Role of Pax6 in pancreatic endocrine cell subtype specification

Dissertation

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Submitted by

Zeeshan Ahmad

Born in
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Dedicated to

My Father

Whose love and support has enabled me to reach this destination

Thesis Supervisor:
Prof. Dr. Ahmed Mansouri

Thesis Committee Members:
Prof. Dr. Ahmed Mansouri
Department of Molecular Cell Biology
Max Planck Institute for Biophysical Chemistry, Göttingen

Prof. Dr. Wolfgang Engel
Department of Human Genetics
Georg-August University, Göttingen

Prof. Dr. Ralf Dressel
Department of Cellular and Molecular Immunology
Georg-August University, Göttingen

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Date of oral examination: May 17, 2013

Affidavit

I hereby declare that my doctoral thesis entitled “Role of Pax6 in pancreatic endocrine cell subtype specification” has been written independently with no other sources and aids than quoted.

Zeeshan Ahmad

Göttingen, March 28, 2013

This work has been carried out at the Max Planck Institute for Biophysical Chemistry (Karl Friedrich Bonhoefer Institute) in Göttingen in the group of Prof. Dr. Ahmed Mansouri (Molecular Cell Differentiation).

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Abbreviations

BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
ChIP	Chromatin immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
dNTPs	Deoxyribonucleoside triphosphates
E	Embryonic day
FGF	Fibroblast growth factor
GFP	Green fluorescent protein
GLP	Glucagon-like peptide
GSIS	Glucose-stimulated insulin secretion
IAPP	Islet amyloid polypeptide
KD	Knockdown
KO	Knockout
LB	Luria-Bertani
MODY	Maturity onset diabetes of the young
OE	Overexpression
PBS	Phosphate buffered saline
PC	Prohormone convertase
PCR	Polymerase chain reaction
PP	Pancreatic polypeptide
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RA	Retinoic acid
RIP	Rat *insulin* II promoter
R26	*Rosa26*
SEM	Standard error of mean
Shh	Sonic hedgehog
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside
YFP	Yellow fluorescent protein

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Zeeshan Ahmad

1. Introduction

1.1 Pancreas: morphology and function

The pancreas is a lobulated gland that is located on the posterior abdominal wall behind the stomach. It is elongated in shape with a head, neck, body, and tail. The head lies towards duodenum, the body runs behind stomach, and its tail contacts the spleen. The pancreas is a gland with dual functions: an endocrine function that most importantly involves the maintenance of glucose homeostasis with the help of peptide hormones released into the blood stream and an exocrine function mediated through the release of digestive enzymes into the small intestine (duodenum), where they aid in the digestion process. Accordingly, the pancreas is comprised of an endocrine compartment and an exocrine compartment. Endocrine portion of the pancreas is arranged into globular structures named as the islets of Langerhans. Each islet is further composed of five different cell types: alpha, beta, delta, PP (pancreatic polypeptide), and epsilon that secrete peptide hormones glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin, respectively (Figure 1.1). Exocrine portion of the pancreas is comprised of acinar cells, that secrete digestive enzymes, and duct cells that form ducts to collect these secretions and empty them into the duodenum along with their own secretion comprised of bicorbonate and mucins (Cleaver and Melton, 2005; Pan and Wright, 2011).

1.2 Endocrine pancreas

Islet of Langerhan is the functional unit of the endocrine pancreas. As mentioned above, each islet is composed of five hormone producing cell types. In mouse islets, beta-cells are mainly arranged at the centre while other cell types are arranged at the periphery. Human islets, on the contrary, possess a mixed architecture. Composition of individual cell types in each islet is also more homogeneous in mouse islets compared to human islets (Brissova et al., 2005; Jeon et al., 2009). Below is a brief description of each endocrine cell type and the relative hormone produced.

1.2.1 Beta-cells

Beta-cell is the most abundant cell type in the islet, constituting 61 to 88 % of the mouse islets (Brissova et al., 2005). Beta-cells produce the peptide hormone insulin. Insulin is

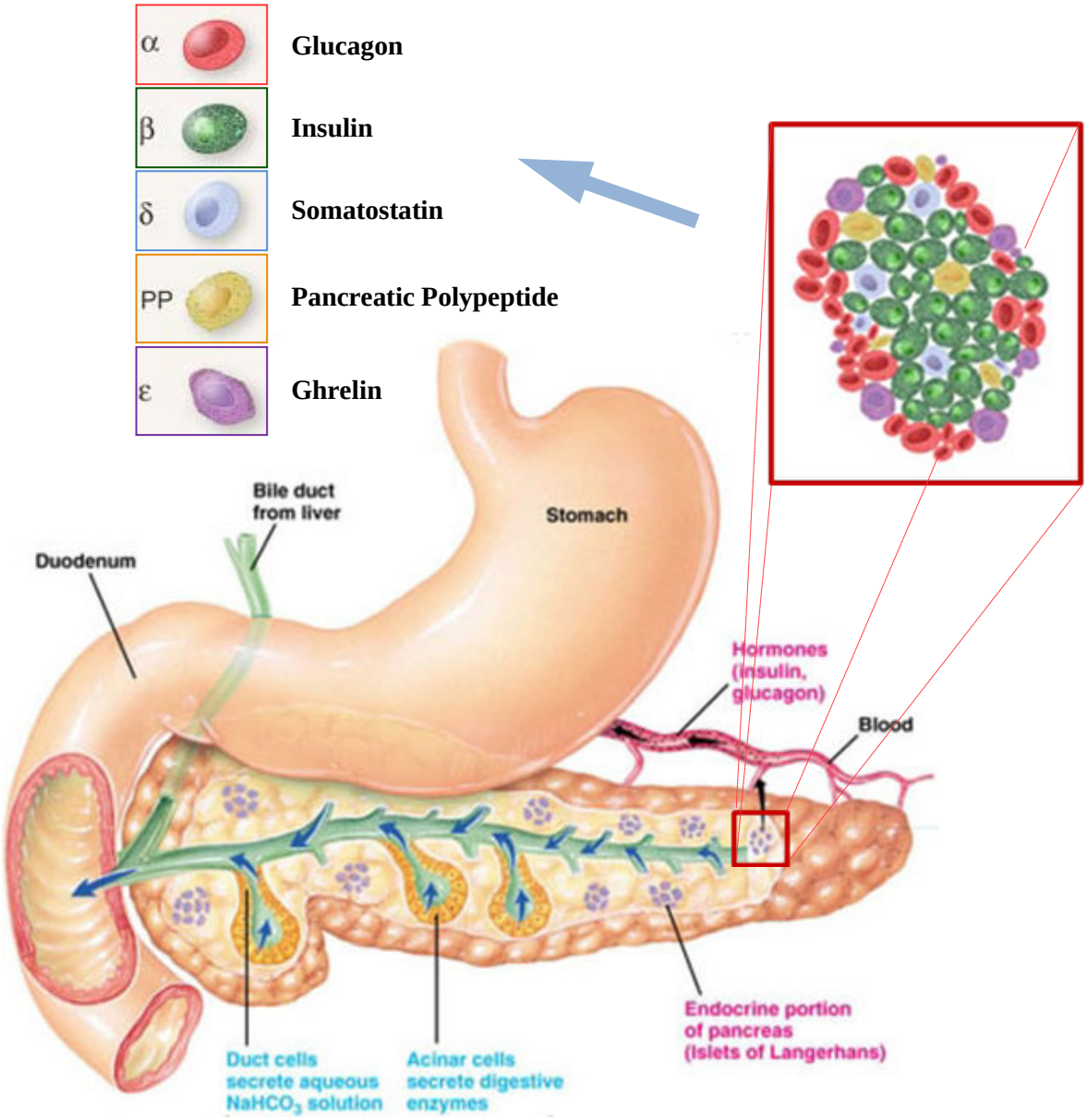

Figure 1.1: The pancreas consists of three main cell types: acinar, duct, and endocrine. Endocrine cells are clustered in the form of islets. Each islet is composed of five different hormone producing cell types. Modified from:

http://www.olivelab.org/the-pancreas-overview.html

http://www.betacell.org/content/articleview/article_id/13?aid=13

synthesized as a prohormone that consists of two chains A and B, and a middle portion called as the connecting peptide (C-peptide). This proinsulin is then cleaved by prohormone convertases (PC1/3 and 2) and carboxypeptidase H to generate the mature hormone. During this cleavage process C-peptide is released as a by-product at an equimolar ratio to insulin (Goodge and Hutton, 2000; Vasic and Walcher, 2012). Insulin is secreted from the beta-cells in response to glucose stimulation and exerts many anabolic effects in the body. It promotes glucose uptake and oxidation, stimulates glycogen synthesis, and inhibits gluconeogenesis. Additionally, it enhances the protein as well as fatty acid synthesis. Insulin action is mediated through insulin receptor (Najjar, 2003). Insulin knockout (KO) mice show growth retardation. They develop hyper-glycemia and die within first two days after birth (Duvillié et al., 1997). Similarly, insulin receptor knockout mice also develop hyperglycemia and die early after birth (Kitamura et al., 2003). In humans, *insulin* gene mutations can cause maturity-onset diabetes of the young type 10 (MODY10) (Molven et al., 2008).

1.2.2 Alpha-cells

Alpha-cells constitute the second largest population in the islet, making 9 to 31 % of the mouse islets (Brissova et al., 2005). Alpha-cells produce the hormone glucagon. Glucagon is also synthesized as a prohormone that is processed by prohormone convertase 2 (PC2) in alpha-cells to generate the mature glucagon. Proglucagon is also expressed in the intestinal L cells where it is differently processed by prohormone convertase 1/3 (PC1/3) to generate two other peptide hormones, glucagon-like peptide 1 and 2 (GLP-1 and 2), instead of glucagon (Wideman and Kieffer, 2009). Glucagon is released from alpha-cells in response to hypoglycemia and mainly acts in opposite to insulin. It promotes glycogenolysis and gluconeogenesis, while it inhibits the glycogen synthesis and glycolysis (Bansal and Wang, 2008). Glucagon action is mediated through the glucagon receptor. Glucagon receptor knockout mice exhibit hypoglycemia and hyperplasia of alpha-cells (Gelling et al., 2003). A similar phenotype is also observed in PC2 knockout mice that have defective proglucagon processing (Furuta et al., 1997).

1.2.3 Delta-Cells

Nearly 5 to 10 % of the islet is composed of delta-cells (Hauge-Evans et al., 2009). Delta-cells produce the hormone somatostatin. Biological actions of somatostatin are mainly of

inhibitory nature. Both insulin and glucagon can induce the release of somatostatin which, in turn, inhibits the release of insulin and glucagon. Somatostatin can also inhibit the exocrine secretions, and the release of ghrelin and pancreatic polypeptide (Kojima et al., 2007; Chanclón et al., 2012). In somatostatin knockout mice the development of endocrine and exocrine pancreas is completely normal. These mice, however, show increased insulin and glucagon secretion after nutrient stimulation (Hauge-Evans et al., 2009).

1.2.4 PP-cells

PP-cells constitute about 6 % of the islet cells (Adeghate and Ponery, 2003). These cells produce the hormone pancreatic polypeptide. Pancreatic polypeptide release is stimulated by food ingestion. It suppresses food intake and inhibits gastric emptying as well as the biliary and pancreatic exocrine secretion. Pancreatic polypeptide may also have a role in increasing the hepatic insulin sensitivity. As expected from its actions, pancreatic polypeptide overexpressing mice are thin and show decreased intake of food and gastric emptying (Banerjee and Onyuksel, 2012; Kojima et al., 2007).

1.2.5 Epsilon-cells

Epsilon-cells constitute a very minute population of endocrine cells in the adult islet. These cells produce the hormone ghrelin (Prado et al., 2004). However, ghrelin is also produced by some endocrine cells (P/D1 cells in humans and X/A like cells in rodents) in the stomach (Inui et al., 2004). Actually, pancreas is the major source of ghrelin during fetal life while stomach produces nearly all of the circulating ghrelin during adult life. Accordingly, the number of epsilon-cells is higher in the developing fetal pancreas and starts to decrease in the early postnatal life. In adult pancreas epsilon-cells are almost undetectable (Wierup et al., 2004; Chanoine and Wong, 2004).

Ghrelin is an orexigenic peptide that is secreted in response to fasting (Inui et al., 2004). Apart from its role in food intake regulation, ghrelin is involved in stimulating the growth hormone release from pituitary (Kojima et al., 1999). It also acts to promote cell proliferation and survival, and to reduce apoptosis (Granata et al., 2006, 2007). In the endocrine pancreas, ghrelin promotes the glucagon secretion and inhibits the secretion of insulin and somatostatin (Chanclón et al., 2012).

Role of ghrelin in the developing pancreas is so far not clear. Epsilon-cell number is tremendously increased when some important pancreatic endocrine transcription factors are knocked out. These include Nkx2.2, Pax4, and Pax6 (Prado et al., 2004; Kordowich et al., 2011). However, in ghrelin knockout mice the pancreatic development is completely intact. Recently, Arnes et al. (2012) have shown by lineage tracing that ghrelin expressing cells are not terminally differentiated cells. Instead, they represent an intermediate progenitor population that would later give rise to a subset of alpha and PP-cells.

1.3 Diabetes mellitus

Diabetes mellitus is a metabolic disease identified by hyperglycemia i.e., the high blood glucose level. It is caused by insufficient insulin production from the beta-cells and/or due to insulin resistance i.e., the inability of the peripheral tissues to correctly respond to insulin. In either case, the result is an elevated blood glucose level. Acute symptoms caused by hyperglycemia include, increase in thirst and hunger, increase in urination, and loss of weight. It can further lead to ketoacidosis that can be fatal. Chronically, hyperglycemia can impair the function of different organs. There are two main types of diabetes, type 1 and type 2. Type 1 diabetes is caused by the cell-mediated autoimmune destruction of the beta-cells that eventually leads to nearly complete loss of insulin production. This form of diabetes is treated by providing external insulin, mostly in the form of injection. On the other hand, type 2 diabetes is initially caused by insulin resistance and a partial insulin deficiency. Accordingly, the treatment involves oral hypoglycemic agents, exercise, and diet changes in the beginning. Only if the insulin deficiency becomes severe at a later stage, the insulin therapy is recommended. Gestational diabetes is a form of type 2 diabetes that results from insufficient insulin production during pregnancy. Rarely, diabetes is also caused by monogenetic disorders (also called maturity onset diabetes of the young MODY). (Buchanan and Xiang, 2005; Stumvoll et al., 2005; Daneman, 2006; Slingerland, 2006)
(http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0002194/).

1.4 Development of the pancreas

During embryonic development of vertebrates, gastrulation results in the specification of three germ layers namely ectoderm, mesoderm, and endoderm (Arnold and Robertson,

2009). The endoderm further undergoes patterning along the anteroposterior axis to generate foregut, midgut, and hindgut domains (Grapin-Botton and Melton, 2000). Two factors that play important roles at this initial step include: fibroblast growth factor 4 (FGF4) and retinoic acid (RA). FGF4 aids in posteriorising the endoderm by promoting the expression of posterior endodermal transcription factors Pdx1 and Cdx1/Cdx2 (Wells and Melton, 2000; Dessimoz et al., 2006). Retinoic acid is important for the initial anterior-posterior patterning as well as for the later pancreas development (Chen et al., 2004; Stafford et al., 2004, 2006; Bayha et al., 2009).

1.4.1 Generation of pancreatic buds

After early patterning, pancreas then develops from the foregut endoderm as two pancreatic evaginations. The dorsal pancreatic bud is first to appear as an outgrowth of the dorsal foregut at the duodenal region. This happens around embryonic day 9.5 (E9.5) in mouse and around gestational day 26 (G26d) in humans. Half a day later in mouse (E10) and six days later in humans (G32d) the ventral pancreatic bud appears as an evagination of the ventral foregut close to the hepatic/biliary region (Gittes, 2009). Dorsal and ventral pancreas development proceeds through different pathways. Dorsal pancreas first receives signals from the notochord, then from the dorsal aorta, and lastly from the pancreatic mesenchyme. Ventral pancreas receives signals from the cardiac mesoderm, lateral plate mesoderm, and the septum transversum mesenchyme (Pan and Wright, 2011).

1.4.1.1 Genesis of dorsal pancreas

In the beginning, prepancreatic dorsal endoderm is in contact with the notochord and receives permissive signals in the form of activin-betaB and FGF2 that may suppress *Sonic hedgehog* (*Shh*) in the endoderm to allow dorsal bud formation (Hebrok et al., 1998). Around E8 then the fusion of dorsal aortas moves the notochord away from the dorsal endoderm. At this time, the aortic endothelial cells may help to induce Pdx1 and Ptf1a expression in the dorsal endoderm. Furthermore, they also promote the survival of dorsal pancreatic mesenchyme. Subsequently, the pancreatic mesenchyme grows and envelops the epithelium. Further pancreatic development then depends on the signals from the mesenchyme. Pancreatic epithelium cultured in the absence of mesenchyme does not develop (Golosow and Grobstein, 1962). Later, it was found that the

mesenchyme is mainly required for the acinar formation while islet formation is the default pathway in the absence of mesenchyme (Gittes et al., 1996). Detailed study further showed that mesenchymal-epithelial contact induces notch-signalling mediated *hes1* expression in the epithelium that would repress *Ngn3* and promote acinar differentiation (Duvillie et al., 2006). Additionally, the FGF signalling from mesenchyme to epithelium promotes epithelial proliferation (Miralles et al., 1999; Elghazi et al., 2002).

1.4.1.2 Genesis of ventral pancreas

Ventral pancreas and liver seem to originate from a common progenitor population located on the ventral side of foregut endoderm (Deutsch et al., 2001). Cardiac mesoderm induces hepatic differentiation from ventral endoderm via FGF signaling (Gualdi et al., 1996; Jung et al., 1999). In the absence of this signal the pancreatic fate is followed that seems to be the "default pathway" for the ventral endoderm (Deutsch et al., 2001). Bone morphogenetic protein (BMP) signals from the septum transversum mesenchyme are also involved in inducing the liver fate at the expense of ventral pancreas (Rossi et al., 2001). Therefore, in order to form the ventral pancreas, a part of the ventral endoderm must escape the hepatogenic signals from the mesoderm. This escape is achieved by movement of a part of the endoderm away from the cardiac mesoderm. It has been shown that this tissue positioning is achieved via *Hex* gene that controls the proliferation rate and, therefore, the proper positioning of the leading edge of endoderm (Bort et al., 2004).

1.4.2 Bud to gland formation

The early dorsal and ventral pancreatic buds (at E10 in mouse) predominantly consist of multipotent pancreatic progenitor cells (MPC) interspersed with a couple of early endocrine cells, including mainly glucagon$^+$ cells. Some insulin-glucagon co-positive cells as well as ghrelin$^+$ cells are also evident at this time point. However, these early endocrine cells do not give rise to the mature endocrine cells and their function is not yet defined. Once generated, the dorsal and ventral bud epithelium proliferates and grows, this leads to elongation of the stalk region and branching at the bud tip. As the development continues, the gut tube rotates and brings the dorsal and ventral buds into close proximity. This leads to their fusion (around E12.5 in mouse and G6w in humans), generating a single definitive pancreas. By this time, along with the morphogenetic development, the pancreatic epithelium also undergoes a clear "tip-trunk" segregation.

The tip region contains multipotential pancreatic cells (MPC), that would later give rise to the progenitors with an acinar fate, and the trunk region contains duct/endocrine bipotential progenitors (Figure 1.2) (Pan and Wright, 2011). It has been suggested that the final size of the adult mouse pancreas is determined by the number of the progenitor cells assigned to the developing pancreas between E9.5 to E12.5 (Stanger et al., 2007).

Around E13.5 in mouse, there is a sudden outburst of growth in the developing pancreas termed as the "secondary transition". During this phase, there is a rapid differentiation and proliferation of acinar cells arising from the tip regions. At the same time, endocrine cells originate from the bipotential progenitors in the trunk regions. Alpha- and beta-cells are first to appear followed by the delta-cells a day later. Finally, PP-cells appear around E18. As the endocrine cells develop, they leave the trunk epithelium and start to cluster into islets (Figure 1.2). In the mouse, further arrangement and maturation of islets continues from late gestation to the first few weeks after birth. Beta-cells form the core of the islets in mouse, while other types of endocrine cells are arranged at the periphery. On the contrary, in mature human islets, alpha- and beta-cells are inter-mingled. Following the secondary transition, pancreatic epithelium continues to expand mainly via proliferation of acinar cells. Expansion of islet cell mass after birth is also predominantly achieved through self-duplication. However, once the mature islet cell mass is achieved the ability of endocrine cells (e.g., beta-cells) to divide is strongly reduced (Collombat et al., 2006; Pan and Wright, 2011).

1.5 Role of transcription factors in developing and adult endocrine pancreas

Gene knockout, overexpression, and misexpression studies in mice have generated a wealth of information on the role of many different transcription factors in the development and function of endocrine pancreas. In the following section, some of the important transcription factors are reviewed. The expression pattern of each transcription factor in the developing and adult pancreas is described, and the published knockout and/or overexpression phenotype is mentioned. In view of the thesis topic, the role of Pax6 is discussed in a separate section in greater detail.

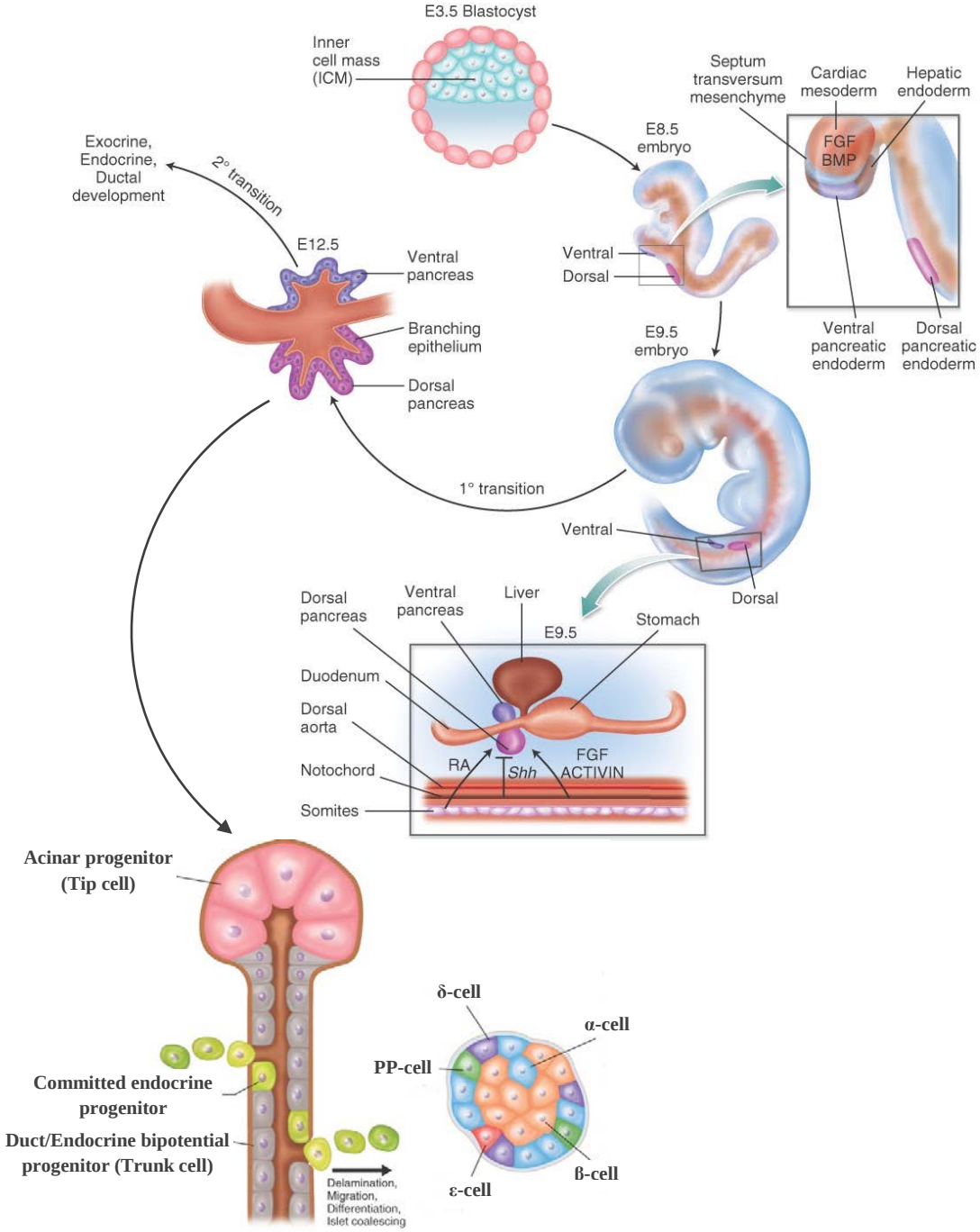

Figure 1.2: Early development of pancreas. Induction of dorsal and ventral bud occurs through different pathways. After the bud epithelium undergoes branching morphogenesis, the tip-trunk segregation becomes apparent. Tip gives rise to acinar cells and trunk contains bipotential progenitors for ductal and endocrine cells. Once a cell becomes committed to endocrine lineage, it leaves the ductal epithelium, undergoes further differentiation, and clusters with other endocrine cells to form an islet (modified from Mastracci and Sussel, 2012).

1.5.1 *Hlxb9*

Hlxb9 expression is detectable in the developing mouse embryo around E8. At this stage, it is expressed in the whole endoderm destined to become pancreas as well as in the notochord. Around E9.5, the *Hlxb9* expression forms a gradient with higher expression to the dorsal side and lower to the ventral side and the expression generally declines towards E12.5 (Sherwood et al., 2009). Later on, during the secondary transition, strong expression comes back and is limited to the $Pax6^+$ endocrine precursors. Finally, *Hlxb9* expression becomes restricted to the beta-cells in the adult pancreas (Harrison et al., 1999; Li et al., 1999). Apart from the pancreas, *Hlxb9* expression is also detected in the developing esophagus, stomach, and small intestine (Li et al., 1999).

In *Hlxb9* knockout mice, dorsal pancreatic bud shows complete agenesis, while the ventral bud development is apparently normal. However, the beta-cells originated from the ventral pancreas are not fully mature as they lack the expression of Glut2, the glucose transporter in beta-cells that plays an important role in the glucose-stimulated insulin secretion (GSIS). Furthermore, *Hlxb9* knockout mice died at birth due to failure of the respiratory system (Thaler et al., 1999). On the other hand, over-expression of *Hlxb9* in the entire *Pdx1* domain disturbs the development of both exocrine and endocrine pancreas, and instead induces an intestinal-like differentiation program in the pancreatic anlage. These *Hlxb9* overexpressing mice also die shortly after birth (Li and Edlund, 2001).

1.5.2 *Pdx1*

Pdx1 expression is first evident at E8.5 in the entire pancreatic-fated region of the endoderm. Around E10 to E11.5, its expression extends to the common bile duct, duodenum, and distal stomach (Jonsson et al., 1994, 1995; Offield et al., 1996). Moreover, lineage-tracing in the pancreas has confirmed that the duct, acinar, and endocrine cells are all generated from the $Pdx1^+$ progenitors (Gu et al., 2002). *Pdx1* expression undergoes dynamic changes during pancreas development. Initially, it is expressed in the entire epithelium at high levels. Then its expression is reduced as the cells are specified to the endocrine, duct, and acinar lineages (Jensen et al., 2000b; Gu et al., 2002; Hale et al., 2005). Subsequently, high level of *Pdx1* expression is re-induced in the beta-cells and is required for their maturity and proper function (McFarlane et al.,

1994; Marshak et al., 1996). A low level of *Pdx1* expression is maintained in the acinar, ductal, and other types of endocrine cells (Guz et al., 1995; Wu et al., 1997).

In *Pdx1* knockout mice, initial budding of the dorsal pancreatic bud occurs but fails to develop further. Additionally, unlike *Hlxb9* mutant mice there is no ventral pancreas development either (Jonsson et al., 1995; Offield et al., 1996). Moreover, it was found that the dorsal pancreatic rudiment in these mutants does contain the first-wave endocrine cells that fail to expand further. These knockout mice died in the first few days after birth due to defects in the pancreatic as well as other gastro-duodenal areas (Larsson et al., 1996; Offield et al., 1996). *Pdx1* expression is critical not only in the beginning but also in the subsequent phases of pancreatic development as shown by the later studies where *Pdx1* was depleted after the initial budding had occurred (Holland et al., 2002; Hale et al., 2005).

As mentioned before, unlike other endocrine cells, *Pdx1* is highly expressed in the mature beta-cells. To uncover the role of Pdx1 in mature beta-cells, Ahlgren et al., (1998) generated knockout of *Pdx1* in beta-cells alone. These mice were initially healthy but developed an overt-diabetic phenotype with age (at 3-5 months), showing the importance of Pdx1 in maintaining the functional identity of beta cells. Consistent with the mouse studies, *PDX1* homozygous null mutation causes pancreatic agenesis (Stoffers et al., 1997a), while a heterozygous mutation causes diabetes (MODY4) in humans (Stoffers et al., 1997b).

To further delineate the role of Pdx1 in pancreatic development, an opposite approach was applied by over-expressing the *Pdx1* in the whole pancreatic anlage (Using *Ptf1a* regulatory domain). This led to the pancreatic hypoplasia with severe defect in the acinar cells. Acinar cells adopted a duct like phenotype and their apoptosis was also increased (Miyatsuka et al., 2006). On the other hand, overexpression of *Pdx1* in the endocrine progenitors (using *Ngn3* regulatory domain) led to the reprogramming of alpha- to beta-cells (Yang et al., 2011).

1.5.3 *Pbx1*

At E10.5, *Pbx1* expression is detected in the pancreatic epithelium and surrounding mesenchyme. Later in development, the expression is maintained in the mesenchyme and reduced in the epithelium. Finally, in the adult pancreas, *Pbx1* is expressed in the acinar, ductal, and endocrine cells. *Pbx1* knockout mice have pancreatic hypoplasia with a severe

defect in both endocrine and exocrine cell differentiation. They die before birth during embryonic development (around E15 to E16). Pbx1 heterozygous mutants are initially healthy, however, they develop glucose intolerance and hypoinsulinemia with age. Furthermore, when *Pbx1* heterozygous mutation is combined with a heterozygous *Pdx1* mutation the resultant phenotype is much more drastic as the mice develop age-related overt diabetes mellitus (Kim et al., 2002). Indeed, Pbx1 and Pdx1 have been shown to interact with each other (Dutta et al., 2001; Swift et al., 1998). In acinar cells, the elastase I expression is activated by a regulatory complex in which Pdx1 interacts with Pbx1 (Liu et al., 2001).

1.5.4 *Ptf1a*

Ptf1a is a member of the heterotrimeric Pancreas Transcription Factor 1 complex (PTF1). This complex comprises of three proteins: Ptf1a (or p48), E2A (or p75), and RBP-J/RBP-JL (Rose et al., 1994; Krapp et al., 1996). *Ptf1a* expression starts around E9.5 in both the dorsal and ventral pancreatic foregut endoderm (Krapp et al., 1998). As the development proceeds, expression is reduced in the endocrine lineage and is maintained in the developing and mature acinar cells of the pancreas. As confirmed by the lineage-tracing in mice, pancreatic progenitors expressing *Ptf1a* in the initial stage of development give rise to acinar cells, nearly all of the ductal, and most of the endocrine cells (Kawaguchi et al., 2002). In *Ptf1a* knockout mice, pancreatic agenesis is evident with almost no ventral bud and a very rudimentary dorsal bud that does not develop beyond the initial outgrowth. These mice die within first few hours after birth. As expected, acinar cells do not develop in the *Ptf1a* knockouts, however, a reduced number of endocrine cells do develop including a significant number of relatively mature beta-cells (Krapp et al., 1998; Burlison et al., 2008). Therefore, it appears that Ptf1a is not required for the endocrine cell specification but is important for the initial allocation and amplification of pancreatic progenitors that would later give rise to the endocrine population. This idea is supported by the fact that *Ptf1a* misexpression together with *Pdx1* can convert duodenum into pancreas with an intact acinar to endocrine cell ratio (Afelik et al., 2006). In accordance to the mouse studies, *PTF1a* null mutation causes permanent neonatal diabetes mellitus in humans due to pancreatic agenesis (Sellick et al., 2004).

1.5.5 *Isl1*

Isl1 expression is first detected in the dorsal pancreatic epithelium and the surrounding mesenchyme around E9 to E9.5. Later, it is expressed in the endocrine cells during development as well as in the adult pancreas. *Isl1* knockout mice do not develop dorsal pancreatic mesenchyme and dorsal pancreatic bud. As they die at E9.5 before the ventral bud emergence, it is not possible to investigate the effect on ventral bud development (Ahlgren et al., 1997). To analyze the role of Isl1 in the later stages of development, Du et al. (2009) generated pancreas-specific *Isl1* knockout mice. These mice are born alive but develop hyperglycemia from early postnatal life onwards that finally leads to their death between 3 to 8 weeks of age. In the mutant pancreata, the number of endocrine cells is significantly declined due to reduced proliferation and increased apoptosis. Moreover, the beta-cells present in the mutant pancreata are not fully mature as they lack the expression of *MafA*. Therefore, Isl1 is initially required for the maintenance of pancreatic mesenchyme and subsequently for the maturation and expansion of endocrine cells.

1.5.6 *Sox9*

Sox9 is expressed around E10.5 in the early pancreatic progenitors. Lineage-tracing experiments have confirmed that these *Sox9* expressing progenitors give rise to the acinar, ductal, and endocrine cells of the pancreas (Akiyama et al., 2005). In the course of secondary transition, *Sox9* expression is confined to the duct/endocrine bipotential progenitors in the developing epithelium. Finally, *Sox9* expression is then restricted to the ductal epithelium of the adult pancreas (Lynn et al., 2007). Pancreas-specific *Sox9* knockout mice show hypoplasia of dorsal as well as ventral pancreatic bud and die within first few days after birth. Moreover, as a cause behind this phenotype, it was found that Sox9 is required for the maintenance of pancreatic progenitors by restricting their premature differentiation, as well as by promoting their survival through increased proliferation and decreased apoptosis (Seymour et al., 2007). In addition to this, Sox9 has been found to regulate the expression of *Ngn3*, and therefore, may play an important role in initiating the endocrine differentiation program (Lynn et al., 2007).

1.5.7 *Ngn3*

Ngn3 is considered as a master regulator in the pancreatic endocrine differentiation program. *Ngn3* expression is first detected in the early endocrine progenitors around E9.5. Later, the expression increases as the endocrine progenitors expand, and peaks around E15.5. From there on, it decreases and finally becomes nearly undetectable in the late embryonic and adult pancreas (Gradwohl et al., 2000; Schwitzgebel et al., 2000). In fact the expression of *Ngn3* in the endocrine progenitors is transient and occurs only in the undifferentiated progenitors. Ngn3 directly activates many of the endocrine specific transcription factors, while it represses its own promoter activity (Huang et al., 2000; Smith et al., 2003, 2004, 2010; Mellitzer et al., 2006). Once the endocrine differentiation program has been initiated in a cell, the expression of *Ngn3* is then repressed (Jensen et al., 2000; Smith et al., 2004). Accordingly, the *Ngn3* expression is biphasic and corresponds to the two phases of endocrine cell generation (primary and secondary). As more endocrine cells are generated in the secondary phase, the *Ngn3* expression also peaks around this time (Villasenor et al., 2008).

Ngn3 knockout mice do not develop any of the endocrine cell types and die within first few days after birth (Gradwohl et al., 2000). Lineage-tracing analysis also confirms the generation of all the endocrine cell types from the *Ngn3* expressing progenitors (Gu et al., 2002). On the other hand, overexpression of *Ngn3* under *Pdx1* domain directs the entire progenitor pool to differentiate into endocrine tissue that consists of predominantly alpha-cells (Apelqvist et al., 1999). Later on, Johansson et al. (2007) found that the ability of Ngn3 to induce various endocrine cell types is associated with the specific developmental time-point. They used *Ngn3*-addback strategy to induce *Ngn3* expression at various time points of pancreatic development in mice that are *Ngn3* deficient. It was found that *Ngn3* activation around E9 generates alpha-cells alone, activation around E11.5 generates alpha, beta, and gamma-cells, and activation around E14.5 generates all four types of endocrine cells. Apart from the timing, the level of *Ngn3* expression is also very important. Endocrine progenitors with a high *Ngn3* expression are truly committed to the endocrine fate, while the progenitors with low *Ngn3* expression can opt for other pancreatic fates as well (Wang et al., 2010). Therefore, progenitors with low *Ngn3* expression may represent an intermediate state where the cell has still a choice to move in either direction.

1.5.8 *Rfx6*

Rfx6 is broadly expressed in the early gut endoderm. At E9, its expression is detected in the dorsal pancreatic bud. Around this time, many Rfx6 positive cells co-express *Pdx1*. Later around E10-12.5, *Rfx6* expression gets more restricted to the differentiating endocrine cells and is excluded from *Pdx1* or *Ptf1a* expressing cells. During the secondary transition, many of the Rfx6 positive cells co-express *Ngn3*. Furthermore, in *Ngn3*$^{-/-}$ mice the expression of *Rfx6* is lost indicating that Rfx6 is Ngn3-dependent transcription factor in the pancreas. Finally, in the adult pancreas, *Rfx6* is expressed only in the endocrine cells of the islet. Rfx6 plays important role in the endocrine pancreas development as in the *Rfx6* knockout mice, pancreatic endocrine cells do not develop except for the PP producing cells (Smith et al., 2010; Soyer et al., 2010).

1.5.9 *NeuroD*

NeuroD expression is first detectable in the endocrine committed cells of the early pancreas around E9.0 (Huang et al., 2000). Later in development, it continues to express in most of the endocrine cell population. Finally, as the islets mature in the adult pancreas, its expression becomes restricted to only beta-cells (Itkin-Ansari et al., 2005). *NeuroD* is one of the direct downstream targets of Ngn3 (Gradwohl et al., 2000; Huang et al., 2000; Jensen et al., 2000) and an important mediator in the induction of endocrine differentiation program (Gasa et al., 2008). This is supported by the fact that both Ngn3 and NeuroD can activate many of the same target genes (Gasa et al., 2008).

In contrast to *Ngn3* knockout mice, *NeuroD* knockout mice can develop all types of endocrine cells. However, the number of endocrine cells is significantly reduced as they fail to expand after initial differentiation. Therefore, *NeuroD* knockout mice also develop hyperglycemia and die within first few days after birth (Naya et al., 1997). To understand the role of NeuroD in beta-cells, Gu et al., (2011) generated beta-cell-specific *NeuroD* knockout mice. These mice are slightly hyperglycemic and develop severe glucose intolerance due to immature beta-cell function. In humans, *NEUROD1* heterozygous mutations are associated with maturity onset diabetes of the young type 6 (MODY6), and homozygous null mutations can cause permanent neonatal diabetes (Malecki et al., 1999; Rubio-Cabezas et al., 2010).

1.5.10 *Nkx2.2*

Nkx2.2 expression begins in the early pancreatic epithelium around E9.0. Initially, it is expressed in most of the epithelial cells and later becomes gradually confined to the endocrine progenitors. *Nkx2.2* expression then persists in beta-cells, and in a subset of alpha- and PP-cells. *Nkx2.2* is not expressed in either developing or mature delta-cells (Sussel et al., 1998; Jørgensen et al., 2007). Accordingly, in *Nkx2.2* knockout mice, beta-cells are lost, alpha- and PP-cells are reduced in number, and delta-cells are not changed. These mice develop hyperglycemia and die shortly after birth (Sussel et al., 1998). Later, it was found that the lost endocrine cells, in *Nkx2.2* mutant mice, are replaced by epsilon-cells expressing ghrelin (Prado et al., 2004). *Nkx2.2* inactivation in pancreas alone produces a similar phenotype (Mastracci et al., 2013). A compound deficiency of *Nkx2.2* and *Arx* leads to upregulation of ghrelin^{+} cells that co-express somatostatin (Kordowich et al., 2011; Mastracci et al., 2011). Nkx2.2 can function both as an activator and a repressor of transcription in the endocrine pancreas depending on the specific cell type or the time-point of development (Raum et al., 2006; Doyle et al., 2007; Papizan et al., 2011). For example, Nkx2.2 can activate *MafA* expression that is required for the beta-cell maturity (Raum et al., 2006) and represses *Arx* expression in beta-cells to maintain their identity (Papizan et al., 2011). On the other hand, Nkx2.2 repressor function is also required for alpha-cell differentiation (Doyle et al., 2007). It has been suggested that Nkx2.2 repressor activity is mainly required during embryonic development of the pancreas, while activator function is more important in mature beta-cells (Doyle et al., 2007; Doyle and Sussel, 2007).

1.5.11 *Nkx6.1*

Nkx6.1 expression is first detected in few cells of the early pancreatic epithelium around E9. Around E10.5, the expression is detected in most of the pancreatic epithelium. Later, the expression becomes restricted to endocrine progenitors and then to beta-cells alone (Sander et al., 2000; Jørgensen et al., 2007). In mature islets, *Nkx6.1* is expressed only in the beta-cells. In *Nkx6.1* knockout mice, beta-cells are significantly reduced in number but other endocrine cell types are not affected. This reduction arises from inefficient beta-cell neogenesis during secondary transition as the earlier population of beta-cells is not affected. Furthermore, analysis of *Nkx6.1/Nkx2.2* double knockout shows a phenotype

similar to *Nkx2.2* single knockout. Accordingly, *Nkx6.1* expression is lost in *Nkx2.2* knockout but *Nkx2.2* expression is maintained in *Nkx6.1* knockout pancreata. It is, therefore, suggested that Nkx6.1 acts downstream of Nkx2.2 (Sander et al., 2000). Inactivation of *Nkx6.1* in endocrine precursors or beta-cells favors non-beta endocrine cell lineages at the expense of beta-cells while overexpression of *Nkx6.1* in endocrine precursors selectively favors beta-cell fate (Schaffer et al., 2013). Therefore, Nkx6.1 is required for beta-cell specification and maintenance of beta-cell fate.

1.5.12 *Pax4* and *Arx*

At E9.5, *Arx* expression is detectable in the pancreatic epithelium. During further development, *Arx* continues to express in the endocrine progenitors and then in the endocrine cells (mainly alpha-cells). In the adult pancreas, *Arx* is expressed in the alpha- and PP-cells (Collombat et al., 2003, 2007). *Pax4* expression also starts in the early pancreas at E9.5. *Pax4* expression then increases and peaks around the time of secondary transition when majority of the endocrine cells are generated. Later, its expression is reduced to nearly undetectable levels and is restricted to beta-cells (Sosa-Pineda et al., 1997; Smith et al., 1999).

Arx and Pax4 are important for the early lineage specification in the endocrine pancreas and an opposing link has been described between them. In *Arx* knockout mice, alpha-cells fail to develop, beta- and delta-cells are increased in number, and PP-cells are not affected (Collombat et al., 2003). On the other hand, in *Pax4* knockout mice, beta- and delta-cells do not develop while alpha-cell numbers are increased (Sosa-Pineda et al., 1997). As expected from these phenotypes, in the *Arx/Pax4* double knockout mice, both alpha- and beta-cells fail to develop but surprisingly the number of delta-cells is highly increased (Collombat et al., 2005). Furthermore, misexpression of *Arx* in the mature beta-cells can convert them to alpha-cells, and that of *Pax4* in alpha-cells can convert them to beta-cells (Collombat et al., 2007, 2009). These studies establish the essential role of Arx in alpha-cell specification, and that of Pax4 in beta-cell specification.

1.5.13 *MafA* and *MafB*

MafB expression is detectable in the early pancreatic epithelium around E10.5. At this time, most of the *MafB* expression is observed in alpha-cells. Subsequently, during secondary transition, *MafB* is expressed in some endocrine progenitors, in most of the

alpha- and many of the beta-cells. Finally, in adult pancreas, *MafB* expression is restricted to alpha-cells only (Artner et al., 2006; Nishimura et al., 2006). *MafB* knockout mice die at birth due to respiratory problems. Pancreatic analysis shows that alpha- and beta-cells are significantly reduced in number, while PP- and delta-cells are not affected in these mice. As compared to wild type mice, the total number of endocrine cells is also not changed. Therefore, the defect seems to be with the maturation of cells rather than their specification. Accordingly, MafB can bind to the promoters of *glucagon*, and *insulin* genes and activates their transcription. MafB may also activate the expression of other factors required for beta-cell maturity, including *MafA*, *Pdx1*, and *Glut2* (Artner et al., 2007). Thus, MafB plays an important role in the development of mature alpha- and beta-cells.

In the developing pancreas, *MafA* expression starts later than *MafB* and is normally restricted to beta-cells. Around E12.5, few MafA positive beta-cells are visible. During secondary transition, the number of beta-cells expressing *MafA* increases (Nishimura et al., 2006). Finally, in the adult pancreas *MafA* is expressed in all of the beta-cells and not in other endocrine cells (Zhang et al., 2005). *MafA* knockout mice have normal pancreas development and appear healthy at birth. However, they develop glucose intolerance and diabetes mellitus with increasing age. Islet architecture, Insulin synthesis, and glucose stimulated insulin secretion is also affected in these mice (Zhang et al., 2005). Hence, MafA plays an important role in the functional maturity of beta-cells.

1.6 *Paired box* genes

The paired box gene family encodes nuclear transcription factors. It comprises of nine members that are characterized by the presence of a highly conserved paired box domain. This domain is named after the Drosophila gene *Paired* (*Prd*) where it was first identified. These nine members are placed into four subgroups based on the presence or absence of an octapeptide, and the presence of a complete, truncated or no homeodomain. Apart from the Paired domain and homeodomain that serve the purpose of DNA binding, all Pax proteins contain a C-terminal transactivation domain that is rich in Proline, Serine, and Threonine. *Pax* genes play an important role in the development and function of many different tissues. Therefore, mutations causing impairment in their function can lead to serious medical ailments (Buckingham and Relaix, 2007; Wang et al., 2008).

1.7 *Paired box gene 6* (*Pax6*)

Pax6 belonging to the group IV of *Pax* gene family is a highly conserved transcription factor among different animal species (Callaerts et al., 1997). The vertebrate *Pax6* gene is organized into 16 exons that are numbered from 0 to 13 with two in between named alpha and 5a. *Pax6* gene is transcribed from three different promoters: P0, P1, and Pα (Figure 1.3). Mammalian Pax6 protein exists in three isoforms: Pax6 canonical, Pax6 5a, and Pax6 pairedless. These three isoforms are generated either by the selection of different promoters or by alternative splicing. Transcription from P0 and P1 promoters generate two different mRNAs that are translated into identical proteins because of the common translation start site. However, transcription from these two promoters shows different spatio-temporal patterns during embryonic development. The function of two different promoters for the expression of identical protein is not clear. However, the presence of different regulatory elements at each promoter may provide differential expression levels in different tissues or in the same tissue at different time points of development. Transcript initiated from P0 and P1 promoter is alternatively spliced to generate either Pax6 canonical or Pax6 5a isoform. Pax6 5a contains an additional exon (5a) that leads to a 14 amino acid insertion in the paired domain and changes the DNA binding properties of the protein. Third promoter Palpha is located within the intron between exon 4 and 5; and transcription from this promoter generates a Pax6 protein without paired domain (Shaham et al., 2012).

1.8 Pax6 in mammalian development

Initially, the role of Pax6 in development has been suggested by its expression in the developing tissues and by analysis of the phenotypes associated with naturally occurring *Pax6* mutations. Later on, the generation of *Pax6* knockout and more importantly the tissue specific *Pax6* knockout mice has helped to understand the finer details of Pax6 role in the development and function of different tissues. *Pax6* is expressed in the mouse eye, central nervous system (CNS), olfactory system, and pancreas (Walther and Gruss, 1991). Mice homozygous for *Pax6* null mutation die early after birth. They lack eyes, and nasal cavities, and the development of CNS and endocrine pancreas is also disturbed (Hill et al., 1991; Ashery-Padan et al., 2004; Osumi et al., 2008), further confirming the role of Pax6 in the development of these organs. Below is a brief description of the Pax6 role in

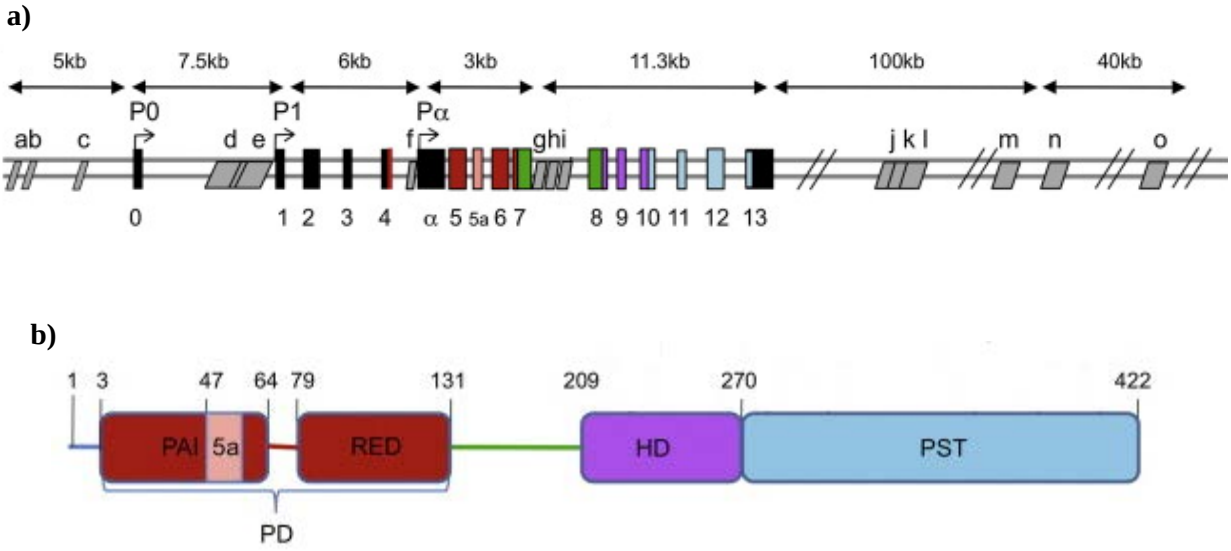

Figure 1.3: *Pax6* gene and protein. (a) *Pax6* gene structure. Colored boxes indicate coding exons and black boxes indicate non-coding exons. Grey boxes indicate various regulatory elements. (b) Pax6 protein domains. Note the additional 5a insertion in the paired domain that is not present in canonical isoform. PD=paired domain, HD=homeodomain, and PST=Proline-Serine-Threonine rich transactivation domain (adapted from Shaham et al., 2012).

eye and brain development. Subsequently, the role of Pax6 in the endocrine pancreas development and function is discussed in greater detail.

1.8.1 Pax6 in eye development

Role of Pax6 in eye development was first to be identified and has been extensively studied. Aniridia is a human disease of the eye that is caused by heterozygous mutations in the *PAX6* gene (Ton et al., 1991). In mouse, a heterozygous *Pax6* mutation results in the "small eye" phenotype and homozygous *Pax6* mutation leads to complete absence of eyes and is lethal in both mouse and human (Hill et al., 1991; Hodgson and Saunders, 1980). Therefore, a diploid dosage of Pax6 is essential for normal eye development. On the contrary, overexpression of *Pax6* can also lead to severe eye defects (Schedl et al., 1996). Mammalian eye develops from three derivatives of ectodermal origin namely the neuroectoderm, head surface ectoderm, and the neural crest. *Pax6* is expressed in the surface and neuroectodermal derivatives of the eye. It is required for the retinal neurogenesis, and for the specification of the ciliary body and iris progenitor pools. It is also required for the lens placode formation, for the lens vesicle detachment from the surface ectoderm, and for the lens fiber differentiation. Moreover, the corneal development is also disturbed in the *Pax6* mutant mice (Shaham et al., 2012).

1.8.2 Pax6 in brain development

In the mouse embryo, *Pax6* expression is first detectable around E8 in the neural plate. Subsequently, after neural tube regionalization, *Pax6* expression is found in certain areas of the forebrain, midbrain, hindbrain, and the spinal cord (Walther and Gruss, 1991; Duan et al., 2013). Pax6 plays multiple roles in the development of central nervous system. It is required for the neural tube patterning, neuronal subtype specification, proper neuronal migration, and also for the neurogenesis itself. Appropriate dosage of Pax6 is required to control the proliferation of neural progenitor cells, the timing of cell cycle exit, and the differentiation into neurons (Osumi et al., 2008; Georgala et al., 2011). In the adult brain, *Pax6* expression continues in the neurogenic niches as well as in certain subtypes of mature neurons. Therefore, Pax6 might be involved in maintaining the neuronal features of some neuronal subtypes (Duan et al., 2013).

1.8.3 Pax6 in Pancreas development

Pax6 expression is first detected around E9 in the developing pancreatic epithelium. Around E10, it is detected in the early glucagon cells and in most of the Isl1 expressing cells in the pancreatic epithelium. Later, *Pax6* expression is detected in the endocrine cells from the secondary transition. It then continues to express in alpha, beta, PP, and delta cells of the islet during later gestation as well as during adult life (Sander et al., 1997; St-Onge et al., 1997; Jensen et al., 2000a). Epsilon-cells, the fifth type of endocrine cells in the islet, may or may not express *Pax6* (Kordowich et al., 2011). The expression pattern of *Pax6* in developing and adult pancreas is suggestive of a vital role of Pax6 in the endocrine pancreas.

1.8.3.1 Pax6 knockout in vivo studies

In 1997, St-Onge et al. published the pancreatic phenotype of *Pax6* KO mice. They found a reduction of endocrine cell population that was the most prominent among alpha-cells. Additionally, the normal islet architecture was disturbed in the mutant pancreata. Due to the fact that *Pax6* KO mice die shortly after birth, it was not possible to analyze the role of Pax6 in adult Pancreas. In the same year, Sander et al. (1997) described a similar phenotype in $Pax6^{sey/sey}$ mutant mice. Moreover, it was found that Pax6 can bind to insulin, glucagon, and somatostatin gene promoters and transactivate the expression of *insulin* and *glucagon*.

In an effort to analyze the role of Pax6 in postnatal pancreas development, Ashery-Padan et al. (2004) used Cre-*loxP* system to generate the conditional inactivation of *Pax6* in *Pdx1* and *Pax6* regulatory domains. The phenotype was similar to the classical *Pax6* KO. Furthermore, they used Z/AP reporter strain to demonstrate that the overall endocrine area in the mutant pancreata was not reduced and, therefore, the islet cell neogenesis was not affected. Similar to the classical KO these conditional KO mice died in a few days after birth leaving the role of Pax6 in adult pancreatic function unresolved.

Later on, Heller et al. (2005) further analyzed $Pax6^{sey/sey}$ pancreata and found that the ghrelin positive cell population was 5 fold increased compared to the wild type. However, increase in ghrelin positive cell population has also been reported in *Pax4* and *Nkx2.2* KO pancreata (Prado et al., 2004). Since these three KO models die shortly after birth it was never possible to analyze the phenotype in adult pancreas. Analysis of ghrelin positive

cells in the embryonic pancreas is further complicated due to the existence of these cells in the wild type pancreas as a separate population as well as being co-positive with alpha-cells. Therefore, the origin of ghrelin positive cells in the embryonic KO pancreas cannot be faithfully defined.

Among the endocrine cells, alpha-cells are the most severely affected ones in the *Pax6* knockout mice. To understand the relative role of different Pax6 domains in the development of alpha-cells, Dames et al. (2010) analyzed the pancreata of *Pax6* mutant mice that carried mutations in either paired domain, homeodomain, or transactivation domain of Pax6. It was found that the activity of paired domain is more important for the development of alpha-cells. On the other hand, mutation in the homeodomain results into least affected phenotype.

Recently, Hart et al. (2013) used tamoxifen inducible ubiquitous Cre line to knockout *Pax6* in adult mice. They observed an overt diabetes and weight loss in knockout animals. Furthermore, the expression of insulin, glucagon, and somatostatin was reduced. In accordance with the previous studies they found an increased expression of ghrelin in the knockout pancreata.

The only in vivo study of *Pax6* overexpression (OE) in mice resulted in diabetes due to apoptosis of beta-cells. In addition, overexpression under *Pdx1* domain resulted in hypoplasia of exocrine pancreatic portion, and pancreatic tumors (Yamaoka et al., 2000).

1.8.3.2 Pax6 knockdown in vitro studies

In order to understand the role of Pax6 in alpha- and beta-cell function at the molecular level, Gosmain et al. (2010, 2012a, 2012b) used siRNA based approach to generate the Pax6 knockdown (KD) in primary rat alpha- and beta-cells. It was found that Pax6 can control the expression of several critical genes involved in the alpha- and beta-cell function. These included *proglucagon, PC2, MafB, c-Maf, NeuroD1/Beta2, GK, GIPR,* and *GPR40* in alpha-cells; and *insulin 1, insulin 2, Pdx1, MafA, Glut2, PC1/3, GK, Nkx6.1, c-Maf, PC2, GLP-1R,* and *GIPR* in beta-cells. Additionally, Pax6 knockdown in alpha-cells imapirs the processing of proglucagon and secretion of glucagon due to decreased expression of the factors involved in proglucagon processing and glucagon secretion (Katz et al., 2009; Gosmain et al., 2012b). Moreover, Liu et al. (2012) showed that Pax6 can bind to the *proSAAS* (*Pcsk1n*) gene promoter and directly downregulate its

expression, thereby controlling the PC1/3 activity and proper insulin processing in beta-cells.

These in vitro studies demonstrate that Pax6 is essential for both alpha- and beta-cell function as it controls the expression of many different genes involved in the synthesis, processing, and secretion of glucagon and insulin.

1.8.3.3 Pax6 in diabetes (human studies)

Several *PAX6* mutations have been reported in humans (Prosser and Heyningen, 1998; Dansault et al., 2007). Heterozygous *PAX6* mutations are a cause of aniridia (an eye disease) and are frequently linked to glucose intolerance (Yasuda et al., 2002). However, the etiology of age-induced glucose intolerance in aniridia patients is not completely understood. Wen et al. (2009) showed that defective proinsulin processing is one of the factors involved in the impairment of glucose tolerance in aniridia patients.

1.9 Aims of the study

As discussed above, the *Pax6* KO mice as well as *Pax6*$^{sey/sey}$ mutant mice die shortly after birth (Hill et al., 1991; St-Onge et al., 1997; Heller et al., 2005). This makes it impossible to analyze the role of Pax6 in adult endocrine pancreas in vivo. Later, the generation of *Pax6* floxed mice allowed the ablation of *Pax6* from the pancreas alone (Ashery-Padan et al., 2004). However, even these pancreas-specific conditional *Pax6* KO mice do not survive beyond early postnatal stage. Recently, a study has been published where *Pax6* was knocked out from adult pancreas with the help of an inducible ubiquitous Cre line (Hart et al., 2013). The ubiquitous Cre, however, removes *Pax6* from all of the endocrine cell types at once and, therefore, does not allow the detection of cell-type-specific effects.

Our study aims at analyzing the role of Pax6 in adult pancreas in a cell-type-specific manner. For this purpose, we carried out conditional *Pax6* KO in alpha- and beta-cells separately. This was done by using the Cre/*loxP* system (Sauer and Henderson, 1989; Rajewsky et al., 1996). Furthermore, *YFP* (yellow fluorescent protein) reporter transgene (Srinivas et al., 2001) was included to specifically mark the KO cells and to trace them over long-period of time. Along with that we also decided to increase the Pax6 dosage by overexpressing it in the whole pancreas, in the beta-cells alone, and in the alpha-cells alone. As an opposite approach to the knockout, overexpression can also provide useful information about the function of a protein.

This study was particularly aimed:

1. To identify and compare the characteristics of *Pax6* KO alpha- and beta-cells. This would help us to know if *Pax6* ablation affects both cell types in a similar or differential way.

2. To study the ghrelin^{+} cell population in both types of KO pancreata, especially focusing on their origin and later fate. Ghrelin upregulation has been linked to *Pax6* ablation in pancreas (Heller et al., 2005; Kordowich et al., 2011) but the origin of ghrelin^{+} cells has not been exactly defined.

3. To investigate the possibility of regeneration in alpha- and beta-cell population in adult pancreas using *Pax6* KO as a model of glucagon and insulin deficiency.

4. To analyze gross changes in the hormonal cell population following *Pax6* overexpression. It would be interesting to know, how increased Pax6 dosage affects pancreas development.

2. Materials and Methods

2.1 Materials

2.1.1 Primary antibodies

Table 2.1: List of primary antibodies used.

Antibody	Manufacturer	Host species	Working dilution
Insulin	Sigma	Mouse	1:1000
Insulin	Dako	Guinea pig	1:1000
Glucagon	Abcam	Mouse	1:1000
Glucagon	Abcam	Rabbit	1:50
Somatostatin	Dako	Rabbit	1:600
PP	Millipore	Rabbit	1:1000
Ghrelin	Santa Cruz	Goat	1:50
Ghrelin	Kindly provided by (1)	Mouse	1:1000
Neurotensin	Abcam	Rabbit	1:200
Pax6	Covance	Rabbit	1:300
Pax6	DSHB	Mouse	1:100
Isl1	Abcam	Rabbit	1:500
Rfx6	Millipore	Rabbit	1:2000
Pdx1	Kindly provided by (2)	Rabbit	1:2000
Nkx6.1	DSHB	Mouse	1:50
MafA	Bethyl Labs	Rabbit	1:500
MafB	Bethyl Labs	Rabbit	1:1000
Arx	Millipore	Rabbit	1:200
Glut2	Millipore	Rabbit	1:1000
GLP-1 receptor	Kindly provided by (3)	Rabbit	1:4000
C-peptide	Cell Signaling	Rabbit	1:100
IAPP	Phoenix Pharma	Rabbit	1:500
PC1/3	Millipore	Rabbit	1:500
PC2	Millipore	Rabbit	1:200

7B2	Abcam	Rabbit	1:5000
7B2	Kindly provided by (4)	Rabbit	1:200
ProSAAS	Kindly provided by (4)	Rabbit	1:50
Ki67	Dako	Rat	1:100
BrdU	Roche	Mouse	1:50
GFP	Abcam	Chicken	1:1000
β-galactosidase	Aves Labs	Chicken	1:5000

1. Dr. C. Tomasetto (Université Louis Pasteur, Strasbourg, France)
2. Dr. C. Wright (Vanderbilt University, Nashville, Tennessee)
3. Dr. J.F. Habener (Harvard Medical School, MA)
4. Dr. I. Lindberg University of Maryland-Baltimore, Maryland

DSHB=Developmental Studies Hybridoma Bank

2.1.2 Secondary antibodies

Table 2.2: List of secondary antibodies used.

Conjugate	**Host species**	**Reactivity**	**Working dilution**	**Manufacturer**
Alexa 488	Goat	Rabbit	1:1000	Invitrogen
Alexa 594	Goat	Rabbit	1:1000	Invitrogen
Alexa 594	Donkey	Rabbit	1:1000	Invitrogen
Alexa 488	Goat	Mouse	1:1000	Invitrogen
Alexa 594	Goat	Mouse	1:1000	Invitrogen
Alexa 594	Donkey	Mouse	1:1000	Invitrogen
Cy5	Sheep	Mouse	1:300	Jackson Immuno-Research Labs, Inc
Alexa 488	Goat	Guinea Pig	1:1000	Invitrogen
Alexa 594	Goat	Guinea Pig	1:1000	Invitrogen
Alexa 594	Goat	Rat	1:1000	Invitrogen
Alexa 488	Chicken	Goat	1:1000	Invitrogen
Alexa 594	Chicken	Goat	1:1000	Invitrogen
Alexa 488	Goat	Chicken	1:1000	Invitrogen
Alexa 594	Goat	Chicken	1:1000	Invitrogen

2.1.3 Quantitative RT-PCR primers

Table 2.3: List of qRT-PCR (quantitative reverse transcription - polymerase chain reaction) primers.

Gene	Primer
Insulin	QuantiTect Primer Assay QT00258083
Ghrelin	QuantiTect Primer Assay QT00137536
Pax6	QuantiTect Primer Assay QT01052786
MafA	QuantiTect Primer Assay QT01037638
Nkx6.1	QuantiTect Primer Assay QT00143318
Pdx1	QuantiTect Primer Assay QT00102235
Nkx2.2	QuantiTect Primer Assay QT00495502
Gusb	QuantiTect Primer Assay QT00176715

2.1.4 PCR primers for chromatin immunoprecipitation (ChIP)

Table 2.4: List of PCR primers used in ChIP.

Primer	Sequence	Promoter-region amplified
Glut2-ChIP-F	CCTAAGACACAGAAAAGTCACAGGG	-415 to -547 (contains proposed Pax6 binding site)
Glut2-ChIP-R	GTGGCCACAGAGTGTGGCAGCATCG	
Ghrelin-ChIP-F	GGAGAAGCCGGTGAGCAGGCACCAC	-335 to -476 (contains proposed Pax6 binding site)
Ghrelin-ChIP-R	CTGAATAATTTAGACCCCGGTGAGC	
MafA-ChIP-F	CACCCCAGCGAGGGCTGATTTAATT	-7750 to -8120 (Raum et al., 2010)
MafA-ChIP-R	AGCAAGCACTTCAGTGTGCTCAGTG	

2.1.5 PCR primers used in cloning

Table 2.5: List of PCR primers used in cloning.

No.	Primer	Sequence
1	Pax6-SalI-forward	TTTTTTTGTCGACATGCAGAACAGTCACAGCGGAGTGAATCAGCTTGG
2	Pax6-SalI-reverse	TTTTTTTGTCGACTTACTGTAATCGAGGCCAGTACTGAGACATGTCAG
3	Pax6-seq-1	CAGTATAAACGGGAGTGCCCTTC
4	Pax6-seq-2	CTTCTCTGGTTCCTCAGTTTCTC

2.1.6 PCR primers used for genotyping

Table 2.6: List of PCR primers used for genotyping.

No.	Primer	Sequence
1	Flp6-primer-83	GCGGTTGAGTAGCTCAATTCTA
2	Flp6-primer-84	AGTGGCTTGGACTCCTCAAGA
3	Flp6-primer-del	CGTGTGCCCCAGCTTCCGGT
4	AM-89-Cre	ATG CTT CTG TCC GTT TGC CG
5	AM-90-Cre	CCT GTT TTG CAC GTT CAC CG
6	GFP-Forward	ACCCTGAAGTTCATCTGCACCA
7	GFP-Reverse	TGGGTGCTCAGGTAGTGGTTGT

2.1.7 Mouse lines used

Table 2.7: List of mouse lines used.

Mouse line	Reference
Pax6 floxed	Ashery-Padan et al., 2000

RIP-CreER	Dor et al., 2004
Glucagon-Cre	Herrera, 2000
Insulin-Cre	Herrera, 2000
Pdx1-Cre	Gannon et al., 2000
R26-YFP reporter	Srinivas et al., 2001

2.1.8 Solutions and Media

Table 2.8: Phosphate buffered saline (PBS).

Component	**Amount (g/L)**
NaCl	8.0
KCl	0.2
$Na_2HPO_4.2H_2O$	1.35
$NaH_2PO_4.2H_2O$	0.1
KH_2PO_4	0.2

Dissolve in Milli-Q water and bring the final volume to 1 litre.

Table 2.9: Luria-Bertani medium (LB medium).

Component	**Amount (g/L)**
Tryptone	10
Yeast extract	5
NaCl	10

Dissolve in 900 mL Milli-Q water, adjust the pH to 7.0 and bring the final volume to 1 liter. The medium was autoclaved before use.

LB agar plates

For LB agar plates, add 15 g/L agar to LB medium before autoclaving. After autoclaving, let the medium cool down to ≈50°C, add the antibiotic (e.g., ampicillin), and pour into sterile plates. After the medium has solidified, invert the plates and store at 4°C in dark.

2.2 Methods

2.2.1 Animal treatments

2.2.1.1 Tamoxifen treatment

Tamoxifen was prepared in corn oil (at 20 mg/ml). For 3 week old mice, 1 mg tamoxifen was administered intraperitoneally for 3 days every other day. For 1.5 month and 2 month old mice, 2 mg tamoxifen was administered intraperitoneally for 4 days every other day.

2.2.1.2 Bromodeoxyuridine (BrdU) treatment

BrdU was given in drinking water (at 0.8 mg/mL) to the mice for 3 days. Water bottles were wrapped in aluminium foil to protect from light-mediated degradation.

2.2.1.3 Blood and urine glucose measurement

For glucose measurements One Touch Glucose meter (Johnson & Johnson) was used. A drop of blood from tail vein or a drop of urine was directly taken on to the test strip.

2.2.2 Histological procedures

2.2.2.1 Pancreas preparation, cryo-embedding, and sectioning

Mice were killed by decapitation (at P0) and by cervical dislocation or CO_2 inhalation (at age of 3 weeks or more). Pancreata were removed and immediately placed in ice-cold PBS (Table 2.8). It was followed by washing in PBS (3x10minutes) at 4°C. After washing the pancreata were fixed in 4% paraformaldehyde (dissolved in PBS; pH 7.4) for 1 hour at 4°C. Fixed pancreata were then washed in PBS (4x20minutes) and placed in 25% sucrose (dissolved in PBS) for overnight at 4°C. After this pancreata were washed two times in Jung Tissue Freezing Medium™ (Leica Microsystems) (2x2hours), and embedded in the same medium on dry ice using cryomolds. Frozen embedded pancreata were stored at -20°C or -80°C.

From the frozen pancreata, 8 µm sections were cut on a cryostat and mounted on SuperFrost® Plus slides (Thermo Scientific). Slides were kept at 30°C for half an hour, to allow the adhesion of sections, before being stored at -20°C or -80°C.

2.2.2.2 Immunofluorescence staining of cryosections

Pancreatic cryosections on each slide were encircled with ImmEdge™ pen. Slides were then washed in PBS (3x5minutes). Next, the sections were blocked with 10% fetal calf serum (in PBS containing 0.1% triton x-100) for 1 hour at room temperature. It was followed by incubation of the sections with primary antibodies (diluted in the same blocking solution) at 4°C for overnight. Next day, the slides were washed in PBS (3x5minutes). After washing, the sections were incubated with the appropriate secondary antibodies (diluted in the same blocking solution) for 1 hour at room temperature. Afterwards, the slides were washed again in PBS (3x5minutes) and mounted with Vectashield® mounting medium containing DAPI (4',6-diamidino-2-phenylindole) (Vector Labs).

2.2.2.3 BrdU staining

Slides with cryosections were washed in PBS for 15 minutes and incubated in 2 N HCl for 30 minutes at 37°C. This was followed by washing of slides in PBS (2x30minutes). Next, the cryosections on each slide were encircled with ImmEdge™ pen and blocked with 10% fetal calf serum (in PBS containing 0.1% triton x-100) for 1 hour at room temperature. It was followed by incubation of the sections with primary antibodies (diluted in the same blocking solution) at 4°C for overnight. Next day, the slides were washed in PBS (2x15minutes). After washing, the sections were incubated with the appropriate secondary antibodies (diluted in the same blocking solution) for 1 hour at room temperature. Afterwards, the slides were washed again in PBS (2x30minutes) and mounted with Vectashield® mounting medium containing DAPI (Vector Labs).

2.2.2.4 TUNEL staining

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay detects apoptotic cells by labeling fragmented DNA resulting from apoptosis. For TUNEL staining, Apop Tag® Red In Situ Apoptosis Detection Kit (Millipore) was used and the procedure was performed according to the manufacturer's instructions.

2.2.2.5 X-Gal staining

For X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) staining, pancreata were prepared and embedded in the same way as mentioned before (section 2.2.2.1). However,

the fixative used was different. These pancreata were fixed in fixative A (1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40, in PBS) for 30 minutes and then in fixative B (1% formaldehyde, 0.2% glutaraldehyde, 0.2% NP-40, 0.1% sodium deoxycholate, in PBS) for 1 hour at 4°C. From the embedded pancreata, 8 μm sections were cut. The cryosections were fixed again in ice-cold 0.2% glutaraldehyde (in PBS) for 10 minutes. Next, the sections were washed in X-Gal staining buffer (2mM $MgCl_2$, 0.02% NP-40, 0.01% sodium deoxycholate, in PBS) at room temperature (3x5minutes). Then the sections were overlayed with X-Gal staining solution (1mg/mL X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% sodium deoxycholate, in PBS) and incubated at 30°C for 24-72 hours in dark. For staining longer than 24 hours, the staining solution was changed after every 24 hours. After completion of staining, the slides were washed in PBS (2x5minutes) and the sections were mounted with Vectashield® mounting medium containing DAPI (Vector Labs). Images of X-Gal staining were acquired on Olympus BX 60 fluorescent microscope at 20x magnification.

2.2.2.6 Imaging

For quantification of islet cell numbers, images were acquired on Olympus BX 60 fluorescent microscope at 20x magnification. All other images were acquired using a 40x oil-immersion objective on Leica TCS SP5 laser scanning confocal microscope.

2.2.2.7 Quantification of islet cell numbers

The whole pancreata were cut into serial sections and every 20^{th} section was stained with antibodies against different hormone/hormone combinations. Cells were counted from all the islets on every section and an average no./islet was calculated. Quantification data is presented as mean±Standard error of mean (SEM). To measure statistical significance, unpaired student's two-tailed t-test was applied and $p<0.05$ was considered as significant.

2.2.3 Cell culture

Mouse insulinoma (Min6) cells were maintained in Dulbecco's modified eagle medium (Gibco®-containing 25 mM glucose and 3.97 mM L-Glutamine) supplemented with 10% fetal calf serum, 1X penicillin/streptomycin (Gibco®-100U/mL penicillin and 100 μg/ml streptomycin) and beta-mercaptoethanol (5 μL/L). The cells were grown at 37°C in the presence of 5% CO_2.

2.2.4 Immunofluorescent staining of cultured cells

For immunofluorescent staining, Min6 cells were cultured in four-chamber slides (Thermo Scientific). Medium was removed and cells were washed once with PBS. Then, the cells were fixed with 3.6% formaldehyde (in PBS) for 20 minutes at room temperature. It was followed by washing with PBS (2x5minutes). Next, the cells were permeabilized with 0.5% triton x-100 (in PBS) for 10 minutes and washed again with PBS (1x5minutes). After washing, the cells were blocked with 10% fetal calf serum (in PBS containing 0.1% triton x-100) for 1 hour at room temperature. Then the cells were incubated with primary antibodies (diluted in the same blocking solution) at 4°C for overnight. Next day, the cells were washed with PBS (3x5minutes). After washing, the cells were incubated with the appropriate secondary antibodies (diluted in the same blocking solution) for 1 hour at room temperature. Afterwards, the cells were washed again with PBS (3x5minutes). The slide chamber was then removed and cells were mounted with Vectashield® mounting medium containing DAPI (Vector Labs).

2.2.5 Molecular Biology

2.2.5.1 Chromatin immunoprecipitation assay

For chromatin immunoprecipitation, EZ-ChIP™ kit (Millipore) was used and the procedure was performed according to manufacturer's instructions. Briefly, Min6 cells were cultured in 10 cm dish to 90% confluency. Formaldehyde was directly added to culture medium (1% final concentration) to fix the cells at room temperature for 10 minutes. Fixation was stopped by adding glycine to the medium (125 mM final concentration) and incubating for 5 minutes at room temperature. All the next steps were performed on ice. Cells were washed twice in PBS and collected in 2 mL PBS containing protease inhibitor cocktail. After centrifugation the pellet was resuspended in 1 mL lysis buffer. This cell suspension was sonicated to shear the crosslinked DNA to a fragment length of ≈200-1000 base pairs. The sonicated suspension was centrifuged to remove the insoluble material. After centrifugation, 100 µL of the supernatant was diluted to 1 mL with dilution buffer containing protease inhibitor cocktail. The diluted chromatin was pre-cleared by incubating with Protein G Agarose beads for 1 hour at 4°C on a rotating platform. At this step, 1% of the chromatin sample was set aside as input. The rest of precleared chromatin was incubated, with 10 µg of rabbit anti-Pax6 antibody or 10 µg of

normal rabbit IgG (Millipore), for overnight at 4°C on a rotating platform. Next day the antibody-protein-DNA complexes were collected by incubating with Protein G Agarose beads for 1 hour at 4°C with rotation. The complexes were then washed through a series of wash buffers and eluted with elution buffer. Finally, the protein-DNA crosslinks were removed and the DNA was purified. This purified DNA was then used for PCR.

To check the binding of Pax6 with various promoter regions, PCR was performed with the primer sets mentioned in Table 2.4. Reaction was carried out in eppendorf Mastercycler®. PCR reaction composition and the program used were as follows:

Reaction component	**Volume (µL)**
DNA	2.0
H_2O	12.6
5X PCR buffer	4
20mM dNTP	0.2
Primer (forward)	0.4
Primer (reverse)	0.4
Go Taq® DNAPolymerase (5U/µL) (Promega)	0.4

PCR program used was as follows:

Initial denaturation 94°C - 3 minutes, 35 cycles (denaturation 94°C – 20 seconds, annealing 59°C – 30 seconds, extension 72°C – 30 seconds), final extension 72°C – 5 minutes.

2.2.5.2 Tail DNA isolation

Genomic DNA was isolated from tail-tip biopsy samples. For tissue lysis, 0.5 mL tail lysis buffer (100 mM Tris-pH 8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, supplemented with 250 µg/ml Proteinase K) was added to each cut-tail taken in a 1.5 mL eppendorf tube. Samples were then incubated at 55°C for overnight on a shaker. Next day, the samples were centrifuged at 13,000 rpm for 6 minutes. The supernatant from each sample was transferred to a new 1.5 mL eppendorf tube. Next, to precipitate the DNA, 0.5 mL isopropanol was added to each sample and inverted several times to mix well. This was followed by centrifugation at 13,000 rpm for 6 minutes to collect the DNA

pellets. Supernatant was discarded and 0.5 mL 70% ethanol was added to each DNA pellet for washing. Next, the samples were centrifuged again at 13,000 rpm for 6 minutes. Supernatant was discarded and pellets were allowed to air-dry. Dried pellets were dissolved in autoclaved Milli-Q water (60 µL per pellet) and stored at 4°C.

2.2.5.3 Genotyping of mice

Genotyping of mouse lines was done by PCR using the DNA isolated from tails. *Pax6*wild-type, floxed allele, and deleted allele was detected by using primer number 1, 2, and 3 (Table 2.6 - as used in Piñon et al., 2008). *Cre* transgene and *YFP* reporter transgene was detected by using primer number 4/5 and 6/7, respectively (Table 2.6). Reactions were carried out in eppendorf Mastercycler®. The same PCR reaction composition was used for all genotyping PCRs but different PCR programs were used as follows:

Reaction component	**Volume (µL) in total of 30 µL**
H_2O	22.3
5X buffer	6.0
dNTPs (20mM)	0.38
Primers (10 µM)	0.16 (each)
Go Taq® DNAPolymerase (5U/µL) (Promega)	0.16
Template DNA	1

Pax6-flox PCR program:

Initial denaturation 95°C - 4 minutes, 30 cycles (denaturation 95°C – 50 seconds, annealing 58°C – 50 seconds, extension 68°C – 50 seconds), final extension 72°C – 10 minutes.

Cre PCR program:

Initial denaturation 95°C - 4 minutes, 30 cycles (denaturation 95°C – 30 seconds, annealing 57°C – 30 seconds, extension 65°C – 30 seconds), final extension 72°C – 10 minutes.

GFP PCR program:

Initial denaturation 95°C - 4 minutes, 35 cycles (denaturation 94°C – 30 seconds, annealing 62°C – 30 seconds, extension 72°C – 30 seconds), final extension 72°C – 10 minutes.

2.2.5.4 Total RNA isolation, cDNA synthesis, and qRT PCR

For RNA isolation, pancreata were placed in RNA*later* (Qiagen) immediately after removal. Pancreata were kept in RNA*later* for overnight at 4°C. Next day they were either used for RNA isolation or transferred to -20°C for long-term storage. For total RNA isolation, pancreatic tissue was first disrupted using Tissuelyser (Qiagen). Total RNA was then extracted from the tissue lysate by using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. RNA quality was checked by spectrometry on NanoDrop (Peqlab) and by agarose gel electrophoresis. For cDNA synthesis, SuperScript II Reverse Transcriptase Kit (Invitrogen) was used according to the manufacturer's instructions. Finally, this cDNA was used for quantitative real-time PCR using primers mentioned in Table 2.3. *Beta-glucuronidase* (*Gusb*) was used as a house keeping control. PCR was carried out in Mastercycler® realplex^2 (Eppendorf) using SYBR Green Master Mix (Qiagen) according to manufacturer's instructions.

2.2.5.5 Generation of calcium competent cells

A single colony of *E. coli* (DH5α) was inoculated into 5 mL LB medium (Table 2.9) and incubated for overnight at 37°C on shaker. Next day this 5 mL pre-culture was added to 50 mL LB medium and incubated further at 37°C on shaker until an OD_{600} of 0.5 was achieved. Then the bacteria were pelleted by centrifugation at 4°C (3000g, 8 minutes). The pellet was resuspended in 25 mL ice-cold $CaCl_2$ (50 mM) and incubated on ice for 30 minutes. It was followed by centrifugation again at 4°C (3000g, 4 minutes). Supernatant was discarded and the pellet was resuspended in 2.5 mL ice-cold $CaCl_2$ (50 mM). For storage, 1 mL of 50% glycerol was added and the competent cells were stored in 200 µL aliquots at -80°C.

2.2.5.6 Transformation of competent cells

Competent cells stored at -80°C were thawed on ice for 10 minutes. Purified plasmid DNA (50 ng) or ligation mixture (10 µL) was added to competent cells and incubated on

ice for 30 minutes. Next, the competent cells were given a heat shock at 42°C for 90 seconds. After heat shock cells were put back on ice for 2 minutes. Then 800 µL LB medium was added to transformed competent cells and the cells were incubated at 37°C for 1 hour on shaker. Finally, the transformed cells were plated on LB agar plates supplemented with appropriate selection antibiotic. The plates were incubated at 37°C for overnight. Next day the plates were observed for the appearance of resistant clones.

2.2.5.7 Plasmid DNA isolation

Plasmid DNA from bacteria was isolated by using the Qiagen Mini prep and High Speed Midi prep kits. DNA concentration was measured by spectrometry using NanoDrop (Peqlab).

2.2.5.8 Agarose gel electrophoresis and gel extraction of DNA fragments

Depending on the size of DNA fragments to be analyzed, a 0.8 – 2% agarose gel was prepared. Agarose (Roti®garose-Roth) was dissolved by melting in 0.5X TBE buffer (44.5 mMTris-base, 44.5 mM boric acid, 1 mM EDTA-pH 8.0). To visulaize the DNA after electrophoresis, ethidium bromide was directly added to the gel at a final concentration of 0.2 µg/mL. DNA was mixed with 6X DNA loading dye (30% v/v glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) before loading. GeneRuler™ 1 kb and 100 bp DNA ladders were used for estimation of band sizes. TBE (0.5X) was used as a running buffer and electrophoresis was performed at 4-5 volts per cm. Afterwards the DNA fragments were visualized under a UV transilluminator. For gel extraction, the DNA band of the required size was cut out of the gel and purified by using Gene Clean® Spin Kit (MPIbio), according to the manufacturer's instructions.

2.2.5.9 Restriction digestion and dephosphorylation

For restriction digestion, 1 to 5 µg of plasmid DNA or purified PCR product was incubated with 20 to 100 units of restriction enzyme (New England BioLabs) and appropriate buffer in the presence or absence of bovine serum albumin. The digestion was carried out at 37°C for 4 to 8 hours. For dephosphorylation of vector backbone antarctic phosphatase (New England BioLabs) and the associated buffer were directly added to the restriction digest mixture (10U/µg of DNA) and incubated for 1 hour more at 37°C.

2.2.5.10 Ligation

Ligation was carried out using T4 DNA ligase at 16°C for overnight. Ligation mixture contained: 2 µL vector DNA (50 ng/µL), 6 µL insert DNA (50 ng/µL), 1 µL ligation buffer (10X), 1 µL T4 DNA ligase (400 U/µL). Next day the ligation mixture was directly used for transformation.

2.2.5.11 Generation of Pax6 overexpression construct

*Pax6*cDNA was amplified by PCR using forward and reverse primers containing SalI restriction site (Table 2.5). CMV-Pax6 plasmid (Walther and Gruss, 1991) was used as a template. Reaction was carried out in eppendorf Mastercycler®. Composition of the reaction mixture and PCR program used was as follows:

Reaction component	**Volume (µL) in total of 50 µL**
H_2O	40.5
10X buffer	5
dNTPs (20mM)	0.5
Primer-forward (10 µM)	1
Primer-reverse (10 µM)	1
Template DNA (100 ng/µL)	1
Taq DNA Polymerase (1U/µL) (Roche)	1

PCR program used:
Initial denaturation 95°C - 4 minutes, 35 cycles (denaturation 95°C – 40 seconds, annealing 60°C – 40 seconds, extension 72°C – 90 seconds), final extension 72°C – 20 minutes.

PCR product was purified by gel extraction and digested with SalI restriction enzyme. The destination vector pJojo (Collombat et al., 2007) was digested with XhoI and dephosphorylated to prevent self-ligation. The digested PCR product and vector backbone were again purified by gel extraction. This was followed by the overnight ligation of PCR product and vector backbone. Next day the ligation mixture was transformed into competent *E. coli* (DH5α) and the transformed cells were plated on LB-agar plates

supplemented with 50 μg/mL ampicillin. The plates were incubated at 37°C for overnight. Next day 10 single colonies were picked and each was inoculated into 5 mL LB medium (supplemented with 50 μg/mL ampicillin) in a sterile tube. The tubes were incubated on shaker at 37°C for overnight. Next day the plasmid DNA was isolated. Finally, the correct orientation and sequence of the insert was confirmed by sequencing with the primers (3/4) mentioned in Table 2.5. For sequencing, Seqlab (Goettingen) sequencing service was used. The clone with the correct orientation and sequence was linearized by digesting with SalI restriction enzyme and purified by gel extraction. This purified linear construct was then used for pronuclear injection to generate the transgenic mouse lines.

3. Results

3.1 Analysis of beta-cell-specific *Pax6* knockout pancreata

3.1.1 Inducible conditional knockout of *Pax6* in the adult pancreatic beta-cells

Pax6 classical as well as pancreas-specific conditional knockout mice die shortly after birth. Death of these mice results from an overt diabetic phenotype that points to the essential role of Pax6 in the maintenance of beta-cell function (Ashery-Padan et al., 2004). However, due to this early postnatal lethality it is impossible to analyze the role of Pax6 in adult beta-cells. For this reason, we decided to generate inducible *Pax6* knockout (KO) mice where *Pax6* is ablated upon tamoxifen induction in beta-cells alone without affecting other endocrine cell types. In order to generate the mice with the required genotype, *Pax6* floxed mouse line ($Pax6^{fl/fl}$) (Ashery-Padan et al., 2000) was crossed with *RIP-CreER* mouse line (Dor et al., 2004). Secondarily, we decided to incorporate the YFP reporter that allows the identification and tracing of Cre-recombined cells (Figure 3.1). This was achieved by including an additional cross with *R26-YFP* reporter mouse line (Srinivas et al., 2001). Mice with the right genotype were injected with tamoxifen and the ablation of *Pax6* from beta-cells was confirmed by double immunofluorescence staining (Figure 3.2a-d). The efficiency of *Pax6* ablation from beta-cells was nearly 95% as indicated by quantification of the $Pax6^{-}$ $insulin^{+}$ cells (Figure 3.2e). A similar labeling efficiency was obtained with the YFP reporter (Figure 3.2f). It was further confirmed by immunofluorescence staining that *Pax6* is specifically deleted from the YFP labeled cells and the YFP^{-} $insulin^{+}$ cells that either escape recombination and/or arise later as a result of regeneration are positive for Pax6 expression (Figure 3.2g-j). Thus, Cre is active in 95% of beta-cells and the ablation of *Pax6* and labeling with the YFP reporter are faithfully linked to each other.

3.1.2 Development of diabetes in the beta-cell-specific *Pax6* KO mice

As Pax6 has been shown to be implicated in the control of beta-cell function, an increase in the level of blood glucose was expected (Ashery-Padan et al., 2004; Gosmain et al., 2012a). Therefore, we monitored the non-fasting blood glucose level in control and beta-cell specific *Pax6* KO mice following the tamoxifen injection. As compared to the control mice, blood glucose level started to rise in the KO mice within a week after first injection

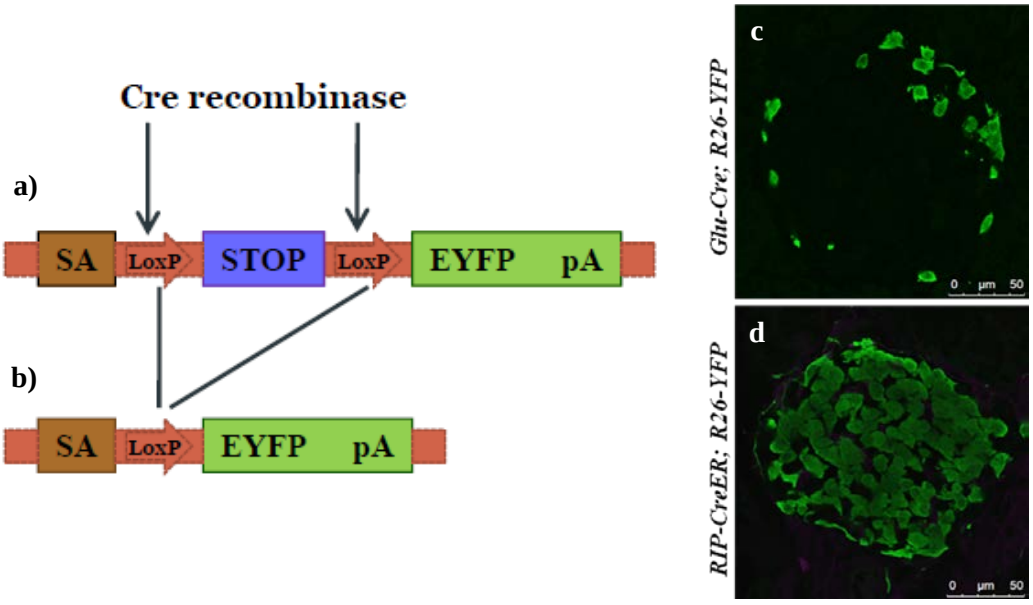

Figure 3.1: Lineage tracing with *R26-YFP* reporter mouse line. (a) The "stop cassette" flanked by loxP sites includes a trascriptional stop that does not allow the expression of YFP in the absence of Cre activity. (b) Following Cre-mediated recombination "stop" is removed allowing the permanent expression of YFP in Cre expressing cells. (c) YFP Labeling of alpha-cells expressing Cre under *glucagon* promoter. (d) YFP labeling of beta-cells expressing Cre under rat *insulin* II promoter (RIP). (*R26=Rosa26*; SA=splice acceptor)

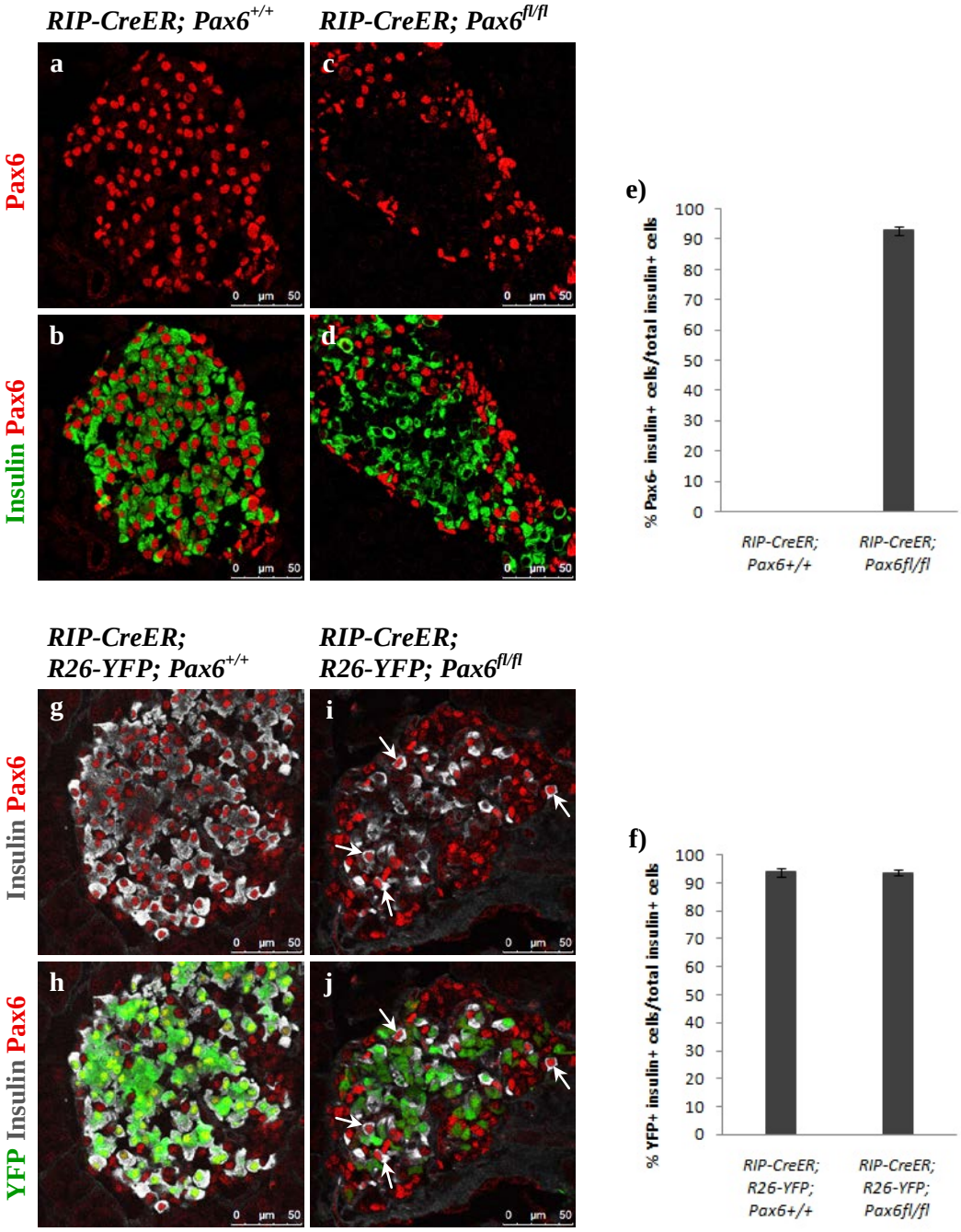

Figure 3.2: Beta-cell-specific ablation of *Pax6*. Double immunofluorescence staining of pancreatic cryosections from 2 month old mice at 4 days (a-d) or 4 weeks (g-j) after tamoxifen induction. Pax6 expression is lost from majority of the insulin$^+$ cells (c, d). Ablation of *Pax6* is specific to YFP labeled cells (i,j) as YFP$^-$ insulin$^+$ cells continue to express Pax6 (arrows i,j). Quantification of Pax6$^-$ insulin$^+$ (e) and YFP$^+$ insulin$^+$ (f) cells in the pancreata of 2 month old mice at 4 days after tamoxifen induction (n=3). Cre-mediated recombination/*Pax6* ablation takes place in nearly 95% of beta-cells. Error bars represent SEM.

of tamoxifen. At four weeks after injection the blood glucose level in the KO mice was ≈450 mg/dL on average compared to ≈100 mg/dL average level in the control mice. At this time point a high glucose level was also detected in the urine of KO mice. Hence, the ablation of *Pax6* from beta-cells leads to an overt diabetic phenotype and supports the essential role of Pax6 in the maintenance of adult beta-cell function.

3.1.3 Expression of pancreatic endocrine hormones in the beta-cell-specific *Pax6* KO mice

In the classical and pancreas-specific conditional *Pax6* KO mice as well as in the *Pax6*$^{sey/sey}$ mutant mice, the number of ghrelin^{+} cells is highly elevated and that of insulin^{+} cells is decreased (Ashery-Padan et al., 2004; Heller et al., 2005; Kordowich et al., 2011). In the adult beta-cell-specific *Pax6* KO mice we also found an increase in the ghrelin^{+} cell population and a decrease in the insulin^{+} cell population. This change was, however, gradual. Following tamoxifen induction, there was a steady increase in the number of ghrelin^{+} cells (Figure 3.3a and 3.4) with a concomitant decrease in the number of insulin^{+} cells over time (Figure 3.3a and 3.5). This inverse relationship over time indicated a possible direct conversion of one cell type into the other. A decrease in the expression of *insulin* and increase in the expression of *ghrelin* was also confirmed by qRT-PCR (Figure 3.3b,c). The number of other endocrine cell types, including glucagon, somatostatin, and PP producing cells, was increased in the pancreata of beta-cell-specific *Pax6* KO mice (Figure 3.6).

3.1.4 Expression of beta-cell related transcription factors in the pancreata of beta-cell-specific *Pax6* KO mice

Apart from Pax6, several other transcription factors are involved in the maintenance of mature beta-cell function. By immunofluorescence staining, we checked the expression of beta-cell related transcription factors MafA, Pdx1, Nkx6.1, Isl1, and Rfx6. MafA, Pdx1, and Nkx6.1 are specifically expressed in mature beta-cells while Isl1 and Rfx6 are expressed in all endocrine cells of the islet (Ahlgren et al., 1997; Ahlgren et al., 1998; Zhang et al., 2005; Gauthier et al., 2007; Smith et al., 2010). In beta-cell-specific *Pax6* KO islets the expression of MafA was absent from most of the insulin^{+} cells (Figure 3.7). Expression of Pdx1, Isl1 and Rfx6 was not affected in the KO islets (Figure 3.7 and 3.8). Expression of Nkx6.1 was not affected in the early days after KO induction but was

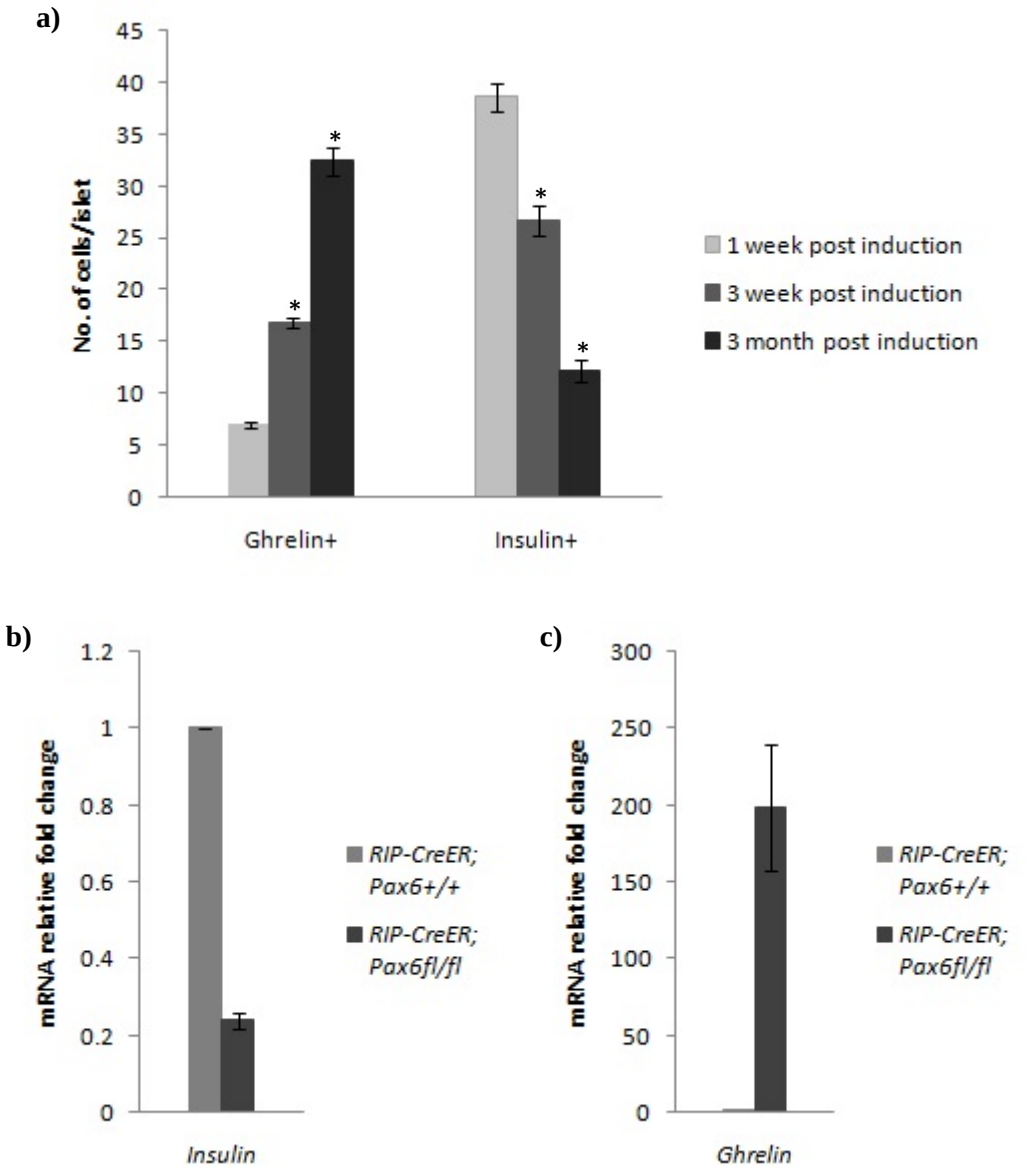

Figure 3.3: Inverse relationship of ghrelin and insullin expression in the pancreata of beta-cell-specific *Pax6* KO mice. (a) Quantification of ghrelin$^+$ and insulin$^+$ cells in the islets of beta-cell-specific *Pax6* KO mice (injected at 1.5 month of age) at different time points after tamoxifen induction (n=3). (b,c) Quantitative RT-PCR of *insulin* and *ghrelin* mRNA in the pancreata of 4.5 month old mice at 3 months after tamoxifen induction (n=2). Error bars represent SEM; *p<0.05.

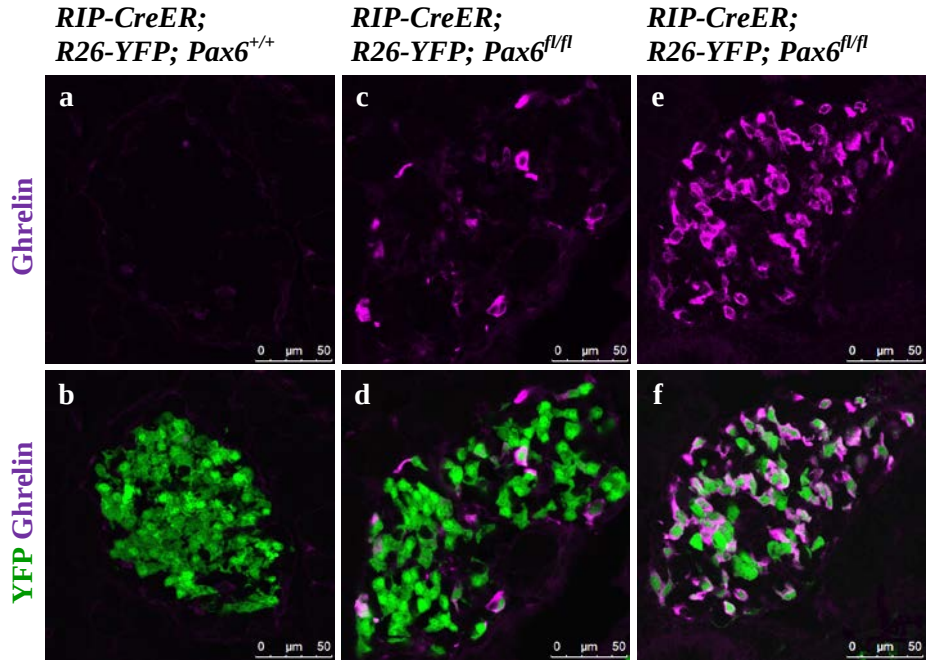

Figure 3.4: Gradual increase in the population of ghrelin expressing cells in the islets of beta-cell-specific *Pax6* KO mice. Immunofluorescence staining of pancreatic cryosections from 4 week old mice at 7 days after tamoxifen induction (a-d) and 9 week old mice at 6 weeks after tamoxifen induction (e,f). (a,b) Ghrelin$^+$ cells are not detected in the control islets. (c,d) At 7 days after tamoxifen induction few YFP$^+$ *Pax6*-deficient cells express ghrelin in the KO islets. (e,f) At 6 weeks after tamoxifen induction majority of the YFP$^+$ *Pax6*-deficient cells express ghrelin in the KO islets.

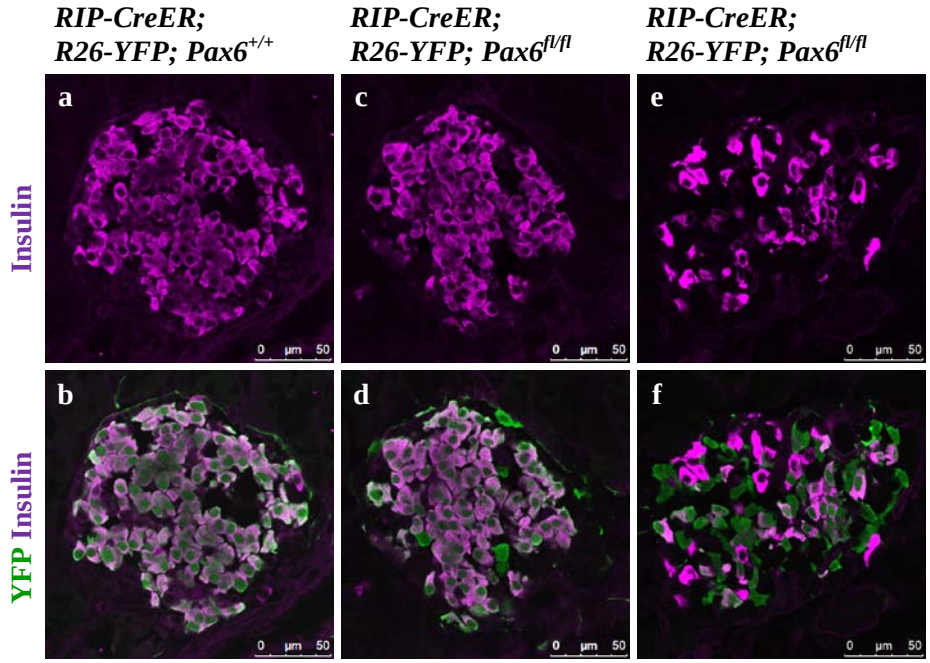

Figure 3.5: Gradual decrease in the population of insulin expressing cells in the islets of beta-cell-specific *Pax6* KO mice. Immunofluorescence staining of pancreatic cryosections from 4 week old mice at 7 days after tamoxifen induction (a-d) and 9 week old mice at 6 weeks after tamoxifen induction (e, f). (a, b) In the control islets, all of the YFP^+ cells express insulin. (c, d) At 7 days after tamoxifen induction few YFP^+ *Pax6*-deficient cells are negative for insulin expression. (e, f) At 6 weeks after tamoxifen induction majority of the YFP^+ *Pax6*-deficient cells are negative for insulin expression.

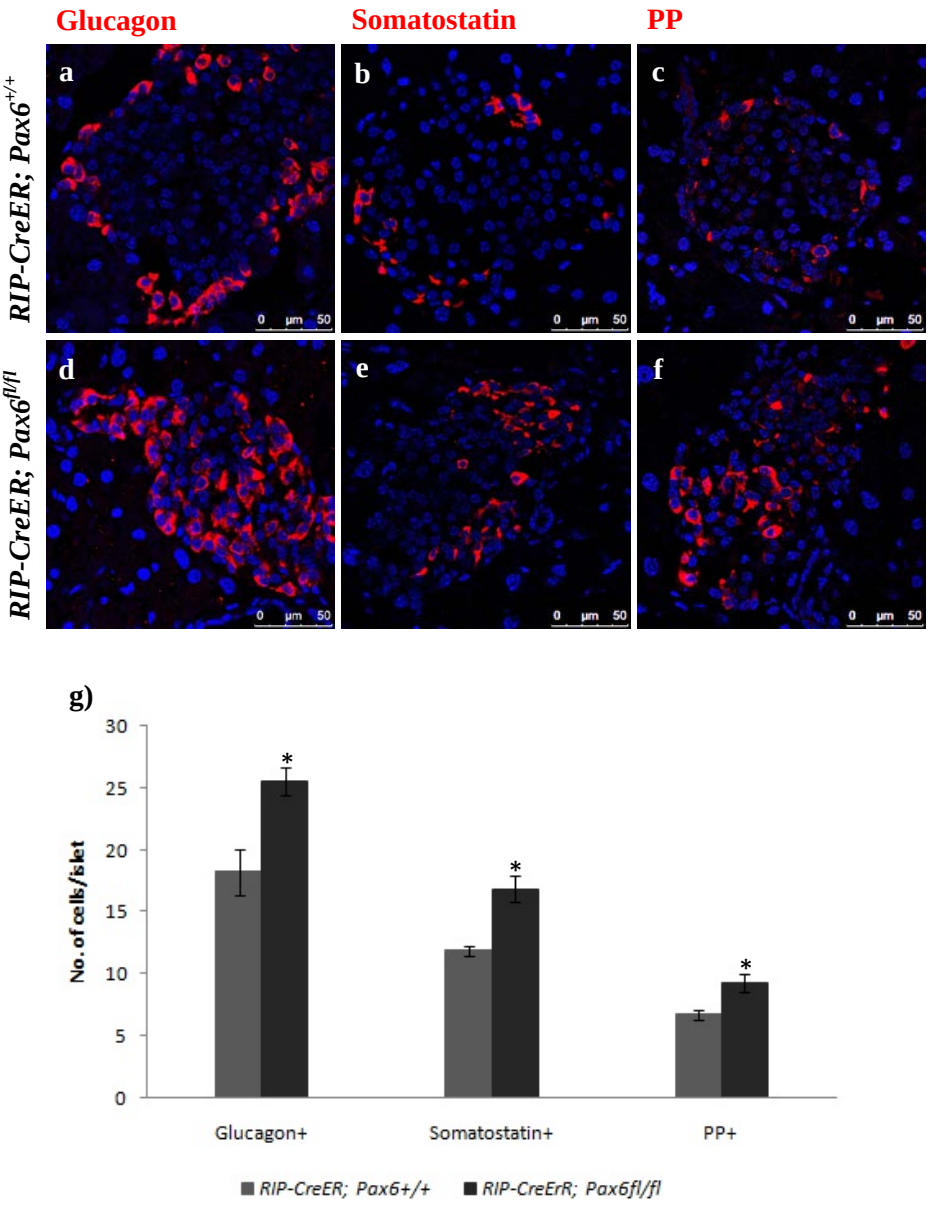

Figure 3.6: Changes in the glucagon$^+$, somatostatin$^+$, and PP$^+$ cell population in the islets of beta-cell-specific *Pax6* KO mice. Immunofluorescence staining of pancreatic cryosections (a-f) and quantification of glucagon$^+$, somatostatin$^+$, and PP$^+$ cells (g) from 2 month old mice at 4 weeks after tamoxifen induction (n=3). Glucagon$^+$, somatostain$^+$, and PP$^+$ cells are increased in number in the beta-cell-specific *Pax6* KO islets. Error bars represent SEM; *p<0.05.

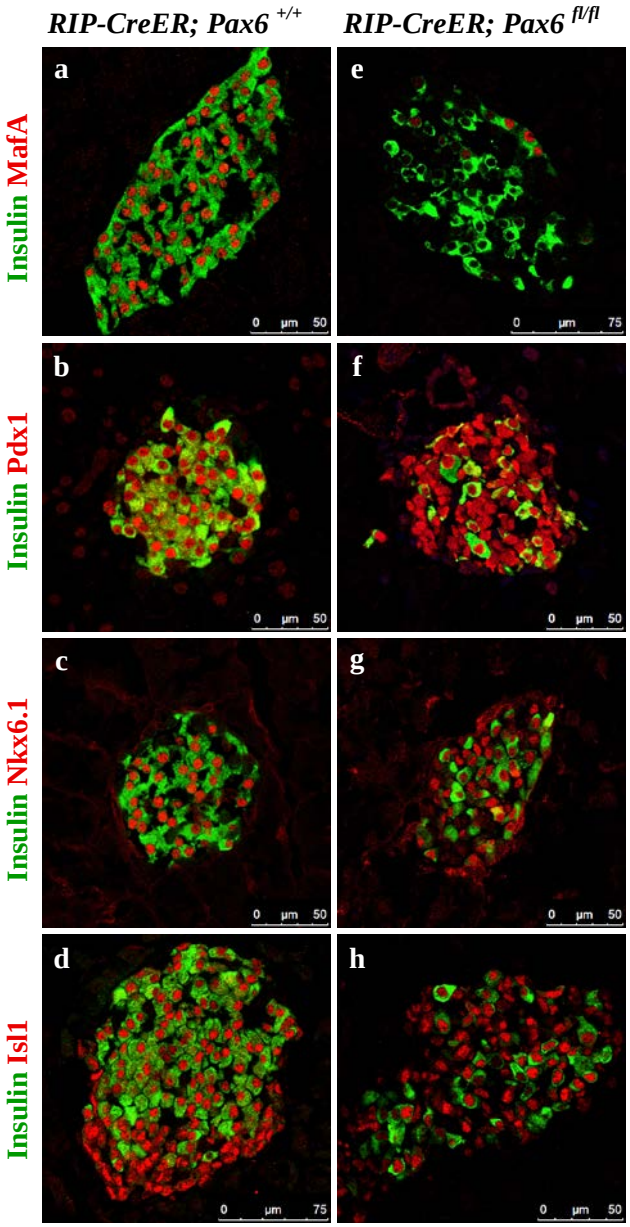

Figure 3.7: Expression of beta-cell related transcription factors in the islets of beta-cell-specific *Pax6* KO mice. Double immunofluorescence staining of pancreatic cryosections from 2.5 month old mice at 2 weeks after tamoxifen induction. Expression of MafA is lost (e) and that of Pdx1, Nkx6.1, and Isl1 (f-h) is maintained in the islets of beta-cell-specific *Pax6* KO pancreata.

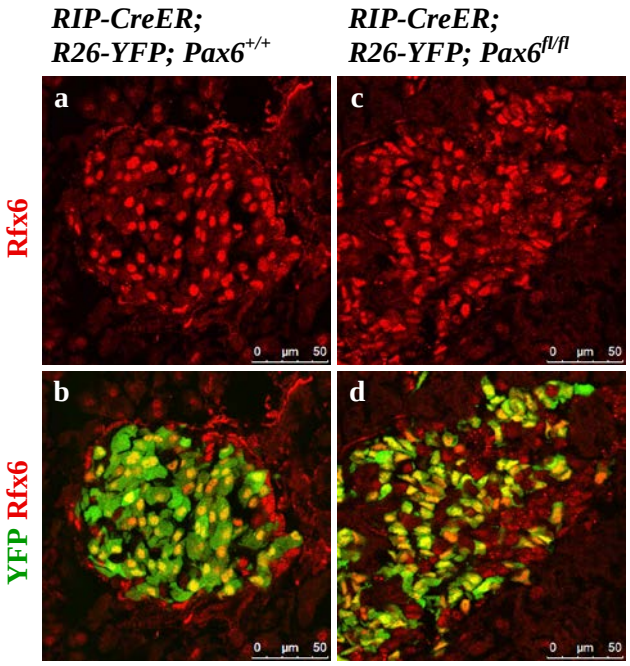

Figure 3.8: Expression of Rfx6 in the islets of beta-cell-specific *Pax6* KO mice. Immunofluorescence staining of pancreatic cryosections from 6 month old mice at 4.5 month after tamoxifen induction. Expression of Rfx6 is maintained in the YFP^{+} *Pax6*-deficient cells of the beta-cell-specific *Pax6* KO islets (c,d).

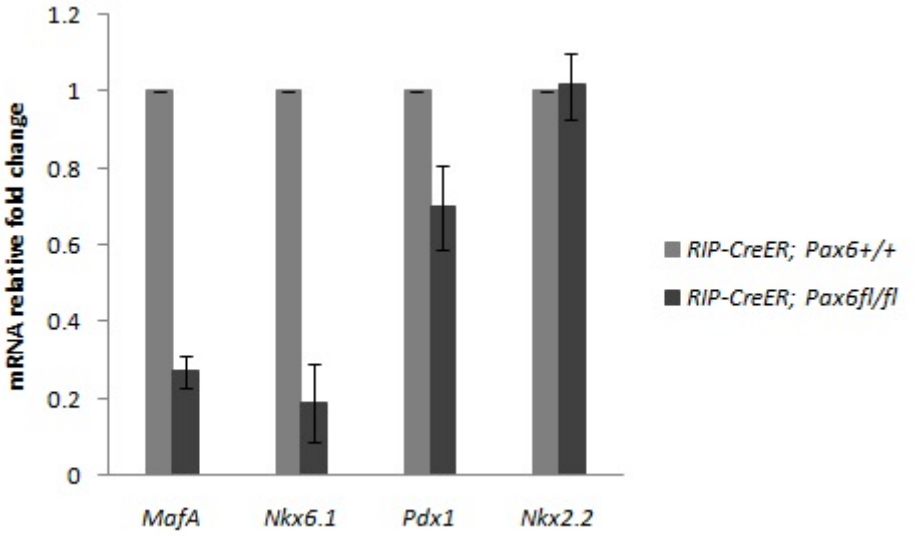

Figure 3.9: Expression of beta-cell related transcription factors in the pancreata of beta-cell-specific *Pax6* KO mice. Quantitative RT-PCR of *MafA*, *Nkx6.1*, *Pdx1*, and *Nkx2.2* mRNA in the pancreata of 4.5 month old mice at 3 months after tamoxifen induction (n=2). Error bars represent SEM.

reduced at the later stage (Figure 3.7 and 3.9). The expression of MafA, Pdx1, Nkx6.1, and Nkx2.2 was also checked by qRT-PCR. Here the expression of MafA and Nkx6.1 was found to be reduced, Pdx1 expression was slightly reduced and that of Nkx2.2 not affected at all (Figure 3.9).

3.1.5 Expression of Glut2 and GLP-1 receptor is lost after ablation of *Pax6* in beta-cells

Glut2 is a glucose transporter in beta-cells that plays an important role in glucose-stimulated insulin secretion. Loss of *Glut2* disturbs the first phase of glucose-stimulated insulin secretion (Guillam et al., 1997). Shortly after tamoxifen induction the expression of Glut2 was found to be lost in the islets of beta-cell-specific *Pax6* KO mice (Figure 3.10). Additionally, when checked in the presence of YFP reporter, Glut2 expression was found to be lost in YFP^+ as well as in the YFP^- $insulin^+$ cell population of the KO islets (Figure 3.11). This indicates that the expression of Glut2 is either affected by *Pax6* ablation directly and/or indirectly by the resulting hyperglycemia. Effect of hyperglycemia on Glut2 expression has been reported before (Thorens et al., 1992).

Glucagon-like peptide 1 (GLP-1) released from the intestinal L cells is known to promote insulin secretion from beta-cells in a glucose dependent manner. The effect of GLP-1 is mediated through the GLP-1 receptor that is expressed on beta-cells (MacDonald et al., 2002). In beta-cell-specific *Pax6* KO islets the expression of GLP-1 receptor was lost. This loss was specific to the YFP labeled *Pax6*-deficient cells and a normal expression was found in the YFP^- $insulin^+$ cells (Figure 3.12). Loss of Glut2 and GLP-1 receptor expression indicates an obvious defect in the glucose-stimulated insulin secretion in the pancreata of beta-cell-specific *Pax6* KO mice.

3.1.6 Defective proinsulin processing in *Pax6*-deficient beta-cells

In beta-cells insulin is synthesized as a prohormone that is cleaved by prohormone convertases 1/3 and 2 (PC1/3 and PC2) to generate mature insulin peptide and C-peptide. C-peptide is released together with insulin at an equimolar concentration (Vasic and Walcher, 2012; Goodge and Hutton, 2000). A defect of proinsulin processing can decrease the amount of C-peptide produced and leads to a concomitant increase in the amount of proinsulin. We checked the expression of PC1/3, PC2, and C-peptide in the islets of beta-cell-specific *Pax6* KO mice. Expression of PC1/3 and PC2 was unchanged

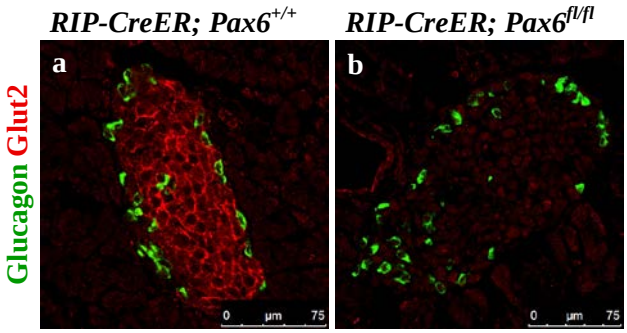

Figure 3.10: Expression of Glut2 in the islets of beta-cell-specific *Pax6* KO mice. Double immunofluorescence staining of pancreatic cryosections from 2.5 month old mice at 2 weeks after tamoxifen induction. Glut2 is expressed in beta-cells of the control islets (a). In the KO pancreata Glut2 expression is lost in the entire islet (b).

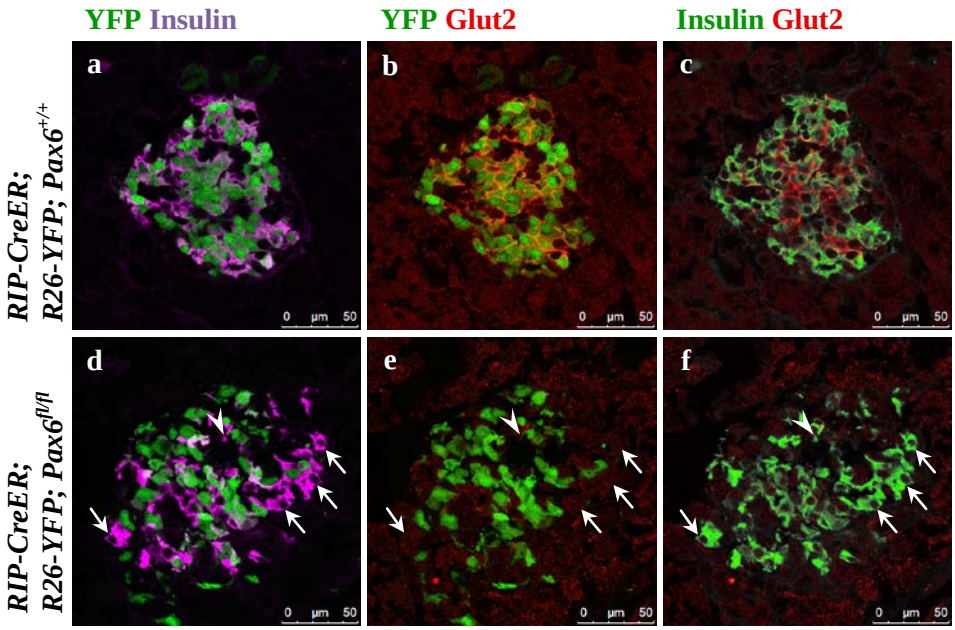

Figure 3.11: Loss of Glut2 expression due to direct and/or indirect effect of *Pax6* ablation in the islets of beta-cell-specific *Pax6* KO mice. Double immunofluorescence staining of pancreatic cryosections from 2 month old mice at 4 weeks after tamoxifen induction. In the control islets, expression of Glut2 is detected in both YFP^+ and YFP^- $insulin^+$ cells (a-c). In the beta-cell-specific *Pax6* KO islets, Glut2 expression is lost in the YFP labeled *Pax6*-deficient cells as well as in majority of the YFP^- $insulin^+$ cells (aroows d-f). Rarely, some YFP^- $insulin^+$ cells do express Glut2 in the KO islets (arrowhead d-f).

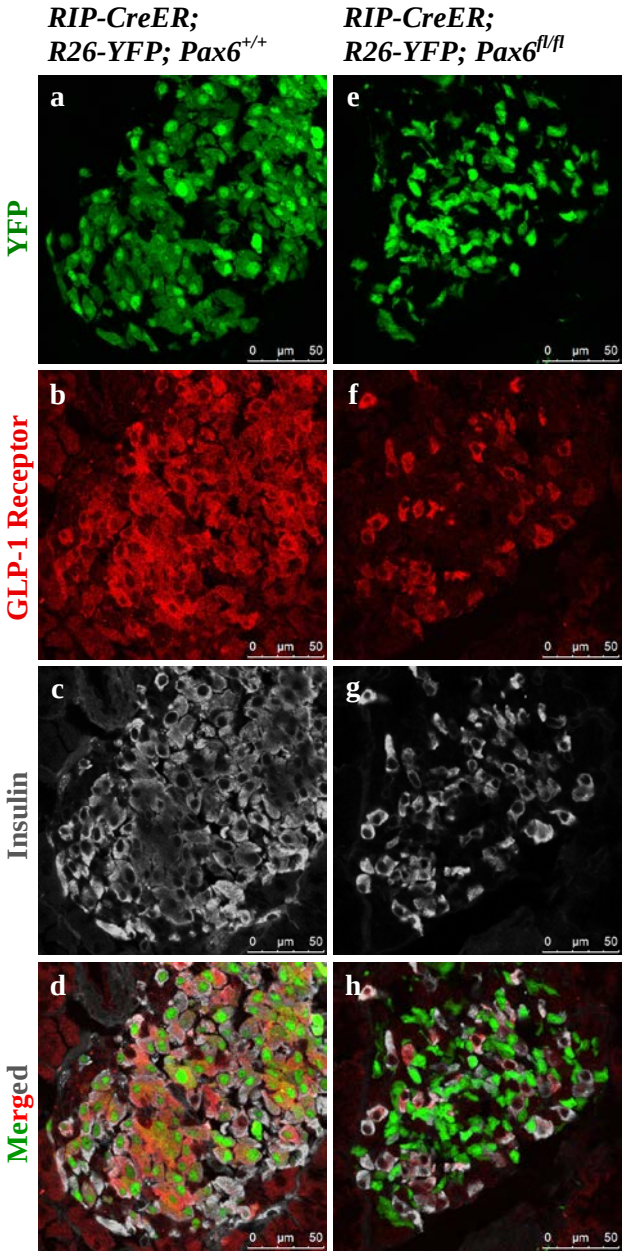

Figure 3.12: Expression of GLP-1 receptor in the islets of beta-cell-specific *Pax6* KO mice. Double immunofluorescence staining of pancreatic cryosections from 4 month old mice at 6 weeks after tamoxifen induction. GLP-1 receptor is expressed in both YFP^+ and YFP^- $insulin^+$ cells in the control islets (a-d). In the beta-cell-specific *Pax6* KO islets, GLP-1 receptor expression is lost from the YFP labeled *Pax6*-deficient cells but maintained in the YFP^- $insulin^+$ cells (e-h).

in the *Pax6*-deficient YFP labeled cells including those cells that had even stopped the expression of insulin (Figure 3.13). Interestingly, however, the expression of C-peptide was lost in the *Pax6*-deficient YFP labeled cells but was maintained in the YFP^- $insulin^+$ cells (Figure 3.14). This may indicate a defect in the normal processing of proinsulin that is not primarily due to an altered expression of prohormone convertases.

To point out the cause of defective proinsulin processing, we then checked the expression of proSAAS and 7B2. ProSAAS and 7B2 are the regulatory peptides of PC1/3 and PC2 respectively. proSAAS has been shown to inhibit the activity of PC1/3 and 7B2 has been shown to increase the activity of PC2 (Helwig et al., 2011; Liu et al., 2012). We found that the expression of both proSAAS and 7B2 was highly upregulated in the *Pax6*-deficient YFP labeled cells of the beta-cell-specific *Pax6* KO islets but the expression was similar to control islets in the YFP^- $insulin^+$ cells (Figure 3.15 and 3.16). Furthermore, this increase in the expression of proSAAS and 7B2 was not immediate but gradual after the induction of *Pax6* ablation. In the early days after tamoxifen induction, only few YFP labeled cells showed upregulated expression of proSAAS and 7B2. However, after long time following the tamoxifen induction nearly all of the YFP labeled cells expressed high levels of proSAAS and 7B2 (Figure 3.17).

Therefore, the defective proinsulin processing in *Pax6*-deficient beta-cells is mainly due to the increased level of proSAAS that would inhibit the activity of PC1/3.

3.1.7 $Ghrelin^+$ cells originate from beta-cells in the beta-cell-specific *Pax6* KO mice

Previous studies have shown that the $ghrelin^+$ cell population is expanded in the *Pax6* KO and $Pax6^{sey/sey}$ pancreata (Heller et al., 2005; Kordowich et al., 2011). This led to the conclusion that Pax6 somehow antagonizes the development of $ghrelin^+$ cells. As compared to the adult pancreas, $ghrelin^+$ cells are normally present at a higher number in the embryonic pancreas and further occur as a ghrelin only or ghrelin-glucagon double positive cell population (Prado et al., 2004). This makes it hard to exactly define the origin of upregulated $ghrelin^+$ cells in the embryonic pancreas of *Pax6* KO mice. Furthermore, as the *Pax6* KO mice die shortly after birth (St-Onge et al., 1997; Ashery-Padan et al., 2004), it makes it impossible to analyze the $ghrelin^+$ cells over a long period of time.

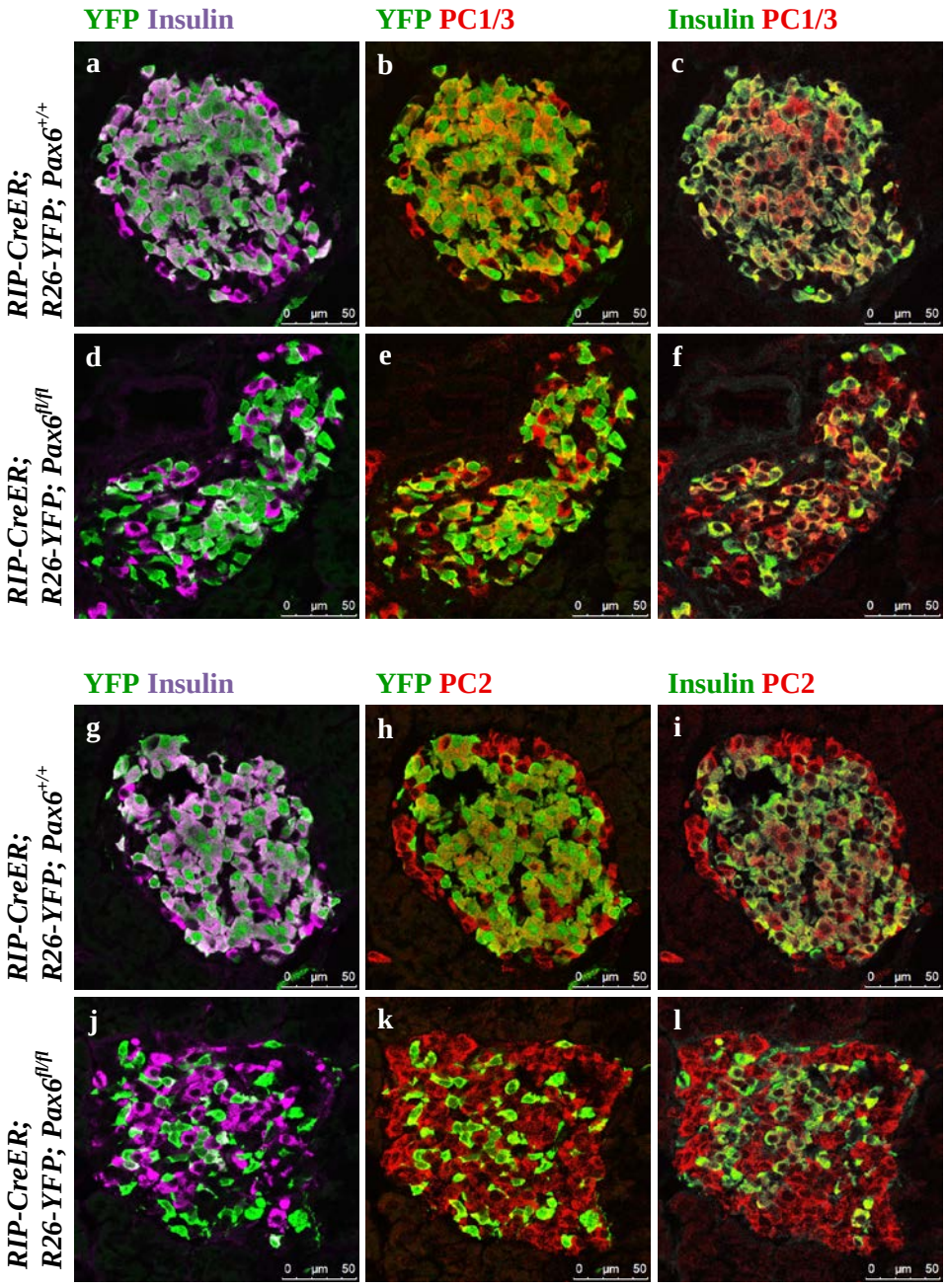

Figure 3.13: Expression of PC1/3 and PC2 in the islets of beta-cell-specific *Pax6* KO mice. Double immunofluorescence staining of pancreatic cryosections from 2 month old mice at 4 weeks after tamoxifen induction. In the control islets, expression of PC1/3 (a-c) and PC2 (g-i) is detected in both YFP^+ and YFP^- $insulin^+$ cells. In the beta-cell-specific *Pax6* KO islets, PC1/3 (d-f) and PC2 (j-l) expression is maintained in the YFP labeled *Pax6*-deficient cells, including those cells that have lost the expression of insulin.

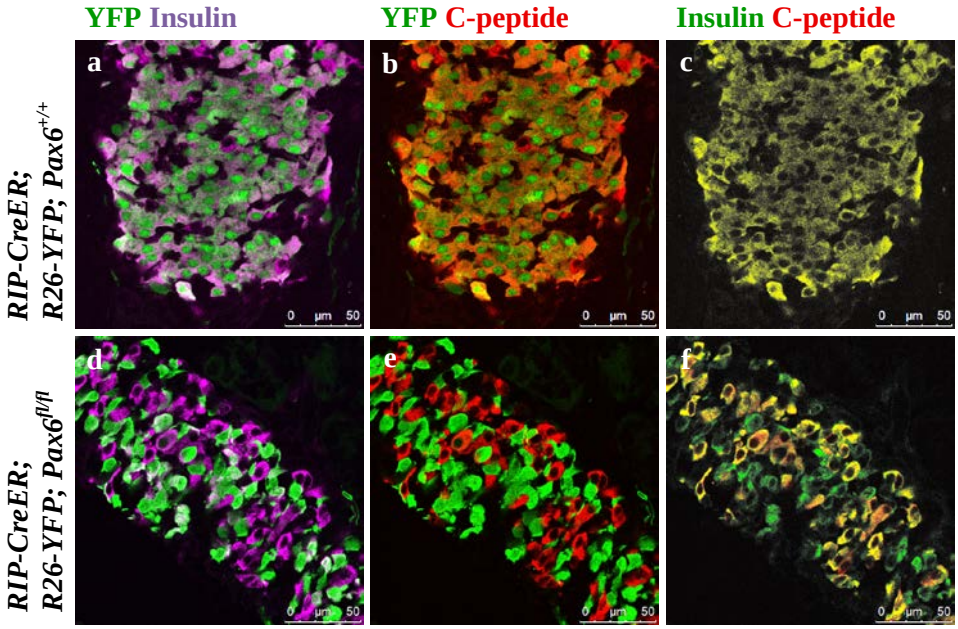

Figure 3.14: Expression of C-peptide in the islets of beta-cell-specific *Pax6* KO mice. Double immunofluorescence staining of pancreatic cryosections from 2 month old mice at 4 weeks after tamoxifen induction. In the control islets, expression of C-peptide is detected in both YFP^+ and YFP^- $insulin^+$ cells (a-c). In the beta-cell-specific *Pax6* KO islets, C-peptide expression is lost from the YFP labeled *Pax6*-deficient cells but maintained in the YFP^- $insulin^+$ cells (d-f).

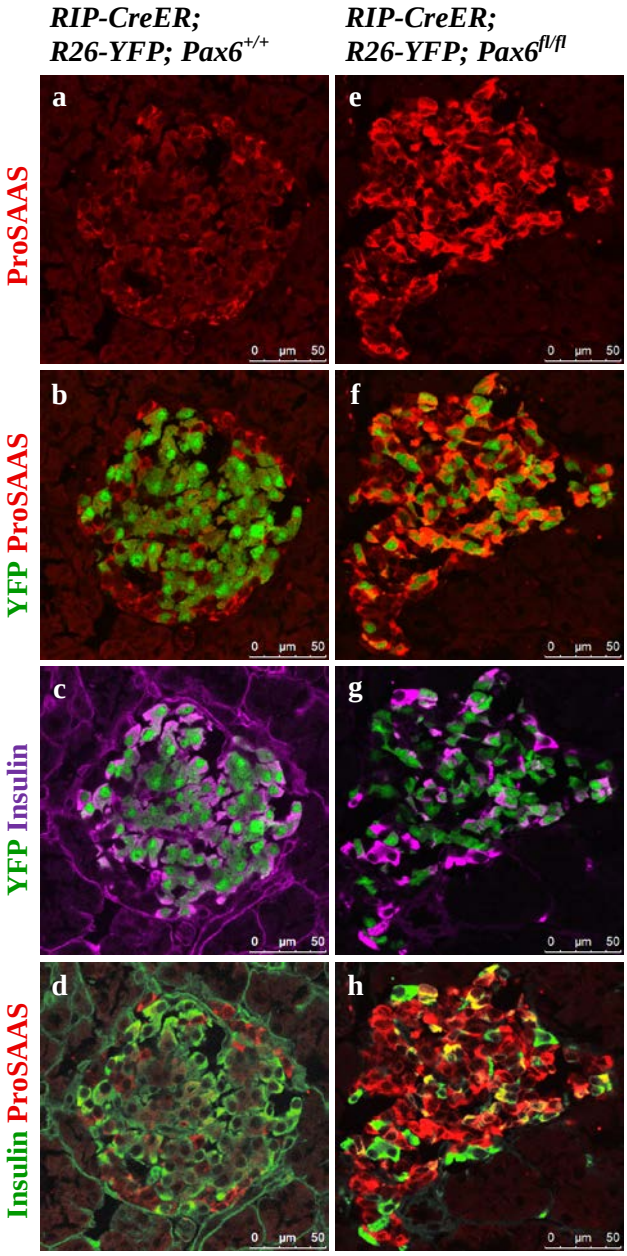

Figure 3.15: Expression of proSAAS in the islets of beta-cell-specific *Pax6* KO mice. Double immunofluorescence staining of pancreatic cryosections from 4 month old mice at 6 weeks after tamoxifen induction. In the control islets, expression of proSAAS is low in the insulin$^+$ cells (a-d). In the beta-cell-specific *Pax6* KO islets, expression of proSAAS is highly upregulated in the YFP labeled *Pax6*-deficient cells (e,f) but remains low in the YFP$^-$ insulin$^+$ cells (g,h).

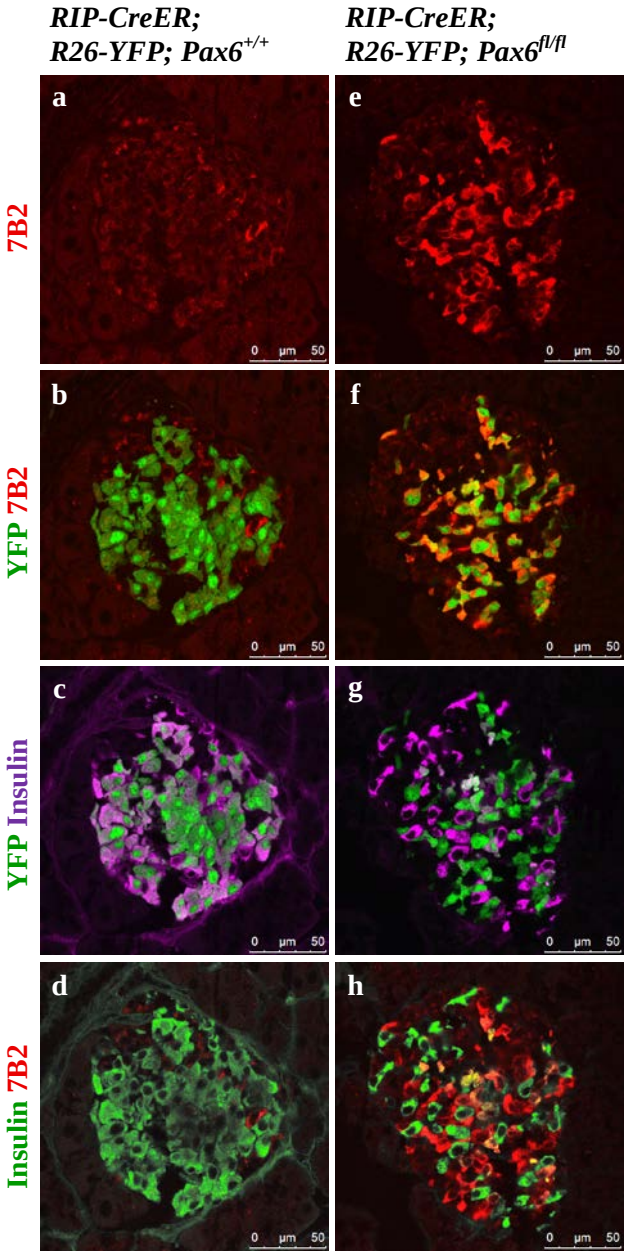

Figure 3.16: Expression of 7B2 in the islets of beta-cell-specific *Pax6* KO mice. Double immunofluorescence staining of pancreatic cryosections from 4 month old mice at 6 weeks after tamoxifen induction. In the control islets, expression of 7B2 is low in the insulin^{+} cells (a-d). In the beta-cell-specific *Pax6* KO islets, expression of 7B2 is highly upregulated in the YFP labeled *Pax6*-deficient cells (e,f) but remains low in the YFP^{-} insulin^{+} cells (g,h).

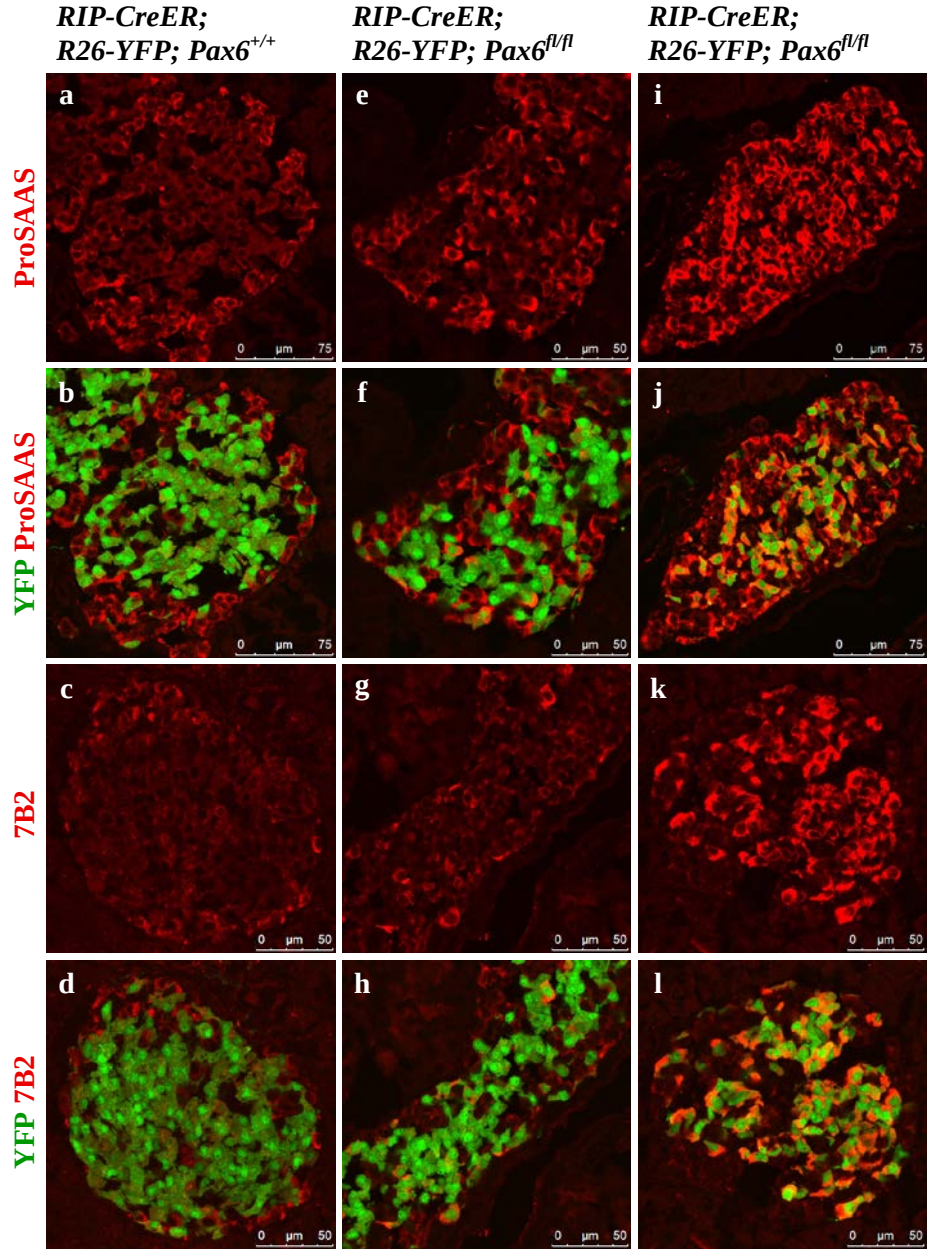

Figure 3.17: Gradual increase in the population of proSAAS and 7B2 expressing cells in the islets of beta-cell-specific *Pax6* KO mice. Immunofluorescence staining of pancreatic cryosections from 4 week old mice at 7 days after tamoxifen induction (a-h) and 9 week old mice at 6 weeks after tamoxifen induction (i-l). Expression of proSAAS (a,b) and 7B2 (c,d) is very low in the YFP labeled beta-cells of the control islets. At 7 days after tamoxifen induction few YFP labeled *Pax6*-deficient cells start to express proSAAS (e,f) and 7B2 (g,h) at a higher level in the KO islets. At 6 weeks after tamoxifen induction majority of the YFP labeled *Pax6*-deficient cells express high levels of proSAAS (i,j) and 7B2 (k,l) in the KO islets.

Therefore, an important aim of generating the adult beta-cell-specific *Pax6* KO mice was to analyze the ghrelin$^+$ cell population in a greater detail. Similar to the previous studies of *Pax6* KO, we found an increase in the number of ghrelin$^+$ cells in the beta-cell-specific *Pax6* KO islets compared to the control islets where ghrelin expression was undetectable. Double immunofluorescence staining of ghrelin with insulin showed some ghrelin-insulin co-positive cells indicating the possible origin of ghrelin$^+$ cells from insulin producing beta-cells (Figure 3.18). To confirm it further, we checked the expression of various beta-cell related factors in the ghrelin$^+$ cells. Ghrelin$^+$ cells expressed Pdx1, Nkx6.1, and Isl1 but were negative for the expression of Pax6 (Figure 3.19). Ghrelin expression was also co-localized with that of islet amyloid polypeptide (IAPP) and PC1/3 but was never co-localized with the expression of glucagon (Figure 3.20). The absence of Pax6 and the presence of several beta-cell related markers in the ghrelin$^+$ cells, therefore, strongly indicated the direct conversion of *Pax6*-deficient beta-cells into ghrelin expressing cells. Additionally, we noticed that the expression of IAPP was completely normal in the islets of beta-cell-specific *Pax6* KO mice (Figure 3.20a,d). IAPP is another peptide hormone released from beta-cells that can modify the secretion and function of insulin (Hull et al., 2004).

Lastly, we took advantage of the YFP reporter system to exactly define the origin of ghrelin$^+$ cells in the beta-cell-specific *Pax6* KO islets. As expected, all of the ghrelin expression co-localized with the YFP labeled *Pax6*-deficient beta-cells. Most of these YFP$^+$ ghrelin$^+$ cells were negative for the expression of insulin but it was possible to find many cells that were ghrelin-insulin-YFP triple positive indicating the intermediate conversion state from insulin to ghrelin expressing cell (Figure 3.21d-f). Based on these observations, we concluded that the ablation of *Pax6* from beta-cells results in the downregulation of insulin expression and upregulation of ghrelin expression in these cells.

3.1.8 Tracing the fate of *Pax6*-deficient beta-cells over long period of time

So far we observed that the beta-cells lose their mature phenotype upon deletion of *Pax6* and start the expression of ghrelin. Now the interesting question is that what happens to these immature *Pax6* deficient beta-cells over time. As mentioned before, the ghrelin expression gradually increased following the ablation of *Pax6* (Figure 3.3a and 3.4).

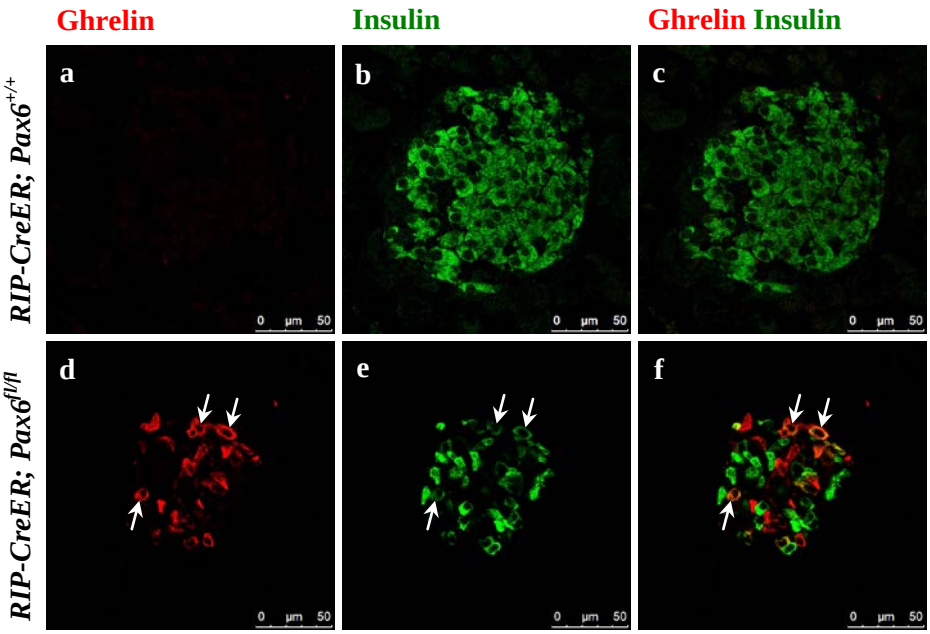

Figure 3.18: Co-expression of ghrelin with insulin in the islets of beta-cell-specific *Pax6* KO mice. Double immunofluorescence staining of pancreatic cryosections from 2.5 month old mice at 2 weeks after tamoxifen induction. Ghrelin^{+} cells are not detected in the control islets (a-c). In the beta-cell-specific *Pax6* KO islets, ghrelin expression is upregulated and some of the ghrelin^{+} cells co-express insulin (arrows d-f).

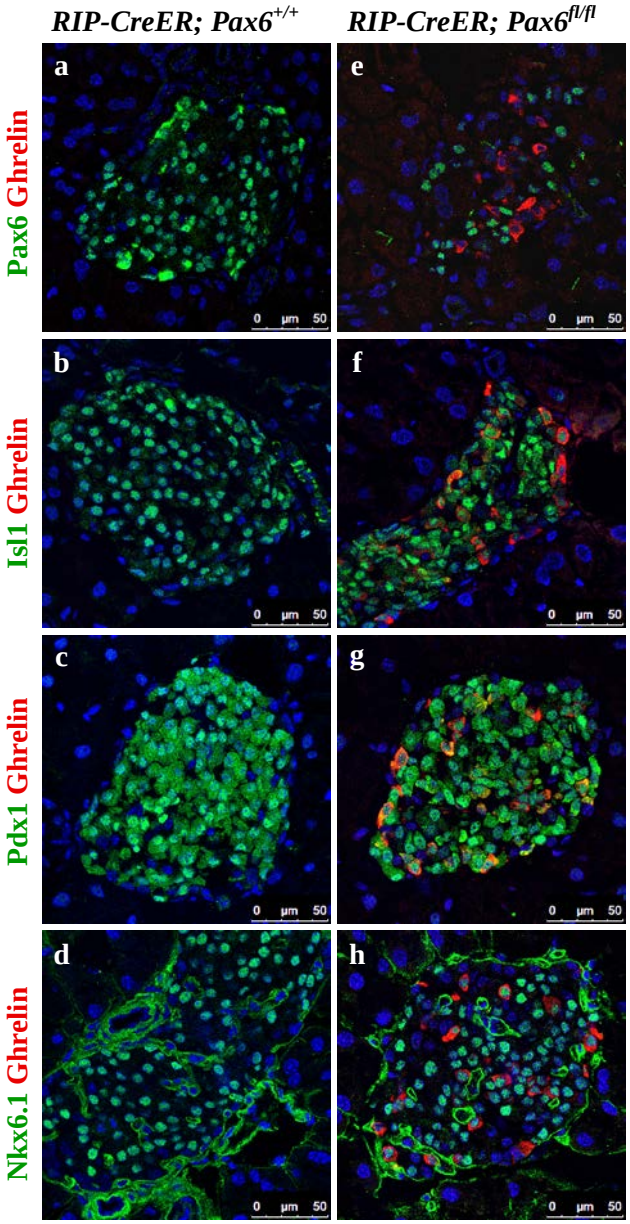

Figure 3.19: Expression of beta-cell related transcription factors in the ghrelin^{+} cell population of the beta-cell-specific *Pax6* KO islets. Double immunofluorescence staining of pancreatic cryosections from 2.5 month old mice at 2 weeks after tamoxifen induction. Ghrelin expression is not detected in the control islets (a-d). In the beta-cell-specific *Pax6* KO islets, ghrelin expression is upregulated and these ghrelin^{+} cells are negative for Pax6 (e) but positive for Isl1 (f), Pdx1 (g), and Nkx6.1 (h) expression.

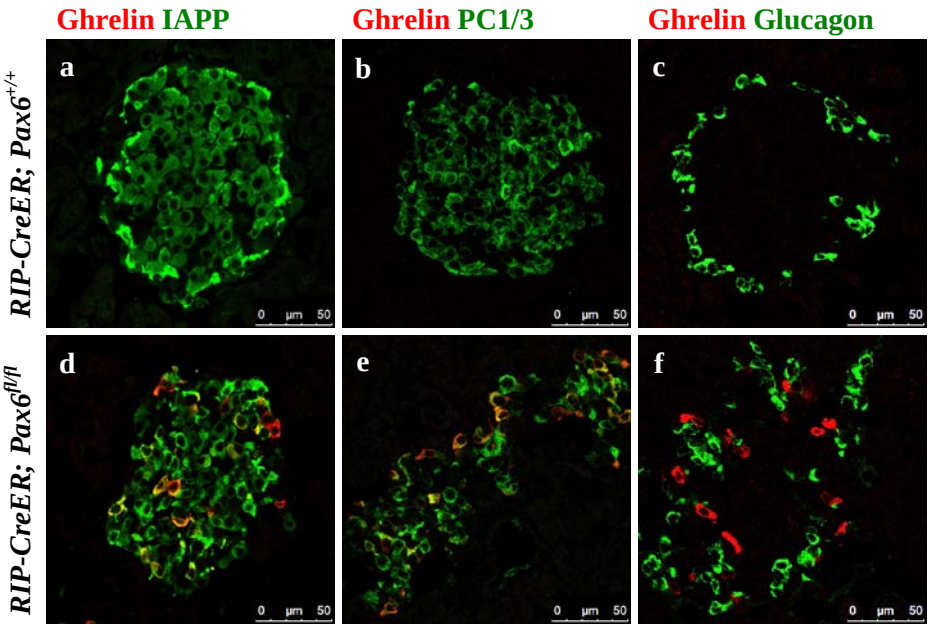

Figure 3.20: Co-expression of ghrelin with beta-cell related factors in the islets of beta-cell-specific *Pax6* KO mice. Double immunofluorescence staining of pancreatic cryosections from 2.5 month old mice at 2 weeks after tamoxifen induction. Ghrelin$^+$ cells are not detected in the control islets (a-c). In the beta-cell-specific *Pax6* KO islets, ghrelin expression is upregulated and these ghrelin$^+$ cells co-express IAPP (d) and PC1/3 (e). Also note that IAPP and PC1/3 expression is maintained in the KO islets (d,e). Ghrelin$^+$ cells never co-express glucagon in the beta-cell-specific *Pax6* KO islets (f).

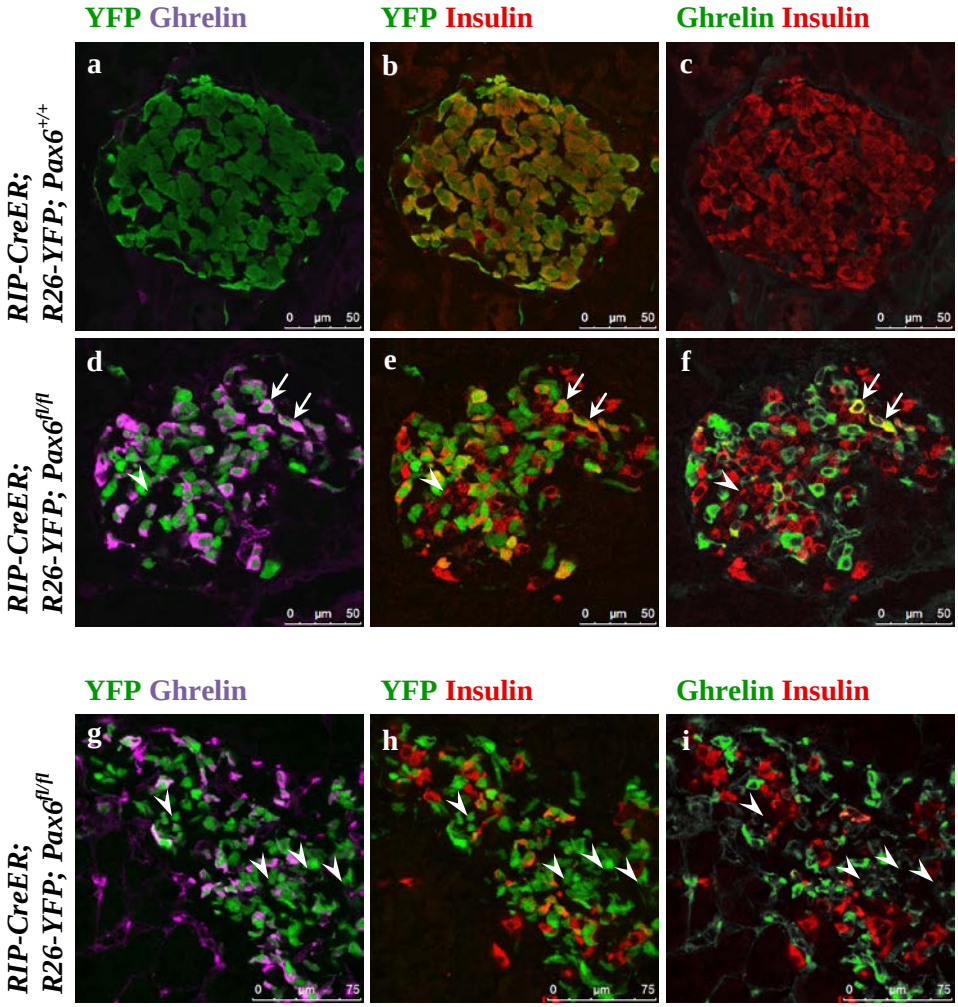

Figure 3.21: Ghrelin$^+$ cells originate from beta-cells in the beta-cell-specific *Pax6* KO islets. Double immunofluorescence staining of pancreatic cryosections from 4 month old mice at 6 weeks after tamoxifen induction (a-f) and 6 month old mice at 4.5 months after tamoxifen induction (g-i). Ghrelin expression is not detected in the control islets (a-c). In the beta-cell-specific *Pax6* KO islets, ghrelin expression is upregulated in the YFP labeled *Pax6*-deficient cells (d-f). Most of the ghrelin$^+$ cells have lost the expression of insulin but some are still insulin$^+$ (arrows d-f). Some YFP$^+$ cells in the KO islets are negative for both insulin and ghrelin (arrowheads d-f). At 4.5 months after tamoxifen induction many of the YFP$^+$ cells are negative for both insulin and ghrelin expression (arrowheads g-i).

Quantification of YFP^+ $ghrelin^+$ cells further clarified that ghrelin expression is upregulated in majority, but not all, of the YFP^+ *Pax6*-deficient beta-cells (Figure 3.22a). Moreover, double immunofluorescence staining with insulin and ghrelin antibodies in the presence of YFP reporter indicated a population of $insulin^-$ $ghrelin^-$ YFP^+ cells at various time points after tamoxifen induction. The number of such cells was, however, higher in the islets of those mice that were kept for the longest time-period after tamoxifen induction (upto 4.5 month) (Figure 3.21g-i and 3.22b). This indicated two things. Firstly, not all of the *Pax6*-deficient beta-cells upregulate ghrelin expression even after they have lost the expression of insulin. Secondly, some of the *Pax6*-deficient beta-cells that upregulate the expression of ghrelin, lose the high level of ghrelin expression over time.

Another possibility is that the *Pax6*-deficient beta-cells may change their fate to another cell type in the pancreas. However, we found that the YFP labeled cells in the beta-cell-specific *Pax6* KO islets were positive for Rfx6, and Pdx1 (Figure 3.7 and 3.8) and they never co-localized with glucagon, somatostatin, or PP expressing cells (data not shown). This means that *Pax6*-deficient beta-cells retain their endocrine character as they are positive for Rfx6 that is an endocrine specific transcription factor in the adult pancreas (Smith et al., 2010; Soyer et al., 2010). Secondly, they do not convert to other endocrine cell types as they continue to express Pdx1 that is specific to beta-cells in the adult pancreas (Ahlgren et al., 1998).

Next, by quantification we found that the number of YFP labeled cells was always less in the beta-cell-specific *Pax6* KO islets compared to the control islets when checked at few weeks post tamoxifen induction. To identify the reason behind this we checked the proliferation and apoptosis in the KO islets. By TUNEL staining we found no increased apoptosis in the KO islets (data not shown). However, we observed that the YFP labeled *Pax6*-deficient cells were never positive for Ki67 compared to the control islets where YFP^+ $Ki67^+$ cells were frequently observed at seven weeks of age (Figure 3.23). From this we concluded that the obvious difference in the number of YFP^+ cells between the control and the KO islets is mainly due to the lack of proliferation in the *Pax6*-deficient beta-cells.

3.1.9 *Ghrelin* and *Glut2* are not the direct downstream targets of Pax6

Ghrelin is upregulated and Glut2 downregulated in *Pax6* KO beta-cells. To see if Pax6 directly regulates the expression of these two genes, we searched for the in silico Pax6

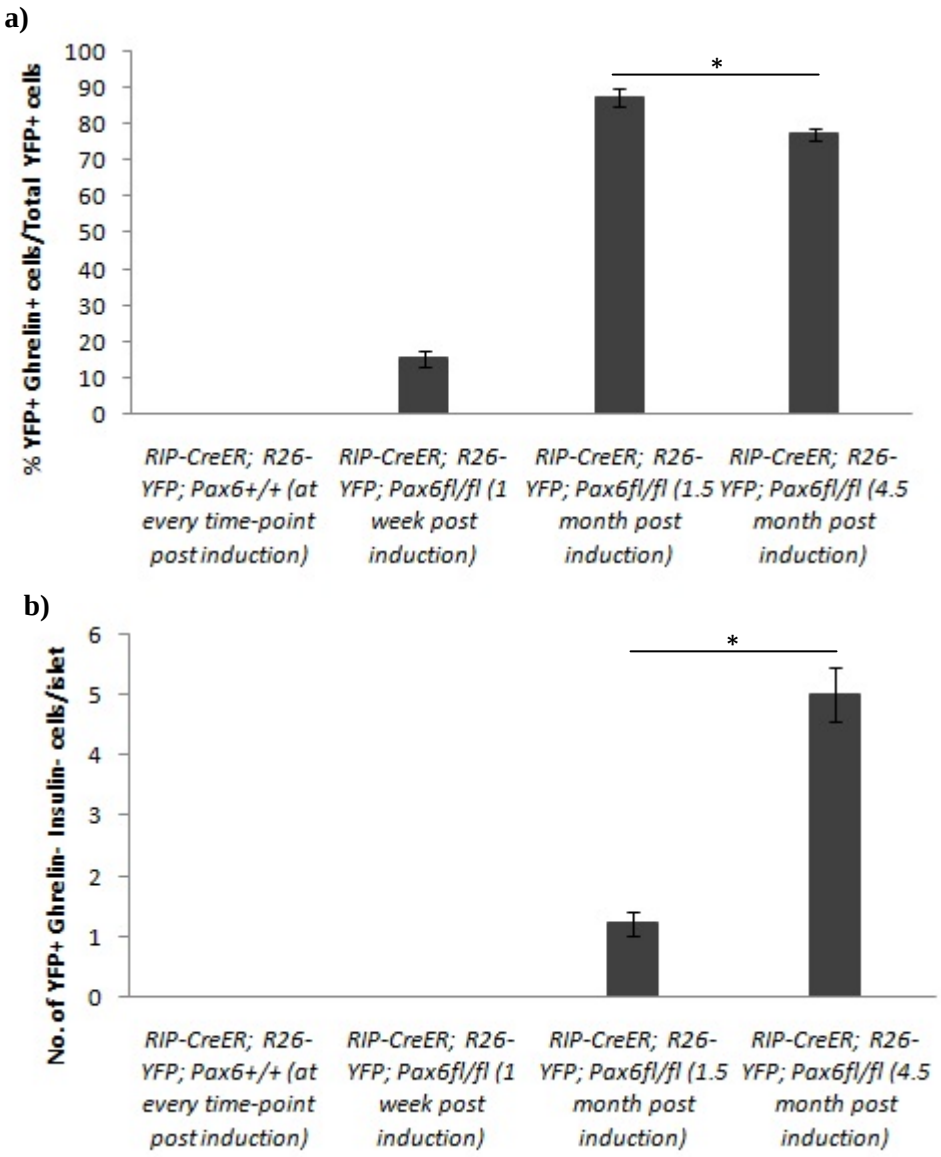

Figure 3.22: Changes in the ghrelin expressing cell population over time in the islets of beta-cell-specific *Pax6* KO mice. Quantification of YFP^+ $ghrelin^+$ cells in relation to the total YFP^+ cells (a) and quantification of YFP^+ $ghrelin^-$ $insulin^-$ cells (b) in the islets of control and beta-cell-specific *Pax6* KO mice injected with tamoxifen at 1.5 months of age and analyzed at various time points indicated (n=3). (a) Ghrelin expressing cell population in the KO islets expands over time but ghrelin expression is never detected in 100% of YFP^+ *Pax6*-deficient cells indicating that a certain number of beta-cells do not express ghrelin after the loss of *Pax6*. (b) The number of YFP^+ $ghrelin^-$ $insulin^-$ cells in the KO islets increases over time indicating the conversion of some $insulin^+/ghrelin^+$ or insulin-ghrelin co-positive cells to hormone negative YFP-only positive cells. Error bars represent SEM; *$p<0.05$.

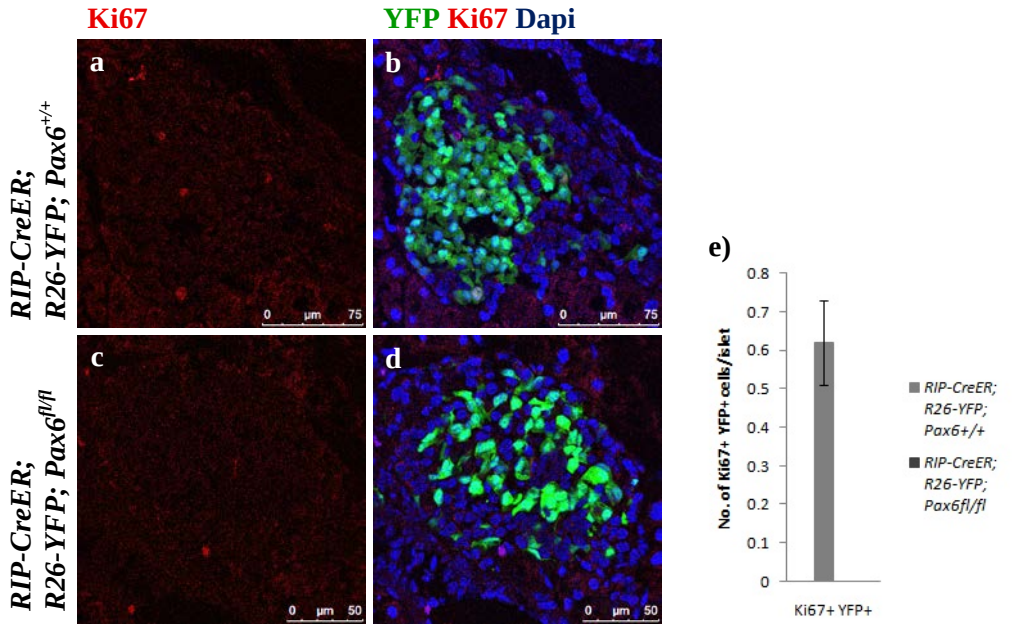

Figure 3.23: *Pax6*-deficient beta-cells do not proliferate. Immunofluorescence staining of pancreatic cryosections from 7 week old mice at 4 weeks after tamoxifen induction. $Ki67^+$ YFP^+ cells are detected in the control islets (a,b) but not in the beta-cell-specific *Pax6* KO islets (c,d). Quantification of $Ki67^+$ YFP^+ cells in the islets of 7 week old mice at 4 weeks after tamoxifen induction (n=3). Error bars represent SEM.

binding sites in the promoters of these two genes by using MatInspector (http://www.genomatix.de/). In silico binding sites were found in both promoters. Next we performed ChIP assay to check the actual binding of Pax6 with the identified promoter regions. The ChIP assay performed on Min6 cells did not show any binding of Pax6 with *ghrelin* or *Glut2* promoter (Figure 3.24). Pax6, however, did show binding to the region3 of MafA promoter that was used as a positive control (Raum et al., 2010). Therefore, the upregulation of ghrelin and loss of Glut2 in *Pax6* KO islets occurs through some indirect pathway.

3.1.10 Beta-cell regeneration in the beta-cell-specific *Pax6* KO islets

Regeneration of beta-cells in the diabetic mouse models is an interesting topic of research. As the beta-cell-specific *Pax6* KO mice developed hyperglycemia, we were also interested in looking for the possibility of beta-cell regeneration. For this purpose, we injected the mice (containing YFP reporter) with tamoxifen at 3 weeks and 1.5 months of age and analyzed the pancreata at 10 weeks and 4.5 months post tamoxifen induction, respectively. During this time period the non-fasting blood glucose level was also monitored over regular intervals. In both cases the blood glucose level started to rise until it reached a plateau. Afterwards, the blood glucose level slightly decreased in the mice that were injected at 3 weeks of age. However, in the mice that were injected at 1.5 months of age, no decrease was observed in the blood glucose level (Figure 3.25a,b). This showed a slight, but not complete, recovery from hyperglycemia in the mice injected at 3 weeks but not in the mice injected at 1.5 months.

To check for the regeneration of beta-cells at the cellular level, immunofluorescence staining was performed with insulin antibody on the pancreatic sections from these 3 weeks and 1.5 months injected mice. Immediately following the tamoxifen injection nearly 5% of the insulin$^+$ cells were YFP$^-$, showing the cells that escaped recombination (data not shown). However, long time after the tamoxifen induction an increase in the number of insulin$^+$ YFP$^-$ cells was observed indicating some regeneration of beta-cells. This increase in the number of insulin$^+$ YFP$^-$ cells was higher in the mice that were injected at 3 weeks of age as compared to the ones injected at 1.5 months of age (Figure 3.26a,b). This result is also in accordance with the levels of blood glucose. Thus, we conclude that following the ablation of *Pax6* some degree of beta-cell regeneration does

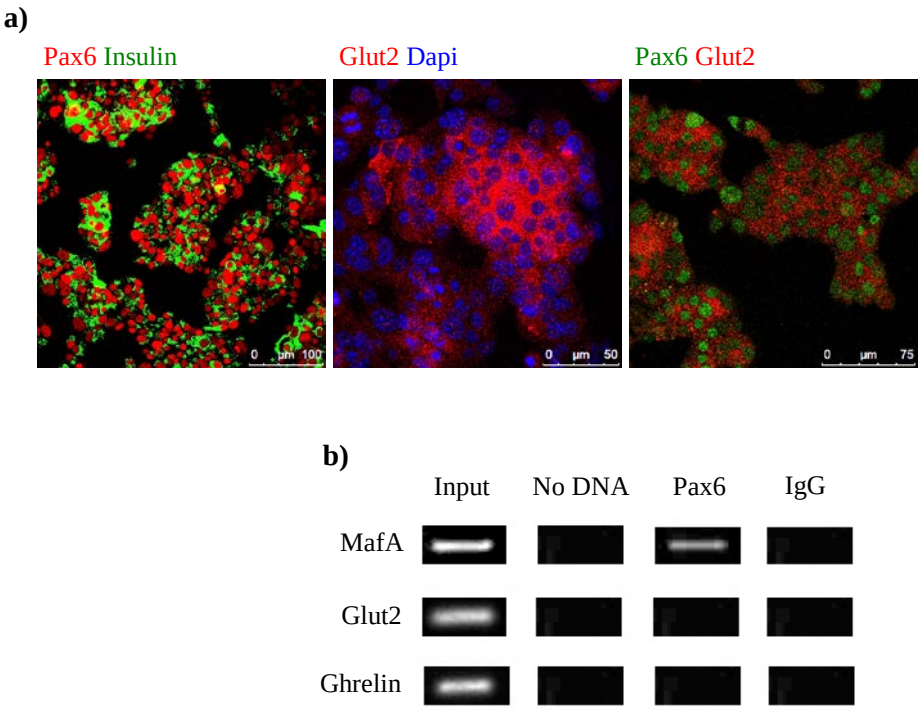

Figure 3.24: Pax6 does not bind to *Glut2* and *ghrelin* promoters. Min6 cells that express Pax6, insulin, and Glut2 were used for ChIP assay (a). Cross-linked chromatin from Min6 cells was precipitated by anti-Pax6 antibody and analyzed by PCR for the corresponding promoter regions (detail in material and methods) (b). Pax6 interacts with *MafA* promoter region 3 but not with *Glut2* and *ghrelin* promoters.

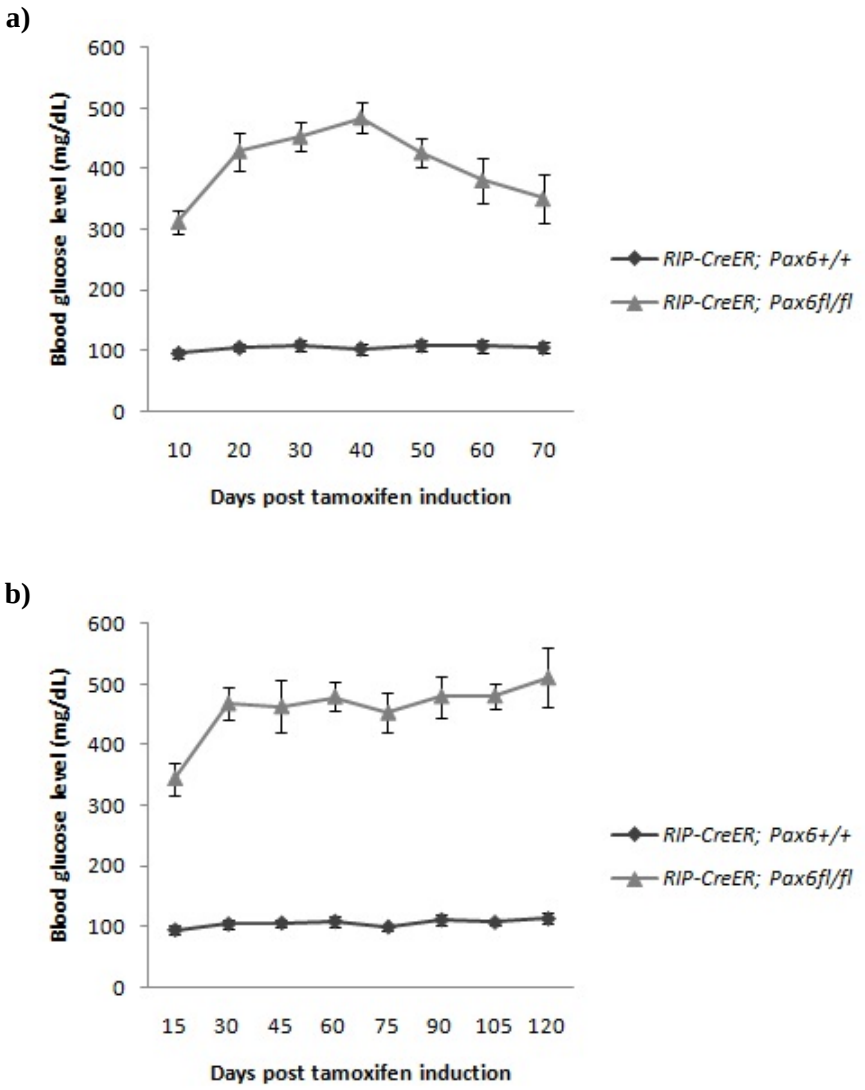

Figure 3.25: Development of diabetes in the beta-cell-specific *Pax6* KO mice. Measurement of blood glucose level in mice after tamoxifen induction at 3 weeks (a) or 1.5 month (b) (n=5). Increase in blood glucose level is observed at either age but a slight decrease in the long term is only seen in young mice. Error bars represent SEM.

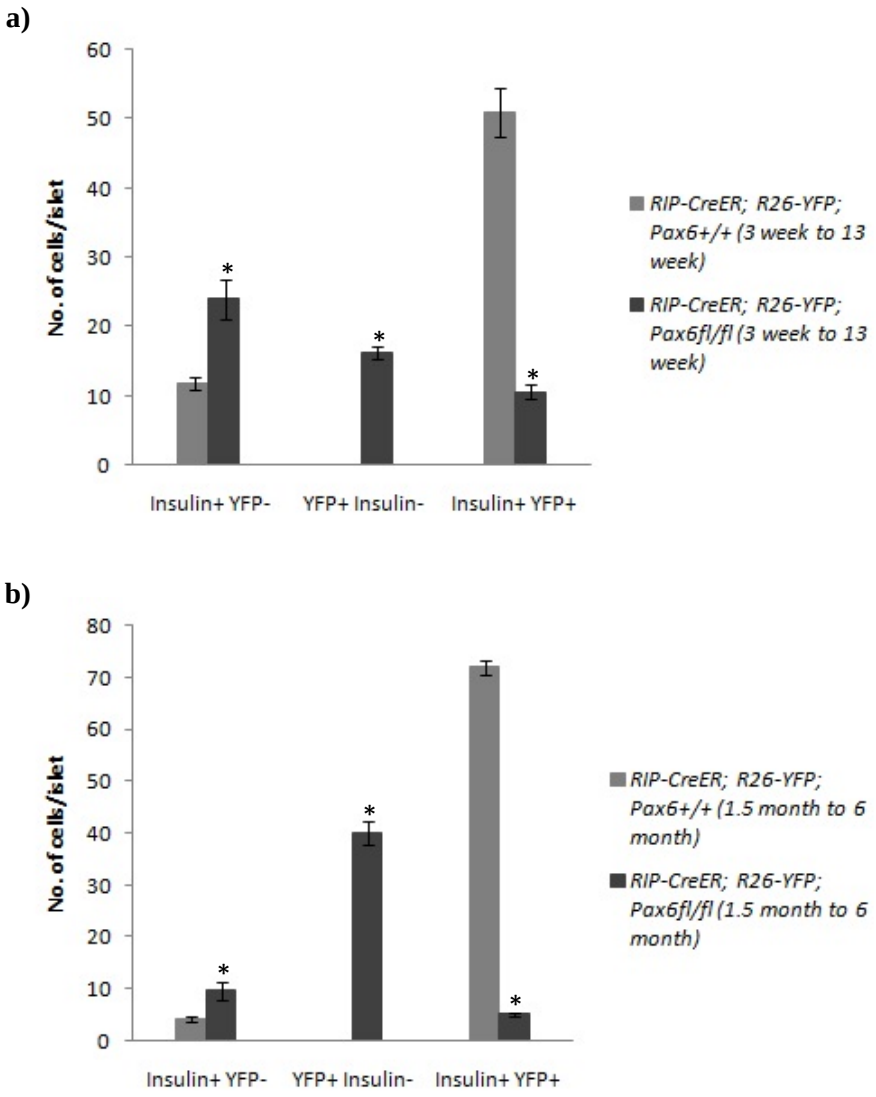

Figure 3.26: Beta-cell regeneration in the beta-cell-specific *Pax6* KO mice. Quantification of insulin$^+$ YFP$^-$, YFP$^+$ insulin$^-$, and insulin$^+$ YFP$^+$ cells in the islets of 13 week old mice at 10 weeks after tamoxifen induction (a) and 6 month old mice at 4.5 month after tamoxifen induction (b) (n=3). Number of insulin$^+$ YFP$^-$ cells is increased at either age indicating some beta-cell regeneration. Error bars represent SEM; *p<0.05.

occur and this is more pronounced in the young 3 week old mice compared to the older 1.5 month old mice.

These new beta-cells may arise from proliferation of existing beta-cells that escaped recombination or by transdifferentiation and/or neogenesis (Ahmad, 2013). As compared to the control islets an increased proliferation was not observed in the KO islets. However, compared to the 1.5 month injected mice an increased proliferation was observed in the YFP^- $insulin^+$ cells of the 3 weeks injected mice at 4 weeks post tamoxifen induction (Figure 3.27). Furthermore, in both young (3 week) and older (1.5 month) injected mice $Pdx1^+$ $glucagon^+$ cells or $MafB^+$ $insulin^+$ cells were occasionally observed (Figure 3.28). Pdx1 expressing $glucagon^+$ cells may indicate alpha-cells transdifferentiating to beta-cells (Thorel et al., 2010). On the other hand, MafB expression in some $insulin^+$ cells can indicate the presence of newly generated $insulin^+$ cells because MafB is expressed in immature $insulin^+$ cells during embryonic development (Artner et al., 2007). Therefore, in both young and older *Pax6* KO mice new beta-cells may arise from alternative sources.

Regeneration of beta-cells in the beta-cell-specific *Pax6* KO mice is, however, not sufficient to allow for the complete recovery from diabetes.

3.2 Analysis of alpha-cell-specific *Pax6* knockout pancreata

3.2.1 Conditional knockout of *Pax6* in the pancreatic alpha-cells

In the classical as well as pancreas-specific conditional *Pax6* KO mice the number of glucagon positive alpha-cells is drastically reduced (St-Onge et al., 1997; Ashery-Padan et al., 2004). It does show the essential role of Pax6 in the development of alpha-cells. However, as these mice die shortly after birth it does not allow analyzing the role of Pax6 in alpha-cells later in life. Furthermore, if *Pax6* is knocked out from the whole islet then the resulting phenotype cannot be specifically linked to the *Pax6*-deficient alpha-cells. Therefore, to investigate the role of Pax6 in alpha-cell population, we decided to ablate *Pax6* from alpha-cells alone. In order to generate the alpha-cell-specific *Pax6* KO mice, *Pax6* floxed mouse line ($Pax6^{fl/fl}$) (Ashery-Padan et al., 2000) was crossed with g*lucagon-Cre* mouse line (*Glu-Cre*) (Herrera, 2000). Additionally, we incorporated the YFP reporter to identify and trace the Cre-recombined cells (Figure 3.1). This was achieved by including an additional cross with *R26-YFP* reporter mouse line (Srinivas et al., 2001).

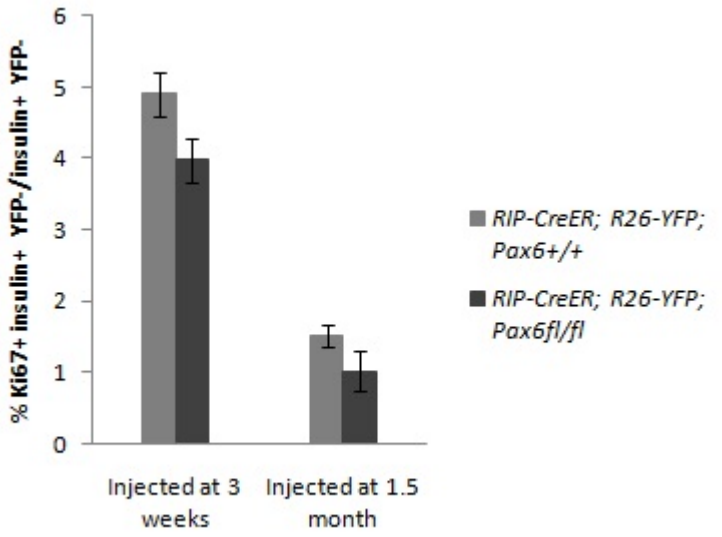

Figure 3.27: Proliferation in the regenerating population of beta-cells in the beta-cell-specific *Pax6* KO islets. Quantification of $Ki67^{+}$ $insulin^{+}$ YFP^{-} cells at 4 weeks after tamoxifen induction (n=3). As compared to the control islets, increased proliferation is not seen in the KO islets. Error bars represent SEM.

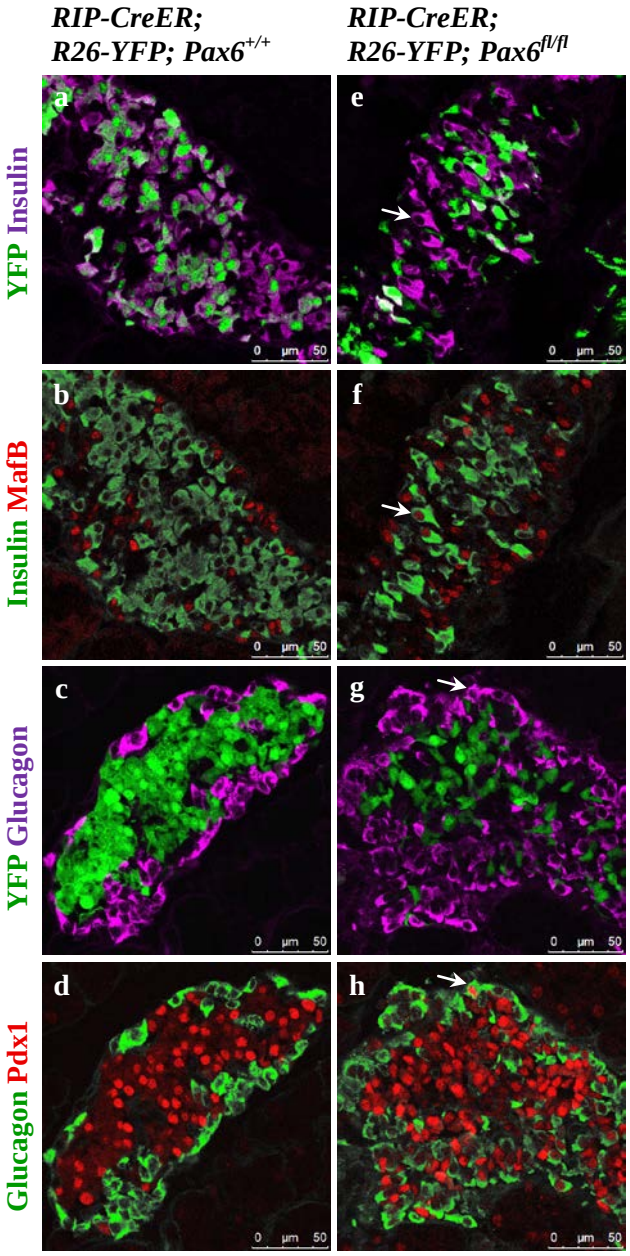

Figure 3.28: Beta-cell regeneration in the beta-cell-specific *Pax6* KO islets may occur via transdifferentiation and/or neogenesis. Double immunofluorescence staining of pancreatic cryosections from 2 month old mice at 4 weeks after tamoxifen induction. In the control islets, MafB is not expressed in the insulin$^+$ cells (a,b) and Pdx1 is not expressed in the glucagon$^+$ cells (c,d). In some of the beta-cell-specific *Pax6* KO islets, few insulin$^+$ cells express MafB (arrow e,f) and few glucagon$^+$ cells express Pdx1 (arrow g,h) that may indicate neogenesis of beta-cells and/or transdifferentiation of alpha- to beta-cells.

Successful ablation of *Pax6* from the Cre-recombined YFP labeled cells was confirmed by double immunofluorescence staining (Figure 3.29a-f). Quantification of YFP^+ $glucagon^+$ cells showed nearly 70% recombination efficiency with *glucagon-Cre* mouse line (Figure 3.29g).

3.2.2 Ghrelin$^+$ cells originate from alpha-cells in the alpha-cell-specific *Pax6* KO pancreata

3.2.2.1 Co-expression of ghrelin with glucagon in the alpha-cell-specific Pax6 KO pancreata

Similar to the previous *Pax6* KO studies (Kordowich et al., 2011; Hart et al., 2013), we found an increase in the number of $ghrelin^+$ cells in the alpha-cell-specific *Pax6* KO mice. As mentioned before, $ghrelin^+$ cells are present at a higher number in the embryonic pancreas. At this stage many of the $ghrelin^+$ cells are co-localized with the $glucagon^+$ cells (Prado et al., 2004). This makes it difficult to relate the ghrelin upregulation with that of *Pax6* ablation. Indeed when we analyzed the control and alpha-cell-specific *Pax6* KO pancreata at P0 we found ghrelin-glucagon double positive as well as ghrelin-glucagon-YFP triple positive cells in both types of pancreata (Figure 3.30a-h). However, even at this stage the number of $ghrelin^+$ cells was increased in the KO pancreata (Figure 3.30i). Furthermore, $ghrelin^+$ YFP^+ $glucagon^-$ cells were only observed in the KO pancreta (Figure 3.30e-h). This indicated the cells that had lost the expression of glucagon as a result of *Pax6* ablation. Loss of glucagon expression in *Pax6*-deficient alpha-cells was expected as Pax6 has been shown to control the transcription of *glucagon* gene (Sander et al., 1997; Gosmain et al., 2010).

As compared to the embryonic or early postnatal pancreas, in the adult pancreas the expression of ghrelin decreases to undetectable level (Wierup et al., 2004). That is why the ghrelin positive cells are very rare to observe in the adult pancreas from control mice. Accordingly, we detected no ghrelin expression in the adult control islets. In comparison to that the number of $ghrelin^+$ cells was increased in the alpha-cell-specific *Pax6* KO islets and nearly all of these cells were co-localized with the YFP labeled cells confirming their origin from Cre-recombined cells (Figure 3.31 and 3.32). Additionally, we observed that majority of these $ghrelin^+$ YFP^+ cells in the KO islets were negative for the glucagon

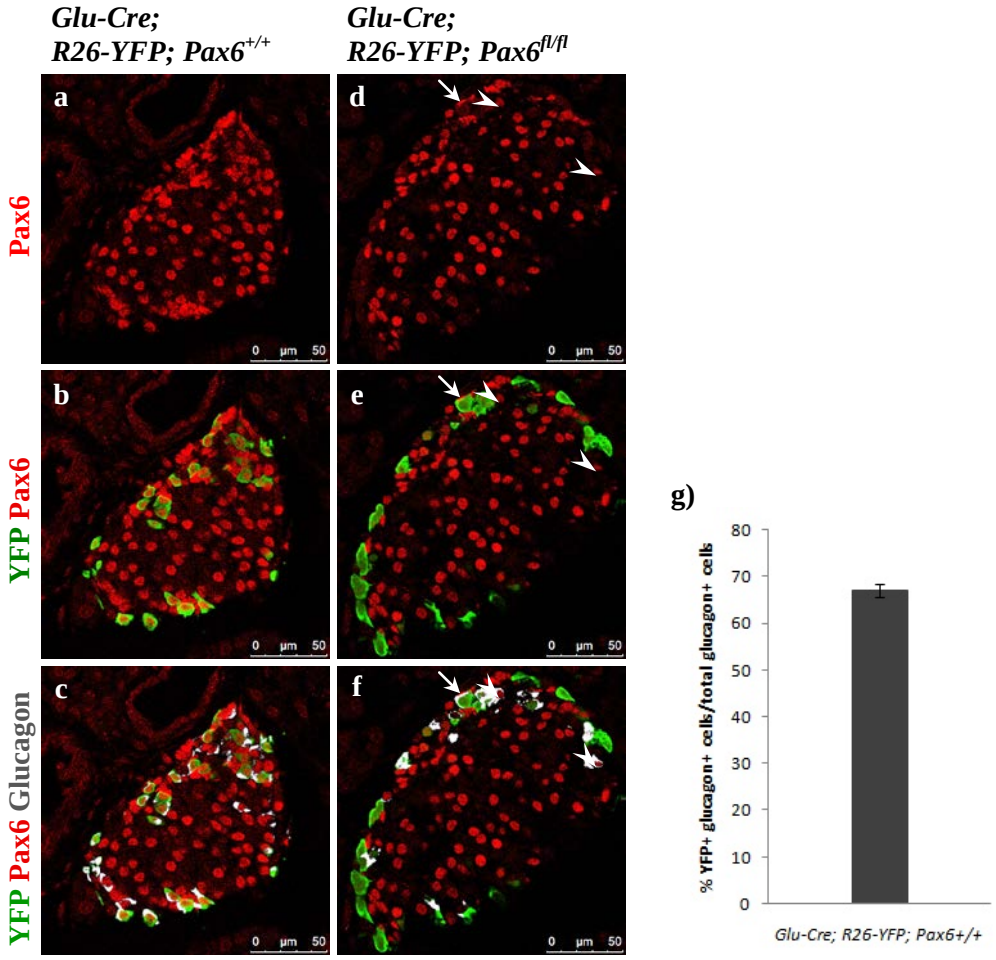

Figure 3.29: Alpha-cell-specific ablation of *Pax6*. Double immunofluorescence staining of pancreatic cryosections from 1 month old mice. In the control islets, all the YFP^+ cells express Pax6 and glucagon (a-c). In the alpha-cell-specific *Pax6* KO islets, most of the YFP^+ cells are negative for Pax6 and have lost the expression of glucagon as well (d-f). YFP^- $glucagon^+$ cells in the KO islets express Pax6 (arrowheads d-f). Rarely YFP^+ $glucagon^+$ $Pax6^-$ cells are also found in the KO islets (arrows d-f). Quantification of YFP^+ $glucagon^+$ cells in relation to the total $glucagon^+$ cells in 1 month old mice (g) (n=3). Nearly 70% of the $glucagon^+$ cells are labeled with YFP. Error bars represent SEM.

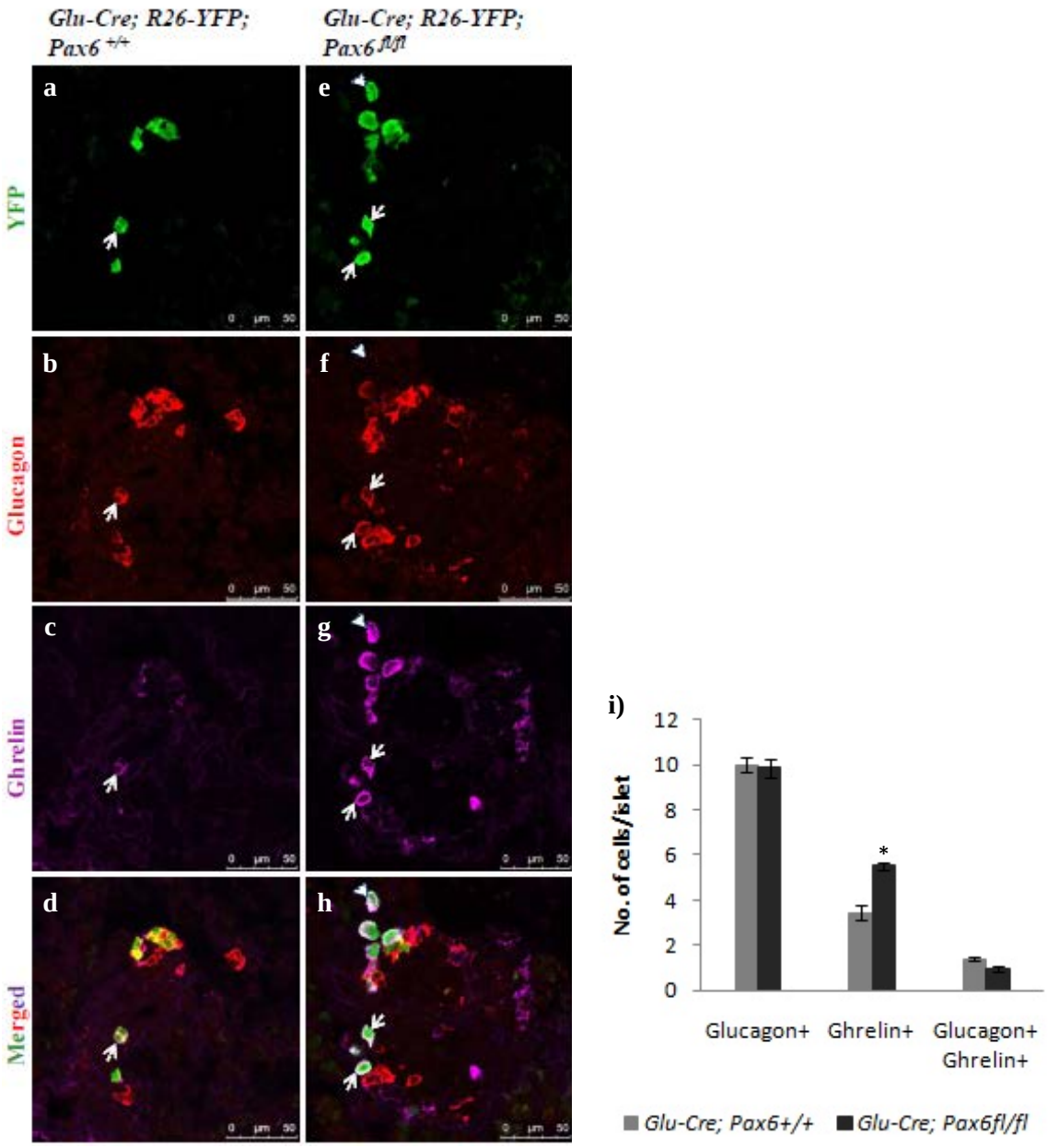

Figure 3.30: A mixed population of ghrelin and glucagon positive cells at P0. Double immunofluorescence staining of pancreatic cryosections at P0. In both the control and alpha-cell-specific *Pax6* KO islets, YFP-glucagon-ghrelin triple positive cells are detectable (arrows a-h). Additionally, in the alpha-cell-specific *Pax6* KO islets, YFP^+ $ghrelin^+$ $glucagon^-$ cells are deteced (arrowhead e-h) that are not found in the control islets. Quantification of $glucagon^+$, $ghrelin^+$, and glucagon-ghrelin co-positive cell population at P0 (i) (n=3). $Ghrelin^+$ cells are increased in the KO pancreata while $glucagon^+$ cell population is not significantly changed. Error bars represent SEM; *$p<0.05$.

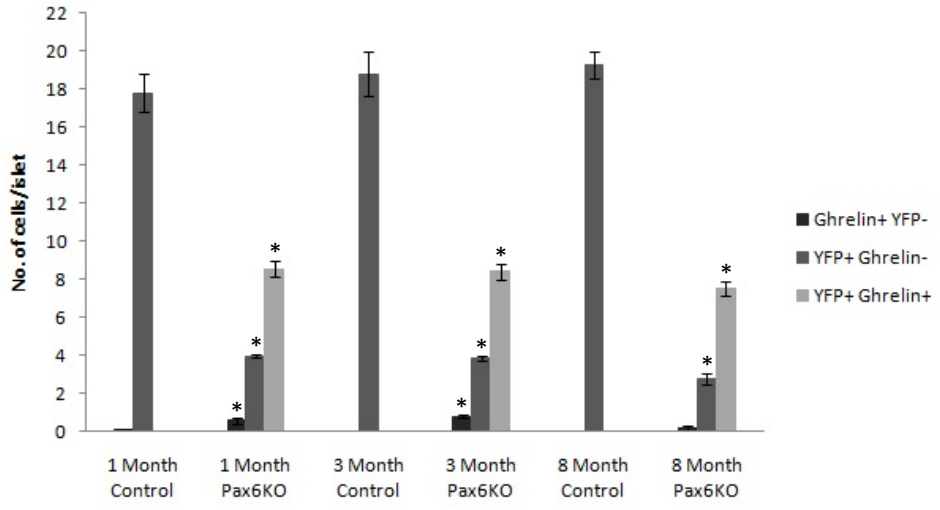

Figure 3.31: Ghrelin$^+$ cell population is increased in the adult alpha-cell-specific *Pax6* KO islets. Quantification of ghrelin$^+$ YFP$^-$, YFP$^+$ ghrelin$^-$, and YFP$^+$ ghrelin$^+$ cells at 1 month, 3 months, and 8 months of age (c) (n=3). As compared to the control islets, number of YFP$^+$ ghrelin$^+$ cells is significantly increased in the KO islets at all the ages analyzed. However, YFP$^+$ ghrelin$^+$ cell population is not significantly changed within the KO group at different ages. Error bars represent SEM; *p<0.05.

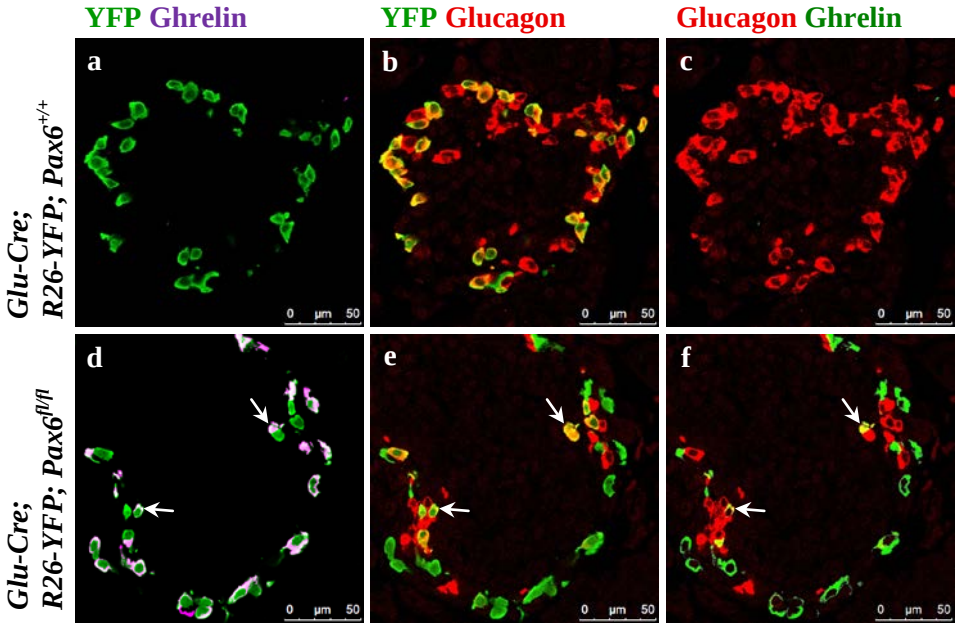

Figure 3.32: Ghrelin^{+} cells originate from alpha-cells in the alpha-cell-specific *Pax6* KO islets. Double immunofluorescence staining of pancreatic cryosections from 1 month old mice. Ghrelin expression is not detected in the control islets (a-c). In the alpha-cell-specific *Pax6* KO islets, ghrelin expression is upregulated in YFP labeled cells (d-f) and rarely ghrelin expression co-localizes with glucagon expression (arrows d-f).

expression but it was possible to observe some ghrelin-glucagon-YFP triple positive cells in the KO islets (Figure 3.32). In the adult islet these cells may indicate an intermediate state of conversion from glucagon to ghrelin expressing cells. Thus, we conclude that alpha-cells lose the expression of glucagon and start to express ghrelin following the ablation of *Pax6*.

3.2.2.2 Expression of alpha-cell related transcription factors in the ghrelin$^+$ cell population of alpha-cell-specific Pax6 KO islets

In the next step we checked the expression of alpha-cell related transcription factors MafB and Arx in the YFP labeled *Pax6*-deficient alpha-cells that now expressed ghrelin instead of glucagon. As compared to the YFP labeled cells in the control islets, YFP labeled ghrelin$^+$ cells in the KO islets were negative for MafB but they still expressed Arx. Additionally, in some KO islets few YFP$^-$ ghrelin$^+$ cells were detected that were also negative for MafB and positive for Arx (Figure 3.33 and 3.34). MafB is specifically expressed in adult alpha-cells and is important for the *glucagon* gene transcription (Artner et al., 2006). On the other hand, expression of Arx defines the alpha-cell lineage (Collombat et al., 2003, 2005, 2007). Therefore, absence of MafB and presence Arx shows the loss of mature alpha-cell function in the *Pax6*-deficient alpha-cells but preservation of the alpha-cell lineage. Furthermore, persistent expression of Arx in YFP labeled ghrelin$^+$ cells is another proof of their origin from alpha-cells.

3.2.3 Regeneration of alpha-cells in the alpha-cell-specific *Pax6* KO islets

Alpha-cell regeneration has been shown in some models of glucagon deficiency (Furuta et al., 1997; Gelling et al., 2003). As *Pax6* KO also led to the loss of glucagon expression, we were interested in looking for the regeneration of alpha-cells. The number of total glucagon$^+$ cells in the KO islets was not significantly different from that in the control islets at either 1 month or 3 months of age but was significantly reduced at 8 months of age (Figure 3.35a). This means that at younger age some alpha-cell regeneration was taking place. To clarify this further, we quantified the number of YFP$^-$ glucagon$^+$ and YFP$^+$ glucagon$^+$ cells in control and KO islets. We found that the number of YFP$^-$ glucagon$^+$ cells was significantly increased in the KO islets at 1 month and 3 months of age but not at 8 months of age (Figure 3.35b).

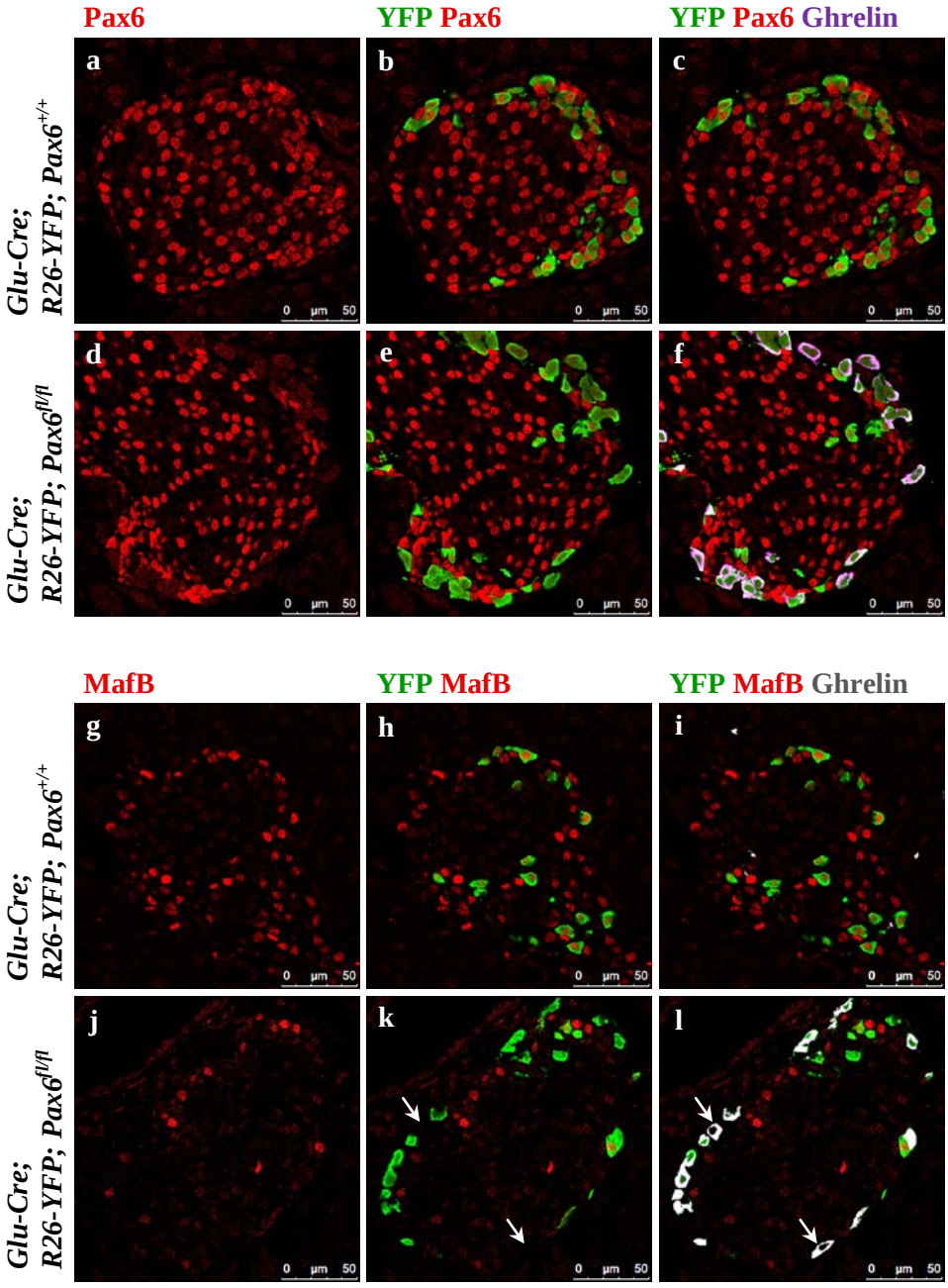

Figure 3.33: Expression of alpha-cell related transcription factors in the ghrelin$^+$ cells of the alpha-cell-specific *Pax6* KO islets. Double immunofluorescence staining of pancreatic cryosections from 1 month old mice. Ghrelin expression is not detected in the control islets (a-c and g-i). In the alpha-cell-specific *Pax6* KO islets, ghrelin expression is upregulated in YFP labeled cells and these ghrelin$^+$ cells are negative for Pax6 (d-f) and MafB (j-l) expression. Rarely some YFP$^-$ ghrelin$^+$ cells are also detected in the KO islets and they are also negative for MafB expression (arrows k,l).

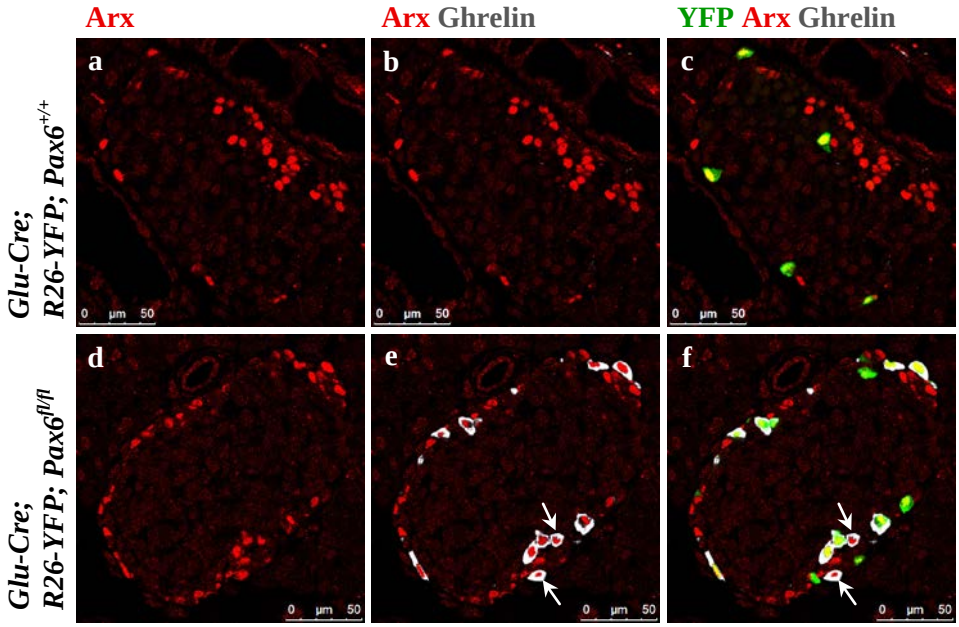

Figure 3.34: Expression of alpha-cell-lineage determinant Arx in the ghrelin$^+$ cells of the alpha-cell-specific *Pax6* KO islets. Double immunofluorescence staining of pancreatic cryosections from 1 month old mice. Ghrelin expression is not detected in the control islets (a-c). In the alpha-cell-specific *Pax6* KO islets, ghrelin expression is upregulated in YFP labeled *Pax6*-deficient cells and these ghrelin$^+$ cells are positive for Arx expression (d-f). Rarely some YFP$^-$ ghrelin$^+$ cells are also detected in the KO islets and they are also positive for Arx expression (arrows e,f).

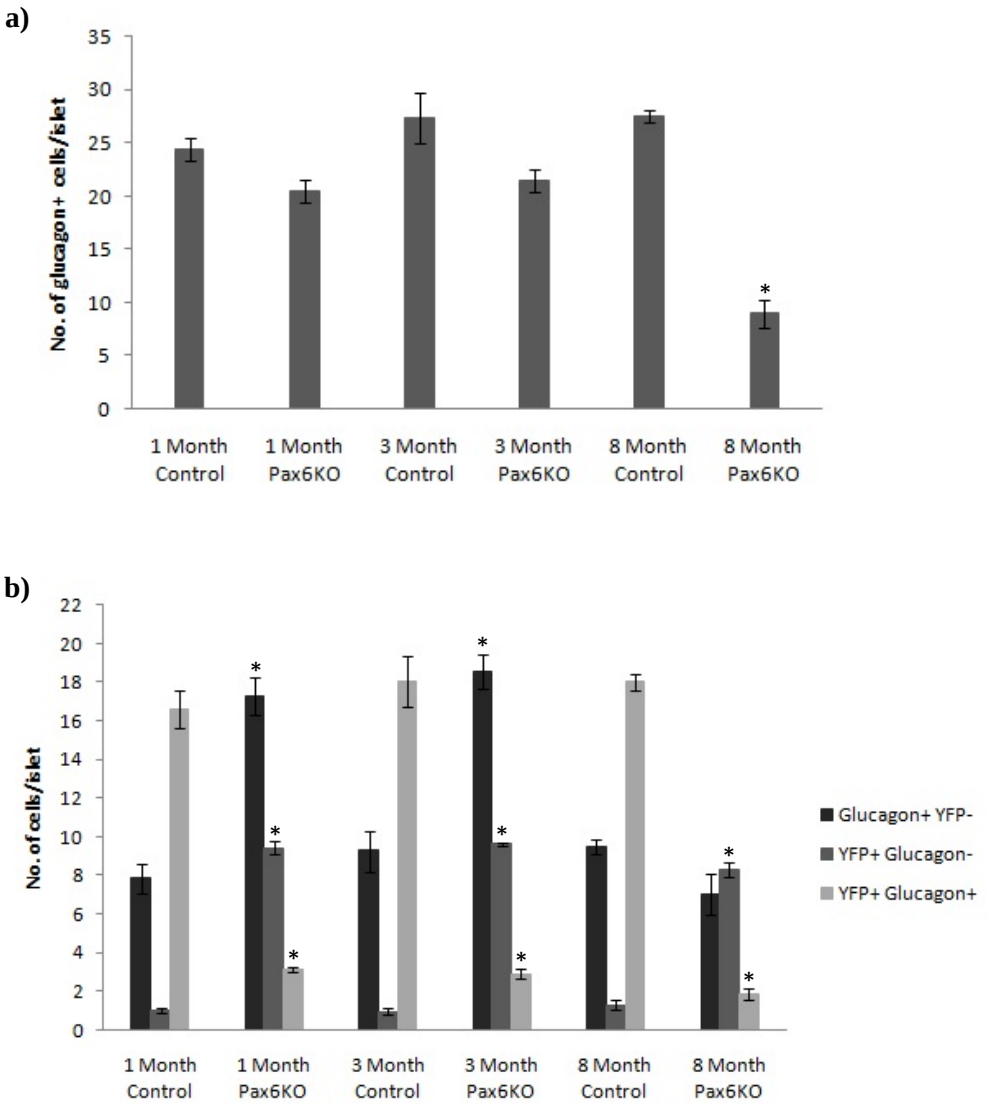

Figure 3.35: Alpha-cell regeneration in the alpha-cell-specific *Pax6* KO islets. Quantification of total glucagon$^+$ cell pouplation at 1 month, 3 months, and 8 months of age (a) (n=3). Number of total glucagon$^+$ cells is significantly reduced in the KO islets at 8 months of age but not at 1 month or 3 months of age. Quantification of glucagon$^+$ YFP$^-$, YFP$^+$ glucagon$^-$, and YFP$^+$ glucagon$^+$ cells at 1 month, 3 months, and 8 months of age (b) (n=3). Number of glucagon$^+$ YFP$^-$ cells is significantly increased in the KO islets at 1 month and 3 months of age but not at 8 months of age. Error bars represent SEM; *p<0.05.

This indicated the regeneration capacity of alpha-cells at young and middle age but a loss of regeneration at older age. However, even the older mice did not show any symptoms of glucagon deficiency because nearly 30% population of alpha-cells that escaped recombination was still present.

3.2.4 7B2 is upregulated and PC2 not affected in the alpha-cell-specific *Pax6* KO islets

Proglucagon, the precursor of glucagon, is processed in alpha-cells by prohormone convertase 2 (PC2) to generate the mature glucagon peptide. The neuropeptide 7B2 is involved in the activation of PC2 (Fortenberry et al., 2002; Katz et al., 2009). To see how proglucagon processing is affected in *Pax6*-deficient alpha-cells, we checked the expression of PC2 and 7B2. We found that in comparison to the control islets the expression of PC2 was not affected and that of 7B2 highly upregulated in the YFP labeled *Pax6*-deficient alpha-cells (Figure 3.36). Therefore, a loss of proglucagon processing via PC2 downregulation does not seem to occur in these cells.

3.3 *Pax6* overexpression

3.3.1 Generation of transgenic mice conditionally overexpressing *Pax6*

For conditional overexpression of *Pax6*, Jojo vector was used (Collombat et al., 2007). *Pax6* cDNA was cloned into this vector to generate the overexpression construct. The construct contained an *EGFP* (enhanced green fluorescent protein) cassette flanked by *loxP* sites under the control of cytomegalovirus (CMV) early enhancer/beta-actin (CAG) promoter that provides high level of constitutive expression. The *EGFP* cassette was followed by *Pax6* cDNA, an internal ribosome entry site (IRES), and beta-galactosidase encoding sequence (Figure 3.37a). In the absence of Cre-mediated recombination only *EGFP* is expressed from this construct because of the transcriptional stop site present after *EGFP*. However, in the presence of Cre, recombination of *loxP* sites removes the *EGFP* cassette along with the transcriptional stop and allows for the expression of *Pax6* along with *beta-galactosidase* in the Cre expressing cells (Figure 3.37b). Expression of beta-galactosidase, therefore, serves as a marker of overexpression.

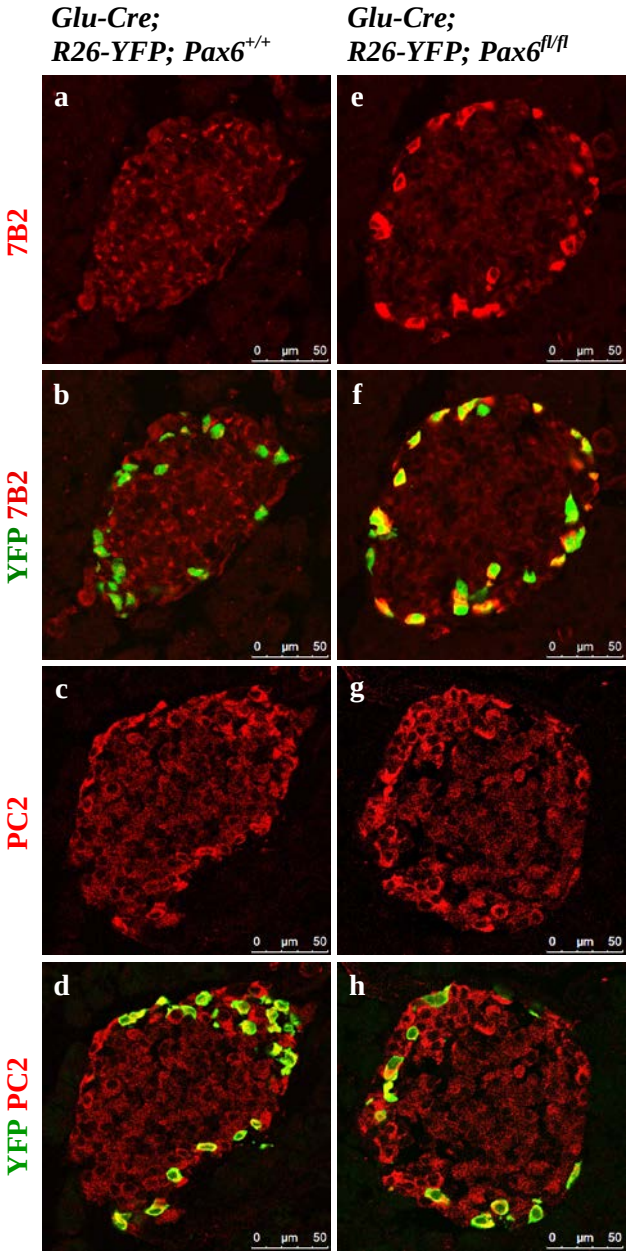

Figure 3.36: Expression of 7B2 and PC2 in the alpha-cell-specific *Pax6* KO islets. Immunofluorescence staining of pancreatic cryosections from 1 month old mice. 7B2 expression is very low in the control islets (a,b). In the alpha-cell-specific *Pax6* KO islets, 7B2 expression is higly upregulated in YFP labeled *Pax6*-deficient cells (e,f). Compared to the control islets, PC2 expression is unchanged in the alpha-cell-specific *Pax6* KO islets (c,d and g,h).

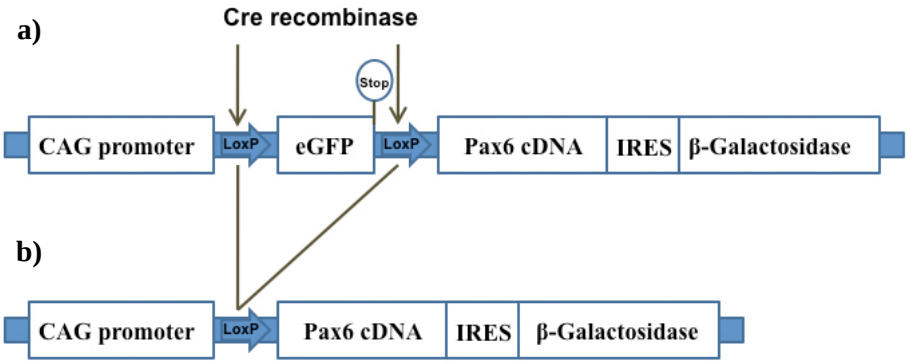

Figure 3.37: Construct used to generate conditional *Pax6*-overexpressing (*Pax6OE*) transgenic mice. (a) Before Cre-mediated recombination, only *GFP* is expressed due to the transcriptional stop present after it. (b) After Cre-mediated recombination, *GFP* cassette (together with the stop) is removed allowing the expression of *Pax6* and *beta-galactosidase* in the Cre expressing cells.

This construct was then used for the pronuclear injection to generate three GFP expressing transgenic mouse lines (termed *Pax6OE*), all of which gave germ-line transmission. Line 3 and 2 were used for all of the experiments. These *Pax6OE* mice were then crossed with different Cre mouse lines including *Pdx1-Cre* (for overexpression in the whole pancreas), *insulin-Cre* (*Ins-Cre*, for overexpression in beta-cells), and *glucagon-Cre* (*Glu-Cre*, for overexpression in alpha-cells) (Gannon et al., 2000; Herrera, 2000).

In the *Pax6OE; Pdx1-Cre* pancreata GFP expression was lost from most of the pancreatic tissue and Pax6 expression was found in the whole pancreas including the exocrine tissue where Pax6 is normally not expressed (Figure 3.38a-f). This indicated the correct functioning of the *Pax6OE* construct. In case *of Pax6OE; Ins-Cre* and *Pax6OE; Glu-Cre* mice the overexpression of Pax6 was confirmed by beta-galactosidase staining and X-Gal staining, respectively (Figure 3.38g-j). Furthermore, overexpression of *Pax6* was also confirmed by qRT-PCR in *Pax6OE; Pdx1-Cre* and *Pax6OE; Ins-Cre* mice (Figure 3.38k).

3.3.2 Pancreatic phenotype of *Pax6* overexpressing mice

There was no difference in the blood glucose level of *Pax6OE; Pdx1-Cre*, *Pax6OE; Ins-Cre*, and *Pax6OE; Glu-Cre* mice as compared to the *Pax6OE* control mice. At the pancreatic level, we observed slight hypoplasia of the pancreatic tissue in the *Pax6OE; Pdx1-Cre* mice as compared to the *Pax6OE* control mice (Figure 3.39). In the endocrine pancreas, islet structure was intact and the number of insulin$^+$ and somatostatin$^+$ cells was not significantly different in the *Pax6OE; Pdx1-Cre* mice as compared to the *Pax6OE* control mice at 3 weeks of age (data not shown). However, the number of glucagon$^+$ and PP$^+$ cells was significantly reduced in *Pax6OE; Pdx1-Cre* mice as compared to the *Pax6OE* control mice at 3 weeks of age (Figure 3.40a). Expression of various beta-cell related factors was also not changed in the *Pax6OE; Pdx1-Cre* mice as compared to the *Pax6OE* control mice (Figure 3.41).

We found in our study that ghrelin expression upregulates in both the alpha- and beta-cell-specific *Pax6* KO pancreata. In relation to that we were interested to see the effect of *Pax6* overexpression on ghrelin$^+$ cell population. In the adult islet, ghrelin$^+$ cell

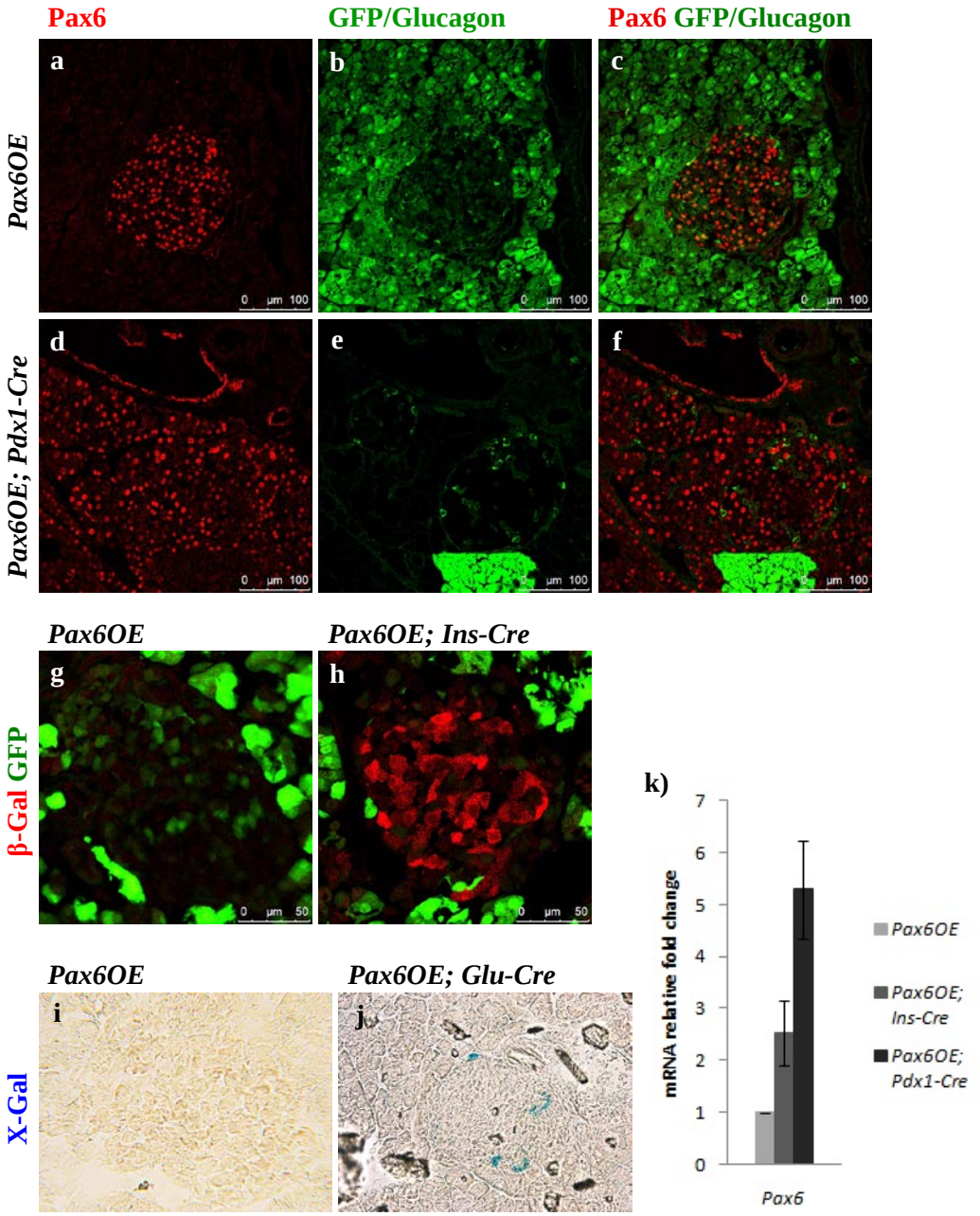

Figure 3.38: *Pax6* overexpression under *Pdx1*, *insulin*, and *glucagon* expression domains. Immunofluorescence staining (a-h) and X-Gal staining (i,j) of pancreatic cryosections from 2 month old mice. In *Pax6OE* control pancreata, Pax6 is expressed only inside the endocrine cells of the islets (a-c). In *Pax6OE; Pdx1-Cre* pancreata, Pax6 is expressed in the whole pancreas including both the endocrine and exocrine tissue (d-f). *Pax6* overexpression indicated by the beta-galactosidase expression in *Pax6OE; Ins-Cre* islets (h), and by the X-Gal staining in *Pax6OE; Glu-Cre* islets (j). Quantitative RT-PCR of *Pax6* mRNA in the pancreata of 2 month old mice shows overexpression in *Pax6OE; Pdx1-Cre* and *Pax6OE; Ins-Cre* mice (k) (n=3). Error bars represent SEM.

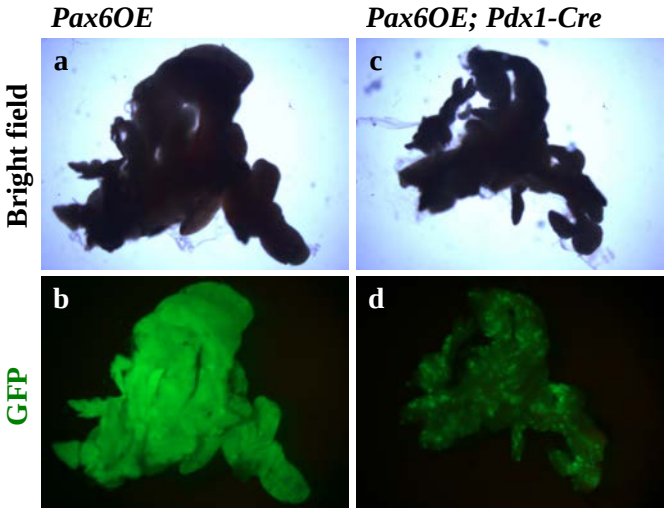

Figure 3.39: Pancreatic hypoplasia in *Pax6OE; Pdx1-Cre* mice. Pancreatic tissue is slightly reduced in size in *Pax6OE; Pdx1-Cre* mice (c,d) compared to the *Pax6OE* control mice (a,b) at 4 months of age. Absence of GFP expression in (d) shows the successful Cre-mediated recombination.

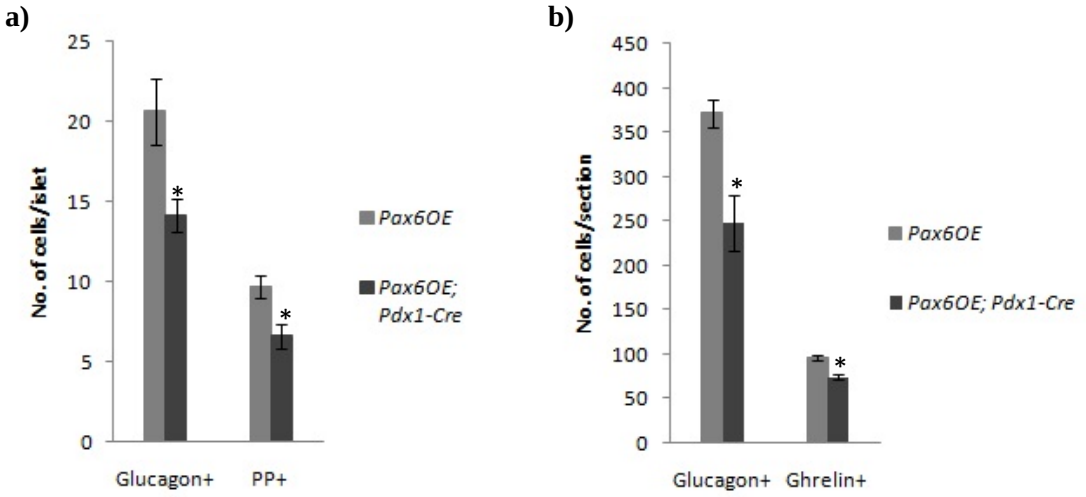

Figure 3.40: Changes in the glucagon$^+$, ghrelin$^+$, and PP$^+$ cell population in the pancreata of *Pax6OE; Pdx1-Cre* mice. Quantification of glucagon$^+$ and PP$^+$ cell pouplation at 3 weeks of age (a), and glucagon$^+$ and ghrelin$^+$ cell pouplation at P0 (b), in *Pax6OE* control and *Pax6OE; Pdx1-Cre* mice (n=3). In *Pax6OE; Pdx1-Cre* pancreata, number of glucagon$^+$, ghrelin$^+$, and PP$^+$ cells is decreased. Error bars represent SEM; *$p<0.05$.

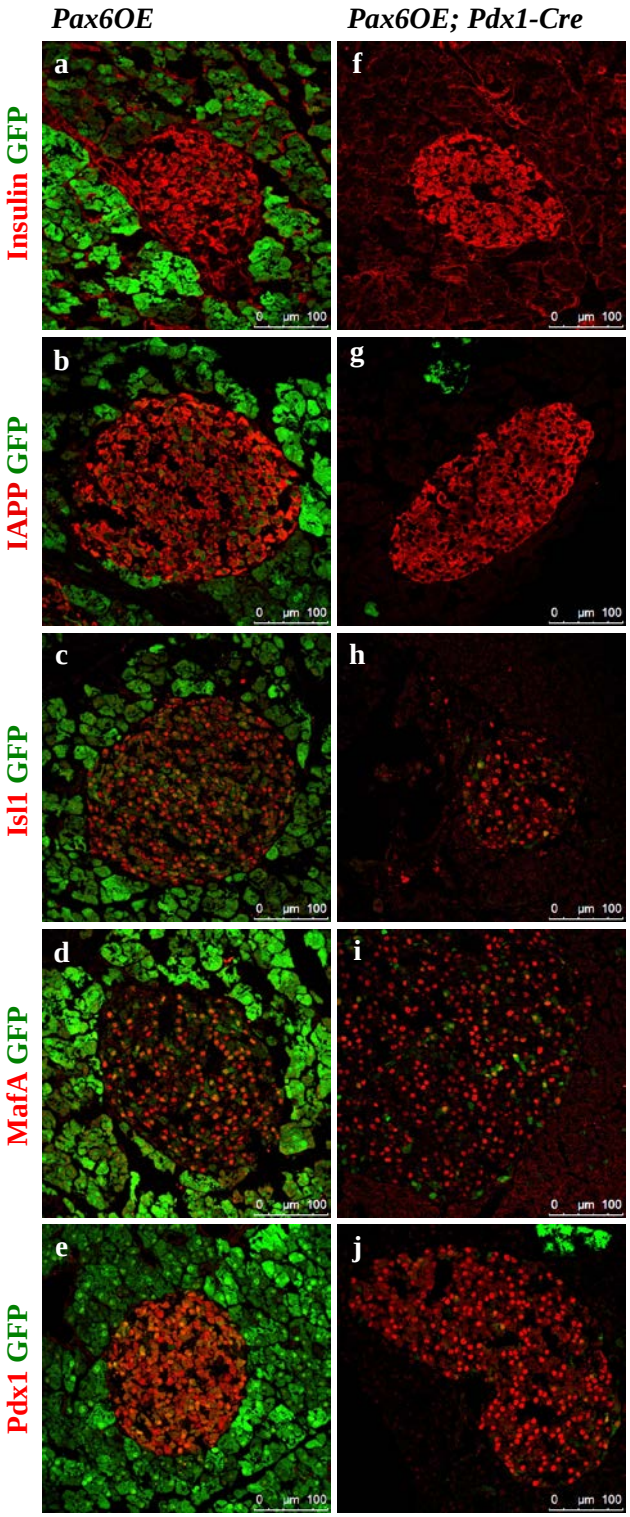

Figure 3.41: Expression of beta-cell related factors in *Pax6OE; Pdx1-Cre* islets. Immunofluorescence staining of pancreatic cryosections from 4 month old mice. As compared to the *Pax6OE* control islets, no change is detected in the expression of insulin (f), IAPP (g), Isl1 (h), MafA (i), and Pdx1 (j) in *Pax6OE; Pdx1-Cre* islets.

population was not detectable in both *Pax6OE* and *Pax6OE; Pdx1-Cre* mice. Therefore, to examine any possible difference we went to the early postnatal stage P0, that is a time-point where ghrelin$^+$ cells are in generally higher in number in the islets (Wierup et al., 2004). By quantification at P0, we found a small but sgnificant decrease in the number of ghrelin$^+$ cells in the pancreata of *Pax6OE; Pdx1-Cre* mice as compared to the *Pax6OE* control mice (Figure 3.40b). Furthermore, the change in other endocrine cells at P0 was similar to that in 3 week old mice (data not shown). As *Pdx1-Cre* is active from the beginning of pancreatic development, we conclude that this reduction in the population of glucagon$^+$, ghrelin$^+$, and PP$^+$ cell population is probably due to the reduced number of progenitors initially specified to these cell types or due to the reduced proliferation during early development. However, at 3 weeks of age no difference in proliferation (determined by BrdU labeling) or apoptosis (determined by TUNEL staining) was detected (data not shown).

In case of *Pax6OE; Ins-Cre* mice, no phenotypic change was observed. As compared to the *Pax6OE* control mice, the number of insulin$^+$ cells, number of islets per section, as well as the proliferation in the insulin$^+$ cell population was not significantly changed (Figure 3.42). Furthermore, the expression of different beta-cell related factors including Pdx1, MafA, Glut2, and IAPP was also not changed in the *Pax6OE; Ins-Cre* mice as compared to the *Pax6OE* control mice (data not shown).

Lastly, in *Pax6OE; Glu-Cre* mice, reduction in the number of glucagon$^+$ cells was observed like the *Pax6OE; Pdx1-Cre* mice. On the contrary, the number of PP$^+$ cells was slightly increased (Figure 3.43). Rest of the endocrine cell population was not changed in these mice (data not shown).

If the pancreatic phenotype of *Pax6OE; Pdx1-Cre*, *Pax6OE; Ins-Cre*, and *Pax6OE; Glu-Cre* mice is compared, it seems that the development of alpha-cells is more sensitive to the level of Pax6 expression as compared to the development of beta-cells.

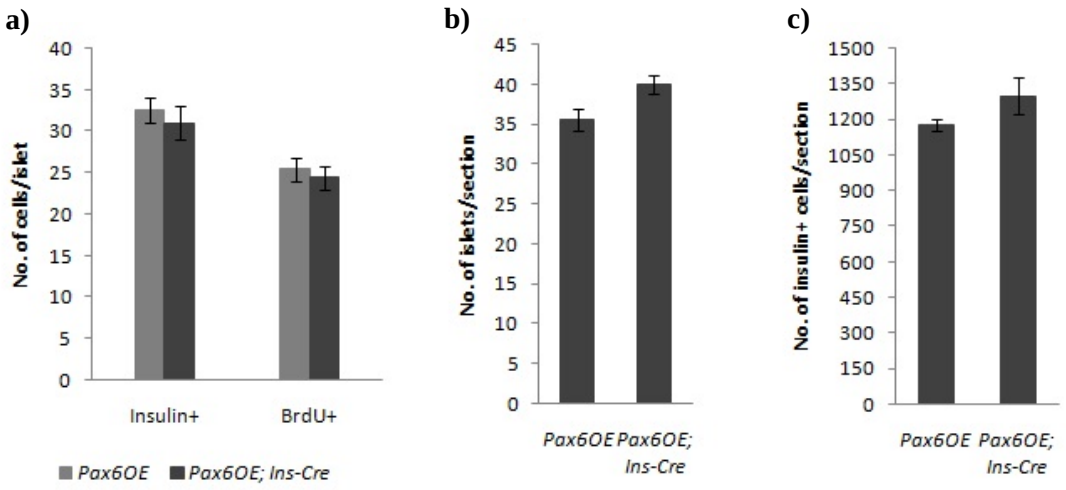

Figure 3.42: *Pax6OE; Ins-Cre* islets do not show any change in the beta-cell population. Quantification of insulin$^+$ and BrdU$^+$ cells (a), islets/section (b), and insulin$^+$ cells/section (c) at 2 months of age shows no significant difference in *Pax6OE* vs. *Pax6OE; Ins-Cre mice* (n=3). Error bars represent SEM.

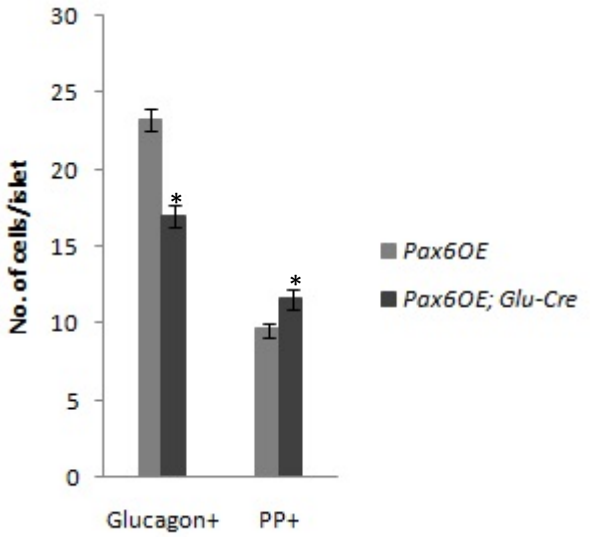

Figure 3.43: Changes in glucagon$^+$ and PP$^+$ cell pouplation in the islets of *Pax6OE; Glu-Cre* mice. Quantification of glucagon$^+$ and PP$^+$ cell pouplation in *Pax6OE* control and *Pax6OE; Glu-Cre* mice at 2 months of age (n=3). In *Pax6OE; Glu-Cre* islets, number of glucagon$^+$ cells is decreased and that of PP$^+$ cells is slightly increased. Error bars represent SEM; *p<0.05.

4. Discussion

Pax6 has been shown to play an important role in the maturation of pancreatic endocrine cells, especially alpha- and beta-cells. The crucial role of Pax6 in this process is evident by the fact that mice lacking a functional Pax6 protein in the pancreas die shortly after birth (St-Onge et al., 1997; Ashery-Padan et al., 2004). Pax6 is one of those transcription factors that are expressed in the endocrine pancreas during development and their expression further continues throughout the adult life-span. This persistent expression during postnatal life indicates the continuous requirement of Pax6 in the endocrine pancreas. This is supported by the fact that in both humans and mice, heterozygous Pax6 mutations lead to glucose intolerance with age (Yasuda et al., 2002; Wen et al., 2009). Hence, there has been a great interest in understanding the role of Pax6 in adult endocrine cell function.

In vitro Pax6 knockdown (KD) studies, in alpha- and beta-cells, have identified several downstream targets of Pax6 that are essential for the functional maturity of these cells (Katz et al., 2009; Gosmain et al., 2010; Gosmain et al., 2012a; Gosmain et al., 2012b). However, a detailed in vivo investigation of *Pax6* knockout (KO) in the adult pancreas is lacking. As both classical and pancreas-specific conditional *Pax6* KO mice die at early postnatal age, the only possible way to investigate the role of Pax6 in adult islets is by utilizing an inducible Cre line that can be activated during the adult life. Using this idea in a study that has been recently published, Hart et al. (2013) knocked out *Pax6* in five month old mice. In this study, many of the same findings were recapitulated as those of the previous KO studies, including the loss of hormone expression and development of diabetes as well as the upregulation of ghrelin expression (Ashery-Padan et al., 2004; Heller et al., 2005). In this study a ubiquitous Cre line was used that led to the removal of *Pax6* from all of the endocrine cell types. Therefore, it did not allow the study of a cell-type-specific effect.

Islets of Langerhans consist of five types of endocrine cells: alpha (glucagon$^+$), beta (insulin$^+$), delta (somatostatin$^+$), PP (pancreatic-polypeptide$^+$), and ε (ghrelin$^+$). Pax6 is expressed in all of them except for a subset of ghrelin$^+$ cells (Ashery-Padan et al., 2004; Kordowich et al., 2011). In our study we aimed at elucidating the role of Pax6 in adult alpha- and beta-cells in vivo by employing the Cre/*loxP* system (Sauer and Henderson, 1989; Rajewsky et al., 1996). Therefore, we utilized the *Pax6* floxed mouse line together

with an inducible Cre mouse line (*RIP-CreER*) for beta-cell-specific *Pax6* ablation, and a non-inducible Cre mouse line (*glucagon-Cre*) for alpha-cell-specific *Pax6* ablation (Ashery-Padan et al., 2000; Dor et al., 2004; Herrera, 2000). This allowed us to study the role of Pax6 in alpha- and beta-cells separately and without affecting the rest of the endocrine pancreas which was not the case in previous studies. We further incorporated the *YFP* reporter transgene (Srinivas et al., 2001) that allowed us to assess the recombination efficiency, to trace the fate of *Pax6* KO cells, and to estimate the relative regeneration in each case.

Apart from the loss-of-function, another useful way to identify the role of a gene product in the developmental and/or functional context is via overexpression of that gene in the specific cell-type of interest (Prelich, 2012). In our study, we generated transgenic mice to conditionally overexpress Pax6. To see the effect of overexpression on pancreatic development, we utilized a wider range of expression domains including the whole pancreas, the beta-cells alone, and the alpha-cells alone. This was achieved by using *Pdx1-Cre*, *insulin-Cre*, and *glucagon-Cre* mouse lines, respectively (Gannon et al., 2000; Herrera, 2000). As the *Pax6* overexpression phenotype was not lethal we used only non-inducible Cre lines for *Pax6* overexpression.

4.1 *Pax6* knockout in beta-cells

4.1.1 Hyperglycemia in beta-cell-specific *Pax6* KO mice and the associated changes in the pancreatic endocrine cell population

To knockout *Pax6* in adult beta-cells, tamoxifen inducible *RIP-CreER* mouse line was employed (Dor et al., 2004). This Cre line gave nearly 95% recombination efficiency. In accordance with this near complete removal of Pax6 from the beta-cell population, a prominent phenotype was observed. *Pax6* KO in beta-cells resulted in severe hyperglycemia, emphasizing the requirement of Pax6 in beta-cells for the establishment of glucose homeostasis. Development of an overt diabetic phenotype in these mice is also in agreement with the previous *Pax6* KO studies (Ashery-Padan et al., 2004; Hart et al., 2013). Consistent with the hyperglycemia, number of insulin$^+$ cells was decreased in the KO pancreata. At the same time all other endocrine cell types (glucagon$^+$, somatostatin$^+$, PP$^+$, and ghrelin$^+$) were increased in number. Decrease in the number of insulin$^+$ cells and increase in the number of glucagon$^+$, somatostatin$^+$, and PP$^+$ cells also occurs in

streptozotocin-induced diabetic mouse models (Adeghate and Ponery, 2003; Zhang et al., 2012). Therefore, an increase in the population of glucagon$^+$, somatostatin$^+$, and PP$^+$ cells seems to be a general phenomenon related to the development of diabetes. However, increase in the ghrelin$^+$ cell population is more unique to the *Pax6* KO phenotype. The difference lies in the fact that in streptozotocin-induced diabetic models beta-cells die but in *Pax6* KO mice beta-cells do not die. Instead, they just lose their mature differentiation status that may cause ghrelin upregulation in the previously insulin$^+$ cells (would be discussed later).

4.1.2 Expression of beta-cell related transcription factors in beta-cell-specific *Pax6* KO pancreata

A detailed immunohistochemical analysis was performed to check the expression status of important beta-cell related factors. Among the beta-cell specific transcription factors, the expression of MafA was undetectable shortly after the KO. MafA is a transcription factor that specifically marks the mature beta cells and has been confirmed as a direct downstream target of Pax6 (Raum et al., 2010; Gosmian et al., 2012a). MafA, together with Pdx1 and Pax6 binds to the insulin promoter and controls its transcription (Sander et al., 1997; Matsuoka et al., 2007; Gosmain et al., 2012a). Indeed, the MafA deficient mice also develop glucose intolerance and diabetes mellitus (Zhang et al., 2005). Therefore, the loss of Pax6 and the concomitant loss of MafA in beta-cells can explain the reduced insulin synthesis that results into diabetes later.

Pdx1 and Nkx6.1 are two more beta-cell specific transcription factors in the adult islet. Pdx1 is important for insulin expression and mature beta-cell function, and its loss from beta-cells can lead to diabetes (Ahlgren et al., 1998; Matsuoka et al., 2007). In some studies, Pdx1 has been shown to be downregulated after *Pax6* KO/KD (Heller et al., 2005; Gosmain et al., 2012a; Hart et al., 2013). On the contrary, it was still found to be expressed after *Pax6* KO in another study (Ashery-Padan et al., 2004). In our beta-cell-specific *Pax6* KO, Pdx1 expression was not changed even at 4.5 months after KO induction. However, as we have seen in our study the expression of Pdx1 alone is not sufficient to rescue the mature beta-cell phenotype. Thus, Pdx1 needs to cooperate with other beta-cell related transcription factors, including Pax6, MafA, and Nkx6.1, to maintain the mature differentiation state of beta-cells.

Nkx6.1 in beta-cells is required to maintain the beta-cell identity by repressing the expression of *glucagon* and *Arx* (Gauthier et al., 2007; Schaffer et al., 2013). A reduction in the Nkx6.1 expression has been reported in *Pax6* KO/KD models (Gosmain et al., 2012a; Hart et al., 2013). In our study, there was a gradual loss of Nkx6.1 expression. Shortly after KO induction, Nkx6.1 was still detectable but after a long time its expression was lost. Nkx6.1 expression is also reduced in beta-cell-specific *Pdx1* KO mice and in turn leads to the ectopic expression of glucagon in beta-cells (Ahlgren et al., 1998). However, in our beta-cell-specific *Pax6* KO mice the loss of Nkx6.1 expression did not lead to upregulation of glucagon, because the expression of glucagon depends on the presence of Pax6 (Sander et al., 1997; Gosmain et al., 2010). Regarding Arx expression following *Nkx6.1* KO, Schaffer et al. (2013) showed that *Nkx6.1* inactivation in endocrine precursors but not in beta-cells leads to the ectopic expression of Arx. Once beta-cells are specified, the repression of *Arx* is independent of Nkx6.1 and occurs via DNA methylation (Dhawan et al., 2011). In accordance with that, we did not observe the ectopic expression of Arx in *Pax6*-deficient beta-cells following Nkx6.1 downregulation (data not shown).

Nkx2.2 is another transcription factor that is expressed in beta-cells, and in a subset of alpha- and PP-cells, and is required for the proper differentiation of these cell types (Kordowich et al., 2011). Previous studies have shown that Nkx2.2 expression is not affected in the *Pax6* KO pancreata (Ashery-Padan et al., 2004; Gosmain et al., 2012a; Hart et al., 2013). Accordingly, in our beta-cell specific *Pax6* KO mice the expression of Nkx2.2 was not changed.

Isl1 and Rfx6 are general pancreatic endocrine specific transcription factors in the adult pancreas. Both of them are expressed in all types of endocrine cells in the islet (Ahlgren et al., 1997; Smith et al., 2010). In mouse, Isl1 is required for the maturation and expansion of islet endocrine cells (Du et al., 2009) and Rfx6 is required for the generation of these cells except for the PP-cells that can develop in the absence of Rfx6 (Smith et al., 2010). In beta-cell-specific *Pax6* KO islets the expression of both Isl1 and Rfx6 was not affected. Isl1 has also been shown to be unaffected in the previous *Pax6* KO studies (Ashery-Padan et al., 2004; Gosmain et al., 2012a; Hart et al., 2013) while Rfx6 has not been evaluated.

Collectively, the expression profile of various beta-cell related transcription factors in *Pax6* KO beta-cells indicates a loss of mature differentiation state (due to the absence of MafA and downregulation of Nkx6.1) but preservation of basic endocrine and partial beta-cell character (due to the presence of Isl1, Rfx6, Nkx2.2, and Pdx1).

4.1.3 Defective glucose-stimulated insulin secretion in beta-cell-specific *Pax6* KO pancreata

Glucose-stimulated insulin secretion (GSIS) from beta-cells is a crucial step in the maintenance of glucose homeostasis. In order for the GSIS to take place, an efficient glucose metabolism is required in the beta-cells. Glut2 is a high capacity (high Km) glucose transporter located in the membranes of beta-cells. At high blood glucose level, it allows an efficient transport of glucose into the cell (e.g., immediately following a meal). This, in turn, leads to an increased glucose metabolism and stimulated insulin secretion (Jensen et al., 2008; Kramer et al., 2009). Loss of GSIS is an important step in the development of type 2 diabetes and is associated with the loss of Glut2 (Thorens et al., 1992). *Glut2* deficient mice develop hyperglycemia and show loss of the first phase of GSIS (Guillam et al., 1997). In our study, following *Pax6* KO, beta-cells quickly lost the expression of Glut2. It took place in the first few days together with the loss of MafA. Loss of Glut2 has been shown in previous *Pax6* KO studies as well (Ashery-Padan et al., 2004). Additionally, we observed that the loss of Glut2 occurred in the whole islet, including the YFP labeled *Pax6*-deficient cells as well as the YFP^- $insulin^+$ cells that still express Pax6. Furthermore, ChIP analysis did not show any binding of Pax6 with the *Glut2* promoter. Collectively, these results suggest an indirect affect of *Pax6* ablation on Glut2 expression. It has been shown before that the diabetic environment can result in the loss of Glut2 expression (Thorens et al., 1992). Therefore, it is quite possible that the loss of Glut2 expression in the islets of beta-cell-specific *Pax6* KO mice also occurs in an indirect way through the development of hyperglycemia.

Another important factor involved in GSIS is glucagon-like peptide 1 (GLP-1). GLP-1 acts through the GLP-1 receptor that is highly expressed on beta-cells. Following nutrient stimulation, GLP-1 is released from intestinal L cells and promotes the insulin secretion from beta-cells. Apart from the role of GLP-1 in GSIS, it has also been shown to promote beta-cell proliferation and neogenesis as well as insulin synthesis (MacDonald et al., 2002). Previously, an in vitro Pax6 KD study has shown Pax6 mediated control of

GLP-1 receptor expression (Gosmain et al., 2012a). In agreement with that, we found that the expression of GLP-1 receptor was specifically lost in the *Pax6*-deficient beta-cells. Loss of both Glut2 and GLP-1 receptor shows an obvious defect in the GSIS in *Pax6*-deficient beta-cells. Additionally, the loss of GLP-1 receptor mediated signaling may also explain the reduced insulin production and the lack of proliferation in these *Pax6*-deficient beta-cells.

Previously, MafA has been shown as a regulator of glucose-stimulated insulin secretion (Zhang et al., 2005). As MafA expression is downregulated in beta-cell-specific *Pax6* KO islets, the impairment in GSIS in these mice may also occur indirectly via Pax6-MafA pathway.

4.1.4 Defective proinsulin processing in beta-cell-specific *Pax6* KO pancreata

Insulin is synthesized as a prohormone in beta-cells. Proinsulin is then processed to generate mature insulin peptide. During this process C-peptide is released as a byproduct at an equimolar concentration. In case of defective proinsulin processing the ratio of proinsulin to C-peptide is increased (Vasic and Walcher, 2012). Beta-cells contain two prohormone convertases for insulin processing, PC1/3 and PC2. This is in contrast to the non-beta-cells of the islet that contain only PC2 (Marcinkiewicz et al., 1994). Although both PC1/3 and PC2 are involved, proinsulin processing is more severely affected in PC1/3 KO mice as compared to PC2 KO mice (Furuta et al., 1997; Zhu et al., 2002). We checked the expression of insulin, C-peptide, PC1/3, and PC2 to evaluate the effect of *Pax6* KO on proinsulin processing. Within few weeks of *Pax6* KO, a lot of beta-cells lost the expression of C-peptide. Some of these C-peptide negative beta-cells were also insulin negative but some continued to express insulin at low level. We speculate that this insulin reactivity was due to the presence of proinsulin that was not completely processed as indicated by the absence of C-peptide. Surprisingly, however, the expression of both PC1/3 and PC2 was unchanged in the C-peptide$^-$ insulin$^+$ cells. PC1/3 and PC2 expression was also found in those cells that had completely lost the expression of insulin (C-peptide$^-$ insulin$^-$ cells). Previous studies show some conflicting data on PC1/3 and PC2 expression. PC1/3 expression has been shown to be not changed in one study (Ashery-Padan et al., 2004) and lost in the other study (Hart et al., 2013) after *Pax6* KO, while it was found to be 30% decreased at protein level after in vitro Pax6 KD (Gosmain et al., 2012a). PC2

expression was found to be not changed after *Pax6* KO (Hart et al., 2013) and a 30% decrease in protein content was found after in vitro Pax6 KD (Gosmain et al., 2012a).

Activities of PC1/3 and PC2 are further modulated by the neuropeptides proSAAS and 7B2, respectively (Fortenberry et al., 2002). We found that both of these peptides were highly upregulated in *Pax6* KO beta-cells. It has been shown before that proSAAS can inhibit the activity of PC1/3 and its overexpression in mice leads to diabetes and obesity (Wei et al., 2004). Moreover, proSAAS is directly repressed by Pax6 and its expression is increased in the *Pax6* heterozygous null mutant mice (Liu et al., 2012). Therefore, the increased level of proSAAS expression is expected in *Pax6* KO beta-cells and is a possible indicator of defective proinsulin processing in these cells. Interestingly, however, we found that the proSAAS expression is in generally higher in alpha-cells in the control islets and does not seem to be affected after alpha-cell-specific *Pax6* ablation (data not shown). Thus, while Pax6 may repress the expression of proSAAS in beta-cells, it does not seem to do so in alpha-cells indicating that the proSAAS expression in alpha- vs. beta-cells is differentially regulated.

Unlike proSAAS, the effect of upregulated 7B2 is hard to explain. 7B2 is a chaperone required for the maturation and activity of PC2 (Westphal et al., 1999). At the same time, C-terminal peptide of 7B2 can inhibit PC2 activity in vitro but apparently not in vivo (Fortenberry et al., 1999). When overexpressed in a beta-cell line, 7B2 did not affect insulin production or secretion, although, it increased the secretion of active PC2 (Helwig et al., 2011). In another study, 7B2 upregulation was linked to obesity and increased PC2 activity without changing the level of PC2 processed peptides (Farber et al., 2008). On the other hand, 7B2 downregulation is found in human medulloblastomas indicating that proper expression of 7B2 is required to suppress the tumor growth and to maintain the neuronal differentiation state (Waha et al., 2007). Finally, in a very recent study, it was shown that 7B2 can also prevent the aggregation of proteins involved in the pathogenesis of Alzheimer's and Parkinson's disease, thereby improving the cell viability (Helwig et al., 2013).

Therefore, on one hand, the increased expression of 7B2 in *Pax6* KO beta-cells may help to increase the PC2 activity that may increase proinsulin processing in the absence of PC1/3 activity. On the other hand, this increased 7B2 expression might also be a result of the immature differentiation status of these cells and help in their survival by acting as a

chaperone. However, the exact role of upregulated 7B2 in *Pax6* KO beta-cells remains elusive.

4.1.5 Ratio of IAPP to insulin is changed in the beta-cell-specific *Pax6* KO islets

Islet amyloid polypeptide (IAPP) (also called amylin) is a peptide hormone produced in beta-cells and secreted together with insulin in a ratio of 1:100 (IAPP to insulin). IAPP is secreted in response to nutrient stimuli together with insulin and may play a role in the regulation of food intake, in the development of insulin resistance (by suppressing the peripheral glucose disposal), and in the inhibition of glucose-stimulated insulin secretion (Leighton and Cooper, 1988; Sowa et al., 1990; Dégano et al., 1993; Hull et al., 2004). At the same time, IAPP is also well known for its pathological role in the development of type 2 diabetes as a part of the amyloid deposits (Kahn et al., 1999). Similar to insulin, IAPP is synthesized as a prohormone (proIAPP). ProIAPP is processed by the same enzymes (PC1/3 and PC2) that process proinsulin. Therefore, a defect in the proinsulin processing can also affect the proIAPP processing. It has been suggested that the unprocessed form of IAPP is more prone to form the amyloid deposits that cause the damage to beta-cells in type 2 diabetes (Marzban et al., 2004).

We found that the expression of IAPP was not affected in the islets of beta-cell-specific *Pax6* KO mice. Firstly, this would change the normal IAPP to insulin ratio (as insulin is decreased in the KO islets) that may negatively affect the insulin secretion and contribute to further impairment of beta-cell function. A change in the IAPP to insulin ratio may also contribute to insulin resistance. Secondly, as the proinsulin processing is defective in the *Pax6* KO islets, proIAPP processing would also be defective due to the same processing machinery involved. This may lead to the accumulation of unprocessed form of IAPP that can cause additional damage to beta-cells.

4.1.6 Long-term tracing of *Pax6* KO beta-cells in relation to ghrelin expression

Ashery-Padan et al. (2004) used hAP (human alkaline phosphatase) reporter in pancreas-specific conditional *Pax6* KO mice to trace the *Pax6* KO endocrine cells. They found that the number of glucagon and insulin expressing cells is decreased but that of somatostatin and PP expressing cells is unchanged in the KO pancreata. Additionally, the hAP labeled endocrine area was not significantly changed in the KO pancreata. This led them to conclude that the KO endocrine cells stay in the pancreas but do not undergo a fate

change to any of the other pancreatic endocrine cells. It was published before the discovery of ghrelin expressing epsilon-cells in the pancreas (Prado et al., 2004). Later on, it was shown by Heller et al. (2005) that ghrelin expressing cells are increased in number in *Pax6*$^{sey/sey}$ pancreata. In this study, it was concluded that Pax6 antagonizes the formation of ghrelin^{+} epsilon-cells.

In the wild-type pancreas, the number of ghrelin expressing cells is highest during the embryonic development. Subsequently, these cells decrease in number and finally become almost undetectable in the adult pancreas (Wierup et al., 2004; Hill et al., 2009). As both the *Pax6* KO and *Pax6*$^{sey/sey}$ mice die shortly after birth (Hill et al., 1991; St-Onge et al., 1997; Ashery-Padan et al., 2004), it was only possible to analyze the ghrelin^{+} cell population at the embryonic stage and the long term fate of the *Pax6*-deficient cells was not identified. Furthermore, due to the absence of Pax6 from the beginning of pancreatic development, the endocrine cells (especially alpha- and beta-cells) never attain the functional maturity. Therefore, an upregulation of ghrelin at this stage can suggest a possible developmental shift to the ghrelin expressing epsilon-cell fate.

Recently, Hart et al. (2013) carried out the whole-body knock out of *Pax6* in the adult mice and found an increase in the ghrelin expressing cell population of the pancreas. In this study, most of the ghrelin^{+} cells found were located at the periphery of the islets and were not shown to be co-positive for any of the other endocrine hormones. Therefore, the authors concluded that these ghrelin^{+} cells belong to the standard epsilon-cell population.

In our study, we knocked out *Pax6* specifically from the beta-cells while leaving the rest of the endocrine cell population unaffected. After the induction of KO, ghrelin expression started to upregulate in beta-cells and gradually expanded as more and more *Pax6*-deficient cells started to produce ghrelin. Initially the *Pax6* KO beta-cells expressed ghrelin together with insulin. At later stages, insulin expression disappeared from many cells converting them to ghrelin only expressing cells. By showing the expression of several other beta-cell related factors in ghrelin^{+} cells we proved that these ghrelin^{+} cells arise from beta-cells. This was additionally confirmed by YFP reporter tracing. Therefore, the ghrelin^{+} cells in our beta-cell-specific *Pax6* KO pancreata do not belong to the standard epsilon-cell population. Instead, they are the beta-cells that have lost their maturity and started to produce higher levels of ghrelin (Figure 4.1).

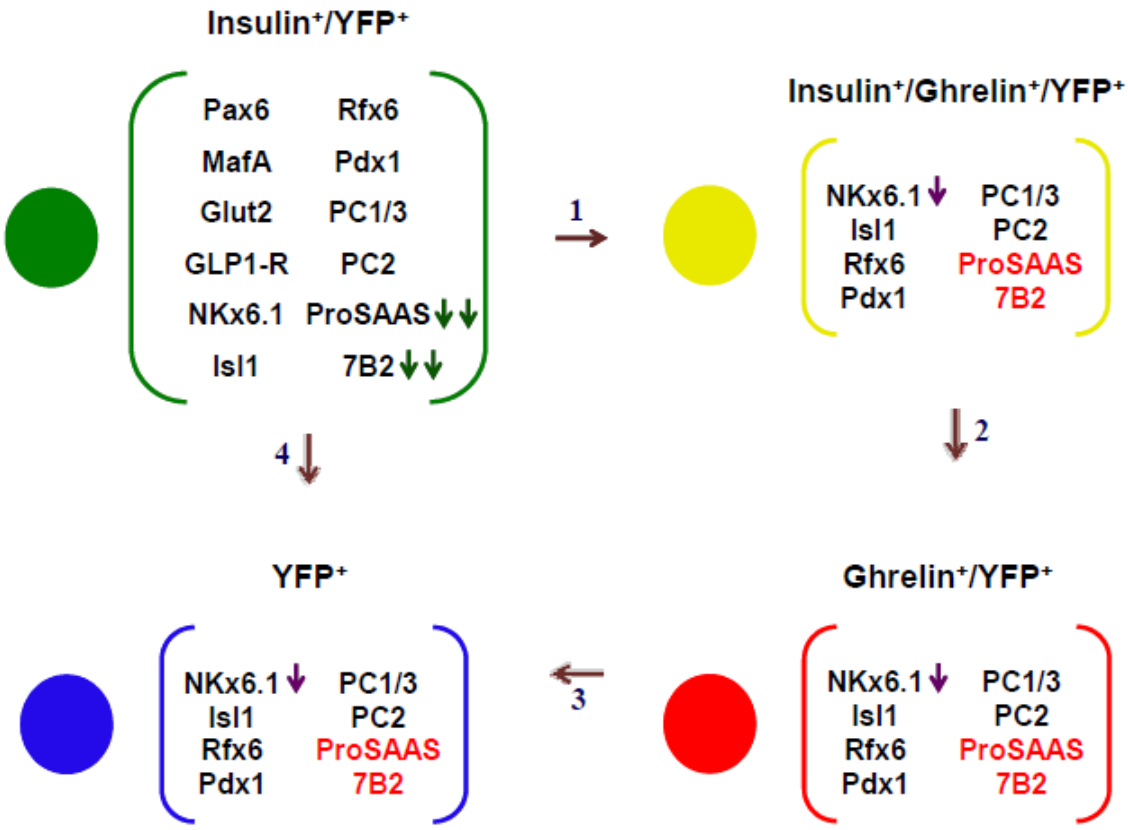

Figure 4.1: Schematic representation of the phenotypic change observed in *Pax6*-deficient beta-cells. Beta-cells in the control mice express insulin, Pax6, Isl1, Rfx6, Pdx1, MafA, Nkx6.1, Glut2, GLP1-R, PC1/3, PC2, and very low levels of 7B2 and proSAAS. Following *Pax6* ablation, the expression of MafA, Glut2, and GLP-1 receptor (GLP1-R) is lost, the expression of Nkx6.1 is reduced, the expression of Isl1, Rfx6, Pdx1, PC1/3, and PC2 is maintained, and the expression of proSAAS and 7B2 is upregulated. Along with that, *Pax6*-deficient cells gradually lose the expression of insulin and gain the expression of ghrelin. During this process these cells pass through a transient state of ghrelin-insulin co-expression before converting to ghrelin only expressing cells (arrows 1 and 2). Later on, some of these ghrelin expressing cells lose the expression of ghrelin as well (arrow 3). Some of the insulin^{+} cells directly convert to insulin^{-} cells after losing *Pax6*, without going through the ghrelin expression route (arrow 4).

Moreover, we found that not all the beta-cells start to produce ghrelin after *Pax6* ablation and many that do produce ghrelin stop to do so over long period of time. However, irrespective of the ghrelin expression these *Pax6*-deficient cells retain their endocrine and beta-cell like character as indicated by the presence of Rfx6 and Pdx1 expression, respectively. Therefore, even after losing the Pax6 expression beta-cells do not change their fate to ductal or exocrine cells that are the Pax6 negative population of cells in the pancreas. Lastly, we found that these *Pax6*-deficient beta-cells neither proliferate nor they undergo apoptosis. They stay as a quiescent population of immature beta-like-cells in the islets. The prevention from apoptosis can be attributed to the antiapoptotic effect of ghrelin and neurotensin (Coppola et al., 2008; Chung et al., 2008). Neurotensin is another hormone that was found to be upregulated in *Pax6* KO islets (data not shown).

The role of ghrelin in the embryonic pancreas development is not clear. *Ghrelin* deficient mice have a completely normal development of endocrine and exocrine pancreas (Hill et al., 2009). Recently, Arnes et al. (2012) used lineage tracing to find the fate of ghrelin^{+} cells in the wild-type pancreas. They found that ghrelin^{+} cells mainly give rise to most of the alpha- and PP-cells in the islets. It was, thus, concluded that ghrelin^{+} cell population represents a multipotent progenitor population in the wild-type pancreas. From this it is also evident that ghrelin expression marks cells that have the potential to differentiate but have not fully differentiated yet, and when they do differentiate they lose the expression of ghrelin.

It could be, therefore, hypothesized that ghrelin expression marks a specific pre-differentiation state of the cell. When beta-cells lose their functional maturity, they go back to a pre-differentiation embryonic-like state. This specific state is marked by an increased expression of ghrelin. However, it must be emphasized here that this pre-differentiation state following *Pax6* ablation is distinct from the normal immature state of beta-cells in the embryonic pancreas. The adult *Pax6* KO beta-cells express neither MafA nor MafB but the early embryonic beta-cells express MafB before they start to express MafA. Furthermore, we never found the co-expression of ghrelin with insulin in the wild-type embryonic pancreas (data not shown).

Finally, a rare population of ghrelin$^+$ cells in the beta-cell-specific *Pax6* KO islets was not YFP$^+$ meaning that these cells do not arise from beta-cells and may represent the genuine epsilon-cell population.

4.1.7 Regeneration of beta-cells in the beta-cell-specific *Pax6* KO islets

Beta-cells constitute a dynamic population that can undergo compensatory changes in relation to the varying metabolic needs. Increase in metabolic demand during pregnancy, obesity, or diabetes can induce an increase in beta-cell mass. This expansion of beta-cell mass may occur via increase in proliferation, decrease in apoptosis, increase in cell size and insulin secretion, and generation of new beta-cells from progenitors (neogenesis) or from other endocrine cells (transdifferentiation) (Ahmad, 2013).

Regeneration of beta-cells has been reported in various diabetic mouse models and can lead to a spontaneous recovery from diabetes (Nir et al., 2007; Thorel et al., 2010; Ahmad, 2013). After *Pax6* ablation in beta-cells, we observed an overt diabetic phenotype. However, when followed over a long time a slight decrease in the blood glucose level was observed in young mice (KO induction at 3 weeks) but not in older mice (KO induction at 1.5 months). Nevertheless, a complete recovery to pre-KO level was never observed. By quantification, an increase in the number of YFP negative insulin$^+$ cells was observed in the KO islets that shows expansion of insulin$^+$ cells that escaped recombination and/or insulin$^+$ cells that appeared as a result of neogenesis. Again this increase was greater at younger age compared to the older age. This difference in young vs. old age is explained by the higher basal proliferation in 3 week old mice compared to 1.5 month old ones.

Apart from proliferation some beta-cells may arise from alternative sources such as via transdifferentiation of alpha-cells (Thorel et al., 2010). Occasionally, we observed some Pdx1 positive alpha-cells in beta-cell-specific *Pax6* KO islets. As Pdx1 is a beta-cell specific transcription factor, its presence in alpha-cells can show an intermediate transition state towards beta-cell generation. Transdifferentiation of alpha- to beta-cells has bee shown as the primary mechanism of beta-cell generation after extreme beta-cell loss (Thorel et al., 2010). Additionally, we observed some MafB positive beta-cells in the beta-cell-specific *Pax6* KO islets. MafB is an alpha-cell specific transcription factor that is also expressed in immature beta-cells during embryonic development (Artner et al.,

2007). Presence of MafB in some beta-cells can, therefore, indicate their immature state and new emergence.

In conclusion, some level of beta-cell regeneration does occur after *Pax6* ablation from beta-cells. This regeneration involves proliferation of pre-existing beta-cells and/or generation of some new beta-cells through other sources.

4.2 *Pax6* knockout in alpha-cells

4.2.1 Loss of glucagon expression in *Pax6* KO alpha-cells

Using the non-inducible *glucagon-Cre* mouse line (Herrera, 2000), we achieved almost 70% recombination efficiency as indicated by the YFP reporter expression. This means that at a given time-point nearly 70% of the alpha-cells would have lost *Pax6*. Labeling with YFP reporter also allowed us to precisely identify and track the *Pax6* KO alpha-cells. The KO alpha-cells did not express glucagon as revealed by immunostaining and were also negative for the expression of MafB. In adult islets, MafB is an alpha-cell specific transcription factor that is a direct downstream target of Pax6 and together with Pax6 it controls the transcription of *Proglucagon* gene (Artner et al., 2006; Gosmain et al., 2010). Therefore, the loss of Pax6 and then MafB explains the inability of these cells to synthesize glucagon.

4.2.2 Ghrelin upregulation in *Pax6* KO alpha-cells

It has been previously shown that ghrelin is upregulated in *Pax6* KO pancreata. However, the exact origin of these ghrelin$^+$ cells has not been clearly identified (Heller et al., 2005; Kordowich et al., 2011). Similar to these preceding studies, we found an increased expression of ghrelin in the mutant islets. Lineage tracing with YFP reporter confirmed that these ghrelin$^+$ cells are originated from previously glucagon$^+$ cells. This conclusion was strengthened by two more observations. Firstly, the presence of some reporter labeled ghrelin-glucagon co-positive cells along with the reporter labeled ghrelin-only$^+$ cell population. Secondly, the expression of Arx, a marker of alpha-cell lineage (Collombat et al., 2003, 2005, 2007), in the ghrelin$^+$ cells. Thus, alpha-cells lost the expression of glucagon following *Pax6* ablation and started to produce ghrelin (Figure 4.2). These ghrelin expressing cells were distinct from the ghrelin$^+$ cells that emerge in the beta-cell-specific *Pax6* KO islets as the later did not express Arx (data not shown).

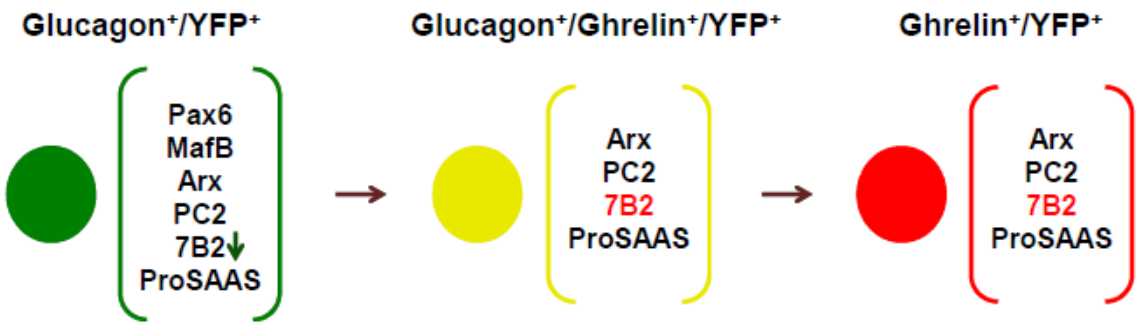

Figure 4.2: Schematic representation of the phenotypic change observed in *Pax6*-deficient alpha-cells. Alpha-cells in the control mice express glucagon, Pax6, MafB, Arx, PC2, proSAAS, and low level of 7B2. Following *Pax6* ablation, the expression of glucagon and MafB is lost, the expression of Arx, PC2, and proSAAS is maintained, and the expression of 7B2 is upregulated. Along with that ghrelin expression is highly upregulated in the *Pax6*-deficient cells, and the cells transiently pass through a glucagon-ghrelin co-positive state before they completely convert into ghrelin expressing cells.

Additionally, we observed some YFP$^-$ ghrelin$^+$ cells in the alpha-cell-specific *Pax6* KO islets that were also Arx$^+$. Hence, a rare population of ghrelin$^+$ cells in the KO islets is not coming from alpha-cells and may represent the cells from genuine epsilon-cell population.

We found the upregulation of ghrelin expression in both *Pax6*-deficient alpha- and beta-cells. This suggests that the molecular mechanism behind this upregulation of ghrelin is probably similar in both of these cell types. By ChIP assay, we found no interaction of Pax6 with the *ghrelin* promoter. Therefore, it seems that the upregulation of ghrelin is not directly due to the loss of Pax6-mediated repression of *ghrelin* expression. It is rather due to the activation of a pathway that is shared between alpha- and beta-cells and is activated when these cells lose their mature differentiation status.

4.2.3 7B2 upregulation in *Pax6* KO alpha-cells

In alpha-cells, glucagon is initially synthesized as proglucagon, a precursor form that is processed by the enzyme prohormone convertase 2 (PC2) to generate the mature glucagon peptide (Furuta et al., 1997). Prohormone convertase 2 is itself synthesized as a proprotein (pro-PC2) that requires the presence of neuroendocrine protein 7B2 for its maturation and activation (Westphal et al., 1999). Previous in vitro studies have shown that proglucagon processing in the Pax6 knockdown alpha-cells is impaired due to the reduced expression of both PC2 and 7B2 (Katz et al., 2009). However, in contrast to this in vitro study we found no change in the PC2 immunoreactivity in *Pax6* KO alpha-cells. Surprisingly, instead of being decreased the expression of 7B2 was highly upregulated in these cells. This increased expression of 7B2 in the KO cells might be in an effort to enhance the PC2 activity in response to the reduced hormone synthesis. This idea is supported by the in vitro study, where 7B2 overexpression in alpha-TC6 cells led to the increased production of glucagon (Helwig et al., 2011). Alternatively, this upregulation of 7B2 might be in relation to the differentiation status of *Pax6*-deficient cells as discussed in the beta-cell section.

As 7B2 is upregulated in both alpha- and beta-cell-specific *Pax6* KO mouse models, this means that a common pathway is affected in both cell types that leads to upregulation of 7B2. This specific pathway is perhaps affected in the intact islet in vivo but not in the isolated cells in vitro. This may explain the difference of 7B2 expression following *Pax6* ablation in vitro vs. in vivo.

4.2.4 Regeneration of alpha-cells in the alpha-cell-specific *Pax6* KO islets

As the function of glucagon is to increase the blood glucose level in response to hypoglycemia, a reduced blood glucose level is expected under conditions of glucagon deficiency (Gelling et al., 2003). In spite of the fact that *Pax6* KO alpha-cells lose the expression of glucagon, we were not able to see any change in the blood glucose level. This can be explained by either the lower recombination efficiency and/or by the regeneration of alpha-cells. Indeed, as the recombination efficiency was 70%, the rest of the 30% alpha-cells may produce enough glucagon to prevent any appreciable change in the blood glucose level. Interestingly, however, the total number of glucagon$^+$ cells was not significantly different in 1 month old control and knockout mice. In contrast to the total population, the number of YFP negative glucagon$^+$ cells was significantly increased in the KO islets. Hence, it is quite probable that alpha-cell regeneration did take place after the *Pax6* KO. Analysis of later stages (3 month and 8 month) showed a decrease in the alpha-cell regeneration at older age (8 months) but not at the middle age (3 months). However, even the older mice that showed no regeneration were healthy, possibly due to the remaining unrecombined population of alpha-cells. Regeneration of alpha-cells has been demonstrated in other models of glucagon deficiency as well. Mice that lack glucagon receptor or PC2 show a massive alpha-cell hyperplasia (Furuta et al., 1997; Gelling et al., 2003). As compared to these models, the regeneration that we observe is only moderate, and this is because of the inability to achieve 100% recombination in alpha-cells.

As the *glucagon-Cre* line used was not inducible, it was not possible to define the age of *Pax6*-deficient alpha-cells. At each of the age analyzed, the number of YFP labeled cells was significantly less in the alpha-cell-specific *Pax6* KO islets compared to the control islets. In contrast to that, same numbers of YFP labeled cells were detected at either 1 month or 8 months of age in the alpha-cell-specific *Pax6* KO islets. If we assume that glucagon$^+$ cells regenerate in the KO islet and convert to ghrelin$^+$ cells following the activation of Cre, this should lead to an increase in the ghrelin$^+$ YFP$^+$ cell population. Otherwise, some of the ghrelin$^+$ YFP$^+$ cells should disappear e.g., via apoptosis. However, apoptosis was not detectable in the KO islets as determined by the TUNEL staining (data not shown). Therefore, it remains unclear that what happens to the ghrelin$^+$ cells that arise from alpha-cells.

4.3 Comparison of *Pax6* KO in alpha- and beta-cells

In both alpha- and beta-cells, the ablation of *Pax6* results in the loss of mature differentiation status and upregulation of ghrelin expression. However, the original character of the cell type is maintained in each case as evident by the continued expression of Arx in *Pax6*-deficient alpha-cells and that of Pdx1 in *Pax6*-deficient beta-cells. Similarly, the ghrelin expressing cells in each type of knockout pancreata are distinct from each other. Ghrelin expressing cells in beta-cell-specific *Pax6* KO islets express Pdx1 and remain negative for Arx and glucagon expression. On the other hand, ghrelin expressing cells in alpha-cell-specific *Pax6* KO islets express Arx and never show co-expression with insulin. Moreover, the alpha-cell derived ghrelin$^+$ cells are located at the periphery of the islets that is the normal location of alpha-cells in mouse islets. In contrast to that, the beta-cell derived ghrelin$^+$ cells are dispersed throughout the islet.

In the normal mouse islet, beta-cells form the core of the islet and other endocrine cell types stay at the periphery (Brissova et al., 2005). As compared to the control mice, the islet architecture in beta-cell-specific *Pax6* KO mice is slightly disturbed as different types of endocrine cells are sometimes mixed into each other. Additionally, the number of glucagon, somatostatin, and PP producing cells is increased in these islets. However, in alpha-cell-specific *Pax6* KO mice no such disturbance is observed. The number of insulin, somatostatin, and PP producing cells is not changed (data not shown) and the islet structure remains intact. For the comparison of cell-type-specific changes in both types of KO mice, see Figures 4.1 and 4.2.

4.4 *Pax6* overexpression

To date, there is only one in vivo study of *Pax6* overexpression done in pancreas. In this study, Yamaoka et al. (2000) overexpressed *Pax6* directly under *Pdx1* and *insulin* promoter. The phenotype was variable with many animals developing diabetes but not all. They found reduction in the number of insulin$^+$ cells due to increased apoptosis. Additionally, when overexpressed under *Pdx1* promoter, Pax6 caused hypoplasia of the pancreatic exocrine tissue. One disadvantage of overexpressing *Pax6* in this way is that both the *Pdx1* and *insulin* promoters would be subject to regulation inside beta-cells that may lead to variation in the phenotype.

We decided to repeat the overexpression of *Pax6* in a different way. In our system *Pax6* was overexpressed under CAG promoter that provides constitutive expression and, therefore, is less sensitive to cellular state. However, this overexpression is dependent on Cre/*loxP* system and can be activated in the desired cell type. Along with the *Pdx1* (whole pancreas), and *insulin* (beta-cells) expression domains we overexpressed *Pax6* in the *glucagon* (alpha-cells) expression domain too. *Pax6OE* mice in any domain did not develop diabetes. Difference in proliferation or apoptosis of beta-cells was also not detectable. However, like this previous study we also found hypoplasia of the exocrine pancreas in *Pax6OE; Pdx1-Cre* mice. Furthermore, we found decrease in the glucagon$^+$, ghrelin$^+$, and PP$^+$ cell population when *Pax6* was overexpressed under *Pdx1* expression domain indicating that compared to insulin$^+$ or somatostatin$^+$ cell population, development of these three cell types is more sensitive to the level of Pax6.

In another study by Kredo-Russo et al. (2012), miR-7 knockdown lead to indirect overexpression of Pax6. In this case also the number of ghrelin$^+$ cells was decreased. However, in contrast to our study, the number of both the insulin$^+$ and glucagon$^+$ cells was increased. This difference may arise from other targets that are affected by miR-7 knockdown along with Pax6. Secondly, the study was performed in explant culture that may behave a bit different from the real in vivo system.

4.5 Comparison of the Pax6 pancreatic phenotype in relation to Pax6 function in brain and eye

Apart from pancreas, Pax6 also plays essential roles in the development of brain and eye (Callaerts et al., 1997). However, the exact function of Pax6 in different tissues as well as in the different cell types of the same tissue can be highly variable. Without going into extensive detail we will note down few interesting examples from eye and brain and compare them with our pancreatic *Pax6* KO /OE phenotype.

During eye development, Pax6 is required in the retinal progenitor cells (RPCs) for the completion of retinal neurogenesis. When *Pax6* is deleted from the late phase optic cup after e10.5, a dual function of Pax6 is revealed. In the peripheral RPCs, loss of Pax6 results in reduced proliferation and beginning of early differentiation that never attains maturity. In the central RPCs, loss of Pax6 leads to a cell-fate change from retinal lineage to amacrine interneurons (Oron-Karni et al., 2008). Along with the retinal neurogenesis,

Pax6 is also required for lens fiber cell differentiation. Here the presence of Pax6 is essential for the early phase of differentiation while downregulation of Pax6 is required for the last stage of lens differentiation (Shaham et al., 2012).

In brain, Pax6 is highly expressed in cortical radial glial cells (neural progenitors). In the absence of Pax6, neural progenitor pool is reduced due to increased cell-cycle exit and premature start of neuronal differentiation (Quinn et al., 2007). Pax6 is also expressed in the developing and postnatal astrocytes that originate from neural progenitors. Cultured astrocytes from *Pax6*$^{sey/sey}$ mice are not fully differentiated and show increased proliferation. Furthermore, the PI3K/Akt pathway, that enhances cell survival and reduces apoptosis, is also activated in these astrocytes as indicated by the increased activity of Akt (Sakurai and Osumi, 2008).

In spite of the variation, it seems that Pax6 derives successful differentiation of the respective cell types. Especially as compared to the *Pax6*$^{sey/sey}$ astrocyte phenotype, we also observed a loss of differentiation in the *Pax6* KO alpha- and beta-cells. Though in our case there was decrease in proliferation rather than increase, that is more similar to the *Pax6* KO in peripheral RPCs in the eye. Moreover, ghrelin that is upregulated in alpha- and beta-cells after *Pax6* KO, may provide cell survival and anti-apoptotic affect by activating PI3K/Akt pathway (Chung et al., 2008).

Pax6 gene dosage is important during development. Like the reduced dosage, an over-dosage of Pax6 also leads to developmental defects. However, again the effect of Pax6 overexpression can vary in different cell types at different time points of development. In eye, overexpression of Pax6 leads to defective iris, ciliary body, and retinal development without inducing apoptosis (Manuel et al., 2008; Davis et al., 2009). In brain, it was shown that Pax6 overexpression causes apoptosis in a set of cortical progenitors. Progenitors that have higher endogenous level of Pax6 expression are more resistant to the effect of Pax6 overexpression. Additionally, overexpression of Pax6 in postmitotic neurons does not cause apoptosis (Berger et al., 2007). Therefore, it seems that the sensitivity of different cell types to Pax6 dosage is different.

In our study, *Pax6* overexpression in the pancreas did not cause apoptosis in the adult pancreas. In the exocrine pancreas, slight hypoplasia was observed. In the endocrine pancreas, glucagon^{+} and PP^{+} cells were reduced in number. Therefore, at the level of

Pax6 expression achieved in these transgenic mice, the development of pancreas does not seem to be drastically affected.

Lastly, it is important to mention that some of the Pax6 target genes are shared among forebrain, lens, and beta-cells. In a recent study, Xie et al. (2013) utilized ChIP-chip approach to identify the targets of Pax6 in forebrain, lens, and beta-cells. Interestingly, they found that 133 target promoters were shared between all three tissue sources. Furthermore, MafA, MafB, Isl1, and Pcsk1n (proSAAS) that are expressed in the endocrine pancreas are also expressed in the eye and regulated by Pax6 (Xie et al., 2013). MafA and MafB are important for the alpha- and beta-cell development/maturation and are also required for lens development (Reza et al., 2002; Cvekl et al., 2004; Zhang et al., 2005; Artner et al., 2007). However, only about 3% of the Pax6-bound promoters are shared between lens, forebrain, and beta-cells (Xie et al., 2013). This means that apart from having some similarity, these three tissue types are only distantly related.

5. Summary

Pax6, a transcription factor from the paired-box family is expressed in the eye, central nervous system, olfactory system, and pancreas, and plays an important role in the development of these tissues (Shaham et al., 2012). In pancreas, Pax6 is required for the development and function of the endocrine pancreas especially alpha- and beta-cells (St-Onge et al., 1997; Ashery-Padan et al., 2004; Gosmain et al., 2010; Gosmain et al., 2012a). However, as the *Pax6* knockout mice die shortly after birth, it does not allow for the elucidation of Pax6 function in adult endocrine pancreas in vivo. Secondly, a cell-type-specific effect of *Pax6* ablation cannot be established if *Pax6* is ablated from the whole pancreas.

For this purpose, we decided to generate *Pax6* konckout (KO) in alpha- and beta-cells, separately. Additionally, we used a tamoxifen-inducible Cre line to ablate *Pax6* from beta-cells in the adult mice. In parallel to that, we overexpressed *Pax6* in the whole pancreas, in beta-cells alone, and in the alpha-cells alone. Following results were obtained in this study:

1. The beta-cell-specific *Pax6* KO mice developed an overt diabetes in a short time-period after tamoxifen induction. In pancreas, the number of insulin$^+$ cells was decreased and that of ghrelin$^+$ cells increased. Lineage tracing with YFP reporter confirmed the origin of these ghrelin$^+$ cells from the insulin$^+$ cells.

2. Following *Pax6* ablation, beta-cells lost their mature differentiation status and function. This was evident by the reduced expression of MafA and Nkx6.1, two mature beta-cell-specific transcription factors. Moreover, *Pax6*-deficient beta-cells were defective in insulin synthesis and glucose-stimulated insulin secretion, indicated by the absence of insulin, Glut2, and GLP-1 receptor expression. Finally, the proinsulin processing was also defective in *Pax6*-deficient beta-cells due to the increased expression of proSAAS that can inhibit PC1/3-mediated proinsulin processing.

3. Long-term tracing of *Pax6*-deficient beta-cells revealed that most of them continue to express ghrelin. These cells did not die or convert to any other cell type. Apparently, they maintained their endocrine and partial beta-cell-like character as shown by the continued expression of Rfx6 and Pdx1, that are

endocrine-specific and beta-cell related transcription factors in the adult pancreas, respectively.

4. The alpha-cell-specific *Pax6* KO mice remained healthy and did not show any change in blood glucose level. In pancreas, the number of glucagon$^+$ cells was unchanged at younger age but decreased at an older age. Ghrelin expression was also upregulated in alpha-cell-specific *Pax6* KO islets. Lineage tracing with YFP reporter confirmed the origin of these ghrelin$^+$ cells from the glucagon$^+$ cells.

5. Following *Pax6* ablation, alpha-cells lost their mature differentiation status and function. This was evident by the loss of glucagon and MafB expression. *Pax6*-deficient alpha-cells, however, maintained their alpha-cell-like character as they continued to express Arx that is alpha-cell lineage determinant.

6. We found the increased expression of ghrelin in both *Pax6*-deficient alpha- and beta-cells. In each case, however, the ghrelin$^+$ cells maintained the character of the cell-type from which they originated. Alpha-cell derived ghrelin$^+$ cells continued to express Arx. On the other hand, beta-cell derived ghrelin$^+$ cells did not express Arx. Instead, they continued to express beta-cell related factors (e.g., Pdx1, IAPP, PC1/3).

7. *Pax6* ablation from alpha- and beta-cells led to the loss of glucagon and insulin expression, respectively. This in turn resulted in the regeneration of the respective cell-type in each case. Regeneration capacity of both alpha- and beta-cells was higher at a younger age. However, in beta-cell-specific *Pax6* KO mice the regeneration of beta-cells was not sufficient to allow for the complete recovery from diabetes even at a younger age.

8. *Pax6* overexpressing mice remained healthy and did not show any change in the blood glucose level. In the pancreas-specific *Pax6* overexpressing mice, slight hypoplasia of the exocrine pancreas was observed. In the endocrine pancreas, numbers of alpha-, PP-, and epsilon-cells were decreased and that of beta- and delta-cells not affected. In the beta-cell-specific *Pax6* overexpressing mice, no change was observed in the pancreas. Lastly, in the alpha-cell-specific *Pax6* overexpressing mice, again a decrease in the number of alpha-cells was observed.

Therefore, the development of alpha-cells appears to be more sensitive to the level of Pax6 expression as compared to that of beta-cells.

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