INVESTIGATIONS INTO ISLET PDX1 ACTIVITY: CONTRIBUTION TO MATURE β-CELL IDENTITY AND THE IDENTIFICATION AND CHARACTERIZATION OF PDX1 COREGULATORS

By

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LIST OF ABBREVIATIONS

ChIP – chromatin immunoprecipitation
CoIP – coimmunoprecipitation
GSIS – glucose stimulated insulin secretion
IP - immunoprecipitation
MPC – multipotent pancreatic progenitor cell
MS – mass spectrometry
PKO – Pdx1 knockout
PLA – proximity ligation assay
ReCLIP – reversible crosslink immunoprecipitation
RT-qPCR – reverse transcriptace quantitative polymerase chain reaction
T1DM – type 1 diabetes mellitus
T2DM – type 2 diabetes mellitus

CHAPTER I

INTRODUCTION

The focus of this dissertation is on the pancreatic and duodenal homeobox 1 (*Pdx1*) gene. *Pdx1* encodes a homeodomain transcription factor of fundamental importance to nearly every stage of pancreas organogenesis and the maintenance of mature β-cell identity and function. The following section provides a general overview of pancreas development and islet function in normal and diabetic conditions, and how Pdx1 and other factors regulate the transcriptional events to modulate these essential processes.

The Pancreas

The pancreas is a compound organ composed of distinct compartments that serve two main functions in regulating the body's response to feeding (Figure 1A). The exocrine compartment produces and secretes digestive enzymes into the duodenum to aid in food digestion. The endocrine compartment supports the maintenance of glucose homeostasis through the production and secretion of hormones such as insulin and glucagon.

The exocrine pancreas makes up ~98% of the overall pancreatic mass and is composed of highly specialized acinar cells that surround a tubular, tree-like branching network of ducts (Figure 1A). Acinar cells secrete proteases, lipases, nucleases, and amylases into the ducts, which serve as a conduit to the duodenum where they aid in food digestion.

The endocrine compartment comprises the remaining ~2% of the pancreatic mass and is made up of small (~100-1000 cells), typically spherical conglomerates of

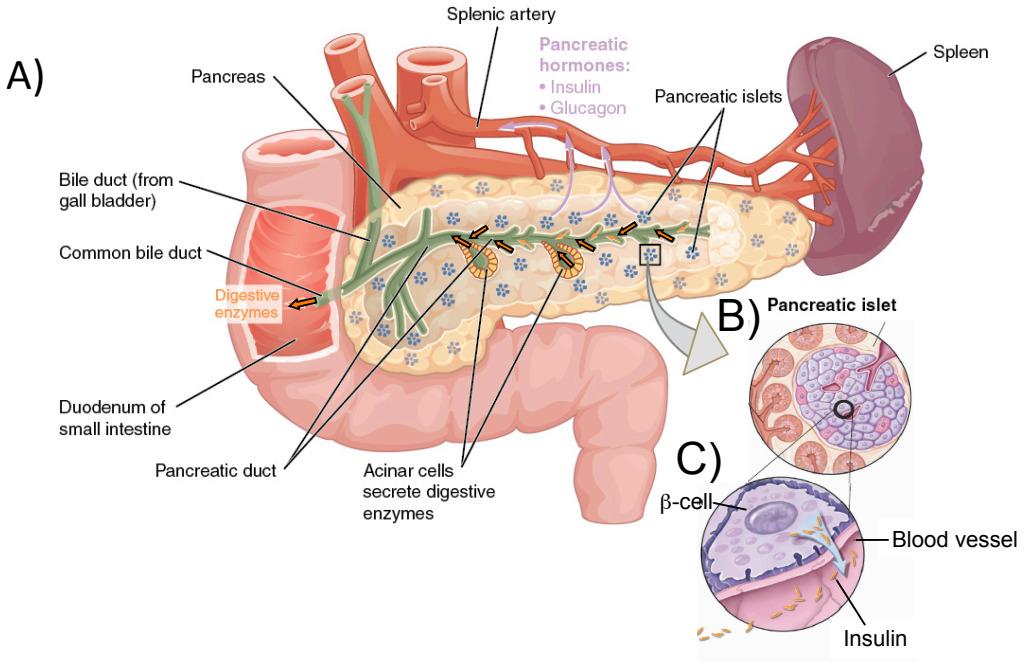

Figure 1. The pancreas. A) Schematic diagram illustrating the gross morphology and location of the adult human pancreas. Orange arrows indicate the secretion of digestive enzymes from the acini, which travel through the ducts and empty into the duodenum. Purple arrows represent the release of hormones (namely insulin and glucagon) from the islets into the bloodstream. B) Magnified view of an islet of Langerhans, which is the functional unit of the endocrine pancreas. C) Within the islets are β-cells, which secrete insulin into the bloodstream in response to changing nutrient conditions.
(Adapted from http://pharmatips.doyouknow.in/Articles/Human-Anatomy/Human-Anatomy-Physiology-Of-Pancreas.aspx and http://www.eurostemcell.org/image/insulin-production-human-pancreas)

endocrine cells called islets of Langerhans (Figure 1B). These micro-organs are found scattered throughout the exocrine tissue and are composed of five subtypes of peptide-hormone producing cells: α-cells producing glucagon, β-cells producing insulin, δ-cells producing somatostatin, and PP-cells and ε-cells producing pancreatic polypeptide and grhelin, respectively. α and β-cells make up roughly 20-25% and 70-80% of the islet cell mass in rodents, respectively, and these are the principle cell types responsible for maintaining glucose homeostasis. After a meal, the rise in blood glucose stimulates β-cells to release insulin into the blood stream (Figure 1C). Circulating insulin acts on peripheral tissues such as skeletal muscle and adipose tissue to cause glucose uptake for energy production, while also promoting glycogen synthesis in muscle and liver. These actions effectively lower blood glucose levels. Conversely, low blood glucose caused by fasting stimulates the secretion of glucagon from α-cells. Circulating glucagon elevates the concentration of glucose in the blood by promoting gluconeogenesis and glycogenolysis primarily in the liver.

Pancreas Development

As mouse pancreas organogenesis is initiated, a region of the foregut endoderm is specified to the pancreatic fate, inducing expression of the *Pdx1* and *Ptf1a* transcription factors (Burlison et al., 2008; Pan and Wright, 2011). Although specification occurs at embryonic day eight (E8) in mice, visual evidence of pancreas formation does not occur until the outgrowth of the dorsal and ventral pancreatic buds at E9 (Figure 2). These distinct pancreatic rudiments are composed of epithelial multipotent pancreatic progenitors (MPCs) that will differentiate into the three lineages of the pancreas (i.e. acinar, endocrine and duct) and an overlying mesenchyme. The epithelial cells of the buds undergo active plexus remodeling, which generates a tree-like, almost fractal

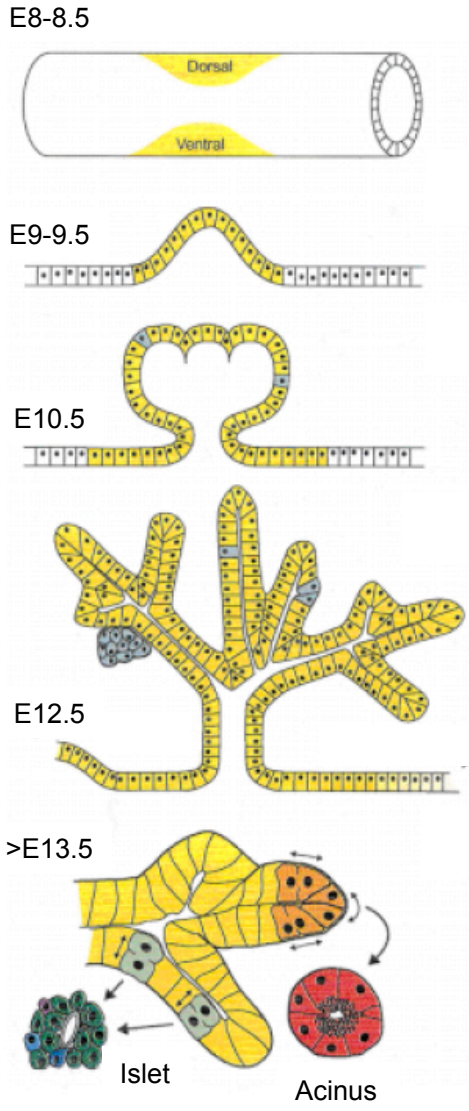

Figure 2. Early mouse embryonic pancreas morphogenesis. Schematic representation of pancreas growth and differentiation during embryonic days (E) 8-13.5 of mouse development. The expression of Pdx1 (depicted in yellow) begins in the primitive gut tube at around E8. One day later, growth and evagination of the epithelium forms the dorsal and ventral pancreatic buds. Active plexus remodeling of the buds generates a complex tubule system of intraepithelial lumens. At E13.5, specification of the endocrine and exocrine lineages begins. (Adapted with permission from Kim and Macdonald, 2002)

tubular system of intraepithelial lumens contiguous with the gut tube. The buds continue to expand independently until around E11.5, when a rotation of the gut tube brings them together to fuse into one unified organ (for comprehensive reviews, see (Pan and Wright, 2011; Villasenor et al., 2010)).

The first insulin-and glucagon-producing cells are observed around E9.5 and E10.5, respectively, in a wave of development termed the “primary transition” (prior to E13.5). However, the physiological role of these cells remains unclear as these cells are not thought to inhabit the islets of the mature pancreas (Herrera, 2000). The emergence of endocrine cells destined to populate the islets is observed during a time of development termed the “secondary transition”. This stage begins around E13.5 and is characterized by massive differentiation and expansion of the endocrine and exocrine lineages. Pancreatic endocrine cells are specified within the pancreatic epithelium by the transient expression of the *Neurogenin3* (*Ngn3*) transcription factor (Gu et al., 2002a). Newly forming endocrine cells aggregate into clusters to form the islets. As they mature, islets expand and become highly vascularized and innervated via vascular endothelial growth factor-A guided endocrine-endothelial signaling (Brissova et al., 2006; Lammert et al., 2003; Reinert et al., 2013, 2014). This intimate association between islets and the vasculature allows for continuous assessment and response to the nutrient conditions of the bloodstream.

Although considerable efforts have defined key developmental events of mouse pancreas organogenesis, due to the extremely limited access to embryonic pancreas specimens, our understanding of developmental events occurring in human pancreas organogenesis remain deficient. Many molecular markers and morphogenic events have been found to be highly similar in mouse and human, yet others are somewhat divergent. For a detailed review of the current knowledge of human pancreas development and its relation to mouse, see (Pan and Brissova, 2014).

β-cells

In the adult pancreas, islet β-cells are a highly specialized cell type whose central function is to produce, store and secrete insulin into the bloodstream in response to changes in nutrient conditions. Secretion of insulin must be precisely regulated so the optimal amount is released at the correct rate to ensure glucose homeostasis is maintained. For example, insufficient secretion (a fundamental cause of diabetes) prohibits glucose clearance, which can have damaging consequences (discussed below). Conversely, unbridled insulin secretion can lead to hypoglycemia, a potentially life-threatening condition. The process by which β-cells sense extracellular glucose and secrete insulin is termed glucose-stimulated insulin secretion (GSIS) (Figure 3). GSIS is biphasic in nature and each phase is regulated by distinct mechanisms. The first phase is characterized by a rapid and robust release of insulin from membrane-docked insulin granules in response to elevated blood glucose. The second phase is a slow and sustained release of insulin from non-membrane docked and newly synthesized insulin granules.

In short, during the first phase, glucose enters β-cells through a specialized membrane glucose transporter, Glut2, by facilitated diffusion (Figure 3) (Hou et al., 2009). Once inside, the glucokinase enzyme phosphorylates glucose to generate glucose-6-phosphate (G6P), which prohibits exit from the cell and marks glucose for further processing (Matschinsky, 1996). The kinetic properties of glucokinase enzymatic activity allows for rapid and precise measurement of extracellular glucose concentrations to by β-cells (Matschinsky et al., 1998). Because it governs the first step in glycolysis and ATP production, glucokinase serves as the rate-limiting step in glucose-stimulated insulin secretion (Matschinsky, 1996; Matschinsky et al., 1998). Following

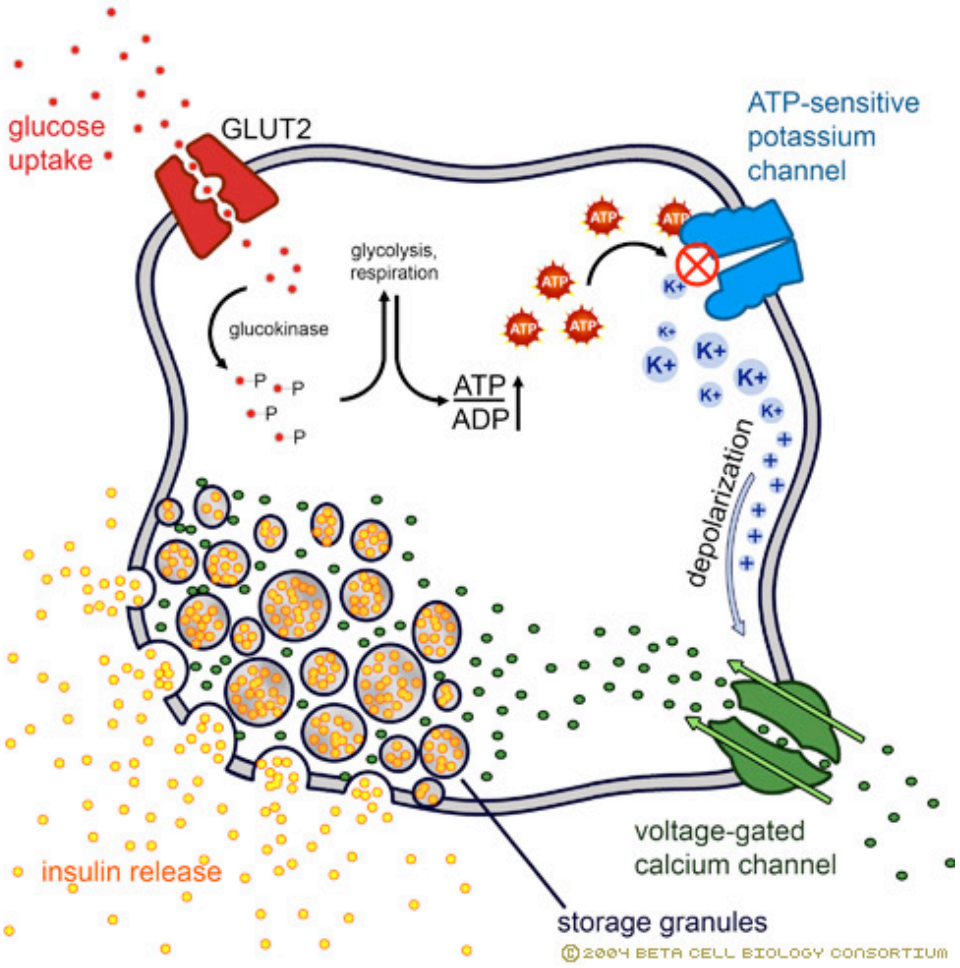

Figure 3. Mechanism of β-cell glucose stimulated insulin secretion. Glucose enters the cell through Glut2 glucose membrane transporters. Once inside the cell the glucokinase enzyme phosphorylates glucose, generating G6P, which is then acted upon by the enzymes of glycolysis and the Krebs cycle to ultimately generate ATP. Transmembrane ATP-sensitive potassium channels close in response to this change in the ATP:ADP ratio, prohibiting outflow of potassium and depolarizing the cell. The change in membrane potential causes voltage-gated calcium channels to open allowing Ca^{2+} influx. Calcium serves as a second messenger, inducing fusion of membrane-docked granules with the cell membrane and exocytosis of insulin and mobilization of cytoplasmic storage granules to the membrane. (modified from http://www.betacell.org/content/article panelview/article _id/1/panel_id/2)

phosphorylation, the enzymes of glycolysis and the citric acid cycle then metabolize G6P, generating ATP. The resulting shift in the ATP:ADP ratio serves as a second messenger, rapidly triggering closure of SUR1/Kir6.2 ATP-dependent potassium channels, which leads to a build-up of K^+ ions within the cell, and thus, depolarization of the cell membrane. Voltage-gated calcium channels sense the change in membrane potential and respond by opening to allow an influx of Ca^{2+} ions. Intracellular calcium signaling, in turn, triggers fusion of "membrane-docked" insulin granules with the cell membrane and insulin exocytosis.

The second phase is thought to be the main effector for lowering blood glucose. Cytoplasmic "reserve" insulin granules are mobilized to the membrane for release during the second phase of insulin secretion (Wang and Thurmond, 2009). Pdx1 and other islet-enriched transcription factors (discussed in detail below) regulate the expression of many of the effectors (e.g. *Glut2, Kir6.2, Glucokinase*) to maintain proper first and second phase GSIS.

Diabetes

The Centers for Disease Control estimate that the prevalence of diabetes in America has reached 29.1 million people, indicating that 9.3% of the population has diabetes (Centers for Disease Control and Prevention). Complications from diabetes include kidney failure, non-traumatic lower-limb amputations, blindness, stroke, and heart disease, making diabetes among the leading causes of morbidity and mortality in the United States. Loss of β-cell mass and/or function are key features that lead to the loss of metabolic control observed in both type 1, type 2, and gestational diabetes.

In type 1 diabetes mellitus (T1DM), the β-cells of the pancreas are identified as foreign by T-cells of the immune system, and thus, are destroyed by an autoimmune attack (Szablewski, 2014). Type 1 diabetes makes up roughly 10% of diagnosed cases

of diabetes and patients are usually identified during childhood and adolescence. Pancreatic β-cells are the body's only source of insulin and type 1 diabetics require life-long administration of exogenous insulin. Thus, prior to the discovery of insulin by Banting and Best in 1921, T1DM was a fatal disorder.

Type 2 diabetes mellitus (T2DM) is often multifactoral in nature with multiple genes and environmental factors conferring susceptibility (Halban et al., 2014). Type 2 diabetes accounts for roughly 90% of the documented cases of the disease, and this form of diabetes typically manifests in adults. Peripheral insulin resistance and consequent β-cell dysfunction are at the core of the disorder. The inability of β-cells to overcome the demand for insulin caused by resistance in peripheral target tissues ultimately results in loss of metabolic control. Although symptoms often present slowly over many years, environmental factors such as age, obesity, lipotoxicity and inflammation can exacerbate disease progression (Donath and Shoelson, 2011).

Genetic analyses have revealed that a minority of diabetes cases (1-2%) are caused by inheritance of single autosomal dominant gene defect associated with one of nine identified genomic loci. This form if diabetes is typically diagnosed in patients less than 25 years of age, and thus, has been termed maturity onset of diabetes of the young (MODY). Seven of the nine susceptibility genes encode transcription factors responsible for islet development and/or function including: Hepatic Nuclear Factor 4α (HNF4α) (MODY1), HNF1α (MODY3), PDX1 (MODY4), HNF1β (MODY5), NEUROD1 (MODY6) Kruppel Like Factor 11 (KLF11) (MODY7), PAX4 (MODY9) (Horikawa et al., 1997; Kristinsson et al., 2001; Neve et al., 2005; Plengvidhya et al., 2007; Stoffers et al., 1997; Yamagata et al., 1996). Two additional MODY genes encode glucokinase (MODY2), which catalyzes the rate-limiting step in β-cell glucose sensing, and intriguingly, carboxyl-ester lipase 2 (MODY8), which is produced and secreted by acinar cells to aid

in the duodenal hydrolysis of cholesterol esters (Froguel et al., 1992; Raeder et al., 2006). Mutations affecting function of all MODY genes results in islet dysfunction and an inability to maintain blood glucose levels within the normal physiological range.

Gestational diabetes mellitus (GDM) is classified as an impairment of glucose homeostasis resulting from insufficient insulin production or signaling, with the first onset of symptoms during pregnancy (DeSisto et al., 2014). The Centers for Disease Control recently reported this condition affects approximately 9% of all pregnancies in the United States (DeSisto et al., 2014). Most women regain glycemic control after giving birth, however, GDM patients have a seven-fold increased risk of developing T2DM later in life (Bellamy et al., 2009). Children whose mothers develop T2DM are also at a higher risk of hyperbilirubinemia, hypocalcaemia, polycythemia and respiratory distress syndrome as neonates, and have greater risk of obesity and impaired glucose tolerance in adulthood (Jr, 2012). Although risk factors such as age, family history of diabetes and obesity are known, GDM appears to be pleiotropic in nature, with numerous genetic and environmental risk factors potentially contributing to disease development (DeSisto et al., 2014). As the use of pharmacological agents to manage glycemia could affect fetal health, treatment of GDM is heavily reliant on diet (Gilmartin et al., 2008). Insulin is administered to patients who cannot maintain normoglycemia with diet alone.

T2DM β-cell Dysfunction

In T2DM, loss of metabolic control occurs gradually over many years to decades and results from progressive insulin resistance and corresponding β-cell dysfunction (Figure 4). There is a strong correlation between obesity and T2DM. Obese individuals exhibit prolonged, low-grade inflammation and activation of the immune system due to increased pro-inflammatory cytokine and chemokine production (Esser et al., 2014). Resistance to the actions of insulin in peripheral target tissues is thought to stem, in part,

from interference in insulin signaling by these inflammatory factors. Initially, an augmentation of β-cell mass and enhanced function are able to compensate for the increased demand for insulin caused by peripheral insulin resistance. However, as the disease progresses the continued stress imposed on β-cells eventually impairs GSIS capacity and functional β-cell mass declines significantly (Halban et al., 2014).

Metabolic stress causes an accumulation of reactive oxygen species in T2DM β-cells, which can induce deleterious modifications to DNA, lipids and proteins (Evans et al., 2002; Khanday et al., 2006; Kondo et al., 2009). In a recent study, the subcellular localization, function, and likely stability of the β-cell enriched transcription factors NKX6.1, PDX1, MAFA and MAFB were found to be compromised in human T2DM islet β-cells and mouse models of T2DM (Guo et al., 2013). The loss of these central factors leads to significant reductions in expression of their gene targets including *Insulin, SLC2A2* and *GLP1R*, all of which are critical to β-cell glucose-sensing and insulin secretion. Exposure of β-cell lines to H_2O_2 mimics disease-stress conditions encountered *in vivo*, and strongly suggests that oxidative is stress a significant contributor to this phenomenon. These experiments also revealed that MAFA and MAFB are highly sensitive to oxidative stress as these proteins were rapidly lost. In contrast, the inactivation of PDX1 and NKX6.1 required much longer exposure to these conditions. It was proposed that β-cells are rendered dysfunctional in a stepwise fashion in T2DM islets, with early loss of MAFA/MAFB potentially manifesting as postprandial glucose intolerance observed in pre-diabetics, and later inactivation of PDX1 and/or NKX6.1 resulting in overt β-cell dysfunction and potentially cell death (Figure 4) (Guo et al., 2013).

A recent report by Dr. Domenico Accilli's group challenges the long-standing paradigm that the decline in β-cell mass is due to cell death (Talchai et al., 2012). In this

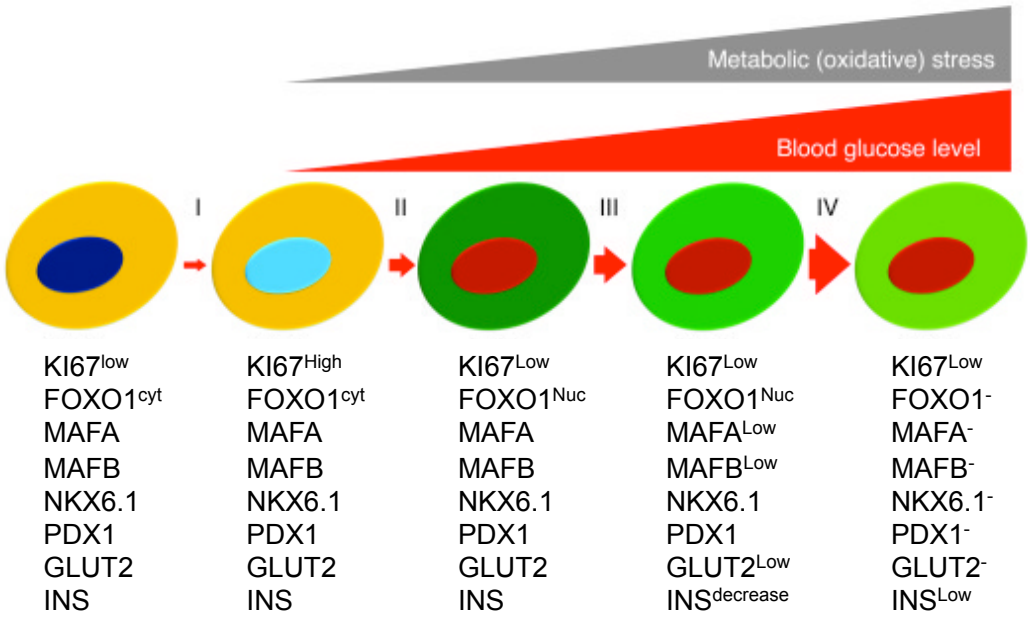

Figure 4. Events leading up to islet β-cell dysfunction in T2DM. Early in disease progression (I) β-cell replication and (II) FOXO1 nuclear localization occurs as an adaptive response to peripheral insulin resistance. However, as the disease progresses (II) FOXO1 nuclear localization/inactivation and sequential loss of (III) MAFA (and/or MAFB), and (IV) NKX6.1 and PDX1 occurs in response to prolonged exposure to hyperglycemia and oxidative stress. These circumstances lead to a decline in β-cell function, also termed “the stunned β-cell”. (Adapted with permission from Guo et al, 2013)

study, lineage tracing was employed to follow the fate of β-cells deficient for *FoxO1*. Strikingly, when these mice faced physiological stress conditions such as multiparity and increased age, cell death was not observed. Instead, cells lost expression of insulin and other β-cell identity markers (i.e. Pdx1, Nkx6.1, MafA, Glut2) and gained expression of progenitor and pluripotency markers such as Ngn3, Oct4, Nanog, L-Myc, and Sox2, indicating a regression to progenitor-like cell types. Intriguingly, a recent study examining another mouse model of β-cell dedifferentiation revealed that cells can be redifferentiated into functional β-cells after insulin therapy (Wang et al., 2014). These results suggest that the exhausted and dedifferentiated β-cells can be reinvigorated if given a chance to rest/recover. Although dedifferentiation has been observed in several mouse models of diabetes, studies from our laboratory did not observe alterations of any above mentioned progenitor markers in human T2DM samples, suggesting this phenomenon may not occur in human islet β-cells (Guo et al., 2013).

The Underappreciated α-Cell

β-cell destruction/dysfunction and resulting insulin deficiency have long been the center of attention, but it is becoming more and more apparent that inappropriately increased α-cell activity and hyperglucagonemia are major contributors to diabetes. Glucagon opposes the actions of insulin in fuel homeostasis by mobilizing hepatic glucose stores to maintain normoglycemia in the fasting state. Upon glucose challenge, paracrine insulin signaling normally acts to suppress glucagon secretion and balance the metabolic needs of the organism. The juxtaposition of α-cells to β-cells within the islets causes α-cells to be exposed to insulin concentrations nearly 100 times higher than any tissue in the body (Unger and Roth, 2015). The absence or shortage of functional β-cells in T1DM patients fail to meet these high levels of insulin required to inhibit glucagon

release (Unger and Cherrington, 2012). In contrast, regardless of the initial excess of circulating insulin, like other peripheral tissues α-cells become resistant to its actions in T2DM, and glucagon hypersecretion ensues (Unger and Cherrington, 2012). Thus, in both T1DM and T2DM, hyperglucagonemia plays a central role in glycemic control. For this reason, bihormonal strategies targeting the secretion and actions of both insulin and glucagon are being developed for the treatment of diabetes.

Current Treatments

The aim for treatment of both T1DM and T2DM is to improve glycemic control, and thus, prevention of complications accompanying the disorder. Multiple daily blood glucose measurements and insulin injections are currently the only treatment option for people with type 1 diabetes. Although injected exogenous insulin meets the needs of most peripheral tissues, it is diluted in the circulation, and fails to duplicate the high concentration required to suppress α-cell glucagon secretion upon reaching the islets. As glucagon hypersecretion is a direct contributor to human diabetic hyperglycemia, strategies implementing hormones such as leptin and amylin to suppress glucagon secretion are currently being explored for the treatment of both T1DM and T2DM (Unger and Cherrington, 2012; Unger and Roth, 2015).

Unlike T1DM, many strategies are implemented for the treatment of T2DM, with some as simple as changes in lifestyle such as exercise and diet (Stephenson et al., 2014). A number of pharmacological interventions that act to increase β-cell function and peripheral tissue glucose uptake can also be used to aid glucose clearance. Examples of drugs that act directly on β-cells include sulfonylureas such as tolbutamide and glubenlamide, and degradation resistant glucagon-like peptide-1 (GLP-1) analogues such as exenatide and liraglutide. Sulfonylureas directly stimulate insulin secretion from

β-cells by inhibition of the SUR1/Kir6.2 ATP-dependent potassium channels (Kaiser and Oetjen, 2014). GLP-1 is an insulin secretagogue that augments β-cell function in the presence of high glucose. Additionally, GLP-1 slows gastric emptying, promotes satiety, and suppresses α-cell glucagon secretion. Drugs such as metformin, on the other hand, act peripherally to lower blood glucose levels by inhibiting hepatic gluconeogenesis and enhancing skeletal muscle glucose uptake (Kaiser and Oetjen, 2014).

Although great strides have been made with regard to these and other drug-based therapies, they still fall short of the accuracy with which endogenous α- and β-cells control glycemia, and come with their own set of side effects. Transplantation surgeries of cadaveric human islets have proven to be an effective strategy for treatment of T1DM, and provide the proof-of-principle that restoring glucose homeostasis can be accomplished by re-establishing a patient's functional α- and β-cell mass (Shapiro et al., 2014). However, as recipient patients typically require the pooling of islets from multiple donors for sufficient numbers, the current demand for donor islets far exceeds the supply. For this reason, substantial efforts are being made to generate renewable sources of β-cells for cell-replacement therapies. Three potential sources of new β-cells are currently being explored as options for generation of such β-cells. These include, inducing the expansion of existing β-cells, reprogramming of terminally differentiated cell types, and guided differentiation of stem or induced pluripotent stem (iPS) cells to a β-cell fate (Figure 5).

Prospective Future β-Cell Replacement Therapies

Expansion of Endogenous β-cell Mass

Replication of islet β-cells is a rare event under normal conditions in adults, and the competence of these cells to replicate appears to significantly decrease with age (Perl et al., 2010). Mouse β-cells display a robust capacity to proliferate in response to conditions such as obesity, injury and pregnancy, all of which increase the demand for insulin (Butler et al., 2007). Therefore, the ability to expand a patient's endogenous β-cell mass as a possible treatment option has sparked research aimed to determine the underlying molecular mechanisms behind β-cell replication (Figure 5C). Efforts have identified a number of mitogens and growth factors such as glucagon-like peptide 1 (GLP-1), hepatocyte growth factor (HGF), lactogens, insulin, and others that can enhance rodent β-cell replication. The signaling pathways activated by these agents impinge upon cell intrinsic factors such as the transcription factors FoxM1, Pdx1 and FoxO1, which integrate these signals to modulate expression of numerous cell cycle regulatory genes (Li et al., 2005a; Okamoto, 2006; Zhang et al., 2010). Unfortunately, most mitogens, growth factors and nutrients found to augment rodent β-cell proliferation have little affect on older mouse β-cells, and more importantly, human β-cells, highlighting the absolute necessity of confirming that findings in mice translate to humans.

Cellular Reprogramming

Reprogramming of a patients own terminally differentiated cell types to β-cells is an enticing prospect for regenerative medicine (Figure 5B). Cellular reprogramming or transdifferentiation involves global removal of epigenetic patterns established during

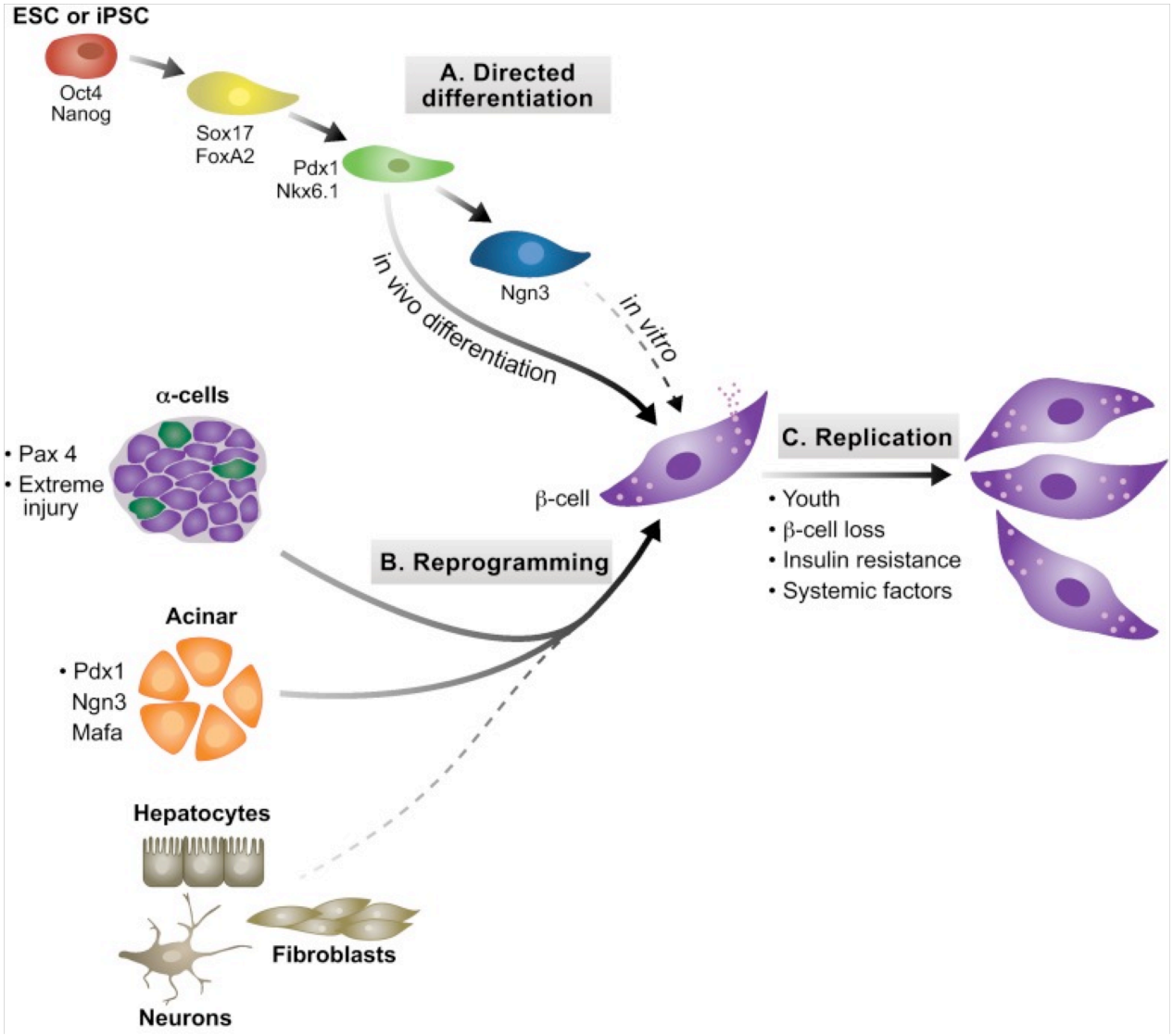

Figure 5. Current strategies to generate new β-cells. A) Directed differentiation of embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) to a β-like cell fate can be accomplished by sequential exposure to growth factors and small molecules. These are designed to mimic developmental conditions, and thus, guide cells through multiple stages of pancreas development (monitored by expression of indicated genes) towards a β-cell fate. B) Reprogramming of mature pancreatic cell types such as acinar and islet cells can be accomplished by overexpression of key β-cell transcription factors (listed) and several pancreatic injury models. Other mature cell types such as hepatocytes, neurons and fibroblasts are also being investigated as potential sources of new β-cells. C) The ability to stimulate replication of existing β-cells through exposure to growth factors and small molecules is currently under investigation (modified with permission from Pagliuca FW, 2013).

development and remodeling of the chromatin landscape to produce gene expression patterns of the desired cell type. All cells of the mature pancreas (acinar, ductal and endocrine) arise from a common population of multipotent pancreatic progenitor cells (MPCs). Genome wide analyses of histone modifications and DNA methylation patterns have revealed that mature pancreatic exocrine and endocrine cells display similar epigenetic patterns (Bramswig et al., 2013). The similarity in epigenetic profiles holds promise to facilitate cellular reprogramming. Consequently, these cell types have received substantial attention as potential sources of new β-cells.

Acinar cells are the most abundant cell type of the pancreas, and therefore, represent a plentiful source of potential β-cells. Zhou et al. (2008) demonstrated that adenoviral-mediated over-expression of key transcription factors involved in normal islet development, *Ngn3*, *Pdx1* and *MafA*, induces acinar-to-β-like cell reprogramming in mice. It is important to note that although these β-like cells possessed many features of *bona fide* β-cells and were shown to significantly reduce hyperglycemia in streptozotocin-induced diabetic animals, the extent of reprogramming and ability to properly respond to glucose remains to be tested. Notably, reprogramming of acinar cells to β-like cells can also occur without the introduction of exogenous factors as a consequence of partial duct ligation and streptozotocin-mediated depletion of endogenous β-cell mass, illustrating the intrinsic plasticity of acinar cells (Pan et al., 2013).

Ductal and endocrine cells arise from common bipotent progenitors during pancreas development, and initiation of the endocrine program hinges upon transient expression of *Ngn3*, a key transcription factor in endocrine lineage specification. Over-expression of the β-cell determinant Pax4 transcription factor in α-cells causes an α-to-β conversion (Collombat et al., 2009). Glucagon deficiency resulting from α-cell depletion

triggers *Ngn3*-mediated α-cell neogenesis from the mature ducts. However, because these newly forming α-cells subsequently convert to β-cells from ectopic Pax4 expression, this study inadvertently identified the ducts as a potential source of new β-cells. This also helped spark the notion of islet cell plasticity, and has led to intense examination of the prospect of facilitated α-to-β cell transdifferentiation.

Numerous studies have revealed that genetic manipulations enforcing expression of β-cell determinants (e.g. *Pdx1, Nkx6.1, Pax4*) or deleting α-cell determinants (e.g. *Arx*) in $Ngn3^{+}$ endocrine progenitors can cause identity conversion to a β-like phenotype of cells fated to other endocrine lineages (Collombat et al., 2009; Schaffer et al., 2013; Yang et al., 2011). Studies have also demonstrated that α-cells possess the intrinsic ability to spontaneously reprogram into β-cells upon near complete (>99%) ablation of endogenous β-cells (Thorel et al., 2010). The ease with which α-cells transdifferentiate is thought to be associated with the large number of genes bivalently marked by both activating H3K4me3 and repressing H3K27me3 histone modifications (Bramswig et al., 2013). Genes possessing this signature remain in a silenced state, yet are poised for activation upon proper signaling, and thus, confer a degree of cellular plasticity. Notably, these marks were found on many genes involved in β-cell transcriptional networks. However, a follow-up study by Chera et al. revealed that the reprogramming of δ-cells, rather than α-cells, reconstitutes β-cell mass of juvenile mice following β-cell ablation (Chera et al., 2014). Intriguingly, unlike the direct α-to-β-cell reprogramming observed in older animals, juvenile δ-cell reprogramming occurred in a stepwise mechanism involving dedifferentiation, proliferation and subsequent re-expression of islet developmental regulators yielding one β– and one δ-cell.

These studies indicate relative plasticity of the terminally differentiated pancreatic cells, and underscore their potential for conversion to β-cells. In all cases, however,

major hurdles remain in fully understanding the molecular mechanisms controlling these identity conversions, and the fine-tuning that is required to generate fully functional reprogrammed β-cells for replacement therapies. More critically, it remains to be tested whether these genetic manipulations will have the same effect in human cells.

Looking outside the pancreas, a recent study aimed to identify which adult tissues are competent to activate an islet β-cell program via transgenic expression of *Pdx1*, *MafA*, and *Ngn3* in a wide spectrum of tissues *in vivo* (pancreas, intestine, gallbladder, skin, spleen, and bone marrow (Chen et al., 2014). Similar to how these factors induce pancreatic acinar cells to become insulin-producing cells, their transient expression rapidly converted intestinal crypt cells into glucose-responsive endocrine-like cells (Chen et al., 2014; Zhou et al., 2008). These cells also coalesced into "neoislets" able to improve glycemic control in diabetic mice. Importantly, conversion of human intestinal epithelial cells to β-like cells was also observed when these factors were ectopically expressed in human intestinal "organoids". This indicates that the intestines (and potentially other human tissues) may serve as an additional source of functional β-cells.

Directed β-Cell Differentiation

Embryonic stem cells (ESC) are pluripotent and have the capacity to differentiate into all cell types of the body (Thomson et al., 1998). The discovery of these progenitor cells provoked extensive investigation into their use for regenerative medicine. To this end, strategies using ESCs have generated functional cardiomyocyte and oligodendrocyte cells able to prevent arrhythmias in injured hearts and restore mobility in rats that have suffered spinal chord injuries, respectively (Keirstead et al., 2005; Shiba et al., 2012). Unfortunately, ethical issues currently surround the use of ESCs, making their

use for treatment options controversial. Tissues generated from ESCs also run the risk of being rejected by recipient patients immune systems, as they will be recognized as non-self. However, the development of induced pluripotent stem cells (iPSC) engineered by reprogramming terminally differentiated mouse and human cells circumvents these roadblocks (Lowry et al., 2008; Takahashi and Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007). The differentiation capacity of iPSCs was demonstrated by producing hematopoietic progenitors from autologous skin cells to effectively treat a mouse model of sickle cell anemia (Hanna et al., 2007). Thus, these cells represent an unparalleled potential to generate patient-specific replacement tissues for diseases such as diabetes.

Strategies have been developed to differentiate human ESCs towards a pancreatic endocrine cell fate *in vitro* (Figure 5A). This is accomplished by sequentially exposing ESCs to differentiation signaling factors to guide them through several precursor populations in a stepwise fashion (D'Amour et al., 2006; Kroon et al., 2008). Previous protocols required engrafting ESC-derived pancreatic endoderm cells as a means to yield glucose-responsive insulin-secreting cells in significant numbers. However, recent advances in methodology have greatly improved *in vitro* protocols to generate glucose-responsive β-like cells capable of rapid reversal of diabetes in animal models (Pagliuca et al., 2014; Rezania et al., 2014).

While these replacement therapies hold great promise, fully understanding the transcriptional networks that underlay β-cell expansion, reprogramming of terminally differentiated cell types, and guided differentiation of stem or induced pluripotent stem (iPS) cells to a β-cell fate remains elusive. Therefore, the study of transcriptional events driving these processes is a fundamental objective of many islet biology researchers.

Transcription

Transcription refers to the process by which the genetic information encoded in specific sequences of DNA (genes) is used to generate a functional gene product. These products are typically proteins, however, functional non-protein RNA, such as transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA), and long non-coding RNAs (lncRNA) are also transcribed from DNA.

Chromatin

Mammalian genomes consist of nearly two meters of linear DNA that is packaged into a 10-micrometer diameter nucleus. To accomplish such an amazing feat, the DNA is complexed with specialized histone proteins to form structures called nucleosomes. Each nucleosome consists of a stretch of 147 base pairs of DNA wrapped 1.67 times around a histone octamer. Nucleosomes are the basic structural units of chromatin, and allow DNA to be compacted into higher ordered structures to accommodate the size of the nucleus. The patterning and packaging of nucleosomes also generates chromatin patterns that can be used to partition regions of the genome into various states, such as “opened” and active euchromatin, or “closed” and inactive heterochromatin structures.

Epigenetic modifications such as DNA methylation, histone modifications, nucleosome positioning, and noncoding RNA interactions alter chromatin structure and gene expression without changing the DNA sequence. These alterations, therefore, can confer information not encoded in the genome to allow genes in the same linear sequences of DNA to be transcribed in a cell type specific manner, and thus generate multiple cellular phenotypes. Some modifications are heritable and allow complex, cell-type-specific traits to be passed on during cellular divisions, while others are transient in nature, allowing for the rapid activation or repression of associated genes in response to

signaling events. Collectively, the distribution and pattern of epigenetic modifications serve to build chromatin landscapes, which alter the accessibility of the underlying DNA. The concerted actions of two specialized classes of proteins called transcription factors and coregulators are responsible for the deposition and removal of epigenetic modifications on chromatin.

Transcription Factors

Transcription factors are proteins that bind DNA in a sequence-specific manner to control the rate of copying particular segments of DNA into RNA (gene expression). The differentiation of all cells occurs through sequential modifications in gene expression, which are tightly regulated by complex networks of transcription factors and recruited coregulator proteins. Strict timing and regulation of these hierarchical arrays of transcription factors is critical to pancreas and β-cell development (Figure 6). The expression of many factors responsible for proper pancreas and β-cell development is maintained in mature β-cells where they take on new roles in proliferation, survival and the maintenance of mature β-cell function.

Pdx1

The Pdx1 transcription factor wields profound regulatory control over pancreas development and β-cell function. For example, all ductal, acinar and islet cells that compose the adult pancreas are derived from a common Pdx1-expressing progenitor pool, with pancreas agenesis manifested in mice and humans unable to produce the functional protein (Gu et al., 2002b; Jonsson et al., 1994; Offield et al., 1996). To investigate the contribution of Pdx1 to pancreas development past the initial bud formation, MacDonald et al. genetically engineered mice in which *Pdx1* expression could

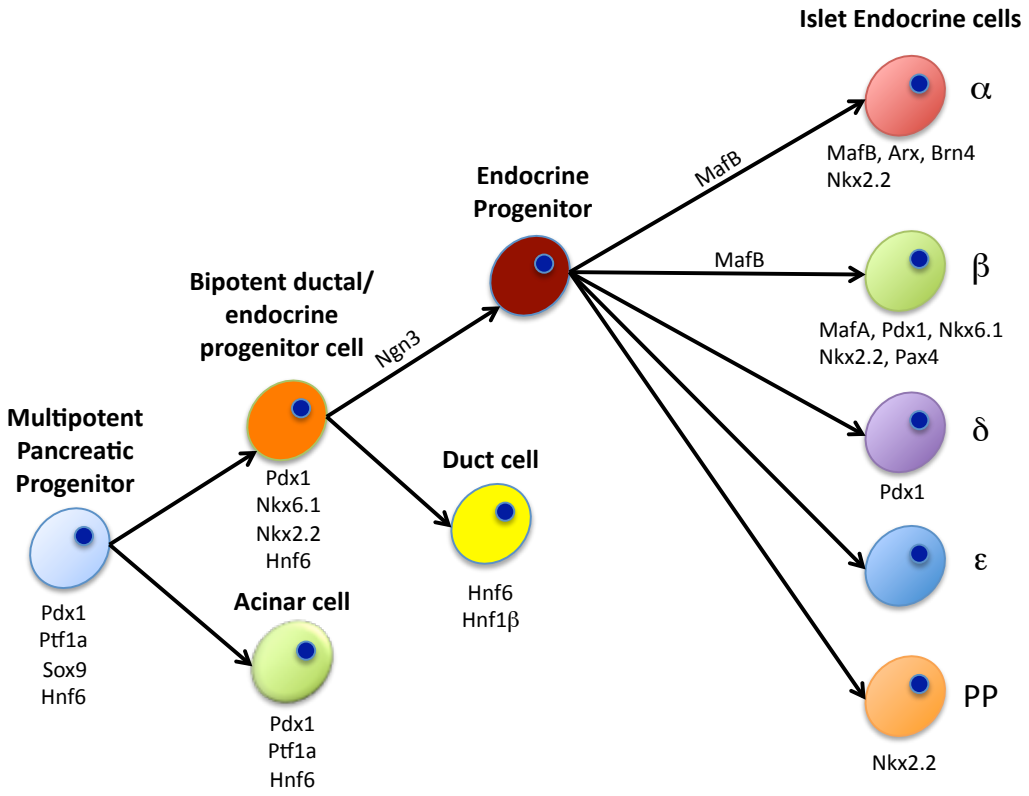

Figure 6. Cascades of key transcription factors drive endocrine cell development in mice. This schematic diagram is a highly simplified model of the transcription factors driving acinar, ductal and endocrine lineage development.

be conditionally repressed by the administration of doxycycline (Figure 7) (Holland et al., 2002). In these mice, the coding region of *Pdx1* was replaced with a tetracycline regulatable transactivator. These mice also harbored a transgene with a tetracycline-regulated promoter linked to a *Pdx1* minigene. In the absence of doxacycline, Pdx1 was expressed in the correct spatiotemporal pattern and the pancreas developed normally. However, removal of Pdx1 at mid-gestation via doxacycline administration was found to halt branching morphogenesis of the epithelium. The resulting truncated ductal tree was composed of immature duct-like cells and a failure to form the acinar and endocrine lineages. Removal of Pdx1 slightly later allows for the initial formation of acinar cell progenitors that fail to fully mature, indicating a role for Pdx1 not only in the specification, but also the maturation of both acinar and endocrine lineages (Hale et al., 2005).

In late gestation and the adult pancreas, Pdx1 is a potent driver of the β-cell program. Forced and persistent expression of *Pdx1* in newly forming $Ngn3^+$ endocrine progenitors causes cells originally destined to become α-cells to postnatally adopt a β-like phenotype (Figure 7) (Yang et al., 2011). *Insulin*-driven Cre-recombinase deletion of *Pdx1* from newly forming β-cells causes a dramatic reduction in β-cell mass, and mutant animals become overtly diabetic soon after birth (Figure 7) (Ahlgren et al., 1998; Gannon et al., 2008). Although β-cell formation was not disrupted, $insulin^+$ cells lacking Pdx1 failed to proliferate or mature causing a dramatic reduction in functional β-cell mass. A concomitant increase of $glucagon^+$ α-cells was also observed, suggesting the appropriate number of β-cells normally dictate islet α-to-β cell ratio. Mice heterozygous for *Pdx1* develop a grossly normal pancreas, however, with age these animals become glucose intolerant due to a progressive loss of β-cell function.

As Pdx1 plays such a critical role in pancreas development and β-cell function, significant effort has been made to elucidate the mechanisms controlling its expression

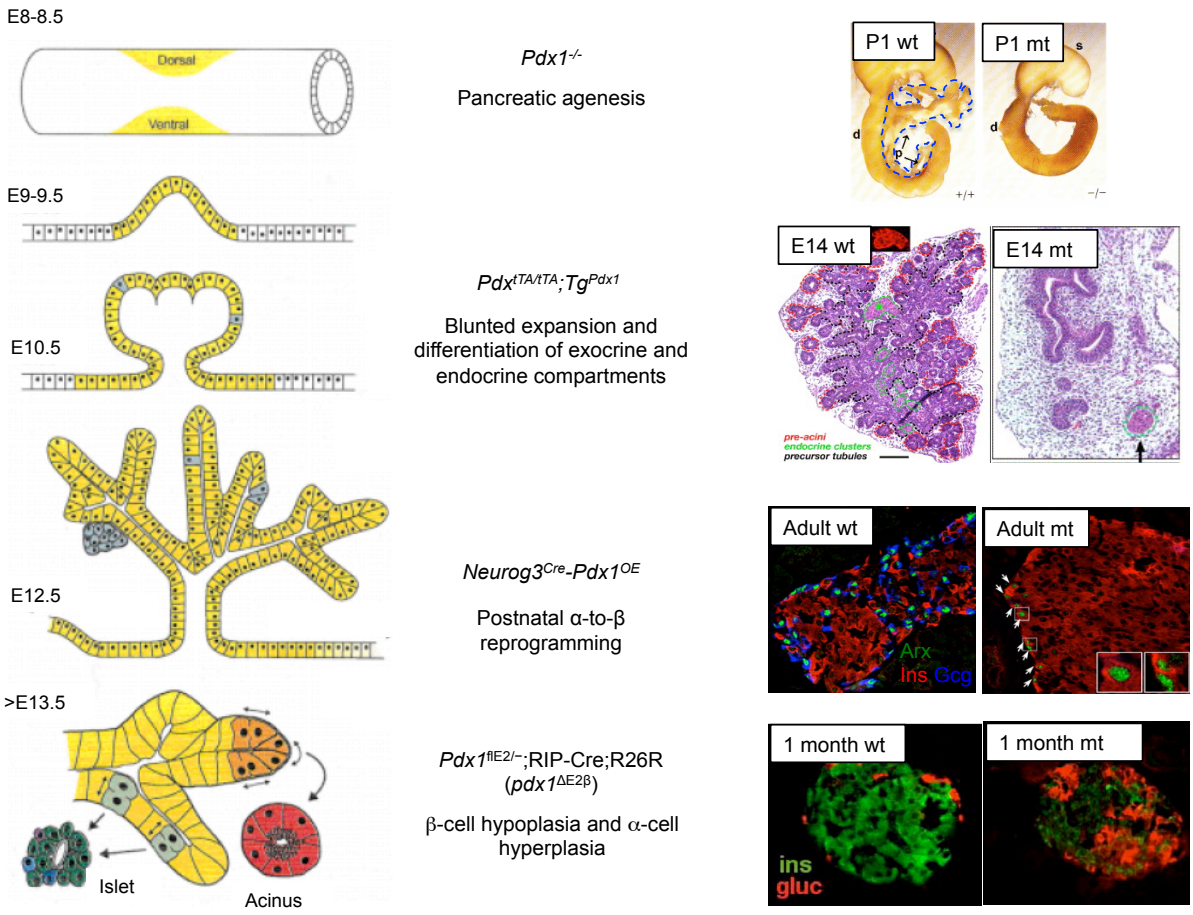

Figure 7. Pdx1 is a critical regulator of pancreas and β-cell development. Schematic representation (Left) of pancreas growth and differentiation during development. The middle column indicates the various genetic models of conditional deletion or overexpression of *Pdx1* that have been used to delineate the contributions Pdx1 makes to pancreas and β-cell development. The effect of each manipulation is described below the italicized model and representative images of each phenotype are shown (Right). (Adapted with permission from Kim and Macdonald, 2002; Jonnson et al., 1995; Hale et al., 2005; Gannon et al., 2008; Yang et al., 2011)

in the developing and mature pancreas. The spatial-temporal expression pattern of Pdx1 is tightly regulated by critical *cis*-acting control regions of DNA located 5' of the coding region. These highly conserved 5' enhancers were termed *Area I* (bp −2761 to −2457), *Area II* (bp −2153 to −1923), *Area III* (bp −1879 to −1600), and *Area IV* (−6530 and −6045 bp) (Gannon et al., 2001; Gerrish et al., 2000, 2004). Each of these regions harbors multiple binding sites for transcription factor proteins such as Ptf1a, FoxA1/2, MafA, and others, which mediate temporal and tissue-specific expression (Samaras et al., 2002; Wiebe et al., 2007).

Analysis of transgenic mice carrying different combinations of the enhancers indicates that *Areas I-III* are essential for the broad expression pattern of Pdx1 in the developing acinar, ductal and endocrine pancreas, while *Areas I* and *II* alone appear to be sufficient for the high level of Pdx1 expression observed in β-cells (Gannon et al., 2001). A recent study from our laboratory examined the consequences of the deletion of Area IV. The germline removal of *Area IV* revealed this enhancer plays important developmental and postnatal roles in *Pdx1* expression. In the absence of *Area IV*, significant reductions in $Ngn3^{+}$ endocrine progenitor cells were observed at embryonic day 15.5 (unpublished data). This phenomenon leads to specific reductions (20%) in β-cells, while α-cell numbers were unaffected. However, at birth, there were no alterations observed for levels of Pdx1 or other β-cell markers including MafA, Glut2 or Nkx6.1, and blood glucose levels were unaltered through weaning. At 4 weeks of age, *Area IV* mutant mice exhibit significant increases in fasting blood glucose with concurrent decline in Pdx1 and other β-cell signature genes *MafA*, *Glut2* and *Nkx6.1*.

Together these studies reveal not only the critical role of *Areas I-IV* in the regulation *Pdx1* expression pattern, but also that the protein level of Pdx1 dictated by these control elements is critical for its proper function. The importance and dose-

dependent nature of Pdx1 activity in pancreas development was illustrated through analysis of transgenic mice carrying various hypomorphic alleles of *Pdx1* (Boyer et al., 2006; Fujitani et al., 2006). In these studies, transgenes harboring different combinations of *Areas I, II and III* to drive *Pdx1* transcription were found to have significantly altered timing and levels of *Pdx1* expression. Disruption of Pdx1 dosage impaired multiple aspects of pancreas development, including commitment of pancreatic precursors, acinar cell differentiation, and endocrine lineage allocation, illustrating how critical the proper level of Pdx1 protein is to these processes.

Two exons comprise the *Pdx1* gene, which code for a highly evolutionarily conserved 283 amino acid protein (Figure 8). The first exon contains the N-terminal activation domain, and the second encodes the DNA-binding homeodomain and the C-terminal repression domain. The Pdx1 homeodomain binds to a core tetranucleotide DNA-binding sequence 5'-TAAT-3', and its consensus recognition element is 5'-CTCTAAT(T/G)AG-3' (Liberzon et al., 2004). The flanking N- and C- terminal domains facilitate protein-protein interactions with various coregulator proteins to modulate transcriptional action (Liu et al., 2004; Mosley and Ozcan, 2004; Qiu et al., 2002).

Pdx1 is subjected to a number of posttranslational modifications, which permit context-dependent coregulator interactions, affect cellular localization and stability (Figure 8). Signaling cascades resulting from changes in extracellular glucose concentrations have been shown to induce phosphorylation, sumoylation and *O*-linked-*N*-acetylglucosamine (*O*-GlcNAc) modifications of Pdx1 (Gao et al., 2003; Kishi et al., 2003; Meng et al., 2010). Although sumoylation and *O*-GlcNAc both appear to augment Pdx1 activity by increasing its nuclear localization and DNA binding capacity, respectively, Pdx1 phosphorylation can either alter activity or protein stability depending on the site of phosphorylation (Al-Quobaili and Montenarh, 2008).

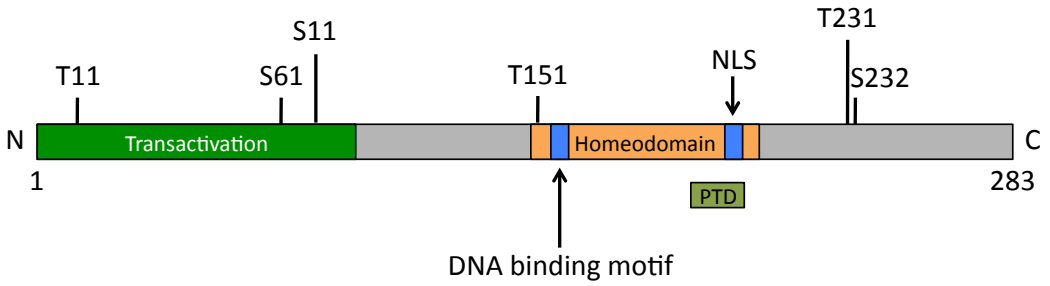

Figure 8. Schematic representation of PDX1 protein. PDX1 is a 283 amino acid transcription factor protein composed of an N-terminal transactivation domain and a central homeodomain. Within the homeobox are the DNA binding motif, nuclear localization signal motif (NLS), and a protein transduction domain/Antennapedia domain (PTD). PDX1 is posttranslationally modified to modulate its function, stability and subcellular location. Numbers indicate serine (S) and threonine (T) phosphorylation sites and functional domains within the protein. These various sites are phosphorylated by different kinases, which include Thr 11 by DNA-PK, Ser 61 and 66 by GSK3beta, Thr 152 by PASK, Thr 231 and Ser 232 by CK2 and Ser 268 by AKT-GSK and HIPK2. Although modifications such as sumoylation and *O*-linked-*N*-acetylglucosamine (*O*-GlcNAc) have also been shown to affect PDX1 activity and subcellular location, the sites of these modifications have not been mapped.

Although the actions of Pdx1 are of the utmost importance for proper pancreas development and β-cell function, it is the concerted actions of complex networks of transcription factors that make these processes possible. The following section describes several key transcription factors, which make both critical and unique contributions to islet cell development and function.

Ngn3

The basic helix-loop-helix Neurogenin3 (Ngn3) transcription factor is among the most important regarding endocrine cell development, as its expression demarcates the specification of the endocrine lineage (Figure 6). Expression can first be detected at E9.5 and peaks during the wave of endocrine cell differentiation in the secondary transition (Rukstalis and Habener, 2009). Although $Ngn3^+$ cells can be detected over a relatively long time window, expression in individual cells is transient and becomes nearly undetectable after birth. However, Ngn3 production is required for endocrine specification, as *Ngn3*-null mice are nearly devoid of any endocrine lineage. Furthermore, lineage-tracing analysis revealed that all endocrine cells are derived from $Ngn3^+$ precursors (Gu et al., 2002b).

Endocrine progenitors appear to go through competence windows, which allow for the sequential production of each cell type (Johansson et al., 2007). Using a transgenic mouse in which the expression of *Ngn3* was dependent on tamoxifen administration, Johansson et al. revealed that expression of *Ngn3* early in the MPC population (~E8.5) almost exclusively generated $glucagon^+$ cells. Later induction of *Ngn3* (E11.5) produced $insulin^+$ and PP^+ cells, while even later (E14.5) induction resulted mainly in production of $somatostatin^+$ and PP^+ cells with decreased capacity to form $glucagon^+$ cells. Although *Ngn3* expression is significantly downregulated, sustained, low levels of expression in hormone-expressing islet cells is important for endocrine cell

maturation and function (Wang et al., 2009). Additionally, reactivation of *Ngn3* appears to be a critical step in α-to-β, δ-to-β, and acinar-to-β transdifferentiation observed in β-cell ablation and partial duct ligation injury models (Chera et al., 2014; Pan et al., 2013; Thorel et al., 2010).

MafA and MafB

MafA and MafB are members of the basic leucine-zipper family of transcription factors. Each contains an N-terminal activation domain and a C-terminal leucine-zipper DNA-binding domain. Although their *in vitro* DNA binding properties are very similar, MafA and MafB make different contributions to islet endocrine cell development. MafB expression can be detected in the pancreatic epithelium around E10.5 and is required for the transcription of *glucagon* and *insulin* in developing α- and β-cells, respectively. Analysis of *MafB*$^{-/-}$ mice revealed that loss of *MafB* results in significant reductions of both α- and β-cell numbers (Artner et al., 2007). This study also indicated that MafB is required for the induction of *MafA* transcription and the sustained expression of other central β-cell genes including *Pdx1* and *Glut2* from E15.5 to birth. However, MafB levels rapidly decline soon after birth in rodent β-cells, and expression becomes restricted to α-cells of the adult islet, where it potently activates α-cell genes involved in glucagon production and secretion (Figure 6, 9) (Artner et al., 2006). Intriguingly, human β-cells retain MAFB expression into adulthood, and its expression is required for the maintenance of β-cell GSIS in human β-cell lines (Dai et al., 2012).

In contrast, *MafA* expression is induced later (E13.5) in development and is strictly confined to insulin^{+} cells in mice (Figure 9). Although similarly restricted to β-cells, human MAFA is not expressed at adult levels until adolescence (Drs. Chris Wright & Al Powers, personal communication). In mice, MafA activity is not required until after birth

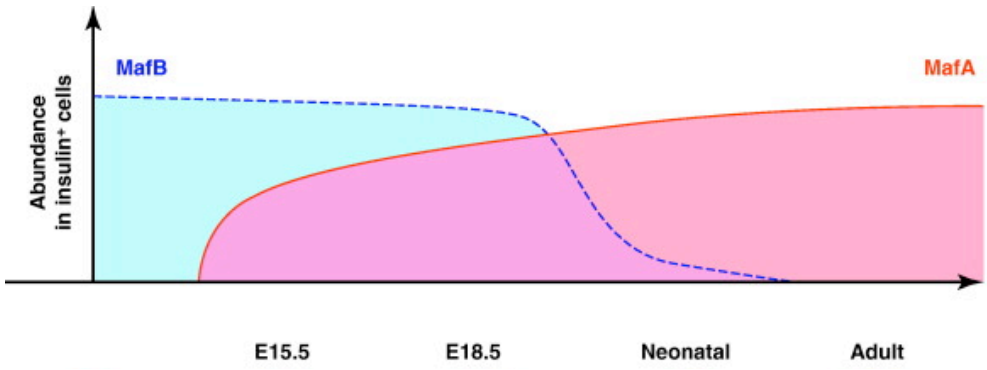

Figure 9. The expression levels of MafA and MafB dynamically change over the course of β-cell development. Diagram depicting the gradual decline in MafB, and concurrent increase in MafA expression over the course of β-cell development. (Adapted with permission from Hang and Stein, 2011)

where it is important for β-cell maturation. Indeed, evaluation of *MafA*$^{-/-}$ and pancreas-specific *MafA* (*Pdx1-Cre;MafA*$^{fl/fl}$ (*MafA*$^{\Delta panc}$)) mutant mice revealed that loss of *MafA* is inconsequential to β-cell formation (Artner et al., 2010; Hang et al., 2014). However, the expression of MafA-regulated genes is significantly reduced in *MafA*$^{\Delta panc}$ by three weeks of age. *MafA* deletion also impairs β-cell mass and function, and disrupts islet architecture. To investigate whether continued expression of MafB could alleviate defects caused by *MafA* deletion, our laboratory has generated transgenic mice in which floxed *MafA* is conditionally deleted by *RIP-Cre*, while a *MafA* promoter-driven transgene maintains *MafB* expression. Preliminary results indicate that in the absence of MafA, MafB is able to partially rescue first and second phase insulin secretion (Dr. Holly Cyphert, personal communication). However, these animals are glucose intolerant, which indicates that MafB is unable to fully compensate for loss of MafA. This indicates that while not important in development, *MafA* appears to play a critical role in β-cell maturation and function.

Pax4 and Arx

Pax4 and Arx act downstream of *Ngn3* and play opposing roles in the regulatory networks that determine endocrine cell type fate allocation (Gécz et al., 2006; Sosa-Pineda, 2004). The expression patterns of these two factors are mutually exclusive in the developing endocrine pancreas, with Pax4 restricted to differentiating insulin$^+$ and somatostatin$^+$ cells, and Arx restricted to glucagon$^+$ and ghrelin$^+$ cells. Arx and Pax4 directly repress each other's expression, and these factors have been shown to play opposing roles in endocrine cell fate allocation (Collombat, 2003, 2005). For instance, mice deficient for *Pax4* fail to develop β- or δ-cells, while their α-cell mass is simultaneously augmented (Collombat, 2003; Sosa-Pineda et al., 1997). In contrast, the

opposite phenotype manifests upon deletion of *Arx*, as these mice lack α-cells and display increased β- and δ-cell numbers (Collombat, 2003). Furthermore, ectopic expression of *Arx* in endocrine precursor and mature β-cells forces cells to adopt α- or PP-like cell features (Collombat et al., 2007a). Conversely, forced expression of *Pax4* in endocrine precursors or α-cell progenitors causes these cells to adopt β-like features at the expense of the α-cell population (Collombat et al., 2009). Proper expression of Pax4 and Arx in endocrine progenitor cells is essential to endocrine cell fate allocation.

Nkx6.1

Nkx6.1 is a member of the NK subfamily of homeodomain transcription factors. Its expression is broadly observed early in pancreas development and subsequently becomes confined to β-cells of the islet. In the multipotent pancreatic epithelium, Nkx6.1 promotes the formation of endocrine and ductal lineages by directly suppressing *Ptf1a*, and thus, acinar cell formation (Schaffer et al., 2010). Later in development, Nkx6.1 becomes both necessary and sufficient for the specification of β-cells. Disruption of *Nkx6.1* in $Ngn3^+$ cells results in an overall decrease in β-cell mass and a concurrent increase in expression of non-β-cell islet genes (Schaffer et al., 2013). Furthermore, like *Pdx1*, forced expression of *Nkx6.1* in endocrine progenitors (via a mouse model allowing conditional *Nkx6.1* transgene overexpression following *Ngn3-Cre* mediated activation) drives β-cell fate specification at the expense of other endocrine cell types (Schaffer et al., 2013). Selective removal of *Nkx6.1* from mature β-cells leads to a rapid onset of diabetes resulting from a loss of functional β-cell mass (Taylor et al., 2013). Lineage tracing indicated that *Nkx6.1*-deleted β-cells lose β-cell markers over time (~6 weeks) and acquire a δ-like identity (Schaffer et al., 2013). Collectively, these studies

indicate critical roles of Nkx6.1 in pancreas development and maintenance of mature β-cell function.

Nkx2.2

Nkx2.2 is another example of an islet NK homeodomain transcription factor. Nkx2.2 is broadly expressed in the embryonic pancreas at E9.5, and becomes restricted to endocrine α-, β- and PP-cells as development progresses, but is not expressed in δ-cells. As its expression pattern would imply, Nkx2.2 is a critical regulator of the commitment and differentiation of α-, β- and PP-cells, and *Nkx2.2*$^{-/-}$ mice mostly lack these endocrine populations (Sussel et al., 1998). In the absence of Nkx2.2, expansion of ghrelin-producing ε-cell occurs at the expense of these other cell types (Prado et al., 2004). Nkx2.2 possesses both activating and repressive transcriptional activities. Ectopic expression of dominant-activator and dominant-repressor derivatives of Nkx2.2 revealed its repressive functions are critical for the α- and β- cell specification, whereas the activating properties of Nkx2.2 are required to form functionally mature β-cells (Doyle and Sussel, 2007; Doyle et al., 2006). A recent study found that Nkx2.2-mediated recruitment of Hdac1, Dnmt3a and Grg3 corepressor proteins to the *Arx* promoter is required to maintain mature β-cell identity. Inhibiting complex recruitment manifests in β-to-α-like cell conversion due to *Arx* derepression (Papizan et al., 2011). This last study highlights the notion that although transcription factors play a pivotal role in gene regulation, without the concerted actions of recruited coregulators most transcription factors are rendered inactive.

Coregulators

Unlike transcription factors, coregulators typically do not bind DNA in a sequence specific manner. Instead, these factors are recruited to target genes by protein-protein interactions with transcription factors to aid in the assembly or disassembly of DNA-protein complexes. The concerted actions of transcription factor:coregulator complexes ultimately cause the underlying DNA to become either more or less accessible to transcription.

Coregulators can be divided into two main groups, coactivators and corepressors, based on their transcriptional activity. Coactivators are factors recruited to target genes that aid transcription factors to increase the rate of transcription, whereas corepressors work to decrease the rate of transcription. Coactivators and corepressors that alter chromatin structure to modulate transcription can do so by both enzymatic and non-enzymatic means. Non-enzymatic coregulators such as KAP-1 harbor protein-protein and protein-DNA/RNA interaction surfaces that influence transcription by altering epigenetic patterns, chromatin compaction, as well as recruitment of distinct cofactors and RNA Polymerase II (Cheng, 2014). The coregulators that function enzymatically can be divided into two main mechanistically distinct classes. First are those that alter chromatin through covalent modifications to DNA (e.g. methylation) and DNA binding proteins (e.g. histones, transcription factors and coregulators by acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and/or glycosylation) (Bhaumik et al., 2007; Chen and Li, 2004; Flotho and Melchior, 2013; Wells et al., 2003). Second are those that use the energy of ATP hydrolysis to destabilize nucleosomes and alter accessibility of DNA to the transcriptional machinery (Sudarsanam and Winston, 2000).

Numerous interesting enzymatic and nonenzymatic coregulators were found to interact with Pdx1 in the studies presented in Chapter III. However, due to its prominent role in developmental and mature cell processes, we focused our analysis on

investigating the contributions the Swi/Snf complex makes to Pdx1 transcriptional activity.

Swi/Snf

The Swi/Snf complex is an ATP-dependent chromatin remodeler, which serves as a molecular motor to alter nucleosome positioning. Although the exact mechanism of histone mobilization remains unknown, Swi/Snf actions generate nucleosome patterns that cause the associated DNA to become either more or less accessible to processes such as transcription, DNA repair, and DNA replication (Dykhuizen et al., 2013; Kwon et al., 2014; Yaniv, 2014).

This complex was first identified from two yeast/genetic screens, which sought to elucidate the genes contributing to mating-type switch (Swi) and sucrose non-fermentable (Snf) phenotypes, which lead to the name Swi/Snf (Winston and Carlson, 1992). At its core, the nearly 2MDa Swi/Snf complex is composed of 12-14 subunits. Homologs of yeast Swi/Snf subunits are conserved throughout all eukaryotes and have been implicated as critical regulators of development in all organisms in which they have been studied (Figure 10). However, as multicellular organisms with increasingly complex body plans emerged, so did a requirement for accurate regulation of equally complex chromatin structures to mediate fine-tuning of cell-type and developmental-stage-specific gene regulation (Wang et al., 1996a). During the long evolutionary transition from single-celled yeast to higher multicellular organisms and eventually mammals, several genes encoding Swi/Snf subunits were lost, others gained, while others expanded into gene families (Figure 10). The evolution of gene families through the process of gene duplication allowed the encoding of multiple alternative subunits, each capable of taking position in the complex in a mutually exclusive manner. This greatly increased the number of possible complex arrangements. In mammals, the original 14 subunits

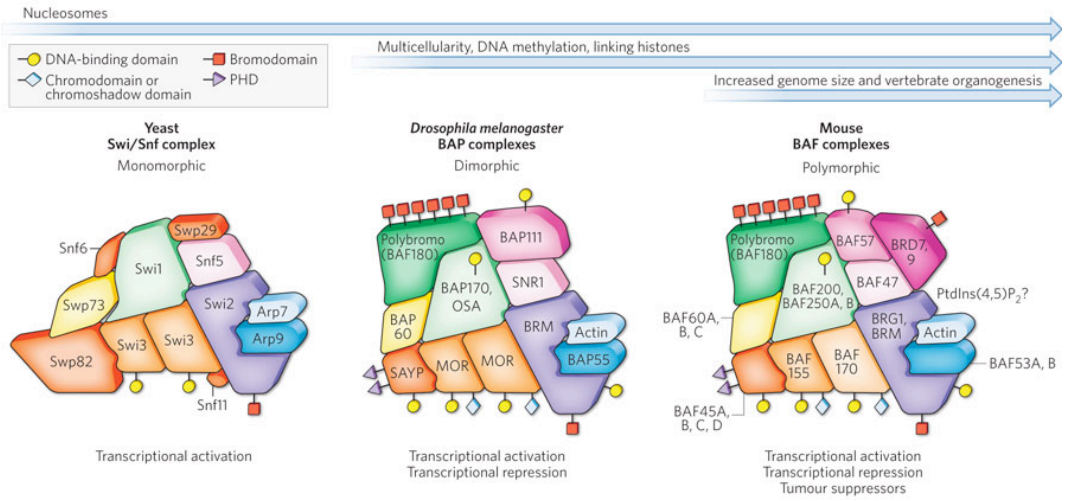

Figure 10. Schematic representation of the evolution of Swi/Snf complexes. The Swi/Snf complex is a highly evolutionarily conserved complex. Homologous subunits conserved between yeast, *Drosophila* and mouse are depicted in the same color. Over the course of evolution several subunits of the Swi/Snf complex have expanded into gene families (e.g. BAF45A, B, C, and D). These subunits incorporate into Swi/Snf complexes in a mutually exclusive manner, with each capable of conferring unique attributes to complex function. (Adapted with permission from Ho and Crabtree, 2010)

present in yeast have now expanded to 28, which allows for over 200 hypothetical Swi/Snf complexes to be formed (Wu et al., 2009). Different combinatorial assemblies of subunits have been illustrated to produce functionally distinct and cell-type-specific Swi/Snf complexes. Consequently, progressive changes or substitutions in subunit composition are now known to play a critical roles in neuronal, cardiac, and smooth muscle cell development.

Known Pdx1 Coregulators

Several coregulator proteins have been identified to modulate Pdx1 transcriptional control. The following section discusses the contributions each previously identified coregulator makes to Pdx1 activity in β-cells.

Set7/9

Set7/9 belongs to the SET family of histone methyltransferase proteins (Marmorstein, 2003). The biochemical activity of Set7/9 is highly specific to the deposition of histone H3K4 dimethylation (H3K4me2) activating marks. Unlike many coregulators, Set7/9 exhibits a tissue-specific expression pattern that consists of brain, muscle, liver, and pancreatic islets (Deering et al., 2009). Pdx1 regulates *Set7/9* expression through a highly conserved β-cell specific enhancer region located between -5768 and -6030 upstream from the promoter. In β-cells, Pdx1 directly recruits Set7/9 to the proximal *Insulin* promoter to coactivate expression via deposition of dimethyl marks on histone H3K4 (Francis, 2005). Knockdown of Pdx1 in INS-1 β-cells leads to a 60% reduction in H3K4me2 at the *Insulin* promoter and an accompanying 50% decline in *Insulin* mRNA. Depletion of Set7/9 from mouse pancreatic islets results in a similar reduction of *Insulin* transcription in addition to glucose responsive genes like *MafA* and

Glut2 (Deering et al., 2009). Set7/9 depletion also impairs recruitment of RNA Pol II, which is attributed to the role of Set7/9 in stabilization of the transcriptional preinitiation complex via methylation of TAF10. Maganti et al. recently investigated the role of this coregulator *in vivo*, finding that mice in which the gene encoding *Set7/9* was conditionally deleted in β-cells become glucose intolerant (Maganti et al., 2015). Moreover, isolated islets exhibit impaired GSIS and reduced expression levels of Pdx1 target genes.

Bridge-1

Bridge-1 is nearly ubiquitously expressed in developing and adult mouse, and human tissues. This coregulator does not possess enzymatic activity, but instead serves as a scaffold, facilitating complex formation through protein-protein interactions. Yeast two-hybrid screening initially identified Bridge-1 as a NeuroD1/E47 coactivator of *Insulin* transcription (Thomas et al., 1999). It was later found that Bridge-1 also interacts with the N-terminal activation domain of Pdx1, and that this interaction significantly augments Pdx1 activity on the *Insulin* promoter (Stanojevic et al., 2005). Interestingly, in a transgenic mouse model designed to overexpress Bridge-1 in the pancreatic compartment, insulin deficiency and diabetes result from reductions in both *Insulin* gene expression and β-cell mass (Volinic et al., 2006).

p300/CBP

p300 and CBP are encoded by two distinct genes, however, due to their high degree of structural and functional similarity are often referred to as one entity (p300/CBP), and thus, will be here. Unlike Set7/9, the p300/CBP coactivator is ubiquitously expressed in both mice and humans. At target promoters, p300/CBP coactivates gene transcription through direct interaction with and stabilization of the

basal transcription machinery and hyperacetylation of histones, which promotes active local chromatin structures (Goodman and Smolik, 2000). In β-cells, p300/CBP binds Pdx1 in combination with NeuroD1/E47 to synergistically activate *Insulin* gene expression (Qiu et al., 2002). Binding to the N-terminal activation domain of Pdx1 and recruitment to the *Insulin* promoter is regulated in a glucose-dependent manner, with elevated glucose levels resulting in histone H4 hyperacetylation to permit enhanced gene expression (Mosley and Ozcan, 2003; Mosley et al., 2004a). In contrast, at low levels of glucose histone *Insulin* promoter H4 acetylation and gene transcription are diminished by the recruitment of histone deacetylases.

Hdac1/2

Histone deacetylases 1 and 2 are ubiquitously expressed proteins that are often part of stable multiprotein complexes (e.g. NuRD, Sin3, CoREST1) (Ahringer, 2000; Roizman, 2011). These corepressors share 82% amino acid sequence identity and exhibit similar biochemical activity (Brunmeir et al., 2009). As the name suggests, Hdac proteins act to remove acetylation marks from histones at target promoters, which promotes nucleosome condensation, and thus, transcriptional repression. In contrast to p300/CBP, Hdac1/2 are recruited to the *Insulin* promoter at low glucose levels to mediate transcriptional repression (Mosley and Ozcan, 2004). It was found that glucose-dependent Pdx1 interaction with p300/CBP is mediated by an unidentified phosphorylation event, whereas, binding to Hdac1/2 requires a dephosphorylation event. The ability of Pdx1 to dynamically interact with both coactivators and corepressors, such as p300/CBP and Hdac1/2, elucidates, in part, its capacity to differentially activate/repress target gene expression.

Pcif1

Pcif1, which stands for PDX C-terminus Interacting Factor-1, was first identified as a Pdx1 binding partner in yeast two hybrid screens (Liu et al., 2004). Pcif1 is expressed in adult islet β-cells where it acts to inhibit Pdx1 transactivation in a dose-dependent manner. The reduced Pdx1 activity, however, was later found to be attributed to a decrease in Pdx1 protein stability. In cooperation with the Cul3 ubiquitin ligase complex scaffolding protein, Pcif1 targets Pdx1 protein for ubiquitination and subsequent proteosomal degradation (Claiborn et al., 2010). Notably, haplosufficiency of Pcif1 in *Pdx1*$^{+/-}$ mouse islets normalizes Pdx1 protein level and augments Pdx1 target gene expression. Thus, in this context, *Pcif1* heterozygosity normalized β-cell mass, improved β-cell function and glucose homeostasis.

Thesis Overview

Previous work from our laboratory and others has identified the Pdx1 transcription factor as a master regulator of pancreas organogenesis and β-cell function. Pdx1 makes critical contributions to the identity and proliferative capacity of newly forming β-cells, but its role in mature β-cells *in vivo* has not been fully examined. In collaboration with Dr. Ben Stanger, I aided in the investigation of the consequence of *Pdx1* deletion from mature β-cells. Chapter II describes the overall findings of these studies and my contribution to this work.

Like most transcription factors Pdx1 relies on its ability to recruit coregulator proteins to mediate its transcriptional action. Although several Pdx1 coregulators have been identified, my preliminary Reversible Cross-Link ImmunoPrecipitation (ReCLIP) analysis revealed that Pdx1 interacted with substantially more proteins than are listed above. Therefore, we hypothesized that the list of known Pdx1-interacting factors

represents only a subset of the coregulators recruited to mediate Pdx1 transcriptional activity. Thus, the main focus of this thesis was to identify and characterize additional Pdx1 coregulators. Chapter III describes the protein isolation/identification strategy used and novel Pdx1 coregulators identified. This chapter also examines the contribution of the Swi/Snf complex to Pdx1 activity and investigates how physiological and pathophysiological conditions affect Pdx1:Swi/Snf complex formation. Chapter IV presents concluding remarks, significance and future directions for the studies described in this dissertation.

CHAPTER II

PDX1 MAINTAINS β-CELL IDENTITY AND FUNCTION BY REPRESSING AN α-CELL PROGRAM

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Introduction

All pancreatic endocrine lineages are derived from $Ngn3^+$ endocrine precursor populations during pancreas development (Gu et al., 2002a). A number of studies have recently emerged that demonstrate a large degree of plasticity in the identity of islet hormone-producing cells, in particular, between α- and β-cells. Experiments designed to manipulate individual genes involved in endocrine cell development and function have revealed key players in the transcriptional programs used to maintain mature cell identity. For instance, forced expression of *Pdx1*, *Pax4* or *Nkx6.1* in endocrine progenitors can re-specify non-β endocrine precursors to a β-like cell fate (Collombat et al., 2009; Schaffer et al., 2013; Yang et al., 2011). Conversely, ectopic *Arx* expression in β-cell progenitors drives these cells to adopt an α-cell phenotype. Similarly, deletion of the DNA methyltransferase 1 (*Dnmt1*) coregulator or disruption of the Nkx2.2/Groucho3/Dnmt3a-represor complex in β-cells (each allowing *Arx* derepression), results in subsequent adoption of α-cell features (Collombat et al., 2007b; Dhawan et al., 2011; Papizan et al., 2011). Thorel et al. recently revealed that islet α-cells possess the intrinsic capacity to transdifferentiate to β-like cells upon near complete β-cell ablation. This study was aimed to investigate the capacity of adult mammals to regenerate β-cell mass after situations of near complete ablation (i.e. T1DM) using a transgenic mouse

model harboring β-cells expressing the diphtheria-toxin receptor. Lineage tracing analysis revealed that upon selective ablation of >99% of adult mouse β-cells via diphtheria toxin administration, a transdifferentiation event occurred where glucagon-producing α-cells spontaneously converted into insulin-producing β-like cells to replenish the functional β-cell mass and restore glucose homeostasis (Thorel et al., 2010). Together these studies illustrate the fine balance between α- and β-cell transcriptional programs.

In the adult mouse pancreas Pdx1 becomes restricted at high levels of expression to islet β-cells where it serves as a potent activator of many β-cell specific genes (Boyer et al., 2006; Khoo et al., 2012). Previous studies have shown that *Pdx1* haploinsufficiency diminishes glucose-stimulated insulin secretion and glucose tolerance, despite normal β-cell mass and pancreatic insulin content (Brissova et al., 2002). Additionally, embryonic deletion of *Pdx1* from newly forming β-cells results in a failure of the β-cell population to expand, and a concurrent hyperplasia of the α-cell population (Gannon et al., 2008). In the current study, *Pdx1* was conditionally and specifically deleted in mature β-cells and their fate was followed with a lineage tracer. As predicted from the earlier experiments using *Insulin*-Cre, *Pdx1* deletion resulted in a loss of β-cell identity (Ahlgren et al., 1998; Gannon et al., 2008). Surprisingly, these cells acquired global transcriptional signatures of β-to-α-like cell reprogramming within days of *Pdx1* deletion. The islet α-cell-enriched MafB transcription factor was among the most significantly up-regulated genes associated with this conversion, whereas induction of other key α-cell transcription factors (i.e. Arx and Brn4) was not observed. We found that Pdx1 normally binds the *MafB* and *Glucagon* promoters in β-cells, and obtained evidence that MafB de-repression in Pdx1-depleted cells was responsible for *Glucagon* gene activation. Significantly, these results highlight the importance of β-cell Pdx1 in

actively inhibiting α-cell identity and provide novel insight into repressive mechanisms involved in regulating islet β-cell identity and function.

Materials and Methods

Immunofluorescence Analysis

Cultured Ins-1 cells were washed with PBS, fixed in 3.2% paraformaldehyde/PBS, permeabilized with 0.2% Triton X-100/PBS, and then immunolabeled overnight with primary antibodies to insulin (1:1000 Dako A0546), glucagon (1:4,000 Sigma g2654-.5 mL), and MafB (1:1000 Bethyl IHC-00351).

Immunoblotting.

Nuclear lysates were fractionated by SDS/PAGE (NuPAGE 10% Bis-Tris gels), transferred to a PVDF membrane, and probed with the following antibodies: goat α-Pdx1 (1:10,000); rabbit α-MafB (1:2,000); Immunoe complexes were visualized usingHRP-conjugated α-rabbit IgG (Promega 1:2,000), HRP-conjugated α-goat IgG (Santa-Cruz, 1:2,000).

Electrophoretic Mobility Shift Assay

Nuclear extract from βTC3 cells was prepared as described previously (Schreiber et al., 1989a), and binding analysis was performed with double-stranded mouse oligonucleotides (MafB Site 1 −1,335 GCTTGGATCAGCTTAATCCTTACAAAACGT −1,306, Site 2 −952 CCCCACCGCATACATTAGCGCAGACAGAGC −923, and Site 2 mutant (MafB site 1 mt: −952 GCTCTGTCTGCGCGCCGGTATGCGGTGGGG −923; mutated bases are underlined). Competition analysis was performed with a 100X molar excess of unlabeled competitor to labeled probe. Antibody supershift analyses were performed by preincubating nuclear extract protein with Pdx1 antibody prior to adding

probe. Samples were electrophoresed on 6% nondenaturing polyacrylamide gels at 150 V for 2 hr in 1X TBE buffer (89 mM Tris-borate, 2 mM EDTA). Gels were then dried and visualized by autoradiography.

Adenoviral Pdx1 Knockdown

7.5×10^5 Ins-1 cells/well were seeded into six-well plates and siRNAs against *MafB* or scrambled control (Dharmacon) were introduced into cells using Lipofectamine 2000 (Invitrogen). Adenovirus expressing GFP or the Pdx1 siRNA was mixed with serum-free Dulbecco's modified Eagle's medium to a concentration of 66.6 MOI and infected for 48 hr before harvesting for immunofluorescence, protein, and RNA analysis.

Chromatin Immunoprecipitation

Mouse Min6 β-cells (~4×10^6 cells) were 1% formaldehyde cross-linked, and the sonicated protein-DNA complexes isolated under conditions described previously (Gerrish et al., 2001). The fragmented chromatin was incubated with α-Pdx1 or species-matched IgG (Bethyl Laboratories), and the immune complexes isolated using BSA- or herring sperm ssDNA (Abcam ab46666)-blocked protein-A Dynabeads (Invitrogen). Crosslinkx were reversed by overnight incubation at 65°C and DNA was purified Qiagen QIAquick PCR Purificatoin Kit following Proteinase K treatment. Quantitative real-time PCR was performed on immunoprecipitated DNA using SYBR Green master mix in a Roche LightCycler 480II. Primers used for amplification were as follows: MafB site1: Forward −938 TTAGCGCAGACAGAGCTACCGAAA −915, Reverse −742 ATACTCTTTACACTCCCACCCTCG −765; Glucagon G1 element: Forward −148 CGTAAAAAGCAGATGAGCAAAGTG −125, Reverse +46 GAACAGGTGTAGACAGAGGGAGTCC +70; Albumin distal TAAT-containing region:

Forward −3,342 TGGGAAAACTGGGAAAACCATC −3,319, Reverse −3,164 CACTCTCACACATACACTCCTGCTG −3,188.

Quantitative RT-PCR Analysis

Total cellular RNA was isolated from Ins-1 cells using the QIAGEN RNeasy Mini Kit. cDNA was prepared from RNA using iScript cDNA Synthesis Kit (Bio-Rad) and quantitative real-time PCR was performed using SYBR Green master mix and a Roche LightCycler 480II. The experimental data were normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) mRNA levels, and relative changes in mRNA were calculated by the comparative ΔCt method.

Results

Pdx1-Deleted β-Cells Rapidly Acquire Global Transcriptional Signatures of β-to-α-like cell Reprogramming

To better understand the role of Pdx1 in the mature β-cell, Dr. Ben Stanger's group generated *RIP-CreER;Pdx1*$^{fl/fl}$*;Rosa*^{YFP} *Pdx1* knockout (PKO) mice. Tamoxifen administration to 1-month-old PKO mice resulted in deletion of *Pdx1* and simultaneous activation of the *YFP* lineage label in β-cells specifically. Shortly after tamoxifen treatment PKO mice became glucose intolerant and had fasting blood glucose levels significantly higher than control animals. Immunofluorescence and RT-qPCR analysis indicated that *Pdx1*-deleted, YFP^{+} cells rapidly lose the expression of β-cell markers *Insulin*, *MafA*, *Nkx6.1*, and *Glut2*. Remarkably, roughly one third of these cells begin expressing α-cell genes *MafB* and *Glucagon*, but not *Arx*. Microarray analysis of fluorescence activated cell sorted (FACS) normal islet α-, β- and reprogrammed PKO β-

cells revealed that PKO cells resemble α-cells at the gene expression level. Among the most significantly upregulated genes was the α-cell enriched *MafB* transcription factor. Interestingly, the expression of other drivers of the α-cell program such as *Arx* and *Brn4* were not changed during the shift toward the α-like phenotype. It was hypothesized that MafB may be the driving-force behind respecification. As Pdx1 and MafB are two principle transcription factors studied in our laboratory, Dr. Stanger approached us to collaborate with his group to perform studies to elucidate the mechanism of the reprogramming phenomenon. The following section describes my efforts to examine Pdx1 control of *MafB* expression in β-cells and the consequences of *MafB* derepression in the absence of Pdx1.

Pdx1 Directly Represses β-Cell Glucagon and MafB Expression

To investigate the hypothesis that the induction of MafB in PKO cells was responsible for the shift towards an α-like phenotype, an adenovirus producing siRNA targeting *Pdx1* was used to deplete rat insulinoma Ins-1 β-cells of Pdx1 protein (Figure 12A). Upon knockdown, both *MafB* and *Glucagon* mRNAs were rapidly upregulated, while *Insulin, MafA, Glut2, and Nkx6.1* expression was significantly attenuated (Figure 11, 12A). Similar to the observations in the PKO cells, immunofluorescence analysis also revealed that neither Arx or Brn4 were upregulated in the absence of Pdx1. We therefore sought to further characterize the role of Pdx1 in *MafB* expression, and the contribution of MafB protein induction on *Glucagon* activation.

ChIP-Seq analysis of Pdx1 binding in Min6 cells indicated enrichment spanning a region between -800 and -1500 bp upstream of the *MafB* coding region (Figure 12B) (Khoo et al., 2012). Bioinformatics analysis identified two putative Pdx1 binding sites within this region located at -1,325/-1,316 bp (i.e., *Site 1*) and -942/-933 bp (*Site 2*). Gel

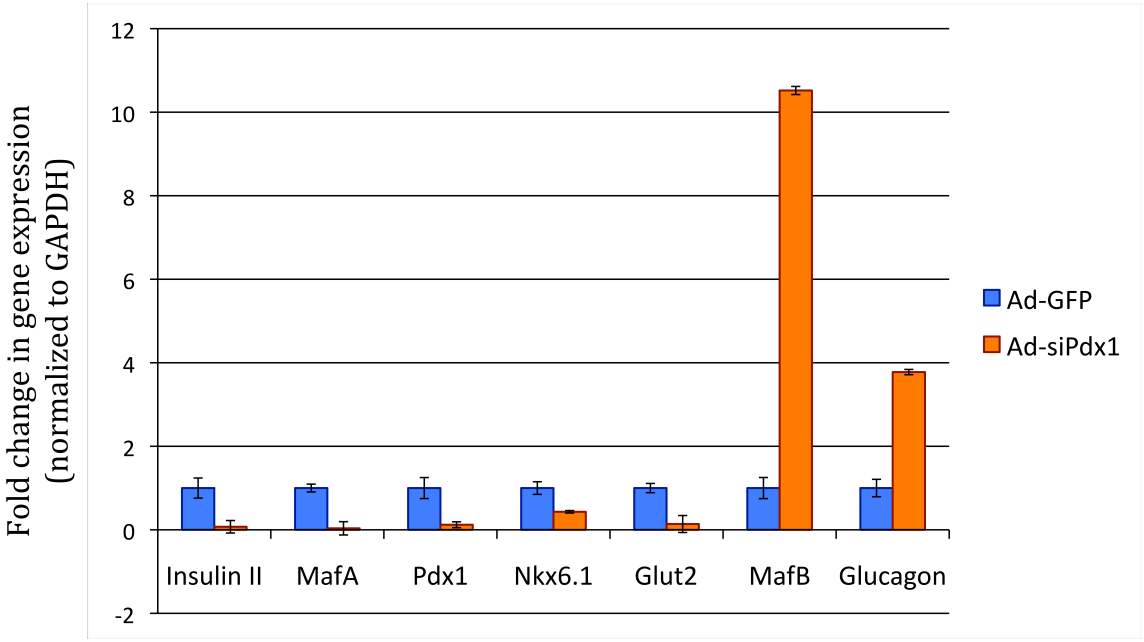

Figure 11. Pdx1 depletion causes Ins-1 cells to lose β-cell identity markers and allows expression of α-cell enriched genes. Adenoviral mediated siRNA knockdown of Pdx1 in Ins-1 cells causes significant reduction in β-cell-specific genes (*Insulin, MafA, Pdx1, Nkx6.1, and Glut2*), and allows for simultaneous activation of α-cell genes (*MafB, Glucagon*).

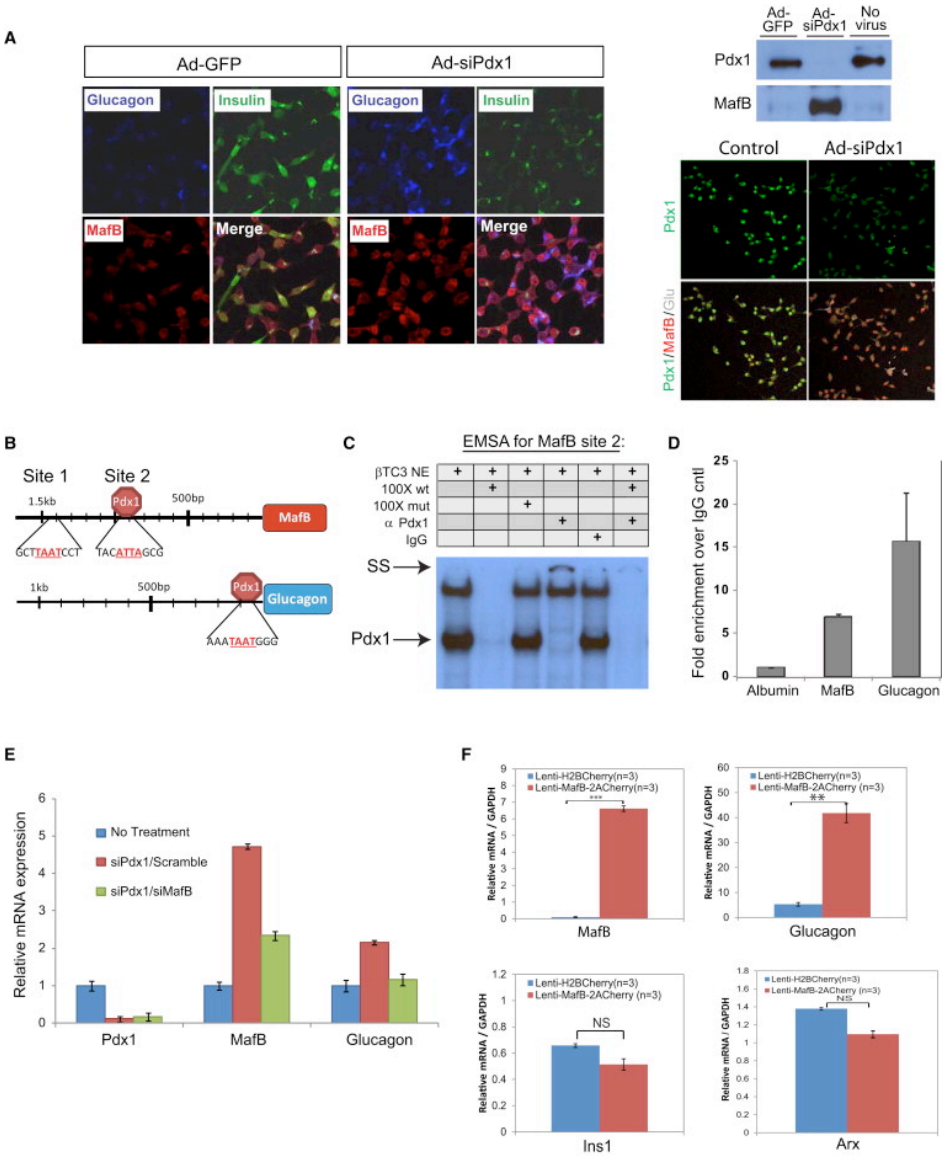

Figure 12. *MafB* and *Glucagon* transcription is stimulated in β-cells by eliminating *Pdx1* promoter binding, with the increased MafB protein levels activating *Glucagon*. A) Immunostaining of Min6 cells infected with adenoviruses expressing GFP or *Pdx1* siRNA illustrating how efficient reduction in Pdx1 protein (assessed by immunofluorescence and western blotting) greatly intensifies MafB and glucagon cellular staining while decreasing insulin staining. B) Schematic view of the two potential Pdx1 binding sites on the *MafB* promoter and *bona fide glucagon* G1 element (Wang et al., 2001). Core homeodomain-protein-binding sequences are designated in red, Pdx1 only bound to *MafB* Site 2 in gel shifts, and both endogenous Site 2 and G1 regions by ChIP. C) Results from EMSA using nuclear extracts (NE) from βTC3 cells showing Pdx1 binding to *MafB* Site 2. Competition with wild-type and mutant Site 2, as well as Pdx1 antibody (α-Pdx1) addition, analysis was performed. The location of the Pdx1:Site 2 and the supershifted (SS) complex is indicated. D) Pdx1 binds to the endogenous *MafB* Site 2 and G*lucagon G1* regions in Min6 cells. ChIP assays and corresponding qPCR reactions were performed in triplicate; control reactions were performed analyzing the IgG immunoprecipitation and *albumin* promoter binding. n≥3. E) Simultaneous knock down of Pdx1 and MafB reveals that MafB is required for *Glucagon* expression in Ins-1 cells. qPCR data from Ins-1 cells following individual or combined knock down of Pdx1 and MafB by siRNA. n ≥3. F) Overexpression of MafB reveals that MafB is sufficient for the induction of endogenous *glucagon* expression in Ins-1 β-cells, as shown by qPCR analysis of transcript levels from either control (Lenti-H2BCherry) or after MafB overexpression (Lenti-MafB-2ACherry). (Adapted with permission from Gao et al., 2014)

mobility shift assays were performed with βTC3 nuclear extracts to determine whether Pdx1 bound to either of these sites (Figure 12C). Antibody addition and wild type and mutant competitor oligo analysis indicated that Pdx1 bound only to *Site 2*. Furthermore, ChIP analysis in Min6 cells showed enrichment for Pdx1 only at the region surrounding *Site 2*, in addition to previously described sequences within the *G1* element of the *Glucagon* promoter associated with Pdx1-mediated repression (Figure12D). These experiments strongly suggest Pdx1 acts as a direct repressor of both *MafB* and *Glucagon* in mature β-cells.

Pdx1-Mediated Repression of MafB is Required for the Maintenance of β-cell Identity

To investigate the role of MafB expression in *Glucagon* activation following Pdx1-depletion from β-cells, we developed a dual siRNA knockdown strategy to simultaneously deplete both Pdx1 and MafB from Ins-1 cells (Figure12E,F). We found that partial attenuation of MafB induction resulted in a failure to activate *Glucagon* expression. These experiments suggest that in the absence of direct Pdx1 repression, reactivation of MafB is sufficient to stimulate *Glucagon* activation, and likely serves as the driving-force behind PKO cell adoption of an α-like identity.

Discussion

Highly complex and dynamic networks of transcription factors control cellular differentiation and the preservation of mature cell phenotypes. In recent years a number of studies have demonstrated the capacity of single factors to alter islet endocrine cell identity when missexpressed. In this study, using a conditional deletion and lineage tracing strategy we reveal a bifunctional role of Pdx1 in maintenance of mature β-cell identity (Figure 13). *Pdx1*-deleted β-cells rapidly lose β-cell identity markers, within days

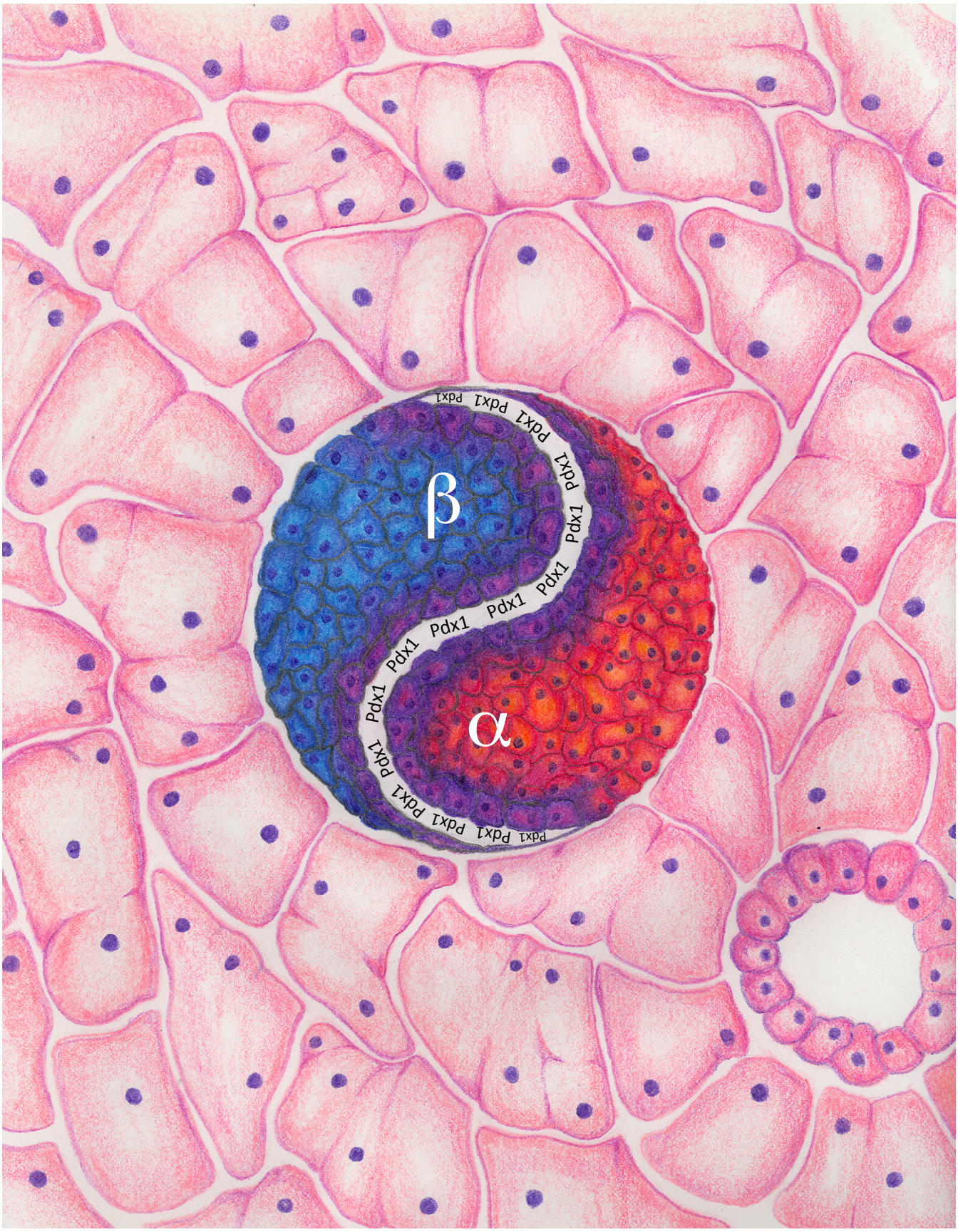

Figure 13. Graphical depiction off the fine balance between islet α- and β-cell identity maintained by Pdx1. (McKenna, 2014)

of *Pdx1* deletion. A large fraction of PKO β-cells were also found to express glucagon and MafB. Most notably, when comparing the global transcription profiles of FACS sorted α-, β- and PKO cells, the *Pdx1*-deleted cells exhibited significant overlap with α-cells, indicating robust reprogramming had occurred. The α-cell enriched *MafB* transcription factor was among the most significantly upregulated genes in PKO cells, and reducing MafB expression inhibited the induction of *Glucagon* expression. Our results strongly suggest that the direct repression of *MafB* by Pdx1 is essential to the maintenance of mature β-cell identity.

β-cell reprogramming to an α-like phenotype following *Pdx1* deletion was reminiscent of recent studies illustrating that deletion of *Dnmt1* from β-cells manifests β-to-α-like lineage conversion as consequence of hypomethylation and derepression of the α-cell determinant, *Arx* (Dhawan et al., 2011). Similarly, a Nkx2.2-repressor complex harboring Dnmt3a, Grg3, and Hdac1 was also found to suppress *Arx* expression, thus inhibiting Arx-mediated β-to-α-like reprogramming during β-cell specification (Papizan et al., 2011). Recent studies have also shown that isolated human β-cells also have the capacity to acquire α-cell features, which again hinged upon derepression of *ARX* (Spijker et al., 2013). Notably, the time course of transdifferentiation was much longer (weeks to months) in the above-mentioned mouse studies, including prolonged periods of intermediate stages of transdifferentiation depicted by cells expressing both α- and β-identity markers (MafA, Nkx6.1, insulin, glucagon). In contrast, α-cell features were rapidly acquired (within days) following *Pdx1* deletion, with only a brief window (five days) where transitional cell states depicted by expression of both α- and β-identity markers were observable. Furthermore, the prior studies examined only a handful of α- and β-cell identity markers, leaving the extent to which such changes in cell phenotype represent global changes in cell identity uncertain.

The in-depth analysis performed in our study revealed that PKO β-cells closely resemble *bona-fide* α-cells on the whole transcriptome level. Importantly, however, these cells lack expression of *Arx*. Because Arx is required to maintain mature α-cell identity, its absence may be the reason why PKO cells fail to achieve a fully functional α-cell phenotype (Courtney et al., 2013). We found that in the absence of direct Pdx1 repression, reactivated MafB was an important driver of α-cell gene expression. Confounding our results, however, are recent studies revealing that human β-cells maintain expression of MAFB into adulthood. Future studies investigating the role of MAFB in human α-cells and β-cells will be important for defining its contribution to transcriptional networks controlling cell identity.

The implications of Pdx1 control of mouse β-cell identity are echoed by a recent study that revealed the expression of PDX1 and a subset of other critical β-cell transcription factors (MAFA, NKX6.1) were selectively compromised in dysfunctional T2DM β-cells (Guo et al., 2013). This is further supported by a prior study indicating that rodent β-cell deletion of the *FoxO1* transcription factor results in hyperglycemia associated with a loss of Pdx1 and other β-cell identity markers. Similar to our study, β-cell apoptosis was not a significant contributor to the decline in β-cell mass in *FoxO1* mutant mice. Instead, *FoxO1*-deleted cells assumed one of two differentiation states upon physiological stress: that of other endocrine cells, particularly Glu^+ cells, or a dedifferentiated state discernible by reactivation of progenitor marker genes (i.e. *Neurogenin3, Nanog, Pou5f11,* and *Mycl1*) (Talchai et al., 2012). Importantly, human T2DM β-cells do not exhibit reactivation of the above mentioned progenitor marker genes. Instead, Guo et al. proposed that the observed inactivation of essential β-cell transcription factors (i.e. MAFA, PDX1, NKX6.1) likely renders these cells dysfunctional rather than dedifferentiated. These studies collectively highlight an important common

theme, as each reveals that loss of β-cell identity and/or function, rather than simply cell death, likely contributes to human T2DM pathogenesis. Notably, a recent report of a separate mouse model of stress-induced β-cell dedifferentiation revealed that insulin therapy allowed "exhausted", dedifferentiated β-cells to redifferentiate into mature insulin^{+} β-cells (Wang et al., 2014). Therefore, future studies aimed to investigate whether exhausted, dysfunctional human β-cells can be rescued to restore mature β-cell function will be of the upmost importance, as they will likely reveal new strategies for the treatment of T2DM. Drugs such as thiazolidinediones (TZDs) have been shown to not only sensitize peripheral tissues to insulin, but also have a major action to preserve β-cell function, which likely occurs due to a decreased demand for β-cell insulin production/secretion (DeFronzo, 2009). For this reason, many investigators feel that TZD treatment, rather than agents that further stimulate β-cell GSIS (i.e. metformin and sulphonylureas), should be used as a first line treatment strategy for T2DM.

CHAPTER III

IDENTIFICATION OF PDX1 TRANSCRIPTIONAL COREGULATORS IN ISLET β-CELLS: RECRUITMENT OF THE SWI/SNF CHROMATIN REMODELER DYNAMICALLY MODULATES ACTIVITY

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Introduction

Like all transcription factors, Pdx1 binds to specific *cis*-acting sequences within target gene control regions, often referred to as enhancer regions (Pasquali et al., 2014a). Transcriptional modulation is then imposed through dynamic changes in chromatin structure that cause the underlying DNA to become more or less accessible to RNA polymerase II. However, even transcription factors like Pdx1, which is required for nearly every step of pancreas development, lack the inherent chromatin-modifying properties and alone cannot modulate gene transcription effectively. Instead, cell-signaling events drive context-dependent protein-protein interactions between these DNA-binding factors and coregulators, which possess transcriptional modifying functions. For example, acute exposure of β-cell lines to glucose, the most important physiological effector of cell function *in vivo*, causes a rapid activator/repressor switch in Pdx1 activity on *Insulin* gene transcription. This involves the differential recruitment of histone acetyltransferases (Hat) coactivators at high, stimulating glucose concentrations, and histone deacetylase (Hdac) corepressors at low, inhibitory glucose levels (Mosley and Ozcan, 2004; Mosley et al., 2004b).

While there are over 250 transcriptional coregulators in mammalian cells, relatively few have been ascribed to Pdx1 specifically (Pcif1, p300, HDAC1/2, Set7/9,

and Bridge1 (Francis, 2005; Liu et al., 2004; Mosley and Ozcan, 2004; Qiu et al., 2002; Stanojevic et al., 2005)) or other islet-enriched transcription factors (Nkx2.2 (Grg3, Hdac1, Dnmt3a (Papizan et al., 2011)), Isl1 (Ldb1/2 (Hunter et al., 2013)), HNF1β (PCAF/CBP (Barbacci, 2004)), NeuroD1 (Bridge1, p300/CBP (Qiu et al., 1998; Thomas et al., 1999)). Significantly, essentially all of these transcription factor associations were made in studies using a small subset of candidate coregulators.

Here I have used an unbiased chemical cross-linking, antibody precipitation, and mass spectrometry strategy to identify endogenous Pdx1-binding proteins in β-cells. Although many new and interesting coregulatory factors were found using this in-cell cross-linking approach, we chose to specifically focus on investigating whether Swi/Snf chromatin remodeling complex recruitment was linked to the positive- and/or negative-acting control properties of Pdx1. My results strongly suggest that Pdx1 interacts with functionally distinct Swi/Snf complexes in a highly dynamic manner in islet β-cells. Hence, Swi/Snf complexes containing the core Brg1 ATPase subunit were demonstrated to be involved in Pdx1-mediated activation, while the Brm ATPase subunit containing complexes imposed transcriptional repression. Evidence is also presented indicating that physiological and pathophysiological conditions influence Pdx1 binding to these distinct complexes in β-cells *in vivo*, and I propose that the antagonistic actions of Brg1-Swi/Snf and Brm-Swi/Snf have significant implications to glucose homeostasis. My findings have identified many different possible coregulators of Pdx1 and shed light on how their recruitment and consequently transcription factor function is impacted normally and in the context of dysfunctional T2DM β-cells.

Materials and Methods

ReCLIP/MS

Mouse βTC-3 and human EndoC-βH1 cells were grown as defined earlier (Nagamatsu and Steiner, 1992; Ravassard et al., 2011a). The ReCLIP procedure was performed as described (Smith et al., 2011) with minor modifications. Briefly, fourteen 15 cm culture plates of βTC-3 cells at 70% confluency (~10^8 cells) were exposed to freshly made DSP (stock 20 mM in DMSO) diluted to a final concentration of 1 mM in phosphate-buffered saline (PBS) at pH 7.4 for 45 minutes at 37°C. Nuclear extract was prepared as described previously (Schreiber et al., 1989b), except that DTT was withheld from the extraction buffer. The nuclear extract was incubated with either goat α-Pdx1 (generated by Dr. Chris Wright (Vanderbilt University)) antibody or goat IgG (control) bound Protein G Dynabeads for 3 hours at 4°C, and then washed with RIPA buffer (100 mM NaCl, 1% Nonidet P40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris/HCl, pH 8.0, and 1 mM EDTA). Pdx1 binding partners were eluted with RIPA buffer supplemented with 200 mM DTT. Eluted proteins were analyzed via MudPIT (Multidimensional Protein Identification Technology) as previously described (Martinez et al., 2012) in the Vanderbilt University Medical Center Proteomics Core. MS analysis was performed on at least three independent ReCLIP preparations, with each showing similar enrichment of specific peptides.

Coimmunoprecipitations and Immunoblotting

βTC-3 nuclear extracts were incubated with either goat α-Pdx1 (generated by Dr. Chris Wright (Vanderbilt University)), rabbit α-Brg1 (Santa-Cruz H88-X), rabbit α-Tif1β (Abcam ab10483), rabbit α-Mi2β (Abcam, ab72418) antibody or species matched IgG (control) bound Protein G Dynabeads for 3 hours at 4°C, as described previously (Hunter et al.,

2013). The immunoprecipitates were fractionated by SDS/PAGE (NuPAGE 10% Bis-Tris gels), transferred on to PVDF membrane, and probed with the following antibodies: goat α-Pdx1 (1:10,000); rabbit α-Brg1 (Santa-Cruz H88-X, 1:5,000); rabbit α-Brm (Cell Signaling 6889S, 1:2,000); rabbit α-Tif1β (Abcam ab10483, 1:2,000); rabbit α-RBBP4 (Bethyl Laboratories A301-206A, 1:2,000); rabbit α-Mi2β (Abcam, ab72418, 1:2,000); rabbit α-Beta-Actin (Cell Signaling 4967S, 1:2,000); HRP-conjugated α-rabbit IgG (Promega 1:2,000), HRP-conjugated α-goat IgG (Santa-Cruz, 1:2,000). The experiments were performed at least 3 times and quantitated using NIH ImageJ software.

Sucrose Gradient Ultracentrifugation and Electrophoretic Mobility Shift Assay (EMSA)

Sucrose gradients were performed as previously described (Guo et al., 2010). Briefly, βTC-3 nuclear extract (1.5-2.5 mg) were separated over a 10-35% sucrose gradient (4.5 mL total volume). Fractions (300 µL each, excluding the first 500 µL) were analyzed by immunoblotting, mouse *MafA Region 3* element binding in electrophoretic mobility shift assays (Guo et al., 2010), and by immunoprecipitation.

Immunofluorescence

Tissue fixation, embedding, and immunofluorescence labeling were performed as previously described (Matsuoka et al., 2003). The primary antibodies used were goat α-Pdx1 (1:15,000), rabbit α-Brg1 (1:300), rabbit α-Brm (Abcam ab72418, 1:300), and guinea pig α-insulin (Dako A0546, 1:500). The secondary Cy2-, Cy3-, or Cy5-conjugated donkey α-rabbit, α-guinea pig, and α-goat IgGs were obtained from Jackson ImmunoResearch Laboratories. Nuclear counterstaining was performed using DAPI (Invitrogen). Immunofluorescence images were acquired by fluorescence microscopy using a Zeiss Axioimager M2 and images processed by NIH ImageJ software.

Proximity Ligation Assay (PLA)

Paraffin embedded human pancreas, mouse embryonic and adult pancreas samples were analyzed by procedures described by the PLA kit manufacturer (OLink Bioscience). Slides were de-waxed/rehydrated as previously described (Matsuoka et al., 2003). Heat antigen retrieval in 1X TEG (25 mM Tris HCl pH 8, 10mM EDTA, 50 mM glucose) was followed by three 10-minute 1xPBS washes. A 1% BSA/PBS blocking solution supplemented with 5% normal donkey serum was applied for 2 hours at room temperature. The following primary antibodies were incubated in a humidity chamber overnight at 4^{o}C: goat α-Pdx1 (1:15,000), rabbit α-Brg1 (1:300), rabbit α-Brm (Abcam ab72418, 1:300), mouse α-glucagon (Sigma g2654-.5mL, 1:4,000), and guinea pig α-insulin (1:500). Immunofluorescence images were acquired on a Zeiss Axioimager M2 (Zeiss) and processed for counting analysis using ImageJ (National Institutes of Health) software. The discrete fluorescent nuclear PLA spots were counted from at least three pancreas sections per experimental animal or human donor. The Gift of Hope Organ Procurement Organization in Chicago generously provided human pancreata, which were obtained from 3 normal and 3 T2DM de-identified cadaver donors: normal, 2 male and 1 female (59.3 ± 8.5 years [range: 51 to 68], BMI 22.6 ± 2.4 [range: 21.1 to 25.4]); T2DM, 2 male, 1 female, (57.0 ± 5.3 years [range: 51 to 61], BMI 25.9 ± 7.1 [range: 21.2 to 34].

Chromatin Immunoprecipitation Assays

Mouse Min6 β-cells (~4 × 10^6 cells) were 1% formaldehyde cross-linked, and the sonicated protein-DNA complexes isolated under conditions described previously (Gerrish et al., 2001). The fragmented chromatin was incubated with α-Pdx1, α-Brg1, α-Brm or species-matched IgG (Bethyl Laboratories), and the complexes isolated with

BSA- or herring sperm ssDNA (Abcam ab46666)-blocked protein-A Dynabeads (Invitrogen). Quantitative real-time PCR was performed using SYBR Green master mix in a Roche LightCycler 480II. The PCR primers were as follows: MafB -742 to -938 (site1): Forward: 5'-TTAGCGCAGACAGAGCTACCGAAA-3', Reverse: 5'-ATACTCTTTACACTCCCACCCTCG-3'; Glucagon +70 to -148 (contains G1 element): Forward: 5'-CGTAAAAAGCAGATGAGCAAAGTG-3', Reverse: 5'-GAACAGGTGTAGACAGAGGGAGTCC-3'; Pax4 -1841 to -1966 (contains β-cell-specific enhancer (Stein 2010 MCB)) Forward: 5'-CCAACGATCCAGGCTCTACATC-3', Reverse: 5'-CGGGTTTGGGGCTAATTGTCC-3'; Pdx1 -2471 to -2598 (contains Area I): Forward: 5'-TGGCTCGGGAAGGCTCTTG-3', Reverse: 5'-CCATCAGGTGGCTAAATCCATTATG-3'; IAPP -97 to -190: Forward: 5'-TCACCCACACAAAGGCACTCAG-3' Reverse: 5'-GGTTTCATTGGCAGATGGAGC-3'; MafA R3: Forward: 5'-CTGGAAGATCACCGCACA-3', Reverse: 5'-ATTTACCAAGCCCCAAACG-3'; Insulin (proximal promoter): Forward: 5'-GCCATCTGCTGACCTACCC-3', Reverse: 5'-CCCCTGGACTTTGCTGTTT-3'; Albumin -3164 to -3342 (distal TAAT-containing region): Forward: 5'-TGGGAAAACTGGGAAAACCATC-3', Reverse: 5'-CACTCTCACACATACACTCCTGCTG-3'. Experiments were performed with at least three independently isolated chromatin preparations.

siRNA Knockdown and RNA Analysis

The monolayer growth pattern of rat INS-1 cells makes them more suitable for siRNA transfection than mouse Min6 or βTC3 cells, which grow in overlapping "piles" of cells. Thus, INS-1 cells ($7.5x10^5$/well) were seeded into 6-well plates and targeting Brg1, Brm or scrambled control siRNAs (Dharmacon) introduced using Lipofectamine 2000 (Invitrogen). RNA and immunoblot protein studies were performed 72 hours following treatment. Cellular RNA was isolated using the QIAGEN RNeasy Mini Kit. cDNA was

prepare63d from RNA using the iScript cDNA synthesis kit (Bio-Rad) and quantitative real-time PCR performed using SYBR Green master mix and a Roche LightCycler 480II. The experimental data were normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) mRNA levels and relative changes calculated by the comparative DCt method (Chakrabarti et al., 2002).

Glucose Treatment Conditions

C57BL/6 mice (Charles River Laboratories) were fasted from 18:00 to 10:00 hours, and then blood glucose measurements were taken from the tail tip using an Aviva glucometer (Accu Chek). Glucose was administered to the 'high' group by intraperitoneal injection of a 20% glucose solution to achieve a final dose of 2 g/kg body weight. Glucose measurements were taken at 0, 15 and 30 minutes post-injection. Pancreata were harvested from each group and tissue embedded as previously described (Matsuoka et al., 2003).

Statistics

Data are expressed as the means ± SEM. P values were calculated with a Student's 2-tailed test. Results were considered significant at $P < 0.05$.

Study approval*:* The IRB at the University of Chicago and Vanderbilt University approved the use of human tissues in these studies. The Vanderbilt University IACUC approved all studies involving animals.

Results

Pdx1 Interacts with many Functionally Distinct Coregulatory Proteins in β-cells

An unbiased strategy utilizing reversible cross-linking, co-immunoprecipitation (ReCLIP (Smith et al., 2011)) and mass spectrometry was used to identify Pdx1-interacting proteins in βTC3 cells, an immortalized murine β-cell line (Figure 14A). Employment of the cell-permeable, lysine-reactive, thiol-cleavable, dithiobis(succinimidyl propionate) (DSP) cross-linking reagent allowed for stringent detergent-based washes, which led to a very clear difference in the proteins obtained from the Pdx1 antibody and control precipitates (Figure 14B, C). As anticipated, established Pdx1-interacting factors such as the MafA transcription factor (Zhao et al., 2005) and the Set7/9 coregulator (Francis, 2005) were found by immunoblot analysis after Pdx1 precipitation (Figure 14D). Additionally, we identified a number of novel candidate Pdx1 binding factors by mass spectrometry (Table 1, Appendix). Many of the candidates are likely linked with Pdx1 transcriptional regulation, while others may be associated with context-dependent recruitment in, for example, DNA repair, proliferation, and apoptosis (Babu et al., 2007; Lebrun et al., 2005).

Several of the transcriptional coregulators were of particular interest because of their potential ability to activate and/or repress Pdx1 *trans*-regulation (e.g. Tif1β, Swi/Snf and NuRD) (Flowers et al., 2009; Iyengar et al., 2011; Miccio et al., 2010). The binding of Tif1β, Swi/Snf and NuRD complex proteins to Pdx1 was independently confirmed in co-IP experiments performed with both mouse and human β-cell lines (Figure 15A). The authenticity of the interactions was further verified by the ability to precipitate Pdx1 with specific coregulator antibodies (Figure 15B). In addition, the roughly 42 kDa Pdx1 protein was found to migrate as a high molecular weight complex after sucrose gradient sedimentation of βTC3 nuclear extracts (Figure 15C), and to bind to Swi/Snf complex

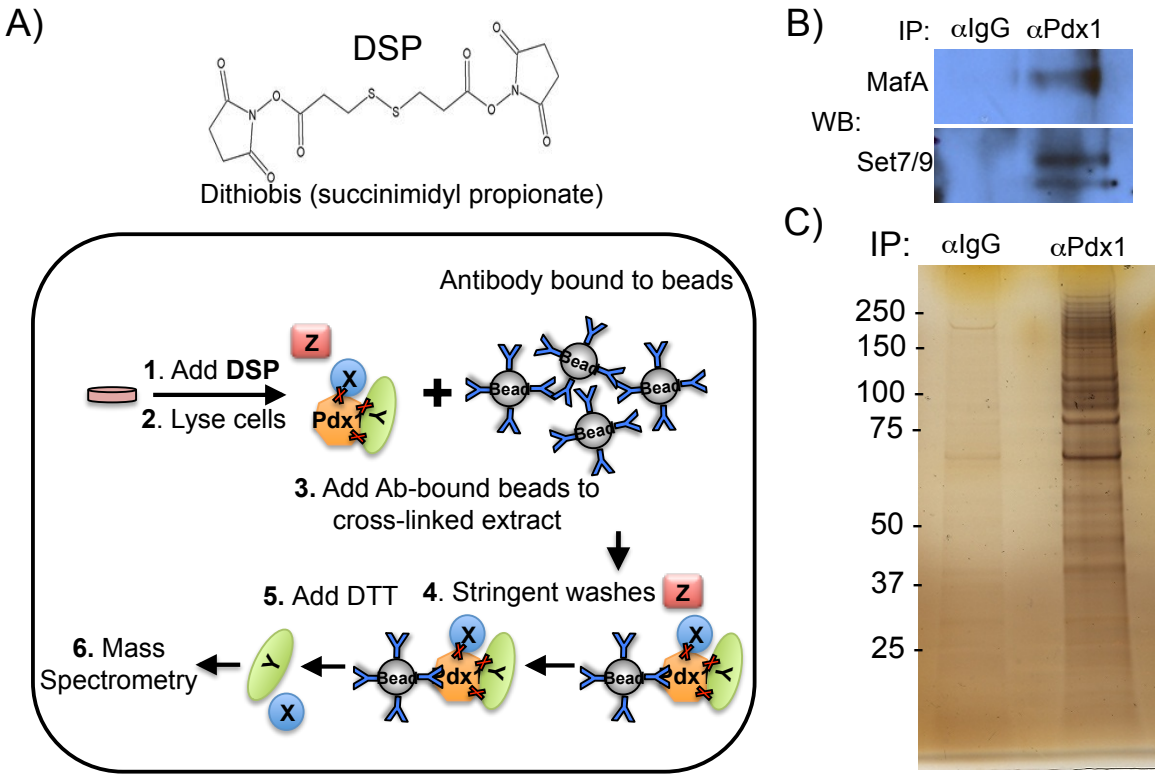

Figure 14. ReCLIP enriches for Pdx1 interacting proteins. A) Diagram of ReCLIP showing the lysine-reactive cross-linking reagent, DSP, that is first incubated with β-cells from which nuclear extract is generated for Pdx1 antibody or IgG immunoprecipitation. The control and α-Pdx1 bound complexes were washed with RIPA buffer and then RIPA+DTT to elute bound cross-linked proteins. The eluted proteins were B) immunoblotted with MafA or Set7/9 antibodies, C) SDS-PAGE separated or submitted for MS analysis (Table 1, Table S1). (Adapted with permission from McKenna et al., 2015)

Factor	% Coverage	Hits	
		IgG control	Pdx1 co-IP
NuRD complex components	RBBp4 = 9 HDAC1 = 4 Mi-2β = 2.5	RBBp4 = 1 HDAC1 = 0 Mi-2β = 0	RBBp4 = 10 HDAC1 = 5 Mi-2β = 7
SWI/SNF complex components	BAF47 = 11 BAF155 = 3 BAF57 = 9 BAF170 = 3 BRG1 = 1.6	BAF47 = 0 BAF155 = 0 BAF57 = 0 BAF170 = 0 BRG1 = 0	BAF47 = 1 BAF155 = 3 BAF57 = 2 BAF170 = 3 BRG-1 = 5
TIF1β	14	0	6

Table 1. Pdx1 bound chromatin remodeling complexes identified by ReCLIP/MS. The coregulator complex is shown as well as the MS results illustrating percent protein subunit coverage and the number of peptides found by IgG or Pdx1 antibody precipitation. These results are representative of data obtained from at least three independently performed IgG and Pdx1 antibody treatments. (Adapted with permission from McKenna et al., 2015)

subunits by co-IP analysis (Figure 15E). Moreover, Pdx1 within these high molecular weight fractions was shown to retain specific *cis*-control element binding properties in electrophoretic mobility shift assays (Figure 15D). Notably, few of the published Pdx1 coregulators and none of the interacting islet-enriched transcription factors were detected by mass spectrometry (Table 1, Appendix). We presume that these represent relatively rare complexes observed by more sensitive analytical methods (e.g. immune-based analysis). Significantly, these experiments identified a number of new, interesting interacting candidate proteins involved in Pdx1 action.

Pdx1 Interacts in the Developing and Adult Pancreas with Brg1 and Brm, the Catalytic ATPase Subunits of the Swi/Snf Complex

The contribution of Swi/Snf to Pdx1 activity was further investigated because of the prominent actions of this coregulator in developing neuron, cardiomyocyte and lymphocyte cell proliferation and lineage decisions (Chi et al., 2003; Lessard et al., 2007a; Lickert et al., 2004). Immunofluorescence analysis revealed that the ATPase subunits of Swi/Snf, Brg1 and Brm, were broadly expressed in the embryonic and adult pancreas, with no observable difference in protein level between cell types within each developmental stage (Figure 16A,B). Because of the unique and dynamic expression pattern of Pdx1 in the pancreas, this transcription factor would be coexpressed with Brm- and Brg1-Swi/Snf within multipotent pancreatic progenitor cells during early embryogenesis (i.e. embryonic day E12.5, (Offield et al., 1996)), and then principally in the insulin^{+} cells of developing (E15.5, E18.5) and adult islet β-cells that express relatively high levels of Pdx1 (termed Pdx1^{High} (Boyer et al., 2006)), and is expressed at low levels (Pdx1^{Low}) in acinar and δ-cells (Guz et al., 1995; Ohlsson et al., 1993).

The proximity ligation assay (PLA) was next used to evaluate Pdx1 interaction with Brg1- and Brm-associated complexes in the forming and adult pancreas. In this

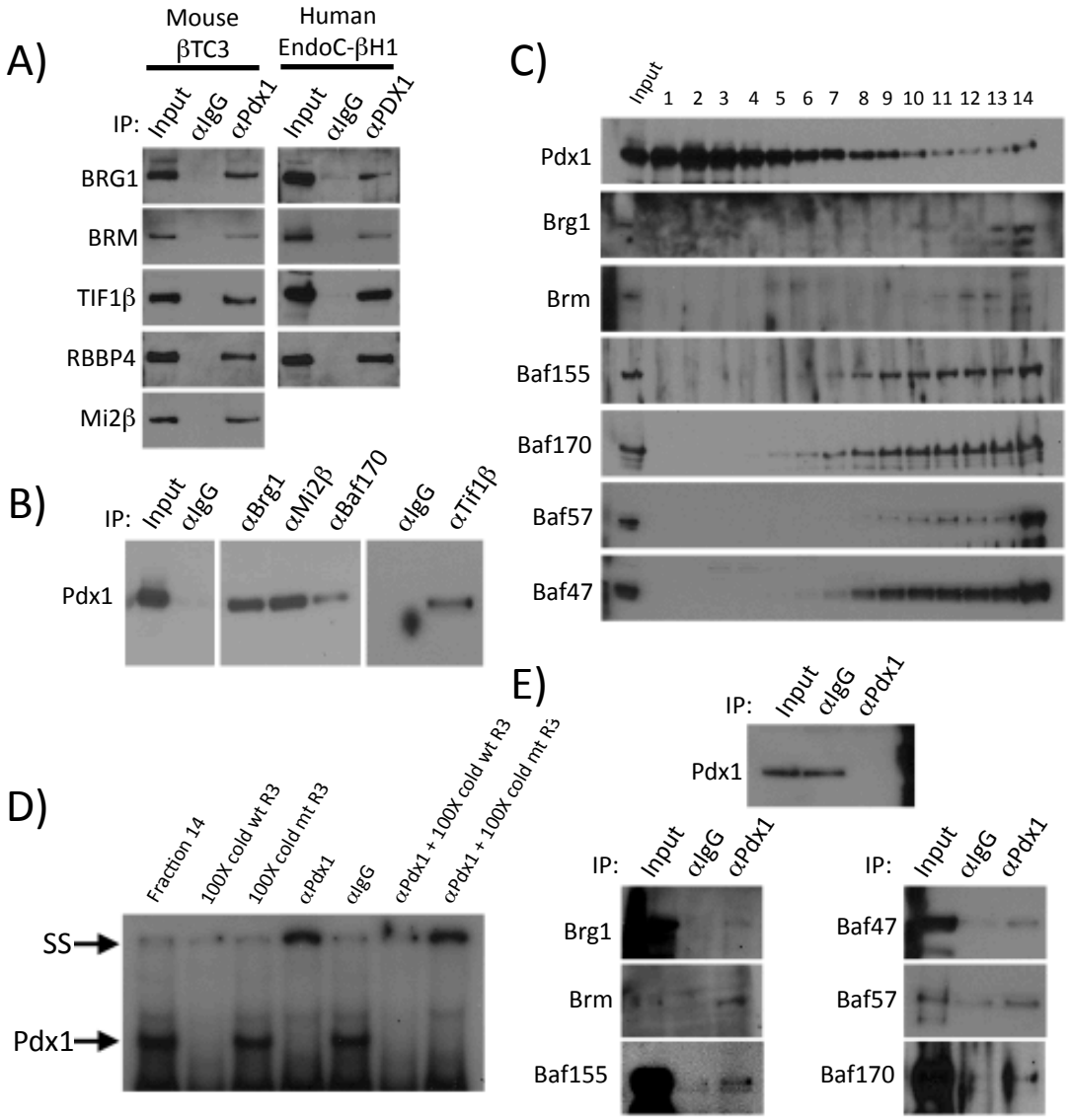

Figure 15. Proteins in NuRD, Tif1β and Swi/Snf immunoprecipitated with Pdx1 in mouse βTC3 and human EndoC-βH1 cells. A) Proteins of the Swi/Snf (Brg1, Brm), NuRD (Rbbp4, Mi2β) complexes, and Tif1β were enriched in Pdx1 antibody ReCLIP precipitations of βTC3 (left) and EndoC-βH1 (right) extracts. B) Reverse co-IPs illustrate Pdx1 enrichment in βTC3 nuclear extracts immunoprecipitated with Tif1β, Swi/Snf and NuRD coregulator subunit antibodies. C,D,E) Sucrose gradient sedimentation demonstrates that the Pdx1 C) migrating in the highest molecular weight fraction retains the ability to D) bind Pdx1 regulated MafA Region 3 (R3) control element DNA and E) coprecipitate with SWI/SNF complex subunits. N≥3 for each co-IP. (Adapted with permission from McKenna et al., 2015)

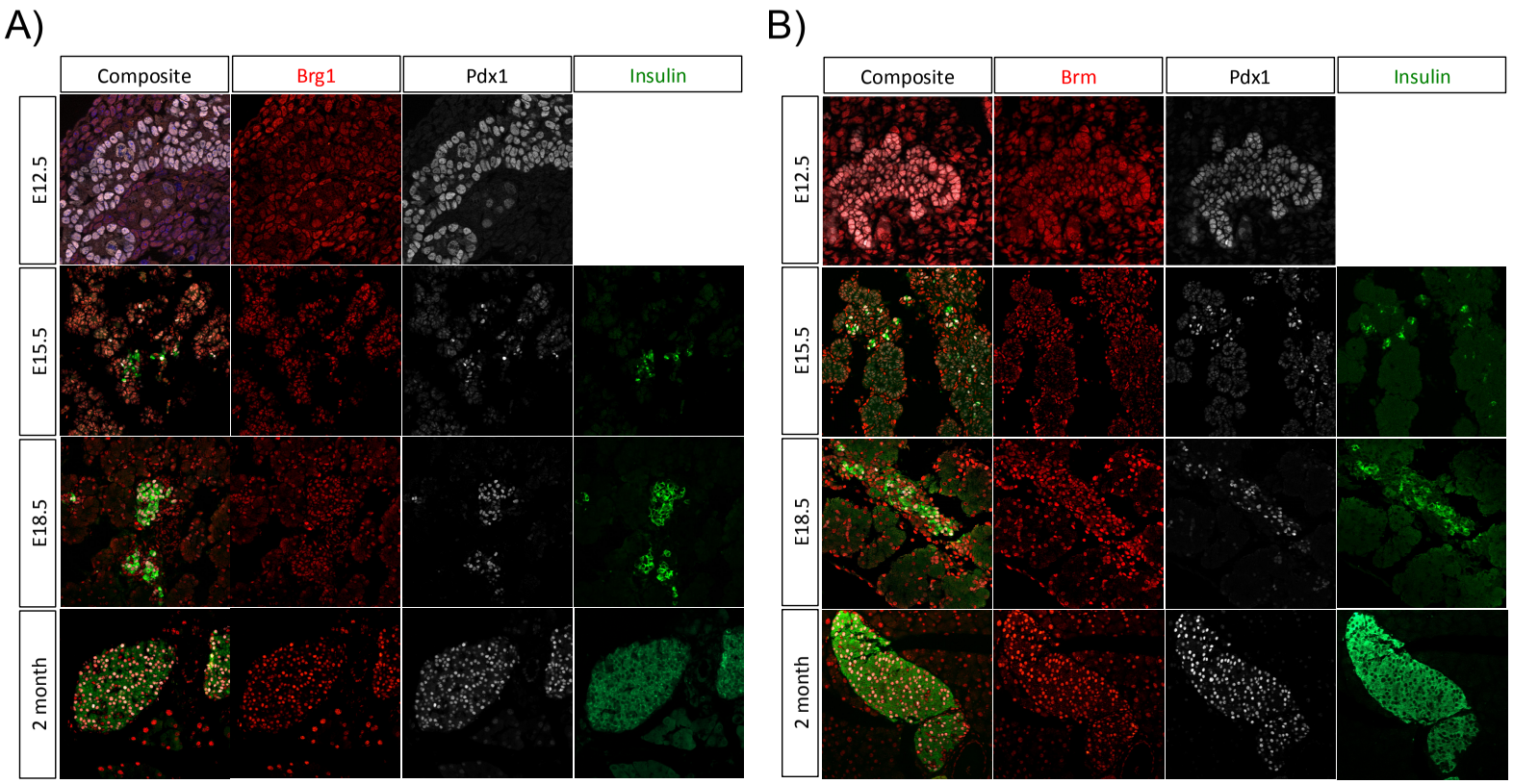

Figure 16. Brg1 and Brm are broadly expressed in the developing and adult mouse pancreas. Immunofluorescence analysis of A) Brg1, B) Brm, insulin, and Pdx1 in embryonic day (E) 12.5, 15.5, 18.5, and 2 month old mouse pancreas sections demonstrating Brg1 and Brm co-expression with Pdx1 in the embryonic and adult mouse pancreas. Scale bars indicate 20mM. (Adapted with permission from McKenna et al., 2015)

assay, a fluorescent signal is generated if Pdx1 is within 30-40 nm of these Swi/Snf proteins, a physical distance only producing a signal for neighboring proteins. However, the signals are not a reflection of the absolute quantity of Pdx1:Brg1-Swi/Snf or Pdx1:Brm1-Swi/Snf complexes in a cell due to antibody quality and/or other vagaries of the assay. Significantly, Pdx1:Brg1 and Pdx1:Brm signals were detected in nearly all $Pdx1^{+}$ pancreatic progenitors of the E12.5 pancreatic epithelium (Figure 17A,B). In contrast, no Brg1 or Brm PLA binding was observed using antibodies specific for Sox9 (Seymour et al., 2007) or Ptf1a (Krapp et al., 1998), other key transcription factors enriched in this multipotent progenitor population (data not shown). Notably, Pdx1:Brg1 and Pdx1:Brm signals became confined to $insulin^{+}$ cells later in development and in islets (Figure 17A,B). These experimental results demonstrate that Pdx1 interacts with the core catalytic subunits of Swi/Snf in the developing and adult mouse pancreas, which we conclude represents greater Swi/Snf complex recruitment. Furthermore, the progressive restriction of binding to $insulin^{+}$ cells supports the specific nature of these interactions, and implies functional relevance upon consideration of the many established roles of Swi/Snf in other cell types (Chi et al., 2003; Lessard et al., 2007a; Lickert et al., 2004).

Blood Glucose Concentration Dynamically Regulates Pdx1:Brg1 Levels in Mouse Islet β-Cells

We next asked whether Pdx1:Swi/Snf complex formation was regulated in a glucose-dependent manner *in vivo* by comparing PLA signal numbers in pancreata prepared from fasted mice with low blood glucose levels to those fasted then given an intraperitoneal injection of a high glucose solution (Figure 18A). Strikingly, the number of Pdx1:Brg1 complexes was significantly increased compared to fasted and ad-lib fed controls 30 minutes after glucose treatment, as shown quantitatively by the specific

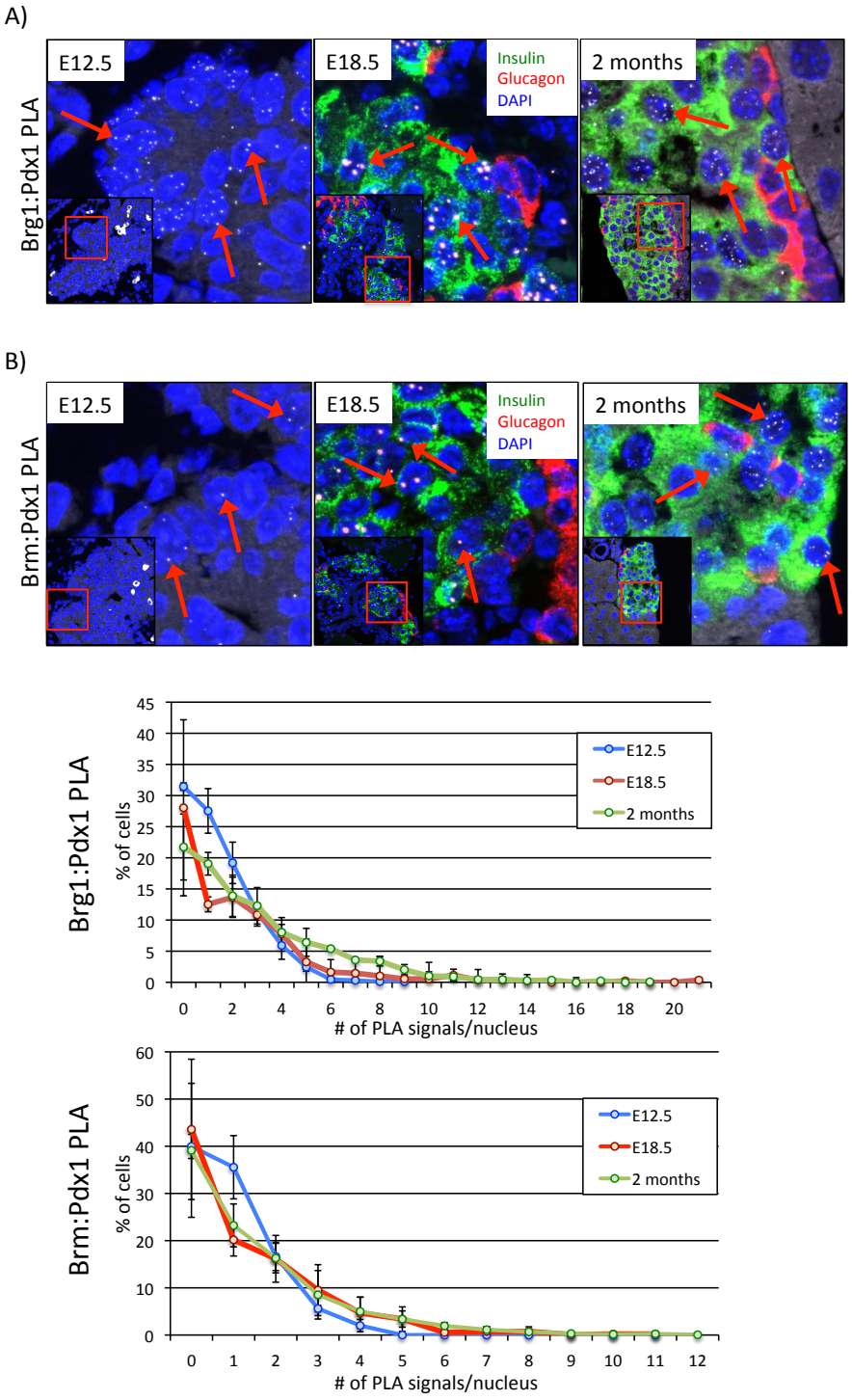

Figure 17. Pdx1 interacts with the Brg1-Swi/Snf and Brm-Swi/Snf complexes in developing and adult mouse pancreas. The PLA was performed with antibodies to Pdx1 and A) Brg1 or B) Brm in E12.5, E18.5, and 2 month old mouse pancreata. The wide cellular distribution of Pdx1:Brg1 or Pdx1:Brm signals reproduces their general distribution in E12.5 pancreatic epithelium, while enrichment at E18.5 and 2 months reflects $Pdx1^{High}$ levels in $insulin^{+}$ cells. Representative images are included for each time point; arrows indicate PLA signals and lower magnification insets are provided to orient each image. Scale bars indicate 10mM. Right panel in A, B) Graphical representations showing the distribution of the PLA signals per cell nucleus. E12.5 quantification includes all pancreatic epithelium, while E18.5 and 2 month quantification is of $insulin^{+}$ nuclei. $N \geq 3$, *$P<0.05$. (Adapted with permission from McKenna et al., 2015)

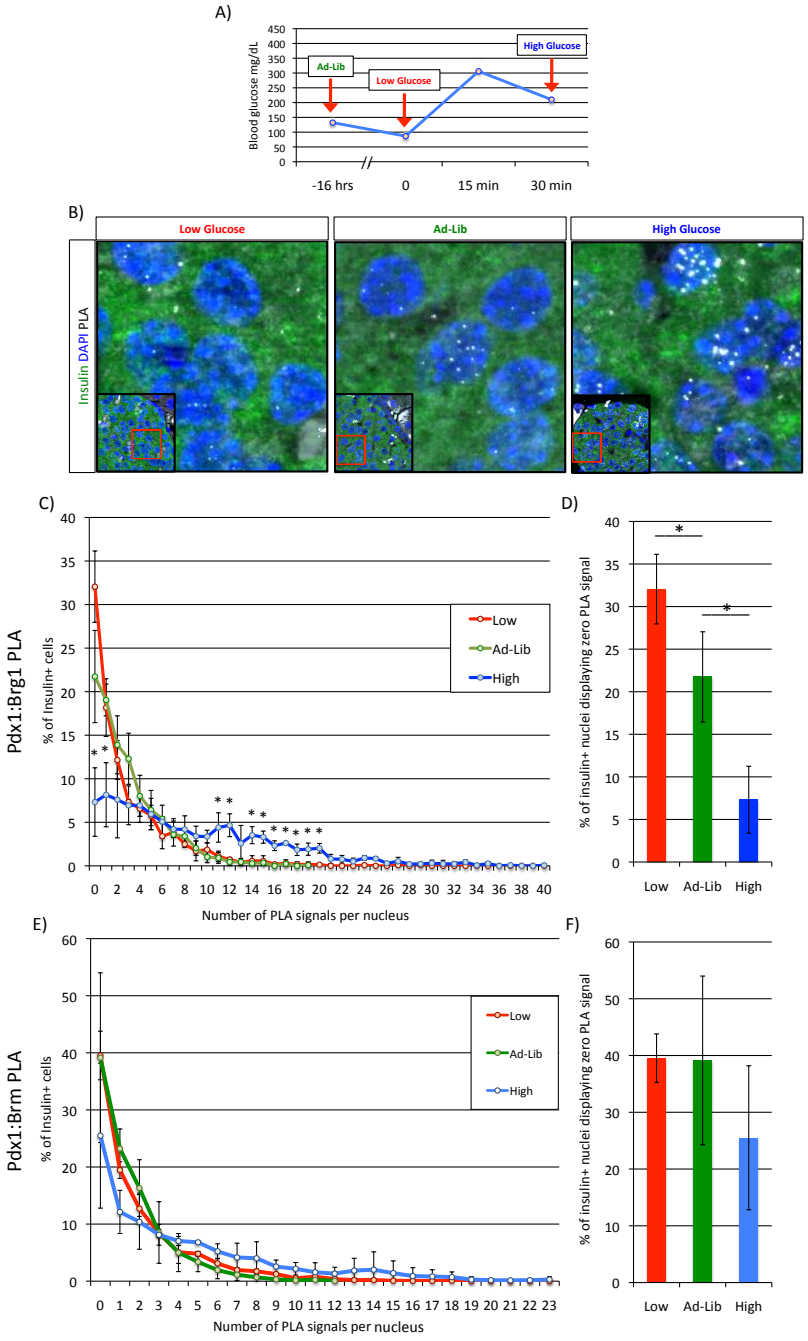

Figure 18. Pdx1:Brg1 complex formation is acutely regulated by blood glucose concentration *in vivo*. A) Diagram showing mouse blood glucose concentration and time of Ad-Lib (AL), Low glucose (16hr fast (LG)) and High glucose (16hr fast + glucose injection (HG)) conditions; arrows represent time of sacrifice and pancreas fixation. B) Pdx1:Brg1 PLA/immunofluorescence analysis of islet β-cell nuclei reveals that Pdx1:Brg1 signals become more abundant with increasing blood glucose concentration. Representative images are shown with Pdx1:Brg1 PLA signals appearing as white spots; lower magnification insets are provided to orient each image. Scale bars indicate 5mM. C) The distribution of Pdx1:Brg1 PLA signals in insulin^{+} cells under high glucose conditions were significantly greater than in low or Ad-Lib, whereas E) Pdx1:Brm PLA signals in this population were essentially unchanged. Islets from high glucose conditions possessed significantly fewer β-cells displaying zero detectible D) Pdx1:Brg1 signals than low or Ad-Lib conditions. This pattern was not observed for F) Pdx1:Brm between glucose conditions. N≥3, *P<0.05. (Adapted with permission from McKenna et al., 2015)

increase per β-cell nucleus after high glucose treatment (Figure 18B, C). Additionally, ad-lib fed and fasted mouse islets had roughly three- and five-fold more β-cells displaying zero detectible PLA signals than glucose-injected animals (Figure 18D). These data illustrate a strong, positive relationship between high glucose conditions that stimulate Pdx1 β-cell activity and Pdx1:Brg1 binding (Figure 18C). Supporting the specific nature of these interactions, no PLA signals were detected under these conditions between Pdx1 and Isl1 transcription factor coregulator, Ldb1 (Hunter et al., 2013) (Figure 19). Interestingly, relatively few of the data points were significantly different between low, ad-lib and high glucose conditions for Pdx1:Brm PLA (Figure 18E). In addition, the number of β-cells harboring zero detectible Pdx1:Brm PLA signals was not altered (Figure 18F). These results indicate that blood glucose signaling dynamically regulates Pdx1 and Brg1-Swi/Snf association.

Brg1 and Brm Play Opposing Roles in the Regulation of β-Cell Specific Genes

Brg1 or Brm are incorporated into Swi/Snf complexes in a mutually exclusive manner (Wang et al., 1996b). These two ATPases share ~74% amino acid sequence identity and have similar *in vitro* biochemical activity (Khavari et al., 1993; Phelan et al., 1999). Brg1 and Brm have the capacity to compensate for one another in heterozygous ATPase subunit mutant mice during development, although their combined gene dosage is critical to function (Bultman et al., 2000; Smith-Roe and Bultman, 2013; Willis et al., 2012a). Null mutants of nearly ubiquitously expressed Brg1 and Brm manifest different phenotypes *in vivo*. Thus, *Brg1*$^{-/-}$ mice die at the preimplantation stage of development (Bultman et al., 2000), while *Brm*$^{-/-}$ mice survive to adulthood with only a roughly 15% increase in body mass, which is attributed to greater bone density (Reyes et al., 1998). Intriguingly, these ATPases have distinct and antagonistic roles in human osteoblast formation, with depletion of BRG1 hindering differentiation and BRM accelerating

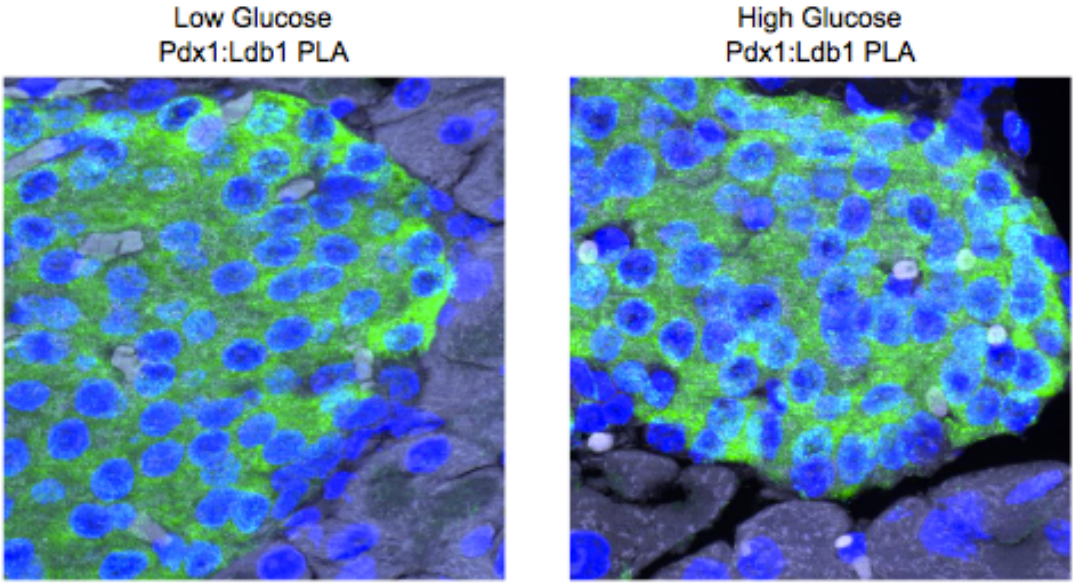

Figure 19. Pdx1 does not interact with the Ldb1 coregulator. No PLA signal was detected under low or high glucose conditions between Pdx1 and the Isl1 transcription factor coregulator, Ldb1. (Adapted with permission from McKenna et al., 2015)

(Flowers et al., 2009). BRM-SWI/SNF represses transcription of differentiation genes such as *osteocalcin* in osteoblast progenitors, at least in part through recruitment of the HDAC1 corepressor. In contrast, BRM and HDAC1 dissociate from the *osteocalcin* promoter during differentiation, allowing activating BRG1-specific complexes to initiate transcription. These experiments reveal how recruitment of BRG1 or BRM containing SWI/SNF complexes can produce different transcriptional outcomes.

Pdx1 both activates and represses gene transcription in islet β-cells (Gao et al., 2014a). Quantitative ChIP-qPCR assays were performed in mouse Min6 β-cells with Brg1 and Brm-specific antibodies to examine their pattern of recruitment to genes that Pdx1 either directly stimulates (i.e. β-cell-enriched *Insulin, MafA* and *Pdx1*) or inhibits (i.e. islet α-cell-enriched *MafB* and *Glucagon* (Artner et al., 2006; Gao et al., 2014a)). Brg1 was recruited to genes stimulated by Pdx1 in β-cells (Figure 20). However, antibody reagent limitations precluded our ability to detect Brm control region binding, as illustrated by our inability to even detect control, *bona fide* Brm binding sites, *E-Cadherin* or *CD44*, in HeLa cells using this assay (Figure 21).

Depletion of Pdx1 from either islet β-cells *in vivo* or β-cell lines causes a rapid loss in cell identity marker expression (e.g. MafA, Glut2, insulin), and activation of islet α-cell-specific genes (e.g. MafB, glucagon) (Gao et al., 2014a). The induction of islet α-cell-enriched MafB transcription factor expression was a driving force in *glucagon* transcription. These studies clearly illustrate the critical role of Pdx1 in maintaining β-cell identity. We therefore sought to investigate how depletion of Brg1 or Brm in rat INS-1 β-cells influenced Pdx1-mediated transcriptional control. Brg1 and Brm siRNA targeting decreased protein levels by roughly 65% (Figure 22A) and 55% (Figure 22C), respectively. As expected from the ChIP results, Brg1 knockdown resulted in a significant reduction in *Insulin, MafA* and *Glut2* transcript levels (Figure 22B). Interestingly, Brm depletion led to increased *Insulin, Glut2, Ucn3,* and *MafB* transcripts

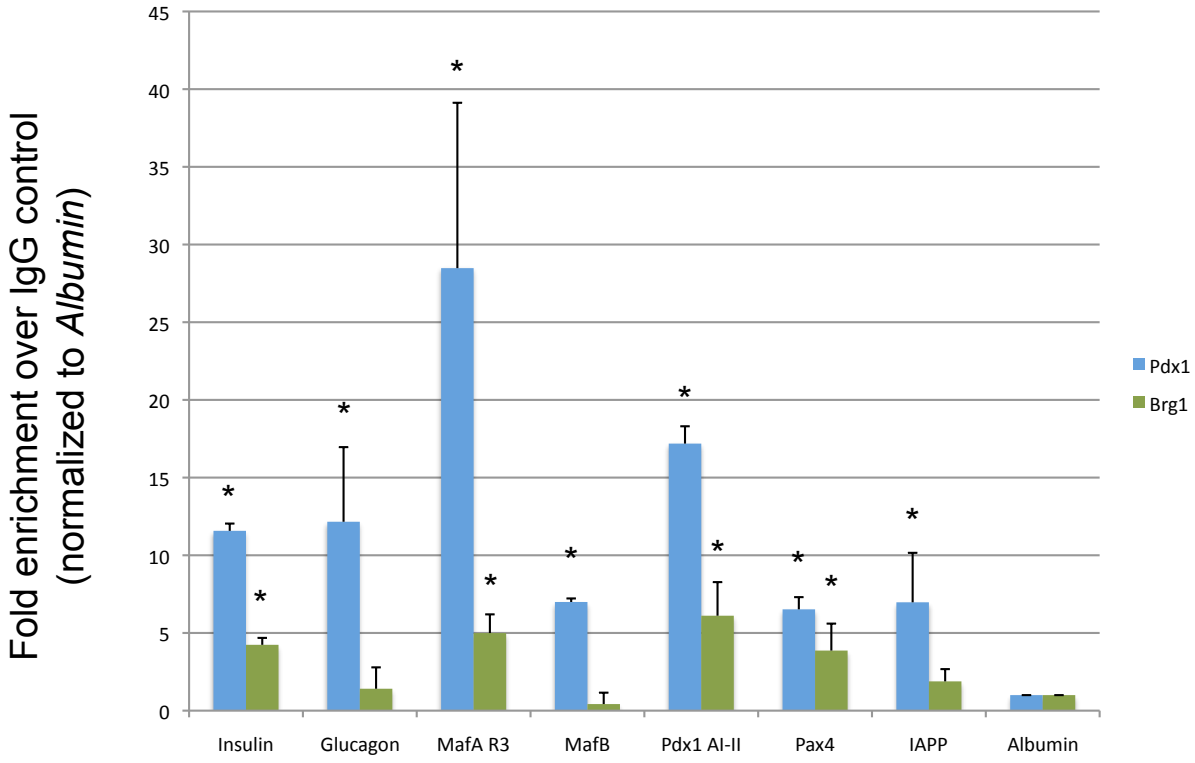

Figure 20. Brg1 only binds to Pdx1 activated genes in Min6 β-cells. ChIP assays illustrating Pdx1 and Brg1 genomic binding, with Brg1 only enriched at activated (*Insulin, MafA R3, Pdx1 AI/II, Pax4*) Pdx1 target genes in Min6 cells. N=4, *P<0.05 compared to IgG control. (Adapted with permission from McKenna et al., 2015)

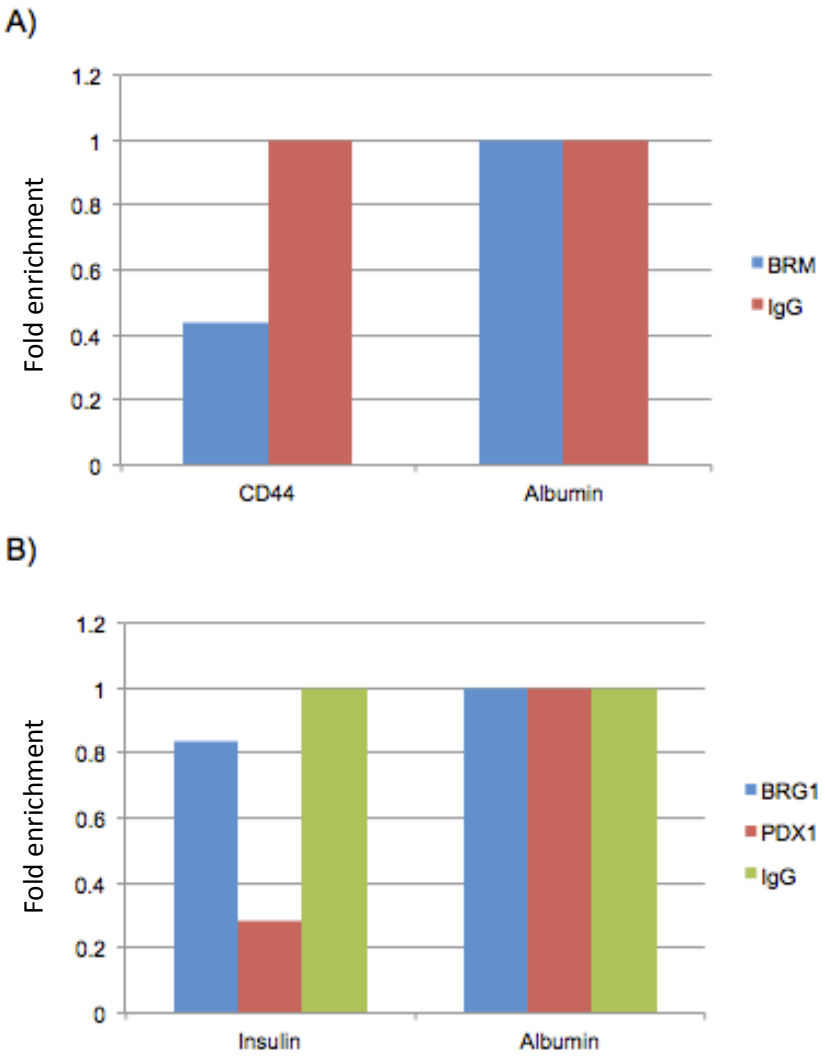

Figure 21. PDX1, BRG1 and BRM antibodies do not enrich for target gene sequences in HeLa cells. A) BRM antibodies failed to immunoprecipitate bona fide target sequences of the *CD44* promoter from HeLa cell chromatin (Banine et al., 2005). B) Neither PDX1 nor BRG1 antibodies immunoprecipitate target sequences of the *INSULIN* or *MAFA* (data not shown) promoters in BRG1+, PDX1- HeLa cells. N=2 for each ChIP. (Adapted with permission from McKenna et al., 2015)

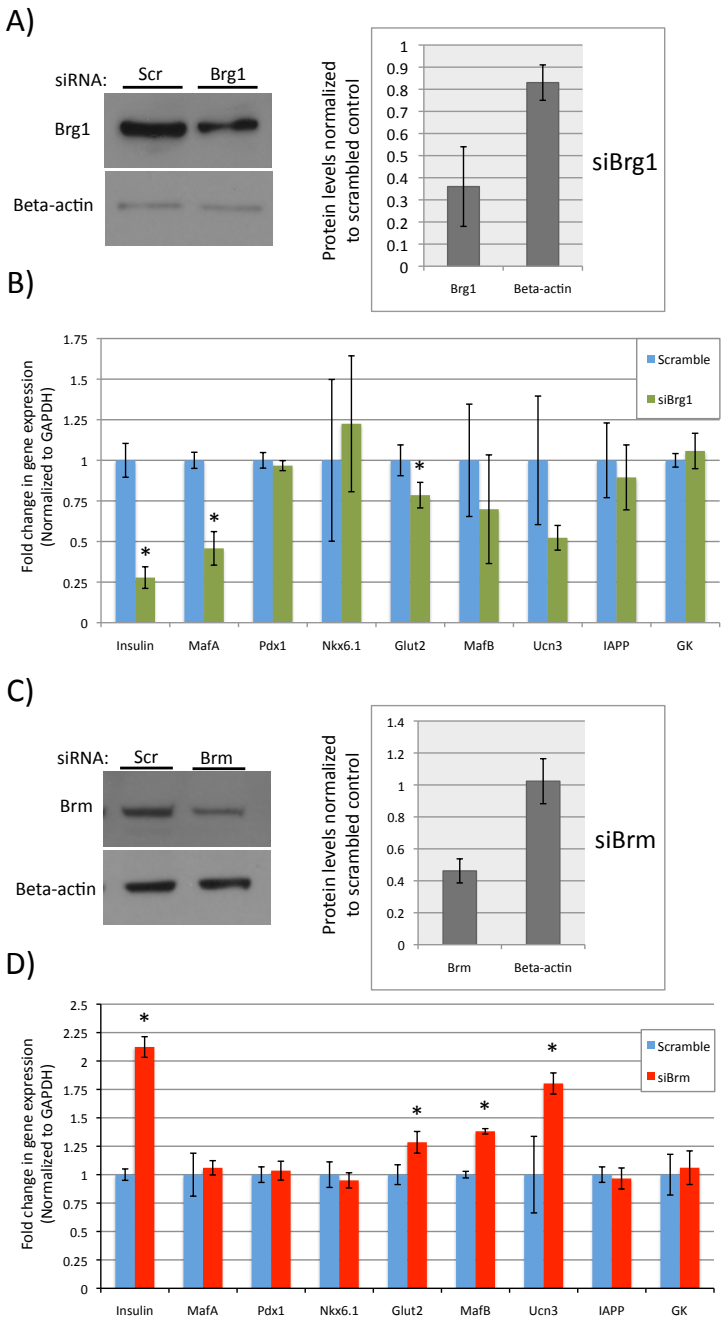

Figure 22. The Pdx1 recruited Brg1- and Brm-containing Swi/Snf complexes appear to play opposing regulatory roles in β-cells. Treatment with A) siBrg1 or C) siBrm effectively reduced protein levels over scrambled RNAs in Ins-1 cells as demonstrated by immunoblot and densitometry analysis. B) Expression of Pdx1 activated *Insulin, MafA* and *Glut2* were significantly compromised following Brg1 knockdown, while D) Brm knockdown had the opposite effect, causing up-regulation of *Insulin*, *Glut2* and non-Brg1 targets (*MafB, Ucn3*). The siRNA transfections were performed in triplicate. N=3, *P<0.05. (Adapted with permission from McKenna et al., 2015)

(Figure 22D). Our data strongly indicate that Brg1-Swi/Snf serves as a coactivator of Pdx1-mediated gene expression in islet β-cells, whereas Brm-Swi/Snf acts in a corepressive manner.

PDX1 Recruitment of BRG1-SWI/SNF is Compromised in Human T2DM Islet β-Cells

Reduced levels of a small subset of islet-enriched transcription factors contribute to T2DM β-cell dysfunction, with PDX1 among the specifically affected factors (Guo et al., 2013). We therefore sought to investigate whether alterations in PDX1:SWI/SNF complex formation could be contributing to diminished β-cell activity. PDX1 binding to BRG1 (Figure 23A) and BRM (Figure 23D) was readily detected in normal human islet β-cell nuclei. However, only insulin^{+} PDX1:BRG1 (Figure 23B) signal numbers were significantly diminished in age-, sex- and BMI-matched T2DM samples, and not PDX1:BRM (Figure 23E). The change in PDX1:BRG1 levels paralleled that of PDX1 in T2DM islet β-cells (Guo et al., 2013), while there is no apparent change in BRG1 or BRM levels compared to normal tissue (Figure 24C, D). In addition, there was a roughly two-fold increase in the number of β-cells with zero PLA signals in the PDX1:BRG1 T2DM samples (Figure 23C), even though PDX1:BRM levels were unaffected (Figure 23F). This suggests that the quantitative change in the amount of PDX1 protein affects BRG1-SWI/SNF coactivator binding in T2DM β-cells, and not the BRM-SWI/SNF corepressor. Collectively, these results strongly indicate that association of PDX1 to the SWI/SNF chromatin remodeler is dynamically regulated under both physiological and pathophysiological conditions in islet β-cells.

Discussion

Modulation of target gene expression by transcription factors is contingent upon their ability to recruit coregulator proteins, such as those with a capacity to modify

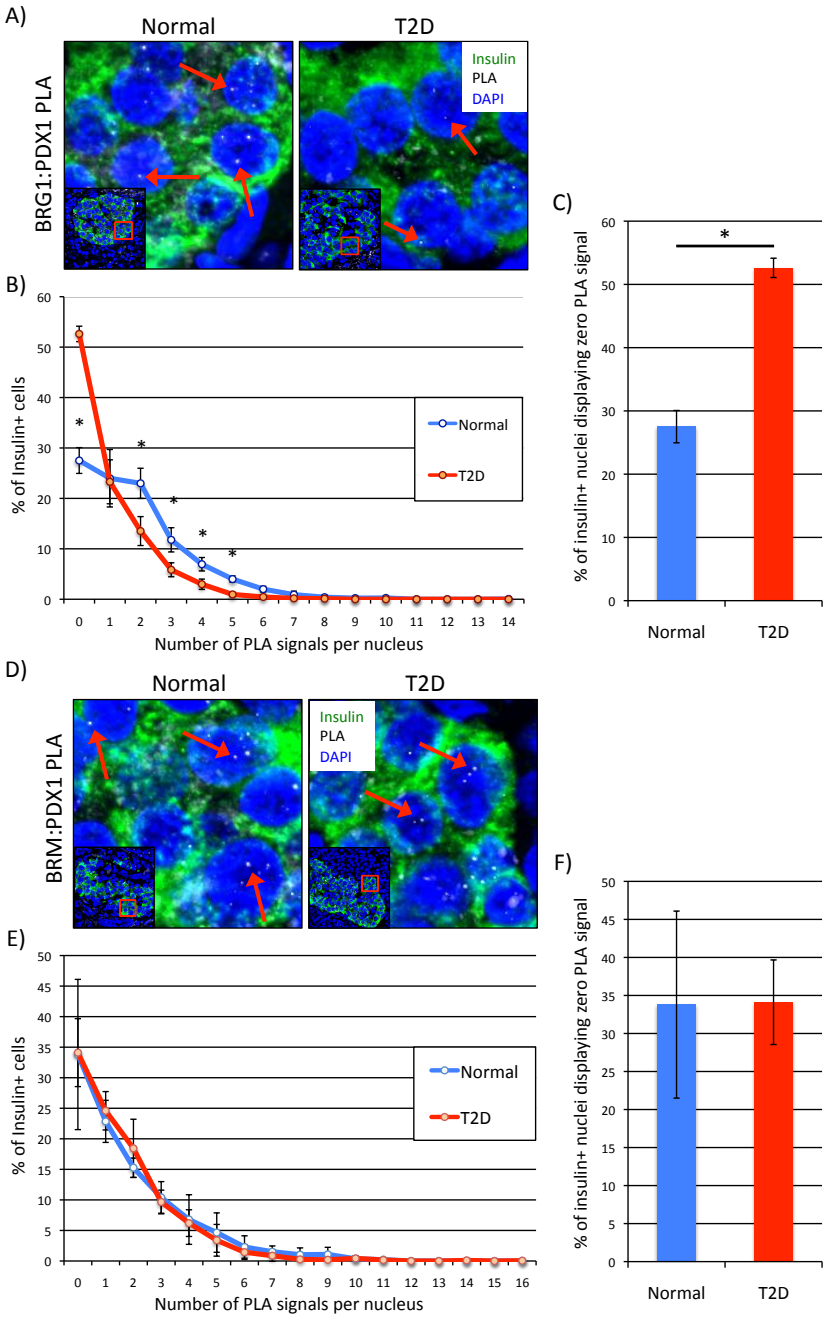

Figure 23. PDX1:BRG1-SWI/SNF activator levels are compromised in T2DM islet β-cells. Representative PLA signals for PDX1 and A) BRG1 or D) BRM in age-, sex- and BMI-matched normal and T2DM human pancreas sections; arrows indicate PLA signals and lower magnification insets are provided to orient each image. Scale bars indicate 5mM. B) PDX1:BRG1, but not E) PDX1:BRM signal levels were significantly attenuated in T2DM β-cell nuclei. C) T2DM islets contain two-fold more β-cells displaying no detectible PDX1:BRG1 signal than normal controls. No significant difference in PDX1:BRM PLA signal E) distribution per β-cell or F) number of β-cells presenting zero was observed between T2DM and normal human islets. Paraffin sections from three individual T2DM and normal donors were independently analyzed and quantitated. *P<0.05. (Adapted with permission from McKenna et al., 2015)

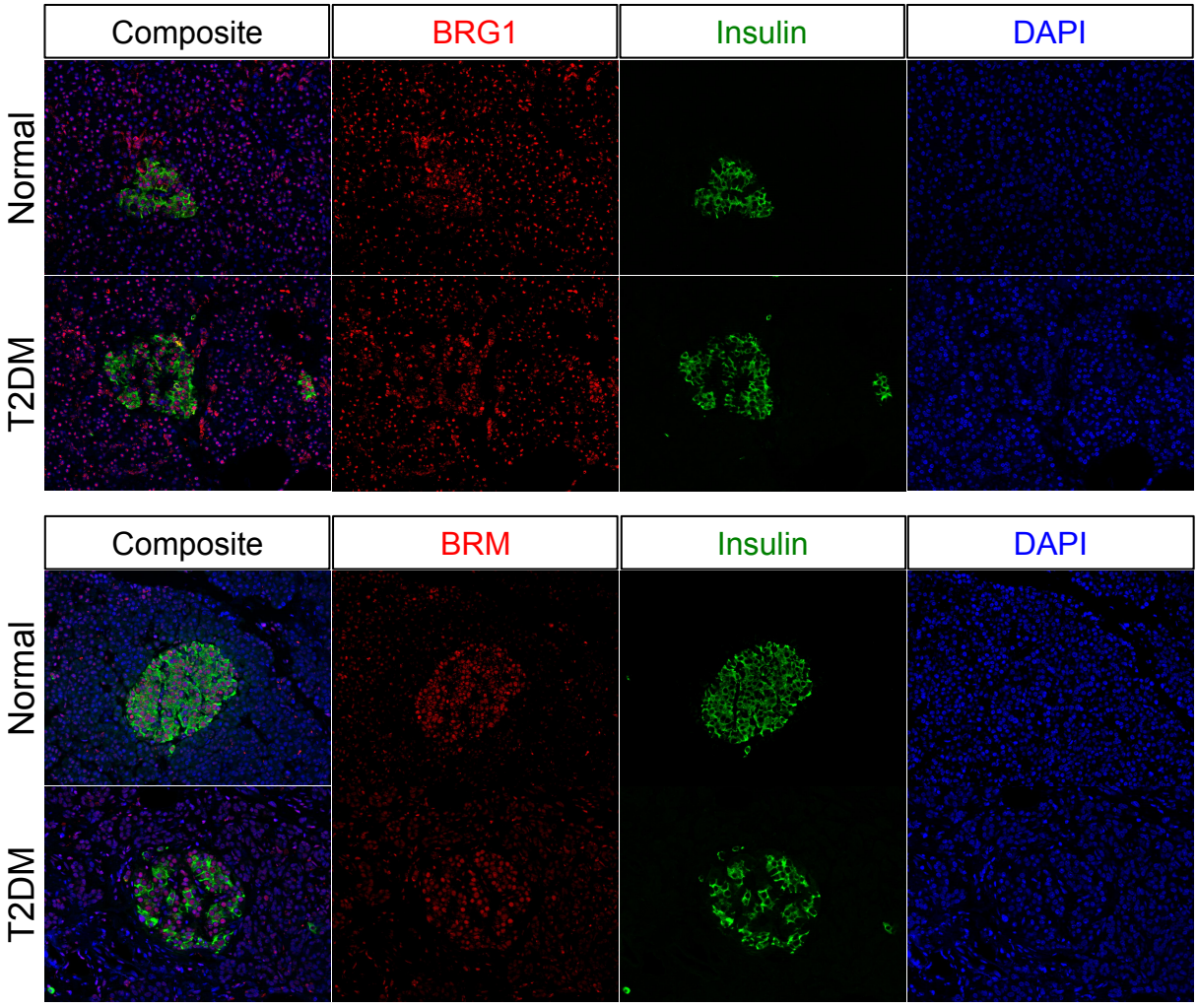

Figure 24. BRG1 and BRM are broadly expressed in the normal and T2DM human pancreas. Representative immunofluorescence staining analysis of insulin, BRG1 (top), and BRM (bottom) in human normal and T2DM pancreas paraffin sections. There was no obvious difference in BRG1 or BRM levels in normal and T2DM islets. Nuclei are counterstained with DAPI (blue). Scale bars indicate 20mM. (Adapted with permission from McKenna et al., 2015)

chromatin structure. Surprisingly, despite the fundamental importance of transcription factors such as Pdx1, MafB, Nkx2.2, and Nkx6.1 to islet β-cell development and function, there is a glaring deficiency in our knowledge of how coregulators influence these, and other islet enriched transcription factors. The goal of this study was to generate an impartial and comprehensive list of coregulators binding to Pdx1 in β-cells. Our ReCLIP/mass spectrometry screen returned a substantial number of candidate coregulators with a vast array of cellular functions, including several capable of mediating the positive and negative transcriptional actions linked to Pdx1 (e.g. TIF1β (Iyengar et al., 2011), DNA methyltransferase 1 (Dnmt1 (Dhawan et al., 2011)), NuRD (Miccio et al., 2010), and Swi/Snf (Flowers et al., 2009)) (Figure 14, Table 1, Appendix). We focused our analysis on determining the significance of the Pdx1:Swi/Snf complex in modulating β-cell activity. Our results strongly suggest that recruitment of Swi/Snf complexes harboring the Brg1 ATPase is essential for Pdx1 transactivation, while the Brm-Swi/Snf complexes mediate repression (Figure 25).

Brg1 and Brm were widely produced throughout the pancreatic epithelium and in the surrounding mesenchyme of the E12.5 pancreas (Figure 16A, B). PLA analyses revealed Pdx1 interacts with both ATPases in nearly all cells of the multipotent $Pdx1^+$ epithelium, but not in the surrounding mesenchyme where Pdx1 is not produced (Figure 17A, B). Notably, a hypoplastic pancreas phenotype was found in mice upon deletion of floxed *Brg1* early in pancreas development by *Ptf1a-Cre* (von Figura et al., 2014). Interestingly, all of the mature pancreatic cell types are formed and *Brg1* mutant animals are otherwise healthy, albeit final pancreas size was reduced by 50%. Because the number of MPCs limits pancreas size (Stanger et al., 2007), we propose that reduced Brg1 decreases $Pdx1^+$/$Ptf1a^+$/$Sox9^+$ progenitor numbers by affecting their proliferative capacity and/or health. Moreover, we further suggest that this is principally due to the actions of Pdx1 recruitment of Swi/Snf to MPC target genes, and not Ptf1a or Sox9, as

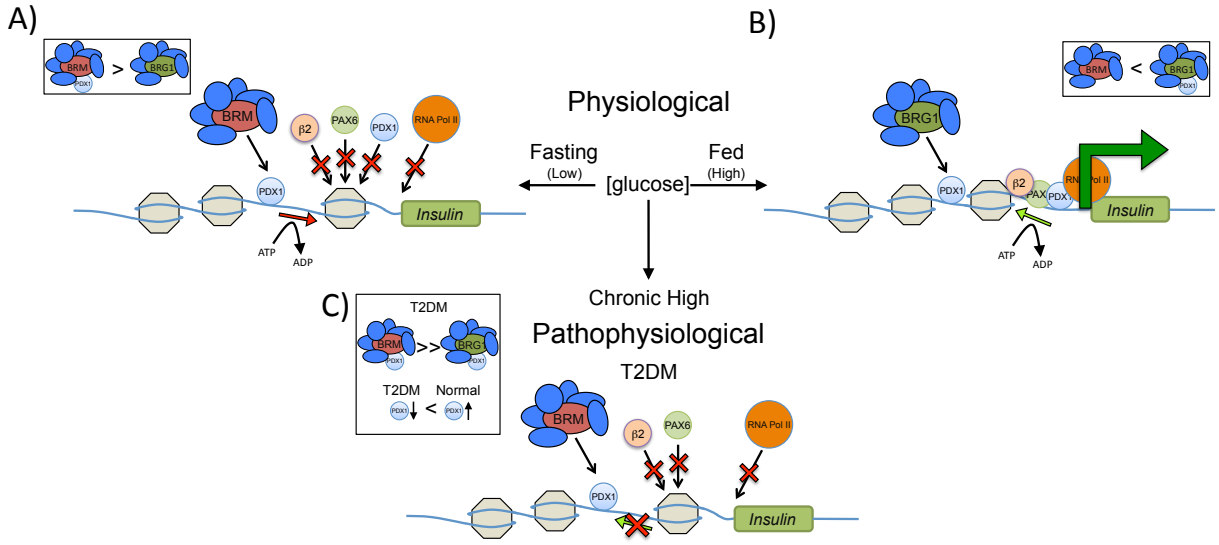

Figure 25. Schematic illustrating how physiological and pathophysiological conditions influence PDX1:SWI/SNF recruitment and *Insulin* gene expression. PDX1 (blue circle) differentially recruits BRG1- (green) or BRM-SWI/SNF (red) complexes to activate or repress target gene genes, respectively. A,B) In response to changes physiological glucose concentrations, PDX1 preferentially recruits BRM-SWI/SNF (inset) at low, fasting levels of glucose, which uses energy from ATP-hydrolysis to mobilize histones and reduce binding/activity of other *trans*-activators (and/or increase *trans*-repressor(s)), consequently reducing gene expression. B) In fed, high glucose stimulating conditions, selective binding of PDX1 to BRG1-SWI/SNF (inset) improves access of *trans*-activators and RNA Pol II to promote gene activation. C) The retention of PDX1:BRM binding over PDX-1:BRG1 in T2DM β-cells (Figure 6) strongly suggests that PDX1 target genes are negatively regulated by BRM-SWI/SNF under the pathophysiological conditions. (Adapted with permission from McKenna et al., 2015)

neither associated with Swi/Snf in this cell population. It will be interesting to determine if Brm-Swi/Snf compensates for the loss of Brg1 in this context and how this coregulator influences Pdx1 action in adult islet β-cells.

In contrast to the extensive interactions of Pdx1:Swi/Snf within the pancreas MPC population, complex formation became restricted to the insulin^{+} cells produced later in development and in islet β-cells (Figure 17A,B). However, as islet somatostatin hormone^{+} δ-cells and acinar cells produce low levels of Pdx1, these results indicate some change within Pdx1 (potentially a post-translational modification) or simply the higher levels in β-cells allows specific recruitment of widely distributed Brg1-Swi/Snf and Brm-Swi/Snf. Strikingly, knockdown of Brg1 and Brm in β-cell lines revealed that Brg1 was important for coactivation of Pdx1 target genes (*Insulin, MafA, Glut2*), and Brm corepression (*Insulin, Glut2, MafB, Ucn3*) (Figure 22A-D). The acute conditions of our analysis presumably enabled detection of this regulatory pattern despite only a 50% reduction in ATPase subunit levels, and not compensation by the unaffected coregulator that has been observed *in vivo* (Bultman et al., 2000; Smith-Roe and Bultman, 2013). Furthermore, Brg1 was found to directly bind within Pdx1 binding control element containing regions of these activated genes in ChIP assays (Figure 20) (Bultman et al., 2000; Smith-Roe and Bultman, 2013; Willis et al., 2012a).

We next examined if Pdx1 binding to Brg1-Swi/Snf and Brm-Swi/Snf was acutely regulated by changes in blood glucose levels *in vivo*, specifically examining low (fasting) to high (fed) conditions that influence Pdx1 activity in β-cell lines. For example, *Insulin* gene transcription is enhanced by recruitment of the p300 coactivator, which catalyzes histone H4 hyperacetylation within the proximal promoter region that correlates with gene activation (Mosley et al., 2004b). Conversely, Hdac1/2 recruitment by Pdx1 at non-stimulating, low glucose levels inhibits transcription (Mosley and Ozcan, 2004). Similarly, Pdx1:Brg1-activator complex formation was rapidly and significantly increased in islet β-

cells by elevated blood glucose levels, while Pdx1:Brm-repressor binding was unaffected (Figure 18A-F). This was observed under circumstances where nuclear Pdx1, Brg1 and Brm immunofluorescence appears unaffected by changing glucose levels (Figure 26). We conclude that high glucose concentrations amplified Pdx1:Brg1-Swi/Snf activator complex formation over Pdx1:Brm-Swi/Snf, causing transcriptional activation by directly influencing nucleosome occupancy and phasing in an ATP-dependent manner (Figure 25A,B). This represents a distinct mechanism from Pdx1 recruited p300 gene activation described above. Collectively, these results highlight how context-dependent recruitment of physiologically regulated and compositionally distinct Swi/Snf complexes dictate Pdx1 action and β-cell function.

Peripheral insulin resistance and islet β-cell dysfunction are the fundamental causes of T2DM, and symptoms often present slowly, after many years of progressive loss of β-cell function and metabolic control. The initial response to peripheral insulin resistance is augmentation of Islet β-cell mass and function (Talchai et al., 2009). However, the increasing metabolic demand imposed on β-cells is strongly linked to their accumulation of destructive stress molecules, such as reactive oxygen species. The resulting oxidative stress conditions induce deleterious posttranslational modifications on proteins, affecting activity, stability and subcellular localization. Indeed, a selective decline in transcription factors central to glucose sensing and insulin secretion is observed in T2DM islet β-cells (i.e. MAFA, MAFB, PDX1, and NKX6.1) (Guo et al., 2013). Notably, MAFA and MAFB are highly susceptible to oxidative stress and their rapid loss is proposed to dictate first phase insulin secretion defects early in disease progression. Persistent insults to PDX1 and/or NKX6.1, however, cause a more prolonged decline (years to decades) in activity, ultimately resulting in overt diabetes manifesting from severe β-cell dysfunction.

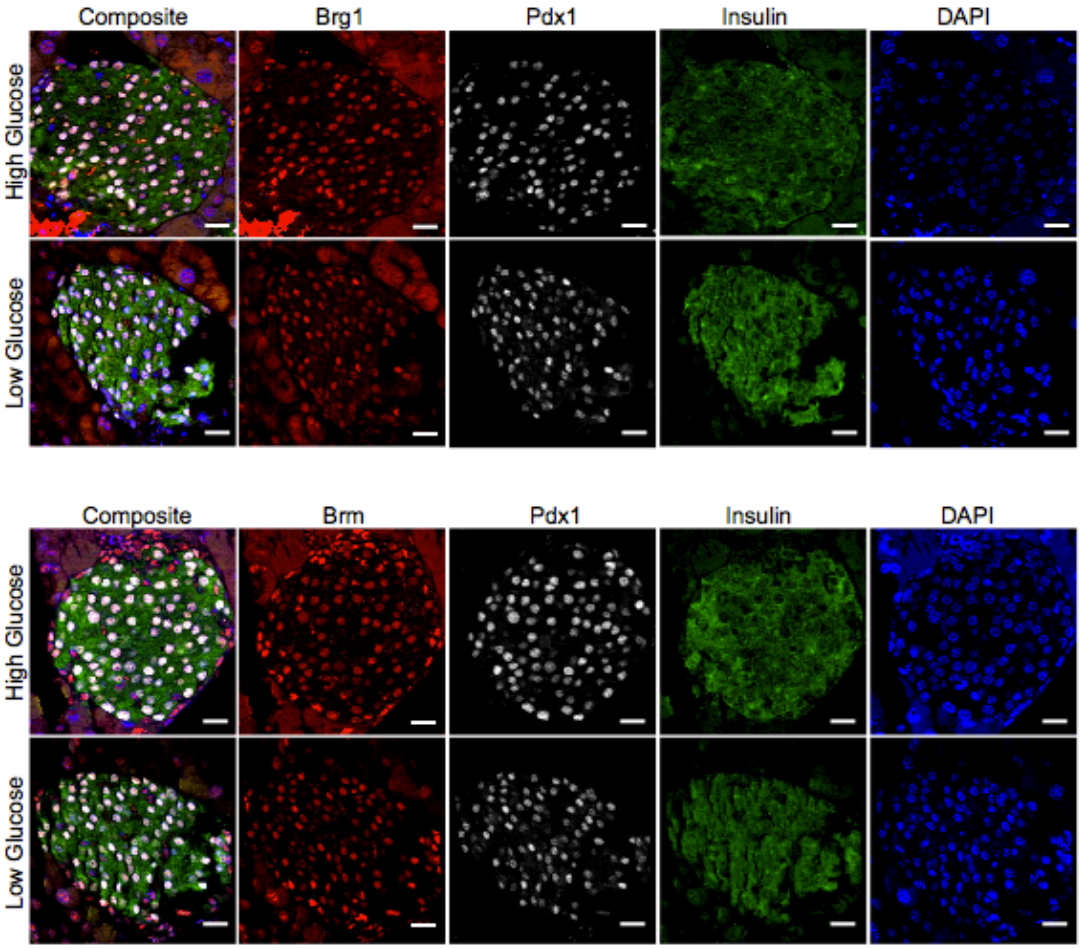

Figure 26. Nuclear Pdx1, Brg1 and Brm protein levels appear unaffected by blood glucose concentration *in vivo*. Immunofluorescence analysis of mouse islet β-cell nuclei reveals that Pdx1, Brg1 (top) and Brm (bottom) protein content is not altered in either low glucose (16hr fast) or high glucose (16hr fast + glucose injection) conditions. Scale bar corresponds to 20μM. (Adapted with permission from McKenna et al., 2015)

Although it remains unclear how these T2DM associated transcription factors are rendered inoperative *in vivo*, we speculate that pathophysiological conditions affect coregulator binding, and consequentially, transcription factor function. Significantly, coregulator catalyzed changes in epigenetic DNA methylation and histone modifications are found in T2DM islets, which influences promoter and enhancer structures driving islet-specific gene expression (Dayeh et al., 2014; Parker et al., 2013). In addition, our PLA results illustrated that PDX1:BRG1-activator binding was diminished in T2DM β-cells, while PDX1:BRM-repressor complex formation was unaffected (Figure 23A-F). Presumably, decreased PDX1:BRG1-activator formation simply reflects the diminished levels of PDX1 protein in T2DM β-cells (Guo et al., 2013), while how BRM binding to PDX1 is selectively retained is unclear. This phenomenon could potentially be explained by PDX1 having higher affinity for BRM-SWI/SNF, and thus, when PDX1 levels decline in T2DM, BRM-SWI/SNF outcompetes BRG1-SWI/SNF for the remaining PDX1. Alternatively, the disease-stress conditions T2DM β-cells encounter may selectively disrupt the signaling pathways required for PDX1:BRG1 association. Posttranslational modifications of PDX1 and/or SWI/SNF subunits likely influence their interactions, since, for example, Pdx1 transcriptional activity (i.e. Hdac1/2 and p300 recruitment (Mosley and Ozcan, 2004; Mosley et al., 2004b)) and protein destruction (Pcif1-Cul3 (Claiborn et al., 2010)) are impacted by such events. We propose that PDX1:BRM-SWI/SNF activity contributes to the loss in T2DM β-cell function (Figure 25). Moreover, conditions affecting PDX1 coactivator and corepressor recruitment occur relatively soon after exposure to insulin resistance in the context of T2DM disease process, and well before overt transcription factor loss.

Overall, our results clearly demonstrate the prominent role of Swi/Snf in regulating Pdx1 activity in β-cells. Furthermore, we have uncovered that Pdx1 recruitment of Brg1-Swi/Snf and Brm-Swi/Snf is influenced by physiological and

pathophysiological settings, which are hypothesized to have significant implications on transcription factor activity and β-cell function. In addition, this study illustrates how a ReCLIP/mass spectrometry strategy can be used to identify coregulators of other islet-enriched transcription factors. Such knowledge will provide valuable mechanistic insight into how these transcription factors regulate islet cell formation, function, and survival.

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

Thesis Summary and Significance

Over the past two decades, studies performed on transcription factors have greatly advanced our knowledge of pancreas development and mature β-cell function. Through this work, Pdx1 has been identified as a master transcriptional regulator of both the aforementioned processes.

Results presented in this dissertation revealed exciting new insight into the contribution of Pdx1 to the maintenance of mature β-cell identity. Consistent with its ability to recruit both coactivator and corepressor proteins, we found that Pdx1 was bifunctional; specifically, Pdx1 1) activates β-cell specific genes and 2) represses the α-cell transcriptional program. Microarray, ultrastructural and physiological analyses indicate that *Pdx1*-deleted β-cells rapidly lose β-cell features, while simultaneously assuming derepressing several aspects of α-cell character. ChIP assays were used to demonstrate that Pdx1 directly represses both α-cell enriched *MafB* transcription factor and *Glucagon* gene expression in β-cells. In the absence of Pdx1, derepression of *MafB* was found to be the driving-force behind the α-cell gene expression and the depletion of both MafB and Pdx1 inhibited the up-regulation of *Glucagon*. Collectively, our results revealed that both the activating and repressive actions of Pdx1 are required to uphold mature β-cell function and identity.

Because of its profound role in pancreas development and mature β-cell function, it is not surprising that Pdx1 has the capacity to both activate and repress target gene expression. Previous studies indicate these functions are attributed to Pdx1 by

recruitment of a seemingly small number of coactivators (e.g. p300, Set7/9, Bridg-1) and corepressors (e.g. Hdac1/2). Thus, the aim of my thesis work was to expand our understanding of how Pdx1 mediates its actions by identifying and characterizing additional Pdx1 coregulator proteins.

The ReCLIP/mass spectrometry protein isolation/identification strategy was implemented to identify Pdx1-interacting proteins in β-cells. Experiments from Chapter III identified proteins possessing a wide range of cellular functions, including, but not limited to, coregulators associated with transcriptional activation/repression, DNA damage response, and DNA replication. Because of its role in controlling developmental and adult cellular events, our analysis focused on the capacity of the Swi/Snf chromatin remodeler to mediate Pdx1 action. Knockdown of the two mutually exclusive core enzymatic ATPase subunits, Brg1 or Brm, differentially affected Pdx1-target gene expression in β-cells. Thus, Brg1 functioned in activation of many glucose-stimulated target genes (e.g. *Insulin*, *MafA* and *Glut2*), and Brm repression (*Insulin*, *Glut2, Ucn3* and *MafB*). Pdx1:Swi/Snf complex formation was also regulated by physiological and pathophysiological conditions in islet β-cells. For example, only Pdx1:Brg1 binding scored by proximity ligation assays was increased upon acute elevation of blood glucose levels in mice, circumstances stimulating Pdx1 activity and increased β-cell function. Moreover, both PDX1:BRG1 and PDX1 levels were decreased in dysfunctional β-cells within human type 2 diabetic (T2DM) islets, whereas PDX1:BRM corepressor formation was unaffected. My thesis work identified a significant number of candidate Pdx1 coregulators with potential functional importance, and thus, illustrates how the ReCLIP/mass spectrometry approach can be applied to identify coregulators utilized by other islet-enriched transcription factors. Furthermore, my analysis of Swi/Snf complexes provides novel mechanistic insight linking the Swi/Snf ATP-dependent chromatin

remodeling complex to Pdx1 control of islet β-cell function under normal and pathophysiological conditions.

Future Directions

Investigating the Role of PDX1 in Human β-Cells

The results presented in Chapter II revealed that Pdx1 maintains adult β-cell identity through both the activation of β-cell genes and repression of the α-cell program. Direct repression of *MafB* by Pdx1 was found to be imperative to inhibiting α-cell *Glucagon* gene expression. However, conclusions drawn from these studies are complicated by immunofluorescence & RNA analyses, which revealed fundamental differences in PDX1 and MAFB expression in human β-cells compared to mouse and rat. In the adult rodent pancreas high levels of Pdx1 ($Pdx1^{Hi}$) are restricted to islet β- and δ-cells, whereas lower levels ($Pdx1^{Low}$) are maintained in acinar cells. In contrast, $PDX1^{Hi}$ and $PDX1^{Low}$ states do not appear to exist in the human pancreas, as roughly 50% of acinar cells harbor similar levels of PDX1 as β-cells (Drs. Chris Wright and Al Powers, unpublished findings). Additionally, rodent β-cell MafB levels decline soon after birth and expression becomes restricted to α-cells, whereas human β-cells maintain MAFB expression into adulthood. These results illustrate the extreme importance of confirming that findings in rodents translate to human. Thus, it is essential to perform studies analogous to my work presented in Chapter II to determine the contribution of PDX1 to the identity and function in human β-cells. This analysis would examine the effect of PDX1 depletion on insulin secretion and gene expression.

Initially, these experiments would be carried out in human EndoC-βH1 & EndoC-βH2 β-cell lines which can be expanded to large numbers, secrete insulin in a dose-

dependent manner upon glucose challenge, and more importantly, express both PDX1 and MAFB (Ravassard et al., 2011b). Although the phenotype and function of EndoC-βH1 cells closely resembles that of human islet β-cells, one major difference is that unlike primary β-cells, EndoC-βH1 cells are continuously proliferating. However, Scharfmann et al. recently developed the conditionally immortalized EndoC-βH2 cell line, in which Cre-mediated deletion of the immortalizing transgene arrests cell proliferation. Transgene excision was also found to elevate expression of β-cell-specific genes (e.g. *MafA, Insulin, Glut2*) and enhances β-cell function to levels which more closely resembling primary β-cells. Thus, because of their availability in large quantities and likeness to endogenous human β-cells, EndoC-βH2 cells would be used to begin exploring the consequences of PDX1 depletion. However, each of the experiments outlined below would ultimately need to be carried out in human islets to ensure that results obtained from cell line experiments translate to primary cells.

As siRNA transfection has been shown to effectively deplete MAFA and MAFB protein from EndoC-βH2 cells, this same approach would also be taken to achieve PDX1 knockdown. Following optimization of protein depletion, examining the expression of candidate genes has traditionally been a first step for this type of analysis to ensure changes in known targets are observable before pursuing more costly RNA-seq analyses. However, subsequent RNA-seq of PDX1-depleted EndoC-βH1 cells would greatly enhance this analysis by granting a global view of gene expression changes associated with PDX1 knockdown. Furthermore, this analysis could then be coupled with existing human islet ChIP-seq datasets to identify genes directly regulated by PDX1.

Notably, the Pdx1 binding site used to mediate direct *MafB* repression in rodent β-cells is not conserved within the human *MAFB* promoter. Moreover, PDX1 was not shown to bind the *MAFB* promoter in human islet ChIP-seq experiments (Khoo et al.,

2012; Pasquali et al., 2014b). This discrepancy, and presumably lack of negative regulation by PDX1, likely allows continued *MAFB* expression in adult human β-cells. Importantly, mice carrying a transgene in which the *MafA* promoter forcibly and persistently drives MafB expression in β-cells exhibit normal β-cell gene expression and function (Dr. Holly Cyphert, personal communication). This suggests that cell-type-specific coregulator/transcription factor interactions may modulate the differential activity of MafB in α- vs. β-cells. Alternatively, MafB may not have the capacity to activate α-cell genes in the presence of dominant Pdx1 repression. Therefore, it will be of particular interest to see whether repressed α-cell gene targets of PDX1 (e.g. *glucagon*) are activated by MAFB in the absence of PDX1.

As inactivating mutations and PDX1 haploinsufficiency cause substantial impairments in glucose tolerance and the development of MODY, it is anticipated that PDX1 depletion will significantly affect the expression of (at least) its direct target genes involved in GSIS (Brissova et al., 2002; Khoo et al., 2012; Stoffers et al., 1997). For this reason, it is also predicted that PDX1 depletion will attenuate EndoC-βH1 cells capacity to secrete insulin in response to elevated glucose. Static incubations in a range of glucose concentrations could be performed to examine this possibility.

Examining BRG1 and BRM Contributions to Human β-Cells

It is noteworthy that PLA analysis of human pancreas samples revealed that PDX1 interacts with both BRG1- and BRM-associated SWI/SNF complexes in adult human β-cells (Chapter III). Unlike PDX1 and BRG1/BRM expression, PDX1:SWI/SNF complex formation was restricted to β-cells in the human pancreas, suggesting functional significance of these interactions. A series of possible experiments including

siRNA knockdowns, ChIP, and GSIS assays could be performed to better understand the consequences PDX1:SWI/SNF binding in human β-cells.

Knockdown studies presented in Chapter III revealed Brg1 and Brm potently and antagonistically regulated the expression of Pdx1 targets in rat Ins-1 β-cell lines. To test whether these control properties translate to human β-cells, the human EndoC-βH2 β-cell line and human islets could be depleted of BRG1 and/or BRM by targeted siRNA transfection. These ATPase depleted cells could be analyzed by RT-qPCR to examine changes in mRNA levels of genes examined in Chapter III (i.e. *Insulin, MAFA, PDX1, NKX6.1, GLUT1, GLUT2, MAFB, UCN3,* and *GCK*). Several potential outcomes exist for such an experiment. One possibility is that BRG1 and BRM knockdown in human β-cells will have the same effect as observed in rodents. It is also feasible that PDX1 utilizes SWI/SNF complexes in humans to regulate a similar as well as a different set of target genes than those identified in mouse. To this end, an approach utilizing RNA-seq could be implemented to more broadly identify genes affected by ATPase knockdown.

Experiments analogous to those proposed above for PDX1-depleted cells could be performed to assess insulin secretion properties of BRG1 and BRM-depleted EndoC-βH2 and human islets. Because Brg1 was found to promote expression of genes involved in insulin secretion in rodents, one would predict BRG1 knockdown would attenuate GSIS—assuming target genes are conserved between mouse and human. In contrast, the BRM knockdown could lead to enhanced insulin secretion, as it was identified as a negative regulator of GSIS genes.

The resulting expression and physiological data could be supplemented with quantitative ChIP-qPCR assays performed with EndoC-βH2 cells and BRG1-specific antibodies. Examining recruitment to genes dysregulated in the above mentioned knockdown experiments would infer direct BRG1 regulation. It is expected that BRG1

will be recruited to the active targets identified in mouse Min6 cell line ChIPs. However, it is also possible that PDX1 recruits other coregulators, and not BRG1, to modulate transcription of these genes in humans.

Although candidate-based ChIP assays indicate Brg1 is recruited to the promoters of *Insulin*, *MafA*, *Pax4, and Pdx1* (which would likely be recapitulated in human EndoC-βH1 ChIPs), these experiments test only a limited number of target genes. Additionally, as the majority of the studies in this thesis have been performed in cell lines, it would be a reasonable next step to further analyze Brg1 recruitment in islet β-cells. Accordingly, to satisfy both of these concerns, mouse and human islet BRG1 ChIP-seq experiments could be performed to gain a comprehensive understanding of genomic BRG1 enrichment *in vivo*. The inclusion of mouse islets in these experiments will complement studies proposed below, which are aimed to investigate the *in vivo* consequences of *Brg1* and *Brm* deletion from mouse islet β-cells. Nonetheless, both mouse and human datasets would be integrated with existing Pdx1 ChIP-seq data to examine the degree of overlap in genomic binding of each factor. Results from Chapter III indicate Brg1 is not recruited to all Pdx1 targets in mouse. Additionally, we must also assume that Pdx1 is not the only Swi/Snf-recruiting transcription factor; thus, we anticipate that numerous unique sites will be identified for both Brg1 and Pdx1.

One concern with this experimental setup is that Brg1 is expressed in other islet cell types. Because islet chromatin will not be generated from a pure β-cell population, upon identifying specific genes of interest, the possibility that Brg1 is recruited to these genomic locations in non-β-cells must be taken into account. Therefore, the use of FACS-sorting strategies may be required to definitively distinguish authentic Brg1 recruitment in β-cells. Alternatively, targets of interest identified in islets could be verified by performing ChIP assays in β-cell lines.

Despite strong evidence supporting Brm-Swi/Snf complexes regulation of Pdx1 targets (*insulin, MafB, Glut2, Ucn3*), the inconclusive ChIP analysis leaves us uncertain if Brm is recruited to directly participate in the expression of these genes. However, binding to *bona fide* BRM targets (CD44 and E-cadherin) also showed no enrichment, suggesting the Brm antibodies used in the ChIP assay were simply not ChIP-grade. Nevertheless, this matter is still worth pursuing, yet will require the acquisition or generation of higher quality reagents. Once this issue is resolved, it would be of interest to reassess the Brm ChIP studies performed in β-cell lines, and then extend these experiments to parallel islet Brg1 ChIP experiments described above to more clearly and definitively determine Brm recruitment.

Pdx1:Swi/Snf Complex Interaction in MPCs

Previous studies indicate the Swi/Snf complex is involved in processes such as embryonic stem cell self renewal, neural stem and progenitor cell proliferation, and activated B-cell expansion, suggesting its importance in cell growth control (Holley et al., 2014; Kidder et al., 2009; Lessard et al., 2007b). As described in Chapter III, removal of floxed *Brg1* early in pancreas development by *Ptf1a-Cre* results in a 50% reduction in final pancreas size (Figure 27). PLA analyses revealed that Pdx1 interacts with both Brg1 and Brm in nearly all E12.5 $Pdx1^{+}/Sox9^{+}/Ptf1a^{+}$ MPCs. Notably, neither Ptf1a or Sox9 bind to Swi/Snf complexes within this population.

The results from the experiments described in my thesis also present strong evidence that Pdx1 transcriptional activity is modulated by direct interactions with Swi/Snf complexes in β-cell lines. Although both Pdx1 and Brg1 affect pancreas size, the consequences of Pdx1:Swi/Snf MPC interactions and the cause of the "small pancreas" phenotype observed with reduced Brg1 remain to be determined. Strategies

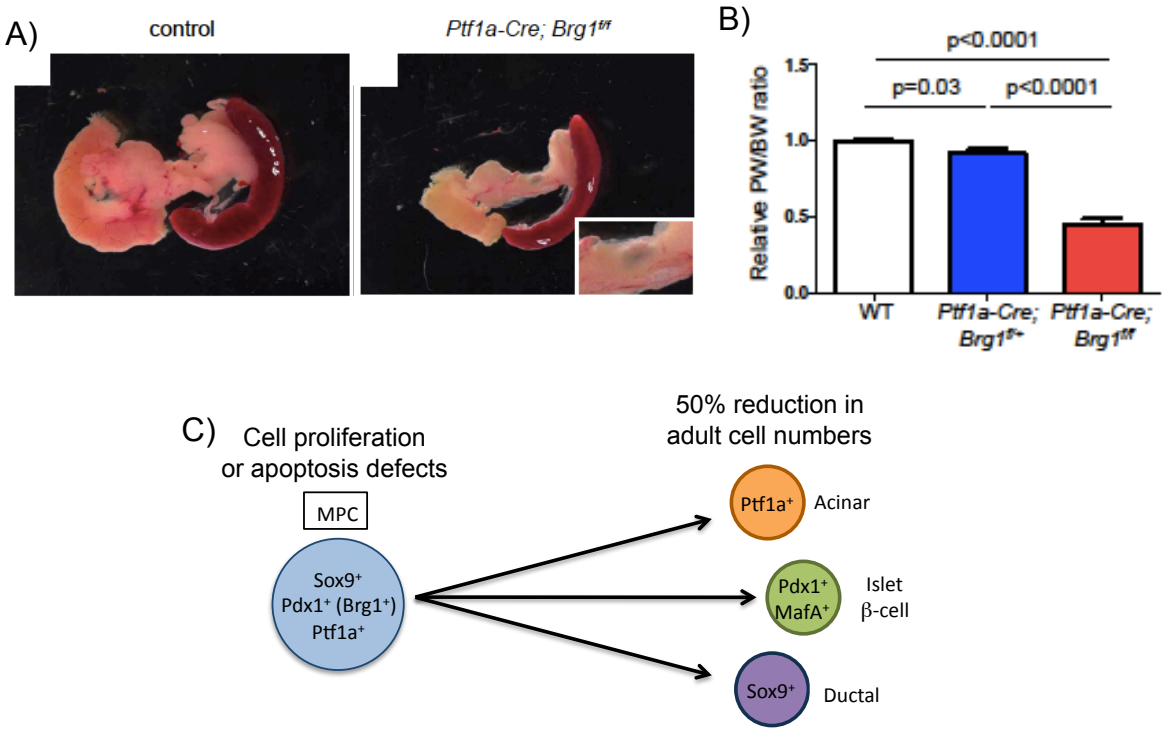

Figure 27. Embryonic deletion of Brg1 reduces final pancreas size by 50%. A) *Ptf1a-Cre;Brg1*$^{f/f}$ mouse pancreata are hypoplastic, exhibiting roughly a B) 50% reduction in pancreas mass compared to controls. C) Schematic diagram indicating the hypothesized reason for pancreas hypoplasia results from MPC proliferation or apoptosis defects following *Brg1* deletion. (Adapted with permission from Figura et al., 2014)

utilizing the mouse *Ptf1a-Cre;Brg1$^{f/f}$* model could be implemented to investigate both of these questions.

Unlike MPC deletion of *Pdx1*, which results in near complete pancreatic agenesis, albeit the 50% reduction in final pancreas size, all mature cell types are formed in the proper proportions in *Brg1* mutant mice. Interestingly, analogous results have been observed by limiting the MPC progenitor pool (Stanger et al., 2007). Specifically, Stanger et. al. generated mice in which the timing of diphtheria toxin-mediated elimination of embryonic Pdx1$^+$ progenitors could be regulated by tetracycline administration. Ablation at the early bud stage revealed that the remaining pancreatic progenitors are able to divide and differentiate into all mature pancreatic cell types, however, are unable to generate a pancreas of normal size by increasing their number of cell divisions. Thus, as final pancreas size is constrained by the number of cells allocated to the MPC pool during development, it seems logical that the hypoplastic pancreas in *Ptf1a-Cre;Brg1$^{f/f}$* mice may result from either limited expansion or aberrant apoptosis of MPCs (Figure 27).

Our laboratory has begun to investigate whether the pancreatic hypoplasia observed in *Ptf1a-Cre;Brg1$^{f/f}$* mice is due to a reduced MPC pool. Immunohistochemical analysis examining the Pdx1$^+$/Sox9$^+$/Ptf1a$^+$ cell population at E12.5 revealed that Cre was not yet expressed, Brg1 remained present, and thus, MPC numbers were unchanged in mutant *Ptf1a-Cre;Brg1$^{f/f}$* embryos. In contrast, Cre expression and *Brg1* deletion are observable at E15.5, and the number of cells staining for Pdx1 or Sox9 is reduced by 50% and 40%, respectively. Importantly, all Pdx1^{Hi} (β-cell progenitor) cells examined at E15.5 retain expression of Brg1, indicating these cells escaped Cre-mediated recombination. Antibody reagent limitations currently preclude Brg1/Sox9 costaining, however, as the mature ducts also retain Brg1 expression in *Ptf1a-Cre;Brg1$^{f/f}$* mice, we presume Sox9$^+$ ductal cell progenitors destined to inhabit the mature pancreas

also escape recombination. These results indicate that a reduction of pancreatic progenitors between E12.5 and E15.5 during organogenesis is the cause of pancreatic hypoplasia. Therefore, E13.5 and E14.5 embryos could next be characterized immunohistochemially for proliferative (e.g. Ki67, phosphohistone H3) and apoptotic (e.g. cleaved caspase 3, terminal deoxynucleotidyl transferase dUTP nick end labeling) markers. If either of these processes are responsible for *Ptf1a-Cre;Brg1$^{f/f}$* pancreatic progenitor deficiency, scoring the total proliferative^{+} marker and/or apoptotic^{+} marker cells within the MPC region relative to control embryos will indicate this change.

Pdx1 has been shown to modulate the expression of numerous cell cycle regulatory genes (e.g. *cyclinD1, cdca5, cyclinE1, cyclinE2*) and pro-apoptotic genes (e.g. *Puma* and *Noxa*) (Hayes et al., 2013; Khoo et al., 2012; Svensson et al., 2007). If altered cellular proliferation or apoptosis is observed in the above analysis, a comparison of global gene expression by RNA-seq of *Ptf1a-Cre;Brg1$^{f/f}$* and control pancreata could be performed. This analysis would broaden our understanding of Pdx1:Brg1 regulation in pancreatic progenitors by identifying genes whose expression is mutually altered between this study and published microarray analysis of *Pdx1$^{-/-}$* pancreatic buds. In conjunction with this gene expression data, direct embryonic targets of Pdx1 and Brg1 could be identified using ChIP-seq analysis. This approach, although lengthy, would help characterize changes in pancreatic progenitor gene expression resulting from *Brg1* deletion in relation to Pdx1 recruitment.

Pancreatic hypoplasia can also result from accelerated MPC differentiation, as seen in *Sox9* or *Hes-1* mutant animals (Jensen et al., 2000; Seymour et al., 2007). In both cases, rather than cell death, the progenitor pool is depleted as a consequence of their accelerated differentiation into post-mitotic endocrine cells. If apoptosis or proliferation defects are not observed in *Brg1* mutant animals, endocrine hormone immunofluorescence analysis could be performed to begin investigating this possibility.

Characterization of Brg1/Brm-Deleted β-Cells

Despite the convincing evidence of Swi/Snf complexes modulating Pdx1 activity, the majority of studies presented in this thesis were performed in β-cell lines. Therefore, it would be logical to further the analysis by investigating Brg1 and Brm contribution to β-cell development and/or function *in vivo*. Previous studies revealed that conditional deletion of *Brg1* in β-cells via generation of *RIP-Cre; Brg1$^{f/f}$* mice, did not alter β-cell formation or function (Dr. Anil Bhushan, personal communication). Interestingly, the only observable phenotype in these *Brg1*-deleted β-cells was up-regulation of the alternate Swi/Snf ATPase, Brm. These results suggest that Brm can compensate for loss of Brg1 in developing β-cells, but not in MPCs. This is not the first study to report Brm gaining the ability to compensate for Brg1 in further differentiated tissues. Brg1-Swi/Snf complexes play a critical role in vascular endothelial cell (VEC) development. Upon conditional deletion of *Brg1* from VEC progenitor cells, proliferative and differentiation defects ensue, as Brm is unable to compensate (Griffin et al., 2008). However, upon differentiation, Brm gains the capacity to compensate for loss of Brg1, as *Brg1* deletion from mature VECs does not alter phenotype or function, yet simultaneous deletion of both ATPases does (Willis et al., 2012b). Because *Brm*-null mice develop normally, this gain in functional redundancy suggests that Brg1-Swi/Snf complexes alone are capable of establishing early pancreas and VEC lineages, whereas both Brg1- and Brm-Swi/Snf complexes can maintain more mature cell phenotypes. Notably, compensation was not observed in the cell line Brg1/Brm knockdown studies presented in Chapter III. This discrepancy is presumably due to the acute nature of the knockdown and immediate analysis thereafter. If Brg1 or Brm depletion were sustained over a longer time-period, upregulation of the alternate ATPase may occur and normalize gene expression.

The fact that *Brm*$^{-/-}$ mice develop normally presents a unique opportunity to circumvent β-cell ATPase compensation. Thus, to perturb all Swi/Snf function in β-cells, *RIP-Cre;Brg1*$^{f/f}$*;Brm*$^{-/-}$*;Rosa-YFP* (double knockout - DKO) mice have been generated in our laboratory. Preliminary analysis indicates that 4-week-old male DKO mice are glucose intolerant (Dr. Jason Spaeth, personal communication). Future experiments with these mice will include examining islet architecture by immunofluorescence, expression changes in islet-specific gene products (e.g. *Insulin, Glucagon, MafA, MafB, Pdx1, Arx*) by qPCR and potentially RNA-seq, and assessing insulin secretion response of isolated islets to glucose and secretagogues (i.e. IBMX, KCl). Due to the observed glucose intolerance, it is expected that genes involved in β-cell function will be dysregulated in DKO β-cells and that isolated islets will display impaired GSIS. Additionally, as deletion of Pdx1 affects proliferation and identity in developing and mature β-cells, respectively, monitoring the fate of DKO β-cells via the YFP reporter will be of particular interest.

As noted above, deletion of *Pdx1* from developing vs. mature β-cells has different consequences. Thus, to examine whether a similar phenomenon occurs with *Brg1* deletion, *RIP-Cre*^{ER} could be utilized to control the timing of *Brg1* deletion. To parallel the *Pdx1*-deleted PKO mice described in chapter II, tamoxifen would be administered to *RIP-Cre*^{ER}*;Brg1*$^{fl/fl}$*;Brm*$^{-/-}$*;Rosa*^{YFP} at postnatal day 30 (Figure 28). Treated and untreated littermate control mice would be monitored daily for alterations in weight, fasting and ad-lib glucose levels. Swi/Snf-deleted β-cells would be subjected to immunofluorescence, RNA-seq, ultrastructural, proliferation, apoptosis, and physiological assays analogous to those performed on PKO β-cells described in Chapter II (Gao et al., 2014b). Overlaying this analysis with data gathered on PKO β-cells will allow an in depth assessment of the overlap in phenotypes resulting from β-cell deletion of *Pdx1* and *Brg1/Brm*. Furthermore, integrating the dataset generated by Brg1 and Brm ChIP-seq experiments (proposed

above) would permit a genome-wide evaluation of transcriptional control afforded to Pdx1 by Swi/Snf recruitment.

Due to our preliminary results in the above mentioned DKO animals and the powerful role these chromatin remodelers play in other cell types, complete deletion of Swi/Snf in mature β-cells is predicted to disrupt β-cell function and potentially viability or identity (Chi et al., 2003; Lessard et al., 2007a; Lickert et al., 2004). It is expected that deletion will significantly impair the transcription of key β-cell genes whose expression is altered by Swi/Snf depletion from β-cell lines (i.e., *MafA, MafB, Insulin, Glut2*). Furthermore, monitoring the survival and identity of these β-cells will be of great interest. Swi/Snf complexes have also been illustrated to play tumor suppressor roles in many tissues and reduced expression of key Swi/Snf subunits is highly associated with pancreatic cancers (von Figura et al., 2014). Thus, it is also quite possible that simultaneous deletion of both Brg1 and Brm may result in the development of pancreatic neuroendocrine tumor formation.

Identifying PDX1 Coregulators in the Developing Human Pancreas

Performing studies analogous to those in Chapter III to identify Pdx1 coregulators in the embryonic pancreas is impeded by the large amount of starting material required for such experiments. However, pancreatic progenitor cells derived from *in vitro* differentiation protocols can be grown in large numbers. These cells were recently subjected to PDX1 ChIP-seq analysis to identify PDX1 transcriptional targets (Teo et al., 2015). Reminiscent to the results presented in Chapter II, this investigation revealed that PDX1 serves a bifunctional role in human pancreatic progenitors by simultaneously activating pancreatic and repressing hepatic developmental programs. To provide mechanistic insight to the dual nature of PDX1 activity, PDX1 ReCLIP/mass

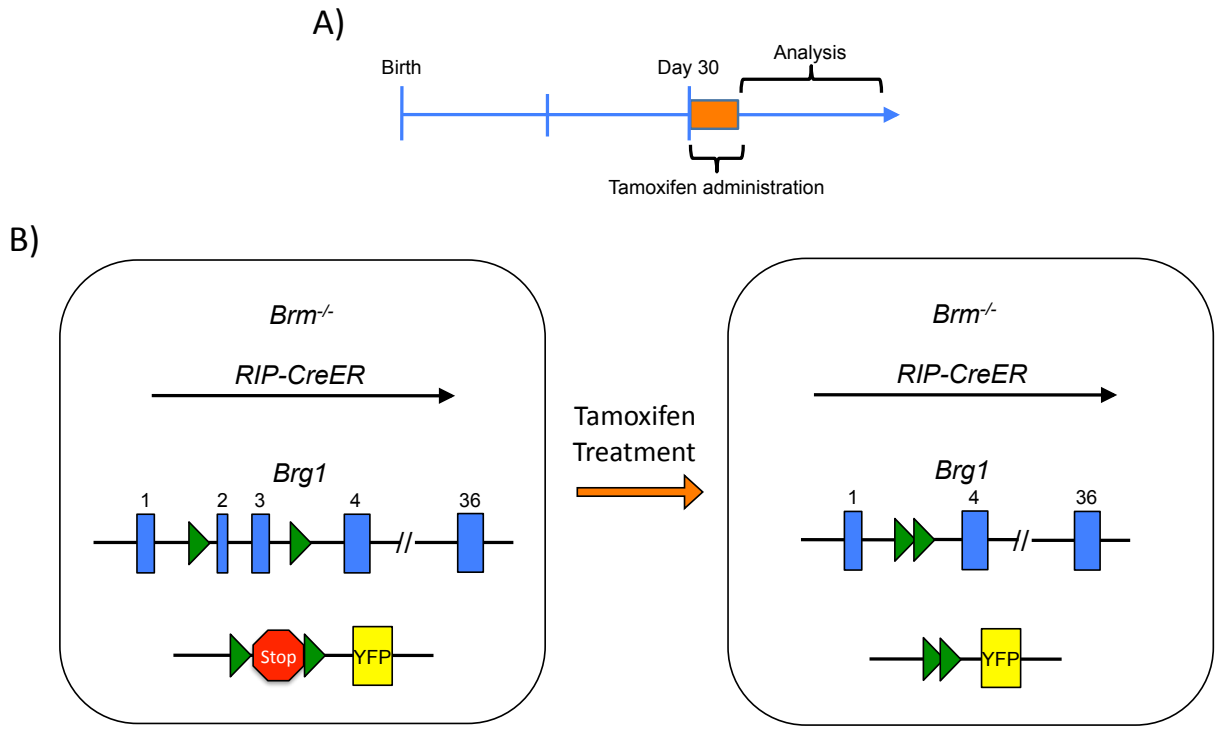

Figure 28. Experimental design for conditional inactivation of Swi/Snf complexes in adult β-cells. A) Schematic depicting the time course of tamoxifen administration and analysis thereafter. B) Schematic illustrating tamoxifen mediated Cre recombinase inactivation of *Brg1* and activation of the YFP reporter in 30 day old *RIP-Cre*^{ER};*Brg1*$^{fl/fl}$;*Brm*$^{-/-}$;*Rosa*^{YFP} mice.

spectrometry analyses could be performed with human *in vitro* differentiated pancreatic progenitors to identify PDX1:coregulator interactions within this population. While it is expected that this analysis will yield some of the same coregulators identified in βTC3 cells, because of the distinct role Pdx1 plays in the embryonic pancreas compared to β-cells, a number of novel factors may emerge as well. However, upon identifying candidates, the possibility that such interactions are artefacts of the *in vitro* differentiation system, which do not occur *in vivo* must be considered. Thus, verifying these PDX1:coregulator interactions in human embryonic pancreas samples by PLA will be a top priority before pursuing further analyses. Following interaction confirmation, a series of possible experiments including ChIP-seq, siRNA knockdown or CRISPR/Cas9 knockout, and RNA-seq analysis could be used to study how these coregulators contribute to PDX1 action in these *in vitro* differentiated cells. The information gained in these experiments could begin to answer the fundamental question of whether what we learn from mice is relevant to man. This analysis may also reveal manipulations to enhance PDX1 activity and further optimize these differentiation protocols.

Investigation of Additional Coregulators Contribution to Pdx1 Activity

In our studies we found Pdx1 interacts with coregulator proteins with an array of cellular functions. The work described in Chapter III explored the interaction of Pdx1 with the Swi/Snf complex; however, the remaining identified coregulators leave a number of unexplored avenues for Pdx1-mediated gene regulation. Of particular interest are the implications of the interactions between Pdx1 with Dnmt1, and MCM2-7 coregulators, as each have known roles in modulation of transcription and other critical cellular processes. However, confirming that interactions between PDX1 and each of these coregulators are preserved in humans would be a fundamental step prior to pursuing any further analyses.

Dnmt1

Dnmt1 is a maintenance methyltransferase, which ensures that cell-type-specific DNA methylation patterns established by *de novo* methyltranferases (*Dnmt3*) are faithfully duplicated during DNA-replication. In doing so, daughter cells retain particular gene expression patterns. Notably, like Pdx1, Dnmt1 was found to play a key role in the maintenance of islet β-cell identity (Dhawan et al., 2011). In this study β-cell-specific deletion of *Dnmt1* resulted in overt diabetes and a β-to-α-like identity switch resulting from de-repression of *Arx*. Additionally, a recent study revealed that DNA methylation patterns and expression of target genes that contribute to insulin secretion are altered in human T2DM islets (Dayeh et al., 2014). As dysregulated DNA methylation clearly represents a potential source of T2DM disease pathogenesis, examining the consequences of DNMT1 recruitment by PDX1 would be of great interest.

Dnmt1-specific antibodies could be used in ChIP assays to investigate recruitment to Pdx1 target genes. Initially these experiments would be performed on chromatin generated from mouse or human β-cell lines. However, once the assay is optimized, analogous islet ChIP and ChIP-seq assays could be performed to provide *in vivo* confirmation of Dnmt1 enrichment at Pdx1 targets and genome wide Dnmt1 recruitment. Combining this analysis with existing RNA-seq data from *Pdx1*- and *Dnmt1*-deleted β-cells would allow for the identification direct targets affected by deletion of either factor. Because Dnmt1 is thought to exclusively act as a corepressor and Pdx1 is a potent activator of many β-cell genes, complete overlap of target genes is not expected (Khoo et al., 2012; Mohan and Chaillet, 2013). Hence, integrating ChIP-seq and microarray datasets will likely identify genes which can be grouped as potential Pdx1-only, Pdx1:Dnmt1 or Dnmt1-only (Pdx1 independent) targets. WebGestalt (WEB-

based GEne SeT AnaLysis Toolkit) analysis performed on genes that exhibit both altered expression and are co-occupied by Pdx1 and Dnmt1 would provide functionally relevant categories to these datasets. Finally, to complement these analyses and provide mechanistic insight to observed gene expression changes, alterations in genome-wide DNA methylation patterns resulting from *Pdx1* and *Dnmt1* deletion could be determined by subjecting methylated DNA isolated FACS-sorted *Pdx1*-deleted, *Dnmt1*-deleted and control β-cells to RNA-seq analysis (Weber et al., 2005).

Epigenetic modifications are dynamic in nature and can be detrimentally altered by exposure to different environmental factors (Malmgren et al., 2013; Thompson et al., 2010). Results from Chapter III of this dissertation revealed that PDX1:BRG1 binding is significantly attenuated in the setting of T2DM, and potentially contributes to PDX1 inactivation and β-cell dysfunction. Therefore, the abnormal DNA methylation patterns observed in T2DM islets may manifest, in part, from compromised PDX1:DNMT1 complex formation and altered DNMT1 genomic recruitment. To test the possibility that pathophysiological conditions alter PDX1:DNMT1 complex formation, PLA assays could be performed on normal and T2DM pancreas sections. If the disease-stress conditions compromise complex formation, a decrease in β-cell PLA signals would be observed. However, it is also possible PDX1:DNMT1 will not be affected in T2DM islet β-cells, as was observed with PDX1:BRM complexes. This result would imply that aberrant recruitment of DNMT1 by other islet enriched transcription factors, and not PDX1, may be responsible for the altered DNA methylation patterns in T2DM islets. Alternatively, this outcome could indicate a DNMT1-independent mechanism, and suggest involvement of other DNA methyltransferase enzymes in the preservation of human islet β-cell methylation patterns.

Dnmt1 has also been illustrated to play a critical role in the development of the mouse pancreas. Deletion of floxed *Dnmt1* from pancreatic progenitors by Cre recombinase under the control of the *Pdx1* promoter causes pancreatic agenesis (Georgia et al., 2013). This phenotype is a result of demethylation and derepression of the tumor suppressor *p53*, and subsequent progenitor cell apoptosis. However, the transcription factor(s) responsible for recruitment of Dnmt1 to targets such as *p53* remain to be identified. As both mice and humans lacking functional Pdx1 also display near complete pancreatic agenesis, Pdx1 would appear to be a prime candidate for Dnmt1 localization. A series of potential experiments could be considered to assess this possibility, including PLA, microarray, ChIP-seq, and analysis of genome-wide DNA methylation patterns.

For the moment, the ReCLIP method is not a feasible approach to investigate Pdx1:Dnmt interactions. The main barrier to all co-IP experiments is the large amount of starting material required for each immunoprecipitation. For example, the ReCLIP studies described in Chapter III were each performed with 800 million cells. Unfortunately, at its peak (~E13.5) the MPC population of the embryonic pancreas only reaches roughly 70 thousand cells. In addition to limited starting material, the ReCLIP technique is most effective when performed on nuclear extracts. In the case of whole tissues, isolating nuclei to generate such extracts represents a technically challenging undertaking that would likely involve cell dispersement and MPC sorting. Collectively, the lack of suitable starting material and technical difficulty makes the ReCLIP method and an unviable option for determining Pdx1 binding to Dnmt1 in MPCs.

Alternatively, Pdx1:Dnmt1 MPC complex formation could be assessed using the PLA on embryonic mouse pancreas sections. This approach was effectively used to determine Pdx1:Swi/Snf complex formation in numerous mouse embryonic stages, in addition to adult mouse and human pancreas sections (Chapter III). Though this analysis

would likely be relatively straightforward, Dnmt1 may be incorporated into multi-protein complexes in β-cells. Therefore, it remains possible that Pdx1 and Dnmt1 are members of a larger complex in which they do not directly interact. In this case, these proteins may be at a physical distance beyond the range of the PLA (30-40nm), potentially rendering this approach inconclusive. An additional concern is that Dnmt1 may not interact with Pdx1 in MPCs, and instead may be recruited by other MPC transcription factors (e.g. Sox9, Ptf1a). However, this matter could likely be addressed using Sox9- and Ptf1a-specific antibody PLAs.

If Pdx1:Dnmt1 interaction is observed, genomic recruitment of Pdx1 and Dnmt1 could be assessed using a ChIP-seq approach. Overlaying the resulting datasets will facilitate identification of targets harboring a Pdx1:Dnmt1 complex or suggest Pdx1-independent Dnmt1 recruitment. For parallel gene transcription analysis, existing microarray gene expression profiles generated from *Pdx1*-null and *Dnmt1*-deleted mouse pancreatic buds could be compared (Svensson et al., 2007). Lastly, genome-wide DNA methylation patterns resulting from *Pdx1* and *Dnmt1* deletion could be determined by subjecting methylated DNA isolated from $Pdx1^{-/-}$, $Pdx1\text{-}Cre;Dnmt1^{fl/fl};ROSA^{YFP}$ and control pancreatic buds to RNA-seq analysis (Weber et al., 2005).

Again, due to the activating properties of Pdx1, complete overlap in targets is not expected (Mohan and Chaillet, 2013; Svensson et al., 2007). However, WebGestalt analysis of Pdx1 and Dnmt1 co-occupied and regulated genes would provide functionally relevant categories to these datasets. The data will likely yield subsets of target genes that group into cell cycle/proliferation, apoptosis and transcriptional regulator genes, among others.

MCM2-7 Complex

DNA polymerase requires single-stranded DNA substrate during the process of DNA replication. The minichromosome maintenance (MCM2-7) complex is a molecular motor responsible for the enzymatic unwinding of double-stranded DNA at replication forks to drive the generation of single-stranded DNA ahead of DNA polymerase (Aparicio 1997 cell). MCM2-7 is a heterohexameric helicase made up of six highly evolutionarily conserved and distinct proteins, MCM2-MCM7, all of which are required for DNA replication elongation *in vivo* (Anthony Schwacha 2008 Microbiol Mol Biol Rev). Additionally, this complex has also been shown to play an essential coregulatory role in transcription (DaFonseca et al., 2001; Snyder et al., 2005; Zhang et al., 1998). Notably, all six MCM2-7 proteins were isolated in my ReCLIP/mass spectrometry analysis. Furthermore, the MCM5 protein was shown to complex with Pdx1 in βTC3 cells by co-immunoprecipitation and PLA analyses (Figure 29A, B). Additionally, MCM5 is co-expressed with Pdx1 in replicating β-cells of 8-week old *db/db* mouse islets (Figure 29C). Thus, gaining a better understanding of the consequences of Pdx1:MCM2-7 interaction may greatly advance our knowledge of the role of Pdx1 in replication, and potentially identify avenues to stimulate β-cell proliferation for replacement therapies.

Many lines of evidence indicate an association between Pdx1 and proliferation of pancreatic cell types, most notably developing β-cells (Gannon et al., 2008; Johnson et al., 2003). Importantly, the MCM2-7 proteins are only expressed in replicating cells of the pancreas (Köhler et al., 2010; Willcox et al., 2010). It is therefore anticipated that Pdx1:MCM2-7 interactions will only be found within replicating cells. To examine this possibility, the high rate of proliferation and known requirement of Pdx1 for progenitor and β-cell expansion makes the embryonic and neonatal pancreas a good starting point to analyze Pdx1:MCM2-7 binding by PLA (Gannon et al., 2008; Holland et al., 2002;

Jonsson et al., 1995). Analysis would include pancreas sections from E12.5, E15.5 and E18.5 embryos, and 1-week neonates to investigate complex formation in proliferating MPCs and acinar, ductal and β-cell progenitors. Although cells expressing MCM2-7 are assumed to be replicating, further validation by immunofluorescence analysis of additional cell proliferation markers (e.g. Ki-67, phosphohistone H3) would be performed. Because deletion of Pdx1 from MPCs and developing endocrine or exocrine tissue impedes expansion, if Pdx1:MCM2-7 modulates proliferation, complexes are expected to be observed in replicating $Pdx1^+$ cells. However, different cell types may utilize alternate routes (i.e. Pdx1:MCM2-7-independent pathways) to drive proliferation and complex formation may not be observed in certain replicating cell types. Additionally, this interaction may be confined to a single stage of the cell cycle (i.e. S-phase). Thus, the use of proliferation markers such as Ki-67, which is present in all active stages of the cell cycle, may identify cells that are proliferating, yet do not harbor Pdx1:MCM2-7 complexes.

Unlike embryonic and neonatal β-cells, adult rodent and human β-cells replication is an extremely rare event (<0.5% replicate per day) (Meier et al., 2008; Teta et al., 2005). However, adult rodent β-cells have the capacity to significantly augment proliferation in response to physiological challenges such as pregnancy, high fat diet, pancreatic injury, peripheral insulin resistance, and high blood sugar (Alonso et al., 2007; Brüning et al., 1997; Cano et al., 2008; Golson et al., 2010; Rieck et al., 2009). Due to the scarcity of proliferating β-cells in normal adult mice, it would be advantageous to use one of these models of induced β-cell proliferation for β-cell Pdx1:MCM2-7 binding analysis. To this end, Pdx1 is required for compensatory β-cell mass expansion in response to diet-induced insulin resistance (Sachdeva et al., 2009). Thus, PLA analysis of mice placed on a high fat diet could be used. Signals would presumably be

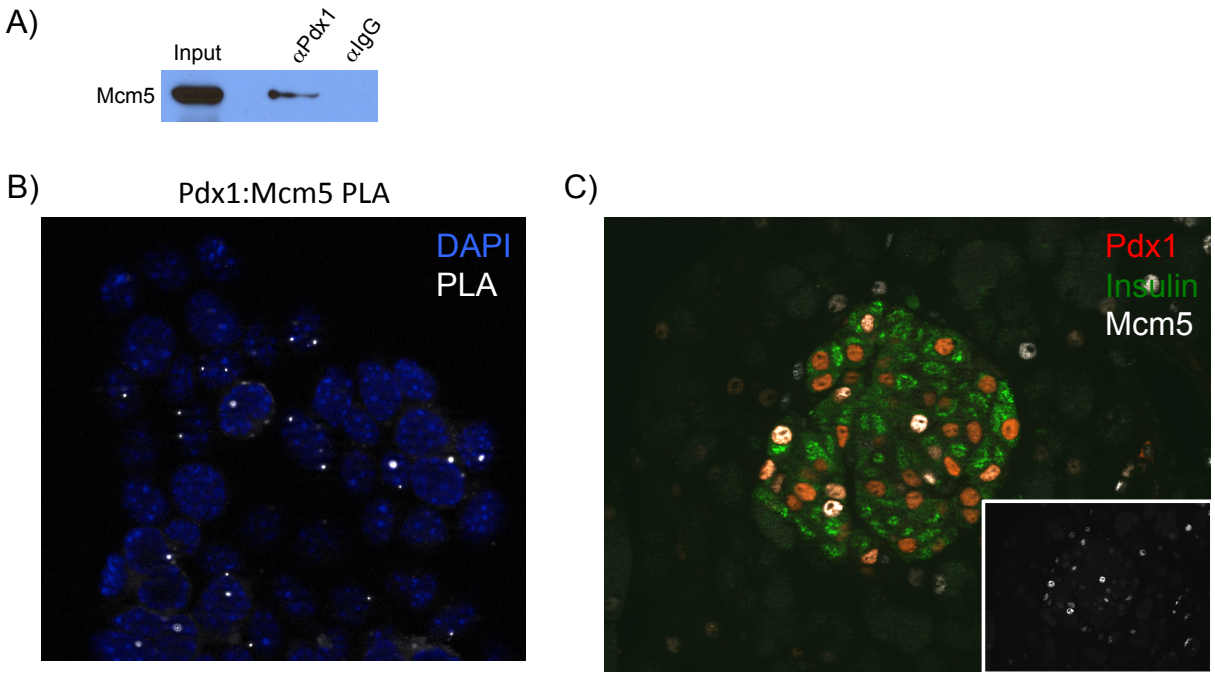

Figure 29. Pdx1 interacts with MCM5 in β-cell lines and is co-expressed in proliferating β-cells. A) Western blot and B) PLA analysis indicating Pdx1 and MCM5 interact in βTC3 nuclei. C) Representative immunofluoresence analysis of Pdx1, Insulin, and MCM5 of 8-week-old *db/db* mouse pancreas illustrating that Pdx1 and MCM5 are coexpressed in presumed proliferating β-cells.

observed in all replicating β-cells. However, the mechanisms controlling β-cell replication in this and other physiologically-driven modes of proliferation are incompletely understood. Therefore, the formation of Pdx1:MCM2-7 complexes may be required to promote proliferation in some contexts of β-cell replication yet their absence inconsequential to others. With this in mind, it may be necessary to examine additional models of adult β-cell mass expansion if no Pdx1:MCM2-7 complexes are found in the high fat diet model.

Pdx1 modulates expression of genes involved in cell-cycle regulation (Khoo et al., 2012). A number of studies also indicate a role for MCM2-7 as a transcriptional coactivator. For example, MCM2-7 proteins are recruited to target genes by the Stat1 transcription factor in response to IFN-γ signaling, and are essential to Stat1-mediated transcription activation (DaFonseca et al., 2001; Snyder et al., 2005; Zhang et al., 1998). To investigate whether Pdx1 recruits MCM2-7 to modulate transcription, ChIP assays with antibodies specific for MCM2-7 proteins would be performed on β-cell line chromatin. Our focus would primarily be to investigate MCM2-7 recruitment to cell-cycle genes regulated by Pdx1 (e.g. *cyclinD1, cdca5, cyclinE1, cyclinE2*). Following this analysis, siRNA knockdown and dominant negative forms of the MCM2-7 proteins coupled with RTqPCR analysis could be used to assess the role of this complex in Pdx1 transcriptional activity (Snyder et al., 2005). As the MCM2-7 appears to predominantly function as a transcriptional coactivator, it is predicted this complex will also serve in this capacity to aid in coactivation of Pdx1 regulated genes. Consequently, although it is expected that recruitment will only be observed at active Pdx1 targets, repressed genes will be analyzed as well, which may potentially serve as a negative control for these experiments.

Transcription factor recruitment of MCM2-7 occurs in a signal-dependent manner (e.g. Stat1/INF-γ gene activation). Numerous mitogens and growth factors (glucagon-like peptide-1, hepatocyte growth factor, lactogens, Insulin, and others) have been shown to induce rodent β-cell proliferation. Moreover, studies suggest that Pdx1 serves as a downstream effector of several factors with mitogenic activity to promote islet β-cell survival and proliferation (Li et al., 2005b; Lombardo et al., 2011). Accordingly, it would be of great interest to investigate whether Pdx1 recruitment of MCM2-7 occurs in response to such stimuli. Incubating isolated mouse islets with growth factors and mitogens known to promote β-cell replication could be used to investigate this possibility. A combination of PLA and immunofluorescence analysis for proliferation markers could then be performed to investigate whether mitogen/growth factor treatment induces Pdx1:MCM2-7 binding in replicating β-cells. If an increase in binding is associated with treatment, ChIP assays coupled with RT-qPCR analysis could be implemented to extend this analysis and investigate whether growth factor/mitogen induced binding is also associated with increases occupancy and expression of target genes. However, although the percent of proliferating β-cells increases significantly in response to mitogens and growth factors, the actual number of proliferating cells is still a relatively small population. In this regard, ChIP assays unfortunately may not be feasible due to limited starting material.

Very little is currently known regarding how β-cell mass is controlled in the human pancreas. Moreover, replication of human β-cells is an extremely rare event and they are refractory to most signaling molecules shown to enhance rodent β-cell proliferation (Kulkarni et al., 2012). These obstacles make studying aspects of human β-cell proliferation such as PDX1:MCM2-7 complex formation a daunting task. However, Raphael Scharfmann's group recently developed a conditionally immortalized human

EndoC-βH2 β-cell line, whose proliferative capacity can be arrested upon Cre-mediated excision of the immortalizing transgenes (Scharfmann et al., 2014). Their likeness to authentic β-cells and unique control over cell proliferation makes these cells an exceptional model to study human β-cell replication. Because these cells can be massively amplified prior to inducing an arrest in cell proliferation, ReCLIP could be a potential strategy to investigate PDX1:MCM2-7 binding in both replicating and arrested cells. A combined PLA and immunofluorescence analysis of proliferation markers (e.g. Ki67, phosphohistone H3) could also be performed to further verify whether complex formation is restricted to replicating cells. If complex formation is observed in replicating cells, a combination of ChIP, siRNA knockdown or dominant negative, and RTqPCR approaches could also be used to extend this analysis and investigate MCM2-7 recruitment and activity on PDX1 targets involved in cell cycle regulation and potentially other aspects of β-cell function. Together, these studies may reveal new insight to the contribution Pdx1 makes to β-cell proliferation.

Collectively, the experiments outlined above have strong potential to significantly increase our understanding of PDX1 transcriptional control of target genes involved in β-cell development, function and proliferation. Such knowledge will almost certainly aid in the improvement of current therapies and may identify new treatment strategies for diabetes.

Appendix

Potential Pdx1-interacting proteins identified by ReCLIP/MS

Protein	# of Identified Peptides IgG	Pdx1	% Coverage
Baf47	0	1	11.00
Baf155	0	3	3.00
Baf57	0	2	9.00
Baf170	0	3	3.00
Brg1	0	5	1.60
Rbbp4	1	10	9.00
Hdac1	0	5	5.00
Mi2beta	0	7	2.50
Tif1beta	0	6	14.00
Spectrin beta chain, brain 1*	1	12	3.76
Myosin-9*	1	14	3.94
Microtubule-associated protein 1B	1	8	1.96
Importin-5	0	8	5.27
Chaperonin subunit 2 (Beta), isoform CRA_a*	2	8	6.07
Glyceraldehyde-3-phosphate dehydrogenase*	2	8	16.44
Stip1	0	7	8.45
14-3-3 protein	1	6	15.22
ATP citrate lyase	1	11	10.93
DNA replication licensing factor MCM2	2	13	8.55
DNA replication licensing factor MCM3	2	10	10.41
DNA replication licensing factor MCM7	1	10	8.29
DNA replication licensing factor MCM6	2	5	6.63
DNA replication licensing factor MCM5	0	7	7.39
DNA replication licensing factor MCM4	1	4	4.59
heterogeneous nuclear ribonuclearprotein U*	2	10	2.58
heterogeneous nuclear ribonuclearprotein K*	3	9	19.96
heterogeneous nuclear ribonuclearprotein A1*	1	3	16.06
heterogeneous nuclear ribonuclearprotein C1/C2*	1	3	7.23
RuvBL1/2	0	5	7.99
Nucleolin*	1	9	14.69
alpha actin*	1	7	8.84
Elongation factor 2	1	4	5.64
Elongation factor 1 alpha	1	4	10.88
Polyadenylate-binding protein *1	1	2	5.47
Serine hydroxymethyltransferase (Shmt2)	0	3	5.67
ATP-dependent RNA helicase Ddx39	0	2	4.40
Actin-like protein 6A	0	2	7.14
DNA methyltransferase 1	0	4	0.79
Peroxiredoxin 1	4	9	24.36
Splicing factor 3B, subunit 3*	1	9	5.65
Cell division control protein 2 homolog	0	8	6.25
E3 ubiquitin-protein ligase NEDD4-like	0	7	2.83
Smc3	0	5	2.02
Serine/threonine-protein phosphatase 2A	1	4	5.91

Triosephosphate isomerase	1	5	11.56
ATPase WRNIP1	1	3	2.51
Delta-1-pyrroline-5-carboxylate synthase	0	6	4.98
Structural maintenance of chromosomes 1A	0	4	1.82
Alanyl-tRNA synthetase	0	5	2.09
Cell division cycle 5-related protein	0	4	4.49
Malate dehydrogenase	0	6	8.21
Heterogeneous nuclear ribonucleoprotein A3*	1	6	12.51
DNA topoisomerase 2-alpha	1	3	1.45
Histone deacetylase 6	0	3	1.53
AP-2 complex subunit beta	1	3	1.32
Adenosylhomocysteinase	0	4	5.92
Sister chromatid cohesion protein PDS5 homolog B	0	3	1.08
U4/U6.U5 tri-snRNP-associated protei	0	3	1.74
Thymopoietin	0	2	3.58
Epb4.1l3 protein	0	3	2.24
Probable ATP-dependent RNA helicase DDX46	0	2	1.90
C-terminal-binding protein 1	0	2	2.72
Ubiquitin carboxyl-terminal hydrolase 7	0	2	0.60
Centrosomal protein of 170 kDa	0	2	0.61
Histone acetyltransferase MYST2	0	2	1.66
NSFL1 cofactor p47	0	2	3.08
Insulin-like growth factor 2 receptor	0	1	0.27
Serrate RNA effector molecule homolog	0	2	3.00

* Proteins found within the CRAPome
(Mellacheruvu et al., 2013 Nature Methods)

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