MECHANISMS OF TRANSCRIPTIONAL REGULATION IN THE MAINTENANCE OF β CELL FUNCTION

Aarthi Vijaykumar Maganti

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Raghavendra G. Mirmira, MD, PhD, Chair

Debbie C. Thurmond, PhD

Doctoral Committee

Paul B. Herring, PhD

December 4, 2014

Carmella Evans-Molina, MD, PhD

Amber L. Mosley, PhD

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The islet β cell is central to the maintenance of glucose homeostasis as the β cell is solely responsible for the synthesis of Insulin. Therefore, better understanding of the molecular mechanisms governing β cell function is crucial to designing therapies for diabetes. Pdx1, the master transcription factor of the β cell, is required for the synthesis of proteins that maintain optimal β cell function such as Insulin and glucose transporter type 2. Previous studies showed that Pdx1 interacts with the lysine methyltransferase Set7/9, relaxing chromatin and increasing transcription. Because Set7/9 also methylates non-histone proteins, I hypothesized that Set7/9-mediated methylation of Pdx1 increases its transcriptional activity. I showed that recombinant and cellular Pdx1 protein is methylated at two lysine residues, Lys123 and Lys131. Lys131 is involved in Set7/9 mediated augmented transactivation of Pdx1 target genes. Furthermore, β cell-specific Set7/9 knockout mice displayed glucose intolerance and impaired insulin secretion, accompanied by a reduction in the expression of Pdx1 target genes. Our results indicate a previously unappreciated role for Set7/9 in the maintenance of Pdx1 activity and β cell function. β cell function is regulated on both the transcriptional and translational levels. β cell function is central to the development of type 1 diabetes, a disease wherein the β cell is destroyed by immune cells. Although the immune system is considered the primary instigator of the disease, recent studies suggest that defective β cells may initiate the autoimmune response. I tested the hypothesis that improving β cell function would reduce immune infiltration of the islet in the NOD mouse, a mouse model of spontaneous type 1 diabetes. Prediabetic NOD mice treated with pioglitazone, a drug that improves β cell function, displayed an improvement in β cell function, a reduction in

β cell death, accompanied by reductions in β cell autoimmunity, indicating that β cell dysfunction assists in the development of type 1 diabetes. Therefore, understanding the molecular mechanisms involved in β cell function is essential for the development of therapies for diabetes.

Raghavendra G. Mirmira, MD, PhD, Chair

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

PP .....Pancreatic Polypeptide

IR ..... Insulin Receptor

Glut4 .....Glucose Transporter 4

IRS ..... Insulin Receptor Substrate

ATP ..... Adenosine Triphosphate

Pdx1 ..... Pancreatic Duodenal Homeobox 1

RNA Pol II ..... RNA polymerase II

HDAC ..... Histone Deacetylase

HAT ..... Histone Acetyltransferase

DNMT ..... DNA methyltransferase

H3K4 .....Histone 3 Lysine 4

LSD-1 ..... Lysine Specific Demethylase-1

Slc2a2 ..... Glucose transporter 2 gene

Gck..... Glucokinase gene

Pbx1 ..... Pre-B cell leukemia 1

pRb ..... Retinoblastoma protein

GSK3β ..... Glycogen Synthase Kinase 3β

NF-κB ..... Nuclear Factor-κB

ERα ..... Estrogen Receptor α

ER ..... Endoplasmic Reticulum

UPR ..... Unfolded Protein Response

Bip ..... Binding IgG Protein

PERK ..... Protein Kinase RNA (PKR)-like ER Kinase

IRE1α ..... Inositol Requiring Enzyme 1α

ATF6 ..... Activating Transcription Factor 6

ATF4 ..... Activating Transcription Factor 4

Wfs1 ..... Wolfram Syndrome 1

TNFα ..... Tumor Necrosis Factor α

IL-1β ..... Interleukin 1- β

CHOP ..... cEBP Homologous Protein

sXbp1 ..... spliced X-box Protein 1

ERAD ..... ER- Associated Degradation

Serca2b ..... Sarcoplasmic Reticulum Calcium Transport ATPase 2b

Trx ..... Thioredoxin

TXNIP ..... Thioredoxin-interacting protein

NLRP3 ..... NOD-like Receptor 3

PPARγ ..... Peroxisome proliferator-activated receptor γ

TZDs ..... Thiazolidinediones

NOD ..... Non-Obese Diabetic

T1D ..... Type 1 Diabetes

T2D ..... Type 2 Diabetes

MODY ..... Maturity Onset Diabetes of the Young

HRP ..... Horse Radish Peroxidase

ChIP ..... Chromatin Immunopreciptation

EMSA ..... Electrophoretic Mobility Shift Assay

CHAPTER 1: Introduction

1.1 Pancreas Structure and Function

The pancreas is a glandular organ located under the stomach and connected to the duodenum via the pancreatic duct. The main functions of the pancreas include the production of digestive enzymes and maintenance of glucose homeostasis. The pancreas consists of three cell types: exocrine, endocrine and ductal cells. The exocrine portion, constituting 90% of the pancreas, consists of acinar cells that synthesize several digestive enzymes, such as proteases, lipases and amylases, which are released along with bicarbonate and water (pancreatic juice) through the pancreatic duct, into the small intestine. The endocrine pancreas consists of spherical clusters of cells called the Islet of Langerhans, which was first described by Paul Langerhans in 1869 (1). The islet functions as an endocrine gland, consisting of five hormone secreting cell types – α, β, δ, ε and the F/PP cells – which synthesize and secrete, respectively, glucagon, insulin somatostatin, ghrelin, and pancreatic polypeptide. The mouse islet consists of a core of β cells, surrounded by a mantle containing the α, δ and F/PP cells, whereas, the human isle consists of β cells interspersed with α, δ and F/PP cells (2).

1.2 The role of pancreatic hormones in glucose homeostasis

Insulin and glucagon have opposing anabolic and catabolic functions that work in concert to maintain glucose homeostasis. A fall in glucose levels stimulates the pancreatic α cells to secrete glucagon, a 31 amino acid hormone. Glucagon binds to its receptor in the liver to promote breakdown of glycogen (the stored form of glucose) and gluconeogenesis (3) and inhibit key enzymes in glycolytic processes, thus contributing to an increase in blood glucose. Glucagon also promotes lipolysis in the liver and adipose

tissue and protein breakdown in the muscle, liberating free fatty acids, ketone bodies and amino acids, all of which can act as alternative sources of fuel for cells.

After ingestion of food, an increase in blood glucose causes insulin to be secreted from the β cells. Insulin signals to other organs in the body, promoting consumption or storage of glucose and thus, decreasing blood glucose to normal levels. As blood from the pancreas travels directly to the liver via the portal vein, the released insulin is first encountered by the liver. In the liver, insulin reduces hepatic glucose output, primarily by inhibition of glycogenolysis (4). Insulin promotes protein and lipid synthesis, decreasing availability of gluconeogenic precursors and consequently decreasing gluconeogenesis. The reduction in blood glucose by insulin is largely achieved through its effect on the liver. Insulin also promotes glucose uptake by skeletal muscle and adipose tissue, where glucose is utilized for protein and triglyceride synthesis respectively (5). Insulin also has other targets, such as the brain, where it has been reported to regulate food intake (6,7) and neuroendocrine counter-regulatory response to hypoglycemia (8) and the α cell, where it is involved in inhibition of glucagon secretion (9,10).

Insulin is a 51 amino acid peptide that binds to is cognate receptor to initiate a cascade of signaling events. The insulin receptor (IR) is a heterotetrameric transmembrane tyrosine kinase that consists of two extracellular α chains and two transmembrane β chains, linked by disulphide bonds. When Insulin binds to the α domain of the IR, a conformational change results in transphosphorylation of the tyrosine kinase domain present in the β chain and subsequent phosphorylation of the downstream insulin receptor substrates IRS 1-4. In the skeletal muscle and fat, the binding of Insulin to the IR activates signaling pathways that result in the localization of

the glucose transporter Glut4 to the plasma membrane, and an increase in protein synthesis, cell proliferation and differentiation. Insulin is absolutely essential in the regulation of glucose homeostasis and maintenance of overall health and therefore, the cell that is responsible for the synthesis and secretion of Insulin, the β cell, is central to glucose homeostasis.

1.3 β cell development

The pancreas, comprised of epithelial cells, is formed by the outgrowth of the foregut endoderm into dorsal and ventral pancreatic 'buds', followed by fusion of the two buds to form a single pancreas at around embryonic day e12.5 in mice. The development of the pancreas is initiated by the expression of homeodomain transcription factors Pdx1 and Ptf1a. Development of the pancreas involves communication between the mesoderm and the foregut endoderm through mesodermally derived signals such as TGFβ (transforming growth factor β), BMP (bone morphogenetic protein), RA (retinoic acid) and FGF (fibroblast growth factor) (11). These mesodermal signals mediate the suppression of endogenous Hedgehog signaling (via Sonic Hedgehog Shh and Indian Hedgehog IHH), which triggers the expression of Pdx1 and Ptf1a at e8.5, leading to the development of the dorsal and ventral pancreatic bud (12–15). The bud cells present in this stage are referred to as multipotent progenitor cells (MPC) and are $Pdx1^{+}Ptf1a^{+}$. Pdx1+ cells, therefore, are the precursor to all pancreatic cell types, including β cells. Homozygous deletion of either Pdx1 (13,16) or Ptf1a (14,15) results in pancreatic agenesis. Expansion of the MPC pool occurs between e10.5 and e12.5. The size of the MPC pool is indicative of the size of the pancreas in adulthood, therefore, predetermining the pancreas's capacity to mount an adequate compensatory response to increased insulin demand. Rotation of the gut tube causes the fusion of the dorsal and

the ventral pancreas to form a single pancreas, following which differentiation of the bud cells into acinar, ductal and endocrine progenitor cells takes place (Figure 1).

Expression of Ptf1a drives the cells towards acinar cell fate, whereas expression of another homeodomain transcription factor, Nkx6.1 drives the cells towards an endocrine/ ductal cell lineage (17). The endocrine cells, marked by the expression of two transcription factors, Ngn3 and NeuroD1 (18–20), further differentiate into the five endocrine cell types (α, β, δ, ε and PP cells). Pdx1 is required for the differentiation of the pancreatic progenitor cell into the mature β cell. Other transcription factors, such as NeuroD1, Isl1, Nkx6.1 expressed in other cell types during development, eventually localize to the mature β cell and maintain the β cell phenotype. Therefore, deletion of these transcription factors can have different phenotypes when depending on when they are deleted (See Table 1). MafA expression is required in the maintenance of β cell identity (21). MafA however, does not crucially affect β cell fate during development, as there is no effect of MafA deletion on any of the endocrine cell types (22,23). Therefore, MafA is considered a marker of the mature β cell. Interestingly, Pdx1 is required for the transcription of most of these transcription factors, reinforcing its importance in the developing and mature β cell (24–26).

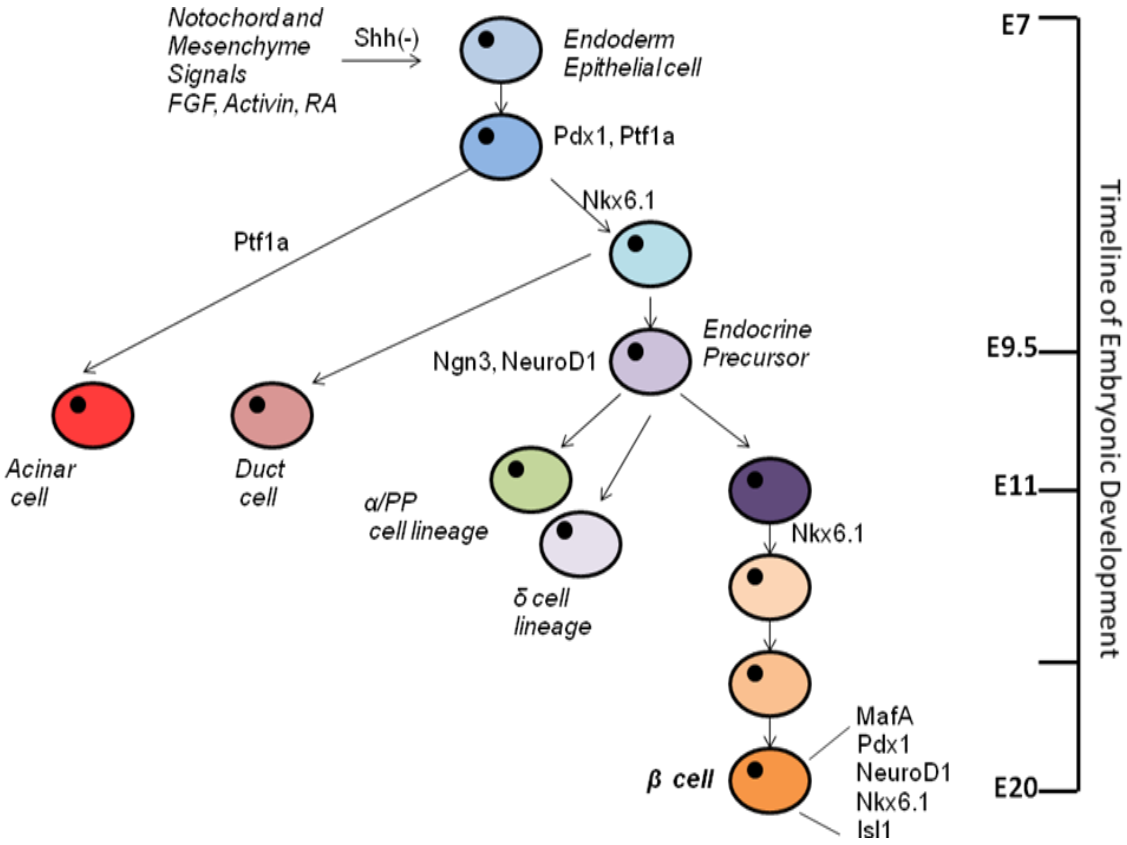

Figure 1. Transcriptional cascade in the developing pancreas. Schematic representation of the transcriptional cascade involved during the differentiation of the pancreatic progenitor cell into the mature β cell.

Table 1: Pancreatic transcription factors

Transcription Factor	Type	Function	Pancreatic phenotype of mutant (development)	Phenotype of mutant (adult)
Pdx1 (MODY4)	Homeodomain (HD)	Required for growth of pancreatic buds, maintenance of adult β cell identity, function and adaptation to stress.	Pancreatic agenesis(13,16)	Overt diabetes, dramatic reduction in insulin^{+} cell area, diminished Glut2 expression (27). Pdx1+/-: glucose intolerance with age, increased β cell apoptosis(28)
Nkx6.1	HD	Specifies MPCs toward an endocrine/ductal fate(17). Required for β cell differentiation(29)	Nkx6.1 null mice show 85% reduction in β cell precursors, and a block in β cell neogenesis(29)	Impaired insulin biosynthesis and secretion, reduced Glut2 expression, decreased β cell proliferation(30)
NeuroD1 (MODY6)	bHLH	Required for normal islet formation; Maintenance of the functional, glucose responsive β-cell phenotype	neonatal diabetes, severe reduction in β cells, mature islets absent. Reduced amylase expression (31).	Glucose intolerance, impaired Insulin secretion. Increased glycolytic metabolism (19)
Isl1	HD	Required for pancreatic epithelial growth and endocrine differentiation (23).	Loss of differentiation of exocrine tissue and islets (32). Decreased endocrine hormones and cells (33).	Impaired Glucose tolerance and insulin secretion. Diminished transcription of Pdx1, MafA, reducing their expression (34)
MafA	Basic Leucine Zipper	Required for maintenance of mature β cells	No differences in endocrine cells or glucose homeostasis.	Glucose intolerance, impaired insulin secretion. Diminished levels of Insulin, NeuroD1, Pdx1, Glut2(22)(23)

1.4 The mature β cell

The main function of the mature β cell is to respond to increasing glucose levels with appropriate insulin secretion in order to maintain glucose homeostasis. As soon as the blood glucose concentration increases beyond a certain threshold level, Glut2, a glucose channel with low affinity (high K_m ca 15-20mM) (35,36) facilitates glucose entry into the cell. Within the cell glucose is converted to glucose-6-phosphate by the key glucose sensing enzyme Glucokinase, and further metabolized through glycolysis and oxidative phosphorylation resulting in the generation of ATP. Glucokinase is a high Km enzyme that is only activated when glucose is abundant and plays an important role in the modulation of insulin secretion (37). An increased [ATP]/[ADP] ratio in the cell causes closure of the ATP-sensitive K^+ channel, depolarizing the membrane. The resultant change in membrane potential causes the L-type voltage-gated Ca^{2+} channel to open, allowing an influx of Ca^{2+} into the cell (38), leading to fusion of insulin secretory granules with the plasma membrane and the release of insulin from the β cell (Figure 2). In rodents, soon after insulin release, insulin stores in the β cell are replenished by translation of *Ins1/2* mRNA, followed by increased transcription of the *Ins1/2* gene (39). The translation of *Ins1/2* mRNA is a highly regulated process. Preproinsulin, is a precursor peptide from which three mature peptides are derived, peptide chains- A, B and C. Within the ER, the signal sequence present at the N terminus of the peptide destined to become the B chain is cleaved and disulphide bonds are formed within the A chain and between the A and B chains. This more mature form, proinsulin, is then packed into transport vesicles that move through the cis and trans Golgi network, to form proinsulin-containing granules. Within these 'immature' granules, proinsulin is cleaved by endopeptidases, such as carboxypeptidase E and pro-hormone convertase 1/3, to

cleave the C (connecting) peptide from the mature A and B chains. These later granules are called ‘mature granules’ and can secrete mature insulin (disulfide bonded A and B chains) and the C peptide upon stimulation. Upon stimulation by glucose mediated depolarization, fusion of the vesicular VAMP proteins and the plasma membrane syntaxin proteins occurs (40), resulting in secretion of insulin and the C peptide. C peptide levels, therefore, are often used to measure endogenous insulin secretion.

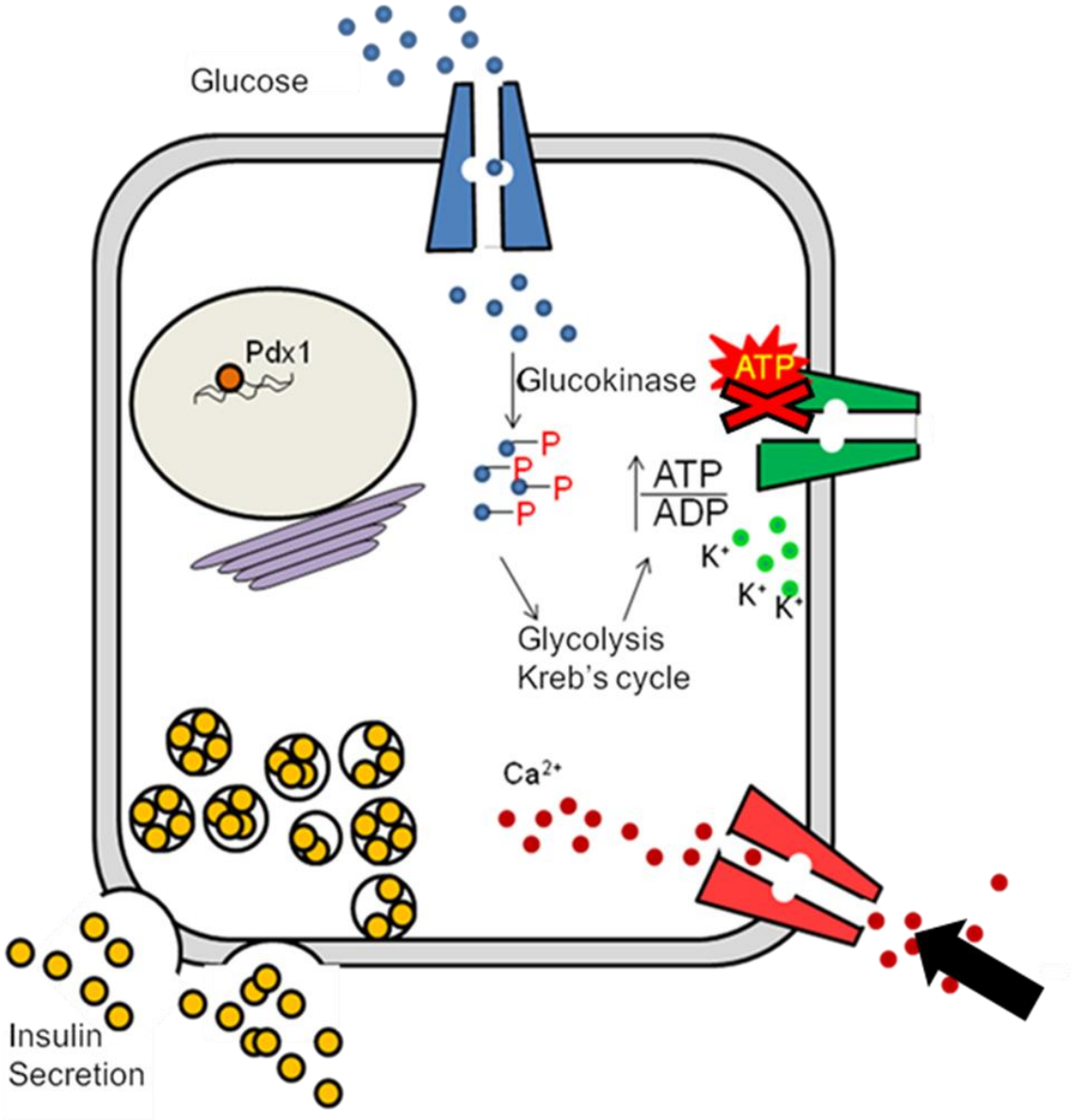

Figure 2. The islet β cell. Glucose enters the β cell through Glut2, and is metabolized through glycolysis and Kreb's cycle, resulting in generation of ATP. An increased ATP:ADP ratio results in closure of the ATP-sensitive K^+ channel and opening of the Voltage gated Ca^{2+} channel. The resulting influx of Ca^{2+} triggers the secretion of Insulin.

1.5 Transcription in the adult β cell

Transcription factors such as Pdx1, Nkx6.1, Isl1, and NeuroD1 are expressed in pancreatic cells during development and later, their expression is restricted to the mature β cell. These transcription factors play very important roles in the maintenance of normal β cell function, as is evidenced by the deletion models described in Table 1. Mice with a postnatal β cell-specific deletion of Pdx1 show a reduction in β cell area, along with impaired insulin secretion and overt diabetes, accompanied by a reduction in *Ins1/2* and *Glut2* mRNAs (27)(41). β cell- specific deletion of *Nkx6.1* in 7 week-old mice results in reduced *Glut2* expression, reduced β cell proliferation, and diminished insulin content. These mice also show impaired insulin secretion, in part due to a decline in mature granules at the plasma membrane and a reduction in *Pcx* (pyruvate carboxylase) transcription, decreasing total cellular ATP levels. Interestingly, *Nkx6.1* deletion also appears to increase the number of $Ngn3^{+}$ cells, indicating that *Nkx6.1* may help maintain β cells in a differentiated state (30)

NeuroD1 plays an important role in the mature islet and therefore, contributes to insulin secretion and glucose homeostasis. *NeuroD1* deletion reduces *Ins1* expression by 50% and disrupts islet architecture. NeuroD1 regulates insulin secretion by facilitating stimulus secretion coupling of insulin secretion, modulating the activity of the K_{ATP} complex, and a few other components of the exocytosis machinery, although the exact mechanism is still unclear (19). Interestingly, islets from NeuroD1-βKO mice upregulate various glycolytic genes, a marker of neonatal cells, suggesting that NeuroD1 also plays a role in maintaining β cell identity (19). As Islet-1 (Isl1) directly activates the transcription of *Pdx1* and *MafA* genes, deletion of *Isl1* in the mature β cell diminishes Pdx1 and MafA levels, reduces insulin content, and impairs insulin secretion (34). MafA,

an adult β cell-specific transcription factor, when deleted in the adult mouse, results in glucose intolerance and impaired insulin secretion at 3 weeks of age. Since MafA regulates cyclin D2, β cell replication is reduced. *MafA* deletion also leads to disrupted islet architecture, decreased insulin content, and a decline in expression of certain exocytosis genes. Furthermore, *MafA* deletion decreases the number of insulin granules docked at the plasma membrane in the first phase of insulin secretion (23). Interestingly, when only one transcription factor e.g. Pdx1 is deleted, expression levels of other transcription factors also decline, indicating that β cell transcription factors appear to form an inter-regulatory transcriptional network, further reinforcing β cell identity and function. Evidence for the role of transcription factors in maintaining β cell function is also provided by several disease models. For example, several models of T2D show reduced expression and reduced DNA binding of MafA (42)(43) and Pdx1 (44)(45).

Since transcription factors play an essential role in maintaining β cell function in normal and disease states, understanding the mechanism of transcriptional activation could improve therapeutic strategies. Transcription factors bind to the promoter of a target gene and regulate the process of transcription. Transcription involves communication between transcription factors and the basal transcriptional machinery (containing RNA pol II), ultimately culminating in the progression of RNA pol II along the coding region of the gene and generation of a mRNA transcript. Transcription of β cell-specific genes is mediated both through several β cell-specific and ubiquitous transcription factors. The insulin promoter contains several cis-regulatory elements, such as A, C and E-box elements (Figure 3A). E47 (or Pan1) is another bHLH transcription factor is ubiquitously expressed, forms a heterodimer with NeuroD1/Beta2 and binds to the E-box elements to promote transcription initiation. Similarly, several A-box elements (5'-TAAT-3') that bind to the β cell Lim-homeodomain transcription factors such as Isl-1,

Lmx1.1, Lmx1.2, and Lmx2 and Pdx1. Out of the 4 or 5 A-box elements, A3 and A4 appear to show more activity (46). MafA appears to be the only transcription factor that binds to the C1-box element (5'-TGCN_7GC-3'). Transcription factors can interact with each other to further enhance transcriptional activation of the target gene. For example, Pdx1 interacts with NeuroD1/E47 to enhance transcription of the insulin minienhancer (47). Even though the binding sites of Pdx1 and NeuroD1 on the insulin promoter are present at distant locations, interaction between the two transcription factors is facilitated by DNA looping (48). MafA has also been shown to interact with both Pdx1 and NeuroD1/E47 to promote transactivation of the insulin promoter (49). In addition, Glis3, a Krüppel-like zinc finger transcription factor has been shown to bind to the Glis binding site in the insulin promoter and promote transcription of insulin. Moreover, Glis3 also interacts with Pdx1, NeuroD1, and MafA to form a complex and enhance the transactivation of insulin by about 20-fold (50). Some of the other ubiquitous transcription factors involved in the regulation of insulin include Sp1 and Creb (51–53) etc. The studies mentioned above determined the role of specific transcription factors using transient transfection assays; therefore, the transcriptional function of a protein was not assessed in the context of chromatin. Therefore, the conclusions drawn may not be indicative of regulation of the endogenous gene. In the following section, I will discuss the role of chromatin in gene transcription.

A. Roles for Co-activators in transcription

In eukaryotes, DNA is arranged in the form of chromatin. A unit of chromatin is referred to as a nucleosome, containing 147 bp of DNA wrapped around a histone "core" octamer comprised of four histones, H2A, H2B, H3 and H4, each in duplicate. The linker histone H1 is present at the DNA entry and exit points and aids in nucleosome

compaction. Adjacent nucleosomes are separated by ‘linker’ DNA, about 80bp in length, which makes the structure of consecutive nucleosomes appear as beads on a string (54). The positively charged lysine and arginine residues of the histone bind to the negatively charged DNA to form a stable complex . The context of the chromosomes can be altered to give two different ‘states’ of chromatin- euchromatin and heterochromatin. Euchromatin is associated with regions of DNA being actively transcribed. Heterochromatin, by contrast, consists of condensed chromatin, wherein the nucleosomes are present in close proximity to one another; these regions are associated with inactive or silenced transcription. The N-terminal tails of histones H3 and H2B protrude out of the nucleosome core and are therefore amenable to post-translational modifications, such as acetylation, methylation, ubiquitination, phosphorylation, and SUMOylation, that can facilitate or repress transcription (55).

A co-activator that has been studied extensively in the context of insulin transcription is p300. p300 is a member of the p300/CBP family of proteins and, by virtue of its several protein-protein interaction domains, acts as an adaptor protein, enabling the assembly of multicomponent transcription complexes. In addition, p300 possesses histone acetyltransferase (HAT) activity, acetylating lysine residues of histones, negating their cationic interactions with DNA, resulting in relaxed chromatin (56). p300 has been shown to enhance NeuroD1-mediated transcription through acetylation of histones and acetylation of NeuroD1 itself (57–59). Furthermore, p300 also augments Glis3/Pdx1/MafA/NeuroD1- and Pdx1-mediated transcription of the insulin promoter through histone acetylation and relaxation of chromatin (50,60). Acetylation is reversible and can be removed by a class of enzymes called histone deacetylases (HDACs). In the presence of low glucose, Pdx1 has been shown to interact with HDAC-1 and HDAC-2, which deacetylate the insulin promoter and inhibit transactivation (61). Therefore, the

balance between HAT and HDAC activity can influence the rate of transcription occurring at any specific gene.

Lysine methylation can occur as mono-, di-, or tri-methylation. DNA is monomethylated on specific cytosine residues within cytosine/guanidine dinucleotides (CpGs). DNA sequences enriched for repetitive CpG clusters are called CpG islands, methylation of which is associated with gene repression. DNA methylation, catalyzed by *de novo* DNA methyltransferases (DNMTs) such as DNMT3a/3b or the maintenance DNMT1, play important roles in embryogenesis, development, and gene regulation (62). Methylation is also found on the lysine and arginine tails of histones. Lysine methylation occurs at K4, K9, K27, K36 and K79 of histone H3, and K20 and K59 of histone H4. Methylation at H3K4 and H3K79 correlates with active transcription, while methylation at K9, K27 and K36 of histone H3 correlates with repressed transcription (63). Methylation alters hydrophobicity (not charge) of the lysine/arginine residue, enabling recruitment of other proteins to specific sites, resulting in modified chromatin structure and altered transcription (64).

For example, H3K9 and H3K27 methylation respectively recruits Heterochromatin Protein 1 (HP1) and Polycomb proteins, which stabilize the formation of heterochromatin (65,66). In yeast, H3K4 methylation results in recruitment of chromodomain 1 (Chd1), a component of the histone acetyltransferases SAGA and SLIK, and subsequent transcriptional activation (67). In human islets, Pasquali et al. showed that active enhancers were occupied by islet-specific transcription factors and were also enriched for H3K4 methylation (25). Lysine demethylation is catalyzed by demethylases classified either as lysine-specific demethylases (LSDs) or Jumonji C domain-containing histone demethylases (JMJCs). During the demethylation reaction, LSD and JMJC demethylases use the cofactors flavin adenine dinucleotide (FAD) and α-

ketoglutarate respectively. LSD-1, a member of the LSD family, can demethylate mono- and dimethylated H3K4. *In vivo*, LSD-1-mediated demethylation can be regulated by protein-protein interactions, as LSD-1 in complex with the transcription factor Co-REST, demethylates histones present within the nucleosome (68). As both methyltransferases (which use S-adenosyl L-methionine or SAM) and demethylases utilize metabolic intermediates to carry out their reactions, recent articles suggest that epigenetic modulation of chromatin occurs not only during development, but also in response to metabolic stimuli within the cell (69,70). Although methyltransferases and demethylases are conventionally known to affect histones, recent studies have begun to appreciate the fact that methyltransferases also methylate a host of non-histone proteins, regulating transcription through alternate mechanisms (71).

B. Pancreatic Duodenal Homeobox-1

Pancreatic Duodenal Homeobox-1 (Pdx1) is the master transcription factor of the β cell. Although Pdx1 is robustly expressed in all pancreatic cells during development, in the mature islet, Pdx1 is mainly expressed in β and δ cells. Pdx1 contributes to the maintenance of β cell identity, promotes β cell survival and proliferation and adaptation to stresses such as endoplasmic reticulum (ER) stress. Pdx1 is essential for the expression of β cell-specific genes such as *Ins1/2* (72), *Slc2a2* (the glucose transporter) (73), *Gck* (the glucose sensing enzyme) (74), *MafA* (75) and *Pdx1* itself (76), indicating that Pdx1 is important for the maintenance of β cell identity (27,41). Gao T et al. showed that Pdx1 maintains β cell identity, in part, through repression of MafB, a transcription factor that is expressed only in postnatal α cells (77). Furthermore, *Pdx1* overexpression in α cells results in their conversion to β cells (78), emphasizing the role of Pdx1 in the maintenance of the adult β cell phenotype. Pdx1 also plays a role in β cell the

proliferative that occurs in response to insulin resistance. Mice that are heterozygous for *Pdx1* and doubly heterozygous for IRS/IR and liver-specific IR knockout mice show an abrogation of β cell compensatory hyperplasia and increased β cell death (79). The decreased proliferation observed in these mice was attributed to reduced nuclear localization of β-catenin, suggesting that Pdx1 could influence cellular proliferation via β-catenin. (79). Moreover, recent studies have demonstrated that islets isolated from Pdx1 heterozygous mice fed on a high fat diet displayed distended ER, a hallmark of ER stress, and increased expression in markers of the unfolded protein response (UPR), the cell's adaptive response to ER stress. Interestingly, Pdx1 activates the transcription of UPR signaling proteins that are required to resolve ER stress, such as *Atf4*, *Wfs1* and *Ero1β.* Therefore, Pdx1 deficiency contributes to unresolved ER stress and β cell death (80,81) (explained in more detail in later sections). Pdx1 also regulates the transcription of mitochondrial transcription factor A, TFAM. TFAM is a transcription factor that is involved in transcription of several mitochondrial DNA (mtDNA) genes including *nd1*, a subunit of the NADH complex I of the mitochondrial respiratory chain. Hence, Pdx1 indirectly regulates mtDNA copy number, ATP synthesis and insulin secretion (82). Therefore, Pdx1, through transcription of its downstream targets, is central to β cell fuction. Not surprisingly, a ChIP-seq study conducted in mouse and human islets revealed that 2824 common genes occupied by Pdx1 associated with endocrine and metabolic disorders, cell survival, and other signaling pathways (83). This study confirmed that we still have only rudimentary knowledge about the downstream targets of Pdx1 and more studies need to be performed to determine its transcriptional network.

C. Regulation of Pdx1 function

The *Pdx1* gene encodes a 283 amino acid, homeodomain transcription factor containing an N-terminal transactivation domain, a DNA-binding homeodomain, and a C-terminal domain (84). The N-terminal region of Pdx1 acts as a transactivation domain, and also interacts with NeuroD1/E47, p300, MafA, and Glis3, as mentioned previously. The Pdx1 amino terminus also interacts with Set7/9, a lysine methyltransferase, (85) and the PDZ domain coactivator Bridge1 to synergistically transactivate the insulin promoter (Figure 3B) (86). The amino terminus contains the highly conserved pentapeptide FPWMK, which is the site of interaction between Pdx1 and the ubiquitous homeodomain transcription factor Pbx1. Pdx1 and Pbx1 co-operatively transactivate elastase (87) and somatostatin (88). Mutated FPWMK pentapeptide disrupts Pdx1:Pbx interaction resulting in a dramatic reduction in pancreatic cell proliferation and death in 3 week-old mice (89). Furthermore, Pbx1 null mice displayed pancreatic hypoplasia along with a reduction in pancreatic differentiation markers such as Isl1, supporting the role of Pbx1 during pancreatic development. Pdx1/Pbx1 transheterozygous mice were more glucose intolerant than Pdx1 or Pbx1 heterozygous mice and had impaired insulin secretion, indicating that the Pdx1:Pbx interaction is important to the mature β cell (90).

The Pdx1 homeodomain, flanked by proline-rich regions on either side, is lysine rich and has strong DNA binding activity toward the TA-rich sequences 5’-TAAT-3’ and 5’-TGATNNAT-3’ (83). This binding specificity is determined by sequences flanking the 5’-TAAT-3’ sequence (91). The homeodomain also contains a highly conserved histidine at amino acid 189 and a conserved motif KIWFQN that are both important for DNA binding (92), in addition to the nuclear localization sequence RRMKWKK (93,94) (Figure 3C). Kim et al. reported that the retinoblastoma protein pRb interacts with the

homeodomain of Pdx1 to regulate its stability. pRb-null embryos display a reduction in Pdx1 protein and Pdx1 target gene expression, along with pancreatic hypoplasia and increased cell death, indicating an important role for the pRb:Pdx1 interaction during development (95). The C terminal region of Pdx1 has also been shown to interact with Pcif1 and an E3 ubiquitin ligase that modulates Pdx1 stability (80). As Pdx1 is key to β cell development and mature β cell function, it is quite likely that Pdx1 interaction is regulated temporally and can vary, depending on the physiological context.

D. Post-translational modifications of Pdx1

Apart from protein-protein interactions, Pdx1 protein levels and function are regulated by post-translational modifications. Pdx1 has been reported to be phosphorylated in several studies, albeit with slightly contradictory results (Figure 3C). Humphrey et al. showed that Pdx1 is phosphorylated at Ser 268 and Ser 272 in isolated rat islets, in low glucose, by glycogen synthase kinase 3β (GSK3β), resulting in Pdx1 degradation. When the cells were stimulated with glucose or insulin, Akt and PASK (96) phosphorylated GSK3β at Ser 9, inactivating GSK3β and increasing Pdx1 protein levels (97). However, homeodomain interacting protein kinase 2 (HIPK2) phosphorylates Pdx1 at Ser 269 in mice (the same as Ser268 in rats), affecting its localization within the nucleus, but not stability (98). Under basal conditions, GSK3β also appears to phosphorylate Pdx1 at Ser 61 and Ser 66 within its amino terminus, causing its association with ubiquitin ligase and subsequent degradation (99,100). In response to radiation, DNA- dependent protein kinase (DNA-PK) phosphorylates Pdx1 at Thr 11, resulting in Pdx1 degradation (101). Previous studies have raised the possibility that Pdx1 phosphorylation occurs in a PI3K- and p38-dependent manner to increase its nuclear localization and transcriptional activity (102,103), however, no residues were

identified. On the contrary, Meng et al. found that casein kinase 2 (CK2) phosphorylates Pdx1 at Thr 231 and Ser 232, leading to a decrease in transcriptional activity (104). Clearly, Pdx1 phosphorylation has context-dependent effects, and its impact on β cell function is nuanced. Pdx1 has been shown to be sumoylated, in the absence of which Pdx1 is localized to the cytoplasm and degraded (105). Lastly, Pdx1 is also glycosylated in response to glucose, resulting in increased DNA binding activity (106) and increased free fatty acid receptor 1 (FFA-1/GPR40) gene transcription (107), both of which augment insulin transcription and insulin secretion. Since *Pdx1* expression and activity is often used as a barometer of β cell health, studying its interactions and post-translational modifications can provide an important gateway into therapeutic options.

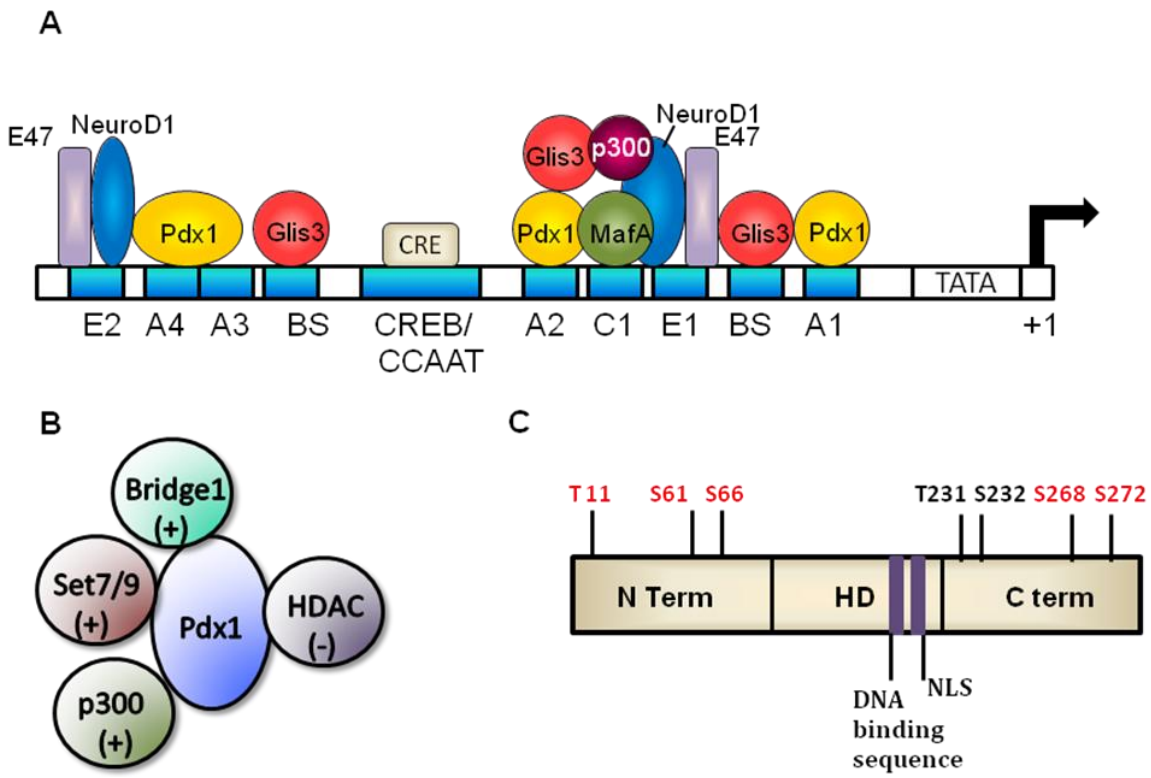

Figure 3. Pdx1 structure and function. (A) Schematic representation of the proximal region of the rat insulin II promoter showing the key elements and trans-activating factors involved in glucose regulation of insulin gene transcription. (B) Pdx1 interacts with several cofactors – Bridge1, Set7/9 and p300 increase; HDAC decreases Pdx1 transactivation on the insulin promoter. (C) Schematic representation of the Pdx1 protein depicting the amino terminus (N term), homeodomain (HD) and the carboxyl terminus (C term). The purple regions highlighted within the homeodomain denote the DNA specificity sequence KIWFQN and the nuclear localization sequence (NLS) RRMKWKK. Residues that are phosphorylated are highlighted in red and black. Red residues are associated with stability and black residues are associated Pdx1 transcriptional activity. Apart from phosphorylation, Pdx1 is also glycosylated and sumoylated.

E. Set7/9

Set7/9, also known as Set7 or Set9 was named based on the presence of an evolutionary conserved 'SET' (Su(var)3-9, Enhancer of Zeste, Trithorax) domain. The *Setd7* gene is present on chromosome 4 in humans and chromosome 3 in mice, and contains 8 exons that code for a 366 amino acid protein (108). Set7/9 was purified and identified as a histone methyltransferase that methylates H3K4. Although Set7/9 methyltransferase activity is carried out by the SET domain and its flanking residues, Set7/9, unlike other methyltransferases, lacks a cysteine-rich pre- and post-SET domain (108). Set7/9-mediated H3K4 methylation has been shown to inhibit H3K9 methylation by SUV39H1, subsequently permitting p300-mediated H3 acetylation. H3K4 methylation also inhibits interaction with the NuRD deacetylase complex and hence, increases transcriptional activity (108,109). Set7/9 null mice are 50% embryonic lethal (110). Interestingly, Set7/9 does not methylate histones in the context of a nucleosome *in vitro*: the implications of this finding *in vivo* is unclear, since it is possible that the nuclear protein milieu may facilitate histone methylation.

The role of Set7/9 in non-histone protein methylation has been a popular topic of investigation in the last few years. Multiple molecular mechanisms may be affected by lysine methylation of non-histone proteins, as exemplified by the following examples. Set7/9 methylates TBP associated factor 10 (TAF 10) and increases its affinity for RNA polymerase II, thereby, affecting the preinitiation complex and resulting in augmentation of transcription of selective target genes (111). Several studies have reported monomethylation of the tumor suppressor gene p53 by Set7/9 at residue K372, during genotoxic stress (112). Methylation of p53 facilitates acetylation of p53 by Tip60 and a subsequent increase in p53 stability and transactivation of its downstream target gene

p21 (110), although this study has also been contradicted (113). Transcriptional activation of p21 also occurs due to acetylation of H4 within the p21 promoter, which follows methylation and acetylation of p53 (114). Therefore, p53 methylation induces cell cycle arrest and prevents cellular transformation. The p65 subunit of NF-κB is also methylated by Set7/9 at residues K314 and K315, resulting in degradation of DNA-bound NF-κB and a subsequent decrease in transcription of NF-κB target genes (115). Interestingly, p65 methylation at K37 by Set7/9 associates with increased DNA binding affinity of p65 and augments transcription of downstream target genes such as TNFα (116). These contrasting results hint at the possibility that differential methylation could be due to the presence of interacting proteins that could influence Set7/9-mediated methylation. Clearly, Set7/9-mediated methylation can affect various aspects of protein function, including stability, DNA binding, transcriptional activation, and protein interactions (see Table 2). Moreover, Set7/9-mediated protein methylation has been shown to be reversible by LSD-1 in certain proteins, such as p53 (117), DNMT1 (118), and HIV Tat protein (119). Hence, protein methylation provides an alternative level of dynamic regulation of protein function.

Table 2: Protein methylation mediated by Set7/9

Substrate	Lysine Residue	Function
P53	K372; K369 in mouse (me1)	Increase in transactivation of p21, increased p53 stability(112), facilitate p53 acetylation(110,114)
TAF10	K189 (me1)	Enhanced affinity to RNA polymerase II, increased transcription of selective target genes(111)
NF-κB (p65 subunit)	K314, K315 (me1) K37 (me1)	In response to TNFα, only DNA bound NF-κB is methylated and degraded. Decreased protein stability. Reduced IL-6 and Nos2 transcription(115) In response to TNFα and IL-1β, methylation enhances NF-κB DNA binding and coactivator recruitment, increases transcription of subset of target genes(116)
Estrogen Receptor (ERα)	302 (me1)	Increased protein stability, Increased recruitment of ERα and transcription of target genes (120).
Androgen receptor (ARα)	630 and/or 632 (me1)	Increased transactivation of target genes. Set7/9 and AR occupancy of target genes was associated with increased H3K4 methylation and p300/CBP recruitment to the promoter (121,122)
FXR	206	Increased transactivation of target genes by increased DNA binding (123)
Tat protein (HIV)	51 (me1)	Stimulation of HIV transcription(124), Monomethylation is followed by acetylation; transcription also enhanced by LSD-1(119).
SUV39H1	105, 123	Down regulation of H3K9 methlyation, heterochromatin relaxation (125)
Dnmt1	K1096	Methylation decreases stability and results in global DNA hypomethylation. LSD1 demethylation enhances Dnmt1 stability(118)

F. Pdx1-Set7/9 interaction

Previous studies from our laboratory have focused on the transcriptional ability of Pdx1 in the context of chromatin. Chakrabarti et al. showed that by combining EMSA and ChIP assays in β and non-β cell lines transfected with the *Ins1/2* promoter and Pdx1, Pdx1 binding and occupancy was higher in β cells, as the chromatin in Pdx1 target genes were more open in β cells compared to non- β cells (126). This finding raises the possibility that chromatin context may restrict Pdx1 binding to some promoters. An increase in the transcription of the *Insulin* promoter associates with increased H3 acetylation, mediated by p300, and H3K4 methylation, mediated by Set7/9 (126,127). Tail vein injection of *Setd7* siRNA resulted in inhibition of Set7/9 in the pancreas and a subsequent reduction in Pdx1 target genes *Ins1/2*, *Slc2a2* and *MafA*, coupled with defects in Ca^{2+} influx and subsequent diminished insulin secretion (128). Moreover, α to β cell conversion was associated with nuclear localization of Set7/9, suggesting that Pdx1-Set7/9 co-localization is associated with the maintenance of β cell identity (78). Pdx1 directly regulates Set7/9 promoter and expression (128,129), which eventually augments transcription of other Pdx1 target genes. A study performed by Francis et al. (85) revealed a possible mechanism underlying Set7/9-mediated increase in Pdx1 target gene expression, showing that Set7/9 was recruited by Pdx1 to the *Insulin* promoter, which underwent H3K4 dimethylation, to increase transcription of the *Insulin* gene. Histone methylation is also associated with conversion of the initiation form of RNA polymerase II (marked by Ser-5-P residue in the carboxyl terminal domain, CTD) to its elongation form (marked by Ser-2-P residue in the CTD), advancing RNA pol II along the coding gene (85). Interestingly, an *in vitro* transcription reaction performed on a chromatinized insulin mini-enhancer template revealed two findings: 1) Pdx1 was required for the occurrence of histone methylation and 2) Pdx1 itself was methylated

(85). This finding and the literature cited above led us to hypothesize that *methylation of Pdx1 by Set7/9 enhances Pdx1 transcriptional activity and promotes β cell function.*

1.6 β cell dysfunction

Since the β cell is so crucial for glucose homeostasis, maintenance of normal β cell function is essential. However, in the presence of several stresses, such as ER stress, oxidative stress and inflammation, β cell function is impaired and results in the development of disease. Therefore, it is important to understand the mechanisms underlying β cell dysfunction.

A. ER stress in the β cell

The β cell is a secretory cell. The entry of proteins into the secretory apparatus, the Golgi network, is through the endoplasmic reticulum. Within the ER, folding of the translated protein is aided by molecular chaperones such as binding Ig protein (Bip), oxidoreductases, and protein isomerases that assist in formation of disulphide bonds. Furthermore, proteins can accumulate post-translational modifications such as glycosylation, which might prevent protein unfolding/misfolding. The endoplasmic reticulum of the β cell translates around 1 million insulin molecules per minute (130). Also, insulin biosynthesis accounts for 0.4% of the total pancreatic protein synthesized, even though islets make up only 1-2% of the pancreas (131). Furthermore, the ER of the β cell is acutely sensitive to external stimuli (such as nutrients), and is responsible for elevating insulin synthesis accordingly. Moreover, protein translation in the β cell is regulated by glucose and free fatty acids (132,133). Quality control in the ER is maintained by the degradation of unfolded proteins either through ERAD (endoplasmic reticulum assisted degradation), where the proteins are ubiquitylated (134–136) and

degraded in the proteasome or through autophagy (137,138). However, insulin resistance, hyperglycemia, or inflammation can trigger a sudden increase in protein synthesis, which in turn could result in the accumulation of unfolded protein, leading to ER stress. ER stress is often associated with a distended ER network, as visualized by electron microscopy. ER stress results in activation of the unfolded protein response (UPR), which is the cell's attempt to resolve/adapt to the ER stress and maintain cell function. However, if the UPR program of the cell is unsuccessful, UPR signaling instead diverts the cell towards apoptosis.

The UPR pathway has three main arms that are mediated by: Perk (Protein kinase RNA (PKR)-like ER Kinase), Ire1α (Inositol Requiring Enzyme 1) and Atf6 (activating transcription factor 6). These three proteins are transmembrane proteins, containing a cytoplasmic and an ER portion, to which the molecular chaperone Bip is bound. Unfolded protein induces release of Bip from the three arms (139) or direct interaction of unfolded proteins with Perk/Ire1α/Atf6 results in their activation (140). Perk is activated by dimerization and trans-autophosphorylation of the kinases, leading to phosphorylation of eukaryotic initiation factor 2 α (eIF2α), a protein that mediates the initiation of translation (Figure 4). Phosphorylated eIF2α inhibits global mRNA translation, but allows translation of only those proteins that are involved in the unfolded protein response (UPR) (141). The activation of the Perk arm of the UPR results in increased translation (142) of *Atf4* (activating transcription factor 4) mRNA, which localizes to the nucleus and activates transcription of target genes, such as the pro-apoptotic protein *Chop* (143,144), molecular chaperones, and genes involved in amino acid biosynthesis and anti-oxidant response, therefore enhancing the protein folding capacity of the cell. Patients with mutations in the gene coding for Perk (called Wolcott Rallison syndrome) are born with permanent insulin-dependent diabetes, osteopenia,

and mental retardation (145). Mutant mice have irremediable ER stress along with distended ER and gradual death of β cells, followed by loss of α cells and exocrine insufficiency (146).

Ire1α, a kinase receptor with endoribonuclease activity, is activated in response to ER stress in a manner similar to Perk. Ire1α mediates the splicing of several mRNA present in the ER, including *X-box protein 1* (*Xbp1*) mRNA and *Insulin* mRNA (147). Spliced Xbp1 (*sXbp1*) mRNA codes for Xbp1 protein, a transcription factor that together with Atf6α, mediates the transcription of molecular chaperones, genes involved in lipid biosynthesis and ER and Golgi biogenesis, to expand the ER and enhance the ability of the cell to resolve ER stress. The Ire1α-Xbp1 pathway enhances transcription of proteins involved in ERAD, such as Edem, ErDj4 and Protein disulfide isomerase (148). The Ire1α-Xbp1 arm facilitates the regulation of proinsulin synthesis and insulin secretion under transient high glucose conditions in a normal β cell (149,150).

The third arm of the UPR is Atf6, a basic leucine zipper transcription factor. During ER stress, the disulfide bonds of the ATF6 dimer are reduced to release Atf6 monomers, which relocate to the Golgi. In the Golgi, Atf6 is cleaved by the proteases S1P and S2P, to give the nuclear fragment, Atf6n, which localizes to the nucleus and mediates transcription of its target genes, including *Xbp1* and *Chop*.

Sustained ER stress results in the induction of cell apoptosis. ChoP, a bZip transcription factor, inhibits transcription of (186) anti-apoptotic Bcl2 proteins (151) and induces pro-apoptotic proteins such as the negative feedback inhibitor of eIF2α phosphorylation, Gadd34 (152), the Akt inhibitor, Trb3 (153) to promote cell apoptosis. During chronic ER stress, interaction between Atf4 and Chop promotes transcription of

genes involved in protein synthesis, depleting cellular ATP, inhibiting transcription of antioxidant genes, worsening ER stress, and increasing ROS and cell death (154). Although, the *Chop*$^{-/-}$ mice exhibit no differences in their phenotype under normal conditions, deletion of *Chop* in models of type 2 diabetes resulted in improved glucose tolerance, β cell function and reduced cell death (155). The Ire1α-Xbp1 pathway also mediates apoptosis during irremediable UPR. In the presence of cytokines, Xbp1 can increase activation of NFκB, resulting in increased β cell death (156). Ire1α interacts with Traf2 (TNFα receptor associated factor), an adaptor protein that facilitates cell apoptosis through: 1) recruitment of Ask1 (157) followed by phosphorylation of JNK (158); 2) activation of caspase-12 (159); 3) interaction of the pro-apoptotic proteins Bax and Bak with Ire1α at the ER membrane, followed by induction of apoptosis (160) and; 4) Increasing the release of ER Ca^{2+}, such that the Ca^{2+} concentration in the mitochondria increases, resulting in membrane depolarization, cytochrome C release and apoptosis.

Apart from the three main arms of the UPR, the sarcoplasmic reticulum calcium transport ATPase 2b (Serca2B) in the β cell regulates Ca^{2+} homeostasis in the ER, that, when perturbed, can cause ER stress since protein folding by chaperones is Ca^{2+} dependent (161). In fact, thapsigargin, a chemical inhibitor of Serca2b, is often used to induce ER stress in cell-line based studies. Wfs1, a transmembrane protein, aids in protein assembly, ERAD (162) and insulin secretion (163) and negatively regulates ATF6 (162). However, in the presence of ER stress, diminished Wfs1 levels can reduce insulin secretion (163). β cell-specific deletion of Wfs1 results in the onset of glucose intolerance at the age of 12 weeks, contributing to ER stress (164). Clearly, an efficient UPR response is crucial to β cell survival.

ER stress, inflammation, and oxidative stress are interlinked, with each process augmenting one another (133,165). Thioredoxin (Trx), a 12KDa ubiquitous protein that possesses disulfide-reducing activity, has two isoforms, Trx1, present in the cytosol, and Trx2, present in the mitochondria. Trx activity is induced by UV radiation, viral infection, ischemia, etc. In the process of reducing other substrate proteins (for example, insulin), disulfide bonds of Trx1 protein itself get oxidized. Thioredoxin reductase helps restore oxidized Trx to its reduced form. Therefore, Trx is important in maintaining the redox status of a cell. TXNIP (thioredoxin-interacting protein) is a protein that interacts with Trx1 in the cytosol and Trx2 in the mitochondria. TXNIP interacts only with the reduced form of Trx to inhibit Trx activity. TXNIP has an arrestin-like domain that interacts with several proteins such as importin α (166), E3 ubiquitin ligase Mybbp1a (167), and NOD-like receptor protein 3 (NLRP3) (168). Activation of TXNIP is sensitive to external signals and can occur in response to oxidative stress, inflammation, obesity and hyperglycemia. TXNIP has been implicated in both type 1 and type 2 diabetes (reviewed in (169)). TXNIP may also be an important intermediate between ER stress and inflammation. TXNIP is activated by IRE1α and the PERK arms of the UPR, resulting in activation of the NLRP3 inflammasome, which produces mature IL-1β, which increases cell death (170,171). More studies need to be conducted to determine the other intermediates between ER stress, oxidative stress and inflammation, so as to design more effective therapeutic strategies to prevent cell death.

Several attempts have been made to ameliorate ER stress. The activation of PPARγ with Pioglitazone, a thiazolidinedione (TZD), has shown promising effects. Evans-Molina et al. reported that in INS1 832/13 cells treated with thapsigargin, an inducer of ER stress, pioglitazone treatment showed a dramatic reduction in ER stress markers. Moreover, db/db mice, a type 2 diabetic mouse model that exhibits ER stress,

when treated with pioglitazone, showed improved glucose tolerance, insulin sensitivity, and insulin secretion, accompanied by a reduction in islet ER stress. (172). In response to pioglitazone treatment, PPARγ binds to its PPRE in the promoter of Serca2b to induce Serca2b expression and reduce β cell death (173). Wfs1 knockout mice, when crossed with C57BL6 mice, have mild obesity and insulin resistance and showed β cell ER stress and apoptosis. Treatment with pioglitazone protected these mice from ER stress and β cell death, further supporting the role of pioglitazone in remediating ER stress (174). PPARγ activation also transcriptionally activates Pdx1 (172,175), which in turn, acts as a transcriptional activator of UPR mediators Atf4, Wfs1 (45) and ER Ca^{2+} homeostasis (176) and helps in augmenting the UPR response and reducing ER stress. PPARγ activation was also shown to reduce oxidative stress-induced β cell death in pioglitazone-treated db/db mice, with activation of antioxidant gene expression and an increase in insulin secretion (177). Hence, pioglitazone treatment can ameliorate β cell function and reduce β cell death, through different mechanisms.

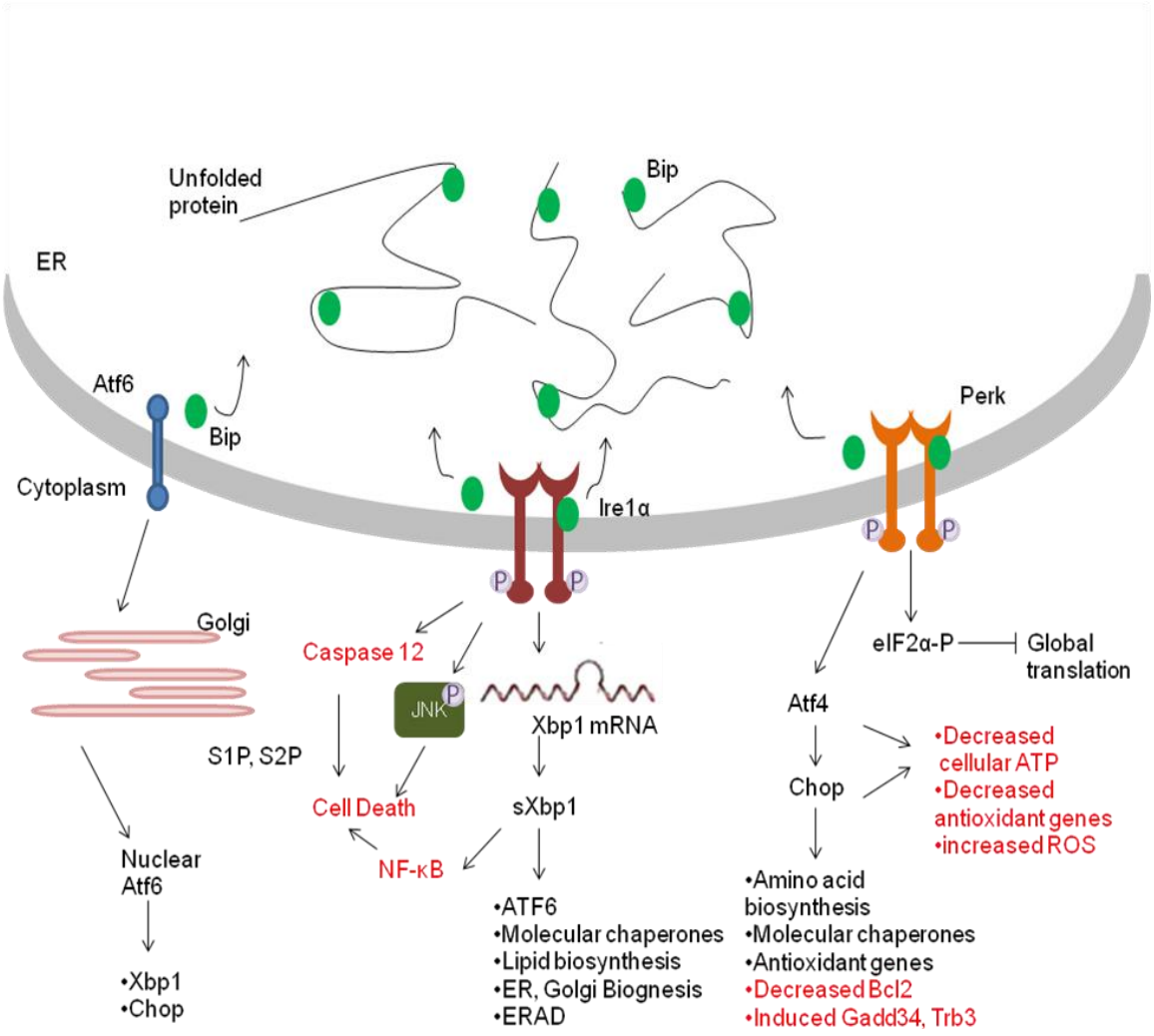

Figure 4. The Unfolded Protein Response (UPR). In response to unfolded protein, Bip (Green Circles), bound to Atf6, Ire1α and Perk is released and aids in protein folding. The release of Bip causes dimerization and trans-autophosphorylation (Purple circles) of Ire1α and Perk. Phosphorylated Perk phosphorylates eIF2α, inhibiting global mRNA translation. Perk also activates Atf4 and Chop, Ire1α splices *Xbp1 mRNA*, resulting in downstream effects. Atf6 is processed in the golgi (cleavage by S1P, S2P proteases) to nuclear Atf6, which acts as a transcription factor for Xbp1 and Chop. Downstream effects labeled in black denote processes that aim to resolve ER stress. Downstream effects labeled in red denote processes occurring during chronic stress that induce cell death.

B. β cell dysfunction in disease

Deficiency of insulin/insulin signaling leads to the development of diabetes. Diabetes mellitus is a group of diseases characterized by chronically elevated blood glucose (>120mg/dl blood glucose). Currently, around 300 million people suffer from diabetes (CDC) and according to current trends, around one in three people in the USA, are projected to develop diabetes by 2050 (ADA). Complications of uncontrolled hyperglycemia include retinopathy, coronary heart disease, nephropathy, cerebrovascular disease and poor blood flow to the extremities (diabetic foot). There are several forms of diabetes: Type 1 Diabetes (T1D), Type 2 Diabetes (T2D) and MODY among others.

Type 1 Diabetes: This form of diabetes is characterized by immune attack of islet β cells, resulting in β cell destruction and hyperglycemia. T1D is diagnosed when low serum levels of insulin are observed together with the presence of autoantibodies against β cell proteins such as Insulin, GAD-65 (Glutamic acid decarboxylase 65), and IA2 (Islet antigen 2) in the serum or plasma of the patient. T1D usually occurs early in life, below 18 years of age and is also called juvenile diabetes. However, T1D can also occur later in life, in a form referred to as latent autoimmune diabetes of adults (LADA). The development of T1D occurs over many years, usually initiated by genetic susceptibility or a viral attack. The appearance of β cell autoantibodies in the serum, along with progressive decline of β cell function and glucose tolerance in the prediabetic stage, eventually leads to the onset of overt diabetes. The most commonly used model for studying T1D is the non-obese diabetic (NOD) mouse model. Female NOD mice spontaneously develop immune infiltration (insulitis) of the islet at around 4 weeks and overt T1D by 12-13 weeks of age.

The mechanism underlying the development of T1D has been investigated for many decades now. Mutations in several genes, such as HLA-DQ/DR, IL-2RA, and CTLA-4 associate with increased susceptibility to T1D (178). A loss of both central and peripheral immune tolerance has also been postulated to contribute to the progression of T1D. During T cell development in the thymus, the transcription factor AIRE in medulla thymic epithelial cells is responsible for the transcription of a host of peripheral proteins, including insulin, that induce tolerance in naïve T cells to self antigens. Deletion of AIRE (179) or mutation of the INS promoter (180) can result in the onset of T1D, demonstrating a role for the loss of central tolerance in bringing about the onset of T1D. An imbalance between T effector cells (T_{eff}) and T regulatory cells (T_{reg}) or a reduction in T_{reg} cells can also cause the inflammatory response mounted by the T_{eff} cells to remain unchecked, and facilitate insulitis to continue. Furthermore, a reduction in the anti-inflammatory cytokines such as IL-10 and TGF-β has also been suggested to contribute to an unabated immune response (reviewed in (178,181)).

Several immunomodulatory drugs with well characterized responses in other autoimmune diseases have been attempted in clinical trials of new-onset T1D subjects. Whereas administration of some of these drugs (such as anti-CD3, anti-CD20, CTLA4-Ig) have led to the preservation of β-cell function (as assessed by C-peptide secretion) for periods of months, subsequent declines in β-cell function paralleled those of placebo (182–185). In no case has true remission, as defined by insulin independence, been observed. These outcomes could be explained by the low replicative capacity of human β cells (186,187), coupled with the possibility that interventions were initiated too late in the disease—at a time in which molecular stress pathways in β cells were so aggressively activated that even a respite from autoimmunity could not prevent β-cell decline.

These considerations have sparked a new perspective in the field, in which greater emphasis has been placed on elucidating the molecular mechanisms within β cells that might initiate or perpetuate autoimmunity and eventual β cell demise. Eizirik et al showed that around 60% of known T1D candidate genes were expressed in human islets exposed to proinflammatory cytokines, demonstrating that the islet β cell plays an active role in the inflammatory process and perhaps, assists in its own demise (188). In addition, several studies have shown the presence of islet autoantibodies (189), β cell dysfunction (190,191) and metabolic alterations (192) years before diagnosis of T1D. Recent reports have shown that a host of alterations occurring in β cells could also contribute to the development of diabetes in the NOD mouse. Age dependent increase in expression of certain miRNA in prediabetic NOD mouse islets predispose the islet to death (193). Other reports have demonstrated the presence of increased ER stress in the new onset Type 1 diabetic β cell in humans (194) and in the prediabetic NOD β cell (172,195). Tersey et al demonstrated that prediabetic mice showed dilated ER, an increase in UPR markers and reduced insulin secretion, possibly resulting in increased β cell death (196). Engin et al showed that pre-diabetic NOD mice treated with a chemical chaperone TUDCA decreases ER stress via ATF6 and therefore decreases β cell death (195). A recent study also showed evidence for autoantibodies against citrullinated GRP78 in prediabetic NOD mice, further strengthening the hypothesis that ER stress in the β cell enables its own death (197). ER stress is also associated with other autoimmune disorders such as colitis, rheumatoid arthritis and idiopathic inflammatory myopathies (198). Collectively, these findings suggest that, genetic susceptibilities of the β cell and/or early inflammation of the pancreas cause ER stress in the β cell, worsening of islet function and resulting in β cell death. Therefore, the β cell then assists in a vicious cycle that leads to its own death (Figure 5) (198,199).

Therapies for T1D currently include administration of exogenous insulin, either in the form of injections or insulin pumps. In a DCCT (Diabetes Concentration and Control) trial, it was reported that patients with decreased insulin secretion were at a higher risk of developing diabetic retinopathy and nephropathy, indicating the importance of maintaining β cell function (200). Other therapeutic strategies such as islet transplantation and pancreatic transplantation could potentially increase endogenous C peptide levels, but these approaches have very low success rates. As T1D patients are known to have elevated β cell death rates (197), an ideal therapeutic approach for T1D would not only ameliorate the inflammatory milieu but also preserve β cell function.

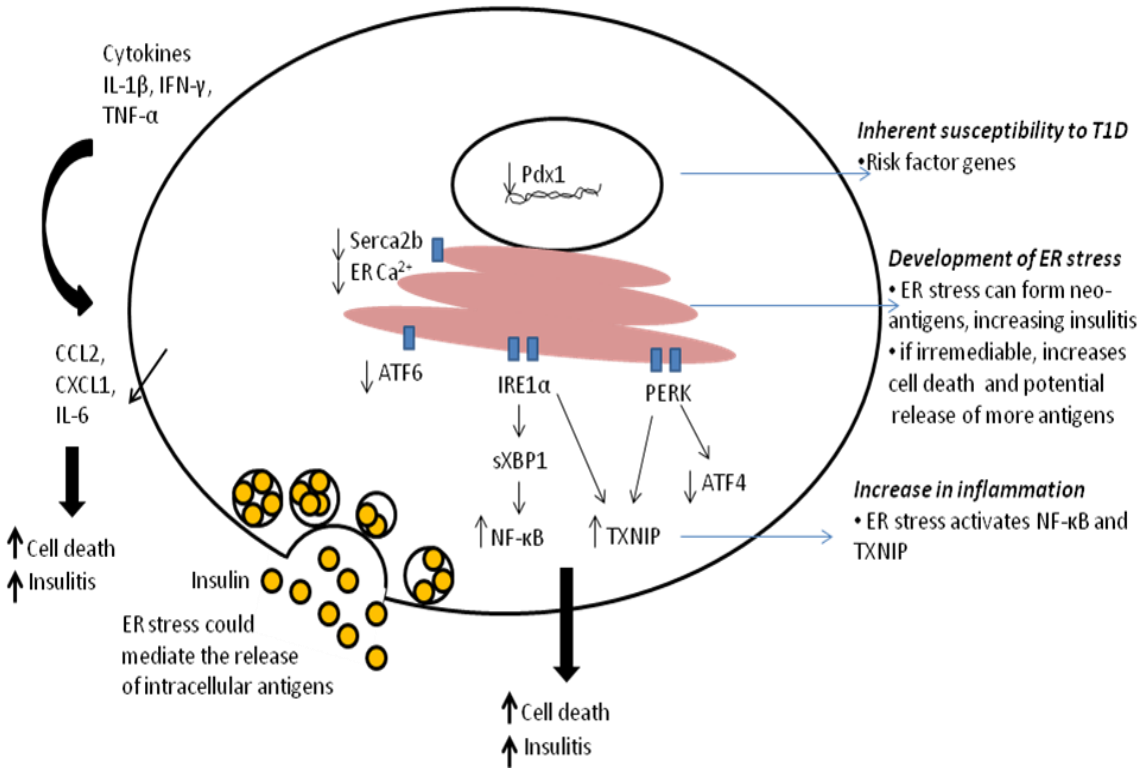

Figure 5. The contribution of ER stress to type 1 diabetes. The prediabetic β cell is susceptible to cell death due to several factors. In response to cytokines, such as IL-1β, IFN-γ and TNF-α, the β cell expresses chemokines such as CCL2, CXCL1 and cytokines such as IL-6, potentially causing enhanced recruitment of immune cells and augmenting β cell death. The inflammatory milieu of the β cell and the presence of inherent genetic factors (such as decreased *Pdx1* and *Serca2b* expression) contribute to the development of ER stress, resulting in the production of neo-antigens, cell death and an exacerbated immune response. Furthermore, ER stress also activates the expression of NF-κB and TXNIP, which, in turn, increase cell death. Therefore, according to this model, the β cell actively assists in its own death.

Type 2 Diabetes: T2D is a metabolic disorder characterized by the presence of insulin resistance along with β cell dysfunction. T2D accounts for around 90% of diabetes cases, with around 300 million people projected to have T2D by 2025 (201). Risk factors such as genetic susceptibility, obesity and insufficient physical activity contribute to the development of T2D. Although insulin resistance was initially believed to be the principal cause of T2D, emerging evidence in the field suggests that β cell dysfunction acts as a key initiator in the onset of the disease. In the presence of chronic excess fuel, the β cell copes or compensates by enhancing insulin secretion or β cell mass. An inability of the β cell to sufficiently compensate can result in the onset of T2D. A loss in β cell function (202) accompanied by around 40-60% loss in β cell mass (203,204) has been shown to precede the development of disease. Furthermore, early loss of β cell function was observed in groups that were at risk to T2D, such as first degree relatives of T2D patients (205), Pima Indians (206) and women with gestational diabetes (207). Several genome wide association studies conducted on diabetic patients revealed more than 30 β cell loci associated with T2D (208–210), supporting the hypothesis that the islet β cell plays a crucial role in the progression of the disease.

Glucolipotoxicity (chronic hyperglycemia and elevated free fatty acid levels in the body) can alter metabolic pathways in β cells, resulting in more malonyl Co-A and long-chain acyl Co-A (LC-CoA) species, subsequently inciting lipogenesis and decreasing β cell function. Hyperglycemia results in a reduction in Insulin and β cell-specific transcription factors such as Pdx1 and MafA, further decreasing β cell function (reviewed in (44,211)). Excessive glucose metabolism results in increased reactive oxygen species production and oxidative stress, which can be toxic as the β cell has low levels of antioxidant enzymes (212–214). The resulting oxidative stress then decreases MafA expression and Pdx1 DNA binding ability (42), among a host of other effects, eventually

causing β cell dysfunction and cell death. Moreover, hyperglycemia and elevated free fatty acids increase the need for β cell insulin secretion, causing the endoplasmic reticulum (ER) to cope with the sudden insulin demand, potentially resulting in ER stress in T2D patients (215). Cytokines secreted by adipose tissue activates inflammatory pathways in the β cell (such as JNK and IKK), exacerbating oxidative stress and ER stress, which, if left unresolved, can result in further β cell death (reviewed in (133,165)). Essentially, the β cell in a type 2 and type 1 diabetic patient, is equivalent to a melting pot, wherein oxidative stress, inflammation, and ER stress pathways collide to instigate and aggravate β cell dysfunction and death. Medications for T2D include drugs that increase insulin sensitivity, such as thiazolidinediones, or enhance β cell function, such as increasing incretin levels by DPP-IV (dipeptidyl peptidase-IV) inhibitors and administration of sulfonylureas, which increase insulin secretion. However, long-term management of blood glucose levels can be difficult. After oral medications fail, insulin injections may become necessary for T2D patients.

MODY: A form of diabetes referred to as maturity onset of diabetes (MODY) occurs in patients with mutations in certain genes expressed in β cells. The onset of MODY occurs around 25 years of age or younger. Patients suffering from MODY usually show impairments in glucose-induced insulin secretion and hyperglycemia without ketoacidosis. MODY2 is due to a mutation in the *Glucokinase* gene, whereas MODY1, 3, and 5 are due to mutations in the genes coding for transcription factors HNF4α, HNF1α and HNF1β. MODY4 and MODY6 are due to loss of function mutations in Pdx1 and NeuroD1. MODY reveals the importance of these transcription factors in β cell function

1.7 Hypothesis:

β cell dysfunction and reduced survival are central to the development of diabetes. Research focusing on maintaining normal β cell function will provide useful insights for novel therapeutic strategies. In this respect, I have undertaken two series of experiments aimed at understanding transcriptional regulation in a normal β cell and translational stress response in type 1 diabetes. I hypothesize the following:

1. *Methylation of Pdx1 by Set7/9 enhances Pdx1 transcriptional activity and promotes β cell function.*
2. *Pioglitazone treatment of prediabetic NOD mice improves β cell function, prevents β cell death and hence, reduces the autoimmune response.*

CHAPTER 2: Materials and Methods

Antibodies: Pdx1 antibody was obtained from Millipore (#07-696, 1:1000). Set7/9 antibody was obtained from Cell Signaling (#2813, 1:1000). Monoclonal Flag M2 antibody was obtained from Sigma-Aldrich (#F1804). Pdx1 K131-methyl antibody was generated in rabbits using the synthetic peptides C-Ahx-TKAHAW[K-Me1]GQWAG-amide (Pdx1 K131me NT or Me-1) and Ac-AHAW[K-Me1]GQWAGGA-Ahx-KKC-amide (Pdx1 K131me CT or Me-2) by contract to 21st Century Biochemicals. Pdx1 K131-methyl antibody was used at a dilution of 1:5000. Gapdh antibody was obtained from Ambion (#AM4300, 1:4000). Other antibodies used are as follows: Bip (Cell Signaling, #3183, 1:1000 dilution), ATF4 (Santa Cruz, #sc-200, 1:500 dilution), Actin (MP Biomedicals, #691001, 1:1000 dilution), GAPDH (Ambion, #AM4300, 1:4000 dilution), Pdx1 (Millipore, #07-696, 1:1000 dilution), and TXNIP (Santa Cruz, #sc33099, 1:500 dilution). Fluorophore-labeled secondary antibodies IRDye 700 and IRDye 800 were obtained from Licor Biosciences.

Vectors: Plasmids were generated using standard recombinant techniques. PCR-generated constructs were verified by automated sequencing. Recombinant protein was expressed using the Escherichia coli expression vectors pET21d, that contained a T7 promoter driving the expression of full length Syrian hamster Pdx1 and truncated mutants Pdx1 homeodomain, Pdx1 homeodomain and C terminal (216). Wildtype human Pdx1 was PCR amplified from the origene cDNA (NM_000209) and then ligated into Nde1 and BamH1 sites in the pET15b vector, which contains an in frame C term 6XHis tag. Recombinant human Pdx1 point mutants were also expressed using the pET15b expression vector. The N terminus Pdx1 protein (1-137 aa) was PCR amplified

from the pET15b vector containing human Pdx1 insert, using primers that incorporated a Nde1 and Sal1 restriction site and stop codon in the forward and reverse primer respectively. The amplified product was cloned into a pCR2.1-TOPO vector (Invitrogen, K4550-01) and then cleaved and ligated into the Nde1/Xho1 sites in pET15b backbone vector. Primers used to clone the N terminus of human Pdx1 were: 5'-CATATGAACGGCGAGGAGCAGTA -3' and 5'-CTCGAGTTATTAGCCGCCTGCCCACTGGCCTT -3'. Point mutants were generated using the Quick Change® site directed mutagenesis kit (Agilent). The following primers were used to make the respective point mutants: K123R: 5'-CAGCTGCCTTTCCCATGGATGAGGTCGACCAAAGCTCAC-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 wildtype and mutant *Pdx1* in HEK293 and NIH3T3 cells. The CMV promoter –driven point mutants were made using the same primers, as mentioned above. However, to make the K123R mutant in the pBAT12 HA vector, the following primers were used: 5'-TCAGCTCCCTTTCCCATGGATGAGATCCACCAAAGCTCA-3' and 5'-TGAGCTTTGGTGGATCTCATCCATGGGAAAGGGAGCTGA-3'. The CMV promoter-driven vector used to drive *Setd7* was described previously (108). The reporter plasmids 5FF and Gal4-UAS is as previously described (85).

Animal Models: 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 *Setd7Loxp*$^{/+}$ 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 (217). For induction of *Cre*-mediated recombination, mice were orally gavaged with tamoxifen dissolved in peanut oil (mixed for 1 hour at 55^0C) at a dose of 5 mg/day/mouse. Pdx1$^{4.7}$-Cre mice were kindly provided by Dr. G. Gu (Vanderbilt University), which were crossed onto the *Setd7*$^{Loxp/+}$ mice on a mixed background to generate pancreas specific Set7/9 knockout mice (Set7/9$^{\Delta panc}$). The expression of Set7/9-FLAG transgene in the pancreas was driven by a 4.7 Kb Pdx1 promoter (218). Two germ line transmissible founders, pSet7/9Tg J and K, having high and low flag expression were phenotyped. Both founder lines were present on a mixed background. pSet7/9Tg J was chosen for further studies. Five week-old female NOD mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were allowed to acclimate for 1 week prior to experimentation. Mice were fed with either a standard chow diet or a standard chow diet containing 0.01 wt% pioglitazone (Harlan-Teklad Global, Indianapolis, IN). This concentration of pioglitazone was designed to deliver approximately 20 mg/kg body weight pioglitazone (based on average daily food intake). Mouse islets were isolated from pancreas (219) and then handpicked and cultured in RPMI medium (11mM glucose) for around 1 hour or overnight for RNA/protein analysis and GSIS assays respectively.

Cell culture: The mouse fibroblast cell line NIH3T3 was maintained in Dulbecco's modified Eagle's medium supplemented with 10% Newborn Calf serum, 1%Sodium pyruvate, 100ug/ml Penicillin and 100ug/mil Streptomycin. The human embryonic kidney cell line, HEK293, and the rat insulinoma cell line INS-1 832/13 were maintained in

RPMI-1640 medium (11mM glucose), supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 50uM β-mercaptoethanol (Ins-1 mix), 10mM HEPES, 100ug/ml Penicillin and 100ug/ml Streptomycin. All cell lines were cultured at 37^0C in humidified chambers with 5% CO_2.

***Reagents*:** Recombinant Set7/9 protein (#ENZ-314) was purchased from Prospec. The inhibitor BP-107-7 (Figure. 10D) was synthesized by employing a similar procedure as described previously (220,221). Overall % yield = 38%. ^{1}H NMR (CD_3OD): δ 7.45-7.27 (m, 20H), 6.67 (s, 2H), 3.78 (t, 4H, J = 5.76 Hz), 3.00-2.94 (m, 8H), 1.72-1.69 (m, 4H), 1.55 (b, 4H), 1.48 - 1.46 (m, 2H). ^{13}C NMR (CD_3OD): δ 141.72, 132.48, 129.62, 128.23, 127.27, 127.07, 61.38, 47.04, 44.50, 40.12, 26.43, 25.83, 25.22, 23.10. HRMS calculated 666.35, found 667.2 ($[M+1]^+$). Melting point is 178-181°C. UPLC retention time is 2.65 min using a Waters® UPLC instrument fitted with an Acquity UPLC BEH C18, 1.7µm column (2.1 X 50 mm), 5-10% acetonitrile in water (0.1% TFA) during a 5 mins run.

Protein Purification: 6X-His tagged Pdx1 wildtype and mutant proteins were overexpressed in BL21DE3/pLysS using the pET expression system (Novagen). Cultures were grown up to log phase at 37^0C, with an OD_{600}= 0.6-1.0 in 1l of Luria Bertani Culture. Over expression of recombinant proteins were induced by the addition of 1mM IPTG. Thereafter, cultures were grown overnight at 30^0C and pelleted by centrifugation at 4000 rpm for 15min. Pellets were resuspended in 25 ml Lysis buffer (50mM NaH2PO4, pH 8.0, 300mM NaCl, 5mM imdiazole, 10mM DTT, 5% glycerol, 40U of lysozyme/ml of culture (novagen) and 25U per 1ml of original culture, novagen) and incubated in ice for 1 hour. The lysates were sonicated at a setting of 10, for 10s bursts, 15 times and then centrifuged at max speed for 10 min at 4^0C. The resulting supernatant

was incubated with 5ml of Ni-NTA resin (Qiagen) for 1 hour at 4^0C, following which it was transferred into a 25ml column (BioRad) and allowed to flow at a gravity rate, and the eluate was collected in 1.5 ml eppendorf tubes. The protein was eluted with an increasing imidzaole gradient (10mM-250mM) of lysis buffer (10ml). Fractions were collected and protein concentrations were determined using Lowry's assay (BioRad). The fractions were resolved on a 12% SDS-PAGE and then stained by coomassie blue

Methylation Assay: 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 MgCl2 , 0.05mg/ml BSA, 1 µM ^{3}H-AdoMet or 50 µM AdoMet in 20µl volume. The reaction was stopped by the addition of 6X SDS gel loading buffer. The samples were then resolved by 10% SDS-PAGE gel, followed by coomassie stain to verify loading. The gel was further incubated with En3hance solution (Perkin Elmer), washed three times with water (10 minutes) and dried under vacuum at 70^0C for 1 hour. The gel was exposed to Kodak XAR film for 24-48 hours at -80^0C. Quantification was done using the ImageJ software.

Immunoblot Assay: Lysis Buffer used contains 0.05% Sodium deoxycholate, 10%NP-40, 0.1% SDS, 0.2% Sarcosyl, 10% Glycerol, 1mM DTT, 1mM EDTA, 10mM Sodium Fluoride, 50mM Tris-HCl (pH 8.0) brought up to 500ml with 1X PBS (Phosphate buffered Saline, pH 7.2). Prior to use, 20ul of 1M $MgCl_2$, 5ul Benzonase, 1 table of min-EDTA inhibitor and protease inhibitor (Roche) were added to 10 ml lysis buffer and mixed. For lysis of isolated islets, the SDS concentration was increased to 2% SDS. Samples were resolved on 10% polyacrylamide gels and were then transferred onto a PVDF membrane (Immobilon-FL, Millipore). Membranes were blocked with Odyssey blocking buffer (Li-Cor Biosciences) for 1 hour at room temperature and exposed to primary

antibody, overnight at 4^0C. The following day, the membrane was washed three times with 0.05% PBS-Tween 20 and exposed to Li-Cor fluor tagged secondary antibody (1:10,000) for 1 hour, following which, the membrane was washed three times with 0.05% PBS-Tween 20 again and visualized and quantified using the Li-Cor Odyssey system. Dot Blot was performed on a PVDF membrane (Millipore), using the Bio-Dot Apparatus (#170-6545) using the Pdx1 methyl antibody and the unmodified Pdx1 peptide, the Pdx1 K131me NT and Pdx1 K131me CT peptide (described under antibodies).

Nuclear Extraction: 10cm plates of cultured cells were washed twice with 1X PBS followed by a 10 min incubation with hypotonic buffer (10mM Hepes, pH 7.9, 10mM KCl, 0.1mM EDTA, protease and phosphotase inhibitors). 25ul of 10% IGEPAL was added to each plate, for 2 min, to disrupt the plasma membrane. The cells were scrapped and centrifuged at 6000rpm for 3 minutes. The supernatant which is the cytoplasm is transferred to another tube. Around 100ul of lysis buffer (described above) was added to the nuclear pellet, followed by incubation at the 4^0C for 2 hours or sonication, followed by centrifugation at maximum speed for 10 minutes. After protein determination, the nuclear lysates is stored at -80^0C or used for further analysis.

Co-immunoprecipitation Assays: For detection of methylation, 10 cm plates cultured with HEK293 cells transfected with 5ug Pdx1 or 5ug Set7/9 expression plasmids or INS-1 832/13 cells treated overnight with BP-107-7 was used. Around 48 hours later, cells were washed twice with ice cold PBS and whole cell extracts were determined for protein. The lysis buffer used was the same as that described for immunoblot, but prepared without DTT and SDS. 1mg of HEK293 whole cell lysates or 500ug of INS-1 832/13 nuclear extract was incubated along with beads and antibody overnight. Prior to

overnight incubation, 20ul of the reaction is collected as input. The beads were subsequently washed three times with lysis buffer and the eluted protein was obtained with boiling SDS sample buffer. The sample was then analyzed by immunoblot analysis. Immunoprecipitation reactions for HA-tagged Pdx1 from HEK293 whole cell lysates was performed using the Pierce HA-tag IP kit (26180) according to manufacturer's instructions. Immunoprecipitation assays for methylated Pdx1 were conducted using 20ul Protein A Dynabeads (Novex, Life technologies), incubated with 4ug anti methyl-lysine antibody (Cayman chemicals, #13727). Co-immunoprecipitation assays from NIH3T3 cells transfected with Pdx1 and Set7/9 were performed using Invitrogen Protein G dynabeads incubated with Flag antibody (Sigma). Nuclear extracts were sonicated to shear DNA and then subjected to co-immunoprecipitation assay. For protein elution from dynabeads, the samples were incubated for ten minutes with 20ul SDS sample buffer at 70^0C. 2% input, along with the eluted protein is analyzed using a 12% SDS-PAGE gel and subjected to immunoblot analysis.

Quantitative Real-Time Reverse Transcriptase RT-PCR: Approximately 10^6 β cells or 75-100 islets were lysed in 350ul of RLT buffer (Qiagen) containing 1% β-mercaptoethanol. Samples were sheared through a 27- guage needle. The Qiagen RNeasy Plus micro kit (#74034) and RNeasy Plus mini kit (#74134) were used for RNA isolation from islet and cell line samples respectively. 5ug of total RNA was reverse transcribed to cDNA at 37^0C for 1 hour using 5ug random hexamers, 0.5mM dNTP, 5X first strand buffer, 0.01mM DTT and 200 units of MMLV reverse transcriptase (Invitrogen) in a final reaction volume of 20ul. The resulting cDNA was diluted with RNase free water in 1:100 ratio. The real-time PCR reaction was performed using Sybr Green, 2.5mM $MgCl_2$, 1X PCR buffer (10Mm Trish HCl, pH 8.3, 50mM KCl), primer mix (0.2uM of each primer) and Taq polymerase (Sigma). The threshold cycle (C_T)

methodology was used to calculate relative quantities of mRNA products from each sample, and all samples were corrected for total RNA by normalizing C_T values to the C_T value of *Actb* message. All data represent the average of triplicate determinations from at least three independent experiments. Primers used for *Pdx1* (QT00102235), *Slc2a2* (QT00103537), *Gck* (QT00140007), *MafA* (QT00298718) were obtained from Qiagen Quantitect Sybr Green kit. Primers used for *Ins1/2*, *Insulin pre*-mRNA and *Actb* are as described previously (72). Other primers described previously include Xbp1s, Atf4 (196), Gpx1 and Sod1 (222). Primer for mouse *Txnip* was 5'-GTGCAGAAGATCAGACCATCC-3' and 5'- AGCCAGGGACACTGACGTA-3'. For rat *Txnip*, the following primers were designed and verified to amplify the expected sequence with appropriate linearity: 5'-TCCATCCACGCTGACTTTGAG -3', and 5'-CCCTGAGATAATGTGATTGCCTCTG-3'. Measurement of methylated and unmethylated preproinsulin DNA in the serum by multiplex PCR was performed as we recently detailed (223).

***Luciferase Assay*:** $5x10^5$ NIH3T3 cells were transfected with 1µg of reporter plasmid (5FF or Gal4-UAS reporter plasmid), 0.1µg of pbat12 or wildtype or mutant Pdx1 transgene and 0.5µg of Set7/9 expression plasmid. Transfections were performed using 6ul of metafectene pro (Biontex) per well. After 48 hours, whole cell extracts were used to assess luciferase activity using a commercially available luciferase assay kit (Promega), as per kit instructions. Whole cell lysates were determined for protein concentration and lysates were analyzed for Pdx1 and Set7/9 levels using immunoblot assay.

Labeling of EMSA probes: Insulin minienhancer probes, containing the E2/A3/A4 element, were used for to determine Pdx1 binding affinity. The top and bottom strand sequences were the following:

GATCCTTCATCAGGCCATCTGGCCCCTTGTTAATAATCTAATTACCCTAGGTCTAA,

GATCTTAGACCTAGGGTAATTAGATTATTAACAAGGGGCCAGATGGCCTGATGAAG

The top strand was 5'- end labeled with γ-32P ATP using T4- poly nucleotide kinase in STE buffer (10mM Tris pH 7.5, 1mM EDTA, 100mM NaCl) and annealed with unlabelled bottom strand at room temperature. Approximately 20000 counts of probe was used for each reaction in the EMSA assay.

Electrophoretic Mobility Shift Assay (EMSA): NIH3T3 cells were transfected with wildtype or mutant Pdx1, with or without Set7/9. Nuclear extracts were sonicated to shear DNA, measured for protein and then quickly frozen in liquid nitrogen and stored at -80^{0}C or used for the EMSA assay. 1ug of nuclear extract was used to perform the EMSA reaction. The EMSA reaction consisted of labeled probe, nuclear extract, 1ug poly-dI/dC, 1ug BSA and binding buffer (50mM Hepes, pH 7.9, 375mM KCl, 12.5mM $MgCl_2$, 0.5mM EDTA, 5mM DTT, 15% Ficoll), with or without increasing concentrations of cold probe. The reaction was incubated at room temperature for 15 minutes and was then resolved on a 5% polyacrylamide gel at 150V and then subsequently dried in a gel drier for 2 h, at 70^{0}C. The gel was exposed to a phosphoimager screen (Molecular Dynamics) overnight and imaged the next day. Competition EMSAs were modeled on the basis of 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 described previously (216)

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 TSK gel 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 (224). 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 SequestR algorithms within Proteome Discoverer (Thermo) against appropriate FASTA sequence databases obtained from UniProt. Database searches included dynamic modification analysis for mono-, di-, and trimethylated Lys residues, and Met residue oxidation.

Tissue Fixation: Pancreata was harvested immediately after the animal was sacrificed. The pancreas was then placed in 4% paraformaldehyde for 2 hours at 4^0C, followed by two 1X PBS washes (15 minutes each) at room temperature. The pancreata are then washed in 25%, 50% and 70% ethanol for 15 minutes each at room temperature, followed by paraffin embedding. The pancreata from the NOD mice were heart perfused with PBS and 4% PFA, followed by transfer of the pancreata to 4% paraformaldehyde overnight at room temperature. The pancreata were further fixed for 1 hour with 4% paraformaldehyde, followed by two 1 hour washes with PBS, a one hour wash each with 50% and 70% ethanol and paraffin embedding. The pancreata were then sectioned into 5 µm sections, with every alternate section being collected for analysis.

Immunhistochemistry: Paraffin was removed from tissue sections by submersion in xylene, 100%, 95%, 70% ethanol and H_2O for 5 minutes each. For staining with HRP secondary antibodies, the endogenous peroxidase activity was quenched by submersion of slides in a mixture of methanol and H_2O_2 for 30 minutes. The antigens were unmasked by heating the slides immersed in unmasking solution (Vector Labs) at 95^0C for 20 minutes. The slides were cooled to room temperature and blocked with 2.5% Goat serum for 30 minutes. After blocking, the slides were incubated with primary antibody overnight at 4^0C. The sections were washed with PBS three times and exposed to Alexa fluor anti-mouse, anti-rabbit, anti-guinea pig (568 and 488) secondary antibodies for 1 hour at room temperature and DAPI (Invitrogen) for 3 minutes. In the case of staining with HRP-conjugated secondary antibody (Vector Labs), the sections were then exposed to either Vector NovaRed or Vector DAB substrate, followed by hemotoxylin staining. The slides were visualized using an LSM 700 confocal microscope (Zeiss, Thornwood, NY).

Insulitis Scoring and β cell area: Pancreatic sections were immunostained for insulin and counterstained with hematoxylin. For insulitis scoring, 3 pancreas sections at least 75 μm apart from 5 animals per group were scored using the following grading scheme (33): grade 1 – no islet-associated mononuclear cell infiltrates; grade 2 – peri-insulitis affecting less than 50% of the circumference of the islet without evidence of islet invasion; grade 3 – peri-insulitis affecting greater than 50% of the circumference of the islet with evidence of islet invasion; grade 4 – islet invasion. The sections were visualized using a Zeiss Axioskop 40 microscope. Three pancreatic sections, 75 μm apart, were stained for insulin using the Vector Labs kit (described above), and evaluated for insulin$^+$ cells. Images were taken with the Axio-observer Z1 microscope (Zeiss) equipped with a Orca ER CCD camera (Hamamatsu).

Glucose tolerance test: Glucose tolerance tests (GTTs) were performed on mice starved overnight. After 2 g/kg glucose was injected intraperitoneally, blood glucose concentration was monitored at 0, 10, 20, 30, 60, 90 and 120 minutes, using an AlphaTrak glucometer (Abbott, Abbott Park, IL). The values are plotted on a graph and Area under the Curve is calculated using Prism 5 Software. To determine insulin secreted *in vivo*, in response to intraperitoneal glucose injection (2g/kg), blood was collected from the tail at 0, 2 and 10 minutes and serum was obtained by spinning the tubes at 5000rpm for 20 minutes, at 4^0C. Serum insulin was measured using an Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL). Serum proinsulin was measured using a Proinsulin Rat/Mouse ELISA kit (Mercodia, Winston Salem, NC).

Insulin Tolerance test (ITT): Random-fed (or free-fed) mice were injected with 1.5U/kg insulin (Humulin R, Eli Lilly) and blood glucose was measured at 0, 10, 20, 30, 40, 50 and 60 minutes. The blood glucose at each time point was calculated as % of basal blood glucose and plotted against time. Area under the curve was calculated taking % of basal blood glucose at 0 minutes as the baseline. Calculations were performed using Prism 5 software.

Glucose stimulated insulin secretion (GSIS): Islets were harvested and allowed to recover overnight in RPMI medium containing 11mM glucose. The next day, islets were transferred to KRB buffer (115mM NaCl, 5.9mM KCl, 1.2mM $MgCl_2$, 1.2mM NaH_2PO_4, 1.2mM Na_2SO_4, 2.5mM $CaCl_2$, 25mM $NaHCO_3$, 0.05% BSA) and first incubated in 2.5 mM D-glucose for 1 hour, followed by 25 mM glucose for 1 hour. Insulin levels in the supernatant and the total cell lysate were measured using a Mouse Insulin ELISA kit (ALPCO, Salem, NH).

DEXA Scanning**:** Lean body mass and fat mass was determined by dual-energy x-ray absorptiometry (DEXA) using a Lunar PIXImus2 Densitometer (GE Medical Systems).

Flow cytometric Analysis of T cells: Spleen and pancreatic lymph node samples were pulverized and passed through a cell strainer (BD Falcon 352350), followed by treatment with red blood cell lysis buffer (155mM NH_4Cl, 12mM $NaHCO_3$, 0.1mM EDTA) for 3 min and two washes with 1X PBS. For T_{reg} cell analyses, equal volumes of the single cell suspensions (at least 10^5 cells) were stained using anti-CD4-fluorescein isothiocyanate (clone RM4-5, eBioscience, San Diego, CA) and anti-CD25-APC (PC61.5, eBioscience) antibodies and fixed overnight before being permeabilized and stained with anti-Foxp3-phycoerythrin (FJK-16s, eBioscience) antibody. For the Th1 and Th17 cell analyses, equal volumes of the single cell suspensions were first incubated with Cell Stimulation Cocktail (eBioscience) for 4 hours prior to staining with anti-CD4 antibody; cells were fixed overnight then permeabilized and stained for IL-17A (eBio17B7) and IFNγ (XMG1.2) according to manufacturer's instructions (eBioscience). Cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software (TreeStar, Ashland, OR).

To assess cell proliferation, single cell suspensions of cells were incubated in 5 μm membrane dye carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 min at 37 °C, diluted with 5 volumes of ice-cold media, and incubated for 5 min on ice. Samples were then washed with PBS three times and subjected to stimulation *in vitro*. For flow cytometric analyses following stimulation *in vitro*, single cell suspensions were made from spleens harvested from mice as described above. 10^6 cells were cultured in the presence of 75 units/ml recombinant human IL-2 (ProSpec), DMSO or 1 or 10uM pioglitazone and activated with anti-CD3/anti-CD28 antibody-coated beads (Invitrogen) at a concentration of one bead/cell for 4 days. Cells were then harvested, nonspecific Fc-mediated interactions were blocked with anti-CD16/32 (clone 93), and cells were stained as described above for the examination of Treg, Th1, and Th17 cell populations.

The LIVE/DEAD® Fixable Violet Dead Cell Stain kit (Invitrogen) allowed for the exclusion of dead cells. Cells were analyzed using a BD LSRII flow cytometer (BD Biosciences) and FlowJo software.

Unmethylation Index: DNA was extracted from 20ul of serum from control and pioglitazone treated NOD mice using the ZR Serum DNA kit (D3013, Zymo Research Corp, Orange, California). All DNA samples underwent bisulfite treatment using the EZ DNA Methylation kit (D5001, Zymo Research). The PCR assay used was a dual fluorescent probe-based multiplex assay (AHMSN8K; Life Technologies, Gaithersburg, Maryland). For amplification of the mouse 2 PPI promoter, the following primers were used: 5′-AATTGGTTTATTAGGTTATTAGGGTTTTTTGTTAAGATTTTA-3′ (forward); 5′-ACTAAAACTACAATTTCCAAACACTTCCCTAA-3′ (reverse). For detection, the following probes were used: 5′-CTCATTAAACGTCAACACC-3′ (VIC); 5′-CTCATTAAACATCAACACC-3′ (FAM). The PCR reaction also contained TaqMan Universal PCR Master Mix, No AmpErase UNG (Life Technologies) and the reactions were cycled 60 times using the following conditions: 92°C for 15 seconds followed by 60°C for 90 seconds. The formula 2^ (Vic C_T- Fam C_T) was used to calculate the unmethylation index.

Statistics: All data are presented as the mean +/- SEM. One-way ANOVA (with Dunnett's post-test) was used for comparisons involving more than two conditions, and a two-tailed Student's t test was used for comparisons involving two conditions. Prism software version 5.0 (GraphPad Software, San Diego, CA) was used for all statistical analyses. Statistical significance was assumed at $P < 0.05$.

CHAPTER 3: Transcriptional Activity of the Islet β Cell Factor Pdx1 is Augmented by Lysine Methylation Catalyzed by the Methyltransferase Set7/9

In this chapter, I explore the impact of the interaction between β cell transcription factor Pdx1 and its cofactor Set7/9 on transcription of Pdx1 target genes and β cell function. In particular, I test the effect of Set7/9 mediated methylation of Pdx1 transcription and investigate the possibility of methylation as another regulatory level of Pdx1 function.

3.1 Introduction

Deficiency of insulin secretion underlies the transition from normoglycemia to hyperglycemia in both type 1 and type 2 diabetes (225,226). 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 (227–229). 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 (230,231). 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 (232,233). By contrast, interaction of Pdx1 with PCIF1 results in degradation of Pdx1 protein by the proteasome and consequent reduction in Pdx1 activity (234). 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 (235) 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 (236–239). 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 (240,241).

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.

3.2 Results

A. 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 (235). As shown in Fig. 6A, ^{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. 6A).

Prior studies suggest that Lys residues in a broad range of sequence contexts can serve as substrates for Set7/9 (242). 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. 6B. 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.

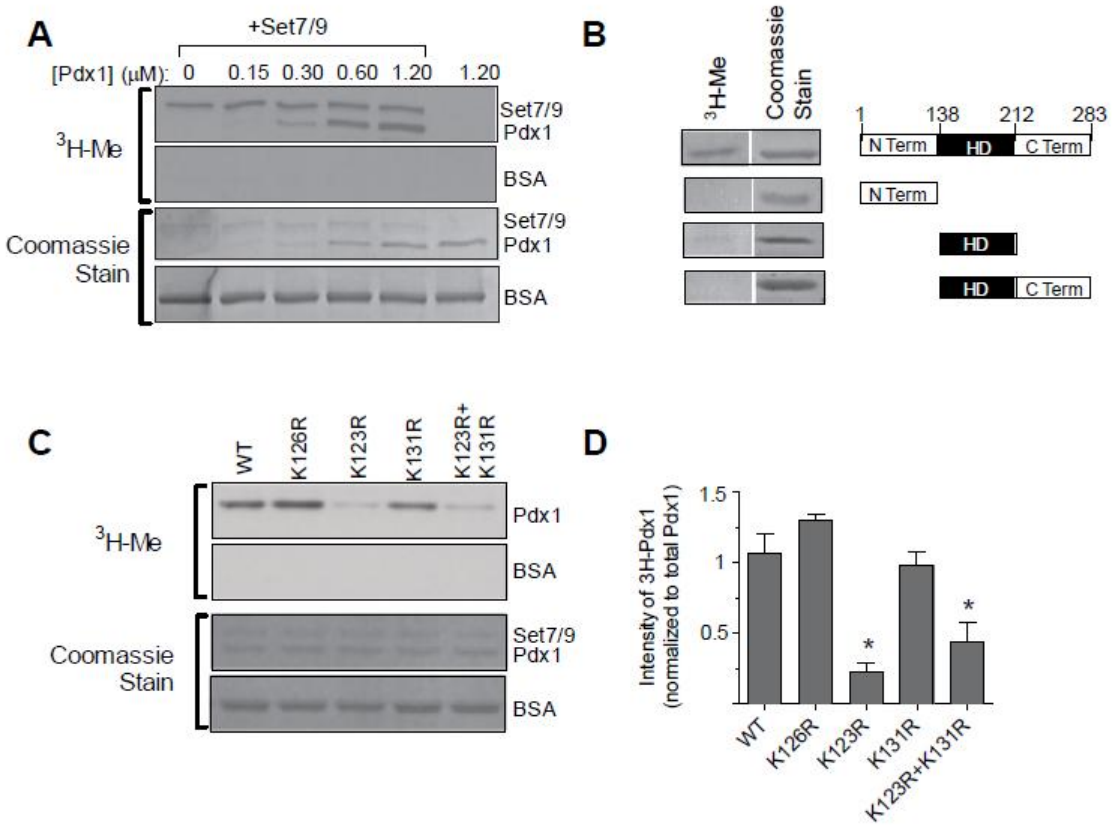

Figure 6. Pdx1 methylation by Set7/9 *in vitro*. *(A)* Methylation assay *in vitro* using recombinant Set7/9, ^{3}H-AdoMet, and increasing concentrations of Pdx1 protein was performed, then reactions were subjected to polyacrylamide gel electrophoresis. *Upper panels* show fluorography for ^{3}H and *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.

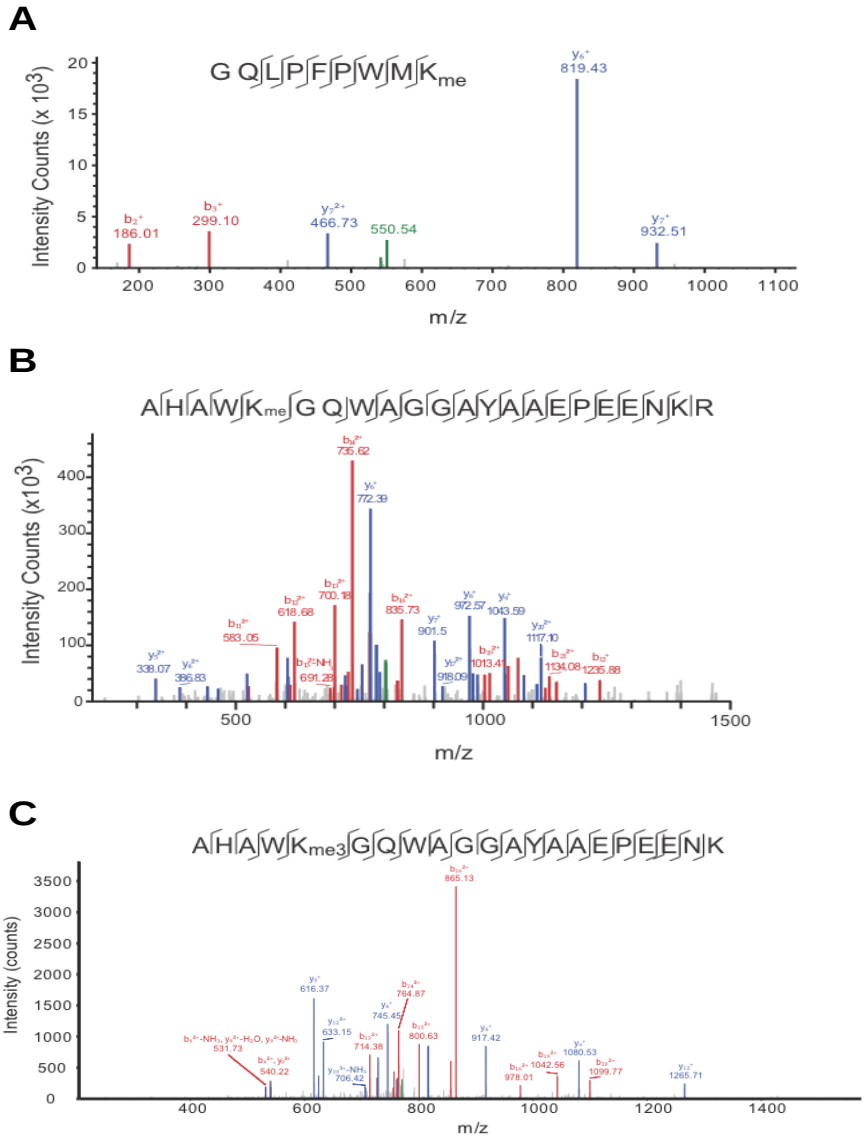

Figure 7. MS/MS spectra of Pdx1 *in vitro* and in MIN6 cells. *(A)* Tandem mass spectrum of the peptide GQLPFPWMK showing modification of K123 with mono-methylation (+14 dalton) following treatment with recombinant Set7/9 *in vitro*. The SEQUEST XCorr for this +2 peptide is 1.83 with a ppm of -1.65; *(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.

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. 7A 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. 6C, 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.

B. 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 (235). Based on our results in Fig. 6, 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. 8A, 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 (233), approximately 12-fold activation of luciferase activity is observed. No

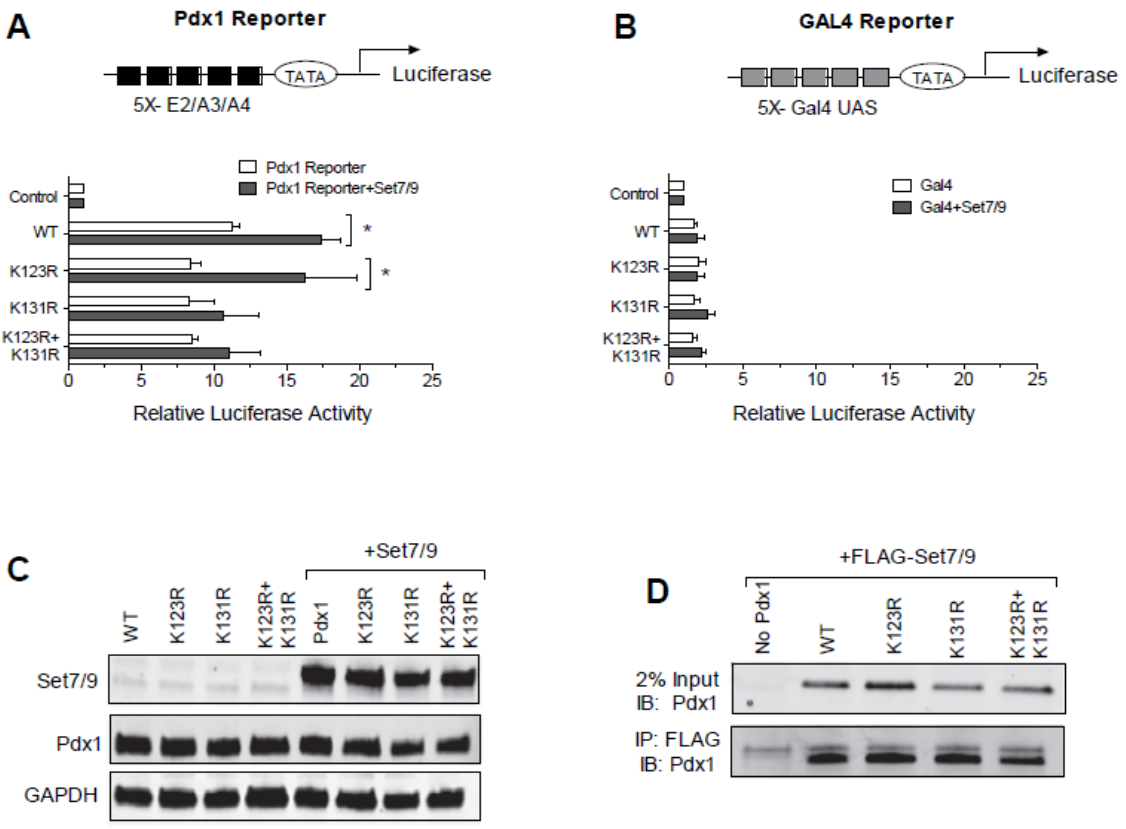

Figure 8. Transcription augmentation by Set7/9 is dependent upon Pdx1 residue K131. NIH3T3 were transiently co-transfected with Set7/9, wild-type (*WT*) and mutant Pdx1 proteins, and either Pdx1 reporter plasmid or Gal4 reporter plasmid. Cells were harvested 48 hours later and whole cell extracts were 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.

activation is observed, however, upon co-transfection of the reporter with a cDNA encoding Set7/9 alone (Fig. 8A). 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. 8A). 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. 8B). 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. 8C). 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 ((241,243)). 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. 8C). 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 8D). 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.

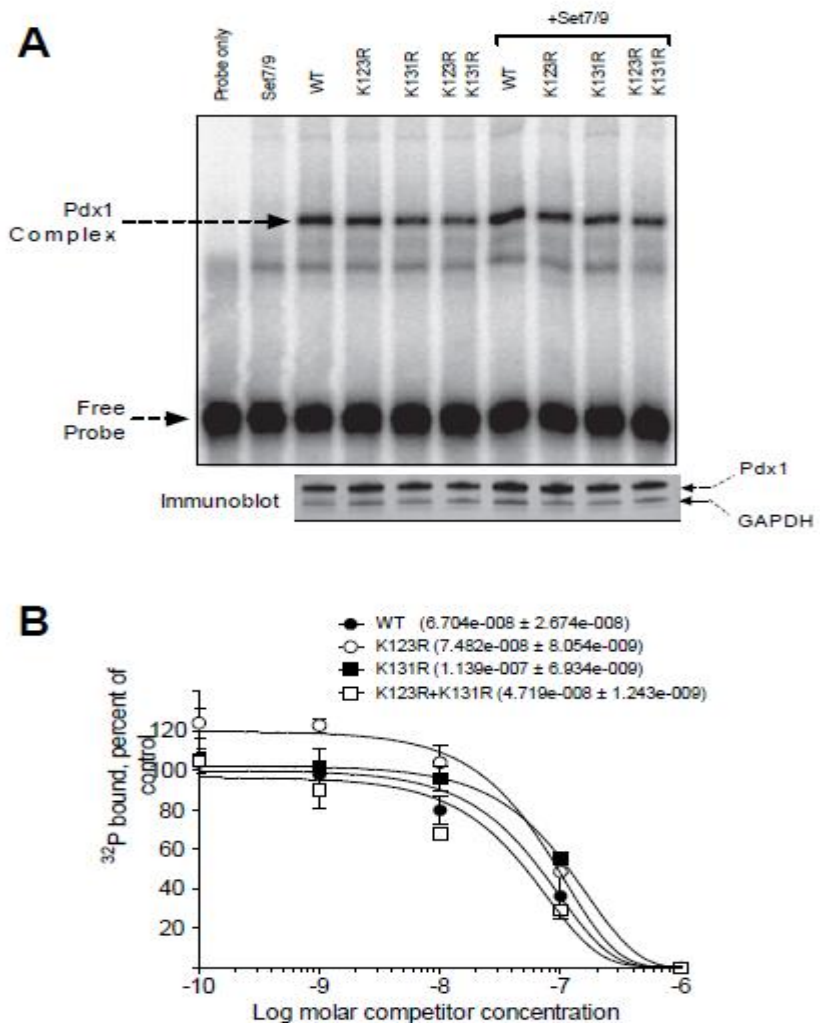

Figure 9. Point mutations do not affect DNA binding by Pdx1. *(A)* NIH3T3 cells were co-transfected with the wild-type (*WT*) or mutant Pdx1 proteins, with or without cotransfection of Set7/9. Nuclear extracts from transfected cells were harvested and subjected to EMSA using ^{32}P-labeled E-A DNA fragment 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 are shown in the inset, with no statistical differences seen. Data shown are from N=3 EMSA experiments.

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 (233,244). No apparent differences in the intensity of the Pdx1-specific complex were observed in the EMSAs (Fig. 9A). To quantify 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. 9B). As shown in the Fig. 9B, none of the mutants exhibited dissociation constants that were different from the wild-type Pdx1 protein. Taken together, the data in Figs. 8 and 9 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 (235).

C. 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. 10A, the antibody exhibited specificity for the Lys-methylated peptides (Fig. 10A). Corresponding specificity of the antibody for methylated full-length Pdx1 was observed using recombinant Pdx1 proteins methylated by Set7/9 *in vitro* (Fig. 10B): 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. 10B). 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. 10C (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. 10D, *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.

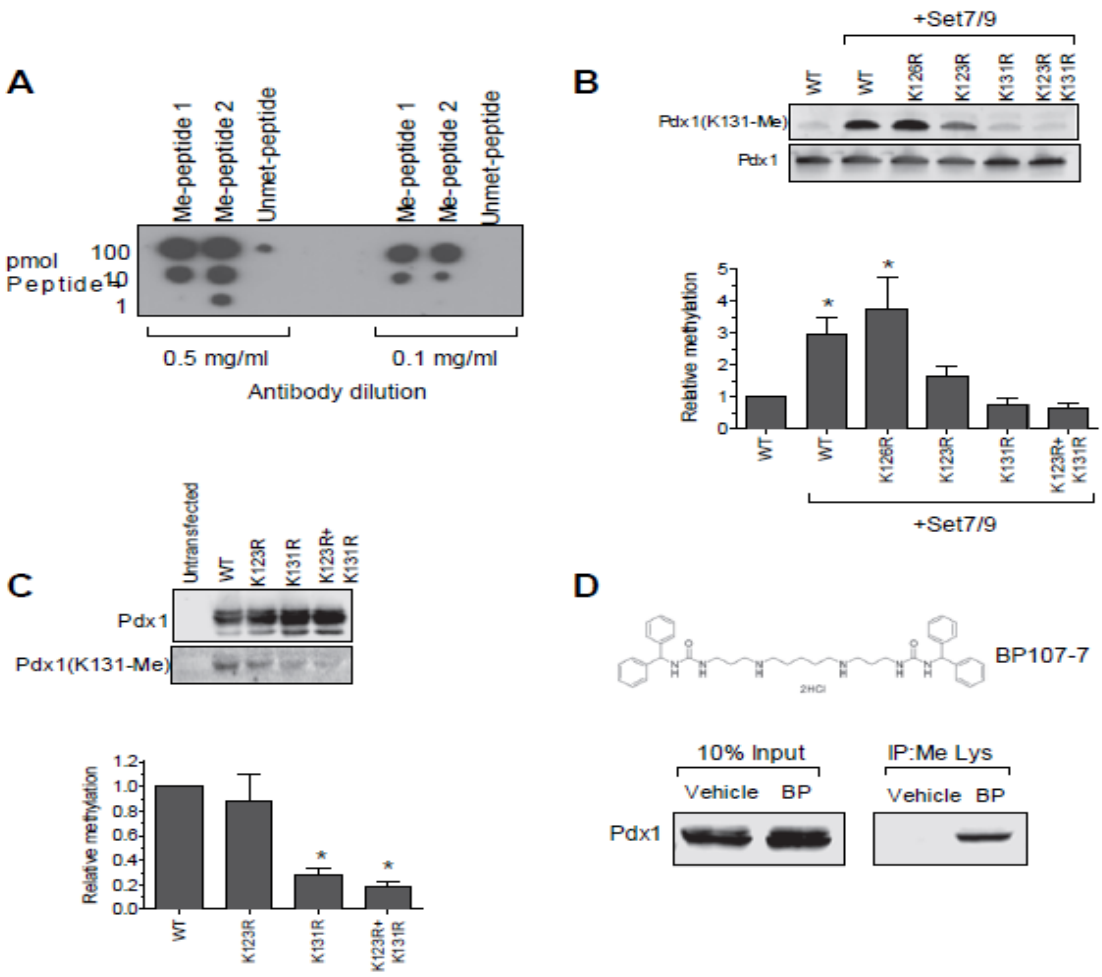

Figure 10. Residue K131 of Pdx1 is methylated in cells. *(A)* Dot blot analysis using two dilutions of anti-Pdx1(K131-Me) antibodies and methylated and unmethylated peptides corresponding to the N-terminus of Pdx1 containing residue K131; *(B)* Representative immunoblots (*top panels*) using anti-Pdx1(K131-Me) 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*). Quantification 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.

As shown in Fig. 10D (*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. 7C shows data for the tri-methylated protein). Collectively, these data suggest that K131 is a target of methylation in cells.

D. 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 (245), 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. 11A). 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-Cre*^{ERT}, ref. (246)). *Setd7*$^{Loxp/Loxp}$*;MIP1-Cre*^{ERT} mice and littermate controls (*Setd7*$^{+/+}$*;MIP1-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

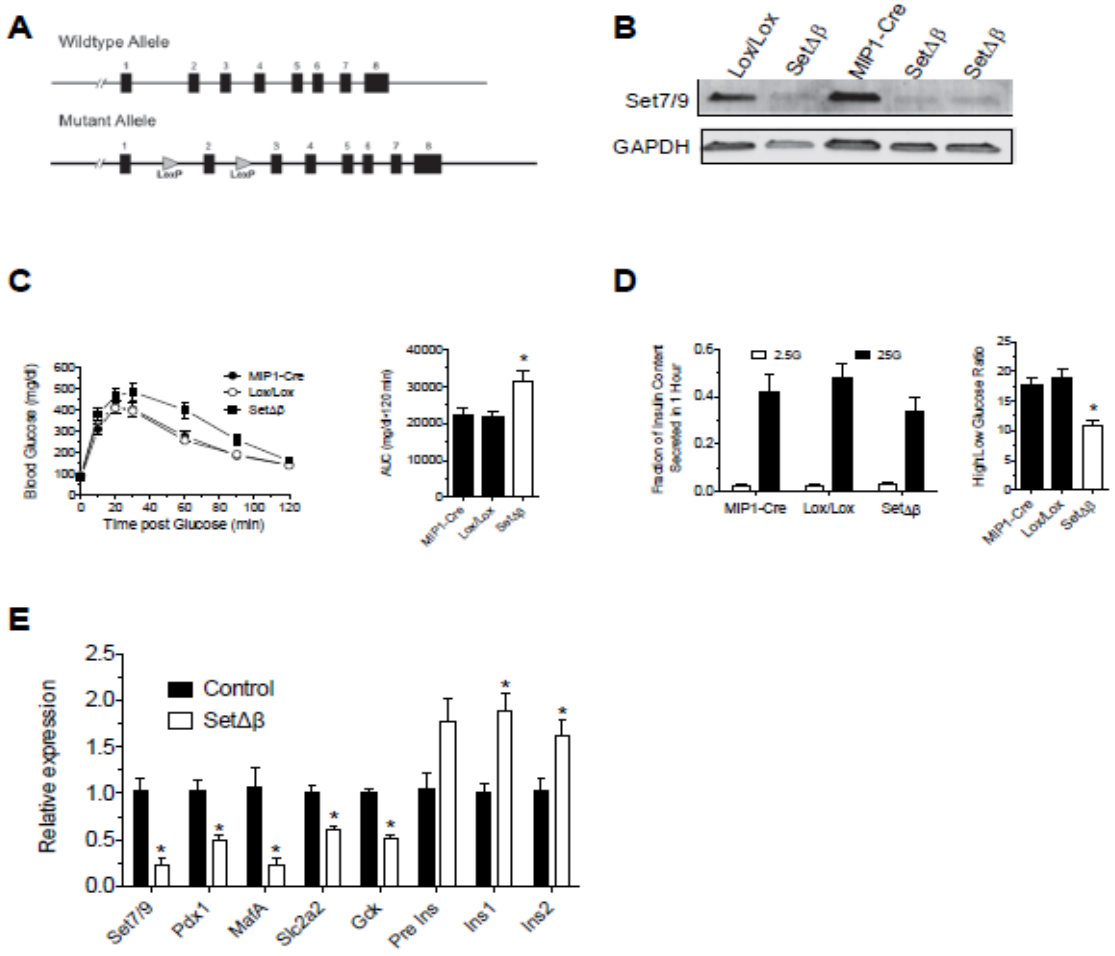

Figure 11. β cell-specific deletion of Set7/9 in mice. *(A)* Schematic representation of WT and mutant alleles present in control and mutant mice, respectively, showing positions of the exons (*numbered*) and the *Loxp* sites; (B) Immunoblot analysis of whole cell lysates of islets isolated from *Setd7*$^{+/+}$*;MIP1-Cre*^{ERT} (*MIP1-Cre*), *Setd7*$^{Loxp/Loxp}$ (*Lox/Lox*), and *MIP1-Cre*^{ERT}*;Setd7*$^{Loxp/Loxp}$ (*Set*^{Δβ}) 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 *Set*^{Δβ} (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*^{Δβ} 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. *Set*^{Δβ} mice. Data are normalized to *Actb* mRNA levels, N=4 independent islet isolations, **P<0.05* compared to control.

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. 11B).*Set*$^{\Delta\beta}$ mice displayed impaired glucose tolerance following an intraperitoneal glucose challenge (Fig. 11C). 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. 11D, 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 (247,248). 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. 11E). 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.

E. Set7/9 plays an important role in β cell proliferation and function from the development.

Since Pdx1 is crucial to pancreatic formation, it is quite likely that Set7/9 also plays an important role in the development and function of the β cell. Yang et al. demonstrated that Set7/9 localizes to the nucleus along with Pdx1 during the conversion of an α cell to a β cell, indicating that Set7/9 may be involved in the differentiation of a β

cell (78). To further elucidate the role of Set7/9 during development, we modulated the levels of Set7/9 using two different mouse models. We used a pancreas specific Set7/9 deletion model, wherein the Cre recombinase expression was driven by a $Pdx1^{4.7}$-Cre promoter. Thus, Set7/9 was deleted from e9.5 specifically in the pancreas and the knockout mice are referred to as $Set7/9^{\Delta panc}$. The $Set7/9^{\Delta panc}$ mice are significantly glucose intolerant, compared to their wildtype littermates (Fig 12A). In addition, compared to wildtype littermates, the knockout mice do not display any variations in insulin sensitivity (data not shown), indicating that deletion of Set7/9 could potentially impair β cell function. Fig 12B shows that isolated islets from $Set7/9^{\Delta panc}$ mice show a dramatic reduction in Set7/9 and around a 50% reduction in Pdx1 protein levels. Interestingly, immunohistochemistry analysis revealed that $Set7/9^{\Delta panc}$ mice displayed a reduction in Pdx1 and MafA protein levels (Fig 12C), providing a possible explanation for the impaired glucose tolerance that we observe in the knockout mice. As Pdx1 and MafA are important for the maintenance of β cell function, a deficiency in these two transcription factors decrease β cell function and impair glucose homeostasis. $Set7/9^{\Delta panc}$ mice also had reduced β cell area (Fig 12D), which could be a consequence of diminished Pdx1 levels and further worsen glucose tolerance. These results suggest that Set7/9 may play an important role in the maintenance of β cell proliferation and function. The other Set7/9 mutant model used was the pancreas specific Set7/9 transgenic mice (pSet7/9Tg), wherein the pancreas-specific expression of a Set7/9-flag transgene was driven by a 4.7Kb murine Pdx1 promoter (Fig 13A). Interestingly, pSet7/9Tg female mice showed better glucose tolerance (Fig 13B), while the males did not. As ERα activation has been shown to improve β cell function (249), and Set7/9 has been reported to increase ERα function (120), the effect of Set7/9 on β cell function could be gender dependent.

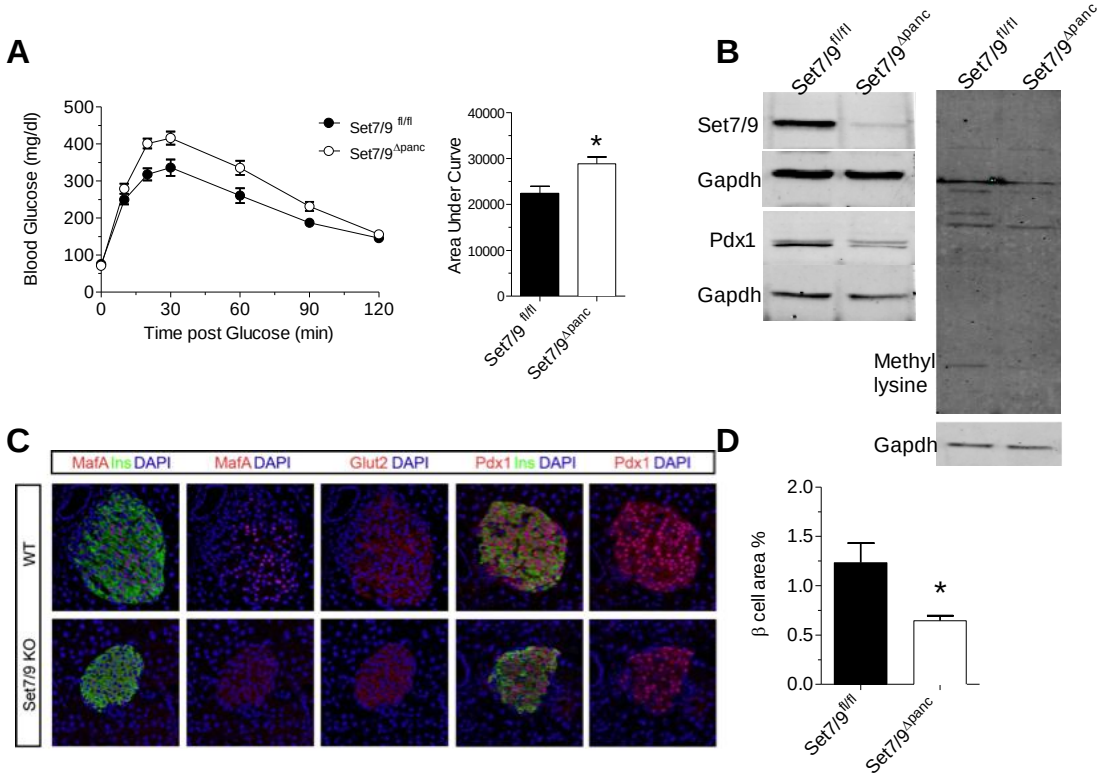

Figure 12. Pancreas-specific deletion of Set7/9 in Set7/9$^{\Delta panc}$ mice: *(A)* Results of intraperitoneal GTT performed on 12 week old *Lox/Lox* (N=15) and *Set7/9$^{\Delta panc}$* (N=17) mice (*left panel*), with corresponding area under the curve (*AUC*) analysis (*right panel*). **P<0.05* compared to wildtype littermate controls; *(B)* Immunoblot analysis of whole cell lysates of islets isolated from 20 week old *Setd7$^{Loxp/Loxp}$* (*Lox/Lox*), and *Set7/9$^{\Delta panc}$* mice for Set7/9, Pdx1 and GAPDH; *(C)* Results of immunofluorescence analysis of pancreatic sections from *Setd7$^{LoxP/LoxP}$* mice and Set7/9$^{\Delta panc}$ mice for MafA, Glut2 and Pdx1 counterstained with Insulin and DAPI; *(D)* Results of β cell area% from 20 week old *Setd7$^{Loxp/Loxp}$* and *Set7/9$^{\Delta panc}$* mice.

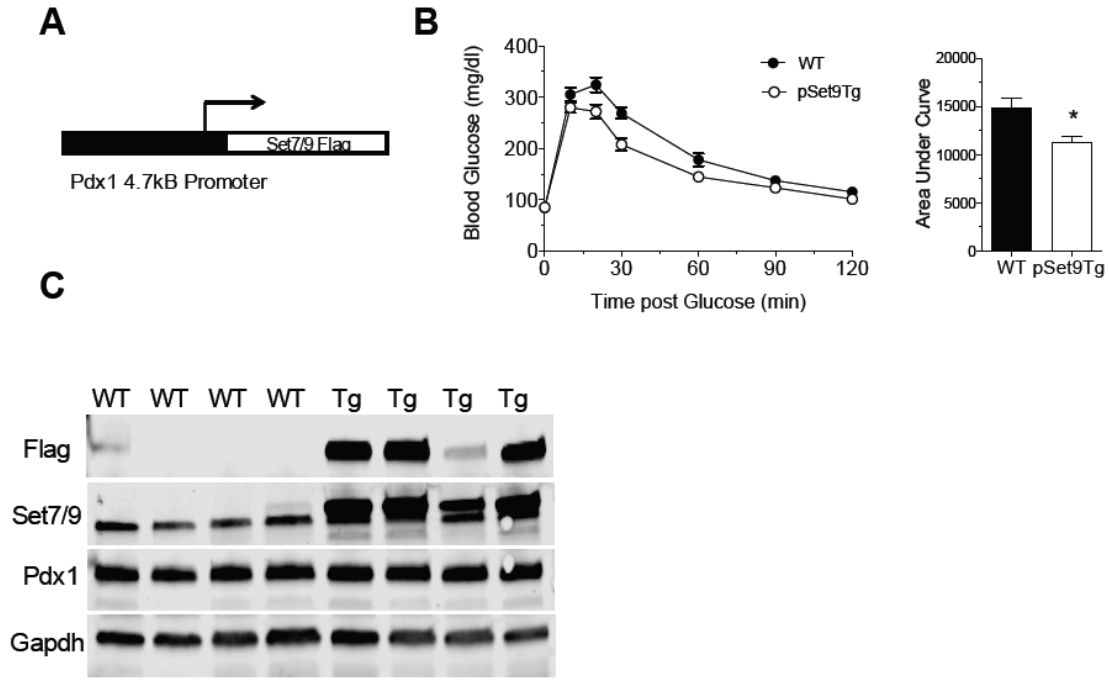

Figure 13. Pancreas-specific expression of Set7/9-FLAG transgene in mice. *(A)* Schematic representation of Set7/9 transgene expressed in the pSet7/9Tg mice, showing the presence of the Pdx1 promoter; *(B)* Results of intraperitoneal GTT on wildtype littermates (N=14) and *pSet7/9Tg* (N=10) mice (*left panel*), with corresponding area under the curve (*AUC*) analysis (*right panel*). **P<0.05* compared to control mice; *(C)* Immunoblot analysis of whole cell lysates of islets isolated from wildtype littermate control mice and pSet7/9Tg mice for Flag, Set7/9, Pdx1 and GAPDH

Although the pSet7/9Tg mice showed a mild, but significant improvement in glucose tolerance (Fig. 8B) expression did not alter Pdx1 protein levels (Fig 13C). Therefore, although Set7/9 deletion affects Pdx1 protein expression and function, Set7/9 overexpression does not. A possible explanation for this finding is that transgenic Set7/9 may modulate only a subset of β cell proteins, therefore enhancing glucose tolerance mildly. Although one of the targets of Set7/9 appears to be Pdx1, as verified by the $Set^{\Delta\beta}$ and the $Set7/9^{\Delta panc}$ mice, the other targets of Set7/9 in the β cell remain to be investigated.

3.3. 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 (245). 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 (250–254), O-GlcNAcylation (255), and sumoylation (256), 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 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, including protein-protein interactions, protein stability, and DNA binding affinity (241,243). Notable proteins that are substrates of Set7/9 Lys

methylation include p53 (236), estrogen receptor-α (238), nuclear factor κB (237,239,257), and TATA box binding protein-associated factor 10 (258), 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 (242,259). 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 (260–263).

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 (264), whereas others suggest it is exclusively a mono-methyltransferase (265). 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 (242); (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 (266); 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 (235). 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 (235,267).

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 (247). 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 (245). 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. However this model does not exclude the possibility (as reported in prior studies) that histone methylation by Set7/9 also contributes to transcriptional activity, this model emphasizes that transcription factor-cofactor interactions can achieve functional transcriptional complexes in many ways.

CHAPTER 4: PPAR-γ Activation Reduces Islet β Cell Stress and β Cell Death in Pre-Type 1 Diabetic Female NOD Mice

In this chapter, I investigate the contribution of β cell survival and function to autoimmune diabetes. To figure out the effect of the β cell in the pathogenesis of type 1 diabetes, prediabetic NOD mice were treated with a PPARγ agonist, known to improve β cell health and function and the mice were assessed for glucose homeostasis and autoimmune response.

4.1 Introduction

Type 1 diabetes (T1D) is characterized by the loss of insulin production, as β cells succumb to targeted autoimmunity. The non-obese diabetic (NOD) mouse spontaneously develops T1D and is used as a model that closely mimics human disease (178). In this model, infiltration of the islet by cells of the immune system (insulitis) is observed as early as 4 weeks of age, with the subsequent development of diabetes in many animals by 12-20 weeks of age (268). The concept that the only defect in T1D lies in the immune system has been challenged in recent years. In recent studies, our group demonstrated that endoplasmic reticulum (ER) stress and resultant insulin secretory defects in the β cell precede the onset of T1D in mice (196). These findings raise the intriguing possibility that ER stress in the β cell might contribute to the aberrant production of “neoantigens” that subsequently invites insulitis (199,269). Dysfunction of the β cell in pre-T1D has been observed in other studies in NOD mice (270) and both β cell ER stress and dysfunction has been observed in humans with early T1D or destined to develop T1D (190,194). Therefore, therapies that directly target β dysfunction and ER stress may prove useful as adjunctive therapies to delay/prevent T1D.

Thiazolidinediones (TZDs) are activators of the nuclear transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) and have been traditionally viewed as insulin sensitizers, owing to their effects on adipose tissue. However, studies in both humans with type 2 diabetes (T2D) and rodent models of T2D suggest that TZDs may also directly enhance β cell function (271,272). The mechanisms underlying the direct effects of TZDs on the β cell include amelioration of ER stress through reduction of oxidative stress (273), restoration of the sarcoplasmic-endoplasmic reticulum calcium ATPase 2 (SERCA2) levels (173), and restoration of levels of the β cell transcription factor Pdx1 and its cofactor Set7/9 (172). In addition to their potential effects on β cells, TZDs have also been implicated in the reduction of inflammation and autoimmunity owing to effects on dendritic cells, macrophages, and T cells of the immune system (274–276)

The potential utility of TZDs in T1D has been limited to only a few studies. In humans, treatment of established T1D with the TZD pioglitazone resulted in, at best, modest improvements in glycemic control (277–279), raising the prospect that intervention with the drug in the pre-diabetic phase might have greater impact. In accordance with this prospect, studies in the NOD mouse have shown that administration of the TZDs rosiglitazone and troglitazone at the time of weaning significantly reduced the incidence of T1D (280,281). These studies in NOD mice did not address the possibility that effects on the β cell conferred by TZDs may have led to reduced stress and improved function early in the pathogenesis of T1D. To address this possibility, we studied the effects of pioglitazone in the pre-diabetic phase in NOD mice. Our results were consistent with a β cell protective effect of pioglitazone independent of effects on insulin sensitivity or body fat distribution, and suggest that reductions in early β cell stress in NOD mice may underlie the reduction in T1D incidence caused by TZDs.

4.2 Results

A. TZD therapy improves glycemic control in pre-diabetic female NOD mice.

Pre-diabetic female NOD mice show progressively impaired glucose homeostasis, impaired β cell function, and increasing markers of ER stress in islets as they age from 6 to 10 weeks (196). To determine if thiazolidinedione therapy can ameliorate β cell stress and dysfunction in the pre-diabetic phase, 6 week-old female NOD mice were fed a standard chow diet containing pioglitazone (0.01 wt%) until 11 weeks of age. The wt% of pioglitazone in the diet was chosen such that the average daily food intake that would result in the delivery of pioglitazone at 20 mg/kg body weight (172). Control mice were fed an identical diet not containing pioglitazone. During the course of the study, none of the animals developed overt diabetes (defined as blood glucose on consecutive occasions exceeding 250 mg/dl). Pioglitazone is known to increase body weight and fat mass in mouse models of T2D (172). However, over the course of this study, pioglitazone-treated NOD mice gained weight at rate identical to controls (Fig. 14A), and demonstrated no differences in body fat composition as determined by dual-energy x-ray absorptiometry scanning at the end of the study (Fig. 14B, C, and D). Nevertheless, at 10 weeks of age, pioglitazone-treated mice exhibited a significant improvement in glycemic control compared to control mice, as judged by an intraperitoneal GTT and corresponding AUC analysis (Fig. 14E and F). To assess if the improved glycemic control in pioglitazone-treated mice was caused by altered insulin sensitivity, an intraperitoneal insulin tolerance test was performed, revealing no gross differences in insulin sensitivity between the two groups (Fig. 14G and H). To further determine if pioglitazone treatment affected insulin signaling, control and pioglitazone mice were injected with saline or insulin (10U/Kg). Liver and Muscle tissues were

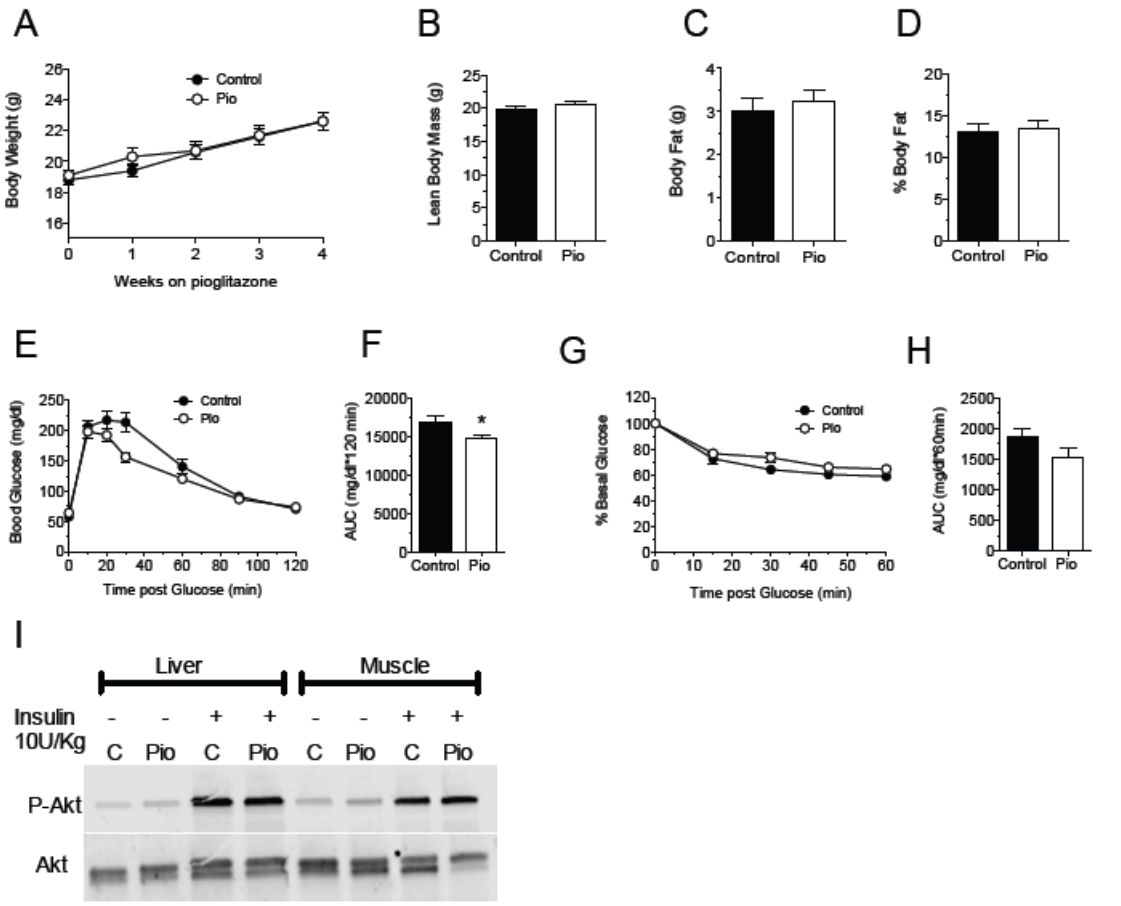

Figure 14. Effect of pioglitazone treatment on metabolic parameters in pre-diabetic NOD mice. 6 week-old pre-diabetic NOD mice (n=10 per group) were placed on either normal chow (*Control*) or chow containing 0.01 wt% pioglitazone (*Pio*). (*A*), body weights during feeding, (*B*) lean body mass as assessed by dual-energy x-ray absorptiometry after 4 weeks of feeding, (*C*) fat mass as assessed by dual-energy x-ray absorptiometry after 4 weeks of feeding, (*D*) percent body fat as assessed by dual-energy x-ray absorptiometry after 4 weeks of feeding, (*E*) results of intraperitoneal GTT after 4 weeks of feeding, (*F*) AUC analysis of GTT shown in panel *E*, (*G*) results of intraperitoneal insulin tolerance test after 4 weeks of feeding, (*H*) AUC analysis of corresponding ITT in panel *G*, (I) Immunoblot analysis of liver and muscle extracts from fasted control and pioglitazone treated mice injected with saline or insulin (10U/Kg) using S473-phospho Akt antibody and Total Akt antibody.*Indicates that the value is significantly different from control by two-tailed *t* test.

extracted 5 minutes post injection and immunoblot assays revealed that there were no differences in Akt phosphorylation between control and pioglitazone treated groups, indicating pioglitazone treatment does not affect insulin sensitivity. Taken together, these results suggest that the improvement in glucose tolerance seen with pioglitazone may be secondary to improvements in β cell function.

B. TZD therapy augments insulin secretion in pre-diabetic female NOD mice

To determine more directly if the improved glycemic control was a consequence of enhanced β cell function, serum insulin levels were measured following an intraperitoneal glucose load. As shown in Fig. 15A, pioglitazone-treated mice displayed significantly higher levels of serum insulin compared to the control group at 10 minutes following the intraperitoneal glucose load. To determine if pioglitazone treatment affected β cell ER stress, we next measured random-fed insulin, proinsulin, and the proinsulin: insulin ratio. Higher proinsulin: insulin ratios have been shown to correlate to ER stress in NOD mice (196). As shown in Fig. 15B-D, whereas the random-fed insulin was a significant 2-fold greater in the treatment group compared to controls, the proinsulin levels were not significantly different, resulting in a proinsulin: insulin ratio that trended lower (*P=0.07*) in the treatment group. In total, the results in Fig. 15 show that pioglitazone treatment causes an improvement in β cell function and a potential reduction in β cell ER stress.

C. Pioglitazone treatment reduces islet ER stress

To assess more directly the possibility that pioglitazone reduced ER stress in NOD mice, we next isolated islets from treated and control mice at the end of the study and measured both mRNA and protein markers of ER stress. ER stress is characterized by activation of three distinct pathways, IRE1α, PERK, and ATF6. In islet β cells, the

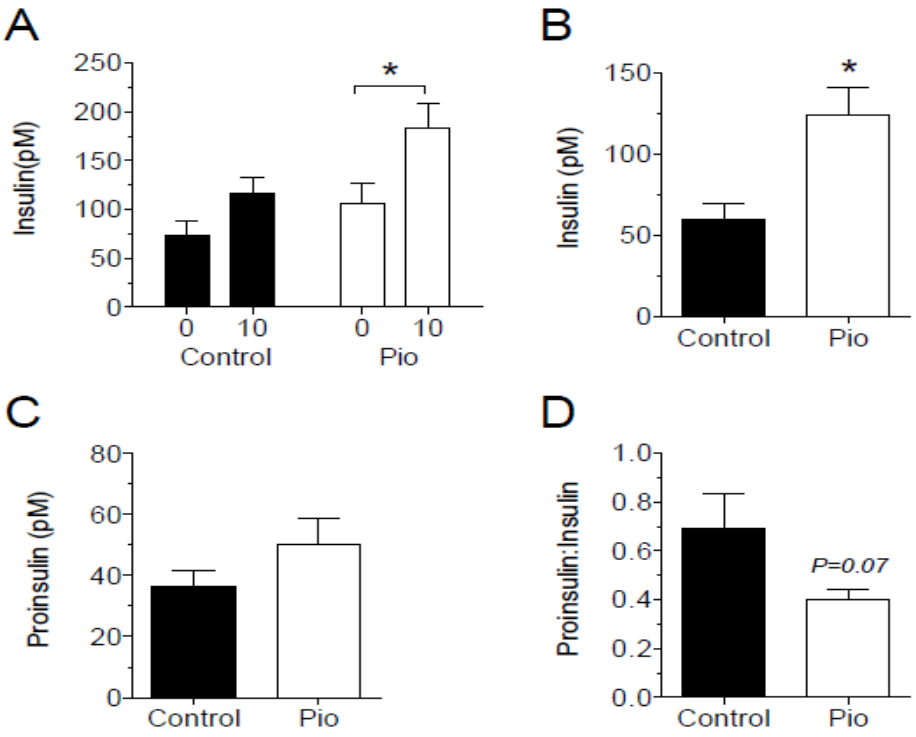

Figure 15. Effect of pioglitazone on insulin secretion in pre-diabetic NOD mice. 6 week-old pre-diabetic NOD mice (n=10 per group) were placed on either normal chow (*Control*) or chow containing 0.01 wt% pioglitazone (*Pio*). (A) serum insulin levels at the indicated time points following intraperitoneal glucose injection (2.0 g/kg body weight) after 4 weeks of feeding, (B) random-fed insulin levels after 4 weeks of feeding, (C) random-fed proinsulin levels after 4 weeks of feeding, (D) random-fed proinsulin:insulin ratio after 4 weeks of feeding. *Indicates value is significantly different compared to time 0 (panel *A*) or Control (panel *B*) by two-tailed *t* test.

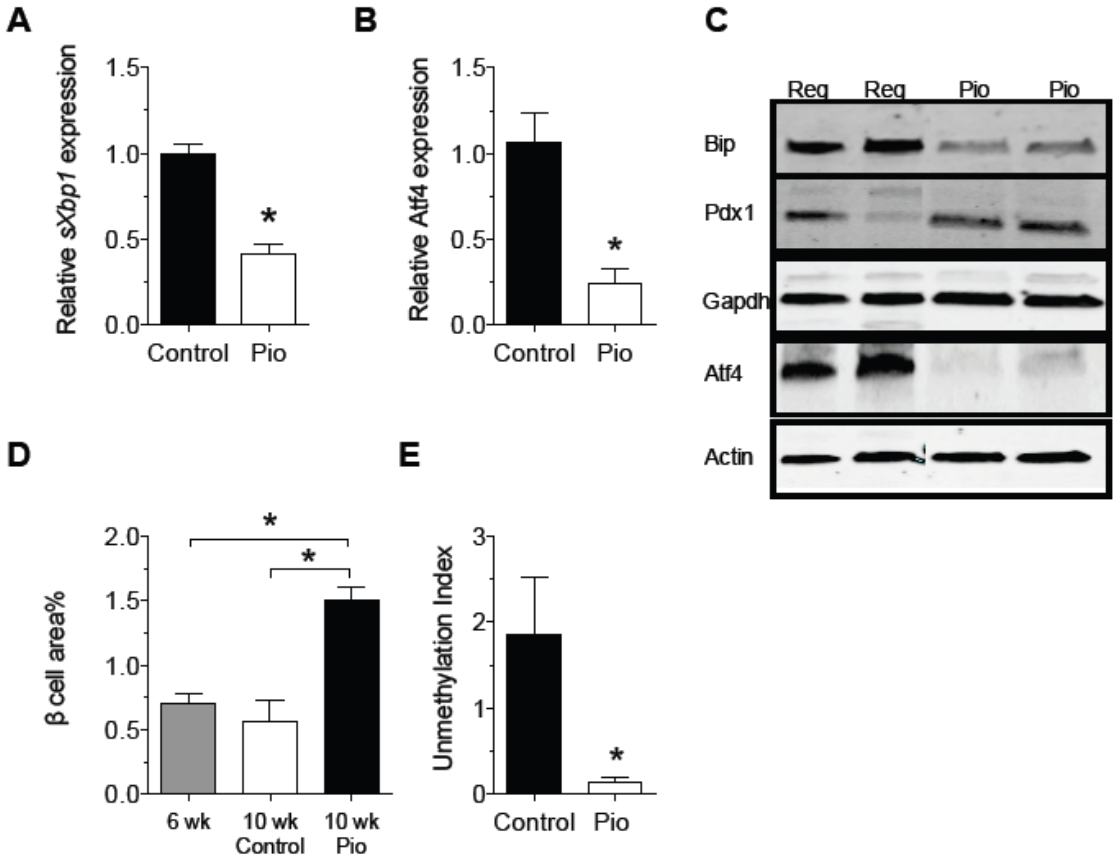

Figure 16. Effect of pioglitazone on β cell ER stress and area% in pre-diabetic NOD mice. 6 week-old pre-diabetic NOD mice were placed on either normal chow (*Control*) or chow containing 0.01 wt% pioglitazone (*Pio*). After 4 weeks of feeding, pancreas was harvested (n=5) or islets were isolated and subjected to RT-PCR (n=3 per group) or immunoblot analysis (n=2-3 per group). (A) spliced *Xbp1* (*sXbp1*) mRNA (relative to *Actb* mRNA), (B) *Atf4* mRNA (relative to *Actb* mRNA), (C) immunoblot analysis for Bip, Pdx1, GAPDH, Atf4 and Actin (D) β cell area as a percentage of total pancreas area for animals at the indicated ages, (E) Unmethylation index from serum of mice (n=4-5 per group). *Indicates that the values are significantly different for the comparisons shown (panel *D*) by one-way ANOVA or compared to Control (panels *A, B,* and *E*) by *t* test.

IRE1α and PERK pathways predominate, and are evident by increases in spliced *Xbp1* mRNA and ATF4 protein levels, respectively (282). As shown in Fig. 16A-B, there were significant reductions in spliced *Xbp1* (*sXbp1*) and *Atf4* mRNA levels in islets from pioglitazone-treated mice compared to controls. The reduction in *Atf4* mRNA was reflected by a reduction in the corresponding ATF4 protein level by immunoblot analysis (Fig. 16C). Fig. 16C also shows a reduction in the levels of the protein folding chaperone Bip. The reduction in islet ER stress (and possible death) with pioglitazone treatment led us to assess relative β cell area in treated and control mice. Fig. 16D shows that β cell area% is increased 2-fold upon pioglitazone treatment. The relatively greater β cell area in pioglitazone-treated mice resulted at least in part from reduced β cell death, as methylated preproinsulin DNA in serum (a biomarker of dying β cells, ref. (223)) was significantly reduced upon pioglitazone treatment at the end of the study (Fig. 16E).

Prior studies have shown that Pdx1 is required for the β cell to appropriately cope with ER stress, as haploinsufficiency of Pdx1 reduces the β cell ER stress threshold in mice and increases β cell apoptosis (45). Because TZDs are known to augment Pdx1 levels (172,175), we also measured Pdx1 protein levels in islets. However, examination of Pdx1 by immunofluorescence of pancreas sections and by immunoblotting of isolated islets revealed no effect of pioglitazone on Pdx1 levels (Fig. 16C), suggesting that the effect of pioglitazone in reducing β cell ER stress was independent of Pdx1 protein levels.

D. Pioglitazone treatment reduces insulitis in NOD mice but does not affect T cell proliferation in vitro

Exposure of β cell antigens, a consequence of β cell ER stress/death, results in eventual activation of autoreactive T cells and development of insulitis (283). We therefore assessed insulitis in histological sections of pancreas from treated and control mice. Fig. 17A and B shows that pioglitazone reduced the incidence and severity of insulitis compared to controls. We next isolated pancreatic lymph nodes from mice and assessed the effect of pioglitazone treatment on CD4+ T cell populations in these tissues. Notably, pioglitazone had no significant effect on Th1, Th17 (CD4+IL17A+), or Treg (CD4+CD25+Foxp3+) cell populations in the pancreatic lymph node (Fig 4C).

The reductions in insulitis without effects on specific CD4+ T cell populations led us to consider if pioglitazone might have caused a generalized reduction in T cell proliferation. Unfractionated splenocytes from NOD mice were loaded with the dye carboxyfluorescein succinimidyl ester (CFSE) and subjected to polyclonal stimulation using a combination of anti-CD3 antibody, anti-CD28 antibody, and IL-2 *in vitro* to mimic antigen-dependent and -independent signals as seen in T1D (284). After 4 days stimulation in the presence or absence of 1 or 10 μM pioglitazone, cells were gated on CD4+ cells and analyzed for CFSE dilution by flow cytometry. Fig 18A shows representative histograms demonstrating dilution of CFSE upon stimulation with anti-CD3/anti-CD28/IL-2, a finding indicative of T cell proliferation. No differences in CFSE dilution were observed with either 1 μM or 10 μM pioglitazone (Fig. 18A and B), indicating that pioglitazone at these concentrations does not alter the proliferative rate of T cells. These data suggest that the reduction in insulitis seen in treated mice is not likely an effect of pioglitazone to directly suppress T cell proliferation.

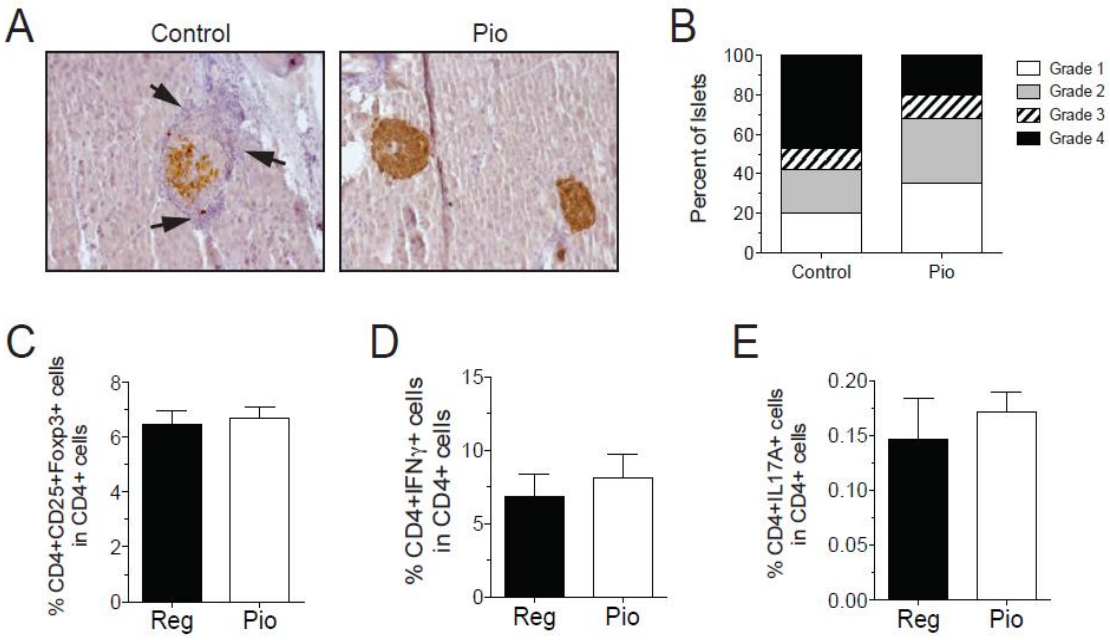

Figure 17. Effect of pioglitazone on the frequency and severity of insulitis in pre-diabetic NOD mice. 6 week-old pre-diabetic NOD mice were placed on either normal chow (*Control*) or chow containing 0.01 wt% pioglitazone (*Pio*). After 4 of weeks feeding, pancreas and pancreatic lymph nodes were harvested from animals (n=5). (*A*) representative images of islets (*brown staining*) from control- and pioglitazone-treated animals; *arrows* indicate infiltrating immune cells (insulitis), (*B*) results of insulitis scoring of control- and pioglitazone-treated animals, (*C*) flow cytometry analysis from pancreatic lymph nodes showing Treg cells (CD4+CD25+Foxp3+) as a percentage of total CD4+ cells, (*D*) flow cytometry analysis from pancreatic lymph nodes showing Th1 cells (CD4+IFN-γ+) as a percentage of total CD4+ cells, (*E*) flow cytometry analysis from pancreatic lymph nodes showing Th17 cells (CD4+IL17A+) as a percentage of total CD4+ cells.

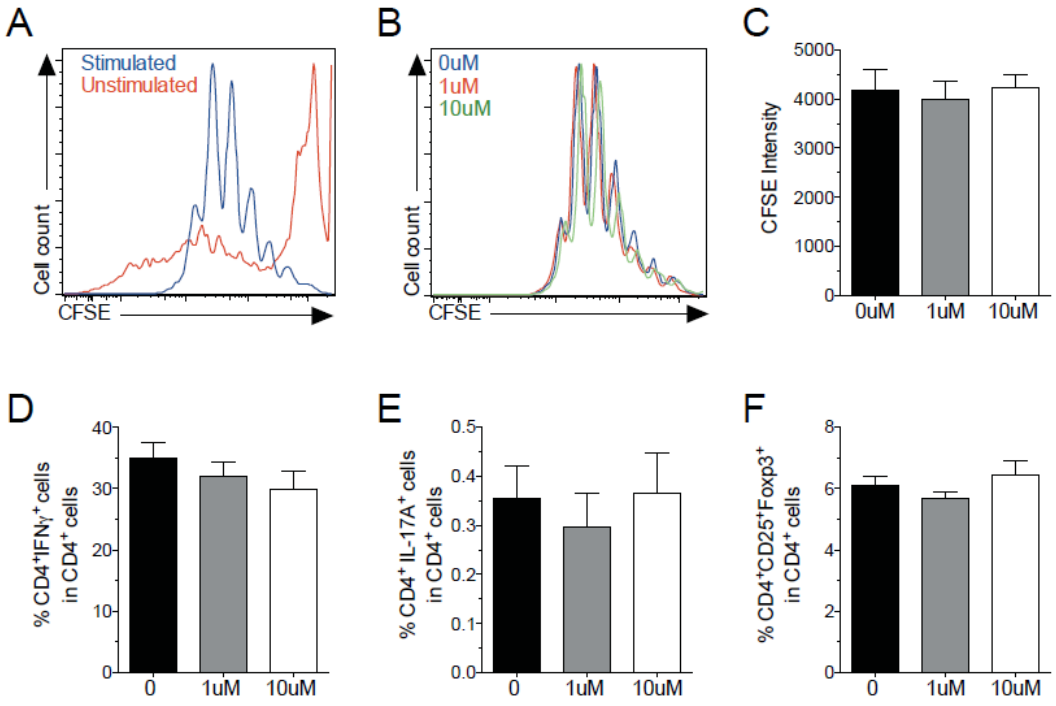

Figure 18. Proliferation of splenocytes in response to pioglitazone treatment *in vitro*. Isolated NOD mouse splenocytes were stimulated *in vitro* with anti-CD3/anti-CD28 and IL-2 for 4 days then gated on CD4+ cells by flow cytometry. (*A*), CFSE dye fluorescence intensity in unstimulated (*red* line) and stimulated (*blue* line) CD4+ cells in the absence of pioglitazone; (*B*), effects of 0 µM (*blue* line), 1 µM (*red* line), and 10 µM (*green* line) pioglitazone on CFSE fluorescence intensity in stimulated CD4+ cells, (*C*), quantitation of median CFSE fluorescence intensity from experiments in *panel B* from 3 independent experiments, (*D*) flow cytometry analysis from 4-day stimulated splenocytes showing Treg cells (CD4+CD25+Foxp3+) as a percentage of total CD4+ cells, (*E*) flow cytometry analysis from 4-day stimulated splenocytes showing Th1 cells (CD4+IFN-γ+) as a percentage of total CD4+ cells, (*F*), flow cytometry analysis from 4-day stimulated splenocytes showing Th17 cells (CD4+IL17A+) as a percentage of total CD4+ cells.

E. Pioglitazone treatment reduces TXNIP mRNA and protein

Previous studies of pioglitazone suggested that its protective effects in β cells in the setting of type 2 diabetes result from reductions in oxidative stress. We therefore examined levels of mRNA encoding anti- and pro-oxidant proteins in islets from control and treated mice. As shown in Fig. 19A, whereas the mRNAs encoding the antioxidant proteins glutathione peroxidase (*Gpx1*) and superoxide dismutase (*Sod1*) were unchanged upon pioglitazone treatment, the mRNA encoding pro-oxidant protein thioredoxin interacting protein (*Txnip*) was reduced more than 5-fold. Thioredoxin interacting protein (TXNIP) was recently shown to mediate ER stress-induced cellular death in islets (170,171). To interrogate in greater detail whether pioglitazone treatment suppresses *Txnip* mRNA and corresponding protein, we employed the INS-1 β cell line. As shown in Fig. 19B, INS-1 cells treated with the ER stress inducer thapsigargin displayed an increase in *Txnip* mRNA, whose levels were significantly reduced upon preincubation with 10 μM pioglitazone. Immunoblots of corresponding incubations in INS-1 cells revealed that TXNIP levels increased upon thapsigargin incubation and were also decreased by pioglitazone (Fig. 19C). Taken together, these data suggest that pioglitazone may promote β cell survival in pre-diabetic NOD mice through the reduction in TXNIP.

4.3. Discussion

Type 1 diabetes (T1D) develops as a disorder of the immune system in which β cell autoantigens, released as a result of β cell death, trigger the eventual selection and proliferation of autoreactive T cell clones (285). Speculation remains as to whether the process in which antigens are released is physiologic or pathologic. It has been speculated that the physiologic early turnover of β cells seen in neonatal mice (and

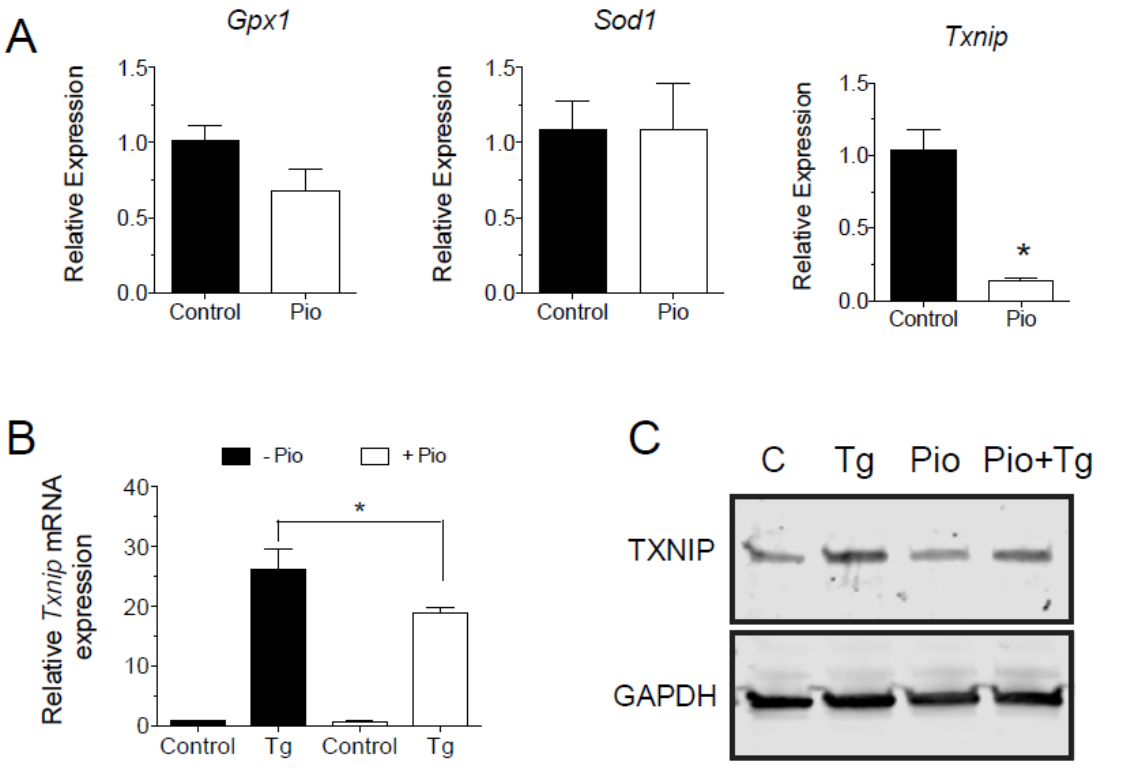

Figure 19. Effects of pioglitazone on TXNIP mRNA and protein expression. (A), abundance of *Gpx1*, *Sod1*, and *Txnip* mRNA (relative to *Actb* mRNA) in islets of 11 week-old NOD mice that were fed a control (*Control*) and pioglitazone (*Pio*)-containing diet, (B) expression of *Txnip* mRNA in INS-1 cells treated with or without thapsigargin (*Tg*) in the presence or absence of pioglitazone (*Pio*), (C) immunoblot analysis of TXNIP and GAPDH from INS-1 cells treated without or without thapsigargin (Tg) in the presence or absence of pioglitazone (Pio). *Indicates that the value is significantly different from Control (*panel A*) or for the comparison shown (*panel B*).

possibly humans, as well) might contribute sufficient antigen exposure to trigger autoimmunity (286), but this possibility was recently challenged in studies of NOD mice (287). A second, more intriguing, hypothesis suggests that stress responses intrinsic to the β result in the pathologic formation of epitopes (as misfolded or postranslationally modified proteins) that are immunogenic (199,269). In this regard, β cell ER stress was recently shown to occur in the early pre-diabetic phase in NOD mice and in humans with new-onset T1D (194,196). Prior studies suggested potential β cell protective effects of TZDs (172,173,273), and because administration of TZDs to NOD mice delayed onset of T1D (280,281), we asked whether TZD administration might directly impact β cell function and survival in NOD mice.

Although TZDs are traditionally viewed as insulin sensitizers, evidence in recent years strongly suggests that they have direct effects on the β cell. These effects include the stimulation of *Pdx1* gene activity and increases in Pdx1 protein levels in β cells (175), reduction in ER stress responses by stimulation of *Serca2* gene activity and protein levels (173), and reductions in β cell oxidative stress (177,273). Moreover, TZD administration was shown to delay the incidence of T1D in NOD mice, although it is unclear if this finding was a result of β cell effects of TZDs. Based on these findings, we asked whether administration of the TZD pioglitazone in the pre-diabetic phase might protect against the early β cell dysfunction and death that contribute to later T1D. Notably, our studies showed that pioglitazone administration resulted in a reduction in islet ER stress indices, including reduced *sXbp1* mRNA, reduced *Atf4* mRNA and its corresponding protein, decreased Bip protein levels, and a trend to reduced proinsulin: insulin ratio. These indices correlated to enhanced insulin secretion and improved glucose tolerance in NOD animals treated with pioglitazone. Because our findings were

not accompanied by changes in body weight, body fat distribution or insulin sensitivity, we believe they suggest a direct effect of pioglitazone on islet β cells.

The mechanism by which pioglitazone reduces ER stress may be multifactorial. Pre-diabetic NOD mice exhibit reductions in *Serca2* mRNA in islets and PPAR-γ stimulation has been shown to activate the *Serca2* gene in β cells (173). Although we did not detect changes to *Serca2* gene expression in our studies, it is possible that its activation was below our threshold of detection or that SERCA2 protein levels might have been enhanced independently of gene activity. Similarly, we did not observe changes in Pdx1 levels in β cells of pioglitazone-treated NOD mice. Pdx1 levels directly correlate to β cell health and to the ability of β cells to remediate ER stress; *Pdx1+/-* mice exhibit glucose intolerance and increased susceptibility to β cell ER stress (45). Based on our findings, we propose that the effects of pioglitazone in reducing β cell ER stress in NOD mice involve a molecular mechanism distinct from those previously suggested in the literature.

A notable outcome in our studies was the increased area of β cells in pioglitazone-treated animals compared to controls. Interestingly, β cell area was also higher in these animals when compared to 6 week-old NOD mice at the start of the study, suggesting that pioglitazone treatment either enhanced the rate of β cell replication or reduced β cell death- or some combination of the two. This result is reminiscent of studies in type 2 diabetic *db/db* mice, in which pioglitazone treatment resulted in increased β cell area that was coincident with improved glycemic control (172). In those studies, pioglitazone did not enhance β cell replication, but rather appeared to reduce cell death. In our case, pioglitazone-treated mice showed a striking reduction in the serum unmethylation index, a sensitive biomarker of β cell death (223). In light of the reduction in β cell death, it is particularly noteworthy that we observed

reductions in *Txnip* mRNA in islets of pioglitazone-treated mice with commensurate reductions in TXNIP levels in pioglitazone-treated INS-1 β cells. In the setting of ER stress, IRE1α stabilizes *Txnip* mRNA, whose encoded protein ultimately activates the Nlrp3 inflammasome and leads to β cell death (170,171). Because pioglitazone reduces IRE1α activity (as judged by the reduced spliced *Xbp1* mRNA), we suggest that β cell death is reduced in NOD mice as a result of reduced TXNIP.

Finally, we should comment on the significant reduction in insulitis we observed upon pioglitazone treatment. Despite the reduction in insulitis, we did not observe differences in relative proportions of CD4+ T cell subtypes in the draining pancreatic lymph node. These findings raise the possibility that pioglitazone may have had a primary effect on reducing proliferation of T cells. We tested this possibility by performing T cell activation assays *in vitro*, wherein no changes in CD4+ cell proliferation or differentiation were observed in response to pioglitazone. Studies have shown stimulatory and inhibitory effects of PPAR-γ on T_{reg} and Th17 cells, respectively (276,278,288), so we cannot rule out the possibility that differences in CD4+ T cell sub-populations occurred in the immediate vicinity of the islets themselves. In the absence of a direct effect of pioglitazone on T cell proliferation, it nevertheless remains possible that the reduction in β cell death with pioglitazone may have diminished the stimulus for autoimmunity.

Certain key limitations to our study must be recognized. First, notwithstanding some evidence to the contrary, we cannot rule out non-β cell effects of pioglitazone in our study, and it remains possible that β cell protective effects may still emanate from indirect effects on adipose tissue, muscle, or immune cells. Second, the implications of our findings to human T1D remain speculative. Whereas administration of TZDs delay onset of T1D in pre-diabetic NOD mice (280,281), their use in humans with established

T1D may have only modest effects on improving glycemic control (277–279). These results leave open the possibility that earlier intervention with pioglitazone (in the pre-diabetic phase) may have greater impact on β cell function in humans, but such studies must await the development of next generation PPAR-γ activators with improved benefit-to-risk ratio. Notwithstanding these limitations, our data support the conclusion that TZDs may have protective effects to reduce β cell stress and death in T1D, and also support the notion that an aggressive approach to β cell function represents an important adjunctive means to control progression or severity of T1D.

CHAPTER 5: Conclusions and Future Work

The β cell is central to the regulation of glucose homeostasis in the body. Several studies have pointed to the fact that the loss of β cell function triggers the development of diabetes (211,289). Therefore, studying the β cell is of primary importance in tackling the diabetes epidemic. In this respect, I have studied the transcriptional and translational mechanisms underlying β cell function.

Pdx1 is undoubtedly, the most widely studied transcription factor in the field of β cell biology. Pdx1 exercises control over β cell phenotype and function by regulating the transcription of a number of key β cell genes. A few studies have demonstrated that Pdx1 interacts with cofactors that regulate chromatin status and influence transcription (reviewed in (84)); however, regulation of Pdx1 protein itself during the process of transcription deserves more attention. In Chapter 3, I have shown that both recombinant and cellular Pdx1 protein is methylated by Set7/9, a lysine methyltransferase, at two lysine residues, Lys 123 and Lys 131 (Fig 6, 7 and 10) . As methylation of endogenous Pdx1 can be captured only when Ins1 832/13 cells are treated with a demethylase inhibitor (Fig 10), it is likely that Pdx1 is also regulated by demethylases. Methylation of Pdx1 at Lys 131 is associated with an increase in transcriptional activity, which could potentially be due to effects of methylation on RNA pol II. For the first time, we also showed that a β cell-specific Set7/9 knockout mice display significant glucose intolerance and impaired insulin secretion in comparison to their wildtype littermates. Additionally, the $Set^{\Delta\beta}$ mice appear to have reductions in expression of Pdx1 and Pdx1 target genes, demonstrating that Set7/9 regulates Pdx1 function (Fig 11). Set7/9 may of course, contribute to the maintenance of normal β cell function by affecting other proteins within the β cell. To determine if the decrease in Pdx1 target gene expression is

specifically due to a decrease in Pdx1 methylation, we would have to create a Lys123 and Lys 131 mutant mouse. Another approach to delineate further the role of Pdx1 methylation in β cell transcription, is to use β cell lines wherein endogenous Pdx1 in knocked down using an adenoviral-siPdx1 vector or a CRISPR/Cas9 kit and wildtype or mutant Pdx1 are transiently expressed, followed by assessment of Pdx1 target gene expression and glucose stimulated insulin secretion.

Interestingly, while incubation of recombinant proteins with radioactive SAM reveals Lys 123 to be the site of methylation (Fig. 6), immunoblot analysis of cellular Pdx1 using methylation specific antibodies and mass spectrometry analysis show that both Lys 123 and Lys 131 are methylated (Fig. 10 and 7). From the experiments performed in this study, Lys 131 seems to be the major target of methylation in cells that are transfected/ transduced with Pdx1 (Fig. 8). Although Lys 131 appears to be the more important target of methylation, another possibility is that we have simply not used the right system to investigate the function of methylation at Lys 123. Lys 123 belongs to the pentapeptide FPWMK, which is the site of interaction between Pdx1 and Pbx1 (88). As described in the introduction, the interaction between these two proteins, when disrupted, dramatically inhibited proliferation of all pancreatic cell types (89). Pdx1: Pbx interaction regulate the transcription of somatostatin (88), elastase (87), Tcf7l2 (associated with β cell proliferation) (83), Isl1 (90) and quite possibly, many other target genes that still need to be investigated. Also, Pbx1 appears to be the most common interacting partner of Pdx1 (83). We think that, perhaps, Set7/9 mediated Lys123 methylation can enhance Pdx1:Pbx mediated transcription, possibly through recruitment of other factors and enhanced stabilization of Pdx1:Pbx complex. In another study, ERα was shown to interact with Pbx1, which associates with increased H3K4 methylation and augmented transcription of ERα target genes (290), further supporting the possibility of a

connection between Pdx1:Pbx1 interaction and Set7/9. Interestingly, the pancreas-specific Set7/9 knockout mice (Set7/9$^{\Delta panc}$) displayed worsened glucose tolerance coupled with reductions in Pdx1 and MafA protein and β cell proliferation. Due to the loss of Set7/9 and possibly reduced Pdx1 function during pancreatic development of Set7/9$^{\Delta panc}$ mice, processes such as β cell proliferation, which mostly occur during development may get impeded. It would be interesting to determine if apart from a reduction in Pdx1 protein, the loss of Set7/9 affects Pdx1:Pbx interaction, which ultimately reduces β cell proliferation. Although there were no differences in the gross phenotype of the Set7/9$^{\Delta panc}$ pancreas (size and weight), one wonders if β cells of the adult Set7/9$^{\Delta panc}$ mice can compensate adequately in response to stresses such as streptozotocin injections or a partial pancreatectomy. As Pdx1 haploinsufficient mice cannot proliferate or function sufficiently in response to stresses such as a high fat diet (45), it is likely that Set7/9$^{\Delta panc}$ mice or Set$^{\Delta \beta}$ mice that have reduced Pdx1 levels may be unable to mount an adequate compensatory response to stress. Moreover, a ChIP seq experiment using Pdx1, Pbx1 and Set7/9 antibodies would be useful to identify all the common target genes and would help us to better understand the role of Set7/9 in the β cell.

Normal β cell function is dictated by optimum β cell transcription and translation. Accumulation of unfolded protein in the ER results in ER stress and activation of the UPR. Unresolved ER stress results in the apoptosis of the cell. Recent reports suggest that early inflammatory conditions cause ER stress, which in turn, causes β cell death and a subsequent increase in recruitment of macrophages and other immune cells, thereby, assisting in its own death (195,196,198,199). Hence, although the immune system was thought to be the main cause for type 1 diabetes, these studies now suggest that the β cell may assist in its own death. In Chapter 4, we tried to address this issue by

asking if an improvement in β cell function and a reduction in β cell death would decrease insulitis. We treated prediabetic 6 week old NOD mice with pioglitazone, a PPARγ activator, for 4-5 weeks and then assessed islet function. We observed that pioglitazone treated NOD mice showed a significantly improved glucose tolerance, which was due to a dramatic increase in insulin secretion. Pioglitazone NOD mice displayed an increase in β cell area, accompanied by a significant decrease in β cell death indicating that pioglitazone treatment enhanced β cell survival. Treated mice also exhibited a decrease in the frequency and extent of insulitis, with no alterations in the size or proliferative capacity of Th1, Th17 and T_{reg} cell populations, indicating that a decrease in β cell death could potentially prevent islet infiltration of immune cells. We then sought the underlying reason for lesser β cell death and found that there was a reduction in ER stress markers *sXbp1* mRNA, Bip and ATF4 protein in islets isolated from pioglitazone treated mice. In addition, pioglitazone diminished *Txnip* mRNA expression, suggesting reduced ER stress mediated TXNIP expression and a potential reduction in subsequent IL-1β maturation contributed to decreased β cell death. This study adds to the growing body of evidence, further supporting the hypothesis that β cell ER stress contributes to inflammation and exacerbates β cell death. While the effects of pioglitazone treatment on the prediabetic β cell seem promising, we accept that there are certain limitations to the interpretation of this study. As pioglitazone is popularly used as an insulin sensitizing agent, we cannot rule out the effect of pioglitazone on other organs. Further experiments should be conducted to determine if pioglitazone affects the proliferation, maturation and antigen presentation ability of macrophage and dendritic cells.

It would be interesting to know if there is an age-dependent decrease in β cell PPARγ expression levels or alterations in phosphorylated PPARγ levels in NOD mice, due to which pioglitazone administration provides a protective response. The exact

mechanism of pioglitazone mediated inhibition of ER stress in the β cell requires further investigation. Rat β cells that have mild ER stress and are exposed to cytokines show higher NF-κB activation, in part due to transcriptional activation by spliced Xbp1 protein (156). PPARγ has been reported to modulate the activity of NF-κB in several reports, including inhibition of NF-κB transcription, increase in IκB transcription and enhanced proteasomal degradation of NF-κB (291,292). In addition, PPARγ expression has been reported to counteract the expression of Chop and subsequently inhibit ER stress mediated activation of NF-κB and synthesis of IL-8, ultimately reducing inflammation in human intestinal epithelial cells (293). NF-κB expression is correlated with the an increase in β cell death (294) and development of immune mediated diabetes (295), therefore, PPARγ could mediate a reduction in ER stress associated cell death through decreased transcription of NF-κB or reduced protein stability. Furthermore, PPARγ has been shown to bind to the *Txnip* promoter and repress Txnip expression in renal cells (296). In β cells, PPARγ agonism could potentially repress *Txnip* expression in a similar manner or may facilitate degradation of miRNA regulating *Txnip* mRNA during ER stress (170). PPARγ can also indirectly contribute to decreased Txnip protein levels by ameliorating ER stress.

As β cell dysfunction is central to the pathogenesis of diabetes, it is important to understand mechanisms regulating β cell function and survival. Elucidation of the transcriptional and translational mechanisms underlying β cell function could potentially provide a number of molecular targets for therapy. Hence, therapies aimed at enhancing the intrinsic function of transcription factors such as Pdx1 and PPARγ or influencing the function of Pdx1/PPARγ regulatory factors (e.g. demethylases) could improve β cell function. Therapies aimed at translational mechanisms of the β cell that could result in

resolution of ER stress have the potential to improve β cell function and survival, ameliorate glucose control and prevent diabetes.

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CURRICULUM VITAE

AARTHI VIJAYKUMAR MAGANTI

A. Education

2009-2015 Doctor of Philosophy, *Cellular & Integrative Physiology,* Indiana University, earned at IUPUI, Indianapolis

2004-2008 Bachelor of Engineering, *Biotechnology,* B.M.S College of Engineering

B. Research Experience

2009-2015 Predoctoral fellow, Department of Cellular & Integrative Physiology, Indiana University, Indianapolis, IN

Advisor: Dr Raghu Mirmira, M D, Ph D

- *Investigating the role of Pdx1 and Set7/9 interaction in normal beta cell function.*
- *Investigating the therapeutic effect of pioglitazone on prediabetic NOD mice.*

2008 – 2009 Volunteer Laboratory Assistant, Cellular Organization and Signaling Program, National Centre for Biological Sciences, Bangalore, India

Advisor: Dr. Sudhir Krishna, MBBS, PhD

- *Investigated the role of NF-kB and TGFb in Epithelial Mesenchymal transition using cervical cancer cell line.*

Jan – May 2008 Volunteer Laboratory Assistant, Department of Biotechnology, Maharani Ammani College and M S Ramaiah Institute of Technology, Bangalore, India

Advisor: Dr M S Baliga, Ph D

- *Investigated the effect of Aegle marmelos on cisplatin-induced toxicity in Swiss albino BRB1 mice.*

C. Honors and Awards

2010 Moenkhaus Physiology Graduate Fellowship Award for Academic Excellence

2011 Indiana Physiological Society Abstract award

2013 Graduate student organization travel award

2013 Midwest Islet Club Conference, Poster Award (3rd place).

D. Abstracts

Maganti A., Tersey SA., Maier B., Hunter C., Stein RW., Mirmira RG. The Lysine methyltransferase Set7/9 is a Pdx1- interacting co-factor that is required for normal islet function. Indiana Physiological Society, 2014. (Accepted for poster)

Maganti A., Tersey SA., Maier B., Hunter C., Stein RW., Mirmira RG. The Lysine methyltransferase Set7/9 is a Pdx1- interacting co-factor that is required for normal islet function. American Diabetes Association Conference, Chicago, 2013. (Talk)

Maganti A., Colvin SC., Tersey SA., Maier B., Mirmira RG. Pioglitazone improves glycemic control in pre-diabetic NOD mice by decreasing insulitis and ER stress. Midwest Islet Club Conference, Ann Arbor, 2013. (Poster)

Maganti A., Tersey SA., Maier B., Hunter C., Stein RW., Mirmira RG. The Lysine methyltransferase Set7/9 is a Pdx1- interacting co-factor that is required for normal islet function. Midwest Islet Club Conference, Ann Arbor, 2013.

Maganti A., Tersey, SA., Liu Y., Maier B., Stein RW., Mirmira RG. The Lysine methyltransferase Set7/9 is a Pdx1- interacting co-factor that is required for normal islet function. Campus Recruitment, Indiana University School of Medicine, 2013.

Maganti A., Tersey, SA., Liu Y., Maier B., Stein RW., Mirmira RG. The Lysine methyltransferase Set7/9 is a Pdx1- interacting co-factor that is required for normal islet function. Midwest Islet Club Conference, Pittsburgh, 2012.

Maganti A. Tersey SA, Gupta D, Maier B, Mirmira RG. Set7/9, a Pdx1 interacting cofactor, is required for normal islet function. Midwest Islet Club Conference, Madison, 2011.

Maganti A, Mirmira RG. The role of Set7/9 in Pdx1 mediated transcription of *insulin* gene. Indiana Physiological Society, Indianapolis, 2011.

E. Publications

Maganti AV, Evans-Molina C, Mirmira RG. From immunobiology to β-cell biology: The changing perspective on type 1 diabetes. Islets. 2014 Apr 29; 6.

Tersey SA, Maier B, Nishiki Y, Maganti A, Nadler J, Mirmira RG. 12-Lipoxygenase Promotes Obesity-Induced Oxidative Stress in Pancreatic Islets. Mol Cell Biol. 2014 Oct 1; 34(19)

Maganti AV, Maier B, Tersey SA, Sampley ML, Mosley AL, Özcan S, Pachaiyappan B, Woster PM, Hunter CS, Stein RW, Mirmira RG. Transactivation by Islet β Cell- Enriched

Pdx1 is augmented by Lysine-Methylation catalyzed by Set7/9. Journal of Biological Chemistry, 2015 Feb 24. pii: M114.616219

Maganti AV, Tersey SA, Colvin SC, Mirmira RG, PPAR-γ Activation Reduces Islet β Cell Stress and Death in Pre-Type 1 Diabetic Female NOD Mice (*In preparation*).