THE METHYLTRANSFERASE SET7/9 MAINTAINS TRANSCRIPTION AND EUCHROMATIN STRUCTURE AT ISLET-ENRICHED GENES

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ABSTRACT

Objective: The activation of β cell genes, particularly of that 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 β 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 α and β cells, as well as occasional cells within ducts. Consistent with these findings, the Set7/9 gene promoter contained an islet-specific enhancer located between −5070 to −6030 bp (relative to the transcriptional start site) that exhibited Pdx1-responsive activation in β 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 Ca²⁺ mobilization and insulin secretion.

Conclusion: We conclude that Set7/9 is required for normal β cell function, likely through the maintenance of euchromatin structure at genes necessary for glucose-stimulated insulin secretion.
It is becoming increasingly clear that the pathogenesis of Type 1 and Type 2 diabetes involves progressive dysfunction at the level of the islet β cell (1). Thus, the most effective therapeutic approaches to diabetes should include efforts to address β cell dysfunction, and further research into the mechanisms underlying β cell gene transcription will be paramount in these efforts. In recent years, a model has been emerging that emphasizes the role of β cell-specific transcription factors such as Pdx1, Nkx6.1, NeuroD1, and Maf factors in the expression and regulation of genes that are crucial to the development and function of β cells (see refs. (2-4) for recent reviews). However, transduction of a variety of non-β cell types with these and other transcription factors has yielded limited success in activating β cell genes, and suggests that this model of β 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 N-terminal histone tails. The roles of chromatin and histone modifications in β 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 β 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 β 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 (MLL) 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 β 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 an 366 amino acid enzyme containing a C-terminal SET domain that harbors methyltransferase activity (12, 13). This methyltransferase activity is not only specific for H3-Lys4, but also for specific 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 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 crucial enzyme in the transcriptional regulation of β cell genes.
novel enzymatic cofactor necessary for maintenance of islet gene transcription.

**RESEARCH DESIGNS AND METHODS**

**Antibodies and cells.** Polyclonal antibody against monomethyl-H3-Lys4 was from Abcam, and antibodies against dimethyl-H3-Lys4, Pdx1, and Set7/9 were from Millipore; anti-RNA polymerase II CTD was from Covance; anti-actin, anti-glucagon, and anti-insulin were from Santa Cruz Biotechnology. 

βTC3 cells, INS-1 (832/13), αTC1.6, and NIH3T3 were maintained as previously described (20, 21). Islets were isolated from C57BL/6 mice following collagenase digestion of pancreatic tissue as described (22). Techniques and protocols were approved by the Indiana University Institutional Animal Care and Use Committee.

**Real-time RT-PCR.** Real time reverse transcriptase (RT)-PCR was performed using forward and reverse primers to amplify insulin pre-mRNA (5´-GGGGAGCGTGCTTCTTCTA-3´ and 5´-GGGGACAGAATTCAGTGGCA-3´) or Actb (5´-AGGTCATCAGTGGCAACGA-3´ and 5´-CCTCACTCATATTGGCAACGA-3´) as described in detail previously (23). Primers used to amplify the Ins1/2 promoter were 5´-TACCTTGCTGCTGAGCTGC-3´ (forward) and 5´-TACCTTGCTGCTGAGCTGC-3´ (reverse); Ins1/2 coding region, 5´-TGGCTTCTTCTACACCCAGAAG-3´ (forward) and 5´-ACATGGGCACCTCATTTCCACATCC-3´ (reverse); Slc2a2 promoter, 5´-ATCTGCTCAGTGGCAATCAT-3´ (forward) and 5´-ATCTGCTCAGTGGCAATCAT-3´ (reverse); Pdx1 promoter, 5´-GAACCTTGACTTTTGCTTGCTGCTTCTGTCTAGG-3´ (forward) and 5´-GAACCTTGACTTTTGCTTGCTGCTTCTGTCTAGG-3´ (reverse); Set7 promoter, 5´-TTGCTGCTGCTGAGCTGC-3´ (forward) and 5´-TTGCTGCTGCTGAGCTGC-3´ (reverse); and Foxa2 promoter, 5´-TACCTTGCTGCTGAGCTGC-3´ (forward) and 5´-TACCTTGCTGCTGAGCTGC-3´ (reverse).

**Immunoblot Assays.** Whole cell extracts were prepared from homogenized tissues, 50 islets, or 1x10⁶ βTC3 cells by lysis in a buffer containing sodium dodecyl sulfate, and 5 µg of extract were 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 (ChIP) Assays.** ChIP assays were performed as described previously (20). Briefly, 10⁷ βTC3 cells or 300 mouse islets were fixed in 1% formaldehyde to crosslink 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, then recovery of co-immunoprecipitated DNA fragments was assessed using SYBR Green-based quantitative real-time PCR. Data are expressed as the percent recovery of co-immunoprecipitated DNA relative to input DNA (prior to immunoprecipitation). Primers used to amplify the Ins1/2 promoter were 5´-TACCTTGCTGCTGAGCTGC-3´ (forward) and 5´-GCAATTTCCACATCATCC-3´ (reverse); Ins1/2 coding region, 5´-TGGCTTCTTCTACACCCAGAAG-3´ (forward) and 5´-ACATGGGCACCTCATTTCCACATCC-3´ (reverse); Slc2a2 promoter, 5´-ATCTGCTCAGTGGCAATCAT-3´ (forward) and 5´-ATCTGCTCAGTGGCAATCAT-3´ (reverse); Pdx1 promoter, 5´-GAACCTTGACTTTTGCTTGCTGCTTCTGTCTAGG-3´ (forward) and 5´-GAACCTTGACTTTTGCTTGCTGCTTCTGTCTAGG-3´ (reverse); Set7 promoter, 5´-TTGCTGCTGCTGAGCTGC-3´ (forward) and 5´-TTGCTGCTGCTGAGCTGC-3´ (reverse); and Foxa2 promoter, 5´-TACCTTGCTGCTGAGCTGC-3´ (forward) and 5´-TACCTTGCTGCTGAGCTGC-3´ (reverse).

**Immunohistochemistry.** Mouse pancreata were fixed by cardiac perfusion with 4% paraformaldehyde, paraffin embedded, and
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Human pancreatic samples were obtained from the University of Virginia Human Islet Transplant Program and 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, 1:2000 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 (Amaza, Inc.) and transfected into 3x10^6 βTC3 mouse insulinoma cells using an Amaza Nucleofector (program D-23) according to the manufacturer’s protocol. Cells were then plated in a 6-well dish containing culture medium for 72 hrs, then harvested for protein, total RNA, or ChIP analysis. siRNA sequences used were: siSet97, 5’-GGUUUAUGUUGCCGACUCTT-3’; siSet98, 5’-GGUAGCAGUUGGACCUAAUTT-3’; siControl, 5’-AAAGUCGACCUUCAGUAAGUU-3’.

GSIS and GSCa studies. For glucose-stimulated insulin secretion (GSIS) studies, 50 islets per condition were incubated in Krebs-Ringer HEPES buffered solution for 1 h at 37°C, and then placed them 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 enzyme-linked immunosorbent assay (Alpco Diagnostics). Glucose-stimulated Ca^{2+} mobilization (GSCa) in mouse islets was measured using the ratiometric Ca^{2+} indicator fura-2 AM as described previously (24). GSCa was defined as the difference between ratio measurements (340/380 nm fluorescence) in 11 mM vs. 3 mM glucose. Data were analyzed with IP Lab® software version 4.0 (Scanalytics).

Cloning of the Setd7 promoter. A genomic clone containing approximately 8000 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 the manufacturer’s instructions. All sequences were confirmed by automated DNA sequencing.

Transfections and reporter assays. 10^6 βTC3/αTC1.6/INS-1 cells or 5x10^5 NIH3T3 cells were seeded in 6-well plates 24 hour before transfection. 2 µg of plasmid diluted into 0.1 ml of PBS was mixed with 6 µl of Metafectene Pro (Biotex) and incubated at room temperature for 15 minutes. The transfection mixture was added to each well of a 6-well plate along with 2 ml of medium without antibiotics. 6 hours after transfection, medium was replaced with fresh medium containing antibiotics. 48 hours after transfection, cells were harvested and...
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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 reverse transcriptase (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, protein levels of Set7/9 do not completely mirror that of 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 post-transcriptional 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. Fig. 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. Co-immunofluorescence 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 co-stained 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).

\(\beta\) cell-specific transcription of the Set7/9 gene. The islet-specific enrichment of Set7/9 suggested to us 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, ref. (25)). These constructs were then transfected into the \(\beta\) cell-derived cell lines \(\beta\)TC3 and INS1, 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 -6584 base pairs (bp) upstream of the transcriptional start site. This enhancement was significantly attenuated when a fragment containing -3131 bp from the transcriptional start site was used, suggesting the potential for a \(\beta\) cell-specific enhancer located between -3131 bp and -6584 bp. To explore this possibility further, we next performed an 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 -3131 bp and -6584 bp exhibited only about 25% identity between these species (data not shown), we identified a region between –5768 bp and –6030 bp that exhibited approximately 68% identity (see 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)) (see 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 activity in \(\beta\)TC3 and INS-1 \(\beta\) cell lines, but not in NIH3T3 cells. Upon co-transfection with a plasmid
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encoding Pdx1, this fragment displayed an approximately 25-fold enhancement in the β cell lines INS-1 and βTC3 (Fig. 2C, D). Mutation of either putative Pdx1 binding site attenuated activity of this fragment in both β cell lines (Fig. 2C, D). Pdx1 appears to occupy the endogenous Setd7 gene in βTC3 cells within the region around -6000 bp, as assessed by the chromatin immunoprecipitation (ChIP) assay, but does not occupy the endogenous gene encoding β-actin (Fig. 2E). Together, these results suggest that the Setd7 gene may contain elements that enhance its expression specifically in β 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 β cell lines and islets. To determine the role of Set7/9 in the regulation of β cell specific genes, we next depleted βTC3 cells of Set7/9 protein and analyzed gene expression by real-time RT-PCR. To knockdown protein, two different siRNAs (siSet-97 and siSet-98) targeted against murine Set7/9, or a control siRNA (siControl), were transfected into βTC3 cells. As shown in Fig. 3A, greater than 90% reductions in Set7/9 protein were seen following transfections with each siRNA compared to 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 β 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 amongst all genes responsible for glucose responsiveness in islets, as those encoding Pdx1 (Pdx1), the ATP-sensitive potassium channel (Kcnj11), sulfonylurea receptor (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 intereference 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 knockdown 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 4 daily intraperitoneal injections of Cy3-labeled double-stranded RNA into male C57BL/6 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 (siSet-1 and siSet-2) or a control siRNA (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, and glucose-stimulated calcium mobilization (GSCa) and glucose-stimulated insulin secretion (GSIS). As shown in Fig. 4B, islet Set7/9 protein was reduced by at least 60% by treatment with either siRNA compared to 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 βTC3 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 upon an intact signaling cascade that begins with the transport of glucose into the β 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 siSet-1 or siSet-2 resulted in GSCa responses (from 3 to 11 mM glucose) that were only about 50% of control. These islets also showed diminished GSIS upon raising glucose from 3 to 11 mM (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 βTC3 cells (10). This observation, coupled with the finding that the Ins1/2 gene is hyper-methylated 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 asked if 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 βTC3 cells using either of two siRNAs (siSet-97 or siSet-98) 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 siSet-1, 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 if Set7/9 could be modifying histones directly at the Slc2a2 gene, we next performed ChIP assays in βTC3 cells to assess recruitment of Set7/9 to this gene. Fig. 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 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 not related 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 asked of the maintenance of Ins1/2 transcription by Set7/9 might be related to Pdx1. As shown in Fig. 8A, knockdown of Set7/9 in βTC3 cells using siSet-97 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 approximately 50% (Fig 8B). These results suggest that Set7/9 action at the Ins1/2
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DISCUSSION

The islet β 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 β 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), but 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 β cells may occur at the level of transcription, as our studies identified a potential conserved β cell-specific enhancer located in the distal Setd7 gene that is responsive to Pdx1. Its strongly nuclear pattern of distribution, particularly in β cells, suggested us a role for Set7/9 in β cell gene regulation.

To identify genes regulated by Set7/9 in β cells, we engaged RNA interference approaches to knockdown Set7/9 in insulinoma-derived β cells (βTC3) 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 GSIS 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 upon binding to accessory proteins (36).

In the absence of endogenous nuclear localization and DNA binding domains, we
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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 βTC3 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, refs. (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 that suggest 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 β cell genes. We propose that Set7/9 is recruited to specific β 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 upon 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 β cell genes in mature cells, the role of Set7/9 in establishing transcriptional patterns during the embryonic development of β cells remains to be determined. In future studies, we will directly address its role in embryonic development using conditional Set7/9 knockout mice.

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Abbreviations: bp, base pairs; ChIP, chromatin immunoprecipitation; GSCa, glucose-stimulated calcium mobilization; GSIS, glucose-stimulated insulin secretion; RT-PCR, reverse transcriptase PCR; siRNA, small interfering RNA
REFERENCES


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Figure 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, 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 10X (C) and 40X (D) magnifications are shown; (E-M), Dual-immunofluorescence staining of representative islets from pancreatic sections showing localization of Set7/9 (E, H, K) with insulin (F, G), glucagon (I, J), or Pdx1 (L, M).
Figure 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 fragment of the Setd7 promoter (-5070 to -6030) 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, D), Setd7 gene fragments (wild-type and mutagenized at potential Pdx1 binding sites) were co-transfected with a cytomegalovirus promoter-driven plasmid (pBAT12) expressing Pdx1 into either βTC3 (C) or INS-1 (D) cells. Luciferase activity was normalized to activity of cells transfected with the backbone pBAT12 plasmid. Data in all panels represent the average of at least 3 independent transfections; (E), results of a ChIP assay using βTC3 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 to 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 3 independent ChIP experiments.
Figure 3. Set7/9 is necessary for the expression of a subset of glucose-responsive genes in βTC3 cells. βTC3 cells were transfected with 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 4 independent transfections, and “*” indicates that the values differ significantly (p<0.05) compared to siControl transfections.
Figure 4. **Set7/9 knockdown in mouse islets.** Male C57BL/6J mice were administered intraperitoneal injections of stabilized siRNAs as detailed 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 β 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 (siSet-1 and siSet-2). Shown are immunoblots of islet, brain, liver, and muscle extracts from siRNA-injected mice for Set7/9 and Actin.
Figure 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 (siSet-1 and siSet-2) as detailed 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 4 independent transfections, and “*” indicates that the values differ significantly (p<0.05) compared to siControl transfections; (B, 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 3 mice injected with each siRNA on a single occasion, and data in (C) represent the average of 50 islets from 6 injected mice. Statistical significance is indicated by the p value (from t test) for the comparisons shown.
Figure 6. Set7/9 is necessary for the maintenance of dimethylated H3-Lys4 at specific genes in βTC3 cells. βTC3 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 prior to 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 3 independent ChIP assays from at least 3 independent siRNA transfections, and “*” indicates that the value is statistically different (p<0.05) from siControl transfections. The amplified region of the genes (in bp) relative to the transcriptional start site is indicated in parentheses below each gene.
**Figure 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 βTC3 cells. (A), Male C57BL/6 mice were injected intraperitoneally with stabilized control siRNA or siRNA against Set7/9 (siSet-1) as detailed 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 3 independent ChIP experiments from mice injected on 3 separate occasions; (B), βTC3 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 3 independent ChIP assays, and “*” indicates that the value is statistically different (p<0.05) from ChIP assays in which normal rabbit serum was used in place of Set7/9 antibody.
Figure 8. Loss of Set7/9 effects Pol II but not Pdx1 recruitment. βTC3 cells were transfected with siControl or siSet-97 and subjected to ChIP using normal rabbit serum (-Ab) or antibodies to Pdx1 or RNA polymerase II (CTD) (+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 prior to ChIP. (A), ChIP experiment using antibody to Pdx1; (B) ChIP experiment using antibody to RNA polymerase II (CTD). Data represent the average of 3 independent ChIP assays from 3 independent siRNA transfections. **” indicates that the value is statistically different (p<0.05) from siControl transfections.