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Transcriptional Activity of the Islet β Cell Factor Pdx1 is Augmented by Lysine Methylation Catalyzed by the Methyltransferase Set7/9*

Aarthi V. Maganti[1], Bernhard Maier[2], Sarah A. Tersey[2], Megan L. Sampley[5], Amber L. Mosley[3], Sabire Özcan[5], Boobalan Pachaiyappan[6], Patrick M. Woster[6], Chad S. Hunter[7,8], Roland Stein[7] and Raghavendra G. Mirmira[1,2,3,4]

From: [1]Department of Cellular and Integrative Physiology, [2]Department of Pediatrics and the Herman B Wells Center for Pediatric Research, [3]Department of Biochemistry and Molecular Biology, [4]Department of Medicine, Indiana University School of Medicine, Indianapolis, IN; [5]Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Lexington, KY; [6]Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, Charleston, SC; [7]Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN, [8]Department of Medicine, University of Alabama at Birmingham, AL (current).

Running Title: *Lys methylation of Pdx1 by Set7/9*
Correspondence: Raghavendra G. Mirmira, 635 Barnhill Drive, MS2031B, Indianapolis, IN 46202, Tel: 317-274-4145, Fax: 317-274-4107; E-mail: rmirmira@iu.edu.
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Background: Pdx1 interacts with the methyltransferase Set7/9 to transactivate β cell genes.
Result: Methylation of Pdx1 residue K131 by Set7/9 augments Pdx1 activity.
Conclusion: The ability of Pdx1 to regulate genes in β cells is partially dependent upon its methylation by Set7/9.
Significance: This study reveals a previously unappreciated role for Lys methylation in the maintenance of Pdx1 activity and β cell function.

SUMMARY

The transcription factor Pdx1 is crucial to islet β cell function, and regulates target genes in part through interaction with co-regulatory factors. Set7/9 is a Lys methyltransferase that interacts with Pdx1. Here, we tested the hypothesis that Lys methylation of Pdx1 by Set7/9 augments Pdx1 transcriptional activity. Using mass spectrometry and mutational analysis of purified proteins, we found that Set7/9 methylates N-terminal residues K123 and K131 of Pdx1. Methylation of these residues occurred only in the context of intact, full-length Pdx1, suggesting a specific requirement of secondary and/or tertiary structural elements for catalysis by Set7/9. Immunoprecipitation assays and mass spectrometric analysis using β cells verified Lys methylation of endogenous Pdx1. Cell-based luciferase reporter assays using wild-type and mutant transgenes revealed a requirement of Pdx1 residue K131, but not K123, for transcriptional augmentation by Set7/9. K131 was not required for high affinity interactions with DNA in vitro, suggesting that its methylation likely enhances post-DNA binding events. To define the role of Set7/9 in β cell function, we generated mutant mice in which the gene encoding Set7/9 was conditionally deleted in β cells (*Set*$^{\Delta\beta}$). *Set*$^{\Delta\beta}$ mice exhibited glucose intolerance similar to Pdx1-deficient mice, and their isolated islets showed impaired glucose-stimulated insulin secretion with reductions in expression of Pdx1 target genes. Our results suggest a previously unappreciated role for Set7/9-mediated methylation in the maintenance of Pdx1 activity and β cell function.

INTRODUCTION

Deficiency of insulin secretion underlies the transition from normoglycemia to hyperglycemia in both type 1 and type 2 diabetes (1, 2). Circulating insulin arises almost exclusively from islet β cells in the pancreas, but our understanding of the molecular mechanisms governing β cell function in health and disease remains incomplete. A key protein that is essential for β cell function is the homeobox

transcription factor Pdx1. During mammalian development, Pdx1 is essential for pancreas organogenesis (3–5). In the adult pancreas, Pdx1 is restricted primarily to β cells and is responsible for the regulation of genes that are essential to β cell function, proliferation, and survival (6, 7). In this context, elucidating the molecular mechanisms of Pdx1 action will be crucial in future attempts to restore β cell function in the setting of diabetes.

The interaction of Pdx1 with other transcription factors and cofactors appears to be important in modulating Pdx1 activity (positively or negatively) at a given target gene. For example, the formation of a transcriptional complex between Pdx1 and the basic helix-loop-helix factor NeuroD1 in β cells results in the formation of a complex with the gene encoding preproinsulin (*Ins1/2*) that permits synergistic activation of transcription (8, 9). By contrast, interaction of Pdx1 with PCIF1 results in degradation of Pdx1 protein by the proteasome and consequent reduction in Pdx1 activity (10). Interactions such as these suggest a model whereby various aspects of Pdx1 activity, including DNA binding affinity, protein stability, and recruitment of basal transcriptional machinery are modulated to achieve homeostatic function of the β cell.

Our laboratory has previously reported that the lysine methyltransferase Set7/9 interacts with Pdx1 to promote transactivation of an *Ins1/2* mini-enhancer (11) that contains the classical Pdx1 binding sequence (5'-TAAT-3') present in elements A3 and A4. Augmentation of transcriptional activity was correlated with an increase in Lys4 methylation of histone 3 (H3) and the conversion of the initiating isoform of RNA polymerase II to its elongating isoform. In recent years, several reports suggested that the methyltransferase activity of Set7/9 is not restricted only to Lys residues on histones, but includes Lys residues of other proteins, such as p53, p65, and estrogen receptor-α, among others (12–15). These varied methylation events were shown to alter the activity or half-life of these proteins, emphasizing that Lys methylation (similar to Ser/Thr phosphorylation or Lys acetylation) modulates transcription factor function (16, 17).

In light of these new perspectives on Lys methylation and Set7/9 action, we asked if the interactions between Set7/9 and Pdx1 might affect Pdx1 activity independently of effects on histones. In this study, our findings reveal a heretofore unappreciated role for Lys-specific methylation of Pdx1 by Set7/9 in the maintenance of normal β cell function.

MATERIALS AND METHODS

Cells, animals, and assays—NIH3T3, HEK293, MIN6 cells, and INS-1 cells were cultured as described previously (11, 18–20). All animal studies were reviewed and approved by the Indiana University Institutional Animal Care and Use Committee. *Setd7*$^{Loxp/+}$ mice, in which *Cre* recombinase recognition sequences (*Loxp*) flank exon 2, were generated by subcontract to Ingenious Targeting Labs. The neomycin selection cassette was removed by crossing *Setd7*$^{Loxp/+}$ mice to the FLPR strain. Mice were backcrossed onto the C57BL/6 background for 10 generations. *MIP1-Cre*^{ERT} mice on the C57BL/6 background were kindly provided by Dr. L. Philipson (21). For induction of *Cre*-mediated recombination, mice were orally gavaged with tamoxifen dissolved in peanut oil (mixed for 1 hour at 55^{0}C) at a dose of 5 mg/day/mouse. Intraperitoneal glucose tolerance tests using 2g/kg body weight glucose proceeded as described previously (22). Islets were isolated from collagenase-perfused pancreata as described previously (23). Static glucose-stimulated insulin secretion assays using islets were performed as previously described (22), and insulin released into the medium was normalized to total islet insulin content. Insulin was measured using a mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Alpco Diagnostics).

Recombinant plasmids, mutagenesis, and PCR—Plasmids were generated using standard recombinant techniques. PCR-generated constructs were verified by automated sequencing. Recombinant protein was expressed using the *E. coli* expression vectors pET21d or pET15b and purified as described previously (24). Recombinant Set7/9 protein was purchased from Prospec. Point mutations were generated using the Quick Change® site directed mutagenesis kit (Agilent). The following primers were used to make the respective point mutants: K123R: 5'-CAGCTGCCTTTCCCATGGATGAGGTCGACC AAAGCTCAC-3' and 5'-

GTGAGCTTTGGTCGACCTCATCCATGGGAAAGGCAGCTG-3'. K126R: 5'-TCCCATGGATGAAGTCTACCAGAGCTCACGCGT-3' and 5'-ACGCGTGAGCTCTGGTAGACTTCATCCATGGGA-3'. K131R: 5'-CCAAAGCTCACGCGTGGAGAGGCCAGTGG-3' and 5'-CCACTGGCCTCTCCACGCGTGAGCTTTGG-3'. CMV promoter-driven vectors (pBAT12) were used to express wild-type and mutant *Pdx1* in HEK293 and NIH3T3 cells, as described previously (9). The CMV promoter-driven vector used to drive *Setd7* was described previously (25). Methods primers for SYBR Green-based real-time RT-PCR was described previously (26).

Antibodies and Demethylase Inhibitor—Pdx1 antibody was obtained from Millipore (#07-696). Set7/9 antibody was obtained from Cell Signaling (#2813). Monoclonal Flag M2 antibody was obtained from Sigma-Aldrich (#F1804). Anti-Pdx1(K131-methyl) antibody was generated in rabbits by contract to 21st Century Biochemicals using the following synthetic peptides: methylated peptide 1: C-Ahx-TKAHAW[K-Me1]GQWAG-amide, and methylated peptide 2: Ac-AHAW[K-Me1]GQWAGGA-Ahx-KKC-amide. Gapdh antibody was obtained from Ambion (#AM4300). Fluorophore-labeled secondary antibodies IRDye 700 and IRDye 800 were obtained from Licor Biosciences. The demethylase inhibitor BP-107-7 was synthesized using a previously described synthetic route (27, 28).-

Methylation Assays In Vitro—Purified Pdx1 protein (150 nM – 1.2 μM) and Set7/9 protein (200 nM) were incubated at 30 °C for 3 hours in a reaction buffer containing 50mM Tris (pH 8.5), 4mM DTT, 5mM $MgCl_2$, 0.05mg/ml BSA, 1 μM ^{3}H-AdoMet, and 50 μM AdoMet in 20 μl volume. The reaction was stopped by the addition of 6X SDS gel loading buffer. Analysis by polyacrylamide gel electrophoresis proceeded as described previously (11).

Co-immunoprecipitation assays—Immunoprecipitations from whole cell lysates using Protein A or Protein G Dynabeads (Life Technologies) proceeded as described previously (11). Immunoprecipitations involving anti-HA antibody, which was performed using the HA-tag IP kit (Pierce). Chromatin immunoprecipitation (ChIP) assays was performed using the Active Motif ChIP-IT® Express Enzymatic kit (#53009), per manufacturer's protocol.

Immunoblot Analysis—Samples were resolved on 10% polyacrylamide gels and were then transferred onto a PVDF membrane (Millipore). Membranes were exposed to primary antibody, overnight at 4 °C and then processed as described previously (29).

Transient transfections and luciferase reporter assays—Cells were transfected using Metafectene Pro (Biontex) according to manufacturer's instructions in 6 well tissue culture plates. After 48 hours, whole cell extracts were used to assess luciferase activity using a commercially available luciferase assay kit (Promega).

Generation and Purification of tandem affinity purification tag (TAP)-Pdx1 from MIN6 Cells—The full-length mouse *Pdx1* cDNA sequence was inserted in-frame into pNTAP-C vector (Stratagene), generating TAP-Pdx1 with the TAP tag fused to the N-terminus of Pdx1. The TAP-Pdx1 sequence was then subcloned into the pAdTrack-CMV shuttle vector, which was used to generate recombinant adenoviruses expressing N-terminal TAP-tagged full-length Pdx1 as described previously (30). MIN6 cells were infected with the purified TAP-Pdx1 or control GFP adenovirus as previously described (30, 31) and lysed after 48 hours later. TAP Pdx1 was purified using the InterPlay TAP purification system (Stratagene) and subjected to mass spectrometry.

Electrophoretic mobility shift assays (EMSAs)[8]—The *Ins* E-A probe 5'-GATCCTTCATCAGGCCATCTGGCCCCTTGTTAATAATCTAATTACCCTAGGTCTAA-3' was labeled with γ^{32}P-ATP using T4 polynucleotide kinase, then duplexed with unlabeled complementary strand (5'-GATCTTAGACCTAGGGTAATTAGATTATTAACAAGGGGCCAGATGGCCTGATGAAG-3'). Nuclear extracts from transfected NIH3T3 cells were isolated as previously described (19), and around 2 μg of total nuclear protein was used in each EMSA reaction with labeled *Ins* E-A probe. Competition EMSAs were performed using unlabeled *Ins* E-A probe. EMSA reactions proceeded at room temperature for 15 min. EMSA buffer and electrophoresis protocol was previously described (32). The gel was visualized and competition EMSAs were quantitated using

using a phosphorimager (Molecular Dynamics). Competition EMSAs were modeled based on single phase exponential dissociation using the non-linear least squares fit algorithm in Prism 5.0 software (GraphPad), and apparent dissociation constants were determined, as previously described (24).

Mass spectrometry analysis—Samples (proteins from in vitro experiments and from MIN6 cells) were first denatured with 8M urea, then reduced with 10 mM DTT in 10 mM ammonium bicarbonate and alkylated with 55 mM iodoacetamide (prepared in 10 mM ammonium bicarbonate), and then digested with trypsin, incubated overnight at 37°C. The samples in vitro were analyzed using Thermo-Fisher Scientific LTQ Orbitrap Velos Pro and Surveyor HPLC. Tryptic peptides were injected onto the C18 column. Peptides were eluted with a linear gradient from 3 to 40% acetonitrile (in water with 0.1% FA) developed over 90 minutes at room temperature, at a flow rate of 60ul/min, and effluent was electro-sprayed into the LTQ mass spectrometer. Blanks were run prior to the sample run to make sure there was no significant signal from solvents or the column. The TSKgel columns (ODS-100 V, 3 μm, 1.0 mm × 50 mm) were used for the Surveyor HPLC system. The trypsin digested Pdx1 immunoprecipitants from MIN6 cells were pressure loaded onto a 12 cm multidimensional protein identification technology (MudPIT) column as previously described (33). Peptides were analyzed by MS/MS on an LTQ XL mass spectrometer following elution during a 10-step MudPIT run. Protein database searches were performed using Sequest® algorithms within Proteome Discoverer (Thermo) against appropriate FASTA sequence databases obtained from UniProt. Database searches included dynamic modification analysis for mono-, di-, and tri-methylated Lys residues, and Met residue oxidation.

Statistical analysis—All data are presented as the mean ± SEM. One-way ANOVA (followed by a Dunnett's post test) was used for comparisons in which two or more conditions were compared, and a two-tailed Student's *t* test was performed when two conditions were compared. Prism 5.0 software (GraphPad) was used for all statistical analyses. Statistical significance was defined as $P<0.05$.

RESULTS

Pdx1 is methylated by Set7/9 in vitro at positions K123 and K131

Recent evidence suggesting that Set7/9 methylates non-histone proteins led us to investigate first if the interaction between Set7/9 and Pdx1 leads to Lys methylation of Pdx1. We performed methylation reactions in vitro using purified Pdx1 and Set7/9 proteins and ^{3}H-S-adenosyl methionine (^{3}H-AdoMet). Incorporation of ^{3}H-methyl group into Pdx1 in this reaction is evidence of protein methylation (11). As shown in Fig. 1A, ^{3}H-methyl was incorporated into Pdx1 in a concentration-dependent manner, and only in the presence of Set7/9. By contrast, no methylation of bovine serum albumin is observed in the same reaction, suggesting that the methylation is specific for Pdx1 (Fig. 1A).

Prior studies suggest that Lys residues in a broad range of sequence contexts can serve as substrates for Set7/9 (34), Within Pdx1, Lys residues occur in both the N-terminal transactivation domain (residues K15, K123, K126, K131) and the DNA binding homeodomain (residues K147, K163, K191, K200, K202, K203, K207, K208). To narrow the possible Lys residues that may be methylated by Set7/9, we next performed methylation assays using different truncated mutants of Pdx1, as shown in Fig. 1B. Surprisingly, none of the truncated mutants were methylated by Set7/9 in vitro, suggesting that methylation of Pdx1 required the structural context of the full-length protein.

To identify the methylated Lys residue(s) in the full-length protein, we next performed mass spectrometry of methylated full-length Pdx1. Broad coverage of the peptides confirmed the mono-methylation of Pdx1 at two Lys residues in the N terminal region of the protein—residues K123 and K131 (Fig. 2A and B). Data from the mass spectrometric analysis was verified using mutated Pdx1 proteins, in which residues K123, K126, and K131 in the N-terminal domain were mutated to Arg (K123R, K126R, and K131R). As shown in Fig. 1C, reductions in methylation of Pdx1 clearly occurred in K123R mutant, but no statistically significant reduction was seen in the K131R mutant. Little residual methylation was observed in the K123R+K131R double mutant. Together, these data suggest that residue K123

appears to be the kinetically preferred site for methylation by Set7/9 *in vitro*, with evidence of lesser methylation occurring at K131.

K131 of Pdx1 is required for transcriptional augmentation by Set7/9

In prior studies, we showed that Set7/9 augments the transcriptional activity of Pdx1 in the context of a Pdx1 reporter (containing 5 tandem Pdx1 binding sites); this result was previously ascribed to Set7/9-mediated methylation of H3 (residue K4) at this reporter (11). Based on our results in Fig. 1, we considered the possibility that this transcriptional augmentation by Set7/9 might instead be a result of Pdx1 Lys methylation. To test this possibility, we performed reporter gene activity assays using wild-type and mutated Pdx1 in the mouse-derived cell line NIH3T3, which is devoid of endogenous Pdx1. As shown in Fig. 3A, when a cDNA encoding wild-type Pdx1 is co-transfected into NIH3T3 cells with a Pdx1 reporter plasmid containing tandem copies of a Pdx1 binding sequence (derived from the rat *Ins1* E2/A4/A3 promoter element) driving luciferase (9), approximately 12-fold activation of luciferase activity is observed. No activation is observed, however, upon co-transfection of the reporter with a cDNA encoding Set7/9 alone (Fig. 3A). When cDNAs encoding both wild-type Pdx1 and Set7/9 are co-transfected, a more-than-additive 18-fold activation of the reporter is observed. Whereas mutation of residue K123 to Arg (K123R) had no effect on reporter gene transactivation, mutation of K131 to Arg (K131R) or the double mutation K123R+K131R resulted in loss of transcriptional augmentation by Set7/9 (Fig. 3A). These effects were specific for the Pdx1 reporter, since no significant effects were observed on the control Gal4 reporter (which contains 5 tandem copies of the yeast Gal4 recognition sequence) (Fig. 3B). Additionally, the effects of the K131R mutation was not caused by changes in Pdx1 levels, since all transfected Pdx1 mutants showed similar intensity on immunoblot analysis (Fig. 3C). These results suggest that although both K123 and K131 appear to be targets for methylation by Set7/9 *in vitro* (with the former being preferred), only K131 appears to be required for the transcriptional augmentation of reporter activity in cells.

Lys methylation has been shown to affect multiple aspects of transcription factor activity, including protein half-life and DNA binding affinity ((17, 35)). We suspected that effects of the K131R mutation on Pdx1 half-life were negligible, since proteins levels of the transfected proteins were identical for wild-type and mutants (Fig. 3C). To test if loss of transcriptional augmentation activity between Pdx1 K131R and Set7/9 was due to decreased interaction between the two proteins, a co-immunoprecipitation assay for Set7/9 and Pdx1 using NIH3T3 cells transfected with wild-type or mutant Pdx1 and Set7/9 was performed (Fig 3D). Neither the K131R mutant nor the K123+K131R double mutant exhibited any differences in their interaction with Set7/9, suggesting that interaction between Pdx1 and Set7/9 is independent of the target Lys residue being methylated.

To ascertain the effects of the Pdx1 mutations on DNA binding affinity, we next performed electrophoretic mobility shift assays (EMSAs). Nuclear extracts from NIH3T3 cells transfected with Pdx1 proteins and Set7/9 were subjected to EMSA with ^{32}P-labeled rat *Ins1* promoter E-A element (9, 36). No apparent differences in the intensity of the Pdx1-specific complex were observed in the EMSAs (Fig. 4A). To quantitate more precisely whether subtle differences in binding affinity exist, we performed competition EMSAs using increasing concentrations of unlabeled E-A probe. Binding competition curves were subsequently calculated and modeled based on single-phase exponential dissociation (Fig. 4B). As shown in the Fig. 4B, none of the mutants exhibited dissociation constants that were different from the wild-type Pdx1 protein. Taken together, the data in Figs. 3 and 4 suggest that the transcriptional augmentation by Set7/9 that is enabled by methylation at K131 likely involves transcriptional events that occur post binding to DNA, such as through the activation of the RNA polymerase II complex at the promoter (11).

Residue K131 of Pdx1 is methylated in cells

We next asked if Pdx1 residue K131 is methylated by Set7/9 in cells. To assess methylation in cells, we generated a peptide-based polyclonal antibody against methylated K131 of Pdx1. Three different peptides (two methylated

and one unmethylated at Lys residues) corresponding to the Pdx1 sequence containing K131 were subjected to dot blots using varying dilutions of the antibody. As shown in Fig. 5A, the antibody exhibited specificity for the Lys-methylated peptides (Fig. 5A). Corresponding specificity of the antibody for methylated full-length Pdx1 was observed using recombinant Pdx1 proteins methylated by Set7/9 *in vitro* (Fig. 5B): as expected, almost complete loss of immunoreactivity was observed with the K131R mutation. However, some cross reactivity of the antibody was observed with methylated K123, since the K123R mutation showed a slight reduction in immunoreactivity (Fig. 5B). These data confirm that K131 is a site for methylation by Set7/9 *in vitro*. This antibody was then used to assess the methylation of Pdx1 proteins immunoprecipitated from cells. Wild-type and mutant Pdx1 constructs containing an N-terminal hemagglutinin (HA) tag were transfected along with Set7/9 into HEK293 cells, which contain no endogenous Pdx1. Pdx1 proteins were immunoprecipitated using an anti-HA antibody, then subjected to immunoblotting using the anti-Pdx1(K131-Me) antibody. As shown in Fig. 5C (lane 2), wild-type Pdx1 was methylated in cells, and the K131R and K123R/K131R double mutants showed clear reduction in methylation. A slight reduction in the K123R mutant is consistent with cross-reactivity of the antibody for methylated K123. Taken together, these data suggest that K131 (and possibly K123) are methylated in cell lines.

To determine if K131 is endogenously methylated in β cells, we immunoprecipitated methylated-Lys proteins from INS-1(832/13) insulinoma cells, then subjected the immunoprecipitate to immunoblotting using an anti-Pdx1 antibody. Initial experiments from INS-1 cells revealed no recovery of methylated Pdx1, raising the concern that β cells may contain demethylases that diminish the ability to detect Pdx1 methylation. To circumvent this possibility, we pretreated INS-1 cells with the Lys demethylase inhibitor BP-107-7 (Fig. 5D, *top panel*). INS-1 cells pretreated with 10 μM BP-107-7 overnight were subjected to immunoprecipitation using anti-Pdx1 antibody and the resulting immunoprecipitate was immunoblotted with anti-methylated Lys antibody. As shown in Fig. 5D (*lower panel*), whereas INS-1 cells treated with vehicle showed no recovery of methylated Pdx1, cells treated with BP-107-7 showed a clear signal for Pdx1. These data suggest that Pdx1 is endogenously methylated and demethylated in β cells. To verify methylation at residue K131 in β cell lines, TAP-tagged Pdx1 was overexpressed in MIN6 β cells using adenoviral gene transfer. The tagged Pdx1 protein was then purified from MIN6 β cells and analyzed by LC/MS/MS. Pdx1 was found to be both di- or tri-methylated at residue K131 (Fig. 2C shows data for the tri-methylated protein). Collectively, these data suggest that K131 is a target of methylation in cells.

Set7/9 is required for the maintenance of normal β cell function and glucose homeostasis in vivo

In prior studies, our group showed that transient knockdown of Set7/9 in islet β cells results in a reduction in several gene targets of Pdx1 (26), yet the link between Set7/9 and β cell function in vivo has not been explored. To address this link more directly, we generated mice in which Cre recombinase recognition (*Loxp*) sites flanked exon 2 of the gene encoding Set7/9 (*Setd7*), such that conditional excision of the exon by Cre recombinase would result in a frame-shift and premature termination of the mRNA (Fig. 6A). This strategy ensures deletion of both the putative Pdx-1 interaction domain (at the N-terminus) and the methyltransferase domain (the SET domain) at the C-terminus. $Setd7^{Loxp/+}$ mice were crossed to mice that express the *Cre* transgene exclusively in islet β cells when treated with tamoxifen ($MIP1\text{-}Cre^{ERT}$, ref. (21)). $Setd7^{Loxp/Loxp}$;$MIP1\text{-}Cre^{ERT}$ mice and littermate controls ($Setd7^{+/+}$;$MIP1\text{-}Cre^{ERT}$ and $Setd7^{Loxp/Loxp}$) were treated with 5 daily doses of tamoxifen at 8 weeks of age to generate β cell-specific Set7/9 knockout mice ($Set^{\Delta\beta}$) and controls, respectively. At 12 weeks of age (~3 weeks after conclusion of tamoxifen treatment), islets isolated from $Set^{\Delta\beta}$ mice showed a clear reduction in Set7/9 levels by immunoblot (Fig. 6B).

$Set^{\Delta\beta}$ mice displayed impaired glucose tolerance following an intraperitoneal glucose challenge (Fig. 6C). To test the possibility that impaired glucose tolerance was caused by β cell dysfunction, we isolated islets from $Set^{\Delta\beta}$ mice and controls and performed glucose stimulated insulin

secretion studies in vitro. As shown in Fig. 6D, islets from $Set^{\Delta\beta}$ mice displayed diminished glucose-stimulated insulin secretion compared to islets from controls, even when corrected for islet insulin content. These data suggest a defect in insulin secretory mechanisms, and is reminiscent of defects seen in *Pdx1*-deficient islets (37, 38). Gene expression analysis of isolated islets revealed reductions in the mRNA levels of key Pdx1-regulated genes, including *MafA*, *Slc2a2*, *Gck* and *Pdx1* itself in $Set^{\Delta\beta}$ islets compared to controls (Fig. 6E). The pre-mRNA and mRNAs encoding preproinsulin showed no change and a ~2-fold increase, respectively, in $Set^{\Delta\beta}$ islets compared to controls, suggesting that the response of this gene may either be delayed compared to the other Pdx1 targets, or that it exhibits more complex regulation in the absence of Set7/9. Taken together, the data from $Set^{\Delta\beta}$ mice suggest a role for Set7/9 in the maintenance of key Pdx1 target genes and overall glucose homeostasis *in vivo*, and parallels the phenotype seen with reductions in Pdx1 itself.

DISCUSSION

Pdx1 is required for both the development and function of islet β cells and, as such, the mechanism underlying its regulation of transcription has been the subject of numerous studies. Nevertheless, the full extent of the mechanisms governing Pdx1 action remains to be elucidated. Set7/9 is a Lys methyltransferase that has been shown to interact with Pdx1 and augment Pdx1 action at several target genes (26). In this study, we provide data suggesting that Set7/9-mediated methylation of Pdx1 augments Pdx1 transcriptional activity. Although other studies have shown that Pdx1 can be regulated by phosphorylation (39–43), O-GlcNAcylation (44), and sumoylation (45), ours is the first study that shows that Pdx1 can be methylated. Moreover, we show the close linkage between Pdx1 and Set7/9 in vivo, where the loss of Set7/9 in β cells closely parallels the phenotype seen with the reduction in Pdx1 levels.

In recent years, the number of identified non-histone targets of Set7/9 methylation has been increasing. Depending upon the protein, Lys methylation appears to affect a variety of functions at the transcriptional level, including protein-protein interactions, protein stability, and DNA binding affinity (17, 35). Notable proteins that are substrates of Set7/9 Lys methylation include p53 (12), estrogen receptor-α (14), nuclear factor κB (13, 15, 46), and TATA box binding protein-associated factor 10 (47), among others. In this study, we show evidence that Pdx1 is methylated at Lys residues in vitro and in β cells. An important finding in vitro was that recombinant Pdx1 was methylated by Set7/9, but occurred only in the context of the full length protein. This requirement for context-dependent methylation seemingly differs from those reported in the literature, where Set7/9 has been shown to methylate Lys residues of specific small peptides, whose sequences could be subsequently used to predict the corresponding full-length protein targets (34, 48). To our knowledge, none of the small peptide sequences presented in those studies correspond to sequences in Pdx1. As such, our studies emphasize that targets of Set7/9 and possibly other SET domain-containing methyltransferases may not be predictable based on peptide sequence specificity. More detailed structural studies will be necessary to identify how specific secondary and tertiary structural folds of Pdx1 allow for its methylation by Set7/9.

Our mass spectrometric and mutational analyses *in vitro* demonstrate that Pdx1 is methylated at two Lys residues in the N terminal transactivation domain, K123 and K131, with K123 being the apparent preferential target for Set7/9 *in vitro*. Interestingly, K131 seems to be the more relevant target of methylation when Pdx1 is expressed in cells, and suggests K131 methylation has greater functional consequences in the context of the cellular milieu. Our finding that Pdx1 methylation becomes more apparent when cells are treated with Lys demethylase inhibitors suggests also that methylation may be transient in cells, or perhaps regulated depending upon the target gene or functions of Pdx1. In this regard, there are several reports that show that proteins methylated by Set7/9 are reciprocally regulated by the demethylase LSD-1 (49–52).

Whereas methylation of Pdx1 by Set7/9 in vitro resulted in mono-methylation of K131 (and K123), our overexpression studies in β cells showed that K131 was di- and tri-methylated. This observation raises an important issue that has been discussed in the literature on the stoichiometry of Lys methylation by Set7/9. Some

studies have suggested that Set7/9 functions as a di-methyltransferase (53), whereas others suggest it is exclusively a mono-methyltransferase (54). These seemingly contradictory observations could be explained by at least three possibilities: (a) mono- vs. di-methylation could be sequence-dependent. In this respect, studies have shown that some peptide sequences are di-methylated by Set7/9 *in vitro*, whereas other peptide sequences are mono-methylated (34); (b) in β cells, interacting proteins may alter the nature of Lys methylation by Set7/9. For example, the methyltransferase G9a exhibits differing stoichiometries of methylation *in vivo* depending the presence of interacting proteins (55); and (c) Set7/9 may be the first in a cascade of different methyltransferases that achieves di- and tri-methylation of Pdx1 residue K131 in cells.

Set7/9 has been previously shown to augment Pdx1 transcriptional activity in reporter gene assays (11). Here, we observed that Pdx1 residue K131 is necessary for this augmentation, since mutation of this residue to Arg (K131R) abrogates transcriptional augmentation. Although the transcriptional augmentation by Set7/9 is only about 40%, the effects of such reductions may have biologically significant consequences in the longer term. We next probed possible reasons for how the K131R mutation abrogates transcriptional augmentation by Set7/9. We found that mutation did not hamper Pdx1-Set7/9 interactions (as determined by co-immunoprecipitation experiments) or DNA binding affinity (based on EMSA analysis). However, It remains possible that binding to target genes in the nucleus may be subtly altered by the mutations. Nevertheless, based on our data, we believe that Pdx1 methylation at K131 may affect the interactions of the Pdx1 transcriptional complex with basal transcriptional machinery. In support of this possibility, we previously showed that Pdx1-Set7/9 interaction was associated with activation of RNA polymerase II (11, 56).

Finally, to interrogate the relationship between Set7/9 and Pdx1 target genes in β cells in vivo, we generated mice with tamoxifen-inducible β cell-specific deletion of Set7/9 ($Set^{\Delta\beta}$). Similar to mice with haploinsufficiency of *Pdx1*, $Set^{\Delta\beta}$ mice exhibited glucose intolerance and β cell dysfunction (37). Several Pdx1 target genes were reduced in $Set^{\Delta\beta}$ mice, consistent with the close functional linkage between Set7/9 and Pdx1. Our results are similar to those we previously published using RNA interference to deplete Set7/9 acutely in islets (26). Although these studies do not definitively prove a link between Set7/9 and methylated Pdx1 (since the loss of Set7/9 may have consequences on broader gene expression), these results do suggest that Pdx1 may be dependent upon the action of Set7/9 for full activity in vivo.

Taken together, our data suggest a previously unappreciated posttranslational modification of Pdx1 that appears to affect its transcriptional activity. Our studies show the methylation of Pdx1 residue K131, the requirement of K131 for the augmentation of gene transcription in reporter assays, the occurrence of K131 methylation in β cell lines, and the requirement for Set7/9 in the maintenance of at least a subset of Pdx1 target genes in vivo. Some limitations and alternative interpretations of our study must be acknowledged. Most notably, it remains possible that the effects observed in our reporter assays or in $Set^{\Delta\beta}$ mice are not directly related to methylation of Pdx1 *per se*, but rather to a separate requirement for residue K131 or Set7/9, respectively, on gene transcription. Gene knock-in studies in mice (using the K131R mutation of Pdx1) may be more appropriate to address this issue in the future. Also, our studies do not exclude the possibility *in vivo* that Set7/9 is *directly* necessary for the maintenance of chromatin structure (histone methylation) at several of the Pdx1 target genes examined, or that it acts in concert with β cell transcription factors other than Pdx1. Nevertheless, our studies provide a framework for understanding better the nuances of transcriptional regulation by Pdx1. We propose a model in which Pdx1 methylation at residue K131 via Set7/9 is required for the maintenance of transcription at selected target genes. Whereas this model does not exclude the possibility (as purported in prior studies) that histone methylation by Set7/9 also contributes to transcriptional activity, it emphasizes that transcription factor-cofactor interactions can achieve functional transcriptional complexes in many ways.

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FOOTNOTES

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[1]Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN
[2]Department of Pediatrics and the Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN
[3]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN
[4]Department of Medicine, Indiana University School of Medicine, Indianapolis, IN
[5]Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Lexington, KY
[6]Department of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, Charleston, SC
[7]Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN

[8]Abbreviations used are: EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; *Set*$^{\Delta\beta}$, β cell-specific Set7/9 knockout; TAP, tandem affinity purification tag

AUTHOR CONTRIBUTIONS

AVM and RGM performed research, designed experiments, contributed to discussion, and wrote the manuscript; BM, SAT, ALM, and CSH performed research and contributed to discussion; MLS, SO, BP, and PMW performed research; RS designed experiments and contributed to discussion. All authors reviewed and edited the manuscript.

FIGURE LEGENDS

Figure 1. Pdx1 methylation by Set7/9 in vitro. *(A)* Methylation assay in vitro using recombinant Set7/9, ^{3}H-AdoMet, and increasing concentrations of Pdx1 protein was performed, then reactions were subjected to polyacrylamide gel electrophoresis. *Upper panels* show fluorography for ^{3}H and *lower panels* show corresponding Coomassie staining of the same gel; *(B)* Methylation assay in vitro using recombinant Set7/9, 3H-AdoMet, and full-length or truncated Pdx1 proteins was performed, followed by polyacrylamide gel electrophoresis. Schematic representation of truncated mutants of Pdx1 is shown in the *right*, and corresponding ^{3}H fluorography and Coomassie stains are shown on the *left*; *(C)* Methylation assays *in vitro* using wild-type (*WT*) and mutated Pdx1 proteins was performed, with corresponding quantitation of methylated Pdx1 protein intensities (normalized to total Pdx1 protein by Coomassie staining). All images shown are representative of at least N=3 experiments. *p<0.05 compared to wild-type Pdx1.

Figure 2. MS/MS spectra of Pdx1 in vitro and in MIN6 cells. *(A)* Tandem mass spectrum of the peptide GQLPFPWMK showing modification of K123 with mono-methylation (+14 dalton) following treatment with recombinant Set7/9 in vitro. The SEQUEST XCorr for this +2 peptide is 1.83 with a ppm of -1.65; *(B)* Tandem mass spectrum of the +3 peptide AHAWKGQWAGGAYAAEPEENKR showing mono-methylation (+14 dalton) of K131 in Pdx1 following treatment with recombinant Set7/9 in vitro. The SEQUEST XCorr for this peptide is 6.19 with a ppm of 0.34; *(C)* Representative MS/MS spectrum from an LTQ ion trap for the +2 peptide AHAWKGQWAGGAYAAEPEENKR showing tri-methylation at K131 in Pdx1 in transduced MIN6 β cells. The SEQUEST XCorr for this peptide is 2.24 with a ppm of 19.25.

Figure 3. Transcription augmentation by Set7/9 is dependent upon Pdx1 residue K131. NIH3T3 were transiently co-transfected with Set7/9, wild-type (*WT*) and mutant Pdx1 proteins, and either Pdx1 reporter plasmid or Gal4 reporter plasmid. Cells were harvested 48 hours later and whole cell extracts were used to assay luciferase activity. *(A)* Luciferase activities (relative to control transfections without Pdx1) with the Pdx1 reporter plasmid, which contains tandem elements of the *Ins* E2/A3/A4 element (schematic diagram of reporter shown at the *top*). N=3 transfections in triplicate, *P<0.05 for the comparisons shown; *(B)* Luciferase activities (relative to control transfections without Pdx1) with the Gal4 reporter plasmid, which contains tandem elements of the yeast Gal4 DNA binding domain (schematic diagram of reporter shown at the *top*). N=3 transfections in triplicate; *(C)* Immunoblots (for the indicated proteins) from whole cell extract from transfected cells. Results are representative of N=3 transfections; *(D)* NIH3T3 cells were transfected with FLAG-Set7/9 and the indicated Pdx1 proteins, then nuclear extracts were subjected to immunoprecipitation using FLAG antibody, the immunoblotted using Pdx1 antibody. Results are representative of N=2 experiments.

Figure 4. Point mutations do not affect DNA binding by Pdx1. *(A)* NIH3T3 cells were co-transfected with the wild-type (*WT*) or mutant Pdx1 proteins, with or without cotransfection of Set7/9. Nuclear extracts from transfected cells were harvested and subjected to EMSA using ^{32}P-labeled E-A DNA fragment containing a Pdx1 binding site derived from the *Ins* E2/A3/A4. The positions of the Pdx1-containing complex and the unbound, free probe (FP) are indicated by the *arrows*. The *bottom panel* shows immunoblots for Pdx1 and GAPDH from the same nuclear extracts used for the EMSA; *(B)* Quantitation and modeling of EMSA reactions (as in *A*), with increasing concentrations of unlabeled E-A probe. For each Pdx1 mutant, the control condition is the fraction of total probe bound in the absence of competitor. The ordinate represents probe binding (at the given competitor concentration) as a percent of the control condition. The apparent dissociation constants of the WT and mutant proteins is shown in the inset, with no statistical differences seen. Data shown are from N=3 EMSA experiments.

Figure 5. Residue K131 of Pdx1 is methylated in cells. *(A)* Dot blot analysis using two dilutions of anti-Pdx1(K131-Me) antibodies and methylated and unmethylated peptides corresponding to the N-terminus of Pdx1 containing residue K131; *(B)* Representative immunoblots (*top panels*) using anti-Pdx1(K131-Me) antibody or anti-Pdx1 antibody on recombinant Pdx1 proteins methylated by Set7/9 in vitro. Quantitation of immunoblots (N=3) is shown in the *bottom panel* bar graph. **P<0.05* compared to Pdx1 alone; (C) HEK293 cells were transfected with Set7/9 and wild-type (*WT*) or mutant HA-tagged Pdx1 proteins, then whole-cell extracted were immunoprecipitated using anti-HA antibody, followed by immunoblot analysis using anti-Pdx1 and anti-Pdx1(K131-Me) antibodies (*top panels*). Quantitation of methylated Pdx1 (relative to total immunoprecipitated Pdx1) (N=3) is shown in the *bottom panel* bar graph. **P<0.05* compared to WT Pdx1; (D) *Top:* chemical structure of the Lys demethylase inhibitor BP-107-7 (*top*). *Bottom:* INS-1 832/13 β cells were treated with vehicle or BP-107-7 (10 μM) overnight, then nuclear extracts were immunoprecipitated using anti-methyl Lys antibody followed by immunoblot using anti-Pdx1 antibody.

Figure 6. β cell-specific deletion of Set7/9 in mice. *(A)* Schematic representation of WT and mutant alleles present in control and mutant mice, respectively, showing positions of the exons (*numbered*) and the *Loxp* sites; (B) Immunoblot analysis of whole cell lysates of islets isolated from $Setd7^{+/+}$;$MIP1\text{-}Cre^{ERT}$ (*MIP1-Cre*), $Setd7^{Loxp/Loxp}$ (*Lox/Lox*), and $MIP1\text{-}Cre^{ERT}$;$Setd7^{Loxp/Loxp}$ ($Set^{\Delta\beta}$) mice for Set7/9 (*upper panel*) and GAPDH (*lower panel*); *(C)* Results of intraperitoneal GTT on *MIP1-Cre* (N=12), *Lox/Lox* (N=9) and *SetΔβ* (N=12) mice (*left panel*), with corresponding area under the curve (*AUC*) analysis (*right panel*). **P<0.05* compared to either *MIP1-Cre* or *Lox/Lox*; *(D)* Results of glucose-stimulated insulin release assays from islets isolated from *MIP1-Cre, Lox/Lox, and* $Set^{\Delta\beta}$ mice (*left panel*), and corresponding ratio of insulin secreted at high glucose relative to low glucose is shown in the *right panel.* N=3, **P<0.05* compared to either *MIP1-Cre* or *Lox/Lox*; (E) Results of real-time RT-PCR for the indicated genes from islets isolated from control *(MIP1-Cre* and *Lox/Lox*) vs. *SetΔβ* mice. Data are normalized to *Actb* mRNA levels, N=4 independent islet isolations, **P<0.05* compared to control.

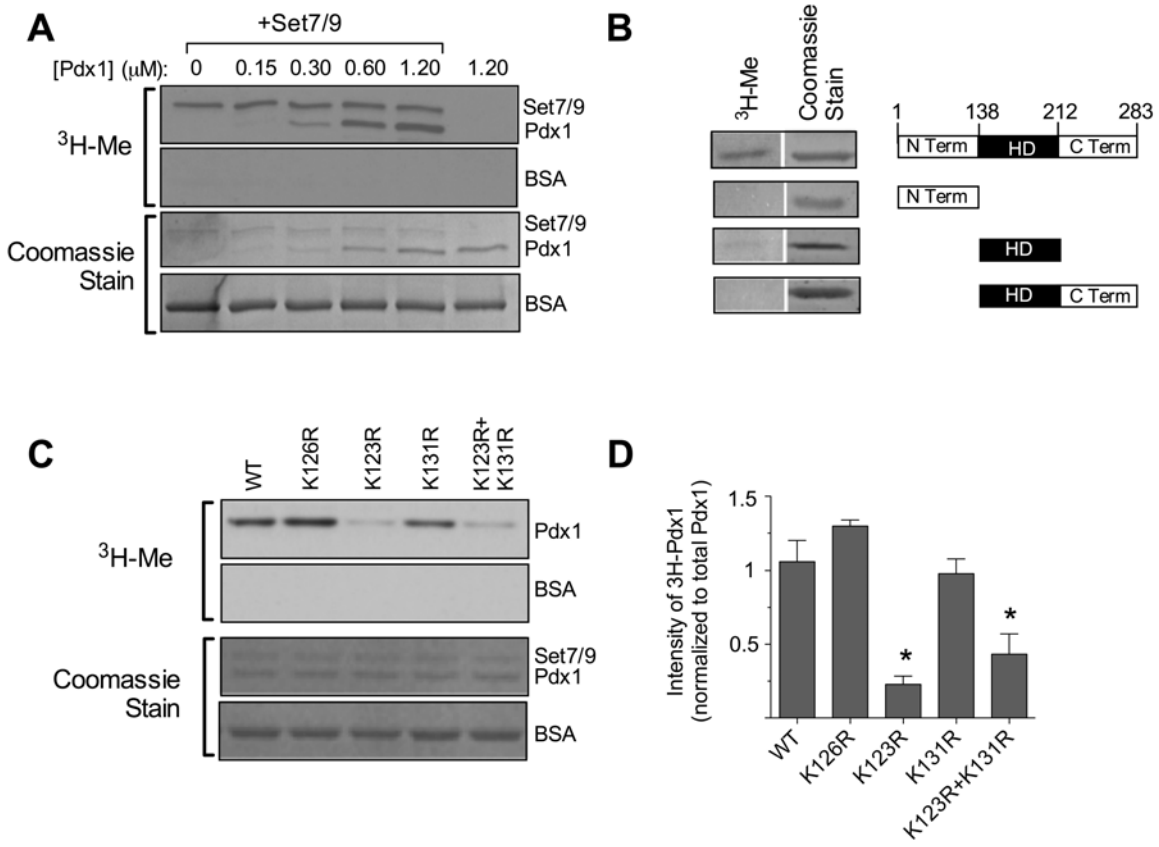

Figure 1

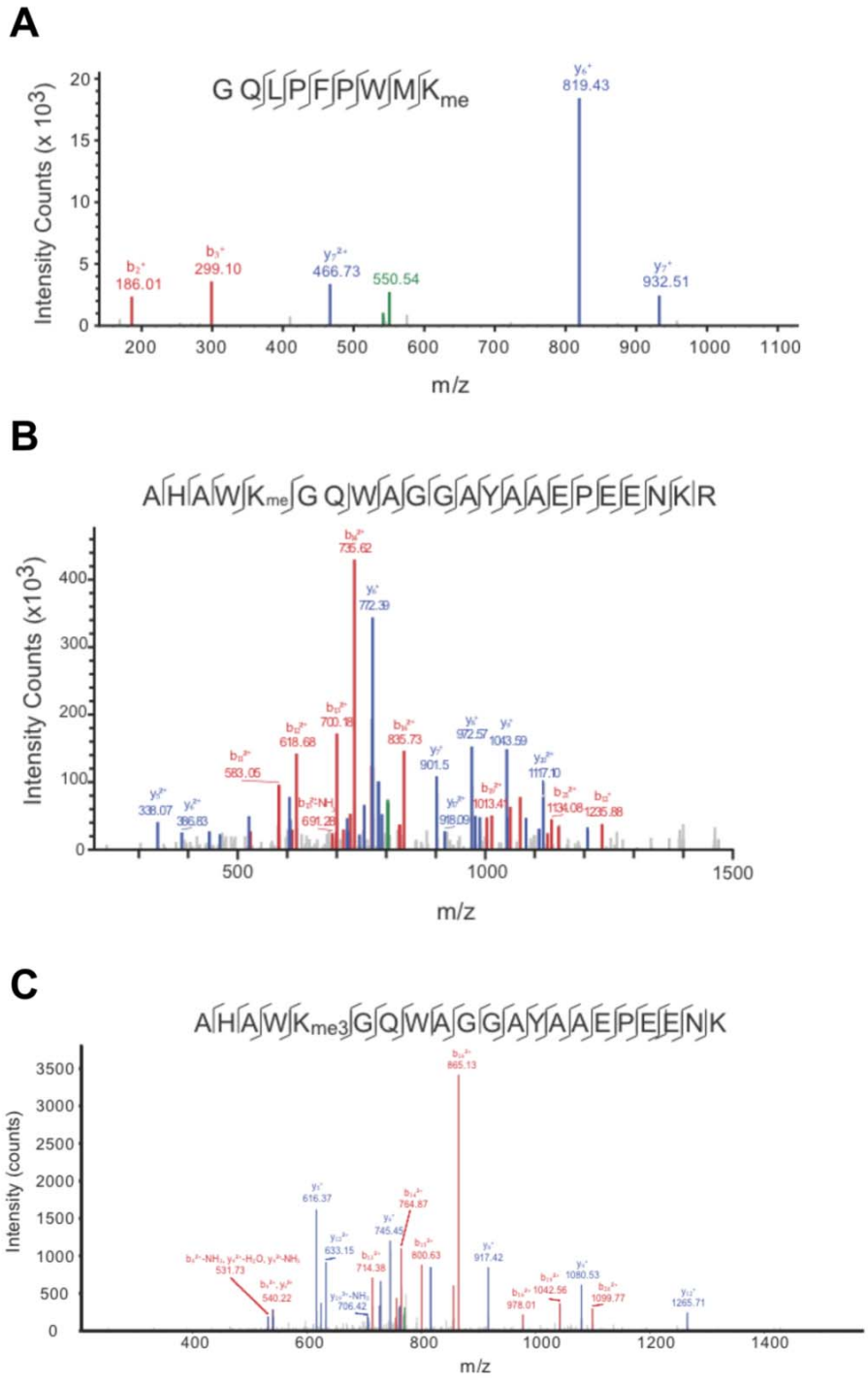

Figure 2

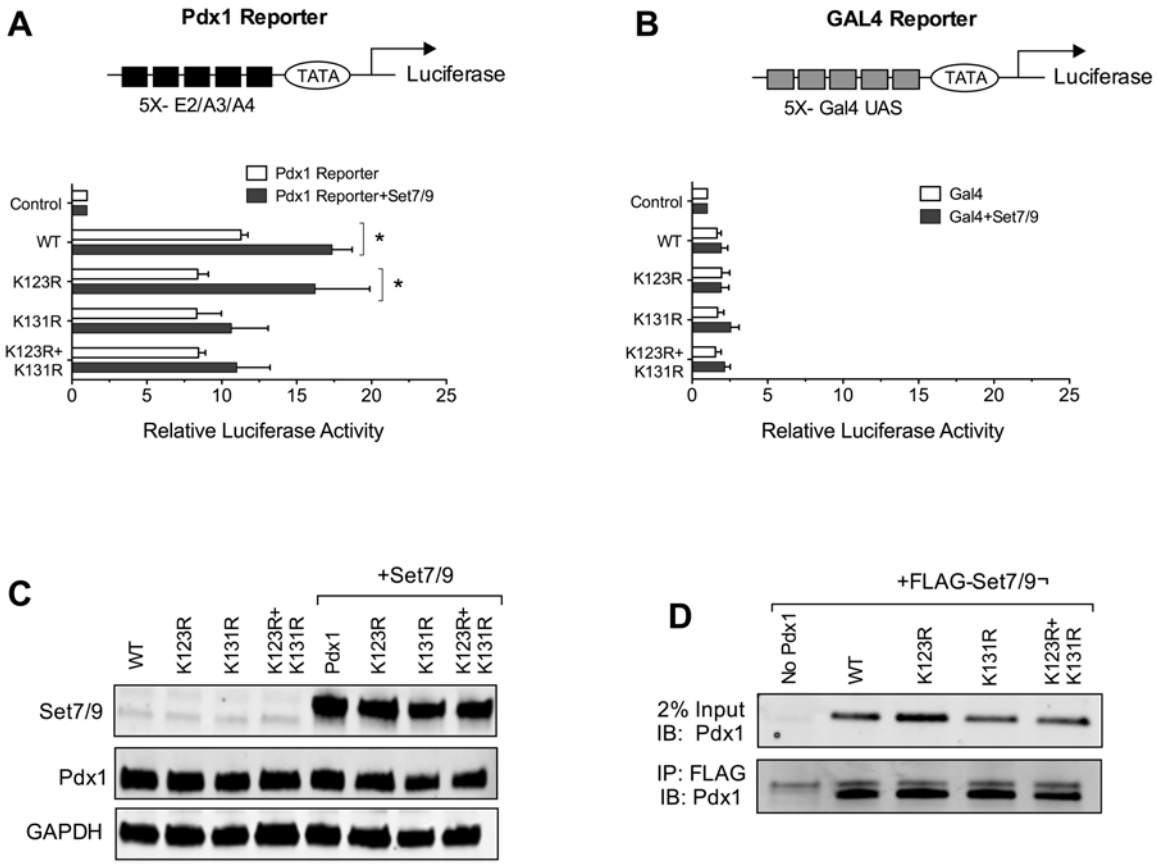

Figure 3

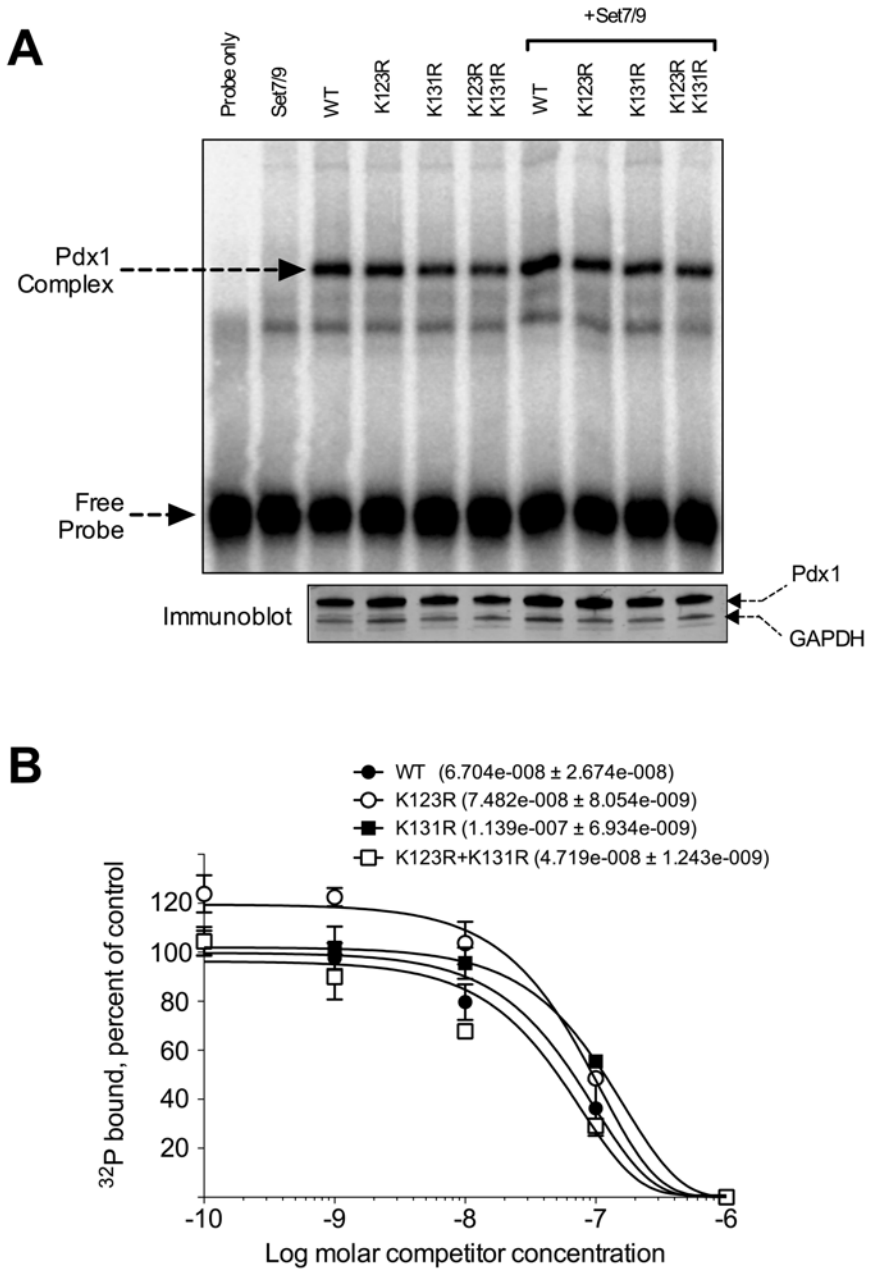

Figure 4

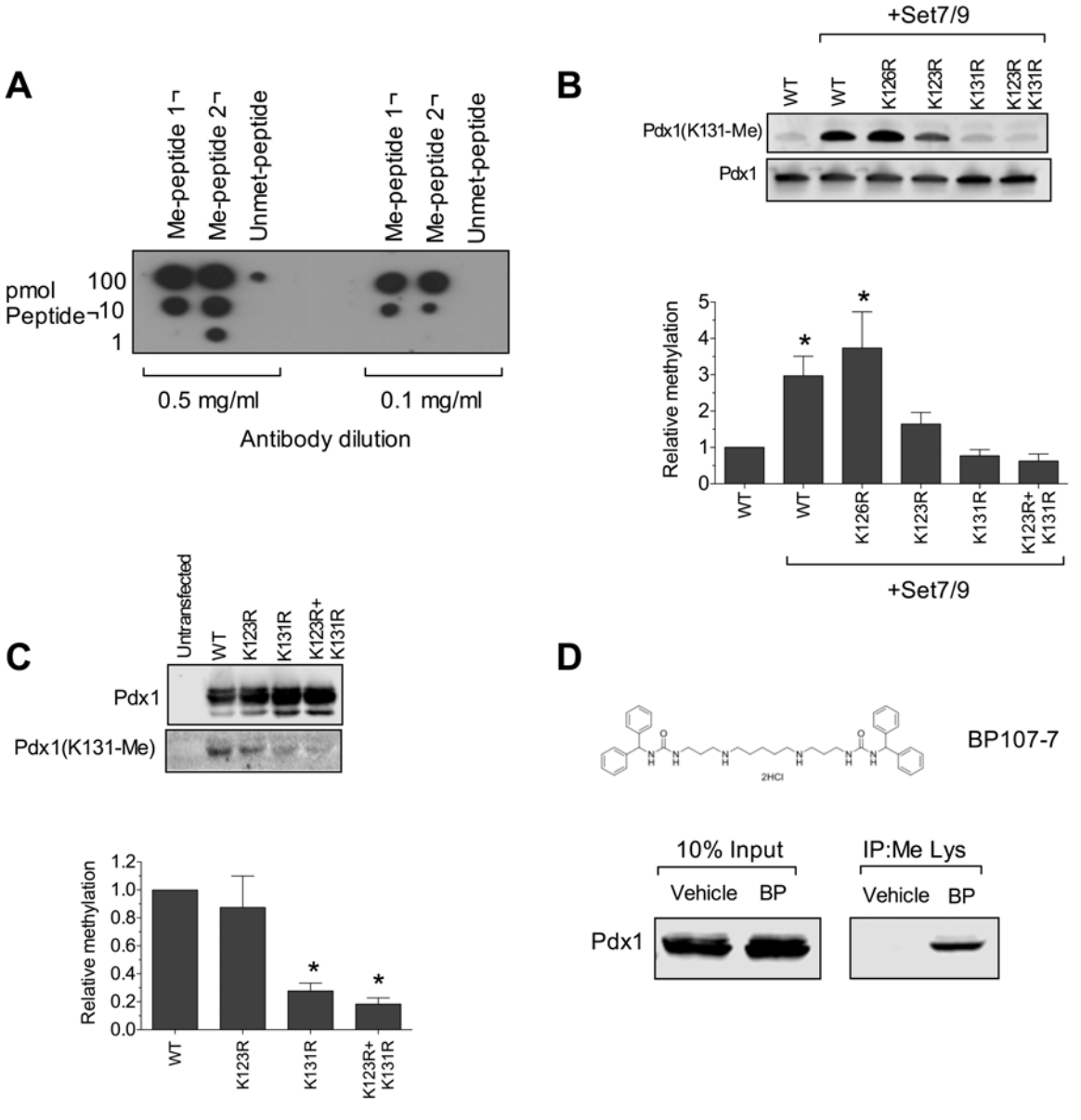

Figure 5

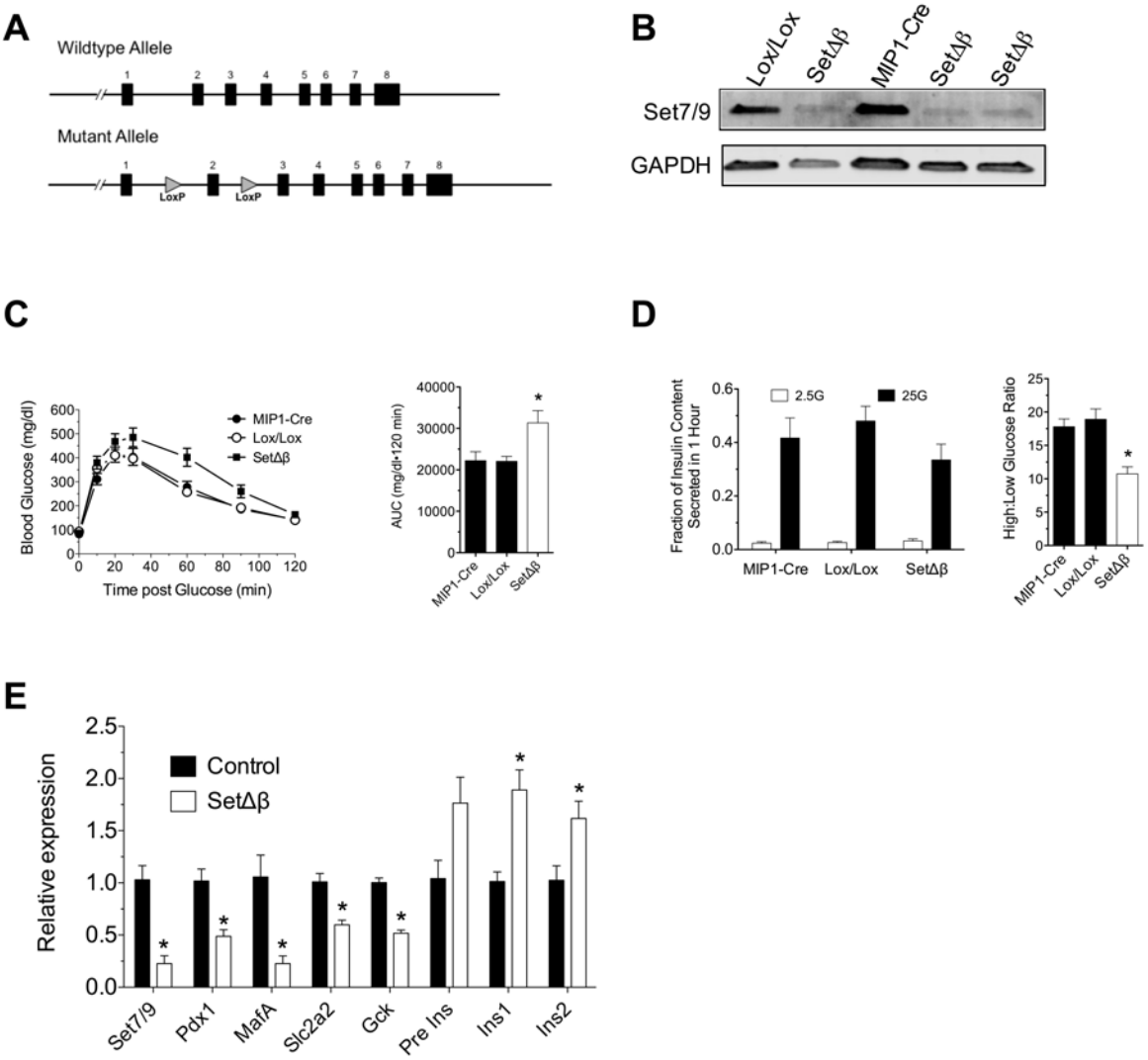

Figure 6

Gene Regulation:

Transcriptional Activity of the Islet β Cell Factor Pdx1 is Augmented by Lysine Methylation Catalyzed by the Methyltransferase Set7/9

Aarthi V. Maganti, Bernhard Maier, Sarah A. Tersey, Megan L. Sampley, Amber L. Mosley, Sabire Ozcan, Boobalan Pachaiyappan, Patrick M. Woster, Chad S. Hunter, Roland Stein and Raghavendra G. Mirmira

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