Spatiotemporal and Mechanistic Analysis of Nkx2.2 Function in the Pancreatic Islet

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ABSTRACT

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Pancreatic beta cell specification is a complex process, requiring proper function of numerous transcription factors. Nkx2.2 is a transcription factor that is crucial for beta cell formation, and is expressed early and throughout pancreatic development. Nkx2.2$^{-/-}$ mice display complete loss of the beta cell lineage and defects in the specification of other endocrine cell types, demonstrating the importance of Nkx2.2 in establishing proper endocrine cell ratios. Recent studies have also demonstrated a role for Nkx2.2 within the mature beta cell to maintain identity and function.

This thesis work investigated the timing of pancreatic beta cell specification and the mechanism of this process. In these studies, Nkx2.2 was ablated specifically within the Ngn3-expressing endocrine progenitor population *in vivo*. These mice displayed defects similar to Nkx2.2$^{-/-}$ mice. Surprisingly, the disruption of endocrine cell specification did not require loss of expression of multiple essential transcription factors known to function downstream of Nkx2.2, including Ngn3, Rfx6, and NeuroD1. While these factors are all necessary for beta cell specification, their preserved expression did not rescue beta cell formation. ChIP-Seq analyses also revealed co-occupancy of Nkx2.2, Rfx6, and NeuroD1 near endocrine-related genes, suggesting Nkx2.2 may cooperate with its downstream targets to regulate beta cell fate. These results have revealed a unique requirement for Nkx2.2 during a critical window of beta cell development.

In addition, the role of a conserved domain of Nkx2.2, the specific domain (SD), was assessed using Nkx2.2^{SDmutant} mice. Transcriptional profiling of Nkx2.2^{SDmutant} endocrine progenitors revealed a critical role for the SD domain in regulating the transcription of endocrine fate genes early in the process of endocrine differentiation. In addition, beta cell-specific deletion of the Nkx2.2 SD domain resulted in hyperglycemia, glucose intolerance and dysregulation of beta cell functional genes. This suggests the SD domain is important for mediating Nkx2.2 function within the beta cell to maintain glucose homeostasis.

Together, these results have elucidated a critical developmental window for beta cell specification and demonstrated an essential role for Nkx2.2 and specifically its SD domain in this process. Furthermore, these studies suggest that beta cell transcription factors may also regulate endocrine fate in a combinatorial manner, and exert changes within the endocrine progenitor lineage. These findings have provided us with a better understanding of *in vivo* pancreatic development, and will improve current research efforts to differentiate beta cells *in vitro* from hPSCs.

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CHAPTER 1
INTRODUCTION

Diabetes

Diabetes is a disease characterized by elevated levels of blood glucose. The pancreatic endocrine compartment is essential for maintaining the blood glucose homeostasis of an organism. Defects present in the endocrine compartment, specifically within the insulin-producing beta cells, can result in hyperglycemia and lead to diabetes. Diabetes was considered to be the seventh leading cause of death in 2010. In the United States, 9.3% of the population was afflicted with diabetes in 2012 (CDC, 2014). Worldwide, there is an estimated 415 million people currently living with diabetes. With increasing prevalence, this number is expected to reach 642 million people in the year 2040 (IDF, 2015).

Diabetes can be classified as type 1 diabetes (T1D), type 2 diabetes (T2D) or gestational diabetes. T1D is caused by autoimmune attack of the pancreatic beta cells, leading to beta cell death and insufficient insulin production. T1D is frequently diagnosed in children in their mid-teens (CDC, 2014). Cases of T1D are thought to comprise 5% of the total number of people diagnosed with diabetes (CDC, 2014). T2D typically arises from insulin resistance and beta cell exhaustion. T2D accounts for 90-95% of the cases of diabetes (CDC, 2014). Lastly, gestational diabetes occurs in pregnant women. It is typically diagnosed in the second or third trimester and is characterized by elevated levels of blood glucose (CDC, 2014). It is estimated that 2-10% of pregnant women will develop gestational diabetes, with most cases resolving after childbirth (CDC, 2014).

There are numerous comorbidities that can occur as a consequence of diabetes. These include cardiovascular diseases: heart attack and stroke, high blood pressure, and high cholesterol (CDC, 2014). Diabetes can also lead to kidney disease and failure, requiring dialysis or transplant. In 2011, 44% of new kidney failure cases in the U.S. were caused by diabetes (CDC, 2014). In addition, blindness can result from diabetic retinopathy. Neuropathy can also occur, sometimes resulting in amputation, typically of the lower-limbs (CDC, 2014; IDF, 2015). In the U.S. alone, it is estimated that $245 billion USD was spent in 2012 on direct and indirect costs of diabetes healthcare (CDC, 2014).

Current treatment options for people with diabetes focuses on maintaining glucose regulation and preventing hyper- or hypo-glycemia. Exogenous insulin injections and numerous medications are currently being used as treatment. In addition, artificial pancreata, islet transplants, and *in vitro* beta cell differentiation protocols are under development as diabetic therapies.

Treatment for T1D patients involves delivery of exogenous insulin through a pump or injection (CDC, 2014; IDF, 2015). This requires close monitoring of blood glucose throughout the day to ensure proper regulation. For people with T2D, there are numerous medications that can be used, such as biguanides and sulfonylureas (IDF, 2015). The most commonly used medication is metformin, a biguanide drug (Inzucchi et al., 2012). Metformin acts by decreasing hepatic glucose synthesis and does not increase the risk of hypoglycemia (Bailey and Turner, 1996; Inzucchi et al., 2012). Sulfonylureas increase insulin secretion from the pancreas (Bryan et al., 2005; Inzucchi et al., 2012). In addition, GLP-1 analogues or DPP-4 inhibitors can be used to regulate blood glucose (Drucker and Nauck, 2006; IDF, 2015). According to the National

Health Interview Survey, between 2010 and 2012, 14% adults with diagnosed diabetes were taking insulin only, 14.7% were taking both insulin and oral medication, and 56.9% were taking oral medication only (CDC, 2014).

In recent years, progress has been made in the development of an artificial pancreas (Battelino et al., 2015; Nimri and Phillip, 2014; Peyser et al., 2014). This system involves continuous glucose monitoring (CGM), calculation of appropriate insulin dosage, and automatic administration of insulin through an insulin pump (Deiss et al., 2006; Pickup 2012). A major concern of the artificial pancreas is the incidence of acute hypoglycemia. This can have serious detrimental effects, such as seizures, loss of consciousness, or death (Peyser et al., 2014). To decrease the risk of hypoglycemia, insulin pump suspension is used to block insulin delivery when the CGM detects glucose at a certain threshold (Bergenstal et al., 2013; Davis et al., 2015; Ly et al., 2013). While the first artificial pancreas models were equipped with insulin delivery only, efforts are being made to incorporate both insulin and glucagon administration to enable counter regulation and precise glycemic control (Haidar et al., 2013).

Cadaver-derived islet transplants have also been successfully performed, resulting in insulin independence (Ryan et al., 2005; Shapiro et al., 2000). However, this procedure is invasive and requires immunosuppression, which can cause ulcers and anemia (Ryan et al., 2005). In addition, there is limited availability of islets for transplantation (Sheehy et al., 2003). Recent efforts are directed at increasing islet yield and improving islet engraftment (Yokoi et al., 2016).

A burgeoning area of research has focused on the *in vitro* differentiation of pancreatic beta cells from human pluripotent stem cells, including both human embryonic stem cells (hESCs) and

induced pluripotent stem cells (iPSCs). A major challenge in this field of research is to differentiate cells that are monohormonal and fully functional. Prior to 2014, the insulin-expressing cells produced *in vitro* (hereinafter referred to as "beta-like cells") co-expressed other pancreatic hormones, did not release insulin in response to glucose, and their transcriptional profiles more closely resembled those of immature beta cells than mature beta cells (D'Amour et al., 2006; Hrvatin et al., 2014; Kroon et al., 2008; Zhang et al., 2009). In the last few years, there have been major advancements in this area of research. By mimicking the events that occur during murine pancreas development, such as incorporating a $Pdx1^{+}Nkx6.1^{+}$ co-positive stage (see below), researchers were able to differentiate monohormonal beta-like cells with greater efficiency (Nostro et al., 2015). To date, the transcriptional profiles of beta-like cells more closely resemble that of mature primary beta cells and are responsive to glucose (Pagliuca et al., 2014; Rezania et al., 2014); however, the protocol still requires additional optimization. Differences in the transcriptional profiles of *in vitro* differentiated beta-like cells compared to adult primary beta cells still remain, possibly reflecting incomplete differentiation (Pagliuca et al., 2014). In addition, these beta-like cells require both vascularization and isolation from the immune system to function and survive *in vivo* following transplantation. Immunoisolation technologies are currently being developed using polymer-encapsulation (Vegas et al., 2016). It will be necessary to optimize these advancements before *in vitro* beta cell differentiation and transplantation can become a viable therapy for patients with diabetes.

Pancreas Development and Establishment of Endocrine Cell Fate

The mature pancreas consists of three main compartments: exocrine, ductal, and endocrine. The exocrine compartment is comprised of acinar cells responsible for the synthesis of digestive enzymes. Ductal cells form networks connected to acinar cells and transport digestive enzymes to the duodenum. The endocrine compartment regulates glucose homeostasis. It is comprised of endocrine cells which form highly vascularized clusters, called islets of Langerhans. The adult pancreas contains four types of endocrine cells, each type producing a single hormone: insulin-producing beta cells, glucagon-producing alpha cells, somatostatin-producing delta cells, and pancreatic polypeptide-producing PP cells (Slack, 1995). Two additional endocrine cell types are confined to embryonic development: ghrelin-producing epsilon cells and gastrin-producing G cells (Arnes et al., 2012a; Prado et al., 2004; Suissa et al., 2013).

Murine pancreas development can be first observed morphologically at embryonic day (E)9.5. Two pancreatic buds emerge from the foregut endoderm, the dorsal pancreatic bud and ventral pancreatic bud. The dorsal bud forms from the dorsal foregut endoderm at E9.5, while the ventral bud can be first observed at E10 from the ventral foregut endoderm (Pan and Wright, 2011). These buds undergo growth via cell proliferation, plexus formation and remodeling. The dorsal and ventral buds eventually fuse together to form a continuous organ beginning at approximately E11.5 (Pan and Wright, 2011; Villasenor et al., 2010). During this time of epithelial remodeling, the cells undergo tip-trunk compartmentalization, with cells in the "tip" region destined to become acinar cells, and cells of the "trunk" region restricted to endocrine or ductal fates (Zhou et al., 2007). Trunk cells destined to become endocrine cells are thought to

undergo a process similar to epithelial-mesenchymal transition (EMT) as they delaminate and migrate from the epithelial trunk, eventually coalescing to form islets of Langerhans (Fig. 1-1) (Rukstalis and Habener, 2007).

$Pdx1^+$ pancreatic progenitor cells

Numerous transcription factors expressed during pancreas development have been identified, and their functional roles elucidated. Pancreatic and duodenal homeobox 1 (Pdx1, aka IPF1 or STF-1) is expressed in the pancreatic progenitor population. These $Pdx1^+$ cells first arise within the foregut endoderm beginning at E8.5, just prior to initial bud formation (Guz et al., 1995; Ohlsson et al., 1993). Lineage tracing studies with Pdx1-Cre and Pdx1-CreER alleles in mice have shown that $Pdx1^+$ cells give rise to all cell types in the mature pancreas: exocrine, ductal, and endocrine cells (Gu et al., 2002; Herrera, 2000).

While Pdx1 is a specific marker of the developing pancreas, studies have also found that Pdx1 expression is functionally essential for pancreas formation. $Pdx1^{-/-}$ mice display pancreatic agenesis and die shortly after birth (Jonsson et al., 1994; Offield et al., 1996). Minimal pancreatic buds are capable of forming in $Pdx1^{-/-}$ mice; however, there is little outgrowth or plexus remodeling (Offield et al., 1996). This demonstrates the specificity and requirement of Pdx1 for pancreas development.

Ectopic Pdx1 expression in chick embryos using *in ovo* electroporation has also demonstrated that Pdx1 is sufficient to repress intestinal, stomach, and liver differentiation markers. In these experiments, Pdx1 expression was induced more broadly throughout the developing endoderm, at a time just after endogenous Pdx1 is activated. While intestine, stomach, and liver cell fates

were repressed, pancreatic endocrine cells did not form. There were no insulin-producing beta cells or glucagon-producing alpha cells present in the ectopic Pdx1 region, indicating additional factors are required for endocrine cell specification (Grapin-Botton et al., 2001).

The $Pdx1^+$ pancreatic progenitor population is also critical for and directly related to final organ size. Stanger et al. (2007) found that the $Pdx1^+$ population present between E8.5 and E12.5 is essential for pancreas formation. They also found a complete lack of compensative growth, suggesting that final organ size is determined by factors intrinsic to the pancreatic progenitor cells (Stanger et al., 2007).

While these developmental studies have been conducted in mouse models, the function of Pdx1 appears to be conserved in humans. Stoffers et al. (1997) identified an inactivating mutation in exon 1 of PDX1, Pro63fsdelC, which was associated with pancreatic agenesis in the patient (Stoffers et al., 1997b). Another mutation in PDX1 has been found, resulting in a cytosine deletion. This mutation was linked to diabetes (maturity-onset diabetes of the young, "MODY") and identified PDX1 as a novel genetic locus associated with diabetes, known as MODY4 (Stoffers et al., 1997a). A compound heterozygous mutation was identified in the homeobox of PDX1 in exon 2 and was also associated with pancreatic agenesis (Schwitzgebel et al., 2003). These studies suggest conserved developmental mechanisms between mouse and human pancreas specification.

$Ngn3^+$ endocrine progenitor cells

Following tip-trunk compartmentalization, there are two waves of endocrine cell differentiation during murine pancreas development. The first wave occurs between E9.5 and E12.5 (Pan and

Wright, 2011). These early endocrine cells are typically glucagon-producing alpha cells (Pictet et al., 1972). The second wave of endocrine cell differentiation occurs from E13.5 to E18.5, and is the major window of endocrine cell formation (Pan and Wright, 2011). Epsilon cells appear beginning at E10.5 (Prado et al., 2004) and G cells at E12.5 (Suissa et al., 2013). Beta cells, delta cells, and PP cells begin to arise at E13.5, E15.5, and E18.5, respectively (Pictet et al., 1972; Slack, 1995). However, the initial formation of PP^+ cells has been controversial due to potential antibody cross reactivity with neuropeptide Y (Npy) and peptide YY (Pyy). Some studies report PP expression as early as E10.5 and others report PP^+ cells present only after birth (Herrera et al., 1991; Jorgensen et al., 2007; Teitelman et al., 1993; Upchurch et al., 1994). Towards the end of embryogenesis, pancreatic beta cells undergo proliferation to increase beta cell mass (Bouwens and Rooman, 2005), and endocrine cells cluster together to form compact islet structures (Herrera et al., 1991; Pictet et al., 1972).

In the expanding epithelial trunk domain, a subset of $Pdx1^+$ cells begin to express Neurogenin 3 (Ngn3, aka Neurog3), the first marker of endocrine cell differentiation (Jensen et al., 2000a; Schwitzgebel et al., 2000). After Ngn3 expression is initiated, these cells become post-mitotic (Gu et al., 2002; Jensen et al., 2000a; Miyatsuka et al., 2011). $Ngn3^+$ cells are thought to undergo EMT-like delamination and migration (Fig. 1-1) (Gouzi et al., 2011; Rukstalis and Habener, 2007), after which Ngn3 is downregulated, and expression of one of the pancreatic hormones is activated (Gradwohl et al., 2000; Gu et al., 2002; Schwitzgebel et al., 2000). Lineage tracing experiments using Ngn3-CreER have shown that Ngn3 expressing cells give rise to all of the endocrine cell types and do not give rise to ductal cells (Gu et al., 2002; Heller et al., 2005; Prado et al., 2004; Suissa et al., 2013). Ngn3 expression is transient, with the peak of

$Ngn3^+$ cells corresponding with the major wave of endocrine cell differentiation occurring at approximately E15.5 (Schwitzgebel et al., 2000).

Initial expression of Ngn3 appears to be controlled by Notch signaling, possibly through a mechanism of lateral inhibition. There are numerous Notch ligands and receptors expressed in the pancreatic epithelium during development (Jensen et al., 2000b; Lammert et al., 2000). Studies manipulating Notch signaling have elucidated a functional role in endocrine fate specification. *Delta-like gene 1* (*Dll1*) and *RBPJk* are both components of the Notch pathway. Mutant *Dll1* and *RBPJk* mice are embryonically lethal at E18.5-9.5 and E10-12, respectively. However, early analysis of endocrine cell specification show that both mutants exhibit increased $Ngn3^+$ cell numbers. *Dll1* mutant mice also show decreased pancreatic bud size (Apelqvist et al., 1999). In mice deficient for *Hes1*, a downstream factor of Notch signaling, there is accelerated endocrine differentiation, particularly of the alpha cell lineage, at the expense of the other pancreatic cell types and pancreas organ size (Jensen et al., 2000b). These findings suggest that Notch signaling may determine cell fate choice between endocrine and ductal lineages within the trunk epithelium.

Ngn3 plays a pivotal function in endocrine specification; it is essential for the formation of all of pancreatic endocrine cell types. $Ngn3^{-/-}$ mice display complete loss of the endocrine cell populations: alpha, beta, delta, epsilon, PP and G cells (Gradwohl et al., 2000; Heller et al., 2005; Suissa et al., 2013). This disruption of endocrine specification results in perinatal lethality due to hyperglycemia (Gradwohl et al., 2000). There is no increase in apoptosis, suggesting the endocrine precursors are mis-specified, rather than abolished (Gradwohl et al., 2000). These

findings demonstrate a strict requirement for Ngn3 to allow endocrine cell fate commitment. While Ngn3 is required for endocrine specification, it is not known when endocrine cell fate is determined. This process may be occurring early within the $Pdx1^+$ pancreatic progenitor, or it may be occurring later in the $Ngn3^+$ endocrine lineage.

The importance of Ngn3 for islet cell specification may be conserved in human pancreas development as well. In a case of permanent neonatal diabetes, compound heterozygous point mutations were identified in NGN3, with each mutation capable of rendering the protein inactive (Rubio-Cabezas et al., 2011). In another study, a homozygous nonsense mutation at amino acid 123 was identified in NGN3 and associated with neonatal diabetes (Pinney et al., 2011). These results suggest NGN3 plays a role in human islet cell specification.

Ectopic and overexpression studies have further elucidated the function of Ngn3 during mouse development. Mice with Pdx1 promoter-driven expression of *Ngn3* show decreased pancreatic bud size with increased endocrine cell numbers as marked by glucagon expression and the endocrine factor Isl1 (Apelqvist et al., 1999). Another study analyzed the effect of ectopic Ngn3 expression driven by the Pdx1 promoter, and reported similar findings (Schwitzgebel et al., 2000). There was increased and early differentiation of endocrine cells in these mutant mice, which were predominantly glucagon-producing alpha cells (Schwitzgebel et al., 2000). Ectopic *Ngn3* expression was also performed in chick embryos via *in ovo* electroporation. This was sufficient to drive endocrine cell differentiation in regions ectopic to normal pancreas development, including the esophagus. These endocrine cells expressed either glucagon or somatostatin and were found in clusters in the adjacent mesenchyme. Insulin-expressing or

pancreatic polypeptide-expressing cells were not found (Grapin-Botton et al., 2001). This is in contrast to the ectopic expression of Pdx1, which was insufficient for endocrine cell fate induction (Grapin-Botton et al., 2001). These studies elucidated a functional role of Ngn3 for the induction of endocrine cell fate, and revealed the requirement for additional factors to differentiate non-alpha cell endocrine identities.

Recently, $Ngn3^{+}$ cell potency has been of particular interest because of the current optimization of *in vitro* beta cell differentiation efficiency. While these cells have traditionally been thought to be multipotent and capable of giving rise to any of the endocrine cell types, recent studies have suggested restriction in their potential (Desgraz and Herrera, 2009; Johansson et al., 2007). The results of these studies are surprising and relevant to *in vitro* differentiation protocols for diabetic replacement therapies. If the $Ngn3^{+}$ cells generated during these protocols are pre-biased in their endocrine fate, then it will be of utmost importance to characterize this heterogeneity and identify discriminating markers to improve beta cell generation efficiency.

The first of these recent studies identified temporal windows of endocrine fate competence throughout developmental progression. Johansson et al. (2007) employed a Ngn3 "addback" approach to enable temporal control of endocrine differentiation. *Ngn3* expression was induced at numerous developmental time points using a Pdx1 promoter-driven Ngn3-ER allele in a $Ngn3^{-/-}$ mouse background. The type of endocrine cell specified at each time point was characterized. Surprisingly, strict windows of endocrine cell competence were found throughout pancreas development. The first cells capable of forming were alpha cells, beginning at E8.7. This is consistent with the Ngn3 ectopic expression studies, which found preferential differentiation of

alpha cells (Apelqvist et al., 1999; Grapin-Botton et al., 2001; Schwitzgebel et al., 2000). Competence to form beta cells and PP cells was acquired between E10.5 and E12.5. Finally, delta cells are able to form after E14.5 (Johansson et al., 2007). This data suggests that progenitor cells acquire competence for different endocrine fates during distinct temporal windows of development. These results are also consist with other studies showing that $Ngn3^+$ endocrine progenitor cell competence is cell autonomous and not due to instructive cues from the surrounding mesenchyme during development (Gittes, 2009).

To directly trace the fate of single $Ngn3^+$ cells, Desgraz and Herrera (2009) used mosaic analyses with double markers (MADM) to allow *in vivo* clonal analysis of endocrine progenitors. They found that single $Ngn3^+$ cells gave rise to single hormone-producing endocrine cells. This is consistent with earlier studies suggesting $Ngn3^+$ cells are quiescent (Gu et al., 2002; Jensen et al., 2000a; Miyatsuka et al., 2011). Adult analysis showed small clusters of labelled cells that all produced the same hormone, indicating minimal postnatal proliferation. Desgraz and Herrera proposed a model in which $Ngn3^+$ cells are unipotent, suggesting the entire $Ngn3^+$ endocrine progenitor population is comprised of multiple unipotent subpopulations (Desgraz and Herrera, 2009).

These studies suggest temporal and cell autonomous mechanisms of endocrine fate regulation; however, the precise mechanism of this process is still poorly understood. Elucidating these specification mechanisms may provide crucial information essential to improve *in vitro* beta cell differentiation.

Rfx6

While Pdx1 and Ngn3 mark the pancreatic and endocrine progenitor populations, respectively, these are just two examples of many transcription factors that play a role in endocrine cell specification (Fig. 1-2). Transgenic mice with mutations in single transcription factors have been characterized and their requirement for beta cell specification is well-established. Many of these factors are also thought to regulate each other in a hierarchical manner. Rfx6, NeuroD1, and Nkx2.2 are three such factors known to be required for beta cell specification.

Rfx6 is a winged helix transcription factor recently identified and implicated in beta cell development. Rfx6 is transiently expressed in the $Pdx1^{+}$ pancreatic progenitors from E9.0 to E10.5. After this time, Rfx6 is expressed in the $Ngn3^{+}$ endocrine progenitors and in alpha, beta, delta, and PP endocrine lineages (Soyer et al., 2010). Soyer et al. (2010) suggested that Rfx6 functions downstream of Ngn3, since its expression is lost in $Ngn3^{-/-}$ animals. In addition, they suggested that Rfx6 is either upstream or independent of NeuroD1 since *Rfx6* gene expression is unchanged in $NeuroD1^{-/-}$ animals (see below) (Naya et al., 1997; Soyer et al., 2010).

Rfx6 morpholino experiments in zebrafish showed disrupted endocrine cell specification: loss of alpha and epsilon cells, decreased delta cells, and a modest decrease in beta cells (Pearl et al., 2011; Soyer et al., 2010). Another study characterized $Rfx6^{-/-}$ mice, which display loss of all endocrine cell types, except PP cells (Smith et al., 2010). The expression of *Ngn3* and *Nkx2.2* were unchanged in these mice, suggesting Rfx6 functions downstream of those factors. However, *NeuroD1* expression was decreased, suggesting Rfx6 regulates NeuroD1 (Smith et al.,

2010). These results reveal a functional role for Rfx6 in endocrine fate determination in both zebrafish and mice.

Mutations in human RFX6 have been identified in cases of neonatal diabetes. Smith et al. (2010) detected missense, splicing, and frameshift mutations in five out of six autosomal recessive cases of neonatal diabetes (Smith et al., 2010). Additional case studies of neonatal diabetes have identified missense mutations in RFX6 which were predicted to disrupt splicing or RFX6 function (Chandra et al., 2014; Concepcion et al., 2014). As observed with PDX1 and NGN3, RFX6 also appears to function in a similar manner across species.

NeuroD1

NeuroD1 (aka BETA2) is a basic helix-loop-helix (bHLH) transcription factor. This factor was first isolated based on its binding to an E-box sequence within the *insulin* gene promoter, and NeuroD1 was found to activate *insulin* expression (Naya et al., 1995). *NeuroD1* expression can be detected in alpha, beta, and delta cells (Naya et al., 1997; Naya et al., 1995). Co-expression analysis of NeuroD1 and Ngn3 in the developing pancreas reveals that 40% of NeuroD1^{+} cells co-express *Ngn3* mRNA at E9.0. This co-expression decreases in frequency at E14.5 and E16.5 but can still be detected (Huang et al., 2000). The specific expression pattern of NeuroD1 and its regulation of *insulin* implicates it in beta cell development.

To explore the function of NeuroD1 in beta cell specification, NeuroD1$^{-/-}$ mice have been analyzed. NeuroD1$^{-/-}$ mice display severe hyperglycemia at birth and die perinatally (Naya et al., 1997). These mice show a large decrease in beta cell numbers by approximately E17.5 and also contain disorganized islet structures and endocrine cell distribution (Naya et al., 1997). The

severity of this phenotype may be dependent on mouse genetic background (Huang et al., 2002). This implicates NeuroD1 as an essential regulator of beta cell differentiation during late pancreas development and is necessary to establish proper beta cell numbers. NEUROD1 frameshift mutations have also been identified in humans presenting with permanent neonatal diabetes (Rubio-Cabezas et al., 2010), and NEUROD1 mutations have been associated with an increased risk for the development of diabetes (Malecki et al., 1999), suggesting conservation of NeuroD1 function.

NeuroD1 is known to function as a transcriptional activator. In addition to its role in regulating *insulin* gene expression, NeuroD1 regulates an important beta cell maturation factor, MafA (Artner et al., 2010; Wang et al., 2006; Zhang et al., 2005). In an *in vitro* beta cell line, NeuroD1 binds an enhancer sequence of *MafA*, which is highly conserved in humans. Mutation of the NeuroD1 binding motif results in decreased activation, suggesting that NeuroD1 directly activates *MafA* in beta cells (Raum et al., 2010).

While NeuroD1 transcriptionally regulates numerous downstream factors essential for beta cell formation and function, many transcription factors lie upstream of NeuroD1. One direct regulator of NeuroD1 is Ngn3. Ngn3 has been found to bind and regulate NeuroD1 directly at its promoter (Anderson et al., 2009a; Huang et al., 2000). Additionally, overexpression of Ngn3 in *Xenopus* and in an *in vitro* beta cell line also results in NeuroD1 induction (Huang et al., 2000). Since *Ngn3* expression is also not changed in NeuroD1$^{-/-}$ mice, this supports a hierarchical model with NeuroD1 downstream of Ngn3 (Schwitzgebel et al., 2000).

Nkx2.2

Nkx2.2 is a homeodomain transcription factor expressed in the central nervous system (CNS), intestine, and pancreas (Arnes et al., 2012b; Price et al., 1992; Sussel et al., 1998). Nkx2.2 was first identified in the developing CNS, in the hindbrain and forebrain (Price et al., 1992). It is involved in the differentiation of V3 interneurons (Briscoe et al., 2000; Ericson et al., 1997), oligodendrocytes (Qi et al., 2001; Zhou et al., 2001), and serotonergic neurons (Briscoe et al., 1999; Pattyn et al., 2003). In the intestine, Nkx2.2 is expressed in many of the enteroendocrine cells and a fraction of the $Ngn3^{+}$ endocrine progenitor population (Desai et al., 2008; Wang et al., 2009). Nkx2.2 is required in the intestine for proper enteroendocrine cell specification (Desai et al., 2008; Wang et al., 2009). Similarly, in the pancreas, Nkx2.2 is necessary for the differentiation of endocrine cell lineages (Sussel et al., 1998).

Nkx2.2 is expressed early in pancreas development, coincident with Pdx1, in the emerging dorsal bud at E8.75 and the ventral bud at E9.5 (Jorgensen et al., 2007; Sussel et al., 1998). Its expression is maintained throughout pancreas development, during endocrine specification, and later in the alpha, beta, PP, and G cells of the pancreas (Suissa et al., 2013; Sussel et al., 1998). Nkx2.2 expression precedes that of Ngn3 (Schwitzgebel et al., 2000).

Nkx2.2 is essential for proper endocrine cell specification and for beta cell formation. $Nkx2.2^{-/-}$ mice display a complete loss of beta cells, a decrease in alpha, G, and PP cells, and a striking increase in epsilon cells (Prado et al., 2004; Suissa et al., 2013; Sussel et al., 1998). These mice are hyperglycemic and die shortly after birth (Sussel et al., 1998). This phenotype demonstrates the importance of Nkx2.2 in establishing proper endocrine cell ratios. However, due to the early

and prolonged expression of Nkx2.2 during pancreas development, the timing of Nkx2.2 influence on endocrine specification is not known.

The precise mechanism of Nkx2.2 action is currently under investigation. The Nkx2.2 protein contains three conserved domains: a homeodomain, tinman (TN) domain, and NK2 specific (SD) domain (Fig. 1-3A). The homeodomain is responsible for the DNA-binding activity of Nkx2.2 (Kim and Nirenberg, 1989; Tsao et al., 1995; Watada et al., 2000), which recognizes the motif T(t/c)AAGT(a/g)(c/g)TT and a second core motif GAGT (Hill et al., 2011; Watada et al., 2000). In addition, recent evidence has suggested that Nkx2.2 frequently binds to distal enhancers for transcriptional regulation (Dominguez Gutiérrez et al., 2016). The TN domain is essential for proper beta cell specification and mediates interaction with Groucho 3 (Grg3), a co-repressor (Doyle et al., 2007; Papizan et al., 2011). Lastly, the SD domain is a feature unique to the NK2 family of proteins. Watada et al. (2000) found that the SD domain does not contribute to DNA binding, and that it may be involved in masking strong activation driven by the C-terminus (Watada et al., 2000). While these studies were confined to *in vitro* cell lines, our lab has investigated the role of the Nkx2.2 SD domain *in vivo* during the development of the pancreas and CNS (Levine et al., in preparation).

To influence endocrine cell fate, Nkx2.2 functions both as a transcriptional activator and a repressor. Mice containing an Nkx2.2-dominant-repressor fusion protein (Nkx2.2hd-EnR) show rescued beta cell differentiation in an Nkx2.2$^{-/-}$ background. However, expression of the mature beta cell factor, MafA, and the beta cell glucose transporter, Glut2, is not rescued (Doyle et al.,

2007). This suggests Nkx2.2 repressor activity is sufficient to specify the beta cell lineage, though Nkx2.2 activating functions are necessary to establish a fully functional beta cell fate.

NKX2-2 has also been implicated in human pancreatic development. Nonsense or frameshift mutations in NKX2-2 were identified in three patients with defects in insulin secretion and hyperglycemia early in life (Flanagan et al., 2014).

Given the early expression of Nkx2.2 and its importance during pancreatic beta cell specification, both in mice and humans, this thesis work focuses on elucidating the timing and mechanism of Nkx2.2 action.

Rfx6, NeuroD1, and Nkx2.2 mutant mice have been thoroughly characterized, and their essential function in beta cell specification is well-established. These beta cell developmental factors are thought to function in a transcriptional hierarchy. Nkx2.2 appears to function upstream of Rfx6 and NeuroD1 since expression of each factor is reduced in Nkx2.2$^{-/-}$ mice (Chao et al., 2007) (Fig. 2-3E), while *Nkx2.2* expression is unchanged in Rfx6$^{-/-}$ and NeuroD1$^{-/-}$ mice (Chao et al., 2007; Smith et al., 2010). Furthermore, Ngn3 and Nkx2.2 have been shown to cooperate for *NeuroD1* activation through direct regulation of the *NeuroD1* promoter, suggesting both Nkx2.2 and Ngn3 function upstream of NeuroD1 (Anderson et al., 2009a). Ngn3 is also believed to function upstream of NeuroD1 since Ngn3^{+} cell numbers are not changed in NeuroD1$^{-/-}$ mice at E18.5 (Schwitzgebel et al., 2000). Nkx2.2 has been shown to affect *Ngn3*, with Nkx2.2$^{-/-}$ mice showing reduced *Ngn3* expression. Although Ngn3^{+} cell numbers are unaffected in Nkx2.2$^{-/-}$ mice (at E15.5 or E18.5), reduced levels of Ngn3 at an individual cell level may influence endocrine potential (Anderson et al., 2009b; Schwitzgebel et al., 2000). Lastly, Nkx2.2 and

NeuroD1 may genetically interact during endocrine cell specification. Nkx2.2$^{-/-}$; NeuroD1$^{-/-}$ compound mutant mice show complete beta cell loss, consistent with the Nkx2.2$^{-/-}$ phenotype. However, alpha, PP, and epsilon cell populations are partially rescued in Nkx2.2$^{-/-}$; NeuroD1$^{-/-}$ mice compared to Nkx2.2$^{-/-}$ mice (Chao et al., 2007). This study revealed an epistatic relationship between Nkx2.2 and NeuroD1 in endocrine specification. Together, these findings suggest that Nkx2.2 is upstream of Rfx6 and NeuroD1, and that these factors may interact directly or indirectly to regulate endocrine cell fate (Fig. 1-3B). Spatiotemporal analysis of such interactions has yet to be investigated.

Human Pancreas Development

As previously described, sequencing of patients with neonatal diabetes has provided insight into the conservation of transcription factor function between mice and humans. Additional studies have explored transcription factor expression patterns during human pancreas development.

Similar to murine pancreas development, PDX1 is expressed in humans in the newly emerging pancreatic bud at 26 days post conception (dpc) (Piper et al., 2004). Additionally, it's been shown that between gestational week 7 (G7w, gestation 7 week) to G21w, PDX1 is expressed in the proliferating epithelial progenitor cells (Jeon et al., 2009).

NGN3 is expressed beginning at G9w and is highly expressed until G17w (Jeon et al., 2009). During this time, islets begin to form at approximately G11w (Pan and Wright, 2011), with large increases in INSULIN, GLUCAGON, and SOMATOSTATIN occurring between G7w and G18w (Jeon et al., 2009; Polak et al., 2000). Early endocrine cells have been found to co-express multiple hormones; however, the frequency of these polyhormonal cells remains controversial

(Polak et al., 2000; Sarkar et al., 2008). This developmental time point is similar to the second wave of endocrine cell differentiation observed in mice between E13.5 and E18.5, with the peak of Ngn3 expression at E15.5 (Pan and Wright, 2011; Schwitzgebel et al., 2000). This expression pattern, with NGN3 expression tightly linked to endocrine cell differentiation, suggests a similar mechanism of endocrine fate induction between species.

NKX2-2 expression has been observed from G7w to G23w; however, its expression earlier than G7w has yet to be identified (Jeon et al., 2009; Sarkar et al., 2008). Additionally, NEUROD1 expression is first observed after NKX2-2, at G15w (Jeon et al., 2009).

One major difference between adult murine and human pancreas lies in the structure of the islet of Langerhans. In adult mice, beta cells are clustered predominantly in the center of the islet, with other endocrine cell types scattered in the periphery (Levetan and Pierce, 2013). In contrast, adult human islets are structured with all endocrine cell types intermingled amongst each other, with no apparent organization resembling the mouse (Levetan and Pierce, 2013). Interestingly, recent studies have discovered human islets show variability in structure, with smaller islets showing arrangements similar to murine organization and larger islets containing intermingled cell types (Bonner-Weir et al., 2015). In addition, human endocrine cells may go through a temporary clustered state during development where $INSULIN^+$ cells are located in the center of the islet and $GLUCAGON^+$ cells are in the periphery at G14w (Jeon et al., 2009). These findings provide additional evidence of shared mechanisms of endocrine cell specification across species, and validate the utility of murine pancreas developmental studies.

Maintenance of Beta Cell Identity and Function

While cell fate was considered to be determined developmentally, recent studies have unexpectedly identified residual plasticity in terminally differentiated cells, including pancreatic beta cells.

Under diabetic conditions, pancreatic beta cells can undergo numerous transformations including loss of function, loss of identity, dedifferentiation, and/or reprogramming. These transformations have been noted in diabetic mouse models, including: beta cell-specific FoxO1 ablation ($FoxO1^{\Delta beta}$), GIRKO, and *db/db* mice. Lineage-tracing in $FoxO1^{\Delta beta}$ mice shows loss of beta cell maturation markers and expression of developmental factors including Ngn3, Oct4, Nanog, and L-Myc. Additionally, these cells can go on to express glucagon. This process of "dedifferentiation" was also observed in GIRKO and *db/db* diabetic mouse models; beta cells showed loss of mature factors, such as MafA, and an increase in Ngn3 (Talchai et al., 2012). Additional studies have demonstrated the loss of mature beta cell factors under stressful conditions similar to the conditions induced during diabetes (Guo et al., 2013). These results demonstrate not only the plasticity of beta cells, but also the relevance of this plasticity during diabetes disease progression.

Beta cell transdifferentiation can also occur when beta cells express other pancreatic hormones. Typically this process does not necessarily involve dedifferentiation, which is characterized by the expression of progenitor factors, such as Ngn3. The process of transdifferentiation has been suggested to occur in cases of both T1D and T2D. In non-obese diabetic (NOD) mice, a mouse model of T1D, and in human islets from patients with T1D, cells co-expressing insulin and

somatostatin can be found, potentially indicative of transdifferentiation (Piran et al., 2014). In newly diagnosed cases of T2D, $INSULIN^{+}GLUCAGON^{+}$ and $INSULIN^{+}SOMATOSTAIN^{+}$ co-expressing cells can also be found (White et al., 2013; Yoneda et al., 2013). These polyhormonal cells were sometimes found to co-express NGN3 (Yoneda et al., 2013). These mechanisms of transdifferentiation, dedifferentiation and loss of beta cell identity appear to be integral components of diabetes progression; therefore, analysis of the mechanisms involved could prove useful for the development of novel disease therapies.

Transcription factors important for beta cell specification during embryonic development have also been implicated in the maintenance of beta cell fate within adult mice. While Pdx1 is expressed in the pancreatic progenitor population, it is also expressed in murine and human beta cells (Jeon et al., 2009; Ohlsson et al., 1993; Piper et al., 2004). Beta cell specific deletion of Pdx1 with rat insulin promoter (RIP)-Cre; $Pdx1^{flox/flox}$ mice reveals an important role for Pdx1 in maintaining beta cell identity and function. These mice show decreased insulin expression, increased glucagon expression and increased $insulin^{+}glucagon^{+}$ co-positive cells. There is also decreased expression of the glucose transporter, Glut2, which is essential for proper glucose-stimulated insulin secretion (GSIS) (Guillam et al., 1997). As a result of these defects, RIP-Cre; $Pdx1^{flox/flox}$ mice develop diabetes, demonstrating a requirement for Pdx1 within the beta cell to maintain its proper function in glycemic control (Ahlgren et al., 1998). Another study induced Pdx1 ablation at 1 month of age in RIP-CreER; $Pdx1^{flox/flox}$ mice. These mice displayed loss of beta cell identity, with decreases in insulin and Glut2 expression. In addition, lineage-tracing revealed beta to alpha cell conversion, with lineage-labeled cells expressing glucagon and other transcription factors important in alpha cell identity, such as MafB and Arx (Gao et al., 2014).

Rfx6 is also necessary within the adult beta cell to maintain function. Piccand et al. (2014) ablated Rfx6 at 8 to 10 weeks of age with tamoxifen administration of Ins1-CreER; Rfx6$^{flox/flox}$ mice. These mice displayed hyperglycemia and glucose intolerance, likely resulting from defective insulin secretion and decreased expression of genes important for GSIS (Piccand et al., 2014). There was also inappropriate activation of genes that are selectively repressed in normal islets (Piccand et al., 2014), which are called "disallowed" genes (Pullen et al., 2010; Thorrez et al., 2011). Rfx6 is also expressed and required in human beta cells. In the EndoC-betaH2 human beta cell line, RFX6 knockdown results in defective insulin secretion (Chandra et al., 2014). These studies highly an important function for Rfx6 in adult beta cells both in mice and humans.

Recently Nkx2.2 has been implicated in maintaining beta cell function and identity. RIP-Cre; Nkx2.2$^{flox/flox}$ and MIP-CreER; Nkx2.2$^{flox/flox}$ mice display glucose intolerance with decreases in insulin, Glut2, Pdx1, and other genes important for beta cell function. These mice also contain polyhormonal cells, with frequent co-expression of insulin with glucagon, somatostatin, or pancreatic polypeptide found in adult islets. In addition, knockdown of NKX2-2 in human islets resulted in similar effects, revealing species conservation (Dominguez Gutiérrez et al., 2016).

Lastly, NeuroD1 is required to maintain functional maturity in beta cells. Beta cell-specific ablation of NeuroD1 in RIP-Cre; NeuroD1$^{flox/flox}$ mice results in glucose intolerance resulting from impaired GSIS. A large increase in insulin^{+}somatostatin^{+} co-positive cells was observed and there was a significant decrease in Glut2 protein expression and insulin content per cell.

Interestingly, Pdx1 and MafA expression was unchanged, indicating NeuroD1 is not important for maintaining expression of these factors (Gu et al., 2010).

These studies have revealed essential roles for Pdx1, Rfx6, Nkx2.2, and NeuroD1 in maintaining adult beta cell identity. It is possible that these transcription factors exert their effects through cooperative binding at enhancer regions. Recently, transcription factor ChIP-Seq experiments (including PDX1 and NKX2.2) were conducted on isolated human islets and cross referenced to histone marks indicative of promoters, active enhancers, and inactive enhancers. These results showed numerous transcription factors, including PDX1 and NKX2.2 binding to enhancers, implicating co-regulation of islet genes. In addition, RFX and bHLH motifs were identified in these enhancer regions, suggesting binding of RFX6 and NEUROD1 is also likely. Moreover, T2D risk variants were frequently found at these distal enhancer regions, suggesting functional relevance of these sites (Pasquali et al., 2014). These findings may reflect a common mechanism of beta cell gene regulation, where co-binding of numerous transcription factors regulate genes through enhancers.

Given the complex regulatory mechanisms associated with the development, differentiation and maintenance of beta cells, there are still many outstanding questions regarding islet cell specification. Overall, this thesis work investigates the timing and mechanism of pancreatic beta cell specification. Previous studies have shown that Nkx2.2 is an essential transcription factor for beta cell formation and establishing proper ratios of endocrine cell fate (Prado et al., 2004; Sussel et al., 1998). By ablating this crucial transcription factor at an intermediate

developmental time point, the temporal regulation of beta cell differentiation is investigated. Through careful analysis of the endocrine cell lineages and comparison to the Nkx2.2$^{-/-}$ phenotype, we identified a critical window of development. In addition, ChIP-Seq datasets for Nkx2.2 (Dominguez Gutiérrez et al., 2016), Rfx6 (Piccand et al., 2014), and NeuroD1 (Tennant et al., 2013) were cross-referenced, identifying co-occupancy among these beta cell transcription factors near endocrine-related genes. These results are indicative of combinatorial interactions and transcription factor cooperation during cell fate specification.

Furthermore, the mechanism of Nkx2.2 function was assessed using mice with mutations in a conserved domain, the Nkx2.2 SD domain. This thesis work revealed an important function for the SD domain in mediating the role of Nkx2.2 in maintaining beta cell function. In addition, the transcriptional profile of endocrine progenitors was assessed, revealing early transcriptional regulation of endocrine cell fate.

These studies have revealed novel processes involved in beta cell specification, in addition to the maintenance of beta cell function. A current area of research for novel diabetic therapies involves the differentiation of beta cells *in vitro* and transplantation of these cells into patients to restore glucose homeostasis. The features of *in vivo* beta cell development that have been explored in this work may prove to be useful for the optimization of *in vitro* beta cell differentiation.

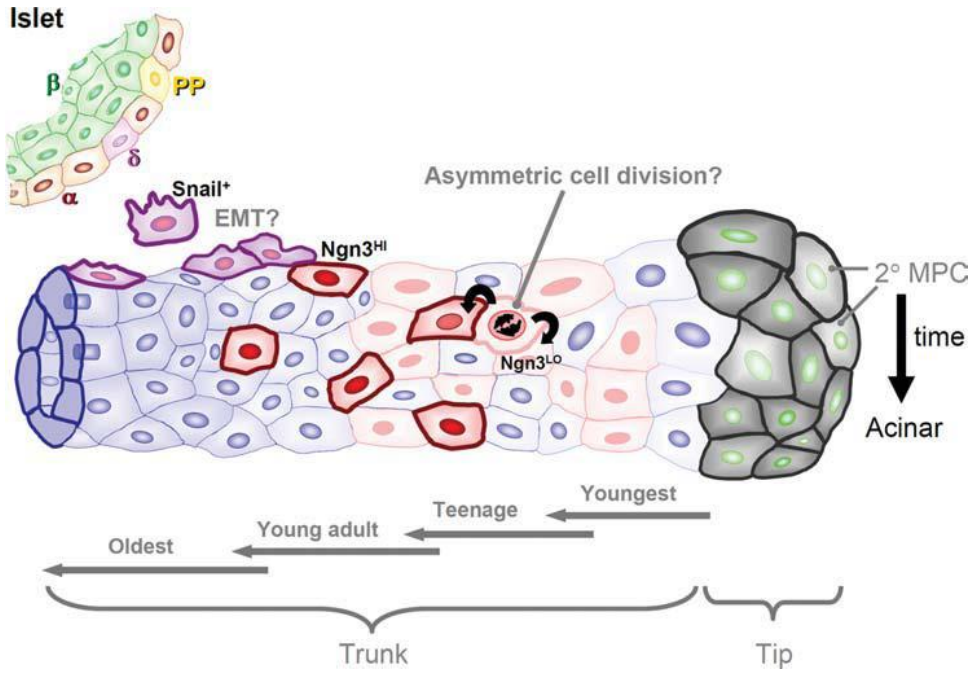


Figure 1-1. Endocrine progenitor delamination and migration. Following Ngn3 expression, endocrine progenitor cells delaminate from the pancreatic trunk epithelium. These cells are thought to undergo a process similar to epithelial-mesenchymal transition (EMT). They migrate and eventually coalesce to form the islets of Langerhans (Pan and Wright, 2011).

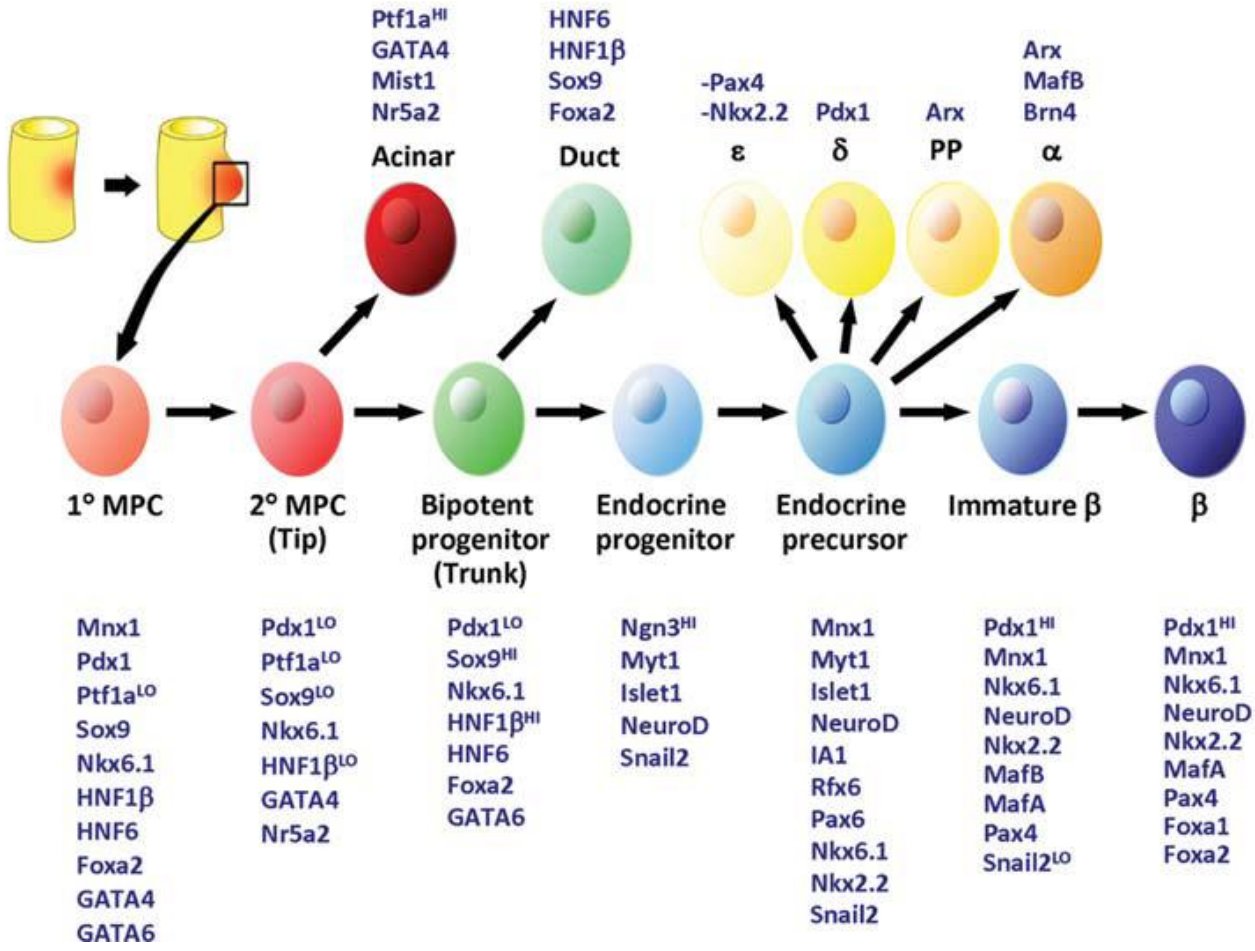


Figure 1-2. Transcription factors involved in pancreas development. Numerous transcription factors are expressed at each stage of pancreas development and beta cell specification (Pan and Wright, 2011).

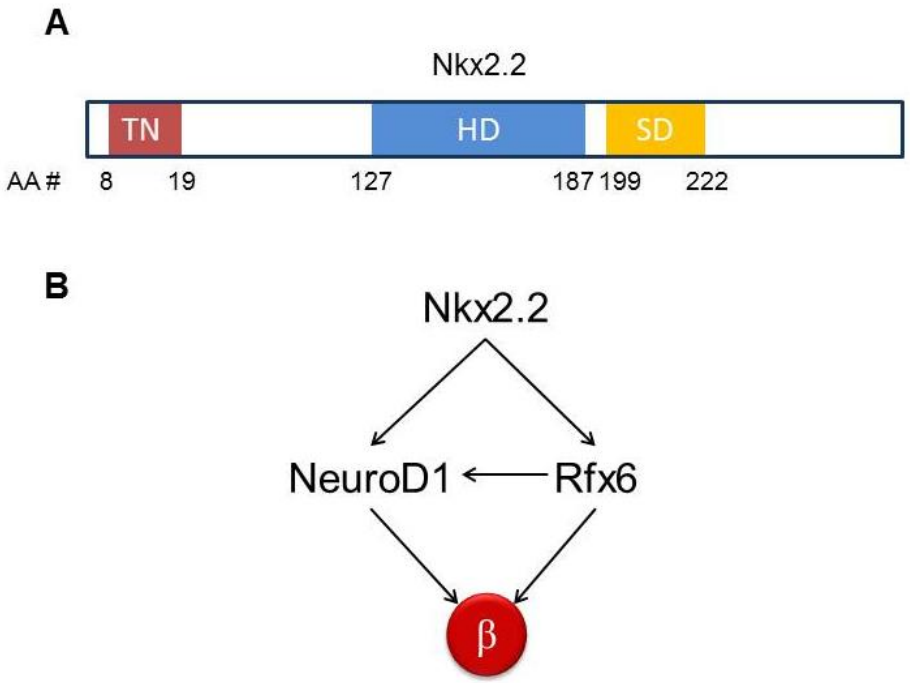


Figure 1-3. Nkx2.2 structure and function. (*A*) Nkx2.2 contains a tinman (TN) domain, homeodomain (HD), and specific domain (SD). (*B*) Nkx2.2 functions upstream of NeuroD1 and Rfx6 for beta cell differentiation.

CHAPTER 2

SUBMITTED MANUSCRIPT: NKX2.2 REPRESENTS AN ESSENTIAL COMPONENT OF A MODULAR REGULATORY PROGRAM TO INDUCE PANCREATIC BETA CELL FATE

Angela Churchill generated all data except for the ChIP-Seq analyses. The Nkx2.2 ChIP-Seq was performed by Giselle Dominguez Gutiérrez (Dominguez Gutiérrez et al., 2016; GSE79725). Bioinformatic analysis and Fisher's Exact Test was conducted by Ruth Singer.

Abstract

Many transcription factors that are essential for islet cell differentiation have been well characterized; however, the precise timing and functional hierarchy by which these factors regulate beta cell fate induction remains unknown. To elucidate the spatiotemporal mechanism of beta cell differentiation, we ablated Nkx2.2, one of the earliest and most broadly expressed islet transcription factors specifically in the Ngn3 endocrine progenitor lineage ($Nkx2.2^{\Delta endo}$). Remarkably, $Nkx2.2^{\Delta endo}$ mice display defective beta cell differentiation and recapitulate the $Nkx2.2^{KO}$ phenotype. Furthermore, we demonstrate that despite the striking defect in beta cell formation, expression of many essential components of the beta cell regulatory network that are regulated by Nkx2.2 in pancreatic progenitor cells remain intact in the $Nkx2.2^{\Delta endo}$ mice. This suggests that within the Ngn3 progenitor Nkx2.2 functions as an obligatory component of a modular regulatory program to induce beta cell fate, and illustrates the combinatorial, rather than hierarchical nature of the beta cell regulatory pathways. Accordingly, a large proportion of Nkx2.2 regulated genes are co-bound by NeuroD1 and Rfx6. These cooperative transcriptional

modules may reflect a fundamental feature of beta cell fate specification, and will have significant implications for the optimization of *in vitro* beta cell differentiation protocols.

Introduction

Pancreatic beta cell specification involves the progressive restriction of progenitor-cell lineage potential through the sequential expression of several essential transcription factors. Pancreatic bud initiation is associated with the expression of pancreatic and duodenal homeobox 1 (Pdx1), a transcriptional regulator that delineates the multipotent pancreatic progenitor population (Jonsson et al., 1994; Offield et al., 1996). These $Pdx1^+$ cells give rise to all cell types of the mature pancreas and deletion of $Pdx1^+$ leads to pancreatic agenesis (Burlison et al., 2008; Gu et al., 2003; Jonsson et al., 1994; Offield et al., 1996). A subset of cells from the $Pdx1^+$ lineage subsequently become restricted to the endocrine lineage with the expression of Neurogenin 3 (Ngn3), which delineates the endocrine progenitor. Ngn3 is essential for the formation of all six pancreatic endocrine cell types: the insulin-producing beta cells, glucagon-producing alpha cells, somatostatin-producing delta cells, pancreatic polypeptide-producing PP cells, gastrin-producing G cells, and ghrelin-producing epsilon cells (Gradwohl et al., 2000; Gu et al., 2002; Heller et al., 2005; Schwitzgebel et al., 2000; Suissa et al., 2013). Mice lacking Ngn3 fail to form these endocrine lineages (Gradwohl et al., 2000; Heller et al., 2005; Suissa et al., 2013).

Although it is well-established that beta cells are derived from the Ngn3 progenitor lineage, there is still some uncertainty about when and where the beta cell lineage is specified during pancreas development. Beta cell fate may be influenced early within the $Pdx1^+$ pancreatic progenitor to specify unipotent populations of $Ngn3^+$ endocrine progenitor cells (Desgraz and Herrera, 2009)

or it may be occurring within the $Ngn3^{+}$ endocrine progenitor lineage after induction of Ngn3. Interestingly, Johansson et al. (2007) demonstrated that pancreatic progenitor cells transition through distinct competence windows in response to Ngn3 expression for each endocrine cell fate, with beta cell competence being acquired during a strict window of development, between E10.5 and E12.5 (Johansson et al., 2007). Although many of the transcription factors essential for beta cell specification have been identified, we still have an incomplete understanding of how these factors regulate the timing and mechanism of beta cell fate induction since they are expressed both early and throughout pancreas development. For example, Glis3, NeuroD1, Nkx2.2 and Rfx6 are all expressed in multipotent pancreatic progenitors *and* endocrine progenitors. All four of these mouse mutants die shortly after birth due to their severe defects in beta cell development (Naya et al., 1997; Prado et al., 2004; Smith et al., 2010; Suissa et al., 2013; Sussel et al., 1998); however, it is unknown exactly when each of these factors exert their functional role in beta cell fate specification.

To gain a better understanding of both the timing and mechanism of beta cell fate induction we chose to focus on the essential islet transcription factor, Nkx2.2. Beta cell fate specification is completely dependent on Nkx2.2; no beta cells are formed in mice carrying a -/- mutation in Nkx2.2 (Sussel et al., 1998). Nkx2.2 is co-expressed with Pdx1 throughout the pancreatic progenitor domain, but its expression becomes restricted to the $Ngn3^{+}$ endocrine progenitor population (Arnes et al., 2012b; Jorgensen et al., 2007; Sussel et al., 1998). Nkx2.2 also appears to function at or near the top of the regulatory cascade of transcription factors to specify beta cell fate: The expression of *Ngn3, Nkx6.1*, *Rfx6* and *NeuroD1* is significantly reduced in $Nkx2.2^{KO}$ progenitor populations (Anderson et al., 2009a; Chao et al., 2007) (Supplemental Table 2-1),

suggesting that Nkx2.2 functions upstream of these factors. The early expression of Nkx2.2 within the multipotent progenitor population, upstream of Ngn3 prompted us to determine whether Nkx2.2 also functioned downstream of Ngn3 to influence islet cell specification. We used Ngn3-Cre; Nkx2.2$^{flox/flox}$ (Nkx2.2$^{\Delta endo}$) mice to ablate Nkx2.2 specifically within the Ngn3^{+} endocrine progenitor lineage. Although Nkx2.2 is expressed upstream of Ngn3 and regulates Ngn3 expression (Anderson et al., 2009a; Sussel et al., 1998), deletion of Nkx2.2 downstream of Ngn3 resulted in severe endocrine defects similar to the Nkx2.2^{KO} phenotype. Notably, Ngn3 expression in the Nkx2.2$^{\Delta endo}$ mice was not affected. This finding identifies a critical window for Nkx2.2 regulation of beta cell specification within the endocrine progenitor cell downstream of Ngn3 activation. Furthermore, unlike the Nkx2.2^{KO} mice, expression of several essential beta cell transcription factors, including Rfx6 and NeuroD1, was unaffected. Co-occupancy analysis revealed significant overlap between Nkx2.2 direct targets and Rfx6 and NeuroD1 bound genes. This indicates that the failure of beta cell formation may not be strictly due to reduction of a linear transcriptional regulatory cascade that functions downstream of Nkx2.2 and implicates Nkx2.2 as an essential component of a modular beta cell transcription network.

Results

Nkx2.2 is efficiently ablated in Nkx2.2$^{\Delta endo}$ mice

Nkx2.2 is expressed at the onset of pancreas development throughout the multipotent progenitor domain, but subsequently becomes restricted to the Ngn3^{+} endocrine progenitor population (Jorgensen et al., 2007). Furthermore, -/- mutations in Nkx2.2 (Nkx2.2^{KO}) lead to reduced Ngn3 expression (Anderson et al., 2009b). To determine whether Nkx2.2 function downstream of Ngn3 is also necessary for endocrine cell specification, *Ngn3-Cre; Nkx2.2$^{flox/flox}$* or *Nkx2.2$^{flox/LacZ}$;*

R26R-Tomato (hereafter referred to as Nkx2.2$^{\Delta endo}$) mice were generated to ablate Nkx2.2 specifically within the endocrine progenitor cells and their descendants (Madisen et al., 2010; Mastracci et al., 2013; Schonhoff et al., 2004). Efficient deletion of *Nkx2.2* in the Nkx2.2$^{\Delta endo}$ mice could be demonstrated by a reduction in *Nkx2.2* mRNA expression at E15.5 and E18.5 (Fig. 2-1A). Residual *Nkx2.2* gene expression at E15.5 likely reflects *Nkx2.2* expression in pancreatic progenitor cells that are still present at this stage of development. Consistent with the decrease in *Nkx2.2* gene expression, Nkx2.2 protein was almost undetectable in the pancreas at E15.5 and postnatal day (P)0 (Fig. 2-1B,C; Supplemental Fig. 2-1A,B). Activation of the *R26R-Tomato* allele was also used to monitor accurate Cre activity within the Nkx2.2$^{\Delta endo}$ mice and, accordingly, Tomato^{+} (Tom^{+}) cells were found specifically within the endocrine compartment (Supplemental Fig. 2-1D,E).

Deletion of Nkx2.2 from the endocrine progenitor population did not affect *Ngn3* expression, nor the Ngn3-expressing endocrine progenitor population (Supplemental Table 2-1, Fig. 2-1D,E). Furthermore, the expression of *Sox9*, a progenitor marker that is expressed upstream of Ngn3 was unaffected and there were no obvious changes in the exocrine lineage, as indicated by amylase expression (Supplemental Fig. 2-1C-E). The area of Tomato expression and *ChromograninA* (*ChgA*), *Isl1*, and *Pax6* gene expression were also unchanged, suggesting endocrine compartment size and pan-endocrine gene expression were unchanged in the absence of Nkx2.2 at E15.5 (Supplemental Fig. 2-1A-C).

Nkx2.2$^{\Delta endo}$ mice display similar endocrine defects as Nkx2.2^{KO} mice

Since Nkx2.2 is necessary for the establishment of proper endocrine cell ratios, we assessed the status of endocrine cell differentiation in the Nkx2.2$^{\Delta endo}$ mice during the course of pancreas development. Similar to the Nkx2.2^{KO} mice (Prado et al., 2004; Sussel et al., 1998), Nkx2.2$^{\Delta endo}$ mice display a reduction of insulin^{+} (Ins^{+}) cells and glucagon^{+} (Gcg^{+}) cells, and increased ghrelin^{+} (Ghrl^{+}) cells at E15.5 and P0 (Fig. 2-2A-H; Supplemental Fig. 2-2). Gastrin^{+} (Gast^{+}) cells are decreased at E15.5 (Fig. 2-2I,J), and no changes were observed in somatostatin^{+} (SST^{+}) cell number at P0 (Fig. 2-2K-L). Unlike the Nkx2.2^{KO} mice which lack all beta cells, we could detect a small number of Ins^{+} cells (Fig. 2-2A-L; Supplemental Fig. 2-2), which is possibly due to the efficiency of the Ngn3-Cre allele. Gene expression at E15.5 and E18.5 corresponded to changes in endocrine cell numbers. *Insulin (Ins1* and *Ins2*) and *glucagon (Gcg)* expression were significantly decreased; there was increased *ghrelin (Ghrl)* expression, and no change in *somatostatin (Sst)* gene expression at E15.5 and E18.5 (Fig. 2-2M). *Gastrin (Gast)* gene expression was also decreased at E15.5, but became normalized by E18.5 (Fig. 2-2M), likely due to the embryo-restricted presence of the G cell population in the pancreas (Suissa et al., 2013). Similar to the Nkx2.2^{KO} mice, the Nkx2.2$^{\Delta endo}$ mice die shortly after birth between P2 and P4 (Fig. 2-2N).

Unexpectedly, we observed a significant increase in (*pancreatic polypeptide*) *Ppy* expression at E15.5 and E18.5 in Nkx2.2$^{\Delta endo}$ embryos (Fig. 2-2M). This is in contrast to our previous studies, in which we had observed a decrease in *Ppy* gene expression and PP^{+} cell numbers (Chao et al., 2007; Prado et al., 2004; Sussel et al., 1998). To confirm the upregulation of *Ppy* in Nkx2.2$^{\Delta endo}$ embryos and directly compare *Ppy* expression in the Nkx2.2^{KO}, we performed qRT-PCR for *Ppy*

expression in E15.5 Nkx2.2$^{LacZ/LacZ}$ (Nkx2.2^{KO}) pancreas (Arnes et al., 2012b). Surprisingly, in this new analysis, *Ppy* expression was upregulated in the Nkx2.2^{KO} (Fig. 2-2O) and it appears that *Ppy* is similarly regulated in the Nkx2.2^{KO} and Nkx2.2$^{\Delta endo}$ embryos, although to a greater magnitude in the Nkx2.2$^{\Delta endo}$ embryos (Fig. 2-2M,O). We could also demonstrate that upregulation of *Ppy* RNA correlated with changes in protein; immunostaining in Nkx2.2$^{\Delta endo}$ mice revealed increased PP^{+} cells at both E15.5 and P0 (Supplemental Fig. 2-3A-F). Quantification of PP^{+} cells normalized to total Tom^{+} cell number also confirmed a > two-fold increase in PP^{+} cells at P0 (Supplemental Fig. 2-3G). While some Ins^{+}PP^{+} cells could be observed (arrow in Supplemental Fig. 2-3D), there was no significant difference between Nkx2.2$^{\Delta endo}$ mice and controls (Supplemental Fig. 2-3G). The surprising observation that *Ppy* is upregulated in the Nkx2.2^{KO} mice may be due to the availability of more specific *Ppy* detection reagents, or could result from differences in mouse strain background (see Discussion).

Nkx2.2$^{\Delta endo}$ and Nkx2.2^{KO} mice share important gene expression changes

It was somewhat unexpected that the restricted deletion of Nkx2.2 in the Ngn3 progenitor cells resulted in a phenotype that was remarkably similar to the Nkx2.2^{KO} mice, which lacked *Nkx2.2* throughout pancreas development. To more precisely determine the similarities and differences between the Nkx2.2$^{\Delta endo}$ and Nkx2.2^{KO} mice during the peak of endocrine cell differentiation, we performed RNA-Seq analysis on E15.5 pancreas derived from Nkx2.2$^{\Delta endo}$ embryos, Nkx2.2^{KO} embryos, and their respective littermate controls. Consistent with their similar phenotypes, there was significant overlap (p-value < 2.2e-16, Fig. 2-3A) between the differentially expressed genes (adjusted p-value < 0.05), including genes encoding pancreatic hormones (Fig. 2-3B) and important alpha and beta cell regulatory factors, such as *MafA* and *MafB* (Fig. 2-3C,D;

Supplemental Table 2-1). Furthermore, several transcription factors expressed in endocrine progenitors and involved in beta cell specification, such as *Nkx6.1*, *Pax4*, and *Pdx1*, that were unchanged Nkx2.2^{KO} embryos were also not affected at E15.5 in Nkx2.2$^{\Delta endo}$ pancreas (Supplemental Fig. 2-4; Supplemental Table 2-1).

Key regulators Ngn3, NeuroD1, and Rfx6 are retained in Nkx2.2$^{\Delta endo}$ mice.

Notably, in sharp contrast to the Nkx2.2^{KO} mice, which have reduced *Ngn3*, *NeuroD1*, and *Rfx6* (Anderson et al., 2009a; Chao et al., 2007), expression of *Ngn3*, *NeuroD1*, and *Rfx6* were unaffected in the Nkx2.2$^{\Delta endo}$ mice (Fig. 2-3E). We performed qRT-PCR analysis to confirm the maintenance of *Ngn3*, *NeuroD1*, and *Rfx6* gene expression in E15.5 and E18.5 Nkx2.2$^{\Delta endo}$ mice (Fig. 2-3F). *Ngn3* mRNA appeared to be increased at E18.5 in the Nkx2.2$^{\Delta endo}$ mice (Fig. 2-3F); however, immunostaining shows no increase in Ngn3^{+} cells at P0 (Fig. 2-3G,H). These results suggest that although Nkx2.2 regulates *Ngn3*, *NeuroD1*, and *Rfx6* expression within the pancreatic progenitor population, regulation of *Ngn3*, *NeuroD1*, and *Rfx6* expression within the endocrine progenitor cells becomes independent of Nkx2.2. Furthermore, this suggests that reduction in *Ngn3*, *NeuroD1*, and *Rfx6* expression in the Nkx2.2^{KO} mice is not a necessary step in the disruption of islet lineage specification.

Nkx2.2 direct targets are co-bound by Rfx6 and/or NeuroD1

The finding that beta cell specification was disrupted, even though *Ngn3*, *NeuroD1* and *Rfx6* expression remained intact, could indicate that Nkx2.2 functions as an obligate co-factor for these proteins in the regulation of beta cell specification. This is supported by our previous observation that Nkx2.2 cooperated with Ngn3 to regulate the expression of NeuroD1 (Anderson et al., 2009a). To determine whether there was a general requirement for a cooperative role for

Nkx2.2 within the known beta cell specification regulatory pathways, we assessed whether Nkx2.2 co-regulated the beta cell program with Rfx6 and NeuroD1. Nkx2.2 bound and regulated genes were identified by cross-referencing the differentially expressed genes in the Nkx2.2$^{\Delta endo}$ RNA-Seq (adjusted p-value < 0.05, Fig. 2-3) with an Nkx2.2 ChIP-Seq performed on the Min6 beta cell line (Dominguez Gutiérrez et al., 2016; GSE79725). These direct targets were compared with an Rfx6 ChIP-Seq performed in Min6 cells (Piccand et al., 2014; GSE62844), and a NeuroD1 ChIP-Seq from adult murine islets (Tennant et al., 2013; GSE30298). Notably, there was significant overlap of Rfx6 binding at Nkx2.2 target genes (p-value < 2.2e-16, Fig. 2-4A). Gene ontology (GO) analysis also revealed a significant representation of genes related to hormone secretion (Fig. 2-4B). Analysis of NeuroD1 bound genes revealed similar overlap with Nkx2.2 targets (p-value < 2.2e-16, Fig. 2-4C), and GO analysis identified significant representation of insulin secretion genes (Fig. 2-4D). Closer inspection of the Nkx2.2, Rfx6, and NeuroD1 ChIP-Seq data revealed numerous combinations of co-occupancy at pancreas-related genes. For example, all three transcription factors show overlapping binding peaks at the *Mlxipl* locus (Fig. 2-4E). At a region upstream of *MafA*, only Nkx2.2 and NeuroD1 are present (Fig. 2-4F); whereas Nkx2.2 and Rfx6 co-bind at the *Gast* locus (Fig. 2-4G). In addition, Nkx2.2, Rfx6, and NeuroD1 all bind near *G6pc2*; however, these peaks are present at distinct loci surrounding the gene (Fig. 2-4H). These co-bound targets, *Mlxipl*, *MafA*, *Gast*, and *G6pc2*, have significantly decreased expression at E15.5 in the Nkx2.2$^{\Delta endo}$ pancreas (Fig. 2-4I), indicating Nkx2.2 activity is necessary for their activation. These results suggest that while Nkx2.2, Rfx6, and NeuroD1 are individually critical for the

regulation of the islet cell program, it is their cooperative function that is required for the appropriate activation of a number of essential beta cell genes.

Discussion

The spatiotemporal progression of beta cell specification is not well understood. While many transcription factors that are essential for this process are well-characterized, their extended expression during development has made it challenging to identify the timing of their function. Nkx2.2 is expressed within the $Pdx1^{+}$ pancreatic progenitor population and its expression is maintained throughout development, gradually becoming restricted to the endocrine cell lineages (Arnes et al., 2012b; Jorgensen et al., 2007; Sussel et al., 1998). Global deletion of *Nkx2.2* causes the reduction of many essential regulatory factors, including *Ngn3, NeuroD1* and *Rfx6* (Anderson et al., 2009a; Chao et al., 2007) (Supplemental Table 2-1). The fact that these mice failed to form beta cells and some of the other islet cell types provided a strong argument for positioning Nkx2.2 at the top of the hierarchy for regulation of beta cell specification. In this study, we ablated Nkx2.2 specifically within the endocrine progenitor lineage to determine whether Nkx2.2 also functions downstream of Ngn3 in regulating islet cell specification. Surprisingly, we found similar endocrine changes in $Nkx2.2^{\Delta endo}$ mice compared to $Nkx2.2^{KO}$ mice, including the failure to form beta cells (Sussel et al., 1998). However, unlike the $Nkx2.2^{KO}$ mice, expression of the essential beta cell transcription factors *Ngn3*, *Rfx6*, and *NeuroD1* were unaffected, suggesting that although Nkx2.2 regulates these factors in the pancreatic progenitor population, reduction of *Ngn3, NeuroD1* and *Rfx6* expression is not an obligate step in the mis-specification of islet fates. This may indicate that although Ngn3,

NeuroD1 and Rfx6 are essential for specifying the beta cell program, Nkx2.2 activity must be preserved to facilitate the functional integrity of these pathways (Fig. 2-5). Furthermore, co-occupancy analysis revealed significant overlap of Nkx2.2 direct targets with Rfx6 and NeuroD1 binding, suggesting cooperative transcriptional regulation of these factors.

Although it was unexpected that deletion of Nkx2.2 from the $Ngn3^{+}$ cell was sufficient to recapitulate the $Nkx2.2^{KO}$ phenotype, it is consistent with a recent study showing that Ngn3-Cre; $Rfx6^{flox/flox}$ mice display beta cell loss similar to the $Rfx6^{-/-}$ mice (Piccand et al., 2014). Similarly, Nkx6.1 functions downstream of Ngn3, with Ngn3-Cre; $Nkx6.1^{flox/flox}$ mice displaying defective beta cell differentiation (Schaffer et al., 2013). These studies are providing increasing evidence that the endocrine progenitor cell, downstream of Ngn3 induction, is the primary site of islet cell specification. However, it is still possible that preprogramming of this population does occur, but that the respective programs can be over-written once the cell has formed. The importance of Nkx2.2 function downstream of Ngn3 expression is also consistent with the timing of NKX2.2 in human islets; unlike mice, NKX2.2 is only first detected after the appearance of NGN3 progenitors (Jennings et al., 2013). However, while Nkx2.2 function within the mouse $Pdx1^{+}$ pancreatic progenitor was not sufficient to allow beta cell formation, further studies would be required to determine whether Nkx2.2 has additional functions at this earlier stage of pancreas development.

We were surprised to observe an increase in the PP population of $Nkx2.2^{\Delta endo}$ mice. Since re-evaluation of *Ppy* expression in the $Nkx2.2^{KO}$ mice revealed a similar phenotype, the increase in PP is likely not due to a unique function of Nkx2.2 in the pancreatic progenitor to specify PP

cells. It is possible that the different PP phenotype in our earlier studies could be explained by the availability of more specific reagents. In the past, cross-reactivity between PP, peptide YY (Pyy), and neuropeptide Y (Npy) antibodies has been observed (Teitelman et al., 1993; Upchurch et al., 1994) and the loss of Pyy or Npy expression in the Nkx2.2^{KO} mice could explain the apparent reduction in PP expression. We no longer have access to the anti-PP antibodies used in our previous studies, but we can demonstrate that the PP antibodies used in this analysis do not cross react with Pyy and Npy (Supplemental Fig. 2-5A-C, data not shown). It is also possible that the discrepant PP phenotype is due to different strain backgrounds, which were changed from a Swiss Black (Taconic) background in the previous analyses to a C57Bl6/J background in this study. The upregulation of PP is also interesting since it parallels the increase we observed with epsilon cells. Consistent with the observation that a subset of the PP population is derived from Ghrl-expressing epsilon cells (Arnes et al., 2012a), PP^{+}Ghrl^{+} cells can be infrequently found in Nkx2.2$^{\Delta endo}$ mice at P0 (Supplemental Fig. 2-5D-E).

Beta cell specification involves the function of numerous transcription factors, some of which are expressed early and throughout pancreas development. Our results identify a critical window for Nkx2.2-mediated regulation of islet cell specification within the Ngn3 lineage following induction of Ngn3. Furthermore, although Nkx2.2 appears to function near the top of the islet transcriptional hierarchy, it appears that maintained Nkx2.2 activity is also a necessary component of the regulatory networks functioning downstream of Ngn3 to induce beta cell specification. Our discovery that different combinations of transcription factors bind and regulate important genes in the beta cell pathways raises important questions about the complex nature of cell-specific gene regulation that may have important implications for beta cell induction,

maturation and function. Furthermore, while previous research has focused on the initial expression of beta cell factors, future studies may benefit by ensuring the prolonged and combinatorial expression of these factors to recapitulate *in vivo* development and allow cooperative induction of target genes.

Acknowledgements

We thank Andrew Leiter (UMass Medical School) for the *Ngn3:Cre* mouse line. We thank members of the Sussel lab for helpful discussions throughout the project. We thank the Columbia University Genome Center for performing the RNA sequencing and assistance with the analysis. Additional core facility support was provided from the Columbia DERC (NIH P30 DK063608) and the Herbert Irving Comprehensive Cancer Center. Funding for the project was provided by NIH R01 DK082590 (LS), NIH T32GM007088 and T32DK007328 (AJC), a CONACYT grant from the Mexican government (GD) and NIH F31 DK107028 (RAS).

Materials and Methods

Animal maintenance

Mice were maintained on a C57BL/6J background (The Jackson Laboratory). *Ngn3-Cre*, *Nkx2.2*$^{flox/flox\ or\ flox/LacZ}$, and *R26R-Tomato* (B6.Cg-*Gt(ROSA)26-Sor*$^{tm14(CAG-tdTomato)Hze}$/J) mice were genotyped as previously published (Arnes et al., 2012b; Madisen et al., 2010; Mastracci et al., 2013; Schonhoff et al., 2004). *Nkx2.2*$^{LacZ/LacZ}$ mice were genotyped as previously published (Arnes et al., 2012b). Both Nkx2.2$^{-/-}$ and Nkx2.2$^{LacZ/LacZ}$ mice are referred to as Nkx2.2^{KO} mice in this paper (Arnes et al., 2012b). Animal maintenance and procedures were conducted in

accordance with a Columbia University Institutional Animal Care and Use Committee approved protocol (AAAG3206).

Immunohistochemistry

Fixation: Samples were fixed in 4% paraformaldehyde (PFA). E15.5 embryos were fixed for 3 hours (h) at 4°C, and P0 neonates were fixed overnight at 4°C. Samples were washed in phosphate-buffered saline (PBS), and then transferred to 30% sucrose/PBS overnight at 4°C. Samples were embedded and frozen in optimum cutting temperature (O.C.T.) and stored at -80°C. 8 µm sections were taken and stored at -80°C.

Staining: Sections were washed in PBS and 0.1% Triton/PBS (PBST) and blocked in 2% donkey serum (DS)/PBST for 30 minutes (min) at room temperature (RT). Primary antibody was diluted in 2% DS/PBST and incubated on sections overnight at 4°C. Sections were washed in PBS and PBST and incubated 2-3 h at RT with secondary antibody (Jackson Immuno Research) diluted in 2% DS/PBST at 1:500. Sections were washed in PBS and PBST and incubated with DAPI (Invitrogen TD21490) diluted in PBS at 1:1000 for 15 min at RT. Sections were washed in PBS and mounted with fluorescent mounting medium (DAKO S3023).

Primary antibodies: guinea pig α-Insulin (DAKO, 1:500), rabbit α-Nkx2.2 (Sigma, 1:400), goat α-Ghrelin (Santa Cruz, 1:500), rabbit α-Glucagon (DAKO, 1:800), rabbit α-Pancreatic Polypeptide (Zymed/Invitrogen, 1:200), guinea pig α-Pdx1 (Beta cell biology consortium, 1:400), rabbit α-Amylase (Sigma, 1:500), rabbit α-Somatostatin (Phoenix Pharmaceuticals, 1:200), rabbit α-Gastrin (Cell Marque, 1:300), guinea pig α-Peptide YY (Research Diagnostics, 1:500).

Microscopy: Images were taken using a Zeiss Confocal LSM 710 microscope and processed with Zen, ImageJ, and Adobe Photoshop software.

Cell Quantification: For each neonate, every twentieth section was analyzed for insulin$^+$, pancreatic polypeptide$^+$, and Tomato$^+$ cell quantification throughout the entire pancreas. Tiled images were taken at 20x objective with a Leica fluorescent microscope (DM5500/MZ16F) and processed with LAF software. Cells were counted using ImageJ software and normalized to total number of Tomato$^+$ cells. All values are expressed as mean ± SEM. Statistical analysis was performed using two-tailed student's unpaired *t*-test. Significance was achieved with p-value < 0.05.

RNA analysis

RNA extraction: Whole pancreata were dissected in PBS and transferred to RNA*later* (Ambion AM7021). Pancreata were incubated at 4°C overnight and stored at -20°C until RNA extraction. Pancreata were homogenized and total RNA was isolated following the Qiagen RNeasy Mini kit.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis: A normalized starting quantity of RNA (200ng-1µg) isolated from whole pancreas and random hexamers were used to synthesize cDNA following the SuperScript III reverse transcriptase protocol (Invitrogen 18080-044). qRT-PCR was performed using 200ng cDNA and master mix (Eurogentec) and analyzed using the BioRad CFX96 Real-Time System. Genes were normalized to *CyclophilinB* (probe 5': tggtacggaaggtggag, forward primer 5': gcaaagttctagagggcatgga, reverse primer 5': cccggctgtctgtcttggt) and to littermate controls. An n ≥ 3 was used at all ages. All values are expressed as mean ± SEM. Statistical analysis was

performed using two-tailed student's unpaired *t*-test. Significance was achieved with p-value < 0.05.

Applied Biosystems TaqMan AODs: *Insulin1* (Mm01950294_s1), *Insulin2* (Mm00731595_gH), *Glucagon* (Mm00801712_m1), *Pancreatic polypeptide* (Mm00435889_m1), *Ghrelin* (Mm00445450_m1), *Somatostatin* (Mm00436671_m1), *Gastrin* (Mm00439059_g1), *ChromograninA* (Mm00514341_m1), *Arx* (Mm00545903_m1), *Pdx1* (Mm00435565_m1), *Peptide YY* (mm00520716), *Neuropeptide Y* (Mm03048253_m1), *Etv1* (Mm00514804_m1), *Pax6* (Mm00443081_m1), *Isl1* (Mm00517585_m1), *Sox9* (Mm00448840_m1), *Pax4* (Mm01159036_m1), *Glucokinase* (Mm00439129_m1), *Glut2* (Mm00446229_m1).

Applied Biosystems Primer/TaqMan Probe sets: *Nkx2.2* (probe 5': ccattgactctgccccatcgctct, forward primer 5': cctccccgagtggcagat, reverse primer 5': gagttctatcctctccaaaagttcaaa), *Neurogenin3* (probe 5': cctgcgcttcgcccacaact, forward primer 5': gacgccaaacttacaaag, reverse primer 5': gtcagtgcccagatgt), *Nkx6.1* (probe 5': tctggttccagaaccgcagga, forward primer 5': cggagagtcaggtca, reverse primer 5': tgcgtgcttctttctc), *MafA* (probe 5': cggcgcacgctcaagaaccg, forward primer 5': catccgactgaaacagaag, reverse primer 5': ctcgctctccagaatgtgccgctgc), *MafB* (probe 5': cgcgtccagcagaaacatcacc, forward primer 5': ccagtcgtgcaggtat, reverse primer 5': tgcgtcttctcgttctc), *NeuroD1* (probe: ABI 185747095, forward primer 5': ccagcccactaccaatttgg, reverse primer 5': gggttctgctcaggcaagaa).

RNA-Sequencing (RNA-Seq): Total RNA concentration and quality were measured using the Agilent Bioanalyzer RNA Nano chip. RNA-Seq was performed by the Columbia Genome

Center with 30 million single end 100bp reads. Dataset overlap was determined with a Fisher's Exact test; significance was achieved with p-value < 0.05. Data is available at GSE80444.

ChIP-Sequencing (ChIP-Seq) comparative analysis: Nkx2.2 (Dominguez Gutiérrez et al., 2016; GSE79725), Rfx6 (Piccand et al., 2014; GSE62844), and NeuroD1 (Tennant et al., 2013; GSE30298) ChIP-Seq datasets were aligned to mm9 and visualized using IGV (Robinson et al., 2011; Thorvaldsdóttir et al., 2013). Nkx2.2 direct targets were determined by cross-referencing the Nkx2.2$^{\Delta endo}$ RNA-Seq data (adjusted p-value < 0.05, GSE80444) with the Nkx2.2 ChIP-Seq data (Dominguez Gutiérrez et al., 2016). Dataset overlap was determined with a Fisher's Exact test; significance was achieved with p-value < 0.05. Rfx6 bound genes contain Rfx6 binding within 100kb of TSS (Piccand et al., 2014; GSE62844). NeuroD1 bound genes contain NeuroD1 binding within 100kb of TSS (Tennant et al., 2013; GSE30298). Gene ontology (GO) analysis was performed (Consortium, 2015).

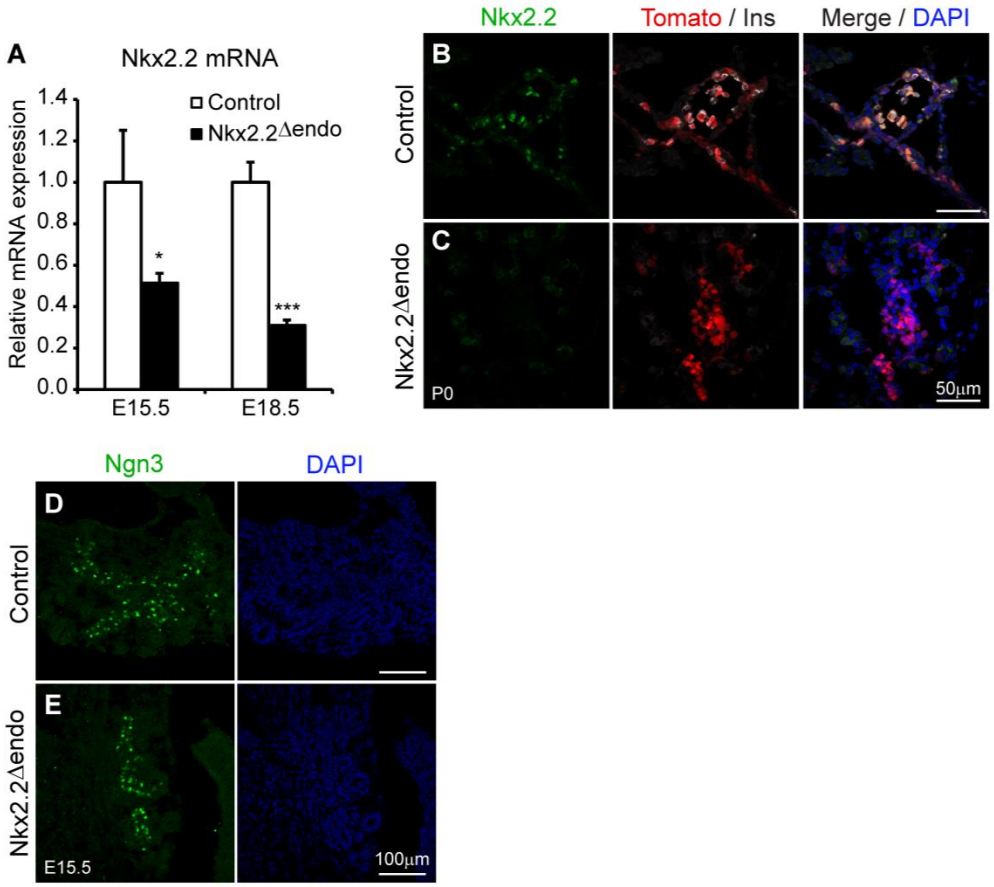


Figure 2-1. Nkx2.2 is ablated specifically in the endocrine lineage in Nkx2.2$^{\Delta endo}$ mice. (A) Nkx2.2 gene expression is decreased at E15.5 (Control n=3, Nkx2.2$^{\Delta endo}$ n=6) and E18.5 (Control n=6, Nkx2.2$^{\Delta endo}$ n=4). (B-C) Immunostaining for Nkx2.2 shows decreased expression in Nkx2.2$^{\Delta endo}$ mice at P0 (n=3). (*D-E*) Immunostaining for Ngn3 shows no apparent changes in the Ngn3^{+} cell population at E15.5 (n=3). (*) $P < 0.05$; (***) $P < 0.001$.

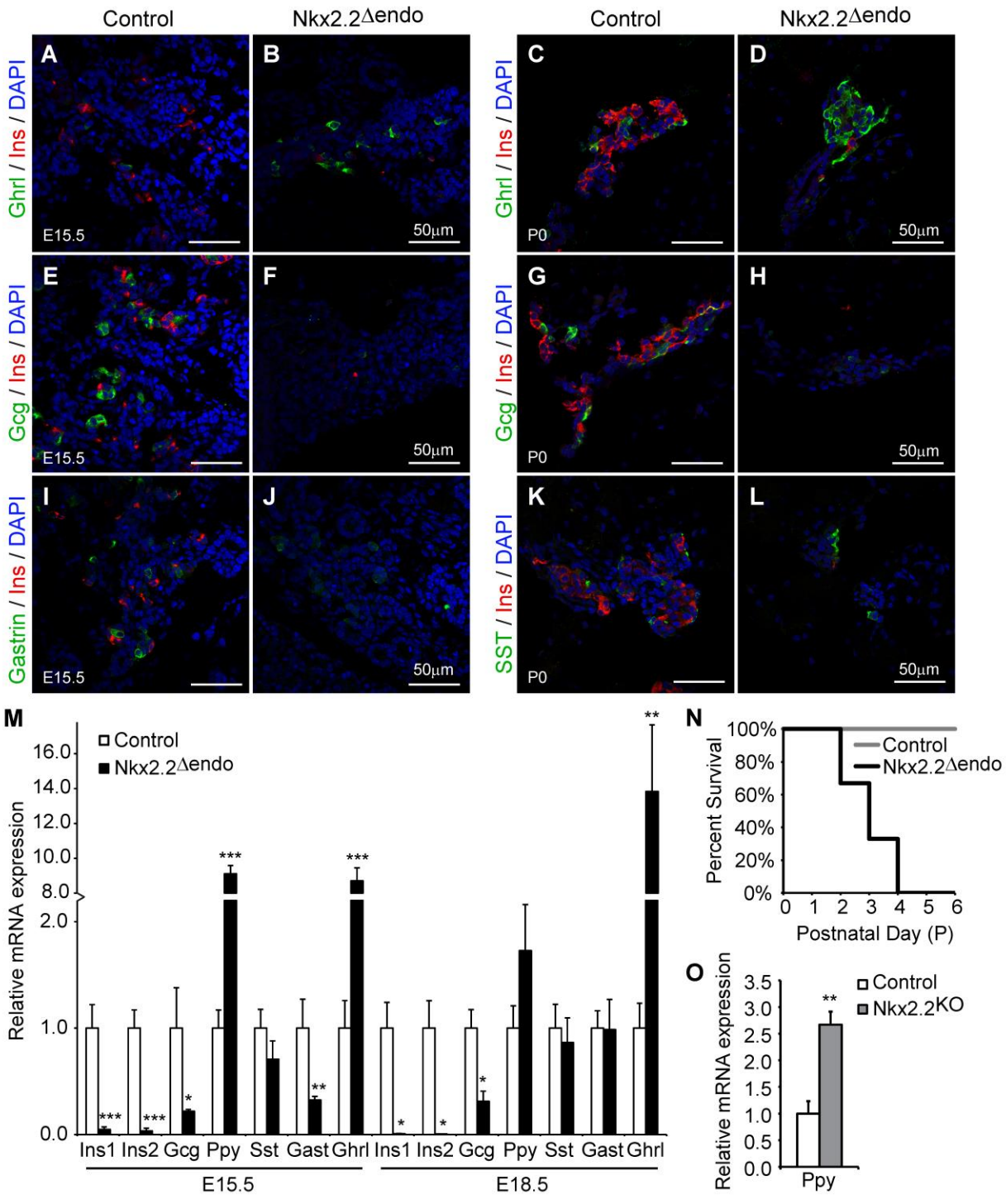

Control
Nkx2.2Δendo
Control
Nkx2.2Δendo
A
B
C
D
Ghrl / Ins / DAPI
E15.5
P0
50μm
E
F
G
H
Gcg / Ins / DAPI
I
J
K
L
Gastrin / Ins / DAPI
SST / Ins / DAPI
M
Control
Nkx2.2Δendo
Relative mRNA expression
16.0
14.0
12.0
10.0
8.0
2.0
1.0
0.0
Ins1 Ins2 Gcg Ppy Sst Gast Ghrl Ins1 Ins2 Gcg Ppy Sst Gast Ghrl
E15.5
E18.5
N
Percent Survival
100%
80%
60%
40%
20%
0%
0 1 2 3 4 5 6
Postnatal Day (P)
O
Relative mRNA expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Nkx2.2KO
Ppy

Figure 2-2. Nkx2.2$^{\Delta endo}$ mice recapitulate the Nkx2.2^{KO} phenotype. (*A-H*) Ghrl^{+} cells are increased, Ins^{+} cells are greatly reduced, and Gcg^{+} cells are decreased at E15.5 and P0 in Nkx2.2$^{\Delta endo}$ mice (n=3). (*I-J*) Gast^{+} cells are reduced in Nkx2.2$^{\Delta endo}$ embryos at E15.5 (n=3). (*K-L*) SST^{+} cells are unchanged in Nkx2.2$^{\Delta endo}$ mice at P0 (n=3). (*M*) Hormone gene expression changes recapitulate Nkx2.2^{KO} mice at E15.5 (Control n=3, Nkx2.2$^{\Delta endo}$ n=6) and E18.5 (Control n=6, Nkx2.2$^{\Delta endo}$ n=4). *Ppy* gene expression is increased at these time points. (*N*) Nkx2.2$^{\Delta endo}$ mice die neonatally. (*O*) qRT-PCR confirms increased *Ppy* at E15.5 in Nkx2.2^{KO} embryos (Control n=3, Nkx2.2^{KO} n=3). (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

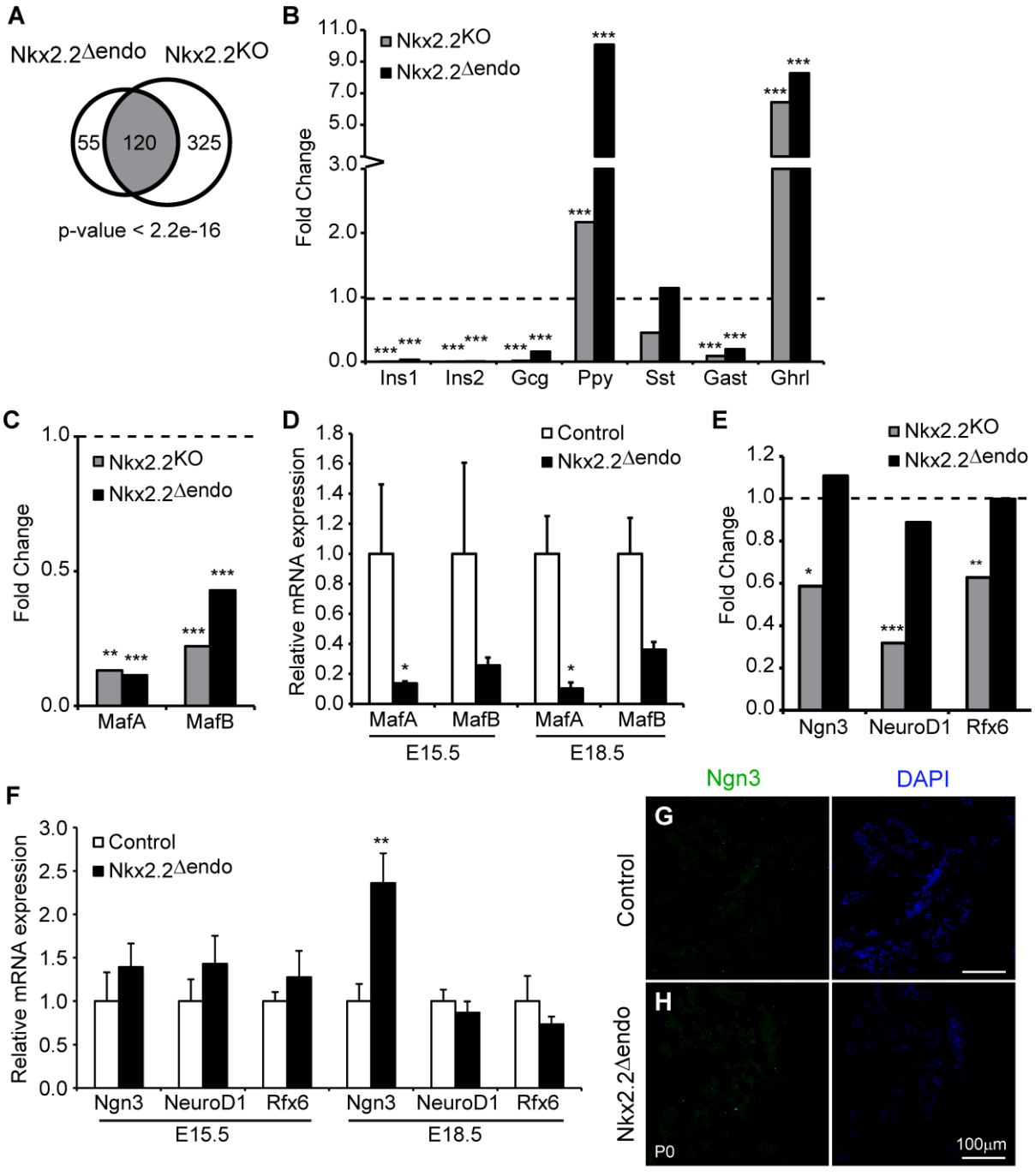
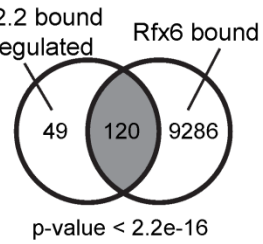

A
Nkx2.2Δendo
Nkx2.2KO
55
120
325
p-value < 2.2e-16
B
Nkx2.2KO
Nkx2.2Δendo
Fold Change
Ins1
Ins2
Gcg
Ppy
Sst
Gast
Ghrl
C
Nkx2.2KO
Nkx2.2Δendo
Fold Change
MafA
MafB
D
Control
Nkx2.2Δendo
Relative mRNA expression
MafA
MafB
MafA
MafB
E15.5
E18.5
E
Nkx2.2KO
Nkx2.2Δendo
Fold Change
Ngn3
NeuroD1
Rfx6
F
Control
Nkx2.2Δendo
Relative mRNA expression
Ngn3
NeuroD1
Rfx6
Ngn3
NeuroD1
Rfx6
E15.5
E18.5
Ngn3
DAPI
G
Control
H
Nkx2.2Δendo
P0
100μm

Figure 2-3. Nkx2.2$^{\Delta endo}$ and Nkx2.2^{KO} embryos have shared and distinct gene expression changes. (*A*) Comparison of Nkx2.2$^{\Delta endo}$ and Nkx2.2^{KO} RNA-Seq experiments shows significant overlap of dysregulated genes with adjusted p-value < 0.05. (*B*) RNA-Seq confirms similar changes in hormone expression in Nkx2.2$^{\Delta endo}$ and Nkx2.2^{KO} embryos, relative to respective littermate controls. (*C*) RNA-Seq shows decreases in *MafA* and *MafB* gene expression in Nkx2.2$^{\Delta endo}$ and Nkx2.2^{KO} embryos, relative to respective littermate controls. (*D*) qRT-PCR confirms decreases in *MafA* and *MafB* at E15.5 (Control n=3, Nkx2.2$^{\Delta endo}$ n=6) and E18.5 (Control n=6, Nkx2.2$^{\Delta endo}$ n=4). (*E*) RNA-Seq shows unchanged *Ngn3*, *NeuroD1*, and *Rfx6* gene expression in Nkx2.2$^{\Delta endo}$ embryos, while Nkx2.2^{KO} embryos show downregulation, relative to respective littermate controls. (*F*) qRT-PCR confirms RNA-Seq results. *Neurod1* and *Rfx6* gene expression are unchanged at E15.5 and E18.5. *Ngn3* gene expression is unchanged at E15.5 and increased at E18.5. (E15.5: Control n=3, Nkx2.2$^{\Delta endo}$ n=3-6; E18.5: Control n=5-6, Nkx2.2$^{\Delta endo}$ n=4). (*G-H*) No Ngn3^{+} cells are detected at P0 (n=3). Adjusted p-values are shown for RNA-Seq data. (*) P < 0.05; (**) P < 0.01; (***) P < 0.001.

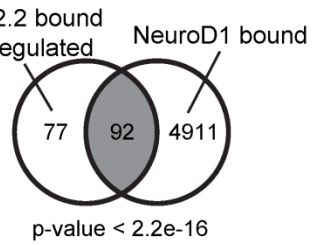
A



B

GO biological process	p-value
Regulation of hormone levels	2.85E-06
Regulation of biological quality	1.02E-05
Cell-cell signaling	4.38E-05
Regulation of peptide hormone secretion	7.12E-05
Regulation of peptide secretion	9.15E-05

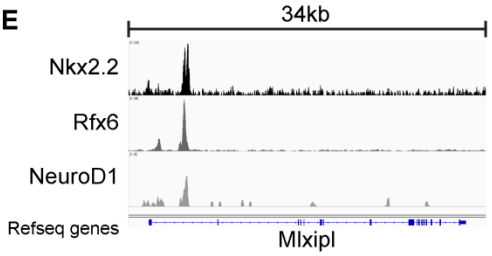
C



D

GO biological process	p-value
Regulation of biological quality	4.18E-06
Regulation of hormone levels	7.48E-06
Cell-cell signaling	1.20E-04
Insulin secretion	1.63E-04
Peptide transport	3.10E-04

E



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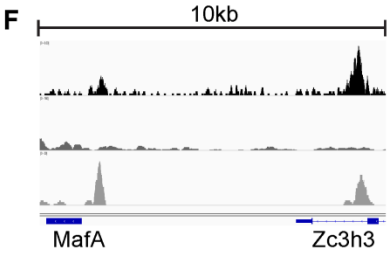
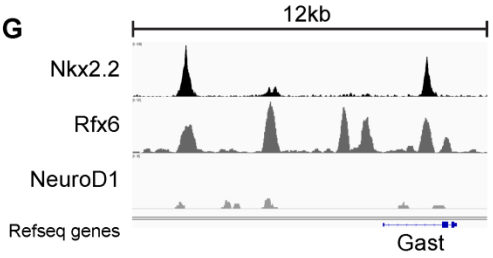
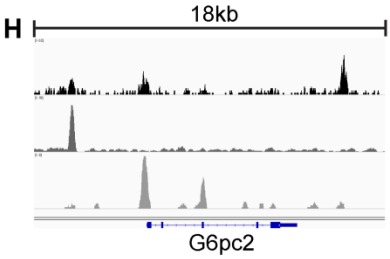


G



H



I

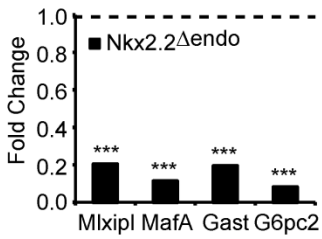


Figure 2-4. Nkx2.2 bound and regulated genes are co-bound by Rfx6 and NeuroD1. (*A*) Comparison of Nkx2.2 bound and regulated genes and Rfx6 bound genes shows significant overlap. (*B*) Gene ontology (GO) analysis of genes with Nkx2.2 binding and regulation and Rfx6 binding. (*C*) Comparison of Nkx2.2 bound and regulated genes and NeuroD1 bound genes shows significant overlap. (*D*) GO analysis of genes with Nkx2.2 binding and regulation and NeuroD1 binding. (*E*) Nkx2.2, Rfx6, and NeuroD1 show overlapping binding peaks at *Mlxipl* locus (mm9 chr5:135,580,783-135,615,792 shown). (*F*) Nkx2.2 and NeuroD1 show overlapping binding peaks at a region upstream of *MafA* (mm9 chr15:75,577,046-75,587,657 shown). (*G*) Nkx2.2 and Rfx6 show overlapping binding peaks upstream of *Gast* and within intron 1 (mm9 chr11:100,186,915-100,199,407 shown). (*H*) Non-overlapping Nkx2.2, Rfx6, and NeuroD1 binding peaks near *G6pc2* locus (mm9 chr2:69,052,146-69,070,781 shown). (*I*) RNA-Seq shows decreased *Mlxipl*, *MafA*, *Gast*, and *G6pc2* expression at E15.5 in Nkx2.2$^{\Delta endo}$ embryos. Adjusted p-values are shown for RNA-Seq data. (***) $P < 0.001$.

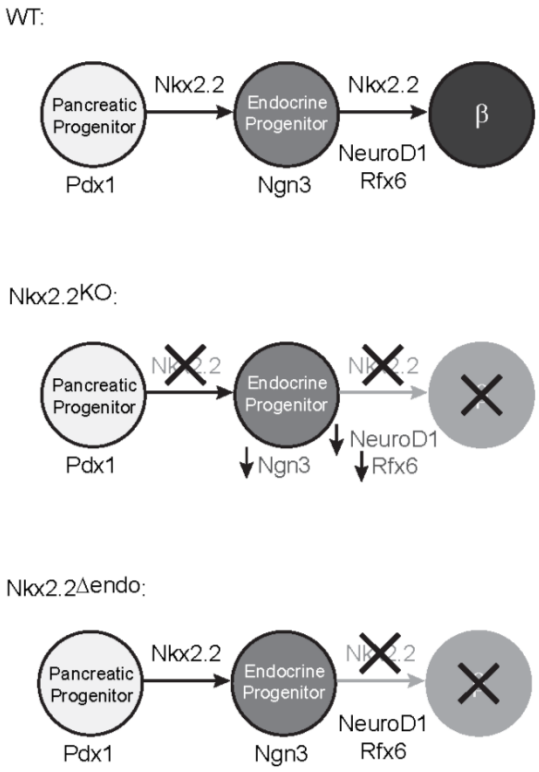
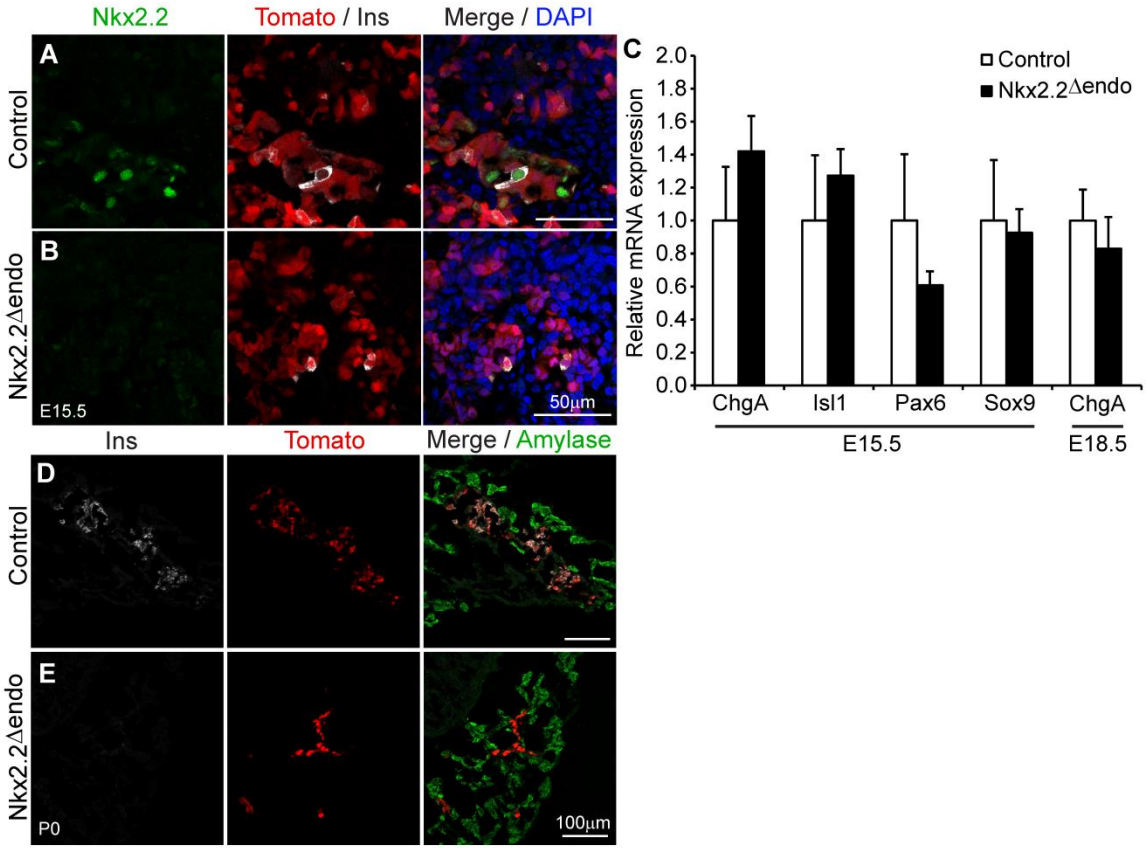
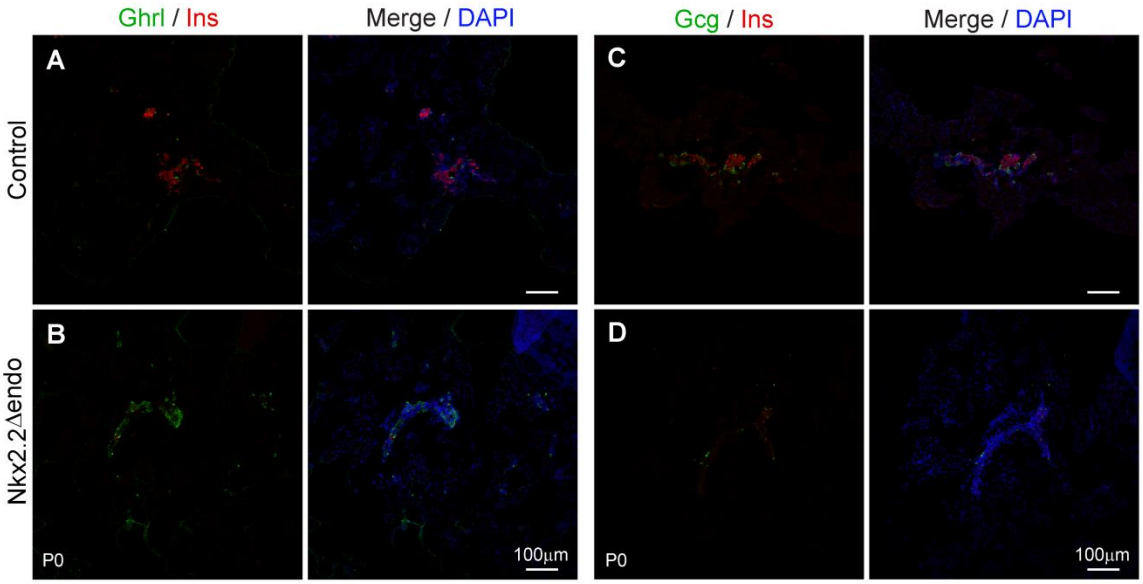


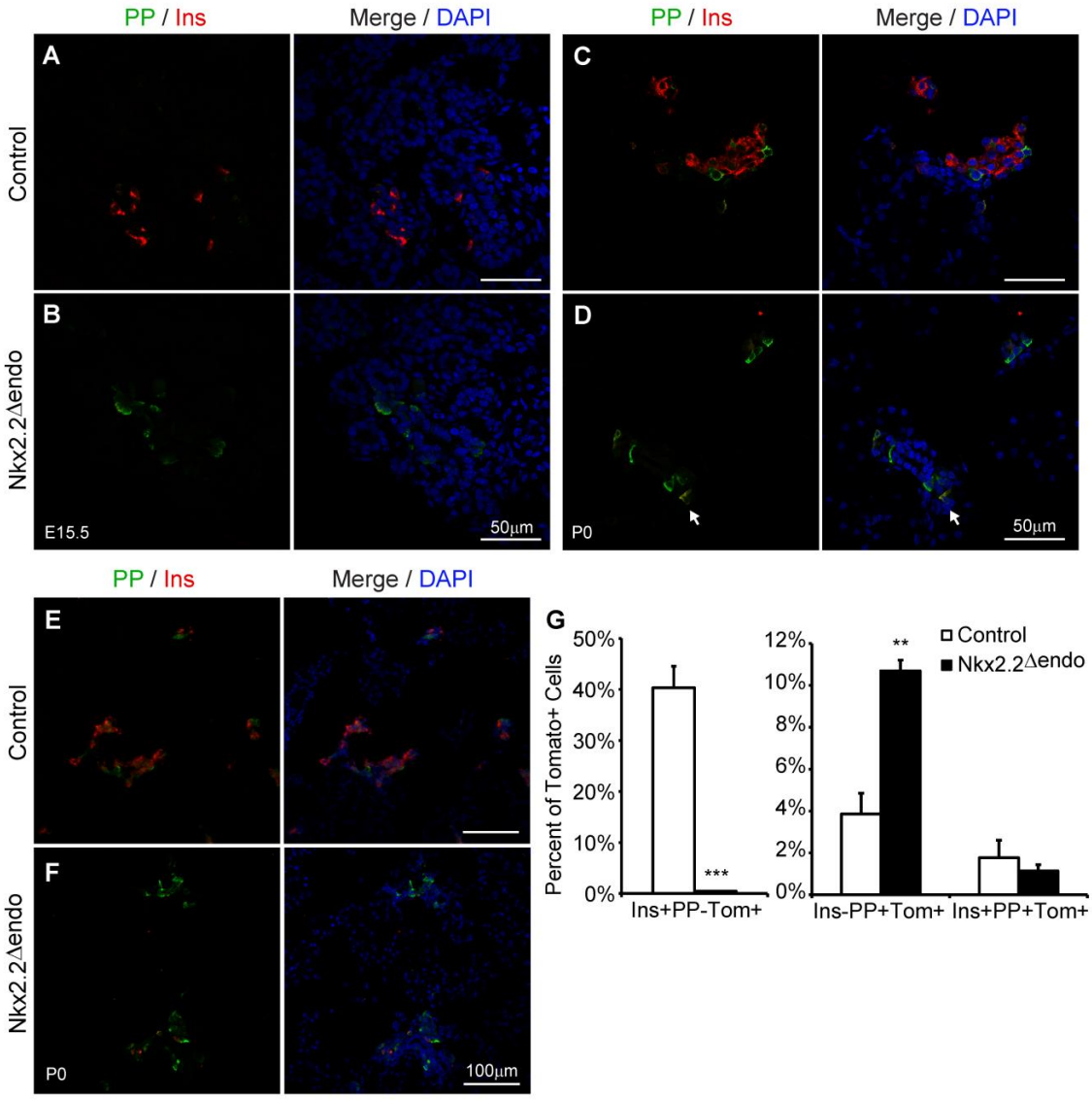
Figure 2-5. Transcriptional networks involved in beta cell specification in wildtype (WT), Nkx2.2^{KO}, and Nkx2.2$^{\Delta endo}$ conditions. Ngn3, NeuroD1, and Rfx6 are not sufficient to allow for beta cell specification in the absence of Nkx2.2 in Nkx2.2$^{\Delta endo}$ mice.



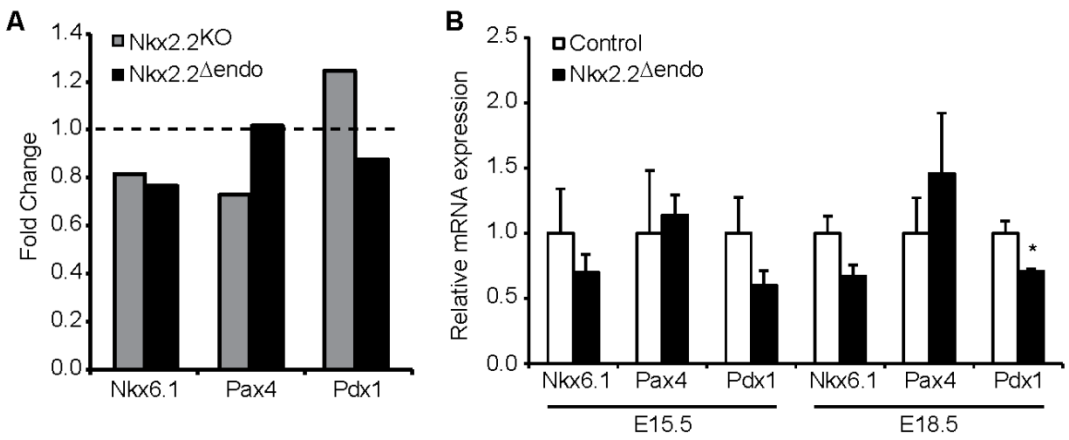
Supplemental Figure 2-1. Exocrine compartment and endocrine compartment size is unaffected in Nkx2.2$^{\Delta endo}$ mice. (A-B) Immunostaining for Nkx2.2 shows decreased expression in Nkx2.2$^{\Delta endo}$ embryos at E15.5 (n=3). (C) Endocrine compartment gene expression is unchanged at E15.5 (Control n=3, Nkx2.2$^{\Delta endo}$ n=6) and E18.5 (Control n=6, Nkx2.2$^{\Delta endo}$ n=4). (D-E) Immunostaining for amylase shows no changes in Nkx2.2$^{\Delta endo}$ mice at P0 (n=3).



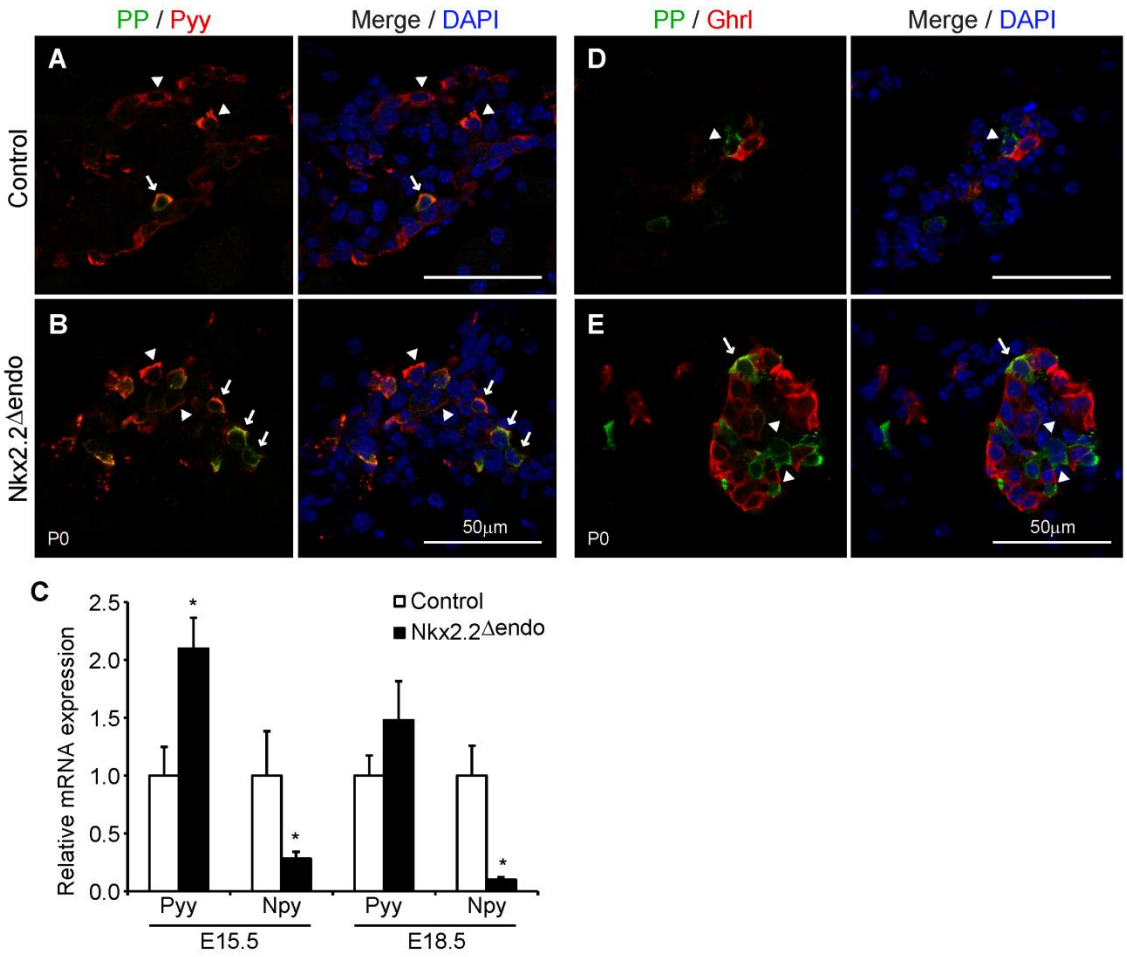
Supplemental Figure 2-2. Nkx2.2$^{\Delta endo}$ mice display endocrine specification defects at P0. (*A-D*) Low magnification images showing increased Ghrl^{+} cells, and decreased Ins^{+} cells and Gcg^{+} cells in Nkx2.2$^{\Delta endo}$ mice at P0 (n=3).



Supplemental Figure 2-3. PP cells are increased in Nkx2.2$^{\Delta endo}$ mice. (*A-D*) Immunostaining for PP shows increased PP^{+} cells at E15.5 and P0 in Nkx2.2$^{\Delta endo}$ mice (n=3). Arrow indicates a PP^{+}Ins^{+} cell. (*E-F*) Low magnification images showing increased PP^{+} cells at P0 in Nkx2.2$^{\Delta endo}$ mice. (*G*) Whole pancreas quantification of Ins^{+} and PP^{+} cells normalized to total number of Tom^{+} cells shows increased Ins^{-}PP^{+}Tom^{+} cells and decreased Ins^{+}PP^{-}Tom^{+} cells in Nkx2.2$^{\Delta endo}$ mice at P0 (n=3). No changes in Ins^{+}PP^{+}Tom^{+} cells are detected. (**) $P < 0.01$; (***) $P < 0.001$.



Supplemental Figure 2-4. Select transcription factor expression in Nkx2.2$^{\Delta endo}$ and Nkx2.2^{KO} mice. (*A*) RNA-Seq at E15.5 shows no changes in *Nkx6.1*, *Pax4*, or *Pdx1* gene expression in Nkx2.2$^{\Delta endo}$ and Nkx2.2^{KO} embryos, relative to respective littermate controls. (*B*) qRT-PCR confirms no changes in *Nkx6.1*, *Pax4*, or *Pdx1* at E15.5 (Control n=3, Nkx2.2$^{\Delta endo}$ n=6), no changes in *Nkx6.1* or *Pax4* at E18.5, and decreased *Pdx1* at E18.5 in Nkx2.2$^{\Delta endo}$ mice (Control n=6, Nkx2.2$^{\Delta endo}$ n=4). (*) $P < 0.05$.



Supplemental Figure 2-5. Analysis of PP-related peptide expression in Nkx2.2$^{\Delta endo}$ mice. (*A-B*) Immunostaining for PP and Pyy is partially overlapping (n=3). Arrows indicate PP^{+}Pyy^{+} cells and arrowheads indicate PP^{-}Pyy^{+} cells. (*C) Pyy* and *Npy* gene expression at E15.5 (Control n=3, Nkx2.2$^{\Delta endo}$ n=6) and E18.5 (Control n=6, Nkx2.2$^{\Delta endo}$ n=4). (*D-E*) PP^{+}Ghrl^{+} cells are found at similar frequency (n=3). Arrows indicate PP^{+}Ghrl^{+} cells and arrowheads indicate PP^{+}Ghrl^{-} cells. (*) $P < 0.05$.

	Fold Change Nkx2.2$^{\Delta endo}$	adjusted p-value	Fold Change Nkx2.2^{KO}	adjusted p-value
Transcription Factors:				
Arx	2.16	3.26E-09	1.57	0.0031
MafA	0.11	3.39E-06	0.13	0.0040
MafB	0.43	1.31E-10	0.22	3.92E-38
NeuroD1	0.89	1	0.32	3.01E-23
Ngn3	1.11	1	0.59	0.0198
Nkx6.1	0.77	1	0.82	0.9684
Pax4	1.02	1	0.73	0.4762
Pax6	0.36	1.07E-06	0.21	2.84E-30
Pdx1	0.88	1	1.25	0.7916
Rfx6	1.00	1	0.63	0.0013
Sox9	0.94	1	1.08	1
Pancreatic Hormones:				
Gast	0.19	2.16E-07	0.09	2.63E-09
Gcg	0.16	2.23E-12	0.01	4.64E-16
Ghrl	8.27	1.94E-31	6.43	6.72E-16
Ins1	0.03	4.84E-24	0.00	4.32E-11
Ins2	0.01	2.99E-55	0.00	1.48E-11
Ppy	10.08	4.99E-42	2.17	7.15E-05
Sst	1.14	1	0.45	0.5130

Supplemental Table 2-1. Nkx2.2$^{\Delta endo}$ and Nkx2.2^{KO} E15.5 RNA-Seq. Gene expression changes in select transcription factors and pancreatic hormones.

CHAPTER 3

REQUIREMENT FOR THE NKX2.2 SD DOMAIN IN THE DEVELOPMENT AND MAINTENANCE OF BETA CELL FUNCTION

Abstract

Nkx2.2 is essential for pancreatic beta cell specification and also for the maintenance of beta cell identity. The SD protein domain of Nkx2.2 mediates Nkx2.2 function during endocrine cell development; however, its role within endocrine progenitors and the beta cell has not been tested. To identify SD domain function in the early transcriptional regulation of endocrine progenitors, the Ngn3-expressing progenitor population was isolated and RNA-Seq was conducted on Nkx2.2^{SDmutant} embryos. Surprisingly, many genes associated with endocrine fate are differentially expressed even at this early stage of endocrine specification. In addition, RIP-Cre; Nkx2.2$^{SD/flox}$ (βSD) mice were used to investigate SD domain function specifically within the beta cell. These mice display hyperglycemia, glucose intolerance, and dysregulation of genes important for beta cell function. Together, these results suggest the Nkx2.2 SD domain plays novel, essential roles in regulating the establishment of appropriate gene transcription early in the endocrine progenitor population and is also important within the beta cell itself.

Introduction

Nkx2.2 is expressed in the CNS, intestine, and pancreas and is important for the proper specification of multiple cell lineages: neuronal populations in the CNS (Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997; Pattyn et al., 2003; Qi et al., 2001; Zhou et al., 2001), enteroendocrine cells in the intestine (Desai et al., 2008; Wang et al., 2009), and pancreatic

endocrine cells (Prado et al., 2004; Sussel et al., 1998). Furthermore, in each of these tissues, Nkx2.2 appears to function both as a transcriptional activator and repressor. For pancreatic endocrine cell development, Nkx2.2 repressor function appears to be responsible for immature beta cell differentiation; however, Nkx2.2 activator function may be required for beta cell maturation (Doyle et al., 2007).

Nkx2.2 exerts its transcriptional regulation through three conserved protein domains: the homeodomain, tinman (TN) domain, and NK2 specific (SD) domain. The homeodomain recognizes and binds DNA (Kim and Nirenberg, 1989; Tsao et al., 1995; Watada et al., 2000). The TN domain mediates interaction between Nkx2.2 and the transcriptional co-repressor Grg3 (Doyle et al., 2007; Papizan et al., 2011). $Nkx2.2^{TNmutant}$ mice display defective beta cell formation and transdifferentiation to the alpha cell lineage, demonstrating the requirement for this domain in establishing beta cell fate (Papizan et al., 2011). Lastly, the SD domain is conserved across species and is the defining feature of the NK2 family of proteins; however, its function has not been well characterized. *In vitro* studies demonstrated that the SD domain may be involved in masking C-terminal-driven transcriptional activation and does not contribute to DNA binding (Watada et al., 2000).

Recent i*n vivo* analysis of mice with a mutated Nkx2.2 SD domain ($Nkx2.2^{SD/SD}$ or $Nkx2.2^{SDmutant}$ mice) has provided insight into the role of the SD domain in mediating Nkx2.2 function (Fig. 3-1) (Levine et al., in preparation). Analysis of $Nkx2.2^{SDmutant}$ mice has revealed a critical role for this domain during pancreatic beta cell specification. These mice display decreased beta cell numbers and polyhormonal cell induction, suggesting the SD domain is

required to establish monohormonal cell identity (Levine et al., in preparation). While the SD domain mediates Nkx2.2 function during beta cell specification, this domain appears to be dispensable for Nkx2.2-mediated cell fate induction in the developing CNS. Nkx2.2 is important for the specification of serotonergic neurons (Briscoe et al., 1999; Pattyn et al., 2003), V3 interneurons (Briscoe et al., 2000; Ericson et al., 1997), and oligodendrocyte maturation (Qi et al., 2001; Zhou et al., 2001). However, these cell fates are unchanged in $Nkx2.2^{SDmutant}$ mice (Levine et al., in preparation). This suggests the SD domain is responsible for exerting pancreas-specific functions of Nkx2.2 transcriptional regulation.

It has recently been discovered that Nkx2.2 is required not only for establishing beta cell fate, but also maintaining beta cell identity. Dominguez Gutiérrez et al. (2016) used RIP-Cre; $Nkx2.2^{flox/flox}$ and MIP-CreER; $Nkx2.2^{flox/flox}$ mice ($Nkx2.2^{\Delta beta}$ mice) to determine the role of Nkx2.2 within the developing and mature beta cell. These mice display glucose intolerance and dysregulation of genes important for beta cell function. $Ins^{+}Gcg^{+}$, $Ins^{+}SST^{+}$, and $Ins^{+}PP^{+}$ polyhormonal cells can also be readily observed in $Nkx2.2^{\Delta beta}$ mice. This suggests Nkx2.2 is necessary for proper expression of genes important for beta cell function, and Nkx2.2 is required for the maintenance of monohormonal cell fate (Dominguez Gutiérrez et al., 2016).

While the Nkx2.2 SD domain is necessary for establishing a monohormonal cell fate during beta cell formation, its transcriptional role specifically within the endocrine progenitor and its function within the beta cell have yet to be assessed. In this study, we sought to identify the early transcriptional targets regulated through the Nkx2.2 SD domain, and also to determine the

contribution of the SD domain in mediating the role of Nkx2.2 in maintaining beta cell function and/or identity.

Results

Transcriptional profiling of Nkx2.2^{SDmutant} endocrine progenitors

Since the Ngn3^{+} endocrine progenitor lineage is a critical window for beta cell specification (Chapter 2), identifying the transcriptional program regulated by the Nkx2.2 SD domain at this stage of pancreas development was of particular interest. To determine the transcriptional profile of endocrine progenitors with mutated Nkx2.2 SD domain, we isolated Ngn3 progenitors from Ngn3-EGFP/+; Nkx2.2$^{SD/LacZ}$ mice (Nkx2.2^{SDmutant} mice) (Arnes et al., 2012b; Lee et al., 2002; Levine et al., in preparation). The Ngn3-EGFP allele marks all *Ngn3*-expressing endocrine progenitor cells with EGFP. Since EGFP has a half-life of over 24h, signal may persist following initial expression (Barrow et al., 2005). EGFP^{+} cells were isolated using FACs from E14.5 and E15.5 embryonic pancreata since this is a time of high Ngn3 expression during development (Fig. 3-2A) (Schwitzgebel et al., 2000). FACs-isolation of the EGFP^{+} cells using this allele likely isolates both Ngn3^{+} endocrine progenitors and their immediate descendants after *Ngn3* becomes repressed.

Since approximately1500-3000 cells could be obtained from each pancreas, EGFP^{+} cells from six pancreata (five E15.5 pancreata and one E14.5 pancreas) of like-genotypes were pooled to gain sufficient quantity for RNA-Seq (n=2).

Clustering of RNA-Seq samples indicated there was separation between control and Nkx2.2^{SDmutant} samples (Fig. 3-2B). RNA-Seq analysis shows a significant decrease in *insulin*

(*Ins1* and *Ins2*) gene expression, and a decrease in the pan-endocrine marker *Chromogranin A* (*Chga*) (Fig. 3-3, Table 3-1). In addition, *Sst* and *Ghrl* gene expression is increased; however, *Gcg* and *Ppy* are unchanged (Fig. 3-3, Table 3-1). While multiple pancreatic hormones are dysregulated, endocrine transcription factors are also differentially expressed. Crucial mature and immature beta cell transcription factors, such as *Nkx6.1*, *MafA*, and *MafB*, show decreased expression (Fig. 3-4, Table 3-1). Furthermore, the alpha cell transcription factor *Arx* is upregulated (Fig. 3-4, Table 3-1). These gene expression changes are consistent with the decreased beta cell population and the polyhormonal phenotype that is observed in Nkx2.2^{SDmutant} mice, indicating the SD domain mediates transcriptional regulation of endocrine genes early in development.

Role of the Nkx2.2 SD domain in the maintenance of beta cell function

To identify Nkx2.2 SD domain function within the beta cell, we generated RIP-Cre; Nkx2.2$^{SD/flox}$; Rosa26-Tomato (βSD) mice. βSD mice display no detectable changes in body weight in males or females (Fig. 3-5A,B). However, these mice display elevated *ad libitum* (*ad lib*) blood glucose levels in males and females, as early as 3 and 2 weeks of age (wk), respectively (Fig. 3-5C,D). βSD males and females also display glucose intolerance at 4wk (Fig. 3-6). These metabolic defects suggest the Nkx2.2 SD domain is important in maintaining beta cell function and glucose homeostasis.

To determine the regulatory pathways within the beta cell that are affected by loss of the SD domain, we performed RNA-Seq analysis on isolated islets in 7.5 week-old βSD mice (Fig. 3-7A). While pancreatic hormones show minor changes (Fig. 3-7B), many genes related to beta

cell function are differentially expressed (Fig. 3-7C). These genes encode important beta cell proteins including beta cell transcription factors and proteins involved in glucose sensing, insulin processing/secretion, insulin granule synthesis, and the glycolytic pathway (Fig 3-7C). Interestingly, unlike the global SD mutant, progenitor cell markers or factors that are important for alternate endocrine cell fates, including *Ngn3*, *Sox9*, *Arx*, and *Hhex*, are not affected (Fig. 3-7D). This indicates that the Nkx2.2 SD domain is necessary for mediating the transcriptional regulation of genes important for beta cell function.

To confirm the RNA-Seq results, I performed qRT-PCR on islets isolated at 4 weeks of age. This time point was chosen to minimize any potential secondary effects that could result from a hyperglycemic state on gene regulation. This analysis revealed minor changes in pancreatic hormone gene expression in βSD mice, including decreased *Ins2* expression and increased *Sst* and *Gast* (Fig. 3-8A). However, immunostaining at 4.5wk revealed no major changes in Ins^+, Gcg^+, PP^+, or SST^+ cell numbers and there was no observed increase in polyhormonal cell frequency (Fig. 3-8B-G). In addition, while there was an increase in *Gast* gene expression, Gast immunostaining shows no $Gast^+$ cells present in βSD or control pancreata despite positive Gast staining in an E15.5 control (Fig. 3-8H-J). This is consistent with previous observations that the G cell population is restricted to the embryonic pancreas (Suissa et al., 2013). In contrast to its role in establishing monohormonal cell fate during embryogenesis, these results suggest the SD domain is dispensable for the Nkx2.2-mediated maintenance of monohormonal beta cell identity.

Consistent with the RNA-Seq analysis, qRT-PCR also confirmed there were no changes in *Ngn3*, *Arx*, or *Hhex* (Fig. 3-9). However, genes that are important for beta cell function were

downregulated in βSD mice. This included decreased in *Insm1*, *Pdx1*, *Nkx2.2*, *Nkx6.1*, and *Glut2* expression (Fig. 3-10). There was also a trending decrease in *MafA* gene expression (Fig. 3-10). These proteins are required for numerous features of beta cell function. For example, Glut2 is the glucose transporter present in pancreatic beta cells, and is therefore essential for glucose-stimulated insulin secretion. Defects in these proteins and the processes of which they are involved could explain the metabolic perturbations observed in βSD mice, including hyperglycemia and glucose intolerance. This data is indicative of a critical role for the Nkx2.2 SD domain in maintaining the expression of proteins important for beta cell function.

Discussion

Nkx2.2 SD domain mediates early transcriptional regulation in endocrine progenitors

The Nkx2.2 SD domain plays an important function in establishing beta cell fate and monohormonal identity. By conducting RNA-Seq on the endocrine progenitor population of $Nkx2.2^{SDmutant}$ mice, the transcriptional profile of this population and early targets of Nkx2.2 could be identified. Surprisingly, changes in pancreatic hormone gene expression were observed at this early stage, likely reflective of the polyhormonal cell phenotype yet to form. In addition, factors important for the proper differentiation of the beta cell lineage were decreased, including *Nkx6.1*, *MafA*, and *MafB*. These results suggest that the Nkx2.2 SD domain is exerting an effect on transcriptional regulation early in the process of endocrine differentiation.

The $Ngn3^{+}$ endocrine progenitor lineage has been implicated as a critical window for beta cell formation (Chapter 2). In addition, the Nkx2.2 SD domain has been found to play a role in establishing monohormonal cell fate (Levine et al., in preparation). This analysis has shown that

the transcriptional changes associated with altered endocrine fate occur very early in the process of endocrine differentiation, shortly following *Ngn3* expression. These results may have implications for optimizing *in vitro* beta cell differentiation protocols which currently enrich for $Ngn3^{+}$ cells and rely on endocrine progenitor potential.

Nkx2.2 SD domain is necessary within beta cells for proper function

Since Nkx2.2 has recently been shown to maintain beta cell identity and function, βSD mice were used to investigate the role of the SD domain in this process. βSD mice display hyperglycemia and glucose intolerance. In addition, many genes important for beta cell function are dysregulated. This is consistent with the phenotype observed in $Nkx2.2^{\Delta beta}$ mice, which also show hyperglycemia, glucose intolerance, and transcriptional changes at a young age (Dominguez Gutiérrez et al., 2016). These results indicate the Nkx2.2 SD domain mediates Nkx2.2 function within the beta cell.

Interestingly, although Nkx2.2 is required within the beta cell to maintain monohormonal fate, the SD domain does not appear to be involved in this function once beta cells are formed. $Nkx2.2^{\Delta beta}$ mice contain polyhormonal cells (Dominguez Gutiérrez et al., 2016); however, βSD mice do not show detectable increases in polyhormonal cell frequency. This is in contrast to the role of the SD domain during embryonic development and its function in establishing monohormonal cell fate (Levine et al., in preparation). These results may be indicative of an essential role for the SD domain in repressing alternate endocrine lineages early during development.

Since the RIP-Cre allele was employed in the βSD mice, it is important to note that recombination is likely occurring in immature beta cells before birth. The metabolic perturbations observed in βSD mice could reflect defects in the beta cell maturation process or they may be reflecting defects acquired after maturation. Future experiments could be conducted using the MIP-CreER allele and tamoxifen to induce recombination in adult mice (Wicksteed et al., 2010). This would elucidate SD domain function within beta cells that have already finished their maturation process.

Beta cell loss of function is thought to occur during diabetes progression (Guo et al., 2013; Talchai et al., 2012). These results show that the Nkx2.2 SD domain may be important in preventing a similar process and ensuring proper expression of genes that are crucial for glucose homeostasis.

Materials and Methods

Animal maintenance

Mice were maintained on a C57BL/6J background (The Jackson Laboratory). *Ngn3-EGFP*, *Nkx2.2*^{LacZ}, and *Nkx2.2*^{SD} mice were genotyped as previously published (Arnes et al., 2012b; Lee et al., 2002; Levine et al., in preparation). *RIP-Cre, Nkx2.2*^{flox}, *Nkx2.2*^{SD}, and *R26R-Tomato* (B6.Cg-*Gt(ROSA)26-Sor*$^{tm14(CAG-tdTomato)Hze}$/J) mice were genotyped as previously published (Herrera, 2000; Levine et al., in preparation; Madisen et al., 2010; Mastracci et al., 2013). Animal maintenance and procedures were conducted in accordance with a Columbia University Institutional Animal Care and Use Committee approved protocol (AAAG3206).

Fluorescence-activated cell sorting (FACs)

Embryonic pancreata were dissected at E14.5 and E15.5 into ice cold PBS. Pancreata were dissociated in Accumax (Invitrogen) with 25µg DNase I (Qiagen) at 37°C for 30min. Reaction was stopped with 2% fetal bovine serum (FBS)/RPMI 1640 with 25µg DNase I (Qiagen). Cells were washed in DPBS without ions and resuspended in 350µL DPBS without ions with 1µL DAPI (Invitrogen TD21490). Cells were FACs-isolated at the Columbia HICCC FACs facility into 750µL Trizol-LS (Invitrogen). Samples were stored at -80°C until RNA extraction.

Endocrine Progenitor RNA analysis

FACs-isolated cells RNA extraction: The total volume of FACs-isolated cells was brought to 1mL with water. 200uL chloroform was added and samples were homogenized via vortexing. Phase lock gel tubes (Eppendorf) were used to isolate the aqueous layer. Total RNA was then isolated using the Qiagen RNeasy Micro kit: cells isolated from six embryonic pancreata were pooled for each n during RNA extraction at the initial column-binding step in the Qiagen RNeasy micro kit protocol. Controls were Ngn3-EGFP/+; Nkx2.2$^{+/+,\ SD/+,\ or\ LacZ/+}$ mice, and mutants ("Nkx2.2^{SDmutant}") were Ngn3-EGFP/+; Nkx2.2$^{SD/LacZ}$ mice.

RNA-Sequencing (RNA-Seq): Total RNA concentration and quality were measured using the Agilent Bioanalyzer RNA Pico chip. Amplified cDNA was synthesized using the Nugen Ovation RNA-Seq System V2 kit (Part No. 7102). Total cDNA concentration and quality were measured using the Agilent Bioanalyzer DNA 1000 chip. RNA-Seq was performed by the Columbia Genome Center with 30 million single end 100bp reads (n=2, each n comprised of six pooled embryonic pancreata). There were 14 genes showing differential expression with an

adjusted *p*-value < 0.05 and 457 genes with an unadjusted *p*-value < 0.05; both sets of genes were considered in subsequent analysis.

Islet RNA analysis

Islet isolation and RNA extraction: Pancreata were perfused through the common hepatic bile duct with 1mg/mL Collagenase P (Roche)/M199 medium (Invitrogen). Pancreata were dissected and incubated at 37°C for 16min. Samples were washed multiple times with 10% fetal bovine serum (FBS; Gemini Bio Products)/M199. Histopaque (Sigma) and M199 were used to separate islets on a gradient and islets were subsequently picked under a dissection microscope. Islets were disrupted via vortexing, and total RNA was isolated following the Qiagen RNeasy Micro kit.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis: A normalized starting quantity of RNA (200ng-1µg) isolated from islets and random hexamers were used to synthesize cDNA following the SuperScript III reverse transcriptase protocol (Invitrogen 18080-044). qRT-PCR was performed using 200ng cDNA and master mix (Eurogentec) and analyzed using the BioRad CFX96 Real-Time System. Genes were normalized to *CyclophilinB* (probe 5': tggtacggaaggtggag, forward primer 5': gcaaagttctagagggcatgga, reverse primer 5': cccggctgtctgtcttggt) and to littermate controls. An n ≥ 3 was used at all ages. All values are expressed as mean ± SEM. Statistical analysis was performed using two-tailed student's unpaired *t*-test. Significance was achieved with p-value < 0.05. Controls were RIP-Cre/+; Nkx2.2$^{flox/+}$; R26R-Tomato/+ (n=1 female) and Nkx2.2$^{flox/+}$;

R26R-Tomato/+ (n=4 females), and mutants (βSD) were RIP-Cre/+; Nkx2.2$^{SD/flox}$; R26R-Tomato/+ (n=4 females).

Applied Biosystems TaqMan AODs: *Insulin1* (Mm01950294_s1), *Insulin2* (Mm00731595_gH), *Glucagon* (Mm00801712_m1), *Pancreatic polypeptide* (Mm00435889_m1), *Ghrelin* (Mm00445450_m1), *Somatostatin* (Mm00436671_m1), *Gastrin* (Mm00439059_g1), *ChromograninA* (Mm00514341_m1), *Arx* (Mm00545903_m1), *Pdx1* (Mm00435565_m1), *Glut2* (Mm00446229_m1), *Insm1* (Mm02581025_s1).

Sybr green primers: *Hhex* (forward primer 5': tcagaatcgccgagctaaat, reverse primer 5': ctgtccaacgcatccttttt).

Applied Biosystems Primer/TaqMan Probe sets: *Nkx2.2* (probe 5': ccattgactctgccccatcgctct, forward primer 5': cctccccgagtggcagat, reverse primer 5': gagttctatcctctccaaaagttcaaa), *Neurogenin3* (probe 5': cctgcgcttcgcccacaact, forward primer 5': gacgccaaacttacaaag, reverse primer 5': gtcagtgcccagatgt), *Nkx6.1* (probe 5': tctggttccagaaccgcagga, forward primer 5': cggagagtcaggtca, reverse primer 5': tgcgtgcttctttctc), *MafA* (probe 5': cggcgcacgctcaagaaccg, forward primer 5': catccgactgaaacagaag, reverse primer 5': ctcgctctccagaatgtgccgctgc).

RNA-Sequencing (RNA-Seq): Total RNA concentration and quality were measured using the Agilent Bioanalyzer RNA Nano chip. RNA-Seq was performed by the Columbia Genome Center with 30 million single end 100bp reads. Controls were RIP-Cre/+; Nkx2.2$^{flox/+}$; R26R-Tomato/+ (n=1 male) and RIP-Cre/+; Nkx2.2$^{flox/+}$; R26R-+/+ (n=1 female, n=1 male), and

mutants (βSD) were RIP-Cre/+; Nkx2.2$^{SD/flox}$; R26R-Tomato/+ (n=2 males) and RIP-Cre/+; Nkx2.2$^{SD/flox}$; R26R-+/+ (n=1 female).

Glucose tolerance test

Mice were fasted for 12-16h. After fasting, mice were given an intraperitoneal (IP) injection of 20% glucose (2mg/g body weight). Blood glucose was measured at 0, 15, 30, 45, 60, 90, 120, 150, and 180 minutes (min) following IP injection. Area under the curve (AUC) was calculated. An $n \geq 3$ was used at all ages. All values are expressed as mean ± SEM. Statistical analysis was performed using two-tailed student's unpaired *t*-test. Significance was achieved with p-value < 0.05.

Immunohistochemistry

Fixation: Adult pancreata were dissected at 4.5 weeks of age. Samples were fixed in 10% neutral-buffered formalin (VWR) at 4°C for 2-4h, and then transferred to 70% ethanol. Samples were embedded in paraffin and 5μm sections were taken and stored at room temperature (RT).

Staining: Sections were subjected to antigen retrieval with 10mM sodium citrate (pH=6), boiling for 20min. Following antigen retrieval, sections were returned to RT for 20min. Sections were washed in PBS and 0.1% Triton/PBS (PBST) and blocked in 2% donkey serum (DS)/PBST for 30min at RT. Primary antibody was diluted in 2% DS/PBST and incubated on sections overnight at 4°C. Sections were washed in PBS and PBST and incubated 2-3h at RT with secondary antibody (Jackson Immuno Research) diluted in 2% DS/PBST at 1:500. Sections were washed in PBS and PBST and incubated with DAPI (Invitrogen TD21490) diluted in PBS

at 1:1000 for 15 min at RT. Sections were washed in PBS and mounted with fluorescent mounting medium (DAKO S3023).

Primary antibodies: guinea pig α-Insulin (DAKO, 1:500), rabbit α-Glucagon (DAKO, 1:800), rabbit α-Pancreatic Polypeptide (Zymed/Invitrogen, 1:200), rabbit α-Somatostatin (Phoenix Pharmaceuticals, 1:200), rabbit α-Gastrin (Cell Marque, 1:200).

Microscopy: Images were taken using a Zeiss Confocal LSM 710 microscope and processed with Zen, ImageJ, and Adobe Photoshop software.

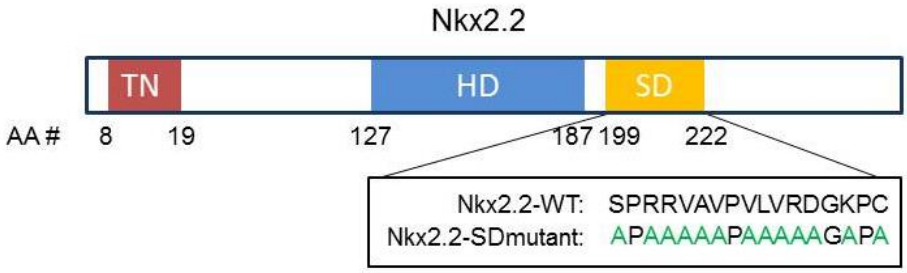


Figure 3-1. Mutation of the Nkx2.2 SD domain. Amino acid substitutions present in Nkx2.2^{SDmutant} mice (Levine et al., in preparation).

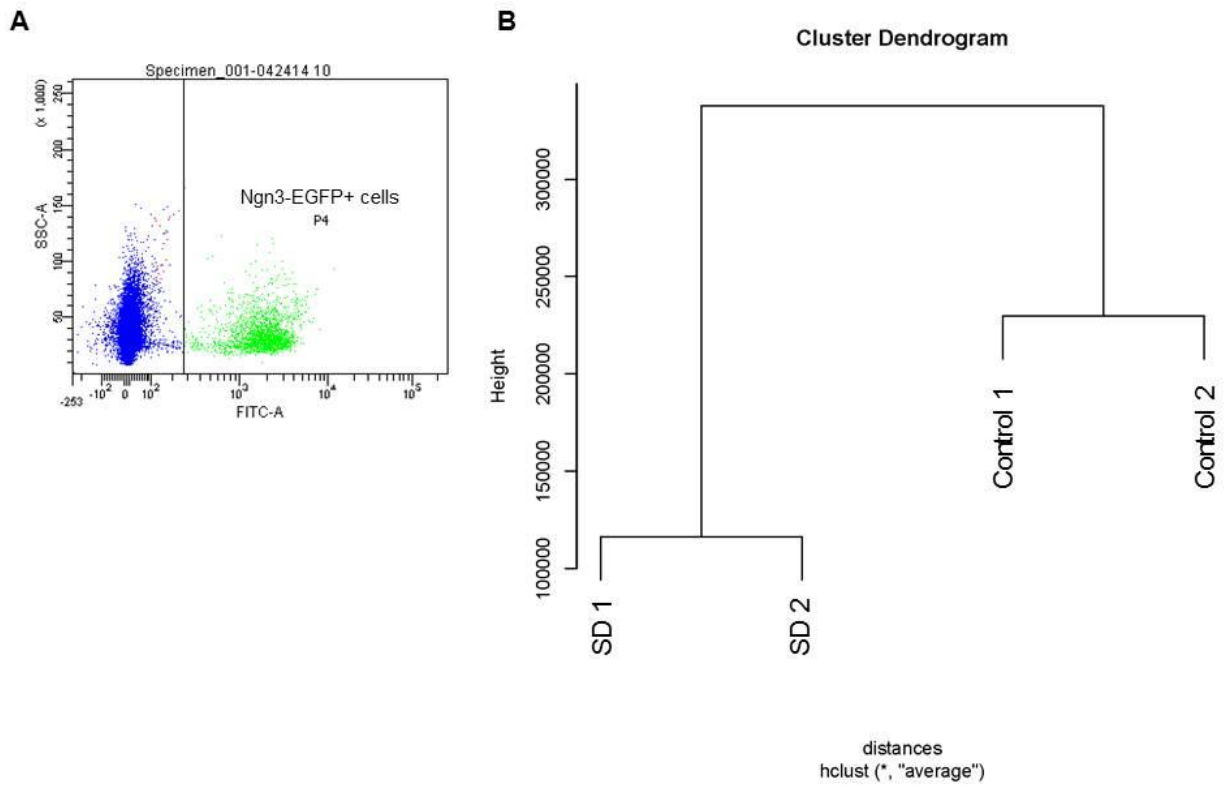


Figure 3-2. Endocrine progenitor isolation and RNA-Seq analysis. (*A*) Representative FACs plot of isolated EGFP^{+} cells. (*B*) Cluster diagram displaying control and Nkx2.2^{SDmutant} (SD) RNA-Seq samples (n=2).

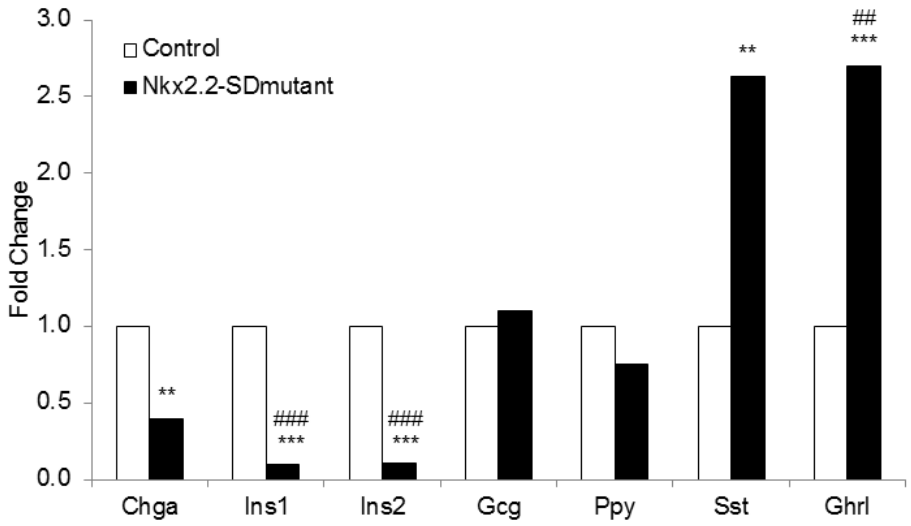


Figure 3-3. Nkx2.2^{SDmutant} endocrine progenitors show changes in pancreatic hormone gene expression. RNA-Seq data showing decreased *Chga*, *Ins1*, and *Ins2* and increased *Sst* and *Ghrl* gene expression. Unadjusted *p*-values: (**) P < 0.01; (***) P < 0.001. Adjusted *p*-values: (##) P < 0.01; (###) P < 0.001.

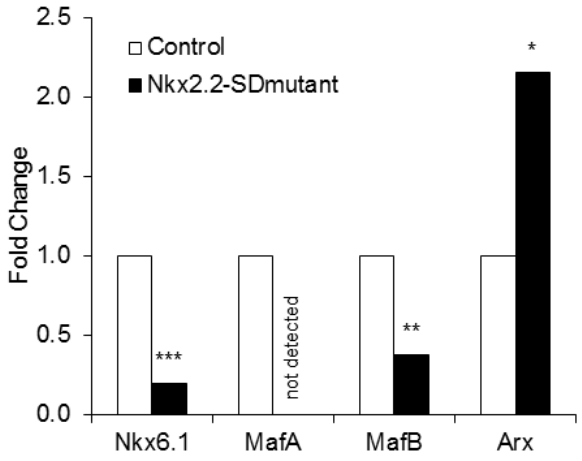


Figure 3-4. Beta and alpha cell transcription factors are changed in Nkx2.2^{SDmutant} endocrine progenitors. RNA-Seq data showing decreased *Nkx6.1*, *MafA*, and *MafB*, and increased *Arx* gene expression. *MafA* gene expression was not detected in Nkx2.2^{SDmutant} samples. Unadjusted *p*-values: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

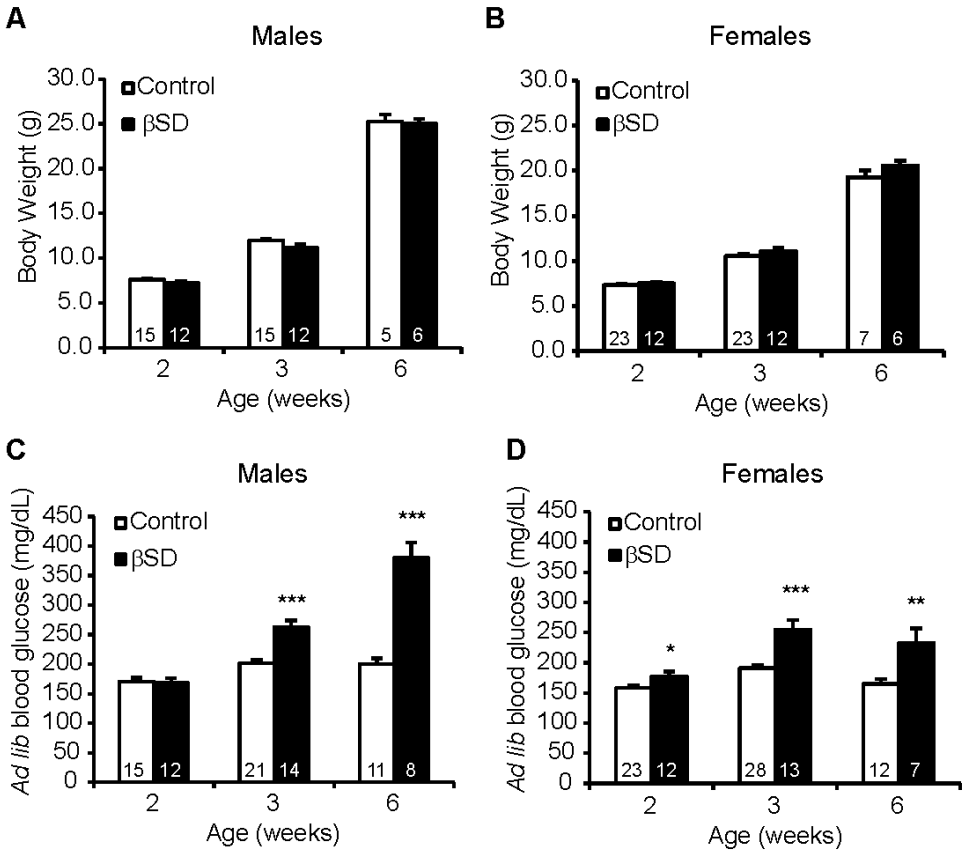


Figure 3-5. βSD mice are hyperglycemic early in life. (*A*) βSD male body weight is not changed. (*B*) βSD female body weight is not changed. (*C*) βSD males display elevated *ad libitum* (*ad lib*) blood glucose levels at 3 and 6 weeks of age. (*D*) βSD females display elevated *ad lib* blood glucose at 2, 3, and 6 weeks of age. The (n) for each condition is displayed at the base of each bar. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

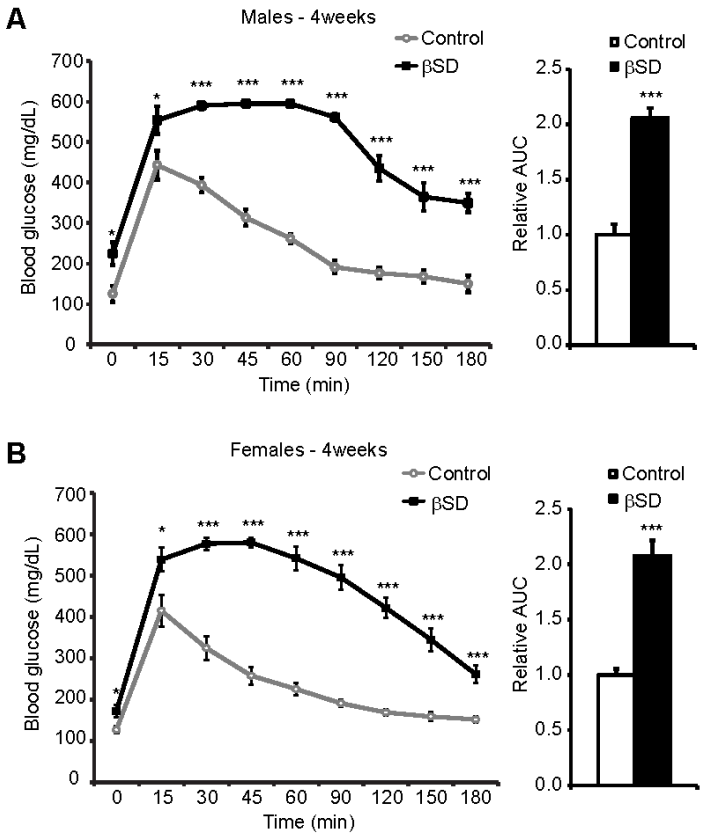
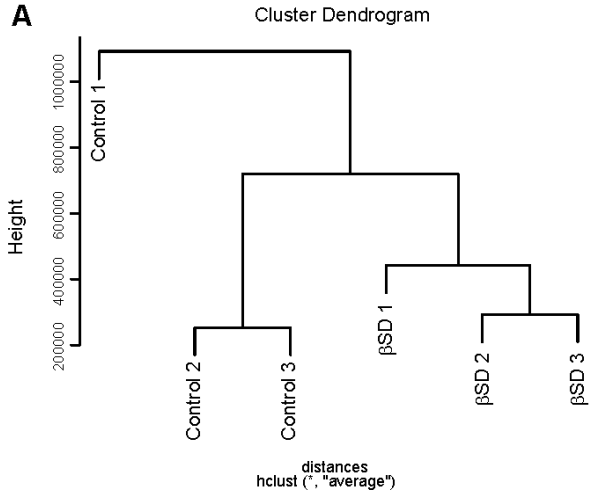


Figure 3-6. βSD mice are glucose intolerant at 4 weeks. (*A*) βSD males are glucose intolerant at 4 weeks of age (Control n=6, βSD n=6). (*B*) βSD females are glucose intolerant at 4 weeks of age (Control n=10, βSD n=6). Area under the curve (AUC) is shown. (*) $P < 0.05$; (***) $P < 0.001$.

A



B

Gene	Fold Change	pval	padj
Hormones:			
Gast	12.67	0.0036	0.0456
Gcg	0.67	0.0146	0.1315
Sst	1.38	0.1521	0.5757
Ghrl	0.69	0.6268	1
Ppy	1.04	0.6893	1
Ins2	0.96	0.7908	1
Ins1	1.00	0.9803	1

C

Gene	Fold Change	pval	padj
β Cell Transcription Factors:			
Isl1	1.89	1.03E-14	1.83E-12
Tle3	0.39	6.79E-10	5.83E-08
MafA	0.63	1.86E-05	0.0006
Nkx6.1	0.58	4.98E-05	0.0014
Insm1	0.73	7.95E-05	0.0021
Pdx1	1.26	0.0084	0.0879
Neurod1	0.89	0.1336	0.5376
Nkx2.2	0.98	0.8066	1
MafB	1.03	0.9233	1
Glucose Sensing, Insulin Processing/Secretion:			
Gipr	0.24	1.19E-13	1.87E-11
Stxbp1	0.65	3.90E-08	2.28E-06
Glut2	0.58	8.51E-07	3.86E-05
Glp1r	0.48	2.43E-06	9.89E-05
Pcsk2	0.75	0.0001	0.0031
Atp2a2	0.73	0.0080	0.0847
Cpe	1.22	0.0242	0.1861
Insulin Granule Features:			
Slc30a8	0.48	1.88E-18	4.73E-16
Sytl4	0.59	0.0026	0.0357
Rab37	0.75	0.0067	0.0739
Ryr3	0.39	0.0201	0.1632
Glycolytic Pathway:			
Ldha	15.40	1.56E-43	1.69E-40
Pcx	0.43	1.63E-16	3.36E-14
Pfkp	0.75	0.0131	0.1221
Gck	1.21	0.0281	0.2050

D

Gene	Fold Change	pval	padj
Additional Transcription Factors:			
Arx	1.46	0.0084	0.0882
Hhex	1.25	0.2289	0.7082
Ngn3	1.71	0.2537	0.7411
Sox9	1.17	0.4289	0.9131

Figure 3-7. RNA-Seq of isolated islets from βSD mice at 7.5 weeks of age. (*A*) Cluster diagram of RNA-Seq samples. (*B*) Pancreatic hormones show minor changes in gene expression in βSD mice. (*C*) β cell transcription factors, glucose sensing, insulin processing/secretion, insulin granule features, and glycolytic pathway genes are differentially expressed in βSD mice. (*D*) Non-beta cell transcription factors are unchanged in βSD mice. (Control n=3, 2 males and 1 female; βSD n=3, 2 males and 1 female). Unadjusted *p*-values (pval) and adjusted *p*-values (padj) are shown.

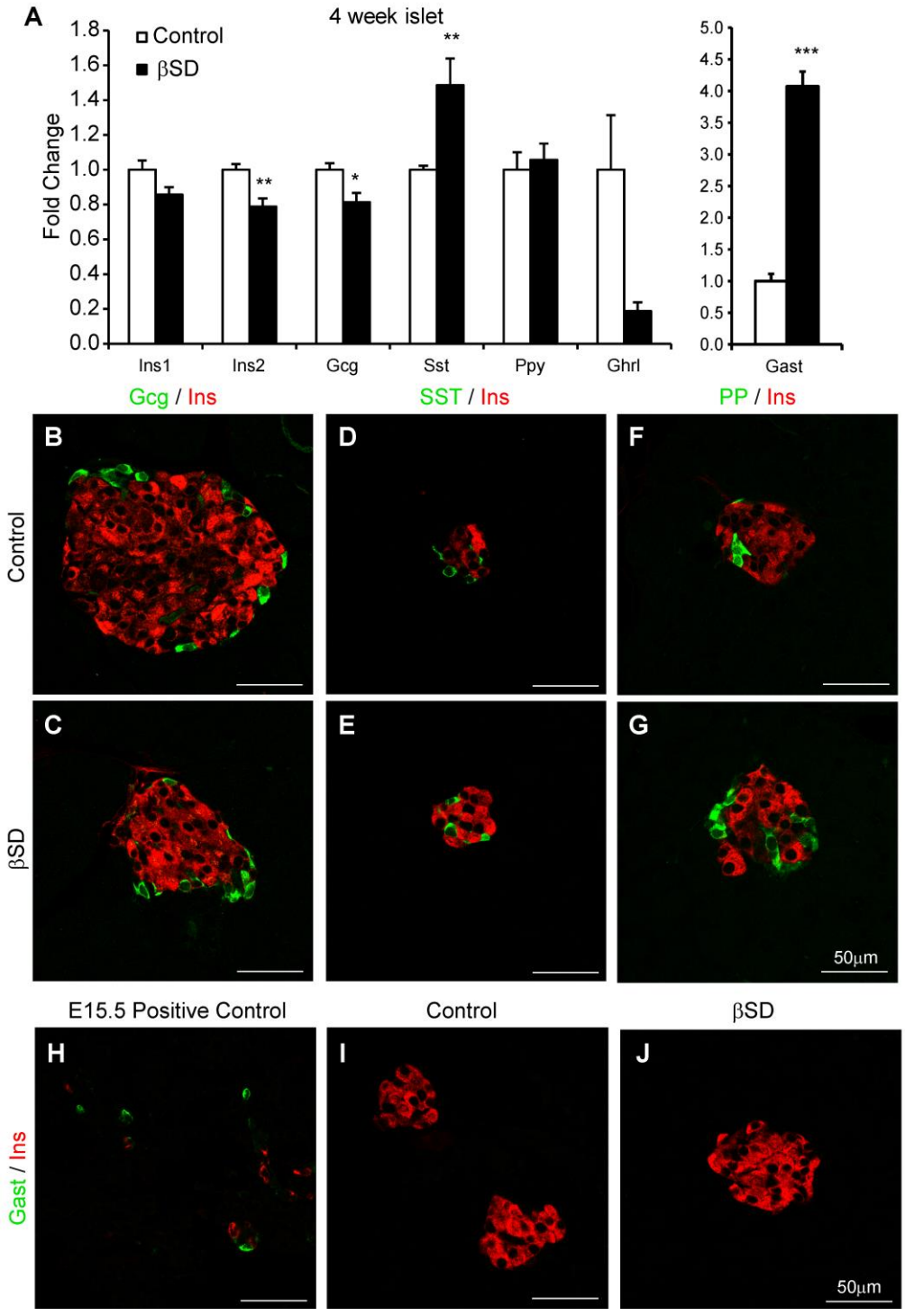

A
4 week islet
Control
βSD
Fold Change
Ins1
Ins2
Gcg
Sst
Ppy
Ghrl
Gast
Gcg / Ins
SST / Ins
PP / Ins
B
D
F
C
E
G
Control
βSD
50μm
E15.5 Positive Control
Control
βSD
H
I
J
Gast / Ins
50μm

Figure 3-8. βSD mice show minor changes in hormone expression. (*A*) qRT-PCR on isolated islets at 4 weeks of age (wk) confirms RNA-Seq results (Control n=5, βSD n=4). (*B-C*) Gcg^+ and Ins^+ cell numbers and Ins^+Gcg^+ co-positive cells appear unchanged in βSD mice at 4.5wk (n=3). (*D-E*) SST^+ cell numbers and Ins^+SST^+ co-positive cells appear unchanged in βSD mice at 4.5wk (n=3). (*F-G*) PP^+ cell numbers and Ins^+PP^+ co-positive cells appear unchanged in βSD mice at 4.5wk (n=3). (*H*) E15.5 pancreas as a positive control for Gast immunostaining (n=3). (*I-J*) Gast+ cells are not detected in control or mice at 4.5wk (n=3). (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

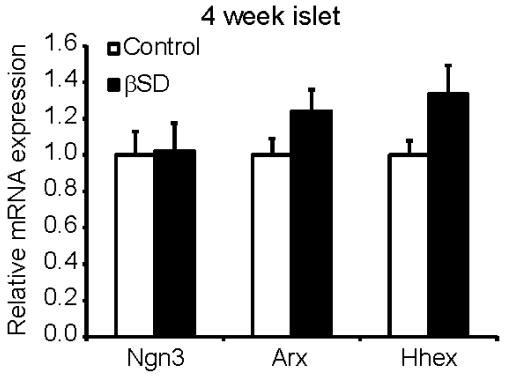


Figure 3-9. Select pancreatic transcription factors are unchanged in βSD mice. qRT-PCR on isolated islets at 4 weeks of age confirms RNA-Seq results (Control n=5, βSD n=4).

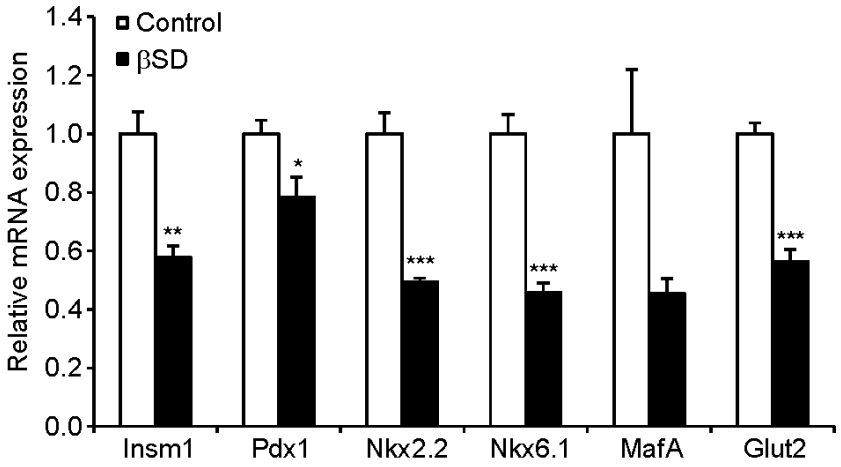


Figure 3-10. β cell factors are decreased in βSD mice. qRT-PCR on isolated islets at 4 weeks of age shows decreased *Insm1*, *Pdx1*, *Nkx2.2*, *Nkx6.1*, and *Glut2* gene expression (Control n=5, βSD n=4).

	Fold Change	pval	padj
Hormones:			
Ins1	**0.10**	1.33E-09	5.27E-06
Ins2	**0.10**	4.31E-24	6.84E-20
Gcg	1.10	0.7633	1
Ppy	0.75	0.4784	1
Sst	**2.63**	0.0019	0.6168
Ghrl	**2.70**	1.08E-06	0.0024
Gast	**1.93**	0.0421	1
Npy	**0.04**	0.0002	0.1436
Pyy	**1.60**	0.0169	1
Endocrine-related factors:			
Chga	**0.40**	0.0010	0.4203
Ngn3	0.87	0.5856	1
Pdx1	0.77	0.5692	1
Nkx6.1	**0.19**	0.0007	0.3248
MafA	0.00	0.6620	1
MafB	**0.38**	0.0010	0.4196
Arx	**2.15**	0.0124	1
Isl1	**2.10**	0.0016	0.5436
Ldha	**6.13**	0.0006	0.3187
Sytl4	**0.03**	1.19E-06	0.0024
Irs4	**8.75**	7.09E-20	5.62E-16
Glp1r	**0.00**	0.0068	1
Mlxipl	**0.60**	0.0412	1

Table 3-1. Nkx2.2^{SDmutant} endocrine progenitor RNA-Seq. RNA-Seq fold change of select pancreas-related genes in Nkx2.2^{SDmutant} endocrine progenitors relative to littermate controls. Unadjusted *p*-values (pval) and adjusted *p*-values (padj) are shown.

CHAPTER 4
CONCLUSIONS AND PERSPECTIVES

My studies into the role of Nkx2.2 and its SD domain have provided insight into the timing and mechanism of beta cell specification, as well as the maintenance of beta cell function. *Ngn3*-driven ablation of Nkx2.2 in Nkx2.2$^{\Delta endo}$ mice revealed a critical window for inducing beta cell fate. Nkx2.2$^{\Delta endo}$ mice displayed defective beta cell differentiation and altered specification of other endocrine lineages; these results were consistent with the Nkx2.2$^{-/-}$ phenotype. However, preserved expression of *Ngn3*, *NeuroD1*, and *Rfx6* has revealed a unique requirement for Nkx2.2 during this developmental window. ChIP-Seq analyses have also revealed co-occupancy of Nkx2.2 with Rfx6 and NeuroD1 at loci near endocrine-related genes. Lastly, the SD domain of Nkx2.2 appears to function within endocrine progenitor cells and beta cells. Early during endocrine specification, the SD domain is necessary for proper transcriptional regulation of endocrine fate genes to establish monohormonal cell fate. Within the beta cell, the SD domain is no longer required to maintain monohormonal cell fate. However, it is necessary for the proper expression of beta cell functional genes, with βSD mice displaying hyperglycemia and glucose intolerance. Overall, these results have implications for *in vitro* beta cell differentiation protocols and reveal processes that may be involved in diabetes progression.

Timing of beta cell specification

Ngn3 marks the endocrine progenitor population, and each Ngn3^{+} cell has traditionally been thought to be multipotent. Recent evidence has suggested there are temporally-regulated endocrine fate competence windows (Johansson et al., 2007), and a recent model has proposed each Ngn3^{+} cell may be unipotent and restricted in its potential (Desgraz and Herrera, 2009).

Since Nkx2.2 is an essential factor for beta cell specification and is expressed early and throughout pancreas development, these thesis studies sought to determine the timing of beta cell specification through conditional ablation of Nkx2.2 within the Ngn3 lineage. The phenotype of $Nkx2.2^{\Delta endo}$ mice suggests Nkx2.2 is required downstream of Ngn3, and suggests that the Ngn3 lineage is a critical developmental window for beta cell specification.

It was surprising that Nkx2.2 function within the pancreatic progenitor population was not sufficient to induce beta cell fate. However, these results are consistent with ablation of other pancreatic transcription factors downstream of Ngn3 in recent studies. Ngn3-Cre; $Rfx6^{flox/flox}$ mice and Ngn3-Cre; $Nkx6.1^{flox/flox}$ mice display defective beta cell differentiation (Piccand et al., 2014; Schaffer et al., 2013). These studies, in combination with the results of this work, demonstrate the importance of the Ngn3 lineage for endocrine specification.

While Nkx2.2 is essential downstream of Ngn3 for beta cell specification, Nkx2.2 function within the $Pdx1^{+}$ pancreatic progenitor has yet to be investigated. Since there is no rescue of the beta cell lineage in $Nkx2.2^{\Delta endo}$ mice, it is clear that Nkx2.2 function within the pancreatic progenitor is not sufficient for beta cell specification. However, Nkx2.2 may still function early in the pancreatic progenitor, upstream of Ngn3, to prime the cell for a particular endocrine fate. Future work could address this question by employing an "addback" approach, where Nkx2.2 expression is induced by an Ngn3-driven allele within an $Nkx2.2^{-/-}$ background. If any defects were present in the beta cell lineage, this would demonstrate that Nkx2.2 is not only playing an early role in beta cell specification, but this would also reveal that endocrine specification can be influenced prior to Ngn3 expression.

These studies have shown that the Ngn3 lineage is essential for beta cell specification and that Nkx2.2 is uniquely required during this window of development. This has implications for optimizing differentiation protocols, where prolonged expression of developmental transcription factors, including Nkx2.2, Rfx6, and Nkx6.1, are likely to be essential to establish a fully functional beta cell *in vitro*.

Nkx2.2, Rfx6, and NeuroD1 co-occupancy

Transcription factors may function in a hierarchical manner, where one transcription factor leads to activation or repression of a downstream transcription factor to influence cell fate. These relationships have been observed in single knockout mice where downstream factors display altered expression in response to loss of an upstream regulator. For example, Nkx2.2$^{-/-}$ mice display decreased expression of *Ngn3*, *NeuroD1*, and *Rfx6* (Chao et al., 2007) (Fig. 2-3E). Since each of these factors is individually required for beta cell specification, it's possible that Nkx2.2 affects beta cell fate via these downstream regulators. While Nkx2.2$^{\Delta endo}$ mice display complete retention of the *Ngn3*, *NeuroD1*, and *Rfx6* expression, beta cell fate is not restored, indicating that Nkx2.2 is uniquely required in the Ngn3 lineage for beta cell specification.

These studies also show significant overlap of Nkx2.2 bound and regulated genes with Rfx6 bound genes and NeuroD1 bound genes. These shared targets are related to endocrine cell fate and function, suggesting Nkx2.2 is not only upstream of Rfx6 and NeuroD1, but also cooperates with them during endocrine specification. Numerous co-binding patterns between Nkx2.2 and Rfx6 or NeuroD1 were observed near target genes. Both overlapping binding peaks and distinct binding peaks were observed, indicating variability in the potential mechanisms underlying co-

occupancy. These results are consistent with motif analysis conducted on the Nkx2.2 ChIP-Seq data (GSE79725). This analysis revealed Rfx4/5/6 and NeuroD1 motifs near Nkx2.2 binding sites (Dominguez Gutiérrez et al., 2016), indicating that Nkx2.2 is likely to share targets with Rfx6 and NeuroD1.

Transcription factor co-occupancy at endocrine related genes also appears to be conserved in humans. A study conducted by Pasquali et al. (2014) characterized pancreatic transcription factor binding in human islets. These binding peaks were cross-referenced with histone modifications, enabling identification of promoter and enhancer regions. Multiple transcription factor ChIP-Seqs were analyzed, including PDX1, NKX2.2, and NKX6.1. This analysis revealed many instances of co-occupancy at enhancer regions. In addition, motifs found near these regions were those of RFX and NEUROD1 (Pasquali et al., 2014). This indicates conservation of pancreatic transcription factor co-occupancy across species.

Although Nkx2.2 and Rfx6/NeuroD1 co-binding suggests cooperation between these transcription factors to regulate gene transcription, the mechanism of such cooperation has not been tested. Functional redundancy may exist, where Nkx2.2, Rfx6, and NeuroD1 function independently of each other to regulate a common target. For this reason, further experiments need to be conducted to first identify the presence of functional cooperation and also to determine the mechanism of such cooperation. Potential manners of cooperative action could include: direct binding in a common complex, factor-dependent recruitment of another (direct or indirect), or independent binding. Simultaneous binding could be determined with re-ChIP

experiments, and high order chromatin structure could be analyzed with chromatin interaction analysis paired-end tag sequencing (ChIA-PET).

These studies reveal co-occupancy of Nkx2.2, NeuroD1, and Rfx6 near endocrine-related target genes. Since each of these factors is individually necessary for beta cell specification (Naya et al., 1997; Smith et al., 2010; Sussel et al., 1998), this co-occupancy implies cooperation to promote beta cell fate. *In vivo* analysis of Nkx2.2$^{\Delta endo}$ mice indicates a unique requirement for Nkx2.2 within the endocrine progenitor lineage, suggesting prolonged expression of Nkx2.2 is necessary for beta cell differentiation. These findings are important to consider for *in vitro* differentiation methods; timing and length of transcription factor co-expression may be crucial for combinatorial regulation of endocrine fate genes.

Role of Nkx2.2 SD domain in establishing endocrine fate

Nkx2.2^{SDmutant} mice display defective beta cell differentiation and polyhormonal cells (Levine et al., in preparation). To determine the transcriptional profile of Nkx2.2^{SDmutant} endocrine progenitors, RNA-Seq was performed on isolated Ngn3-EGFP^{+} cells. This analysis revealed many transcriptional changes in genes related to endocrine fate, including pancreatic hormones and beta cell functional genes. These changes are consistent with the endocrine defects observed in Nkx2.2^{SDmutant} mice. This analysis reveals an important role for the SD domain and Nkx2.2 in the early regulation of endocrine genes, either prior to or shorty after *Ngn3* expression. These results are also consistent with our other studies identifying the Ngn3^{+} endocrine progenitor lineage as a critical window for beta cell specification (Chapter 2).

Future experiments will determine the mechanism of Nkx2.2 transcriptional regulation during early endocrine development. Wang et al. (2015) recently identified poised enhancers near lineage-specific genes in the gut tube stage of hESC differentiation. They also found these poised enhancer states were predictive of transcriptional response, and suggested pioneer transcription factors, such as FOXA1/2, may affect chromatin accessibility and cell fate prior to lineage acquisition (Wang et al., 2015). A similar phenomenon may be occurring with Nkx2.2-mediated transcriptional regulation. Nkx2.2 and its SD domain may prime the chromatin landscape for a particular endocrine cell fate, thereby enabling the rapid transcriptional changes observed in Ngn3 endocrine progenitors. Cell fate choice may be dictated by the open or closed chromatin regions established by Nkx2.2 and/or by the cofactors present in a particular cell.

An additional regulatory mechanism could involve the Nkx2.2 SD domain mediating repression of alternative endocrine fate genes. If the SD domain is responsible for repressing non-beta cell fate genes within developing beta cells, then in Nkx2.2^{SDmutant} mice, this repressive regulation could be lost, leading to polyhormonal cell formation.

Gaining a better understanding of the timing and mechanism of beta cell fate induction, with respect to *Ngn3* expression, has implications for *in vitro* differentiation research. Ngn3^{+} cells are commonly considered to be multipotent for all endocrine lineages; however, their chromatin landscape may already be primed toward particular endocrine fates.

Role of Nkx2.2 SD domain in maintaining beta cell function

Dominguez Gutiérrez et al. (2016) have recently shown that Nkx2.2 is required within the mature beta to maintain its monohormonal identity and function (Dominguez Gutiérrez et al.,

2016). The Nkx2.2 SD domain is also important in establishing monohormonal beta cell fate during development and mediating the pancreas-specific functions of Nkx2.2 (Levine et al., in preparation). In these studies, SD domain function within the mature beta cell was explored using βSD mice. These mice displayed defective glucose homeostasis, indicating the SD domain is required within the beta cell for proper function. In addition, many genes related to beta cell function were also dysregulated, suggesting the SD domain mediates Nkx2.2 transcriptional regulation of targets important for beta cell function. These results are consistent with a model where the SD domain mediates pancreas-specific functions of Nkx2.2, in contrast to Nkx2.2 function in neuronal fate decisions in the CNS.

Surprisingly, βSD mice do not display polyhormonal cells. This is in contrast to the Nkx2.2$^{\Delta beta}$ mice, indicating the SD domain is dispensable for maintaining monohormonal cell fate. This also suggests that the defective regulation of blood glucose observed in Nkx2.2$^{\Delta beta}$ mice is likely not contingent on its polyhormonal phenotype. These results also reveal a context-dependent role of the Nkx2.2 SD domain, where this domain is required for establishing but not maintaining monohormonal endocrine cell fate. This context-dependency could be controlled by the different chromatin landscape or by the different cofactors expressed in endocrine progenitors versus beta cells. As proposed earlier, the SD domain could establish monohormonal beta cell fate by repressing non-beta cell genes. These genes, once repressed, may not require active repression within the beta cell once formed. In Nkx2.2$^{\Delta beta}$ mice, the polyhormonal cells observed could be caused by inappropriate expression of transcriptional activators, which release the repressive marks established by the SD domain during development.

The Nkx2.2 SD domain may exert its function by mediating interactions between Nkx2.2 and other proteins. There is evidence that suggests the SD domain is necessary for interaction between Nkx2.2 and the DNA methyltransferase Dnmt1 (Levine et al., in preparation). Dnmt1 is thought to be a maintenance methyltransferase since it binds preferentially to hemi-methylated DNA (Fatemi et al., 2001; Goyal et al., 2006; Leonhardt et al., 1992). Maintenance of methylation is important during cell cycle progression, since this process involves DNA replication and synthesis of nascent DNA strands. Without propagation of methylation marks, this epigenetic information could be lost during cell cycle progression (Goyal et al., 2006; Hermann et al., 2004). Both pancreatic progenitors and newly formed beta cells undergo proliferation. Pancreatic progenitors proliferate early in pancreas development to expand the progenitor population and establish proper organ size (Stanger et al., 2007). Newly formed beta cells also proliferate to expand the beta cell population during the end of gestation and also in neonatal life (Bouwens and Rooman, 2005). Given these instances of proliferation during development and cell fate determination, Nkx2.2 and its SD domain may be responsible for recruiting Dnmt1 to maintain methylation near endocrine genes. This interaction may be required both before and after beta cell formation to establish monohormonal cell fate and to maintain proper expression of beta cell function genes.

It is also likely that the SD domain facilitates interactions with other proteins as well. Recently, mass spectrometry (mass spec) analysis was conducted to identify interactors with Nkx2.2-WT and Nkx2.2-SDmutant protein in the Min6 β cell line (Balderes, unpublished data). Preliminary analysis has identified Nkx2.2 interactions with chromatin remodeling proteins, many of which are members of the nucleosome remodeling and deacetylase complex (NuRD) complex,

including Chd3/4/5, Ddx5, Hdac1/2, Mta1/2/3, Rbbp4/7, and Gatad2a/2b (Balderes, unpublished data). Nkx2.2-SDmutant protein appears to have reduced interactions with Chd3/5, Mta1/2/3, Rbbp4/7, and Gatad2a/2b (Balderes, unpublished data); however, these mass spec experiments were not quantitative. Any observed reduction in interactions with Nkx2.2-SDmutant protein compared to Nkx2.2-WT protein must be confirmed through other methods. Co-immunoprecipitation experiments can be conducted to confirm these interactions, and requirement for the SD domain can be assessed.

Future experiments can also dissect the role of the SD domain in mature beta cells. The MIP-CreER allele can be used to distinguish between SD domain function during beta cell maturation and in fully mature beta cells (Wicksteed et al., 2010). The RIP-Cre allele used in βSD mice is activated shortly after beta cell formation and therefore cannot distinguish between these two stages. If the primary function of the SD domain is to mediate interaction with Dnmt1 to maintain epigenetic information during proliferation, perhaps mice with the MIP-CreER would no longer display metabolic defects since adult beta cells are primarily quiescent. However, these mice may display defects when challenged with high fat diet (HFD) or pregnancy, since these conditions stimulate beta cell proliferation (Bouwens and Rooman, 2005), and may reveal defects in the propagation of epigenetic marks.

Mouse models of diabetes can involve loss of beta cell function (Guo et al., 2013; Talchai et al., 2012). This analysis of Nkx2.2 SD domain function has given us a better understanding of the mechanisms required to establish and maintain beta cell fate. Similar mechanisms may be dysregulated in a diabetic state and may lead to novel therapeutic targets for diabetic therapy.

Concluding Remarks

Diabetes is a serious condition involving beta cell dysfunction and loss of glycemic control; this disease is increasing in prevalence worldwide (IDF, 2015). A current area of therapeutic research has focused on the *in vitro* differentiation of pancreatic beta cells, with the goal of transplanting these cells back into patients to restore glucose homeostasis. In this thesis work, a critical window for beta cell specification has been identified, with a unique requirement for the transcription factor Nkx2.2. In addition, co-occupancy between Nkx2.2, Rfx6, and NeuroD1 at endocrine-related genes suggests novel cooperation among these beta cell transcription factors. Rather than functioning in a strict hierarchy, there may be combinatorial functions that are necessary for endocrine commitment. Lastly, the SD domain of Nkx2.2 protein appears to not only be involved in early regulation of pancreatic specification genes, but also within the beta cell for maintenance of beta cell fate. These studies provide a better understanding of pancreatic beta cell differentiation and also the mechanisms involved in maintaining beta cell function. These *in vivo* mechanisms may prove to be important for the optimization of *in vitro* beta cell differentiation protocols and successfully maintaining beta cell function following beta cell transplantation for diabetic therapies.

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