-1) transcription factor is known to play an indispensable role in \( \beta \) 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 \textit{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 \( \text{Ca}^{2+} \) regulation, and activation of \( \beta \) cell ER stress signaling pathways in diabetes.

**EXPERIMENTAL PROCEDURES**

\textbf{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 \textit{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, 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin (Invitrogen) and incubated overnight at 37 °C with 5% \( \text{CO}_2 \). 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/m². 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/m².

\textbf{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% \( \text{CO}_2 \) in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin (38). For \textit{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′-GAGATAGAGATAGAAA-3′, and the random siRNA sequence was 5′-GACCTCTATCCGTGATTA-3′. After 24–48 h, RNA or protein was isolated for the indicated analyses, or cells were used for \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) imaging or RNA or protein isolation. To simulate hyperglycemic and proinflammatory conditions \textit{in vitro}, INS-1 cells were treated with 25 mM glucose and 5 ng/ml IL-1β for 16–24 h using a protocol established previously (19).

\textbf{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% deoxycholate, 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 MgCl₂, 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-Protein
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× 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 Ca\(^{2+}\) was measured using the ratiometric calcium indicator Fura-2/AM (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% CO\(_2\) in 4 mM caffeine or 1 mM 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 ×10 or ×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 Ca\(^{2+}\) levels (45, 46). The probe was created by replacing the Ca\(^{2+}\) binding domain of the D1ER construct with D4 to provide lower Ca\(^{2+}\) 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 ×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: \(F_{\text{FRET}} = 1 - \left(\frac{\tau_\text{D}}{\tau_\text{T}}\right)\), where \(\tau_\text{T}\) and \(\tau_\text{D}\) 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 μg 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 ~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′-TGGTGTCCTTGGC-TGGCCTC-3′ (reverse) and the rat insulin 1 (INS1) promoter with the sequences 5′-TCAGCCAAAGATGAAGAAGGT-CTC-3′ (forward) and 5′-GCATTTCACATCATTC-CCC-3′ (reverse).

Statistical Analysis—Differences between groups were analyzed for significance using an 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.
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**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 heterozygous 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 and 5 ng/ml IL-1β (HG + IL-1β) 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, pre-insulin, 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 ± S.E.

The transcriptional effects of Pdx-1 loss. As anticipated, decreased preinsulin mRNA expression was observed with HG + IL-1β 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-1β treatment (Fig. 1E).

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 (R²) 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. 1F).

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...
between Pdx-1 and SERCA2b. Pdx-1 haploinsufficient mice and wild-type littersmates were fed a normal chow diet containing 17% of calories from fat. At 11 weeks of age, intraperitoneal glucose tolerance tests (IPGTTs) were performed in 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 ± S.E.

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**FIGURE 2.** SERCA2b is decreased in islets isolated from Pdx-1 haploinsufficient mice. Pdx-1 haploinsufficient mice (\( \text{Pdx-1}^{+/−} \)) and wild-type littersmates (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 \( \text{Pdx-1}^{+/−} \) 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 ± 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 Δ4ER 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 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 (F0) according to the formula ΔF/F0, as indicated in Fig. 5B. Indeed, rapid Ca\(^{2+}\) release following caffeine and thapsigargin treatment was detected (Fig. 5, C and E). Notably, the ΔF/F0 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
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 normal chow 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 significantly in HFD-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 Ca2+ 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 ΔF/F0 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-
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creatic 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.
Our previous work has shown that altered SERCA2b expression leads to altered insulin secretion, activation of ER stress signaling pathways, and decreased 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 cell development and the maintenance of postnatal 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 cell adaptation to metabolic stress, and Pdx-1 haploinsufficiency superimposed on a background of severe insulin resistance leads to impaired compensatory 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-(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-
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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 ± S.E. The indicated comparisons are significantly different. *, p < 0.05; **, p < 0.01; n.s., not significant.

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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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![Graph of SERCA2b expression](image)

**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 ΔF/Δt, 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 ± S.E. The indicated comparisons are significantly different. **,** p < 0.01.

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 an 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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