Université catholique de Louvain

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# ROLES OF THE TRANSCRIPTION FACTORS HNF6 AND SOX9 IN THE INITIATION OF METAPLASTIC AND NEOPLASTIC LESIONS OF THE PANCREAS

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# Summary

Pancreatic ductal adenocarcinoma originates from acinar cells that undergo acinar-to-ductal metaplasia (ADM) and evolve to pancreatic intraepithelial neoplasia (PanIN), and eventually to invasive cancer. Ductal transcription factors are induced in ADM but the roles of these transcriptional regulators, in particular Hepatocyte Nuclear Factor (HNF) 6 and SRY-related HMG-box (Sox) 9, have not yet been investigated.

Here we show that HNF6 and SOX9 are expressed in human and mouse ADM. Using *in vitro* and *in vivo* gain- and loss-of-function experiments, we demonstrate that both factors are essential for ADM induced by pancreatic duct ligation-mediated pancreatitis or by exposure to the carcinogen 9,10-dimethyl-1,2-benzanthracene. HNF6 and, to a lesser extent, Sox9 are required for repression of acinar genes, for modulation of ADM-associated changes in cell polarity, and for activation of ductal genes in metaplastic acinar cells.

SOX9, but not HNF6, is expressed in human and mouse PanIN lesions. Using *in vitro* and *in vivo* loss-of-function experiments, we show that Sox9 is required for development of PanIN from acinar cells harboring an oncogenic Kras mutation and exposed to an inflammatory stimulus. Sox9 stimulates expression of several metaplastic genes (cytokeratin 19, clusterin, ezrin) to promote ADM. It further stimulates the activity of the Erythroblastic leukemia viral oncogene homolog (ErbB) signaling pathway, which is required for initiation and progression of pancreatic ductal adenocarcinoma.

Together, our findings demonstrate that HNF6 and Sox9 promote pancreatitis-driven ADM and are biomarkers of ADM. Also, Sox9 acts as an oncogene in pancreatic cancer by promoting tumor progression via stimulation of the ErbB signaling pathway.

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# Abbreviations

| | |
|---|---|
| ADM | acinar-to-ductal metaplasia |
| AREG | amphiregulin |
| CAC | centroacinar cells |
| CD | cluster of differentiation |
| CDK | cyclin dependent kinase |
| CCK | cholecystokinin |
| CFTR | cystic fibrosis transmembrane conductance regulator |
| CK19 | cytokeratin 19 |
| CPA | carboxypeptidase |
| ErbB | Erythroblastic leukemia viral oncogene homolog |
| EGFR | epidermal growth factor receptor |
| FAMMM | familial atypical multiple mole melanoma |
| GAP | GTPase activating protein |
| GEF | guanine nucleotide exchange factor |
| GM-CSF | granulocyte-macrophage colony stimulating factor |
| HB-EGF | heparin-binding EGF |
| Hh | hedgehog |
| HMG | high mobility group |
| HNF | hepatocyte nuclear factor |
| Hras | Harvey rat sarcoma |
| ICAM-1 | intercellular adhesion molecule 1 |
| IKK2 | ikB kinase 2 |
| IL | interleukin |
| KO | knockout |
| Kras | Kirsten rat sarcoma |
| LSL | lox-stop-lox |
| MDSCs | myeloid-derived suppressor cells |
| MMP | matrix metalloproteinase |
| MPC | multipotent progenitor cells |
| MT | metallothioneine |
| NF-κB | nuclear factor-kappa B |

| | |
|---|---|
| Ngn3 | neurogenin 3 |
| Nkx6.1 | Nk6 homeobox 1 factor |
| Nr5a2 | nuclear receptor subfamily 5, group A, member 2 |
| Nras | neuroblastoma rat sarcoma |
| NRG | neuregulin |
| OC | onecut |
| PanIN | pancreatic intraepithelial neoplasia |
| PDAC | pancreatic ductal adenocarcinoma |
| Pdx1 | pancreatic and duodenal homeobox 1 |
| Prox1 | prospero homeobox protein 1 |
| PP | pancreatic polypeptide |
| PSC | pancreatic stellate cells |
| Ptf1a | pancreas-specific transcription factor 1a |
| Rbpj | recombining binding protein suppressor of hairless |
| ROS | reactive oxygen species |
| shRNA | short hairpin RNA |
| siL-6R | soluble form of IL-6 receptor |
| Sox | SRY-related HMG box |
| SRY | sex-determining region Y |
| TNF-$\alpha$ | tumor necrosis factor-alpha |
| TP | tumor protein |

# I. Introduction

## A. Pancreas

### 1. Architecture and functional organization of a mature pancreas

The pancreas is an endoderm-derived organ located in the upper abdomen of vertebrates, under the stomach, between the duodenum and the spleen. It constitutes an amphicrine gland, with both an endocrine and exocrine function. Anatomically, the pancreas can be divided in three parts, the head, the body and the tail. The exocrine pancreas, which represents around 95% of the pancreatic cells, is equally represented in these three parts, whatever the species. In contrast, in humans, but not in rodents, the endocrine cells (1-2% of the pancreatic cells) are mainly found in the body and tail of the pancreas (Figure 1).

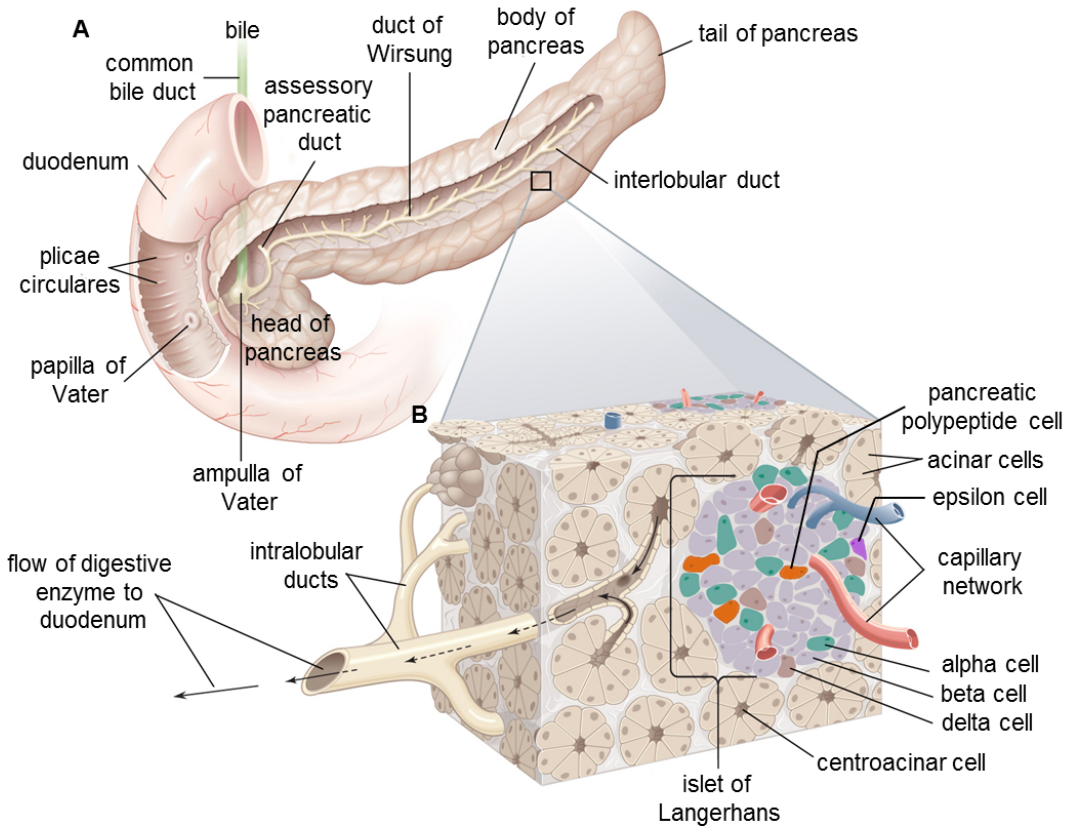


**Figure 1. Architecture of the human pancreas** (adapted from Encyclopaedia Britannica, Inc 2010). Schematic representation of the pancreas (**A**) and of the organization of the different pancreatic cell types (**B**). See text for additional details.

The endocrine pancreas is a central regulator of glucose and metabolic homeostasis. Endocrine cells are organized in clusters called Islets of Langerhans and produce several hormones. Islets are located within the pancreatic parenchyma, intimately associated with blood vessels, which allows distribution of hormones in the

body. The endocrine cell types, namely alpha, beta, delta, epsilon, and pancreatic polypeptide (PP)-cells, secrete glucagon, insulin, somatostatin, ghrelin and PP hormones, respectively, in response to metabolic and chemical cues from the local blood supply (Figure 1B).

The exocrine pancreas secretes the pancreatic juice that contains digestive enzymes, like proteolytic enzymes (trypsin, elastase, carboxypeptidase (CPA)) and hydrolases (pancreatic amylase, ribonucleases, and lipases) which contribute to the digestion of nutrients in the intestine (Rutter et al., 1968). The exocrine compartment contains acinar and centroacinar cells (CAC) interconnected by an epithelial-lined ductal network whose cells constitute the third exocrine cell type (Figure 1A-B). Acinar cells form discrete units called acini, which, in cross-section, represent irregular circular clusters of pyramidal-polarized cells organized around a small concentric lumen. Apical portions of acinar cells face the middle of these units, adjacent to the centrally positioned centroacinar cells. The latter are located at the extremity of the ducts linked to the acinar cells.

In response to the ingestion of food or drink, the upper intestine releases secretin and cholecystokinin (CCK), which induces acinar secretion of inactive forms of digestive enzymes called zymogens. The latter are activated in the acidic duodenal environment; hence, concomitant release of bicarbonate-rich fluid by ductal cells maintains an alkaline microenvironment in the ducts and prevents activation of digestive enzymes prior to exiting the pancreas. Lumens of the acinar units drain into the network of ducts. This network is successively composed of the intercalated, intralobular, and interlobular ducts lined by cuboidal epithelial cells. The interlobular ducts anastomose to the Wirsung duct lined by a simple columnar epithelial, which drains acinar secretions into the duodenum (Reichert and Rustgi, 2011) (Figure 1A-B).

Acini are organized in lobular complexes delineated by mesenchyme-derived stromal cells, composed of endothelial cells and quiescent pancreatic fibroblasts. In normal condition, this stroma represents less than 1% of the pancreas. It supports normal function of the epithelium, but also stores Vitamin A, regulates the immune system, and maintains endothelial cell turnover (MacDonald et al., 2010). Communication between the stromal and epithelial compartments ensures homeostasis within the organ.

Perturbed pancreatic homeostasis contributes to the development of several pathologies. Endocrine function is affected in type 1 and 2 diabetes. Various stimuli, including alcohol abuse or smoking, generate inflammation of the exocrine pancreas called pancreatitis, which is a risk factor for pancreatic cancer. Molecular mechanisms contributing to diabetes and pancreatitis often implicate molecular pathways involved in pancreas development. Thus, a detailed characterization of these diseases requires understanding of pancreas differentiation and morphogenesis in the embryo. For this reason, the key steps and players in development of the pancreas will be described in the next chapter.

## 2. Pancreas development

### *a. Concepts in differentiation and morphogenesis*

Development of the pancreas is summarized in Figure 2. The initial step consists of the formation of endodermal pre-pancreatic dorsal and ventral buds at the foregut-midgut junction, approximately at embryonic day (e) 9 in mice or at the 26$^{th}$ day of gestation in humans (Gittes, 2009). Cells in these buds are highly proliferative; they are called multipotent progenitor cells (MPC) and have the potential to form the three pancreatic cell lineages characteristic of the mature gland, the acinar, ductal, and endocrine cells (Gu et al., 2003; Kawaguchi et al., 2002). Around e12 in mice and e40 in humans, the dorsal and ventral buds fuse and branching morphogenesis and cell differentiation are initiated. Tubular structures consist initially of a multi-lumen tubular plexus, which coordinately extends and remodels into a ramifying, single-lumen ductal system (Pierreux et al., 2010; Villasenor et al., 2010). Acinar differentiation and morphogenesis are associated with an increase in the expression of enzyme genes, development of large amounts of rough endoplasmic reticulum and formation of zymogen granules. In parallel, endocrine cells arise (Pictet et al., 1972), and the pancreas becomes progressively depleted in MPC while acquiring a mature architecture.

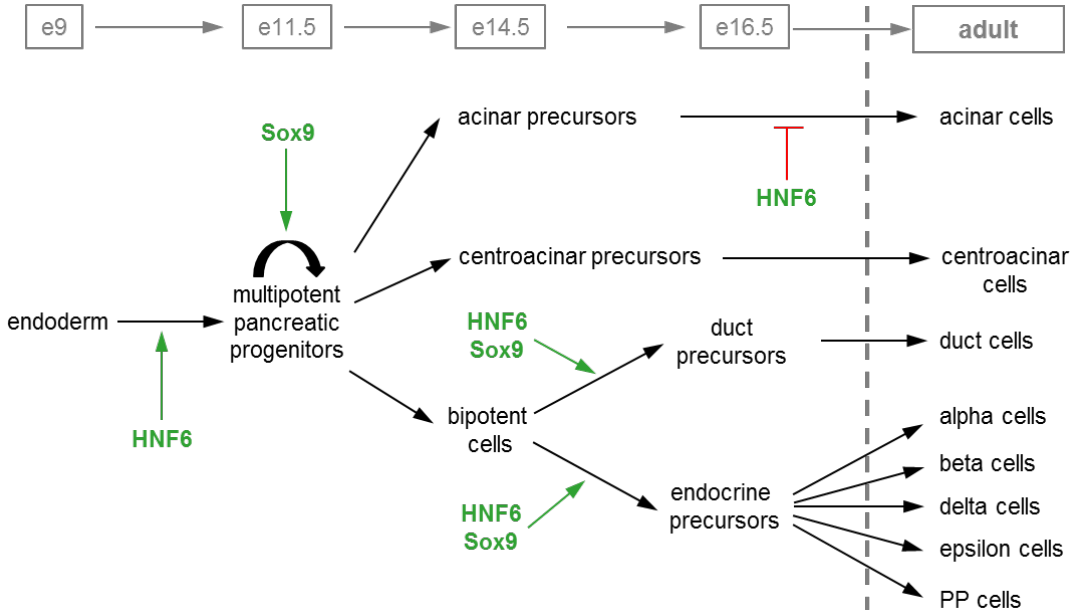


**Figure 2. Schematic representation of mouse pancreatic embryogenesis.** Key roles of HNF6 and Sox9 during pancreas development are highlighted in green and red.

Until e12.5 MPC have the ability to generate the three epithelial lineages of the pancreas (Kopp et al., 2011; Solar et al., 2009). Beyond e12.5, the differentiation capacity of the MPC is progressively restricted; they give rise to a population of bipotent cells located in the pancreatic trunks and to a population of unipotent pre-acinar cells located at the tips of the pancreatic branches. Between e13.5 and e18.5, the bipotent trunk cells give rise to endocrine and duct cells (Solar et al., 2009). In the

trunk cells, those expressing high levels of Neurogenin 3 (Ngn3) become committed to an endocrine fate, at the expense of the ductal fate (Magenheim et al., 2011; Wang et al., 2010) (Figure 3B). From e18.5 onwards, the trunk cells develop only into duct cells (Solar et al., 2009). Interestingly, from e14.5, cells co-expressing SRY-related HMG-box transcription factor 9 (Sox9) and Pancreas-specific transcription factor 1a (Ptf1a) are observed at the junction between the trunk and the tip populations. This intermediate population seems to be the earliest manifestation of centroacinar lineage (Schaffer et al., 2010; Seymour et al., 2007). The role of the CAC is still not clear but some report suggests that they contain stem cells involved in pancreas regeneration after injury (Gasslander et al., 1992; Hayashi et al., 1999; Seymour et al., 2007).

### *b. Transcription factors in exocrine pancreas*

Pancreatic ductal adenocarcinoma is the main topic of the thesis. Since it originates in exocrine tissues, the focus of the present chapter is on exocrine development.

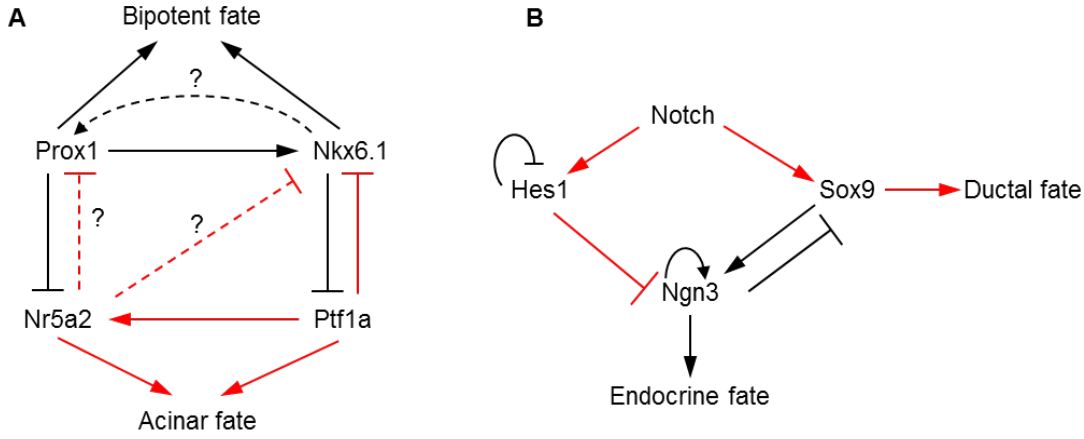


**Figure 3. Regulatory interactions among transcription factors involved in the exocrine fate choice** (MacDonald et al., 2010; Shih et al., 2012). Mutual antagonism of Nr5a2/Prox1 and Ptf1a/Nkx6.1 in multipotent progenitor cells leads to two alternative choices. Nr5a2 and Ptf1a are required for acinar fate whereas Prox1 and Nkx6.1 are required to generate bipotent ductal/endocrine cells (**A**). In bipotent cells, the level of Notch signaling controls the ductal fate (**B**). Dashed lines with question marks indicate lack of experimental evidence (**A**). Red lines indicate regulations promoting acinar fate (**A**) and ductal fate when Notch signaling is high (**B**).

Differentiation of the exocrine cell types requires a temporally- and spatially-controlled cascade of transcription factors. Separation of the trunk and tip cell populations depends on a double negative feedback loop between Ptf1a and the Nk6 homeobox 1 factor (Nkx6.1). Co-expression of Ptf1a and of the Recombining binding protein suppressor of hairless (Rbpj) inhibits *Nkx6.1* gene expression and activates a self-reinforcing gene expression network locking cells into a fixed state of acinar cell (Masui et al., 2007; Schaffer et al., 2010) (Figure 3A). Similarly, prospero homeobox

protein 1 (Prox1) and the nuclear receptor subfamily 5, group A, member 2 (Nr5a2) inhibit each other to drive a bipotent or acinar fate, respectively (MacDonald et al., 2010; Qin et al., 2004; Rausa et al., 1999; Steffensen et al., 2004; Wang et al., 2005) (Figure 3A). Importantly, these inhibitory mechanisms also contribute to prevent premature differentiation of MPC.

In acinar precursor cells, Ptf1a forms a complex with Rbpj, to induce expression of RbpjL, a paralog of Rbpj. This leads to progressive replacement of Rbpj by RbpjL in the complex with Ptf1a, concomitantly with acinar cell maturation (Beres et al., 2006). In late pancreatic development, the RbpjL/Ptf1a complex and the transcription factor Mist1 induce acinar-specific genes. After birth, Mist1 contributes to stabilize the mature acinar phenotype by controlling the establishment of the secretory machinery and by limiting proliferation of mature cells (Jia et al., 2008; Luo et al., 2005; Masui et al., 2008; Pin et al., 2001; Rodolosse et al., 2004).

As mentioned above, bipotent cells in the pancreatic trunk give rise to endocrine and ductal cells. While endocrine fate is determined by high expression of Ngn3, the ductal fate seems to be acquired by “default” in the absence of extracellular instructive signals. However, loss of function experiments targeting pancreatic and duodenal homeobox 1 (Pdx1), a factor expressed since the earliest stage of pancreas development, show an absence of duct development (Wescott et al., 2009). In early pancreas Pdx1 inhibits expression of cytokeratin 19 (CK19), a marker of duct cells, to allow proliferation of MPC and bipotent cells. Later, Pdx1 expression progressively decreases while CK19 increases, suggesting that this regulation participates to the establishment of the duct cells fate (Deramaudt et al., 2006). Beyond its function in cell fate decision, Pdx1 initiates branching morphogenesis in the developing pancreas by activating a program of cytoskeletal reorganization and cell migration in the duct cells (Wescott et al., 2009).

Two other transcription factors, Hepatocyte Nuclear Factor-6 (HNF6; also called Onecut 1) and Sox9 regulate duct development, and their function is at the core of the thesis. Therefore, they are described in detail in the following separate sections.

### *c. Hepatocyte Nuclear Factor 6*

HNF6 is a member of the Onecut (OC) family, which also comprises OC2 and OC3, in mammals. Members of the Onecut family are characterized by a bipartite DNA binding domain, composed of a single cut domain and of a divergent homeodomain. This homeodomain has a phenylalanine and a methionine at positions 48 and 50 of the homeodomain respectively, two amino acids which are never found at these positions in other homeodomains (Lemaigre et al., 1996). Both the cut domain and the homeodomain are bifunctional; they are required for DNA binding and for transcriptional stimulation (Lannoy et al., 1998) (Figure 4A). The Onecut factors bind to DNA as monomers, on the consensus sequence 5’ A/G A/T TC A/G AT A/G/C (Laudadio et al., 2012).

The Onecut proteins have restricted and partially overlapping expression patterns. HNF6, OC2 and OC3 are expressed in liver, skin, pancreas, testis and brain. OC2 and OC3 are also expressed in intestine and stomach (Jacquemin et al., 2000; Vanhorenbeeck et al., 2007). During pancreas organogenesis, HNF6 is expressed in the endoderm as early as e8 at the foregut-midgut junction (Landry et al., 1997; Rausa et al., 1997). Later, HNF6 is expressed in MPC. At e15.5, the highest expression is observed in differentiating ductal cells. From this stage onwards, HNF6 expression progressively decreases in acinar cells, and it is never detected in endocrine cells. From e17.5, pancreatic expression of HNF6 is restricted to intra-, interlobular and intercalated ducts, and to CAC (Pierreux et al., 2006).

The function of HNF6 was initially investigated by analyzing *HNF6* knockout (*HNF6* KO) mice. Most of the *HNF6* KO mice die within one or two weeks after birth. However, about 25% of the KO mice survive until adulthood. They develop a pleiotropic phenotype that affects pancreas, liver and nervous system (Audouard et al., 2012; Clotman et al., 2002; Jacquemin et al., 2000). The pancreas of the *HNF6* KO embryos is severely hypoplastic, as result of delayed onset of Pdx1 expression in the pre-pancreatic endoderm (Jacquemin et al., 2000; Vanhorenbeeck et al., 2007). At the onset of endocrine cell specification, HNF6 is critically required for Ngn3 expression. Consequently, the number of endocrine cells is reduced in *HNF6* KO mice and the mice become diabetic (Jacquemin et al., 2000). Moreover, *HNF6* KO mice show severe defects in inter- and intralobular ducts morphogenesis: the primary cilium is lacking at the apical pole of the duct cells, the duct epithelium is dysplastic, and the ducts are cystic. This is associated with reduced expression of HNF-1β and of ciliogenic genes (Pierreux et al., 2006). The cystic morphology of the ducts may also result from re-differentiation of endocrine cells toward a ductal fate, thereby creating an excess of duct cells. Indeed, in mice lacking Ngn3 in the pancreas, the endocrine cells revert to a duct phenotype thereby generating cysts due to accumulation of duct cells (Magenheim et al., 2011). HNF6 has also a role in acinar differentiation. In this cell type, the level of HNF6 expression is controlled by two microRNAs, Let-7b and miR-495. In the absence of these microRNAs, HNF6 expression is upregulated, leading to reduced expression of acinar genes and ectopic induction of hepatic gene (Prevot et al., 2013) (Figure 2). These different results indicate that HNF6 is a key factor for pancreas development and differentiation.

Mice knockout for *OC2* or *OC3* show no pancreatic phenotype. However, analysis of *HNF6* and *OC2* double knockout embryos revealed a redundant role for HNF6 and OC2 in pancreas specification and endocrine differentiation. Indeed, in double *HNF6* and *OC2* knockouts hypoplasia and endocrine deficiency is more pronounced than in *HNF6* KO mice (Vanhorenbeeck et al., 2007).

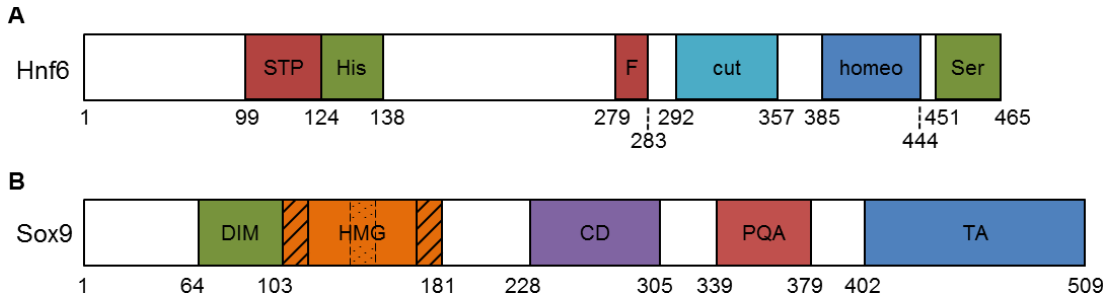


**Figure 4. Schematic structure of the transcription factors HNF6 and Sox9**. Colored boxes represent functional domains. In HNF6, the serine/threonine- and proline-rich sequence (STP) and the conserved sequence MRDER (F) display a transcriptional activating activity whereas the polyhistidine tract (His) and the polyserine tract (Ser) have transcriptional inhibitory activity. The cut domain (cut) and the homeodomain (homeo) are essential for DNA binding, and the homeodomain is also involved in transcriptional regulation by interacting with the coactivator CREB-binding protein (**A**). In Sox9, the dimerization domain (DIM), the high mobility group domain (HMG), a conserved domain (CD), a proline-glutamine-alanine rich domain (PQA) and the transactivation domain (TA) are indicated. The nuclear localization and nuclear export signals are hatched and dotted, respectively (**B**).

### *d. SRY-related HMG-box transcription factor 9*

Since discovery of the first sex-determining region Y (SRY) box (SOX) transcription factor in 1990, 20 SOX factors have been identified in mammals (Schepers et al., 2002). Members of the SOX family share a highly conserved high-mobility-group (HMG) DNA binding domain which fulfills the functions of DNA binding, DNA bending, protein-protein interactions, and nuclear import or export (Wilson and Koopman, 2002) (Figure 4B). The SOX factors bind the minor groove of DNA; this likely allows assembly of large multi-protein complexes, which bring distant transcriptional activators or repressors in contact with target promoters (Ferrari et al., 1992; Weiss, 2001). Interestingly, all Sox proteins are capable of recognizing the same DNA consensus sequence, 5' A/T A/T CAA A/T G 3', suggesting redundant roles (Harley et al., 1994; Pevny and Lovell-Badge, 1997).

To date, 20 *SOX* genes have been identified in mice and humans and classified into 9 subgroups based on HMG domain phylogeny and genomic organization: Sox A (SRY), Sox B1 (SOX1, SOX2, SOX3), Sox B2 (SOX14, SOX21), Sox C (SOX4, SOX11, SOX12), Sox D (SOX5, SOX6, SOX13), Sox E (SOX8, SOX9, SOX10), Sox F (SOX7, SOX17, SOX18), Sox G (SOX15), Sox H (SOX30). SOX factors are detected in nearly every embryonic and adult tissue. In embryonic pancreas, a function has been identified for Sox4, Sox9, Sox10, Sox11 and Sox17 (McDonald et al., 2009). While Sox4 appears to be the most highly expressed Sox protein in both developing pancreas and adult islets, SOX9, which is, expressed both in fetal and adult human/mouse pancreas is the most studied (Seymour et al., 2007; Wilson et al., 2005). In mice, Sox9 is detected in MPC from e9.5 onwards (Seymour et al., 2008). Later, Sox9 expression is maintained in the duct cells while it progressively decreases in acinar cells. Sox9 is not expressed in precursor and mature endocrine cells. In adult pancreas, Sox9

expression is restricted to the duct cells, including the CAC, according to a pattern highly reminiscent to that of HNF6.

In humans, mutations in the *SOX9* gene lead to the campomelic dysplasia syndrome associated with XY sex reversal. Campomelic dysplasia is a rare skeletal malformation characterized by short stature, deformed limbs, typical facial appearance and macrocephaly (Chen et al., 2012). Interestingly, analysis of patients with campomelic dysplasia also reveals abnormal pancreatic morphology (Piper et al., 2002). In mice, heterozygous deletion of *Sox9* leads to a perinatal lethality with cleft palate, as well as hypoplasia and bending of many skeletal structures derived from cartilage precursors (Bi et al., 2001). Consequently, conditional deletion of *Sox9* gene with a floxed allele is required to study the role of Sox9 (Kist et al., 2002).

Pancreatic inactivation of *Sox9* demonstrated that this protein is a key factor for pancreas development and differentiation. Sox9 is initially essential for maintenance of the MPC; in the absence of Sox9, severe pancreatic hypoplasia resulting from cell death and MPC depletion is observed (Seymour et al., 2007). At a later stage, Sox9 is also required for pancreatic endocrine cell formation (Seymour et al., 2008). These roles can be explained by the observation that Sox9 directly binds to the *HNF6*, *HNF1β*, *HNF3β*, and *Ngn3* genes and regulates the expression of these genes which are also important for pancreas development (Lynn et al., 2007; Seymour et al., 2007). Similar to *HNF6* KO, the conditional deletion of *Sox9* in pancreatic epithelial cells leads to the formation of cystic ductal structures (Shih et al., 2012). This role is controlled by Notch. In fact, by regulating Sox9 expression, Notch renders bipotential progenitors competent to adopt ductal or endocrine fates: in the presence of high levels of Notch, Ngn3 is repressed and cells undergo ductal fate conversion (Seymour et al., 2007; Shih et al., 2012) (Figure 3B).

# B. Pancreatitis

## 1. Pathophysiology

Pancreatitis, or pancreas inflammation, most often results from premature activation of zymogens in the pancreas. Activated digestive enzymes lyse the pancreas leading to necrosis and apoptosis. Pancreatitis is acute or chronic. Gallstones, which cause an obstruction of pancreatic ducts, and alcohol, are the most common etiology (80% of cases) of acute and chronic pancreatitis, respectively (Lowenfels et al., 1994; Volzke et al., 2005). Other less common causes are toxins (scorpion venom), drugs (corticosteroids, metformin…), infectious agents (coxsackie virus, hepatitis B, legionella, ascaris…), or some genetic predispositions like activation of trypsinogen (*PRSS1*), inhibition of trypsin inhibitor (*SPINK1*), or disruption of the cystic fibrosis transmembrane conductance regulators (*CFTR*) (Bhatia et al., 2002; Chandak et al., 2004; Drenth et al., 2002; Keim et al., 2001; Yadav and Lowenfels, 2013). Also, the risk factor for pancreatitis is increased 2.3-fold in patients with type 2 diabetes mellitus (Girman et al., 2010; Noel et al., 2009).

In most cases, acute pancreatitis does not cause long-term sequels when appropriately treated using antibiotics, anti-inflammatory drugs, and maintenance of energy and fluid homeostasis. However, in some cases, dehydration and shock lead to heart, lung or kidney failure and eventually death. The average mortality in patients with acute pancreatitis is approximately 9% (Singh et al., 2011; van Santvoort et al., 2011).

Chronic pancreatitis is a long-term disease most frequently found in people between 30 and 40 years old, evolving over 15-20 years. It results either from multiple subclinical or clinically evident bouts of acute pancreatitis or from a single irritating and often severe injury that establishes a perpetuating condition. Extreme consumption of alcohol and smoking leads to a progressive fibrosis of the pancreatic parenchyma and to a slow destruction of the exocrine compartment. At a later stage of chronic pancreatitis, the composition of the islets of Langerhans evolves, leading to functional impairment of endocrine function (Brock et al., 2013; Kloppel et al., 1978). This destruction and the associated inflammation generate complications, such as diabetes, digestive hemorrhages, pseudocyst formation and development of pancreatic ductal adenocarcinoma (PDAC) (Yadav and Lowenfels, 2013). Fibrosis is characterized by excessive deposits of extracellular matrix. In chronic pancreatitis, this dynamic process is essentially driven by the pancreatic stellate cells, which in normal condition maintain the synthesis and degradation of extracellular matrix (see below for detail) (Aghdassi et al., 2011).

## 2. Rodent models of pancreatitis

To identify the mechanisms underlying pancreatitis, models of acute and chronic pancreatitis have been generated in several animal species, the most popular

models being developed in mouse and rat. It is important to keep in mind that none of these models display all features of human pancreatitis with exocrine and endocrine cell mass loss, distinct patterns of chronic inflammation, formation of intraductal protein plugs, calcification, sensitization to pain and pancreatic fibrosis. Moreover, in rodents, strain, age, sex gender and the protocol used to induce inflammation can modulate pancreatitis. We describe and discuss here the most widely used rodent models of pancreatitis (Lerch and Gorelick, 2013).

### *a. Models of acute pancreatitis*

The most popular model is based on intraperitoneal injections of cerulein, which is a synthetic decapeptide derived from the skin of the Australian frog *Litorea caerulea*. Cerulein is similar in composition and action to the secretagogue cholecystokinin (CCK). Overstimulation by cerulein, which binds to the CCK1 or CCK2 receptors, increases protein synthesis and transport of newly synthesized proteins through the secretory pathway. This accumulation leads to premature intrapancreatic activation of zymogens and induces rapid development of edema characterized by increased vascular permeability and hydrostatic pressure. Pancreatic stellate cells seem to be key actors in progression of pancreatitis and fibrosis. Bacteria, macrophages, neutrophils, acinar cells and platelets activate pancreatic stellate cells, which then transdifferentiate in myofibroblast-shaped cells and acquire the capacity to migrate and secrete extracellular matrix proteins to promote fibrosis (Aghdassi et al., 2011; Phillips et al., 2003). Moreover, activated pancreatic stellate cells produce TGF-$\beta$, TNF-$\alpha$ and IL-1 to stimulate themselves by an autocrine loop (Aoki et al., 2006) (Figure 5B).

Injections of cerulein also induce activation of the NF-$\kappa$B signaling pathway, which upregulates the expression of intercellular adhesion molecule 1 (ICAM-1) in pancreatic acinar cells; this transmembrane protein contributes to the adhesion of neutrophils to the acinar cells, a process contributing to the inflammatory response (Zaninovic et al., 2000). Activation of NF-$\kappa$B by reactive oxygen species leads to the production of inflammatory IL-1$\beta$, IL-6 and TNF-$\alpha$ cytokines by the acinar cells which initiates fibrosis and participates in its progression (Apte et al., 2009; Gukovskaya et al., 2002; Kim, 2008). Finally cerulein-CCK1/2 binding leads to activation of the Jak2/Stat3 pathway, which induces the inflammatory cytokine IL-1$\beta$, thereby amplifying the inflammatory process (Kim, 2008) (Figure 5A).

Interestingly, when cerulein injections are stopped, the acinar tissue regenerates within a few days through the activation of several signaling pathways, including Notch, Wnt and Shh (Puri and Hebrok, 2010).

Another experimental model to induce acute pancreatitis consists in ligating the main pancreatic duct. By blocking the flow of pancreatic secretions, this model is hypothesized to recapitulate the events occurring when gallstones generate pancreatitis in human. Here also, enzyme accumulation is suspected to produce auto-digestion of the pancreas (Watanabe et al., 1995). One day after pancreatic duct

ligation, the pancreas volume increases as a result of interstitial edema and infiltration of neutrophils. Three days after pancreatic duct ligation, the exocrine tissue undergoes atrophy, leading to a decrease in the volume of the pancreas (Hamamoto et al., 2002). Moreover, macrophage activity leads to degeneration and death of acinar cells by apoptosis, in contrast to the situation seen in the cerulein model where acinar death is mainly due to necrosis (Walker et al., 1993).

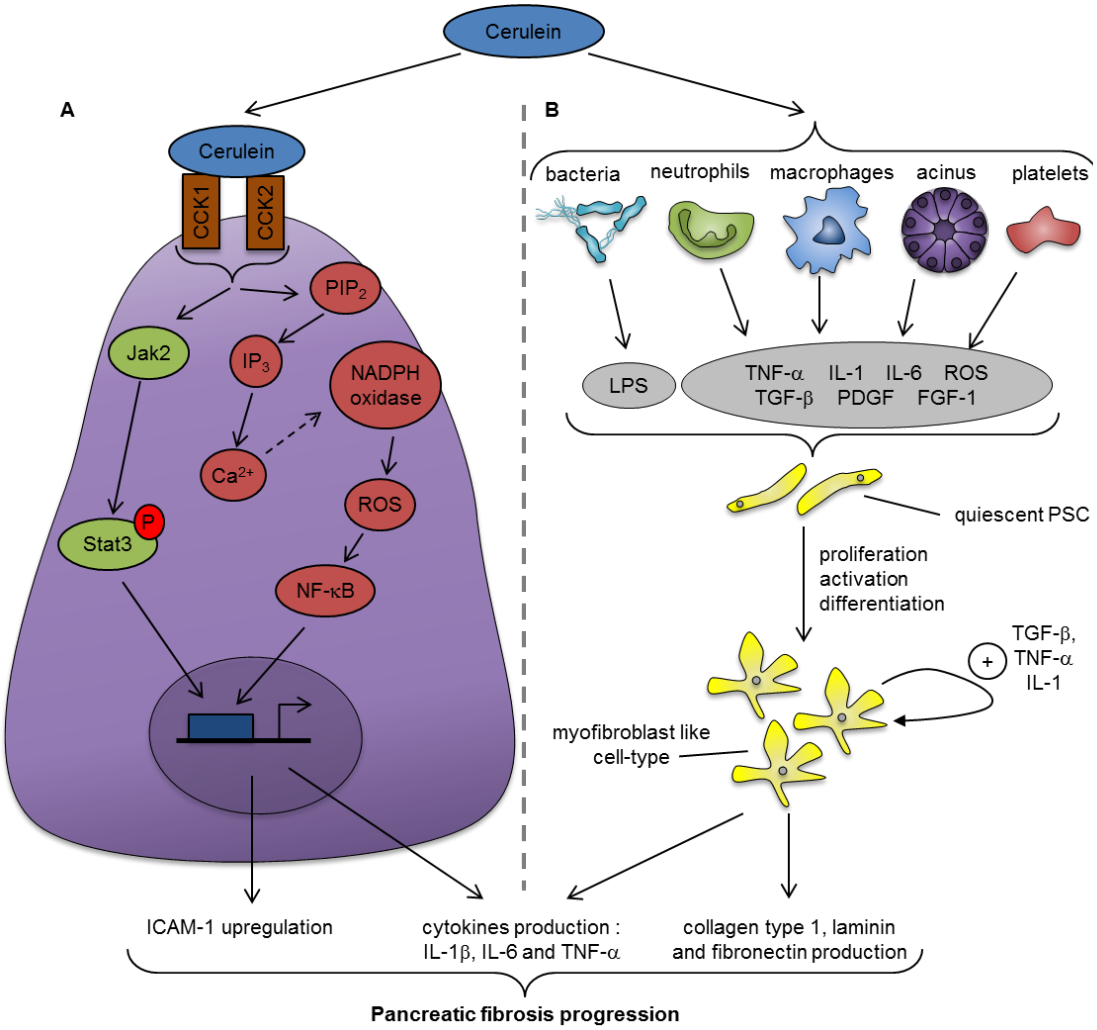


**Figure 5. Effects of cerulein in an inflamed pancreas** (adapted from Kim 2008; Aghdassi 2001). In acinar cells, cerulein induces ICAM-1 transcription and cytokine production through activation of the Jak2/Stat3 and the NF-κB pathways (**A**). Acinar cells, neutrophils, macrophages and platelets stimulate pancreatic stellate cells (PSC) in a paracrine manner through production of growth factors, cytokines and reactive oxygen species (ROS). Bacteria produce lipopolysaccharide (LPS) to activate PSC. Activated stellate cells promote pancreatic fibrosis by producing cytokines and extracellular matrix (**B**). CCK1, 2: cholecystokinin receptor 1, 2.

*b. Models of chronic pancreatitis*

Ten percent of chronic alcoholics develop chronic pancreatitis and represent 80% of all cases of chronic pancreatitis cases (Shimizu, 2008). It appears that rodent pancreas is more resistant to alcohol than human pancreas, repeated alcohol feeding leads to mild changes in pancreatic morphology and lack of chronic pancreatitis (Li et al., 2008). To overcome this problem, ethanol was combined with cerulein treatment. This led to faster development of chronic pancreatitis than in the presence of cerulein alone, indicating that alcohol cannot initiate pancreatitis but sensitizes pancreas to chronic injury (Deng et al., 2005; Gukovsky et al., 2008).

A convenient model to study chronic pancreatitis is to inject cerulein over a longer period of time than that is required to generate acute pancreatitis. Long-term pancreatic duct ligation also mimics the development of chronic pancreatitis. In both cases, like in acute pancreatitis, a massive loss of acinar tissues is observed with edema development. With time, the fibrotic tissue is replaced by a fatty tissue. The latter is a characteristic of chronic pancreatitis (Kimura, 1989; Otsuki et al., 2010; Watanabe et al., 1995). Long-term injections of cerulein have been also used to study the development of preneoplastic lesions in transgenic mice (Guerra et al., 2007).

While pancreatic activation of digestive enzymes, in particular trypsinogen, is important in the initiation of pancreatic injury during acute pancreatitis, chronic pancreatitis does not require intra-acinar activation of trypsinogen (Dawra et al., 2011; Sah et al., 2013). In chronic pancreatitis, the activation of inflammatory pathways by recurrent genetic or environmental injuries seems important. Thus mutations in different genes can contribute to chronic pancreatitis. This was investigated using genetic models. Disruption of the *cystic fibrosis transmembrane conductance regulators* (*CFTR*) gene results in a proinflammatory state which contributes, when associated with cerulein, to the development of a more severe pancreatitis than in wild-type mice, like in humans (Dimagno et al., 2005). A oncogenic form of Kras ($Kras^{G12D}$) is found in 27% of the patients with chronic pancreatitis and its expression in acinar cells give rise to pancreatitis by NF-κB-mediated activation of Cox-2 (Daniluk et al., 2012; Ji et al., 2009). Constitutive activity of the ikB kinase 2 (IKK2), the activator of NF-κB, also produces chronic pancreatitis (Huang et al., 2013). This indicates a predominant role of NF-κB in the inflammatory response driving pancreatitis.

## 3. Acinar-to-ductal metaplasia

Pancreatitis is associated with massive loss of exocrine tissue and development of fibrosis. Structures called tubular complexes appear. They are derived from the acinar cells and are composed of a monolayer of duct-like cells (Bockman et al., 1982). Following pancreatic duct ligation or cerulein injections, lineage-tracing experiments confirmed the acinar origin of these tubular complexes (Pan et al., 2013; Pinho et al., 2011; Shi et al., 2013; Strobel et al., 2007). They are formed by the appearance of

lumina in the center of the acini and the adoption by the acinar cells of a cuboidal shape characteristic of the duct cells. These morphologic changes are associated with a decreased expression of digestive enzymes (elastase, amylase, CPA, lipase) and acinar-specific transcription factors (Mist1, Ptf1a) and by the ectopic expression of ductal markers (CFTR, carbonic anhydrase 2 and CK19) (Pan et al., 2013; Pin et al., 2001; Shi et al., 2013). These changes in gene expression and morphology convert the acinar cells into duct-like cells and are called acinar-to-ductal metaplasia (ADM) (Figure 6). Recently it was shown that this process, originally characterized in rodents, operates also in human acinar cells (Houbracken et al., 2011).

Functional studies have identified factors controlling ADM. Mist1 inhibition induces ADM, indicating that Mist1 is required to maintain acinar cell identity (Zhu et al., 2004). Conversely, ectopic expression of the progenitor marker Pdx1 in acinar cells induces ADM through Stat3 activation (Miyatsuka et al., 2006) (Figure 6). Other works have shown that the TGF-$\alpha$ and EGFR signaling pathways are also essential to induce ADM (De Lisle and Logsdon, 1990; Means et al., 2003; Sandgren et al., 1990). In line with these findings, inhibition of MAPK signaling prevents ADM, both in humans and rodents (Houbracken et al., 2011; Shi et al., 2013).

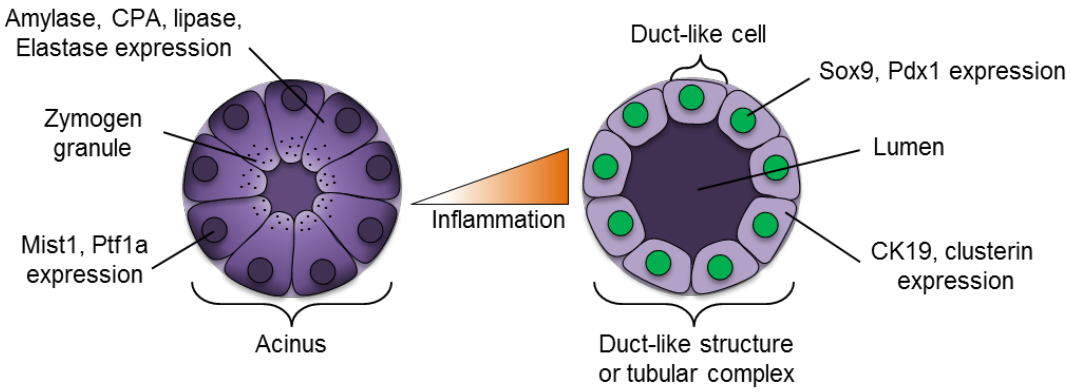


**Figure 6. Schematic representation of acinar-to-ductal metaplasia.** In left, a normal acinus shows nuclear expression of the acinar-specific transcription factors Mist and Ptf1a and amylase, CPA, lipase, and elastase expression is found in cytoplasm. Zymogen granules are represented by dark dots at the apical pole of the cell. Inflammation leads to several morphologic changes in acinar cells (see text for details). After inflammation, a phenotypic switch transforms acinar cells into duct-like cells. Duct-like cells are cuboidal and are organized in tubular complexes around a large lumen. These cells express the ductal markers CK19 and Sox9, and re-express the embryonic markers Pdx1 and Clusterin.

# C. Pancreatic ductal adenocarcinoma

## 1. Pathophysiology

Pancreatic cancer accounts for only a weak percentage of all types of cancer diagnosed in the developed world; however, it is the fourth leading cause of cancer death, indicating that this cancer is almost invariably fatal (Raimondi et al., 2009). The term “pancreatic cancer” groups different types of cancer; the more frequent (95%) is the adenocarcinoma from exocrine cells. In this condition, the pancreatic ductal adenocarcinoma (PDAC) is the most studied form and often referred to as “pancreatic cancer”. In this section, I will essentially describe PDAC.

Among the risk factors for PDAC, cigarette smoking is the principal risk factor of pancreatic tumors (20%) and cancers from smokers carry more genetic mutations than those from non-smokers (Blackford et al., 2009). Importantly, most cases of PDAC occur after the age of 60, and obesity, diabetes mellitus, occupational exposures to chlorinated hydrocarbonates solvents and nickel, and high fat diet (high red meat, low vegetables, high sugar-sweetened drinks) are known to increase the risk of PDAC (Vincent et al., 2011). Besides these risk factors, a family history is present in 7-10% of cases (Raimondi et al., 2009; Shi et al., 2009). Germline mutations in the *BRCA2*, *PALB2*, *CDKN2A*, *LKB1* and *PRSS1* genes constitute an inherited basis. Most mutations implicate genes coding for a tumor suppressor protein. Also, a family history of familial atypical multiple mole melanoma (FAMMM) increases the risk by 47-fold. However, the inherited sensitivity to PDAC remains unexplained. Thus, consensus guidelines have not yet been established to run genetic testing for inherited susceptibility to PDAC.

PDAC is distinguished for its aggressiveness, desmoplastic stromal responses, immunosuppression and resistance to therapies. Inflammation takes a preponderant place for the PDAC initiation through a chronic inflammation and for the PDAC progression through tumor-associated inflammation. In mice, experiments have shown that 49 % of the cells in the tumor mass express the cluster of differentiation (CD) 45, which is specific for the leucocytes while only 7.5 % of leucocytes are normally found in a healthy pancreas (Clark et al., 2007). The role of inflammation is described in detail in section I.C.3.b. and I.C.4.a.

The high mortality rate of PDAC is explained by the absence of symptoms before pancreatic cancer has reached an advanced state. Common symptoms are obstructive jaundice, abdominal or mid-back pain, weight loss due to anorexia, malabsorption and cachexia, and/or appearance of diabetes in 25% of patients (Vincent et al., 2011). Upon diagnosis, 85% of patients harbor an unresectable tumor, with 60% metastatic tumors, yielding an overall survival of no more than 6 months (Stathis and Moore, 2010; Vincent et al., 2011). The treatment is symptomatic with palliative chemotherapy to improve quality of life and to gain a modest survival benefit of few months. When the tumor is resectable, curative surgical treatment is attempted by the Whipple procedure when cancer affects the head of the pancreas, or by a distal pancreatectomy if the tumor is located in the tail of the pancreas. The Whipple

procedure is the most common curative surgery; it consists of removing the pancreatic head and the curve of the duodenum (pancreato-duodenectomy) to create a bypass for food from stomach to jejunum, and drainage of bile from liver to the intestine via an explanted intestinal loop. Surgery procedures are followed by chemotherapy, most often using gemcitabine, a nucleoside analog inducing cell apoptosis. However, even with surgery the average survival of PDAC patients is only increased by 5 years (Raimondi et al., 2009).

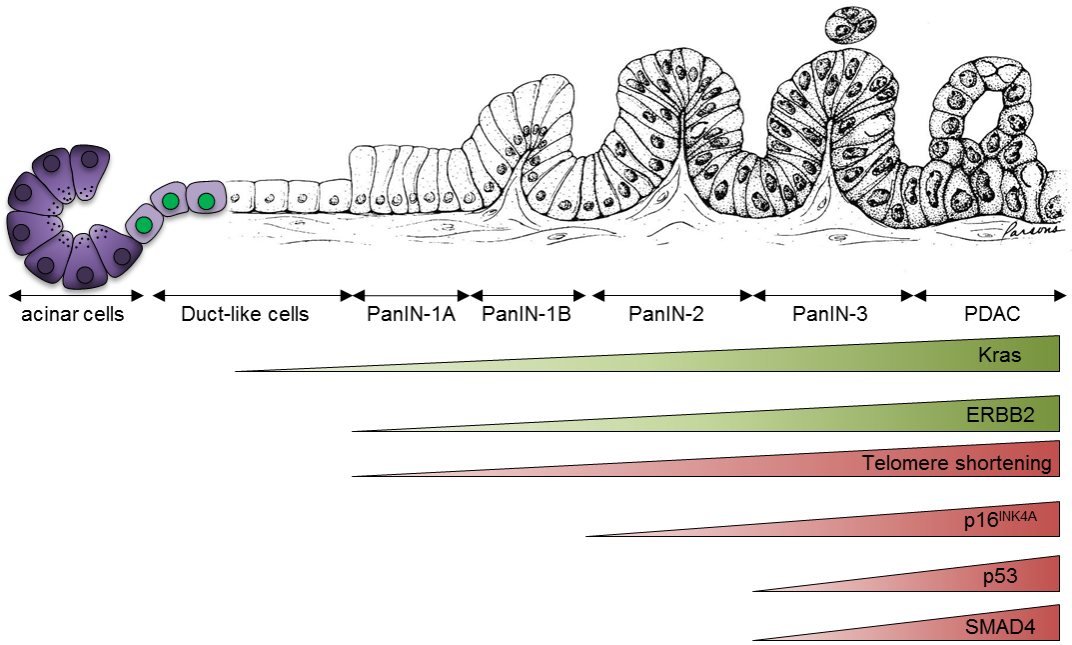


**Figure 7. Model of PanIN progression** (adapted from Aghdassi et al., 2011; Hruban et al., 2000)). Acinar cells evolve into duct-like cells which give rise to PDAC through a series of histologically defined precursor stages called PanIN (see text for details). Mutations in tumor suppressors, amplifications of oncogenes, and telomere shortening further contribute to PanIN evolution.

From a histopathological point of view, progression of PDAC is sequential with the initial appearance of neoplastic lesions called pancreatic intraepithelial neoplasia (PanIN). These are frequent microscopic lesions (< 0.5 cm) not detectable by imaging. A biopsy is needed to demonstrate their presence. PanIN are classified from PanIN-1A to PanIN-3, and this depends on the degree of architectural and cytological atypia (Hruban et al., 2001). PanIN-1A is the earliest form with a flat (non-papillary) epithelial structure composed of tall columnar mucin-containing cells. At this stage, almost no nuclear atypia is observed. When these lesions acquire a papillary, micro-papillary or basally pseudo-stratified architecture, lesions are called PanIN-1B. PanIN-2 are papillary or non-papillary epithelial lesions with moderate cytological atypia, including loss of polarity, nuclear crowding, nuclear enlargement, pseudo-stratification and nuclear hyper-chromatism. Finally PanIN-3 are the latest stage before invasive carcinoma and are characterized by a papillary or micro-papillary epithelial structure with a cribriform growth and the budding-off of small clusters of epithelial cells into the lumen. Severe nuclear atypia are observed with loss of polarity, loss of differentiated cytoplasmic features, cellular and nuclear pleomorphism, and the presence of mitoses

(Figure 7). All along the initial development of PDAC, PanIN appear and evolve with the activation of Kras as result of mutation or amplification, or expression of *ERBB2/EGFR* associated with telomere shortening. At later stages, inhibition of tumor suppressors (*P16*$^{INK4A}$, *TP53*, *SMAD4*) contributes to PDAC progression (Hruban et al., 2001; Sharif et al., 2008) (Figure 7).

## 2. Genetics of PDAC

### *a. Kras*

Kras, also called Kirsten rat sarcoma (ras) viral homolog, is a member of the ras family which comprises three members, each of them sharing oncogenic functions. The two other members are Harvey ras (Hras) and Neuroblastoma ras (Nras). Ras proteins are small GTPases that act as transducers of receptor signals to intracellular effectors. Like all G proteins, ras functions as a switch between “on” and “off” states, regulated by the binding to GTP and GDP, respectively. Guanine nucleotide exchange factors (GEF) activate ras by stimulating the release of GDP to allow GTP binding whereas GTPase activating proteins (GAP) inactivates it by controlling the reverse process (Pylayeva-Gupta et al., 2011) (Figure 8).

In human, ras mutations are found in 30% of all tumors (Fernandez-Medarde and Santos, 2011). Remarkably, Kras is the member of the ras family which is the most frequently mutated (25%) in tumors (Fernandez-Medarde and Santos, 2011; Forbes et al., 2011). In pancreas diseases, Kras is mutated in 30% of patients with chronic pancreatitis and in 90% of the PDAC samples. In neoplastic lesions, Kras mutations increase from 35% in PanIN-1 to 86% in PanIN-3 (Wilentz et al., 2000). Kras mutation, in parallel to telomere shortening, is the earliest genetic event contributing to PanIN formation (Hruban et al., 2000; Hruban et al., 1993; van Heek et al., 2002).

In pancreatic cancer, Kras is predominantly mutated in codon 12 (Hruban et al., 1993; Jones et al., 2008). Glycine is replaced by aspartic acid (G12D, 41% of cases), valine (G12V, 24%) or cysteine (G12C, 15%) (Pylayeva-Gupta et al., 2011). These substitutions prevent the interaction between ras and GAP and reduce GTP hydrolysis. This results in persistent ras-GTP binding and to constitutive activity of Kras and its downstream effector pathways (Scheffzek et al., 1997).

In normal condition, Kras protein has multiple roles within the cell, with main effects on proliferation and apoptosis (pro-apoptotic and anti-apoptotic roles are found). Kras can be activated by several receptors: these include G protein coupled receptors when bound to interleukins or serotonin, receptor tyrosine kinase when bound to members of the EGF family, or Integrins. The effects of Ras on proliferation and cell survival are mediated by Ral-guanine nucleotide dissociation stimulator (GDS), Raf and PI3K signaling. Kras-promoted apoptosis is mediated by Raf, RassF1 and JNK pathways, and depends on ligand activation. Interestingly, a tumorigenic phenotype is characterized by suppression of the apoptotic effects of Kras (Pylayeva-Gupta et al., 2011). Kras is also implicated in cell polarity, motility and adhesion via Tiam1

activation, and in membrane trafficking through Ral-GDS (Camonis and White, 2005; Malliri and Collard, 2003) (Figure 8).

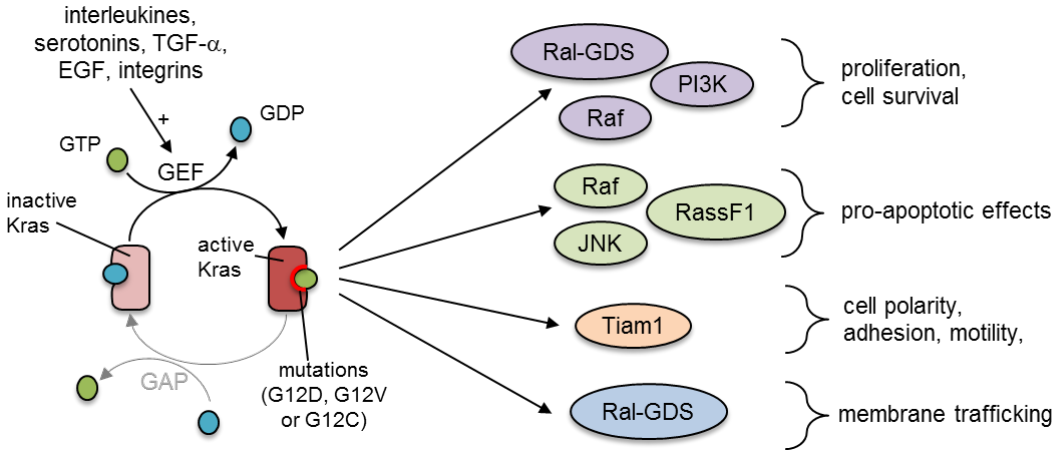


**Figure 8. Schematic representation of Kras activation and downstream pathways activated by Kras.**

### *b. Other oncogenes*

Screening of pancreatic tumors identified mutations or amplifications of the *BRAF*, *ERBB2*, *MYB*, *CTNNB1* and *AKT2* genes (Jones et al., 2008; Zavoral et al., 2011). These mutations or amplifications lead to increased protein expression or to the activation of several pathways important for PanIN and PDAC (see below) (Dancer et al., 2007; Moore et al., 2007). Interestingly, overexpression of several proteins is observed in PDAC without amplification of the respective gene. Such overexpressions promote development of PDAC and function as oncogenes. This is the case for EGFR, which is overexpressed in 65% of PDAC (Dancer et al., 2007).

Recently, a study using a large short hairpin (sh) RNA screen identified 18 genes essential for proliferation and survival of pancreatic cancer cell lines. These genes are potential pancreatic oncogenes (Cheung et al., 2011). Of particular importance for our work, *SOX9* was one of these 18 genes.

### *c. Tumor suppressors*

With the exception of Kras, other genes frequently mutated in PDAC are tumor suppressor genes. The most frequently affected are *CDKN2A*, *TP53* and *SMAD4* which are mutated at a frequency of 95, 60, and 55%, respectively (Hong et al., 2011). Mutation of these genes is essential to support the evolution of PanIN into PDAC (see below) (Figure 7).

*CDKN2A* gene, also called *P16*$^{INK4A}$, codes for a cyclin-dependent kinase (CDK) inhibitor, the protein (p) 16. P16 is implicated in cell cycle regulation by inactivating the CDK that phosphorylates and inactivates the retinoblastoma protein. Inactivation of *P16*$^{INK4A}$, from PanIN-2, due to either a homologous deletion of both alleles (41%), an intragenic mutation in one allele with loss of the second allele (40%), or promoter hyper-methylation (15%) induces an acceleration of the cell cycle by activating the G1-S transition and cell proliferation (Caldas et al., 1994; Hong et al., 2011; Schutte et al., 1997; Ueki et al., 2000).

The protein (p) or tumor-protein (TP) 53 is involved in multiple processes, including regulation of the cell cycle, apoptosis, senescence, and DNA repair. *TP53* mutations are found in more advanced neoplastic lesions (PanIN-3) and generally combine an intragenic mutation of one allele with loss of the other allele (Hong et al., 2011; Redston et al., 1994; Scarpa et al., 1993). In contrast to the *P16*$^{INK4A}$ and *SMAD4* genes, *TP53* mutation frequently results in expression and accumulation of a stable p53$^{R172H}$ protein rather than complete loss of protein expression (Morton et al., 2010). This situation contributes to cell survival and division of damaged-DNA in cells that accumulate genetic abnormalities (Morton et al., 2010; Vogelstein and Kinzler, 2004).

Smad4 has a crucial role in differentiation, apoptosis, embryonic development and cell cycle. It is activated by TGF-β ligands, and one of its targets is the cell cycle inhibitory factor p21. *SMAD4* mutations are essentially observed starting from the PanIN-3 stage (Iacobuzio-Donahue et al., 2000). Smad4 inactivation results from a homozygous deletion or intragenic mutation with loss of the other allele, and causes activation of the cell cycle (Hahn et al., 1996; Iacobuzio-Donahue et al., 2000).

## 3. Mouse models of PDAC

### *a. Genetics models*

Generation of transgenic mouse models that faithfully recapitulate the characteristics of human cancers has been extremely useful to better understand how cancers develop. This has also been the case for pancreatic cancer. Starting from the observation that the large majority of PDAC have *KRAS* mutations, transgenic models aimed at recapitulating KRAS mutations. The most frequently used models contain a knock-in insertion of an oncogenic form of Kras (G12D or G12V) in the endogenous *Kras* locus. This knock-in insertion is flanked by a removable transcriptional termination stop element called Lox-Stop-Lox (LSL). This stop cassette can be eliminated by Cre-mediated recombination, which removes the Stop cassette located between the loxP sites (Guerra et al., 2003; Jackson et al., 2001). This model was initially described in pancreas using a Cre recombinase driven by the promoters of the *Pdx1* or *Ptf1a* genes, which are both active in the pancreatic progenitor cells. For this reason, Kras$^{G12D}$ is expressed in the MPC and, after birth, in all the pancreatic lineages. Interestingly, the corresponding mice develop the full spectrum of human PanIN (Hingorani et al., 2003). However, in these mice, PDAC develops with a low frequency and a long latency, questioning the requirement of additional events to generate efficiently PDAC. Like in

humans, combining-activating mutations of Kras with inactivation of tumor suppressor genes *p53* or *p16*$^{INK4A}$ allows development of PDAC at high frequency; metastastic tumors are also observed (Aguirre et al., 2003; Hingorani et al., 2005). Interestingly, mutation of *p53* or *p16*$^{INK4A}$ alone does not lead to a PanIN or PDAC (Morton et al., 2010; Qiu et al., 2011). These results demonstrate that mutant Kras is required to initiate development of pancreatic cancer.

Further work showed that oncogenic Kras is also required for maintenance of pancreatic cancer. This was demonstrated by the use of a transgenic system, which allows the inducible, pancreas-specific, and reversible expression of oncogenic Kras. Inactivation of oncogenic Kras in PanIN and PDAC leads to regression of the neoplastic and cancer lesions. This indicates that oncogenic Kras is required for tumor cell survival (Collins et al., 2012). Moreover, senescence induced by Kras inhibits progression of PanIN *in vivo* (Guerra et al., 2011). Senescence is due to the induction of the cell cycle regulators p16, p53, and p21. Thus, inactivation of tumor suppressor *p16*$^{INK4A}$ and/or *p53* prevents senescence and promotes development of PDAC (Collado et al., 2005; Guerra et al., 2011; Morton et al., 2010). Moreover, accumulation of mutated p53 drives metastasis by conferring invasive properties to the tumor cells (Morton et al., 2010).

### b. *Role of inflammation in development of PDAC and cellular origin(s) of PDAC*

In the first models of PDAC, the onset of oncogenic Kras expression occurs in embryonic pancreas. This does not reflect the reality of PDAC as, in humans; this cancer is mainly observed in aged people. Therefore, models with postnatal induction of oncogenic Kras were developed. One model resorted to the use of the promoter of the ductal gene CK19 to drive the expression of oncogenic Kras in ducts. Surprisingly, no PanIN developed in this setting (Brembeck et al., 2003) (Figure 9).

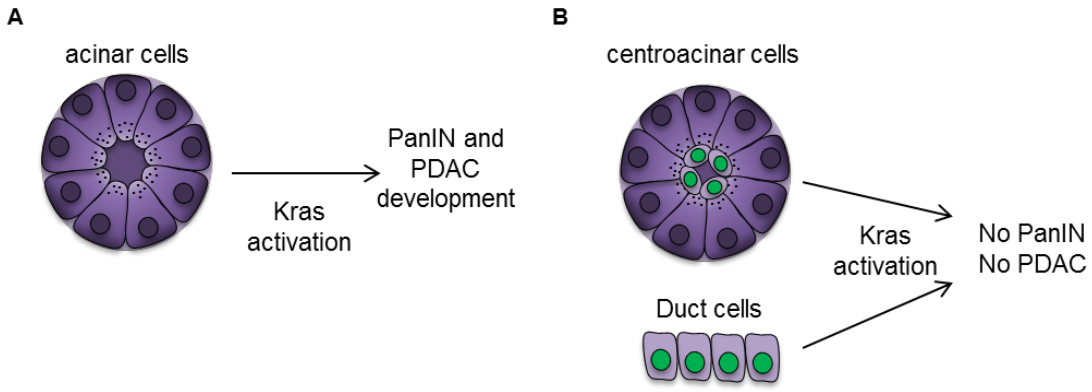


**Figure 9. Summary of the origin cell of pancreatic cancer.** Following Kras activation, acinar cells give rise to PanIN lesions through acinar-to-ductal metaplasia and PDAC (**A**). Centroacinar cells and pancreatic ductal cells cannot give rise to PanIN or PDAC even upon Kras activation (**B**).

Based on the observation that acinar cells are plastic and undergo ADM, several groups drove conditional expression of oncogenic Kras in the acinar cells using the inducible CreER system. They observed that, in young mice, aged from 2 to 6 weeks, expression of oncogenic Kras in acinar cells consistently promotes PanIN formation (Habbe et al., 2008). However, 8 weeks after birth, adult acinar cells become resistant to PanIN formation, even if oncogenic Kras expression is associated with *p53* or $p16^{INK4A}$ deletion (Guerra et al., 2011; Guerra et al., 2007; Perez-Mancera et al., 2012). However, if pancreas is exposed to cerulein-induced inflammation, PanIN develop from adult acinar cells. In this condition, *p53* and $p16^{INK4A}$ deletion contribute to PDAC development. Reduction of pancreatitis-induced inflammation interferes with PanIN development and facilitates tissue repair (Guerra et al., 2011; Guerra et al., 2007). Recently, genetic lineage tracing confirmed that ductal cells are refractory to transformation by oncogenic Kras and that PDAC originates from acinar cells. This indicates that formation of PDAC initially relies on an acinar-to-ductal reprogramming (Kopp et al., 2012). These results contribute to explain why pancreatitis is a risk factor for PDAC and identifies ADM as a precursor lesion for PDAC.

## 4. Transcription factors and signaling pathways in PDAC

### *a. Transcriptional regulation and intercellular communication in PDAC*

Ninety percent of the tumor volume is composed of stromal cells, indicating that the developing tumor often co-opts the host immune system for its own tumor-promoting purposes. Stroma normally plays a supportive role in tissues or organs. In PDAC, stroma is composed of reactive cancer-associated fibroblasts, activated pancreatic stellate cells, scattered inflammatory cells, partially collapsed microvasculature, nerve fibers, and dense connective tissue, which secrete numerous cytokines and growth factors (Evans and Costello, 2012). From ADM and low grade PanIN, a layer of reactive stroma surrounds the epithelial structures and seems to contribute to their neoplastic development. While the presence of the stroma has been taken aside for a long time, recent reports have identified pathways involved in the molecular dialogue between stroma and tumors cells.

Aberrant expression of Hedgehog (Hh) ligands is observed at high frequency in human and mouse PanIN and PDAC (Nolan-Stevaux et al., 2009; Thayer et al., 2003). Moreover, the transcriptional mediator of the canonical Hh signaling, Gli is also expressed in PDAC cells. This suggests that the Hh pathway drives PDAC. In these conditions, it was surprising that deletion of smoothened, a transmembrane receptor essential for Gli activity, does not affect PDAC development (Nolan-Stevaux et al., 2009). In contrast, Gli is required for pancreatic tumorigenesis (Rajurkar et al., 2012). These results indicate that, in PDAC, Gli activity is independent of Hh signaling (Nolan-Stevaux et al., 2009). In this condition, Gli transcription is regulated by Kras and TGF-β signaling and oncogenic activity of Gli seems to be controlled by a mechanism

involving NF-κB (Dennler et al., 2007; Nolan-Stevaux et al., 2009; Rajurkar et al., 2012). If tumors cells are refractory to the canonical Hh signaling, the aberrant expression of ligands by tumors cells activates the Hh signaling in others cells like PSC and cancer-associated fibroblasts to promote their proliferation (Bailey et al., 2008; Hwang et al., 2012; Yauch et al., 2008). While the role of cancer-associated fibroblasts cells is not yet elucidated, these cells seem to protect the tumors against anti-tumoral signals since inhibition of smoothened produces an expansion of tumor vasculature and leads to an increased intratumoral delivery of gemcitabine (Olive et al., 2009).

Wnt/β-catenin signaling has been shown to play opposite roles during pancreatic cancer progression. After cerulein-induced ADM, β-catenin signaling is required for regeneration of the injured acinar cell compartment: this activity antagonizes the effect of oncogenic Kras that promotes ADM (Morris et al., 2010). However, at later stages, the Wnt/β-catenin signaling is important for PDAC progression (Morris et al., 2010; Zhang et al., 2013a). These results obtained essentially in mice are corroborated by other data showing that the Wnt/β-catenin signaling is activated in human PDAC (Morris et al., 2010; Pasca di Magliano et al., 2007). The importance of Wnt signaling in PDAC progression is supported by another study showing that inactivating mutations of RNF43, a negative regulator of Wnt signaling, confers Wnt dependency to pancreatic cancer cell lines (Jiang et al., 2013).

A characteristic of PanIN and PDAC lesions is the presence of infiltrated inflammatory cells, in particular leucocytes: mast cells, myeloid derived suppressor cells (MDSCs), macrophages and immunosuppressive T lymphocytes (Clark et al., 2007). Relationships between immune system and tumoral cells are very complex and still controversial: the current view is that the leucocytes infiltrating PDAC lesions induce a local immune suppression. How the inflammatory cells are recruited during PanIN formation and are implicated in PDAC progression is still poorly understood. However, recent results revealed that tumor cells secrete granulocyte-macrophage colony stimulating factor (GM-CSF): this cytokine drives the accumulation of MDSCs, which in turn suppress cytotoxic T cells (Bayne et al., 2012; Pylayeva-Gupta et al., 2012). GM-CSF is not the only cytokine induced during PDAC development. Others have shown that the pro-inflammatory cytokines IL-1β, VEGF, and IL-6 are targets of oncogenic Kras and are also present in PDAC (Ancrile et al., 2008; Coppe et al., 2008; Roshani et al., 2013). The role of IL-6 has been well characterized during PanIN progression and maintenance (Zhang et al., 2013b). IL-6 is secreted by infiltrating immune cells, in particular by macrophages (Lesina et al., 2011). Secreted IL-6 binds to a soluble form of IL-6 receptor (siIL-6R), and this interaction induces Stat3 activation in the tumor cells (Lesina et al., 2011). Stat3 activation contributes to the matrix metalloproteinase (MMP) 7 induction, both factors being essential for PDAC development and metastasis (Fukuda et al., 2011).

Another pathway implicated in PDAC is Notch signaling. Notch and especially its target Hes1 are expressed throughout pancreatic cancerogenesis from metaplastic lesions to PDAC. In normal pancreatic tissue, Notch 1 is expressed in acinar cells whereas Notch 2 is expressed in duct and centroacinar cells. While Notch1 plays a role in acinar regeneration after acute pancreatitis, its expression is not required for PanIN

development (Avila et al., 2012; Siveke et al., 2008). Expression of Notch 2, like others ductal markers, is increased and required during PanIN progression and PDAC development (De La et al., 2008; Mazur et al., 2010). Interestingly Notch 1 and 2 inactivations do not decrease Hes1 expression suggesting a Notch-independent signaling (Nakhai et al., 2008; Siveke et al., 2008).

In many tissues, the NF-κB pathway controls cell survival and inflammation (Buday and Downward, 2008). This is also the case in pancreas: in the presence of cerulein-induced inflammation, the level of NF-κB activation correlates with severity of pancreatitis (Huang et al., 2013). Along the same line, when oncogenic Kras is present, inflammatory stimuli trigger an NF-κB-mediated positive feedback mechanism involving inflammation mediators, like Cox-2, which amplifies Ras activity to pathological levels. IKK2 plays a central role in this process (Daniluk et al., 2012). This feedback mechanism also includes IL-1α: $Kras^{G12D}$-activated AP-1 induces IL-1α, which, in turn, activates NF-κB and its target genes IL-1α, to sustain NF-κB activity (Ling et al., 2012). NF-κB and Notch signaling pathways synergize to promote pancreatic cancer progression: this synergy is obtained by suppressing anti-inflammatory protein expression (Maniati et al., 2011).

Besides the various pathways described above, the EGFR pathway was recently shown to be required for PDAC development. This pathway is described in the next section.

### *b. The EGFR pathway*

The EGFR family contains 4 members that function like tyrosine kinase receptors: ErbB (erythroblastic leukemia viral oncogene homolog) 1/EGFR, ErbB2/ Human Epidermal Growth Factor Receptor (HER) 2, ErbB3 and ErbB4. They possess a common extracellular ligand-binding domain composed of two cysteine-rich regions, a single-span transmembrane region, a cytoplasmic tyrosine kinase domain and a C-terminal signaling tail (Burgess et al., 2003; Ferguson, 2008). ErbB receptors are widely expressed in several cell types. Ligand binding induces homo or hetero-dimerization of the receptors, which stimulates the tyrosine kinase activity and leads to auto-phosphorylation of specific tyrosine residues in the cytoplasmic domain. Interestingly, ErbB2 and ErbB3 differ from the other ErbB members: ErbB2 has no known ligand whereas ErbB3 has an impaired tyrosine kinase domain (Garrett et al., 2003; Jura et al., 2009; Shi et al., 2010). Both need to heterodimerize with other ErbB members to induce signaling. In fact ErbB1, ErbB3, and ErbB4 heterodimerize preferentially with ErbB2, and heterodimers show increased activity as compared to ErbB homodimers (Graus-Porta et al., 1997; Hartman et al., 2012; Tzahar et al., 1996; Yarden and Sliwkowski, 2001). Numerous ErbB ligands activate ErbB receptors in a specific manner. All these ligands display an EGF-like domain and three disulphide-bonded intramolecular loops (Jones et al., 1999). Briefly, TGF-α, EGF, epiregulin, β-cellulin, heparin-binding EGF (HB-EGF), and amphiregulin (AREG) bind to ErbB1; epiregulin, HB-EGF, and neuregulin (NRG) 1-4 bind to ErbB4; and NRG1 and 2 are the

only ligands of ErbB3 (Jones et al., 1999) (Figure 10). By indirect signaling, cytokines, lysophosphatic acid and thrombin activate also ErbB receptors (Yarden and Sliwkowski, 2001). Depending on the cellular context, ErbB receptors promote cell division, migration, adhesion, differentiation and apoptosis.

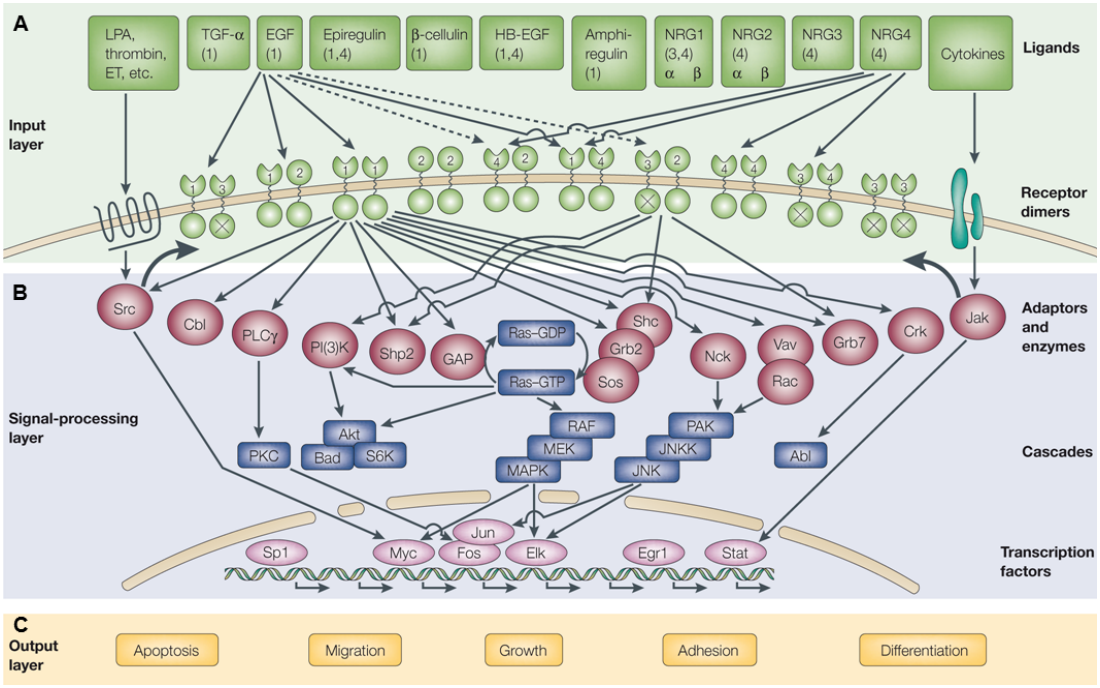


**Figure 10. The ErbB signaling network** (Yarden and Sliwkowski, 2001). Combinations between ligands and ErbB receptors are shown in the input layer. Numbers in each ligand block indicate the respective high-affinity ErbB receptors. The crossed kinase domain represents the inactive catalytic domain of ErbB3 (**A**). Signaling of ErbB1 homodimers and ErbB2-ErbB3 heterodimers are shown in the signal-processing layer. Only some of the downstream pathways and transcription factors are represented in this layer (**B**). Functional effects of ErbB signaling on cells are represented in the output layer (**C**). EGF, epidermal growth factor; NRG4, neuregulin 4; LPA, lysophosphatidic acid; ET, endothelin; GAP, GTPase activating protein; HB-EGF, heparin-binding EGF; Jak, janus kinase; PKC, protein kinase C; PLCγ, phospholipase Cγ; Shp2, Src homology domain-2-containing protein tyrosine phosphatase 2; Stat, signal transducer and activator of transcription.

Constitutive deletion of ErbB2, ErbB3 or ErbB4 leads to early embryonic lethality due to cardiac malformation (Erickson et al., 1997; Gassmann et al., 1995; Lee et al., 1995). The early lethality (e10.5) of the ErbB2 and ErbB4 embryos prevents to study their role in pancreas development. ErbB3 KO embryos, which die at e13.5, display pancreatic hypoplasia resulting from defective mesenchymal signaling: ErbB3 expression is indeed detected in pancreatic mesenchyme but not in pancreatic epithelium (Erickson et al., 1997; Gassmann et al., 1995; Lee et al., 1995). EGFR KO embryos survive until 1 week after birth and present with impaired branching morphogenesis, reduced proliferation of the pancreatic epithelium, and delayed endocrine cell differentiation (Miettinen et al., 2000). Beyond these phenotypic descriptions, the molecular mechanisms controlled by ErbB receptors during pancreas embryogenesis are unknown (Kritzik et al., 2000). In adult mouse and human pancreas, expression of ErbB receptors is hardly detectable in healthy tissue (Ardito et al., 2012).

While ErbB receptors are critical regulators of normal growth and development, positive or negative deregulation has been implicated in development and progression of cancer (Baselga and Swain, 2009; Hynes and MacDonald, 2009; Jaiswal et al., 2013). ERBB2 has a prominent role in various cancers, especially breast cancer. Amplification or overexpression of the *ERBB2* gene occurs in up to 30% of breast cancers and is associated with poor prognosis (Slamon et al., 1989). This overexpression gives rise to spontaneous dimerization and auto-activation of ERBB2, or to a stabilization of ERBB2 heterodimers that drives to malignancy and tumor development by maintaining a high level of proliferation (Harari and Yarden, 2000; Hartman et al., 2012). This important role was the starting point for pharmacological targeting of the extracellular domain of ERBB2 with the monoclonal antibody Herceptin$^{TM}$, which is successfully used in treatment of ERBB2-positive breast cancer (De Mattos-Arruda and Cortes, 2012). Mutations that lead to EGFR overexpression or to constitutive activation have been found in a number of cancers, including lung and colorectal cancer (Markman et al., 2010; Shigematsu and Gazdar, 2006). This led to further development of anti-EGFR drugs, which act either as inhibitory monoclonal antibody or as small molecule kinase inhibitor, gefitinib or erlotinib.

EGFR is rarely mutated in PDAC (<3%) (Oliveira-Cunha et al., 2012). Gene amplification of *ERBB2* is observed in only 2.1% of PDAC (Chou et al., 2013). However some reports up this percentage to 25% (Dancer et al.; Sharif et al., 2008). Nevertheless elevated expression of EGFR, ERBB2 and ERBB3 is found in PDAC, and two EGFR ligands (EGF and TGF-$\alpha$) show increased expression as well (Friess et al., 1996; Sharif et al., 2008). More precisely, EGFR and ERBB2 expression is found from PanIN-1A to PDAC lesions (Hingorani et al., 2005; Ueda et al., 2004). While the role of ERBB2 in PDAC formation is still undetermined, that of EGFR is better understood. Experiments in cultured acinar cells or in a mouse model expressing TGF-$\alpha$ or oncogenic Kras in the acinar cells showed that activation of the EGFR signaling is sufficient and required for induction of metaplasia (De Lisle and Logsdon, 1990; Means et al., 2005; Means et al., 2003; Navas et al., 2012; Sandgren et al., 1990). Interestingly, and in sharp contrast with lung and colon cancer, EGFR is essential for Kras-induced pancreatic tumorigenesis (Ardito et al., 2012; Navas et al., 2012). In this process, ADAM17, a metalloproteinase that sheds EGFR ligands like TGF-$\alpha$ and AREG, is required for the activity of the EGFR pathway and consequently, for Kras-driven pancreatic tumorigenesis. In human PDAC, elevated expression of ADAM17 is observed (Ringel et al., 2006). Importantly, without EGFR, active ras levels are not sufficient to induce robust MEK/ERK activity. EGFR activates the PI3K and Stat3 pathways: inhibition of the latter prevents proliferation of the pancreatic cancer cells. Finally, loss of p53 made pancreatic tumors independent of EGFR signaling (Navas et al., 2012) (Figure 10). Cooperations between EGFR and other pathways have been observed. EGFR and Hh cooperate to promote transformation and proliferation of pancreatic cancer cells. Together, both pathways regulate synergistically the expression of several genes required for controlling tumor initiation and growth (Eberl et al., 2012). Also, EGFR signaling induces TGF-$\alpha$ expression, which activates $\gamma$-secretase-dependent activation of Notch, and Notch is required for ADM (Miyamoto et al., 2003). Together, these results show that EGFR has a predominant role in the initiation and progression of PDAC.

### *c. The transcription factors HNF6 and SOX9 in cancer*

As indicated above, HNF6 and SOX9 are essential for proper embryonic development of the pancreas. While carcinogenesis is frequently linked to the reactivation of embryonic markers, only limited evidence is available to establish a functional role for these two transcription factors in tumorigenesis. In human lung adenocarcinoma, HNF6, E-cadherin and P53 expression are highly correlated. These correlations are explained by the fact that HNF6 directly regulates P53 to maintain an epithelial phenotype. So, expression of HNF6 suppresses migration and invasive growth in lung cancer cell line (Yuan et al., 2013). Similarly, in PDAC and pancreatic cancer cell lines, HNF6 in not expressed. Interestingly, overexpression of HNF6 induces HNF1β expression and reduces invasiveness (Jiang et al., 2008). These reports suggest a tumor suppressor function for HNF6.

High expression of SOX9 is observed in a wide range of human tumors, including pancreatic cancer. In colon cancer, SOX9 facilitates tumor growth and progression. It exerts its function by controlling the expression of the polycomb protein Bmi1, a repressor of *P16*$^{INK4A}$ (Matheu et al., 2012). In human breast cancer, SOX9 expression correlates with poor prognosis: this observation could be linked with data showing that SOX9 enhances Wnt/β-catenin signaling to tumorigenesis (Wang et al., 2013). Conversely, in prostate cancer, Wnt/β-catenin stimulates SOX9 to promote tumor growth and invasion (Wang et al., 2008). In lung adenocarcinoma, SOX9 is expressed and required for the tumor progression, possibly via downregulation of the cell cycle inhibitor p21 (Jiang et al., 2010). Moreover, Ling and colleagues show that urothelial injury promotes expression of SOX9 through EGFR, thereby leading to urothelial cancer formation (Ling et al., 2011). In basal cell carcinoma and pancreatic cancer cells, SOX9 is also induced by the cooperative action of EGFR and Hh and plays a role in the growth of these cancer cells (Eberl et al., 2012). Finally, an essential role for SOX9 in proliferation and survival of pancreatic cancer cell lines was demonstrated (Cheung et al., 2011; Eberl et al., 2012), and recently, while the thesis was in progress, Kopp et al. published that SOX9 is essential for development of PDAC (Kopp et al., 2012). This will further be analyzed in the discussion.

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# II. Aims of the thesis

Pancreatic ductal adenocarcinoma originates from acinar-derived metaplastic lesions that evolve in neoplastic lesions (PanIN) and then in cancer. In metaplasic lesions (called acinar-to-ductal metaplasia, or ADM), which can be considered pre-cancerous lesions, acinar cells are phenotypically converted to duct-like cells.

The host lab and others have shown earlier that the transcription factors HNF6 and Sox9 are expressed in the duct cells and are required for duct differentiation. Therefore, we hypothesized that HNF6 and Sox9 participate in the phenotypic conversion of acinar cells, and at later stages of tumorigenesis, in pancreatic cancer development.

In the first part of this thesis, we specifically addressed the role of HNF6 and Sox9 in ADM. To answer this question, we performed gain- and loss-of-function experiments, *in vitro* and *in vivo*. The results of these experiments are compiled in an article published in "Gut", which constitutes the first chapter of the "Results" section.

In the second chapter of my results, we studied the role of Sox9 in PanIN formation, beyond the stage of ADM, again using loss-of-function approaches, *in vitro* and *in vivo*. These results constitute my personal contribution to a paper which will include additional data on humans obtained by collaborators, and which is in the final stage of preparation. The available human data from our collaborators are presented in the appendix.

# III. Results

## A. Role of the ductal transcription factors HNF6 and Sox9 in pancreatic acinar-to-ductal metaplasia

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In this article I contributed to study concept and design, to acquisition, analysis and interpretation of data for Figures 1, 4, 5, 6 and Supplemental Figures 1, 2, 3 and 5, and to drafting of the manuscript.

### Summary

**Objective** Growing evidence suggests that a phenotypic switch converting pancreatic acinar cells to duct-like cells can lead to pancreatic intraepithelial neoplasia and eventually to invasive pancreatic ductal adenocarcinoma. Histologically, the onset of this switch is characterised by the co-expression of acinar and ductal markers in acini, a lesion called acinar-to-ductal metaplasia (ADM). The transcriptional regulators required to initiate ADM are unknown, but need to be identified to characterise the regulatory networks that drive ADM. In this study, the role of the ductal transcription factors hepatocyte nuclear factor 6 (HNF6, also known as Onecut1) and SRY-related HMG box factor 9 (Sox9) in ADM was investigated.

**Design** Expression of HNF6 and Sox9 was measured by immunostaining in normal and diseased human pancreas. The function of the factors was tested in cultured cells and in mouse models of ADM by a combination of gain and loss of function experiments.

**Results** Expression of HNF6 and Sox9 was ectopically induced in acinar cells in human ADM as well as in mouse models of ADM. HNF6 and, to a lesser extent, Sox9 were required for repression of acinar genes, for modulation of ADM-associated changes in cell polarity and for activation of ductal genes in metaplastic acinar cells.

**Conclusions** HNF6 and Sox9 are new biomarkers of ADM and constitute candidate targets for preventive treatment in cases when ADM may lead to cancer. This work also shows that ectopic activation of transcription factors may underlie metaplastic processes occurring in other organs.

# B. Sox9 acts as an oncogene in pancreatic cancer by stimulating the ErbB pathway

The results in this chapter describe the role of Sox9 in a mouse model of pancreatic cancer. They are part of a collaborative study that integrates data obtained in human pancreatic cancer samples (summarized in Appendix 1) and whose collection is being finalized. An article containing the complete mouse and human data is in preparation. In this chapter I contributed to study concept and design, acquisition of data, analysis and interpretation of data and drafting of the manuscript; Cécile Augereau performed and analyzed the pancreas explant experiments; Christine Sempoux (Department of Pathology, Cliniques universitaires St. Luc) helped to analyze the PanIN lesions.

## 1. Chapter summary

We have shown in the previous chapter that the pancreatic ductal transcription factor Sox9 promotes acinar-to-ductal metaplasia, yet its role in pancreatic tumorigenesis is still uncharacterized. Here we show that Sox9 is required for the initiation of premalignant lesions called pancreatic intraepithelial neoplasia (PanIN) originating from acinar-to-ductal metaplasia. In a mouse model of pancreatic cancer driven by the expression of an oncogenic form of Kras ($Kras^{G12D}$), Sox9 deletion blocks development of PanIN. The absence of Sox9 reduces expression of members of the v-erb-b erythroblastic leukemia viral oncogene homolog (ErbB) pathway, including epidermal growth factor receptor (Egfr), and ErbB2. Using pancreatic explant cultures, we demonstrate that this results in the lack of activity of the ErbB pathway, which is known to be required for pancreatic tumor development. Further experiments in cultured cells also show how ErbB2 is involved in this process, suggesting new therapeutic routes to treat pancreatic cancer. All together, these results reveal a key oncogenic role for Sox9 in development of pancreatic cancer.

## 2. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most deadly cancers. High mortality rate results from insufficient understanding of the molecular mechanisms driving tumor initiation and progression, leading to late diagnosis and an inefficient response to conventional therapies. PDAC is nearly always associated with activating mutations in the proto-oncogene *KRAS* as recently confirmed by exome sequencing of 99 informative tumors (Biankin et al., 2012).

Analyses of genetically modified mouse models suggest that acinar cells undergo acinar-to-ductal metaplasia (ADM), a process in which the acinar cells are phenotypically converted to duct-like cells (Guerra and Barbacid; Kopp et al., 2012;

Rooman and Real, 2012). In the presence of activating *KRAS* mutations, metaplastic lesions give rise to non-invasive ductal lesions called pancreatic intraepithelial neoplasia (PanIN), which upon activation of other proto-oncogenes or loss of tumor suppressor genes, eventually evolve to malignant lesions and PDAC (Guerra and Barbacid; Hingorani et al., 2005; Jones et al., 2008). This model of tumorigenesis is further supported by the observation in humans that pancreatitis, an inflammatory disease of the pancreas with pronounced ADM, is associated with increased risk of PDAC (Pinho et al., 2013; Rooman and Real, 2012). Therefore, it is essential to unravel the mechanisms that underlie the early stages of pancreatic tumorigenesis, namely ADM and PanIN.

Loss of acinar differentiation, recapitulation of embryonic features typical of ductal differentiation, inflammation and oncogenic Kras expression are intimately linked in the oncogenic process (Collins et al., 2012; Guerra et al., 2007). Recent observations indicate that the EGFR pathway contributes to ADM and is essential for Kras-induced pancreatic tumorigenesis (Ardito et al., 2012; Navas et al., 2012). EGFR signaling stimulates RAS, leading to robust ERK activation, which is required for pancreatic transformation (Ardito et al., 2012).

The HMG-box transcription factor SOX9 plays pleiotropic roles during embryonic development and is involved in various steps of pancreatic ontogenesis (Kopp et al., 2011). SOX9 expression is initially detected in pancreatic multipotent progenitors and later becomes restricted to the ductal and centroacinar cells. Recently, we observed that SOX9 is present in human PanIN and PDAC cells. In addition, we found that in inflammatory conditions, SOX9 is expressed in metaplastic acinar cells and contributes to ADM (Prevot et al., 2012), thereby suggesting a role of this factor in PDAC.

To address this hypothesis, we manipulated SOX9 levels in cultured cell lines and used transgenic mice models combining experimental pancreatitis, inducible expression of oncogenic Kras, and acinar-specific inactivation of Sox9 in adult pancreas. We found that Sox9 is required for PanIN formation by regulating the expression of Egfr and ErbB2, as well as the activity of the ErbB pathway. Therefore, our work establishes a functional link between two key players in PDAC tumorigenesis, namely SOX9 and EGFR signaling.

## 3. Results

### *a. Cerulein-induced inflammation induces expression of Sox9 in acinar cells and in PanIN*

As a first step to study the role of Sox9 in pancreatic tumorigenesis, we characterized the expression of this factor in *wild-type* (*WT*) mice and in mice expressing an oncogenic form of Kras ($Kras^{G12D}$) treated with cerulein (Hingorani et al., 2003). In the latter, induction of $Kras^{G12D}$ expression was restricted to adult acinar cells using a tamoxifen-inducible Cre recombinase driven by the elastase promoter

(Elastase CreER mice) (Desai et al., 2007). The effects of cerulein-induced inflammation were studied both in an acute and a chronic set-up (Figure 1A. See Methods for details).

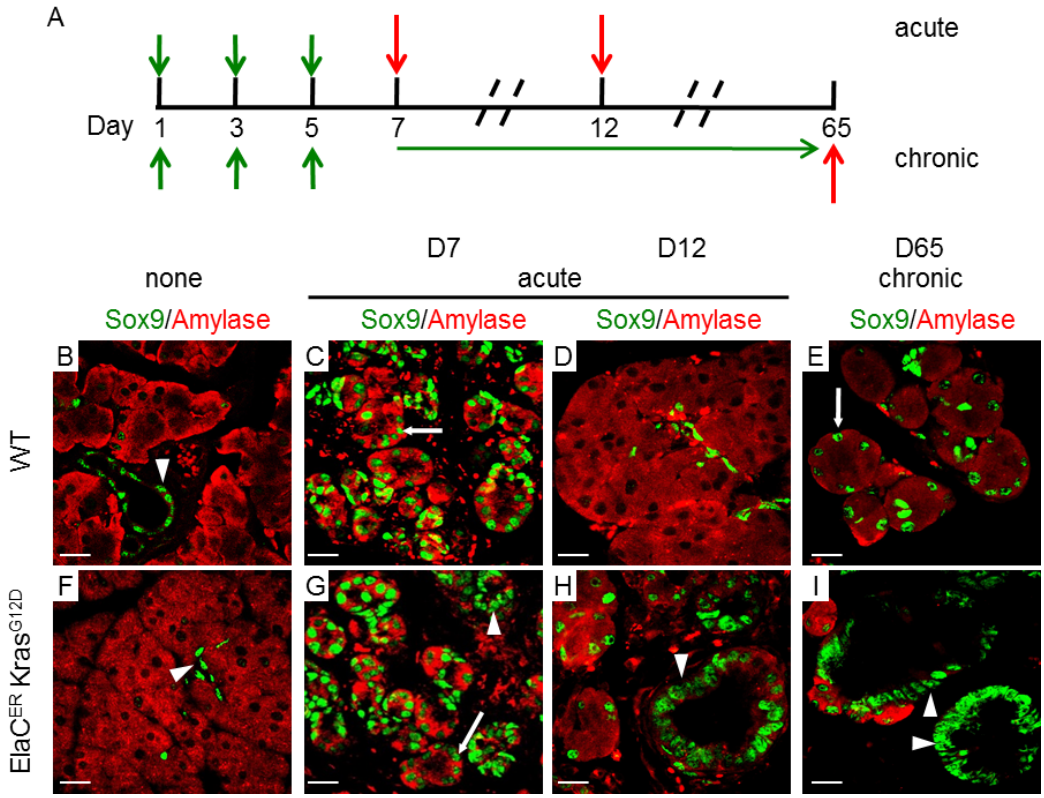


**Figure 1. Sox9 is expressed in metaplastic acini and PanIN.** (**A**) Scheme of the cerulein treatment. Six hourly intraperitoneal injections were performed every other day, for 5 days (vertical green arrow). In an acute set-up, pancreatic tissues were analyzed (red arrow) 7 (D7) and 12 days (D12) after the first injection. In a chronic setting, after the first week of treatment, the mice received one injection of cerulein per day, 5 days per week (horizontal green arrow), for 58 days, before tissue analysis (D65). (**B-I**) Immunofluorescent labeling of Amylase and Sox9 performed on *WT* and *ElaC*$^{ER}$ *Kras*$^{G12D}$ pancreas before (none) or after (D7, D12, D65) cerulein treatment. Without cerulein treatment, Sox9 expression is found in ducts (arrowhead, **B** and **F**). Cerulein treatment induces expression of Sox9 in metaplastic acinar cells of *WT* mice (arrow, **C**). In *WT* pancreas, expression of Sox9 is no longer seen in acinar cells at D12 during post-pancreatitis regeneration cells (**D**). In the chronic set-up, the expression of Sox9 is partially induced in *WT* pancreas (arrow, **E**). In *ElaC*$^{ER}$ *Kras*$^{G12D}$ mice, Sox9 expression is induced by cerulein in metaplastic acinar cells and in tubular complexes at D7 (arrow and arrowhead, respectively, **G**). Sox9 was also detected in PanIN at D12 and D65 (arrowhead, **H** and **I**). Scale bars = 50 $\mu$m.

In *WT* adult pancreas, expression of Sox9 was found in ductal and centroacinar cells; Sox9 was absent in most of the acinar cells, except for rare acinar cells expressing Sox9 at a low level (Figure 1B). 2 days after 5 days of cerulein treatment expression of Sox9 was detected in a large proportion of acinar cells (Figure 1C), like in pancreatitis induced by duct ligation (Prevot et al., 2012). When treatment was interrupted at day 5, regeneration took place at D12, and Sox9 was again restricted to

the ductal compartment (Figure 1D). In contrast, in the chronic set-up cerulein treatment was prolonged to day 65, and Sox9 expression was maintained in the acinar cells (Figure 1E).

In *Elastase CreER/LSL Kras*$^{G12D}$ (*ElaC*$^{ER}$ *Kras*$^{G12D}$) mice, in the absence of pancreatitis, the expression pattern of Sox9 was identical to that of *WT* mice (Figure 1F). In contrast, with cerulein, Sox9 was found at high level in most metaplastic acinar cells (Figure 1G) and this expression persisted for 7 days after the end of the cerulein injections (Figure 1H). Sox9 was also present in PanIN found in *ElaC*$^{ER}$ *Kras*$^{G12D}$ pancreata after chronic cerulein treatment. In the latter, Sox9 labeling was observed in most nuclei in the neoplastic lesions, but cytoplasmic expression was also occasionally detected (Figure 1I), in line with our earlier data in humans (Prevot et al., 2012). This expression pattern suggests that Sox9 plays a role in the formation of metaplastic and neoplastic lesions.

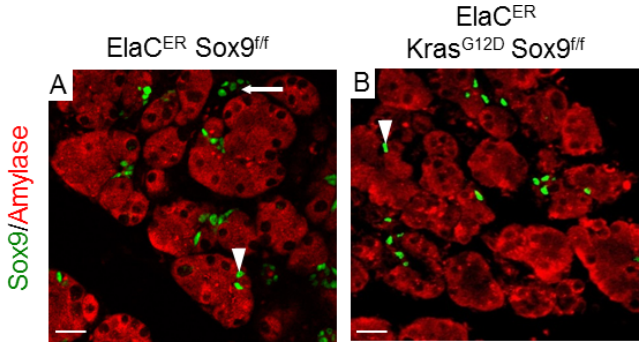


**Figure 2. Sox9 is efficiently deleted in acinar cells of cerulein-treated *ElaC*$^{ER}$ *Sox9*$^{f/f}$ and *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ mice.** (**A**, **B**). Immunofluorescent labeling of Sox9 and Amylase in *ElaC*$^{ER}$ *Sox9*$^{f/f}$ and *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ mice at D7 of acute pancreatitis. Sox9 is restricted to the duct (arrow) and centroacinar (arrowhead) cells. Scale bars = 50 $\mu$m.

### *b. Sox9 is required for PanIN formation*

To test the role of Sox9 in pancreatitis and at the onset of tumor formation, we inactivated the *Sox9* gene by crossing *ElaC*$^{ER}$ mice with mice bearing floxed alleles of *Sox9* (*ElaC*$^{ER}$ *Sox9*$^{f/f}$ mice) and treated the mice with tamoxifen to induce Cre recombinase activity (see materials and methods for details) (Kist et al., 2002). The mice were exposed to acute and chronic cerulein treatment as above. Immunolabeling revealed that Sox9 was efficiently depleted from acinar cells in *ElaC*$^{ER}$ *Sox9*$^{f/f}$ mice (Figure 2). At D7, pancreatitis was similar in mutant and *WT* mice: edema and inflammatory infiltrates were observed (Figure 3A and 3D). Regeneration was identical in *WT* and *ElaC*$^{ER}$ *Sox9*$^{f/f}$ mice at D12 (Figure 3B and 3E). In chronic pancreatitis, mild inflammation was found in both genotypes at D65 (Figure 3C and 3F). These results did not reveal any role of Sox9 in cerulein-driven pancreatitis.

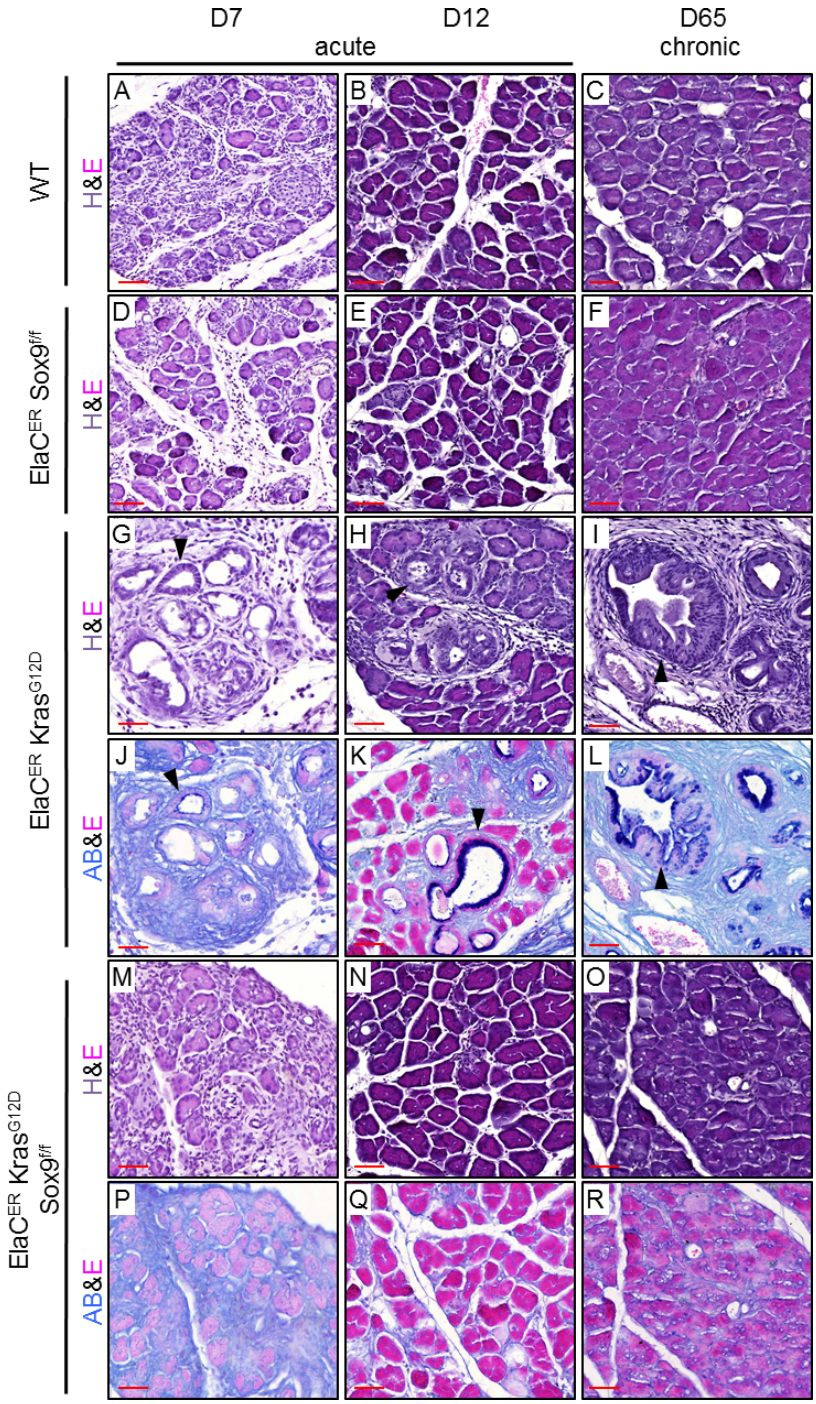


**Figure 3. PanIN do not develop in *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreas.** (**A**-**R**) Haematoxylin-eosin (H&E) staining was performed in *WT* (**A**-**C**), *ElaC$^{ER}$ Sox9$^{f/f}$* (**D**-**F**), *ElaC$^{ER}$ Kras$^{G12D}$* (**G**-**I**), and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* (**M**-**O**) mice at D7, D12 and D65. Alcian blue and eosin (AB&E) staining was performed in *ElaC$^{ER}$ Kras$^{G12D}$* (**J**-**L**) and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* (**P**-**R**) mice at D7, D12 and D65. At D7, inflammation is observed in all the genotype (**A**, **D; G; M**). Cerulein treatment induces tubular complex formation in *ElaC$^{ER}$ Kras$^{G12D}$* pancreas (arrowhead, **G**); at this stage these complexes are either negative or only weakly positive (arrowhead) for AB staining (**J**). In *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreas, inflammation is present (**M**) but no AB staining is observed (**P**). At D12, acinar regeneration is observed in mice of the different genotypes (**B**, **E**, **H**, **N**) but PanIN lesions are only present in *ElaC$^{ER}$ Kras$^{G12D}$* mice (arrowhead, **H** and **K**). In the chronic set-up, mild inflammation is seen in *WT*, *ElaC$^{ER}$ Sox9$^{f/f}$* and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreata (**C**, **F**, **O**) whereas in the ElaC$^{ER}$ Kras$^{G12D}$ mice, inflammation persists and PanIN develop (arrowhead, **I**, **L**). No PanIN lesion is observed in *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* mice (**R**). Scale bars = 50 $\mu$m.

The lack of obvious role of Sox9 in pancreatitis does not rule out that this factor is involved in development of neoplastic lesions. To address this issue, cerulein was injected in *ElaC$^{ER}$ Kras$^{G12D}$* and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* mice, and PanIN development was compared between both genotypes. In *ElaC$^{ER}$ Kras$^{G12D}$* mice, 7 days of acute cerulein treatment induced ADM with formation of tubular complexes, some of which being slightly positive for Alcian blue staining (Figure 3G). The latter detects intestinal mucins expressed ectopically in pancreatic neoplastic lesions (Figure 3J). In these mice, acinar tissue had regressed and edema and inflammatory infiltrates were amplified as compared to *WT* mice treated with cerulein. At D12, regeneration was less efficient (Figure 3H) and Alcian blue-positive PanIN lesions were observed (Figures 3K and 4A). In chronic pancreatitis at D65, a large number of neoplastic lesions, ranging from PanIN1A to PanIN3, were detected in *ElaC$^{ER}$ Kras$^{G12D}$* pancreata (Figure 3I and 3L).

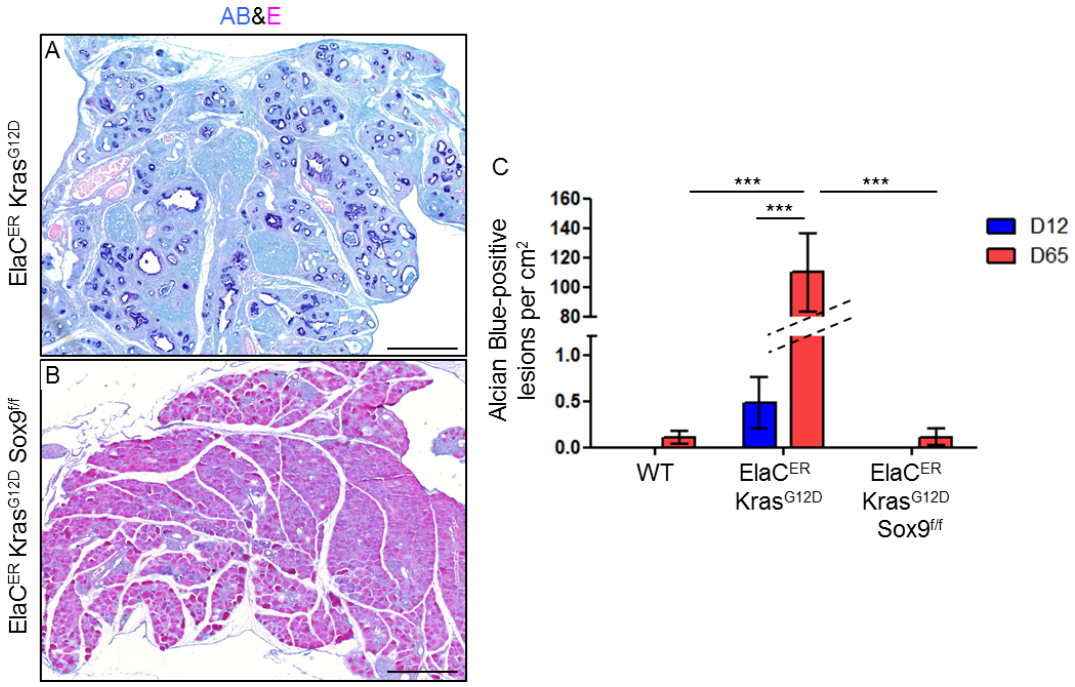


**Figure 4. Quantification of Alcian blue-positive lesions in acute and chronic pancreatitis.** (**A**, **B**) Low magnification pictures of Alcian blue and Eosin (AB&E) staining on sections of *ElaC$^{ER}$ Kras$^{G12D}$* (**A**) and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* (**B**) pancreas at D65. (**C**) Alcian blue-positive lesions were quantified by dividing the number of Alcian blue-positive lesions in randomly chosen pictures of *WT*, *ElaC$^{ER}$ Kras$^{G12D}$* and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* mice at D12 and D65 by the surface of the corresponding pancreatic tissue. Data are means ± SEM (two-way ANOVA, F[2,22]=14,8, p<0.001 with Bonferroni posttest p<0.001, n=4). Scale bars = 500 $\mu$m.

Strikingly, in *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreata, no Alcian blue-positive lesion was observed at any time (Figures 3P-R and 4B). At D7, edema and inflammatory infiltrates (Figure 3M) were similar to those seen in *WT* inflamed pancreata. At D12, the extent of regeneration was also similar in both genotypes (Figure 3N). Finally, even when cerulein was injected for 65 days, virtually no PanIN lesion was observed in *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreata (Figure 3O). Quantification of the number of PanIN lesions in *WT*, *ElaC$^{ER}$ Kras$^{G12D}$*, and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreata confirmed the interpretation of the data (Figure 4C).

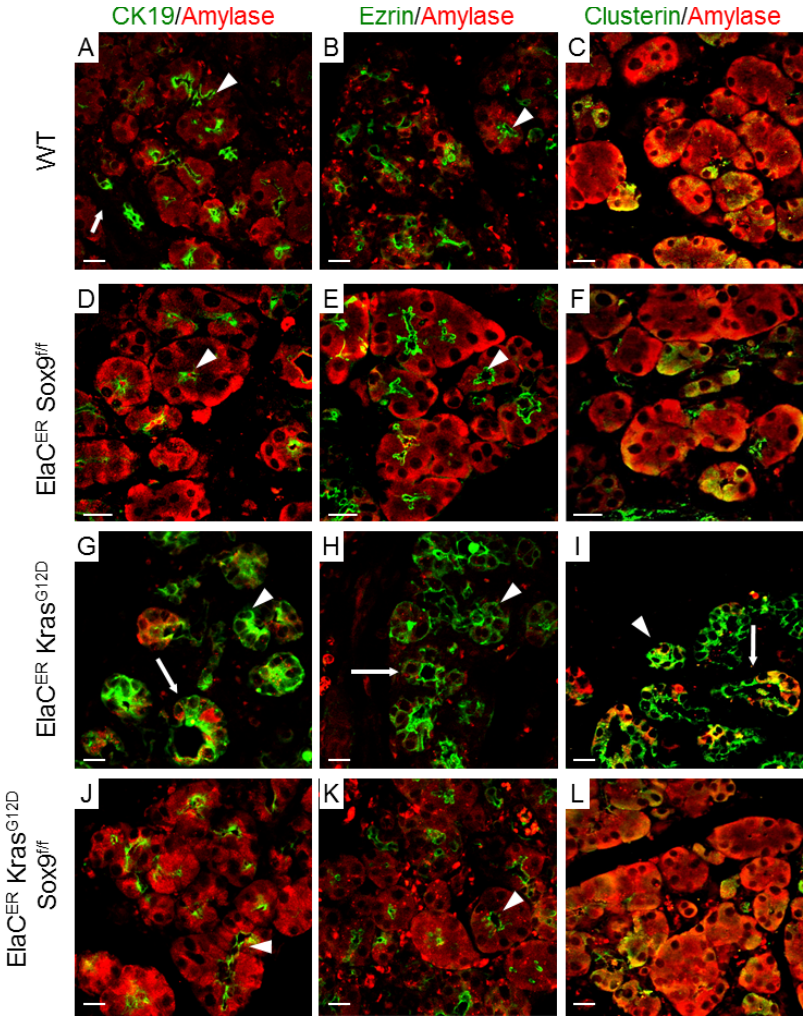


Figure 5. **Metaplastic markers are not induced in *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreas at day 7 of acute pancreatitis.** (**A-L**) Immunofluorescent labeling of Cytokeratin (CK) 19, Ezrin, Clusterin, and Amylase in *WT* (**A-C**), *ElaC$^{ER}$ Sox9$^{f/f}$* (**D-F**), *ElaC$^{ER}$ Kras$^{G12D}$* (**G-I**), and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* (**J-L**) pancreata at D7. In *ElaC$^{ER}$ Kras$^{G12D}$* pancreas, CK19, Ezrin, and Clusterin expression is seen in the cytoplasm of metaplastic acinar cells (arrowhead, **G, H, I**) and in tubular complexes (arrow, **G, H, I**). Compared to *ElaC$^{ER}$ Kras$^{G12D}$* pancreas, the extent of metaplasia is limited in *WT, ElaC$^{ER}$ Sox9$^{f/f}$* and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreas. After cerulein treatment, in *WT* pancreas, expression of CK19 is seen not only in duct cells (arrow, **A**) but also at the apical pole of the acinar cells (arrowhead, **A**). Apical expression of CK19 is also detected in acinar cells of *ElaC$^{ER}$ Sox9$^{f/f}$* (arrowhead, **D**) and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreas (arrowhead, **J**). Ezrin expression is restricted to apical pole of the acinar cells and ducts cells in *WT, ElaC$^{ER}$ Sox9$^{f/f}$* and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreas (arrowhead, **B**, **E**, **K**). Clusterin expression is mainly ductal in these genotypes (**B**, **C**, **E**). Scale bars = 50 $\mu$m.

To study if Sox9 controls the emergence of a metaplastic program in the acinar cells of *ElaC$^{ER}$ Kras$^{G12D}$* mice, we tested the expression of metaplastic markers at D7 of acute pancreatitis. In *WT* untreated pancreas, expression of Cytokeratin (CK) 19 and Clusterin was restricted to ducts whereas expression of Ezrin was found both in duct cells and at the apical pole of the acinar cells (Morris et al., 2010; Prevot et al., 2012). After cerulein treatment, *WT* mice showed a slight induction in CK19 and Clusterin in

the acini, as well as increase of Ezrin in the cytoplasm of the acinar cells (Morris et al., 2010) (Figure 5A-C). This did not differ in cerulein-treated *ElaC$^{ER}$ Sox9$^{f/f}$* (Figure 5D-F). In contrast, *ElaC$^{ER}$ Kras$^{G12D}$* pancreas treated with cerulein showed elevated expression of CK19, Clusterin and Ezrin and reduced expression of Amylase in the cytoplasm of metaplastic acinar cells and tubular complexes (Figure 5G-I). In *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreas, however, Amylase expression was maintained and the expression patterns of CK19, Ezrin, and Clusterin looked similar to that in cerulein-treated *ElaC$^{ER}$ Sox9$^{f/f}$* and *WT* pancreas (Figure 5J-L). We concluded that the extent of the metaplasia driven by Kras is limited in the absence of Sox9.

Together, these results indicate that Sox9 is required for the initiation and the development of neoplastic lesions driven by oncogenic Kras in cerulein-induced pancreatic inflammation.

### *c. Sox9 is required for expression of members of the ErbB pathway in the presence of oncogenic Kras*

We next tried to identify the mechanism of action of Sox9 in pancreatic cancer initiation. Mouse pancreata lacking Egfr in an oncogenic Kras background are resistant to ADM and PanIN development (Ardito et al., 2012; Navas et al., 2012), suggesting that lack of Egfr or Sox9 exerts similar effects and that Sox9 acts through the ErbB pathway. Moreover, gene expression analysis performed on a large collection of human PDAC samples indicated that high expression of SOX9 correlated with high expression of different members of this pathway (Ilse Rooman, personal communication. See Appendix I, for details), including ERBB2, EGFR, and ERBB2 interacting protein (ERBB2IP) also known as ERBIN, a protein important for ERBB2 activity (Borg et al., 2000).

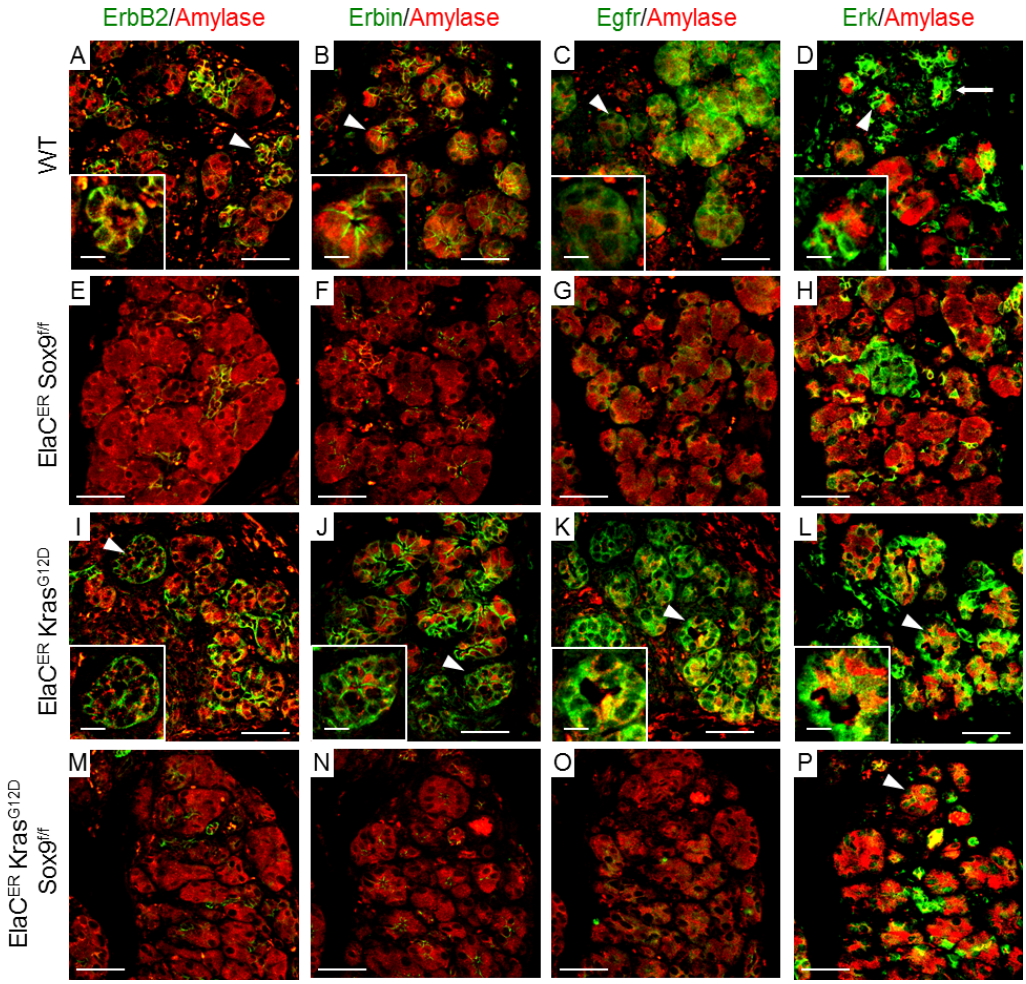


**Figure 6. Sox9 controls expression of members of the ErbB pathway during acute pancreatitis**. (**A-P**) Immunofluorescent labeling of Amylase, ErbB2, Erbin, Egfr, and Erk in *WT* (**A-D**), *ElaC$^{ER}$ Sox9$^{f/f}$* (**E-H**), *ElaC$^{ER}$ Kras$^{G12D}$* (**I-L**), and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* (**M-P**) mice at D7 of acute pancreatitis. The expression of ErbB2, Erbin, Egfr, and Erk is strongly induced in metaplastic acini and tubular complexes in the *ElaC$^{ER}$ Kras$^{G12D}$* pancreas (**I-L**) while it is only weakly induced in the *WT* metaplastic acinar cells (**A-D**). In *ElaC$^{ER}$ Sox9$^{f/f}$* (**E-H**) and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* (**M-P**) pancreas, this expression is restricted to the apical pole of the acinar cells for Erbin (**F**, **N**) or to the ducts, for ErbB2 (**E**, **M**) and Egfr (**G**, **O**). In both genotypes, Erk expression is weakly observed in acinar cells (**H**, **P,** arrowhead). Scale bars = 50 $\mu$m.

From these data, we hypothesized that expression of these proteins could be reduced in *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* mice and tested their expression in the mouse models by immunofluorescent labeling. The experiments also included labeling of Erk, a downstream target of the ErbB pathway important for PanIN development, and also a potential target of Sox9 (Ardito et al., 2012; Oh et al., 2010). In normal pancreas, these proteins were not detectable in acinar cells whereas they were weakly expressed in duct and centroacinar cells (data not shown). At D7 of pancreatitis, some *WT* metaplastic acini expressed low levels of ErbB2 and Erk (Figure 6A and D) whereas expression of Erbin and Egfr was seen in a larger proportion of metaplastic acini (Figure 6B-C). Strong induction of ErbB2, Erbin, Egfr, and Erk was seen in *ElaC$^{ER}$ Kras$^{G12D}$* mice; typically, their expression was detected in metaplastic acini showing

decreased expression of Amylase and in tubular complexes (Figure 6I-L). Importantly, ErbB2 labeling distinguished between duct and acinar-derived tubular complex, since Amylase was absent in the former and low in the latter (Figure 7A-C). In *ElaC$^{ER}$ Sox9$^{f/f}$* and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* pancreas, only weak ductal expression of ErbB2 and Egfr was seen and weak acinar expression of Erbin and Erk was detected (Figures 6E-H and 6M-P). At D12 and D65, expression of ErbB2, Erbin, Egfr, and Erk was mainly restricted to the ductal compartment in the various genotypes, with the notable exception of *ElaC$^{ER}$ Kras$^{G12D}$* mice in which the proteins were expressed at high levels in developing PanIN and in a subset of metaplastic acini (Figure 7D-F, 8A-P and 9A-P).

These experiments show that Sox9 is required to stimulate expression of ErbB signaling components during Kras-induced tumorigenesis.

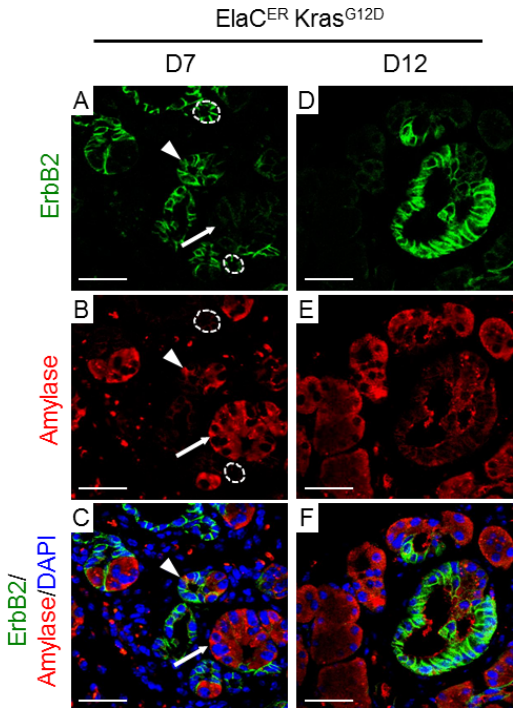


**Figure 7. Expression pattern of ErbB2 in *ElaC$^{ER}$ Kras$^{G12D}$* pancreas.** (**A-F**) Immunofluorescent labeling for Amylase and ErbB2 in *ElaC$^{ER}$ Kras$^{G12D}$* pancreas at D7 (**A-C**) and D12 of acute pancreatitis (**D-F**). Ducts (dotted lines) express ErbB2 but are devoid of Amylase. Acini in which Amylase is not reduced express only ErbB2 at a very low level (arrow) whereas acini in which Amylase is reduced show ErbB2 expression (arrowhead) (**A-C**). PanIN express ErbB2 at high levels and Amylase at low, but still detectable, levels (**D-F**). Scale bars = 50 $\mu$m.

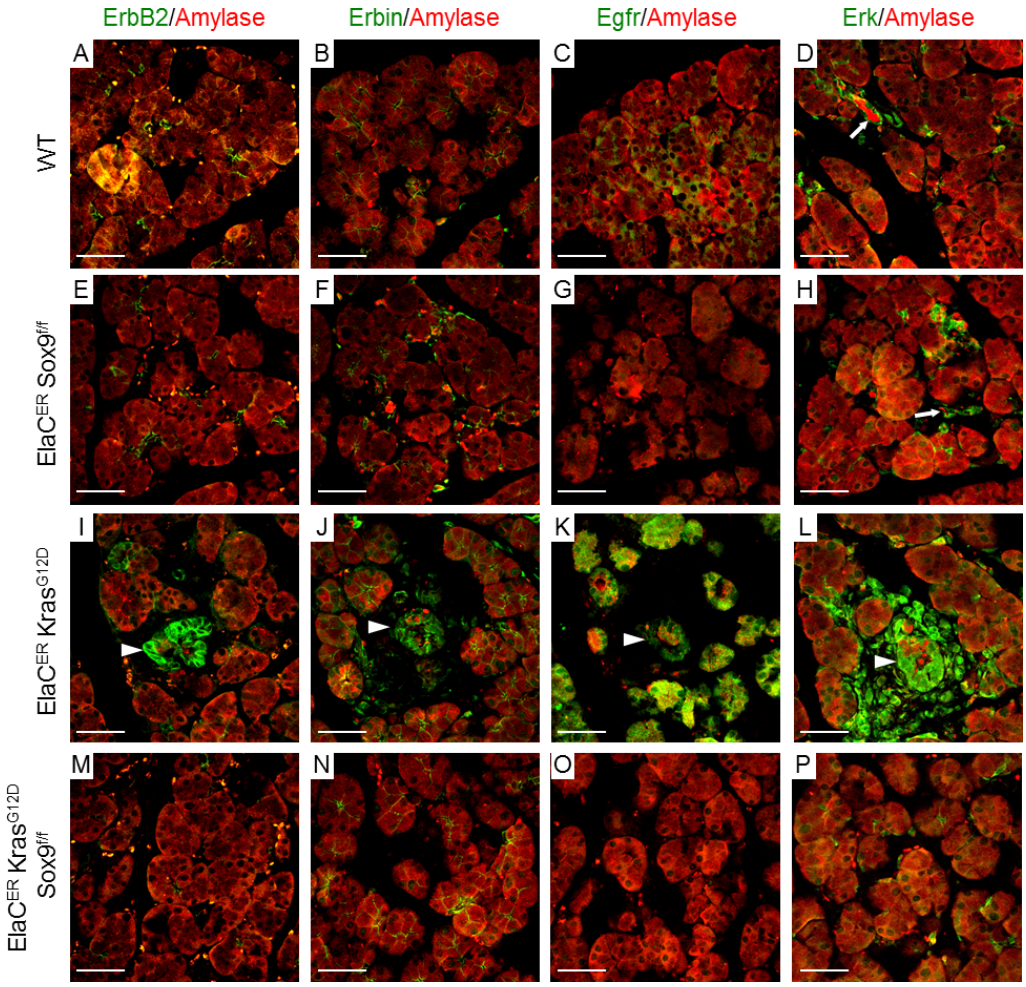


**Figure 8. Expression of members of the ErbB pathway is reduced during post-pancreatitis regeneration** (**A-P**) Immunofluorescent labeling of Amylase, ErbB2, Erbin, Egfr, and Erk in *WT* (**A-D**), *ElaC$^{ER}$ Sox9$^{f/f}$* (**E-H**), *ElaC$^{ER}$ Kras$^{G12D}$* (**I-L**), and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* (**M-P**) mice at D12. High expression of ErbB2, Erbin, Egfr, and Erk is observed in PanIN (arrowhead) in the *ElaC$^{ER}$ Kras$^{G12D}$* pancreas (**I-L**) while it is restricted to the ductal cells (arrow) in the *WT* pancreas (**A-D**), *ElaC$^{ER}$ Sox9$^{f/f}$* (**E-H**), and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* (**M-P**) pancreas. Scale bars = 50 $\mu$m.

### *d. Reduced ErbB pathway activity contributes to the absence of PanIN in ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$ mice*

The reduced expression of ErbB components in *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* mice suggests that this pathway has little or no activity in these mice. Phosphorylation of Egfr and Erk is associated with activation of the ErbB pathway and was taken for further investigation of this pathway activity. In the absence of pancreatitis, weak staining for P-Egfr and P-Erk was only detected in the ducts of the tested genotypes (data not shown). At D7 of acute pancreatitis, in *WT* mice, P-Egfr and P-Erk were detected in a subset of metaplastic acinar cells (Figure 10A-B). In *ElaC$^{ER}$ Sox9$^{f/f}$* mice, the phosphorylated forms were not detected in the acinar compartment (Figure 10E-F). In contrast, in *ElaC$^{ER}$ Kras$^{G12D}$* mice, strong levels of P-Egfr and P-Erk were found in

metaplastic acini and tubular complexes (Figures 10I-J). In *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ mice, no P-Egfr and weak level of P-Erk were detected in acinar cells (Figure 10M-N). In the chronic setup, at D65, Erk was weakly phosphorylated in *WT*, *ElaC*$^{ER}$ *Sox9*$^{f/f}$ and *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ mice, whereas high level of phosphorylated Erk was seen in PanINs of *ElaC*$^{ER}$ *Kras*$^{G12D}$ mice. P-Egfr was detected only in PanINs of *ElaC*$^{ER}$ *Kras*$^{G12D}$ mice (Figure 10K-L).

These data indicate that P-Egfr, and P-Erk are strongly reduced in the absence of Sox9 and suggest that the activity of the EGFR pathway is Sox9-dependent.

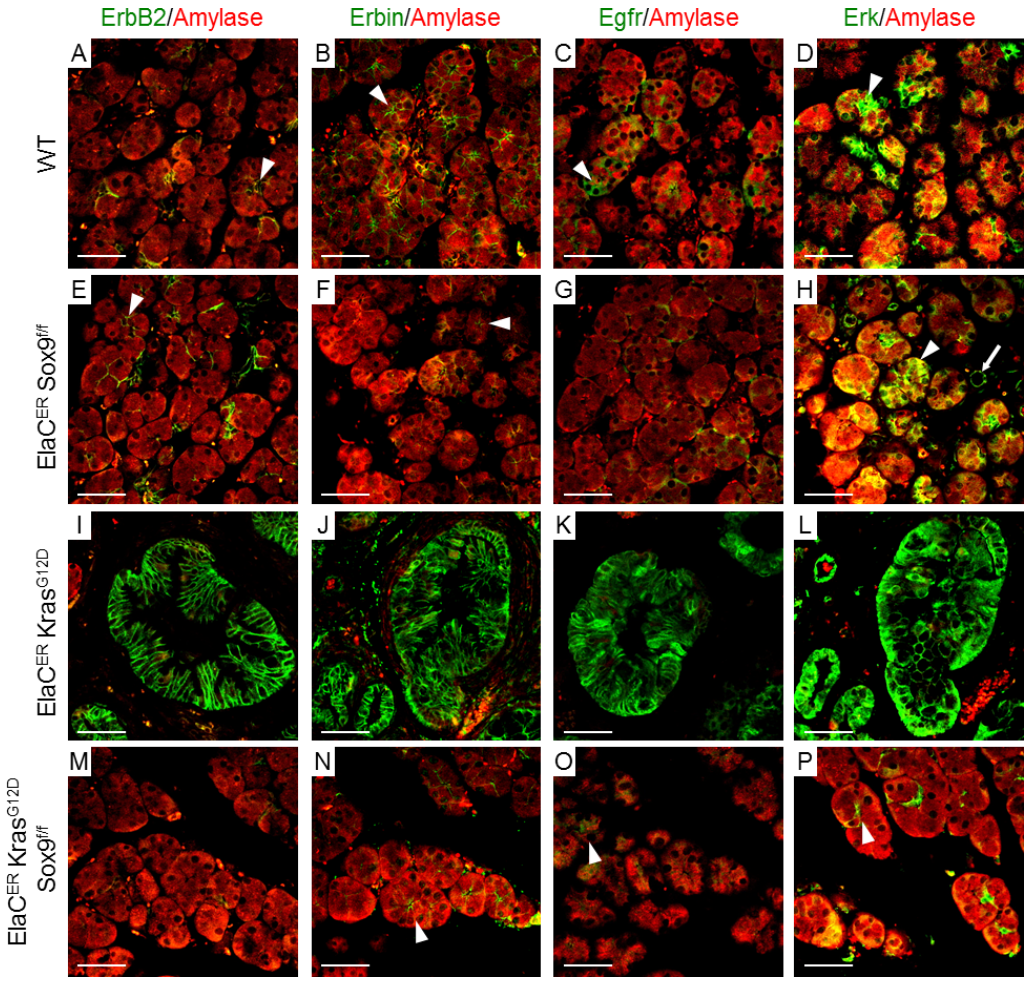


**Figure 9. Sox9 controls expression of members of the ErbB pathway in chronic pancreatitis**. (**A-P**) Immunofluorescent labeling of Amylase, ErbB2, Erbin, Egfr, and Erk in *WT* (**A-D**), *ElaC*$^{ER}$ *Sox9*$^{f/f}$ (**E-H**), *ElaC*$^{ER}$ *Kras*$^{G12D}$ (**I-L**), and *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ (**M-P**) mice at D65. High expression of ErbB2, Erbin, Egfr, and Erk is observed in PanIN in the *ElaC*$^{ER}$ *Kras*$^{G12D}$ pancreas (**I-L**) while it is mainly observed in the ductal cells in the *WT* pancreas (**A-D**), *ElaC*$^{ER}$ *Sox9*$^{f/f}$ (**E-H**), and *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ (**M-P**) pancreas; arrowheads point to weak expression of Erbb2, Erbin, Egfr and Erk in acinar cells (A-F, H, N-P). Scale bars = 50 $\mu$m.

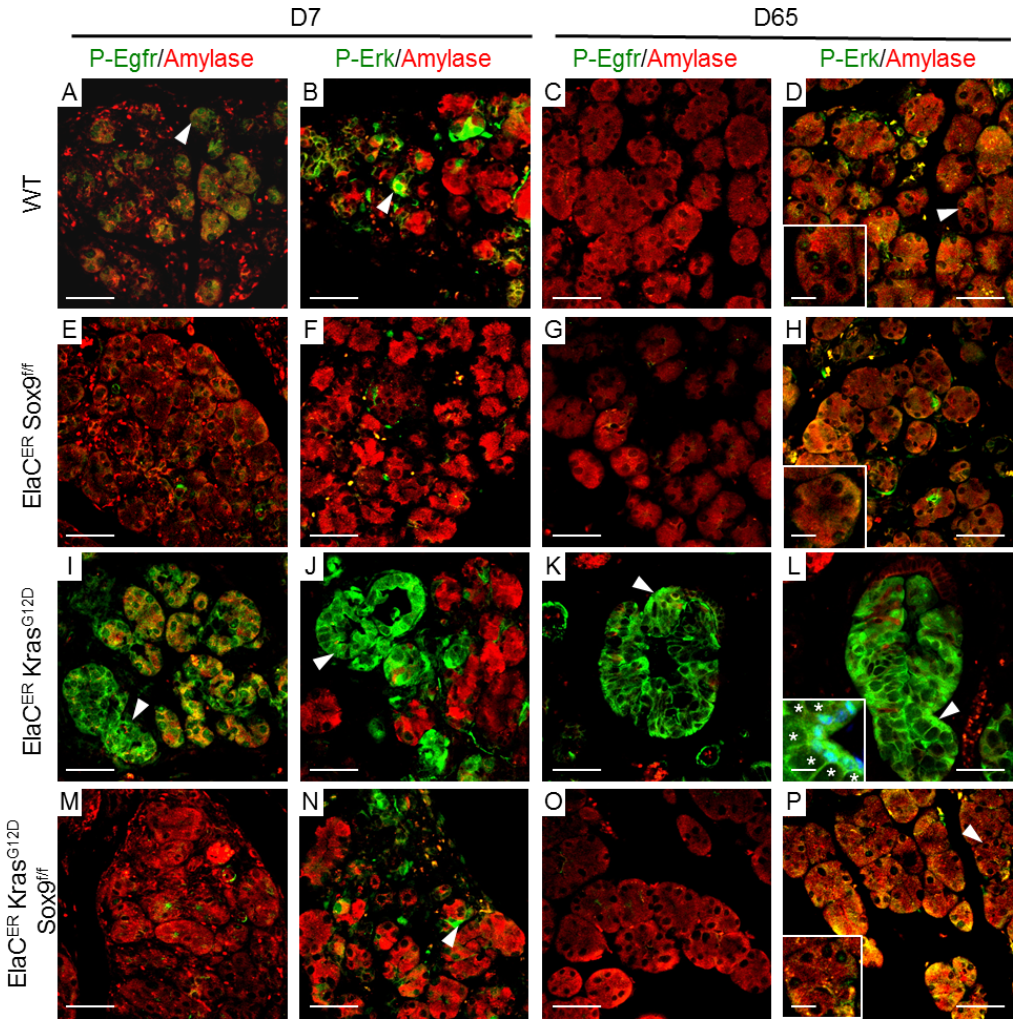


**Figure 10. The absence of Sox9 reduces EGFR signaling activity during pancreatitis**
(**A**-**P**) Immunofluorescent labeling for Amylase, P-Egfr, and P-Erk in *WT*, *ElaC*$^{ER}$ *Sox9*$^{f/f}$, *ElaC*$^{ER}$ *Kras*$^{G12D}$ and *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ mice at D7 of acute pancreatitis and D65 of chronic pancreatitis. At D7, the activity of Egfr and Erk is strongly induced in tubular complexes in *ElaC*$^{ER}$ *Kras*$^{G12D}$ mice (**I**-**J**) whereas this activity is reduced in *WT* (**A**-**B**), *ElaC*$^{ER}$ *Sox9*$^{f/f}$ (**E**-**F**) and *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ (**M**-**N**) mice; arrowheads point to acini showing P-Egfr and P-Erk labeling (A, **B**, **I**, **J**, **N**). At D65, the activity of Egfr and Erk is elevated in PanIN (**K**-**L**) in *ElaC*$^{ER}$ *Kras*$^{G12D}$ mice whereas the EGFR pathway is strongly reduced or inactive in *WT*, *ElaC*$^{ER}$ *Sox9*$^{f/f}$ and *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ mice (**C**, **D**, **G**, **H**, **O**, **P**). Insets show magnifications that reveal P-Erk labeling in nuclei: low levels of P-Erk were observed in nuclei of *WT*, *ElaC*$^{ER}$ *Sox9*$^{f/f}$ and *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ metaplastic acinar cells (C, D, F), in comparison to the high levels of P-Erk present in the nuclei of *ElaC*$^{ER}$ *Kras*$^{G12D}$ PanIN cells (labeled in blue by DAPI staining) (E). Star: mucin vesicle. Scale bars = 50 $\mu$m.

To further test the function of the EGFR pathway in the absence of Sox9, pancreatic cell clusters, obtained by enzymatic digestion, were embedded in collagen gel and cultured in the absence or in the presence of EGF. In such conditions, acinar cells undergo a metaplastic change and convert into duct-like cysts in an EGF-dependent process (Means et al., 2005). In the absence of EGF, we observed a few small cysts dispersed among clusters of acinar cells in the *WT* and *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ tissue culture, whereas larger cysts formed in cultured *ElaC*$^{ER}$ *Kras*$^{G12D}$ tissue (Figure 5A, C, E). This confirmed the propensity of oncogenic Kras to produce

metaplastic structures in these culture conditions (Shi et al., 2013). When EGF was exogenously added to the culture, the size of cysts increased in *WT* cultures and this process was exacerbated in *ElaC*$^{ER}$ *Kras*$^{G12D}$ culture (Figure 5A-D). In contrast, in *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ cultures, the size of cysts did not increase (Figure 5E-F). From these tissue culture experiments, we conclude that lack of Sox9 abrogates EGF response in a *Kras*$^{G12D}$ background.

All together, these experiments indicate that the ErbB pathway is inactive in *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ pancreas and that Sox9 stimulates the ErbB pathway in Kras-induced ADM. Thus, a deficiency in the ErbB signaling most probably explains the absence of PanIN in *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ pancreas.

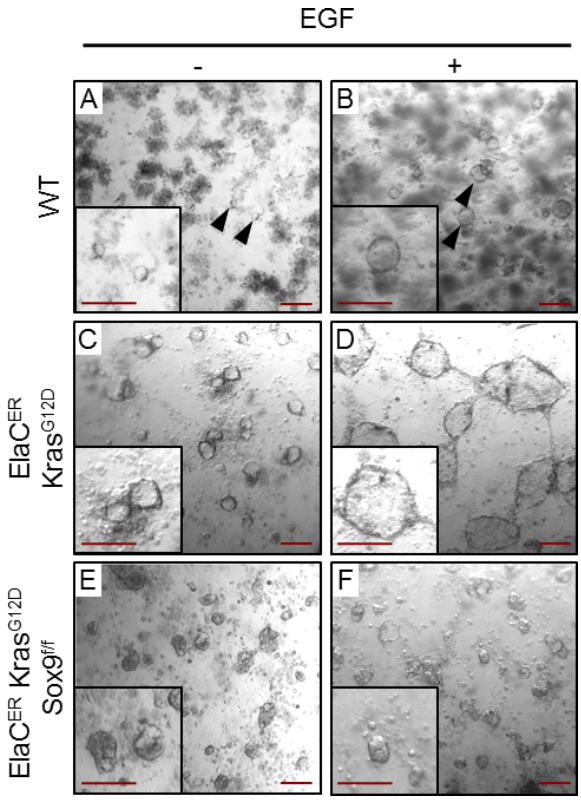


**Figure 11. The formation of EGF-induced duct-like cysts in collagen cultures of *ElaC*$^{ER}$ *Kras*$^{G12D}$ pancreata is inhibited in the absence of Sox9.** (**A-F**) Digested pancreata is cultured in the absence (-) or in the presence (+) of EGF. Phase contrast microscopy shows cyst formation in acinar cell cultures. In cultures of WT pancreas, the size of cysts is slightly increased after addition of EGF (arrowheads, **A**-**B**) requires addition of EGF in cultures of WT pancreas. Cysts spontaneousy form in *ElaC*$^{ER}$ *Kras*$^{G12D}$ pancreas, and are strongly enlarged after addition of EGF (**C**-**D**). The absence of Sox9 in *ElaC*$^{ER}$ *Kras*$^{G12D}$ *Sox9*$^{f/f}$ culture prevents EGF-mediated cyst enlargement (**E**-**F**). Magnifications of cysts are shown in the insets. Scales Bars = 50 $\mu$m.

### *e. ERBB2 is required for pancreatic cancer cell growth*

To deepen the relation between Sox9 and ERBB2, we manipulated both pancreatic cancer HPAF2 cell lines and murine PanIN cell lines (Hingorani et al., 2005). Downregulation of ERBB2 expression by stably transducing both cell lines with ERRB2 targeting shRNA did not affect the protein expression of SOX9. In contrast, stably infecting HPAF2 cells with shRNA for SOX9 resulted in less ERBB2 expression (21%) as compared to a control scramble shRNA (Figure 12A). Expression of ERBIN and ERK was not affected in the absence of SOX9. Interestingly EGFR expression tends to decrease in shSOX9 and shERBB2 HPAF2 cells while in the murine PanIN cell line, expression of EGFR is increased in the absence of ERBB2. Several compensatory mechanisms could explain these differences with the mouse models.

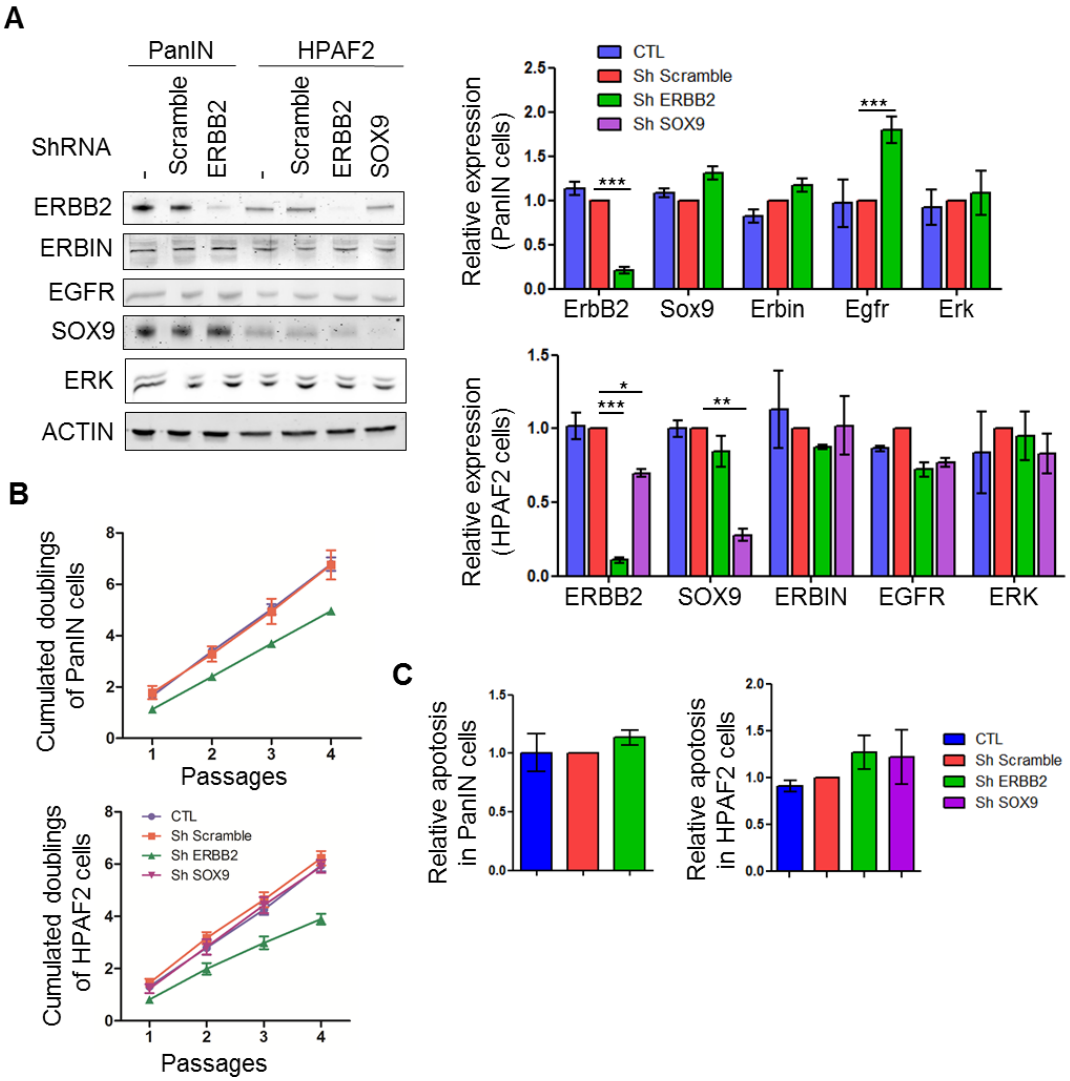


**Figure 12. SOX9 controls the expression of ERBB2, a stimulator of pancreatic cancer cell growth.** SOX9 and ERBB2 shRNAs were used to reduce the expression of SOX9 and ERBB2 in PanIN and HPAF2 cells (**A**-**C**). Western blots were performed to detect (left) and to quantify (right) the expression of SOX9, ERBB2, EGFR, ERBIN, and ERK. The shRNAs led to an important reduction of expression of the corresponding proteins. Reduction of Sox9 expression was associated with a reduction of ERBB2 expression (Bonferroni test *** $p<0.001$, ** $p<0.01$, n=3 and * $p< 0.05$ n=6) (**A**). Reduced expression of ERBB2 decreased growth of PanIN and HPAF2 cells (Bonferroni test *** $p<0.001$ at passages 4, n=3) (**B**). Reduced expression of SOX9 or ERBB2 did not cause increased apoptosis, as shown using an Annexin V Apoptosis Detection assay (n=3) (**C**).

Next we studied how ERBB2 influences growth of pancreatic cancer cell lines. To this end, ERBB2 shRNA was used in HPAF2 cells and in murine PanIN cells. Reduced expression of ERBB2 (89% and 80% in HPAF2 and PanIN cells, respectively) was observed (Figure 12A); this was associated with a significant decrease in cell growth in both lines (Figure 12B). In contrast, the downregulation of SOX9 by shRNA in HPAF2 do not modify the cell growth rate (Figure 12B). Lower growth rate was not associated with an increased cell apoptosis (Figure 12C). These experiments confirm that SOX9 controls the expression of ERBB2 and indicate that ERBB2 is important for pancreatic cancer cell growth.

*f. Sox9 is dispensable for development of PanIN when the EGFR pathway is constitutively active.*

The EGFR pathway is required for the development of PDAC, and can be activated by TGFα ligand (Ardito et al., 2012; Navas et al., 2012). To further investigate the link between Sox9 and the EGFR pathway, we turned to the *MT-TGFα* mouse model, which expresses TGFα under control of the metallothionein-1 (MT) promoter when zinc is provided ad libitum in drinking water (Sandgren et al., 1990). MT is produced in pancreatic exocrine cells and its expression increases during pancreatitis (Milnerowicz et al., 2004). In *WT* adult mice treated during 6 weeks with zinc, no phenotype was observed, whereas *MT-TGFα* pancreases showed fibrosis and PanIN (Figure 13). In the absence of Sox9 (*ElaC Sox9$^{f/f}$ MT-TGFα* mice), the pancreas was also fibrotic, and PanIN lesions negative for Sox9 were detected (Figure 13). This indicates that ectopic activation of the ERBB pathway can compensate by itself for the lack of Sox9, and suggests that the role of Sox9 on PanIN development is not dependent on another unidentified pathway.

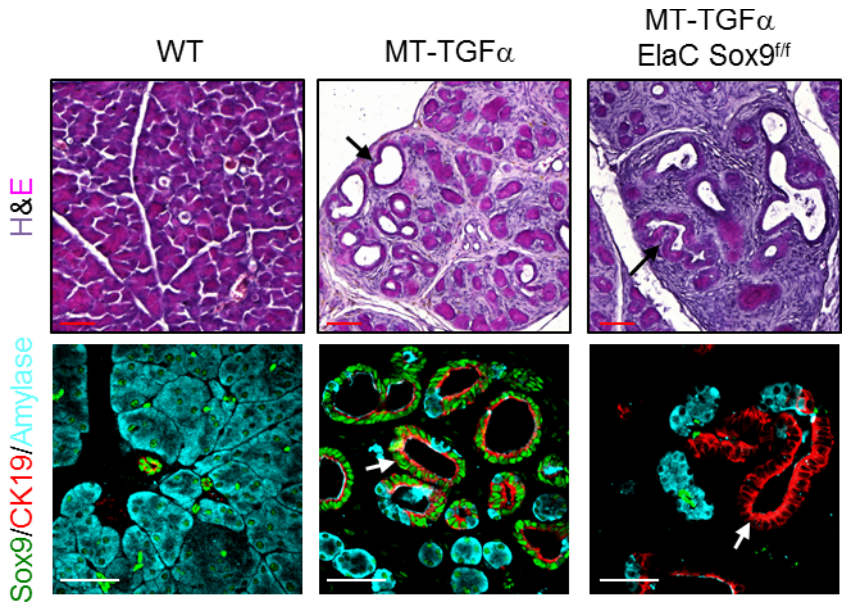


**Figure 13. Sox9 is dispensable for PanIN formation when the EGFR pathway is constitutively active.** Haematoxylin-eosin (H&E) and immunofluorescent labeling Sox9, CK19 and Amylase in *WT*, *MT-TGFα* and *ElaC Sox9$^{f/f}$ MT-TGFα* adult mice after 6 weeks of zinc treatment. After zinc treatment, no phenotype is observed in WT pancreas while fibrosis and several PanIN are found in *MT-TGFα* and *ElaC Sox9$^{f/f}$ MT-TGFα* mice (dark and white arrow). In the former, PanIN express both Sox9 and CK19 while in the latter they express CK19 but not Sox9. Scale bars = 50 μm.

### g. *Inflammatory response is reduced in ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$ mice*

Development of Kras$^{G12D}$-driven PanIN is associated with edema and inflammatory infiltrates in the injured pancreas: macrophages and T lymphocytes are the two main leukocyte types in the infiltrates (Guerra et al., 2011). To characterize the inflammatory response in the *WT*, *ElaC$^{ER}$ Kras$^{G12D}$* and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* mice, we first quantified the surface covered by edema in sections of pancreata. Seven days after beginning cerulein treatment (D7), this surface was about 2-fold larger in *ElaC$^{ER}$ Kras$^{G12D}$* mice than in the other two genotypes (Figure 14A). We then quantified the number of T lymphocytes and macrophages and found that their number was again 2.3- and 5.3- fold higher respectively in *ElaC$^{ER}$ Kras$^{G12D}$* pancreas than in *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* mice (Figure 14B and C). These differences increased when the inflammatory process was sustained over a longer period (data not shown).

These results indicate that Sox9 participates to development of inflammation in a mutant Kras background and to the recruitment of inflammatory cells in the initial steps of pancreatic neoplasia.

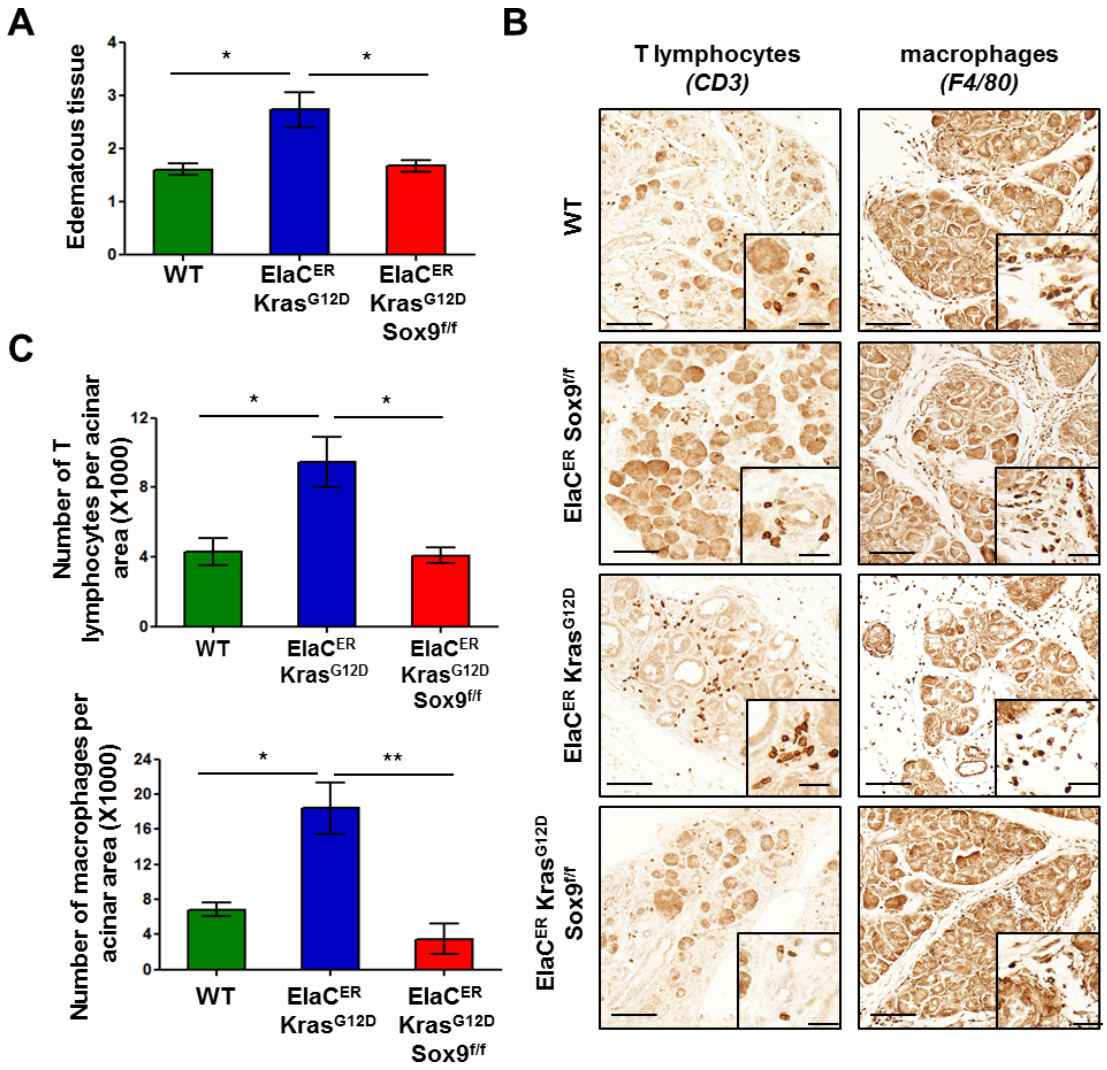


**Figure 14. Sox9 contributes to the Kras-induced recruitment of inflammatory cells.** Quantification of the edematous tissue in *WT*, *ElaC$^{ER}$Kras$^{G12D}$* and *Kras$^{G12D}$ Sox9$^{f/f}$* mice. Data are means ± SEM (One-way ANOVA, F[2,8]=8,9, p<0.05 with Tukey's Multiple comparison posttest, p<0.05, n=3) (**A**). Immunohistochemistry labeling of T lymphocytes (CD3) and macrophages (F4/80) in *WT*, *ElaC$^{ER}$ Sox9$^{f/f}$*, *ElaC$^{ER}$ Kras$^{G12D}$* and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* mice, at seven days after beginning cerulein treatment (D7). Magnifications are shown in the insets. Scales bars = 100 *μ*m and 25 *μ*m for high magnification (**B**). Quantification of the number of T lymphocytes and macrophages per acinar area in *WT*, *ElaC$^{ER}$ Kras$^{G12D}$* and *ElaC$^{ER}$ Kras$^{G12D}$ Sox9$^{f/f}$* mice, at D7. Data are means ± SEM (One-way ANOVA, F[2,8]=9,5, p<0.05, with Tukey's Multiple comparison posttest, p<0.05, n=3; One-way ANOVA, F[2,8]=14,9, p<0.05, with Tukey's Multiple comparison posttest, p<0.05, n=3) (**C**).

## 4. Discussion

Identifying new pancreatic oncogenes and characterizing their oncogenic properties is essential to better understand pancreatic tumorigenesis and to develop new therapeutic strategies. Transgenic mouse models recently revealed that PDAC originates from acinar cells that undergo ADM, prompting the need for proper

understanding of ADM (Kopp et al., 2012). In the previous chapter, we hypothesized that ductal transcription factors ectopically expressed in metaplastic acinar cells control this process and found that SOX9 is required for ADM (Prevot et al., 2012). The present chapter underscores that Sox9 is important beyond ADM in neoplastic progression: Sox9 is required for PanIN development and in the absence of Sox9, metaplastic markers associated with neoplasia are not induced in oncogenic Kras pancreas.

In *WT* pancreas, we surprisingly found that Sox9 is not required for cerulein-driven ADM, in contrast to what we observed when ADM is induced by pancreatic duct ligation (Prevot et al., 2012). The potential discrepancy could result from different molecular mechanisms driving ADM in the two models. Further investigation of the two injury models is expected to reveal distinct mechanisms driving inflammation and distinct roles of SOX9 in KRAS-driven pancreatic cancer.

Our results position Sox9 as an upstream regulator of the ErbB pathway. Strikingly, previous reports have shown that SOX9 is a downstream target of EGFR. This is the case in urothelial, basal cell, and pancreatic carcinoma cell lines (Eberl et al., 2012; Ling et al., 2011). Therefore a positive feedback loop involving EGFR and SOX9 functions to initiate PDAC. Such loop is not necessarily functional in all tissues co-expressing SOX9 and EGFR, since oncogenic KRAS-driven lung and intestinal tumors, which both express SOX9, do not require EGFR signaling, in contrast to pancreatic tumors (Navas et al., 2012). Combining our data with others (Ardito et al., 2012; Navas et al., 2012) allows us to propose that when KRAS is mutated in a context of pancreatitis, a “SOX9-ERBB” axis contributes to induce ADM and PanIN. Later, when additional mutations occur, PanIN evolve into PDAC; at this advanced stage, when P53 becomes inactivated, pancreatic tumors become independent of EGFR signaling (Navas et al., 2012). Thus, a patient with PDAC could present both EGFR-insensitive, advanced lesions and EGFR-sensitive, metaplastic and neoplastic lesions.

Data from chromatin immunoprecipitation experiments showed that SOX9 binds to the EGFR and ERK genes (Oh et al., 2010), suggesting that SOX9 controls the expression of these two genes at the transcriptional level. In the present work, we also searched for Sox9 binding sites in the promoter region of mouse ErbB2 gene. We found a site matching very closely with the Sox9 consensus binding sequence. However, transient transfection experiments with a reporter plasmid indicated that Sox9 was not sufficient to transactivate the ErbB2 promoter region.

ERBB2 is the preferred dimerization partner of EGFR and the EGFR/ERBB2 heterodimer binds EGF with a 7-fold higher affinity than the EGFR homodimer (Li et al., 2012). ERBB2 has known oncogenic functions in several types of cancer and it was recently shown that antibody-mediated inhibition of ErbB2 inhibits PDAC progression in animal models (Maron et al., 2013). Therefore our work contributes to the understanding of a molecular pathway that has become an important and potential target of preventive and curative therapy.

## 5. Materials and Methods

*Mice*

Mice received humane care according to the criteria listed by the National Academy of Sciences. *LSL Kras$^{G12D}$*, *ElaCre$^{ER}$, Sox9$^{f/f}$* and *MT-TGFα* mice have been described (Desai et al., 2007; Hingorani et al., 2003; Kist et al., 2002; Sandgren et al., 1990). All strains were maintained in an enriched CD1 background.

*Tamoxifen and 4-hydroxytamoxifen (4OHT) treatments*

Six week-old mice were treated with tamoxifen and 4OHT (Sigma, St-Louis, MO, USA) dissolved in corn oil (Sigma) at a concentration of 30 mg/ml and 0.3 mg/ml respectively. Treatment consisted of an intraperitoneal (IP) injection of 100 µl of 4OHT combined with gavage of 100 µl of tamoxifen, every other day, for 5 days. Mice were always treated with tamoxifen at least 10 days before beginning of the cerulein treatment.

*Cerulein and zinc treatments*

Acute pancreatitis was induced by 6 hourly IP injections of cerulein (Sigma, 125µg/kg), every other day, for 5 days. The initial day of treatment was considered day 1 (D1). Mice were sacrificed on day 7 (D7) and day 12 (D12). To induce chronic pancreatitis, at the end of the acute cerulein treatment, the mice continued to receive a daily injection of cerulein, 5 days per week. Mice were then sacrificed on day 65 (D65).

Zinc was diluted at a concentration of 7 g/L in water and given ad libitum in drinking water for 6 months. Mice were sacrificed at the end of the treatment.

*Immunofluorescence and immunohistochemistry*

Mouse tissue samples were fixed overnight in PBS 4% paraformaldehyde at 4° C, and embedded in paraffin. Serial paraffin sections, 9 µm thick, were mounted on glass slides, then deparaffinised and re-hydrated. Antigen retrieval was performed by boiling the slides for 10 min in 10 mM citrate buffer pH 6 in a microwave oven, or by incubating them for 20 min at 90°C in a Lab Vision PT module (Thermo Scientific, Waltham, MA, USA). Permeabilization was performed by incubation of the slides in PBS / Triton X-100 0.3% for 5 min and then by blocking in PBS / Triton X-100 0.3% / BSA 10% / milk 3% for 45 min at RT.

The primary antibodies were diluted in blocking buffer and incubated with the samples overnight at 4° C. Details on the primary antibodies used are given in Table 1.

Secondary fluorescent antibodies and streptavidin-POD conjugate (1/1000) were diluted in PBS / Triton X-100 0.3% / BSA 10% and incubated at 37° C for one hour. DAB (Dako, Glostrup, Denmark) was used for immunostaining detection. Pictures were taken using a Cell Observer Spinning Disk confocal microscope (Zeiss, Oberkochen, Germany) for the fluorophore labeling, or with a Mirax imaging system (Zeiss) for histological staining.

*Cell lines*

HPAF2 cells, a human pancreatic adenocarcinoma cell line derived from peritoneal ascitic fluid of a 44 year old Caucasian male with primary pancreatic adenocarcinoma and metastases to the liver, diaphragm and lymph nodes, were grown in modified Eagle medium (MEM) (Gibco, Basel, Switzerland) supplemented with fetal bovine serum (FBS) 10%, L-glutamine 1%, and antibiotics. Murine PanIN cells are a pre-invasive cell line derived from *Pdx1-Cre LSL-Kras*$^{G12D/+}$ pancreas (Hingorani et al., 2005). These cells were grown in Dulbecco's MEM (DMEM) /F12 (Gibco) supplemented with FBS 10%, 2.5 g sterile filtered dextrose/500ml, and antibiotics. Human embryonic kidney cells (HEK 293T) were grown in DMEM (Gibco) supplemented with FBS 10%, L-glutamine 1%, and antibiotics. For all experiments, cells were used between passages 5 to 8 after thawing, unless otherwise stated, and maintained under 37°C/$CO_2$ 5% atmosphere.

*Lentiviral vectors*

Plasmid constructs (pLKO vector) expressing Scramble, ERBB2 (clone TRCN0000234101, MissionShRNA, Sigma) or SOX9 (clone TRCN0000342886, MissionShRNA, Sigma) shRNAs were used to prepare lentiviral vectors. ERBB2 shRNA targets both the human and mouse sequences whereas SOX9 shRNA targets the mouse sequence. Viral packaging was performed by HEK 293T transfection using $Ca_2PO_4$, the above plasmids and three other plasmids encoding proteins required for viral packaging (pRSV-REV, pHCMV-G and pMDLG/pRRE). The next day, medium was changed. Culture supernatants containing lentiviral particles were collected 48 hours after transfection, pooled if needed and filtered through a 40 µm nylon mesh (BD Biosciences, San Jose, CA, USA) to remove cell debris. Viral particles were concentrated by using Centricon Plus-70 Centrifugal Filter, Ultracel-PL Membrane (Merk Millipore, Billerica, MA, USA).

*Infection of PanIN and HPAF2 with lentiviral particles*

PanIN and HPAF2 cells were infected using Polybrene (4 µg/ml) and an equal amount of Scramble or ERBB2 shRNA lentiviral particles. HPAF2 were also infected with SOX9 shRNA lentivirus. After six hours, culture medium was replaced with fresh

media. Cells were selected 48 hours after infection with puromycin (Gibco) during 2 days.

*Proliferation and apoptosis assays*

After puromycin selection, control (no shRNA), Scramble shRNA-, ERBB2 shRNA-infected PanIN cells and control (no shRNA), Scramble shRNA-, ERBB2 shRNA-, SOX9 shRNA-infected HPAF2 cells were counted with a Bürker cell and $10^5$ cells were plated on 6-well plates in triplicate. Every two days, cells were trypsinized, counted and $10^5$ cells were plated on 6-well plates.

For apoptosis assay culture media containing cells in suspension were collected 2 days after plating. Adherent cells were trypsinized and collected separately. Cell preparation and staining were performed using PE Annexin V Apoptosis Detection Kit I (BD Biosciences). Briefly, cells were washed twice with cold PBS (Lonza, Basel, Switzerland) and resuspended in Binding Buffer at the concentration of 1 x $10^6$ cells/ml. 100 µl (1 x $10^5$ cells) were stained with 5 µl of PE Annexin V and 5 µl of 7-AAD during 15 min at RT in the dark. 400 µl of Binding Buffer were then added and analysis was performed using Facscalibur flow cytometer (BD Biosciences) and the CellQuest software (Version 3.3, BD Biosciences). Final analysis of the data was performed using FlowJo software (version 9.6.4 mac, Tree Star).

*In vitro ADM assay*

This protocol was adapted from Wagner et al. and Shi et al. (Shi et al., 2013; Wagner et al., 2002). Briefly, pancreas was rinsed in cold Hank's balanced solution (HBSS, Invitrogen, Carlsbad, CA, USA) and cut into small pieces with scissors. Pancreas was digested in collagenase P 0.25 mg/ml (Roche, Basel, Switzerland) / collagenase type IV 0.35 mg/ml (Invitrogen) / dispase 1 mg/ml (Gibco) at 37°C for 20 min. Isolated acini were washed three times in cold HBSS/FBS 5% and then filtered sequentially through 500 and 105 µM nylon meshes (Spectrum Laboratories, Rancho Dominguez, CA, USA). Cell suspensions were carefully layered on the top of a HBSS/ FBS 30% cushion and acini were collected by centrifugation (1000 rpm, 2 min, 4°C). Then cells were resuspended with 6 ml of 3D culture base medium (RPMI, FBS 10%, soybean trypsin inhibitor 0.1 mg/ml, dexamethasone 1 µg/mL and antibiotics) and mixed with collagen (8 volumes of rat tail collagen stock solution at a concentration of 3.56 mg/ml and one volume of 10x concentrated MEM, neutralized with one volume of NaOH 1M) in a 1:1 ratio. The cell suspension was then plated (0,5 ml/well) in 24-well tissue culture plates coated with 250 µl of collagen at least 1 h before acini isolation. The cell-collagen mix was allowed to solidify for 1 h at 37°C before adding 1 ml 3D culture media. Medium was changed on days 1 and 3. Pictures were performed at day 5 with Axiovert 200 microscopy (Zeiss).

*Western Blotting*

Proteins were extracted from infected PanIN and HPAF2 cells with RIPA buffer (NaCl 150 mM, PMSF 1mM, EDTA 1 mM, Triton X-100 1%, SDS 0.1%) complemented with protease and phosphatase inhibitors (Roche).

For western blot analysis, protein extracts were subjected to electrophoresis on 10% or 7.5% polyacrylamide gels (SDS-PAGE) and transferred onto a nitrocellulose Hybond-LFP membrane (Amersham Biosciences, Peapack, NJ, USA). The membrane was incubated overnight at 4°C with primary antibodies (Table 1) diluted in StartingBlock blocking buffer (Thermo Scientist). The membrane was then incubated with fluorescent antibodies anti-Rabbit IRDye 800 CW (Li-cor, Lincoln, NE, USA) or anti-Goat-680 (Invitrogen) at 1/10000 dilution in StartingBlock solution complemented with Tween 0.1% for 1 h at RT. The fluorescent signals were visualized and measured with Odyssey (Li-Cor).

*Quantification procedures*

For PanIN quantification, glass slide composed of two pancreas sections per mouse (n=4, for each genotype), separated by 180 $\mu$m, were digitalized with Mirax imaging system (Zeiss). Acinar surface was delineated and quantified using Mirax Viewer (Zeiss) and Alcian blue positive lesions were counted manually.

For quantification of edematous tissue, T lymphocytes and macrophages, ten randomly chosen sections were taken per mouse (n=3, for each genotype). The whole tissue area was measured and divided by the acinar tissue area. Both were measured using ImageJ software (http://rsbweb.nih.gov/ij/). T lymphocytes and macrophages were counted using Adobe Photoshop CS2 and the resulting numbers were divided by the acinar tissue area. Graphics and statistical analysis for all quantifications were performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com).

**Table 1. List of the antibodies used in the present study**.

| Antibodies | Origin | Reference | Dilution | |
|---|---|---|---|---|
| | | | IF | WB |
| Sox9 | Rabbit | Millipore (ab5535) | 1/1500 | 1/1000 |
| EGFR | Rabbit | Santa Cruz (sc-03) | - | 1/200 |
| EGFR | Rabbit | Cell Signaling (#4267) | 1/50 | - |
| P-Y845-EGFR | Rabbit | Invitrogen (#4478G) | 1/100 | - |
| Amylase | Goat | Santa Cruz (sc-12821) | 1/500 | - |
| CK19 | Rat | Developmental Studies Hybridoma Bank | 1/50 | - |
| Ezrin | Mouse | NeoMarkers (MS-661-P1) | 1/200 | - |
| Clusterin-α | Goat | Santa Cruz (sc-6420) | 1/200 | - |
| ErbB2 | Rabbit | Dako (#A0485) | 1/600 | 1/1000 |
| Erbin non-purified sera (E2/2) | Rabbit | L. Mei's lab | 1/2000 | 1/1000 |
| ERK | Rabbit | Cell Signaling (31F5) | 1/200 | 1/1000 |
| P-T202/Y204-ERK | Rabbit | Cell Signaling (#43705) | 1/200 | - |
| Actin | Goat | Santa Cruz (sc-1615) | - | 1/1000 |
| CD3-ε | Goat | Santa Cruz (sc-1127) | 1/200 (IHC) | - |
| F4/80 | Rat | Abcam (ab6640-200) | 1/300 (IHC) | - |

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# C. Role of HNF6 and Sox9 in acinar versus ductal cell fate decision in an oncogenic Kras background.

## 1. Introduction

At the beginning of the thesis we started investigating the role of Kras and transcription factors in a mouse model in which Kras is activated prior to birth. As mentioned in the introduction (see chapter I.C.3), embryonic Kras activation does not faithfully mimic the human acute pancreatitis. However, our initial experiments showed that activation of Kras during pancreas development affected cell differentiation. These results revealed an unexpected role of Kras in cell fate decision and are presented in this chapter.

## 2. Results

To induce Kras activation, we turned to the *Mist1-Kras$^{G12D}$* model in which Kras is knocked-in in the Mist1 locus and to an inducible activation of Kras by Elastase-Cre-mediated recombination of the LSL-Kras$^{G12D}$ transgene. We first characterized the expression of HNF6 and Sox9 in the *Mist-Kras$^{G12D}$* mouse during the pancreas development. Wild-type pancreas from e15.5 to 18.5 showed no expression of HNF6 and Sox9 in acinar precursors, as expected from the work by others. In contrast, in *Mist-Kras$^{G12D}$* pancreas, developing acinar cells expressed both factors (Figure 1A and data not shown). Moreover the developing acini presented large lumina and a small cytoplasm. Therefore, when Kras is activated in acinar cells, which have not yet reached full maturity, the cells display a combination of acinar and ductal characteristics.

In the absence of HNF6 in *Mist1-Kras$^{G12D}$ HNF6$^{-/-}$* mouse, at e18.5, smaller lumina were observed. Their average diameter was 19,1 $\mu$m in the absence of HNF6, and 38,8 $\mu$m in the presence of this factor (Figure 1A and B). Similarly, the absence of Sox9 in *ElaC$^{ER}$ Sox9$^{f/f}$ Mist1-Kras$^{G12D}$* mouse was also associated with a decrease in the luminal diameter (Figure 1A and C). These results indicate that persistent expression of HNF6 and Sox9 in *Mist1-Kras$^{G12D}$* mouse contributes to the ductal-like phenotype of developing acinar cells.

Next, we investigated if the hybrid acinar-ductal cells could further evolve to PanIN. *ElaC LSL-Kras$^{G12D}$* mice developed a limited number of PanIN lesions starting 30 days after birth (Figure 2). Deletion of Sox9 in this embryonic model (*ElaC LSL-Kras$^{G12D}$ Sox9$^{f/f}$*) did not inhibit PanIN formation (Figure 2). Indeed, several PanIN lesions devoid of Sox9 and positive for CK19 and Alcian Blue were observed (Figure 2B and data not shown). Therefore, when Kras is activated in the embryonic period,

hybrid acinar-ductal cells are generated, and in contrast to mice with postnatal activation of Kras, Sox9 is dispensable for development of PanIN.

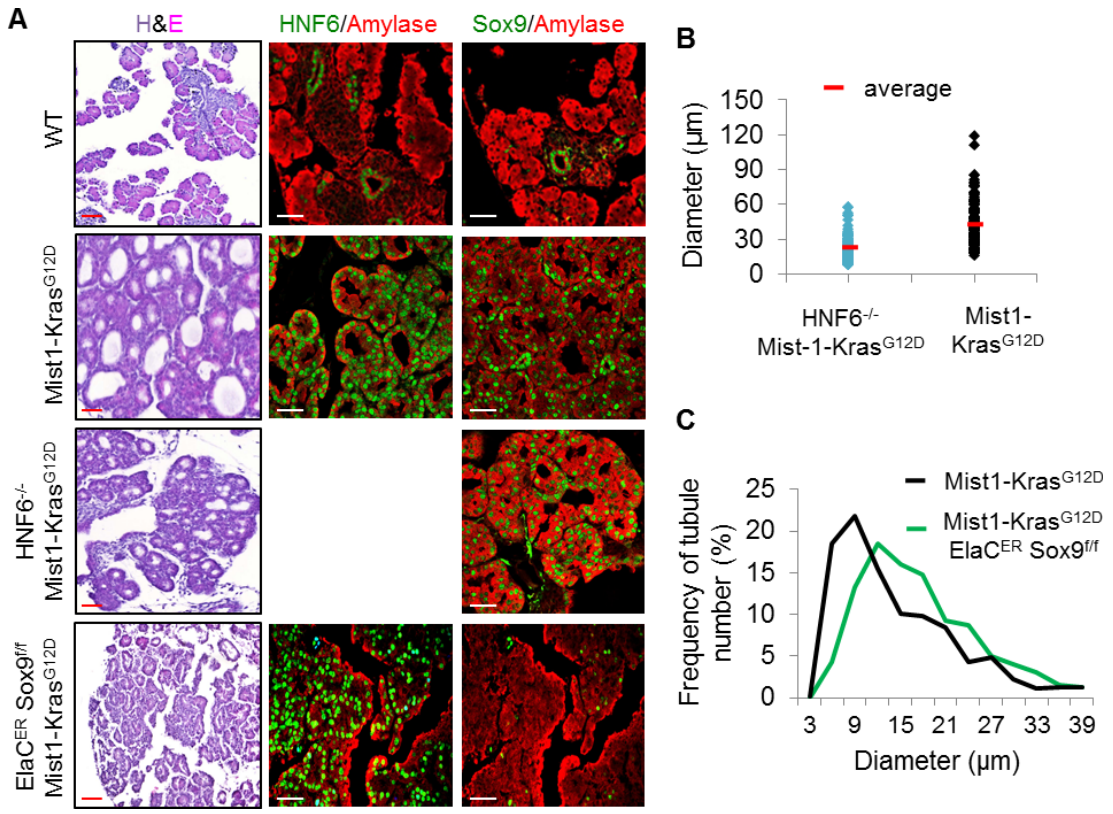


**Figure 1. HNF6 and Sox9 control tubular-complex formation induced by oncogenic Kras.** Haematoxylin-eosin (H&E) and immunofluorescent labeling of HNF6, Sox9 and Amylase in *WT*, *Mist1-Kras$^{G12D}$*, *HNF6$^{-/-}$ Mist1-Kras$^{G12D}$* and *ElaC$^{ER}$ Sox9$^{f/f}$ Mist1-Kras$^{G12D}$* pancreata at e18.5. Scale bars = 50 µm (**A**). Quantification of the luminal diameter in *HNF6$^{-/-}$ Mist1-Kras$^{G12D}$* (blue dots, n=178) and *Mist1-Kras$^{G12D}$* (black dots, n=198) pancreas (**B**). Quantification of the luminal diameters between *Mist1-Kras$^{G12D}$* (black line) and *ElaC$^{ER}$ Sox9$^{f/f}$ Mist1-Kras$^{G12D}$* (green line) pancreas (**C**).

## 3. Discussion

This result point to differential sensitivity of acinar cells to activated Kras, depending on the maturation stage. When Kras is activated in the embryo, acinar cells fail to properly mature and become hybrid. Acquisition of ductal characteristics depends on HNF6 and Sox9, but how Kras allows persistent expression of these factors is unknown. Our observations also reveal that accurate Kras activation is required for normal maturation of acinar cells.

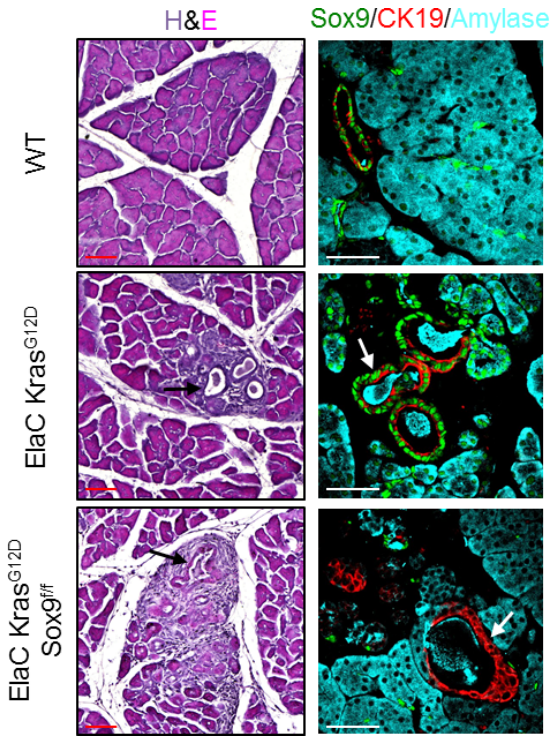


**Figure 2. Sox9 is not required for PanIN formation in Kras embryonic activation.** Haematoxylin-eosin (H&E) and immunofluorescent labeling of Sox9, CK19 and Amylase in *WT*, *ElaC LSL-Kras$^{G12D}$* and *ElaC Sox9$^{f/f}$ LSL-Kras$^{G12D}$* mice at postnatal day 30. At postnatal day 30, PanIN indicated with dark and white arrows are observed in *ElaC LSL-Kras$^{G12D}$* and *ElaC Sox9$^{f/f}$ LSL-Kras$^{G12D}$* mice. In the former, PanIN express both Sox9 and CK19 while in the later they express CK19 but not Sox9. Scale bars = 50 $\mu$m.

The hybrid cells developing in embryos with activated Kras can evolve to PanIN in the absence of Sox9, revealing their ability to bypass the need for Sox9 to generate such lesions. This is in contrast to mature acinar cells, which can only develop into PanIN when Sox9 is expressed. The reason for the differential Sox9-dependence of mature acinar versus hybrid acinar-ductal cells is not known. However, our work indicates that there is more than one route to generate PanIN lesions from the acinar lineage.

## 4. Materials and Methods

*Mice*

Mice received humane care according to the criteria listed by the National Academy of Sciences. *LSL-Kras$^{G12D}$*, *ElaCre$^{ER}$*, *ElaC*, *Sox9$^{f/f}$*, *HNF6$^{-/-}$* and *Mist1-Kras$^{G12D}$* mice have been described (Desai et al., 2007; Grippo et al., 2002; Hingorani et al., 2003; Jacquemin et al., 2000; Kist et al., 2002; Shi et al., 2009). All strains were maintained in an enriched CD1 background.

*Tamoxifen treatment*

Pregnant female were treated at e15.5 with tamoxifen (Sigma, St-Louis, MO, USA) dissolved in corn oil (Sigma) at a concentration of 30 mg/ml. Treatment consisted

of an intraperitoneal (IP) injection of 100 µl of tamoxifen combined with gavage of 100 µl of tamoxifen.

*Immunofluorescence*

Please see previous chapter (III.B.5) for details.

*Quantification procedures*

For lumen quantification, glass slides with three pancreas sections per mouse (n=4, for each genotype), separated by 180 *µ*m, were digitalized with Mirax imaging system (Zeiss). Diameter of lumen was quantified and measured using Adobe Photoshop CS2.

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# IV. Conclusion, general discussion and perspectives

Experimental evidence from transgenic mouse models indicates that PDAC can arise from acinar cells. This implies that in an initial phase of PDAC development acinar cells transdifferentiate into duct-like cells, a process called ADM. At the start of the thesis, the mechanisms controlling ADM were only poorly known. In the present work I demonstrated that the ductal transcription factors HNF6 and SOX9 play a critical role in duct ligation-induced ADM. Expression of both proteins leads to repression of acinar genes and induction of ductal genes. Interestingly, HNF6 is no longer expressed when ADM has progressed to PanIN lesions, whereas SOX9 persists in ADM, PanIN and PDAC. While SOX9 is not critically required in inflammation-induced ADM, it becomes essential in ADM associated with Kras mutations, provided that Kras is activated after birth. In the latter context, SOX9 stimulates the activity of the ERBB signaling pathway.

## A. Differential sensitivity of the pancreas to distinct inflammatory stimuli

We demonstrated that HNF6 is required for development of ADM after pancreatic duct ligation (PDL). Moreover, ectopic expression of HNF6 can induce metaplasia in acinar cells through induction of ductal markers like Sox9 and CK19. Immunofluorescence stainings in human tissue also suggest that HNF6 is induced prior to SOX9 in metaplastic acinar cells. However, in the cerulein-induced acute pancreatitis model we found that Sox9 expression precedes that of HNF6 (data not shown). The differential expression profiles of HNF6 and Sox9 in distinct models of pancreatitis indicate that the genetic regulatory cascade differs after PDL and cerulein treatment. The inflammatory response may diverge in the two models. PDL mimics gallstone-induced acute pancreatitis. Depending on the location of the ligation, acute pancreatitis may be associated with systemic inflammation, acute lung injury, multi-organ dysfunction and death (Samuel et al., 2010). In cerulein-induced acute pancreatitis, systemic inflammation has not been monitored. Still, a comparative study between the two models has not yet been performed.

While our work was in progress, Kopp et al. showed that the Sox9-dependent acinar-to-ductal reprogramming is the main mechanism for PDAC development in a context of oncogenic Kras activation (Kopp et al., 2012). In addition to the data of Kopp et al., we further demonstrated that Sox9 stimulates ErbB signaling during the ADM- and PanIN-induced development by mutated Kras. Consistent with their data, we also found that Sox9 has no obvious role in the ADM in a wild-type Kras context. Again, this difference with the results obtained in the PDL model could be explained by the variable inflammatory response discussed above when addressing the role of HNF6.

## B. Distinct roles of HNF6 and Sox9 depending on inflammatory stimulus and activation of signaling pathways

After acute cerulein treatment, the highest expression of HNF6 coincides with the onset of acinar cell regeneration (data not shown). The potential role of HNF6 in regeneration has not been yet elucidated. Our unpublished data further show that postnatal deletion of HNF6 (*ElaC$^{ER}$ LSL-Kras$^{G12D}$ HNF6$^{f/f}$*) does not impact on cerulein-induced ADM and PanIN when Kras is activated (*HNF6$^{f/f}$* mouse was generated and generously given by F. Clotman, M. Goulding, unpublished). This is at odds with the requirement of HNF6 in development of ADM after PDL. Again, we speculate that PDL- and cerulein-induced inflammations differ, leading to differential genetic response of the acinar cells.

We demonstrated that Sox9 activates ErbB signaling pathways during PanIN formation. However persistent activation of the EGFR signaling bypasses the requirement of Sox9, as suggested in the experiments using the *MT-TGF$\alpha$* mice. We hypothesize that SOX9 is not required in patients with activating mutation of EGFR or amplification of EGFR or ERBB2. Confirmation of this hypothesis could lead to personalized therapy.

## C. Regulation of Sox9 and HNF6

The identification of Sox9 as an essential factor for PanIN development begs the question about the mechanisms allowing expression of Sox9 in acinar cells. MicroRNAs are regulators of gene expression. They participate to many biological processes, including cancer, and several microRNAs affect PDAC development (Papaconstantinou et al., 2013; Park et al., 2011). Thus, miRNA-mediated modulation of Sox9 expression may influence initiation of pancreatic cancer. In this context, miR-145 stands out as a good candidate. MirR-145 represses SOX9 in chondrocytes and in head and neck cancer, and expression of miR-145 is decreased in PDAC patients (Martinez-Sanchez et al., 2012; Papaconstantinou et al., 2013; Yu et al., 2013). Moreover, miR-145 also represses ADAM17, the EGFR ligand sheddase essential for PanIN development, suggesting that a decrease in miR-145 allows induction of SOX9 and ADAM17, which are two activators of the EGFR pathway (Ardito et al., 2012; Yu et al., 2013). Interestingly, SOX9 binds directly to the ADAM17 promoter in head and neck squamous cell carcinomas (Yu et al., 2013). In pancreatic cancer cells, Kras inhibits miR-145 via the zinc finger transcription factor RREB1, and conversely, miR-145 inhibits proliferation and activates apoptosis in pancreatic cells by directly repressing Kras and RREB1 (Kent et al., 2010). Altogether, these interactions suggest the existence of a SOX9- and EGFR-regulated network in PDAC (Figure 1). Targeting one or several members of this network may constitute a potential therapeutic approach. However, this still needs further experimental evidence. Interestingly, expression of

SOX9 is low and that of miR-145 is high in pancreatic cancer cell lines that have wild-type alleles of Kras (Ilse Rooman, personal communication; (Kent et al., 2010). Conversely, miR-145 expression is low or absent in pancreatic cell lines with oncogenic mutations of Kras. Overexpressing SOX9 or oncogenic Kras in the appropriate cell line is expected to provide information supporting the model defined in Figure 4. If the result fits with the model, confirming the existence of this network *in vivo* opens perspectives to target PDAC by miRNA-mediated therapy.

Besides the direct regulation of miRNAs on Sox9, indirect regulation has been demonstrated. In metaplasia, inhibition of miR-495 by the immune response induces expression of HNF6 and increases expression of Sox9. Interestingly, inhibition of miR-495 and HNF6 do not lead to a total decrease of Sox9 expression indicating that other activators of Sox9 play a role (Prevot et al., 2013). If the model proposed is confirmed, constitutive activation of Kras could lead to repression of miR-145, and indirectly miR-495, and thus to a constitutive expression of HNF6 and Sox9. This may also explain why activation of Kras in the embryo (chapter III.C) may upregulate HNF6 and Sox9, leading to generation of hybrid acinar-ductal cells.

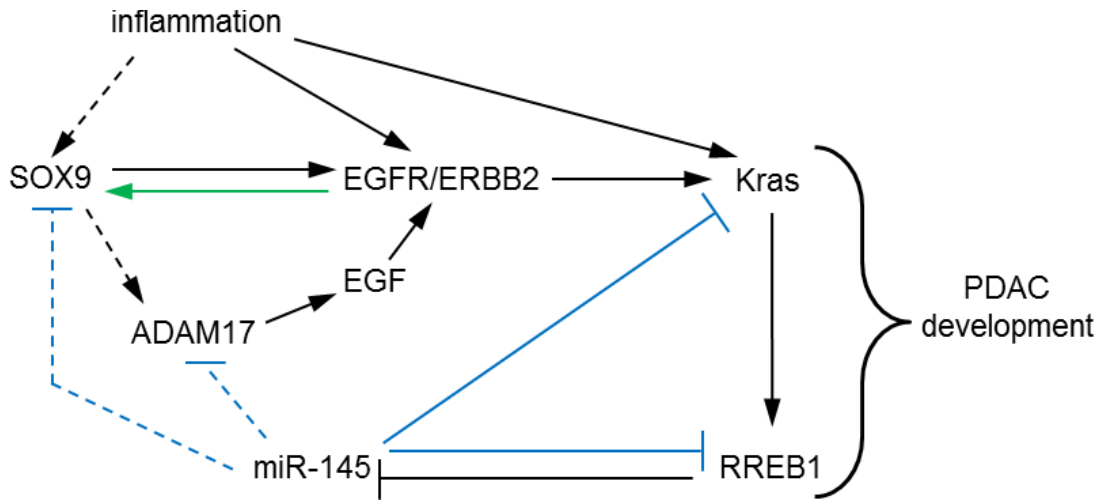


**Figure 1. Model of Sox9 regulation in pancreatic tumorigenesis**

Epigenetic modifications of the *SOX9* gene might also contribute to modulate SOX9 expression. The expression of a number of epigenetic regulators is positively correlated with that of SOX9 in PDAC samples. BCOR (BCL6 corepressor), KDM3B (a lysine-specific demethylase), and HDAC1 (histone deacetylase 1) belong to this category. The Human Protein Atlas (http://www.proteinatlas.org) reveals that KDM3B is exclusively expressed in human normal ductal cells. In PDAC tissue sample, KDM3B is expressed in metaplastic acinar cells and PanIN lesions. Therefore, SOX9 and KDM3B are expressed all along the development of PDAC. We hypothesize that SOX9 activates expression of KDM3B or that SOX9 is regulated by KDM3B to promote cancer progression. Investigation of this potential link is expected to improve our understanding of the role of SOX9 in PDAC.

The NF-κB p65 subunit, which is activated in inflammation, binds to the *SOX9* promoter in pancreatic cancer stem cells. These cells self-renew, differentiate into cells composing the bulk of the tumors and are associated with the invasive phenotype of the tumors. Interestingly, *SOX9* needs to be demethylated to allow binding of NF-κB p65 (Sun et al., 2013). However, how demethylation occurs remains an open question. We conclude and propose that epigenetic, transcriptional and post-transcriptional regulators combinatorially activate SOX9 in ADM and tumor progression. Unraveling this network will increase the list of potential targets for preventive and curative therapy.

## D. EGFR pathway in pancreatic tumorigenesis

EGFR signaling is essential for PDAC development (Ardito et al., 2012; Navas et al., 2012). In chapter III.B, we demonstrated that ErbB2 is induced in ADM and PanIN lesions. Moreover, depletion of this protein reduces growth of PanIN and of pancreatic cancer cell lines. To our knowledge, this is the first evidence for a role of ErbB2 in pancreatic cancer. Further unraveling of the function of the EGFR pathway should focus on ERBB3. ERBB3 expression has been observed in several cancers and it is essential for proliferation in ERBB2-expressing breast cancer cells (Holbro et al., 2003; Jiang et al., 2007; Liles et al., 2010; Nagumo et al., 2009; Reschke et al., 2008; van der Horst et al., 2005; Yoon et al., 2009). ERBB3 is overexpressed in pancreatic cancer and high expression correlates with advanced stage and decreased overall survival (Friess et al., 1995; Lemoine et al., 1992). ERBB3 can heterodimerize with EGFR, ERBB2 or ERBB4. Interestingly ERBB3/EGFR heterodimerization activates PI3K-AKT and stimulates cellular proliferation *in vitro* and *in vivo* (Liles et al., 2010). Considering the growing importance of ERBB2 and ERBB3 in cancer, there is a need to investigate the expression and formation of ERBB heterodimers in PanIN. This may pave the way for therapeutic interventions using antibody-mediated therapy targeting the ERBB family in pancreatic cancer.

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# V. Appendix

## Study of SOX9 in human PDAC

The group of Ilse Rooman and Andrew Biankin (Garvan Institute, Sidney, Australia), participates to an international project devoted to sequence the exome and genome of over hundred human PDAC samples. They identified a large number of genes mutated in pancreatic cancer (Biankin et al., 2012), and are currently finalizing collection of data related to the SOX9 gene. These data are summarized below and will be combined with our mouse data (see chapter III.B) in a manuscript.

### A. Copy number gains of SOX9 gene are frequently detected in PDAC samples

While searching for aberrations of the *SOX9* gene in PDAC samples, Rooman and co-workers performed a *SOX9* centric analysis in a cohort of 90 early (pre-operative clinical stages I and II) sporadic PDAC from the Australian Pancreatic cancer Genome Initiative (APGI). Analysis of the exome sequencing data and SNP arrays revealed one case with a point mutation. Copy number gain, loss of one copy and copy neutral loss of heterozygosity (LOH) of the *SOX9* gene were also detected in 5, 4, and 4 cases, respectively (Figure 1A).

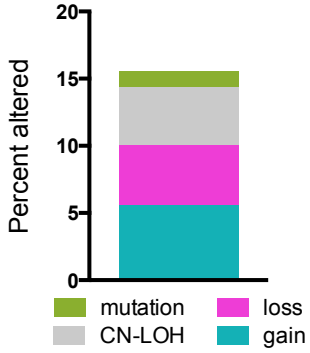



**Figure 1. Genetic aberrations in the SOX9 gene are frequently found in a cohort of human PDAC** Frequency of SOX9 somatic mutations and copy number variants in the APGI cohort (n=90).

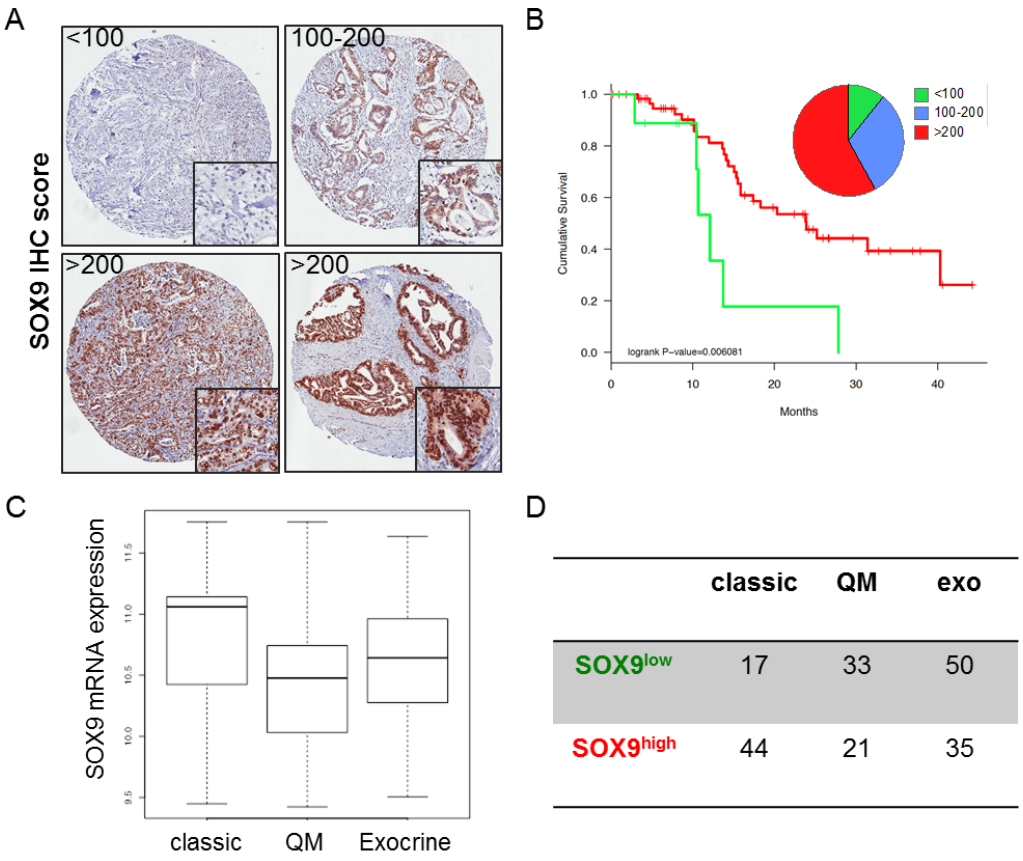
### B. SOX9 is highly expressed in human PanIN and PDAC tumors.

Analysis of SOX9 protein expression in tissue microarrays comprising 32 PanIN lesions and 112 surgically resected PDAC tumors from the APGI cohort showed a heterogeneous expression pattern in PanIN, ranging from strongly positive to negative, with occasionally both situations found within the same lesion (Figure 2A). Heterogeneity persisted in the PDAC samples, with around 60% showing strong

nuclear SOX9 expression (SOX9$^{high}$) and 10% scoring low (SOX9$^{low}$) (Figure 2A and B). These observations extended the results recently found in an independent study (Kopp et al., 2012).

## C. SOX9$^{High}$ tumors are mostly classical subtype with better survival outcomes

Further investigation of the differential characteristics between SOX9$^{high}$ and SOX9$^{low}$ tumors revealed that SOX9 expression did not correlate with tumor size, tumor grade, invasion of the lymph nodes, or vascular space (data not shown). However, SOX9$^{low}$ tumors have poorer disease-specific survival (Figure 2B), which is also reflected when examining SOX9 mRNA expression.




| | classic | QM | exo |
|---|---|---|---|
| SOX9$^{low}$ | 17 | 33 | 50 |
| SOX9$^{high}$ | 44 | 21 | 35 |

**Figure 2. SOX9 is highly expressed in PDAC and Sox9$^{High}$ tumors are associated with better survival outcomes.** Immunohistochemical analysis of SOX9 protein in tissue microarrays of X stage I and II PDAC samples. Representative pictures are shown for samples according to their IHC score (<100, 100-200 and >200) (A). (B) Kaplan-Meier curve for disease specific survival in groups with an IHC score <100 (n=9) versus IHC score >200 (n=62), respectively the SOX9$^{low}$ and SOX9$^{high}$ group; P<.01. Pie chart represents the proportion of samples within each group. (C) SOX9 mRNA expression levels, obtained from Illumina microarray analysis, in the different subtypes of PDAC (classical, quasimesenchymal (QM) and exocrine). SOX9 expression was higher in the classical subtype. (D) Representation of subtypes within the SOX9$^{low}$ and SOX9$^{high}$ group, expressed as percentage of total samples in the analysis.

Rooman et al. then verified if tumors with distinct levels of SOX9 matched with PDAC subtypes as defined by Collisson et al., who made a distinction between classical, quasi-mesenchymal (QM) and exocrine subtypes (Collisson et al., 2011). $SOX9^{low}$ tumors were underrepresented in the classical type, with 50% in exocrine, 33% in QM and 17% in classical type. Conversely, the $SOX9^{high}$ tumors were mainly representing the classical type (44%), versus 35% exocrine and 21% QM (Figure 2C and D).

Therefore $SOX9^{high}$ tumors mostly match with the classical type, which is associated with better survival and responsiveness to EGF-directed therapy. In contrast, $SOX9^{low}$ tumors are mostly of non-classical subtypes, characterized by lower survival rate and poor response to EGFR-directed therapy.

## D. High expression of SOX9 correlates with expression of members of the ERBB signaling pathway

To gain further mechanistic insights in the role of SOX9 in PanIN development, they re-analyzed the expression microarray data of the whole PDAC cohort and considered the top 100 genes of which expression correlated with SOX9. Among these, they found HNF1β, another typical pancreatic ductal transcription factor (Maestro et al., 2003), HES1, an upstream regulator of SOX9 in adult pancreas (Shih et al., 2012), FOXA2, a suppressor of EMT in PDAC (Song et al., 2010), tight junction proteins (MARVELD2, Occludin, Zona Occludens 2), and members of the ERBB signaling pathway (see below), as well as Src, another protein that cooperates with EGFR (Biscardi et al., 1999). To better capture the differences between 'extreme SOX9 expressors', the 5 samples with highest expression of SOX9 were compared with the 5 samples showing the lowest expression in the APGI cohort. A top 30 heat map of the 5 $SOX9^{high}$ tumors *versus* the 5 $SOX9^{low}$ tumors identifies co-regulation of SOX9 and ERBB2 (Figure 3). Using gene set enrichment analysis (GSEA), SOX9 was found to be associated with several pathways involved in cancer biology. The two major themes are protein kinase activity, especially MAP kinase and EGFR/ERBB, as well as cell cycle control, and regulation of gene expression. Besides ERBB2, other components of the ERBB pathway, e.g. EGFR and ERBB2IP/ERBIN, a protein that interacts with ERBB2 and is important for its activity, also showed core enrichment (not shown) (Borg et al., 2000; Tao et al., 2009).

These data linking SOX9 and ERBB2 in humans constituted core information for our analysis of the ErbB pathway in mouse (chapter III.B).

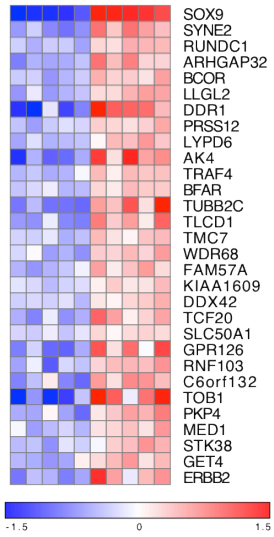



**Figure 3. SOX9 expression correlates with ERBB2 expression.** Heat map of top 30 genes that have correlative expression with SOX9 comparing the five PDAC samples with the lowest SOX9 expression (blue) versus the five PDAC samples with the highest SOX9 expression (red) from the APGI cohort.

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