

# Methyltransferase Set7/9 Maintains Transcription and Euchromatin Structure at Islet-Enriched Genes

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**OBJECTIVE**—The activation of  $\beta$ -cell genes, particularly of those encoding preproinsulin, requires an appropriate euchromatin (or “open”) DNA template characterized by hypermethylation of Lys4 of histone H3. We hypothesized that this modification is maintained in islet  $\beta$ -cells by the action of the histone methyltransferase Set7/9.

**RESEARCH DESIGN AND METHODS**—To identify the role of Set7/9, we characterized its expression pattern and gene regulation and studied its function using RNA interference in both cell lines and primary mouse islets.

**RESULTS**—Within the pancreas, Set7/9 protein shows striking specificity for islet cells, including  $\alpha$ - and  $\beta$ -cells, as well as occasional cells within ducts. Consistent with these findings, the Set7/9 gene promoter contained an islet-specific enhancer located between  $-5,768$  and  $-6,030$  base pairs (relative to the transcriptional start site) that exhibited Pdx1-responsive activation in  $\beta$ -cells. To study Set7/9 function, we depleted insulinoma cells and primary mouse islets of Set7/9 protein using siRNA. Following siRNA treatment, we observed striking repression of genes involved in glucose-stimulated insulin secretion, including *Ins1/2*, *Glut2*, and *MafA*. These changes in transcription were accompanied by loss of dimethylated H3 Lys4 and RNA polymerase II recruitment, particularly at the *Ins1/2* and *Glut2* genes. Consistent with these data, depletion of Set7/9 in islets led to defects in glucose-stimulated  $\text{Ca}^{2+}$  mobilization and insulin secretion.

**CONCLUSIONS**—We conclude that Set7/9 is required for normal  $\beta$ -cell function, likely through the maintenance of euchromatin structure at genes necessary for glucose-stimulated insulin secretion. *Diabetes* 58:185–193, 2009

It is becoming increasingly clear that the pathogenesis of type 1 and type 2 diabetes involves progressive dysfunction at the level of the islet  $\beta$ -cell (1). Thus, the most effective therapeutic approaches to diabetes should include efforts to address  $\beta$ -cell dysfunction, and further research into the mechanisms underlying

$\beta$ -cell gene transcription will be paramount in these efforts. In recent years, a model has been emerging that emphasizes the role of  $\beta$ -cell-specific transcription factors such as Pdx1, Nkx6.1, NeuroD1, and Maf factors in the expression and regulation of genes crucial to the development and function of  $\beta$ -cells (recent reviews in 2–4). However, transduction of a variety of non- $\beta$ -cell types with these and other transcription factors has yielded limited success in activating  $\beta$ -cell genes and suggests that this model of  $\beta$ -cell gene activation requires further refinement. In this regard, chromatin structure is becoming recognized as a crucial factor in modulating eukaryotic gene expression. Covalent modifications of the histone proteins H2A, H2B, H3, and H4 that make up the fundamental unit of chromatin have been shown to directly or indirectly lead to the formation of euchromatin (“open,” or active, chromatin) or heterochromatin (“closed,” or inactive, chromatin) (5–7). These modifications include methylation, acetylation, phosphorylation, and ubiquitination of specific amino acid residues in the  $\text{NH}_2$ -terminal histone tails. The roles of chromatin and histone modifications in  $\beta$ -cell gene transcription have received attention only in recent years.

Our laboratory and others have been particularly interested in the role of methylation of Lys4 of H3 in the activation of  $\beta$ -cell genes. For example, recent studies have demonstrated that H3-Lys4 methylation at the control region of the genes encoding cell cycle inhibitors p27Kip1 and p18Inc4c is crucial in their activation and subsequent suppression of  $\beta$ -cell tumorigenesis (8). Maintenance of this methylation state appears to involve a complex containing the protein menin and members of the SET methyltransferase family, the mixed lineage leukemia proteins (9). Similarly, our laboratory has demonstrated that mono- and dimethylation of H3 Lys4 is enriched in the control region of the gene encoding preproinsulin (*Ins1/2*) and that the maintenance of dimethylated H3 Lys4 by a transcriptional protein complex involving Pdx1 is crucial to the ongoing activation of this gene in  $\beta$ -cell lines (10,11). Because Pdx1 contains no methyltransferase activity, we proposed that another member of the SET protein family, Set7/9, may be recruited to a complex with Pdx1 to mediate H3 methylation at the *Ins1/2* gene (10). Set7/9 is a 366 amino acid enzyme containing a COOH-terminal SET domain that harbors methyltransferase activity (12,13). This methyltransferase activity is specific not only for H3 Lys4 but also for particular Lys residues in proteins such as p53, TAF10, and the estrogen receptor (14–19). However, consistent with a direct role in gene transcription in the islet, our prior studies using chromatin-reconstituted reporters in vitro showed that the interaction of Pdx1 with Set7/9 leads to synergistic gene activation in a methyltransferase-dependent manner (11). These data have led us to hypothesize that Set7/9 may represent a novel chromatin-modifying protein that functions in part

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See accompanying commentary, p. 28.

through its recruitment to target genes by cell-specific transcription factors such as Pdx1. In this study, we show that Set7/9 is strongly enriched in islets relative to other pancreatic cell types and that it is important in the maintenance of transcription and chromatin structure at genes necessary for glucose-stimulated insulin secretion in primary islets. Taken together, our data identify Set7/9 as a novel enzymatic cofactor necessary for maintenance of islet gene transcription.

## RESEARCH DESIGN AND METHODS

**Antibodies and cells.** Polyclonal antibody against monomethyl-H3 Lys4 was from Abcam, and antibodies against dimethylated H3 Lys4, Pdx1, and Set7/9 were from Millipore; anti-RNA polymerase II COOH-terminal domain was from Covance; anti-actin, anti-glucagon, and anti-insulin were from Santa Cruz Biotechnology.  $\beta$ TC3 cells, INS-1 (832/13),  $\alpha$ TC1.6, and NIH3T3 were maintained as previously described (20,21). Islets were isolated from C57BL/6J mice following collagenase digestion of pancreatic tissue as previously described (22). Techniques and protocols were approved by the Indiana University Institutional Animal Care and Use Committee.

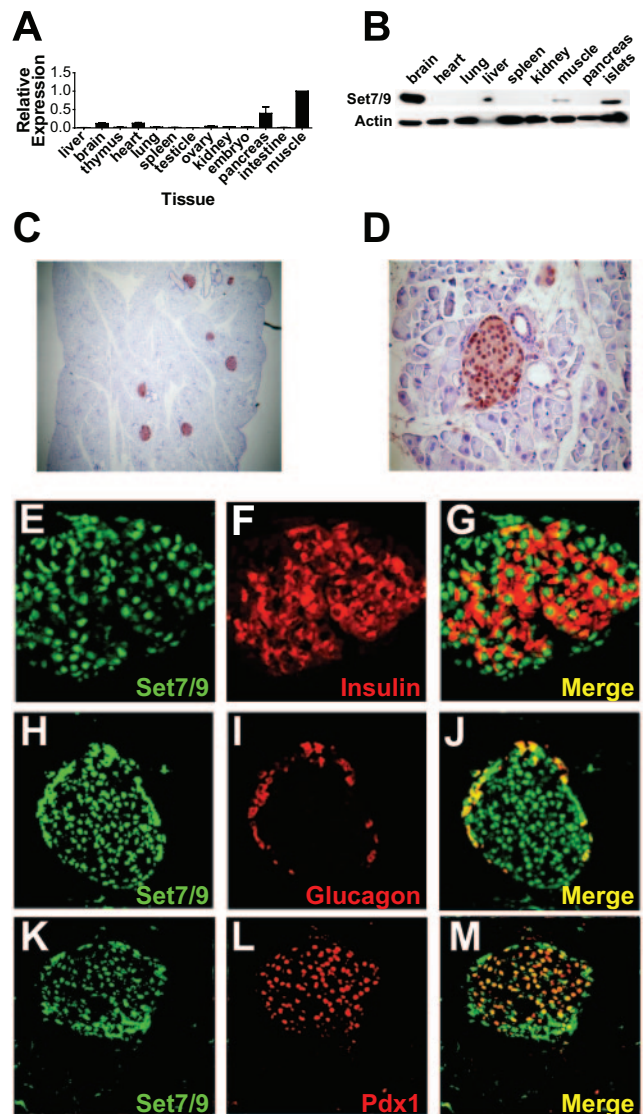
**Real-time RT-PCR.** Real time RT-PCR was performed using forward and reverse primers to amplify insulin premRNA (5'-GGGGAGCGTGGCTTCTCTCTA-3' and 5' GGGGACAGAATTCAGTGGCA-3') or *Actb* (5'-AGGTCATCACATTTGGCAACGA-3' and 5'-CACTTCATGATGGAATTGAATGTAGTT-3') as previously described in detail (23). Primers for *Setd7*, *Sle2a2*, *Gck*, *Nkx6-1*, *NeuroD1*, *Foxo1*, *Pax6*, *Iapp*, *Mafa*, *Ipf1*, *Kcnj11*, *Abcc8*, and *Foxa2* were obtained from Qiagen (Quantitech primer kit catalog nos. QT00139727, QT00103537, QT00140007, QT00143318, QT00251265, QT00116186, QT01052786, QT00101052, QT01037638, QT00102235, QT00305319, QT01042300, and QT00242809, respectively). PCR products were subcloned and sequenced to verify amplification of the correct gene product. All values are normalized to *Actb* mRNA levels.

**Immunoblot Assays.** Whole-cell extracts were prepared from homogenized tissues, 50 islets, or  $1 \times 10^6$   $\beta$ TC3 cells by lysis in a buffer containing sodium dodecyl sulfate, and 5  $\mu$ g extract was resolved by electrophoresis on a 12% SDS-polyacrylamide gel followed by immunoblot with anti-Set7/9 or anti-actin antisera. Immunoblots were visualized using the ECL-Plus system (Amersham).

**Chromatin immunoprecipitation assays.** Chromatin immunoprecipitation (ChIP) assays were performed as previously described (20). Briefly,  $10^7$   $\beta$ TC3 cells or 300 mouse islets were fixed in 1% formaldehyde to cross-link proteins to DNA, and cells were lysed and chromatin sheared to 400–600 base pairs (bp) by sonication. Antibodies were used to subsequently immunoprecipitate the protein of interest and then recovery of coimmunoprecipitated DNA fragments was assessed using SYBR green–based quantitative real-time PCR. Data are expressed as the percent recovery of coimmunoprecipitated DNA relative to input DNA (before immunoprecipitation). Primers used for amplification were as follows: for the *Ins1/2* promoter, 5'-TACCTGTGCTGCTGAGTTCTGC-3' forward and 5'-GCATTTTCCACATCATCCCC-3' reverse; the *Ins1/2* coding region, 5'-TGGCTTCTTACACACCAAG-3' forward and 5'-ACAATGCCACGCTTCTGCC-3' reverse; the *Sle2a2* promoter, 5'-ATCTGGCTCCGCACTCTCATCTTG-3' forward and 5'-CCCTGTGACTTTTCTGTGCTTAGG-3' reverse; the *Pdx1* promoter, 5'-AGCCTGGACTACATCTTGAGTTGC-3' forward and 5'-TTACAAAACCGCTCCAAATGAG-3' reverse; and the *Setd7* promoter, 5'-TCCTGTTGCCATCATCAAAA-3' forward and 5'-ACATGAAGGCACCACATGAA-3' reverse.

**Immunohistochemistry.** Mouse pancreata were fixed by cardiac perfusion with 4% paraformaldehyde, paraffin embedded, and sectioned at 5- $\mu$ m intervals. Human pancreatic samples were obtained from the University of Virginia Human Islet Transplant Program, fixed for 2 h in 4% paraformaldehyde, and processed similarly to mouse pancreata. Immunohistochemical analysis of insulin (anti-insulin, 1:500 dilution), Pdx1 (anti-Pdx1, 1:2,000 dilution), glucagon (anti-glucagon, 1:500 dilution), and Set7/9 (anti-Set7/9, 1:50), was performed as previously described (11). For immunofluorescence, secondary antibodies were goat anti-rabbit–conjugated to Alexa Fluor 555 and donkey anti-mouse–conjugated to Alexa Fluor 488 (Molecular Probes). Images were acquired using a Zeiss Z1 microscope equipped with Apotome optical sectioning hardware.

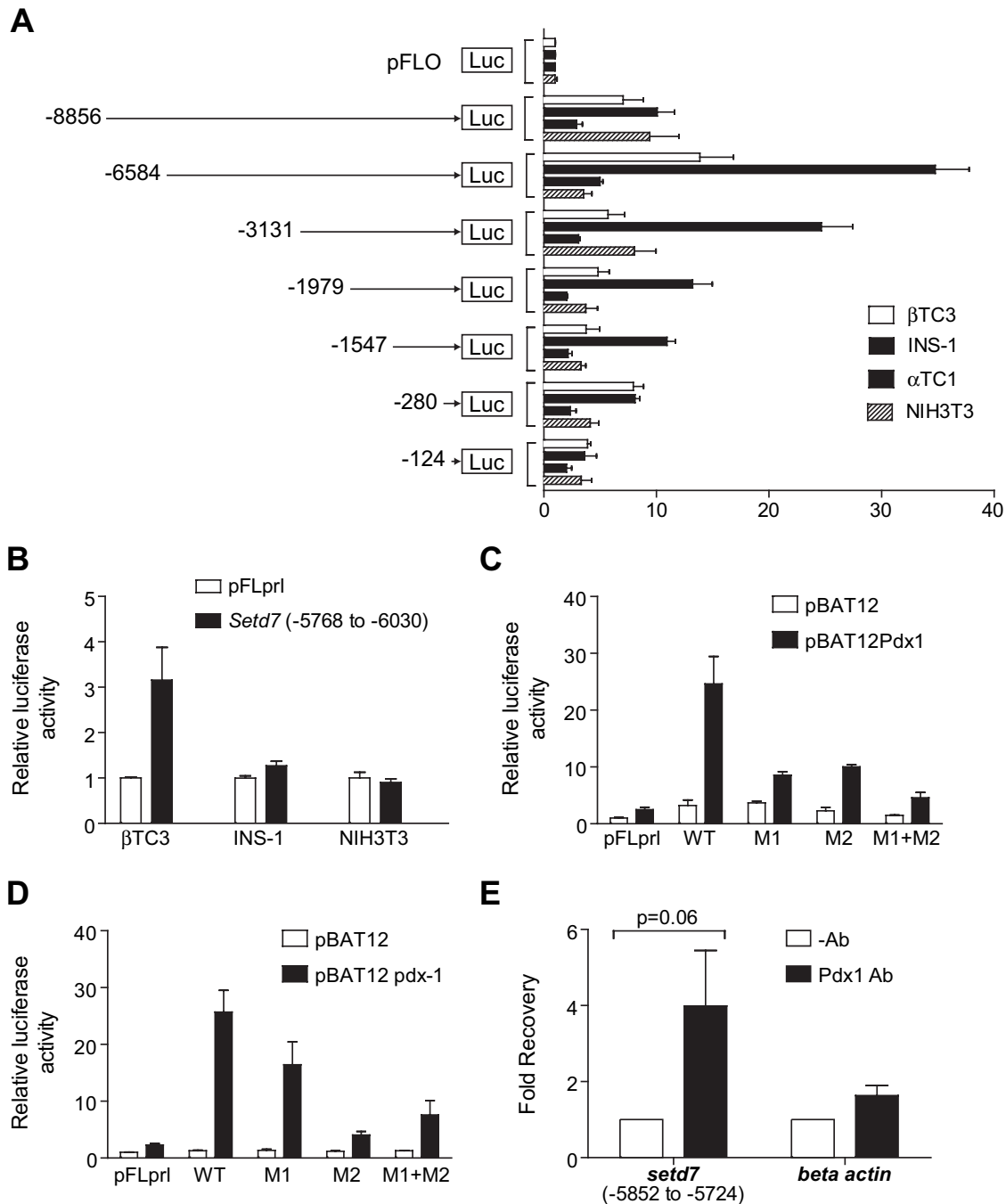
**Transfection of cell lines with siRNA.** siRNAs against Set7/9 or a scrambled control were mixed with Reagent V (Amaxa, Inc.) and transfected into  $3 \times 10^6$   $\beta$ TC3 mouse insulinoma cells using an Amaxa Nucleofector (program D-23) according to the manufacturer's protocol. Cells were then plated in a six-well dish containing culture medium for 72 h and then harvested for protein, total RNA, or ChIP analysis. siRNA sequences used were as follows: siSet97, 5'-GGUUUAUGUUGCCGACUCCTT-3'; siSet98, 5'-GGUAGCAGUUGGACCUAAUTT-3'; and a control siRNA (siControl), 5'-AAAGUCGACCU-CAGUAAGUU-3'.



**FIG. 1.** Tissue expression pattern of Set7/9. Mouse tissues were harvested for either RNA or protein, and mouse pancreatic tissue was harvested for immunohistochemistry. **A:** Quantitative real-time RT-PCR for the gene encoding Set7/9 in the mouse tissues indicated. Data shown are corrected for *Actb* mRNA levels and normalized to the levels seen in muscle. **B:** Immunoblot analysis of Set7/9 protein and actin proteins from mouse tissues indicated. **C** and **D:** Mouse pancreas was harvested, fixed, and stained using primary antibodies to Set7/9 and secondary horseradish peroxidase–linked anti-rabbit antibodies. Representative images of a pancreas at 10 $\times$  (**C**) and 40 $\times$  (**D**) magnifications are shown. **E–M:** Dual-immunofluorescence staining of representative islets from pancreatic sections showing localization of Set7/9 (**E**, **H**, and **K**) with insulin (**F** and **G**), glucagon (**I** and **J**), or Pdx1 (**L** and **M**). (Please see <http://dx.doi.org/10.2337/db08-1150> for a high-quality digital representation of this figure.)

**In vivo delivery of siRNA.** Stabilized siRNAs were obtained from Dharmacon and diluted in 0.9% saline. Eight-week-old male C57BL/6J mice were injected intraperitoneally with 1.6 mg/kg mouse wt siRNA daily for 4 days. Islets were then harvested, handpicked and allowed to recover overnight and then subjected to glucose-stimulated insulin secretion (GSIS)/glucose-stimulated  $Ca^{2+}$  mobilization (GSCa) studies (see below), protein isolation, or RNA isolation. siRNA sequences used were as follows: siSet1, 5'-CGGACAGAUUU-GAGGGCAAUU-3'; siSet2, 5'-GGGUUUUAUGUUGCCGACUCUU-3; and siControl, 5'-AAAGUCGACCUUCAGUAAGUU-3'.

**GSIS and GSCa studies.** For GSIS studies, 50 islets per condition were incubated in Krebs-Ringer HEPES buffered solution for 1 h at 37 $^{\circ}$ C and then placed in Krebs-Ringer HEPES buffered solution containing 3 or 11 mmol/l glucose for 1 h. Insulin released into the medium was assayed using a two-site immunospecific ELISA (Alpco Diagnostics). GSCa in mouse islets was measured using the ratiometric  $Ca^{2+}$  indicator Fura-2AM as described previously



**FIG. 2.** Regulation of the mouse *Setd7* gene. **A:** Fragments of the *Setd7* promoter in plasmid pFoxLuc1 (pFLO) were transfected into the cell lines indicated. Luciferase activity was measured 48 h after transfection, corrected for total protein, and normalized to the activity of the promoterless backbone plasmid. **B:** An AT-rich DNA fragment of the *Setd7* promoter (-5,768 to -6,030) was subcloned into a luciferase reporter plasmid driven by the prolactin minimal promoter (pFLprl) and transfected into the cell lines indicated. Luciferase activity was normalized to the activity of pFLprl-transfected cells. **C and D:** *Setd7* gene fragments (wild-type and mutagenized at potential Pdx1 binding sites) were cotransfected with a cytomegalovirus promoter-driven plasmid (pBAT12) expressing Pdx1 into either  $\beta$ TC3 (**C**) or INS-1 (**D**) cells. Luciferase activity was normalized to the activity of cells transfected with the backbone pBAT12 plasmid. **E:** Results of a ChIP assay using  $\beta$ TC3 cell chromatin and either normal rabbit serum (-Ab) or primary antibody to Pdx1 (*Pdx1* Ab). Recovery of either the *Setd7* or *Actb* promoters were quantitated by real-time PCR, and data are expressed as fold recovery compared with the data for normal rabbit serum alone. The amplified region of the *Setd7* gene (in bp) relative to the transcriptional start site is indicated in parentheses. Data represent the average from three independent ChIP experiments.

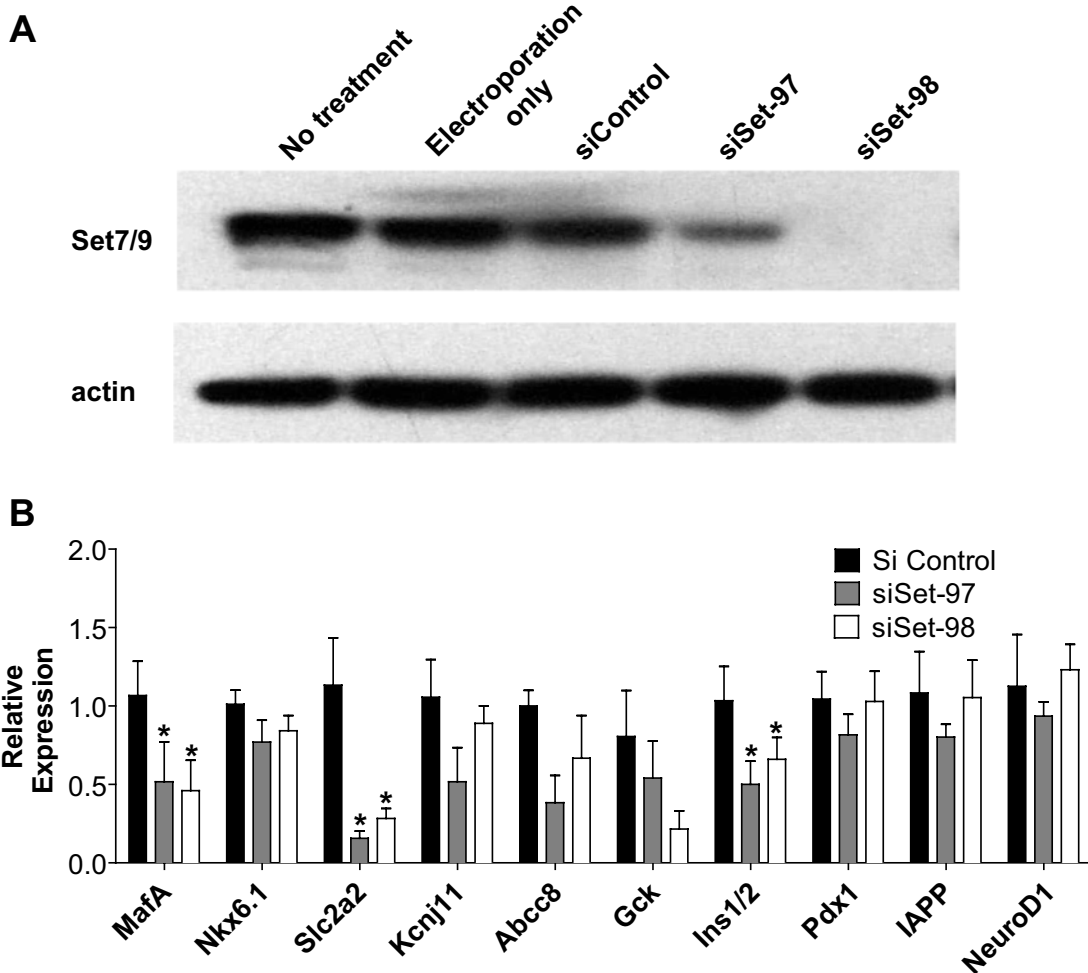
(24). GSCa was defined as the difference between ratio measurements (340/380 nm fluorescence) in 11 mmol/l vs. 3 mmol/l glucose. Data were analyzed with IP Lab software, version 4.0 (Scanalytics).

**Cloning of the *Setd7* promoter.** A genomic clone containing ~8,000 bp of the *Setd7* gene was used as a template in PCR reactions to generate various fragments of the *Setd7* gene, which were then subcloned upstream of the luciferase coding sequence in plasmid pFoxLuc (25). Mutagenesis of the *Setd7* gene was performed using the QuickChange Site-Directed Mutagenesis Kit

(Stratagene) according to the manufacturer's instructions. All sequences were confirmed by automated DNA sequencing.

**Transfections and reporter assays.**  $10^6$   $\beta$ TC3/ $\alpha$ TC1.6/INS-1 cells or  $5 \times 10^5$  NIH3T3 cells were seeded in six-well plates 24 h before transfection. Plasmid (2  $\mu$ g) diluted into 0.1 ml PBS was mixed with 6  $\mu$ l of Metafectene Pro (Biotex) and incubated at room temperature for 15 min. The transfection mixture was added to each well of a six-well plate along with 2 ml medium without antibiotics. At 6 h after transfection, the medium was replaced with





**FIG. 3.** Set7/9 is necessary for the expression of a subset of glucose-responsive genes in  $\beta$ TC3 cells.  $\beta$ TC3 cells were transfected with the siRNAs indicated, and protein and total RNA were harvested. **A:** Immunoblots for Set7/9 and actin. **B:** Quantitative real-time RT-PCR for each of the genes indicated. Data shown are corrected for *Actb* mRNA levels and normalized to the levels seen with siControl transfections. Data represent the average of at least four independent transfections. \*Values differ significantly ( $P < 0.05$ ) from those of siControl transfections.

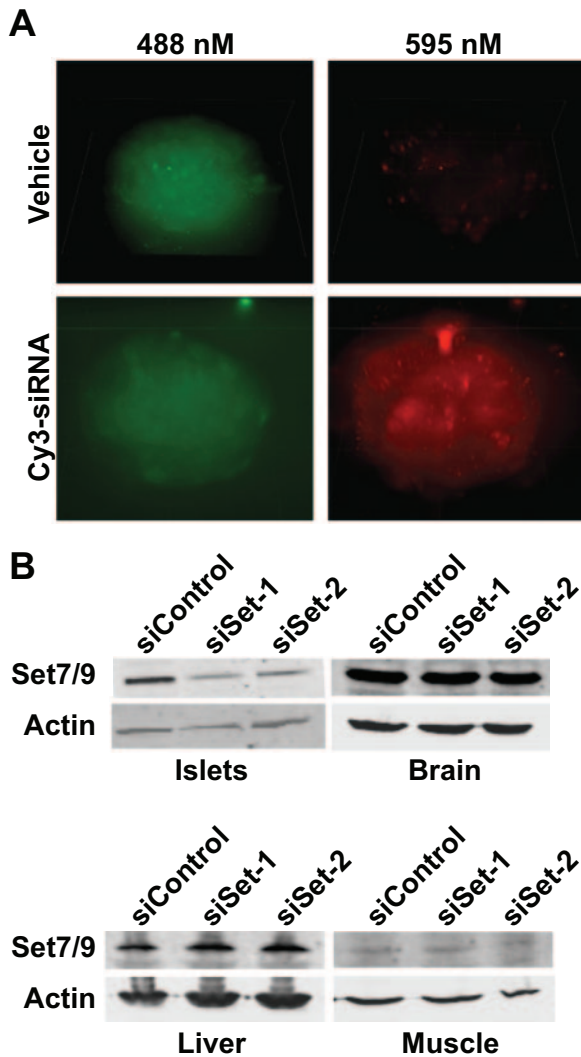
fresh medium containing antibiotics. At 48 h after transfection, cells were harvested and a luciferase assay was performed utilizing a commercially available luminometric kit (Promega). Luciferase activity was normalized to protein concentration as measured using the Bio-Rad Protein Assay reagent (Bio-Rad) according to manufacturer's instructions.

## RESULTS

**Set7/9 mRNA and protein are enriched in pancreatic islets.** To evaluate the tissue distribution of Set7/9 in adult mice, Set7/9 mRNA and protein were assayed in different tissues by real-time RT-PCR and immunoblot, respectively. As shown in Fig. 1A, mRNA levels demonstrate a pattern of tissue-specific enhancement, with the highest relative levels seen in muscle, brain, heart, and pancreas. Interestingly, Set7/9 protein levels do not completely mirror Set7/9 mRNA levels (Fig. 1B), as no protein was detectable in either heart or total pancreas and very little was detectable in muscle. These data suggest the potential for posttranscriptional regulation of Set7/9 expression. However, within the pancreas, there was clear enrichment of Set7/9 protein within islets as determined by immunoblotting (Fig. 1B). To directly visualize the distribution of Set7/9 among pancreatic cell types, we next performed immunohistochemistry of pancreatic sections from both mice and humans. Figure 1C and D shows that Set7/9 is strikingly enriched in islets of mouse pancreas, with a predominantly nuclear pattern in most cells of the central islet.

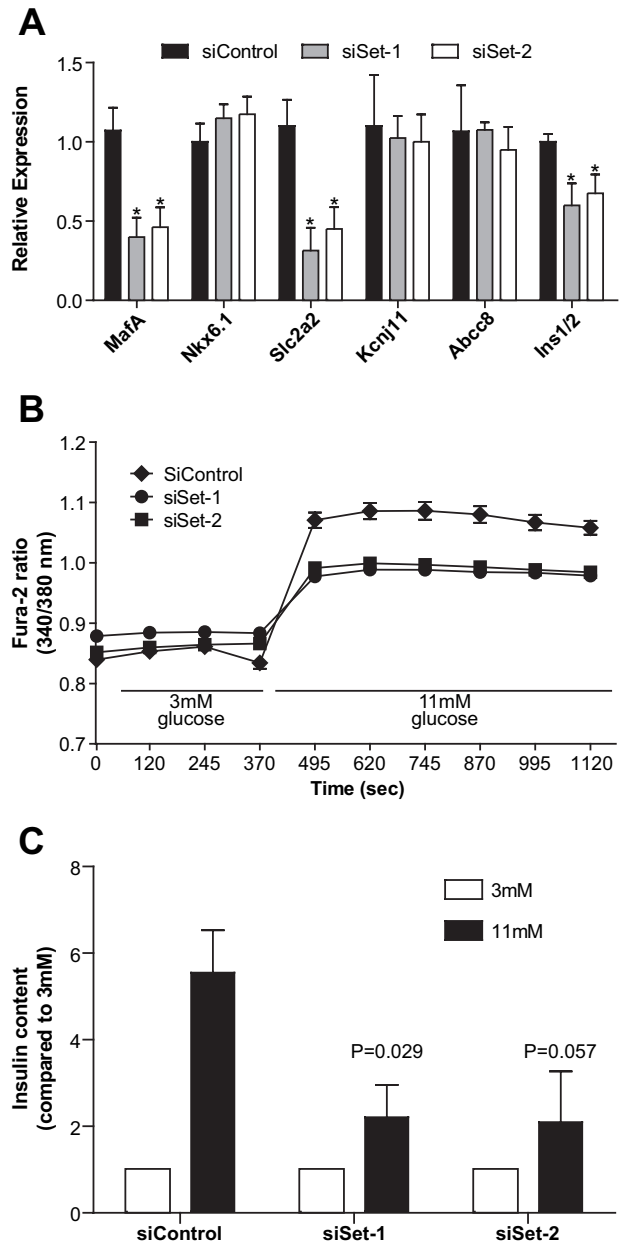
Coimmunofluorescence revealed that Set7/9 exhibited a nuclear pattern in insulin-producing  $\beta$ -cells (Fig. 1E–G) and both a nuclear and cytoplasmic pattern in glucagon-producing  $\alpha$ -cells (Fig. 1H–J). All Pdx1-positive cells of the islet costained for Set7/9 (Fig. 1K–M). Human pancreas sections demonstrated a Set7/9 staining pattern identical to that seen in mouse sections (supplemental Fig. S1, available in an online appendix at <http://dx.doi.org/10.2337/db08-1150>).

**$\beta$ -Cell-specific transcription of the *Setd7* gene.** The islet-specific enrichment of Set7/9 suggested the potential for regulation at the transcriptional level. To explore this possibility, we placed a range of DNA fragments from the 5' regulatory region of the gene encoding Set7/9 (*Setd7*) upstream of the *luciferase* reporter gene (in plasmid pFoxLuc [25]). These constructs were then transfected into the  $\beta$ -cell-derived cell lines  $\beta$ TC3 and INS-1, the  $\alpha$ -cell-derived cell line  $\alpha$ TC1.6, and the fibroblast-derived line NIH3T3. As shown in Fig. 2A,  $\beta$ -cell lines displayed enhancement of luciferase activity with a DNA fragment containing  $-6,584$  bp upstream of the transcriptional start site. This enhancement was significantly attenuated when a fragment containing  $-3,131$  bp from the transcriptional start site was used, suggesting the potential for a  $\beta$ -cell-specific enhancer located between  $-3,131$  and  $-6,584$  bp. To further explore this possibility, we next performed an



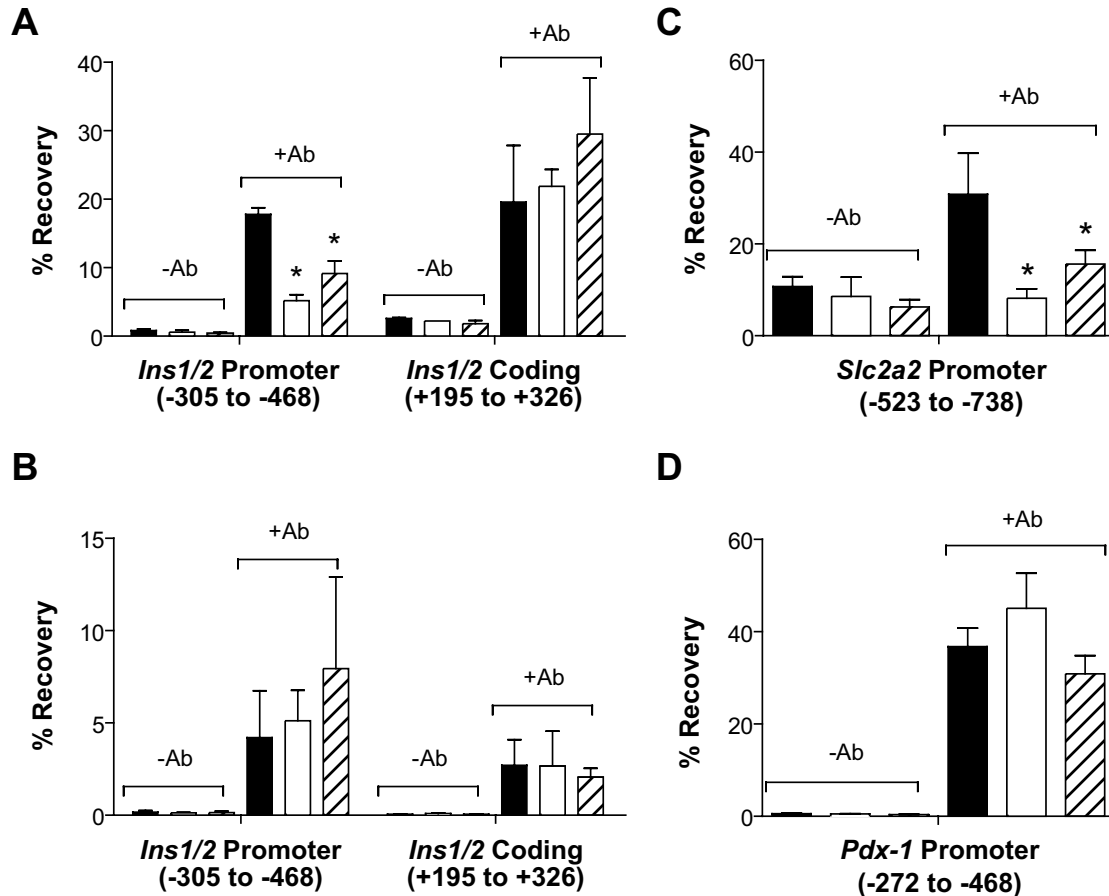
**FIG. 4.** *Set7/9* knockdown in mouse islets. Male C57BL/6J mice were administered intraperitoneal injections of stabilized siRNAs as described in RESEARCH DESIGN AND METHODS. **A:** Confocal microscopic imaging of representative islets isolated from mice injected with either unlabeled control siRNA or Cy3-labeled control siRNA. The panel shows islets imaged upon excitation at 488 nm (autofluorescence of islet  $\beta$ -cells) and the same islets imaged upon excitation at 595 nm (corresponding to the Cy3 label). **B:** Male C57BL/6J mice were injected intraperitoneally with stabilized control siRNA or siRNAs against *Set7/9* (siSet1 and siSet2). Shown are immunoblots of islet, brain, liver, and muscle extracts from siRNA-injected mice for *Set7/9* and actin. (Please see <http://dx.doi.org/10.2337/db08-1150> for a high-quality digital representation of this figure.)

alignment of human, mouse, and rat *Setd7* genes to identify regions of homology that might suggest conserved  $\beta$ -cell-specific enhancer regions. Although the entire region between -3,131 and -6,584 bp only exhibited ~25% identity between these species (data not shown), we identified a region between -5,768 and -6,030 bp that exhibited ~68% identity (supplemental Fig. S2). Within this conserved region, we identified two potential binding sites for the  $\beta$ -cell-specific transcription factor Pdx1 (consensus: 5'-TAAT-3' [refs. 20,26]) (supplemental Fig. S2). To directly test whether this region of the *Setd7* gene might be regulated in  $\beta$ -cells, a DNA fragment containing this conserved region was placed upstream of the prolactin minimal promoter driving luciferase and used in reporter gene analysis studies. As shown in Fig. 2B, this fragment displayed relative enhancement of luciferase



**FIG. 5.** *Set7/9* is necessary for normal islet function. Male C57BL/6J mice were injected intraperitoneally with stabilized control siRNA or siRNAs against *Set7/9* (siSet1 and siSet2) as described in RESEARCH DESIGN AND METHODS. Islets were then harvested. **A:** Islets from injected mice were subjected to quantitative real-time RT-PCR for each of the genes indicated. Data shown are corrected for *Actb* mRNA levels and normalized to the levels seen with siControl transfections. Data represent the average of at least four independent transfections. \*Values differ significantly ( $P < 0.05$ ) from those of siControl transfections. **B** and **C:** Islets from injected mice were subjected to GSCa (**B**) or GSIS (**C**) studies at the indicated glucose concentrations. Data in **B** represent the average of at least 10 islets pooled from three mice injected with each siRNA on a single occasion, and data in **C** represent the average of 50 islets from six injected mice. Statistical significance is indicated by the  $P$  value (from  $t$  test) for the comparison with siControl at 11 mmol/l glucose.

activity in  $\beta$ TC3 and INS-1  $\beta$ -cell lines, but not in NIH3T3 cells. Upon cotransfection with a plasmid encoding Pdx1, this fragment displayed an ~25-fold enhancement in the  $\beta$ -cell lines INS-1 and  $\beta$ TC3 (Fig. 2C and D). Mutation of either putative Pdx1 binding site attenuated activity of this fragment in both  $\beta$ -cell lines (Fig. 2C and D). Pdx1 appears to occupy the endogenous *Setd7* gene in  $\beta$ TC3 cells within



**FIG. 6.** Set7/9 is necessary for the maintenance of dimethylated H3 Lys4 at specific genes in  $\beta$ TC3 cells.  $\beta$ TC3 cells were transfected with the siRNAs indicated and subjected to ChIP using either normal rabbit serum (*-Ab*) or antibodies to methylated histones. Recovery of the gene fragments was assessed by real-time PCR. Data are reported as recovery of the indicated gene (*Ins1/2* or *Slc2a2*) following ChIP as a percent of the input levels of the gene before ChIP. **A:** Percent recovery of dimethylated H3 Lys4 at the *Ins1/2* promoter and coding regions. **B:** Percent recovery of monomethylated H3 Lys4 at the *Ins1/2* promoter and coding regions. **C:** Percent recovery of dimethylated H3 Lys4 at the *Slc2a2* proximal promoter region. **D:** Percent recovery of dimethylated H3 Lys4 at the *Pdx1* proximal promoter region. Data represent the average of at least three independent ChIP assays from at least three independent siRNA transfections. \*Statistically different ( $P < 0.05$ ) from data of siControl transfections. The amplified region of the genes (in bp) relative to the transcriptional start site is indicated in parentheses below each gene. ■, siControl; □, siSet97; ▨, siSet98.

the region around  $-6,000$  bp, as assessed by the ChIP assay, but does not occupy the endogenous gene encoding  $\beta$ -actin (Fig. 2E). Together, these results suggest that the *Setd7* gene may contain elements that enhance its expression specifically in  $\beta$ -cells and that the islet transcription factor Pdx1 may be a necessary component of a transactivation complex on the *Setd7* gene.

**Set7/9 is necessary for the expression of a subset of glucose-responsive genes in  $\beta$ -cell lines and islets.** To determine the role of Set7/9 in the regulation of  $\beta$ -cell-specific genes, we next depleted  $\beta$ TC3 cells of Set7/9 protein and analyzed gene expression by real-time RT-PCR. To knock down protein, two different siRNAs (siSet97 and siSet98) targeted against murine Set7/9 or siControl were transfected into  $\beta$ TC3 cells. As shown in Fig. 3A,  $>90\%$  reductions in Set7/9 protein were seen following transfections with each siRNA compared with those seen in control transfections. Use of two different siRNAs ensured that any effects on gene transcription were unlikely a result of off-target effects of these siRNAs. A survey of islet-specific genes involved in glucose responsiveness showed that the genes encoding the  $\beta$ -cell-specific transcription factor MafA (*MafA*), the glucose transporter Glut2 (*Slc2a2*), and preproinsulin (*Ins1/2*) were reduced significantly (Fig. 3B) in cells depleted of

Set7/9. However, reduction was not seen uniformly among all genes responsible for glucose responsiveness in islets, as those encoding Pdx1 (*Pdx1*), the ATP-sensitive  $K^+$  channel (*Kcnj11*), sulfonylurea receptors (*Abcc8*), Nkx6.1 (*Nkx6.1*), and NeuroD1 (*NeuroD1*) appeared statistically unaffected by acute reductions in Set7/9 (Fig. 3B).

To confirm our findings in primary cells, we next performed knockdown experiments in mouse islets. However, a major challenge in RNA interference studies in primary rodent islets is their relatively poor transfection efficiency in vitro, which typically necessitates use of viral-based approaches (27). Instead, we sought to knock down Set7/9 in islets by delivering chemically stabilized siRNAs in vivo. A similar technique has been used to target other proteins for systemic knockdown (28,29). To test the efficacy of this approach in islets, we performed four daily intraperitoneal injections of Cy3-labeled double-stranded RNA into male C57BL/6J mice and subsequently isolated islets from these animals on day 5. As shown in Fig. 4A, fluorescent microscopic images of isolated islets revealed penetration of the Cy3 label into the islets, suggesting islet entry of siRNA. Two different stabilized siRNAs targeted against Set7/9 (siSet1 and siSet2) or siControl were then delivered to mice using the same approach. Following the injections, islets from mice were then harvested and

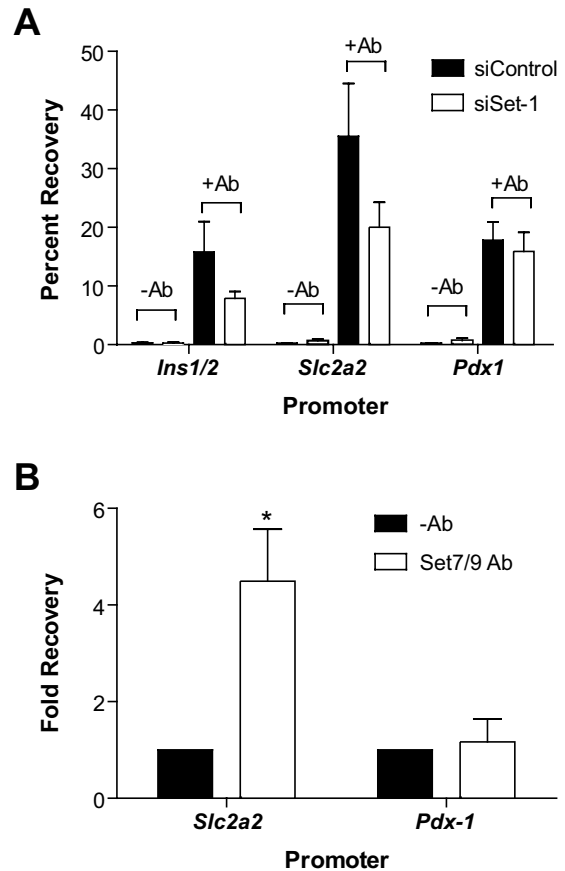
studied by immunoblot, real-time RT-PCR, GSCa, and GSIS. As shown in Fig. 4B, islet Set7/9 protein was reduced by at least 60% by treatment with either siRNA compared with control. Interestingly, similar knockdown was not observed in liver, brain, or muscle, suggesting that for the specific protocol employed, targeting of islets appeared to be most efficient.

Consistent with the data observed in  $\beta$ TC3 cells, depletion of Set7/9 in primary islets resulted in decreases in *MafA*, *Slc2a2*, and *Ins1/2* (Fig. 5A). Because decreases in these genes would be predicted to result in loss of glucose responsiveness in islets, we tested islet functionality by GSCa. The GSCa assay is a Fura-2AM dye-based assay that measures the intracellular mobilization of  $Ca^{2+}$  in response to glucose and is dependent on an intact signaling cascade that begins with the transport of glucose into the  $\beta$ -cell by GLUT2 and ending in the activation of voltage-gated  $Ca^{2+}$  channels; intracellular  $Ca^{2+}$  mobilization through this cascade is closely linked to insulin secretion (30). As shown in Fig. 5B, reductions in islet Set7/9 protein using either siSet1 or siSet2 resulted in GSCa responses (from 3 to 11 mmol/l glucose) that were only ~50% those associated with control. These islets also showed diminished GSIS upon raising glucose from 3 to 11 mmol/l (Fig. 5C). Taken together, these results suggest that Set7/9 is necessary for the transcription of a subset of islet genes that are required for the maintenance of normal islet function.

**Set7/9 is necessary for the maintenance of H3-Lys4 dimethylation at the *Ins1/2* and *Slc2a2* genes.** In prior ChIP studies, we demonstrated that Set7/9 occupies the *Ins1/2* genes in  $\beta$ TC3 cells (10). This observation, coupled with the finding that the *Ins1/2* gene is hypermethylated at H3 Lys4 (10,11), suggested to us that Set7/9 may be directly responsible for the maintenance of this euchromatin histone modification at this gene. We therefore wondered whether the reduction in *Ins1/2* gene activity observed upon knockdown of Set7/9 in this study could be secondary to loss of H3-Lys4 methylation. As shown in Fig. 6A, depletion of Set7/9 in  $\beta$ TC3 cells using either of two siRNAs (siSet97 or siSet98) led to 50–80% reductions in dimethylated H3 Lys4 in the *Ins1/2* promoter region as determined by ChIP assay but did not affect dimethylated H3 Lys4 in the coding region of the gene. Importantly, we did not observe any changes to monomethylated H3 Lys4 in either the promoter or coding regions of the *Ins1/2* gene (Fig. 6B), suggesting that Set7/9 is associated with a very specific dimethylation effect.

To ascertain the specificity of Set7/9, we next examined the genes *Slc2a2* (whose transcription is reduced by loss of Set7/9) and *Pdx1* (whose transcription is unaffected by loss of Set7/9). As shown in Fig. 6C and D, we observed a specific loss of dimethylated H3 Lys4 at the proximal *Slc2a2* promoter but not at the proximal *Pdx1* promoter. Similar results were obtained in primary mouse islets depleted of Set7/9 using siSet1, although the decreases in dimethylated H3 Lys4 at the *Ins1/2* and *Slc2a2* genes did not quite approach statistical significance (Fig. 7A). These results are consistent with gene-specific effects of Set7/9.

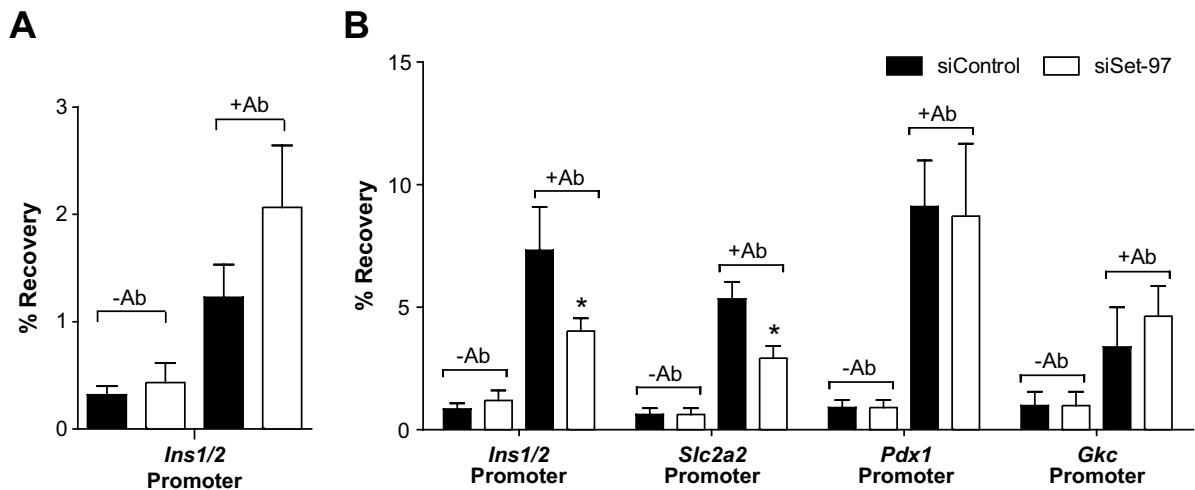
To determine whether Set7/9 could be modifying histones directly at the *Slc2a2* gene, we next performed ChIP assays in  $\beta$ TC3 cells to assess recruitment of Set7/9 to this gene. Figure 7B shows that Set7/9 occupies the *Slc2a2* gene within its proximal control region (–738 to –523 bp relative to the transcriptional start site) but was not detected at the proximal *Pdx1* promoter. These results



**FIG. 7.** Set7/9 may be necessary for maintenance of dimethylated H3 Lys4 at the *Ins1/2* and *Slc2a2* genes in mouse islets and occupies the *Slc2a2* gene in  $\beta$ TC3 cells. **A:** Male C57BL/6 mice were injected intraperitoneally with stabilized control siRNA or siRNA against Set7/9 (siSet1) as described in RESEARCH DESIGN AND METHODS. Islets were subsequently harvested and subjected to ChIP using either normal rabbit serum (–Ab) or antibody to dimethylated H3 Lys4 (+Ab). Data are reported as percent recovery of the indicated gene promoter relative to input. Data represent the average of three independent ChIP experiments from mice injected on three separate occasions. **B:**  $\beta$ TC3 cells were subjected to ChIP analysis using normal rabbit serum (–Ab) or antibody against Set7/9 (Set7/9 Ab). Recovery of the proximal *Slc2a2* and *Pdx1* promoters are reported as fold recovery relative to normal rabbit serum. Data represent the average of three independent ChIP assays. \*Statistically different ( $P < 0.05$ ) from data of ChIP assays in which normal rabbit serum was used in place of Set7/9 antibody.

suggest that the chromatin effects observed at the *Slc2a2* gene are the result of Set7/9 recruitment to its promoter. **Set7/9 is necessary for the recruitment of RNA polymerase II to the *Ins1/2* gene.** Pdx1, like Set7/9, is required for the full activation of the *Ins1/2* gene (23,31,32). This effect of Pdx1 is apparently related not to the recruitment of RNA polymerase II to the *Ins1/2* gene but, rather, to defects in the conversion of RNA polymerase II from its initiation to elongation isoforms (11,23). We therefore wondered whether the maintenance of *Ins1/2* transcription by Set7/9 might be related to Pdx1. As shown in Fig. 8A, knockdown of Set7/9 in  $\beta$ TC3 cells using siSet97 led to no changes in Pdx1 occupancy at the *Ins1/2* gene. However, we observed that RNA polymerase II recruitment to the promoter region of *Ins1/2* (and *Slc2a2*) was diminished by ~50% (Fig. 8B). These results suggest that Set7/9 action at the *Ins1/2* gene may be distinct from that of Pdx1 and that either Set7/9 itself or its associated dimethylated H3-Lys4 modification is linked to RNA polymerase II recruitment.





**FIG. 8.** Loss of Set7/9 affects Pol II but not Pdx1 recruitment.  $\beta$ TC3 cells were transfected with siControl or siSet97 and subjected to ChIP using normal rabbit serum (*-Ab*) or antibody to RNA polymerase II COOH-terminal domain (*+Ab*). Recovery of the gene fragments was assessed by real-time PCR. Data are reported as recovery of the indicated genes following ChIP as a percent of the input levels of the genes before ChIP. **A:** ChIP experiment using antibody to Pdx1. **B:** ChIP experiment using antibody to RNA polymerase II COOH-terminal domain. Data represent the average of three independent ChIP assays from three independent siRNA transfections. \*Statistically different ( $P < 0.05$ ) from data of siControl transfections.

## DISCUSSION

The islet  $\beta$ -cell derives many of its characteristics, particularly stimulus-secretion coupling, from the transcription of a unique subset of genes that is controlled by a group of cell-specific transcription factors and cofactors. In this report, we identify a new member of this cohort of islet-specific factors, Set7/9, and demonstrate its role in the maintenance of gene transcription and chromatin structure in  $\beta$ -cell lines and primary islets. Set7/9 was originally identified as a histone H3-Lys4-specific methyltransferase (12,13). Subsequently, Set7/9 was shown to methylate Lys residues in a variety of proteins including TAF10 (18), TAF7 (17), p53 (14–16), the estrogen receptor (19), and possibly Pdx1 (11). To date, however, its role in the maintenance of histone H3–Lys4 methylation at islet genes has only been implied (11) and not directly studied.

We show that Set7/9 is distributed in a limited number of tissues in the mouse but includes those derived from all three major germ layers, including ectoderm (brain), mesoderm (muscle), and endoderm (pancreas). Within the pancreas of both the mouse and human, Set7/9 exhibits a striking islet-specific distribution pattern. The enrichment of Set7/9 in  $\beta$ -cells may occur at the level of transcription, as our studies identified a potential conserved  $\beta$ -cell-specific enhancer located in the distal *Setd7* gene that is responsive to Pdx1. Its strongly nuclear pattern of distribution, particularly in  $\beta$ -cells, suggested to us a role for Set7/9 in  $\beta$ -cell gene regulation.

To identify genes regulated by Set7/9 in  $\beta$ -cells, we engaged RNA interference approaches to knock down Set7/9 in insulinoma-derived  $\beta$ -cells ( $\beta$ TC3) and primary mouse islets. Successful knockdown using multiple different siRNAs demonstrated reductions in key genes that mediate stimulus-secretion coupling, including *Slc2a2*, *MafA*, and *Ins1/2*. Consistent with these reductions, we observed impaired GSCa and GISIS in islets depleted of Set7/9. We note that our knockdown studies were of short duration by design in order to obtain information on the most immediate and potentially direct genetic targets of Set7/9. Thus, we cannot rule out the possibility that other genes may contribute to the dysfunctional islet phenotype observed or that more prolonged knockdown may not

have broader effects on islet function, replication, or survival. Importantly, effects on islet survival are possible, given that recent studies suggest a role for Set7/9 in the maintenance of inflammatory genes in macrophages (33).

As noted earlier, Set7/9 exhibits specific methyltransferase activity toward histone H3 Lys4. Some studies suggest that it may function as a dimethyltransferase (34), whereas others suggest that it functions exclusively as a monomethyltransferase in vitro (35). Although our data are compatible with the role of Set7/9 as a dimethyltransferase in cells, they do not rule out the possibility that it may indeed function as a monomethyltransferase. For example, it is possible that Set7/9 is very closely linked to a second methyltransferase that completes the dimethylation reaction in cells, such that transient alterations in monomethylation are not detectable in our system. Alternatively, it is possible that catalytic activities observed in vitro are modified in the cellular milieu as a result of the complexity of protein-protein interactions. In this context, recent studies of the H3-Lys9 methyltransferase G9a show that it exhibits differing stoichiometries of methylation in vivo depending on binding to accessory proteins (36).

In the absence of endogenous nuclear localization and DNA binding domains, we presume that Set7/9 must be chaperoned to both the nucleus and target genes through an interaction with transcription factors. We have previously demonstrated that Set7/9 forms an immunoprecipitable complex with Pdx1 (11). In those studies, we also showed that depletion of Pdx1 in  $\beta$ TC3 cells leads to diminution of H3-Lys4 dimethylation at the *Ins1/2* gene—an observation that our present studies suggest could be caused by disruption of the Pdx1-Set7/9 complex. Interestingly, however, whereas the depletion of Pdx1 led to defective conversion of RNA polymerase II to its elongation isoform (and not its recruitment to the promoter [11,23]), we observed here that depletion of Set7/9 causes impairments in RNA polymerase II recruitment to the promoter. Our findings are therefore consistent with studies suggesting that Set7/9 stabilizes the transcriptional preinitiation complex by methylation of TAF10 (18).

Taken together, our results suggest a model for Set7/9 action at target  $\beta$ -cell genes. We propose that Set7/9 is



recruited to specific  $\beta$ -cell genes (e.g., *Ins1/2* and *Glut2*) through interaction with factors such as Pdx1. This recruitment subsequently leads to dimethylation of H3 Lys4 within the promoter region (either directly by Set7/9 or through interaction with another methyltransferase) and recruitment of RNA polymerase II. The subsequent conversion of RNA polymerase II to its elongation isoform requires other factors that are dependent on Pdx1. The preponderance of studies in the literature suggest that dimethylation of H3 Lys4 appears to be crucial for laying a euchromatic template at target genes, thereby leaving genes poised for transcription. Although we have demonstrated an important role for Set7/9 in the transcriptional regulation of key  $\beta$ -cell genes in mature cells, the role of Set7/9 in establishing transcriptional patterns during the embryonic development of  $\beta$ -cells remains to be determined. In future studies, we will directly address its role in embryonic development using conditional Set7/9 knock-out mice.

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