UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of Nkx6.1 in maintenance of pancreatic beta cell identity and function

A dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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Professor Seth Field
Professor Martyn Goulding
Professor Pamela Mellon
Professor Miles Wilkinson

2013

The Dissertation of Brandon Lawrence Taylor is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2013

DEDICATION

Dedicated to my family

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LIST OF ABBREVIATIONS

Abcc8	ATP-binding cassette, sub-family C (CFTR/MRP), member 8
Ad	Adenovirus
ADP	Adenosine di-phosphate
AMP	Adenosine monophosphate
Arx	Aristaless related homeobox
ATP	Adenosine tri-phosphate
Brn4	Brain transcription factor 4
BSA	Bovine serum albumin
Chga	Chromogranin A
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation with massively parallel sequencing
CMV	Cytomegalovirus
Cn/NFAT	Calcineurin/nuclear factor of activated T cells
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT1	DNA methyl transferase 1
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbant assay
EMSA	Electromobility gel shift assay
Endo	Endocrine
Ero1lb	Endoplasmic reticulum oxidoreductase 1 like beta
ES cells	Embryonic stem cells
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FC	Fold change
FDR	False discovery rate
Flox	Flanked by loxP sites
Flpe	Flippase
FoxO1	Forkhead box O1
Frt	Flippase recognition target
G6pc2	Glucose 6 phosphate carboxylase 2
Glc	Glucagon
Glp1r	Glucagon-like-peptide 1 receptor
Glut2	Glucose transporter 2
GO	Gene ontology
GSIS	Glucose stimulated insulin secretion
GWAS	Genome wide associate study
HBSS	Hanks buffered salene solution
HDAC1	Histone deacetylase 1
Ins	Insulin
IPGTT	Intraperitoneal glucose tolerance test
Isl1	Islet 1 transcription factor
K_{ATP} channel	ATP sensitive potassium channel
kb	Kilobases
Kcnj11	Potassium inwardly rectifying channel, subfamily J, member 11

LoxP	Locus of crossover, P1 phage
MADM	Mosaic analysis with double markers in mice
MafA	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A
MafB	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B
Min6	Mouse insulinoma cell line 6
mM	Millimolar
Mnx1	Motor neuron and pancreas homeobox 1
MODY	Maturity onset diabetes of the young
MPC	Multipotent pancreatic progenitor cell
mRNA	Messenger ribonucleic acid
Neo^r	Neomycin resistance
NeuroD	Neurogenic differentiation 1
ng	Nanograms
Ngn3	Neurogenin 3
Nkx2.2	NK2 homeobox 2
Nkx6.1	Nk6 Homeobox-1
$Nkx6.1^{\Delta\beta}$	*Nkx6.1* deletion in beta cells
$Nkx6.1^{\Delta adult\beta}$	*Nkx6.1* deletion in adult beta cells
$Nkx6.1^{OE}$	Nkx6.1 conditional overexpressing mice
P0	Postnatal day 0
pAMPK	Phosphorylated adenosine mono-phosphate kinase
Pax4	Paired box gene 4
Pax6	Paired box gene 6
PBS	Phosphate buffered salene
PC1	Prohormone convertase 1/3
Pcsk1	Prohormone convertase 1/3
Pcx	Pyruvate carboxylase
Pdx1	Pancreatic and duodenal homeobox 1
PFA	Paraformaldehyde
PGK	Phosphoglycerate kinase 1
PP	Pancreatic polypeptide
Prm	Protamin 1
Ptf1a	Pancreas specific transcription factor, 1a
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
R26	Rosa 26
Rfx6	Regulator factor X 6
RIP	Rat insulin 2 promoter
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute media
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Slc30a8	Solute carrier family 30 (zinc transporter), member 8
Som	Somatostatin
Sox9	SRY-box containing gene 9
STZ	Streptozotocin
Sytl4	Synaptotagmin-like 4
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

TEM	Transmission electron microscopy
Tle3	Transducin like enhancer of split 3
TM	Tamoxifen
TSA	Tyramide signal amplification
TSS	Transcriptional start site
TTS	Transcriptional termination site
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Ucn3	Urocortin 3
Wks	Weeks
YFP	Yellow fluorescence protein
μm	Micrometer/Micron

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The work presented in this thesis is the culmination of shared ideas and critical discussions between many people, which without this support would not have been possible.

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Chapter 3, in full, is a reprint of the material as it appears in Taylor, B.L.*, Liu, F., and Sander, M.A. (2013). Nkx6.1 is essential for maintaining the functional state of pancreatic beta cells. Cell Reports. The dissertation author was the primary investigator and author of this study.

Chapter 4 includes material that is currently being prepared for submission as a manuscript. Taylor, B.L., Benthuysen, J., and Sander, M.A. (In preparation). Nkx6.1 is required for postnatal beta cell mass expansion. The dissertation author was the primary investigator and author of this study.

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Guo S, Dai C, Guo M, Taylor BL, Sander M, Harmon J, Powers A, Robertson RP, Stein R. *Inactivation of specific beta cell transcription factors in type 2 diabetes. The Journal of Clinical Investigation* (2013).

Taylor BL, Liu F, Sander M. *Nkx6.1 is essential for maintaining the functional state of pancreatic beta cells. Cell Reports* (2013).

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Taylor BL, Liu F, Benthuysen J, Yuan W, Magnuson MA, Sander M. Nkx6.1 is required for maintenance of beta cells and beta cell function, 40th Keystone Symposia, Advances in Islet Biology, Monterey, CA, March 2012. Workshop Oral Presentation and Poster.

Taylor BL, Liu F, Benthuysen J, Yuan W, Magnuson MA, Sander M. Nkx6.1 is required to maintain pancreatic beta cell function. Center for Molecular Medicine Lunch Talks, San Diego, CA, November 2011. Oral Presentation.

Taylor BL, Liu F, Benthuysen J, Yuan W, Magnuson MA, Sander M. Nkx6.1 is critical for beta cell proliferation and maintenance of beta cell identity, 71st ADA Scientific Sessions, San Diego, CA, June 2011. Oral Presentation.

Taylor BL, Liu F, Benthuysen J, Yuan W, Magnuson MA, Sander M. Investigating the temporal requirements for Nkx6.1 in maintenance of beta cell identity and function, Western Regional Islet Study Group (WRISG), UCLA Retreat Center, CA, April 2011. Oral Presentation.

Taylor BL, Liu F, Benthuysen J, Yuan W, Magnuson MA, Sander M. Insulin producing beta cells require the transcription factor Nkx6.1 for maintenance of cell identity and function, Center for Molecular Medicine Student and Postdoc Seminar Series, San Diego, CA, March 2011. Oral Presentation.

Taylor BL, Liu F, Benthuysen J, Yuan W, Magnuson MA, Sander M. Investigating the temporal requirements for Nkx6.1 in maintenance of beta cell identity and function, San Diego Beta Cell Society, San Diego, CA, November 2010. Oral Presentation.

Taylor BL, Schaffer A, Sosa-Pineda B, Yuan W, Magnuson MA, Sander M. Conditional Inactivation of Nkx6.1 in Mice Reveals a Requirement for Nkx6.1 Downstream of Ngn3 in Beta-Cell Development, Beta Cell Biology Consortium, Bethesda, MD, February 2010. Poster.

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ABSTRACT OF THE DISSERTATION

The role of Nkx6.1 in maintenance of pancreatic beta cell identity and function

by

Brandon Lawrence Taylor

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2013

Professor Maike Sander, Chair

The insulin producing beta cells of the pancreas are essential for maintaining blood glucose levels. With either the destruction or dysfunction of beta cells leading to the onset of diabetes mellitus, it is necessary to understand the factors that maintain beta cells and their function. Nkx6.1 is a homeodomain transcription factor that is required for beta cell development. It is expressed in pancreatic progenitors and once pancreatic development is complete, Nkx6.1 expression becomes restricted to insulin producing beta cells. Although the functional importance of Nkx6.1 during the earliest steps of pancreatic development is known, it is less clear how Nkx6.1 regulates beta cell development and whether Nkx6.1 is required to maintain adult beta cells. Therefore, we developed mice with a conditional *Nkx6.1* loss of function allele to examine the spatial and temporal requirements for Nkx6.1.

By conditionally ablating *Nkx6.1* in pancreatic endocrine progenitors of mice, we found that Nkx6.1 promotes beta cell development at the expense of non-beta endocrine cell subtypes. Nkx6.1 achieves this through direct repression of genetic lineage determinants of non-beta endocrine cells. Once specified, Nkx6.1 continues to be required in beta cells to repress delta cell gene programs. Therefore, Nkx6.1 promotes and maintains beta cell identity through repression of alternative endocrine lineages.

In addition to regulating beta cell identity, we determined that Nkx6.1 is also a master regulator of mature beta cell function. By directly regulating genes required for central beta cell functions, Nkx6.1 maintains insulin biosynthesis and glucose import and metabolism. Consequently, deletion of *Nkx6.1* in beta cells of adult mice causes the rapid onset of diabetes. By maintaining glucose import, Nkx6.1 also indirectly influences glucose stimulated beta cell proliferation. This role for Nkx6.1 is essential, as loss of Nkx6.1 in neonatal beta cells results in decreased beta cell mass expansion. Finally, we show that Nkx6.1 maintains identity of adult beta cells, as Nkx6.1-deficient beta cells adopt delta cell characteristics over time.

Overall, our studies uncover the functional importance of a beta cell specific transcription factor, Nkx6.1, as a central regulator of pancreatic beta cell identity and cellular function.

CHAPTER 1:

Introduction of the dissertation

Pancreatic beta cells are highly specialized cells required for producing and secreting insulin in response to elevated glucose levels. A large body of work has identified a combinatorial network of transcription factors that is required for development and maintenance of pancreatic beta cells (Seymour and Sander, 2011; Shih et al., 2013). The Sander laboratory has identified a transcription factor, Nkx6.1, which is necessary for beta cell development and remains beta cell-specific in the adult pancreas. *In vitro* findings suggest that Nkx6.1 may also regulate mature beta cell function, including insulin secretion. Therefore, the work in this thesis explored how Nkx6.1 regulates beta cell development and whether Nkx6.1 maintains beta cell function and identity in the adult mouse pancreas. The introduction of this thesis aims to familiarize the reader with the general biology of the pancreas, transcriptional regulation of pancreatic development, and the mechanisms of beta cell function.

The biology of the pancreas

The pancreas is intimately situated in the digestive system, located below the liver and attached to the stomach, duodenum, and spleen [Reviewed in (Shih et al., 2013; Slack, 1995)]. It contains two separately functioning cell compartments composed of exocrine and endocrine tissue. The majority of the pancreas (~95%) is composed of exocrine tissue, which includes the acinar cells that produce and secrete digestive enzymes as well as the ductal network that carries these enzymes into the duodenum. The other ~5% of the pancreas consists of highly vascularized clusters of endocrine cells comprising the islets of Langerhans, which regulate blood glucose homeostasis.

Pancreatic islets are composed of five endocrine subtypes at birth, which include insulin-producing beta cells, glucagon-producing alpha cells, somatostatin-

producing delta cells, pancreatic polypeptide (PP)-producing cells, and ghrelin-producing epsilon cells (Slack, 1995). Beta cells secrete insulin to reduce levels of and store glucose in tissues including the liver, muscle and fat, while alpha cells secrete glucagon under fasting conditions for the release of stored glucose. Somatostatin and pancreatic polypeptide do not directly regulate glucose levels, but act in a paracrine fashion on alpha and beta cells to modulate secretion of glucagon and insulin (Hauge-Evans et al., 2009). The fifth hormone type ghrelin, which regulates satiety, is produced in newly differentiated islets of the pancreas, however it is not maintained in the adult pancreas (Arnes et al., 2012).

Elevated blood glucose levels and diabetes are the direct result of the loss or dysfunction of pancreatic endocrine cells, particularly the insulin-producing beta cells. Since beta cell loss or dysfunction can be solely responsible for the onset of diabetes, it is critical to understand how beta cells function as well as how they are maintained.

Transcriptional regulation of pancreatic development

Although there are distinct functional domains within the mature pancreas, each pancreatic cell subtype originates from a pool of common pancreatic progenitor cells, termed multipotent progenitor cells (MPCs) (Figure 1.1) [Reviewed in (Seymour and Sander, 2011; Shih et al., 2013)]. This pool of cells is identified by its distinct signature of transcription factor expression, which includes Pdx1, Nkx6.1, Ptf1a, and Sox9. Notably, *Pdx1*, *Ptf1a*, and *Sox9* are each critical for maintenance of this progenitor cell pool, as null mutations in any of these genes results in severe pancreatic agenesis (Burlison et al., 2008; Kawaguchi et al., 2002; Krapp et al., 1998; Offield et al., 1996; Seymour et al., 2008; Seymour et al., 2007). Furthermore, genetic lineage analysis of MPCs demonstrates that they have the potential to give rise to all

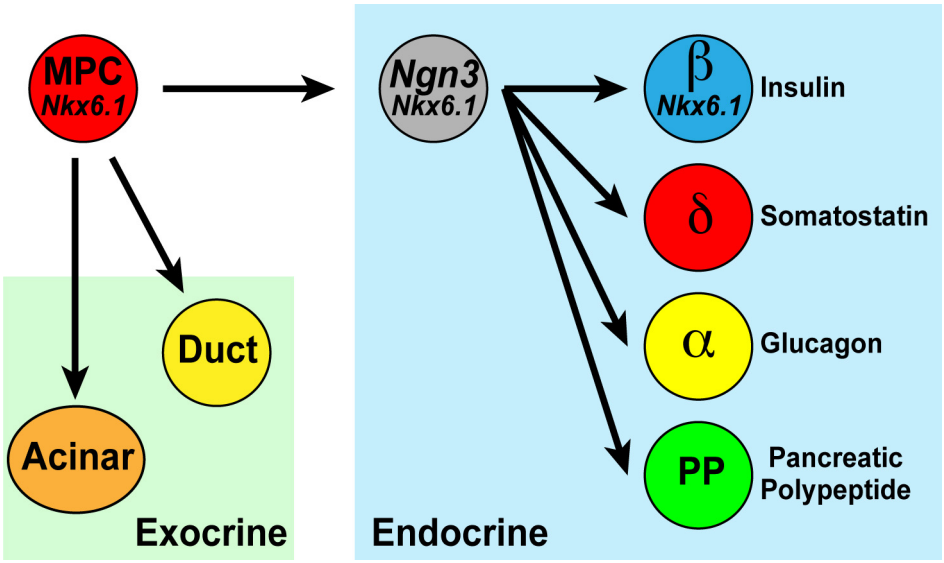


Figure 1.1. Nkx6.1 expression during pancreatic development. Nkx6.1 expression is initiated in the multipotent progenitor cell (MPC) and is subsequently restricted to Neurogenin3 (Ngn3) expressing endocrine progenitors. After pancreatic differentiation is complete, Nkx6.1 expression in the pancreas is restricted to insulin producing beta (β) cells.

pancreatic subtypes. Therefore, transcription factors regulate the maintenance of MPCs, which are critical for the development of the pancreas.

The lineage potential of progenitor cells becomes limited later in development as they commit to endocrine or exocrine cell fates (Zhou et al., 2007). During this stage of pancreatic development, exocrine and endocrine progenitor cells segregate into functionally distinct regions in either the outer “tip” or the inner “trunk” domain of the developing pancreas, respectively. During this period, differential transcription factor expression is observed between the developing endocrine and exocrine progenitors (Zhou et al., 2007). Of critical importance to this segregation is the transcription factor Neurogenin 3 (Ngn3), which is restricted to pancreatic endocrine progenitor cells and is required for progenitor maintenance. Therefore, *Ngn3*-null mutations result in an exclusive and complete loss of endocrine cells in the pancreas (Gradwohl et al., 2000). Genetic lineage analysis further confirmed their developmental potential, as the progeny of $Ngn3^{+}$ endocrine progenitors are restricted to an endocrine cell fate (Gu et al., 2002; Schonhoff et al., 2004). Recently, a study utilizing mosaic analysis with double markers in mice (MADM), which is a genetic lineage analysis method that has the ability to distinguish between symmetric or asymmetric division of a progenitor cell (Zong et al., 2005), showed that $Ngn3^{+}$ endocrine progenitors are unipotent and non-proliferative (Desgraz and Herrera, 2009). Thus, each endocrine progenitor cell develops into a single endocrine cell. Although this suggests that each endocrine progenitor cell is pre-fated to become a single endocrine type, it remains unclear whether cell fate can change downstream of Ngn3 expression. Recent evidence suggests that endocrine progenitor cells remain plastic, as misexpression of the transcription factor Pdx1 in $Ngn3^{+}$ cells results in the allocation of endocrine progenitors pre-fated to become alpha cells to the beta cell

lineage (Yang et al., 2011). Therefore, differential transcription factor expression has a critical role in development of endocrine cells from endocrine progenitors.

Nkx6.1 is a homeodomain transcription factor that is expressed throughout pancreatic development (Figure 1.1) (Henseleit et al., 2005; Sander et al., 2000a). Nkx6.1 expression is initiated in MPCs and as development proceeds, becomes increasingly restricted to $Ngn3^{+}$ endocrine progenitors. Once beta cells differentiate from endocrine progenitors, Nkx6.1 expression becomes restricted to insulin-producing beta cells. Nkx6.1 is selectively required for beta cell development, as the *Nkx6.1*-null mutation results in an 85% decrease in beta cell numbers, without affecting the non-beta endocrine cells (Henseleit et al., 2005; Nelson et al., 2007; Sander et al., 2000b). Notably, Nkx6.1 is required early in pancreatic development for beta cell specification as sustained expression of Nkx6.1 in the MPC domain specifies endocrine cell progenitor cells at the expense of acinar progenitor cells (Schaffer et al., 2010). Although the role of Nkx6.1 in early pancreatic development has been extensively characterized, it remains unclear how Nkx6.1 regulates beta cell specification later in development. In particular, it is unknown how Nkx6.1 maintains beta cell fate specification downstream of Ngn3 expression. Therefore, the studies presented in Chapter 2 of this dissertation aim to address this question using a conditional mutant $Nkx6.1^{flox}$ allele.

Beta cell function in the adult pancreas

The insulin-producing beta cells of the pancreas secrete insulin in a regulated fashion in response to blood glucose levels. Since beta cells are critical for maintenance of blood glucose homeostasis, it is important to understand what maintains beta cells and their function. One of the defining characteristics associated

with functional beta cells is a distinct expression pattern of transcription factors [Reviewed in (Oliver-Krasinski and Stoffers, 2008; Seymour and Sander, 2011)]. This includes the beta cell-enriched transcription factors Nkx6.1 (Sander et al., 2000b), MafA (Kataoka et al., 2002), Pdx1 (Ohlsson et al., 1993), and NeuroD (Beta2) (Naya et al., 1995). Suggesting that transcriptional regulators have essential roles in beta cell function, mutations in genes encoding beta cell enriched transcription factors are associated with a form of diabetes called maturity onset diabetes of the young (MODY) (Malecki et al., 1999; Stoffers et al., 1997). Furthermore, it has been recently identified that expression of beta cell enriched transcription factors is compromised in mouse models of type 2 diabetes and in T2DM patients (Guo et al., 2013). Therefore, the functional characterization of beta cell transcription factors is critical to the understanding of normal beta cell function as well as the process of beta cell failure in T2DM.

Beta cells devote a large amount of transcriptional resources to the production of *insulin*, as *insulin* is the most transcribed gene in these cells (45% of total mRNA transcripts) (Moran et al., 2012). Early *in vitro* studies have implicated that beta cell-enriched transcription factors such as Nkx6.1, MafA, NeuroD1, and Pdx1 regulate *insulin* transcription (Aramata et al., 2005; Kataoka et al., 2002; Matsuoka et al., 2003; Melloul et al., 2002; Mirmira et al., 2000; Ohlsson et al., 1993; Olbrot et al., 2002; Petersen et al., 1998; Sharma and Stein, 1994; Taylor et al., 2005; Zhao et al., 2005). In addition to these initial *in vitro* studies, the advent of advanced mouse genetic tools has allowed investigators to demonstrate that islet-enriched transcription factors are required for *insulin* transcription *in vivo*. For example, deletion of *NeuroD* in mature beta cells of mice results in the selective loss of *Ins1* expression (rodents contain two separate *insulin* genes contributing to insulin levels equally (Davies et al.,

1994; Gu et al., 2010). Therefore, beta cell-enriched transcription factors are central to beta cell function as they initiate and maintain *insulin* gene expression.

Although beta cell-specific transcription factors regulate insulin at the level of gene expression, insulin requires further processing once translated. The immediate protein product from the *insulin* gene is a non-functional and full-length product called pre-proinsulin. This protein requires significant processing, as a signal peptide has to be cleaved in the endoplasmic reticulum to form proinsulin, followed by oxidative folding and packaging into secretory vesicles (Orci et al., 1987; Zito et al., 2010). Within secretory vesicles, proinsulin is further processed into a functional heterodimer through cleavage of a region called C-peptide by prohormone convertases (Furuta et al., 1997; Orci et al., 1985; Zhu et al., 2002). This processing is essential, as failure to either oxidatively fold proinsulin or cleave C-peptide results in severe defects in insulin biosynthesis. Exemplifying the importance of this process, a single amino acid substitution in insulin2 that blocks folding of proinsulin results in processing defects and subsequently a severe lack of insulin (Wang et al., 1999). Although many of the enzymes and regulators of this process have been identified, not many transcription factors have been associated with insulin biosynthesis. One identified transcriptional regulator of insulin biosynthesis is Pdx1, as *Pdx1* heterozygous mutations result in decreased expression of Ero1-lb, a protein involved in oxidative folding of proinsulin. Therefore, decreased Ero1-lb expression in *Pdx1* heterozygotes results in reduced insulin protein biosynthesis under conditions of increased metabolic demand for insulin (Khoo et al., 2011). From these findings, it is evident that beta cell-enriched transcription factors are not only required for regulation of *insulin* expression, but are also necessary to maintain the downstream factors essential for post-translational processing of insulin.

The response of beta cells to glucose is central to their function. Elevated blood glucose levels are coupled to the secretion of insulin in a process termed glucose stimulated insulin secretion (GSIS) (Muoio and Newgard, 2008) (Figure 1.2). To achieve this, beta cells must link glucose import and metabolism to insulin secretion. Murine beta cells import glucose through a high K_m (17 mM) glucose transporter, Glut2 (Guillam et al., 2000; Guillam et al., 1997; Johnson et al., 1990; Thorens et al., 1988). Following glucose import, glucokinase (the rate-limiting enzyme of glycolysis) is required for phosphorylation of glucose, which is eventually metabolized into pyruvate (Postic et al., 1999; Vionnet et al., 1992). Pyruvate can then undergo oxidative metabolism in the mitochondria, resulting in elevated levels of intracellular ATP and closure of the outwardly rectifying K_{ATP} channel (Cook and Hales, 1984). This process leads to global membrane depolarization of the beta cell and stimulation of calcium influx through an L-type voltage-gated calcium channel, resulting in secretion of insulin from vesicle stores (Figure 1.2) (Schulla et al., 2003). Secreted insulin then causes glucose uptake in peripheral tissues, which lowers blood glucose levels. Therefore, elevated blood glucose can stimulate insulin secretion for the return of glucose levels to non-stimulatory conditions. In addition to insulin secretion, glucose can also directly stimulate the translation of insulin, thus allowing beta cells to replenish insulin stores after secreting insulin in the presence of high glucose levels (Wicksteed et al., 2003). Together, this demonstrates that beta cells intimately link signals from the increased metabolic demand for insulin (elevated glucose levels) to essential beta cell functions (insulin biosynthesis and secretion).

Previous studies suggest that the beta cell-specific transcription factor Nkx6.1 may serve as a regulator of mature beta cell function. Analysis of *Nkx6.1*-null mice revealed that the remaining Nkx6.1-deficient beta cells do not express Glut2,

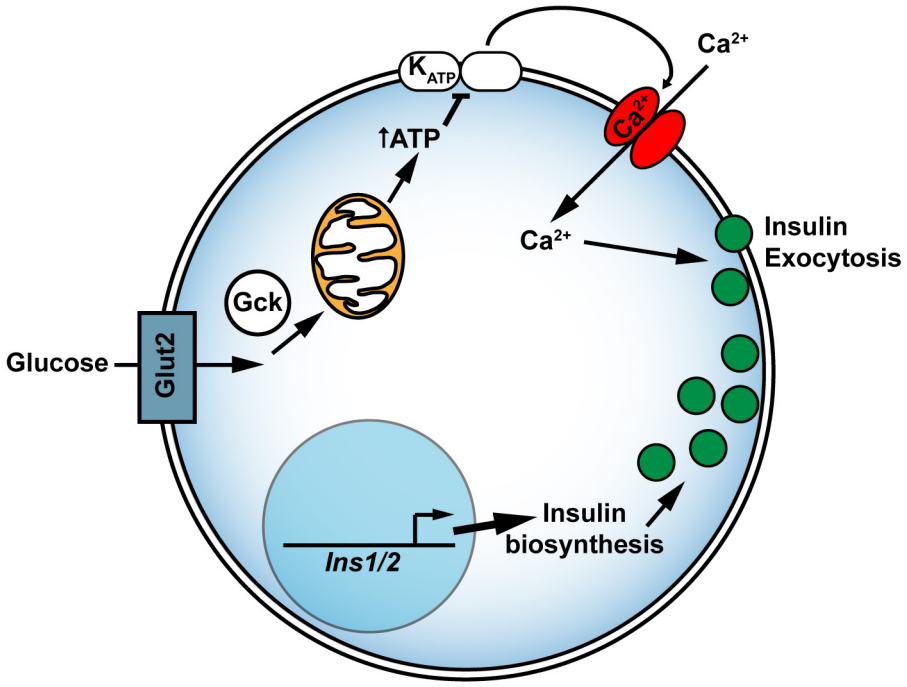


Figure 1.2. Schematic of pancreatic beta cell function. Glucose is coupled to the secretion of insulin. First, glucose is imported into beta cells by the glucose transporter, Glut2. It is subsequently phosphorylated by glucokinase (Gck) where it can be further processed into pyruvate, which is then metabolized in the mitochondria into ATP. Increased ATP levels result in closure of the outwardly rectifying K_{ATP} channel, which then leads to global membrane depolarization, calcium influx, and secretion of insulin.

suggesting that Nkx6.1 may maintain glucose import in mature beta cells (Nelson et al., 2007). Furthermore, studies in beta cell lines and isolated islets suggest that Nkx6.1 regulates glucose stimulated insulin secretion (Schisler et al., 2005). Lastly, *in vitro* studies suggest that Nkx6.1 may regulate *insulin* transcription by virtue that it binds to the *insulin* promoter using EMSA analysis (Taylor et al., 2005). Together, Nkx6.1 is suggested to be associated with several mature beta cell functions. However, none of these studies have addressed whether Nkx6.1 regulates these functions *in vivo* or identified the mechanisms by which Nkx6.1 controls these functions. Therefore, in Chapter 3 of this dissertation, we address whether Nkx6.1 is a true master regulator of beta cell function *in vivo* by utilizing a conditional loss of function allele for *Nkx6.1*.

Maturation and expansion of beta cells in the postnatal period

After specification during pancreatic development, beta cells must undergo a postnatal “maturation” process in which multiple aspects of beta cell function are finely tuned so that they may properly regulate blood glucose levels. This process takes place during the immediate postnatal period and is associated with increased insulin biosynthesis (Blum et al., 2012; Bruin et al., 2008) and regulated insulin secretion (Blum et al., 2012; Hellerstrom and Swenne, 1991; Jermendy et al., 2011). Insulin is initially secreted at both low glucose levels (2.7mM) and high glucose levels (16.7mM), which resolves to stimulated secretion only at high glucose levels (16.7mM) (Blum et al., 2012; Hellerstrom and Swenne, 1991; Jermendy et al., 2011). Because of this change in function, many studies have sought to identify the factors associated with the process of beta cell maturation. One recent study utilizing global gene expression analysis to compare beta cells of newborns and adult animals

identified a factor that increases in expression with the gain of beta cell function (Blum et al., 2012). This factor was identified to be urocortin 3 (Ucn3), which is a peptide normally required for potentiation of insulin secretion (maintaining a robust and sustained insulin secretory response) (Li et al., 2007). This finding implies that the increased expression of peptides produced by the beta cell may aid in its maturation. In addition to changes in peptides that contribute to insulin secretion, other labs have shown that changes in transcription factor expression are associated with beta cell maturation (Takano et al., 1988). In mice, insulin-producing beta cells initially co-express the transcription factors MafA (beta cell specific) and MafB (expressed in beta and alpha cells at birth). As postnatal maturation continues, MafA expression is maintained in beta cells, whereas MafB becomes restricted to alpha cells (Artner et al., 2010). Furthermore, MafA is necessary in newly specified beta cells for maturation of regulated insulin secretion (Zhang et al., 2005). Therefore, MafA has been implicated as a transcriptional regulator of beta cell maturation. Overall, these studies demonstrate that there are distinct transcriptional changes that are necessary for the functional maturation of beta cells during the early postnatal period.

In addition to promoting the functional maturation of beta cells during the early postnatal period, transcription factors also play a critical role in maintaining beta cell identity. To achieve this, transcription factors promote the expression of beta cell specific genes as well as repress non-beta endocrine cell lineage programs (Ahlgren et al., 1998). There are several mechanisms by which transcription factors repress non-beta cell genes. One direct mechanism is through competition with activators of non-beta cell genes by binding to non-beta enriched gene promoters/enhancers (Schaffer et al., 2013). In addition to this function, transcription factors have been

shown to indirectly repress non-beta genes by recruiting histone and DNA modifiers, such as histone deacetylases (HDACs) and DNA methyl transferases (DNMT), respectively (Papizan et al., 2011). Exemplifying the importance of indirect regulation, deletion of *DNMT1* in newly differentiated beta cells results in an exclusive conversion of beta cells to alpha cells because of de-repression of the alpha cell specific transcription factor Arx (Dhawan et al., 2011). Furthermore, it is necessary for transcription factors to recruit DNA modifiers, as deleting the DMNT interacting domain in a beta cell enriched transcription factor, Nkx2.2, results in subsequent beta to alpha transdifferentiation (Papizan et al., 2011). In summary, beta cell identity is not fully established immediately after specification and requires a distinct set of transcription factors to aid in the repression of non-beta cell genes through both indirect and direct mechanisms.

In conjunction with postnatal maturation and stabilization of cell identity, beta cells also undergo a period of rapid proliferation in the first few weeks after birth (Teta et al., 2005). This is essential for establishing beta cell mass in adult animals, as failure to do so can result in diabetes (Georgia and Bhushan, 2004). Several studies using lineage-tracing analysis of beta cells determined that beta cell proliferation is solely responsible for establishing cell mass in adult animals (Brennand et al., 2007; Dor et al., 2004; Teta et al., 2007). By labeling all beta cells at birth with a genetic lineage reporter, these studies determined that no other cell population contributed to beta cell mass in adult mice, as all new beta cells acquired a lineage label from pre-existing beta cells. Furthermore, deletion of key *cyclin* genes, including *Cyclin D1* and *Cyclin D2*, show that proliferation is responsible for beta cell mass expansion in the postnatal period (Georgia and Bhushan, 2004; Kushner et al., 2005). Together these

findings demonstrate that beta cells must replicate in order to establish sufficient beta cell mass adult animals.

Although many of the intrinsic cues for beta cell proliferation have been identified (i.e. *Cyclin* expression), it has not been until recently that the functional importance of extrinsic signals has been definitively demonstrated. Glucose metabolism, the key signal for insulin secretion, was found to also induce beta cell proliferation. Through glycolysis and stimulation of calcium influx, glucose influx leads to increased *Cyclin* expression and subsequent proliferation (Alonso et al., 2007; Heit et al., 2006; Porat et al., 2011; Salpeter et al., 2010; Salpeter et al., 2011). Notably, this process is largely dependent on calcium signaling through the Calcineurin/Nuclear Factor of Activated T Cells (Cn/NFAT) pathway, as defects in this pathway lead to decreased beta cell mass expansion (Heit et al., 2006). Overall, these studies demonstrate that a combination of both intrinsic and extrinsic cues promote beta cell proliferation in the early postnatal period.

In summary, multiple events occur postnatally that are necessary for establishing a functional beta cell mass in adult animals. These processes include the maturation of beta cell function, stabilization of cell identity, and expansion of cell numbers. Although each of these processes are seemingly separate and are modulated by different signaling pathways, their occurrence in conjunction with one another suggests that they may be coordinately regulated. Notably, the beta cell specific transcription factor Nkx6.1 has been implicated in many of these essential processes for postnatal maturation of the beta cell. Nkx6.1 may maintain beta cell identity, as during pancreatic development it inhibits alternative lineage programs of the pancreas to promote the beta cell fate (Schaffer et al., 2010). Furthermore, Nkx6.1 has also been suggested to regulate insulin secretion in the adult beta cell,

therefore it may promote maturation of beta cell function during this period (Schisler et al., 2005). Finally, Nkx6.1 may also modulate postnatal expansion of beta cells, as recent *in vitro* studies suggest that Nkx6.1 regulates pro-proliferative gene expression and beta cell replication (Schisler et al., 2008). Therefore, it is possible that a single transcription factor, Nkx6.1, may link these separate processes together during the postnatal period. Whether Nkx6.1 regulates these functions is addressed in Chapters 2, 3 and 4 of this dissertation.

Significance to type 2 diabetes

Type 2 diabetes (T2DM) is the result of either insulin resistance of peripheral tissues or relative insulin deficiency due to insufficient beta cell mass and function (Butler et al., 2003; Muoio and Newgard, 2008). Beta cell dysfunction is a core component of T2DM as islets in T2DM patients have a decreased capacity for insulin secretion and a reduced insulin secretory response to glucose (Cerasi and Luft, 1967; Hosker et al., 1989). Several causes of beta cell dysfunction have been identified including exposure to elevated free fatty acids (Muoio and Newgard, 2008), mitochondrial dysfunction (Supale et al., 2012), and chronic hyperglycemia leading to oxidative stress (Guo et al., 2013; Robertson, 2004). Additionally, genome wide association studies (GWAS) further demonstrated that beta cell dysfunction is a major contributing factor to T2DM, as they identified mutations in genes required for beta cell function in T2DM patients (Ridderstrale and Groop, 2009). These include a gene encoding a potassium channel subunit (*Kcnj11*) and a gene required for import of Zn^{2+} into insulin secretory granules (*Slc30a8*) (Saxena et al., 2007; Tamaki et al., 2013). Overall, these combined defects compromise beta cell function and result in the relative insulin deficiency observed in T2DM. In addition to loss of beta cell

function, the exposure of beta cells to chronic hyperglycemia in T2DM further reduces insulin levels by resulting in beta cell apoptosis (Butler et al., 2003; Matveyenko and Butler, 2008). Therefore, a combination of both insulin secretory dysfunction and beta cell death contributes to the pathogenesis of T2DM.

The progression of diabetes has been recently associated with changes in transcription factor expression. Specifically, expression of the beta cell-enriched transcription factors MafA, Pdx1, and Nkx6.1 were found to be reduced in both rodent models of obesity as well as in human T2DM subjects (Guo et al., 2013). Since these genes have been implicated in maintaining mature beta cell function, these findings suggested that deleterious gene expression changes secondary to altered transcription factor function may underlie T2DM.

Objective of the dissertation

The work presented in this dissertation is based on several critical observations about the beta cell-specific transcription factor Nkx6.1: (1) *Nkx6.1* is essential for the development of beta cells, (2) Nkx6.1 has been shown to regulate beta cell proliferation and insulin secretion *in vitro*, (3) in the adult pancreas Nkx6.1 is exclusively expressed in beta cells, and (4) Nkx6.1 expression is reduced in type 2 diabetes. Therefore, the objective of my dissertation was to examine how Nkx6.1 regulates development and maturation of beta cells, beta cell proliferation, and beta cell function and identity in the adult. Specifically: Chapter 2 focuses on the roles of Nkx6.1 in specification of beta cells from endocrine progenitor cells during pancreatic organogenesis as well as the role of Nkx6.1 in maintenance of the beta cell fate after specification is complete; Chapter 3 focuses on the role of Nkx6.1 in maintenance of genes that control beta cell function and identity in the adult pancreas; and chapter 4

examines the role of Nkx6.1 in maintenance of beta cells after specification and focuses on how Nkx6.1 regulates beta cell mass expansion in the early postnatal period.

CHAPTER 2:

Nkx6.1 controls a gene regulatory network required for establishing and maintaining pancreatic beta cell identity

ABSTRACT

All pancreatic endocrine cell types arise from a common endocrine precursor cell population; yet the molecular mechanisms that establish and maintain the unique gene expression programs of each endocrine cell lineage have remained largely elusive. Such knowledge would improve our ability to correctly program or reprogram cells to adopt specific endocrine fates. Here, we show that the transcription factor Nkx6.1 is both necessary and sufficient to specify insulin-producing beta cells. Heritable expression of *Nkx6.1* in endocrine precursors of mice is sufficient to respecify non-beta endocrine precursors towards the beta cell lineage, while endocrine precursor- or beta cell-specific inactivation of *Nkx6.1* converts beta cells to alternative endocrine lineages. Remaining insulin$^+$ cells in conditional *Nkx6.1* mutants fail to express the beta cell transcription factors Pdx1 and MafA and ectopically express genes found in non-beta endocrine cells. By showing that Nkx6.1 binds to and represses the alpha cell determinant *Arx*, we identify *Arx* as a direct target of Nkx6.1. Moreover, we demonstrate that Nkx6.1 and the *Arx* activator Isl1 regulate *Arx* transcription antagonistically, thus establishing competition between Isl1 and Nkx6.1 as a critical mechanism for determining alpha *versus* beta cell identity. Our findings establish Nkx6.1 as a beta cell programming factor and demonstrate that repression of alternative lineage programs is a fundamental principle by which beta cells are specified and maintained. Given the lack of Nkx6.1 expression and aberrant activation of non-beta endocrine hormones in human embryonic stem cell (hESC)-derived insulin$^+$ cells, our study has significant implications for developing cell replacement therapies.

AUTHOR SUMMARY

Diabetes is a disease caused by the loss or dysfunction of insulin-producing beta cells in the pancreas. Recent studies suggest that modification of the beta cells' differentiation state is among the earliest events marking the progressive failure of beta cells in diabetes. Currently, very little is known about the factors that instruct cells to adopt beta cell characteristics and maintain the differentiated state of beta cells. We have discovered that a single transcription factor can instruct precursor cells of other endocrine cell types to change their identity and differentiate into beta cells. Conversely, inactivation of the transcription factor in endocrine precursors prevents their differentiation into beta cells and results in excess production of other endocrine cell types. When the factor is specifically inactivated in beta cells, beta cells lose their identity and adopt characteristics of other endocrine cell types, similar to what is seen in animal models of diabetes. Thus, we have identified a single factor that is both sufficient to program beta cells and necessary for maintaining their differentiated state. This factor could be an important target for diabetes therapy and could help reprogram other cell types into beta cells.

INTRODUCTION

Innovative strategies for diabetes therapy aim to replace lost insulin-producing beta cells by reprogramming other cell types or by deriving beta cells from pluripotent cells. Ectopic expression of the transcription factors Pdx1, Neurogenin 3 (encoded by the *Neurog3* gene; Ngn3), and MafA has been shown to reprogram pancreatic exocrine acinar cells into beta-like cells (Zhou et al., 2008). Similarly, some success in reprogramming of liver cells into beta cells has been reported after misexpression of Pdx1, Ngn3, MafA, NeuroD, or Nkx6.1 (Ferber et al., 2000; Gefen-Halevi et al.,

2010; Kojima et al., 2003; Song et al., 2007; Yechoor et al., 2009). Moreover, recent studies have demonstrated that pancreatic endocrine alpha cells can spontaneously convert into beta cells after near complete ablation of beta cells in adult mice (Thorel et al., 2010). Conversely, loss of beta cell identity and partial conversion of beta cells into other endocrine cell types has recently been identified as an early event marking beta cell failure in diabetes (Talchai et al., 2012). Thus, substantial plasticity exists between pancreatic cell types, and this plasticity could potentially be exploited to halt diabetes progression or to replenish beta cells in diabetic individuals. However, little is still known about the factors that control this plasticity.

During embryonic development, all endocrine cell types are derived from a common endocrine precursor population marked by the transcription factor Ngn3 (Desgraz and Herrera, 2009; Gu et al., 2002). Ngn3 activity is required for the specification of all endocrine cells (Gradwohl et al., 2000) and the expression of Arx and Pax4, two transcription factors that control endocrine subtype choices downstream of *Ngn3*. *Arx*-deficient mice display a loss of alpha cells and concomitant increase in beta and delta cells, while *Pax4*-deficiency results in the opposite phenotype of reduced beta and delta cells but increased alpha cells (Collombat et al., 2003; Sosa-Pineda et al., 1997). Strikingly, forced expression of *Pax4* in endocrine precursors and their differentiated progeny imparts a beta-like cell identity to differentiating precursors, resulting in hyperplastic islets with an excess of beta-like cells at the expense of the other endocrine cell types (Collombat et al., 2009). However, despite their increased beta cell mass, mice misexpressing *Pax4* eventually become diabetic and succumb prematurely, suggesting that sustained expression of Pax4 is not compatible with normal beta cell function. Since Pax4 is normally absent from beta cells and only transiently expressed in endocrine precursors during

embryogenesis (Wang et al., 2004), it is possible that proper beta cell development and maturation requires Pax4 downregulation. Similar to Pax4, misexpression of Pdx1 in endocrine precursors has also been shown to favor a beta cell fate choice over other endocrine cell types (Yang et al., 2011). Unlike ectopic Pax4 expression, forced expression of Pdx1 did not reduce the numbers of delta and PP cells, but selectively affected the ratio between beta and alpha cells. Therefore, Pdx1 activity appears to primarily control the alpha *versus* beta cell fate decision, which is consistent with its expression in both beta and delta cells (Sosa-Pineda et al., 1997). Nkx2.2 has recently been identified as a beta cell maintenance factor and stabilizes beta cell fate by repressing the alpha cell fate determinant *Arx* (Papizan et al., 2011). While these studies have provided insight into the factors involved in endocrine cell type specification and maintenance, still little is known about how these factors interact to establish and maintain gene expression programs characteristic of each endocrine cell type. In particular, it is unclear which molecular mechanisms operate in beta cell precursors to ensure that alternative endocrine lineage programs are repressed, while beta cell-specific programs are activated. Given the simultaneous initiation of multiple endocrine subtype programs in one cell with current human embryonic stem cell (hESC) differentiation protocols (D'Amour et al., 2006; Kroon et al., 2008), such knowledge is critical for refining these protocols to support the differentiation of mature and functional beta cells *in vitro*.

In addition to MafA and Mnx1 (also called Hb9) (Harrison et al., 1999; Li et al., 1999; Matsuoka et al., 2004), in the adult pancreas Nkx6.1 is among the few transcription factors exclusively detected in beta cells. During development, Nkx6.1 is first expressed in multipotent pancreatic progenitors, where it specifies an endocrine identity by repressing the pre-acinar transcription factor Ptf1a (Schaffer et al., 2010).

At later developmental stages, Nkx6.1 expression persists in common progenitor cells for the ductal and endocrine cell lineages before becoming eventually restricted to the beta cell lineage (Henseleit et al., 2005). Whether or not Nkx6.1 plays a role in beta cell specification and maintenance remains unknown, largely due to the lack of appropriate genetic models to study this question. Excessive early acinar cell specification and reduced numbers of $Ngn3^{+}$ cells in *Nkx6.1*-null mutant mice preclude their utility for such studies.

To determine the function of Nkx6.1 in endocrine cell type specification and beta cell maintenance, we generated novel genetic mouse models to conditionally inactivate or misexpress *Nkx6.1* after endocrine precursors have been specified. Our studies reveal that Nkx6.1 is both necessary and sufficient to specify the beta cell lineage. Nkx6.1 prevents alpha cell specification in cooperation with Pdx1 by directly repressing *Arx* through competition with the *Arx* gene activator Isl1. Furthermore, inactivation of *Nkx6.1* in beta cells causes loss of beta cell identity and conversion into delta cells. Our findings identify Nkx6.1 as a beta cell programming factor and uncover a transcriptional network that initiates and maintains beta cell-specific gene expression programs, while repressing programs of alternative endocrine lineages.

RESULTS

Heritable expression of *Nkx6.1* in endocrine precursors favors a beta cell fate choice at the expense of other islet cell types.

During pancreatic development, Nkx6.1 is expressed in a subset of $Ngn3^{+}$ cells and is then exclusively maintained in beta cells (Nelson et al., 2007), suggesting that the specification of non-beta endocrine cell types might require *Nkx6.1* downregulation. To explore whether expression of *Nkx6.1* in all, or at least the

majority of, Ngn3$^+$ cells is sufficient to allocate precursors to the beta cell lineage, we heritably expressed Nkx6.1 in Ngn3$^+$ cells, utilizing a mouse line that allows for conditional overexpression of Nkx6.1 after expression of Cre recombinase (*Nkx6.1*^{OE} mice). In *Nkx6.1*^{OE} mice, concomitant expression of Nkx6.1 and enhanced green fluorescent protein (eGFP) is induced by Cre recombinase-mediated excision of a *lacZ* expression cassette flanked by *loxP* sites. The *Nkx6.1*^{OE} transgene design is analogous to the *Z/EG* transgene, in which Cre recombinase induces expression of GFP by recombining *loxP* sites flanking a *lacZ* cassette. Therefore, *Z/EG* mice were used as controls for the *Nkx6.1*^{OE} strain (Figure 2.1A, B). We induced transgene recombination with *Ngn3-Cre* and compared the relative contribution of recombined GFP$^+$ cells to each of the five endocrine cell types in *Ngn3-Cre;Nkx6.1*^{OE} and *Ngn3-Cre;Z/EG* control mice. Notably, due to mosaic expression of the transgenes, not all hormone$^+$ cells expressed GFP. At postnatal day (P) 2, 55.1±1.7% of the GFP$^+$ cells expressed insulin in *Ngn3-Cre;Z/EG* control mice, while 86.1±2.5% of the GFP$^+$ cells were insulin$^+$ in *Ngn3-Cre;Nkx6.1*^{OE} mice (Figure 2.1C, H, M; P<0.001), suggesting that Nkx6.1 favors a beta cell fate choice. Consistent with this notion, *Nkx6.1*-expressing endocrine precursor cells displayed a significantly decreased propensity to differentiate into glucagon$^+$, somatostatin$^+$, pancreatic polypeptide (PP)$^+$, or ghrelin$^+$ cells compared to endocrine precursor cells from control mice (Figure 2.1D-G, I-M). Since *Nkx6.1* expression did not affect cell replication, as shown by analysis of the proliferation marker Ki67 (Figure 2.1N), or survival (Schaffer et al., 2010), these data indicate that Nkx6.1 promotes beta cell differentiation from endocrine precursors at the expense of alternative endocrine fates.

Similar to Nkx6.1, conditional expression of *Pax4* in mouse endocrine precursors results in beta cell specification at the expense of all other endocrine cell

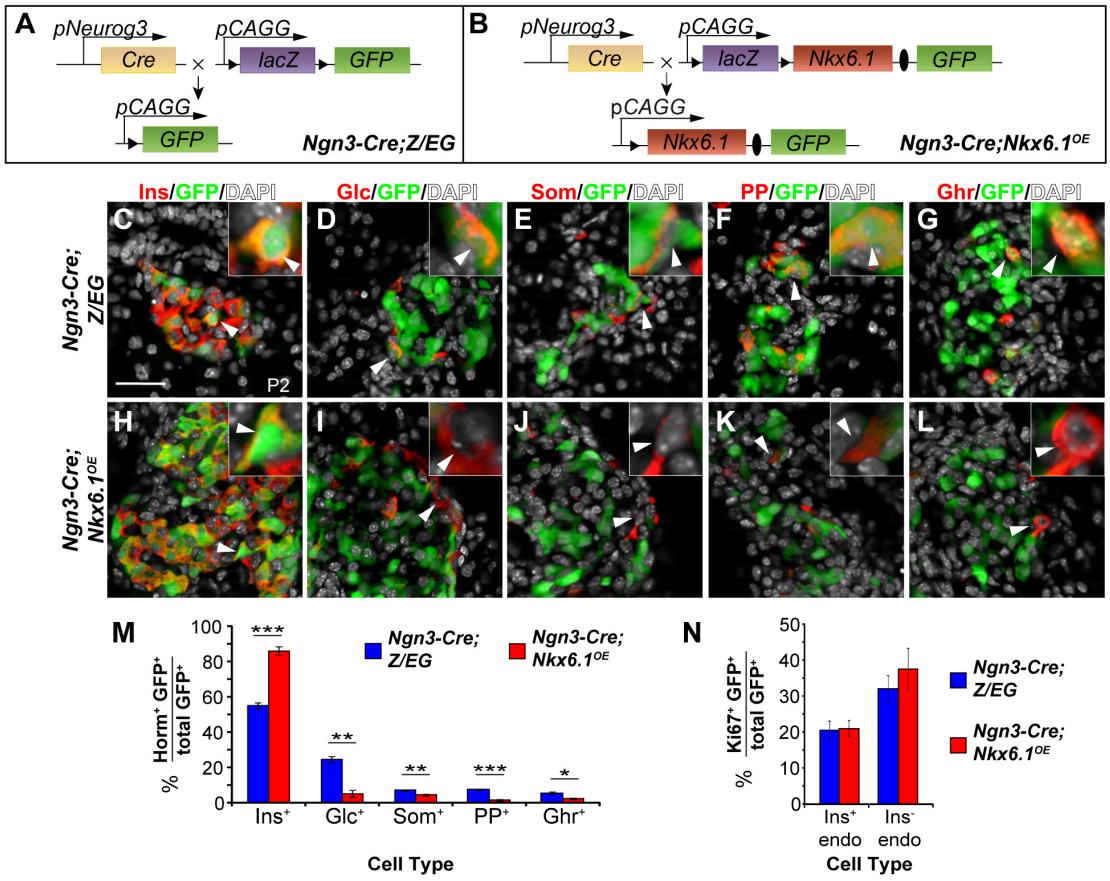
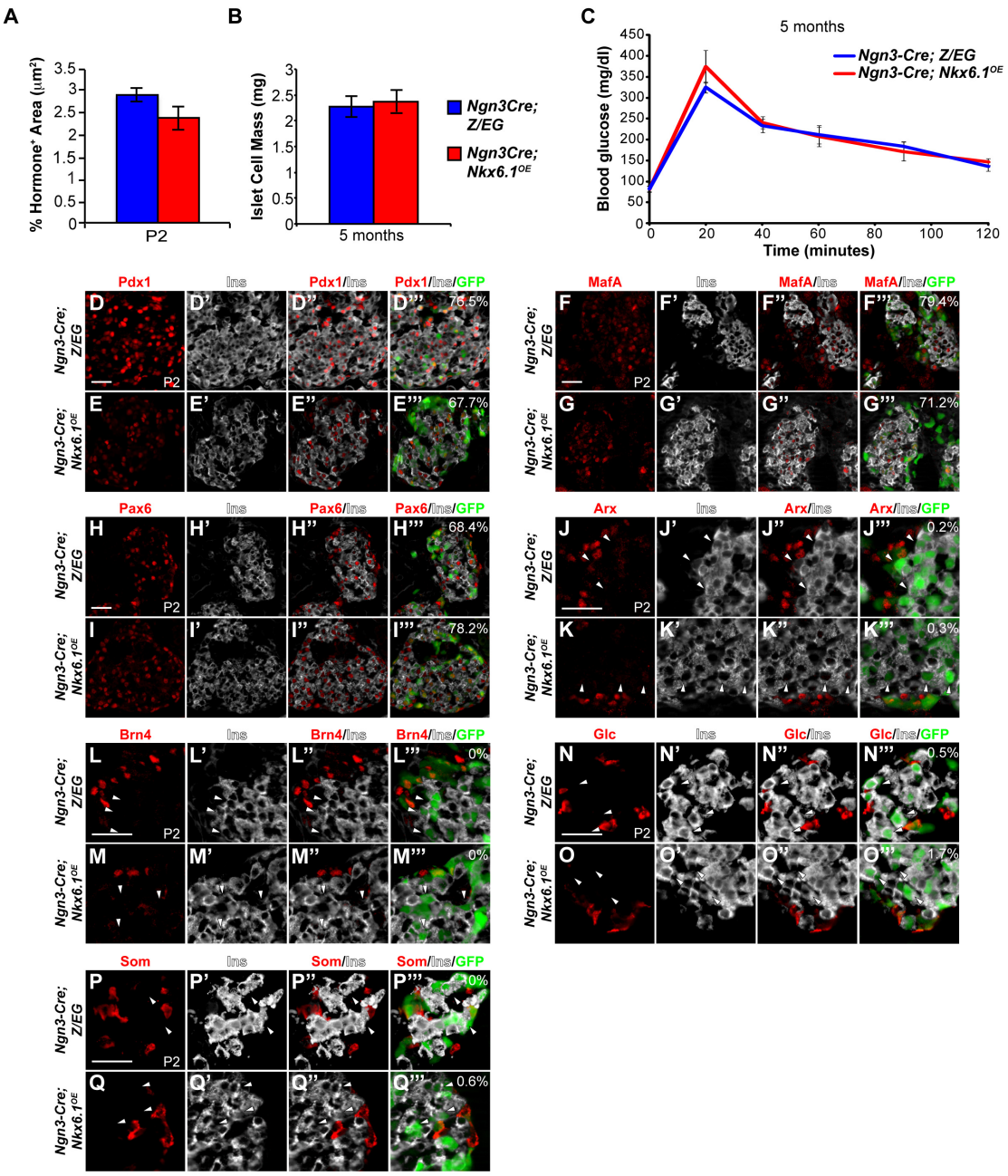


Figure 2.1. Forced *Nkx6.1* expression favors the beta cell fate choice. (A, B) Schematic diagram of the transgenes for conditional *Nkx6.1* misexpression and cell lineage tracing; Triangles, *loxP* sites; Ovals, *internal ribosomal entry site* (IRES). (C-L) Immunofluorescence staining of pancreata from *Ngn3-Cre;Z/EG* and *Ngn3-Cre;Nkx6.1^{OE}* mice at postnatal day (P) 2 for GFP together with each of the five endocrine hormones. The insets display higher magnification images. Arrowheads point to GFP^{+} cells expressing each of the five hormones in *Ngn3-Cre;Z/EG* mice and insulin, but not glucagon, somatostatin, pancreatic polypeptide, or ghrelin in *Ngn3-Cre;Nkx6.1^{OE}* mice. Quantification of hormone^{+}GFP^{+} (M) or Ki67^{+}GFP^{+} (N) co-positive cells as a percentage of all GFP-expressing cells in pancreata of *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* and *Ngn3-Cre;Z/EG* mice at P2 (n=4). Forced expression of *Nkx6.1* in endocrine precursors favors a beta cell fate choice over all other non-beta endocrine cell fate choices. Horm, hormones; Ins, insulin; Glc, glucagon; Som, somatostatin; PP, pancreatic polypeptide; Ghr, ghrelin; endo, endocrine. Scale bar = 50 μm. Error bars represent S.E.M; *p<0.05, **p<0.01, ***p<0.001.

types (Collombat et al., 2009). The expression of Pax4 leads to oversized islets and is eventually accompanied by beta cell dysfunction and diabetes. To determine whether transgenic *Nkx6.1* expression similarly causes islet and beta cell hyperplasia, we compared islet size in *Ngn3-Cre;Nkx6.1*^{OE} and control mice at P2 and at 5 months of age. Consistent with our observation that Nkx6.1 overexpression in adult beta cells does not stimulate beta cell expansion (Schaffer et al., 2011), *Ngn3-Cre;Nkx6.1*^{OE} mice displayed normal islet cell mass (Figure 2.2A, B). Furthermore, 5-month-old *Ngn3-Cre;Nkx6.1*^{OE} mice exhibited normal glucose tolerance (Figure 2.2C), showing that sustained expression of the *Nkx6.1* transgene does not perturb glucose homeostasis.

To next explore the extent of endocrine precursor reprograming and to assess the maturity of beta cells in *Ngn3-Cre;Nkx6.1*^{OE} mice, we analyzed insulin^{+} progeny of targeted endocrine precursors for the expression of critical beta cell markers and possible ectopic expression of non-beta endocrine cell markers. As expected, insulin^{+}GFP^{+} cells in *Ngn3-Cre;Nkx6.1*^{OE} mice expressed the beta cell marker Pdx1, MafA, and Pax6 at P2, showing that transgenic *Nkx6.1* expression in endocrine precursors and their progeny does not impair beta cell maturation (Figure 2.2D-I). Next, to determine whether Nkx6.1 is sufficient to fully repress alternative endocrine lineage programs during endocrine cell differentiation, we analyzed insulin^{+} cells in *Ngn3-Cre;Nkx6.1*^{OE} mice for expression of Arx, Brn4, glucagon, and somatostatin. At P2, targeted GFP^{+} cells in *Ngn3-Cre;Nkx6.1*^{OE} mice were largely indistinguishable from their counterparts in control mice and rarely displayed coexpression of insulin with any of these non-beta endocrine markers (Figure 2.2J-Q; arrowheads), suggesting that Nkx6.1 is effective in fully establishing a beta cell expression program

Figure 2.2. Forced *Nkx6.1* expression results in beta cell programming without altering islet cell mass. Morphometric analysis of hormone$^+$ cell area at postnatal day (P) 2 (A) or islet cell mass at 5 months of age (B) shows no difference between *Ngn3-Cre;Z/EG* and *Ngn3-Cre;Nkx6.1^{OE}* mice (n=3). (C) Misexpression of *Nkx6.1* in all endocrine cell types does not alter glucose tolerance. (D-Q) Immunofluorescence staining of pancreata from *Ngn3-Cre;Z/EG* and *Ngn3-Cre;Nkx6.1^{OE}* mice at P2. Quantification of the average percentage of insulin$^+$GFP$^+$ cells expressing the displayed marker is shown in each panel. Recombined insulin$^+$GFP$^+$ cells in *Ngn3-Cre;Nkx6.1^{OE}* mice express the beta cell markers Pdx1 (D, E), MafA (F, G), and Pax6 (H, I) as in control *Ngn3-Cre;Z/EG* mice. Recombined, insulin$^+$GFP$^+$ cells rarely express the alpha cell markers Arx (J, K) and Brn4 (L, M), showing that the majority of recombined cells have no hybrid alpha/beta identity. Likewise, recombined, insulin$^+$GFP$^+$ cells seldom express glucagon (N, O) or somatostatin (P, Q). Arrowheads point to insulin$^+$ cells that have recombined the *Nkx6.1^{OE}* transgene. Ins, insulin; Glc, glucagon; Som, somatostatin. Scale bar = 50 μm. Error bars represent S.E.M.


A
% Hormone+ Area (µm²)
P2
B
Islet Cell Mass (mg)
5 months
Ngn3Cre; Z/EG
Ngn3Cre; Nkx6.1OE
C
5 months
Ngn3-Cre; Z/EG
Ngn3-Cre; Nkx6.1OE
Blood glucose (mg/dl)
Time (minutes)
Pdx1
Ins
Pdx1/Ins
Pdx1/Ins/GFP
76.5%
67.7%
MafA
MafA/Ins
MafA/Ins/GFP
79.4%
71.2%
Pax6
Pax6/Ins
Pax6/Ins/GFP
68.4%
78.2%
Arx
Arx/Ins
Arx/Ins/GFP
0.2%
0.3%
Brn4
Brn4/Ins
Brn4/Ins/GFP
0%
0%
Glc
Glc/Ins
Glc/Ins/GFP
0.5%
1.7%
Som
Som/Ins
Som/Ins/GFP
10%
0.6%
Ngn3-Cre; Z/EG
Ngn3-Cre; Nkx6.1OE

Forced *Nkx6.1* expression is not sufficient to reprogram differentiated non-beta endocrine cells into beta cells.

We next sought to determine whether *Nkx6.1* acts by inducing cell fate conversion during differentiation of Ngn3^{+} endocrine precursors or by converting already differentiated non-beta endocrine cells. Since we observed residual glucagon^{+}, somatostatin^{+}, pancreatic polypeptide (PP)$^{+}$, and ghrelin^{+} cells misexpressing Nkx6.1 at P2 (Figure 2.1M), we first examined whether these cells convert into beta cells during postnatal life, as observed after Pdx1 expression in Ngn3^{+} cells (Yang et al., 2011). Different from *Ngn3-Cre;Pdx1^{OE}* mice, we found that targeted non-beta endocrine cells persisted in 5-month-old *Ngn3-Cre;Nkx6.1^{OE}* mice (Figure 2.3A-H). Notably, the endocrine cell type ratios observed in *Ngn3-Cre;Nkx6.1^{OE}* mice at P2 are largely maintained at 5 months of age (Figure 2.3I), suggesting that additional non-beta-to-beta cell fate conversion does not occur postnatally. To directly test whether forced expression of Nkx6.1 in differentiated alpha cells triggers their conversion into beta cells, we induced recombination of the *Nkx6.1^{OE}* transgene with *Glucagon-Cre (Glc-Cre)*. Consistent with the persistence of targeted non-beta endocrine cells in adult *Ngn3-Cre;Nkx6.1^{OE}* mice, we failed to observe insulin^{+} cells expressing GFP (Figure 2.4). These findings pinpoint Nkx6.1 beta cell programming activity to a period between the Ngn3^{+} state and activation of hormone gene expression.

Nkx6.1* is required for beta cell specification downstream of *Ngn3.

Since global loss of *Nkx6.1* impairs the generation of Ngn3^{+} endocrine precursors (Schaffer et al., 2010), it has remained unclear whether beta cell development requires Nkx6.1 activity downstream of *Ngn3*. To investigate a potential

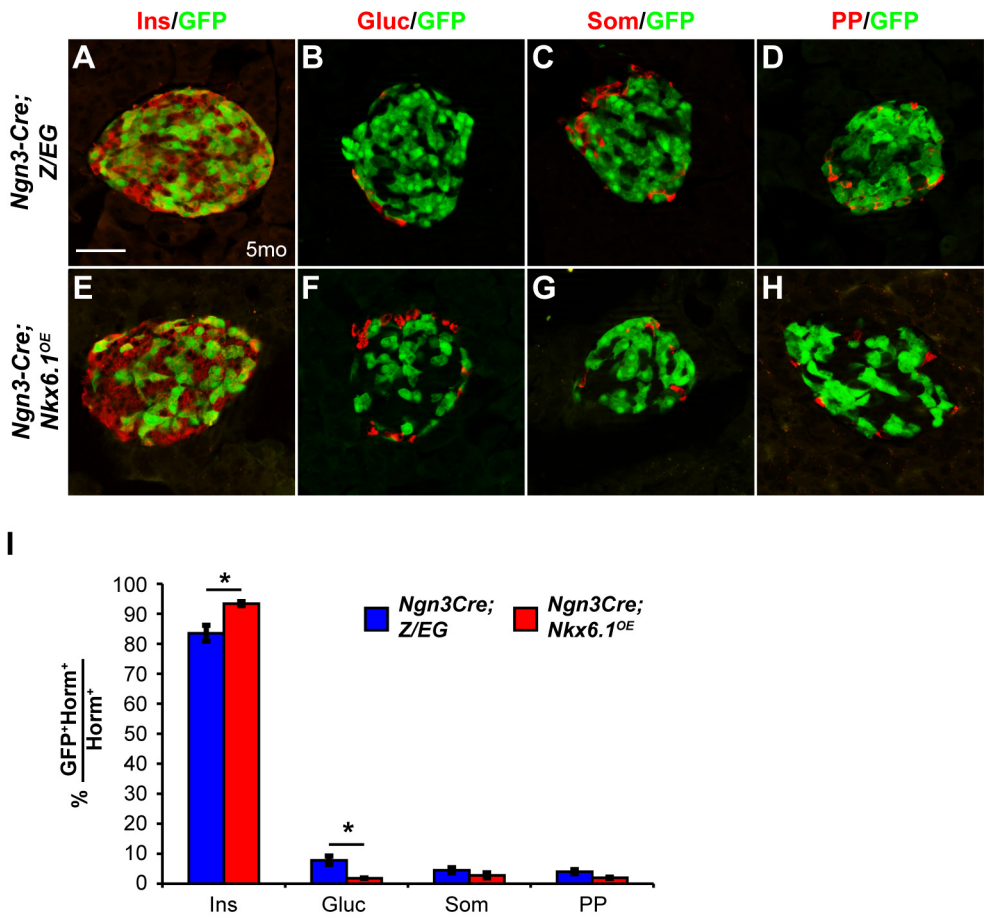


Figure 2.3. Stable expression of Nkx6.1 in endocrine precursors and their progeny results in persistent increase of beta cells and decrease of alpha cells in adult mice. Immunofluorescence staining of pancreata from 5-month-old *Ngn3-Cre;Z/EG* and *Ngn3-Cre;Nkx6.1*^{OE} mice for GFP with each of the endocrine hormones (A-H). Quantification of the percentage of lineage-labeled progeny of *Ngn3*-expressing cells that express insulin, glucagon, somatostatin, or pancreatic polypeptide at 5 months of age (I) (n=3). Ins, insulin; Glc, glucagon; Som, somatostatin; PP, pancreatic polypeptide; Horm, hormone; mo, month. Scale bar = 50 μm. Error bars represent S.E.M.; *p<0.05.

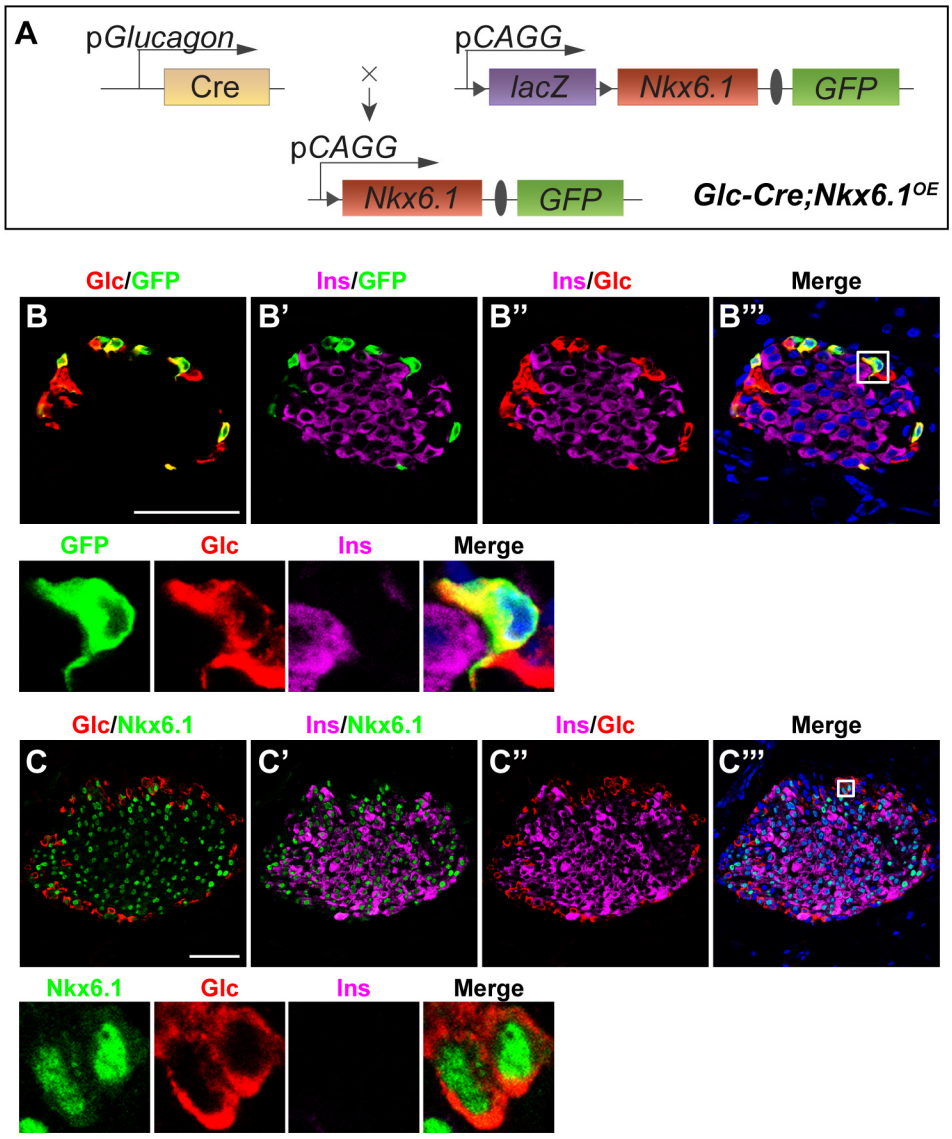


Figure 2.4. Forced expression of *Nkx6.1* in alpha cells does not cause alpha-to-beta cell conversion. (A) Schematic of the transgenes used for conditional *Nkx6.1* misexpression and cell tracing; Triangles, *loxP* sites; Ovals, *internal ribosomal entry sites* (IRES). (B, C) Immunofluorescence staining of pancreata from *Glc-Cre;Z/EG* and *Glc-Cre;Nkx6.1*^{OE} mice at 4 months of age for GFP together with glucagon (Glc) and insulin (Ins) (B) or Nkx6.1 with glucagon and insulin (C). The insets display higher magnification images. Nkx6.1 is ectopically expressed in glucagon^{+} cells, but the GFP lineage label is not detected in insulin^{+} cells. Scale bar = 50 μm.

requirement for *Nkx6.1* in this process, we constructed a conditional mutant allele for *Nkx6.1* by flanking exon 2 with *loxP* sites (Figure 2.5A). Cre recombinase-mediated deletion of exon 2 eliminates a large portion of the DNA-binding homeodomain and additionally introduces a frameshift, resulting in three premature stop codons in exon 3, which cause termination of translation (Rudnick et al., 1994). Importantly, mice heterozygous or homozygous for the *Nkx6.1*^{flox} (*Nkx6.1*^{f}) allele show no abnormalities, suggesting that the floxed allele of *Nkx6.1* is fully functional. To verify that Cre-mediated recombination of the *Nkx6.1*^{f} allele generates a null allele, we intercrossed *Nkx6.1*$^{f/+}$*;Prm1-Cre* and *Nkx6.1*$^{+/-}$ mice to induce recombination of the *Nkx6.1*^{f} allele in germ cells (*Nkx6.1*$^{\Delta f/-}$ allele). As expected, these *Nkx6.1*$^{\Delta f/-}$ mice phenocopied *Nkx6.1* germline null mutant mice (Sander et al., 2000a), and died immediately after birth, manifesting paralysis of their upper extremities and asphyxia (Figure 2.5B). Western blot analysis of Nkx6.1 protein expression in pancreata from *Nkx6.1*$^{\Delta f/-}$ embryos at embryonic day (e) 14.5 showed a complete absence of Nkx6.1 (Figure 2.5C). The pancreas of *Nkx6.1*$^{\Delta f/-}$ embryos was of normal size, but displayed a drastic reduction in insulin^{+} cells at e18.5 (Figure 2.5D-F), phenocopying *Nkx6.1*$^{-/-}$ mice (Sander et al., 2000b).

To determine whether *Nkx6.1* is required for beta cell formation from Ngn3^{+} precursors, we utilized *Ngn3-Cre* to simultaneously induce recombination of the *Nkx6.1*^{f} allele and the *Z/EG* reporter transgene for stable lineage tracing of all progeny derived from Ngn3^{+} cells. In *Nkx6.1*$^{f/-}$*;Ngn3-Cre;Z/EG* embryos, Cre recombines the *loxP* sites in both the *Nkx6.1*^{f} allele and the *Z/EG* transgene to produce cells that are deficient for *Nkx6.1* and express eGFP (Figure 2.6A, B). At e15.5, when *Ngn3* expression peaks (Gradwohl et al., 2000), Nkx6.1 was detected in a large subset of Ngn3^{+} and GFP^{+} cells derived from the *Ngn3*-expressing domain in

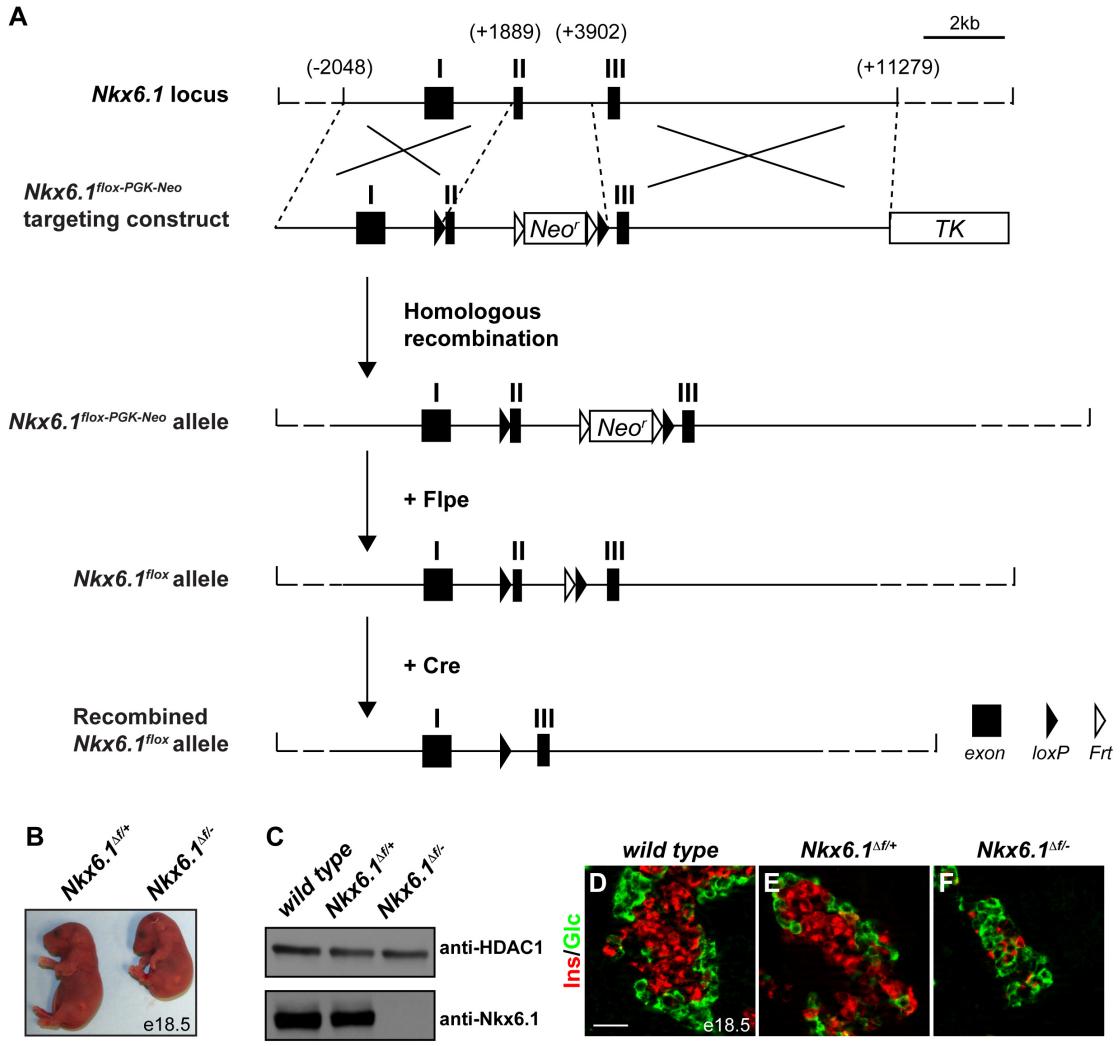


Figure 2.5. Generation of the *Nkx6.1*^{flox} allele. (A) Schematic of the gene targeting strategy to generate the *Nkx6.1*^{flox} allele. Cre recombinase-mediated recombination of the two *loxP* sites removes exon 2 (closed triangles = *loxP* sites, open triangles = *Frt* sites). (B) The gross morphology of *Nkx6.1*$^{f/-}$*;Prm-Cre* (*Nkx6.1*$^{\Delta f/-}$) embryos at e18.5 is identical to *Nkx6.1*-null mutants. (C) Western blot analysis of pancreatic lysates from e14.5 *wild type, Nkx6.1*$^{\Delta f/+}$, and *Nkx6.1*$^{\Delta f/-}$ embryos demonstrates absence of Nkx6.1 protein in lysates from *Nkx6.1*$^{\Delta f/-}$ embryos. HDAC1 was used as a loading control. Immunofluorescence staining for insulin and glucagon on pancreatic sections from *wild type* (D), *Nkx6.1*$^{\Delta f/+}$ (E), and *Nkx6.1*$^{\Delta f/-}$ (F) embryos at e18.5 shows marked reduction in beta cells upon *Nkx6.1* deletion. Ins, insulin; Glc, glucagon. Scale bar = 50 μm.

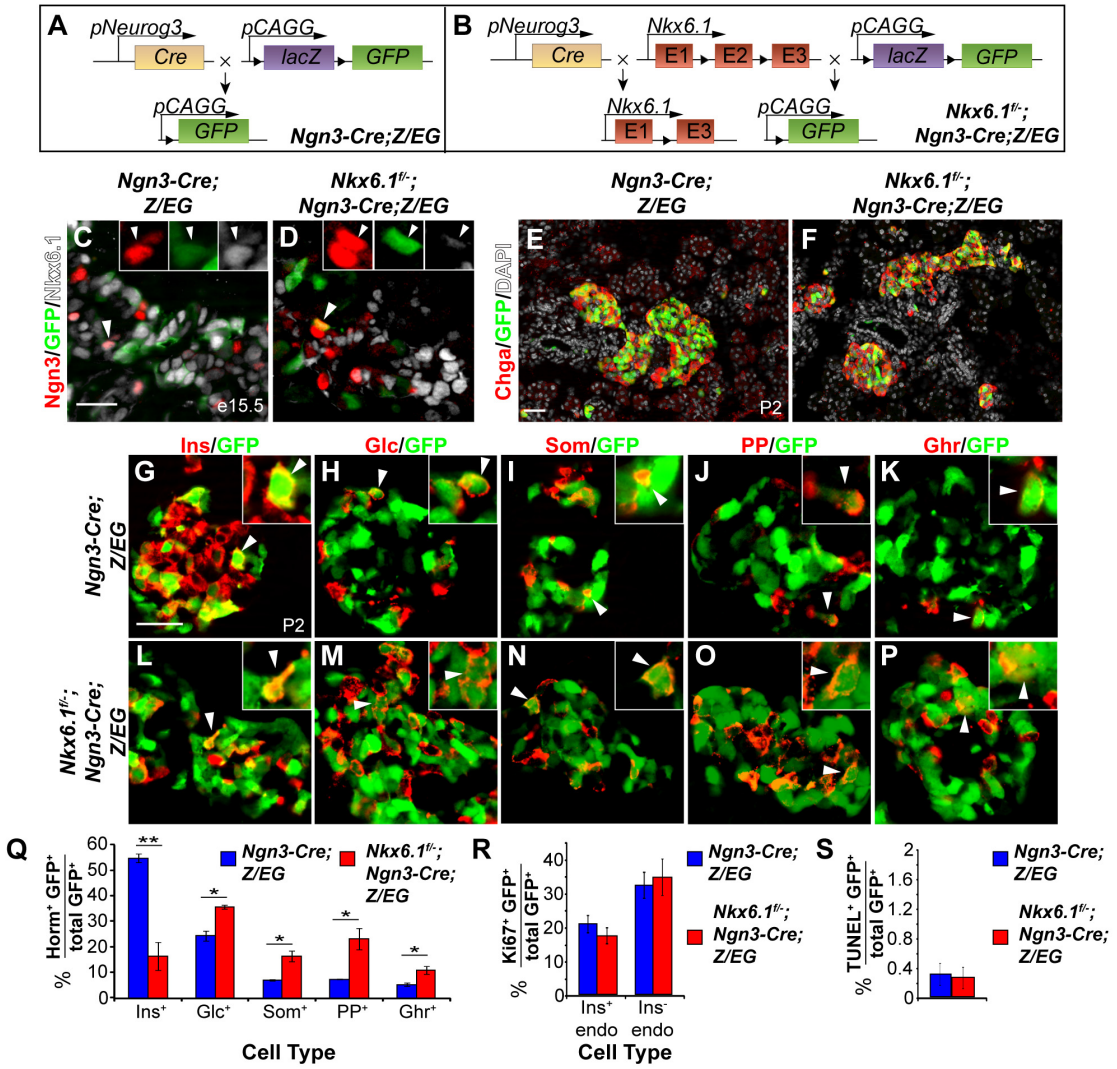


Figure 2.6. *Nkx6.1* is required for beta cell specification downstream of Ngn3. (A, B) Schematic of the alleles and transgenes for *Nkx6.1* inactivation and lineage tracing; Triangles, *loxP* sites. Immunofluorescence staining of pancreata at e15.5 (C, D) or postnatal day (P) 2 (E-P). Recombined, GFP$^+$ cells are restricted to the endocrine compartment (antibody against the pan-endocrine marker Chromogranin A, Chga) in control (E) and *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice (F). The insets show higher magnifications and arrowheads point to GFP$^+$ cells expressing Ngn3 (C, D) or hormones (G-P). Quantification of hormone$^+$GFP$^+$ (Q), Ki67$^+$GFP$^+$ (R), or TUNEL$^+$GFP$^+$ (S) co-positive cells as a percentage of all GFP-expressing cells in pancreata of *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* and *Ngn3-Cre;Z/EG* mice at P2 (n=4). Loss of *Nkx6.1* in endocrine precursors favors alternative, non-beta endocrine cell fate choices over beta cell fate. Horm, hormones; Ins, insulin; Glc, glucagon; Som, somatostatin; PP, pancreatic polypeptide; Ghr, ghrelin; endo, endocrine. Scale bar = 50 μm. Error bars represent S.E.M; *p<0.05, **p<0.01.

Ngn3-Cre;Z/EG control embryos (Figure 2.6C). In *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice, GFP^{+} cells were devoid of Nkx6.1 (Figure 2.6D, Figure 2.7B**)**, showing the *Ngn3-Cre* transgene efficiently deletes *Nkx6.1* in Ngn3^{+} cells and their progeny. Ngn3 was similarly expressed in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* and control embryos (Figure 2.6C, D), demonstrating that loss of *Nkx6.1* in endocrine precursors does not affect Ngn3 expression.

Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG mice were born at the expected Mendelian frequency, but died within the first few days after birth from dehydration and hyperglycemia; a phenotype indicative of a beta cell defect. To determine whether loss of *Nkx6.1* affects the cell fate choice of endocrine precursors, we analyzed the fate of Ngn3^{+} cells in *Ngn3-Cre;Z/EG* and *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice. Based on our previous finding that Nkx6.1 prevents acinar cell fate specification (Schaffer et al., 2010), we first examined whether loss of *Nkx6.1* in Ngn3^{+} cells allocates endocrine precursors to the acinar lineage. The ability of Ngn3^{+} cells to undergo endocrine-to-acinar cell fate conversion has been previously demonstrated in conditions of reduced *Ngn3* gene dosage or impaired Notch signaling activity (Cras-Meneur et al., 2009; Wang et al., 2010). At P2, we found GFP^{+} cells to be exclusively restricted to endocrine islets in both *Ngn3-Cre;Z/EG* and *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice (Figure 2.6E, F), revealing that *Nkx6.1* deletion in Ngn3^{+} cells does not cause endocrine-to-acinar fate conversion. Thus, unlike multipotent pancreatic progenitors, which adopt an acinar cell identity in the absence of Nkx6.1 activity (Schaffer et al., 2010), Ngn3^{+} endocrine precursors are no longer competent to activate acinar gene expression programs after deletion of *Nkx6.1*.

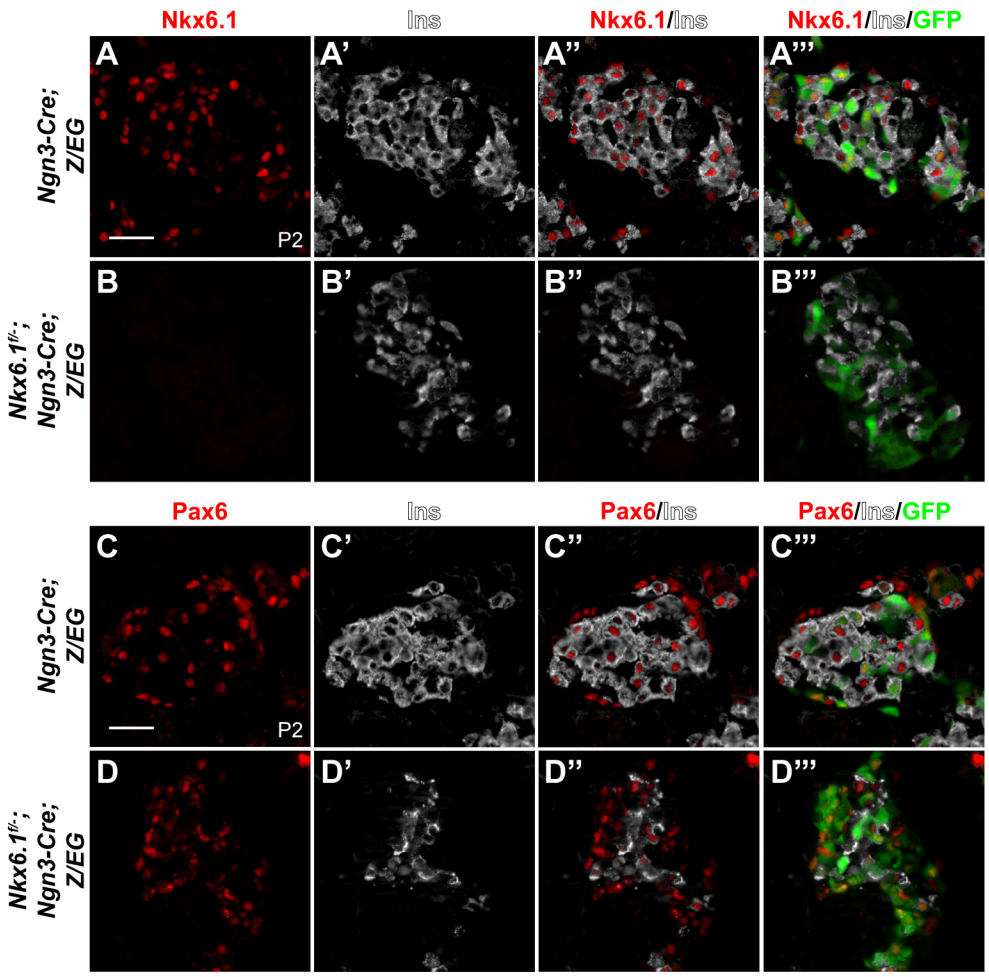


Figure 2.7. Expression of Pax6 is maintained in *Nkx6.1*-deficient cells. Immunofluorescence staining for Nkx6.1 (A, B) and Pax6 (C, D) in pancreata from *Ngn3-Cre;Z/EG* and *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice at postnatal day (P) 2 shows absence of Nkx6.1 and normal expression of Pax6 in *Nkx6.1*-deficient, recombined, insulin^{+}GFP^{+} cells. Ins, insulin. Scale bar = 50 μm.

Because *Nkx6.1*-deficient endocrine precursors differentiate into endocrine cells (Figure 2.6F), we next sought to determine whether loss of *Nkx6.1* affects the relative proportion of the different endocrine cell types arising from Ngn3$^+$ cells. To examine the endocrine cell fate choice of Ngn3$^+$ cells, we quantified how many of the recombined *Ngn3*-expressing cells were allocated to each endocrine cell lineage by co-staining for GFP as a lineage marker of Ngn3-cell progeny together with each of the five hormones individually. At P2, 55.1±1.7% of recombined cells were insulin$^+$ in *Ngn3-Cre;Z/EG* control mice, while only 16.6±5.4% of recombined cells were insulin$^+$ in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice (Figure 2.6G, L, Q; P<0.01), suggesting that *Nkx6.1*-deficient precursors have a lower propensity to differentiate into insulin$^+$ cells. To test whether *Nkx6.1*-deficient Ngn3$^+$ cells instead adopt non-beta endocrine cell identities, we compared the percentage of Ngn3$^+$ cells that contributed to each non-beta endocrine cell lineage in *Ngn3-Cre;Z/EG* and *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice. *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice at P2 exhibited significantly more glucagon$^+$GFP$^+$ (35.9±0.8% vs. 24.6±2.0%; P<0.05), somatostatin$^+$GFP$^+$ (16.6±2.1% vs. 7.2±0.2%; P<0.05), PP$^+$GFP$^+$ (23.3±4.1% vs. 7.5±0.1%; P<0.025), and ghrelin$^+$GFP$^+$ (11.1±1.5% vs. 5.5±0.7%; P<0.05) cells than *Ngn3-Cre;Z/EG* control mice (Figure 2.6H-K, M-Q). Together, these findings suggest that endocrine precursor cells require Nkx6.1 activity to differentiate into beta cells and that Nkx6.1 prevents precursors from adopting non-beta endocrine fates. To ascertain that the differences in islet cell type composition between *Ngn3-Cre;Z/EG* and *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice are indeed the result of preferential precursor cell fate choices and not due to different proliferation or survival rates, we analyzed GFP$^+$ cells in each genotype for their rates of proliferation and apoptosis at P2. Both insulin$^+$GFP$^+$ and insulin$^-$GFP$^+$ endocrine cells displayed similar proliferation and apoptotic rates in *Ngn3-Cre;Z/EG* and

Nkx6.1$^{f/-}$*;Ngn3-Cre;Z/EG* mice (Figure 2.6R, S), demonstrating that loss of *Nkx6.1* does not affect proliferation or survival. Together, we show that Nkx6.1 controls the fate choice between beta and non-beta endocrine cell lineages in endocrine precursor cells, without favoring any one non-beta endocrine cell type in particular.

Nkx6.1-deficient insulin^{+} cells are polyhormonal and ectopically express alpha cell markers.

To investigate whether Nkx6.1 mediates beta cell specification downstream of Ngn3 by regulating transcription factors necessary for beta cell development, we analyzed expression of the beta cell progenitor markers Pax4, MafB, and Pdx1 in *Ngn3-Cre;Z/EG* and *Nkx6.1*$^{f/-}$*;Ngn3-Cre;Z/EG* embryos during the peak period of beta cell differentiation at e15.5. Confirming our previous findings in *Nkx6.1*-null mutant embryos (Henseleit et al., 2005), Pax4 expression was not affected in *Nkx6.1*$^{f/-}$*;Ngn3-Cre;Z/EG* embryos (Figure 2.8A, B; 9.6% of GFP^{+} cells expressed Pax4 in control mice *vs.* 10.1% in *Nkx6.1*$^{f/-}$*;Ngn3-Cre;Z/EG* mice). In contrast, the marker of newly-born alpha and beta cells, MafB, was absent from the majority of targeted cells in *Nkx6.1*$^{f/-}$*;Ngn3-Cre;Z/EG* embryos (Figure 2.8C, D; 76.2% of GFP^{+} cells expressed MafB in control mice *vs.* 20.7% in *Nkx6.1*$^{f/-}$*;Ngn3-Cre;Z/EG* mice), suggesting that MafB, similar to its homolog MafA (Matsuoka et al., 2004), is controlled by Nkx6.1. Likewise, while a large percentage of Ngn3^{+} cell progeny were Pdx1^{+} in control embryos, only a small percentage expressed Pdx1 in *Nkx6.1*-deficient embryos (Figure 2.8E, F; 81.0% of GFP^{+} cells expressed Pdx1 in control mice *vs.* 18.6% of GFP^{+} cells in *Nkx6.1*$^{f/-}$*;Ngn3-Cre;Z/EG* mice). Thus, Nkx6.1 controls the expression of MafB and Pdx1, but not Pax4 in embryonic beta cell precursors.

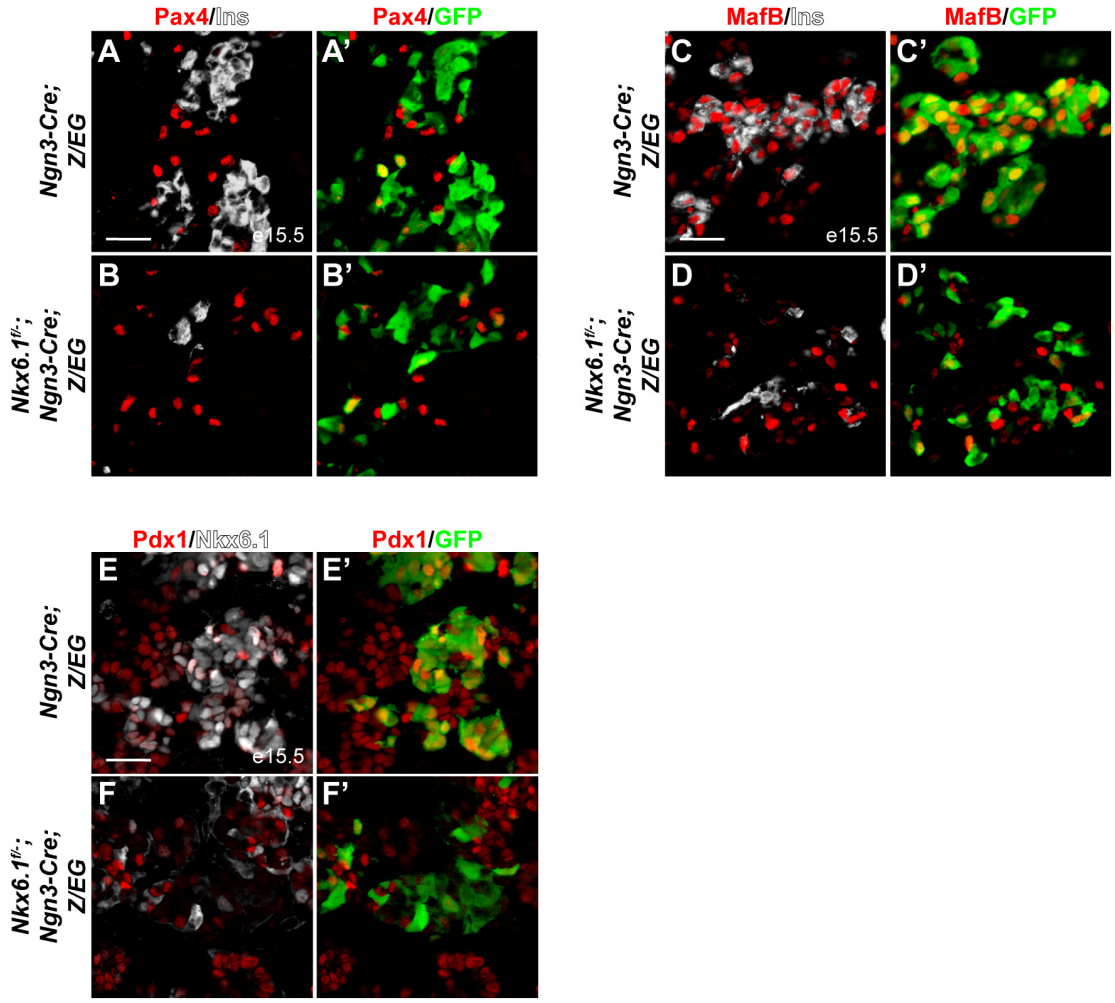
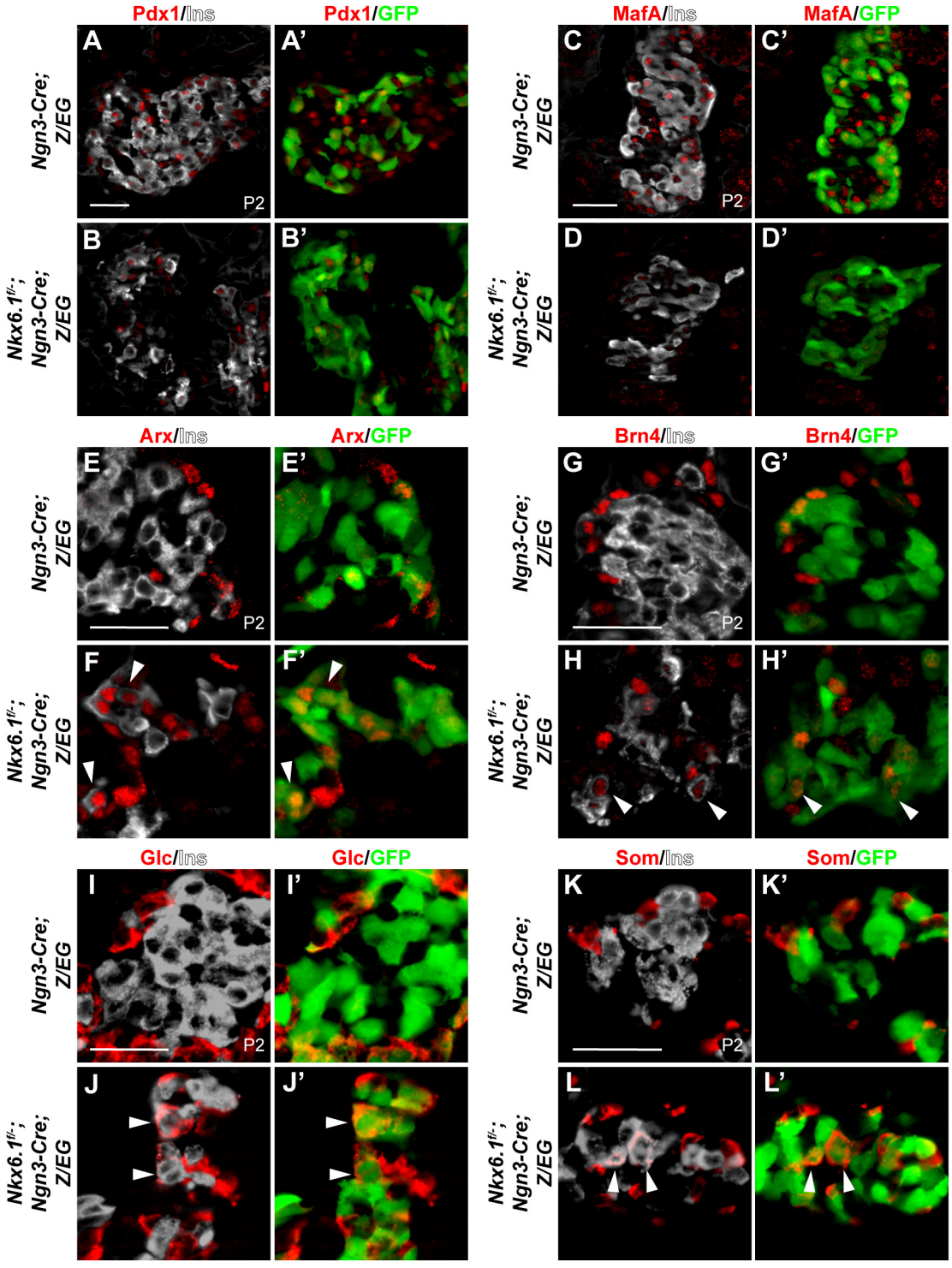


Figure 2.8. *Nkx6.1* controls Pdx1 and MafB expression. Immunofluorescence staining of pancreata at e15.5 shows no difference in Pax4 expression (A, B) in recombined, GFP$^+$ cells between *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* embryos and control *Ngn3-Cre;Z/EG* embryos, while MafB (C, D) and Pdx1 (E, F) expression is reduced in *Nkx6.1*-deficient, GFP$^+$ cells compared to control embryos. Ins, insulin. Scale bar = 50 μm.

Although reduced in numbers, we still observed targeted GFP$^+$ cells expressing insulin in neonatal *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice, raising the question of whether these *Nkx6.1*-deficient insulin$^+$ cells properly differentiate into beta cells. To investigate how lack of Nkx6.1 affects beta cell gene expression programs, we analyzed insulin$^+$ cells in neonatal *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice for the expression of Pax6, Pdx1, and MafA. Expression of the islet cell marker Pax6 was not affected in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice (Figure 2.7C, D; 68.4% of insulin$^+$GFP$^+$ cells expressed Pax6 in control mice *vs.* 72.7% in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice). By contrast, confirming our findings at embryonic stages (Figure 2.8E, F), Pdx1 expression was markedly reduced in *Nkx6.1*-deficient insulin$^+$ cells at P2 (Figure 2.9A, B; 76.5% of insulin$^+$GFP$^+$ cells expressed Pdx1 in control mice *vs.* 30.2% in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice). Similarly, the mature beta cell marker MafA was absent from insulin$^+$ cells in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice (Figure 2.9C, D; 79.3% of insulin$^+$GFP$^+$ cells expressed MafA in control mice *vs.* 0% in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice). This demonstrates that beta cells require Nkx6.1 activity during their differentiation to initiate MafA expression and to maintain high levels of Pdx1. These findings are consistent with the phenotype of *Nkx6.1*-null mutant mice, in which limited numbers of insulin$^+$ cells lacking MafA are observed (Henseleit et al., 2005; Matsuoka et al., 2004). We conclude that insulin expression can still be initiated in the absence of Nkx6.1, but that these insulin$^+$ cells lack key features of normal beta cells.

The lack of beta cell-specific markers in *Nkx6.1*-deficient insulin$^+$ cells raised the question of whether these insulin$^+$ cells also carry features of alternative endocrine lineages. To determine whether loss of *Nkx6.1* in endocrine precursors results in the activation of mixed endocrine gene expression programs, we analyzed insulin$^+$ cells in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice for coexpression of the alpha cell

Figure 2.9. Loss of *Nkx6.1* in endocrine precursors results in activation of non-beta endocrine genes. (A-L) Immunofluorescence staining of pancreata from *Ngn3-Cre;Z/EG* and *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice at postnatal day (P) 2 shows reduced Pdx1 (A, B), absence of MafA (C, D), and ectopic expression of Arx (E, F), Brn4 (G, H), glucagon (Glc; I, J), and somatostatin (Som; K, L) in *Nkx6.1*-deficient, recombined, insulin^{+}GFP^{+} cells. Arrowheads point to insulin^{+} cells ectopically expressing non-beta endocrine markers. Ins, insulin. Scale bar = 50 μm.


Pdx1/Ins
Pdx1/GFP
MafA/Ins
MafA/GFP
Arx/Ins
Arx/GFP
Brn4/Ins
Brn4/GFP
Glc/Ins
Glc/GFP
Som/Ins
Som/GFP
A
A'
B
B'
C
C'
D
D'
E
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F
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G
G'
H
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I
I'
J
J'
K
K'
L
L'
P2
Ngn3-Cre; Z/EG
Nkx6.1fl-; Ngn3-Cre; Z/EG

lineage determinants Arx and Brn4. As expected, in *Ngn3-Cre;Z/EG* control mice virtually no colocalization of Arx and Brn4 with insulin was observed at P2 (Figure 2.9E, G; 0.2% of insulin$^+$GFP$^+$ cells expressed Arx and 0% Brn4 in control mice). In contrast, a subset of recombined insulin$^+$GFP$^+$ cells in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice also expressed Arx and Brn4 (Figure 2.9F, H; arrowheads; 26.1% of insulin$^+$GFP$^+$ cells expressed Arx and 26.5% Brn4 in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice), showing aberrant activation of alpha cell differentiation genes. Notably, *Nkx6.1* deletion or misexpression did not affect Arx expression in the immediate progeny of endocrine precursors at e15.5 (Figure 2.10A-D), pinpointing Nkx6.1-mediated regulation of Arx to a time window between e15.5 and birth.

At P2, loss of Nkx6.1 activity was also associated with aberrant expression of glucagon in insulin$^+$ cells (Figure 2.9I, J; arrowheads in J; 0.5% of insulin$^+$GFP$^+$ cells expressed glucagon in control mice *vs.* 29.8% in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice). Moreover, we found that many of the targeted insulin$^+$ cells ectopically expressed somatostatin in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice (Figure 2.9K, L; arrowheads in L; 0% of insulin$^+$GFP$^+$ cells expressed somatostatin in control mice *vs.* 19.6% in *Nkx6.1$^{f/-}$;Ngn3-Cre;Z/EG* mice). These findings demonstrate that Nkx6.1 is critical for repressing alternative endocrine lineage programs and that beta cell-specific programs can only be induced to a limited extent when *Nkx6.1* is lost.

Previous studies have shown that the number of glucagon$^+$ cells is increased in *Pdx1* heterozygous mutant mice (Dutta et al., 1998; Johnson et al., 2003). Furthermore, beta cells lose Nkx6.1 expression upon *Pdx1* deletion in beta cells (Ahlgren et al., 1998). Combined with our observation that *Nkx6.1* maintains Pdx1 expression during beta cell differentiation, these findings raise the possibility that Pdx1 and Nkx6.1 cooperate through a positive feedback loop to establish and

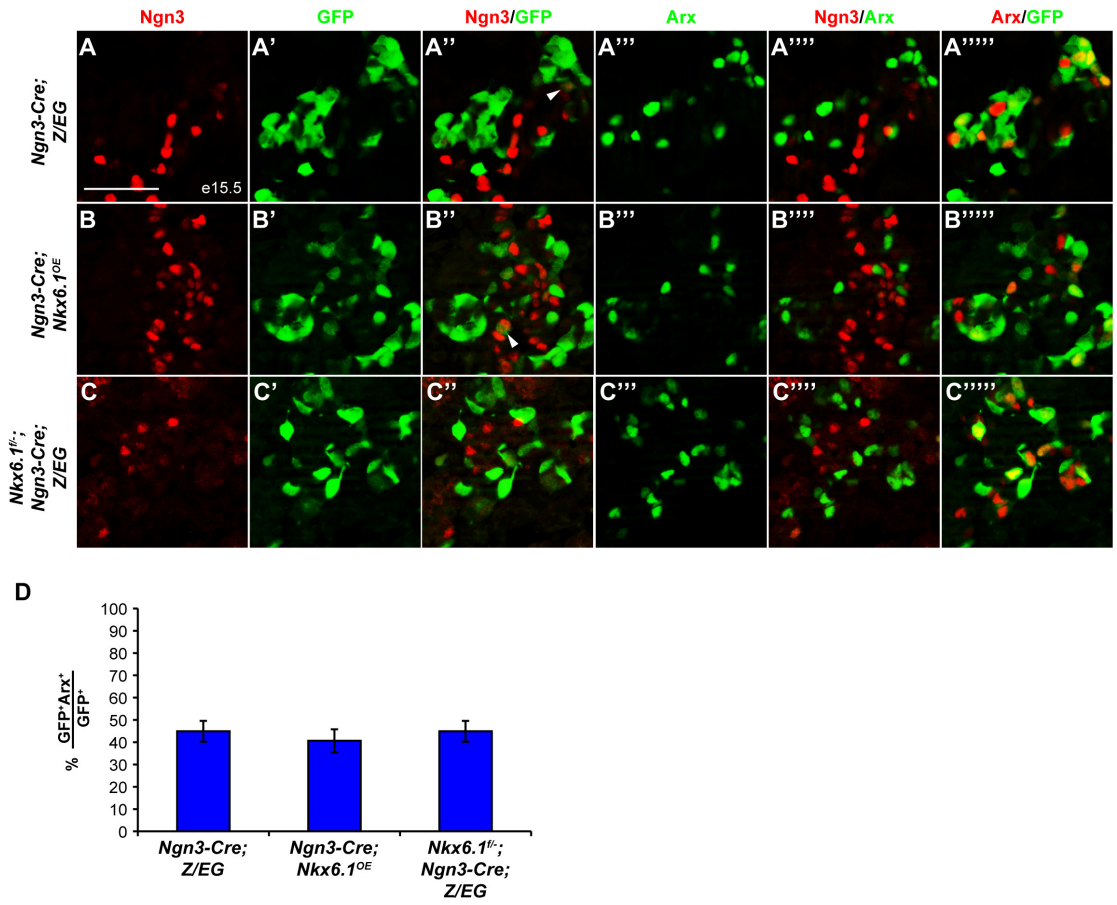


Figure 2.10. *Nkx6.1* gain- or loss-of-function does not affect Arx expression at e15.5. Immunofluorescence staining for GFP, Ngn3, and Arx on pancreata from *Ngn3-Cre;Z/EG* (A), *Ngn3-Cre;Nkx6.1*^{OE} (B), and *Nkx6.1*$^{f/-}$*;Ngn3-Cre;Z/EG* (C) mouse embryos at e15.5 reveals a small subset of GFP^{+} cells expressing Ngn3 (arrowheads in A" and B"), but no coexpression of Arx and Ngn3 (A"", B"", C""). In *Ngn3-Cre;Nkx6.1*^{OE} and *Nkx6.1*$^{f/-}$*;Ngn3-Cre;Z/EG* mice GFP^{+} cells express Arx (B""", C"""). (D) Quantification of the percentage of lineage-labeled *Ngn3*-expressing cells that express Arx in *Ngn3-Cre;Z/EG*, *Ngn3-Cre;Nkx6.1*^{OE}, and *Nkx6.1*$^{f/-}$*;Ngn3-Cre;Z/EG* mice at e15.5 (n=3). Scale bar = 50 μm.

maintain beta cell identity and to repress non-beta endocrine lineage programs. To test this idea, we analyzed *wild-type*, *Nkx6.1$^{f/+}$;Ngn3-Cre, Pdx1$^{+/-}$,* and compound heterozygous *Nkx6.1$^{f/+}$;Ngn3-Cre;Pdx1$^{+/-}$* mice for the ectopic expression of non-beta endocrine hormones in insulin^{+} cells at P2. While we saw no coexpression of somatostatin or PP with insulin in any of the four genotypes (Figure 2.11A-D; data not shown), glucagon and insulin co-positive cells were occasionally detected in all genotypes, including *wild-type* mice (Figure 2.11A-D, I). Quantification of the percentage of insulin^{+} cells also expressing glucagon revealed significantly more dual hormone-positive cells in compound heterozygous *Ngn3-Cre;Nkx6.1$^{f/+}$;Pdx1$^{+/-}$* mice than in either single heterozygous mutant or in *wild-type* mice (Figure 2.11A-D, I). As previously reported (Dutta et al., 1998; Johnson et al., 2003), *Pdx1$^{+/-}$* mice displayed an increase in the glucagon to insulin cell ratio that was also seen in compound heterozygous *Ngn3-Cre;Nkx6.1$^{f/+}$;Pdx1$^{+/-}$* but not in *Nkx6.1$^{f/+}$;Ngn3-Cre* mice (Figure 2.11J). Thus, haploinsufficiency for *Pdx1* but not *Nkx6.1* increases alpha cell numbers, which may reflect a non-cell autonomous effect on alpha cell proliferation, as recently shown in a mouse model of conditional deletion of *Pdx1* in beta cells (Gannon et al., 2008). To determine whether the mixed lineage identity of insulin^{+}glucagon^{+} cells is associated with the expression of Arx, we co-stained pancreatic sections of mice at P2 from all genotypes for insulin, glucagon, and Arx. The majority of insulin^{+}glucagon^{+} cells expressed Arx in all genotypes, although occasional insulin^{+}glucagon^{+}Arx^{-} cells were also detected (Figure 2.11E-H; arrowheads). The observed increase in cells exhibiting mixed alpha/beta cell identity and Arx expression in *Nkx6.1*/*Pdx1* compound heterozygous mice supports the notion that Pdx1 and Nkx6.1 cooperate in beta cell fate specification by preventing

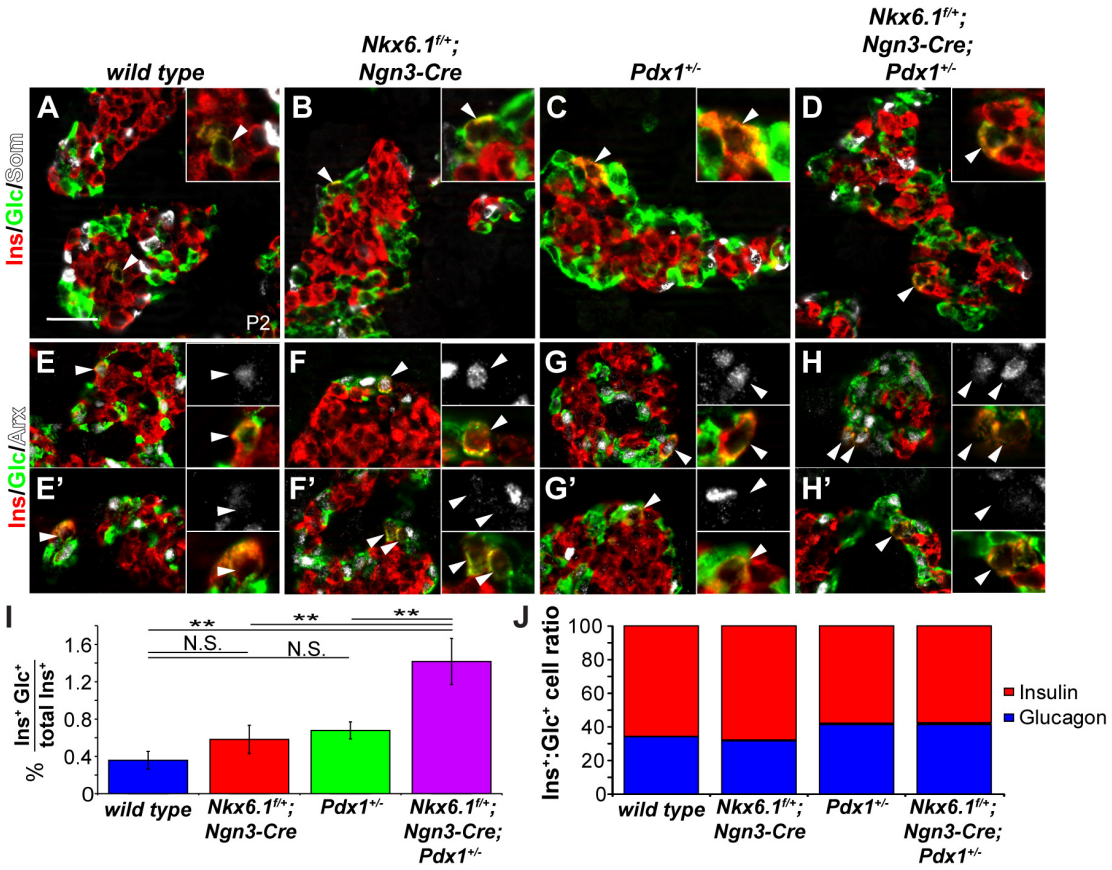


Figure 2.11. *Nkx6.1* and *Pdx1* collectively stabilize beta cell identity. (A-H) Immunofluorescence staining of pancreata from *wild type*, *Nkx6.1*$^{f/+}$*;Ngn3-Cre*, *Pdx1*$^{+/-}$, and *Nkx6.1*$^{f/+}$*;Ngn3-Cre;Pdx1*$^{+/-}$ mice at postnatal day (P) 2 reveals occasional coexpression of insulin with glucagon but not with somatostatin in all genotypes (A-D; arrowheads and insets). Both Arx^{+} (E-H) and Arx^{-} (E'-H') insulin^{+}glucagon^{+} cells (arrowheads and insets) are found in all genotypes. (I) Quantification of the percentage of insulin^{+} cells co-expressing glucagon at P2 reveals significantly more insulin^{+}glucagon^{+} cells in *Nkx6.1*$^{f/+}$*;Ngn3-Cre*;*Pdx1*$^{+/-}$, *Nkx6.1*$^{f/+}$*;Ngn3-Cre,* and *Pdx1*$^{+/-}$ mice compared to *wild-type* controls. In addition, *Nkx6.1*$^{f/+}$*;Ngn3-Cre*;*Pdx1*$^{+/-}$ mice show more insulin^{+}glucagon^{+} cells than either single heterozygous mutant (n=3). (J) Quantification of insulin^{+} and glucagon^{+} cell numbers in P2 pancreata shows an increase in glucagon^{+} cells in *Nkx6.1*$^{f/+}$*;Ngn3-Cre;Pdx1*$^{+/-}$ and *Pdx1*$^{+/-}$ mice compared to *Nkx6.1*$^{f/+}$*;Ngn3-Cre* and *wild-type* mice demonstrating that loss of a single *Nkx6.1* allele does not significantly affect alpha cell numbers (n=3). Ins, insulin; Glc, glucagon; Som, somatostatin. Scale bar = 50 μm. Error bars represent S.E.M; **p<0.01, N.S. = not significant.

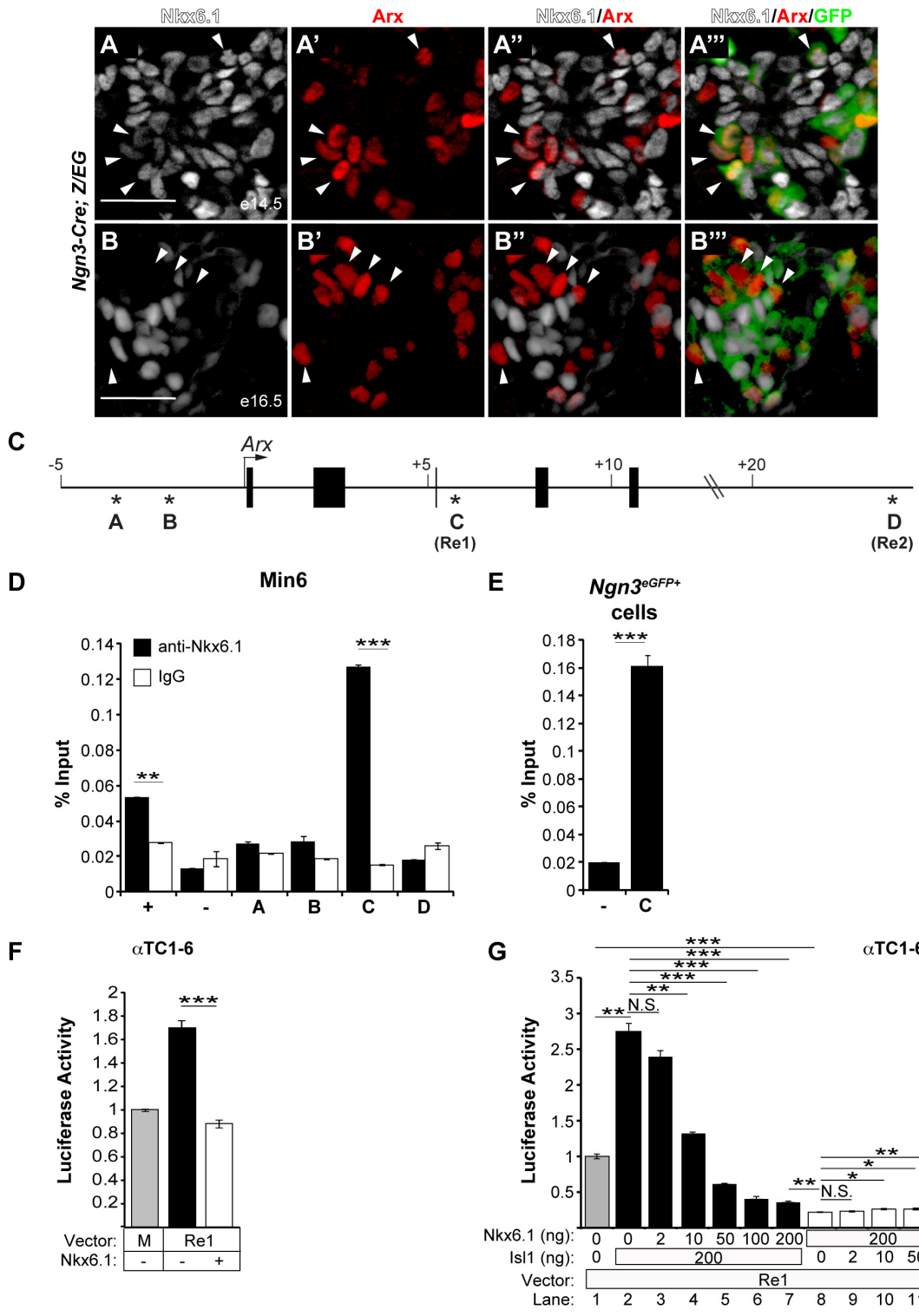
activation of alpha cell-specific gene expression programs during endocrine cell differentiation.

Nkx6.1 is a direct repressor of *Arx* and competes with the *Arx* activator Isl1 at an *Arx* intronic enhancer.

To further explore how Nkx6.1 prevents endocrine precursors from adopting alpha cell identity, we examined the relationship of the Nkx6.1 and Arx expression domains in progeny of *Ngn3*-expressing cells during development. Consistent with the dependence of Arx expression on *Ngn3* (Collombat et al., 2003), Arx was confined to a domain that marks descendants of *Ngn3*-expressing cells (Figure 2.12A'", B'"; note, occasional $Arx^{+}GFP^{-}$ cells in A and B can be explained by mosaic expression of the *Z/EG* and/or *Ngn3-Cre* transgenes). At e14.5, preceding the onset of the major wave of beta cell differentiation, the majority of Arx^{+} cells also expressed Nkx6.1 and $Arx^{+}Nkx6.1^{-}$ cells were rare (Figure 2.12A; arrowheads). By e16.5, however, when large numbers of beta cells arise (Johansson et al., 2007), GFP^{+} cells seldom coexpressed Nkx6.1 and Arx (Figure 2.12B; arrowheads). Thus, endocrine precursors initially activate Nkx6.1 and Arx concomitantly, but their expression domains become mutually exclusive during beta cell differentiation. Together with our finding that Nkx6.1 regulates Arx during this time window, the observed expression pattern of Arx and Nkx6.1 raised the possibility that Nkx6.1 functions as a transcriptional repressor of *Arx*.

To explore this hypothesis, we tested whether Nkx6.1 occupies *Arx* regulatory sequences and is capable of repressing *Arx* transcription. We identified two conserved Nkx6.1 binding motifs within 5 kb of the 5' end flanking region from the *Arx* transcriptional start site (Figure 2.12C; site A and B) as well as 12 potential Nkx6.1

Figure 2.12. Nkx6.1 and Isl1 function as antagonistic transcriptional regulators of the *Arx Re1* enhancer. Immunofluorescence staining of pancreata from *Ngn3-Cre;Z/EG* mice at e14.5 (A) and e16.5 (B) for Nkx6.1, Arx, and GFP shows that the majority of progeny of Ngn3-expressing cells (GFP^+) co-express Arx and Nkx6.1 at e14.5 (arrowheads in A), while the Arx^+ and $Nkx6.1^+$ domains are distinct at e16.5 (arrowheads in B point to $GFP^+Arx^+Nkx6.1^-$ cells). (C) Schematic of the *Arx* locus; asterisks indicate phylogenetically-conserved Nkx6.1 binding motifs and numbers indicate the distance from the transcriptional start site. Nkx6.1 binds to site C (*Re1* element) in the *Arx* locus in chromatin from Min6 cells (D) and FACS-sorted GFP^+ cells (E) from e15.5 pancreata of *Neurog3*^{eGFP} embryos analyzed by ChIP with antibodies against Nkx6.1 or control immunoglobulin G (IgG). Mouse *glucagon* promoter and intergenic primers were used as positive (+) and negative (-) controls, respectively. (F) Co-transfection of αTC1-6 cells with the *Arx Re1* enhancer-luciferase construct, the *CMV-Renilla* expression construct, and with or without the *CMV-Nkx6.1* expression construct. Lane one (M) represents basal luciferase expression of the minimal promoter. Luciferase activity was quantified relative to the expression of the minimal promoter. Activity of the *Re1* enhancer is repressed by Nkx6.1. (G) Co-transfection of αTC1-6 cells with the *Arx Re1* enhancer-luciferase construct, *CMV-Renilla*, and with different concentration of *CMV-Nkx6.1* and *CMV-Isl1*, as indicated. Nkx6.1 prevents activation of the *Arx Re1* enhancer by Isl1 in a dose-dependent manner (lanes 2-7). Luciferase activity was quantified relative to the expression of the *Re1* enhancer. Increasing concentrations of Isl1 are not sufficient to restore baseline activity of the *Re1* enhancer in the presence of 200ng of *CMV-Nkx6.1* (lanes 8-12). Scale bar = 50 μm. Error bars represent S.E.M; *$p<0.05$, **$p<0.01$, ***$p<0.001$.


Nkx6.1
Arx
Nkx6.1/Arx
Nkx6.1/Arx/GFP
Ngn3-Cre; Z/EG
A
A'
A''
A'''
B
B'
B''
B'''
e14.5
e16.5
C
Arx
-5
+5
+10
+20
A
B
C
(Re1)
D
(Re2)
D
Min6
anti-Nkx6.1
IgG
% Input
**
***
+
-
A
B
C
D
E
Ngn3eGFP+
cells
% Input
***
-
C
F
αTC1-6
Luciferase Activity
***
Vector:
Nkx6.1:
M
Re1
G
αTC1-6
Luciferase Activity
N.S.
Nkx6.1 (ng):
Isl1 (ng):
Vector:
Lane:
0 0 2 10 50 100 200 200
0 200 0 2 10 50 100
Re1
1 2 3 4 5 6 7 8 9 10 11 12

binding sites located in two previously characterized enhancers within the third intron of the *Arx* genomic sequence (Figure 2.12C; site C, *Re1*) and the 3' flanking region (Figure 2.12C; site D, *Re2*) (Liu et al., 2011). Recently, the *Arx Re1* and *Re2* enhancers have been shown to be required for Isl1-mediated activation of *Arx* in alpha cells, suggesting that these two enhancers are critical for *Arx* transcription (Liu et al., 2011). Chromatin immunoprecipitation (ChIP) analyses for Nkx6.1 revealed that Nkx6.1 directly and specifically associates with the *Arx Re1* enhancer (site C) in the Min6 beta cell line (Figure 2.12D) and in embryonic endocrine precursors isolated by fluorescence-activated cell sorting (FACS) from *Ngn3*$^{eGFP/+}$ embryonic pancreata at e15.5 (Figure 2.12E). Discordant with a previous report, which reported binding of Nkx6.1 to site B in a beta cell line (Papizan et al., 2011), no association was observed with the other sites (sites A, B, or D) containing Nkx6.1 motifs (Figure 2.12D). Transfection of the αTC1-6 alpha cell line with an expression plasmid for *Nkx6.1* and a luciferase reporter construct containing the *Arx Re1* enhancer sequence revealed that Nkx6.1 significantly reduced reporter gene activity (Figure 2.12F). Confirming previous findings (Liu et al., 2011), transfection of an expression plasmid for *Isl1* activated the *Re1* enhancer (Figure 2.12G). Co-transfection of *CMV-Nkx6.1* abolished the ability of *Isl1* to activate the *Re1* enhancer in a dosage-dependent manner, showing that Nkx6.1 and Isl1 regulate *Arx* antagonistically through competition at the *Re1* enhancer. However, in the presence of *Nkx6.1*, addition of *CMV-Isl1* was not sufficient to revert Nkx6.1-mediated repression (Figure 2.12G), indicating dominance of Nkx6.1 repressive over Isl1 activator activity. While our experiments show that Nkx6.1 is able to repress the Arx *Re1* enhancer in alpha cell lines, Nkx6.1 cannot evoke an alpha-to-beta cell fate change when misexpressed in differentiated alpha cells (Figure 2.4). Thus, Nkx6.1-dependent *Arx* repression

through the *Re1* enhancer appears to be functionally most relevant during endocrine cell type specification, when Nkx6.1 prevents initiation of *Arx* expression (Figure 2.9E, F).

***Nkx6.1* is necessary for maintaining beta cell identity.**

It has recently been shown that Nkx2.2 is an obligatory repressor of *Arx* in differentiated beta cells and that the absence of Nkx2.2 repressor activity causes beta-to-alpha cell conversion in mice (Papizan et al., 2011). Since Nkx2.2 is expressed in both beta and alpha cells, it has been speculated that beta cell-specific repression of *Arx* might depend on Nkx6.1 (Papizan et al., 2011). To directly test this hypothesis, we deleted *Nkx6.1* selectively in beta cells, using the rat *insulin* promoter II (*RIP*)-*Cre* transgene to recombine the *Nkx6.1*^{f} allele and the *Rosa26* (*R26)-YFP* reporter allele. As expected, the YFP lineage label was largely confined to beta cells in control *Nkx6.1*$^{f/+}$*;RIP-Cre;R26-YFP* mice at 6 weeks of age (Figure 2.13A-E, O). In striking contrast, only a few *Nkx6.1*-deficient, YFP-labeled cells expressed insulin in *Nkx6.1*$^{f/-}$*;RIP-Cre;R26-YFP* mice (Figure 2.13F, G, O), suggesting that cells that once activated the *insulin* promoter no longer expressed insulin. Analysis of YFP expression in conjunction with glucagon, somatostatin, and PP revealed that *Nkx6.1*-deficient beta cells adopted delta cell identity, but did not convert into alpha or PP cells (Figure 2.13H-J, O). Thus, loss of *Nkx6.1* in differentiated beta cells no longer causes activation of glucagon and PP, as observed after *Nkx6.1* inactivation in Ngn3^{+} endocrine precursors. These findings suggest that *Nkx6.1* is necessary to repress delta cell-specific genes in beta cells, but that expression of alpha and PP cell-specific genes are inhibited through an Nkx6.1-independent mechanism once beta cells have formed.

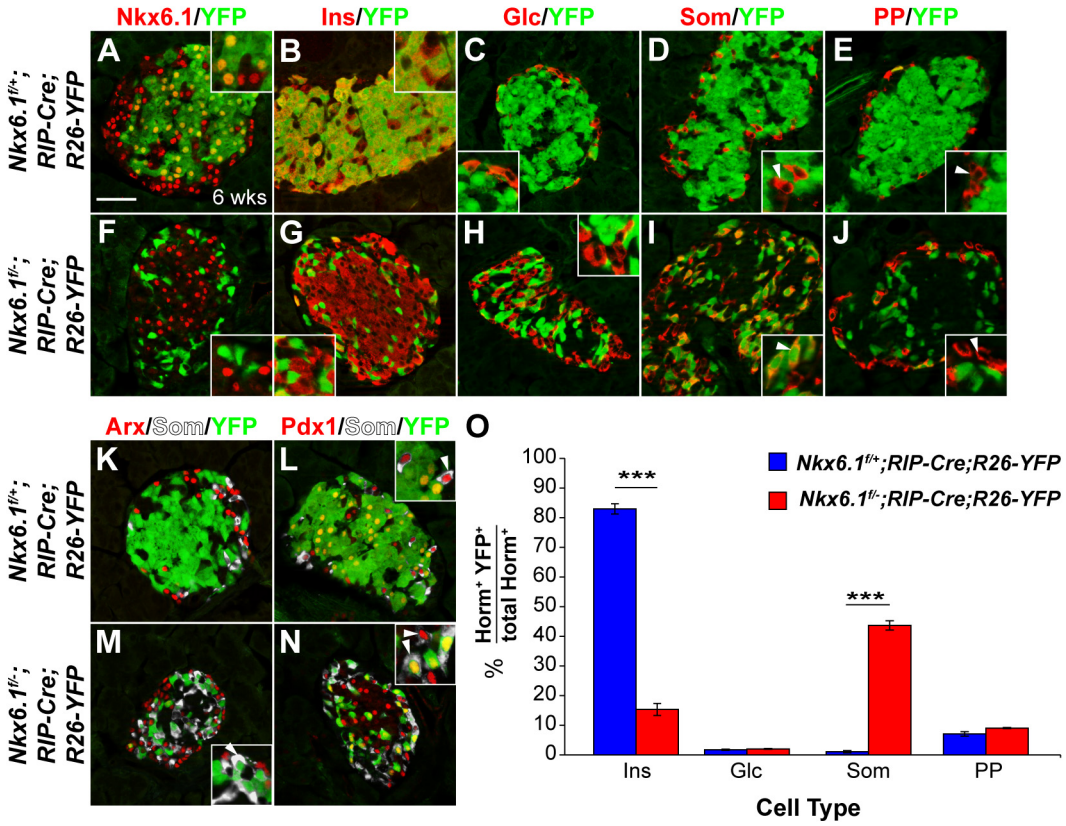


Figure 2.13. Loss of *Nkx6.1* in beta cells causes beta-to-delta cell conversion. Immunofluorescence staining of pancreata from *Nkx6.1$^{f/+}$;RIP-Cre;R26-YFP* and *Nkx6.1$^{f/-}$;RIP-Cre;R26-YFP* mice at 6 weeks of age shows Nkx6.1 (A) and insulin (B) expression in YFP$^+$ cells of *Nkx6.1$^{f/+}$;RIP-Cre;R26-YFP* control mice, but loss of Nkx6.1 (F) and insulin (G) in YFP$^+$ cells of *Nkx6.1$^{f/-}$;RIP-Cre;R26-YFP* mice. The insets display higher magnification images. YFP$^+$ cells do not express glucagon (C, H) and rarely express pancreatic polypeptide (E, J; insets, arrowheads) in either genotype. While YFP$^+$ cells are somatostatin$^-$ in *Nkx6.1$^{f/+}$;RIP-Cre;R26-YFP* mice (D; insets, arrowheads), YFP-labeled cells are mostly somatostatin$^+$ in *Nkx6.1$^{f/-}$;RIP-Cre;R26-YFP* mice (I; insets, arrowheads), suggesting beta-to-delta cell conversion. Arx expression is similar in both genotypes and absent from lineage-labeled YFP$^+$ cells (K, M; inset, arrowhead), showing that loss of *Nkx6.1* in beta cells does not activate *Arx*. Pdx1$^+$somatostatin$^+$ cells are found in both genotypes (L, N; insets, arrowheads), but express YFP only in *Nkx6.1$^{f/-}$;RIP-Cre;R26-YFP* mice (L; inset, arrowhead). (O) Quantification of the percentage of hormone$^+$YFP$^+$ cells relative to all hormone$^+$ cells for each islet cell type shows reduced numbers of insulin$^+$YFP$^+$ cells and increased numbers of in somatostatin$^+$YFP$^+$ cells in *Nkx6.1$^{f/-}$;RIP-Cre;R26-YFP* mice compared to *Nkx6.1$^{f/+}$;RIP-Cre;R26-YFP* mice at 6 weeks (n=3). Wks, weeks; Ins, insulin; Glc, glucagon; PP, pancreatic polypeptide; Som, somatostatin; Horm, hormones. Scale bar = 50 μm. Error bars represent S.E.M; ***p<0.0001.

To further test whether *Nkx6.1* deficiency in beta cells could lead to partial activation of an alpha cell gene expression program, we examined YFP^+ cells for Arx expression. YFP^+ cells rarely expressed Arx in both control and *Nkx6.1$^{f/-}$;RIP-Cre;R26-YFP* mice (Figure 2.13K, M), demonstrating that Nkx6.1 is no longer necessary for *Arx* repression after beta cells have differentiated. Likewise, Pdx1 expression in adult islet cells was Nkx6.1-independent, as somatostatin$^+$ cells that arose from *Nkx6.1*-deficient insulin-expressing cells were $Pdx1^+$ (Figure 2.13L, N). Our data reveal that gene regulation by Nkx6.1 is highly context-dependent. While Nkx6.1 is necessary for *Arx* repression and Pdx1 activation in beta cell precursors, both genes are regulated by Nkx6.1-independent mechanisms in mature beta cells.

DISCUSSION

The specification of pancreatic endocrine cell types is governed by the transcription factors Pdx1, Pax4, and Arx (Collombat et al., 2003; Sosa-Pineda et al., 1997; Yang et al., 2011). However, as none of these transcription factors control the development of solely one endocrine cell type, the molecular mechanisms that confer lineage specificity have remained largely elusive. Here, we demonstrate that the beta cell-specific transcription factor Nkx6.1 is both necessary and sufficient to specify the beta cell lineage (Figure 2.14A). We show that Nkx6.1 and Isl1 antagonistically regulate *Arx* expression in endocrine precursors through a direct transcriptional mechanism (Figure 2.14B). Thus, our study identifies Nkx6.1 as a critical beta cell programming factor that promotes the beta cell fate choice by simultaneously inducing beta cell genes and repressing non-beta endocrine genes.

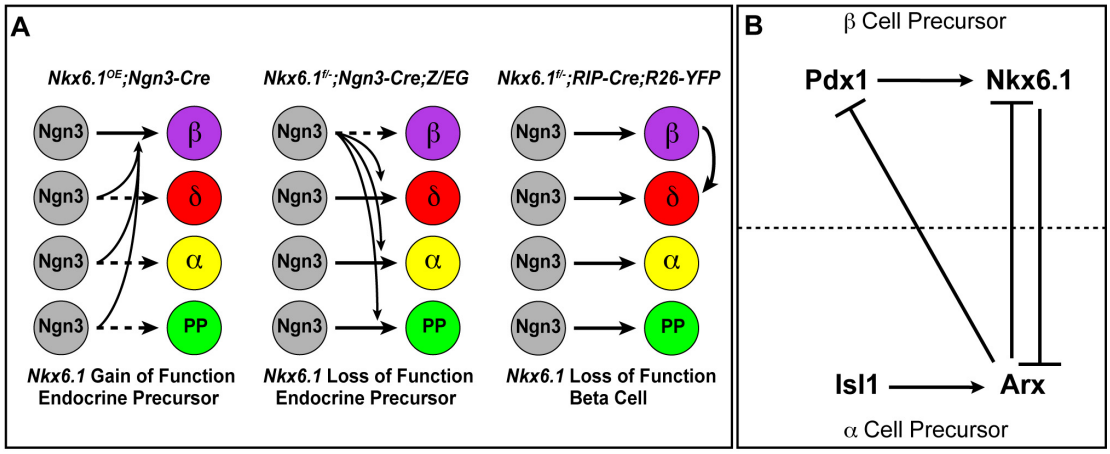


Figure 2.14. Model of Nkx6.1 function in endocrine precursor cells. (A) Expression of *Nkx6.1* results in allocation of precursors from all non-beta endocrine lineages to the beta cell lineage. Deletion of *Nkx6.1* in endocrine precursors has the opposite effect. When *Nkx6.1* is deleted in beta cells, beta cells convert into delta cells, but not into alpha or pancreatic polypeptide (PP)-producing cells. (B) Our study suggests that in endocrine precursors, Nkx6.1 and Isl1 compete for repression and activation, respectively, of the alpha cell fate determinant *Arx*. We also demonstrate that the expression of Pdx1 in endocrine precursors depends on Nkx6.1. In conjunction with previous studies, showing repression of Pdx1 and Nkx6.1 by Arx (Collombat et al., 2007) and activation of Nkx6.1 by Pdx1 (Ahlgren et al., 1998), our data support a model whereby cross-repression between Arx and Nkx6.1 confers alpha *versus* beta cell precursor identity. In beta cell precursors, Nkx6.1 expression is reinforced by Pdx1, which is repressed by Arx in alpha cell precursors.

Nkx6.1: a master regulator of the beta cell fate choice.

We found that forced expression of Nkx6.1 allocates endocrine precursors to the beta cell lineage. While a similar activity has been described for Pdx1 (Yang et al., 2011), Nkx6.1 and Pdx1 display different kinetics of beta cell programming. As evidenced by cells coexpressing insulin, glucagon, and Arx at birth (Yang et al., 2011), forced expression of Pdx1 in endocrine precursor cells initially produces cells with mixed alpha and beta cell identity. In contrast to *Ngn3-Cre;Pdx1*^{OE} mice, we rarely observed cells exhibiting both alpha and beta cell features in neonatal *Ngn3-Cre;Nkx6.1*^{OE} mice, suggesting that Nkx6.1 plays a critical role early during cell fate specification of endocrine precursors. This notion is consistent with our finding that Nkx6.1 acts as a direct transcriptional repressor of the *Arx* gene. While Pax4 has also been shown to act as a direct repressor of *Arx* (Collombat et al., 2005), our observation that Pax4 expression is not affected by *Nkx6.1* ablation suggests that Pax4 alone is not sufficient to repress *Arx* in beta cell precursors. Given the dependence of Nkx6.1 expression on Pax4 activity (Wang et al., 2004), it is possible that the observed derepression of Arx and glucagon in *Pax4* mutant mice (Collombat et al., 2005; Collombat et al., 2003) is a consequence of Nkx6.1-deficiency.

In addition to repressing alpha cell-specific genes through the regulation of *Arx*, Nkx6.1 also reallocated delta, PP, and epsilon cell precursors to the beta cell lineage. Likewise, inactivation of *Nkx6.1* in endocrine precursors resulted in increased production of cells of all non-beta endocrine cell types and ectopic expression of non-beta cell hormones in insulin^{+} cells. The phenotype observed upon conditional activation or deletion of *Nkx6.1* in endocrine precursors identifies Nkx6.1 as a potent general repressor of non-beta endocrine gene expression programs. While our study establishes Nkx6.1 as a direct repressor of the alpha cell fate determinant *Arx*,

Nkx6.1 likely represses additional cell fate determinants critical for the specification of delta, PP, and epsilon cells. As little is known about the transcription factors mediating non-beta endocrine cell fate choices, identification of additional direct target genes for Nkx6.1 in endocrine cell fate specification will have to await future studies.

Similar to Nkx6.1, forced expression of Pax4 also conferred beta cell identity to precursors of all endocrine cell lineages (Collombat et al., 2009). However, in contrast to *Pax4* misexpressing mice (Collombat et al., 2009), we did not observe oversized islets or diabetes in adult mice misexpressing *Nkx6.1*. Our observations in *Ngn3-Cre;Nkx6.1*^{OE} mice are consistent with our previous study, showing that transgenic overexpression of Nkx6.1 in beta cells does not stimulate beta cell proliferation or perturb beta cell function (Schaffer et al., 2011). Thus, despite their shared property as a direct repressor of *Arx* (Collombat et al., 2005), Nkx6.1 and Pax4 must also have distinct targets in endocrine cells. The lack of adverse effects on beta cell function makes Nkx6.1 an excellent candidate for beta cell programming strategies.

The transcriptional network that specifies beta cells.

Unlike Pax4 expression, which we found to be independent of Nkx6.1, maintenance of Pdx1 expression downstream of *Ngn3* required Nkx6.1 activity. Previous genetic studies in mice have shown that the expression of Nkx6.1 in beta cells also depends on Pdx1 activity (Ahlgren et al., 1998), suggesting that these two "pro-beta" transcription factors reinforce each other's expression. Since both Pdx1 and Nkx6.1 control the expression of critical genes for beta cell function, such as *MafA* and *Glut2* ((Ahlgren et al., 1998; Matsuoka et al., 2004; Nelson et al., 2007);

this study), the positive regulatory loop between Pdx1 and Nkx6.1 might be critical for initiating and stabilizing beta cell-specific gene expression programs during endocrine cell differentiation. The mutual reinforcement in gene expression between these two transcription factors also explains why the combined deletion of one copy of *Pdx1* and *Nkx6.1* was sufficient to destabilize the beta cell fate choice and to cause ectopic expression of glucagon in insulin$^+$ cells. Nkx6.1 could regulate *Pdx1* either directly or indirectly by repressing an inhibitor of Pdx1 expression. Since Nkx6.1 directly represses *Arx* and *Arx* has previously been shown to repress both Pdx1 and Nkx6.1 (Collombat et al., 2007), loss of Pdx1 in *Nkx6.1*-deficient precursors is most likely the consequence of *Arx* derepression (Figure 2.14B).

Competence window of beta cell programming.

We found that expression of Nkx6.1 did not force all Ngn3$^+$ cells to differentiate into beta cells and that small numbers of alpha, delta, PP, and epsilon cells expressing Nkx6.1 persisted during adulthood. This implies that the competence of precursors to adopt a beta cell fate upon *Nkx6.1* activation is limited to a short period during development when cells are still plastic and lineage-specific gene expression programs have not been fully established. Our observation that misexpression of Nkx6.1 in alpha cells failed to induce conversion of alpha into beta cells supports the notion that cells quickly lose the competence to respond to Nkx6.1 repressive cues as they undergo endocrine differentiation.

Our study shows that Nkx6.1 occupies the *Arx* enhancer in beta cells (Figure 2.12D), but unlike its inactivation in endocrine precursors, *Nkx6.1* inactivation in beta cells does not activate Arx. This finding suggests that Nkx6.1 is only capable of repressing *Arx* during endocrine cell differentiation. One potential mechanism that

could account for Nkx6.1-independent *Arx* repression in beta cells is DNA methylation, which has recently been shown to occur at the *Arx* locus (Dhawan et al., 2011). Interestingly, deletion of the DNA methyltransferase *Dnmt3a* or *Dnmt1* in beta cells results in *Arx* derepression and spontaneous conversion of beta into alpha cells (Dhawan et al., 2011; Papizan et al., 2011), suggesting that DNA methylation alone is sufficient to keep *Arx* repressed in beta cells. Dnmt3a is recruited to *Arx* by Nkx2.2 and loss of Nkx2.2 repressor activity causes spontaneous conversion of beta into alpha cells (Papizan et al., 2011), demonstrating that Arx expression in beta cells can be readily induced when Nkx2.2 repressor function is removed. Whereas Nkx2.2 forms a complex with Dnmt3a in the 5' regulatory region of *Arx* (Papizan et al., 2011), we show that Nkx6.1 occupies an intronic *Arx* enhancer, where it competes with Isl1 and prevents Isl1 from activating *Arx*. Thus, it appears that DNA methylation-dependent repression of *Arx* is particularly important for keeping Arx repressed in beta cells, whereas Nkx6.1 prevents *Arx* activation in differentiating beta cell precursors. Overall, the role of DNA methylation in restraining cell plasticity during development is still poorly understood. Knowledge of how islet cell type-specific genes are epigenetically modified as cells differentiate and how this process can be reversed will prove important for devising effective cell programming and reprogramming strategies.

Our study highlights the importance of Nkx6.1 as a beta cell programming factor during endocrine cell differentiation and shows that insulin expression can be initiated independent of Nkx6.1 and Pdx1. Strikingly, the insulin^{+}Nkx6.1^{-}Pdx1^{-} cells observed after conditional inactivation of *Nkx6.1* in Ngn3^{+} cells display a similar molecular profile as insulin^{+} cells generated *in vitro* with current hESC differentiation protocols. Like *Nkx6.1*-deficient insulin^{+} cells in *Nkx6.1$^{f/-}$;Ngn3-Cre* mice, hESC-

derived insulin^{+} cells are polyhormonal and fail to express Nkx6.1, Pdx1, MafA, and critical glucose transporters [(D'Amour et al., 2006; Kroon et al., 2008); Sander laboratory, unpublished data], which suggests that Nkx6.1 is required for complete beta cell programming in mice and humans. Our studies now pave the way for exploring the effectiveness of Nkx6.1 in (re)-programming strategies to generate functional beta cells for diabetes therapy.

MATERIALS AND METHODS

Nkx6.1 gene targeting, mice and glucose tolerance test.

CAG-Bgeo,-Nkx6.1,-eGFP (*Nkx6.1*^{OE}) (Schaffer et al., 2010), *Nkx6.1*$^{+/-}$ (Sander et al., 2000b), *Pdx1*$^{+/-}$ (Offield et al., 1996), *Neurog3*^{eGFP} (Lee et al., 2002), *Prm1-Cre* (Matsumura et al., 2004), *Ngn3-Cre* (Schonhoff et al., 2004), *RIP-Cre* (Postic et al., 1999), *Rosa26-YFP* (Srinivas et al., 2001), *CAG-Bgeo,-eGFP* (*Z/EG*) (Novak et al., 2000), and *Glc-Cre* mice (Herrera, 2000) have been previously described. To create the *Nkx6.1*^{flox} (*Nkx6.1*^{f}) allele, a targeting vector consisting of two *loxP* sites inserted into the first and second introns of *Nkx6.1* was generated (Figure 2.5A). The *herpes simplex virus-thymidine kinase* gene was placed outside of the *Nkx6.1* gene homology region for negative selection. After electroporation of 129S6-derived mouse embryonic stem cells, 375 clones survived *neomycin*^{R} selection. Southern blotting identified 14 clones as correctly targeted. Two clones carrying the *Nkx6.1*$^{flox\text{-}PGK\text{-}Neo}$ allele were independently injected in mouse blastocysts, and chimeric mice bred with C57BL/6J mice for germline transmission screening. The FRT-flanked *neomycin*^{R} gene in intron 2 was subsequently removed by crossing *Nkx6.1*$^{flox\text{-}PGK\text{-}Neo}$ mice with *ActB-FlpE* mice (JAX; more information at http://www.mmrrc.org/strains/29994/029994.html).

Midday on the day of vaginal plug appearance was considered e0.5. Glucose tolerance tests were performed as previously described (Schaffer et al., 2011).

Immunohistochemistry, morphometry, and cell quantification.

Tissue preparation, immunofluorescence and TUNEL staining were performed as previously described (Seymour et al., 2008). For detection of nuclear antigens, antigen retrieval was performed in pH 6.0 citrate buffer and sections were permeabilized in 0.15% Triton X-100 in PBS. The following primary antibodies were used at the given dilutions: mouse anti-insulin (Sigma), 1:5000; guinea pig anti-insulin (Dako), 1:2000; guinea pig anti-glucagon (Sigma), 1:2000; rabbit anti-PP (Dako), 1:2000; goat anti-ghrelin (Santa Cruz), 1:1000; rabbit anti-somatostatin (Dako), 1:3000; goat anti-chromogranin A (Santa Cruz), 1:500; rat anti-GFP (C. Kioussi), 1:1000; mouse anti-Nkx6.1 (BCBC clone #2023; against C-terminal part of Nkx6.1), 1:500; guinea pig anti-Ngn3 (Henseleit et al., 2005), 1:2000; rabbit anti-Pax6 (Chemicon), 1:1000; rabbit anti-Brn4 (M. Rosenfeld), 1:500; rabbit anti-Ki67 (Lab Vision), 1:500; rabbit anti-MafB (Bethyl Labs), 1:1000; rabbit anti-MafA (Bethyl Labs), 1:1000; rabbit anti-Pax4 (B. Sosa-Pineda), 1:100; rabbit anti-Arx (P. Collombat), 1:500; rabbit anti-Arx (K. Morohashi), 1:250; guinea-pig anti-Pdx1 (C. Wright), 1:10,000. Staining with antibodies raised in mice was conducted using the M.O.M. Kit (Vector Labs) in conjunction with streptavidin-conjugated secondary antibodies (Jackson ImmunoResearch). When necessary, nuclei were counterstained with Hoechst 33342 (Invitrogen) at 10 μg/ml. Primary antibodies were detected with donkey-raised secondary antibodies conjugated to Cy3, Cy5, DyLight488 (Jackson ImmunoResearch) or Alexa488 (Molecular Probes) at 1:1500 dilution (1:500 for Cy5). ApoTome images were captured on a Zeiss Axio Observer Z1 microscope with Zeiss

AxioVision 4.8 and figures prepared with Adobe Photoshop/Illustrator CS4. Where necessary, the Cy5 channel was pseudo-colored white. Images were processed in accordance with the *Journal of Cell Biology* figure manipulation guidelines.

For all morphometric analyses and cell quantifications, a total of 10 sections per mouse from at least three mice per genotype were analyzed. For TUNEL, proliferation, and cell lineage analyses, the number of $GFP^+Hoechst^+marker^+$ cells was manually counted, divided by the total number of $GFP^+Hoechst^+$ (*Ngn3-Cre*-mediated lineage tracing) or $marker^+Hoechst^+$ (*RIP-Cre*-mediated lineage tracing) cells, and multiplied by 100. For the analysis of *Pdx1* and *Nkx6.1* single and compound heterozygous mice, sections were stained for insulin, glucagon, and somatatostatin and all $marker^+$ cells (on average 2100 cells per pancreas) were manually counted. $Insulin^+$ cells were analyzed for the expression of glucagon or somatostatin. For quantification of $GFP^+marker^+$ cells, on average 500 GFP^+ cells from at least five different sections per mouse were counted.

For islet cell mass measurements, images covering an entire pancreas section were tiled using a Zeiss Axio Oberver Z1 microscope with the Zeiss ApoTome module. The $hormone^+$ area and total pancreas area were measured using ImagePro Plus 5.0.1 software (Media Cybernetics) and islet cell mass was calculated as follows: ($hormone^+$ area/total pancreatic area) multiplied by pancreatic weight.

Western blot, ChIP, and reporter gene assays.

Western blot analysis using anti-Nkx6.1 (P. Serup) and anti-HDAC1 (Santa Cruz) antibodies was performed as previously described (Schaffer et al., 2011).

Based on previously identified motifs (Jorgensen et al., 1999; Mirmira et al., 2000), a custom positional weight matrix was used to identify putative Nkx6.1 binding

sites. ChIP assays were performed as described (Gerrish et al., 2001), using Nkx6.1 (P. Serup, 1:250) or rabbit IgG antisera. Immunoprecipitations were performed on Min6 cells (ATCC) or on 1.5 x 10^6 GFP^+ cells isolated from 300 pancreata of *Neurog3*$^{eGFP/+}$ embryos at e15.5 by fluorescence activated cell sorting. Each ChIP assay was quantified in triplicate by qPCR. The following primer sequences were used: (site A) 5'-CAT CCG GTG ATA CTG GAA GCC C -3' and 5'-GTC TTT ATC TGA GGG GGG GCT G -3'; (site B) 5'-GCA GAG GGG GGA GGA GGG -3' and 5'-CGG CAG GGA AAT CCA CAA AAC -3'; (site C; Re1) 5'- CCA TTT GAA GGC AAA ATG CT -3' and 5'- GTA TGG GCT GCA AAC ACC TT -3'; (site C; Re2) 5'- TGA AGT GGC TGA ATG AGA GC -3' and 5'- AGT TGG AGC GCG TTT TGT AG -3'; *glucagon* 5'- AAG CAG ATG AGC AAA GTG AGT G -3' and 5'- AGG CTG TTT AGC CTT GCA GAT A -3'; and intergenic control 5'- CAC TCA GAT CCT GAG CCA CA -3' and 5'-GCT CTC TGC CTT CCA CTT TG -3' .

The *Isl1 Re1* enhancer and *CMV-Isl1* constructs as well as procedures for transient transfections and luciferase assays have been described previously (Liu et al., 2011). Unless indicated otherwise, 0.1 µg of *CMV-Nkx6.1* was transfected. Luciferase and Renilla expression were measured 48 hours post transfection. For each data point relative luciferase activity was quantified as the total luciferase units divided by the total Renilla units. All reporter gene analyses were performed in triplicate.

Statistical analysis.

All values are shown as mean ± standard error of the mean (SEM); *p*-values were calculated using student's 2-tailed *t*-test; $P<0.05$ was considered significant.

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Chapter 2, in full, is a reprint of the material as it appears in Schaffer, A.E.*, Taylor, B.L.*, Benthuysen, J.R., Liu, J., Thorel, F., Yuan, W., Jiao, Y., Kaestner, K.H., Herrera, P.L., Magnuson, M.A., May, C.L., and Sander, M.A. (2013). Nkx6.1 controls a gene regulatory network required for establishing and maintaining pancreatic Beta cell identity. PLoS Genet *9*, e1003274. (* denotes equal contribution). The dissertation author contributed equally with Ashleigh Schaffer as the primary investigators and authors of this study.

CHAPTER 3:

Nkx6.1 is essential for maintaining the functional state of pancreatic beta cells

ABSTRACT

Recently, loss of beta cell-specific traits has been proposed as an early cause of beta cell failure in diabetes. However, the molecular mechanisms that underlie this loss of beta cell features remain unclear. Here, we identify an Nkx6.1-controlled gene regulatory network as essential for maintaining the functional and molecular traits of mature beta cells. Conditional *Nkx6.1* inactivation in adult mice caused rapid-onset diabetes and hypoinsulinemia. Genome-wide analysis of Nkx6.1-regulated genes and functional assays further revealed a critical role for Nkx6.1 in the control of insulin biosynthesis, insulin secretion and beta cell proliferation. Over time, Nkx6.1-deficient beta cells acquired molecular characteristics of delta cells, revealing a molecular link between impaired beta cell functional properties and loss of cell identity. Given that Nkx6.1 levels are reduced in human type 2-diabetic beta cells, our study lends support to the concept that loss of beta cell features could contribute to the pathogenesis of diabetes.

INTRODUCTION

Type 2 diabetes mellitus (T2D) is characterized by reduced insulin sensitivity of insulin target tissues and impaired insulin secretion by pancreatic beta cells. Although both of these factors play a role, genetic studies suggest that the ability of beta cells to respond to metabolic stressors is the predominant factor in determining the predisposition to T2D (Muoio and Newgard, 2008).

In T2D, beta cells exhibit an impaired capacity to compensate for increased insulin demand (Cerasi and Luft, 1967), a defect that has been ascribed to both inadequate cellular capacity to secrete insulin (Hosker et al., 1989) and beta cell death (Butler et al., 2003). Among the earliest defects observed in T2D patients is a reduced ability of beta cells to secrete insulin in response to elevated blood glucose levels (Hosker et al., 1989). This impairment in glucose-stimulated insulin secretion has been attributed to defects in glucose sensing (Froguel et al., 1992), mitochondrial dysfunction (Supale et al., 2012), as well as to oxidative stress (Robertson, 2004). Thus, mounting evidence suggests that defects in multiple cellular processes can compromise beta cell function and could be a factor in T2D development. Furthermore, hyperglycemia has been shown to impair the expression of genes important for beta cell identity (Jonas et al., 1999). More recently, Talchai et al. (Talchai et al., 2012) described a loss of beta cell features, characterized by a decline in insulin production, acquisition of progenitor-like characteristics, and fate conversion into other endocrine cell types in mouse models of T2D, suggesting that loss of the differentiated beta cell state also contributes to beta cell failure in T2D. However, it is currently unknown whether the loss of beta cell functional properties, namely regulated insulin secretion, and loss of beta cell identity are linked during T2D progression. A simultaneous loss of beta cell function and identity could be explained

by reduced expression of a central transcriptional regulator controlling genes involved in both processes.

Several lines of evidence suggest that the beta cell-enriched transcription factor Nkx6.1 could have a role in T2D. First, genome wide association studies suggest that variants of *Nkx6.1* associate with T2D (Yokoi et al., 2006). Second, decreased Nkx6.1 expression has been shown to accompany the development of T2D in rodents and humans (Guo et al., 2013; Talchai et al., 2012). Third, *in vitro* studies in beta cell lines and isolated islets suggest a possible role for Nkx6.1 in the regulation of glucose-stimulated insulin secretion as well as beta cell proliferation (Schisler et al., 2008; Schisler et al., 2005). Additionally, we have recently shown that Nkx6.1 is necessary and sufficient to confer beta cell identity to differentiating endocrine precursors in the embryo (Schaffer et al., 2013), raising the possibility that Nkx6.1 could also help maintain the differentiated state of adult beta cells. Together, these findings suggest that Nkx6.1 may be a regulator of beta cell function and identity in adult animals.

To explore the role of Nkx6.1 in mature beta cells, we ablated *Nkx6.1* specifically in beta cells of adult mice and identified Nkx6.1 target genes in beta cells by combining gene expression profiling and chromatin immunoprecipitation with massively parallel sequencing (ChIP-seq). We found that loss of Nkx6.1 causes rapid onset diabetes due to defects in insulin biosynthesis and secretion. The observed loss in insulin production and beta cell functional properties was later accompanied by ectopic activation of delta cell genes in beta cells. Thus, by impairing beta cell function and destabilizing beta cell identity, reduced Nkx6.1 levels, as seen in T2D, could contribute to the pathogenesis of T2D.

RESULTS

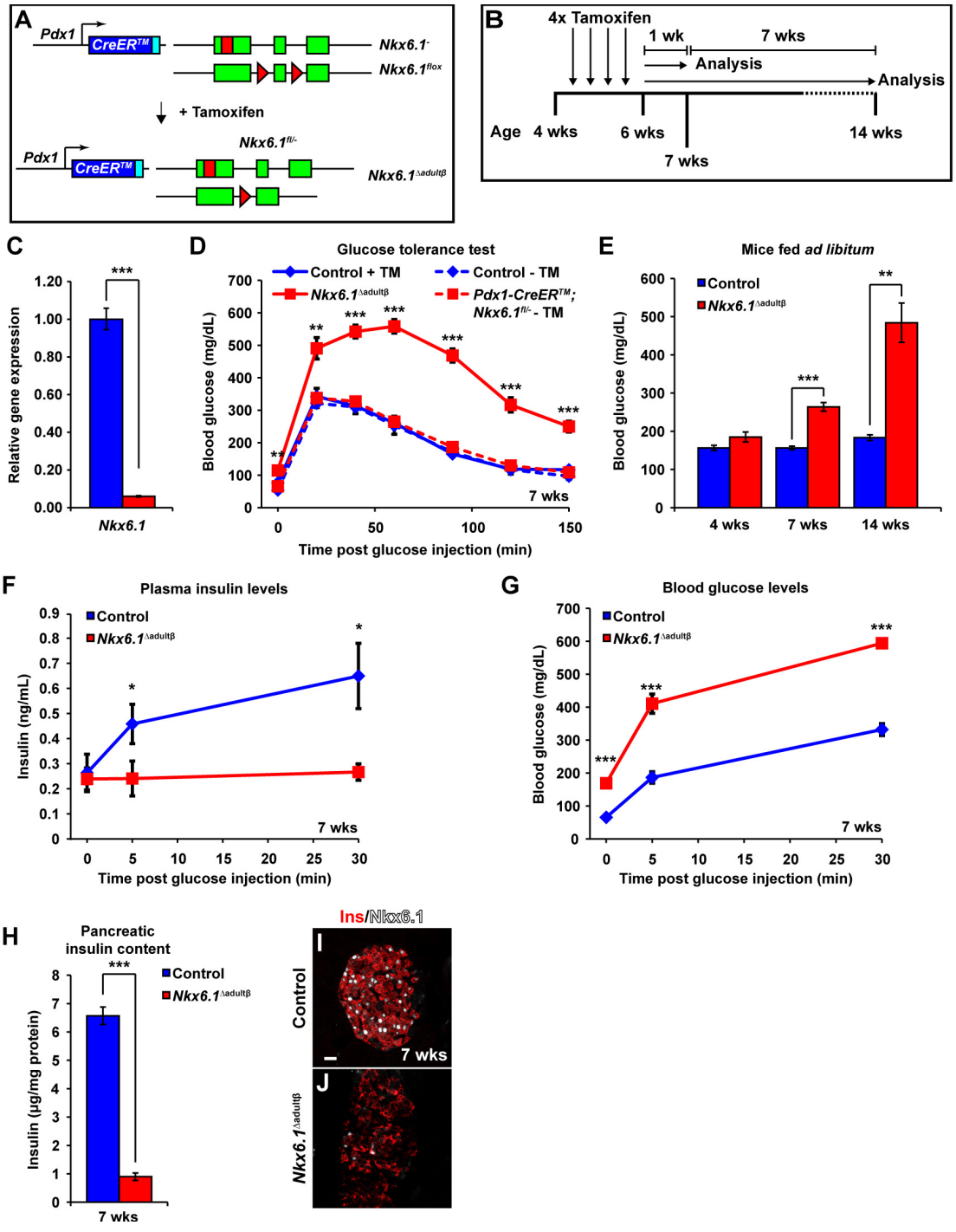
Loss of *Nkx6.1* in mature beta cells causes diabetes and reduced insulin production.

To examine Nkx6.1 function in mature beta cells in vivo, we conditionally inactivated *Nkx6.1* in islet cells of adult mice by triggering recombination of an *Nkx6.1*^{flox} (*Nkx6.1*^{fl}) allele with the tamoxifen-inducible *Pdx1-CreER*^{TM} transgene. *Pdx1-CreER*^{TM}*;Nkx6.1*$^{fl/-}$ and *Pdx1-CreER*^{TM}*;Nkx6.1*$^{fl/+}$ mice were injected with tamoxifen between 4 and 6 weeks of age to produce *Nkx6.1*$^{\Delta adult\beta}$ and control mice, respectively (Figure 3.1A,B). Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and immunofluorescence staining for Nkx6.1 demonstrated efficient recombination of the *Nkx6.1*^{fl} allele in beta cells (Figure 3.1C,I,J).

To determine whether *Nkx6.1* deletion affects beta cell function, we conducted glucose tolerance tests and measured blood glucose levels. Glucose tolerance tests performed one week after the last tamoxifen injection revealed elevated fasting blood glucose levels and glucose intolerance in male *Nkx6.1*$^{\Delta adult\beta}$ mice when compared to tamoxifen-treated and non-tamoxifen-treated control mice (Figure 3.1D). Likewise, blood glucose levels were significantly elevated in *Nkx6.1*$^{\Delta adult\beta}$ mice fed *ad libitum*, with levels reaching near 500 mg/dL within eight weeks after the last tamoxifen injection (Figure 3.1E). Female *Nkx6.1*$^{\Delta adult\beta}$ mice also became diabetic, but the phenotype developed slightly later and was less severe than in males (Figure 3.2A-C). Thus, loss of Nkx6.1 in adult beta cells causes rapid development of diabetes.

To investigate whether diabetes in *Nkx6.1*$^{\Delta adult\beta}$ mice is caused by insulin insufficiency, we measured plasma insulin levels after glucose administration. As expected, control mice responded to a glucose bolus with a rapid increase in plasma insulin levels within 5 minutes of glucose administration (Figure 3.1F). By contrast,

Figure 3.1. Deletion of *Nkx6.1* in adult beta cells results in diabetes and loss of pancreatic insulin. (A) Schematic of alleles and transgenes used to inactivate *Nkx6.1* in adult beta cells. Rectangles, coding sequences; Triangles, *loxP* sites. (B) Schematic of experimental design. (C) qRT-PCR analysis of isolated islets shows a significant reduction of *Nkx6.1* in *Nkx6.1*$^{\Delta adult\beta}$ mice (n=3). (D) Intraperitoneal glucose tolerance test reveals glucose intolerance in male *Nkx6.1*$^{\Delta adult\beta}$ mice compared to non-injected and tamoxifen (TM)-treated control mice at 7 wks (n=6). Solid lines, post-TM treatment; Dashed lines, without TM treatment. (E) Blood glucose measurements of mice fed *ad libitum* show diabetes in male *Nkx6.1*$^{\Delta adult\beta}$ mice (n=6). (F,G) *Nkx6.1*$^{\Delta adult\beta}$ mice have lower plasma insulin levels and elevated blood glucose after a glucose stimulus compared to control mice (n=6). (H) Pancreatic insulin content normalized to protein concentration is reduced in *Nkx6.1*$^{\Delta adult\beta}$ mice (n=6). (I,J) Immunofluorescence staining reveals almost complete absence of Nkx6.1 and reduced insulin expression in *Nkx6.1*$^{\Delta adult\beta}$ mice at 7 wks. Scale bar = 20 μm. Ins, insulin; Wk, week. Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.


A
Pdx1
CreER™
Nkx6.1-
Nkx6.1flox
+ Tamoxifen
Nkx6.1fl/-
Nkx6.1Δadultβ
B
4x Tamoxifen
1 wk
7 wks
Analysis
Analysis
Age 4 wks
6 wks
14 wks
7 wks
C
Relative gene expression
Nkx6.1
D
Glucose tolerance test
Control + TM
Control - TM
Nkx6.1Δadultβ
Pdx1-CreER™; Nkx6.1fl/- - TM
Blood glucose (mg/dL)
7 wks
Time post glucose injection (min)
E
Mice fed ad libitum
Control
Nkx6.1Δadultβ
Blood glucose (mg/dL)
4 wks
7 wks
14 wks
F
Plasma insulin levels
Control
Nkx6.1Δadultβ
Insulin (ng/mL)
7 wks
Time post glucose injection (min)
G
Blood glucose levels
Control
Nkx6.1Δadultβ
Blood glucose (mg/dL)
7 wks
Time post glucose injection (min)
H
Pancreatic insulin content
Control
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7 wks
J
Nkx6.1Δadultβ

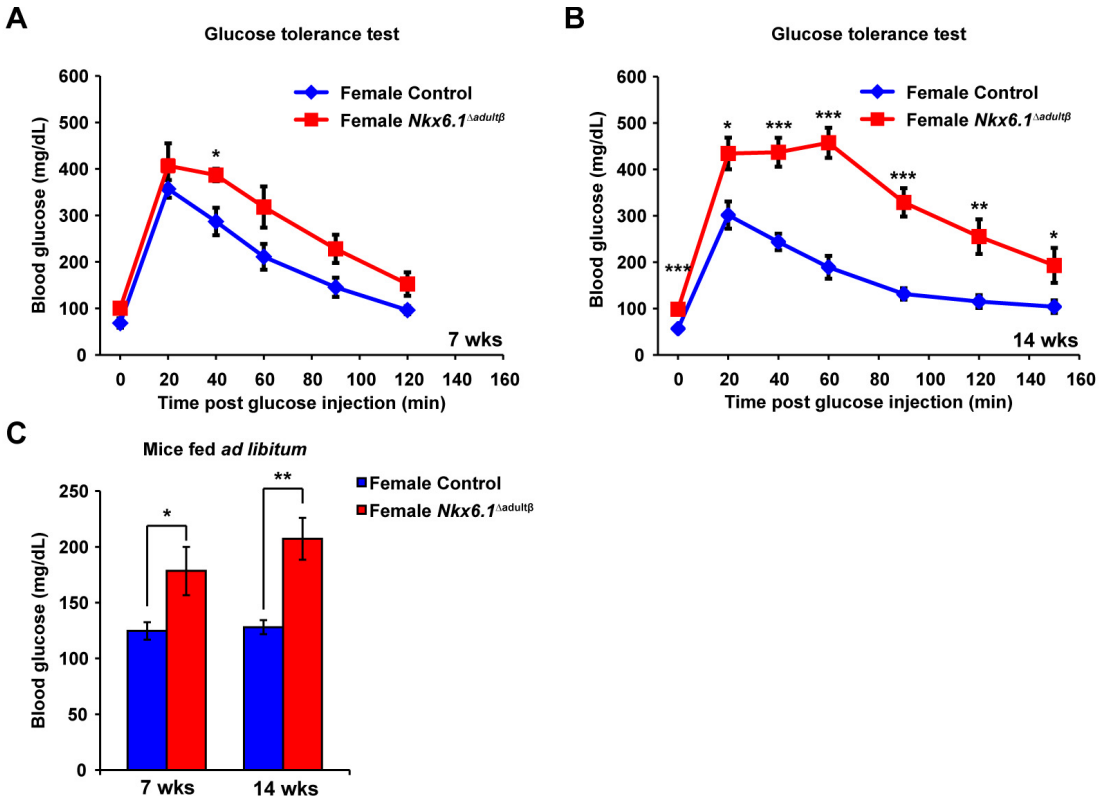


Figure 3.2. Female *Nkx6.1*$^{\Delta adult\beta}$ mice progress to diabetes less rapidly than males. (A-C) Intraperitoneal glucose tolerance tests (A,B) and blood glucose measurements of mice fed *ad libitum* (C) reveal glucose intolerance in female *Nkx6.1*$^{\Delta adult\beta}$ mice at 14 wks (n=6). Wk, week. Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

insulin levels did not increase in *Nkx6.1*$^{\Delta adult\beta}$ mice and blood glucose levels were significantly higher than in control mice (Figure 3.1F,G). A striking reduction in pancreatic insulin content in *Nkx6.1*$^{\Delta adult\beta}$ mice (Figure 3.1H) further demonstrated decreased overall pancreatic insulin production. To determine whether the reduction in pancreatic insulin levels in *Nkx6.1*$^{\Delta adult\beta}$ mice is a result of beta cell loss, we next examined beta cell survival and quantified beta cell mass. *Nkx6.1*$^{\Delta adult\beta}$ mice did not show increased beta cell apoptosis (Figure 3.3A-D) or reduced mass of endocrine or beta cells (Figure 3.3E-H), suggesting that diabetes in *Nkx6.1*$^{\Delta adult\beta}$ mice is caused by decreased insulin biosynthesis rather than beta cell loss. Consistent with a possible defect in the cellular production of insulin, the insulin fluorescence signal was dramatically reduced in Nkx6.1-deficient beta cells (Figure 3.1I,J). Notably, reduced insulin production did not appear to be accompanied by a change in endocrine cell type identity, as insulin^{+} cells in *Nkx6.1*$^{\Delta adult\beta}$ mice did not co-express other pancreatic hormones (Figure 3.3I-N). These data suggest that diabetes following *Nkx6.1* inactivation is initially caused, at least in part, by loss of insulin production but not cell death or conversion into other endocrine cell types.

Nkx6.1 directly regulates islet transcription factors and genes involved in glucose metabolism and insulin biosynthesis.

To more globally understand how loss of Nkx6.1 impacts beta cell gene expression and to identify molecular pathways immediately affected following *Nkx6.1* inactivation, we next conducted transcriptional profiling of *Nkx6.1*$^{\Delta adult\beta}$ and control islets 3 days after completion of tamoxifen-induced *Nkx6.1* ablation (Figure 3.4A). At this time point, *Nkx6.1*$^{\Delta adult\beta}$ mice were only mildly glucose intolerant and blood glucose levels of mice fed *ad libitum* were still below 250 mg/dL (Figure 3.5A,B).

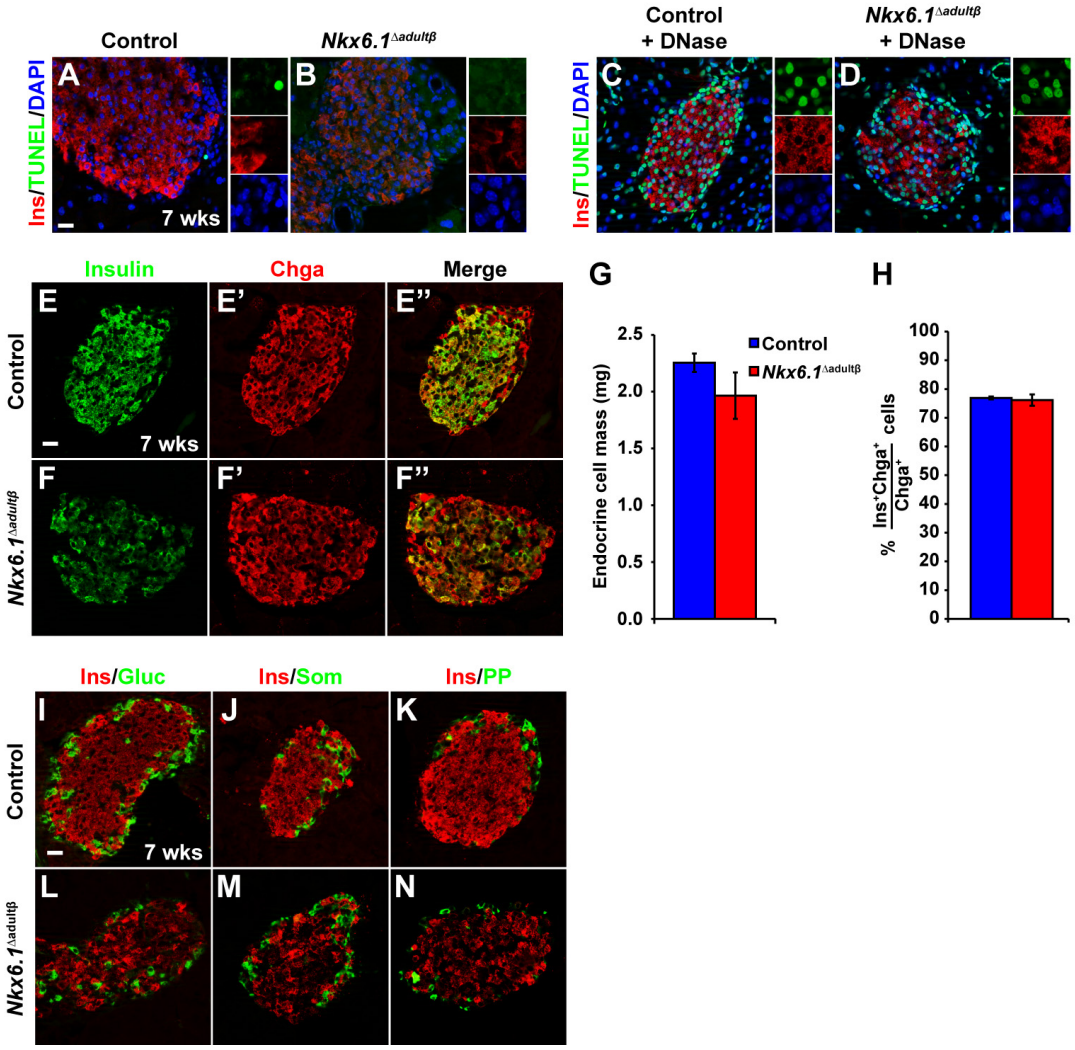
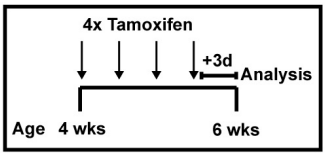


Figure 3.3. *Nkx6.1*$^{\Delta adult\beta}$ mice have normal beta cell viability, pancreatic endocrine cell mass, and beta cell numbers. (A-D) TUNEL assay combined with immunofluorescence staining for insulin and DAPI staining shows that beta cells in *Nkx6.1*$^{\Delta adult\beta}$ and control mice are not apoptotic at 7 wks. Pancreatic sections were treated with DNaseI as a positive control for the TUNEL assay (C,D). Right row shows higher magnification images and split color channels. (E-F) Immunofluorescence staining for Chga and insulin in *Nkx6.1*$^{\Delta adult\beta}$ and control mice at 7 wks. (G,H) Quantification of endocrine cell mass based on Chga staining (G) and insulin/Chga co-positive relative to total Chga positive cell numbers (H) shows no difference between *Nkx6.1*$^{\Delta adult\beta}$ and control mice (n=3). (I-N) Immunofluorescence staining reveals that insulin-expressing cells do not co-express glucagon, somatostatin, or pancreatic polypeptide in *Nkx6.1*$^{\Delta adult\beta}$ mice at 7 wks. Scale bar = 20μm. Ins, insulin; TUNEL, terminal deoxynucleotidyl transferase dUTP nicked end labeling; DAPI, 4',6-diamidino-2-phenylindole; Chga, chromogranin A; Gluc, glucagon; Som, somatostatin; PP, pancreatic polypeptide; Wk, week. Data are shown as mean ± SEM.

A



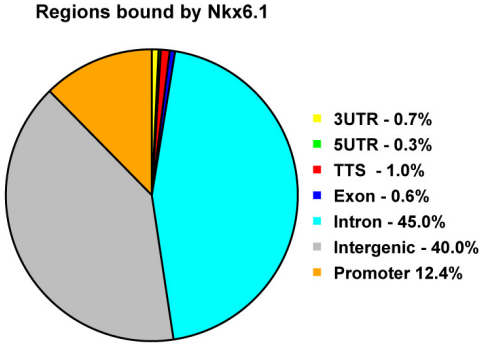
B

Gene ontology of significantly regulated biological processes

Category	Bonferonni P-Value	Gene Symbol
Ion transport	4.54E-09	*Ptger3, Kcnj13, Mcoln3, Trpm5, Slc5a10, Kcnf1, Kcnj5, Slc26a1, Kcnj12, Mfi2, Kcnmb4, Slc4a7, Scnn1a, Kcns3, Adora1, Slc22a2, Atp4a, Cnnm1, Trpm1, Scn3a, Kcnq1**, Slc30a8***
Regulation of secretion	4.44E-08	*Sytl4*, Maob, Cartpt, Adora1, Ucn3*, Kcnq1**, Prkca, Vegfc, Rbp4, Hadh, Cadps2, Chrna4, Adora3, Aacs, Syt9, Niacr1, Agt, Snca, Glul, Snap23*
Oxidation reduction	9.67E-03	*Cybrd1, Cyp4f39, Nox4*, Aldh1a3*, Gpx3*, Maob, Pam, Ero1lb*, Moxd1, Bckdhb, Aldh1l2, Kdm2b, Hsd17b13, Slc1a3, Fmo1, Aass, Dhrs4, Adh1, Egln3, Steap1*
Insulin secretion	1.09E-02	*Sytl4*, Cartpt, Ucn3*, Kcnq1**, Hadh, Slc30a8**, Gpr119, Aacs, Syt9, Glul, Fkbp1b, Rfx6*, Inhbb, Oit1, Glp1r*, Nkx6.1, Oxct1, Adra2a, Irs1**
Hexose biosynthesis	3.48E-02	*Aldob, Rbp4, Pcx*, G6pc2*, Gnpda1, Pck1, Fbp2, Fpgt*

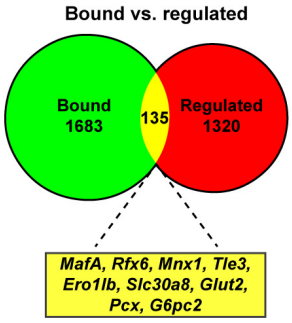
* = Known metabolic function in mice, ** = Associated with type 2 diabetes in GWA studies

C

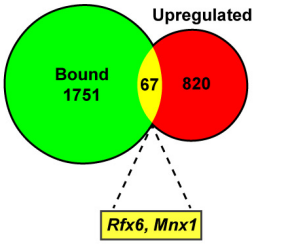


D

E



F



G

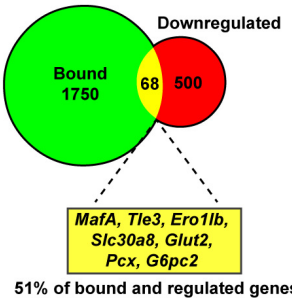


Figure 3.4. Nkx6.1 regulates important beta cell genes. (A) Schematic of experimental design for microarray analysis. (B) Gene ontology analysis of differentially expressed genes as identified by cDNA microarray analysis of *Nkx6.1*$^{\Delta adult\beta}$ and control islets. (C) Distribution of Nkx6.1 binding peaks from ChIP-seq analysis within the genome. (D) *De novo* motif analysis of Nkx6.1 binding peaks identifies a consensus Nkx6.1 binding motif. (E) Venn diagram of genes bound and regulated by Nkx6.1 in mature islets. (F,G) Analysis of Nkx6.1-occupied genes reveals Nkx6.1 target genes that are up- and down-regulated after *Nkx6.1* deletion. Yellow boxes represent Nkx6.1-bound and regulated genes with known function in beta cells. TTS, transcriptional termination site.

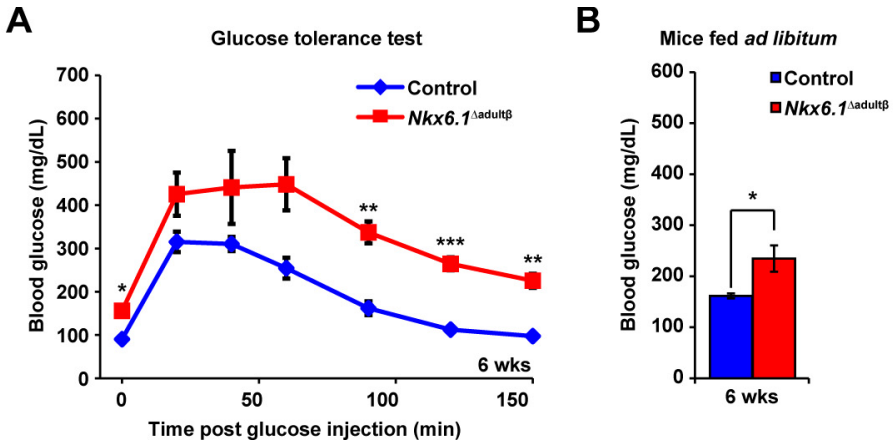


Figure 3.5. *Nkx6.1*$^{\Delta adult\beta}$ mice are not overtly diabetic one week after completion of tamoxifen-mediated *Nkx6.1* inactivation. (A,B) Intraperitoneal glucose tolerance test (A) and random blood glucose measurements (B) in male *Nkx6.1*$^{\Delta adult\beta}$ and control mice at 6 weeks (wks) (n=6). Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Comparison of gene expression profiles between *Nkx6.1*$^{\Delta adult\beta}$ and control islets revealed significant differences in the expression of 1455 genes (FDR <0.01 and fold change (FC) >1.5; File 3.1), of which 887 were upregulated and 568 downregulated. To define the cellular processes affected by *Nkx6.1* inactivation, we performed Gene Ontology (GO) analysis of the differentially expressed genes. Consistent with the diabetic phenotype, Nkx6.1-regulated genes showed association with biological processes that are critical for beta cell function, such as ion transport, regulation of secretion, oxidation reduction, insulin secretion, and hexose biosynthesis (Figure 3.4B). These data suggest that reduced insulin production may not be the only cause of hyperglycemia following *Nkx6.1* deletion, but that simultaneous impairment of multiple processes required for proper beta cell function could contribute to the development of diabetes in *Nkx6.1*$^{\Delta adult\beta}$ mice.

To distinguish between direct transcriptional target genes of Nkx6.1 and genes indirectly affected by *Nkx6.1* inactivation, we performed ChIP-seq for Nkx6.1 on primary mouse islets to identify Nkx6.1-occupied genes. We detected a total of 6771 Nkx6.1 binding regions throughout the murine genome (FDR < 0.001; File 3.2), of which 4066 were near genes or intronic (Figure 3.4C). D*e novo* motif analysis revealed a TAAT core motif and two flanking nucleotides on each side as the sequence motif preferentially occupied by Nkx6.1 (Figure 3.4D). Notably, the TAAT core of the Nkx6.1 *de novo* binding motif has been previously identified by *in vitro* EMSA analysis (Jorgensen et al., 1999). The TAAT motif is shared among many homeodomain transcription factors (Wilson et al., 1996), demonstrating binding of Nkx6.1 to the core homeodomain-binding motif.

To next determine the overlap between those genes occupied by Nkx6.1 in beta cells and those whose expression is affected by *Nkx6.1* loss, we analyzed which

of the 1988 Nkx6.1 binding sites within 10kb of a transcriptional start site were associated with genes significantly regulated in Nkx6.1-deficient islets. Surprisingly, only 8% of Nkx6.1-occupied genes (135/1818) were also regulated by Nkx6.1 (Figure 3.4E). Similarly, of the 1455 genes with statistically significant changes in expression only 9% were bound by Nkx6.1 (Figure 3.4E), indicating that only a fraction of genes affected by *Nkx6.1* inactivation directly depends on transcriptional input by Nkx6.1. Statistical analysis using hypergeometric distribution (Bhinge et al., 2007) revealed that this overlap between Nkx6.1-bound and regulated genes was still significantly greater than randomly expected ($P<0.05$). Interestingly, an equal percentage of Nkx6.1-bound and regulated genes were upregulated as were downregulated (Figure 3.4F,G), suggesting that Nkx6.1 can act as both a transcriptional repressor and activator.

Nkx6.1 was found to directly regulate various critical beta cell genes, including genes involved in glucose uptake and metabolism (*Slc2a2* (Glut2), *Pcx*, and *G6pc2*), insulin processing (*Ero1lb* and *Slc30a8*), as well as transcriptional regulators with known roles in islet development and/or beta cell function (*MafA*, *Rfx6*, *Mnx1,* and *Tle3*) (Figure 3.4E). These results suggest that Nkx6.1 exerts its function by transcriptionally regulating mediators of multiple beta cell processes.

The insulin secretory response is impaired after *Nkx6.1* inactivation.

The insulin secretory response of beta cells is regulated by the coupling of glucose metabolism to insulin secretion (Muoio and Newgard, 2008). Glycolysis results in an increase in the ATP:ADP ratio, which serves as the key trigger for closure of ATP-sensitive potassium channels (K_{ATP} channels), ultimately stimulating calcium influx and insulin secretion. Because Nkx6.1 directly regulates the glucose

metabolic genes *Glut2*, *Pcx*, and *G6pc2* (File 3.1; Figure 3.6A-C), we hypothesized that glucose uptake, glycolytic flux and energy production could be impaired in Nkx6.1-deficient beta cells. Supporting that the downregulation of Glut2 is physiologically significant, we found that *Nkx6.1*$^{\Delta adult\beta}$ mice were resistant to streptozotocin-induced beta cell death (Figure 3.7), which depends on Glut2-mediated uptake of streptozotocin (Schnedl et al., 1994).

To investigate whether the changes in expression of glucose metabolic genes are associated with defects in energy production, we stained pancreata for phospho-AMP kinase (p-AMPK), a sensitive indicator of cellular energy depletion (low ATP:AMP ratio) (Porat et al., 2011). *Nkx6.1*$^{\Delta adult\beta}$ islets displayed strikingly more intense p-AMPK staining than control islets (Figure 3.6D-E'), indicating that loss of Nkx6.1 causes reduced glycolytic flux and energy stress. Further supporting this conclusion, intracellular ATP content was also significantly decreased in *Nkx6.1*$^{\Delta adult\beta}$ islets (Figure 3.6F). We conclude that despite increased blood glucose levels, Nkx6.1 deficiency results in energy-depleted beta cells. Since energy depletion has been shown to impair insulin secretion and cause diabetes in mice (Piston et al., 1999; Porat et al., 2011; Terauchi et al., 1995), the defect in energy production in *Nkx6.1*$^{\Delta adult\beta}$ mice could lead to a severely impaired insulin secretory response.

In addition to affecting ATP production, *Nkx6.1* deletion also led to reduced expression of *Sytl4*, a vesicle-associated protein implicated in the modulation of insulin secretion (Gomi et al., 2005), as well as *Ucn3* and *Glp1r* (Figure 3.6G-I), which are involved in peptide-mediated potentiation of insulin secretion (Li et al., 2007; Preitner et al., 2004). These changes in gene expression suggest that Nkx6.1 also has glucose metabolism-independent roles in insulin secretion. Notably, core components of the stimulus-secretion-coupling mechanism (e.g. *Abcc8*, *Kcnj11*, and

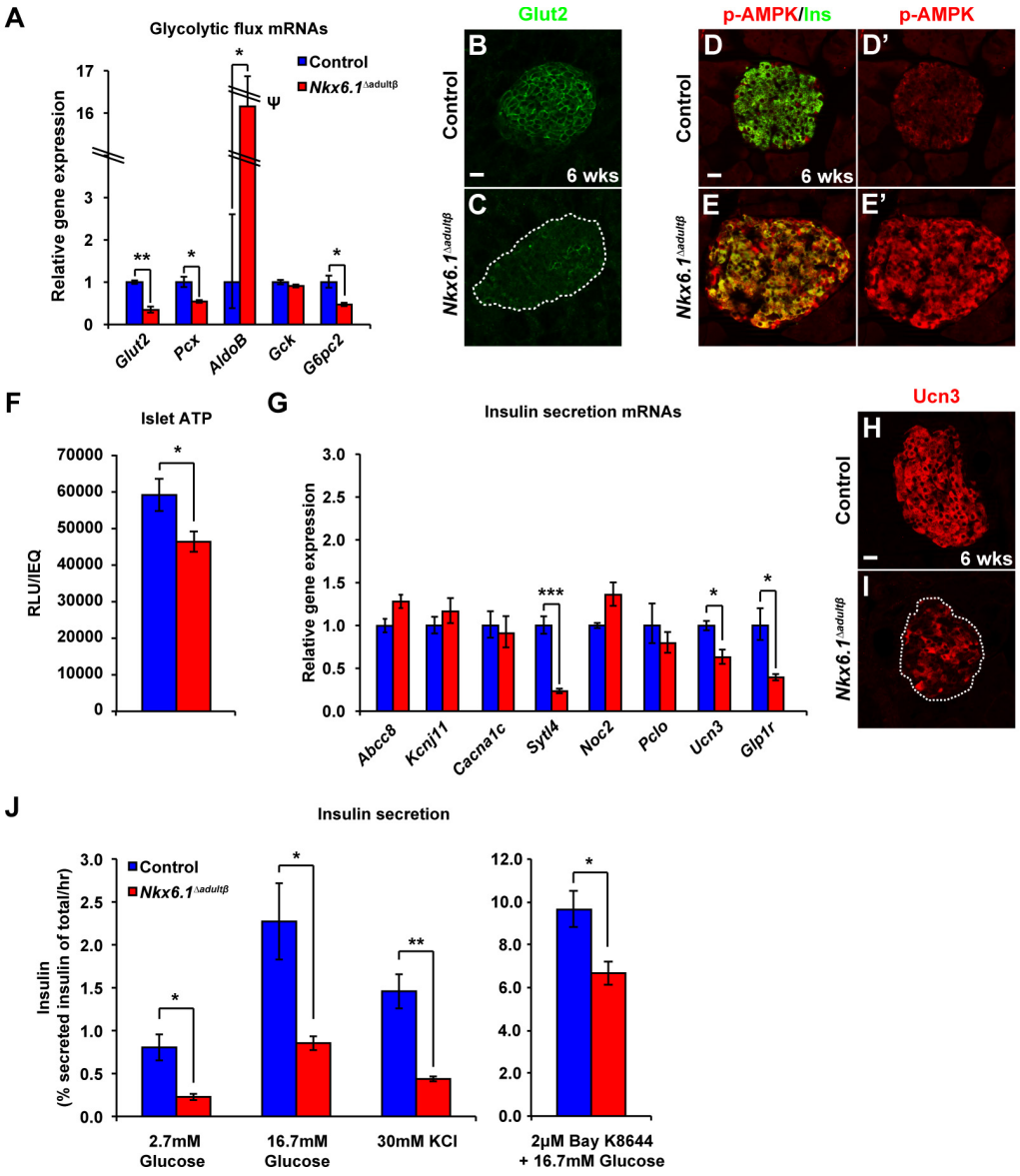


Figure 3.6. Islets from *Nkx6.1*$^{\Delta adult\beta}$ mice exhibit reduced insulin secretion *in vitro*. (A) qRT-PCR analysis of islets from *Nkx6.1*$^{\Delta adult\beta}$ and control mice at 6 wks for genes involved in glycolytic flux (n=3). (B-E') Immunofluorescence staining of pancreata from *Nkx6.1*$^{\Delta adult\beta}$ and control mice at 6 wks. (F) ATP measurement in islets from *Nkx6.1*$^{\Delta adult\beta}$ and control mice at 6 wks. (G) qRT-PCR analysis of islets from *Nkx6.1*$^{\Delta adult\beta}$ and control mice at 6 wks for genes involved in insulin secretion (n=3). (H,I) Immunofluorescence staining for Ucn3 in *Nkx6.1*$^{\Delta adult\beta}$ and control pancreata at 6 wks. (J) Static incubation of islets from *Nkx6.1*$^{\Delta adult\beta}$ and control mice with 2.7mM glucose, 16.7mM glucose, 30mM KCl, or 2μM Bay K8644 for 1 hour reveals that islets from *Nkx6.1*$^{\Delta adult\beta}$ mice secrete less of their total insulin content per hour than control islets (n=6). Scale bar = 20 μm. Dashed lines in C and H represent islet area. Ins, insulin; p-AMPK, phospho-AMP kinase; Wk, week; Hr, hour. Data are shown as mean ± SEM. Ψ = 16.27 with an SEM of ± 2.93. Slashes in A = change in Y axis scale. *p<0.05, **p<0.01, ***p<0.001.

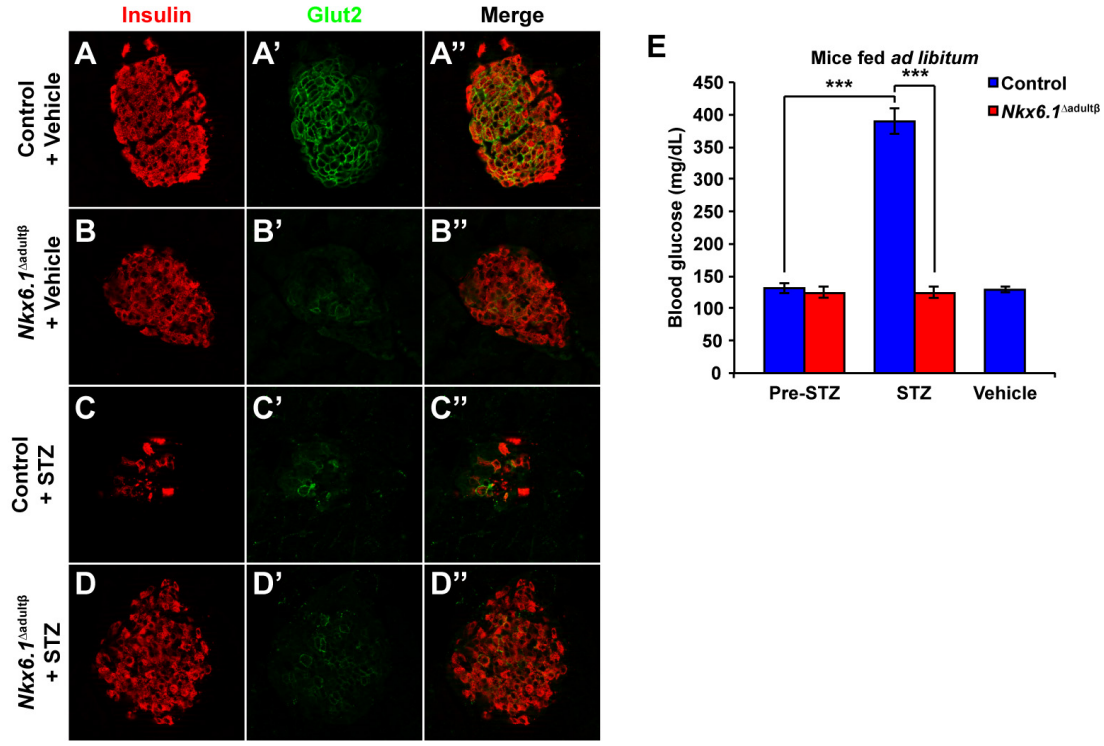


Figure 3.7. *Nkx6.1*$^{\Delta adult\beta}$ mice are resistant to streptozotocin. (A-E) *Nkx6.1*$^{\Delta adult\beta}$ and control mice were treated with either vehicle or 200mg/kg streptozotocin (STZ) at 6 weeks of age. (A-D'') 48 hours after treatment, immunofluorescent staining of pancreatic sections for insulin and Glut2 revealed that control mice treated with STZ had a severe reduction in beta cell numbers (C-C'') compared to vehicle-treated control mice (A-A'') and *Nkx6.1*$^{\Delta adult\beta}$ mice treated with either vehicle (B-B'') or STZ (D-D''). (E) Control mice treated with STZ developed diabetes while STZ-treated *Nkx6.1*$^{\Delta adult\beta}$ mice remained normoglycemic.

Cacna1c) and genes encoding proteins important for vesicle docking (e.g. *Pclo* and *Noc2*) were normally expressed in Nkx6.1-deficient islets (Figure 3.6G).

To directly test whether the observed changes in gene expression affect insulin secretion at a functional level, we performed in vitro glucose stimulated insulin secretion (GSIS) assays on isolated islets from *Nkx6.1*$^{\Delta adult\beta}$ and control mice. To account for decreased insulin content of Nkx6.1-deficient beta cells, we calculated insulin secretion as a percentage of total islet insulin content. Islets from *Nkx6.1*$^{\Delta adult\beta}$ mice secreted less of their total insulin than control islets under conditions of basal (2.7 mM) and high (16.7 mM) glucose concentrations (Figure 3.6J), showing that insulin secretion is impaired after *Nkx6.1* deletion. However, stimulation of secretion by glucose appeared to be unaffected by *Nkx6.1* deletion, as there was a similar increase in insulin secretion in *Nkx6.1*$^{\Delta adult\beta}$ and control islets between low and high glucose conditions (3.75-fold increase between 2.7 mM and 16.7 mM glucose in *Nkx6.1*$^{\Delta adult\beta}$ islets *versus* 3.06-fold increase in control islets). The insulin secretory pattern of *Nkx6.1*$^{\Delta adult\beta}$ islets is highly similar to the pattern observed in Glut2-deficient islets (Guillam et al., 2000), suggesting that loss of Glut2 in *Nkx6.1*$^{\Delta adult\beta}$ beta cells has a major contribution to the insulin secretory defect. Notably, additional defects downstream of K_{ATP} channel-mediated membrane depolarization also appear to exist, as insulin secretion in Nkx6.1-deficient islets was also reduced in response to membrane depolarization and calcium influx (30 mM KCl and 2 μM Bay K8644, respectively; Figure 3.6J). Together, these results imply that impaired insulin secretion is a major contributor to the diabetic phenotype of *Nkx6.1*$^{\Delta adult\beta}$ mice.

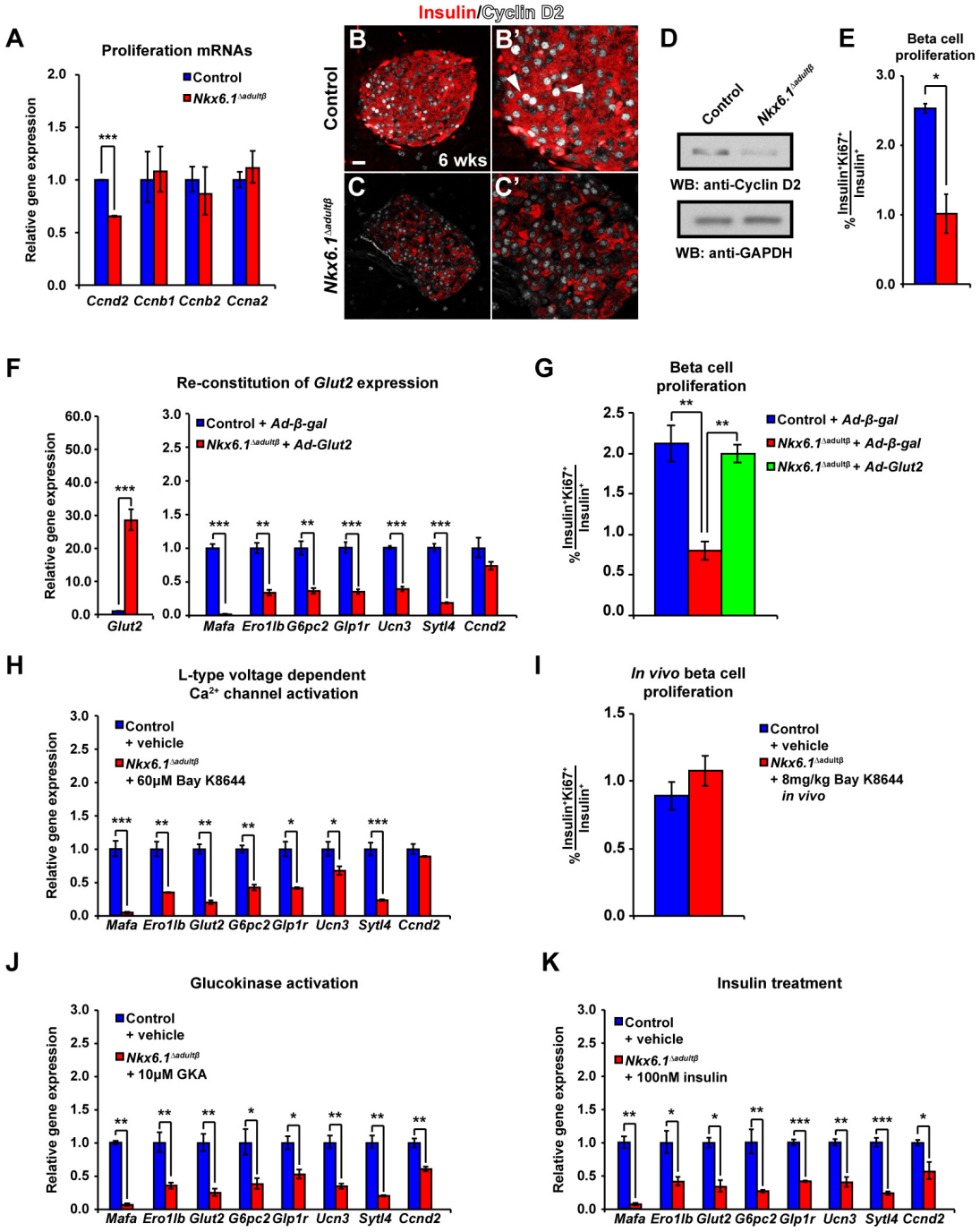
Decreased beta cell proliferation in *Nkx6.1*$^{\Delta adult\beta}$ mice.

It has been suggested that glycolytic flux and ATP production serve as a trigger for beta cell replication (Porat et al., 2011). Specifically, glucose metabolism is thought to control beta cell proliferation by regulating expression of *Cyclin D2* (*Ccnd2*) (Salpeter et al., 2010; Salpeter et al., 2011), which is a critical regulator of beta cell mass in mice (Georgia and Bhushan, 2004; Kushner et al., 2005). Since Nkx6.1-deficient beta cells have defects in energy production, we examined whether *Nkx6.1* deletion affects *Ccnd2* mRNA levels. We found that *Ccnd2* mRNA levels were indeed decreased in *Nkx6.1*$^{\Delta adult\beta}$ islets (File 3.1; Figure 3.8A). Strikingly, *Nkx6.1* inactivation specifically affected *Ccnd2*, while mRNA levels of other *cyclins* were unaffected (File 3.1; Figure 3.8A). Immunofluorescence staining (Figure 3.8B-C') and Western blot analysis (Figure 3.8D) further demonstrated significantly reduced Cyclin D2 protein levels in beta cells of *Nkx6.1*$^{\Delta adult\beta}$ mice. Similar to the phenotype of *Ccnd2*-null mutant mice (Georgia and Bhushan, 2004; Kushner et al., 2005), Nkx6.1-deficient beta cells exhibited a reduction in the number of beta cells expressing the proliferation marker Ki67 (Figure 3.8E), showing that beta cell proliferation is impaired after *Nkx6.1* inactivation. Notably, Nkx6.1 did not occupy *Ccnd2* regulatory sequences (File 3.2), suggesting that the regulation of *Ccnd2* by Nkx6.1 could be indirect.

Restoring glucose import reinstates *Ccnd2* expression in *Nkx6.1*$^{\Delta adult\beta}$ islets.

To explore whether limited glucose uptake capacity due to loss of Glut2 could be the main cause of reduced *Ccnd2* expression in Nkx6.1-deficient islets, we investigated whether restoring Glut2-mediated glucose import could increase *Ccnd2* levels after *Nkx6.1* inactivation. To examine this, we reconstituted Glut2 expression in

Figure 3.8. Nkx6.1 maintains Cyclin D2 expression and beta cell proliferative capacity through regulation of glucose uptake. (A) qRT-PCR analysis of islets shows a decrease in *Ccnd2* expression in *Nkx6.1*$^{\Delta adult\beta}$ compared to control mice at 6 wks (n=3). (B-C') Immunofluorescence staining for insulin and Cyclin D2 shows a decrease in Cyclin D2 expression in beta cells of *Nkx6.1*$^{\Delta adult\beta}$ mice at 6 wks. B' and C' are higher magnification images of B and C, respectively. White arrowheads point to Cyclin D2^{high} cells. (D) Immunoblot analysis of whole cell islet lysates confirms reduced Cyclin D2 expression in *Nkx6.1*$^{\Delta adult\beta}$ mice. (E) Quantification of the percentage of insulin^{+} cells expressing Ki67 shows decreased beta cell proliferation in *Nkx6.1*$^{\Delta adult\beta}$ mice at 6 wks (n=3). (F) qRT-PCR analysis of genes with decreased expression in islets from *Nkx6.1*$^{\Delta adult\beta}$ mice after adenoviral infection of *Nkx6.1*$^{\Delta adult\beta}$ islets with *Ad-CMV-Glut2* (*Ad-Glut2*) and control islets with *Ad-CMV-β-gal* (*Ad-β-gal*) (n=3). *Ad-Glut2* restores *Ccnd2* expression to levels observed in control islets infected with *Ad-β-gal*. (G) Quantification of the percentage of insulin^{+} cells expressing Ki67 after infection of *Nkx6.1*$^{\Delta adult\beta}$ and control islets with *Ad-Glut2* or *Ad-β-gal* shows that *Ad-Glut2* restores the number of Ki67^{+} beta cells in *Nkx6.1*$^{\Delta adult\beta}$ islets to control values (n=3). (H) qRT-PCR analysis after a three-hour treatment of *Nkx6.1*$^{\Delta adult\beta}$ islets with the calcium channel activator Bay K8644 and control islets treated with vehicle (n=3). (I) Quantification of the percentage of insulin^{+} cells expressing Ki67 after injection of *Nkx6.1*$^{\Delta adult\beta}$ mice with 8mg/kg Bay K8644 or control mice injected with vehicle (n=3). Stimulation of calcium influx restores *Ccnd2* expression and beta cell proliferation in *Nkx6.1*$^{\Delta adult\beta}$ mice. (J,K) qRT-PCR analysis of islets from *Nkx6.1*$^{\Delta adult\beta}$ mice treated with 10 μM glucokinase activator (GKA) (J) or 100 nM insulin (K) for 3 hours and islets from control mice treated with vehicle (n=3). Scale bar = 20 μm. Wk, week. Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.


A
Proliferation mRNAs
Control
Nkx6.1Δadultβ
Relative gene expression
Ccnd2 Ccnb1 Ccnb2 Ccna2
Insulin/Cyclin D2
B
B'
C
C'
Control
Nkx6.1Δadultβ
6 wks
D
Control
Nkx6.1Δadultβ
WB: anti-Cyclin D2
WB: anti-GAPDH
E
Beta cell proliferation
%Insulin+Ki67+/Insulin+
F
Re-constitution of Glut2 expression
Control + Ad-β-gal
Nkx6.1Δadultβ + Ad-Glut2
Relative gene expression
Glut2
Mafa Ero1lb G6pc2 Glp1r Ucn3 Sytl4 Ccnd2
G
Beta cell proliferation
Control + Ad-β-gal
Nkx6.1Δadultβ + Ad-β-gal
Nkx6.1Δadultβ + Ad-Glut2
H
L-type voltage dependent Ca2+ channel activation
Control + vehicle
Nkx6.1Δadultβ + 60μM Bay K8644
Relative gene expression
Mafa Ero1lb Glut2 G6pc2 Glp1r Ucn3 Sytl4 Ccnd2
I
In vivo beta cell proliferation
Control + vehicle
Nkx6.1Δadultβ + 8mg/kg Bay K8644 in vivo
J
Glucokinase activation
Control + vehicle
Nkx6.1Δadultβ + 10μM GKA
Relative gene expression
Mafa Ero1lb Glut2 G6pc2 Glp1r Ucn3 Sytl4 Ccnd2
K
Insulin treatment
Control + vehicle
Nkx6.1Δadultβ + 100nM insulin
Relative gene expression
Mafa Ero1lb Glut2 G6pc2 Glp1r Ucn3 Sytl4 Ccnd2

Nkx6.1$^{\Delta adult\beta}$ islets using an adenovirus containing *Glut2* cDNA (*Ad-Glut2*), which resulted in a 25-fold increase in *Glut2* mRNA levels compared to *Ad-β-gal-treated* control islets as well as restored Glut2 protein expression (Figure 3.8F; Figure 3.9). Glut2 reconstitution increased *Ccnd2* expression to levels of control islets, while expression of other Nkx6.1-regulated genes remained significantly reduced (Figure 3.8F). Glut2 reconstitution in *Nkx6.1*$^{\Delta adult\beta}$ islets also restored the number of insulin^{+} cells expressing Ki67 to control values (Figure 3.8G), indicating that Glut2 re-expression rescues the proliferation defect. These findings demonstrate that *Ccnd2* expression does not depend on direct transcriptional input from Nkx6.1, but that Nkx6.1 controls *Ccnd2* and beta cell proliferation indirectly by regulating glucose import.

Consistent with the notion that glycolytic flux regulates *Ccnd2* expression via the stimulus-secretion-coupling pathway (Salpeter et al., 2010; Salpeter et al., 2011), increasing calcium influx by treating islets with the L-type dependent calcium channel opener, Bay K8644, similarly restored *Ccnd2* expression in *Nkx6.1*$^{\Delta adult\beta}$ islets (Figure 3.8H). Significantly, Bay K8644 administration to mice increased the number of insulin^{+} cells expressing Ki67 to control values (Figure 3.8I), providing *in vivo* evidence that beta cell proliferation can be rescued by stimulating calcium influx. In contrast, culture of Nkx6.1-deficient islets in the presence of an activator for the rate-limiting enzyme of glycolysis, glucokinase, or the beta cell mitogen, insulin (Paris et al., 2003), failed to restore *Ccnd2* expression (Figure 3.8J,K; also compare to Figure 3.8A). These findings illustrate that the proliferative capacity of beta cells is coupled to glucose metabolism and that Nkx6.1 controls this process by regulating *Glut2* expression. Because beta cells have a low turnover rate in adult mice (Teta et al., 2005), the observed reduction in beta cell proliferation did not result in decreased

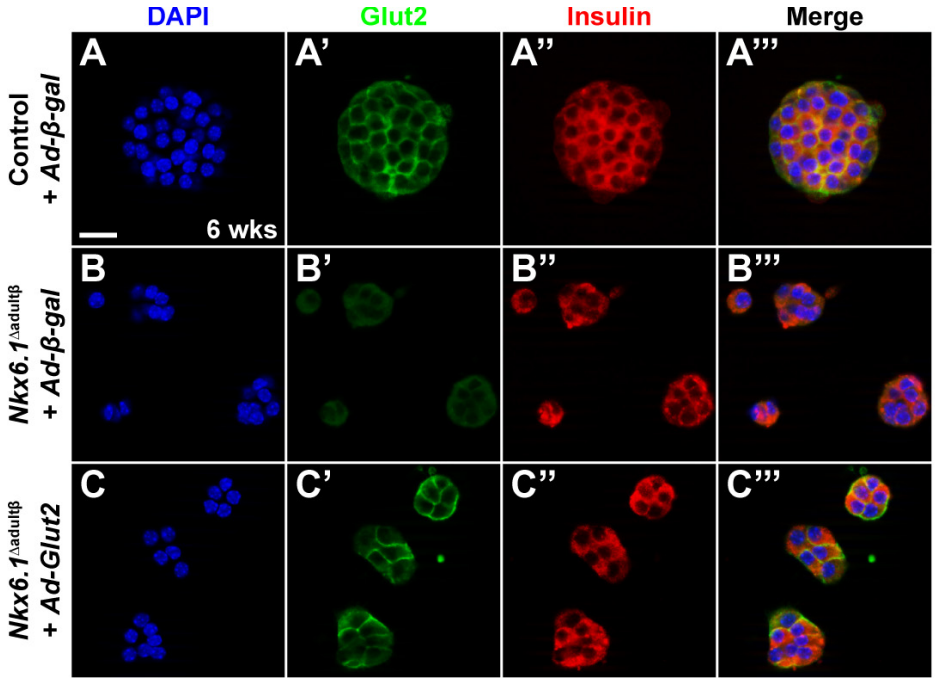


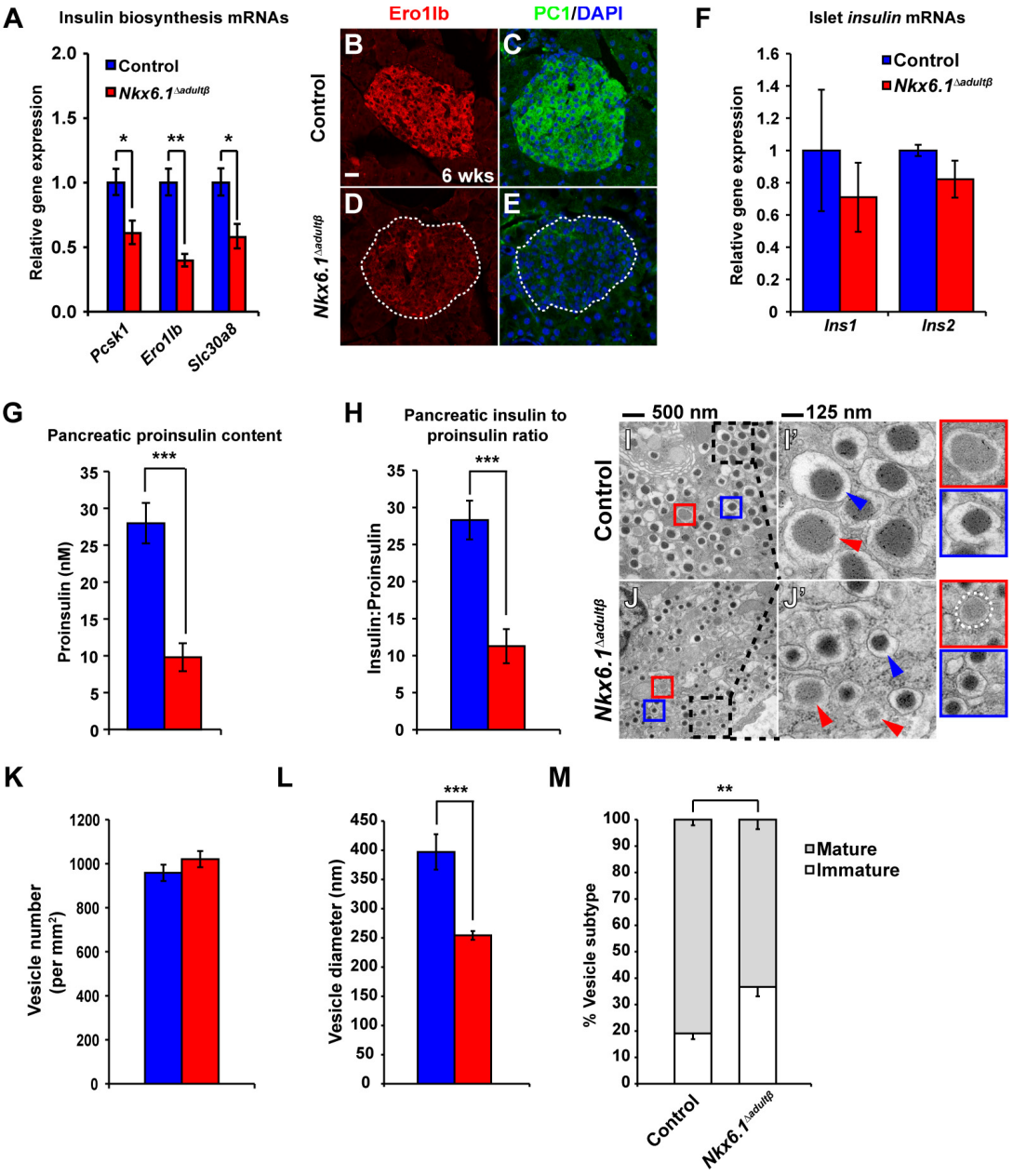
Figure 3.9. Glut2 protein expression is restored in *Nkx6.1*$^{\Delta adult\beta}$ islets after infection with *Glut2* expressing adenovirus. (A-C''') Immunofluorescent staining of dispersed islets for DAPI, Glut2 and insulin after infection of *Nkx6.1*$^{\Delta adult\beta}$ and control islets with *Ad-Glut2* or *Ad-β-gal* reveals that Glut2 protein expression is restored in *Nkx6.1*$^{\Delta adult\beta}$ islets. Mice were 6 weeks (wks) old. Scale bar = 20 μm.

beta cell mass in our acute *Nkx6.1* deletion model (Figure 3.3G,H). However, the decreased proliferative capacity could become metabolically relevant when beta cells need to undergo adaptive expansion under conditions of increased insulin demand.

Nkx6.1$^{\Delta adult\beta}$ mice have posttranscriptional defects in insulin biosynthesis.

Our gene expression and ChIP-seq analysis revealed that Nkx6.1 also controlled genes required for insulin biosynthesis, which could explain the reduction in pancreatic insulin levels in *Nkx6.1*$^{\Delta adult\beta}$ mice. Most notably, expression of the T2D-associated zinc transporter *Slc30a8*, the oxidoreductase *Ero1lb*, and the proinsulin to insulin convertase *Pcsk1* (PC1) was severely reduced in Nkx6.1-deficient beta cells (File 3.1; Figure 3.10A-E). These proteins have established roles in insulin processing and/or maturation of insulin secretory vesicles (Bellomo et al., 2011; Zhu et al., 2002; Zito et al., 2010), implying a role for Nkx6.1 in multiple aspects of the insulin biosynthesis pathway. Notably, the finding that *ins1* and *ins2* mRNA levels were not significantly changed (File 3.1; Figure 3.10F), suggests that reduced insulin production in *Nkx6.1*$^{\Delta adult\beta}$ mice is mainly caused by posttranscriptional defects in insulin biosynthesis. To further define which steps in insulin biosynthesis are affected by *Nkx6.1* inactivation, we measured pancreatic proinsulin content and calculated the insulin to proinsulin ratio in *Nkx6.1*$^{\Delta adult\beta}$ islets. Compared to control mice, pancreatic proinsulin levels and the ratio of insulin to proinsulin were significantly reduced in *Nkx6.1*$^{\Delta adult\beta}$ mice (Figure 3.10G,H). While the defect in proinsulin to insulin processing was expected based on the observed decrease in *Slc30a8*, *Ero1lb*, and *Pcsk1* expression, it is less clear why loss of Nkx6.1 impairs proinsulin biosynthesis.

Figure 3.10. *Nkx6.1* is necessary for insulin biosynthesis. (A) qRT-PCR analysis of islets reveals reduced expression of genes involved in insulin biosynthesis in *Nkx6.1*$^{\Delta adult\beta}$ compared to control mice at 6 wks (n=3). (B-E) Immunofluorescence staining of pancreata from *Nkx6.1*$^{\Delta adult\beta}$ and control mice at 6 wks. Dashed lines represent islet area. (F) qRT-PCR analysis of *Nkx6.1*$^{\Delta adult\beta}$ and control islets from mice at 6 wks shows no significant difference in *ins1* or *ins2* expression (n=3). (G) Proinsulin content normalized to protein concentration of whole pancreatic lysates (n=6). (H) The pancreatic insulin to proinsulin ratio is reduced in *Nkx6.1*$^{\Delta adult\beta}$ mice (n=6). (I,J) Transmission electron microscopy of pancreatic sections reveals smaller vesicle size and an increase in immature vesicles (red arrowheads) in *Nkx6.1*$^{\Delta adult\beta}$ compared to control mice. Dashed boxes indicate area of magnification in I' and J'. Blue arrowheads point to vesicles containing mature insulin dense core granules. Insets framed red show a representation of a typical immature vesicle and insets framed blue a typical mature vesicle. (K-M) Quantification of vesicle numbers (K), vesicle diameter (L), and the percentage of mature and immature vesicles (M) in *Nkx6.1*$^{\Delta adult\beta}$ and control mice (n=10). In G-M, mice were analyzed at 7 wks. PC1, prohormone convertase 1/3; Wk, week. Data are shown as mean ± SEM for A,F,G,H and ± SD for K-M. *p<0.05, **p<0.01, ***p<0.001.


A
Insulin biosynthesis mRNAs
Relative gene expression
Control
Nkx6.1Δadultβ
Pcsk1
Ero1lb
Slc30a8
Ero1lb
PC1/DAPI
Control
Nkx6.1Δadultβ
6 wks
B
C
D
E
F
Islet insulin mRNAs
Ins1
Ins2
G
Pancreatic proinsulin content
Proinsulin (nM)
H
Pancreatic insulin to proinsulin ratio
Insulin:Proinsulin
500 nm
125 nm
I
I'
J
J'
K
Vesicle number (per mm²)
L
Vesicle diameter (nm)
M
% Vesicle subtype
Mature
Immature

Because glucose is a direct stimulator of proinsulin translation (Wicksteed et al., 2003), we examined whether decreased Glut2 expression in *Nkx6.1*$^{\Delta adult\beta}$ mice limits intracellular availability of glucose and in turn reduces proinsulin production. However, restoring *Glut2* expression in *Nkx6.1*$^{\Delta adult\beta}$ dispersed islets had no effect on proinsulin or insulin levels (Figure 3.11A,B), suggesting that not glucose import, but reduced expression of insulin biosynthetic enzymes limits proinsulin synthesis in *Nkx6.1*$^{\Delta adult\beta}$ mice.

To determine whether the defect in insulin biosynthesis affects the formation of insulin secretory vesicles, we examined secretory vesicle numbers and morphology in beta cells from *Nkx6.1*$^{\Delta adult\beta}$ and control mice. According to the guidelines established by Pictet et al. (Pictet et al., 1972), secretory vesicles were considered immature if they had a homogenous light gray appearance similar to the electron density of the cytoplasm or mature if the vesicles contained an electron dense granule darker than the density of the cytoplasm. Transmission electron microscopy (TEM) showed that the overall number of secretory granules was unchanged (Figure 3.10I-K), suggesting that loss of Nkx6.1 does not affect vesicle formation or stability. In accordance with the observed defect in insulin biosynthesis, examination of vesicle morphology revealed smaller overall vesicle size in beta cells of *Nkx6.1*$^{\Delta adult\beta}$ mice (Figure 3.10I',J',L). Reduced vesicle size has been similarly observed in the *MODY* (*Ins2*^{Akita}) mouse model of impaired insulin biosynthesis (Wang et al., 1999). In addition to their reduced size, secretory granules in beta cells of *Nkx6.1*$^{\Delta adult\beta}$ mice exhibited a smaller halo around the dense core of mature insulin (Figure 3.10I',J'; blue arrowheads); a feature reflecting reduced processing of proinsulin to insulin (Orci et al., 1984). Moreover, the proportion of immature vesicles was significantly increased in Nkx6.1-deficient beta cells (Figure 3.10M), representing

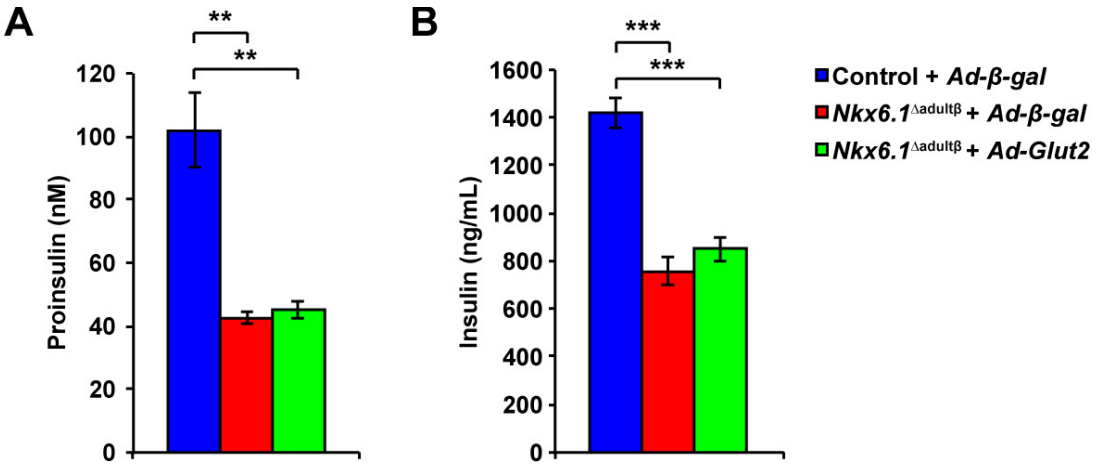


Figure 3.11. Expression of Glut2 in *Nkx6.1*$^{\Delta adult\beta}$ islets does not restore proinsulin or insulin content. (A,B) Measurements of proinsulin (A) and insulin content (B) after infection of *Nkx6.1*$^{\Delta adult\beta}$ and control islets with *Ad-Glut2* or *Ad-β-gal* shows that adenoviral expression of *Glut2* in *Nkx6.1*$^{\Delta adult\beta}$ islets does not increase either proinsulin or insulin levels (n=6). Data are shown as mean ± SEM. **p<0.01, ***p<0.001.

another feature of impaired insulin processing. These findings demonstrate that the changes in the expression of insulin biosynthesis-associated genes after *Nkx6.1* deletion manifest in defects in insulin processing and mature insulin secretory vesicle formation. Given previous evidence that deletion of *Ero1lb* and Slc30a8 in mice perturbs glucose homeostasis (Nicolson et al., 2009; Zito et al., 2010), these defects in insulin biosynthesis together with the impaired insulin secretory response are likely the predominant cause of diabetes in *Nkx6.1*$^{\Delta adult\beta}$ mice.

***Nkx6.1* inactivation destabilizes beta cell identity.**

In T2D mouse models of metabolic stress, reduced beta cell insulin production is associated with a decrease in *Nkx6.1*, *Pdx1* and *NeuroD* expression as well as increased expression of the pancreatic progenitor cell marker Ngn3 and the pluripotency markers Oct4, Nanog and L-Myc (Talchai et al., 2012). Because a subset of metabolically stressed beta cells eventually adopts other endocrine fates, it has been proposed that beta cell dedifferentiation followed by conversion into other endocrine cell types could cause beta cell failure in T2D (Talchai et al., 2012). While *Nkx6.1* deletion did not affect the expression of *Pdx1*, *NeuroD* or pluripotency markers (File 3.1; Figure 3.12A,C,E), we observed robust induction of Ngn3 expression in beta cells similar to what has been observed in models of metabolic stress (File 3.1; Figure 3.12A,B,D,F). To investigate whether the upregulation of Ngn3 is associated with destabilization of beta cell identity, we examined pancreata from *Nkx6.1*$^{\Delta adult\beta}$ mice at 14 weeks of age (8 weeks after *Nkx6.1* deletion) for co-expression of insulin with other pancreatic hormones. We did not observe co-expression of insulin with glucagon or pancreatic polypeptide in *Nkx6.1*$^{\Delta adult\beta}$ or control islets, but found a significant number of insulin^{+} cells co-expressing somatostatin in

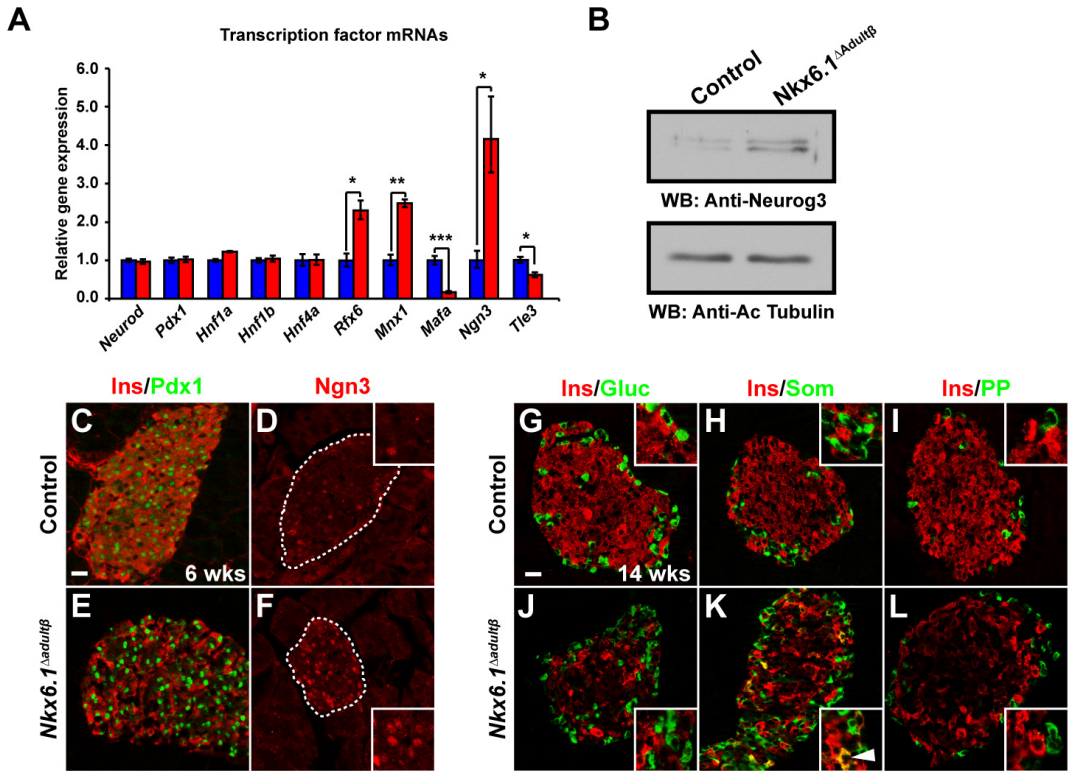


Figure 3.12. Increased expression of the progenitor marker Ngn3 in Nkx6.1-deficient beta cells. (A,B) qRT-PCR (A) and Western blot analysis (B) of islets from *Nkx6.1*$^{\Delta adult\beta}$ and control mice at 6 wks for multiple transcription factor genes (A) or Ngn3 (B) (n=3). (C-L) Immunofluorescence staining of pancreata from *Nkx6.1*$^{\Delta adult\beta}$ and control mice at 6 wks (C-F) and 14 wks (G-L). Dashed lines represent islet area. Insets are magnifications of selected areas. Arrowhead in (K) points to a cell co-expressing insulin (Ins) and somatostatin (Som). Scale bar = 20 μm. Gluc, glucagon; PP, pancreatic polypeptide; Wk, week. Data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

Nkx6.1$^{\Delta adult\beta}$ mice (Figure 3.12G-L). This finding is consistent with our previous observation of a beta-to-delta cell fate switch after *Nkx6.1* ablation in embryonic beta cells (Schaffer et al., 2013) and suggests that although not immediately, *Nkx6.1* inactivation in adult beta cells causes beta cells to adopt delta cell identity over time. In conjunction with our previous findings, these data strongly suggest that loss of *Nkx6.1* in adult mice destabilizes beta cell identity, eventually leading to fate conversion of beta into delta cells.

Together, our analysis demonstrates that Nkx6.1 is a critical regulator of insulin biosynthesis and secretion, as well as proliferative capacity and cell identity in adult beta cells. The severe beta cell defects observed after *Nkx6.1* inactivation suggest that restoring Nkx6.1 levels could be a therapeutic strategy in T2D.

DISCUSSION

It is widely recognized that beta cell dysfunction, specifically the inability of beta cells to properly secrete insulin in response to high blood glucose levels is among the earliest clinical features during progression to T2D (Ferrannini, 2010). The ability to sense glucose levels and to couple this information to an insulin secretory response is bestowed upon beta cells by specialized transporters and enzymes. While the mechanisms that underlie glucose-mediated insulin secretion are fairly well understood, it has remained unclear which transcriptional regulators initiate and maintain the expression of genes that enable beta cells to perform their highly specialized function. In this study, we show that the transcription factor Nkx6.1 is a master regulator of genes that define the functional beta cell state; a role that is consistent with its exclusive expression in beta cells of the adult pancreas.

We show that conditional inactivation of *Nkx6.1* in beta cells of adult mice results in overt diabetes within days of *Nkx6.1* ablation. Loss of Nkx6.1 activity had an immediate and dramatic impact on the expression of genes that impart upon beta cells their unique ability to synthesize and release insulin in a regulated fashion. We found that genes involved in insulin biosynthesis (*Slc30a8* and *Ero1lb*), glucose import (*Glut2*), and glucose metabolism (*Pcx*) are direct transcriptional target genes of Nkx6.1. In addition, *Nkx6.1* ablation indirectly affected the expression of numerous genes important for beta cell function and interestingly, also beta cell proliferation (Figure 3.13). The finding that islet *Ccnd2* levels and beta cell proliferation were decreased in *Nkx6.1* conditional mutant mice was somewhat surprising, as several studies have shown that hyperglycemia has a stimulatory effect on beta cell *Ccnd2* expression and self-renewal (Alonso et al., 2007; Bonner-Weir et al., 1989; Salpeter et al., 2011). We found that reduced availability of the beta cell mitogen insulin (Paris et al., 2003) had no apparent contribution to the reduced proliferative capacity of beta cells after *Nkx6.1* ablation. Instead, our results suggest that the proliferative capacity of Nkx6.1-deficient beta cells is limited by reduced intracellular availability of glucose due to loss of *Glut2* expression (Figure 3.13). These findings demonstrate an intricate link between the beta cell's ability to import glucose and its proliferative capacity, which lends further support to the emerging concept that glucose metabolism plays a critical role in the regulation of beta cell proliferation (Porat et al., 2011; Salpeter et al., 2010; Salpeter et al., 2011). Combined with the finding that Nkx6.1 levels are reduced in T2D models of metabolic stress (Talchai et al., 2012), our work suggests that Nkx6.1 acts as a metabolic sensor that modulates both insulin secretion and proliferative capacity in response to metabolic stress. By preventing the proliferation of beta cells that have lost glucose responsiveness, the cell autonomous coupling of

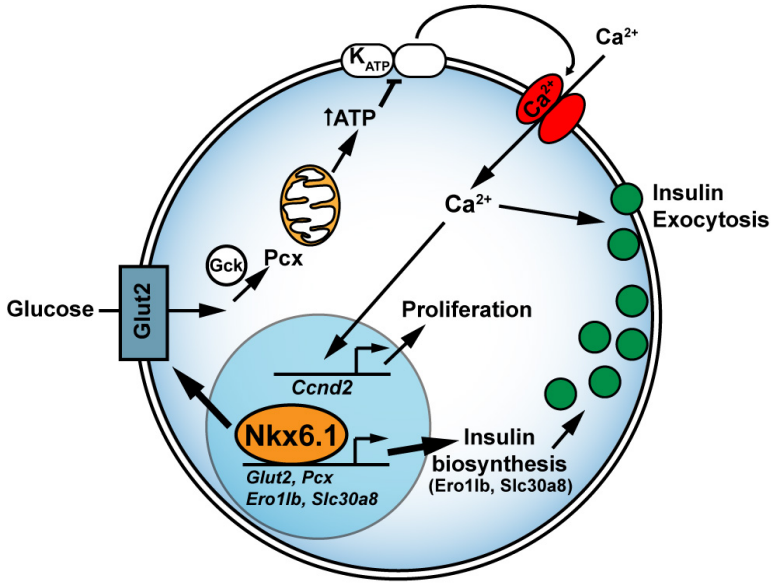


Figure 3.13. Nkx6.1 function in adult beta cells. Nkx6.1 directly regulates transcription of genes encoding proteins involved in glucose uptake and metabolism (Glut2 and Pcx) and insulin biosynthesis (Ero1lb and Slc30a8). Reduced expression of these Nkx6.1 target genes affects beta cell function threefold: First, decreased glucose uptake and metabolism diminishes ATP production, leading to impaired insulin secretion via the stimulus-secretion coupling pathway. Second, by enabling glucose uptake through Glut2 regulation, Nkx6.1 indirectly controls beta cell proliferative capacity. In the absence of Nkx6.1, expression of *Ccnd2,* which encodes the critical beta cell mitogen Cyclin D2, is reduced and beta cell proliferation is decreased. Reconstituting Glut2 expression in Nkx6.1-deficient beta cells restores *Ccnd2* levels and beta cell proliferation. Third, insulin biosynthesis is severely impaired, leading to reduced production of mature insulin and an overabundance of immature secretory vesicles. Glut2, glucose transporter 2; Gck, glucokinase; Pcx, pyruvate carboxylase; K_{ATP}, ATP-sensitive potassium channel.

glucose import to beta cell proliferation might provide an inherent selection mechanism for healthy beta cells.

Our study also reconciles previously reported, seemingly contradictory findings about the role of Nkx6.1 in beta cell proliferation. We recently reported that transgenic overexpression of *Nkx6.1* in beta cells of adult mice *in vivo* had no positive effect on beta cell proliferation or beta cell mass (Schaffer et al., 2011). By contrast, virus-mediated expression of Nkx6.1 in cultured islets had pro-proliferative activity (Schisler et al., 2008). A possible explanation for this apparent contradiction is that *in vitro* culture of islets compromises Nkx6.1 expression levels. It is known that once removed from their niche and put into culture beta cells quickly lose Glut2 expression and cease to proliferate (Weinberg et al., 2007), indicating that expression of upstream Glut2 regulators, including Nkx6.1 could also be compromised. Thus, the observed pro-proliferative effect of Nkx6.1 *in vitro* may be explained by virally expressed *Nkx6.1* restoring reduced Nkx6.1 levels and in turn also Glut2 and Ccnd2 levels in cultured islets. By contrast, increasing Nkx6.1 levels above normal in healthy beta cells *in vivo* appears to have no further stimulatory effect on glucose import and beta cell proliferation.

After *Nkx6.1* deletion, we observed an extremely rapid decline in beta cell insulin content. The loss of insulin was not associated with beta cell death, revealing a striking similarity between Nkx6.1-deficient beta cells and “empty” beta cells observed in mouse models of T2D (Talchai et al., 2012). Furthermore, as reported under conditions of metabolic stress (Talchai et al., 2012), we found that reduced *Nkx6.1* expression was also accompanied by de-repression of the endocrine progenitor cell marker Ngn3. Based on the observation that beta cells undergo fate conversion into non-beta endocrine cell types in T2D models (Talchai et al., 2012),

the loss of beta cell features and gain of Ngn3 expression has been proposed to render beta cells plastic and more prone to changing their identity.

Consistent with this notion, we observed that a subset of Nkx6.1-deficient beta cells ectopically expressed somatostatin eight weeks after *Nkx6.1* deletion. Previously, we have shown with lineage tracing studies that *Nkx6.1* deletion in embryonic beta cells leads to a rapid beta-to-delta cell fate switch (Schaffer et al., 2013). However, conversion of beta cells into other non-beta endocrine cell types was not observed after *Nkx6.1* inactivation in embryonic beta cells. Similarly, after *Nkx6.1* deletion in adult beta cells, we observed co-expression of insulin exclusively with somatostatin but no other pancreatic hormones. Thus, loss of Nkx6.1 leads to selective de-repression of delta cell genes in both embryonic and adult beta cells. However, fate conversion is more complete and occurs more rapidly when *Nkx6.1* is inactivated in immature beta cells. Therefore, a sequential loss of beta cell traits preceding the adoption of alternative endocrine cell fates seen after adult *Nkx6.1* inactivation closely mirrors the gradual loss of functional beta cell mass observed in T2D models (Talchai et al., 2012).

Our study provides support for an evolving concept that transcription factors, such as Nkx6.1 and FoxO1 (Talchai et al., 2012), are critical for maintaining beta cells in their differentiated state. A key question that requires further exploration is whether a destabilized beta cell state is observed in humans and possibly contributes to the pathogenesis of T2D. The observation that NKX6.1 expression is decreased in beta cells from humans with T2D (Guo et al., 2013) suggests that findings in rodent models might indeed be relevant to human disease. Future studies will need to explore which aspects of the rodent phenotype are also found in humans and how loss of beta cell features relates to disease progression. Such knowledge could

identify a window for therapeutic intervention, during which the functional beta cell state can be restored before beta cells convert into other endocrine cell types.

MATERIALS AND METHODS

Mouse Strains.

The following mouse strains were utilized in this study: *Pdx1CreER^{TM}* (Gu et al., 2002), *Nkx6.1$^{+/-}$* (Sander et al., 2000a), and *Nkx6.1^{flox}* mice (Schaffer et al., 2013). All animals carrying the *Nkx6.1^{flox}* allele were maintained on a mixed 129Sv/C57Bl6/J genetic background. Unless otherwise stated in the text, male mice were used for experiments. Tamoxifen (Sigma) was dissolved in corn oil at 20mg/mL and 2mg was injected subcutaneously four times over a two week period. All animal experiments were approved by the Institutional Animal Care and Use Committees of the University of California, San Diego.

Glucose tolerance tests, insulin, and proinsulin measurements.

Glucose tolerance tests, GSIS assays, and insulin measurements were performed as previously described (Schaffer et al., 2011). Proinsulin measurements were performed on whole pancreatic lysates using a mouse proinsulin ELISA (ALPCO).

For glucose tolerance tests, mice were fasted overnight for 16 hours and blood glucose levels were recorded (Bayer Contour glucometer; Bayer, Tarreytown, NJ) before an intraperitoneal injection of a 1.5mg/g body weight dextrose solution in sterile water. Blood glucose levels were recorded at 20, 40, 60, 90, 120, and 150 minutes post injection. Cohorts of at least six mice of the same sex per genotype were used to perform glucose measurements. To measure plasma insulin levels,

mice were fasted overnight and blood was collected from tails into low retention microcentrifuge tubes (Eppendorf) before and 5 and 30 minutes after an intraperitoneal glucose injection. Collected blood was then centrifuged and serum was removed to assay for insulin using an insulin (mouse) ultrasensitive ELISA (ALPCO). To determine pancreatic insulin content, mice were fasted for four hours before whole pancreata were homogenized in 4 mL of a 2% acid:80% ethanol solution. Clarified samples were diluted 1:3000 in PBS before performing a mouse insulin ELISA (ALPCO). Insulin content was normalized to the protein concentration of pancreatic lysates (Pierce). To determine pancreatic proinsulin content, acid/ethanol extracts were diluted 1:1000 in PBS, measured by the mouse proinsulin ELISA (ALPCO) and values normalized to protein concentration. To determine the insulin to proinsulin ratio of pancreata, normalized proinsulin and insulin measurements were compared within each biological sample.

Tissue preparation, immunohistochemistry, and morphometric analysis.

Tissue preparation, immunofluorescence staining, TUNEL assays, and morphometry were performed as previously described (Schaffer et al., 2010; Schaffer et al., 2013). Mouse pancreata were fixed in 4% paraformaldehyde (Fisher Scientific) at 4ºC overnight. After fixation, samples were washed three times with PBS and then incubated in 30% sucrose at 4ºC overnight. Pancreata were embedded with Optimal Cutting Temperature Compound (O.C.T) (Tissue-Tek), frozen, and sectioned at 10 µm using a Cryostat (Leica). Sections were washed with PBS for 30 min and permeabilized in 0.15% Triton X-100 in PBS for 1 hour. For detection of nuclear antigens, antigen retrieval was performed at 37ºC in 10 mM sodium citrate buffer, pH 6. For detection of Cyclin D2, adult pancreata were embedded in paraffin and

subjected to antigen retrieval using a BioCare pressure cooker as previously described (Salpeter et al., 2011). Primary and secondary antibodies are listed in the Table 3.1 and Table 3.2, respectively. Immunodetection of p-AMPK required amplification of the primary signal using the TSA Kit (Invitrogen, Carlsbad, CA, USA). Staining using mouse primary antibodies was conducted using the mouse on mouse (M.O.M.) kit (Vector Labs) in conjunction with streptavidin/biotin blocking (Vector Labs) and streptavidin-conjugated secondary antibodies. When necessary, nuclei were counterstained with DAPI (Sigma) at 0.1 µg/ml. Images were captured using a Zeiss Axio Observer Z1 microscope with an apotome module on Zeiss AxioVision 4.8 and figures were prepared with Adobe Photoshop and Illustrator CS5.1.

Endocrine cell mass and area measurements were performed as previously described (Schaffer et al., 2011). Briefly, immunohistochemistry for insulin or chromogranin A was performed on six evenly spaced sections throughout the entire pancreas. To calculate endocrine cell mass, tiled images were taken from each section, fluorescent area for chromogranin A was determined as a percentage of the total pancreatic area (ImagePro Plus 5.0.1), and multiplied by the weight of the pancreas. To determine the percentage of endocrine cells expressing insulin, the total number of insulin$^+$chromogranin A$^+$ cells was divided by the total number of chromogranin A$^+$ cells. For examination of apoptosis, TUNEL analysis was performed as specified by the manufacturer (Millipore). For all quantifications, at least 50 islets were examined per mouse.

Microscopy and image analysis.

All immunofluorescent images were acquired using a Zeiss AxioOberver.Z1 microscope (Carl Zeiss, New York, NY) with the Zeiss ApoTome module and

processed in Zeiss AxioVision Release 4.8 and Adobe Photoshop CS5.1. Only brightness and contrast was adjusted in images in accordance with the *Journal of Cell Biology* figure manipulation guidelines.

Islet isolation, culture, and adenoviral infection.

Islet isolations were performed as previously described (Schaffer et al., 2011) with Liberase TL (Roche). Briefly, Liberase TL (Roche) was perfused into pancreata at a working concentration of 0.655 units/mL through the common hepatic bile duct. Pancreata were then removed and dissociated at 37ºC for 15 minutes. Islets were separated onto a gradient composed of HBSS (Cellgro) and Histopaque (Sigma) layers. Purified islets were then hand picked twice under a dissection microscope to minimize acinar contamination.

For Western blot analysis, islets were washed in sterile HBSS twice to remove residual BSA from islet isolations. For RNA isolation, islets were washed twice in DPBS (Calcium, magnesium, and RNase free) (Cellgro) and then lysed in buffer RLT (Qiagen) containing beta-mercaptoethanol and stored in -80ºC until RNA purification.

For incubation of islets with chemical compounds, RPMI supplemented with 2.7mM glucose and 1% BSA was used. *Nkx6.1*$^{\Delta adult\beta}$ islets were incubated with 100nM recombinant insulin (Sigma), 60μM +/- Bay K8644 (Sigma), or 10μM glucokinase activator (GKA) (EMD Calbiochem) and control islets were incubated with DMSO (ATCC) for three hours. For infection of islets with *Ad-Glut2* and *Ad-β-gal*, islets were dispersed and plated before infection as described previously (Fiaschi-Taesch et al., 2009). *Ad-Glut2* and *Ad-β-gal* viruses were kindly provided by C. Newgard (Duke University). Adenoviruses were expanded, purified, and titered as plaque forming units (PFU) per mL by ViraQuest Inc. Adenoviral infection of dispersed islets was

adapted from a previously described protocol (Fiaschi-Taesch et al., 2009). Briefly, on the day of islet isolation, islets were dispersed in 0.05% Trypsin/EDTA (Invitrogen) with gentle agitation for 5 minutes at 37ºC, washed in RPMI 1640 containing 10% FBS, and allowed to adhere overnight in 6 well tissue culture treated dishes coated with poly-l-lysine (Sigma Aldrich). 300 islet equivalents (~1000 cells per islet) pooled from two mice were used per biological sample for infections. On the day of infection, media was replaced with RPMI 1640 containing 2% FBS and islets were infected with a viral MOI of 100 (100 PFU/cell) for 2 hours before cells were washed and media was replaced with RPMI containing 10% FBS. 72 hours after viral infection, RNA was harvested from infected islets as described or immunofluorescence staining was performed as described on dispersed islets plated on poly-l-lysine coated coverslips (BD).

For determination of proinsulin and insulin content 72 hours after viral infection, dispersed islets were equilibrated in RPMI 1640 supplemented with 2.7mM glucose for 1 hour at 37ºC. Media was then replaced with RPMI 1640 supplemented with 16.7mM glucose for 2 hours at 37ºC. After incubation, dispersed islets were washed in ice cold DPBS (Ca^{2+} and Mg^{2+} free) followed by extraction of proinsulin and insulin with a 2% acid:80% ethanol solution. Proinsulin and insulin content was measured by ELISA (Alpco) and normalized by protein concentration determined by BCA (Pierce).

Incubation of islets with chemical compounds.

Mouse islets were isolated and washed in RPMI 1640 supplemented with 2.7mM glucose and 1% BSA. Islets were then incubated for three hours immediately after isolation in media supplemented with either a DMSO vehicle for control mice or

100nM recombinant insulin (Sigma), 60μM +/- Bay K8644 (Sigma), or 10 μM glucokinase activator (EMD Calbiochem) for *Nkx6.1*$^{\Delta adult\beta}$ mice. After incubation, islets were washed in HBSS and lysed in 350μL RLT containing beta-mercaptoethanol for subsequent RNA isolation.

Analysis of Proliferation after treatment with Bay K8644.

To examine beta cell proliferation after administration of Bay K8644, mice were administered 8mg/kg Bay K8644 dissolved in 20% DMSO, 79% Salene, and 1% Tween 80 or vehicle control (Salpeter et al., 2011). Pancreata were harvested 24 hours after injection and processed for immunostaining.

Glucose Stimulated Insulin Secretion (GSIS) assays.

GSIS assays were performed as previously described (Schaffer et al., 2011). Briefly, islets were isolated from 6 mice per genotype and were incubated overnight in RPMI 1640 supplemented with 8mM glucose, 10% FBS, 2mM L-glutamine, 100u/mL Pen/Strep, 1mM sodium pyruvate, 10mM HEPES, and 0.25μg/mL amphoterecin B. The next day, islets were washed and pre-incubated for 1 hour in a 2.7mM glucose solution. Afterwards, groups of 10 islets were transferred to a 96-well dish into solutions of 2.7mM glucose, 16.7mM glucose, 30mM KCl with 2.7mM glucose, or 2μM Bay K8644 with 16.7mM glucose. After incubation for 1 hour, supernatant was collected and islets were lysed overnight in a 2% acid:80% ethanol solution. Insulin was then measured in supernatants and lysates using the mouse insulin ELISA (ALPCO). Secreted insulin was calculated as percentage of total insulin content per hour.

ATP Measurement.

ATP measurements were performed on isolated islets as described previously (Zhang et al., 2011). Briefly, on the day of islet isolation, isolated islets were equilibrated in RPMI 1640 supplemented with 2.7mM glucose for one hour. After incubation, 20 islet equivalents per mouse were washed in ice cold DPBS and subsequently lysed in 5% trichloroacetic acid (TCA) for 5 minutes at room temperature. TCA was then neutralized with 90 µL of 1% Triton X-100, 100mM Tris-Acetate pH 8.0. ATP levels were measured using the Enliten ATP assay kit (Promega, Madison, WI) and expressed as relative light units (RLU) per islet equivalent.

Electron microscopy.

For electron microscopy sample preparation, a fixative containing 2% paraformaldehyde, 2.5% glutaraldehyde, 3µM CaCl2 prepared in 0.1M sodium cacodylate buffer to a final pH 7.4 was used. Pancreata were perfused through the common hepatic bile duct with 200µL of fixative before being removed from the mice. Pancreata were then immersed in fixative and islets were microdissected using fine razors at room temperature. Samples were incubated in fixative for at least 4 hours at 4ºC, postfixed in 1% osmium tetroxide in 0.15 M cacodylate buffer for 1 hour and stained en bloc in 1% uranyl acetate for 1 hour. Samples were dehydrated in ethanol, embedded in Durcupan epoxy resin (Sigma-Aldrich), sectioned at 50 to 60 nm, and picked up on Formvar and carbon-coated copper grids. Sections were then stained with 2% uranyl acetate for 5 minutes and Sato's lead stain for 1 minute. Grids were viewed using a Tecnai G2 Spirit BioTWIN transmission electron microscope equipped with an Eagle 4k HS digital camera (FEI, Hilsboro, OR). To quantify vesicle diameter,

a total of 300 vesicles in 10 image planes were randomly selected for measurement. Vesicle diameter was measured as the longest distance between two points of a vesicle containing a mature insulin granule. To quantify vesicle number per area, blood vessel area was subtracted from total area, and vesicle numbers were quantified on at least 10 randomly selected 2900x image planes. To quantify vesicle subtype, total vesicle numbers were quantified on at least 10 randomly selected 2900x image planes and vesicles classified as either immature or mature were calculated as a percentage of total vesicles. At least 50 beta cells and 4000 vesicles were analyzed per genotype.

Western blot analysis.

To determine Cyclin D2 and Neurogenin 3 protein levels, islets were lysed in RIPA buffer and 30µg of protein was loaded onto a 10% Tris-HCl SDS-polyacrylamide gel. Protein was then transferred to a nitrocellulose membrane and membranes were blocked in 5% milk PBS/0.1% tween followed by incubation with primary antibodies in blocking solution overnight at 4ºC and secondary antibodies for 1 hour at room temperature the following day. Primary and secondary antibodies are listed in Table 3.1 and Table 3.2, respectively.

Microarray analysis and statistical methods.

To identify Nkx6.1-regulated genes in islets, we isolated islets from six mice per genotype four days after the final tamoxifen injection and then pooled islets from two mice per genotype for a total of three independent biological replicates. Each individual RNA sample was prepared as per the manufacturer's instructions (Qiagen Micro RNeasy isolation kit). RNA was quantified using a NanoDrop-1000

spectrophotometer and quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Approximately 150 ng of total RNA was amplified and labeled with Cy3 using the QuickAmp Low Input Labeling Kit, One-Color (Agilent Technologies). This labeling reaction produced 1650 ng of Cy3-labeled cRNA (anti-sense), by first converting mRNA primed with an oligo (d)T-T7 primer into dsDNA with MMLV-RT. The sample was then amplified using T7 RNA Polymerase in the presence of Cy3-CTP. Three independent biological samples of each genotype were hybridized to Agilent Whole Mouse Genome Oligo Microarray G2519F chips. Agilent microarray and data analysis was performed with support from the UCSD BioMedical Genomics Microarray (BIOGEM) Facility (San Diego, CA). Expression changes were considered significant if they had a false discovery rate (FDR) less than 0.01 and a fold change of at least 1.5. A table of significantly changed genes is provided in File 3.1.

Reverse transcription and quantitative PCR.

Total mRNA was purified from isolated islets from individual mice with the RNAeasy Micro kit (Qiagen). Subsequent cDNA synthesis was performed using the iScript Reverse Transcription Supermix for RT-qPCR (BioRad). qPCR reactions were run in biological triplicates and in technical replicates with 0.5 to 5 ng cDNA per reaction. A CFX96 real-time system (BioRad) was used to acquire data from qRT-PCR reactions. The housekeeping gene *GAPDH*, verified to remain unchanged between samples from normalized microarray data, was used as an endogenous control. Primers used for verifications are listed in Table 3.3.

Chromatin immunoprecipitation and sequencing.

Chromatin immunoprecipitation (ChIP) was performed as previously described (Schaffer et al., 2013) with rabbit anti-Nkx6.1 antiserum (1:250) on sheared chromatin obtained from ~10,000 islet equivalents (1000 cells per islet) isolated from C57BL/6J mice. ChIP-seq libraries were prepared as per Illumina's instructions (http://www.illumina.com). Sequencing was performed on an Illumina/Solexa Genome Analyzer II in accordance with the manufacturer's protocols. Data analysis was performed using Hypergeometric Optimization of Motif EnRichment (HOMER) (Heinz et al., 2010). Peak annotation and *de novo* motif analysis were performed using HOMER and venn diagrams were generated using BioVenn (Hulsen et al., 2008).

Nkx6.1-enriched regions were identified with the findpeaks parameter from the HOMER package. A p-value of 1e-4, peak size of 150, and a six-fold enrichment over input was used to compare Nkx6.1 ChIP reads to input reads for identification of significant Nkx6.1 binding peaks. Binding peaks were annotated with the gene with the nearest transcriptional start site within 10kb of an identified Nkx6.1 binding peak. To identify genes that were bound and regulated by Nkx6.1, the list of significantly changed genes in islets from *Nkx6.1*$^{\Delta adult\beta}$ mice was compared to the gene list of annotated peaks from the Nkx6.1 ChIP-seq experiment using BioVenn (Hulsen et al., 2008). To identify *de novo* Nkx6.1 binding motifs, the HOMER parameter findmotifsgenome.pl was used. Lists of annotated peaks and comparison of ChIP-seq and microarray data is provided in Tables 3.2 and 3.3.

Statistics.

Unless otherwise stated, all values are shown as mean ± SEM; P-values were calculated using an unpaired Student's t-test in Microsoft Excel; Hypergeometric

distribution was determined using R. $P<0.05$ was considered significant.

Accession numbers.

The GEO (http://www.ncbi.nlm.nih.gov/geo/) accession number for the microarray data set reported in this paper is GSE40470. The GEO (http://www.ncbi.nlm.nih.gov/geo/) accession number for the ChIP-seq data set reported in this paper is GSE40975.

Table 3.1. List of primary antibodies used in immunofluorescence staining and Western blot analysis.

Primary Antibodies				
Antigen	**Host**	**Dilution**	**Source**	**Catalogue #**
Nkx6.1	Rabbit	1:800	BCBC	AB1069
Insulin	Guinea Pig	1:2000	Dakocytomation	A0564
Glucagon**	Mouse	1:5000	Sigma	G2654
Somatostatin	Rabbit	1:3000	Dakocytomation	A0566
Pancreatic Polypeptide	Rabbit	1:2000	Dakocytomation	A0619
GFP	Rat	1:1000	C. Kioussi, Oregon State University	Gift
Pdx1	Guinea Pig	1:10000	C. Wright, Vanderbilt	Gift
MafA	Rabbit	1:1000	Bethyl Labs	IHC-00352
PC1/3	Rabbit	1:2000	D. Steiner, University of Chicago	Gift
ERO1-Beta	Rabbit	1:300	D. Ron, Cambridge University	Gift
GLUT2	Rabbit	1:1000	Millipore	07-1402
Chromogranin A	Goat	1:500	Santa Cruz	SC-1488
p-AMPK*	Rabbit	1:100	Cell Signaling Technology	#2535
Cyclin D2	Rabbit	1:200	Santa Cruz	SC-593
Ki-67	Rabbit	1:500	Lab Vision	#RM-9106-S0
GAPDH	Mouse	1:1000	Ambion	4300
Urocortin 3	Rabbit	1:1000	M. Huising, Salk Institute	Gift
Ngn3	Guinea Pig	1:1000	M. Sander	(Henseleit et al., 2005)

*: Tyramid signal amplification (TSA) was required (Invitrogen)
**: Used with M.O.M. kit (Vector Labs)

Table 3.2. List of secondary antibodies used in immunofluorescence staining and Western blot analysis.

Secondary Antibodies			
Antigen	**Conjugation**	**Dilution**	**Source**
Rabbit/Goat/Guinea Pig/Rat	Alexa-488	1:2000	Jackson Immunoresearch
Rabbit/Goat/Guinea Pig/Rat	Cy3	1:2000	Jackson Immunoresearch
Rabbit/Goat/Guinea Pig/Rat	Cy5	1:500	Jackson Immunoresearch
Mouse	Biotinylated		Vector laboratories
Mouse	HRP	1:5000	GE Healthcare
Rabbit	HRP	1:5000	GE Healthcare
Guinea Pig	HRP	1:5000	Jackson Immunoresearch

Table 3.3. List of primer sequences used for qRT-PCR analysis.

Primer	Primer sequence (5'-3')
Abcc8 Fw	TCAACTTGTCTGGTGGTCAGC
Abcc8 Rv	GAGCTGAGAAAGGGTCATCCA
Aldob Fw	AGAAGGACAGCCAGGGAAAT
Aldob Rv	GTTCAGAGAGGCCATCAAGC
Cacna1c Fw	ATGAAAACACGAGGATGTACGTT
Cacna1c Rv	ACTGACGGTAGAGATGGTTGC
Ccna2 Fw	GCCTTCACCATTCATGTGGAT
Ccna2 Rv	TTGCTGCGGGTAAAGAGACAG
Ccnb1 Fw	GCGTGTGCCTGTGACAGTTA
Ccnb1 Rv	CCTAGCGTTTTTGCTTCCCTT
Ccnb2 Fw	AGCTCCCAAGGATCGTCCTC
Ccnb2 Rv	TGTCCTCGTTATCTATGTCCTCG
Ccnd2 Fw	GAGTGGGAACTGGTAGTGTTG
Ccnd2 Rv	CGCACAGAGCGATGAAGGT
Ero1lb Fw	ACCCTGAGCTTCCTCTCAAGT
Ero1lb Rv	AAAGGACATGGTCGTTTCAGATT
G6pc2 Fw	CCTACTACGTGTGAAACAGGC
G6pc2 Rv	CAGAAAGGACCAGGTCAGTCT
Gapdh Fw	CATGTTCCAGTATGACTCCACTC
Gapdh Rv	GGCCTCACCCCATTTGATGT
Gck Fw	CTGTTAGCAGGATGGCAGCTT
Gck Rv	TTTCCTGGAGAGATGCTGTGG
Glp1r Fw	ACGGTGTCCCTCTCAGAGAC
Glp1r Rv	ATCAAAGGTCCGGTTGCAGAA
Hnf1a Fw	GTGGCGAAGATGGTCAAGTC
Hnf1a Rv	GCGTGGGTGAATTGCTGAG
Hnf1b Fw	GCCTGAACCAATCCCACCTC
Hnf1b Rv	TGACTGCTTTTGTCTGTCATGT
Hnf4a Fw	TAACACGATGCCCTCTCACCT
Hnf4a Rv	GGCAGGAGCTTGTAGGATTCA
Ins1 Fw	GACCAGCTATAATCAGAGACCATC
Ins1 Rv	GTAGGAAGTGCACCAACAGG
Ins2 Fw	GGCTTCTTCTACACACCCAT
Ins2 Rv	CCAAGGTCTGAAGGTCACCT
Kcnj11 Fw	AAGGGCATTATCCCTGAGGAA
Kcnj11 Rv	TTGCCTTTCTTGGACACGAAG
Mafa Fw	GAGGAGGTCATCCGACTGAAA
Mafa Rv	GCACTTCTCGCTCTCCAGAAT
Mnx1 Fw	GAACACCAGTTCAAGCTCAACA
Mnx1 Rv	GCTGCGTTTCCATTTCATTCG
NeuroD Fw	GCCCAGCTTAATGCCATCTTT
NeuroD Rv	CAAAAGGGCTGCCTTCTGTAA
Ngn3 Fw	CAGTCACCCACTTCTGCTTC
Ngn3 Rv	GAGTCGGGAGAACTAGGATG
Nkx6.1 Fw	CTTCTGGCCCGGAGTGATG
Nkx6.1 Rv	GGGTCTGGTGTGTTTTCTCTTC
Noc2 Fw	GCAGTGGAAATGATCAGTGG
Noc2 Rv	TCAGGCACTGGCTCCTCCTC
Pclo Fw	TACTCGGACCCATTTGTGAA

Table 3.3. List of primer sequences used for qRT-PCR analysis (Continued).

Primer	Primer sequence (5'-3')
Pclo Rv	TACTGTTTGATTCCACTCGGGATT
Pcsk1 Fw	AGTTGGAGGCATAAGAATGCTG
Pcsk1 Rv	GCCTTCTGGGCTAGTCTGC
Pcx Fw	CTGAAGTTCCAAACAGTTCGAGG
Pcx Rv	CGCACGAAACACTCGGATG
Pdx1 Fw	CTTAACCTAGGCGTCGCACAA
Pdx1 Rv	GAAGCTCAGGGCTGTTTTTCC
Rfx6 Fw	TGCCAGTGCATACTCGACAAT
Rfx6 Rv	AACAGGATTTTCAAGCAGGGG
Glut2 Fw	TTCCAGTTCGGCTATGACATCG
Glut2 Rv	CTGGTGTGACTGTAAGTGGGG
Slc30a8 Fw	CAGAGAACTTCGACAGAAGCC
Slc30a8 Rv	CTTGCTTGCTCGACCTGTT
Sytl4 Fw	ATCATTTAGTGTGCCGAGAATGC
Sytl4 Rv	CCTGTTCGGTAATCAAAGCGA
Tle3 Fw	AGCACGAACAATTCCGTGTCA
Tle3 Rv	CCATCGCTATCGTATCTGCTG
Ucn3 Fw	GCTGTGCCCCTCGACCT
Ucn3 Rv	TGGGCATCAGCATCGCT

SUPPLEMENTAL MATERIAL LEGENDS

File 3.1. List of significantly up- and down-regulated genes in *Nkx6.1*$^{\Delta adult\beta}$ mice. Genes with an FDR<0.01 and a fold change of at least 1.5 are listed. FDR, false discovery rate; f(mut/ctr), fold change of mutant samples relative to control.

File 3.2. List of Nkx6.1 ChIP-seq binding peaks in islets. ChIP-seq peaks were defined as 6-fold higher than input signal. Peaks within 10kb of a transcriptional start site (TSS) were annotated with the respective gene. PeakID, unique peak identification name; Chr, chromosome; Distance to TSS, distance to transcriptional start site.

File 3.3. List of genes bound and regulated by Nkx6.1. Nkx6.1-bound genes were defined as having an Nkx6.1 ChIP-seq peak within 10kb of a transcriptional start site. Nkx6.1-regulated genes were defined as mRNAs having an FDR<0.01 and a fold change of at least 1.5 in *Nkx6.1*$^{\Delta adult\beta}$ compared to control islets. The total number of genes within each category is listed in parentheses.

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Chapter 3, in full, is a reprint of the material as it appears in Taylor, B.L.*, Liu, F., and Sander, M.A. (2013). Nkx6.1 is essential for maintaining the functional state of pancreatic beta cells. Cell Reports. The dissertation author was the primary investigator and author of this study.

CHAPTER 4:

Nkx6.1 is required for postnatal beta cell mass expansion

ABSTRACT

Pancreatic beta cells undergo a period of rapid expansion in the postnatal period, required for establishing beta cell mass in adults. Many of the factors required for this highly proliferative period have been identified, however few studies have identified what transcriptionally maintains the pro-proliferative response of beta cells to external factors. Here, we report that the beta cell specific transcription factor Nkx6.1 is essential for postnatal beta cell mass expansion. Mosaic inactivation of *Nkx6.1* in newly specified beta cells results in a cell autonomous decrease in beta cell proliferation, leading to reduced beta cell mass and glucose intolerance in adult mice. Beta cell proliferation was not immediately affected in neonatal beta cells, but was significantly reduced within a few days after birth. This proliferative defect correlates with the transition from placental nutrition to postnatal feeding, indicating that Nkx6.1 is required for the response of beta cells to systemic factors associated with feeding. In accordance with this observation, Nkx6.1 deficient beta cells lacked key nutrient sensors including Glut2 and Glp1r. Therefore, we identify a cell autonomous role for Nkx6.1 to maintain nutrient-stimulated beta cell proliferation in the perinatal period.

INTRODUCTION

The formation and maintenance of pancreatic beta cell mass is essential for the regulation of blood glucose homeostasis, as reductions in beta cell numbers results in diabetes (Thorel et al., 2010). To form beta cell mass in adult animals, it is well established that beta cells undergo a highly proliferative phase during perinatal development (Finegood et al., 1995; Georgia and Bhushan, 2004; Kushner et al., 2005; Teta et al., 2005). Beta cells do not arise from specialized progenitors during this period, but are derived from self-duplication (Brennand et al., 2007; Dor et al.,

2004; Teta et al., 2007). Furthermore, proliferation is directly responsible for formation of beta cell mass, as loss of pro-proliferative genes including *Cyclin D1* or *D2* results in reduced beta cell proliferation and beta cell mass in adult animals (Georgia and Bhushan, 2004; Kushner et al., 2005). Overall, these studies establish that beta cells are highly proliferative in the perinatal period and that proliferation rapidly declines once beta cell mass is fully formed in adult animals and humans (Teta et al., 2005). Therefore, many studies are aimed at identifying the extrinsic and intrinsic factors that stimulate beta cell proliferation and pro-proliferative gene expression during this period.

Recently, there have been significant advances in the understanding of how pro-proliferative genes in the beta cell are regulated by external stimuli. Previous studies have suggested that glucose may be a key pro-proliferative systemic factor for beta cells, as glucose infusion results in elevated proliferative rates of beta cells (Alonso et al., 2007; Bonner-Weir et al., 1989). Furthermore, recent studies directly demonstrated that glucose metabolism in beta cells is both necessary and sufficient for *cyclin* gene expression and subsequently stimulation of beta cell proliferation (Porat et al., 2011; Salpeter et al., 2010; Salpeter et al., 2011). Overall, these studies link feeding and increases in blood glucose levels to beta cell proliferation. Since this pathway is specialized to beta cells in the pancreas, it suggests that beta cell enriched transcription factors may maintain this signaling pathway.

Several studies have reported seemingly contradictory roles for the beta cell specific transcription factor Nkx6.1 as a regulatory factor of pancreatic beta cell proliferation. *In vitro* overexpression of *Nkx6.1* in islets was found to be sufficient to stimulate beta cell proliferation (Schisler et al., 2008). However, *in vivo* Nkx6.1 overexpression was not capable of achieving the same results (Schaffer et al., 2011).

Our laboratory recently resolved these conflicting reports by using an *in vivo* model to inactivate *Nkx6.1* in beta cells. We found that Nkx6.1 is in fact necessary for beta cell proliferation and *Cyclin D2* expression, however this regulation is indirect (Taylor et al., 2013). Specifically, Nkx6.1 is necessary for expression of the glucose transporter, Glut2, and subsequently the stimulation of calcium influx and expression of *cyclin D2* by glucose. Therefore, Nkx6.1 regulates beta cell proliferation in response to fluctuations in glucose concentrations, which explains why Nkx6.1 is necessary for beta cell proliferation in adult mice. However, due to the relatively low proliferative rates of adult beta cells (Teta et al., 2005), it is not clear whether beta cell mass is affected by proliferative defects in Nkx6.1-deficient beta cells.

To directly determine whether Nkx6.1 mediated beta cell proliferation affects formation of beta cell mass, we examined the role of Nkx6.1 in highly proliferating beta cells during postnatal beta cell mass expansion. We show that deletion of *Nkx6.1* in newly differentiated beta cells causes decreased beta cell proliferation, which subsequently results in reduced beta cell mass in adult animals. Furthermore, mosaic inactivation of *Nkx6.1* in this model also revealed that Nkx6.1 regulates beta cell proliferation in a cell autonomous manner, as non-targeted cells proliferate. Finally, we identify that Nkx6.1 is only required for beta cell proliferation after newborn mice have been separated from placental nutrients and transitioned to feeding.

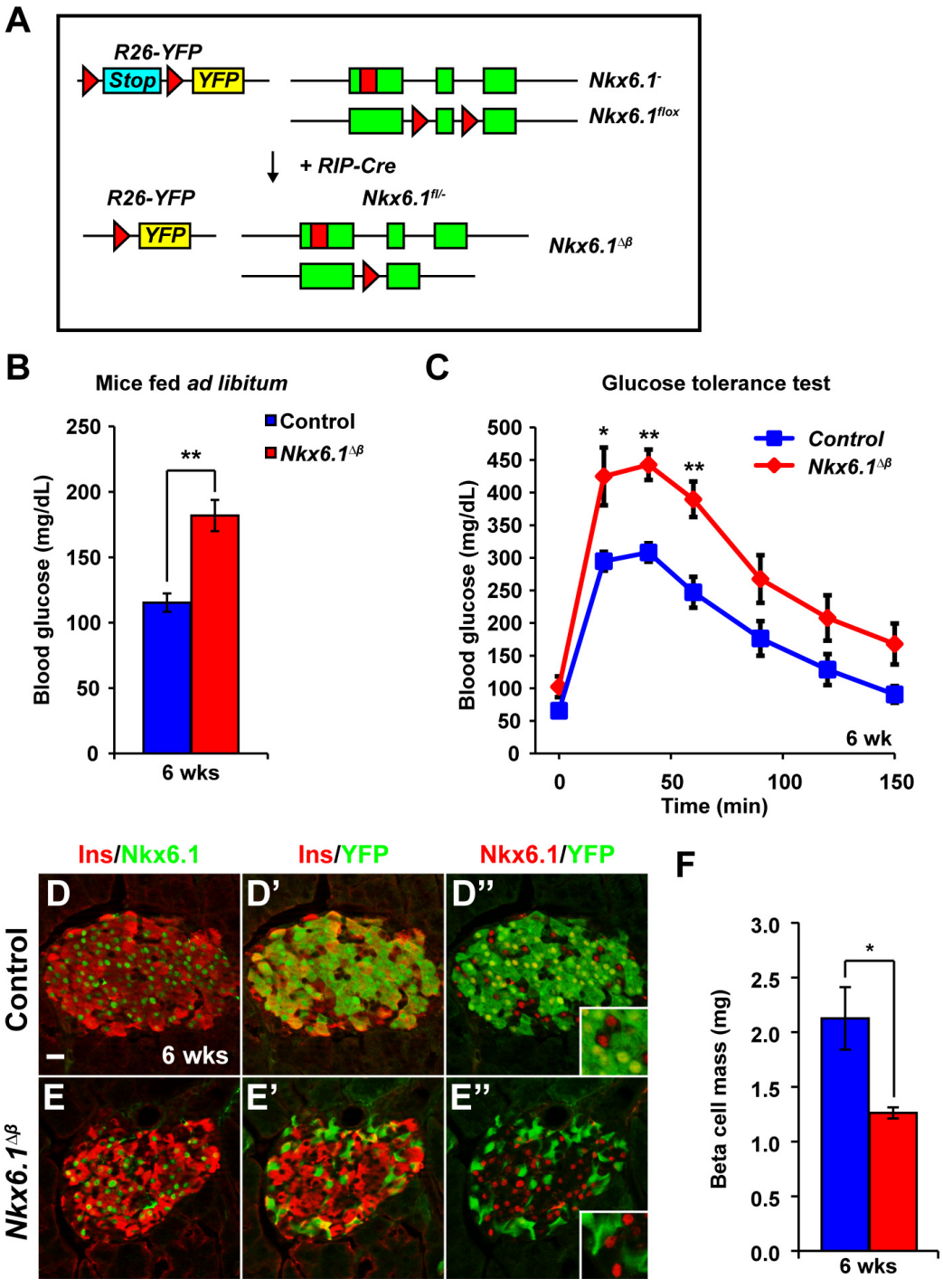
RESULTS

Inactivation of *Nkx6.1* in immature beta cells results in decreased beta cell mass in adult mice.

Due to the relatively low proliferative rates of adult beta cells (Teta et al., 2005), it is not clear whether beta cell mass is affected by Nkx6.1-regulated beta cell proliferation (Taylor et al., 2013). Therefore, to examine whether loss of Nkx6.1 affects beta cell mass, we sought to inactivate *Nkx6.1* in highly proliferative beta cells of neonatal mice. To inactivate *Nkx6.1* in this domain, we utilized mice that express *Cre* recombinase under the control of the rat *insulin2* promoter (*RIP-Cre*) (Postic et al., 1999). This results in recombination of the *Nkx6.1* conditional loss of function allele ($Nkx6.1^{flox}$) in beta cells at the onset of *insulin2* gene transcription (Schaffer et al., 2013). Additionally, we intercrossed a conditional YFP genetic lineage reporter targeted to the *Rosa-26* allele (*R26-YFP*), which expresses YFP upon Cre-mediated recombination of a translational stop signal (Srinivas et al., 2001). Therefore in *RIP-Cre;*$Nkx6.1^{flox/-}$*;R26-YFP* ($Nkx6.1^{\Delta\beta}$) mice, YFP will label all cells in which *Nkx6.1* has been inactivated (Figure 4.1A) and in *RIP-Cre;*$Nkx6.1^{flox/+}$*;R26-YFP* (control) mice, cells in which Cre recombinase is expressed.

Since mice rapidly develop diabetes after deletion of Nkx6.1 in mature beta cells (Taylor et al., 2013), we expected that deletion of *Nkx6.1* in newly differentiated beta cells would result in overt diabetes, regardless of changes in beta cell mass. Surprisingly, $Nkx6.1^{\Delta\beta}$ mice at six weeks of age were only mildly hyperglycemic with blood glucose levels of 180±12 mg/dL, compared to 114±7 mg/dL in control mice (Figure 4.1B). Furthermore, $Nkx6.1^{\Delta\beta}$ mice were only slightly glucose intolerant compared to control mice with significantly higher blood glucose levels at 20, 40, and

Figure 4.1. Deletion of Nkx6.1 in neonatal beta cells results in glucose intolerance in adult mice. (A) Schematic diagram of alleles and transgenes utilized to inactivate *Nkx6.1* in neonatal beta cells. Red triangles, loxP sites; rectangles, coding sequence; red rectangle, DsRed insertion. (B) Blood glucose levels of mice fed *ad libitum* were significantly increased in six week old *Nkx6.1*$^{\Delta\beta}$ mice (red bars) compared to control mice (blue bars) (n=6). (C) Six week old *Nkx6.1*$^{\Delta\beta}$ mice (red lines) were glucose intolerant compared to control mice (blue lines) (n=6). (D-E'') Immunofluorescent staining of pancreatic sections from control and *Nkx6.1*$^{\Delta\beta}$ mice at six weeks of age reveals efficient deletion of Nkx6.1 in YFP^{+} cells of *Nkx6.1*$^{\Delta\beta}$ mice and decreased YFP^{+} cell numbers (insets D'', E''). (F) Beta cell mass is decreased at six weeks of age in *Nkx6.1*$^{\Delta\beta}$ mice (red bars) compared to control mice (blue bars) (n=3). Scale bars = 20 μm. Ins, insulin; YFP, yellow fluorescent protein; wks, weeks. Data shown as mean ± SEM. *p<0.05, **p<0.0


A
R26-YFP
Stop
YFP
Nkx6.1-
Nkx6.1flox
+ RIP-Cre
R26-YFP
YFP
Nkx6.1fl/-
Nkx6.1Δβ
B
Mice fed ad libitum
Control
Nkx6.1Δβ
Blood glucose (mg/dL)
6 wks
C
Glucose tolerance test
Control
Nkx6.1Δβ
Blood glucose (mg/dL)
Time (min)
6 wk
Ins/Nkx6.1
Ins/YFP
Nkx6.1/YFP
Control
Nkx6.1Δβ
D
D'
D''
E
E'
E''
6 wks
F
Beta cell mass (mg)
6 wks

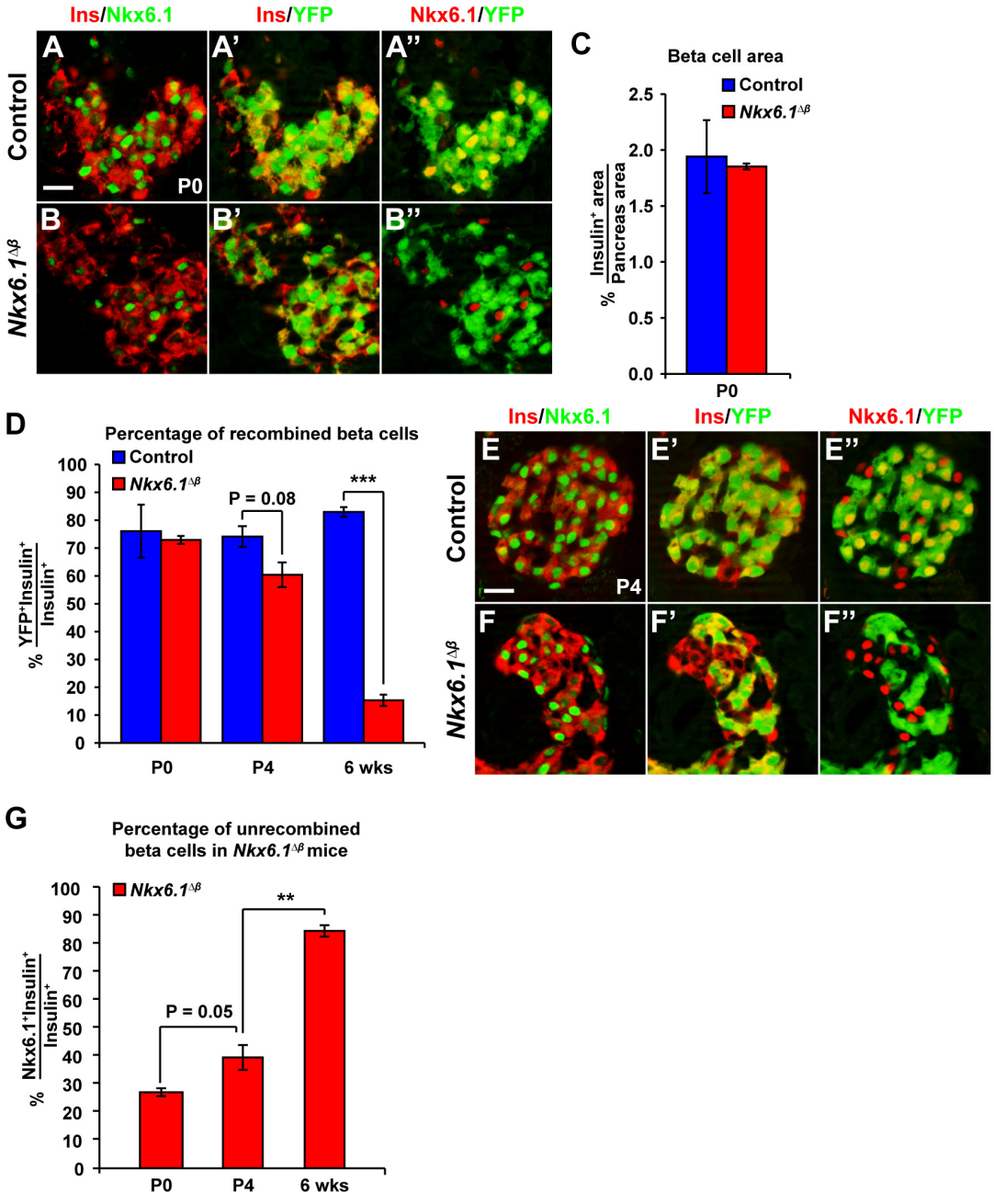
60 minutes after a glucose stimulus (Figure 4.1C). Therefore, this suggested that *Nkx6.1* may not have been efficiently deleted in *Nkx6.1*$^{\Delta\beta}$ mice.

Due to the inherent mosaic expression of the *RIP-Cre* transgene (Postic et al., 1999), it is possible that *Nkx6.1*$^{\Delta\beta}$ mice were not diabetic because of the presence of untargeted Nkx6.1^{+} beta cells. To examine this possibility, we performed immunofluorescent staining for insulin, Nkx6.1, and YFP in pancreatic sections of control and *Nkx6.1*$^{\Delta\beta}$ mice at six weeks of age (Figure 4.1D-E''). Consistent with this idea, we observed a high percentage of YFP^{-} and Nkx6.1^{+} beta cells in *Nkx6.1*$^{\Delta\beta}$ mice (Figure 4.1D-E''). Quantification of beta cell mass further revealed a 40% reduction in *Nkx6.1*$^{\Delta\beta}$ mice (1.26+/-0.05 mg compared to 2.13+/-0.29 mg in control mice) (Figure 4.1F). Since the majority of insulin^{+} cells in *Nkx6.1*$^{\Delta\beta}$ mice were Nkx6.1^{+}, it is therefore likely that *Nkx6.1*$^{\Delta\beta}$ mice were only hyperglycemic and not diabetic because of reduced beta cell mass (Thorel et al., 2010).

Deletion of *Nkx6.1* results in the postnatal decrease of Nkx6.1 deficient beta cells.

The reduced presence of YFP^{+}insulin^{+} cells in adult *Nkx6.1*$^{\Delta\beta}$ mice may be the result of decreased recombination efficiency in *Nkx6.1*$^{\Delta\beta}$ mice. To directly examine this, we analyzed pancreata from control and *Nkx6.1*$^{\Delta\beta}$ mice at P0, before we observed decreased YFP^{+}insulin^{+} cells (Figure 4.2A-B''). Nkx6.1 was absent in the majority of insulin^{+}YFP^{+} cells in *Nkx6.1*$^{\Delta\beta}$ mice, indicating that *Nkx6.1* was efficiently deleted by P0 (Figure 4.2B-B''). Additionally, there appeared to be an equal percentage of insulin^{+}YFP^{+} cells at P0 between control and *Nkx6.1*$^{\Delta\beta}$ mice (76+/-9.5% vs. 73+/-1.4%, respectively) (Figure 4.2D) and a similar insulin^{+} area in control (1.94+/-0.32%) and *Nkx6.1*$^{\Delta\beta}$ mice (1.85+/-0.03%) (Figure 4.2C). Together these data

Figure 4.2. Nkx6.1 maintains beta cells during postnatal beta cell mass expansion. (A-B'') Immunofluorescence staining for insulin, Nkx6.1, and YFP reveals efficient deletion of Nkx6.1 in pancreata of *Nkx6.1*$^{\Delta\beta}$ mice at P0. (C) Quantification of insulin immunofluorescent area at P0 reveals no significant difference in beta cell area in pancreata of control and *Nkx6.1*$^{\Delta\beta}$ mice (n=3). (D) Quantification of the percent of insulin^{+} cells expressing YFP at P0, P4, and 6 wks reveals a trending decrease at P4 and significant decrease at 6 wks in *Nkx6.1*$^{\Delta\beta}$ mice (n=3). (E-F'') Immunofluorescent staining for insulin, Nkx6.1, and YFP at P4 reveals a decrease in YFP and insulin co-positive cells in *Nkx6.1*$^{\Delta\beta}$ mice. (G) Quantification of the percent of insulin^{+} cells expressing Nkx6.1 in *Nkx6.1*$^{\Delta\beta}$ mice reveals an increase after P0 (n=3). Scale bar = 20 μm. Ins, insulin; YFP, yellow fluorescent protein; wks, weeks. Data shown as mean +/- SEM. **, $p<0.01$; ***, $p<0.001$.


Ins/Nkx6.1
Ins/YFP
Nkx6.1/YFP
Control
Nkx6.1Δβ
A
A'
A''
B
B'
B''
P0
C
Beta cell area
Control
Nkx6.1Δβ
% Insulin+ area / Pancreas area
2.5
2.0
1.5
1.0
0.5
0.0
P0
D
Percentage of recombined beta cells
Control
Nkx6.1Δβ
% YFP+Insulin+ / Insulin+
P = 0.08
***
P0
P4
6 wks
E
E'
E''
F
F'
F''
P4
G
Percentage of unrecombined beta cells in Nkx6.1Δβ mice
Nkx6.1Δβ
% Nkx6.1+Insulin+ / Insulin+
P = 0.05
**
P0
P4
6 wks

suggest that Cre-mediated recombination occurs at equal frequencies between control and *Nkx6.1*$^{\Delta\beta}$ mice at P0.

Because the number of insulin^{+}YFP^{+} cells is drastically lower in *Nkx6.1*$^{\Delta\beta}$ mice at six weeks of age, but is equal to controls at birth, it suggests that there is a selective reduction in Nkx6.1-deficient beta cells after birth. Consistent with this observation, immunofluorescent staining revealed a decrease in the percentage of insulin^{+}YFP^{+} cells in *Nkx6.1*$^{\Delta\beta}$ mice by p4 [60.5±4.4% compared to control mice (74.2+/-3.7%)], which further decreases at 6 wks when we observed lower beta cell mass [15+/-2.02% compared to 83+/-1.72% in control mice, reported in (Schaffer et al., 2013)] (Figure 4.2D, E-F''). Notably, the decreased percentage of insulin^{+}YFP^{+} cells in *Nkx6.1*$^{\Delta\beta}$ mice was accompanied by a significant increase in the percentage of Nkx6.1^{+}insulin^{+} cells in *Nkx6.1*$^{\Delta\beta}$ mice (Figure 4.2F-F'', quantified in G). Therefore, this data suggests that Nkx6.1^{+} cells are positively selected for over Nkx6.1-deficient beta cells in the postnatal period.

Deletion of Nkx6.1 results in decreased beta cell proliferation.

The selective decrease in Nkx6.1-deficient beta cells after birth suggests that Nkx6.1 is required for either proliferation or survival of beta cells during postnatal development. To investigate whether survival of Nkx6.1-deficient beta cells was affected, we performed a terminal deoxynucleotidyl transferase dUTP nicked end labeling (TUNEL) assay on pancreatic sections at P4 to detect apoptosis. Although we observed a reduction in Nkx6.1-deficient beta cells (Figure 4.3A-B') at P4, we did not observe a reciprocal increase in TUNEL and YFP co-positive cells in *Nkx6.1*$^{\Delta\beta}$ compared to control mice (Figure 4.3A, B). This suggests that beta cells do not

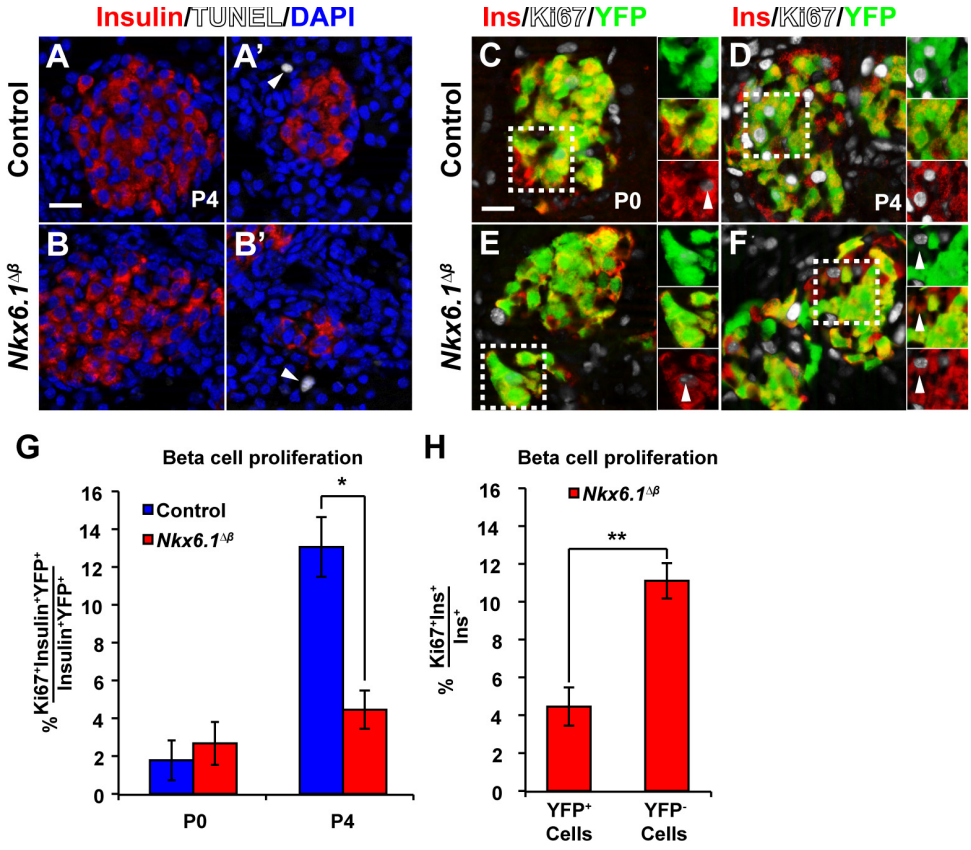


Figure 4.3. Nkx6.1 is required for postnatal beta cell proliferation. (A-B') TUNEL combined with immunofluorescent staining for insulin and DAPI reveals that beta cells are not apoptotic in either control or *Nkx6.1*$^{\Delta\beta}$ mice (Arrowheads, example of a TUNEL^{+} cell in pancreatic tissue of control and *Nkx6.1*$^{\Delta\beta}$ mice). (C-F) Immunofluorescent staining for insulin, Ki67, and YFP reveals a significant decrease in the percentage of YFP^{+}insulin^{+} cells that express Ki67 in *Nkx6.1*$^{\Delta\beta}$ mice at P4, but not in YFP^{-}insulin^{+} cells of *Nkx6.1*$^{\Delta\beta}$ mice at P4 (F, insets, arrowhead). (G) Quantification of the percent of insulin^{+}YFP^{+} cells that express Ki67 reveals a decrease in beta cell proliferation of *Nkx6.1*$^{\Delta\beta}$ mice at P4, but not at P0 (n=3). (H) Quantification of the percent of insulin^{+} cells that were Ki67^{+} in *Nkx6.1*$^{\Delta\beta}$ mice reveals a selective decrease in proliferation of YFP^{+} cells, but not YFP^{-}insulin^{+} cells (n=3). Insets are color channel separations of areas denoted by dashed boxes. Scale bar = 20 μm. Ins, insulin; YFP, yellow fluorescent protein. Data shown as mean ± SEM. *,p<0.05; **,p<0.01.

undergo programmed cell death after inactivation of *Nkx6.1*, indicating that Nkx6.1-deficient beta cells decrease by another mechanism such as reduced proliferation.

To examine whether Nkx6.1-deficient beta cells in *Nkx6.1*$^{\Delta\beta}$ mice had proliferative defects, we performed immunofluorescence staining for Ki67, insulin, and YFP in control and *Nkx6.1*$^{\Delta\beta}$ pancreata. Staining revealed no significant difference in the percent of Ki67^{+}YFP^{+} beta cells between control (1.78+/-1.05%) and *Nkx6.1*$^{\Delta\beta}$ mice (2.67+/-1.14%) at P0 (Figure 4.3C, E, arrows, and G). However, when we initially observe a decrease in Nkx6.1-deficient beta cells at P4, we observed a significant decrease in the percent of Ki67^{+}YFP^{+} beta cells between control (13.00+/-1.58%) and *Nkx6.1*$^{\Delta\beta}$ mice (4.48+/-1.01%) (Figure 4.3D,F,G). Therefore, our data suggest that Nkx6.1-deficient beta cells have proliferative defects at P4, resulting in decreased insulin^{+}YFP^{+} cells in *Nkx6.1*$^{\Delta\beta}$ mice. Notably, we observed a higher percentage of Ki67^{+}YFP^{-} cells (11+/-0.93%) compared to Ki67^{+}YFP^{+} cells (4.48+/-1.01%) in *Nkx6.1*$^{\Delta\beta}$ mice at P4 (Figure 4.3F arrow, H). This indicates that Nkx6.1^{+} cells in *Nkx6.1*$^{\Delta\beta}$ mice proliferate, suggesting that Nkx6.1 maintains beta cell proliferation in a cell autonomous manner.

Deletion of Nkx6.1 in newly differentiated beta cells results in loss of Glut2 and Glp1r expression.

We observed that Nkx6.1-deficient beta cells display proliferative defects by P4, but not at birth. This suggested that Nkx6.1 may be necessary to respond to a biological change that occurs during this period. Since mice are transitioning from placental nutrients to postnatal feeding at birth (Fowden and Hill, 2001), it is possible that Nkx6.1 is necessary for stimulation of beta cell proliferation by nutrition. Previously, we have identified that Nkx6.1 indirectly controls beta cell proliferation in

adult beta cells by maintaining glucose import through regulating *Glut2* expression (Taylor et al., 2013). Therefore, we expected that beta cells in *Nkx6.1*$^{\Delta\beta}$ mice may not be capable of responding to diet-induced mitogenic signals that change within the postnatal period. To examine this possibility, we performed immunohistochemistry for Glut2 in conjunction with insulin and YFP in control and *Nkx6.1*$^{\Delta\beta}$ mice at P4 (Figure 4.4A, C). We observed a selective loss of *Glut2* expression in Nkx6.1-deficient beta cells in *Nkx6.1*$^{\Delta\beta}$ mice (Figure 4.4C, arrowheads). Notably, Glut2 expression was maintained in the nonrecombined beta cells in *Nkx6.1*$^{\Delta\beta}$ mice. This suggests that changes in blood glucose levels in the perinatal period may stimulate beta cell proliferation, as Glut2-deficient beta cells proliferate less in *Nkx6.1*$^{\Delta\beta}$ mice.

Another mitogenic signal induced by feeding is glucagon-like peptide-1 (Glp1), an incretin hormone secreted from the enteroendocrine cells in the gut that is a potent stimulator of beta cell proliferation (Xu et al., 1999). To examine the potential of beta cells to respond to incretins in *Nkx6.1*$^{\Delta\beta}$ mice, we performed immunofluorescence staining for insulin, YFP, and glucagon-like peptide-1 receptor (Glp1r) (Figure 4.4B,D). As with Glut2 expression, YFP^{+} cells in *Nkx6.1*$^{\Delta\beta}$ mice were devoid of Glp1r expression (Figure 4.4B,D, arrows). Together, this data demonstrates that Nkx6.1-deficient beta cells lack key metabolic sensors for signals that are known to have pro-proliferative roles in beta cells, which could account for defects in proliferation in Nkx6.1-deficient beta cells.

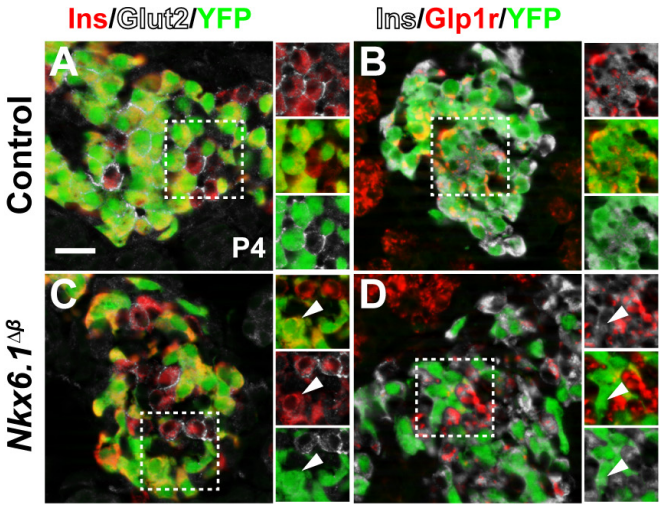


Figure 4.4. Nkx6.1 deficient beta cells have defects in glucose and Glp1 sensing. (A,C) Immunofluorescent staining for insulin, Glut2, and YFP reveals loss of Glut2 expression in $YFP^{+}insulin^{+}$ cells (arrows) of *Nkx6.1*$^{\Delta\beta}$ mice at P4. Insets are color channel separations of areas marked by dashed boxes. (B,D) Immunofluorescent staining for insulin, Glp1r, and YFP reveals loss of expression of Glp1r expression in $YFP^{+}insulin^{+}$ cells (arrows) of *Nkx6.1*$^{\Delta\beta}$ mice at P4. Insets are color channel separations of areas marked by dashed box.

DISCUSSION

The postnatal expansion of pancreatic beta cells is largely maintained by self-renewal of pre-existing beta cells (Brennand et al., 2007; Dor et al., 2004; Teta et al., 2007). Although it is well understood that cyclin expression is critical for this process, the transcriptional regulators that maintain this pro-proliferative program are not clearly defined. Here we demonstrate an essential role for the transcription factor Nkx6.1 in maintaining beta cell proliferation during postnatal beta cell mass expansion. Using mosaic inactivation of *Nkx6.1* in newly differentiated beta cells, we show a selective decrease in Nkx6.1-deficient beta cells. This decrease was due to reduced proliferation and ultimately resulted in reduced beta cell mass and glucose intolerance in adult animals. Finally, we show that Nkx6.1 temporally regulates beta cell proliferation, during the switch from placental nutrition to perinatal feeding. Therefore, our study identifies a transcriptional regulator essential for the expansion of functional beta cells in response to changes in nutrition.

Due to the extremely low rate of adult beta cell proliferation (Teta et al., 2005), we were previously unable to determine whether deletion of *Nkx6.1* in adult beta cells affects beta cell mass (Taylor et al., 2013). In this study, we examined Nkx6.1 function in beta cells that are highly proliferative, which allowed us to examine whether deletion of Nkx6.1 affects beta cell mass. As with deletion of *Nkx6.1* in adult beta cells, deletion of *Nkx6.1* caused a loss of pro-proliferative marker expression (Ki67), but also resulted in a reduction of beta cell mass. Therefore, in this study we demonstrate that deletion of Nkx6.1 in beta cells is sufficient to affect beta cell proliferation and expansion of cell mass. Notably, by using *RIP-Cre* mediated inactivation of *Nkx6.1* in this study, we were able to maintain a small population of beta cells that express Nkx6.1 due to mosaic expression of the *RIP-Cre* transgene.

Compared to our previous study in which we completely ablated Nkx6.1 expression in adult islets (Taylor et al., 2013), mosaic inactivation in this study allowed us to demonstrate that Nkx6.1 regulates beta cell proliferation in a cell autonomous manner.

We have previously observed that *RIP-Cre* mediated deletion of *Nkx6.1* results in an exclusive beta to delta fate switch by six weeks of age (Schaffer et al., 2013). However, in this study we only observe insulin and YFP co-positive cells in *Nkx6.1*$^{\Delta\beta}$ mice at P0 and P4, suggesting that the beta to delta fate switch occurs later in development. Although Nkx6.1-deficient beta cells maintained insulin expression at P4, they did display a loss of key beta cell features including Glut2 and Glp1r expression. This suggests that deletion of Nkx6.1 in newly differentiated cells initially results in the destabilization of beta cell identity with loss of key beta cell features (Glut2, Glp1r) and beta cell functions (proliferation) before adoption of an alternative endocrine lineage identity. Notably, this is highly similar to ablation of *Nkx6.1* in adult beta cells, in which beta cell function and gene expression is initially affected before the adoption of a delta cell identity (Taylor et al., 2013).

Since Nkx6.1 is an indirect regulator of beta cell proliferation (Taylor et al., 2013), our current study suggests that deletion of *Nkx6.1* affects the ability of beta cells to respond to external signals that exist at P4, which are not present immediately after birth. This signal may be nutritional, as our phenotype coincides with the switch from placental derived nutrients to feeding. Consistent with this idea, we recently identified that Nkx6.1 indirectly regulates stimulation of beta cell proliferation in adult mice by maintaining glucose import through Glut2 expression (Taylor et al., 2013). Furthermore, glucose metabolism in beta cells has been demonstrated to have a critical role in stimulation of beta cell proliferation in adult

animals (Porat et al., 2011; Salpeter et al., 2010; Salpeter et al., 2011). In the perinatal period, Nkx6.1 is only required for proliferation of beta cells after feeding for days. Therefore, it is possible that changes in blood glucose levels in the perinatal period are essential for initiating beta cell mass expansion. We also observed that Nkx6.1-deficient beta cells have decreased Glp1r expression. Therefore, in addition to defective proliferation due to decreased glucose import, Nkx6.1-deficient beta cells may also lose responsiveness to incretin-mediated stimulation of beta cell proliferation. Although, *Glp1r* deficient mice do not have significant decreases in postnatal beta cell mass expansion (Ling et al., 2001), Glp1 is a potent stimulator of beta cell proliferation (Xu et al., 1999). Therefore, our findings suggest that the switch from placental nutrients to feeding may be a key factor in initiation of pancreatic beta cell mass expansion and that Nkx6.1 is necessary to maintain the responsiveness of beta cells to this change.

One such occurrence of a phenotype in the perinatal period induced by feeding was previously observed in *Pax4* and *Arx* double null-mutant mice (Collombat et al., 2005). Collombat et al observed an increase in somatostatin and pancreatic polypeptide co-expressing cells 12 hours after birth in *Pax4*$^{-/-}$*Arx*$^{-/-}$ mice. However, this phenotype was only observed in mice that were fed, as *Pax4*$^{-/-}$*Arx*$^{-/-}$ mice that were immediately separated before feeding did not have somatostatin and pancreatic polypeptide co-expression. This suggested that nutrients derived from perinatal feeding were responsible for their observed phenotype in *Pax4*$^{-/-}$*Arx*$^{-/-}$ islets. Similarly, we only observe proliferative defects in Nkx6.1-deficient beta cells after mice have been feeding. Together, this suggests that the signals derived from postnatal feeding are critical for islet development.

Our data demonstrates that Nkx6.1 is essential for maintenance of postnatal beta cells in a cell autonomous manner. Selection against Nkx6.1-deficient beta cells may serve as a mechanism to maintain healthy beta cells during postnatal maturation of the pancreas. Cell competition based on health has been a revisited concept because of the generation and availability of genetic tools for lineage analysis. A recent study using lineage reporters specific to Wnt levels, demonstrated that cells with decreased Wnt levels in the developing embryo were out-competed by cells that expressed Wnt normally (Claveria et al., 2013). This was previously more difficult to accomplish without the inclusion of complex genetics and lineage reporters. However, these novel genetic lineage tools can now directly demonstrate that “healthy” cells outcompete “unhealthy” cells. By using lineage reporters to examine the selective pressure for Nkx6.1-expressing beta cells in our study, we identify a potential way in which the islet has evolved to minimize the amount of defective cells that develop. Therefore, Nkx6.1 may serve as a molecular sensor of beta cell health, promoting cell expansion by maintaining a nutrient responsive state.

MATERIALS AND METHODS

RIP-cre (Postic et al., 1999), *Nkx6.1*^{flox} (Schaffer et al., 2013), and *R26-YFP* mice (Srinivas et al., 2001) have been previously described. Tissue preparation, immunofluorescence and TUNEL staining have been previously described (Schaffer et al., 2013). For detection of nuclear antigens, antigen retrieval was performed in pH 6.0 citrate buffer and sections were permeabilized in 0.15% Triton-X100 in PBS. The following primary antibodies were used: guinea pig anti-insulin (Dako), 1:2000; mouse anti-Nkx6.1, (BCBC #2023), 1:500; rabbit anti-Glut2 (Millipore), 1:1000; rabbit anti-Glp1r (S. Heller, Novo Nordisk), 1:2000, TSA amplification; rat anti-GFP (C. Kioussi,

Oregon State University), 1:1000; rabbit anti-Ki67 (Lab Vision), 1:500. Staining with antibodies raised in mice was performed using the M.O.M. Kit (Vector Labs). When necessary, nuclei were counterstained with DAPI (Sigma) at 0.1 µg/ml. Primary antibodies were labeled with donkey-raised secondary antibodies conjugated to Cy3, Cy5, or Alexa 488 (Jackson ImmunoResearch). Beta cell mass and area were determined as previously described (Schaffer et al., 2013). Images were captured on a Zeiss Axio Observer Z1 microscope with an Apotome module and processed with Zeiss Axiovision 4.8 software. All images were processed in accordance with the *Diabetes* journal's guidelines and only brightness and contrast was adjusted.

Glucose tolerance tests and blood glucose measurements were performed as previously described (Taylor et al., 2013). For glucose tolerance tests, a 1.5 g/kg intraperitoneal injection of glucose was administered after fasting overnight. Blood glucose levels were measured on a Bayer Contour glucometer (Bayer).

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California.

All values are shown as mean ± standard error of the mean (SEM); p-values were calculated using a two-tailed student's t-test in Microsoft Excel. $P<0.05$ was considered significant.

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CHAPTER 5:

Conclusions, open questions, and future directions

The major goals of this dissertation were: (1) To identify whether the beta cell enriched transcription factor Nkx6.1 stabilizes beta cell identity in developing, maturing, and fully mature beta cells; and (2) To determine whether Nkx6.1 regulates pancreatic beta cell function in the adult pancreas. The resulting work in this thesis identified that Nkx6.1 is a master regulator of beta cell identity and function during all stages of beta cell development and maturation. At all developmental stages, Nkx6.1 promotes the beta cell fate at the expense of non-beta endocrine cell lineages. In the mature beta cell, Nkx6.1 directly binds to and regulates genes critical for beta cell functional properties, including insulin biosynthesis and glucose import and metabolism. Additionally, through maintenance of glucose import, Nkx6.1 also controls proliferation of both mature and immature beta cells. Since each chapter of this thesis concludes with a discussion of these findings in relation to the literature, this section will focus on the open questions and future directions that result from this work in relation to the disease diabetes.

Could Nkx6.1 be a target for therapeutic intervention in diabetes?

Recent therapeutic approaches for diabetes aim to restore functional beta cell mass. Therefore, it is critical to identify the factors that produce and maintain beta cells in order to generate replacement cells *in vivo* or *in vitro* for therapies for diabetes. Our studies demonstrate the importance of transcription factors, in particular Nkx6.1, in both the regulation of beta cell development and the maintenance of cell function and identity in adults (Schaffer et al., 2013; Taylor et al., 2013). Therefore, our studies in mice indicate that inducing and maintaining Nkx6.1 expression will be critical for generating functional beta cells for diabetes therapies.

The loss of transcription factor expression, including Nkx6.1, in human beta cells is associated with beta cell dysfunction and the onset of diabetes (Guo et al., 2013). Notably, re-expression of transcription factors has also been implicated in restoration of beta cell function in type 2 diabetics. This is exemplified by diabetic patients that have undergone roux-en-y gastric bypass surgery, in which restoration of beta cell function is observed in conjunction with re-expression of beta cell enriched transcription factors (Rabiee et al., 2011; Salehi et al., 2011). Together with our data, it is reasonable to believe that targeted re-expression of beta cell transcription factors including Nkx6.1 could restore beta cell function in diabetic patients. Therefore, identification of a small molecule inducer of *Nkx6.1* expression could be highly beneficial for treatment of diabetes and restoration of functional beta cell mass.

Relevance of Nkx6.1 to type 1 diabetes and beta cell replacement based therapies

In type 1 diabetics, greater than 90% of beta cells are destroyed by the immune system at the time of diagnosis (Gepts and De Mey, 1978). Therefore, it would be more feasible to replace beta cells entirely rather than to expand the remaining beta cells in these patients. To achieve this, many efforts have sought to derive beta cells through the differentiation of ES cells (D'Amour et al., 2006; Kroon et al., 2008). Although ES cells can be efficiently differentiated into pancreatic progenitor cells *in vitro*, they require additional *in vivo* maturation to produce functional beta cells (D'Amour et al., 2006; Kroon et al., 2008). A concern of this approach is that improperly generated progenitor cells may have the potential to become tumorigenic once implanted. Therefore, *in vitro* differentiation of progenitor cells to beta cells

would be more ideal. However, this approach is limited, as current ES cell differentiation protocols cannot produce functional beta cells *in vitro.* The insulin$^+$ cells derived from *in vitro* differentiation of ES cells lack many characteristics of mature beta cells *in vivo*. They exhibit defects in insulin biosynthesis, glucose stimulated insulin secretion, and co-express other non-beta endocrine hormones. This suggests that the genes required for establishing beta cell identity and function are not induced with use of this differentiation protocol. Importantly, Nkx6.1 is one of the factors not expressed insulin$^+$ cells derived from this protocol, suggesting that lack of its induction accounts for this phenotype (D'Amour et al., 2006; Kroon et al., 2008; Xie et al., 2013). Since we demonstrate that Nkx6.1 is necessary to promote beta cell formation at the expense of non-beta endocrine lineages (Schaffer et al., 2013; Schaffer et al., 2011) and Nkx6.1 is required to maintain beta cell function and identity in adults (Taylor et al., 2013), induction of Nkx6.1 with a small molecule from the pancreatic progenitor stage and onward may correct *in vitro* differentiation of beta cells. Particularly, the function of Nkx6.1 during pancreatic development to repress alternative endocrine lineages may be used to suppress aberrant alpha gene expression. Supporting the idea that Nkx6.1 is critical for derivation of beta cells from ES cells, *in vitro* generated pancreatic progenitors are more efficient at producing functional beta cells *in vivo* when they express high levels of Nkx6.1 (Rezania et al., 2013). Therefore, the developmental and maintenance functions of Nkx6.1 may be exploited for the generation of functional beta cells *in vitro*.

An additional approach that investigators are seeking to treat type 1 diabetes is to convert non-beta pancreatic endocrine cells into functional beta cells *in vivo*. Under conditions of severe beta cell ablation in mice (>99%), alpha cells spontaneously transdifferentiate into beta cells (Thorel et al., 2010). This

reprogramming event is associated with the gain of beta cell enriched transcription factor expression in alpha cells before adopting a beta cell identity. Notably, misexpression of transcription factors in alpha cells is sufficient for this cell fate conversion as forced expression of Pdx1 in alpha cells results in conversion of alpha to beta cells (Yang et al., 2011). Therefore, some adult endocrine cells retain plasticity and could be potentially used as a source of beta cells in type 1 diabetics. However, what remains to be determined is whether the ability of cells to convert into beta cells is limited to alpha cells or whether other non-beta endocrine subtypes can give rise to beta cells in the adult pancreas.

We determined that deletion of *Nkx6.1* in either immature (Schaffer et al., 2013) or mature beta cells (Taylor et al., 2013) results in an exclusive beta-to-delta cell fate switch. Since *Nkx6.1*-deficient beta cells readily adopt a delta cell fate, it suggests that there is plasticity between beta and delta cells in the adult pancreas. However, it is still unknown whether delta cells can give rise to beta cells in the adult pancreas following challenge or genetic manipulation. To first address this question, it would be necessary to examine whether a delta-to-beta cell fate conversion occurs after severe beta cell ablation. Although it is known that alpha cells give rise to beta cells after severe ablation, lineage reporters have not been used to examine delta cells (Thorel et al., 2010). Therefore, inclusion of delta cell lineage analysis in this mouse model may reveal that delta cells are capable of converting to beta cells. Additionally, it would be critical to examine whether delta cells also induce beta cell enriched transcription factor expression, similar to alpha cells converting to beta cells. Together, this would uncover whether delta cells have the potential to convert into beta cells.

In addition to conversion of delta cells after severe beta cell ablation, it may also be possible to cause a delta-to-beta cell conversion by misexpressing transcription factors. Notably, misexpression of Nkx6.1 alone in alpha cells does not result in an alpha-to-beta cell fate switch even in the absence of injury (Schaffer et al., 2013). However, since delta cells express the critical beta cell transcription factor Pdx1 (Schaffer et al., 2013), delta cells may more responsive to Nkx6.1 expression. Due to the function of Nkx6.1 in both immature and mature beta cells to prevent delta cell gene expression, conditional misexpression of *Nkx6.1* in adult delta cells will allow us to determine whether delta cells are capable of giving rise to beta cells. Overall, these experiments will elucidate the possibility of utilizing delta cells as alternative sources of beta cells during extreme beta cell loss, as seen in T1DM.

Relevance of Nkx6.1 to type 2 diabetes and rodent models of beta cell dysfunction

Recent evidence suggests that loss of beta cell enriched transcription factor expression, including Nkx6.1, precedes beta cell dysfunction and the onset of type 2 diabetes (T2DM) (Guo et al., 2013; Talchai et al., 2012). This concept stems from the observation that transcription factor expression is reduced under oxidative stress conditions, a key causative factor for beta cell failure and type 2 diabetes (Guo et al., 2013; Harmon et al., 2009; Poitout and Robertson, 2008; Robertson and Harmon, 2007). Furthermore, obese mouse models that develop diabetes [leptin receptor deficient *db/db* mice (Hummel et al., 1966; Ingalls et al., 1950)] also have decreased expression of beta cell enriched transcription factors (Guo et al., 2013; Harmon et al., 2009). Lastly, expression of antioxidant enzymes in *db/db* mice is sufficient to restore transcription factor expression and beta cell function (Harmon et al., 2009). Although,

changes in transcription factor expression are tightly associated with beta cell dysfunction and diabetes, it remains unclear whether restoration of transcription factors is sufficient to restore beta cell function and reverse the symptoms of diabetes.

Our studies demonstrate that Nkx6.1 is a direct regulator of genes critical for beta cell function, including insulin biosynthesis, insulin secretion, and glucose sensing (Taylor et al., 2013). Since Nkx6.1 regulates multiple beta cell functions, it is likely that loss of Nkx6.1 in type 2 diabetes causes beta cell dysfunction. To examine this possibility in mice, ongoing studies are aimed at conditionally expressing *Nkx6.1* in beta cells of *db/db* mice. Briefly, Nkx6.1 can be temporally induced before loss of its endogenous expression or after diabetes develops in *db/db* mice. These studies would determine: (1) the extent by which Nkx6.1 loss contributes to the diabetic phenotype in *db/db* mice and (2) whether established diabetes can be reversed through therapeutic rescue of Nkx6.1 expression. One caveat to this approach is that conditions of oxidative stress due to chronic hyperglycemia in type 2 diabetics results in nuclear to cytoplasmic translocation of Nkx6.1 in beta cells. Since exogenous antioxidant enzyme expression in beta cells of *db/db* mice is sufficient to restore beta cell function and Nkx6.1 gene expression (Guo et al., 2013; Robertson, 2004), it may also be beneficial to treat mice with antioxidants in conjunction with Nkx6.1 activation. Overall, such studies will reveal the role of Nkx6.1 in the progression of diabetes in the *db/db* mouse model.

Although examining Nkx6.1 function in *db/db* mice would be a useful model to measure the therapeutic potential of Nkx6.1 in treatment of type 2 diabetes, there are additional changes not observed in *db/db* mice that occur in T2DM. In *db/db* mice, of the beta cell enriched transcription factors, Nkx6.1 and MafA are selectively reduced.

However, islets in human T2DM patients are additionally deficient for Pdx1 (Guo et al., 2013), which is a critical upstream regulator of genes required for beta cell function including *Nkx6.1* (Ahlgren et al., 1998). Since Pdx1 expression is not affected in islets of *db/db* mice, it may be informative to examine Nkx6.1 function in mice in which Pdx1 expression is also lost. Notably, deletion of Pdx1 in beta cells results in a decrease in both Nkx6.1 and MafA expression, thereby generating a mouse model of the transcriptional defects observed in T2DM (Ahlgren et al., 1998). Therefore, re-expressing Nkx6.1 after deletion of Pdx1 in islets would elucidate both the Nkx6.1-dependent and –independent defects observed in T2DM. Furthermore, this experiment would also uncover whether re-expression of Nkx6.1 would be sufficient to restore beta cell function in Pdx1 independent manner. Overall, these future studies will demonstrate whether induced re-expression of Nkx6.1 with small molecules could be considered as a therapeutic approach to restore multiple beta cell functions simultaneously in type 2 diabetics.

Conclusions

In summary, our studies in mice demonstrate the importance of Nkx6.1 in maintenance of beta cell identity and function. Due to the critical role of Nkx6.1, our findings implicate that Nkx6.1 could be a potential therapeutic target for cell based replacement strategies or as a drug target in beta cells of diabetics. Since Nkx6.1 is not expressed in ES cell derived insulin$^+$ cells and beta cells of type 2 diabetics, one therapeutic approach to correct beta cells in these models would be to induce Nkx6.1 expression with a small molecule. For ES cell based beta cell replacement strategies, inducing Nkx6.1 expression by a small molecule would be highly feasible because a whole population of cells could be targeted *in vitro*. Furthermore, screening for

induction of Nkx6.1 would be easily achievable. However, *in vivo* restoration of beta cell function using this approach may be more difficult, as a small molecule may have effects on non-beta cells. A second issue for small molecule induction of Nkx6.1 expression would be whether this compound would have to be administered chronically to maintain Nkx6.1 expression. Since hyperglycemia is strongly linked to beta cell dysfunction and Nkx6.1 expression (Guo et al., 2013), it may be possible that once a non-diabetic state is achieved that Nkx6.1 expression would be self-sustaining. Nonetheless, Nkx6.1 expression is essential for beta cells as it must be maintained or induced to promote beta cell function and identity. If not a suitable drug target, at the very least, our findings strongly support that Nkx6.1 expression in beta cells serves as an accurate bio-marker of beta cell health and function.

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