STRUCTURE-FUNCTION ANALYSIS OF THE ESSENTIAL ISLET REGULATORY FACTOR NKX2.2

JAMES PAPIZAN

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy under the Executive Committee of the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2013
ABSTRACT

STRUCTURE-FUNCTION ANALYSIS OF THE ESSENTIAL ISLET REGULATORY FACTOR NKX2.2

JAMES PAPIZAN

The specification and differentiation of the pancreatic beta cell lineage requires guidance by spatiotemporally regulated signaling cues and a highly orchestrated set of transcription factors. Defining the factors and their regulatory functions that are required for proper beta cell development will enhance our ability to recapitulate these developmental events in vitro to generate beta cells from alternate cell sources. The homeodomain transcription factor Nkx2.2 is essential for pancreatic endocrine cell development; Nkx2.2−/− mice lack all beta cells and have reductions in alpha and pancreatic polypeptide (PP) cells. In place of these cell populations, the Nkx2.2−/− null islet is replete with ghrelin-producing epsilon cells. An Nkx2.2-repressor fusion protein derivative (Pdx1:Nkx2.2-EnR) expressed in the Nkx2.2−/− background can fully rescue the alpha cell population, but can only specify a few immature beta cells, suggesting that Nkx2.2 must contain both repressor and activator functions to properly guide beta cell development. Accordingly, Nkx2.2 has been shown to be an activator of several beta-cell targets. It has also been demonstrated that the corepressor Grg3 is expressed in the endocrine population and can physically interact with Nkx2.2, which points toward a mechanism by which Nkx2.2 confers transcriptional repression; however, the genes targeted by Nkx2.2/Grg3 are unknown. Additionally, how Nkx2.2 can both repress and activate genes in the same cellular context, and differentially regulate the same gene in different cellular contexts, is not understood. In this dissertation, I sought to determine the regulatory role of Nkx2.2 in the developing pancreas and its dependence on Grg interactions, and to elucidate whether post-translational modifications
play a role in modulating Nkx2.2 regulatory activities. By analyzing mice carrying knock-in mutations in the Nkx2.2 Grg-interaction domain (Nkx2.2^T/Nmut), I show that the interaction between Nkx2.2 and Grg protein is required at two developmental stages of beta cell development: 1) Grg-mediated Nkx2.2 repression is necessary for correct beta-cell specification, and 2) the recruitment of Grg by Nkx2.2 is required to repress Arx in the beta cells to prevent beta-to-alpha cell reprogramming. Additionally, by analyzing the Nkx2.2^T/Nmut and Nkx2.2^T/Nmut;Ins:Cre;Arx^fl/fl mice, I have identified several additional genes that may be regulated by Grg-mediated Nkx2.2 repression. Finally, I also present data to suggest that Nkx2.2 protein is phosphorylated, and that the phosphorylation state determines whether Nkx2.2 functions as an activator or a repressor in a promoter-specific context. These studies have begun to elucidate the complex regulatory roles that Nkx2.2 plays in specifying and maintaining the beta-cell lineage. Future analyses will help us to better understand the spatiotemporal regulatory activities that are required to make and maintain functional beta cells.
# TABLE OF CONTENTS

1. INTRODUCTION 1

DIABETES 1

ALTERNATE SOURCES OF PANCREATIC BETA CELLS 2

PANCREAS DEVELOPMENT 5

Pancreas Specification and Outgrowth 5
NGN3 and Endocrine-Cell Commitment 9
Nkx2.2 15
ARX 18
Groucho Proteins 20

MECHANISM OF REPRESSION 22
ROLE IN PANCREAS DEVELOPMENT 23

TRANSCRIPTIONAL REGULATION 24

Repression Mechanisms Controlling Pancreas Development and Maintenance 25

POST-TRANSLATIONAL MODIFICATIONS 27

2. PUBLISHED MANUSCRIPT: Nkx2.2 Repressor Complex Regulates Islet BetaCell Specification and Prevents Beta-To-Alpha Cell Reprogramming 33

INTRODUCTION 33

RESULTS 36

Mutation of the Nkx2.2 TN Domain Disrupts the In Vivo Interaction Between Nkx2.2 and the Grg3 Corepressor and Leads to Diabetes in Adult Nkx2.2^{TNmut/TNmut} Mice 34

Nkx2.2 TN Domain Is Required for Appropriate Islet Cell Development 34

Islet Cell-Specific Transcription Factor Expression Is Disrupted in Nkx2.2^{TNmut/TNmut} Pancreas 35

Mutation of the Nkx2.2 TN Domain Results in a Beta-To-Alpha Cell Conversion Due to the Ectopic Expression of Arx in Beta Cells 36

Nkx2.2 Directly Binds and Represses the Methylated Arx Promoter in Beta Cells 38
BETA-CELL-SPECIFIC DELETION OF DNMT3A ALSO RESULTS IN A BETA-TO-ALPHA CELL CONVERSION 40

REMOVAL OF ARX FROM Nkx2.2^{TMMUT/TMMUT} BETA CELLS IS SUFFICIENT TO PREVENT BETA-CELL REPROGRAMMING, BUT FAILS TO RESTORE BETA-CELL MATURATION AND FUNCTION 40

DISCUSSION 40

MATERIALS AND METHODS 44

REFERENCES 45

SUPPLEMENTARY DATA 48

3. TRANSCRIPTIONAL PROFILING OF Nkx2.2^{TMMUT/TMMUT} AND Nkx2.2^{TMMUT/TMMUT};INS:CRE;ARX^{FL/FL} PANCREAS 63

INTRODUCTION 63

RESULTS 65

DISCUSSION 69

MATERIALS AND METHODS 72

4. SERINE PHOSPHORYLATION REGULATES Nkx2.2 FUNCTION 79

INTRODUCTION 79

RESULTS 82

DISCUSSION 86

MATERIALS AND METHODS 90

5. CONCLUSIONS AND PERSPECTIVES 100

GRG-MEDIATED Nkx2.2 REPRESSION 100

Nkx2.2, Histone Modifications and Epigenetics 101

Prominin-2, IRS4 and RBP4 104

Nkx2.2 Phosphorylation 105

Concluding Remarks 107

REFERENCES 109
LIST OF FIGURES AND TABLES

1. INTRODUCTION

Figure 1: Tip-Trunk Compartamentalization in the Developing Pancreas 12

2. PUBLISHED MANUSCRIPT: NKX2.2 Repressor Complex Regulates Islet Beta Cell Specification and Prevents Beta-to-Alpha Cell Reprogramming

Figure 1: The NKX2.2 TN Domain Mutation Disrupts the NKX2.2-Grg3 Interaction 35

Figure 2: NKX2.2<sup>TN<sub>MUT</sub>/TN<sub>MUT</sub></sup> Mice Develop Diabetes 36

Figure 3: NKX2.2<sup>TN<sub>MUT</sub>/TN<sub>MUT</sub></sup> Mice Display Decreased Insulin Expression and Increased Glucagon and Ghrelin Expression 37

Figure 4: Mutations in the NKX2.2 TN Domain Cause Arx Misexpression in Beta Cells and Beta-to-Alpha Cell Reprogramming 38

Figure 5: NKX2.2 Directly Represses Arx in Beta-TC6 Cells but not Alpha-TC1 Cells 39

Figure 6: Beta-Cell-Specific Loss of Arx in the NKX2.2<sup>TN<sub>MUT</sub>/TN<sub>MUT</sub></sup> Mice Rescues Glucagon and Arx Expression 41

Figure 7: Model of NKX2.2 Regulation of the Arx Promoter in Beta Cells 43

3. TRANSCRIPTIONAL PROFILING OF NKX2.2<sup>TN<sub>MUT</sub>/TN<sub>MUT</sub></sup> AND NKX2.2<sup>TN<sub>MUT</sub>/TN<sub>MUT</sub>;INS:CRE;ARX<sup>FL/FL</sup></sup> Pancreas 63

Figure 1: Global Gene Expression Changes in P0 Pancreatic RNA 74

Figure 2: qRT-PCR Confirmation of Gene Expression Changes in NKX2.2<sup>TN<sub>MUT</sub>/TN<sub>MUT</sub></sup> Mice 75

Figure 3: NKX2.2 Binds the Prominin-2 Promoter in Beta-TC6 Cells 76

Figure 4: NKX2.2 and Grg3 Bind the Rbbp4 Promoter in Beta-TC6 Cells 77

Figure 5: qRT-PCR Confirmation of Gene Expression Changes in NKX2.2<sup>TN<sub>MUT</sub>/TN<sub>MUT</sub></sup> Mice 78

4. SERINE PHOSPHORYLATION REGULATES NKX2.2 FUNCTION

Figure 1: NKX2.2 S11 Is Predicted To Be Phosphorylated and Is Well Conserved 93

Figure 2: NKX2.2 S11 Is Phosphorylated by PKC 94

Figure 3: Transfected and Endogenous Levels of NKX2.2 in Beta-TC6 Cells 95

Figure 4: S11 Mutations Affect Recruitment of Cofactors to the Arx Promoter 96
FIGURE 5: S11 Mutations Affect Local Epigenetic Marks on the NeuroD Promoter 97

FIGURE 6: Gene Expression Changes in Response to NKX2.2 S11 Mutations 98

FIGURE 7: Proposed Model of Cell-Specific Regulation by NKX2.2 S11 Phosphorylation 99
ACKNOWLEDGEMENTS

I would like to begin by thanking Lori Sussel for her endless support and guidance. I feel very fortunate to have been accepted into her lab as her first Columbia graduate student, who did not have any background in development, transcription factors or gene regulation. Lori has maintained an open door and has always been willing to entertain any of my questions and ideas. Because of Lori, my biological interests have translocated from the cytoplasm to the nucleus, a subcellular location that used to intimidate me. Her support, encouragement and influence have guided me on a career path that I will pursue with vigor and enthusiasm.

I also would like to extend a great deal of gratitude toward the Sussel lab members. Being able to come into a supportive, convivial atmosphere, made every day enjoyable. When I joined the lab, Jonathon Hill was always happy to answer my incessant questions and assist me with lab techniques. I thank Jonathon, as his help was indispensable as I learned the skills I would use throughout my graduate studies.

Finally, I thank my wife Ansley. I have pulled Ansley away from home and family, and yet she continues to unconditionally love and support me. Her encouragement is more than what I feel I deserve.
DEDICATION

I dedicate this work to my wife Ansley and my daughter Ruby.
CHAPTER 1
INTRODUCTION

Diabetes

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from defects in insulin action, insulin production, or both. According to data from the 2011 National Diabetes Fact Sheet, there are 25.8 million Americans with diabetes. In 2010 there were 1.9 million new cases diagnosed in individuals aged 20 years or older. These numbers emphasize that there is a current, escalating diabetes pandemic, which exacerbates healthcare problems when considering diabetes increases the risk of heart disease and stroke, high blood pressure, blindness, kidney disease, neuropathy and amputation; diabetes and its complications resulted in a staggering $174 billion in healthcare expenditure in the United States in 2007. But diabetes is no longer a Western problem; the International Diabetes Federation has estimated that there are 336 million individuals with diabetes worldwide, and 4.6 million people aged 20-79 died in 2011 from diabetes and/or diabetes-related issues, which equates to one death every seven seconds.

Type 1 diabetes (T1DM) is an autoimmune disease that results in beta cell destruction, and symptoms are generally presented early in life. T1DM accounts for 5-10% of all diabetes cases, and patients remain dependent on exogenous insulin life-long. Type 2 diabetes (T2DM), the most prevalent form of diabetes, results from a peripheral insulin resistance and insufficient insulin production by beta cells. In addition to genetic components, factors such as age, pregnancy and obesity have been shown to influence the progression of T2DM. The early stages of T2DM can be controlled by diet, but ultimately, as the disease progresses and beta cell failure ensues, patients become insulin-dependent. However, even with insulin treatment, many patients, both T1DM and T2DM, have difficulty maintaining correct blood glucose levels at all times.
Additionally, insulin treatment increases the risk of dangerous episodes of hypoglycemia, especially when in the hands of young and/or uneducated individuals. These problems have led physicians and researchers to explore other avenues of treatment.

The first pancreas transplant was carried out in 1967 (Kelly et al., 1967). However, due to risks associated with major surgery, the isolation and transplantation of pancreatic islets was introduced (Ballinger and Lacy, 1972; Scharp et al., 1990), avoiding the need for major laparotomy. Further refinements to this protocol (Shapiro et al., 2000) revealed that pancreatic islet transplantation was a viable option for T1DM treatment (Ryan et al., 2005; Shapiro et al., 2006). Islet transplantation has its limitations, however. Not only do recipients require ongoing immunosuppressive therapy to prevent rejection, cadaveric donor islets are severely lacking in number (Sheehy et al., 2003), and live donors are subjected to risky surgery and are put at risk for developing diabetes themselves (Matsumoto et al., 2006; Matsumoto et al., 2005). These impediments have led researchers to investigate other sources of mature, glucose-responsive beta cells for the treatment of diabetes.

**Alternate sources of pancreatic beta cells**

The liver: In order to circumvent the aforementioned problems with islet transplantation (immunorejection and cadaveric islet availability), many investigators in developmental biology and regenerative medicine have focused their efforts on generating functional beta cells from alternate sources such as liver, exocrine pancreas, induced pluripotent stem (iPS) cells or embryonic stem (ES) cells. The liver is an attractive candidate from which to derive beta cells since it is an endoderm-derived organ that shares the nascent developmental program with the pancreas (Xu et al., 2011).
Many attempts to directly transdifferentiate liver cells to beta cells have been made (Ber et al., 2003; Kojima et al., 2003; Li, 2009; Nagaya et al., 2009; Tang et al., 2006); however, this approach is not achieved without the use of viruses or transgenes and, moreover, the beta-like cells that were generated were lacking in numbers and mature, functional markers.

iPS cells: Zhou and colleagues were able to show the transdifferentiation of pancreatic acinar cells into beta-like cells by introducing three key transcription factors, known for individual essential roles in proper beta-cell development, into the exocrine pancreas (Zhou et al., 2008). This exciting result demonstrated that highly specialized cells in the exocrine pancreas retain enough plasticity that when manipulated with just three transcription factors, can change directly into another highly specialized cell in vivo. However, the small number of beta-like cells that were generated did not coalesce with the other transdifferentiated beta-like cells to form islets. Although, these cells did establish intimate contact with the vasculature, suggesting they had the capacity to sense fluctuating changes in blood glucose levels. Additionally, this approach was achieved by the use of adenoviruses, which may not be a realistic therapeutic option when considering the problems associated with the use of viral vectors (Hasbrouck and High, 2008).

In 2006, Takahashi and Yamanaka made a momentous contribution to the field of stem cell biology when they demonstrated that terminally differentiated cells can be reprogrammed to pluripotency with the addition of a defined set of transcription factors (Takahashi and Yamanaka, 2006). In theory, these iPS cells could then be directed toward the beta-cell lineage in culture to amass large quantities of beta cells. The use of iPS cells is an appealing alternate source of beta cells, in that they circumvent the political and ethical struggles associated with the use of human ES cells. iPS cells could also be used to dissect the developmental steps that go awry in many genetic diseases, including diabetes. Despite the exciting progress, questions still remain
regarding the use of iPS cells: for example, are iPS cells functionally equivalent to embryonic stem cells? A recent report illustrates that iPS cells retain an epigenetic memory of the donor tissue, which favors their differentiation along the lineage of the donor cell and impedes the differentiation along other lineages (Kim et al., 2010). Reports such as these warrant a more critical evaluation of iPS cells relative to ES cells.

Human ES cells: By far, the greatest advances in generating beta cells from alternate sources have been made by utilizing human ES cells. The most successful protocol was outlined by D’Amour and colleagues, in which human ES cells were first differentiated to definitive endoderm (D'Amour et al., 2005). When ES-cell-derived endoderm was further differentiated toward the endocrine lineage in vitro, only a small percentage of cells were insulin-positive, they were not glucose-responsive, many cells were poly-hormonal, and the majority of the cells were ghrelin-positive (D'Amour et al., 2006). Subsequent refinements to the protocol led to the development of strategies to induce ES cells to differentiate into endodermal cells capable of generating glucose-sensitive, insulin-secreting cells when transplanted into mice (Kroon et al., 2008). In spite of this compelling work, insulin positive, glucose-sensing cells have yet to be generated in vitro. The generation and purification of mature beta cells in vitro is paramount for transplantation applications in order to avoid recipient teratoma formations, which occur after implantation of multipotent progenitor endoderm (Kroon et al., 2008). However, this exciting work by Kroon et al. begs the question: What is happening in vivo that directs the transplanted endoderm to ostensibly functioning beta cells? What are these in vivo potentiating and maturation signals that promote the differentiation of the transplanted endoderm down the beta-cell lineage, and which seem to be lacking or improperly applied in the in vitro protocols?

Although the advances in stem-cell directed differentiation are in many ways directly attributable
to our increased understanding of pancreatic development, the inability to generate a fully
functional beta cell in vitro suggests that there is still much to be learned regarding how
developmental processes guide pancreatic specification, endocrine differentiation and beta cell
maturation.

**Pancreas development**

Pancreas specification and outgrowth

The pancreas is a bivalent organ with both exocrine and endocrine functions. The exocrine tissue
consists of acinar cells that synthesize and secrete digestive juices into the duodenum by way of
pancreatic ducts, while the endocrine pancreas is made up of islets of Langerhans that sense
fluctuations in blood glucose and release hormones via fenestrated connections with the islet
vascular capillary bed into the bloodstream to maintain glucose homeostasis.

The development of the mature pancreas involves a carefully organized series of embryological
events that consist of appropriately timed external signaling cascades and a hierarchy of
transcriptional regulation. The first morphological evidence of pancreas development occurs
around embryonic day 8.5 (e8.5) as buds of thickening mesenchyme on the dorsal and ventral
aspects of the primitive gut tube, just distal to the stomach. Around e9.0, the underlying
endoderm evaginates into the mesenchyme with subsequent plexus formation, which eventually
expands to a fully differentiated, functional organ. The position and competence of early
pancreatic buds are influenced by specific inductive and permissive interactions from
neighboring tissues (Zaret and Grompe, 2008), as long as the endoderm is competent to respond
to these signals; therefore, pancreas specification is dependent upon endodermal patterning itself
(Grapin-Botton and Melton, 2000). Proper endodermal patterning will refine gene expression
domains along the anterior-posterior (A-P) and dorsal-ventral axes that distinguish the pancreas lineage from other endodermal-derived organs, such as the lungs, liver, thyroid, thymus, biliary system and digestive tract.

The definitive endoderm begins as a flat sheet of cells at the beginning of gastrulation, and multiple studies using different developmental systems have shown critical roles for Nodal (a TGF-beta superfamily member with powerful dose-dependent inductive effects) in the development of endoderm. In *Xenopus* (Clements et al., 1999; Green and Smith, 1990) and zebrafish (Shen, 2007) Nodal has been shown to initiate gastrulation and provide a necessary gradient for the segregation of mesoderm and endoderm. During gastrulation the endoderm establishes the A-P axis, by way of a mechanism that today remains quite vague. Interestingly, the endoderm remains plastic along its A-P axis, in that anterior endoderm transplanted to a posterior location will acquire a posterior fate, and the same is true when posterior endoderm is transplanted into anterior endoderm (Wells and Melton, 2000). The posterior endoderm plasticity is lost, however, by early somite stages. This was shown by the inability of the posterior endoderm to become anteriorized when transplanted, while the anterior endoderm retained the ability to become posteriorized (Kumar et al., 2003). While the understanding of early molecular mechanisms controlling endodermal patterning remains incomplete, several signaling pathways have been identified that play important roles in this process and pancreas specification, which are discussed below.

Studies in *Xenopus* have illustrated that pancreas induction is prompted by broad suppression of mesodermal WNT signaling in the anterior endoderm, while active mesodermal WNT signaling in the posterior endoderm suppresses pancreas induction (McLin et al., 2007; Wells and Melton, 2000). McLin et al. demonstrated that suppression of WNT is required for the expression of the
anterior endoderm marker *Hhex*, and when WNT is ectopically expressed, there is a loss of anterior derived organs, namely the pancreas and liver. It has further been demonstrated that *Sfrp5*, a secreted WNT antagonist, is required to maintain low WNT signaling levels in the anterior endoderm, a condition conducive to pancreas specification (Li et al., 2008). However, it remains to be determined if pancreas specification requires the complete absence of anterior endodermal WNT signaling, or if a low-to-high gradient is needed along the A-P axis.

During this early specification time window, the dorsal and ventral pancreatic domains are exposed to different inductive stimuli. From the time of gut tube formation to about e8.0, Sonic hedgehog (Shh) is broadly expressed in the developing endoderm but is excluded from the dorsal pre-pancreatic primordium, which is in close apposition with the notochord. This proximity to the notochord has been demonstrated to be essential in dorsal bud differentiation; removal of the notochord prior to e8.0 results in improper dorsal pancreas formation (Kim et al., 1997). From these and other studies combined, it has been concluded that the notochord dictates dorsal pancreatic differentiation most likely by Shh suppression in the pancreatic endoderm. Although Shh is secreted from the notochord, the TGFβ signaling molecule Activin-βB is also secreted, which has been shown to repress Shh and induce *Pdx1* in the dorsal endoderm (Hebrok et al., 1998). Grafting experiments have shown that notochord can suppress Shh signaling in the lateral and ventral endoderm, while notochord deletion results in ectopic Shh expression, accompanied by failure of pancreas formation (Hebrok et al., 1998; Hebrok et al., 2000). Accordingly, Shh expression driven by the promoter elements of the pancreatic/duodenal progenitor marker, *Pdx1*, severely impairs pancreatic endocrine and exocrine formation (Apelqvist et al., 1997). Around e8.0, the paired dorsal aortae fuse at the midline, coming between the notochord and dorsal foregut. Lammert et al. have shown a role for this aortic intervention, in that the aortic
endothelial cells can induce pancreatic bud-like structures as well as Pdx1 and subsequent insulin expression in the adjacent endoderm (Lammert et al., 2001). While the dorsal pancreatic domain receives signals from the notochord and dorsal aorta, the formation of the ventral pancreatic tissue is under control of the adjacent cardiac mesenchyme and septum transversum mesenchyme. During these early events, FGF from the cardiac mesoderm and bone morphogenetic protein (BMP) from the septum transversum mesenchyme work in a time- and dose-dependent manner to specify the liver fate while suppressing pancreas induction. It is only during foregut closure, when ventral endodermal cells migrate caudally to distance themselves from the cardiac mesodermal tissue, that these cells escape FGF signals and are allowed to initiate ventral pancreas specification (Bort et al., 2004). However, it was recently shown that FGF10 signaling through, most likely, the Fgfr2b receptor was necessary to maintain pancreas fate and to prevent liver induction in pancreatic Pdx1+ progenitor cells (Seymour et al., 2012). This conclusion was derived from conditionally inactivating Sox9 in Pdx1+ cells, which led to an upregulation in liver-specific genes and a concomitant decrease in Fgfr2b and Fgfr4, suggesting that Sox9 is required to properly regulate Fgfr expression to maintain pancreas identity. Supporting evidence using Ptf1a:Cre to excise Sox9, which resulted in a mosaic Sox9 deletion due to sensitivity issues at the Sox9FL locus, illustrated that only the cells that lacked Sox9 lacked Fgfr2, while Sox9+ cells retained Fgfr2. The authors also demonstrated that FGF10 null mice have a phenotype similar to the Pdx1:Cre;Sox9fl/fl mice, which may suggest a Sox9/FGF feed-forward loop. To substantiate these claims a closer molecular analysis is needed as well as possible genetic rescue experiments, such as overexpressing Sox9 at various times within the FGF10 null background. Regardless, the paper presents convincing genetic data and merits further investigations into the FGF-mediated pancreas specification mechanism.
Retinoic acid (RA) signaling is required for proper development of many organs, and multiple systems have demonstrated an essential role for RA in pancreas specification, including zebrafish (Stafford and Prince, 2002), Xenopus (Chen et al., 2004; Stafford et al., 2004) and mouse (Martin et al., 2005; Molotkov et al., 2005). These investigations have shown that the RA-synthesizing enzyme, retinaldehyde dehydrogenase 2 (Raldh2), is required for Pdx1 induction and dorsal pancreas development. Raldh2 is expressed as early as e7.5 in the mesendoderm and becomes localized to lateral plate and paraxial mesoderm during segmentation. Kumar et al., using chick embryo in vitro tissue recombination, have shown that signals coming from lateral plate mesoderm instruct the adjacent endoderm toward a pancreatic fate. They further demonstrated that RA is sufficient to induce Pdx1 expression in endoderm, but only when co-cultured with mesoderm, suggesting RA is acting indirectly through the mesoderm or response to RA-signaling depends on signals from the mesoderm (Kumar et al., 2003). However, Stafford et al. show RA, from paraxial mesoderm, signals directly to zebrafish endoderm by using a cell transplantation technique in which the ability of SOX32 to confer endodermal identity was used to selectively target reagents to the endoderm (Stafford et al., 2006). More recently, Pan et al., using tissue recombination experiments in Xenopus, demonstrated that RA signals to the endoderm via direct and indirect mechanisms (Pan et al., 2007). A case for RA signaling directly to the endoderm is strengthened in a recent study where the RA-degrading enzyme Cyp26, which is expressed in the anterior endoderm, is required for the proper size and location of the pre-pancreatic anlage (Kinkel et al., 2009). In this study, the authors demonstrate that the loss of Cyp26 in zebrafish results in a dramatic expansion of pancreatic cell types anteriorly, and conclude that the primary role of Cyp26 is to set the boundary of the anterior pancreatic field.

Ngn3 and endocrine cell commitment
Both dorsal and ventral pancreatic buds consist of multipotent progenitor cells (MPCs) marked by Pdx1, Ptf1a, Sox9, Nkx6.1/2 and Hnf1β (Haumaitre et al., 2005; Henseleit et al., 2005; Kawaguchi et al., 2002; Seymour et al., 2007), which give rise to all adult pancreatic cells types. Endocrine cell production first begins in these early buds, generating mainly alpha cells, with subsequent formation of beta, delta, PP and epsilon cells (Henseleit et al., 2005; Herrera et al., 1991; Johansson et al., 2007; Pictet et al., 1972; Prado et al., 2004; Schwitzgebel et al., 2000).

The fate of these early “first wave” endocrine cells remains a debated issue. While some investigators have claimed these first wave endocrine cells are fugacious and do not contribute to the adult islet (Herrera, 2000), others have suggested that at least some of them persist and, indeed, contribute to the adult islet (Gu et al., 2002; Johansson et al., 2007). If they do persist throughout pancreatic development, a remaining question is: how do they become incorporated into the orchestrated architecture of the “tip-trunk” branching of the secondary transition, which will now be discussed.

The secondary transition is a period of massive endocrine cell differentiation. As the pancreatic epithelium branches, a pro-acinar domain is formed at the growing tips, and a bi-potential, ductal/endocrine compartment is acquired in the extending trunks (Schaffer et al., 2010; Zhou et al., 2007). The tip domain is defined by the transcription factors Ptf1a, Cpa1 and Pdx1, while the trunk domain expresses Sox9, Nkx6.1, Hnf1β and Pdx1 (Figure 1). The mechanism controlling the segregation of the pancreatic epithelium into the tip-trunk domains remains obscure; however, evidence illustrating the mutual repressive events between the tip-specific factor Ptf1a and the trunk-specific factor Nkx6.1, suggest a mechanism responsible for the development of the tip-trunk compartmentalization (Krapp et al., 1998; Schaffer et al., 2010). There are additional reports of Sox9, Nkx6.1 and Hnf1β all having the ability to bind regulatory regions in Ptf1a.
(Haumaitre et al., 2005; Lynn et al., 2007; Schaffer et al., 2010), providing further evidence that the compartmentalization of the tip-truck domains is acquired through the direct cross repressive activities of transcription factors.

The “second wave” endocrine cells are derived from the bi-potential trunk domain when the endocrine progenitor marker Ngn3 is expressed, leading to the subsequent delamination and coalescing of these cells into the developing islets (Gu et al., 2002). Notch signaling has been shown to play a critical role in this process. The classical lateral inhibition model proposes that Ngn3+ cells express the Notch ligand, Delta-like 1 (Dll1), whereas neighboring cells express the Notch1 receptor. Upon activation of the Notch receptor via neighboring Ngn3+/Dll1+ cells, the intracellular domain of the Notch1 receptor goes through a series of proteolytic cleavages, which result in the release of the activated Notch1 intracellular domain (NICD) and its translocation to the nucleus where it regulates its target genes, chiefly Hes1 (Apelqvist et al., 1999; Jensen et al., 2000a; Jensen et al., 2000b). As a strong transcriptional repressor, Hes1 inhibits endocrine cell differentiation by binding to and repressing the Ngn3 promoter (Gradwohl et al., 2000; Gu et al., 2002; Lee et al., 2001). In accordance with the Notch-mediated lateral inhibition model, loss-of-function studies have demonstrated that the absence of Notch signaling components leads to the premature development of endocrine cells (Ahnfelt-Ronne et al., 2012; Apelqvist et al., 1999; Jensen et al., 2000b). Conversely, the forced expression of the NICD blocks endocrine cell formation (Hald et al., 2003; Murtaugh et al., 2003). In addition, Hes1+ and Ngn3+ cells are found in a mosaic expression pattern throughout the pancreatic epithelium (Ahnfelt-Ronne et al., 2007; Esni et al., 2004). While this lateral inhibition model has remained essentially unchallenged for over a decade, recent reports have suggested alternatives and additional complexities to this model.
Figure 1. Tip-trunk compartmentalization in the developing pancreas. Immunostaining of e14.5 epithelial chords in the mouse demonstrate that Ptf1a is predominantly restricted to the tips, while Nkx6.1, Hnf1b and Sox9 are localized to the trunk domain (Kopp et al., 2011).
Ahnfelt-Ronne et al. provide data showing that Ptf1a is a transcriptional activator of Dll1 expression in MPCs and that Hes1 is required for maintaining high levels of Ptf1a, suggesting that Hes1 acts upstream and downstream of Dll1. Additionally, the authors show that Notch may be subject to cis-inhibition by high levels of Dll1, which is induced by Ngn3 (Ahnfelt-Ronne et al., 2012). Another study illustrates a pro-endocrine role for Notch. Shih and colleagues show that, in addition to the Notch-mediated activation of Hes1 and subsequent repression of Ngn3, Notch is also responsible for the activation of Sox9, which is necessary for endocrine and ductal cell differentiation (Shih et al., 2012), establishing a novel pro-endocrine mechanism for Notch (that is, the opposite of its normal role in sustaining the undifferentiated progenitor condition). These studies clearly highlight the complex role of Notch signaling and warrant further investigations to explore at a cellular and carefully temporally resolved level the expression of the Notch family receptors and ligands.

While a clear, mechanistic role for Notch-mediated regulation of Ngn3+ endocrine progenitors remains incondite, it is clear that Ngn3 is sufficient to promote endocrine development; when high levels of Ngn3 are expressed in early pancreas development, the entire pancreas develops into small clusters of glucagon-positive cells (Apelqvist et al., 1999; Johansson et al., 2007; Schwitzgebel et al., 2000). These studies clearly highlight the ‘trigger nature’ of Ngn3 in dictating the endocrine lineage; however, it had been unclear for years whether a single Ngn3 positive cell has the capacity to generate any one of the five embryonic endocrine cell types, or if each Ngn3 positive cell has been previously specified to become a specific endocrine cell type. In order to address these issues, Desgraz et al. used clonal analysis by labeling single Ngn3+ endocrine precursors to illustrate that Ngn3+ cells are unipotent, meaning they can only give rise to one cell type (Desgraz and Herrera, 2009). Furthermore, the authors suggest that Ngn3+
endocrine precursors may be pre-committed to a particular endocrine cell fate, given that *Nkx6.1* expression is required before *Ngn3* expression for the acquisition of the beta-cell fate (Nelson et al., 2007). These findings raise another issue: if Nkx6.1 is required before the expression of *Ngn3* for the differentiation along the beta-cell lineage, are there endocrine-cell-specific niches in the trunk domains at the onset of the second wave of endocrine cell formation? One could imagine the possibility that, prior to *Ngn3* expression, the presence or absence, or the correct combination of transcription factors could generate a gene regulatory network that is responsible for initiating a particular endocrine cell lineage. These gene regulatory networks, while being specific to a particular endocrine cell lineage in each niche, would all be sufficient to activate *Ngn3* expression. The obvious question then is: what would be the spatiotemporal signals that would provide instructive or permissive cues for the proper allocation of the correct set of transcription factors that would create the necessary gene regulatory network? One study has begun to answer this question by providing evidence that the temporal context within which the *Ngn3*+ cells arise governs the endocrine cell fate. Johansson et al. used *Ngn3* null mice and added back *Ngn3* function via a tamoxifen-inducible *Ngn3* fusion transgenic approach to add back *Ngn3* at various time points of pancreatic development using an inducible *Pdx1* promoter (Johansson et al., 2007). The authors demonstrated that the early endocrine precursors (e8.75) are sufficient to produce alpha cells, whereas endocrine precursors do not acquire competence to develop beta cells until e11.5, with beta-cell formation peaking at e14.5. The authors further illustrated that *Ngn3*+ cells gained competence to produce PP and delta cells at e12.5 and e14.5, respectively. While this study heightens our understanding of the temporal regulation of endocrine lineage commitment, a detailed, comprehensive map of transcription factor expression in pre-committed endocrine cells is lacking. Having such a map would, potentially, enable us to
dissect mechanisms controlling MPC allocation, tip-trunk compartmentalization and endocrine cell commitment, and therefore, may facilitate how we approach stem cell differentiation protocols.

**Islet transcription factors**

There is a complex array of transcription factors that controls islet-cell-type lineage decisions. These transcription factors can generally be categorized into four groups based on their null phenotype. The first group, including *Islet1* and *NeuroD*, affects all endocrine cells, causing either a complete loss or reduction of all islet cell types. Group two consists of *Nkx2.2, Pax4* and *Pax6* and affects most endocrine cells. The third group, comprising *Arx, Nkx6.1* and *Hlxb9*, affects only one endocrine cell type, while the last group, including *MafA*, affects postnatal endocrine cell function (Table 1). This is not an exhaustive list, and there are many comprehensive reviews covering the pancreatic transcription factor gamut (Gittes, 2009; Oliver-Krasinski and Stoffers, 2008; Pan and Wright, 2011). For the purpose of this thesis, I will focus on two important regulatory factors, Nkx2.2 and Arx.

**Nkx2.2**

Nkx2.2 is a member of the NK family of homeodomain proteins (originally identified in *Drosophila* (Kim and Nirenberg, 1989)) and, like many pancreatic transcription factors, was first identified for its role in neuronal differentiation (Price et al., 1992). Cloning strategies later revealed the expression of Nkx2.2 in multiple alpha and beta cell lines (Rudnick et al., 1994), and this was confirmed in the first *in vivo* analysis of Nkx2.2 in the developing pancreas (Sussel et al., 1998). It was demonstrated that Nkx2.2 is expressed as early as e9.5 and is present in alpha, beta and PP cells, but excluded from delta cells. Furthermore, Sussel and colleagues
demonstrated that a null mutation for Nkx2.2 results in hyperglycemia and lethality shortly after birth. Closer inspection of the pancreas revealed that Nkx2.2 mutant mice have a complete absence of beta cells and a severe reduction in alpha and PP cells. It was also noted that the mutant mice retain a large population of cells with endocrine characteristics, but lack mature markers of beta cells. At that time, the authors attributed this phenotype to the arrested development of beta cells; that is, Nkx2.2 is not required for the specification of beta cells, but is necessary for the differentiation and maturation of insulin expressing beta cells. That fact that some alpha and PP cells remained suggested that another factor could possibly compensate for Nkx2.2 in the development of these cells (Sussel et al., 1998). Later studies showed that this interpretation was incorrect, and that the absence of Nkx2.2 causes endocrine progenitors to become diverted from the beta cell fate to a newly identified epsilon cell, characterized by the expression of the hormone ghrelin (Prado et al., 2004). These ghrelin-positive cells, which are normally present in the gastric pits of the stomach (Kojima et al., 2001), were shown to be highly upregulated and in place of the beta cells in the Nkx2.2 null mouse. Remarkably, ghrelin was also shown to be transiently present, albeit in small numbers, in the normal embryonic mouse islet, defining a new pancreatic islet cell type. These data point to a role for Nkx2.2 in specifying and maintaining the beta cell lineage while suppressing the epsilon cell lineage.

Doyle et al. extended these findings by determining whether Nkx2.2 is acting as a transcriptional activator or repressor during pancreas development. The creation of an Nkx2.2-engrailed fusion repressor or Nkx2.2-VP16 fusion activator construct expressed under the control of the Pdx1 promoter in the Nkx2.2 null background demonstrated that Nkx2.2 repressor activity was sufficient for alpha cell differentiation, while repressing the epsilon cell lineage (Doyle et al., 2007). Although some beta cells were rescued with Nkx2.2 repressor activity, these cells lacked
mature markers of bona fide, glucose-sensing beta cells, i.e., MafA and Glut2. This finding may reflect the requirement of Nkx2.2 transcriptional activator functions for the beta cell maturation process, which would be consistent with previous data illustrating Nkx2.2 as a transcriptional activator of MafA (Raum et al., 2006). Alternatively, the incomplete rescue of beta cells could be attributed to the lack of Nkx2.2 protein regulatory domains in the repressor derivative transgene; the Nkx2.2-engrailed fusion repressor transgene contained only the Nkx2.2 homeodomain fused to the engrailed repressor domain. The authors attempted to address this issue by expressing the full-length Nkx2.2 cDNA from the Pdx1 promoter, but were unable to establish any transgenic lines. The full rescue of the alpha-cell population is quite intriguing in that Pdx1, which was used to drive the Nkx2.2 repressor derivative, is strongly downregulated in the normal development alpha cells. This suggests that Nkx2.2 repressor activity is only needed in the early Pdx1+ progenitor population to establish the alpha cell differentiation program.

In the developing CNS, Nkx2.2 functions as a repressor by way of its interaction with the corepressor Groucho 4 (Grg4) (Muhr et al., 2001), and given that the Nkx2.2 seems, at least partially, to function as a repressor in the developing islet, Doyle et al. investigated the expression of Grg family members during pancreas organogenesis. Grg4 expression was undetectable in the pancreatic epithelium. In contrast, Grg1 and 2 were expressed at low levels, while Grg3 had the highest expression. RNA in situ analyses further determined that Nkx2.2 and Grg3 had similar expression patterns in early (e12.5) and late (e16.5) pancreas development, and a combination of RNA in situ hybridization and immunodetection revealed that the Grg3 expression domain overlapped with the endocrine compartment and was excluded from the exocrine pancreas. Additional biochemical studies showed that Nkx2.2 was able to physically interact with Grg3, and this interaction required the N-terminal decapeptide TN domain,
previously shown essential for the Grg4 interaction in neural cells (Doyle et al., 2007; Muhr et al., 2001). Doyle and Sussel expanded these findings by expressing the Nkx2.2 repressor derivative in the adult beta cell, which resulted in the loss of Mafa and Glut2 expression, as well as endogenous Nkx2.2 (Doyle and Sussel, 2007), suggesting a self-regulating and activating function. These reports provide a great example of the diverse roles that a transcription factor can play at various times and in different cellular contexts during development. While these studies have improved our understanding of the molecular mechanism underlying Nkx2.2 function in the endocrine cell ontogeny, there are caveats worth noting. The Pdx1 promoter, which is expressed in a similar but not identical spatiotemporal domain to Nkx2.2, was used to drive the expression of the transgenes. In addition, only the Nkx2.2 homeodomain was present in the transgenes. This could potentially disrupt the interaction with co-regulators that interact with Nkx2.2 through well-conserved regulatory protein domains, such as the NK2-SD domain, the defining domain of the Nkx2 family of transcription factors. However, the fact that the Nkx2.2 repressor derivative can partially restore islet cell differentiation, and especially cause a full rescue of alpha cells, indicates that it has at least some capacity to replace normal endogenous functions, which begets the question: when, and in which cell, and on which genes is Nkx2.2 repressor activity required? To help answer these questions, a major focus of this dissertation is to define the exact role of Grg-mediated Nkx2.2 repression during mouse pancreatic development.

Arx

Another transcription factor required for specific endocrine cell lineage is Arista-less homeobox (ARX) gene. Mice carrying a null mutation for Arx exhibit a complete loss of mature alpha cells with a corresponding increase in the beta and delta-cell populations, so that the total endocrine cell count is unaltered. This shift in cell fate seems to be due to unopposed Pax4 expression,
which is required for beta-cell specification (Collombat et al., 2005; Collombat et al., 2003).

Consistent with these findings, the overexpression of Arx in Pdx1+ progenitor cells was sufficient to shunt most beta and delta cells toward the alpha and PP cell lineages. This divergence from beta cells to alpha or PP cells can be recapitulated when Arx is overexpressed specifically in beta cells using Ins:Cre. Finally, when Arx is overexpressed in four-week-old mice using an inducible Pdx1:CreER, which is restricted to beta cells at this time, Arx is adequate to reprogram the beta cell to either an alpha or PP cell (Collombat et al., 2007). A closer, molecular analysis of Arx regulation revealed an epigenetic component involving DNA methylation of the Arx promoter that is required for its repression specifically in beta cells. In this study, beta cells deficient for the maintenance DNA methyltransferase, Dnmt1, were directly reprogrammed to alpha cells. The Arx promoter was shown to be normally hypermethylated in beta cells, preventing its expression, while being hypomethylated in alpha cells. Upon deletion of Dnmt1 specifically in beta cells, the Arx promoter lost the methylated signature, expression was de-repressed and an alpha-to-beta cell conversion ensued (Dhawan et al., 2011). This is consistent with the previous studies that report Arx is sufficient to divert the beta-cell lineage to the alpha-cell lineage, and ectopic expression of Arx can reprogram beta cells to alpha cells. A greater discussion about the regulation of the Arx promoter, and its relationship with Nkx2.2, will be presented in chapter 2.

The phenotypes of the Arx null mice and Nkx2.2 null mice share a common thread; they both have either a severe or total loss of alpha cells, and recent reports have described genetic interactions between Arx and Nkx2.2. Despite a severe reduction in alpha cells in the Nkx2.2 null mice, the expression of Arx is unchanged (Chao et al., 2007). Mastracci et al. have confirmed this finding and demonstrated that Arx was localized to the ghrelin cells that become massively increased in number in the absence of Nkx2.2. Interestingly, the compound Nkx2.2/Arx double
mutant (in this case, *Arx* was deleted specifically in the pancreas) revealed that *Arx* is necessary for *ghrelin* expression but not Ghrelin cell specification. The double mutant mouse had the expected increase in ghrelin⁺ cells, which was not statistically different from *Nkx2.2* null mice; however, *ghrelin* expression was significantly decreased. Immunofluorescent detection of ghrelin in double-mutant mice displayed a much weaker signal compared to *Nkx2.2* null mice. Since the ghrelin signal was not decreased in *Arx* single mutants compared to wild-type mice, the authors suggested that *Arx* may only be able to regulate *ghrelin* expression in the absence of *Nkx2.2* (Mastracci et al., 2011). The inverse was shown for delta cell and *somatostatin* expression. As previously mentioned, *Arx* deletion results in an increase in delta cells and *somatostatin* expression. In the *Nkx2.2/Arx* double mutant the increase in delta cells persists, yet *somatostatin* expression increases nearly 3-fold. This phenomenon was attributed to Ghrelin/Somatostatin co-positive cells that arise as early as e12.5 in the *Nkx2.2/Arx* double mutant, but are undetectable in the wild type and *Nkx2.2* null mice (Mastracci et al., 2011). The finding that *Nkx2.2/Arx* compound deficiency leads to ghrelin/somatostatin co-positive cells was independently confirmed (Kordowich et al., 2011). These studies merit further investigations to dissect the molecular components that regulate the crosstalk between these transcription factors that governs early endocrine cell specification and lineage commitment.

**Groucho proteins**

The *Drosophila groucho (gro)* gene was first identified from a viable mutation resulting in clumps of supernumerary bristles above the eye, a characteristic reminiscent of Groucho Marx. The gro protein is the prototype of a family of co-repressors known as Transducin-like enhancer of split (TLE) 1-4 in humans, and groucho-related gene (Grg) 1-4 in mice. (Hereafter, I will be referring to groucho proteins, in general, using the mouse nomenclature, Grg). Grg proteins are
broadly expressed and are recruited to DNA by interacting with a diverse group of transcription factors. Correspondingly, many developmental phenotypes are compromised in the loss of Gro in the fly (Delidakis et al., 1991; Paroush et al., 1994), while embryonic lethality occurs in the mouse (Metzger et al., 2012). Structural studies have determined that the WD-repeat (WDR) domain at the C-terminus of Grg acts as the protein interaction interface for most transcription factors. The WDR domain interacts with one of two short polypeptide motifs: 1) WRPW and related tetrapeptide motifs and 2) engrailed homology 1 (eh1) domains (Fisher et al., 1996; Goldstein et al., 2005; Jimenez et al., 1997; Muhr et al., 2001; Paroush et al., 1994). Because of its ability to interact with an array of transcription factors containing these peptide motifs, as well as other proteins harboring different peptide sequences (Aronson et al., 1997; Daniels and Weis, 2005), Grg is involved in many developmental signaling pathways, including Notch and WNT.

The most notable effectors of Notch signaling are the bHLH factors of the Hes family (Bray, 2006), factors for which Grgs have been shown to act as co-repressors (Ju et al., 2004; Nuthall et al., 2004). In Drosophila, Gro was shown to operate at a functional intersection between the Notch and EGF signaling pathways. Specifically, in response to Notch signaling, Gro antagonizes vein formation and bristle patterning through Hes-mediated repression, and Hasson et al. demonstrated that EGFR-mediated Gro phosphorylation by MAPK is sufficient to relieve the Gro/Hes-mediated repression of downstream Notch targets, allowing for proper vein formation and bristle patterning (Hasson et al., 2005).

WNT signaling has a well appreciated role in patterning and cell determination events (Logan and Nusse, 2004). The central player in canonical WNT signaling is beta-catenin, a co-activator for Tcf/Lef factors. In the absence of WNT signaling, cytoplasmic beta-catenin is phosphorylated, marking it for proteolysis. Conversely, active WNT signaling inhibits the destruction of beta-
catenin, leading to its cytoplasmic accumulation and translocation to the nucleus, where it binds to Tcf/Lef factors, activating downstream target genes (Clevers, 2006). Interestingly, in the absence of WNT signaling, Tcf/Lef factors recruit Grgs to repress target genes (Barolo and Posakony, 2002). Correspondingly, Grg deficiencies in both Drosophila and vertebrates manifest phenotypes similar to that of WNT signaling overexpression (Cavallo et al., 1998; Roose and Clevers, 1999).

Mechanisms of repression

Several mechanistic models of Grg-mediated repression have evolved, one of which is the well-characterized histone deacetylase (HDAC) recruitment (Arce et al., 2009; Chen and Courey, 2000; Watson et al., 2000; Winkler et al., 2010). However, HDAC recruitment and histone deacetylation cannot be the only mechanism by which Grgs repress gene expression, as shown by deletion mutants (Fisher et al., 1996), deacetylase inhibitors (Yao et al., 2001), and anti-HDAC antibodies (Dasen et al., 2001). In the context of WNT signaling, it has been determined that Grg can out-compete beta-catenin for Tcf/Lef binding and vice versa. Therefore, high levels of WNT activity, and consequently beta-catenin accumulation, will out-compete Grg for Tcf/Lef binding, relieving repression. Thus, the level of WNT activity will dictate the binding partner of Tcf/Lef, leading to either repression (which in this case is the displacement of co-activators) or activation (Daniels and Weis, 2005). Another study determined that phosphorylation of the paired-box transcription factor Pax2 by c-Jun N-terminal kinase (JNK), a modification that enhances Pax2-dependent transcription, is inhibited by its interaction with Grg4, causing Pax2-dependent repression (Cai et al., 2003). This group expanded on these findings to show that the recruitment of Grg4 to Pax2 DNA binding sites is sufficient to displace an H3K27me complex (an epigenetic mark of active chromatin, discussed later in this chapter) and to subsequently
recruit the arginine methyltransferase PRMT5, an enzyme responsible for H4R3 dimethylation and recruitment of Polycomb proteins (Patel et al., 2012). Another mechanism of repression involves the targeted recruitment of Grg by DNA-binding proteins, and the subsequent oligomerization of Grg, spanning three to four nucleosomes. In this scenario, Grg both condenses the chromatin and forms aggregates, thereby preventing activator recruitment and repressing transcription (Sekiya and Zaret, 2007). While Grg proteins have a well-appreciated repressor function, it is important to keep in mind that Grg has also been shown in certain contexts to act as a co-activator (Villanueva et al., 2011). Grg3 was shown to integrate the PPARγ and Wnt pathways in adipogenesis by acting as a co-activator of PPARγ target genes, while antagonizing Wnt signaling. These findings suggest that Grg may not be functioning as a co-repressor in all contexts, and its role as a repressor or activator may depend on cell- or promoter-specific binding partners.

**Role in pancreas development**

Grg has been well characterized in neuronal development and differentiation (Gasperowicz and Otto, 2005); however, very little is known about its role in pancreas development. The first paper ascribing a role for Grg in proper pancreatic endocrine formation was from Doyle and colleagues, who reported that Grg1, 2 and 3 were expressed in the developing pancreas (Doyle et al., 2007). The authors further determined that Grg3 exhibited a similar expression profile to Nkx2.2 and, through co-immunoprecipitation analyses, demonstrated that Nkx2.2 and Grg3 physically interact. These findings were extrapolated to attribute the mechanism by which Nkx2.2 repressor function can partially rescue the Nkx2.2 null phenotype. A more recent study attempted to investigate Grg function during pancreas development by analyzing the pancreas defects in a Grg3 null condition. Since the non-tissue-specific loss of Grg3 results in embryonic lethality by
e14.5, possibly due to placental defects, Metzger et al. used e12.5 pancreatic explants to dissect roles for Grg3 ex vivo (Metzger et al., 2012). Their findings highlighted a role for Grg3 in proper delamination of endocrine precursors from the trunk epithelium. Consequently, a reduction in all endocrine cell types was observed, because of the loss of Grg3-mediated E-cadherin downregulation, which is a de-adhesion step apparently critical for the proendocrine cell delamination from the trunk epithelium (Gouzi et al., 2011). While this study provides evidence of a role for Grg3 in pancreas development, the experimental methods make it difficult to ascribe specific roles for Grg3, i.e., Grgs interact with many factors, and by using a knock out model, the function of multiple Grg-interacting regulatory factors may potentially be disrupted. Secondly, the embryonic lethality caused by the global Grg3 inactivation constrains the investigators to an ex vivo approach, which may be significantly different and confounding as compared to a fully in vivo analysis. Nevertheless, these studies provide data that warrant more elegant explorations into deciphering roles for Grg proteins in pancreas development.

**Transcriptional regulation**

Genes need to be regulated in a context-dependent manner for proper cell fate determination. Many years of study have yielded the unequivocal conclusion that gene repression is as influential as activation mechanisms; indeed, repression might be the predominant mode of transcriptional modulation, especially for tuning levels and timing of gene expression to sophisticated cell differentiation programs. Fundamental potent controllers of the activated or repressed state include DNA methylation (Suzuki and Bird, 2008), histone modifications (Wang et al., 2004) and chromatin remodeling (Ho and Crabtree, 2010). Understanding how these processes control gene regulation in cell-type-specific and developmental-stage-specific contexts
is essential to comprehend the underlying processes controlling pancreatic cell type
differentiation.

Repression mechanisms controlling pancreas development

Developmental processes, including cell specification, are often controlled by the spatial
expression of gene activity, during which boundaries of gene expression are determined by the
restricted activity of transcriptional repressors. In the pancreas, evidence exists for a cross-
repressive mechanism controlling the tip-trunk compartmentalization (Haumaitre et al., 2005;
Lynn et al., 2007; Schaffer et al., 2010), which demarcates the bi-potential ductal/endocrine
progenitors from the acinar progenitors. Similar scenarios exist in other systems, such as the
CNS, where cross-repressive interactions between homeodomain proteins refine and maintain
progenitor domains (Briscoe et al., 2000). Repression mechanisms are also used to distinguish
one organ anlage from another, which is the case for ventral foregut endoderm, as it undergoes a
fate choice between liver or ventral pancreas progenitors. Xu et al. determined that H3K27me3
and the PcG protein, Ezh2, were enriched on the \( Pdx1 \) enhancer elements in early endodermal
cells, and these features were absent from liver regulatory elements. Inducing the loss of \( Ezh2 \)
from within the early endoderm results in an expanded \( Pdx1^+ \) ventral pancreatic domain. This
expansion occurs at the expense of liver development, and the result suggests \( Ezh2 \) acts
permissively to allow liver development by restraining ventral pancreas development (Xu et al.,
2011). As pancreatic development proceeds, proper beta-cell-lineage commitment requires the
repression of non-beta-cell factors; aberrant expression of non-beta-cell factors, such as \( Arx \),
diverts the beta-cell lineage to an alpha/PP lineage (Collombat et al., 2005; Collombat et al.,
2003). More recently, molecular studies analyzing the \( Arx \) promoter revealed an integrated
mechanism controlling \( Arx \) repression in beta cells (Dhawan et al., 2011). DNA methylation is
required to correctly repress *Arx* in beta cells and to maintain their identity. This methylation was not observed on the *Arx* promoter in alpha cells, which is the cell type within which it is normally expressed as an alpha-cell specification factor. *Arx* promoter methylation recruits the methyl-binding protein, MeCP2, which recruits PRMT6, leading to H3R2 methylation and repression. The beta-cell-specific deletion of *Dnmt1* led to the gradual loss of *Arx* promoter methylation, and its subsequent de-repression, which was sufficient to reprogram the beta cell to an alpha cell. The phenotype exhibited by beta-cell-specific loss of *Dnmt1* was not immediate, but gradual, caused by the property of Dnmt1 being required for maintaining methylation only after cell division occurs, and normal beta cells replicate at an extremely low rate (Teta et al., 2005). However, this work shows the importance of properly inherited patterns of repression of alpha-cell lineage determination factors for normal development and maintenance of beta cells.

The observed decrease in beta-cell replication with advancing age (in both mice and humans) is another process controlled by repressive mechanisms. Normal beta-cell replication is required for proper islet size, maintaining homeostasis and for adapting islet function in the face of metabolic demand, such as diet fluctuation, pregnancy, and others (Dor et al., 2004; Kulkarni et al., 2004; Okamoto et al., 2006; Zhong et al., 2007). Normal healthy mice show a precipitous drop in the number of replicating beta cells occurring just after birth, and this number steadily declines in advancing age (Teta et al., 2005). In young animals, *p16/Ink4a* is repressed in beta cells by the presence of Bmi-1 and Ezh2-induced H3K27me3. With advancing age, the H3K27me3 mark and the Bmi-1 chromatin regulatory protein are replaced with H3K4me3 and MLL1, allowing for *p16/Ink4a* de-repression. p16 is a cyclin-dependent kinase inhibitor and a negative regulator of the cell cycle. Thus, the de-repression of *p16/Ink4a* in the beta cell inhibits beta-cell proliferation.

It has also been illustrated that the beta-cell-specific deletion of *Ezh2* led to a precocious
decrease in beta cell replication. Furthermore, the regeneration and replication of beta cells following STZ-induced beta-cell destruction was mediated by an upregulation of Ezh2 and subsequent repression of \(p16/\text{Ink4a}\), which was not observed in beta-cell-specific \(Ezh2\) null mice (Chen et al., 2009a; Dhawan et al., 2009). These data highlight that gene repression is as important as gene activation in the correct specification, development and maintenance of pancreatic beta cells.

**Post-transcriptional modification**

Many transcription factors carry out the complex task of differentially regulating multiple genes; within a cell, a transcription factor may aid in repressing one gene while activating another. In *Drosophila* neural stem cells, Prospero (Prox1 in vertebrates) has been shown to repress genes required for self-renewal and activate genes necessary for differentiation (Choksi et al., 2006). Often times this “binary switch” involves differential recruitment of cofactors. But how does a transcription factor choose to interact with, for example, a co-repressor on gene \(A\) and a co-activator on gene \(B\), when all three are coexpressed in the same cell? Another interesting scenario is the ability of a transcription factor to repress a gene in a progenitor cell, but then activate the same gene as the cell differentiates.

Many studies have demonstrated that post-translational modification (PTM) of transcription factors can directly flip a protein between activator and repressor functions, perhaps driving acute switching between potent coactivators/corepressors. c-Myc PTMs that influence its functional activities have been especially well characterized (Luscher and Vervoorts, 2012). Recently, Uribesalgo et al. provided a mechanism by which c-Myc phosphorylation mediates whether retinoic acid receptor \(\alpha\) (RAR\(\alpha\)) represses or activates its target genes (Uribesalgo et al.,
In the absence of retinoic acid (RA), RARs associate with co-repressors and constitutively repress target genes. Upon stimulation with RA, a conformational change occurs in the RAR and co-repressors are exchanged for co-activators, resulting in gene transcription. Uribesalguo et al. show that c-Myc regulates RA-response element (RARE)-containing genes by binding to RARα. The co-repressors HDAC3 and N-CoR were associated with RARα and enriched at RAREs in the presence of dephosphorylated c-Myc. Conversely, constitutively phosphorylated c-Myc, even in the absence of RA, disrupts c-Myc binding to RARα and causes an exchange in the co-repressor complex for the histone acetyltransferase CBP leading to increased histone acetylation and gene activation.

Another phenomenon is the ability of a transcription factor to switch between repressor and activator modes over time as a cell moves through ontogeny. Ju et al. used an in vitro system to analyze the regulation of Mash1 in neural differentiation and illustrate how Hes1 changes from repressing to activating Mash1. Treatment of neural stem cells with PDGF initiates development along a neural pathway, which is characterized by the expression of Mash1. Repression of the Mash1 promoter is maintained in neural stem cells by a Hes1/Parp1/TLE1 complex, but within hours of PDGF treatment, Mash1 expression is de-repressed, and co-repressors are exchanged for co-activators; however, Hes1 and Parp1 remained. PDGF treatment also stimulates expression of CaMKIIδ, which was shown to phosphorylate Parp1 and Hes1. This phosphorylation is necessary for the exchange of cofactors; loss of CaMKIIδ resulted in stable repression of Mash1 even in the presence of PDGF (Ju et al., 2004).

Many other transcription factors have been shown to differentially regulate gene expression in response to PTM. For example, protein kinase A phosphorylation of NF-κB creates CBP/p300
binding sites, leading to NF-κB target gene activation (Zhong et al., 1998), and Pax2 phosphorylation by c-Jun N-terminal kinase precludes binding with Grg, allowing for Pax2-dependent transactivation (Cai et al., 2003). However, very little is known about PTM of pancreatic factors and how they might contribute to gene regulation in pancreas development. Multiple groups have demonstrated that Pdx1 contains several different PTMs, including phosphorylation, glycosylation and sumoylation (Boucher et al., 2006; Elrick and Docherty, 2001; Gao et al., 2003; Kishi et al., 2003), some of which are responsible for selective interactions with cofactors (Mosley et al., 2004; Mosley and Ozcan, 2004) Most recently, Frogne et al. provided convincing evidence that the majority of Pdx1 phosphorylation is occurs at serine 61 (S61) (Frogne et al., 2012). While this study did not determine a function for S61 phosphorylation, it was demonstrated, in contrast to other studies of Pdx1 PTM, that S61 phosphorylation was not regulated by glucose. Another pancreatic factor, NeuroD, can also be phosphorylated, and this PTM was demonstrated to regulate NeuroD in a context-dependent manner. In Xenopus over-expression gain-of-function experiments, the S266A and S274A mutations in NeuroD, which mimic dephosphorylation, act as strong activators of multiple neuronal program genes, and greatly increase ectopic neurogenesis (Dufton et al., 2005). But these same alterations cause and inhibitory effect, compared to wild-type NeuroD, in a pancreatic beta cell line (Khoo et al., 2003b).

Collectively, these studies illustrate the importance of transcription factor PTM. Despite the large amount of collective work over the last 20 years on unraveling the transcriptional cascades of endocrine lineage allocation, it is surprising that so little attention has been focused on such a potent functional influence. PTM can modulate many aspects of protein function, including cofactor interactions, cytoplasmic/nuclear shuttling, DNA binding, and protein turnover.
The overall goal of my Ph.D. thesis research was to define regulatory domains within the Nkx2.2 protein and possible PTMs that contribute to its apparent variation (amongst cell types and over time) of functions, specifically focusing on the idea that there could be a large degree of back-and-forth activator-repressor switching. I began by analyzing mice with a knock-in mutation of the Nkx2.2-TN domain. This domain contains homology to the core eh1 region of the Engrailed repressor and has previously been characterized as essential for interaction with Grg proteins in the CNS (Muhr et al., 2001). Our lab has previously shown that Nkx2.2 repressor activity can partially rescue the Nkx2.2-null phenotype, and since Grg3 was shown to be expressed in the endocrine pancreas and interact with Nkx2.2, we predicted that by mutating the Nkx2.2-TN domain, we would disrupt the interaction with Grg3, and therefore, the role of Grg-mediated Nkx2.2 repression in pancreas development could be defined. This is a practical and elegant approach, as opposed to a pancreas- or beta-cell-specific loss of Grg, which would demonstrate a more global role for Grgs in the pancreas. Instead, we are trying to define precise, Grg-mediated Nkx2.2 functions.

My careful phenotypic analyses led to the determination that mice carrying a homozygous TN mutation (Nkx2.2\textsuperscript{TNmut/TNmut}) have aberrant islet hormone expression beginning at e13.5, characterized with an increase in ghrelin expression and a slight decrease in insulin expression. As the embryos developed, this phenotype worsened, and by e18.5 glucagon expression and alpha-cell numbers were significantly increased. The expression of cell-type-specific transcription factors paralleled these changes, with decreases in beta-cell factors and increases in alpha-cell factors. However, Arx, and alpha-cell lineage determinant, was significantly elevated before increases in alpha cells or increases in glucagon expression were observed. I discovered that Arx was ectopically expressed in beta cells, which suggested Grg-mediated Nkx2.2
regulation was necessary for beta-cell-specific repression of $Arx$. Moving to a more molecular level, I used cell lines to determine that Nkx2.2 is present in a repressor complex on the methylated $Arx$ promoter specifically in beta cells, but this complex was absent in alpha cells. I further demonstrated that the ectopic expression of $Arx$ in Nkx2.2$^{TNmut/TNmut}$ mice was necessary to reprogram the beta cells to alpha cells, and this reprogramming occurred both embryonically and postnatally. Since beta-cell-specific loss of $Arx$ in the Nkx2.2$^{TNmut/TNmut}$ mice rescued the beta-cell reprogramming, I concluded that the ectopic expression of $Arx$ was solely responsible for the beta-to-alpha transdifferentiation. However, beta-cell-specific deletion of $Arx$ was not sufficient to rescue lineage specification defects in Nkx2.2$^{TNmut/TNmut}$ mice, and these adult mice remained hyperglycemic. Interestingly, the Nkx2.2$^{TNmut/TNmut}$ mice do not have an early phenotype as is seen in the Nkx2.2-null mice. These findings illustrate the importance of the spatiotemporal regulation of lineage commitment decisions by cofactors and highlight a role for epigenetic and transcription factor interactions for proper gene regulation.

To determine if PTMs affect Nkx2.2 function, I began by examining the Nkx2.2 peptide sequence for putative PTM sites. In silico analysis revealed several serine and lysine residues that were predicted to be phosphorylated and acetylated, respectively. Mutation of these residues affected Nkx2.2 target gene expression in vitro, most likely by an inability of Nkx2.2 to properly recruit cofactors to target genes. These data warrant further investigations of Nkx2.2 PTM and the effect on its highly flexible transcriptional regulatory properties.

Collectively, my thesis studies have provided insight into how regulatory domains and PTMs contribute to Nkx2.2 function. Further understanding the molecular events that mediate transcription factor function in spatiotemporal regulation of pancreas development will aid our protocols to induce beta cells from alternate sources.
CHAPTER 2

PUBLISHED MANUSCRIPT: NKX2.2 REPRESSOR COMPLEX REGULATES ISLET β-CELL SPECIFICATION AND PREVENTS β-TO-α-CELL REPROGRAMMING

James Papizan generated all data presented in this chapter except for the data included in Figures 5B,C,E,F; Supplemental Figure 7B (S. Tschen, S. Dhawan, A. Bhushan), Figure 3A-C; and Supplemental Figures 1D-J; 2E-H (R. Singer). R. Singer generated the Arx-pGL3 vector, J.M. Friel created the Nkx2.2TNmut construct, and members from the M. Magnuson laboratory generated the Nkx2.2TNmut ES cells.
Nkx2.2 repressor complex regulates islet β-cell specification and prevents β-to-α-cell reprogramming

James B. Papizan,1 Ruth A. Singer,1,4 Shuen-Ing Tschen2,4 Sangeeta Dhawan,2,4 Jessica M. Friel,1 Susan B. Hipkens,5 Mark A. Magnuson,6 Anil Bhushan,7 and Lori Sussel1,5

1Department of Genetics and Development, Institute of Human Nutrition, Columbia University, New York, New York 10032, USA; 2Department of Medicine, University of California at Los Angeles, Los Angeles, California 90095, USA; 3Department of Molecular Physiology and Biophysics, Center for Stem Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, USA

Regulation of cell differentiation programs requires complex interactions between transcriptional and epigenetic networks. Elucidating the principal molecular events responsible for the establishment and maintenance of cell fate identities will provide important insights into how cell lineages are specified and maintained and will improve our ability to recapitulate cell differentiation events in vitro. In this study, we demonstrate that Nkx2.2 is part of a large repression complex in pancreatic β cells that includes DNMT3a, Grg3, and HDAC1. Mutation of the endogenous Nkx2.2 tinman (TN) domain in mice abolishes the interaction between Nkx2.2 and Grg3 and disrupts β-cell specification. Furthermore, we demonstrate that Nkx2.2 preferentially recruits Grg3 and HDAC1 to the methylated Aristaless homeobox gene [Arx] promoter in β cells. The Nkx2.2 TN mutation results in ectopic expression of Arx in β cells, causing β-to-α-cell transdifferentiation. A corresponding β-cell-specific deletion of DNMT3a is also sufficient to cause Arx-dependent β-to-α-cell reprogramming. Notably, subsequent removal of Arx in the β cells of Nkx2.2⁠[TNmut/TNmut] mutant mice reverts the β-to-α-cell conversion, indicating that the repressor activities of Nkx2.2 on the methylated Arx promoter in β cells are the primary regulatory events required for maintaining β-cell identity.

[Keywords: islet development; β-cell reprogramming; Nkx2.2; DNMT3a; transcriptional repression; DNA methylation]

Supplemental material is available for this article.

Received June 25, 2011, revised version accepted September 22, 2011.

In the developing embryo, cell fates are achieved through the regulated activation and repression of transcription factors, which act as intermediaries in response to the secretion of inductive signals. The acquisition of cell fate is accomplished by a series of differentiation steps that were once thought to be irreversible. More recently, however, studies in multiple systems have demonstrated that dedifferentiation and transdifferentiation are mechanisms commonly used during developmental programs and in paradigms of regeneration (Tsomis et al. 2004; Mirskey et al. 2006; Jopling et al. 2010, 2011). The ability to reprogram somatic cells into pluripotent cells by the expression of transcription factors has also demonstrated the feasibility of direct reprogramming that does not require an intermediate dedifferentiation step (Takahashi and Yamanaka 2006, Boland et al. 2009). Following this breakthrough, there have been many examples of tissue reprogramming, including the transdifferentiation of fibroblasts into functional cardiomyocytes or multilineage blood progenitors (Ieda et al. 2010; Selvaraj et al. 2010; Szabo et al. 2010).

Similar to most other developmental systems, lineage determination and cell specification in the developing pancreas involve an organized series of events consisting of appropriately timed extrinsic signaling that regulates hierarchies of transcriptional networks (Gittes 2009). In addition, recent studies have exploited the known islet transcriptional networks to transdifferentiate nonpancreatic or exocrine tissue into endocrine cells. Ectopic expression of different combinations of pancreatic transcription factors—including Pdx1, Ngn3, NeuroD, MafA, and Nkx6.1—promotes liver-to-pancreatic β-cell reprogramming (Chan et al. 2003; Kosima et al. 2003; Song et al. 2007; Delisle et al. 2009; Nagaya et al. 2009; Gefen-Halevi et al. 2010). Similarly, ectopic expression of a defined set of transcription factors in adult pancreatic acinar cells results in an acinar-to-β-cell conversion (Zhou et al.)
Recent studies have also demonstrated a previously unappreciated plasticity between the different islet cell types in embryonic and adult pancreas. Misexpression of the α-cell-specific transcription factor Aristaless homeobox gene (Arx) in fetal β cells is sufficient to cause a β-to-α-cell conversion (Collombat et al. 2007). Furthermore, Thorel et al. [2010] have shown that mice with a >90% reduction in β cells are able to replenish the lost β cells through the up-regulation of β-cell factors in α cells to induce α-to-β-cell transdifferentiation. Most recently, epigenetic manipulations have also demonstrated the importance of DNA methylation and histone modifications in maintaining β-cell identity and driving islet cell differentiation (Haumaitre et al. 2009; Dhawan et al. 2011). These groundbreaking studies have underscored the potential of using reprogramming methods to generate novel sources of islet cells.

The homeodomain transcription factor Nkx2.2 is required for cell fate decisions in the pancreatic islet and cell patterning in the ventral neural tube [Sussel et al. 1998; Briscoe et al. 1999]. It has previously been shown that Nkx2.2 acts as a transcriptional repressor to regulate ventral neural patterning through its interaction with the corepressor Groucho-4 (Grg4) (Muhr et al. 2001). This interaction is mediated by a motif called the tinman (TN) domain, which shares sequence homology with the core region of the engrailed homology-1 domain in the transcriptional repressor Engrailed and through which Grg/TLE proteins interact [Lints et al. 1993; Smith and Jaynes 1996; Jimenez et al. 1997]. In the developing pancreas, Nkx2.2 appears to function as either a transcriptional repressor or an activator, depending on the temporal- or cell-specific environment (Watada et al. 2000; Cissell et al. 2003; Raum et al. 2006; Doyle and Sussel 2007; Doyle et al. 2007; Anderson et al. 2009).

To further investigate the regulatory role of Nkx2.2 in the developing pancreas and its dependence on Grg interactions, we generated mice containing amino acid substitutions in the highly conserved TN domain of the interactor, we generated mice containing amino acid substitutions in the highly conserved TN domain of the interacting factor Arx in fetal β cells (Fig. 1A). Presence of the mutant allele was monitored using allele-specific primers (Fig. 1B; Materials and Methods). Heterozygous Nkx2.2^{TNmut/+} and homozygous Nkx2.2^{TNmut/TNmut} mice display gross normal pancreatic morphology and histology at birth (Fig. 1D, data not shown). Importantly, mutation of the TN domain does not appear to affect Nkx2.2 mRNA or protein expression in Nkx2.2 containing amino acid substitutions within the core sequence of the TN domain [Nkx2.2^{TNmut/TNmut}; Fig. 1A]. Presence of the mutant allele was monitored using allele-specific primers [Fig. 1B; Materials and Methods]. Heterozygous Nkx2.2^{TNmut/+} and homozygous Nkx2.2^{TNmut/TNmut} mice do survive postnatally, but the male mice begin to display overt hyperglycemia by 3.5 wk of age [Supplemental Fig. 1A,B]. By 6 wk, both male and female Nkx2.2^{TNmut/TNmut} mice are severely diabetic in fed and fasted conditions, with compromised fertility (Fig. 2A; Supplemental Fig. 1C). The Nkx2.2^{TNmut/TNmut} mice do not survive beyond 8 wk of age. Histological analysis of the 6-wk-old Nkx2.2^{TNmut/TNmut} pancreas indicates that total islet cell numbers are unchanged, but the mutant islets are smaller and more disorganized than in wild-type littermate controls (Fig. 2B,C,F). Consistent with the phenotype of the Nkx2.2^{−/−} mice, which form fewer α and no β cells, the Nkx2.2^{TNmut/TNmut} islets have fewer insulin-producing β cells (Fig. 2D). Surprisingly, however, the number of glucagon-producing α cells appears to be increased (Fig. 2E). The Nkx2.2^{TNmut/TNmut} mice do not display any apparent defects in δ, pancreatic polypeptide (PP), or acinar cell populations (data not shown).

**Results**

**Mutation of the Nkx2.2 TN domain disrupts the in vivo interaction between Nkx2.2 and the Grg3 corepressor and leads to diabetes in adult Nkx2.2^{TNmut/TNmut} mice**

In the pancreas, Grg2 and Grg3 are coexpressed with Nkx2.2 (Doyle et al. 2007; Hoffman et al. 2008), and Grg3 interacts with Nkx2.2 via the conserved TN domain (Doyle et al. 2007). To determine whether the physical interaction between Nkx2.2 and Grg proteins is necessary for appropriate pancreatic islet development and/or function, we generated mice that express a mutant allele of Nkx2.2 containing amino acid substitutions within the core sequence of the TN domain [Nkx2.2^{TNmut/TNmut}; Fig. 1A]. Presence of the mutant allele was monitored using allele-specific primers [Fig. 1B; Materials and Methods]. Heterozygous Nkx2.2^{TNmut/+} and homozygous Nkx2.2^{TNmut/TNmut} mice do survive postnatally, but the male mice begin to display overt hyperglycemia by 3.5 wk of age [Supplemental Fig. 1A,B]. By 6 wk, both male and female Nkx2.2^{TNmut/TNmut} mice are severely diabetic in fed and fasted conditions, with compromised fertility (Fig. 2A; Supplemental Fig. 1C). The Nkx2.2^{TNmut/TNmut} mice do not survive beyond 8 wk of age. Histological analysis of the 6-wk-old Nkx2.2^{TNmut/TNmut} pancreas indicates that total islet cell numbers are unchanged, but the mutant islets are smaller and more disorganized than in wild-type littermate controls (Fig. 2B,C,F). Consistent with the phenotype of the Nkx2.2^{−/−} mice, which form fewer α and no β cells, the Nkx2.2^{TNmut/TNmut} islets have fewer insulin-producing β cells (Fig. 2D). Surprisingly, however, the number of glucagon-producing α cells appears to be increased (Fig. 2E). The Nkx2.2^{TNmut/TNmut} mice do not display any apparent defects in δ, pancreatic polypeptide (PP), or acinar cell populations (data not shown).

**Nkx2.2 TN domain is required for appropriate islet cell development**

Nkx2.2 is required for the primary induction of most endocrine cell lineages, in Nkx2.2^{−/−} embryos, ghrelin-expressing
cells form at the expense of all β cells and the majority of α and PP cells [Sussel et al. 1998]. To determine whether the disrupted islet structure and abnormal islet cell ratios observed in the Nkx2.2 TNmut/TNmut adult mice are caused by pancreatic defects manifest during embryonic development, we assessed endocrine cell formation in Nkx2.2 TNmut/TNmut embryos. Unlike the Nkx2.2-/- embryos, which already display endocrine cell differentiation phenotypes by E9.5, the Nkx2.2 TNmut/TNmut embryos display no apparent defects in α- and β-cell numbers at E12.5 [Fig. 3A–C]. In agreement with the cell quantification (Fig. 3I–L), these phenotypes persist through the end of gestation (Fig. 3I–L).

Interestingly, the Nkx2.2-/- phenotype differs substantially from that observed in the Nkx2.2-/- embryos. While the less severe β-cell and α-cell phenotypes could be attributed to mutations in the TN domain, having a more moderate reduction in Nkx2.2 function than the null allele, the resulting increase in α-cell numbers is more difficult to interpret. To begin to characterize the underlying molecular changes that may be contributing to the Nkx2.2 TNmut/TNmut endocrine cell...
Interestingly, although the general changes in expression levels of the α- and β-cell-specific genes corresponded well with the observed alterations in the α- and β-cell numbers in the Nkx2.2TNmut/TNmut embryos, we could detect a threefold to fourfold increase in the expression of the α-cell transcription factor Arx at E15.5, prior to a significant increase in the number of glucagon+ cells [Fig. 4C]. To identify the cellular source of increased Arx expression, we performed immunofluorescence analysis to correlate Arx expression with the different pancreatic populations. At E15.5, we observe a large increase in the number of Arx-expressing cells that do not coexpress glucagon in the Nkx2.2TNmut/TNmut [Fig. 4D,E]. Surprisingly, at the same stage of development, we begin to observe a number of insulin-producing cells that ectopically express Arx [Fig. 4F,G]. By the end of gestation, the Nkx2.2TNmut/TNmut pancreas contains Arx+, insulin− β cells and Arx+, glucagon− α cells, whereas pancreata from wild-type littermates show mutually exclusive expression of Arx and insulin [Fig. 4H,L].

**Mutation of the Nkx2.2 TN domain results in a β-to-α-cell conversion due to the ectopic expression of Arx in β cells**

Previous studies have demonstrated that misexpression of Arx in embryonic β cells is sufficient to convert β cells into α cells [Collombat et al. 2007]. Since we observe elevated Arx expression and Arx+ insulin-producing cells at E15.5, prior to a significant increase in α-cell numbers, we postulated that mutation of the Nkx2.2 TN domain may cause aberrant derepression of the Arx gene in β cells to trigger their transdifferentiation into α cells. To test this hypothesis, we generated Nkx2.2TNmut/TNmut mice carrying the Ins:Cre transgene [Herrera 2000] and either the Rosa26:tomato or Rosa26:LacZ reporter alleles [Soriano 1999; Madisen et al. 2010] to allow us to lineage trace the mutant β cells. While Rosa26 reporter expression is restricted to the insulin-expressing population in control Nkx2.2+/-;Ins-Cre;Rosa26:LacZ/tomato islets [Fig. 4J, Supplemental Fig. 4A,B], we detect Rosa26:tomato reporter expression in >2% of the glucagon-expressing cells of the Nkx2.2TNmut/TNmut Ins-Cre; Rosa26:LacZ/tomato islets at postnatal day 0 [P0] [Fig. 4K, Supplemental Fig. 4C]. Many of the glucagon and tomato double-positive cells coexpress Arx, supporting the idea that Arx is upregulated in the β-cell-derived glucagon-expressing population that appears in the absence of functional Nkx2.2 [Fig. 4N]. Consistent with the discovery that β cells are reprogrammed to an α-cell fate in the Nkx2.2TNmut/TNmut mice, expression of a number of β-cell transcription factors—including Pdx1, MafA, and Nkx6.1—can be detected in a small population of cells that are in the process of converting to glucagon-expressing α cells [Supplemental Fig. 4D–F]. The insulin- and glucagon-coexpressing population present in the Nkx2.2TNmut/TNmut pancreas may also represent this transitioning population [Fig. 4E,F]. It should be noted that containing of tomato with ghrelin cannot be detected, indicating, in
contrast to the α-cell lineage, the observed increase in ε cells is not due to β-cell reprogramming [Supplemental Fig. 4K,L]. Since the Nkx2.2 TNmut/TNmut pups are not born with a metabolic phenotype but continue to lose β cells and become increasingly hyperglycemic with age, we wished to determine whether the β-to-ε-cell conversion continues to occur in postnatal animals. For this analysis, we generated Nkx2.2 TNmut/TNmut mice carrying the Pdx1.CreER transgene [Gu et al. 2002] and the Rosa26: tomato reporter allele. To avoid outcomes that could be associated with a hyperglycemic environment, we induced 3-wk-old mice with tamoxifen prior to the onset of overt hyperglycemia [Supplemental Fig. 1]. Five days following tamoxifen induction, we can detect tomato reporter expression in glucagon-producing ε cells in Nkx2.2 TNmut/TNmut; Pdx1:CreER; Rosa26:tomato islets (n = 3), indicating that β-to-ε-cell reprogramming continues to occur postnatally (Supplemental Fig. 5A–D). Glucagon expression and tomato reporter expression were mutually exclusive in control Nkx2.2 TNmut/+; Pdx1.CreER, Rosa26:tomato littermates receiving tamoxifen. Nkx2.2 TNmut/TNmut; Pdx1.CreER; Rosa26:tomato islets that did not receive tamoxifen were devoid of

Figure 3. Nkx2.2 TNmut/TNmut mice display decreased insulin expression and increased glucagon and ghrelin expression. (A–L) Characterization of insulin-, glucagon-, and ghrelin-expressing cells in Nkx2.2 TNmut/TNmut mice (n = 3–5). (A–C,L) Hormone-positive cell numbers and expression were unaltered at E12.5. (D–H,L) By E15.5, Nkx2.2 TNmut/TNmut mice display increased ε-cell numbers, slightly elevated α-cell numbers, and decreased numbers of β cells. (I–L) By E18.5, the glucagon population is significantly increased, while the insulin and ghrelin populations remain decreased and increased, respectively. [*] P < 0.05. Arrowheads indicate glucagon/insulin containing in F and J.
Nkx2.2 directly binds and represses the methylated Arx promoter in β cells

Given the dramatic increase in Arx expression in the Nkx2.2 TNmut/TNmut β cells, it is possible that disruption of the interaction between Nkx2.2 and Grg3 interferes with repression of the Arx promoter in β cells. To determine whether Arx is a direct target of Nkx2.2, we performed in silico analysis of the Arx promoter to identify a highly conserved Nkx2.2-binding site located ~1.4 kb upstream of the transcriptional start site [Supplemental Fig. 5A; Hill et al. 2011]. Chromatin immuno-precipitation (ChIP) of this region with Nkx2.2 antibody in islet cell lines demonstrated that endogenous Nkx2.2 normally occupies this region of the Arx promoter in both α and β cells, although binding is significantly enriched in βTC6 cells [Fig. 5A]. Furthermore, Grg3 and HDAC1 are preferentially recruited to the Arx promoter in β cells, supporting the idea that Nkx2.2 may function to recruit Grg3 to repress Arx expression in the β-cell population. Further analysis of the Arx promoter revealed the presence...
of CpG islands surrounding the conserved Nkx2.2-binding site, which, if methylated, could also contribute to the mechanism of Arx repression in β cells. Accordingly, bisulfite analysis for the Nkx2.2-binding region of the Arx promoter identified differential methylation patterns in β versus α cells; the Arx promoter is hypermethylated in β cells [Fig. 5B]. Since the de novo DNA methyltransferase Dnmt3a has been shown to be important for establishing methylation patterns during development and promotes site-specific methylation through its interaction with transcription factors (Hervouet et al. 2009), we investigated whether Dnmt3a was also differentially present on the
Arx promoter. Consistent with a role for Dnmt3a in the cell-specific regulation of Arx promoter methylation, Dnmt3a occupies the Arx promoter specifically in β cells (Fig. 5A). Furthermore, communoprecipitation analysis demonstrates that Flag-tagged Dnmt3a can form a protein complex with Nkx2.2, but not other β-cell factors such as Pax4 and Isl1 (Fig. 5C). Grg3 and HDAC1 are also part of this complex, suggesting that Dnmt3a and Nkx2.2 cooperate to facilitate recruitment of a repressor complex to the Arx promoter in β cells. Interestingly, the Nkx2.2 TN domain appears to be dispensable for a direct interaction between Nkx2.2 and Dnmt3a [Supplemental Fig. 6B], suggesting that the two factors interact through another Nkx2.2 domain and/or through other proteins within the complex.

To investigate whether Nkx2.2 specifically represses the Arx promoter in β cells, we subcloned a 2.5-kb region of the Arx promoter located upstream of the transcriptional start site into the pG3 basic promoter-less luciferase reporter vector. Cotransfection of pG3-Arx and Nkx2.2 into αTC1 cell lines resulted in a significant induction of Arx transcriptional activity, whereas cotransfection of pG3-Arx and Nkx2.2 into βTC6 cell lines elicited a 65% repression of transcription [Fig. 5D]. These data suggest that Nkx2.2 represses Arx activity in β cells through its interaction with Dnmt3a and Grg3 and preferential recruitment of HDAC1 to the Arx promoter in β cells. These findings are consistent with the in vivo results, suggesting that the Nkx2.2 TN domain is essential for the recruitment of Grg3 and maintaining repression of Arx in the β-cell population.

β-Cell-specific deletion of Dnmt3a also results in a β-to-α-cell conversion

Given the enrichment of Dnmt3a on the Arx promoter in β cells and its presence in the Nkx2.2, Grg3, and HDAC1 repressor complex, we wished to determine whether Dnmt3a is critical for proper islet cell type maintenance, similar to Nkx2.2. Interestingly, mice with a β-cell-specific deletion of Dnmt3a [Ins:Cre; Dnmt3a-α-lo] phenotype, the Nkx2.2 TTNmut/TNmut mice, α cells are increased with a corresponding loss of β cells [Fig. 5E]. Furthermore, genetic lineage tracing in Ins:Cre;Dnmt3a-α-lo;Rosa26:LacZ mice demonstrates that the β-cell loss associated with deletion of Dnmt3a results in the ectopic expression of Arx specifically from the Nkx2.2 TTNmut/TNmut β cells. Wild-type mice do not normally express Arx in β cells, and, accordingly, Ins:Cre; Arx-α-lo mice develop normal islets and are indistinguishable from wild-type mice [Supplemental Fig. 7A]. Alternatively, deletion of Arx from the β cells of Nkx2.2 TTNmut/TNmut mice returned Arx to wild-type expression levels, confirming that the elevated Arx expression was due primarily to its ectopic expression in β cells [Fig. 6G]. Furthermore, removal of Arx from Nkx2.2 TTNmut/TNmut β cells normalized both glucagon mRNA levels and α-cell numbers [Fig. 6B,D,F,G]. Lineage tracing of the β cells in the Nkx2.2 TTNmut/TNmut;Ins:Cre; Arx-α-lo mice did not identify any β-cell-derived glucagon-expressing cells [Fig. 6J,K], suggesting that the deletion of Arx is sufficient to prevent the β-to-α-cell transdifferentiation. On the other hand, the β-cell deletion of Arx is not sufficient to restore β-cell numbers or insulin expression at E18.5, and adult Nkx2.2 TTNmut/TNmut; Ins:Cre;Arx-α-lo mice remained hyperglycemic [Fig. 6A,C,E,G-I]. This suggests that the TN domain regulates additional Nkx2.2 targets, which are responsible for β-cell formation, maintenance, and function. Similarly, elevated ghrelin levels are unaffected by the removal of Arx from the Nkx2.2 TTNmut/TNmut β cells, demonstrating that Arx-independent Nkx2.2 TN domain functions are also required for the e- versus β-cell fate choice during embryogenesis [Fig. 6G].

To investigate whether the Ins:Cre; Dnmt3a-α-lo phenotype is also dependent on Arx, we tested whether simultaneous deletion of Dnmt3a and Arx could rescue the β-to-α-cell transdifferentiation process. siRNAs targeting Dnmt3a, Arx, or a combination of the two genes were transfected into Min6 cells. Similar to the Ins:Cre; Dnmt3a-α-lo mice, knockdown of Dnmt3a in Min6 cells resulted in β-to-α-cell reprogramming: Expression of insulin, Pdx1, and Pdx4 was reduced, with a concomitant up-regulation of glucagon, Arx, and MatB. Combined siRNA knockdown of Arx with Dnmt3a was able to prevent the corresponding alterations in α- and β-cell marker expression, indicating that, similar to Nkx2.2, Dnmt3a-mediated reprogramming is dependent on Arx misexpression [Supplemental Fig. 7B].

Discussion

Transcriptional repression is widely appreciated for its role in determining lineage and cell specification, as repressive mechanisms are known to be key regulators of cellular commitment in many tissues and organisms [Gray et al. 1995; Gray and Levine 1996; Laslo et al. 2006; Jepsen et al. 2007; Rajasekhar and Begemann 2007; Li and Davidson 2009; Nishi et al. 2009]. Nkx2.2 has been shown to act as a transcriptional repressor to regulate ventral neural patterning through its interaction with the corepressor Grg4 [Muh et al. 2001]. The ability of Nkx2.2 to act as a transcriptional repressor in the developing pancreas has also been shown [Doye et al. 2007]. Grg2 and Grg3, rather than Grg4, are coexpressed with Nkx2.2 in the pancreas and can also interact with Nkx2.2 through the groucho interaction TN domain [Doye et al. 2007; Hoffman et al. 2008]. In this study, we mutated the Nkx2.2 TN domain to determine the role
of Grg-mediated Nkx2.2 repressor activity in the developing pancreas. We demonstrate that mutation of the Nkx2.2 TN domain abolishes the interaction between Nkx2.2 and Grg3 protein in vivo, and this is sufficient to cause a shift from the β-cell to the ε-cell lineage. Furthermore, many of the remaining β cells are reprogrammed into α cells due to the β-cell-specific derepression of the α-cell regulator Arx. Remarkably, it appears that Arx is the primary downstream target of Nkx2.2 in the maintenance of β-cell identity; removal of Arx from the Nkx2.2TNmut/TNmut β cells appears to completely inhibit the β-to-α-cell conversion.

The phenotypes associated with Nkx2.2TNmut/TNmut suggest that TN domain-mediated functions are required in two distinct processes within the pancreas: primary islet cell fate decisions and maintenance of β-cell identity. Initially, the TN domain appears to influence the competency of the Ngn3ε endocrine progenitor to differentiate into ε cells versus β cells, similar to what we observed in the Nkx2.2ε−/ε− mice. A proposed defect in cell fate choice regulation is supported by the observation that the corresponding alterations of β-cell and ε-cell ratios occurs at the onset of the secondary transition, which marks the first major wave of β-cell differentiation. Furthermore, we demonstrated that the increase in ghrelin-producing cells is not due to reprogramming of the β-cell population, since we did not observe ghrelin cells that were derived from the lineage-labeled β-cell population (Supplemental Fig. 4L). Although the Nkx2.2TNmut/TNmut and Nkx2.2ε−/ε− mice both display an increase in ε-cell numbers, coupled with a corresponding decrease in β cells, the phenotype is much more dramatic in the Nkx2.2ε−/ε− mice, which have a complete absence of β cells at all developmental stages. The less severe phenotype associated with the Nkx2.2TNmut/TNmut mice could indicate that the interaction between Nkx2.2 and Grgs is not completely disrupted, and residual activity is sufficient to allow for the formation of a small number of β cells. Alternatively, it is possible that other domains of the Nkx2.2 protein are responsible for β-cell formation during the primary transition stage of pancreas development, which would be consistent with the absence
of a β-cell phenotype in the Nkx2.2TNmut/TNmut mice prior to E12.5 (Fig. 3A–C). A third possibility is that additional Nkx2.2 protein domains can compensate for the loss of TN domain activity to allow the formation of a small number of β cells throughout development. These possibilities are not mutually exclusive and will be explored in future studies to gain a more complete understanding of the role of Grg-mediated regulation of Nkx2.2 function in β-cell lineage decisions.

The Nkx2.2 TN domain also appears to play a distinct role in maintaining the β cells that are able to form in the Nkx2.2TNmut/TNmut mice. We show that by E18.5, there is an additional reduction in β cells that corresponds to an unexpected increase in α-cell numbers. Unlike the early changes we observed in β-cell versus α-cell ratios, we do not observe a significant increase in α-cell numbers until the end of gestation. The observation that α-cell genes are becoming ectopically expressed in insulin-producing populations, combined with the β-cell lineage-tracing analysis, indicates that many of the α cells are derived from insulin-producing cells. This implies that Nkx2.2 is functioning within the β cell to maintain its cellular identity and prevent transdifferentiation into the α-cell fate. Interestingly, in the Nkx2.2TNmut/TNmut mice, we do not observe immediate and simultaneous reprogramming of all β cells in response to dysregulation of Arx. Instead, the transdifferentiation event occurs gradually over time and continues in the postnatal animal. This would suggest that, in addition to the activation of α-cell lineage inducers, such as Arx, a number of additional (perhaps more resistant) cellular events, such as global chromatin remodeling, must occur to allow reprogramming to be initiated. Further epigenetic analyses and gene expression profiling of FACs-purified β-cell populations derived from the Nkx2.2TNmut/TNmut mice during intermediate stages of transdifferentiation may elucidate the β-cell-determining features that must be overcome to allow β-to-α-cell reprogramming.

Interestingly, the Nkx2.2TNmut/TNmut mice also differ quite significantly from the Nkx2.2−/− phenotype with regards to PP- and α-cell formation. Unlike the Nkx2.2−/− mice, which display an ~50% reduction in PP cells, there is no apparent change in PP-cell numbers or PPY expression in the Nkx2.2TNmut/TNmut mice, suggesting that PP-cell formation is not regulated by TN domain functions. Even more puzzling was the unexpected α-cell phenotype associated with the Nkx2.2TNmut/TNmut mice. We previously demonstrated that the Pdx1:Nkx2.2 heterozygous repressor derivative was sufficient to fully rescue α-cell numbers in the Nkx2.2−/− mice (Doyle et al. 2007), suggesting that Nkx2.2.2 repressor function is sufficient for α-cell formation. However, removal of the Nkx2.2 TN repressor domain does not appear to negatively affect the α-cell population at any stage of development, and instead we observe an increase in α-cell numbers. This may indicate that Grg-dependent repression is dispensable for the α-cell lineage specification or perhaps that other regions of the Nkx2.2 protein can compensate for the loss of this interaction and/or provide an alternative mechanism of repressive activity. We are currently exploring the possibility that the conserved NK2-SD domain, the defining domain of the Nkx2 family, can contribute to Nkx2.2 repressor activity in vivo, as has been suggested previously (Watada et al. 2000). The data presented here now give us a greater understanding of a previously unanticipated role for the Nkx2.2 TN domain in maintaining β-cell identity through its repression of the Arx promoter in β cells. This function would not be revealed in Nkx2.2−/− mice, since there are no β cells available to reprogram. This unexpected result not only begins to elucidate the distinct functions of Nkx2.2 in different islet cell types and at different stages of development, but also emphasizes the usefulness of generating hypomorphic and/or domain-specific alleles to understand the nuances of a protein’s function in vivo.

Our study also re-emphasizes the importance of maintaining the appropriate regulation of Arx expression in the different islet cell types. Previous studies have demonstrated that Arx is essential for specifying the α- and PP-cell lineages (Collombat et al. 2003, 2005, 2007). Disruption of Arx leads to reduced numbers of α cells coupled with a corresponding increase in β and δ cells (Collombat et al. 2003). Alternatively, misexpression of Arx in β cells is sufficient to convert β cells to α or PP cells (Collombat et al. 2007). Interestingly, although we recapitulate the β-to-α-cell reprogramming phenomenon with the derepression of Arx in β cells, we are unable to detect significant changes in PP expression. It is possible that the increased levels of Arx caused by the Nkx2.2 TN mutation are sufficient for the α-cell fate but are not sufficient for conversion to a PP-cell fate. This is consistent with studies showing that protein expression levels and dosing requirements of transcription factors are important for controlling islet cell development and islet cell fate choices (Puntani et al. 2006; Wang et al. 2009). Alternatively, removal of the Nkx2.2 TN domain likely affects additional downstream effectors that may function to counter the Arx regulation of the PP fate.

Differential expression of transcription factors can allow for tissue- and cell-specific gene regulation; however, when a factor is expressed in more than one cell type, higher-order regulation will be required to control cell- and/or promoter-specific regulation. Differential promoter regulation can be achieved by cell-specific cofactor interactions or at the level of DNA accessibility or both. In the case of Nkx2.2, we demonstrated that it preferentially binds the Arx promoter in β cells, although Nkx2.2 is expressed and functions in both α and β cells. The differential binding ability of Nkx2.2 may be influenced by the methylation state of the Arx promoter that surrounds the Nkx2.2 consensus binding site and is possibly facilitated by the DNA modifications induced by Dnmt3a. A cooperative role between these factors for maintaining β-cell-specific Arx repression and β-cell identity is supported by the similar reprogramming phenotypes displayed by the β-cell-specific deletion of Dnmt3a and the Nkx2.2 TN mutant mice. Therefore, it is possible that differential DNA methylation and the presence of Dnmt3a on the Arx promoter...
provide a cell-specific platform for Nkx2.2 binding on the Arx promoter in β cells. However, Dnmt3a is also expressed in both α and β cells, suggesting that its preferential recruitment to the Arx promoter in β cells must also rely on a β-cell-specific factor. Nkx6.1 is a possible candidate for mediating β-cell-specific recruitment of these repressor complexes can be facilitated by sequence-specific transcription factors (Kehle et al. 1998; Liang et al. 2008; Fu et al. 2011). Since Nkx2.2 preferentially recruits Grg3 and HDAC1 to the Arx promoter in β cells, it is possible that Nkx2.2 cooperates with methyl-binding proteins to recruit histone deacetylases and achieve repression of Arx. Since it appears that the initiation and maintenance of Arx expression in β cells is crucial for maintaining β-cell identity, it is likely that a combination of these mechanisms is necessary to cooperatively repress Arx and retain β-cell integrity (schematized in Fig. 7A). Additionally, given the small but significant recruitment of Nkx2.2 and Grg3 to the Arx promoter in α cells (Fig. 5A) and the Nkx2.2-mediated induction of Arx in α cells (Fig. 5D), we cannot rule out the possibility that Nkx2.2 and Grg3 may be facilitating the activation of Arx in α cells. While Grgs are generally described as corepressors, a recent study demonstrated that Grg3 is able to function as both an activator and a repressor during adipogenesis (Villanueva et al. 2011). Lineage-specific gene expression patterns rely on a complex interplay between transcription factors, cofactors, and DNA accessibility. This process becomes much more complicated when a single factor has differential functions across development and within unique cell types. Our analysis highlights the complex regulation of lineage

Figure 7. Model of Nkx2.2 regulation of the Arx promoter in β cells. (A) Our data, in combination with the study by Dhawan et al. (2011), support a model whereby a repressor complex comprised of Nkx2.2, Grg3, and Dnmt3a preferentially recruits HDAC1 to the Arx promoter to silence Arx expression specifically in the pancreatic β cells to prevent β-to-α cell conversion. We can also detect the presence of Nkx6.1 bound to this promoter region, which possibly confers cell-specific recruitment of the Nkx2.2/Dnmt3a complex to the Arx promoter. Since Nkx2.2^TNmut/TNmut mice no longer retain a Nkx2.2/Grg3 interaction, Grg3 and HDAC1 are no longer recruited to the Arx promoter, allowing ectopic expression of Arx in β cells. It is not yet known whether Nkx2.2^TNmut/TNmut mice affect Dnmt3a recruitment and subsequent methylation of the Arx promoter (B) Grg3-mediated Nkx2.2 repression controls two developmental stages of pancreas development. We first observe aberrations in the proper specification of β and c cells at E13.5 in the Nkx2.2^TNmut/TNmut mice, when an increased number of c cells are forming at the expense of β cells. The remaining β cells then go on to transdifferentiate to α cells. This transdifferentiation is evident by E18.5 and continues to occur postnatally.
commitment within the endocrine pancreas and draws attention to the need to dissect out the cell-specific functions of each regulatory factor to fully understand lineage specification and maintenance. The phenotype of mice carrying a disruption of the Nkx2.2 TN domain has revealed that the Grg-mediated repressor activity of Nkx2.2 controls two stages of development (schematized in Fig. 7B): (1) Grg-mediated Nkx2.2 repression is not required for the early commitment of \( \beta \) versus \( \alpha \) cells, but is required for the correct balance of \( \beta \) and \( \epsilon \) cells that form at the beginning of the secondary transition. [2] The functions mediated by this interaction are also critical for the maintenance of \( \beta \)-cell identity by preventing ectopic expression of Arx in these cells. This emphasizes the importance of understanding the spatial and temporal organization of cofactors in lineage commitment decisions and underscores the potential importance of epigenetic and transcription factor interactions to further regulate promoter occupancy. Our studies, coupled with those of Dhawan et al. (2011), suggest that Nkx6.1, Nkx2.2, Dmnt3a, and MeCP2 converge on methylated DNA of the Arx promoter specifically in \( \beta \) cells to recruit histone modifiers (HDAC1 and PRMT6, respectively), in order to silence Arx expression (schematized in Fig. 7). Further understanding of the precise molecular events of islet cell-specific transcriptional networks on individual promoters will aid in formulating effective protocols to induce and maintain appropriate endocrine lineage commitment from alternative cell sources, such as embryonic stem cells or induced pluripotent cells, for the treatment of diabetes.

**Materials and methods**

**Generation of Nkx2.2\(^{TN\text{mut}}\) mice**

The Nkx2.2 TN domain mutant (Nkx2.2\(^{TN\text{mut}}\)) mice were generated using the two-step recombination-mediated cassette exchange (RMCE) technology (Chen et al. 2011) to insert amino acid substitutions in the endogenous Nkx2.2 locus. To do so, we first engineered an Nkx2.2 loxed cassette acceptor (Nkx2.2\(^{L\text{CA}}\)) allele using the strategy and plasmids described in Chen et al. (2011) [L. Arnes, K. Leclerc, J. Friel, S. Hipkens, M.A. Magnuson, and L. Sussel, in prep.]. Second, to generate the Nkx2.2\(^{TN\text{mut}}\) allele, we then made a basal exchange Nkx2.2 targeting vector containing a 5.1-kb genomic (129-S6) DNA fragment encompassing the two Nkx2.2 coding exons. PCR-mediated site-directed mutation of the TN domain was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene) to replace the conserved TN domain core SVKD\(^{14-15}\) amino acids with four alanine residues and the invariant leucine \( L \) with valine, an equally hydrophobic amino acid. We also introduced a Sall restriction site for genotyping purposes. All mutations were verified by DNA sequencing. The Nkx2.2TNmut exchange vector was used to perform RMCE using mouse embryonic stem cells containing the Nkx2.2\(^{L\text{CA}}\) allele. A dual, positive-negative selection strategy was used to identify clones that had undergone cassette exchange (Chen et al. 2011), which were then confirmed by DNA PCR. Positive clones were further analyzed by Southern blot analysis for the presence of the novel Sall restriction site. Nkx2.2\(^{TN\text{mut}}\) embryonic stem cells were injected into blastocysts obtained from the mating of C57Bl/6 mice, then transferred to pseudopregnant C57Bl/6 mice. Male chimeras were bred to C57Bl/6 female mice, and agouti offspring were PCR-genotyped using Nkx2.2 allele-specific primers that distinguish between the wild-type and Nkx2.2\(^{TN\text{mut}}\) allele (Fig. 1B): cKL6 [FWD, wild type], GTCAAGGACATCTTGG ACCTTCGG, cKL1 [FWD, Nkx2.2\(^{TN\text{mut}}\)], TTTGGAGGCCGCAATC, and cKL5 (REV, wild type and Nkx2.2\(^{TN\text{mut}}\)), ATCGG GATGGCACTTTGGACATT. The FRT-flanked hygromycin cassette was subsequently removed by mating to FlpE mice [Rodriguez et al. 2005]. All procedures to generate the Nkx2.2\(^{TN\text{mut}}\) mice were approved by the Vanderbilt University Animal Care and Use Committee.

**Animal maintenance**

All mice were maintained on a Swiss Black (Taconic) background and housed and treated according to the Columbia University Institutional Animal Care and Use Committee approval protocol. Genotyping of Nkx2.2\(^{TN\text{mut}}\) mice and embryos was performed using Nkx2.2 allele-specific primers (described above).

**Immunohistochemistry**

Tissues were fixed in 4% PFA for 4 h or overnight, washed in cold PBS, incubated in 10% sucrose, and cryopreserved. Immunofluorescence was performed on 7 \( \mu \)M sections. See Supplemental Table 1 for a list of antibodies used. Fluorescent images were obtained with a Nikon Eclipse 80i microscope, Q-image camera, and ImagePro software [Media Cybernetics]. Confocal images were captured using a Zeiss LSM 510 upright (pinhole = 1). For quantification purposes, stained cells were counted manually on every fourth section (E12.5 and E13.5) and every eighth section (E13.5) throughout the entire pancreas for the wild type (\( n = 3 \)) and mutant (\( n = 3 \)). For animals at E18.5, the number of stained cells, which were counted on every eighth section, was divided by total pancreas area. All values are expressed as mean \( \pm \) SEM. Statistical analysis was performed using a two-tailed Student's unpaired \( t \)-test. Results were considered significant when \( P < 0.05 \).

**RNA analysis**

Total RNA was isolated from whole pancreatic tissue from the respective embryonic ages (RNeasy, Qiagen), and cDNA was prepared using the SuperScript III kit [Invitrogen] and random hexamer primers. Quantitative PCR was performed using 200 ng of cDNA, PCR master mix [Eugenotech], and TaqMan probes [ABI Assays on Demand]. See Supplemental Table 2 for a list of primers used. All genes were normalized to Cyclophilin B and were quantified with ABI prism software. An \( n \geq 4 \) was obtained for all mouse populations. All values are expressed as mean \( \pm \) SEM. Statistical analysis was performed using a two-tailed Student's unpaired \( t \)-test. Results were considered significant when \( P < 0.05 \).

**ChIP**

\( \alpha \)-TC1 and \( \beta \)-TC6 cells were grown to \( ~90\% \) confluency in a 15-cm culture dish and were formaldehyde-cross-linked according to the ChIP-IT Express kit [Active Motif]. Cross-linked chromatin was fragmented by sonication using a Diagenode BioRupter \( \beta \) min to 30 sec on/off. Three micrograms of mouse \( \alpha \)-Nkx2.2 [Developmental Studies Hybridoma Bank [DSHB]],
rabbit α-TLE3 (Santa Cruz Biotechnologies), rabbit α-HDAC1 (Santa Cruz Biotechnologies), rabbit α-NKx6.1 (Beta Cell Biology Consortium), or mouse α-IgG (AbCam) was added to the sheared chromatin. The antibody/chromatin complexes were left to rotate end to end overnight at 4°C. Antibody/chromatin complex regions were pulled down using ChIP-grade protein G magnetic beads (Cell Signaling). Chromatin was washed, eluted, and reverse-cross-linked, followed by protease treatment. Chromatin fragments were then analyzed by quantitative PCR using SYBR Green fluorescence with the following primers: Arx (FWD) TCCTCACCACATGGAGCGTA, and (REV) GCAACCTTGAGCGGTACAGA. All values are expressed as mean ± SEM. Statistical analysis was performed using a two-tailed Student’s unpaired t-test. Results were considered significant when P < 0.05.

Coimmunoprecipitation and Western

Nuclear extraction was performed on excised pancreata from P0 mouse pups using the Nuclear Complex Co-IP kit (Active Motif). Nuclear extract was pooled from five wild-type or five mutant animals. One-hundred fifty micrograms of nuclear extract and 5 μg of rabbit anti-TLE3 (Santa Cruz Biotechnologies), mouse α-NKx2.2 (DSHB), or rabbit anti-IgG (Chemicon) were incubated overnight at 4°C, rotating end to end. Protein/antibody complexes were pulled down using protein G DynaBeads (Invitrogen). Protein/antibody complexes were washed, eluted, and run on a NuPAGE Novex 10% Bis-Tris gel (Invitrogen). Protein/antibody complexes were washed, eluted, and run on a NuPAGE Novex 10% Bis-Tris gel (Invitrogen). Proteins were transferred onto a nitrocellulose membrane (GE Healthcare) and probed using mouse anti-NKx2 or rabbit α-Dnmt1a. For immunoprecipitation control, the membrane was stripped, washed, and reprobed with the antibody that was used for the initial immunoprecipitation.

Luciferase assays

To generate the Arx-Luciferase reporter, a 2.5-kb fragment of the Arx genomic region upstream of the transcriptional start site was PCR-amplified from mouse tail genomic DNA using the following primers: oRS18 (FWD), ACGCGTTGCACCTCCCCCTTTA and 3′-end, respectively, to facilitate cloning into the pGL3 basic promoter-less luciferase reporter vector using MluI and BglII cloning sites. The resulting plasmid was verified by DNA sequencing. Luciferase assays were performed in oTC6 cell lines using the Dual-Luciferase Reporter Assay System (Promega) as described previously (Hill et al. 2010).

Tamoxifen treatment in PdxCreER,R26R:Tomato mice

PdxCreER was induced with a 5-mg intraperitoneal injection of tamoxifen (Sigma) in 3-week-old mice every 48 h for 4 d. On the fifth day, pancreata were harvested and fixed in paraformaldehyde, as described above, and immunostained for indicated hormones.

In vitro siRNA-mediated knockdown

Knockdown of Dmnt3a, Arx, and Dmnt3a2 Arx in Min6 cells was performed by transfection of specific-targeting small inhibitory RNAs or scrambled controls (Dharmacon Research, Inc.) using Lipofectamine-2000 (Invitrogen), according to the manufacturer’s instructions, in OPTI-MEM medium. Min6 cells were transfected with appropriate siRNAs or scrambled controls every 3 d (average transfection efficiency, 65%–80%), and samples were harvested at 4 d post-transfection, as indicated. RNA was isolated from cells using TRI Reagent (MRC) and treated with DNase (Ambion) according to the manufacturers’ instructions. One microgram of RNA was used for preparation of single-stranded cDNA using SuperScript III reverse transcriptase (Invitrogen) by the oligo(dT) priming method. Real-time RT–PCRs were performed with the Fast SYBR Green Master Mix (Applied Biosystems) and the 7900HT Fast Real-Time PCR equipment (Applied Biosystems). The expression levels of each transcript were normalized to the housekeeping gene Cyclophilin. Each real-time PCR experiment shown is a representative from at least three independent experiments. Primers for RT–PCR are listed in Supplemental Table 2.

Acknowledgments

We thank Catherine Lee May (CHOP) and Jeffrey Golden (CHOP) for providing the Arxdeleted mice, and Kanako Miyabayashi (Kyushu University, Fukuoka, Japan) for providing Arx antibody. We thank Jonathan Hill for identifying the conserved Nkx2.2 binding site within the Arx promoter. We thank Drs. Lori Zehser and Stephanie Padilla for critical reading of the manuscript. Ms. Kathy D. Shelton for assembling the gene targeting vector, and the staff of the Vanderbilt Transgenic/ES Cell Shared Resource for their expert performance of the blastocyst microinjection experiments. This work was supported by NIH grants DK0272504 (to L.S.), DK082590 (to L.S. and J.P.), DK0242502 (to M.A.M.), DK072473 (to M.A.M.), and DK068763 (to A.B.). Additional support was provided by the Columbia University DERC (P30 DK66608) and CTS grants.

References


Dhawan S, Georga S, Toshen SI, Fan G, Bhusan A. 2011. Pancreatic β cell identity is maintained by DNA methyla-


Gray S, Levine M. 1996. Transcriptional repression in develop-


Jopling C, Boue S, Izpisua Belmonte JC. 2011. Dedifferentiation, transdifferentiation and reprogramming: three routes to regen-


Raum JC, Gerrish K, Attner I, Henderson E, Guo M, Sussel L, Schissel JC, Newgard CB, Stein R. 2006. FoxA2, Nkx2.2, and


Supplemental figure 1. Nkx2.2^{TNmut/TNmut} mice are hyperglycemic. Fed blood glucose levels (A-C) (n = 3-6). Neither male nor female Nkx2.2^{TNmut/TNmut} mice are diabetic at weaning (3 weeks old) (A). Male Nkx2.2^{TNmut/TNmut} mice become diabetic shortly after weaning (3.5 weeks old) (B). Both male and female Nkx2.2^{TNmut/TNmut} mice are diabetic by 7 weeks old (C).

Characterization of insulin, glucagon and ghrelin expressing cells in Nkx2.2^{TNmut/TNmut} mice (n = 3) (Petit et al.).*: p<0.05, ***: p<0.001.
Glucose levels in 3 week old mice

- A
- B

Glucose levels in 3.5 week old mice

- C

Glucose levels in 7 week old mice

- D

e13.5 hormone* cell numbers

- E
- F
- G
- H
- I
- J

*WT* vs *TN/TN*

**p = 0.061**
Supplemental figure 2. Beta cells do not undergo apoptosis and α cells do not proliferate in Nkx2.2<sup>TNmut/TNmut</sup> mice. Immunofluorescence showing exclusive staining of insulin and caspase3 at e12.5, a time point just prior to detection of changes in beta cell numbers (A,B). At e15.5, when beta cell numbers are significantly decreased in Nkx2.2<sup>TNmut/TNmut</sup> mice, caspase3 staining is still excluded from beta cells (C,D). Insets in (B,D) show caspase3 staining in liver tissue present in the same tissue section. Immunofluorescence showing exclusive staining of glucagon and PHH3 at e12.5, prior to changes in alpha cell numbers (E,F). At e15.5, as alpha cells are beginning to increase in the Nkx2.2<sup>TNmut/TNmut</sup> mice, PHH3 staining is not detected in alpha cells (G,H) (n = 3).
WT | TN/TN
---|---
A | B
12.5
C | D
15.5
E | F
12.5
G | H
15.5
Supplemental figure 3. MafB localization in Nkx2.2^{TNmut/TNmut} mice. MafB expression is restricted to glucagon expressing cells (A,B) and excluded from insulin expressing cells (C,D) in 4 week old animals (n =3).
<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>TN/TN</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>![Image](5x258 to 503x700)</td>
<td>![Image](5x258 to 503x700)</td>
</tr>
<tr>
<td>B</td>
<td>![Image](5x258 to 503x700)</td>
<td>![Image](5x258 to 503x700)</td>
</tr>
<tr>
<td>C</td>
<td>![Image](5x258 to 503x700)</td>
<td>![Image](5x258 to 503x700)</td>
</tr>
<tr>
<td>D</td>
<td>![Image](5x258 to 503x700)</td>
<td>![Image](5x258 to 503x700)</td>
</tr>
</tbody>
</table>

Glucagon MaB

Insulin MaB
Supplemental figure 4. a cells acquire beta cell marker expression as beta cells transdifferentiate to alpha cells. The R26Rtomato reporter expression under the control of Ins:Cre, is restricted to beta cells (A). Using the R26RLacZ reporter, beta cell-derived α cells can be detected in Nkx2.2$^{TNmut/TNmut}$ mice (B,C). By e15.5, as alpha cell numbers are beginning to increase, Nkx6.1 is misexpressed in alpha cells of the Nkx2.2$^{TNmut/TNmut}$ mice (D,G). At P0, the beta cell markers MafA (E,H) and Pdx1 (F,I) are commonly found in alpha cells of Nkx2.2$^{TNmut/TNmut}$ mice. Approximately 2.5% of glucagon expressing cells are labeled with the Tomato reporter at P0 (J). Although there is an increase in ghrelin expression and cell number, lineage tracing using Ins:Cre confirms that the increased ghrelin cells are not beta cell-derived (K,L). (n = 3).
Supplemental figure 5. Beta-to-alpha cell reprogramming continues postnatally. 3 week old TN/TN;Pdx1:CreER;TOM mice show no Tomato expression in the absence of Tamoxifen treatment (A). Upon Tamoxifen treatment, Tomato expression is restricted from alpha cells in 3 week old TN/+;Pdx1:CreER;TOM mice (B). However, Tamoxifen treatment induces Tomato expression in glucagon expressing cells in 3 week old TN/TN;Pdx1:CreER;TOM mice (C) (n = 3). Arrowheads in (C) indicate co-positive cells.
Supplemental figure 6. *Arx* promoter conservation and regulation. The *Arx* promoter contains a highly conserved Nkx2.2 binding site at -1.4 kb (A). Coimmunoprecipitation analysis showing interaction between Nkx2.2 and Dnmt3a in WT and Nkx2.2^{TNmut/TNmut} mice (B). Chromatin immunoprecipitation demonstrates an enrichment of Nkx6.1 on the *Arx* promoter specifically in beta cells (C). ***: p < 0.001.
GENESDEV/2011/173039 Papizan et al., Supplemental figure 6

**A**

![DNA alignment and conservation](image)

**B**

**IP**

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>Dnmt3a</th>
<th>IgG</th>
<th>Nkx2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dnmt3a</td>
<td>WT</td>
<td>WT</td>
<td>TN/TN</td>
<td></td>
</tr>
<tr>
<td>Nkx2.2</td>
<td>WT</td>
<td>WT</td>
<td>TN/TN</td>
<td></td>
</tr>
</tbody>
</table>

**C**

**Arx promoter occupancy**

[Bar chart showing fold enrichment for Alpha TC1 and Beta TC6]
Supplemental figure 7. beta cell-targeted loss of Arx. Wild type mice with beta cell-specific loss of Arx form normal islets (A) (n = 3). Real-time RT-PCR analyses comparing the transcript levels of Dnmt3a, Pdx1, Pax4, Insulin (Ins), Glucagon (Glu), MafB and Arx in Min6 cells, after 4 days of transfection with scrambled (Scr), Dnmt3a, Arx or Dnmt3a+Arx siRNAs, showing a requirement for Arx in cell fate conversion upon loss of Dnmt3a (n=3) (B). *: p<0.05 vs Scr siRNA.
A

WT;Ins:Cre;Arx^{fl/fl}

P28

Insulin Glucagon

B

Relative transcript levels

Scr siRNA Arx siRNA
Dnmt3a siRNA Dnmt3a+Arx siRNAs

Dnmt3a Pdx1 Pax4 Ins Arx MafB Glu
### Supplementary Table 1

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit α-glucagon</td>
<td>1:1000</td>
<td>Phoenix Pharmaceuticals</td>
</tr>
<tr>
<td>Guinea pig α-glucagon</td>
<td>1:1000</td>
<td>Linco</td>
</tr>
<tr>
<td>Guinea pig α-insulin</td>
<td>1:1000</td>
<td>Linco</td>
</tr>
<tr>
<td>Rabbit α-ghrelin</td>
<td>1:500</td>
<td>Phoenix Pharmaceuticals</td>
</tr>
<tr>
<td>Rabbit α-PP</td>
<td>1:500</td>
<td>Linco</td>
</tr>
<tr>
<td>Rabbit α-somatostatin</td>
<td>1:500</td>
<td>Phoenix</td>
</tr>
<tr>
<td>Rabbit α-MafA</td>
<td>1:500</td>
<td>Bethyl</td>
</tr>
<tr>
<td>Rabbit α-MafB</td>
<td>1:500</td>
<td>Bethyl</td>
</tr>
<tr>
<td>Rabbit α-Nkx6.1</td>
<td>1:500</td>
<td>BCBC</td>
</tr>
<tr>
<td>Rabbit α-Arx</td>
<td>1:500</td>
<td>K. Miyabayashi, Fukuoka, Japan</td>
</tr>
<tr>
<td>Rabbit α-caspase3</td>
<td>1:250</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Rabbit α-phospho-histone-H3</td>
<td>1:500</td>
<td>Millipore</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy2 donkey α-guinea pig</td>
<td>1:250</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Cy3 donkey α-rabbit</td>
<td>1:250</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Cy2 donkey α-rabbit</td>
<td>1:250</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Cy3 donkey α-guinea pig</td>
<td>1:250</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Cy5 donkey α-guinea pig</td>
<td>1:250</td>
<td>Jackson ImmunoResearch</td>
</tr>
</tbody>
</table>

### Supplementary Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>AOD</th>
<th>FWD</th>
<th>REV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin1</td>
<td>Mm01950294_s1</td>
<td>GCAAAGTTCTA</td>
<td>CCCGGCTG</td>
</tr>
<tr>
<td>Insulin2</td>
<td>Mm00731595_gH</td>
<td>GAGGGCATGGA</td>
<td>TCTGTCTTGGT</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Mm00801712_m1</td>
<td>GCAAAGTTCTA</td>
<td>CCCGGCTG</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>Mm00445450_m1</td>
<td>GAGGGCATGGA</td>
<td>TCTGTCTTGGT</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Mm00436671_m1</td>
<td>GCAAAGTTCTA</td>
<td>CCCGGCTG</td>
</tr>
<tr>
<td>Pancreatic polypeptide</td>
<td>Mm00435889_m1</td>
<td>GAGGGCATGGA</td>
<td>TCTGTCTTGGT</td>
</tr>
<tr>
<td>Nkx2.2</td>
<td>Mm00839794_m1</td>
<td>GCAAAGTTCTA</td>
<td>CCCGGCTG</td>
</tr>
<tr>
<td>Nkx6.1</td>
<td>Mm00545962_m1</td>
<td>GCAAAGTTCTA</td>
<td>CCCGGCTG</td>
</tr>
<tr>
<td>Pdx1</td>
<td>Mm0035565_m1</td>
<td>GCAAAGTTCTA</td>
<td>CCCGGCTG</td>
</tr>
<tr>
<td>NeuroD</td>
<td>Mm01280117_m1</td>
<td>GCAAAGTTCTA</td>
<td>CCCGGCTG</td>
</tr>
<tr>
<td>Irx2</td>
<td>Mm01340316_m1</td>
<td>GCAAAGTTCTA</td>
<td>CCCGGCTG</td>
</tr>
<tr>
<td>Arx</td>
<td>Mm00545903_m1</td>
<td>GCAAAGTTCTA</td>
<td>CCCGGCTG</td>
</tr>
<tr>
<td>MafB</td>
<td>Mm00627481_s1</td>
<td>GCAAAGTTCTA</td>
<td>CCCGGCTG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene</th>
<th>FWD</th>
<th>REV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclphilin B</td>
<td>GCAAAGTTCTA</td>
<td>CCCGGCTG</td>
</tr>
<tr>
<td></td>
<td>GAGGGCATGGA</td>
<td>TCTGTCTTGGT</td>
</tr>
<tr>
<td>MafA</td>
<td>CTCCTAGAGGAACGCCAGGAGAA</td>
<td>CCTCCCCCAGTCGAGTATAGC</td>
</tr>
<tr>
<td>Dnmt3a</td>
<td>AACGGAAACGGGTAGTAGTG</td>
<td>ACTGCAATTACCTGGCTTGT</td>
</tr>
<tr>
<td>Pdx1</td>
<td>GAAATTCACCAAAAGCTCAGC</td>
<td>CCGGGTCTCAGTGTCAAG</td>
</tr>
<tr>
<td>Pax4</td>
<td>TCCAGGCTATTCTCCACAC</td>
<td>TATGAGGAGAAGCCACAGG</td>
</tr>
<tr>
<td>Ins1</td>
<td>GCAAGGCAGTCATTGTCCCAAC</td>
<td>AAGCGTGGGTTGGT</td>
</tr>
<tr>
<td>MafB</td>
<td>CACACGGCTACCAGTACAGCCA</td>
<td>GCCGAGTTTCGCAGCTTGA</td>
</tr>
<tr>
<td>Arx</td>
<td>TCCAGAAGAGACACTACC</td>
<td>TGTGAGCTCAGTCCCAT</td>
</tr>
<tr>
<td>Glucagon</td>
<td>CATCAGACAGGACTACAGCAGA</td>
<td>TCTGAACAGCTGCACAAATCT</td>
</tr>
<tr>
<td>Cyclphilin</td>
<td>GTTGCCAGGCTGTGGCACAG</td>
<td>CAGGCTGGTGAGCAGGCTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3

TRANSCRIPTIONAL PROFILING OF NKX2.2\textsuperscript{TNmut/TNmut} AND NKX2.2\textsuperscript{TNmut/TNmut};INS:CRE;ARX\textsuperscript{FL/FL} PANCREAS

Microarray analysis was performed by the University of Pennsylvania School of Medicine’s IDOM-DRC Functional Genomics Core. James Papizan generated all other data.

Abstract

The Nkx2.2\textsuperscript{TNmut/TNmut} mice are hyperglycemic and exhibit defects in islet cell fate specification and beta cell maintenance. The phenotype, in part, is due to the de-repression and ectopic expression of *Arx* in beta cells, resulting in beta-to-alpha-like cell reprogramming. Interestingly, several aspects of the Nkx2.2\textsuperscript{TNmut/TNmut} islet phenotype cannot be rescued by the beta-cell-specific *Arx* deletion, and the mice remain hyperglycemic due to defects in beta cell formation and function. To identify the dysregulated genes responsible for the persistent beta-cell phenotype in the Nkx2.2\textsuperscript{TNmut/TNmut};Ins:Cre;Arx\textsuperscript{fl/fl} mice, we compared global gene expression analysis of the pancreata of wildtype, Nkx2.2\textsuperscript{TNmut/TNmut}, and Nkx2.2\textsuperscript{TNmut/TNmut};Ins:Cre;Arx\textsuperscript{fl/fl} (i.e., Arx rescued) neonatal mice. The data generated from this analysis identified many genes whose altered expression is independent of ectopic *Arx* expression, and which have diverse roles ranging from chromatin remodeling to metabolic functioning. This information provides insight into the molecular mechanisms by which Grg-mediated Nkx2.2 function normally regulates beta cell specification, maturation and function.

Introduction

Pancreogenesis is a tightly controlled process that requires the correct spatiotemporal regulation of many genes. In regenerative medicine, many investigators are drawing on the knowledge
gained from pancreas development studies that have begun to characterize the gene regulatory networks required for proper allocation of differentiated endocrine cell types, in an attempt to treat diabetes with stem cell-derived beta cell replacement therapy. Over the past twenty years, a great amount of research has begun to unravel the transcriptional networks required for pancreatic endocrine cell lineage determination, which has directly contributed to enhancements in stem cell-derived beta cells. However, functional ES cell-derived beta cells have yet to be produced. The most recent in vitro protocol yields only a small percentage of insulin positive cells, which are not glucose responsive. Additionally, many cells generated were poly-hormonal, and the majority were ghrelin positive (D’Amour et al., 2006). This is particularly interesting when considering the phenotypes of the Nkx2.2 null mice, which have a full replacement of beta cells with ghrelin-positive epsilon cells (Prado et al., 2004), and the Nkx2.2-SD mutant mice, which exhibit many poly-hormonal cells (J. Levine and L. Sussel, unpublished data). This suggests that transcription factors play a pivotal role in determining endocrine cell identity, and that the expression of these factors is not optimal in the ES-cell derived beta cells. Thus, fully understanding the consequences of a transcription factor’s action, i.e., stage and cell type expression, activator/repressor functions and locus-specific co-regulatory recruitment, will greatly enhance our knowledge of endocrine cell ontogeny at a molecular level and assist in enhancing ES-cell-based differentiation protocols.

To identify dysregulated genes in the Nkx2.2<sup>TNmut/TNmut</sup> mice, we generated a microarray data set from P0 pancreatic RNA. We have previously demonstrated that Arx ectopic expression in Nkx2.2<sup>TNmut/TNmut</sup> mice causes a beta-to-alpha-like-cell transdifferentiation, and beta-cell-specific loss of Arx (Nkx2.2<sup>TNmut/TNmut</sup>;Ins:Cre;Arx<sup>fl/ft</sup>) was sufficient to rescue this reprogramming event; however, Nkx2.2<sup>TNmut/TNmut</sup>;Ins:Cre;Arx<sup>ft/fl</sup> mice retained a beta-cell specification defect and
remained hyperglycemic. Our microarray analysis identified several intriguing genes involved in very disparate aspects of cell biology, including chromatin remodeling and metabolic control, that are altered in Nkx2.2\textsuperscript{TNmut/TNmut} mice and not rescued in the Nkx2.2\textsuperscript{TNmut/TNmut};Ins:Cre;Arx\textsuperscript{fl/fl} mice. The data presented here will assist in the identification of novel mediators of endocrine cell specification and metabolic function.

Results

The purpose of this analysis was to identify genes that are changed in Nkx2.2\textsuperscript{TNmut/TNmut} mice and not rescued in the Nkx2.2\textsuperscript{TNmut/TNmut};Ins:Cre;Arx\textsuperscript{fl/fl} mice, which could identify novel transcriptional pathways regulated by Nkx2.2. These genes would most likely be responsible for the pancreatic defects that persist in Nkx2.2\textsuperscript{TNmut/TNmut};Ins:Cre;Arx\textsuperscript{fl/fl} mice and are independent of Arx and the beta-to-alpha transdifferentiation phenomenon. To identify these genes, we isolated P0 pancreatic RNA from wild type, Nkx2.2\textsuperscript{TNmut/TNmut}, and Nkx2.2\textsuperscript{TNmut/TNmut};Ins:Cre;Arx\textsuperscript{fl/fl} mice (n = 3 per genotype) for microarray analysis. Using a minimum fold cutoff of 1.5, our microarray analysis identified 9,924 genes with altered expression in Nkx2.2\textsuperscript{TNmut/TNmut} mice compared to wild type. When Nkx2.2\textsuperscript{TNmut/TNmut} mice were compared to Nkx2.2\textsuperscript{TNmut/TNmut};Ins:Cre;Arx\textsuperscript{fl/fl} mice, there were 12,076 changed genes (Figure 1A). The majority of these genes were changed in the opposite direction, suggesting beta-cell-specific loss of Arx rescued many of the changes in gene expression seen in Nkx2.2\textsuperscript{TNmut/TNmut} mice (Figure 1B). Accordingly, only 278 genes remained upregulated in the Nkx2.2\textsuperscript{TNmut/TNmut};Ins:Cre;Arx\textsuperscript{fl/fl} mice when compared to wild type. Our studies identify Prominin-2, Irs4 and Rbbp4 as significantly upregulated in Nkx2.2\textsuperscript{TNmut/TNmut} mice and not rescued with beta-cell-specific deletion of Arx. We initially chose to focus on these three genes,
since they are potentially involved in processes that are important for formation and maturation of beta cells, including chromatin remodeling, cell-cell communication and metabolic functions.

Prominin-2

Prominin-2 is a cholesterol-binding pentaspan membrane glycoprotein that is structurally related to Prominin-1 (CD-133). Prominin-1 has garnered much interest, as it has been described to be an important cell surface marker capable of identifying and isolating stem cells from the kidney and the haematopoietic and central nervous systems (Bitan et al., 2005; Bussolati et al., 2005; Lee et al., 2005). Recent studies demonstrate that prominin-1 is expressed within the pancreatic acinar rosettes, but is excluded from a Ngn3+ progenitor population, suggesting that prominin-1 is most likely not a marker for endocrine progenitors (Hald et al., 2012) While prominin-1 is expressed in both epithelial and non-epithelial cells, prominin-2 is restricted to epithelial cells and is expressed in the kidney tubules, pancreas and abundantly throughout the digestive tract (Florek et al., 2007). Other than a cholesterol-binding, membrane protein, no clear functional role has been described for prominin-2.

Our microarray identified prominin-2 as having an 18-fold increased expression in wild-type mice compared to Nkx2.2TNmut/TNmut mice. This change in expression was unaltered in the Nkx2.2TNmut/TNmut;Ins:Cre;Arxfl/fl mice, demonstrating prominin-2 regulation is independent of Arx and may be a direct target of Nkx2.2. Prominin-2 expression was unchanged at e14.5 (Figure 4A), a time when the first changes are seen in hormone expression in the Nkx2.2TNmut/TNmut mice; however, by P0 prominin-2 was significantly elevated, in congruence with the microarray findings (Figure 4B). This suggests that prominin-2 is not involved in the immediate and direct effects of the Nkx2.2TNmut/TNmut early phenotype, since its expression is unchanged during a time
when abnormalities are occurring in the Nkx2.2\textsuperscript{TNmut/TNmut} mice, but rather could be eliciting later defects in beta cell function.

*In silico* analysis of the *prominin*-2 promoter identified a small area of conservation about 1.8 kb upstream of the TSS containing the lower affinity Nkx2.2 core motif GAGT (opposed to AAGT) (Figure 5A), which has been described previously (Hill et al., 2011). Furthermore, this site was predicted to be an Nkx2.2 binding site by PBM mapping analysis (personal communication, Jonathon Hill). To test whether Nkx2.2 directly binds this region of *prominin*-2, I performed ChIP and qRT-PCR with primers surrounding the putative Nkx2.2 binding motif, which revealed a strong enrichment of Nkx2.2. As a positive and negative control, Nkx2.2 ChIP is shown using primers specific to *Arx* and *Gapdh*, respectively (Figure 5B). This finding suggests that *prominin*-2 is a direct target of Nkx2.2, but since its expression is unaltered at e14.5 in the Nkx2.2\textsuperscript{TNmut/TNmut} mice, it may not be responsible for the early specification defects observed at that time.

*Insulin receptor substrate 4 (Irs4)*

IRS proteins are major docking proteins of both the IGF-1 and insulin receptors and undergo rapid tyrosine phosphorylation upon ligand binding (Kahn et al., 1993). Most of insulin’s actions are propagated through tyrosine phosphorylation of the IRS proteins 1-4. While functions for all IRS proteins have been described, most of the insulin-induced anabolic and nutritional homeostatic responses are mediated through IRS1 and 2 (White, 2003). Irs4 was discovered by anti-phosphotyrosine affinity chromatography following insulin treatment of Hek293 cells (Lavan et al., 1997). Its tissue distribution was detected by RT-PCR of its isolated message and revealed strong expression in skeletal muscle, brain, heart, liver and kidney, and very weak
expression was detected in the pancreas (Fantin et al., 1999). In accordance with Irs1 and 2 being the main mediators or insulin action, Irs4<sup>−/−</sup> mice have a very subtle phenotype, exhibited by a slightly smaller body size and lower glucose levels in the fed and fasted state. While glucose tolerance tests in the Irs4<sup>−/−</sup> mice were moderately impaired, suggesting problems with glucose clearance (Fantin et al., 2000), functional studies have suggested that Irs4 antagonizes insulin-stimulated Irs1 and 2 activity (Tsuruzoe et al., 2001), which is consistent with the reduced fed and fasted glucose levels in the Irs4<sup>−/−</sup> mice, but inconsistent with the impaired glucose tolerance tests.

Irs4 was upregulated 18 fold in the Nkx2.2<sup>TNmut/TNmut</sup> mice and remained elevated in the Nkx2.2<sup>TNmut/TNmut</sup>;Ins:Cre;Arx<sup>fl/fl</sup> mice, suggesting the upregulation of Irs4 is independent of Arx. Interestingly, Irs4 has previously been predicted by PBM mapping and validated by EMSA and ChIP as being a direct target of Nkx2.2 in a pancreatic beta cell line (Hill et al., 2011). These data suggest that the Nkx2.2/Grg3 repressor complex is required to repress Irs4 in pancreatic beta cells to allow for proper insulin receptor/Irs1-2 activity.

Retinoblastoma binding protein 4 (Rbbp4)

Retinoblastoma binding proteins are ubiquitously expressed nuclear proteins and were originally identified as the major interacting proteins with retinoblastoma (Qian et al., 1993). Based on the fact that Rbbps interact with the core histones H2A and H4 and are part of large complexes involved in modifying core histones and remodeling nucleosome structure (Verreault et al., 1998), Rbbps are believed to function as bridges that link histone modifying enzymes with their targets. Rbbp4, also known as RbAp4, is a core component of several histone modifying/nucleosome remodeling complexes such as NuRD, PRC2 and Sin3. Our microarray
identified $Rbbp4$ having a 33-fold increased expression in the Nkx2.2$^{TNmut/TNmut}$ mice compared to wild type, and this expression was only partly rescued in the Nkx2.2$^{TNmut/TNmut;Ins:Cre;Arx^{fl/fl}}$ mice. Additionally, the Champion ChIP Transcription Factor Search Portal, which is based on SABioscience’s proprietary database, DECODE, predicted an Nkx2.2 binding motif upstream of the $Rbbp4$ transcriptional start site. The Nkx2.2 binding site is also within a CpG island, an epigenetic signature that has been associated with Nkx2.2 binding (Papizan et al., 2011) (Figure 3A). Furthermore, the Nkx2.2 motif is a predicted binding site with a score of .32 when analyzed with PBM-mapping (personal communication, Jonathon Hill) (Hill et al., 2011).

To determine if Nkx2.2 directly binds to the $Rbbp4$ promoter, and if Grg3 is also recruited, I performed a ChIP analysis in beta TC6 cells with antibodies against Nkx2.2 or Grg3. I subsequently amplified the associated DNA with 3 sets of primers spanning a 700 bp region (Regions 1-3). The ChIP analysis suggests that Nkx2.2 and Grg3 are both present on the $Rbbp4$ promoter in a beta TC6 cell line (Figure 3B). However, I was unable to validate the microarray findings with qRT-PCR at e14.5 or P0 (Figure 2A,B), which could be attributed to differences in primer sets used in the array versus the set I generated. Accordingly, the Agilent microarray uses two probes to assess $Rbbp4$ expression: probe A_52_P424767 shows the increase in expression, while probe A_51_P307325 shows no change. While the ChIP analysis suggests that Nkx2.2 and Grg3 are binding the $Rbbp4$ promoter and perhaps repressing its transcription in beta cells, it will be useful to generate primers similar to the microarray probe used to identify the change in $Rbbp4$ expression to confirm the change with qRT-PCR.

Discussion
We have previously determined that the Nkx2.2 TN mutation de-represses Arx in beta cells and results in beta-to-alpha cell reprogramming. Beta cell-specific deletion of Arx in the Nkx2.2^TNmut/TNmut background prevented cellular reprogramming, but was insufficient to rescue the beta cell specification defect and beta cell function, suggesting other Nkx2.2/Grg-dependent genes are responsible for these functions. To identify genes important for beta cell specification and function, we compared gene expression of Nkx2.2^TNmut/TNmut mice with Arx-rescued mice. The microarray analysis identified many genes that are affected by the Nkx2.2 TN mutation, many of which are involved in pancreas development, but also genes important in metabolic pathways, including insulin and growth factor signaling. Notably, Ingenuity Pathway Analysis identified the top functional pathways that were altered in the Nkx2.2^TNmut/TNmut mice were involved in the hematological system, immune cell trafficking, cell-to-cell communication and inflammatory responses (Figure 1B). Given that the Nkx2.2^TNmut/TNmut mice have lost the Grg-mediated Nkx2.2 repressor capacity, and most of the effectors of these pathways are downregulated, the changes in these functional pathways may be viewed as secondary effects.

The primary transition of pancreas development, which occurs from e9.5 to e12.5, involves thickening of the endoderm, proliferation of pancreatic progenitors and branching morphogenesis. The onset of the secondary transition at e12.5-13 marks the beginning of a major wave of endocrine cell differentiation, primarily that of beta cells. The first changes in the Nkx2.2^TNmut/TNmut mice are seen at e13.5 with decreases in insulin and increases in ghrelin expression. Increases in Arx are not observed until e15.5, with subsequent changes in glucagon expression occurring at e18.5. Interestingly, deletion of Arx prevents the beta-to-alpha cell transdifferentiation, but the beta cell population is not fully restored. Future experiments will focus on identification of changes in the expression of genes occurring at or before e13.5 since
genes whose expression is changed at this time could be responsible for the primary effects causing the beta-to-epsilon cell fate shift. Unfortunately, it was difficult to obtain enough quality RNA at e13.5 for the microarray; instead we chose to assess gene expression at P0, reasoning that these candidate genes will still be changed at this time point.

The fact that Nkx2.2<sup>TNmut/TNmut</sup>;Ins:Cre;Arx<sup>fl/fl</sup> mice retain beta cells, although at a lower mass compared to wild type, and remain hyperglycemic, suggests that the beta cells that are formed are dysfunctional. Mice lacking the insulin receptor specifically in the beta cells exhibit impairments in insulin secretion and glucose tolerance but maintain normal beta cell development (Kulkarni et al., 1999). Additionally, compound beta cell-specific loss of the insulin and IGF1 receptor suggests that insulin plays a major roll in beta cell proliferation (Ueki et al., 2006). Irs4 has been shown to antagonize insulin and IGF1 signaling by impairing IRS1 and 2 activity (Tsuruzoe et al., 2001). Thus, the increase in Irs4 expression in the Nkx2.2<sup>TNmut/TNmut</sup> mice, which is not rescued in the Nkx2.2<sup>TNmut/TNmut</sup>;Ins:Cre;Arx<sup>fl/fl</sup> mice, may cause functional defects in the beta cell similar to those observed in beta cell-specific IR and/or IGF1 knockout mice.

Hald et al. have shown that prominin-1 is expressed in the acinar rosettes and restricted from the Ngn3<sup>+</sup> endocrine precursor population (Hald et al., 2012). However, an inspection of pancreatic prominin-2 protein expression has yet to be thoroughly investigated. If the Nkx2.2/Grg3 complex were directly repressing prominin-2 in the endocrine population, the TN mutation would presumably be responsible for the elevated prominin-2 expression in the Nkx2.2<sup>TNmut/TNmut</sup> mice. Being a pentaspan membrane protein, the de-repression of prominin-2 in the endocrine cells could then wreak havoc with the appropriate cell-cell interactions/communications that may be
required for proper endocrine cell formation and maintenance. Therefore, functional and immunohistochemical analyses of prominin-2 throughout pancreatic development is warranted.

The effects due to dysregulation of Irs4, Rbbp4 or Prominin-2 individually is unlikely to account for beta cell specification defects and dysfunction in the Nkx2.2\textsuperscript{TNmut/TNmut};Ins:Cre;Arx\textsuperscript{fl/fl} mice. But the combinatorial action resulting from their dysregulation could interfere with both beta cell specification and function. These data presented here highlight the many roles Nkx2.2 may play in endocrine cell ontogeny, and may lead to new therapeutic targets of diabetes and a better understanding of stem cell-directed differentiation protocols.

**Materials and Methods**

**RNA isolation, expression and microarray**

Total RNA was isolated from whole pancreatic tissue from e14.5 or P0 mouse pups (RNeasy, Quiagen), and cDNA was prepared using the SuperScript III kit (Invitrogen) and random hexamer primers. Quantitative PCR was performed using 200 ng cDNA and SYBR Green fluorescence with the following primers: \textit{Rbbp4} (FWD) – TCTGTTTGGGTAGCTG, and (REV) – AACTGAGTGGCTTGGTTTGG; \textit{Prominin2} (FWD) – ACAACTTTTCCATATCCCAAGG, and (REV) – ACAAGGACAAAGGACAGGAAAG; and \textit{CycloB} (FWD) – GCAAAGTTTCTAGAGGGCAGTTGG, and (REV) – CCCGGCTGTCTGTCTTG. The single color Agilent whole genome array analysis was performed by the University of Pennsylvania School of Medicine’s IDOM-DRC Functional Genomics Core using P0 whole pancreatic RNA.

**Chromatin Immunoprecipitation**
Expression vectors containing 3Xmyc-Nkx2.2 cDNA or Grg3 cDNA were transfected into betaTC6 cells using X-tremeGENE HP (Roche) according to the provider’s protocol. Chromatin was prepared using the ChIP-IT express kit (Active Motif). Immunoprecipitation was performed using the isolated chromatin diluted in ChIP dilution buffer (16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton-X 100) with 5 ug of mouse anti-myc (Kelloff et al.) or rabbit anti-TLE3 M201X (Santa Cruz Biotechnology) antibody while rotating overnight at 4° C. The following day antibody/chromatin complexes were pulled down using ChIP grade protein G magnetic beads (Cell Signaling). The beads were washed once with 1 mL each of the following wash buffers in order: TSE I (20 mM Tris-HCl (ph 8.1), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100); TSE II (20 mM Tris-HCl (pH 8.1), 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100); ChIP Buffer III (10 mM Tris-HCl (pH 8.1), 0.25 M LiCl, 1 mM EDTA, 1% NP-40, 1% deoxycholic acid); TE (10 mM Tris-HCl (pH 8.1), 1 mM EDTA). The antibody/chromatin complexes were eluted from the beads with the addition of 100 uL of freshly made elution buffer (1% SDS, 0.1 M NaHCO₃) and allowed to rotate at room temperature for 15 minutes. 4 uL 5 M NaCl was added to the eluate and incubated at 65° C overnight. The following day 4 uL 1 M Tris-HCl (pH 7.5), 4 uL 0.5 M EDTA and 1 uL 10 mg/mL proteinase K were added and allowed to incubate at 37° C for 1 hour. Samples were then purified using the QIAquick PCR purification kit (Qiagen). Quantitative analysis of ChIP products was performed using 2 uL of PCR purified ChIP DNA and SYBR Green fluorescence with the following primers: Rbp4 region 1 (FWD) – GACCAATGAGAAAGCGGGGT, and (REV) AAGTCTCCGGACTGTGGTGGG; Rbp4 region 2 (FWD) – CCACCCCCACCACACGTCCG, and (REV) – TCGCTACCCTGAGGGAGTTGTCAGC; Rbp4 region 3 (FWD) – GCTGACAACTCCCTCAGGGTAGCGA, and (REV) –
AGGGAGTCAGTTAAAAGACGCCACCTTGAAT; *Prominin2* (FWD) – 
AGTTCTCCTAGAGTTGGGGGTG, and (REV) – CTTCCCTCACATACCCAGAGCTGG; 
*Gapdh* (FWD) – CTCCACGACATACCTCAGCACC, and (REV) – TCAACGGCAGCTAGCAGGC.

**Figure 1: Global gene expression changes in P0 pancreatic RNA.** A) Gene expression changes in wild type vs. Nkx2.2^{TNmut/TNmut};Ins:Cre;Arx^{fl/fl} (ARX-WT, top row), wild type vs. Nkx2.2^{TNmut/TNmut} (TN-WT, middle row), and Nkx2.2^{TNmut/TNmut} vs. Nkx2.2^{TNmut/TNmut};Ins:Cre;Arx^{fl/fl} mice (ARX-TN, bottom row). B) Ingenuity Pathway Analysis of top functional pathways altered in Nkx2.2^{TNmut/TNmut} mice. Blue coloring indicates downregulated pathways in Nkx2.2^{TNmut/TNmut} mice compared to wildtype (top), and orange
indicates upregulated pathways in Nkx2.2\textsuperscript{TNmut/TNmut};Ins:Cre;Arx\textsuperscript{fl/fl} compared to Nkx2.2\textsuperscript{TNmut/TNmut} mice (Boden et al.).

Figure 2: qRT-PCR confirmation of gene expression changes in Nkx2.2\textsuperscript{TNmut/TNmut} mice. A) Prominin 2 pancreatic mRNA levels at e14.5 and B) P0 (n = 5). (*) $p < 0.05$. 


Figure 3: Nkx2.2 binds the *prominin-2* promoter in beta-TC6 cells. A) *In silico* analysis of the *prominin* 2 gene. The Nkx2.2 core motif is highlighted in yellow (inset). B) ChIP analysis demonstrating Nkx2.2 is highly enriched on the *prominin* 2 promoter in beta TC6 cells. Nkx2.2 enrichment on *Arx* is shown as a positive control and *gapdh* as a negative control. The primers used for qRT-PCR analysis of ChIP for *prominin* 2 are shown as a PCR amplicon above the conservation map in (A). (n = 3) (**) $P < 0.01$, (***) $P < 0.001$. 
Figure 4: Nkx2.2 and Grg3 bind the *rbbp4* promoter in beta-TC6 cells. A) *In silico* analysis of the *rbbp4* promoter. B) ChIP analysis demonstrating Nkx2.2 and Grg3 are highly enriched on the *rbbp4* promoter in beta TC6 cells. The primers used for qRT-PCR analysis of ChIP for region 1 are shown as a PCR amplicon above the conservation map in (A). Regions 2-3 are successively 5-prime in 200 bp intervals. (*P < 0.05, **P < 0.01.*
Figure 5: qRT-PCR confirmation of gene expression changes in Nkx2.2<sup>TNmut/TNmut</sup> mice. A) Rbbp4 pancreatic mRNA levels at e14.5 and B) P0 (n = 5).
CHAPTER 4

SERINE PHOSPHORYLATION REGULATES NKX2.2 FUNCTION

James Papizan generated all data presented in this chapter

Abstract

Nkx2.2 is expressed in multiple cell types and has the ability to activate and repress gene activity (Anderson et al., 2009; Papizan et al., 2011; Raum et al., 2006). In order for Nkx2.2 to properly regulate its target genes in a spatiotemporal manner, a high degree of regulation must be in place for correct cofactor interaction and DNA binding. Many factors employ post-translational modifications (PTMs) as a mechanism to regulate their function. In such cases, the PTM acts as a binary switch that dictates cofactor binding. Here, we investigate the possibility of PTMs as a mechanism by which Nkx2.2 protein can acquire the ability to differentially interact with cofactors and regulate gene expression in a spatial context. Nkx2.2 has highly conserved serine and lysine residues, which based on bioinformatic PTM prediction algorithms, are predicted to be phosphorylated or acetylated, respectively. Here, I demonstrate that mutations that alter the phosphorylation status of Nkx2.2 serine residues cause changes in the recruitment of cofactors to Nkx2.2 regulatory elements, and I show these changes are associated with alterations in the epigenetic signature. These findings highlight the importance of PTMs in regulating Nkx2.2 in vitro activities and warrant further investigations into how they might affect in vivo Nkx2.2 function, which enzymes are responsible for the modifications, and how these modifications are temporally regulated during pancreas development.

Introduction
Nkx2.2 is a homeodomain-containing protein required for correct pancreatic islet cell
development (Sussel et al., 1998) and neuronal differentiation (Briscoe et al., 1999). During
pancreatic development, Nkx2.2 is expressed during the primary transition at e9.5 and becomes
restricted to alpha, beta and PP cells, and excluded from delta cells. Mice carrying a null
mutation for Nkx2.2 exhibit a complete absence of beta cells and a drastic reduction in alpha
cells (Sussel et al., 1998). In place of these cell types, the Nkx2.2⁻/⁻ islet is populated with
ghrelin⁺ epsilon cells (Prado et al., 2004). These data point to a role for Nkx2.2 in specifying and
maintaining the alpha and beta cell lineages while suppressing the epsilon cell lineage.
Subsequent studies demonstrated that expression of an Nkx2.2-engrailed fusion repressor
construct from the Pdx1 regulatory elements was sufficient to partially rescue the Nkx2.2 null
phenotype, suggesting that Nkx2.2 is functioning as a repressor to suppress the epsilon cell fate,
while specifying the alpha cell population (Doyle et al., 2007). Interestingly, while some beta
cells were rescued with Nkx2.2 repressor activity, they lacked mature markers of mature, glucose
sensing beta cells, i.e., MafA and Glut2, which may reflect the requirement of Nkx2.2
transcriptional activator functions for the beta cell maturation process. This hypothesis is
consistent with previous data that demonstrated Nkx2.2 functions as a transcriptional activator of
important beta cell genes, such as MafA and NeuroD (Anderson et al., 2009; Raum et al., 2006).
In addition, a recent study using algorithmic predictions identified 111 novel Nkx2.2 binding
sites in the proximal promoters of 30 genes differentially expressed in wild type and Nkx2.2 null
pancreas (Hill et al., 2011). These studies highlight the multitude of functions Nkx2.2 must
execute for proper islet cell development and function, but do not address how Nkx2.2 can
simultaneously repress and activate different genes in the same cellular context, or how it can
activate and repress the same gene in different cellular contexts.
PTM is mechanism that can provide a layer of regulation in a tissue-, cell- or gene-specific context. Several beta cell factors are known to be post-translationally modified, including NeuroD phosphorylation, which has been shown to be required for transcriptional activation of the *insulin* gene upon glucose stimulation (Khoo et al., 2003a). NeuroD phosphorylation has also been demonstrated to induce its translocation into the nucleus in response to glucose stimulation. This subcellular localization is MEK-ERK dependent and is lost when NeuroD serine-274 (S274) is mutated to an alanine (Petersen et al., 2002). NeuroD phosphorylation can also inhibit its activity in neurons. Studies in *Xenopus* reveal that formation of N-tubulin positive neurons is inhibited when GSK3β, a kinase that is presumably responsible for phosphorylating NeuroD S274, is overexpressed, and that co-injection of GSK3β and NeuroD mRNAs inhibits ectopic neurogenesis, which is induced by NeuroD mRNA alone (Marcus et al., 1998). Collectively, these studies suggest that phosphorylation-induced NeuroD activity is cell type specific.

PTMs of Pdx1, another essential pancreatic transcription factor with multiple functions, include phosphorylation, glycosylation and sumoylation (Boucher et al., 2006; Elrick and Docherty, 2001; Gao et al., 2003; Kishi et al., 2003). Like NeuroD, the majority of Pdx1 phosphorylation is glucose-dependent and regulates its interactions between histone deacetylases and histone acetyltransferases (Mosley et al., 2004; Mosley and Ozcan, 2004). Recently, Frogne et al. provided thorough evidence that the majority of Pdx1 phosphorylation is occurring at serine 61 (S61) and is not glucose dependent, contrary to other sites of Pdx1 PTMs (Frogne et al., 2012). Although a function for S61 phosphorylation was not determined, these studies suggest that Pdx1 is differentially phosphorylated in response to metabolic changes and other unknown stimuli, which alters Pdx1 regulatory activity by way of cofactor exchange (Mosley et al., 2004), subcellular localization (Elrick and Docherty, 2001), and DNA binding (Wu et al., 1999).
Here, we sought to determine whether PTMs regulate Nkx2.2 activity in a context dependent manner. We demonstrate that Nkx2.2 contains several highly conserved serine and lysine residues predicted to be phosphorylated and acetylated, respectively. Point mutations to S11 alter the ability of Nkx2.2 to properly recruit cofactors to promoter regions in beta and alpha cell lines. These changes are also associated with alterations in the local epigenetic landscape at these promoters. Further characterization of phosphorylation-induced Nkx2.2 regulatory activity, and the responsible kinase(s) and phosphatase(s), will enhance our understanding of the complexities of cell specific gene regulatory events and cell differentiation. These findings could also lead to the identification of small molecules that could help promote the differentiation of hES cells toward the beta cell lineage.

Results

Nkx2.2 is expressed in multiple cell types and has the ability to activate and repress gene activity in a context-dependent manner (Anderson et al., 2009; Papizan et al., 2011; Raum et al., 2006; Sussel et al., 1998). The observation that ventral nervous defective (Vnd), the Nkx2.2 Drosophila homolog is phosphorylated (Zhang et al., 2008), suggests that PTMs might provide an additional component of regulation required for context dependent Nkx2.2 activity.

To determine which amino acid residues are sites of modification, the murine Nkx2.2 peptide sequence was subjected to a sequence-based PTM prediction analysis (Blom et al., 1999). Multiple serine, threonine and tyrosine residues were predicted to be phosphorylated, but only four serine residues reached a score greater than 0.99 (1.0 being the greatest), with S11 having the greatest score, 0.998 (Figure 1A). No threonine or tyrosine residues reached a score greater than 0.9, and these residues were not pursued in further analyses. Interestingly, serine-11 resides
within the Grg-interacting TN domain. This residue is conserved from *Homo sapiens* to *Xenopus* (Figure 1B), and a serine is present in all Nkx2 family members at either the 11, 12 or 13 position, which is surrounded by a similar motif, in mouse and human (Figure 1C). The fact that S11 is predicted to be phosphorylated and retains such a high degree of conservation, points to a functional role for this residue. Additional bioinformatic analysis predicted that PKC is the responsible kinase for S11 phosphorylation (Figure 2A) (Blom et al., 2004). To examine whether S11 is phosphorylated, we used site directed mutagenesis to convert S11 to an alanine (Nkx2.2$^{S11A}$), mimicking the dephosphorylated state, and introduced the mutant protein into beta TC6 cells. Nkx2.2$^{S11A}$ was immunoprecipitated and subjected to Western analysis using a PKC-phospho-specific primary antibody. In comparison to wild type Nkx2.2 protein, Nkx2.2$^{S11A}$ exhibited a dramatic decrease in the amount of phosphorylation, even in the presence of phosphatase inhibitors (Figure 2B).

The PKC family of serine/threonine kinases is composed of ten isoforms and can be divided into three groups: the conventional PKCs (cPKC) include PKCα, β and γ; the novel PKCs (nPKC) include PKCδ, ε, θ and η; and the atypical PKCs (aPKC) include PKCζ, τ and λ (Mellor and Parker, 1998). Analysis of isoform expression in alpha TC1 and beta TC6 cell lines, indicated the mRNAs of PKCε and PKCζ, which are important in beta cell biology (Furukawa et al., 1999; Hennige et al., 2002; Schmitz-Peiffer et al., 2007), were the most abundant family members. These data suggest that Nkx2.2 is phosphorylated at S11, possibly by PKCε or PKCζ.

We next wanted to determine the function of Nkx2.2 phosphorylation. Since the S11 residue is located within the TN (Grg-interaction) domain, it seemed plausible that phosphorylation at this site would affect binding with Grg proteins. To address this, we generated Nkx2.2 proteins that represented constitutive S11 phosphorylation (Nkx2.2$^{S11D}$) or dephosphorylation (Nkx2.2$^{S11A}$).
Western blot analysis demonstrated that transfected myc-tagged Nkx2.2 cDNA constructs were expressed, although at lower levels compared to endogenous Nkx2.2 (Figure 3). Due to technical difficulties, protein interaction could not be assessed, and phosphorylation-dependent protein interactions are currently being pursued. However, we were able to determine whether the phosphorylation status of S11 affected cofactor recruitment to gene promoters regulated by Nkx2.2 by assessing the appropriate recruitment of Grg3 and HDAC1 to the Arx promoter in alpha TC1 versus beta TC6 cell lines. We had previously shown that Nkx2.2 forms a repressor complex with Grg3 and HDAC1 on the Arx promoter preferentially in beta cells (Papizan et al., 2011). In beta cells Nkx2.2S11A did not affect Grg3 recruitment; however, Nkx2.2S11D resulted in a significant decrease in Grg3 enrichment (Figure 4A). Grg proteins have the ability to bind and recruit HDACs (Chen and Courey, 2000), but surprisingly, the disruption of Grg3 recruitment was not correlated with a decrease in HDAC1 recruitment in beta cells, suggesting that in this context, HDAC1 may be recruited independently of Grg3. Examination of the Arx promoter in alpha cells, where HDAC1 is normally absent, revealed that Nkx2.2S11A caused a significant increase in HDAC1 recruitment, which was not associated with an increase in Grg3 (Figure 4B). The fact that Nkx2.2S11A does not result in an enrichment of Grg3 in alpha cells could be a reflection of endogenous Grg3 expression levels. Supporting this, it was recently demonstrated that only 53% of glucagon-expressing alpha cells expressed Grg3 in e15.5 embryos, and that percentage decreased to 22% in neonatal mice (Metzger et al., 2012), indicating that Nkx2.2 can recruit HDAC1 in a Grg3-independent mechanism.

Since HDACs and Grgs are known to be involved in chromatin remodeling complexes (Hayakawa and Nakayama, 2011; Patel et al., 2012), we next wanted to determine if changes in their recruitment were associated with the underlying chromatin signature by examining activator and
repressor histone marks. The beta cell-specific Nkx2.2S11D-induced decrease in Grg3 recruitment was associated with a decrease in the repressive mark H3K27me3, and the alpha cell-specific Nkx2.2S11A-induced increase in HDAC1 was associated with an increase in H3K27me3 (Figure 4C). Additionally, the increases in HDAC1 and H3K27me3 in alpha cells correlated with a modest decrease in Arx expression (Figure 4D). These results suggest that regardless of cell type, Nkx2.2S11D is associated with the loss of repressive marks, while Nkx2.2S11A is associated with the gain of repressive marks on the Arx promoter. Thus, Nkx2.2 S11 phosphorylation may serve to activate Arx, while S11 dephosphorylation might facilitate its repression.

We next wished to determine whether Nkx2.2 S11 phosphorylation is functioning in a similar manner with regards to a beta-cell transcriptional target. Deletion of NeuroD demonstrated its requirement for maintenance of functional beta cells; NeuroD−/− mice have reduced insulin production and a decrease in beta cell mass due to apoptosis (Naya et al., 1997). While NeuroD is first expressed in the early (e9.5) alpha cell population, by birth its expression becomes predominantly restricted to the insulin-expressing beta cells. (Itkin-Ansari et al., 2005; Naya et al., 1997). Nkx2.2 has previously been shown to activate the expression of NeuroD and to be required for maintaining high levels of NeuroD in mouse and zebrafish beta cells (Anderson et al., 2009), while repressing its expression in alpha cells (K.R. Anderson, unpublished data). Therefore, we hypothesized that Nkx2.2 may be recruiting co-repressors to the NeuroD promoter in alpha cells and co-activators in beta cells. Interestingly however, neither Grg3 nor HDAC1 were recruited to the NeuroD promoter in either cell line, indicating that Nkx2.2 may be regulating NeuroD through an alternative mechanism (Figure 5A,B). Conversely, we were able to detect a strong enrichment in the activator mark, H3K4me3 to the NeuroD promoter in beta
cells. H3K4me3 was also present on the promoter in alpha cells, although at a significantly lower amount (Figure 5C). This differential recruitment appears to depend on Nkx2.2 phosphorylation state; the expression of \( \text{Nkx2.2}^{S11A} \) in alpha cells caused a significant increase in the amount of H3K4me3 at the NeuroD promoter, while expression of \( \text{Nkx2.2}^{S11D} \) in beta cells reduced H3K4me3 levels (Figure 5C). The repressive H3K27me3 mark was undetectable in both alpha and beta cells, suggesting that the promoter is actively induced rather than derepressed (Figure 5D).

To determine if the changes in histone marks were sufficient to alter gene expression, we tested whether changes in H3K4me3 were associated with NeuroD promoter activity. Co-transfection of \( \text{Nkx2.2}^{S11A} \) and a NeuroD-luciferase construct resulted in an increase in promoter activity, and conversely, co-transfection of \( \text{Nkx2.2}^{S11D} \) and NeuroD-luciferase resulted in a loss of activity (Figure 6A). However, no changes in endogenous NeuroD expression levels were detected in either alpha or beta cells with either mutant (Figure 6B, C). This may be attributed to the additional requirements of other genomic factors to regulate NeuroD expression. Since Nkx2.2 and NeuroD have both been ascribed roles for regulating insulin expression, we additionally examined whether the Nkx2.2 S11 mutants altered its expression. While insulin expression remained undetectable in alpha cells, neither mutant altered its expression in beta cells (Figure 6D). These results suggest that \( \text{Nkx2.2}^{S11D} \) is associated with the loss of active marks, while \( \text{Nkx2.2}^{S11A} \) is associated with the gain of active marks on the NeuroD promoter. Collectively, since we observe that \( \text{Nkx2.2}^{S11D} \) is associated with activation, and \( \text{Nkx2.2}^{S11A} \) is associated with repression on the Arx promoter, these findings imply that two different regulatory mechanisms are in place on each promoter (Figure 7).

**Discussion**
PTMs can act as a binary switch that dictates cofactor binding to transcriptional complexes. For example, Nf-κB phosphorylation allows binding to CBP/p300, leading to Nf-κB target gene activation (Zhong et al., 1998), and Pax2 phosphorylation obstructs its interaction with Grg4, allowing Pax2 target gene activation (Cai et al., 2003). Here, we demonstrate that Nkx2.2 is phosphorylated at S11, and this phosphorylation potentially acts as a context-dependent regulator of Nkx2.2 function. We demonstrate that Nkx2.2 S11 phosphorylation may serve to activate Arx and repress NeuroD. Thus, since these genes are exclusively expressed in the mature islet, Nkx2.2 S11 phosphorylation must be cell type-specific, e.g., in the beta cell, Nkx2.2 is presumably dephosphorylated where it can recruit active marks to the NeuroD promoter, while recruiting a repressive complex (Grg3, HDAC1 and H3K27me3) to the Arx promoter. Conversely, in the alpha cell, Nkx2.2 would be phosphorylated leading to a decrease in active marks on the NeuroD promoter and the dismissal of the repressive complex on the Arx promoter.

In our studies, we show that Nkx2.2 S11 is predicted to be phosphorylated by PKC; however, we have not determined which isoform is responsible. We demonstrate that PKCε is the most highly expressed isoform in the beta and alpha cell lines used in these studies, observations that are interesting since the loss of PKCε has recently been reported to improve whole body glucose tolerance, and normalize glucose tolerance in high fat-fed mice, which was due to an enhancement of insulin availability (Schmitz-Peiffer et al., 2007). Presumably, the loss of PKCδ would result in the dephosphorylated form of Nkx2.2, leading to NeuroD activation and Arx repression. Given that NeuroD is required for maintenance of beta cells and insulin production, dephosphorylated Nkx2.2 – induced activation of NeuroD could be a component of the mechanism by which PCKε/− mice have improvements in GSIS and whole body glucose tolerance. However, while we were able to see an induction of the NeuroD-luciferase construct
with the addition of \( \text{Nkx2.2}^{S11A} \), we did not observe any expression changes in endogenous \( \text{NeuroD} \). The requirement of other genomic factors and/or the competing endogenous Nkx2.2 in the cell lines could possibly explain why gene expression changes were not altered. In support of this, we show that endogenous levels were much greater than transfected mutants (Figure 3). Ideally, these experiments would best be performed after efficient knock down of endogenous Nkx2.2.

We have previously performed CoIPs with transfected Nkx2.2 and Grg3 in Hek293 cells, which may be more amenable to either transfection or Nkx2.2 expression; however, we wished to determine Nkx2.2 function in relevant cell lines that contain all of the appropriate interacting partners and cell components. We would like to have determined if the PTMs affect Nkx2.2 protein interactions; however, we were unable to efficiently overexpress enough Nkx2.2 protein in alpha or beta cells for CoIP experiments, which also presented difficulties in generating enough Nkx2.2 protein from alpha and beta cells for mass spectrometry analysis of PTMs. The effects of Nkx2.2 phosphorylation on protein interaction are currently being pursued using \textit{in vitro} translated Nkx2.2.

This study demonstrates that the \( \text{NeuroD} \) promoter contains the active H3K4me3 mark in both alpha and beta cells, which appears to be regulated by Nkx2.2 phosphorylation; S11 phosphorylation reduces H3K4me3 levels. How Nkx2.2 regulates H3K4me3 is unknown. The observation that the mixed lineage leukemia protein-1 (MLL1) complex contains the WD40-repeat protein, WDR5, an effector of H3K4 methylation (Wysocka et al., 2005), suggests that the MLL1 complex could be a potential candidate. Interestingly, it is the Grg3 WD-repeat domain that interacts with eh1 motif-containing proteins, which includes the Nkx2.2 TN domain. A scenario could exist in the beta cell where the dephosphorylated Nkx2.2 TN domain could
interact with WDR5/MLL1 on the NeuroD promoter and with Grg3 on the Arx promoter. In this case, other context-specific DNA binding factors could influence the choice made by dephosphorylated Nkx2.2 to interact with the MLL1 complex or the Grg3 complex. Alternatively, other Nkx2.2 PTMs could dictate interacting partners.

That Grg3 nor HDAC1 are present on the NeuroD promoter in alpha cells, suggests that Nkx2.2 may be repressing its transcription by an unknown alternative mechanism. Other factors could be recruited, such as DNA methyltransferases, resulting in DNA methylation and silencing of the NeuroD promoter in alpha cells. In support of this, we have previously shown that Nkx2.2 is involved in the differential methylation of the Arx promoter, where the promoter is methylated in beta cells and hypomethylated in alpha cells (Papizan et al., 2011). A similar phenomenon could exist on the NeuroD promoter, in that phosphorylated Nkx2.2 could have the ability to directly or indirectly recruit DNA methyltransferases specifically to the NeuroD promoter in alpha cells, while on the Arx promoter, the absence or presence of promoter specific DNA binding factors would preclude the recruitment of DNA methyltransferases. Studies of cell type-specific NeuroD promoter methylation are ongoing.

These studies also highlight the importance of understanding the temporal regulation of Nkx2.2 phosphorylation, which may help to unveil the mechanisms by which the Ngn3 positive endocrine progenitor makes the decision to become an alpha cell or a beta cell. Some studies have suggested that Ngn3 positive cells have been pre-determined to become a particular islet cell type (Desgraz and Herrera, 2009). In this scenario, it is likely that Ngn3 positive cells, in which Nkx2.2 is phosphorylated, may be destined to become an alpha cell. Understanding these cell fate decisions is of great importance when trying to coax hES cells towards a particular lineage, such as pancreatic beta cells. In this context, deciphering Nkx2.2 function is particularly
intriguing, in that the endocrine cell phenotype resulting from Nkx2.2 mutations looks strikingly similar to the hormone positive cells generated from hES cells. The majority of hES cell–derived endocrine cells are ghrelin positive, and many others are polyhormonal (D'Amour et al., 2006). Similarly, loss of Nkx2.2 leads to the replacement of beta cells with ghrelin positive cells (Prado et al., 2004), and mutation to the Nkx2.2-SD domain results in the generation of many polyhormonal cells (J. Levine and L. Sussel, unpublished). It is also important to understand whether it is absolutely necessary to recapitulate every in vivo event to generate hES cell-derived beta cells. A concept proposed by Rieck et al, suggests that instead of manually inducing multiple steps of a differentiation protocol, it may be feasible that the addition of a factor or set of local signals may be sufficient to initiate a gene regulatory network that could influence the cell differentiation toward a particular lineage (Rieck et al., 2012). Accordingly, it has been recently demonstrated that the small molecule (−)-indolactam V (ILV) enhanced the directed differentiation of hES cells to Pdx1 positive cells (Chen et al., 2009b). It was further demonstrated that ILV functioned at the stage of differentiation from gut tube endoderm to Pdx1 positive pancreatic progenitors by acting as a PKC agonist, although the PKC isoform was undetermined.

These findings demonstrate the complexities of context-dependent gene regulation and highlight how a PTM may confer a level of regulation upon a transcription factor, so that differential gene regulation can be properly executed in the same cellular context. Additionally, further explorations into the function of Nkx2.2 phosphorylation may reveal shortcuts in hES cell directed differentiation protocols that could be taken by addition of PKC modulators that would direct cells toward the beta cell lineage.

Materials and methods
**RNA isolation and expression**

Total RNA was isolated from beta TC6 or alpha TC6 cells (RNasey, Quiagen), and cDNA was prepared using the SuperScript III kit (Invitrogen) and random hexamer primers. Quantitative PCR was performed using 200 ng cDNA, PCR master mix (Eurogentec) and the following TaqMan probes (ABI Assays on Demand): NeuroD - Mm01280117_m1; Ins2 - Mm00731595_gH; and Arx - Mm00545903_m1. CyclophilinB probe - FAM-TGGTACGGAAGGTGGAG, (FWD) - GCAAAAGTTCTAGAGGGCATGGA, (REV) - CCCGGCTGTCTGTCTTTGGT. Primers for PKC isoforms are from (Berdiev et al., 2002)

**Western blot**

250 ng of the 3Xmyc-Nkx2.2 cDNA expression vector was transfected into beta TC6 cells using X-tremeGENE HP (Roche) according to the provider’s protocol. The nuclear extract of transfected and non-transfected beta cells was isolated using the nuclear extract kit (Active Motif). 6, 10, 20 or 40 ug of nuclear extract protein was run on a NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen). Proteins were transferred onto a nitrocellulose membrane (GE Healthcare) and probed using mouse anti-Nkx2.2 (DSHB) or anti-myc (Kelloff et al.).

**Generation of mutant constructs**

Point mutations were made to 3xmyc-tagged Nkx2.2 cDNA using the QuickChange II Site Directed Mutagenesis kit (Agilent Technologies) with the following primers S-11-A: (FWD) – CAACACAAAGACGCGTGGTGCTGTCAAGGACATCTTTGGAC, (REV) – GTCCAAGATGTCCTTGACAGCAAAACCCGCTTTTGTGTGTTG [oJP 29,30]; S-11-D: (FWD) – CAACACAAAGACGCGTGGTGATGTCGAAGGGACATCTTTGGAC, (REV) – GTCCAAGATGTCCTTGACATCAAAACCCGCTTTTGTGTGTTG [oJP 33,34].
**Chromatin Immunoprecipitation**

Expression vectors containing the according mutant versions of 3Xmyc-Nkx2.2 cDNA, Grg3 cDNA and FLAG-HDAC1 cDNA were transfected into beta TC6 or alpha TC6 cells using X-tremeGENE HP (Roche) according to the provider’s protocol. Chromatin was prepared using the ChIP-IT express kit (Active Motif), and immunoprecipitation was performed as in Chapter 3. 5 ug of rabbit anti-TLE3 M201X (Santa Cruz Biotechnology), mouse anti-FLAG (Kelloff et al.), mouse anti-H3K27me3, or mouse anti-H3K4me3 (Abcam) antibodies were used to immunoprecipitate chromatin. Quantitative analysis of ChIP products was performed using 2 uL of PCR-purified ChIP DNA and SYBR Green fluorescence with the following primers: Gapdh (FWD) – CTCCACGACATACTCAGCACC, and (REV) – TCAACGGCACAGTCAAGGC; NeuroD (FWD) – AAAGGGTTAATCTCTCCTGCGGT, and (REV) – CATGCGCCATATGGTCTTCCCGGT; and Arx (FWD) – TCCTCCACCATTTGAGGGTA, and (REV) - GCAACCTTGAGGGGTACAGA.
Figure 1: Nkx2.2 S11 is predicted to be phosphorylated and is well conserved. A) NetPhos 2.0 Server predictions of mouse Nkx2.2 serine phosphorylation. Where the highest achievable score = 1.00, S11 scores 0.998. B) S11 is conserved in Nkx2.2 among species. C) A serine is present at the 11, 12 or 13 position in mouse and human Nkx2 family members.
Figure 2: Nkx2.2 S11 is phosphorylated by PKC. A) Predictions made by NetPhosK 1.0 Server of the kinase that may be governing Nkx2.2 phosphorylation. S11 is predicted to be phosphorylated by PKC. B) Co-immunoprecipitation of 3Xmyc-Nkx2.2 followed by western analysis using an anti-PKC-specific phosphoserine antibody, which recognizes phosphorylated serines within a PKC motif. In the presence of phosphatase inhibitors, wild-type Nkx2.2 retains more phosphorylation compared to Nkx2.2 without inhibitors. When S11 is mutated to an alanine, almost all phosphorylation is ablated, even in the presence of phosphatase inhibitors. C) In beta and alpha TC6 cells, PKCε and PKCζ are most highly expressed.
Figure 3: Transfected and endogenous levels of Nkx2.2 in beta-TC6 cells. The endogenous levels of Nkx2.2 in beta TC6 cells are much higher than the transfected 3xmyc-Nkx2.2 levels.
Figure 4: Nkx2.2 S11 mutations affect recruitment of cofactors to the Arx promoter. A) Nkx2.2$^{S11D}$ causes a decrease in Grg3 recruitment to the Arx promoter in beta TC6 cells. B) Nkx2.2$^{S11A}$ causes an increase in HDAC1 recruitment to the Arx promoter in alpha TC6 cells. C) On the Arx promoter Nkx2.2$^{S11A}$ causes increases in H3K27me3 in alpha cells, and Nkx2.2$^{S11D}$ causes decreases in H3K27me3 in beta cells. D) In alpha TC6 cells, where Arx is expressed, Nkx2.2$^{S11A}$ causes a modest decrease in Arx expression.
Figure 5: Nkx2.2 S11 mutations affect local epigenetic marks on the NeuroD promoter. Neither Grg3 (A) nor HDAC1 (B) is present on the NeuroD promoter in alpha or beta TC6 cells. C) The active H3K4me3 mark is present on the NeuroD promoter in alpha and beta cells, although at significantly lower levels in alpha cells. Nkx2.2\textsuperscript{S11A} causes an increase in H3K4me3 in alpha cells to levels indistinguishable from beta cells. Conversely, Nkx2.2\textsuperscript{S11D} causes a decrease in H3K4me3 in beta cells to levels comparable to alpha cells. D) The repressive H3K27me3 mark is not present on the NeuroD promoter in alpha or beta TC6 cells.
Figure 6: Gene expression changes in response to Nkx2.2 S11 mutations. A) Nkx2.2$^{S11A}$ induces the activation of the full-length NeuroD promoter fused to luciferase in alpha TC6 cells. Endogenous expression of NeuroD was not altered in beta (B) or alpha TC6 cells (C). D) Expression of insulin was unchanged in beta TC6 cells and was undetectable in alpha TC6 cells (not shown).
Figure 7: Proposed model of cell specific regulation by Nkx2.2 S11 phosphorylation. Since Nkx2.2$^{S11A}$ is associated with the activation of NeuroD and the repression of Arx, we hypothesize that Nkx2.2 would be dephosphorylated in the beta cell to properly regulate these two genes. Conversely, since Nkx2.2$^{S11D}$ is associated with the repression of NeuroD and the activation of Arx, Nkx2.2 would be phosphorylated in the alpha cell. However, we cannot rule out, for example, that the phosphorylation of Nkx2.2 might be required for the activation of other beta-cell targets, such as MafA, which would require Nkx2.2 to be differentially phosphorylated in the same beta cell. Additional studies are needed to determine these scenarios and to elucidate other promoter-specific factors that may help confer specificity.
CONCLUSIONS AND PERSPECTIVES

These studies have shown that the domain-dependent regulatory activities of the homeobox protein Nkx2.2 function in a spatial and temporal manner to control beta cell development and function. Mutation of the Nkx2.2 TN domain disrupts the interaction with Groucho-related gene (Grg) co-repressors to demonstrate that Grg-dependent Nkx2.2 regulation is required for correct beta cell specification and maintenance of differentiated beta cells. These studies have also shown that Nkx2.2 is associated with H3K27me3 modification at multiple loci, suggesting a role for Nkx2.2 in guiding chromatin modulators to sites of remodeling necessary for development. That Nkx2.2 is phosphorylated may reveal a mechanism by which Nkx2.2 gains cell- and promoter-specific functions. The implications of these findings are discussed below.

Grg-mediated Nkx2.2 repression

Grg2 and 3 are coexpressed with Nkx2.2 in the pancreas and can interact with Nkx2.2 through the Grg-interaction TN domain. In these studies the role of Grg-mediated Nkx2.2 regulatory activities was analyzed by creating mice carrying a mutation in the Nkx2.2 TN domain. In contrast to the Nkx2.2−/− mice, which lack all beta cells and are hyperglycemic at birth, the Nkx2.2TNmut/TNmut phenotype manifests later and is less severe. Several reasons could account for this. First, the mutation may not have completely disrupted the Nkx2.2/Grg interaction, and residual activity is sufficient for these early functions. Secondly, it is possible that other Nkx2.2 protein domains can compensate for the loss of TN activity, allowing the development of a small number of beta cells. Accordingly, the NK2-SD domain has been reported to contain repressor activity (Watada et al., 2000). Studies determining the role of the Nkx2.2 SD domain are ongoing. Lastly, Nkx2.2 function prior to the secondary transition may not require Grg protein
interactions. In support of this, a recent study documenting Grg3 protein expression in foregut endoderm tissues demonstrated that Grg3 is not detected until e12.5 (Metzger et al., 2012). However, the phenotype of Nkx2.2−/− mice, and the rescue of the Nkx2.2−/− mice using an Nkx2.2 dominant repressor, indicated that Nkx2.2 repressor activity is functioning prior to e12.5 (Doyle et al., 2007). This suggests that Nkx2.2 may be interacting with other repressive complexes during the primary transition. Since a connection between Nkx2.2 and HDAC1 has been determined (Chapter 2), the HDAC-containing complexes Sin3 or NuRD are possible candidates for Nkx2.2-mediated repression during the primary transition. Additionally, genomic regions where Nkx2.2 is known to bind and repress are associated with H3K27me3 marks (Chapter 4); therefore, PRC2 is another possible interacting candidate. We can also speculate that Nkx2.2 uses Grg-independent repressive mechanisms to specify the alpha cell population. This is supported by the observations that the Nkx2.2 dominant repressor is sufficient to fully rescue the alpha cell population that is lacking in the Nkx2.2−/− mice, yet the Nkx2.2TNmut/TNmut mice have no decreases in glucagon, Irx2, Arx, Brn4 or alpha cell numbers at any developmental time point. Thus, Grg-independent Nkx2.2 repressor activity is needed to specify alpha cells. These studies highlight the need to identify additional Nkx2.2 co-repressors, specifically in a spatiotemporal context, which would yield a better understanding of the transcriptionally repressive networks required to promote the beta cell fate at the expense of other endocrine cell types.

**Nkx2.2, histone modifications and epigenetics**

It has been previously demonstrated that Grg4 is recruited to Pax2 binding sites, displacing components of the activating complex MLL4. Grg4 then recruits the arginine methyltransferase Prmt5, resulting in H4R3 di-methylation, which further recruits PRC2 and H3K27me3 to the region (Patel et al., 2012). This study demonstrated how a single DNA binding factor, in this
case Pax2, can act as either a repressor or activator, based on the presence of Grg4. Similarly, on the Arx promoter in beta cells, Nkx2.2 is associated with Grg3, Prmt6 and H3K27me3 (Dhawan et al., 2011) and (Chapter 4). The difference between these otherwise analogous Nkx2.2/Grg3 and Pax2/Grg4 mechanisms is DNA methylation. While Patel et al. did not detect the presence of Dnmt3a, which has been reported to interact with Prmt5 (Zhao et al., 2009), we have shown the Nkx2.2/Grg3 complex contains Dnmt3a on the methylated Arx promoter (Chapter 2). DNA methylation is thought to silence gene expression by inhibiting transcription factor binding or by recruiting methyl-binding domain (MBD) proteins, which recruit chromatin-modifying complexes (Klose and Bird, 2006). The latter is most likely the case for beta cell-specific Arx repression, in that the transcription factors Nkx2.2 and Nkx6.1 are present, as well as the MBD protein MeCP2 (Chapter 2; Dhawan et al., 2011). Some studies also suggest that DNA methylation is not the primary event in gene silencing, but rather acts to secure the silent state (Payer and Lee, 2008). Perhaps this is the case for Nkx2.2-induced repression of the Arx promoter as well, since the Nkx2.2 TN mutation retains the ability to interact with Dnmt3a, suggesting that even though the TN mutation causes de-repression of Arx, the promoter may still be methylated. This would suggest that DNA methylation alone is not sufficient to repress Arx. Therefore, a model could exist whereby Nkx2.2/Grg3 binds the Arx promoter, which would induce H3K27me3 (Chapter 4), presumably by PRC2. The de novo DNA methyltransferases would then recognize the modified histones, i.e., unmethylated H3K4 (Ooi et al., 2007) and H3K27me3 (Mohn et al., 2008) to initiate DNA methylation. This DNA methylation may be in place to inhibit the re-deposition of H3K4 methylation (Dhawan et al., 2011; Mohn et al., 2008), which may have been in place at one time during differentiation.
But how can \textit{Arx}, which is silenced by DNA methylation, HDAC, Grg, H3K27me3, be de-repressed in a system where a single transcription factor is carrying point mutations that causes the dissociation with Grg3? This suggests the mutations in the Nkx2.2 TN domain could additionally disrupt interactions with other co-repressors. Alternatively, and more likely, Grg3 has the ability to recruit other co-repressors that are being lost in the Nkx2.2\textsuperscript{TNmut/TNmut} mice. As mentioned above, in the nervous system, Prmt5 is required for recruiting Polycomb proteins in a Pax2/Grg4-dependent manner (Patel et al., 2012). Correspondingly, the disruption of Grg3 on the \textit{Arx} promoter may cause the disassembling of the complex, leading to a loss of H3K27me3 and DNA methylation, which may give rise to the re-establishment of active marks. Notably, there are other genes that are upregulated in the Nkx2.2\textsuperscript{TNmut/TNmut} mice that normally contain H3K27me3 marks in their promoter regions in FACs-purified beta cells: \textit{Irs4}, which is discussed in chapter 3, and \textit{Nkx6.2} (van Arensbergen et al., 2010).

In support of the idea that loss of Grg3 causes disruption of the entire complex, a similar scenario has been shown in yeast, where loss of recruiters causes a repressor complex to disassemble, resulting in gene re-activation. This was seen even in G\textsubscript{1} or G\textsubscript{2}/M-arrested cells. The authors further demonstrated that re-activation was blocked with overexpression of Sir3p (a histone de-acetylase), which was incorporated into silent chromatin even in G\textsubscript{1}-arrested cells. That the silencers are continuously maintained, illustrates the unexpectedly dynamic nature of heterochromatin (Cheng and Gartenberg, 2000). Taken together, my data point to a broad role for the Nkx2.2/Grg complex in mediating histone modifiers and epigenetic silencing of non-beta cell components. Further epigenetic studies in FACs-purified beta cells from Nkx2.2\textsuperscript{TNmut/TNmut} mice derived at different time points during beta cell reprogramming will help us better
understand the features that must be overcome to initiate de-repression and cellular reprogramming.

**Prominin-2, Irs4 and Rbbp4**

Prominin-2 is an interesting candidate for mediating some of the beta cell functional defects in the Arx rescued mice, in so much as it has yet to be characterized in the pancreas, and Nkx2.2 seems to be directly regulating its repression. Given its role as a transmembrane protein, it could presumably be disrupting cell-cell communication or other signaling events mediated through membrane receptors. However, I have not determined if prominin-2 protein is expressed in the Nkx2.2\(^{TNmut/TNmut}\) beta cells. This could be done with immunofluorescence or Western analysis from FACs-purified cells. To determine a role for prominin-2, beta cell specific deletion most likely would not yield much information, since presumably prominin-2 is either expressed at very low levels in the beta cell or not expressed at all. Instead, to mimic part of the Nkx2.2\(^{TNmut/TNmut}\) phenotype and to determine if prominin-2 impairs beta cell function, prominin-2 should be overexpressed specifically in the beta cells. Additionally, crossing a prominin-2 floxed allele into the Nkx2.2\(^{TNmut/TNmut}\);Ins:Cre;Arx\(^{fl/fl}\) mice would demonstrate if the combined loss of Arx and prominin-2 is sufficient for a more pronounced Nkx2.2\(^{TNmut/TNmut}\) rescue than what is seen with Arx\(^{fl/fl}\) alone. Similar experiments could also be done with Irs4. It has been determined that Irs4 antagonizes Irs1 and 2 activity, which are essential mediators of insulin action (Tsuruzoe et al., 2001), and loss of the insulin receptor specifically in beta cells leads to defects in insulin secretion and glucose intolerance (Kulkarni et al., 1999). That Nkx2.2 directly binds the Irs4 promoter (Hill et al., 2011) and its expression is highly upregulated in the Nkx2.2\(^{TNmut/TNmut}\) mice, suggests Nkx2.2 is playing a role in maintaining the function of the mature beta cell by repressing Irs4.
I chose to follow up on the change in \textit{Rbbp4} expression for two reasons. 1) Our microarray showed that the increase in \textit{Rbbp4} was not rescued with beta cell-specific \textit{Arx} deletion, and 2) \textit{Rbbp4} is a core component of chromatin remodeling complexes such as Sin3, PRC2 and NuRD, which suggested that Nkx2.2 is involved in global chromatin remodeling. However, I was not able to confirm the increase in \textit{Rbbp4} with the primer set I generated. It is interesting, though, that the microarray demonstrated that \textit{Rbbp4} was highly upregulated with one probe set, and unchanged with another. This should be resolved to unequivocally determine \textit{Rbbp4}'s expression in the Nkx2.2\textsuperscript{TNmut/TNmut} mice. If \textit{Rbbp4}, a component of many repressor complexes, is indeed upregulated, it may explain the fact that so many genes are suppressed in a mouse model that presumably has lost the ability to repress Nkx2.2-target genes (Figure1A,B Chapter 3). In contrast to Arx and presumably Irs4 and Prominin 2, which are not normally expressed in the beta cell, beta cell-specific deletion of \textit{Rbbp4} would probably cause additional problems since it is such a gregarious protein. Even though the microarray demonstrates its up-regulation, and I have shown Nkx2.2 directly binds its promoter, which suggests \textit{Rbbp4} is repressed in the beta cell, \textit{Rbbp4} repression is perhaps intermittent, allowing for the correct levels of expression. Conversely, beta cell-specific overexpression of \textit{Rbbp4} might determine if the many pathways that are down-regulated in the Nkx2.2\textsuperscript{TNmut/TNmut} (Figure 1B, Chapter 3), are mediated by \textit{Rbbp4}-dependent repression.

\textbf{Nkx2.2 phosphorylation}

The observation that Nkx2.2 is phosphorylated is a novel and exciting finding; however, more detailed analyses need to be completed. Admittedly, technical problems precluded some experiments I had planned to perform. I would like to have determined whether constitutive phosphorylation or dephosphorylation, afforded by serine-to-aspartate or –alanine mutations,
respectively, affected the interaction between Nkx2.2 and other cofactors such as Grg3, HDAC, Dnmt3a or CBP/p300. However, I was able to determine that constitutive phosphorylation and dephosphorylation affected recruitment of cofactors to Nkx2.2 target genes in a cell-specific context. These changes were accompanied by alterations in histone modifications. Thus, I hypothesize that phosphorylation inhibits the interaction with Grg3, which in turn, disrupts the assemblage of a repressor complex that may include components of PRC2, given changes in H3K27me3. In my model I predict that Nkx2.2 might be phosphorylated in alpha cells and dephosphorylated in beta cells (Figure 7, Chapter 4). Serine 11 is predicted to be phosphorylated by PKC, and I observed that the isoforms PKCe and PKCζ are the most highly expressed in the cells lines I used. Although PKCe−/− mice have a beta-cell protective effect, which would be consistent with my prediction (that Nkx2.2 is dephosphorylated in beta cells), I found PKCζ to be expressed about two-fold higher in alpha cells (Figure 2, Chapter 4), which would be consistent with my prediction that Nkx2.2 is phosphorylated in alpha cells. Direct in vitro assays that detect incorporation of radio-labeled phosphate into Nkx2.2 in the presence of the appropriate isoform could correctly identify which kinase is phosphorylating Nkx2.2. To confirm my hypothesis, this kinase could then be overexpressed specifically in the beta cell. This would presumably phosphorylate Nkx2.2 and cause the repressor complex to disassemble. The caveat would be that this kinase would most likely have other targets, which would cause unexpected changes, possibly obscuring the predicted effects. A more elegant approach might be to identify the kinase in vitro and determine co-expression and interaction with this kinase in vivo. Then, to demonstrate the differential requirement of Nkx2.2 phosphorylation, Nkx2.2S11A (dephosphorylated) could be expressed in the alpha cell using Glu:Cre, which may cause the repression of Arx and activation of NeuroD, or Nkx2.2S11D (phosphorylated) could be expressed
in the beta cell using Ins:Cre, which might cause the derepression of Arx and repression of NeuroD (Chapter 4). Identifying the kinase and at which developmental stage phosphorylation is required could strengthen the ability to guide stem cell differentiation to the beta cell fate. Additionally, determining how phosphorylation might affect Nkx2.2 protein stability, subcellular localization and response to metabolic flux remains to be determined, but could possibly reveal new therapeutic targets of diabetes.

**Concluding remarks**

Developmental programs are guided by transcription factors and chromatin modulators, which maintain context-specific gene expression by remodeling chromatin to either facilitate repression or activation. Histone modifiers have no intrinsic DNA-binding ability and no specificity for a particular histone residue versus one on another nucleosome. Thus, gene regulation is guided by DNA binding transcription factors that recruit either activating or repressive complexes to enhancers. To determine which enhancers contribute to the development of the beta cell lineage, an atlas of transcription factor binding is needed. This is being facilitated by ongoing experiments in the lab, which make use of in vivo tagged-Nkx2.2 to enhance ChIP-seq. A tagged version of Nkx2.2 will provide the ability to perform ChIP-seq in small cell populations, such as FACs-purified pancreatic progenitors, Ngn3⁺ progenitors and individual endocrine cell types. Determining where Nkx2.2 binds throughout the genome in a spatiotemporal manner will increase our understanding of how Nkx2.2 guides endocrine cell fate and maintains cellular function. Moreover, it is important to understand which external signals guide the regulated activity of transcription factors and when these signals are needed. I have shown that Nkx2.2 is phosphorylated, and this phosphorylation seems to be cell-type specific, i.e., phosphorylation is pro-alpha cell, while de-phosphorylation is pro-beta cell. As PKC family members are well
known to be mediators of receptor signaling cascades, identifying the signals that promote the activation of the responsible PKC and phosphorylation of Nkx2.2 could help in understanding how the Ngn3^+ endocrine progenitor determines whether to become an alpha versus beta cell. Correctly applying these signals to ES cell differentiation cocktails could then aid in diverting the cells toward the beta cell lineage. In summary, I have shown how a single transcription factor can control beta cell development, maintenance and function by utilizing post-translational modifications and interactions with different cofactors. Expansion of these studies will help develop more precise ES cell differentiation protocols and identify novel therapeutic targets for the treatment of diabetes.
References


Gao, Y., Miyazaki, J., and Hart, G.W. (2003). The transcription factor PDX-1 is post-translationally modified by O-linked N-acetylglucosamine and this modification is correlated with its DNA binding activity and insulin secretion in min6 beta-cells. Archives of biochemistry and biophysics 415, 155-163.


extensively phosphorylated and forms multiple complexes in embryos. The FEBS journal 275, 5062-5073.


