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# Regulation of pancreatic and intestinal endocrine cell differentiation and function: roles of Pak3 and Rfx6

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**IGBMC – CNRS UMR7104 – Inserm U964 - UDS**

**THÈSE** présentée par :

**Julie PICCAND**

soutenue le : 18 septembre 2012

pour obtenir le grade de : **Docteur de l'Université de Strasbourg**

Discipline/ Spécialité : Biologie cellulaire et développement

**REGULATION OF PANCREATIC AND  
INTESTINAL ENDOCRINE CELL  
DIFFERENTIATION AND FUNCTION:**

**Roles of Pak3 and Rfx6**

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## THESE

*Pour l'obtention du grade de*  
**Docteur en Sciences de l'Université de Strasbourg**  
Discipline: Sciences de la Vie  
Spécialité: Biologie cellulaire et développement

Présentée par  
***Julie PICCAND***

# **Regulation of pancreatic and intestinal endocrine cell differentiation and function: Roles of Pak3 and Rfx6**

Soutenue publiquement le 18 septembre 2012

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# REGULATION OF PANCREATIC AND INTESTINAL ENDOCRINE CELL DIFFERENTIATION AND FUNCTION: Roles of Pak3 and Rfx6

## Résumé

Les hormones sécrétées par les cellules endocrines pancréatiques et intestinales participent à la régulation de l'homéostasie énergétique. Leur différenciation repose sur des programmes génétiques similaires contrôlés par le facteur de transcription Ngn3. Peu de choses sont connues sur les gènes activés par *Ngn3* et leurs implications dans les mécanismes contrôlant la spécification et la maturation des cellules endocrines. Par conséquent, le transcriptome des progéniteurs endocrines a été déterminé dans l'équipe. Parmi les gènes fortement enrichis dans le lignage endocrine, j'ai caractérisé l'expression et la fonction de la kinase *Pak3* et j'ai continué l'étude de la fonction pancréatique et intestinale du facteur de transcription *Rfx6*. J'ai montré que *Pak3* est exprimé dans le lignage endocrine pendant le développement et chez l'adulte. Avec des expériences de perte de fonction, j'ai montré que ce gène inhibe la prolifération des progéniteurs endocrines et des cellules bêta durant l'embryogénèse. De plus, une étude métabolique a montré que les souris mutantes pour *Pak3* sont intolérantes au glucose. En parallèle, en utilisant une souris conditionnelle pour *Rfx6*, j'ai montré que *Rfx6* est nécessaire en aval de *Ngn3* pour la différenciation des cellules endocrines pancréatiques et intestinales. Finalement, des expériences dans les souris adultes suggèrent que *Rfx6* est nécessaire pour maintenir les cellules bêta, renouveler les cellules entéroendocrines et absorber les lipides dans l'intestin. En conclusion, ces études révèlent deux nouveaux gènes clés dans la régulation de la différenciation des cellules endocrines et de l'homéostasie énergétique dans le pancréas et l'intestin.

Mots Clés : Pancréas, Intestin, Différenciation, Rfx6, Pak3, Développement, Endocrine, Cellule bêta

## Résumé en anglais

Pancreatic and intestinal endocrine cells, and their secreted hormones, contribute to the regulation of energy homeostasis. Their differentiation relies on similar genetic programs controlled by the proendocrine transcription factor Ngn3. However, our knowledge of the endocrinogenic programs implemented by Ngn3 is still fragmentary. Therefore, the transcriptome of endocrine progenitors has been determined in the lab. Among the genes which showed a strong enrichment in the endocrine lineage, I studied the expression and function of *Pak3*, a serine/threonine kinase and further pursued the dissection of the function of the transcription factor *Rfx6* in the pancreas and the intestine. I showed that *Pak3* is expressed throughout pancreas development and maintained in adult islets. Using *ex vivo* loss of function experiments and *in vivo* characterisation of the *Pak3*-deficient mice, I identified *Pak3* as an inhibitor of islet progenitors and beta-cell proliferation in the embryonic mouse pancreas. Furthermore, we performed metabolic studies which revealed that *Pak3*-deficient mice have an impaired glucose homeostasis, especially under challenging high fat diet. In parallel, using a conditional knockout mouse for *Rfx6*, we showed that *Rfx6* is necessary downstream of *Ngn3* for endocrine cell differentiation in the pancreas as well as in the intestine. Finally, additional experiments in adult mice suggest that *Rfx6* is necessary to maintain pancreatic beta-cells, enteroendocrine cell turnover and intestinal lipid absorption. In conclusion, these studies revealed two novel key players in the regulation of endocrine cell differentiation and energy homeostasis in the pancreas and the intestine.

Key Words: Pancreas, Intestine, Differentiation, Rfx6, Pak3, Development, Endocrine, beta-cell



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***On se lasse de tout sauf de comprendre.***

Virgile

***Success is not final, failure is not fatal:  
It is the courage to continue that counts.***

Winston Churchill



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# Abbreviations

<b>5HT</b>	Serotonin
<b>AID</b>	Auto-Inhibitory Domain
<b>Arx</b>	Aristaless related homeobox
<b>Ascl2</b>	Achaete-scute complex homolog 2
<b>Atoh1</b>	Atonal homolog 1
<b>ATP</b>	Adenosine Triphosphate
<b>bHLH</b>	Basic Helix Loop Helix
<b>BrdU</b>	Bromodeoxyuridine
<b>BMP</b>	Bone morphogenetic protein
<b>cKO</b>	Conditional knockout
<b>c-myc</b>	Myelocytomatosis oncogene
<b>CCK</b>	Cholecystokinin
<b>Cdc42</b>	Cell division cycle 42
<b>CDK</b>	Cyclin-dependant kinase
<b>Cdx</b>	Caudal type homeobox
<b>ChgA</b>	ChromograninA
<b>CREB</b>	cAMP response element binding
<b>CRIB</b>	Cdc42/Rac1 Interacting Binding
<b>Dll</b>	Delta-like
<b>DNA</b>	Desoxyribonucleic acid
<b>E</b>	Embryonic day
<b>EC</b>	Enterochromaffine
<b>EE</b>	Enteroendocrine
<b>Eph</b>	Ephrin
<b>ERK</b>	Extracellular signal regulated kinase
<b>FGF</b>	Fibroblast Growth Factor
<b>Fox</b>	Forkhead box
<b>Gata</b>	GATA binding protein
<b>Gck</b>	Glucokinase
<b>GEF</b>	Guanine Exchange Factor
<b>Gfi1</b>	Growth factor independent 1
<b>GIP</b>	Gastric Inhibitory Polypeptide
<b>GLP</b>	Glucagon Like Peptide
<b>GSIS</b>	Glucose stimulated insulin secretion
<b>GTP</b>	Guanosine Triphosphate
<b>H</b>	Hour
<b>Hes1</b>	Hairy and enhancer of split 1
<b>HH</b>	Hedgehog
<b>Hnf1b</b>	Hepatocyte Nuclear Factor 1b
<b>Hox</b>	Homeobox
<b>Insm1</b>	Insulinoma associated 1
<b>IP</b>	Intraperitoneal

<b>IPGTT</b>	Intraperitoneal Glucose Tolerance Test
<b>IPS</b>	Induced Pluripotent Stem
<b>KO</b>	Knockout
<b>LIM</b>	Lin11, Isl-1 & Mec-3
<b>LTP</b>	Long-Term Potentiation
<b>Maf</b>	V-mafmusculoaponeurotic fibrosarcoma oncogene family, protein (avian)
<b>MAP</b>	Mitogen activated protein
<b>MEK</b>	MAP kinase kinase or MAP enzyme kinase
<b>MHC</b>	Major Histocompatibility Complex
<b>MPC</b>	Multipotent Progenitor Cell
<b>Myr</b>	Myristylation signal
<b>NeuroD1</b>	Neurogenic differentiation 1
<b>NFkB</b>	Nuclear factor kappa B
<b>Ngn3</b>	Neurogenin3
<b>NICD</b>	Notch intracellular domain
<b>Nkx</b>	Nirenberg-Kim homeobox
<b>OGTT</b>	Oral Glucose Tolerance Test
<b>P</b>	Postnatal day
<b>PAK</b>	p21 protein (Cdc42/Rac)-activated kinase
<b>Pax</b>	Paired box gene
<b>PKA</b>	Protein Kinase A
<b>Pdx1</b>	Pancreatic and duodenal homeobox 1
<b>PIX</b>	PAK Interacting exchange Factor
<b>PP</b>	Pancreatic Polypeptide
<b>PTF1</b>	Pancreas Transcription Factor 1
<b>PYY</b>	Peptide Tyrosine Tyrosine or Pancreatic Peptide YY
<b>Rac1</b>	RAS-related C3 botulinum substrate 1
<b>Ras</b>	Resistance to audiogenic seizures
<b>Rbpj</b>	Recombination signal binding protein for immunoglobulin kappa J
<b>Rbpjl</b>	Recombination signal binding protein for immunoglobulin kappa J region-like
<b>RFX</b>	Regulatory Factor X
<b>RIPE3b</b>	Rat insulin promoter element 3b
<b>RNA</b>	Ribonucleic Acid
<b>RT</b>	Retro-transcription
<b>SD</b>	Standard Deviation
<b>SEM</b>	Standard Error of the Means
<b>SHH</b>	Sonic Hedgehog
<b>siRNA</b>	Small Interfering Ribonucleic Acid
<b>Sox</b>	SRY-box containing gene
<b>Src</b>	Rous sarcoma oncogene
<b>Tcf</b>	T cell factor
<b>Tcf7l2</b>	Transcription factor 7-like 2, T-cell specific, HMG-box
<b>TGF</b>	Transforming Growth Factor
<b>VEGF</b>	Vascular Endothelial Growth Factor
<b>Wnt</b>	Wingless-related MMTV integration site
<b>YFP</b>	Yellow Fluorescent Protein

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# I. Introduction

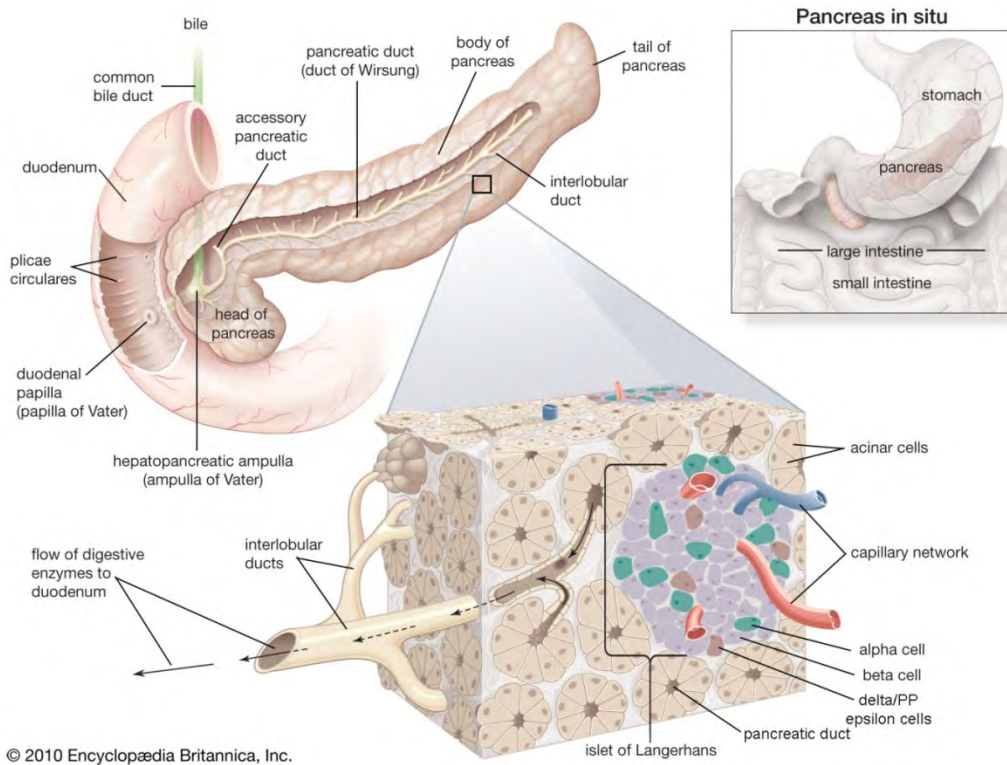
The pancreas develops from the endoderm and is composed of endocrine, acinar and ductal cells. Endocrine cells are clustered in islets which are composed mainly of four endocrine cell types: alpha-, beta-, delta- and PP-cells secreting respectively the hormones glucagon, insulin, somatostatin and pancreatic polypeptide. The main role of islets is the regulation of glycaemia. Ingestion of nutrients triggers a complex hormonal response which stimulates glucose uptake in the liver, muscles and adipose tissue to minimise the increase in blood glucose level. Insulin secretion by pancreatic beta-cells plays a major role in this process. Even though the secretory response of beta-cells is mainly controlled by blood glucose level, some gut hormones secreted in response to food intake have an important part in the regulation of glucose-stimulated insulin secretion. These hormones are called gluco-incretins and are able to effect on both insulin secretion and production. In type 2 diabetes patients, where the pancreas is able to produce insulin but either not enough or the body is not responding to it, gluco-incretins are used as therapeutic drugs (Holst, 2002). However this cannot be used in patients with type 1 diabetes where beta-cells are killed by an autoimmune reaction.

Type 1 diabetes is an autoimmune disease which ultimately leads to the destruction of pancreatic beta-cells (Gianani and Eisenbarth, 2005). This loss causes a progressive decrease in insulin secretion and thus an increase of the glycaemia. This disease is well spread in Europe, North America, Australia and New-Zealand where about 20-40 cases per 100'000 persons are reported each year (Zipris, 2009). The world global incidence of type 1 diabetes is constantly increasing; 70.000 new cases are expected each year. The treatment for type 1 diabetes is daily injections of insulin, however in some cases this does not allow to regulate glycaemia. On top of that, regular insulin injection does not always prevent secondary complications due to hyperglycaemic episodes (retinopathy, nephropathy, neuropathy, ...) (Shapiro et al., 2000). Cellular therapy of diabetes, by grafting of islets of Langerhans, appeared these last years like a potential substitute for insulin-therapy (Maffi et al., 2011). In 2008, 13% of transplanted patients were normoglycaemic without insulin injection 84 months after the transplantation and 67% were secreting C-peptide with a reduction of hypoglycaemic events (Fiorina et al., 2008). Other studies have been showing insulin-independence after islet transplantation but with a shorter follow up of only about 2 to 4 years post-transplantation (Maffi et al., 2011; Shapiro et al., 2006; Vantyghem et al., 2009). Despite encouraging results, there are many obstacles to the wide usage of cell therapy for diabetes, among them immunosuppressive treatments, surgery complications and lack of donors. To overcome the spare number of donors, one promising solution is the generation of beta-cells *in vitro* from pluripotent cells. However, to produce beta-cells one needs to reproduce in a dish what is happening *in vivo* during embryonic differentiation. Thus, it is crucial to understand the different steps giving rise to mature and functional beta-cells. To date, the cells that are produced are immature and not able to appropriately secrete insulin in response to glucose (Van Hoof et al., 2009). In this context, the strategy of the lab is to find effectors downstream of the proendocrine gene *Ngn3*. Using a transcriptomic approach (Soyer et al., 2010) we have identified many potential candidate genes, among them *Pak3* and *Rfx6*. Thus, the objectives of my thesis were (1) to analyse the expression and function of *Pak3*, a kinase implicated in cell-cycle exit and differentiation, (2) to characterise the precise role of *Rfx6* in pancreatic and intestinal endocrine cell differentiation and function using mice models.

## A. Anatomy and function of the pancreas

### 1. Pancreas anatomy and histology

The pancreas is an organ of the digestive tract that is found in all vertebrates. It is a long and narrow **gland** located in the epigastric region behind the stomach and the spleen. The pancreas is both an exocrine and endocrine gland (Figure 1).



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**Figure 1: Anatomy and histology of the human pancreas.** (Adapted from Encyclopaedia Britannica, 2010)

The **exocrine** pancreas accounts for 90-95% of the total pancreatic volume. It is composed mainly of **acinar** and **ductal** cells (Edlund, 2002). Moreover, centroacinar cells can be found at the junction between acinar and ductal cells (Stanger and Dor, 2006). The acinar cells, which are arranged in clusters called acini at the end of ducts, secrete the diverse digestive enzymes (see I.A.2.1.1) of the pancreatic juice into the ducts. The pancreatic interlobular ducts eventually merge into duct of Wirsung which links the pancreas to the duodenum where the pancreatic juice is delivered to the small intestine to participate in the digestion of nutrients. Centroacinar cells as well as duct cells are secreting the electrolytic components, mainly  $\text{HCO}_3^-$  ions, of the pancreatic juice (Garcia et al., 2008).

The **endocrine** pancreas represents only about 1-2% of the mature pancreas (Githens, 1988). It is composed of endocrine cells clustered in **islets of Langerhans** which are disseminated throughout the exocrine pancreas and intermingled with blood vessels and neurons. Pancreatic islets are highly vascularised thus allowing secreted **hormones** to enter the bloodstream. The five endocrine cell types composing the islets (see I.A.2.2) are organised differently depending on the species; in rodents, beta-cells are clustered at the centre of the islet and surrounded by a heterogeneous layer of alpha-, delta- and PP-cells.

## 2. Epithelial cells of the pancreas and their functions

### 2.1. Exocrine cells

The function of the exocrine pancreas relies on the properties of the two major components of the pancreatic juice: **bicarbonate ions** and **digestive enzymes**. The exocrine secretions are mainly regulated by duodenal and stomachal hormones like secretin and gastrin (see I.B.2.4.2). However, it has been shown that the pancreatic polypeptide produced by the PP-cells can reduce exocrine enzymes secretions (Murphy and Bloom, 2006).

#### 2.1.1. Ductal cells

Ductal cells secrete an aqueous solution made of diverse **electrolytic compounds** that are added to the digestive enzymes produced by the acinar cells to form the “pancreatic juice”. Thanks to the presence of **bicarbonate ( $\text{HCO}_3$ ) ions** the pancreatic juice is able to neutralise the gastric chime which is enriched in hydrochloric acid secreted by the parietal cells of the stomach. Moreover, mature ducts also drain the pancreatic juice towards the duodenum. Secretin induces the secretion of electrolytic compounds by pancreatic ductal cells (Chey and Escoffery, 1976).

#### 2.1.2. Acinar cells

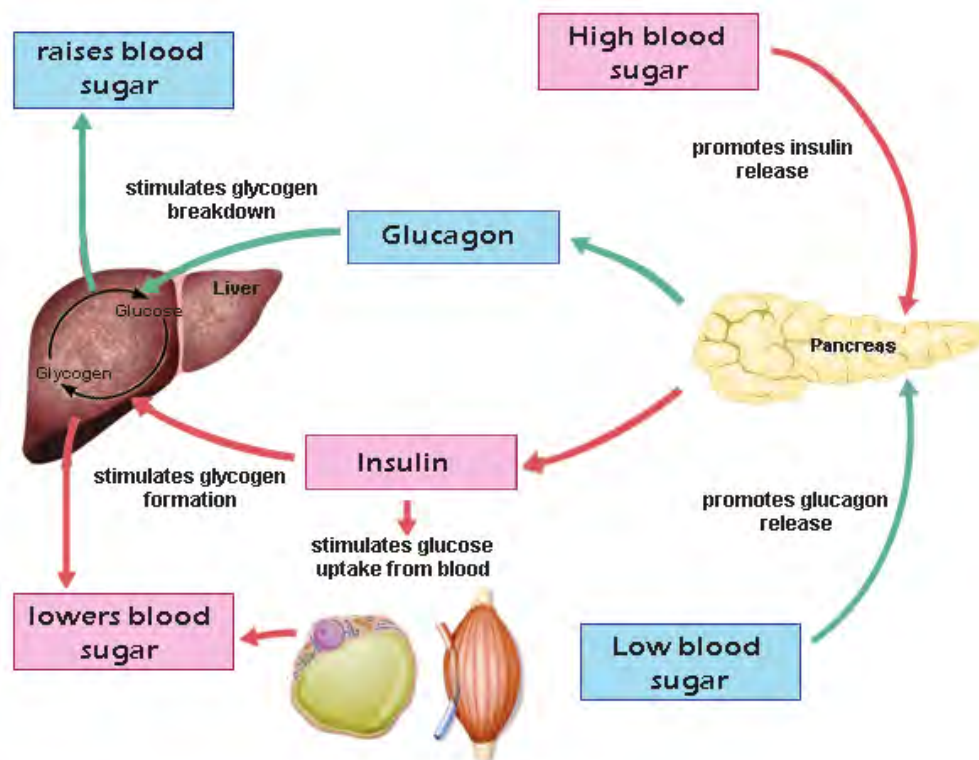
Acinar cells secrete the **pancreatic enzymes** that carry on the metabolisation of the chime to generate simple substances like carbohydrates, lipids and proteic compounds which can be absorbed by the intestine. The pancreatic enzymes are divided into five categories based on their function: **lipolytic**, **proteolytic**, **glycolytic**, **nucleolytic** and **inhibitors**. Lipase, a lipolytic enzyme, hydrolyses triglycerides into fatty acid and glycerol. Among the proteolytic enzymes, trypsin participates in the hydrolysis of proteins and elastase cuts the peptidic bond after a neutral non-aromatic amino acid with an aliphatic lateral chain. Among the glycolytic enzymes the  $\alpha$ -amylase hydrolyses bonds between glucose polymers. The nucleolytic enzymes are endonucleases which can degrade RNA or DNA. Finally, the inhibitory enzymes are very important to prevent pancreas auto-digestion by trypsin or other enzymes secreted in their active form.

### 2.2. Endocrine cells

In this section I will describe alpha-, delta-, PP- and epsilon-cells, their secretory products and their role in the **regulation of glycaemia**. Beta-cells will be described in a separated chapter.

#### 2.2.1. Alpha-cells

Alpha-cells represent about 15% of the islet cells and secrete **glucagon** in response to low blood sugar level. The regulation of glycaemia by islet cells relies on the equilibrium between the secretory activities of alpha- and beta-cells. Insulin increases the formation of glycogen in the liver, inhibits hepatic neoglucogenesis and glycogenolysis (Michael et al., 2000) and induces the absorption of glucose by muscles and adipocytes (Bergman and Ader, 2000). On the other side, glucagon secretion stimulates glucose synthesis by inducing glycogenolysis in the liver and lipolysis in the adipocytes, freeing glycerol which is then metabolised into glucose (Figure 2) to maintain a sufficient level of glucose in the blood.



**Figure 2: Regulation of glucose homeostasis by insulin and glucagon release.** High blood sugar promotes insulin release by the beta-cells. Insulin induces glycogen formation in the liver and stimulates glucose uptake by adipocytes and muscles. Inversely, low blood sugar promotes glucagon release by the alpha-cells. Glucagon stimulates glucose synthesis in the liver and induces lipolysis in the adipocytes thus liberating glycerol which will be further metabolised in glucose by the liver.

### 2.2.2. Delta-cells

Delta-cells secrete **somatostatin** a hormone implicated in the local control of alpha- and beta-cells. For example, somatostatin can have an **inhibitory effect on insulin secretion**. This hormone has been shown to inhibit the acid secretions of the stomach directly or via the inhibition of ghrelin and gastrin secretions (Schubert, 2003). Delta-cells represent less than 5% of the islet cells.

### 2.2.3. PP-cells

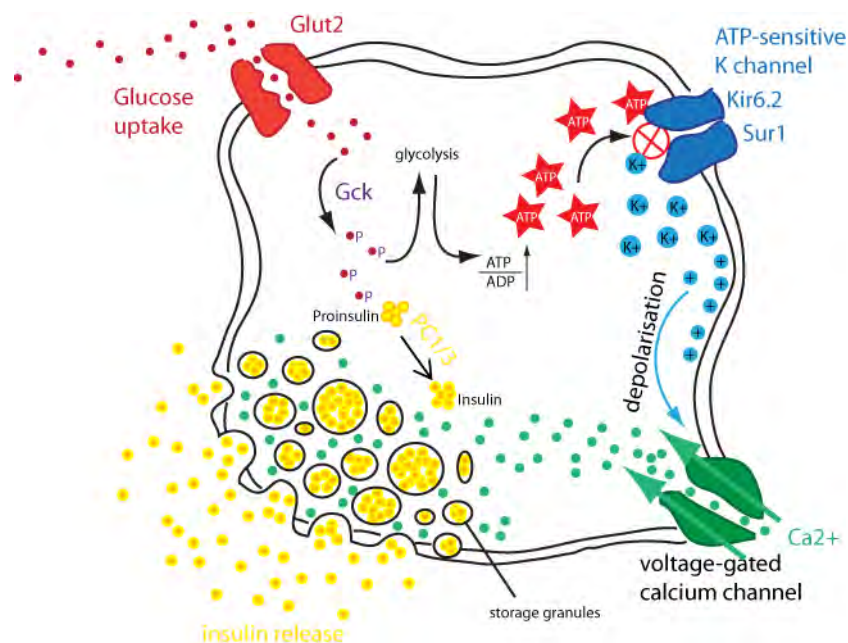
PP-cells produce and secrete the **pancreatic polypeptide**, a peptide which physiological role has not been found so far in the pancreas. However, it has been shown that it can **reduce exocrine enzymes secretions**, gastric acid secretions and intestinal motility (Murphy and Bloom, 2006). PP-cells are rare as they account for less than 1% of the total islet population.

### 2.2.4. Epsilon-cells

In the pancreas, **ghrelin** is produced by alpha- and epsilon-cells the latter only recently identified in mice and human as a fifth endocrine cell type deriving from  $\text{Ngn3}^+$  endocrine progenitors (Date et al., 2002; Heller et al., 2005; Prado et al., 2004). Epsilon-cells are lost from the pancreas after birth but ghrelin is also produced by cells from the stomach and intestine. Ghrelin stimulates gastric emptying, inhibits satiety state and might play an inhibitory role in the **control of insulin secretion** (Dezaki et al., 2004; Murphy and Bloom, 2006).

### 2.3. Beta-cells: functions, key actors and insulin release

Beta-cells are the major population of the islet, representing about 80% of the cells in mice. Diverse players are necessary to achieve proper beta-cell function especially in the control of glucose homeostasis. Among them I will describe the roles of prohormone convertases 1/3, Glut2, Gck, Kir6.2 and Sur1 in insulin secretion in response to glucose stimuli (Figure 3).



**Figure 3: Main beta-cell players in insulin secretion.** Glucose enters the cell via the glucose transporter Glut2 and is transformed into glucose-6-phosphate by glucokinase (Gck) during glycolysis. Glycolysis generates multiple ATP molecules thus increasing the ATP/ADP ratio in the cell, closing the ATP-dependent potassium channel (composed of 2 subunits Sur1 and Kir6.2). This closure causes depolarisation of the cell membrane and opens the voltage-gated calcium channel to allow calcium to flow into the cell. Insulin is synthesised as proinsulin and transformed into mature insulin by the prohormone convertase PC1/3 and stored into granules. When the concentration of calcium is high it triggers docking of the granules on the plasma membrane and release of insulin in the bloodstream. (Modified from betacellbiology.org)

#### 2.3.1. Functions

Beta-cells synthesise, stock and release **insulin** a hormone composed of two polypeptidic chains (A and B) of respectively 21 and 30 amino acids linked together by 2 disulphide bonds and a C-peptide bridge. Initially, insulin is synthesised as preproinsulin in pancreatic beta-cells; it is then processed to proinsulin which then undergoes maturation into active insulin through the action of the **prohormone convertases** (PC1/3 and PC2, also known as Pcsk1 and Pcsk2 respectively) which cleave off C-peptide from insulin (Figure 3). Mature insulin is subsequently packaged inside granules and released when necessary.

Insulin **stimulates glucose uptake** in muscles and adipose tissue and enhances glycolysis and glycogen synthesis thus increasing the uptake of glucose in hepatocytes (Figure 2). Briefly, insulin exerts its **hypoglycaemic** role by increasing the usage or uptake of glucose and inhibiting its production. Moreover, the presence of nutrients in the gut induces the secretion of other regulators of glycaemia. These hormones, known as gluco-incretins, are synthesised and secreted by L and K cells of the intestine and act on beta-cells by amplifying insulin secretion in response to glucose (see I.B.2.4.1). Other substances known to stimulate insulin release include the amino acids arginine and leucine, acetylcholine and sulfonylurea but this will not be discussed here.



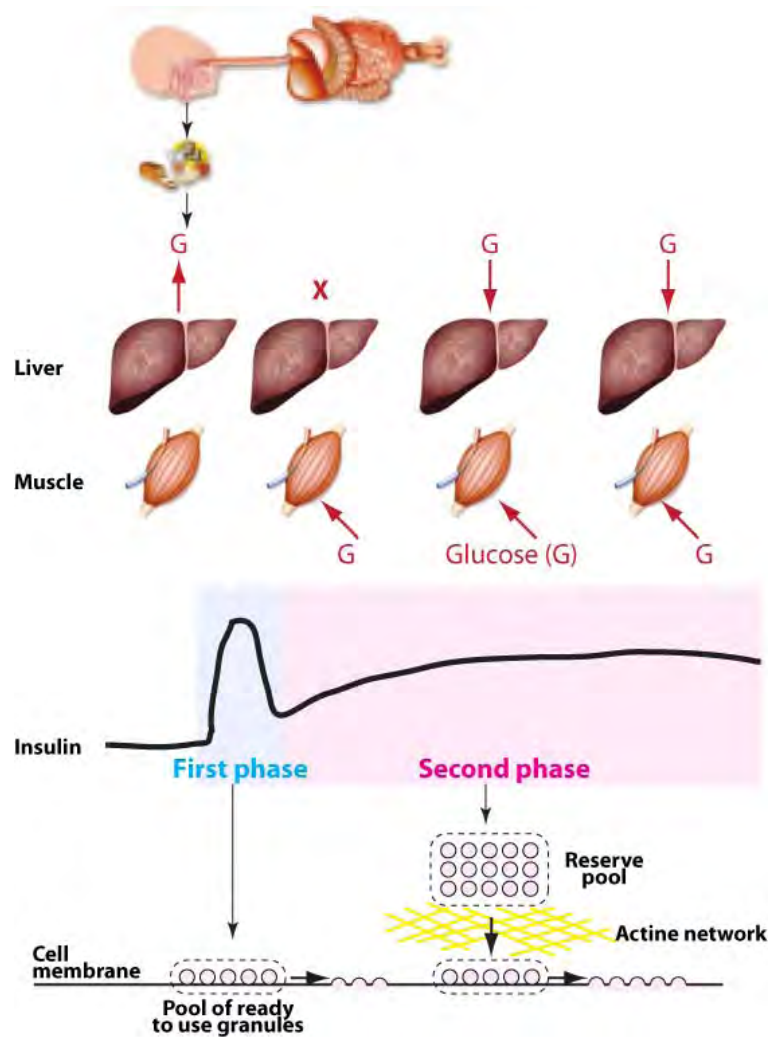
The major role of beta-cells is the regulation of glycaemia via the secretion of insulin. However recent studies points to other functions of beta-cells for example in proliferation. It is known that in addition to its role in growth and expansion of multipotent pancreatic progenitors, *Pdx1* is also required for beta-cell proliferation and survival. Deletion of *Pdx1* in embryonic beta-cells leads to a loss of beta-cells accompanied by an increase in alpha- and delta-cells. However, with lineage tracing experiments, Gannon and colleagues showed that this increase in alpha- and delta-cells comes from an increased proliferation and not from the redirection of beta-cells. Thus the authors propose that beta-cells could **inhibit proliferation of adjacent islet cell types** (Gannon et al., 2008).

### 2.3.2. Insulin release

Beta-cells **secrete insulin in two phases** (Figure 4). The first phase is **rapidly triggered** when blood glucose level is high. The second phase is a **more sustained**, slow release of insulin. The first phase can be described as follows (Figure 3): (1) glucose enters the cell via the glucose transporter **Glut2** (or Slc2a2); (2) glucose enters glycolysis and is transformed into glucose-6-phosphate by **glucokinase** (Gck). During glycolysis, multiple ATP molecules are produced by oxidation, (3) increased ATP:ADP ratio closes the ATP-dependent potassium channel composed of the 2 subunits **Sur1/Kir6.2** (or Abcc8/Kcnj11) causing cell membrane depolarisation, (4) after depolarisation voltage-controlled calcium channels open and calcium flows into the cells, (5) increased intracellular calcium level activates phospholipase C which generates inositol triphosphate (IP3) that will bind receptors on the endoplasmic reticulum and allow the release of more calcium, (6) high amount of calcium in the cell finally causes the release of previously synthesised insulin.

The second phase of insulin secretion relies mainly on actin remodelling as the reserve pool of insulin granules is separated from the membrane by a barrier of F-actin (Figure 4). The **actin cytoskeleton** is a very dynamic and complex structure which is remodelled in response to various stimuli, thus the dynamics of insulin granules is likely to be affected by actin remodelling. Indeed, it has been shown that glucose stimulation can trigger transient F-actin remodelling to mobilise granules at the plasma membrane (Wang and Thurmond, 2009). A recent study showed that the inhibition of RhoGDI could enhance the second phase of glucose-stimulated insulin secretion (GSIS) via the activation of Cdc42 (Wang and Thurmond, 2010). Since Cdc42 is able to promote actin polymerisation it is in favour of a role of actin dynamics in the second phase of GSIS.

Several **kinases** have been implicated in beta-cell function. Among them **LKB1**, a kinase implicated in the control of cell polarity and energy metabolism. *In vivo*, *LKB1*-deficient mice show a great increase in insulin secretion which is, at least in part, regulated via **AMPK** signalling (Granot et al., 2009). The authors have placed LKB1 as a coordinator of beta-cell size, polarity, form and function. Moreover, insulin release can also be regulated at the level of exocytosis. In this context, it has recently been shown that mice lacking the mitogen-activated protein kinase (MAPK) **p38delta** exhibit an improved glucose tolerance due to an increased insulin secretion (Sumara et al., 2009). The authors showed that *p38delta* deletion results in the activation of protein kinase D (**PKD**) which they have identified as a regulator of insulin exocytosis. Thus phosphorylation of PKD by p38delta suppresses PKD-mediated stimulated insulin secretion. Additionally, insulin signalling can also be regulated by other MAPK signals, including ERKs and JNKs reviewed recently (Gehart et al., 2010) or by islet capillaries signals (Zaret, 2006).



**Figure 4: The two phases of insulin secretion.** Upon ingestion of nutrients, the liver releases glucose, increasing the concentration of glucose in the blood and triggering insulin secretion. The first phase (blue) of insulin secretion is rapid and results from a ready-to-use pool of insulin granules that are docked on the cell membrane which will fuse and secrete insulin. Secretion of insulin will act on the liver to stop the release of glucose and on the muscles to favour glucose uptake. The second phase (pink) is more sustained and comes from a reserve pool of granules located further away from the membrane; they are recruited upon stimulation, go through the F-actin barrier, are docked and fused to the plasma membrane to secrete insulin. (Scheme adapted from Serino et al., 2011)

### 2.3.3. Turnover, renewal and proliferation in the adult

The **turnover** of beta-cells in aged mice has been quantified with BrdU experiments and the authors report that only 1 in 1.500 adult beta-cells replicates per day, suggesting that adult beta-cells are very long lived (Teta et al., 2005). In contrast, in the first year of life in rodents, beta-cell mass has to increase dramatically to compensate for increasing metabolic demands (Montanya et al., 2000). During development, beta-cells are produced mainly by **neogenesis** from endocrine progenitors whereas shortly before birth and in healthy adult mice (and humans), new beta-cells are generated mainly by **replication of pre-existing beta-cells** (Dor et al., 2004; Georgia and Bhushan, 2004; Meier et al., 2008). Interestingly, in a model of injured adult mouse pancreas, it has been shown that beta-cells can be generated from endogenous progenitors by a process which relies on the reactivation of high levels of Ngn3 (Xu et al., 2008). Different signals and molecules have been implicated in the regulation of adult beta-cell proliferation including (1) regulators of cell cycle progression, (2) growth factors, (3) transcription factors and (4) epigenetic components (see for review Heit et al., 2006).



In mammalian cells, cell cycle progression is controlled by the expression and function of three main classes of proteins: cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs). Perturbing the expression or function of these cell cycle regulators has a strong impact on beta-cell mass (Table 1). Briefly, **Cyclin-dependent kinase 4** (*Cdk4*) has been shown to be a positive regulator of beta-cell proliferation as mice lacking *Cdk4* develop diabetes due to a reduction in beta-cell mass whereas over-expression of *Cdk4* in pancreatic islets significantly increases beta-cell mass (Marzo et al., 2004; Rane et al., 1999). Moreover, in a regeneration model, *Cdk4* was shown to directly regulate the recruitment of quiescent beta-cells to reconstitute beta-cell mass (Lee et al., 2010). Similarly, **Cyclin D1** and **Cyclin D2** positively regulate beta-cell mass and are the likely partners of *Cdk4* to promote cell cycle progression in adult beta-cells (Georgia and Bhushan, 2004; Kushner et al., 2005). Inversely, cyclin-dependent kinase inhibitors including *p27<sup>kip1</sup>*, *p57<sup>kip2</sup>* and *p21<sup>cip</sup>* are important inhibitors of beta-cell proliferation (Table 1). **p27** prevents beta-cell division thus maintaining the quiescent state of beta-cells both during development and in the adult (Georgia and Bhushan, 2006; Zhong et al., 2007). Interestingly, adult mice with *p27* deficiency are able to re-enter cell cycle at a greater frequency than wild-type mice after streptozotocin-induced diabetes, establishing *p27* as a potential target to facilitate beta-cell regeneration in diabetic patients. During mouse development, **p57** has been shown to regulate the proliferation of endocrine progenitors to prevent premature differentiation (Georgia et al., 2006). Whether *p57* has a role in adult beta-cells is not yet known. Even though **p21**-deficient mice have normal beta-cell mass, *p21* seems to participate in the repression of division as *p21* overexpression in beta-cells has been shown to inhibit proliferation and lead to apoptosis (Yang et al., 2009).

Cellular signals sufficient or necessary for beta-cell proliferation have been identified even though it is not always known how they activate the cell cycle machinery (Table 1). Recently, Porat and colleagues have proposed that beta-cell mass is controlled directly by **glucose metabolism** (Porat et al., 2011). They showed by genetic and pharmacologic manipulations that glucose is able to induce beta-cell replication via glucokinase and closure of the  $K_{ATP}$  channels, demonstrating that beta-cell mass can be regulated by metabolic demand (Porat et al., 2011). Other signals regulating beta-cell proliferation or neogenesis include amino acids, insulin, prolactin, placental lactogen, **glp1**, **gastrin**, growth hormones, hepatic growth factors, parathyroid hormone-related proteins, platelet-derived growth factors, epidermal growth factors, insulin-like growth factors and calcium signalling. These regulators have been extensively reviewed (Heit et al., 2006; Sachdeva and Stoffers, 2009; Vasavada et al., 2006) and will be discussed in more details later (see I.A.2.3.5). Of note, **pregnancy** also stimulates beta-cell mass either via transcriptional response (Rieck and Kaestner, 2010; Rieck et al., 2009) or via serotonin signalling (Kim et al., 2010) for example.

Regulators of beta-cell proliferation	
Cell Cycle regulators	Cdk4, Cyclin D1, Cyclin D2, p27, p57, p21
Signals	Glucose, insulin, growth factors, hormones, calcium signalling
Transcription factors	E2F1, E2F2, FoxO1, Pdx1, Sox9, FoxM1, CREB
Epigenetic factors	Ezh2, Bmi1

**Table 1 : Selected regulators of beta-cell proliferation.** Growth factors and hormones are detailed in Table 3.

Several transcription factors have a role in the maintenance of adult beta-cells (Table 1), including but not restricted to E2F, FoxO1, FoxM1 and CREB. For a comprehensive overview, please refer to the review by Bernardo and colleagues (Bernardo et al., 2008). **E2F1** and **E2F2** have an important role in regulating beta-cell proliferation (Fajas et al., 2004; Iglesias et al., 2004). Indeed, *E2F1*<sup>-/-</sup> mice fail to undergo postnatal islet expansion, resulting in reduced beta-cell mass and glucose intolerance (Fajas et al., 2004). However, surprisingly, the loss of both *E2F1* and *E2F2* resulted in increased proliferation but reduced beta-cell mass which could at least partially be explained by increased apoptosis (Iglesias et al., 2004). **FoxO1** has been implicated in the transduction of signals from the insulin-signalling pathway and was recently shown to regulate beta-cell proliferation by controlling *Pdx1* and *MafA* (Kikuchi et al., 2012; Kitamura et al., 2002; Kobayashi et al., 2012). Additionally, mice in which **CREB** has been inactivated specifically in beta-cells develop diabetes in the adult due to reduced beta-cell proliferation and increased apoptosis (Jhala et al., 2003). **FoxM1** appears to affect beta-cell proliferation in adult mice but not in embryos. Mice with pancreatic inactivation of FoxM1 were born with normal beta-cell mass; however they exhibited a gradual decline in beta-cell mass with age (Zhang et al., 2006). Interestingly, it was recently shown that FoxM1 is essential for placental lactogen-induced beta-cell proliferation during pregnancy (Zhang et al., 2010). Other transcription factors, which have important roles during endocrine cell differentiation, have been recently suggested to take part in the maintenance and proliferation of beta-cells. Briefly, Gannon and colleagues showed that deletion of **Pdx1** in early beta-cells leads to a strong reduction in the number of insulin cells beginning at late gestational stages and causing early-onset diabetes (Gannon et al., 2008). Similarly, **Sox9** has also been shown to regulate beta-cell proliferation as mice with pancreatic *Sox9* haploinsufficiency exhibit impaired glucose tolerance and about two-fold decrease in beta-cell mass at birth (Dubois et al., 2011). However by six weeks of age, beta-cell mass in *Sox9* heterozygous mutant mice had raised to 68% of controls suggesting a compensatory increased proliferation (Dubois et al., 2011).

Finally, it has recently been shown that the **epigenetic** factors Ezh2 and Bmi1 (Table 1) can regulate the *Ink4a/Arf* locus to control beta-cell proliferation (Chen et al., 2009; Dhawan et al., 2009).

#### 2.3.4. Pdx1, NeuroD1 and MafA regulate insulin transcription in adult beta-cells

Several regulatory sequences can be found in the promoter of the insulin gene with binding site for different transcription factors (Table 2). In general, A-boxes (TAAT motif) are bound by **Pdx1**, E-boxes (CANNTG motif) by **NeuroD1** and C-boxes (cytosine-rich sequence) to **MafA** (Melloul et al., 2002). Synergistic activation of insulin promoter is induced by simultaneous binding of these three factors.

Regulatory sequences	Transcription factors	Selected references
E boxes	E2A and NeuroD1	(Naya et al., 1995)
A boxes	Pdx1	(Iype et al., 2005)
C boxes	MafA	(Olbro et al., 2002; Zhao et al., 2005)
PISCES (only in rat <i>Ins1</i> )	Pax6	(Sander et al., 1997)
AT-rich (A-boxes and G1)	Pax6	(Wolf et al., 2010)

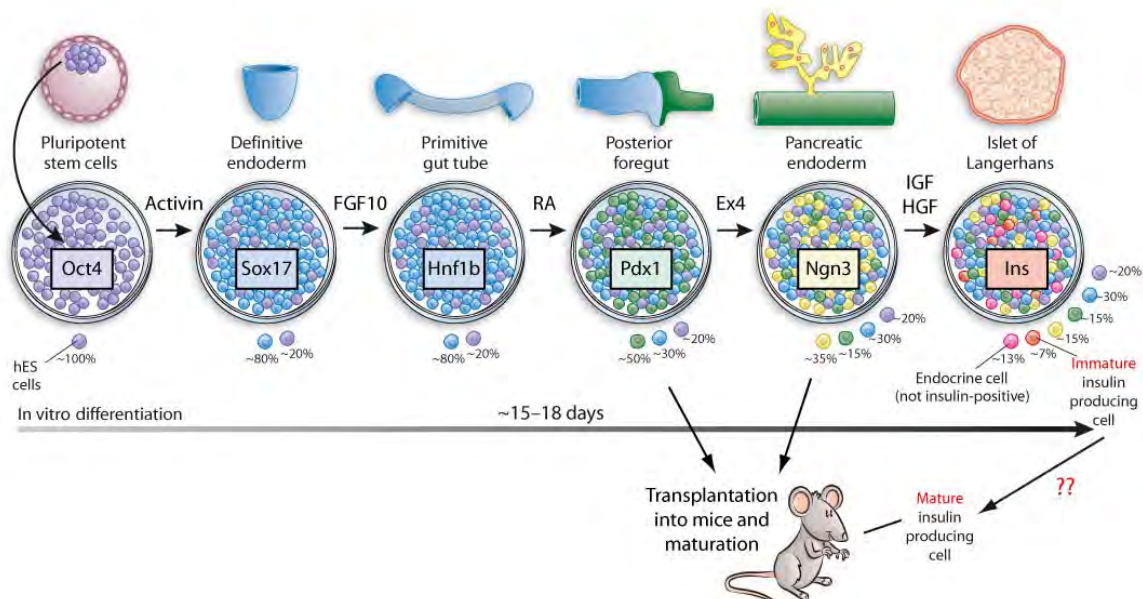
Table 2: Regulatory sequences on the insulin gene and corresponding binding transcription factors.

**Pdx1** regulates insulin transcription in adult beta-cells through the formation of a complex with transcriptional co-activators on the proximal insulin promoter (Iype et al., 2005) and acts synergistically with *NeuroD1* and *MafA* (Zhao et al., 2005). **MafA** regulates insulin gene transcription by direct binding to the RIPE3b1 element whereas **NeuroD1** binds on E-boxes (Table 2). These three genes are the major regulators of insulin gene transcription. However, to ensure fine tuning of this regulation, these genes are in turn regulated by other transcription factors including **FoxA2**, **Hnf1 $\alpha$** , **Pax4** and **Pax6** (Cerf, 2006). For example, it has been shown that **Pax6** is able to repress insulin gene activation by *NeuroD* and *Pdx1* in rat insulinoma cells (Wolf et al., 2010). Moreover, *Pax6* is also important for the maintenance of beta-cell function and is involved in the regulation of *Glut2*, *PC1/3*, *Pdx1*, *MafA* and *NeuroD* expression (Sander et al., 1997; Wen et al., 2009).

### 2.3.5. Cell therapy: (re)programming beta-cells or preserving beta-cell mass

Despite encouraging results, wide usage of **cell therapy** for diabetes is confronted to a major obstacle which is the **grafts availability**. To date, the only source of beta-cells for transplantation comes from pancreas given by donors. However for one graft many pancreases are required. To overcome this limitation, it is important to look for new sources of beta-cells. There are two major approaches: **(re)programming** cells *in vitro* toward beta-cells or **increasing/preserving the beta-cell mass** *in vivo*.

Researchers have developed protocols to convert **human embryonic stem cells** (hESC) to beta-cells capable of synthesising insulin (Baetge, 2008; D'Amour et al., 2006) by mimicking pancreatic development (Figure 5). However, the process is very inefficient (only 7% of insulin-producing cells) and the beta-cells generated are not functional as they do not reliably secrete insulin in response to glucose. Interestingly, if the differentiated cells are transplanted into mice at the pancreatic progenitor or endocrine progenitor stage, they can mature *in vivo* and give rise to functionally mature beta-cells which are glucose-responsive and can rescue diabetes in mice (Kroon et al., 2008; Rezanian et al., 2012). This indicates that there is a need to better understand the developmental processes giving rise to functional beta-cells, especially the late steps of differentiation and maturation. Recently, beta-cell maturation was shown to be marked by the expression of the gene urocortin3 (Blum et al., 2012). This gene can be used as a marker of mature beta-cells in screens for factors that induce *bona fide* beta-cells *in vitro*. To overcome the ethical problems related to the use of hESCs many labs are trying to generate beta-cells from induced pluripotent stem cells (iPSC) (Kunisada et al., 2012), adult skin (Guo et al., 2009) or adipose-tissue (Chandra et al., 2009) derived stem cells or adult somatic cells like human skin fibroblasts (Tateishi et al., 2008), **exocrine cells** of the pancreas (Zhou et al., 2008), alpha-cells (Yang et al., 2011), liver cells (Yechor et al., 2009), gall bladder cells or **intestinal cells** (Talchai et al., 2012).



**Figure 5 : Differentiation of hES cells to insulin-producing cells by mimicking embryonic development.** Up: Key stages of pancreatic endocrine development, Down: Stepwise differentiation of hES cells. Cells are cultured in five successive conditions (main added signal is indicated) to give rise to about 7% of insulin-producing cells. The maturing hESC were found to recapitulate expression in the correct order of a series of key endodermal and pancreatic marker genes (boxed) known to be activated during mouse development. If cells expressing Pdx1 or Ngn3 are transplanted into mice for maturation, then they are able to produce *bona fide* insulin-secreting cells. Question marks indicate that we do not know yet the signals governing the maturation of beta-cells. RA: retinoic acid, Ex4: exendin4 (Glp1-R agonist), IGF: insulin-like growth factor, HGF: hepatic growth factor. (Adapted from Madsen and Serup, 2006)

The alternative approach for cell therapy is to preserve or increase the beta-cell mass with small molecules. These molecules have been extensively reviewed (Baggio and Drucker, 2006; Bonner-Weir et al., 2010) and are summarised in Table 3. Most existing therapeutic agents lower blood glucose but do not directly reverse the decline in beta-cell mass. However, emerging potential new drugs rely on the **incretins** which have been shown to regulate beta-cell mass in rodents (see I.B.2.4.1). Briefly, in mice injection of Glp1 receptor agonist, exendin4, not only lowers blood glucose but also signals in pathways that lead to the stimulation of beta-cell replication and inhibition of apoptosis (Li et al., 2003). Similarly, activation of Gip receptor stimulates insulin secretion, enhances beta-cell proliferation and reduces apoptosis in INS1 cells (Kim et al., 2005). Complementary approaches involve injections of combinations of factors, like **EGF and gastrin**, which promote beta-cell neogenesis and improve diabetes in rodents (Suarez-Pinzon et al., 2005). The ability of these molecules to enhance beta-cell survival and stimulate proliferation in rodents suggests that they could provide non-invasive means to preserve/restore beta-cell mass in diabetic patients.

Stimulate neogenesis or proliferation	Reduce apoptosis
Glp1/Gip/ DPP (dipeptidyl-peptidase)-IV inhibitors	Glp1/Gip/ DPP-IV inhibitors
Growth hormone	Growth hormone
Hepatocyte GF (HGF)/ Insulin-like GF (IGF)	Hepatocyte GF (HGF)/ Insulin-like GF (IGF)
Parathyroid hormone-related peptides	Parathyroid hormone-related peptides
Epidermal growth factor (EGF)/gastrin	
Human placental lactogen	
Prolactin	

**Table 3: Selection of molecules which improve or preserve beta-cell mass.** GF: growth factor. (Baggio and Drucker, 2006)

### 3. Diabetes: The main pancreas dysfunction

**Diabetes is a world-leading disease** of the pancreas which is induced by a deregulation of blood glucose level. The poor regulation of glycaemia and the repeated insulin injections can lead to various complications including retinopathies, nephropathies, neuropathies and amputations. In 2011, World Health Organisation (WHO) reported 346 million people suffering from diabetes which represent a prevalence of 10% in adults. It is now considered as pandemic with 552 million diabetic patients predicted for 2030 (WHO). Diabetes is a complex disease caused by a variety of inherited as well as environmental and lifestyle-related factors. According to the American Diabetes Association, there are four main types of diabetes; **Type 1**, **Type 2**, **Gestational** Diabetes Mellitus and other specific types of diabetes. Within this last category are found some rare diabetes of exclusively genetic origins, denoted as **monogenic** diabetes.

#### 3.1. Monogenic forms of diabetes

Monogenic forms of diabetes represent 1-2% of all cases of diabetes (Eide et al., 2008) and are primary genetic disorders of the insulin-secreting beta-cells (Murphy et al., 2008). Depending on when the disease arises, monogenic diabetes have been classified into Maturity Onset Diabetes of the Young (**MODY**) and **Neonatal diabetes** (ND). However, as more and more patients are being sequenced, some genes eventually turned out to be the cause of both MODY and ND depending on the mutation. So far mutations in about 20 genes that affect pancreas development/differentiation or normal beta-cell physiology have been identified (Table 4).

Gene/Protein	Function	Type of MD
ABCC8/SUR1	Subunit of $K_{ATP}$ -channel regulating insulin secretion	PNDM or TNDM
B lymphoid TK	Kinase stimulating insulin synthesis and secretion	MODY
CEL	Enzyme involved in cholesterol hydrolysis and absorption	MODY
GCK	Phosphorylation of glucose to glucose-6-phosphate	MODY or PNDM
HNF1A	Beta-cell function	MODY
HNF1B	Pancreas development / Induction of Ngn3	MODY or PNDM
HNF4B	Beta-cell function (GSIS)	MODY
INS	Hormone regulating blood glucose levels	PNDM or MODY
KCNJ11/KIR6.2	Subunit of $K_{ATP}$ -channel regulating insulin secretion	PNDM
KLF11	Insulin gene transcription	MODY
NEUROD1	Endocrine cell differentiation / Beta-cell function	MODY or PNDM
PAX4	Endocrine subtype specification toward beta-/delta-cells	MODY
PDX1	Pancreas development / Beta-cell function	MODY or PNDM
PTF1A	Pancreas development	PNDM
RFX6	Endocrine cell differentiation	PNDM
GLIS3	Beta-/delta-cell differentiation / Insulin gene transcription	PNDM
GATA4	Pancreas development	PNDM
PAX6	Beta-cell function / alpha-cell differentiation	PNDM
NGN3	Endocrine cell differentiation	PNDM or MODY

**Table 4: Selection of genes causing monogenic diabetes.** MD: monogenic diabetes. TK: tyrosine kinase, PNDM: permanent neonatal diabetes mellitus, TNDM: transient neonatal diabetes mellitus. (Adapted from Molven and Njolstad, 2011)

### 3.1.1. MODY

The MODY concept describes a group of patients who have non-ketotic, autoantibodies-independent diabetes with **autosomal dominant inheritance** and primary pancreatic beta-cell defects (Molven and Njolstad, 2011). The first MODY gene identified encodes *glucokinase (GCK)* and now more than 500 inactivating mutations in different genes have been discovered. Mutations in *GCK* is a very frequent cause of MODY, however subjects with mutations in this gene only have mildly elevated fasting glucose with almost no risk to develop the usual complications associated with diabetes (cardiovascular problems, kidney failure, retinopathy and neuropathy). Mutations in *HNF1A* and *HNF4A* lead to progressive loss of beta-cell function. With increasing population studies, mutations in other transcription factors have been identified (Table 5). However, except for *HNFs*, each transcription factor has only been found mutated in a few families.

MODY type	Gene mutated	Prevalence
MODY1	HNF4A	Frequent
MODY2	GCK	Very frequent
MODY3	HNF1A	Very frequent
MODY4	PDX1	Rare
MODY5	HNF1B	Frequent
MODY6	NEUROD1	Rare
MODY7	KLF11	Rare
MODY8	CEL	Rare
MODY9	PAX4	Rare
MODY10	INS	Rare
MODY11	BLK	Rare
MODY12	NGN3	Rare

**Table 5: The different subtypes of MODY.** (Adapted from Molven and Njolstad, 2011)

### 3.1.2. Neonatal Diabetes

By definition, neonatal diabetes arises in the **first six months** of life. As insulin is a growth factor, neonatal diabetes is often associated with low weight at birth. More than 50% of all neonatal diabetes cases are now solved genetically. The most common mutations are in the *ABCC8* and *KCNJ11* genes which encode the subunits of the beta-cell ATP-sensitive potassium channel (Molven and Njolstad, 2011). Neonatal diabetes can be **permanent or transient** although transient neonatal diabetes often relapses as type 2 diabetes later in life. The transient form can be caused by mutations in genes that usually give PNDM but most frequently, the TNDM is due to various dosages imbalances that are associated with chromosome *6q24* (Molven and Njolstad, 2011). In very rare cases, mutations in transcription factors can lead to neonatal diabetes. This is the topic of a review we are currently writing in the lab (Piccand, Spizzo and Gradwohl, in preparation). Mutations causing neonatal diabetes have been reported so far for *HNF1B*, *NEUROD1*, *NGN3*, *PAX6*, *GLIS3*, *PDX1*, *PTF1A*, *GATA4* and *RFX6* (Table 4 and I.E.2.7.2 for *RFX6* mutations).



### 3.2. Type 1 Diabetes

Type 1 diabetes or **insulin-dependent** diabetes represents about 10% of all diabetes. It is due to an **autoimmune reaction**, in which the immune system **attacks its own insulin-producing cells**, preventing the pancreas to produce the insulin it needs. The reason why this happens is not completely understood but may be due to changes in environmental risk factors, early events in the womb, diet or viral infections. It can affect people of any age, but mainly occurs in children and young adults (Eizirik et al., 2009). Type 1 diabetes can usually be diagnosed before any hyperglycaemia arises by the appearance of autoantibodies against islet cells (ICA), insulin, glutamic acid decarboxylase (GAD) and insulinoma-associated2 (IA2). To compensate for the massive production of glucose due to the absence of insulin, patients need daily insulin injections to stabilise blood glucose level. With accurate dosage, healthy diet and regular physical exercise, patients with type 1 diabetes can lead normal, healthy lives. For people who cannot regulate their diabetes with insulin injections and suffer from complications like nephropathies, a graft of pancreas or islets can be considered. However, this is strongly limited by the lack of donors. To overcome this problem, researchers are studying the possibility to make functional beta-cells from stem cells or IPS cells (see I.A.2.3.5).

### 3.3. Type 2 Diabetes

Type 2 diabetes is **non-insulin-dependent**; it is often linked to obesity and/or genetic predisposition. Type 2 diabetes is the most common type of diabetes (90%) and is due mainly to peripheral insulin resistance. It usually occurs in adults older than 40 but is increasingly seen in children and adolescents due to changes in lifestyle. In type 2 diabetes patients, the pancreas is able to produce insulin, but it is either not sufficient or the body is not responding to its effects, leading to an increase amount of glucose in the blood. Unfortunately people with type 2 diabetes can remain unaware of their disease for a long time because the symptoms are not recognised. During this time, the body is being irreversibly damaged by excess blood glucose. Although, the causes for developing type 2 diabetes are not all known, several risk factors have been identified including: obesity, poor diet, lack of physical activity, aging, genetic predisposition, ethnicity. In contrast to people with type 1 diabetes, the majority of patients with type 2 diabetes do not require injections of insulin to survive. They are mostly prescribed to change to a more healthy diet and increase their physical activity. In obese people, the body is making the muscles use the fatty acids to make energy, thus the glucose cannot enter the cells, and beta cells will produce more and more insulin to force glucose uptake. Little by little, beta-cells will eventually run out and stop producing insulin (Doria et al., 2008).

### 3.4. Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is diagnosed when women are suffering from diabetes during pregnancy. It normally occurs late and arises because the body is unable to make and use enough insulin for the pregnancy. Because the baby is already well-developed, the risk of malformation (or even death) is low. However, women with GDM still have to control blood glucose levels to minimise risks for the baby growth. GDM normally disappears after birth. However, both mother and babies have higher risk of developing type 2 diabetes later in their life.

## B. Anatomy and function of the intestine

### 1. Intestinal anatomy and histology

The intestine is an organ of the digestive tract which main function is the **absorption of nutrients**. It is the direct link between the stomach (where food is digested mechanically and chemically) and the rectum (where wastes are carried out of the body). The intestine is divided in five segments which are different from their histology and function: the small intestine divided in **duodenum**, **jejunum** and **ileum** and the large intestine subdivided in **colon** and **rectum** (Figure 6).

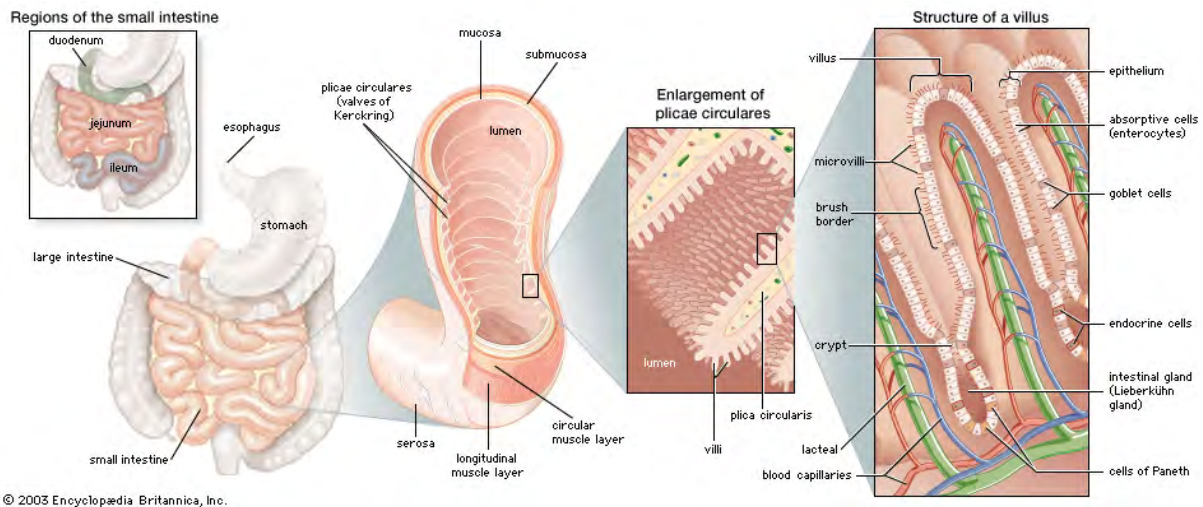


Figure 6: Anatomy and histology of the human small intestine. (From Encyclopaedia Britannica, 2010)

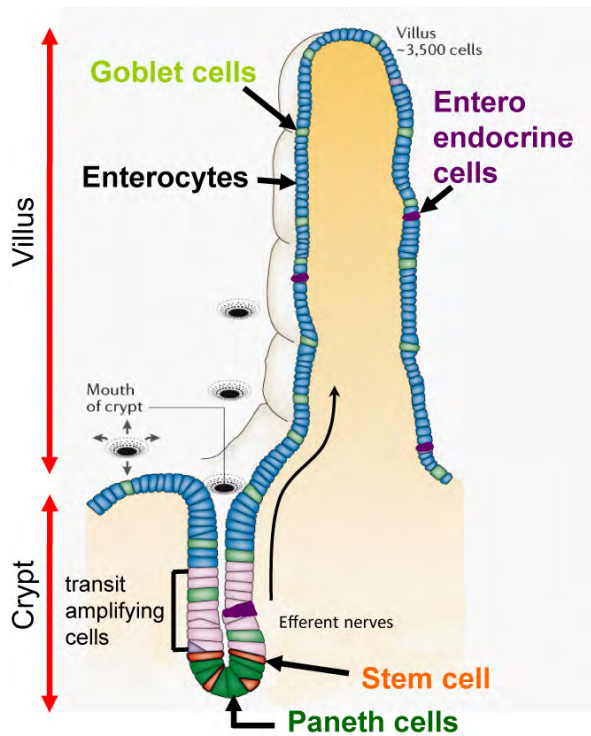
From the periphery toward the lumen, the intestine wall is made of serosa, musculosa, submucosa and mucosa (Figure 6). The musculosa is made of two muscle layers, a superficial layer of longitudinal muscle fibres and a deeper layer of circular muscles. The submucosa is a layer of connective tissue which supports the arterial, venous and lacteal networks. Finally the mucosa is formed by the lamina propria and a monostratified epithelium. Mucosa and submucosa are separated by a thin layer of muscular cells.

The epithelium of the small intestine is divided into two compartments, **crypts** and **villi** (Figure 7). Crypts are invaginations of the epithelium into the connective tissue of the mucosa. They are composed of **stem cells**, **progenitor cells** (for all the intestinal lineages) and **differentiating cells**. The crypt cells are mostly proliferative. Only one differentiated cell type lays into the crypts: the Paneth cells. The differentiating cells migrate from the crypt toward the villus where they all become post-mitotic. At the end of their life, cells are extruded from the top of the villus into the intestinal lumen. The large intestine is structurally different. There is no villus and the crypts form glands which secrete mucus. The organisation is otherwise similar and cells are discarded into the gut lumen after they emerge onto the exposed flat surfaces around the mouth of crypts.



## 2. Epithelial cells of the intestine and their functions

The intestinal stem cells of the gut epithelium differentiate mainly into absorptive and secretive cells. Enteroendocrine (EE), goblet and Paneth cells are the three secretory lineages of the intestine whereas the absorptive lineage is made of enterocytes (Figure 7).



**Figure 7: Distribution of the epithelial cell types in the mouse intestine.** Stem cells lie near the crypt base with the Paneth cells. Above the stem cells are transit-amplifying cells (dividing progenitors, some already partially differentiated); and above these on the villus, lie post-mitotic differentiated cells (enterocytes, goblet cells and enteroendocrine cells). Please note that in the colon there are no villus, but the organisation is otherwise similar; cells are discarded into the gut lumen after they emerge onto the exposed flat surfaces around the mouth of crypts. (Adapted from Crosnier et al., 2006)

### 2.1. Absorptive cells

The absorptive cells, also called **enterocytes**, represent more than 90% of the intestinal population (Dauca et al., 1990). They participate to the final steps of intestinal digestion by **absorption of nutrients**. Their apical surface is made by a brush border composed of microvilli covered by numerous digestive enzymes including disaccharidases (lactase, sucrase-isomaltase, maltase-glucoamylase) and peptide hydrolases (Lentze, 1995).

### 2.2. Goblet cells

Mucus cells, also called goblets, are responsible for the **secretion of mucus** which is composed of acidic and neutral mucopolysaccharides. Their number increases from the duodenum to the colon. On top of facilitating the food bowl transit and protecting the intestinal walls from the digestive enzymes, mucosa is also a reserve of antibodies and antimicrobial peptides (Stappenbeck, 2009).

### 2.3. Paneth cells

The Paneth cells are the only differentiated cells which do not migrate toward the villus, instead they migrate down the crypt. These cells have the slowest turnover of all intestinal cells (>30 days in mice). Their primary function is to **secrete antimicrobial enzymes** including lysozymes and cryptidins (Stappenbeck, 2009).

## 2.4. Enteroendocrine cells

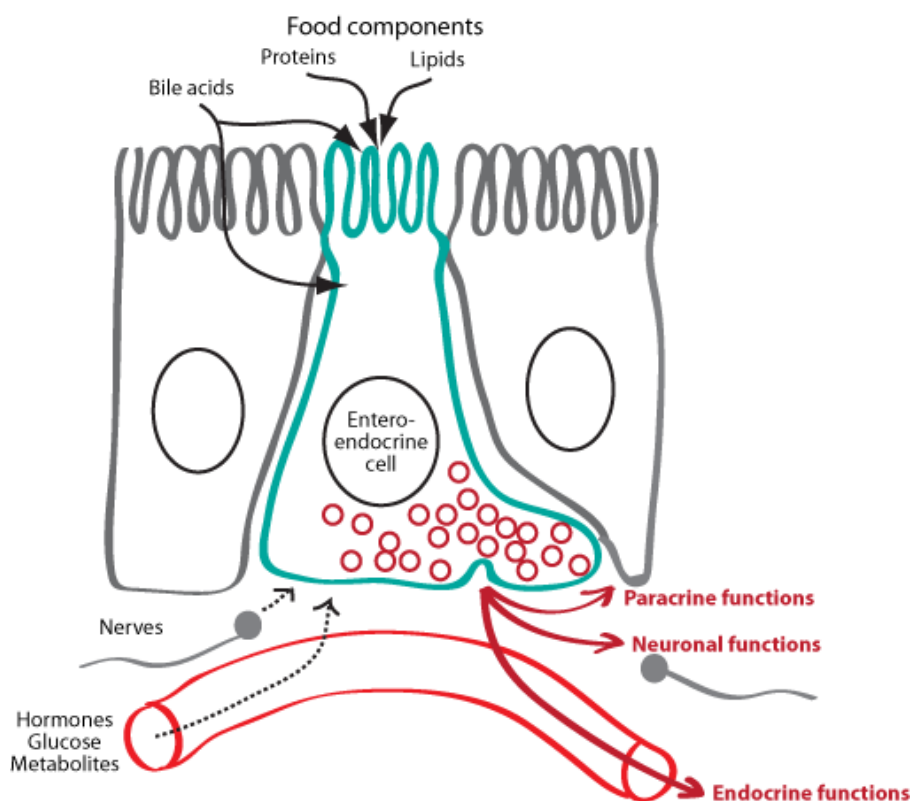
On contrary to many endocrine glands, enteroendocrine cells are scattered individually in the gut epithelium. They represent less than 1% of the intestinal epithelium but are the main endocrine organ by their number. The enteroendocrine system consists of at least 12 different cell types that can be classified based on their secretory products and ultrastructure (Rindi et al., 2004). The different enteroendocrine lineages have different expression patterns along the intestine and can secrete one or more hormone/hormone-like peptide/amine (Table 6). Enteroendocrine cells play an important role in **intestinal energy homeostasis** and **regulation of digestion** and **glycaemia** (Drucker, 2007; Mellitzer et al., 2010).

It has recently been shown that the total loss of enteroendocrine cells in mice impairs lipid absorption, causes diarrhoea, reduces weight gain and improves glucose homeostasis (Mellitzer et al., 2010). *Ngn3<sup>Δint</sup>* mice were deficient for all enteroendocrine cells and hormones and frequently died during the first week of life. Interestingly, mutant mice showed an enlarged proliferative crypt compartment and accelerated cell turnover, unravelling the role of enteroendocrine cells in maintenance of the intestinal epithelium (Mellitzer et al., 2010). In humans, *NGN3* mutations lead to the loss of enteroendocrine cells and have been reported as the cause of congenital malabsorptive diarrhoea (Wang et al., 2006).

Cell type	Peptide/Amine	Pancreas	Duodenum	Jejunum	Ileum	Colon
Alpha	Glucagon	+				
Beta	Insulin	+				
PP	PP	+	e			
D, Delta	Somatostatin	+	+	+	f	f
Epsilon	Ghrelin	e, f	f	f	f	f
EC	5-HT	f	+	+	+	+
L	Glp1, Glp2, Pyy		f	+	+	+
K	Gip		+	+	+	f
G	Gastrin		+			
CCK	Cholecystokinin		+	+	f	
S	Secretin, 5-HT		+	+		
M	Motilin		+	+	f	
N	Neurotensin		f	+	+	

**Table 6: Distribution of endocrine cells in the pancreas and intestine of humans.** +: presence of cells; f: presence of few cells; e: presence of cells during development and shortly after birth; EC: enterochromaffin cell; 5-HT: 5-hydroxytryptamine=serotonin. (Adapted from Rindi et al., 2004)

Enteroendocrine cells are conically shaped with a small apical pole decorated with microvilli facing the gut lumen and a boarder basal pole where the hormones are released from secretory granules (Figure 8). Among the different enteroendocrine subtypes, I will describe in more details Glp1/Gip and CCK/Gastrin for their roles in regulating glucose homeostasis and insulin secretion.



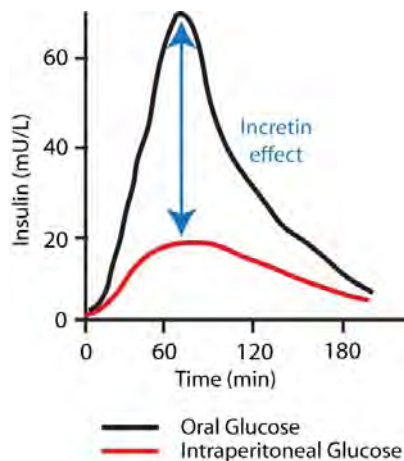
**Figure 8: Schematic overview of sensory and secretory functions of enteroendocrine cells.** Between two enterocytes, is represented a conical enteroendocrine cell (green) with its microvillus decorated apical pole reaching the gut lumen and with hormone-filled secretory granules at the base. The secreted hormones have three main modes of action: paracrine, neuronal and endocrine. At the top, the microvilli can sense food components and secretions present in the gut lumen. (Adapted from Engelstoft et al., 2008)

#### 2.4.1. Gip / Glp1 and the incretin effect

**Gip** (Gastric Inhibitory Peptide or Glucose-dependent Insulinotropic Polypeptide) was first identified as a peptide that inhibits gastric motility in dogs, it was subsequently shown to have a role in glucose-stimulated insulin secretion (Drucker, 2007). In the intestine, Gip is expressed mainly in K cells of the proximal small intestine (Table 6). Gip secretion is stimulated by nutrient ingestion. Its main action is the stimulation of glucose-stimulated insulin secretion (GSIS) via improved exocytosis. Moreover, it has also been shown to **promote insulin biosynthesis** and to **activate anti-apoptotic** pathways thus enhancing beta-cell survival (reviewed in Drucker, 2007). Mice deficient for *GipReceptor* have a defective insulin secretion after oral glucose administration (Miyawaki et al., 1999). Using these mice, the same team later deciphered a role for Gip in adipocytes biology. *Gipr*-null mice have less fat, use fat as preferred energy, are resistant to diet-induced obesity and have improved insulin sensitivity (Miyawaki et al., 2002). Hence Gip action on the beta-cell **improves insulin secretion**, whereas it **promotes energy storage** and reduces insulin action via effects on adipocytes. Therefore it is not clear whether activation or blockade of Gip signalling would be the best strategy for a drug against diabetes.

**Glp1** (Glucagon-Like Peptide 1) and glucagon are both synthesised as proglucagon, then the precursor is transformed in proglucagon which, by the action of proteases, undergoes maturation giving rise to glucagon, Glp1 and Glp2. Paradoxally, glucagon and Glp1 are two hormones, hyperglycaemic and hypoglycaemic respectively, which come from the expression of the same gene. Glp1 is secreted by L cells (Table 6) upon nutrient ingestion, reception of neuronal signals and stimulation by Gip. *Glp1r* (*Glp1 receptor*)-null mice are glucose intolerant and have defective glucose-stimulated insulin secretion and fasting hyperglycaemia (Scrocchi et al., 1996). Glp1 **increases insulin synthesis and secretion, stimulates somatostatin secretion, reduces glucagon secretion and enhances beta-cell survival and proliferation** (reviewed in Drucker, 2007). Even though, the signalling pathways through which Glp1 mediate its proliferative effects are not completely defined, it involves PI3K, EGFR and p38 MAPK.

There is a strongly increased release of insulin in response to oral glucose administration as compared to injection of the same amount of glucose intraperitoneally. This is the “**incretin effect**” and results from the release of incretin peptides by the gastrointestinal tract following ingestion of food. It has been estimated to account for at least 50-70% of the total insulin secretion (Baggio and Drucker, 2007). The incretin hormones, Glp1 and Gip, stimulate insulin release, beyond what is normally released by the direct effect of blood glucose concentration on beta-cells (Figure 9).



**Figure 9: The incretin effect.** Both incretin hormones, Glp1 and Gip are responsible for about 70% of the total insulin response in healthy patients. This is called the “incretin effect”.

#### 2.4.2. CCK / Gastrin in glucose homeostasis

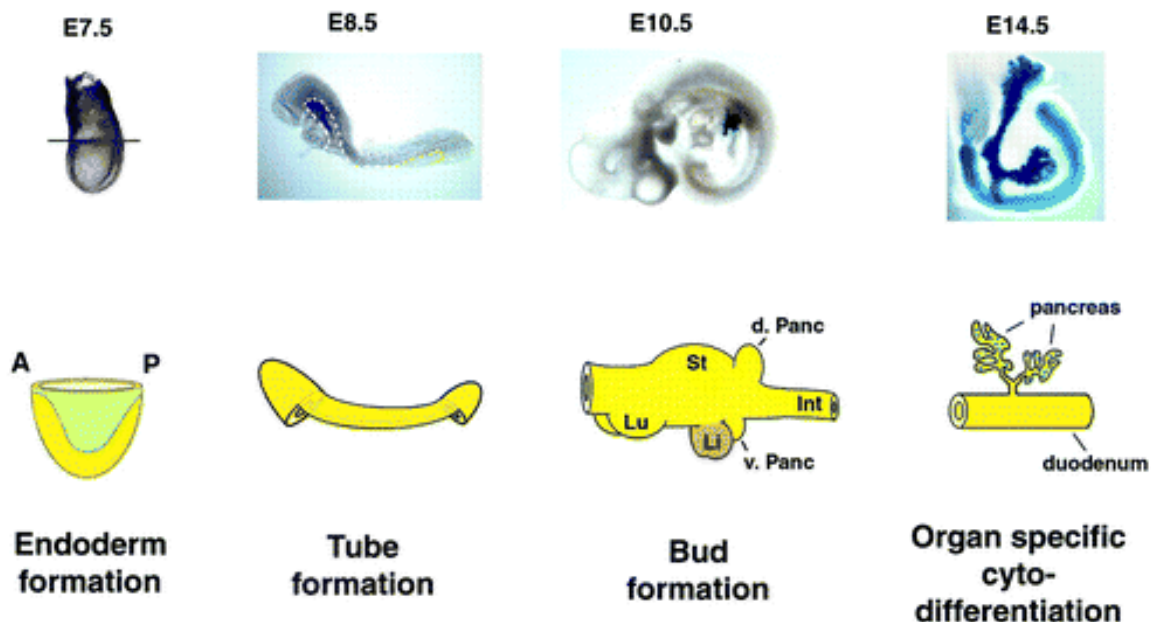
**CCK** and **gastrin** regulate gall bladder contraction, satiety and pancreatic and gastric secretions. CCK induces the secretion of digestive enzymes by acinar cells (Polak et al., 1975) whereas gastrin induces the secretion from the whole exocrine pancreas (digestive enzymes and bicarbonate ions). Interestingly, a few studies have uncovered actions of these hormones which could be relevant to the control of **glucose homeostasis**. Both gastrin and CCK have been shown to stimulate glucagon release from human islets *in vitro* and CCK is also able to stimulate insulin secretion in rodents in a glucose-dependant manner (Drucker, 2007). Although glucose homeostasis seems normal in mice with impaired CCK signalling, *gastrin*-deficient mice exhibit a mild hypoglycaemia and defective glucagon secretion (Boushey et al., 2003). Several studies (reviewed in Drucker, 2007) have suggested a role of both CCK and gastrin on the proliferation of beta-cells especially in regeneration models.

## C. Pancreatic and intestinal development

### 1. Endoderm regionalisation

The gut endoderm becomes grossly regionalised into distinct organ fields by a series of **antero-posterior** (A-P) and dorso-ventral (D-V) **patterning events** which are not yet fully elucidated. Regionalisation is mediated by extrinsic signals from the adjacent mesoderm as well as by intrinsic programs controlled by factors expressed within the endoderm. The **mesoderm** produces various signalling molecules including **FGF, BMP, Wnt, Retinoic acid, Hh** and **Notch** that play **dynamic** and multiple **stage-specific** roles during endoderm patterning. It is thus crucial to precisely control spatiotemporally these interactions for proper gut tube formation and pancreas organogenesis.

Starting around E7.5, the endoderm is regionalised to give the ventral pancreas, lungs and liver for the ventral part of the foregut; oesophagus, dorsal pancreas and stomach for the dorsal part of the foregut (Figure 10). The middle gut will give the jejunum and ileum whereas the posterior gut will form the colon (Wells and Melton, 1999). Wells and Melton showed that *Fibroblast Growth Factor 4* (**Fgf4**) is implicated in this process of determination of the antero-posterior identity of the endoderm (Wells and Melton, 2000). Later work revealed that a gradient of *Fgf4* influences the regionalisation of genes like *Hex1* (*Hematopoietically expressed homeobox 1*), *Pdx1* (*Pancreatic and duodenal homeobox 1*) and *CdxB* (*Caudal type homeobox B*) marking respectively the anterior endoderm, the posterior stomach, pancreas and duodenum and the posterior endoderm (Dessimoz et al., 2006).



**Figure 10: Stages of mouse endoderm development.** The top panels show stages of development of the gastrointestinal tract in mouse embryos (E7.5-E14.5) which are illustrated in the lower panels. At the end of gastrulation (E7.5), the endoderm is a one cell-layer, which covers the mesoderm and ectoderm. Within 24h, a series of morphogenetic processes transforms the endoderm into a tube. At E8.5, the first *Pdx1*-expressing cells (blue) appear in the endoderm. At E10.5, the organs bud. At E14.5, *Pdx1* is expressed in the pancreas, the distal part of the stomach and the duodenum. (Lu: lung, Li: liver, St: stomach, D.Panc: dorsal pancreatic bud, V.Panc: ventral pancreatic bud, Int: intestine. (From Wells and Melton, 1999)



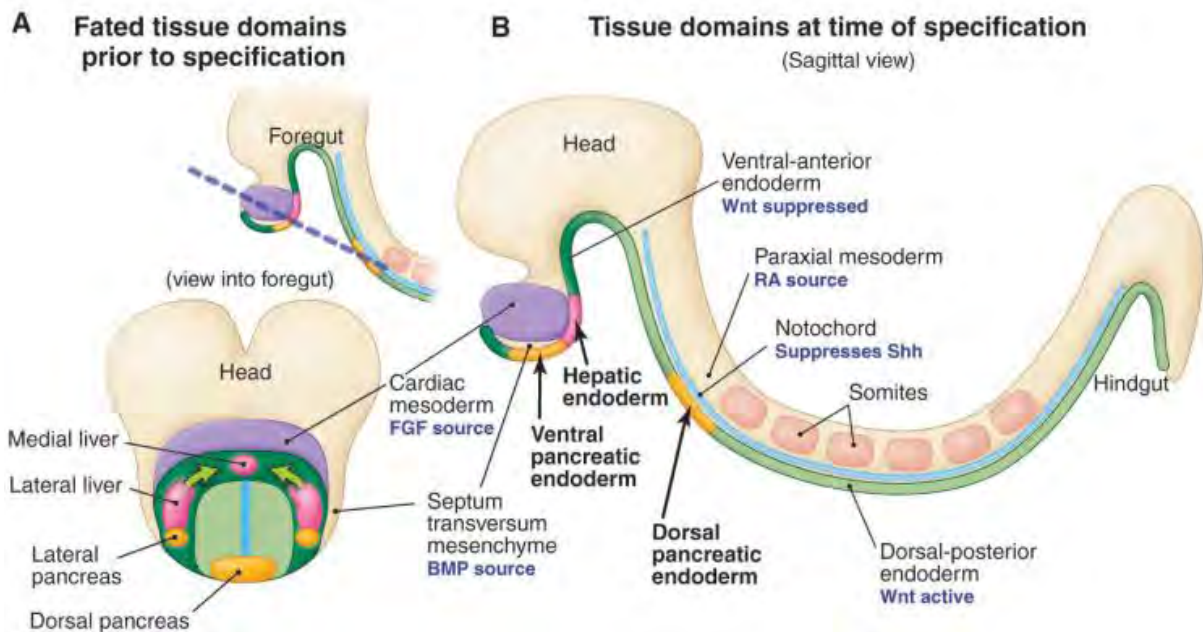
The Hox (Homeobox) genes expressed in the mesoderm and the ParaHox genes in the endoderm are important for the antero-posterior regionalisation of the endoderm. The ***Pdx1*** (previously known as *Insulin-promoter factor 1, Ipf1*) gene is expressed in the anterior part of the endoderm which is at the origin of the distal part of the stomach, pancreas and duodenum whereas *Caudal type homeobox 2 (Cdx2)* is expressed in the caudal part giving rise to the intestine. These two genes are necessary to form the pancreas (Jonsson et al., 1994) and the intestine (Gao et al., 2009) respectively.

## 1.1. Pancreas specification

During embryogenesis, the prepancreatic endoderm is into contact with mesodermal tissues, which function **instructively** and **permissively** in the specification, proliferation, differentiation and morphogenesis of the pancreatic epithelium. I will describe some signals and transcription factors that are important for dorsal and ventral pancreas specification.

### 1.1.1. Signals

The *Fibroblast growth factor (FGF)*, *Bone morphogenetic protein (BMP)*, *Transforming growth factor beta (TGF $\beta$ )*/*Activin* and *retinoic acid (RA)* signalling pathways have roles in endoderm regionalisation (Figure 11). Activation of these signals at the **appropriate time** and **location** is crucial for proper pancreas development. They are differentially required for dorsal and ventral pancreas, it is thus important to keep in mind that extrinsic cues affecting tissue specification need to be considered as a highly dynamic, context- and level-dependent process.



**Figure 11: Signals specifying the hepatic and pancreatic regions of the endoderm. (A)** Fate map of progenitor cell domains prior tissue induction at E8.25. Dashed line indicates plane view. **(B)** Sagittal view of a mouse embryo several hours after A, showing the positions of the newly specified liver and pancreas tissue domains. Main signals and cell sources that pattern the endoderm are shown. (Zaret and Grompe, 2008)

Early during development, **Shh** is expressed strongly in the stomach and duodenum, but is excluded from the pancreatic endoderm. It has been suggested that the expression of **FGF** and activin by the notochord is repressing the expression of *Sonic hedgehog (Shh)* in the dorsal endoderm allowing the development of the dorsal pancreas (Hebrok et al., 1998; Kim et al., 1997; Zaret and Grompe, 2008). However, in the ventral part of the endoderm, the secretion of FGF by the cardiac mesoderm induces expression of *Shh*, thus inhibiting the pancreatic differentiation to facilitate the hepatic fate (Deutsch et al., 2001). During the closure of the ventro-lateral endoderm, cells which migrate caudally escape from FGF signals thus allowing ventral pancreas differentiation (Zaret, 2008). While inhibition of *Shh* is required for early pancreas specification, it is later necessary for pancreatic epithelium expansion and regulation of *insulin* gene expression in beta-cells (Lau and Hebrok, 2010). Adding to the FGF inhibitory signals, **TGF $\beta$**  also represses ventral pancreas differentiation at the advantage of liver (Wandzioch and Zaret, 2009). The **BMP** pathway has an action on liver and pancreas development that changes with time. Early, BMP activates the hepatic function at the expense of the pancreatic differentiation. However, at later stages BMP signalling is necessary to activate the proliferation of *Pdx1*<sup>+</sup> progenitors (Wandzioch and Zaret, 2009).

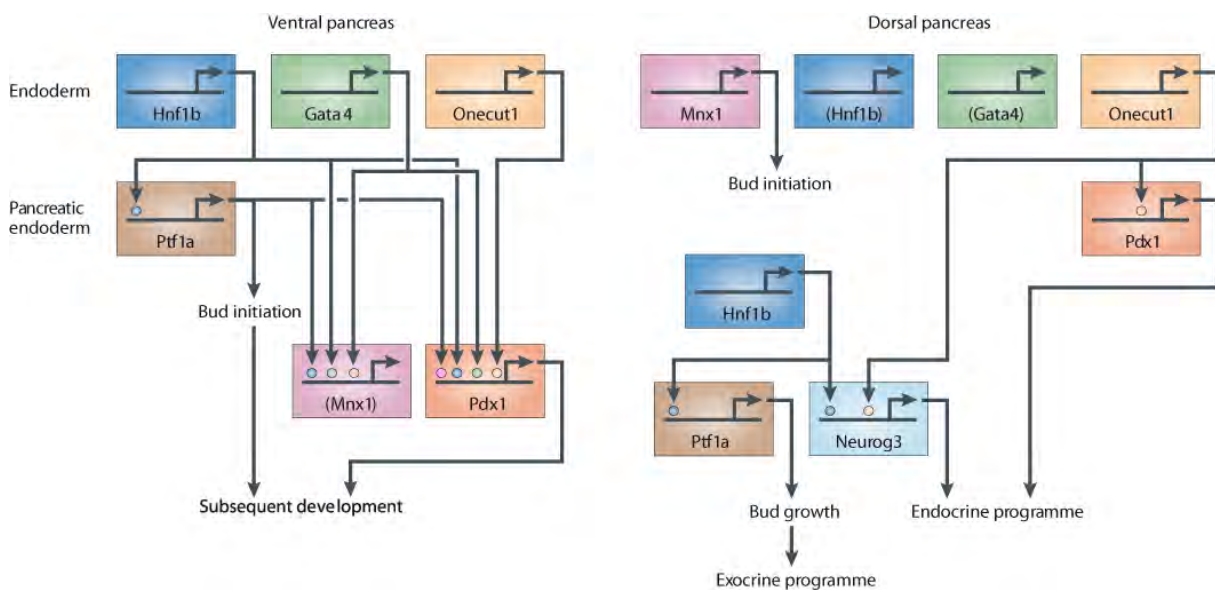
**Retinoic acid (RA)** produced by the mesoderm is also a known regulator of A-P patterning as it posteriorises gut endoderm and promotes pancreas allocation during development. Blocking RA signalling inhibits pancreas specification (Bayha et al., 2009; Kumar et al., 2003). Also, in mice, retinoic acid signalling is necessary for the formation of the dorsal pancreas. More precisely, in *RALDH2* null mice, which lack a RA-synthesising enzyme, the dorsal pancreas does not bud and early glucagon cells do not develop (Martin et al., 2005; Molotkov et al., 2005). In mouse and chick, RA is also required to maintain the pancreatic progenitors and to promote their differentiation into endocrine progenitors expressing *Ngn3* (Bayha et al., 2009; Ostrom et al., 2008).

*In vitro* co-culture of endoderm with or without the dorsal aorta showed that **endothelial cells** are necessary for pancreas **development** as they can induce *Pdx1* and *insulin* expression (Lammert et al., 2001). On the other hand, loss-of-function of *Vascular Endothelial Growth Factors (Vegf)* receptor or *Kinase Insert Domain Protein Receptor (Kdr or Flk1)* showed that endothelial cells are required only for the initiation of pancreatic development (Yoshitomi and Zaret, 2004). Moreover, using *in vivo* ablation of *VegfA*, Pierreux and colleagues have shown that endothelial cells can regulate exocrine cell differentiation by repressing *Ptf1a* expression. With explant cultures they also provided evidences that *VegfA* signalling is required (but not sufficient) to induce endocrine differentiation (Pierreux et al., 2010). Aortic signals are also suggested to promote the survival of dorsal mesenchyme, thereby maintaining *Fgf10* levels, inducing the expression of *Ptf1a* and expanding the pancreatic progenitor pool (Jacquemin et al., 2006). Along the same line, mice deficient for *sphingosin-1-phosphate (S1P)*, blood vessel derived molecule) exhibit pancreatic growth defects due to deficient proliferation of *Pdx1*<sup>+</sup> pancreatic progenitors without affecting endocrine cell differentiation (Sand et al., 2011). Altogether these data show that endothelial cells can provide signals that are essential for pancreatic growth and maintenance. However, in a different study Magenheim et al. showed that blood vessels **restrain pancreas branching** and differentiation (both endocrine and exocrine) (Magenheim et al., 2011). These findings suggest that the well-established notion that blood vessels are positive regulators of tissue growth might need refinement. Indeed, whereas blood vessels are necessary for **progenitor pool maintenance**, they can also regulate **renewal versus differentiation decision**.

The ventral pancreatic endoderm is directly adjacent to the **lateral plate mesoderm (LPM)** which produces signals establishing the ventral pancreatic domain. Using quail-chick tissue recombination assays, it has been shown that instructive signals from the LPM (which could be BMP, RA or activin) regionalise the endoderm (Kumar et al., 2003). It is proposed that liver and ventral pancreas fates may come from an early common population of bipotential progenitors, then FGF signals from the mesoderm induce hepatic differentiation whereas, pancreatic fate is initiated in the absence of FGF (Deutsch et al., 2001). Finally, the septum transversum mesenchyme produces BMPs which are also required for liver specification as they allow some cells of the ventral foregut endoderm to escape pancreatic fate instruction. Moreover, BMPs positively regulate the endodermal expression of *Gata4* a gene which has a role in the activation of *Pdx1* (Rossi et al., 2001).

### 1.1.2. Transcription Factors

**Sox17** is a major regulator of endoderm formation which loss-of-function leads to biliary agenesis and ectopic pancreas formation. Inversely, misexpression of *Sox17* represses pancreas development by promoting ectopic biliary tissue in the posterior foregut region (Spence et al., 2009). The *Motorneuron and pancreas homeobox1 (Mnx1*; also known as *Hlxb9*), *Hepatocyte nuclear factor 1 B (Hnf1b)*, *Gata binding protein 4 (Gata4)* and *Pancreas specific transcription factor 1a (Ptf1a)* genes are expressed in the presumptive pancreatic region and take part in the activation of *Pdx1*. They are differently required for the formation of the ventral or dorsal pancreas. Whereas ***Mnx1*** is strictly necessary for dorsal pancreas formation, ***Hnf1b*, *Gata4* and *Ptf1a*** are required for initiation of the ventral bud (Figure 12). The transcription factors *Forkhead box A1 (FoxA1)* and *Forkhead box A2 (FoxA2)* present in the presumptive pancreas also participate to the **activation of *Pdx1*** (Gao et al., 2008). *Gata4* null mice have a normal dorsal pancreatic bud whereas the ventral pancreas is not specified. ***Gata6*** mutants have a similar but less severe phenotype as a few *Pdx1*<sup>+</sup> cells are found in the ventral pancreas compared to none in the *Gata4* KO (Watt et al., 2007).



**Figure 12: Transcription factors regulating pancreas specification.** The genes expressed in the top line are expressed in the endoderm first, followed by genes on the second and subsequent lines. Arrows between genes or functions indicate a positive regulatory relationship. Genes in brackets are expressed at the stages shown but their mutation does not yield a phenotype at that time. (Adapted from Zaret, 2008)

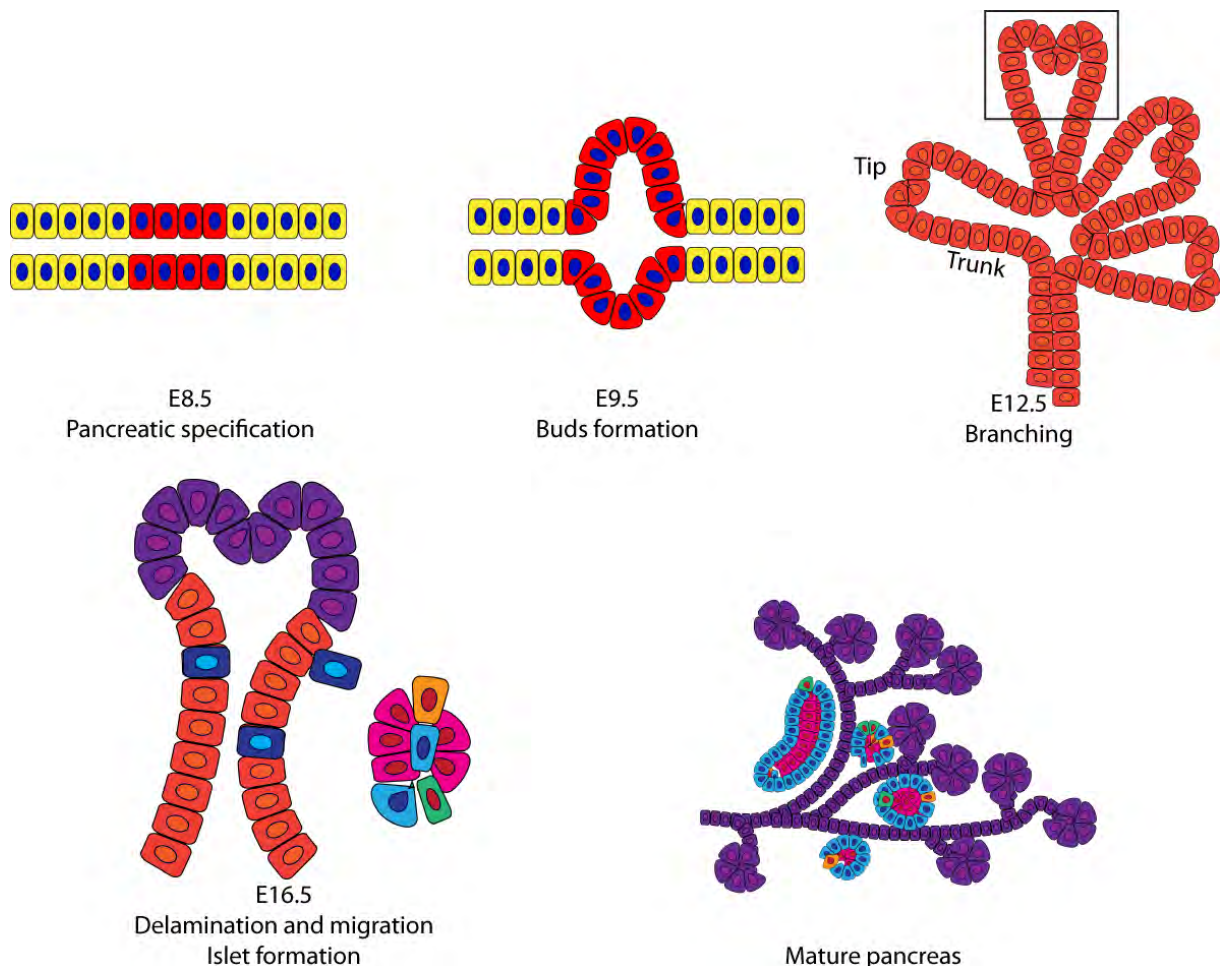


## 1.2. Intestinal specification

The intestine forms from the posterior region of the endoderm expressing *Shh* (Hebrok et al., 1998; Kim et al., 1997). In the posterior part of the gut, the expression of Hox genes, including *Hoxd13* and the expression of *BMP4* by the mesoderm take part in colon specification (Wells and Melton, 1999). *Wnt* (Wingless-related MMTV integration site) signalling is also required for proper gut formation. In *Xenopus*, Wnt signalling represses liver and pancreas development thus favouring the intestine by repressing *Hematopoietically expressed homeobox (Hhex)* (McLin et al., 2007).

## 2. Mouse pancreas development

Pancreatic development has been intensively studied and numerous recent reviews are describing it (Jorgensen et al., 2007; Murtaugh, 2007; Oliver-Krasinski and Stoffers, 2008; Pan and Wright, 2011; Puri and Hebrok, 2010; Seymour and Sander, 2011). I will only present some of the steps that are important to understand my work.



**Figure 13: Scheme of pancreas morphogenesis.** Around E8.5 the pancreatic domain is specified by the expression of *Mnx1*, forming a bulge of pancreatic multipotent progenitor cells (red). About a day later, the duodenal epithelium (red cells) evaginates forming a dorsal and a ventral bud. Bud expansion and branching rely on different signals and genes, including *Shh*, *Fgf10*, *Tgfβ* and signal from the surrounding mesenchyme. Differentiation begins around E10.5 with glucagon-expressing cells (blue) coming first, followed by somatostatin-expressing cells (green), insulin-expressing cells (magenta), pancreatic polypeptide cells (orange) and exocrine cells (purple). Endocrine cells are created in the epithelium and then delaminate and migrate in the mesenchyme and aggregate into islets of Langerhans which are scattered in the ductal/acinar tree of the mature pancreas.

## 2.1. Morphogenesis

The pancreatic primordium forms from the **endoderm** around E8.5 (Wells and Melton, 1999). The first sign of dorsal pancreas budding is the thickening and invagination of dorsal midline endoderm caudal to the stomach around E9.5, whereas ventral budding posterior to the liver is evident only about 12h later (Figure 10). During the **primary transition** (between E9.5-12.5), an epithelial bud with accurate number of progenitors is generated. This early pancreatic epithelium comprises mainly **multipotent pancreatic progenitor cells** (MPC) and a few early-differentiated endocrine cells (mainly glucagon). It has been reported that some of the early endocrine cells co-express insulin and glucagon (Teitelman et al., 1993) whereas later born endocrine cells never do (Herrera, 2000). Formation of these early endocrine cells does not require the same factors than the later ones. More precisely, endocrine cells born during the primary transition do not require the expression of *Pdx1* (Ahlgren et al., 1996). During the **secondary transition** (starting around E13), the epithelial bud goes through programs of proliferation, morphogenesis into an arbour and cascade of cellular differentiation along the duct, acinar and endocrine fates. This will eventually give a correctly sized organ with a tubular-like shape composed of ducts with acinar tips and scattered islets between the branches (Figure 13).

### 2.1.1. Tubulogenesis: roles of Cdc42 and EphB signalling

Starting from simple buds of unpolarised cells, the pancreatic epithelium stratifies, then reacquires polarity and forms microlumens which will eventually fuse to form the *luminal plexus* that will then be remodelled into a complex tubular network (Kesavan et al., 2009; Villasenor et al., 2010). **Tubulogenesis** is a critical event during pancreas morphogenesis because it is the tubular network that sets up the initial tip (future acinar cells) versus trunk compartmentalisation. Progenitors in close proximity to the mesenchyme receive a longer exposure to extracellular matrix and will acquire an acinar fate. Whereas progenitors receiving less mesenchymal signals more likely will give rise to endocrine cells. Several players of pancreas tubulogenesis have been identified. Among them, it has been shown that altering Rho-GTPase **Cdc42** function lead to defect in tube formation and cell fate specification. Deficits in tube formation were caused by a lack of expansion of polarisation thus preventing lumen formation (Kesavan et al., 2009). **EphB** signalling is also required for proper pancreatic epithelium morphogenesis. EphB2/B3 receptors are expressed in the epithelium and their ligands (Ephrin B1/B2) in the mesenchyme and arteries (Villasenor et al., 2010). Mice deficient for the ephrin receptors have disrupted epithelial rosettes and microlumen formation and delayed remodelling, all these leading to a deficient arbour. These mutants have less endocrine/exocrine mass due to the formation of less Ptf1a<sup>+</sup> multipotent pancreatic cells (Villasenor et al., 2010).

### 2.1.2. Tip / Trunk compartmentalisation

Proper tubulogenesis is required for the accurate **tip/trunk compartmentalisation**, indeed the developing epithelial arbour can be considered to be composed of two distinct compartments with different functions. Multipotent pancreatic progenitor (MPC) cells are heterogeneous through the bud but the cells with the “best” MPC state are redistributed to the **tips** of the developing branches. These cells have been shown to maintain the expression of *Pdx1* and *Ptf1a* as well as **Cpa1** (an enzyme that is regulated by *Ptf1a*). With lineage tracing experiments, Zhou and colleagues showed that at E12.5 these cells can produce all pancreatic cell types, thus being true MPC (Zhou et al., 2007). They also suggested that tip MPC proliferation is extending the arbour and leaving behind the trunk cells which have a slower proliferative rate. Around E14.5 however, the tip cells lose their MPC identity and switch toward an acinar fate (Masui et al., 2010; Zhou et al., 2007).

The **trunk** epithelium contains a pool of cells with duct/endocrine bipotency (see I.C.2.2.3). Scattered cells within this structure will eventually up-regulate **Ngn3** expression and commit to become endocrine cells. Endocrine precursors then **delaminate** during the secondary transition, further differentiate and migrate to form islets of Langerhans. Delamination has been suggested to be initiated by *Ngn3* (Gouzi et al., 2011) which triggers a cascade leading to the degradation of E-cadherin and delamination. Recently, *Groucho3* (*Grg3*) which is highly expressed in *Ngn3*<sup>+</sup> progenitors has been shown to suppress *E-cadherin* gene expression, thereby allowing delamination of endocrine cells from the trunk epithelium (Metzger et al., 2012). The cells remaining in the trunk epithelium (positive for **Hnf1b**, **Sox9** and **Nkx6.1**) will become duct cells (Schaffer et al., 2010; Seymour et al., 2007; Solar et al., 2009).

### 2.1.3. Mesenchymal / epithelial crosstalk

On top of the role of mesenchymal signals during pancreas specification (described above I.C.1.1.1), it has been reported that epithelial proliferation, morphogenesis and differentiation also depend on interactions with the surrounding mesenchyme.

Mice deficient for **Fgf10** display pancreatic hypoplasia (Bhushan et al., 2001). Moreover, *Fgf10* misexpression in *Pdx1*<sup>+</sup> cells led to increased proliferation of pancreatic progenitors and strongly reduced differentiation of both endocrine and exocrine cells. This effect again relies on Notch signalling like during pancreas specification. Excessive *Fgf10* leads to sustained Notch activation and promotes proliferation at the progenitor state (Hart et al., 2003; Norgaard et al., 2003).

**BMP** signals derived from the mesenchyme are also critical for pancreatic epithelium expansion and branching morphogenesis. Loss of BMP signalling, either by over-expressing Noggin or with a dominant-negative BMP receptor leads to severe pancreas hypoplasia, reduced branching and excessive endocrine differentiation (Ahnfelt-Ronne et al., 2010). Unpublished work from the lab of Anne Grapin-Botton to which I participated during my Master suggests that BMP signalling is also necessary for the proliferation of endocrine cells during development and after birth (Gésina et al., in preparation).

## 2.2. Multipotent pancreatic cells

Early **MPC** can remain **plastic** for a time but as the bud grows, MPC progressively commit to the **pancreatic fate** and become highly proliferative. MPC are at the origin of all different pancreatic epithelial lineages (Figure 18), namely acinar, ductal and endocrine cells (Zhou et al., 2007). These cells are characterised by the expression of several transcription factors important for pancreas development. Some of these regulators work in the regional allocation of pancreas fate (like Gata4 and Gata6 for example) while others promote progenitors proliferation (Hnf1b) and suppress differentiation during the primary transition. It is important to note that similarly to the **spatio-temporal** requirement of signals described earlier, transcription factors also play multiple context-dependent roles during pancreas development. I decided to describe here some MPC markers: *Pdx1*, *Ptf1a*, *Hnf1b*, *Nkx6.1* and *Sox9* (*SRY-box containing gene 9*) to show that even though MPC express the same markers, they represent a heterogeneous domain of cells, which have diverse potentialities depending on their localisation and developmental stage.

### 2.2.1. Pdx1

**Pdx1** is considered to be the first expressed pancreatic marker. It was the first gene shown to be cell-autonomously required for the earliest step of pancreas development in mice and humans (Jonsson et al., 1994; Stoffers et al., 1997). Pdx1 protein is expressed in the dorsal and ventral bud at E8.5 and its expression is maintained in differentiated beta- and delta-cells (Jorgensen et al., 2007). In addition to total **pancreas agenesis**, *Pdx1*-deficient mice have defects throughout the posterior foregut region and defective endocrine cell differentiation (Ahlgren et al., 1996; Boyer et al., 2006; Fujitani et al., 2006; Jepeal et al., 2005; Larsson et al., 1996; Offield et al., 1996). However, its expression is not restricted to the pancreas. At E9.5, *Pdx1* is also expressed in the presumptive duodenal endoderm and its expression expands to the stomach from E11.5 (Offield et al., 1996). Later, lineage tracing experiments showed that *Pdx1*-expressing progenitors produce acini, ducts and endocrine cells of the mature pancreas (Gu et al., 2002).

### 2.2.2. Ptf1a

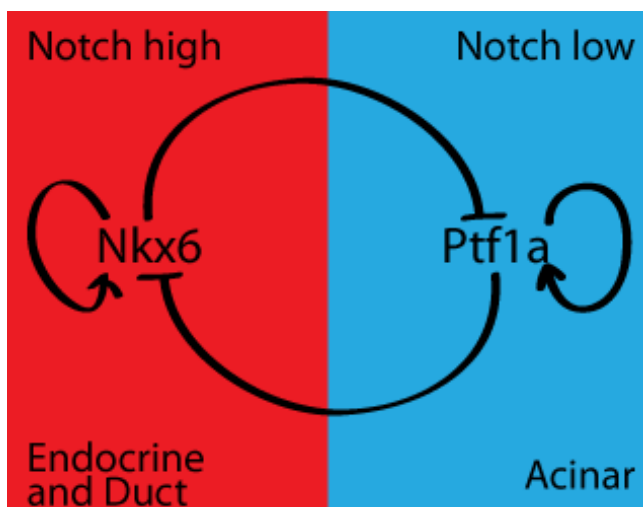
The trimeric *Pancreas Transcription Factor 1 complex (PTF1)* was first identified as an acinar enzyme gene activator (Krapp et al., 1996) and comprises a tissue-specific bHLH (basic helix loop helix) protein **Ptf1a**; an ubiquitous bHLH protein E2A and the mammalian Suppressor of Hairless (Rbpj) protein (Beres et al., 2006). Mice deficient for *Ptf1a* have a less severe phenotype than *Pdx1*-deficient mice; they show **no exocrine tissue and only a rudiment of dorsal endocrine bud** (Krapp et al., 1998). *Ptf1a* is expressed as early as E9.5 in most cells of the nascent pancreatic buds. The expression of Ptf1a is completely lost in the ventral bud of *Pdx1*-deficient mice. However, its expression in the dorsal bud is independent of *Pdx1* (Burlison et al., 2008). Lineage tracing experiments showed that *Ptf1a* is at the origin of most differentiated pancreatic cells (Kawaguchi et al., 2002). Moreover, *Ptf1a* is necessary for the specification of the ventral bud; loss of *Ptf1a* expression leads to **ventral pancreas agenesis** and redirection of ventral pancreatic cells toward the epithelial lineage of the gut (Burlison et al., 2008; Kawaguchi et al., 2002). It has recently been shown with RNA profiling and ChIP-seq that *Ptf1a* stabilises pancreas progenitor identity via the direct control of *Mnx1* and a network of other transcription factors including *Pdx1*, *Nkx6.1* and *Onecut* (Thompson et al., 2012).

### 2.2.3. Hnf1b

**Hnf1b** is an endoderm **patterning regulator** which also has a role in **pancreatic progenitor expansion**. It has recently been shown that *Hnf1b*-expressing cells are multipotent and can give cells from all three pancreatic lineages (Solar et al., 2009). It is expressed broadly in the foregut endoderm at E8 and then maintained in the developing liver and pancreas. During the pancreatic secondary transition, Hnf1b becomes restricted to the **duct/endocrine bipotent** progenitor domain and finally confined to the ducts after birth (Solar et al., 2009). *Hnf1b* null mice form a transient dorsal pancreas expressing *Pdx1* but these progenitors fail to expand. Moreover, similarly to *Ptf1a* KO, the ventral bud is absent in these mice (Haumaitre et al., 2005). It has been shown that *Ptf1a* expression is lost in *Hnf1b* null mice and that there is putative Hnf1b-binding sites on the *Ptf1a* regulatory element, suggesting a direct relationship. In addition to *Ptf1a*, *Hnf1b* also seem to form a transcriptional complex with *Hnf6* to control pancreatic progenitor generation and endocrine progenitor induction (Poll et al., 2006)

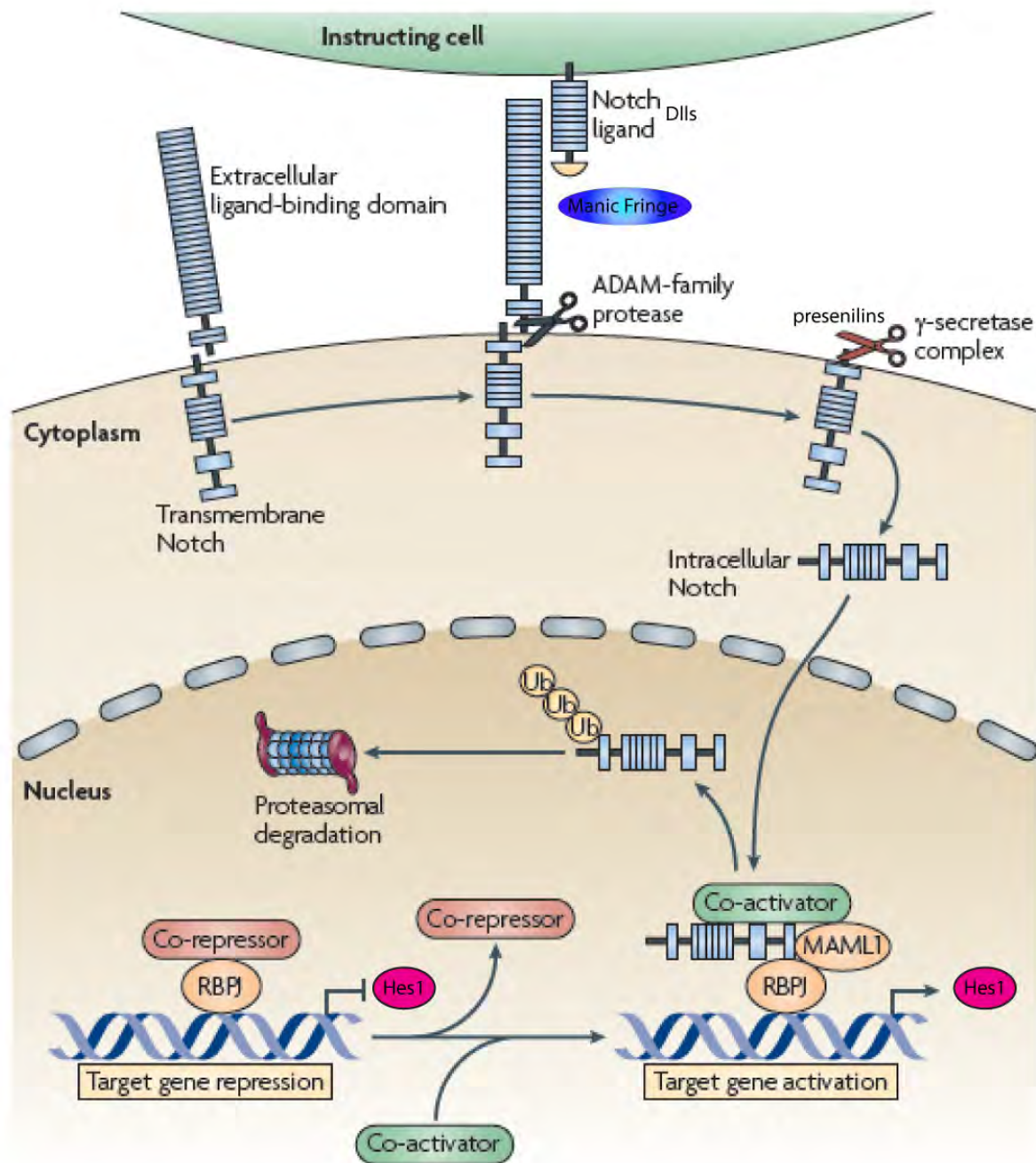
### 2.2.4. Nkx6

Recently, Schaffer and colleagues have proposed an antagonistic relationship between **Nkx6.1** and *Ptf1a* (Figure 14). They showed that *Ptf1a* is required for tip formation, while trunk formation requires Nkx6.1, each transcription factor being sufficient to repress the alternative lineage program (Schaffer et al., 2010). This “**bistable switch**” occurs during a critical time window prior any lineage commitment, when progenitors are still multipotent. Nkx6.1 is able to bind *Ptf1a* elements in cell lines, suggesting that this switch could happen by direct transcriptional repression. Moreover, their experiments also showed that high level of Notch favours the expression of Nkx6 biasing the cells toward an endocrine/duct stage (see I.C.2.3.1). Further illustrating the different potentialities of the MPC along development, Beucher et al. showed that failed endocrine progenitors can adopt acinar or ductal fate at early bud stage but eventually differentiate only into duct cells in the branching pancreas (Beucher et al., 2012b). These data support the idea that endocrine progenitor cells arise from bipotent precursors already committed to the duct/endocrine lineages.



**Figure 14: Scheme of MPC specification by Nkx6 and Ptf1a.** Schaffer et al. showed that there is a bistable switch between Nkx6 and *Ptf1a* that predetermines the cells for an endocrine versus acinar fate. High level of Notch favours the expression of Nkx6 which can repress *Ptf1a*. In turn, low level of Notch favours *Ptf1a* which can inhibit Nkx6 creating a regulatory circuit. Positive auto-regulatory feedback creates bistability of the fate choice. (Schaffer et al., 2010)





**Figure 15: The Notch pathway.** Notch is expressed on the cell surface as a heterodimer that consists of an extracellular ligand-binding domain that is non-covalently associated with a transmembrane polypeptide. Signalling is initiated by the interaction of Notch with *Delta-like ligands (Dlls)* or *Jagged* ligands on the surface of instructing cells. Following ligand binding, sequential cleavages occur in the transmembrane region of Notch, the first mediated by an ADAM (a disintegrin and metalloproteinase-family protease) and subsequently by a gamma-secretase complex. This final cleavage results in the release of the NICD from the plasma membrane and its translocation to the nucleus, where it binds to recombination-signal-binding protein for immunoglobulin-kappa j region (RBPJ) transcription factors that are bound to the promoters of Notch target genes. At these sites, intracellular Notch displaces co-repressors from RBPJ and recruits co-activators, including Mastermind-like 1 (MAML1) to activate the expression of target genes. The activity of intracellular Notch is terminated by its polyubiquitylation (Ub) and subsequent proteasomal degradation. NICD: Notch IntraCellular Domain. (Amsen et al., 2009)

### 2.2.5. Sox9

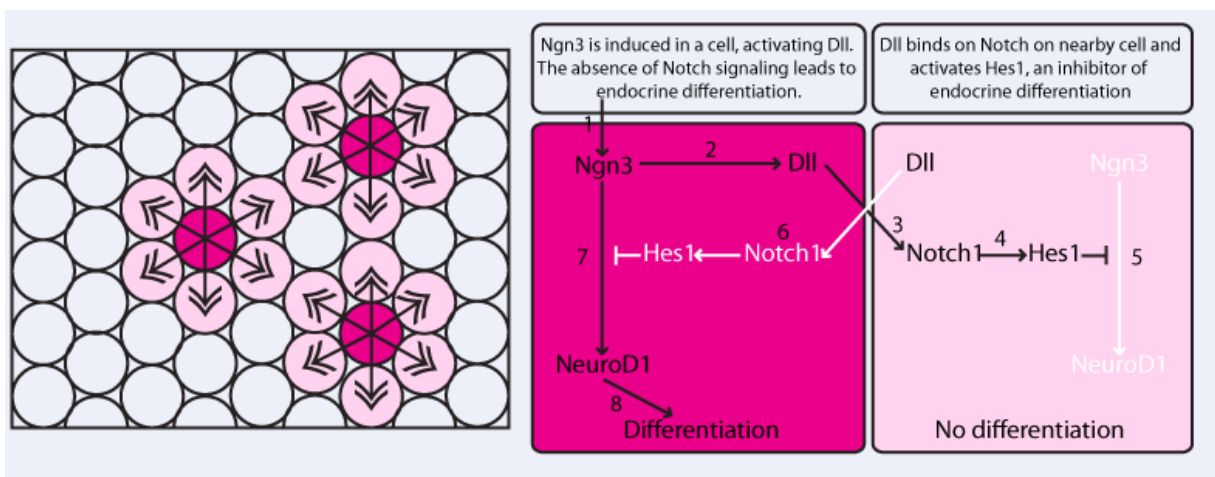
**Sox9** is a member of the SRY/HMG box (Sox) family which is important for **proliferation**, survival and maintenance of pancreatic progenitors (Lioubinski et al., 2003; Seymour et al., 2007). *Sox9* expression is first detected around E10.5 in the early pancreatic MPC, and lineage tracing experiment revealed that *Sox9*<sup>+</sup> progenitors produce cells of all three pancreatic lineages (Akiyama et al., 2005). *Sox9* expression then persists in the endocrine/duct bipotent progenitor pool during the secondary transition (Seymour et al., 2007). *Sox9* is also a known regulator of *Ngn3* expression (Lynn et al., 2007) suggesting a role in initiation of endocrine differentiation. For the relation between *Sox9* and Notch signalling please refer to the next chapter (I.C.2.3.1).

## 2.3. Endocrine commitment

### 2.3.1. Predetermining the endocrine fate via Notch signalling

The apparition of endocrine progenitor cells from MPC expressing *Pdx1* is tightly regulated by the **Notch signalling** pathway (Figure 15). The pathway was studied by gain- and loss-of-function of its different components. Basically these studies revealed that the **spatio-temporal regulation** of Notch is critical to avoid **precocious differentiation** of the MPC and to allow the coordination of epithelial outgrowth and proliferation with the accurate production of endocrine and acinar cells.

Multiple Notch ligands and receptors as well as the mediator *Rbpj* and the downstream target *Hes1* are expressed in the early pancreatic epithelium. This includes *Notch1-4*, *Dll1*, *Dll3*, *Jagged1* and *Jagged2*. The inactivation of *Delta-like1* (*Dll1*), *Rbpj*, or *Hairy and enhancer of split1* (*Hes1*) induces a **premature differentiation** of MPC into endocrine progenitors expressing *Ngn3* (Apelqvist et al., 1999; Jensen et al., 2000b). Other studies showed that *Ngn3* is capable of inducing the expression of *Dll1* (Heremans et al., 2002), thus Notch signalling could act by **lateral inhibition** (Figure 16).

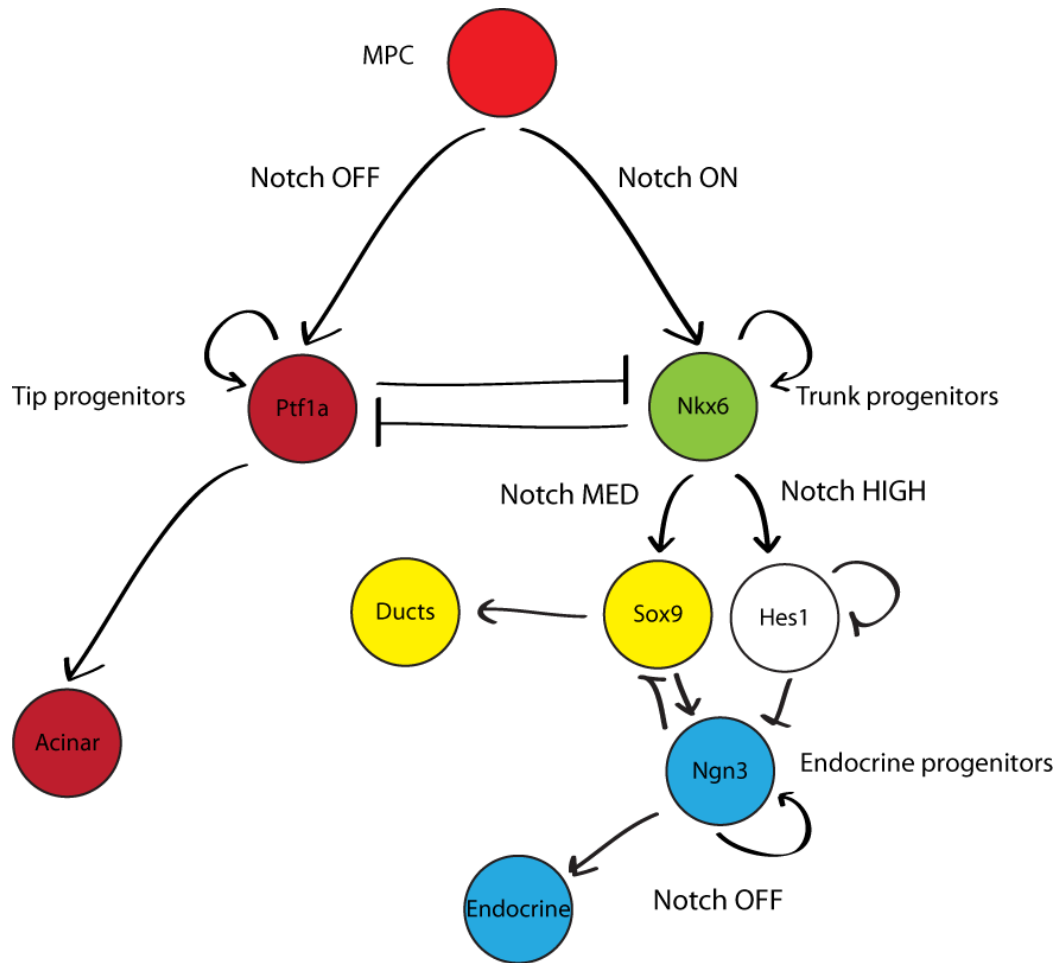


**Figure 16: Model for lateral inhibition by the Notch signalling pathway.** Left panel: General scheme of lateral inhibition. Right panel: Lateral inhibition in endocrine differentiation. (1) *Ngn3* expression is induced in a cell. (2) *Ngn3* transactivates Notch ligands of the Dll family. (3) *Dll* binds Notch receptor on a neighbouring cell. (4) The receptor is cleaved and the NICD translocates into the nucleus and activates *Hes1*. (5) *Hes1* represses *Ngn3* subsequent endocrine differentiation. (6) The absence of Notch stimuli in the cell expressing *Ngn3* leads to the activation of *NeuroD1* (7) and allows for endocrine differentiation (8). NICD: Notch IntraCellular Domain. (Adapted from Jensen, 2004)

Gain-of-function experiments by over-expressing the intracellular domain of Notch (NICD) confirmed that Notch **inhibits endocrine differentiation** (Ahnfelt-Ronne et al., 2007; Hald et al., 2003; Murtaugh et al., 2003). *Manic Fringe (Mfng)* which codes for a glycosyltransferase which increases the ability of Notch to bind Dll, is expressed in endocrine progenitors but not in pancreatic multipotent progenitors nor in mature endocrine cells (Golson et al., 2009; Xu et al., 2006b). However, Svensson and colleagues showed that *Mfng* was not necessary for endocrine cells differentiation. Mice deficient for *Mfng* have a normal number of endocrine cells (Svensson et al., 2009). Along the same line, it has been shown that the inactivation of *presenilins*, members of the gamma-secretase complex responsible for Notch cleavage, turn Ngn3<sup>+</sup> cells into exocrine cells (Cras-Meneur et al., 2009). The authors also observed that a down-regulation of *presenilins* due to a loss of *Notch2* expression leads to the same phenotype. However, in mice depleted of both *Notch1* and *Notch2*, Ngn3<sup>+</sup> cells can differentiate into endocrine cells (Nakhai et al., 2008). These results suggest that the transcriptional effect of *Notch1* and *2* represses endocrine differentiation but that a certain amount of NICD is necessary for endocrine differentiation. Thus, Notch could control the **number** of endocrine **progenitors** generated during development. In parallel, Notch signalling via *Hes1*, could keep multipotent pancreatic progenitors in a proliferative state by inhibition of the Cyclin-dependent Kinase (CDK) inhibitor *p57<sup>kip2</sup>* (Georgia and Bhushan, 2006). Recent studies have further unravelled the roles of Notch during pancreas development showing that Notch does not function in an on/off mode but rather that a gradient of Notch activity produces distinct cellular states during pancreas development. Shih et al. showed that Notch renders progenitors competent to differentiate into ductal and endocrine cells via the expression of *Sox9* (Shih et al., 2012). Similarly, in zebrafish, different levels of Notch signalling drive distinct behaviours in the progenitor population. High levels of Notch induce quiescence, whereas lower levels promote progenitor amplification (Ninov et al., 2012). Other recent studies showed that Notch mediates patterning and cell fate allocation of the MPC (Afelik et al., 2012; Horn et al., 2012). Basically, Notch-suppressed cells lose trunk marker genes and gain expression of tip marker genes and subsequently differentiate into acinar cells at the expense of endocrine cells. This could be explained by the requirement of Notch for the expression of the trunk determination gene *Nkx6.1* (see I.C.2.2.4) and indeed the authors found that *Rbpj* can directly bind the proximal promoter of *Nkx6.1* (Afelik et al., 2012; Schaffer et al., 2010) (Figure 17).

Numerous papers showed that Notch could also regulate **exocrine cell differentiation**. Inhibition of exocrine differentiation was reported in NICD gain-of-function in multipotent pancreatic progenitors (Ahnfelt-Ronne et al., 2007; Hald et al., 2003; Murtaugh et al., 2003) and when *Rbpj* is inactivated in these same cells (Fujikura et al., 2006; Nakhai et al., 2008). However, it has been shown that *Rbpj* which binds to NICD to activate Notch target genes could also be part of the PTF1 complex in pancreatic progenitors (Beres et al., 2006; Masui et al., 2007). The PTF1 complex which is also composed of the transcription factor *Ptf1a* activates exocrine differentiation (Masui et al., 2007). Thus, *Rbpj* inactivation impairs the Ptf1 complex and thus subsequent exocrine differentiation. Similarly, over-expression of NICD causes a competition between *Ptf1a* and NICD for *Rbpj*, reducing the amount of available *Rbpj* to form the Ptf1 complex and thus alters exocrine differentiation. This modification of Notch signalling is probably not responsible for the inhibition of exocrine differentiation observed in mice deficient for *Rbpj* or over-expressing NICD.





**Figure 17: Schematic representation of the spatio-temporal roles of Notch on pancreas differentiation.** In MPC, Notch mediates a dynamic patterning into tip/trunk domains. The loss of Notch within MPC leads to a tip fate and localisation, whereas sustained Notch signalling favours trunk localisation by the activation of Nkx6. At high Notch activity Hes1-mediated Ngn3 repression prevents Sox9 from inducing Ngn3. At intermediate Notch levels, Hes1 is not maintained allowing Sox9 to enable a molecular circuitry that drives endocrine differentiation. Negative feedback of Ngn3 on Sox9 ensures exit from the bipotent duct/endocrine state and terminal endocrine commitment. (Adapted from Afelik et al., 2012 and Shih et al., 2012)

### 2.3.2. Endocrine specification is achieved by the expression of Ngn3

The commitment of pancreatic progenitor cells to the **endocrine lineage** is done by the expression of the bHLH transcription factor **Neurogenin3** (*Ngn3*). Inputs from several transcription factors, including *Sox9*, *Hnf1b*, *Hnf6*, *FoxA2* and *Pdx1* which have been shown to bind on the *Ngn3* regulatory region (Lynn et al., 2007; Oliver-Krasinski et al., 2009) are necessary to activate *Ngn3*. *Ngn3*<sup>+</sup> progenitors appear between E9.5 and E18.5 before cells start making hormones (Gradwohl et al., 2000; Jensen et al., 2000a; Schwitzgebel et al., 2000) and the inactivation of *Ngn3* leads to the loss of all pancreatic endocrine cells, even the ones from the primary transition (Gradwohl et al., 2000). It has been shown that the expression of *Ngn3* in pancreatic progenitors is sufficient to induce endocrine differentiation (Apelqvist et al., 1999; Schwitzgebel et al., 2000). The number of *Ngn3*<sup>+</sup> cells is higher at the secondary transition. At this stage, *Ngn3*<sup>+</sup> cells are localised in the epithelium or in contact with the ducts (Gradwohl et al., 2000; Schwitzgebel et al., 2000). After birth, cells expressing high levels of *Ngn3* almost totally disappear, however low expression of *Ngn3* can be observed in mature beta-cells (Gu et al., 2002; Wang et al., 2009a).

The tracing of *Ngn3*<sup>+</sup> cells using the Cre/loxP system in a transgenic mouse expressing the Cre recombinase under the control of the *Ngn3* promoter, showed that *Ngn3*<sup>+</sup> cells are at the origin of all endocrine cells from the pancreas (Gu et al., 2002). Moreover, a very small population (less than 1%) of exocrine cells is often labelled, suggesting that some cells expressing *Ngn3* can eventually differentiate into exocrine cells and that *Ngn3* levels are important to secure the endocrine destiny (Beucher et al., 2012b; Gu et al., 2002; Schonhoff et al., 2004; Wang et al., 2009b). Moreover, Beucher et al. recently showed that this competence of *Ngn3*<sup>+</sup> progenitors to **give rise to exocrine** cells is changing during development. Early during development, failed endocrine progenitors (cells which have been instructed to turn on the *Ngn3* gene but cannot adopt an endocrine destiny because they fail to express *Ngn3* protein) can adopt acinar or ductal fate, whereas later they do not contribute to the acinar lineage but only to the ductal one (Beucher et al., 2012b).

In endocrine progenitors, the transient expression of *Ngn3* induces the endocrine differentiation program. *Ngn3* transactivates directly *Neurogenic differentiation 1 (NeuroD1)* (Huang et al., 2000) and *Insulinoma-associated 1 (Insm1)* (Breslin et al., 2007), two genes required for the differentiation and/or function of endocrine cells (Gierl et al., 2006; Gu et al., 2010; Mellitzer et al., 2006; Naya et al., 1997). Several experiments showed that only about 1-2% of *Ngn3*<sup>+</sup> cells are proliferating (Jensen et al., 2000a; Miyatsuka et al., 2011), whereas more differentiated cells expressing *NeuroD1* were all **post-mitotic** (Jensen et al., 2000a). It was recently shown that *Ngn3* itself inhibits proliferation by inducing *Cdkn1a* a cyclin-dependent kinase inhibitor (Miyatsuka et al., 2011). The apparition of endocrine cells during pancreas development is asynchrone and gain-of-function of *Ngn3* in *Pdx1*<sup>+</sup> pancreatic progenitors revealed that the competences of pancreatic progenitors change over time (Johansson et al., 2007). More precisely, between E8.5 and E12.5, appearing endocrine cells are essentially alpha-cells. However, after E14.5, progenitors differentiate essentially into beta-, delta- and PP-cells. Recently, it has also been shown that *Ngn3* is the initiating factor for delamination of differentiating endocrine cells during pancreas development (Gouzi et al., 2011).

## 2.4. Transcription factors regulating endocrine differentiation

Endocrine differentiation happens in two phases. Between E9 and E12.5 it is the *primary transition*; the first endocrine cells form, mainly expressing glucagon. The *secondary transition* starts around E13 in mice and is a period of extensive epithelial expansion and differentiation of endocrine, ductal and acinar cells. During this period the “second wave” endocrine cells are born from the trunk epithelium upon the expression of *Ngn3*. There are a lot of transcription factors acting downstream of *Ngn3* that are necessary for the setting up of a functional endocrine program or to specify the progenitors toward the different endocrine lineages (Figure 18); including *NeuroD1*, *Insm1*, *Aristaless related homeobox (Arx)*, *Paired box gene 4 (Pax4)*, *Paired box gene 6 (Pax6)*, *V-mafmusculoaponeurotic fibrosarcoma oncogene family, protein A (avian) (MafA)*, *V-mafmusculoaponeurotic fibrosarcoma oncogene family, protein B (avian)(MafB)*, *NK2 transcription factor related, locus 2 (Nkx2.2)*, *NK6 homeobox 1 (Nkx6.1)*, *NK6 homeobox 2 (Nkx6.2)*, *ISL1 transcription factor*, *LIM/homeodomain (Isl1)* and *POU domain, class 3, transcription factor 4 (Pou3f4* also known as *Brn4*) (Collombat et al., 2006; Oliver-Krasinski and Stoffers, 2008). Sequential triggering to all these transcription factors (and others) is necessary to generate accurate numbers of functional (mono-) hormonal endocrine cells.

#### 2.4.1. Generic transcriptional programs of endocrine differentiation

Generic endocrine transcription factors are classified in this category as they facilitate the formation of **accurate endocrine cell numbers**. In this category I will describe briefly *NeuroD1*, *Insm1*, *Rfx6* (more details will be found in the RFX chapter of this introduction), *Isl1* and *Pax6*.

Naya and colleagues reported that **NeuroD1**-deficient mice stop endocrine development during the secondary transition and thus have a globally reduced number of endocrine cells. Moreover, the remaining cells are not able to form islets (Naya et al., 1997). Analysis of **Pax6** mutant mice revealed that *Pax6* is necessary for global endocrine cell differentiation. Indeed, two different studies report a strong reduction of all endocrine subtypes in *Pax6* mutants (Sander et al., 1997; St-Onge et al., 1997). Moreover, an almost complete loss of alpha-cells was observed in the full KO (St-Onge et al., 1997). Similar defects are observed in *Insm1*, *Rfx6* and *Isl1* mutants. Loss of **Rfx6** or **Insm1** prevents the differentiation of endocrine progenitors towards hormones-expressing cells (Gierl et al., 2006; Mellitzer et al., 2006; Smith et al., 2010; Soyer et al., 2010). Interestingly, in *Insm1* KO, the endocrine differentiation defect is associated with an accumulation of *Ngn3*<sup>+</sup> progenitors which may be explained by the role of *Insm1* in cell cycle progression (Gierl et al., 2006). **Isl1**, on top of its role in the mesenchyme for dorsal pancreas induction, has been shown to be necessary for survival and proliferation of differentiating endocrine cells during the secondary transition (Du et al., 2009). *Insm1*, *Isl1*, *Pax6* and *Rfx6* are maintained in adult islets but their function at this point is not known. Deciphering the role of *Rfx6* in mature beta-cells is one of the objectives of my thesis.

#### 2.4.2. Subtype-specific transcriptional programs

Lineage allocation is specified by the combined action of *Arx/Pax4* and this will be the main focus of this chapter. It is however important to note that other transcription factors like for example *Nkx2.2* could have a role in endocrine subtype specification: *Nkx2.2* is expressed from E9.5 in pancreatic buds and maintained in alpha-, beta- and PP-cells. Mice deficient for **Nkx2.2** have an almost normal number of endocrine cells but the beta-cells and most alpha- and PP-cells are replaced by ghrelin-producing cells (Prado et al., 2004; Sussel et al., 1998). *Nkx6.1* and *Nkx6.2* are also necessary for endocrine differentiation. After E14.5, **Nkx6.1** is expressed in a subpopulation of *Ngn3*<sup>+</sup> cells and in mature beta-cells and its inactivation leads to an altered endocrine differentiation as only few beta-cells are formed (Sander et al., 2000). Even though *Nkx6.1* is expressed downstream of *Ngn3*, its expression in *Pdx1* progenitors is also important for beta-lineage specification. Indeed, the expression of *Nkx6.1* under the control of *Pdx1*, but not under the control of *Ngn3*, is able to correct the altered endocrine phenotype and lead to normal beta-cell differentiation in *Nkx6.1*-deficient mice (Nelson et al., 2007). **Nkx6.2** is also expressed in the embryonic pancreas but on contrary to *Nkx6.1*, its expression is slowly restricted to alpha-cells (Henseleit et al., 2005). *Nkx6.2*-null mice do not have an alpha-cell phenotype, suggesting that *Nkx6.1* can compensate the lack of *Nkx6.2*. Accordingly, mice deficient for both *Nkx6.1* and 6.2 exhibit a reduced number of alpha- and beta-cells (Henseleit et al., 2005).

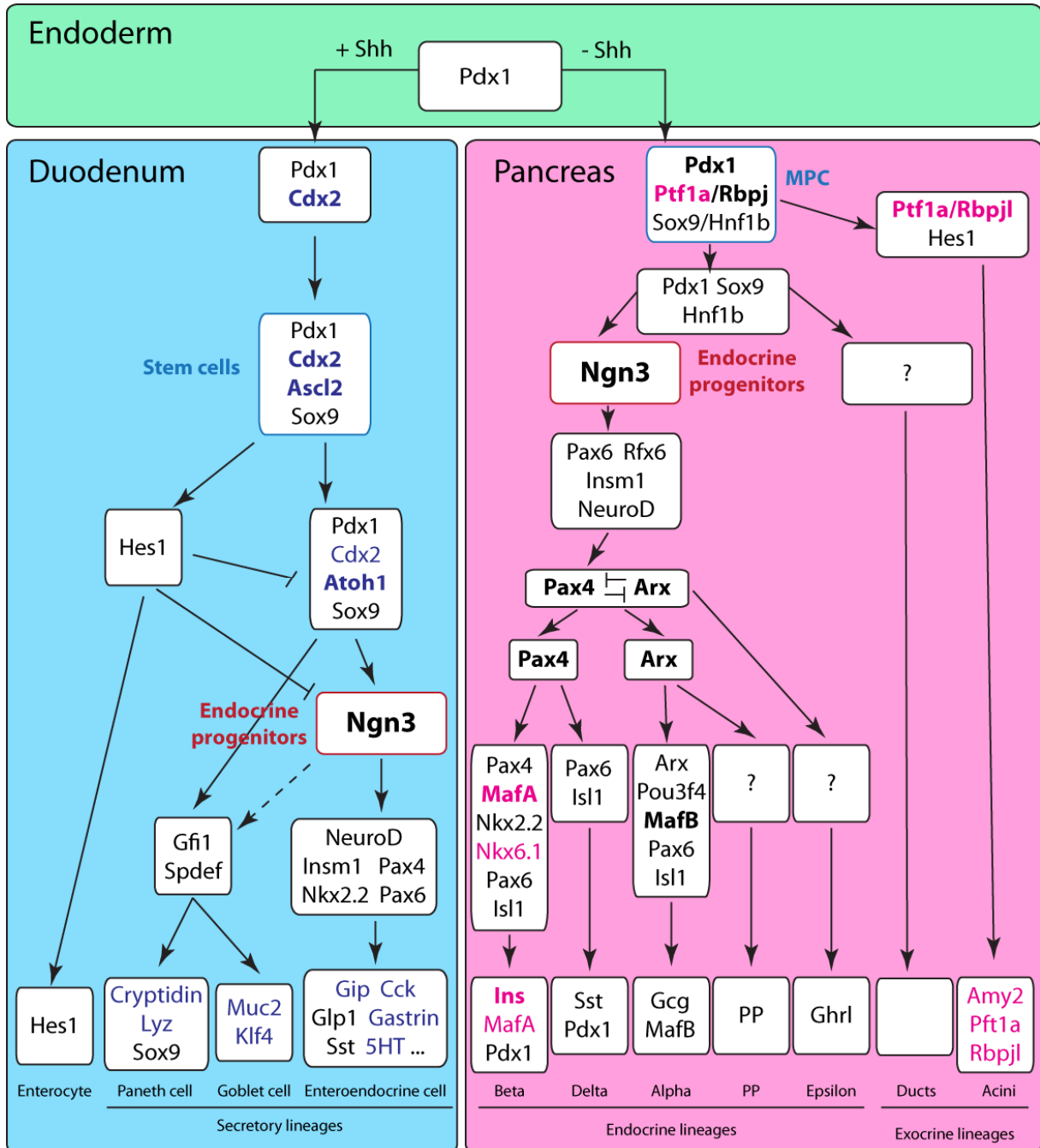
The transcription factors **Arx** and **Pax4** are necessary for endocrine subtype specification. **Pax4** loss-of-function leads to the differentiation of endocrine progenitors in alpha-cells and the loss of beta- and delta-cells (Sosa-Pineda et al., 1997). Moreover, it has been reported that **Pax4** also inhibits *ghrelin* expression (Wang et al., 2008). Inversely, the loss of **Arx** is characterised by the absence of alpha-cells at the benefit of beta- and delta-cells (Collombat et al., 2003). The inverse effect is observed when **Arx** or **Pax4** is over-expressed in endocrine progenitors or differentiated endocrine cells; **Arx** over-expression favouring alpha- and PP-cells (Collombat et al., 2007) and **Pax4** over-expression converting alpha-cells to beta-cells (Collombat et al., 2009). The absence of **Arx** expression is required for delta-cells specification because mice depleted for **Arx** and **Pax4** do not make alpha- and beta-cells but somatostatin-expressing cells are not lost (Collombat et al., 2005). **Arx** being able to inhibit **Pax4** and inversely, endocrine subtype specification seems to rely on the expression levels of these two transcription factors in the early endocrine progenitors. Thus differentiation of endocrine progenitors towards the alpha-, PP- and epsilon- or beta- and delta-lineages is controlled by the combined action of **Arx** and **Pax4**. The conversion of mature alpha-cells toward beta-cells upon misexpression of **Pax4** (and vice-versa with **Arx** misexpression) (Collombat et al., 2007; Collombat et al., 2009) suggests that differentiated mature endocrine cells keep a certain **plasticity**. Supporting this idea, a recent study by the lab of Pedro Herrera showed that mature alpha-cells can be converted to beta-cells after extreme beta-cell loss (Thorel et al., 2010).

#### 2.4.3. Transcription factors necessary for endocrine cell maturation

**Arx** plays a crucial role in initiation of alpha-cell differentiation (Collombat et al., 2003) but other transcription factors are required for the terminal differentiation including the winged-helix transcription factors *Forkhead box A1* (**FoxA1** formerly known as *HNF3a*) and *A2* (**FoxA2** formerly known as *HNF3b*). The FoxA factors play an important role in the proper development of multiple endoderm-derived organs, including the pancreas (Kaestner, 2010). **FoxA1**-deficient mice die soon after birth due to severe hypoglycaemia due to a decreased expression of several islet genes including *glucagon* (Kaestner et al., 1999; Shih et al., 1999). Interestingly, FoxA1 binds to and transactivates the G2 element of the preproglucagon promoter (Kaestner et al., 1999). All together, these data show an important role for FoxA1 in **preproglucagon gene transcription** and therefore in the maintenance of alpha-cell function. In addition, FoxA1 and FoxA2 are required as well for preproglucagon gene expression in the intestine (I.C.3.2.3) and for the **maintenance of mature beta-cells function** (Gao et al., 2010). **FoxA2** null embryos die shortly after gastrulation thus requiring a conditional ablation to study its role during development (Weinstein et al., 1994). The specific deletion of **FoxA2** in the endoderm using a *FoxA3-Cre* driver causes severe hypoglycaemia and early lethality. Similar to what was described in **FoxA1** mutants, a reduction in plasmatic glucagon levels was observed with a dramatically reduced number of mature alpha-cells and decreased postnatal preproglucagon mRNA levels (Lee et al., 2005). Nevertheless, initial specification of alpha-precursor cells was normal, stressing the important role of **FoxA2** during terminal, but not initial alpha-cell differentiation. Preproglucagon transcription is also regulated by other genes including *Pax6*, *Brn4*, *Isl1* and *MafB*. For more details please refer to this comprehensive review on alpha-cell differentiation (Bramswig and Kaestner, 2011). Mutant mice with a specific ablation of **FoxA2** in beta-cells exhibit disorganised islet architecture and misregulated insulin secretion because of a reduction in the expression of *Kir6.2* and *Sur1* and die shortly after birth from hypoglycaemia (Sund et al., 2001).

Among the *basic leucine zipper* transcription factors from the Maf family, three are expressed in the pancreas (*MafA*, *MafB* and *c-Maf*) and **regulate insulin transcription** (Matsuoka et al., 2003; Olbrot et al., 2002). ***MafB*** is expressed in immature alpha- and beta-cells and then restricted to alpha-cells in new-borns where it participates to the **activation of glucagon transcription** (Artner et al., 2006). *MafB* inactivation leads to the total loss of alpha-cells and half of the beta-cells as well as some delayed beta-cell development (Artner et al., 2007; Artner et al., 2010). Unlike *Pax4* or *Arx* mutants, cells depleted for *MafB* are not redirected to another lineage; they are stuck at an earlier differentiation point and do not express glucagon nor insulin. ***MafA*** is the factor that binds the RIPE3b element on the promoter of the *insulin* gene (Aramata et al., 2007; Matsuoka et al., 2003; Olbrot et al., 2002). Like *Pdx1* and *NeuroD1*, *MafA* is able to **transactivate the insulin promoter**. Unlike *MafB*, *MafA* is expressed only in beta-cells and its expression is lost in *Nkx6.1* mutants (Matsuoka et al., 2004). *MafA*-deficient mice have a decreased insulin expression (Zhang et al., 2005a) and progressively develop diabetes after birth due to progressive loss of beta-cells. Thus *MafA* is essential for the maturation of beta-cells. However, induced expression of *MafA* in pancreatic progenitors is detrimental for pancreatic development (Nishimura et al., 2009), showing once more that timing is very important in the function of transcription factors during pancreas development. Artner and colleagues also showed that *MafA/B* are necessary to activate genes important for beta-cell function including *Pdx1*, *Glut2* and *Nkx6.1* (Artner et al., 2010). To summarise, whereas *MafA* function is beta-cell specific, *MafB* is required for both alpha- and beta-cell differentiation and maturation.

Recently, a role for ***NeuroD1*** in the maturation of beta-cells was proposed. Gu and colleagues have inactivated *NeuroD1* expression specifically in mature beta-cells and showed that *NeuroD1*-deficient islets respond poorly to glucose and have a metabolic profile similar to immature beta-cells with increased expression of glycolytic genes and lactate dehydrogenase, elevated basal insulin secretion and oxygen consumption. Therefore, *NeuroD1* is necessary for the **complete maturation** of beta-cells and the maintenance of adequate glucose response (Gu et al., 2010). In the same paper, they interestingly showed that the insulin produced in the mutants was exclusively derived from *Ins2* as *Ins1* expression was almost extinguished.



**Figure 18: Simplified scheme of intestinal and pancreatic lineages specification.** In blue, genes expressed specifically in the intestine, in magenta pancreas-specific genes. (Adapted from A. Beucher)

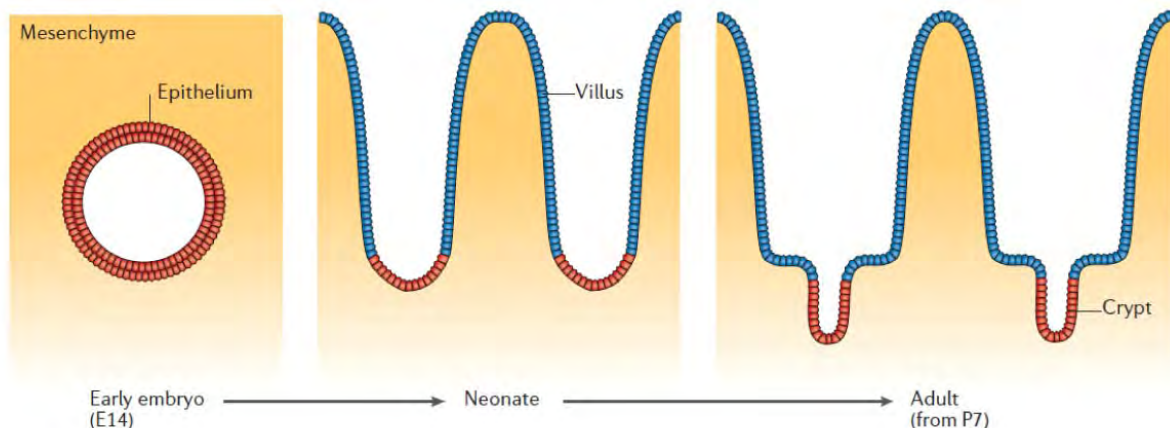


### 3. Mouse intestinal development

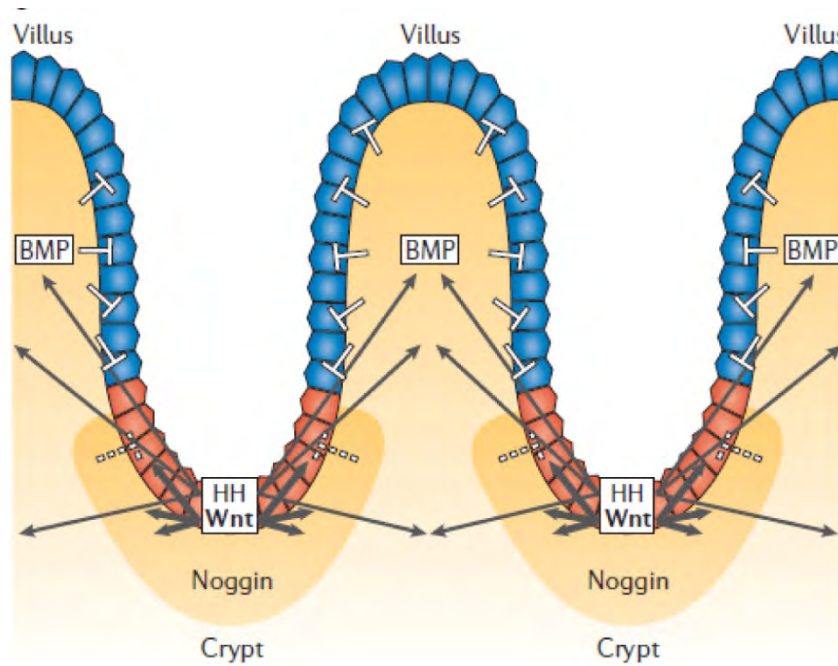
In the adult, the gastrointestinal tract is lined with a monolayer of cells that undergo continuous and rapid renewal from stem cells located in the crypts.

#### 3.1. Morphogenesis and homeostasis maintenance

At E8.5 the intestinal epithelium is already specified. *Cdx2* expression is restricted to the posterior part of the endoderm at the origin of the intestine. Gut morphogenesis takes place in **two steps**. Between E10.5 and E11.5 the intestine is **elongated**. The stratified cuboidal epithelium slowly becomes monostratified (Scoville et al., 2008). At this step, Wnt signalling via *Wnt5a* is necessary to reallocated correctly each cell in the monostratified epithelium (Cervantes et al., 2009). At this developmental stage, beta-catenin is not expressed in the intestinal epithelium, thus Wnt signalling does not go via the canonical pathway (Clevers, 2006). Nuclear beta-catenin is only observed after E16.25 in the epithelium (Kim et al., 2007). Canonical Wnt pathway is thus not necessary for the early intestinal morphogenesis but is required later for the maintenance of intestinal homeostasis. From E12.5, *Cdx2* expression is vital for proper morphogenesis and differentiation into the different intestinal lineages. Abnormal caecum and absent colon is the phenotype of mice deficient for *Cdx2* (Gao et al., 2009). The second step of intestinal morphogenesis is the **formation of crypts and villi** (Figure 19). Intestinal villi form from E15.5, whereas crypts only appear after birth, around P7 (Crosnier et al., 2006). The interaction between epithelium and mesenchyme via HH (Hedgehog) and BMP pathways plays an important role in the formation of crypts and villi (Figure 20).



**Figure 19: Morphogenesis of the mouse small intestine.** The digestive tract originates from the folding of an endodermal sheet, which undergoes remodelling to form a tube that is lined with stratified epithelium. Until around E14, all of the intestinal epithelial cells, which are still undifferentiated, proliferate actively. Villus morphogenesis starts at E15 and involves a reshaping of the mesenchyme (orange) that underlies the intestinal epithelium; the mesenchyme seems to drive the formation of protrusions into the gut lumen. This process is accompanied by marked effects on the epithelial cells (red and blue cells): the epithelium becomes monolayered and epithelial proliferation (red) becomes restricted to the intervillus pockets, and ultimately to crypts. The crypts themselves develop relatively late, beginning around P7, by invagination of the intervillus epithelium. (Crosnier et al., 2006)



**Figure 20: Signalling pathways controlling intestinal morphogenesis.** A model of how the HH, BMP and Wnt signalling pathways combine to organise the pattern of villi and crypts. Epithelial cells in each crypt form a signalling centre, which functions as a source of long-range inhibition through the HH-BMP relay, and short-range auto-activation through Wnt signalling. HH signalling activates the expression of BMP in the mesenchyme. BMP feeds back on the intestinal epithelium to repress Wnt signalling. The expression of the BMP inhibitor noggin in the neighbourhood of the crypts counteracts the effect of BMP so that Wnt activity is maintained in the crypt epithelium. (Crosnier et al., 2006)

*Shh* and *Indian Hedgehog (Ihh)* are expressed in the gut epithelium from E10.5. At E15.5 *Shh* expression is restricted to the intervilli territory whereas *Ihh* is only observed at the top of the villi (Kolterud et al., 2009). Mice deficient for the HH pathway **do not make villus**; instead a pseudostratified epithelium forms (Madison et al., 2005). These mice have **no proliferative cells** and enterocytes maturation is altered which is associated with aberrant Wnt signalling. In the intestine, the HH signalling is paracrine and necessary for the correct development of the stroma and mucosa (Madison et al., 2005).

BMP signalling in the intestine is mostly mediated by **BMP4** which is expressed by the mesenchyme in the intervillus and intercrypt regions (Haramis et al., 2004; He et al., 2004) in response to Shh. Cells responsive to BMP signals express the phosphosmads 1, 5, 8 and are essentially **post-mitotic**. BMP inactivation by the ectopic expression of its inhibitor *Noggin* in the intestinal epithelium leads to a massive disorganisation of the villi with an aberrant localisation of proliferative cells in the villus in 3 months old mice. Similarly to the inactivation of HH pathway, BMP inhibition causes the activation of genes from the Wnt pathway in the villi, like *myelocytomatosis oncogene (c-myc)* and *EPH receptor (EphB3)*. BMP signalling is thus important for the maintenance of the **structure of crypts and villi**.

Growth factors are also important for the formation of crypt and villus. *Platelet derived growth factor alpha (PDGF-A)*, has been identified as a growth factor necessary for a normal differentiation of the stroma (Karlsson et al., 2000). The inactivation of PDGF-A or its receptor causes a decrease in proliferation associated with malformation and reduction in the number of crypts and villi.



Ever since the first villus forms, the intestinal epithelium is divided into two compartments. The proliferative cells concentrate at the base of the villus, also called intervillus region, which will form the crypts after birth. The villus contains post-mitotic cells which migrate toward the top as they differentiate. Each lineage is generated in adequate proportions from **stem cells** located into the crypts. The equilibrium between stem cell self-renewal, progenitor proliferation, differentiation and migration is tightly regulated. Adding to HH and BMP already described, **Wnt** and Notch are also necessary for the maintenance of gut epithelium homeostasis. Inactivation of Wnt signalling leads to a reduced number of villi due to the loss of the proliferative compartment (Korinek et al., 1998; Pinto et al., 2003). Oppositely, the over-expression of the Wnt pathway in the intestine increases proliferation and leads to the formation of aberrant crypts (Andreu et al., 2005; Sansom et al., 2004). Wnt signalling thus plays a capital role in the **maintenance of the proliferative cell pool**. Moreover, when Wnt signalling is altered, crypts are lost and Paneth cells, normally located at the bottom of the crypts are then found in the villi (Andreu et al., 2005; Korinek et al., 1998; Pinto et al., 2003; Sansom et al., 2004). Thus Wnt signalling controls the **correct positioning of the different cell types** in the intestinal epithelium. Finally, Wnt signalling is also necessary for the **specification of the different lineages** of the gut epithelium. Inhibition of Wnt signalling leads to the loss of all secretory lineages and their progenitors (Pinto et al., 2003; van Es et al., 2005a). Wnt activates the transcription of *Sox9* in crypt cells via the beta-catenin/Tcf complex (Blache et al., 2004). *Sox9* inactivation showed that its expression is required for the formation of Paneth cells (Bastide et al., 2007; Mori-Akiyama et al., 2007). Oppositely, Wnt signalling does not seem to be necessary for the specification of the endocrine lineage downstream of *Ngn3*<sup>+</sup> as a normal number of endocrine cells form in mice where beta-catenin is inactivated in *Ngn3* cells (Wang et al., 2007a). To summarise, on top of its importance in the control of proliferation and migration of intestinal epithelial cells, Wnt signalling is vital for the proper differentiation of goblet and Paneth cells.

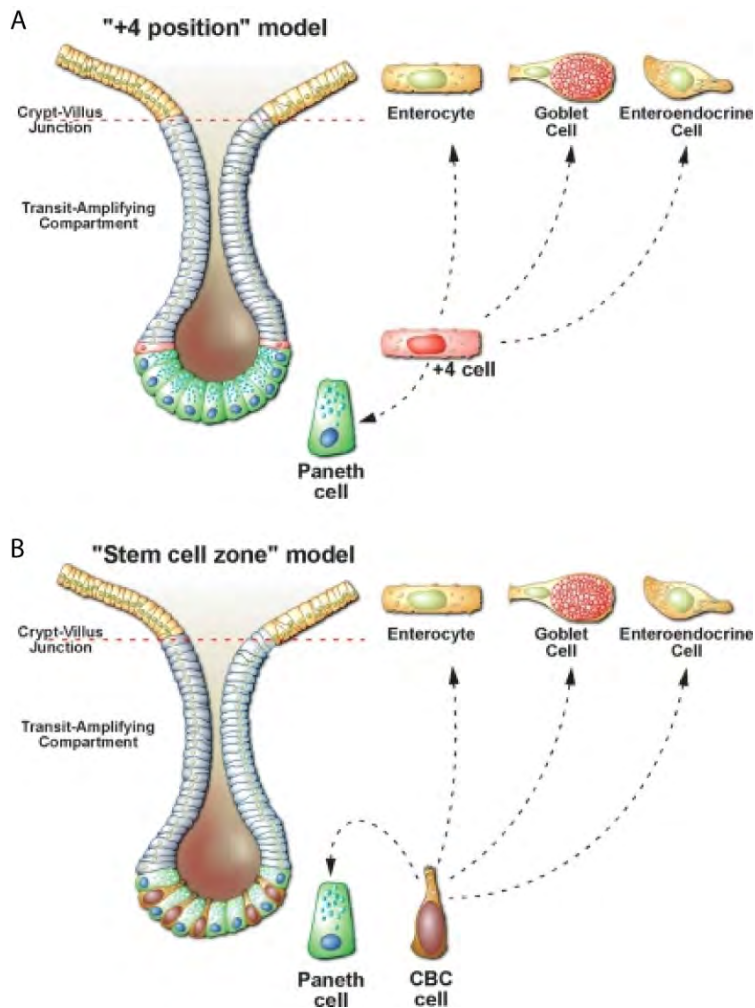
**Notch** signalling controls proliferation and lineage specification similarly to Wnt signalling. The loss of Notch function in the intestinal epithelium causes a **diminution of proliferation** and the replacement of transit amplifying cells by post-mitotic cells without affecting Wnt signalling (van Es et al., 2005b). Notch seems necessary to maintain transit amplifying cells in a proliferative state via *Hes1* by inhibiting the CDK inhibitors *p27<sup>kip1</sup>* and *p57<sup>kip2</sup>* (Riccio et al., 2008). In addition to converting transit amplifying cells to post-mitotic cells, Notch loss-of-function favours differentiation toward the secretory lineages at the expense of enterocytes. In mice inactivated for *Rbpj*, *Notch1* and *Notch2* or *Hes1* the number of goblet and enteroendocrine cells increases whereas the amount of enterocytes decreases (Jensen et al., 2000b; Riccio et al., 2008; van Es et al., 2005b). These results can be explained by the absence of *Atonal homolog 1 (Drosophila)* (*Atoh1* also called *Math1*) and *Ngn3* repression by *Hes1*. These two bHLH transcription factors are expressed respectively by secretory and endocrine progenitors (Jenny et al., 2002; Shroyer et al., 2007; Yang et al., 2001). The opposite effect is observed in intestinal Notch gain-of-function; the number of goblet and enteroendocrine cells decreases at the benefit of enterocytes (Fre et al., 2005; Stanger et al., 2005). However, when Notch is over-expressed, enterocytes localised in the villus exhibit maturation defects. In parallel, Notch signalling controls the maturation of goblet cells as over-expression of NICD in the intestinal epithelium speeds up the maturation of post-mitotic mucus cells (Zecchini et al., 2005). Notch signalling is thus an important component in the control of **proliferation/differentiation** and **specification** of the different intestinal lineages.

## 3.2. Intestinal epithelium differentiation

### 3.2.1. Intestinal stem cells

Intestinal stem cells are defined as cells able to generate all intestinal lineages and to self-renew. Each intestinal crypt contains four to six stem cells; however their exact identity is still somehow controversial. The first putative stem cells have been described to be localised at the bottom of the crypt in-between Paneth cells. These cells have been named *crypt-base columnar* (CBC, Figure 21b). Clonal analysis by mutagenesis showed that these cells were capable of generating several lineages (Cheng and Leblond, 1974). However no clone at the origin of all the lineages has been found. Later, cells located right on top of Paneth cells, position +4 relative to the bottom of the crypt, have been proposed to be the intestinal stem cells (Figure 21a). The particularity of these cells to retain a DNA label on the long term has given them the name of *label-retaining cells* (LRCs) (Potten et al., 2002). More recently, two groups have identified stem cell specific markers which have been validated with the Cre/LoxP system. The use of transgenic mice expressing the inducible Cre recombinase (CreER) downstream of the marker, crossed with the Rosa R26R reporter line allowed to trace cells expressing the marker. Thus, the *Leucin rich repeat containing G protein coupled receptor 5* (**Lgr5**) gene coding for an orphan receptor activated by the beta-catenin/Tcf complex was identified as a stem cell marker (Barker et al., 2007). Cells expressing *Lgr5* correspond to CBCs (Figure 21b) and are present in the **whole intestine**. Once labelled, *Lgr5*<sup>+</sup> cells colonise half of a crypt and villus unit in about 1 week, staining all cell types and maintaining with time. The other group showed that *Bmi1 polycomb ring finger oncogene* (**Bmi1**) a gene of the *polycomb* family has similar properties (Sangiorgi and Capecchi, 2008). Like for *Lgr5*, the progeny of a cell expressing *Bmi1* colonise a crypt/villus unit and the staining is maintained. Surprisingly, cells expressing *Bmi1* do not correspond to cells expressing *Lgr5* but to cells in +4 position (Figure 21a). *Bmi1* cells follow a **decreasing gradient** from the duodenum to the ileum and are absent from the colon. The identification of a second type of stem cells thus restarted the debate on the real position of stem cells in the intestine. However, *Lgr5* cells are expressed in the whole intestine and can generate all intestinal cell type also *in vitro* (Sato et al., 2009). Thus *Lgr5* is a more accurate stem cell marker than *Bmi1*.

Other markers, including **Prominin1** (*CD133*) have been shown to be expressed in stem cells (Montgomery and Shivdasani, 2009) however its expression is also found in early progenitors (Snippert et al., 2009). The identification of other Wnt signalling targets allowed for the discovery of more stem cell markers. One of these genes is the bHLH transcription factor *Achaete-scute complex homolog 2* (*Drosophila*) (*Ascl2*). **Ascl2** is expressed in CBCs and controls the fate of stem cells (van der Flier et al., 2009b). Mice with gain of *Ascl2* function exhibit intestinal hyperplasia due to an increase of the proliferative compartment. On the other hand, mice deficient for *Ascl2* have no stem cells. Another Wnt target **Olfactomedin4** (*OLFM4*) is expressed in intestinal stem cells (van der Flier et al., 2009a). To date, the transcriptional program giving stem cell properties to these cells is not known. However, the identification of stem cell specific transcription factors like *Ascl2* and their targets might elucidate the mechanisms controlling self-renewal and differentiation of stem cells.



**Figure 21: Controversial position of stem cells in the crypt.** The exact identity of the intestinal stem cells has proven controversial over the last 30 years, with two opposing models dominating the literature. **(A)** The +4 position model proposed by Potten and colleagues assumed that the crypt base is exclusively populated by terminally differentiated Paneth cells and the stem cells must therefore be located just above the Paneth cells at the +4 position. These cells are able to retain a DNA staining and were thus called *label-retaining cells* (Potten et al., 2002). Recently, *Bmi1* has been identified as a marker of these cells (Sangiorgi and Capecchi, 2008). **(B)** This model, proposed by Cheng and Leblond in the seventies (Cheng and Leblond, 1974) states that small, undifferentiated, cycling cells intermingled with the Paneth cells and called *crypt base columnar cells* are the true stem cells. Recently these cells have been shown to express *Lgr5*, *Olfactomedin4* and *Ascl2* (Barker et al., 2007; van der Flier et al., 2009a; van der Flier et al., 2009b). (From Barker et al., 2008)

### 3.2.2. Specification of secretory and endocrine progenitors

Intestinal stem cells are at the origin of all different intestinal lineages. To do so, stem cells will give rise to progenitors, some shared between different lineages (Figure 18). A total absence of secretory cells is observed after deletion of the bHLH transcription factor ***Atoh1*** (Shroyer et al., 2007; VanDussen and Samuelson, 2010; Yang et al., 2001). Furthermore, it has been shown through lineage tracing studies that all **secretory lineages** in the intestine are derived from *Atoh1*-expressing precursor cells (Yang et al., 2001), suggesting a common progenitor for goblet, Paneth and enteroendocrine cells. However, clonal analysis by mutagenesis performed by Bjerknes and Cheng has failed to prove the evidence of a common secretory progenitor (Bjerknes and Cheng, 1999). Recently, a study using an intestine-specific deletion of *Atoh1* in adult mice revealed its role in the balance between enterocytes and enteroendocrine cells, as intestinal precursors preferentially differentiate into enterocytes at the expense of secretory lineages in *Atoh1*-deficient intestine (Shroyer et al., 2007).

Analysis of the Wnt-responsive transcription factor ***Spdef*** showed that this transcription factor was only required for the maturation of **goblet and Paneth cells** without affecting enteroendocrine cells (Gregorieff et al., 2009; Noah et al., 2010). However *Spdef* cannot be considered as a Paneth/goblet progenitor as these cells are not completely lost in *Spdef*-deficient mice.

The link between secretory and endocrine lineages has been reinforced with results obtained with the inactivation of *Growth factor independent 1 (Gfi1)* (Shroyer et al., 2005). *Gfi1*-deficient mice show an increase of **enteroendocrine** cells at the expense of mucus and Paneth cells suggesting a redirection. Expression of lineage-specific transcription factors such as *Sox9* (Mori-Akiyama et al., 2007) for Paneth cells, *Klf4* (Katz et al., 2002) for goblet cells and *Ngn3* (Jenny et al., 2002; Mellitzer et al., 2010) for enteroendocrine cells is required for the final differentiation of these secretory cells.

***Ngn3*** is expressed essentially in crypts and its inactivation leads to the agenesis of the enteroendocrine lineage without significantly affecting the others (Jenny et al., 2002; Mellitzer et al., 2010). Finally, *Ngn3* gain-of-function in the intestinal epithelium favours endocrine differentiation at the expense of goblet cells (Lopez-Diaz et al., 2007). *Ngn3* is thus a good marker of intestinal endocrine progenitors (similarly to the pancreas). Somehow in contradiction with this finding, lineage tracing experiments showed that progenitors expressing *Ngn3* also can give rise to goblet and Paneth cells (Schonhoff et al., 2004). However, adult mice with intestinal-specific deletion of *Ngn3* showed an enlarged proliferative crypt compartment, accelerated cell turnover but no change to the number of Paneth and goblet cells (Mellitzer et al., 2010).

All together these data show that the specification of each intestinal lineage is highly complex. Even if goblet and Paneth cells seem to derive from a common progenitor downstream of *Atoh1*, it has not yet been identified. However, the expression of *Sox9* is required only for Paneth cell differentiation (Bastide et al., 2007; Mori-Akiyama et al., 2007), whereas *Kruppel-like factor 4 (Klf4)*, *FoxA1* and *FoxA2* are implicated in goblet cells differentiation (Katz et al., 2002; Ye and Kaestner, 2009). Enteroendocrine cells require the expression of *Ngn3* but enterocytes seem to differentiate “by default” from *Atoh1* progenitors (Shroyer et al., 2007; Yang et al., 2001).

### 3.2.3. Differentiation of enteroendocrine subtypes

Intestinal endocrine differentiation follows a program very similar to what has been shown in the pancreas (Figure 18). Numerous genes previously shown to be required for islet cell differentiation also have a role in the differentiation of enteroendocrine cells (Li et al., 2011). Like in the pancreas, differentiation of intestinal endocrine cells requires the proendocrine gene ***Ngn3*** both during development (Jenny et al., 2002) and in the adult (Mellitzer et al., 2010). *Ngn3* induces the endocrine transcription program in all enteroendocrine cells. However the transcription factors downstream of *Ngn3* are differentially required depending on the endocrine subtype (Table 7).

***NeuroD1*** is necessary for CCK (Cholecystokinin) and S (Secretin) cells (Naya et al., 1997). ***Insm1*** is also expressed in the intestine (Mellitzer et al., 2006) and its inactivation leads to the loss of N (Neurotensin) cells and cells secreting substance P, associated with a reduction of enterochromaffin (EC) cells secreting serotonin, CCK and PYY (PP-like peptide tyrosine tyrosine) cells (Gierl et al., 2006).

The inactivation of ***Nkx2.2*** leads to the reduction of many enteroendocrine cells including, CCK, EC, GIP (Gastric Inhibitory Polypeptide), G (Gastrin), L (Glucagon-Like Peptide, GLP1), D (Somatostatin) and N cells (Desai et al., 2008). Mice deficient for ***Pax6*** develop less GIP cells in the duodenum (Larsson et al., 1998). ***Pdx1*** is necessary for the differentiation of some subtypes but only in the duodenum. Mice deficient for *Pdx1* have less EC, CCK and S cells in the duodenum (Offield et al., 1996). Recently, the Kaestner lab showed that ***FoxA1*** and ***FoxA2*** were required for the differentiation of L and D cells (Ye and Kaestner, 2009).

The homeodomain-containing transcription factors **Arx** and **Pax4** are also necessary to control enteroendocrine subtype specification in the intestine (Beucher et al., 2012a; Du et al., 2012; Larsson et al., 1998). Very recently, Beucher and colleagues showed that differentiation of Glp1, Gip, Cck, secretin, gastrin and ghrelin cells was severely affected in *Arx*-deficient mice. They reported a redirection of *Arx*-deficient progenitor toward the D-cell lineage with a concomitant increase of somatostatin cells. In *Pax4* mutants, the differentiation of serotonin, somatostatin, Gip and gastrin cells is impaired whereas *Arx* is up-regulated thus increasing the number of Glp1 cells (Beucher et al., 2012a). This shows that differently from what is happening in the pancreas where *Arx* and *Pax4* have an antagonistic function, in the intestine only the specification of a subset of enteroendocrine subtypes (L and D cells) relies on both *Arx* and *Pax4*, whereas others depend only on *Arx* or *Pax4*.

The transcription factors implicated in endocrine cell differentiation are differently required depending on the subtype and the location in the intestine. It however remains largely unknown whether and to what extent these transcription factors control hormone expression in the adult. Answering this question will require the use of conditional mutants and inducible Cre lines. Moreover, the description of the different enteroendocrine subtypes is still controversial. Last month, Fiona Gribble's group showed by transcriptional profiling and flow cytometry that there is a strong overlap of endocrine hormone expression between L, K and I cells, suggesting that they may rather comprise a **single cell type with specific hormonal expression** which could rely on location along the intestine and exposure to nutrients (Habib et al., 2012).

Knock-outs	Somatostatin	Ghrelin	5-HT	GLP1	PYY	GIP	Gastrin	Cholecystokinin	Secretin	Neurotensin
<b>Ngn3</b>	-	-	-	-	-	-	-	-	-	-
<b>NeuroD1</b>	NA	?	NA	NA	NA	NA	?	-	-	?
<b>Insm1</b>	?	?	↓	?	↓	?	?	↓	NA	-
<b>Nkx2.2</b>	↓	↑	↓	↓	NA	↓	↓	↓	NA	↓
<b>Pdx1</b>	?	?	↓*	?	?	?	↓*	↓*	↓*	?
<b>Pax4</b>	↓	↑	↓	↑	↑°	↓	↓ <sup>+</sup>	NA	↓ <sup>+</sup>	↓ <sup>+</sup>
<b>Arx</b>	↑	↑ <sup>+</sup>	NA	↓	↓	↓	↓ <sup>+</sup>	↓	↓	↓
<b>Pax6</b>	?	?	NA	NA	↓	?	?	NA	?	?
<b>FoxA1/2</b>	↓	?	NA	-	↓	NA	NA	NA	NA	?

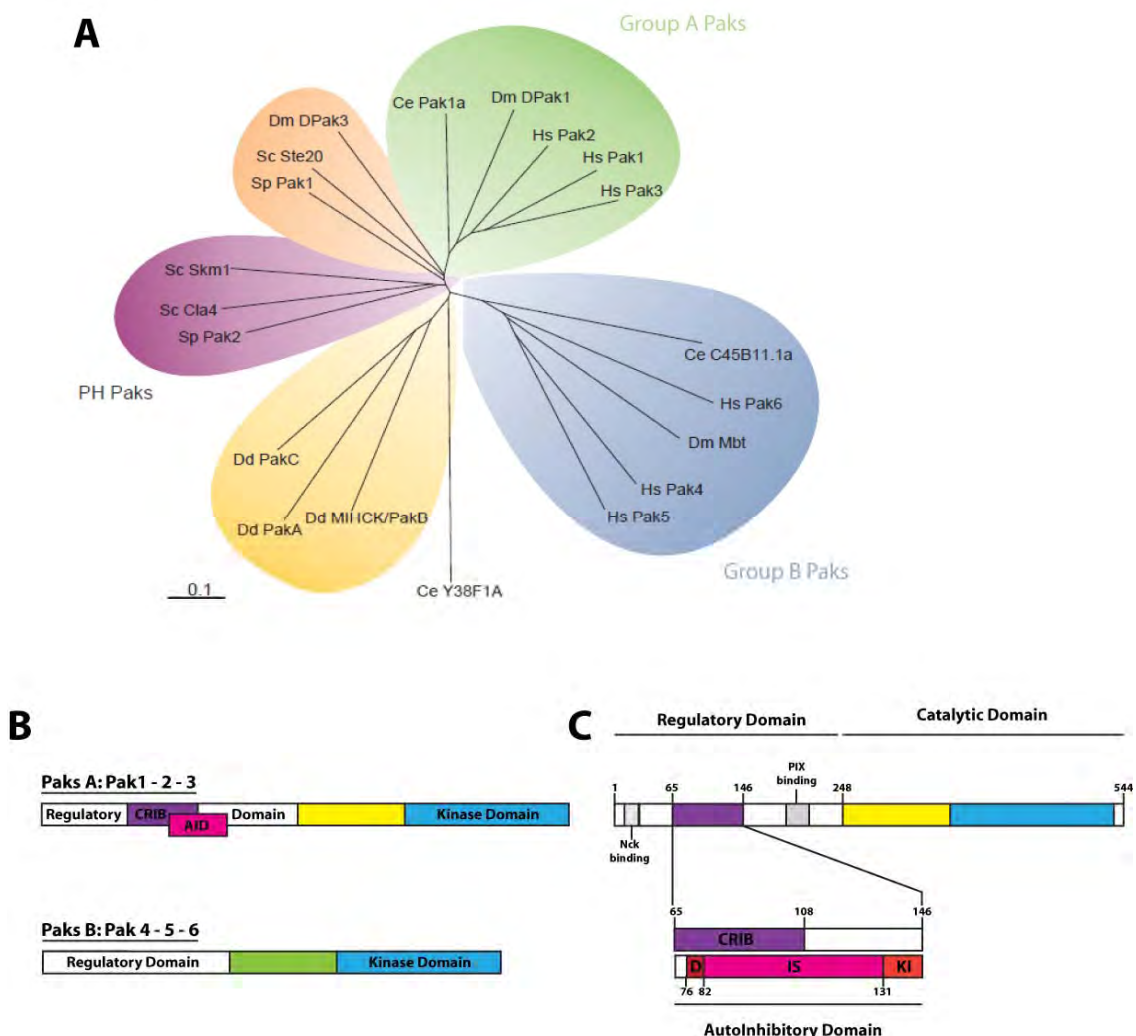
**Table 7: Enteroendocrine phenotype of mice lacking some transcription factors.** (-) loss, NA (not affected), ? (not determined), ↓ (decreased), ↑ (increased), (\*) only in duodenum, normal elsewhere in the intestine, (†) not affected in colon, (°) only in colon, normal elsewhere in the intestine. (Table filled with results from Offield et al., 1996, Naya et al., 1997, Larsson et al., 1998, Jenny et al., 2002, Gierl et al., 2006, Desai et al., 2008, Ye and Kaestner, 2009 and Beucher et al., 2012a)



## D. The PAK gene family

### 1. The PAK kinases belong to a large kinase family called STE20

The serine/threonine kinases PAK belong to the **kinase** family *STE20* which is composed of about fifty kinases in humans (Dan et al., 2001). In *C. Elegans*, *D. Melanogaster* and mammals, PAKs are divided into **2 groups** with distinct structure and function (Figure 22A). In mammals, the group A is composed of *PAK 1, 2* and *3* and the group B of *PAK 4, 5* and *6*. PAKs are **effectors of the Rho GTPases *Rac1* and *Cdc42***. All the eukaryotes, except plants, have one or more PAK genes, which suggest the functional relevance of these kinases. All PAKsA contain a N-terminal regulatory region, which includes the binding domain for the GTPases, so called CRIB (*Cdc42* and *Rac1* interacting binding) domain and a very well conserved C-terminal kinase domain (Figure 22B).



**Figure 22 : Phylogenetic tree and structure of the PAK family. (A)** The PAKs from *C. Elegans*, *D. Melanogaster* and mammals cluster in group A (green) and B (blue). Ce: *Caenorhabditis Elegans*; Dd: *Dictyostelium Discoideum*; Hs: *Homo Sapiens*; Mm: *Mus Musculus*; Sc: *Saccharomyces Cerveisiae*; Sp: *Schizosaccharomyces Pombe*. (From Hofmann et al, 2004) **(B)** The members of group A and B have different structures. The regulatory region contains a binding domain for Rac1 and Cdc42 (CRIB) and an autoinhibitory domain (AID). **(C)** Detailed structure of Pak3. The regulatory region contains the CRIB and AI domains characteristic from the group A as well as many proline-rich regions (grey rectangles) necessary for interaction with PIX and Nck. The AID is subdivided in 3 regions: D (dimerisation interface), IS (inhibitory switch) and KI (kinase inhibitory) which allow dimerisation and inhibition of the catalytic domain. Numbers correspond to the amino acids in Pak3 sequence. (Adapted from Lei et al, 2000)

## 2. PAKs from group A: PAK 1, 2, 3

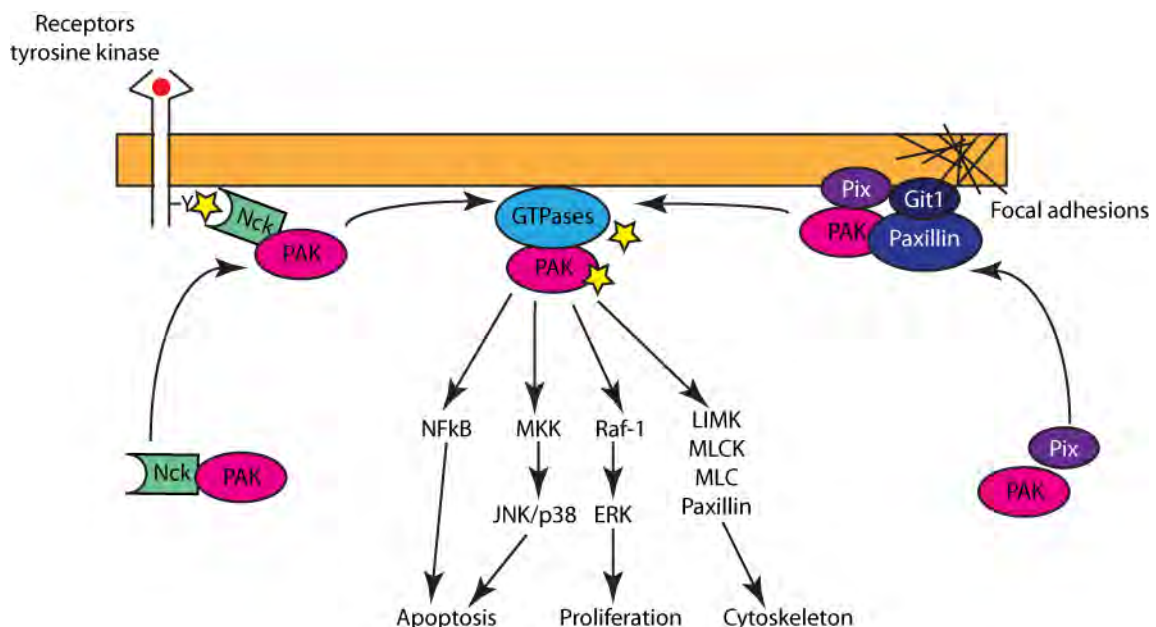
### 2.1. Structure

The 3 kinases from the group A have a very well conserved N-terminal region containing an auto-inhibitory domain (AID) which is included into the **CRIB domain** (Figure 22B) and many proline-rich regions necessary for the interaction with adaptor molecules Nck/PIX (non-catalytic region of tyrosine kinase adaptor protein/Pak interacting exchange factor) which are the exchange factors for *Rac1* and *Cdc42* (Daniels and Bokoch, 1999; Lei et al., 2000).

### 2.2. Regulation of PAKs

#### 2.2.1. Recruitment to the membrane

The general mode of activation for the group A PAKs is the following: PAK is **recruited to the membrane** where it is activated by the Rho GTPase *Cdc42* and *Rac1*. The interaction with the small GTPase allows the removal of the inhibition on the catalytic domain by the AID. This allows the phosphorylation on serines and threonines and subsequent phosphorylation of its substrates. Briefly, 2 main paths allow the recruitment of PAKs to the membrane. (1) The first pathway relies on the activation of the **tyrosine kinase receptors** localised into the membrane. Phosphorylation of these receptors creates site of interaction for the adaptor Nck. In the cytoplasm, Nck interacts via its SH3 domain on the first proline-rich domain of PAK. The Nck/PAK complex is then recruited to the membrane (Bagrodia et al., 1995). (2) A second way to recruit PAK to the membrane relies on the **exchange factor PIX** (also called Rho guanine nucleotide exchange factor (GEF7)). PIX brings PAK to the membrane at the level of focal adhesions (Bagrodia et al., 1998) (Figure 23).



**Figure 23: The two pathways to recruit PAK to the membrane.** After activation of the tyrosine kinase receptor, PAK is recruited to the membrane via the adaptor Nck. PAK can then be activated by the GTPases which are membrane-anchored. A second way to recruit PAK to the membrane relies on the exchange factor PIX which brings PAK at the focal adhesions.

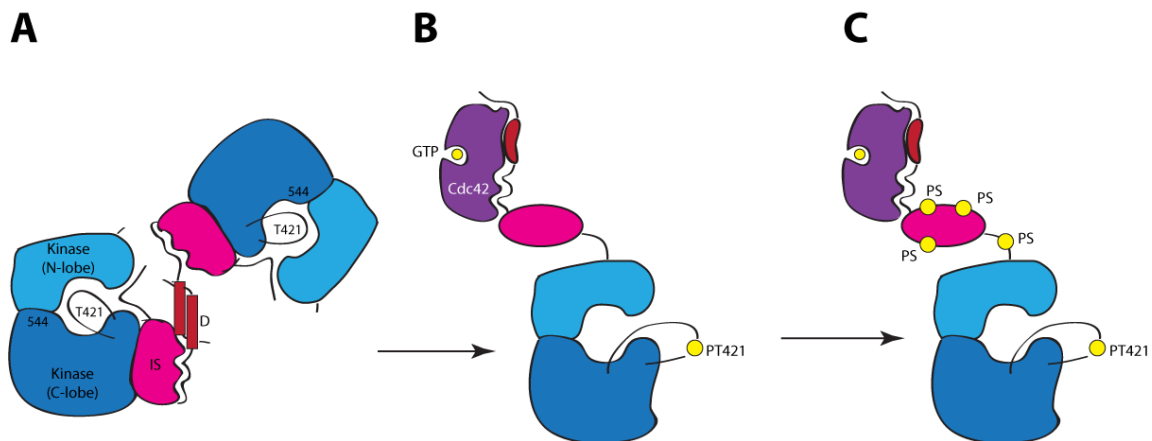


### 2.2.2. The CRIB domain allows the binding of PAKs to Rho GTPases

The CRIB domain is necessary for the **interaction** of the kinase with the active GTP-bound form of the **Rho GTPases**. This domain is composed of about forty amino acids (Lei et al., 2000). The minimal motif for the interaction with GTPases is called CRIB and contains sixteen amino acids (Burbelo et al., 1995) and is found in almost all the effectors of *Rac* and *Cdc42*. The whole CRIB domain contains other important residues including a lysine-rich region before the CRIB motif (Knaus et al., 1998).

### 2.2.3. The autoinhibitory domain maintains the kinase in an inactive state

In 1998, Zhao et al. showed that a sixty-seven amino acids polypeptide from the N-terminal region of PAK inhibits its activation from *Cdc42* (Zhao et al., 1998). Since then others have shown that mutations in the **AID** prevents the binding of PAK with its catalytic region (Tu and Wigler, 1999). The crystal structure of Pak1 showed that in the basal situation, the PAKs are dimeric, the N-terminal region interacting with the catalytic region (Lei et al., 2000) (Figure 24). The binding of the inhibitory **switch domain** (IS) to the C-lobe of the kinase permits for the **kinase inhibitory** (KI) segment to come in the catalytic cleft to prevent its activation. This dimerisation requires different regions to interact, in particular, the beta1-sheet of the **dimerisation** (D) domain and the IS domain interacting with the catalytic region. The binding of *Cdc42* to the CRIB domain leads to a conformational change of the IS domain, removing the inhibition on the KI and dissociating the dimer and subsequently activates the kinase (Figure 24). Further biochemical experiences, showed that the inhibition of the PAK1 homodimers is achieved by a trans-regulation: the N-terminal part of a PAK1 monomer inhibiting the catalytic domain of the other (Parrini et al., 2002).



**Figure 24: Activation mechanisms of the group A PAKs.** (A) In the auto-inhibited conformation, PAK is an asymmetric dimer. The inhibitory switch (IS) domain associates tightly with the C-lobe of the kinase domain. The kinase inhibitory (KI) segment occupies the cleft of the kinase domain and stabilises a disabled catalytic site. (B) Binding of the GTP-bound form of *Cdc42* (or *Rac1*) with the CRIB domain disrupts the dimer and unfolds the IS domain. The conformational change withdraws the KI segment from the catalytic cleft and releases the activation loop. Phosphorylation of Thr421 (PT421, on PAK3) will activate the enzyme. (C) Once the Thr421 has been phosphorylated, PAK can auto-phosphorylate on several serines (phosphoserine: PS). These modifications prevent the kinase from reverting to an inactive conformation. (Lei et al., 2000)

#### 2.2.4. Phosphorylation allows PAK activation and maintenance in its active form

During PAK activation, a number of sites are **phosphorylated**, among them six serine residues in the amino-terminal region and the threonine 421 in the catalytic region (Chong et al., 2001). The phosphorylation of T421, located in the **activation loop** plays a fundamental role. T421 can be either auto-phosphorylated or by an external kinase like PDK1. The main role of the phosphorylation of the serine residues is to prevent the folding of the KI domain in its closed configuration. To add more complexity, some other sites are targets for other regulatory kinases, for example, the phosphorylation of PAK1 by Cdk5 is implicated in PAK inhibition (Nikolic et al., 1998).

#### 2.2.5. Binding of other factors can modulate the activity of PAKs

The PAK kinases are phosphoproteins which activity is finely tuned by many factors. A membrane targeting sequence contributes to the activation of PAKs by a GTPases-independent mechanism (Bokoch et al., 1998). More precisely, some lipids into the membrane, mainly **sphingosine derivatives**, are capable of activating PAK by blocking the binding of the AID and inducing the same kind of phosphorylation than the canonical activation by GTPases. The **Exchange Factors** alpha- and beta-PIX are also modulators of PAK activity. Alpha-PIX interacts directly with PAK and activates it by mechanisms dependent or independent of its Guanine Exchange Factor (GEF) activity (Daniels et al., 1999). Beta-PIX can inhibit PAK activity thanks to the presence of a region of 18 amino acids called T1, this region could inhibit the GEF activity of PIX and block the access to other regulators to the active site of PAK (Feng et al., 2002). Moreover, some PAK substrates are capable to activate it by direct binding, for example **filamin A** with the CRIB domain of PAK (Vadlamudi et al., 2002) or **DSCAM** (Down Syndrome Cell Adhesion Molecule) with the kinase domain (Li and Guan, 2004).

### 3. PAKs from group B: PAK 4, 5, 6

The structure of the group B PAK is very different than the PAKs of the group A (**Erreur ! Source du renvoi introuvable.**B) as they do not have CRIB or AID domains. The N-terminal region shows less than 40% identity with group A but about 60% among the group B (Jaffer and Chernoff, 2002). These kinases also have a binding domain for GTPases; however the binding of GTPases does not modify the kinase activity. Even though the regulatory mechanisms are not yet known, members of the group B are mainly implicated in **filopodia formation** (Hofmann et al., 2004).

### 4. Biological functions of PAK kinases

Because of the numerous interacting proteins and substrates for PAK, these kinases play important roles in many different cellular processes. For an extensive review of PAK signalling in neurophysiology please refer to the review from Kreis and Barnier (Kreis and Barnier, 2009) as I will here only briefly describe the main results and important functions.

## 4.1. PAKs regulate cytoskeleton dynamic

Actin filaments, microtubules and intermediary filaments are the 3 major components of the cytoskeleton. The **cytoskeleton** is a “skeleton” made out of proteins that plays important roles in intracellular transports (guiding vesicles and organelles) and cellular division. Actin is divided in two different types of structures. (1) Bundles of parallel actin filaments present in stress fibres and filopodia. Filopodia are fine protrusions which sense the extracellular environment seeking for attractant or repulsive signals. The stress fibres are composed of actin-myosin fibres which link focal points together to allow the communication between cytoskeleton and extracellular environment. Focal adhesions are proteic complexes which link the extracellular matrix to the actin cytoskeleton via the transmembrane receptors of the integrin family. (2) Actin networks from the cellular periphery are webbed together to form large protrusions called lamellipodia.

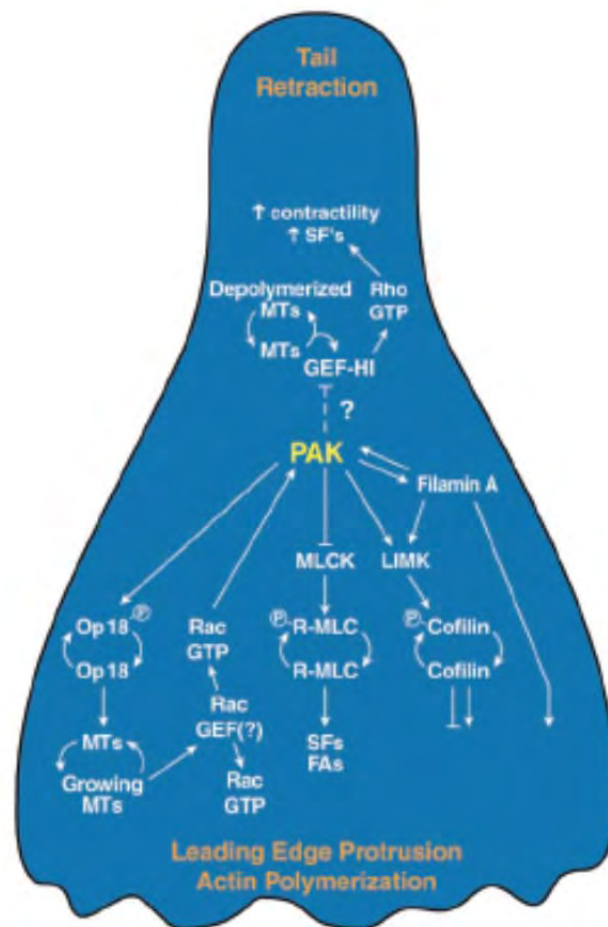
The PAKs are concentrated in **actin-rich regions**. PAKs over-expression induces membrane extensions, mostly **lamellipodia and filopodia** as well as dissociation of stress fibres and focal adhesions (Nayal et al., 2006; Sells et al., 1997). These effects can be dependent or independent from the kinase activity. PAKs act on the cytoskeleton by phosphorylation of different signal proteins, in fact, these kinases can: (1) control **actin polymerisation** by regulation of LIM Kinases 1 and 2 which phosphorylate and inhibit cofilin, a protein necessary for actin depolymerisation (Asano et al., 2009; Edwards et al., 1999). (2) **regulate myosin II** to increase cellular contraction or its regulatory kinase MLCK (myosin light chain kinase) to inhibit contraction (Bokoch, 2003). (3) **act on microtubules** by regulation of regulatory proteins (Daub et al., 2001), PAK1 also phosphorylates tubulin cofactor B to generate new microtubules (Vadlamudi et al., 2005). (4) regulate the structure of **focal adhesions** by interaction with paxillin (Nayal et al., 2006). (5) regulate **membrane folding** by phosphorylation of filamin A, a protein linking the orthogonal actin filaments and integrins (Vadlamudi et al., 2002).

## 4.2. PAKs in neuronal differentiation, guidance and synaptic plasticity

From their role in cytoskeleton regulation, the PAK kinases are also fundamental for shape and **cellular movements**, in particular during cell **migration**, neuritic **growth** and axonal **guidance**. Many studies also suggest that PAK kinases have fundamental roles in **synaptic plasticity** and morphogenesis of dendritic spines.

### 4.2.1. Cell migration

**Cell migration** is essential during embryonic development and its deregulation is a characteristic of metastatic cells. Fibroblast migration requires lamellipodia extension, binding of the cytoskeleton to the extracellular matrix and a force to propel the cell ahead. **Actin polymerisation** is responsible for the formation and extension of lamellipodia, myosin brings the necessary force to induce cellular movement and microtubules form a network which allows the organelles and proteins to move inside the cell (Etienne-Manneville, 2004). The expression of an active or inactive form of PAK modifies cellular mobility. Active PAK localises at the migratory front to inhibit contractility and facilitate stress fibres and focal **adhesion dissociations** to increase the movement of the cell (Bokoch, 2003; Sells et al., 2000). The absence of active PAK at the rear of the cell allows the retraction of filopodia and maintains the directionality (Figure 25).

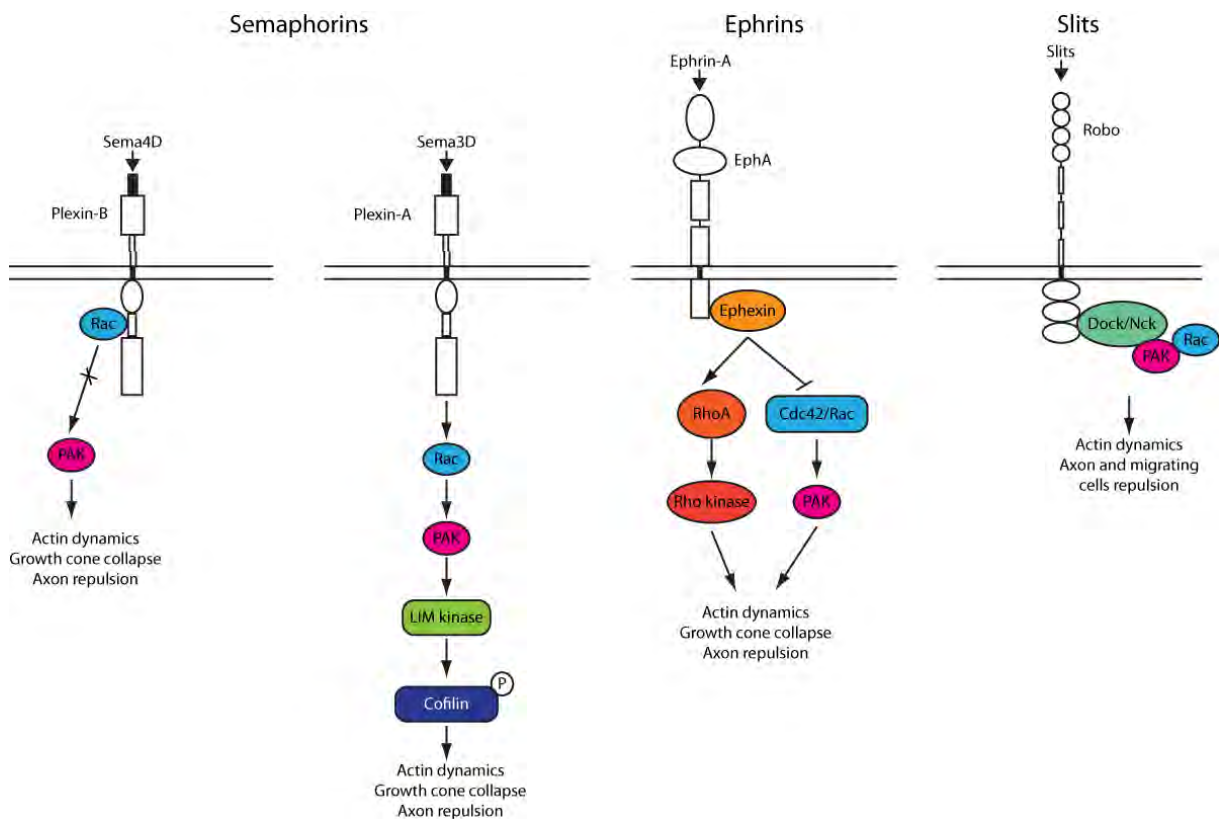


**Figure 25: Model for the role of PAK in cell migration.** FA: focal adhesions, MT: microtubules, SF: stress fibres. At the leading edge protrusion contraction is inhibited, SF and FA are dissolved and MT assemble to reinforce cell polarity. At the tail retraction, MT are dissolved and contractility is reinforced in association with activation of Rho GTPase. The exchange factor Rho-specific, GEF-H1 is phosphorylated by PAK and could be the link between PAK and contractility. (Bokoch, 2003)

During embryonic development, the vertebrate brain undergoes important changes in length and shape which necessitate cellular movements of great amplitude. Interneuron cell migration in the cortex is a complex process which relies on numerous proteins and signalling cascades regulated mainly by transcription factors. It has been shown that the transcription factors *Dlx1* (*distal-less homeobox*) and *Dlx2* play an important role for the migration of GABAergic interneurons in the cortex by repressing some genes important for **cytoskeleton regulation** like Pak3 (Cobos et al., 2007). The authors showed that *Pak3* expression is low or absent in migrating interneurons, but very high in interneurons which reached the cortex. Moreover, **Pak3 over-expression** is enough to induce **migration arrest**. More recently, *Pak3* was shown to be over-expressed in neuroendocrine tumours and participate in cell migration (Liu et al., 2010).

#### 4.2.2. Axonal guidance

Axonal growth is guided and oriented by small molecules present in the environment. The growing extremity of an axon (growth cone) is made of filopodia and lamellipodia which explore the surrounding environment in response to guidance molecules (Luo, 2002). There are four different types of **guidance molecules**: semaphorins, ephrins, slits and netrins. Activated *RhoA* reduces growth cone motility and induces neurite retraction whereas *Rac1* and *Cdc42* stimulate the formation of lamellipodia and filopodia thus inducing neurite formation (Govek et al., 2005). Studies in *Drosophila* indicate that *Pak* and its adaptor *Dock* (*Dreadlocks*, *Nck* homologue) are central for axonal guidance as mutations in *Pak* or *Dock* induce a **disorganisation of axonal projections** on photoreceptors (Hing et al., 1999). During axonal guidance PAK kinases act at different levels (Figure 26). They compete with *Rac1* for binding on *Plexin-B1*, a semaphorin receptor as when *Rac* is bound to *Plexin-B1* it cannot activate PAK (Vikis et al., 2002). It has also been shown that **growth cone collapse**, a vital event for axonal guidance induced by semaphorins, depends on the phosphorylation of cofilin which is achieved by LIM kinase, itself phosphorylated by PAK (Aizawa et al., 2001). The PAKs also take part in axonal guidance driven by ephrins. PAK is activated by *Rac1* via *ephexin* a GEF bound constitutively to some ephrin receptors like *EphA*. This exchange factor is implicated in growth cone collapse during axonal guidance. It has been shown that stimulation of *EphA* by *ephrins* modulates the activity of *ephexin* to activate *RhoA* and inhibit *Cdc42/Rac1* (Shamah et al., 2001). Finally, PAK is also important in the **signalling of the guidance molecule *Slit***. In *Drosophila*, *Slit* stimulates the receptor *Robo* (*Roundabout*) inducing the recruitment of *Dock* and *Pak* thus increasing *Rac* activity to regulate the repulsive effects of axonal guidance in the central nervous system (Fan et al., 2003).

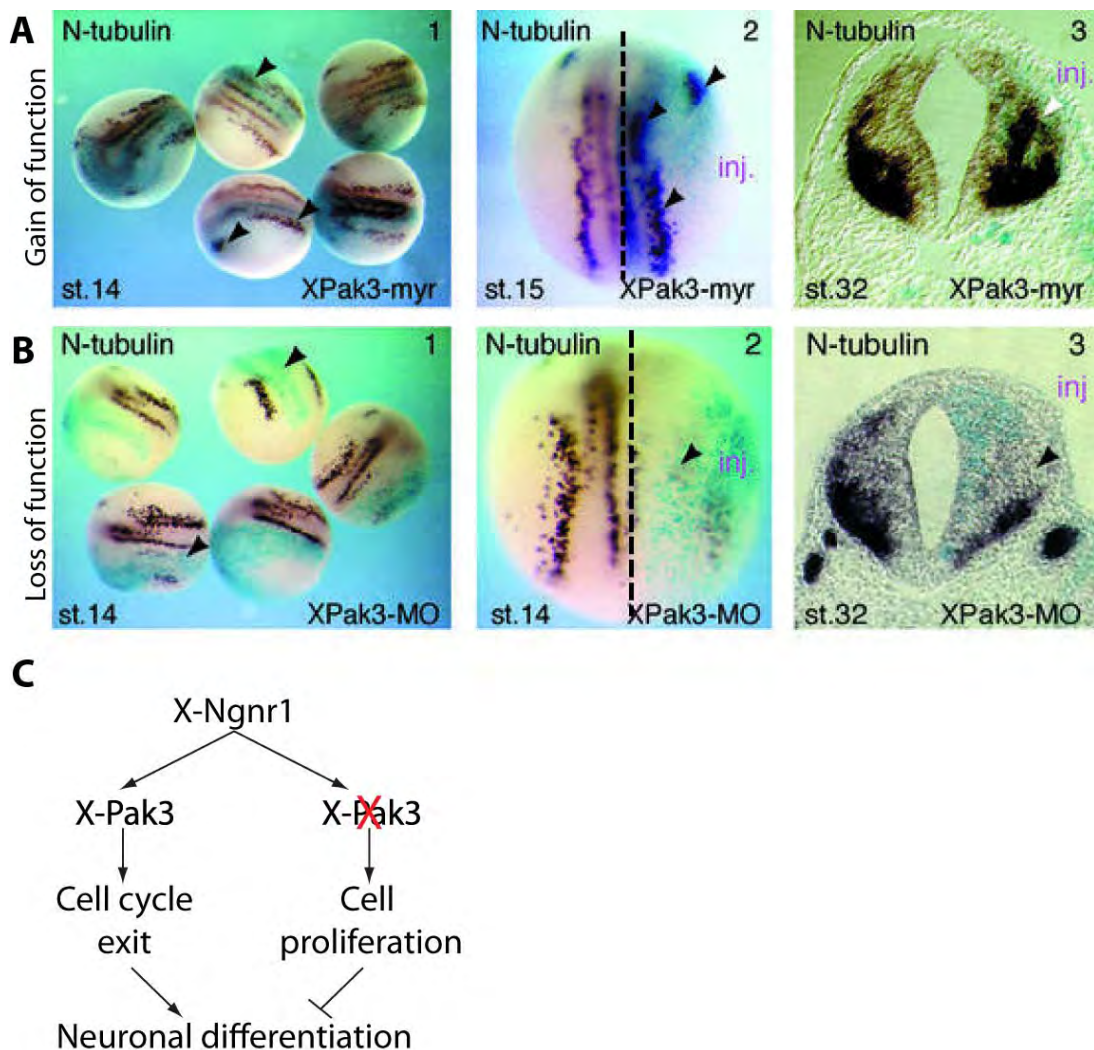


**Figure 26: PAK signalling in axonal guidance driven by semaphorins, ephrins and slits.** Guidance cues are read by growth cone receptors and downstream signal transduction pathways converge onto Rho GTPases to elicit changes in cytoskeletal organization that determine which way the growth cone will turn. Sema: Semaphorin, PAK: p21-activated kinases, LIM kinases: Lin-11, *Isl-1*, and *Mec-3 kinase*, *Robo*: *Roundabout*, *Dock*: *Dreadlocks*. (Adapted from Govek et al, 2005)



#### 4.2.3. Neuronal differentiation

PAKs are implicated in **neuronal specification and differentiation** via the Notch pathway. After the cleavage of the transmembrane receptor Notch, the intracellular part of the protein is translocated into the nucleus where it is responsible for the activation of target genes by removing co-repressors like the protein SHARP. *Pak1* is responsible for Sharp phosphorylation, thus increasing its repressor effect (Vadlamudi et al., 2005). Moreover, *Pak3* is implicated in **cell cycle exit** during neuronal differentiation in the *Xenopus* (Souopgui et al., 2002). More specifically, *Pak3* down-regulation correlates with hyperproliferation and inhibition of neuronal differentiation. In contrary, a constitutively active form of *Pak3* induces cell cycle arrest and premature neuronal differentiation (Figure 27). In addition, Notch activity inhibits *x-Pak3* expression during embryogenesis via inhibition of X-Ngnr1 (Souopgui et al., 2002).



**Figure 27: X-Pak3 is a regulator of neuronal differentiation in *Xenopus*.** (A-B) Modulation of XPak3 activity influences neuronal differentiation. *Xenopus* embryos were injected into one blastomere at the two-cell stage with (A) a constitutively active form of *Pak3* RNA, XPak3-myr, (B) a morpholino against *Pak3*, XPak3-MO all with LacZ RNA as tracer. Neurula and tailbud stages embryo were fixed, stained with X-gal (light blue) and analysed for neuronal differentiation using N-tubulin expression. (A1-2) XPak3-myr injection results in increased N-tubulin expression as indicated by arrowheads. (A3) In the neural tube, XPak3-myr injections expand/shift the expression domain of N-tubulin. (B1-3) XPak3-MO injections strongly inhibit N-tubulin expression. (Souopgui et al., 2005) (C) Scheme representing XPak3 function during primary neurogenesis in *Xenopus*.

#### 4.2.1. Many signalling pathways implicating PAKs regulate spine morphogenesis

**Upstream** of Pak, the **exchange factor Pix** plays an important role in the **formation of spines**. Loss of *alpha-Pix* by siRNA leads to an alteration of the morphology of the dendritic spines consisting of a decrease in mushroom type spines and increase of filopodial type spines. It has been shown that this can be rescued by the expression of a constitutively active form of *Pak3* (Node-Langlois et al., 2006). More recently, *Pak3* was shown to regulate spine morphogenesis via *Cdc42*. Altogether these data suggest that *alpha-Pix* and *Pak3* are implicated in the same signalling cascade regulating spine morphogenesis. Strengthening this idea, it has been shown that spine morphology defects associated with *Pak3* loss of expression by siRNA is not restored by expression of alpha-Pix.

**Downstream** of Pak is the LIM kinase-cofilin pathway which regulates actin polymerisation. Mice deficient for **LIM kinase** have anomalies in dendritic spine morphology without modifying their density (Meng et al., 2002).

Another regulatory pathway for the cytoskeleton dynamic by PAK relies on the phosphorylation of the light chain of myosin II (myosin II regulatory light chain, MLC), which increases the ATP activity of myosin and the **cytoplasmic contraction**. Thus, the signalling cascade Git1/Pix/Rac/Pak regulates morphogenesis and synapse formation via MLC (Zhang et al., 2005b).

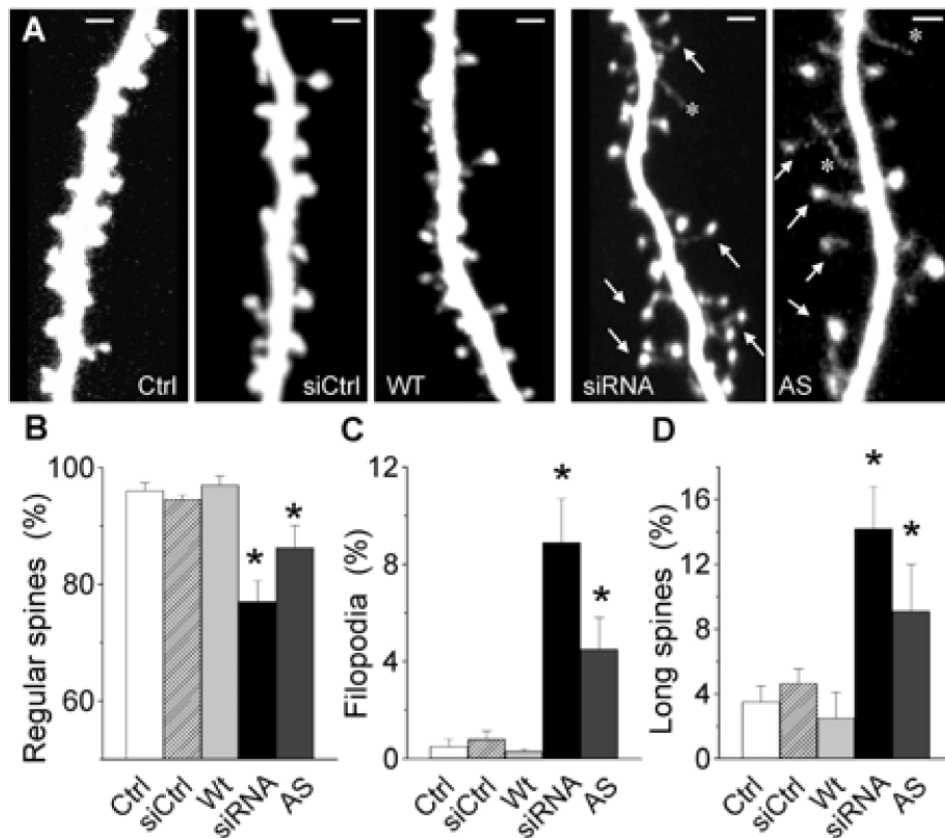
#### 4.2.2. Pak deficits and Alzheimer disease

**Alzheimer disease** (AD) is characterised by a neurodegenerescence responsible for the progressive loss of cognitive functions along with anomalies in spine morphology and synaptic defects (Jacobsen et al., 2006). It was shown that the over-expression of wild-type *APP* (amyloid beta precursor protein) in neurons leads to apoptosis and expression of mutated *APP* leads to DNA synthesis and apoptosis (McPhie et al., 2003). A dominant-negative form of *Pak3* inhibits apoptosis and DNA synthesis induced by *APP* expression (McPhie et al., 2003). This suggests that *Pak3* is specifically implicated in the reinitialisation of DNA synthesis in post-mitotic neurons. Besides, Alzheimer disease is accompanied by defaults in the expression, distribution and phosphorylation of proteins implicated in actin cytoskeleton regulation like cofilin and Pak. This could be the origin of the synaptic defects of Alzheimer disease (Zhao et al., 2006).

#### 4.2.3. A specific role for Pak3 in synaptic plasticity

*Pak3* is different from the other kinases of the group A, by its expression restricted to the central nervous system and its implication in non-syndromic mental retardation. *Pak3* is expressed in neurons and is localised in the cellular body and dendrites, in particular in dendritic spines (Boda et al., 2004; Hayashi et al., 2004; Meng et al., 2005). Diverse data show that the group A kinases and mainly *Pak3* have a fundamental role in dendritic spine formation and neuronal plasticity. The over-expression of a kinase-dead form of *Pak3* decreases dendritic spines density whereas the loss of *Pak3* expression by siRNA increases the number of filopodial spines and decreases normal spines and long-term potentiation (LTP, Figure 28) (Boda et al., 2004; Zhang et al., 2005b). Surprisingly, mice deficient for *Pak3* do not suffer from dendritic spines morphology defects (Meng et al., 2005). However these mice show **defects in learning, late LTP** and **CREB phosphorylation** is reduced. It was recently shown that *Pak3* regulates **synaptic transmission** via its interaction with the adaptor *Nck2* (Thevenot et al., 2011).





**Figure 28: Changes in spine morphology induced by antisense or siRNA down-regulation of *Pak3* expression.** (A) Illustration of the spine morphology obtained from cells co-transfected with GFP and an empty vector (Ctrl), a control non-sense siRNA (siCtrl), wild-type *Pak3* (WT), *Pak3* siRNA (siRNA) or *Pak3* antisense (AS). Scale bars: 2 $\mu$ m. Note the numerous elongated spines (arrows) and filopodia-like protrusions (\*) present in siRNA- and antisense-transfected cells. (B) Changes in the proportion of regular spines (<2 $\mu$ m in length) observed under the indicated conditions. Data are mean  $\pm$  SEM of measurements obtained from 7-11 experiments (260-891 protrusions analysed). (C) Changes in the proportion of filopodia observed in the same group of experiments. (D) Changes in the proportion of elongate spines (>2 $\mu$ m in length) observed in the same group of experiments. (Boda et al., 2004)

### 4.3. Role of PAKs in cell proliferation, apoptosis and cancer

PAKs have a role in the control of **cell proliferation** and transformation (Bokoch, 2003). The active form of Pak1 is not able to induce **transformation**. However, a dominant-negative form of Pak can inhibit the transformation induced by the oncogene Ras (Tang et al., 1997). PAK is a regulator of the MAP kinase ERK cascade by phosphorylation of Raf1 and MEK1. These phosphorylations are necessary but not sufficient for Raf1 and MEK1 activation by Ras (Hofmann et al., 2004). Similarly, many studies have shown that Pak1 is essential for transformation induced by Ras, Rac1, Rac3 and Cdc42, thus being at the centre of the transformation pathway induced by small proteins G (Kumar et al., 2006). The PAKs receive signals from many signalling cascades and are regulated by seven major regulators Akt (thymoma viral proto-oncogene 1), Pdk1 (pyruvate dehydrogenase kinase, isoenzyme 1), Cdk5, Cdc2, Src, Abl (Abelson murine leukemia viral oncogene) and PKA (protein kinase A) (Bokoch, 2003). Downstream, PAKs are regulator of many cascades implicated in cell proliferation.

#### 4.3.1. Regulation of cell cycle

Cell division depends on the coordination of a series of key events requiring microtubules dynamic and actin cytoskeleton modulations. The PAKs play an important role in **cell cycle progression**. The expression of a dominant-negative Pak1 **inhibits progression into G1** phase (Thullberg et al., 2007). Pak1 phosphorylates Histone H3 allowing cell cycle progression (Li et al., 2002) and stimulates the expression of CyclinD1, an important regulator of cell cycle. Inversely, the AID of Pak1 is able to inhibit cell cycle progression by repressing CyclinD1 (Nheu et al., 2004; Thullberg et al., 2007). In *Xenopus*, X-Pak1 is able to block G2/M progression (Faure et al., 1999). Last, Pak3 participates to **S-phase initiation** in post-mitotic neurons (McPhie et al., 2003).

#### 4.3.2. Apoptosis and cell survival

It has been shown that PAKs can participate in both **pro- and anti-apoptotic** pathways. The **pro-apoptotic** pathway is specific of **Pak2**. In response to external stimuli inducing apoptosis, Pak2 is cleaved by caspase3 allowing for GTPases-independent activation (Rudel and Bokoch, 1997). Early activation of **Pak1 allows cell survival**. In the absence of survival factors, the pro-apoptotic protein BAD (Bcl-2 antagonist of cell death) interacts with diverse partners like Bcl-2 (B-cell lymphoma-2) to inhibit their activity. The phosphorylation of BAD by Pak1 allows the dissociation of BAD/Bcl-2 complexes, freeing Bcl-2 which exercises its anti-apoptotic action (Schurmann et al., 2000). The anti-apoptotic function of Paks is implicated in the **physiopathology of infection by HIV**. Thus, Nef, an accessory viral protein expressed early in the replicative cycle of HIV, associates with PAK kinases. The Nef/PAK complex phosphorylates BAD preventing the apoptosis of T cells (Wolf et al., 2001). Cell survival depending on PAK relies on the activation of the transcription factor **NFkB** (nuclear factor kappa B) implicated in expression of anti-apoptotic genes (Frost et al., 2000).

#### 4.3.3. PAKs and cancer

PAKs are implicated in the control of **cell proliferation, apoptosis, cell migration, cell adhesion and angiogenesis**; deregulation of one of these processes leads to apparition of cancers (Kumar et al., 2006). It has been shown that the increase of PAK activity is correlated to **tumour progression and invasiveness** of breast cancer (Gururaj et al., 2005), melanoma (Jung et al., 2004), lung cancer (Regala et al., 2005), colorectal cancer (Carter et al., 2004) and pancreatic cancer (Mahlamaki et al., 2004). PAK is also implicated in the regulation of *Merlin*, a protein encoded by NF2 (Neurofibromatosis type 2). It has been shown that the loss of NF2 is responsible for cerebral tumours (Xiao et al., 2002). The increase of PAK activity in cancers might be due to an alteration of the kinase regulation or an increase in gene expression (Kumar et al., 2006). More recently, a Rac1/Pak1 cascade was shown to activate beta-catenin in colon cancer cells (Zhu et al., 2011). Moreover, Pak1 is able to **phosphorylate Snail**, a well-known regulation of **epithelial-to-mesenchymal transition** (EMT). Pak1 phosphorylation of Snail promotes Snail's nuclear accumulation and consequently its repressor activity in the nucleus. Thus Pak1 signalling might constitute a modifier of EMT by directly phosphorylating Snail (Yang et al., 2005). For these reasons PAKs are presently therapeutical targets in cancer treatments. The analysis of kinome mutations in hundreds of tumours allowed to identify a mutation in the *Pak3* gene (Greenman et al., 2007). This mutation, localised in the activating loop of the kinase domain of Pak3 could lead to constitutive activation, making Pak3 a new potential oncogene.

## 4.4. Role of PAKs in the pancreas

### 4.4.1. Indirect evidence

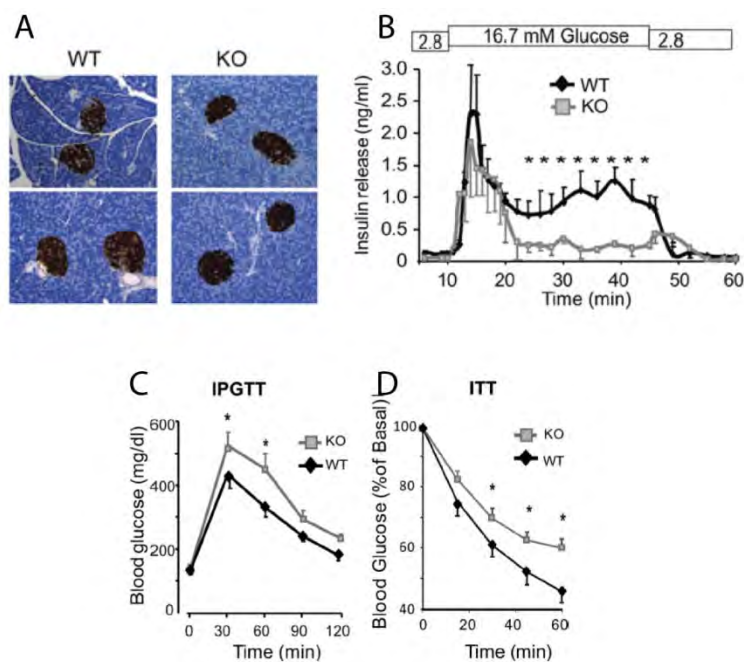
As mentioned earlier, PAKs are known effectors of the small Rho GTPases *Rac1* and *Cdc42*. It has been shown that *in vitro* glucose-stimulated **Cdc42** signalling is essential for the second phase of **insulin secretion** (Wang et al., 2007b). *Cdc42* depletion in Min6 cells ablates glucose-induced *Rac1* activation and results in the selective loss of second-phase insulin release (Wang et al., 2007b). Paks could be mediators of this mechanism. A few years ago the group of Henrik Semb showed that *Cdc42*, a PAK effector was necessary for proper **pancreas tubulogenesis** and progenitor cell specification (Kesavan et al., 2009). Later, the same group and others showed that one of the other PAK effector, **Rac1** was important for pancreatic **islet morphogenesis** and beta-cell function probably by regulating **insulin exocytosis** (Greiner et al., 2009; Kowluru, 2011). However it is not known if these effects of *Cdc42* and *Rac1* in the pancreas rely on PAKs function.

PAKs have been shown to be critical regulators of cell cycle progression and exit in different organisms (I.D.4.3.1). Previous work on different **cell cycle** regulators in the pancreas showed that proper cell cycle regulation is necessary for **endocrine differentiation** and/or quiescence of adult beta-cells and/or better recovery after induced diabetes (Georgia and Bhushan, 2006; Georgia et al., 2006; Yang et al., 2009); a role for PAKs in these mechanisms has not been studied yet.

In the last years, some studies have identified a role for the **Rho-Rock** (Rho associated kinase) signalling pathway in the regulation of pancreas function. The authors showed that **insulin secretion** is inhibited by this pathway via its impact on the cytoskeleton of beta-cells and that blockade of the Rho-Rock signalling results in increased insulin secretion (Hammar et al., 2009).

### 4.4.2. Direct evidence

There are accumulating evidences pointing to an eventual role for **PAK1** in processes that are crucial for regulation and maintenance of **glucose homeostasis**, namely insulin release by beta-cells and glucose clearance by skeletal muscles. Insulin secretion is impaired *in vitro* by *Pak1* depletion in Min6 cells, probably due to the function of *Pak1* in cytoskeleton remodelling to mobilise insulin granules to the plasma membrane (Wang et al., 2007b). In L6 skeletal myotubes, *Pak1* is thought to have an effect in *Rac1* mediated **translocation of Glut4 vesicles** to the cell surface to enable glucose uptake (Chiu et al., 2010). Very recently, the first direct evidence of an eventual role for PAKs *in vivo* in the pancreas was unravelled by the Thurmond team (Wang et al., 2011). They showed that *Pak1* was necessary *in vivo* for proper regulation of glucose homeostasis (Figure 29). Of relevance, they showed that human islets of type 2 diabetic donors contained about 80% less PAK1 protein compared with non-diabetic patients. This could suggest a role for PAK1 in islet signalling/scaffolding functions. To answer this question they have studied the physiology of mice deficient for *Pak1*. Islets from *Pak1* knockout mice exhibited defects in the **second-phase of insulin secretion**. Analyses in human and mouse pancreas revealed that PAK1 activation was **dependent on Cdc42 quantity**, crucial to activate ERK1/2 signalling but dispensable for the phosphorylation of cofilin, a known PAK target in other tissues.



**Figure 29: Pak1 is required *in vivo* for proper regulation of glucose metabolism. (A)** Insulin staining of WT and Pak1 KO pancreas sections showed equivalent islet density, size and beta-cell mass. **(B)** Isolated Pak1 KO and WT mouse islets were perfused in 2.8mM glucose and 16.7mM glucose, curves represent the average  $\pm$  SEM of three independent paired experiments, \* $p < 0.01$  versus WT. **(C)** IPGTT of Pak1 KO and WT mice was performed by intraperitoneal injection of D-glucose (2g/kg body weight) into 7 pairs of male mice (age 4-6 months) fasted for 18h; \*  $p < 0.05$  versus WT. **(D)** Insulin tolerance testing of Pak1<sup>-/-</sup> KO (n=6) and WT male mice (n=7) was performed by intraperitoneal injection of insulin (0.75 units/kg body weight) into male mice (age 4-6 months) fasted for 6h. Blood glucose levels were normalised to basal=100% for each animal for calculation of the mean percentage  $\pm$  SEM; \*  $p < 0.05$  versus WT. (Wang et al., 2011)

#### 4.5. PAK expression, loss of function and associated phenotypes

The three group A kinases have a very similar structure however they differ by their expression pattern. *Pak1* is expressed in the brain, muscles and spleen, *Pak2* is ubiquitous and *Pak3* is expressed uniquely in the brain (Kreis et al., 2008). Among the group B, *PAK4* is expressed in most tissues but mainly in the prostate, testis and colon. Similar to *Pak3*, *Pak5* is brain specific. *Pak6* is detected in the kidney, placenta, testis and prostate (Jaffer and Chernoff, 2002). Genetic studies of the *PAK* are more and more common. The KO mice for *Pak1*, *Pak3*, *Pak5* and *Pak6* are viable, whereas loss of *Pak2* or *Pak4* is embryonic lethal. Main phenotypes of Pak KO are presented in Table 8.

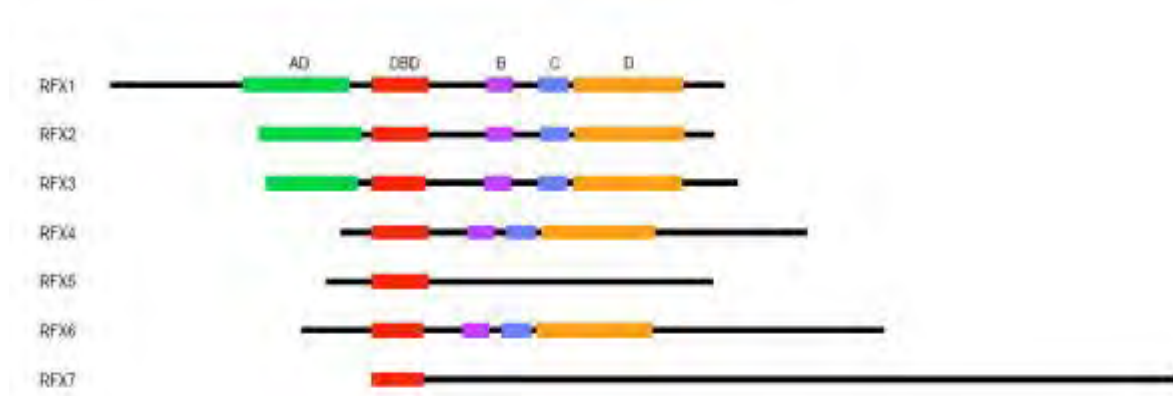
Knock-out	Main phenotypes	References
Pak1	Immune Defects, Synaptic plasticity defects Glucose intolerance	(McDaniel et al., 2008) (Asrar et al., 2009) (Wang et al., 2011)
Pak2	Lethal (E8)	(Marlin et al., 2011)
Pak3	Learning and memory defects	(Meng et al., 2005)
Pak4	Lethal, cardiac and neuronal defects	(Qu et al., 2003)
Pak5	No phenotype	(Li and Minden, 2003)
Pak6	No phenotype	(Nekrasova et al., 2008)
Pak1/Pak3	Impaired post-natal brain growth	(Huang et al., 2011)
Pak5/Pak6	Deficits in learning and locomotion	(Nekrasova et al., 2008)

**Table 8: Phenotypes associated with mouse PAK knock-outs.**

## E. The RFX gene family

### 1. The RFX gene family

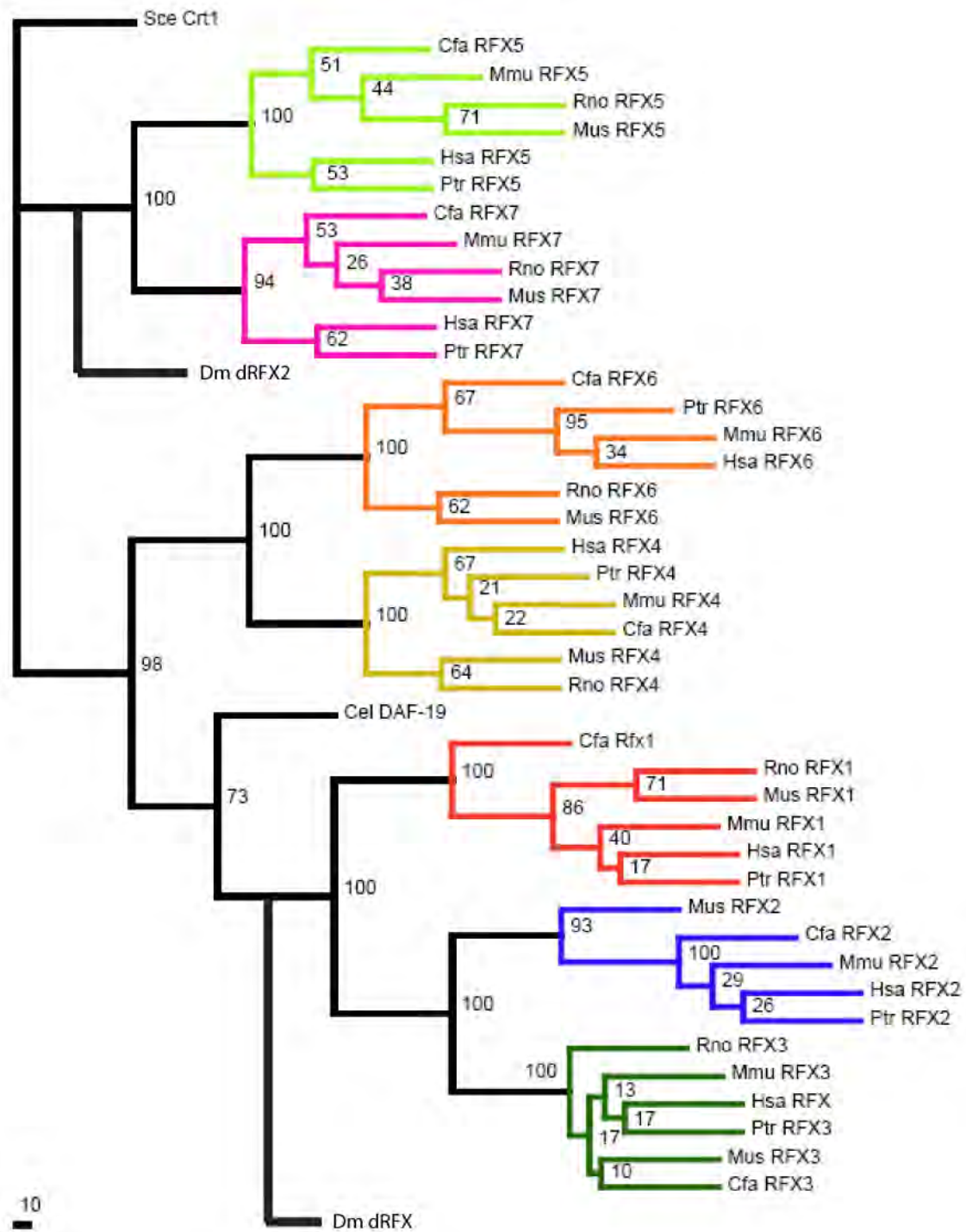
The Rfx (Regulatory Factor X) gene family contains seven **transcription factors** in mice: *Rfx1*, *Rfx2*, *Rfx3*, *Rfx4*, *Rfx5*, *Rfx6* and *Rfx7* (Aftab et al., 2008). It was described for the first time in the late eighties for their ability to bind on the **X boxes** of DNA. The canonical X box specific fourteen nucleotides sequence is GTNRCCNRRGYAAC (Laurencon et al., 2007). These transcription factors are characterised by the presence of a X box DNA binding domain (Reith et al., 1990). This binding domain is of the **winged-helix** type (Gajiwala and Burley, 2000; Gajiwala et al., 2000). RFX1, RFX2, RFX3, RFX4 and RFX6 proteins, also have a dimerisation domain (domain D), a domain B and a domain C (Emery et al., 1996). These three domains have roles in the dimerisation of the RFX proteins (Katan-Khaykovich and Shaul, 1998). RFX5 and RFX7 differ from the other RFX as they contain the DNA binding domain but no other conserved domain (Figure 30). The *RFX* genes are implicated in many different processes during development, notably in the brain and the testis.



**Figure 30: Functional domains of human RFX genes.** The functional domains AD (RFX Activation Domain), DBD (RFX DNA Binding Domain), B, C and D (Dimerisation Domain) are indicated using color-coded boxes. Genes are represented using horizontal lines, which are proportional to the human protein lengths. The domain lengths and positions are also proportional to their actual lengths. Alignment is based on the position of the DBDs. (Aftab et al., 2008)

The Rfx proteins are **conserved** from *S. Cerevisiae* to mammals (Figure 31) and the major findings concerning the function of these proteins come from studies in invertebrates (Aftab et al., 2008; Chu et al., 2010). Their functions are also mainly conserved during evolution. For example, *daf-19* (the unique *Rfx* gene in *C. elegans*) has been shown to be a crucial regulator of ciliogenesis (Swoboda et al., 2000). This function in ciliogenesis is maintained in *Drosophila* (Dubruille et al., 2002), zebrafish (Liu et al., 2007) and mouse (Ait-Lounis et al., 2007).





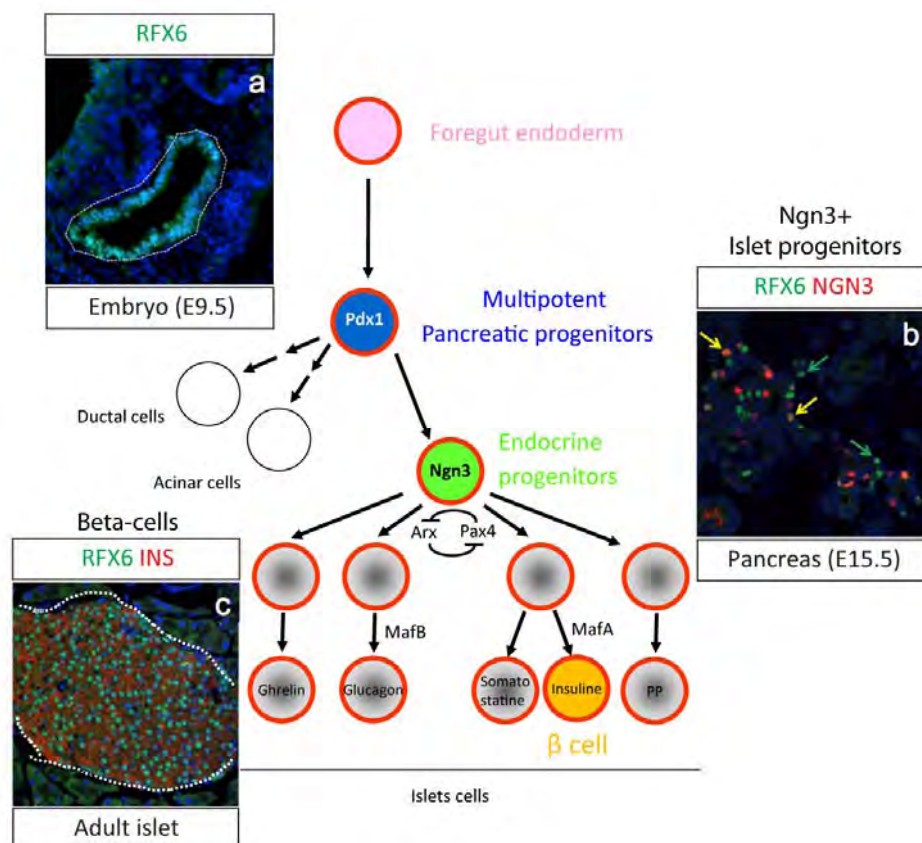
**Figure 31: Phylogenetic tree of RFX genes.** Each orthologous group is coloured differently. *Sce*: Yeast (*Saccharomyces cerevisiae*); *Mus*: Mouse (*Mus Musculus*); *Rno*: Rat (*Rattus Norvegicus*); *Cel*: Worm (*Caenorhabditis elegans*); *Cfa*: Dog (*Canis familiaris*); *Dm*: Drosophila (*Drosophila Melanogaster*); *Ptr*: Chimpanzee (*Pan troglodytes*); *Mmu*: Monkey (*Macaca mulatta*) and *Hsa*: Human (*Homo Sapiens*). (Modified from Aftab et al., 1998)

## 2. RFX expression and functions

### 2.1. RFX expression, loss of function and associated phenotypes

**Rfx1** and **Rfx7** are found expressed in **all organs** but with higher levels in the brain (Aftab et al., 2008). **Rfx2** is expressed primarily in **testis** but a recent study suggests that it could be expressed in ciliated tissues as well (Horvath et al., 2004; McClintock et al., 2008). **Rfx3** is expressed in numerous ciliated tissues, including the **pancreas** and the **brain** (Ait-Lounis et al., 2007; Bonnafe et al., 2004). **Rfx4** has six different isoforms, three are expressed specifically in the **testis**, one (*Rfx4 v3*) is **brain** restricted and two have been identified in glial tumours (Matsushita et al., 2005). In the brain, *Rfx4* has also been shown to be expressed in the suprachiasmatic nuclei (the circadian clock of the brain), where its expression is directly induced by light (Araki et al., 2004). **Rfx5** is expressed at high levels in the **brain** and at lower levels in all other organs except muscles, placenta and skin (Aftab et al., 2008).

Early during development **Rfx6** is expressed broadly in the primitive foregut **endoderm** and becomes progressively restricted to the stomach, intestinal endoderm and pancreas around E10.5. In the developing pancreas *Rfx6* expression is restricted to the **endocrine lineage** where it colocalises with a subset of  $\text{Ngn3}^+$  cells and differentiating endocrine cells (Figure 32). *Rfx6* is maintained in the **adult** endocrine cells (Smith et al., 2010; Soyer et al., 2010)(Figure 32). In the embryo, *Rfx6* mRNA has been found also in the stomach, lungs, testis and intestine (Soyer et al., 2010).



**Figure 32: Scheme summarising the pancreatic expression of Rfx6.** Early during development *Rfx6* is expressed broadly in the foregut endoderm (a). In the pancreas, *Rfx6* is found in scattered cells corresponding to a subset of endocrine progenitors (b) and differentiated endocrine cells. *Rfx6* is maintained in the adult islet cells (c). Red circles indicate expression of *Rfx6*. (Images: Josselin Soyer/Anthony Beucher)



Table 9 presents a summary of the main phenotypes of RFX KO in mouse.

Knock-out	Main phenotypes	References
Rfx1	Embryonic lethal (E2.5)	(Feng et al., 2009)
Rfx3	Left-right asymmetry due to cilia malformation Hydrocephalus Endocrine differentiation defects	(Bonnafe et al., 2004) (Baas et al., 2006) (Ait-Lounis et al., 2007)
Rfx4 v3	Hydrocephalus Ependymal cilia malformation	(Blackshear et al., 2003) (Zhang et al., 2007)
Rfx5	Decrease or loss of MHCII complexes	(Clausen et al., 1998)
Rfx6	Loss of endocrine cells (except PP) and perinatal death	(Smith et al., 2010; Soyer et al., 2010)

**Table 9: Phenotypes associated with RFX mouse knock-outs.**

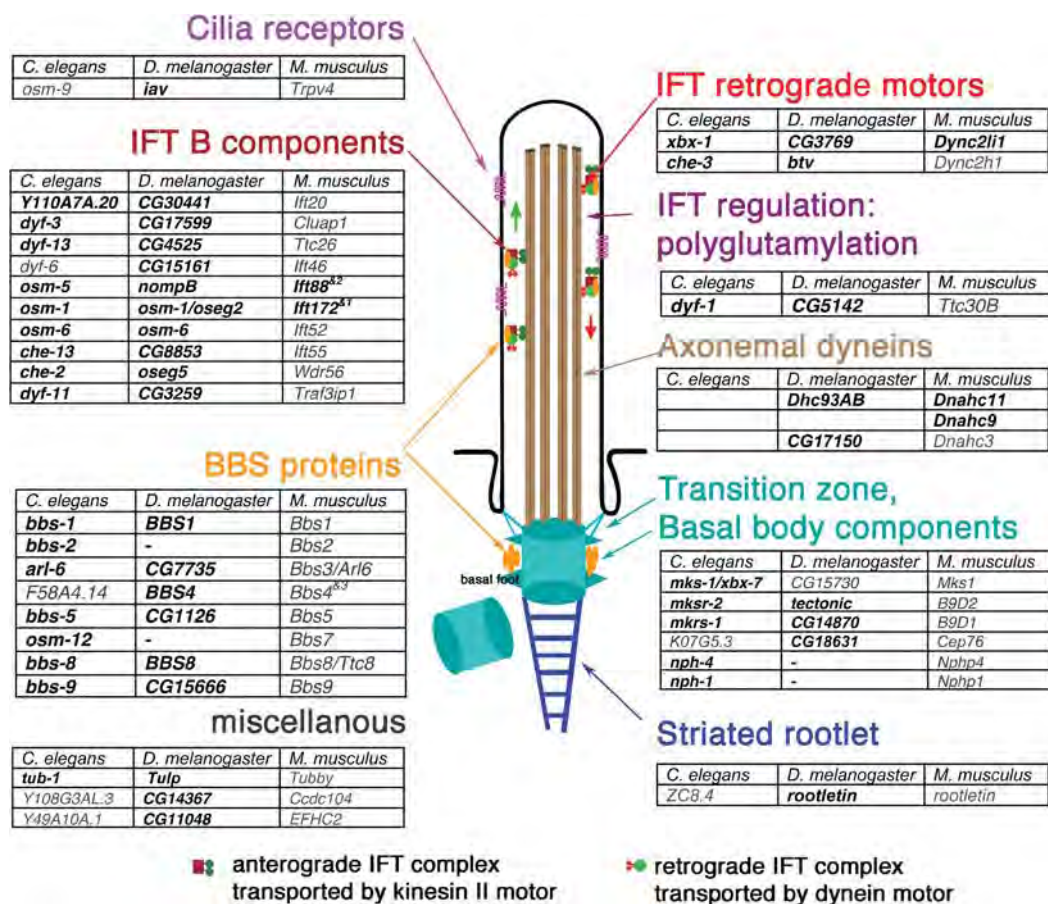
## 2.2. RFX and cilia

**Cilia** are sensory organelles found almost ubiquitously in vertebrates which role is to relay extracellular signals affecting the development and physiological response of cells. Mutations in cilia genes are responsible for human genetic disorders called ciliopathies (Gerdes et al., 2009).

RFX proteins have been shown to be necessary to assemble both primary and motile cilia in metazoans, in particular by regulating genes involved in intraflagellar transport (Figure 33). **Intraflagellar transport** is a bidirectional transport along microtubules that is essential for the formation and maintenance of cilia. The function of Rfx in cilia is conserved among evolution; the loss of *dRfx* and *Daf19* function, respectively homologues of the Rfx in *Drosophila* and *C. Elegans*, induces cilia malformation on sensory neurons (Dubruille et al., 2002; Swoboda et al., 2000).

A recent study on genes expressed in highly ciliated tissues from the olfactory epithelium, trachea and lungs suggested that *Rfx2* is expressed in ciliated tissues (McClintock et al., 2008). *Rfx3* is also expressed in many ciliated tissues and it has been shown that *Rfx3* loss of function leads to **left-right asymmetry** due to malformation of the nodal cilium which are necessary for proper organs positioning (Bonnafe et al., 2004). Another study showed that *Rfx3* is specifically lost in *Noto* mutants, in which nodal cilia are malformed and not functional (Beckers et al., 2007). Loss of *Rfx3* function is also linked to a reduction of cilia number in the ependymal cerebral ventricles, even though the structure of cilia does not seem to be affected. These defects in the developing brain lead to hydrocephalus associated with ependymal cell differentiation defects (Baas et al., 2006). *Rfx3* is also necessary for growth and beating efficiency of motile cilia in mouse and controls the expression of some genes involved in human ciliopathies (El Zein et al., 2009). The loss-of-function of *Rfx4 v3* (brain specific variant) induces **hydrocephalus** similarly to what is observed in the *Rfx3* mutants (Blackshear et al., 2003). The subcommissural organ does not develop, affecting the circulation of the cerebrospinal fluid. It has also been shown that the loss of *Rfx4 v3* induces malformation of ependymal cilia (Zhang et al., 2007). *Rfx4* also has a role in the formation of the cerebral cortex (Zarbalis et al., 2004).

Ait-Lounis et al. have shown that in *Rfx3*-deficient mice, **cilia on pancreatic endocrine cells** are smaller and less numerous (Ait-Lounis et al., 2007). However work by other labs, including the Hebrok lab, showed that the loss of cilia during pancreas development or in beta-cells has no effect on the formation or function of the pancreas (Cano et al., 2006). Somehow in contradiction, the same lab showed a few years before that the loss of *Ift88* (*Intraflagellar Transport 88*) has a role in the organisation of the pancreatic acinar tissue (Cano et al., 2004). This shows that the role of cilia in the pancreas is still controversial and whether or not the endocrine phenotype observed in *Rfx3*-deficient mice (see I.E.2.7.1) comes from the loss of cilia remains debatable. Two hypotheses can reconcile these results: (1) the endocrine phenotype is independent from cilia function or (2) no effect on pancreas differentiation was observed in *Ift88* knockout because of an incomplete or timely inappropriate deletion of cilia with the Cre.



**Figure 33: RFX target genes known to be involved in ciliogenesis.** Scheme of a primary cilium and its basal body. The basal body is derived from the mother centriole of the cells and harbours specialised structures, such as the basal foot. The basal body is anchored to the plasma membrane by the transition fibres. The region between the basal body and the axoneme is the transition zone. In *C. Elegans* and *Drosophila*, RFX proteins control all genes encoding components of the IFTB complex and all BBS (Bardet-Biedl Syndrome) orthologues. RFX regulates the dynein motor of retrograde IFT and is also involved in the control of tubulin polyglutamylation, which have been shown to be important for IFT in *C. Elegans* and zebrafish. RFX proteins regulate genes involved in basal body anchoring and channels found to be localised on cilia and several proteins known to be associated with cilia function, but of which the mechanism is still to be understood. **Bold:** genes found to be controlled by RFX in the different organisms. **Grey:** orthologues not tested in the different organisms. &1: *Ift172* is down-regulated in the *Rfx3* KO node, pancreas and in *Rfx4* KO nervous system. &2: *Ift88* is down-regulated in the *Rfx3* KO pancreas but not in the node of E7.5 embryos. &3: *Bbs4* was not found to be regulated by Rfx3 in ependymal cells. (From Thomas et al., 2010)

### 2.3. RFX and the major histocompatibility complex of class II

*Rfx1* has been first described for its action on the expression of genes from the MHCII complex (Reith et al., 1990). However *Rfx5* is the gene that has been studied the most due to its role in the **bare lymphocyte syndrome**, which is a hereditary immune monogenic autosomal recessive disease. It is characterised by the absence of expression of the MHCII. As a consequence, patients suffer from multiple infections frequently leading to death during childhood. The bare lymphocyte syndrome is due to the loss of expression of one of the four genes inducing the expression of the MHCII, including *Rfx5* (Reith and Mach, 2001).

### 2.4. RFX and testis development

*Rfx2* is the less studied gene of the Rfx family; it has only been attributed one function. It is expressed mainly in testis and more specifically in spermatocytes and spermatids (Horvath et al., 2004; Reith et al., 1994). A study on the development of testis showed that *Rfx2* can **bind** the promoter of **histone H1t** (a histone H1 variant), suggesting that *Rfx2* can regulate *H1t* expression (Horvath et al., 2004; Wolfe et al., 2004). *Rfx4* has six isoforms and three of them are expressed specifically in the testis (Matsushita et al., 2005; Morotomi-Yano et al., 2002). It has been shown that like *Rfx2*, *Rfx4* is specifically expressed in spermatocytes and can directly bind the promoter of the histone *H1t* and dimerise with *Rfx2* (Wolfe et al., 2006; Wolfe et al., 2008). Altogether, these data suggest that *Rfx4* and *Rfx2* interact in order to regulate H1t expression.

### 2.5. RFX and hepatitis and cancer

*Rfx1* has been shown to be necessary for the expression of genes from the **hepatitis B** virus and functions as transactivator of the hepatitis B virus enhancer (Siegrist et al., 1993). Two of the *Rfx4* variants have been observed in **glial tumours** (Matsushita et al., 2005). *Rfx5* is also implicated in the regulation of the expression of collagen (Sengupta et al., 2002). *Rfx5* in complex with histone deacetylase 2 and a transcriptional repressor is able to inhibit the expression of collagen alpha2(I) (Xu et al., 2006a).

### 2.6. RFX and DNA lesions and repair

It has been shown that *Rfx1* has a role in the **response to DNA damage** (Lubelsky et al., 2005). From this point of view, the function of *Rfx1* would be similar to *Rfx1p*, a gene from the RFX family in *S. Cerevisiae*. *Rfx1p* has been shown to be implicated in the regulation of DNA replication caused by DNA damage (Huang et al., 1998).

## 2.7. RFX and pancreas differentiation

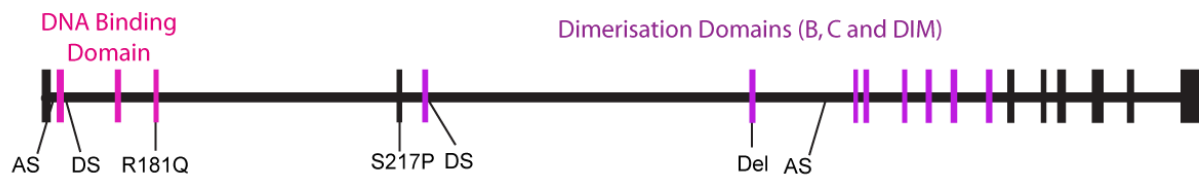
### 2.7.1. Roles Rfx3 and Rfx6 during pancreas differentiation in mice

*Rfx3* has recently been given a role in pancreatic **endocrine cells differentiation**. It has been shown that mice deficient for *Rfx3* have endocrine differentiation defects leading to an important reduction of the number of alpha- and beta-cells and an increase of PP-cells (Ait-Lounis et al., 2007). Later, the same group showed with a conditional knockout that *Rfx3* regulates **beta-cell differentiation and function** at least in part by its direct **binding to the glucokinase** gene (Ait-Lounis et al., 2010).

*Rfx6* is an islet-specific transcription factor (Figure 32). We and other have shown that *Rfx6* is necessary for proper **endocrine cell development** in the pancreas of zebrafish, mouse and human (Smith et al., 2010; Soyer et al., 2010). Loss-of-function studies in zebrafish revealed that *Rfx6* is necessary for the differentiation of glucagon-, ghrelin- and somatostatin-expressing cells. Beta-cells however were affected in their organisation and no longer clustered in compact islets (Pearl et al., 2011; Soyer et al., 2010). The phenotype is even stronger in *Rfx6*-deficient mice as these mutants failed to generate any of the normal islet cell types except for pancreatic-polypeptide-producing cells. However, surprisingly, the total number of endocrine cells (expressing the pan-endocrine marker chromograninA) was not affected (Smith et al., 2010). The increase in PP-cells is not sufficient to maintain the total number of endocrine cells, leaving the identity of these cells unknown so far. Studies of different transcription factors implicated in endocrine cell differentiation in mice lacking *Rfx6* showed that whereas *Rfx6* regulate factors involved in **beta-cell maturation and function** (reduced *MafA* and *NeuroD* for example), it restricts the expression of the beta-cell differentiation and specification genes and thus the beta-cell fate choice (e.g. increase of *Pax4*). The endocrine phenotype observed in *Rfx6*-deficient mice is **not a consequence of cilia defects** (Smith et al., 2010). The differences in phenotypes between *Rfx3*- and *Rfx6*-deficient mice suggest that these two genes can cooperate in regulating a set of genes involved in endocrine differentiation but not in cilia formation. Expression of *Rfx1*, *Rfx2* and *Rfx5* has also been reported in the embryonic pancreas (Smith et al., 2010) and might partially compensate for the loss of *Rfx3* thus explaining the milder phenotype of *Rfx3* knockout mice. Another explanation is that on contrary to *Rfx3*, *Rfx6* is able to direct gene expression as homodimers and not only as heterodimers with *Rfx3* (Smith et al., 2010).

### 2.7.2. RFX6 human mutations

Recently, by genetic mapping and sequencing of human infants with syndrome of **neonatal diabetes**, several mutations (Figure 34 and Table 10) in the human *RFX6* gene have been identified (Smith et al., 2010; Spiegel et al., 2011). The phenotype of these patients is remarkably similar to what was observed in mice. The probands have normal exocrine pancreas with clusters of chromogranin<sup>A</sup> cells but no staining for insulin, glucagon or somatostatin (Mitchell et al., 2004). In addition, the patients suffer from **small bowel obstruction**, hypoplastic gall bladder and **diarrhoea**. All these defects are now known as Mitchell-Riley syndrome. Depending on the mutations and number of normal copies of *RFX6* the prognosis differs. Patients homozygous for respectively, a loss of the donor site in intron 2 (IVS2+2T > C), an out-of-frame deletion in exon 7 (OF del E7) and a missense mutation involving a highly conserved arginine in the DNA-binding domain (R181Q), all died in the first months of life. One patient, still alive at the age of 9, was a compound heterozygote for a donor-site loss in intron 6 (IVS6+2T>G) and disruption of an acceptor site in intron 1 (IVS1-12A>G) (Smith et al., 2010). The last patient, still alive at the age of 6, had a homozygous missense mutation in exon 6 (S217P) (Smith et al., 2010; Spiegel et al., 2011). More recently a new homozygous mutation was identified in a patient who is still alive at 21 months (Spiegel et al., 2011) it consists of a deletion of nine nucleotides (AGGTTGATA) and an insertion of one nucleotide (G) which deletes a conserved acceptor site in intron 7 (c781-2\_787 del insG). *In silico* analysis revealed that this mutation is very likely to cause aberrant splicing of exon 8.



Acceptor Splice (AS) Donor Splice (DS)

**Figure 34: Position of the mutations on the human *RFX6* gene.**

Mutations	Phenotypes
IVS2+2T > C	Neonatal diabetes, duodenal and jejunal atresia, hypoplastic gall bladder, diarrhoea, parenteral nutrition, liver failure, died at 5 months
IVS1-12A>G/ IVS1-12A>G	Neonatal diabetes, duodenal atresia, hypoplastic gall bladder, no diarrhoea, no parenteral nutrition, no liver failure, alive at 9 years
OF del E7	Neonatal diabetes, duodenal and jejunal atresia, gall bladder agenesis, no diarrhoea, parenteral nutrition, no liver failure, died at 3 months
R181Q	Neonatal diabetes, duodenal atresia, liver failure, died at 2.5 months
S217P	Neonatal diabetes, duodenal and anal atresia, gall bladder agenesis, diarrhoea, no parenteral nutrition, no liver failure, alive at 6 years
c781-2_787 del insG	Neonatal diabetes, duodenal and jejunal atresia, gall bladder agenesis, diarrhoea, parenteral nutrition, no liver failure, alive at 21 months

**Table 10: Mutations in the human *RFX6* gene and associated phenotypes.**





## II. Results and discussion

### A. Role of Pak3 in pancreatic endocrine cell differentiation and function

*Ngn3* is a bHLH transcription factor which is indispensable for endocrine cell formation in the pancreas. As such, in mice deficient for *Ngn3*, no endocrine cells develop and pups die from diabetes shortly after birth (Gradwohl et al., 2000). This gene has been called proendocrine as its expression in multipotent pancreatic progenitors specifies these cells toward the endocrine lineage (Apelqvist et al., 1999; Schwitzgebel et al., 2000). However, despite the importance of *Ngn3* in the guiding of MPC toward a restricted endocrine fate, we only have few clues about the genes downstream of *Ngn3* and their importance in the mechanisms controlling endocrine subtype specification and maturation. During the last 15 years, researchers have established a transcription factors cascade regulating pancreas organogenesis and endocrine cell differentiation (Figure 18). In spite of new important findings in the understanding of programs controlling endocrine cell differentiation downstream of *Ngn3* many questions remains to be elucidated. Which mechanisms regulate cell cycle? What drives endocrine cell delamination and migration? How is the choice of endocrine subtype integrated? How do committed endocrine cells complete maturation and become functional? In order to answer those questions, the lab performed a microarray analysis of genes expressed differentially in *Ngn3*<sup>+</sup> endocrine progenitors versus *Ngn3*<sup>-</sup> cells in the pancreas at E15.5. This approach leads to the identification of genes which could potentially have a role in these mechanisms. Among those genes, the lab has already shown the importance and roles of *Insm1* and *Rfx6* (Mellitzer et al., 2006; Soyer et al., 2010) in endocrine cell differentiation. Along this line of research one of my tasks was to study the expression and role of another candidate; the serine/threonine *p21 protein-activated kinase 3*, *Pak3*, in endocrine cell differentiation and function.

We decided to focus on *Pak3* for many reasons; the main one being its role in neuronal cell differentiation. It has been shown in *Xenopus* that X-*Pak3* is downstream of the *neurogenin* gene *Ngnr1* and is necessary to promote cell cycle exit of neuronal progenitors and their subsequent differentiation (Souopgui et al., 2002). We thus hypothesised that *Pak3* could have a similar role in endocrine cell differentiation, promoting cell cycle exit of *Ngn3*<sup>+</sup> progenitors and endocrine cell differentiation. Briefly, I showed that *Pak3* is expressed throughout pancreas development in the endocrine lineage and maintained in adult islet cells. *Pak3* is strongly decreased in *Ngn3*-deficient mice suggesting an endocrine role downstream of *Ngn3*. This pattern of expression suggested that *Pak3* could have a role both in development and function of endocrine cells. To determine its role during development, I carried out a loss-of-function study using morpholinos in culture of embryonic pancreatic explants and studied the differentiation in *Pak3*-deficient embryos. The results show that around E15.5 inactivation of *Pak3* leads to an accumulation of *Ngn3*<sup>+</sup> progenitors together with an increased proliferation of endocrine cells, ultimately leading to impaired islet cell differentiation. No effect related to *Pak3* deficiency was found at later embryonic stages. Accordingly, *Pak3* KO mice survive well and are not diabetic. To understand the role of *Pak3* in adult endocrine cells, we carried out a metabolic study in collaboration with the Mouse Clinic Institute (ICS, Illkirch). We found that *Pak3* KO mice are glucose intolerant when challenged with a High Fat Diet (HFD) while insulin sensitivity and secretion are not affected.



In the next pages, you will find the manuscript we are currently writing containing the major findings about *Pak3* expression and role in endocrine cell differentiation and function. Then I will describe some additional experiments that we did not put in the paper and finally I will briefly discuss the relevance of this work.

## **1. Manuscript in preparation**

**Pak3 prevents proliferation and promotes differentiation of endocrine cells in the embryonic pancreas and is necessary to maintain glucose homeostasis in adult mice**

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## ABSTRACT

*Aims/hypothesis:* We showed previously that the bHLH transcription factor neurogenin 3 (Ngn3/Neurog3) controls islet cell fate specification in the mouse embryonic pancreas. It is thought that Ngn3 triggers both generic and islet subtype specific programs leading to differentiated islet cells. However little is known about the genetic programs implementing Ngn3 endocrinogenic function such as the mechanisms coupling cell cycle regulation and differentiation. In this context, we identified the p21-activated kinase Pak3, known to be implicated in X-linked intellectual disability, as a novel effector of Ngn3. Here we study the role of Pak3 in islet cell development and function.

*Methods:* We used *in situ* hybridisation and immunofluorescence to determine the expression profile of *Pak3* in the embryonic and adult pancreas. We performed loss-of-function studies both in mouse embryonic pancreas explant cultures using *Pak3* antisense morpholinos as well as in *Pak3*-deficient mice.

*Results:* *Pak3* expression is initiated in subsets of Ngn3-positive endocrine progenitor cells and next maintained in maturing hormones-expressing islet cells during pancreas development. *Pak3* depends on Ngn3 but not on NeuroD, a downstream target of Ngn3. The proliferation of Ngn3 cells as well as developing beta-cells was increased in *Pak3*-deficient embryos. The differentiation of beta-cells was impaired at E15.5 but resumed at late embryonic stages. In the adult pancreas, Pak3 is found exclusively in islet cells, including beta-cells. *Pak3*-deficient mice do not develop overt diabetes but are glucose intolerant under high fat diet.

*Conclusions/Interpretation:* First, our data support a role for Pak3, downstream of Ngn3, in islet cell development. We propose that Pak3 would favour endocrine differentiation by promoting cell cycle exit of endocrine progenitors and maintenance of a quiescent stage in developing hormone-positive islet cells. Second, our findings demonstrate that Pak3 is required for the control of glucose homeostasis under challenging high fat diet.

## INTRODUCTION

Understanding the mechanisms which control the progressive restriction of the fate of pancreatic progenitor cells and their differentiation into highly specialised hormone-secreting cells is a major issue for future cell-based therapies of type 1 diabetes. Despite the report of successful restoration of insulin production and glycaemic stability in patients with type 1 diabetes mellitus after cadaveric islet transplantation (Shapiro et al., 2000), major limitations have still to be overcome such as the lack of donors. In the last 10 to 20 years, significant knowledge has been acquired on the transcriptional regulation and signals controlling beta-cell development during mouse embryogenesis (Pan and Wright, 2011; Seymour and Sander, 2011). These lead to important progress in the generation of insulin-producing cells from human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) by recapitulating embryonic differentiation programs (D'Amour et al., 2006). However, to date, the cells which have been produced are immature and genetically different from normal glucose-responsive single-hormone-positive beta-cells (Kroon et al., 2008). This points to the fact that it is of crucial importance to carry on basic research to gain a highly detailed knowledge of the developmental programs giving rise to functional beta-cells.

During mouse pancreas development, the basic helix-loop-helix (bHLH) transcription factor Neurogenin3 (Ngn3) is the master gene controlling endocrine cell fate decisions in multipotent pancreatic progenitors. Ngn3 is expressed in endocrine progenitors before they start expressing hormones (Gradwohl et al., 2000; Schwitzgebel et al., 2000). In *Ngn3*-deficient mice, all endocrine cells fail to develop and mice die from diabetes shortly after birth (Gradwohl et al., 2000). Of note, ectopic expression of *Ngn3* is sufficient to generate all islet-cell types *in vivo* in mice (Johansson et al., 2007). Along the same line, lineage tracing experiments demonstrated that all pancreatic endocrine cells derive from Ngn3-positive progenitors cells (Gu et al., 2002; Schonhoff et al., 2004). Ngn3 activates directly or indirectly important downstream target genes, including *Pax4* and *Arx*, the regulatory switch of the beta- versus alpha-cell fate (Collombat et al., 2003; Sosa-Pineda et al., 1997). Other genes known to be downstream of Ngn3 includes transcription factors such as *NeuroD1* (Huang et al., 2000), *Insm1* (Mellitzer et al., 2006) or *Rfx6* (Smith et al., 2010; Soyer et al., 2010). However, our knowledge of the programs downstream of Ngn3, mainly those controlling cell cycle exit, migration and islet subtype specification, is only fragmental. Therefore, we have previously performed gene expression profiling of islet cell progenitors and identify potential novel downstream effectors of Ngn3 (Soyer et al., 2010). Among those, we will present here our findings on the role of the *p21* protein (*Cdc42/Rac1*)-activated kinase 3 (Pak3) in endocrine cell differentiation and glucose homeostasis.

Pak3 is a serine/threonine kinase of the PAK family which plays important roles in many cellular processes including cytoskeleton dynamic, neuronal differentiation and cell cycle regulation (Bokoch, 2003). PAKs are divided into two groups with distinct structure and function. Pak3 is part of the group A which members are effectors of the Rho GTPases Rac1 and Cdc42 (Manser et al., 1994). The mouse *Pak3* gene is located on position qF2 on mouse X chromosome and contains 16 exons. Pak3 has been mostly studied in the brain because of its role in X-linked non syndromic mental retardation (Meng et al., 2005). The role of Pak3 in the pancreas has not been studied so far. However, recent accumulating evidences point to a potential role of Pak1, another member of the PAK family group A in processes key to the regulation and maintenance of glucose homeostasis: pancreatic beta-cell insulin release and skeletal muscle glucose clearance (Chiu et al., 2011; Wang et al., 2011; Wang et al., 2007). In this report, we provide the first evidence that Pak3 might be necessary for endocrine cells differentiation and glucose homeostasis in mice.

## RESULTS

### Identification of *Pak3* in Neurog3-positive islet progenitor cells.

To identify the panel of genes activated specifically in islet progenitor cells, we previously determined the genes differentially expressed in Ngn3<sup>+</sup>/eYFP<sup>+</sup> versus Ngn3<sup>-</sup>/eYFP<sup>-</sup> cells purified from E15.5 Ngn3<sup>eYFP/+</sup> pancreata (Mellitzer et al., 2004; Soyer et al., 2010). Importantly, due to the greater stability of eYFP protein compared to Ngn3, the eYFP<sup>+</sup> population includes Ngn3<sup>+</sup> progenitor cells as well as their endocrine descendants. Interestingly, we found that the p21 protein-activated kinase Pak3 was enriched by 75 fold in Ngn3<sup>+</sup>/eYFP<sup>+</sup> cells suggesting that this kinase might be an important effector of Ngn3 function. To confirm the microarray data we first performed RT-qPCR experiments on sorted eYFP<sup>+</sup> and eYFP<sup>-</sup> pancreatic cells from E15.5 transgenic embryos (Fig. 1A). *Ngn3* transcripts are detected exclusively in eYFP<sup>+</sup> cells supporting efficient purification of Ngn3<sup>+</sup> cells. As expected *Pak3* followed a similar expression profile suggesting that this gene is expressed either in islet progenitors or in hormone-expressing cells or both. In contrast to *Pak3*, *Pak1* and *Pak2* transcripts are found both in endocrine and non-endocrine cells. Four splice variants of *Pak3* (Fig. 1B) have been described in the brain (Kreis et al., 2008; Rousseau et al., 2003) and we have been able to detect all of them by RT-qPCR on E15.5 wild-type pancreata (Fig. 1C). To our knowledge *Pak3* expression had never been reported in the endocrine pancreas we thus decided to pursue the study of this potential novel regulator of islet cell development and/or function.

### *Pak3* is maintained in hormone-expressing endocrine cells in the embryonic pancreas.

To characterise *Pak3* expression during mouse embryogenesis and pancreas development, we performed a series of *in situ* hybridisation and immunohistochemistry experiments. At E10.5, *Pak3* transcripts can be detected in few cells in the pancreatic bud (Fig. 2A). The number of *Pak3*-expressing cells then increases in the pancreatic epithelium at E12.5 (Fig. 2B) and peaks at E15.5, in a pattern reminiscent of developing endocrine cells (Fig. 2C). At E18.5 *Pak3* transcripts are low but still detectable in forming islets (Fig. 2D). In absence of a working anti-Pak3 antibody we performed *in situ* hybridisation followed by immunohistochemistry for endocrine markers to further characterise *Pak3*-expressing cells. As expected *Pak3* transcripts can be found in some Ngn3<sup>+</sup> endocrine progenitors (Fig. 2E arrows). *Pak3* is next maintained in more differentiated descendant cells, here stained for *Insm1* a direct target of Ngn3 (Fig. 2F arrows), as well as in hormone-positive endocrine cells (glucagon<sup>+</sup> in Fig. 2G and insulin<sup>+</sup> in Fig. 2H). *Pak3* was not found in developing acinar or ductal cells in wild-type embryonic pancreas (Fig. 2I-L). Together these data suggest that *Pak3* is expressed throughout pancreas development, first in Ngn3<sup>+</sup> endocrine progenitors and next in maturing hormone-positive islet cells.

### **Pak3 is expressed in pancreatic islets in adult mice.**

Next we explored the expression of *Pak3* in the adult pancreas. As mentioned above, for technical reasons we could not perform immunohistochemistry. Thus we first carried out RT-qPCR on purified adult islets. *Pak3* was found strongly enriched (FC 24.3) in islets compared to the exocrine tissue as well as, to a lower extent, *Pak1* (FC 2.3) (Fig. 3A). In contrast, *Pak2* was found equally distributed between the islet and exocrine compartments. To determine whether the PAK genes were expressed in beta-cells we took advantage of *Ins1*-mRFP mice generated in the lab (Merle et al., unpublished). In this model, the monomeric Red Fluorescent Protein is expressed in beta-cells under the control of *Ins1* regulatory elements. *Ins1*<sup>+</sup>/mRFP<sup>+</sup> cells were FACS sorted from purified islets. As expected *Ins1* transcripts were strongly enriched in mRFP<sup>+</sup> cells supporting efficient purification of beta-cells (Fig.3F). *Pak3* transcripts were readily detected in adult mouse beta-cells however; the qPCR data suggest that higher levels of *Pak3* mRNA are found in non-beta islet cells (Fig. 3E). *Pak1* and *Pak2* were similarly expressed in beta versus other islet cells (Fig. 3C-D). In agreement with the presence of *Pak3* in beta-cells, Pak3 protein is detected as a single 62 kDa peptide in the beta-cell lines bTC3 and Min6b1 (Fig. 3B). Taken together these data indicated that Pak3 is expressed in adult islet cells suggesting that this kinase might regulate pancreatic endocrine cell function.

### **Pak3 is specific of the endocrine lineage and independent of NeuroD.**

The strong enrichment of *Pak3* in purified Ngn3<sup>+</sup> endocrine progenitors suggested that Pak3 is specific of the endocrine lineage and downstream of Ngn3. To verify this hypothesis we analysed *Pak3* transcripts in *Ngn3*-deficient embryonic pancreas, which lack islet cells (Gradwohl et al., 2000). *Pak3* was strongly down-regulated but unexpectedly about 30% of *Pak3* transcripts can be detected in *Ngn3* mutant mice compared to controls (Fig. 4A). In contrast *Pak1* and *Pak2* are not affected in *Ngn3*-deficient mice. This result may suggest that while Pak3 lies downstream of Ngn3, Pak3 could also be found in upstream pancreatic progenitors or in non-endocrine cells. The scattered expression of *Pak3* in embryonic pancreata at E10.5 and E12.5 (Fig. 2A-B) excluded the first hypothesis, since a much broader expression would have been expected if *Pak3* was in pancreatic progenitors. We next determined which cells were expressing *Pak3* at E15.5 in the absence of Ngn3. In *situ* hybridisation experiments followed by immunohistochemistry showed that *Pak3* is not found in Tuj1<sup>+</sup> neuronal cells (Fig. 4B left column). Surprisingly, *Pak3* signal seems confined to acinar cells as suggested by co-expression with *Cpa1* (Fig. 4B right column, arrows) and *Ptf1a* (not shown). Importantly acinar expression was never observed in the wild-type situation (Fig. 2L) in agreement with the absence of *Pak3* in Ngn3<sup>-</sup>/YFP<sup>-</sup> sorted cells.



Recently we showed that, before E12.5, pancreatic cells where *Ngn3* gene has been induced but which cannot adopt an endocrine fate because they lack Ngn3 protein, can adopt an acinar destiny (Beucher et al., 2012). Thus our hypothesis is that *Pak3/Cpa1*<sup>+</sup> cells arise from *Ngn3*-deficient progenitors. We next tried to determine the position of *Pak3* in the hierarchy of transcription factors controlling islet development. At E15.5, *Pak3* expression (as well as other *PaksA*) was unaffected (Fig. 4C-D) in the pancreas of *NeuroD*-deficient mice (Naya et al, 1997). These data suggest that *Pak3* acts in the endocrine lineage either upstream or in parallel of NeuroD1.

### ***Pak3* promotes endocrine differentiation in the embryonic mouse pancreas.**

To determine the role of *Pak3* in endocrine cell development we first performed loss-of-function studies in embryonic pancreatic explant cultures using antisense oligonucleotides. Briefly, the pancreatic rudiments were isolated from E12.5 wild-type CD1 embryos. Explants were depleted from their surrounding mesenchyme and incubated with *Pak3*-specific antisense oligonucleotides of the morpholino-type. Two antisense oligonucleotides were designed (sequences under Material and Methods) to impair differently the translation of the *Pak3* mRNA. Morpholino1 is acting by binding to the ATG (*Pak3*-MO<sub>ATG</sub>) while Morpholino2 is preventing the correct splicing of the mRNA (*Pak3*-MO<sub>SP</sub>). We validated the uptake of the biotinylated morpholinos by immunostaining and observed a homogenous distribution of the morpholinos in the explants (Fig. 5A-B). As compared to non-treated explant cultures, the control morpholino oligonucleotide did not alter growth rate or normal cell differentiation (not shown). However, while differentiation of exocrine cells was not affected by the antisense morpholinos, as shown by RT-qPCR for *Amylase2* and *Ptf1a*, *Ins1* and *Gcg* transcripts exhibited a significant decrease of respectively 40% and 50% with both anti-*Pak3* oligos (Fig. 5C). This decrease is likely a consequence of the reduced number of cells immunoreactive for insulin and glucagon (Fig. 5E) suggesting impaired endocrine differentiation. Interestingly, the total number of *Ngn3*<sup>+</sup> endocrine progenitor cells doubled (Fig. 5D). However the doubling of the *Ngn3* population could not be confirmed by RT-qPCR (not shown), probably because of the strong dilution of *Ngn3*-expressing cells in the whole explant (5/1000 and 10/1000 cells in control and treated explants respectively), we were not in the resolution range of this technique.

These results prompted us to characterise the pancreatic phenotype of *Pak3* knockout mice (Meng et al., 2005). Analysis of *Pak3*-deficient embryos, at E15.5, confirmed our observations in pancreas explant cultures. Indeed, we observed a small, but significant, decrease in the number of insulin<sup>+</sup> and glucagon<sup>+</sup> cells together with a higher number of *Ngn3*<sup>+</sup> cells (Fig. 6A-B). A decrease in the amount of *Ins1* mRNA was also revealed by RT-qPCR (Fig. 6C). We next characterised the expression of *Arx* and *Pax4*, two transcription factors known to regulate alpha- and beta-cell fates downstream of *Ngn3* (Collombat et al., 2003). We noticed a small decrease of *Pax4* transcripts in

*Pak3*-deficient mice which may be the cause of impaired beta-cell development or the consequence of the loss of beta-cells (Fig. 6C). *Arx* was unchanged. Importantly, when we analysed *Pak3*-deficient pancreata at E13.5 or E18.5 (Fig. S1) we did not observe any alterations of islet cell development but the transcription of *Pak2* was up-regulated at E18.5. Altogether these data suggest that *Pak3* deficiency leads to impaired alpha-and beta-cell development, however this effect is only transient and differentiation resumed at late embryonic stages.

### ***Pak3* inhibits the proliferation of islet progenitors and beta cells in the embryonic mouse pancreas.**

*Pak3* expression is initiated in *Ngn3*<sup>+</sup> endocrine progenitors which are mainly postmitotic (Miyatsuka et al., 2011). Accordingly, *Pak3* is excluded from dividing cells in the embryonic pancreas (Fig. 6D). In addition a previous study showed that *Pak3* promotes cell cycle exit downstream of Neurogenin during primary neurogenesis in *Xenopus laevis* (Souopgui et al., 2002). Thus we speculated that the increased number of *Ngn3*<sup>+</sup> cells observed in the above *Pak3* loss-of-function studies might result from failure to exit cell cycle. To test this hypothesis we evaluated the impact of *Pak3* down-regulation on cell proliferation in cultures of embryonic pancreas. Immunofluorescence and quantification of the number of *BrdU*<sup>+</sup> cells revealed that in the absence of *Pak3*, the number of proliferating *Ngn3*<sup>+</sup> cells is increased (Fig. 5F, Table 1). Concomitantly we observed as well an increase in the total number of *Pdx1*<sup>+</sup>/*Ki67*<sup>+</sup> cells (Fig. 5G, Table 1). *Pdx1* is expressed at high levels in developing beta-cells (*Pdx1*<sub>high</sub>) as well as more moderately in multipotent pancreatic progenitors and developing acinar cells (*Pdx1*<sub>low</sub>). Only the percentage of *Pdx1*<sub>high</sub><sup>+</sup>/*Ki67*<sup>+</sup> cells was augmented suggesting that beta-cells proliferate more when *Pak3* is down-regulated (Figure 5G, Table 1). These observations are confirmed *in vivo* as well since we showed that in E15.5 *Pak3*-deficient embryos both the number of dividing *Ngn3*<sup>+</sup> progenitors and *Ins*<sup>+</sup> cells increased compared to controls (Fig. 6E-F). Together these data suggest that during embryogenesis *Pak3* down-regulation with morpholinos in pancreatic explants as well as *Pak3* loss-of-function *in vivo* promotes islet progenitors and beta-cell proliferation.

### **Upregulation of endocrine transcription factors and stimulation of cell division upon *Pak3* inhibition in Min6B1 cells.**

To study the role of *Pak3* in mature beta-cells we took advantage of the Min6B1 cells, a mouse beta-cell line which secrete insulin in response to glucose (Lilla et al., 2003). We transfected these cells with a pool of 4 siRNA against *Pak3* and tested for efficiency and specificity by RT-qPCR. 48h after transfection, *Pak3* was specifically down-regulated by ~50% whereas *Pak1* and *Pak2* were not affected (Fig. 7A). After down-regulation of *Pak3*, we observed an increased expression of several key transcription factors such as *NeuroD1*, *Insm1* and *Pax4* known to control islet cell development downstream of *Ngn3* in the embryo, or islet cell function in the adult (Fig. 7B). However, despite this

increase of TFs levels, markers of functional beta-cells, such as *Glut2* or *Gck* or *Ins1* a target of NeuroD1 were not affected (Fig. 7C). Notably, we observed a small but significantly higher percentage of Min6B1 cells in S phase when Pak3 was knocked-down compared to control cells suggesting that like in the embryo Pak3 controls cell cycle *in vitro* (Fig. 7D). These results suggest that Pak3 might be required to maintain beta-cell identity by controlling the appropriate levels of specific transcription factors as well as repressing replication.

#### **Impaired glucose homeostasis in *Pak3*-deficient mice.**

The expression of *Pak3* in adult islets prompted us to determine the role of this gene in the control of glucose homeostasis. *Pak3* KO mice have been shown to be fertile and exhibit a normal lifespan (Meng et al., 2005) however glucose homeostasis has not been explored in these mice. We thus generated 2 cohorts of *Pak3*-deficient and control adult males and performed metabolic studies under normal or high fat diet (summarised in Fig. 8 and Table S1). Mice did not develop overt diabetes and no difference in body weight (Fig. 8A/F) was observed in any diet. Under classical diet, oral glucose tolerance (Fig. 8B) as well as insulin sensitivity (Fig. 8D) are comparable between the two groups. Mutant animals are slightly glucose intolerant when given intraperitoneally (Fig. 8C) which may be due to the bypass of Glp1 insulin stimulation. However blood insulin level is not affected during the test (Fig. 8E). Together these data suggest that glucose homeostasis is mildly perturbed under normal diet conditions. Nevertheless, when challenged with HFD, *Pak3* KO males are clearly glucose intolerant both when given intraperitoneally or orally (Fig. 8G-H). However, no difference in insulin sensitivity (Fig. 8I) or secretion (Fig. 8J) has been detected. Taken together these results suggest that *Pak3* is necessary to maintain glucose homeostasis particularly under challenging conditions.

## DISCUSSION

In this study, we characterised for the first time the pancreatic expression and function of a potential downstream effector of Ngn3, the p21-protein activated kinase Pak3 which we found highly enriched in Ngn3<sup>+</sup> endocrine progenitors. These studies provide evidences that in the embryo Pak3 might promote cell cycle exit and then quiescence in developing islet cells and control their differentiation. In the adult mice Pak3 is necessary for normal glucose homeostasis.

### **PAK kinases in the mouse pancreas.**

p21-protein (Cdc42/Rac1) activated kinases (PAK) are serine/threonine protein kinases whose activity is stimulated by the binding of the active form of Rac1 and Cdc42 GTPases. PAKs are divided into two distinct groups: PAKsA includes Pak 1-3 and PAKsB includes Pak 4-6. Although they have also been termed PAKs, Pak 4-6 differ significantly in their structural organisation and regulation (Jaffer and Chernoff, 2002). The PAK family plays significant roles in modulating a range of biological activities (Bokoch, 2003). Until now, Pak3 was thought to be a brain-specific PAK (Kreis et al., 2008) but here we report for the first time the expression of *Pak3* in the mouse endocrine pancreas, both during development and in the adult. We showed that *Pak3* is expressed in the endocrine lineage throughout embryogenesis and maintained in adult islets. Pancreatic expression of the other members of the PakA family has not been reported. However, very recently, it has been shown that Pak1 loss-of-function in mice and in human islets resulted in impaired insulin secretion (Wang et al., 2011). Here, we showed that, in addition to Pak3, all the other PaksA are expressed in the mouse pancreas. In the embryo we found that *Pak1* and *Pak2* transcripts are present in both Ngn3<sup>+</sup> endocrine progenitor cells and Ngn3<sup>-</sup> pancreatic cells whereas *Pak3* is found only in Ngn3<sup>+</sup> cells. Thus only *Pak3* is strictly endocrine specific even though *Pak1* and *Pak2* are enriched in islet progenitor cells (60% and 50% respectively). In the adult pancreas, and in agreement with the embryonic expression, *Pak3* is highly enriched in islets compared to the exocrine compartment, while *Pak1* and *Pak2* are distributed in the exocrine and endocrine pancreas. Interestingly, isolation of beta-cells from purified islets suggests that *Pak1* and *Pak2* are distributed evenly among islet cell types. Surprisingly, these experiments also revealed that *Pak3* expression is higher in non-beta cells. However, the fact that *Pak3* is found in beta-cell lines bTC3 and Min6b1 (this study) as well as in induced beta-cells resulting from reprogramming of acinar cells (Zhou et al., 2008) supports a role for Pak3 in beta-cells.

Altogether these data suggest that Pak3 could (1) act during endocrine cell differentiation in the embryo, (2) regulate the function of adult endocrine cells, (3) help to generate beta-cells by reprogramming. However, given that Pak 1-3 are very closely related sequence- and functionally-wise, one cannot exclude that they could cooperate to regulate common pathways in developing and adult mouse islets and this point could be addressed with studies of double or triple PAKs KO.

### **Pak3 is induced by Ngn3 in the endocrine lineage.**

In microarray experiments, we found that Pak3 was enriched by 75 fold in Ngn3<sup>+</sup> endocrine progenitors compared to Ngn3<sup>-</sup> pancreatic cells at E15.5 suggesting a role for Pak3 in the endocrine lineage downstream of Ngn3. Accordingly, we showed that *Pak3* transcripts were restricted to the endocrine pancreas in the wild-type embryos (Fig. 2E-L and 4B upper row). Further supporting the idea that Pak3 is induced by Ngn3 in the embryonic mouse pancreas, *Pak3* transcripts were found to be increased (FC 7.2) in a study designed to identify Ngn3 downstream targets by rescuing *Ngn3* expression and endocrine differentiation in *Ngn3*-deficient mice (Johansson et al., 2007). Of note, in the same study a 2-fold increase of Pak6 was reported. However, Pak6 was not found enriched in Ngn3<sup>+</sup> cells, and given the structural differences between Pak3 and Pak6 we do not believe that Pak6 could functionally replace Pak3. Although these data support that Pak3 is a downstream target of Ngn3, we found that *Pak3* expression was not completely lost in Ngn3 KO mice (Fig. 4A), in sharp contrast to other endocrine genes such as *insulin* (not shown). Expression studies in wild-type embryos ruled out that Pak3 could be expressed upstream of Ngn3 in multipotent pancreatic progenitors or in ductal, acinar or neuronal cells. However, a characteristic acinar pattern, together with co-labelling of *Pak3* with *Cpa1* at E15.5 (never observed in wild-type embryos), hints that Pak3 could be found in some acinar cells in the absence of Ngn3. Importantly, we recently showed that, before E12.5, failed endocrine progenitors in which *Ngn3* transcription was induced but which lack Ngn3 protein, could adopt an acinar destiny (Beucher et al., 2012). Thus we hypothesised that *Pak3/Cpa1*<sup>+</sup> cells could arise from *Ngn3*-deficient cells. This would imply that although *Pak3* is specific of the endocrine lineage in the wild-type context, *Pak3* could actually be expressed independently of Ngn3 in acinar cells that derive from *Ngn3*-deficient cells. As *Pak3* is not affected in NeuroD KO but down-regulated (FC1.5) in *Rfx6*-deficient embryos at E15.5 (Piccand et al., in preparation), we suggest that Pak3 lies downstream of Ngn3 and Rfx6 and either upstream or in parallel of NeuroD1 in the control of endocrine differentiation (Fig. 9).

### **Pak3 a novel regulator of endocrine cell proliferation and differentiation.**

Several studies have shown that Ngn3 controls cell cycle exit in endocrine progenitors. The most direct evidence has been provided by the tracing and analysis of *Ngn3*-deficient cells in the embryonic pancreas which were shown to continue to proliferate (Beucher et al., 2012; Miyatsuka et al., 2011). In this regard, the cyclin-dependent kinase inhibitor 1a (Cdkn1a) has been demonstrated to play a critical role downstream of Ngn3 in cell cycle control (Miyatsuka et al., 2011). In the current study we observed an increased number of proliferating endocrine progenitor cells when Pak3 was inactivated providing evidence that Pak3 might contribute as well to cell cycle exit and thus maturation of islet cells. Interestingly both studies link transcription and signal transduction however it is not known whether Cdkn1a and Pak3 operate in the same pathway. Further supporting a role of Pak3 in cell cycle regulation, a previous study by Souopgui et al., showed that in *Xenopus* XPak3 is necessary for cell cycle exit of Ngn<sup>+</sup> neuronal progenitors (Souopgui et al., 2002). The authors described that XPak3 is expressed during neurogenesis in a pattern comparable to neuronal differentiation markers like N-tubulin and that it is induced by neurogenin. Using constitutively active form of Pak3 in *Xenopus* embryos they further showed that ectopic activation of XPak3 leads to cell cycle arrest and to premature neuronal differentiation. Conversely, inhibition of XPak3 with antisense morpholino results in increased cell proliferation and inhibition of neuronal differentiation (Souopgui et al., 2002). Based on the similarities between the two studies, we propose that Pak3 could regulate cell cycle exit and proliferation in the context of endocrine cells in the mouse pancreas. Notably we also observed an increased number of proliferating beta-cells in the pancreas of *Pak3*-deficient embryos suggesting that Pak3 is required in maturing beta-cells to repress proliferation. The role of Pak3 in cell cycle regulation of adult islet cells remains to be studied.

Despite the increased proliferation of islet progenitors and beta-cells we counted slightly less differentiated endocrine cells at embryonic stage E15.5 in Pak3 KO. This result can seem somehow contradictory as one would believe that the proliferation should compensate for the loss of differentiation. We would argue that the proliferation observed is not sufficient to compensate the differentiation defect at least at E15.5. At later embryonic stages it is known that beta-cells reenter cell cycle to increase beta-cell mass (Bouwens and Rooman, 2005). This process would then eventually compensate the differentiation defect explaining why we do not see a reduction in differentiation at later stages. We do believe that these results could indicate two different actions of Pak3. First, acting on Ngn3<sup>+</sup> progenitors, loss of Pak3 preventing them to exit cell cycle and mature which would explain the reduction in differentiated endocrine cells. Second, acting on differentiating cells Pak3 would be necessary to keep them in a quiescent stage to complete differentiation. The reduction of *Pax4* in Pak3 KO embryos, a transcription factor essential for beta-cell destiny, further supports a role

of Pak3 in beta-cell differentiation. It is however important to keep in mind that functional redundancy with Pak1 and Pak2 as well as with other cell cycle inhibitors such as Cdkn1a might explain the relatively mild increase in the proliferation of developing islet cells observed in *Pak3*-deficient mice.

#### **Impaired regulation of glucose homeostasis in Pak3 KO mice.**

In a recent study, it has been demonstrated that Cdc42-Pak1 signalling is essential for glucose-induced second-phase insulin secretion in mouse and human islets. Pak1 heterozygous and KO mice are glucose intolerant and this is linked to impaired second-phase insulin secretion (Wang et al., 2011). Interestingly, the Pak1 KO mice also showed peripheral insulin resistance due to defective Glut4 translocation in the skeletal muscle of these mice (Wang et al., 2011). Under normal diet, Pak3 KO mice show very mild differences in the control of glucose homeostasis compared to wild-types. This phenotype could be explained by the redundant function of the remaining group A members that we also found to be expressed in the adult islets. Pak1 and Pak2 compensation is however not sufficient under challenging condition as Pak3 KO mice are glucose intolerant under a high fat diet. Further studies are needed to understand the origin of this intolerance as insulin secretion and sensitivity seem normal. Of note we cannot exclude that a difference in the first phase of insulin secretion or a mild effect in the second phase of insulin secretion may not have been detected. Islets purification and insulin/glucagon secretion tests might answer this question as these tests have an increased sensitivity. Pak1 KO and Pak3 KO mice phenotypes are fairly different especially since we showed that Pak3 KO mice do not develop peripheral insulin resistance even under HFD. However, when we performed OGTT, the defects in glucose regulation appear very early, suggesting that glucocretins could have a role. Of note, we know from so far unpublished experiments that Pak3 is also present in the endocrine lineage of the intestine. Thus *Pak3*-deficiency in the intestine might impair enteroendocrine cell differentiation, including GLP1- and GIP-producing cells which might contribute to the glucose intolerance observed in the Pak3 KO mice. Moreover, we showed that Pak3 seems to be expressed at higher levels in islet non-beta cells than in beta-cells, thus one could hypothesise that *Pak3*-deficient mice have defects in alpha-cells or glucagon secretion and thus the observed phenotype might result from a combination of defects in alpha- and beta-cells. The function of Pak1 in glucose metabolism relies on Cdc42 activation, but how Pak3 is activated and what his downstream targets are, are so far not known. PAKs have been shown to signal in different pathways, among them MAPK and LIMK-cofilin have been shown to impact insulin secretion. In the MAPK pathway, ERK1/2 has been implicated in the later phase of insulin release as it phosphorylates cytoplasmic proteins implicated in exocytosis of insulin granules such as synapsin I (Longuet et al., 2005; Rondas et al., 2011). Cofilin is an actin-binding protein which disassembles actin filaments and



has been shown to be necessary for Glut4 vesicles translocation in skeletal muscles. Wang et al. showed that Pak1 signaling in islet beta-cells relies primarily on ERK1/2 activation and not on cofilin. Whether Pak3 signals similarly or not in the pancreas remains an open question. Of note, in *Pak3*-deficient mice no difference in the phosphorylation of cofilin or MAP kinases has been observed in the brain (Meng et al., 2005). Since CREB phosphorylation was strongly diminished in the brain of these mice, one can hypothesise that Pak3 may act via CREB in the pancreas as well. Indeed, CREB has been shown to be a key transcription factor for the maintenance of an efficient glucose sensing, insulin exocytosis, insulin gene transcription and beta-cell survival (Dalle et al., 2011). CREB activates the transcription of target genes within the beta-cells including insulin, cyclin D1 or IRS2. If Pak3 participates in CREB phosphorylation on Ser133 in the pancreas like in the brain, the absence of Pak3 could be detrimental for CREB-mediated transcription.

In summary, the results presented in this study provided evidence that Pak3 is a new regulator of the proliferation of endocrine progenitors and beta-cells in the embryo. We also showed that Pak3 is necessary to maintain glucose homeostasis in adult mice. Additional analysis of Pak3 KO mice, including proliferation of other endocrine cell types, role of Pak3 in the skeletal muscle or liver and search for downstream targets, may provide important clues for elucidating the mechanisms leading to impaired glucose tolerance in these mice.

## **MATERIAL AND METHODS**

### **Mouse strain and genotyping.**

The Ngn3 knockout mice were generated in the lab (Gradwohl et al., 2000) and kept on a mixed background. The Pak3 knockout mice (Meng et al., 2005) are a kind gift of Dr. Zhengping Jia (Hospital for Sick Children, Toronto, Ontario, Canada) and were kept on a mixed background. Embryos were genotyped using the following primers (Dr. Jean-Viannay Barnier, Laboratory of Cellular and Molecular Neurobiology, Gif-sur-Yvette): P1 5'-GAGTCAATTGCTTCACCAGCTG-3'; P2 5'-GGGGAACTTCCTGACTAGG-3'; P3 5'-GAAGTATGTGCGCACCAATGAC-3'. BAC-Ins1-mRFP1 mice were generated in collaboration with the Mouse Clinical Institute (Merle et al., unpublished) and used for purification of beta-cells.

### **Immunohistochemistry and Immunofluorescence.**

Explants or tissues of mice were fixed in 4% paraformaldehyde at 4°C, washed in PBS, equilibrated in 20% sucrose at 4°C and embedded in Shandon Cryomatrix (Thermo Scientific) and 7µm sections were used for explants and 10µm for tissues. Slides were hydrated in PBS, treated in blocking buffer (5% donkey serum in PBS, 0.1% Triton X-100) for 1h at RT and incubated with primary antibodies in blocking buffer overnight at 4°C. After washing in PBS 0.1% Triton X-100 (PBST), the secondary antibodies were added for 1 h at RT in PBST. For immunofluorescence assays, nuclei were stained with Dapi and mounted in Aqua-poly/mount (Polysciences). For immunohistochemistry analysis, endogenous peroxidase activity was blocked by incubation in 0.5% H<sub>2</sub>O<sub>2</sub> diluted in Methanol and signal was revealed using the Vectastain Elite ABC Kit (Vector Laboratories) and the DAB chromogen (DakoCytomation) supplemented with 0.02% H<sub>2</sub>O<sub>2</sub>. Slides were dehydrated and mounted in Eukitt (Euromedex). For BrdU detection assays, BrdU was injected to pregnant females at 50 mg/kg body weight, 2h prior sacrifice. If required, antigen retrieval was performed by incubating slides in 10mM Sodium Citrate pH6 and microwaving 4min at 1000W and 15min at 300W. Slides were then let to cool down to room temperature for at least 30 min.

Primary antibodies used: Ngn3, guinea pig anti-ngn3 at 1:1000 (kind gift of Dr Maïke Sander, University of California – San Diego, La Jolla, CA, USA); Insulin, guinea pig anti-insulin at 1:1000 (Linco), mouse anti-insulin at 1:1000 (Sigma); Glucagon, guinea pig anti-glucagon at 1:2000 (Linco); Ki67, mouse anti-Ki67 at 1:100 (Novo Castra); BrdU, mouse anti-BrdU at 1:100 (Boehringer Mannheim), rat anti-BrdU at 1:10 (AbD Serotec); Pdx1, rabbit anti-pdx1 at 1:2000 (kind gift of Dr Chris Wright, Vanderbilt University, Nashville, TN, USA); Sox9, rabbit anti-sox9 at 1:500 (AbCys); Insm1, guinea pig anti-insm1 at 1:500 (kind gift of Dr Carmen Birchmeier, Max-Delbrück-Centrum for Molecular Medicine, Berlin, Germany).

Secondary antibodies conjugated to DyLight488, DyLight549 and DyLight649 (JacksonImmunoResearch) were used at 1:500. For Ki67 and BrdU, signal amplification was performed using biotin anti-mouse coupled antibody at 1:500 (JacksonImmunoResearch) and streptavidin-Cy3 conjugate at 1:500 (Molecular Probes).

#### **Fluorescence quantification and statistics.**

Quantification was performed on every other section of whole explants. On nucleic staining, number of cells was counted manually using ImageJ software. For cytoplasmic stainings, the immunopositive area was reported to the total area of the explants using Metamorph software.

Values are presented as mean  $\pm$  SD. p-values were determined using the 2-tailed Student t test with unequal variance.  $p < 0.05$  was accepted as statistically significant.

#### ***In situ* hybridisation.**

*In situ* hybridisation was carried out on 10 $\mu$ m cryostat sections as previously described (Cau et al., 1997) and in some cases followed by immunohistochemistry. cRNA probes specifically recognised Ngn3 and NeuroD1 (Gradwohl et al., 2000) and Pak3 (transcribed from a 2.2kb mouse cDNA; Image clone 30060082)

#### **Quantitative RT-PCR analyses.**

Total RNA from Min6B1 cells, embryos, pups, sorted cells or purified islets was isolated in Tri Reagent (Invitrogen). 1  $\mu$ g of total RNA was used for cDNA synthesis using the Transcriptor Reverse Transcriptase (Roche). Quantitative PCR was performed using mouse-specific TaqMan primers and probes recognising *Ngn3* (Mm00437606\_s1), *Pak3* (Mm00435482\_m1), *Pak1* (Mm00440612\_m1) and *Pak2* (Mm01170646\_m1), *Ins1* (Mm01259683\_g1), *Ins2* (Mm00731595\_gH), *Gcg* (Mm01259683\_g1), *Sst* (Mm00436671\_m1), *Ppy* (Mm00435889\_m1), *Slc2a2* (Mm00446228\_m1), *Gck* (Mm00439129\_m1), *Abcc8* (Mm00803450\_m1), *Kcnj11* (Mm00440050\_s1), *Pcsk1* (Mm00479023\_m1), *Nkx6.1* (Mm00454962\_m1), *Pax4* (Mm01159036\_m1), *Arx* (Mm00545903\_m1), *Pdx1* (Mm00435565\_m1), *Insm1* (Mm02581025\_s1), *Rfx6* (Mm00624115\_m1), *NeuroD1* (Mm01280117\_m1) with TaqMan Light Cycler 480 Probes Master mix (Roche) on Light Cycler 480 (Roche). Gene expression results were normalised to *Rplp0* (Mm01974474\_gH) expression levels.

### **Culture and manipulation of embryonic pancreas.**

Dorsal pancreata were dissected from E12.5 CD1 embryos and pancreatic epithelium was separated from its surrounding mesenchyme as described (Mellitzer et al., 2004) and cultured on membrane filter (FHLC 0.45µm, Millipore) at the air/medium interface. The medium was DMEM/Ham-F12 supplemented with 10% FCS heat inactivated, pen/strep 1%, gentamycine 0.04mg/ml and 2mM L-glutamine. Epithelium was supplemented with biotinylated Pak3 antisense 1 (5'-CTTCGTTATCCAAGCTGTGTCAGACAT-3'), Pak3 antisense 2 (5'-CCCGGCCCTGATACTTTACTTTTA-3') or standard missense (5'-CCTCTTACCTCAGTTACAATTTATA-3') oligonucleotides (20µM) of the morpholino-type (GeneTools, LCC) partial medium changes were performed daily.

### **Cell culture and siRNA treatment.**

Min6B1 cells were maintained in culture as previously described (Lilla et al., 2003; Wang et al., 2007). At 30-50% confluence, cells were transfected with 66nM of siRNA oligonucleotides (PAK3 siGENOME SMART pool, Dharmacon) using Lipofectamine2000 (Invitrogen) to obtain ~70-80% transfection efficiency. A non-targeting RNA (Dharmacon) was used as control. Cells were harvested in Tri Reagent (Invitrogen) for RNA extraction.

### **Mouse islet purification.**

Mouse islet purification was performed as previously described (Carter et al., 2009). Briefly mice were euthanised with a cocktail of ketamine and injected with Type V Collagenase (Sigma C9263) solution directly into the common bile duct to perfuse the pancreas. Pancreas was dissected out and digested and islets were handpicked after several purification steps and kept in culture O/N before harvesting for FACS sorting or RNA isolation.

### **Metabolic studies.**

For the OGTT, after a 16-hours overnight fast, 17-weeks-old males received glucose by intragastric gavage (2g/kg body weight of 15% D-glucose). For the IPGTT, after a 4-hours fasting period, 18 weeks-old males received glucose by intraperitoneal injection (2g/kg body weight of 15% D-glucose). For the OGTT and IPGTT, circulating blood glucose was measured in tail blood at 0, 5, 15, 30, 45, 60, 90 and 120 minutes using a Glucofix Sensor (A. Menarini Diagnostics). For the IPIST, 6-hours fasted 20 weeks-old males were given an intraperitoneal injection of human insulin (1 IU/kg; Umluline, Lilly). Circulating blood glucose was measured in tail blood at 0, 15, 30, 45, 60 and 90 minutes using a Glucofix Sensor (A. Menarini Diagnostics). Insulin secretion was measure in tail blood during the IPGTT at 0, 15, 30 minutes. Blood analyses were done on 23 weeks-old males and were performed by the ICS (<http://www.ics-mci.fr>).

**Preparation of single-cell suspensions from dissected pancreata or purified islets and flow cytometric cell sorting and cell cycle analysis.**

Dissected pancreata from E15.5 Pak3 WT and Pak3 KO embryos were prepared as previously described (Mellitzer et al., 2004). Flow cytometric sorting was performed on a FACS ARIA (Becton-Dickinson). Sorted cells were then either harvested for RNA extraction or kept at 4°C in EtOH until processed for cell cycle analysis. For cell cycle analysis suspended cells were stained with propidium iodide (15µg/ml) and processed on a FACS Calibur (Becton-Dickinson) and results analysed with Modfit Software.

## REFERENCES

- Beucher, A., Martin, M., Spenle, C., Poulet, M., Collin, C. and Gradwohl, G.** (2012). Competence of failed endocrine progenitors to give rise to acinar but not ductal cells is restricted to early pancreas development. *Dev Biol* **361**, 277-85.
- Bokoch, G. M.** (2003). Biology of the p21-activated kinases. *Annu Rev Biochem* **72**, 743-81.
- Bouwens, L. and Rooman, I.** (2005). Regulation of pancreatic beta-cell mass. *Physiol Rev* **85**, 1255-70.
- Carter, J. D., Dula, S. B., Corbin, K. L., Wu, R. and Nunemaker, C. S.** (2009). A practical guide to rodent islet isolation and assessment. *Biol Proced Online* **11**, 3-31.
- Cau, E., Gradwohl, G., Fode, C. and Guillemot, F.** (1997). Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development* **124**, 1611-21.
- Chiu, T. T., Jensen, T. E., Sylow, L., Richter, E. A. and Klip, A.** (2011). Rac1 signalling towards GLUT4/glucose uptake in skeletal muscle. *Cell Signal* **23**, 1546-54.
- Collombat, P., Mansouri, A., Hecksher-Sorensen, J., Serup, P., Krull, J., Gradwohl, G. and Gruss, P.** (2003). Opposing actions of Arx and Pax4 in endocrine pancreas development. *Genes Dev* **17**, 2591-603.
- D'Amour, K. A., Bang, A. G., Eliazer, S., Kelly, O. G., Agulnick, A. D., Smart, N. G., Moorman, M. A., Kroon, E., Carpenter, M. K. and Baetge, E. E.** (2006). Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol* **24**, 1392-401.
- Dalle, S., Quoyer, J., Varin, E. and Costes, S.** (2011). Roles and regulation of the transcription factor CREB in pancreatic beta -cells. *Curr Mol Pharmacol* **4**, 187-95.
- Gradwohl, G., Dierich, A., LeMeur, M. and Guillemot, F.** (2000). neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A* **97**, 1607-11.
- Gu, G., Dubauskaite, J. and Melton, D. A.** (2002). Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* **129**, 2447-57.
- Huang, H. P., Liu, M., El-Hodiri, H. M., Chu, K., Jamrich, M. and Tsai, M. J.** (2000). Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. *Mol Cell Biol* **20**, 3292-307.
- Jaffer, Z. M. and Chernoff, J.** (2002). p21-activated kinases: three more join the Pak. *Int J Biochem Cell Biol* **34**, 713-7.
- Johansson, K. A., Dursun, U., Jordan, N., Gu, G., Beermann, F., Gradwohl, G. and Grapin-Botton, A.** (2007). Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types. *Dev Cell* **12**, 457-65.
- Kreis, P., Rousseau, V., Thevenot, E., Combeau, G. and Barnier, J. V.** (2008). The four mammalian splice variants encoded by the p21-activated kinase 3 gene have different biological properties. *J Neurochem* **106**, 1184-97.
- Kroon, E., Martinson, L. A., Kadoya, K., Bang, A. G., Kelly, O. G., Eliazer, S., Young, H., Richardson, M., Smart, N. G., Cunningham, J. et al.** (2008). Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol* **26**, 443-52.
- Lilla, V., Webb, G., Rickenbach, K., Maturana, A., Steiner, D. F., Halban, P. A. and Irminger, J. C.** (2003). Differential gene expression in well-regulated and dysregulated pancreatic beta-cell (MIN6) sublines. *Endocrinology* **144**, 1368-79.

- Longuet, C., Broca, C., Costes, S., Hani, E. H., Bataille, D. and Dalle, S. (2005).** Extracellularly regulated kinases 1/2 (p44/42 mitogen-activated protein kinases) phosphorylate synapsin I and regulate insulin secretion in the MIN6 beta-cell line and islets of Langerhans. *Endocrinology* **146**, 643-54.
- Manser, E., Leung, T., Salihuddin, H., Zhao, Z. S. and Lim, L. (1994).** A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* **367**, 40-6.
- Mellitzer, G., Bonne, S., Luco, R. F., Van De Castele, M., Lenne-Samuel, N., Collombat, P., Mansouri, A., Lee, J., Lan, M., Pipeleers, D. et al. (2006).** IA1 is NGN3-dependent and essential for differentiation of the endocrine pancreas. *EMBO J* **25**, 1344-52.
- Mellitzer, G., Martin, M., Sidhoum-Jenny, M., Orvain, C., Barths, J., Seymour, P. A., Sander, M. and Gradwohl, G. (2004).** Pancreatic islet progenitor cells in neurogenin 3-yellow fluorescent protein knock-add-on mice. *Mol Endocrinol* **18**, 2765-76.
- Meng, J., Meng, Y., Hanna, A., Janus, C. and Jia, Z. (2005).** Abnormal long-lasting synaptic plasticity and cognition in mice lacking the mental retardation gene Pak3. *J Neurosci* **25**, 6641-50.
- Miyatsuka, T., Kosaka, Y., Kim, H. and German, M. S. (2011).** Neurogenin3 inhibits proliferation in endocrine progenitors by inducing Cdkn1a. *Proc Natl Acad Sci U S A* **108**, 185-90.
- Pan, F. C. and Wright, C. (2011).** Pancreas organogenesis: from bud to plexus to gland. *Dev Dyn* **240**, 530-65.
- Rondas, D., Tomas, A., Soto-Ribeiro, M., Wehrle-Haller, B. and Halban, P. A. (2011).** Novel mechanistic link between focal adhesion remodeling and glucose-stimulated insulin secretion. *J Biol Chem* **287**, 2423-36.
- Rousseau, V., Goupille, O., Morin, N. and Barnier, J. V. (2003).** A new constitutively active brain PAK3 isoform displays modified specificities toward Rac and Cdc42 GTPases. *J Biol Chem* **278**, 3912-20.
- Schonhoff, S. E., Giel-Moloney, M. and Leiter, A. B. (2004).** Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. *Dev Biol* **270**, 443-54.
- Schwitzgebel, V. M., Scheel, D. W., Conners, J. R., Kalamaras, J., Lee, J. E., Anderson, D. J., Sussel, L., Johnson, J. D. and German, M. S. (2000).** Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development* **127**, 3533-42.
- Seymour, P. A. and Sander, M. (2011).** Historical perspective: beginnings of the beta-cell: current perspectives in beta-cell development. *Diabetes* **60**, 364-76.
- Shapiro, A. M., Lakey, J. R., Ryan, E. A., Korbitt, G. S., Toth, E., Warnock, G. L., Kneteman, N. M. and Rajotte, R. V. (2000).** Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* **343**, 230-8.
- Smith, S. B., Qu, H. Q., Taleb, N., Kishimoto, N. Y., Scheel, D. W., Lu, Y., Patch, A. M., Grabs, R., Wang, J., Lynn, F. C. et al. (2010).** Rfx6 directs islet formation and insulin production in mice and humans. *Nature* **463**, 775-80.
- Sosa-Pineda, B., Chowdhury, K., Torres, M., Oliver, G. and Gruss, P. (1997).** The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. *Nature* **386**, 399-402.
- Souopgui, J., Solter, M. and Pieler, T. (2002).** XPak3 promotes cell cycle withdrawal during primary neurogenesis in *Xenopus laevis*. *EMBO J* **21**, 6429-39.



**Soyer, J., Flasse, L., Raffelsberger, W., Beucher, A., Orvain, C., Peers, B., Ravassard, P., Vermot, J., Voz, M. L., Mellitzer, G. et al.** (2010). Rfx6 is an Ngn3-dependent winged helix transcription factor required for pancreatic islet cell development. *Development* **137**, 203-12.

**Wang, Z., Oh, E., Clapp, D. W., Chernoff, J. and Thurmond, D. C.** (2011). Inhibition or ablation of p21-activated kinase (PAK1) disrupts glucose homeostatic mechanisms in vivo. *J Biol Chem* **286**, 41359-67.

**Wang, Z., Oh, E. and Thurmond, D. C.** (2007). Glucose-stimulated Cdc42 signaling is essential for the second phase of insulin secretion. *J Biol Chem* **282**, 9536-46.

**Zhou, Q., Brown, J., Kanarek, A., Rajagopal, J. and Melton, D. A.** (2008). In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature* **455**, 627-32.

## LEGENDS TO FIGURES

Figure 1: **Expression of *Pak3* and its isoforms in mouse embryonic pancreas.** (A) RT-qPCR on sorted E15.5 *Ngn3*/eYFP cells revealed that *Pak3* is restricted to eYFP<sup>+</sup> cells whereas *Pak1* and *Pak2* are found in both populations. #: not detected, \*\*  $p < 0.01$ , \*  $p < 0.05$ . (B) mRNA structure of the four *Pak3* mouse transcripts identified in the brain (Kreis et al., 2008). Arrows represent primers position for RT-PCR. (C) RT-PCR revealing the presence of all known *Pak3* isoforms in E15.5 wild-type embryonic pancreas.

Figure 2: ***Pak3* is expressed in endocrine cells in the embryonic pancreas.** (A-D) *In situ* hybridisation (blue) for *Pak3* mRNA throughout pancreas development. Dotted line delineate pancreatic domain. (E-L) *In situ* hybridisation (blue) for *Pak3* mRNA followed by immunohistochemistry (brown) for different endocrine (E-H) and exocrine (I-L) markers in E15.5 wild-type embryos. Arrows indicate co-staining. Scale bar 50 $\mu$ m (A-D) and 20 $\mu$ m (E-L).

Figure 3: ***Pak3* is maintained in adult islet cells.** (A) RT-qPCR for the *PaksA* on purified islets and exocrine tissues from adult CD1 mice. (B) Western blot for *Pak3* on protein extracts from bTC3 and Min6B1 beta-cell lines. *Pak3* protein is detected as a single band of 62kD. (C-F) RT-qPCR for *PaksA* on *Ins*/mRFP sorted cells from adult purified islets. *Ins1* showed as a control of efficient sorting of insulin cells. (A/C-F) data are represented as mean  $\pm$  SD, \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

Figure 4: ***Pak3* depends on *Ngn3* but is independent of *NeuroD*.** (A) RT-qPCR for *PaksA* and *Ngn3* on E15.5 pancreata from *Ngn3* KO and control littermates. (B) *In situ* hybridisation for *Pak3* (blue) followed by ICC (brown) for the pan neuronal marker (*Tuj1*) or the acinar marker (*Cpa1*) in E15.5 *Ngn3*-deficient and control embryos. Scale bars 50 $\mu$ m. (C) RT-qPCR for *PaksA* and *NeuroD1* on E15.5 pancreata from *NeuroD* KO and control littermates. (D) *In situ* hybridisation for *Pak3* and *NeuroD1* in E15.5 *NeuroD1*-deficient and control embryos. Scale bars 20 $\mu$ m. (A/C) data are represented as mean  $\pm$  SD, # not detected, \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

**Figure 5: Pak3 regulates endocrine cell proliferation and differentiation in embryonic pancreatic explants.** Explants were dissected from E12.5 wild-type embryos and cultured on filter floating on medium supplemented with the morpholinos during four days before analysis. **(A-B)** Immunofluorescence staining for E-cadherin (green) and biotin (red) showing efficient intracellular absorption of Pak3 morpholino into the explants. **(C)** RT-qPCR for the exocrine markers *Ptf1a* and *Amylase2* and the endocrine markers *Insulin1* and *Glucagon* in pancreatic explants cultured with the different Pak3 or control morpholinos. **(D)** Quantification of the number of Ngn3<sup>+</sup> cells per explants (n=20). **(E)** Quantification of the Ins<sup>+</sup>/Gcg<sup>+</sup> area per explants (n=20). **(F)** Quantification of the number of proliferative Ngn3<sup>+</sup> endocrine progenitor cells in pancreatic explants (n=17). **(G)** Quantification of the percentage dividing Pdx1<sup>+</sup> cells in explants treated with morpholinos (n=18). Data are represented as mean ± SD, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

**Figure 6: Promotion of islet progenitors and beta cell proliferation together with impaired islet cell differentiation in Pak3-deficient embryos.** **(A)** Quantification of the insulin<sup>+</sup> and glucagon<sup>+</sup> area in E15.5 pancreas from *Pak3*-deficient and control embryos (n=3). **(B)** Quantification of the number of Ngn3<sup>+</sup> cells in E15.5 pancreas from *Pak3*-deficient and control embryos (n=3). **(C)** RT-qPCR for *PaksA* and different endocrine markers in *Pak3* KO and control pancreas from E15.5 embryos. **(D)** *In situ* hybridisation for *Pak3* (blue) followed by ICC for BrdU (brown) on wild-type E15.5 embryos. **(E)** Percentage of BrdU<sup>+</sup>/Ngn3<sup>+</sup> endocrine progenitors in E15.5 pancreas from *Pak3* KO and wild-type littermates (n=3). **(F)** Quantification of the proliferation of insulin<sup>+</sup> cells in E15.5 pancreas from *Pak3*-deficient and control embryos (n=3). Data are represented as mean ± SD, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

**Figure 7: Upregulation of endocrine transcription factors upon Pak3 inhibition in Min6B1 cells.** RT-qPCR for the *PaksA* **(A)**, markers of differentiated beta-cells **(B)** and pancreatic transcription factors **(C)** on Min6B1 cells treated with non-targetting siRNA or *Pak3* siRNA for 48 hours. **(D)** Cell cycle analyses of Min6B1 cells treated with siNT or si*Pak3* by Flowcytometry. Data are represented as mean ± SD, \* p<0.05, \*\* p<0.01, \*\*\* p< 0.001.

**Figure 8: Impaired glucose homeostasis Pak3-deficient mice.** Metabolic exploration of adult *Pak3* KO (n=13) and *Pak3* WT (n=13) males under normal diet **(A-E)** or high fat diet **(F-J)**. Body weight measure **(A/F)**, oral glucose tolerance test after 16 hours fasting **(B/G)**, intraperitoneal glucose tolerance test after 4 hours fasting **(C/H)**, intraperitoneal insulin sensitivity test after 6 hours fasting **(D/I)** and blood insulin levels during the IPGTT **(E/J)**. Data are represented as mean ± SEM, \* p<0.05, \*\* p<0.01, \*\*\* p< 0.001.

**Figure 9: Model for the role of Pak3 in beta-cell differentiation and proliferation in the embryo.** Proliferating multipotent pancreatic progenitors eventually turn on Ngn3. Ngn3<sup>+</sup> endocrine progenitors activate Pak3 which participate in the control of cell cycle exit. Pak3 is then maintained in hormone<sup>+</sup>/ Ngn3<sup>-</sup> differentiating endocrine cells. In differentiated embryonic beta-cells Pak3 acts on the regulation of quiescence. However whether Pak3 has to be turned down in order for beta-cells to proliferate remains unknown. Green circles represent cells with Pak3 expression.

**Table 1: Ex vivo quantification of the effect of Pak3 down-regulation on pancreatic explants.** Table summarising the effect of Pak3 on proliferation and endocrine differentiation of E12.5 wild-type pancreatic explants cultured during 4 days with morpholinos. Table contains number and proliferation of Ngn3<sup>+</sup> cells, hormones<sup>+</sup> area, number of Pdx1<sub>high</sub><sup>+</sup> cells, proliferative Pdx1<sup>+</sup> cells, number and proliferation of Sox9<sup>+</sup> cells and total number of cells per explants (n>20).

**Figure S1: Analysis of pancreatic endocrine differentiation in E13.5 and E18.5 Pak3-deficient embryos. (A)** RT-qPCR for *PaksA* and different endocrine *markers* on E13.5 pancreas of Pak3 KO and WT embryos. **(B-C)** Quantification of the insulin<sup>+</sup> and glucagon<sup>+</sup> area **(B)** and Ngn3<sup>+</sup> cells **(C)** in E13.5 pancreas from *Pak3*-deficient and control embryos. **(D)** Quantification of the proliferation of Ngn3<sup>+</sup> cells in E13.5 pancreas from Pak3 KO and wild-type littermates. **(E)** RT-qPCR for *PaksA* and different endocrine *markers* on E18.5 pancreas of Pak3 KO and WT embryos. Data are represented as mean ± SD, \* p<0.05, \*\* p<0.01.

**Table S1: Blood chemistry of Pak3 mutant and control males.** Blood was collected by retro-orbital puncture on 23 weeks old males after a 4h fasting period and analysed for the following components: glucose, urea, total proteins, albumin, bilirubin, total cholesterol, HDL (high density lipid) cholesterol, LDL (low density lipid) cholesterol, triglyceride (TG), free fatty acids (FFA), glycerol, ketone bodies (beta hydroxybutyrate, BHBA), bile acids, creatinine, insulin and leptin. Results are represented at mean ± SD, \* p<0.05, \*\* p<0.01.

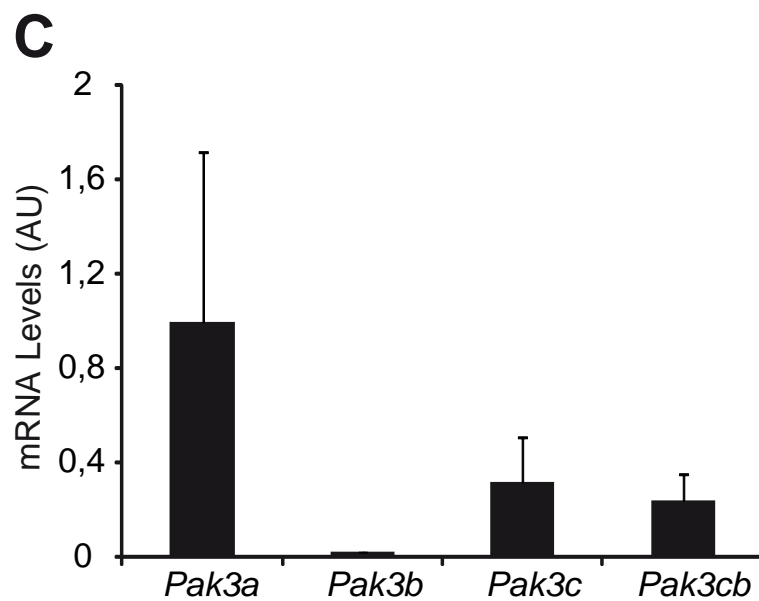
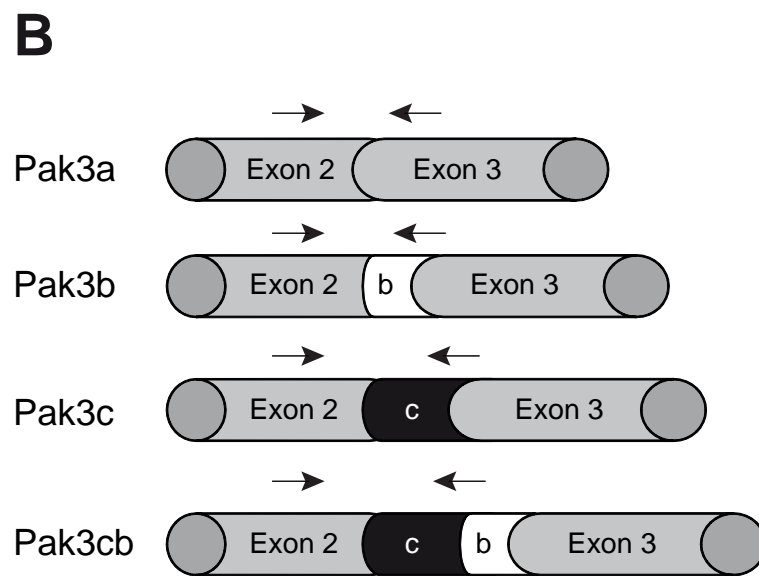
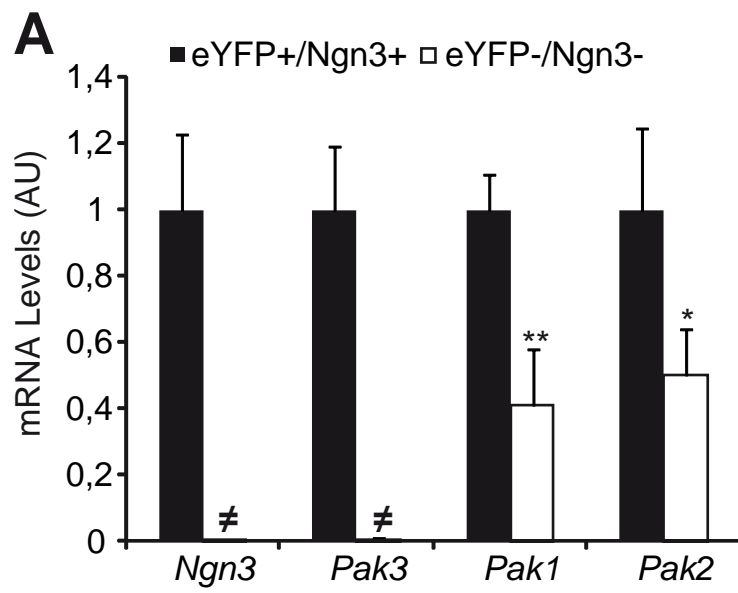


Fig.1

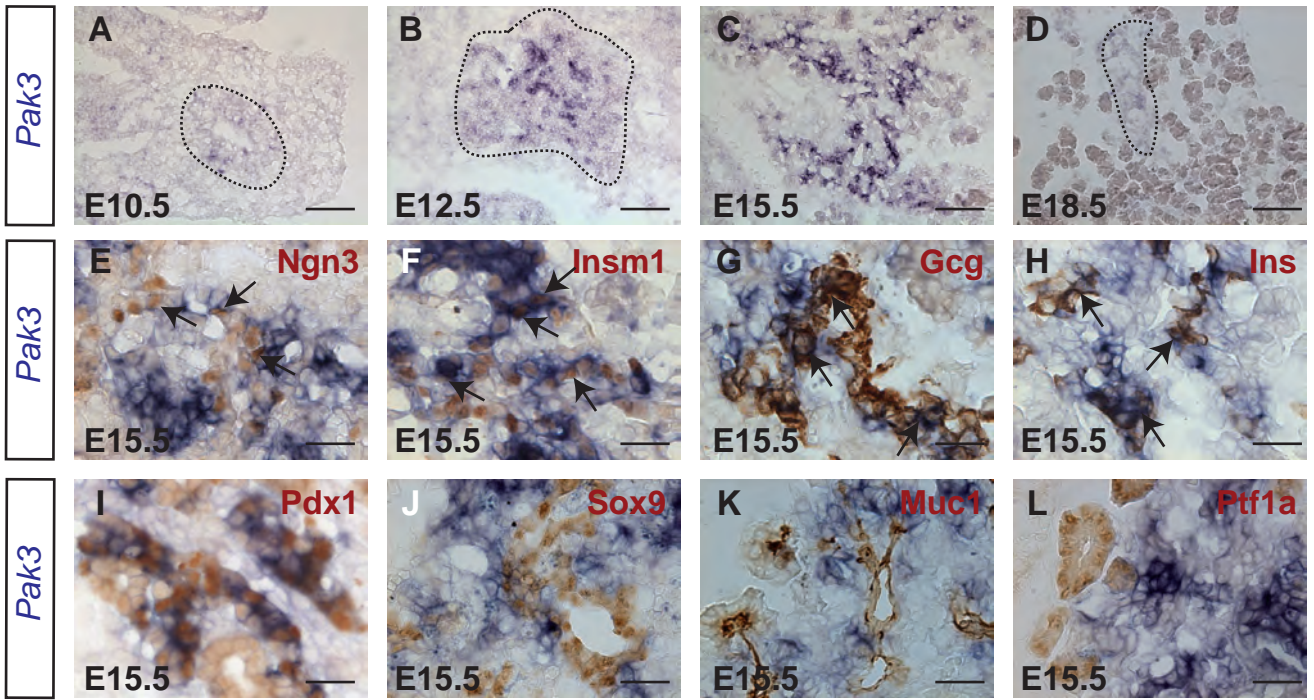


Fig.2

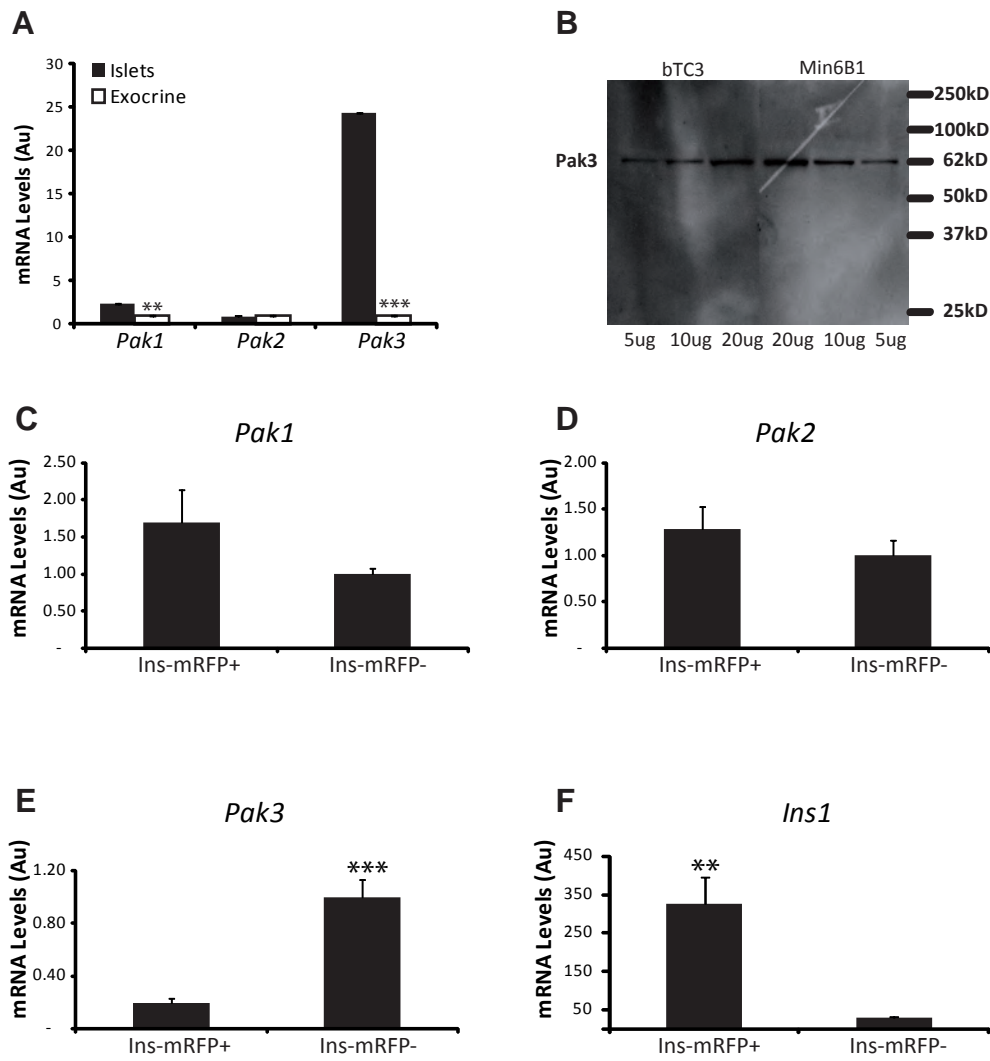


Fig.3



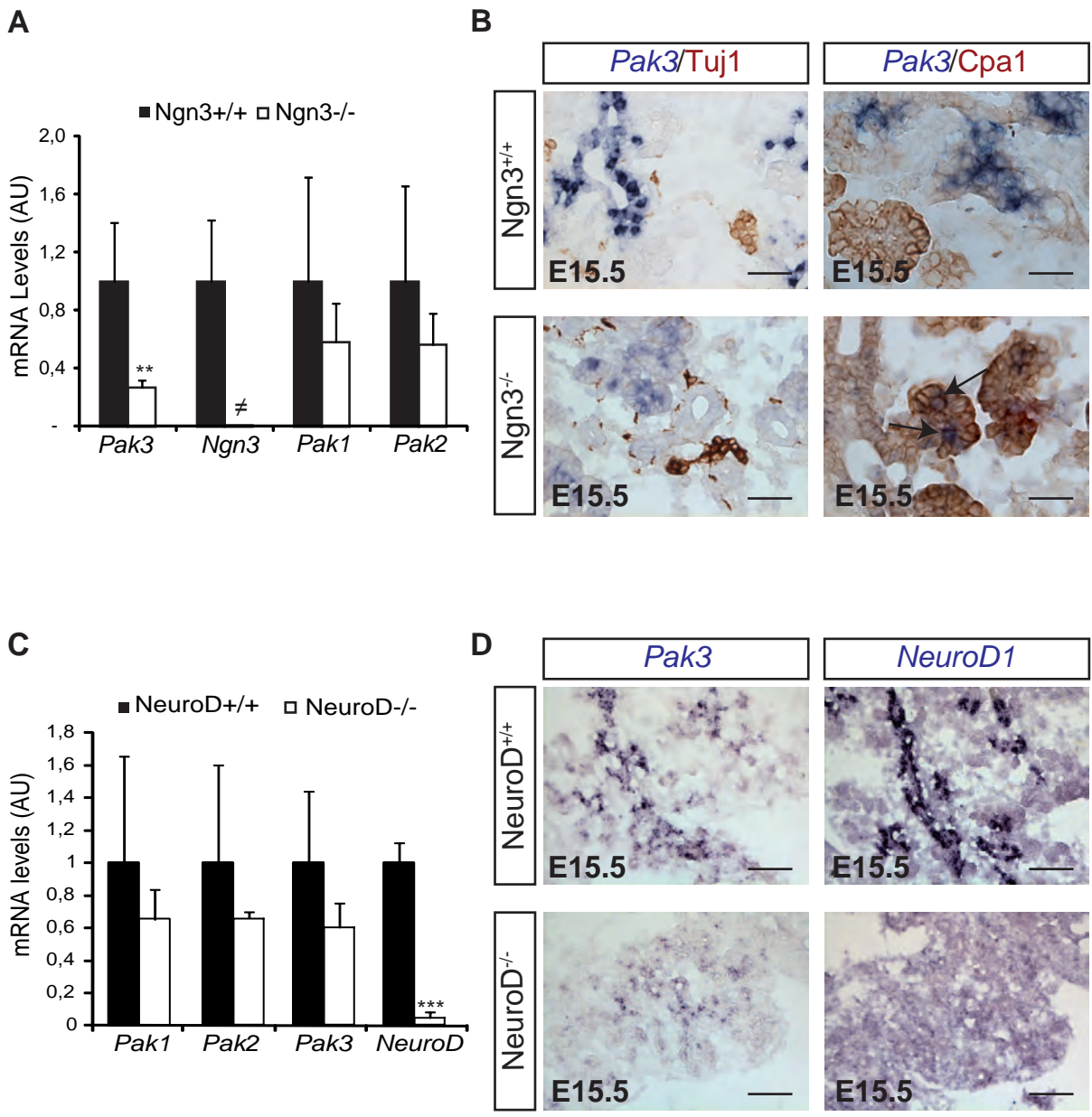


Fig.4

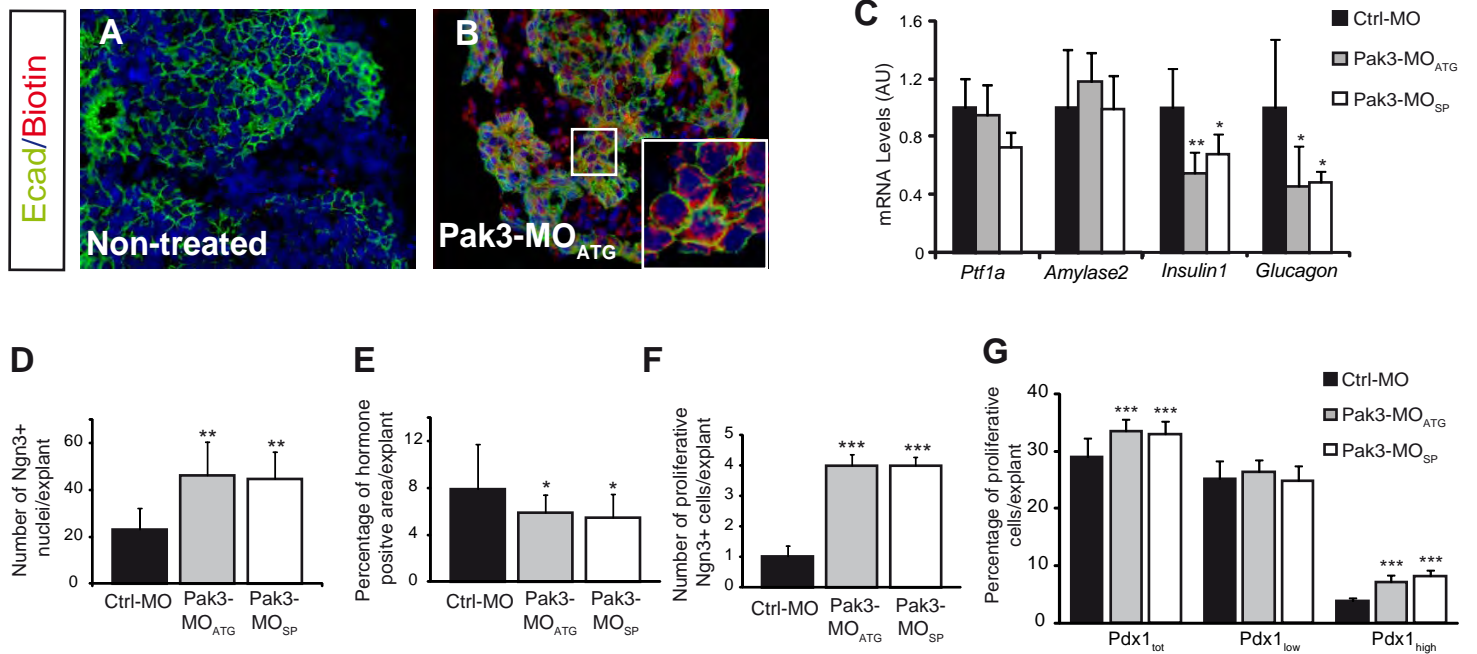


Fig.5

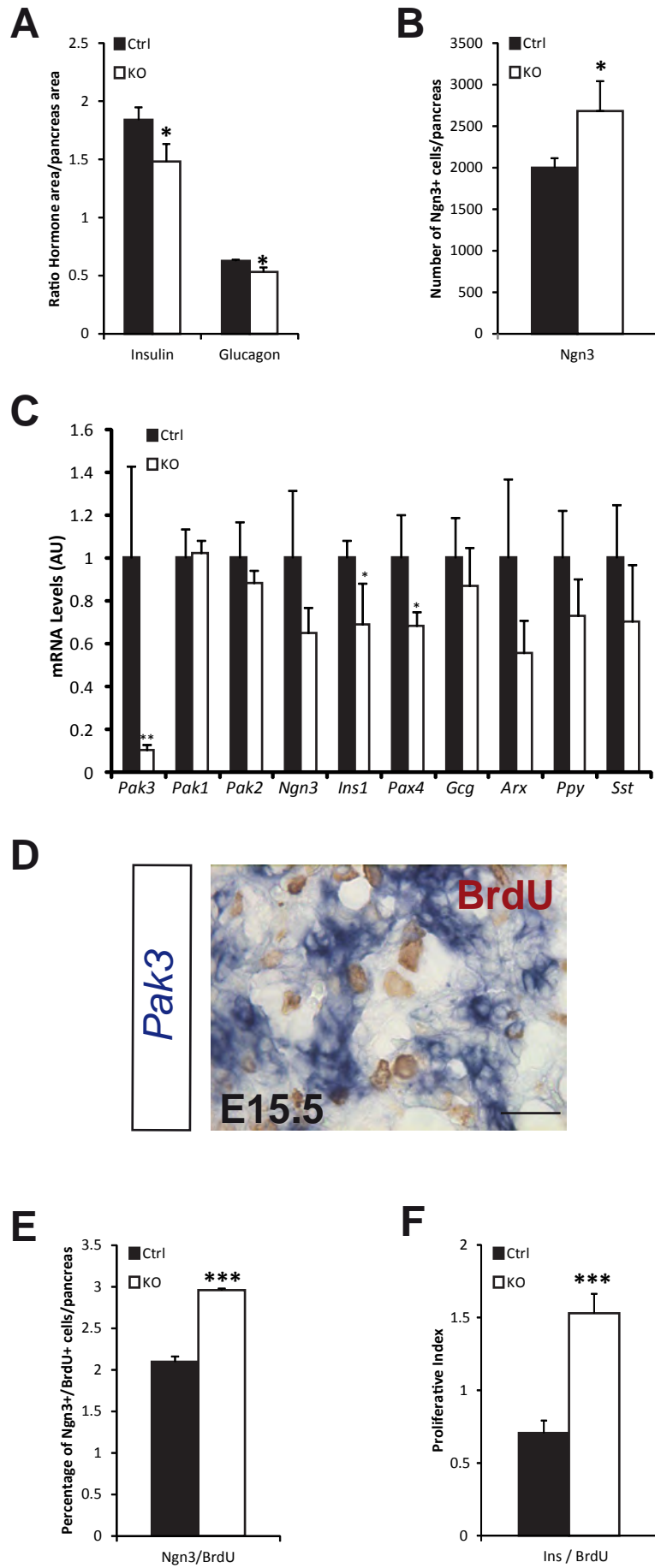


Fig.6

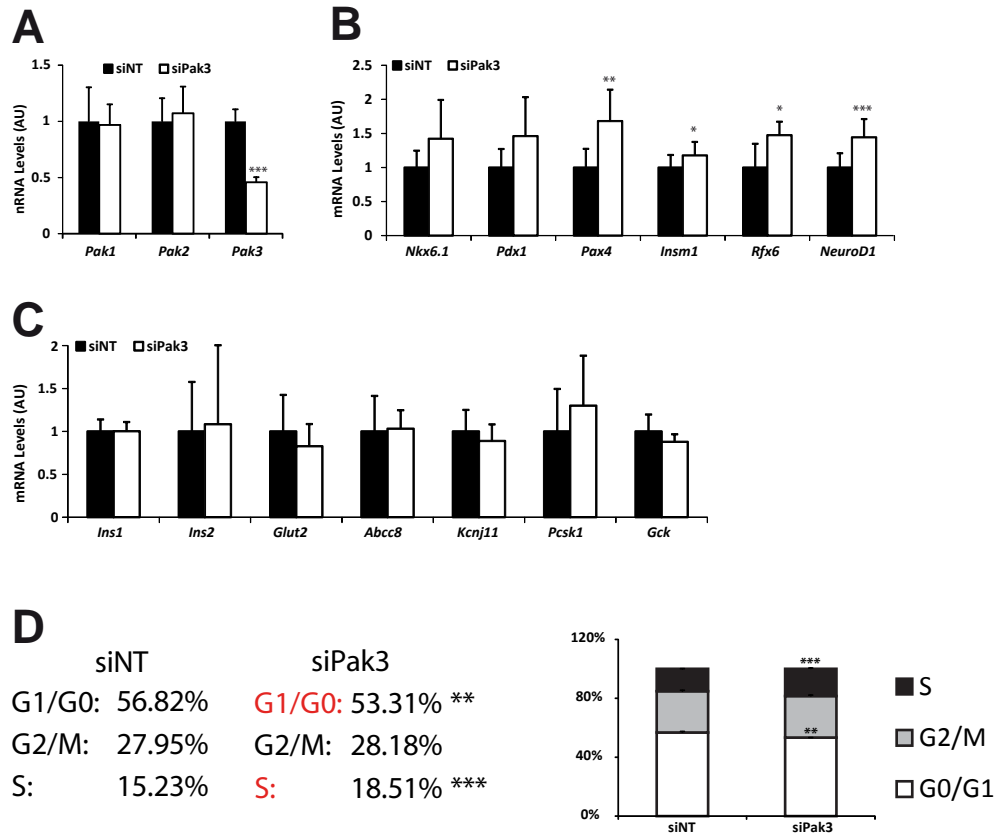


Fig.7

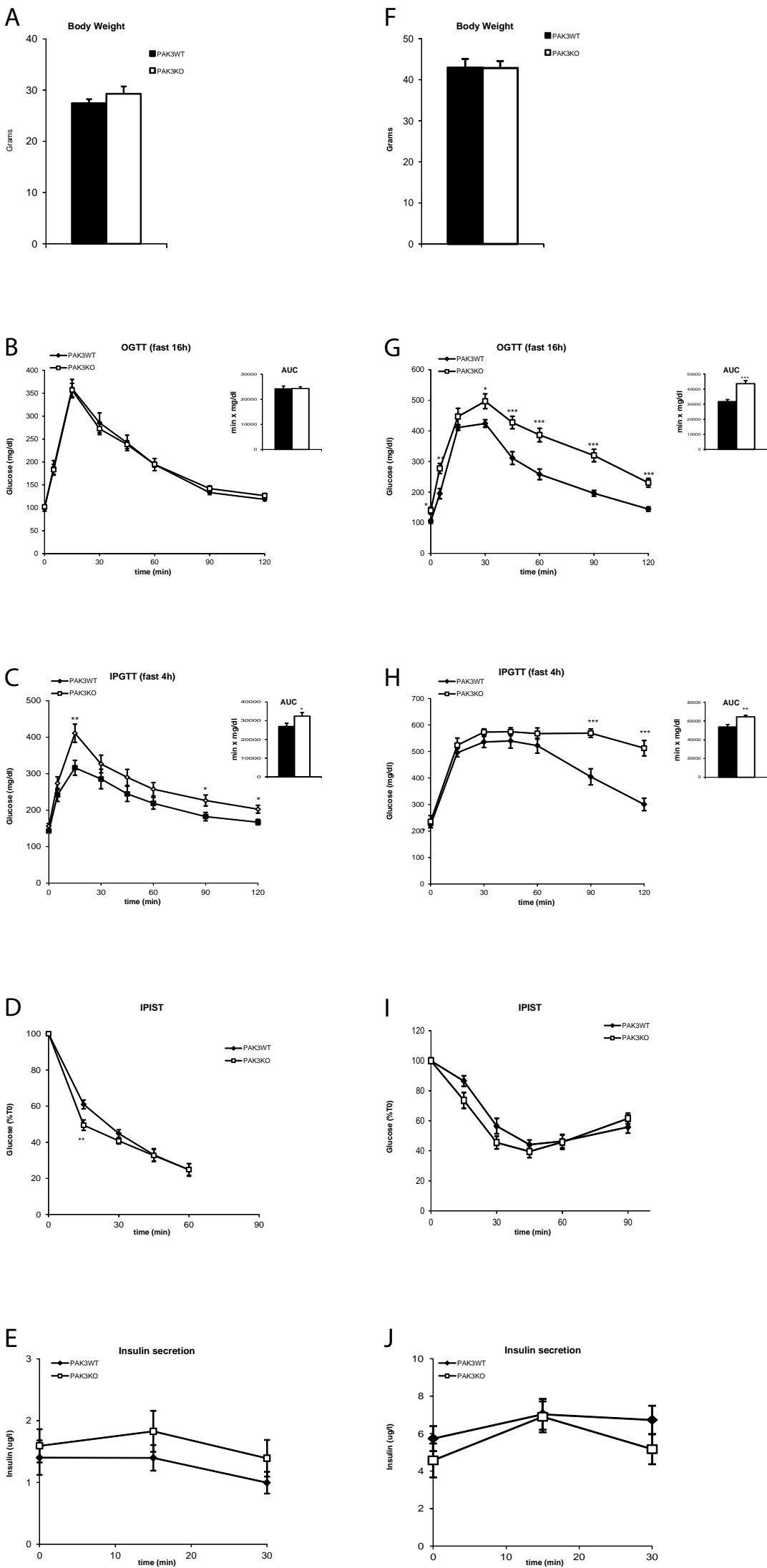


Fig.8

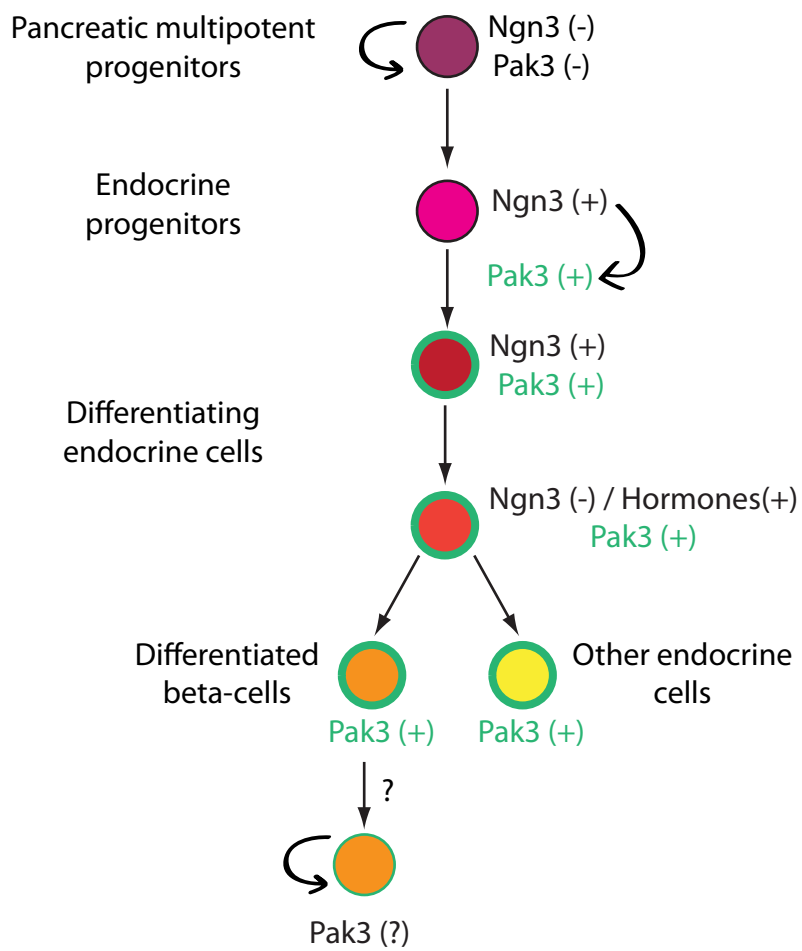


Fig. 9

Table 1: *Ex Vivo* quantification of the effect of Pak3 downregulation

	Ctrl-MO	Pak3-MO <sub>ATG</sub>	Pak3-MO <sub>SP</sub>
Number of Ngn3 cells per explant	23 ± 19	46 ± 28 p=0.008	45 ± 25 p=0.007
Number of proliferative Ngn3 cells per explants	1 ± 0.34	4 ± 0.38 p=0.0007	4 ± 0.29 p=0.001
Percentage of Hormone positive area per explant	7.89 ± 3.82	5.86 ± 2.09 p=0.05	5.47 ± 1.42 p=0.02
Number of Pdx1 <sub>high</sub> cells per explant	643 ± 145	515 ± 177 p=0.02	520 ± 131 p=0.007
Percentage of proliferative Pdx1 <sub>tot</sub> cells per explant	29.04 ± 3.27	33.53 ± 2.19 p=1E-05	32.96 ± 2.24 p=6E-05
Percentage of proliferative Pdx1 <sub>high</sub> cells per explant	3.91 ± 0.47	7.17 ± 1.22 p=4E-11	8.14 ± 1.15 p=6E-15
Percentage of proliferative Pdx1 <sub>low</sub> cells per explant	25.13 ± 3.22	26.36 ± 2.12 p=0.2	24.82 ± 2.57 p=0.7
Number of Sox9 cells per explant	1722 ± 555	1553 ± 474 p=0.3	1623 ± 415 p=0.52
Percentage of proliferative Sox9 cells per explant	33.29 ± 2.69	32.48 ± 2.89 p=0.3	33.61 ± 2.09 p=0.7
Number of cells (Dapi) per explant	4027 ± 669	4284 ± 549 p=0.2	4390 ± 640 p=0.1

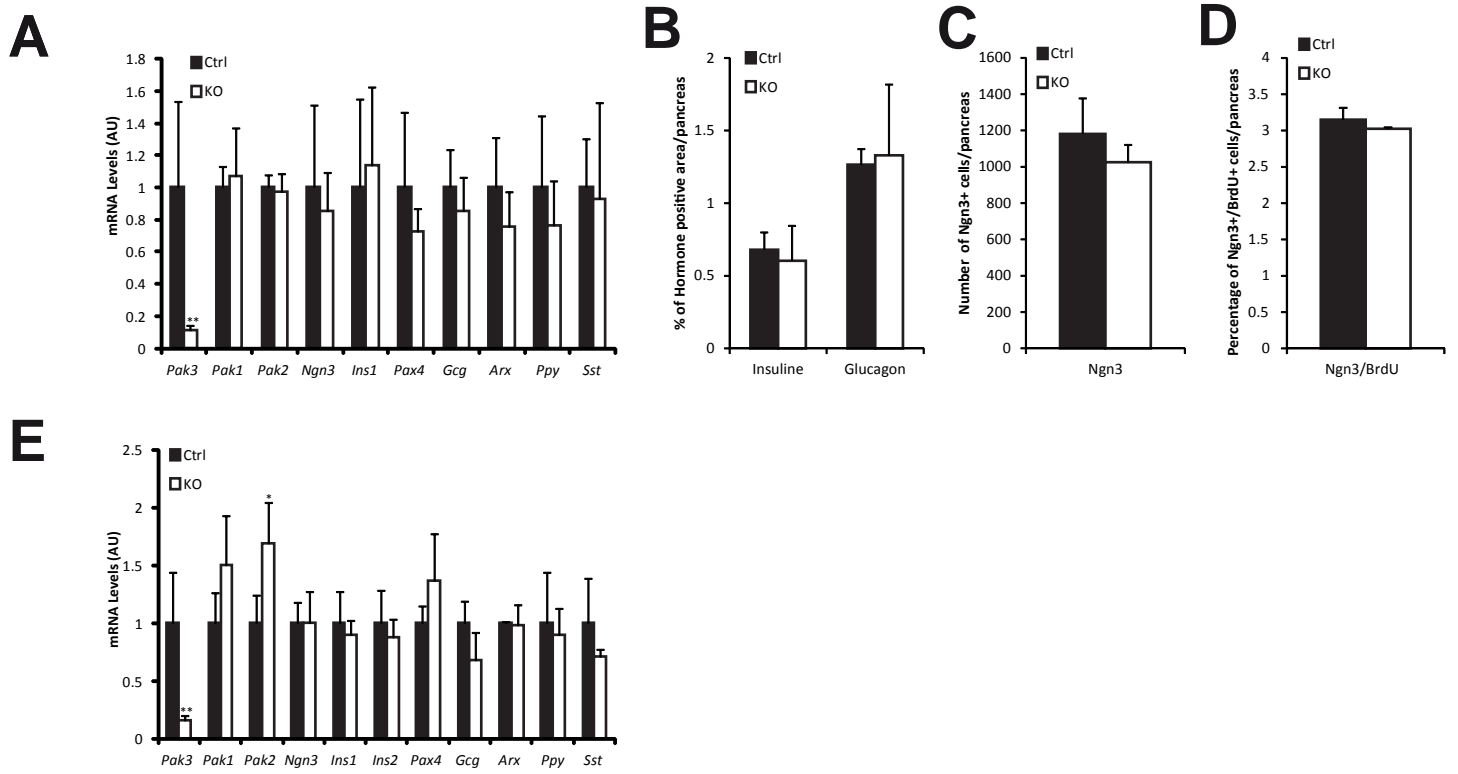


Fig.S1



Table S1: Blood chemistry of Pak3 mutant and control animals

Normal diet	Glucose	Urea	T. proteins	Albumin	T. bilirubin	T. Chol.	HDL Chol.	LDL Chol.	TG	FFA	Glycerol	bHBA	Bile acids	Creatinine	Insulin	Leptin
	mmol/l	mmol/l	g/l	g/l	μmol/l	mmol/l	mmol/l	mmol/l	mmol/l	mEq/l	μmol/l	mmol/l	μmol/l	μmol/l	μg/l	ng/ml
Mean KO	16.085	8.3	53.2	29.7	2.46	2.867	2.129	0.6	0.804	1.134	289.12	0.207	3.201	10.186	1.17	2.2678
SD KO	2.09854	1.66199	3.0840089	1.88856	0.3596294	0.36212	0.25929	0.06749	0.1334	0.125	33.2764	0.0593	3.702811	1.7204663	0.683	2.0418
Mean WT	17.985	8.07	50.7	27.7	2.37	2.783	2.064	0.626	0.945	1.071	268.74	0.237	2.309	9.428	0.533	1.959
SD WT	2.29618	1.31745	1.7669811	0.94868	0.4321779	0.24023	0.20189	0.07321	0.2478	0.12	22.7794	0.0894	1.426421	0.8885669	0.331	1.8681
p value	0.06932	0.73561	<b>0.0391666</b>	<b>0.00781</b>	0.6188595	0.54867	0.5395	0.41979	0.1306	0.265	0.12742	0.3883	0.491199	0.2316637	<b>0.021</b>	0.7348
			*	**											*	

HF diet	Glucose	Urea	T. proteins	Albumin	T. bilirubin	T. Chol.	HDL Chol.	LDL Chol.	TG	FFA	Glycerol	bHBA	Bile acids
	mmol/l	mmol/l	g/l	g/l	μmol/l	mmol/l	mmol/l	mmol/l	mmol/l	mEq/l	μmol/l	mmol/l	μmol/l
Mean KO	22.0842	6.61667	54.083333	28.9167	2.1916667	4.52167	3.35917	0.65667	1.0708	0.745	306.192	0.1792	0.825833
SD KO	3.75484	0.66856	1.6764862	1.24011	0.4737823	0.52277	0.35101	0.14393	0.2364	0.124	103.459	0.0646	0.488382
Mean WT	20.7067	7.55	54.833333	29.5833	2.4916667	4.99917	3.59833	0.77667	1.1458	0.632	226.75	0.2792	0.884167
SD WT	2.6315	0.97561	1.9462474	1.50504	0.2906367	1.12237	0.66356	0.28474	0.2898	0.113	36.6754	0.0661	0.374735
p value	0.30931	<b>0.01212</b>	0.3228127	0.24896	0.0748914	0.20078	0.28538	0.21074	0.4945	<b>0.029</b>	<b>0.02541</b>	<b>0.0011</b>	0.745818
		*								*	*	**	

## 2. Additional results

### 2.1. PaksA expression in the embryonic pancreas

#### 2.1.1. Context

Previous microarray results (Soyer et al., 2010) and RT-qPCR experiments I performed on E15.5 wild-type and Ngn3 KO embryos showed that *Pak3* was strongly enriched in Ngn3<sup>+</sup>/eYFP<sup>+</sup> cells (FC 75) and mostly endocrine specific (only 30% residual expression in Ngn3 KO embryos). These experiments also suggested that *Pak1* and *Pak2* were expressed in the embryonic pancreas. To confirm these results and decipher their expression patterns, I performed a series of *in situ* hybridisation (ISH) on E15.5 wild-type embryos.

#### 2.1.2. Methods

I generated *Pak1* and *Pak2* specific cRNA probes for ISH. To do so, I cloned PCR products in TOPO vector (Figure 35) using TA cloning kit (Invitrogen). These plasmids were then linearised and reverse transcribed and *in situ* hybridisation was carried out on 10µm cryostat sections as previously described (Cau et al., 1997). Pak3 probe is described in the manuscript. Primers used for PCR: *Pak1*: Pak1F 5'-CCCCTCCGATGAGAAACACC-3', Pak1R 5'-CTGGCATCCCCGTAACCTCC-3', *Pak2*: Pak2F 5'-CTACACAAGGTCTGTCATCGACC-3', Pak2R 5'-GCTTGATAGCAACCTCTTGCCC-3'.

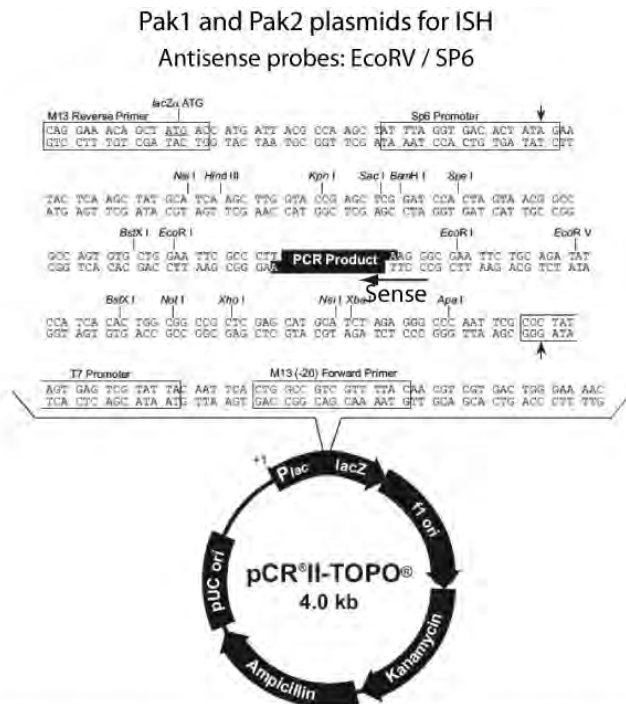
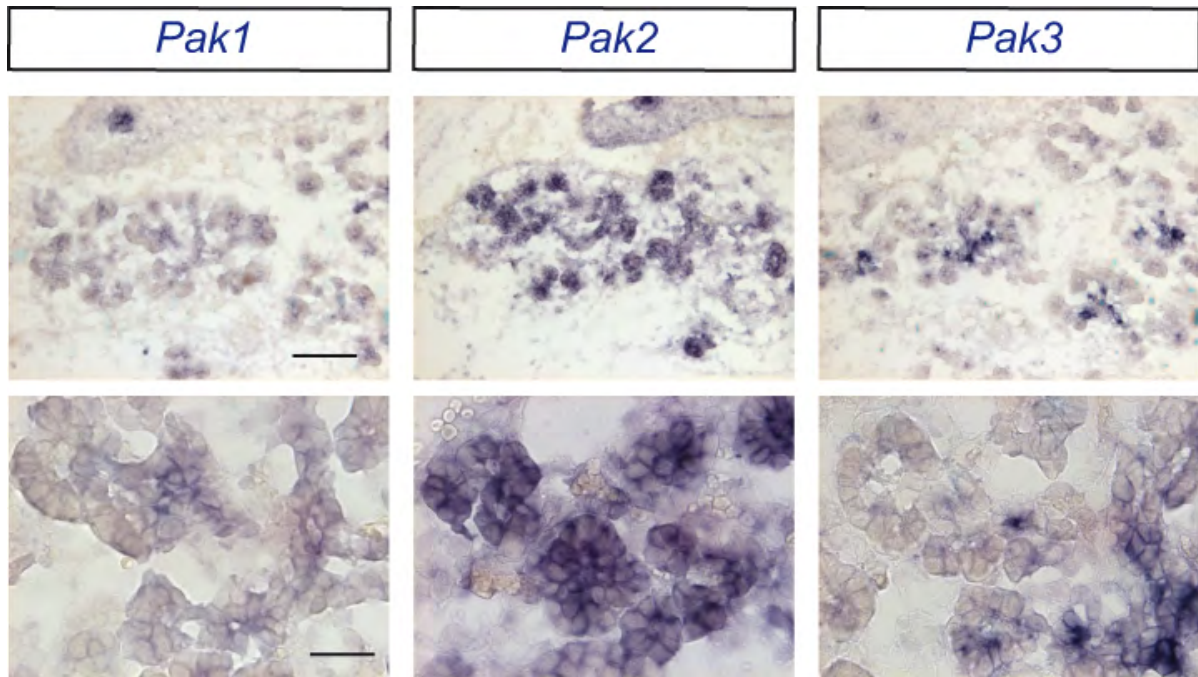


Figure 35: Pak1 and Pak2 plasmids map.

### 2.1.3. Results

As expected from the microarray and RT-qPCR experiments on Ngn3<sup>+</sup>/eYFP<sup>+</sup> cells, we found the expression of *Pak1* and *Pak2* in the embryonic pancreas. Accordingly to their expression in both Ngn3<sup>+</sup> and Ngn3<sup>-</sup> cells by RT-qPCR, *Pak1* and *Pak2* domains are broader than *Pak3* even though *Pak1* expression seems weaker. Thus only *Pak3* is specific to the endocrine lineage. Of note, *Pak2* is expressed to a much higher level than *Pak1* (Figure 36).



**Figure 36: All the PaksA are expressed in the embryonic pancreas but only Pak3 presents a scattered staining reminiscent of developing endocrine cells.** *In situ* hybridisation for *Pak1*, *Pak2* and *Pak3* on wild-type E15.5 adjacent pancreatic sections. Scale bar 50µm (upper row) and 20µm (lower row).

## 2.2. Cell Cycle analysis of Ngn3<sup>+</sup>/eYFP<sup>+</sup> cells on Pak3 KO background

### 2.2.1. Context

We showed that *ex vivo* as well as *in vivo*, Pak3 has a role on the proliferation of Ngn3<sup>+</sup> endocrine progenitors and that *Pak3* down-regulation or absence leads to accumulation of those cells. Because of this increased proliferation observed in *Pak3*-deficient mice, we wanted to characterise the repartition into the different cell cycle phases of Ngn3<sup>+</sup> cells in Pak3 WT compared to Pak3 KO embryos.

### 2.2.2. Methods

To do so, we took advantage of the Ngn3-eYFP mouse line and crossed them with Pak3 WT or Pak3 KO mice. As a single embryo does not give enough cells to perform cell cycle analysis, we decided to generate separately WT and KO litters using brothers and sisters. This avoided the need for genotyping and allowed to pool the embryos directly and yielded to a significantly higher number of sorted cells for cell cycle analysis. To obtain the knockout embryos we crossed Pak3<sup>-/-</sup>; Ngn3<sup>eYFP/eYFP</sup> males with Pak3<sup>-/-</sup>; Ngn3<sup>eYFP/eYFP</sup> females and for the controls we crossed Pak3<sup>+/-</sup>; Ngn3<sup>eYFP/eYFP</sup> males with Pak3<sup>+/-</sup>; Ngn3<sup>eYFP/eYFP</sup> females (Figure 37). Embryos were harvested at E15.5, the pancreas was dissected and digested in trypsin (0.05%) to obtain single cells suspension. These cells were then sorted for YFP fluorescence on BD FACS Aria II using the BD FACS DIVA acquisition Software (version 6.1.3) and kept in 70% EtOH until a sufficient amount of cells was collected. Even though only 20.000 cells are necessary for cell cycle analysis, we eventually figured out that we had to sort about 400.000 cells because of the high loss during sorting and in subsequent centrifugations. When we had sorted enough cells, we stained them for 30 min in propidium iodide (15µg/ml) and ran them on FACS Calibur using Cell Quest Pro acquisition software (version 5.2.1). Cell cycle analysis was performed with ModFit LT 3.2 (Verity Software) and data are reported as mean ± SD.

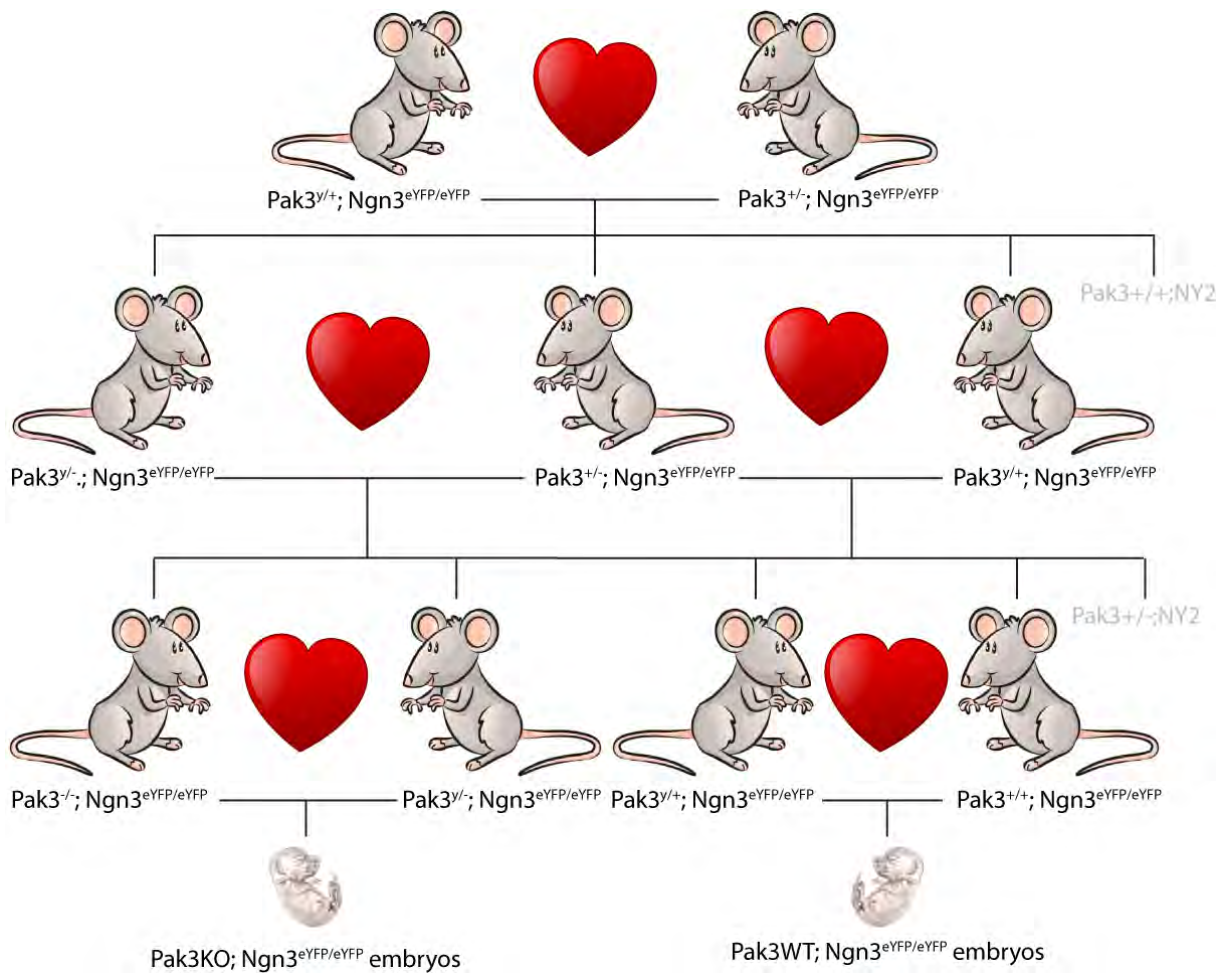
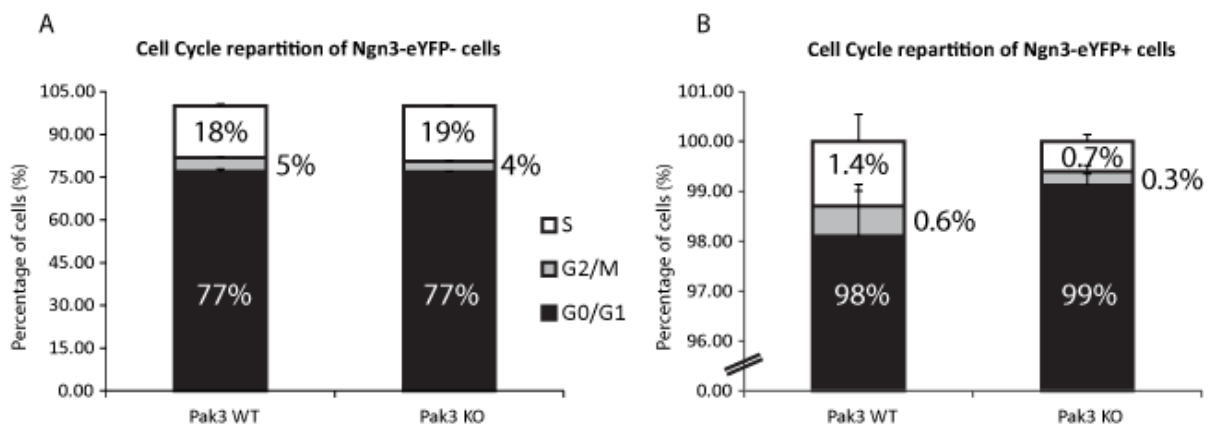


Figure 37: Breeding scheme to obtain Pak3 WT and Pak3 KO embryos.

### 2.2.3. Results

Cell cycle repartition of wild-type Ngn3<sup>+</sup>/eYFP<sup>+</sup> and Ngn3<sup>-</sup>/eYFP<sup>-</sup> cells was as expected from previous experiments done in the lab (Martin, unpublished). Namely around 1-2% of Ngn3<sup>+</sup>/eYFP<sup>+</sup> cells are in S phase and about 18-20% of the Ngn3/eYFP<sup>-</sup>. Since Pak3 is not expressed in the Ngn3<sup>-</sup>/eYFP<sup>-</sup> cells (Pak3 manuscript Figure 1) we did not expect to see any change in cell cycle repartition of Ngn3<sup>-</sup>/eYFP<sup>-</sup> cells and this is what we saw (Figure 38A). However, because of the role of Pak3 in the proliferation of endocrine progenitors and endocrine cells we were expecting a higher amount of cells in G2/M and/or S phase in the Pak3 KO background. To our surprise no significant change was detected in the repartition of Ngn3<sup>+</sup>/eYFP<sup>+</sup> cells in the cell cycle (Figure 38B). Of note, this technic does not allow to discriminate between quiescent cells in G0 and dividing cells in G1 thus these results cannot exclude that more cells have actually entered cell cycle since we cannot detect cells going from G0 to G1.



**Figure 38: Cell cycle repartition of Ngn3<sup>+</sup>/eYFP<sup>+</sup> cells is not affected in the absence of Pak3. (A)** Cell cycle repartition of Ngn3<sup>-</sup>/eYFP<sup>-</sup> sorted cells in Pak3 WT and Pak3 KO backgrounds. **(B)** Cell cycle repartition of Ngn3<sup>+</sup>/eYFP<sup>+</sup> sorted cells in Pak3 WT and Pak3 KO backgrounds. The cell cycle analysis is a summary of 4 different sorting experiments.

## 2.3. Culture of pancreatic explants with PaksA inhibitors

### 2.3.1. Context

Using explants culture and *Pak3* morpholinos we showed that *Pak3* was necessary for endocrine cell differentiation *ex vivo*. However the effect was only mild and *in vivo* experiments showed that differentiation resumes at later stage. We hypothesised that because of the high similarity between the 3 PaksA, namely *Pak1*, *Pak2* and *Pak3*, there could be a certain redundancy in the function of these genes. We looked in the literature and found that a small molecule, IPA-3, which directly and noncompetitively inhibits PaksA activity (Deacon et al., 2008) has been successfully used to block PaksA signalling in the brain (Rex et al., 2009). Thus we decided to test this compound on our explant system to study how PaksA affect endocrine cell differentiation.

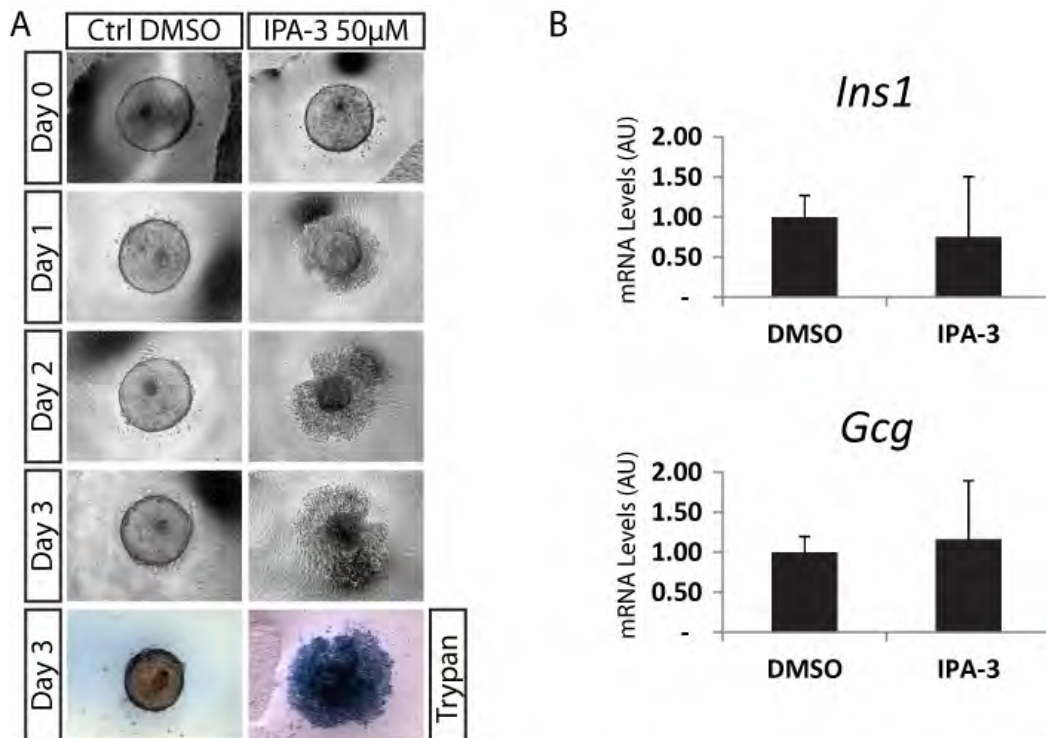


### 2.3.2. Methods

Pancreata were dissected from E11.5 embryos and explants were cultured without mesenchyme during 7 days. We added IPA-3 (range: 5-50 $\mu$ M) directly in the culture medium and changed it every day. At different time points, we performed trypan blue staining to check for cell viability. At the end of the culture time, we collected explants either for RNA extraction and RT-qPCR or for western blotting. Antibodies used: goat@Pak3 1:200 (Santa Cruz), rabbit@phospho-Pak1,2,3 (pThr402) 1:500 (Thermo Scientific), goat@rabbitHRP 1:20.000 (IGBMC) and donkey@goatHRP 1:20.000 (Santa Cruz).

### 2.3.3. Results

We realised that at the suggested dose of 50 $\mu$ M explants were dying within the first 2-3 days of culture (Figure 39A). We thus tested a gradient of concentrations (from 5 $\mu$ M to 50 $\mu$ M) and realised that only at very low dose (5 $\mu$ M), the epithelium survived the whole culture time (not shown). After 7 days we harvested the explants and performed RT-qPCR because they were too fragile for inclusion and subsequent immunofluorescence experiments. We observed no differences in the mRNA of *Ins1* and *Gcg* (Figure 39B). Because of this result we wanted to determine if the activity of PaksA was down-regulated using a phospho-specific PaksA antibody. However we never succeeded in collecting enough material to obtain a convincing signal on western blots even when we pooled up to 20 explants, we thus decided at that time to stop the cultures and focus on the *in vivo* data.



**Figure 39: PaksA inhibitor, IPA-3, is lethal for epithelium explant cultures. (A)** Brightfield microscope images of selected explants during culture in the presence or not of IPA-3. Last row shows trypan blue staining showing cell death in epithelium cultured with 50 $\mu$ M IPA-3 compared to control treated with DMSO. **(B)** RT-qPCR for *Ins1* and *Gcg* on whole pancreatic explants after 7 days of culture with or without IPA-3 (5 $\mu$ M) n=3, each n being a pool of 10-20 explants.

### 3. Discussion/Perspectives/Conclusion

The major discussion points have been addressed in the manuscript; however in this section I want to summarise the results, place them in the current context and give some perspectives.

During my thesis, I characterised the expression of the kinase *Pak3* and showed that it is expressed in the pancreas in a sub-population of  $\text{Ngn3}^+$  cells but mainly in differentiating endocrine cells during development and maintained in adult islets. I identified *Pak3* as a new gene implicated in the coupling of cell cycle exit and endocrine cell differentiation both *ex vivo* and *in vivo*. In the adult, even though *Pak3*-deficient mice show no obvious metabolic defects under normal diet, I showed that *Pak3* KO mice are glucose intolerant under HFD suggesting a role for *Pak3* in islet cell function.

However, the **downstream targets phosphorylated** by *Pak3* in these processes are not known and will need to be investigated for example by phosphoproteomic. Pancreas from *Pak3* WT and KO mice are lysed and enzymatically digested, then peptides are separated using ion exchange chromatography, phosphopeptides are enriched with phosphospecific antibodies and analysed using mass spectrometry and finally sequenced and analysed. Comparing data from WT and KO should give us a list of the molecules implicated in the phenotype of *Pak3*-deficient mice.

To understand the function of *Pak3* in the maintenance of quiescence in endocrine cells, we will further characterise the **proliferation of beta-cells** by quantifying dividing beta-cells perinatally as it is known that quiescent developing beta-cells re-enter cell cycle and proliferate massively to expand beta-cell mass **perinatally**. Moreover, quantification of the proliferation in the **adult** might help us to understand the phenotype of *Pak3* KO mice. We also showed that *Pak3* is expressed at higher levels in non-beta cells in adult islets, thus it will also be necessary to investigate the **proliferation of alpha-cells** both in the embryo and in the adult.

Interestingly, in our metabolic study, we did not find any differences in the insulin levels in the blood of *Pak3* KO compared to *Pak3* WT males. However, looking at blood insulin during the IPGTT is not very sensitive and technical limitations prevented us to investigate the first phase of insulin secretion (between 0-15 minutes). To overcome this problem, we will perform **insulin (and glucagon) secretion tests** in response to glucose on purified islets. Moreover, blood chemistry revealed an increased blood concentration of free fatty acids, glycerol and ketone bodies (bHBA) in *Pak3*-deficient mice under HFD suggesting defects in **liver metabolism**. Thus it is important to look at *Pak3* expression in the liver and in other organs related to glucose homeostasis like the intestine, adipocytes and skeletal muscles.

Of note, we found in a microarray experiment where we compared  $\text{Ngn3}^+/\text{eYFP}^+$  cells of the duodenum to other  $\text{Ngn3}^-/\text{eYFP}^-$  duodenal cells, that *Pak3* was strongly enriched (FC 50) in  $\text{Ngn3}^+/\text{eYFP}^+$  cells. Because of the numerous similarities between endocrine cells of the pancreas and their enteroendocrine cousin in the intestine, and also because the phenotype of *Pak3* KO mice may involve the incretins (Glp1 and Gip), we believe that *Pak3* could have a role in **enteroendocrine cell differentiation** and function. To explore this hypothesis, we will characterise by *in situ* hybridisation the expression of *Pak3* and the intestine and study the role of *Pak3* on enteroendocrine cell development by immunofluorescence. Altogether these additional experiments should help us understand the phenotype of *Pak3* KO mice.

## B. Role of Rfx6 in pancreatic and intestinal endocrine cell differentiation and function

Future strategies for cell therapies strongly depend on our knowledge of the precise mechanisms which control the differentiation of multipotent progenitor cells into highly specialised beta-cells. Along this line, the goal of the lab of Gérard Gradwohl where I did my thesis is to understand how pancreatic and intestinal endocrine cells are generated from Ngn3<sup>+</sup> progenitors both during embryogenesis and in the adult (Figure 18). The focus is mainly on the role of transcription factors and signals that control cell fate choices and acquisition of specific properties of islet and enteroendocrine cells during development. In 2010 in the lab, Soyer and colleagues identified for the first time the expression of Rfx6 in the mouse pancreas and characterised its function during zebrafish development (Soyer et al., 2010). Briefly, they showed that very early Rfx6 is expressed in the whole primitive foregut endoderm and then becomes progressively restricted to the endocrine lineage in the pancreas from E10. Rfx6 is also expressed in differentiated islet cells during development and in the adult (Soyer et al., 2010). In collaboration with the lab of Marianne Voz in Liege, they studied the endocrine function of Rfx6 in zebrafish and showed that a reduced expression of zRfx6 with morpholinos prevents the differentiation of endocrine progenitors, causing a depletion of the number of glucagon, somatostatin and ghrelin cells and a disorganisation of the beta-cells (Soyer et al., 2010). Later that year, this gene was shown to be important in mice and human where it directs the formation of islets and the production of insulin. Mice deficient for *Rfx6* develop no hormone-secreting endocrine cells, except the cells producing the pancreatic polypeptide and die at P2 (Smith et al., 2010). Human patients with RFX6 mutations have neonatal diabetes caused by the absence of insulin, glucagon and somatostatin cells. In addition, the syndrome also involves hypoplastic gall bladder, intestinal atresia and diarrhoea (Smith et al., 2010).

Because of its particular expression pattern (early in the whole foregut endoderm and then restricted to the endocrine lineage in the pancreas) which suggests different functions of Rfx6 during development and in the adult, we decided to study its function using a conditional KO mouse. We generated in collaboration with the ICS (Mouse Clinical Institute, Illkirch), a conditional KO mouse for Rfx6 by flanking the exon 3 in the DNA binding domain by two loxP sites. Thus, upon Cre mediated recombination, the exon 3 is deleted and no functional Rfx6 protein can be made. To study the function of Rfx6 in different cell types, I crossed the conditional KO with different Cre lines: CMV-Cre to generate a full KO; Ngn3-Cre for the endocrine function (pancreas and intestine); Ins1-CreER<sup>T2</sup> for the function in adult beta-cells; Gcg-CreER<sup>T2</sup> for the function in adult alpha-cells; Villin-Cre for the early intestinal function; and Villin-CreER<sup>T2</sup> for the function in the adult intestine.

In the next pages, you will find the results I obtained on the function of Rfx6 during pancreatic endocrine cell differentiation, in the adult beta-cells as well as in the developing and adult intestine. The results are written in the form of a manuscript even though much work remains to be done, especially on the metabolic consequences of Rfx6 deletion and the gene regulatory network downstream of Rfx6 before it can be submitted.



## **1. Manuscript in preparation**

**Rfx6 mediates Ngn3-dependent endocrinogenic program in the islet and enteroendocrine lineages and is required for the maintenance of beta-cells in the adult mice**

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## ABSTRACT

*Aims/hypothesis:* The winged helix transcription factor Rfx6 is expressed in the early gut and pancreatic endoderm and later becomes restricted to developing and adult islet cells. Previous studies showed that Rfx6 is required during embryogenesis for proper islet cell development in zebrafish, mouse and human. The objective of this work was to unravel the role of Rfx6 in intestinal endocrine cells as well as in adult beta-cells.

*Research design and methods:* Rfx6-floxed mice were generated and crossed with Ngn3-Cre, Villin-Cre, Villin-CreER<sup>T2</sup> and Ins1-CreER<sup>T2</sup> mice to specifically and conditionally inactivate Rfx6, in the pancreatic and intestinal endocrine lineages, in the intestinal epithelium and in adult beta-cells. Immunofluorescence studies and quantitative RT-qPCR experiments were used to evaluate the differentiation of hormone-expressing cells, the expression of transcription factors regulating islet and enteroendocrine cell differentiation as well as the expression of markers of mature beta- and enteroendocrine cells.

*Results:* In the intestine Rfx6 is expressed in Ngn3<sup>+</sup> endocrine progenitors and maturing enteroendocrine (EE) cells. Mice lacking Rfx6 in endocrine progenitors are diabetic and die at P2-P3. They fail to generate hormone-expressing cells except PP-cells in the pancreas and serotonin-cells in the intestine. Constitutive deletion of Rfx6 exclusively in the intestine is lethal and EE cell differentiation is impaired. Rfx6 removal in the adult intestine leads to diarrhoea, weight loss and defective lipids absorption consecutively to impaired enteroendocrine cell differentiation. Rfx6 removal specifically in adult beta-cells severely reduces the transcription of *insulin* genes as well as of other key regulators of beta-cell function (e.g *Glut2*, *Gck*, *Kir6.2*, *Sur1*).

*Conclusion:* Rfx6 is essential to implement the Ngn3-dependent endocrinogenic program in the developing pancreas and intestine. Endocrine defects result from Rfx6 function downstream of Ngn3 and not from an earlier role in the endoderm. In the adult, Rfx6 is required for proper EE cell differentiation, intestinal absorption and maintenance of beta-cells.

## INTRODUCTION

Diabetes mellitus is a world-leading disease that cannot be cured to date. The Edmonton study provided hope that cell-based therapy could one day be a cure for diabetes (Shapiro et al., 2000). To overcome the lack of donors, many researchers have been trying to recapitulate *in vitro* the developmental programs of endocrine cell differentiation with the aim to generate beta-cells able to respond accurately to glucose stimuli. However, to date these cells have to be transplanted into mice to achieve proper maturation (Kroon et al., 2008; Rezanian et al., 2012). This points to the fact that we need to keep research going to achieve a more in-depth knowledge of the cascades regulating beta-cell development during embryogenesis.

In the mouse embryo, the pancreas appears as clusters of cells on the primitive foregut endoderm. All pancreatic cell types differentiate from these clusters of pancreatic progenitors (Pan and Wright, 2011). The expression of the pro-endocrine bHLH transcription factor *Ngn3* is necessary and sufficient to drive these progenitors to an endocrine fate and mice lacking *Ngn3* fail to develop endocrine cells (Apelqvist et al., 1999; Gradwohl et al., 2000; Schwitzgebel et al., 2000). As in the pancreas, the differentiation of endocrine cells of the intestine relies on *Ngn3* (Jenny et al., 2002). Recently we showed that mice with an intestinal-specific ablation of *Ngn3* were deficient for all enteroendocrine cells and hormones and frequently died during the first week of life (Mellitzer et al., 2010). Mutant mice were growth retarded and had impaired lipid absorption. Interestingly, these mice also exhibited an enlarged proliferative crypt compartment and accelerated turnover of the intestinal epithelium (Mellitzer et al., 2010). Both in the intestine and in the pancreas, the transient expression of *Ngn3* triggers the expression of transcription factors, including *NeuroD1*, *Pax4*, *Arx*, *Insm1* and others, which then direct the differentiation into the different endocrine cell types (Beucher et al., 2012; Gierl et al., 2006; Naya et al., 1997). Mutations in several genes controlling pancreatic endocrine differentiation in mice, including *NGN3* and its downstream targets, can cause neonatal or childhood diabetes demonstrating their key function in humans as well (Molven and Njolstad, 2011; Rubio-Cabezas et al., 2010). Thus, deciphering the genetic programs controlling beta-cell differentiation in the mouse embryo provides important clues to generate surrogate beta-cells from multipotent stem cells to treat diabetes mellitus. Despite these findings, our knowledge of *Ngn3*-regulated programs is still incomplete. Particularly, we do not understand if or how this transcription factor integrates the generic program of endocrine differentiation with the programs that specify the different islet cell types. In this context, we identified recently the winged helix transcription factor *Rfx6* as a novel *Ngn3*-dependent transcription factor controlling pancreatic islet cell development (Soyer et al., 2010). In human, several mutations in the *RFX6* gene have been identified in an autosomal recessive syndrome characterised by neonatal diabetes, small bowel

obstruction and biliary abnormalities associated with severe intestinal malabsorption and diarrhoea (Smith et al., 2010; Spiegel et al., 2011). Autopsies of two cases and analysis of the pancreas revealed clusters of chromograninA<sup>+</sup> cells but absence of staining for insulin, glucagon and somatostatin (Mitchell et al., 2004). Similarly, in *Rfx6*-deficient mice, with a constitutive deletion of the gene, islet cell development is initiated but not completed. Mice thus fail to develop any hormone-expressing islet cells except for pancreatic-polypeptide-producing cells and die at P2 (Smith et al., 2010). A similar even though less severe phenotype was observed in mice deficient for *Rfx3*, another member of the RFX family (Ait-Lounis et al., 2007; Ait-Lounis et al., 2010). Just before birth, *Rfx3*<sup>-/-</sup> islets contain less insulin-, glucagon- and ghrelin-producing cells, whereas PP<sup>+</sup> cells are markedly increased (Ait-Lounis et al., 2007). Most mutant mice die in utero from left-right asymmetry defects. However, rare surviving adults have smaller islets, defective insulin secretion and are glucose intolerant (Ait-Lounis et al., 2007). Furthermore *Rfx3* was shown to directly bind the promoter of the *glucokinase* gene (Ait-Lounis et al., 2010).

*Rfx6* is essential for islet cell development in mouse, human and zebrafish. However, the early expression of this transcription factor in the gut and pancreatic endoderm, then in developing pancreatic endocrine cells and next in adult islet cells does not allow the precise dissection of *Rfx6* function in mice with a constitutive deletion of the gene. Furthermore, the postnatal lethality of *Rfx6*-deficient mice precludes any metabolic study in adults. Finally, *Rfx6* function in the differentiation of gut endocrine cells has not been studied so far. Therefore we decided to generate *Rfx6*-floxed mice to decipher the role of *Rfx6* specifically in endocrine cell differentiation as well as in mature beta-cells and in the adult intestine.

In this study, the deletion of *Rfx6* uniquely in *Ngn3*<sup>+</sup> cells and descendants demonstrates that it is the function of *Rfx6* downstream of *Ngn3* that is responsible for the loss of endocrine cells in the full knockout and not an early role in the endoderm. Conditional deletion in adult beta-cells supports a role for *Rfx6* expression in the maintenance of beta-cells. Moreover, we report for the first time the expression and function of *Rfx6* in the enteroendocrine lineage of the intestine and show that similarly to the pancreas, the expression of *Rfx6* in the intestine is necessary for enteroendocrine cell differentiation. Mice lacking *Rfx6* in the gut, fail to develop enteroendocrine hormones-expressing cells, except serotonin cells and die within the first week of life.

## RESULTS

### **Ngn3-dependent Rfx6 function is necessary for endocrine cell development in the pancreas.**

The special expression pattern of Rfx6, namely its broad expression very early in the gut and pancreas endoderm and then restricted to endocrine cells in the pancreas from E10.5 and maintained in adult islets (Smith et al., 2010; Soyer et al., 2010) strongly suggested that Rfx6 could have diverse functions at different stages and in different organs. To identify the different roles of Rfx6, we generated in collaboration with the Mouse Clinic Institute (ICS, Illkirch, France) a conditional KO mouse for Rfx6 by flanking the exon 3 of the DNA binding domain by 2 loxP sites (Figure 1A). Upon Cre-mediated recombination, exon 3 is deleted and no functional Rfx6 protein can be made (Figure 1B). Of note, a hypothetical protein could be made from an ATG present in the exon 8 of *Rfx6*. However this ATG is in another open-reading-frame and the putative protein has no known functional domain. To validate our approach, we generated a full KO using a CMV-Cre line and we were able to recapitulate the loss of all endocrine cells except the PP-expressing cells described in another study of a constitutive KO (Smith et al., 2010) but not the intestinal atresia (data not shown).

To determine the role of Rfx6 in the endocrine lineage, we crossed our conditional KO with a Ngn3-Cre line (Yoshida et al., 2004) and reported by immunofluorescence efficient deletion of Rfx6 in Ngn3-Cre; Rfx6<sup>fl/fl</sup> mutant animals (Figure 1C-D). For simplicity we called these mutants  $\Delta$ Endo thereafter.  $\Delta$ Endo pups are diabetic (not shown) and die between 2-3 days after birth. Interestingly,  $\Delta$ Endo mice did not have any intestinal atresia. We showed by immunofluorescence (Figure 1C-H) and RT-qPCR (Figure 1I) that endocrine cells are formed in  $\Delta$ Endo mutants but the development of hormone-expressing cells was severely impaired, except for PP-cells. Interestingly, at P0 we found a previously not reported decrease of *chromograninA* transcripts (Figure 1I). We further characterised the role of Rfx6 downstream of Ngn3 by exploring the expression levels of several important transcription factors. Interestingly and not previously reported, we showed that *Ngn3* transcripts were elevated by 4-fold in the pancreas of  $\Delta$ Endo mutants at birth (Figure 2 bottom right). However, because of the high variability in the numbers of remaining Ngn3<sup>+</sup> cells at birth, we were not able to confirm this increase by quantification of the number of cells. Our results also showed that subtype specification and endocrine cell maturation was affected in  $\Delta$ Endo pups compared to their control littermates (Figure 2). More specifically, *Arx* transcripts were strongly diminished whereas *Pax4* was found to be highly elevated. Gene coding for transcription factors controlling beta-cell maturation and insulin transcription such as *MafA* or *NeuroD1* were reduced. However, some endocrine markers, like *Nkx6.1* and *Insm1*, were not affected suggesting that an endocrine differentiation program has been implemented but cannot be completed.

Altogether, these data suggest that Rfx6 could act on subtype specification by repressing beta-cell fate via *Pax4*. These results, similar to what was reported in the Rfx6 full KO (Smith et al., 2010), show that it is Rfx6 function downstream of Ngn3 which is responsible for the endocrine phenotype observed in mutant mice and not the expression of Rfx6 early in the endoderm.

#### **Rfx6 is necessary for proper enteroendocrine cell differentiation during development.**

Because pancreatic and intestinal endocrine cells share many molecular, cellular and functional characteristics, we also investigated whether Rfx6 was expressed in the intestine. We performed a series of immunofluorescence experiments on adult intestinal tissue from CD1 wild-type mice and found that Rfx6 was expressed in a subset of Ngn3<sup>+</sup> endocrine progenitors (Figure 3A-F) and maintained in all differentiating enteroendocrine cells, except gastrin, in the crypt and at the bottom of the villus (Figure 3G-O). However, in contrast to islet cells, Rfx6 staining fades as enteroendocrine cells differentiate and migrate toward the villus top (Figure 3L).

To decipher the role of Rfx6 in enteroendocrine cells we took again advantage of the  $\Delta$ Endo mutants where Cre-mediated recombination occurs in pancreatic but also in gastro-intestinal endocrine progenitors. Using RT-qPCR on small intestine (from beginning of duodenum to end of ileum) and colon samples from P0 pups, we first showed that deletion of *Rfx6* was efficient (Figure 4). Similarly to what was observed in the pancreas, endocrine cell differentiation is severely perturbed in the intestine of  $\Delta$ Endo mutants (Figure 4). More specifically, transcripts for intestinal hormones are almost gone (*Glp1*, *Gip*, *Cck*, *Pyy*, *Nts*, *Gast*, *Sst* and *Ghrl*) or decreased (*Sct*), except for *serotonin* suggesting that Rfx6 is important for the enteroendocrine lineage. This phenotype almost phenocopies what we observed in mice with an intestinal deletion of *Ngn3* (Mellitzer et al., 2010) suggesting that Rfx6 lies downstream of Ngn3. Of note however, in  $\Delta$ Endo pups *chromograninA* transcript is not affected. Among the transcription factors important for enteroendocrine cell differentiation and function, we showed that *Pax6* and to a lesser extent *Arx* are decreased but not for example *Pax4* or *Insm1*, suggesting that differentiation starts but does not proceed towards hormone-expressing cells. Very interestingly, we showed a strong increase of *Ngn3* transcripts (about 10-fold) in the small intestine and colon of  $\Delta$ Endo pups (Figure 4). This result is correlated with an increase (between 2.5-3.5-fold) in the number of Ngn3<sup>+</sup> cells per mm<sup>2</sup> in all different segments of the intestine at P0 in  $\Delta$ Endo pups (Figure 5).

To confirm these results, we also crossed Rfx6 conditional KO mice with the Villin-Cre line which drives recombination in the developing and adult intestinal epithelium (el Marjou et al., 2004). These mutants, called  $\Delta$ Intestine for simplicity, die between 3-5 days after birth in most cases (only 3 surviving mice in 14 litters). Deletion of Rfx6 in the whole intestine gave approximately the same phenotype than the endocrine-specific deletion (Figure 6). Taken together these data suggest that Rfx6 is essential to implement the Ngn3-dependent endocrinogenic program in the developing intestine leading to hormone-expressing cells.

**Rfx6 removal in the adult intestine leads to diarrhoea, weight loss, impaired lipids absorption associated to defective enteroendocrine cell differentiation.**

As mentioned above, very few  $\Delta$ Intestine mutants survived. These mice exhibited severe diarrhoea with yellowish stools, which could be a sign of excess fat in the faeces and steatorrhoea (Figure S3G-I) as it was described for the intestinal deletion of Ngn3 (Mellitzer et al., 2010). Mutants were sacrificed and analysed. Intestinal deletion of Rfx6 in these mutants was efficient as judged by immunofluorescence (Figure S3A-B) suggesting that survival was not due to defective Rfx6 removal. However, in contrast to what was observed in the intestinal deletion of Ngn3,  $\Delta$ Intestine mutants exhibited no obvious defects in proliferation of crypt progenitors or intestinal crypt/villi organisation in the small intestine (Figure S3C-F and data not shown). From these experiments we cannot conclude whether the phenotype observed in the adult mice results from embryonic *Rfx6*-deficiency or reflects Rfx6 function in the adult intestine. Indeed, in contrast to the pancreas, the endocrine lineage is permanently renewed in the adult intestine by differentiation from stem cells in a mechanism depending on Ngn3 (Jenny et al., 2002; Schonhoff et al., 2004).

Thus, to determine the role of Rfx6 in the adult intestine we crossed Rfx6 conditional KO mice with the Villin-Cre<sup>ERT2</sup> line (el Marjou et al., 2004). RT-qPCR results suggest efficient deletion of Rfx6 in the different segments of the adult intestine (Figure 7). Mice deleted for *Rfx6* start losing weight very rapidly after tamoxifen gavage. Out of the 4 treated mice so far, 1 mutant died twelve days after the gavage. The other mutants stopped losing weight but never caught up with the controls (Figure S4A). Four weeks after the tamoxifen treatment mutant mice had lost about 30-35% of their initial weight (Figure S4C). By RT-qPCR we showed that similarly to when we removed Rfx6 during development, *Rfx6* inactivation in the adult intestine causes a dramatic decrease of enteroendocrine hormone-expressing transcripts (except *serotonin* which increase). Similarly as well, *Pax6* and *Arx* transcripts were severely down-regulated whereas *Ngn3* transcripts were up-regulated. Interestingly, an induction of *Pax4* was revealed in the adult, which was not the case at P0 (Figure 7).



Reminding what we observed in surviving  $\Delta$ Intestine mutants, *Rfx6* deletion in the adult intestine causes diarrhoea. Increased Oil red staining of lipid droplets in the colon and faeces suggests that defective intestinal fat absorption might be the cause of diarrhoea (Figure 8). *Muc2* and *Defa1* transcripts were unchanged suggesting that *Rfx6* removal does not affect goblet and Paneth cell differentiation respectively (Figure 7). The effect of *Rfx6* on crypt homeostasis as well as a more in-depth analysis of goblet and Paneth cells remain to be studied.

### ***Rfx6* is necessary to maintain beta-cells in the adult mouse pancreas.**

We and others showed previously that *Rfx6* is maintained in the adult islets (Smith et al., 2010; Soyer et al., 2010). To determine *Rfx6* role in adult beta-cells we crossed the conditional KO mice with an inducible insulin-specific Cre line (*Ins1-CreER<sup>T2</sup>*) generated at the ICS (Illkirch, France). We showed by immunofluorescence and RT-qPCR that *Rfx6* is efficiently deleted in *Insulin*<sup>+</sup> cells 3 weeks after tamoxifen treatment (Figure 9A-B and I). We verified by RT-qPCR that no differences were observed at the level of the transcription of genes encoding other islet-specific hormones cells (Figure 9I). As expected *glucagon* and *somatostatin* were not affected, quite unexpectedly *Ppy* was decreased. We are currently verifying the specificity of the *Ins1-CreER<sup>T2</sup>* by tracing of recombined cells with *Rosa-YFP* mice, looking particularly at PP-cells. Although *Ins1* and *Ins2* transcripts (Figure 9I) were decreased by more than 60%, insulin peptide is still detected in beta-cells lacking *Rfx6* three weeks after gene deletion (Figure 9A-B) and mice do not develop overt diabetes. Interestingly, transcripts of genes necessary for beta-cell function like the glucose transporter *Glut2*, the 2 subunits of the  $K_{ATP}$  channel *Abcc8* and *Kcnj11*, the proconvertase *PC1/3* and the *glucokinase* are also decreased (Figure 9J). Similarly, transcription factors important for beta-cell maturation (*Ucn3*), function (*Nkx6.1*, *FoxA2*) or insulin transcription (*MafA*, *NeuroD1*) are down-regulated at the transcript level (Figure 9J-L). However, at the protein level *Glut2*, *MafA* and *Pdx1* are still expressed although it is difficult to determine whether they are decreased or not as immunofluorescence is not quantitative (Figure 9C-H). Taken together these results suggest that *Rfx6* is necessary for normal beta-cell function.

## DISCUSSION

To decipher the function(s) of Rfx6 during pancreatic and intestinal endocrine cell differentiation as well as in the adult beta-cells and intestine, we have generated a conditional knockout mouse for Rfx6. Our data further extend our knowledge on the function of Rfx6 (Soyer et al., 2010, Smith et al., 2010) owing to the specific deletion of Rfx6 in different tissues and at different time points. In the present study we have characterised the function of Rfx6 in the pancreatic and intestinal endocrine lineages downstream of Ngn3, as well as in the adult beta-cells.

### **Rfx6 expression downstream of Ngn3 drives endocrine differentiation.**

It has been previously shown that mice with a constitutive deletion of Rfx6 fail to generate any of the normal islet cell types except for pancreatic-polypeptide-expressing cells (Smith et al., 2010). However, because Rfx6 is expressed first broadly in the endoderm and then restricted to the endocrine cells of the pancreas, it was not known whether this phenotype was due to an early role of Rfx6 in the endoderm or to its function downstream of Ngn3. To answer this question we generated mice with a specific deletion of Rfx6 in the endocrine lineage. Interestingly, our  $\Delta$ Endo mice exhibited the same phenotype than the full KO even though it was slightly less severe as we have been able to observe rare hormone-expressing cells. The  $\Delta$ Endo mice have a strong reduction of insulin-, glucagon- and somatostatin-cells and transcripts. The remaining hormone<sup>+</sup> cells can either come from cells which escape deletion of Rfx6 (very rare) or from cells which deleted Rfx6 too late and were already differentiating or, finally from a putative Rfx6-independent differentiation.

At the transcriptional level  $\Delta$ Endo mice are also very similar to the full KO even though the stages analysed were different (E17.5 in Smith et al. and P0 in this study). Similarly we observed a decrease of *Arx* with a concomitant increase of *Pax4*; *Pdx1*, *MafA* and *MafB* were also strongly down-regulated. However, in contradiction with Smith et al., we have observed no difference in *Insm1* expression at P0, placing *Rfx6* either downstream or in parallel of *Insm1*. At the mRNA level, we have shown a 4-fold increase in *Ngn3* transcripts at P0 however this could not be confirmed at the protein level. As this increase was not observed at the embryonic stage (Smith et al., 2010 and Figure S1), it is possible that this increase is revealed only in the perinatal period. Taken together, these very similar phenotypes between the total deletion of *Rfx6* and the specific deletion of *Rfx6* downstream of Ngn3, demonstrate that it is the role of Rfx6 in Ngn3<sup>+</sup> cells that is responsible for the phenotype of *Rfx6* null mice.

What is the role of Rfx6 in the early gut endoderm thus remains unknown. Importantly, the presence of Rfx6 in the endoderm is not conserved in zebrafish (Soyer et al., 2010). The early function of Rfx6 might also be compensated by other RFX members for example by Rfx3 which seem to be expressed also in the endoderm (J. Soyer, unpublished to be confirmed). Interestingly, a recent study by Wang et al., showed that *Rfx6* was enriched in Sox17<sup>+</sup> endoderm compared to undifferentiated hESC suggestive that like in mice, *hRFX6* is expressed in the definitive endoderm (Wang et al., 2011). The role of Rfx6 in the early endoderm could be studied taking advantage of FoxA3-Cre mice (Lee et al., 2005). Of note, in contradiction to what was reported in the full KO by Smith et al. or in human mutations for RFX6, we have never observed small bowel obstructions or intestinal occlusions, a phenotype that could be related to defective development of the gut endoderm. We do not have any obvious explanations for this difference except a potential effect of the genetic background.

#### **Deletion of Rfx6 in the intestine leads to an increased number of Ngn3-positive cells.**

In the intestine the absence of Rfx6 strongly increases *Ngn3* expression as well as the number of Ngn3<sup>+</sup> cells. These data suggest that Ngn3-progenitor cells accumulate as they cannot differentiate further or alternatively that *Ngn3* expression is not shut off. The regulation of *Ngn3* expression in the intestine has not been studied extensively. In the pancreas, *Ngn3* transcription is activated by several pancreatic transcription factors (Pdx1, Sox9, Hnf1b, Hnf6,...) and inhibited by Notch signalling through Hes1 (Jacquemin et al., 2000; Lee et al., 2001; Lynn et al., 2007; Oliver-Krasinski et al., 2009). It has been suggested that Ngn3 can bind and repress its own promoter *in vitro* (Smith et al., 2004). However, in human duct cells, it has recently been shown that exogenous *Ngn3* is able to activate endogenous Ngn3 suggesting auto-activation of this gene (Swales et al., 2012). Whether similar regulations operate in the intestine is not known. One hypothesis could be that first *Rfx6* is induced by Ngn3 in the intestine (directly or indirectly), and then Rfx6 would repress *Ngn3* to stimulate the progression of enteroendocrine cell differentiation. In the absence of Rfx6, Ngn3 is not turned down and cells cannot differentiate. Interestingly, Ngn3<sup>+</sup> cells were frequently found close to each other in the intestine of mutants (Figure S2), suggesting impaired Notch signalling. Indeed, it has been suggested that Notch signalling was acting by lateral inhibition in the intestine because Ngn3<sup>+</sup> cells were never close to each other (Jensen et al., 2000). One hypothesis could be that Rfx6 contributes to lateral inhibition via the activation of the transcription of Notch ligands. Thus the increased number of Ngn3<sup>+</sup> cells could also be explained by ectopic expression of *Ngn3* resulting from defective Notch-mediated lateral inhibition.

### **Rfx6 controls endocrine subtype specification in the pancreas and intestine by regulating Arx and Pax4 expressions.**

Arx and Pax4 are essential regulators of alpha versus beta/delta destinies in the embryonic pancreas (Collombat et al., 2003; Collombat et al., 2009). Similarly in the intestine, while Arx and Pax4 are necessary for the development of L (GLP-1) and D (Somatostatin) cells respectively they conversely restrict D- and L-cells fates suggesting antagonistic functions at least in D/L cell allocation (Beucher et al., 2012; Du et al., 2012; Larsson et al., 1998). Importantly in the current study we observed a down-regulation of Arx with a concomitant up-regulation of Pax4, both in the intestine and pancreas of *Rfx6*-deficient mice. These data suggest first that Arx and Pax4 might be downstream of Rfx6. Second that in both organs Rfx6 could promote Arx expression in some endocrine progenitors but repress Pax4 in others and thus contribute to subtype specification (Figure 10). Ectopic Pax4 expression in the pancreas would however not be sufficient to promote beta-cell differentiation as reported previously in gain-of-function studies (Collombat et al., 2009) because Rfx6 might have additional roles in beta-cell function including the transcription of insulin genes. In contrast, increased Pax4 transcripts in the intestine may explain the increased transcripts of *serotonin* as these cells do not rely on Rfx6 (this study) neither on Arx (Beucher et al., 2012; Du et al., 2012) but depend on Pax4 (Beucher et al., 2012; Larsson et al., 1998) (Figure 10).

### **Rfx6 is necessary for lipid absorption in the adult intestine.**

Comparably to what we observed in mice lacking Ngn3 in the intestine (Mellitzer et al., 2010) and what was reported in human with mutations in NGN3 (Rubio-Cabezas et al., 2011; Wang et al., 2006) or RFX6 genes (Smith et al., 2010; Spiegel et al., 2011), the loss of Rfx6 in mouse intestine induces diarrhoea. Our data suggest that like in *Ngn3* mutants this diarrhoea is caused by defective lipid absorption. Although gut endocrine cells have not yet been explored in patients with mutations in RFX6, a common feature of *Ngn3*- and *Rfx6*-deficient mice, as well as of human mutations of NGN3 is an impaired development of enteroendocrine cells. Exocrine function has not been tested here but is unaltered in human and mice with deficient *Ngn3* (Gradwohl et al., 2000; Wang et al., 2006) or *Rfx6* (Smith et al., 2010). Thus one can speculate that one or several of the missing enteroendocrine hormones promote intestinal lipid absorption. Of note it has been shown recently that removal of Arx results as well into chronic diarrhoea associated with defects in lipid absorption in the neonatal intestine (Du et al., 2012). Interestingly in *Arx*-deficient mice only a subset of enteroendocrine hormones is lacking (Beucher et al., 2012; Du et al., 2012). It would thus be interesting to test whether it is possible to rescue *Rfx6*-dependent malnutrition and diarrhoea by administration of defined cocktails of enteroendocrine hormones.

### **Rfx6 is necessary for the maintenance of *insulin* and key regulators of beta-cell function.**

Rfx6 removal specifically in adult beta-cells impairs transcription of insulin genes. Expression of genes necessary for insulin synthesis (*PC1/3*), glucose sensing/processing (*Glut2*, *Gck*), insulin secretion (*Kir6.2*, *Sur1*), insulin transcription (*Pdx1*, *NeuroD1*, *MafA*) is also strongly reduced. These results strongly suggest a key function for Rfx6 in the regulation of beta-cell function. Surprisingly, a reduction of up to 60% of these key beta-cell transcripts could not be revealed by immunofluorescence and insulin peptide is readily detected in *Rfx6*-negative beta-cells 3 weeks after tamoxifen treatment. It has been suggested that both *insulin* transcripts and peptides are very stable (Lee et al., 2000). Thus remaining insulin could have been generated before *Rfx6* deletion or reflect translation of residual transcripts. To determine active insulin synthesis we will assess *preproinsulin* levels by RT-qPCR or C-peptide expression by immunofluorescence. Reduced insulin, *Glut2* and *Gck* was also described in *Rfx3*-deficient mice (Ait-Lounis et al., 2010). *Rfx3* has been suggested to heterodimerise with *Rfx6* to regulate gene expression. Although *Rfx3* is down-regulated in islets with a beta-cell specific removal of *Rfx6*, remaining *Rfx3* could thus contribute to partially compensate *Rfx6* absence. The decrease of several beta-cell specific transcripts could also reflect an overall decrease in beta-cell number. We will thus evaluate cell death and beta-cell mass in these mice. However, nine months after tamoxifen injections mice did not develop diabetes suggesting that sufficient beta-cells are remaining. We will however evaluate whether glucose tolerance is altered in mice lacking *Rfx6* specifically in beta-cells.

This study places *Rfx6* as a major regulator of endocrine cell differentiation both in the pancreas and in the intestine. Furthermore, our findings support an essential role of *Rfx6* in beta-cell function. Finding direct targets of this gene is now required to further clarify the molecular mechanisms underlying *Rfx6* role in the control of glucose homeostasis function in beta-cells. The gained knowledge might in turn be useful to scrutinise or generate functional beta-cells for diabetic patients.

## **MATERIALS AND METHODS**

### **Generation of animals with a conditional Rfx6 allele.**

Homologous recombination in ES cells and generation of chimeric mice were performed at the Institut Clinique de la Souris (ICS), Illkirch, France. All mice were housed in an animal facility licensed by the French Ministry of Agriculture (agreement no. B67-218-5) and all animal experiments were approved by the Direction des Services Vétérinaires in compliance with the European legislation on care and use of laboratory animals. The final targeting vector (Figure 1A) was linearised and electroporated into the BD10 ES cell line derived from mouse strain C57BL/6N. Homologous recombination events were detected in geneticin resistant colonies by Southern blot. Chimeras were generated by standard procedures. Germline transmission was obtained by crossing the chimeras with C57BL/6N females, and the colony was amplified by crossing heterozygous animals with C57BL/6N mice (Taconic). Heterozygous females were crossed with CMV-Flip males to excise the Neomycin selection cassette.

### **Mouse strain, genotyping and manipulations.**

Animal experiments were supervised by G. Gradwohl (agreement N° C67-59 approved by the direction des Services Vétérinaires, Strasbourg, France) in compliance with the European legislation on care and use of laboratory animals. Ngn3-Cre mice are a gift from Dr. Shosei Yoshida (Yoshida et al., 2004), Villin-Cre and Villin-Cre<sup>ERT2</sup> were generously given by Dr. Sylvie Robine (el Marjou et al., 2004), Ins1-Cre<sup>ERT2</sup> were generated at the ICS. Genomic tail DNA was analysed by PCR, using the following primers: Ngn3-F (CTGCAGTTTAGCAGAACTTCAGAGGGA), Villin-F (ATAGGAAGCCAGTTTCCCTTC), Cre-R (ATCAACGTTTTGTTTTCGGA), ERT-F (GCATTACCGGTCGATGCAACGAGTGATGAG), ERT-R (AGGATCTCTAGCCAGGCACA), Rfx6L2-F (GAAGGTGCACCCATAAAAGC), Rfx6 $\Delta$ -F (GCTCCGACTTCTGATCATCC) and Rfx6-R (TATAAGCCACCCAGGGTCAG). Band size was the following: Ngn3-Cre: 800bp, Villin-Cre: 200bp, ERT: 1200bp, Rfx6L2: 350bp, Rfx6WT: 250bp, Rfx6L: 500bp. To achieve recombination in inducible mutants, the mice were given Tamoxifen (Sigma), either intraperitoneally once a day during 3 consecutive days for the Ins1-Cre<sup>ERT2</sup> or by gavage 3 times per day every second day during 5 days for the Villin-Cre<sup>ERT2</sup>.

## **Immunohistochemistry and Immunofluorescence.**

Tissues of mice were fixed in 4% paraformaldehyde at 4°C and embedded in paraffin or Shandon Cryomatrix (Thermo Scientific) and 10µm sections were used. Slides were hydrated in PBS, treated in blocking buffer (5% donkey serum in PBS, 0.1% Triton X-100 (PBST)) for 1h at RT and incubated with primary antibodies in blocking buffer overnight at 4°C. After washing in PBST, the secondary antibodies were added for 1h at RT in PBST. For immunofluorescence assays, nuclei were stained with Dapi and mounted in Aqua-poly/mount (Polysciences). For immunohistochemistry analysis, endogenous peroxydase activity was blocked by incubation in 0.5% H<sub>2</sub>O<sub>2</sub> diluted in Methanol and signal was revealed using the Vectastain Elite ABC Kit (Vector Laboratories) and the DAB chromogen (DakoCytomation) supplemented with 0.02% H<sub>2</sub>O<sub>2</sub>. Slides were dehydrated and mounted in Eukitt (Euromedex). For BrdU detection assays, BrdU was injected to pregnant females at 50 mg/kg body weight, 2h prior sacrifice. If required, antigen retrieval was performed by incubating slides in 10mM Sodium Citrate pH6 and microwaving 4min at 1000W and 15min at 300W. Slides were then let to cool down to room temperature for at least 30min.

Primary antibodies used: Ngn3, guinea pig anti-ngn3 at 1:1000 (kind gift of Dr Maïke Sander, University of California – San Diego, La Jolla, CA, USA); Insulin, guinea pig anti-insulin at 1:1000 (Linco), mouse anti-insulin at 1:1000 (Sigma); Glut2, rabbit anti-glut2 at 1:500 (Chemicon); Glucagon, guinea pig anti-glucagon at 1:2000 (Linco); PP, guinea pig anti-PP at 1:1000 (Linco); Somatostatin, rat anti-somatostatin at 1:500 (Chemicon) or goat anti-somatostatin at 1:200 (Santa Cruz); Ghrelin, mouse anti-ghrelin at 1:1500 (C. Tomasetto, IGBMC, Strasbourg, France); ChromogranineA, goat anti-chromogranineA at 1:500 (Santa Cruz); CCK, goat anti-CCK at 1:50 (Santa Cruz); GLP1, goat anti-glp1 at 1:100 (Santa Cruz); GIP, goat anti-Gip at 1:100 (Santa Cruz); Gastrin, goat anti-gastrin at 1:50 (Santa Cruz); Secretin, goat anti-secretin at 1:50 (Santa Cruz); Serotonin, goat anti-serotonin at 1:2000 (Abcam); Lysozyme, rabbit anti-lysozyme AB at 1:200 (Dako); Ki67, mouse anti-Ki67 at 1:100 (Novo Castra); rat anti-BrdU at 1:10 (AbD Serotec); Pdx1, rabbit anti-pdx1 at 1:2000 (kind gift of Dr Chris Wright, Vanderbilt University, Nashville, TN, USA); Insm1, guinea pig anti-insm1 at 1:500 (kind gift of Dr Carmen Birchmeier, Max-Delbrück-Centrum for Molecular Medicine, Berlin, Germany); MafA, rabbit anti-MafA at 1:2000 (Bethyl); Rfx6, rabbit anti-Rfx62766 at 1:500 (IGBMC).

Secondary antibodies conjugated to DyLight488, DyLight549 and DyLight649 (JacksonImmunoResearch) were used at 1:500. For Ki67 and BrdU, signal amplification was performed using biotin anti-mouse coupled antibody at 1:500 (JacksonImmunoResearch) and streptavidin-Cy3 conjugate at 1:500 (Molecular Probes).

Goblets cells were stained for mucin using PAS (Periodic Acid Schiff) reaction. Lipids were stained on cryosections or faeces using Oil Red O staining.

### **Real time PCR analysis.**

Total RNA from pancreas, purified islets, whole small intestine (pups), duodenum (adults), jejunum (adults), ileum (adults) or colon was extracted using TRI Reagent (Invitrogen). Reverse transcription was performed using Transcriptor Reverse Transcriptase (Roche). Quantitative PCRs were performed using mouse-specific TaqMan primers and probes (Applied Biosystems) recognizing Ngn3 (Mm00437606\_s1), Ins1 (Mm01259683\_g1), Ins2 (Mm00731595\_gH), Slc2a2 (Mm00446228\_m1), Pcsk1 (Mm00479023\_m1), Gck (Mm00439129\_m1), Abcc8 (Mm00803450\_m1), Kcnj11 (Mm00440050\_s1), Ppy (Mm00435889\_m1), ChgA (Mm00514341\_m1), Arx (Mm00545903\_m1), Pax4 (Mm01159036\_m1), Pax6 (Mm00443081\_m1), Pdx1 (Mm00435565\_m1), Nkx6.1 (Mm00454962\_m1), Foxa1 (Mm00484713\_m1), Foxa2 (Mm00839704\_mH), Insm1 (Mm02581025\_s1), NeuroD1 (Mm00520715\_m1), MafB (Mm0062748\_s1), Pyy (Mm00520715\_m1), Nts (Mm00481140\_m1), Cck (Mm00446170\_m1), Sct (Mm00441235\_g1), Gip (Mm00433601\_m1), Gcg/Glp1 (Mm00801712\_m1), Tph1 (Mm00493794\_m1), Gast (Mm00772211\_g1), Sst (Mm00436671\_m1), Ghrl (Mm00445450\_m1), Muc2 (Mm00458299\_m1), Defa1 (Mm02524428\_g1) or UPL probes #10 (Roche) for MafA (5'primer ctccagagccaggtggag, 3'primer gtacaggtcccgtccttg), #20 for Nkx2.2 (5'primer gcagcgacaaccctaca, 3'primer atttgagctcgagctcttg), #34 for Nkx6.3 (5'primer aactgctgggcagacacag, 3'primer actcaggggctctggagtg), #70 for Nkx6.2 (5'primer ccttagtatccctgcttctctc, 3'primer agaggaccgacggctgtt), #83 for Rfx6WT (5'primer tgcaggaaagagaaactggag, 3'primer ggaaatnttggcgaattgtc), #105 for Ucn3 (5'primer gacctgagcatttccactcc, 3'primer cagaagtggcagcaggaagt), #108 for Glis3 (5'primer cacgtgaaggcacattcttc, 3'primer ggagctcagtgctagaccgta), with Light Cyclers 480 Probes Master Mix (Roche) on Light Cyclers 480 (Roche). Gene expression levels were normalised to Rplp0 (Mm01974474\_gH).

### **Morphometric analysis.**

Ngn3<sup>+</sup> cells were counted after immunostaining on approximately 50 sections of duodenum, 30 sections of jejunum, ileum and colon at P0 on 3 controls and 3 mutant samples. The numbers of Ngn3<sup>+</sup> cells were normalised according to the area of the sections estimated by the surface of DAPI staining.

### **Statistics.**

Values are presented as mean  $\pm$  SD. p-values were determined using the 2-tailed Student t-test with unequal variance. p value of less 0.05 was accepted as statistically significant. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.



**Mouse Islet purification.**

Mouse Islet purification was performed as previously described (Carter et al., 2009). Briefly mice were euthanised with a cocktail of ketamine and injected with Type V Collagenase (Sigma C9263) solution directly into the common bile duct to perfuse the pancreas. Pancreas was dissected out and digested and islets were handpicked after several purification steps and kept in culture O/N before harvesting for RNA isolation.

## REFERENCES

- Ait-Lounis, A., Baas, D., Barras, E., Benadiba, C., Charollais, A., Nlend Nlend, R., Liegeois, D., Meda, P., Durand, B. and Reith, W.** (2007). Novel function of the ciliogenic transcription factor RFX3 in development of the endocrine pancreas. *Diabetes* **56**, 950-9.
- Ait-Lounis, A., Bonal, C., Seguin-Estevez, Q., Schmid, C. D., Bucher, P., Herrera, P. L., Durand, B., Meda, P. and Reith, W.** (2010). The transcription factor Rfx3 regulates beta-cell differentiation, function, and glucokinase expression. *Diabetes* **59**, 1674-85.
- Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., Hrabe de Angelis, M., Lendahl, U. and Edlund, H.** (1999). Notch signalling controls pancreatic cell differentiation. *Nature* **400**, 877-81.
- Beucher, A., Gjernes, E., Collin, C., Courtney, M., Meunier, A., Collombat, P. and Gradwohl, G.** (2012). The homeodomain-containing transcription factors arx and pax4 control enteroendocrine subtype specification in mice. *PLoS One* **7**, e36449.
- Carter, J. D., Dula, S. B., Corbin, K. L., Wu, R. and Nunemaker, C. S.** (2009). A practical guide to rodent islet isolation and assessment. *Biol Proced Online* **11**, 3-31.
- Collombat, P., Mansouri, A., Hecksher-Sorensen, J., Serup, P., Krull, J., Gradwohl, G. and Gruss, P.** (2003). Opposing actions of Arx and Pax4 in endocrine pancreas development. *Genes Dev* **17**, 2591-603.
- Collombat, P., Xu, X., Ravassard, P., Sosa-Pineda, B., Dussaud, S., Billestrup, N., Madsen, O. D., Serup, P., Heimberg, H. and Mansouri, A.** (2009). The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells. *Cell* **138**, 449-62.
- Du, A., McCracken, K. W., Walp, E. R., Terry, N. A., Klein, T. J., Han, A., Wells, J. M. and May, C. L.** (2012). Arx is required for normal enteroendocrine cell development in mice and humans. *Dev Biol* **365**, 175-88.
- el Marjou, F., Janssen, K. P., Chang, B. H., Li, M., Hindie, V., Chan, L., Louvard, D., Chambon, P., Metzger, D. and Robine, S.** (2004). Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. *Genesis* **39**, 186-93.
- Gierl, M. S., Karoulias, N., Wende, H., Strehle, M. and Birchmeier, C.** (2006). The zinc-finger factor Insm1 (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells. *Genes Dev* **20**, 2465-78.
- Gradwohl, G., Dierich, A., LeMeur, M. and Guillemot, F.** (2000). neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A* **97**, 1607-11.
- Jacquemin, P., Durviaux, S. M., Jensen, J., Godfraind, C., Gradwohl, G., Guillemot, F., Madsen, O. D., Carmeliet, P., Dewerchin, M., Collen, D. et al.** (2000). Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. *Mol Cell Biol* **20**, 4445-54.
- Jenny, M., Uhl, C., Roche, C., Duluc, I., Guillermin, V., Guillemot, F., Jensen, J., Kedinger, M. and Gradwohl, G.** (2002). Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium. *EMBO J* **21**, 6338-47.
- Jensen, J., Pedersen, E. E., Galante, P., Hald, J., Heller, R. S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P. and Madsen, O. D.** (2000). Control of endodermal endocrine development by Hes-1. *Nat Genet* **24**, 36-44.

- Kroon, E., Martinson, L. A., Kadoya, K., Bang, A. G., Kelly, O. G., Eliazar, S., Young, H., Richardson, M., Smart, N. G., Cunningham, J. et al.** (2008). Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol* **26**, 443-52.
- Larsson, L. I., St-Onge, L., Hougaard, D. M., Sosa-Pineda, B. and Gruss, P.** (1998). Pax 4 and 6 regulate gastrointestinal endocrine cell development. *Mech Dev* **79**, 153-9.
- Lee, C. S., Sund, N. J., Behr, R., Herrera, P. L. and Kaestner, K. H.** (2005). Foxa2 is required for the differentiation of pancreatic alpha-cells. *Dev Biol* **278**, 484-95.
- Lee, J. C., Smith, S. B., Watada, H., Lin, J., Scheel, D., Wang, J., Mirmira, R. G. and German, M. S.** (2001). Regulation of the pancreatic pro-endocrine gene neurogenin3. *Diabetes* **50**, 928-36.
- Lee, M. S., Zhu, Y. L., Sun, Z., Rhee, H., Jeromin, A., Roder, J. and Dannies, P. S.** (2000). Accumulation of synaptosomal-associated protein of 25 kDa (SNAP-25) and other proteins associated with the secretory pathway in GH4C1 cells upon treatment with estradiol, insulin, and epidermal growth factor. *Endocrinology* **141**, 3485-92.
- Lynn, F. C., Smith, S. B., Wilson, M. E., Yang, K. Y., Nekrep, N. and German, M. S.** (2007). Sox9 coordinates a transcriptional network in pancreatic progenitor cells. *Proc Natl Acad Sci U S A* **104**, 10500-5.
- Mellitzer, G., Beucher, A., Lobstein, V., Michel, P., Robine, S., Kedinger, M. and Gradwohl, G.** (2010). Loss of enteroendocrine cells in mice alters lipid absorption and glucose homeostasis and impairs postnatal survival. *J Clin Invest* **120**, 1708-21.
- Mitchell, J., Punthakee, Z., Lo, B., Bernard, C., Chong, K., Newman, C., Cartier, L., Desilets, V., Cutz, E., Hansen, I. L. et al.** (2004). Neonatal diabetes, with hypoplastic pancreas, intestinal atresia and gall bladder hypoplasia: search for the aetiology of a new autosomal recessive syndrome. *Diabetologia* **47**, 2160-7.
- Molven, A. and Njolstad, P. R.** (2011). Role of molecular genetics in transforming diagnosis of diabetes mellitus. *Expert Rev Mol Diagn* **11**, 313-20.
- Naya, F. J., Huang, H. P., Qiu, Y., Mutoh, H., DeMayo, F. J., Leiter, A. B. and Tsai, M. J.** (1997). Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev* **11**, 2323-34.
- Oliver-Krasinski, J. M., Kasner, M. T., Yang, J., Crutchlow, M. F., Rustgi, A. K., Kaestner, K. H. and Stoffers, D. A.** (2009). The diabetes gene Pdx1 regulates the transcriptional network of pancreatic endocrine progenitor cells in mice. *J Clin Invest* **119**, 1888-98.
- Pan, F. C. and Wright, C.** (2011). Pancreas organogenesis: from bud to plexus to gland. *Dev Dyn* **240**, 530-65.
- Rezania, A., Bruin, J. E., Riedel, M. J., Mojibian, M., Asadi, A., Xu, J., Gauvin, R., Narayan, K., Karanu, F., O'Neil, J. J. et al.** (2012). Maturation of Human Embryonic Stem Cell-Derived Pancreatic Progenitors into Functional Islets Capable of Treating Pre-existing Diabetes in Mice. *Diabetes*.
- Rubio-Cabezas, O., Jensen, J. N., Hodgson, M. I., Codner, E., Ellard, S., Serup, P. and Hattersley, A. T.** (2011). Permanent Neonatal Diabetes and Enteric Anendocrinosis Associated With Biallelic Mutations in NEUROG3. *Diabetes* **60**, 1349-53.
- Rubio-Cabezas, O., Klupa, T. and Malecki, M. T.** (2010). Permanent neonatal diabetes mellitus--the importance of diabetes differential diagnosis in neonates and infants. *Eur J Clin Invest* **41**, 323-33.
- Schonhoff, S. E., Giel-Moloney, M. and Leiter, A. B.** (2004). Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. *Dev Biol* **270**, 443-54.

- Schwitzgebel, V. M., Scheel, D. W., Conners, J. R., Kalamaras, J., Lee, J. E., Anderson, D. J., Sussel, L., Johnson, J. D. and German, M. S.** (2000). Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development* **127**, 3533-42.
- Shapiro, A. M., Lakey, J. R., Ryan, E. A., Korbitt, G. S., Toth, E., Warnock, G. L., Kneteman, N. M. and Rajotte, R. V.** (2000). Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* **343**, 230-8.
- Smith, S. B., Qu, H. Q., Taleb, N., Kishimoto, N. Y., Scheel, D. W., Lu, Y., Patch, A. M., Grabs, R., Wang, J., Lynn, F. C. et al.** (2010). Rfx6 directs islet formation and insulin production in mice and humans. *Nature* **463**, 775-80.
- Smith, S. B., Watada, H. and German, M. S.** (2004). Neurogenin3 activates the islet differentiation program while repressing its own expression. *Mol Endocrinol* **18**, 142-9.
- Soyer, J., Flasse, L., Raffelsberger, W., Beucher, A., Orvain, C., Peers, B., Ravassard, P., Vermot, J., Voz, M. L., Mellitzer, G. et al.** (2010). Rfx6 is an Ngn3-dependent winged helix transcription factor required for pancreatic islet cell development. *Development* **137**, 203-12.
- Spiegel, R., Dobbie, A., Hartman, C., de Vries, L., Ellard, S. and Shalev, S. A.** (2011). Clinical characterization of a newly described neonatal diabetes syndrome caused by RFX6 mutations. *Am J Med Genet A* **155A**, 2821-5.
- Swales, N., Martens, G. A., Bonne, S., Heremans, Y., Borup, R., Van de Castele, M., Ling, Z., Pipeleers, D., Ravassard, P., Nielsen, F. et al.** (2012). Plasticity of adult human pancreatic duct cells by neurogenin3-mediated reprogramming. *PLoS One* **7**, e37055.
- Wang, J., Cortina, G., Wu, S. V., Tran, R., Cho, J. H., Tsai, M. J., Bailey, T. J., Jamrich, M., Ament, M. E., Treem, W. R. et al.** (2006). Mutant neurogenin-3 in congenital malabsorptive diarrhea. *N Engl J Med* **355**, 270-80.
- Wang, P., Rodriguez, R. T., Wang, J., Ghodasara, A. and Kim, S. K.** (2011). Targeting SOX17 in human embryonic stem cells creates unique strategies for isolating and analyzing developing endoderm. *Cell Stem Cell* **8**, 335-46.
- Yoshida, S., Takakura, A., Ohbo, K., Abe, K., Wakabayashi, J., Yamamoto, M., Suda, T. and Nabeshima, Y.** (2004). Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. *Dev Biol* **269**, 447-58.

## LEGENDS TO FIGURES

**Figure 1: Deletion of Rfx6 downstream of Ngn3 induces the loss of differentiated hormone-expressing cells except PP. (A)** Rfx6 targeted locus. The exon 3 in the DNA binding domain (pink) is flanked by two LoxP sites. **(B)** Rfx6 recombined locus. Upon Cre-mediated recombination, the exon 3 is lost and no functional Rfx6 protein can be made. **(C-H)** Immunofluorescence experiments on pancreata from P0 controls and  $\Delta$ Endo pups. **(C-D)** Staining for insulin and glucagon (green) and Rfx6 (red) showing efficient deletion of Rfx6 and strong reduction of insulin- and glucagon-expressing cells in  $\Delta$ Endo mutants. **(E-F)** Staining for PP (green) and Rfx6 (red) revealed that PP is not dependent on Rfx6. **(G-H)** Staining for hormones (insulin, glucagon, PP, somatostatin) in green and the pan-endocrine marker chromograninA in red suggests that endocrine cells which do not express any of the hormones are found in the pancreas of  $\Delta$ Endo pups. **(I)** RT-qPCR experiments for *Rfx6*, *ChromograninA*, *Insulin1*, *Glucagon*, *PP* and *Somatostatin* supporting the efficient deletion of Rfx6 and the strong reduction of all hormone-expressing cells except PP in  $\Delta$ Endo pups. Data are represented as mean  $\pm$  SD on n=4 samples; \*\*\* p< 0.001, \*\* p<0.01.

**Figure 2: Rfx6 deletion in Ngn3<sup>+</sup> endocrine progenitors perturbs the transcriptional cascade of endocrine cell differentiation in the postnatal pancreas.** RT-qPCR experiments for different transcription factors on pancreata from Ctrl and  $\Delta$ Endo P0 pups for *Arx*, *Pax4*, *Pdx1*, *Nkx6.1*, *Insm1*, *MafA*, *MafB*, *NeuroD1* and *Ngn3*. Data are represented as mean  $\pm$  SD on n=4 samples; \*\*\* p< 0.001, \*\* p<0.01, \*p<0.05.

**Figure 3: Rfx6 is expressed in a subset of enteroendocrine progenitors and in differentiating enteroendocrine cells in the adult intestine. (A-F)** Immunofluorescence for Ngn3 (green) and Rfx6 (red), yellow arrows point to double-immuno-positive cells, green arrows to Ngn3<sup>+</sup>/Rfx6<sup>-</sup> cells and red arrows to Ngn3<sup>-</sup>/Rfx6<sup>+</sup> cells. **(G-O)** Immunofluorescence staining for Rfx6 (red) and different enteroendocrine markers (green): ChromograninA (G-H), GLP1 (I), GIP (J/L), Somatostatin (K), Ghrelin (M), CCK (N) and Gastrin (O).

**Figure 4: In the intestine, the loss of Rfx6 in the endocrine lineage strongly impairs enteroendocrine cell differentiation.** RT-qPCR experiments on P0 small intestine and colon of controls and  $\Delta$ Endo pups for several hormonal markers (*Glp1*, *Gip*, *Cck*, *Pyy*, *Nts*, *Gast*, *Sct*, *Tph1*, *Sst*, *Ghrl* and *ChgA*) and transcription factors (*Arx*, *Rfx6*, *Ngn3*, *Pax4*, *Insm1*, *Pax6*, *NeuroD1*, *Nkx2.2*, *FoxA1*, *FoxA2*, *MafB*, and *Pdx1*). Data are represented as mean  $\pm$  SD on n=4 samples; \*\*\* p< 0.001, \*\* p<0.01, \*p<0.05.

**Figure 5: Increase in the number of Ngn3<sup>+</sup> cells upon loss of Rfx6 in the enteroendocrine lineage of the intestine.** Graph representing the quantification of the number of immuno-positive Ngn3 cells per mm<sup>2</sup> along the different intestinal segments on controls and  $\Delta$ Endo pups at P0. Data are represented as mean  $\pm$  SD on n=3 samples; \*\*\* p< 0.001, \*\* p<0.01.

**Figure 6: The loss of Rfx6 in the intestinal epithelium strongly impairs enteroendocrine cell differentiation.** RT-qPCR experiments on P3 small intestine and colon of controls and  $\Delta$ Intestine pups for several hormonal markers (*Glp1*, *Gip*, *Cck*, *Pyy*, *Nts*, *Gast*, *Sct*, *Tph1*, *Sst*, *Ghrl* and *ChgA*), transcription factors (*Arx*, *Rfx6*, *Ngn3*, *Pax4*, *Insm1*, *Pax6*, *NeuroD1* and *Nkx2.2*), Paneth cells (*Defa1*) and goblet cells (*Muc2*). Data are represented as mean  $\pm$  SD on n=4 samples; \*\*\* p< 0.001, \*\* p<0.01, \*p<0.05.

**Figure 7: Rfx6 is necessary in the adult intestine to maintain enteroendocrine cells renewal.** RT-qPCR experiments on adult jejunum and colon of controls and tamoxifen treated Rfx6<sup>fl/fl</sup>; Villin-CreER<sup>T2</sup> animals 1 month after tamoxifen or vehicle gavage. Mice were given tamoxifen 3 times per day every second day during 5 days. Samples were analysed for several hormonal markers (*Glp1*, *Gip*, *Cck*, *Pyy*, *Nts*, *Gast*, *Sct*, *Tph1*, *Sst*, *Ghrl* and *ChgA*), transcription factors (*Arx*, *Rfx6*, *Ngn3*, *Pax4*, *Insm1*, *Pax6*, *NeuroD1*, *FoxA1*, *FoxA2* and *Pdx1*), Paneth cells (*Defa1*) and goblet cells (*Muc2*). Data are represented as mean  $\pm$  SD on n=3 samples; \*\*\* p< 0.001, \*\* p<0.01, \*p<0.05.

**Figure 8: The loss of Rfx6 in the adult intestine perturbs lipid absorption. (A-J)** Oil Red O staining on cryosections from the duodenum (A-B), jejunum (C-D), ileum (E-F), colon (G-H) and on fresh faeces (I-J) from mutant and control animals 1 month after gavage. The + indicates the relative amount of lipid droplets per segment.

**Figure 9: Rfx6 is necessary to maintain adult beta-cells. (A-H)** Immunofluorescence staining on Ins1-CreERT2;Rfx6<sup>fl/fl</sup> adults 3 weeks after tamoxifen or vehicle injections. Mice were injected once a day during 3 consecutive days. Pictures show islets stained for staining for insulin (green) / Rfx6 (red) in A-B; Glut2 in C-D, MafA in E-F and Pdx1 (red) / PP (green) in G-H. **(I-L)** Islets were purified from Ins1-CreERT2;Rfx6<sup>fl/fl</sup> adults 3 weeks after tamoxifen or vehicle injections and analysed by RT-qPCR for *Rfx6* and *Hormones* (*Ins1*, *Ins2*, *Gcg*, *Ppy* and *Sst*) in I; markers of beta-cell function in J (*Gck*, *Abcc8/Sur1*, *Kcnj11/Kir6.2*, *Slc2a2/Glut2*, *Pcsk1/PC1/3* and *Ucn3*) and for transcription factors in K-L (*Pdx1*, *MafA*, *Nkx6.1*, *FoxA2*, *Pax6*, *Ngn3*, *Rfx3*, *Glis3*, *NeuroD1*, *Nkx6.2*, *Nkx2.2* and *MafB*). Data are represented as mean  $\pm$  SD on n=4 samples; \*\*\* p< 0.001, \*\* p<0.01, \*p<0.05.

Figure 10: **Model of enteroendocrine subtype specification: roles of Arx, Pax4 and Rfx6.** Gast-, GIP-, Nts-, Sct-, CCK-, and GLP1-expressing cells arise from endocrine progenitors expressing Ngn3 then Rfx6 and subsequently Pax4 and Arx. Sst- and serotonin (5-HT)-expressing cells are generated from progenitors expressing Ngn3 then Rfx6 and subsequently Pax4. Upon Rfx6 inactivation the differentiation of Gast-, GIP-, Nts-, Sct-, CCK-, GLP1-, Sst-expressing cells is impaired whereas serotonin-expressing cells and Ngn3<sup>+</sup> enteroendocrine progenitors are increased. Key transcription factors controlling intestinal cell destiny are also indicated.

Figure S1: **Rfx6 is necessary during embryogenesis for the formation of endocrine cells.** Pancreas from  $\Delta$ Endo and controls E15.5 embryos were dissected and analysed by RT-qPCR for *Rfx6*, *Ngn3*, *Ins1*, *Glucagon*, *Ppy*, *Sst*, *Pax4*, *Arx*, *Insm1* and *NeuroD1*. Data are represented as mean  $\pm$  SD on n=4 samples; \*\* p<0.01, \*p<0.05.

Figure S2: **Number of Ngn3<sup>+</sup> cells is increased in all intestinal segments upon Rfx6 deletion in the enteroendocrine lineage.** Immunofluorescence staining for Ngn3 on controls and  $\Delta$ Intestine mutants at P0 in duodenum (A-B), jejunum (C-D), ileum (E-F) and colon (G-H). Arrows points to single Ngn3<sup>+</sup> cells showing that in the mutants the individual Ngn3 cells are in closer vicinity.

Figure S3: **Rare Rfx6  $\Delta$ intestin surviving mice show no obvious defects in Paneth cells or proliferative compartment but have lipid absorption problem and diarrhoea.** (A-B) Immunofluorescence staining for Rfx6 shows efficient deletion in mutant mice. (C-D) Paneth cell lineage, stained with lysozyme, is not affected in mutant mice. (E-F) The proliferative compartment (stained with Ki67) of mutant and control mice is grossly similar. (G-H) Oil Red O staining of lipids revealed the presence of lipids in the faeces of mutant animals. (I) Photography showing that mutants have yellowish stools.

Figure S4: **Adult mice with intestinal deletion of Rfx6 are losing weight.** (A) Ten days after tamoxifen gavage, mice which received tamoxifen have lost weight and are smaller than vehicle treated animals. (B) Mutant males show evident sign of irritation around the anus compared to vehicle treated animals. (C) Graph representing the weight curves of controls and tamoxifen fed animals. Tamoxifen was administered at D0, D2 and D4. At day 12 the mutant with the smaller weight (14g) was dead.

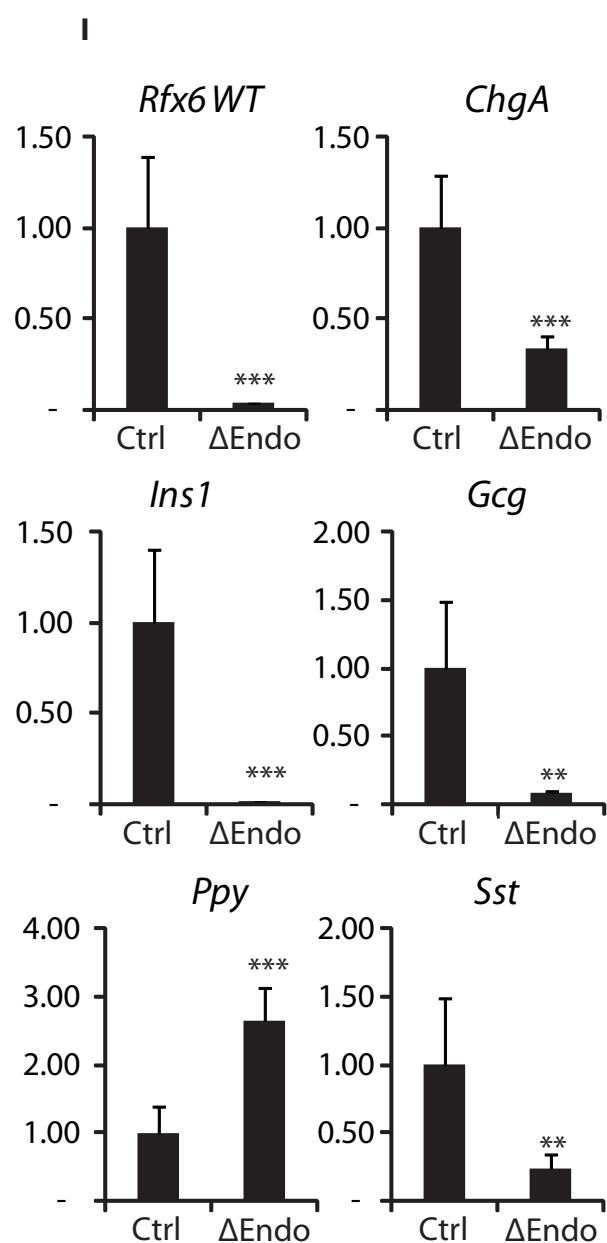
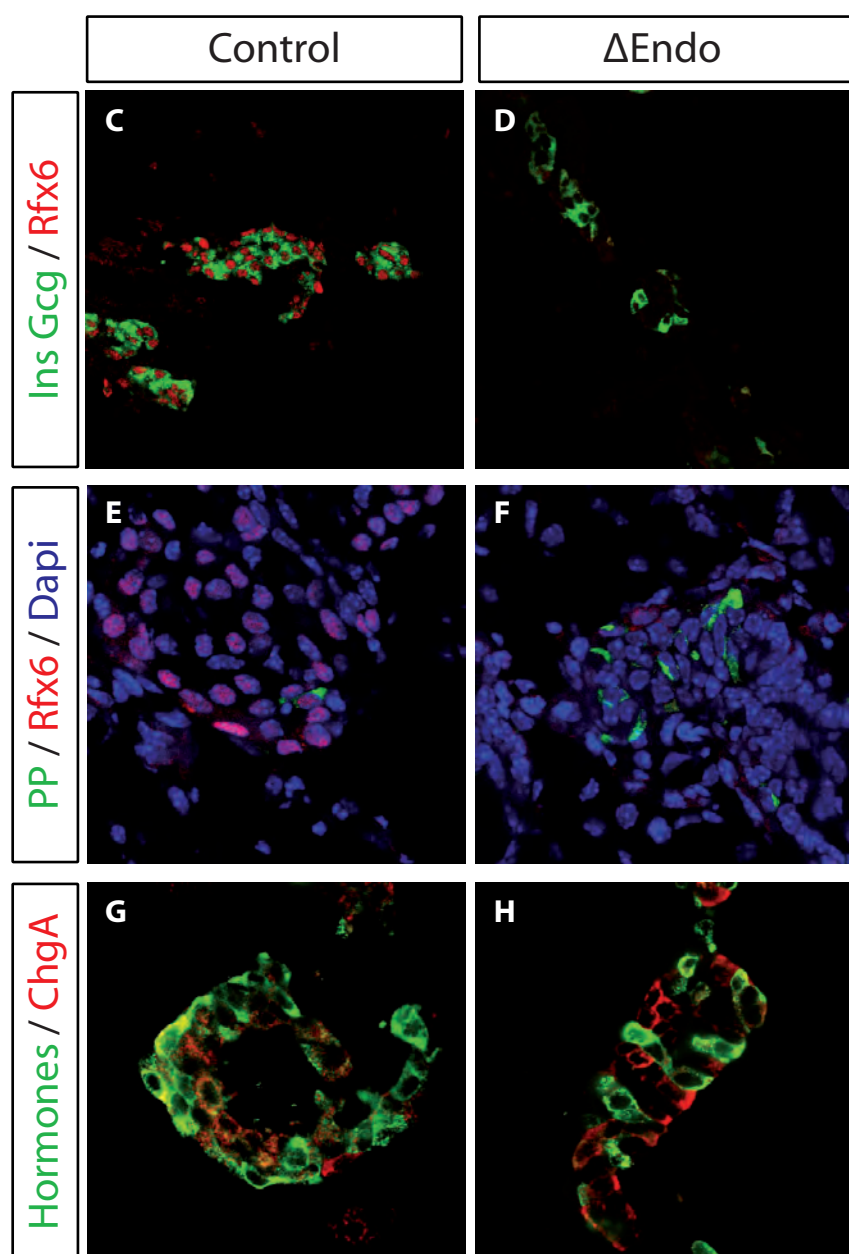
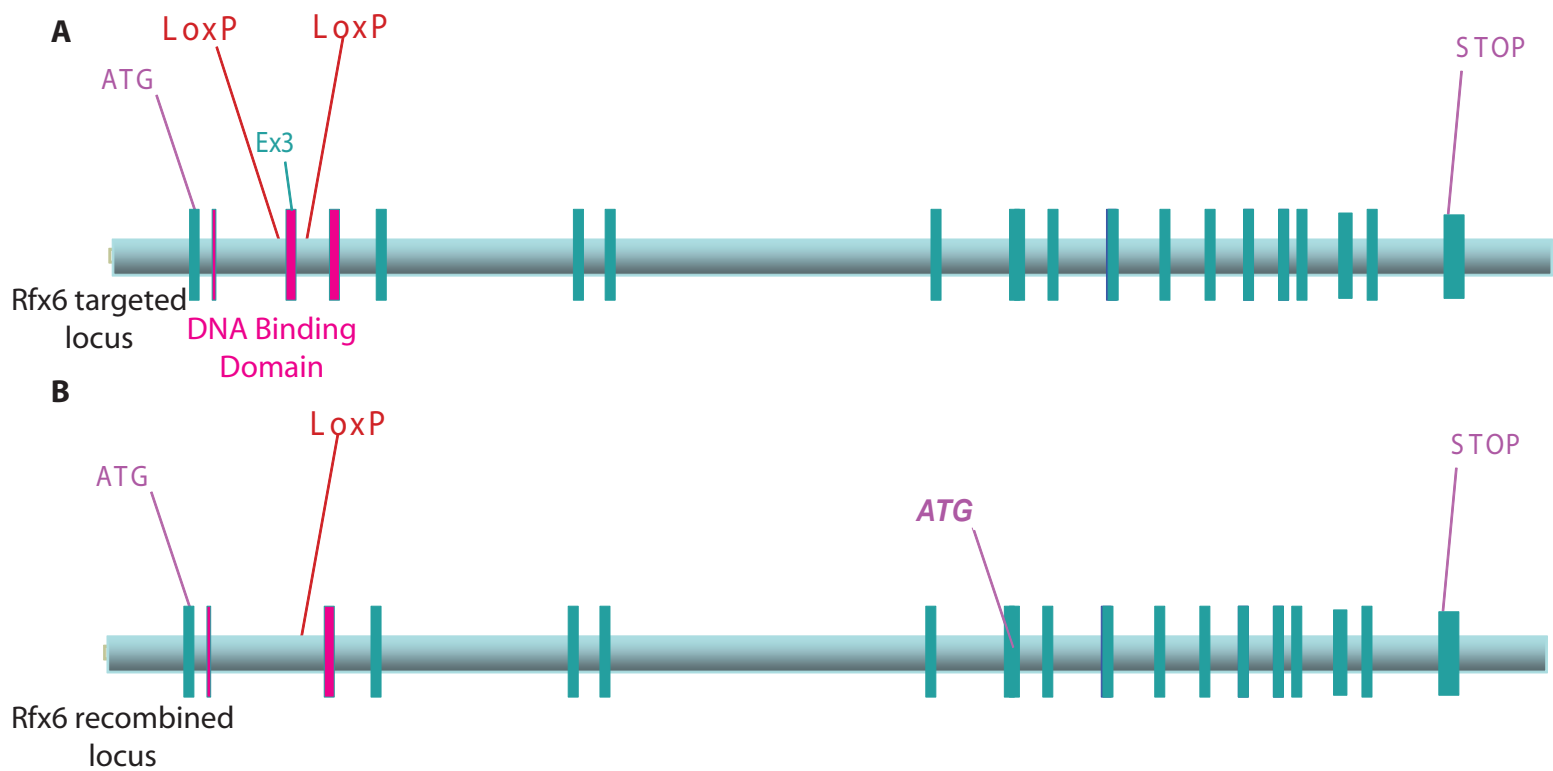


Figure 1



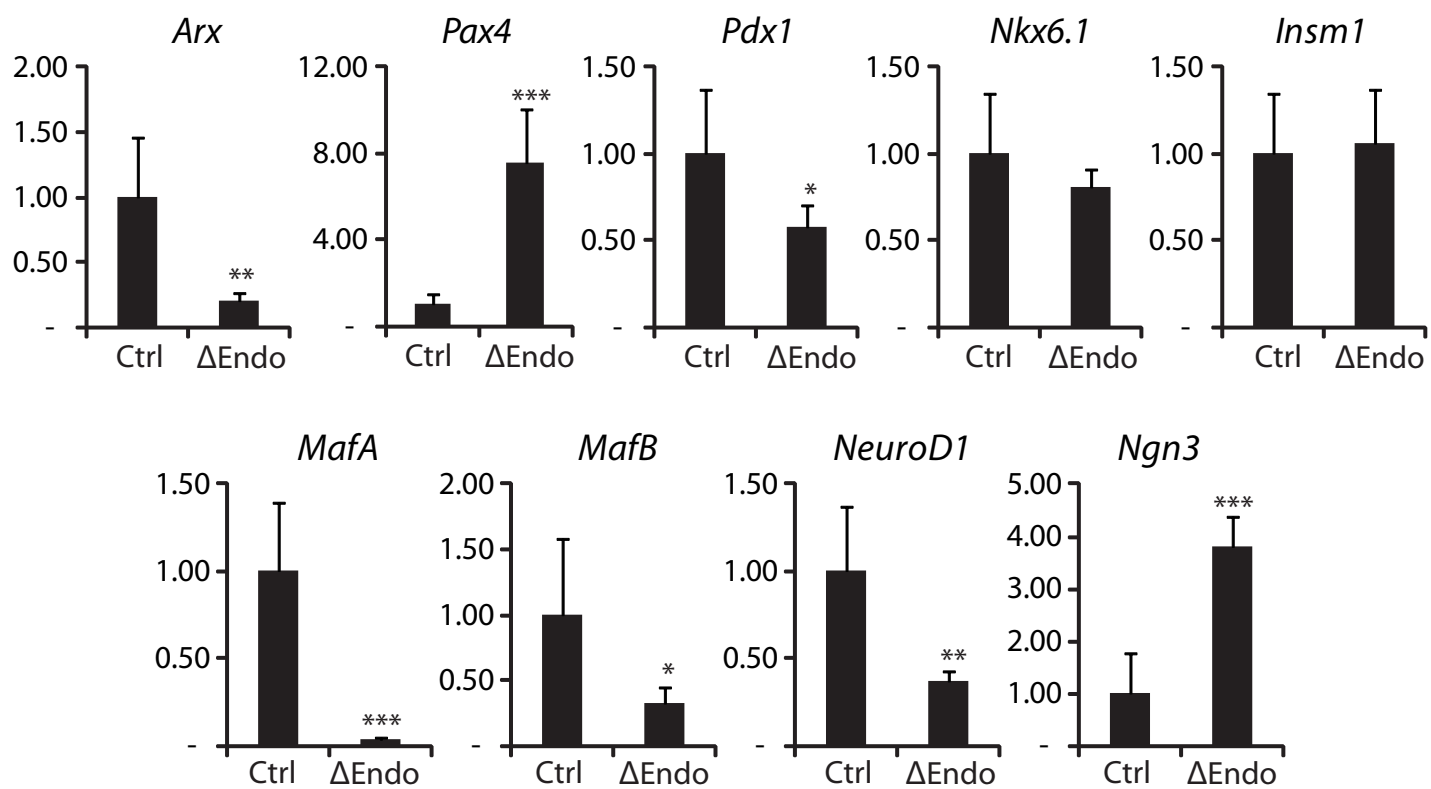


Figure 2

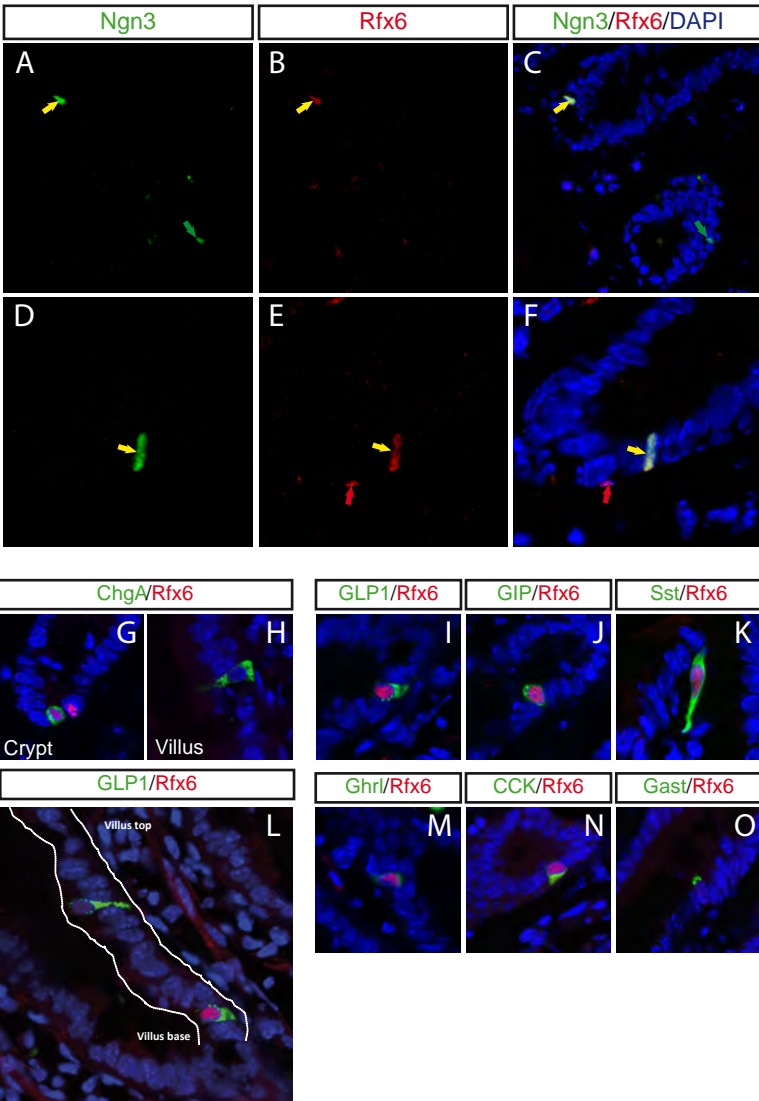
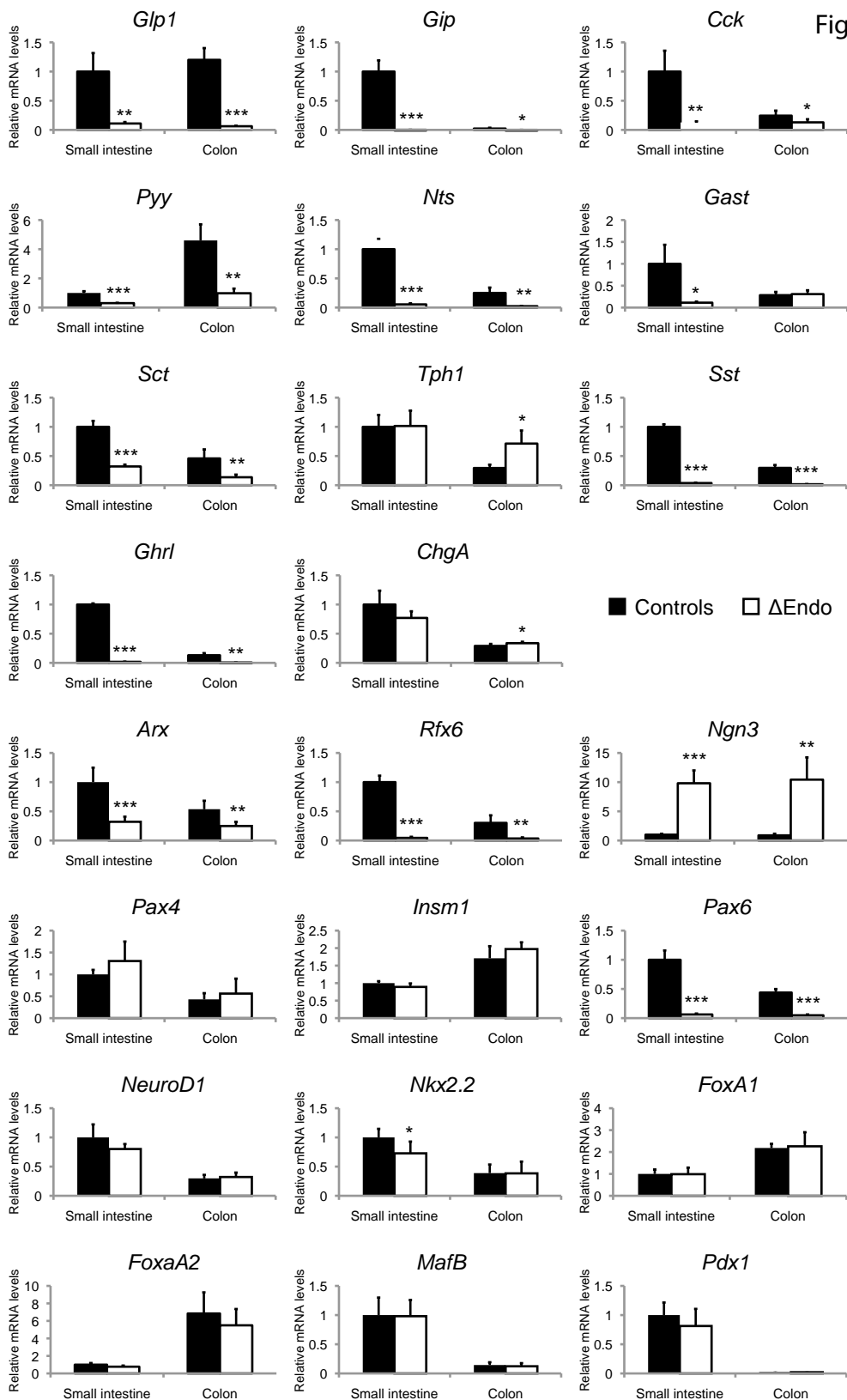


Figure 3



## IF quantification on P0 intestinal segments

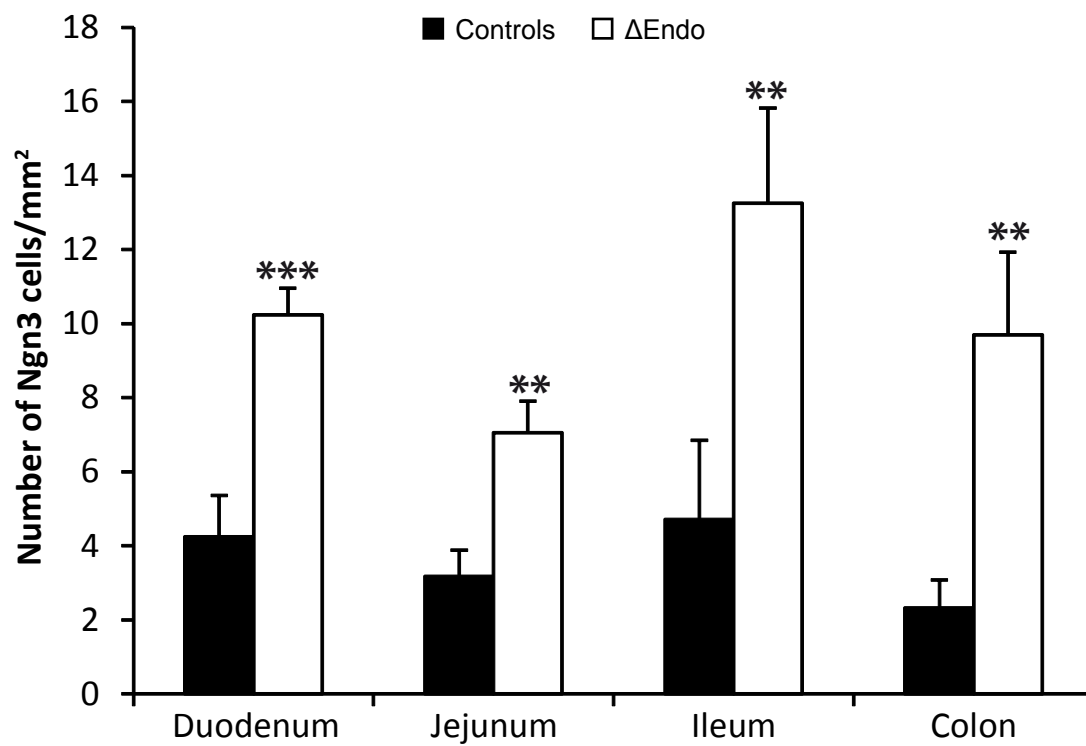
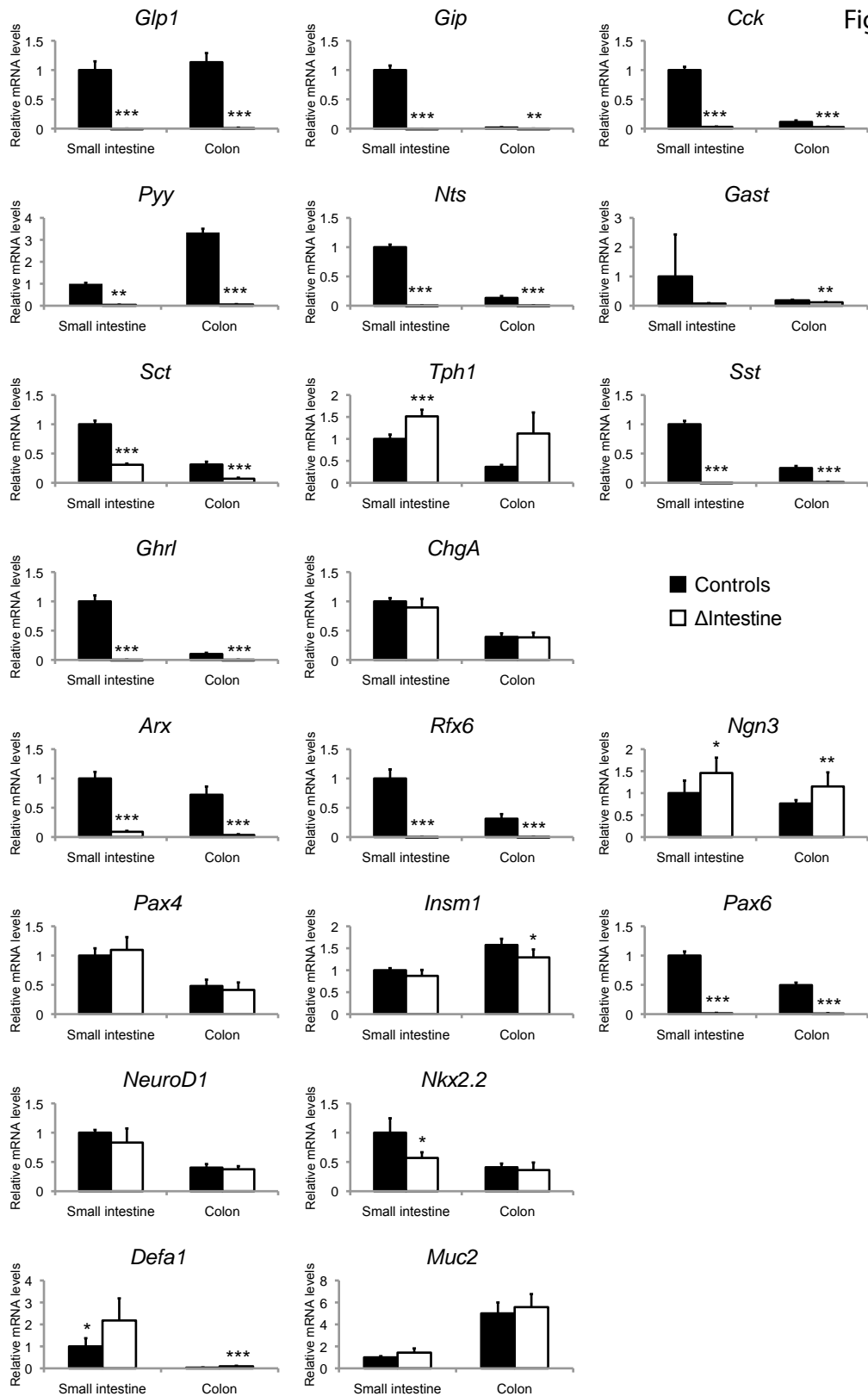
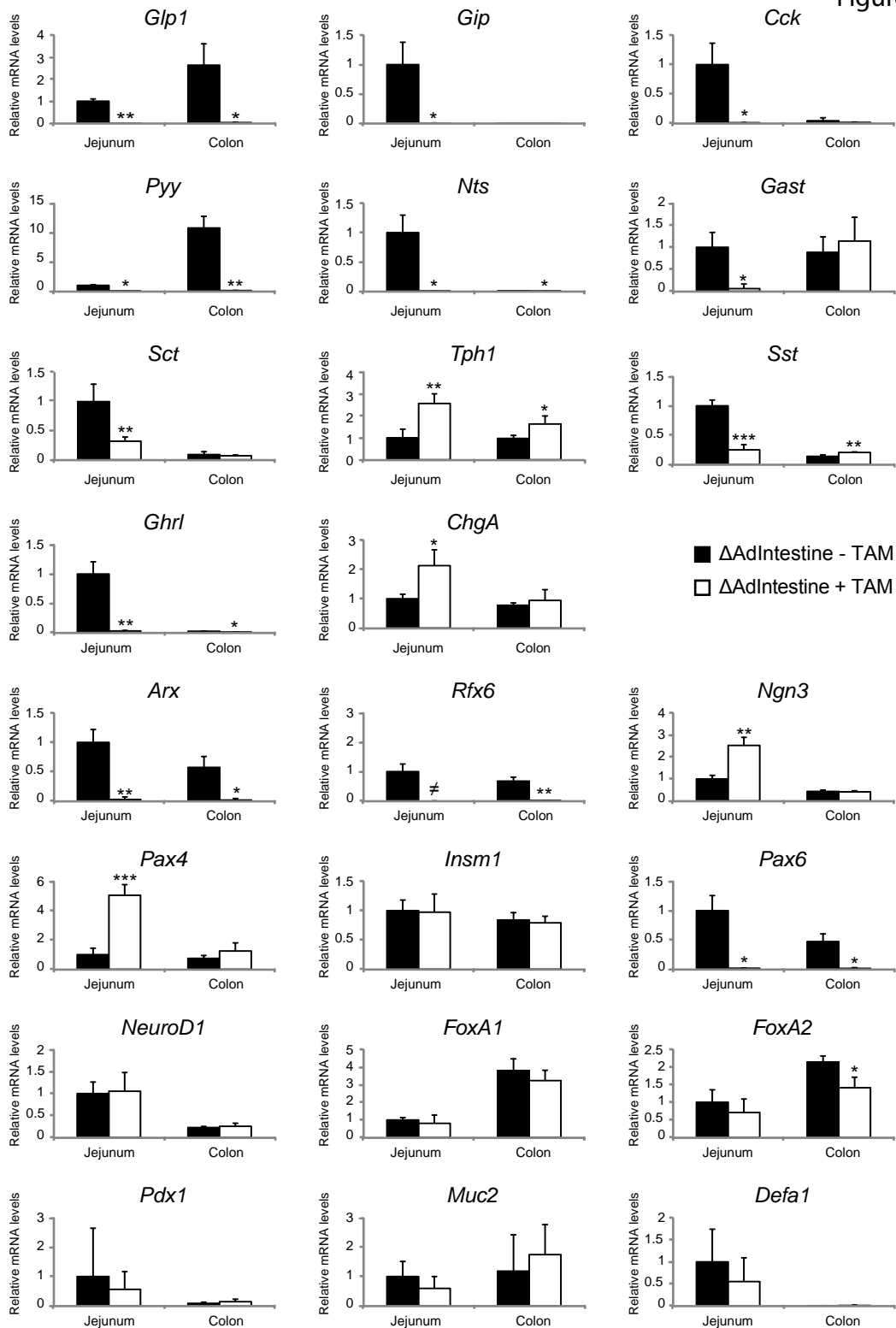


Figure 5





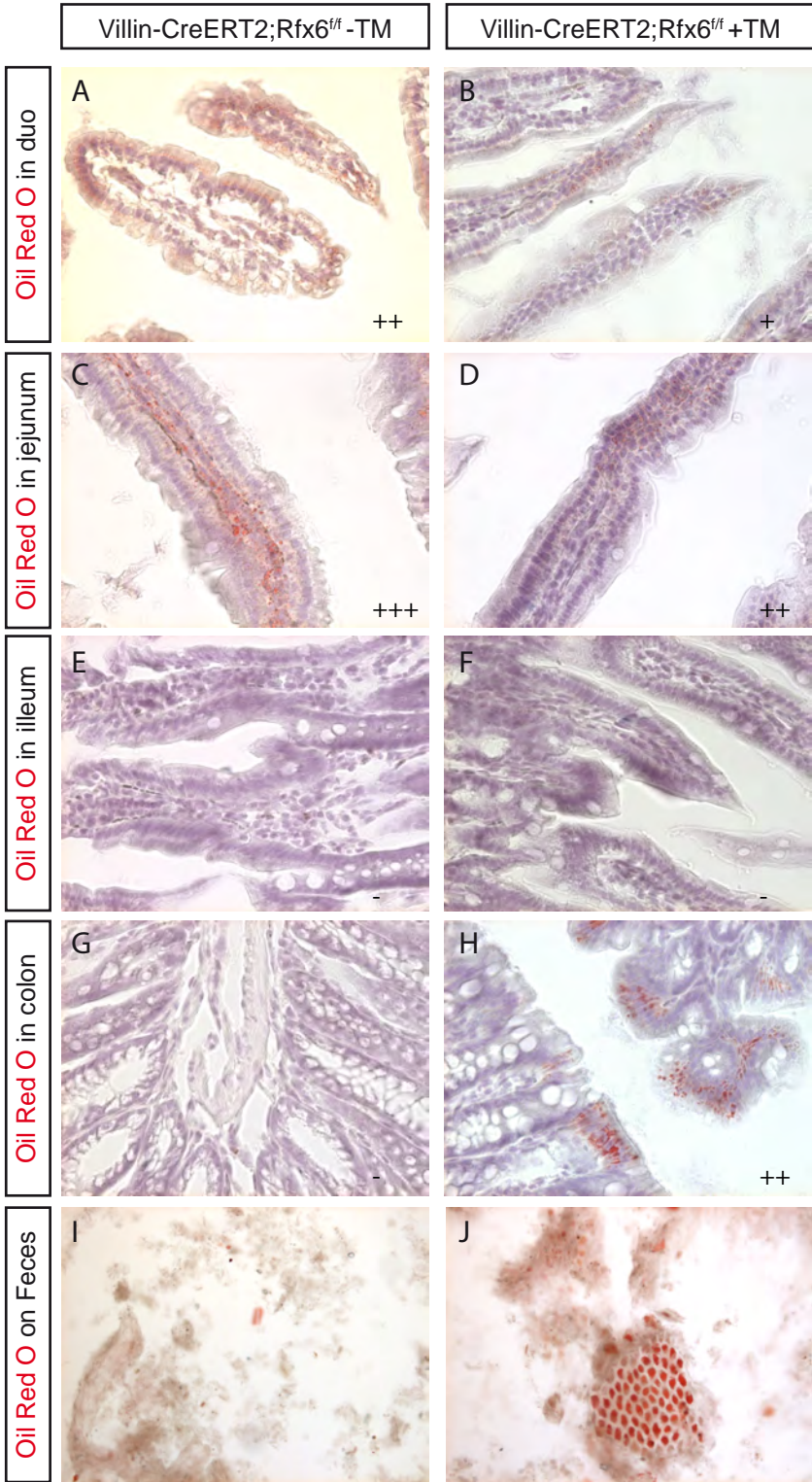


Figure 8

Ins1-CreERT2;Rfx6<sup>fl/fl</sup>

-TAM

+TAM

RT-qPCR on purified adult islets 3 weeks post tamoxifen injections

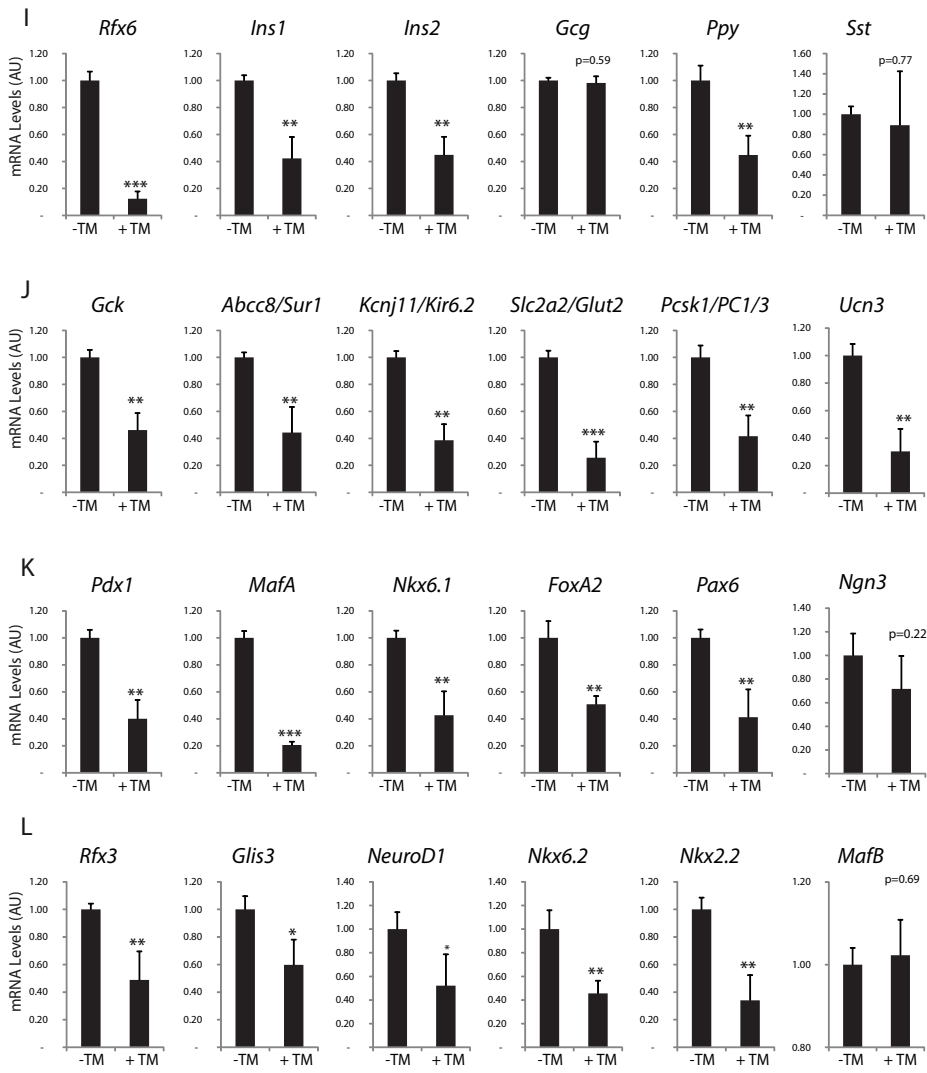
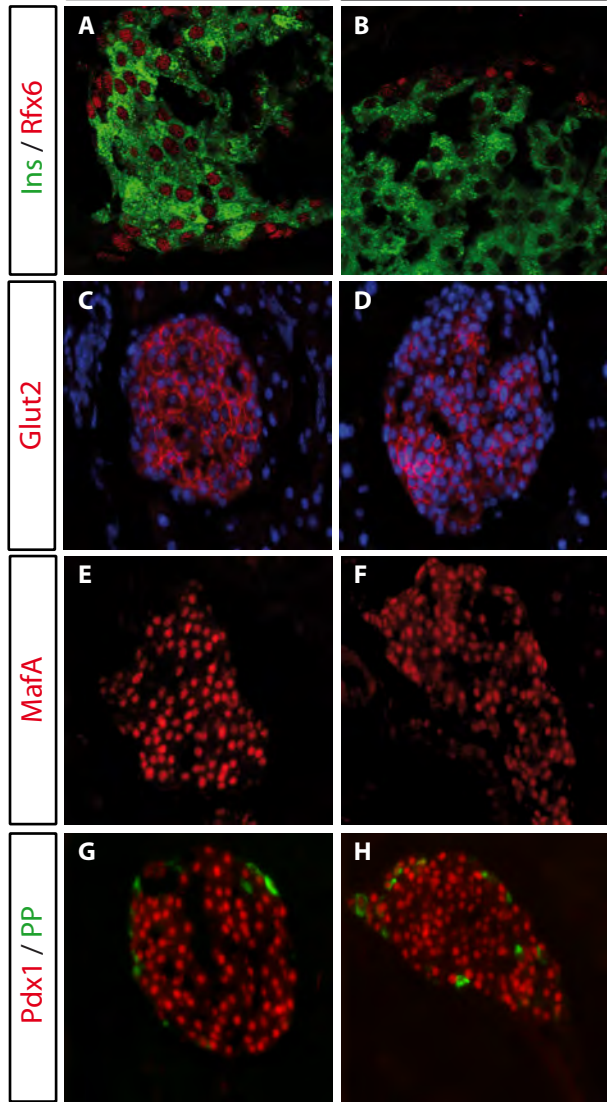


Figure 9



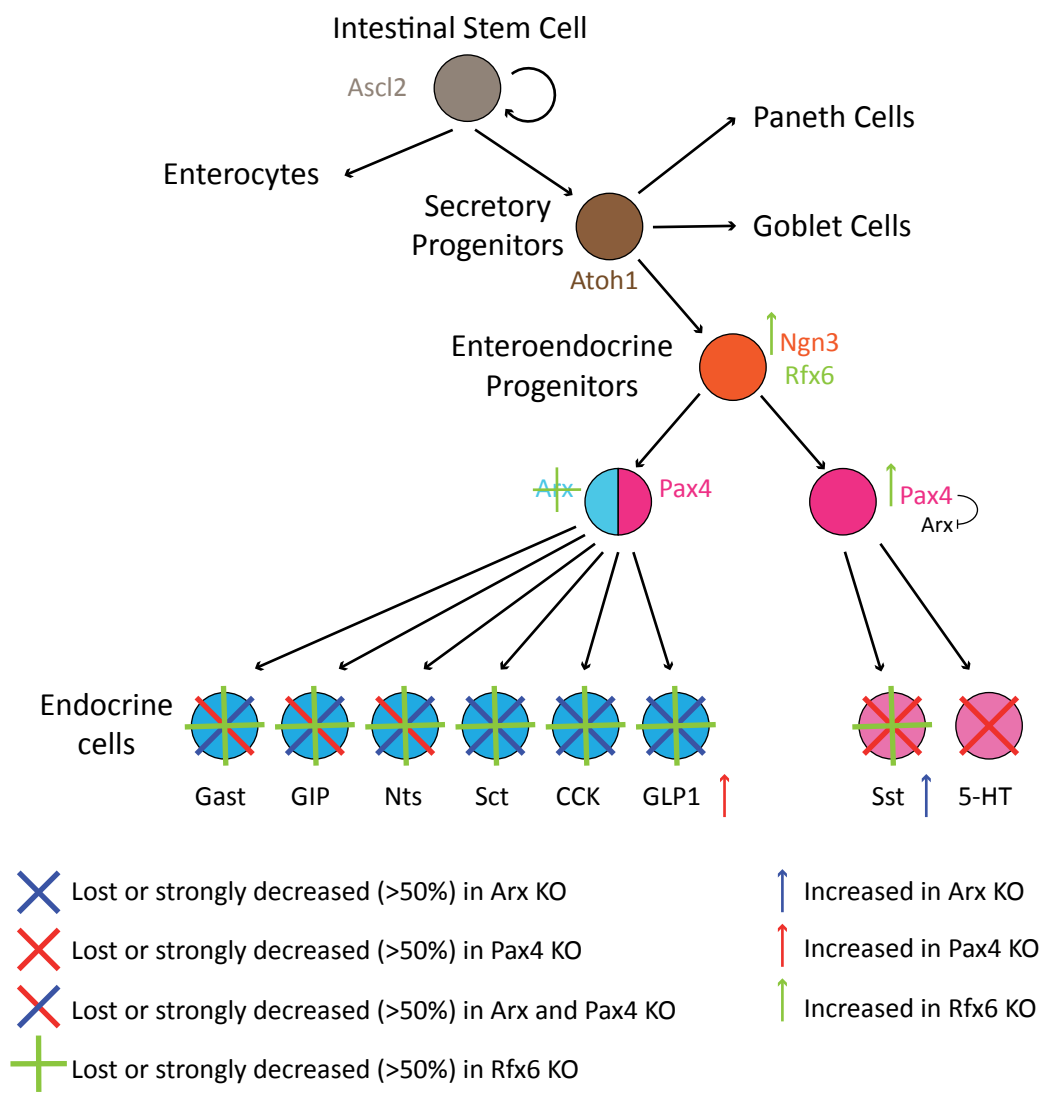


Figure 10

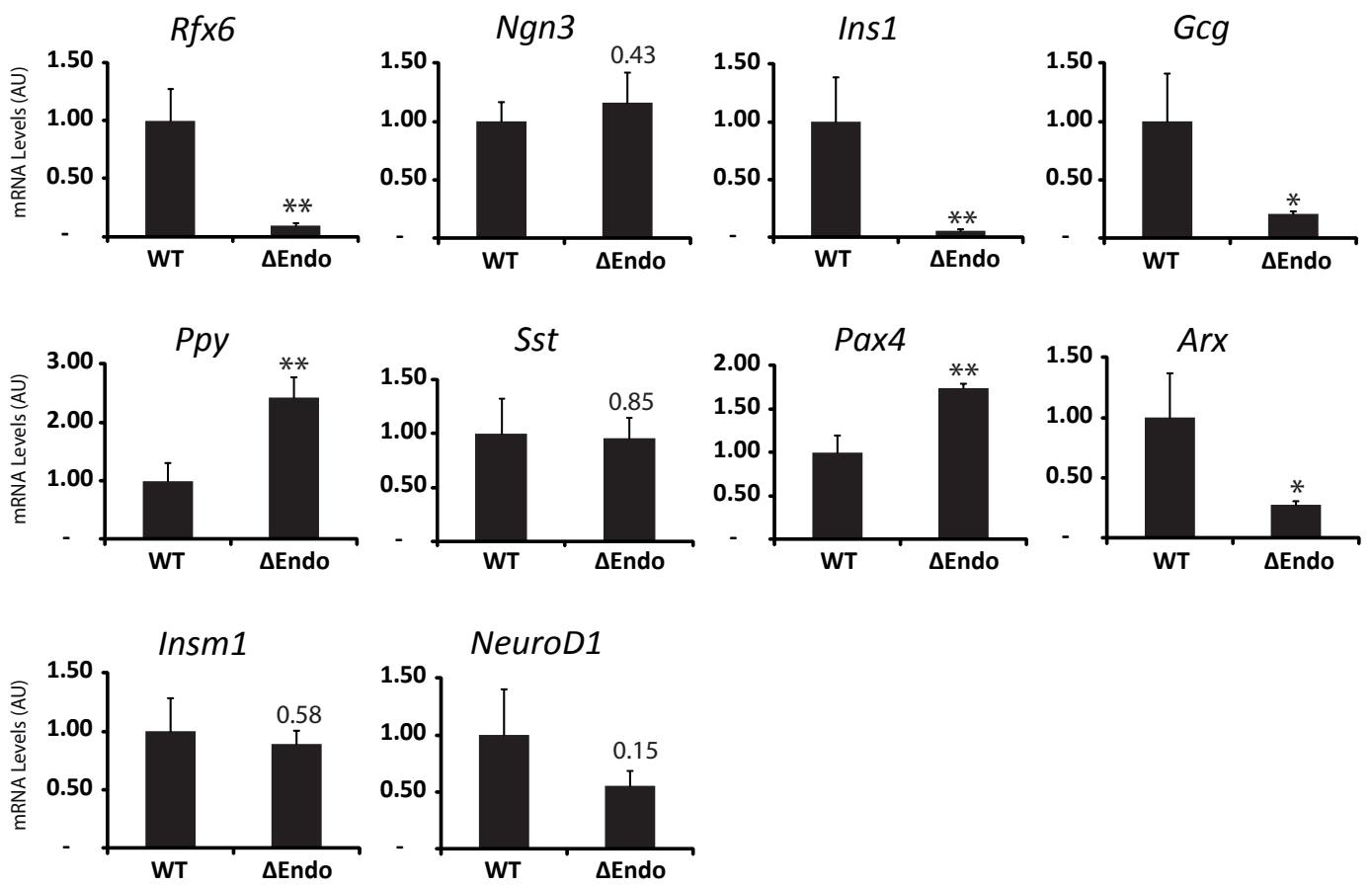


Figure S1

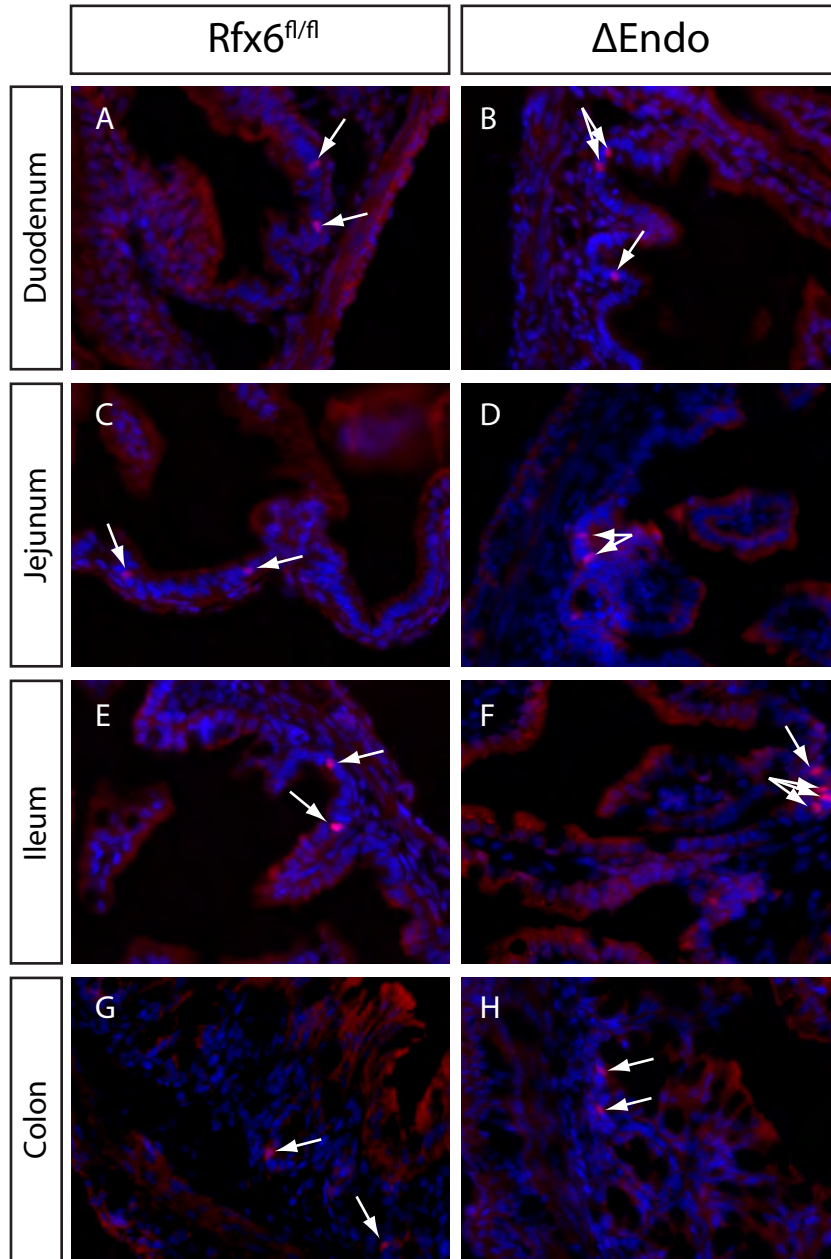
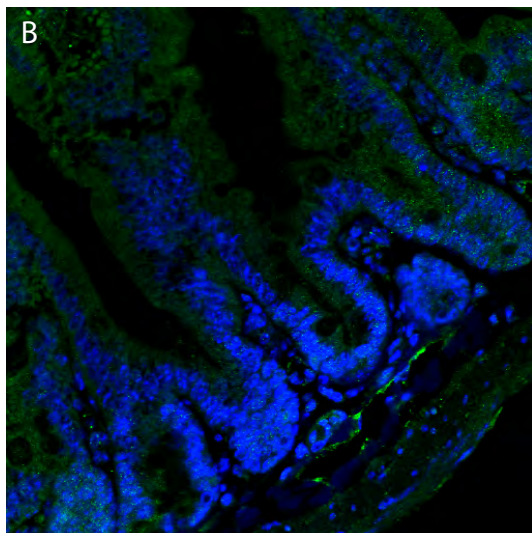
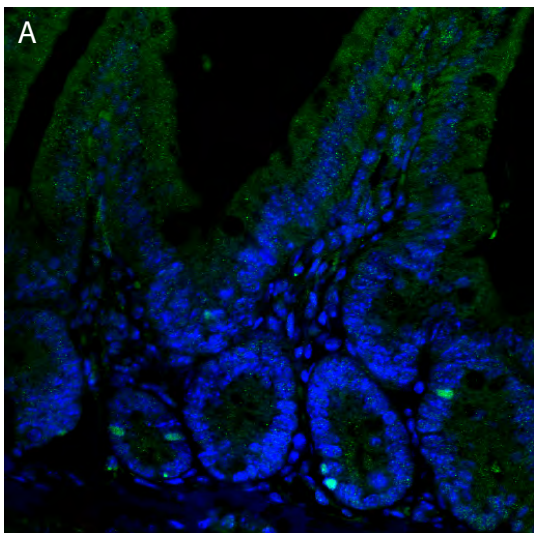


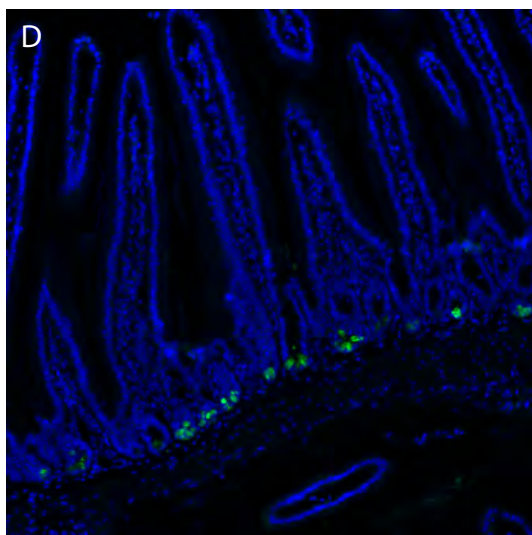
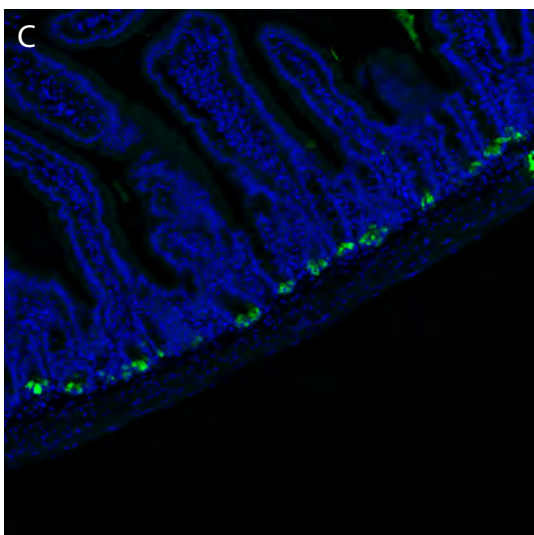
Figure S2

Rfx6<sup>f/f</sup> $\Delta$ Intestine

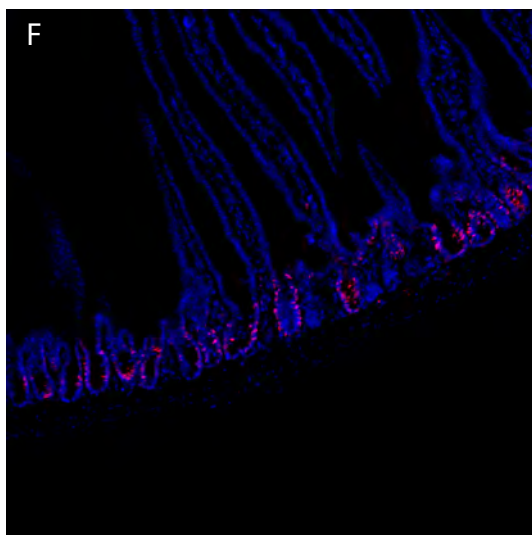
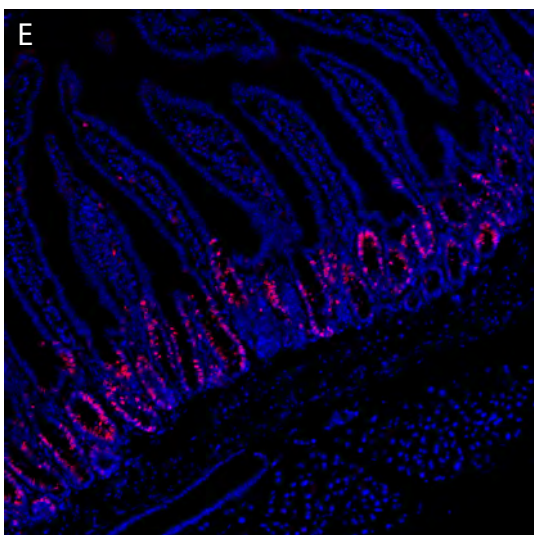
Rfx6 / Dapi



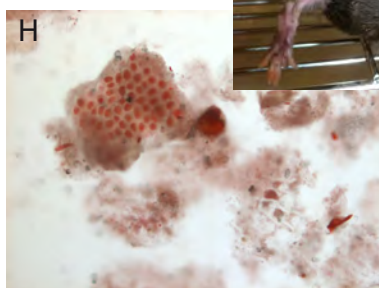
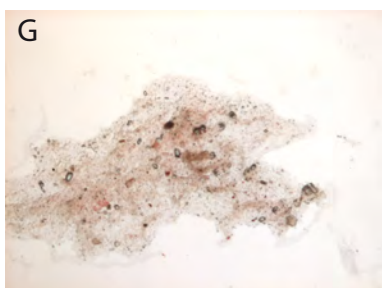
Lysozyme / Dapi



Ki 67 / Dapi



Oil Red O



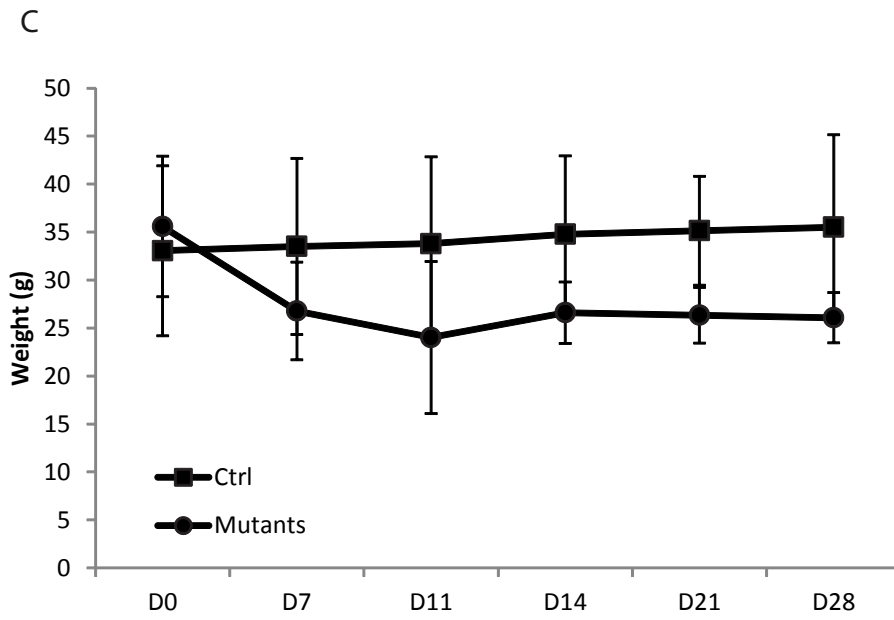
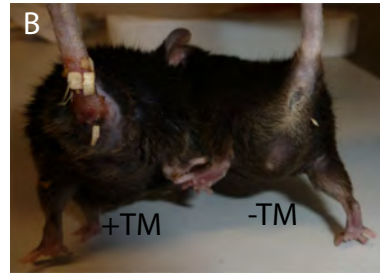
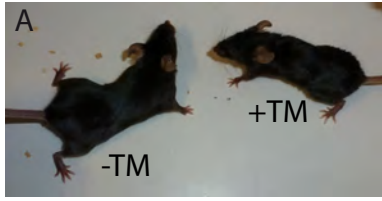


Figure S4



## 2. Additional results

### 2.1. Deletion of exon 3 of Rfx6 is sufficient to knockout Rfx6

#### 2.1.1. Context

In order to validate our approach which consisted of deleting the exon 3 of Rfx6 (Figure 41A) which is part of the DNA binding domain, we crossed our Rfx6 cKO with a CMV-Cre line to obtain the deletion of Rfx6 in all tissues and compare our phenotype with the one observed by Smith et al.

#### 2.1.2. Methods

We realised that the deletion with the CMV-Cre was not 100% efficient in all tissues (data not shown). Thus we had to select for mice which had a germline deletion of Rfx6 by doing a backcrossing on B6J/N wild-type mice (Figure 40). This way we have been able to get rid of the CMV-Cre allele and make sure that Rfx6 is totally deleted. We then crossed heterozygous mice together to obtain Rfx6 KO embryos and pups. Genotyping was performed using the strategy described in Figure 41C with the following primers: **4160** 5'-GCTCCGACTTCTGATCATCC-3'; **4162** 5'-GAAGGTGCACCCATAAAAGC-3'; **4163** 5'-TATAAGCCACCCAGGGTCAG-3'.

RT-qPCR were performed as described in the manuscript with the following primers: Rfx6ex9F 5'-GGCCATGGAGACTCAATTTAAC-3', Rfx6ex10R 5'-GGCAGCTTTACTCGCATCC-3', Rfx6ex3F 5'-TGCAGGAAAGAGAACTGGAG-3', Rfx6ex4R 5'-GGAAATTTTGGCGAATTGTC-3'.

Western Blot was performed with the antibody generated using the human/mouse Rfx6 peptide located at the end of the Rfx6 protein (see II.B.2.2).

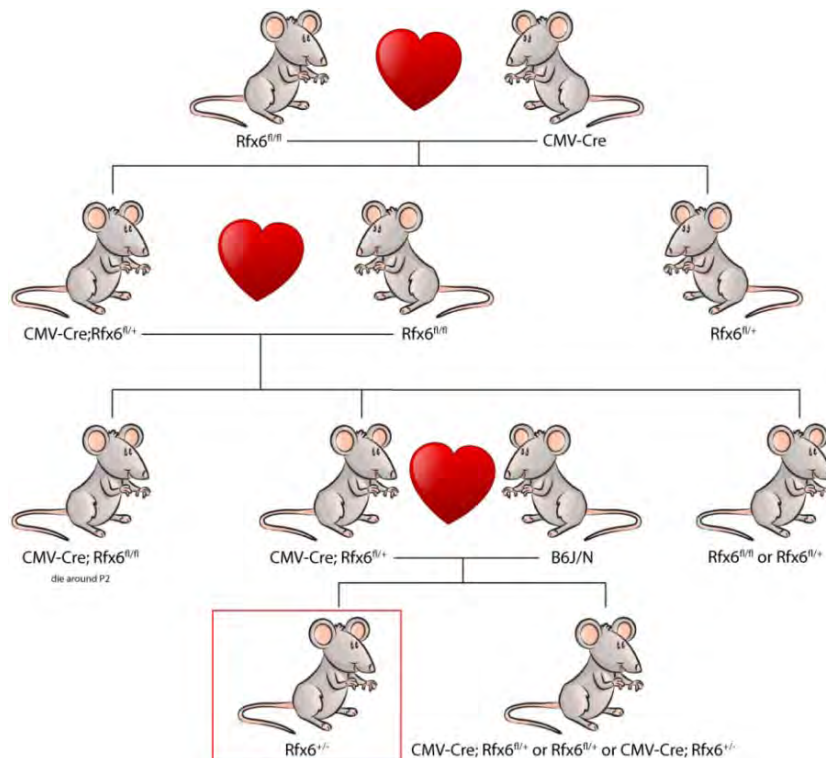


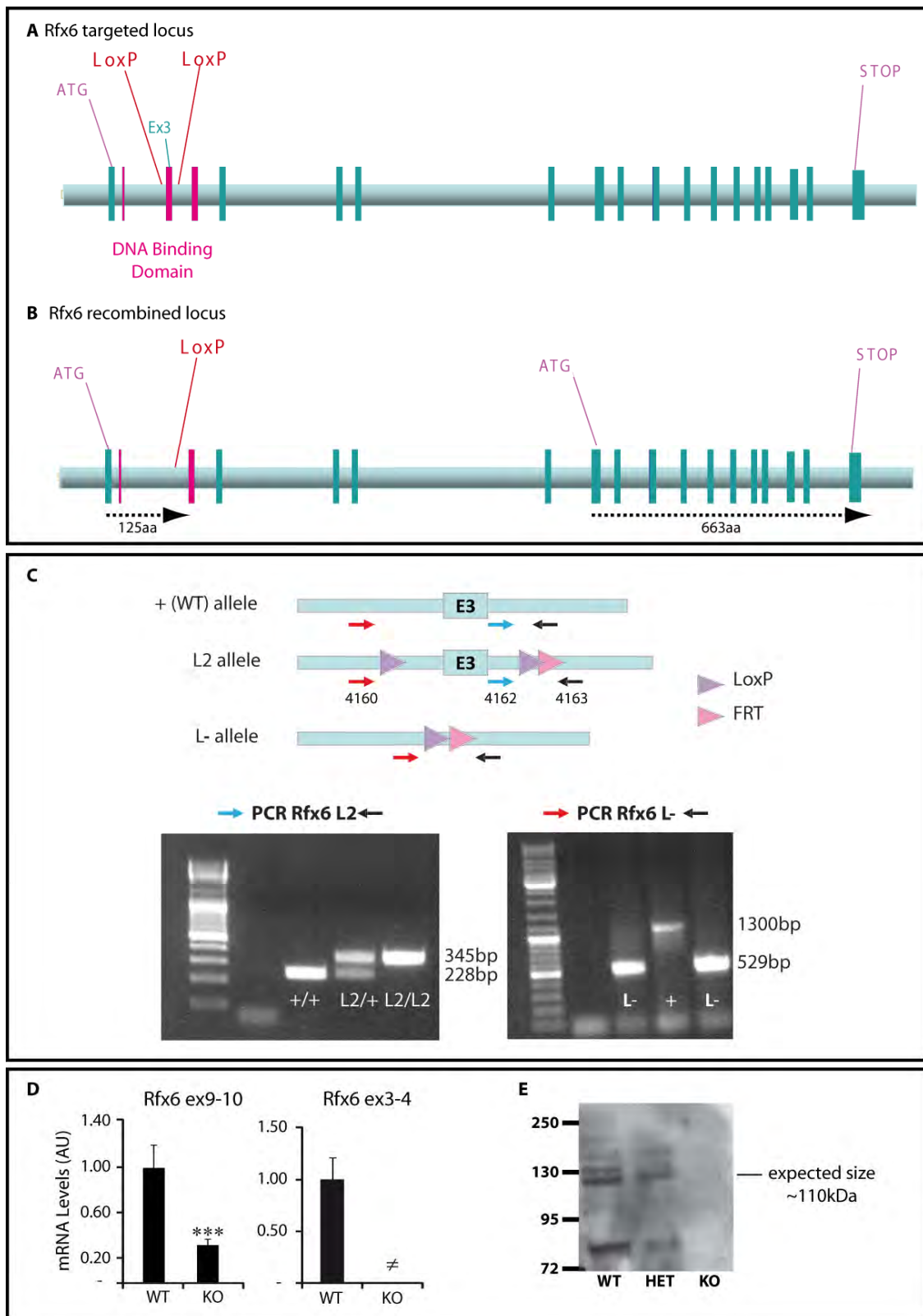
Figure 40: Breeding scheme to obtain Rfx6 heterozygous mice.

### 2.1.3. Results and discussion

The construction we used could give rise to two putative proteins (Figure 41B). The first one between the ATG and the exon 4 of 125 amino acids contains no putative conserved domain. The second one corresponds to the Ensembl! transcript ENSMUST00000050455 which is generated from an ATG in exon 8 which is in another open-reading-frame. This putative protein has no similarity with *Rfx6* and contains no putative conserved domain. We checked for the total loss of *Rfx6* by both RT-qPCR and Western Blot (Figure 41D-E). Using primers located after the ATG in exon 8, we observed that mRNA was being produced (Figure 41D left); probably corresponding to the transcript described earlier (ENSMUST00000050455). However, using primers before exon 8, we showed that the deletion of *Rfx6* was complete (Figure 41D right). Similarly no *Rfx6* protein was found in the pancreas of E18.5 knockout embryos (Figure 41E). In the wild-type, the band for *Rfx6* was expected around 110kDa; surprisingly our antibody also detects smaller and larger bands on Western Blot. We believe that these bands are specific as their intensity is reduced in heterozygotes and lost in the knockout. They may correspond to either uncharacterised isoforms or degradation products of *Rfx6*.

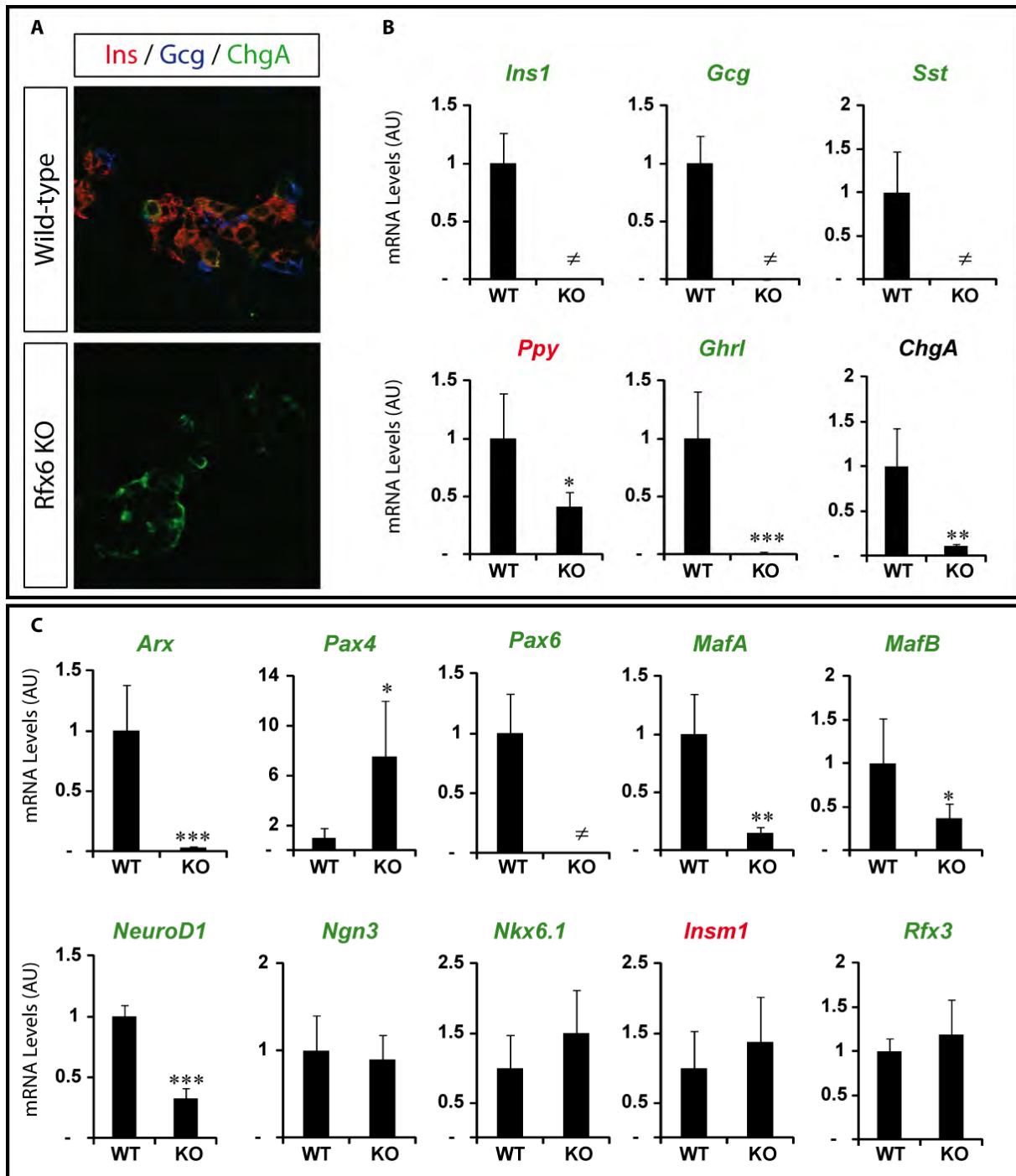
We then characterised the phenotype of our knockout mice and compared it with what was previously published by the lab of Mike German. Even though our construction is different from the one published by Smith et al., as our strategy deletes only the exon 3 when they replaced exons 1-5 by a eGFP-Cre cassette, the phenotypes are fairly similar and mutants die shortly after birth. We showed by measuring glycosuria and glycaemia that the mutant mice are diabetic (not shown) which was not reported previously. We further characterised the phenotype of the *Rfx6*-deficient mice by immunofluorescence and RT-qPCR at P0 (Figure 42). In both studies, *Rfx6* depleted mice initiate endocrine cell differentiation, outlined by the presence of chromograninA<sup>+</sup> and PP<sup>+</sup> cells but fail to develop any insulin-, glucagon- or somatostatin-expressing cells (Figure 42A-B and data not shown). In line with Smith et al., the absence of *Rfx6* did not affect *Ngn3*, *Nkx6.1* and *Rfx3*. Altogether these data show that the deletion of the exon 3 of *Rfx6* is sufficient to inactivate its function, thus validating our approach.

Interestingly, in our experiments *Insm1* expression is not affected (2-fold decrease in Smith et al.) suggesting that *Rfx6* acts either downstream or in parallel of *Insm1*. Another difference between the two studies is that *Ppy* is still present but slightly down-regulated in this study whereas the number of PP-cells was increased (mRNA not affected) in the study by Smith and colleagues. We believe that the effect on the level of *Ppy* might be stage-dependent. Supporting this hypothesis, we observed an increase in *Ppy* expression at E15.5 (not shown). Interestingly, we report a decrease in *chromograninA* transcripts that was not reported by Smith et al. as *chromograninA* probe is not present on the TaqMan array they used. Both studies show that chromograninA<sup>+</sup> cells are still present in the pancreas but it has not been quantified and thus we do not know whether they are decreased or not. It is important to note that the two studies were performed at different stages (Smith et al: E17.5, us: P0) which might also account for some of the differences. However, the major contradiction between the two studies lies in the intestinal morphology. Smith and colleagues reported in both mice and humans that *Rfx6* loss-of-function induces small bowel obstruction. We have never observed any intestinal atresia in our mutants. At least two hypotheses can explain this difference: (1) the insertion of the Cre-eGFP cassette in the construction by Smith et al. could generate a protein or disrupt enhancer sites which could interfere with intestinal development; (2) this effect might be related to the genetic background.



**Figure 41: Generation of a conditional knockout for *Rfx6*.** (A) *Rfx6* targeted locus. (B) *Rfx6* recombined locus. Dashed lines indicate putative proteins which might be expressed after Cre recombination. (C) Genotyping strategies. Primers were design to discriminate three kinds of alleles: WT, L2 (floxed), L- (deleted). In the PCR Rfx6L2, the L2 allele gives a band at 345bp and the WT allele a band at 228bp. In the PCR Rfx6L-, the L- allele gives a band at 529bp and the WT allele a band at 1300bp. In this PCR, the WT band is not always visible in heterozygous mice; in case of doubts perform the PCR Rfx6L2 as well. (D) RT-qPCR on P0 pups showing efficient deletion of *Rfx6* RNA in the pancreas. *Rfx6* ex9-10 corresponds to primers located in exons 9-10 and *Rfx6*ex3-4 to primers in exons 3-4. Data are represented as mean  $\pm$  SD, \*\*\* $p$ <0.001. (E) Western blot analysis on E18.5 pancreata showing the loss of *Rfx6* protein in the KO. Expected band size for *Rfx6* protein: 110kDa.





**Figure 42: Deletion of the exon 3 of *Rfx6* is sufficient to recapitulate *Rfx6* KO mice endocrine phenotype.** (A) IF staining on P0 pups for insulin (red), glucagon (blue) and chromograninA (green) showing that *Rfx6*-deficient mice do not develop insulin- or glucagon-expressing cells but do have endocrine cells as shown with chromograninA. (B) RT-qPCR analysis for hormones on P0 pancreata showing the loss of all hormones mRNA except *Ppy*. (C) RT-qPCR analysis for a subset of pancreatic transcription factors on P0 pancreata. (B-C) Genes in green are affected similarly in our study and the study by Smith et al., genes in red are affected differently in both studies, genes in black have not been studied by Smith and colleagues. Data are represented as mean  $\pm$  SD, \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

## 2.2. Generation of a mouse / human Rfx6 antibody

### 2.2.1. Methods

I started by finding all the antigenic sites of the human and mouse Rfx6 proteins using the online program *Antigenic* (EMBOSS). *Antigenic* predicts potentially antigenic regions of a protein sequence. Data from diverse experiments revealed that the hydrophobic residues Cys, Leu and Val, if they occur on the surface of a protein, are more likely to be part of antigenic sites. Kolaskar and Tongaonkar designed a semi-empirical method to predict antigenic determinants on proteins (Kolaskar and Tongaonkar, 1990). Application of this method to a large number of protein showed that this method can predict antigenic determinants with about 75% accuracy. Using this program I found respectively 37 and 36 antigenic sites on the human (Q8HWS3) and the mouse (OTTMUSP00000029881) Rfx6 proteins. Among those 21 were conserved between both species with 1 or no mismatch. The next step was to check that these peptides were specific to Rfx6 and not conserved in the other RFX. Thus I performed a sequence alignment of Rfx1-7 (P48377, P48379, ENSMUSP00000038760, ENSMUSP00000051107, OTTMUSP00000026691, OTTMUSP00000029881, ENSMUSP00000091338) using *Clustal W* and looked for the antigenic peptides. Among the 21 sites that were conserved between mouse and human, 13 were specific to Rfx6. Among these 13 only 2 were soluble and suitable for peptide synthesis. The peptide synthesis was carried on by Pascal Eberling from the peptide platform of IGBMC. One of the peptide is located between the DNA binding domain and the B domain and the other one at the very end of the protein (Table 11).

Peptides	Mouse position	Human position	Comment
GTLLPEFPSAQHLVYQGC	237-254	238-255	
CNQHVSIVISSIRSLPPYSD	818-835	819-836	Mismatch in mouse 11I→11V

Table 11: Rfx6-specific antigenic peptides conserved between human and mouse.

We coupled the peptides with ovalbumin using the IMJECT\* MALEIMIDE ACTIVATED OVALBUMIN kit (PIERCE). Briefly, maleimide activated ovalbumin was solubilised in water and mixed with 2mg of the peptide and incubated for 2h at room temperature and then dialysed overnight against PBS. The harvested coupled peptides were quantified by Bradford. 500µg was injected per rabbits in two injections of respectively 300µg and 200µg for the boost. Rabbits are two months old female White Zew-Zeland strain. First blood sample is collected from the median artery of the ear to produce the preimmune serum, and then rabbits are injected with the antigen solution on 40 to 60 different locations. One month after immunisation, blood sample is collected each week for a one-month period (Fractions 1-4). If the immune response is not positive, a boost is performed and blood is collected after 12 days (Fraction boost). Nineteen days after the boost, rabbits are sacrificed and blood is collected by intracardiac puncture (Fraction Final Bleed). The different fractions obtained were tested on Min6b1 protein extracts by Western Blot.

### 2.2.2. Results

Fractions 1-4 never gave a signal in Western Blot (WB) thus we did boost the immune response with a second injection of the coupled peptides. The boost and final bleeding gave a signal but the background noise was very high on WB performed on Min6b1 extracts. Thus we purified part of the sera and did WB again. Unfortunately, to date we have not been able to obtain reproducible results, either due to degradation of the antibody or technical problems. We are investigating these hypotheses with immunofluorescence and new WB solutions. In parallel we will test the antibodies on human proteins on WB with extracts from a human beta-cell line (Ravassard et al., 2011).

## 2.3. Identification of Rfx6-regulated genes by microarrays

### 2.3.1. Context

The deletion of Rfx6 in the endocrine lineage gave a similar phenotype than the total deletion of Rfx6 showing that the loss of pancreatic and intestinal endocrine cells was essentially due to the function of Rfx6 downstream of Ngn3. We then wanted to analyse two different aspects. (1) First, the **targets of Rfx6 in the endocrine lineages** of pancreas and the intestine. To answer this question, we generated two different sets of microarray using  $\Delta$ Endo mice, one in the pancreas at E15.5 and one in the intestine at E18.5 comparing controls to  $\Delta$ Endo embryos. (2) Second, we generated two other sets of microarrays using full KO mice, in the pancreas as well as in the intestine at E15.5 and E18.5 respectively. Genes affected in this experiment and not in (1) should give us insight to the **endodermal function of Rfx6**.

### 2.3.2. Methods

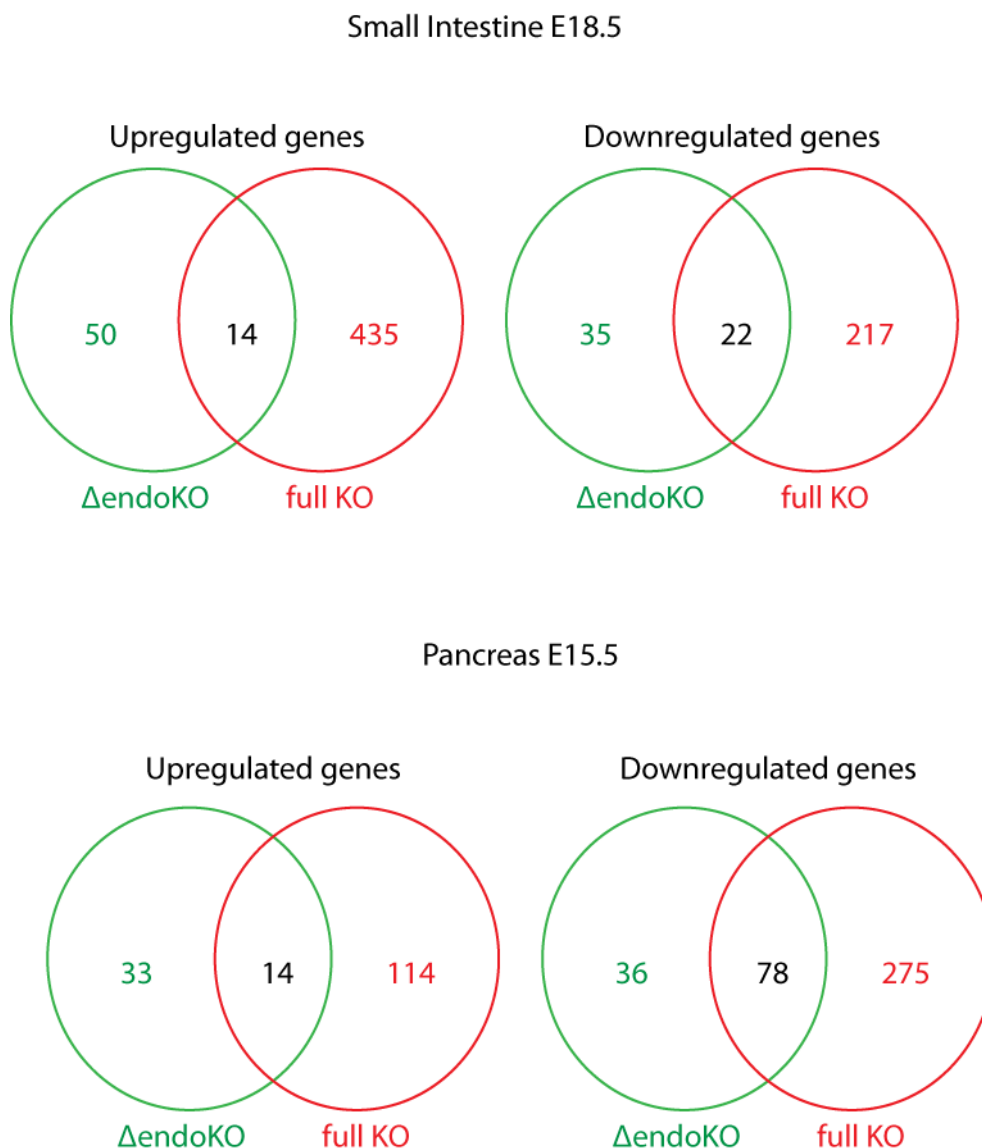
Embryos were dissected at E15.5 for the pancreas and E18.5 for the intestine. Individual pancreata were harvested and dissociated into 1ml of Tri Reagent (Sigma) with a 26G needle. Small intestines were homogenised in 2ml of Tri Reagent using a Polytron (Capitol Scientific). Samples were snap frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  until genotyped. RNA extraction was then performed as described in the manuscript. RNA for 4 different samples was then hybridised on Agilent microarrays (Gene Expression Microarray, 8x60K : SurePrint G3 Mouse GE 8x60K G4852A) by the Biopuce platform of the IGBMC. Signal intensity values were extracted with the LOWESS method using the Agilent software *Feature Extraction* (version 10.10.1.1) and then normalised by the quantile method using *Naomi* (v3, in-house software created by Doulaye Dembele). Data were then summarised in an Excel file which was used for further analysis. We generated four different studies (Table 12) and analysed the data similarly. Briefly, when every sample gave a signal lower than 70 the probeset was considered as not expressed, fold change was calculated on the signal intensity comparing mutants versus controls. P values were determined on the  $\log_2$  values of the signal intensity and considered as statistically significant when smaller or equal to 0.05.

Organ	Stage	Study
Pancreas	E15.5	Full KO vs. WT
Pancreas	E15.5	$\Delta$ Endo KO vs. CTRL (Rfx6 <sup>fl/+</sup> or Rfx6 <sup>fl/fl</sup> )
Small Intestine	E18.5	Full KO vs. WT
Small Intestine	E18.5	$\Delta$ Endo KO vs. CTRL (Rfx6 <sup>fl/+</sup> or Rfx6 <sup>fl/fl</sup> )

Table 12: Design of the four microarrays experiments.

## 2.4. Results and discussion of the microarray data

In the full knockout we obtained 688 (449 up-regulated and 239 down-regulated) and 481 (128 up-regulated and 353 down-regulated) genes differentially expressed in the intestine and the pancreas respectively. In the  $\Delta$ Endo, we obtained 121 (64 up-regulated and 57 down-regulated) and 161 (47 up-regulated and 114 down-regulated) genes differentially expressed in the intestine and the pancreas respectively (Figure 43). Genes were considered differentially expressed when fold-change was higher than 1.5 or lower than -1.5 and  $p < 0.05$ . Fold change in the full KO varied between -565 (*Ins1*) and +9 (*Ppy*) in the pancreas and -75 (*Ghrl*) and +1000 (*Gkn2*) in the intestine. In the  $\Delta$ Endo, fold change varied between -21 (*Ghrl*) and +8 (*Tac1*) in the pancreas and -50 (*Gip*) and +25 (*Amy2a5*) in the intestine.



**Figure 43 : Number of genes differentially expressed in the intestine and the pancreas by microarrays on *Rfx6* full KO or  $\Delta$ Endo embryos.** Genes were considered as differentially expressed if the fold-change was higher than 1.5 or lower than -1.5 and  $p < 0.05$ .

#### 2.4.1. Is Rfx6 an activator or a repressor?

Interestingly, in our microarrays we have found genes both up- and down-regulated. The question arises whether Rfx6 is an activator or a repressor. Just using microarrays we cannot conclude if Rfx6 is an activator or a repressor, as the differences we observed may be due to indirect effects. For example, Rfx6 could be activating an inhibitor of gene X thus leading to the decrease of X even though Rfx6 is an activator.

Generally, RFX are mostly repressors (Hsu et al., 2010) however some Rfx have been shown to act as transcriptional activators for example for MHC class II genes or IL-5R activation (Iwama et al., 1999). Whether Rfx6 act as an activator or a repressor may also rely on the presence of **co-factors** in the environment. Supporting this idea, Rfx6 seems to have an activating role in the pancreas (71% and 73% of down-regulated genes in the  $\Delta$ Endo and full KO respectively) whereas in the intestine it seems to be both a repressor and an activator (53% and 65% of up-regulated genes in the  $\Delta$ Endo and full KO respectively). In order to be able to classify Rfx6 it will be necessary to identify its direct targets in both tissues (see Perspectives/Conclusion).

#### 2.4.2. Genes differentially expressed in the pancreas

Among the genes differentially expressed in the pancreas of both the Rfx6 full KO and  $\Delta$ Endo most were related to the function of endocrine cells (*Ins1*, *Ins2*, *Ghrl*, *Arx* ...). Table 13 contains a short-list of genes differentially expressed which could represent interesting Rfx6 targets. Among them *Npy* (FC -9 in both KO) and *Vdr* (FC -3 in  $\Delta$ Endo; FC -2 in full KO) were in the top10 down-regulated genes and *Tac1* (FC 8 in  $\Delta$ Endo; FC 4 in full KO) was the top1 up-regulated, I will briefly discuss these genes here as we believe they could be interesting potential *Rfx6* targets with a role in endocrine cell differentiation and function and as such it would be of interest to characterise them in the pancreas.

Gene Name	Change	Functions (GO)
Inhba	UP	G1/S transition of mitotic cell cycle, gonad development
Kcnc2	UP	Voltage-gated potassium channel activity
Slc35d3	UP	Carbohydrate transport
Sult1d1	UP	Metabolic process
Tac1	UP	Synaptic transmission, Substance P receptor binding
Cdhr1	DOWN	Calcium ion binding
Hopx	DOWN	Trophectodermal cell differentiation, Regulation of heart contraction
Npy	DOWN	Positive regulation of appetite
Vdr	DOWN	Skeletal system development, Negative regulation of cell proliferation

**Table 13:** Short-list of genes differentially expressed in the pancreas in both the  $\Delta$ Endo and the full KO at E15.5. GO: Gene Ontology.

**a) Genes up-regulated in the pancreas of the full and  $\Delta$ Endo KO**

In the pancreas, among the genes up-regulated in both knockouts 64% were endocrine related, either hormones or genes necessary for proper endocrine function (e.g. *Chgb*, *Pax4*, *Ppy*). The other genes were either not annotated or not studied in the pancreas but implicated in transport (e.g. *Kcnc2*, *Kcnh6*) or differentiation (e.g. *Inhba*) in other organs (based on Gene Ontology GO).

**Tac1** which codes for substance P, neurokinin A, neuropeptide K and neuropeptide gamma was the gene affected in both KO with the highest enrichment (FC 8 and 4). It has never been directly studied in the pancreas before. However, it has been demonstrated that substance P affects protein kinase C signalling in adipocytes and as such has a role in insulin resistance and could be important for type 2 diabetes (Karagiannides et al., 2011). Further studies will tell which pancreatic cells secrete substance P and what its role is. Interestingly, it has been shown that intra-arterial injection of substance P in the pancreas of NOD (non-obese diabetic) mice reverses abnormal insulin resistance and diabetes for weeks (Razavi et al., 2006).

**b) Genes down-regulated in the pancreas of the full and  $\Delta$ Endo KO**

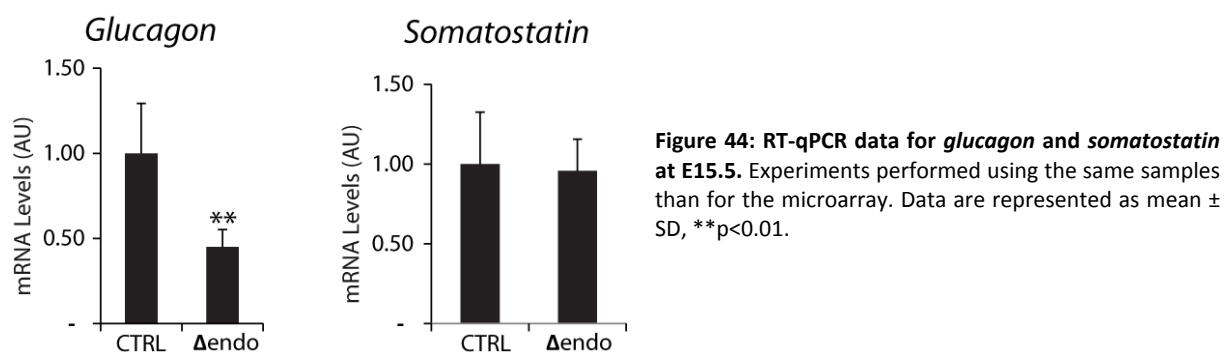
About 35% of the down-regulated genes have been shown to have a role in the endocrine function (e.g. *Arx*, *Ghrl*, *Iapp*, *Ins1*, *MafB*, *Mlxipl*, *Vdr*, *G6pc2*), 27% are putative genes which have not been annotated yet, the others 38% have roles in differentiation (e.g. *Hopx*, *Th*), cell cycle regulation (e.g. *Scg2*), migration (e.g. *Adra2a*, *Mapt*), transport (e.g. *Abcb9*, *Ap1s2*, *Slc2a5*) or exocytosis (e.g. *Syt14*) in other organs (Table 13).

**Neuropeptide Y** is a neurotransmitter found in the brain and autonomic nervous system. It regulates energy homeostasis and has been involved in learning and memory. When the levels of *Npy* are high food intake is increased and physical activity decreased. In the hypothalamus, *Npy* is secreted by NPY neurons to increase the proportion of energy stored as fat (Padilla et al., 2010). It has recently been shown in the neuroendocrine hypothalamus that *Ngn3* inhibits the development of NPY neurons in the arcuate nuclei whereas it promotes the development of the POMC neurons (Pelling et al., 2011). Interestingly, it has been shown that insulin secretion is increased in pancreatic islets from *Npy*-deficient mice (Imai et al., 2007). We could thus imagine that *Rfx6* could be necessary downstream of *Ngn3* to promote the differentiation of *Npy*-secreting cells. Supporting this idea, it has been shown that beta-cells (as well as alpha- and delta-cells) can synthesise *Npy* (Whim, 2011). However, the precise function of *Npy* in the pancreas remains to be studied.

**Vitamin D Receptor** (*Vdr*) is expressed on pancreatic beta-cells and it has been shown that insulin secretory capacity was impaired in mice lacking a functional *Vdr* (Zeitl et al., 2003). Vitamin D is a major regulator of calcium metabolism (Xue and Fleet, 2009) however it is not known whether the impaired insulin secretion is related to the defects in calcium metabolism. Thus further studies would be required to identify the role of *Vdr* in the pancreas and its downstream targets in the regulation of insulin secretion.

Surprisingly, in the  $\Delta$ Endo microarray we have not been able to detect a differential expression of *glucagon* ( $p < 0.09$ ) and *somatostatin* at E15.5. However, when I performed RT-qPCR experiments on the samples we used for the microarray *glucagon* was down-regulated by 60% (Figure 44). This discrepancy between the microarray and the RT-qPCR cannot be explained by the position of the oligo on the gene as different probsets covering the *glucagon* gene are present on the array. One might argue that alpha-cells might not be lost at this stage. However, *ghrelin* which is also expressed by alpha-cells at E15.5 is down-regulated in the microarray, suggesting that alpha-cells are affected. To secure the hypothesis that the number of alpha-cells is decreased, we will perform immunofluorescence staining in the embryo. This discrepancy between RT-qPCR and microarray may suggest that other genes might be differentially expressed and missed in the microarray. Thus it would be interesting to look by RT-qPCR at the expression of genes with a fold-change of 2 and a p-value between 0.1 and 0.05.

*Somatostatin* is not decreased in the  $\Delta$ Endo KO at E15.5 whereas it is almost lost at P0 (Figure 1 in the manuscript). This could be explained by the reduced number of somatostatin-expressing cells at E15.5 which may preclude detection of any differences by RT-qPCR or microarray.



### 2.4.3. Genes differentially expressed in the intestine

Among the genes with a differential expression in the small intestine of the full KO and the  $\Delta$ Endo we short-listed ten genes which have not been associated with a precise function in the intestine so far (Table 14). I will briefly discuss *Bglap2* (FC-2) which was in the top10 down-regulated and *Fgf15* (FC 6 in  $\Delta$ Endo and 39 in full KO), *Apoa2* (FC 4 in  $\Delta$ Endo and 3 in full KO) and *Fabp6* (FC 4 in  $\Delta$ Endo and 18 in full KO) which were in the top15 up-regulated.

Gene Name	Change	Functions (GO)
<i>Apoa2</i>	UP	Immune response, fatty acid metabolism, response to glucose stimulus
<i>Aqp3</i>	UP	Immune system, water transport, response to retinoic acid
<i>Atp6v0a4</i>	UP	Ion transport, ATP hydrolysis coupled proton transport
<i>Fabp6</i>	UP	Bile acid metabolic process, lipid binding
<i>Fgf15</i>	UP	Cell migration, heart development, cell proliferation, glucose import
<i>Bglap2</i>	DOWN	Regulation of insulin secretion, calcium ion binding, lipid metabolism
<i>Cml5</i>	DOWN	Cell adhesion
<i>Dpep1</i>	DOWN	Proteolysis
<i>Trpa1</i>	DOWN	Calcium channel activity, sensory perception of pain

**Table 14: Short-list of genes differentially expressed in the small intestine in both the  $\Delta$ Endo and the full KO at E18.5.** GO: Gene Ontology.



**a) Genes up-regulated in the intestine of the full and  $\Delta$ Endo KO**

In the intestine, among the genes up-regulated in both knockouts only 21% were endocrine related, either hormones or genes necessary for proper endocrine function (e.g. *Clstn3*, *Fga*, *Fgf15*, *Pck1*, *Pdk4*), 36% were implicated in water absorption (e.g. *Aqp3*) or lipid metabolism (e.g. *Apoa2*, *Apom*, *Fabp6*). The other genes were either not annotated or not studied in the intestine but implicated in ion transport (e.g. *Atp6v0a4*) for example (based on Gene Ontology GO).

*Fgf* signalling plays important roles in organs formation (including in the intestine) during development. In addition, in the adult intestine, ***Fgf15*** (also known as *Fgf19*) is synthesised and released when bile acids are taken up into the ileum (Kir et al., 2011). Moreover, mice lacking *Fgf15* fail to maintain normal blood glucose concentration and liver glycogen. Interestingly, *Fgf15* treatment restored the loss of glycogen in diabetic animals lacking insulin, suggesting that *Fgf15* activates insulin-independent pathways which could regulate hepatic glycogen metabolism (Kir et al., 2011). During development *Fgf15* has been shown to promote neurogenesis by suppressing proliferation (Borello et al., 2008). Thus in the intestine, *Rfx6* might have a role in regulating *Fgf15* levels to allow proper enteroendocrine cell differentiation. To validate this hypothesis, we need to study the proliferation of enteroendocrine progenitors and differentiated cells in *Rfx6* mutant mice.

***Apolipoprotein A-II*** (*Apoa2*) is a high density lipoprotein which has been linked to type 2 diabetes in humans (Warden et al., 1993). *Apoa2* null mice exhibit a reduction of total cholesterol, free fatty acids, insulin and glucose levels (Castellani et al., 2001) showing that *Apoa2* promotes insulin resistance and has diverse effects on fat homeostasis. Thus *Apoa2* could be a player in the lipid absorption phenotype which we have observed in inducible  $\Delta$ intestine mutants.

Similarly, ***Fatty acid binding protein 6, ileal*** (*Fabp6*) could also have a role in this phenotype as FABPs have roles in fatty acid uptake, transport and metabolism (Barley et al., 2003). Interestingly, *Fabp6* expression is normally restricted to the distal part of the intestine and regulated, at least in part, by binding of Cdx2 which is also expressed at high levels in the distal ileum and proximal colon (Barley et al., 2003). *Rfx6* could be repressing *Fabp6* expression in the proximal small intestine.

**b) Genes down-regulated in the intestine of the full and  $\Delta$ Endo KO**

About 64% of the down-regulated genes have been shown to have a role in endocrine function (e.g. *Arx*, *Bglap2*, *Cck*, *Nts*), 18% are putative genes which have not been annotated, the others 18% have never been studied in the intestine but have roles in ion transport (e.g. *Dpep1*, *Trpa1*).

***Bone gamma-carboxyglutamate protein 2*** (*Bglap2*) is also known as *osteocalcin* and is normally secreted by osteoblasts. It has been recently suggested that osteoblasts participate in the regulation of glucose metabolism through the secretion of osteocalcin. Indeed, FoxO1 deficiency in osteoblasts increases the expression of osteocalcin and mice lacking FoxO1 only in osteoblasts had increased beta-cell proliferation and insulin secretion and sensitivity (Rached et al., 2010). FoxO1 is also expressed in the intestine and it was shown that *FoxO1* deficiency leads to the generation of insulin-secreting cells in the intestine (Talchai et al., 2012). Mice deficient for *Bglap1* and *Bglap2* exhibit decrease insulin in the blood and impaired glucose tolerance (Lee et al., 2007). It would be of particular interest to determine if enteroendocrine cells are the source of osteocalcin in the intestine.



#### 2.4.4. Genes differentially expressed in the full KO

Many genes are affected only in the full KO (652 in the intestine and 389 in the pancreas) and not in  $\Delta$ Endo embryos. Among them I short-listed those affected similarly in the intestine and the pancreas (Table 15) and those inversely affected in both organs (Table 16). I will not discuss genes affected only in the pancreas, or genes down-regulated in the intestine here as the fold changes were not very important. However I will describe some interesting genes which were highly up-regulated in the intestine of the *Rfx6*-deficient mice at E18.5.

Gene Name	Change	Functions (GO)
Cited4	UP	Development and differentiation of blood, endothelial and epithelial cells
Cym	UP	Endopeptidase
Paqr5	UP	Steroid membrane receptor
Slc26a9	UP	Bicarbonate transmembrane transporter
Tesc	UP	Regulation of cell adhesion
Ryr1	DOWN	Calcium channel
Serpina3g	DOWN	Serine protease inhibitor 2A inhibits caspase-independent cell death
Slc2a5	DOWN	Glucose transporter
Tmem27	DOWN	Proteolysis

**Table 15 : Short-list of genes differentially expressed in the small intestine (E18.5) and pancreas (E15.5) of the full KO.**  
GO: Gene Ontology.

##### a) Genes up-regulated only in the full KO in the pancreas and the intestine

Among the genes up-regulated in the full KO in both the intestine and the pancreas I will describe shortly *Cym* as it was the common gene with the highest enrichment.

*Cym* was enriched 8-fold in the pancreas and 103-fold in the intestine of *Rfx6*-deficient embryos. It codes for chymosin an aspartic protease that is used to promote milk clotting in cheese manufacturing (Palmer et al., 2010). To our knowledge nothing has been described for the expression or function of chymosin in mouse however, the strong enrichment observed in the intestine of *Rfx6* mutants embryos (and to a lesser extent in the pancreas) suggests a potential function of this gene which should be further studied.

##### b) Genes down-regulated only in the full KO in the pancreas and the intestine

Between the genes down-regulated in the full KO in both the intestine and the pancreas I will describe shortly *Tmem27* as it was the gene with the strongest down-regulation.

*Tmem27*, also called *collectrin*, was down-regulated 5-fold in the pancreas and 3-fold in the small intestine. *Tmem27* is a transmembrane protein which has been shown to be expressed in pancreatic islets (Hald et al., 2012). *Collectrin* is a known downstream target of *Hnf1a* and mice deficient for *collectrin* exhibit increased insulin sensitivity (Malakauskas et al., 2009). It is not known which cells express *collectrin* in the intestine or whether it has a role. However, *Hnf1a* has been shown to control terminal differentiation and cell fate commitment in the gut epithelium together with *Hnf1b* (D'Angelo et al., 2010). It would be interesting to know if *Tmem27* is a downstream effector of *Hnf1a* in this process.

**c) Genes up-regulated only in the full intestinal KO**

Very interestingly, the genes which were affected the most in our different microarrays experiments were found in the intestine of the full KO embryos: *Gkn2*, enriched 1033-fold, *Tff1* enriched 634-fold and *Tff2* enriched 22-fold.

In the stomach **Gkn2** (also called blottin) has been shown to bind to **Tff2** and to a lesser extend to **Tff1** (Otto et al., 2006). Trefoil factor family (Tff) proteins are the major secretory products of mucous epithelia and have been shown to play a role in cytoprotection, apoptosis and immune response in the gastrointestinal tract (Taupin et al., 2000). To our knowledge, expression of *Gkn2* has not been reported in the intestine before. This very striking increase in *Gkn2* and *Tff1* suggests that *Rfx6* could have a role in repressing these genes in the intestine. It will be important to study whether this increase is also true at the protein level and to characterise the expression of these genes in the intestinal epithelium. To do so we are setting up a series of immunofluorescence experiments.

**d) Genes affected inversely in the pancreas and the intestine of the full KO**

In order to find some genes which might be important for the pancreatic versus intestinal fate in the early endoderm, I looked for genes that were only differentially expressed in the full knockout and up-regulated in the pancreas and down-regulated in the intestine and vice-versa (Table 16). The comparison may not be optimal as the two organs were analysed at different stages and these stages might be late to detect differences in the endoderm.

Gene Name	FC SI	FC Panc	Functions (GO)
Anxa1	3.7	-1.7	Inflammation, Calcium binding, Cell Cycle, Insulin secretion
Bace2	2.7	-1.5	Beta-cell function and mass, APP processing
Bdh2	-1.7	1.7	Fatty acid oxidation, Proteolysis
Gpx3	1.6	-1.5	Peroxidase
Il18r1	1.6	-1.7	Innate Immune response
Mal	83.6	-1.6	Apoptosis, Myelinisation, Vesicular trafficking
Myh14	-1.5	1.5	Cytoskeleton, EMT, Secretion
Nkx6-3	-4.0	3.0	Transcription
Peg10	1.5	-1.6	Adipocyte differentiation, Apoptosis

**Table 16: List of genes inversely regulated in the pancreas and in the intestine of *Rfx6*-deficient mice.** SI: Small intestine; Panc: Pancreas; GO: Gene Ontology; FC: Fold Change.

The only transcription factor which was affected differentially in the pancreas and the intestine of *Rfx6*-deficient mice was *Nkx6.3*. Its expression was found to be enriched 3-fold in the pancreas and decreased 4-fold in the intestine of *Rfx6* mutant embryos, thus being an interesting candidate for the specification of pancreatic versus intestinal fates.

**Nkx6.3** belongs to the Nkx family which is essential for normal development of many different tissues including the pancreas. Three members of the Nkx6 family have been shown to be expressed in the mouse endoderm of which *Nkx6.1* and *Nkx6.2* are also expressed in the pancreatic epithelium (Pedersen et al., 2005). *Nkx6.3* is not found in the pancreas but is expressed in the stomach and proximal duodenum (Pedersen et al., 2005). *Rfx6* could be implicated in the inhibition of *Nkx6.3* in the pancreatic epithelium to promote a pancreatic versus duodenal fate. *Nkx6.3*<sup>-/-</sup> mice develop and grow normally but show a markedly reduced number of gastrin-producing cells and an increase in somatostatin-producing cells in the stomach (Choi et al., 2008), interestingly, these two cell types require *Ngn3* expression to differentiate (Jenny et al., 2002) suggesting a role for *Nkx6.3* downstream of *Ngn3* in the endocrine lineage of the stomach. The role of *Nkx6.3* in the duodenum is not yet known but it might participate in patterning or differentiation. Of note, over-expression of *Nkx6.3* in the pancreas might give insights in the early transcriptional differences between pancreas and intestine specification.

#### 2.4.5. Genes down-regulated in all four KO

Only seven genes were found affected in all four experiments out of them four (***Arx***, ***Gcg***, ***Ghrl*** and ***Pyy***) were endocrine genes.

One is a receptor of the GDNF family (***Gfra3***) which we also identified in the profiling of the endocrine progenitors and Laure Nivlet in the lab is currently investigating its function during endocrine cell development.

The last two genes (*Fam183b* and *Scg2*) have no known function in the pancreas or the intestine. ***Fam183b*** has recently been identified as a target of *FoxA2* in the notochord even though its function is not known (Tamplin et al., 2011).

***Scg2*** is a member of the MAPKKK cascade which functions in endothelial cell proliferation and belongs to the chromogranin/secretogranin family (by GO), moreover this transcript has been found enriched in the pancreas compared to other tissues in a study published in 2008 (Hoffman et al., 2008).

### 3. Perspectives/Conclusion

After the first study on the *Rfx6* gene published in 2010 in the lab, I continued the characterisation of the role of this transcription factor. The expression pattern of this gene suggested different functions that I tried to understand with a conditional KO. I showed that it is the expression of *Rfx6* downstream of *Ngn3* that is responsible for the loss of hormone-secreting cells observed in *Rfx6*-deficient mice. I also showed that *Rfx6* is necessary for the maintenance of beta-cells. I characterised the expression pattern of *Rfx6* in the wild-type adult intestine and showed that mice with an intestinal deletion of *Rfx6* do not produce enteroendocrine hormones except serotonin. The deletion of *Rfx6* in the adult intestine also leads to the loss of enteroendocrine cells, accompanied by diarrhoea and lipid absorption problems.

### 3.1. Role of Rfx6 in energy homeostasis and glucose metabolism

**Metabolic studies** will be required to characterise the role of Rfx6 in adult beta-cells and intestine. Similarly to what was done to study the function of *Ngn3* in the intestine, we will perform in collaboration with the ICS (Illkirch) different metabolic tests, including glucose tolerance (oral and IP) and insulin sensitivity tests; measures of cholesterol, triglycerids, free fatty acids and hormones blood levels, food transit and energy expenditure; either on normal diet or on high fat diet. Moreover, a careful investigation of the intestinal homeostasis in the adult (proliferation in the crypts, cell turnover) will also be needed.

### 3.2. Function of Rfx6 in endocrine cell differentiation

Interestingly, from our and others data, the differentiation of PP-cells does not seem to depend on *Rfx6*. A possible explanation could be that *Rfx6* is necessary to **repress PP** in the other endocrine cells. The IGBMC will soon acquire a machine which will allow performing RT-qPCR on single cells, using this tool we will be able to determine if the expression of *Rfx6* is heterogenous among the different endocrine cell types thus gaining more insight into how Rfx6 could direct the endocrine progenitors toward one fate at the expense of others.

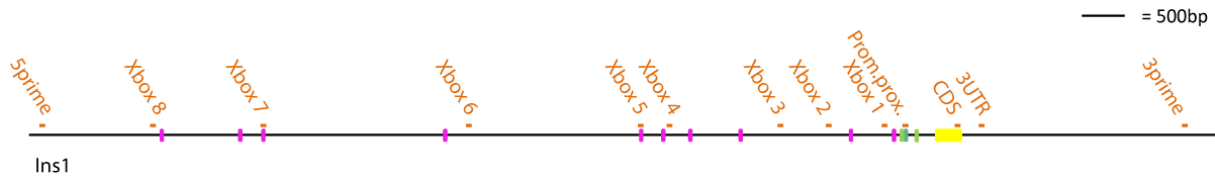
Moreover, it would be interesting to set up gain-of-function (GOF) experiments to study if Rfx6 is sufficient to induce endocrine cell differentiation. Either *in vivo* by crossing Rfx6GOF mice (Figure 45) with a *Ngn3*-Cre line in a *Ngn3* KO background or *ex vivo* for example by infecting pancreatic explants from *Ngn3*-deficient mice with Rfx6-adenovirus.



**Figure 45: Construction to generate a transgenic mouse for *in vivo* gain-of-function study of Rfx6.** CAGS: cytomegalovirus enhancer/human  $\beta$ -actin; IRES: Internal ribosome entry site;  $\beta$ -gal:  $\beta$ -galactosidase.

### 3.3. Looking for Rfx6 direct targets

To understand how Rfx6 works in promoting endocrine cell differentiation, it is now crucial to identify direct target genes of Rfx6. Using bioinformatics tools, we have looked for the presence of X-boxes in the regulatory sequences of genes enriched in *Ngn3*<sup>+</sup> endocrine progenitors. We have identified X-boxes in many hormone genes, including *Ins1* (Figure 46). This information has been used to generate primers for ChIP experiments on Min6 cells and in the mouse embryo. As no Rfx6 targets are known so far, we also want to perform **ChIP-seq** experiments in these two models. This project is the work of Perrine Strasser in the lab. We are currently generating transgenic mice with a 3-HA tagged version of Rfx6 fused to the mCherry fluorescent protein for ChIP experiments. This tool will allow us to use an anti-HA antibody to compensate for the lack of a ChIP-efficient anti-rfx6 antibody. Moreover we will also be able to sort Rfx6-expressing cells using the mCherry fluorescence. These mice will thus allow us to perform ChIP-seq on different tissues and find tissue-specific direct Rfx6 targets *in vivo*.



**Figure 46 : Localisation of X-boxes and ChIP primers in the *Ins1* promoter region.** Orange: position of primer pairs, Pink: X-boxes, Green: NeuroD binding sites, Blue: MafA binding site, Yellow: coding DNA sequence (CDS), Prom.prox.: promoter proximal, UTR: UnTranslated Region. (Image Perrine Strasser)

### 3.4. Further analysis of the microarray data

Microarrays generated a lot of data which need further analysis. For example, we have not found enrichment of intestinal genes in the pancreas of *Rfx6*-deficient mice. However this was based on Gene Ontology, more careful literature mining will be required to secure this statement.

Moreover, crossing our data on genes affected in *Rfx6*-deficient mice with previous experiments on *Ngn3*-deficient mice could provide interesting insight into *Ngn3*-independent *Rfx6* functions.

### 3.5. Role of *Rfx6* in the formation of cilia on endocrine cells

RFX genes have been shown to be master regulators of **ciliogenesis** (see I.E.2.2) however immunofluorescence experiments for acetylated beta-tubulin (primary cilia marker) and RT-qPCR for *Dync2lic* or *Ift88* (two genes expressed in primary cilia) performed by us and others (Smith et al., 2010) suggest that *Rfx6* is not implicated in the formation or maintenance of cilia on endocrine cells. Nevertheless, the defect might be mild and a more careful analysis could prove necessary. Purification of adult islets followed by immunofluorescence for a cilia marker (for example acetylated beta-tubulin) and 3D reconstruction will permit to measure the size and orientation of cilia on endocrine cells.

### 3.6. Function of *Rfx6* in other tissues

*Rfx6*, like *Ngn3*, is also expressed in the **testis** (J. Soyer, unpublished results) but its function is not known. In the testis, *Ngn3* has been shown to be expressed in undifferentiated spermatogonia and give rise to all the spermatogenesis in mature testis (Yoshida et al., 2004). Using the conditional *Rfx6* knockout, it would be possible to study if *Rfx6* is downstream of *Ngn3* in the testis and what is his role.

*Ngn3* has been shown to regulate **glucose homeostasis** in different tissues, including the **pancreas, intestine**, stomach and hypothalamus. It would be of interest to know whether this regulation relies on *Rfx6* in these tissues. On-going metabolic studies (see II.B.3.1.1) on mice with deletion of *Rfx6* in beta-cells or in the adult intestine will improve our knowledge of the role of *Rfx6* in energy homeostasis in the pancreas and the intestine.

In the **stomach**, *Ngn3* is not required for the differentiation of all endocrine subtypes (Jenny et al., 2002; Lee et al., 2002). Indeed, in the stomach of *Ngn3*-deficient mice, glucagon-, gastrin- and somatostatin-secreting cells are lost whereas serotonin-, histamine- and ghrelin-expressing cells are still present. It will be important to know if *Rfx6* is required for gastric endocrine cell differentiation.

In the **hypothalamus**, *Ngn3* is differentially required for the differentiation of POMC and NYP neurons which are antagonist regulators of feeding behaviour (Pelling et al., 2011). We could look whether *Rfx6* is expressed in the hypothalamus and if it participates in the regulation of feeding behaviour.

Finally, we hope that our studies on *Rfx6* will contribute to the development of a cell-based therapy in diabetes, as well as to understand the mechanisms underlying the pathophysiology of islet and enteroendocrine hormone failure in human.



### III. Conclusion

The aim of my PhD was to characterise the expression and/or function of two different genes downstream of Ngn3, the *p21 protein-activated kinase 3*, *Pak3* and the *Regulatory Factor X 6*, *Rfx6* in pancreatic and intestinal endocrine cell differentiation and function both during development and in the adult. We hoped that understanding the function of these genes would give us more insights into the programs downstream of Ngn3 which eventually give rise to mature, functional endocrine cells both in the pancreas and in the intestine and could have important biomedical outcome for example in cell therapy.

Enhancing survival or preventing apoptosis of beta-cells is promising not only to improve islet transplantation outcomes as a treatment for type 1 diabetes but also to protect beta-cells against effect of hyperglycaemia in type 2 diabetes. In this context, my work identified *Pak3* as a new gene implicated in pancreatic endocrine cell proliferation and differentiation. Deciphering the downstream effectors of Pak3 function in the maintenance of quiescence in beta-cells might lead to new potential therapeutic drugs to improve the proliferative capacity of beta-cells in type 2 diabetes patients. However the question of how Pak3 works in this process is still not elucidated. A promising putative downstream effector of Pak3 function is the transcription factor cAMP-responsive element binding protein (CREB). Indeed, in the brain Pak3 has been shown to impact on the phosphorylation of CREB on Ser133 (Meng et al., 2005). In the pancreas, CREB is emerging as a key transcriptional element for the maintenance of glucose sensing, insulin exocytosis, *insulin* gene transcription and beta-cell survival (recently reviewed in Dalle et al., 2011). Among the different signalling pathways that phosphorylate and activate CREB in beta-cells, PKA is a known activator of PAK and ERK1/2 is a downstream target of Pak3 (Bokoch, 2003). Thus, Ngn3-dependent Pak3 activation could lead to the phosphorylation of PKA and/or ERK1/2 which will subsequently phosphorylate CREB on Ser133 and regulate for example proliferation or beta-cell survival. As such Pak3 could be an interesting novel drug target for strategies aimed at improving the survival and proliferation of beta-cells in diabetic patients.

Moreover, our strategy to study genes enriched in Ngn3<sup>+</sup> progenitors proves fruitful as *Pak3* is the third gene we identified as new regulator of endocrine cell differentiation after *Insm1* (Mellitzer et al., 2006) and *Rfx6* (Soyer et al., 2010). Interestingly, Pak3 was shown to regulate both the proliferation of endocrine progenitors and beta-cells, applying this new finding in the right time-frame to current protocols aimed at generating beta-cells *in vitro* might improve the yield of Ngn3<sup>+</sup> endocrine progenitors produced. Similarly, the appropriate use of Pak inhibitors might improve the proliferative capacity of the beta-cells obtained from these protocols.

The study of Rfx6 function in intestinal enteroendocrine cells revealed that this gene is necessary for the differentiation of hormone-expressing cells. We are now aware that part of these cells is necessary for lipid absorption; as such Rfx6 might be able to rescue defects observed in *Ngn3*-deficient mice or patients with mutation in NGN3. Similarly, cocktails of enteroendocrine cells agonists might help to cure diarrhoea in these patients.



Interestingly, during development Rfx6 seems to have a dual function downstream of Ngn3 both in generic and subtype-specific programs. Indeed, *Rfx6*-deficient mice as well as human with RFX6 mutations fail to generate any hormone-expressing cells except PP-cells in the pancreas and serotonin-cells in the intestine, suggesting that Rfx6 acts in the generic transcription program. However, Rfx6 also seems to control subtype specification programs, favouring the alpha-cell destiny by inhibiting *Pax4* the beta-cell fate gene. As such, down-regulation of Rfx6 at the right time might improve the yield of beta-cells generated in differentiation protocols.

To conclude, I believe that my work, helped to consolidate the knowledge on the differentiation and maintenance of endocrine cells with the hope, one day, to translate this expertise to be able to develop *in vitro* mature beta-cells capable of secreting insulin in response to glucose stimuli or new therapies to control energy metabolism.

## IV. References

- Afelik, S., Qu, X., Hasrouni, E., Bukys, M. A., Deering, T., Nieuwoudt, S., Rogers, W., Macdonald, R. J. and Jensen, J. (2012). Notch-mediated patterning and cell fate allocation of pancreatic progenitor cells. *Development* **139**, 1744-53.
- Aftab, S., Semenec, L., Chu, J. S. and Chen, N. (2008). Identification and characterization of novel human tissue-specific RFX transcription factors. *BMC Evol Biol* **8**, 226.
- Ahlgren, U., Jonsson, J. and Edlund, H. (1996). The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. *Development* **122**, 1409-16.
- Ahnfelt-Ronne, J., Hald, J., Bodker, A., Yassin, H., Serup, P. and Hecksher-Sorensen, J. (2007). Preservation of proliferating pancreatic progenitor cells by Delta-Notch signaling in the embryonic chicken pancreas. *BMC Dev Biol* **7**, 63.
- Ahnfelt-Ronne, J., Ravassard, P., Pardanaud-Glavieux, C., Scharfmann, R. and Serup, P. (2010). Mesenchymal bone morphogenetic protein signaling is required for normal pancreas development. *Diabetes* **59**, 1948-56.
- Ait-Lounis, A., Baas, D., Barras, E., Benadiba, C., Charollais, A., Nlend Nlend, R., Liegeois, D., Meda, P., Durand, B. and Reith, W. (2007). Novel function of the ciliogenic transcription factor RFX3 in development of the endocrine pancreas. *Diabetes* **56**, 950-9.
- Ait-Lounis, A., Bonal, C., Seguin-Estevez, Q., Schmid, C. D., Bucher, P., Herrera, P. L., Durand, B., Meda, P. and Reith, W. (2010). The transcription factor Rfx3 regulates beta-cell differentiation, function, and glucokinase expression. *Diabetes* **59**, 1674-85.
- Aizawa, H., Wakatsuki, S., Ishii, A., Moriyama, K., Sasaki, Y., Ohashi, K., Sekine-Aizawa, Y., Sehara-Fujisawa, A., Mizuno, K., Goshima, Y. et al. (2001). Phosphorylation of cofilin by LIM-kinase is necessary for semaphorin 3A-induced growth cone collapse. *Nat Neurosci* **4**, 367-73.
- Akiyama, H., Kim, J. E., Nakashima, K., Balmes, G., Iwai, N., Deng, J. M., Zhang, Z., Martin, J. F., Behringer, R. R., Nakamura, T. et al. (2005). Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc Natl Acad Sci U S A* **102**, 14665-70.
- Amsen, D., Antov, A. and Flavell, R. A. (2009). The different faces of Notch in T-helper-cell differentiation. *Nat Rev Immunol* **9**, 116-24.
- Andreu, P., Colnot, S., Godard, C., Gad, S., Chafey, P., Niwa-Kawakita, M., Laurent-Puig, P., Kahn, A., Robine, S., Perret, C. et al. (2005). Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. *Development* **132**, 1443-51.
- Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., Hrabe de Angelis, M., Lendahl, U. and Edlund, H. (1999). Notch signalling controls pancreatic cell differentiation. *Nature* **400**, 877-81.
- Araki, R., Takahashi, H., Fukumura, R., Sun, F., Umeda, N., Sujino, M., Inouye, S. T., Saito, T. and Abe, M. (2004). Restricted expression and photic induction of a novel mouse regulatory factor X4 transcript in the suprachiasmatic nucleus. *J Biol Chem* **279**, 10237-42.
- Aramata, S., Han, S. I. and Kataoka, K. (2007). Roles and regulation of transcription factor MafA in islet beta-cells. *Endocr J* **54**, 659-66.
- Artner, I., Bianchi, B., Raum, J. C., Guo, M., Kaneko, T., Cordes, S., Sieweke, M. and Stein, R. (2007). MafB is required for islet beta cell maturation. *Proc Natl Acad Sci U S A* **104**, 3853-8.

- Artner, I., Hang, Y., Mazur, M., Yamamoto, T., Guo, M., Lindner, J., Magnuson, M. A. and Stein, R.** (2010). MafA and MafB regulate genes critical to beta-cells in a unique temporal manner. *Diabetes* **59**, 2530-9.
- Artner, I., Le Lay, J., Hang, Y., Elghazi, L., Schisler, J. C., Henderson, E., Sosa-Pineda, B. and Stein, R.** (2006). MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells. *Diabetes* **55**, 297-304.
- Asano, Y., Jimenez-Dalmaroni, A., Liverpool, T. B., Marchetti, M. C., Giomi, L., Kiger, A., Duke, T. and Baum, B.** (2009). Pak3 inhibits local actin filament formation to regulate global cell polarity. *HFSP J* **3**, 194-203.
- Asrar, S., Meng, Y., Zhou, Z., Todorovski, Z., Huang, W. W. and Jia, Z.** (2009). Regulation of hippocampal long-term potentiation by p21-activated protein kinase 1 (PAK1). *Neuropharmacology* **56**, 73-80.
- Baas, D., Meiniel, A., Benadiba, C., Bonnafe, E., Meiniel, O., Reith, W. and Durand, B.** (2006). A deficiency in RFX3 causes hydrocephalus associated with abnormal differentiation of ependymal cells. *Eur J Neurosci* **24**, 1020-30.
- Baetge, E. E.** (2008). Production of beta-cells from human embryonic stem cells. *Diabetes Obes Metab* **10 Suppl 4**, 186-94.
- Baggio, L. L. and Drucker, D. J.** (2006). Therapeutic approaches to preserve islet mass in type 2 diabetes. *Annu Rev Med* **57**, 265-81.
- Baggio, L. L. and Drucker, D. J.** (2007). Biology of incretins: GLP-1 and GIP. *Gastroenterology* **132**, 2131-57.
- Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J. and Cerione, R. A.** (1995). Identification of a mouse p21Cdc42/Rac activated kinase. *J Biol Chem* **270**, 22731-7.
- Bagrodia, S., Taylor, S. J., Jordon, K. A., Van Aelst, L. and Cerione, R. A.** (1998). A novel regulator of p21-activated kinases. *J Biol Chem* **273**, 23633-6.
- Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P. J. et al.** (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**, 1003-7.
- Barley, N. F., Taylor, V., Shaw-Smith, C. J., Chakravarty, P., Howard, A., Legon, S. and Walters, J. R.** (2003). Human ileal bile acid-binding protein promoter and the effects of CDX2. *Biochim Biophys Acta* **1630**, 138-43.
- Bastide, P., Darido, C., Pannequin, J., Kist, R., Robine, S., Marty-Double, C., Bibeau, F., Scherer, G., Joubert, D., Hollande, F. et al.** (2007). Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. *J Cell Biol* **178**, 635-48.
- Bayha, E., Jorgensen, M. C., Serup, P. and Grapin-Botton, A.** (2009). Retinoic acid signaling organizes endodermal organ specification along the entire antero-posterior axis. *PLoS One* **4**, e5845.
- Beckers, A., Alten, L., Viebahn, C., Andre, P. and Gossler, A.** (2007). The mouse homeobox gene Noto regulates node morphogenesis, notochordal ciliogenesis, and left right patterning. *Proc Natl Acad Sci U S A* **104**, 15765-70.
- Beres, T. M., Masui, T., Swift, G. H., Shi, L., Henke, R. M. and MacDonald, R. J.** (2006). PTF1 is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or its paralogue, RBP-L. *Mol Cell Biol* **26**, 117-30.
- Bergman, R. N. and Ader, M.** (2000). Free fatty acids and pathogenesis of type 2 diabetes mellitus. *Trends Endocrinol Metab* **11**, 351-6.

- Bernardo, A. S., Hay, C. W. and Docherty, K.** (2008). Pancreatic transcription factors and their role in the birth, life and survival of the pancreatic beta cell. *Mol Cell Endocrinol* **294**, 1-9.
- Beucher, A., Gjernes, E., Collin, C., Courtney, M., Meunier, A., Collombat, P. and Gradwohl, G.** (2012a). The homeodomain-containing transcription factors Arx and Pax4 control enteroendocrine subtype specification in mice. *PLoS One* **7**, e36449.
- Beucher, A., Martin, M., Spenle, C., Poulet, M., Collin, C. and Gradwohl, G.** (2012b). Competence of failed endocrine progenitors to give rise to acinar but not ductal cells is restricted to early pancreas development. *Dev Biol* **361**, 277-85.
- Bhushan, A., Itoh, N., Kato, S., Thiery, J. P., Czernichow, P., Bellusci, S. and Scharfmann, R.** (2001). Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. *Development* **128**, 5109-17.
- Bjerknes, M. and Cheng, H.** (1999). Clonal analysis of mouse intestinal epithelial progenitors. *Gastroenterology* **116**, 7-14.
- Blache, P., van de Wetering, M., Duluc, I., Domon, C., Berta, P., Freund, J. N., Clevers, H. and Jay, P.** (2004). SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. *J Cell Biol* **166**, 37-47.
- Blackshear, P. J., Graves, J. P., Stumpo, D. J., Cobos, I., Rubenstein, J. L. and Zeldin, D. C.** (2003). Graded phenotypic response to partial and complete deficiency of a brain-specific transcript variant of the winged helix transcription factor RFX4. *Development* **130**, 4539-52.
- Blum, B., Hrvatin, S. S., Schuetz, C., Bonal, C., Rezanja, A. and Melton, D. A.** (2012). Functional beta-cell maturation is marked by an increased glucose threshold and by expression of urocortin 3. *Nat Biotechnol* **30**, 261-4.
- Boda, B., Alberi, S., Nikonenko, I., Node-Langlois, R., Jourdain, P., Moosmayer, M., Parisi-Jourdain, L. and Muller, D.** (2004). The mental retardation protein PAK3 contributes to synapse formation and plasticity in hippocampus. *J Neurosci* **24**, 10816-25.
- Bokoch, G. M.** (2003). Biology of the p21-activated kinases. *Annu Rev Biochem* **72**, 743-81.
- Bokoch, G. M., Reilly, A. M., Daniels, R. H., King, C. C., Olivera, A., Spiegel, S. and Knaus, U. G.** (1998). A GTPase-independent mechanism of p21-activated kinase activation. Regulation by sphingosine and other biologically active lipids. *J Biol Chem* **273**, 8137-44.
- Bonnafé, E., Touka, M., AitLounis, A., Baas, D., Barras, E., Ucla, C., Moreau, A., Flamant, F., Dubruille, R., Couble, P. et al.** (2004). The transcription factor RFX3 directs nodal cilium development and left-right asymmetry specification. *Mol Cell Biol* **24**, 4417-27.
- Bonner-Weir, S., Li, W. C., Ouziel-Yahalom, L., Guo, L., Weir, G. C. and Sharma, A.** (2010). Beta-cell growth and regeneration: replication is only part of the story. *Diabetes* **59**, 2340-8.
- Borello, U., Cobos, I., Long, J. E., McWhirter, J. R., Murre, C. and Rubenstein, J. L.** (2008). FGF15 promotes neurogenesis and opposes FGF8 function during neocortical development. *Neural Dev* **3**, 17.
- Boushey, R. P., Abadir, A., Flamez, D., Baggio, L. L., Li, Y., Berger, V., Marshall, B. A., Finegood, D., Wang, T. C., Schuit, F. et al.** (2003). Hypoglycemia, defective islet glucagon secretion, but normal islet mass in mice with a disruption of the gastrin gene. *Gastroenterology* **125**, 1164-74.
- Boyer, D. F., Fujitani, Y., Gannon, M., Powers, A. C., Stein, R. W. and Wright, C. V.** (2006). Complementation rescue of Pdx1 null phenotype demonstrates distinct roles of proximal and distal cis-regulatory sequences in pancreatic and duodenal expression. *Dev Biol* **298**, 616-31.

- Bramswig, N. C. and Kaestner, K. H.** (2011). Transcriptional regulation of alpha-cell differentiation. *Diabetes Obes Metab* **13 Suppl 1**, 13-20.
- Breslin, M. B., Wang, H. W., Pierce, A., Aucoin, R. and Lan, M. S.** (2007). Neurogenin 3 recruits CBP co-activator to facilitate histone H3/H4 acetylation in the target gene INSM1. *FEBS Lett* **581**, 949-54.
- Burbelo, P. D., Drechsel, D. and Hall, A.** (1995). A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J Biol Chem* **270**, 29071-4.
- Burlison, J. S., Long, Q., Fujitani, Y., Wright, C. V. and Magnuson, M. A.** (2008). Pdx-1 and Ptf1a concurrently determine fate specification of pancreatic multipotent progenitor cells. *Dev Biol* **316**, 74-86.
- Cano, D. A., Murcia, N. S., Pazour, G. J. and Hebrok, M.** (2004). Orpk mouse model of polycystic kidney disease reveals essential role of primary cilia in pancreatic tissue organization. *Development* **131**, 3457-67.
- Cano, D. A., Sekine, S. and Hebrok, M.** (2006). Primary cilia deletion in pancreatic epithelial cells results in cyst formation and pancreatitis. *Gastroenterology* **131**, 1856-69.
- Carter, J. H., Douglass, L. E., Deddens, J. A., Colligan, B. M., Bhatt, T. R., Pemberton, J. O., Konicek, S., Hom, J., Marshall, M. and Graff, J. R.** (2004). Pak-1 expression increases with progression of colorectal carcinomas to metastasis. *Clin Cancer Res* **10**, 3448-56.
- Castellani, L. W., Goto, A. M. and Lusis, A. J.** (2001). Studies with apolipoprotein A-II transgenic mice indicate a role for HDLs in adiposity and insulin resistance. *Diabetes* **50**, 643-51.
- Cau, E., Gradwohl, G., Fode, C. and Guillemot, F.** (1997). Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development* **124**, 1611-21.
- Cerf, M. E.** (2006). Transcription factors regulating beta-cell function. *Eur J Endocrinol* **155**, 671-9.
- Cervantes, S., Yamaguchi, T. P. and Hebrok, M.** (2009). Wnt5a is essential for intestinal elongation in mice. *Dev Biol* **326**, 285-94.
- Chandra, V., G, S., Phadnis, S., Nair, P. D. and Bhonde, R. R.** (2009). Generation of pancreatic hormone-expressing islet-like cell aggregates from murine adipose tissue-derived stem cells. *Stem Cells* **27**, 1941-53.
- Chen, H., Gu, X., Su, I. H., Bottino, R., Contreras, J. L., Tarakhovsky, A. and Kim, S. K.** (2009). Polycomb protein Ezh2 regulates pancreatic beta-cell Ink4a/Arf expression and regeneration in diabetes mellitus. *Genes Dev* **23**, 975-85.
- Cheng, H. and Leblond, C. P.** (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cell. *Am J Anat* **141**, 461-79.
- Chey, W. Y. and Escoffery, R.** (1976). Secretion cells in the gastrointestinal tract. *Endocrinology* **98**, 1390-5.
- Chiu, T. T., Patel, N., Shaw, A. E., Bamburg, J. R. and Klip, A.** (2010). Arp2/3- and cofilin-coordinated actin dynamics is required for insulin-mediated GLUT4 translocation to the surface of muscle cells. *Mol Biol Cell* **21**, 3529-39.
- Choi, M. Y., Romer, A. I., Wang, Y., Wu, M. P., Ito, S., Leiter, A. B. and Shivdasani, R. A.** (2008). Requirement of the tissue-restricted homeodomain transcription factor Nkx6.3 in differentiation of gastrin-producing G cells in the stomach antrum. *Mol Cell Biol* **28**, 3208-18.
- Chong, C., Tan, L., Lim, L. and Manser, E.** (2001). The mechanism of PAK activation. Autophosphorylation events in both regulatory and kinase domains control activity. *J Biol Chem* **276**, 17347-53.

- Chu, J. S., Baillie, D. L. and Chen, N.** (2010). Convergent evolution of RFX transcription factors and ciliary genes predated the origin of metazoans. *BMC Evol Biol* **10**, 130.
- Clausen, B. E., Waldburger, J. M., Schwenk, F., Barras, E., Mach, B., Rajewsky, K., Forster, I. and Reith, W.** (1998). Residual MHC class II expression on mature dendritic cells and activated B cells in RFX5-deficient mice. *Immunity* **8**, 143-55.
- Clevers, H.** (2006). Wnt/beta-catenin signaling in development and disease. *Cell* **127**, 469-80.
- Cobos, I., Borello, U. and Rubenstein, J. L.** (2007). Dlx transcription factors promote migration through repression of axon and dendrite growth. *Neuron* **54**, 873-88.
- Collombat, P., Hecksher-Sorensen, J., Broccoli, V., Krull, J., Ponte, I., Mundiger, T., Smith, J., Gruss, P., Serup, P. and Mansouri, A.** (2005). The simultaneous loss of Arx and Pax4 genes promotes a somatostatin-producing cell fate specification at the expense of the alpha- and beta-cell lineages in the mouse endocrine pancreas. *Development* **132**, 2969-80.
- Collombat, P., Hecksher-Sorensen, J., Krull, J., Berger, J., Riedel, D., Herrera, P. L., Serup, P. and Mansouri, A.** (2007). Embryonic endocrine pancreas and mature beta cells acquire alpha and PP cell phenotypes upon Arx misexpression. *J Clin Invest* **117**, 961-70.
- Collombat, P., Hecksher-Sorensen, J., Serup, P. and Mansouri, A.** (2006). Specifying pancreatic endocrine cell fates. *Mech Dev* **123**, 501-12.
- Collombat, P., Mansouri, A., Hecksher-Sorensen, J., Serup, P., Krull, J., Gradwohl, G. and Gruss, P.** (2003). Opposing actions of Arx and Pax4 in endocrine pancreas development. *Genes Dev* **17**, 2591-603.
- Collombat, P., Xu, X., Ravassard, P., Sosa-Pineda, B., Dussaud, S., Billestrup, N., Madsen, O. D., Serup, P., Heimberg, H. and Mansouri, A.** (2009). The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells. *Cell* **138**, 449-62.
- Cras-Meneur, C., Li, L., Kopan, R. and Permutt, M. A.** (2009). Presenilins, Notch dose control the fate of pancreatic endocrine progenitors during a narrow developmental window. *Genes Dev* **23**, 2088-101.
- Crosnier, C., Stamatakis, D. and Lewis, J.** (2006). Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nat Rev Genet* **7**, 349-59.
- D'Amour, K. A., Bang, A. G., Eliazar, S., Kelly, O. G., Agulnick, A. D., Smart, N. G., Moorman, M. A., Kroon, E., Carpenter, M. K. and Baetge, E. E.** (2006). Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol* **24**, 1392-401.
- D'Angelo, A., Bluteau, O., Garcia-Gonzalez, M. A., Gresh, L., Doyen, A., Garbay, S., Robine, S. and Pontoglio, M.** (2010). Hepatocyte nuclear factor 1alpha and beta control terminal differentiation and cell fate commitment in the gut epithelium. *Development* **137**, 1573-82.
- Dan, I., Watanabe, N. M. and Kusumi, A.** (2001). The Ste20 group kinases as regulators of MAP kinase cascades. *Trends Cell Biol* **11**, 220-30.
- Daniels, R. H. and Bokoch, G. M.** (1999). p21-activated protein kinase: a crucial component of morphological signaling? *Trends Biochem Sci* **24**, 350-5.
- Daniels, R. H., Zenke, F. T. and Bokoch, G. M.** (1999). alphaPix stimulates p21-activated kinase activity through exchange factor-dependent and -independent mechanisms. *J Biol Chem* **274**, 6047-50.
- Date, Y., Nakazato, M., Hashiguchi, S., Dezaki, K., Mondal, M. S., Hosoda, H., Kojima, M., Kangawa, K., Arima, T., Matsuo, H. et al.** (2002). Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. *Diabetes* **51**, 124-9.



- Daub, H., Gevaert, K., Vandekerckhove, J., Sobel, A. and Hall, A.** (2001). Rac/Cdc42 and p65PAK regulate the microtubule-destabilizing protein stathmin through phosphorylation at serine 16. *J Biol Chem* **276**, 1677-80.
- Dauca, M., Bouziges, F., Colin, S., Kedinger, M., Keller, M. K., Schilt, J., Simon-Assmann, P. and Haffen, K.** (1990). Development of the vertebrate small intestine and mechanisms of cell differentiation. *Int J Dev Biol* **34**, 205-18.
- Deacon, S. W., Beeser, A., Fukui, J. A., Rennefahrt, U. E., Myers, C., Chernoff, J. and Peterson, J. R.** (2008). An isoform-selective, small-molecule inhibitor targets the autoregulatory mechanism of p21-activated kinase. *Chem Biol* **15**, 322-31.
- Desai, S., Loomis, Z., Pugh-Bernard, A., Schrunk, J., Doyle, M. J., Minic, A., McCoy, E. and Sussel, L.** (2008). Nkx2.2 regulates cell fate choice in the enteroendocrine cell lineages of the intestine. *Dev Biol* **313**, 58-66.
- Dessimoz, J., Opoka, R., Kordich, J. J., Grapin-Botton, A. and Wells, J. M.** (2006). FGF signaling is necessary for establishing gut tube domains along the anterior-posterior axis in vivo. *Mech Dev* **123**, 42-55.
- Deutsch, G., Jung, J., Zheng, M., Lora, J. and Zaret, K. S.** (2001). A bipotential precursor population for pancreas and liver within the embryonic endoderm. *Development* **128**, 871-81.
- Dezaki, K., Hosoda, H., Kakei, M., Hashiguchi, S., Watanabe, M., Kangawa, K. and Yada, T.** (2004). Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca<sup>2+</sup> signaling in beta-cells: implication in the glycemic control in rodents. *Diabetes* **53**, 3142-51.
- Dhawan, S., Tschén, S. I. and Bhushan, A.** (2009). Bmi-1 regulates the Ink4a/Arf locus to control pancreatic beta-cell proliferation. *Genes Dev* **23**, 906-11.
- Dor, Y., Brown, J., Martinez, O. I. and Melton, D. A.** (2004). Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* **429**, 41-6.
- Doria, A., Patti, M. E. and Kahn, C. R.** (2008). The emerging genetic architecture of type 2 diabetes. *Cell Metab* **8**, 186-200.
- Drucker, D. J.** (2007). The role of gut hormones in glucose homeostasis. *J Clin Invest* **117**, 24-32.
- Du, A., Hunter, C. S., Murray, J., Noble, D., Cai, C. L., Evans, S. M., Stein, R. and May, C. L.** (2009). Islet-1 is required for the maturation, proliferation, and survival of the endocrine pancreas. *Diabetes* **58**, 2059-69.
- Du, A., McCracken, K. W., Walp, E. R., Terry, N. A., Klein, T. J., Han, A., Wells, J. M. and May, C. L.** (2012). Arx is required for normal enteroendocrine cell development in mice and humans. *Dev Biol* **365**, 175-88.
- Dubois, C. L., Shih, H. P., Seymour, P. A., Patel, N. A., Behrmann, J. M., Ngo, V. and Sander, M.** (2011). Sox9-haploinsufficiency causes glucose intolerance in mice. *PLoS One* **6**, e23131.
- Dubruille, R., Laurencon, A., Vandaele, C., Shishido, E., Coulon-Bublex, M., Swoboda, P., Couble, P., Kernan, M. and Durand, B.** (2002). Drosophila regulatory factor X is necessary for ciliated sensory neuron differentiation. *Development* **129**, 5487-98.
- Edlund, H.** (2002). Pancreatic organogenesis--developmental mechanisms and implications for therapy. *Nat Rev Genet* **3**, 524-32.
- Edwards, D. C., Sanders, L. C., Bokoch, G. M. and Gill, G. N.** (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. *Nat Cell Biol* **1**, 253-9.

- Eide, S. A., Raeder, H., Johansson, S., Midthjell, K., Sovik, O., Njolstad, P. R. and Molven, A.** (2008). Prevalence of HNF1A (MODY3) mutations in a Norwegian population (the HUNT2 Study). *Diabet Med* **25**, 775-81.
- Eizirik, D. L., Colli, M. L. and Ortis, F.** (2009). The role of inflammation in insulinitis and beta-cell loss in type 1 diabetes. *Nat Rev Endocrinol* **5**, 219-26.
- El Zein, L., Ait-Lounis, A., Morle, L., Thomas, J., Chhin, B., Spassky, N., Reith, W. and Durand, B.** (2009). RFX3 governs growth and beating efficiency of motile cilia in mouse and controls the expression of genes involved in human ciliopathies. *J Cell Sci* **122**, 3180-9.
- Emery, P., Durand, B., Mach, B. and Reith, W.** (1996). RFX proteins, a novel family of DNA binding proteins conserved in the eukaryotic kingdom. *Nucleic Acids Res* **24**, 803-7.
- Etienne-Manneville, S.** (2004). Actin and microtubules in cell motility: which one is in control? *Traffic* **5**, 470-7.
- Fajas, L., Annicotte, J. S., Miard, S., Sarruf, D., Watanabe, M. and Auwerx, J.** (2004). Impaired pancreatic growth, beta cell mass, and beta cell function in E2F1 (-/-) mice. *J Clin Invest* **113**, 1288-95.
- Fan, X., Labrador, J. P., Hing, H. and Bashaw, G. J.** (2003). Slit stimulation recruits Dock and Pak to the roundabout receptor and increases Rac activity to regulate axon repulsion at the CNS midline. *Neuron* **40**, 113-27.
- Faure, S., Vigneron, S., Galas, S., Brassac, T., Delsert, C. and Morin, N.** (1999). Control of G2/M transition in *Xenopus* by a member of the p21-activated kinase (PAK) family: a link between protein kinase A and PAK signaling pathways? *J Biol Chem* **274**, 3573-9.
- Feng, C., Xu, W. and Zuo, Z.** (2009). Knockout of the regulatory factor X1 gene leads to early embryonic lethality. *Biochem Biophys Res Commun* **386**, 715-7.
- Feng, Q., Albeck, J. G., Cerione, R. A. and Yang, W.** (2002). Regulation of the Cool/Pix proteins: key binding partners of the Cdc42/Rac targets, the p21-activated kinases. *J Biol Chem* **277**, 5644-50.
- Fiorina, P., Vergani, A., Petrelli, A., D'Addio, F., Monti, L., Abdi, R., Bosi, E., Maffi, P. and Secchi, A.** (2008). Metabolic and immunological features of the failing islet-transplanted patient. *Diabetes Care* **31**, 436-8.
- Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D. and Artavanis-Tsakonas, S.** (2005). Notch signals control the fate of immature progenitor cells in the intestine. *Nature* **435**, 964-8.
- Frost, J. A., Swantek, J. L., Stippec, S., Yin, M. J., Gaynor, R. and Cobb, M. H.** (2000). Stimulation of NFkappa B activity by multiple signaling pathways requires PAK1. *J Biol Chem* **275**, 19693-9.
- Fujikura, J., Hosoda, K., Iwakura, H., Tomita, T., Noguchi, M., Masuzaki, H., Tanigaki, K., Yabe, D., Honjo, T. and Nakao, K.** (2006). Notch/Rbp-j signaling prevents premature endocrine and ductal cell differentiation in the pancreas. *Cell Metab* **3**, 59-65.
- Fujitani, Y., Fujitani, S., Boyer, D. F., Gannon, M., Kawaguchi, Y., Ray, M., Shiota, M., Stein, R. W., Magnuson, M. A. and Wright, C. V.** (2006). Targeted deletion of a cis-regulatory region reveals differential gene dosage requirements for Pdx1 in foregut organ differentiation and pancreas formation. *Genes Dev* **20**, 253-66.
- Gajiwala, K. S. and Burley, S. K.** (2000). Winged helix proteins. *Curr Opin Struct Biol* **10**, 110-6.
- Gajiwala, K. S., Chen, H., Cornille, F., Roques, B. P., Reith, W., Mach, B. and Burley, S. K.** (2000). Structure of the winged-helix protein hRFX1 reveals a new mode of DNA binding. *Nature* **403**, 916-21.
- Gannon, M., Ables, E. T., Crawford, L., Lowe, D., Offield, M. F., Magnuson, M. A. and Wright, C. V.** (2008). pdx-1 function is specifically required in embryonic beta cells to generate appropriate numbers of endocrine cell types and maintain glucose homeostasis. *Dev Biol* **314**, 406-17.



- Gao, N., Le Lay, J., Qin, W., Doliba, N., Schug, J., Fox, A. J., Smirnova, O., Matschinsky, F. M. and Kaestner, K. H.** (2010). Foxa1 and Foxa2 maintain the metabolic and secretory features of the mature beta-cell. *Mol Endocrinol* **24**, 1594-604.
- Gao, N., LeLay, J., Vatamaniuk, M. Z., Rieck, S., Friedman, J. R. and Kaestner, K. H.** (2008). Dynamic regulation of Pdx1 enhancers by Foxa1 and Foxa2 is essential for pancreas development. *Genes Dev* **22**, 3435-48.
- Gao, N., White, P. and Kaestner, K. H.** (2009). Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2. *Dev Cell* **16**, 588-99.
- Garcia, M., Hernandez-Lorenzo, P., San Roman, J. I. and Calvo, J. J.** (2008). Pancreatic duct secretion: experimental methods, ion transport mechanisms and regulation. *J Physiol Biochem* **64**, 243-57.
- Gehart, H., Kumpf, S., Ittner, A. and Ricci, R.** (2010). MAPK signalling in cellular metabolism: stress or wellness? *EMBO Rep* **11**, 834-40.
- Georgia, S. and Bhushan, A.** (2004). Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. *J Clin Invest* **114**, 963-8.
- Georgia, S. and Bhushan, A.** (2006). p27 Regulates the transition of beta-cells from quiescence to proliferation. *Diabetes* **55**, 2950-6.
- Georgia, S., Soliz, R., Li, M., Zhang, P. and Bhushan, A.** (2006). p57 and Hes1 coordinate cell cycle exit with self-renewal of pancreatic progenitors. *Dev Biol* **298**, 22-31.
- Gerdes, J. M., Davis, E. E. and Katsanis, N.** (2009). The vertebrate primary cilium in development, homeostasis, and disease. *Cell* **137**, 32-45.
- Gianani, R. and Eisenbarth, G. S.** (2005). The stages of type 1A diabetes: 2005. *Immunol Rev* **204**, 232-49.
- Gierl, M. S., Karoulias, N., Wende, H., Strehle, M. and Birchmeier, C.** (2006). The zinc-finger factor Insm1 (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells. *Genes Dev* **20**, 2465-78.
- Githens, S.** (1988). The pancreatic duct cell: proliferative capabilities, specific characteristics, metaplasia, isolation, and culture. *J Pediatr Gastroenterol Nutr* **7**, 486-506.
- Golson, M. L., Le Lay, J., Gao, N., Bramswig, N., Loomes, K. M., Oakey, R., May, C. L., White, P. and Kaestner, K. H.** (2009). Jagged1 is a competitive inhibitor of Notch signaling in the embryonic pancreas. *Mech Dev* **126**, 687-99.
- Gouzi, M., Kim, Y. H., Katsumoto, K., Johansson, K. and Grapin-Botton, A.** (2011). Neurogenin3 initiates stepwise delamination of differentiating endocrine cells during pancreas development. *Dev Dyn* **240**, 589-604.
- Govek, E. E., Newey, S. E. and Van Aelst, L.** (2005). The role of the Rho GTPases in neuronal development. *Genes Dev* **19**, 1-49.
- Gradwohl, G., Dierich, A., LeMeur, M. and Guillemot, F.** (2000). neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A* **97**, 1607-11.
- Granot, Z., Swisa, A., Magenheim, J., Stolovich-Rain, M., Fujimoto, W., Manduchi, E., Miki, T., Lennerz, J. K., Stoeckert, C. J., Jr., Meyuhos, O. et al.** (2009). LKB1 regulates pancreatic beta cell size, polarity, and function. *Cell Metab* **10**, 296-308.
- Greenman, C., Stephens, P., Smith, R., Dalgliesh, G. L., Hunter, C., Bignell, G., Davies, H., Teague, J., Butler, A., Stevens, C. et al.** (2007). Patterns of somatic mutation in human cancer genomes. *Nature* **446**, 153-8.

- Gregorieff, A., Stange, D. E., Kujala, P., Begthel, H., van den Born, M., Korving, J., Peters, P. J. and Clevers, H.** (2009). The ets-domain transcription factor Spdef promotes maturation of goblet and paneth cells in the intestinal epithelium. *Gastroenterology* **137**, 1333-45 e1-3.
- Greiner, T. U., Kesavan, G., Stahlberg, A. and Semb, H.** (2009). Rac1 regulates pancreatic islet morphogenesis. *BMC Dev Biol* **9**, 2.
- Gu, C., Stein, G. H., Pan, N., Goebbels, S., Hornberg, H., Nave, K. A., Herrera, P., White, P., Kaestner, K. H., Sussel, L. et al.** (2010). Pancreatic beta cells require NeuroD to achieve and maintain functional maturity. *Cell Metab* **11**, 298-310.
- Gu, G., Dubauskaite, J. and Melton, D. A.** (2002). Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* **129**, 2447-57.
- Guo, W., Miao, C., Liu, S., Qiu, Z., Li, J. and Duan, E.** (2009). Efficient differentiation of insulin-producing cells from skin-derived stem cells. *Cell Prolif* **42**, 49-62.
- Gururaj, A. E., Rayala, S. K. and Kumar, R.** (2005). p21-activated kinase signaling in breast cancer. *Breast Cancer Res* **7**, 5-12.
- Habib, A. M., Richards, P., Cairns, L. S., Rogers, G. J., Bannon, C. A., Parker, H. E., Morley, T. C., Yeo, G. S., Reimann, F. and Gribble, F. M.** (2012). Overlap of endocrine hormone expression in the mouse intestine revealed by transcriptional profiling and flow cytometry. *Endocrinology* **153**, 3054-65.
- Hald, J., Galbo, T., Rescan, C., Radzikowski, L., Sprinkel, A. E., Heimberg, H., Ahnfelt-Ronne, J., Jensen, J., Scharfmann, R., Gradwohl, G. et al.** (2012). Pancreatic islet and progenitor cell surface markers with cell sorting potential. *Diabetologia* **55**, 154-65.
- Hald, J., Hjorth, J. P., German, M. S., Madsen, O. D., Serup, P. and Jensen, J.** (2003). Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development. *Dev Biol* **260**, 426-37.
- Hammar, E., Tomas, A., Bosco, D. and Halban, P. A.** (2009). Role of the Rho-ROCK (Rho-associated kinase) signaling pathway in the regulation of pancreatic beta-cell function. *Endocrinology* **150**, 2072-9.
- Haramis, A. P., Begthel, H., van den Born, M., van Es, J., Jonkheer, S., Offerhaus, G. J. and Clevers, H.** (2004). De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science* **303**, 1684-6.
- Hart, A., Papadopoulou, S. and Edlund, H.** (2003). Fgf10 maintains notch activation, stimulates proliferation, and blocks differentiation of pancreatic epithelial cells. *Dev Dyn* **228**, 185-93.
- Haumaitre, C., Barbacci, E., Jenny, M., Ott, M. O., Gradwohl, G. and Cereghini, S.** (2005). Lack of TCF2/vHNF1 in mice leads to pancreas agenesis. *Proc Natl Acad Sci U S A* **102**, 1490-5.
- Hayashi, M. L., Choi, S. Y., Rao, B. S., Jung, H. Y., Lee, H. K., Zhang, D., Chattarji, S., Kirkwood, A. and Tonegawa, S.** (2004). Altered cortical synaptic morphology and impaired memory consolidation in forebrain-specific dominant-negative PAK transgenic mice. *Neuron* **42**, 773-87.
- He, X. C., Zhang, J., Tong, W. G., Tawfik, O., Ross, J., Scoville, D. H., Tian, Q., Zeng, X., He, X., Wiedemann, L. M. et al.** (2004). BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nat Genet* **36**, 1117-21.
- Hebrok, M., Kim, S. K. and Melton, D. A.** (1998). Notochord repression of endodermal Sonic hedgehog permits pancreas development. *Genes Dev* **12**, 1705-13.
- Heit, J. J., Karnik, S. K. and Kim, S. K.** (2006). Intrinsic regulators of pancreatic beta-cell proliferation. *Annu Rev Cell Dev Biol* **22**, 311-38.

- Heller, R. S., Jenny, M., Collombat, P., Mansouri, A., Tomasetto, C., Madsen, O. D., Mellitzer, G., Gradwohl, G. and Serup, P. (2005). Genetic determinants of pancreatic epsilon-cell development. *Dev Biol* **286**, 217-24.
- Henseleit, K. D., Nelson, S. B., Kuhlbrodt, K., Hennings, J. C., Ericson, J. and Sander, M. (2005). NKX6 transcription factor activity is required for alpha- and beta-cell development in the pancreas. *Development* **132**, 3139-49.
- Heremans, Y., Van De Castele, M., in't Veld, P., Gradwohl, G., Serup, P., Madsen, O., Pipeleers, D. and Heimberg, H. (2002). Recapitulation of embryonic neuroendocrine differentiation in adult human pancreatic duct cells expressing neurogenin 3. *J Cell Biol* **159**, 303-12.
- Herrera, P. L. (2000). Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development* **127**, 2317-22.
- Hing, H., Xiao, J., Harden, N., Lim, L. and Zipursky, S. L. (1999). Pak functions downstream of Dock to regulate photoreceptor axon guidance in *Drosophila*. *Cell* **97**, 853-63.
- Hoffman, B. G., Zavaglia, B., Witzsche, J., Ruiz de Algora, T., Beach, M., Hoodless, P. A., Jones, S. J., Marra, M. A. and Helgason, C. D. (2008). Identification of transcripts with enriched expression in the developing and adult pancreas. *Genome Biol* **9**, R99.
- Hofmann, C., Shepelev, M. and Chernoff, J. (2004). The genetics of Pak. *J Cell Sci* **117**, 4343-54.
- Holst, J. J. (2002). Therapy of type 2 diabetes mellitus based on the actions of glucagon-like peptide-1. *Diabetes Metab Res Rev* **18**, 430-41.
- Horn, S., Kobberup, S., Jorgensen, M. C., Kalisz, M., Klein, T., Kageyama, R., Gegg, M., Lickert, H., Lindner, J., Magnuson, M. A. et al. (2012). Mind bomb 1 is required for pancreatic beta-cell formation. *Proc Natl Acad Sci U S A* **109**, 7356-61.
- Horvath, G. C., Kistler, W. S. and Kistler, M. K. (2004). RFX2 is a potential transcriptional regulatory factor for histone H1t and other genes expressed during the meiotic phase of spermatogenesis. *Biol Reprod* **71**, 1551-9.
- Hsu, Y. C., Liao, W. C., Kao, C. Y. and Chiu, I. M. (2010). Regulation of FGF1 gene promoter through transcription factor RFX1. *J Biol Chem* **285**, 13885-95.
- Huang, H. P., Liu, M., El-Hodiri, H. M., Chu, K., Jamrich, M. and Tsai, M. J. (2000). Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. *Mol Cell Biol* **20**, 3292-307.
- Huang, M., Zhou, Z. and Elledge, S. J. (1998). The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. *Cell* **94**, 595-605.
- Huang, W., Zhou, Z., Asrar, S., Henkelman, M., Xie, W. and Jia, Z. (2011). p21-Activated kinases 1 and 3 control brain size through coordinating neuronal complexity and synaptic properties. *Mol Cell Biol* **31**, 388-403.
- Iglesias, A., Murga, M., Laresgoiti, U., Skoudy, A., Bernales, I., Fullaondo, A., Moreno, B., Lloreta, J., Field, S. J., Real, F. X. et al. (2004). Diabetes and exocrine pancreatic insufficiency in E2F1/E2F2 double-mutant mice. *J Clin Invest* **113**, 1398-407.
- Imai, Y., Patel, H. R., Hawkins, E. J., Doliba, N. M., Matschinsky, F. M. and Ahima, R. S. (2007). Insulin secretion is increased in pancreatic islets of neuropeptide Y-deficient mice. *Endocrinology* **148**, 5716-23.
- Iwama, A., Pan, J., Zhang, P., Reith, W., Mach, B., Tenen, D. G. and Sun, Z. (1999). Dimeric RFX proteins contribute to the activity and lineage specificity of the interleukin-5 receptor alpha promoter through activation and repression domains. *Mol Cell Biol* **19**, 3940-50.

- Iype, T., Francis, J., Garmey, J. C., Schisler, J. C., Nesher, R., Weir, G. C., Becker, T. C., Newgard, C. B., Griffen, S. C. and Mirmira, R. G.** (2005). Mechanism of insulin gene regulation by the pancreatic transcription factor Pdx-1: application of pre-mRNA analysis and chromatin immunoprecipitation to assess formation of functional transcriptional complexes. *J Biol Chem* **280**, 16798-807.
- Jacobsen, J. S., Wu, C. C., Redwine, J. M., Comery, T. A., Arias, R., Bowlby, M., Martone, R., Morrison, J. H., Pangalos, M. N., Reinhart, P. H. et al.** (2006). Early-onset behavioral and synaptic deficits in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A* **103**, 5161-6.
- Jacquemin, P., Yoshitomi, H., Kashima, Y., Rousseau, G. G., Lemaigre, F. P. and Zaret, K. S.** (2006). An endothelial-mesenchymal relay pathway regulates early phases of pancreas development. *Dev Biol* **290**, 189-99.
- Jaffer, Z. M. and Chernoff, J.** (2002). p21-activated kinases: three more join the Pak. *Int J Biochem Cell Biol* **34**, 713-7.
- Jenny, M., Uhl, C., Roche, C., Duluc, I., Guillermin, V., Guillemot, F., Jensen, J., Kedinger, M. and Gradwohl, G.** (2002). Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium. *EMBO J* **21**, 6338-47.
- Jensen, J., Heller, R. S., Funder-Nielsen, T., Pedersen, E. E., Lindsell, C., Weinmaster, G., Madsen, O. D. and Serup, P.** (2000a). Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation. *Diabetes* **49**, 163-76.
- Jensen, J., Pedersen, E. E., Galante, P., Hald, J., Heller, R. S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P. and Madsen, O. D.** (2000b). Control of endodermal endocrine development by Hes-1. *Nat Genet* **24**, 36-44.
- Jepeal, L. I., Fujitani, Y., Boylan, M. O., Wilson, C. N., Wright, C. V. and Wolfe, M. M.** (2005). Cell-specific expression of glucose-dependent-insulinotropic polypeptide is regulated by the transcription factor PDX-1. *Endocrinology* **146**, 383-91.
- Jhala, U. S., Canettieri, G., Sreaton, R. A., Kulkarni, R. N., Krajewski, S., Reed, J., Walker, J., Lin, X., White, M. and Montminy, M.** (2003). cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. *Genes Dev* **17**, 1575-80.
- Johansson, K. A., Dursun, U., Jordan, N., Gu, G., Beermann, F., Gradwohl, G. and Grapin-Botton, A.** (2007). Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types. *Dev Cell* **12**, 457-65.
- Jonsson, J., Carlsson, L., Edlund, T. and Edlund, H.** (1994). Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* **371**, 606-9.
- Jorgensen, M. C., Ahnfelt-Ronne, J., Hald, J., Madsen, O. D., Serup, P. and Hecksher-Sorensen, J.** (2007). An illustrated review of early pancreas development in the mouse. *Endocr Rev* **28**, 685-705.
- Jung, I. D., Lee, J., Lee, K. B., Park, C. G., Kim, Y. K., Seo, D. W., Park, D., Lee, H. W., Han, J. W. and Lee, H. Y.** (2004). Activation of p21-activated kinase 1 is required for lysophosphatidic acid-induced focal adhesion kinase phosphorylation and cell motility in human melanoma A2058 cells. *Eur J Biochem* **271**, 1557-65.
- Kaestner, K. H.** (2010). The FoxA factors in organogenesis and differentiation. *Curr Opin Genet Dev* **20**, 527-32.
- Kaestner, K. H., Katz, J., Liu, Y., Drucker, D. J. and Schutz, G.** (1999). Inactivation of the winged helix transcription factor HNF3alpha affects glucose homeostasis and islet glucagon gene expression in vivo. *Genes Dev* **13**, 495-504.

- Karagiannides, I., Bakirtzi, K., Kokkotou, E., Stavrakis, D., Margolis, K. G., Thomou, T., Giorgadze, N., Kirkland, J. L. and Pothoulakis, C. (2011). Role of substance P in the regulation of glucose metabolism via insulin signaling-associated pathways. *Endocrinology* **152**, 4571-80.
- Karlsson, L., Lindahl, P., Heath, J. K. and Betsholtz, C. (2000). Abnormal gastrointestinal development in PDGF-A and PDGFR-(alpha) deficient mice implicates a novel mesenchymal structure with putative instructive properties in villus morphogenesis. *Development* **127**, 3457-66.
- Katan-Khaykovich, Y. and Shaul, Y. (1998). RFX1, a single DNA-binding protein with a split dimerization domain, generates alternative complexes. *J Biol Chem* **273**, 24504-12.
- Katz, J. P., Perreault, N., Goldstein, B. G., Lee, C. S., Labosky, P. A., Yang, V. W. and Kaestner, K. H. (2002). The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. *Development* **129**, 2619-28.
- Kawaguchi, Y., Cooper, B., Gannon, M., Ray, M., MacDonald, R. J. and Wright, C. V. (2002). The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat Genet* **32**, 128-34.
- Kesavan, G., Sand, F. W., Greiner, T. U., Johansson, J. K., Kobberup, S., Wu, X., Brakebusch, C. and Semb, H. (2009). Cdc42-mediated tubulogenesis controls cell specification. *Cell* **139**, 791-801.
- Kikuchi, O., Kobayashi, M., Amano, K., Sasaki, T., Kitazumi, T., Kim, H. J., Lee, Y. S., Yokota-Hashimoto, H., Kitamura, Y. I. and Kitamura, T. (2012). FoxO1 gain of function in the pancreas causes glucose intolerance, polycystic pancreas, and islet hypervascularization. *PLoS One* **7**, e32249.
- Kim, B. M., Mao, J., Taketo, M. M. and Shivdasani, R. A. (2007). Phases of canonical Wnt signaling during the development of mouse intestinal epithelium. *Gastroenterology* **133**, 529-38.
- Kim, H., Toyofuku, Y., Lynn, F. C., Chak, E., Uchida, T., Mizukami, H., Fujitani, Y., Kawamori, R., Miyatsuka, T., Kosaka, Y. et al. (2010). Serotonin regulates pancreatic beta cell mass during pregnancy. *Nat Med* **16**, 804-8.
- Kim, S. J., Winter, K., Nian, C., Tsuneoka, M., Koda, Y. and McIntosh, C. H. (2005). Glucose-dependent insulinotropic polypeptide (GIP) stimulation of pancreatic beta-cell survival is dependent upon phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) signaling, inactivation of the forkhead transcription factor Foxo1, and down-regulation of bax expression. *J Biol Chem* **280**, 22297-307.
- Kim, S. K., Hebrok, M. and Melton, D. A. (1997). Notochord to endoderm signaling is required for pancreas development. *Development* **124**, 4243-52.
- Kir, S., Beddow, S. A., Samuel, V. T., Miller, P., Previs, S. F., Suino-Powell, K., Xu, H. E., Shulman, G. I., Klierer, S. A. and Mangelsdorf, D. J. (2011). FGF19 as a postprandial, insulin-independent activator of hepatic protein and glycogen synthesis. *Science* **331**, 1621-4.
- Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs, W. H., 3rd, Wright, C. V., White, M. F., Arden, K. C. and Accili, D. (2002). The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J Clin Invest* **110**, 1839-47.
- Knaus, U. G., Wang, Y., Reilly, A. M., Warnock, D. and Jackson, J. H. (1998). Structural requirements for PAK activation by Rac GTPases. *J Biol Chem* **273**, 21512-8.
- Kobayashi, M., Kikuchi, O., Sasaki, T., Kim, H. J., Yokota-Hashimoto, H., Lee, Y. S., Amano, K., Kitazumi, T., Susanti, V. Y., Kitamura, Y. I. et al. (2012). FoxO1 as a double-edged sword in the pancreas: analysis of pancreas- and beta-cell-specific FoxO1 knockout mice. *Am J Physiol Endocrinol Metab* **302**, E603-13.
- Kolaskar, A. S. and Tongaonkar, P. C. (1990). A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Lett* **276**, 172-4.



- Kolterud, A., Grosse, A. S., Zacharias, W. J., Walton, K. D., Kretovich, K. E., Madison, B. B., Waghray, M., Ferris, J. E., Hu, C., Merchant, J. L. et al.** (2009). Paracrine Hedgehog signaling in stomach and intestine: new roles for hedgehog in gastrointestinal patterning. *Gastroenterology* **137**, 618-28.
- Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J. and Clevers, H.** (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat Genet* **19**, 379-83.
- Kowluru, A.** (2011). Friendly, and not so friendly, roles of Rac1 in islet beta-cell function: lessons learnt from pharmacological and molecular biological approaches. *Biochem Pharmacol* **81**, 965-75.
- Krapp, A., Knofler, M., Frutiger, S., Hughes, G. J., Hagenbuchle, O. and Wellauer, P. K.** (1996). The p48 DNA-binding subunit of transcription factor PTF1 is a new exocrine pancreas-specific basic helix-loop-helix protein. *EMBO J* **15**, 4317-29.
- Krapp, A., Knofler, M., Ledermann, B., Burki, K., Berney, C., Zoerkler, N., Hagenbuchle, O. and Wellauer, P. K.** (1998). The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. *Genes Dev* **12**, 3752-63.
- Kreis, P. and Barnier, J. V.** (2009). PAK signalling in neuronal physiology. *Cell Signal* **21**, 384-93.
- Kreis, P., Rousseau, V., Thevenot, E., Combeau, G. and Barnier, J. V.** (2008). The four mammalian splice variants encoded by the p21-activated kinase 3 gene have different biological properties. *J Neurochem* **106**, 1184-97.
- Kroon, E., Martinson, L. A., Kadoya, K., Bang, A. G., Kelly, O. G., Eliazar, S., Young, H., Richardson, M., Smart, N. G., Cunningham, J. et al.** (2008). Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat Biotechnol* **26**, 443-52.
- Kumar, M., Jordan, N., Melton, D. and Grapin-Botton, A.** (2003). Signals from lateral plate mesoderm instruct endoderm toward a pancreatic fate. *Dev Biol* **259**, 109-22.
- Kumar, R., Gururaj, A. E. and Barnes, C. J.** (2006). p21-activated kinases in cancer. *Nat Rev Cancer* **6**, 459-71.
- Kunisada, Y., Tsubooka-Yamazoe, N., Shoji, M. and Hosoya, M.** (2012). Small molecules induce efficient differentiation into insulin-producing cells from human induced pluripotent stem cells. *Stem Cell Res* **8**, 274-84.
- Kushner, J. A., Ciemerych, M. A., Sicinska, E., Wartschow, L. M., Teta, M., Long, S. Y., Sicinski, P. and White, M. F.** (2005). Cyclins D2 and D1 are essential for postnatal pancreatic beta-cell growth. *Mol Cell Biol* **25**, 3752-62.
- Lammert, E., Cleaver, O. and Melton, D.** (2001). Induction of pancreatic differentiation by signals from blood vessels. *Science* **294**, 564-7.
- Larsson, L. I., Madsen, O. D., Serup, P., Jonsson, J. and Edlund, H.** (1996). Pancreatic-duodenal homeobox 1 -role in gastric endocrine patterning. *Mech Dev* **60**, 175-84.
- Larsson, L. I., St-Onge, L., Hougaard, D. M., Sosa-Pineda, B. and Gruss, P.** (1998). Pax 4 and 6 regulate gastrointestinal endocrine cell development. *Mech Dev* **79**, 153-9.
- Lau, J. and Hebrok, M.** (2010). Hedgehog signaling in pancreas epithelium regulates embryonic organ formation and adult beta-cell function. *Diabetes* **59**, 1211-21.
- Laurencon, A., Dubruille, R., Efimenko, E., Grenier, G., Bissett, R., Cortier, E., Rolland, V., Swoboda, P. and Durand, B.** (2007). Identification of novel regulatory factor X (RFX) target genes by comparative genomics in Drosophila species. *Genome Biol* **8**, R195.

- Lee, C. S., Perreault, N., Brestelli, J. E. and Kaestner, K. H.** (2002). Neurogenin 3 is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity. *Genes Dev* **16**, 1488-97.
- Lee, C. S., Sund, N. J., Behr, R., Herrera, P. L. and Kaestner, K. H.** (2005). Foxa2 is required for the differentiation of pancreatic alpha-cells. *Dev Biol* **278**, 484-95.
- Lee, J. H., Jo, J., Hardikar, A. A., Periwal, V. and Rane, S. G.** (2010). Cdk4 regulates recruitment of quiescent beta-cells and ductal epithelial progenitors to reconstitute beta-cell mass. *PLoS One* **5**, e8653.
- Lee, N. K., Sowa, H., Hinoi, E., Ferron, M., Ahn, J. D., Confavreux, C., Dacquin, R., Mee, P. J., McKee, M. D., Jung, D. Y. et al.** (2007). Endocrine regulation of energy metabolism by the skeleton. *Cell* **130**, 456-69.
- Lei, M., Lu, W., Meng, W., Parrini, M. C., Eck, M. J., Mayer, B. J. and Harrison, S. C.** (2000). Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. *Cell* **102**, 387-97.
- Lentze, M. J.** (1995). Molecular and cellular aspects of hydrolysis and absorption. *Am J Clin Nutr* **61**, 946S-951S.
- Li, F., Adam, L., Vadlamudi, R. K., Zhou, H., Sen, S., Chernoff, J., Mandal, M. and Kumar, R.** (2002). p21-activated kinase 1 interacts with and phosphorylates histone H3 in breast cancer cells. *EMBO Rep* **3**, 767-73.
- Li, H. J., Ray, S. K., Singh, N. K., Johnston, B. and Leiter, A. B.** (2011). Basic helix-loop-helix transcription factors and enteroendocrine cell differentiation. *Diabetes Obes Metab* **13 Suppl 1**, 5-12.
- Li, W. and Guan, K. L.** (2004). The Down syndrome cell adhesion molecule (DSCAM) interacts with and activates Pak. *J Biol Chem* **279**, 32824-31.
- Li, X. and Minden, A.** (2003). Targeted disruption of the gene for the PAK5 kinase in mice. *Mol Cell Biol* **23**, 7134-42.
- Li, Y., Hansotia, T., Yusta, B., Ris, F., Halban, P. A. and Drucker, D. J.** (2003). Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. *J Biol Chem* **278**, 471-8.
- Lioubinski, O., Muller, M., Wegner, M. and Sander, M.** (2003). Expression of Sox transcription factors in the developing mouse pancreas. *Dev Dyn* **227**, 402-8.
- Liu, R. X., Wang, W. Q., Ye, L., Bi, Y. F., Fang, H., Cui, B., Zhou, W. W., Dai, M., Zhang, J., Li, X. Y. et al.** (2010). p21-activated kinase 3 is overexpressed in thymic neuroendocrine tumors (carcinoids) with ectopic ACTH syndrome and participates in cell migration. *Endocrine* **38**, 38-47.
- Liu, Y., Pathak, N., Kramer-Zucker, A. and Drummond, I. A.** (2007). Notch signaling controls the differentiation of transporting epithelia and multiciliated cells in the zebrafish pronephros. *Development* **134**, 1111-22.
- Lopez-Diaz, L., Jain, R. N., Keeley, T. M., VanDussen, K. L., Brunkan, C. S., Gumucio, D. L. and Samuelson, L. C.** (2007). Intestinal Neurogenin 3 directs differentiation of a bipotential secretory progenitor to endocrine cell rather than goblet cell fate. *Dev Biol* **309**, 298-305.
- Lubelsky, Y., Reuven, N. and Shaul, Y.** (2005). Autorepression of rfx1 gene expression: functional conservation from yeast to humans in response to DNA replication arrest. *Mol Cell Biol* **25**, 10665-73.
- Luo, L.** (2002). Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. *Annu Rev Cell Dev Biol* **18**, 601-35.
- Lynn, F. C., Smith, S. B., Wilson, M. E., Yang, K. Y., Nekrep, N. and German, M. S.** (2007). Sox9 coordinates a transcriptional network in pancreatic progenitor cells. *Proc Natl Acad Sci U S A* **104**, 10500-5.

- Madison, B. B., Braunstein, K., Kuizon, E., Portman, K., Qiao, X. T. and Gumucio, D. L.** (2005). Epithelial hedgehog signals pattern the intestinal crypt-villus axis. *Development* **132**, 279-89.
- Maffi, P., Scavini, M., Socci, C., Piemonti, L., Caldara, R., Gremizzi, C., Melzi, R., Nano, R., Orsenigo, E., Venturini, M. et al.** (2011). Risks and benefits of transplantation in the cure of type 1 diabetes: whole pancreas versus islet transplantation. A single center study. *Rev Diabet Stud* **8**, 44-50.
- Magenheim, J., Ilovich, O., Lazarus, A., Klochendler, A., Ziv, O., Werman, R., Hija, A., Cleaver, O., Mishani, E., Keshet, E. et al.** (2011). Blood vessels restrain pancreas branching, differentiation and growth. *Development* **138**, 4743-52.
- Mahlamaki, E. H., Kauraniemi, P., Monni, O., Wolf, M., Hautaniemi, S. and Kallioniemi, A.** (2004). High-resolution genomic and expression profiling reveals 105 putative amplification target genes in pancreatic cancer. *Neoplasia* **6**, 432-9.
- Malakauskas, S. M., Kourany, W. M., Zhang, X. Y., Lu, D., Stevens, R. D., Koves, T. R., Hohmeier, H. E., Muoio, D. M., Newgard, C. B. and Le, T. H.** (2009). Increased insulin sensitivity in mice lacking collectrin, a downstream target of HNF-1alpha. *Mol Endocrinol* **23**, 881-92.
- Marlin, J. W., Chang, Y. W., Ober, M., Handy, A., Xu, W. and Jakobi, R.** (2011). Functional PAK-2 knockout and replacement with a caspase cleavage-deficient mutant in mice reveals differential requirements of full-length PAK-2 and caspase-activated PAK-2p34. *Mamm Genome* **22**, 306-17.
- Martin, M., Gallego-Llamas, J., Ribes, V., Kedingner, M., Niederreither, K., Chambon, P., Dolle, P. and Gradwohl, G.** (2005). Dorsal pancreas agenesis in retinoic acid-deficient Raldh2 mutant mice. *Dev Biol* **284**, 399-411.
- Marzo, N., Mora, C., Fabregat, M. E., Martin, J., Usac, E. F., Franco, C., Barbacid, M. and Gomis, R.** (2004). Pancreatic islets from cyclin-dependent kinase 4/R24C (Cdk4) knockin mice have significantly increased beta cell mass and are physiologically functional, indicating that Cdk4 is a potential target for pancreatic beta cell mass regeneration in Type 1 diabetes. *Diabetologia* **47**, 686-94.
- Masui, T., Long, Q., Beres, T. M., Magnuson, M. A. and MacDonald, R. J.** (2007). Early pancreatic development requires the vertebrate Suppressor of Hairless (RBPJ) in the PTF1 bHLH complex. *Genes Dev* **21**, 2629-43.
- Masui, T., Swift, G. H., Deering, T., Shen, C., Coats, W. S., Long, Q., Elsasser, H. P., Magnuson, M. A. and MacDonald, R. J.** (2010). Replacement of Rbpj with Rbpjl in the PTF1 complex controls the final maturation of pancreatic acinar cells. *Gastroenterology* **139**, 270-80.
- Matsuoka, T. A., Artner, I., Henderson, E., Means, A., Sander, M. and Stein, R.** (2004). The MafA transcription factor appears to be responsible for tissue-specific expression of insulin. *Proc Natl Acad Sci U S A* **101**, 2930-3.
- Matsuoka, T. A., Zhao, L., Artner, I., Jarrett, H. W., Friedman, D., Means, A. and Stein, R.** (2003). Members of the large Maf transcription family regulate insulin gene transcription in islet beta cells. *Mol Cell Biol* **23**, 6049-62.
- Matsushita, H., Uenaka, A., Ono, T., Hasegawa, K., Sato, S., Koizumi, F., Nakagawa, K., Toda, M., Shingo, T., Ichikawa, T. et al.** (2005). Identification of glioma-specific RFX4-E and -F isoforms and humoral immune response in patients. *Cancer Sci* **96**, 801-9.
- McClintock, T. S., Glasser, C. E., Bose, S. C. and Bergman, D. A.** (2008). Tissue expression patterns identify mouse cilia genes. *Physiol Genomics* **32**, 198-206.
- McDaniel, A. S., Allen, J. D., Park, S. J., Jaffer, Z. M., Michels, E. G., Burgin, S. J., Chen, S., Bessler, W. K., Hofmann, C., Ingram, D. A. et al.** (2008). Pak1 regulates multiple c-Kit mediated Ras-MAPK gain-in-function phenotypes in Nf1<sup>+/-</sup> mast cells. *Blood* **112**, 4646-54.



- McLin, V. A., Rankin, S. A. and Zorn, A. M.** (2007). Repression of Wnt/beta-catenin signaling in the anterior endoderm is essential for liver and pancreas development. *Development* **134**, 2207-17.
- McPhie, D. L., Coopersmith, R., Hines-Peralta, A., Chen, Y., Ivins, K. J., Manly, S. P., Kozlowski, M. R., Neve, K. A. and Neve, R. L.** (2003). DNA synthesis and neuronal apoptosis caused by familial Alzheimer disease mutants of the amyloid precursor protein are mediated by the p21 activated kinase PAK3. *J Neurosci* **23**, 6914-27.
- Meier, J. J., Butler, A. E., Saisho, Y., Monchamp, T., Galasso, R., Bhushan, A., Rizza, R. A. and Butler, P. C.** (2008). Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. *Diabetes* **57**, 1584-94.
- Mellitzer, G., Beucher, A., Lobstein, V., Michel, P., Robine, S., Keding, M. and Gradwohl, G.** (2010). Loss of enteroendocrine cells in mice alters lipid absorption and glucose homeostasis and impairs postnatal survival. *J Clin Invest* **120**, 1708-21.
- Mellitzer, G., Bonne, S., Luco, R. F., Van De Castele, M., Lenne-Samuel, N., Collombat, P., Mansouri, A., Lee, J., Lan, M., Pipeleers, D. et al.** (2006). IA1 is NGN3-dependent and essential for differentiation of the endocrine pancreas. *EMBO J* **25**, 1344-52.
- Mellitzer, G., Martin, M., Sidhoum-Jenny, M., Orvain, C., Barths, J., Seymour, P. A., Sander, M. and Gradwohl, G.** (2004). Pancreatic islet progenitor cells in neurogenin 3-yellow fluorescent protein knock-add-on mice. *Mol Endocrinol* **18**, 2765-76.
- Melloul, D., Marshak, S. and Cerasi, E.** (2002). Regulation of insulin gene transcription. *Diabetologia* **45**, 309-26.
- Meng, J., Meng, Y., Hanna, A., Janus, C. and Jia, Z.** (2005). Abnormal long-lasting synaptic plasticity and cognition in mice lacking the mental retardation gene Pak3. *J Neurosci* **25**, 6641-50.
- Meng, Y., Zhang, Y., Tregoubov, V., Janus, C., Cruz, L., Jackson, M., Lu, W. Y., MacDonald, J. F., Wang, J. Y., Falls, D. L. et al.** (2002). Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. *Neuron* **35**, 121-33.
- Metzger, D. E., Gasperowicz, M., Otto, F., Cross, J. C., Gradwohl, G. and Zaret, K. S.** (2012). The transcriptional co-repressor Grg3/Tle3 promotes pancreatic endocrine progenitor delamination and beta-cell differentiation. *Development* **139**, 1447-56.
- Michael, M. D., Kulkarni, R. N., Postic, C., Previs, S. F., Shulman, G. I., Magnuson, M. A. and Kahn, C. R.** (2000). Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol Cell* **6**, 87-97.
- Mitchell, J., Punthakee, Z., Lo, B., Bernard, C., Chong, K., Newman, C., Cartier, L., Desilets, V., Cutz, E., Hansen, I. L. et al.** (2004). Neonatal diabetes, with hypoplastic pancreas, intestinal atresia and gall bladder hypoplasia: search for the aetiology of a new autosomal recessive syndrome. *Diabetologia* **47**, 2160-7.
- Miyatsuka, T., Kosaka, Y., Kim, H. and German, M. S.** (2011). Neurogenin3 inhibits proliferation in endocrine progenitors by inducing Cdkn1a. *Proc Natl Acad Sci U S A* **108**, 185-90.
- Miyawaki, K., Yamada, Y., Ban, N., Ihara, Y., Tsukiyama, K., Zhou, H., Fujimoto, S., Oku, A., Tsuda, K., Toyokuni, S. et al.** (2002). Inhibition of gastric inhibitory polypeptide signaling prevents obesity. *Nat Med* **8**, 738-42.
- Miyawaki, K., Yamada, Y., Yano, H., Niwa, H., Ban, N., Ihara, Y., Kubota, A., Fujimoto, S., Kajikawa, M., Kuroe, A. et al.** (1999). Glucose intolerance caused by a defect in the entero-insular axis: a study in gastric inhibitory polypeptide receptor knockout mice. *Proc Natl Acad Sci U S A* **96**, 14843-7.
- Molotkov, A., Molotkova, N. and Duester, G.** (2005). Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. *Dev Dyn* **232**, 950-7.

- Molven, A. and Njolstad, P. R.** (2011). Role of molecular genetics in transforming diagnosis of diabetes mellitus. *Expert Rev Mol Diagn* **11**, 313-20.
- Montanya, E., Nacher, V., Biarnes, M. and Soler, J.** (2000). Linear correlation between beta-cell mass and body weight throughout the lifespan in Lewis rats: role of beta-cell hyperplasia and hypertrophy. *Diabetes* **49**, 1341-6.
- Montgomery, R. K. and Shivdasani, R. A.** (2009). Prominin1 (CD133) as an intestinal stem cell marker: promise and nuance. *Gastroenterology* **136**, 2051-4.
- Mori-Akiyama, Y., van den Born, M., van Es, J. H., Hamilton, S. R., Adams, H. P., Zhang, J., Clevers, H. and de Crombrughe, B.** (2007). SOX9 is required for the differentiation of paneth cells in the intestinal epithelium. *Gastroenterology* **133**, 539-46.
- Morotomi-Yano, K., Yano, K., Saito, H., Sun, Z., Iwama, A. and Miki, Y.** (2002). Human regulatory factor X 4 (RFX4) is a testis-specific dimeric DNA-binding protein that cooperates with other human RFX members. *J Biol Chem* **277**, 836-42.
- Murphy, K. G. and Bloom, S. R.** (2006). Gut hormones and the regulation of energy homeostasis. *Nature* **444**, 854-9.
- Murphy, R., Ellard, S. and Hattersley, A. T.** (2008). Clinical implications of a molecular genetic classification of monogenic beta-cell diabetes. *Nat Clin Pract Endocrinol Metab* **4**, 200-13.
- Murtaugh, L. C.** (2007). Pancreas and beta-cell development: from the actual to the possible. *Development* **134**, 427-38.
- Murtaugh, L. C., Stanger, B. Z., Kwan, K. M. and Melton, D. A.** (2003). Notch signaling controls multiple steps of pancreatic differentiation. *Proc Natl Acad Sci U S A* **100**, 14920-5.
- Nakhai, H., Siveke, J. T., Klein, B., Mendoza-Torres, L., Mazur, P. K., Algul, H., Radtke, F., Strobl, L., Zimmer-Strobl, U. and Schmid, R. M.** (2008). Conditional ablation of Notch signaling in pancreatic development. *Development* **135**, 2757-65.
- Naya, F. J., Huang, H. P., Qiu, Y., Mutoh, H., DeMayo, F. J., Leiter, A. B. and Tsai, M. J.** (1997). Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev* **11**, 2323-34.
- Naya, F. J., Stellrecht, C. M. and Tsai, M. J.** (1995). Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. *Genes Dev* **9**, 1009-19.
- Nayal, A., Webb, D. J., Brown, C. M., Schaefer, E. M., Vicente-Manzanares, M. and Horwitz, A. R.** (2006). Paxillin phosphorylation at Ser273 localizes a GIT1-PIX-PAK complex and regulates adhesion and protrusion dynamics. *J Cell Biol* **173**, 587-9.
- Nekrasova, T., Jobs, M. L., Ting, J. H., Wagner, G. C. and Minden, A.** (2008). Targeted disruption of the Pak5 and Pak6 genes in mice leads to deficits in learning and locomotion. *Dev Biol* **322**, 95-108.
- Nelson, S. B., Schaffer, A. E. and Sander, M.** (2007). The transcription factors Nkx6.1 and Nkx6.2 possess equivalent activities in promoting beta-cell fate specification in Pdx1+ pancreatic progenitor cells. *Development* **134**, 2491-500.
- Nheu, T., He, H., Hirokawa, Y., Walker, F., Wood, J. and Maruta, H.** (2004). PAK is essential for RAS-induced upregulation of cyclin D1 during the G1 to S transition. *Cell Cycle* **3**, 71-4.
- Nikolic, M., Chou, M. M., Lu, W., Mayer, B. J. and Tsai, L. H.** (1998). The p35/Cdk5 kinase is a neuron-specific Rac effector that inhibits Pak1 activity. *Nature* **395**, 194-8.
- Ninov, N., Borius, M. and Stainier, D. Y.** (2012). Different levels of Notch signaling regulate quiescence, renewal and differentiation in pancreatic endocrine progenitors. *Development* **139**, 1557-67.

- Nishimura, W., Bonner-Weir, S. and Sharma, A.** (2009). Expression of MafA in pancreatic progenitors is detrimental for pancreatic development. *Dev Biol* **333**, 108-20.
- Noah, T. K., Kazanjian, A., Whitsett, J. and Shroyer, N. F.** (2010). SAM pointed domain ETS factor (SPDEF) regulates terminal differentiation and maturation of intestinal goblet cells. *Exp Cell Res* **316**, 452-65.
- Node-Langlois, R., Muller, D. and Boda, B.** (2006). Sequential implication of the mental retardation proteins ARHGEF6 and PAK3 in spine morphogenesis. *J Cell Sci* **119**, 4986-93.
- Norgaard, G. A., Jensen, J. N. and Jensen, J.** (2003). FGF10 signaling maintains the pancreatic progenitor cell state revealing a novel role of Notch in organ development. *Dev Biol* **264**, 323-38.
- Offield, M. F., Jetton, T. L., Labosky, P. A., Ray, M., Stein, R. W., Magnuson, M. A., Hogan, B. L. and Wright, C. V.** (1996). PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* **122**, 983-95.
- Olbrot, M., Rud, J., Moss, L. G. and Sharma, A.** (2002). Identification of beta-cell-specific insulin gene transcription factor RIPE3b1 as mammalian MafA. *Proc Natl Acad Sci U S A* **99**, 6737-42.
- Oliver-Krasinski, J. M., Kasner, M. T., Yang, J., Crutchlow, M. F., Rustgi, A. K., Kaestner, K. H. and Stoffers, D. A.** (2009). The diabetes gene Pdx1 regulates the transcriptional network of pancreatic endocrine progenitor cells in mice. *J Clin Invest* **119**, 1888-98.
- Oliver-Krasinski, J. M. and Stoffers, D. A.** (2008). On the origin of the beta cell. *Genes Dev* **22**, 1998-2021.
- Ostrom, M., Loffler, K. A., Edfalk, S., Selander, L., Dahl, U., Ricordi, C., Jeon, J., Correa-Medina, M., Diez, J. and Edlund, H.** (2008). Retinoic acid promotes the generation of pancreatic endocrine progenitor cells and their further differentiation into beta-cells. *PLoS One* **3**, e2841.
- Otto, W. R., Patel, K., McKinnell, I., Evans, M. D., Lee, C. Y., Frith, D., Hanrahan, S., Blight, K., Blin, N., Kayademir, T. et al.** (2006). Identification of blottin: a novel gastric trefoil factor family-2 binding protein. *Proteomics* **6**, 4235-45.
- Padilla, S. L., Carmody, J. S. and Zeltser, L. M.** (2010). Pomc-expressing progenitors give rise to antagonistic neuronal populations in hypothalamic feeding circuits. *Nat Med* **16**, 403-5.
- Palmer, D. S., Christensen, A. U., Sorensen, J., Celik, L., Qvist, K. B. and Schiott, B.** (2010). Bovine chymosin: a computational study of recognition and binding of bovine kappa-casein. *Biochemistry* **49**, 2563-73.
- Pan, F. C. and Wright, C.** (2011). Pancreas organogenesis: from bud to plexus to gland. *Dev Dyn* **240**, 530-65.
- Parrini, M. C., Lei, M., Harrison, S. C. and Mayer, B. J.** (2002). Pak1 kinase homodimers are autoinhibited in trans and dissociated upon activation by Cdc42 and Rac1. *Mol Cell* **9**, 73-83.
- Pearl, E. J., Jarikji, Z. and Horb, M. E.** (2011). Functional analysis of Rfx6 and mutant variants associated with neonatal diabetes. *Dev Biol* **351**, 135-45.
- Pedersen, J. K., Nelson, S. B., Jorgensen, M. C., Henseleit, K. D., Fujitani, Y., Wright, C. V., Sander, M. and Serup, P.** (2005). Endodermal expression of Nkx6 genes depends differentially on Pdx1. *Dev Biol* **288**, 487-501.
- Pelling, M., Anthwal, N., McNay, D., Gradwohl, G., Leiter, A. B., Guillemot, F. and Ang, S. L.** (2011). Differential requirements for neurogenin 3 in the development of POMC and NPY neurons in the hypothalamus. *Dev Biol* **349**, 406-16.

- Pierreux, C. E., Cordi, S., Hick, A. C., Achouri, Y., Ruiz de Almodovar, C., Prevot, P. P., Courtoy, P. J., Carmeliet, P. and Lemaigre, F. P. (2010). Epithelial: Endothelial cross-talk regulates exocrine differentiation in developing pancreas. *Dev Biol* **347**, 216-27.
- Pinto, D., Gregorieff, A., Begthel, H. and Clevers, H. (2003). Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev* **17**, 1709-13.
- Polak, J. M., Bloom, S. R., Rayford, P. L., Pearse, A. G., Buchan, A. M. and Thompson, J. C. (1975). Identification of cholecystokinin-secreting cells. *Lancet* **2**, 1016-8.
- Poll, A. V., Pierreux, C. E., Lokmane, L., Haumaitre, C., Achouri, Y., Jacquemin, P., Rousseau, G. G., Cereghini, S. and Lemaigre, F. P. (2006). A vHNF1/TCF2-HNF6 cascade regulates the transcription factor network that controls generation of pancreatic precursor cells. *Diabetes* **55**, 61-9.
- Porat, S., Weinberg-Corem, N., Tornovsky-Babaey, S., Schyr-Ben-Haroush, R., Hija, A., Stolovich-Rain, M., Dadon, D., Granot, Z., Ben-Hur, V., White, P. et al. (2011). Control of pancreatic beta cell regeneration by glucose metabolism. *Cell Metab* **13**, 440-9.
- Potten, C. S., Owen, G. and Booth, D. (2002). Intestinal stem cells protect their genome by selective segregation of template DNA strands. *J Cell Sci* **115**, 2381-8.
- Prado, C. L., Pugh-Bernard, A. E., Elghazi, L., Sosa-Pineda, B. and Sussel, L. (2004). Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proc Natl Acad Sci U S A* **101**, 2924-9.
- Puri, S. and Hebrok, M. (2010). Cellular plasticity within the pancreas--lessons learned from development. *Dev Cell* **18**, 342-56.
- Qu, J., Li, X., Novitch, B. G., Zheng, Y., Kohn, M., Xie, J. M., Kozinn, S., Bronson, R., Beg, A. A. and Minden, A. (2003). PAK4 kinase is essential for embryonic viability and for proper neuronal development. *Mol Cell Biol* **23**, 7122-33.
- Rached, M. T., Kode, A., Silva, B. C., Jung, D. Y., Gray, S., Ong, H., Paik, J. H., DePinho, R. A., Kim, J. K., Karsenty, G. et al. (2010). FoxO1 expression in osteoblasts regulates glucose homeostasis through regulation of osteocalcin in mice. *J Clin Invest* **120**, 357-68.
- Rane, S. G., Dubus, P., Mettus, R. V., Galbreath, E. J., Boden, G., Reddy, E. P. and Barbacid, M. (1999). Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. *Nat Genet* **22**, 44-52.
- Ravassard, P., Hazhouz, Y., Pechberty, S., Bricout-Neveu, E., Armanet, M., Czernichow, P. and Scharfmann, R. (2011). A genetically engineered human pancreatic beta cell line exhibiting glucose-inducible insulin secretion. *J Clin Invest* **121**, 3589-97.
- Razavi, R., Chan, Y., Afifiyan, F. N., Liu, X. J., Wan, X., Yantha, J., Tsui, H., Tang, L., Tsai, S., Santamaria, P. et al. (2006). TRPV1+ sensory neurons control beta cell stress and islet inflammation in autoimmune diabetes. *Cell* **127**, 1123-35.
- Regala, R. P., Weems, C., Jamieson, L., Khor, A., Edell, E. S., Lohse, C. M. and Fields, A. P. (2005). Atypical protein kinase C iota is an oncogene in human non-small cell lung cancer. *Cancer Res* **65**, 8905-11.
- Reith, W., Herrero-Sanchez, C., Kobr, M., Silacci, P., Berte, C., Barras, E., Fey, S. and Mach, B. (1990). MHC class II regulatory factor RFX has a novel DNA-binding domain and a functionally independent dimerization domain. *Genes Dev* **4**, 1528-40.
- Reith, W. and Mach, B. (2001). The bare lymphocyte syndrome and the regulation of MHC expression. *Annu Rev Immunol* **19**, 331-73.

- Reith, W., Ucla, C., Barras, E., Gaud, A., Durand, B., Herrero-Sanchez, C., Kobr, M. and Mach, B.** (1994). RFX1, a transactivator of hepatitis B virus enhancer I, belongs to a novel family of homodimeric and heterodimeric DNA-binding proteins. *Mol Cell Biol* **14**, 1230-44.
- Rex, C. S., Chen, L. Y., Sharma, A., Liu, J., Babayan, A. H., Gall, C. M. and Lynch, G.** (2009). Different Rho GTPase-dependent signaling pathways initiate sequential steps in the consolidation of long-term potentiation. *J Cell Biol* **186**, 85-97.
- Rezania, A., Bruin, J. E., Riedel, M. J., Mojibian, M., Asadi, A., Xu, J., Gauvin, R., Narayan, K., Karanu, F., O'Neil, J. J. et al.** (2012). Maturation of Human Embryonic Stem Cell-Derived Pancreatic Progenitors into Functional Islets Capable of Treating Pre-existing Diabetes in Mice. *Diabetes*.
- Riccio, O., van Gijn, M. E., Bezdek, A. C., Pellegrinet, L., van Es, J. H., Zimmer-Strobl, U., Strobl, L. J., Honjo, T., Clevers, H. and Radtke, F.** (2008). Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27Kip1 and p57Kip2. *EMBO Rep* **9**, 377-83.
- Rieck, S. and Kaestner, K. H.** (2010). Expansion of beta-cell mass in response to pregnancy. *Trends Endocrinol Metab* **21**, 151-8.
- Rieck, S., White, P., Schug, J., Fox, A. J., Smirnova, O., Gao, N., Gupta, R. K., Wang, Z. V., Scherer, P. E., Keller, M. P. et al.** (2009). The transcriptional response of the islet to pregnancy in mice. *Mol Endocrinol* **23**, 1702-12.
- Rindi, G., Leiter, A. B., Kopin, A. S., Bordi, C. and Solcia, E.** (2004). The "normal" endocrine cell of the gut: changing concepts and new evidences. *Ann N Y Acad Sci* **1014**, 1-12.
- Rossi, J. M., Dunn, N. R., Hogan, B. L. and Zaret, K. S.** (2001). Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes Dev* **15**, 1998-2009.
- Rudel, T. and Bokoch, G. M.** (1997). Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. *Science* **276**, 1571-4.
- Sachdeva, M. M. and Stoffers, D. A.** (2009). Minireview: Meeting the demand for insulin: molecular mechanisms of adaptive postnatal beta-cell mass expansion. *Mol Endocrinol* **23**, 747-58.
- Sand, F. W., Hornblad, A., Johansson, J. K., Loren, C., Edsbacke, J., Stahlberg, A., Magenheimer, J., Ilovich, O., Mishani, E., Dor, Y. et al.** (2011). Growth-limiting role of endothelial cells in endoderm development. *Dev Biol* **352**, 267-77.
- Sander, M., Neubuser, A., Kalamaras, J., Ee, H. C., Martin, G. R. and German, M. S.** (1997). Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. *Genes Dev* **11**, 1662-73.
- Sander, M., Paydar, S., Ericson, J., Briscoe, J., Berber, E., German, M., Jessell, T. M. and Rubenstein, J. L.** (2000). Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates. *Genes Dev* **14**, 2134-9.
- Sangiorgi, E. and Capecchi, M. R.** (2008). Bmi1 is expressed in vivo in intestinal stem cells. *Nat Genet* **40**, 915-20.
- Sansom, O. J., Reed, K. R., Hayes, A. J., Ireland, H., Brinkmann, H., Newton, I. P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I. S. et al.** (2004). Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. *Genes Dev* **18**, 1385-90.
- Sato, T., Vries, R. G., Snippert, H. J., van de Wetering, M., Barker, N., Stange, D. E., van Es, J. H., Abo, A., Kujala, P., Peters, P. J. et al.** (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262-5.



- Schaffer, A. E., Freude, K. K., Nelson, S. B. and Sander, M.** (2010). Nkx6 transcription factors and Ptf1a function as antagonistic lineage determinants in multipotent pancreatic progenitors. *Dev Cell* **18**, 1022-9.
- Schonhoff, S. E., Giel-Moloney, M. and Leiter, A. B.** (2004). Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. *Dev Biol* **270**, 443-54.
- Schubert, M. L.** (2003). Gastric secretion. *Curr Opin Gastroenterol* **19**, 519-25.
- Schurmann, A., Mooney, A. F., Sanders, L. C., Sells, M. A., Wang, H. G., Reed, J. C. and Bokoch, G. M.** (2000). p21-activated kinase 1 phosphorylates the death agonist bad and protects cells from apoptosis. *Mol Cell Biol* **20**, 453-61.
- Schwitzgebel, V. M., Scheel, D. W., Conners, J. R., Kalamaras, J., Lee, J. E., Anderson, D. J., Sussel, L., Johnson, J. D. and German, M. S.** (2000). Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development* **127**, 3533-42.
- Scoville, D. H., Sato, T., He, X. C. and Li, L.** (2008). Current view: intestinal stem cells and signaling. *Gastroenterology* **134**, 849-64.
- Scrocchi, L. A., Brown, T. J., McClusky, N., Brubaker, P. L., Auerbach, A. B., Joyner, A. L. and Drucker, D. J.** (1996). Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. *Nat Med* **2**, 1254-8.
- Sells, M. A., Knaus, U. G., Bagrodia, S., Ambrose, D. M., Bokoch, G. M. and Chernoff, J.** (1997). Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Curr Biol* **7**, 202-10.
- Sells, M. A., Pfaff, A. and Chernoff, J.** (2000). Temporal and spatial distribution of activated Pak1 in fibroblasts. *J Cell Biol* **151**, 1449-58.
- Sengupta, P. K., Fargo, J. and Smith, B. D.** (2002). The RFX family interacts at the collagen (COL1A2) start site and represses transcription. *J Biol Chem* **277**, 24926-37.
- Seymour, P. A., Freude, K. K., Tran, M. N., Mayes, E. E., Jensen, J., Kist, R., Scherer, G. and Sander, M.** (2007). SOX9 is required for maintenance of the pancreatic progenitor cell pool. *Proc Natl Acad Sci U S A* **104**, 1865-70.
- Seymour, P. A. and Sander, M.** (2011). Historical perspective: beginnings of the beta-cell: current perspectives in beta-cell development. *Diabetes* **60**, 364-76.
- Shamah, S. M., Lin, M. Z., Goldberg, J. L., Estrach, S., Sahin, M., Hu, L., Bazalakova, M., Neve, R. L., Corfas, G., Debant, A. et al.** (2001). EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. *Cell* **105**, 233-44.
- Shapiro, A. M., Lakey, J. R., Ryan, E. A., Korbitt, G. S., Toth, E., Warnock, G. L., Kneteman, N. M. and Rajotte, R. V.** (2000). Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* **343**, 230-8.
- Shapiro, A. M., Ricordi, C., Hering, B. J., Auchincloss, H., Lindblad, R., Robertson, R. P., Secchi, A., Brendel, M. D., Berney, T., Brennan, D. C. et al.** (2006). International trial of the Edmonton protocol for islet transplantation. *N Engl J Med* **355**, 1318-30.
- Shih, D. Q., Navas, M. A., Kuwajima, S., Duncan, S. A. and Stoffel, M.** (1999). Impaired glucose homeostasis and neonatal mortality in hepatocyte nuclear factor 3alpha-deficient mice. *Proc Natl Acad Sci U S A* **96**, 10152-7.

- Shih, H. P., Kopp, J. L., Sandhu, M., Dubois, C. L., Seymour, P. A., Grapin-Botton, A. and Sander, M.** (2012). A Notch-dependent molecular circuitry initiates pancreatic endocrine and ductal cell differentiation. *Development* **139**, 2488-99.
- Shroyer, N. F., Helmrath, M. A., Wang, V. Y., Antalffy, B., Henning, S. J. and Zoghbi, H. Y.** (2007). Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis. *Gastroenterology* **132**, 2478-88.
- Shroyer, N. F., Wallis, D., Venken, K. J., Bellen, H. J. and Zoghbi, H. Y.** (2005). Gfi1 functions downstream of Math1 to control intestinal secretory cell subtype allocation and differentiation. *Genes Dev* **19**, 2412-7.
- Siegrist, C. A., Durand, B., Emery, P., David, E., Hearing, P., Mach, B. and Reith, W.** (1993). RFX1 is identical to enhancer factor C and functions as a transactivator of the hepatitis B virus enhancer. *Mol Cell Biol* **13**, 6375-84.
- Smith, S. B., Qu, H. Q., Taleb, N., Kishimoto, N. Y., Scheel, D. W., Lu, Y., Patch, A. M., Grabs, R., Wang, J., Lynn, F. C. et al.** (2010). Rfx6 directs islet formation and insulin production in mice and humans. *Nature* **463**, 775-80.
- Snippert, H. J., van Es, J. H., van den Born, M., Begthel, H., Stange, D. E., Barker, N. and Clevers, H.** (2009). Prominin-1/CD133 marks stem cells and early progenitors in mouse small intestine. *Gastroenterology* **136**, 2187-2194 e1.
- Solar, M., Cardalda, C., Houbracken, I., Martin, M., Maestro, M. A., De Medts, N., Xu, X., Grau, V., Heimberg, H., Bouwens, L. et al.** (2009). Pancreatic exocrine duct cells give rise to insulin-producing beta cells during embryogenesis but not after birth. *Dev Cell* **17**, 849-60.
- Sosa-Pineda, B., Chowdhury, K., Torres, M., Oliver, G. and Gruss, P.** (1997). The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. *Nature* **386**, 399-402.
- Souopgui, J., Solter, M. and Pieler, T.** (2002). XPak3 promotes cell cycle withdrawal during primary neurogenesis in *Xenopus laevis*. *EMBO J* **21**, 6429-39.
- Soyer, J., Flasse, L., Raffelsberger, W., Beucher, A., Orvain, C., Peers, B., Ravassard, P., Vermot, J., Voz, M. L., Mellitzer, G. et al.** (2010). Rfx6 is an Ngn3-dependent winged helix transcription factor required for pancreatic islet cell development. *Development* **137**, 203-12.
- Spence, J. R., Lange, A. W., Lin, S. C., Kaestner, K. H., Lowy, A. M., Kim, I., Whitsett, J. A. and Wells, J. M.** (2009). Sox17 regulates organ lineage segregation of ventral foregut progenitor cells. *Dev Cell* **17**, 62-74.
- Spiegel, R., Dobbie, A., Hartman, C., de Vries, L., Ellard, S. and Shalev, S. A.** (2011). Clinical characterization of a newly described neonatal diabetes syndrome caused by RFX6 mutations. *Am J Med Genet A* **155A**, 2821-5.
- St-Onge, L., Sosa-Pineda, B., Chowdhury, K., Mansouri, A. and Gruss, P.** (1997). Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. *Nature* **387**, 406-9.
- Stanger, B. Z., Datar, R., Murtaugh, L. C. and Melton, D. A.** (2005). Direct regulation of intestinal fate by Notch. *Proc Natl Acad Sci U S A* **102**, 12443-8.
- Stanger, B. Z. and Dor, Y.** (2006). Dissecting the cellular origins of pancreatic cancer. *Cell Cycle* **5**, 43-6.
- Stappenbeck, T. S.** (2009). Paneth cell development, differentiation, and function: new molecular cues. *Gastroenterology* **137**, 30-3.

- Stoffers, D. A., Thomas, M. K. and Habener, J. F.** (1997). Homeodomain protein IDX-1: a master regulator of pancreas development and insulin gene expression. *Trends Endocrinol Metab* **8**, 145-51.
- Suarez-Pinzon, W. L., Yan, Y., Power, R., Brand, S. J. and Rabinovitch, A.** (2005). Combination therapy with epidermal growth factor and gastrin increases beta-cell mass and reverses hyperglycemia in diabetic NOD mice. *Diabetes* **54**, 2596-601.
- Sumara, G., Formentini, I., Collins, S., Sumara, I., Windak, R., Bodenmiller, B., Ramracheya, R., Caille, D., Jiang, H., Platt, K. A. et al.** (2009). Regulation of PKD by the MAPK p38delta in insulin secretion and glucose homeostasis. *Cell* **136**, 235-48.
- Sund, N. J., Vatamaniuk, M. Z., Casey, M., Ang, S. L., Magnuson, M. A., Stoffers, D. A., Matschinsky, F. M. and Kaestner, K. H.** (2001). Tissue-specific deletion of Foxa2 in pancreatic beta cells results in hyperinsulinemic hypoglycemia. *Genes Dev* **15**, 1706-15.
- Sussel, L., Kalamaras, J., Hartigan-O'Connor, D. J., Meneses, J. J., Pedersen, R. A., Rubenstein, J. L. and German, M. S.** (1998). Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. *Development* **125**, 2213-21.
- Svensson, P., Bergqvist, I., Norlin, S. and Edlund, H.** (2009). MFng is dispensable for mouse pancreas development and function. *Mol Cell Biol* **29**, 2129-38.
- Swoboda, P., Adler, H. T. and Thomas, J. H.** (2000). The RFX-type transcription factor DAF-19 regulates sensory neuron cilium formation in *C. elegans*. *Mol Cell* **5**, 411-21.
- Talchai, C., Xuan, S., Kitamura, T., DePinho, R. A. and Accili, D.** (2012). Generation of functional insulin-producing cells in the gut by Foxo1 ablation. *Nat Genet* **44**, 406-12, S1.
- Tamplin, O. J., Cox, B. J. and Rossant, J.** (2011). Integrated microarray and ChIP analysis identifies multiple Foxa2 dependent target genes in the notochord. *Dev Biol* **360**, 415-25.
- Tang, Y., Chen, Z., Ambrose, D., Liu, J., Gibbs, J. B., Chernoff, J. and Field, J.** (1997). Kinase-deficient Pak1 mutants inhibit Ras transformation of Rat-1 fibroblasts. *Mol Cell Biol* **17**, 4454-64.
- Tateishi, K., He, J., Taranova, O., Liang, G., D'Alessio, A. C. and Zhang, Y.** (2008). Generation of insulin-secreting islet-like clusters from human skin fibroblasts. *J Biol Chem* **283**, 31601-7.
- Taupin, D. R., Kinoshita, K. and Podolsky, D. K.** (2000). Intestinal trefoil factor confers colonic epithelial resistance to apoptosis. *Proc Natl Acad Sci U S A* **97**, 799-804.
- Teitelman, G., Alpert, S., Polak, J. M., Martinez, A. and Hanahan, D.** (1993). Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. *Development* **118**, 1031-9.
- Teta, M., Long, S. Y., Wartschow, L. M., Rankin, M. M. and Kushner, J. A.** (2005). Very slow turnover of beta-cells in aged adult mice. *Diabetes* **54**, 2557-67.
- Thevenot, E., Moreau, A. W., Rousseau, V., Combeau, G., Domenichini, F., Jacquet, C., Goupille, O., Amar, M., Kreis, P., Fossier, P. et al.** (2011). p21-Activated kinase 3 (PAK3) protein regulates synaptic transmission through its interaction with the Nck2/Grb4 protein adaptor. *J Biol Chem* **286**, 40044-59.
- Thompson, N., Gesina, E., Scheinert, P., Bucher, P. and Grapin-Botton, A.** (2012). RNA profiling and chromatin immunoprecipitation-sequencing reveal that PTF1a stabilizes pancreas progenitor identity via the control of MNX1/HLXB9 and a network of other transcription factors. *Mol Cell Biol* **32**, 1189-99.
- Thorel, F., Nepote, V., Avril, I., Kohno, K., Desgraz, R., Chera, S. and Herrera, P. L.** (2010). Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. *Nature* **464**, 1149-54.



- Thullberg, M., Gad, A., Beeser, A., Chernoff, J. and Stromblad, S.** (2007). The kinase-inhibitory domain of p21-activated kinase 1 (PAK1) inhibits cell cycle progression independent of PAK1 kinase activity. *Oncogene* **26**, 1820-8.
- Tu, H. and Wigler, M.** (1999). Genetic evidence for Pak1 autoinhibition and its release by Cdc42. *Mol Cell Biol* **19**, 602-11.
- Vadlamudi, R. K., Barnes, C. J., Rayala, S., Li, F., Balasenthil, S., Marcus, S., Goodson, H. V., Sahin, A. A. and Kumar, R.** (2005). p21-activated kinase 1 regulates microtubule dynamics by phosphorylating tubulin cofactor B. *Mol Cell Biol* **25**, 3726-36.
- Vadlamudi, R. K., Li, F., Adam, L., Nguyen, D., Ohta, Y., Stossel, T. P. and Kumar, R.** (2002). Filamin is essential in actin cytoskeletal assembly mediated by p21-activated kinase 1. *Nat Cell Biol* **4**, 681-90.
- van der Flier, L. G., Haegerbarth, A., Stange, D. E., van de Wetering, M. and Clevers, H.** (2009a). OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. *Gastroenterology* **137**, 15-7.
- van der Flier, L. G., van Gijn, M. E., Hatzis, P., Kujala, P., Haegerbarth, A., Stange, D. E., Begthel, H., van den Born, M., Guryev, V., Oving, I. et al.** (2009b). Transcription factor achaete scute-like 2 controls intestinal stem cell fate. *Cell* **136**, 903-12.
- van Es, J. H., Jay, P., Gregorieff, A., van Gijn, M. E., Jonkheer, S., Hatzis, P., Thiele, A., van den Born, M., Begthel, H., Brabletz, T. et al.** (2005a). Wnt signalling induces maturation of Paneth cells in intestinal crypts. *Nat Cell Biol* **7**, 381-6.
- van Es, J. H., van Gijn, M. E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D. J., Radtke, F. et al.** (2005b). Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435**, 959-63.
- Van Hoof, D., D'Amour, K. A. and German, M. S.** (2009). Derivation of insulin-producing cells from human embryonic stem cells. *Stem Cell Res* **3**, 73-87.
- VanDussen, K. L. and Samuelson, L. C.** (2010). Mouse atonal homolog 1 directs intestinal progenitors to secretory cell rather than absorptive cell fate. *Dev Biol* **346**, 215-23.
- Vantyghem, M. C., Kerr-Conte, J., Arnalsteen, L., Sergent, G., Defrance, F., Gmyr, V., Declerck, N., Raverdy, V., Vandewalle, B., Pigny, P. et al.** (2009). Primary graft function, metabolic control, and graft survival after islet transplantation. *Diabetes Care* **32**, 1473-8.
- Vasavada, R. C., Gonzalez-Pertusa, J. A., Fujinaka, Y., Fiaschi-Taesch, N., Cozar-Castellano, I. and Garcia-Ocana, A.** (2006). Growth factors and beta cell replication. *Int J Biochem Cell Biol* **38**, 931-50.
- Vikis, H. G., Li, W. and Guan, K. L.** (2002). The plexin-B1/Rac interaction inhibits PAK activation and enhances Sema4D ligand binding. *Genes Dev* **16**, 836-45.
- Villasenor, A., Chong, D. C., Henkemeyer, M. and Cleaver, O.** (2010). Epithelial dynamics of pancreatic branching morphogenesis. *Development* **137**, 4295-305.
- Wandzioch, E. and Zaret, K. S.** (2009). Dynamic signaling network for the specification of embryonic pancreas and liver progenitors. *Science* **324**, 1707-10.
- Wang, J., Cortina, G., Wu, S. V., Tran, R., Cho, J. H., Tsai, M. J., Bailey, T. J., Jamrich, M., Ament, M. E., Treem, W. R. et al.** (2006). Mutant neurogenin-3 in congenital malabsorptive diarrhea. *N Engl J Med* **355**, 270-80.
- Wang, Q., Elghazi, L., Martin, S., Martins, I., Srinivasan, R. S., Geng, X., Sleeman, M., Collombat, P., Houghton, J. and Sosa-Pineda, B.** (2008). Ghrelin is a novel target of Pax4 in endocrine progenitors of the pancreas and duodenum. *Dev Dyn* **237**, 51-61.

- Wang, S., Jensen, J. N., Seymour, P. A., Hsu, W., Dor, Y., Sander, M., Magnuson, M. A., Serup, P. and Gu, G.** (2009a). Sustained Neurog3 expression in hormone-expressing islet cells is required for endocrine maturation and function. *Proc Natl Acad Sci U S A* **106**, 9715-20.
- Wang, S., Yan, J., Anderson, D. A., Xu, Y., Kanal, M. C., Cao, Z., Wright, C. V. and Gu, G.** (2009b). Neurog3 gene dosage regulates allocation of endocrine and exocrine cell fates in the developing mouse pancreas. *Dev Biol* **339**, 26-37.
- Wang, Y., Giel-Moloney, M., Rindi, G. and Leiter, A. B.** (2007a). Enteroendocrine precursors differentiate independently of Wnt and form serotonin expressing adenomas in response to active beta-catenin. *Proc Natl Acad Sci U S A* **104**, 11328-33.
- Wang, Z., Oh, E., Clapp, D. W., Chernoff, J. and Thurmond, D. C.** (2011). Inhibition or ablation of p21-activated kinase (PAK1) disrupts glucose homeostatic mechanisms in vivo. *J Biol Chem* **286**, 41359-67.
- Wang, Z., Oh, E. and Thurmond, D. C.** (2007b). Glucose-stimulated Cdc42 signaling is essential for the second phase of insulin secretion. *J Biol Chem* **282**, 9536-46.
- Wang, Z. and Thurmond, D. C.** (2009). Mechanisms of biphasic insulin-granule exocytosis - roles of the cytoskeleton, small GTPases and SNARE proteins. *J Cell Sci* **122**, 893-903.
- Wang, Z. and Thurmond, D. C.** (2010). Differential phosphorylation of RhoGDI mediates the distinct cycling of Cdc42 and Rac1 to regulate second-phase insulin secretion. *J Biol Chem* **285**, 6186-97.
- Warden, C. H., Daluiski, A., Bu, X., Purcell-Huynh, D. A., De Meester, C., Shieh, B. H., Puppione, D. L., Gray, R. M., Reaven, G. M., Chen, Y. D. et al.** (1993). Evidence for linkage of the apolipoprotein A-II locus to plasma apolipoprotein A-II and free fatty acid levels in mice and humans. *Proc Natl Acad Sci U S A* **90**, 10886-90.
- Watt, A. J., Zhao, R., Li, J. and Duncan, S. A.** (2007). Development of the mammalian liver and ventral pancreas is dependent on GATA4. *BMC Dev Biol* **7**, 37.
- Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M. and Darnell, J. E., Jr.** (1994). The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. *Cell* **78**, 575-88.
- Wells, J. M. and Melton, D. A.** (1999). Vertebrate endoderm development. *Annu Rev Cell Dev Biol* **15**, 393-410.
- Wells, J. M. and Melton, D. A.** (2000). Early mouse endoderm is patterned by soluble factors from adjacent germ layers. *Development* **127**, 1563-72.
- Wen, J. H., Chen, Y. Y., Song, S. J., Ding, J., Gao, Y., Hu, Q. K., Feng, R. P., Liu, Y. Z., Ren, G. C., Zhang, C. Y. et al.** (2009). Paired box 6 (PAX6) regulates glucose metabolism via proinsulin processing mediated by prohormone convertase 1/3 (PC1/3). *Diabetologia* **52**, 504-13.
- Whim, M. D.** (2011). Pancreatic beta cells synthesize neuropeptide Y and can rapidly release peptide co-transmitters. *PLoS One* **6**, e19478.
- Wolf, D., Witte, V., Laffert, B., Blume, K., Stromer, E., Trapp, S., d'Aloja, P., Schurmann, A. and Baur, A. S.** (2001). HIV-1 Nef associated PAK and PI3-kinases stimulate Akt-independent Bad-phosphorylation to induce anti-apoptotic signals. *Nat Med* **7**, 1217-24.
- Wolf, G., Hessabi, B., Karkour, A., Henrion, U., Dahlhaus, M., Ostmann, A., Giese, B., Fraunholz, M., Grabarczyk, P., Jack, R. et al.** (2010). The activation of the rat insulin gene II by BETA2 and PDX-1 in rat insulinoma cells is repressed by Pax6. *Mol Endocrinol* **24**, 2331-42.
- Wolfe, S. A., van Wert, J. and Grimes, S. R.** (2006). Transcription factor RFX2 is abundant in rat testis and enriched in nuclei of primary spermatocytes where it appears to be required for transcription of the testis-specific histone H1t gene. *J Cell Biochem* **99**, 735-46.

- Wolfe, S. A., Vanwert, J. M. and Grimes, S. R.** (2008). Transcription factor RFX4 binding to the testis-specific histone H1t promoter in spermatocytes may be important for regulation of H1t gene transcription during spermatogenesis. *J Cell Biochem* **105**, 61-9.
- Wolfe, S. A., Wilkerson, D. C., Prado, S. and Grimes, S. R.** (2004). Regulatory factor X2 (RFX2) binds to the H1t/TE1 promoter element and activates transcription of the testis-specific histone H1t gene. *J Cell Biochem* **91**, 375-83.
- Xiao, G. H., Beeser, A., Chernoff, J. and Testa, J. R.** (2002). p21-activated kinase links Rac/Cdc42 signaling to merlin. *J Biol Chem* **277**, 883-6.
- Xu, X., D'Hoker, J., Stange, G., Bonne, S., De Leu, N., Xiao, X., Van de Casteele, M., Mellitzer, G., Ling, Z., Pipeleers, D. et al.** (2008). Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. *Cell* **132**, 197-207.
- Xu, Y., Sengupta, P. K., Seto, E. and Smith, B. D.** (2006a). Regulatory factor for X-box family proteins differentially interact with histone deacetylases to repress collagen alpha2(I) gene (COL1A2) expression. *J Biol Chem* **281**, 9260-70.
- Xu, Y., Wang, S., Zhang, J., Zhao, A., Stanger, B. Z. and Gu, G.** (2006b). The fringe molecules induce endocrine differentiation in embryonic endoderm by activating cMyt1/cMyt3. *Dev Biol* **297**, 340-9.
- Xue, Y. and Fleet, J. C.** (2009). Intestinal vitamin D receptor is required for normal calcium and bone metabolism in mice. *Gastroenterology* **136**, 1317-27, e1-2.
- Yang, J., Zhang, W., Jiang, W., Sun, X., Han, Y., Ding, M., Shi, Y. and Deng, H.** (2009). P21cip-overexpression in the mouse beta cells leads to the improved recovery from streptozotocin-induced diabetes. *PLoS One* **4**, e8344.
- Yang, Q., Bermingham, N. A., Finegold, M. J. and Zoghbi, H. Y.** (2001). Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. *Science* **294**, 2155-8.
- Yang, Y. P., Thorel, F., Boyer, D. F., Herrera, P. L. and Wright, C. V.** (2011). Context-specific alpha- to-beta-cell reprogramming by forced Pdx1 expression. *Genes Dev* **25**, 1680-5.
- Yang, Z., Rayala, S., Nguyen, D., Vadlamudi, R. K., Chen, S. and Kumar, R.** (2005). Pak1 phosphorylation of snail, a master regulator of epithelial-to-mesenchyme transition, modulates snail's subcellular localization and functions. *Cancer Res* **65**, 3179-84.
- Ye, D. Z. and Kaestner, K. H.** (2009). Foxa1 and Foxa2 control the differentiation of goblet and enteroendocrine L- and D-cells in mice. *Gastroenterology* **137**, 2052-62.
- Yehoor, V., Liu, V., Espiritu, C., Paul, A., Oka, K., Kojima, H. and Chan, L.** (2009). Neurogenin3 is sufficient for transdetermination of hepatic progenitor cells into neo-islets in vivo but not transdifferentiation of hepatocytes. *Dev Cell* **16**, 358-73.
- Yoshida, S., Takakura, A., Ohbo, K., Abe, K., Wakabayashi, J., Yamamoto, M., Suda, T. and Nabeshima, Y.** (2004). Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. *Dev Biol* **269**, 447-58.
- Yoshitomi, H. and Zaret, K. S.** (2004). Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor Ptf1a. *Development* **131**, 807-17.
- Zarbalis, K., May, S. R., Shen, Y., Ekker, M., Rubenstein, J. L. and Peterson, A. S.** (2004). A focused and efficient genetic screening strategy in the mouse: identification of mutations that disrupt cortical development. *PLoS Biol* **2**, E219.
- Zaret, K. S.** (2006). Pancreatic beta cells: responding to the matrix. *Cell Metab* **3**, 148-50.
- Zaret, K. S.** (2008). Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. *Nat Rev Genet* **9**, 329-40.

- Zaret, K. S. and Grompe, M.** (2008). Generation and regeneration of cells of the liver and pancreas. *Science* **322**, 1490-4.
- Zecchini, V., Domaschenz, R., Winton, D. and Jones, P.** (2005). Notch signaling regulates the differentiation of post-mitotic intestinal epithelial cells. *Genes Dev* **19**, 1686-91.
- Zeitz, U., Weber, K., Soegiarto, D. W., Wolf, E., Balling, R. and Erben, R. G.** (2003). Impaired insulin secretory capacity in mice lacking a functional vitamin D receptor. *FASEB J* **17**, 509-11.
- Zhang, C., Moriguchi, T., Kajihara, M., Esaki, R., Harada, A., Shimohata, H., Oishi, H., Hamada, M., Morito, N., Hasegawa, K. et al.** (2005a). MafA is a key regulator of glucose-stimulated insulin secretion. *Mol Cell Biol* **25**, 4969-76.
- Zhang, D., Zeldin, D. C. and Blackshear, P. J.** (2007). Regulatory factor X4 variant 3: a transcription factor involved in brain development and disease. *J Neurosci Res* **85**, 3515-22.
- Zhang, H., Ackermann, A. M., Gusarova, G. A., Lowe, D., Feng, X., Kopsombut, U. G., Costa, R. H. and Gannon, M.** (2006). The FoxM1 transcription factor is required to maintain pancreatic beta-cell mass. *Mol Endocrinol* **20**, 1853-66.
- Zhang, H., Webb, D. J., Asmussen, H., Niu, S. and Horwitz, A. F.** (2005b). A GIT1/PIX/Rac/PAK signaling module regulates spine morphogenesis and synapse formation through MLC. *J Neurosci* **25**, 3379-88.
- Zhang, H., Zhang, J., Pope, C. F., Crawford, L. A., Vasavada, R. C., Jagasia, S. M. and Gannon, M.** (2010). Gestational diabetes mellitus resulting from impaired beta-cell compensation in the absence of FoxM1, a novel downstream effector of placental lactogen. *Diabetes* **59**, 143-52.
- Zhao, L., Guo, M., Matsuoka, T. A., Hagman, D. K., Parazzoli, S. D., Poitout, V. and Stein, R.** (2005). The islet beta cell-enriched MafA activator is a key regulator of insulin gene transcription. *J Biol Chem* **280**, 11887-94.
- Zhao, L., Ma, Q. L., Calon, F., Harris-White, M. E., Yang, F., Lim, G. P., Morihara, T., Ubeda, O. J., Ambegaokar, S., Hansen, J. E. et al.** (2006). Role of p21-activated kinase pathway defects in the cognitive deficits of Alzheimer disease. *Nat Neurosci* **9**, 234-42.
- Zhao, Z. S., Manser, E., Chen, X. Q., Chong, C., Leung, T. and Lim, L.** (1998). A conserved negative regulatory region in alphaPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1. *Mol Cell Biol* **18**, 2153-63.
- Zhong, L., Georgia, S., Tschen, S. I., Nakayama, K. and Bhushan, A.** (2007). Essential role of Skp2-mediated p27 degradation in growth and adaptive expansion of pancreatic beta cells. *J Clin Invest* **117**, 2869-76.
- Zhou, Q., Brown, J., Kanarek, A., Rajagopal, J. and Melton, D. A.** (2008). In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature* **455**, 627-32.
- Zhou, Q., Law, A. C., Rajagopal, J., Anderson, W. J., Gray, P. A. and Melton, D. A.** (2007). A multipotent progenitor domain guides pancreatic organogenesis. *Dev Cell* **13**, 103-14.
- Zhu, G., Wang, Y., Huang, B., Liang, J., Ding, Y., Xu, A. and Wu, W.** (2011). A Rac1/PAK1 cascade controls beta-catenin activation in colon cancer cells. *Oncogene* **31**, 1001-12.
- Zipris, D.** (2009). Epidemiology of type 1 diabetes and what animal models teach us about the role of viruses in disease mechanisms. *Clin Immunol* **131**, 11-23.

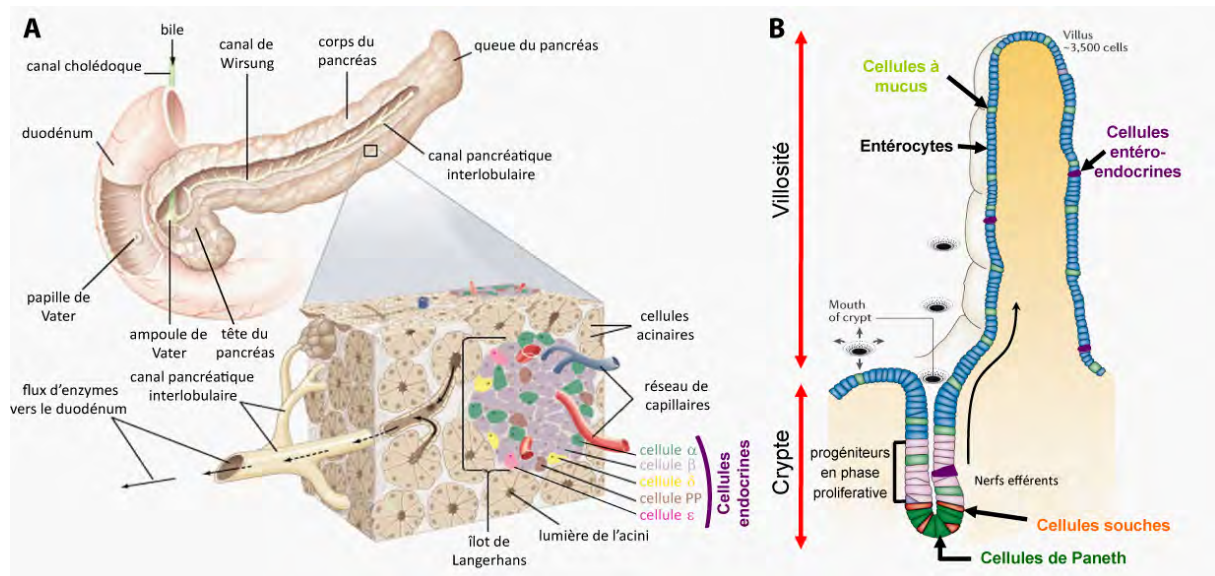


## V. Annexes

### A. Résumé en français

#### 1. Introduction

Le pancréas est une glande mixte composée de deux compartiments : le pancréas exocrine et le pancréas endocrine qui ont des fonctions très différentes (Figure 47A). Le tissu exocrine est composé de cellules canalaire et acinaires, ces dernières produisent et sécrètent des enzymes digestives qui sont transportées via un système de canaux dans la lumière de l'intestin où elles participent à l'absorption des nutriments. Le tissu endocrine, représentant moins de 1% de la masse totale du pancréas, est composé de cinq types cellulaires qui, durant l'embryogénèse et juste après la naissance se regroupent en unités fonctionnelles appelées îlots de Langerhans. Les îlots de Langerhans sont composés de cellules alpha, bêta, delta, PP et epsilon produisant respectivement les hormones glucagon, insuline, somatostatine, polypeptide pancréatique et ghréline.



**Figure 47 : Organisation du pancréas et de l'intestin chez l'adulte. (A)** Le pancréas adulte est abouché au duodénum par la papille de Vater. Le pancréas exocrine est composé de canaux pancréatiques et d'acini. Ces derniers produisent et sécrètent des enzymes digestives qui sont transportées jusqu'au duodénum par un système de canaux. Le pancréas endocrine est composé d'îlots de Langerhans qui sont disséminés dans le pancréas exocrine. Les îlots sont constitués de cinq types cellulaires : les cellules alpha, bêta, delta, PP et epsilon sécrétant respectivement l'insuline, le glucagon, la somatostatine, le polypeptide pancréatique et la ghréline. (Adaptée de l'Encyclopédie Britannica) **(B)** L'intestin grêle est composé de villosités et de cryptes. Les cellules souches se trouvent à la base des cryptes avec les cellules de Paneth. Au-dessus des cellules souches se trouvent les progéniteurs en phase proliférative et plus haut, le long des villosités, les cellules post-mitotiques différenciées suivantes : entérocytes (cellules absorbatives), cellules à mucus et les cellules entéroendocrines qui sont disséminées dans l'épithélium. (Adaptée de Crosnier et al., 2006)



Ces hormones sont sécrétées dans le sang où elles participent au contrôle de l'homéostasie du glucose. Une perte ou dysfonctionnement des cellules bêta provoque le diabète, maladie actuellement incurable. Depuis quelques années, la greffe d'îlots de Langerhans est proposée à certains patients diabétiques de type 1 (dont les cellules bêta sont détruites par un processus auto-immun) qui n'arrivent pas à réguler leur glycémie par injections régulières d'insuline. Si cette thérapie pouvait être généralisée, elle pourrait éviter de graves complications à long terme telles que la rétinopathie, la néphropathie ou la neuropathie. Cependant, le manque de greffons ne permet pas de faire face à la demande, et il faut donc trouver d'autres sources de cellules bêta pour envisager une thérapie cellulaire du diabète dans le futur. Une des alternatives possibles serait de générer des cellules bêta à partir de cellules souches embryonnaires humaines (hESC) ou de cellules pluripotentes induites (iPSC). Cependant, pour (re)programmer *in vitro* des cellules souches multipotentes, des iPSC ou même des cellules progénitrices en cellules bêta productrices d'insuline, nous avons besoin de comprendre en détail les cascades de signalisation et les programmes génétiques contrôlant leur différenciation.

L'intestin participe aussi à la régulation de l'homéostasie du glucose. Des cellules endocrines, très proches de celles présentes dans le pancréas, se trouvent dans l'intestin et sont appelées cellules entéroendocrines (EE). Ces cellules, rares et éparpillées dans l'épithélium intestinal, sécrètent divers peptides et hormones (Figure 47B). Plusieurs types de cellules entéroendocrines ont été identifiés et classés en fonction de leur principal produit de sécrétion : cholecystokinine (cellules CCK), sécrétine (cellules S), Glp1/Glp2/Pyy (cellules L), Gip (cellules K), somatostatine (cellules D), neurotensine (cellules N), motiline (cellules M), ghréline (cellules epsilon), gastrine (cellules G), sérotonine (cellules EC) (Li et al., 2011). Les cellules EE perçoivent les nutriments dans la lumière intestinale, sécrètent leurs peptides (ou amines) dans le sang ou activent des circuits neuronaux pour coordonner l'absorption et le stockage du glucose dans les tissus périphériques tels que le foie, les muscles squelettiques ou les tissus adipeux (Drucker, 2007). Les hormones dites incrétines, Glp1 et Gip, ont un rôle clé dans la régulation de la glycémie car elles stimulent la sécrétion d'insuline des cellules bêta pancréatiques en réponse au glucose. Ainsi, comprendre comment ces cellules EE sont générées et ce qui les différencie de leurs analogues pancréatiques (Figure 48) est aussi important pour produire des cellules bêta de substitution *in vitro*.

Dans les quinze dernières années, principalement grâce à l'analyse de souris génétiquement modifiées, les chercheurs ont établi une hiérarchie de facteurs de transcription régulant l'organogénèse du pancréas et la différenciation des cellules endocrines (Figure 48). Par exemple, l'inactivation des gènes *Arx*, *Pax4*, *Pax6*, *Isl1*, *Nkx2.2*, *Nkx6.1*, *Insm1*, *NeuroD1* et *Rfx6* entraîne une diminution d'un ou plusieurs types cellulaires endocrines matures (Pan and Wright, 2011). Dans ce contexte, l'équipe du Dr. Gérard Gradwohl qui m'accueille pour mon doctorat, s'attache à disséquer les mécanismes moléculaires et cellulaires de régulation au cours du développement des cellules bêta chez la souris. Une percée majeure dans ce domaine a été l'identification et la caractérisation de la fonction du facteur de transcription à domaine bHLH *Neurogénine3* (*Ngn3*). En effet, il a été montré antérieurement dans l'équipe que les souris déficientes pour *Ngn3* n'expriment aucune hormone ni aucun des marqueurs de différenciation endocrine et meurent de diabète peu après la naissance (Gradwohl et al., 2000). Ces résultats, complétés par des études de gain de fonction réalisées par d'autres équipes suggèrent que *Ngn3* contrôle la destinée endocrine des progéniteurs pancréatiques multipotents et donne naissance à toutes les cellules endocrines.

Cependant, malgré l'importance de *Ngn3* dans l'instruction des progéniteurs multipotents vers une destinée endocrine plus restreinte, on ne connaît que peu de chose sur les gènes activés par *Ngn3* et leur implication dans les mécanismes contrôlant la spécification et la maturation des sous-types cellulaires endocrines. Malgré de nouvelles avancées importantes dans l'élucidation des programmes qui contrôlent la différenciation des cellules endocrines en aval de *Ngn3*, beaucoup de questions restent sans réponse. Quels mécanismes intègrent et coordonnent la sortie du cycle cellulaire ? Quels sont les signaux qui déclenchent la délamination et la migration des cellules endocrines ? Comment est intégré le choix du sous-type cellulaire endocrine ? Comment une cellule endocrine complète sa maturation et devient fonctionnelle ? Pour comprendre tout ceci un peu mieux, l'équipe a réalisé un criblage par puce à ADN des gènes exprimés différemment dans les progéniteurs endocrines *Ngn3*<sup>+</sup> par rapport aux cellules *Ngn3*<sup>-</sup> dans le pancréas embryonnaire sauvage à E15.5. Parmi ces gènes, l'équipe a déjà montré l'importance et le rôle de *Insm1* et *Rfx6* (Mellitzer et al., 2006; Soyer et al., 2010) dans la différenciation des cellules endocrines. L'objectif de ma thèse a été de continuer l'exploitation de ces résultats en étudiant l'expression et/ou le rôle de deux autres gènes enrichis dans cette étude. J'ai donc caractériser l'expression et le rôle de la kinase sérine/thréonine *p21 protein-activated kinase 3 (Pak3)* et poursuivi l'étude de la fonction du facteur de transcription *Regulatory factor X 6 (Rfx6)* dans le pancréas et l'intestin avec des approches génétiques, cellulaires, moléculaires et transcriptomiques.

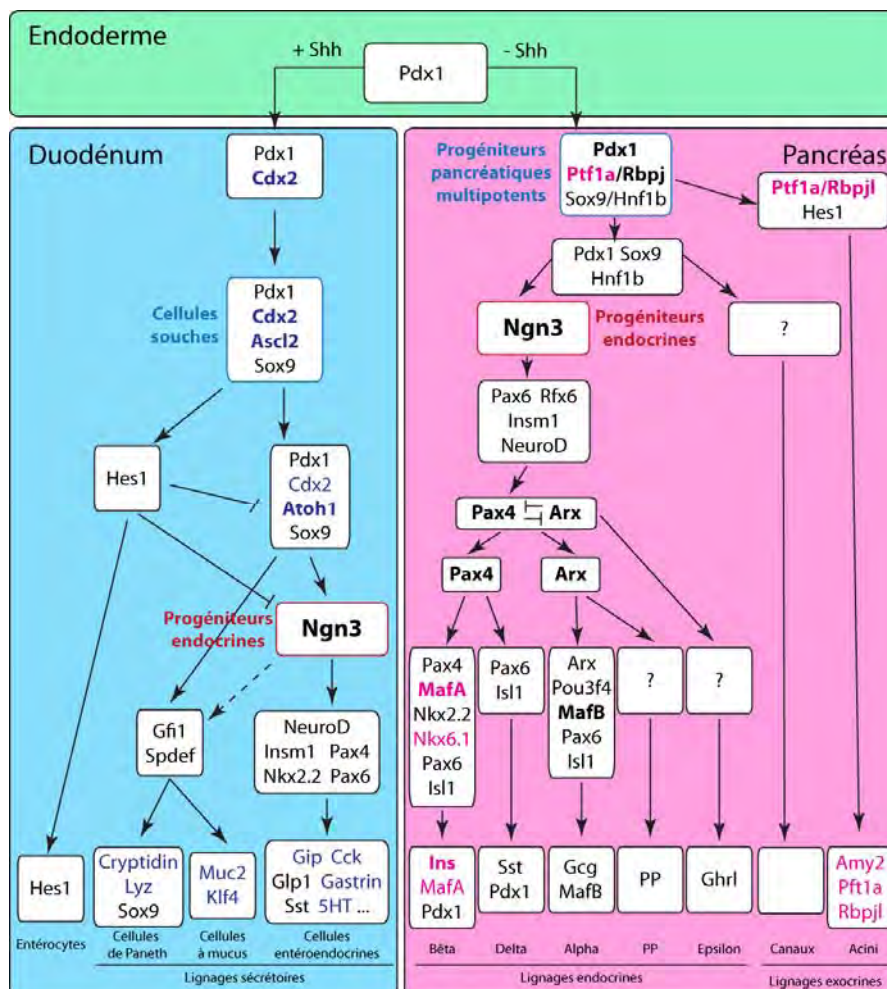


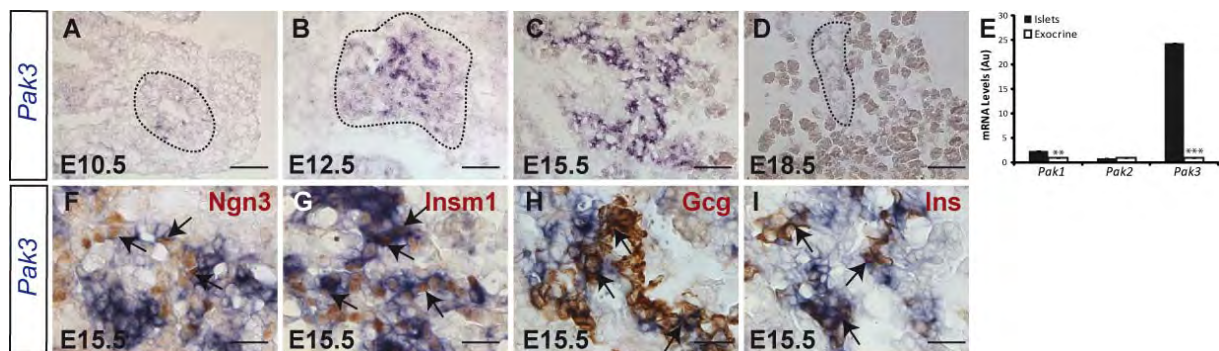
Figure 48 : Cascades de transcription régulant la spécification des différents sous-types cellulaires pancréatiques et intestinaux. En bleu, les gènes exprimés uniquement dans l'intestin, en rose, les gènes exprimés uniquement dans le pancréas. (Adaptée de A. Beucher)



## 2. Résultats

### 2.1. Expression et fonction de Pak3 dans la différenciation des cellules endocrines

Nous avons choisi d'étudier *Pak3* pour plusieurs raisons. La principale raison étant qu'il a été montré que dans le système nerveux du Xénope, *Pak3* était en aval d'un autre membre de la famille neurogénine, *Ngnr1*. En effet, des expériences de perte de fonction durant la neurogénèse du Xénope ont révélé que *XPak3* est nécessaire en aval de *Ngnr1* pour une différenciation neuronale normale (Souopgui et al., 2002). De plus, notre analyse transcriptomique a révélé un enrichissement de 75 fois de *Pak3* dans les cellules *Ngn3*<sup>+</sup> par rapport aux cellules *Ngn3*<sup>-</sup> dans le pancréas à E15.5. Notre hypothèse de départ était que similairement à son rôle dans la différenciation neuronale, *Pak3* pourrait avoir une fonction comparable au cours de la différenciation endocrine dans le pancréas de souris. *Pak3* appartient à la famille des PAK qui sont effecteurs des Rho GTPase Cdc42 et Rac1. Les membres de cette famille sont impliqués dans une grande variété de processus cellulaires tels que la migration, la réorganisation du cytosquelette et la prolifération (Bokoch, 2003). Durant ma thèse, j'ai d'abord caractérisé l'expression de *Pak3* dans le pancréas, puis tenté de comprendre son rôle dans la différenciation endocrine en utilisant des expériences de perte de fonction *ex vivo* et enfin étudié le phénotype endocrine des souris déficientes pour *Pak3*. J'ai montré par hybridation *in situ* et RT-qPCR que *Pak3* est exprimé dans le pancréas tout au long du développement, et maintenu dans les îlots adultes (Figure 49A-E). Dans les souris déficientes pour *Ngn3*, *Pak3* est perdu, suggérant un rôle endocrine en aval de *Ngn3*. Des expériences d'immunohistochimie après hybridation *in situ* montrent que *Pak3* est exprimé dans certains progéniteurs *Ngn3*<sup>+</sup>, mais surtout dans les cellules embryonnaires plus différenciées exprimant *Insm1*, l'insuline ou le glucagon (Figure 49F-I). Des expériences de tri cellulaire avec des souris *Insuline*-mRFP révèlent que *Pak3* est également exprimé dans les cellules bêta adultes (et encore plus fortement dans les autres cellules endocrines). Ces résultats suggèrent donc un rôle de *Pak3* dans le développement et la fonction des cellules bêta.



**Figure 49 : *Pak3* est exprimé dans les cellules endocrines pancréatiques au cours du développement et dans les îlots adultes.** (A-D) Hybridation *in situ* pour l'ARNm de *Pak3* (bleu) tout au long du développement pancréatique. Les lignes pointillées délimitent le domaine pancréatique. (E) RT-qPCR pour les *Paks* du groupe A sur des îlots et du tissu exocrine purifiés de souris sauvages adultes. Les données sont représentées en moyenne  $\pm$  SD sur  $n=3$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$  (F-I) Hybridation *in situ* pour l'ARNm de *Pak3* (bleu) suivie d'une immunohistochimie pour différents marqueurs endocrines (brun) sur des embryons de souris sauvages à E15.5 (F-I). Les flèches indiquent un double marquage. Echelle : 50 $\mu$ m (A-D) and 20 $\mu$ m (F-I).

Pour déterminer la fonction de Pak3 lors du développement pancréatique, j'ai réalisé une étude de perte de fonction à l'aide de morpholinos, bloquant soit la traduction, soit l'épissage de Pak3. J'ai utilisé une technique de culture d'explants pancréatiques embryonnaires permettant de récapituler la différenciation endocrine observée *in vivo* (Mellitzer et al., 2004). Les résultats montrent que l'inactivation de Pak3 entraîne une accumulation des progéniteurs Ngn3<sup>+</sup> ainsi qu'une augmentation de la prolifération des cellules endocrines, affectant ainsi leur différenciation. Ainsi, ces résultats suggèrent que Pak3 pourrait réguler le cycle cellulaire et la différenciation des cellules endocrines durant l'embryogénèse. Afin de déterminer la fonction de *Pak3 in vivo*, j'ai caractérisé le phénotype des souris déficientes pour *Pak3* (Meng et al., 2005). Des expériences d'immunofluorescence et de RT-qPCR réalisées à E13, E15 et E18 montrent que Pak3 participe à la régulation du cycle cellulaire des progéniteurs endocrines à E15 (Figure 50A-D). J'ai essentiellement pu observer (comme *ex vivo*) une augmentation de la prolifération des progéniteurs endocrines Ngn3<sup>+</sup> et une diminution des cellules sécrétrices d'insuline et de glucagon. Aucun effet de l'absence de Pak3 n'a été observé à des stades plus tardifs, suggérant que les animaux mutants peuvent restaurer la différenciation endocrine par un mécanisme compensatoire pouvant impliquer Pak2. Confirmant cette hypothèse, les souris Pak3 KO survivent et ne sont pas diabétiques dans des conditions de nutrition standard. Cependant, par une étude métabolique menée en collaboration avec la Clinique de la Souris (ICS, Illkirch), nous avons montré que lorsque ces souris sont soumises à un régime riche en graisses (HFD), elles deviennent intolérantes au glucose bien que la sensibilité et la sécrétion d'insuline ne soient pas affectées. En résumé, lorsque l'expression *Pak3* est diminuée ou perdue, les progéniteurs endocrines Ngn3<sup>+</sup> s'accumulent, continuent de proliférer et ne parviennent pas à se différencier de manière normale pendant le développement. Cependant, aucun effet de la perte de Pak3 n'a été observé à des stades plus tardifs de développement, suggérant que la différenciation est rétablie. Chez l'adulte, les souris déficientes pour *Pak3* sont intolérantes au glucose sous HFD suggérant ainsi un rôle de Pak3 dans la fonction des îlots adultes (Figure 50E-F).

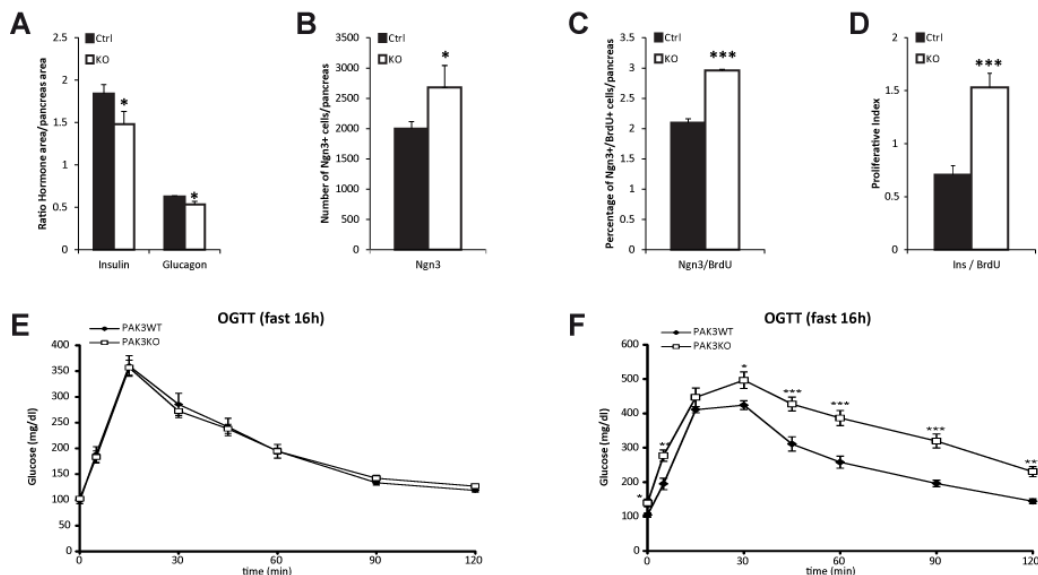


Figure 50 : **Augmentation de la prolifération des progéniteurs endocrines et des cellules bêta chez les embryons n'exprimant pas *Pak3*.** Quantification sur des pancréas (n=3) d'embryons mutants pour *Pak3* et contrôle à E15.5 de l'aire insuline+ et glucagon+ (A), du nombre de cellules Ngn3<sup>+</sup> (B), du pourcentage de progéniteurs endocrines en prolifération (BrdU<sup>+</sup>/Ngn3<sup>+</sup>) (C) et de la prolifération des cellules à insuline (D). Les données sont représentées en moyenne ± SD, \* p<0.05, \*\*\* p<0.001. **Les souris Pak3 KO sont intolérantes au glucose.** Test oral de tolérance au glucose sur des mâles Pak3 KO et WT (n=13) avec un régime normal (E) ou un régime riche en graisses (F). Les données sont représentées en moyenne ± SEM, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

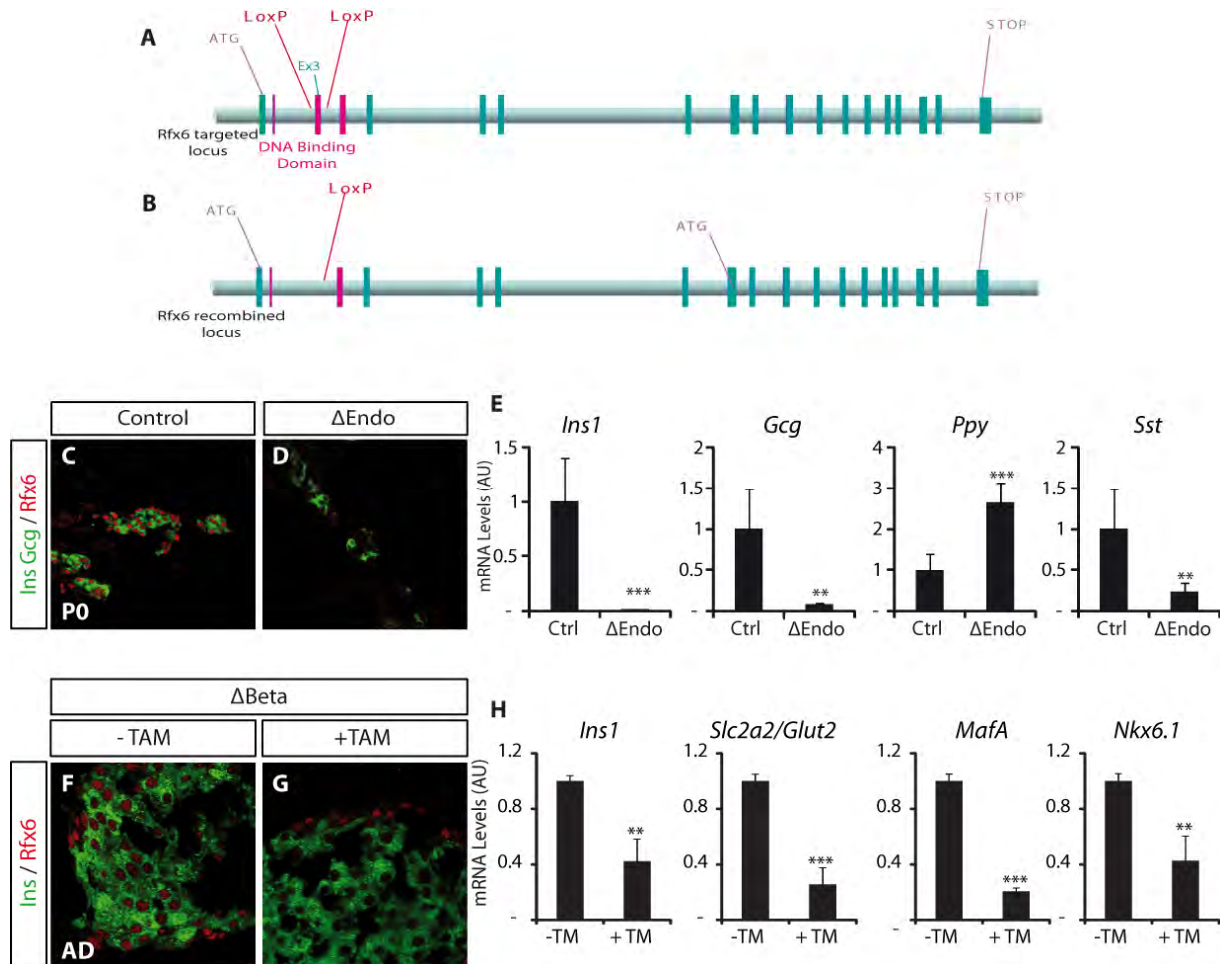
## 2.2. Fonction de Rfx6 dans les cellules endocrine pancréatiques et intestinales

J'ai en parallèle, étudié le gène *Rfx6*, un facteur de transcription à domaine « winged-helix » de la famille Regulatory Factor X. Les RFX sont connus principalement pour leur rôle dans la ciliogénèse. Cependant, un membre de cette famille, Rfx3, est aussi un régulateur de la différenciation endocrine (Ait-Lounis et al., 2007; Ait-Lounis et al., 2010). *Rfx6* a été identifié dans le laboratoire et intervient en aval de Ngn3. Il est exprimé de manière précoce dans l'endoderme intestinal primitif puis, dans le pancréas, son expression se restreint au lignage endocrine à partir de E10. Il est exprimé aussi bien dans les cellules endocrines en développement que dans les cellules endocrines matures (Soyer et al., 2010). Sa fonction endocrine a déjà été étudiée chez le poisson zèbre, où une diminution de l'expression de *zRfx6* par des morpholinos empêche la différenciation des progéniteurs endocrines, entraînant une réduction du nombre de cellules à glucagon, somatostatine et ghréline ainsi qu'une désorganisation des cellules bêta (Soyer et al., 2010). Ce gène est également important chez la souris et l'humain où il dirige la formation des îlots et la production d'insuline. Les souris déficientes pour *Rfx6* meurent à P2 et ne développent aucune cellule endocrine sécrétrice d'hormone, excepté les cellules produisant le polypeptide pancréatique (Smith et al., 2010).

De par de son profil d'expression particulier (tôt dans tout l'endoderme intestinal puis restreint au lignage endocrine) qui suggère différentes fonctions de Rfx6 au cours du développement et chez l'adulte, nous avons décidé d'étudier sa fonction avec un knockout conditionnel. Nous avons généré en collaboration avec l'ICS (Institut Clinique de la Souris, Illkirch) une souris conditionnelle pour Rfx6 en encadrant l'exon 3 du domaine de liaison à l'ADN de deux sites loxP (Figure 51A-B). Ainsi, après recombinaison avec la Cre recombinase, l'exon 3 est perdu et aucune protéine Rfx6 fonctionnelle ne peut être produite. Pour étudier la fonction de Rfx6 dans différents types cellulaires, j'ai croisé les souris KO conditionnelles avec différentes lignées Cre. CMV-Cre pour générer un KO total, Ngn3-Cre pour la fonction endocrine (pancréatique et intestinale), Ins1-CreER<sup>T2</sup> pour la fonction dans les cellules bêta adultes, Gcg-CreER<sup>T2</sup> pour la fonction dans les cellules alpha adultes, Villin-Cre pour la fonction dans l'intestin précoce et Villin-CreER<sup>T2</sup> pour la fonction dans l'intestin adulte. Les souris KO total que nous avons générées présentent grossièrement le même phénotype que les souris déficientes pour *Rfx6* publiées par Smith et al, validant notre approche. Les souris *Rfx6*<sup>fl/fl</sup> Ngn3-Cre ( $\Delta$ Endo) sont diabétiques, meurent entre P2 et P4 et montrent une perte presque totale des cellules endocrines hormis les cellules PP (Figure 51C-E). Ces résultats montrent que le phénotype endocrine de Rfx6 est dû à sa fonction en aval de Ngn3 et pas à son rôle précoce dans l'endoderme intestinal primitif. Une analyse de puce à ADN des souris *Rfx6* KO total et *Rfx6*  $\Delta$ Endo a confirmé cette observation puisque les gènes endocrines sont affectés de manière similaire dans les deux KO.

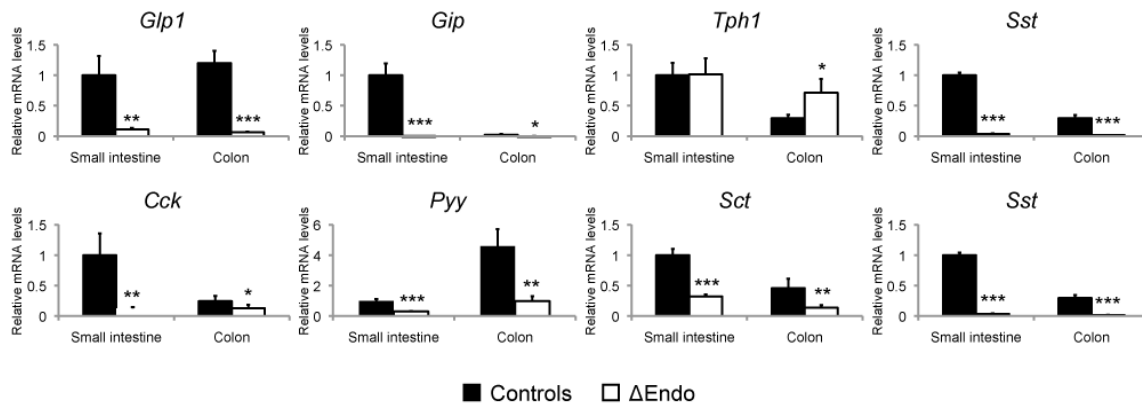
En résumé, les souris déficientes pour *Rfx6* ne développent pas de cellules sécrétrices alpha, bêta, delta et epsilon, sont diabétiques et meurent entre 2 et 3 jours après la naissance. Avec une délétion de Rfx6 spécifiquement dans le lignage endocrine, j'ai montré que c'est sa fonction en aval de Ngn3 qui est responsable de ce phénotype et pas son rôle précoce dans l'endoderme.

J'ai aussi commencé la caractérisation de la fonction de Rfx6 dans les cellules bêta adultes. Des résultats encore préliminaires montrent une diminution de 85% des transcrits *Rfx6* dans les îlots adultes purifiés de souris *Rfx6<sup>fl/fl</sup> Ins1-CreER<sup>T2</sup>* trois semaines après injection de tamoxifène suggérant une délétion efficace. Bien que les transcrits *Ins1* et *Ins2* soient diminués de plus de 60%, la protéine insuline est toujours détectée dans les cellules bêta déficientes pour *Rfx6* et ces souris ne deviennent pas diabétiques (Figure 51F-H).



**Figure 51 : Rfx6 est nécessaire durant l'embryogénèse pour la différenciation des cellules endocrines en aval de Ngn3 et chez l'adulte pour le maintien de l'identité de la cellule bêta. (A)** Locus ciblé de Rfx6. L'exon 3 dans le domaine de liaison à l'ADN (rose) est entouré de deux sites LoxP. **(B)** Locus recombinaison de Rfx6. Après recombinaison par la Cre, l'exon 3 est perdu et aucune protéine Rfx6 fonctionnelle ne peut être faite. **(C-D)** Des expériences d'immunofluorescence sur des pancréas de nouveaux-nés *Rfx6* ΔEndo et contrôles montrent que la délétion de Rfx6 (rouge) est efficace et que l'insuline et le glucagon (vert) sont fortement diminués en l'absence de Rfx6. **(E)** Les résultats de RT-qPCR pour *Insuline1*, *Glucagon*, *Polypeptide pancréatique (Ppy)* et *Somatostatine* supportent la perte quasi totale des cellules endocrines pancréatiques sauf les cellules exprimant le polypeptide pancréatique. **(F-G)** Des expériences d'immunofluorescence sur des pancréas de souris adultes 3 semaines après injection de tamoxifène, montrent que la délétion de Rfx6 (rouge) est efficace dans les cellules bêta mais que la production d'insuline n'est pas affectée (vert). **(H)** Les résultats de RT-qPCR sur des îlots purifiés de souris adultes ΔBêta 3 semaines après injection de tamoxifène pour *Insuline1*, *Glut2*, *MafA* et *Nkx6.1* montrent que l'identité de la cellule bêta est fortement perturbée. Les données sont représentées en moyenne ± SD sur n=4, \* p<0.05, \*\*p<0.01, \*\*\* p<0.001.

Dans l'intestin, j'ai montré avec l'aide d'Anthony Beucher (ancien post-doctorant dans l'équipe) que Rfx6 est exprimé dans une sous-population de progéniteurs entéroendocrines Ngn3<sup>+</sup> ainsi que dans les cellules entéroendocrines (EE) naissantes mais pas matures. La différenciation de toutes les cellules EE, hormis les cellules produisant la sérotonine, est sévèrement affectée dans les souris avec une délétion intestinale de Rfx6 (Figure 52). Ensemble, ces résultats montrent que Rfx6 est nécessaire dans l'intestin et le pancréas pour une différenciation (entéro) endocrine normale.



**Figure 52 : Rfx6 est nécessaire pour la formation des cellules entéroendocrines dans l'intestin.** Des résultats de RT-qPCR sur des nouveaux-nés ΔEndo et contrôles pour les gènes codant les hormones intestinales montrent que la différenciation de ces cellules est sévèrement affectée en absence de Rfx6. Les données sont représentées en moyenne ± SD sur n=3, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.

### 3. Conclusion et perspectives

En résumé, durant ma thèse, j'ai étudié la fonction endocrine de deux gènes en aval de Ngn3 dans le pancréas et l'intestin au cours du développement mais aussi chez l'adulte.

J'ai caractérisé l'expression de la kinase *Pak3* et montré qu'elle est exprimée dans le pancréas dans une sous-population de progéniteurs endocrines Ngn3<sup>+</sup>, mais surtout dans les cellules endocrines plus différenciées durant le développement, et aussi maintenue dans les cellules endocrines adultes. J'ai identifié *Pak3* comme un nouveau gène participant à la différenciation endocrine *ex vivo* et *in vivo*. Ce gène joue aussi un rôle dans le maintien de la glycémie chez l'adulte en particulier dans une situation de régime riche en graisses. D'autres expériences seront réalisées après ma thèse pour déterminer le rôle de *Pak3* dans les cellules entéroendocrines de l'intestin.

Après une première étude du gène Rfx6 publiée par l'équipe en 2010, j'ai continué la caractérisation de la fonction de ce facteur de transcription. Le profil d'expression de ce gène suggérait plusieurs fonctions pour Rfx6 que j'ai tenté d'élucider avec une souris KO conditionnelle. J'ai montré que c'est l'expression de Rfx6 en aval de Ngn3 qui est responsable de la perte des cellules hormone-sécrétrices chez les souris déficientes pour *Rfx6*. J'ai également montré que Rfx6 est nécessaire pour le maintien de l'identité des cellules bêta adultes.



J'ai commencé en parallèle l'étude de la fonction de Rfx6 dans l'intestin. J'ai caractérisé le patron d'expression de Rfx6 dans l'intestin adulte sauvage et montré que les souris avec une délétion intestinale de Rfx6 ne produisent pas de cellules entéroendocrines sécrétrices d'hormones à l'exception des cellules à sérotonine. La délétion de Rfx6 dans l'intestin adulte entraîne également une perte des cellules entéroendocrines accompagnée d'une diarrhée et de problèmes d'absorption des lipides. D'autres études sont actuellement en cours pour explorer les conséquences métaboliques de l'inactivation de *Rfx6* dans le pancréas et l'intestin ainsi que pour identifier des gènes cibles de Rfx6 dans ces deux tissus.

Ainsi, ce travail a permis de consolider les connaissances sur la différenciation et la fonction des cellules bêta, dans l'espoir de pouvoir un jour transférer ce savoir pour développer *in vitro* des cellules bêta de substitution capables de sécréter de l'insuline en réponse à des stimuli de glucose.

## 4. Références

**Ait-Lounis, A., Baas, D., Barras, E., Benadiba, C., Charollais, A., Nlend Nlend, R., Liegeois, D., Meda, P., Durand, B. and Reith, W.** (2007). Novel function of the ciliogenic transcription factor RFX3 in development of the endocrine pancreas. *Diabetes* **56**, 950-9.

**Ait-Lounis, A., Bonal, C., Seguin-Estevez, Q., Schmid, C. D., Bucher, P., Herrera, P. L., Durand, B., Meda, P. and Reith, W.** (2010). The transcription factor Rfx3 regulates beta-cell differentiation, function, and glucokinase expression. *Diabetes* **59**, 1674-85.

**Bokoch, G. M.** (2003). Biology of the p21-activated kinases. *Annu Rev Biochem* **72**, 743-81.

**Drucker, D. J.** (2007). The role of gut hormones in glucose homeostasis. *J Clin Invest* **117**, 24-32.

**Gradwohl, G., Dierich, A., LeMeur, M. and Guillemot, F.** (2000). neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A* **97**, 1607-11.

**Li, H. J., Ray, S. K., Singh, N. K., Johnston, B. and Leiter, A. B.** (2011). Basic helix-loop-helix transcription factors and enteroendocrine cell differentiation. *Diabetes Obes Metab* **13 Suppl 1**, 5-12.

**Mellitzer, G., Bonne, S., Luco, R. F., Van De Castele, M., Lenne-Samuel, N., Collombat, P., Mansouri, A., Lee, J., Lan, M., Pipeleers, D. et al.** (2006). IA1 is NGN3-dependent and essential for differentiation of the endocrine pancreas. *EMBO J* **25**, 1344-52.

**Mellitzer, G., Martin, M., Sidhoum-Jenny, M., Orvain, C., Barths, J., Seymour, P. A., Sander, M. and Gradwohl, G.** (2004). Pancreatic islet progenitor cells in neurogenin 3-yellow fluorescent protein knock-add-on mice. *Mol Endocrinol* **18**, 2765-76.

**Meng, J., Meng, Y., Hanna, A., Janus, C. and Jia, Z.** (2005). Abnormal long-lasting synaptic plasticity and cognition in mice lacking the mental retardation gene Pak3. *J Neurosci* **25**, 6641-50.

**Pan, F. C. and Wright, C.** (2011). Pancreas organogenesis: from bud to plexus to gland. *Dev Dyn* **240**, 530-65.

**Smith, S. B., Qu, H. Q., Taleb, N., Kishimoto, N. Y., Scheel, D. W., Lu, Y., Patch, A. M., Grabs, R., Wang, J., Lynn, F. C. et al.** (2010). Rfx6 directs islet formation and insulin production in mice and humans. *Nature* **463**, 775-80.

**Souopgui, J., Solter, M. and Pieler, T.** (2002). XPak3 promotes cell cycle withdrawal during primary neurogenesis in *Xenopus laevis*. *EMBO J* **21**, 6429-39.

**Soyer, J., Flasse, L., Raffelsberger, W., Beucher, A., Orvain, C., Peers, B., Ravassard, P., Vermot, J., Voz, M. L., Mellitzer, G. et al.** (2010). Rfx6 is an Ngn3-dependent winged helix transcription factor required for pancreatic islet cell development. *Development* **137**, 203-12.







## Summary

Pancreatic and intestinal endocrine cells, and their secreted hormones, contribute to the regulation of energy homeostasis. During embryogenesis their differentiation relies on similar genetic programs controlled by the proendocrine transcription factor Ngn3. However, our knowledge of the endocrinogenic programs implemented by Ngn3 is still fragmentary. Therefore, the transcriptome of endocrine progenitors has been determined in the lab. Among the genes which showed a strong enrichment in the endocrine lineage, I studied the pancreatic expression and function of *Pak3*, a serine/threonine kinase and further pursued the dissection of the function of the transcription factor *Rfx6* in the pancreas and in the intestine. I showed that *Pak3* is expressed throughout pancreas development and maintained in adult islet cells. Using *ex vivo* loss of function experiments and *in vivo* characterisation of the *Pak3*-deficient mice, I identified *Pak3* as an inhibitor of islet progenitors and beta-cell proliferation in the embryonic mouse pancreas. Furthermore, we performed metabolic studies which revealed that *Pak3*-deficient mice have an impaired glucose homeostasis, especially under challenging high fat diet. In parallel, using a conditional knockout mouse for *Rfx6*, we showed that *Rfx6* is necessary downstream of Ngn3 for proper endocrine cell differentiation in the pancreas as well as in the intestine. Finally, additional experiments in adult mice suggest that *Rfx6* is necessary to maintain pancreatic beta-cells, enteroendocrine cell turnover and intestinal lipid absorption. In conclusion, these studies revealed two novel key players in the regulation of endocrine cell differentiation and energy homeostasis in the pancreas and the intestine.

## Résumé

Les cellules endocrines pancréatiques et intestinales, ainsi que les hormones qu'elles sécrètent, participent à la régulation de l'homéostasie énergétique. Pendant l'embryogénèse, leur différenciation repose sur des programmes génétiques similaires contrôlés par le facteur de transcription proendocrine Ngn3. Cependant, on ne connaît que peu de choses sur les gènes activés par *Ngn3* et leurs implications dans les mécanismes contrôlant la spécification et la maturation des différentes cellules endocrines. Par conséquent, le transcriptome des progéniteurs endocrines a été déterminé dans le laboratoire. Parmi les gènes présentant un fort enrichissement dans le lignage endocrine, j'ai caractérisé l'expression et la fonction pancréatique de la kinase serine/thréonine *Pak3* et j'ai continué l'étude de la fonction pancréatique et intestinale du facteur de transcription *Rfx6*. J'ai montré que *Pak3* est exprimé dans le lignage endocrine pendant le développement et maintenu dans les îlots chez l'adulte. Avec des expériences de perte de fonction *ex vivo* et en caractérisant les souris mutantes pour *Pak3*, j'ai montré que ce gène inhibe la prolifération des progéniteurs endocrines et des cellules bêta durant l'embryogénèse. De plus, nous avons montré par une étude métabolique que les souris mutantes pour *Pak3* sont intolérantes au glucose particulièrement lorsqu'elles ont un régime riche en graisses. En parallèle, en utilisant une souris conditionnelle pour *Rfx6*, nous avons montré que *Rfx6* est nécessaire en aval de Ngn3 pour une différenciation appropriée des cellules endocrines pancréatiques et intestinales. Finalement, des expériences supplémentaires dans les souris adultes suggèrent que *Rfx6* est nécessaire pour le maintien des cellules bêta pancréatiques, le renouvellement des cellules entéroendocrines et l'absorption intestinale des lipides. En conclusion, ces études révèlent deux nouveaux gènes clés dans la régulation de la différenciation des cellules endocrines et de l'homéostasie énergétique dans le pancréas et l'intestin.