ICAT: A NOVEL PTF1a/p48 PARTNER THAT MODULATES ACINAR GENE EXPRESSION

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ABSTRACT

Ptf1a/p48 is a pancreas specific bHLH transcription factor that is required at early stages of embryonic development for pancreas formation and, during adulthood, for the proper exocrine pancreatic function. P48 also exerts an antiproliferative effect, which may exert a tumor suppressor activity. In this study, based on a yeast two-hybrid approach, we have identified new p48 partners that modulate the activity of p48. Among the newly identified putative interactors we found p/CAF, which is a coactivator that potentiates its transcriptional activity, and ICAT, an inhibitor of the β-catenin/TCF signaling pathway. ICAT binds to p48 and is coexpressed with it in the pancreas during development and postnatally. Using different cellular models, increasing the levels of ICAT in acinar tumor cells resulted in changes of the pancreatic specific gene expression pattern. Furthermore, high levels of ICAT inhibited the interaction between p48 and p/CAF. While this hetero-oligomeric complex is required for the acinar gene expression, ICAT itself is shown to be present in a reconstituted PTF1 complex. Importantly, altered ICAT expression is demonstrated in several histological types of pancreatic tumors, possibly contributing to their differentiation phenotype and neoplastic properties.

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This thesis is dedicated to my family, for all the love and share. For being so many, for being so special and for being so much of my self. To Ró, Xico, Kikó, Tates, Bernardo, Marcela e Afonso. Obrigada! A Gianni, que tan cerca me ha atado…

ABBREVIATIONS

^{35}S, Sulfur-35

ACC, Acinar cell carcinomas
Amyl, Amylase
AP, Anterior-posterior
Apc, Adenomatous polyposis coli

β-gal, Beta-galactosidase
bHLH, Basic-helix-loop-helix

CBP, CREB-binding protein
cK, Cytokeratin
CMV, Cytomegalovirus
CP, Chronic pancreatitis
CPA, Carboxypeptidase
Ct, Threshold cycle
CtrB, Chymotrypsinogen B

DBD, DNA-binding domain

Elas, Elastase
EMSA, Electrophoretic mobility shift assays

FACS, Flow cytometry
FBS, Fetal bovine serum
FOP, Far-from-optimal Tcf-binding site

Gcn5, General control non-derepressible 5
GFP, Green fluorescent protein
GST, Glutathione-S-transferase

HAT, Histone acetyltransferase
His, Histidine

IP, Immunoprecipitation
IPMT, Intraductal papillary mucinous tumors
IPTG, Isopropyl β-D-1thiogalactopyranoside
IRDye, Infrared dye

LB, Luria-bertani media
Leu, Leucine

LiAc, Lithium acetate

MK, Midkine

PanIN, Pancreatic intraepithelial neoplasia
PCR, Polymerase chain reaction
PDAC, Pancreatic ductal adenocarcinoma
PEN, Pancreatic endocrine neoplasms
PFA, Paraformaldehyde
PP, Pancreatic-polypeptide
PTF1, Pancreas transcription factor 1
PSC, Pancreatic stellate cells

qRT-PCR, Quantitative reverse-transcription polymerase chain reaction

SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA, Short hairpin ribonucleic acid
SPT, Solid pseudopapillary tumors

TOP, Optimal Tcf-binding site
TRP, Tryptophan
TU, Titer units

Y2H, Yeast two-hybrid.

INTRODUCTION

1.The Pancreas

1.1. Histology

The adult mammalian pancreas is a mixed gland comprised by two distinct compartments: endocrine and exocrine. The endocrine compartment is made up of Langerhans' islets and is responsible for monitoring the glucose blood levels and for the release of specific hormones into the bloodstream. Langerhans' islets comprise at least four distinct cell types (α, β, δ, and PP), responsible for the production of hormones such as glucagon, insulin, somatostatin and pancreatic polypeptide, respectively. The islets are scattered throughout the exocrine compartment, usually found in close contact to capillaries.[1] The exocrine pancreas corresponds to about 95% of the organ and it is organized in acinar structures and a ductular branching system that converges in the main pancreatic duct, which – in some species – merges with the common bile duct.

The acinar cells are the production center for the digestive enzymes (i.e. amylase, elastase, and carboxypeptidase) that will be released as zymogens into the glandular lumina. The duct system, made of ductal cells, allows the flow of the acinar secretion towards the main duct. In addition, ductal cells produce bicarbonate (that neutralizes the acidity of the gastric juice) and mucins.

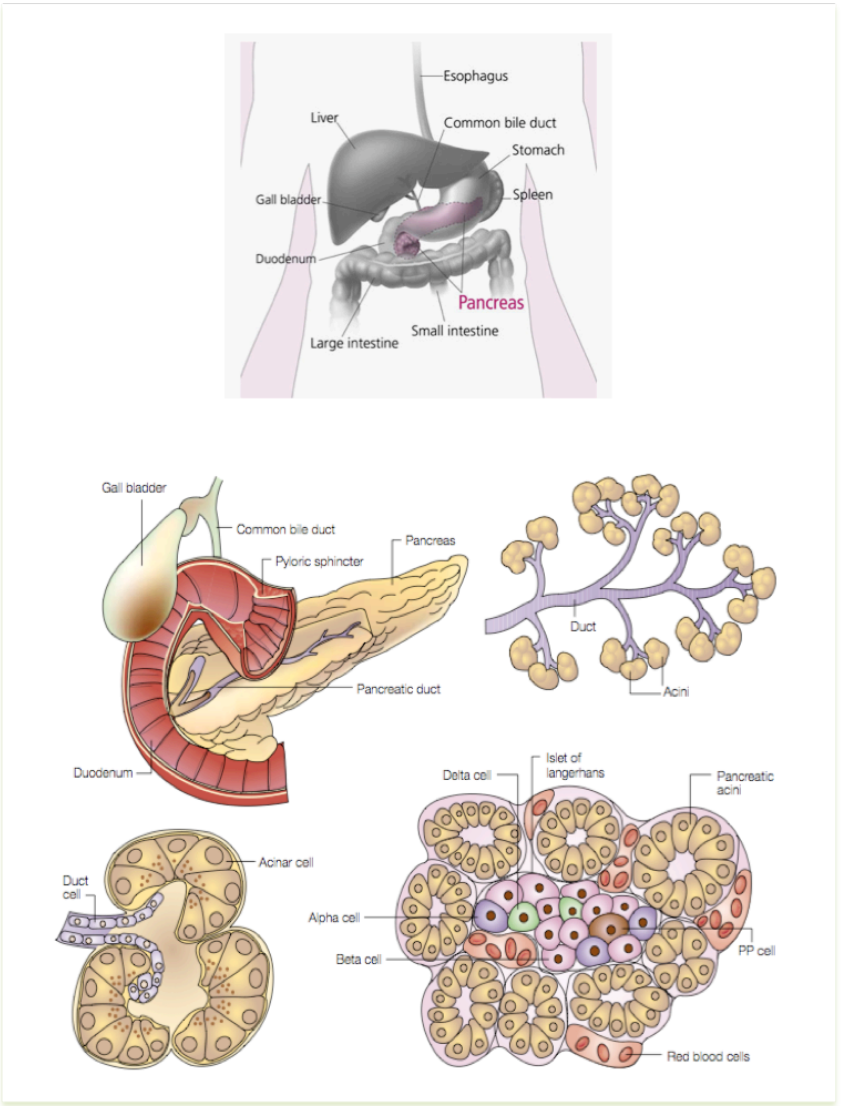

Figure 1.
A) Human pancreas and the surrounding organs; **B)** Representation of pancreatic histology in the adult. (Extracted from Bardeesy, N. & DePinho, R.A. (2002))

Furthermore, there are some evidences suggesting that, in the adult, ductal cells may constitute a reservoir for stem cells.[2] Centroacinar cells represent the terminal compartment of the ductal systems. Their biology is not well understood but it has also been proposed that they are important in pancreatic homeostasis and that they harbour stem cell potential.[3] All endocrine and exocrine cells are derived from the endodermal embryonic epithelium.[4,5]

1.2. Dynamics of Pancreatic Development

1.2.1. Morphogenesis

In mice, pancreatic organogenesis starts at embryonic day E8-8.5 when two independent buds develop in the foregut epithelium, one on the ventral side - right after the hepatic diverticulum - and the other one dorsally, on the opposite side of the foregut.[1] The budding happens asynchronically, with the dorsal bud arising slightly earlier than the ventral bud. Two transition steps occur during organogenesis: the primary transition (E8), when the cells from the endodermal primitive gut tube become committed to a pancreatic fate, and the secondary transition (E13.5-E15.5), when cellular differentiation occurs during the budding and branching processes.[6] In time, the two pancreatic buds develop separately until the gut rotation takes place by day E12.5. The rotation of the stomach and the duodenum forces the ventral bud to move around until it is side to side with the dorsal bud. Eventually, the two buds fuse and assemble yielding the final morphology of the pancreas.[2,7,8] The ventral bud will form the posterior part of the pancreatic head and the dorsal bud will form the remainder organ. Recent studies have described several morphogenetic signals involved in pancreatic formation, such as those from retinoic acid (RA), Fibroblast Growth Factor (FGF), members of the Transforming Growth Factor beta (TGF) family, Vascular Endothelial Growth Factor (VEGF), and the Hedgehog signaling pathway (Hh).[9,10,11,12] The ductal epithelium contains all the pancreatic progenitor cells which start to differentiate as soon as day E13.5.[13] These

multipotent progenitors are confined to the tip of the developing branches, and as these dig up, the differentiated progeny cells are left behind in the trunk.[13] At the end of this secondary transition stage, few pancreatic progenitor cells are left and pancreatic expansion occurs by proliferation of differentiated cells. The last step of the organogenesis process occurs with the detachment of the endocrine progenitor cells from the formed duct and migration within the surrounding mesenchyme, where they cluster and give rise to Langerhans' islets – "isletogenesis". From day E15.5 until birth, the cells undergo additional growth and maturation.

1.2.2. Pancreatic Specification: The Surroundings

Behind morphological pancreatogenesis, a network of intercellular and intracellular factors is involved in organ determination and proper cellular

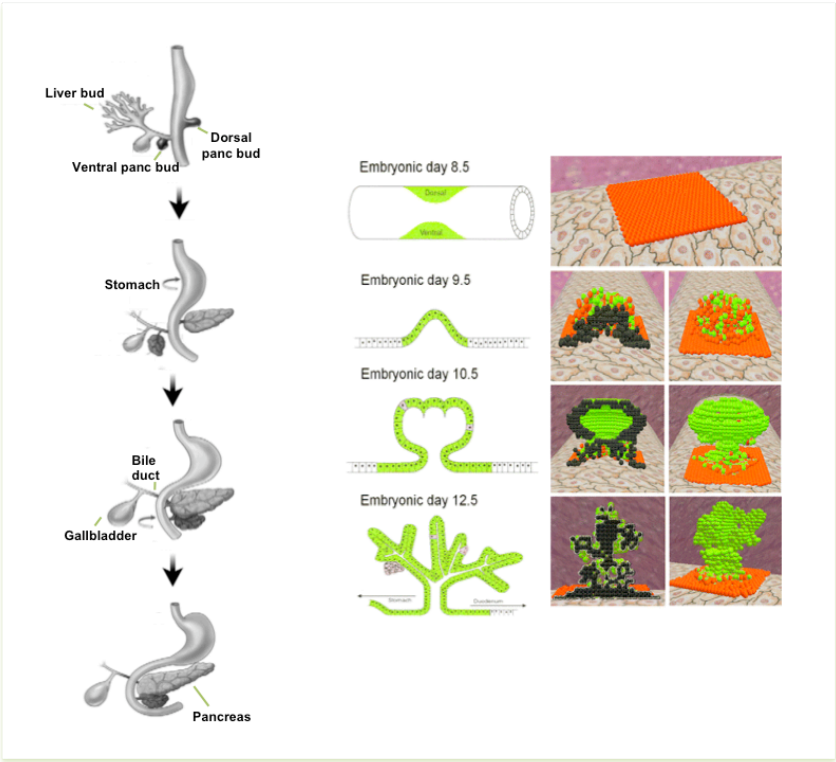

Figure 2. Stages of pancreatic morphogenesis.
A) Representation of pancreatic development and gut rotation during embryogenesis; **B)** 4-Dimensional Model of the developing pancreatic buds. (Adapted from Cano, D.A., Hebrok, M. & Zenker, M. (2007), and from; Setty, Y. et al. (2008))

differentiation. The intercellular signals are crucial for the guidance of endodermal cells towards a pancreatic fate, by triggering intracellular processes such as gene expression.

At the beginning of gastrulation, the endodermal layer becomes sensitized to the surrounding inductive signals after an anterior-posterior prepatterning process. Recent experiments have shown that **RA** plays a role in the anterior-posterior patterning of the vertebrate endoderm, therefore also influencing the endoderm that becomes pancreatic. For instance, ectopic application of RA induces the pancreas specification, and disruption of the RA-synthesizing enzyme Raldh2 results in the absence of the dorsal pancreatic bud.[14,15,16] Explant experiments in which the pancreatic buds are induced *in vitro* revealed that RA is associated with the early expression of the transcription factor Pdx1 (pancreatic and duodenal homeobox 1).[17]

Before specification towards pancreatic fate, the endoderm consists of a monolayer of epithelial cells. The prospective dorsal pancreatic endoderm is in direct contact with the notochord, a mesoderm-derived structure whose signals induce the pancreatic fate. The ventral pancreas is adjacent to the splanchnic mesoderm and to the aortic endoderm, but not in direct contact with the notochord. Chick embryo manipulation experiments whereby the notochord was removed from the prepancreatic endoderm neighborhood eliminated the expression of specific pancreatic marker genes.[18] When the notochord was placed in direct contact with the posterior endoderm, it failed to induce the pancreatic fate in this region. These experiments suggest that the endoderm is prepatterned and that the notochord confers a permissive signal to pancreatic development.[10] The means by which the notochord exerts such influence is the secretion of growth factors such as **activin-βB** (a TGF-β family member) and **FGF2**. These factors induce the expression of pancreas-specific genes by repressing the expression of Sonic Hedgehog (**Shh**) in the dorsal pancreatic epithelium.[11,18] Additionally, increasing the activin and TGF-β levels induced endocrine cell development and disrupted epithelial branching and acinar formation, whereas **Follistatin** (an TGF-β antagonist) promoted exocrine cell

differentiation.[19] Together with the embryonic transplant experiments, where removing the pancreatic mesenchyme impaired pancreatic development, these evidences highlight the requirement for specific epithelial-mesenchymal interactions for the proper specification of the anlagen and the endocrine or exocrine cell fate.

Recently, blood vessel signaling has also been shown to induce the expression of some transcription factors essential for pancreatic organogenesis. The aorta and the vitelline veins are near the forming dorsal and ventral buds, respectively and the aortic endothelial cells have been shown to be able to induce dorsal bud formation as well as the expression of the transcription factors Pdx1 and Ptf1a/p48 (pancreas specific transcription factor, 1a).[20]

1.2.3. Pancreatic Specification: Intercellular Signaling

Until today, it remains unclear how the factors participating in pancreatic organogenesis interact. The mesodermal inductors are required, as well as the expression of some already defined transcriptional factors, but which mesodermal cell type is activating which pancreatic transcription factor, and by

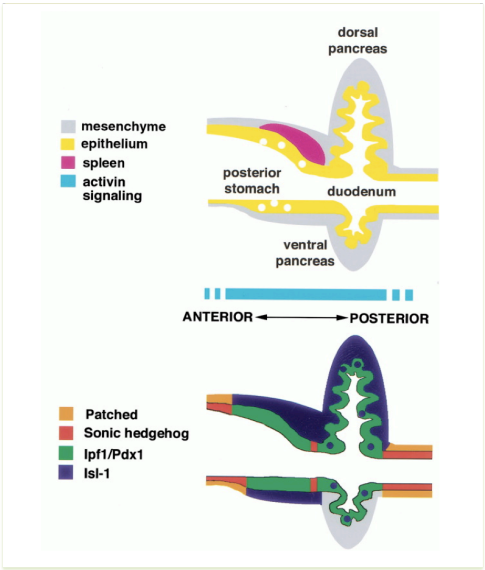

Figure 3. Cell interactions and intercellular signaling in pancreas development. (Extracted from Kim, S.K. & Hebrok, M. (2001))

which molecular signaling pathway, is still currently unknown.

The studies mentioned above reveal the existence of an anterior-posterior prepatterning of the endoderm. For the mesoderm and ectoderm layers, the usual patterning determinants are the Hox transcriptional factors. However, few Hox genes are expressed in the endodermal layer, and until now no associations have been proven between misexpression of Hox genes and alterations in the AP patterning. In the endoderm, a genomic cluster of Hox-related genes has been identified (ParaHox family) and **Hlxb9** - the homeobox gene member HB9 - has been shown to be required for dorsal bud formation and for the subsequent expression of the Pdx1 homeobox transcription factor.[21,22] Hlxb9 is normally expressed transiently until day E10.5 and sustained overexpression of Hlxb9 for longer periods during embryogenesis biases pancreatic development, forcing the presumptive pancreatic cells to acquire an intestinal phenotype, reminiscent of the Ptf1a knock out mouse phenotype.[23,24]

Pdx1 expression is a cell autonomous requirement for pancreatic specification. Pdx1 knock out results in pancreatic agenesis, distorted gastro-duodenal junction, loss of Brunner's glands, and deficiency of enteroendocrine differentiation in the stomach and duodenum.[25] Subsequent lineage tracing studies of the primitive buds have revealed that all the endodermally derived cells of the pancreas arise from Pdx-1 progenitor cells.[4,26,27] At the onset of the secondary transition, the insulin-producing β-cells upregulate Pdx1 expression and the exocrine acinar and ductal cells downregulate it.[28] Importantly, Pdx1 expression is induced by RA and is lost when the mesenchymal factor FGF10 is inactivated, highlighting the importance of mesenchymal inductive signals for the expression of the required cell-autonomous factors.[29,30]

Among Pdx1-expressing cells, neurogenin3 (**Ngn3**) is expressed in a small subset of cells of the developing endoderm and these cells will never express either insulin or glucagon. Ngn3 inactivation results in the lack of endocrine progenitors and accumulation of exocrine cells, suggesting that these cells are endocrine progenitors.[31] A recent study has shown that these Ngn3 progenitors

depend on the γ-secretase activity to acquire an endocrine phenotype, and only when Pax-6 expression starts the cells become completely endocrine committed. Until then, a degree of plasticity is preserved, where Notch2 competes with p48 for the RBP-Jκ co-factor that would be determining for the cellular descendants fate. The authors speculate that during the normal embryonic development, Notch2 contributes to Ngn3/E12 complex formation by sequestering RBP-Jκ and, by doing so, preventing the formation of the p48/E12/RBP-Jκ, favoring an endocrine outcome.[32]

Lineage tracing from the Ptf1a locus revealed that Ptf1a is also expressed within the undifferentiated precursor cells as early as day E9.5. *Ptf1a* knock out results in pancreatic agenesis, with a shift from pancreatic to a duodenal fate and the relocation of some individual differentiated endocrine cells into the spleen.[33,24] Interestingly, reduced expression of Ptf1a in pancreatic progenitors resulted in pancreatic hypoplasia with drifting of some of the progenitors to a common bile duct and duodenal cell type. Pancreatic morphogenesis and exocrine differentiation was reduced and delayed.[34] These studies have shown that the mature pancreatic cells arise from a common progenitor domain in the foregut endoderm specified by the expression of Pdx1 and Ptf1a. Indeed, lineage-tracing experiments in *Hes1*$^{-/-}$ mice have pointed out that Ptf1a is sufficient to guide the formation of all pancreatic cell types within a Pdx1 expressing undifferentiated endoderm.[35] This supports the concept that cells expressing Pdx1 and Ptf1a are the embryonic pancreatic stem cells. Whether Pdx1/Ptf1a-expressing stem cells exist in the adult pancreas and knowledge on the signaling activation required for their concomitant expression would be of great interest to elucidate the mechanisms involved in the specification and maintenance of the pancreatic homeostasis in the adult.

Since Ptf1a plays an important role later in life in the maintenance of the exocrine program, it is thought that, at some stage during the embryonic development, progenitor cells will choose between a pro-exocrine versus a pro-endocrine status. This decision-making would be the result of unknown mesenchymal signals that would sensitize the progenitor cells to overexpress

either Ptf1a or Ngn3, given the fact that later during embryogenesis the expression of these proteins is mutually exclusive, and assuming that early on there are undifferentiated precursors that co-express both of them.[6]

Mist1 (basic helix-loop-helix family, member a15) is another bHLH transcription factor that is strongly expressed in the pancreas, starting at day E10.5. As other bHLH transcription factors, Mist1 heterodimerizes with the bHLH transcription factor E2A and binds to E-boxes in the genomic regulatory regions.[36] Mist1-null mice exhibit extensive disorganization of exocrine tissue and intracellular enzyme activation, which is accompanied by molecular alterations resembling chronic pancreatic injury.[37] Therefore, Mist1 is required for maintaining the stable exocrine identity.

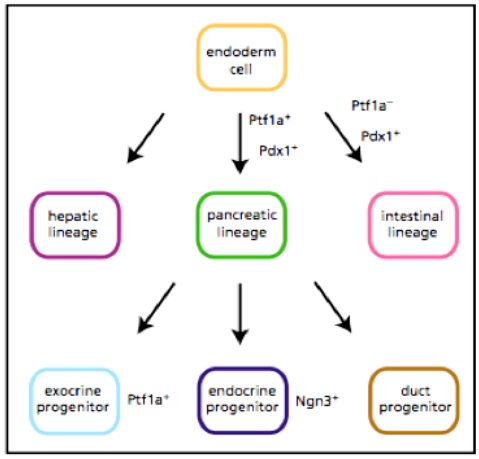

Figure 4. Regulatory transcription factors determining the fate of the endodermal cell lineages. (Extracted from Bort (2001))

1.2.4. Pancreatic Development: Notch Signaling

During embryonic development, Ngn3-positive cells appear with a scattered distribution, individually or in clusters. This observation was suggestive of a Notch lateral specification.[38] Neurogenin expression leads to the production of

the extracellular Delta, Serrate, or Jagged ligands that activate Notch receptors in adjacent cells. After ligand-receptor binding, NotchIC (the intracellular portion of the Notch receptors) binds the DNA-binding RBP-Jκ transcription factor and together they activate the expression of hairy/enhancer-of-split (*Hes*) and *Hey* genes. The Hes bHLH transcription factors repress the expression of neurogenin, preventing adjacent cells from differentiating, and the PTF1 transcriptional complex by binding p48.[39] Mice deficient for Dll1 (delta-like 1) or RBP-Jκ (recombination signal binding protein for immunoglobulin kappa J region) have an accelerated differentiation of Ngn3- and glucagon- expressing cells, whereas Hes1 deficiency results in a precocious development of the endocrine cells and in exocrine cell defects.[38,40] Moreover, *Ngn3* inactivation results in the lack of endocrine progenitors and accumulation of exocrine cells.[31]

Pancreatic precursor cells from day E11-E12 express Notch1 and Notch2, leading to activation of Hes1 expression in these cells, and few of them express

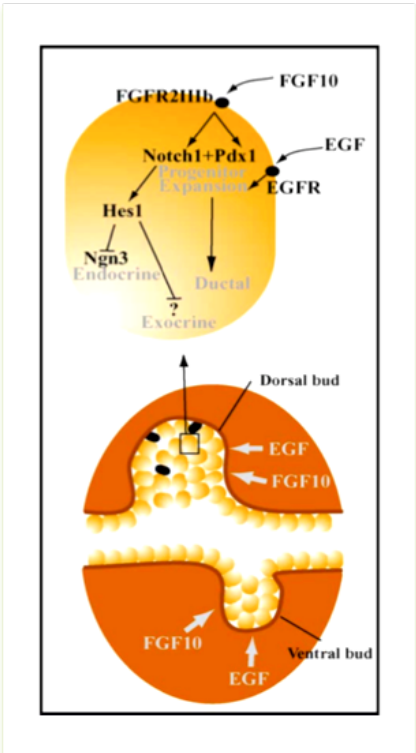

Figure 5. Inductive signals during pancreatic morphogenesis and the Notch pathway.
(Extracted from Grapin-Botton, A. (2005))

Ngn3. If Ngn3 expression is abolished, Hes1 expression is maintained. This is indicative that Notch signaling may be beyond the lateral specification model.[6] This alternative role may be related to the maintenance of the precursor status of the cells and may be under the control of the mesenchymal inductive signals. In mice in which FGF10 is overexpressed in cells expressing Pdx1, an upregulation of both Notch1 and Notch2 is observed, together with the upregulation of Hes1 and the downregulation of Ngn3 expression.[30] So, mesenchymal FGF10 signaling induces Notch signaling in the endoderm and represses differentiation. This is reiterated by early activation studies of the Notch pathway that prevents the differentiation of both endocrine and exocrine pancreas, by keeping the progenitor cells trapped in their status.[41]

1.2.5. Pancreatic Development: Wnt Signaling

Canonical Wnt signaling and β-catenin play important roles during development, in the maintenance of the stem cell niche, and during tumorigenesis. Several Wnt, Frizzled, and secreted frizzled-related proteins (sFRP) have been detected in the mesenchyme and pancreatic epithelium during embryonic development. Early forced expression of some Wnt proteins (namely Wnt1 and Wnt5a molecules) in Pdx1-expressing cells results in severe pancreatic defects and, in some cases, shifts in the anterior-posterior endoderm patterning. These results suggest that Wnt signaling may also play a role in the specification of regional identity within the foregut endoderm.[42,43] Recent studies took profit from different transgenic mice strains to better understand the precise regulation of Wnt signaling during early embryonic development and its impact on pancreatic organogenesis.

The canonical Wnt signaling pathway is activated through the recognition of Wnt ligands by their seven transmembrane domain Frizzled receptors or LRP (low density lipoprotein-related protein) co-receptors, which results in the inactivation of the so-called destruction complex, composed by adenomatous polyposis coli (Apc), Axin and the serine/threonine kinase Gsk3-beta. The role

of this complex is to phosphorylate β-catenin and lead it to its consequent proteolysis. When Wnt ligands bind their receptors, they cause Disheveled (Dsh) to inhibit Gsk3-mediated β-catenin phosphorylation. Thus, with the destruction complex inactivated upon Wnt signaling, active unphosphorylated β-catenin accumulates in the cytosol and translocates to the nucleus where, in collaboration with Lef/Tcf transcription factors, it activates the expression of certain target genes such as c-Myc and cyclin D1.[44] Lef/Tcf components of the complex are responsible for the DNA binding, whereas the β-catenin counterpart contributes with its transactivation domain located in its carboxy-terminal region. To this β-catenin-transactivation domain binds p300, a known transcriptional coactivator involved in cellular events such as chromatin remodeling and protein acetylation.[45,46] Actually, p300 acetylates β-catenin which increases its binding affinity to Tcf4.[47] The p/CAF (lysine acetyltransferase) transcription coactivator also acetylates β-catenin, increasing its stability and promoting its nuclear translocation.[48] In the absence of Wnt signal, the Lef/Tcf transcription factors recruit transcriptional repressors such as Groucho/TLE that promote histone deacetylation and chromatin compaction, preventing the expression of the Wnt target genes.

β-catenin is also a cytoskeletal component of the E- cadherin-mediated cell-cell adhesion system, and there are some evidences that the two β-catenin functions are mutually exclusive.[49]

In the last few years, new insights into the variety of β-catenin complexes and functions have emerged. For instance, in neural precursors FGF signals through phosphatidylinositol-3 kinase (PI3K) and inactivates Gsk3β, allowing the nuclear accumulation of β-catenin in a Wnt-independent manner.[50] In this model, β-catenin is found in complex with the Notch intracellular domain and, together, they positively regulate Hes1 expression, which is essential for neural stem cell maintenance. Another study has shown that the LKB1 serine/threonine kinase acts upstream of β-catenin by directly inhibiting Gsk3β.[51] So, apart from Wnt ligand signaling, other molecules can regulate the pathway by interfering

with its effectors. Liver receptor homolog 1 (LRH-1, or NR5A2) is one additional β-catenin interactor that increases the specific induction of cyclin D1, a Wnt target gene, promoting cellular proliferation.[52] In another important study, a physical interaction between β-catenin and the bHLH transcription factor MyoD (myogenic differentiation factor) was shown to be required for myogenic differentiation. This interaction, and not the β-catenin/Tcf complex, would increase MyoD binding to the E-boxes and specifically raise its activity.[53] Given the homology among bHLH factors, it is conceivable that – similarly - β-catenin could regulate the transcriptional activity of other tissue-specific bHLH factors. Hence, Wnt and β-catenin are definitely major cellular regulators of differentiation interacting with a wide variety of other signaling molecules, depending on the initiation signals and the interactors involved.

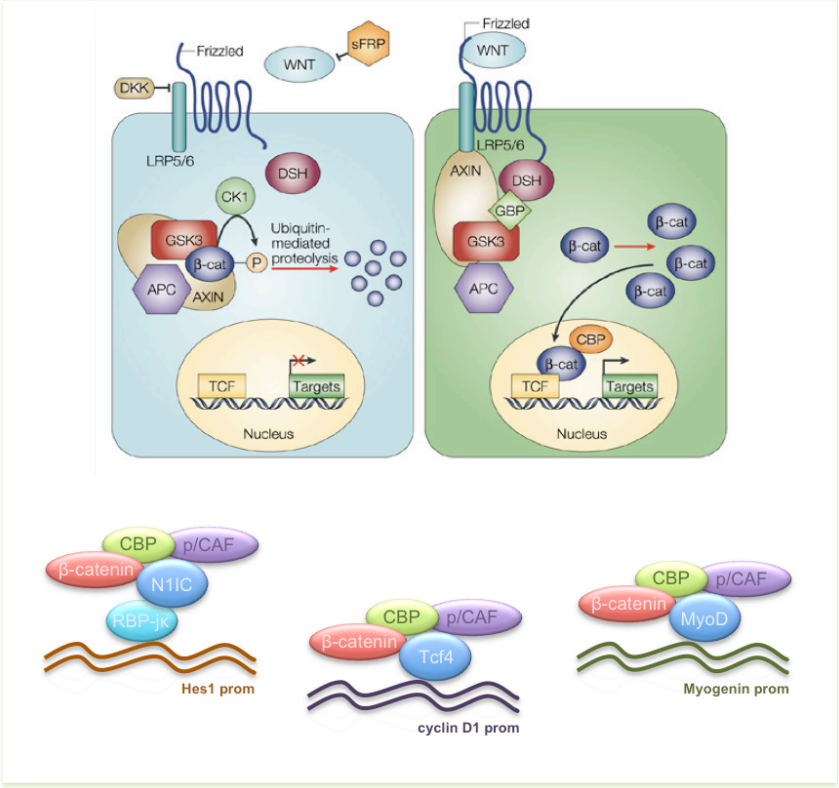

Figure 6. Schematic representation of the canonical Wnt signaling pathway in its inactive and active status, and three putative β-catenin complexes known to target distinct genes. (Adapted from Moon, R.T. et al. (2004))

Wnt signaling in pancreatic development.

During embryonic development, the early pancreatic epithelium expresses mainly unphosphorylated β-catenin, which is a feature of activation of the canonical Wnt signaling pathway. In fact, β-catenin appears to be a cell-autonomous requirement for the specification of acinar cells during embryonic development, since its early abrogation results in an almost complete absence of acinar cells.[54,55] β-catenin deletion in adult acinar cells does not affect the exocrine compartment or its functions.[55] Indeed, these studies indicated that β-catenin signaling was required for the proliferation of the early Ptf1a+ pancreatic progenitor cells. However, lack of β-catenin did not result in premature differentiation or apoptosis, suggesting that the canonical Wnt signaling is involved in the specification or differentiation of the acinar cells. Since these cells comprise the majority of the pancreas, mice in which β-catenin has been disrupted have pancreatic hypoplasia but, surprisingly, unaltered endocrine function.[54,55] Furthermore, in mice in which Apc was specifically deleted in Pdx1 expressing cells - allowing the free accumulation of β-catenin and consequent Wnt signaling activation - an acinar hyperplasia was observed with no significant changes in the islets.[56] Consistently, conditional inactivation of c-Myc - a key Wnt target gene - results in a reduction of the pancreatic developing buds and the consequent pancreatic hypoplasia, with a slightly accelerated differentiation of pancreatic epithelial cells into endocrine cells in the early embryo. This suggests that c-Myc is required for expansion of the exocrine progenitors.[56,57] Likewise, a recent study has shown that conditional c-Myc inactivation in the early developing pancreas promotes transdifferentiation whereby acinar epithelial cells become mesenchymal adipocytes.[58]

Finally, Wnt signaling activity may as well influence endocrine cell proliferation.[59] By selectively inducing an active β-catenin expression or deleting it from the insulin producing β-cells, it has shown that in this cellular context Wnt signaling stimulates the expression of multiple cell cycle regulators such as the cyclins D1 and D2 through the Pitx2 factor, enhancing cell

proliferation. Accordingly, another study has shown that pancreas-specific β-catenin deletion in developing mice results in a reduced number of Langerhans' islets. However, β-catenin signaling does not seem to be active in the adult pancreas and is not required for neither β-cell function nor homeostasis.[60]

Therefore, an accurate spatiotemporal regulation of Wnt signaling activity is required for proper pancreas formation and to maintain the pancreatic endoderm identity. Also during embryogenesis, cell-autonomous β-catenin expression is a requisite for acinar and endocrine cellular development.

Wnt pathway regulation.

The Wnt/β-catenin signaling is regulated at different steps of the pathway. For instance, the Secreted Frizzled-related proteins (sFRP) and the Wnt inhibitory protein WIF bind Wnt extracellularly which results in a signaling downregulation;[61,62] the DKK proteins are LRPs ligands that specifically antagonize the Wnt signaling,[63] and; the Shisa are transmembrane proteins found in the endoplasmic reticulum that bind Fz before the receptors reach the cell surface, preventing Wnt pathway activation.[64] At the destruction complex level, the PP1 serine/threonine phosphatase dephosphorylates Axin disassembling the complex,[65] whereas Diversin binds Axin and facilitates the β-catenin phosphorylation by recruiting CK1ε.[66] Once β-catenin is phosphorylated and translocated to the nucleus, different cellular mechanisms prevent its activity. For example, the endogenous dominant-negative dnTCF1/dnLEF1 isoforms lack the β-catenin binding domain and antagonize Tcf4.[67,68] A number of nuclear β-catenin antagonists have also been described; those bind it and prevent β-catenin to complex with TCF or with its coactivators, such as the β-catenin interacting protein ICAT, Duplin, or Chibby.[69,70,71]

2. Pancreatic acinar cells

Acinar cells are highly specialized in synthesizing the mRNAs coding for digestive enzymes, which are stored intracellularly in zymogen granules as proenzymes. The high hydrolytic activity of these enzymes needs to be tightly controlled and this is done so because many of the enzymes need to be processed in order to be active and thanks to the presence on intracellular and extracellular inhibitors of trypsin, the first enzyme involved in zymogen activation. Acinar cells respond to secretagogues and secrete zymogen granule contents thus initiating the digestive process in the intestinal lumen.

2.1. The regulatory elements of genes coding for digestive enzymes

The expression of the genes coding for the digestive enzymes (i. e. amylase, elastase) is under the control of transcriptional complexes that interact with conserved sequences localized in their promoters, which are active exclusively in the pancreas.[72,73] The expression of the corresponding mRNAs starts as early as day E10.5 during murine embryonic development, and transcriptional control is the principal mechanism regulating their differential accumulation in acinar cells.[74] Footprint analysis of the 5'-flanking regions of some of the pancreatic enzyme genes showed multiple sites of protein-DNA interaction.[75] For instance, in the elastase I promoter, the region between -72 and -205 comprises three elements that are necessary for the normal expression of these genes in acinar cells, in vitro and in vivo.[76] A 13-nucleotide motif is conserved between the 5'-flanking regions of the pancreatic enzymes genes.[77] This region is known as the "A" element, and it is a bipartite sequence that has a TC box (TTTCCC) and an E box (CACCTG), the latter being a consensus sequence for the binding of bHLH proteins.[76] This 5'-regulatory element can confer acinar specificity to gene expression. Two components from the PTF1 (Pancreas Transcription Factor 1) heterotrimeric oligomer complex – Ptf1a and RBPL/RBPJ - bind to

and mediate the activity of the "A" element.[78] The "B" element has a binding sequence for the HOX-like factor Pdx1, present both in the acinar and the endocrine β-cells of the adult pancreas. In acinar cells Pdx1 is found in complex with the Pbx1b and MEIS2b TALE class homeodomain factors on the B element of the elastase I gene, whereas in the endocrine cells Pdx1 binds to B element-like sequences to regulate the expression of genes such as insulin.[79] The PDX1/Pbx1b/MEIS2 trimer complex increases the activity of the PTF1 complex on digestive enzyme promoters in acinar cells. The activity of the elastase I promoter requires the functional cooperation between the trimer bound to the "B" element and the PTF1 complex bound to the "A" element.[80,81] The "C" element is a non-specific enhancer that increases the activity driven by the other elements, probably by binding a general transcription factor.[79,82,83] Upstream in the 5' flanking region, from -206 to 500, a repressor domain is found that silences the B element in the endocrine pancreas, therefore contributing to the acinar cell-specific expression (Figure 7).[79]

Figure 7.
A) Scheme of the general regulatory elements located in the promoter regions of the acinar specific genes, such as the digestive enzymes;
B) The two known compositions of the PTF1 complex.
(Adapted from Beres, T.M. et al. (2006) and Liu, Y., MacDonald, R.J. & Swift, G.H. (2001))

2.2. The PTF1 transcriptional complex

The PTF1 complex was firstly described to comprise two ubiquitous class-A bHLH transcription factors (HEB or E2A) and a tissue-specific bHLH, Ptf1a/p48. The group of R. MacDonald showed that, indeed, the PTF1 complex comprises only one ubiquitous class-A bHLH (either E2A or HEB), Ptf1a/p48, and a third protein, RBP-Jκ.[72,84] More recently, it was shown that the composition of the complex could also consist of RBP-L instead of RBP-Jκ. The former is homologous to RBP-Jκ but, unlike it, is unable to interact with Notch IC.[85] The two distinct compositions of the complex are related to the different roles played by Ptf1a/p48 during lifetime. As it will be addressed in the next section, RBP-Jκ is though to be involved in the early functions of the complex during embryonic development, whereas RBP-L would be required later for the full expression of the acinar differentiation genes.

Ptf1a/p48 expression is restricted to the pancreas in the adult and it is necessary, although not sufficient, for the activation of the acinar differentiation program.[86] Overall, the data indicate that Ptf1a/p48 is required for exocrine differentiation.

2.3. The dynamic role of Ptf1a/p48

Ptf1a (Pancreas-specific transcription factor 1a, or p48 as it will be designated hereafter) is a unique class II bHLH transcription factor, highly conserved among mammalians and with homologues found as early as in the zebrafish and fugu genomes but not in invertebrates.[87] It is a 328 aa protein that shares homology only with other members of the bHLH-class transcription factor family in its DNA-binding domain, comprised by the amino acid residues 165-220. The amino- and the carboxy-regions of p48 share no homology with other known proteins apart from its orthologs.

In humans, the p48 gene is located in chromosome 10, in the 10p12.2 chromosomal band. Its sequence is coded by two exons. The NCBI accession numbers are NM_178161.2 and NP_835455 for mRNA and protein, respectively. During embryonic development, p48 is found expressed in the dorsal part of the neural tube, in the pancreas, and in the central nervous system in the retina and cerebellum. (For reference go to the following webpage: http://www.ncbi.nlm.nih.gov; PTF1A pancreas specific transcription factor, 1a - GeneID: 256297).

As mentioned before, p48 is required for the activation of the expression of the genes coding for the digestive enzymes. In addition to its role in the adult pancreas, p48 is now known to be required for pancreas formation, since *p48*$^{-/-}$ mice lack both exocrine and endocrine pancreas. Pancreatic precursor cells lacking p48 expression acquire an intestinal fate.[24] Furthermore, a recent study

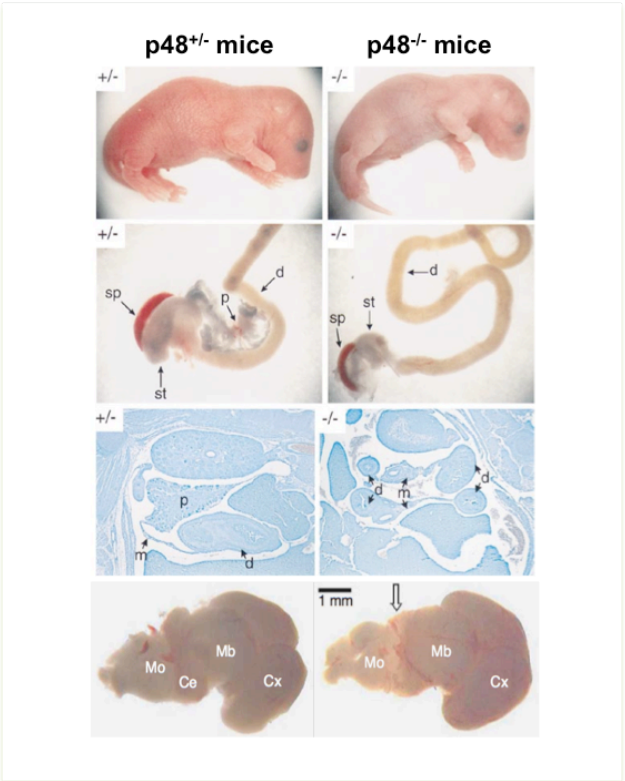

Figure 8. *Ptf1a/p48* heterozygous and null mice, showing the complete lack of pancreas and cerebellum. (Adapted from Krapp, A. et al. (1998), and from Sellick, G. S. et al. (2004))

has shown that the correct *p48* gene dosage is crucial for the proper pancreatogenesis: transgenic mice with a hypomorphic allele for *p48* had small pancreatic developing buds, with less progenitor cells, a decrease in the β-cell number, and a disorganization of the Langerhans' islets.[34] These findings support the notion that p48 plays a double role: first in the specification and expansion of pancreatic precursors, and later in exocrine differentiation.

Recent analyses of *p48*$^{-/-}$ mice have also shown that Purkinje cells do not develop, leading to cerebellar hypoplasia.[88] In agreement with these observations, two mutations in the carboxy-region of *p48* have been found in association with the development of permanent neonatal diabetes mellitus in humans, and the agenesis of both pancreas and cerebellum.[89] Moreover, during the embryonic development p48 is also expressed in the spinal cord, the hindbrain, and the retina.[87,90] P48 induces a GABAergic phenotype, playing a key role in the specification of a GABAergic versus glutamatergic neuronal cell fate.[91,92,93] p48 is expressed in the precursor neuron cells, and its interaction with RBP-Jκ is required to induce the generation of the GABAergic inhibitory neurons.[91,94] Therefore, p48 is required for pancreatic specification and acinar cell differentiation, for cerebellar organogenesis and for GABAergic neuron specification.

In the developing murine pancreas, p48 expression starts in the nascent buds at embryonic day E9. The very first event that leads to its expression is currently unknown but, once it is produced, it binds to a ubiquitous bHLH-type factor (either HEB or E2A) and to RBP-Jκ. This "PTF1-J" form of the complex then binds to a 5'-regulatory region of the p48 gene and enhances p48 expression in a positive feedback loop, in an autoregulatory fashion. For instance, once p48 expression is triggered, this autoregulatory loop is sufficient to maintain p48 expression throughout the early days of embryonic development. Later, during the secondary transition at around E13.5, p48 expression strongly increases alongside with the induction of RBP-L driven by means of the PTF1-J complex. In time, RBP-L will replace the RBP-Jκ form in the complex, and this ultimate

"PTF1-L" complex will be the one in charge for the maintenance of the expression of the acinar specific genes.[95]

Since p48 and RBP-Jκ can be found bound to the area III of the *Pdx1* regulatory region, the PTF1-J complex can regulating not only the expression of p48 but also the Pdx1 transcription factor in pancreatic precursors.[96,97] In this manner, p48 plays a crucial role in the specification of $Pdx1^{+}p48^{+}$ pancreatic progenitors. Area III of the *Pdx1* promoter has been shown to be responsible for its early embryonic expression. Interestingly, in progenitor pancreatic cells from transgenic mice with reduced *p48* gene dosage, a decrease in the Pdx1 expression levels is seen.

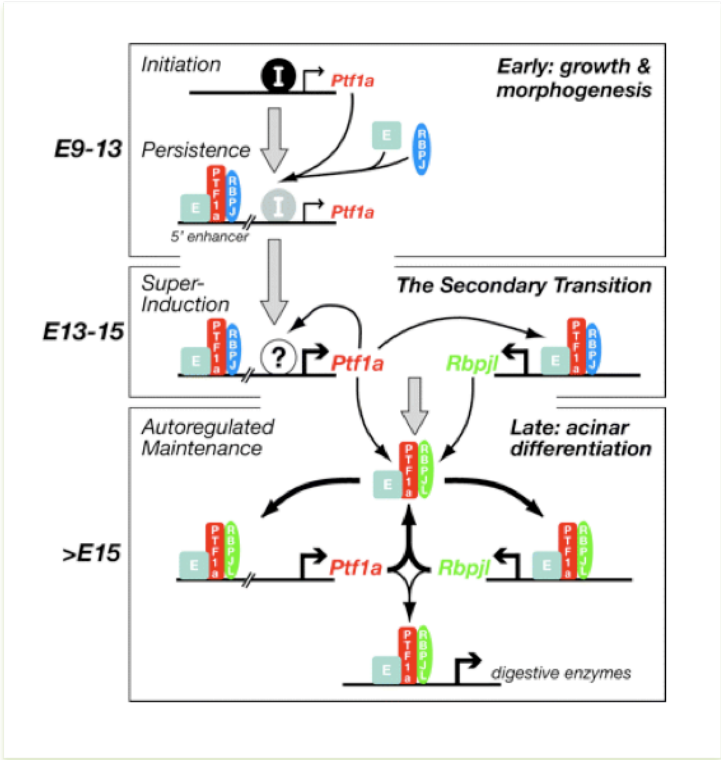

Figure 9. The PTF1-J and PTF1-L complexes.
The molecular events that initiate p48 expression are currently unknown (I), but once p48 is expressed, it binds a class E bHLH protein (E) and RBP-Jκ, forming the PTF1-J complex. Through this complex, p48 autoregulates its expression levels and maintains them after the loss of the first inductive signal. During the secondary transition, RBP-Jκ is exchanged by RBP-L, constituting the PTF1-L complex that will burst the transcription of the acinar specific digestive enzymes. (Extracted from Masui, T. et al. (2008))

In the adult the only established role for p48 is the maintenance of acinar gene expression in the pancreas, though this conclusion awaits the development of a conditional p48 knock out mice model. Work from our lab has also shown that p48 is downregulated during acinar-to-ductal transdifferentiation in vitro and in vivo, and that ectopic p48 expression results in reduced cell proliferation, suggesting that this protein couples cell differentiation and proliferation in the exocrine pancreas. The antiproliferative effects do not seem to depend on its transcriptional activity: the p48 C-terminus lacking the bHLH domain is necessary and sufficient for this effect while it acts as a dominant negative for the transcriptional activity of the PTF1 complex on the elastase promoter.[98]

3. Pancreatic Tumors

3.1. Pancreatic Ductal Adenocarcinoma (PDAC)

Pancreatic Ductal Adenocarcinoma (PDAC) is the most frequent type of pancreatic tumor, nowadays being the 4^{th} – 5^{th} cause of mortality due to cancer in the occidental world. PDAC is known for its extremely poor prognosis and survival data place it as the tumor with poorest prognosis.[99] The mean survival of patients with PDAC is 5-6 months and the 5-year survival stands between 3%-5%. The lethality of this neoplasm relates to its rapid dissemination to the lymphatic system and other distant organs. The reasons for such an aggressive behavior are presently unknown and may rely on the anatomical localization of the pancreas, its histological structure, the associated genetic alterations or a combination of these factors. PDAC is essentially a fatal disease and cure is only possible when the cancer is detected very early, at a stage when it is still circumscribed within the pancreas and, therefore, suitable for complete resection. Even when small tumors of 2 cm diameter are diagnosed, long-term survival is only of 20%. Strategies to detect PDAC early and to identify new drugs are desperately needed.

3.1.1. PDAC: Histology and Progression

Pancreatic ductal adenocarcinoma was named based on the presence of the duct-like morphology of the tumor glands. It commonly arises in the head of the pancreas and infiltrates the surrounding lymphatic system, the spleen, and the peritoneal cavity, and metastasizes to the liver and lungs. The tumor is characterized by a strong desmoplasia with a dense stroma of fibroblasts and inflammatory cells.

The putative preneoplastic lesions that may give rise to the PDAC have been called Pancreatic Intraepithelial Neoplasia (PanIN hereafter) and they display

variable degrees of cellular atypia and differentiation.[100] There are three distinct types of PanIN lesions (PanIN-1, -2, and -3) ranging from low-grade to high-grade dysplasia. The low-grade PanIN-1 lesions may be found in association with PDAC and other less common types of pancreatic tumors, such as acinar cell carcinomas, mucinous cystic neoplasms, serous cystoadenomas, solid pseudopapillary neoplasms and pancreatic endocrine tumors. These lesions can also be found in the pancreas of individuals without pancreatic cancer, especially in association with ageing. PanIN-1A and -1B lesions have a flat or papillary mucinous epithelial monolayer, respectively. PanIN-2 lesions display an increased degree of cellular atypia and PanIN3 correspond to carcinoma in

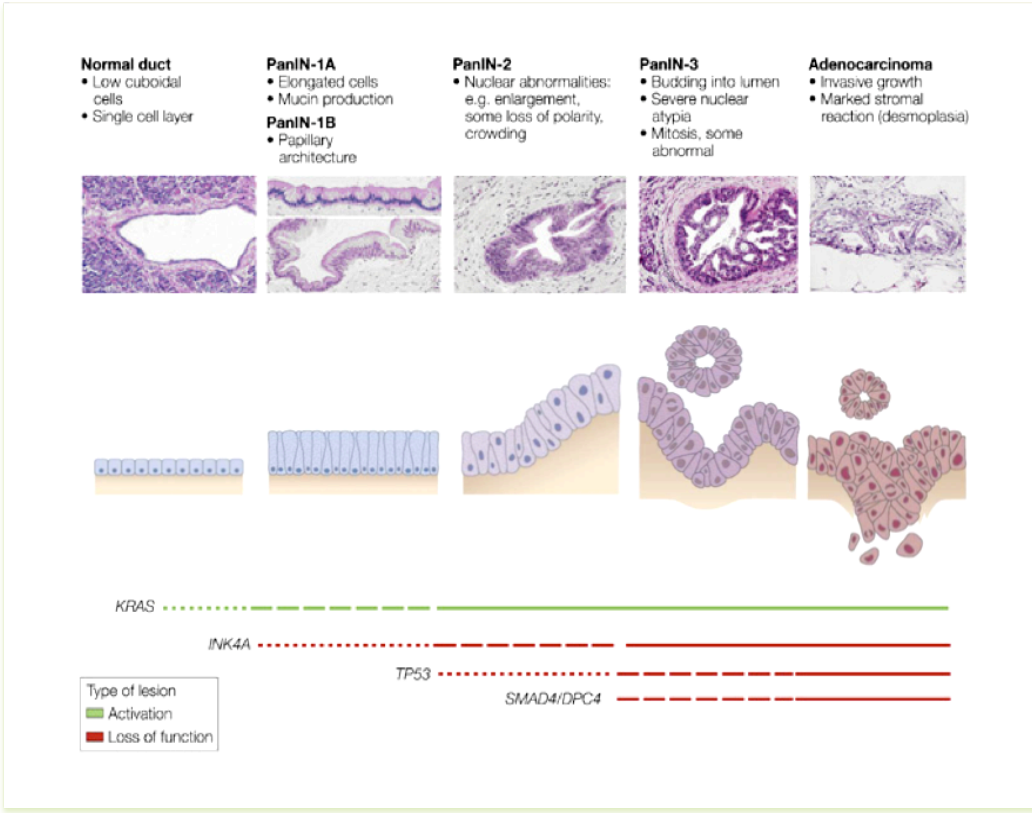

Figure 10. A model for Pancreatic Ductal Adenocarcinoma progression depicting the most common genetic alterations associated with the different neoplastic lesions.
(Adapted from Bardeesy, N. & DePinho, R.A. (2002) and Hezel, A.F. et al. (2006))

situ and are only exceptionally found in the absence of overt cancer.[100] The occurrence of PanIN lesions nearby pancreatic tumors and the molecular phylogenetic analysis of human PDAC are arguments in favor of a linear progressive model for the PDAC. However, a re-evaluation of this model may be needed and an "alternative hypothesis" has been recently proposed. In this model, it is hypothesized that early PanINs are actually hyperplastic lesions that display very low potential for progression and are in an "arrested state", protected from progressing into PDAC by oncogenic-induced senescence. By contrast, this model proposes that K-ras mutations occurring in cells displaying preexisting genetic changes in tumor suppressors might lead "directly" to pancreatic cancer.[101]

3.1.2. PDAC: Epidemiology

Epidemiologic studies have shown that the incidence of PDAC increases with age and is associated with lifestyle and environmental factors such as smoking, obesity and diabetes.[102] In addition, an increased risk of PDAC is associated with sporadic and hereditary chronic pancreatitis (CP). Inherited alterations in the cationic trypsinogen gene *PRSS1* - which has been implied in acute pancreatitis - has been associated with the development of the neoplasia.[103] Inactivating mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) are another risk factor for the appearance of pancreatic insufficiency risk. This clinical condition is characterized by tissue damage as a consequence of the permanent inflammation, and it increases the risk of neoplasic transformation and PDAC development. Germline mutations in a few additional genes can also contribute to increase the risk of PDAC in a small number of patients (see below).

Chronic pancreatitis appears as a consequence of recurrent bursts of necroinflammatory pancreatitis. Histologically, the stroma of CP and PDAC share some features: both consist of cell-poor tissue, with an extracellular matrix rich in collagen, and an abundant inflammatory cell infiltration.

Pancreatic stellate cells (PSC) are major contributors to the fibroblastic proliferation and the fibrosis in both chronic pancreatitis and PDAC. These cells resemble the liver vitamin-A storing stellate cells, they are normally quiescent, and when activated they express α-SMA, desmin, glial fibrillary acidic protein (GFAP), collagen I and III, laminin, and fibronectin. Pancreatic stellate cells produce both matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), important for extracellular matrix remodeling.[104] PSC are now established to play a central role in fibrogenesis during pancreatic injury, upon activation both by toxic factors such ethanol or its metabolites, or by cytokines released during the inflammatory processes.[105,106] Furthermore, Transforming (TGF), Platelet-Derived (PDGF), and Vascular-Endothelial (VEGF) Growth Factors are released by pancreatic cancer cells and also activate the PSC, contributing to the stromal reaction in the pancreas cancer.[107]

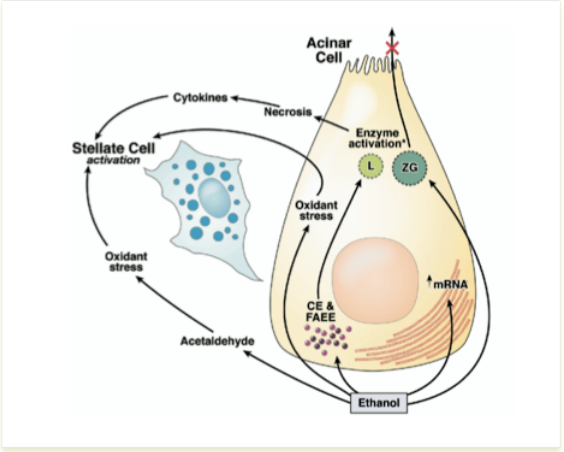

Figure 11. Acinar and pancreatic stellate cells cross talk in pancreatitis. Ethanol, its metabolites, or oxidative stress induce pancreatic necroinflammation and abnormal activation of the pancreatic stellate cells. (Extracted from Witt, H. et al (2007))

3.1.3. PDAC: Cellular origin

PDAC cells have morphological characteristics that are similar to those of the normal pancreatic ducts. However, the cells in which these tumors arise have not been conclusively identified yet, at least in humans. Some evidences suggest that they arise in ductal complexes originated through a transdifferentiation

event whereby acinar cells acquire a ductal phenotype, then becoming targets for the carcinogenesis process. This acinar-to-ductal transdifferentiation has been described in vitro and in vivo; the latter typically occurs in the context of chronic pancreatitis. In fact, in primary cultures of human exocrine pancreas a downregulation of the acinar-specific genes and an upregulation of ductal markers is seen in few days, meaning that these cells in culture rapidly acquire a ductal phenotype.[108,109] In this acinar-to-ductal transdifferentiation process, acinar cells first dedifferentiate into nestin expressing precursor-like cells and afterwards start to express ductal markers such as cytokeratins.[109] In patients with chronic pancreatitis, especially those with hereditary chronic pancreatitis, a substitution of the acinar compartment by ductular structures is often observed. Both the sporadic and hereditary conditions are associated with an increased risk of PDAC and in the latter clinical group, it has been estimated as a 60-fold increase in risk.[103] A role for transdifferentiation events in PDAC generation has also been supported by studies of chemically induced tumors in mice. This is the case of the model where TGF-alpha is overexpressed under the control of the metalothionein promoter (MT) and a massive acinar-ductal metaplasia is produced.[110,111,112] A definitive and very important recent research has

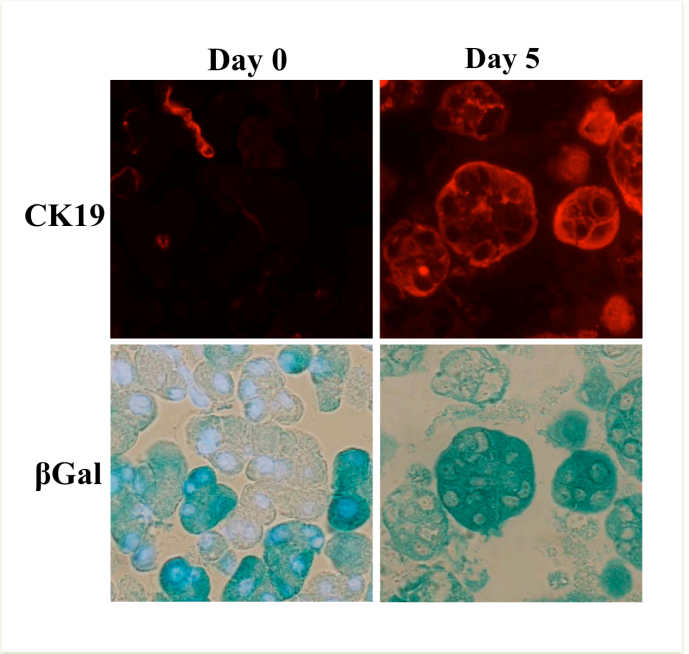

Figure 12. Acinar-to-ductal metaplasia in primary cultures of Ela-CreERT2; R26R-LacZ mouse pancreas, at days 0 and 5. Acinar cells are stained in blue, corresponding to the Ela-CRE transgene expression. Upregulation of the ductal marker cytokeratin 19 is observed. (A. Pinho and I. Rooman, unpublished data).

demonstrated that activation of the oncogene *K-Ras*(G12V) in acinar cells can lead to PanIN and PDAC, resulting from a transdifferentiation phenomenon from acinar to ductal-like phenotype, specially during embryonic development. Moreover, experimental induction of a chronic pancreatitis-like condition, together with K-Ras activation in the adult, promoted the full spectrum of PanIN lesions and PDAC.[113] Therefore, a transdifferentiation process may participate in the first steps toward pancreatic tumor development, and a detailed understanding of the mechanisms that control cell differentiation and homeostasis in the pancreas is required.

3.1.4. PDAC: Common Genetic Alterations

PDAC is thought to arise through the accumulation of genetic alterations. Four genes have been identified that play a fundamental role in the development of PDAC.[100,114,115,116] Activating mutations of the *K-Ras* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) gene are found in 80-90% of invasive PDAC, in 75% of PanIN-3 lesions, in 39% of the early PanIN-1 lesions, and also in the pancreas from patients with chronic pancreatitis.[117,118] Because of their occurrence in preneoplastic lesions, *K-Ras* mutations are considered to be an initial event in the progression of the tumor. Mutation, deletion of one or both alleles, or promoter hypermethylation of the *INK4A* locus have been seen in advanced PanIN lesions with variable levels of dysplasia.[119] Moreover, germline mutations in *INK4A* have been associated with the Familial Atypical Mole-Malignant Melanoma (FAMMM) syndrome, whose patients are at a 13-fold increased risk of developing PDAC.[120,121] Recent murine models have shown a genetic cooperation between *K-Ras* and *Ink4a* inactivation in the induction of pancreatic neoplasia.[122] The INK4A locus codes for another tumor suppressor gene: *ARF*. Mechanistically, $p16^{INK4A}$ arrests cell cycle through an inhibition of cyclin-dependent kinase (CDK)-mediated RB phosphorylation, whereas $p19^{ARF}$ inhibits MDM2-mediated p53 proteolysis. *Tp53* is another tumor suppressor gene found frequently mutated or deleted in PDAC.[123] p53 is normally expressed at low levels in normal cells, and it is upregulated in response to

DNA-damage and other cellular stresses, inducing cell cycle arrest, DNA repair, senescence, and apoptosis.[124,125,126] *Tp53* mutations are found almost exclusively in PanIN-3 and PDAC, indicating that they are a late event.

SMAD4 (SMAD family member 4) inactivation has also been associated with progression of the neoplasia, with 90% of PDAC showing loss of heterozygosity for its locus, and up to 50% of the PDAC with homozygous deletions and approximately 10% having additional inactivating mutations.[127] Because SMAD4 is a player in the TGF-beta signaling pathway, its mutational inactivation may imply the loss of response to the antiproliferative regulatory signals of TGF-beta.

A recent pancreatic cancer genome analysis identified 12 cellular processes that are altered in the majority of the pancreatic cancers. All of the analyzed tumors showed at least one alteration in one of the genes coding for a member on such processes, among them: apoptosis; regulation of the G_1/S cell cycle phase; Hedgehog; KRAS; TGFβ; and Wnt/Notch signaling. Although each individual mutation was not frequently found amongst the tumors, this study demonstrated that the pancreas cancer results from a variety of genetic alterations that predominantly affect a small number of cellular pathways.[128]

3.1.5. Wnt signaling in pancreas cancer

About 65% of pancreatic cancers have an aberrant β-catenin activation.[129,130,131] In ductal and acinar pancreatic adenocarcinoma cell lines, the Wnt signaling activity has been shown to be low but detectable.[132] More recently, a study of a large cohort of pancreatic ductal adenocarcinoma patients identified an aberrant β-catenin expression in 78% of the cases (13% with nuclear accumulation and 65% presenting loss of membranous β-catenin expression and/or increased cytoplasmic expression).[133] The same research team later described that activation of β-catenin within a small number of cells of the early pancreatic epithelium in a transgene mice model induces pancreas tumor formation.

Surprisingly, when the β-catenin activation occurs in the presence of an activating mutation in *K-Ras*, the usually observed pancreatic intraepithelial neoplasia (PanIN) lesions are absent and the mice develop distinct ductal neoplasms.[134]

Moreover, functional blockade of *Smad4*, a gene whose inactivation is associated with the later stages of pancreatic cancer progression, was demonstrated to downregulate the β-catenin activity by inducing its ubiquitination and proteasomal degradation. This mechanism was also associated with a decrease of the tumorigenic potential.[135]

3.1.6. Intraductal Papillary Mucinous Tumors (IPMT)

Intraductal papillary mucinous tumors (IPMT) are epithelial neoplasms, usually with papillary projections, that grow inside the ductal system. The epithelium is tall and columnar, containing mucin-producing cells.[136] IPMT are multifocal, and histologically they may range from adenoma, to dysplasia, to carcinoma in situ, to invasive carcinoma. Typically, IPMT involve the main pancreatic duct, however, branch-duct IPMT also occur and are less aggressive than the main-duct IPMTs.[137,138] Although they account for only 1% of all the pancreatic cancers, they have been associated with PDAC as well as endocrine tumors. The 5-year survival for noninvasive IPMT is of 80%, whereas for malignant invasive IPMT it drops to 50%.[139] Global gene expression analysis of IPMT revealed that some of the overexpressed genes in these tumors are also upregulated in PDAC. In fact, *K-Ras* activating mutations are early events in IPMT, p16 loss is common in high-grade tumors, and *Tp53* inactivation occurs in some severe/high grade IPMTs.[140]

3.2. Other Pancreatic Tumors

Because of their low incidence, the less common tumors of the pancreas have not been so intensely studied as PDAC. However, due to their histological and molecular diversity, to study the molecular mechanisms involved therein may unveil properties that are key for pancreatic homeostasis.

3.2.1. Acinar Cell Carcinoma (ACC)

Acinar cell carcinomas account for approximately 1% of all pancreatic neoplasias and they are less aggressive than PDAC, with a mean survival of 19 months. These carcinomas may arise in any part of the organ, tend to be unifocal and well circumscribed, and are usually large in size (10 to 15 cm in diameter).[141] Histologically, they are solid, differentiated tumors with scanty stroma, which systemically secrete pancreatic enzymes such as trypsin, lipase or chymotrypsin, and have higher mitotic rates than normal acini. Interestingly, some studies revealed low levels of amylase found in the ACC.[142,143] The tumor cells maintain an acinar-like organization, nuclei with minimal pleiomorphism, and cytoplasm with fine zymogen granules. Acinar-endocrine mixed tumors and pancreatoblastomas are also tumors displaying acinar differentiation, with a similar clinical outcome in the adult in terms of long-term survival, metastases, and treatment response.[144] Unlike in PDAC, genetic alterations of the Wnt/β-catenin signaling pathway (namely, high frequence of *β-catenin* mutations and an even higher frequency of nuclear β-catenin accumulation) have been found in ACC. However, no relationship has been established with the common genetic alterations of PDAC such as *K-Ras*, *Tp53*, or *SMAD4*.[142,145]

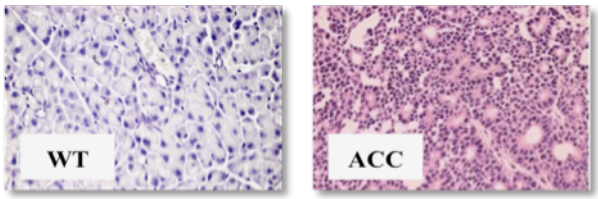

Figure 13. Histology of normal pancreas (WT) and acinar cell carcinoma (ACC).

3.2.2. Pancreatoblastoma

Pancreatoblastoma is a solid malignant neoplasm with most prominently an acinar differentiation pattern. It is the most common pancreatic neoplasia in childhood. Assessment of the prognosis is difficult due to its low incidence rate. As ACC, pancreatoblastomas may arise in any location of the pancreas, are usually well circumscribed, and are large in size. Moreover, a similar genetic signature is found, with mutations in the *β-catenin* in 50%-80% of the cases.[145] Pancreatoblastoma differs from ACC particularly because of the presence of squamoid nests and they may also be distinguished by an extensive lobulation, a stroma with high cellular density, and the maintenance of some endocrine and ductal components.[141]

3.2.3. Solid Pseudopapillary Tumors (SPT) of the Pancreas

Solid pseudopapillary tumors are another rare type of pancreatic neoplasia, with a higher incidence among young women. They are mostly localized to the pancreas with 10 to 15% of the patients presenting liver or peritoneal metastases. This clinical pattern of presentation results in a better outcome and a higher long-term survival of the patients.[146] They may have a solid and a cystic component, they present pseudopapillae typical for these lesions, and often show necrosis and hemorrhagic foci.[147] The tumors do not show any type of pancreatic cellular differentiation and their cellular origin is currently unknown. SPTs have high cellularity, with cells being uniform and polygonal, and non-cohesive. The most common features of these tumors are the loss of E-cadherin and the translocation of β-catenin to the nucleus.[148,149,150] It has been also reported that they display an increase in cyclin D1 expression, which is expected as a result of the β-catenin nuclear accumulation. The mitotic rates, however, are generally low, as in the normal pancreas.[151]

3.2.4. Pancreatic Endocrine Neoplasms (PEN)

Deriving from the neuroendocrine gut system, Pancreatic Endocrine Neoplasms account for 1% to 2% of all pancreatic tumors. Their incidence has been estimated to be of 0.3-1.6% but a systematic evaluation concluded that PENs can be detected in up to 10% of the autopsied pancreas.[152] Among other features, PENs can secrete a variety of hormones depending on the differentiation of the tumor cells. PENs appear to be asymptomatic, though it has been shown by immunohistochemistry that they all produce at least one hormone.[153] Indeed, these neoplasms are named after the hormone they produce. For instance, insulinomas (42% of PENs) produce insulin, gastrinomas (24%) produce gastrin, glucagonomas (14%) produce glucagon, VIPomas (10%) produce vasoactive intestinal peptide, and somatostatinomas (6%) produce somatostatin.[141] Usually, PENs contain scattered cells that express weakly acinar differentiation markers such as trypsin and chymotrypsin. The diversity of PENs is also reflected in the histological analysis. PENs can be small and circumscribed, or larger and multinodular, soft or sclerotic. The amount of surrounding stroma also varies. Some may show hemorrhage, necrosis, and cysts. From the different neuroendocrine pancreatic neoplasms, the poorly differentiated endocrine carcinoma is the less common but the most aggressive one. Their genetic alterations and the inherited component to them are completely different from those involved in exocrine tumors, both PDAC and the acinar-related neoplasms.

HYPOTHESIS

Our starting hypothesis was that different domains of p48 are involved in different functions, and that those functions are accomplished through two distinct types of mechanisms: some being dependent, and the others being independent, of the bHLH domain. The effects of p48 would rely not only on its interaction with the other members of the PTF1 complex but also with other proteins that regulate cellular proliferation and/or differentiation.

OBJECTIVES

The major objectives of this thesis project were:

1. To identify and characterize novel p48 partners that may be implicated in its functions in pancreatic organogenesis and/or exocrine differentiation;
2. To dissect the roles of the N- and the C- domains of p48;
3. To determine the role of ICAT as a modulator of p48 function.

MATERIAL AND METHODS

1. Plasmids

1.1. Yeast Two-Hybrid plasmids

For the LexA- yeast two-hybrid assays, the baits were constructed by insertion of the cDNA of interest into the EcoRI/BamHI restriction site of the pBTM116 plasmid (TRP marker), in-frame with a LexA DNA-binding domain.[154] The cDNAs coding for full length p48 and its fragments were amplified by PCR, using the following primers: p48-fw 5' TAT GAA TTC ATG GAC GCC GTG CTC CTG GA 3'; $p48^{\Delta N}$-fw 5' TAT GAA TTC ATG CAG CTG CGA CAA GCG GCC AA 3'; $p48^{\Delta N+bHLH}$ -fw 5' TAT GAA TTC ATG ATT AAC TTC CTC AGC GAG CTG 3'; p48-rev 5' TAT GGA TTC TCA GGA CAC AAA CTC AAA AGG TGG TT 3'; $p48^{\Delta C}$ -rev 5' CCC GGA TCC TCA CAG CTC GCT CGC TGA GGA AGT TAA TGT AGC C 3'. The designed primers add an EcoRI restriction site at the 5' end and a BamHI restriction site at the 3' end to the PCR products, which were cut with both enzymes and cloned into the pBTM116 plasmid (TRP marker) directly in frame with the LexA DNA-binding domain.[154] For the GAL4- yeast two-hybrid assays, the $p48^{\Delta N}$ coding cDNA was subcloned from the $p48^{\Delta N}$pBTM116 plasmid. To do so, this plasmid was

digested with EcoRI and SalI, the excised cDNA was fractionated by agarose gel electrophoresis, purified, and ligated into the digested pBD-GAL4 Cam phagemid (Stratagene), in frame with the GAL4 binding domain. The ability of the resulting fusion proteins to activate transcription by themselves was tested prior to selection as baits for the yeast two-hybrid assays.

1.2. Bacterial expression vectors

The plasmids encoding the ICAT and B-myc proteins fused to GST were a kind gift from Cara J. Gottardi (Northwestern University, Chicago, USA)[155] and Stephen R. Hann (Vanderbilt University, Nashville TN, USA)[156], respectively. The β-catenin expressing vectors were a kind gift from Antonio García de Herreros (IMIM-Hospital del Mar, Barcelona, Spain).

1.3. Mammalian expression vectors

For the Pull-Down assays, the plasmids coding for MEIS2b, Pbx1b, and Pdx1 were a kind gift from Raymond J. MacDonald (Department of Molecular Biology, The University of Texas Southwestern Medical Center, Dallas TX, USA). Midkine cDNA was obtained from RZPD (RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin, Germany) and subcloned into the pCMV2 plasmid (Stratagene, La Jolla, CA). HA-tagged p48 was expressed from a pcDNA3 plasmid (Invitrogen), Flag-tagged p48 was expressed from a pFLAGCMV2 plasmid (Sigma), and untagged p48 was expressed from a pIRESneo vector.[98,157] Plasmids were prepared that encoded for different p48 deletion mutants, and the cDNAs were amplified by PCR using the primers: p48-fw 5' TAT GAA TTC ATG GAC GCC GTG CTC CTG GA 3'; $p48^{296}$-rev 5' TAA GGA TCC GGC TAG CCA CC 3'; $p48^{270}$-rev 5' TAA GGA TCC AGT CAC GGG TCG C 3'; $p48^{236}$-rev 5' CCC GGA TCC TCA ACG AAT AAT AT 3'. After EcoRI/BamHI digestion, they were inserted into the pIRESneo plasmid (Clontech).

For co-localization experiments, a p48GFP (Green Fluorescent Protein) expressing plasmid was generated in our laboratory by Annie Rodolosse.[157]

The luciferase reporter plasmids were described elsewhere: $6xA_{26}$-luc[157], Ela200-luc and Ela500-luc, TOP- and FOP-Flash[158], p21-luc.[159]

Because 266 cells bear an elastase I/neomycin resistance transgene, the ICAT cDNA had to be subcloned to a mammalian expression vector that would confer another resistance suitable for the cloning selection. To generate cells stably expressing ICAT-Flag, the ICAT cDNA was PCR-amplified from the pcDNA3-FLAGICAT (Cara J. Gottardi) with the following primers: fw- 5' GGC TCG

AGA TGG ACT ACA AAG ACG ATG ACG ACA AG 3'; rv- 5' AAG GAT CCC AGC TAC TGC CTC CGG TCT TC 3'. After XhoI and BamHI restriction, the purified PCR product was inserted into the $pREP_4$ vector, which additionally codes for the hygromycin resistance gene (Invitrogen).

A bi-cistronic expressing vector coding for ICAT and GFP was prepared through a PCR cloning strategy using pGEX4T3.ICAT vector (Cara J. Gottardi) as template. ICAT was first amplified with the primers fw-kozac-ICAT- 5' GCC GCC ACC ATG AAC CGC GAG GGA GC 3' and rv-ICAT-FlagM2-5' CTT GTC GTC ATC GTC TTT GTA GTC CAT CTG CCT CCG GTC TTC CG 3'. By doing so, a Flag epitope was added to the carboxy-terminus. A second round of PCR amplification was performed with a new set of primers, in order to add EcoRI and XhoI restriction sites at the 5' and 3' end, respectively. The following primers were used: fw- 5'AAA GAA TTC GCC GCC ACC ATG AAC CG 3' and rv- 5' CGG CTC GAG CTT GTC GTC ATC GTC TTT GTA GTC 3'. After EcoRI and XhoI digestion, the PCR product was inserted into the pIRES-hrGFP-2a vector (Stratagene).

For the electrophoretic mobility shift assays (EMSA), the RBP-L coding vector used was a kind gift of Raymond J. MacDonald, and the p/CAF-Flag coding vector was a kind gift of H. Santos-Rosa (The Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Cambridge, U.K.).

The Flag-ICAT cDNA was PCR-amplified from the pcDNA3-FLAGICAT with the following primers: fw- 5' AAG GCT AGC ATG GAC TAC AAA GAC GAT GAC 3'; rv- 5' AAC CTC GAG TTA CTG CCT CCG GTC TTC 3'. After NheI and XhoI restriction, the purified PCR product was inserted into the pBI-L vector (Clontech) between the restriction sites NheI and SalI.

1.4. Lentiviral delivery vectors

To produce lentivirus coding for ICAT, its cDNA was PCR amplified using the following primers: fw-kozac-ICAT- 5' GCC GCC ACC ATG AAC CGC GAG GGA GC 3', fw- 5' AAA GAA TTC GCC GCC ACC ATG AAC CG 3' and rv- 5' AAC CTC GAG TTA CTG CCT CCG GTC TTC 3'. After the two-round PCR amplification, the PCR product was digested with EcoRI and XhoI and was cloned into the FG12 vector.[160] This vector also codes for the GFP protein, allowing to follow the lentiviral transduction either by FACS analysis or direct fluorescence microscopy. Because lentivirus are integrated into the cellular genome, the lentiviral expression is usually constantly maintained after cellular transduction.

2. Yeast two-hybrid interaction screenings

Two libraries were used in the screening for cDNAs encoding p48-binding proteins: a 20-22 week human fetal brain library (Clontech) was a kind gift of Susana de la Luna (Centre de Regulació Genòmica, Barcelona, Spain) and a mouse whole embryo E9.5 cDNA library was obtained from Dr. Junko Obata (Nara Institute of Science and Technology, Japan). The cDNA libraries, cloned into the $pACT_2$ vector (Clontech), were introduced by LiAc transformation into the L40 reporter strain [MATa trp1-901 leu2-3, 112 his3-D200 ade2 LYS:: (LexAop)4-HIS3 URA3:: (LexAop)8-LacZ; 3][154] expressing the fusion protein LexA.$p48^{\Delta N}$. Yeast transformants were selected on Trp- Leu- His- plates and clones were isolated during the 10 days of incubation following transformation. For the screening with the human fetal brain library, $2x10^5$ clones were analyzed; for the assay performed with the mouse whole E9.5 embryo cDNA library, $9.5x10^5$ clones were screened. After isolation on Trp- Leu- His- plates, clones were assayed for β-galactosidase activity by a semi-quantitative filter assay using X-gal as the substrate. For those clones clearly showing β-galactosidase activity (blue colour), the plasmid DNA was amplified and sequenced with primers $pACT_2$-fw- 5' CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CC 3' and $pACT_2$-rev- 5' AGT GAA CTT GCG GGG TTT TTC AGT ATC TAC GA 3'. The sequences obtained were used to identify the genes from which they came by using standard features of the Basic local alignment search tool (BLAST), and comparing to the PROTEIN database and the TRANSLATED database from the NCBI web site.

3. Cell culture

All the cells used in this work were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, L-glutamine, non-essential amino acids, 100 units/ml penicillin and 100 mg/ml streptomycin (Gibco, Gaithesburg, MD). HEK293 and Cos7 cells were obtained from the American Type Culture Collection (Rockville, MD). AR42J cells were established from an azaserine-induced pancreatic acinar tumor and were obtained from N. Vaysse (IFR Louis Bugnard, Toulouse, France); 266-6 cells are mouse epithelial cells derived from a pancreatic tumor induced by transgenesis with an elastase I/SV-40 T antigen fusion gene and were obtained from R. MacDonald.[161] These cells retain a partially differentiated phenotype, expressing detectable levels of some digestive pancreatic enzymes. The RWP-1 cell line was established from a human PDAC metastatic to the liver[162], and was obtained from N. Vaysse.

4. Proliferation assays

To generate pseudostable 266-6 cells expressing ICAT, $5x10^5$ cells were seeded overnight in 6-well plates and transfected in triplicate with 1 μg of the corresponding plasmid, using lipofectamine (Lipofectamine Plus Reagent, GIBCO, Gaithsburg, MD) following the manufacturer's instructions. Transfectants were selected in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS and 350 μg/mL of hygromycin until analyzed. At the end of selection, the total number of viable cells in each well was counted after staining with trypan blue.

To determine the proportion of cells in S-phase, $5x10^5$ Cos-7 cells were seeded in 100mm Ø dishes in complete DMEM plus 10% FBS. After seeding (24h), cells were transfected overnight with FuGENE6 reagent (ROCHE) with 2 μg of $pCMV_2$.p48-Flag expression vector and 2 μg of pCX.p/CAF-Flag plasmid, together with the FG12 plasmid coding for EGFP. After transfection (16h), the culture medium was changed and the cells were kept for 36h in DMEM without FBS. Sixteen h prior to analysis, FBS was re-added to the medium and – one hour prior to collection - cells were incubated with 10 μM of EdU component A (Click-iTTM EdU Flow Cytometry Assay Kits, Invitrogen). Cells were trypsinized, washed once with PBS, and separated using a Flow Citometry sorter FACSAria. The number of cells in S-phase was analysed as described by the manufacturer.

5. Pull-down assays

Glutathione-S-transferase (GST) fusion proteins were purified from IPTG-induced BL21 E. coli strain transformed with the corresponding plasmids (pGEX4T3.ICAT; pGEX2T.B-myc; pGEX6T3.p48). A 20 mL pre-inoculum culture was prepared overnight in LB medium, at 37 ºC with shaking at 200 rpm. The pre-inoculum culture was transferred to 200 mL of fresh LB and allowed to grow for 2h at 37 ºC, 200 rpm. Bacteria were induced by adding 0.3 mM IPTG to the growing culture from a 0.1 M stock stored at -20 ºC. The induced cultures were incubated for 3h at 37ºC and 200rpm, and afterwards pelleted by centrifugation for 10 min at 3000 rpm, at 4 ºC. The pellets were ressuspended in 10 mL of pre-chilled autoclaved STE buffer (10 mM Tris-HCl, 1 mM EDTA and 100 mM NaCl) plus protease inhibitors and left on ice for 15 min. Sonication was performed at a 35% potency, for 50 seconds with 2 seconds intervals each 10 seconds. Triton X-100 (0.1%) was added to the samples and they were incubated for additional 25 min on ice. Sepharose beads in STE buffer (1 mL of 50% slurry) were added to the samples and incubated 45 min at

room temperature or overnight at 4 ºC, with rotation. After binding, the beads were pelleted by centrifugation for 5 min at 1000 xg, the supernatant was stored at 4 ºC and the beads were washed twice in STE buffer. The beads were then resuspended in 10% of glycerol in STE buffer, aliquoted, and stored at -20 ºC.

The fusion proteins were purified using glutathione-Sepharose beads (Amersham Biosciences) and then dialyzed overnight at 4 ºC in Phosphate Buffered Saline (PBS).

The TnT® Quick Coupled Transcription/Translation Systems kit (Promega) was used to prepare *in vitro* translated proteins, following the manufacturer's specifications. Redivue™ L-[^{35}S]-methionine (Amersham Biosciences) was used to radiolabel proteins.

To test the ability of p48 to interact with putative partners in vivo, subconfluent HEK293T cells ($2x10^6$) were cultured overnight in 100 mm plates and transiently transfected using lipofectamine (Lipofectamine Plus Reagent, GIBCO, Gaithsburg, MD) with pcDNA3 p48-HA (5 μg). Cells were lysed 24 h later by incubating cells for 30 min on ice with lysis buffer (20 mM Tris-Cl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 10 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin and 50 mM phenylmethylsulfonyl fluoride); lysates were cleared by 15 min centrifugation at 4 ºC at 13,000 rpm. Cell lysates (1 mg protein) were incubated with 50 μL of 50% glutathione-Sepharose beads in PBS and 25 μg of GST for 2 h at 4 ºC with continuous end-over-end mixing. After preclearing, lysates were incubated overnight at 4 ºC with 50 μL of glutathione-Sepharose beads and 10 μg of either GST or 10 μg of the GST fusion probe proteins, with continuous end-over-end mixing. The beads were washed four times with 1 mL of ice-cold lysis buffer, resuspended in a 4x reducing loading buffer (40% glycerol, 200 mM Tris-HCl pH 6.8, 4% SDS, 200 mM β-mercaptoethanol and bromophenol blue) and boiled for 5 min. p48-associated proteins were detected by SDS-PAGE followed by immunoblotting using a rabbit anti-p48 primary antibody[163] or an anti-HA monoclonal antibody (Sigma; Saint Louis, Missouri USA).

6. Co-Immunoprecipitation assays

HEK293T cells were seeded at 50-70% of confluence in 100 mm dishes, cultured overnight, and transfected using lipofectamine as described above. After transfection (24h or 48h), cells were lysed in 50 mM HEPES pH 7,4, 5 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, 2 mM EGTA, 1% Triton X-100 supplemented with protease inhibitors (Complete, EDTA-free protease inhibitor cocktail, Roche Diagnostics, Germany). Cells were incubated for 15 min in lysis

buffer on ice, centrifuged for 8 min at 8000 rpm, and the supernatant was recovered. Fresh cell lysates (1 mg) were incubated for 2 h at 4 ºC with non-specific antibody or preimmune serum and 50 µL Protein G-Agarose (Roche Diagnostics, Germany), centrifuged for 2 min at 4000 rpm, and the supernatant was recovered. Antibodies (control and specific) were added to the supernatants together with 20 µL of Protein G-Agarose and were incubated overnight at 4 ºC with rotation. Immune complexes were pelleted by centrifugation and washed 4 times with lysis buffer supplemented with 0.1% SDS. Loading buffer (20 µL of 2x buffer) was added to the samples that were then boiled for 5min at 100 ºC. A fraction of this material (15-20 µL) was loaded onto a SDS-PAGE gel and this was followed by western blotting analysis with relevant antibodies.

7. Western blotting

Cell lysates were prepared by incubating cells with lysis buffer (20 mM Tris-HCl , 200 mM NaCl, 1 mM EDTA pH 8.0, 0.5% Nonidet P-40, 10 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin and 50 mM phenylmethylsulfonyl fluoride) for 30 min on ice and clearing by 15 min centrifugation at 4 ºC. Proteins were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes. After blocking for 1h in 5% skim milk in TBST solution (100 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1% Tween 20), the membranes were incubated with the primary antibodies at room temperature for 1h. The following antibodies were used: affinity-purified rabbit anti-p48 (0.39 µg/mL)[163]; mouse anti-Flag M2 monoclonal antibody (0.43 µg/mL) (Sigma, Saint Louis, Missouri USA), and mouse anti-HA monoclonal antibody (0.47 µg/mL) (Sigma); anti-ICAT affinity-purified polyclonal serum (a kind gift from Cara J. Gottardi, Northwestern University, Chicago, IL - verificar); mouse anti β-catenin monoclonal antibody (0.125 µg/mL) (BD Biosciences Pharmingen, San Diego, CA, USA). After washing once with 1% skim milk in TBST and 2x with TBST solution, the membranes were incubated for 1h with the corresponding peroxidase-conjugated secondary antibody (DAKO) diluted in 1% skim milk in TBST. Reactions were developed using chemoluminiscence (ECL detection reagents, Amersham Pharmacia Biotechnology, Uppsala, Sweden).

8. Luciferase reporter assays

For promoter reporter activity assays, HEK293 ($0.8x10^5$), 266-6 ($2.0x10^5$), or AR42J ($2.0x10^5$) cells were seeded and cultured overnight in wells of 24-well plates and then transfected with 0.1 µg of the luciferase reporter construct of

interest, 15 ng of pRL-TK vector (Promega, Madison, WI), and 0.2 μg of the appropriate plasmids as well as with the corresponding empty plasmids The pRL-TK vector was included as an internal control for normalization of the transfection efficiency. Transfections were performed in triplicate using the FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis). After transfection (24-48h), cells were lysed and the luciferase activity was determined using the Dual Luciferase Assay system (Promega), following the manufacturer's specifications. Firefly and Renilla luciferase activities were measured using a luminometer. Results are shown after normalization for Renilla luciferase activity.

9. Immunofluorescence and immunohistochemical analysis

Cos7 and 266-6 cells were seeded in 24-well plates on ethanol-sterilized coverslips. Cells were fixed in 4% PFA in PBS for 10 min at room temperature. The coverslips were washed twice for 5 min with PBS, permeabilized for 20 min in 0.3% Triton X-100 in PBS, washed twice with PBS, and blocked in 1% BSA in 0.3% Triton X-100 PBS for 45 min at room temperature. The antibodies were then diluted in the blocking solution and cells were incubated with the primary antibody for 1 h at room temperature, washed twice in PBS, and incubated with the secondary antibodies for 40 min in the dark. After washing twice in PBS and water, the slides were mounted with Fluoromount (SouthernBiotech, Birmingham, USA). The following antibody dilutions were used: monoclonal anti-Flag M2 (1:1000); anti-ICAT polyclonal antibody (1:100); mouse anti β-catenin monoclonal antibody (1:200).

For immunohistochemistry on paraffin-embedded tissues, sections were deparaffinized, rehydrated, and antigen retrieval was performed by boiling in citrate buffer pH6.0 for 10 min and leaving for an additional 10 min to cool down. The sections were rinsed twice with PBS, blocked for 15 min in 1%BSA in 0,3% Triton X-100 in PBS, rinsed for 5 min in PBS, and incubated for 45 min in 10% slim milk in PBS. After blocking, the sections were incubated for 2h with affinity-purified rabbit anti-ICAT (1:20). Reactions were developed by incubating with the Envision secondary reagent (Dako) for 40 min, followed by washing with PBS. Diaminobenzidine was used as a chromogen for 5 min. After washing, sections were stained lightly with hematoxylin and dehydrated.

10. Generation of stably transfected cell lines

The human ICAT tagged with a FLAG-epitope at its amino-terminal region was cloned into the $pREP_4$ vector, which allows the establishment of stable transfected cells by growing them in complete medium with hygromycin selection. 266-6 and HEK293 cells were therefore transfected either with the $pREP_4$-empty vector or with the $pREP_4$.Flag-ICAT vector and were selected for 14 days in DMEM-complete medium with 10% FBS and 350 μM of hygromycin. The resistant cells were then cloned by minimal dilution at 0.3 cells/well in a 96 well plate, and single clones were chosen for further work. All the culture procedures hereafter were done in DMEM-complete medium maintaining the hygromycin selection.

11. RNA extraction and qRT-PCR

Total RNA was isolated using the Gen-elute™ mammalian total RNA miniprep kit (Sigma). RNA concentration was measured by spectophotometry in a NanoDrop instrument and all the concentrations were normalized with Rnase-free water. The RNA was then treated with the DNaseI enzyme (DNA-free™, from Ambion, Austin TX, USA) and the cDNAs were prepared according to the manufacturer's specifications, using the TaqMan® Reverse transcription Reagents (Applied Biosystems, Roche).

The expression of the different transcripts of interest was analyzed by using the SYBR Green PCR Master Mix and the ABIPRISM 7900HT instrument (Applied Biosystems) for detection and real-time quantification. All the RT-PCR reactions were prepared in duplicates with 40 ng of cDNA in a final volume of 20 μL and the conditions used were the following: 30 min at 50 ºC, 15 min at 95ºC, 40 thermal cycles of 15 seconds at 94 ºC, 30 seconds at 55 ºC, and 30 sec at 72 ºC. A dissociation step was done at the end of each run, to evaluate the amplified PCR product. For the stable cell line studies, three independent RNA extractions were prepared and analyzed in separate, for each clone. The expression levels of the transcripts were compared by the comparative Ct method, according to the manufacturer's manual. As an internal control, changes in the expression of HPRT (Hypoxanthine-guanine phosphoribosyltransferase) mRNA were monitorized. A list with the sequence of the used primers is provided in table 1.

Primer Name	Sequence
HPRT	fw - GGC CAG ACT TTG TTG GAT TTG rv - TGC GCT CAT CTT AGG CTT TGT
ICAT (endogenous)	fw - ATG AAC CGC GAG GGA GCA CC rv - CTA CTG CCT CCG GTC TTC CG
Flag-ICAT (exogenous)	fw- TAC AAA GAC GAT GAC GAC AAG rv - ATC ACC ACG TCC TCT GCA C
p48	fw - ACA AGC CGC TAA TGT GCG AGA rv - TTG GAG AGG CGC TTT TCG T
RBP-L	fw - ATG CCA AGG TGG CTG AGA AAT rv - CTT GGT CTT GCA TTG GCT TCA
Amyl2	fw - TGG CGT CAA ATC AGG AAC ATG rv - AAA GTG GCT GAC AAA GCC CAG
Ela1	fw - CGT GGT TGC AGG CTA TGA CAT rv - TTG TTA GCC AGG ATG GTT CCC
CtrB1	fw - GCA AGA CCA AAT ACA ATG CCC rv - TGC GCA GAT CAT CAC ATC G
Cpa1	fw - TAC ACC CAC AAA ACG AAT CGC rv - GCC ACG GTA AGT TTC TGA GCA
cK7	fw - CAC GAA CAA GGT GGA GTT GGA rv - TGT CTG AGA TCT GCG ACT GCA
Pdx1	fw - AAA TCC ACC AAA GCT CAC GC rv - CGG TCA AGT TCA ACA TCA CTG C
Ngn3	fw - ACAGGCCCAAGAGCGAGTT rv - GCCGAGTTGAGGTTGTGCAT
Pax6	fw - AAC AAC CTG CCT ATG CAA CC rv - ACT TGG ACG GGA ACT GAC AC
Ins1	fw - TAG TGA CCA GCT ATA ATC AGA G rv - ACG CCA AGG TCT GAA GGT CC
PPy	fw- CCT GTT TCT CGT ATC CAC TT rv - AAG TCC ACC TGT GTT CTC C
RBP-jκ	fw - GTG TTC CTC AGC AAG CGG ATA rv - TGC CAC CTT CGT TCC TGA A
Hes1	fw - TCC AAG CTA GAG AAG GCA GAC A rv - CGT TCA TGC ACT CGC TGA A
Sox9	fw - CGT GCA GCA CAA GAA AGA CCA rv -GCA GCG CCT TGA AGA TAG CAT
c-Myc	fw - CCT AGT GCT GCA TGA GGA GAC A rv - CCT CAT CTT CTT GCT CTT CTT CAG A
p21	fw - GCA GAC CAG CCT GAC AGA TTT C rv - GGC ACT TCA GGG TTT TCT CTT G
NeuroD1	fw - ATC CCT ACT CCT ACC AGT CC rv - TTG AAA GAG AAG TTG CCA TT
Glucagon	fw -TCA AGA CAC AGA GGA GAA CC rv - ATG CCT CTC AAA TTC ATC AT
Ghrl	fw - ATC TGC AGT TTG CTG CTA CT rv -GCT CCT CCT CTG TCT CTT CT
Nestin	fw - GGA GAG TCG CTT AGA GGT G rv - TGA CTC TGT AGA CCC TGC TT
Nkx2.2	fw - CCC TTA AGA GCC CTT TCT AC rv - GGT CTC CTT GTC ATT GTC C
cK19	fw - CCT CCC GAG ATT ACA ACC ACT rv - GGC GAG CAT TGT CAA TCT GT
CyclinD1	fw - CCC TCC GTA TCT TAC TTC AA rv - GGA ATG GTC TCC TTC ATC TT
CyclinD2	fw - CAG AAG GAC ATC CAA CCG TAC AT rv - CAC TTT TGT TCC TCA CAG ACC TCT AG
p/CAF	fw - TGG AGA AGA AGC CGC CAT TT rv - TCG TTG TCT GCC TCT CTT TCG
MDK	fw - GAA TTT GGA GCC GAC TGC AA rv - TTG ACT TGG TCT TGG AGG TGC

Table 1. RT-qPCR primers list and respective sequence (fw, forward; rv, reverse).

12. Lentiviral constructs: preparation and delivery

HEK293FT cells were used to prepare lentiviral particles for cell infection procedures. Prior to transfection (24h), HEK293FT packaging cells were seeded in 150 mm dishes so that they would reach 80-90% confluence at the time of transfection. In order to produce virus, cells were transfected with 22.5 µg of the FG12 empty plasmid or the FG12.ICAT plasmid together with 7.9 µg of the corresponding helper vectors: pRSVrev, pHCMV-G, and pMDLg/pRRE. Transfections were done overnight using Lipofectamine^{TM} 2000 (Invitrogen). After transfection (14-16h), the culture medium was replaced by 14 mL of pre-heated fresh medium and, for the next two days, the medium containing the released lentiviral particles was collected and stored at

4ºC for further purification/concentration. The collected supernatants were then pooled, centrifuged for 5 min at 1500 rpm to remove cell debris, and filtered using 0.22 µm cut-off filters. The lentiviral particles were purified by ultracentrifugation at 47.000 xg for 2 h at 16 °C in a swinging rotor, resuspended in 100-200 µL of sterile PBS, and stored in aliquotes al -80 ºC (for the production and titration of the lentiviral vectors see the tronolab.epfl.ch webpage). The lentiviral titer was determined in HEK293FT cells and this value was used to calculate the necessary TU of lentiviral particles needed to infect 100% of the seeded cells. 293T cells ($3x10^4$) were seeded per well in a 24-well plate, in 1 mL of medium/well. 24h after seeding the total number of cells in one well was counted. Four 4-fold serial dilutions of the lentivirus in 250 µL total volume of fresh medium were used to transduce the cells, taking 1 µL of the stock vector as a first dilution. The following day, 1 mL of fresh medium was added to each well. One day later, the cells were split with trypsin and the percentage of fluorescent cells was determined by fluorescent flow cytometry analysis. The lentivirus titer corresponds to the number of cells transduced by a given volume of stock virus, counted on day 2 post-transduction, and the values are given in TU/mL (titer units per mililiter). The importance of this titering step is related to the very low efficiency rates of transfection or infection of 266-6 cells. Lentiviral infections were preformed by directly adding the viral-containing stock to the culture medium and incubating overnight. Transduction efficiency was always monitorized by direct fluorescence microscopy.

13. Electrophoretic mobility shift assays (EMSA)

For electrophoretic mobility shift assays, nuclear extracts were prepared according to the protocol described below, adapted from Dignam *et al.*[164] HEK293T cells were tranfected with plasmids coding for the three elements of

the PTF1 complex ($pCMV_2$.p48-Flag, $pcDNA_3$-RBP-L, and E2A), in the presence or absence of ICAT-Flag. After transfection (48h), cells were washed once with PBS and resuspended in 5 packed cell pellet volumes of Buffer A (10 mM HEPES, pH7.4; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT) plus protease inhibitors. Lysates were incubated for 10 min on ice, centrifuged for 10 min at 4 ºC, and resuspended in 2 packed cell pellet volumes of Buffer A plus protease inhibitors. Cells were carefully homogeneized with the help of a douncer and lysates were centrifuged for 10 min at 2000 rpm to pellet the nuclei. After supernatant removal, the pellets were further centrifuged for 20 min at 13200 rpm to completely remove the cytoplasmic fraction. The pellets were resuspended in 1 packed cell volume of Buffer B (20 mM HEPES pH7.4, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA pH8) and incubated for 30 min on ice with occasional strong vortexing. Lysates were then centrifuged for 20 min at 13200 rpm and the nuclear supernatant fraction was recovered. Protein content was measured by spectrophotometry and the nuclear extracts were divided in aliquots and stored at -80 ºC at a concentration of 2 µg/µL. Final concentrations were adjusted with Buffer C (20 mM HEPES pH7.4, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA).

A set of probes was designed for the EMSA assays. As a control to ensure the adequacy of the nuclear extracts, oligonucleotides corresponding to the AP-1 binding site were used as probes: sense CGC TTG ATG ACT CAG CCG GAA (sense) and GCG AAC TAC TGA GTC GGC CTT (antisense) (modification: IRDye700). Additional probes used (IRDye 700-modified) include:
- optimal TCF/LEF probe: CCC TTT GAT CTT ACC (sense), GGG AAA CTA GAA TGG (antisense);
- Amylase2.1 (GI: NW_001030737.1): CAC AGC TGA AGG TTC TTC AGA AA (sense), GTG TCG ACT TCC AAG AAG TCT TT (antisense);
- Carboxypeptidase1 (GI: NW_001030802.1): CCA CCT GCC TTG TTC CCT GAT ACT (sense), GGT GGA CGG AAC AAG GGA CTA TGA (antisense);
- Chymotrypsinogen1 (GI: NW_001030904.1): CAG AGC AGC TGT CCT TTT CCC ATG GG (sense), GTC TCG TCG ACA GGA AAA GGG TAC CC (antisense);
- E-Box C>T mutant (amylase): CAC A GT TTG AAG GTT CTT CAG AAA (sense), GTG TCA ACT TCC AAG AAG TCT TT (antisense).

To prepare the EMSA oligonucleotides in working solution, dehydrated oligos were resuspended in 50 µL in 1xTE buffer to a final concentration of 20 µM. The corresponding forward and complementary oligonucleotides were incubated in a 1:1 proportion, and annealed by placing them for 5 min at 100 ºC and were allowed to slowly cool to room temperature. The annealed IRDye oligonucleotides were diluted 1:200 in ddH_2O and the unlabeled oligonucleotides were diluted 1:5 in ddH_2O.

For the binding reactions, the buffer was prepared by adding components in the following order: 20 mM Tris-HCl, 60 mM KCl, 5% glycerol, 500 nM DTT, 2 mM EDTA). The EMSA reactions were prepared by adding 5 µg of nuclear extracts to 4.5 µL of binding buffer plus 1 µL of polyd(I-C) and 2 µL of IRDye oligo in the presence or absence of 2 µL of unlabeled competitor oligonucleotide. The binding reactions were prepared on ice. For the competition reactions, the unlabeled oligonucleotide was added first and the mix was incubated on ice, prior to adding the IRDye corresponding oligonucleotide. For the supershift assays, the antibodies were added at the same time as the unlabeled competitor oligonucleotides. The EMSA reactions were then incubated for an additional 20 min at room temperature. Orange Dye Loading buffer (2 µL of 10x) was added to the reactions and the mix was loaded onto 5% native polyacrylamide gels (5% wt/vol gel pre-run for 1h at 180V in 1xTAE buffer). After a 2h run at 4 ºC, the gels were scanned using the Odyssey Infrared Imaging System (Li-COR, Lincoln, NE, USA), with the focus set at 1.5 mm and at a starting intensity of F8.

RESULTS

1. Screening for proteins interacting with p48

1.1. Setting up the Yeast Two-Hybrid system

To identify new putative p48 interactors, four different baits were designed coding for different regions of the p48 protein: the N-terminus lacking the bHLH domain (amino acid residues from 1-163), the C-terminus with and without the bHLH domain (amino acid residues 162-326 and 211-326, respectively) and the full-length p48 protein (amino acid residues from 1-326). The baits comprised the mentioned regions of the p48 cDNA fused to the LexA-binding domain, in the pBTM116 plasmid. Figure 14 shows the constructs used for the control experiments. In control experiments, the L40 yeast strain - which carries the HIS3 and LacZ reporter genes under the control of LexA binding sites - did not grow in medium lacking His and showed no β-galactosidase activity. As a control for the activity of the bait-prey interactions, L40 yeast cells were co-transformed with plasmids coding for RBP-Jκ – a known p48

interactor[165] - fused to a GAL4 transcription activation domain and with the LexA-p48$^{\Delta N+bHLH}$ bait. Both reporter genes were effectively activated, indicating that the system was suitable for the isolation of novel p48 interacting proteins.

When the bait constructs were transformed in L40 yeast to determine their ability to activate the reporter genes, the N-terminus of p48 and the full-length p48 gave a strong positive β-galactosidase activity in the absence of prey (Figure 14 B). This result indicated that this reporter gene was activated in the presence of these baits alone, suggesting that the N-terminus domain could comprise a transcription activation domain and that these two bait constructs were not suitable for a yeast two-hybrid screening. The remaining bait constructs did not activate, by themselves, the reporter genes.

1.2. The N-terminal region of p48 contains two transactivating domains (Paper 1)

The functions of the N- and C-terminal domains of p48 were unknown when this work this initiated. The β-galactosidase activity assays performed during the yeast two-hybrid experiments suggested that the amino-terminal region of p48 is a regulator of its transcriptional activity. As introduced before, the expression of genes coding for pancreatic enzymes is under control of the PTF1 complex, formed by p48, RBP-L, and a class A bHLH transcription factor. However, the mechanisms by which the activity of the complex is regulated are unknown. The yeast two-hybrid result suggested that the amino-terminal region of p48 contained a transcriptional activation domain. To explore this hypothesis, p48 deletion mutants were fused to a Gal4 DNA-binding domain and assayed for their ability to activate the transcription of an artificial Gal4-reporter system. The results confirmed this hypothesis and allowed dissecting the p48 regions involved in this function. The p48 N-terminal contains at least two domains that positively regulate its transcriptional function that are located between amino acid residues 1-43 and 43-138. The experiments also suggest that the C-terminal

domain of p48 potentiates the transactivation promoted by the N-terminal of p48 through yet unknown mechanisms.

Because ubiquitous co-factors with histone acetyl transferase activity (HAT), such as CBP/p300, p/CAF and Gcn5, are involved as co-activators of gene expression[166], a physical interaction between these factors and p48 was then assayed. Co-immunoprecipitation assays confirmed that p48 specifically interacts both with p/CAF and Gcn5 transcriptional co-activators.

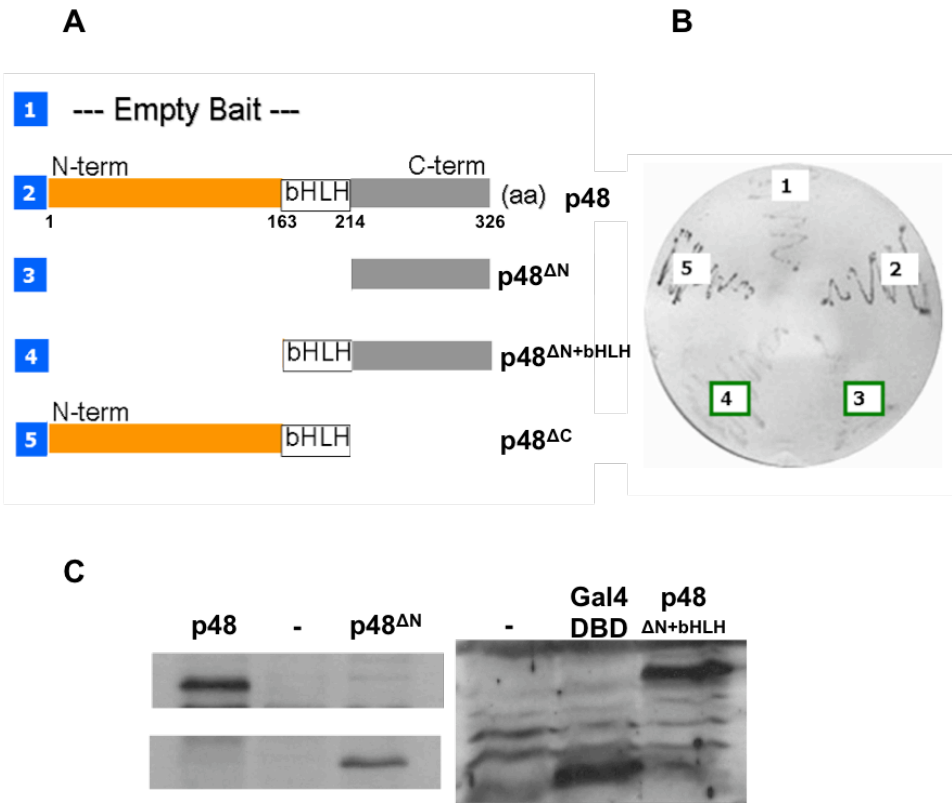

Figure 14.
A) Different regions of p48 used as baits for the Yeast Two-Hybrid screening.
B) β-galactosidase Colony-lift Filter assay showing L40 yeast cells sequentially transformed with the pBTM116 plasmid and the different bait constructs. 1 – pBTM116 empty plasmid; 2 – pBTM116.p48; 3 - pBTM116. $p48^{\Delta N}$; 4 - pBTM116. $p48^{\Delta N+bHLH}$; 5 - pBMT116.$p48^{\Delta C}$. Colonies 2 and 5 are positive for the LacZ reporter gene expression and 1, 3, and 4 colonies are negative. (in grey scale, strong black corresponds to the blue color and faded grey to the white color in the β-galactosidase activity assay). **C)** Western blot anti-p48 and anti-Gal4DBD showing the protein expression of the baits used in the library-scale of the Yeast Two-Hybrid assays.

Additional experiments carried out in the laboratory indicated that p/CAF potentiates the transcriptional activity of p48 through: 1) the promotion of the nuclear accumulation of p48, and 2) the acetylation of p48 on Lys^{200}, required for its functional collaboration with RBP-L to ensure the full activity of the PTF1 complex. Importantly, chromatin immunoprecipitation assays confirmed *in vivo* the presence of p/CAF on the promoter region of acinar specific enzymes, such as carboxypeptidase A, chymotrypsinogen and elastase. No cooperation was observed between p/CAF and p48 regarding its antiproliferative activity.

Thus, this study describes the regulatory mechanism that may drive the upregulation of genes under control of the PTF1-complex. p48 is required to recruit transcriptional co-activators with intrinsic HAT activity.[157]

This work resulted in a publication attached as Supplementary Material (Rodolosse et al.).

1.3. Selecting a suitable cDNA library for the Yeast Two-Hybrid screenings

The following reasoning was applied to select a library for the Y2H screening. During development, p48 is first detectable at day E9.5. A library of pancreatic epithelium at this stage would be the most interesting tool to identify partners of p48 involved in pancreatic development. However, due to the reduced size of the pancreatic bud at E9.5, to prepare such a library is a formidable task. To identify p48 partners involved in its function in differentiated acinar cells, an adult pancreas library would be most interesting. However, the very high RNAse content of the pancreas makes this also a formidable task and achieving negative results with such a library would be no warranty of the lack of novel interactors. p48 also plays an important role in the generation of GABAergic neurons in the central nervous system. We finally chose to use a whole E9.5

mouse embryo library and a library from human fetal brain of 20-22 weeks. The latter was also chosen because it is often recognized that fetal brain is a good source of expression of a wide range of tissue-specific mRNAs.

1.4. Scale-up assays

L40 yeast cells were sequentially transformed with the LexA-p48$^{\Delta N+bHLH}$ plasmid and the human fetal brain cDNA library cloned into the pACT2 vector. From a total of $2x10^5$ transformants, 283 colonies grew in selective medium lacking His and 106 of them were also β-galactosidase positive. After plasmid rescue, the cDNA was amplified by PCR and 55 different products were obtained, purified and sequenced. To determine the identity of the cDNAs corresponding to the obtained sequences, BLAST search was performed against the PROTEIN database and the TRANSLATED database from the NCBI web site. Sequences were checked for coding translated regions and for being in frame with the transcription-activating domain. A list of the cDNAs recovered is shown in Table 2.

When the whole mouse E9.5 cDNA library was transformed into L40/LexA-p48$^{\Delta N+bHLH}$ strain, a total of $9.5x10^5$ clones were screened. Of those, 82 grew on selective medium and 63 expressed the LacZ reporter gene. Sixty-three PCR products were sequenced, compared to the databases as indicated above, and the findings are shown in Table 2. Among the isolated clones recovered in the two screenings, the following cDNAs were identified:

RBP-Jκ – which appeared once in frame and provided evidence that the assay worked adequately;
Midkine - which appeared in 4 different clones - and **Pleiotrophin** (a midkine family member) - that appeared once, both proteins in frame. The fact that Midkine appeared in several independent clones in the screening performed with the whole mouse embryo library and that Pleiotrophin appeared once using

YEAST TWO-HYBRID SCREENING		Prey cDNA	Frequency	Seq. frame
Yeast strain	**L40**	Meis3	1	No
Bait	**LexA-p48$^{\Delta N}$**	HIP (Huntington Interacting Protein)	1	No
Library	**Human Fetal Brain, 20-22 weeks**	E2F transcription factor 2	2	Yes
Transformants analyzed	**2x10^5**	EEF1A1	1	Yes
Total cfu	**283**	PTN (Pleiotrophin)	1	Yes
β-gal activity	**106 positive cfus**	PTPRD	2	No
Recovered plasmids	**55**	CPT2 (carnitine palmitoyltransferase II)	1	No
Analized sequences	**46**	Shwachman-Bodian-Diamond syndrome	1	No
		CXCL12 (chemokine C-X-C ligand 12)	1	No
		hIRH (human intercrine alpha)	1	Yes
		Calmodulin 1	1	No
		Stathmin-like 2 (STMN2)	1	No
		MARCKS	1	No
		CD63 antigen (melanoma1 antigen)	1	No
		Hnrpf	1	No
		ZAK	1	Yes
		Acetyl CoEnzyme A acyltransferase 1	1	Yes
		NADH dehydrogenase sub4L	3	No
Yeast strain	**L40**	Midkine	4	Yes
Bait	**LexA-p48$^{\Delta N}$**	B-myc	1	Yes
Library	**Mouse whole embryo, E9,5**	β-catenin interacting protein ICAT	1	Yes
Transformants analyzed	**9,5x10^5**	Nestin	1	Yes
Total cfu	**82**	eEF-1B gamma	1	N.D.
β-gal activity	**45 positive cfus**	psmb4	1	N.D.
Recovered plasmids	**63**	psmb6	1	N.D.
Analized sequences	**57**	COP9 signalosome subunit B	1	Yes
		eIF3, subunit5 / epsilon / p47 subunit	1	No
		Hemoglobin 2, beta-like embryonic chain	1	No
		Sjogren's syndrome	1	No
		RPL19	1	Yes
		Single strand DNA binding protein 3	1	Yes

YEAST TWO-HYBRID SCREENING		Prey cDNA	Frequency	Seq. frame
Yeast strain	**AH109**	E2A transcription factor (TcfE2A, E47)	3	Yes
Bait	**pCAM-p48$^{\Delta N+bHLH}$**	Splicing factors, arginine/serine-rich 6	2	N.D.
Library	**Mouse whole embryo, E9,5+E10,5**	Filamin, alpha, transcript variant 16	1	No
Transformants analyzed	**3,63x10^{5}**	Anaphase-promoting complex subunit 5	1	Yes
Total cfu	**142**	Transcription factor 12 (Tcf12)	2	Yes
β-gal activity	**17 positive cfus**	Guanylate kinase 1 (GUK1)	1	Yes
Recovered plasmids	**17**	Tyrosine monooxigenase/tryptophan 5-5 monooxigenase activation protein	1	Yes
Analized sequences	**17**	RPL4	1	Yes
		Polimerase (DNA directed), epsilon	1	No
		Leucine-rich repeat containing protein 14	1	Yes
		Ankyrin repeat and BTB (POZ) domain containing2	1	Yes
		Grb10 interacting GYF protein2	1	N.D.
Yeast strain	**AH109**	Transcription factor 12 (Tcf12)	2	N.D.
Bait	**pCAM-p48$^{\Delta N+bHLH}$**	E2A transcription factor (TcfE2A, E47)	4	N.D.
Library	**Mouse whole embryo, E9,5+E10,5**	myeloid/lymphoid or mixed lineage-leukemia translocation to 1 homolog (Drosophila) (Mllt1)	1	N.D.
Transformants analyzed	**6,22x10^{5}**	Guanylate kinase 1 (GUK1)	1	N.D.
Total cfu	**11**			
β-gal activity	**11 positive cfus**			
Recovered plasmids	**11**			
Analized sequences	**10**			

Table 2.
Yeast Two-hybrid screening results.
cfu - colony forming units; β-gal activity refers to the colonies with blue color that appeared during the β-galactosidase filter assay; frequency correspond to the number of times a prey coding for the same protein was recover in the screening; N.D.- not determined.

a different library, placed Midkine as a strong candidate for binding the C-terminus of the p48 protein;
β-catenin-interacting protein (ICAT) - which appeared once in frame;
B-myc - which appeared once in frame, and;
MEIS3 - which appeared once, although not in frame.

Because the number of recovered positive clones was relatively small, two additional yeast two-hybrid screenings using the pCAM-p48$^{\Delta N}$ bait (consisting of the bHLH region plus the carboxy-region of p48 fused to a Gal4DNA-binding domain) were carried out. For these experiments, a whole mouse embryo library of E9.5 + E10.5 was used; this library had yielded many positive clones in a parallel screening being carried out in the laboratory with a different bait. In total, through both assays, $1x10^6$ clones were screened. As might have been expected, given that this bait contained the p48 bHLH domain, the cDNAs coding for **E2A** and **Tcf12/HEB** bHLH-transcription factors - already described as being part of the PTF1 complex - were identified. Furthermore, sequences corresponding to **GUK1**, a guanylate kinase, were recovered in frame in two different clones, one in each Y2H screening. Both recovered clones coded for the carboxy-terminal most region of GUK1 (Table 2).

GENE NAME	GI NUMBER	PREY SEQUENCE
MEIS3	Q99687	TPPGGDVCSSDSFNEDIAAFAKQVRSERPLFSSNP ELDNLMIQAIQVLRFHLLELEKVHDLCDNFCHRYITC LKGKMPIDLVIEDRDGGCREDFEDYPASCPSLPDQ NNMWIRDHEDSGSVHLGTPGPSSGGLASQSGDNS SDQGDGLDTSVASPSSGGEDEDLDQERRRNKKRG IFPKVATNIMRAWLF
Pleiotrophin	NP_002816	MQAQQYQQQRRKFAAAFLAFIFILAAVDTAEAGKKE KPEKKVKKSDCGEWQWSVCVPTSGDCGLGTREG TRTGAECKQTMKTQRCKIPCNWKKQFGAECKYQF QAWGECDLNTALKTRTGSL
Midkine	NP_034914	MQHRGFFLLALLALLVVTSAVAKKKEKVKKGSECSE WTWGPCTPSSKDCGMGFREGTCGAQTQRVHCKV PCNWKKEFGADCKYKFESWGACDGSTGTKARQG TLKKARYNAQCQETIRV
B-myc	NP_075815	DSVQPYFMCDDEEEDVHHQQPPQPPAPSEDIWKK FELLPTPRPSPGHAGLYSPPCEAVAVSFAPRDHDG DSFSIADLPELPGGDAVKQSFVCDPDDETFVKNIILQ DCMWNGFSASAKLVSKLDPYQAVRKEGTGVSLAA DVEP
ICAT	NP_075954	MNREGAPGKSPEEMYIQQKVRVLLMLRKMGSNLTA SEEEFLRTYAGVVSSQLSQLPQHSIDQGAEDVVMA FSRSETEDRRQ
E2A	AAH18260.1	LPSQPSSLPDLSQRPPDSYSGLGRAGTTAGASEIK REEKEDEEIASVADAEEDKKDLKVPRTRTSPDEDED DLLPPEQKAEREKERRVANNARERLRVRDINEAFKE LGRMCQLHLSSEKPQTKLLILHQAVAVILS
HEB	Q61286	KERRMANNARERLRVRDINEAFKELGRMCQLHLKS EKPQTKLLILHQAVAVILSLEQQVRERNLNPKAACLK RREEEKVSAASAEPPSTLPGAHPGLSESTNPM
GUK1	NP_032219.1	RLRLRNTETEESLAKRLAAARTDMESSKEPGLFDLV IINDDLDKAYATLKQALSEEIKKAQGTGHA

Table 3.
GI number and sequence of the prey proteins, corresponding to the coding sequences of the putative involved interaction domains.

2. Experimental validation of the putative p48 partners identified in the Y2H screen

To confirm the binding of p48 to its putative partners described above, in vitro pull-down assays with recombinant proteins and co-immunoprecipitation of interacting proteins in transfected cells were used.

2.1. MEIS3

The potential interaction of p48 with MEIS3 was particularly interesting given that the PTF1 complex cooperates with a complex binding to the "B" element of acinar gene promoters that is composed by the trimer formed by PDX1.Pbx1b.MEIS2. Because the region coded by the recovered prey shares a high homology (80%) with MEIS2b (amino acid residues from 107 to 297, see Figure 15) and the latter is essential for the formation of the PDX1.Pbx1b.MEIS2 trimer, we postulated that MEIS2b and p48 might also physically interact, contributing to the functional cooperation between both complexes on acinar gene promoters. To clarify the nature of this interaction, MEIS2b and Pbx1b proteins were *in vitro* translated and radiolabeled with ^{35}S. Their ability to directly interact with the purified p48-GST fusion protein was assayed by pull-down. As shown in Figure 16A, it was not possible to assess the interaction between MEIS2b and p48 with this technique, since the ^{35}S-labelled MEIS2 was non-specifically pulled down with control GST. This interaction could not be further confirmed by co-immunoprecipitation experiments. In the course of these studies, an interaction was detected between p48 and the Pbx1b. Although this was a serendipitous finding, this interaction was confirmed by co-immunoprecipitation assays. Figure 16B and 16C shows that Pbx1 was pulled-down from HEK293 total cell lysates with purified p48-GST fusion protein and that p48 co-immunoprecipitated with Pbx1-Flag. An interaction between p48 and Pdx1 was not detected. Hence, p48 is able to interact with Pbx1 and this

interaction may account for the cooperation of transcriptional complexes binding to the A and B elements in acinar gene enhancer/promoters. Whether additional interactions of p48 with Meis factors take place needs to be further studied.

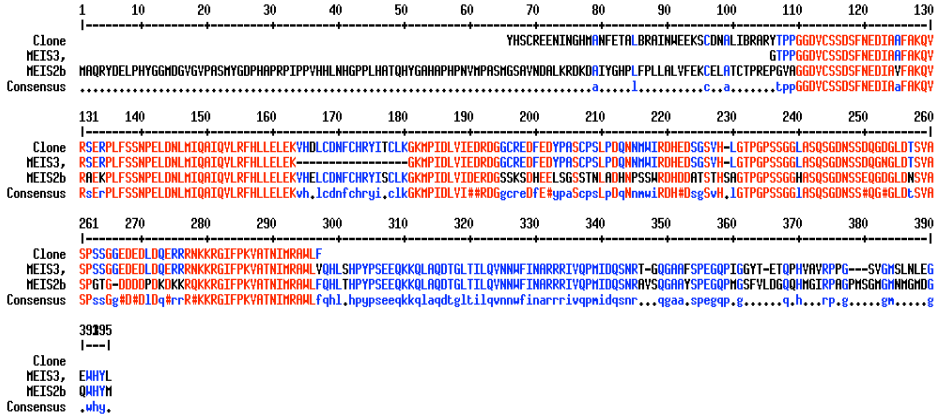

Figure 15. Nucleotide alignment of the prey cDNA sequence recovered by Yeast Two-Hybrid assay and the MEIS2b and MEIS3 mRNA sequences. Black characters correspond to no similarities, Blue characters to similar alignment between two of the sequences analyzed, and Red characters to complete homology between the prey cDNA sequence and both MEIS mRNAs.

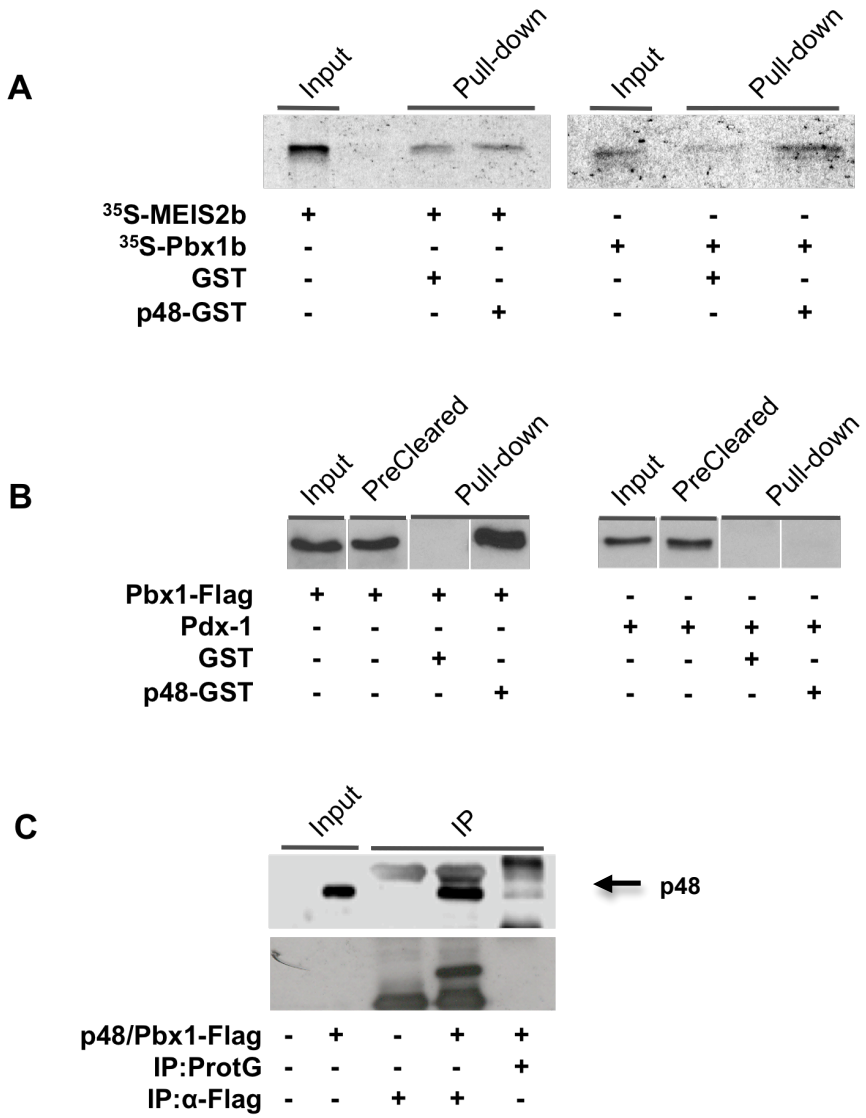

Figure 16. p48 interaction with the Pdx1.Pbx1.MEIS2b trimer elements.
A) Pull-down assay using p48-GST fusion protein or GST purified protein and the *in vitro* translated proteins MEIS2b and Pbx1b, radiolabelled with ^{35}S. MEIS2b is nonspecifically bound to the GST protein alone, whereas Pbx1b is found specifically pulled down with the p48-GST protein. **B)** Pull-down assays using HEK293 cell lysates overexpressing the Pbx1 or Pdx1, showing again the specific interaction between Pbx1 and p48 and the absence of interaction between p48 and Pdx1. The anti-Flag antibody and an affinity-purified polyclonal anti-Pdx1 serum were used to detect the pulled down proteins. **C)** Co-Immunoprecipitation assay with lysates from HEK293T cells overexpressing p48 and Pbx1 tagged with a Flag-epitope, showing p48 specifically in complex with Pbx1.

2.2. Midkine

Midkine (MK) is a 13.4KDa secreted protein that has been reported as a secreted heparin-binding growth/differentiation factor[167], although it can also been found intracellularly in the nucleus.[168] Tsutsumi *et al* reported that, in normal pancreas, MK was weakly expressed in the islets and was absent from ductal and acinar cells.[169] However, in chemically induced PDAC, increasing levels of MK were found associated to more advanced stages of the neoplastic progression, and in all of the analyzed pancreatic cancers analyzed MK was localized in the cytoplasm.

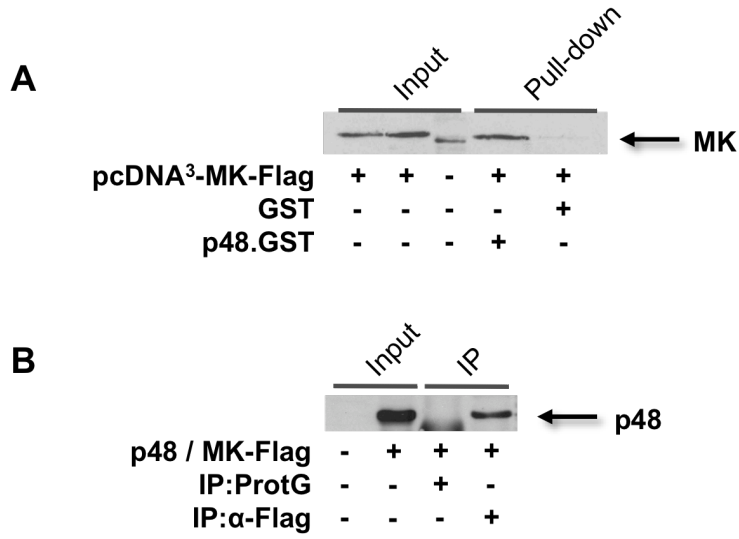

Figure 17. p48 interaction with Midkine.
A) Pull-down assay using p48-GST fusion protein or GST purified protein and HEK293 cell lisates overexpressing the MK protein tagged with a Flag epitope. MK is found specifically pulled down with the p48-GST protein. **B)** Co-Immunoprecipitation assay with lysates from HEK293T cells overexpressing p48 and MK-Flag, showing p48 specifically in complex with MK.

The interaction between MK and p48 was confirmed using both pull-down and co-immunoprecipitation assays. For the pull-down experiments, total cell lysates from 293T cells transfected with a pCMV2-MK-Flag plasmid were incubated with purified p48-GST fusion protein and analyzed as described in the Methods section. Figure 17A shows that MK-FLAG strongly binds p48-GST while it does not bind to GST alone. Freshly prepared lysates from HEK293T cells co-transfected with both the pCMV-MK-Flag and the pIRES-p48 vectors were incubated with an anti-Flag M2 antibody and the immune complexes were analyzed by western blotting; p48 specifically bound to MK-Flag was detected using α-p48 antibodies (Figure 17B).

2.3. B-myc

B-myc is a member of the *myc* gene family coding for proteins playing an important role in proliferation, apoptosis, transformation and differentiation. Although B-myc shares high homology with c-Myc, the two proteins appear to be antagonists at the functional level. B-myc is considerably smaller than c-Myc (approximately 20kDa and 64kDa, respectively) and contains two myc boxes, sharing high similarity with the N-terminal-myc-boxes-containing region of the c-Myc protein. It has been postulated that B-myc may indeed function as a dominant-negative protein, since c-Myc is usually associated with increased rates of proliferation whereas B-myc is described to exert an antiproliferative role.[170]

Again, pull-down assays using purified GST or B-myc-GST fusion protein and total cell lysates from HEK293T cells transfected with a vector coding for p48-HA confirmed the interaction of the two proteins (Figure 18B).

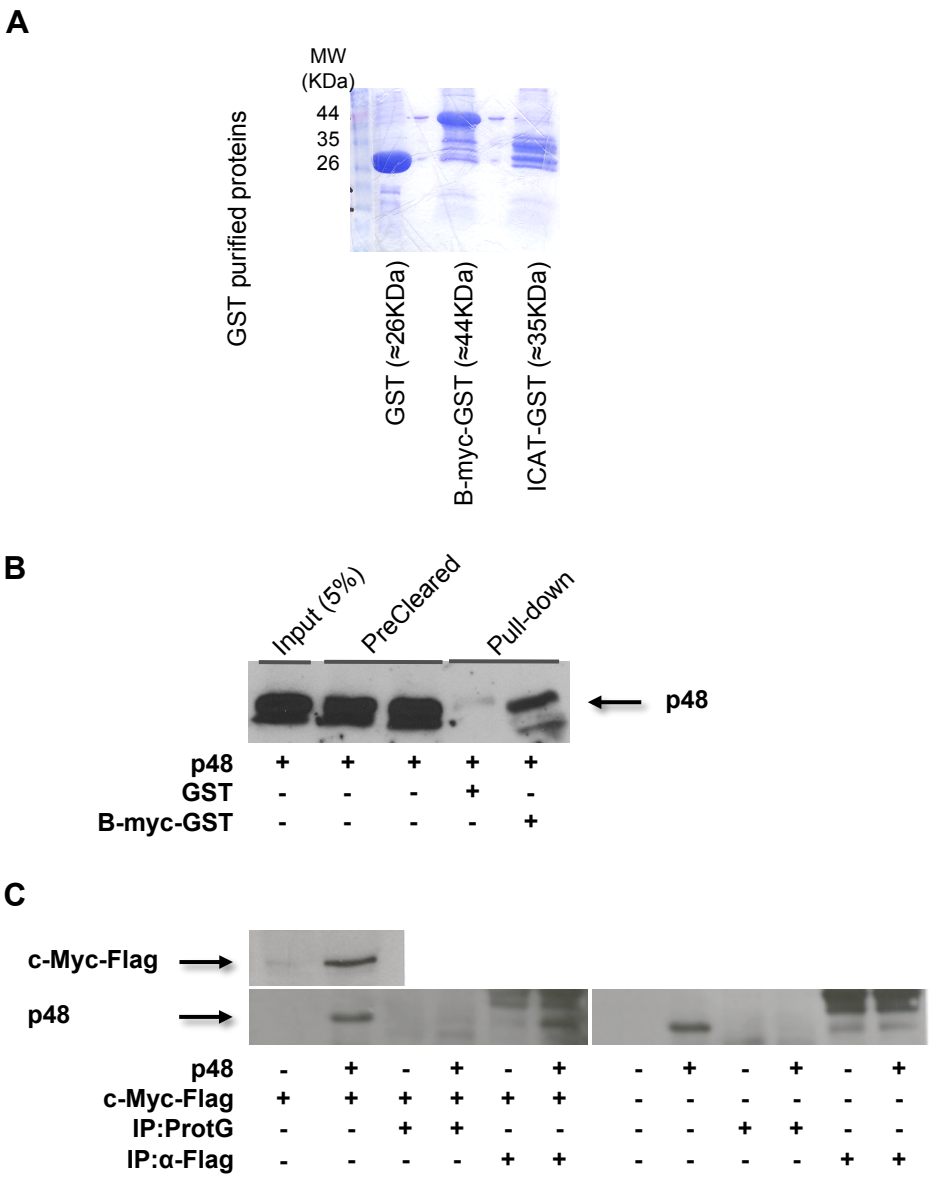

Figure 18. p48 interaction with B-myc.
A) Coommassie staining of a SDS-PAGE gel loaded with the purified GST-fusion proteins: GST; B-myc-GST, and; ICAT-GST. **B)** Pull-down assay using B-myc-GST fusion protein or GST purified protein and HEK293 cell lysates overexpressing the p48. p48 is found specifically pulled down with the B-myc-GST protein. **C)** Co-Immunoprecipitation assay with lysates from HEK293T cells overexpressing p48 and c-Myc tagged with a Flag-epitope, showing p48 specifically in complex with c-Myc.

2.4. ICAT

ICAT is a 9 KDa polypeptide that negatively regulates Wnt signaling by preventing complex formation between TCF and β-catenin. This inhibition is achieved through its direct binding to β-catenin.[69] To confirm the ability of p48 to interact with ICAT, the same strategy described above for MK was applied. As shown in Figure 19A, binding of p48 to ICAT was confirmed using pull-down assays. Since ICAT is a known interactor of β-catenin, its ability to interact with p48 in the presence of increasing amounts of β-catenin was also addressed. Total lysates from HEK293T cells overexpressing p48 and/or β-catenin were incubated with ICAT-GST and p48 was detected by western blotting. As shown in Figure 19A, high levels of β-catenin appeared to reduce, but not abolish, the ICAT/p48 interaction. The co-immunoprecipitation assays, performed using HEK293T cells transiently co-transfected with plasmids coding for p48-HA and ICAT-Flag, confirmed the interaction of ICAT with full-length p48 (Figure 19B).

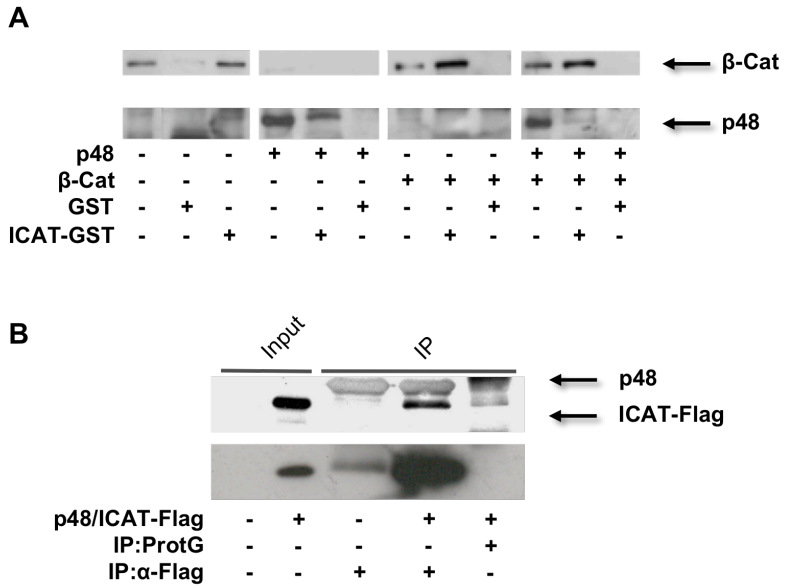

Figure 19. p48 interaction with ICAT.
A) Pull-down assay using ICAT-GST fusion protein or GST purified protein and HEK293 cell lysates overexpressing the p48 and/or β-catenin. Both p48 and β-catenin are found specifically pulled down with the ICAT-GST protein. When both are present, the p48 interaction appears less intense. **B)** Co-Immunoprecipitation assay with lysates from HEK293T cells overexpressing p48 and ICAT-Flag, showing p48 specifically in complex with ICAT.

3. ICAT as a new partner of p48

3.1. Mapping the p48 region required for its interaction with ICAT

To better define the region of the p48 carboxy-terminus involved in its interaction with ICAT, plasmids coding for different p48 deletion mutants were used. Total cell lysates from HEK293T cells expressing these mutants were incubated with ICAT-GST and their ability to interact was examined by western blotting. $p48^{\Delta 270\text{-}326}$ and $p48^{\Delta 296\text{-}326}$ proteins retained the ability to bind ICAT whereas the $p48^{\Delta 214\text{-}326}$ mutant was unable to interact with p48 (Figure 20), indicating that residues 214-270 are required for p48 to bind ICAT.

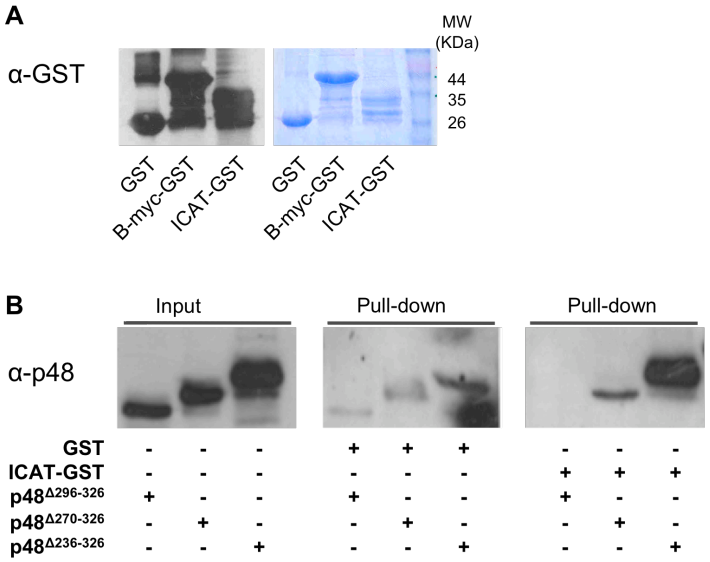

Figure 20. p48 carboxy-region involved in its interaction with ICAT.
A) Coommassie staining of a SDS-PAGE gel loaded with the purified GST-fusion proteins, and the corresponding Western Blot using an antibody that specifically recognizes GST. **B)** Pull-down assay using ICAT-GST fusion protein or GST purified protein and HEK293 cell lysates overexpressing the different p48 deleted mutants. By deleting from the 236 aa residue until the last 326 aa residue the p48 interaction with ICAT is lost.

3.2. Expression of ICAT in the murine pancreas

To examine whether ICAT and p48 are co-expressed in the same cell types in the pancreas, immunohistochemical assays were performed using an affinity-purified polyclonal anti-ICAT serum. ICAT was found to be expressed in the nucleus of epithelial and interstitial cells of the embryonic developing pancreas at day E15.5. At 1 month of age, ICAT was detected at low levels in the nucleus of acinar cells, at intermediate levels in the pancreatic ducts, and at higher in islet cells (Figure 21). Therefore, ICAT and p48 share the same location both in the developing cellular lineages of the embryonic pancreas and in the nucleus of the adult acinar cells.

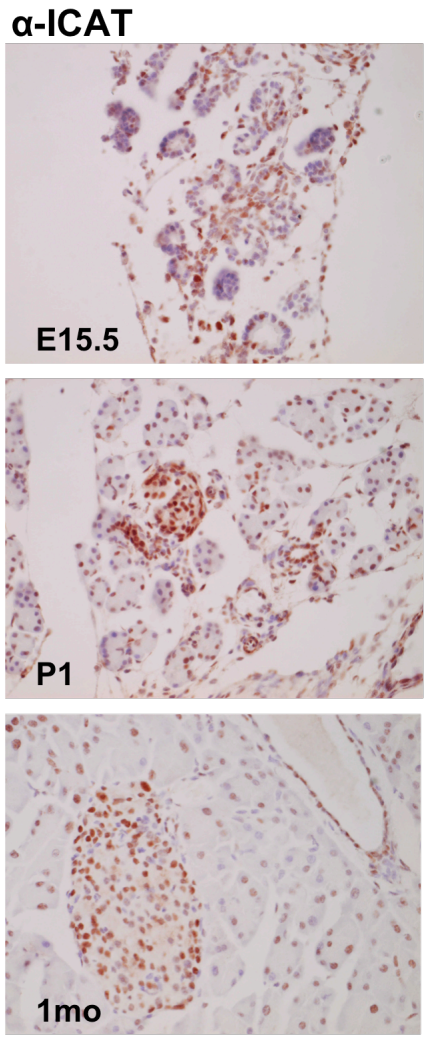

Figure 21. ICAT expression in mouse pancreas.
Sections of pancreas at different developmental stages and in the adult were incubated with affinity-purified anti-ICAT antibody. ICAT is expressed in the embryonic pancreas as well as in the adult organ. Expression levels are low in normal acinar cells, intermediate in ductal cells, and high in islet cells.

3.3. ICAT interferes with the transcriptional activity of the PTF1 complex

To determine whether ICAT affects PTF1 complex transcriptional activity, promoter assays were performed in HEK293T and acinar cells. HEK293T cells were transiently co-transfected with the p48 and RBP-L expression vectors as well as with the $6xA_{26}$-luc reporter. When increasing amounts of ICAT were co-transfected in these cells, the transcriptional activity of the reporter decreased in a significant and dose-dependent manner (Figure 22). The same behavior was observed when these reporter assays were performed in AR42J and 266, two tumor cell lines displaying acinar differentiation. Similar results were also obtained using the $6xA_{26}$-luc artificial reporter and the endogenous Ela200/Ela500 luciferase reporters (Figure 22). Endogenous β-catenin is mainly localized in the membrane/cytoplasm of these cells and there is no evidence for its nuclear accumulation. To determine whether β-catenin could be involved in the effects observed on the acinar specific reporters, increasing amounts of a plasmid coding for β-catenin were transfected in these assays. No changes in the $6xA_{26}$-luc reporter activity could be consistently demonstrated. Moreover, overexpressing β-catenin together with ICAT did not affect the repressive effect of ICAT exert on the reporter. Therefore, ICAT interferes with the PTF1 complex activity and in a manner that is β-catenin independent.

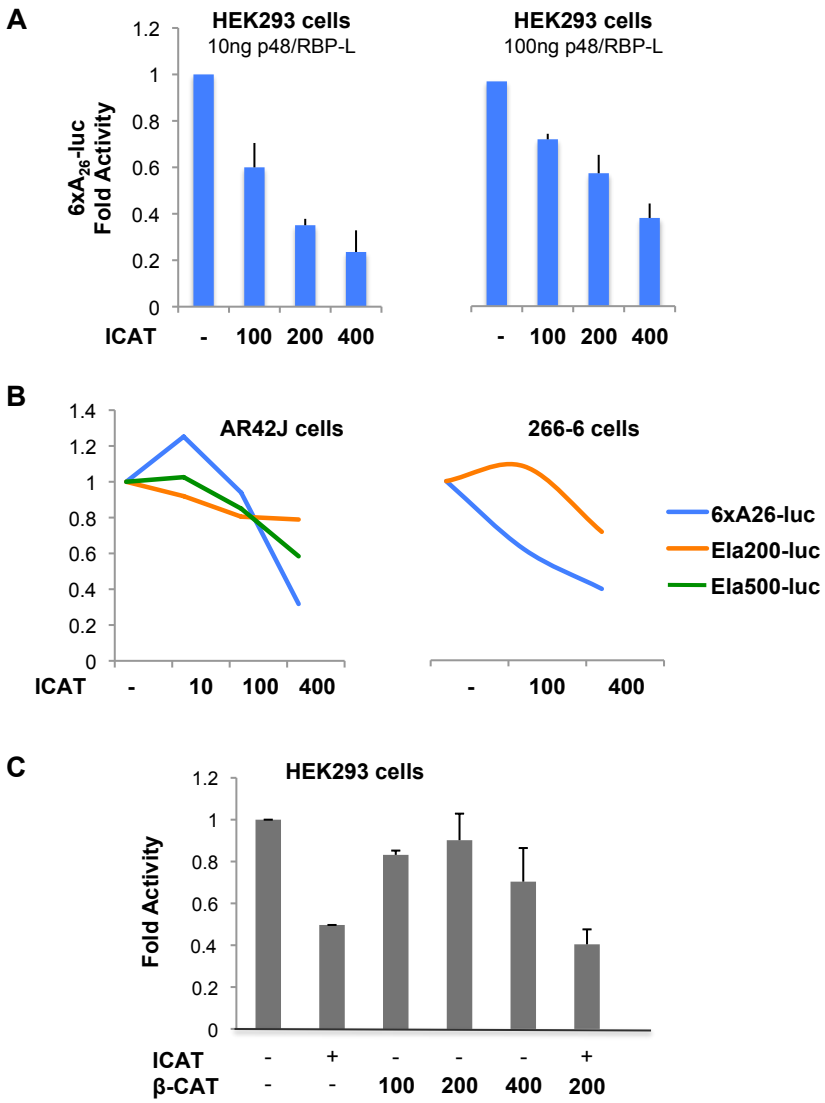

Figure 22. Luciferase Reporter assays.
A) Increasing amounts of ICAT decreased the activity of the artificial 6xA26-luc reporter in a doses-dependent manner, in HEK293 cells. **B)** The same decrease in the activity of the 6xA26 artificial reporter was observed in both AR42J and 266-6 pancreatic acinar-like cell lines. Moreover, two reporters consisting on the promoter regulatory region of the elastase gene were affected in a similar way upon ICAT overexpression. **C)** In order to define whether the β-catenin signaling was implied in the effects seen on the PTF1 transcriptional activity, increasing amounts of β-catenin were transfected in HEK293 cells together with the 6xA26-luc reporter. No significant changes were seen in the reporter activity, and importantly, increasing the amount of β-catenin did not revert the ICAT inhibitory effect on the promoter activity. The reporters activity was measured in triplicates, in three independent transfection assays, and the error bars represent the corresponding SEM value.

3.4. Effects of ICAT overexpression on the acinar differentiation program in vitro

To determine the effects of ICAT overexpression on cell differentiation in exocrine pancreatic cells, experiments were carried out with 266-6 acinar cells. The human ICAT-Flag cDNA was cloned into the $pREP_4$ vector, which allows the establishment of stable transfectants upon hygromycin selection. Four 266-6 independent clones overexpressing ICAT-Flag, as well as four control clones, were selected. Western blotting and immunofluorescence using both the anti-Flag and anti-ICAT antibodies confirmed ICAT expression in the clones stably transfected with $pREP_4$.ICAT.Flag. ICAT mRNA levels were also analyzed by qRT-PCR by using primers designed to detect both endogenous and exogenous ICAT mRNA (Figure 23). All the confirmation experiments were done using as controls four different 266-6 clones transfected with the $pREP_4$-empty vector.

As shown in Figure 24, ICAT localized both in the cytosol and in the nucleus of 266 cells. Because ICAT binds β-catenin, its subcellular localization was also analyzed by indirect immunofluorescence: no changes in the membrane/cytoplasmic β-catenin localization were observed. Accordingly, the β-catenin transcriptional activity was also not affected, as shown by transient promoter assays using the TOP-Flash and FOP-Flash luciferase reporters (Figure 24).

qRT-PCR analysis to detect expression of a panel of transcripts from pancreatic genes was carried out to examine whether ICAT could modulate the activity of the PTF1 complex. As shown in Figure 24, the clones overexpressing ICAT showed altered expression of some pancreas-specific genes. For instance, while p48 transcript levels were unchanged, Pdx1 mRNA levels were upregulated in all four clones analyzed. ICAT overexpression also resulted in the downregulation of amylase and chymotrypsinogen B1 transcripts, while it had no effect on the levels of elastase mRNA. In addition, carboxypeptidase A transcript levels increased 2-10 fold in ICAT overexpressing clones. Because it

is now established that the digestive enzyme gene expression is regulated in a complex manner that does not involve a single regulatory module,[171] these results support the notion that ICAT modulates acinar differentiation.

To further explore the hypothesis that increasing levels of ICAT could modulate the differentiation status of acinar cells, the mRNA levels of some transcription factors involved in early stages of the pancreatic embryonic development, such as RBP-Jκ, RBP-L, HES1, and Sox9, were analyzed. ICAT overexpression did not affect the mRNA levels of RBP-Jκ, RBP-L, and HES1. By contrast, Sox9 mRNA levels consistently increased up to 3-fold in all clones analyzed. To examine whether ICAT overexpression affected the expression of Wnt target genes in these cells, c-Myc, cyclin D1, and p21 mRNA levels were analyzed; all of them were unchanged in all of the assayed clones, supporting the idea that Wnt signaling levels are unchanged in these cells (Figure 25).

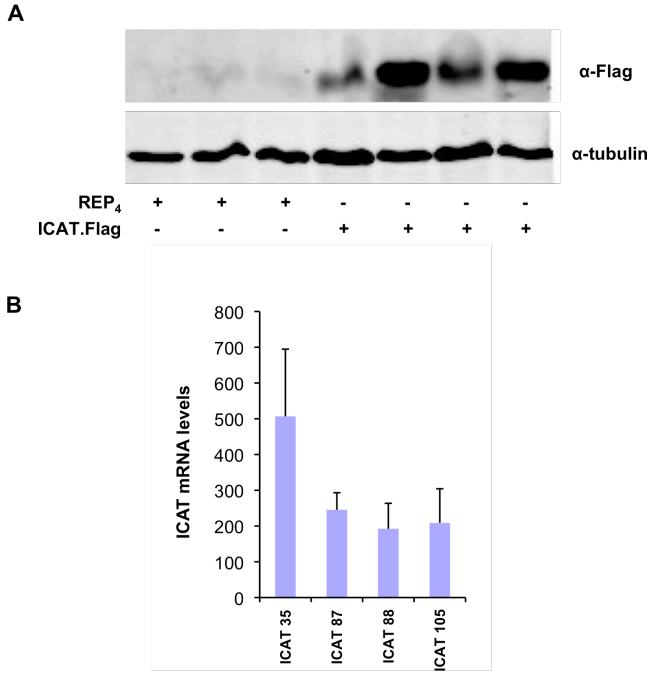

Figure 23.
A) Western Blot using an anti-Flag specific antibody, showing the ICAT-Flag overexpression in four different clones. **B)** qRT-PCR analysis amplifying the transgene ICAT-Flag on the four 266-6 stable clones selected for further analysis.

The Isl-1 and ngn3 mRNA levels were also analyzed but no significant changes were observed in comparison to the control cell clones. Pax6 was shown to be downregulated in the ICAT overexpressing clones. These genes are associated with endocrine differentiation. The expression levels of endocrine markers such as insulin-1 and pancreatic-polypeptide were found unchanged between clones.

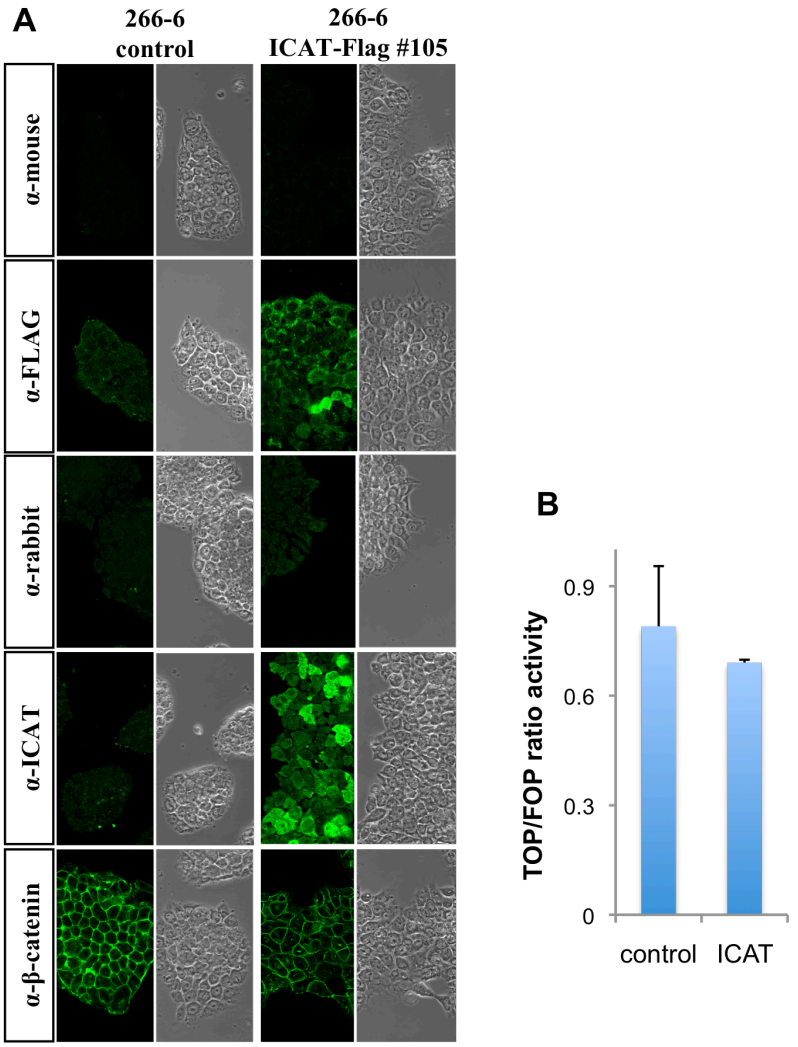

Figure 24.
A) Immunostaining characterization of the ICAT overexpressing stable clones and the respective control. Both anti-Flag antibody and anti-ICAT serum have shown the overexpression of the transgene. Moreover, the cellular localization of β-catenin was confirmed to be mainly membranar in both cell types, with no detection ever found in the nucleus. **B)** The cells were transfected in triplicate with the TOP-Flash and FOP-Flash reporter vectors, and according to its localization the β-catenin activity was low and also not affected in the ICAT overexpressing clones.

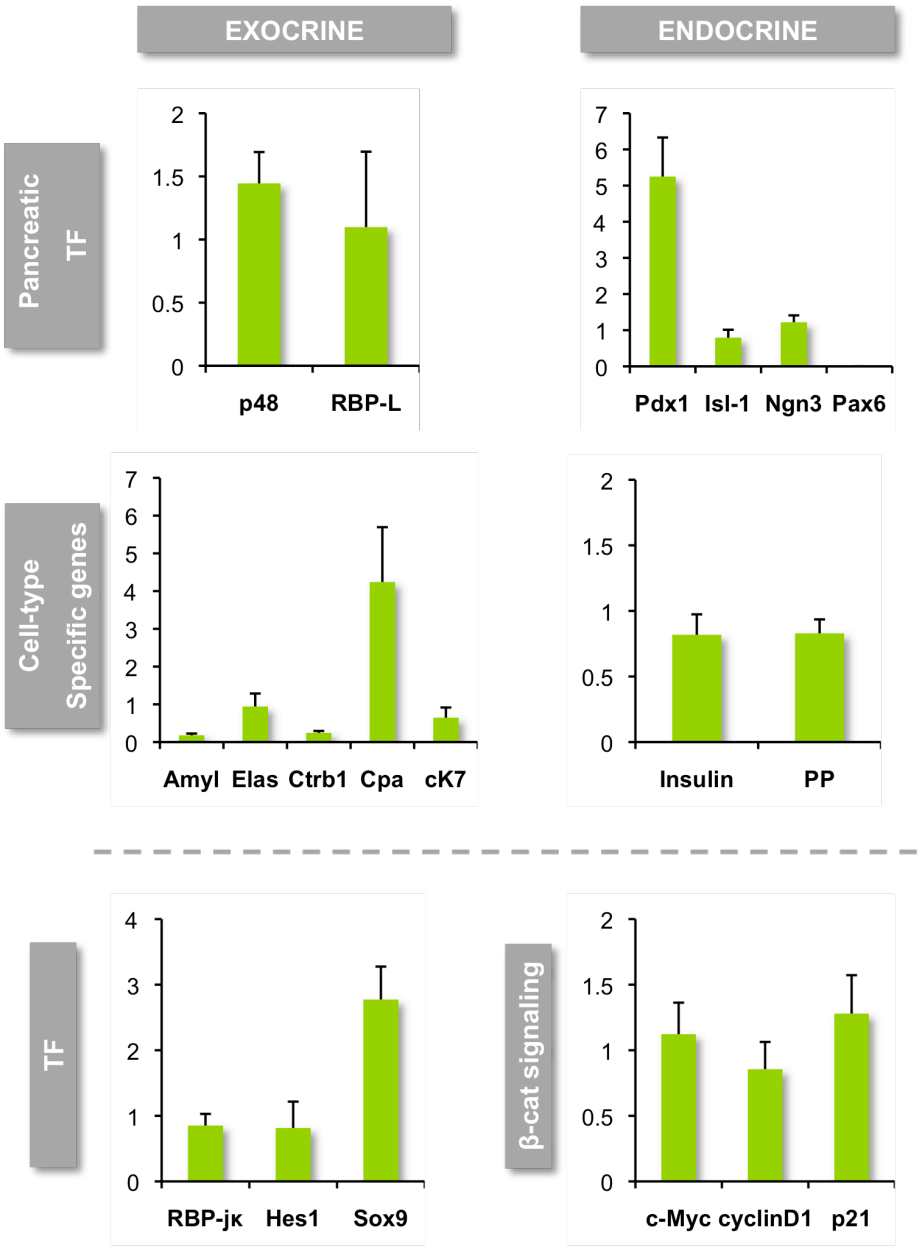

Figure 25.
qRT-PCR analysis of a set of pancreatic transcription factors and cell-type specific genes such as digestive enzymes or the insulin hormone, either exocrine or endocrine specific. Transcritpion factors known for playing a role in the pancreatic determination where also analyzed, such as RBP-jk, Hes1 and Sox9, as well as the c-Myc and p21 transcript levels. The columns represent the mean changes on the respective transcript levels of four different ICAT overexpressing clones in comparison to four independent negative control clones. The results correspond to the analysis of three independent mRNA extractions, and the qPCR were done in duplicates. Error bars correspond to the SEM value.

Because stable transfection and clonal analysis are bound to artifacts derived from selection and adaptation over long time periods, a lentiviral-delivering strategy to overexpress ICAT in 266 acinar cells was used. The human ICAT

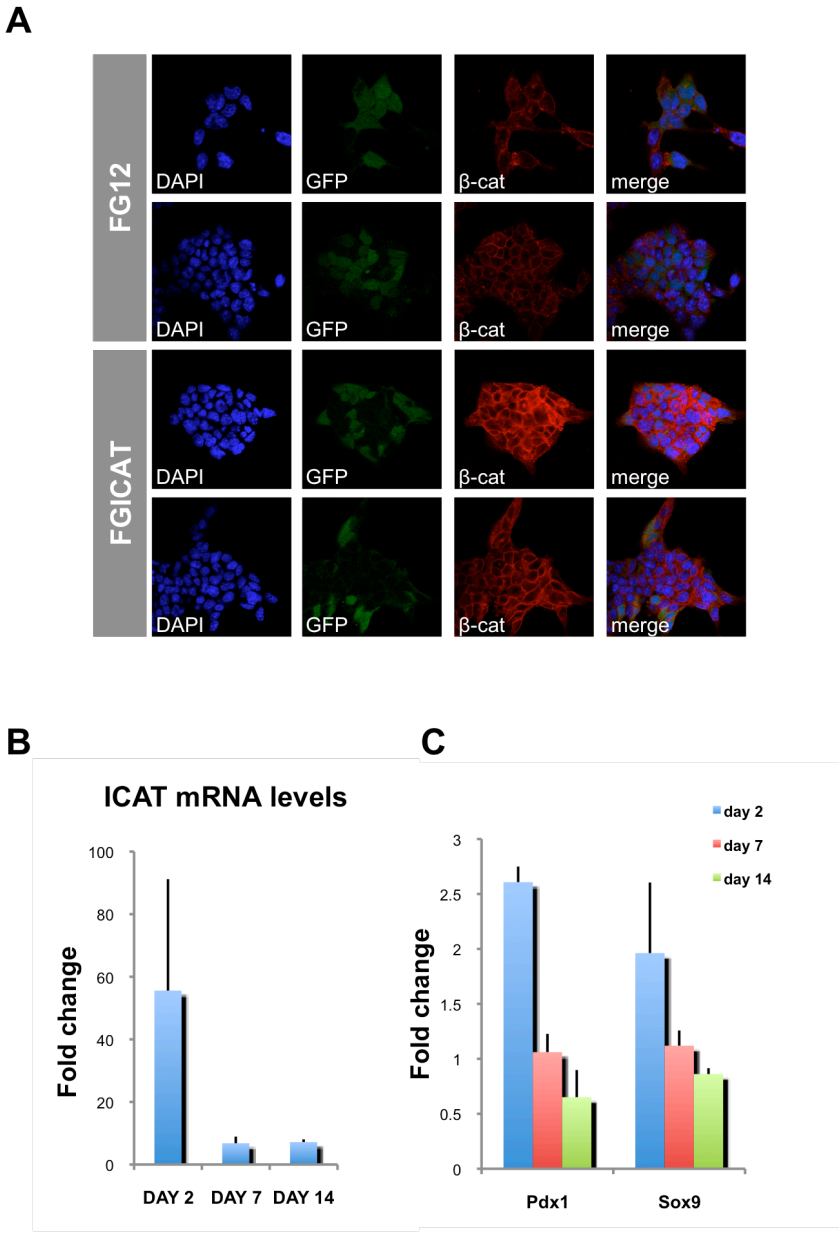

Figure 26.
A) Immunostaining for β-catenin on 266-6 cells infected with the FG12 empty lentivirus or the FG12.ICAT lentivirus. β-catenin is maintained at the cellular membrane. **B)** ICAT mRNA transduced levels. **C)** Expression profile of the Pdx1 and Sox9 transcripts at 2, 7 and 14 days after lentiviral infection. The changes were analyzed in comparison to the 266-6 control population.

cDNA was cloned into a lentiviral vector that would concomitantly express ICAT and GFP, allowing to follow the lentiviral infection efficiency directly by live fluorescence microscopy. ICAT was overexpressed in 266 cells as soon as 48h after infection. Again, ICAT protein and mRNA levels were assayed by western blotting and qRT-PCR. The subcellular localization of β-catenin was assessed by indirect immunofluorescence and it was confirmed to remain mostly membrane-restricted (Figure 26A). Wnt transcriptional activity was assayed using TOP-Flash and FOP-flash luciferase reporters with no significant changes being observed, though the ICAT overexpressing levels are shortly and moderately increased in these cells.

The qRT-PCR analysis of the infected cell populations showed that the virus were effectively transduced in the 266 cell line, and the ICAT transcript is overexpressed as soon as 48h after lentiviral infection. However, at day 7 after infection, the ICAT overexpression has droped already to near 5 times the endogenous levels (Figure 26B). Two days after transduction both Pdx1 and Sox9 transcripts appeared as being upregulated in the 266 cells with increased ICAT expression (Figure 26C). When the ICAT levels decrease, 7 days after the lentiviral delivery, the Pdx1 and Sox 9 levels are also comparable to the levels on the control cell population.

In summary, in 266-6 cells, overexpression of ICAT was associated with up-regulation of Pdx1 and Sox9 mRNA levels, and a complex modulation of expression of acinar digestive enzymes.

3.5. ICAT is present in a reconstituted PTF1-complex

To determine whether ICAT can bind to the PTF1 complex and thus regulate acinar gene expression, a set of oligonucleotides corresponding to its binding sites on the promoter of acinar genes was used for EMSA. The selected sequences were taken from the murine amylase2, the carboxypeptidase1, the chymotrypsinogen B1, and elastase1 promoters. The mobility of IRDye-labeled oligos was assayed in the presence of nuclear extracts from HEK293T cells transfected with plasmids coding for p48 and RBP-L. As expected, when the PTF1 components were introduced, a specific shift of the CtrB1-, Cpa1-, and amylase2-IRDye oligos was detected. This shift was inhibited by prior incubation of nuclear extracts with non-labeled competitor oligos. When nuclear extracts from cells transfected with the PTF1 complex components plus ICAT were used, the PTF1 complex also bound to the oligonucleotides in the presence of ICAT (Figure 26).

Supershift assays were done using the CtrB1-IRDye oligonucleotide since it yielded stronger PTF1 binding. When a monoclonal ICAT-specific antibody was incubated with the nuclear extracts and CtrB1-IRDye oligo, a dose-related decrease in the amount of complex formed was observed (Figure 27). This effect was not seen when non-specific mouse IgGs were used. Thus, overexpressed ICAT is present in a reconstituted PTF1-complex.

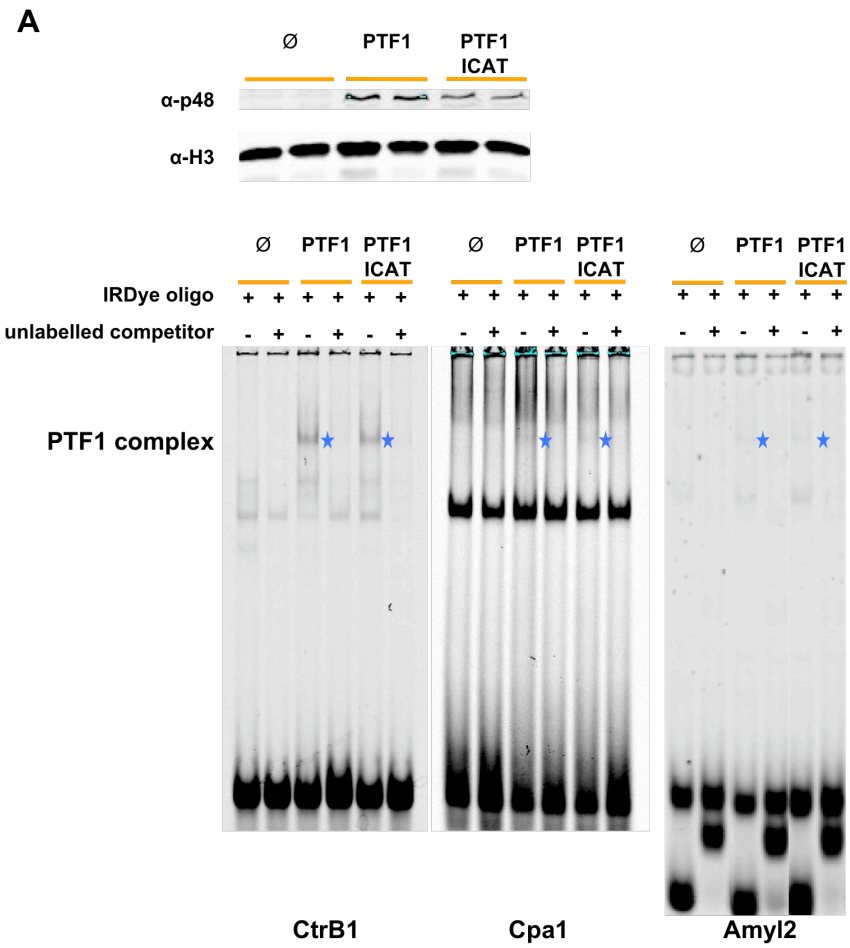

Figure 26.
A) Western Blot anti-p48 and anti-Histone3 on two independent nuclear extractions, showing the correct expression of p48 in HEK293 cells overexpressing the three elements of the PTF1 complex: p48, RBP-L, and E2A. **B)** Electrophoretic Mobility Shift Assays. The blue stars identify the shifted band corresponding to the binding of the PTF1 complex to the different IRDyed oligo probes, corresponding to the PTF1 binding conservative regions on the promoter of the chymotrypsinogen, the carboxypeptidase, and the amylase genes. Unlabelled oligos were used in competition reactions to confirm the specificity of the shifted band.

3.6. ICAT inhibits the binding of p/CAF to p48

The inhibitory regulation of β-catenin signaling by ICAT is achieved by two independent domains of ICAT: an extended region that prevents its interaction with the Tcf/Lef DNA-binding factors and a helical domain that prevents its binding to the CBP/p300 transcriptional co-activator. By displacing CBP/p300 from the promoter complexes, Tcf factors are then repressed by Groucho/TLE and histone deacetylases.[172]

We have shown that p/CAF is a coactivator of the PTF1 complex that effects on the activity of acinar gene promoters.[157] In view of the known effects of ICAT on Tcf/β-catenin transcriptional activity through CBP/p300 coactivators, we hypothesized that ICAT might affect the ability of p48 to bind p/CAF, thus leading to effects on the levels of acinar gene expression.

Co-immunoprecipitation assays were performed with HEK293T cells overexpressing p48-Flag, p/CAF-Flag, and increasing amounts of ICAT-Flag. Total cell lysates were incubated with affinity-purified rabbit anti-p48 antibodies. Figure 28 show that increasing ICAT levels diminish the interaction between p48 and p/CAF in a dose-related manner. Therefore, together with the results shown in the previous section, we propose that the changes in the p48 transcriptional activity due to ICAT overexpression may result from the disruption of the p48 interaction with its transcriptional co-factor p/CAF.

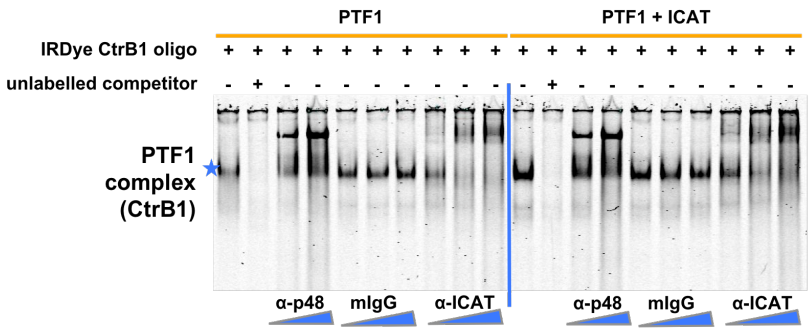

Figure 27. Supershift of the CtrB1oligo probe in the presence of increasing amounts of a monoclonal antibody anti-ICAT (0.5, 1, or 2 µg of mIgGs).

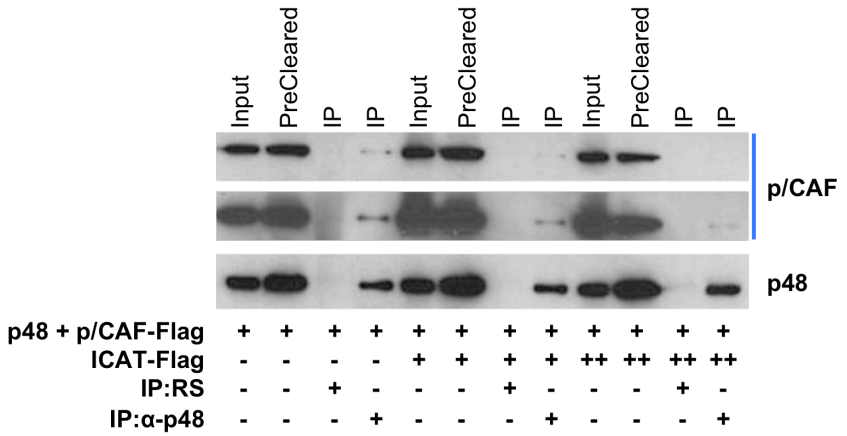

Figure 28. Western Blot anti-Flag showing the decreased amount of p/CAF that co-immunoprecipitate with p48 in the presence of increasing amounts of ICAT. 3 µg of p48 and p/CAF plasmids were used, and either 0 µg, 3 µg, or 6 µg of the ICAT-Flag plasmid were transfected into HEK293FT cells. The fresh lysates were prepared as described before, and the immunoprecipitations were done using a specific serum that recognizes p48 or the corresponding preimmunization serum. Two ECL-developing times are shown to allow the proper visualization of the p/CAF both in the inputs and in the precipitates. The anti-p48 antibody was used to confirm that the p48 precipitation was equivalent in all the immunocomplexes.

3.7. ICAT overexpression increases the p21$^{CIP1/WAF1}$ promoter activity

Until now, the only established function of p48 is its transcriptional activity. However, previous results from our laboratory have shown that p48 also exerts antiproliferative effects that contribute to the quiescence of acinar cells in vivo.[98] Furthermore, loss of p48 during pancreatic carcinogenesis may release a negative growth mechanism that may poise cells to hyperproliferation and malignant transformation. To assess whether ICAT could contribute to the

ability of p48 to regulate cell proliferation, 266 acinar cells were transfected with the $pREP_4$-ICAT-Flag construct or the empty vector. As shown in Figure 29, ICAT overexpression modestly decreased cellular proliferation. This effect was also seen in Cos7 fibroblasts that were transfected with the pcDNA3-ICAT-Flag constructs together with the plasmid coding for p48-GFP, and the proliferation rates measured by flow cytometry. Cells overexpressing p48 showed a reduction of S-phase cells of approximately 50% whereas those overexpressing ICAT had a decrease of 20%, when compared to control cells co-transfected with the $pcDNA_3$ and the GFP empty vectors. Co-transfection of p48 did not affect the modest reduction of cell proliferation induced by ICAT overexpression (Figure 29B). Rodolosse et al have described that the decrease in the G1-S transition produced by p48 in pancreatic RWP-1 ductal cells was associated with an increase of the activity of the $p21^{CIP1/WAF1}$ promoter, a cyclin-dependent kinase inhibitor (CDKI). Using RWP-1 cells and the 2.4-kb $p21^{CIP1/WAF1}$ promoter reporter, ICAT and p48 separately increased 1.5-fold promoter activity and similar effects were observed when the cDNAs for both proteins were co-transfected (Figure 29C). We have found no evidence for synergy in the effects of p48 and ICAT on cell proliferation.

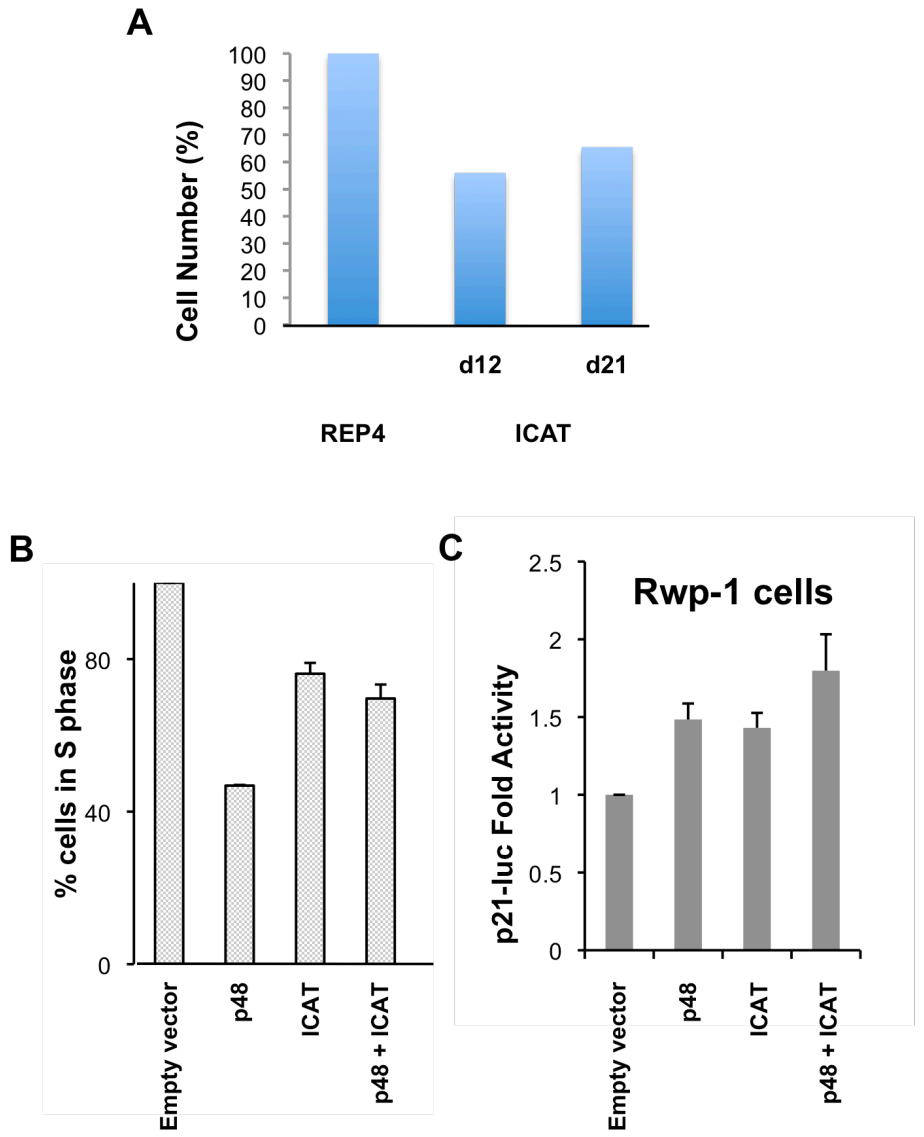

Figure 29.
A) Relative cell number of 266-6 pseudo-stable cells overexpressing ICAT-Flag in comparison to the respective control cells. 12 days and 21 days after transfection and hygromycin selection, the increase of ICAT results in a sustained slow down of the cellular proliferation.
B) Percentage of Cos7 cells in the S phase, upon overexpression of p48, ICAT, or both. ICAT itself decreases the percentage of cells in the S phase, but this effect is not sinergistic with p48.
C) Both p48 and ICAT induce the activity of the p21-luciferase reporter, in Rwp1 cells. The results shown represent three independent assays. The error bar represents the respective SEM value.

3.8. ICAT is differential expressed in an APC$^{-/-}$ transgenic mice model (Paper 2)

While this work was being performed, we became aware that A. Strom in the laboratory of P. Herrera had analyzed the effects of conditional inactivation of *Apc* in the mouse pancreas by crossing *Apc* floxed mice with transgenic mice in which Cre recombinase is under the control of the Pdx1 promoter. [26,173,174] In these mice, β-catenin is allowed to freely accumulate from day E10.5 on in the pancreas. A collaboration ensued which led to the published article attached in Supplementary Material (Strom et al.).

Apc$^{P-/-}$ mice showed a normal development until the third week after birth. Between the first and the sixth month of age, an acinar overproliferation lead to pancreatomegaly with a 5-fold increase in pancreatic mass. Simultaneous deletion of c-*Myc* reverted the pancreatomegaly phenotype, suggesting that the effects of β-catenin on pancreatic size result are dependent on c-Myc upregulation.

Despite the oversize, all the pancreatic cell types were histologically normal in young animals, maintaining normal exocrine and endocrine functions. However, because the number of acinar cells is higher than in the normal pancreas, a similar absolute amylase activity may reflect a decreased amylase content per cell or a failure in the amylase release, suggestive of a defective acinar cell differentiation.

In 1 year-old mice, hypertrophic acinar cells with dysplastic nucleus were focally found, but no tumor formation was observed. Moreover, although β-catenin remained in the nucleus of the acinar cells, the observed upregulation of c-Myc and cell proliferation was no longer observed. Therefore, adult mice displayed a "resistance" to the transcriptional effects derived from nuclear accumulation of β-catenin. A candidate gene to play a role in acquiring the "resistance" to β-catenin was ICAT, especially in the view of our finding of its

expression in the pancreas and its interaction with p48. In both normal and *Apc*$^{P-/-}$ mice, and accordingly to what was previously described, ICAT expression was high in islet and ductal cells throughout life. In young *Apc*$^{P-/-}$ acinar cells, the ICAT expression levels are similar to those of their control littermates. Later, ICAT acinar expression is downregulated in wild type aged animals whereas it remains upregulated in *Apc*$^{P-/-}$ acini. Thus, the pattern of ICAT expression is consistent with the changes in the β-catenin signaling activity observed in the acinar cells.[56]

3.9. ICAT expression in pathological conditions in the pancreas

Our findings suggest that changes in ICAT expression levels may affect cell differentiation, as well as Wnt signaling activity, in the pancreas. Therefore, we analyzed ICAT expression by immunohistochemistry in a human pancreata from diverse pathological conditions (Figure 30).

We analyzed the expression of ICAT in a panel of tissue microarrays containing normal pancreas and samples from pancreatic ductal adenocarcinomas, acinar cell carcinomas, and pancreatic endocrine neoplasms. In all the neoplasic lesions is observed an upregulation of ICAT, which sub-cellular localization remains mainly nuclear. Interestingly, among the pancreatic endocrine neoplasms subtypes, the insulinoma has a different pattern of ICAT distribution than the non-functional and carcinoid PETs. Upregulation is seen in only 1 out of 9 insulinoma cases, whereas ICAT downregulation appears as a more common event.

In ACC, unlike in normal acinar cells, ICAT is expressed at high levels in the majority of the tumors assayed, although no correlation was seen between the ICAT overexpression and the β-catenin nuclear accumulation or mutation status (see Table 4 and Figure 31).

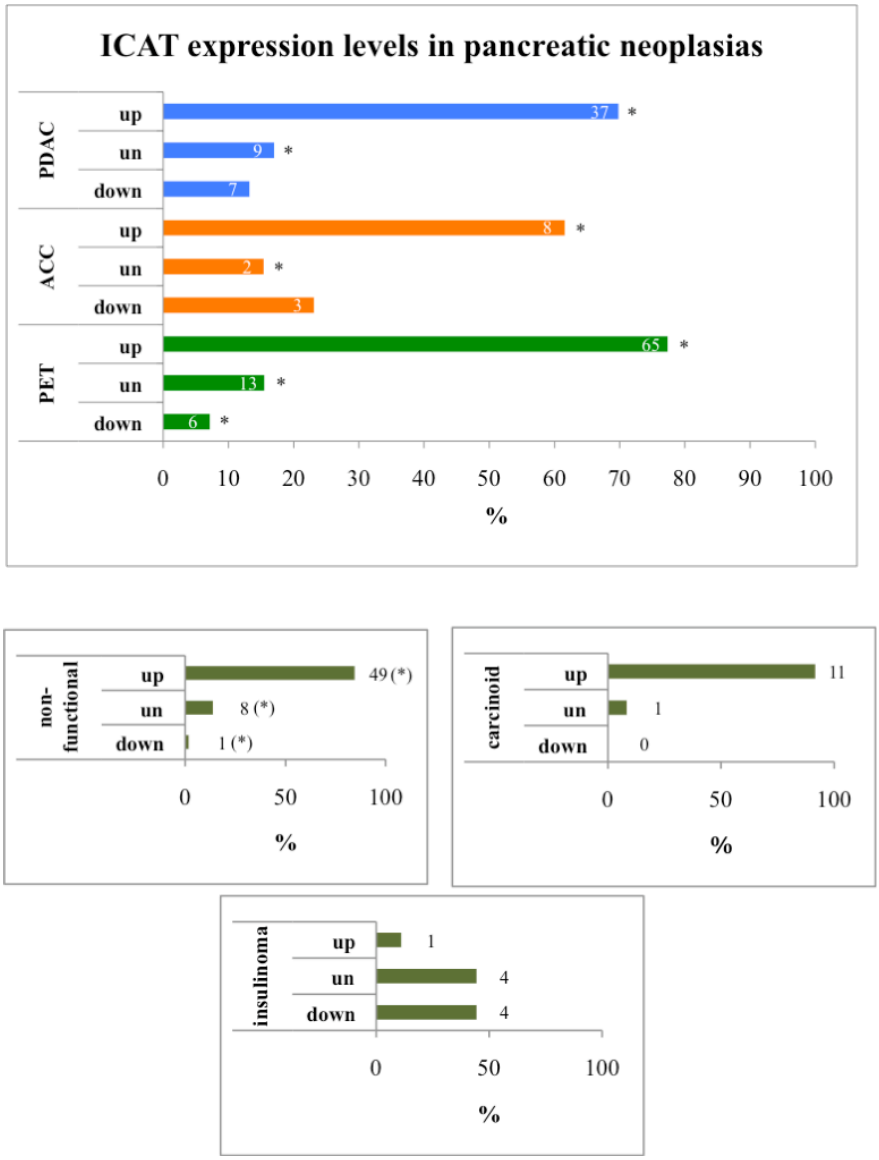

Figure 30.
Distribution graphics of ICAT expression levels in pancreatic ductal adenocarcinomas, acinar cell carcinomas, and pancreatic endocrine neoplasms, accordingly to a qualitative analysis parameters set. Non-functional, carcinoid and insulimonas are pancreatic endocrine neoplasm subtypes. The figure in each bar indicates the n (number of cases), and the % corresponds to the percentage of cases with specific upregulation, downregulation, or unaltered ICAT expression. Binomial probability mass function (*) <0.01.

ICAT expression in acinar tumors in relationship to beta-catenin alterations

	ICAT levels	ICAT distribution	beta-Catenin distribution	beta-Catenin mutation
normal pancreas	++	nucleus	membrane, cytoplasm	
ACC 1	+++	nucleus	membrane, cytoplasm	
ACC2	++++	nucleus	membrane, cytoplasm, and nucleus	
ACC3	-	n.r.	membrane	
ACC4	-	n.r.	membrane, cytoplasm, and nucleus	no mut.
ACC5	+++++	nucleus and cytoplasm	NV	
ACC6	+	nucleus	membrane, cytoplasm, and nucleus	ACC-ATC T41I
ACC7	+++	nucleus and cytoplasm	membrane, cytoplasm, and nucleus	no mut.
ACC8	++++	nucleus	membrane, cytoplasm	
ACC9	+++++	nucleus	membrane, cytoplasm	
ACC10	+++++	nucleus	membrane, cytoplasm, and nucleus	no mut.
ACC11	++++	nucleus	membrane, cytoplasm, and nucleus	no mut.
ACC12	+	nucleus	membrane, cytoplasm	

Table 4.
Analysis of ICAT expression levels and subcellular distribution in a panel of acinar cell carcinomas, and its relation to the β-catenin localization and mutational condition. The ICAT levels are represented accordingly to a qualitative analysis parameters set, where – corresponds to no expression and +++++ to all the pancreata nucleus stained for ICAT. (n.r., not relevant)

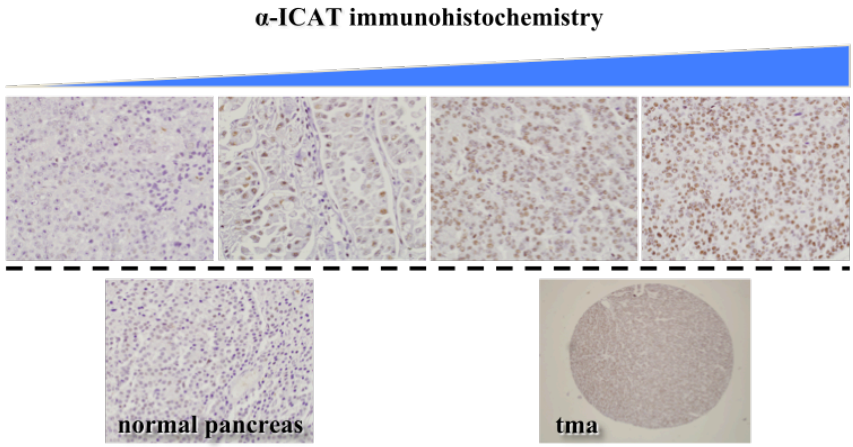

Figure 31.
Representative pictures of the human acinar tumor microarray (tma), showing the variability of the ICAT expression levels between study cases. An affinity-purified anti-ICAT antibody was used to detect ICAT.

DISCUSSION

p48 is a bHLH transcription factor required for the maintenance of the adult pancreatic exocrine compartment homeostasis, the formation of the embryonic pancreas[33,89], and for the generation of GABAergic neurons at multiple sites in the nervous system including the cerebellum, the spinal cord, and the retina.[88,90,91,93,175] The requirement of p48 for the morphogenesis of these cell types and organs highlights that its functions are not limited to the activation of expression of acinar pancreatic digestive enzymes, as initially thought. The functions of p48 are spatially and temporally regulated and while the PTF1 complex is required for its transcriptional activity in the adult pancreas, little is known about the partners of p48 in other cell types or at other developmental stages. Indeed, recent research work has focused on a better characterization of the PTF1 complex and its putative multiple constituents, which vary at different time points in the pancreas, as well as to determine their specific function throughout the lifetime. Cellular and biochemical experiments have shown that p48 also exerts an antiproliferative effect and that this function appears to be independent of the bHLH DNA binding domain, revealing that at least some of the p48 roles are independent of this domain and may not be related to the p48

transcriptional activity in the PTF1 complex on the expression of acinar-specific genes.

In this work, we hypothesized that different domains of p48 may be implied in regulating its different functions by physically interacting with other yet unidentified proteins, different from those involved in the formation of the PTF1 complex. These interactors would contribute to activate the expression of target genes as member of transcription complexes, and to exert/relieve its antiproliferative cellular effect. Here, I have carried out experiments aimed at identifying proteins that are able to interact with the p48 transcription factor, taking advantage of the yeast two-hybrid technique.

The choice of the yeast two-hybrid system for the identification of new partners, rather than a proteomic approach, relied on the fact that this strategy had already proven suitable for the detection of p48 interactors. For instance, RBP-Jκ transcription factor was identified as a new p48 co-factor using this technique.[165] This interaction was afterwards used as an internal control in our assays and the fact that it was pulled out in a library-scale yeast two-hybrid screening using p48 as bait, provides trustworthiness to the p48 interactors identified in this work. In addition, a proteomic search for additional interactors not involved in the PTF1 complex of the adult pancreas would have required the use of large amounts of protein from the early embryonic pancreas, a formidable task that was unlikely to be successful.

Next, we will discuss the newly identified putative p48 interactors that may cooperate with it in its different functions in the pancreas.

1. The N-amino domain of p48 as a co-activator for transcription: the p/CAF interaction

In the course of the experiments required to confirm the specificity of the yeast two-hybrid screenings, we found that the N-terminal region of p48 might actually consist of a transactivation domain by using a β-galactosidase assay preformed with different regions of p48 used as baits. This reporter assay control was done in order to reduce future false positive colonies at the time of the library-scale screening for new partners. Typically, a type of transcriptional activating domains consists of regions rich in acidic amino acids that recruit co-activators such as p/CAF or its homologue Gcn5, which have an intrinsic histone acetyltransferase (HAT) activity. Acetylation neutralizes the positive-charged lysines at the histone amino-terminal chains, decreasing their binding to the underlying DNA, changing the nucleosomal conformation and enhancing the exposure of regulatory sequences to *trans*-acting factors.[166] Therefore, histone modifications are involved in the generation of chromatin domains that are permissive for gene expression.[176] The fact that the expression of the reporter gene was activated by the presence of the amino-terminal residues of p48 led us to investigate the role of this domain in the p48 transcriptional activity. This region of p48 was shown to be able to recruit both transcription co-activators indicated above. p/CAF acetylates both histone and non-histone substrates that may be architectural DNA binding factors (such as high-mobility group – HMG – proteins), general transcription factors, and site-specific DNA-binding factors. Post-transcriptional modifications of the latter - such as phosphorylation, glycosylation, or acetylation - can affect both the DNA-binding activity and transcriptional functions.[177] Furthermore, acetylation may also interfere with protein-protein interactions, as TCF CBP-mediated acetylation inhibits its bond to the co-activator β-catenin.[178]

When the co-activation of p/CAF was assayed in reporter experiments, we demonstrated that indeed p/CAF increased the transcriptional activity of p48 plus RBP-L-mediated activation and had no effect upon the p48 plus RBP-Jκ

activity. Because p/CAF enhanced the p48 transcriptional activity by itself but not that of RBP-L, the coactivity of p/CAF is therefore mediated through p48. Further experiments proved that the p/CAF-acetyltransferase activity promoted the nuclear accumulation of p48. To comprehend how the p/CAF acetyltransferase activity could affect p48, the eight lysines found in p48 were mutated into arginine and assayed for their transcriptional activity and ability to complex with p/CAF, RBP-L, and the bHLH E47 transcription factor. Lysine 200 is located in the p48 bHLH domain, and its mutation completely abolishes the p48 transcriptional activity although it did not interfere with the complex formation, again indicating that the loss of activity is due to a loss of the acetylation modification. This phenomenon of p/CAF acetylation in a lysine residue located in the bHLH domain is similar to that observed in Beta2 and TAL1 acetylation, and sequence comparison between the bHLH domains of this factors indicates that p/CAF acetylates in highly conserved regions of the bHLH transcription factors.[179,180] Another transcription factor whose function is regulated by lysine acetylation is the myogenic bHLH MyoD.[181,182,183]

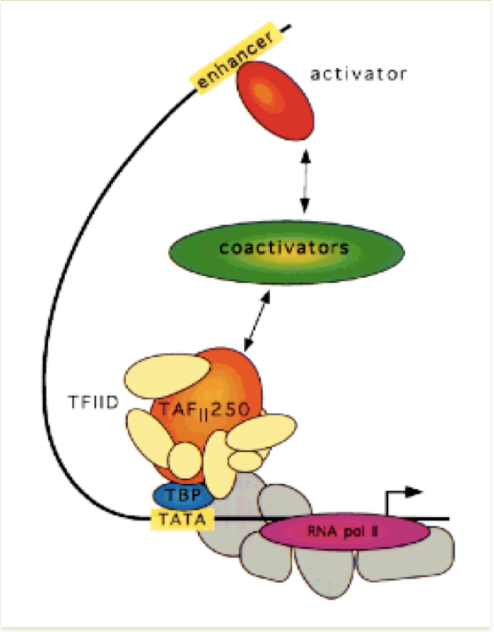

Figure 32: Components of transcriptional regulation: a conceptual model based on physical interactions of activators and coactivators. The curved thick line represents DNA at the 5' end of a gene regulated via a core promoter (TATA) and an enhancer element. (Extracted from Mizzen, C.A. & Allis.)

This finding contributes to a better understanding of the mechanisms through which high levels of the acinar specific genes are reached in the pancreas, a hallmark of the accomplishment of the fully acinar differentiation. We have shown that p/CAF contributes to the nuclear accumulation of the p48 factor, and that its acetylation on the Lysine200 is required for transcriptional co-activation. To further understand the involvement of p/CAF in the cellular differentiation in the pancreas and in the nervous system, where p48 is determinant, conditional inactivation of p/CAF and/or Gcn5 should be studied. Because p/CAF silencing experiments in an acinar cell line had no apparent effect on the p48 transcriptional function, it is possible that Gcn5 is also able to acetylate p48. Due to this redundancy, a very valuable model would be to conditionally inactivate both acetyltransferases p/CAF and Gcn5 in the pancreas.

2. p48 directly interacts with Pbx1, an element of the trimer complex binding to the B element in the enhancer of acinar specific genes

Another important finding that may shed light on the mechanisms involved in transcriptional activation of the acinar specific genes is the physical interaction between p48 and the Pbx1 factor of the Pdx1-Pbx1b-MEIS2b trimer, binding to region B of the enhancer. As indicated earlier, this trimer has been shown to be a functional co-activator of the PTF1 complex on the expression of genes such as elastase.[80] The fact that a specific interaction may occur between the factors comprising the two complexes binding to the A and B elements of acinar genes provides a physical mechanism contributing to the transcriptional coactivation.[81]

Previous work has underlined that Pdx1 is involved not only in the activation of the insulin gene in pancreatic β-cells but in activation of the exocrine program, as well. In acinar cells, the role of Pdx1 consists of binding to the B element as

part of the Pdx1-Pbx1b-MEIS2b co-active trimer. In β-cells, Pdx1 acts as a transcriptional factor involved in the expression of genes such as insulin and Glut2, among others. In the insulin promoter, Pdx1 binds to the A1 and A3/A4 regulatory boxes. When the activity of an artificial reporter containing a repeat of the acinar B element was assayed in β-cells, Pdx1 per se was able to activate it. Of note, in this β-cell context the trimer may not form since Pbx1 and MEIS2b proteins are not endogenously expressed and when their expression was forced, the transcriptional Pdx1 activity was downregulated. By contrast, in acinar cells in the absence of Pbx1 and MEIS2b, Pdx1 by itself could not increase the reporter activity of the B element from the Ela1 enhancer, being unable to co-activate the expression of the acinar specific genes.[80] Therefore, for an endocrine role Pdx1 suffices, but for an exocrine enhancing activity it needs to be in complex with Pbx1b and MEIS2. Pdx1 is expressed both in the acinar cells and in β-cells and it has been proposed that the association of Pbx1 plus MEIS2b with Pdx1 in the former cells swaps its activity from activating the expression of endocrine markers to enhancing acinar-specific gene expression. This suggests that the use of Pdx1 in two distinct cell types may drive the expression of different sets of genes that mark either the endocrine or the exocrine phenotype. In the embryonic pancreas, where both Pdx1 and p48 are present and determine the cell pancreatic lineage fate, it would be of relevance to confirm whether p48 defines the exocrine program by interacting with the Pbx1b and MEIS2 factors, while in the absence of p48 these factors become dispensable and Pdx1 alone suffices for β-cell determination. In fact, Pbx1 expression starts at day E9.5 in the early embryonic development of the mouse and it is required for delimitating the pancreatic anlage in a more defined way than Pdx1 itself. Pbx1 inactivation results in pancreatic hypoplasia and marked defects in both exocrine and endocrine cell differentiation, before death occurs at day E15.5.[184,185]

We hypothesize that, in the adult, the physical bond between p48 and Pbx1b may bring together the machinery required to increase the expression of the acinar genes, such as the digestive enzymes, in a macromolecular complex (see Figure 33 and 34).

3. p48 binds to Midkine

Midkine (MK) is a heparin-binding growth/differentiation factor that, together with Pleiotrophin (PTN), forms the *Midkine* family.[167,186] MK is the product of a gene regulated by retinoic acid[167] and, while it is a secreted peptide, it may also be found intracellularly in the nucleus.[168] MK is a 13KDa mature polypeptide rich both in Cys and basic amino acids, and MK and PTN share 46% of sequence identity, all Cys residues being conserved and both proteins having very similar three-dimensional structures.[167,187,188]

N-syndecam, PTPζ, anaplastic lymphoma kinase (ALK), and LDL receptor-related protein (LRP) are the known putative cellular receptors for MK and PTN. These receptors might be differentially used for the biological activities of MK and PTN, or might cooperate to induce intercellular signaling pathways.[188]

A broad range of functions have been attributed to MK and PTN during development and differentiation, namely to have neurotrophic activity and to be a mitogen for fibroblasts.[189,190,191,192] MK also promotes the survival and outgrowth of neurites from embryonic neuronal cells and is expressed at high levels in the brain during the early stages of experimental cerebral infarction.[193]

During mouse development, MK expression starts at day E7 of gestation and peaks at day E9. MK is strongly expressed in all germ layers and at day E11 its expression decreases becoming gradually restricted to nervous tissues, epithelia with mesenchymal interactions such as the pancreas, and to the kidney. The anterior lobe of the hypophysis and the retina also display high levels of MK. In the developing pancreas, MK shows strong expression at the basement membrane and a subsequent down-regulation upon pancreatic maturation, being expressed in budding cells and in islets forming from E15.5 on.[194] High levels of expression of MK are found in the tips of the developing acini at E14.5 (www.genepaint.org), suggestive of co-localization with p48. These findings are

consistent with a role for MK in cell growth/differentiation during early pancreas development. Interestingly, although PTN shares homology and at least some functionality with MK, its expression pattern is very different. For instance, PTN is weakly expressed between days E12-E15 and in the adult it is strongly expressed in the brain. [187,190]

MK and PTN expression are induced in various carcinoma tissues. For instance, MK is detected in colorectal, gastric, gall bladder, pancreas, breast and lung carcinomas.[188] Particularly in G401 Wilm's tumor cells, a truncated version of MK has been detected[195], a variant also detected in the developing human bile ducts.[196] Masahiro et al reported the expression of MK in normal and neoplastic hamster pancreas. In normal pancreas, islets had a weak positive signal for MK and ductal and acinar cells were negative, and MK levels increased progressively throughout the evolution of the neoplastic lesions. All of the analyzed pancreatic cancers were positive for MK that was localized in the cytoplasm.[169]

In our assays, we addressed the question whether p48 and MK could co-localize and whether a physical interaction between the two proteins could take place. For biochemical analyses, the model we chose consisted in co-transfecting a p48-GFP fused protein vector together with a vector coding for MK, or the respective empty control, into Cos-7 cells. The subcellular localization of p48 in these cells had already been described to be mainly, though not exclusively, nuclear.[157] Preliminary results have shown that the co-transfection with MK did not change the p48 subcellular localization, nor vice-versa, an observation that would suggest that a co-localization between the proteins and a physical bond between them could take place. (See Supplementary Results).

We also asked whether MK could affect the p48 functions by assessing the effects on its best-described function: the transcriptional activation of elastase. This activity was measured by the specific luciferase activity of the artificial $6xA_{26}$-luc promoter, as described previously. Unfortunately, due to technical reasons, it has not been possible to conclude on these effects because both the

$6xA_{26}$-luciferase reporter as well as the renilla normalization reporter were negatively affected upon MK overexpression. As a result, the ability of MK to modulate p48 activity remains to be conclusively established. Further work is going on in our laboratory to establish the functional meaning of p48 and MK co-expression in both normal and neoplastic pancreas.

4. Myc proteins as new p48 partners

B-myc is a member of the *myc* gene family coding for proteins playing an important role in proliferation, apoptosis, transformation and differentiation.[197] Thus, the interaction between p48 and a member of this family is very appealing and might represent a case of partnership between a bHLH transcription factor and a *myc* family gene. B-myc is a small protein that shares high homology with the c-Myc N-terminal comprising the transcriptional regulatory domain and the Myc homology boxes MBI and MBII.[198,199] Therefore, B-myc lacks the region corresponding to the bHLH/LC domain, required for MAX dimerization and DNA binding, which may explain the inhibitory effects it plays on the c-Myc transactivation, transforming, and proliferative abilities. [156,170] Until now, no human B-myc orthologue have been found. B-myc has been described to be highly expressed both in fetal tissues and in the adult, especially in hormonally-controlled tissues and in the brain. In the adult mouse pancreas, B-myc was not detectable by Northern blotting.[156] Since we have found B-myc as a putative p48 partner, it would be interesting to determine whether B-myc is expressed in the developing pancreas and whether it plays a role, together with p48, in the balance of cellular proliferation/differentiation.

Because B-myc shares high homology with the N-terminal region of the c-Myc protein, we examined whether p48 was also able to interact with c-Myc. Preliminary co-immunoprecipitation results have pointed out that p48 could indeed interact also with c-Myc (Figure 18C). A recent study has shown that

conditional c-Myc inactivation in the developing pancreas results in a smaller organ due to a decreased in the proliferation rate of the acinar progenitor cells.[57] Since p48 expression is retained in the c-Myc-deficient pancreata, c-Myc does not seem to be required for the acinar specification and determination, but rather possibly for the expansion of the early pancreatic epithelium. This study is in agreement with the description of a multipotent pancreatic progenitor domain expressing c-Myc consisting of fast-proliferating cells.[13] The mechanism of c-Myc to promote the cell cycle reentry in the developing pancreas likely involves CDK4 upregulation, although other c-Myc-dependent factors should be involved but are currently unknown. c-Myc is a target gene of the Wnt signaling pathway,[200] and consequently its expression has been confirmed in the developing pancreas, where the pathway is active.[60] c-Myc can also act as a potent oncogene in embryonic acinar cells, since c-Myc overexpression under the control of the elastase promoter induces mixed acinar/ductal adenocarcinomas in mice.[201]

The new finding that myc proteins interact with p48 is therefore of potential relevance and should be further studied.

5. p48 binds ICAT, establishing a new link between the p48 function and the β-catenin signaling pathway

ICAT is a 9 kDa polypeptide that negatively regulates Wnt signaling by preventing complex formation between TCF and β-catenin. This inhibition is achieved through its direct competition to bind β-catenin.[69] The crystal structure obtained from ICAT purified with the armadillo repeat of β-catenin has shown that ICAT consists of two regions that bind β-catenin. The ICAT helical domain binds to the β-catenin arm repeats 11 and 12, to which the coactivators CBP/p300 bind, whereas the ICAT extended carboxy-terminal binds to the arm repeats 5 to 10 and is the region responsible for the disruption of the β-catenin

complex with Tcf/Lef.[172] High levels of ICAT are found in the intestinal villi, the nonproliferative region of the intestinal epithelium where the β-catenin/TCF signaling is switched off, and it localizes both in the cytoplasm and nucleus.[155] It has been proposed that ICAT may function as a buffer for cytosolic β-catenin. Actually, ICAT bound to β-catenin does not inhibit the binding between β-catenin and the APC destruction complex, meaning that the turnover of the protein is not affected.[155] When ICAT was overexpressed in cells with aberrantly high levels of β-catenin, it inhibited cell proliferation through induced G2 cell cycle arrest and cell death.[202]

Here, we identify ICAT as a new p48 partner that may modulate some of its functions. Because of the relevance of the Wnt pathway in exocrine pancreas differentiation and in the development of a subset of exocrine tumors, we focused this Ph.D. thesis work on its analysis.

At the biochemical level, this work has shown that ICAT is able to directly interact with p48. The yeast two-hybrid assay identified ICAT as a putative interactor for p48, and further pull-down assays demonstrated that the p48 residues 214 to 270 are required for the interaction. No other known p48 interactors are described to bind to this region. Notably, the p48 C1 box to where RBP-L binds is only 8 residues apart, from residue 278 to 282, whereas the C2 box to where RBP-Jκ binds is further away, from residue 300 to 306 in the rat p48 sequence.[85] I have also demonstrated that increased levels of ICAT lead to a decreased expression of some acinar-specific genes such as the digestive enzymes. This modulation of activity was verified both by luciferase reporter assays and in a cellular model where ICAT was stably overexpressed in an acinar-like cell line. For the expression of the acinar specific genes such as elastase, p48 and RBP-L bind to the enhancer region in their promoters and cooperate on the transcription activation. In the reporter assays, the transcriptional high levels obtained when both p48 and RBP-L were co-transfected were significantly decreased by the presence of ICAT, in a β-catenin-independent way.

While the in vivo relevance of this finding remains to be established (see below), we have shown in collaboration with the laboratory of P. Herrera in Geneva that, in *Apc*$^{-/-}$ mice, ICAT levels increase in the adult acinar pancreas in this mouse strain (see below). This is accompanied by a reduced amylase content per cell in comparison to control littermates. Therefore, ICAT overexpression is associated with a downregulation of at least some of the enzyme acinar genes. Whether ICAT plays a causal role in these effects remains to be established.

5.1. ICAT inhibits the efficient transcriptional activating capacity of the PTF1 complex

By electrophoretic mobility shift assays, we found that ICAT does not prevent the binding of an orthologous PTF1 complex to the corresponding DNA binding site on the promoter region of the digestive enzymes. However, the result was consistent with ICAT being present in the complex, as shown when a specific anti-ICAT antibody was pre-incubated in the binding reactions. Therefore, the mechanism by which ICAT downregulates the transcriptional activity of p48 is not by preventing the PTF1 complex formation upon its consensus-binding site in the promoter regions.

Because we have shown before that p/CAF functions as a coactivator in the expression of acinar-specific genes, we assessed whether ICAT could disrupt the physical/functional interaction between the p/CAF co-activator and p48, inhibiting the acinar program. Indeed, increasing levels of ICAT interfere with the formation of the p48-p/CAF complex, which may mechanistically explain its downregulatory effect of the transcriptional function of p48 on acinar genes. Likewise, it was previously shown that ICAT is not able to prevent the interaction between β-catenin and the transcription factor Lef-1 upon the DNA, even though the transcription was effectively repressed by ICAT. This experiment lead the authors to postulate that apart from preventing the protein-

protein complex formation, ICAT would be able to recognize and block the activity of the complexes that have previously bound to chromatin, possibly by disrupting the interactions between β-catenin and other transcriptional coactivators.[203]

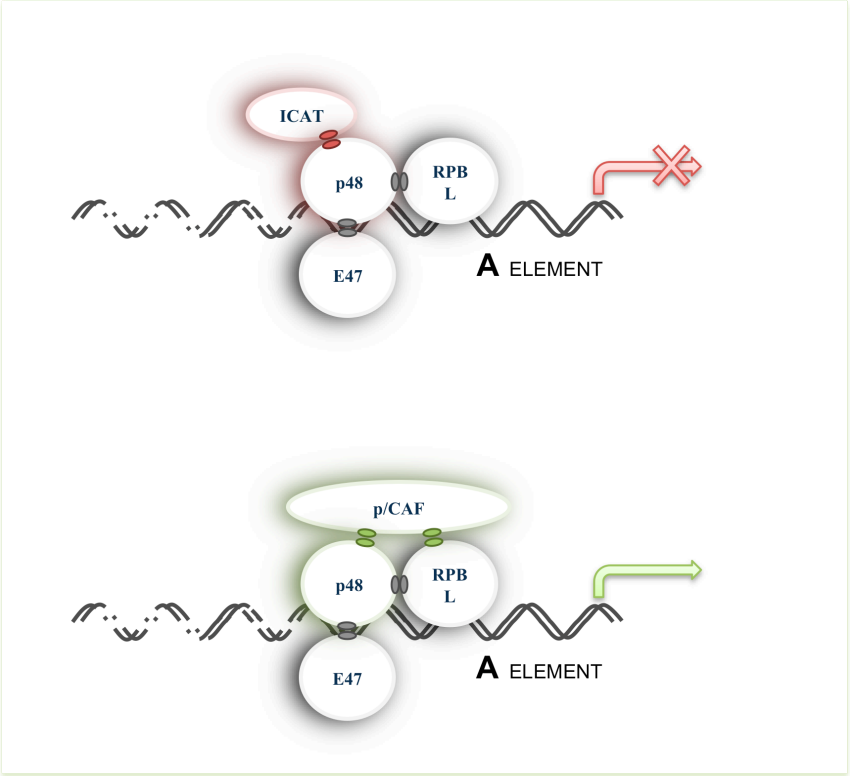

Figure 33. Model of the ICAT inhibitory effect on acinar-specific gene expression. ICAT binds p48 and inhibits the transcriptional activity of the PTF1 complex. In the absence of ICAT, a fully transcriptional active complex is formed, where the p/CAF contributes to the high levels of expression of the acinar digestive enzymes. In the presence of ICAT the effect of p/CAF is markedly reduced.

5.2. The spatiotemporal distribution of ICAT is consistent with the function of both p48 and β-catenin in the pancreas

The immunohistochemical analysis of pancreas sections has revealed that ICAT shares the same cellular compartment of p48, both proteins being present in the epithelial cells of the developing pancreas as well as in the nucleus of acinar cells in the adult. However, the levels of ICAT appear to be specifically decreased in the acinar cells of the adult pancreas. Interestingly, potential p48-binding sites have been discovered in the promoter region of ICAT (R. MacDonald, personal communication). It is conceivable that a negative feedback exists that spatiotemporally regulates ICAT expression in the pancreas. Because of the specific changes of the ICAT expression pattern in acinar cells, p48 could be an ICAT gene regulator. For instance, during embryonic development ICAT could be bound to p48 promoting the undifferentiated status. Later, during development and after birth, the ICAT levels are maintained downregulated.

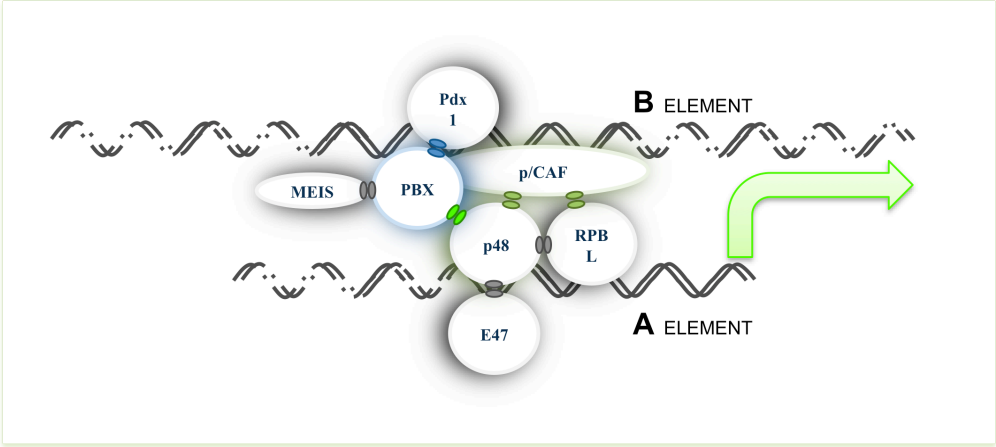

Figure 34. Based on the fact that p/CAF is required for the high transcriptional levels of the PTF1-L complex as well as the functional co-activator trimer Pdx1.Pbx1b.MEIS2b bound upstream on the 5'-enhancer B element, we hypothesize that p48 links all the active factors in a macromolecular complex that binds to the promoter region of the acinar specific enzyme genes.

The findings included in the second paper here in attachment have led to postulate that the young pancreas is sensitive to the β-catenin induced proliferation but the adult pancreas is refractory to this effect of β-catenin. This blockade of responsiveness occurs even in the presence of nuclear β-catenin, suggesting the existence of other regulatory elements such as β-catenin signaling inhibitors. Remarkably, although inactivation of *Apc* occurred efficiently in all ductal, endocrine, and acinar cell lineages, the "sensitive-window" was acinar-restricted with no changes observed in endocrine function and cellular proliferation rate. This result was the first evidence that the β-catenin signaling is facilitated in the adult acinar cells. In fact, acinar cells overproliferated in the early months of life, causing the pancreatomegaly. Accordingly, during this time an increase of c-Myc transcript levels was observed in comparison to the control littermates. With aging, both c-Myc levels and the cellular proliferation rates were normalized, defining the unresponsive phase. The onset of this "β-catenin-resistant stage" suggests the existence of adaptation after continuous β-catenin signaling. Hence, we studied the differential expression of the β-catenin competitor ICAT in the $Apc^{P-/-}$ pancreas and in the normal controls. In both cases, and in agreement with what was previously described in the results, ICAT expression was high in islet and ductal cells throughout life. In young $Apc^{P-/-}$ acinar cells, the ICAT expression levels are similar to those of the control littermates. Later, ICAT acinar expression is dowregulated in aged wild type animals, whereas it is upregulated in $Apc^{P-/-}$ acini. Thus, the pattern of ICAT expression is consistent with the changes in the β-catenin signaling activity observed in acinar cells, and its high expression in islet and ductal cells may account for their cell-specific β-catenin signaling resistance.[56]

The ICAT expression pattern throughout lifetime is also consistent with the functional effects it plays on the p48 transcriptional activity. In light of our results, we hypothesize that ICAT levels have to be downregulated in order for the acinar cells to produce high levels of digestive enzymes. Therefore, ICAT interaction with p48 may serve: (1) to maintain low p48 transcriptional activity

in the embryonic developing pancreas, and/or (2) to drive p48 to accomplish a different function early in the lifetime. Indeed, the expression analysis upon stable ICAT overexpression in 266 acinar cells resulted in changes of the expression of some genes in a pattern that seems to be coherent with this early involvement. For instance, induction of ICAT led to an upregulation of both Pdx1 and Sox9, two markers of pancreatic progenitors.

5.3. The effects of ICAT on p48 in 266 cells are β-catenin independent

One important issue when discussing the observed effects relates to the interaction between ICAT and β-catenin: to which extent these observations are related to which of the ICAT partners. As it was shown by immunofluorescence both in the 266 cellular model and in the adult pancreas, β-catenin is found only in the membrane/cytoplasm. Moreover, in the reporter assays performed, the TOP- and FOP-Flash activity was always very low, a feature that does not support (but does not completely rule out) the existence of an active β-catenin signaling activity in these cells. Therefore, it appears that the effects of ICAT on enzyme transcript expression in these cells are β-catenin independent. However, it is intriguing that when assaying the β-catenin interactions, both by pull-down and co-immunoprecipitation, we found that p48 is also able to bind to β-catenin, opening the possibility of a more complex interaction network between these proteins. To fully describe the dynamics of these protein-protein interactions, more experiments should be done taking advantage of additional ICAT and β-catenin mutants. I have designed and performed pull-down experiments with purified p48 and a set of different ICAT mutant proteins. So far, I have not found an ICAT mutant that binds p48 and not β-catenin or vice versa. Such an ICAT mutant, which would interact only with p48, would be the best tool to discriminate if the effects observed are due to the physical interaction between p48 and ICAT and/or β-catenin.

5.4. ICAT and pancreatic carcinogenesis in humans

Upregulation of the ICAT transcript levels has been described in human colorectal tumors, suggesting that ICAT may play a role in the development/progression of this tumor.[204] In human pancreatic acinar tumors, changes on the ICAT expression levels were observed. Whether these changes are involved in the neoplastic origin or are a consequence of the cellular adaptation to an oncogenic condition should be further investigated. However, given the fact that mysregulation of the β-catenin signaling is the most frequent alteration in this type of tumors, the second option is the most likely. Interestingly, stabilization of the β-catenin signaling in the pancreas promotes the development of tumors that resemble the human solid pseudopapillary tumors.[134] Aberrantly activated *K-Ras* results in PanIN lesions but, strikingly, these lesions were overcome when β-catenin was stabilized, meaning that the sequence in which the oncogenic signals are acquired impact on the outcome of the developed neoplasia.[134] The interrelationship between β-catenin, ICAT and p48 may, therefore, establish a required equilibrium to maintain the pancreatic acinar homeostasis.

Within this study we have characterized the ICAT expression levels in different type of pancreatic tumors. ICAT was seen upregulated in most of them, with exception for the pancreatic endocrine insulinomas, a rare pancreatic tumor growing from β-cells of islets of Langerhans. Although the adult endocrine cells normally express high levels of ICAT, in insulinomas ICAT is rather downregulated or kept unchanged. The reasons for such divergence remain unknown, but it would be of importance to scrutinize whether changes on the ICAT levels associate with the type and prognosis of pancreatic neoplasias.

6. Future studies

To have a better understanding of the role of ICAT in the pancreatic development/function, during the last years we have taken different approaches such as shRNA gene silencing, lentiviral transduction systems, and use of genetically modified mice. None of these strategies has so far yielded conclusive results but a description of the current status of these studies is worthwhile.

For **shRNA gene silencing**, four ICAT conserved sequences were used as silencing targets using lentivirus. The effectiveness in silencing the ICAT expression was confirmed in 293HEK cells. However, because the ICAT endogenous expression levels in the acinar cell lines 266-6 and AR42J were very low, we were unable to study a cellular model of acinar cells with silenced ICAT.

A **lentiviral strategy** was developed in order to infect the pancreas of adult mice using a surgical procedure. The lentiviral vector coded both for ICAT and GFP, which would allow us to confirm the proper delivery/infection of the pancreatic cells. Similar to what was recently shown by other laboratory (I. Rooman, unpublished data), the murine pancreas was not efficiently infected by this type of virus. Therefore, we found our work in need of an efficient method to study ICAT functions *in vivo* in mice.

To this end, we have generated a **transgene vector** based on the pBI-L Tet Vector from Clontech, which contains a bidirectional tet-responsive promoter that can be used in the Tet-On/Tet-Off systems. The Tet-responsive element (TRE) consists of seven copies of the tet operon (tetO) sequence and is in between two minimal CMV promoters without the enhancer element. Thus, in the Tet-On system the promoter is only active when the reverse tetracycline-controlled transactivator (rTtA) is bound to the tetO sequence, which controls the expression of ICAT and the luciferase reporter. The use of this Tet-On

system would allow us to tightly control the ICAT overexpression upon doxycycline (a tetracycline derivative) administration to the mice. Figure 35 shows the correctly expression of the cloned ICAT-Flag by two different established Tet-On cell lines, in the presence of doxycycline. Moreover, the luciferase activity was measured and it was consistent with the observed ICAT-expression (data not shown).

This construct has been microinjected in ooccytes and we have three founder lines positive for the construct; our laboratory is currently testing whether these lines transmit the transgene to the progeny. The group is planning to use these mice to induce the expression of ICAT specifically in the pancreas. To have a tight control on the ICAT overexpression and to be able to guide it to specific cell types, we plan first to cross the *Tet-ICAT* mice with the *Rosa-rTtA-IRES-EGFP* and with the *Rosa LSL-rTtA-IRES-EGFP* strains, available at the CNIO facilities (E. Wagner's lab). The first cross would allow testing the effects of ICAT overexpression in all tissues at the desired point in time. The second cross

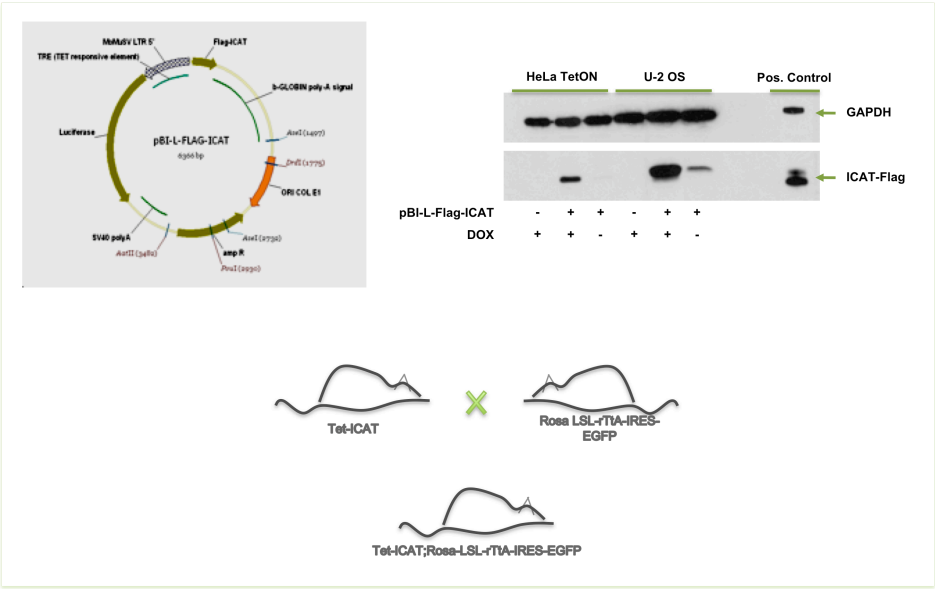

Figure 35. Map of the pBI-L-Flag-ICAT vector constructed to generate transgenic mice overexpressing ICAT in an inducible manner. Western blotting using a specific antibody against the Flag-tag demonstrates the correct regulation of the transgene. HeLa TetON and U-2 OS inducible cell lines were transfected with the pBI-L-Flag-ICAT vector and grown in the presence or absence of doxycycline.

would allow to obtain tissue-specific inducible ICAT expression upon an additional cross with a pancreas-specific deletor strain (i.e. p48-Cre) since expression of the rtTA is under the regulation of a LoxP-STOP-LoxP cassette. In the presence of the Cre-recombinase, the sequences between the LoxP sites are deleted and the STOP signal is excised, activating the expression of the rTtA protein and inducing the ICAT expression upon doxycycline administration. In addition, this model will allow to cross the *Tet-ICAT;Rosa-LSL-rTtA-IRES-EGFP* with any strain coding for the Cre-recombinase under the control of a specific promoter, being able to guide the ICAT expression into any specific cell type of interest. Such an inducible ICAT system may be of broader interest to the scientific community since ICAT has been implied in other organs, as well as during tumorigenesis.

These studies will allow to determine more precisely the role of ICAT in pancreatic physiology and in pancreatic cancer, its relationship with p48, as well as the role of ICAT in a wider variety of tissue types.

CONCLUSIONS

1. We have identified new putative p48 interactors using a variety of strategies, including p/CAF, Pbx1, Midkine, B-myc, and ICAT.

2. p/CAF binds to the N-terminal domain of p48 and is a co-activator for the p48-dependent transcription of the acinar-specific genes, such as the digestive enzymes.

3. ICAT binds to the C-terminal of p48; this interaction relies upon different residues than those to which RBP-L or RBP-Jκ bind.

4. ICAT can bind the PTF1 complex and inhibit its transcriptional activity, possibly by diminishing the p48 interaction with its p/CAF co-activator.

5. In acinar cells, ICAT overexpression is associated with the upregulation of markers of pancreatic progrenitors such as Pdx-1 and Sox9.

6. ICAT is expressed in the developing and adult pancreas. In the adult organ, highest levels are found in islet and ductal cells.

7. ICAT is abnormally overexpressed in a fraction of pancreatic tumors.

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ANNEXES

1. SUPPLEMENTARY RESULTS:
Functional studies of the Midkine-p48 interaction

In order to determine whether midkine could co-localize with p48, Cos-7 cells were transiently co-transfected with a plasmid coding for MK-Flag and a plasmid coding for p48 fused to a Green-Fluorescent-Protein (GFP). As expected, p48-GFP was detected mainly in the nucleus and weakly in the cytoplasm. MK was present mainly in the cytoplasm, with some cells displaying nuclear or perinuclear expression. This pattern was more consistent with that of a secreted protein. Co-transfection of both proteins did not change their subcellular localization. (Figure 36).

To explore whether MK could affect the transcriptional activity of the PTF1 complex, luciferase reporter assays were performed using an artificial reporter construct containing luciferase under the control of a hexamer of the "A" element of the rat elastase-1 promoter ($6xA_{26}$-luc). HEK293T cells were co-

transfected with plasmids coding for p48, RBP-L, and increasing amounts of the plasmid coding for MK. A plasmid coding for the firefly renilla was also co-transfected. Increasing amounts of MK affected the activity of both reporters, raising the possibility of artifactual interpretation of the assays. Therefore, we cannot conclude as whether MK modulates the p48 function as part of the transcriptional complex PTF1. Other experiments should be designed to further investigate whether the p48 interaction with MK can indeed influence its multiple functions, either during the pancreatic embryogenesis or in the adult organ.

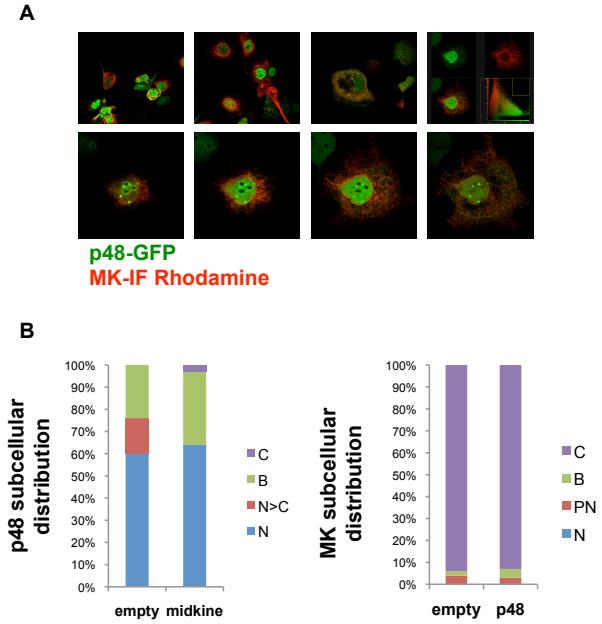

Figure 36.
A) Immunofluorescent picture of Cos7 cells co-expressing p48-GFP and Midkine tagged with a Flag-epitope. p48 is directly detected due to the fused Green Fluorescent Protein, and MK is detected by indirect immunofluorescence using a specific monoclonal antibody against the Flag epitope. **B)** Subcellular localization of both p48 and MK when in the presence or absence of each other. At least 100 cells were counted for each condition and respective controls. C - cytoplasmic; B - both; N - nucleus; PN - perinuclear.

2. SUPPLEMENTARY MATERIAL:

(Paper 1)

p/CAF modulates the activity of the transcription factor p48/Ptf1a involved in pancreatic acinar differentiation

Reference:

Rodolosse A, **Campos ML**, Rooman I, Lichtenstein M, Real FX. **p/CAF modulates the activity of the transcription factor p48/Ptf1a involved in pancreatic acinar differentiation.**

Biochem J **418**, 463-73(2009).

My specific contributions to this manuscript were:

- setting up the yeast two-hybrid assay using the N-terminus region of p48 as bait, identifying for the first time the putative transactivation domain for p48 in this region;
- the determination of the interaction between p/CAF and RBP-L by pull down assays, another element of the PTF1 complex (unpublished data);
- the luciferase reporter assays demonstrating the relevance of each Lys acetylation event for the p48 transcriptional activity;
- the analysis of the effect of p/CAF on the antiproliferative activity of p48;
- the luciferase reporter assays aiming to detect the effects of p/CAF overexpression on the $p21^{CIP1/WAF1}$ promoter activity.

Rodolosse A, Campos ML, Rooman I, Lichtenstein M, Real FX. p/CAF modulates the activity of the transcription factor p48/Ptf1a involved in pancreatic acinar differentiation. Biochem J. 2009; 418(2): 463-73.

3. SUPPLEMENTARY MATERIAL:

(Paper 2)

Unique mechanisms of growth regulation and tumor suppression upon Apc inactivation in the pancreas

Reference: **Strom A**, Bonal C, Ashery-Padan R, Hashimoto N, **Campos ML**, Trumpp A, Noda T, Kido Y, Real FX, Thorel F, Herrera PL. **Unique mechanisms of growth regulation and tumor suppression upon Apc inactivation in the pancreas.**
Development **134**, 2719-2725(2007).

My specific contribution to this article consisted in the identification of ICAT as a putative regulatory inhibitor for the β-catenin signaling expressed in the acinar compartment of the pancreas. By immunohistochemistry, we confirmed the up-regulation of ICAT in the adult acinar cells upon inactivation of Apc in the pancreas.

Strom A, Bonal C, Ashery-Padan R, Hashimoto N, Campos ML, Trumpp A, et al. Unique mechanisms of growth regulation and tumor suppression upon Apc inactivation in the pancreas. Development. 2007; 134(15): 2719-25.