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Study of the role of Pax6b and CREB signaling on pancreas differentiation in zebrafish (*Danio rerio*)

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Abbreviations

A-MSH	*α-melanocyte stimulating hormone*
AC	*Adenylyl cyclase*
AKAPs	*PKA-anchoring proteins*
AP	*anterioposterior*
ARX	*Aristaless Related Homeobox*
bHLH	*basic helix-loop-helix*
BMP	*Bone Morphogenetic Protein*
CAM	*calmodulin*
cAMP	*Cyclic adenosin 3', 5' monophosphate*
CBP	*CREB-binding protein*
CRE	*cAMP-response element*
CREM	*cAMP-responsive modulator*
CREB	*cAMP-response element binding protein*
dpf	*Day post fertilization*
ECL	*External cell layer*
FFAs	*Free fatty acids*
FGF	*Fibroblast Growth Factor*
FGFR	*Fibroblast Growth Factor Receptor*
GFP	*Green Fluorescent Protein*
GEF	*Guanine-nucleotide-exchange factor*
GLP1	*Glucagon Like Peptide 1*
GPCR	*G-protein-couple receptors*
Hb9	*Gene Homeobox 9*
HD	*Homeodomain*
Hh	*Hedgehog*
Hnf6	*Hepatic nuclear factor 6*
hpf	*Hour post fertilization*
Isl1	*Islet 1*
LPM	*Lateral plate mesoderm*
MAPK	*Mitogen-activated protein kinase*
MC1R	*Melanocyte 1 receptor*
mib	*Mind bomb*
Ngn	*Neurogenin*
Nkx	*NK Drosophila gene cluster-related homeobox*
OC-1	*One-Cut*
Pax	*Paired Box Containing Gene*
PD	*Paired domain*
PDE	*Phosphodiesterase*
pdx1	*Pancreatic and duodenal homeobox 1*

PP2B	*calcineurin*
PKA	*Protein kinase A*
PKB	*Protein kinase B*
PKC	*Protein kinase C*
PKi	*Protein kinase inhibitor*
PLC	*Phospholipase C*
PP	*Pancreatic polypeptide*
PST	*Prolin-serin-threonin*
ptf1a	*Pancreatic specific transcription factor 1*
RA	*Retinoic acid*
shh	*Sonic hedgehog*
Sox	*SRY type HMG-Box*
TNF	*Tumor Necrosis Factor*

SUMMARY

The vertebrate endocrine pancreas has the crucial function of maintaining blood sugar homeostasis. This role is dependent upon the development and maintenance of pancreatic islets comprising appropriate ratios of hormone-producing cells.

Pax6 is a well-conserved transcription factor that contains two DNA-binding domains, and plays a key role in the development of eye, brain and pancreas in vertebrates. Within the endocrine progenitor pool, a variety of transcription factors influence cell fate decisions. Here we show that in zebrafish, in absence of pax6b, there is a loss of insulin-producing cells, a decrease of somatostatin-expressing cells, a decrease of glucagon-expressing cells and a strong increase of ghrelin-expressing cells. We propose a model where, in absence of pax6b, cells that should become insulin-positive become ghrelin-expressing cells.

In the second part of the work we describe the generation and characterization of zebrafish transgenic lines responsive to CREB signaling. These lines could represent useful tools for studying specific human pathologies.

SOMMARIO

Il pancreas endocrino nei vertebrati svolge la cruciale funzione di mantenere l'omeostasi della glicemia. Questo ruolo dipende dallo sviluppo e dal mantenimento di isole endocrine comprendenti appropriate proporzioni tra cellule ormone-producenti.

Pax6 è un fattore di trascrizione molto conservato, contenente due domini leganti il DNA, che gioca un ruolo chiave nello sviluppo di occhi, encefalo e pancreas nei vertebrati.

Nell'ambito del serbatoio dei progenitori endocrini, una serie di fattori di trascrizione influenza le decisioni sul destino cellulare.

In questa tesi dimostriamo che in zebrafish, in assenza di pax6b, vi è una perdita di cellule insulina-producenti, una riduzione di cellule esprimenti somatostatina, una riduzione di cellule esprimenti glucagone e un forte aumento di cellule grelina-positive. Proponiamo un modello dove, in assenza di pax6b, cellule destinate ad essere insulina-producenti diventano invece cellule esprimenti grelina.

Nella seconda parte di questa tesi, descriviamo la produzione e la caratterizzazione di linee transgeniche di zebrafish rispondenti alla via di segnale CREB. Queste linee potrebbero rappresentare degli utili strumenti per lo studio di specifiche patologie umane.

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INTRODUCTION

1. Pancreas

1.1. Pancreas structure

The pancreas is a mixed exocrine and endocrine gland that controls many homeostatic functions. The exocrine pancreas produces and secretes digestive enzymes, whereas the endocrine compartment consists of five distinct hormone-producing cell types. The cell types are α-, β-, δ-, Ɛ- and pancreatic polypeptide (PP) cells. α-cells (red) secrete **glucagon** and compose 15–20% of the endocrine pancreas. **β-cells** (green) secrete **insulin** and compose 60–80% of the endocrine pancreas. **δ-cells** (yellow) secrete **somatostatin** and compose 5–10% of the endocrine pancreas, whereas **PP** cells (blue) secrete PP and compose less than 2% of the endocrine pancreas. Epsilon cells, a recently discovered cell type, secrete **ghrelin**.

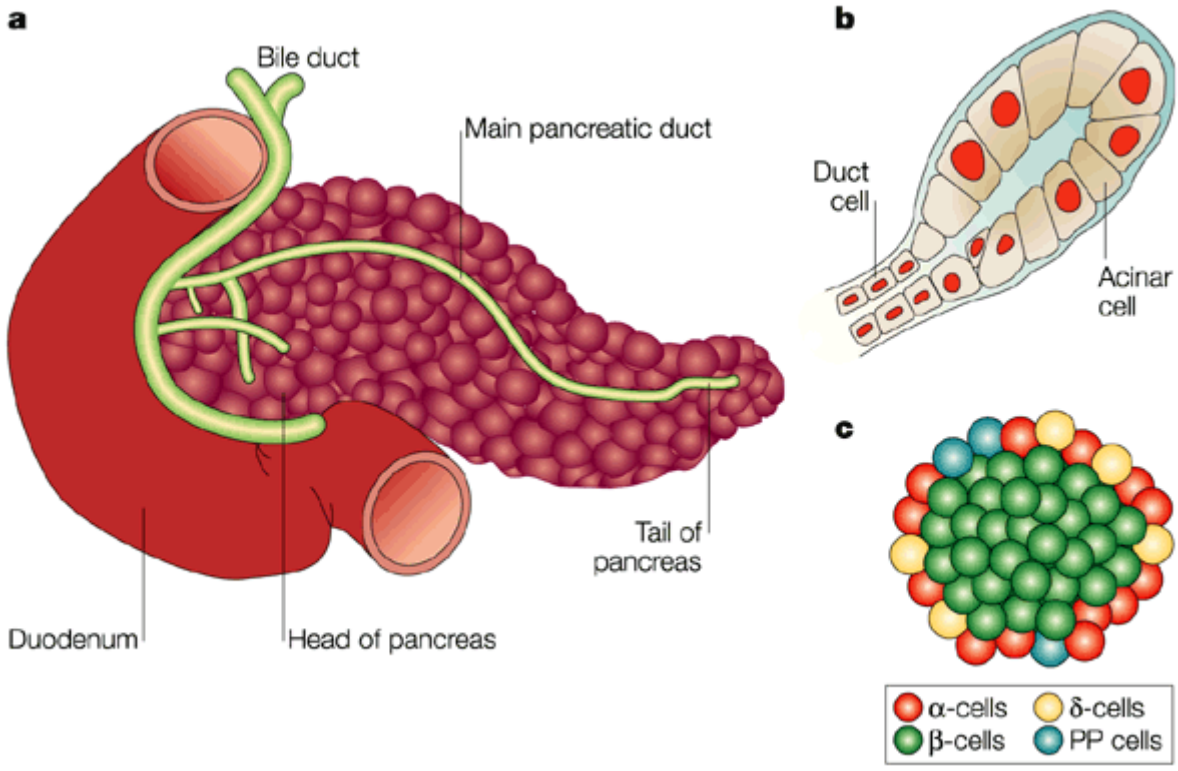

Fig. 1: Schematic representation of the mouse pancreas. a The mature pancreas is adjacent to the duodenum the most anterior part of the small intestine. **b** The function of the exocrine pancreas is to supply the gut with digestive enzymes; these are produced and secreted by acinar cells and subsequently transported to the intestine via the pancreatic ductal system.
c The endocrine pancreas consists of four hormone-producing cell types (Edlund, 2002).

The **exocrine pancreas** is a lobulated gland composed of acinar cells and of the pancreatic ductal tissue (Fig. 1). The acinar cells present a morphology characteristic of secretory cells: pyramidal shaped with basal nuclei, a developed Golgi apparatus, regular arrays of rough endoplasmic reticulum and numerous zymogen granules containing the digestive enzymes (Slack, 1995) (Fig. 2).

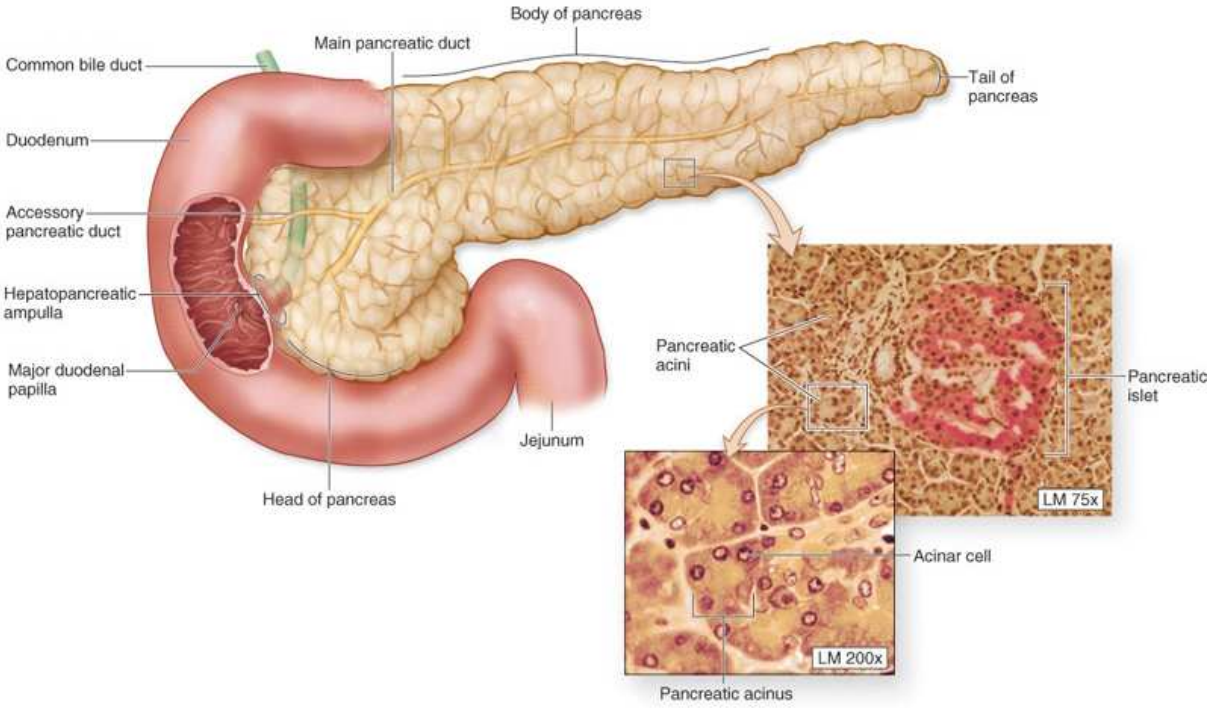

Fig. 2: Anatomy of the human adult pancreas. A drawing of the adult pancreas showing the organ's location in the gut and connection to the small intestine via the common bile duct, shared with the liver and gallbladder. A hematoxylin and eosin-stained paraffin section through the adult pancreas, below, shows an islet of Langerhans, its associated vasculature, and surrounding acinar tissue.

The acinar cells synthesize and release in the duodenum at least 22 digestive enzymes, notably amylases, proteases, lipases and nucleases. These enzymes compose the so-called pancreatic juice, which comprises also non-enzymatic components, such as the bicarbonate. These components are secreted by low cuboidal centroacinar cells located at the junction between the acini and the ducts (Fig. 2). The highly branched ductal epithelium, also belonging to the exocrine compartment, conveys the pancreatic juice to the duodenum where the pancreatic digestive enzymes, first produced as inactive precursors, are activated.

The **endocrine pancreas** represents only 1-2% of the pancreatic mass (Fig. 1). It is composed of the highly innervated and vascularised Langerhans islets, compact spheroidal cell clusters embedded in the exocrine tissue (Fig. 2). In vertebrates, there are five different pancreatic endocrine cell types, each of them producing a specific hormone. These hormones are mainly involved in the glucose homeostasis. The β-cells are the most abundant (around 70% of the islet cell populations) and produced insulin. The α-, δ-, ε- and PP-cells respectively produce glucagon, somatostatin, ghrelin and the pancreatic polypeptide. In mouse and zebrafish, the core of the islets is mainly composed of β-cells, the other cell types surrounding them; while less sharp, this segregation is also present in humans (Kumar & Melton, 2003).

1.2. Organogenenesis of the pancreas

During the last fifteen years, the zebrafish has been used along the mouse to decipher vertebrate development. A few laboratories including ours, have initially characterized the zebrafish pancreas during its development (Argenton et al, 1999; Biemar et al, 2001; Pack et al, 1996). Preliminary data about pancreatic gene expression and function have suggested an evolutionary conservation of the developmental mechanisms for pancreas specification and differentiation, validating the zebrafish as a relevant model for pancreas development studies. The zebrafish pancreas displays an organization comparable to Mammals, with its endocrine tissue embedded in the exocrine tissue (marked by *carboxypeptidase* expression in Fig.3) and composed of a core of β-cells surrounded by α- and δ-cells type (Fig. 1). The existence of ε-cells in zebrafish was demonstrated in our laboratory (Pauls et al, 2007). While the presence of the PP protein was previously mentioned (Biemar et al, 2001), its detection by *in situ* hybridization has not yet been published.

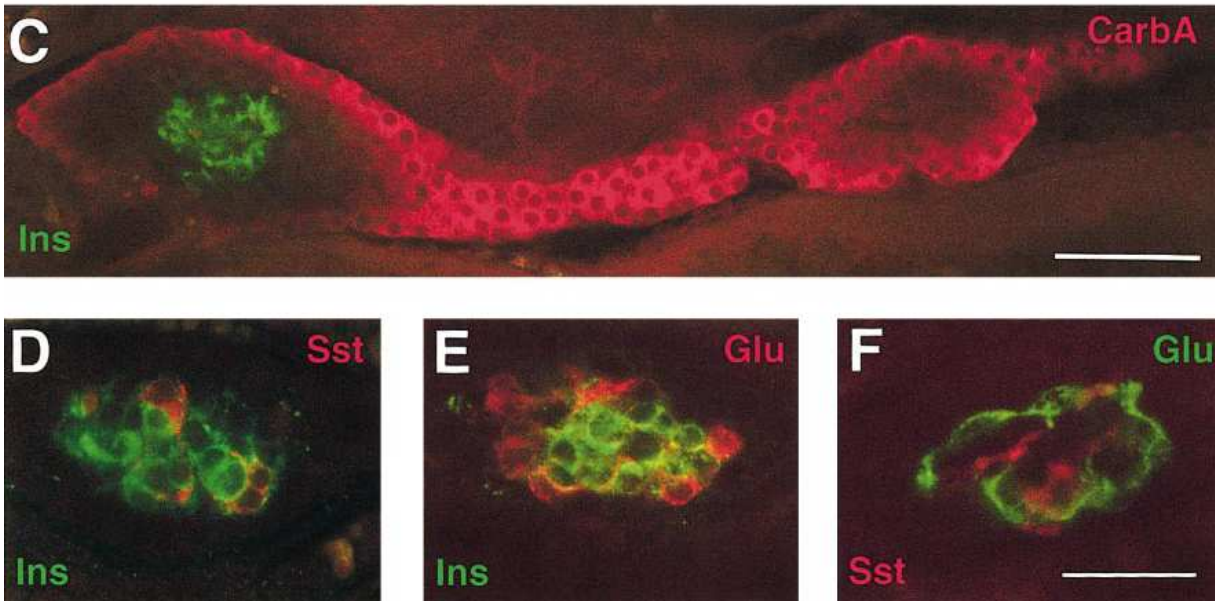

Fig. 3: Morphology of the exocrine and endocrine pancreas in the zebrafish larva. (C–F) Confocal images of pancreatic islet tissue showing whole-mount immunofluorescence staining performed on (C) 5.5-day-old, (D) 4-day-old, and (E, F) 3-day-old embryos with a combination of (C) anti-carboxypeptidase A and anti-insulin; (D) anti-insulin and anti-somatostatin; (E) anti-insulin and anti-glucagon; (F) anti-somatostatin and anti-glucagon antisera. Adapted from (Biemar et al, 2001).

1.2.1. Origin of the pancreatic tissue

The vertebrate pancreas is shaped by the union of dorsal and ventral buds arising from the endoderm (Andrew, 1976; Fontaine & Le Douarin, 1977; Percival & Slack, 1999). In mice like in zebrafish, there are two buds, a dorsal one arising first followed by a ventral and more anterior bud.

Mouse pancreas development begins with the emerging of the dorsal pancreatic bud from the posterior foregut endoderm, at E9, after the closure of the anterior endoderm: the ventral bud arises one day later and they fuse at E17.5 to form the definitive pancreas (Fig. 4) (Slack, 1995)

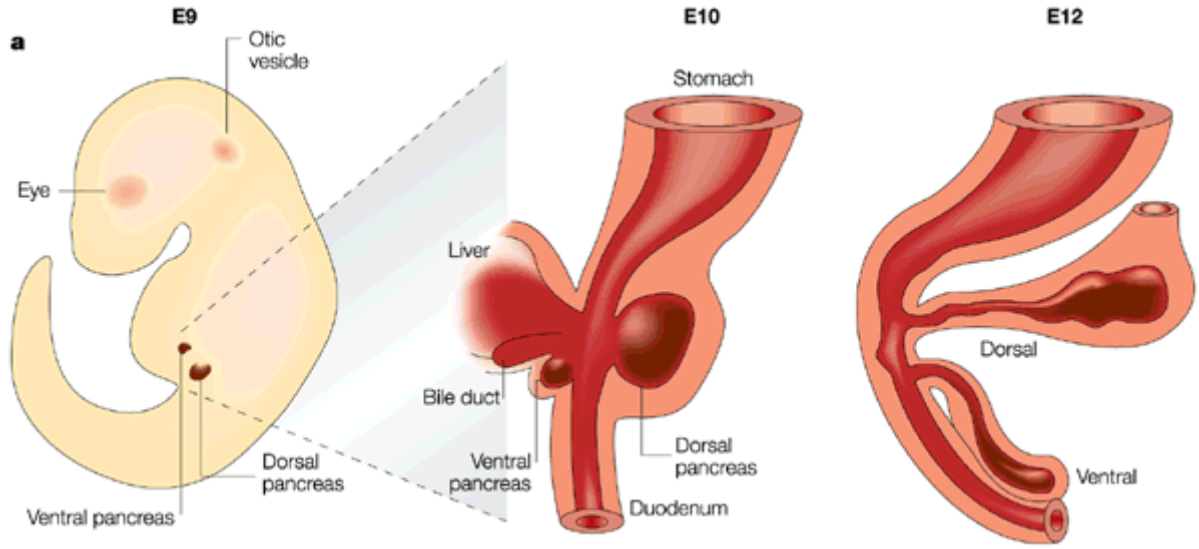

Fig. 4: The pancreas develops as evaginations of the primitive gut epithelium. Schematic representation of the pancreas at embryonic day E9, E10 and E12 of a mouse embryo. Adapted from (Edlund, 2002).

In zebrafish, the pancreas also forms from two distinct buds: the dorsal bud protrudes at 24 hpf, at the level of the third somite (showed at 34 hpf in Fig. 5., labeled by the arrowhead). At 40 hpf, a second bud appears, ventrally to the gut and more anterior compared to the dorsal bud (Fig. 5, labeled by the arrow). Between 24 hpf and 48 hpf, the dorsal bud relocates on the right side of the gut and the two buds fuse at 52 hpf to form the mature pancreas, the exocrine tissue surrounding the endocrine islet (Fig. 5) (Field et al, 2003). While the appearance of the dorsal bud at 24 hpf is the first morphological sign of the zebrafish pancreas development, it is important to note that cells expressing pancreatic markers can be observed at earlier stages. The pancreatic marker *pdx1* (Pancreatic and Duodenal homeobox gene 1) is expressed in a bilateral domain as soon as the 10-somites stage (14 hpf), and *insulin* expression is observable at the 12-somites stage (15 hpf) (Biemar et al, 2001).

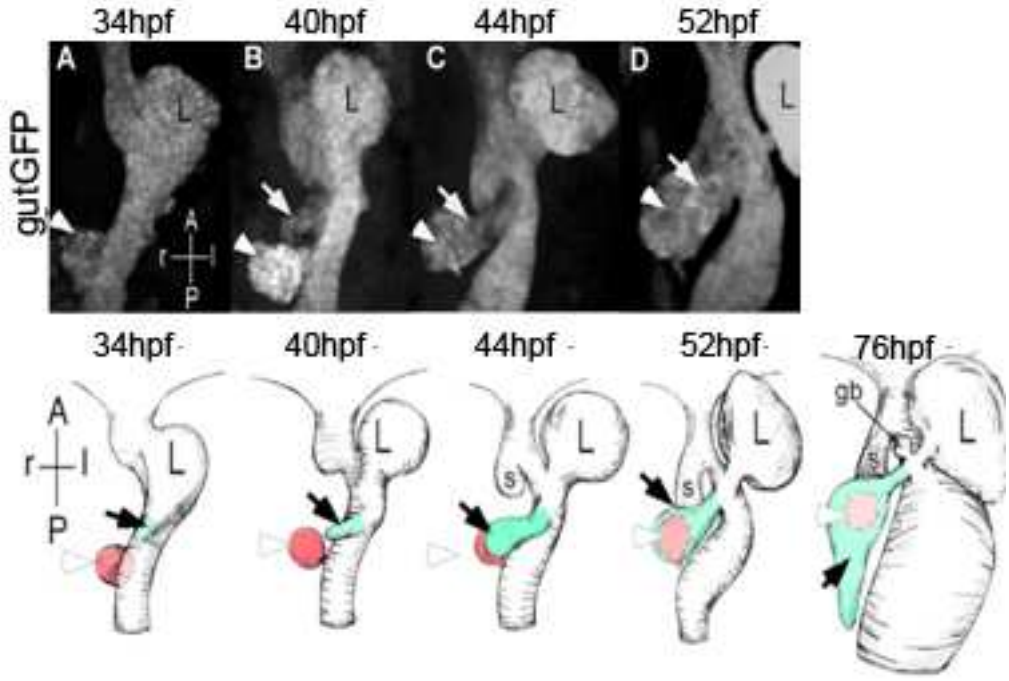

Fig. 5: Pancreas morphogenesis in zebrafish. A. ventral view of the pancreatic region of a zebrafish transgenic embryo gut-GFP. B Schematic representation of the model. Arrow heads show the dorsal bud and arrows show the ventral bud. L: Liver, gb: gall bladder, S: swim bladder. Adapted from (Field et al, 2003).

While this morphogenesis is similar to what is observed in mice, the zebrafish development presents an exciting particularity: while in mice the two buds give rise to both endocrine and exocrine cells, the zebrafish dorsal bud generates only endocrine cells and the ventral bud mostly exocrine cells (Field et al, 2003).

1.2.2. Specification factors of the pancreas

In amniotes, the gut endoderm gives rise to different organs, such as pharynx, liver, the pancreas and intestine. The first step of their organogenesis is the regionalization of the gut endoderm in different territories. Each territory has specific competences that permit them to go through the following differentiation and organogenesis. Budding of the dorsal and ventral pancreas is then induced in the prospective pancreatic territory, followed by the growth of the pancreatic buds and their merging. The pancreas ontogenesis is thus a stepwise process, which was shown to be influenced by soluble factors, produced by the contiguous structures (Wells & Melton, 2000).

1.2.2.1. Regionalization of the endoderm

1.2.2.1.1. Retinoic acid (RA)

The retinoic acid (RA) is a factor patterning the antero-posterior axis in the three germ layers (endoderm, mesoderm and ectoderm). Mutant mice with an impaired expression of *Raldh2 (*Retinaldehyde dehydrogenase 2, the enzyme synthesizing RA), present a dorsal pancreas agenesis (Martin et al, 2005; Molotkov et al, 2005). Similarly, the *neckless* mutant zebrafish, with an impaired *raldh2* expression (Begemann et al, 2001), lacks expression of pancreatic and hepatic markers. Inversely, ectopic RA treatments lead to a severe anterior shift of all pancreatic markers. Timed treatments with a pan-RA receptor inhibitor indicate that RA signaling is required between 9 hpf and 12 hpf, at the end of gastrulation (Stafford & Prince, 2002). It was demonstrated that the RA signaling is a direct signal from the mesoderm to the endoderm. Indeed, while *raldh2* is expressed in the whole mesendoderm, transplantation experiments have determined that the RA derived from the anterior paraxial mesoderm is sufficient to induce pancreas development in the endoderm. Moreover, the pancreas development requires expression of the RA receptors only in the endoderm (Stafford et al, 2006). Up till now, RA is synthesized in an anteroposterior (AP) region of the mesoderm considerably larger than the pre-pancreatic domain. In conclusion, the RA signal from the mesoderm participates in the regionalization of the endoderm and in the specification of the pre-pancreatic domain at the end of the gastrulation.

1.2.2.1.2. Bone morphogenetic proteins (BMP)

BMP signaling is involved in the dorsoventral axis patterning of the embryo. In particular, BMP-4 is a ventralizing factor antagonised by the dorsalizing effect of chordin. Comparable results were obtained in zebrafish; the discovery of the dorsalized *swirl* (*bmp2*) and ventralized *chordino* (*chordin*) zebrafish mutants have confirmed the role of the BMP signaling in the dorsoventral patterning but has also highlighted its implication in the AP patterning of the endoderm. Indeed, these two mutants present respectively a reduced or an expanded pancreas (Tiso et al, 2002)

1.2.2.1.3. Fibroblast growth factor (FGF)

The Fibroblast Growth Factors (FGFs) were shown to be involved in the patterning of the endoderm through various studies. *In vitro,* FGF4 patterns cultured E7.5 mouse endoderm in a concentration-dependent manner (Wells & Melton, 2000). Moreover, *in vivo* gain- and loss-of-function experiments in chicken demonstrated that FGF signaling represses posterior and promotes anterior endoderm fate. Local application of FGF shifted anteriorly the expression domain of *Pdx1* (Dessimoz et al, 2006). Interestingly, expression of the FGF receptors (FGFRs) 1 and 2 was observed in the pancreatic islets, together with the ligands FGF1, 2, 4, 5, 7 and 10 (Hart et al, 2000).

In zebrafish, a FGF signal sent by the lateral plate mesoderm (LPM) is crucial for the pancreatic ventral bud specification. In zebrafish, the left and right LPM migrate asymmetrically toward the left side of the embryo between 26 hpf and 30 hpf. At that point of the development, LPM contacts only the ventral endoderm and intercommunicate by FGF signaling. First, Fgf24 secreted by the pancreatic endoderm patterns the adjacent LPM between 24 and 30 hpf, leading to *fgf24*, *isl1* (islet-1) and *meis3* expression in the LPM. Secondly, between 30 and 32 hpf, the pancreatic LPM instructs the endoderm to induce *ptf1a* expression, thereby specifying the ventral pancreatic bud. Finally, from 32 hpf onwards, FGF signaling from the pancreatic LPM promotes the pancreatic ventral bud growth (Manfroid et al, 2007).

1.2.2.2. Induction of dorsal and ventral pancreatic buds

1.2.2.2.1. The hedgehog signaling

Shh is an intercellular patterning signal of the Hedghehog (Hh) family. In mouse, whereas largely expressed mouse and chicken gut, *Shh* is not expressed in the future ventral and dorsal pancreatic endoderm (Apelqvist et al, 1997; Hebrok et al, 1998). Moreover, its repression is required for pancreas development, as shown by the transformation of the pancreatic endoderm into intestinal tissue following ectopic *Shh* expression (Apelqvist et al, 1997; Ramalho-Santos et al, 2000).

The ventral endoderm is first constituted by a hepatic/pancreatic bipotential population, and the FGF signal emitted by the cardiac mesoderm activates *Shh* expression

in cells close to it and diverts their default pancreatic cell fate to a hepatic cell fate (Deutsch et al, 2001).

Thus, the repression of *Shh* expression in the dorsal foregut allows the induction of the dorsal pancreatic bud whereas the induction of *Shh* expression in the ventral endoderm diverts the hepatic/pancreatic bipotential cells from their default pancreatic fate and converts them into hepatic cells.

In zebrafish, the role of the Hh signaling in pancreas development is somehow contradictory compared to the role of Hh signaling in mice. While *Shh* over-expression prevents mice pancreas development, the zebrafish pancreas requires *shh* expression to develop (Roy et al, 2001). Moreover, an ectopic *shh* expression in duodenum significantly increases the number of pancreatic endocrine cells (Roy et al, 2001). Treatments with cyclopamine (inhibitor of hedgehog signaling pathway) have demonstrated that Hh signal is required during gastrulation for proper pancreatic islet development.

This apparent species-specific difference has to be nuanced. Firstly, while *Shh* over-expression impairs mouse pancreas development, the effect of its knockout on this development can-not be assessed, as *Smo* mutant mice die early before pancreas specification (Zhang et al, 2001). Consequently, it is not known if the mouse pancreas development has also an early requirement for notochord Hh signaling. Secondly, the timing of *Shh* expression is different between these species; while the endoderm of the mouse foregut expresses *Shh* before pancreas specification and requires this expression to be repressed in the prospective pancreas domain, the zebrafish endoderm begins to express *Shh* long after the appearance of the pancreatic markers (~24 hpf) (Roy et al, 2001). Transplantation studies demonstrated that in zebrafish the Shh receptor Smo function is not required in the β-cells progenitors themselves but rather in their neighboring cells, which will become exocrine pancreas, intestine or liver cells (Chung & Stainier, 2008).

So, Hh signaling would play sequential roles in pancreas development: the Hh signaling sent by the notochord would play an early positive role while the *Shh* expression in the endoderm would play a later negative role. Consistent with this hypothesis is the observation that the zebrafish *tcf2* mutants display both a *Shh* over-expression in the endoderm at 30 hpf and a severe *pdx1* expression decrease (Sun & Hopkins, 2001).

1.2.3. Growth and proliferation

1.2.3.1. Notch signaling

In pancreas, repression of a premature differentiation is provided by the lateral inhibition process, mediated by the Delta-Notch signaling (Fig. 6).

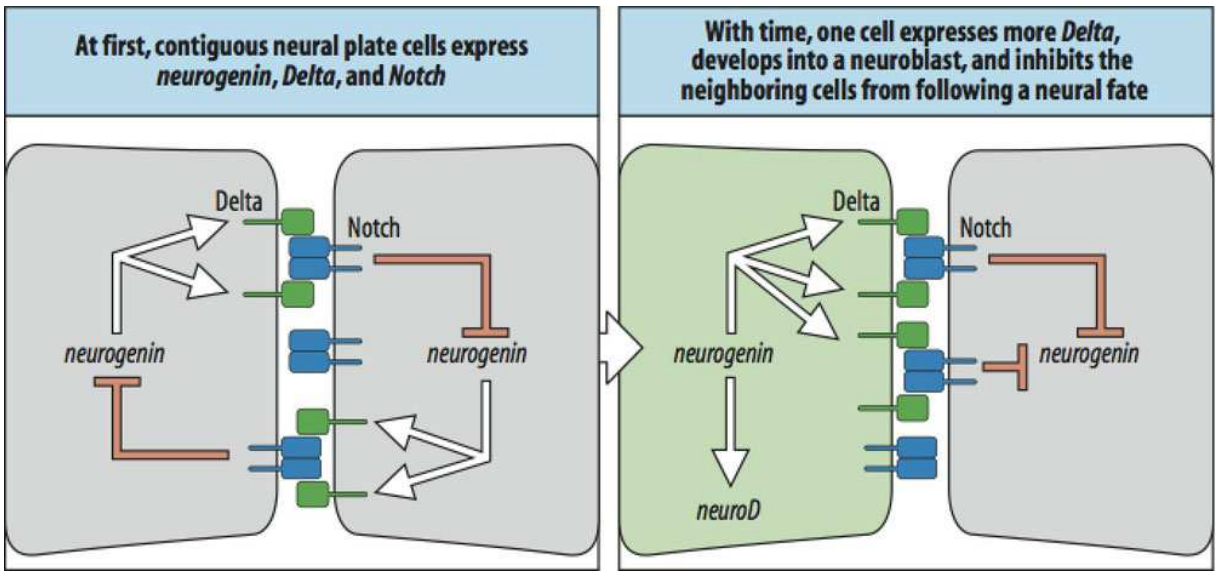

Fig. 6: Notch-mediated lateral inhibition. Left panel: at first, all the progenitor cells present similar quantity of the Delta and Notch inter-membrane proteins; the interaction between them represses *Ngn3* (neurogenin 3) expression, which activates *Delta* expression. Right panel: with time, one cell expressing more *Delta* will be able to stimulate more Notch receptors in its neighbor cells and thus to repress more strongly their *Ngn3* expression and consequently their *Delta* expression. This cell will be able to increase its *Ngn3* levels. *Ngn3* induces *NeuroD* expression, allowing the cell to adopt an endocrine fate.

In mice, the four known Notch genes are expressed in the pancreatic epithelium or in the surrounding mesenchyme (Lammert et al, 2000). Mice genetically altered at several steps of the Notch signaling (Hes, Delta-like gene 1, RBP-Jκ) present a decrease of the pancreas size, due to an up-regulation of the *Ngn3* pro-endocrine gene and to the consequent depletion of pancreatic progenitors (Apelqvist et al, 1999; Jensen et al, 2000). Misexpression of constitutively active Notch prevents both endocrine and exocrine differentiation and seems to trap both early and late progenitors in an undifferentiated state (Hald et al, 2003; Murtaugh et al, 2003).

Similar results have been obtained in zebrafish. The *mind bomb* (*mib*) mutant lacks a Delta-ubiquitin ligase, which results in the failure to release the Delta-mediated Notch signaling. At 24 hpf, their pancreas presents an excess of β- and δ-cells along with a reduced number of α-cells (Zecchin et al, 2007). Differently from mice, the α-cells are the

last pancreatic endocrine cell type to differentiate in zebrafish; their absence in *mib* mutants is thus most likely due to a depletion of the pancreatic progenitor pool caused by the excessive differentiation of β- and δ-cells (Zecchin et al, 2007). Conversely, ectopic expression of Notch in zebrafish leads to a complete lack of *insulin* expression (Esni et al, 2004).

1.2.4. Transcriptions factors controlling pancreas development

The diverse signals emitted by mesoderm-derived structures induce, during pancreas development, a network of transcription factors. Many of them belong to the homeodomain or the basic helix-loop-helix (bHLH) families. These transcription factors were identified because of their participation in the regulation of *insulin* and *somatostatin* expression, the promoters of these genes possessing several homeodomain or bHLH binding sites. Several knockout studies have demonstrated the implication of these genes in pancreas development.

1.2.4.1. Initial pancreatic specification

1.2.4.1.1. Pdx1

Pdx1 is the first factor that was demonstrated as required for pancreas development. At E8.5, this homeobox factor is expressed in the whole pancreatic epithelium. Cell lineage studies demonstrated that all endocrine and exocrine pancreatic cells derived from cells expressing *Pdx1* throughout early embryogenesis (Gu et al, 2002; Herrera, 2000). At later stages, *Pdx1* expression becomes restricted to β- and δ-cells, which is coherent with the fact that Pdx1 was first identified as a transcription factor activating *insulin* and *somatostatin* expression (Guz et al, 1995; Leonard et al, 1993; Miller et al, 1994; Offield et al, 1996; Ohlsson et al, 1993).

Targeted deletion of *Pdx1* does not impair the initial induction of the pancreatic fate, early *glucagon*- and *insulin*-expressing cells being observed in the *Pdx1*$^{-/-}$ mice; however, the subsequent progression of the pancreatic differentiation as well as the pancreatic morphogenesis are impaired in these embryos and consequently the neonatal *Pdx1*$^{-/-}$ mice display pancreatic agenesis (Ahlgren et al, 1996; Offield et al, 1996).

The zebrafish ortholog of *Pdx1* was identified (Milewski et al, 1998) and presents very similar expression pattern and function. As soon as the 10-somite stage, it is expressed in two bilateral rows of cells adjacent to the midline that then converge to form the pancreatic primordium. Later, it is also expressed in the duodenum (Biemar et al, 2001). The knockdown of *pdx1* in zebrafish leads to a severe reduction of endocrine and exocrine compartments but not a complete ablation (Huang et al, 2001; Yee et al, 2001).

1.2.4.1.2. Hnf6

The One-Cut homeobox (OC-1) factor Hnf-6 is largely expressed in the pancreatic epithelium at early stages, before becoming restricted to acinar and ductal cells (Landry et al, 1997; Rausa et al, 1997). Hnf-6 fulfils different roles in pancreas development, the first of them being the control of pancreas specification via positive regulation of *Pdx1* expression. Hnf-6 has been indeed shown to directly regulate *Pdx1* expression and *Hnf6*-null mice present a delay of *Pdx1* expression onset (Jacquemin et al, 2003).

Up till now, implication of one-cut factor has not been demonstrated in the zebrafish pancreas development.

1.2.4.1.3. Ptf1a

The basic helix-loop-helix (bHLH) Ptf1a/p48 factor is part of the PTF1 complex,(Cockell et al, 1989; Sommer et al, 1991).

ptf1a is expressed in the pancreas at E9.5 and a cell lineage study demonstrated that it is in fact expressed in the progenitors of exocrine, endocrine and ductal cells. *Ptf1a* is also necessary to the acquisition of pancreatic fate by the undifferentiated endoderm(Kawaguchi et al, 2002). The role of *Ptf1a* in the whole pancreas development was also highlighted by analysis of the *Ptf1a* hypomorphic mutant mice that display a *Ptf1a* dosage-dependant pancreatic hypoplasia (Fukuda et al, 2008).

Besides, Ptf1a is also specifically required for the exocrine differentiation. Mice deficient in *p48* expression display a total lack of exocrine cells (Krapp et al, 1996; Krapp et al, 1998).

In zebrafish, the *ptf1a* expression is limited to the ventral bud, which gives rise to exocrine compartments. Its expression in exocrine cells is visible from 32 hpf. Its implication in the ventral bud differentiation was demonstrated by loss-of-function studies that showed that embryos with an impaired *ptf1a* expression do not express *trypsin* anymore, indicating a loss of the exocrine tissue (Zecchin et al, 2004). More recently, it

was demonstrated that in zebrafish low levels of *pft1a* expression promote endocrine fate while high levels promotes exocrine fate. This indicates that in addition to its known roles in pancreas specification and exocrine differentiation, Ptf1a could be involved in the endocrine versus exocrine fate decision (Dong et al, 2008).

1.2.4.2. Endocrine differentiation

1.2.4.2.1. Ngn3

Ngn3 is a bHLH transcription factor. It is the key factor of the endocrine pancreas, being expressed in all endocrine progenitor cells and required for their differentiation. Its pancreatic expression is transient; it begins at E9, reaches a peak at E15.5 and then decreases, *Ngn3* being no more expressed in pancreas at birth (Apelqvist et al, 1999; Jensen et al, 2000; Schwitzgebel et al, 2000; Sommer et al, 1996). *Nng3* is not expressed in hormone-expressing cells, yet permanent tracing of the *Ngn3*$^{+}$-cells has shown that their progeny comprises all endocrine cell types (Gu et al, 2002). In other words, all endocrine progenitor cells transiently express *Ngn3* during their differentiation process.

Different loss- or gain-of-functions studies have confirmed these results. Mice presenting a homozygote null mutation of *Ngn3* die perinatally due to absence of pancreatic endocrine tissue and the resulting diabetes (Gradwohl et al, 2000). Conversely, ectopic expression of *Ngn3* in the *Pdx1*-expressing domain leads to a precocious endocrine differentiation, depleting the progenitor pool and resulting in a reduced endocrine pancreas (Apelqvist et al, 1999; Schwitzgebel et al, 2000). Moreover, ectopic *Ngn3* expression in the non-pancreatic gut endoderm is sufficient to turn these cells into endocrine cells that migrate in the mesenchyme where they form islets expressing *glucagon* and *somatostatin*.

In zebrafish, a functional ortholog for the mouse *Ngn3*, islet cell fate decisions, has not been yet characterized, although a zebrafish *ngn3* gene has been found to be transiently expressed in the pancreatic ventral bud, at around 70 hours post-fertilization (N. Tiso, personal communications).

1.2.4.2.2. NeuroD

Initially isolated as a transcriptional activator of *insulin* expression, the bHLH *NeuroD1* factor is a direct target of *Ngn3* (Gradwohl et al, 2000; Huang et al, 2000; Naya et al, 1997; Naya et al, 1995; Schwitzgebel et al, 2000). Its expression is first detected in the pancreatic domain at E9.5 and is then observed exclusively in the endocrine pancreas, in all endocrine cell types. *NeuroD*-deficient mice die perinatally due to severe diabetes; the number of their endocrine cells is markedly reduced and the few cells left fail to form proper islets (Naya et al, 1997). Conversely, ectopic *NeuroD1* expression (as well as *Ngn3* ectopic expression), driven by *Pdx1* promoter, leads to a premature endocrine differentiation (Schwitzgebel et al, 2000). These results indicate that *NeuroD1* is specifically involved in pancreas endocrine development after the initiation of the endocrine program, in the maintenance and/or proliferation of endocrine cells.

It is worthy to note that another NeuroD gene, *NeuroD2*, was detected in the pancreas. Its expression peaks between E12.5 and E17.5 and it seems to be a direct target of *Neurogenin3*. It is able to activated *in vitro* endocrine cell lineage. Yet, NeuroD2 null mice present normal islet differentiation, suggesting that other bHLH genes (especially *NeuroD1*) could be redundant and exert compensatory roles (Gasa et al, 2008).

In zebrafish, *neuroD1* has been first identified by Korzh and co-workers and was shown to be expressed in the pancreas (Korzh et al, 1998; Mavropoulos et al, 2005).

1.2.4.2.3. Islet 1

The Islet1 (Isl1) transcription factor belongs to the LIM homeodomain subfamily. It was first identified as a transcription factor binding to the *Insulin* promoter (Karlsson et al, 1990) but is expressed in all adult islet cells, soon after they have left the cell cycle (Ahlgren et al, 1997). Impairment of *Isl1* expression leads to a complete loss of pancreatic endocrine cells and to impaired dorsal bud exocrine cell differentiation. This effect on exocrine differentiation could be due to the fact that in $Isl1^{-/-}$ mutant mice, the organization of the dorsal mesenchyme (that normally expresses *Isl1*) is impaired (Thor et al, 1991). Indeed, *in vitro* recombination of $Isl1^{-/-}$ dorsal bud with wild-type dorsal mesenchyme is able to restore exocrine differentiation. Isl1 is therefore required for endocrine cells differentiation and normal dorsal mesenchyme development, indicating an implication of

the dorsal mesenchyme in the development of the pancreatic dorsal bud (Ahlgren et al, 1997).

In zebrafish, *isl1* expression is detected in the pancreatic anlage as early as the 12-somite stage and persists until at least 24 hpf; at this stage, its expression is restricted to the endocrine dorsal bud (Biemar et al, 2001). From 28 hpf, *isl1* starts to be expressed in the lateral plate mesoderm (LPM), which is required for the ventral bud development (Manfroid et al, 2007). Consistently, knockdown of *isl1* expression results in a partial loss of the exocrine tissue.

1.2.4.3. Endocrine cell type specification

1.2.4.3.1. Arx and Pax4

Arx and Pax4 are two key factors in the establishment of α- or β-cell fate, fulfilling antagonist roles. The homeobox Arx factor is expressed in the pancreas epithelium from E9.5 and is later found in mature α- and δ-cells (Collombat et al, 2003). *Arx*-deficient mice present a loss of mature α-cells concomitant with an increase in the number of β-cells and an even more marked increased in the δ-cell number. As the total endocrine cell number is unaffected, it is assumed that there is a replacement of the mature α-cells by β- and δ-cells (Collombat et al, 2005).

Concerning *Pax4*, it is transiently expressed in the endocrine pancreas, with a peak of expression between E13.5 and E15.5 corresponding to the peak of endocrine cell genesis (Dohrmann et al, 2000; Sosa-Pineda et al, 1997). *Pax4* ectopic expression in pancreas progenitors or in mature α-cells forces them to adopt a β-cell fate (Collombat et al, 2009). Moreover, the *Pax4*$^{-/-}$ mutant mice reveal a complete lack of β- and δ-cells and a proportional increase of the α-cells number, suggesting that, in absence of Pax4, β-cells precursors adopt an α-cell fate (Sosa-Pineda et al, 1997; Wang et al, 2004).

Arx and Pax4 have therefore opposite functions in endocrine pancreas development, the former promoting α-cell fate while the latter promotes β-cell fate. Their antagonism is reinforced by the fact that the two factors repress each other expression. Moreover, mice carrying deficiencies in both genes present a loss of both α- and β-cells, with a proportional increase of the δ-cell number (Collombat et al, 2005).

1.2.4.3.2. Sox4

The involvment of the sox4 factor in pancreas development was in fact first demonstrated in zebrafish. Due to ancient duplication, the zebrafish genome possesses two sox4 genes, but only *sox4b* is expressed in the pancreas. Expression of *sox4b* is detected in endodermal cells in the prospective pancreatic region as soon as the 5-somite stage; *sox4b* transcripts are detected in the dorsal bud until at least 40 hpf; the gene is not expressed in the ventral bud (Mavropoulos et al, 2005). At the 18-somite stage, *sox4b* is perfectly co-expressed with *neuroD*, which indicates that it is expressed in the progenitor of all endocrine cell lineages (Soyer et al, 2010). Yet, its knockdown leads only to a severe decrease of the α-cells, the other cell types being not affected (Mavropoulos et al, 2005).

In mouse, *Sox4* expression was also identified in pancreas. Its pancreatic expression peaks between E12.5 and E14.5 and is still observable in adult pancreas. The *Sox4*$^{-/-}$ mutant mice present a decrease of β-cells (Wilson et al, 2005). This contrasts with the observations made in zebrafish.

1.2.4.3.3. Nkx2.2

At E9.5, ***Nkx2.2*** is expressed in the pancreatic epithelium; later, its expression becomes restricted to all β-cells, and to most α- and PP-cells, but is excluded of δ- and ε-cells. When *Nkx2.2* expression is disrupted in mice, the β-cells can-not reach their differentiation; there is also a decrease of the α- and PP-cell number (Sussel et al, 1998). In contrast, the pancreas of *Nkx2.2*-deficient mice displays a large increase of ε-cells (Prado et al, 2004).The Nkx2.2 factor possesses both activator and repressor activities, and it has been demonstrated that its repressor activity is sufficient to rescue the lack of α-cells in the *Nkx2.2*-deficient mice, but not the terminal differentiation of β-cells (Doyle et al, 2007).

In zebrafish, *nkx2.2a* is also largely expressed in the pancreas, and its expression is required for α- and β-cell differentiation; moreover, it is expressed in the intrapancreatic duct and is necessary for its morphogenesis (Biemar et al, 2001; Pauls et al, 2007).

1.2.4.3.4. Nkx6

Studies on *Nkx6.1*$^{-/-}$, *Nkx6.2*$^{-/-}$ and *Nkx6.1*$^{-/-}$*;Nkx6.2*$^{-/-}$ mutant mice revealed that both factors share similar functions in the differentiation of α- and β-cells. *Nkx6.1*$^{-/-}$ mice show a markedly reduced number of β-cells (Sander et al, 2000), whereas the pancreas appears normal in *Nkx6.2*$^{-/-}$ mice (Henseleit et al, 2005).

In zebrafish *nkx6.1* and *nkx6.2* are expressed in the dorsal pancreatic bud at 24 hpf; nkx6.1 and nkx6.2 share similar functions in α-cell differentiation (Binot et al, 2010).

1.2.4.3.5. Pax6

The transcription factor Pax6 is well conserved among metazoa. The protein contains two different DNA-binding domains, a paired domain (PD) and a paired-like homeodomain (HD), and the C-terminal proline-, serine-, and threonine-rich (PST) region, which is acting as a transcriptional activation domain.

This factor plays important roles in the development of different organs, including the eye, brain, pituitary, and pancreas (Choi et al, 2008; Quiring et al, 1994). Its pancreatic expression begins at E9 in a few cells (Fig. 7) and then expands in all endocrine cell types, in which it is still expressed in newborn mice (St-Onge et al, 1997).

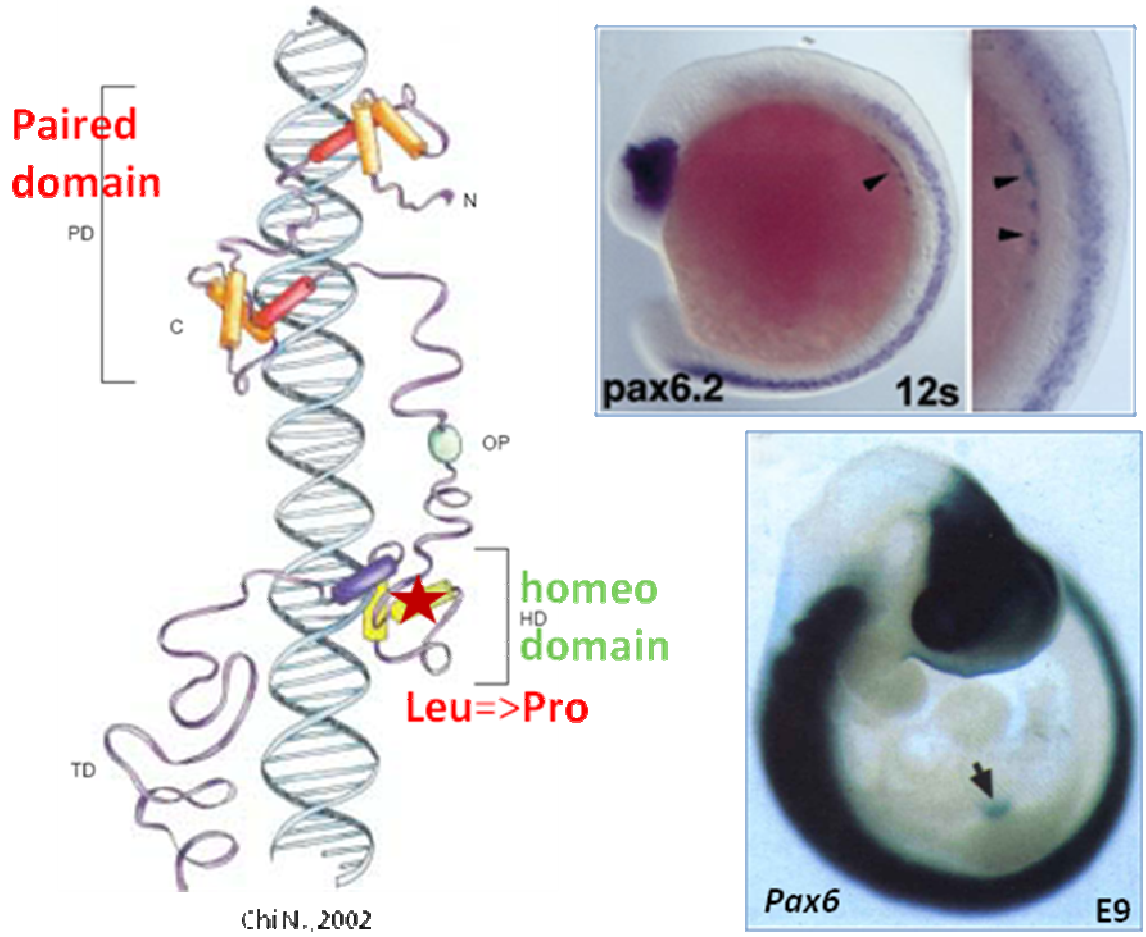

Fig. 7: The transcription factor Pax6 is well conserved among metazoan for its structure and expression pattern (Biemar et al, 2001; St-Onge et al, 1997).

Studies of the *Pax6* functions rely on the study of the *Pax6*^{Sey} (Small eye) and *Pax6*$^{Sey\text{-}Neu}$ mutant mice that display pancreatic phenotypes largely similar to that of the *Pax6* knockout mice (Heller et al, 2004; Sander et al, 1997; St-Onge et al, 1997). Analysis of these mice revealed that the *Pax6* inactivation leads to a large decrease of β- and δ-cells as well as to an almost total loss of α-cells, and an increase of *ghrelin*$^{+}$ *glucagon*$^{-}$ ε-cells was observed in Sey^{NEU} mice (Heller et al, 2005). Finally, Pax6 function was shown to be required for maintaining normal β-cells functions after birth (Ashery-Padan et al, 2004). Because of its genome duplication, the zebrafish possesses two pax6 genes but only *pax6b* is expressed in endocrine pancreas.

Fig. 8: Expression pattern of the two pax6 in zebrafish. Embryos at 24 hpf. The upper panel represents the expression of pax6a, which is detectable in the eyes and nervous system. The lower panel represents pax6b, which is expressed also in the pancreas. *In situ* hybridizations adapted from (Thisse et al, 2004; Thisse & Thisse, 2005).

Both are active in ocular and brain structures, but only one, *pax6b*, is expressed in pancreatic endocrine cells because of sequence divergence within the pancreatic cis-regulatory regions.

As in mice, it is expressed in all endocrine cell types (Delporte et al, 2008; Kleinjan et al, 2008).

1.2.4.3.6. Hb9

Hb9 is a homeodomain protein; only one hb9 gene is known in mammals but two related genes hb9 and mnr2 were identified in chicken. They both belong to the mnx protein family. Hb9 in vertebrates has conserved function in motoneuron differentiation and pancreas development (Wendik et al, 2004). In all vertebrates the expression pattern of Hb9 genes is conserved in motoneurons, the pancreatic endoderm, differentiating beta-cells and in different mesodermal structures including the posterior notochord (Harrison et al, 1999; Thaler et al, 1999; Wendik et al, 2004).

1.3. Endocrine pancreas function and dysfunction

1.3.1. Glucose homeostasis

The endocrine pancreas main function is the regulation of the glucose homeostasis; the two principal hormones involved in this process are insulin and glucagon, which possess opposite actions. Food intake increases the blood glucose level. In response to this

increase, the pancreatic β-cells secrete insulin that will lessen the glycaemia by inhibiting hepatic gluconeogenesis and glycogenolysis and stimulating glucose intake by the liver, the skeletal muscles and the fat cells (Fig. 9). Besides these punctual releases due to food intake, small insulin quantities are continuously released in the plasma, the β-cells having an intrinsic pacemaker that generates an insulin release every ~15 min (Chou et al, 1992; Kahn et al, 2009).

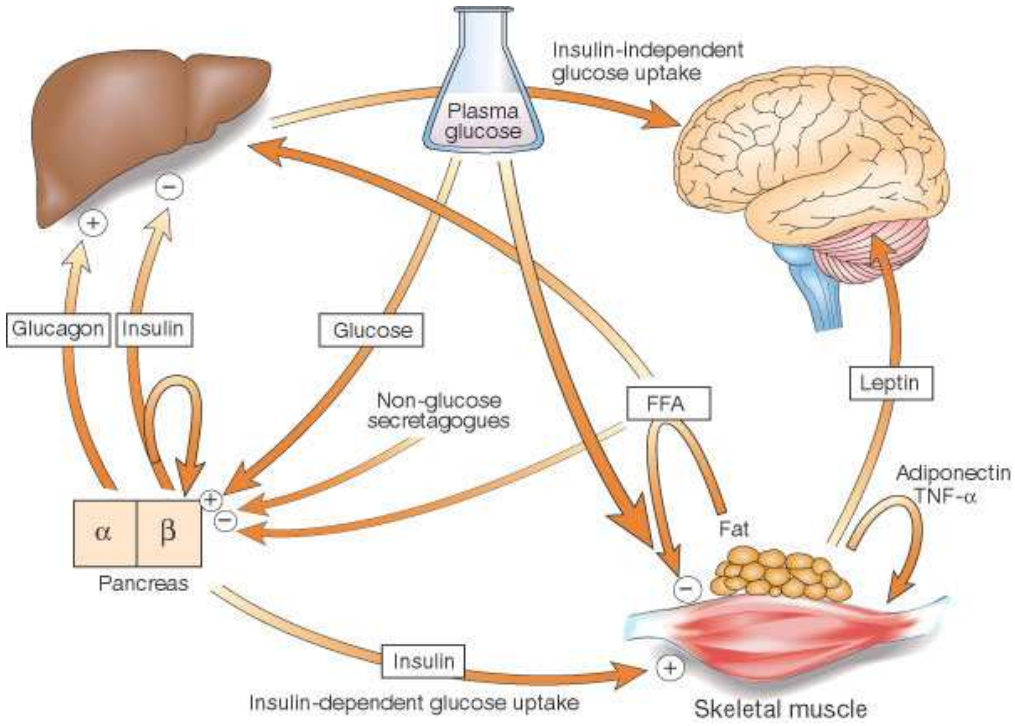

Fig. 9: Insulin is secreted from the β-cells of the pancreas in response to elevations in plasma glucose. The hormone decreases glucose production from the liver, and increases glucose uptake, utilization and storage in fat and muscle. The fat cell is important in metabolic regulation, releasing Free fatty acids (FFAs) that reduce glucose uptake in muscle, insulin secretion from the β-cell, and increase glucose production from the liver. The fat cell can also secrete hormones such as leptin, adiponectin and TNF, which regulate food intake, energy expenditure and insulin sensitivity. Adapted from (Saltiel & Kahn, 2001).

Contrary to insulin, glucagon secretion is stimulated by low blood glucose levels. This hormone promotes gluconeogenesis and glycogenolysis and its release leads thus to an elevation of the blood sugar.

The three other pancreatic hormones have indirect effects on the regulation of the blood glucose levels. Somatostatin inhibits secretions of both endocrine and exocrine pancreas. The pancreatic polypeptide inhibits the enzymatic secretion of the pancreas. Mainly produced by the gastric mucosa, ghrelin has been recently found to be also expressed by the ε-cells of the pancreas (Kageyama et al, 2005; Prado et al, 2004); it is an orexigenic

(i.e. that increases the appetite) hormone, the only known hormone that powerfully increases the food intake (Tschop et al, 2000). Moreover, ghrelin decreases insulin secretion (Cummings et al, 2007).

1.3.2. Diabetes

The diabetes mellitus, commonly referred as diabetes, is a group of pancreatic disorders characterized by a hyperglycaemia due to impaired insulin secretion or impaired insulin sensitivity. Moreover, this disease is frequently associated with various kinds of micro- (nephro-, neuro- and retinophathy) and macro- (coronary artery diseases and peripheral vascular diseases) vascular complications.

Mainly because of environmental factors (sedentary lifestyle and overfeeding), the prevalence of diabetes in the western societies has reached epidemic proportions, especially the type 2 diabetes: in 2014, almost 52 millions of European (7.9% of the population) lived with diabetes and the number of diabetic patients in the world will rise from 287 to 492 million between 2014 and 2035. Moreover, 1 person died every 7 seconds from diabetes in 2014 and this number is expected to double by 2035 (statistics from Internal Diabetes Federation, www.idf.org; the Juvenile Diabetes Research Foundation International, www.jdrf.org and the World Health Organization, www.who.int/topics/diabetes_mellitus/en).

There are different forms of diabetes, the most common forms being the type1 and type 2 diabetes. Along these, a form of the disease due to a single gene mutation called the MODY also exists.

2. Cyclic AMP (cAMP) signaling

Induction of cAMP second messenger pathway promotes β-cell growth and survival by GLP-1 receptor activation (Hui et al, 2003). cAMP stimulates the expression of severql target genes via PKA-mediated phosphorylation of CREB and its paralogs ATF- and CREM (Gonzalez & Montminy, 1989) Glucose and GLP1 converge on CREB, via calcium and cAMP to regulate β-cell gene expression. The Montminy lab demonstrated a requirement for CREB function in glucose homeostasis and islet-cell survival, using transgenic mice that were deficient in CREB activity in β-cells (Jhala et al, 2003).

2.1. Cyclic adenosine 3′,5′-monophosphate (cAMP)

Cyclic adenosine 3′,5′-monophosphate (cAMP) was the first “second messenger” to be identified and plays fundamental roles in cellular responses to many hormones and neurotransmitters (Sutherland & Rall, 1958).

The intracellular levels of cAMP are regulated by the balance between the activities of two enzymes (Fig. 10): adenylyl cyclase (AC) and cyclic nucleotide phosphodiesterase (PDE). Different isoforms of these enzymes are encoded by a large number of genes, which differ in their expression patterns and mechanisms of regulation, generating cell-type and stimulus-specific responses (McKnight, 1991).

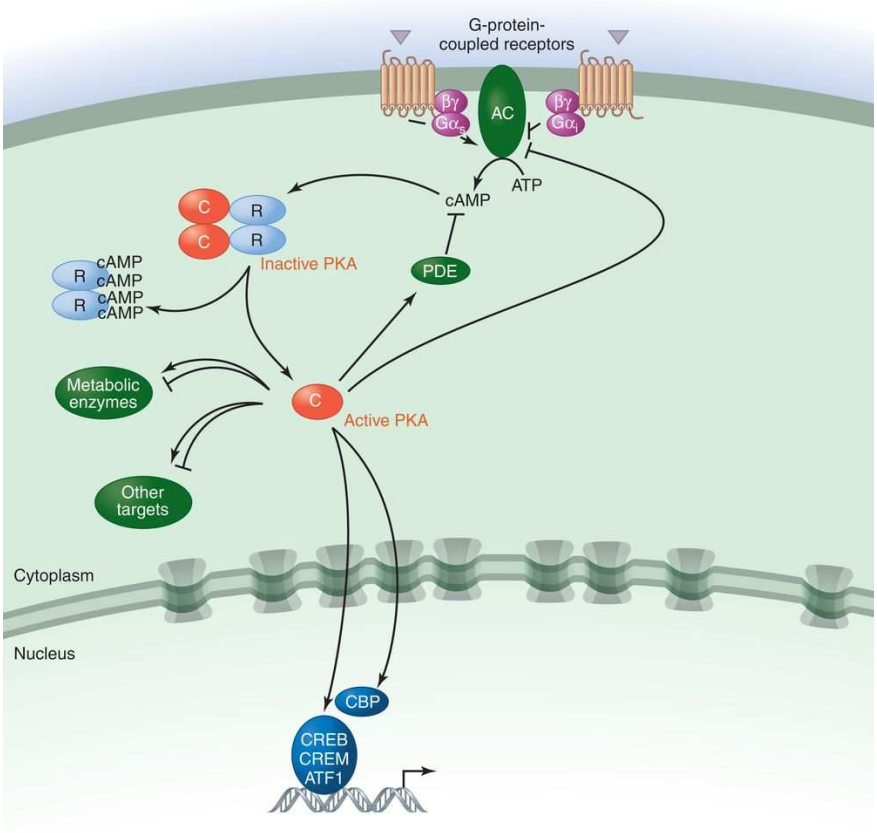

Fig. 10: Regulation of PKA adapted from (Fimia & Sassone-Corsi, 2001).

2.2. cAMP Signaling activation

Most ACs (soluble bicarbonate-regulated ACs are the exception) are activated downstream of G-protein-coupled receptors (GPCRs) such as the β adrenoceptor by interactions with the α subunit of the G_s protein (α_s). α_s is released from heterotrimeric αβγ G-protein complexes following binding of agonist ligands to GPCRs (e.g., epinephrine in the case of β adrenoceptors) and binds to and activates AC. The βγ subunits can also stimulate some AC isoforms. cAMP generated as a consequence of AC activation can activate several effectors, the most well studied of which is cAMP-dependent protein kinase (PKA; Fig. 10) (Pierce et al, 2002).

2.3. cAMP Signaling termination

Otherwise, AC activity can be inhibited by ligands that stimulate GPCRs coupled to G_i and/or cAMP can be degraded by PDEs. Indeed both ACs and PDEs are regulated positively and negatively by numerous other signaling pathways (Fig. 11), such as calcium signaling (through calmodulin [CaM], and calcineurin [also known as PP2B]), subunits of

other G proteins (e.g., α_i, α_o, and α_q proteins, and the $\beta\gamma$ subunits in some cases), inositol lipids (by PKC), and receptor tyrosine kinases (through the ERK MAP kinase and PKB) (Bruce et al, 2003; Goraya & Cooper, 2005; Yoshimasa et al, 1987). Crosstalk with other pathways provides further modulation of the signal strength and cell-type specificity, and feedforward signaling by PKA itself stimulates PDE4.

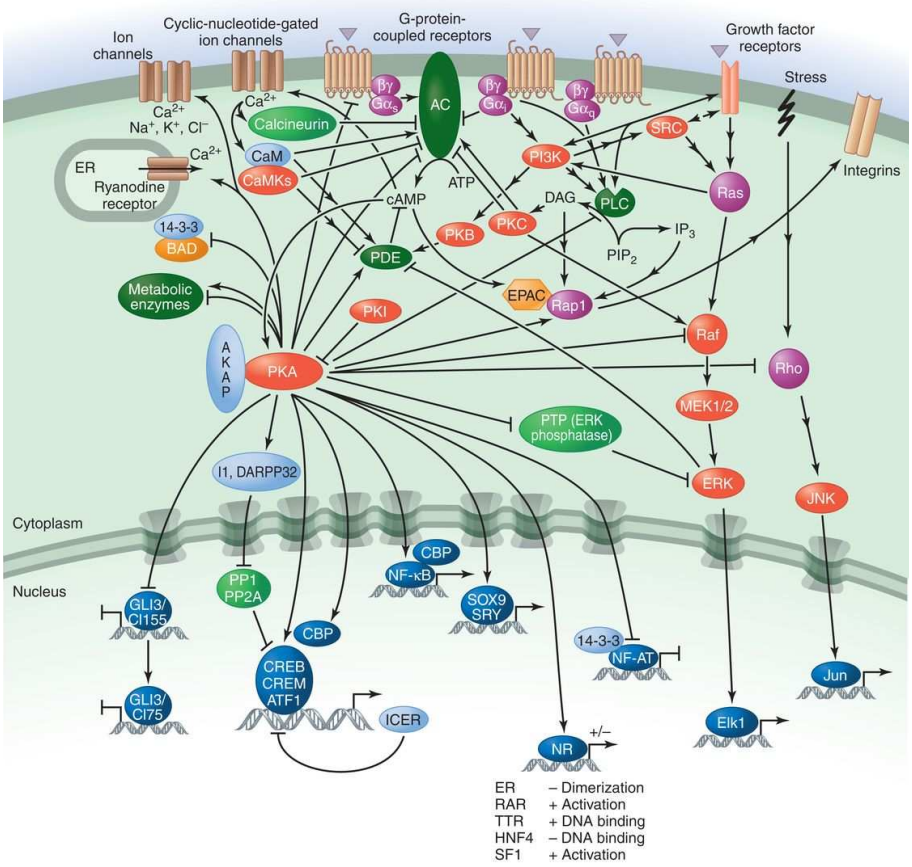

Fig. 11: the cAMP/PKA pathway adapted from(Fimia & Sassone-Corsi, 2001).

2.4. cAMP signaling effectors

There are three main effectors of cAMP: PKA, the guanine-nucleotide-exchange factor (GEF) EPAC (exchange proteins activated by cAMP) and cyclic-nucleotide-gated ion channels. Protein kinase (PKA), the best-understood target, is a symmetrical complex of two regulatory (R) subunits and two catalytic (C) subunits (there are several isoforms of both subunits). It is activated by the binding of cAMP to two sites on each of the R subunits, which causes their dissociation from the C subunits (Taylor et al, 1992).The catalytic activity of the C subunit is decreased by a protein kinase inhibitor (PKI), which

can also act as a chaperone and promote nuclear export of the C subunit, thereby decreasing nuclear functions of PKA. PKA-anchoring proteins (AKAPs) provide specificity in cAMP signal transduction by placing PKA close to specific effectors and substrates. They can also target it to particular subcellular locations and anchor it to ACs (for immediate local activation of PKA) or PDEs (to create local negative feedback loops for signal termination (Wong & Scott, 2004).

A large number of cytosolic and nuclear proteins have been identified as substrates for PKA (Tasken et al, 1997). PKA phosphorylates numerous metabolic enzymes, including glycogen synthase and phosphorylase kinase, which inhibits glycogen synthesis and promotes glycogen breakdown, respectively, and acetyl CoA carboxylase, which inhibits lipid synthesis. PKA also regulates other signaling pathways. For example, it phosphorylates and thereby inactivates phospholipase C (PLC) β2. In contrast, it activates MAP kinases; in this case, PKA promotes phosphorylation and dissociation of an inhibitory tyrosine phosphatase (PTP). PKA also decreases the activities of Raf and Rho and modulates ion channel permeability. In addition, it regulates the expression and activity of various ACs and PDEs.

In addition to the negative regulation by signals that inhibit AC or stimulate PDE activity, the action of PKA is counterbalanced by specific protein phosphatases, including PP1 and PP2A. PKA in turn can negatively regulate phosphatase activity by phosphorylating and activating specific PP1 inhibitors, such as DARPP32. PKA-promoted phosphorylation can also increase the activity of PP2A as part of a negative feedback mechanism.

Another important effector for cAMP is EPAC, a GEF that promotes activation of certain small GTPases (e.g., Rap1). A major function of Rap1 is to increase cell adhesion via integrin receptors (how this occurs is unclear) (Bos, 2003).

2.5. cAMP signaling transcription factors

Regulation of transcription by PKA is mainly achieved by direct phosphorylation of the transcription factors cAMP-response element-binding protein (CREB), cAMP-responsive modulator (CREM), and ATF1. Phosphorylation is a crucial event because it allows these proteins to interact with the transcriptional coactivators CREB-binding protein (CBP) and p300 when bound to cAMP-response elements (CREs) in target genes (Mayr & Montminy, 2001). The *CREM* gene also encodes the powerful repressor ICER, which

negatively feeds back on cAMP-induced transcription (Sassone-Corsi, 1995). Note, however, that the picture is more complex, because CREB, CREM, and ATF1 can all be phosphorylated by many different kinases, and PKA can also influence the activity of other transcription factors, including some nuclear receptors.

2.6. cAMP modulates ion channels

Finally, cAMP can bind to and modulate the function of a family of cyclic-nucleotide-gated ion channels. These are relatively nonselective cation channels that conduct calcium. Calcium stimulates CaM and CaM-dependent kinases and, in turn, modulates cAMP production by regulating the activity of ACs and PDEs (Zaccolo & Pozzan, 2003). The channels are also permeable to sodium and potassium, which can alter the membrane potential in electrically active cells.

AIM OF THE WORK

In this work, we are studying zebrafish development. This animal model is really interesting in development studies. Taking advantage of its external development and translucide embryos, we can use it to characterize the involvement of genes during developmental life. Moreover, its development is faster compared to mammals, and the key regulators in development process are conserved between mice and zebrafish. So zebrafish is the perfect model to study organogenesis.

In our laboratory, we are interested to study the pancreas development. Indeed the pancreas, composed by two tissues, the exocrine and the endocrine components, is an organ affected by several pathologies, including cancer and diabetes. The cancer is more associated with the exocrine pancreas. The diabetes is mainly caused by a dysregulation in endocrine pancreas and, more precisely, in β-cells. Given the importance of diabetes in the world, it is indispensable to understand the β-cells differentiation process, in order to develop news therapeutics agents. So the goal of this project is to determine the process of pancreatic differentiation.

Previously, several studies have shown that Pax6b is a key regulator in pancreas development and β-cell differentiation in mice and zebrafish (Heller et al, 2004; Sander et al, 1997; St-Onge et al, 1997; Verbruggen et al, 2010). By taking advantage of this knowledge, we aim to determine all the cascades activating this transcription factor and therefore pancreas development. In this regard, several studies have also shown the involvement of CREB signalling in β-cell differentiation in mice and humans. Thus, in this thesis we also aim to generate CREB reporter transgenic zebrafish lines that can be helpful in the dissection of molecular interactions inside the pancreatic tissue and at the whole-animal level.

MATERIALS & METHODS

1. Embryos

Zebrafish (*Danio rerio*) were raised and cared for according to standard protocols. Wild-type embryos of the Giotto Leo and Umbria strains were used and staged according to Kimmel (Kimmel et al, 1995). Homozygous mutants were obtained by mating heterozygous fish for the *pax6b mutant*^{sa0086} from the Sanger Institute.

2. Whole-mount *in situ* hybridizations (WISHs)

Single and double *in situ* hybridizations were performed as described (Hauptmann and Gerster, 1994; Mavropoulos et al., 2005). Anti-sense RNA probes were prepared by transcribing linearized cDNA clones with SP6, T7 or T3 polymerases using digoxigenin labeling mix (Roche) or DNP-11-UTP (TSA™ Plus system, Perkin Elmer). They were subsequently purified on NucAway spin columns (Ambion) and ethanol-precipitated. The riboprobes used were: *neuroD (Korzh et al, 1998), glucagon (Argenton et al, 1999), insulin (Milewski et al, 1998), somatostatin (Argenton et al, 1999), ghrelin (Pauls et al, 2007), sox4(Mavropoulos et al, 2005), pdx1 (Milewski et al, 1998), pax6b(Nornes et al, 1998) , isl1 (Korzh et al, 1993)*. The NBT/BCIP staining was carefully monitored in order to avoid an over-staining which would have prevented us from visualizing the individual cell boundaries.

3. Immunohistochemistry

Fluorescent immunochemistry was performed on embryos fixed for two hours in PFA 2%, using chicken anti-GFP (Life Technologies) as primary antibody (1/100) and Alexa-488 anti-chicken (1/100) (Molecular Probes) as secondary antibody. The washes were performed in PBS/Triton 0.3% and the antibody fixation in PBS/Triton 0.3%/BSA 4%. Yolks were roughly removed following fixation.

4. Generation of Tg(6XCRE:GFP) and Tg(6XCRE:nlsmCherry) lines

A cassette containing six multimerized CREB response elements (- 5’ TGACGTCA 3’ -) (Montminy et al 1986, Short et al 1986, Comb et al 1986) were placed upstream of a rabbit beta-globulin minimal promoter (Fortin et al., 2009). The original plasmid, containing 6XCRE-Luc, was kindly provided from the laboratory of Alan S. Kopin. These repeated elements (6XCRE) were then cloned in a 5’-entry vector, following the manufacturer's guidelines (Invitrogen Multisite Gateway System, CA). The resulting Gateway 5’ entry vector was recombined with a middle entry vector containing a reporter gene (EGFP, nls-mCherry), pMEvector (pME-EGFP,pME-nlsmCherry), and a 3’ entry vector containing the SV40-polyA sequence (p3E-polyA) (Kwan et al, 2007). 25–50 pg of the obtained Tol2 vector, containing the CREB- responsive transgene, was co-injected together with 25 pg of *in vitro* synthesized Tol2 Transposase mRNA into one-cell stage wild-type embryos. Mosaic transgenic fish were selected at approximately 24 h post-fertilization (hpf) for fluorescent expression and raised to the adult stage for screening.

Positive founders were selected for the fluorescence level of their offspring in areas of suspected CREB activity, and by checking responsiveness of reporter expression to CREB signaling modulators (8-Br-cAMP, 8-Cl-cAMP and noradrenaline).

5. Morpholino micro-injection

Morpholino oligos (MO) were synthesized by Gene Tools (Corvalis, OR). Each MO was resuspended in Danieau’s solution at the stock concentration of 1 mM or 2 mM. For injection, this stock solution was diluted as specified in Danieau’s solution. Rhodamine dextran was added at 0.5% to the samples, to allow checking the injection efficiency.

Micro-injections of MO targeting *creb3l2* were carried out by injecting morpholinos previously tested by Melville *et al*. (2011). MO-*creb3l2* targeting intron3-exon4 boundary: 5’-CAGACCTGGACAACAGCATGACACT-3’. Three ng of MO-*creb3l2* were injected into one- to two-cell stage embryos. A standard control MO (MO cont) has also been designed by Gene Tools in a way that it should have no target and no significant biological activity.

6. Chemical treatments

The following drugs were used to modify CREB signaling:

8-Cl-cAMP (SIC, Italy): cAMP agonist; stock solution 25 mM; final concentration 250 µM diluted in water (Weissinger et al, 2004).
8-Br-cAMP (Sigma Aldrich): cAMP antagonist; stock solution 25 mM; final concentration 250 µM diluted in water (Gjertsen et al, 1995).

Noradrelanine (Sigma Aldrich): cAMP agonist; stock solution 100 mM; final concentration 100 µM diluted in water.

All substances have been solubilized in water. The drugs have been diluted in zebrafish embryo medium containing 2 mM 1-phenyl-2-thiourea (PTU), to prevent pigmentation.
The larvae were treated for 48 hpf, starting from 3 dpf, and analyzed at 5 dpf.

7. Confocal microscopy analysis and co-localization measurements

Fluorescence was visualized using a Leica M165FC dissecting microscope, and then acquired using a Nikon C2H600L confocal microscope. For *in vivo* analyses, embryos and larvae were anesthetized with tricaine and mounted in 0.8% low melting agarose gel. EGFP and mCherry fluorescence were visualized by using 488 and 561 nm lasers, respectively, through 20x and 40x immersion objectives (Nikon). All images were analyzed with Nikon software.

8. Western blot analysis

The embryos were lysed in lysis buffer and heated at 95°C for 10 min. Equal amounts of protein were resolved by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes according to the manufacturer's protocol. The blots were blocked for 1 h at room temperature with 5% BSA in Tris-buffered saline with 0.1% Tween 20 and probed overnight at 4°C with 1/1000 rabbit anti-CREB (Cell Signaling). After three washes with Tris-buffered saline containing 0.1% Tween 20, the appropriate secondary antibody at a 1/3000 dilution was added for 1 h at room temperature. The bands were

visualized by enhanced chemiluminescence with an ECL kit (Pierce). Quantification was performed with ImageJ software.

RESULTS

PART I: ROLE OF PAX6b IN PANCREAS DEVELOPMENT

1. Study of the role of the transcription factor Pax6b on pancreas differentiation in zebrafish

The developmental dynamics of zebrafish pancreas are similar to mammals: the organ arises from two buds, a ventral and a dorsal one (Field et al, 2003), and the endocrine compartment produces all the different hormones: insulin, ghrelin, glucagon, somatostatin and pancreatic polypeptide (Argenton et al, 1999; Biemar et al, 2001). Notably, during vertebrate evolution, the signalling pathways leading to pancreas development have been highly conserved. Most of the regulatory genes, identified in human and genetically characterized in mouse, are also involved in zebrafish pancreas development with little or no modification in their genetic interactions. Therefore, the zebrafish is a good model to efficiently study signalling pathways involved in pancreas development.

In the first part of my work, performed in the Molecular Biology and Genetic Engineering Lab (Liège), I have been interested on the role of *pax6b* in pancreas development in *Danio rerio*

By injecting splicing morpholino targeting *pax6b* in zebrafish embryos, Verbruggen showed the crucial involvement of this gene in endocrine pancreas development. Indeed, in morphant embryos they observe a loss of insulin cells, reduction of somatostatin cells and an increase of ghrelin cells (Verbruggen et al, 2010). In order to confirm this peculiar pancreatic phenotype, we decided to use *pax6b* mutants.

1.1. Pancreatic phenotype of the pax6b mutant

The *pax6b* mutant, considered in this thesis, was identified at the Zebrafish Mutant Resource (available on the World Wide Web). Its allele (*sa0086*) harbors a C to A substitution changing codon 109 (Tyr) to a premature stop codon. Consequently, this allele encodes a Pax6b protein possessing a truncated paired domain and lacking the whole homeodomain plus the transactivation C-terminal domain (Fig. 12 A, B). Based on the *pax6* allelic mutant series described in mammals and in *Drosophila*, the *pax6b sa0086* allele is predicted to correspond to a null mutation.

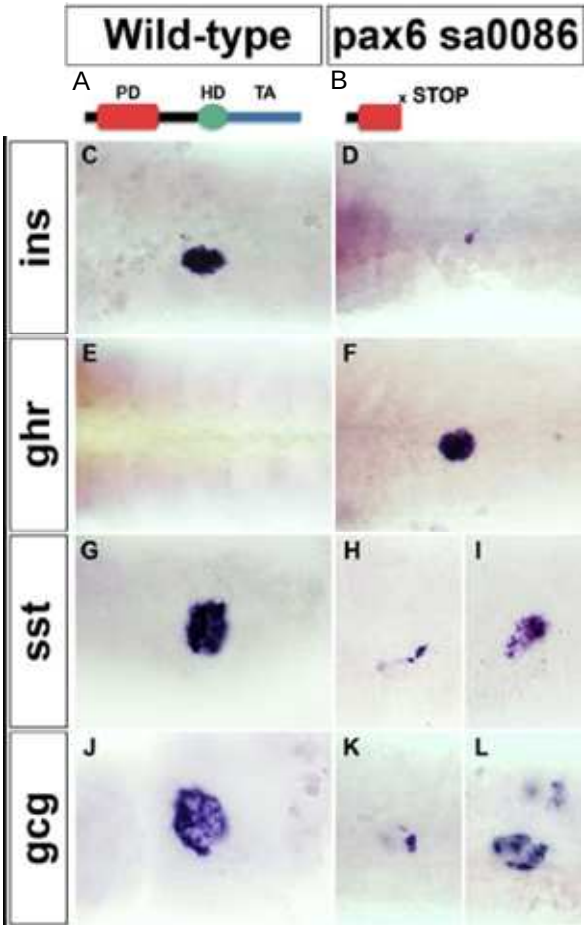

Fig.12: Pancreatic endocrine differentiation is perturbed in *sa0086 pax6b*-/- zebrafish mutants. A, B: schematic drawing of Pax6 structures in wild type and *sa0086* mutant. *C–L*, phenotype comparison of wild-type (left column) and *sa0086* mutant (right column) 3 dpf larvae. *C–L*, expression of pancreatic hormones by *in situ* hybridization. Ventral views of the pancreatic area are shown with the anterior side to the left. *ins*, insulin; *gcg*, glucagon; *sst*, somatostatin; *ghr*, ghrelin. The number of insulin and somatostatin cells is significantly reduced in the mutants, whereas ghrelin cells increase dramatically. A high variability is observed for glucagon expression among mutants.

The pancreatic phenotype of the homozygous *pax6bsa0086* mutants was similar to the morphants with almost an absence of beta cells and a strong increase of ghrelin cells in all homozygous mutants (Fig. 12, C–F). A clear reduction of somatostatin expressing cells was also detected, as in morphants, but the level of reduction varied between mutant embryos, one mutant displaying a complete lack of delta cells, nine mutants containing 1–5 delta cells, and three possessing 6–10 delta cells (Fig. 12, G–I). With regards to glucagon-expressing alpha cells, some mutants were presenting with an almost normal level or a slight decrease of alpha cells, while other mutants displayed a severe reduction (Fig. 12, J–L). Thus, the analysis of the *pax6bsa0086* mutant strengthens the knockdown data,

confirming the crucial role of Pax6b in the differentiation of endocrine cells in the zebrafish pancreas.

1.2. Establishment of the *pax6b* mutant x *pax6:GFP* transgenic line

To see if the transcription factor pax6b has an autoregulatory loop, we checked if the pax6:GFP transgene is affected in the *pax6b* sa0086 mutant. For this purpose, we crossed the pax6b mutant line with a pax6:GFP line and we looked at the progeny.

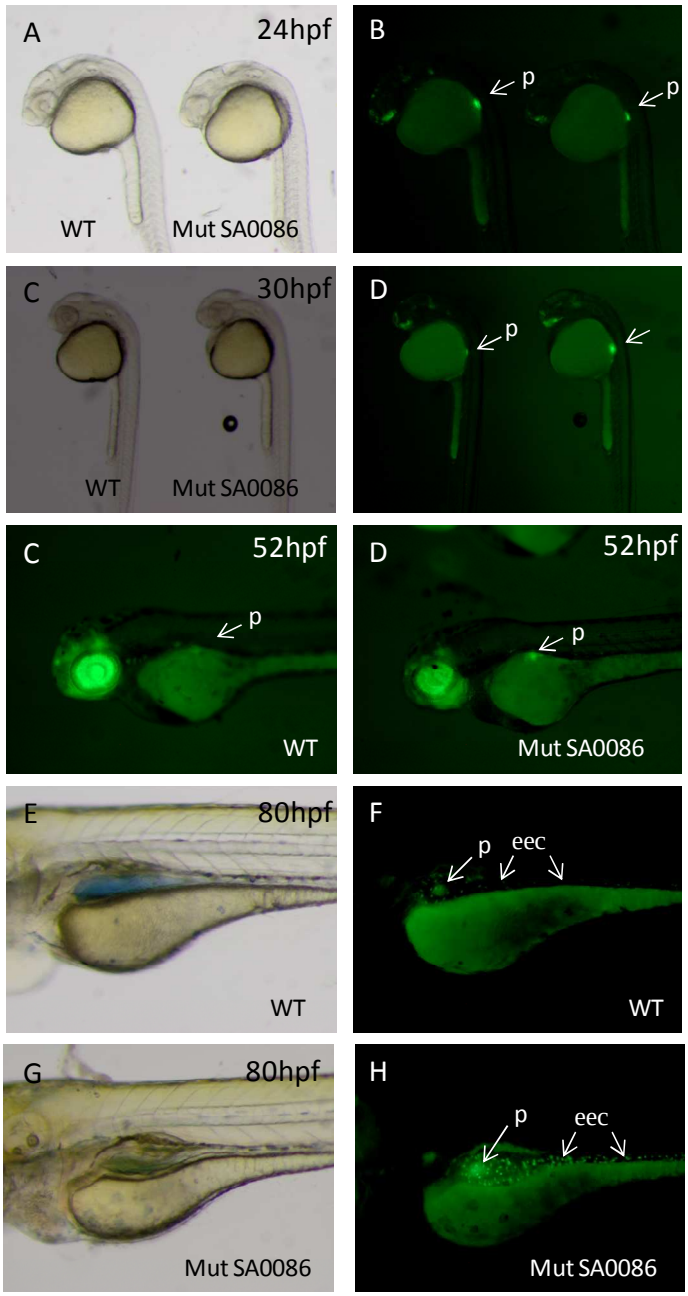

Fig. 13: Expression of the pax6:GFP transgene in the *pax6b* mutant. Pax6:GFP expression in WT embryos and *pax6b* mutants at 24 hpf (A,B), 30 hpf (C,D), 52 hpf (E,F), 80 hpf (G,H). p: pancreas, eec: enteroendocrine cells.

As we can see in Figure 13, the GFP is still expressed in the pax6 mutant. From 1 day to almost 80 hpf the fluorescence is not affected. We can still see also the enteroendocrine cells. Thus, there is no autoregulation of the pax6 transcription factor on itself.

1.3. Expression analysis of different pancreatic transcription factors in the pax6b mutant

Given the strong pancreatic phenotype in *pax6b* mutants, we wondered if *pax6b* controls the expression of transcription factors known to be involved in pancreatic endocrine cell differentiation. Thus, we analyzed their expression in the *pax6b* mutants. We performed *in situ* hybridization on fixed embryos at 25 and 48 hpf, using different probes (*pdx1, hb9, isl1, neuroD, sox4b, pax*4, *nkx2.2*).

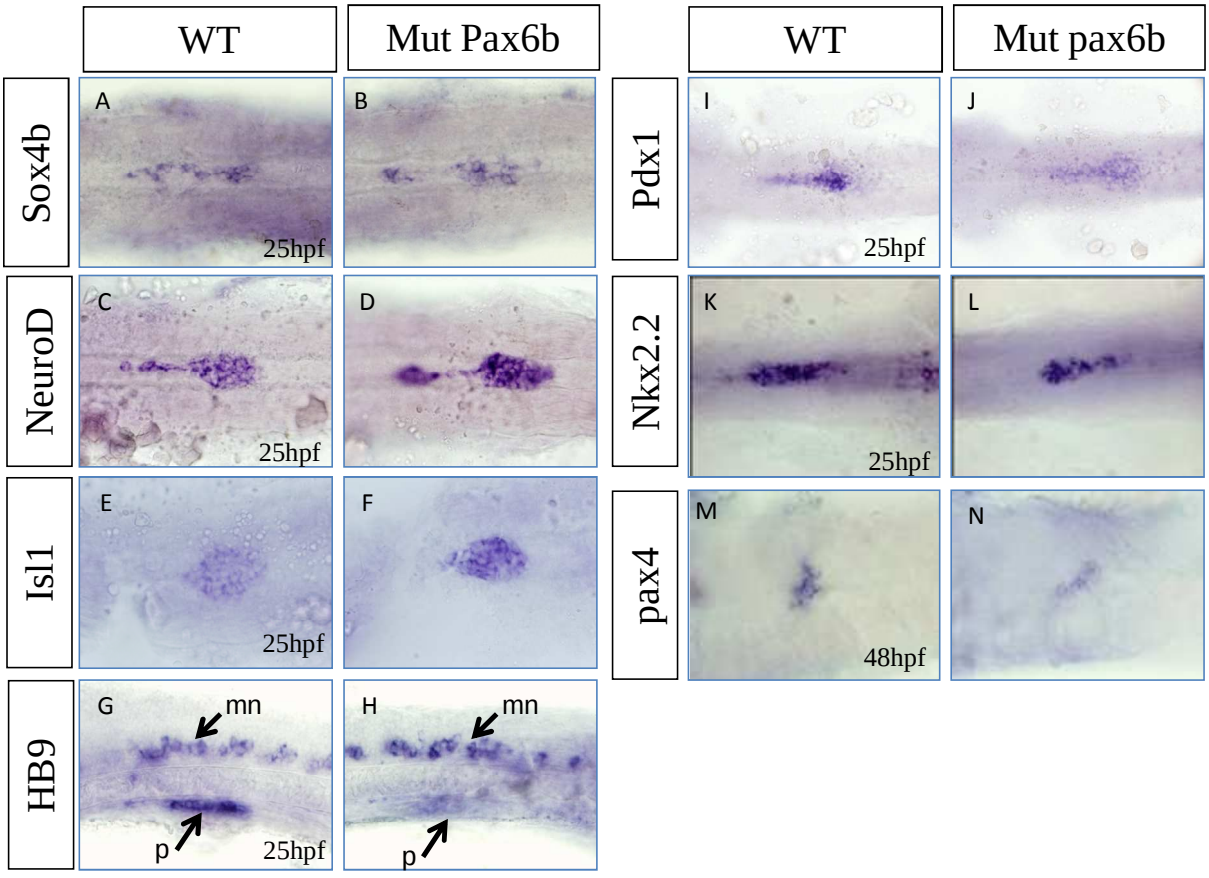

Fig. 14: Pancreatic transcription factor expression in *pax6b* mutants. Transcription factor expression in the pancreatic region of wild type embryos (A,C, E, G, I, K, M) and *pax6b* mutants (B, D, F, H, J, L, N) at 25 hpf (A-L) and 48 hpf (M-N). Expression of *sox4b* (A, B), *neuroD* (C, D), *isl1* (E, F), *hb9* (G, H), *pdx1* (I, J), *nkx2.2* (K, L), *pax4* (M, N). A-F and I-N are ventral views, G-H are lateral views, all with anterior to the left. p: pancreas, mn: motor neurons.

We observed a slight increase of the expression level of *NeuroD* and *isl1* in the mutants. The *sox4b* expression is not affected. We observed also a slight decrease of *pdx1*. We observed a strong decrease of the expression of *hb9* in the pancreas, whereas its expression remains the same in motor neurons of *pax6b* mutant and WT embryos.

1.4. *hb9* expression is initiated but not maintained in *Pax6b*$^{-/-}$ mutants

We noted that the expression of *hb9* is strongly affected in the pancreas of *pax6b* mutants, so we wondered from when and until when the expression of *hb9* is affected. To do this we performed *in situ* hybridization with the *hb9* RNA probe at different developmental stages of pancreas development: 18-somite stage, 25 hpf, 30 hpf, 35 hpf.

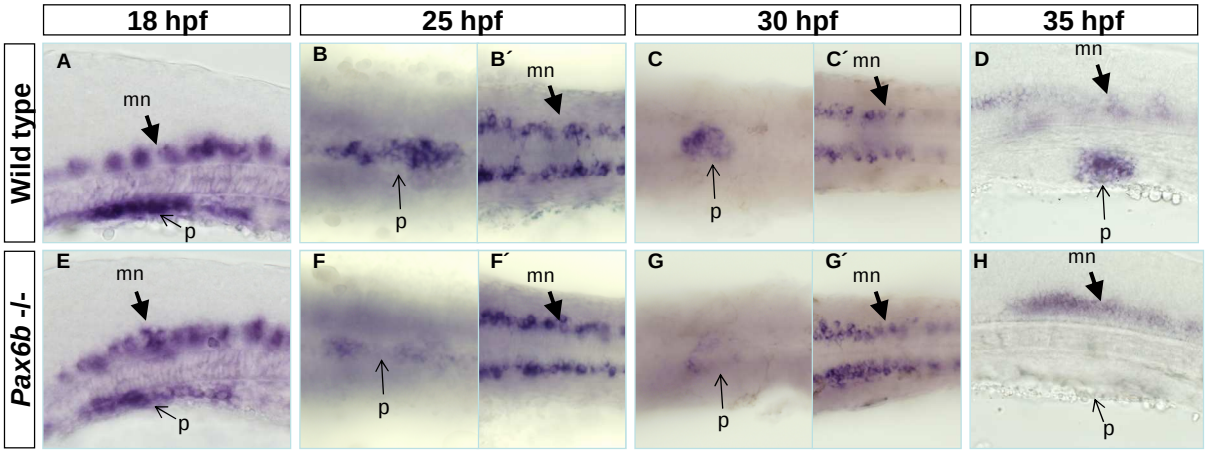

Fig. 15: *hb9* expression is initiated but not maintained in *Pax6b* deficient embryos. *hb9* expression in wild type embryos (A-D) and *pax6b* mutants (E-H) at 18 hpf (A, E), 25 hpf (B, F), 30 hpf (C, G) and 35 hpf (D, H). Note that *hb9* expression in pancreas (arrow) decreases during development in mutants, whereas expression in motor neurons (big black arrowhead) is not affected. Embryos in A,E,D,H are shown in lateral view with anterior to the left, and embryos in B, F, E, G are ventral views. B, F, C, G are focused on the pancreas; B', F', C', G' are focused on the motor neurons.

The pax6b mutants showed a reduced number of hb9 expressing pancreatic cells, and the expression levels per cell appear much weaker as revealed by comparing staining intensities in the spinal cord and pancreas of mutants and wild type embryos (Fig. 15). At 18 hpf, hb9 expression in pax6b mutants is similar to wild type embryos (Fig. 15 A, E), whereas between 25 hpf and 30 hpf absence of Pax6b leads to the progressive reduction of hb9 expression in the pancreas (Fig. 15 B, C, F, G). At 35 hpf no hb9 expression could be detected in the endocrine pancreas (Fig. 15 D, H). Importantly, at all analyzed stages, motor neuron hb9 expression in pax6b$^{-/-}$ mutants was not affected (Fig. 15 A-H), revealing the specific role for Pax6b in regulating hb9 in the endocrine pancreas.

1.5. Establishment of the pax6b mutant x hb9:GFP transgenic line

We had previously observed a decrease of the expression of the Hb9 mRNA in the pancreas of the *pax6b* zebrafish mutants. We thought that it could be interesting to follow *in vivo* the expression of the transcription factor Hb9, during embryogenesis and larval growth. To achieve this goal, we crossed *pax6b* mutant heterozygous fish with transgenic fish *hb9:GFP*. After genotyping and cross of the *pax6b*$^{+/-}$ transgenic line, we followed at the microscope the expression of the transgene in homozygous transgenic embryos (Fig 16). Surprisingly, in contrast with the endogenous expression of *hb9*, we did not observe difference between WT and p*ax6b* mutants in the pancreas. At 25 hpf *hb9*:GFP expression in *pax6b* mutants is similar to that in wild type embryos (Fig. 16 A, B). At 48 hpf *hb9*:GFP expression in *pax6b* mutants is also similar to that in wild type embryos (Fig. 16 C, D). At 72 hpf *hb9*:GFP expression in pax6b mutants clearly decreases in the pancreas, but it is still present (Fig. 16 E, F).

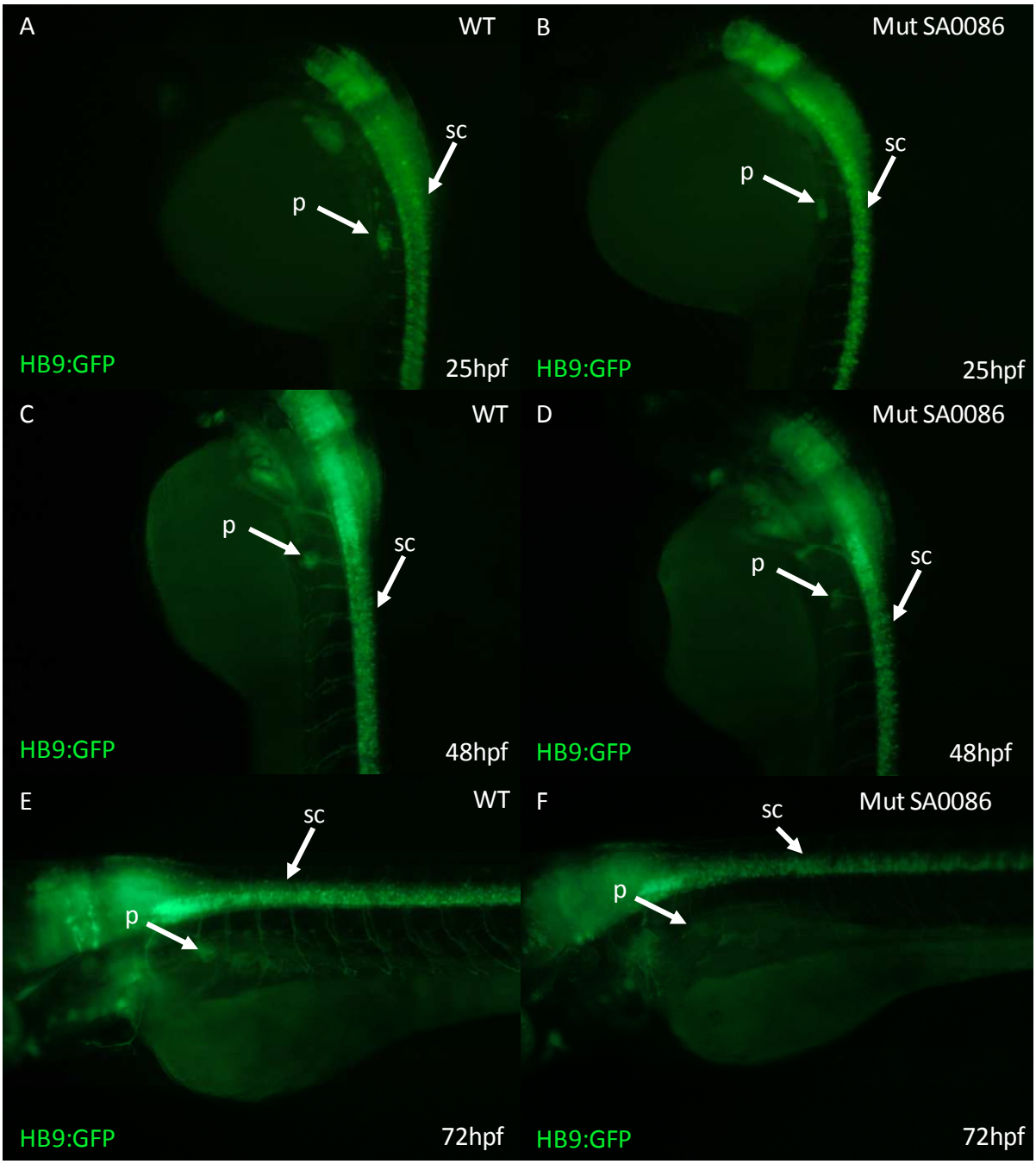

Fig. 16: *hb9:*GFP expression is maintained in *Pax6b* deficient embryos. *hb9:GFP* expression in wild type embryos (A, C, E) and *pax6b* mutants (B, D, F) at 25 hpf (A, B), 48 hpf (C, D) and 72 hpf (E, F). Note that *hb9* expression in pancreas (p) slowly decreases during development in mutants, whereas expression in the spinal cord (sc) is not affected.

The difference of the GFP protein and *hb9* mRNA expression levels in the pancreas of 24 hpf *pax6b* mutants could be due to the fact that the GFP is a very stable protein that remains in the cells even when mRNA expression has stopped. To test this, we performed

in situ hybridization with a GFP RNA probe at different developmental stages of pancreas development: 18-somite stage, 25 hpf, 30 hpf and 35 hpf in *pax6b* mutant embryos (Fig. 17).

At 18 hpf, *hb9* expression in pax6b mutants is similar to that in wild type embryos (Fig. 17 A, D), whereas between 25 hpf and 30 hpf absence of Pax6b leads to the progressive reduction of *hb9* expression in the pancreas (Fig. 17 B, C, E, F). Notably, at all considered stages, motor neuron *hb9* expression in pax6b$^{-/-}$ mutants was not affected (Fig. 17 A-F), revealing a similar expression of *hb9* mRNA and GFP mRNA from the *hb9*:GFP transgenic line.

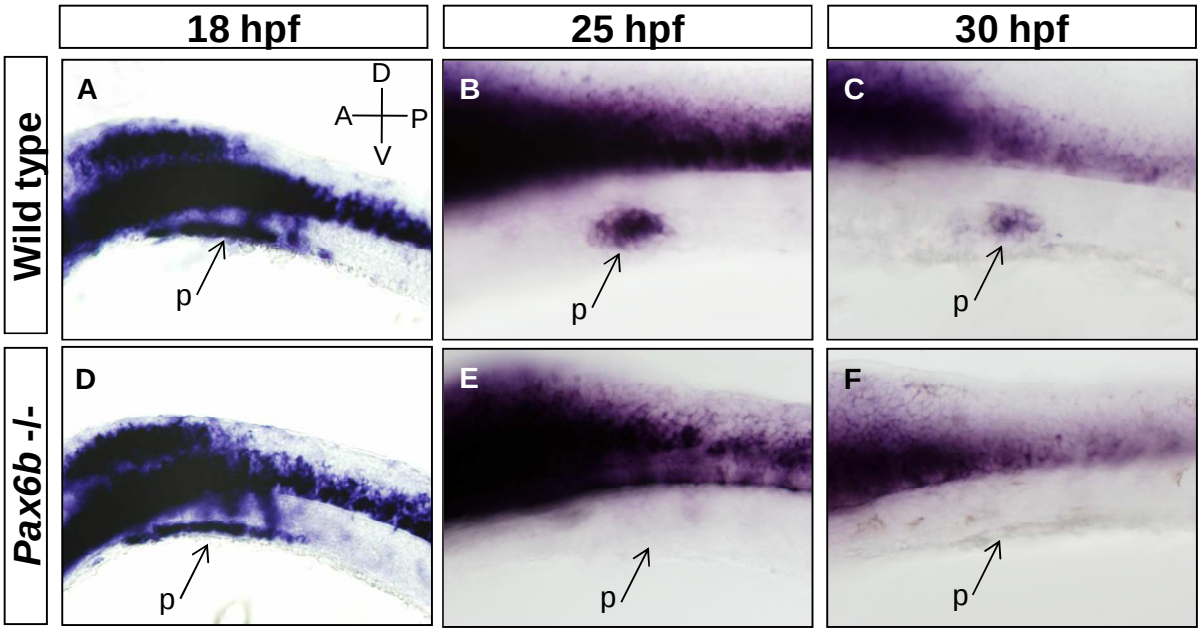

Fig. 17: GFP mRNA expression is initiated but not maintained in *Pax6b* deficient embryos. GFP mRNA expression in wild type embryos (A-C) and *pax6b* mutants (D-F) at 18 hpf (A, D), 25 hpf (B, E), and 30 hpf (C, F). All lateral views, with anterior to the left.

1.6. Beta cell fate progenitors take on an epsilon fate in absence of pax6b

Due to the fact that the GFP is a very stable protein that remains in the cells even when mRNA expression has stopped, we tested the hypothesis that the absence of pax6b causes progenitors cells that are fated to become beta cells to instead differentiate as epsilon cells. To enable recognition of cells that would normally differentiate as beta cells we used the hb9:GFP x pax6b mutant line. At 30 hpf the endogenous hb9 gene is no longer expressed in *pax6b* deficient embryos but the GFP in *hb9*:GFP is still detectable in *pax6* deficient embryos. Hb9 at 24 hpf and 48 hpf is exclusively expressed in insulin-expressing cells (Arkipova et al 2012). Thus, we used the GFP to track cells that should be insulin-producing cells in *pax6b* mutants.

We performed an *in situ* hybridization/immunostaining against the GFP (Fig. 18 A-R) and different hormones: glucagon (A-F), somatostatin (G-L), ghrelin (M-R).

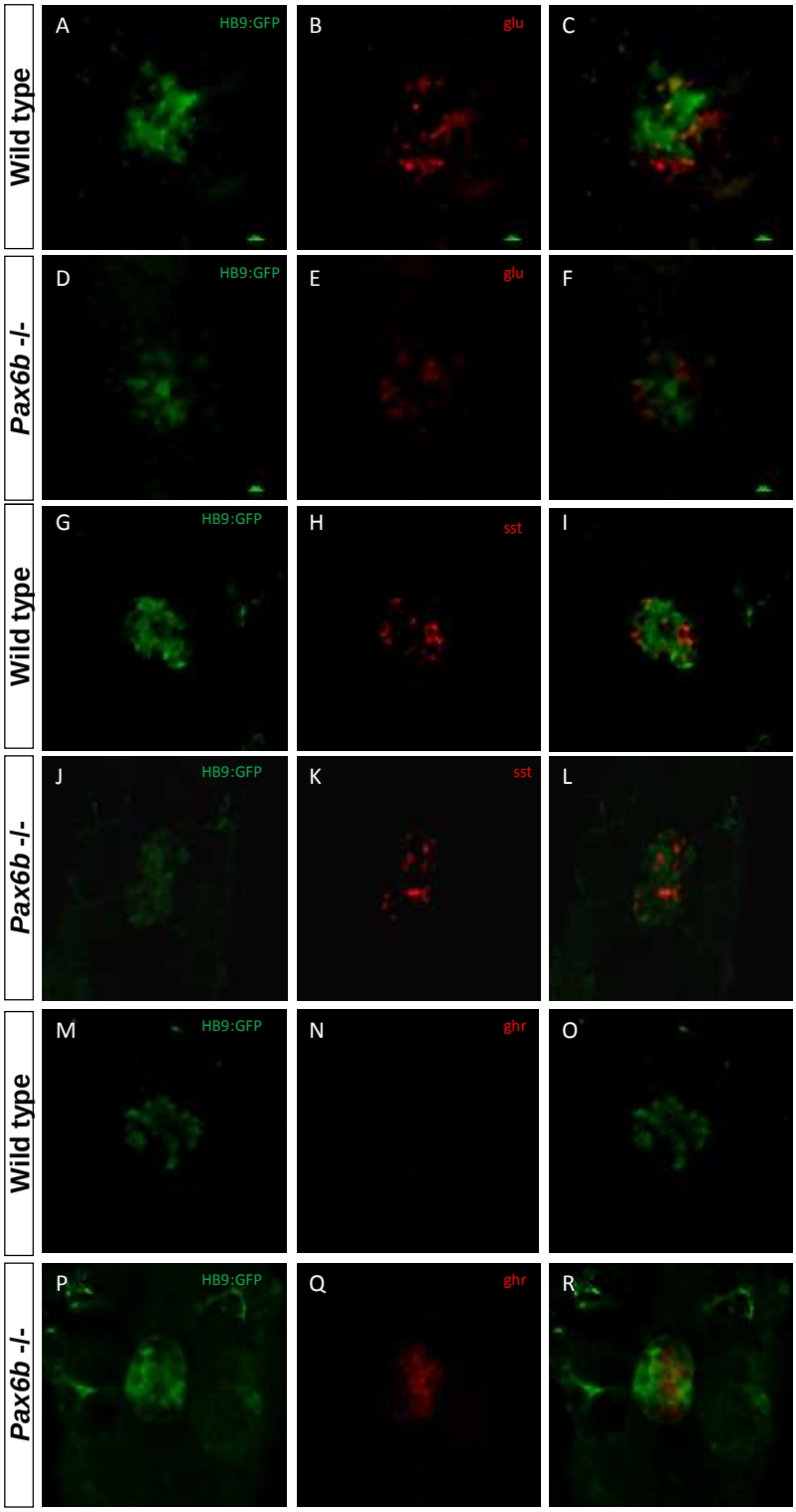

Fig. 18: Beta cell fated progenitors take on an epsilon fate in absence of Pax6b. Confocal images of 30 hpf *hb9*:GFP embryos WT (A, B, C, G, H, I, M, N, O) and *pax6b* mutants (D, E, F, J, K, L, P, Q, R). Whole mount immunolabeling for GFP (green) and *in situ* hybridization for the hormones (red): glucagon (A-F), somatostatin (G-L), ghrelin (M-R).

Analysis of the GFP positive cells in WT confirmed that these cells do not express glucagon (Fig 18 A-C), do not express somatostatin (Fig 18 G-I), and do not express ghrelin (Fig 18 M-O). By contrast, in pax6 mutants the ghrelin probe co-localizes with the GFP. This suggests a cell fate change where beta cell precursors give rise to ghrelin-expressing cells.

PART II: CHARACTERISATION OF A CREB SIGNALING REPORTER

1. Generation of a cAMP-CREB pathway reporter transgenic line.

We have generated a transgenic fish line expressing *in vivo* fluorescent reporter proteins under the control of CREB signaling pathway responsive elements.

To achieve this goal, we took advantage of *ad hoc* transgenesis methods. The Tol2 technology has revolutionized the transgenesis approaches for aquatic vertebrates, such as zebrafish. This method relies on the microinjection into fertilized eggs of a transposon-donor plasmid named Tol2, and a synthetic mRNA encoding the transposase, to catalyze DNA excision and recombination within the targeted genome. To generate the transgene, we used a multimerized CREB responsive element (6X 5' **TGACGTCA**-3'). We generated a 5'-entry vectors, containing a multimerized cAMP response element (6XCRE: 6X cAMP Responsive Elements, -5' TGACGTCA 3'-), ligated upstream of the rabbit beta-globin minimal promoter. The first plasmid, containing 6XCRE-Luc, was kindly provided from the laboratory of Alan S. Kopin (Fortin et al, 2009). These repeated elements (6XCRE) were then cloned upstream of a **cytoplasmic GFP** or a **nuclear mCherry** coding sequence in the pTol2-Destination vector.

The transgenic reporter line is responsive through the transcription factor-mediated activation of the regulatory elements located upstream to the coding sequences of the reporter protein. The major goal of this second part of my PhD thesis has been the validation and the characterization of these cAMP/CREB transgenic zebrafish lines.

2. Analysis of the CREB reporter lines

The first step for the validation of the CRE reporter line is the spatiotemporal characterization of the expression pattern of the reporter protein.

The CREB response element (CRE) is activated maternally

Being CREB signaling highly involved in many developmental events, including gametogenesis processes, we verified if this pathway could be maternally activated in zebrafish. In our animal model, the zygotic transcription begins at the mid-blastula transition (4hpf). Before that stage, embryonic transcripts and proteins are maternally provided. So, in order to determine whether the CREB transgene is translated before the beginning of the zygotic transcription, we crossed one female carrier of the CREB transgene with one wild type male. As shown in Figure 19, we can observe that both GFP and mCherry CREB reporter proteins are already expressed at 3 hpf, before zygotic transcription. So the CREB response element (CRE) is maternally activated in the zebrafish.

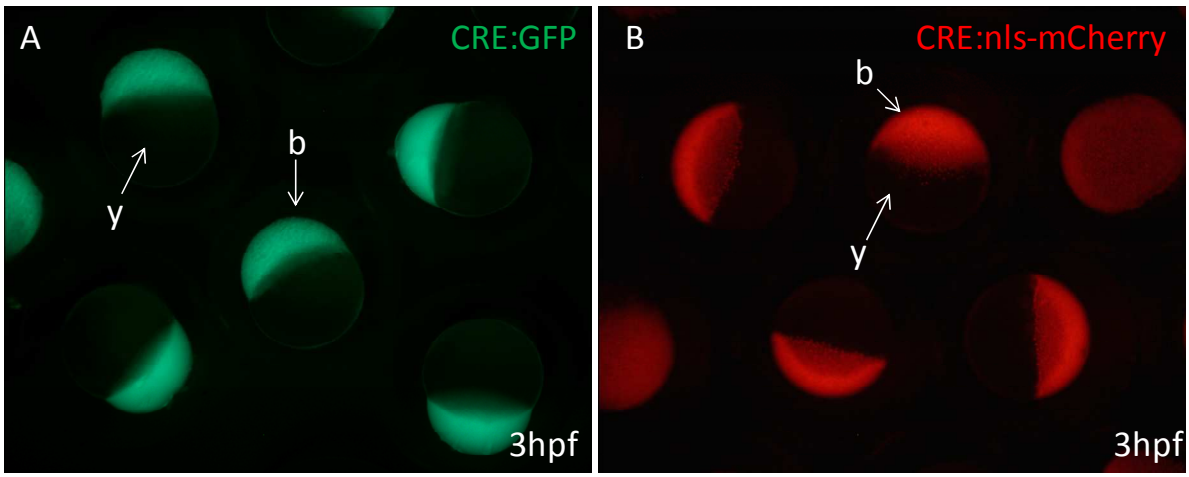

Fig. 19: Maternal expression of the CRE transgenes. CRE:GFP (A) and CRE:nls-mCherry (B) embryos at blastula stage (3dpf). The embryos come from an outcross between a CRE carrier female and a WT male. b: blastoderm, y: yolk.

1.1. CRE reporter expression in zebrafish embryos

Next, we analyzed the expression pattern of the CREB response element at 24 hpf, stage where main organ *primordia* are formed. At 24 hpf the embryo autonomously transcribes its genome. As shown in Figure 20, the CRE reporters GFP and mCherry proteins are expressed in muscles, anal region and eyes.

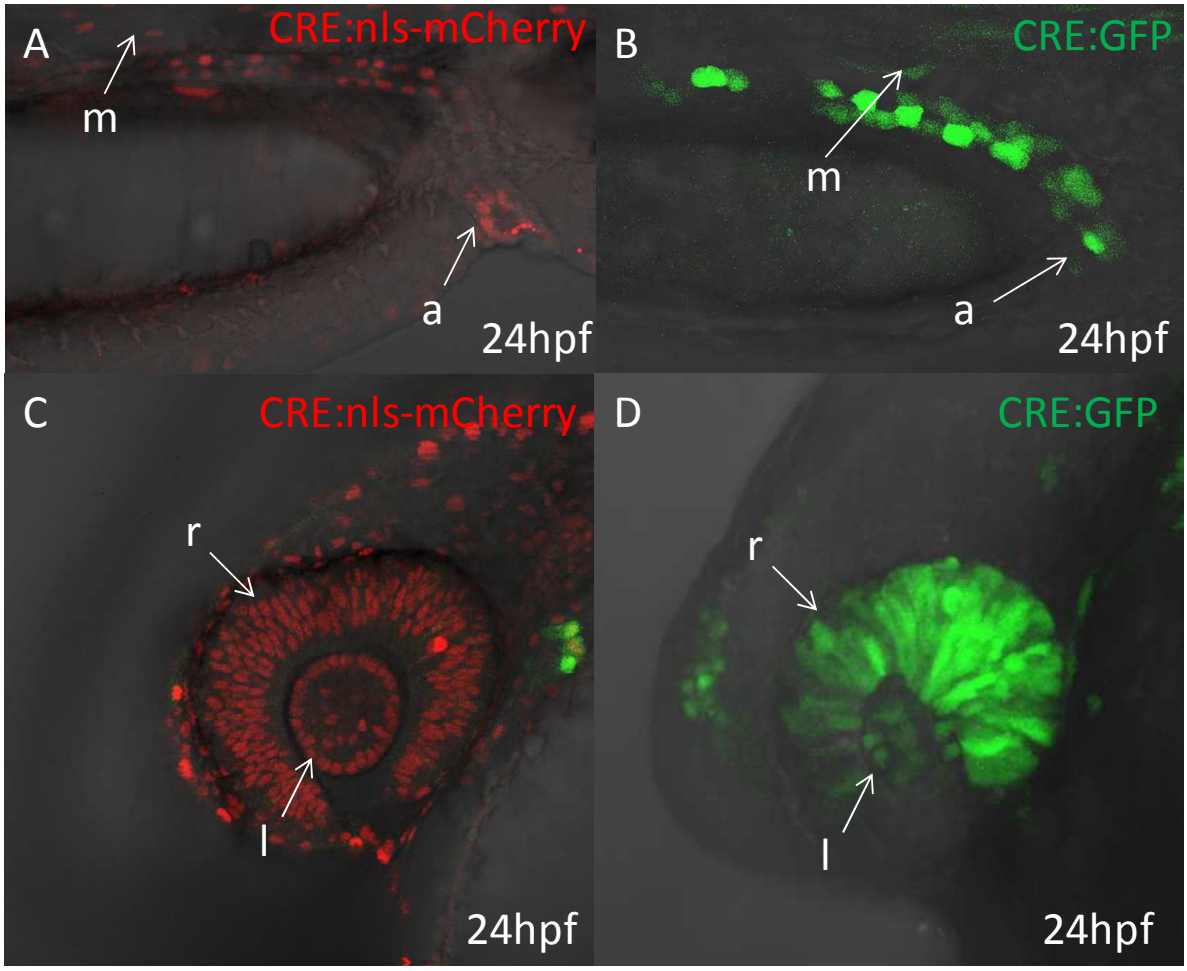

Fig. 20: **Reporter expression in the anal region and in the eye**. CRE:GFP (B, D) and CRE:nls-mCherry (A, C) embryos at 24 hpf. Lateral views. m: muscles, a: anus, l: lens, r: retina.

In the eyes, the reporter proteins are expressed in the lens (particularly in the lens epithelium) and, more strongly, in the retinal cells (Figure 20).

In the muscles, the expression is not homogeneous throughout the entire myotome. Figure 21 shows the muscular region of a 9 dpf larval trunk (fig 21 A), where the red reporter (mCherry) nuclear expression is compared with the green fluorescence of a Shh reporter (GFP), labeling the adaxial part of the myotome (source of Shh-dependent slow

fibers). Observing a transverse section of the same region (Fig 21 B), we can note that the CRE reporter expression appears located in the peripheral part of the myotome, suggesting that these cells may correspond to the external cell layer (ECL) of the myotome, a thin cell sheet giving rise to myogenic progenitors and myoblasts. Comparison of the CREB reporter with ECL markers such as *pax3* or *pax7* could prove or exclude this hypothesis.

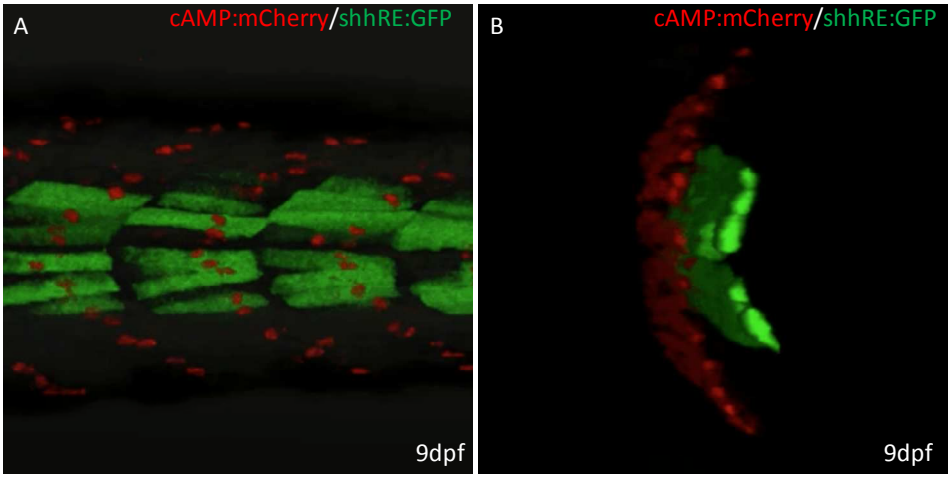

Fig. 21: Muscle expression of CRE:GFP and ShhRE:mCherry in a zebrafish larva at 9 dpf. A: lateral view of the trunk, B: transverse section of the trunk.

Then, we investigated the CREB reporter expression pattern at 48 hpf. First, we analyzed the expression of the reporter protein in the whole head of the embryos. As shown in Figure 22A, the CRE-nls-mCherry is highly expressed in olfactory pits (op), epiphysis (e) and nervous system (ns). As the skin epithelium (s) expresses highly the reporter (Fig. 4A), we decided to analyze the expression profile in one z-plan of the embryo (Fig. 22B). As shown in Figure 22B, we can also appreciate that CRE-nls mCherry is also expressed in pharyngeal arches (pa) and in the heart (h). As shown in Figure 22A, we observe also an expression in the skin, olfactory pits, epiphysis and nervous system (Figure 22B).

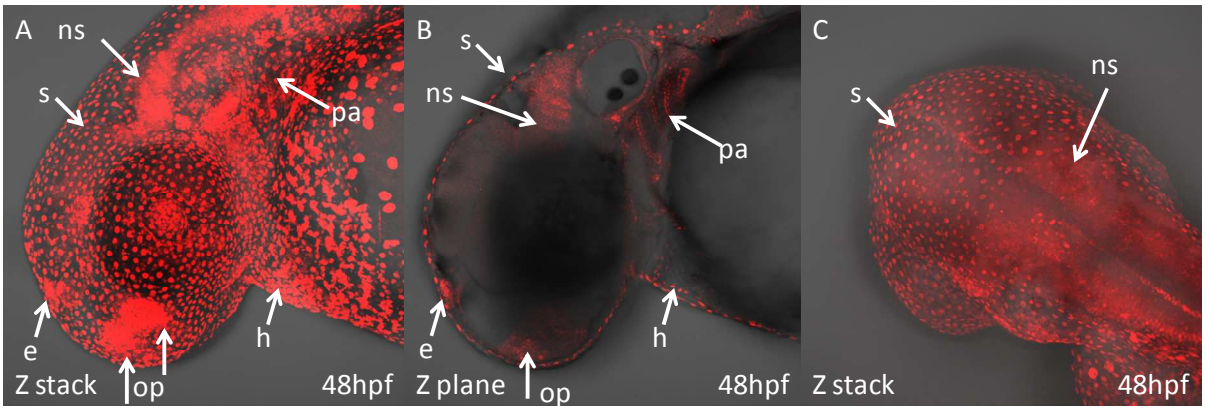

Fig. 22: Expression of the CRE:mCherry reporter at 48 hpf in the head region. A: Lateral view of a 48 hpf embryo, Z stack picture. B: Lateral view of a 48 hpf embryo, Z plane picture C: Dorsal view of a 48 hpf embryo, Z stack picture. s: skin epithelium, ns: nervous system, e: epiphysis, op: olfactory pits, pa: pharyngeal arches, h: heart.

Next, we analyzed the CRE reporter expression pattern in the trunk of the embryo (Figure 23). In this region, the CRE reporter is also expressed in the skin (s). Moreover, it is expressed in neural tube (nt), in somitic muscles (m), in the hypocord (h), in the floor plate (fp) and cloacal region (cr). Moreover, the CRE reporter is also weakly expressed in the blood vessels at 48 hpf (Figure 23 A-C). We can also observe that the CRE reporter is not expressed in the notochord (n) (Figure 23 B)

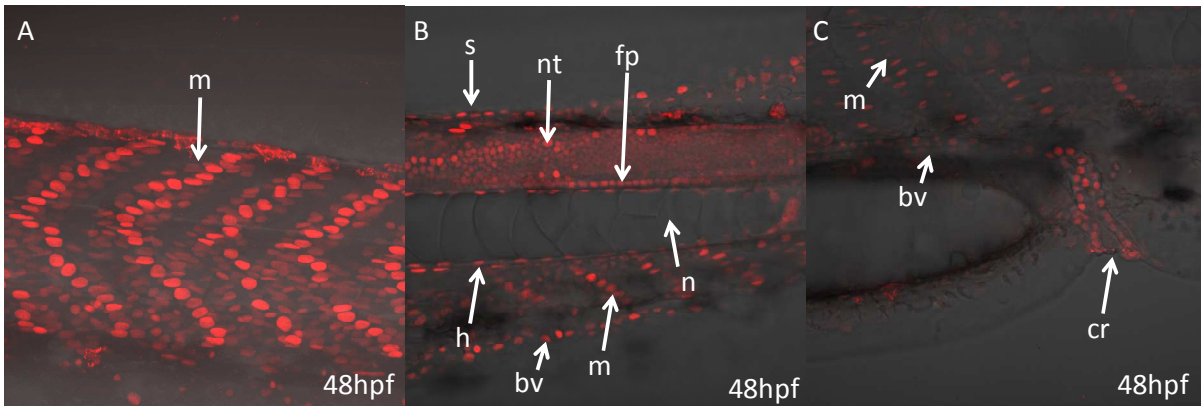

Fig. 23: Expression of the CRE:mCherry reporter at 48 hpf. A: lateral view of the trunk, muscle region, B: lateral view of the trunk, notochord region, C lateral view of the anal region. s: skin epithelium, nt: neural tube, fp: floor plate, m: muscles, bv: blood vessels, h: hypocord, cr: cloacal region, n: notochord.

In order to validate the two reporter lines (CRE:GFP and CRE:nls mCherry), we compared their profiles between each other. As said, both transgenic lines are CRE reporters but one has cytoplasmic expression (CRE:GFP) while the other one is expressed

in cell nuclei (CRE:nls-mcherry). As observed in CRE:nls-mCherry embryos at 48 hpf, the GFP reporter protein is also expressed in the epidermis, muscles, pharyngeal arches, floor plate, blood vessels and pronephric duct (Figure 24 A-H).

Based on these results, we can consider that the two CRE transgenic lines are equivalent in terms of expression profiles.

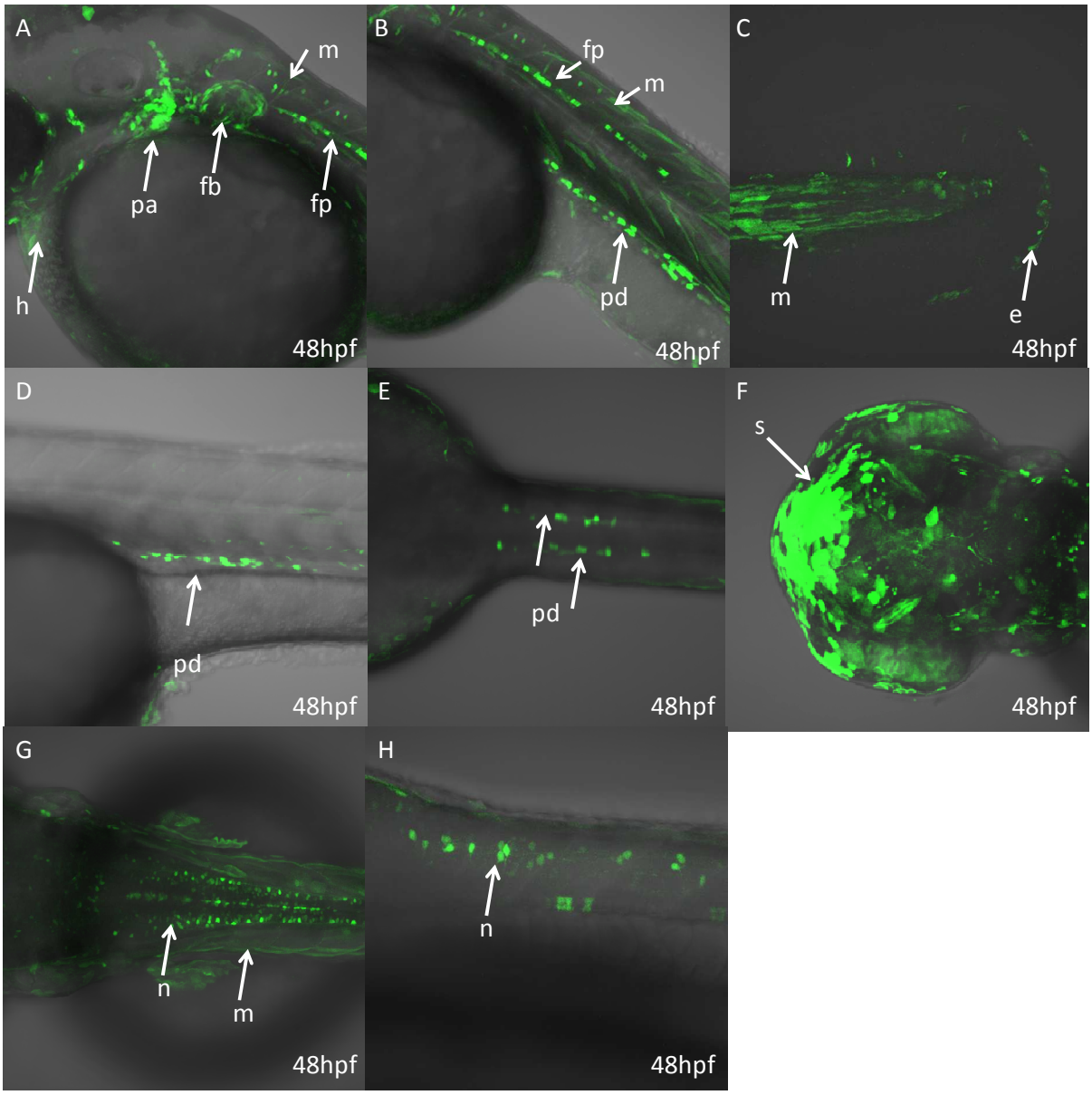

Fig. 24: Expression of the CRE:GFP reporter at 48 hpf. A, B, C, D, H are lateral views; E, F, G are dorsal views. e: epidermis, fp: floor plate, m: muscles, h: heart, fb: fin bud, pa: pharyngeal arches, s: skin epithelium, n: neurons, pd: pronephric duct.

1.2. CRE reporter expression in zebrafish larva

Subsequently, in order to follow the expression pattern of CRE reporters during development, we analyzed its expression in the zebrafish larva (5 dpf). At this stage, we focused our attention on the head of the larva. As shown in Figure 25, the CRE reporter proteins are highly expressed in the orbital muscles and craniofacial skeletal muscles (A). Moreover, we see moderate expression in heart (h), blood vessels (bv) and thyroid (t).

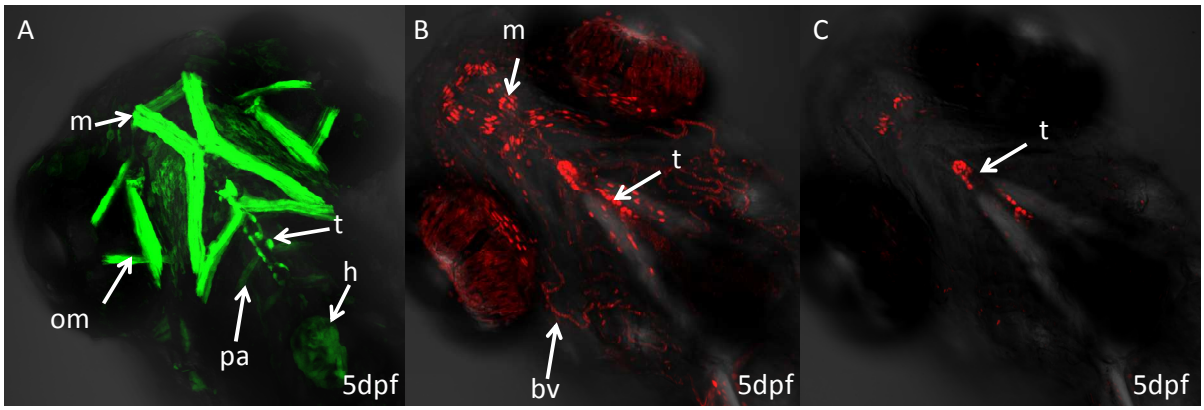

Fig. 25: Expression of CRE:GFP and CRE:mCherry in the larval head. A: dorsal view of the head of a CRE:GFP larva at 5 dpf. B: dorsal view of the head of a CRE: mCherry larva at 5 dpf. C: ventral view of the head of a CRE:mCherry larva at 5 dpf focused on the thyroid plane. m: muscles, bv: blood vessels, h: heart, t: thyroid, pa: pharyngeal arches, bv: blood vessels, om: orbital muscles.

One of the major goals of the generation of cAMP reporter zebrafish lines was the study of pancreatic and hepatic development. First, we investigated the CRE:GFP expression in the liver. For this purpose, we crossed the CRE:GFP reporter with the Lipan zebrafish line. Lipan (liver-pancreas) transgenic fish express the GFP in the pancreas (elastase:GFP) and the DsRed protein in the liver (Fatty acid binding protein:DsRed). As shown in Figure 26, in the green channel we can see the liver (L) and the pancreas (P) in green. The weak green signal is due to the CRE transgene. In the red channel we can see the hepatic transgene of the Lipan line. When we merge the two channels, we can see co-localization of the CRE:GFP with the red hepatic marker, thus proving that the CRE reporter is indeed expressed in the liver.

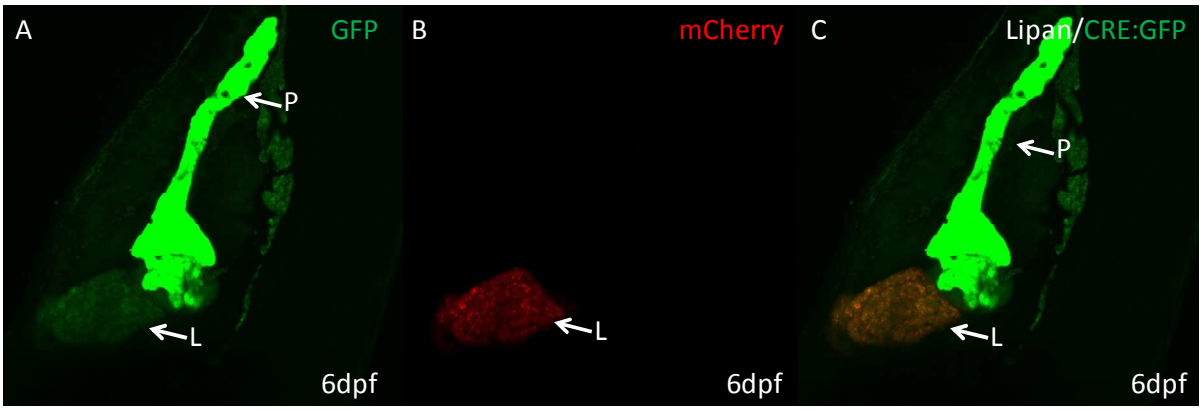

Fig 26: Expression of CRE:GFP in the liver. A. Green channel with the Lipan:GFP (elastase promoter) in the exocrine pancreas (P) and CRE:GFP in the liver (L). B: Red channel with the hepatic Lipan transgene (fabp:mDsRed). C: merge. The displayed larva is at 6 dpf.

Given that the Lipan line has a green pancreas, we cannot study the CRE:GFP pancreatic expression by using it. So, in order to investigate this issue, we crossed the CRE:mCherry reporter with an insuline:GFP transgenic zebrafish line. Unfortunately, we did not observe any CRE:mCherry expression in both exocrine and endocrine pancreas (including β-cells), at least at the considered stage of 48 hpf (Figure 27). These data have been confirmed by crossing Lipan zebrafish with CRE:mCherry (data not shown).

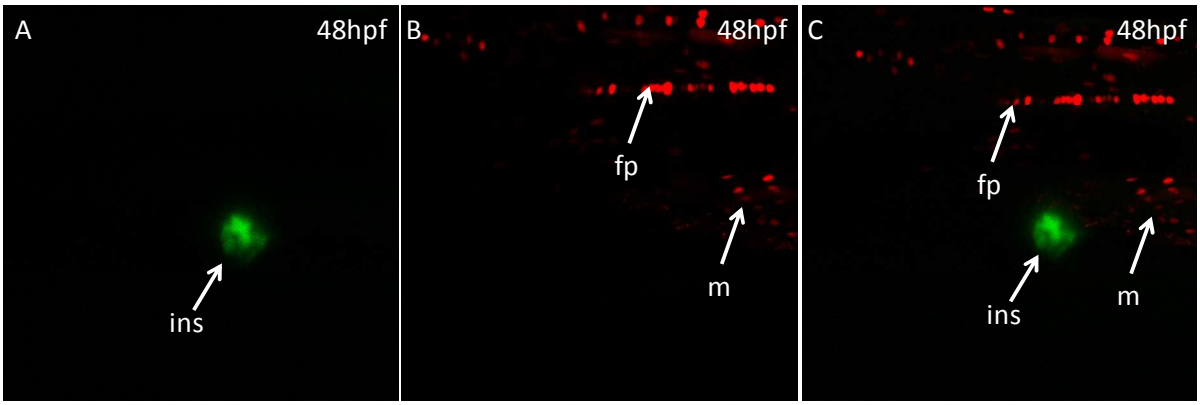

Fig. 27: The CRE:mCherry reporter is not expressed in the pancreas at 48 hpf. A: green channel with ins:GFP; B: red channel with CRE:mCherry; C: merge. fp: floor plate, ins: insulin cells, m: muscles.

SOX9, transcription factor highly involved in cartilage tissue-specific gene expression and chondrocyte differentiation, is activated by the CREB signaling pathway (Tsuda et al, 2003). So, we decided to analyze if our CRE:GFP reporter is expressed in the cartilage. For this purpose, we observed the CRE:GFP expression in the zebrafish larva at 7 dpf, when cartilage is already formed. As shown in Figure 28B, the reporter GFP is

indeed detectable in the cartilage of the head. Moreover, in Figure 28A, we can appreciate that at the 7 dpf stage the reporter CRE:GFP is still activated in the optics muscles, pharyngeal arches and thyroid.

Concerning the expression in the thyroid, we can note that the fluorescence is not homogeneous, but gradually decreasing, following an antero-posterior gradient (Figure 28C)

The fin muscles (fm) are also highly positive to the CRE:GFP reporter in the 7 dpf larva (Figure 28A, D).

Fish and amphibians are characterized by the presence of the Mauthner cells. Mauthner cells are a pair of big neurons (one on each half of the body) located in the hindbrain and responsible for a very fast escape reflex. Interestingly, these Mauthner cells show a high CREB reporter activity (Figure 28E).

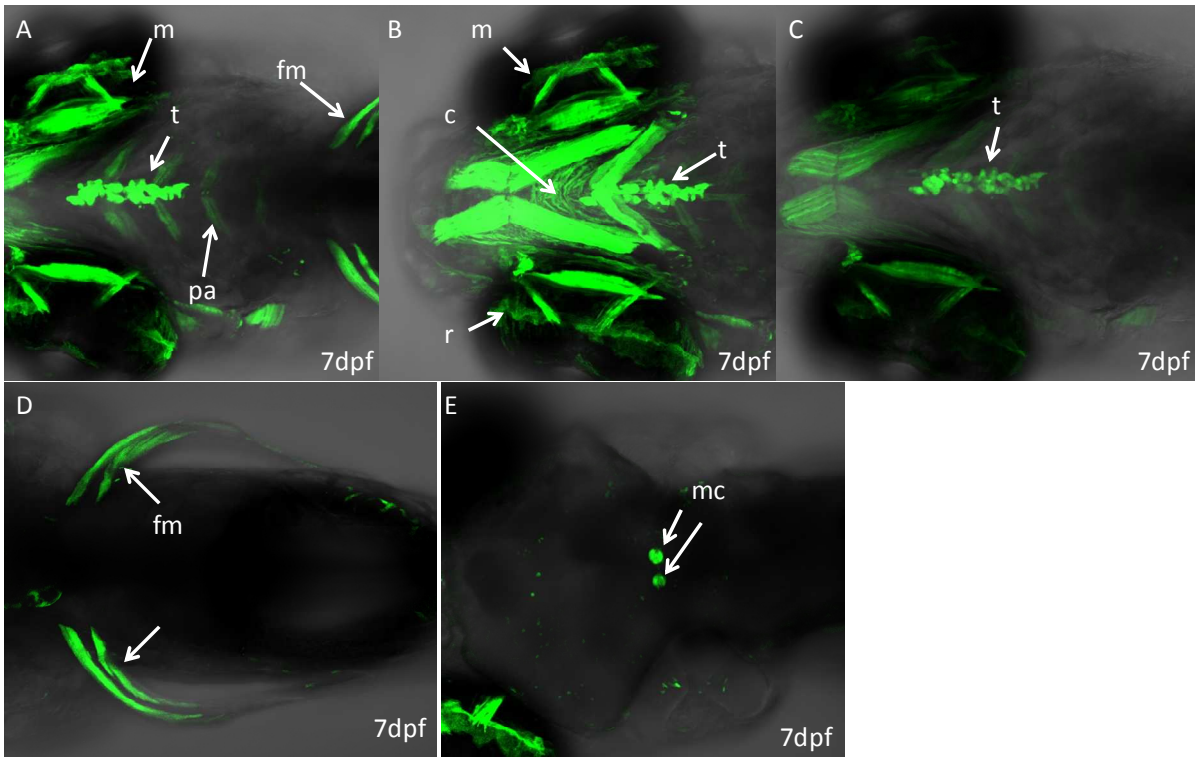

Fig. 28: Expression of the CRE:GFP reporter in the head of a 7 dpf larva. A: head of a CRE:GFP larva at 7 dpf, focused on the pharyngeal arches (pa). B: focus on head cartilage (c) and muscles (m) C: focus on the thyroid follicles (t). r: retina. D: fin muscles (fm). E: Mauthner cells (mc). All views are ventral, except for the dorsal view in E. Anterior to the left.

1.3. CRE reporter expression in the zebrafish adult

One of the goals in generating pathway reporter transgenic lines, as the CREB reporter in our case, is the characterization of the signaling pathway in the adulthood, which represents a very powerful tool to study the consequences of the deregulation of a transduction pathway in different pathologies, including cancer formation.

First of all, we wondered if the CREB pathway reporter is still activated in adult zebrafish. To answer this question, we analyzed whole CRE:GFP adult zebrafish (males and females). As shown in Figure 29 (A-D), the CRE:GFP is strongly expressed in fin joints (fj). We did not observe any difference in CRE:GFP expression pattern in males and females.

In addition, we did not detect CRE:GFP expression in adult eyes nor in the skin, in spite of being anatomical districts strongly positive to the transgene during embryonic development.

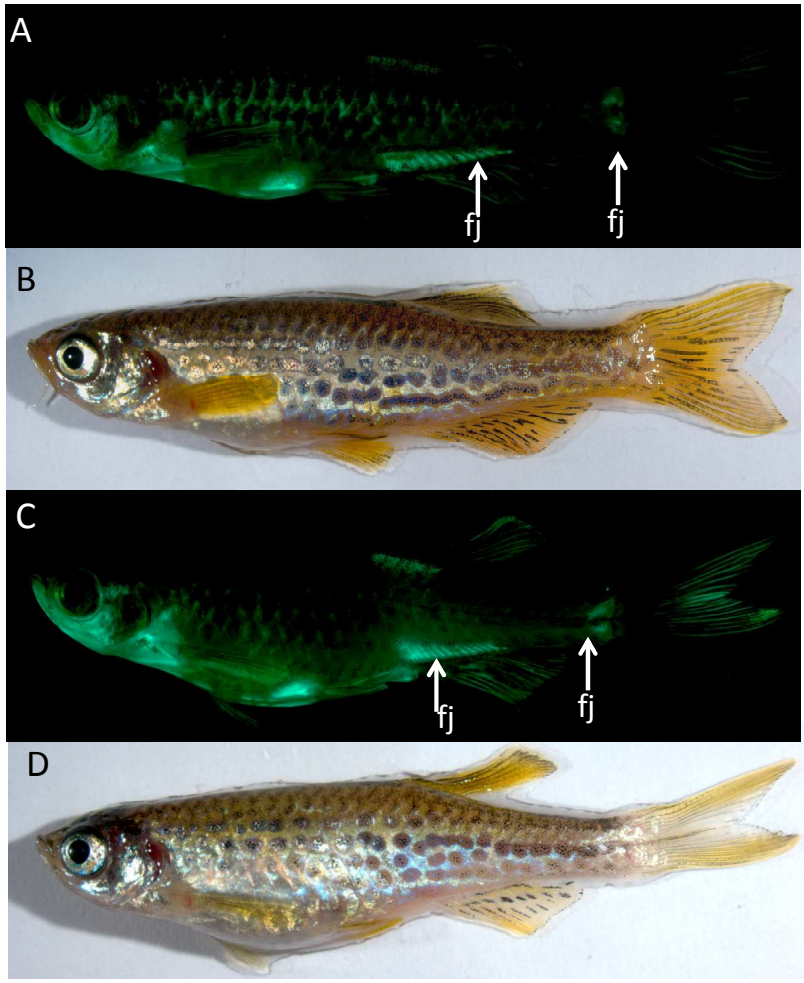

Fig. 29: Expression of the CREB reporter in whole adult fishes. A,B: adult male. C,D: adult female. The reporter is strongly expressed in the fin joints (fj) and in other cartilaginous parts of the body. A,C: green channel; B,D: bright field.

Then, we wondered whether the internal organs still express the CRE:GFP reporter and therefore if CREB signaling is involved in organ maintenance and function. So, we euthanized and dissected CRE:GFP adult zebrafish and observed the GFP expression. As reported in Figure 30, CRE:GFP is highly expressed in the heart ventricle (A), pharyngeal region (B), optic muscles (C), brain (D), kidney (E), ovary (F) and testis (G).

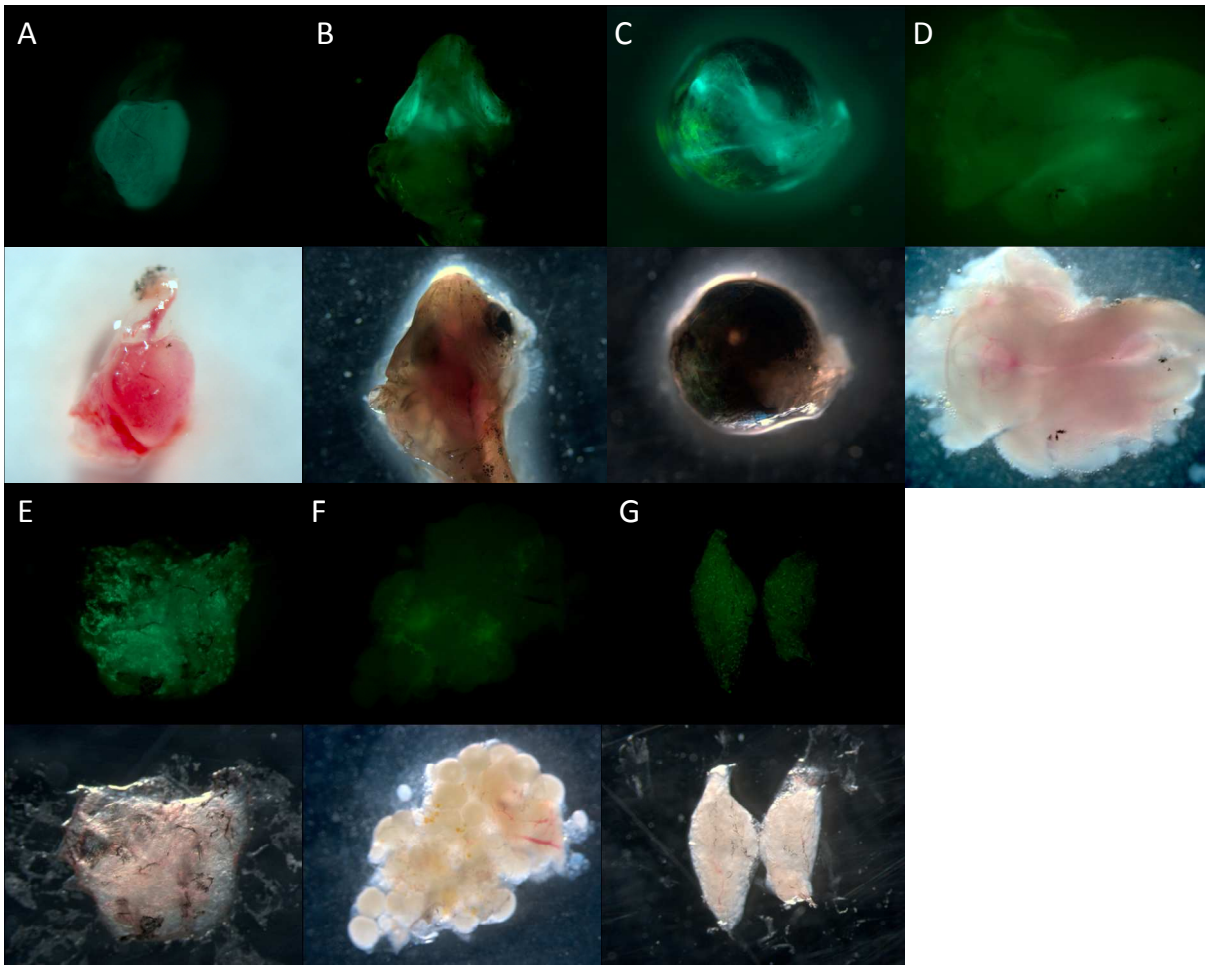

Fig. 30: Expression of the CRE:GFP reporter in adult organs. A: heart ventricle, B: pharyngeal region, C: eye muscles, D: brain, E: kidney, F: ovary, G: testis.

In order to analyze more precisely the GFP expression in internal organs, we observed the organs by confocal microscopy. As reported in Figure 30 A, the cardiac fibers in the heart express GFP. In the testis, the GFP expression is not homogeneous, being stronger in the spermatogenic cysts (B). GFP expression in ovary is very strong in the egg cells (C). In the kidney, the glomerulus is strongly positive for the GFP expression (D).

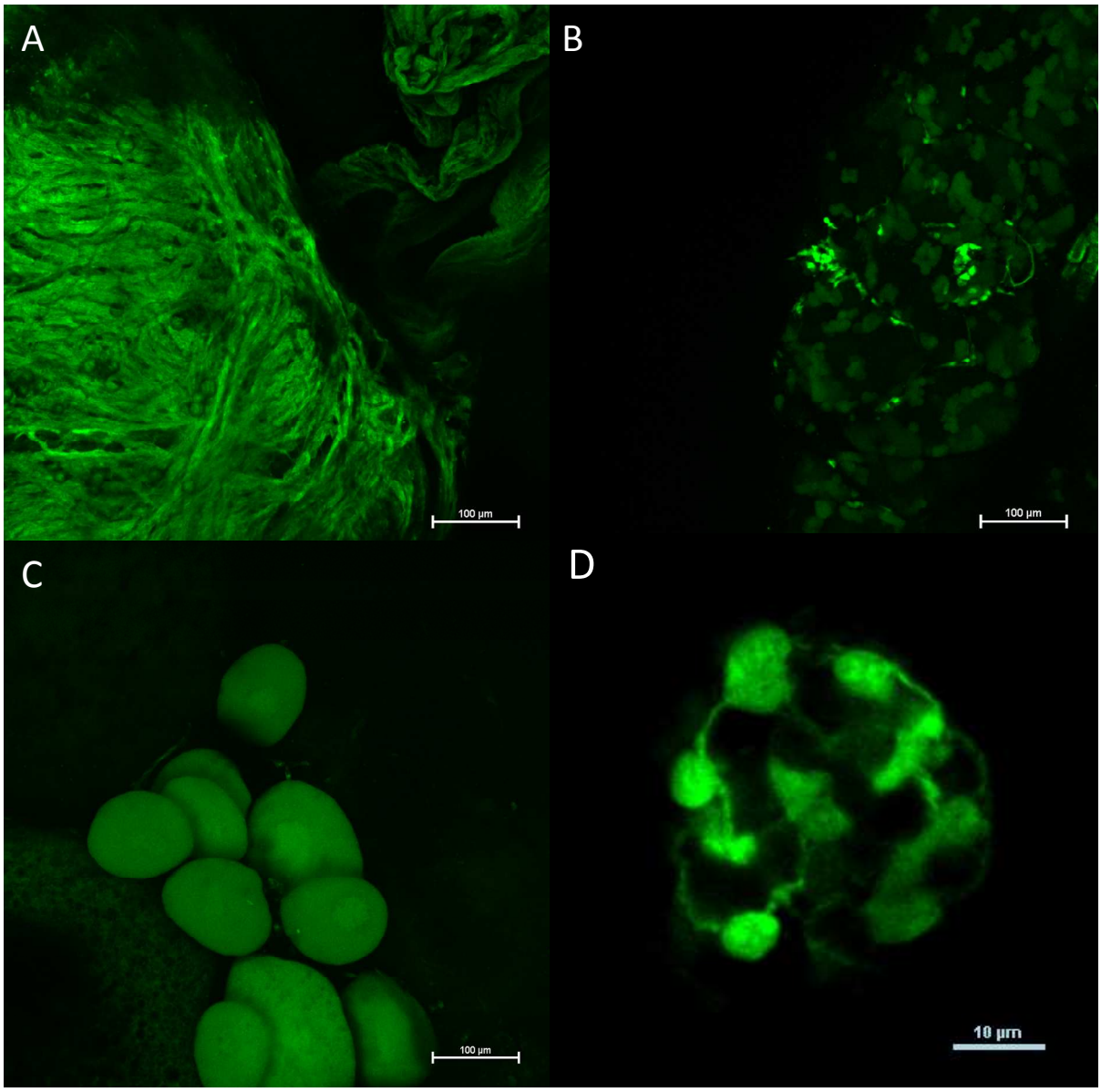

Fig. 30: Confocal view of adult organs of CRE:GFP fish. A: heart myocardium, B: testis, C: ovary. D: kidney glomerulus.

2. Functional analysis of CREB reporter zebrafish lines.

2.1. Comparaison of CREB signaling and BMP signaling in the heart.

One of the aims in the generation of these CREB reporter zebrafish lines is the analysis of crosstalks among different signaling pathways.

Bone morphogenetic proteins (BMPs), which are members of the TGF-β superfamily, have been demonstrated to be important candidates that regulate expression of some cardiac-enriched transcription factors such as Nkx2.5 or GATA4, and induce cardiomyocytes differentiation. In Drosophila, it has been demonstrated that expression of the *tinman* gene (equivalent to vertebrate Nkx2.5) is restricted to the dorsal part of the mesoderm by the ectodermally expressed dpp, wich is related to vertebrate BMP2 or 4 (Evans et al, 1995). Moreover, experiments using chicken embryos have demonstrated that expression of BMP2/BMP4 is detected in the ectoderm and endoderm adjacent to the precardiac mesoderm and that ectopic expression of BMP2 induces differentiation of nonprecardiac mesodermal cells into beating cardiomyocytes (Schultheiss et al, 1997), suggesting a pivotal role of BMPs in the induction of vertebrate cardiac development. Moreover, BMP2 and BMP4 knockout mice present an impairement of cardiac development (Winnier et al, 1995; Zhang & Bradley, 1996).

Given the cardiac expression of our CREB reporters, in order to compare CREB and BMP signaling in the heart we decided to cross the CRE:GFP line with a id1:mCherry BMP reporter line (Collery & Link, 2011). ID1 is a transcription factor induced by the BMP pathway; ID1 protein responsive elements have been used as promoter sequences to drive the BMP-responsive reporter. In the id1:mCherry zebrafish line the heart shows a nuclear red fluorescence.

We can see, in Figure 31, that cytoplasmic CREB and nuclear BMP reporters are both expressed in the cardiac ventricle and atrium of a zebrafish larva at 5 dpf. Interestingly, the intensity of the BMP reporter is the same in ventricle and atrium, while the CREB reporter shows a higher intensity in the ventricle, compared to the atrium.

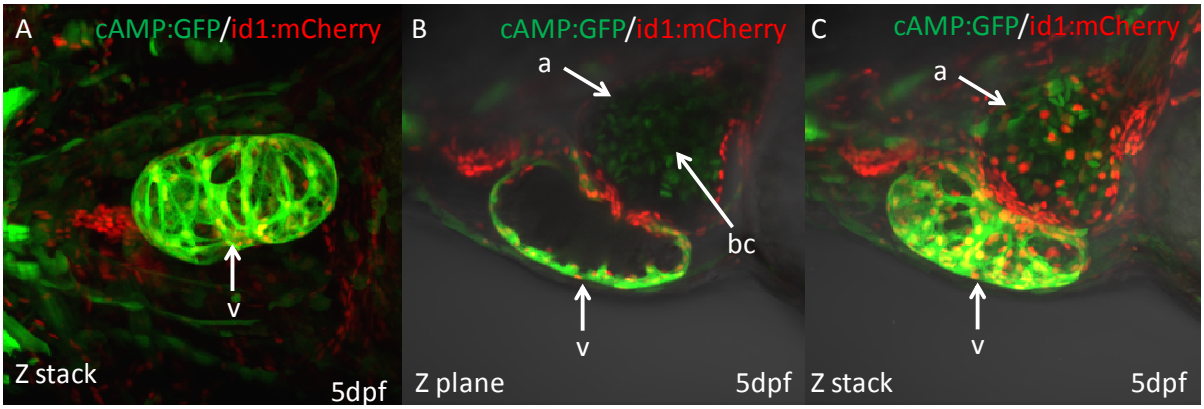

Fig. 31: Heart expression of CRE:GFP and id1:mCherry in a zebrafish larva at 5 dpf . A: ventral view of the heart, Z stack. B: lateral view, Z plane in the heart. C: lateral view. Z stack. a: atrium, v: ventricle, bc: blood cells.

3. *Pharmacological and genetic validation of CRE:GFP and CRE mCherry lines*

Analysis of the regulation of CREB signaling

Finally, we wanted to determine if we can study the regulation of CREB signaling by using these CRE reporter zebrafish lines. We used different pharmacological and genetic approaches to demonstrate the specificity of our CREB responsive transgenic lines.

For a genetic validation of the reporter line, we used an antisense morpholino oligo against the CREB protein creb3l2 (Melville et al 2011), to determine if our reporter is sensitive to a decrease of CREB protein levels. By western blotting, we show that the creb3l2 morpholino reduces CREB protein levels (74% of reduction) after 72 h from the injection (Fig. 32 A and B). Then, by looking at the expression of the CRE:GFP mRNA by *in situ* hybridization, we observed a significant decrease of reporter expression in the morphant embryo (Fig. 32 D) compared to the control (Fig 32 C). With this technique, we demonstrated that a genetic knock-down of CREB activity coherently reduces the CREB reporter expression levels.

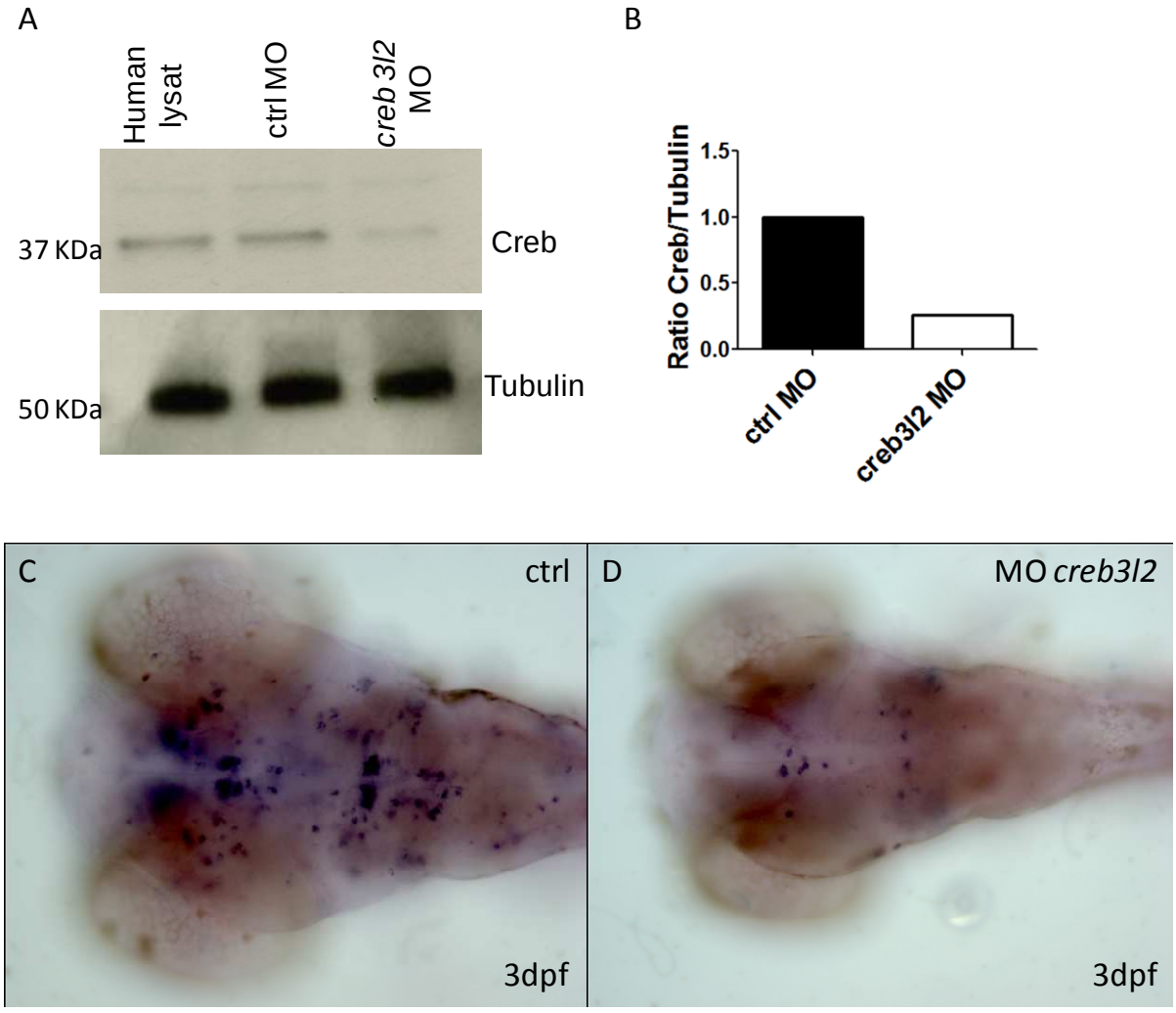

Fig. 32: The creb3l2 knock-down decreases CREB reporter expression. A: Western blot showing the decrease of CREB protein in the creb3l2 morphant compared to the control. B: quantification of the efficiency of the morpholino. C: expression of the reporter GFP mRNA in a control embryo at 3 dpf. D: expression of the reporter GFP mRNA in a creb3l2 morphant embryo at 3dpf.

For validation experiments using pharmacological approaches, both CRE:GFP and CRE:mCherry lines were tested from 2 to 5 dpf, using the agonists 8-Cl-cAMP and noradrenalin, or the antagonist 8-Br-cAMP. Fig. 33 shows the results for the CRE:GFP line, which demonstrates a coherent response of the reporter to all considered CREB-specific treatments.

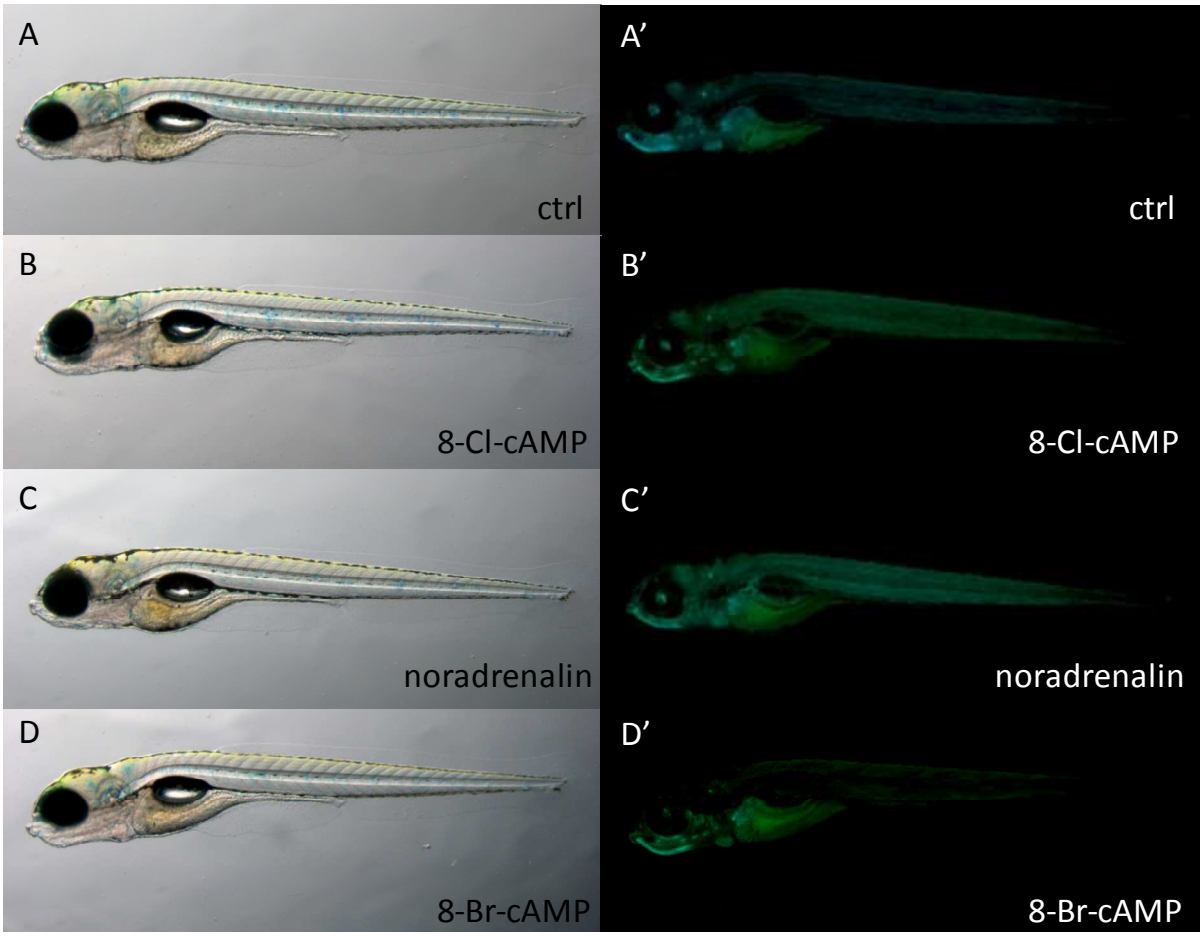

Fig. 33: The CREB reporter coherently responds to specific pharmacological treatments. A, A': control larva, B B': 8-Cl-cAMP (agonist) treated larva, C C': noradrenalin (agonist) treated larva, D D': 8-Br-cAMP (antagonist) treated larva. All larvae are at 5 dpf, in lateral view, anterior to the left.

DISCUSSION

In this work we used the zebrafish as animal model for our experimental setup. The zebrafish (*Danio rerio*) is an animal model commonly used in developmental studies because it offers a lot of advantages: external fertilization and embryonic development, eggs transparency, fast development, short generation time (getting a lot of eggs in one clutch), easy microinjection of fertilized eggs, possibility to realize random mutagenesis, and transgenesis using transposons. Gene expression can be easily analyzed during embryonic development by *in situ* hybridization experiments; moreover, gene function can be easily studied by mutant analysis or by "knock down", consisting in injecting antisense "morpholinos" specifically blocking the studied factor in injected embryos, called "morphants".

Pax6 has been shown to be involved in pancreatic differentiation in mice. St Onge demonstrated that Pax6 mutant mice have a strong decrease of insulin- and PP-expressing cells, an increase of ghrelin-expressing cells, and almost a total loss of glucagon-expressing cells (Heller et al, 2004; Sander et al, 1997; St-Onge et al, 1997).
Verbruggen and colleagues showed by morpholino-mediated experiments that pax6b is involved in pancreas differentiation in zebrafish. They noted a loss of insulin-expressing cells, a drastic decrease of somatostatin-expressing cells and an increase of ghrelin-expressing cells in pax6b morphants. Thus, the pax6 deficiency phenotype appears not conserved in vertebrates, at the insulin and glucagon level. In fish we have a loss of insulin cells whereas in mouse a loss of glucagon cells. To check if the role of pax6 is species-specific or methodology-dependent, we decided to look at the pancreatic phenotype in zebrafish *pax6b* stable mutants, instead of transient morphants. We obtained the sa0086 mutant allele from the Sanger Institute. In this pax6b mutant we confirmed the morphant phenotype. The only difference we noted between *pax6b-sa0086* mutants and *pax6b* morphants is a reduction of glucagon-expressing cells, which was observed in 40% of mutants, whereas alpha cells were not significantly affected after morpholino injection. This slight divergence might be due to a small residual expression of Pax6b in morphants that could be sufficient to drive alpha cell differentiation. On the other hand, the fact that α cell differentiation is not strongly affected in about 60% of *pax6b-sa0086* mutants suggests that a Pax6b-independent pathway may also be used to generate glucagon cells.

The expression of the progenitor marker *sox4b* does not change in pax6 mutants compared to WT siblings. That means that the progenitor cells are not affected.

We looked at the expression of the *nkx2.2* factor in pax6 mutants because the knock down of *nkx2.2* in zebrafish (Pauls et al, 2007) is associated with an increase of ghrelin cells. So we wondered if nkx2.2 was acting downstream or in parallel with pax6 in the pancreas. No significant difference was found, so nkx2.2 is likely acting upstream or in parallel with pax6 in pancreas development.

We found that the expression level of *isl1* is slightly increased, but not the number of the cells. That means that the total number of endocrine cells remains the same in *pax6b* deficient embryos compared to WT.

We showed by *in situ* hybridization that *hb9* mRNA is expressed at the 18-somite stage in the pancreas of *pax6b* deficient embryos. While in the pancreas there is a strong decrease of *hb9* mRNA expression at 25 hpf, at 30 hpf and later on, this factor is still expressed in the motoneurons. We conclude that pax6b is required to maintain the expression of *hb9* in the pancreas, but not to turn on its expression.
By crossing the *hb9*:GFP line with the *pax6b* mutant we saw that from 1 dpf to 3 dpf the GFP is still detectable in the pancreas, whereas the endogenous hb9 disappears in the pancreas at 1 dpf. This can be due to the half-life of the GFP. To confirm this, we looked at the expression pattern of the mRNA of the GFP of the *hb9*:GFP transgene in *pax6b* deficient embryos. This experiment confirmed that the GFP persistence was due to the stability of the protein.
All of the above mentioned data indicate that in pax6b deficient embryos we have a loss of insulin-expressing cells, a decrease of glucagon and somatostatin, and an increase of ghrelin-expressing cells. The total number of endocrine cells does not change. Thus, we hypothesized that cells that should become insulin-positive become ghrelin-positive in absence of pax6b. We used the stability of the GFP protein in the *hb9*:GFP line to mark the insulin precursor cells in pax6 deficient embryos. Then, we looked at the expression of glucagon, somatostatin and ghrelin. Arkipova et al. showed that the GFP perfectly co-localizes with the insulin immuno-responsive cells. We saw that there is no co-localization between the GFP and somatostatin, ghrelin or glucagon in WT embryos. In pax6 deficient

embryos we didn't see co-localization with GFP and somatostatin or glucagon. On the contrary, we saw co-localization with ghrelin-expressing cells, but not with all ghrelin-expressing cells. This led us to propose a model represented in Fig. 34. In *pax6b* deficient embryos, precursor cells that are normally destined to differentiate as beta cells instead take on an epsilon cell fate. In summary, our findings suggest that *pax6b* functions to promote beta and suppress epsilon cell fate during zebrafish pancreatic development.

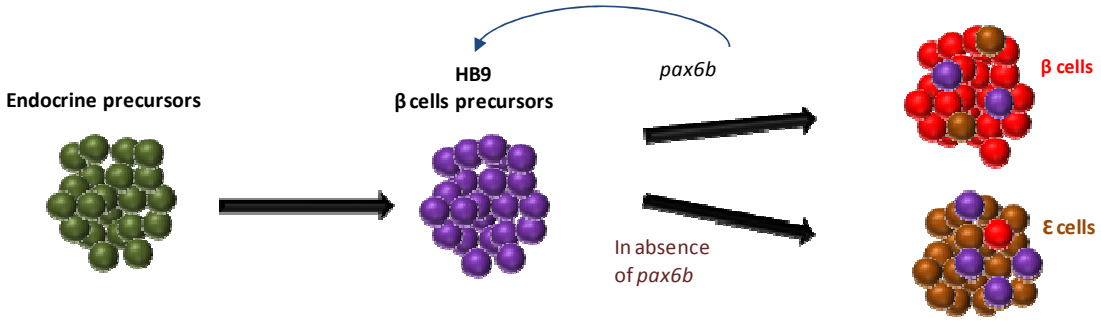

Fig. 34: Model showing that, in *pax6b* deficient embryos, precursor cells that are normally destined to differentiate as beta cells instead take on an epsilon cell fate.

The results obtained in the first part of this work show a primary role of Pax6b in endocrine cells differentiation and endocrine cell fate. In particular, the absence of Pax6b leads to the formation of ghrelin-producing cells at the expenses of insulin-producing cells. Our preliminary results suggest that this effect might be due to a trans-fate of beta cell precursors. However, to further support this hypothesis, cell death and cell proliferation analyses would be required to demonstrate that this trans-fate occurs without total cell number modification. The molecular mechanism by which Pax6b regulates endocrine cell differentiation has not yet been identified. Thus, our future goal will be to determine the effects of Pax6 ablation on different signalling pathways known to be active in the pancreatic region, such as Bmp, Shh, or Notch.

Given that CREB signalling is highly involved in pancreas development, we decided to generate a cAMP reporter zebrafish transgenic line. Indeed, a study published in 2003 showed that induction of CREB signalling pathway promotes □-cell growth and survival by GLP-1 receptor activation (Hui et al, 2003). Moreover, cAMP stimulates the expression of several genes via PKA-mediated phosphorylation of CREB and its paralogs

ATF- and CREM (Gonzalez & Montminy, 1989; Gonzalez et al, 1989). Another study shows that glucose and GLP1 induces □-cell gene expression by activating calcium and CREB signalling (Jhala et al, 2003).

So, we decided to generate CREB signalling reporter zebrafish lines by injecting the transgene 6X cAMP Responsive Element (5' TGACGTCA 3') CRE:GFP (or mCherry) into zebrafish eggs. As a first result, we noted that the CREB reporter is maternally activated. This observation suggests that CREB signaling plays a key role during early development.

Then, we characterized the CRE transgenic lines by following the expression of the fluorescent proteins during development and in the different organs. In the embryos, we observed that CREB signaling is activated in the eye (lens and retina), skin, nervous system, epiphysis, olfactory pits, pharyngeal arches, heart, floor plate, muscles and fin bud. On the contrary, cAMP is not activated in the notochord.

In the larva, CREB reporters were activated in orbital muscles, heart, blood vessels, thyroid and liver. These results suggest that CREB signaling is involved in the growth of many different organs. At larval stage, CREB signaling activation starts to decrease in the ocular region.

Unfortunately, in our CREB lines the reporter does not seem to be activated in the pancreas. This result is surprising given the importance of CREB signaling in pancreas development and endocrine cells differentiation. Indeed, induction of CREB signaling promotes beta-cell growth and survival (Hui et al, 2003). Moreover, glucose, by activating CREB signaling, induces beta-cell gene expression (Jhala et al, 2003). However, it must be said that these data were collected from humans or mouse models. To date, in the zebrafish, there are no studies showing a role of CREB signaling in pancreas development, differentiation or growth.

To explain the divergent results, we postulate some hypotheses: i: the role of CREB signaling in pancreatic development is species-specific; ii: the lack of CRE:GFP or CRE:mCherry expression in pancreas is due to the position of the transgene insertion inside the genome; iii: the expression levels of the reporters in the pancreas are below the instrumentation sensitivity; iv: the analysis has been focused only on the Brockmann body, the first endocrine islet appearing in fish. To discriminate which hypothesis is correct, it

could be interesting to analysis pancreas development in zebrafish after specific cAMP signaling activation or inhibition. Moreover, the analysis should be extended to later developmental stages (eg: from 5 dpf onwards), when secondary endocrine islets start to appear in the exocrine pancreatic mass.

We noted that in the larva the CREB reporters are also activated in the cartilage . This result is really interesting. Indeed, mutations in the gene encoding CBP were found to cause the Rubinstein-Taiby syndrome (Petrij et al, 1995). The CBP +/- mice exhibited the clinical features of Rubinstein-Taiby syndrome, including skeletal abnormalities (Yao et al, 1998). Moreover, Sox9, a transcription factor identified as a key molecule in chondrocyte differentiation, interacts with CBP in vitro and in vivo. By interacting with Sox9, CBP regulates its transcriptional activity (Tsuda et al, 2003). So the phenotype of Rubinstein-Taybi syndrome could be, at least in part, explained by the role of CBP in transcriptional activity of Sox9. The determination of the molecular function of CBP with Sox9 will be helpful for our understanding of skeletal development and has the potential to identify new therapeutic approaches.

One of the goals in generating pathway reporter trangenic lines is the characterization of the signaling pathway in the adulthood, which represents a very powerful tool to study the consequences of the dysregulation of a transduction pathway in different pathologies, including cancer formation. Interestingly, we found that CREB signalling is still activated in numerous organs in the adult fish, including heart, brain, genital organs, kidney, pharyngeal region and fin joints. This indicates that in adult zebrafish CREB signaling is still involved in organ maintenance and/or function. Notably, CREB signalling is not anymore activated in the skin of adult fish, differently from the embryonic stages. These results suggest that CREB signalling plays a major role during skin formation rather than in its maintenance. On this regard, it could be interesting to analyse if CREB signalling is activated in studies of skin injury and regeneration.

In addition, it is known that melanocortin-1 receptor (MC1R), a G-protein coupled receptor (GPCR), plays a crucial role in melanocyte development, proliferation and differentiation. Activation of the MC1R by the □-melanocyte stimulating hormone (□-MSH) leads to the activation of the CREB signalling pathway that is mainly associated with differentiation and hormone production. Some polymorphisms of MC1R impair

CREB signalling and pigmentary phenotypes such as red head colour and fair skin phenotype that is usually associated with higher risk for melanoma development. Despite its importance in melanocyte biology, the role of CREB signalling on cutaneous melanoma is not yet understood. Melanoma is primary driven by mutations in components of MAPK pathway. Now, increasing evidences suggest that CREB signalling plays also a important part in melanoma formation (Rodriguez & Setaluri, 2014). So, by taking advantage of our CRE reporter lines, combined with zebrafish models for melanoma, we could determine at which stage of melanoma development and progression cAMP signalling might be involved.

Another major goal in generating pathway reporter transgenic lines is the determination of crosstalk between different signalling pathways. In this work we were interested on the potential crosstalk between CREB and BMP signaling. BMPs have been demonstrated to be important candidates that regulate expression of some cardiac-enriched transcription factors including Nkx2.5 or GATA4, and induce cardiomyocyte differentiation (Monzen et al, 1999). Moreover, BMP2 and BMP4 knockout mice present an impairment of cardiac development (Winnier et al, 1995; Zhang & Bradley, 1996). Given the cardiac expression of our CREB reporters, and the finding of concomitant activation of CREB and BMP pathways in the cardiac ventricle, we plan to study a possible crosstalk between this two signalling pathways. To this purpose, we will study the effect of the activation or inhibition of the CREB pathway on BMP signalling and *vice versa*.

Moreover, we will use the same strategy to determine the molecular link between Sox9 and CREB signaling in chondrocyte development. Finally, in order to determine at which stage the CREB pathway could play a role in melanoma formation, we will compare its activation in relation to the MAPK signaling cascade.

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