# Pancreas Development in *Xenopus laevis*

**PhD Thesis**

**in partial fulfilment of the requirements**

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**in the Molecular Biology Program**

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**Faculty of Biology**

**submitted by**

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**born in**

**Takoradi, Ghana**

**2005**

**Affidavit**

Herewith I declare, that I prepared the PhD thesis

`` Pancreas Development in *Xenopus laevis´´*

on my own and with no other sources and aids than quoted.

Solomon Afelik .....

Date of submission .....

# List of Publications

1. Afelik, S., Chen, Y., and Pieler, T. 2004. Pancreatic protein disulfide isomerase (XPDIp) is an early marker for the exocrine lineage of the developing pancreas in Xenopus laevis embryos. *Gene Expr. Patterns*. **4:** 71– 76.

2. Chen, Y., Pan, F. C., Brandes, N., Afelik, S., Sölter, M., and Pieler, T. 2004. Retinoic acid signalling is essential for pancreas development and promotes endocrine at the expense of exocrine cell differentiation in *Xenopus. Dev. Biol.* **271:** 144-160.

3. Afelik, S., Chen, Y., and Pieler, T. Combined ectopic expression of Pdx1 and Ptf1a/p48 results in the stable conversion of posterior endoderm into endo- and exocrine pancreatic tissue. (Submitted)

# Contents

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# List of Figures

# Abstract

Previous studies on pancreas development have mainly relied on the use of mouse as a model system in gene targeting experiments. In this study, *Xenopus laevis* is employed for the analysis of pancreatic organogenesis, as part of an effort to establish *Xenopus laevis* as a model system for the study of pancreatic organogenesis. *Xenopus* pancreatic protein disulfide isomerase (XPDIp) has been identified as a molecular marker of the early exocrine pancreatic lineage. Moreover, the roles of the pancreas transcription factors Ptf1a/p48 and the *Xenopus* homologue of Pdx1 (XlHbox8) in early pancreas development have been analysed. These findings strongly suggest that a combination of two transcription factors, XPtf1a/p48 and XlHbox8, is sufficient to convert non-pancreatic endodermal cells into pancreatic precursor cells.

# 1. Introduction

## 1.1 Morphology and function of the pancreas

The pancreas consists primarily of two functionally distinct cell populations, the exocrine and the endocrine cells. The exocrine pancreas consists of acinar cells arranged into subunits of acini, which secrete digestive enzymes. The acini of the exocrine pancreas are connected to a highly branched network of ductal cells, which drain secreted enzymes from the acinar cells into the duodenum. Pancreatic ducts also secrete bicarbonate to neutralise stomach acidity (Slack, 1995). The endocrine cells of the pancreas consist of five different hormone secreting cell types, α, β, δ, ε and PP cells, which aggregate to form a cluster of cells (the islets of Langerhans) embedded in the exocrine tissue. The β-cells which constitute about 90% of the islet mass, secrete insulin as well as the anorectic hormone called amylin, the α-cells secrete glucagon, the δ-cells somatostatin, the ε-cells ghrelin and PP cells secrete pancreatic polypeptide (Figure 1.1) (Slack, 1995; Prado et al., 2004; Lutz, 2005) . Despite the functional diversity of the endocrine and exocrine pancreatic compartments, both cell lineages derive from a common precursor population in the foregut endoderm.

During embryogenesis, development of the pancreas becomes evident in the form of one dorsal and two ventral epithelial evaginations of the endoderm at the junction of the presumptive stomach and duodenum at stage 35 in *Xenopus laevis* and embryonic day 8.5 in mouse (Figure 1.2) (Kelly and

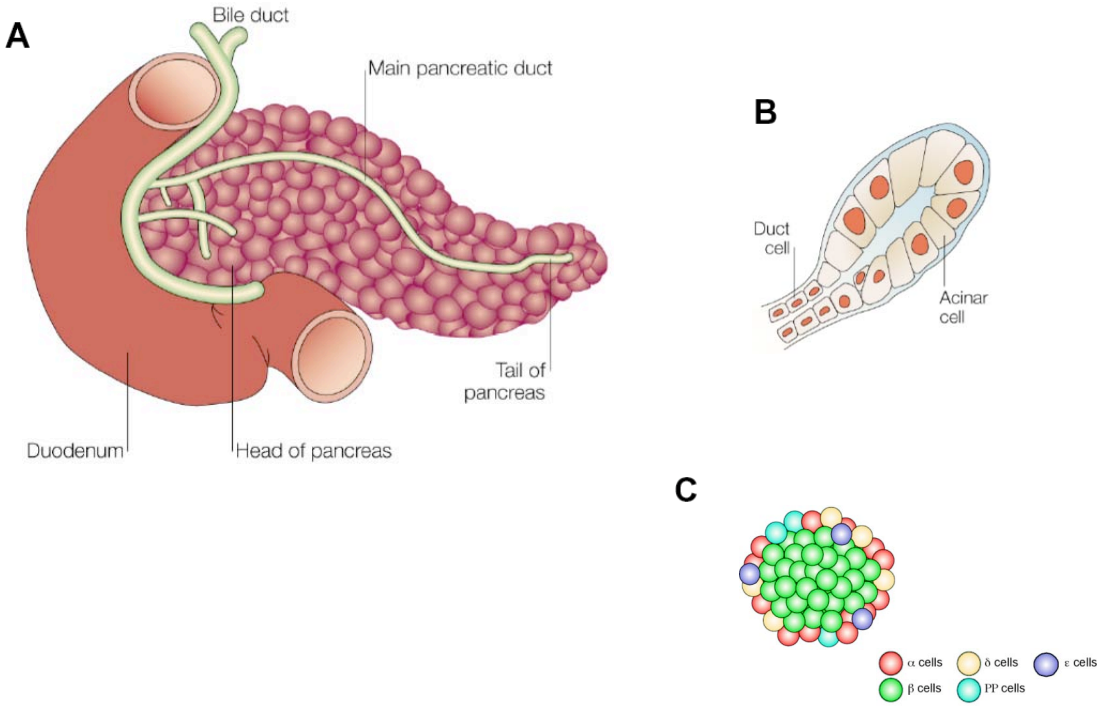



**Figure 1.1** Anatomy of the adult pancreas. (A) The mature pancreas adjacent to the duodenum. (B) A mature pancreatic acinus. (C) A pancreatic islet. (from Edlund, 2002 with modifications)

Melton, 2000). In the course of development, the pancreatic buds grow, branch and eventually fuse to form the definitive pancreas (Slack, 1995). Pancreas development, as observed during mouse development, follows two distinct waves of differentiation. The first wave, referred to as the primary transition, occurs between E9.5 and 13.5 and is marked by the appearance of glucagon and insulin expressing cells which do not contribute to the formation of the mature islets (Wilding and Gannon, 2004; Herrera, 2000). The second wave of differentiation, the secondary transition, initiates between E14.5 and E16.5 and results in acinar cells and hormone positive endocrine cells that form the mature islets (Wilding and Gannon, 2004; Prasadan et al., 2002).

Specification of the pancreatic rudiments, like other endoderm derived organs that arise along the gut tube in a stereotyped anterior-posterior and dorsal-ventral pattern, is the result of an early endodermal prepattern leading to the establishment of molecular boundaries demarcating the various organ primordia. Subsequent development of the presumptive pancreatic endoderm requires a series of permissive interactions with neighbouring mesodermal tissues.

Previous analysis of pancreas development has mainly been done using the mouse as a model system. Gene targeting experiments have led to the identification of a number of transcription factors involved in the various stages of pancreas development. Tissue recombination experiments in the chick have also been employed to verify the role of mesoderm derived signals in pancreas development. In this study, *Xenopus laevis* is used as a model system to study pancreas development. The availability of large numbers of eggs, which can easily be grown and manipulated *in vitro*, the relative ease in the study of gene function by gain or loss of function experiments, as well as its fast development make *Xenopus* a suitable system.

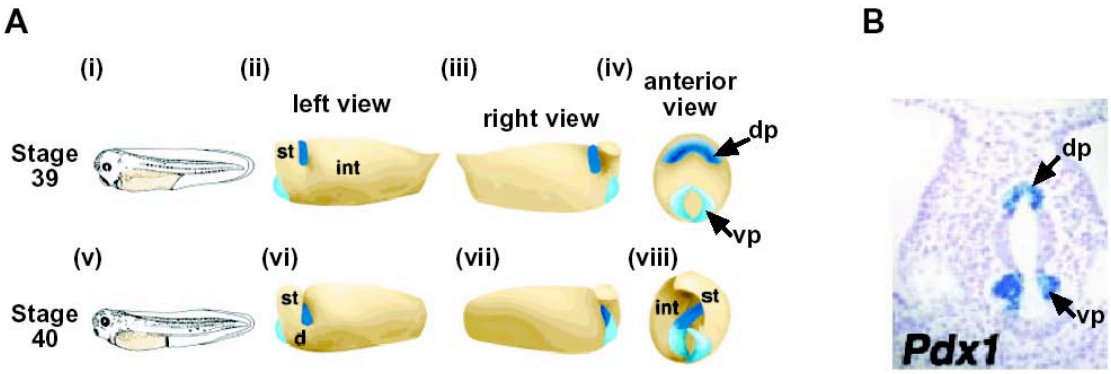



**Figure 1.2** Embryonic pancreas of Xenopus and mouse. (A) A model of the embryonic pancreatic buds before and after fusion in *Xenopus* embryos. (i) and (v) are whole embryos. (ii)-(iv) and (vi)-(viii) show the whole endoderm from the embryos in (i) and (v) (from Kelly and Melton 2000). (B) Transverse section showing the pancreatic buds of 9.75 dpc mouse embryos. The pancreatic buds are marked by the expression of Pdx1 in blue, as indicated by arrows (taken from Lammert et al., 2001 with modifications). Abbreviations: d, duodenum; dp, dorsal pancreas; vp, ventral pancreas; int, intestine; st, stomach.

## 1.2 Regional specification of the endoderm

Classical recombination experiments with *Xenopus* embryos have clearly demonstrated asymmetry in the presumptive endodermal cells already at the blastula stage. In these studies it was shown that dorsal endoderm from blastula stage embryos induced dorsal mesoderm when recombined with prospective ectoderm, whereas recombination with ventral endoderm induced ventral mesoderm in prospective ectoderm (Nieuwkoop, 1973; Orgi, 1967,1969). Subsequent transplantation experiments showed that dorsal vegetal cells cultured in isolation express anterior endodermal markers, like XlHbox8, while ventral vegetal cells would later express posterior endodermal genes, like IFABP. The expression of XlHbox8 is abolished when embryos are

ventralised by UV treatment (Henry at al., 1996). Zorn and colleagues have also shown that maternal Wnt/β-catenin signalling in the dorsal vegetal blastomeres, resulting from cortical rotation, in conjunction with vegetally localised TGF-β signals are involved in the specification of anterior endomesoderm (AE), a subset of endoderm cells fated to form liver and foregut (Zorn et al., 1999). Thus, it seems that anteriorposterior patterning of the endoderm is under the influence of cortical rotation, which leads to the establishment of maternal Wnt/β-catenin signalling on the presumptive dorsal side of the early *Xenopus* embryo.

Events during gastrulation are also crucial in the regional patterning of the endoderm. In both *Xenopus* and zebrafish, retinoic acid signalling during gastrulation is essential for specification of the pancreas. Inhibition of retinoic acid signaling results in a complete loss of the pancreas in zebrafish, while a similar treatment results in a specific loss of the dorsal pancreas in *Xenopus* embryos (Stafford and Prince, 2002; Chen et al., 20004). Conversely, exogenous retinoic acid applied during gastrulation leads to an expansion of endocrine cells of the dorsal pancreas at the expense of the exocrine pancreatic compartment in *Xenopus* embryos (Chen et al., 20004). In zebrafish, exogenous retinoic acid results in an anterior expansion of the pancreas, consisting of both endocrine and exocrine cell types (Stafford and Prince, 2002). Besides the effects of retinoic acid, studies with zebrafish embryos have shown that BMP signalling plays a role in the control of the A-P patterning of the endoderm during gastrulation. Mutant zebrafish embryos deficient in BMP2b develop an expanded anterior endoderm, and since the

zebrafish pancreas derives from the posterior endoderm, this results in a reduction of the pancreatic domain, whereas *chordino* mutants, which lack the Bmp antagonist Chordin, have reduced anterior endoderm and an enlarged posterior endoderm with an enlarged pancreatic primordium (Tiso et al., 2002). Further evidence that the region-specific induction of endodermal organ primordia is dependent on early events of an endodermal prepattern comes from in vitro tissue recombination studies using chick-quail chimeras. Transplantation of presumptive pancreatic endoderm to more anterior positions revealed that, although the pancreatic marker Pdx1 becomes detectable at the 9- to 10- somite stage, pancreatic fate is already determined by the six-somite stage through instructive signals derived from the lateral plate mesoderm underlying the Pdx1 expression domain (Kumar et al., 2003).

## 1.3 Mesoderm-pancreatic epithelial interaction is essential for pancreas development

In the course of embryogenesis, the prepancreatic endoderm comes into contact with various mesodermal tissues, which have been shown to play a crucial role in the commitment, proliferation, differentiation and morphogenesis of the pancreatic epithelium. Before budding occurs, the dorsal pancreatic endoderm is closely associated with the notochord until 8.5 days post coitum (dpc), when fusion of the paired dorsal aortae displaces the notochord, bringing the dorsal pancreatic endoderm into contact with vascular endothelium (Slack, 1995). By 9.5 dpc, condensation of the dorsal

mesenchyme results in the evagination of the dorsal pancreatic rudiment (Figure 1.3) (Slack, 1995). In the ventral endoderm, the pancreatic endoderm makes contact with cardiac mesoderm and the septum transversum mesenchyme (Rossi et al., 2001; Deutsch et al., 2001; Figure 1.3).

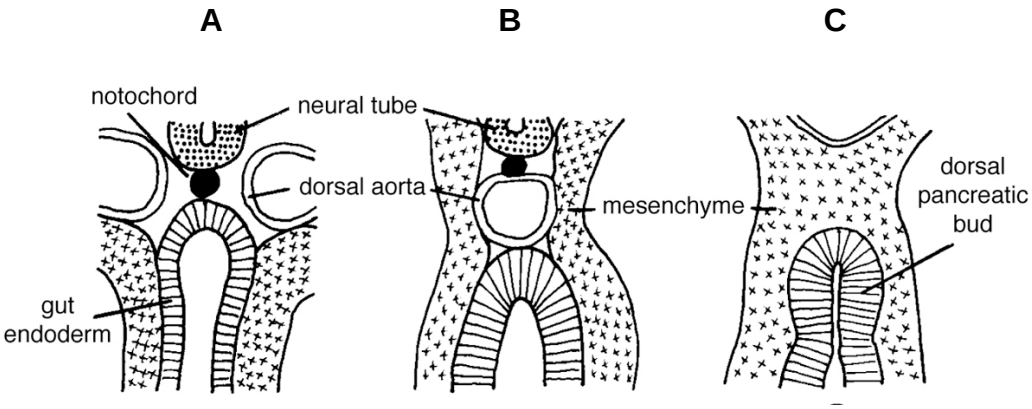



**Figure 1.3** Early stages of mouse pancreatic organogenesis. (A) The notochord contacts the endoderm at the 15 somite stage. (B) Fusion of the paired dorsal aorta at the 20 somite stage places the aorta between the endoderm and the notochord. By the 28 somite stage mesenchyme surrounds the endoderm as the dorsal pancreatic buds form (taken from Slack et al., 1995).

### 1.3.1 The role of the notochord

In vitro tissue recombination studies with early chick endoderm have shown that early signals from the notochord are required for morphogenesis of the dorsal pancreas. The early pancreatic endoderm has no sonic hedgehog expression in contrast to the uniform expression of sonic hedgehog in non-pancreatic endoderm (Ahlgren et al., 1997; Apelqvist et al., 1997; Kim et al., 1997). Premature separation of the notochord from the endoderm results in

ectopic expression of sonic hedgehog in the pancreatic endoderm, resulting in the loss of pancreatic marker gene expression, while in vitro recombination of pre-pancreatic endoderm with notochord is sufficient to restore pancreatic marker gene expression in the endoderm (Kim et al., 1997; Hebrok et al., 1998). The inability of the notochord to induce pancreatic gene expression in non-pancreatic endoderm however indicates that the signals from the notochord are permissive rather than instructive to pancreas development. Activin-βB and Fibroblast growth factor 2 (FGF2), both expressed in the notochord, are sufficient to mediate repression of sonic hedgehog expression in pancreatic endoderm, leading to the expression of pancreatic marker genes (Hebrok et al., 1998). Though the ventral pancreatic endoderm makes no contact with the notochord, it does not express sonic hedgehog (Kim and Hebrok 2001).

### 1.3.2 The role of blood vessel endothelium

Budding of the dorsal and the two ventral pancreatic primordia occurs precisely where the endoderm makes contact with blood vessel endothelium; the dorsal bud contacts the fused dorsal aorta (Figure 1.3) whereas both ventral buds contact the vitelline veins. Removal of the dorsal aorta in *Xenopus* embryos results in the loss of dorsal pancreatic marker gene expression, and while mouse pre-pancreatic endoderm cultured alone fails to differentiate, recombination with dissected aorta or other endothelial cells is sufficient to initiate pancreas differentiation (Lammert et al., 2001).

Furthermore, mutant mice lacking endothelial cells fail to express the pancreas specific transcription factor Ptf1a/p48; however, the expression Pdx1 is initiated but not maintained (Yoshitomi and Zaret, 2004). These studies therefore implicate vascular endothelial cells as a source of permissive signals for pancreas development.

### 1.3.3 The role of the mesenchyme

A number of experiments have pointed out that, subsequent proliferation, morphogensis and differentiation of the pancreatic epithelium is dependent on its interaction with the mesenchyme. Initial studies by Golosow and Grobstein (1962) showed that in vitro culture of E 11 pancreatic epithelium free of the adjacent mesenchyme fails to grow and differentiate, whereas recombination of the naked pancreatic epithelium with pancreatic mesenchyme restores growth and morphogenesis. The ability of non-pancreatic mensenchyme to support growth and morphogensis of the pancreatic epithelium in culture suggests that the pancreatic fate is specified by E11 and the mesenchyme derived signals are permissive rather than instructive to pancreas development (Golosow and Grobstein, 1962). Earlier analyses of the importance of the mensenchyme in pancreas development focused largely on the ability of the mesenchyme to promote epithelial branching and the appearance of zymogen granules. Later studies equipped with molecular markers for the analysis of the effects of the mesenchyme on the differentiation of the various components of the pancreas suggest that the

default fate of the embryonic pancreatic epithelium is to form endocrine cells, a fate which is repressed by signals from the mesenchyme in favour of an exocrine fate. In these studies, growth of pancreatic epithelia without mesenchyme in vitro, or under kidney capsules, resulted in the formation of dense aggregates of pure islets with little or no acinar or ductal cells; on the contrary, in the presence of mesenchyme, the pancreatic epithelium developed predominantly into acinar structures with much less endocrine cells (Gittes et al., 1996; Miralles et al., 1998). Miralles et al. (1998) went further to show that follistatin, a soluble factor from the embryonic pancreatic mesenchyme, can mimic the effect of the mesenchyme by promoting exocrine cell development at the expense of endocrine cell differentiation in cultured embryonic pancreatic epithelium.

Besides follistatin, the Fgf10/Fgfr2b pathway has been shown to mediate mesenchymal-epithelial signalling in the developing pancreas. During early pancreatic organogenesis, the fibroblast growth factor receptor 2b (Fgfr2b) is expressed in the pancreatic epithelium, whereas its high affinity ligand Fgf10 is expressed in the pancreatic mesenchyme (Hart et al., 2003; Bhushan et al., 2001; Miralles et al., 1999). Mutant mice that express a dominant-negative form of Fgfr2b under the control of the inducible metallothionein promoter, or mice that lack either Fgf10 or Fgfr2b display pancreatic hypoplasia (Celli et al., 1998; Revest et al., 2001; Bhushan et al., 2001). Furthermore, overexpression of Fgf10 in the pancreatic epithelium results in increased proliferation of the pancreatic epithelium at the expense of differentiation (Norgaard et al., 2003; Hart et al., 2003)

Further genetic evidence for the growth-promoting role of the pancreatic mesenchyme is derived from genetic inactivation of the LIM homeodomain protein islet1, as well as the cell adhesion molecule N-cadherin, both of which are expressed in the pancreatic mesenchyme. Targeted disruption of either of these genes results in a failure of the mesenchyme to condense around the dorsal pancreatic bud, resulting in dorsal pancreas agenesis, an effect which can be rescued by recombination of the mutant pancreatic epithelium with pancreatic mesenchyme from wild type embryos (Ahlgren et al., 1997; Esni et al., 2001).

### 1.3.4 The role of septum transversum mesenchyme in ventral pancreas development

In mouse embryos, precursors of the ventral pancreas, as marked by the expression of Pdx1, become visible at embryonic day 8.5 directly adjacent to hepatic precursor cells in the ventral foregut endoderm (Gannon and Wright, 1999; Gauldi et al., 1996; Deutsche et al., 2001). The embryonic liver primordium is as well directly adjacent to cardiac mesoderm, and tissue explant studies have shown that liver development is dependent on fibroblast growth factor signalling derived from the cardiac mesoderm (Jung et al., 1999). In addition BMP signalling from the septum transversum mesenchyme has been shown to promote liver development (Rossi et al., 2001). Isolation of the ventral foregut endoderm from the sources of these hepatic inductive

signals results in the differentiation of the presumptive liver domain into pancreas. Moreover, when ventral foregut endoderm explants are cultured in the presence of Fgf, pancreas differentiation is inhibited and the entire ventral foregut endoderm explants differentiate into liver (Rossi et al., 2001; Deutsch et al., 2001). These findings suggest that the default fate of the ventral foregut endoderm is to form pancreas.

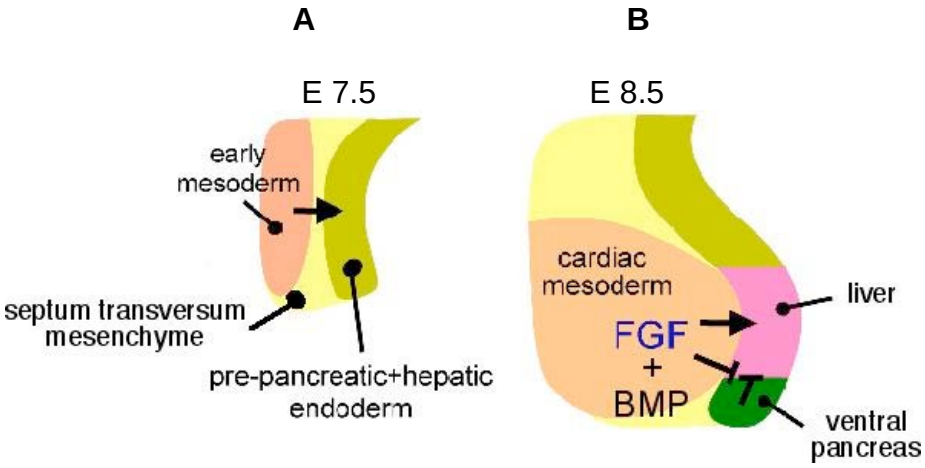



**Figure 1.4** A model showing the common origin of the mouse ventral pancreas and the liver. (A) Early patterning by the mesoderm (arrow) leads to pancreas-liver bipotentiality of the ventral foregut endoderm. (B) FGF and BMP signalling from the cardiac mesoderm and septum transversum mesenchyme divert proximal endoderm from a pancreatic to a hepatic fate (modified from Deutsch et al., 2001).

## 1.4 The common pancreatic precursor

### Pdx1

Specification of the pancreatic buds results in the establishment of pancreatic precursor cell populations in the dorsal and ventral buds that have the potential to differentiate into all pancreatic cell types of the exocrine and endocrine lineages. The early pancreatic precursor cells express Pdx1, a homeodomain protein which is also expressed in adjacent presumptive posterior stomach and duodenum (Ohlsson et al., 1993; Jonsson et al., 1994). Lineage tracing studies have indicated that the Pdx1 expressing cells represent precursor cells that contribute to the formation of both endocrine and exocrine lineages of the mature pancreas (Gu et al., 2002). In Pdx1 homozygous mutant mice, pancreatic buds are arrested in their development; however, early glucagon and insulin expressing cells, which normally do not express Pdx1, can still be detected (Ahlgren et el., 1996; Offield et el., 1996). Besides the pancreas, the role of Pdx1 in duodenal and posterior stomach development is evidenced in the lack of development of Brunners glands in the duodenum and impaired specification of gastrin-producing stomach endocrine cells (Jonsson et al., 1994; Offield et al., 1996).

### Hlxb9

Hlxb9 encodes the homeodomain protein Hb9, which displays two distinct phases of expression in the developing mouse pancreas. It is initially expressed broadly in both dorsal and ventral pancreatic buds, starting from

E8.0, diminishes by E10.0 and reappears later, but only in differentiated β-cells. The expression of Hb9 in the dorsal pancreatic rudiment precedes that of Pdx1 and in Hlxb9 null mice, no dorsal pancreatic programme is initiated. However, the ventral pancreas forms and differentiates to give rise to all pancreatic cell types, albeit with a reduced number of insulin positive cells and an increase in somatostatin positive cells (Li et al., 1999; Harrison et al., 1999). Thus, whereas Hb9 is required for specification of the dorsal pancreas, its activity appears to be required only for β-cell differentiation in the ventral pancreas, an instance that reflects the different molecular mechanism involved in the specification of the dorsal and ventral pancreatic rudiments.

Although the early expression of Hb9 is essential for dorsal pancreas development, its downregulation by E10 is critical for subsequent pancreatic differentiation. A sustained high level expression of Hb9 in the developing pancreas under the promoter of Pdx1 results in embryos with pancreatic hypoplasia, an effect which was observed to initiated already by E11.5; prior to this stage, at E10.5, pancreatic development appear normal (Li and Edlund, 2001). Hb9 activity is therefor required only transiently at early stages (E8.0-E10) for dorsal pancreas development, while at subsequent developmental stages it is restricted to and required for proper β-cell differentiation.

**Ptf1a/p48**

Ptf1a/p48 is a basic helix-loop-helix transcription factor, which was originally identified as a part of a heterotrimeric pancreas transcription factor complex, referred to as PTF1; it consists of the bHLH factors p64 and p75 in addition to Ptf1a/p48, and it activates transcription of exocrine specific pancreatic genes in the mature pancreas (Cockell et al., 1989). p64 mediates nuclear import of the complex, but does not bind DNA, while p48 and p75 make direct contact with their target DNA (Cockell et al., 1989; Roux et al., 1989). Mice bearing a null mutation of Ptf1a/p48 are completely devoid of exocrine pancreas, while the endocrine pancreas still develops, but is found to translocate to the spleen (Krapp et al., 1998). Using a combination of knock out and cell lineage tracing strategies, Kawaguchi et al., have shown that, Ptf1a/p48 is expressed in pancreatic precursor cells that contribute to both exocrine and endocrine pancreatic lineages, and in Ptf1a/p48 deficient mice, pancreatic precursor cells adopt a duodenal fate (Kawaguchi et al., 2002).

## 1.5 Notch signalling and exocrine-endocrine cell segregation

Notch signaling is initiated by intracellular cleavage of the Notch receptor following activation by a ligand from a neighbouring cell. The released intracellular domain of the receptor interacts with the DNA-binding protein RBP-jk in the nucleus to activate transcription of the bHLH genes of the Hairy Enhancer of Split (HES) family. HES family members generally act as

transcriptional repressors of pro-differentiation factors such as neurogenin3 (ngn3) (Lee et al., 2001).

Both endocrine and exocrine pancreatic cells develop from a common precursor cell population in the foregut endoderm. Endocrine precursor cells appear scattered within the pancreatic progenitor pool and are marked by a high level of ngn3. Ectopic expression of ngn3 in most of the pancreatic precursors results in a massive, premature differentiation of these pancreatic progenitors into endocrine cells, with a concomitant loss of exocrine differentiation (Apelqvist et al., 1999; Schwitzgebel et al., 2000). This observation implicated Notch signaling to be involved in endocrine–exocrine segregation from the pancreatic precursor pool. The involvement of Notch signaling in pancreatic cell segregation was confirmed by examining mice deficient in the Notch receptor delta-like ligand-1 (Dll1) or the intracellular mediator RBP-jk. Dll1 mutant mice show a reduced level of HES-1 in the dorsal pancreatic epithelium and an increase in ngn3. These mice develop a relatively small pancreatic bud consisting primarily of differentiated endocrine cells (Apelqvist et al., 1999). RBP-jk mutant mice as well show an increased number of ngn3 positive cells in the dorsal pancreatic epithelium (Apelqvist et al., 1999). Furthermore, HES-1 deficient mice show pancreatic hypoplasia as a result of increased endocrine differentiation (Jensen et al., 2000). The above studies portray a lateral inhibition model, in which ngn3 positive cells (endocrine progenitor cells) represent the primary fate of the developing pancreas and Notch signaling acts to reserve a population of the precursor pool for proliferation and subsequent development into a secondary fate, which would be the exocrine fate.

Indeed, constitutive activation of notch signaling by selective misexpression of the intracellular domain of Notch1 (Notch-ICD) in ngn3 positive cells is sufficient to inhibit them from differentiating into endocrine cells whereas fully differentiated endocrine cell cannot be dedifferentiated by missexpression of Notch-ICD (Murtaugh et al., 2003). Furthermore, a broader expression of Notch-ICD in the developing pancreas under the Pdx1 promoter results in an arrest of both endocrine and exocrine differentiation, leaving cells in a progenitor state (Hald et a.,2003; Murtaugh et al., 2003). This suggests that, for subsequent exocrine pancreas differentiation, notch signaling needs to be downregulated.

### 1.5.1 Transcription factor regulation of exocrine pancreas differentiation

Compared to the endocrine pancreas, not many transcription factors have been shown to be involved in the development of the exocrine compartment of the pancreas. As stated above (section 1.4) the bHLH transcription factor Ptf1a/p48 is required for the development of the exocrine pancreas, and the entire exocrine pancreas is lost in Ptf1a/p48 null embryos (Krapp et al., 1998).

**Mist1**

Mist1 is a bHLH transcription factor which is expressed in the exocrine pancreas in addition to its expression in exocrine cells of the salivary glands and chief cells of the stomach (Pin et al., 2000). Mice deficient in Mist1 develop exocrine pancreatic cells that coexpress both, ductal and exocrine marker genes, suggesting that Mist1 might play a role in the maintenance of a stable exocrine cell identity (Pin et al., 2001).

### 1.5.2 Transcription factor regulation of endocrine pancreas differentiation

**Ngn3**

The basic helix-loop-helix (bHLH) transcription factor ngn3 is expressed in a subset of pancreatic precursor cells starting from E9.0, its expression reaches a peak at E15.5 and then diminishes to barely detectable levels at birth (Apelqvist et al., 1999 ; Jensen et al., 2000 ; Schwitzgebel et al., 2000). Immunohistochemical analysis indicates that ngn3 is expressed in islet cell progenitors (Schwitzgebel et al., 2000). The requirement of Ngn3 for endocrine pancreas differentiation is demostrated by the coplete loss of all endocrine cell types in ngn3 null mice (Gradwohl et al., 2000). However, ectopic expression of ngn3 in pancreatic precursor cells results in premature differentiation of predominantly $\alpha$ cells, suggesting that other factors may be

required for the full complement of endocrine cells (Apelqvist et al., 1999 ; Schwitzgebel et al., 2000).

**NeuroD/Beta2**

NeuroD/Beta2 is another bHLH transcription factor that is expressed in all pancreatic endocrine cells (Lee et al., 1995 ; Naya et al., 1995). Coexpression of NeuroD and ngn3 indicates that, NeuroD expressing cells derive from cells that have downregulated ngn3 expression, such that the expression of both factors does not overlap (Naya et al., 1997). The fact that ngn3 null mice lack epxression of NeuroD is a further indication that NeuroD expressing cells derive from ngn3 postive lineage. The expression of NeuroD precedes markers of differentiated endocrine cells leading to a model whereby ngn3 positive pancreatic cells that become commited to differentiation express NeuroD prior to the appearance of differentiation markers.

**Differentiation of endocrine panceatic cell types**

**Nkx2.2**

The NK-homeodomain factor Nkx2.2 is initially expressed broadly in the pancreatic epithelium at E8.5-9.0 and becomes restiricted to $\alpha$-, $\beta$-, and PP cells in the mature pancreas (Sussel et al., 1998). Despite its broad

expression in undifferentiated pancreatic epithelium, Nkx2.2 mutant embryos only display an islet-specific phenotype. These mutant embryos develop smaller islets which completly lack insulin-producing β-cells and exhibit a reduced number of glucagon and PP cells. Consistent with the lack of Nkx2.2 expression in δ-cells, somatostatin expression remains normal in Nkx2.2 deficient embryos (Sussel et al., 1998). Recent studies have shown that, in Nkx2.2 null mice all β-cells and a portion of the α cells are replaced by ghrelin-expressing cells, which therefore define a novel ε cell population in the islets (Prado et al., 2004).

**Nkx6.1**

Nkx6.1 is another NK homeodomain transcription factor that is expressed in the developing pancreas. Similar to Nkx2.2, Nkx6.1 displays an initial broad expression in the undifferentiated pancreatic buds, though excluded from the early insulin producing cell, but becomes restricted to only β-cells after the secondary transition (Sander et al., 2000 ;Jensen et al., 1996 ;Oster et al., 1998 ; Rudnick et al., 1994). Homozygous mutation of Nkx6.1 results in a dramatic reduction in the number of β-cells formed during the secondary transition (Sander et al., 2000 ). In Nkx6.1 mutant embryos, the number of endorine pancreatic precursors, as marked by the expression of ngn3, remains normal. While the expression of Nkx2.2 is unaffected in Nkx6.1 mutant embryos, Nkx2.2 null embryos lose Nkx6.1 expression; moreover, analysis of the Nkx6.1 promoter suggest binding sites for Nkx2.2 and Pdx1,

thus placing Nkx6.1 downstream of Nkx2.2 and Pdx1 (Sussel et al., 1998; Sander et al., 2000; Watada et al., 2000).

The phenotypes observed in the mutations of the NK homeodomain proteins Nkx2.2 and Nkx6.1 suggest a model whereby Nkx2.2 is required to ensure the progression of endocrine precursor cells to the β-cell lineage by inducing Nkx6.1 expression. In the absence of Nkx2.2 these cells adopt an alternative fate and differentiate into ghrelin produncing ε-cells (Figure 1.5).

**Pax6**

Pax6 is a paired box transcription factor that is expressed as early as E9.0 in cells of the endocrine lineage, a pattern which is maintained in the adult pancreas (Sander et al., 1997 ; St-Onge et al., 1997). Homogygous deletion of Pax6 in mice results in a complete loss of α-cells , while the remaining endocrine cells fail to form the typical islet structure (St-Onge et al., 1997). This effect suggest that Pax6 activitity is required specifically for α-cell differentiation.

However, analysis of the pancreas in mutant mice bearing a point mutation in the Pax6 alleles, resulting in truncated Pax6 proteins (referred to as small eye-*Sey*$^{Neu}$) reveals that Pax6 might play a role in the differentiation of all islet cell types where it has been shown to be expressed. These mice show a significant reduction in all endocrine cells, although α-cells appear to be the

most affected (Sander et al., 1997). This observation is in agreement with the observation that Pax6 binds to a conserved regulatory element common to the promoters of the glucagon, insulin and somatostatin genes (Sander et al., 1997). The possible reason for the discrepancy in the pancreatic phenotype observed in homozygous *Sey*$^{Neu}$ and in Pax6 knock out mice remains to be resolved, as both mutant mice show an identical phenotype in the eye and the brain, where Pax6 activity is also required (Dohrmann et al., 2000).

**Pax4**

Pax4 is another paired box transcription factor that is stcructurally related to Pax6; both factors posses a homeodomain and a conserved octapeptide in addition to the paired domain (Dahl et al., 1997). During murine pancreas development, expression of Pax4 becomes detectable by E9.5, with a peak level during the secondary transition (E13.5-E15.5), and it drops to barely detectable levels at birth. The expression of Pax4 has been shown to colocalise with endocrine marker genes like ngn3, Nkx2.2 and Pax6. (Sosa-pineda, 2004). Initial analysis of Pax4 homozyogous null mice revealed a loss of insulin and somatostatin producing cell with a resulting increase in the number of glucagon-producing cells (Sosa-pineda et al., 1997). The requirement of Pax4 for β-cell fate determination has also been shown in recent studies in which β cells are replaced by ε cells in Pax4 mutant mice (Prado et al., 2004). These findings suggest that the activity of Pax4 is

required for the commitment of β and δ cell fates in pancreatic precursor cells that would otherwise form α and ε cells (Figure 1.5).

In line with the specific loss of β/δ cells in Pax4 mice and α cells in Pax6 null mice, a combined knock out of both genes results in a loss of α, β, δ and PP cells, although PP cells are not affected in the knock out of either gene. This suggest complementary roles of Pax6 and Pax4 for the differentiation of all four endocrine endocrine cell types (St-Onge et al., 1997). An effect of this mutation on ε-cells remains to be shown.

**Arx**

Arx is a homeodomain transcription factor which has recently been shown to be expressed in mouse embryonic pancreas under the control of ngn3. Targeted disruption of Arx results in loss of α cells with an increased level of Pax4 and a corresponding increase in β- and δ-cells. Conversly, examination of Pax4 mutants revealed an increased level of Arx transcripts, suggesting an oppossing effect of Arx and Pax4 on each others expression in the determination of α and β/δ cell fates respectively (Collombat et al., 2003).

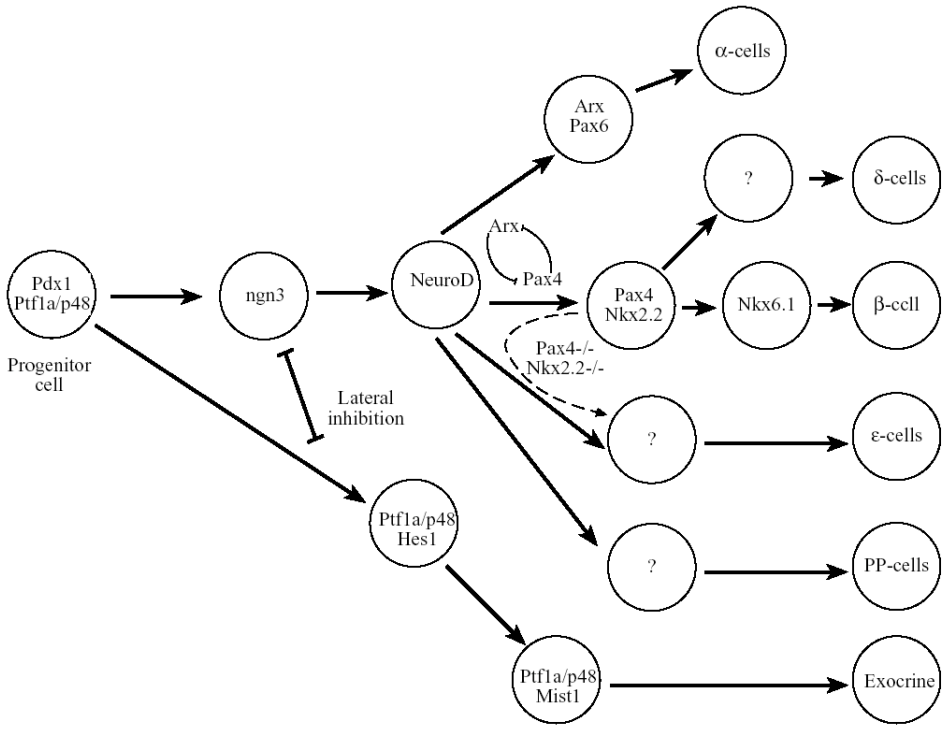



**Figure 1.5** A simplified scheme of gene network involved in determination of the various pancreatic cell lineages. Circles represent cell clusters. Arrows represent cell lineages. Blunt arrows indicate inhibition of a particular lineage. Broken arrows represent the alternative lineage of cells in the absence of the genes indicated. Question marks indicate instances where the gene(s) involved in a particular lineage determination is unknown (the above scheme is based on the studies discussed above).

## 1.3 Aim of this Work and Most Important Conclusions

As outlined above, previous studies of pancreas development have been carried out mainly in the mouse. In an effort to study pancreas development in *Xenopus laevis* embryos, a previous screening of *Xenopus laevis* pancreas cDNA library had been carried out in order to identify marker genes expressed in the embryonic pancreas. This resulted in the isolation of, among other marker genes, pancreatic protein disulfide isomerase (XPDIp). XPDIp is expressed exclusively in the embryonic pancreas before fusion of the pancreatic buds. In continuation of these studies, the present work aimed at a

detailed characterisation of XPDIp in order to define the pancreatic cell type marked by its expression, and as such establish it as a definitive molecular marker for use in studying pancreas development in *Xenopus laevis*.

Studies on pancreas development as carried out in mice, have to a large extent employed loss of function strategies leading to an understanding of the crucial roles of individual factors at different stages of pancreas development. It remains to be clarified, however, what constitutes the basic complement of factor(s) required and sufficient for a given stage of pancreas development. The second part of this study is focussed on the characterisation of the role of Ptf1a/p48 in early pancreas development in *Xenopus laevis*. Data obtained in a gain of function experiments with Ptf1a/p48 suggest that the activities of both Ptf1a/p48 and XlHbox8, the *Xenopus* homologue of Pdx1 are required for pancreas development.

# 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Chemicals

| | |
|---|---|
| Acetic anhydride | (Sigma) |
| 10 X PCR buffer | (Perkin – Elmer ) |
| Acetic acid | (Merck) |
| Acetone | (Merck) |
| Agarose | (Gibco - BRL) |
| Ammonium Persulfate | (Serva) |
| Ampicillin | (Biomol) |
| BCIP | (Boehringer Mannheim) |
| Boehringer block | (Sigma) |
| Boric acid | (Merck) |
| Bovine Albumin | (Sigma) |
| BSA | (Sigma) |
| $Ca(NO_3)_2$ | (Merck) |
| $CaCl_2$ | (Merck) |
| CHAPS | (Sigma) |
| Chloroform | (Merck) |
| Dexamethasone | (Sigma) |
| Dextran sulfate | (Merck) |

| | |
|---|---|
| DMSO | (Merck) |
| DTT | (Gibco) |
| EDTA | (Paesel & Lorei) |
| EGTA | (Sigma) |
| Ethanol | (Merck) |
| Ethidium Bromide | (Roth) |
| Ficoll | (Sigma) |
| Ficoll 400 | (Pharmacia) |
| Formaldehyde | (Merck) |
| Formamid | (Merck) |
| Glutaraldehyde | (Sigma) |
| Glycerol | (Merck) |
| Glycogen, Molecular Biology grade | (Boehringer Mannherim) |
| Goat Serum | Gibco – BRL |
| $H_2O_2$ | (Baker), (ROTH, $N^r$ 2014) |
| HCl | (Merck) |
| Heparin | (Sigma) |
| HEPES | (Sigma) |
| Human Chorionic gonadotropin (HCG) | (Sigma) |
| Isopropanol | (Merck) |
| $K_3Fe(CN)_6$ | (Sigma) |
| $K_4Fe(CN)_6$ | (Sigma) |
| KCl | (Merck) |
| L-Cysteinhydrochloride | (Fluka) |
| Methanol | (Merck) |

| | |
|---|---|
| $MgSO_4$ | (Merck) |
| Na acetate | (Roth) |
| Na citrate | (Fluka) |
| $Na_2HPO_4$ | (Merck) |
| NaCl | (Merck) |
| $NaH_2PO_4$ | (Merck) |
| $NaHCO_3$ | (Merck) |
| $NaN_3$ | (Sigma) |
| NaOH | (Merck) |
| NBT | (Boehringer Mannherim) |
| $NH_4$ acetate | (Merck) |
| Nile blue chloride | (Sigma) |
| Paraffin | (Polysciences inc.) |
| Phenol | (Merck) |
| SDS | (Biomol) |
| Streptomycin | (Sigma) |
| Sucrose | (BRL) |
| TEMED | (Serva) |
| Triethanolamine | (Sigma) |
| Tris | Paesel & Lorei) |
| Triton-X-100 | (Sigma) |
| Tween-20 | (Sigma) |
| Xylene | (Roth) |

### 2.1.2 Solutions, buffers and media

#### 2.1.2.1 In situ hybridization

**Alkaline Phosphatase Buffer (APB)**

| | |
|---|---|
| Tris-HCl 1 M, pH 9.5 | **:** 50 ml (100 mM) |
| $MgCl_2$ 1 M | **:** 25 ml (50 mM) |
| NaCl 5 M | **:** 10 ml (100 mM) |
| Tween-20, 20 % | **:** 2.5 ml (0.1 %) |
| $dH_2O$ | **:** to 500 ml |

**Boehringer Block (BMB) 10 % (autoclaved)**

| | |
|---|---|
| 5 X MAB | **:** 20 ml (1 X) |
| BMB* | **:** 10 g (10 %) |
| $dH_2O$ | **:** to 100 ml |

***** Takes about 1 h at 60 °C to dissolve.

Autoclave and store at – 20 °C.

**Denhart´s 100 X solution**

| | |
|---|---|
| BSA | **:** 2 g (2 %) |
| PVP | **:** 2 g (2 %) |
| Ficoll 400 | **:** 2 g (2 %) |
| $dH_2O$ | **:** to 100 ml |

Store at – 20 °C.

**Hybridization solution**

| | |
|---|---|
| Deionized formamid* | **:** 50 ml (50 %) |
| 20 X SSC | **:** 5 ml (5 X) |
| Torula RNA 50 mg / ml | **:** 2 ml (1 mg / ml) |
| Heparin 10 mg / ml | **:** 1 ml (100µg / ml) |
| Denhart´s 100 X | **:** 1 ml (1 X) |
| Tween-20, 20 % | **:** 0.5 ml (0.1 %) |
| CHAPS 10 % | **:** 1 ml (0.1 %) |
| EDTA 0.5 M | **:** 2 ml (10 mM) |
| DEPC $dH_2O$ | **:** to 100 ml |

Store at – 20 °C.

***** To deionize formamid: Add 50 g of mixed bead resin (BioRad) to 500 ml formamid, mix on magnetic stirrer for 2 h and filter on Whatman paper. Reuse resin.

**Heparin 10 mg / ml**

| | |
|---|---|
| Heparin | **:** 100 mg (10 mg / ml) |
| DEPC $dH_2O$ | **:** to 10 ml |

Store at – 20 °C.

**HEPES buffer pH 7.5 (autoclaved)**

| | |
|---|---|
| HEPES | : 238.3 g (1M) |
| $dH_2O$ | : to 1 l |
| pH | : 7.5 |

Autoclave.

**5 X MAB solution (autoclaved)**

| | |
|---|---|
| Maleic acid | : 29 g (500 mM) |
| NaCl | : 21 g (750mM) |
| $dH_2O$ | : to 500 ml |
| pH | : 7.5 |

Autoclave.

**$MgCl_2$, 1 M**

| | |
|---|---|
| $MgCl_2$, 6 H2O | : 203.3 g (1 M) |
| $dH_2O$ | : to 1 l |

**5 X NaCl (autoclaved)**

| | |
|---|---|
| NaCl | : 292.2 g (5 M) |
| $dH_2O$ | : to 1liter |

Autoclave.

**1 X PTw**

| | |
|---|---|
| 10 X PBS | : 50 ml (1 X) |
| Tween-20, 20 % | : 2.5 ml (0.1 %) |

| | |
|---|---|
| $dH_2O$ | : to 500 ml |

**PTw / Proteinase K solution**

| | |
|---|---|
| PTw | : 20 ml |
| Proteinase K 20 mg / ml | : 20 µl |

**2 X SSC / RNase A and RNase T1 solution**

| | |
|---|---|
| 20 X SSC | : 1 ml (2 X) |
| RNase A 10 mg / ml | : 20 µl (20 µg / ml) |
| RNase T1 20,000 U / ml | : 5 µl (10 U / ml) |
| $dH_2O$ | : to 10 ml |

**20 X SSC (autoclaved)**

| | |
|---|---|
| NaCl | : 175.3 g (0.3 M) |
| Sodium citrate | : 88.2 g (0.3 M) |
| $dH_2O$ | : to 1 l |
| pH | : 7.0 |

**Staining solution : NBT / BCIP**

| | |
|---|---|
| APB (1ml) | |
| NBT 75mg/ml in 70% dimethylformamide | : 2 µl |
| BCIP 50mg/ml in 100% dimethylformamide | : 3,5 µl |

**Torula RNA 10 mg / ml**

| | |
|---|---|
| Torula RNA | **:** 100 mg (10 mg / ml) |
| DEPC - $dH_2O$ | **:** to 10 ml |

Store at – 20 °C.

**Tris buffer, pH 9.5 (autoclaved)**

| | |
|---|---|
| Tris-HCl | **:** 121.1 g (1 M) |
| $dH_2O$ | **:** to 1 l |
| pH | **:** to 9.5 |

**Tween – 20, 20%**

| | |
|---|---|
| Tween – 20 | **:** 20 ml (20 %) |
| $dH_2O$ | **:** to 100 ml |

Store at – 20 °C.

### 2.1.2.2 Vibratome sectioning

**Gelatin-Albumin**

| | |
|---|---|
| PBS (10x) | **:** 22.5 ml |
| Gelatine | **:** 1.1 g |
| Albumin | **:** 67.5 g |
| Sucrose | **:** 45.0 g |

Add water up to 225 ml

Stir at 60°C till well disollved. Store at –20°C

**Moviol- Mounting solution**

5 g moviol was dissolved in 20 ml PBS (takes about 16 hours to dissolve). 10 ml of 100% glycerol was then added to the dissolved moviol stirred, aliquoted and stored at –20°C.

### 2.1.2.3 Gel electrophoresis

**Ficoll loading buffer**

| | |
|---|---|
| Tris-HCl 1 M, pH 7.5 | : 0.5 ml (10 mM) |
| EDTA 0.5 M | : 0.1 ml (1 mM) |
| Bromphenol blue | : 0.025 % |
| Xylencyanol | : 0.025 % |
| Ficoll 400 | : 5 g (10 %) |
| $dH_2O$ | : to 50 ml |

**Glycerol loading buffer**

| | |
|---|---|
| Tris-HCl 1 M, pH 7.5 | : 0.5 ml (10 mM) |
| EDTA 0.5 M, pH 8 | : 1 ml (10 mM) |
| Bromphenol blue | : 0.025 % |
| Xylencyanol | : 0.025 % |
| Glycerol 99 % | : 15.15 ml (30 %) |
| $dH_2O$ | : to 50 ml |

**Formamid loading buffer**

| | |
|---|---|
| EDTA 0.5 M, pH 8 | : 0.2 ml (10 mM) |
| Bromphenol blue | : 0.025 % |
| Xylene cyanol FF | : 0.025 % |
| Formamid 99 % | : to 10 ml |

**10 X TBE buffer**

| | |
|---|---|
| Tris | : 108 g (0.89 M) |
| Boric acid | : 55 g (0.89 M) |
| EDTA 0.5 M, pH 8 | : 40 ml (20 mM) |
| $dH_2O$ | : to 1 l |

**EDTA solution**

| | |
|---|---|
| $Na_2EDTA, 2H_2O$ | : 186.12 g (0.5 M) |
| $dH_2O$ | : to 1 l |
| pH | : 8 |

### 2.1.2.4 Plasmid DNA preparation

**TELT buffer**

| | |
|---|---|
| Tris-HCl 1 M, pH 7.5 | : 2.5 ml (50 mM) |
| EDTA 0.5 M, pH 8 | : 0.1 ml (1 mM) |
| LiCl, 10 M | : 16 ml (3.2 M) |
| Triton-X-100, 20 % | : 1.25 ml (0.5 %) |

| | |
|---|---|
| $dH_2O$ | : to 50 ml |

**TE buffer**

| | |
|---|---|
| Tris-HCl 1 M, pH 7.5 | : 1 ml (10 mM) |
| EDTA 0.5 M | : 200 µl (1 mM) |

**Lysozyme solution**

| | |
|---|---|
| Lysozyme | : 100 mg (10 mg / ml) |
| $dH_2O$ | : to 10 ml |

Store at – 20 °C

**Tris buffer (autoclaved)**

| | |
|---|---|
| Tris-HCl | : 121.1 g (1 M) |
| dH2O | : to 1 l |
| pH | : to 7.5 |

**Triton-X-100, 20 % solution**

| | |
|---|---|
| Triton-X-100 | : 20 ml (20 %) |
| $dH_2O$ | : to 100 ml |

### 2.1.2.5 Media and Antibiotics

All the media were autoclaved for at least 15 min at 120 °C, under 1.5 bar.

**LB – medium**

| | |
|---|---|
| Bacto Trypton | : 10 g (1 %) |
| Bacto Yeast Extract | : 5 g (0.5 %) |
| NaCl | : 10 g (17.1 mM) |
| $dH_2O$ | : to 1 l |
| pH | : to 7.5 |

**LB –Agar medium**

| | |
|---|---|
| Bacto Trypton | : 10 g (1 %) |
| Bacto Yeast Extract | : 5 g (0.5 %) |
| NaCl | : 10 g (17.1 mM) |
| Agar | : 15 g (1.5 %) |
| $dH_2O$ | : to 1 l |
| pH | : to 7.5 |

**Tetracyclin stock solution**

| | |
|---|---|
| Tetracyclin | : 250 mg (25 mg / ml) |
| Ethanol 100 % | : to 10 ml |
| Store at – 20 °C | |
| Working concentration | : 12.5 µg / ml |

**Ampicillin stock solution**

| | |
|---|---|
| Ampicillin | **:** 1000 mg (100 mg / ml) |
| $dH_2O$ | **:** to 10 ml |

Store at – 20 °C

| | |
|---|---|
| Working concentration | **:** 75 µg / ml |

### 2.1.2.6 RNA isolation

**Diethylpyrocarbonat (DEPC) – $dH_2O$ (autoclaved)**

| | |
|---|---|
| Diethylpyrocarbonat | **:** 0.5 ml (0.1 %) |
| $dH_2O$ | **:** 500 ml |

Incubate 2 h at 37 °C and autoclave.

**2 X lysis buffer**

| | |
|---|---|
| SDS 20 % | **:** 5 ml (1 %) |
| EDTA 0.5 M, pH 8.0 | **:** 2 ml (10 mM) |
| Tris – HCl 1 M, pH 7.5 | **:** 10 ml (100 mM) |
| NaCl 5 M | **:** 2 ml (100 mM) |

**Lysis buffer**

| | |
|---|---|
| 2 X lysis buffer | **:** 5 ml |
| DEPC – $dH_2O$ | **:** 4.5 ml |
| Proteinase K* (20 µg / µl) | **:** 0.5 ml |

***** Added immediately before use.

**10 X DNase buffer**

| | |
|---|---|
| Tris – HCl | : 400 mM, pH 7.9 |
| NaCl | : 100 mM |
| $MgCl_2$ | : 60 mM |
| $CaCl_2$ | : 1 mM |

**DNase I digestion mix**

| | |
|---|---|
| 10 X DNase buffer | : 5 µl |
| DTT 20 mM | : 2.5 µl |
| RNasin (40 U / µl) | : 1 µl |
| DNase I (10 U / µl) | : 1.5 µl |
| RNA sample | : 40 µl |

**Phenol – Chloroform mix**

| | |
|---|---|
| Ratio | : 1 : 1 (v / v) |
| pH | : 8 – 8.3 |

**Ammonium acetate ($NH_4Ac$) 7.5 M**

| | |
|---|---|
| $NH_4Ac$ | : 57.8 g (7.5 M) |
| $dH_2O$ | : to 100 ml |

### 2.1.3 Kits

| | |
|---|---|
| Big dye terminator Cycle sequencing kit | (PE Applied Biosystems) |
| SP6 Message Machine *in vitro* transcription | (Ambion) |
| Qiagen Plasmid Midi kit | (Qiagen) |
| Qiagen PCR Purification kit | (Qiagen) |
| RNA PCR Core kit | (Perkin Elmer) |
| RNeasy kit | (Qiagen) |
| $T_NT^{®}$ Coupled Reticulocyte Lysate System | (Promega) |

### 2.1.4 Enzymes

| | |
|---|---|
| Restriction enzymes (with supplied buffers) | (NEB, Promega) |
| Proteinase K | (Merck) |
| RNase A | (Worthington) |
| RNase T1 | (Sigma) |
| RNasin (Ribonuclease Inhibitor) | (Promega) |
| RNase – free DNase I (2000 U / ml) | (Ambion) |
| Taq polymerase | (Perkin Elmer) |
| T4 DNA ligase | (Promega) |
| SP6, T3 and T7 RNA polymerases | (Stratagene) |

### 2.1.5 Others

| | |
|---|---|
| Salmon sperm DNA | (Sigma) |
| RNA molecular weight markers | (Gibco BRL) |
| 1 kb molecular weight marker mix | (Gibco BRL) |

**Microscopes**

LSM 510 confocal microscope (Zeiss).

**Computer**

**Hardware** : Apple Macintosh OS 9.1

**Software** :

Adope Photoshop 5.0;

DNA-Star software;

Free Hand 9.0;

Image Quant 2.0;

MS Win Word 6.0

MacMolly Tetra, Version 3.1

## 2.2 Methods

### 2.2.1 Handling and manipulation of embryos

Albino X*enopus laevis* embryos were obtained by hormone-induced egg laying and *in vitro* fertilization. Eggs were collected from *X. laevis* females, which had been injected with 400 – 800 U of human chorionic gonadotrophin (HCG) approximately 10 hours prior to eggs collection. Eggs were fertilised with minced testis in 0.1 X MBS, dejellied with 2 % cystein hydrochloride, pH 8.2 and cultured in 0.1 X MBS. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

**Embryo microinjection**

For knockdown experiments, 2 pmoles of morpholino antisense oligonucleotides (MO, Gene Tools, LLC), either directed against XPtf1a/p48 (5´-CCAACTGCTCCAGGACCGTTTCCAT-3´), or against XlHbox8 (5´-TATTGGTCATCAGCATTCATGGCGA-3´) were injected per embryo. In the case of combined injections of both MOs, 1 pmole of each was injected per embryo. For all mRNA injections, a total of 1.6 ng was injected per embryo. In coinjection experiments of XPtf1a/p48GR together with XlHbox8GR, 0.8 ng of each was injected. For single injections, 0.8 ng of the respective mRNA was injected together with 0.8 ng of β-galactosidase RNA. Capped mRNAs were transcribed in vitro with the SP6 mMESSAGE mMACHINE$^{TM}$ Kit (Ambion). All mRNAs and MOs were injected into the four vegetal cells of 8-cell stage embryos from the vegetal pole. For the activation of the GR-fusion proteins, dexamethasone (Sigma) was prepared as 5 mM stock solutions in 100%

ethanol and applied to the control and mRNA injected embryos at desired stages in a concentration of 10 μM in 0.1 × MBS. Embryos were kept in dexamethasone up to stage 41.

### 2.2.2 Preparation of Electrocompetent Bacteria

xL1 blue bacteria was grown on agar plate overnight and a single colony picked and grown overnight in 10 ml of LB medium containing tetracylin. The cell culture obtained was used to inoculate 1L of LB medium without antibiotic in a 2L conical flask. The cells were grown at 37°C until 0.8 OD was attained after which they were placed on ice for 30 minutes. The cells were pelleted in autoclaved centrifuge bottles by centrifuging half of the volume for 15 minutes at 5000rpm in a GSA rotor, the supernatant was discarded and the other half was addded to the pellet and centrifuged for 7 minutes at 7000rpm. The pellet was resuspended in 10 ml of autoclaved distilled water, centrifuged for 7 minutes at 7000 rpm, the supernatant was discarded and the pellet resuspended in 10 ml of glycerin and centrifuged for 15 minutes at 7000 rpm. The pellet was completely resuspended in 1.2 ml of 10% glycerin (600 μl glycerin/ L of the initial culture volume) and 40 μl aliqouted into 1.5 eppendorf tubes prechilled on ice. The aliqouted cells were chilled in liquid Nitrogen for a while and stored at –70°C.

### 2.2.3 DNA Methods

#### 2.2.3.1 Plasmid mini-preparation (TELT)

The selected clones were subcultured in LB medium overnight; each selected clone was subcultured separately in 1.5 ml LB medium containing 50 μg/ml Kanamycin using 2.5μl of the bacterial colony from the library. The bacteria was then grown overnight at 37°C.

The subcultured bacterial colonies were collected into separate eppendorf tubes (1.5ml of each bacterial colony). These were centrifuged at room temperature at 6000 rpm for 5 minutes in a bench centrifuge. The supernatant was completely sucked off, and the pellet well resuspended in 150 μl of TELT (ref.appendix), 15 μl of 10mg/ml lysozyme added to each tube, vortexed, and incubated at room temperature for 5 minutes. The bacterial cell suspension was heated at 100°C for 2 minutes, and transferred onto ice for 5 minutes. The cell lysate was centrifuged at 14000 rpm for 8 minutes at room temperature. The pellet was removed with a sterilized toothpick, and 100ul isopropanol added, vortexed and centrifuged at 14000 rpm for 15 minutes at room temperature. The supernatant was sucked off with a pipett, and the pellet washed with 200 μl of 70% ethanol by centrifuging at 14000 rpm for 5 minutes at room temperature. The supernatant was sucked off and the pellet heated at 56°C for 5 minutes to dry, and then dissolved in 30 μl of TE buffer with RNAase A (1ml TE+1ul RNAase A), heated again at 56°C to completely dissolve the pellet and centrifuged briefly to collect the solution at the bottom of the tube.

### 2.2.3.2 Plasmid Midi-Preparation (QIAGEN)

Plasmids concentrations of 1μg or more were prepared using the ´´Plasmid Midi kit``(Qiagen) which is based on a modified alkaline lysis procedure followed by binding of plasmid DNA to an anion-exchange resin under low salt and pH conditions. RNA, protiens and low molecular weight molecules are then removed by a medium-salt wash and the DNA eluted in a high-salt buffer and subsequently desalted by isopropanol precipitation.

Bacteria transformed with the appropriate plasmid were grown by inoculating 50 ml of LB containing the appropriate selective antibiotic and incubated overnight at37°C (225 rpm). The bacterial cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C. All traces of the supernatant was removed and the bacteria pellet resuspended in 4 ml of Buffer P1. The cells were then lysed by adding 4 ml of Buffer P2 and mixed by inverting 4-6 times and incubated for 5 minutes at room temperature. In the next step, 4 ml of chilled buffer P3 was added mixed by inverting 4-6 times and incubated on ice for 15 minutes. The lysate was then filtered through a filter paper onto a QIAGEN-tip 100 column pre-equilibrated with 4 ml of Buffer QBT. The cleared lysate was allowed to flow through the the resin by gravity. The column was then washed twice with 10 ml of Buffer QC each time. The DNA was eluted with 15 ml of Buffer QF, and precipitated by adding 3.5 ml of isopropanol, mixed and centrifuged at 15000 x g for 30 minutes at 4°C. The supernatant was decanted and the pellet washed with 2 ml of 70% ehtanol and centrifuged at 14000 x g for 10 minutes in a bench centrifuge. The pellet was dried for 5 minutes on a 65°C block and dissolved in 50 μl of Buffer TE.

**Buffer P1:** 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.1 mg/ml RNase A

**Buffer P2:** 200 mM NaOH, 1% SDS (w/v)

**Buffer P3:** 3 M Potassium-Acetate (pH 5.5)

**Buffer QBT**: 750 mM NaCl, 50 mM MOPS (pH 7.0), 15% Ethanol, 0.15% Triton X-100

**Buffer QC:** 1 M NaCl, 50 mM MOPS (pH 7.0), 15% Ethanol

**Buffer QF:** 1.25 M NaCl, 50 mM Tris-HCl (pH 8.5), 15% Ethanol

**Buffer TE:** 10 mM Tris-HCl (pH 8.0), 1 mM EDTA

#### 2.2.3.3 DNA Restriction Digest

Plasmid DNA or PCR generated DNA restriction digest was performed with 2 to 5 U of the appropriate restriction enzyme per μg DNA and incubated in the appropriate reaction buffer at 37°C overnight.

#### 2.2.3.4 Agarose-Gel Electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments of different sizes and also for fragment size determination. Preparative and analytical gels of 1.2% and 1.5%(w/v) respectively of agarose in TBE-Buffer were used, 0.5μg/ml of ethidium bromide was added before the gel solidified. DNA samples were mixed with equal volume of loading buffer and loaded in the

slots of the gels immersed in TBE Buffer contained in a horizontal gel chamber and run under a voltage of 80 to 150 V. DNA bands were visualised under UV-light and documented with a video camera. The size of fragments were determined by comparison to a 1 kb DNA ladder (Gibco) which was run alongside the loaded samples.

**DNA-loading buffer (2 x):** 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.025% Bromphenolblue, 0.025% Xylencyanol, 30% (v/v) Glycerin

**"1 kb Ladder":** 1 x DNA-Probe buffer, 1/20 Vol. Boehringer Buffer H (10 x), 0.4 Vol. TE, 50 ng/µl "1 kb Ladder" (Gibco, 1 µg/µl)

### 2.2.4 RNA Methods

#### 2.2.4.1 Tissue Total RNA Isolation

**Homogenisation**

The tissue samples as well as a mortar and pestle were pre-chilled in liquid Nitrogen. The tissues were then ground to powder with the pre-chilled mortar and pestle. The tissue (50 – 100 mg) was then homogenized in 1.0 ml of TRIZOL reagent by passing it 10 or more times through needles (0.8, 0.55, 0.33 diameter) fitted to an RNase-free syringe.

**Phase Separation**

200 μl of chloroform was added to the homogenized lysate and mixed by inverting 15 seconds and allowed to stand at room temperature for 2 – 3 minutes. The sample was then centrifuged for 15 seconds at 10000 rpm in a bench centrifuge at 4°C. The centrifugation resulted in two phases; an upper aqueous phase and a lower phenol-chloroform phase.

**RNA Precipitation**

The aqueous upper phase containing the RNA was transferred into a fresh tube. The RNA was precipitated from the aqueous phase by mixing it with 0.5 ml of isopropanol and left to stand at room temperature for 10 minutes. It was then centrifuged at 10000 rpm for 10 minutes at 4°C.

**RNA Wash**

The supernatant was removed and the pellet washed with 75% ethanol and centrifuged at 10 000 rpm for 5 minutes at 4°C in a bench centrifuge. The RNA pellet was dried briefly on a 37% block for about 5 minutes. The pellet was then dissolved in 90 μl of DEPC water.

**DNase Digestion**

Genomic DNA in the RNA sample was digested as follows:

| | |
|---|---|
| 5x Transcription buffer | 12.0 μl |
| RNA sample | 45.0 μl |
| RNasin | 1.0 μl |
| DNase I | 2.0 μl |

The reaction mix was incubated at 37°C for 20 minutes and purified by QIAGEN RNA purification kit.

#### 2.2.4.2 Embryonic Total RNA Isolation (QIAGEN)

Embryos (2-3) were collected into a 1.5 ml eppendorf tube and sucked dry of water. They were then homogenised in 350 μl of lysis buffer (10μl β-mecaptoeth1ml buffer RTL) by passing it 10 or more times through needles (0.8, 0.55, 0.33 diameter) fitted to an RNase free syringe. The homogenate was centrifuged at 14000 rpm for 3 minutes, the supernatant was transferred into a fresh tube containing 350 μl of 70 % ethanol and mixed by pipetting. The resulting solution was loaded onto a column and centrifuged at 10000 rpm for 15 seconds. The column was washed with 350 μl of buffer RW1 and centrifuged at 10000 rpm for 15 seconds. DNase digestion was performed on the column by pipetting 80 μl of DNase I solution (70 μl buffer RDD + 10 μl DNase I stock) onto it and incubated at 30°C for 30 minutes. The column was washed with 700 μl of buffer RW1 and centrifuged at 10000 rpm for 15 seconds, 700 μl of buffer RPE and centrifuged at 10000 rpm for 15 seconds. The last washing step was repeated, but centrifuged at 14000 rpm for 2 minutes. The RNA was eluted from the column in two steps, each with 20 μl of DEPC water and centrifuged at 10000 rpm for 1 minute.

### 2.2.5 Determination of Nucleic acid Concentration

The concentration of nucleic acids (DNA, RNA, and Oligonucleotides) was determined by measuring their absorbances with a spectrophotometer at the wavelenght of 260 nm and their repective concentrations were determined as follows:

ds DNA : 1 $OD_{260}$ = 50 μg/ml

RNA : 1 $OD_{260}$ = 40 μg/ml

Oligonucleotides : 1 $OD_{260}$ = 33 μg/ml

The Dig-labelled antiesnse RNA generated above was then used for whole mount *in situ* hybridization .

### 2.2.6 Sequence reaction

The sequence reaction consisted of 5 μl of sequence buffer (seq. mix); 1μl of primer-CMV-F and 5ul of mini-plasmid preparation (0.5 μg),this was then incubated in the same way as in PCR.

**Purification after sequence reaction**

The sequence reaction product was added to 90ul of PCR water, 10ul 3M NaAc, pH 5.2, and 275 μl of 100% ethanol. This was centrifuged at 14000rpm for 30 minutes in the cold room. The supernatant was sucked off and the pellet redissolved in 250ul of70% ethanol and centrifuged at 14000 rpm for 5 minutes. The pellet was dried by heating at 65°C for 5 minutes, it was then

dissolved in 3 μl of pink formamid and submitted for it to be ran on a gel and the nucleotide sequence determined.

### 2.2.7 In situ Hybridization

#### 2.2.7.1 Dig-labelled antisense RNA transcription

The antisense probes for insulin, XPDIp, amylase, elastase, Cyl18, XHex, XPtf1a/p48 and XlHbox8 were prepared as described previously (Afelik et al., 2005; Chen et al., 2004). The antisense probe for HNF3α was prepared from the full-length cDNA clone in pCS2+, which was cloned by RT-PCR based on the sequence from the data bank (GenBank accession numbers: M93658, BC047130). The antisense probes for En2 (Hemmati-Brivanlou et al., 1991) and Krox20 (Bradley et al., 1993) were prepared as described.

Dig-labelled antisense RNA was transcribed for each of the above genes using the reaction mix below:

| | | |
|---|---|---|
| 1x | Transcription buffer | 5.0μl |
| *Dig-NTP mix | | 2.0μl |
| DTT (0.75uM) | | 1.0μl |
| RNasin | | 0.5μl |
| DEPC-Water | | 11.0μl |
| T 7 RNA polymerase | | 0.5μl |

Template (plasmid) 5.0μl

The Dig-labelled antiesnse RNA generated above was then used for whole mount *in situ* hybridization .

*The Dig-NTP mix consist of the following:

| | |
|---|---|
| dATP | 2nM |
| dCTP | 2nM |
| dGTP | 2nM |
| dTTP | 1.9nM |
| Dig-11-dUTP | 0.1nM |

*The Dig-NTP mix consist of the following:

| | |
|---|---|
| dATP | 2nM |
| dCTP | 2nM |
| dGTP | 2nM |
| dTTP | 1.9nM |
| Dig-11-dUTP | 0.1nM |

This was incubated at 37°C for 4 hours and the template DNA plasmid was digested by adding 1 μl of DNase1 to the reaction and incubated at 37°C for 15 minutes.

The Dig labelled RNA was purified by adjusting the volume to 100 μl with RNase free water, 350 μl of buffer RTL was added and mixed thoroughly, 250 μl of 100% ethanol was then added and mixed by pipetting. The sample was applied onto a spin column and centrifuged at 1000 rpm for 15 seconds. The column was washed twice with 500μl of buffer RPE and cetrifuged at 15 seconds and the RNA eluted with 50 μl of RNase free water.

The Dig-labelled anti sense RNA was then used for either whole mount in situ hybridization or in situ hybridization of sectioned materials.

### 2.2.7.2 Whole-mount in situ hybridization

**Rehydration of embryos**

Embryos of the various stages, prefixed in MEMFA, were rehydrated as shown in the table below (the total volume in each case was 5ml):

| | Ethanol (%) | Buffer (%) | Incubation (min) | No. of times |
|---|---|---|---|---|
| 1. | Ethanol 100 % | | 5 | 1 x @ |
| 2. | Ethanol 75 % | $dH_2O$ 25% | 5 | 1 x @ |
| 3. | Ethanol 50% | $dH_2O$ 50% | 5 | 1 x @ |
| 4. | Ethanol 25 % | PTw 75 % | 5 | 1 x @ |
| 5. | | PTw 100% | 5 | 4 x @ |

@=End over end;PTw=1xPBS+0.1% Tween-20

**Proteinase-K treatment**

Up to this step, embryos of the same stage were kept together in one tube. Each stage was kept in 1ml PTw containing 10ug/ml Proteinase K. Embryos of gastrula, neurula and tail bud stages were incubated for 12,18 and 25 minutes respectively at room temperature, and tadpole stage embryos incubated at 37°C for 35 minutes.

**Refixing Embryos**

Proteinase K treatment, though renders the embryos permeable to the Dig-labelled antisense RNA probes, this makes the embryos fragile and can affect histology negatively. So in the next step, proteinase K activity is stopped by treatment with acetanhydride, and the embryos refixed with PTw and formaldehyde. The steps are as shown below:

| | Buffer | Other components | Incubation (min) | No. of times |
|---|---|---|---|---|
| 1. | 0.1 M Triethanolamine pH 7.5 | | 5 | 2 x @ |
| 2. | 0.1 M Triethanolamine | 12.5 μl Acetanhydride | 5 | 1 x @ |
| 3. | | 12.5 μl Acetanhydride | 5 | 1 x @ |
| 4. | PTw | | 5 | 2 x @ |
| 5. | PTw + 4% FA | | 20 | 1 x @ |
| 6. | PTw | | 5 | 5 x @ |

**Hybridization**

At this stage embryos of all the stages were distributed into tubes such that each tube for each of the Dig-labelled probes contained all the stages. The embryos were kept in 1ml PTw to which was added 250ul of Hybridization-Mix*, sucked off and 1ml Hybridization-Mix added and incubated at 65°C for 10 minutes, this was again sucked off and 1ml of fresh Hybridization-Mix added and incubated at 60°C for 6 hours. The Hybridization -Mix was replaced with 1ml of new Hybridization buffer to which was added 1μg/ml of the respective Dig-labelled antisense RNA probes and incubated at 60°C overnight.

*Hybridization-Mix (for 100ml)

| | | | |
|---|---|---|---|
| Formamid | 50% | 50 ml | (BRL 5515UB) |
| SSC | 5x | 25 ml | 20x-stock-Lsg. |
| Torula RNA | 1mg/ml | 2 ml | 50mg/ml-stock-Lsg |
| Heparin | 100ug/ml | 10mg | (sigma H-3125) |
| Denhardt´s | 1x | 1ml | 100x-stock-lsg |
| Tween-20 | 0.1% | 0.1g | (sigma P-1379) |
| CHAPS | 0.1% | 0.1g | (Sigma C-3023) |
| EDTA | 10mM | 5ml | 0.2 M-stock-Lsg. |
| DEP-water | | 100 ml | DEPC-water |

Denhart´s 100x=2% BSA,2% PVP,2% Ficoll 400 in double distilled water.

Torula RNA=inDEPC-water, frozen in 1ml aliquots.

## Washing

| | Buffer | Temperature(°C) | Incubation(min) | No. of times |
|---|---|---|---|---|
| 1. | Hybridization buffer (500 µl) | 60 | 10 | 1 x |
| 2. | 2 x SSC | 60 | 20 | 3 x |
| 3. | 2 x SSC with RNasen* | 37 | 30 | 2 x |
| 4. | 2 x SSC | RT | 10 | 1 x |
| 5. | 0.2 x SSC | 60 | 30 | 2 x |
| 6. | MAB | RT | 15 | 2 x |

*RNAse A 20µg/ml (Sigma R-5000)-dissolved in TE 10mg/ml,100°C 10 min)

RNAse T1 10 U/ml (Sigma R-8251)

Aliquoted and stored at -20°C

## Antibody -Incubation

In this step the embryos were incubated with Alkaline Phosphatase(AP)-linked anti Dig-antibody.

| | Buffer | Temperature (°C) | Incubation (min) | No. of times |
|---|---|---|---|---|
| 1. | MAB + 2% BMB | RT | 60 | 1 x — |
| 2. | MAB + 2% BMB 20% Serum | RT | 60 | 1 x — |
| 3. | MAB + 2% BMB 20% Serum + Antibody*(1 : 5.000) | RT | 240 | 1 x — |
| 4. | MAB (Washing) | RT | 30 | 2 x — |
| 5. | MAB (Washing) | 4 | over night | 1 x — |

*Sheep-Anti-Dig antibody linked to Alkaline Phosphatase

**Colour Reaction**

NBT/BCIP is the substrate for Alkaline Phosphatase, which converts it to a coloured product.

| | Buffer | Temperature (°C) | Incubation(min) | No. of times |
|---|---|---|---|---|
| 1. | MAB | RT | 60 | 1 x |
| 2. | APB | RT | 5 | 2 x |
| 3. | APB + NBT/BCIP** | RT | 5 min - 24 h | 1 x |

**1.5 µl NBT/ml APB; 3.5 µl BCIP/ml APB

After NBT/BCIP treatment the embryos were incubated once in MEMFA at room temperature for 15 minutes, followed by an overnight incubation in MEMFA at room temperature.

### 2.2.7.3 In situ hybridization on sectioned material

**Tissue sample preparation**

Embryos were fixed for 1 hour in 4% MEMFA and transferred into 100% ethanol for storage. For paraffin embedding, embryos were incubated in Xylene (2x 20 minutes), followed by incubation in molten paraffin (2x 1 hour at 60°C) and then embedded in paraffin. A microtome was used to prepare 5 µm tissue sections and fixed onto precoated glass slides.

**Dewaxing and rehydration**

Sectioned material was dewaxed in Xylene (2x 10 minutes) and rehydrated in a decreasing series of ethanol (100%, 95%, 80%, 70% and 40% ethanol respectively). Dehydrated tissue sections were then refixed for 20 minutes using 4% Formaldehyde in 1x PBS, pH 8.0.

**Prehybridisation treatments**

Rehydrated tissue samples were prepared for hybridisation as follows:

1 Rinse slide in 2x SSPE

2 Incubate slides in Proteinase K solution at 37°C for 30 minutes
(3μg/ml Prot.K in o.1M Tris pH 7.5, 10mM EDTA)

3 Rinse slides in 2x SSPE

4 Incubate slides in 0.2M HCl for 15 minutes at RT

5 Rinse slides in 2x SSPE

6 Transfer slides to 0.1M triethanolamine, pH 8.0
Add 0.25% acetic anhydride while agitating slides; repeat after 5 minutes

7 Rinse slides in 2x SSPE

8 Put 300μl hybridisation buffer on each slide. Incubate in a humid chamber for aprroximately 2 hours.

**Hybridisation**

1 Heat probes for 5 minutes at 70°C

2 Drain off excess hybridisation buffer. AD 1μg/ml probe solution and return slides to humid chamber and incubate overnight.

**Post Hybridisation**

1 Rinse slides in 2x SSPE (65°C)

2 Add 300μl hybridisation buffer to each slide. Incubate at 65°C for 10 minutes

3 Drain slides and add 300 μl 50% hybridisation buffer; 50% 2x SSPE + 0.3% CHAPS. Incubate at 65°C for 10 minutes.

4 Add 500 μl 2x SSPE + 0.3% CHAPS. Incubate at RT for 30 minutes

5 Rinse slides in 2x SSPE for 30 minutes

6 Incubate slides in 0.02 μg/ml Rnase A in 4x SSPE at 37°C for 30 minutes

7 Wash slides with 50% formamide in 2x SSPE at 65°C for 45 minutes

8 Drain slides and add 500 μl of 2x SSPE + 0.3% CHAPS. Incubate at RT for 10 minutes.

9 Rinse slides 3x, 10 minutes each in *buffer1* (100mM Tris-HCl pH 7.5, 150 mM NaCl).

10 Add 500 μl 1% BMB/20% serum in *buffer1* to each slide. Incubate at RT for 2 hours.

11 Drain slides and add 200 μl 1/1000 anti-Dig-AP or anti-Flu-AP fragments in *buffer 1*. Incubate at RT for 1 hour.

12 Rinse slides 3x 10 minutes in *buffer 1*

13 Rinse slides for 10 minutes in APB

14 Add 1ml colour development solution to each slide. Develop in dark at RT.

15 Rinse in water to stop reaction, rinse quickly in methanol

16 Fix in MEMFA for 40 minutes. Rinse in 1x PBS/Tween for 3x 7 minutes each before mounting in PBS glycerol (1:10).

For double *in situ* hybridisation on sections, the second staining was perfomed by starting from step 9.

### 2.2.8 Vibratome sectioning

Whole mount in situ hybridisation processed embryos were embedded in gelatin-albumin. Gelatin-albumin was polymerised by mixing 2 ml of it with 105 μl of 25% glutaldehyde. 30 μm vibratome sections were made using a Leica VT1000S Vibratome. Sectioned tissue samples were mounted on glass slides using mowiol.

### 2.2.9 Immunohistochemistry

Embryos fixed in 4% MEMFA for 1 hour were dehydrated in 100% ethanol and transferred to xylene before embedding in paraffin. Transverse sections were made (5 μm) and fixed to precoated glass slides. Tissue samples were dewaxed in xylene and rehydrated through a decreasing series of ethanol, and finally in 1 × PBS. The following primary antibodies were used: mouse anti glucagon (1:1000; Sigma) and rabbit anti-somatostatin (1:300; Dako Cytomation). The following secondary antibodies were used: FITC-conjugated anti-rabbit (1:1000; Sigma) and Cy3-conjugated anti-mouse (1:500; Dianova). The fluorescence images were documented with a LSM 510 confocal microscope (Zeiss).

### 2.2.10 RT- PCR Analysis

Reverse transcriptase-polymerase chain reactions were used to analyse the temporal expression of the following genes at various stages of embryogenesis.

#### 2.2.10.1 RT-PCR Primer sequences

XPDIp (27 cycles): forward: 5´-GGAGGAAAGAGGGACCAA-3´ and reverse: 5´-GCGCCAGGGCAAAAGTG-3´; Amylase (32 cycles), forward: 5´-CAAGCTCTGCACTCGTTCTG-3´ and reverse: 5´-GTTTTCTATTTCGCCACTGC-3´; Elastase (28 cycles), forward: 5´-CAGCGCCTCCTTGAACAGTA-3´ and reverse: 5´-TCACCACCAGCGCACACCAT Trypsinogen (30 cycles), forward: 5´-CTGGCTGGGGCAACACTCTC-3´ and reverse: 5´-TAGCCCCAGGACACCACACC-3´; Insulin (35 cycles), forward: 5´-ATGGCTCTATGGATGCAGTG-3´ and reverse: 5´-AGAGAACATGTGCTGTGGCA-3´ (Henry et al., 1996); XlHbox8 (33 cycles), forward: 5´-AATCCACCAAATCCCACACCT-3´ and reverse: 5´-GCCTCAGCGACCCAATAGAA-3´; Histone H4 (26 cycles), forward: 5´-CGGGATAACATTCAGGGTATCACT-3´ and reverse: 5´-ATCCATGGCGGTAACTGTCTTCCT-3´ (Niehrs et al., 1994).

### 2.2.10.2 RT- PCR-Reaction

**Reverse transcription**

| Component | Volume | Final Concentration |
|---|---|---|
| 10x PCR Buffer (contains no MgCl2) | 1.0 µl | 1x |
| MgCl2 | 2.0 µl | 5mM |
| dATP | 1.0 µl | 1mM |
| dCTP | 1.0 µl | 1mM |
| dGTP | 1.0 µl | 1mM |
| dTTP | 1.0 µl | 1mM |
| Random Hexamers/ Oligo d(T)16 primer | 0.5 µl | 2.5µM |
| RNase inhibitor | 0.5 µl | 1U/µl |
| MuLV Reverse transcriptase | 0.5 µl | 2.5U/µl |
| RNA | 1.5 µl | 20ng/µl |
| Total Volume | 10.0 µl | |

**Thermocycling**

| | |
|---|---|
| 22°C | 10 mins |
| 42° C | 50 mins |
| 99° C | 5 mins |

**PCR**

| Component | Volume | Final Concentration |
|---|---|---|
| 10x PCR Buffer (contains no MgCl2) | 2.0 μl | 1x |
| MgCl2 | 0.5 μl | 1.5mM |
| primer pair (7.5 μM each) | 0.75 μl | 0.225μM |
| Water | 16.65 μl | |
| DNA Taq polymerase | 0.1 μl | 0.5U/25μl |
| cDNA (obtained from RT-PCR) | 5.0 μl | |
| **Total Volume** | **25.0μl** | |

**Thermocycling**

| | | |
|---|---|---|
| 1. | 95°C | 2 minutes |
| 2. | 95°C | 45 seconds |
| 3. | 60°C | 45 seconds |
| 4. | 72°C | 4 minutes |
| 5. | 72°C | 30 minutes |
| 6. | 12°C | ∞ |

The number of reaction cycles, from step 4 to step 2, as stated above for each gene.

### 2.2.11 Transformation

Electrocompetent cell (40 μl) was thawed on ice, 1.0 μl of ligation reaction was added to the competent cell and mixed by pippeting, avoiding air bubbles. The cell ligation mix was pipetted into a prechilled 2 cm cuvette, tapped gently to ensure that the level of the suspension was even from both ends of the cuvette. The pulser was set to 2.4kV, the cuvette was placed in the chamber slid and pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber. The cells were pulsed by holding on the twin pulse buttons until there was a beep. 500 μl of LB medium was immediatly transferred into the cell suspension and mixed by pipetting. 250 μl of the cell suspension was plated on agar plates containing ampicillin and preplated with 40 μl of xGal first plated followed by 40 μl of IPTG after 2 minutes. Single colonies (white colonies for pGEMT cloning) were picked and grown for 4 hours in 100 μl of LB containing ampicillin in 96 microtitre plate (37°C, 225rpm )

**PCR Screening of Transformants**

Components for 20 μl reaction volume

| | |
|---|---|
| 10x PCR Buffer | 02.00 μl |
| dNTP mix (25μM) | 00.16 μl |
| Gene specific primer pair (15 μM) | 00.75 μl |
| PCR grade water | 16.75 μl |
| DNA Polymerase | 00.10 μl |

A pippet tip was dipped into each colony and and swirled in the corresponding PCR reaction. The reaction was then thermocycled as follows:

| | |
|---|---|
| 95°C | 2 mins |
| 95°C | 45 secs |
| 60° C | 45 secs |
| 72°C | 4 mins |
| 72°C | 30 mins |
| 12°C | ∞ |

### 2.2.12 Isolation and construction of inducible XPtf1a/p48 and XlHbox8 proteins

Degenerate primers (5´-TCYATYAACGAYTTCGAG-3´ and 5´-GTCCTCKGGKGTCCASCCYT-3´) based on the conserved domains of the human, rat, mouse and zebrafish XPtf1a protein sequences (GenBank accession numbers: CAI12668, NP_446416, NP_061279 and AAO92259 respectively) were used to generate a partial *Xenopus* Ptf1a/p48 (XPtf1a/p48) cDNA fragment by RT-PCR. The 5´- and 3´- extended sequences were isolated from an adult *Xenopus* pancreas λZAP Express phage cDNA library (Afelik et al., 2004) by PCR screening. The full length XPtf1a/p48 open reading frame was cloned into the pCS2+ vector (Rupp et al., 1994; Turner

and Weintraub, 1994), fused with the human glucocorticoid receptor (GR) ligand binding domain (Gammill and Sive, 1997) and is referred to as XPtf1a/p48GR. A corresponding murine Ptf1a/p48-GR fusion construct in pCS2+ was generated using the mouse Ptf1a/p48 cDNA clone (Obata et al., 2001). The full length XlHbox8 open reading frame was amplified by RT-PCR using the data bank sequence (GenBank accession number: X16849) and similarly cloned into the pCS2+ vector containing GR ligand binding domain.

### 2.2.13 In vitro transcription-translation assay (TNT)

In vitro transcription–translation assay was used to analyse the ability of XlHbox8-MO and XPtf1a/p48-MO to inhibit translation of XlHbox8 and XPtf1a/p48 respectively. As template circular pCS2+ plasmid containing XlHbox8GR or XPtf1a/p48, downstream of the SP6 promoter was used. Reaction components is as indicated below:

| | |
|---|---|
| $T_NT^{®}$ Rabbit reticulocyte lysate | 6.25 μl |
| $T_NT^{®}$ Reaction buffer | 0.5 μl |
| Amino acid mixture, minus Methionine, 1 mM | 0.25 μl |
| RNasin® ribonuclease inhibitor (40 u/μl) | 0.25 μl |
| $^{35}$S Methionin (1, 000Ci/mmol at 10mCi/ml) | 0.5 μl |
| $T_NT^{®}$ SP6 RNA Polymerase | 0.25 μl |
| DNA template (1.0 μg/μl) | 1.0 μl |
| Morpholino (appropriate dilution) | ——— |
| Nuclease free water to a final volume of | 12.5 μl |

The reaction was incubated at 30°C for 2 hours.

### 2.2.14 Analysis of translation products by Polyacrylamide gel electrophoresis

Proteins generated in vitro transcription translation assays were separated on 12% polyacrylamide gels. Equal volumes of protein samples and loading buffer (7.5 μl each) was mixed and heated at 100°C, cooled down, before loaded onto the gel. The gel was run at 30 mA, 200V through the starking gel, and then run at 50mA, 200V. After electrophoresis, gels were dried at 70°C for 2 hours. Dried gels were exposed to a phosphoscreen for 2-24 hours and the image developed with a phosphoimager (Typhoon 9400, Amasham biosciences).

Loading buffer consist of: 50mM Tris-HCl (pH6.8), 2% SDS, 0.1% glycerol, 100mM dithiothreitol.

1L volume of Running buffer consists of: 30g Tris base, 144g glycine, 100ml 10% SDS.

**12% Acrylamide gel**

Running gel: Water, 6.45 ml; 30% acrylamide 8.35 ml; 1.5 M Tris-8.0 5ml;

10% SDS 200 μl; 10% APS 100μl; TEMED 20 μl.

Starking gel: Water, 6.0 ml; 30% acrylamide 1.30 ml; 1.5 M Tris-8.0 2.5ml;

10% SDS 100 μl; 10% APS 60μl; TEMED 12 μl.

# 3. Results

## 3.1 Pancreatic protein disulfide isomerase (XPDIp) is an early marker for the exocrine lineage of the developing pancreas in *Xenopus laevis* embryos

XPDIp was initially isolated in a previous screen of an adult Xenopus pancreas cDNA library for pancreatic marker genes as a marker of the embryonic pancreatic buds before and after fusion of the pancreatic bud (Afelik, M.Sc. thesis, 2002; Figure 3.1). The expression of XPDIp reflects the morphology of the embryonic pancreatic buds, clearly portraying the dynamics of their fusion. PDIp has previously been shown to be expressed exclusively in the adult pancreas of human, canine and mouse (DeSilva et al., 1996, 1997; Volkmer, 1997). In humans it has been shown to be expressed only in the acinar cells, the functional subunits of the exocrine pancreas, but not in the islets (DeSilva et al., 1997). This present study was aimed at a detailed characterisation of the expression of XPDIp to clearly define whether its expression in the embryonic pancreas marks all pancreatic precursor cells or a sub-population of cells in the developing pancreas.

XPDIp/insulin co-staining by in situ hybridisation on paraffin serial sections of adult *Xenopus* pancreas shows that XPDIp is expressed in the acinar cells, excluding the insulin expressing cells (Figure 3.2). Moreover, XPDIp/insulin co-staining as well as staining on neighbouring sections of the embryonic

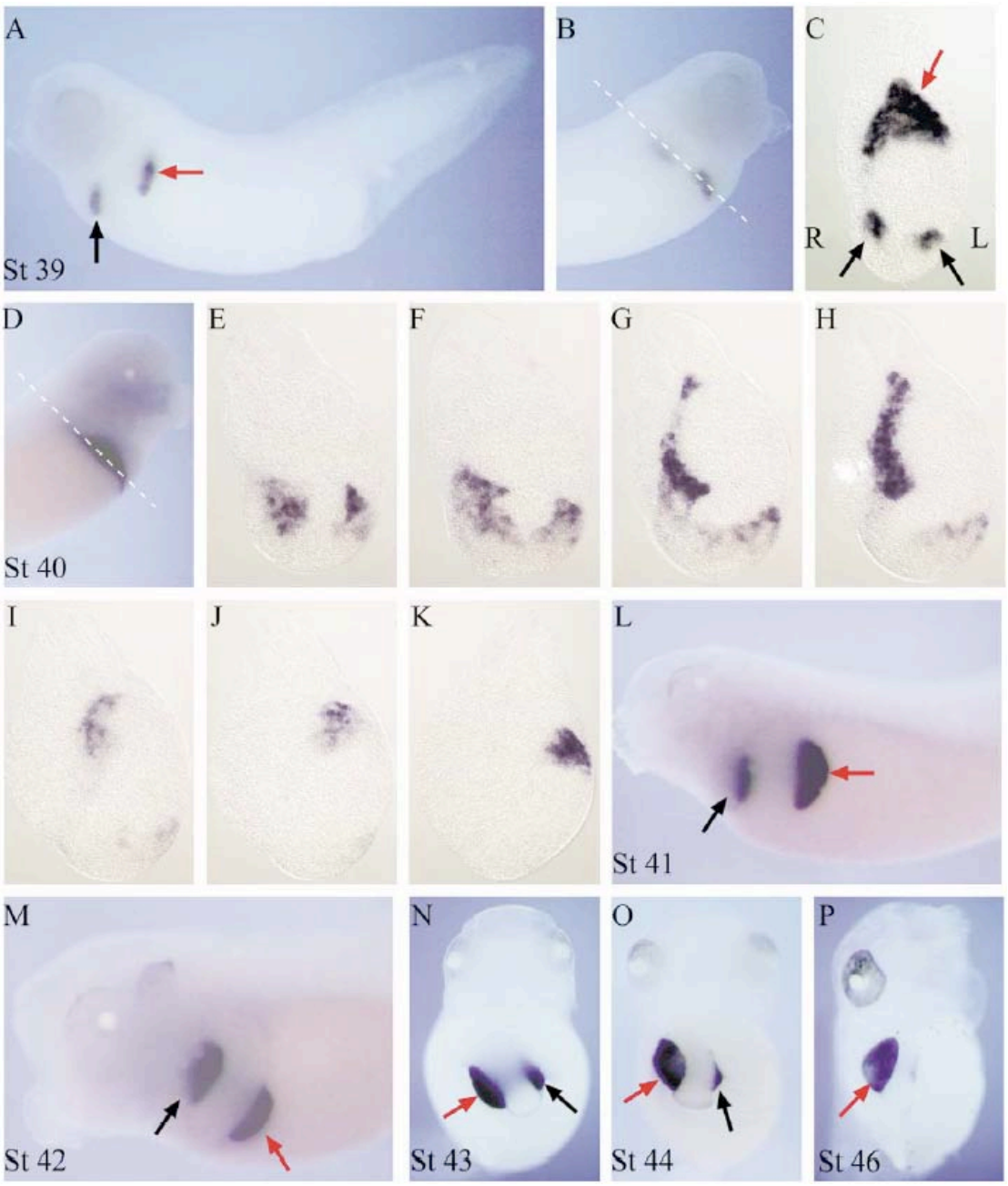



**Figure 3.1** Expression of XPDIp in *Xenopus* embryos between stages 39 to 46. (A) A left side view with expression of XPDIp in the dorsal (red arrow) and ventral (black arrow) pancreatic buds. (B) A right side view of the same embryo as shown in panel A; note the proximity of the dorsal and ventral pancreatic buds on this side of the embryo. The dim appearance of the dorsal bud in right side view as compared to the left side view is as a result of a slight shift of the dorsal bud to the left of the embryo. (C) A cross section of a stage 39 embryo at the level indicated by dotted lines in panel (B) showing the dorsal pancreatic bud (red arrow) and the two ventral buds (black arrows) prior to fusion. Note the slight ventral extension of the dorsal bud on the right side, and the corresponding dorsal shift of the right ventral bud. R and L indicate the right and left side of the embryo, respectively. (D) A right side view showing the fused dorsal and ventral pancreatic buds at stage 40. (E-K) Serial sections, from anterior to posterior, of the embryo shown in panel D. Note the fusion of the two ventral buds in panel F. Inspection of panel G reveals that a part of the right half of the dorsal pancreas is fused to the right ventral pancreas. Panel H

illustrates the dorsal bud in the medial position of the embryo as it extends from the right to the left half; the left half of the dorsal bud is not visible in G and H because it is positioned more posteriorly. Panel I-K reveal the left half of the dorsal pancreas, which lies posterior to the right half. (L – P) Expression of XPDIp from stages 41 to 46. L, M are left lateral views; N, O are antero-ventral views and P a right-ventral view. Dorsal pancreas is indicated with a red arrow and ventral pancreas with a black arrow. (Panels A, B, and N-P are from Afelik, Master thesis, 2002).

pancreas indicates that XPDIp is largely excluded from insulin expressing cells of stage 39 and 44 embryos (Figure 3.2). Thus the expression of XPDIp marks cells of the exocrine pancreatic lineage.

Unlike insulin, which is expressed exclusively in the dorsal bud, or the exocrine pancreatic marker genes amylase, elastase and trypsinogen, which are first detected solely within the ventral pancreas at stage 41 before spreading to the dorsal pancreas by stages 42-44 (Horb and Slack, 2002), XPDIp is expressed simultaneously in both ventral and dorsal pancreatic buds. This implies the presence of cells of the exocrine lineage in both dorsal and ventral pancreatic buds of stage 39 *Xenopus* embryos before fusion. The early and simultaneous expression of XPDIp in the dorsal and ventral pancreas appears to rule out the possibility that the delayed expression of other exocrine pancreas genes in the dorsal pancreas is a result of a delayed specification of exocrine precursor cells in the dorsal pancreas as compared to the ventral pancreas.

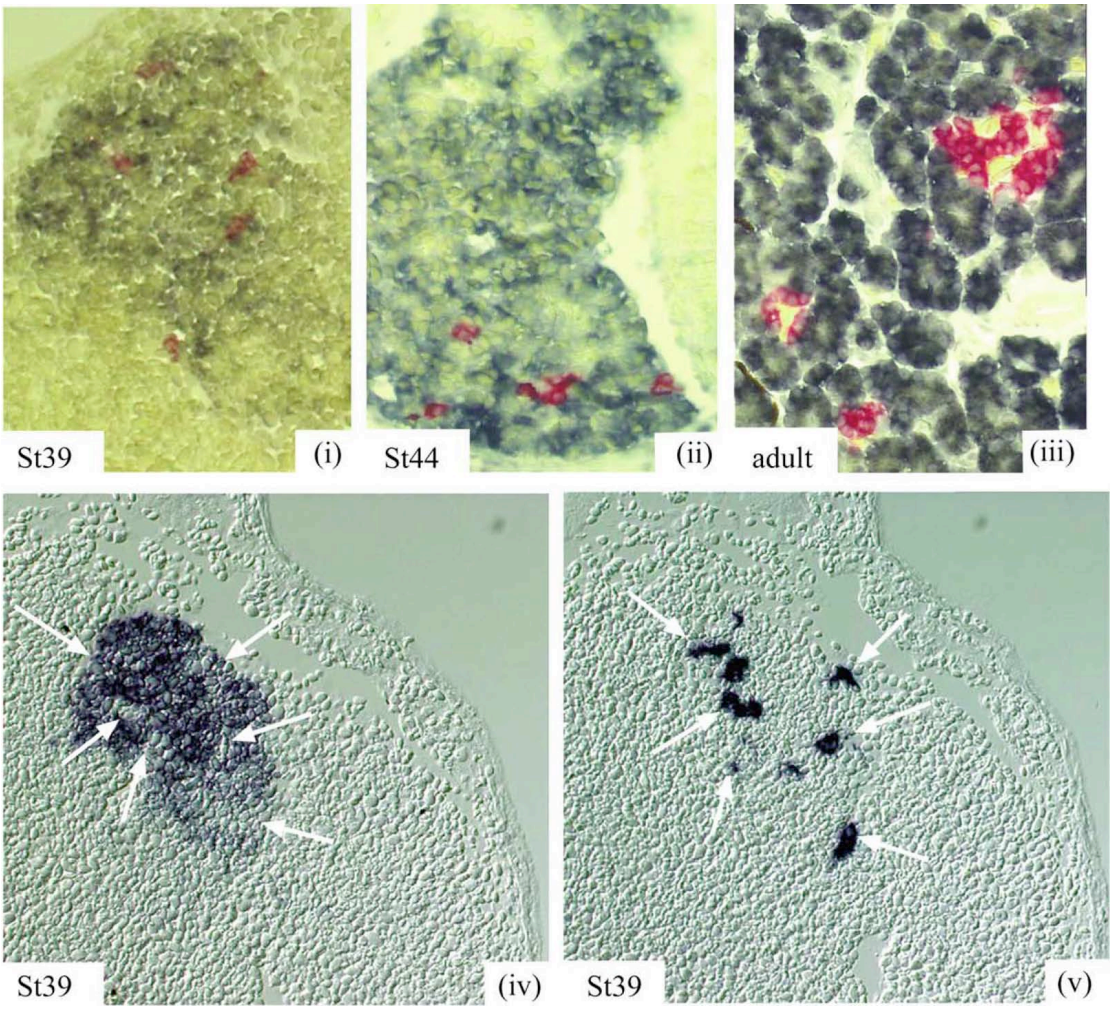



**Figure 3.2** In situ hybridization showing XPDIp and insulin expressing cells in embryonic and adult pancreas. (i)-(iii) Double in situ hybridization staining of insulin (red) and XPDIp (blue) expressing cells in the adult and embryonic pancreas. XPDIp/insulin co-staining of stage 39 dorsal embryonic pancreas, stage 44 embryonic pancreas as well as the adult *Xenpous* pancreas shows that expression of XPDIp is largely restricted to the exocrine pancreatic lineage. (iv) and (v) show the expression of XPDIp and insulin respectively on neighbouring section of stage 39 embryo. The two sections are 5 μm apart. The arrows indicate groups of cells, which express insulin (v) and the corresponding exclusion of XPDIp expression in such cells (iv).

The temporal expression pattern of XPDIp mRNA was analysed by RT-PCR in comparison with amylase, elastase, trypsinogen, insulin and XlHbox8. RT-PCR, being a more sensitive method, reveals small but significant amounts of XPDIp already at stage 35/36, while the exocrine pancreas enzymes amylase, elastase and trypsinogen only become detectable from stage 41 onwards. However, XlHbox8 and insulin can both be detected as early as stage 30/31 (Figure 3.3). Thus, although XPDIp expression can be observed as early as stage 35/36, its activation is significantly later than the one of XlHbox8 and insulin.

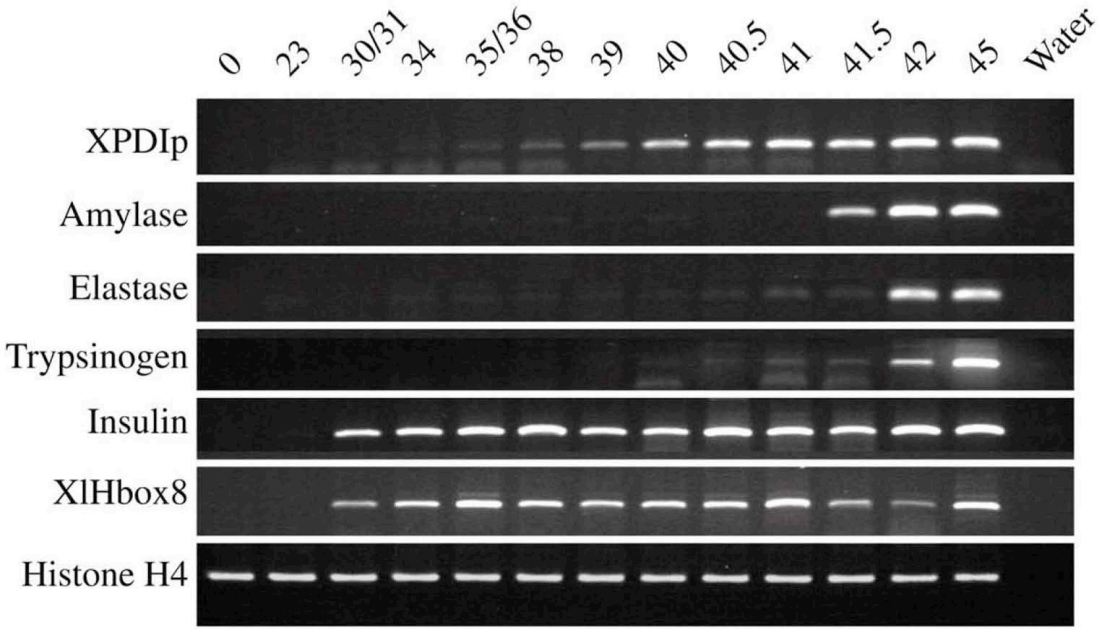



**Figure 3.3** Comparison of the temporal expression patterns of XPDIp, amylase, elastase, trypsinogen, insulin and XlHbox8 by RT-PCR. Onset of XPDIp is detectable at stage 35/36, Amylase at stage 41.5, Elastase and Trypsinogen at stage 42. Both Insulin and XlHbox8 are detected at stage 30/31.

### 3.2.1 Xenopus Ptf1a/p48 displays a high sequence identity to other vertebrate orthologues

The *Xenopus* Ptf1a/p48 was cloned by degenerative PCR based on the conserved domains of the human, rat, mouse and zebra fish Ptf1a/p48 sequences (see Materials and Methods for details). Amino acid sequence comparison of the *Xenopus* Ptf1a/p48 and its orthologues in human, rat, mouse and zebrafish reveals a high degree of sequence conservation across these species, with the zebrafish and frog Ptf1a/p48 being most closely related. Sequence alignment identifies three highly conserved domains, with the most conserved one being the basic helix-loop-helix (bHLH) domain followed by the C-terminal domain. The N-terminal domain is also well conserved, especially in a comparison between the *Xenopus* and zebrafish sequences (Figure 3.4). Linker regions that vary in length and sequence separate the three conserved domains.

**A**

```
X-PTF1a/p48         1   METVL.EQL.AGLESFPSPYFDEDDFFTDHSSRDAL.DADDFLEDD...VDFLAGQIQDY.YRDSR.............
Z-PTF1a/p48         1   MDTVL.DPF.TGLDSFSSSYFDDDDFFTDHSSRDHL.DTDDFLDED...VDFLTNQIQEY.YKDSR.............
M-PTF1a/p48         1   MDAVLLEHFPGGLDTFPSPYFDEEDFFTDQSSRDPLEDSDELLGDEQAEVEFLSHQLHEYCYRDGACLLLQPAPSAAPHA
R-PTF1a/p48         1   MDAVLLEHFPGALDTFPSSYFDEEDFFTDQSSRDPLEDGDELLGDEQAEVEFLSHQLHEYCYRDGACLLLQPAPSAAPHA
H-PTF1a/p48         1   MDAVLLEHFPGGLDAFPSSYFDEDDFFTDQSSRDPLEDGDELLADEQAEVEFLSHQLHEYCYRDGACLLLQPAPPAAPLA

X-PTF1a/p48        81   ...........VLHT..DDDYC.....DAGNFSFSSSSSGG.FPYECG..DGGC......DLSPGMKGGSLV..MKRRRR
Z-PTF1a/p48        81   ...........I.SQ..DGDYC.....DVGNFSFSSSSST..FSYGCA..DSTS......ELSPHRDGG..L..LKRRRR
M-PTF1a/p48        81   LAPPPLGDPGEP.ED..NVSYCCDAGAPLAAFPYSPGSPPSCLAYPCA..AVLSPGARLGGLNGAAAAA..A..ARRRRR
R-PTF1a/p48        81   LAPPPLGDPGEP.ED..SGSYCCDAGAPLGAFPYSPGSPPSCLAYPCT..AVLSPGTRLRGLNGAAAAA..AAAARRRRR
H-PTF1a/p48        81   LAPPSSGGLGEP.DDGGGGGYCCETGAPPGGFPYSPGSPPSCLAYPCAGAAVLSPGARLRGLSGAAAAA..A...RRRRR

X-PTF1a/p48       161   LRSDAEMQQLRQAANVRERRRMQSINDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSEMVQSDLPLRNPNSDS..
Z-PTF1a/p48       161   MRSEVEMQQLRQAANVRERRRMQSINDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLAELVQSDMPIRNPHSDA..
M-PTF1a/p48       161   VRSEAELQQLRQAANVRERRRMQSINDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLHGSGAGGCG
R-PTF1a/p48       161   VRSEAELQQLRQAANVRERRRMQSINDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLRGSGTGGCG
H-PTF1a/p48       161   VRSEAELQQLRQAANVRERRRMQSINDAFEGLRSHIPTLPYEKRLSKVDTLRLAIGYINFLSELVQADLPLRGGGAGGCG

X-PTF1a/p48       241   .............GNQPKKVIICHRGTRSPSPSDPDYGLPPLAGHSLSWTDEKQLRDQNVVRTAKVWTPEDPRKLN.KSP
Z-PTF1a/p48       241   .............LNQPKKVIICHRGTRSPSPNDPDYGLPPLAGHSLSWTDEKQLKDQNIIRTAKVWTPEDPRKLHLKSS
M-PTF1a/p48       241   GPGGSRHLGEDSPGNQAQKVIICHRGTRSPSPSDPDYGLPPLAGHSLSWTDEKQLKEQNIIRTAKVWTPEDPRKLN.SKS
R-PTF1a/p48       241   GPGGSRHLGGDSPGNQAQKVIICHRGTRSPSPSDPDYGLPPLAGHSLSWADEKQLKEQNIIRTAKVWTPEDPRKLN.SKS
H-PTF1a/p48       241   GPGGGGRLGGDSPGSQAQKVIICHRGTRSPSPSDPDYGLPPLAGHSLSWTDEKQLKEQNIIRTAKVWTPEDPRKLN.SKS

X-PTF1a/p48       321   .FSNIENEPPLTLCLDM
Z-PTF1a/p48       321   .INNIENEPPFN..FIS
M-PTF1a/p48       321   .FDNIENEPPFE..FVS
R-PTF1a/p48       321   .FDNIENEPPFE..FVS
H-PTF1a/p48       321   SFNNIENEPPFE..FVS
```

**B**


| | Percentage identity to X-PTF1a/p48 | | | | | |
|---|---|---|---|---|---|---|
| Z-PTF1a/p48 | 76 | 76 | 80 | 96 | 88 | 81 |
| M-PTF1a/p48 | 54 | 16 | 73 | 98 | 18 | 86 |
| R-PTF1a/p48 | 56 | 15 | 73 | 98 | 18 | 82 |
| H-PTF1a/p48 | 59 | 15 | 73 | 98 | 18 | 78 |

**Figure 3.4** Sequence comparison of *Xenopus* Ptf1a/p48 to orthologues in other vertebrates. (A) Alignment of *Xenopus*, zebrafish, mouse, rat and human Ptf1a/p48 proteins sequences. *Xenopus* Ptf1a/p48 is 270 amino acids in length and shows considerable sequence identity with its orthologues. The sequence alignment in (A) highlights the most conserved domains. Besides the three long stretches of conserves domains, namely the N-terminal, bHLH domain and the C-terminal domain, a short but highly conserved domain can be found N-terminal to the bHLH domain. Linker sequences between the most conserved domains are well conserved between *Xenopus* and zebrafish but vary between *Xenopus* and the other three vetebrate species. Percentage sequence identities are depicted in (B).

### 3.2.2 Xenopus Ptf1a/p48 is expressed in the developing pancreas, retina and hindbrain

Using whole mount in situ hybridization, the expression of XPtf1a/p48 is first detected at embryonic stage 20 along two parallel longitudinal stripes representing the neural folds (Figure 3.5, panel A). Neural expression becomes restricted to the hindbrain in tailbud stage (stage 26) embryos, with the anterior end defined by the midbrain-hindbrain boundary and the posterior limit with rhombomere 5. At the same stage, expression of XPtf1a/p48 is also becoming detectable in the developing retina (Figure 3.5, panels B, E and F). During later phases of development, retinal expression is confined to the proliferating precursor cells of the ciliary marginal zone and expression in the neural tube to dorsal elements (Figure 3.5, panel H).

Within the endoderm, expression of XPtf1a/p48 is specifically restricted to the entire dorsal and ventral pancreatic anlagen, initiating as a faint signal at stage 27 (data not shown) and becoming more prominent at stage 28 (Figure 3.5, panel C). A transverse section of a stage 35 embryo depicts expression of XPtf1a/p48 in the dorsal and the two ventral pancreatic rudiments (Figure 3.5, panel G). While the endodermal expression of XPtf1a/p48 is restricted to the pancreas, expression of XlHbox8 also includes adjacent regions of the presumptive stomach and duodenum (Figure 3.5, panels J, K).

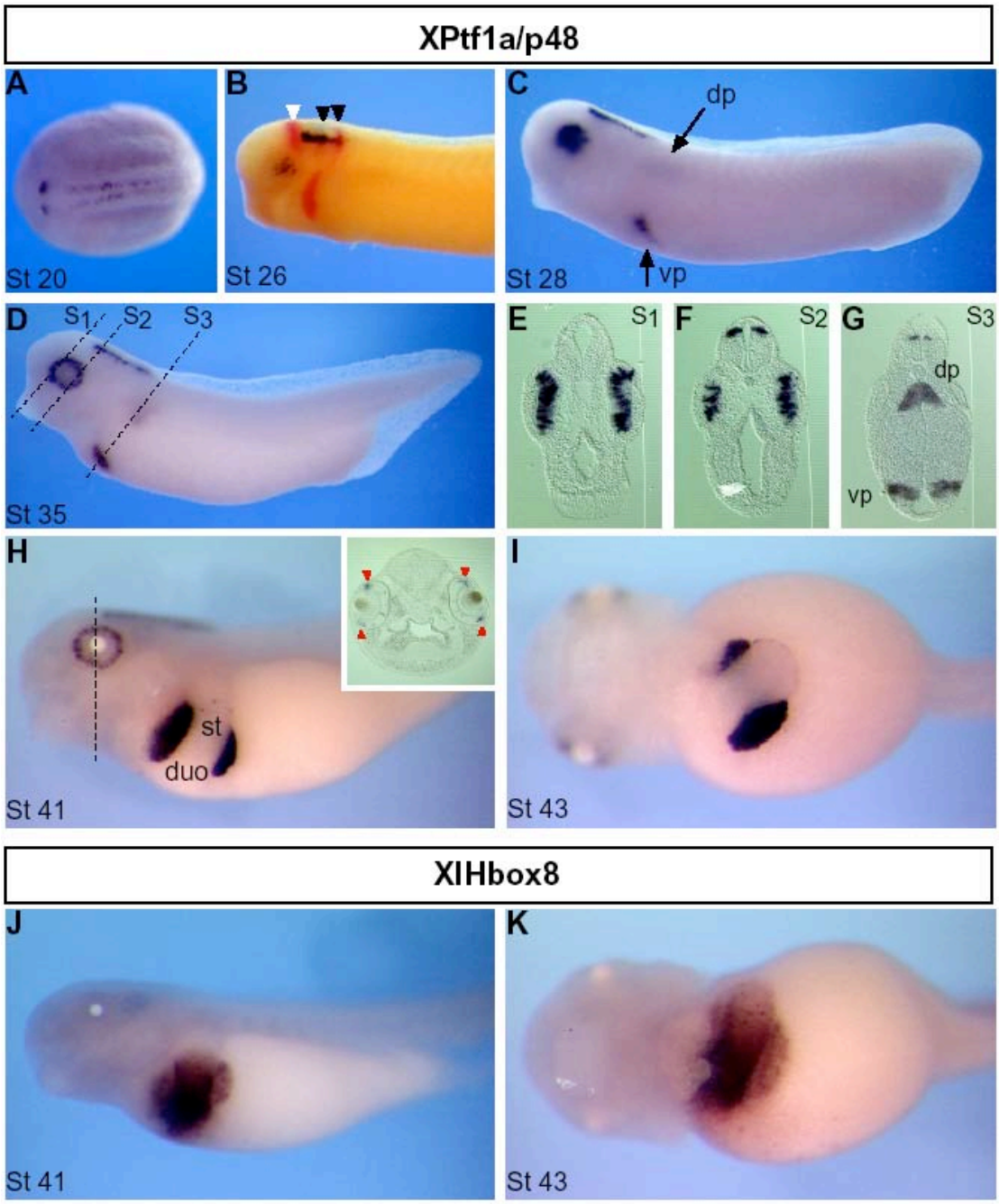



**Figure 3.5** Whole mount in situ hybridization analysis of XPtf1a/p48 expression during Xenopus embryogenesis and in comparison to XlHbox8. (A) Dorsal view of a stage 20 embryo, anterior towards the left. (B) Double-staining in situ hybridization using En2 (red) as midbrain-hindbrain boundary marker (white arrowhead) and Krox20 (red) as a marker for rhombomeres 3 and 5 (black arrowheads) mapping XPtf1a/p48 (dark blue) expression in the hindbrain; lateral view with the head towards the left. (C, D) Lateral view with head towards the left. (E-G) Transverse sections (S1, S2, S3) of a stage 35 embryo at the levels indicated in panel D, dorsal to the top. (H) Lateral view. Insert in panel H is a transverse section, at the level indicated by the dashed line, depicting the expression of Ptfa/p48 in the ciliary marginal zone (red arrowheads) (I) Ventral view. (J, K) Lateral and ventral view of XlHbox8 expression. Abbreviations: duo, duodenum; dp, dorsal pancreatic bud; st, stomach; vp, ventral pancreatic buds.

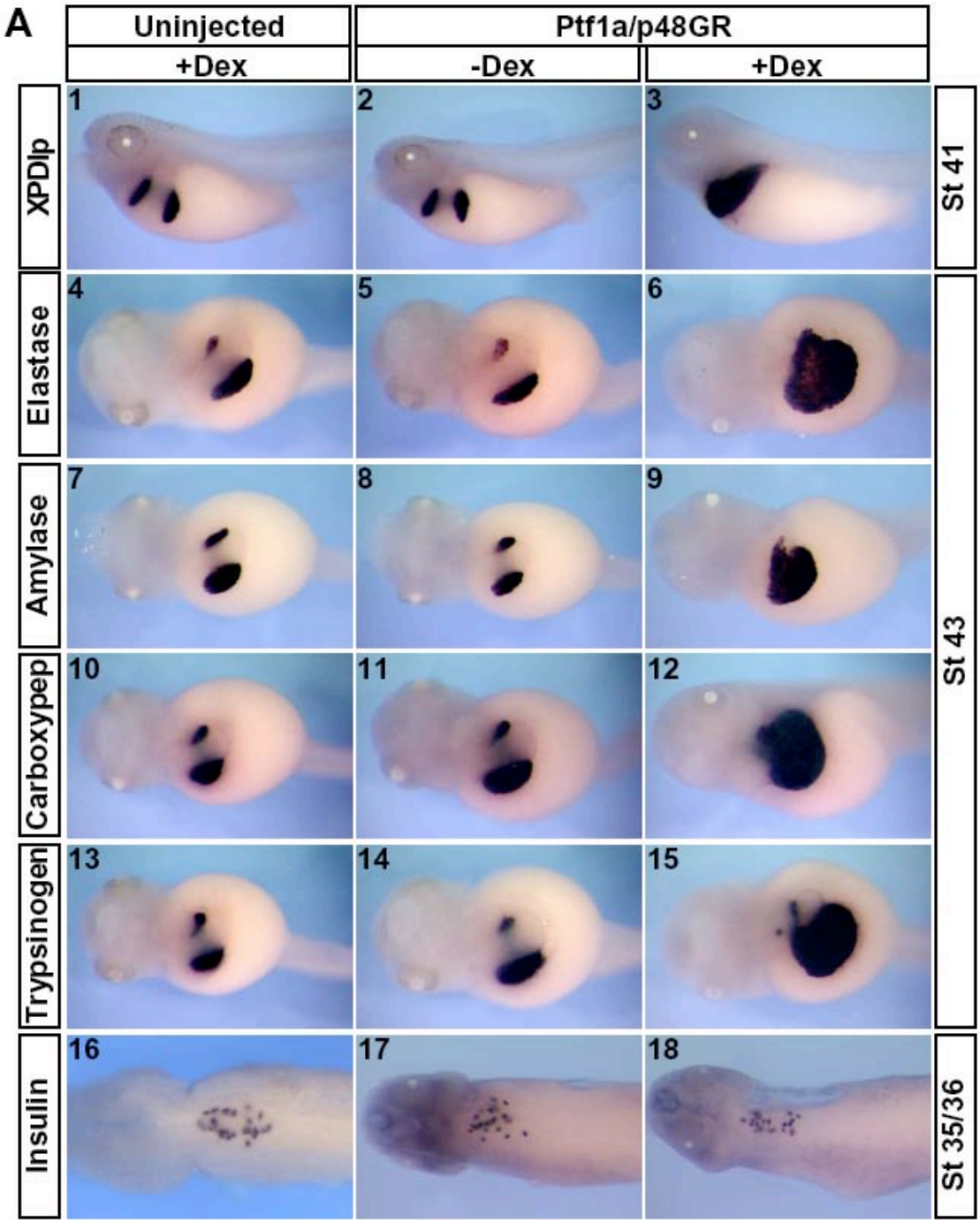
### 3.2.3 XPtf1a/p48 induces ectopic pancreatic differentiation within the limits of the XlHbox8 expression domain

To test for the function of XPtf1a/p48 in the context of early pancreas development, mRNA encoding a dexamethasone inducible variant of XPtf1a/p48, referred to as XPtf1a/p48GR, was injected into all four vegetal blastomeres of eight-cell stage *Xenopus* embryos. Following the temporal expression profile of the endogenous gene, dexamethasone treatment was performed at embryonic stage 27. Co-injection of β-galactosidase encoding mRNA indicates that the injected RNA is evenly distributed throughout the entire endoderm at tadpole stages (data not shown). As a result, ectopic expression of exocrine pancreatic differentiation markers, such as pancreatic protein disulphide isomerase (XPDIp), elastase, amylase, carboxypeptidase, and trypsinogen was observed (Figure 3.6 A, panels 3, 6, 9, 12 and 15). Embryos that were injected with the XPtf1a/p48GR mRNA but not treated with dexamethasone showed no effect, and were indistinguishable from uninjected embryos treated with dexamethasone (Figure 3.6 A panels 2, 5, 8, 11 and 14). Strikingly, the ectopic expression of exocrine pancreatic genes is specifically restricted to the territory of prospective stomach and duodenum, adjacent to the developing pancreas.

During early embryonic stages, insulin expression is first detected exclusively in the dorsal pancreas by stage 32 and represents the only endocrine differentiation gene expressed until stage 46 (Kelly and Melton, 2000; Horb and Slack, 2002). Insulin was therefore used to monitor the effect of

overexpressing XPtf1a/p48GR on endocrine cell differentiation in early embryos. Insulin expression remained unaffected (Figure 3.6 A panels 16, 17, 18). Similarly, examination of embryos of stages 41 and 43 showed no obvious effect on insulin expression (data not shown).

In normal embryos, the Forkhead transcription factor HNF3α is expressed in the stomach and duodenum but excluding the pancreas, as well as in the proctodaeum (Figure 3.6 B panel 1 and 4). Upon ectopic activation of XPtf1a/p48 in the endoderm, expression of HNF3α is lost specifically at the site of ectopic pancreatic gene expression, whereas its expression in the proctodaeum remains unaffected (Figure 3.6 B panels 3 and 6). These findings suggests adaptation of a pancreatic fate with a concomitant loss of duodenal and stomach identities within the XlHbox8 positive parts of stomach and duodenum.


A
Uninjected
+Dex
Ptf1a/p48GR
-Dex
+Dex
XPDIp
Elastase
Amylase
Carboxypep
Trypsinogen
Insulin
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
St 41
St 43
St 35/36

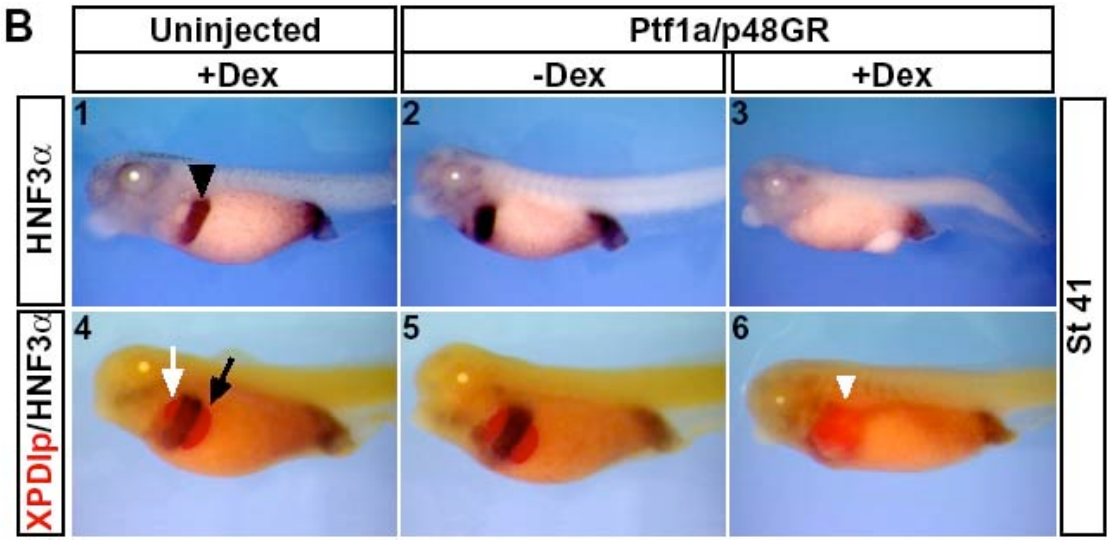



**Figure 3.6** A combination of XPtf1a/p48 and XlHbox8 induces ectopic pancreatic differentiation (A) Whole-mount in situ hybridization analysis reveals that ectopic expression of XPtf1a/p48 results in expression of exocrine pancreatic marker genes in regions of the stomach and duodenum. (1-3) Lateral view. 93% of the embryos examined showed effects as in panel 3 (n = 62). (4-15) Ventral view. Phenotype in panel 6, 87%, n = 24; panel 9, 69%, n = 29;panel 12, 64%, n = 25; panel 15, 76%, n = 25 (16-18) Dorsal view (head towards the left) with somites, neural tube and notochord being removed. As shown in panel 18, none of the embryos examined exhibited altered insulin expression (n = 25). (B) Ectopic expression of XPtf1a/p48 leads to a loss of stomach and duodenal marker gene expression. (1-6) Lateral view. The black arrowhead in panel 1 indicates stomach and duodenum expression of HNF3α. Phenotype in panel 3, 91%, n = 35. (4-6) Double-staining in situ hybridization with HNF3α in dark blue and XPDIp in red. Black and white arrows indicate dorsal and ventral pancreas respectively. The white arrowhead in panel 6 indicates ectopic expression of XPDIp and loss of HNF3α expression (85%, n = 67).

### 3.2.4 A combination of XPtf1a/p48 and XlHbox8 is sufficient to induce pancreatic differentiation in posterior endoderm

In agreement with previous studies (Grapin-Botton et al., 2001), ectopic expression of Pdx1 in the endoderm of *Xenopus* embryos was found to be insufficient to cause ectopic pancreas differentiation (data not shown). The extent of ectopic gene induction observed for exocrine pancreatic genes in XPtf1a/p48GR injected embryos was restricted to regions of the foregut endoderm corresponding to the territory of stomach and duodenum, reminiscent of the expression domain for XlHbox8 (Figure 3.5 panels J and K). This observation prompted us to test whether or not, the combined activities of XPtf1a/p48 and XlHbox8 are sufficient to convert non-pancreatic into pancreatic endoderm. Co-injection of XPtf1a/p48GR with XlHbox8GR results in an induction of exocrine specific differentiation genes also outside of stomach and duodenal regions, now expanding into the more posterior portions of the endoderm (Figure 3.7 panel 3). The expression of insulin in the dorsal pancreas of early (stage 36) embryos remained again unaffected (Figure 3.7 panel 6). In all cases of ectopic pancreatic gene induction, the onset of expression in the ectopic domain followed the normal temporal pattern, as observed in uninjected control embryos (data not shown).

In order to analyse if regions of ectopic pancreatic gene expression lose their normal molecular identity, the expression of the intestinal differentiation marker Cyl18 was examined (Chen et. al., 2003). In control embryos Cyl18 is expressed in the entire intestinal region (Figure 3.7, panel 10), with no

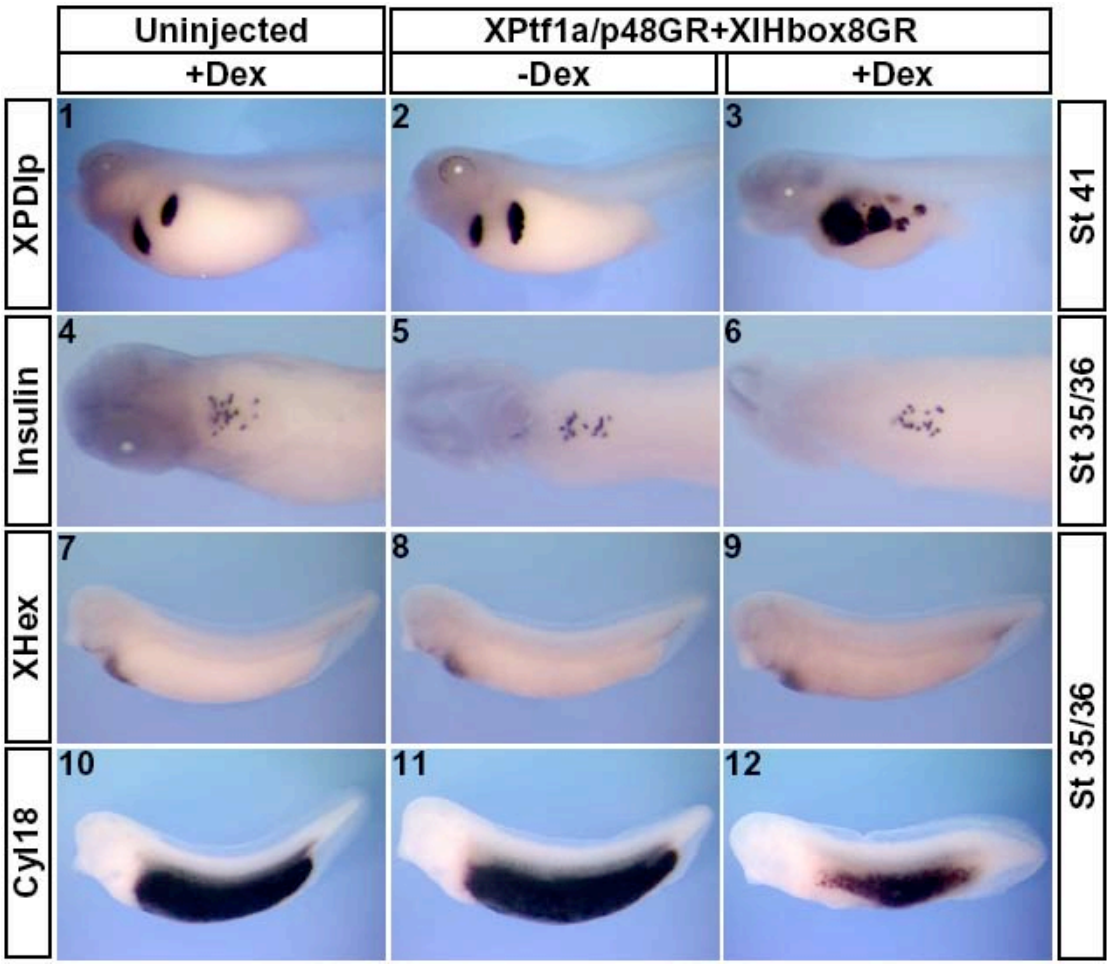



**Figure 3.7** Overexpresssion of both XPtf1a/p48 and XlHbox8 leads to a loss of intestinal marker gene expression and concomitant ectopic expression of exocrine marker genes in the presumptive intestine. (1-3) Lateral view. Phenotype in panel 3, 82%, n = 45. (4-6) Dorsal view (head towards the left) with somites, neural tube and notochord being removed (panel 6, 100%, n = 23). (7-12) Lateral view. Phenotype in panel 9, 100%, n = 39, in panel 12, 70%, n = 53.

expression in the stomach and duodenum. Ectopic activation of XPtf1a/p48GR together with XlHbox8GR results in a loss of Cyl18 expression in regions of the intestine posterior to the normal position of the pancreas (Figure 3.7, panel 12), while expression of the liver marker XHex was unaffected (Figure 3.7, panel 9). Thus, results obtained with both, injection of XPtf1a alone or together with XlHbox8, are compatible with the idea that it is a combination of both activities that defines pancreatic precursor cells in the

endoderm of *Xenopus* embryos that have the ability to differentiate accordingly.

### 3.2.5 Ectopic activation of XPtf1a/p48 and XlHbox8 at pancreatic precursor cell stages results in the stable induction of a giant pancreas with supernumerary endo- and exocrine cells

The above embryo injection experiments had employed dexamethasone treatment at embryonic stage 27 to induce ectopic activities when the corresponding endogenous genes start to be expressed in the developing pancreas. In order to test when exactly ectopic activation of XPtf1a/p48 is required for the expansion of the pancreatic territory, a systematic variation of the timepoint of hormone treatment was performed (Figure 3.8). Induction from embryonic stage 15 up to embryonic stage 27 results in pancreatic expansion for the vast majority of the embryos analysed. If hormone treatment is performed later, the percentage of embryos with a strong phenotype drops significantly, and upon induction at stage 36 or later results in a loss of effects. The practical limitations of the experimental approach employed here do not allow for the exclusion of the possibility that the amount of ectopic XPtf1a/p48 per cell becomes limiting during these later stages. In conclusion, ectopic XPtf1a/p48 operates most efficiently during pancreatic precursor cell stage, which is when endogenous XlHbox8 and XPtf1a/p48 are present, but before pancreatic differentiation markers start to be expressed.

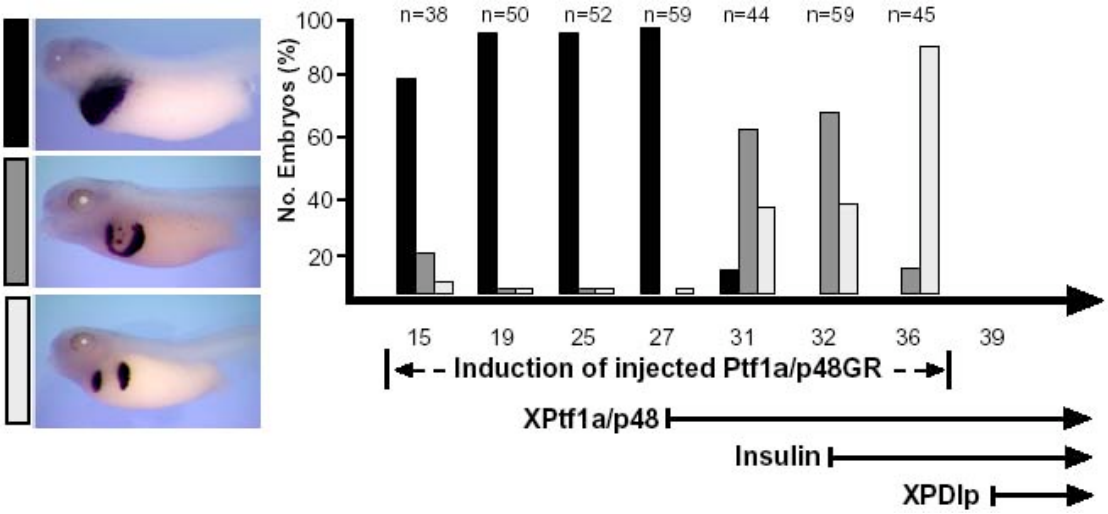



**Figure 3.8** Only uncommitted XlHbox8-expressing endodermal cells respond to XPtf1a/p48 by pancreatic differentiation. Dexamethasone treatment of XPtf1a/p48GR-injected embryos was performed at different time points in between stage 15 and stage 36 as indicated. The effects on pancreas development were evaluated by statistical analysis of three different degrees of ectopic XPDIp expression, as illustrated on the left hand side. The lower right part illustrates the temporal expression profile of endogenous XPtf1a/p48, insulin and XPDIp.

In order to test if the effects observed are transient or stable, embryos injected with either XPtf1a/p48GR alone or in combination with XlHbox8GR were grown to late tadpole stages (stage 48) and analysed for the expression of XPDIp and insulin. Morphology and marker gene expression reveal a dramatic expansion of the pancreas (Figure 3.9). Conversely, stomach and duodenum are deformed and reduced in size. The giant pancreas consists mainly of exocrine cells with interspersed endocrine cells, similar to the pancreas in control embryos. Interestingly, coinjection of XPtf1a/p48GR and XlHbox8GR results in a significant increase in the number of insulin positive, i.e. endocrine cells. In summary, the ectopic expression of XPtf1a/p48GR and XlHbox8GR results in a conversion of non-pancreatic endodermal cells to both exocrine and endocrine pancreatic cells at these late stages, even though the number

of the first insulin expressing cells (see Figures 3.6 and 3.7) was found to remain constant under the same experimental conditions.

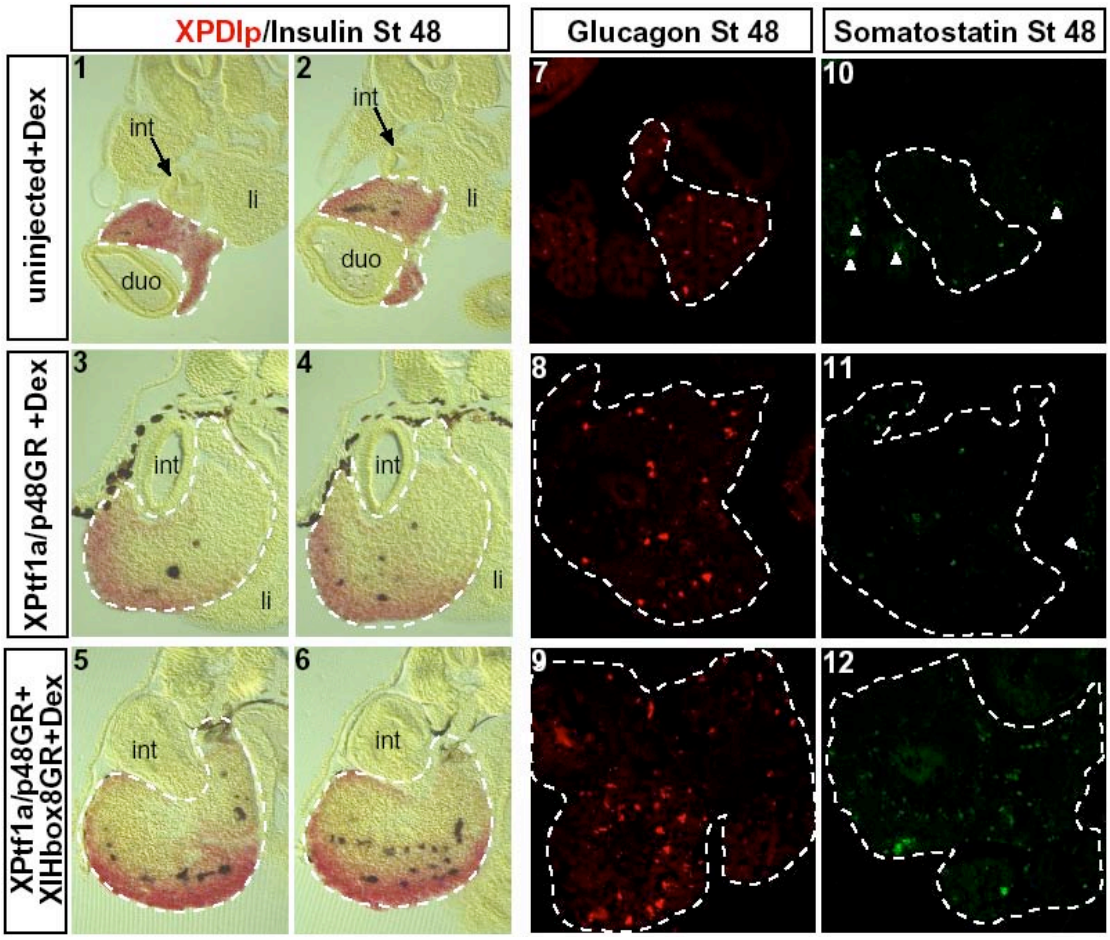



**Figure 3.9** Giant pancreata containing both differentiated endocrine and exocrine cells at late tadpole stage (stage 48), as generated by ectopic expression of XPtf1a/p48 and XlHbox8 and induction at tailbud stage (stage 27). (1-6) Vibratome sections of embryos after double staining whole-mount in situ hybridization (insulin in dark blue, XPDlp in red). Panels 1/2, 3/4, and 5/6 represent two neighboring sections each. Due to incomplete probe penetration into the giant pancreas, the inner part remains refractory to the staining procedure; the boundaries of pancreatic tissue (broken line) were identified by microscopic analysis. (7-9) Glucagon expression, as revealed by immunohistochemistry. Dotted lines demarcate the pancreas. Abbreviations: duo, duodenum; int, intestine; li, liver.

### 3.2.6 Knock down of *Xenopus* Ptf1a/p48 results in a specific loss of the exocrine pancreas

The requirement for XPtf1a/p48 in the context of pancreas development in *Xenopus* was tested by microinjection of the corresponding morpholino antisense oligonuleotide. Manipulated embryos displayed a complete loss of exocrine differentiation gene expression, such as XPDIp, amylase and trypsinogen (Figure 3.10 B, panels 2 and 6, and data not shown). Conversely, early insulin expression was maintained under the same experimental conditions in the morpholino injected embryos, but the insulin expressing cells were clustered and not dispersed as in the control embryos (Figure 3.10 B, panel 1).

Coinjection of the *Xenopus* XPtf1a/p48 morpholino antisense oligonucleotide (MO) with mRNA encoding mouse XPtf1a/p48GR which is not a target for the MO revealed that expression of XPDIp was restored in a pattern reminiscent of the results obtained upon ectopic expression of XPtf1a/p48 (Figure 3.10 B, panel 8). These observations not only confirm the specificity of the MO effect, but they also suggest that the function of XPtf1a/p48 has been highly conserved in evolution.

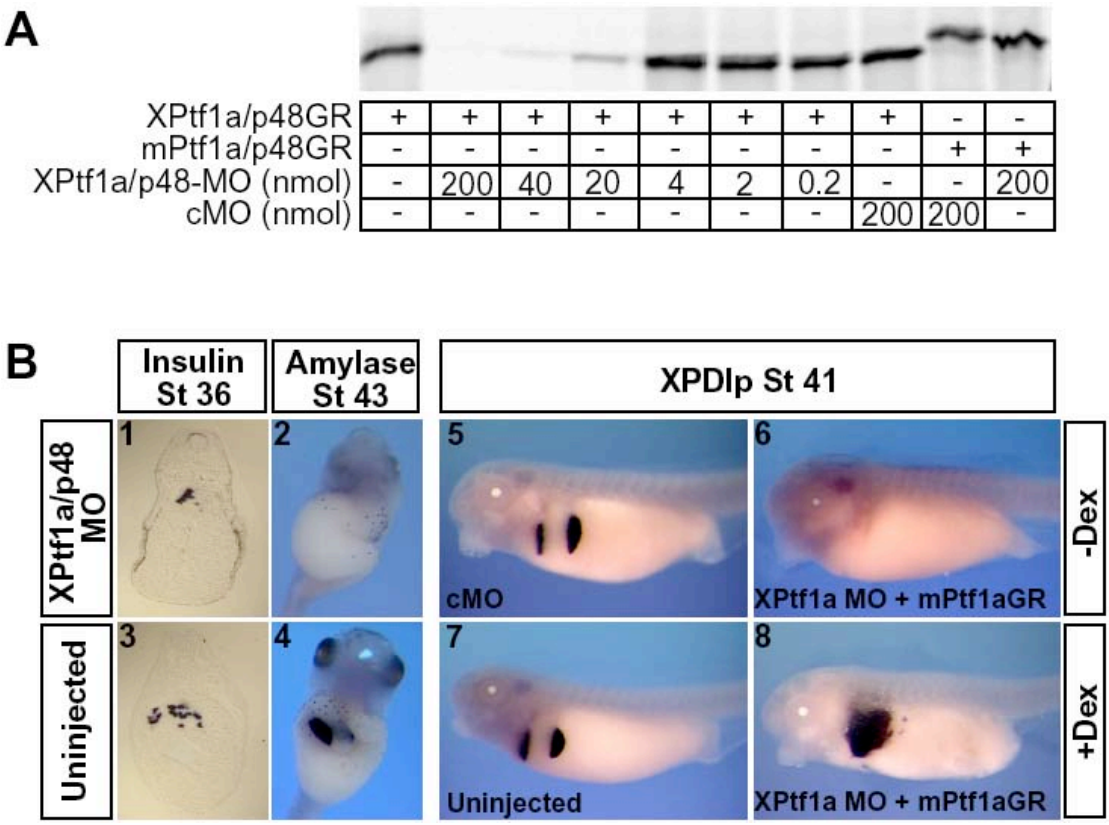



**Figure 3.10** Knockdown of XPtf1a/p48 results in a loss of exocrine pancreatic gene expression, while early dorsal insulin expression remains unaffected. (A) In vitro test for morpholino mediated inhibition of XPtf1a/p48 translation. In vitro trlanslation of XPtf1a/p48 is inhibited in a concentration dependent manner by the XPtf1a/p48 morpholino, whereas control morpholino shows no effect on translation. The XPtf1a/p48 morpholino has no effect on translation of the mouse Ptf1a/p48 (mPtf1a/p48). (B) XPtf1a/p48 morpholino effect in whole embryos. (1, 3) Transverse vibratome sections (panel 1, 100%, n = 85). (2, 4) Ventral view, head towards the top (panel 2, 77%, n = 27). (5-8) Lateral view. Panel 8 shows the rescue of the XPtf1a/p48 morpholino effect with a mouse the Ptf1a/p48GR. Phenotype in panel 5: 100%, n = 30; panel 6: 81%, n = 22; panel 8: 81%, n = 48.

In an effort to provide further experimental evidence for the notion that the pancreatic precursor cells in the endoderm are the result of the combined activities of XPtf1a/p48 and XlHbox8, embryos were also injected with MO directed against XlHbox8. Similar to the results obtained with the XPtf1a/p48 MO, such embryos had also lost exocrine marker gene expression, while dorsal expression of insulin was maintained (Figure 3.11 B, panels 2 and 5).

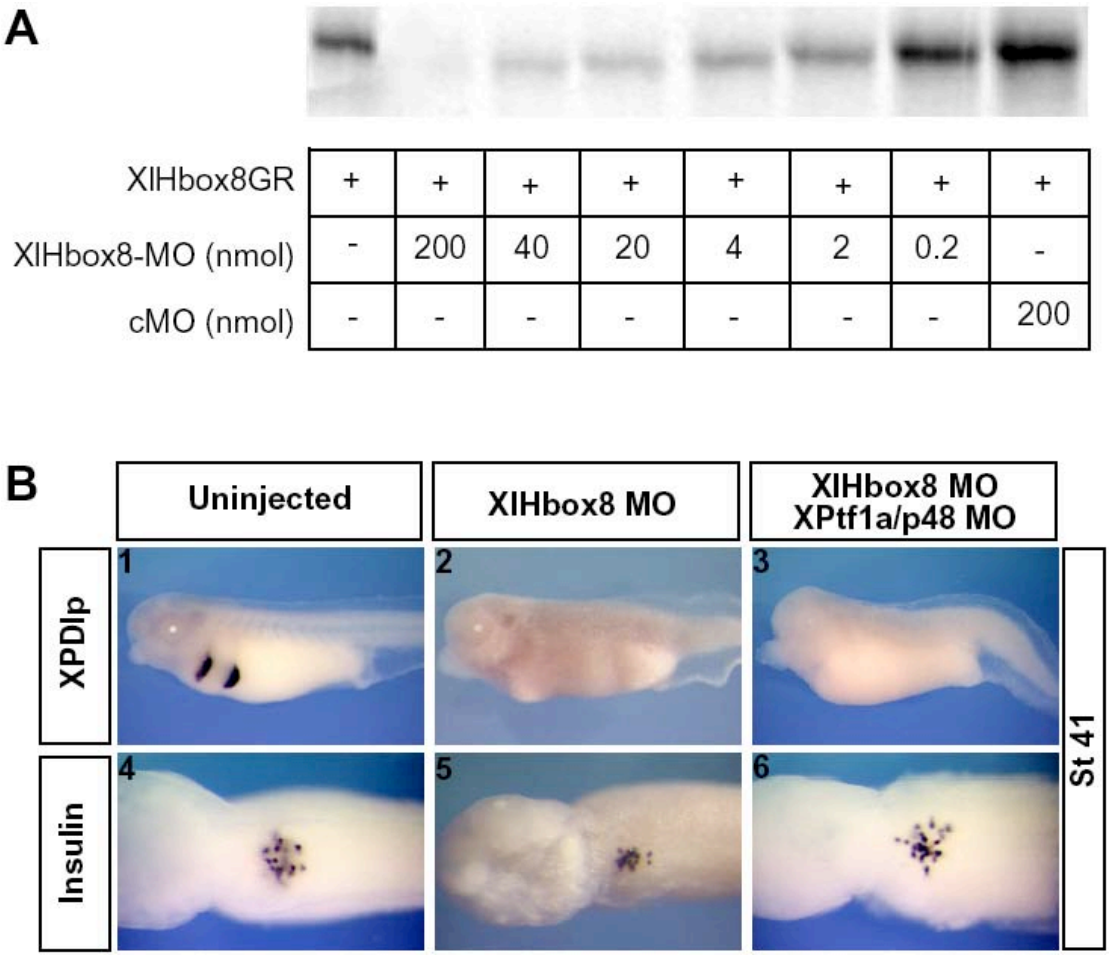



**Figure 3.11** Knockdown of XlHbox8 and XPtf1a/p48 alone and in combination. (A) In vitro test for morpholino mediated inhibition of XlHbox8 translation. In vitro trlanslation of XlHbox8 is inhibited in a concentration dependent manner by the XlHbox8 morpholino, whereas control morpholino shows no effect on translation. 1.0μg of XlHbox8 plasmid DNA was transcribed and translated *in vitro* in the presence of different concentrations of XlHbox8 morpholino oligonucleotides. (B) Knock down of XlHbox8 alone or in combination with XPtf1a/p48 in whole embryos. (1-3) Lateral view (panel 2, 100%, n = 25; panel 3, 100%, n = 30). (4-6) Dorsal view, with somites, neural tube and notochord being removed. (panel 5, 100%, n = 29; panel 6, 100%, n = 30).

Even more unexpectedly, microinjection of a combination of both XlHbox8 and XPtf1a/p48 MOs again results in the same situation, i.e. loss of exocrine and maintenance of early insulin expression (Figure 3.11 B, panels 3 and 6). Taken together, these observations indicate that both XPtf1a/p48 and XlHbox8 are needed to induce pancreatic genes of the exocrine lineage within

the endoderm. It appears however, as though the early insulin expression commencing at stage 36 is independent of the same two proteins, also providing an explanation for the absence of early but not late ectopic insulin expressing cells when both XPtf1a/p48 and XlHbox8 are applied.

### 3.2.7 Functional interdependence between XlHbox8 and XPtf1a/p48

The data present thus far clearly suggest that the activities of both XlHbox8 and XPtf1a/p48 are required for the commitment of endodermal precursor cells to the pancreatic fate. In an effort to verify whether these factors, XlHbox8 and XPtf1a/p48, require each other for their expression, the expression of each of them was examined in embryos lacking the other factor. Morpholino mediated knock down of XPtf1a/p48 results in no effect on the expression of XlHbox8 (Figure 3.12, panel D), an indication that the expression of XlHbox8 is independent of XPtf1a/p48 function.

Conversely, morpholino mediated knock down of XlHox8 results in maintenance of Ptf1a/p48, although its expression domain is reduced (Figure 3.12, panel B). Furthermore the expression of XPtf1a/p48 in XlHbox8 morpholino injected embryos reflects separate pancreatic buds at embryonic stage 42, compared to the fused pancreatic buds in control uninjected embryos. Previous genetic studies in mice have shown that, in Pdx1 (mouse orthologue of XlHbox8) null mice, though the pancreatic buds are formed, they fail to proliferate and differentiate. Thus the observed reduction in the expression domain of XPtf1a/p48 in *Xenopus* embryos lacking XlHbox8 might

reflect a similar phenotype of Pdx1 null mice, reduced pancreatic buds. Since the endodermal expression of XPtf1a/p48 is restricted to the pancreas, a reduced pancreatic bud size would result in a reduction in the expression domain of XPtf1a/p48.

These effects therefore suggest that the expression of XPtf1a/p48 is not subject to XlHbox8 control and, conversely, that of XlHbox8 is independent of XPtf1a/p48 function.


**Figure 3.12** XlHbox8 is not required for Ptf1a/p48 expression; similarly the expression of XlHbox8 is independent of Ptf1a function. All embryos are shown in lateral view, head towards left. (panel B, 100%, n = 24; panel D, 100%, n = 19).

# 4 Discussion

During *Xenopus* embryogenesis, the endodermal expression of the transcription factor Ptf1a/p48 is restricted to the developing pancreas, with its onset corresponding to pancreatic precursor stages. Therefore, in an effort to study early events of pancreas development in *Xenopus laevis*, the role of the transcription factor Ptf1a/p48 has been characterised. Prior to this, a detailed characterisation of the expression of XPDIp within the developing pancreas has been carried out to define which cell types it is expressed in. Thus enabling the use of XPDIp as a molecular marker for the study of pancreas development.

## 4.1 XPDIp as a marker of the exocrine pancreatic lineage

Pancreatic protein disulfide isomerase (XPDIp), was isolated in a previous screen of an adult *Xenopus* cDNA library for embryonic pancreas marker genes (Afelik, M.Sc. thesis, 2002). A detailed expression analysis of XPDIp carried out in this study has shown that its expression is confined to cells of the exocrine lineage in the embryonic and adult pancreas. Previous analysis of the exocrine enzymes amylase, elastase and trypsinogen, as molecular markers of the exocrine pancreas during *Xenopus* embryogenesis, has shown that these genes are detectable solely in the ventral pancreas at stage 41 before spreading to the dorsal pancreas by stage 42-44 (Horb and Slack, 2002). Contrary to these genes, the non-differential expression of XPDIp in both dorsal and ventral pancreatic buds before fusion thus rules out the

absence of exocrine cells in the dorsal pancreas until stage 42, as might be implied from the expression of the exocrine enzymes previously employed to mark the developing exocrine pancreas. Furthermore, the earlier expression of XPDIp at stage 39 compared to the other exocrine pancreatic makers makes it useful for the analysis of effects on these cells in experimental conditions which result in the death of embryos before exocrine pancreatic enzymes become detectable.

PDIp has previously been shown to be expressed exclusively in the adult pancreas of human, canine and mouse (DeSilva et al., 1996, 1997; Volkmer, 1997). In humans it has been shown to be expressed only in the acinar cells, the functional subunits of the exocrine pancreas, but not in the islets (DeSilva et al., 1997). It is therefore likely that in human, canine and mouse the embryonic expression of XPDIp is as well restricted to the exocrine pancreatic lineage as observed for *Xenopus* embryos. Studies in mouse and human indicate that PDIp is structurally and functionally related to the ubiquitously expressed protein disulfide isomerase (PDI). Both proteins are retained in the endoplasmic reticulum (ER), where they are involved in proper disulfide bond formation, and have been shown to posses chaperoning activity *in vitro* (DeSilva et al., 1997; Volkmer et al., 1997). The exocrine pancreas specific expression of PDIp therefore suggest a role in proenzyme maturation. A possible role of PDIp in development remains to be analysed.

## 4.2. Pancreatic fate determination by Ptf1a/p48 and XlHbox8

Patterning of the embryonic endoderm into distinct sets of precursor cells giving rise to the different major intestinal organs involves the precisely regulated activities of key transcriptional regulators. *Xenopus* Ptf1a/p48 is expressed in pancreatic precursor cells in a pattern overlapping with the one of XlHbox8. Results reported here reveal that the ectopic expression of a combination of both activities is sufficient to expand the pancreatic precursor cell population within the endoderm of *Xenopus* embryos, resulting in the formation of a giant pancreas that carries both endocrine and exocrine cells. Inhibition of Ptf1a/p48 activity results in a specific loss of the exocrine pancreas.

### 4.2.1 The pancreatic endoderm is defined by the expression of Ptf1a/p48 and Pdx1/XlHbox8

The onset of *Xenopus* Ptf1a/p48 expression corresponds in time with the expression of XlHbox8, i.e. when cells in the embryonic foregut endoderm become committed to a pancreatic fate. However, while the expression domain of XPtf1a/p48 is restricted to the pancreatic primordia, the XlHbox8 positive area expands into the adjacent parts of stomach and duodenum. As shown in this study, ectopic expression of XPtf1a/p48 alone results in the expansion of pancreatic differentiation into the XlHbox8 positive territory outside of the pancreatic rudiments that normally develops into stomach and duodenum. Conversely, ectopic expression of XlHbox8 fails to induce ectopic pancreas differentiation in *Xenopus* embryos. This observation suggests that

it is a combination of both activities that defines the pancreatic precursor cell status in the anterior endoderm of vertebrates. The expression characteristics of these two transcription factors within the endoderm of *Xenopus* embryos as observed in this study is similar to that observed mouse embryos. During early stages of mouse pancreas development, the expression domain of Pdx1 encompasses the posterior stomach and duodenum, compared to the restricted expression of Ptf1a/p48 to the pancreatic rudiments (Offield et al., 1996; Kawaguchi et al., 2002,). Although previous genetic studies in mouse have shown an important role of Pdx1 in pancreas development, as demonstrated by the lack of exocrine and endocrine cell differentiation in Pdx1 null mice (Jonsson et al., 1994), the expression of Pdx1 in non-pancreatic endoderm suggest that it is not alone sufficient for commitment of cells to the pancreatic fate, as indicated by the failure of overexpressed XlHbox8 alone to induce ectopic pancreatic gene expression. Very much in line with this observation, previous studies using in ovo electroporation of chick embryos had shown that ectopic expression of Pdx-1, the homologue of XlHbox8, results in the initiation of epithelial budding, but these structures fail to complete pancreatic cytodifferentiation (Grapin-Botton et al., 2001).

On the other hand, evidence presented in this work, together with previous studies, suggests that the activity of Ptf1a/p48 alone is also not sufficient to initiate pancreas development. Overexpression experiments performed in this study are such that Ptf1a/p48 is ectopically expressed in the entire endoderm as well as non endodermal cells of the embryo, nevertheless, ectopic pancreatic gene induction is observed only within the endogenous expression

domain of XlHbox8, which is the posterior stomach and duodenum. Moreover, the ability of Pdx1-/- mice to drive expression from the mouse Ptf1a/p48 promoter (Kawaguchi et al., 2002) is an indication that Ptf1a/p48 is also expressed in Pdx1-/- mice. It is therefore conceivable that the activity of Ptf1a/p48 without that of XlHbox8 is not sufficient to support pancreatic differentiation.

As a further demonstration that within the developing endoderm the activities of Ptf1a/p48 and XlHbox8 are sufficient to initiate pancreas development, ectopic expression of both factors results in an expanded ectopic pancreatic domain which extends into posterior endodermal regions which normally develop into intestine. Thus the expression pattern and activities of Ptf1a/p48 and XlHbox8 suggest that both factors act together and are sufficient to initiate pancreas differentiation within the developing endoderm. The similarity in expression characteristics of the factors between *Xenopus* and mouse suggests that they may act similarly in murine pancreas development.

### 4.2.2 Non-pancreatic endoderm possesses competence for pancreatic fate

Previous studies have shown that the endoderm is prepatterned early in development, resulting in the typical anterior to posterior pattern of organ primordia along the gut tube during organogesis. In particular the specifcation of the pancreatic primordia has been shown to initiate as early as during gastrulation and is subsequently reinforced through interaction with

neighbouring tissues. At embryonic stage 27 (when ectopic gene activation is performed in this study) the gut is already prepatterned and the various organ primodia have reached their final positions and have undergone contact with the appropriate neighbouring tissues. The induction of ectopic pancreatic fate by ectopic expression Ptf1a/p48 and XlHbox8 at stage 27 therefore suggests that the primordia of the duodenum, pancreas and intestine share common core endodermal factors, but differ from each other by the expression of Ptf1a/p48 and XlHbox8. Thus, it appears that the combinatorial expression of these two factors is utilised to determine different organ primordia such that, expression of XlHbox8 alone, XlHbox8 and Ptf1a/p48 together, and neither of the two are utilised to deteremine duodenum, pancreas and intestine respectively.

### 4.3.3 Ptf1a/p48 and XlHbox8 act to recruit cells to the pancreatic precursor state

The observed activity of Ptf1a/p48 together with XlHbox8 in the recruitment of cells to the pancreatic programme is restricted to early embryogenesis, until stage 28, which is an indication that Ptf1a/p48 and XlHbox8 are involved in the determination of the pancreatic fate in uncommitted cell. This is in agreement with the early expression of the intestinal differentiation marker IFABP at stage 30, an indication of intestinal differentiation by this stage (Horb and Slack, 2001). Ectopically induced pancreatic cells follow the same temporal expression of pancreatic marker genes as in normal embryos, which is an indication that these cells get committed to a pancreatic fate and go

through the normal maturation steps of pancreatic precursor cells. Thus, this early function of Ptf1a/p48 is distinct from its later role in the differentiation of the exocrine part of the pancreas, in that cells recruited to the pancreatic programme yield differentiated cells of both exocrine and endocrine lineages.

It has been reported that adenovirus mediated Pdx-1 gene transfer into the adult liver induces both endo- and exocrine pancreatic gene expression in the mouse (Ferber et al., 2000; Ber et al., 2003; Kojima et al., 2003), and that an activated version of Pdx1 (Pdx1-VP16), under the control of the liver specific transthyretin promoter, is sufficient to transform the liver into ectopic pancreatic tissue in transgenic *Xenopus* embryos (Horb et al., 2003). Such transdifferentiation events are conceptually different from what is observed in this study; in the former, ectopic Pdx-1 activity is activated in differentiated cells rather than in multipotent embryonic precursor cells. In this present study, liver development was not found to be affected by the early embryonic activation of a combination of Ptf1a/p48 and XlHbox8, neither was pancreatic gene expression observed in the embryonic liver. RNA injected into cleavage stage embryos, as well as the proteins encoded, are not likely to survive until tadpole stages of embryogenesis when the transthyretin promoter is active.

### 4.2.4 Regulation of Ptf1a/p48 and XlHbox8 expression

The expression patterns and activities of Ptf1a/p48 and XlHbox8 as described in this study suggest that the pancreatic determination state is defined by the expression of these factors within the endoderm. This suggests that the

specification of the pancreatic endoderm, which is preceded by a series of inductive interactions between various mesodermal tissues and the presumptive pancreatic endoderm, culminate in the expression of these two factors. Indeed, previous studies provide evidence that the endodermal expression of both factors is under the influence of signals derived from neighbouring tissues. The endodermal expression domain of Pdx1 requires FGF mediated signalling from the pancreatic mesenchyme, as demonstrated by the loss of Pdx1 expression in Fgf10 null mice. Furthermore, ectopic expression of Fgf10 in the pancreas results in a sustained Pdx1 expression and the arrest of cells in a precursor cell state (Jensen et al., 2003). Using mouse embryos that lack endothelial cells together with tissue recombination assays, Yoshitomi and Zaret have shown that the expression of Ptf1a/p48 in the dorsal pancreatic endoderm is induced by signals derived from endothelial cells (Yoshitomi and Zaret, 2003). Thus it seems that both Ptf1a/p48 and XlHbox8 are directly induced by mesodermally derived signals that have previously been shown to be essential for pancreas development.

As shown in this study, each of these factors (Ptf1a/p48 and XlHbox8) does not require the other for its expression. However, it has been shown that the expression of both Pdx1 and Ptf1a is autoregulated (Gerrish et al., 2001; Chakrababarti et al., 2002; Lin et al., 2004). This implies that the initiation of their expression in the endoderm results in an intrinsic stability of the pancreatic fate. Notably, in this study ectopic expression of both factors by injection of their corresponding mRNAs fused to a glucocorticoid receptor

ligand binding domain into cleavage stages of *Xenopus* embryos, followed by induction at stage 27, results in a stable induction of ectopic pancreas.

### 4.2.5 The early dorsal insulin expressing cells require neither Ptf1a/p48 nor XlHbox8

Antisense morpholino oligonucleotide mediated inhibition of Ptf1a/p48 translation in *Xenopus* embryos results in the specific loss of the entire exocrine pancreas, while expression of early dorsal pancreatic insulin can still be detected. Similar results were previously reported in corresponding loss of function experiments in mice and zebrafish (Krapp et al., 1998; Kawaguchi et al., 2002; Zecchin et al., 2004; Lin et al., 2004), providing clear indications for a conserved role of Ptf1a/p48 in pancreas development. However, the exact nature of the surviving insulin expressing cells in *Xenopus* embryos needs to be reflected. Both endocrine and exocrine specific gene activities can be detected in the giant pancreata induced by ectopic activation of Ptf1a/p48 and XlHbox8, but no ectopic endocrine cell differentiation is detectable before embryonic stage 48. With the exception of the early dorsal insulin, this late expression of endocrine marker genes in ectopic pancreatic tissue correlates well with the normal induction of the same endocrine genes. Therefore, the early dorsal insulin expressing cells are distinct from the other differentiated pancreatic cells. This is also portrayed in their persistence upon inhibition of XlHbox8 activity by anti-sense morpholino oligonucleotides, a finding that is reminiscent of the survival of early insulin and glucagon expressing cells in Pdx1-/- mice (Ahlgren et. al., 1996). Moreover, the early dorsal insulin

expressing cells in *Xenopus* embryos can be detected as early as stage 32, which is prior to pancreas morphogenesis (Kelly and Melton, 2000). It seems therefore, that the second wave of pancreas differentiation, which involves the vast majority of endo- and exocrine marker genes, has to be treated separately from the events that result in the dorsal, early insulin expressing cells in *Xenopus* embryos.

### 4.2.6 Molecular mechanisms underlying the activities of Ptf1a/p48 and XlHbox8

The data presented here clearly indicate that during embryogenesis, both Ptf1a/p48 and XlHbox8 are required for the initiation of the pancreatic programme, but it remains unclear weather these two factors interact physically to contact the same promoter elements or if they activate the expression of different factors that in turn act together toward the common goal of initiating pancreas development. It has been shown that, in the mature pancreas, Ptf1a/p48 and Pdx1 act together to activate transcription of elastase1. In this case, the trimeric complex of Pdx1, Pbx1b and Meis2 bound to the B element and the dimeric complex of p48 and p64 on the A element of the elastase enhancer interact to co-operatively activate transcription (Rose et al., 2001). It is not clear whether p48 requires the same cofactors for its function during early pancreogenesis as in the fully differentiated exocrine pancreas.

### 4.2.7 Possible application of Ptf1a/p48 and XlHbox8 for generating pancreatic cells in vitro

The ability of Ptf1a/p48 and XlHbox8/Pdx-1 to expand the pancreatic precursor cell population of the endoderm in whole embryos as presented here raises the question if similar effects can be generated with pluripotent precursor cells in tissue culture. For Pdx-1, this obvious possibility has been tested with some success using embryonic stem cells (Blyszczuk et al., 2003; Miyasaki et al., 2004); it will be interesting to find out what activities a combination of Ptf1a/p48 and Pdx-1 may exert in these systems. Furthermore, embryonic expression of Ptf1a/p48 exhibits two domains in addition to the developing pancreas: the embryonic hindbrain and eye. Most recently, mutations in Ptf1a/p48 were also described to result in cerebellar agenesis (Sellick et al., 2004). The role of Ptf1a/p48 in retinogenesis remains to be elucidated.

## 4.3 Summary and Conclusion

*Xenopus laevis* has been used to study organogenesis of the pancreas. As part of this effort, the pancreas specific protein disulfide (PDIp) has been characterised as a molecular marker of the exocrine pancreatic lineage. This marker, as utilised in this study, allows for the analysis of experimental effects on the exocrine lineage early in development.

Furthermore, the role of the transcription factors XPtf1a/p48 and XlHbox8 in early events of pancreas development has been studied. Data obtained in this

study reveal that the activities of both of these factors are required and sufficient for the determination of a pancreatic precursor state in endodermal precursor cells. This is clearly demonstrated by the ectopic induction of a pancreatic fate in presumptive duodenal and intestinal cells by the combined activities of XPtf1a/p48 and XlHbox8. The ectopically induced pancreatic precursors develop normally to yield both endocrine and exocrine pancreatic cell types. These findings provide an insight into why XlHbox8, which is normally expressed in presumptive posterior stomach and duodenum, as well as ectopic expression of XlHbox8 alone are not sufficient to induce a pancreatic fate.

As part of this study, the early dorsal insulin cells in *Xenopus laevis* were found not to require the function of either XlHbox8 or Ptf1a/p48. This observation is reminiscent of the early glucagon and insulin expressing cells in mice which normally do not require the function of Pdx1.

In conclusion, this study has resulted in the novel finding that the definitive pancreatic precursor fate is defined by the combined activities of XlHbox8 and XPtf1a/p48. These findings also suggest that the presence of these two factors defines a key difference between precursor cells of the pancreas, duodenum and intestine. Besides the novel contributions of this study to our understanding of pancreas development, a comparison of the observed effects to previous studies of pancreas development indicates that the molecular events involved in pancreas development in *Xenopus* and mouse are quite identical.

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