Involvement of DLX Genes in the Regulation of Insulin Gene(s) Transcription

by

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Abstract

Even though extensive research over recent decades has increased our understanding of the key steps in pancreatic development and gene regulatory networks involved in pancreatic cell specification and maturation, it is well accepted that a more detailed understanding of pancreatic development is needed for successful early diagnosis and treatment of pancreatic diseases such as diabetes mellitus and pancreatic cancer. The endocrine portion of the pancreas, known as the islets of Langerhans, consist of 5 main cell types: α cells, β cells, δ cells, PP cells and Epsilon cells. The α and β cells each secrete a regulatory hormone into the bloodstream. Insulin and glucagon are the main pancreatic hormones secreted by β- and α- cells, respectively. Several homeodomain-containing transcription factors, such as PDX1, NKX2.2 and PAX4, have been identified that not only regulate the expression of insulin in β-cells, but also participate in pancreatic development and pancreatic islet cell differentiation. Some of these transcription factors have been associated with the pathogenesis of diabetes. The Dlx1 and Dlx2 homeodomain-containing transcription factors are highly expressed in the developing pancreas as early as E14.5 and are co-expressed with pancreatic hormones and transcription factors such as PDX1. Previously, the Eisenstat lab showed that neonatal pancreas from Dlx1/2 double knockout mice shows reduced expression of glucagon and insulin compared with wild-type littermates at P0. Based on these observations, I hypothesized that DLX2 plays a role in pancreatic islet cell development by direct transcriptional regulation of insulin and glucagon gene expression and that loss of function of Dlx1/Dlx2 results in islet-cell-specific defects. ChIP-qPCR of mouse E18 pancreas showed DLX2 enrichment at putative DLX2 binding sites on INS1, INS2 and proglucagon 5'-regulatory regions. The direct binding of DLX2 to the INS2 regulatory region was confirmed by electrophoretic mobility shift assays *in vitro*. Co-transfection of Dlx2 expression plasmids with PGL3-INS2 promoter regions

resulted in a significant increase in luciferase reporter gene activity. qRT-PCR showed a reduction in mRNA levels of INS1 and INS2 in the Dlx1/2 DKO pancreas tissue compared to wild-type littermates at E18.5, supporting the activating effects of DLX2 on INS1 and INS2 transcription. However, INS1 and INS2 mRNA levels were significantly elevated in Dlx2-siRNA treated Beta-TC 6 cells, which was in contrast with our luciferase reporter assay results, where DLX2 activated the expression of INS1and INS2 genes in HEK923 cells, and also our *in vivo* results where a decrease in mRNA levels of INS1 and INS2 in the Dlx1/2 DKO pancreas tissue was observed. Overall, this project provides more information about pancreatic islet cell development and gene regulation, and contributes to our understanding of pancreatic islet cell function in health and disease, including diabetes.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

Arx	Aristaless related homeobox
bHLH	Basic helix-loop-helix
Brn4	Octamer-Binding Transcription Factor 9
BSA	Bovine serum albumin
CDX2	Caudal Type Homeobox 2
ChIP	Chromatin Immunoprecipitation
CRE	cAMP-Response Element
CPE	Carboxypeptidase E
DLX	Distal-less Homeobox
DKO	Double Knockout
EMSA	Electrophoretic Mobility Shift Assay
E	Embryonic day
FGF10	Fibroblast Growth Factor 10
Foxa2	Forkhead Box Protein A2
Gcg	Proglucagon gene
GWAS	Genome-Wide Association Studies
GRPP	Glicentin Related Pancreatic Polypeptide
GLP-1	Glucagon Like Peptide 1
Hnf1b	Hepatocyte nuclear factor-1-beta
HES1	Hairy and Enhancer Of Split 1
hESCs	Human Embryonic Stem Cells
IgG	Immunoglobulin G
IAPP	Islet Amyloid Polypeptide
INS1	Preproinsulin1
INS2	Preproinsulin2
iPSC	Induced Pluripotent Stem Cells
Isl1	Insulin gene enhancer protein ISL-1
IR	Insulin Receptor
IRS	Insulin Receptor Substrates
IP-1	Intervening Peptide-1
MafB	V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog B
Mist1	Muscle, Intestine and Stomach Expression 1
MPF	Major Proglucagon Fragment
MODY	Maturity Onset Diabetes of the Young
mRNA	Messenger RNA
MPCs	Multipotent Pancreatic Progenitor Cells
MSC	Mesenchymal Stem Cells
NDM	Neonatal Diabetes Mellitus
Neurog3	Neurogenin 3

NeuroD1	Neuronal Differentiation 1
Nkx6.1	Homeobox Protein NK-6 Homolog A
Nr5a2	Nuclear Receptor Homolog-1 Variant 2
Pax4	Paired Box 4
Pax6	Paired Box 6
Ptf1a	Pancreas associated Transcription Factor 1a
Pdx1	Pancreatic and Duodenal Homeobox 1
PP	Pancreatic Polypeptide cells
PFA	Paraformaldehyde
PPIA	Peptidylprolyl Isomerase A
PNDM	Permanent neonatal diabetes mellitus
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PIP2	Phosphatidylinositol 4,5-bisphosphate
PDK1	Phosphoinositide-Dependent protein Kinase 1
PIC	Protease Inhibitor Cocktail
PKB	Protein Kinase B
PKC	Protein Kinase C
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
Rfx6	Regulatory Factor X6
Sox9	Sex-determining region Y-Box 9
siRNA	Small Interfering RNA
TCA	Tricarboxylic Acid Cycle
tRNA	Transfer ribonucleic acid
TNDM	Transient Neonatal Diabetes Mellitus
TSS	Transcription Start Site
UTR	Untranslated Region
WT	Wild type

Chapter 1: Introduction

1.1 The Pancreas

The pancreas, an endoderm-derived glandular organ, consists of 2 major components: the exocrine and endocrine cells. The exocrine compartment is composed of acinar cells that produce digestive enzymes, and ductal cells that neutralize these enzymes by secreting bicarbonate and deliver them to the duodenum (Reichert & Rustgi, 2011). The endocrine portion is organized into small islands of cells, called the islets of Langerhans. These islets consist of 5 cell types: α (20–30%) producing hormone glucagon, β (~60%) producing insulin, δ (~10%) producing somatostatin, Pancreatic Polypeptide cells (PP) (<5%) producing pancreatic polypeptide and Epsilon cells (~1%) producing ghrelin. Dysfunction of pancreatic islet cells can lead to diseases such as diabetes mellitus (Dassaye, Naidoo, & Cerf, 2016; Slack, 1995). The main hormones secreted by pancreatic islets are insulin and glucagon; the main function of these two hormones is to maintain glucose homeostasis (Ojha, Ojha, Mohammed, Chandrashekar, & Ojha, 2019). Somatostatin has been shown to inhibit the secretion of almost every exocrine and endocrine factor in different organs, including glucagon and insulin in the pancreas (Huang, 1997). Pancreatic polypeptide was shown to reduce pancreatic exocrine secretion (Williams, 2014). Ghrelin is known to be involved in the regulation of pancreatic cell development and β-cell proliferation. This hormone also has an inhibitory effect on pancreatic exocrine and endocrine secretion (T. Napolitano et al., 2018).

1.2 Development of pancreas in mice

Pancreas development starts with the specification of dorsal and ventral pancreatic buds from the dorsal and ventral foregut. These two independent pancreatic buds have a stratified epithelial structure, consisting of Multipotent Pancreatic Progenitor Cells (MPCs) marked by Pancreatic and Duodenal Homeobox 1 (Pdx1) and Pancreas associated Transcription Factor 1a (Ptf1a) (Burlison, Long, Fujitani, Wright, & Magnuson, 2008; Villasenor, Chong, Henkemeyer, & Cleaver, 2010).

The size of the progenitor cell pool defines the final size of the adult pancreas (Stanger, Tanaka, & Melton, 2007). The proliferation and maintenance of MPCs is regulated by several factors, such as the Notch/Delta signaling pathway, the Fibroblast Growth Factor 10 (FGF10) signaling pathway and Pdx1 (Ahlgren, Jonsson, & Edlund, 1996; Bhushan et al., 2001; Hald et al., 2003).

Pancreatic development can be separated into three main stages: the primary transition, from embryonic day (E) 9.0 to E12.5, the secondary transition from E12.5 to E16.5, and the tertiary transition from E16.5 to the early postnatal period (Dassaye et al., 2016). The primary transition stage of pancreatic development is characterized by proliferation of pancreatic progenitor cells, microlumen formation and establishment of the tip-trunk domain. The secondary transition is characterized by extensive pancreatic epithelial expansion and differentiation of endocrine, acinar, and ductal cells (C. a. F. Pin, M, 2017a). During this step, gut rotation occurs which brings the dorsal and ventral buds together for their future conjoining into a single organ (Gittes, 2009). During the tertiary transition, differentiated pancreatic cells undergo additional remodeling and maturation, including apoptosis, replication and neogenesis, thereby establishing a mature organ (Dassaye et al., 2016) (Figure 1.1).

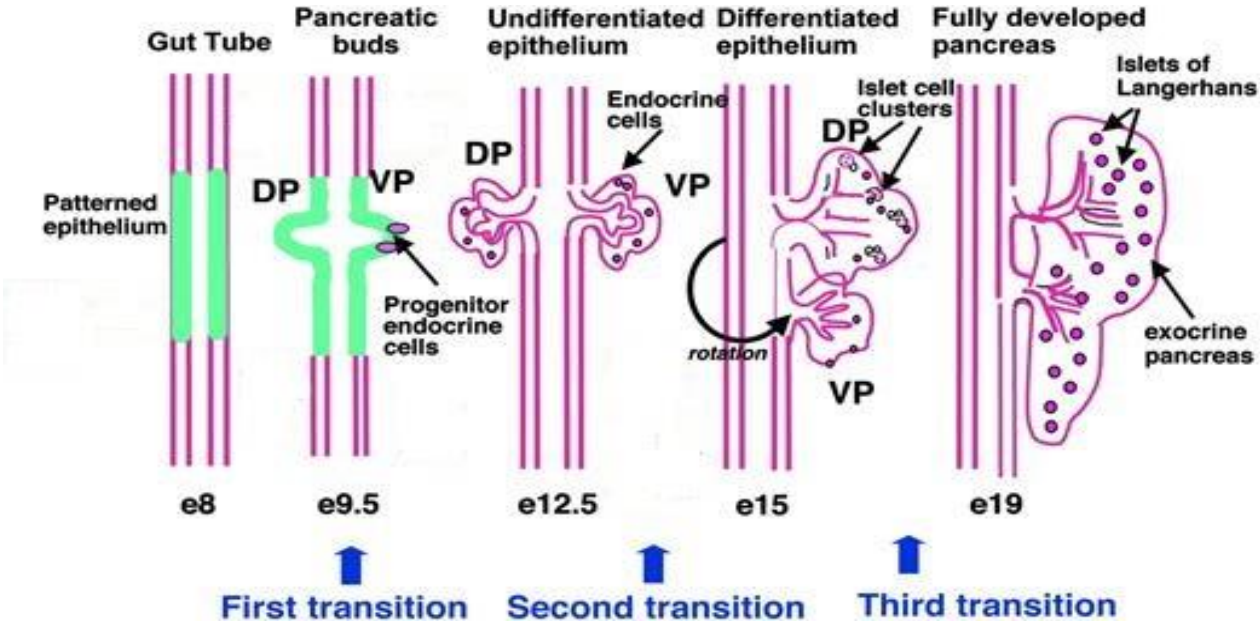


Figure 1.1: Summary of stages of pancreas formation in mice

Pancreas development can be divided into three main developmental stages: the primary transition (E9.0 to E12.5), the secondary transition (E12.5 to E16.5), and the tertiary transition (E16.5 to postnatal). DP; dorsal pancreas, VP; ventral pancreas. Modified from (Habener, Kemp, & Thomas, 2005). Permission for use is provided by an Oxford University Press License (License No. 4726101153506, issued Jan. 24, 2020).

1.3 Lineage Decisions during Pancreas Development

Starting at E11.5, the mouse pancreatic epithelium containing multipotent progenitors segregates into tip or trunk domains. Progenitor cells located at the trunk domain generate endocrine and ductal cells and are characterized by NK6 Homeobox 1 (Nkx6.1), Sex-determining region Y-Box 9 (Sox9), Hepatocyte nuclear factor-1-beta (Hnf1b) and Pdx1 expression. Progenitor cells in the tip domain give rise to acinar cells and are marked by Ptf1a and Nuclear Receptor Homolog-1 Variant 2 (Nr5a2) expression (Arda, Benitez, & Kim, 2013; Bastidas-Ponce, Scheibner, & Lickert, 2017; Solar et al., 2009; Q. Zhou et al., 2007).

1.3.1 Exocrine lineage

Development of the exocrine compartment starts at E11.5-12.5 (Marty-Santos & Cleaver, 2015). Several transcription factors, signaling pathways and epigenetic markers are involved in the development and differentiation of exocrine cells. For instance, follistatin and FGF10 production and secretion by surrounding mesenchyme induce exocrine differentiation (Benitez, Goodyer, & Kim; Miralles, Czernichow, & Scharfmann, 1998). Moreover, knocking out Wnt signaling components resulted in extensive loss of exocrine tissue in mice (James M. Wells et al., 2007). Loss-of-function mutations in Notch pathway genes induces ductal cell specification and represses acinar cell differentiation (Hald et al., 2003). Muscle, Intestine And Stomach Expression 1 (Mist1) and Ptf1a have a crucial role in acinar cell specification and maturation; mice lacking Ptf1a showed a complete absence of exocrine pancreatic tissue (Krapp et al., 1998; C. L. Pin, Rukstalis, Johnson, & Konieczny, 2001). Epigenetic markers such as histone deacetylases influence exocrine cell fate. Treatment of rat pancreas with histone deacetylase inhibitors suppress and induce acinar and ductal differentiation, respectively (Haumaitre, Lenoir, & Scharfmann, 2008).

1.3.2 Endocrine subtype formation, Differentiation, and maturation

Sox9+ bipotent progenitor cells become specified to the endocrine lineage upon expression of the transcription factor, Neurogenin 3 (Neurog3). The expression of many endocrine specific genes such as the Insulin gene enhancer protein ISL-1 (Isl1), Paired Box 4 (Pax4), Paired Box 6 (Pax6), and Neuronal Differentiation 1 (NeuroD1) is regulated by Neurog3 (Gradwohl, Dierich, LeMeur, & Guillemot, 2000). The number of Neurog3+ cells and threshold of Neurog3 expression are important factors in endocrine lineage segregation; pancreatic progenitor cells that produce low levels of Neurog3 default to an acinar or ductal fate (S. Wang et al., 2010). The Neurog3+ cells give rise to five endocrine cell types: α-cells, β-cells, δ-cells, PP-cells and Epsilon cells (Gu, Dubauskaite, & Melton, 2002). It has been suggested that timing of Neurog3 expression plays a role in the specification of Neurog3+ cells toward different endocrine cell types; activation of the Neurog3 gene at E7.5 or E11.5 induces the formation of Glucagon+ cells (α-cells) or Insulin+ cell (β-cells) and PP-cells, respectively (Johansson et al., 2007).

Differentiation of Neurog3+ progenitor cells towards specific endocrine subtypes involves the expression of various endocrine specific transcription factors as well as signaling pathways derived from surrounding tissues. Aristaless related homeobox (Arx) is a transcription factor required for α-cell differentiation and maintenance (Collombat et al., 2007); Arx mutant mice showed a significant decrease in the number of α-cells (Hancock, Du, Liu, Miller, & May, 2010). Arx loss-of-function mice do not develop α-cells, show acute hypoglycemia and die two days after birth (Collombat et al., 2003). Other transcription factors such as Pax6, Octamer-Binding Transcription Factor 9 (Brn4), Forkhead Box Protein A2 (Foxa2), V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog B (MafB) and Regulatory Factor X6 (Rfx6) are also involved in α-cell specification (Bramswig & Kaestner, 2011; Teresa L. Mastracci & Sussel, 2012). Foxa2

mutant mice show a significant reduction in the number of mature α-cells and glucagon expression and die shortly after birth (Lee, Sund, Behr, Herrera, & Kaestner, 2005). Pax6 mutant mice display a significant reduction in the numbers of hormone-producing cells and hormone production, predominantly glucagon (Sander et al., 1997). The glucagon-producing cells are absent in the pancreas of Pax6 null mice (Scott Heller et al., 2004; St-Onge, Sosa-Pineda, Chowdhury, Mansouri, & Gruss, 1997). Brn4, a pancreatic α-cell-specific transcription factor, is expressed in glucagon-expressing cells as early as E9.5 (Jorgensen et al., 2007). However, Brn4 null mutant mice show normal levels of glucagon production and normal pancreas morphology (Scott Heller et al., 2004). MafB protein is expressed in both α-cells and β-cells during pancreas development. However, it is exclusively expressed in α-cells in the adult pancreas (I. Artner et al., 2006). MafB (-/-) mouse embryos display reduced numbers of insulin and glucagon-positive cells during development (I. Artner et al., 2007). Pax4 is first detected around embryonic day 9.5 and the number of Pax4+ cells increases with the onset of the secondary transition. After E18 it is only expressed in β-cells. This transcription factor is required for development of β-cells and δ-cells (Tiziana Napolitano et al., 2015; Beatriz Sosa-Pineda, Kamal Chowdhury, Miguel Torres, Guillermo Oliver, & Peter Gruss, 1997; J. Wang et al., 2004); Pax4 null mutant mice lack mature β and δ-cells (Beatriz Sosa-Pineda et al., 1997).

Pdx1 and Nkx6.1 transcription factors are crucial for β-cell formation. Nkx6.1 expression becomes restricted to β-cells during development (Binot et al., 2010). Nkx6.1 mutant mice display a significant reduction in β-cell numbers; however, α-cells develop normally (Henseleit et al., 2005; Sander et al., 2000). Pdx1 expression starts in the foregut endoderm at E8.5. During the early stages of pancreas development, Pdx1 is expressed in all pancreatic cell types. However, its expression becomes limited to β-cells and a subset of δ-cells after the completion of the secondary

transition (Gao et al., 2008; Guz et al., 1995). Pdx1 expression is essential for maintaining β cell identity by inducing or repressing insulin and Islet Amyloid Polypeptide (IAPP) expression or glucagon expression, respectively. Knocking out Pdx1 in β cells can cause diabetes in mice (Ahlgren, Jonsson, Jonsson, Simu, & Edlund, 1998). Mutations in Pdx1 can lead to maturity-onset diabetes of the young (MODY) in humans (Hani et al., 1999).

Nkx2.2 is another transcription factor expressed in α-, β- and PP-cells (Churchill et al., 2017; Sussel et al., 1998). Nkx.2.2 expression starts at E9.5 in the dorsal bud of the pancreas in mice and it is limited to endocrine progenitor cells at E14.5 (Bramswig & Kaestner, 2011). Nkx2.2 is essential for the final differentiation of β-cells; null mutations of Nkx2.2 lead to an absence of insulin producing β-cells in mice and reduced numbers of glucagon-producing α cells and PP cells (Sussel et al., 1998). However, there is an increase in the number of ε-cells in mice lacking Nkx2.2 (Prado, Pugh-Bernard, Elghazi, Sosa-Pineda, & Sussel, 2004). Nkx2.2 induces the specification of α-cells and β- cells by repressing or activating the NeuroD gene, respectively (T. L. Mastracci, Anderson, Papizan, & Sussel, 2013).

NeuroD, an important regulator of the insulin gene, is expressed in pancreas as early as E9.5 in mice and is limited to endocrine cells at E17 (Bramswig & Kaestner, 2011; Naya, Stellrecht, & Tsai, 1995). Homozygous NeuroD mutant mice show a significant decrease in the number of insulin producing β-cells and severe diabetes, and die 3 to 5 days after birth (Naya et al., 1997) (Figure 1.2).

The NOTCH signaling pathway is involved in ductal cell, endocrine lineage and trunk cell specification (S. Afelik et al., 2012; C. a. F. Pin, M, 2017b; Shih et al., 2012). NOTCH suppression in mouse pancreatic progenitor cells results in loss of trunk marker gene expression, and cells adopt a tip fate (S. Afelik et al., 2012). Hung *et al.* suggested that the level of NOTCH signaling

activity is an important factor in producing different cell lineages during pancreas development. High levels of NOTCH signaling pathway activity induce the expression of HES1 (Hairy Enhancer of Split), a repressor of Neurog3. Inhibition of Neurog3 expression by Hes1 prevents endocrine lineage specification. However, medium levels of NOTCH signaling activity lead to a lower level or absence of Hes1, with subsequently higher levels of Neurog3 and induction of endocrine differentiation (Shih et al., 2012).

The β-catenin/Wnt and Wnt/PCP signaling pathways have been shown to be involved in endocrine cell development. β-catenin loss-of-function mice have reduced numbers of β-cells. This reduction in β-cell number is because of the early loss of multipotent pancreatic progenitor cells and not a defect in β-cell differentiation (Baumgartner, Cash, Hansen, Ostler, & Murtaugh, 2014). Deletion of β-catenin also has a negative effect on exocrine compartment expansion: β-catenin null mice display a significant reduction in the numbers of acinar cells (Baumgartner et al., 2014; J. M. Wells et al., 2007). The Wnt/PCP signaling pathway is involved in β-cell maturation in humans and mice (Bader et al., 2016), although the underlying mechanism is not clear yet (Bastidas-Ponce et al., 2017).

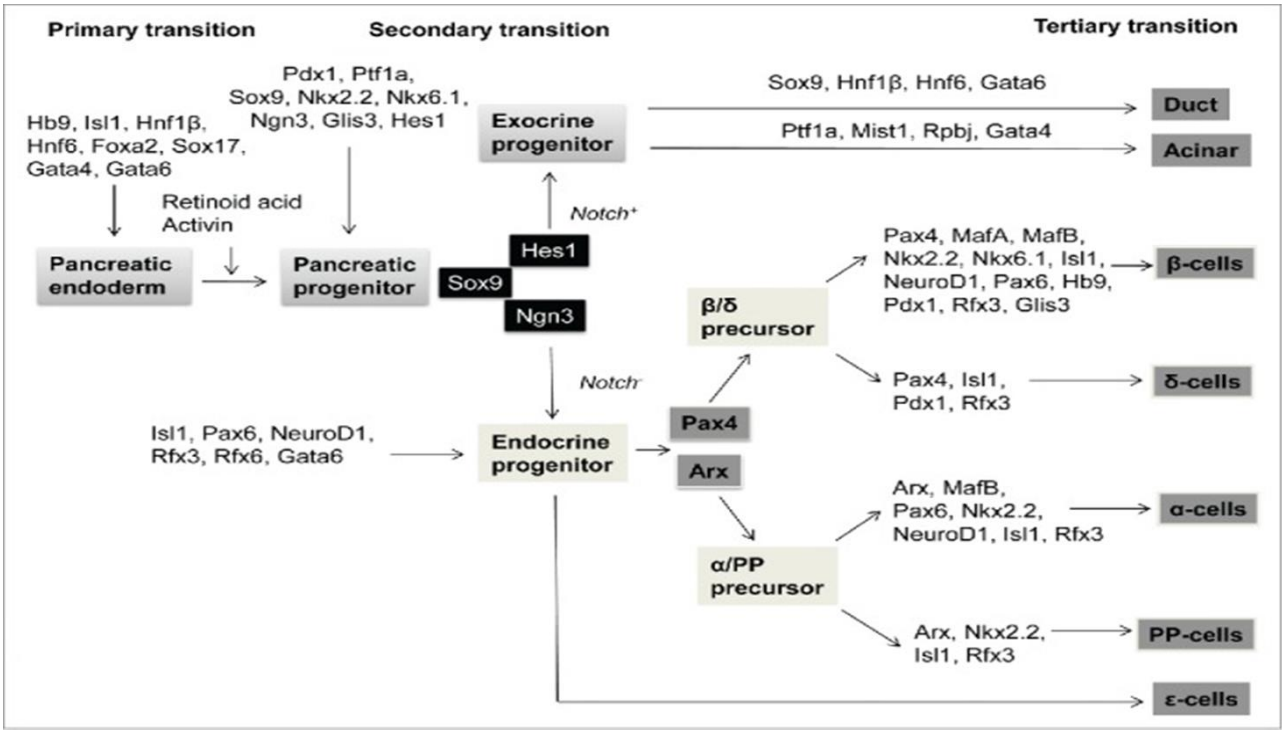


Figure 1.2 Transcription factors involved in the development of mouse pancreas

Endocrine subtype formation is initiated by the expression of the Ngn3 transcription factor in all the endocrine progenitor cells. Ngn3 expressing cells have the potential to become either α- /PP-cells, or β-/ δ-cells based on the expression of ARX and Pax4, respectively. Adapted from (Dassaye et al., 2016). Permission for use of this figure is provided by the publishers of the journal *Islets*, Taylor and Francis (www.tandfonline.com) issued Dec. 13, 2019.

1.4 Insulin

Insulin, a crucial regulator of metabolism, is secreted by pancreatic β-cells in response to elevated blood glucose concentration (Z. Fu, E. R. Gilbert, & D. Liu, 2013). The first step in the biosynthesis of mature insulin is the transcription of the insulin gene into preproinsulin messenger RNA (mRNA). This mRNA is then translated into a single chain precursor called preproinsulin, composed of four polypeptide chains designated as signal peptide, B chain, C-peptide, and A chain. The preproinsulin molecule is led by its signal peptide and is translocated from the cytoplasm into the endoplasmic reticulum. At this step, the signal peptide of preproinsulin is excised, forming proinsulin. Proinsulin is folded, exits the endoplasmic reticulum and is transported to the Golgi complex. Proinsulin then enters into the immature secretory granules, where it undergoes proteolytic cleavage by Prohormone Convertases (PC1/3 and PC2) and Carboxypeptidase E (CPE) and forms mature insulin and C-peptide. Insulin and free C-peptide are packaged in the secretory granules and secreted upon stimulation (M. Liu et al., 2015; Thompson & Kanamarlapudi, 2013).

1.5 Preproinsulin1 and Preproinsulin2

Unlike other mammals with one copy of the insulin gene, including humans, mice and rats have two non-allelic insulin genes, Preproinsulin1 (INS1) and Preproinsulin2 (INS2) (Wentworth et al., 1992). The INS2 gene is an orthologue of the human insulin gene. Ins1 is a retrogene, originated from the partly processed INS2 mRNA. Both genes are expressed in the pancreas, transcribed into preproinsulin and encode proinsulin (Shiao, Liao, Long, & Yu, 2008) (Figure 1.3).

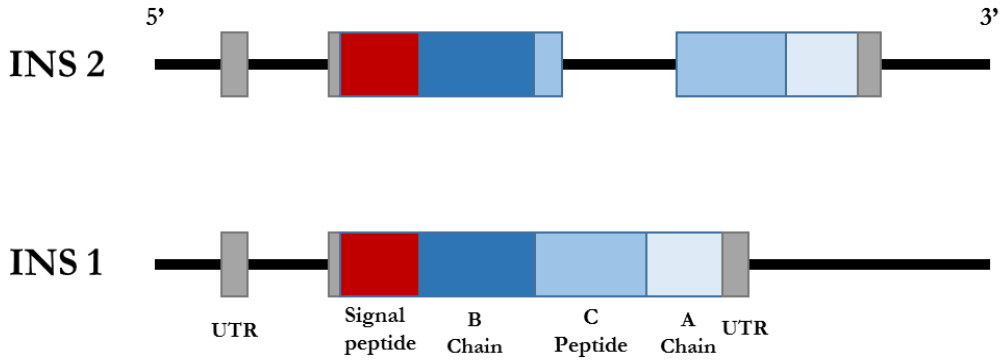


Figure 1.3 Gene structure of the mouse Ins1 and Ins2 genes.

While INS2 has three exons and two introns, INS1 has only one intron and two exons. Both genes contain 3'- and 5' untranslated regions (shown as gray boxes). Boxes indicate exons and black lines indicate introns. Modified from (Shiao et al., 2008). Permission for use is provided by a license agreement from the publisher of *Genetics*, the Genetics Society of America (License ID. 1015040-1, issued Jan. 24, 2020).

In the mouse embryo, the expression of INS2 starts at embryonic day 8.5 in the foregut area. However, INS1 mRNA is not detected until embryonic day 9.5, which shows an independent regulation of these genes during development (Deltour et al., 1993). The peptides produced by INS1 and INS2 differ by two amino acids in the B chain at B9 and B29. Also, there are two amino acids missing in the C peptide of preproinsulin produced by INS1. The expression of INS1 has been shown to be restricted to β-cells; however, human insulin and INS2 are expressed not only in β-cells but also in the brain. However, the specific site of expression and function of insulin produced within the brain remain unclear (Deltour et al., 1993; Li et al., 2016; M de la Monte, 2012; Arya E. Mehran et al., 2012; A. E. Mehran, Templeman, Hu, & Johnson, 2015; Soares et al., 1985).

Ins1 knockout mice and Ins2 knockout mice show different phenotypes related to insulin deficiency. A reduction in the level of insulin was observed in Ins1+/+, Ins2-/- and Ins1+/-, Ins2-/- mice; however, no reduction in insulin levels was observed in Ins1 knockout mice carrying the Ins2 gene (Babaya et al., 2006).

1.6 Regulation of insulin synthesis

Insulin biosynthesis and secretion are precisely regulated transcriptionally, translationally and post-translationally (Jahr, Schroder, Ziegler, Ziegler, & Zuhlke, 1980; Kulkarni et al., 2011). The expression of the insulin gene in β-cells is regulated differently during development than it is postnatally. After birth, the expression and secretion of insulin are regulated by glucose; however, embryonic insulin levels are not glucose dependent (Hernández-Sánchez, Mansilla, de la Rosa, & de Pablo, 2006; Leibiger et al., 1998; Perez-Villamil, de la Rosa, Morales, & de Pablo, 1994).

Several cis-regulatory sequences within the 5′ flanking region of the insulin gene have been discovered. These cis-acting elements are essential in regulating insulin gene transcription levels. Several transcription factors such as PDX-1, MafA, NeuroD1 and PAX-6, which interact with these regulatory motifs, have been identified and isolated. Mouse knockout studies have revealed the importance of these trans-regulatory elements not only in the regulation of the insulin gene transcription, but also in β-cell differentiation and maturation (Isabella Artner & Stein, 2008).

There is almost 65% homology between the human and mouse insulin gene promoters (Hay & Docherty, 2006). Some of the most critical cis-acting regulatory elements involved in transcriptional regulation of the insulin gene(s) are the A, C, and E elements (Poitout et al., 2006). A elements are A/T rich elements located in the promoter region of the insulin gene(s) (M. German et al., 1995). There is at least one homeodomain binding motif within A elements (Z. Fu et al., 2013). Several homeodomain-containing transcription factors such as PDX-1 (Ohlsson, Karlsson, & Edlund, 1993), Cdx2/3 (M. S. German, Wang, Chadwick, & Rutter, 1992) and Isl-1 (Karlsson, Thor, Norberg, Ohlsson, & Edlund, 1990) bind to the A elements and regulate insulin gene transcription. Protein dimers formed by an ubiquitous class A basic helix-loop-helix (bHLH) protein such as E47, and E2/5, or HEB, and NeuroD1/BETA2 which is a basic bHLH transcription factor enriched in pancreatic islets, bind to E elements (5′CANNTG) located within the regulatory regions of the insulin gene(s) and activate its transcription (Naya et al., 1995; Ohneda, Ee, & German, 2000).

There are two C elements located in the promoter of the insulin gene. The pancreatic beta-cell-specific transcriptional activator MafA binds to the C1 element and activates insulin gene transcription (Zhuo Fu, Elizabeth R. Gilbert, & Dongmin Liu, 2013; Kataoka et al., 2002; Matsuoka et al., 2004; Matsuoka et al., 2003; Olbrot, Rud, Moss, & Sharma, 2002). The C2

element is a binding site for PAX6, a crucial transcription factor for normal expression of insulin and pancreatic islet development (Sander et al., 1997). PAX4 can also bind to the C element during early pancreas development, but not in mature β-cells (Zhuo Fu et al., 2013; Sosa-Pineda, 2004; B. Sosa-Pineda, K. Chowdhury, M. Torres, G. Oliver, & P. Gruss, 1997).

The 5' Untranslated Region (UTR) of preproinsulin mRNA has an essential role in the regulation of proinsulin translation and preproinsulin mRNA stability (Tillmar, Carlsson, & Welsh, 2002; B. Wicksteed et al., 2001). Glucose is one of the factors promoting proinsulin translation through controlling the interaction between trans-regulatory factors and cis-regulatory elements within the UTR of preproinsulin mRNA (Barton Wicksteed et al., 2007).

1.7 Glucose stimulated insulin secretion

Elevated blood glucose concentration is the primary stimulus for the secretion of insulin into the blood by pancreatic β-cells (Z. Fu et al., 2013). Glucose transporter 2 (GLUT2) is a membrane protein found on rodent pancreatic β-cells. GLUT2 facilitates the entry of glucose into the β-cells in mice. After entry of glucose into β-cells, a phosphate from ATP is transferred to glucose by glucokinase, generating glucose 6-phosphate. It is then converted into pyruvate through the glycolytic pathway. Pyruvate is then converted to acetyl CoA, which enters the citric acid cycle (TCA cycle). Progress through the TCA cycle leads to an increase in the ATP/ADP ratio, resulting in the closure of ATP-sensitive potassium channels (K_{ATP}) and opening of Ca^{2+} channels. Ca^{2+} influx leads to the exocytosis of insulin granules and insulin secretion (Gohring & Mulder, 2012; Jitrapakdee, Wutthisathapornchai, Wallace, & MacDonald, 2010; Mukhuty, Fouzder, Das, & Chattopadhyay, 2019).

Amino acids, free fatty acids and various hormones such as estrogen, melatonin, growth hormone, glucagon like peptide-1 and leptin are other stimuli involved in the control of insulin secretion (Z. Fu et al., 2013).

The primary role of insulin is to maintain blood glucose levels within ~4.0–6.0 mmolL when fasting and under 7.8 mmol/L postprandially ("Blood Sugar Level Ranges,"). Insulin initiates its action through binding to the tyrosine kinase transmembrane insulin receptor (IR) on target tissues such as adipose tissue, liver and muscle. The interaction between insulin and IR leads to a cascade of phosphorylation events. Intracellular Insulin Receptor Substrates (IRS) proteins are tyrosine-phosphorylated by the activated insulin receptors and interact with PI3-kinase (PI3K) to mediate the phosphorylation of Phosphatidylinositol 4,5-bisphosphate (PIP2) to synthesize Phosphatidylinositol (3,4,5)-triphosphate (PIP3). Subsequently, PIP3 activates 3-Phosphoinositide-Dependent Protein Kinase 1 (PDK1), which activates Protein Kinase B (PKB)/ Akt and Protein Kinase C (PKCs). AS160, an Akt substrate, is phosphorylated by PKB/Akt, mediates the translocation of glucose transporters from the cytoplasm onto the cell membrane, and consequently, glucose flows into the cell. PKB/Akt is also involved in other insulin actions such as inhibition of lipolysis in adipocytes, glycogen synthase and accumulation in the liver, gluconeogenesis, protein synthesis, and cell cycle and survival (Boucher, Kleinridders, & Kahn, 2014; Świderska et al., 2018). PKC, once activated by PDK1, mediates insulin-stimulated glucose transport (Sajan, Rivas, Li, Standaert, & Farese, 2006). Insulin also regulates cell proliferation through the Ras/ERK pathway (Boucher et al., 2014).

1.8 The proglucagon gene

The proglucagon gene (Gcg), located on chromosome 2, is expressed in a tissue-specific manner in pancreatic α-cells, enteroendocrine cells (L-cells), large intestine and neuronal cells of the central nervous system (Han et al., 1986; Raffort et al., 2017). It encodes the proglucagon protein which is a precursor prohormone. Proglucagon is post-translationally modified by prohormone convertase 1/3 and 2, producing various peptides with different biological functions. Glucagon, Glicentin Related Pancreatic Polypeptide (GRPP), Major Proglucagon Fragment (MPF) and Intervening Peptide-1 (IP-1) are produced by prohormone convertase 2, and Glucagon Like Peptide 1 (GLP-1) production is mediated by prohormone convertase 1/3 (Raffort et al., 2017).

1.8.1 Transcriptional regulation of the proglucagon gene

Transcriptional regulation of the proglucagon gene occurs via its 5'-upstream flanking region. Six cis regulatory regions; an upstream promoter region G1, four enhancer regions (G2–G5) and a cAMP-Response Element (CRE), have been identified within approximately 300 bp 5′ of the proglucagon gene transcription start site (Jin, 2008; Sandoval & D'Alessio, 2015; Sharma et al., 2005). Many transcription factors have been shown to bind to these regulatory elements, regulating cell-specific expression of the proglucagon gene. It has been suggested that the G1 element plays a crucial role in conferring α-cell-specific expression of proglucagon (Morel, Cordier-Bussat, & Philippe, 1995). The G1 element is located adjacent to the TATA box and contains two TAAT motifs (homeobox proteins binding site) and three AT-rich regions. Several transcription factors such as Foxa1/2 and heterodimers Pax6/cMaf and Pax6/MafB bind these AT-rich regions and mediate the expression of proglucagon in α-cells (Gauthier et al., 2002; Gosmain, Avril, Mamin, & Philippe, 2007; Gosmain, Cheyssac, Heddad Masson, Dibner, & Philippe, 2011). It has also been suggested that β-cell specific transcription factors such as Nkx6.1, Pdx1 and Pax4 can

regulate the transcription of the proglucagon gene. Nkx6.1, Pdx1 and Pax4 can bind to the Pax6 binding site within the G1 regulatory element and prevent the transcription of proglucagon by interfering with Pax6 (Gauthier, Gosmain, Mamin, & Philippe, 2007; Ritz-Laser et al., 2002; Ritz-Laser et al., 2003). Pax6 binding to the G1 regulatory element is necessary for the expression of the proglucagon gene in pancreatic α-cells (Sander et al., 1997).

Studies on a clonal hamster islet cell line have shown the inhibitory effects of insulin on islet proglucagon gene transcription *in vitro* (J. Philippe, 1989). Insulin-dependent inhibition of proglucagon gene expression is mediated by G3 regulatory elements (J. Philippe, 1991; Jacques Philippe, Morel, & Cordier-Bussat, 1995). However, whether insulin mediates the inhibition of proglucagon gene expression *in vivo* has been disputed (Dumonteil et al., 1998; Magnan et al., 1995).

1.9 Glucagon

Glucagon was first discovered in 1923 as a toxic precipitant in insulin extracts when Kimball and Murlin were working on methods of insulin concentration and isolation. They first named it (GLUCose-AGONist), a second pancreatic hormone which causes an increase in blood glucose levels and death in de-pancreatized dogs (Fisher, 1923; Kimball & Murlin, 1923).

The top three sources of circulating glucose are glycogenolysis, a metabolic process that results in (1) the breakdown of glycogen in the liver, gluconeogenesis; (2) the generation of new glucose from lactate, amino acids and glycerol during periods of starvation; and (3) glucose absorption from the intestine (Aronoff, Berkowitz, Shreiner, & Want, 2004). Glucagon is released from α-cells when the concentration of glucose decreases below basal levels. It elevates the levels of

glucose output by facilitating gluconeogenesis and glycogenolysis (Aronoff et al., 2004). Several *in vitro* and *in vivo* studies have shown the negative regulatory effects of insulin on glucagon secretion (Maruyama, Hisatomi, Orci, Grodsky, & Unger, 1984; Muller, Faloona, & Unger, 1971; J. Philippe, 1989). Glucagon has been found to be elevated in most patients with diabetic ketoacidosis and diabetic patients with hyperglucagonemia required higher amounts of insulin than those without hyperglucagonemia (Magnan et al., 1995; Muller, Faloona, & Unger, 1973). Higher plasma glucagon levels were observed in some cases of type 2 diabetes (Baron, Schaeffer, Shragg, & Kolterman, 1987; Reaven, Chen, Golay, Swislocki, & Jaspan, 1987; Unger, Aguilar-Parada, Müller, & Eisentraut, 1970).

1.10 Diabetes

Diabetes mellitus (diabetes) is a group of metabolic diseases that are characterized by blood glucose levels, chronically elevated above the normal range. Undiagnosed, or poorly controlled, diabetes can lead to complications such as blindness, renal insufficiency, stroke, and amputation of extremities. Diabetes is associated with a decrease in life expectancy (Malecki, 2005). There are various types of diabetes mellitus. From a genetic perspective, they can be categorized into two types: (1) monogenic, including Maturity Onset Diabetes of the Young (MODY) and Neonatal Diabetes Mellitus (NDM); and (2) polygenic, including type 1 and type 2 diabetes (X. Wang et al., 2016). Type 1 and type 2 diabetes are the two most common types, resulting from autoimmune destruction of β-cells or peripheral insulin resistance and β-cell dysfunction, respectively (S. J. Willmann et al., 2016). In recent years many research studies such as Genome-Wide Association Studies (GWAS) have shown the polygenic nature of type 2 diabetes, indicating the role of multiple genetic variants interacting together along with environmental factors to cause diabetes

(Bonnefond & Froguel, 2015). However, the contribution of each of these diabetes-associated mutations is small and none of them alone are sufficiently penetrant to cause type 2 diabetes (Bonnefond & Froguel, 2015).

Monogenic forms of diabetes affect up to 5% of diabetic patients. MODY is an autosomal dominant disorder that usually first occurs during early adulthood (before 25 years of age). Fourteen different types of MODY caused by mutations in fourteen different genes have been identified (Table 1). MODY1, MODY2 and MODY3 resulting from mutations in the HNF4A, GCK and HNFIA genes respectively, are the more common types of MODY. Treatment, clinical features and severity vary, depending on the type of MODY (Sanyoura, Philipson, & Naylor, 2018). NDM happens in newborns in the first 6 to 12 months of life. There are two types of NDM: Permanent Neonatal Diabetes Mellitus (PNDM) and Transient Neonatal Diabetes Mellitus (TNDM). Mutation in the KCNJ11 and ABCC8 genes, which encode subunits of the KATP channel, and the INS gene, are the most common mutations resulting in permanent neonatal diabetes mellitus (Babenko et al., 2006; Edghill et al., 2008; Gloyn et al., 2004; Kanakatti Shankar et al., 2013).

The primary treatment for type 1 diabetes is the administration of insulin (Dassaye et al., 2016). However, it does not provide complete glycemic control and daily insulin injections are needed (Hao et al., 2006; Stefanie J. Willmann et al., 2016). Restoration of β- cell mass by transplantation of isolated islets or whole pancreas is one way to circumvent the shortcomings of available diabetes treatments (Larsen et al., 2004; Shapiro et al., 2000). However, this treatment is also limited because of the shortage of donors and the possibility of immune responses and organ rejection (Dassaye et al., 2016; Ryan et al., 2005).

To overcome the shortcomings of currently available treatments for diabetes, new alternate strategies aiming at the restoration of β-cell mass are under investigation (Johnson, 2016). Several β-cells regeneration models developed in rodents have been used to reprogram various cell types into β-cells by genetic modification of key signaling pathways and transcription factors (Solomon Afelik & Rovira, 2017). Currently, potential sources for restoration of insulin-producing β-cell are: induced pluripotent stem cells (iPSC) (Manzar, Kim, & Zavazava, 2017; Mayhew & Wells, 2010; Zhang et al., 2009), Human Embryonic Stem Cells (hESCs) (D'Amour et al., 2006; Jiang et al., 2007), Mesenchymal Stem Cells (MSCs) (Cao, Han, Zhao, & Liu, 2014), reprogramming of various non β-cell types (Akinci, Banga, Greder, Dutton, & Slack, 2012; Lima, Docherty, Chen, & Docherty, 2012; Talchai, Xuan, Kitamura, DePinho, & Accili, 2012; Q. Zhou, Brown, Kanarek, Rajagopal, & Melton, 2008) and induction of replication of existing β cells (Orlando et al., 2014; Tesemma Sileshi Chala, 2016). Even though extensive research over recent decades has increased our understanding of the key steps in pancreatic development, and of the gene regulatory networks and signaling pathways involved in pancreatic cell specification and maturation, it is well accepted that a more detailed understanding of pancreas development is needed to generate fully functional and mature human β-cells (Johnson, 2016).

MODY 1	HNF4A
MODY 2	GCK
MODY 3	HNF1A
MODY 4	PDX1
MODY 5	HNF1B
MODY 6	NEUROD1
MODY 7	KLF11
MODY 8	CEL
MODY 9	PAX4
MODY 10	INS
MODY 11	BLK
MODY 12	ABCC8
MODY 13	KCNJ11
MODY 14	APPL1

Table 1: Genes Known to Harbour Mutations Causing MODY.

Modified from (Bonnefond & Froguel, 2015). Permission for use is provided by a license agreement from the publishers of *Cell Metabolism*, Elsevier Science & Technology, License ID 1015120-1, issued Jan. 24, 2020).

1.11 DLX genes

The Distal-less Homeobox (DLX) gene family encodes transcription factors that contain a 60 amino acid helix-turn-helix DNA binding domain called the homeodomain. Homeodomain containing transcription factors can bind to regulatory regions of other genes through ATTA/TAAT tetranucleotide DNA-binding motifs and repress or activate the transcription of target genes (Kraus & Lufkin, 2006). In humans and mice, the Dlx family consists of six genes: Dlx1, Dlx2, Dlx3, Dlx4, Dlx5 and Dlx6. These six genes are grouped into pairs and each of these pairs is linked to a Hox cluster; Dlx1 and Dlx2 are located near HoxD, Dlx3 and Dlx4 are located near HoxB, and Dlx5 and Dlx6 are located near HoxA (McGuinness et al., 1996; Maria I. Morasso & Radoja, 2005).

The roles of Dlx genes in vertebrate development have been assessed through loss and gain of function studies, mainly in mice. The expression of Dlx genes in the apical ectodermal ridge during limb morphogenesis, facial mesenchyme and branchial region shows the importance of these genes in the development of the craniofacial structures and limbs (Bulfone et al., 1993; Dollé, Price, & Duboule, 1992; M. Qiu et al., 1997; Robinson & Mahon, 1994). Several studies have shown the expression of Dlx1, Dlx2, Dlx5 and Dlx6 in the central nervous system (Eisenstat et al., 1999; J. K. Liu, Ghattas, Liu, Chen, & Rubenstein, 1997; Yang et al., 1998). The expression of DLX3 and DLX4 genes has been shown in the placenta (Quinn, Johnson, Nicholl, Sutherland, & Kalionis, 1997; Quinn, Latham, & Kalionis, 1998). Extreme forms of the phenotype have been observed in transgenic mice with the deletion of two DLX genes. The Dlx5/Dlx6–/– embryos die around embryonic day 18 probably due to extreme craniofacial abnormalities and defect of the central ray of the hind limbs which is a deformity that occurs in split hand/foot malformation type I disorder. The Dlx5/Dlx6+/- mice are born without abnormalities in the limbs (Merlo et al., 2002; M. I.

Morasso, Grinberg, Robinson, Sargent, & Mahon, 1999; Robledo, Rajan, Li, & Lufkin, 2002). The Dlx3 −/− mouse embryos die mid-gestation due to placental defects (M. I. Morasso et al., 1999).

My study is focused on the DLX1 and DLX2 genes. Both DLX1 and DLX2 are located on human and mouse chromosome 2 and contain three exons and two introns. The region encoding the homeobox is split between exons 2 and 3. The intergenic region located between DLX1 and DLX2 contains shared enhancer elements (McGuinness et al., 1996) (Figure1.4). The Dlx1/Dlx2 double knockout mice die a few hours after birth. They have several craniofacial abnormalities such as lacking maxillary molars and abnormal proximal first and second branchial arches (M. Qiu et al., 1997). Enteric nervous system defects were also observed in Dlx1/Dlx2 double knockout mice (M Qiu et al., 1997).

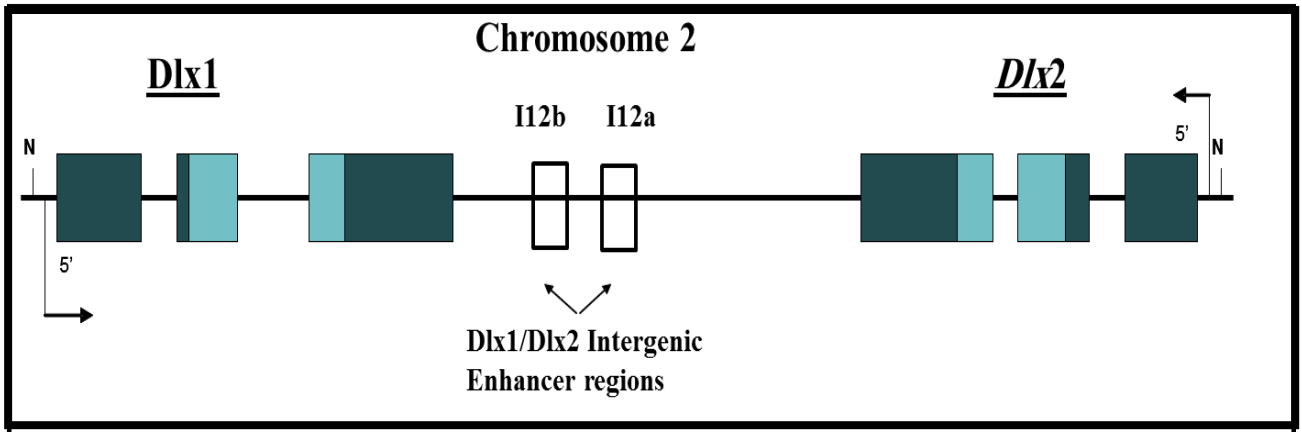


Figure 1.4 Genomic organization of the Dlx1 and Dlx2 bigenic cluster

DLX1 and DLX2 are located on chromosome 2 of both humans and mouse. As with the other DLX genes, they are arranged in a bigenic cluster. They both contain two introns and three exons (shown as boxes). Regions encoding the homeodomain are shown as light blue boxes. Modified from (Q. P. Zhou et al., 2004). Permission for use is provided by an Oxford University Press License (License No. 4755490951026, issued Jan. 24, 2020).

1.11.1 DLX2 expression in the pancreas

The onset of DLX2 expression is the pancreas is after E14.5 (Figure 1.5). Previously, the Eisenstat lab showed co-expression of DLX2 with insulin, glucagon, somatostatin, and pancreatic polypeptide at E18.5 (Figure 1.6). The Eisenstat lab has shown the co-expression of DLX2 with insulin and glucagon, in human islet cultures obtained through collaboration (Figure 1.7). Cell counting using immunofluorescence studies performed on E18.5 cryosections demonstrates significantly reduced insulin and glucagon expression in the Dlx1/Dlx2 Double Knockout (DKO) islets compared to controls (Figure 1.8). Radioimmunoassay studies of samples obtained at P0 show plasma insulin levels that are significantly reduced in Dlx1/Dlx2 DKO compared to wild type littermates (Figure 1.9).

DLX1 & DLX2 may play a role in pancreatic islet cell development by direct transcriptional regulation of insulin and glucagon expression. Previously, the Eisenstat lab showed that DLX2 binds to the mouse INS1 and proglucagon promoters in E18.5 pancreas, respectively, *in vivo* (Figure 3.3 & 3.11) and confirmed the specificity of binding *in vitro* using EMSA (Figures 1.10 & 1.11). Dlx2 co-expression activates transcription of the INS1 promoter *in vitro* (Figure 1.12)

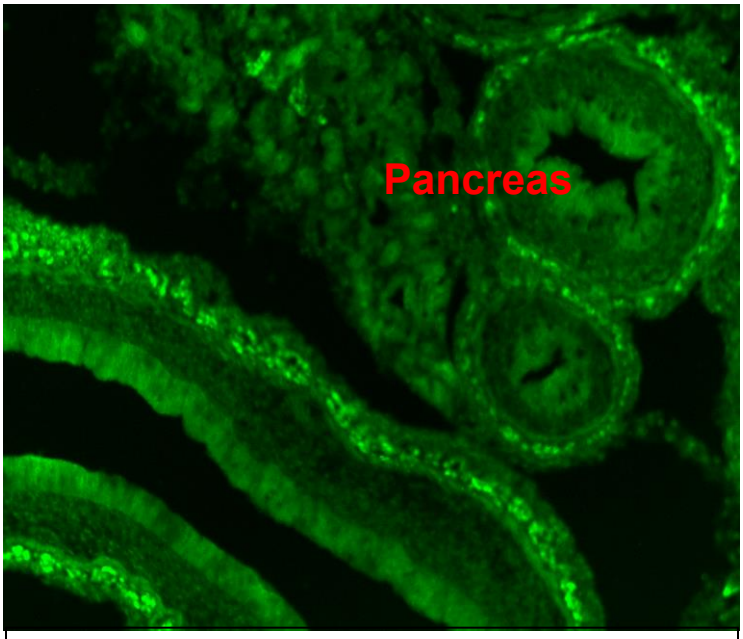


Figure 1.5 Expression of DLX2 appears in the pancreas at E14.5

(Niki Boyko and D. Eisenstat, unpublished).

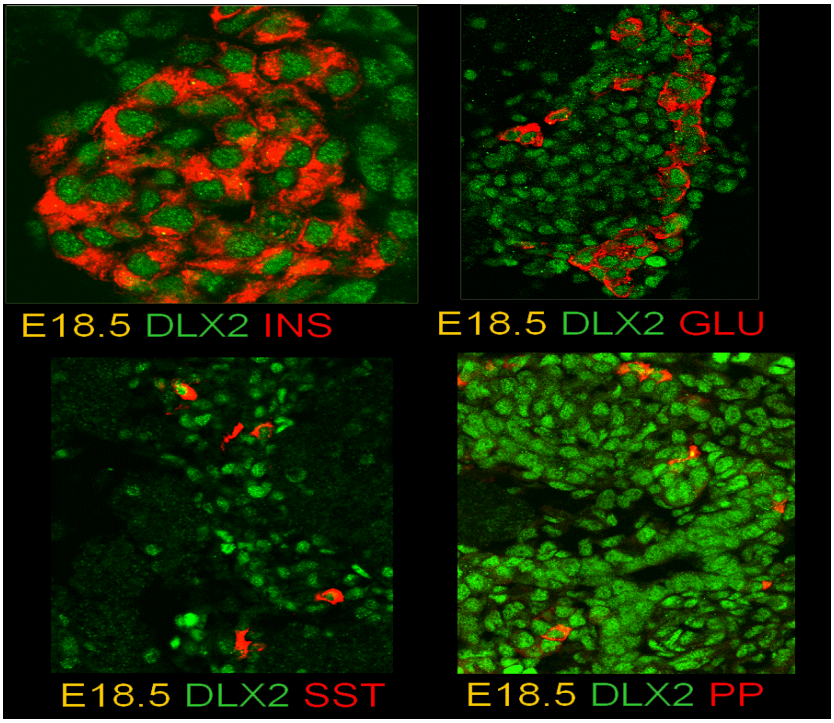


Figure 1.6 Dual immunofluorescence studies of DLX2 with insulin (INS), glucagon (GLU), somatostatin (SST) and pancreatic polypeptide (PP).

DLX2 is co-expressed with insulin, glucagon, pancreatic polypeptide and somatostatin in a subset of islet cells at E18.5 in the mouse (Andrew Ho and D. Eisenstat, unpublished).

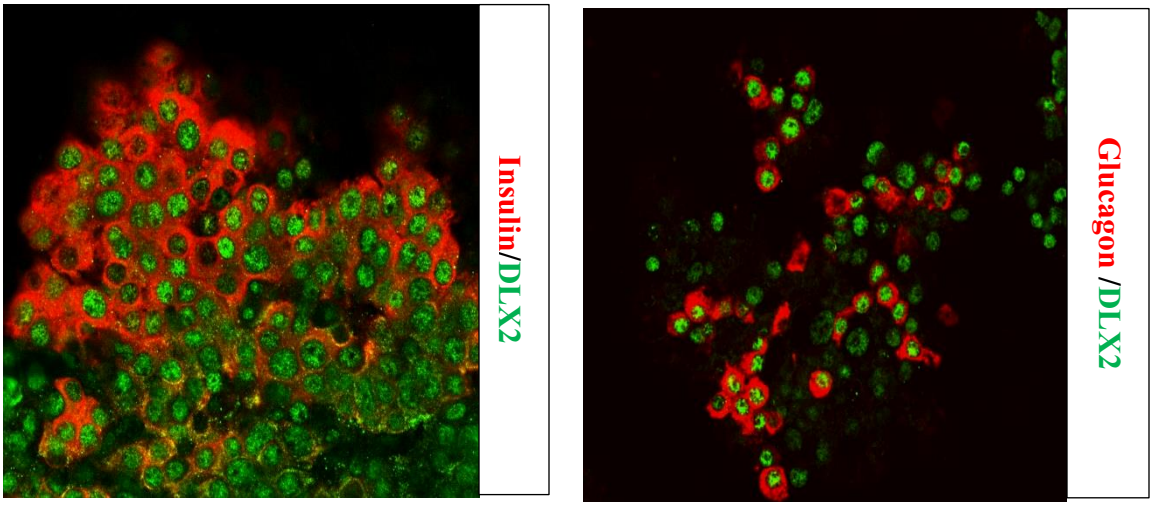


Figure 1.7 Dual immunofluorescence of DLX2 with INS or glucagon.

Cultured human islet cells co-express DLX2 and insulin (left) or glucagon (right) (Andrew Ho, L. Rosenbloom and D. Eisenstat, unpublished).

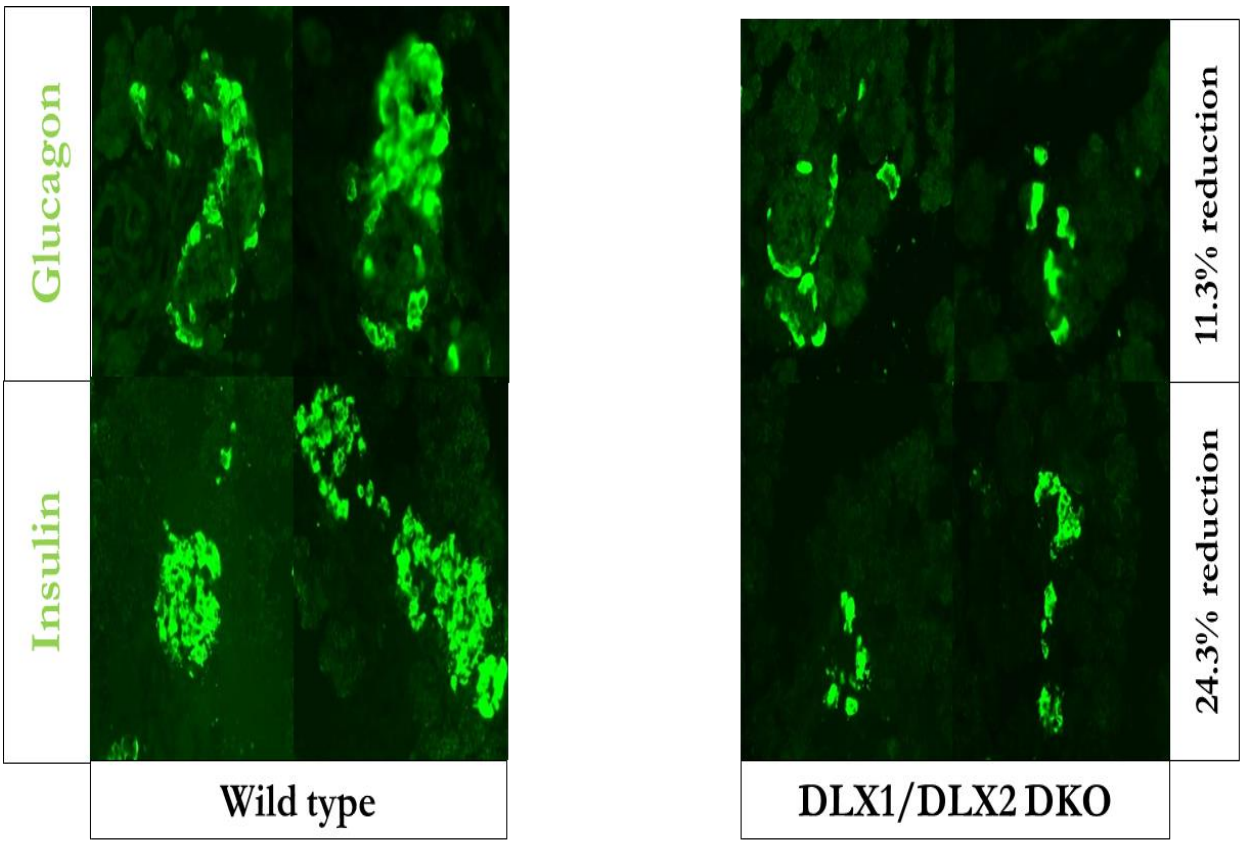


Figure 1.8 Reduced expression of glucagon and insulin in the Dlx1/2 DKO compared with wild type littermates at P0.

Quantification shows a 24.3% reduction in insulin positive cells ($p < 0.05$) and an 11.3% reduction in glucagon positive cells ($p < 0.05$) in the neonatal pancreas from Dlx1/2 DKO mice compared with wild type littermate controls at P0 (X. Qiu and D. Eisenstat, unpublished).

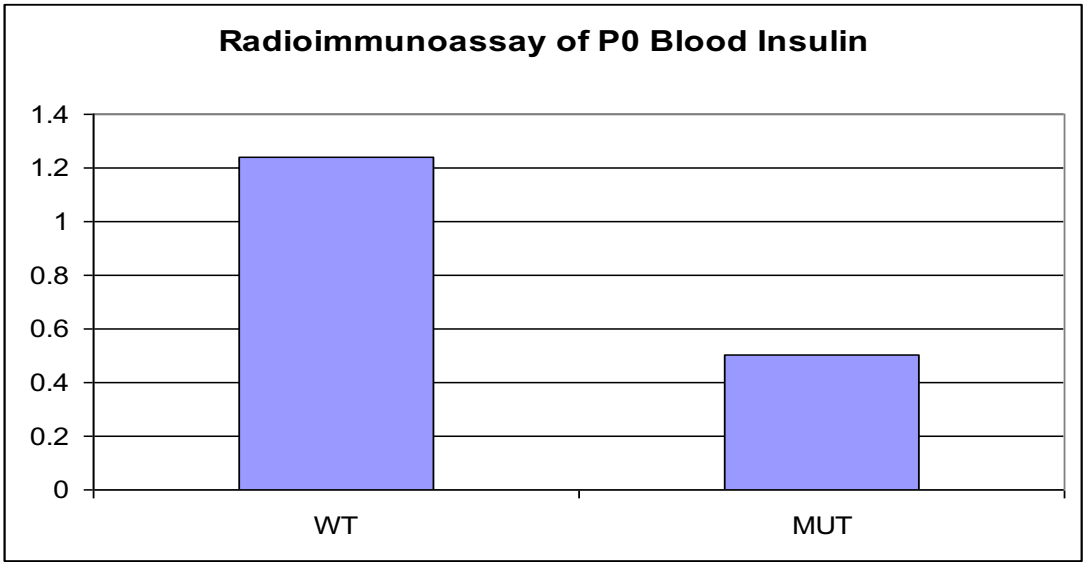


Figure 1.9 Radioimmunoassay of P0 blood insulin.

Radioimmunoassay of samples obtained at P0 before feeding show that insulin levels are significantly reduced in Dlx1/Dlx2 DKO mice compared to wild type littermates ($p<0.05$) (J. Silha, L. Murphy and D. Eisenstat, unpublished).

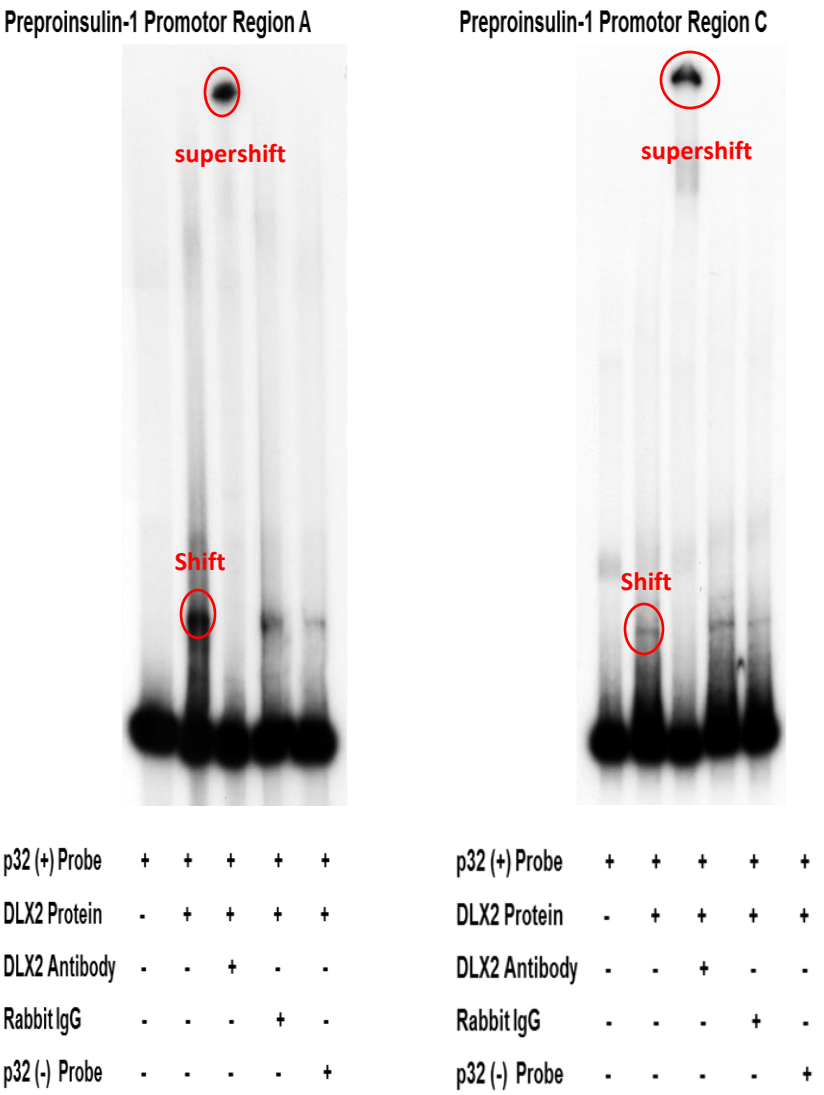


Figure 1.10 Direct binding of DLX2 at INS1 region A and region C.

Direct binding of DLX2 to radiolabelled INS2 region A and region C resulted in shifted bands. The DLX2 antibody, recombinant DLX2 protein and labelled probe complex resulted in a supershift band, confirming the specificity of DLX2 binding (Miten Dhruve and D. Eisenstat, unpublished).

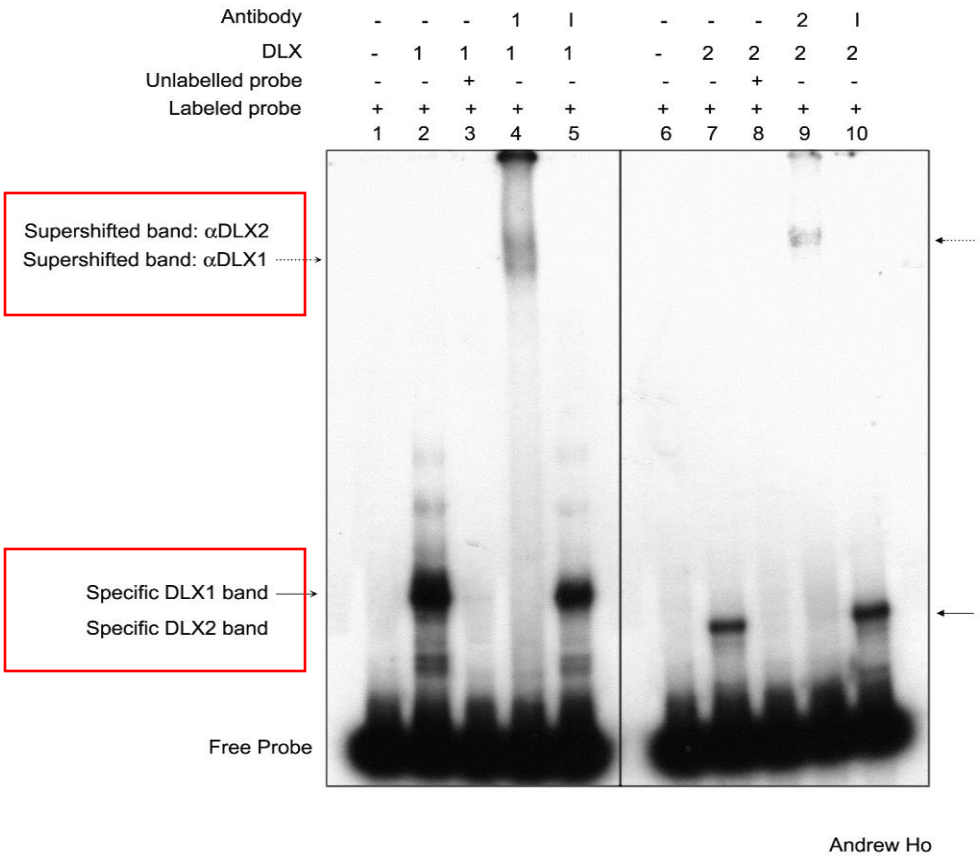


Figure 1.11 Direct binding of DLX1 and DLX2 to the proglucagon promoter.

Direct binding of DLX1 and DLX2 to radiolabelled proglucagon promoter sequences resulted in shifted bands. The DLX1 or DLX2 antibody, DLX1 or DLX2 recombinant protein, and labelled probe complex resulted in a supershift band, confirming the specificity of DLX1 or DLX2 binding (Andrew Ho and D. Eisenstat, unpublished).

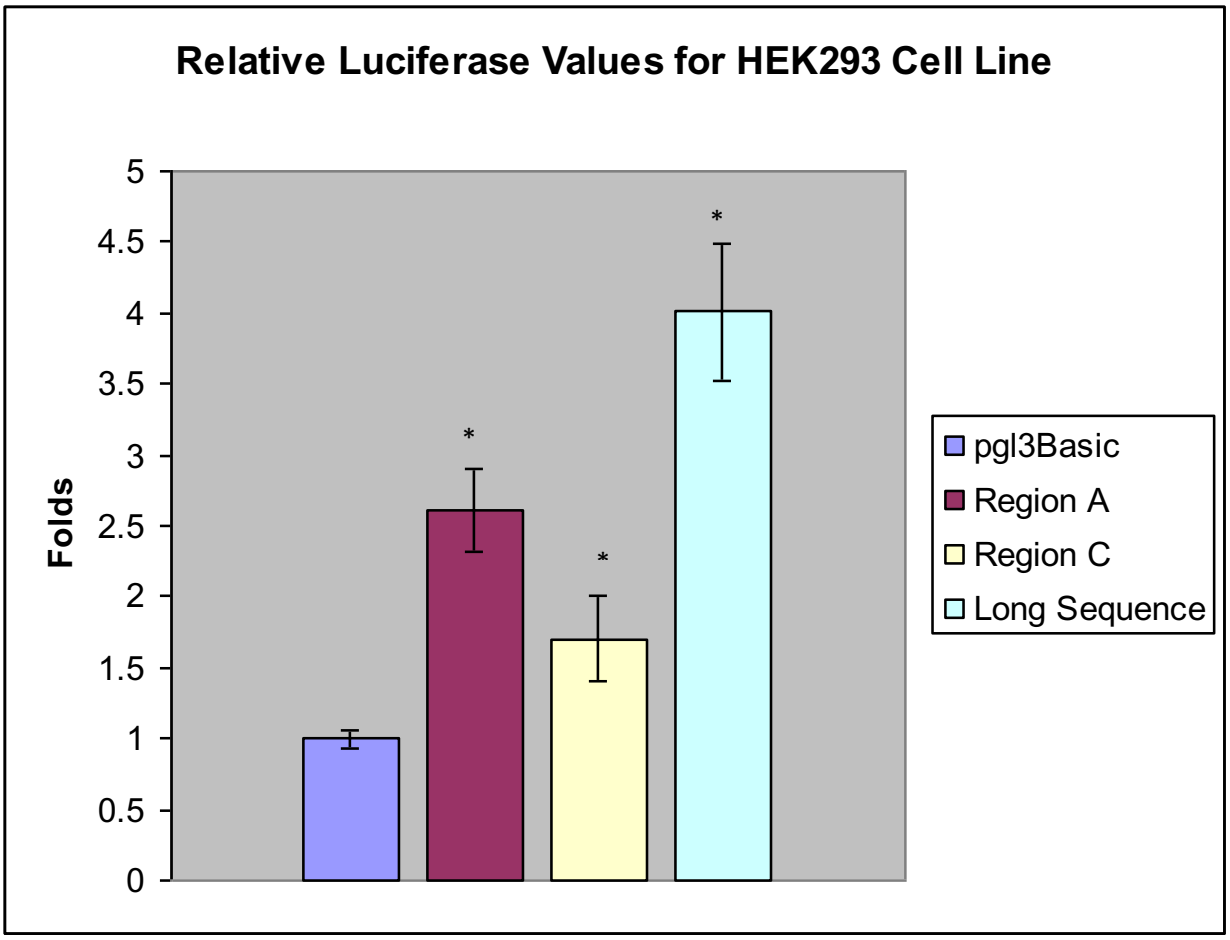


Figure 1.12: Dlx2 co-expression activates transcription of the INS1 promoter *in vitro*.

Co-transfection of Dlx2-pcDNA3 with PGL3- INS1 regulatory regions resulted in significant upregulation of luciferase expression compared to empty pCDNA3 control. Error bars shows standard error of the mean (Miten Dhruve and D. Eisenstat, unpublished).

1.12. Research Aim and Hypotheses

My research is aiming to determine whether *Dlx1* & *Dlx2* are essential for normal pancreatic development and if the loss of function of *Dlx1/Dlx2* results in islet-cell-specific defects.

Hypothesis 1:

DLX2 plays a role in pancreatic islet cell development by direct transcriptional regulation of insulin and glucagon expression.

Questions:

1. Does DLX2 bind to the INS1, INS2 and proglucagon promoters *in vitro* and *in vivo* (E18.5)?
2. Does DLX2 promote transcriptional repression or activation of INS2 expression during late pancreas development?

Hypothesis 2:

Dlx1 and Dlx2 genes are essential for normal pancreatic islet development and loss of Dlx1/Dlx2 function results in islet cell specific defects.

Question:

Does loss of *Dlx1/Dlx2* gene function alter INS1 and INS2 mRNA levels in the pancreas at E18.5?

Chapter 2. Materials and Methods

2.1 Animals and Tissue collection

Timed-pregnant wild-type CD-1 strain (Charles River Laboratory) and Dlx1/Dlx2 DKO transgenic mice (originally produced by Dr. John Rubenstein (UCSF, CA, USA)) propagated on a CD-1 background were sacrificed by cervical dislocation at day 18.5 of pregnancy. The presence of a vaginal plug in the female mouse indicated day 0.5 of pregnancy. Pancreas tissues were then immediately dissected from E18.5 embryos in phosphate buffered saline (PBS). Dlx1/Dlx2 DKO mice die a few hours after birth, therefore, phenotypically normal heterozygous mice were crossed to generate a Dlx1/Dlx2 DKO colony (Anderson, Qiu et al. 1997, Qiu, Bulfone et al. 1997). To verify Dlx1/2 DKO genotype in the littermates, tails were collected from embryos and PCR amplification (Qiagen, catalog #: 203601) with specific primers for Dlx2 and Neo was performed on genomic DNA extracted from samples (Table 2).

All experiments using Dlx1/2 DKO or CD-1 mice were performed using protocols approved by the University of Alberta Animal Care and Use Committee.

2.2 Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) assay was performed on E18.5 WT CD-1 embryo whole pancreas. Pancreas tissue was dissected as described above. Since embryonic hindbrain does not express any type of Dlx gene, this tissue was used as a negative control in ChIP-PCR experiments. Dissected tissue was washed with cold 1XPBS and dispersed by pipetting several times followed by a 2-hour incubation in 1% paraformaldehyde (PFA) with 25X Protease Inhibitor Cocktail (PIC) at 4°C. After being washed twice with PBS, 400µl lysis buffer (1% SDS, 10mM Tris-HCl pH 8.1, 10mM EDTA) was added to the cells. Sonication (60 Sonic Dismembrator) was performed on ice

at 40% output strength (15s sonication followed by 30s rests) to shear the DNA into 300 to 500 bp fragments. The size of the DNA fragments was confirmed by running the resulting lysate on 1% agarose gel. 20µl of sample was set aside as Total Input (represents the amount of chromatin used in the ChIP) and kept in -80°C for use in subsequent analysis.

In order to remove Immunoglobulin G (IgG) from the sample, Pierce UltraLink Protein A/G beads (Thermo Fisher, Catalog #: 53132) was used. Beads need to be washed twice by 1ml of dilution buffer (0.01% SDS, 1% Triton X-100, 1.2mM EDTA, 167mM NaCl, 16.7mM Tris-HCl pH8.1) and 25X PIC before adding them to the samples. 2 sets of primed beads were prepared; 500 µg/ml of bovine serum albumin (BSA) and Transfer ribonucleic acid (tRNA) were added to one of them.

60µl of the primed beads (without BSA and tRNA) were added to each sample followed by a 1-hour incubation at 4°C with rotation. To collect the supernatant, samples were centrifuged at 2000 rpm for 5 min. Beads were discarded at this step. Supernatant was divided in 2 tubes and 500 µg/ml of BSA and tRNA was added to each. 5 µl of DLX2 antibody (D. Eisenstat and J. Rubenstein) was added to one of the tubes (+ Antibody) and 10µl IgG antibody was added to the other one (Control). Samples were incubated at 4°C with rotation. The next day, beads containing BSA and tRNA were added to the samples followed by an overnight incubation at 4°C with rotation. To pellet beads, samples were centrifuged at 2500 rpm for 5 minutes. Beads were then washed with 1ml of 4 different solutions with rotation at 4°C: low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8.1 and 150mM NaCl) for 5 minutes, high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8.1 and 500mM NaCl) for 30 minutes, LiCl buffer (0.25M LiCl, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl (pH8.1) and 1% NP-40) for 30 minutes and TE buffer pH 8.0 for 5 minutes. To elute the DNA-protein complexes, 250 µl of pre-heated freshly made Elution Buffer (1% SDS, 0.1M NaHCO3) was added to the beads followed

by a 15-minutes incubation at room temperature with agitation. Samples were then centrifuged at 13000 rpm for 5 minutes at 4°C. The supernatant, which contains DLX2 antibody-DNA- protein complexes, was transferred to a fresh tube and elution steps were repeated one more time and the two supernatants were combined. 25µl 5M NaCl and 1µl of RNaseA were added to the supernatant to degrade RNA and remove proteins that were bound to the DNA. Samples were incubated at 65°C overnight. To degrade the protein, 20µl of 1M Tris-HCl (pH 6.5), 10µl of 0.5M EDTA, and 2µl of Proteinase K were added to the samples followed by a 2-hour incubation at 65°C. QiaQuick PCR purification kit (Qiagen, catalog #: 28104) was used to purify the DNA. PCR and q-PCR with primers for regulatory regions of preproinsulin 1, preproinsulin 2 and proglucagon with potential DLX2 binding sites was performed to identify DLX2 binding sites within these regulatory regions. Primers used for ChIP are listed in Table 3.

2.3 ChIP-qPCR

ChIP-qPCR was performed on E18.5 WT pancreas tissue on a LightCycler 96 System (Roche) using the FastStart SYBR Green Master System (Roche, catalog #: 03003230001). 20µl reaction containing 1µl of each Forward and Reverse primer, 10µl Master mix, 7µl PCR grade water and 1µl ChIP sample (+ Antibody, +IgG) was added into a Roche Lightcycler 480 multi 96 well.

The fold enrichment method was used to normalize ChIP-qPCR data. Fold enrichment shows the results relative to the IgG sample (Bradburn). The IgG samples was given a value of ‘1‘ and +DLX2 antibody samples were then determined as a fold change of the IgG samples.

First the Nonspecific adjustment was calculated:

(Ct + Antibody) - (Ct IgG)

Next, Fold enrichment was calculated:

(2^- (Nonspecific adjustment))

ChIP experiments were performed in technical and biological triplicates.

2.4 Molecular cloning

Regulatory regions identified by ChIP experiments were cloned into the pGL3-Basic Vector reporter plasmid (Promega, catalog #: E1751) to be used later for EMSA and luciferase experiments. To create overlapping ends, appropriate restriction sites were added to the 5' and 3' ends of primers that were used in the ChIP assay (Table 4). Regulatory regions were PCR amplified using HotStarTaq DNA polymerase (Qiagen, Catalog# 203205) and purified using a QIAquick PCR Purification Kit. To digest the DNA and empty pGL3, 2 units of restriction enzymes, 4µl of NEB Buffer and DNase/RNase free water were added to 500ng of DNA (total reaction volume of 40µl) and incubated at 65°C for 2 hours. To isolate the digested plasmid, it was run on 1% agarose gel, and cleaned with Illustra GFX PCR DNA and Gel Band Purification Kits (GE Healthcare Life Sciences). Regulatory regions were purified using a QIAquick PCR Purification Kit. Digested regulatory regions were inserted into the pGL3 vector using T4 DNA ligase (NEB, catalog #: M0202S). 3 µl of the ligation mixtures were then added to the competent DH5α E. coli cells followed by a 30-minute incubation on ice, 1-minute heat shock at 42°C and another 5-minute incubation on ice. 1 ml Lysogeny Broth (LB) was then added to the cells and incubated for 2 hours at 37°C with rotation. 200µl of cells was spread onto a Carbenicillin (50mg/ml) containing agar plate and incubated overnight at 37°C. Single colonies were picked and added to 4ml LB containing 50mg/m Carbenicillin and incubated overnight at 37°C with rotation. QIAprep Spin

Miniprep Kit (Qiagen, catalog #: 27106) was used for plasmid isolation. Insertion of regulatory regions into the plasmid was confirmed using Sanger sequencing (TAGC, University of Alberta).

2.5 Electrophoretic Mobility Shift Assay (EMSA)

25-30 bp oligonucleotides for each TAAT/ATTA motif of the INS2 gene promoter were designed. Sequences of the oligonucleotides are provided in Table 5. [γ-32P]-dGTP was used to radiolabel oligonucleotides at their 5' ends. For labelling, 5pmol of oligonucleotide, 1 µl T4 polynucleotide kinase, 4µl 5X exchange buffer and 1-2 µl [γ-32P]-dGTP was mixed and incubated for 30 minutes at RT. The reaction was terminated using 1µl of 0.5M EDTA. Micro-Spin G-25 columns (Healthcare Illustra) was used to purify labelled oligonucleotides. An LS 6500 scintillation counter was used to measure the radioactivity levels. 80,000 counts per million/µl was used for carrying out EMSA. For sample preparation for the assay, 5X Binding Buffer, 1mg/ml Poly (dI-dC), 200ng recombinant DLX2 protein was mixed followed by a 30-minute incubation at RT. After 30 minutes, labelled oligonucleotides were added to the samples followed by another 30-minutes incubation. There were three controls for this assay: free probe, where no DLX2 protein or DLX2 antibody was added to the solution, supershift where DLX2 antibody was added to the solution, and cold competition where unlabelled probe was added to the solution.

The binding reaction was then run on a 4% acrylamide gel at 300V for an hour in 0.5X TBE buffer. The gel was then dried using a gel dryer and a HydraTech vacuum pump for an hour at 80°C. The gel was then exposed to X-ray film (Kodak) in Biorad autoradiography cassettes overnight at -80°C. Films were then developed at the dark room using a Mini-Medical 90 film developer.

2.6 Luciferase gene reporter assay

Luciferase reporter assays were performed on regulatory regions of INS2 that showed interaction with DLX2 *in vitro*. These regulatory regions were cloned into the pGL3-Basic Vector containing the luciferase gene, as described in the previous section. HEK293 cells were grown in DMEM media (Gibco) with 10% Fetal Bovine Serum (Sigma) until they reached 90% confluence. Cells were then detached from the flask using 0.25% Trypsin-EDTA (1X) (Gibco). Cells were counted using a Countess™ II FL Automated Cell Counter and 175,000 cells/ml was added to each well of a 12 well plate. Cells were incubated overnight at 37°C, 5% CO_2. The following day, transfections were carried out using Lipofectamine 2000 (Invitrogen). Renilla vector (Promega) was used a control for transfection efficiency. The following table shows the plasmid mixtures added to each well of the 12 well plates.

pGL3 + pCDNA3 + Renilla vector	pGL3 + pCDNA3-DLX2 expression vector + Renilla vector	pGL3 plasmid containing regulatory region + pCDNA3 + Renilla vector	pGL3 plasmid containing regulatory region + pCDNA3-DLX2 expression vector + Renilla vector

- 0.5µg for pGL3- regulatory region, pGL3, pCDNA3-Dlx2, pCDNA3
- 4ng Renilla vector

First, plasmids were mixed in 4 different 1.5ml tubes. Opti-MEM (Gibco) was added to each plasmid mixture up to 400µl. 20µl of Lipofectamine was added to 2ml of Opti-MEM (Gibco) (1µl of Lipofectamine for every 100µl of Opti-MEM) and incubated for 5 minutes at RT. 400µl of this mixture was added to each of the plasmid mixtures. 300µl of final mixture was added to each well of 12 well plates. After a 4-hour incubation, media was replaced with a fresh DMEM with 10% FBS and cells were incubated for 48 hours at 37°C, 5% CO2. After 48 hours, 100 µl of 1X reporter lysis buffer, prepared from 5X reporter lysis buffer (Promega, catalog #: E4030) was added to each

well followed by a 15-minutes in -80°C. 20µl of cell lysates were then added into wells of two 96-well plates. A SpectraMax L Microplate Reader was used for measuring luciferase and renilla activity. This experiment was performed in three technical and biological replicates. Statistical significance was calculated using the Student's unpaired t-Test.

2.7 Dlx2 knockdown using Small Interfering RNA (siRNA)

To investigate the expression levels of INS1 and INS2 after Dlx2 knockdown, Beta-TC 6; Pancreatic Beta Cells; Mouse (Mus musculus) were used (ATCC). Beta-TC 6 cells were cultured with low glucose DMEM Media supplemented with 15% FBS. $1x10^6$ was added to each well of a 12 well plate and incubated for 24 hours to reach 90% confluence before transfection. Two different double strand siRNAs were used. Dlx2-siRNA1 (Ambion): sense 5'GGAAGACCUUGAGCCUGAATT3', antisense 5'UUCAGGCUCAAGGUCUUCCTT3'; Dlx2-siRNA2: sense 5'CCUGAAAUCCGAAUAGUGATT3', antisense 5'UCACUAUUCGGAUUUCAGGCT3'. Cells were transfected with either Dlx2-siRNA1 or Dlx2-siRNA2 or Silencer® Negative Control (Ambion) or transfection reagent only (mock-transfected control). Transfection was carried out using Lipofectamine 2000. Cells were then incubated for 48 hours at. at 37°C, 5% CO2. After 48 hours, cells were collected and RNA was extracted using an RNA isolation kit (Qiagen, catalog #: 74104). Using the Superscript III Reverse Transcriptase (Invitrogen, catalog #: 18080-044), cDNA was synthesized from mRNA. qRT-PCR was then performed to quantify expression levels of INS1 and INS2 using INS1 and INS2 primers. Peptidylprolyl Isomerase A (PPIA) primers were used as an internal control. Primers are listed in (Table 6).

2.8 Reverse Transcription Quantitative PCR (RTq-PCR)

In order to assess the effects of DLX1 and Dlx2 absence on mRNA expression levels of INS1 and INS1 *in vivo,* I performed qRT-PCR on E18.5 Dlx1/2 DKO and WT whole pancreas tissue and compared the levels of INS1 and INS2 mRNA expression between Dlx1/2 DKO and WT. RNA was extracted immediately after tissue dissection using TRIzol (Invitrogen, catalog #:15596026). The concentration and purity of RNA was measured using a NanoDrop Spectrophotometer. Using the Superscript III Reverse Transcriptase (Invitrogen, catalog #: 18080-044), cDNA was synthesized from mRNA. 500ng of RNA, 1µl of Oligo(dT) and 1µl d NTP were mixed and incubated at 65 °C for 5 minutes, followed by at least 1-minute incubation on ice. 1µl RNaseOUT, 4µl 5X First-Strand Buffer, 1µl DTT and 1µl Superscript III RT were added to the mixture, followed by an hour incubation at 50°C and a 15-minute incubation at 70°C. The FastStart SYBR Green Master System and a LightCycler 96 System (Roche) was used to perform qRT-PCR. PPIA primers were used as an internal control. The method used for data analysis was the delta delta Ct method. The statistical significance was determined using the Student's unpaired t-Test. The sequences of primers for qRT-PCR are provided in (Table 6).

Table 2 List of primers used for Dlx1/Dlx2 DKO colony genotyping

Primer name	Primer sequence (5' to 3')
Dlx2	F- TCCGAATAGTGAACGGGAAGCCAAAG R- CAGGGTGCTGCTCGGTGGGTATCTC
Neo	F- CAAGATGGATTGCACGCAG R- CATCCTGATCGACAAGAC

Table 3 List of primers used for qPCR-ChIP

Primer name	Primer sequence (5' to 3')
INS1 Region 1	F- CTACGTGCCAGGACACAAG R- GAATGAGCTACTTTGAGGGACA
INS1 Region 2	F- CCCTCAAAGTAGCTCATTCTCT R- CCCAAAGTATCAGACCTCAGAA
INS1 Region C	F- GGTTTGTGAAAGGAGAGATGGA R-AGGGAGGAGGAAAGCAGAA
INS2 Region A	F- ATGCACTAACAGATGGAGACAG R- CATCCTCCTGCAGTATCCAATAA
INS2 Region B	F- TGGAAAGAGAGATAGAGGAGGAG R- CTCTCTCTCAGAGGTAGAAGGAA
INS2 Region C	F- CCC TGC TGT GAA CTG GT R- TCT GCA GAA AGC GCT CAT
INS2 Region D	F- CCACCTGGAGCCCTTAAT R- CCACTACCTTTATAGACCAAAGC
Proglucagon promoter	F- AAGCAGATGAGCAAAGTGAGT R- GGCTGTTTAGCCTTGCAGATA

Table 4 List of cloned regions primers

Primer name	Primer sequence (5' to 3')
INS2 Region A-F- *HINDIII*	ATAAAGCTT ATGCACTAACAGATGGAGACAG
INS2 Region A-R- *BG1II*	GTAAGATCT CATCCTCCTGCAGTATCCAATAA
INS2 Region B-F- *HINDIII*	ATAAAGCTT TGGAAAGAGAGATAGAGGAGGAG
INS2 Region B-R- *BG1II*	ATAAGATCT CTCTCTCTCAGAGGTAGAAGGAA
INS2 Region C-F- *HINDIII*	ATAAAGCTT CCC TGC TGT GAA CTG GT
INS2 Region C-R- *BG1II*	ATAAGATCT TCT GCA GAA AGC GCT CAT
INS2 Region D-F- *HINDIII*	ATAAAGCTT CCACCTGGAGCCCTTAAT
INS2 Region D-R- *KPN1*	ATAGGTACC CCACTACCTTTATAGACCAAAGC

Table 5 Sequence of oligonucleotides used in EMSA

Primer name	Primer sequence (5' to 3')
INS2 Region A	F- TGAAGCAAGTATTACATATGGAGAC R- GTCTCCATATGTAATACTTGCTTCA
INS2 Region B	F- AGGAGGGACCATTAAGTGCCTTGC R- GCAAGGCACTTAATGGTCCCTCCT
INS2 Region C	F- TTGTTAAGACTCTAATTACCCTAGGACTA R- TAGTCCTAGGGTAATTAGAGTCTTAACAA
INS2 Region D	F- CACCTGGAGCCCTTAATGGGTCAAACA R- GCTGTTTGACCCATTAAGGGCTCCAGG

Table 6 List of primers used in qRT-PCR

Primer name	Primer sequence (5' to 3')
INS1	F- TTAATGGGCCAAACAGCAAAG R- CTCTGATTATAGCTGGTCACTAAGG
INS2	F- AGCGTGGCATTGTAGATCAG R- GTGGGTCTAGTTGCAGTAGTTC
PPIA	F- CGCGTCTCCTTCGAGCTGTTTG R-TGTAAAGTCACCACCCTGGCACAT

Chapter 3. Results

3.1 INS1 and INS2 regulation by DLX2

3.1.1 The INS1 and INS1 proximal regulatory region contains several potential binding sites for DLX2

To identify potential DLX2 binding sites on the INS1 and INS2 proximal promoter, the 1 kilobase pair (kbp) region upstream of the INS1 and INS2 transcription start sites were screened for homeodomain binding sites (ATTA/TAAT). INS1 and INS2 contain 9 and 4 potential DLX2 binding sites, respectively. To make the experiments more convenient, the proximal promoters were arbitrarily divided into different regions, with each region containing one or more DLX2 potential binding sites (Figure 3.1).

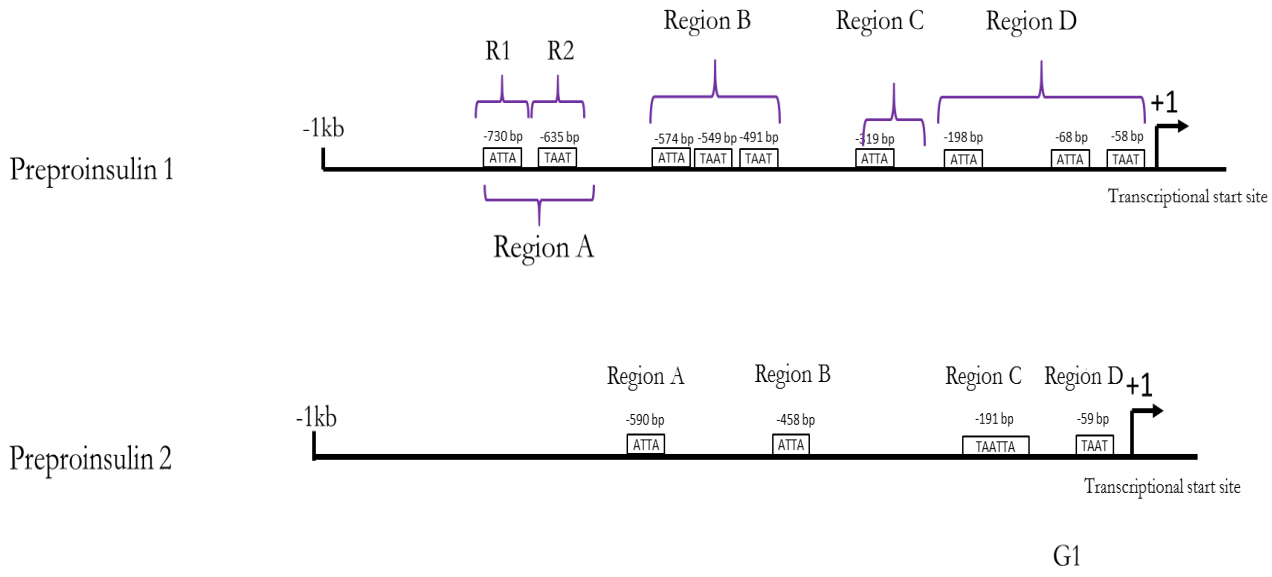


Figure 3. 1 Schematic diagram of the INS1 and INS2 proximal promoters showing potential DLX2 binding sites.

All the homeodomain binding sites (ATTA/TAAT) present in the proximal promoter 1Kb upstream of INS and INS2 genes were assigned as potential DLX2 binding sites. INS1 and INS2 contain 9 and 4 potential DLX2 binding sites, respectively. The INS1 promoter was divided into 4 regions, Region A to Region D, each containing one or more DLX2 potential binding sites. The INS2 proximal promoter was also divided into 4 regions, each region containing one DLX2 potential binding site.

3.1.2 DLX2 binds to INS1 and INS2 regulatory regions in mouse at embryonic day 18.5

To investigate the regulatory function of DLX2 on the INS1 and INS2 genes at E18.5, the first step was to determine whether DLX2 occupies INS1 and INS2 regulatory regions at that developmental time point. Towards that goal, I performed ChIP-qPCR on E18.5 WT CD-1 embryo whole pancreas. DLX2 protein-DNA complexes were isolated using the DLX2 polyclonal antibody. After reverse cross-linking, primers for regulatory regions of INS1 and INS2 were used for the amplification of DLX2 bound regulatory regions of INS1 and INS2.

ChIP-qPCR results showed that all 4 regulatory regions of the INS2 gene are highly enriched by DLX2 in the pancreatic tissues at E18.5 (Figure 3.2).

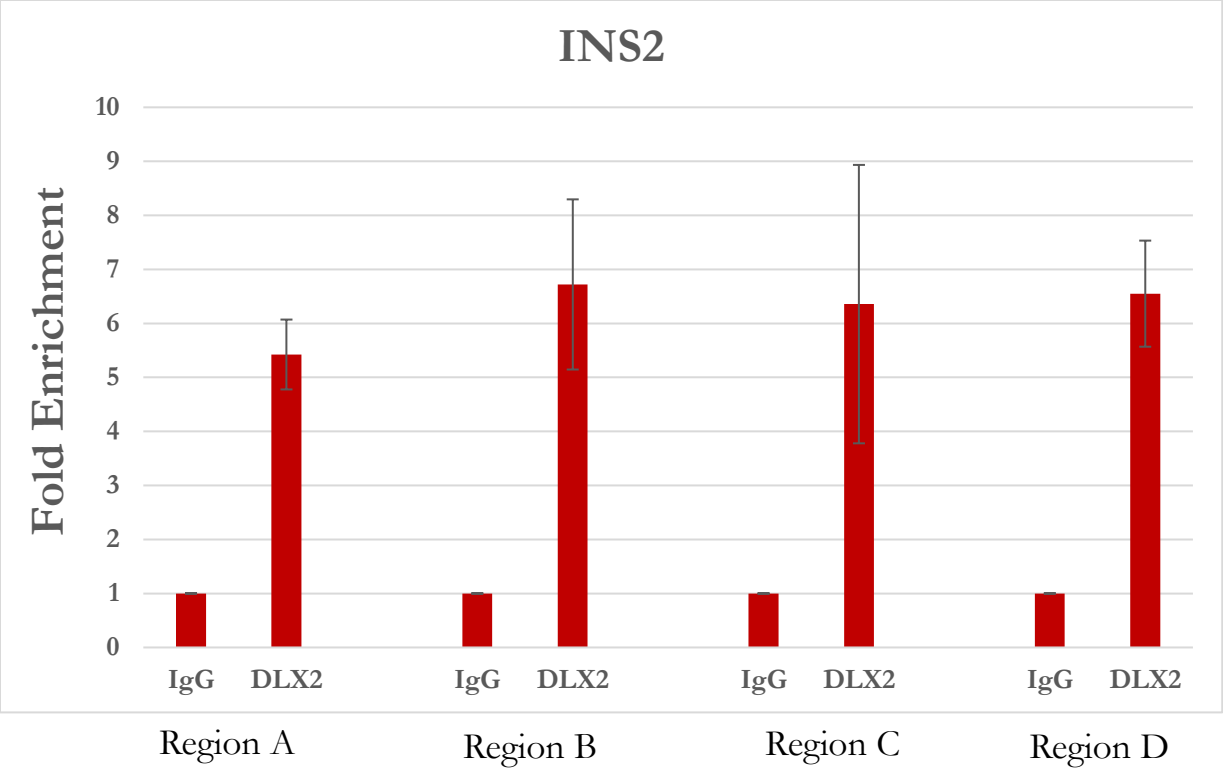


Figure 3.2 DLX2 binds to INS2 regulatory regions in mouse pancreas at embryonic day 18.5.

INS2 regions A to D were enriched by DLX2 in the presence of DLX2 polyclonal antibody relative to the IgG control. Fold enrichment represents the results relative to IgG (non-specific bindings), i.e. the signal over background. The IgG was given a value of '1'and DLX2 enrichment was a fold change of IgG sample. Error bars represent standard error of the mean. N=3

Previously, the Eisenstat lab showed that DLX2 binds to the mouse INS1 promoter at region A and region C at E18.5 pancreas using ChIP-PCR (M. Dhruve and D. Eisenstat, unpublished) (Figure 3.3). I performed ChIP-qPCR to confirm DLX2 enrichment at INS1 promoter region A and region C. In order to design improved primers for ChIP-qPCR, I divided region A into 2 separate regions (region 1 and region 2), each with one potential DLX2 binding site. ChIP-qPCR results show the enrichment of DLX2 at region 1, region 2 and region C in the pancreas tissues at E18.5 (Figure 3.3).

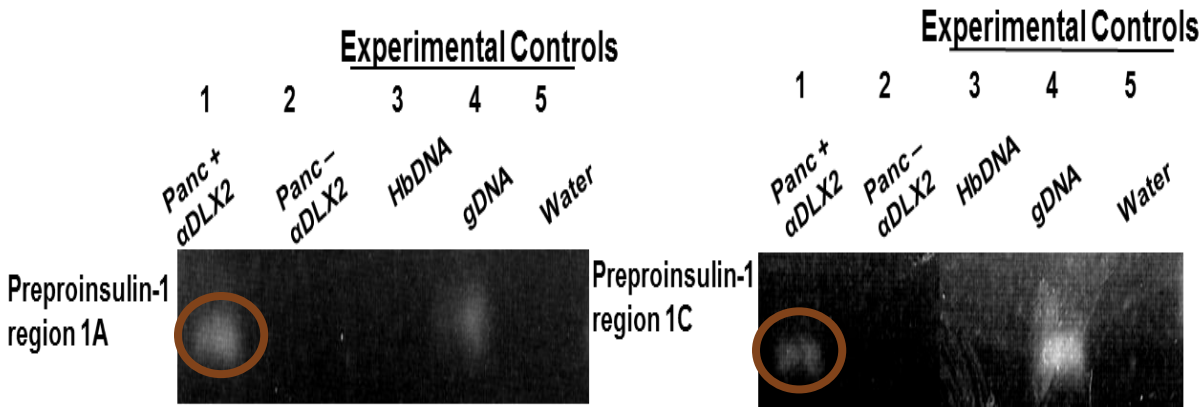


Figure 3.3 DLX2 binds to INS1 promoter region A and region C in pancreatic tissue of E18.5 mouse embryos. (M. Dhruve and D. Eisenstat, unpublished)

Panc+DLX2 = The presence of DLX2 antibody, Panc-DLX2 = The presence of DLX2 antibody, Hb = hindbrain, negative tissue control, gDNA = genomic DNA, positive control, Water = negative control

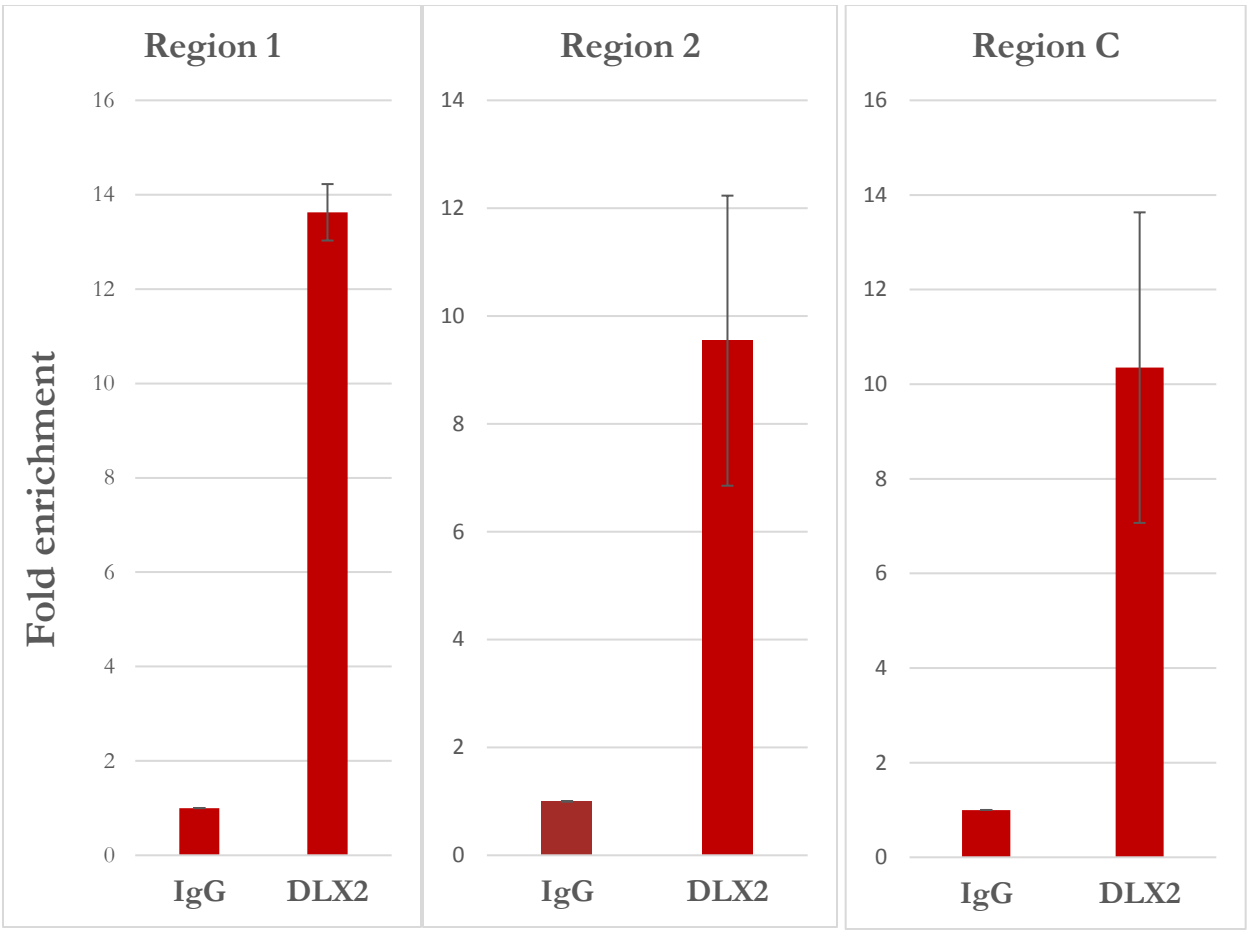


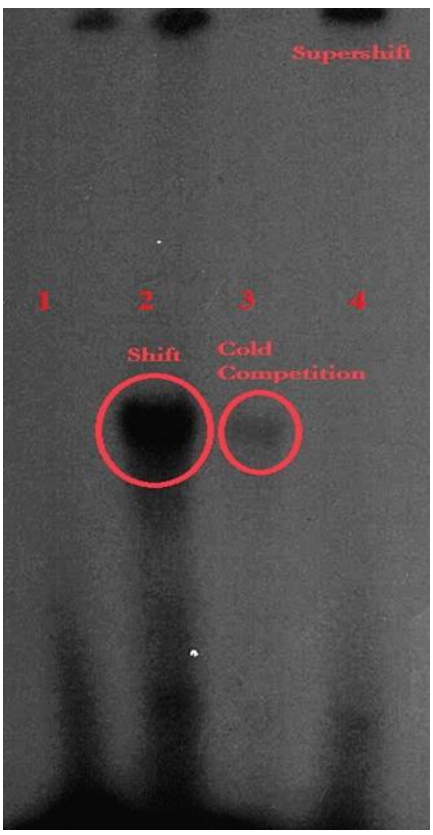
Figure 3.4 DLX2 binds to INS1 regulatory regions 1, 2 and C in mouse pancreas at embryonic day 18.5.

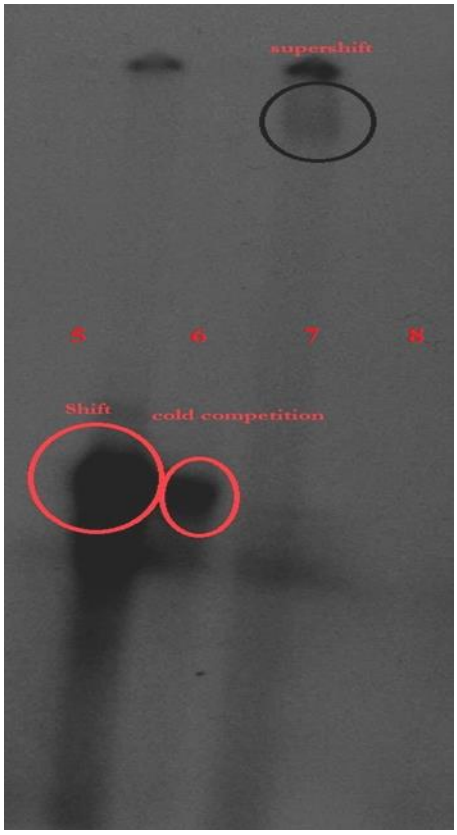
INS1 regions 1, 2 and C were enriched by DLX2 in the presence of DLX2 polyclonal antibody relative to the IgG control. Fold enrichment represents the results relative to IgG (non-specific bindings). Binding enrichment of DLX2 is shown relative to the IgG control. The IgG was normalized to '1'and DLX2 enrichment was a fold change of IgG sample. Error bars represent standard error of the mean. N=3

3.1.3 DLX2 binds directly to the INS2 regulatory region *in vitro*

ChIP assays show the enrichment of DLX2 at the specified regulatory region; however, it does not give us any information about the manner of occupancy. In order to assess the specificity of DLX2 binding to the INS2 regulatory region, EMSA assays were performed. INS2 regulatory elements were radiolabelled with [γ-32P]-dGTP, incubated with recombinant DLX2 protein and size separated on a 4% acrylamide gel. Direct binding of DLX2 to radiolabelled INS2 region B and region C resulted in shifted bands since DLX2 protein-INS2 region complexes have a higher molecular weight compared to the free labelled probes (Figure 3.5, lanes 2 & 5). Addition of unlabelled INS2 oligonucleotides competitively decreased binding of radiolabelled INS2 probes to DLX2, resulting in weaker shifted bands, confirming the specificity of DLX2 binding to the INS2 region B and region C (Figure 3.5, lanes 3 & 6). The DLX2 antibody, DLX2 protein and labelled probe complex should move slower in the acrylamide gel than the DLX2 protein-labelled probe complex alone, resulting in a supershift band (Figure 3.5, lanes 4 & 8). However, sometimes, the antibody obstructs the DNA-binding motifs of the protein, resulting in the disappearance of the supershift band (Figure 3.5, Lane 4) (Deckmann, Rorsch, Geisslinger, & Grosch, 2012). Direct binding of DLX2 was not observed for INS2 region A and region D (Figure 3.6).

INS2 Region B **INS2 Region C**



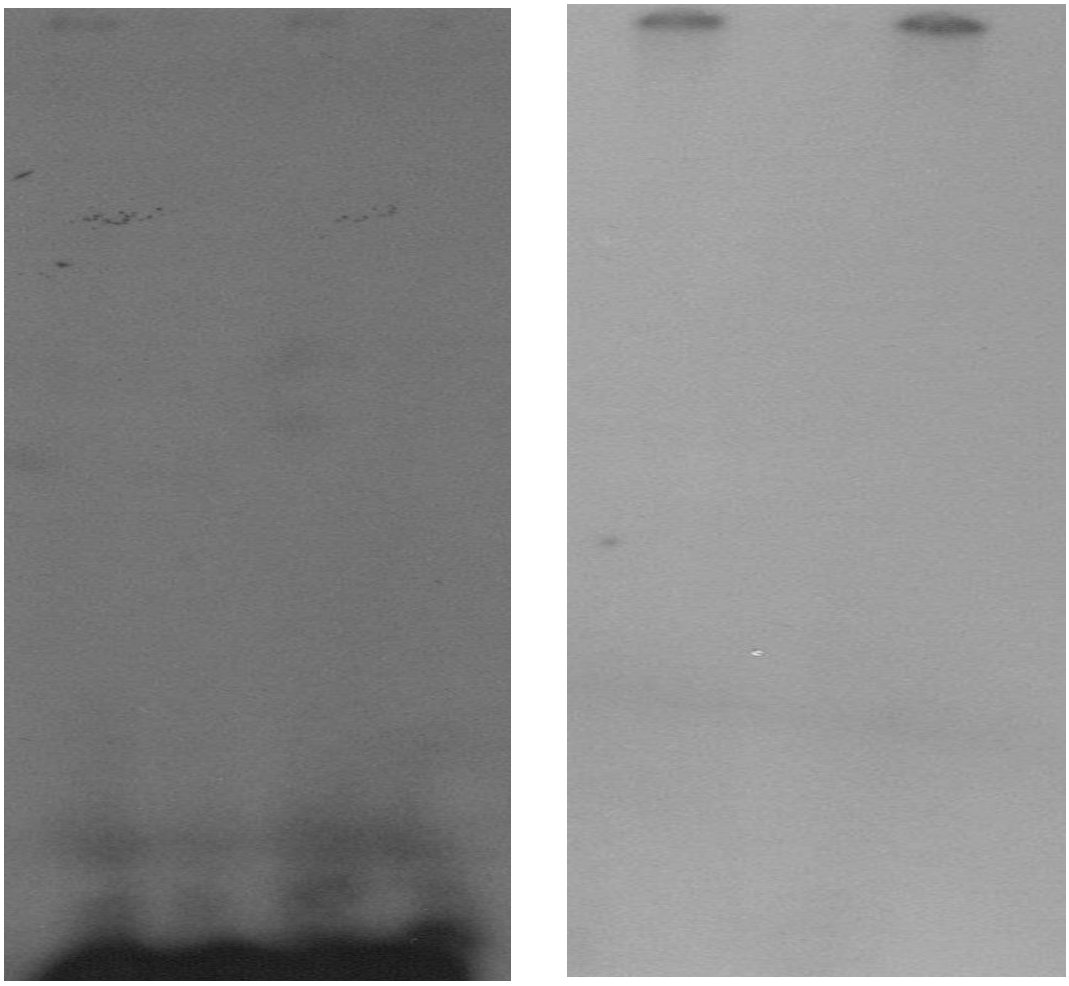


	Free Probe							
p32 (+) Probe	+	+	+	+	+	+	+	+
DLX2 Protein	-	+	+	+	+	+	+	-
DLX2 Antibody	-	-	-	+	-	-	+	-
p32 (-) Probe	-	-	+	-	-	+	-	-

Figure 3.5 direct binding of DLX2 at INS2 region B and region C.

Direct binding of DLX2 to radiolabelled INS2 region B and region C resulted in shifted bands (Lanes 2 and 5). Controls for the experiment were included: free probe where DLX2 protein was not added (Lines 1 and 8), cold competition where excess unlabelled probe was added resulting in a weaker band (Lines 3 and 6), and supershift where DLX2 antibody was added to the binding solution resulted in a supershifted band (Line 4 and 7).

INS2 Region A **INS2 Region D**



p32 (+) Probe	+	+	+	+	+	+	+	+
DLX2 Protein	-	+	+	+	+	+	+	-
DLX2 Antibody	-	-	-	+	-	-	+	-
p32 (-) Probe	-	-	+	-	-	+	-	-

Figure 3.6 Direct binding of DLX2 was not observed for INS2 region A and region D.

Controls for the experiment were included: free probe where DLX2 protein was not added (Lines 1 and 8), cold competition where excess unlabelled probe was added (Lines 3 and 6), and supershift where DLX2 antibody was added to the binding solution (Lines 4 and 7).

3.1.4 DLX2 activates the expression of INS2 gene *in vitro*

Binding of DLX2 to regulatory regions of INS2 does not necessarily mean there is a functional consequence of DLX2 binding to the INS2 promoter. In order to look at possible functional consequences *in vitro*, luciferase reporter gene assays were performed. I sub-cloned INS2 ChIP-positive regulatory regions up-stream of a luciferase gene in a PGL3 vector. PGL3- INS2 promoter regions and pCDNA3-DLX2 expressing vector were co-transfected into HEK 293 cells. Co-transfection of Dlx2 expression plasmids with PGL3- INS2 promoter regions resulted in a significant increase in luciferase activity relative to co-transfection with pGL3- INS2 promoter regions and empty pCDNA3 vector (Figure 3.7). These results suggest that DLX2 has an activating effect on INS2 gene expression *in vitro.* The results were normalized to Renilla activity.

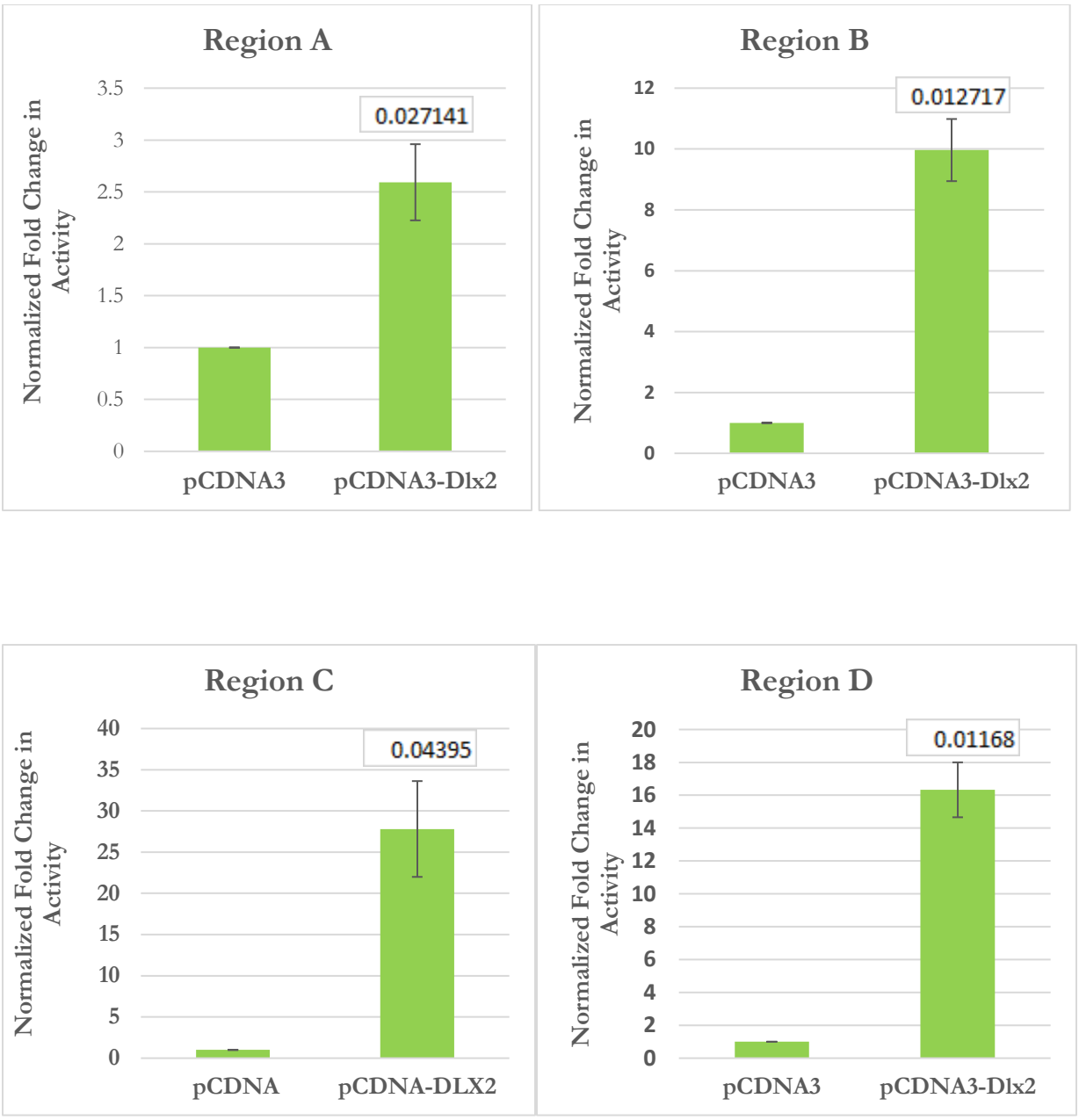


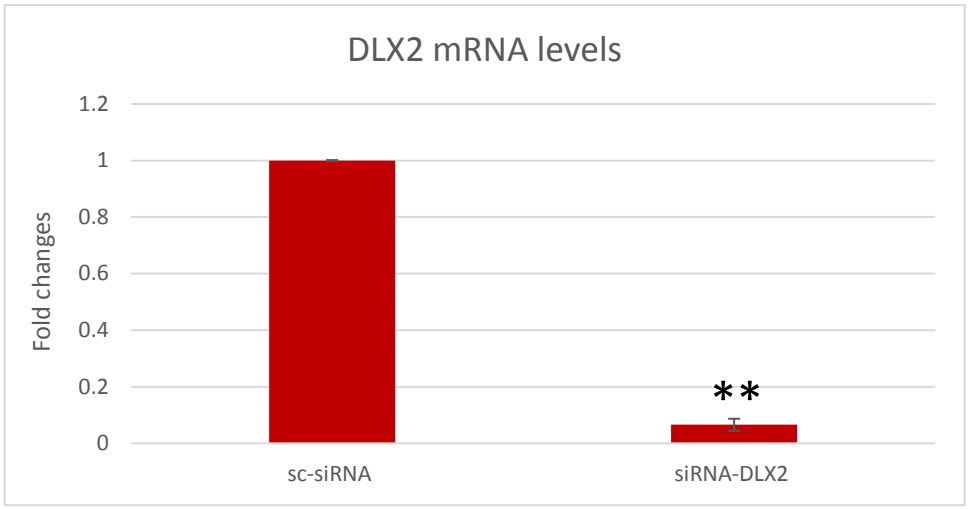
Figure 3.7 Luciferase reporter gene activity is increased upon Dlx2 expression *in vitro*.

Co-transfection of Dlx2-pcDNA3 with PGL3- INS2 regulatory regions A, B, C and D resulted in significant upregulation of luciferase expression compared to empty pCDNA3 control. Error bars shows standard error of the mean. N=3.

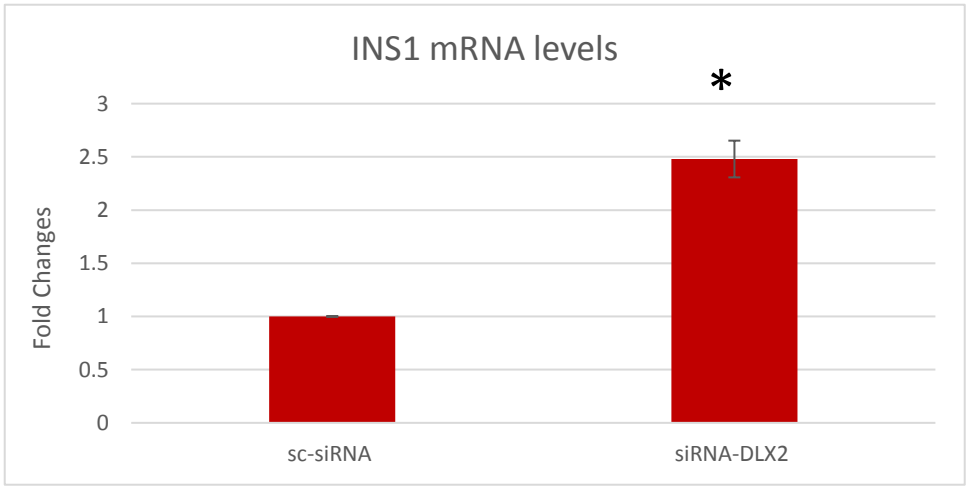
3.1.5 INS1 and INS2 mRNA levels are significantly increased in Dlx2-siRNA treated Beta-TC6 cells

siRNA knockdown experiments were performed to confirm that DLX2 activates INS1 and INS2 gene transcription *in vitro*. Dlx2-siRNA was used to knockdown DLX2 in Beta-TC6 cells (Figure 3.8A). Unexpectedly, knockdown of Dlx2 expression resulted in a significant increase in the expression levels of INS1 and INS2 in Beta-TC6 cells (Figure 3.8B). These results were in contrast with our luciferase reporter gene data, where DLX2 activated the expression of INS1 (data not shown) and INS2 genes in HEK923 cells *in vitro*.

A.


DLX2 mRNA levels
Fold changes
1.2
1
0.8
0.6
0.4
0.2
0
**
sc-siRNA
siRNA-DLX2

B.


INS1 mRNA levels
Fold Changes
3
2.5
2
1.5
1
0.5
0
*
sc-siRNA
siRNA-DLX2

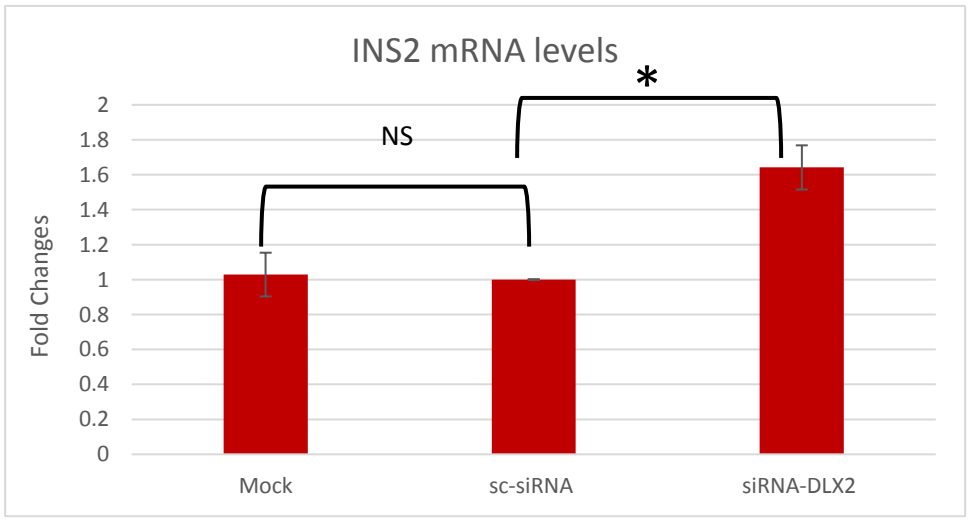

INS2 mRNA levels
Fold Changes
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NS
*
Mock
sc-siRNA
siRNA-DLX2

Figure 3.8 INS1 and INS2 mRNA levels in Dlx2-siRNA treated Beta TC6 cells

qRT-PCR showing the mRNA levels of DLX2 in Beta TC6 cells treated with Dlx2-siRNA or Silencer® Negative Control (A). INS1 and INS2 mRNA levels were significantly elevated in Dlx2-siRNA treated Beta TC6 cells (B). Error bars represent standard error of the mean.

*= P value ≤ 0.05, **= P value ≤ 0.0005. N=3

3.1.6 The mRNA level of INS1 and INS2 is reduced in the *Dlx1/2* DKO in E18.5 pancreas tissues

In order to assess the mRNA levels of INS1 and INS2 in Dlx1/2 DKO and WT E18.5 whole pancreas tissue, qRT-PCR was performed. INS1 and INS2 mRNA levels were decreased in the Dlx1/Dlx2 DKO pancreas tissue compared to WT (Figure 3.9). These results are consistent with the luciferase reporter gene assay results where we showed DLX2 has an activating effect on INS1 (Figure 1.12) and INS2 expression *in vitro.* However, these results were in contrast with the *in vitro* results where INS1 and INS2 mRNA levels significantly increased in Dlx2-siRNA treated Beta-TC 6 cells compared to the Silencer-siRNA treated cells.

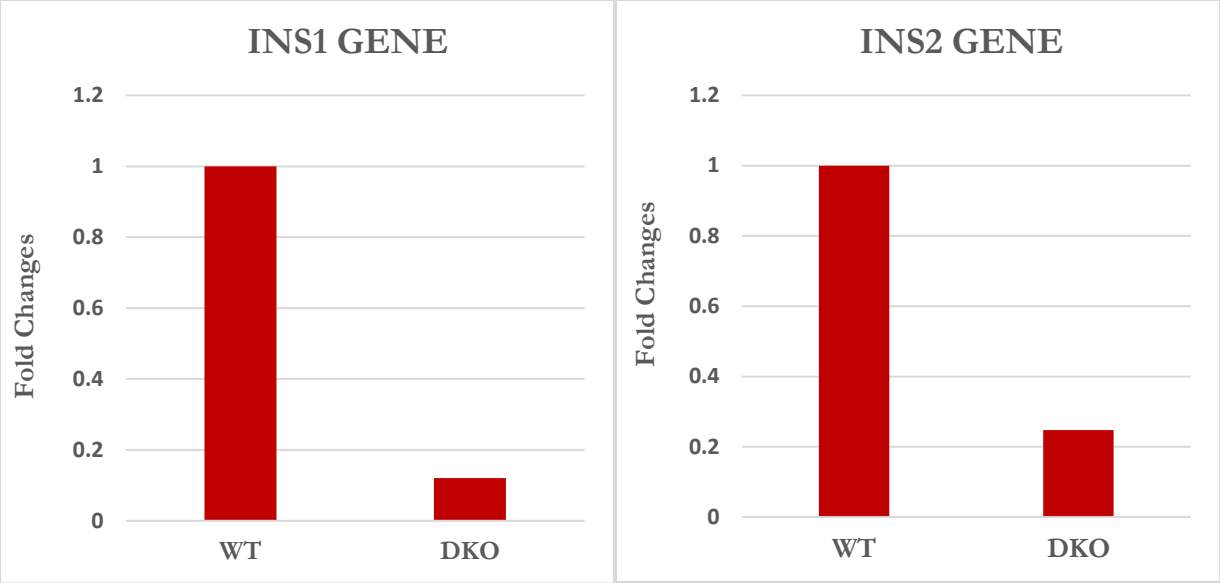


Figure 3.9 INS1 and INS2 mRNA levels of E18 Dlx1/Dlx2 DKO and WT pancreas tissue were quantified using qRT-PCR.

mRNA levels of INS1 and INS2 are reduced in the Dlx1/Dlx2 DKO in E18.5 pancreas tissues. N=2. WT: wild-type, DKO: Dlx1/Dlx2 double knockout

3.2 Proglucagon regulation by DLX2

3.2.1 The proglucagon promoter contains two potential binding sites for DLX2

The G1 element is located adjacent to the TATA box and contains two potential binding sites for DLX2 (Figure 3.10).

Figure 3.10 Schematic diagram of the proglucagon promoter showing potential DLX2 binding sites.

Two homeodomain binding sites (ATTA/TAAT) present in the proglucagon G1 promoter were assigned as potential DLX2 binding sites.

3.2.2 DLX2 binds to the proglucagon promoter in mouse pancreas at embryonic day 18.5

Previously, the Eisenstat lab showed that DLX1 and DLX2 bind to the proglucagon G1 promoter in pancreatic tissue of E18.5 mouse using ChIP-PCR (Andrew Ho and D. Eisenstat, unpublished) (Figure 3.11). I performed ChIP-qPCR to confirm DLX2 enrichment at the proglucagon G1 promoter. ChIP-qPCR results showed the binding enrichment of DLX2 at this region in the pancreas tissues at E18.5 (Figure 3.12).

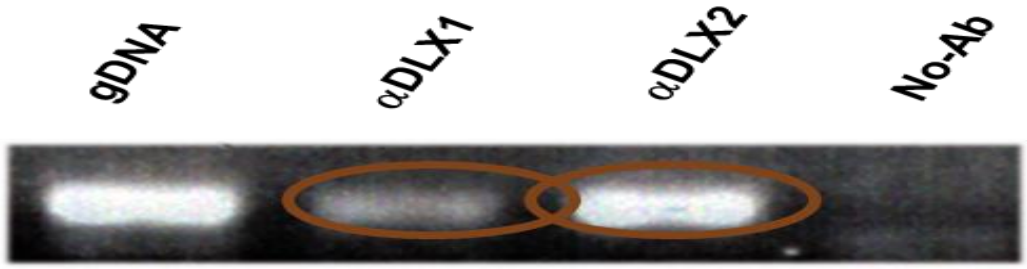


Figure 3.11 DLX1 and DLX2 bind to the proglucagon G1 promoter in pancreatic tissue of E18.5 mouse embryos (A. Ho and D. Eisenstat, unpublished)

gDNA = genomic DNA, positive control.

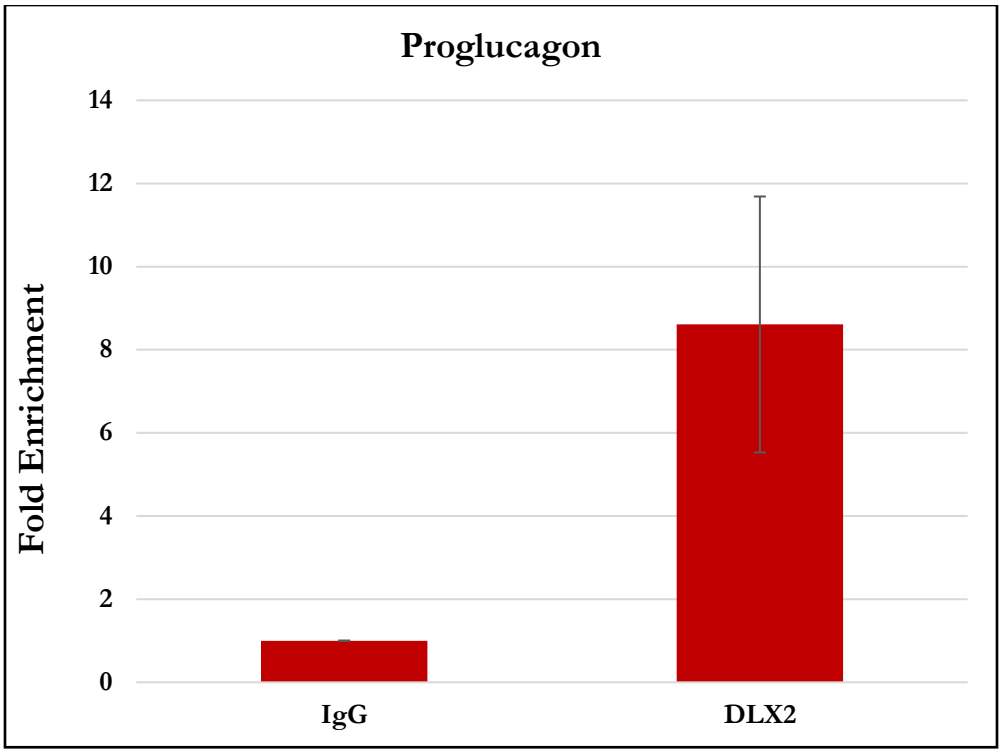


Figure 3.12 DLX2 binds to the proglucagon G1 promoter in pancreatic tissue of E18.5 mouse embryos

The proglucagon G1 promoter was enriched by DLX2 in the presence of DLX2 polyclonal antibody relative to the IgG control. Fold enrichment represents the results relative to the IgG (non-specific binding); it means the signal over background. The data was normalized such that the abundance of proglucagon promoter DNA pulled down by the control IgG was given a value of 1 and DLX2 enrichment was a fold change of the IgG sample result. N=3

Chapter 4. Discussion

A large effort is underway to identify transcription factors that not only regulate the tissue- and temporal-specific expression of insulin gene in β-cells, but also participate in the development and proper function of pancreatic islet cells. Some of these transcription factors have been identified to be associated with both monogenic and polygenic forms of diabetes (Bonnefond & Froguel, 2015). My research was aiming to determine whether DLX2 plays a role in pancreatic islet cell development by direct transcriptional regulation of insulin and glucagon gene expression and if the loss of function of Dlx1/Dlx2 results in islet-cell-specific defects.

4.1 Role of DLX2 in transcriptional regulation of INS1, INS2 and proglucagon gene expression

DLX2 is a homeodomain-containing transcription factor, known to regulate transcriptional activation or repression of target genes through binding to TAAT/ATTA motifs in their regulatory regions (Kraus & Lufkin, 2006). All the TAAT/ATTA motifs within 1kb of the transcription start sites of INS1 and INS2 were interrogated as potential binding sites for DLX2. The ChIP-qPCR assay revealed that DLX2 occupied these binding sites in the regulatory regions of INS1 and INS2. Immune enrichment for DLX2 was also seen in the promoter region of the proglucagon gene. The ChIP assay shows the enrichment of DLX2 at INS1, INS2 and proglucagon regulatory regions; however, it does not give us any information about the manner of promoter occupancy. DLX2 might bind the regulatory regions of INS1, INS2 and proglucagon either directly or indirectly through heterodimerization with other transcription factors. For instance, DLX2 might bind to TAAT/ATTA motifs in the regulatory region of INS2 through heterodimerization with another homeodomain containing transcription factor such as PDX1. In order to determine if DLX2 acts directly on the INS1, INS2 and proglucagon regulatory regions, EMSA was performed. DLX2

binds directly to TAAT/ATTA motifs at region B and region C of INS2. PDX1 is another transcription factor that binds directly to region C and activates the expression of INS2 (Arcidiacono et al., 2015). It is possible that DLX2 and PDX1 interact on the promoter of INS2 and activate its expression. PDX1 is one of the most important transcription factors in the development of pancreas, and its expression starts at the early stages of pancreas development around E8.5 and continues until after birth (Gao et al., 2008; Guz et al., 1995). PDX1 also regulates expression of the insulin gene in β-cells. Mutations in PDX1 are associated with both monogenic (MODY4) and polygenic forms of diabetes (Bonnefond & Froguel, 2015). DLX2 is co-expressed with PDX-1 at E18.5 (data not shown). Whether or how DLX2 and PDX1 interact to regulate β-cell formation and function is not yet clear. They might develop a protein-protein interaction or an indirect cooperativity such as co-binding to the same cofactor complex. It is also possible that binding of one of them to the INS2 regulatory region primes the regulatory region for the binding of the other transcription factor (Spitz & Furlong, 2012).

Islet 1 (Isl1) is also a transcription factor that is critical for pancreas development, as well as INS gene regulation. Isl1 expression starts at the primary transition and continues until after birth. This transcription factor is one of the few transcription factors that is expressed in all the pancreatic endocrine cells at the tertiary transition (Dassaye et al., 2016). Some transcription factors can specify different cell fates based on the presence of different interaction partners. The interaction of a transcription factor with a cell-type specific transcription factor can induce different cell types (Spitz & Furlong, 2012). Given that DLX2 is also expressed in all the islet cell types at E18.5 (DLX2 is co-expressed with insulin, glucagon, somatostatin and pancreatic polypeptide at E18.5), it is possible that DLX2 and Isl1 interact with each other or/and with other cell-type specific transcription factors or cofactors and play a role in formation and function all of the endocrine cell

types. Moreover, Isl1 plays a role in the activation of the INS gene by binding to A elements on its promoter (Ohneda et al., 2000). DLX2 binds to the INS gene promoter through A elements as well.

After showing that DLX2 directly binds to the regulatory region of the INS2 gene, the luciferase gene reporter assay was performed to investigate the functional effects of DLX2 on INS2 transcriptional regulation. Luciferase activity was significantly higher in the presence of DLX2 when INS2 regulatory regions were cloned upstream of the luciferase gene in HEK923 cells, which shows DLX2 activates INS2 expression when it binds to the promoter of this gene. Based on the EMSA and luciferase assays performed by Andrew Ho and Miten Dhruve, DLX2 binds directly to the promoter region of proglucagon and INS1 genes and represses or activates their expression, respectively (Figures 1.10, 1.11 & 1.12 and data not shown). Given the above-mentioned results, it is possible that DLX2 plays a role in α- and β-cell differentiation and function by being involved in the regulation of proglucagon and INS1/2 genes, respectively, during pancreas development. However, *in vivo* there are other transcription factors present in the cell and they might have higher affinity for these binding sites and binding of DLX2 can be influenced by them. Moreover, DLX2 might be expressed at different levels at different stages of development and sometimes there is a linear correlation between the concentration of transcription factors and occupancy of their binding sites. Moreover, transcription factors can have other functions beside directly regulating the levels of gene expression. For instance, the binding of a transcription factor might label a regulatory region to be used subsequently by another transcription factor. Such contributions cannot be observed in the *in vitro* assays such as EMSA and luciferase reporter experiments since the regulatory region is not situated in its normal environment (Spitz & Furlong, 2012).

In order to assess the effects of Dlx2 loss of function on INS1 and INS2 expression, Dlx2-siRNA was used to knockdown DLX2 in Beta-TC 6 cells (mouse Pancreatic Beta Cells). Surprisingly, INS1 and INS2 mRNA levels significantly increased in Dlx2-siRNA treated Beta-TC 6 cells compared to the scrambled-siRNA treated cells, which was in direct contrast with our luciferase reporter gene assay results, where DLX2 activated the expression of INS1 and INS2 genes in HEK923 cells *in vitro*. This discrepancy might be because we used two different cell types for luciferase reporter (HEK923) and DLX2-siRNA (Beta-TC6) experiments. A transcription factor might be able to both activate and repress the same gene based on the presence of cell-specific transcription factors and cofactors. It is likely that DLX2 activator and repressing effects were observed due to the availability of different sets of cofactors in those two different cell types. Even in the same cell type, a transcription factor can have both activating and repressing effects depending on the availability of cell-specific transcription factors and cofactors expressed at different time points (Spitz & Furlong, 2012).

In addition, for the luciferase reporter gene assay, the regulatory region upstream of the INS2 transcription start site was divided into 4 regions, each containing some of the regulatory elements. These regulatory elements might interact with each other or flanking sequences *in vivo* or in Beta-TC6 cells, and consequently have different effects on gene regulation compared to when the elements are separated. For instance, when I cloned a DNA region containing some of the regulatory elements up-stream of the luciferase reporter gene and overexpressed Dlx2 in HEK923 cells, the DNA configuration might have changed in a way that resulted in an activating effect on INS2 transcription. However, in Beta-TC6 cells, the regulatory region upstream of INS gene contains all of the regulatory elements, and DLX2 might interact with other transcription factors

that bind to another regulatory element on the INS2 promoter or flanking sequences, and result in changes in promoter configuration in a way that the expression of INS gene is decreased. For instance, E elements and A elements by themselves do not have any effects on transcriptional activity in β-cells; however, together they can enhance the activity of the INS promoter (Ohneda et al., 2000). Therefore, one of the future directions for this project will be sub-cloning the whole INS2 regulatory region upstream of a luciferase gene and co-transfection into Beta-TC6 cells with a pCDNA3-DLX2 expressing vector.

4.2 Role of DLX2 in pancreatic islet cell development through INS gene(s) regulation

Not all the *in vitro* results correlate with a biologically relevant phenotype. Since Dlx1/Dlx2 DKO mice die at birth, it is not yet possible to assess their phenotype postnatally and then later in life during adulthood.

What is the relative importance of DLX1 and DLX2 in the overall regulation of INS gene(s) transcription? Is the lack of DLX1 and DLX2 sufficient to make any changes in the expression levels of insulin gene(s) *in vivo*? In order to assess the overall effects of DLX1 and Dlx2 loss of function on INS1 and INS2 transcription *in vivo*, I performed qRT-PCR for INS1 and INS2 on E18.5 Dlx1/Dlx2 DKO and WT whole pancreas tissue and compared the levels of INS1 and INS2 mRNA expression between Dlx1/Dlx2 DKO and WT. The mRNA levels of both INS1 and INS2 are downregulated in Dlx1/Dlx2 DKO mice. However, I could not obtain N=3 for this experiment due to some difficulties we have been recently encountering when genotyping the Dlx1/Dlx2 DKO

mouse line. This data is in contrast with the *in vitro* data where INS1 and INS2 were up-regulated in Dlx2 knockdown in Beta-TC 6 cells.

Given the complex process of transcriptional regulation during development where time, DNA configuration and co-factors play a vital role, it is possible that DLX2 regulates INS gene(s) in β-cells at E18.5, which are immature β-cells in a different way than it does in Beta-TC 6 cells which are more similar to a mature β-cell and secrete insulin in response to glucose. Moreover, *in vivo*, β-cells are in a complex environment where endocrine islets cells (α, δ, pp and Epsilon cells) and other components such as vascular endothelial cells, neurotransmitter and islet macrophages interactions influence β-cell functions and insulin secretion (Banaei-Bouchareb et al., 2004; Caicedo, 2013; Hogan & Hull, 2017; Jain & Lammert, 2009; Rodriguez-Diaz, Menegaz, & Caicedo, 2014). This could explain in part why the *in vitro* study (Dlx2 knockdown in Beta-TC 6 cells) provided results that do not correspond with the *in vivo* (Dlx1/Dlx2 DKO mouse) results.

4.3 DLX1/DLX2, potential risk factors for diabetes

Many transcription factor and other mutations have been shown to increase the risk of type 2 diabetes. The contribution of each of these diabetic-associated mutations is small and none of these mutations alone are sufficiently penetrant to cause type 2 diabetes (Bonnefond & Froguel, 2015). Additional mutations in any gene involved in the regulation of insulin or glucagon seems to increase the risk of diabetes. In this study, we provided evidence for DLX2 regulation of INS gene(s), and showed that Dlx1/Dlx2 loss of function results in the dysregulation of INS gene transcription *in vitro* and *in vivo*. Data obtained from this study shows that disruptions to DLX1 and/or DLX2 could be considered as potential risk factors in the occurrence of diabetes.

One way to investigate this hypothesis further is to look at the DLX1/DLX2 protein and mRNA levels in the pancreas of diabetic mice to see if these two genes are dysregulated in diabetic conditions. Also, Dlx1/Dlx2 could be knocked down singly or together in diabetic or hyperglycemic mice, to assess the effects of decreased Dlx1/Dlx2 function on the severity of diabetic or hyperglycemic conditions.

Due to the death of Dlx1/Dlx2 DKO mice immediately after birth, the effects of Dlx1/Dlx2 loss of function on the overall levels of insulin, glucagon and blood glucose after birth and complete maturation of pancreatic cells are still unanswered questions. In order to overcome this limitation, a mouse line with conditional Dlx1/Dlx2 deletion in the pancreas could be generated, such as by crossing Pdx1-Cre and either Dlx2 single floxed or Dlx1/Dlx2 double floxed mice. If persistent hyperglycemia is observed in neonatal mice, it can be hypothesized that DLX1 and DLX2 are associated with neonatal diabetes. If early signs of diabetes occurred without any signs of obesity, it is possible that Dlx1/Dlx2 loss of function is associated with MODY.

Chapter 5. Conclusion and Future Directions

My data support the hypothesis that DLX2 plays a role in mouse pancreatic islet cell development by direct transcriptional regulation of INS1 and INS2 expression. Where DLX2 first takes place in the transcription factor cascade is unclear. Dlx1 and Dlx2 downregulate Notch signaling in forebrain (Yun et al., 2002). The expression of DLX1 and DLX2 starts at E13.5 (secondary transition), where inhibition of Notch signaling promotes endocrine cell fate. It is possible that DLX1 and DLX2 are negative regulators of Notch signaling during the secondary transition, with a role in inducing endocrine cell fate. Another role for DLX2 might also take place during the tertiary transition and has an effect on maintaining β-cells identity by inducing insulin expression.

What is the etiology of the Dlx1/Dlx2 deletion phenotype observed at P0 (lower level of glucagon and insulin)? Does knocking out both Dlx1 and Dlx2 affect the numbers of pancreatic exocrine cells, endocrine progenitor cells (Ngn3+ cells) or α/β cells? Do Dlx1 and/or Dlx2 play a role in endocrine cell subtype differentiation and maturation? These questions could be answered by the analysis of pancreatic cell differentiation at different time points in mice with conditional Dlx2 deletion in the pancreas using fluorescence-activated cell sorting (FACS) to sort, isolate and count pancreatic cell subtypes.

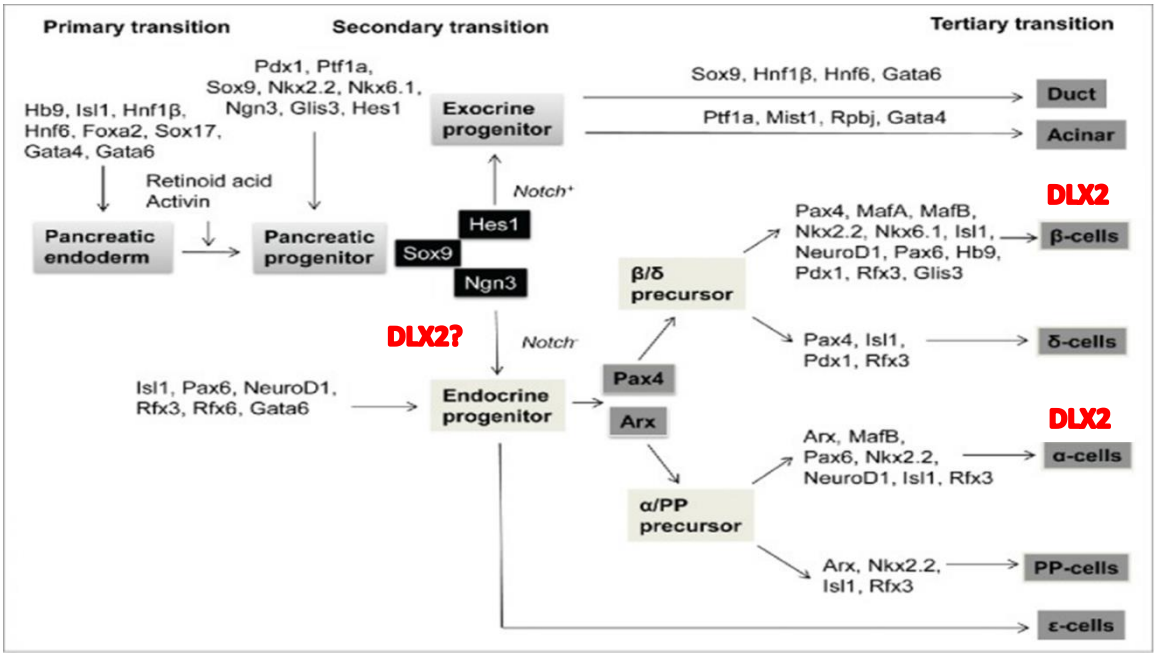


Figure 5.1. Reconsidering the role of DLX2 during the three main transition stages of pancreatic development.

The results of this study provide evidence that Dlx1/Dlx2 loss of function results in α/β cell specific defects which contribute to decreased INS1 and INS2 mRNA levels in Dlx1/Dlx2 DKO mice. However, it is not clear if the observed phenotype occurs only because of disruption of direct binding of DLX1 or DLX2 to INS gene(s). DLX2 might not only regulate INS gene(s), but also other transcription factors involved in the regulation of INS gene(s) such as PDX1 or Isl-1 (DLX2 is co-expressed with PDX1, data not shown). One of the best ways to investigate this possibility is to perform ChIP-seq with a DLX2 antibody on E18.5 pancreas tissue, to assess all the proximal and distal regulatory regions enriched by DLX2. Furthermore, performing RNA-seq on Dlx1/Dlx2 DKO and WT pancreas tissue can give us substantial data about differentially expressed genes that are potentially affected by Dlx1/Dlx2 loss of function. Analyzing RNA-seq data will help us to investigate whether the expression levels of any of the transcription factors involved in the regulation of INS gene(s) or pancreas development are effected by Dlx1/Dlx2 loss of function. The enrichment of DLX2 on the regulatory regions of the differentially expressed genes can be investigated using ChIP-seq results.

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