# POLYCOMB GROUP PROTEINS BMI1 AND RING1B ARE INVOLVED IN CELL PLASTICITY AND TUMORIGENESIS OF THE PANCREAS

A thesis presented by **CARLES MARTÍNEZ ROMERO**

in partial fulfilment of the requirements for

the degree of *Doctor of Philosophy* in Biomedicine at

the Department of Experimental and Health Sciences,

Faculty of Health and Life Sciences

**Universitat Pompeu Fabra**


This thesis was performed under the supervision of

**Dr. Mª Inmaculada Hernández Muñoz** in the Cancer Research Programme,

**Institut Municipal d'Investigació Mèdica (IMIM-Hospital del Mar)**


**Barcelona, December 2009**

**Carles Martínez Romero**
**(PhD Candidate)**

**Mª Inmaculada Hernández Muñoz**
**(Thesis Supervisor)**

*A la meva àvia i a la Uka,*

**ACKNOWLEDGEMENTS**

*Un bon amic em va dir una vegada que s'han de viure totes les experiències que et dóna la vida al màxim, tant si són bones com si són dolentes. Considero que aquest consell resumeix molt bé la essència de realitzar una tesi doctoral i de la sensació que sento en el moment d'escriure aquestes línies: una amalgama de bons records al laboratori, frustració davant un experiment que no acaba de funcionar, alegria al veure la banda que tant esperaves obtenir tot subjectant la pel·lícula encara calenta del revelat, discussions i reunions que t'han ajudat a resoldre dubtes i saber per on continuar...*

*Malgrat la investigació sigui una feina molt personal i individual, no puc oblidar-me de la gent sense la qual no hauria estat possible arribar fins on he arribat. No només voldria agrair als membres del tribunal que s'hagin brindat a compartir aquests moments amb mi, amb bons consells i savis punts de vista, sinó també a la meva directora de tesi, la Inma, pels coneixements compartits i per ajudar-me a millorar com a investigador i com a persona. Al Paco, per confiar en mi al principi d'aquesta aventura i per haver-me guiat durant els primers compassos. A l'Anna, per haver-me introduït en aquest món i per haver-me ajudat a pujar el primer esgraó. No voldria perdre la oportunitat d'agrair el suport incondicional dels meus pares, que els estaré eternament agraït per haver estat sempre al meu costat compartint la seva experiència i vivint amb mi tant els bons moments com els dolents. A la meva àvia Antonia, que me la he estimat i me l'estimaré sempre.*

*També voldria fer menció de la gent del laboratori per tots els moments inoblidables que hem passat junts. En especial, el Fabien, per aportar el*

*seu "savoir-faire" al laboratori, amb el seu gran sentit de l'humor (vive le Cantal!). El Mohammad, per les interessants converses i per descobrir-me el "gaz". La Mireia, per compartir les primeres impressions a l'estabulari i per ser la persona més bona i amb més paciència que he conegut mai. El David i l'Elena, per haver-me ajudat en moments de dubte i per haver-me fet descobrir noves fronteres musicals. La Pilar, una persona excel·lent que sempre està disposada a ajudar-te sense demanar res a canvi. La Neus, un exemple a seguir com a persona i com a investigadora. La Laura, la Raquel i la Marta, que malgrat estar a l'altra punta de la galeria sempre m'he sentit molt unit a elles i amb les que he compartit estones molt entranyables. Les festes compartides, les tardes al "Bitácora", les converses a la sala de cultiu o fent un cafè, les preparacions prèvies a les diferents tesis... Tot són records inoblidables que caminaran amb mi per sempre. Molta sort a tothom i us desitjo el millor.*

*No voldria oblidar-me del Víctor i de la seva imprescindible ajuda a l'antic IMIM, dels que ja van marxar del laboratori i dels nouvinguts, de la gent de Veterinària, dels meus amics de tota la vida, del Chicho i de tot el club, i de totes les persones que malgrat no els he dedicat unes línies, tenen un lloc en el meu cor.*

*I finalment a l'Almu, per compartir tota mena de bons records i responsabilitats comunes, i per créixer junts tant professional com personalment.*

*Barcelona, 26 d'octubre de 2009*

## ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers. To improve early diagnosis, research efforts are focused in characterising early events of cancer formation like preneoplastic lesions and deciphering the cell origin of the malignancy. Polycomb proteins constitute a family of epigenetic silencers found in a variety of solid tumours. The main hypothesis is that Polycomb might play a role in preneoplastic states in the pancreas and in tumour development and progression. The expression of Bmi1 and Ring1B was analysed during pancreatic development, in pancreatic tissue from mouse models of disease and in human pancreatic tissue samples. Mechanistic insights of Bmi1 were performed using *in vitro* models and with induced Bmi1 depletion. Bmi1 and Ring1B were expressed in pancreatic exocrine precursors during early development and in ductal and islet cells, but not in acinar cells, in the adult pancreas. Bmi1 was induced in acinar cells during acute injury, in acinar–ductal metaplastic lesions, in pancreatic intraepithelial neoplasia (PanIN) and PDAC. In contrast, Ring1B was significantly increased in high-grade PanINs and in PDAC. Bmi1 knockdown in acinar cell line changed the expression of pancreatic digestive enzymes.

The results of this project suggest that Bmi1 and Ring1B contribute differently to tumour development in the pancreas.

L'adenocarcinoma ductal pancreàtic (PDAC) és un dels càncers més letals. Per tal de millorar el diagnòstic precoç, s'estan investigant les etapes inicials de la formació del càncer, com és el cas de les lesions preneoplàstiques, i es vol desxifrar l'origen cel·lular de la malaltia. Les proteïnes Polycomb constitueixen una família de silenciadors epigenètics que es troben en una varietat de tumors sòlids. La hipòtesi principal és que Polycomb pot estar participant en els processos preneoplàstics del pàncreas i en l'aparició i progressió del tumor. La expressió de Bmi1 i Ring1B fou analitzada durant el desenvolupament del pàncreas, en teixit pancreàtic de diferents models murins de la malaltia i en mostres humans de teixit pancreàtic. Es va dur a terme l'anàlisi del mecanisme de Bmi1 mitjançant models *in vitro* i induint la depleció de Bmi1. Bmi1 i Ring1B s'expressaren en precursors pancreàtics durant etapes primerenques del desenvolupament i en cèl·lules ductals i dels illots, però no en els acins, en el pàncrees adult. Bmi1 s'induí en cèl·lules acinars durant lesió aguda, en lesions metaplàstiques acinoductals, en neoplàsies intraepitelials pancreàtiques (PanIN) i en PDAC. Ring1B s'incrementà significativament en PanINs de grau alt i en PDAC. La disminució dels nivells de Bmi1 en la línia cel·lular acinar canvià l'expressió dels enzims digestius pancreàtics.

Els resultats d'aquest projecte suggereixen que Bmi1 i Ring1B contribueixen de maneres distintes en la progressió tumoral pancreàtica.

## A

**AP**: Alkaline Phosphatase

**APC**: Adenomatosis Polyposis Coli

**ATM**: Ataxia Telangiectasia Mutated

ATR: Ataxia Telangiectasia and Rad3 Related

## B

**bHLH-zip**: basic Helix–Loop–Helix leucine zipper

**Bmi1**: Bmi1 polycomb ring finger oncogene

**BNIP3**: BCL2/adenovirus E1B 19kDa Interacting Protein 3

**BRCA2**: Breast Cancer 2, early onset

**BSA**: Bovine Serum Albumin

## C

**CCK**: Cholecystokinin

**CDH13**: Cadherin 13, H-cadherin (heart)

**CDKN2A**: Cyclin-Dependent Kinase inhibitor 2A

**Cdk4**: Cyclin-Dependent Kinase 4

**cDNA**: complimentary DNA

**CMYC**: v-myc avian myelocytomatosis viral oncogene homolog

**CpG**: Cytosine Phosphate Guanine

**CSC**: Cancer Stem Cell

## D

**DAB**: 3,3'-Diaminobenzidine

**Dhh**: Desert hedgehog

**DLL**: Delta-like

**DMEM**: Dulbecco's Modified Eagle Medium

**DNA**: Deoxyribonucleic Acid

**DNMT**: DNA Methyltransferase

**DPC4**: Deleted in Pancreatic Carcinoma 4

## E

**EED**: Embryonic Ectoderm Development

**EGF**: Epidermal Growth Factor

**ERK**: Extracellular signal-Regulated Kinase

**EZH2**: Enhancer of Zeste Homolog 2

**E2F**: Electro-acoustic 2 Factor

## F

**FBS**: Foetal Bovine Serum

## G

**GDP**: Guanine Diphosphate

**GEF**: Guanine nucleotide-Exchange Factor

**GSK-3**: Glycogen Synthase Kinase 3

**GTP**: Guanine Triphosphate

## H

**HBBS**: Hank's Balanced Salt Solution

**HAT**: Histone Acetyltransferases

**HEK293**: Human Embryonic Kidney 293 cells

**Hh**: Hedgehog

**HRP**: Horseradish Peroxidase

**HPC**: Human Polycomb

**HPH**: Human Polyhomeotic

**HPRT**: Hypoxanthine-guanine Phosphoribosyltransferas

**H2AK119**: ubiquitylation of lysine 199 of histone H2A tail

**H3K27me$^3$**: tri-methylation of lysine 27 at the histone H3 tail

## I

**ICD-O**: International Classification of Diseases for Oncology

**Ihh**: Indian hedgehog

**IPMN**: intraductal Papillary Mucinous Neoplasm

## J

**JAG**: Jagged

## K

**KRAS**: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

## M

**MAPK**: Mitogen-Activated Protein Kinase

**MCN**: Mucinous Cystic Neoplasm

**Meis2b**: Meis homeobox 2

**MyoD**: Myogenic Differentiation

**MUC**: Mucin

## N

**NeuroD**: Neurogenic Differentiation

## P

**PanIN**: Pancreatic Intraepithelial neoplasia

**PBS**: Phosphate Buffered Saline

**Pbx1b**: Pre-B-cell leukemia homeobox 1

**PcG**: Polycomb Group of proteins

**PDAC**: Pancreatic Ductal Adenocarcinoma

**PDX1**: Pancreatic and Duodenal homeobox 1

**PET**: Pancreatic Endocrine Tumour

**PI3K**: Phosphatidylinositol 3-kinase

**PP**: Pancreatic Polypeptide

**pRb**: Retinoblastoma

**PRC**: Polycomb Repressive Complex

**Ptch**: Patched

**PTF1A**: Pancreas specific Transcription Factor 1a

## R

**RAF**: v-raf-1 murine leukemia viral oncogene homolog

**Rbp-l**: Recombination signal binding protein for immunoglobulin kappa J region-like

**Ring1B**: Ring finger protein 2

**RIPA**: Radioimmuno-precipitation Assay

**RNA**: Ribonucleic acid

**RT**: Room Temperature

**RTK**: Receptor Tyrosine Kinase

## S

**SDS**: Sodium Dodecyl Sulfate

**Shh**: Sonic hedgehog

**SMAD4**: SMAD family member 4

**Smo**: Smoothened

**SPINK1**: Serine Protease Inhibitor, Kazal type 1

**STK11/LKB1**: Serine/Threonine Kinase 11

**SUZ12**: Suppressor of Zeste 12 homolog

**SV40**: Simian Virus 40

## T

**T-ALL**: T-cell Acute Lymphoblastic Leukaemia

**TGF**: Transforming Growth Factor

**TrxG**: Trithorax Group of proteins

## W

**WHO**: World Health Organization

**Wnt**: Wingless-type MMTV integration site family

# 1. Cancer

## 1.1 Definition of cancer and the cancer stem cell theory

The current definition of cancer, or malignant neoplasia, describes a class of diseases in which a group of cells display uncontrolled growth, ability to invade adjacent tissues and sometimes metastatic properties. A common misconception is to consider cancer and neoplasia – the latter commonly known as tumour – as synonyms. Indeed, malignant neoplasias differ from benign neoplasias, which do not possess neither invasive nor metastatic capabilities. In addition to different tumour subtypes, cancers present different types of cells within, which exhibit distinct proliferative and differentiative capacities, interacting between each other and also with their niche to promote stromal affinity and angiogenesis.

Pioneering studies in acute myeloid leukaemia in mice reported a minor subpopulation of tumour cells which had self-renewal capacity and was responsible of sustaining tumour growth [1]. Thus, a "cancer stem cell" (CSC) hypothesis was proposed, in which a small group of self-renewing, undifferentiated and pluripotent tumour cells is in charge of maintaining the neoplastic lesion by replenishing every cell type found in the lesion [2]. The existence of CSCs became evident in liquid tumours when a subpopulation of leukaemic cells was reported to have self-renewal and tumorigenic capacities [3]. Later on, evidence of CSCs was found in a wide range of epithelial tumours: colon [4], lung [5], breast [6], pancreas [7] and prostate [8] among others.

Normal stem cells are relatively quiescent, resistant to drugs and toxins, bearing an active DNA-repair capacity and resistance to apoptosis [9]. CSCs are thought to be sharing many of these properties, thus conventional chemotherapy and radiotherapy treatments, which target rapidly cycling cells, will lead to tumour reduction but the CSCs themselves will remain unaffected, allowing the tumour to relapse. The efficiency of cancer treatments is often measured by means of the reduction of tumour mass. However, as the proportion of CSCs is very small compared to total cancer cells, relapse of the tumour may happen even there is a considerable reduction of its mass. Therefore, there is a consensus to find specific treatments against CSCs to improve the outcome of cancer patients, especially those with recurrent cancer or with metastasis.

Many questions are still to be answered about CSCs. For example, there is an ongoing debate regarding the origin of these cells, whether they might come from altered stem cells which cannot control proliferation, or they may represent a differentiated population of cells which gained self-renewal characteristics.

## 1.2 Genes involved in tumorigenesis

There is a group of genes which have been reported to be altered in several types of cancers, possibly due to their crucial role in important signalling pathways [10]. These genes are usually distributed in two groups depending on their role: oncogenes and tumour-suppressor genes.

### 1.2.1. Oncogenes commonly involved in cancer

An oncogene is defined as a gene which, when mutated or overexpressed, induces tumorigenesis. In normal conditions, proto-oncogenes are involved in signalling transduction pathways controlling cell growth, differentiation and apoptosis. A mutation in the DNA sequence, an increase in protein concentration or any chromosomal abnormality can induce a proto-oncogene to become an oncogene. As a result, oncogenes allow the transformation of a normal cell into a cancer cell.

Paradigms of oncogenes are the KRAS and the CMYC genes.

#### 1.2.1.1. KRAS

The v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) is a member of the RAS superfamily of guanine-nucleotide binding proteins, which is involved in cell growth, differentiation and survival. Along with KRAS, two more members of the RAS family – HRAS and NRAS – have been found to be working as a binary signal switch in pathways related to cell growth. In a resting cell, inactive RAS protein is bound to GDP.

Upon binding of growth factors like EGF to receptor tyrosine kinases (RTK), RTK become phosphorylated and activated. Consequently, adaptor molecules localize to RTKs followed by recruitment and activation of guanine nucleotide-exchange factors (GEFs). GEFs catalyse the transition from GDP-bound, inactive RAS to GTP-bound, active RAS. RAS-GTP interacts with more than a dozen effector molecules to regulate a variety of biological processes. GTPase-activating proteins (GAPs) allosterically stimulate the intrinsic GTPase activity of RAS, leading to GTP hydrolysis and RAS inactivation. To activate the MAPK signalling cascade, RAS recruits Raf to the cell membrane, where Raf is activated and

subsequently forms complexes with MEK, ERK and scaffolding proteins. Raf then phosphorylates MEK, which in turn phosphorylates ERK. ERK both activates cytosolic substrates and translocates to the nucleus to stimulate diverse gene expression programs through transcription factors such as JUN and ELK1.

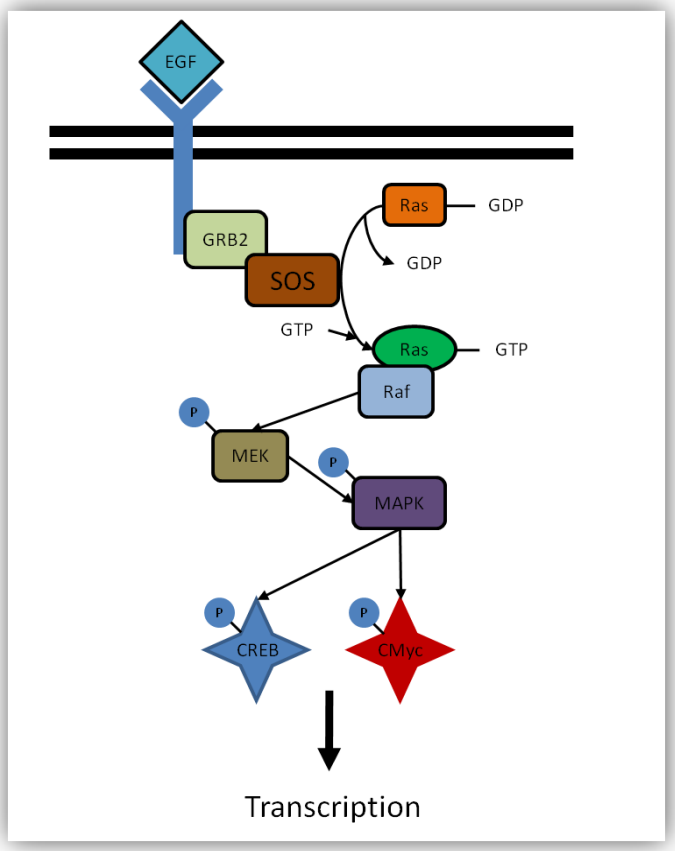



**Figure I.1. The MAPK/ERK pathway.** Ras has a crucial role in the phosphorylation cascade of the pathway which culminates in the regulation of several transcription factors, like CREB and CMyc.

It is well documented that oncogenic forms of H, K and NRAS are preferentially detected in certain tumor types. For instance, more than 80% of pancreatic adenocarcinomas harbor a mutated KRAS gene [11], whereas in myeloid leukemia, the NRAS gene is most frequently mutated

[12]. Nevertheless, while some neoplasias do not show specificity in any mutated RAS gene, differences in the function of RAS proteins still remain elusive.

As mentioned above, KRAS possesses an intrinsic inactivating enzymatic activity that cleaves bound GTP converting it to GDP. Point mutations in the codifying sequence usually disrupt the GTP/GDP switch which renders KRAS constitutively active, acquiring oncogenic properties. Indeed, activating mutations of KRAS have been reported in thyroid, colorectal, lung and pancreatic carcinomas among others [13].

#### 1.2.1.2. CMYC

One of the quintessential oncogenes, CMYC, is a basic helix–loop–helix leucine zipper (bHLH-Zip) transcription factor which induces the expression of around 15% of all known genes [14] through consensus sequences like E-boxes and by recruiting histone acetyltransferases (HATs). Different signalling pathways like Wnt, Shh or the MAPK/ERK induce CMYC expression, which has been reported to promote cell proliferation but at the same time it also has a role in regulating cell growth [15], apoptosis [16] and stem cell self-renewal [17].

Abnormal expression of CMYC is thought to be necessary for tumorigenesis, as it has been reported in almost 70% of human cancers [18]. Overexpression of CMYC is achieved through translocations, amplifications, or enhanced translation or protein stability [19-23]. This highly abnormal expression has been reported to promote tumour angiogenesis, which is crucial for cancer development [24-26]. Nevertheless, expression of CMYC can also be increased by altered signalling pathways that induce or repress its transcription. Indeed, CMYC expression is persistently induced as a primary response of

virtually all signal transduction pathways known to be altered in cancer, like those associated to tyrosine kinase growth factor receptors such as the MAPK/ERK pathway [18] among others [27-28].

### 1.2.2. Tumour-suppressor genes involved in cancer

Tumour-suppressor genes exert a protective activity upon tumorigenesis, normally, although not exclusively, limiting the growth of tumours. Tumour-suppressor genes generally codify for proteins which repress cell cycle progression and promote cell death. Thus, they act as brakes to the cycle of cell growth, DNA replication and cell division, collaborating with the DNA damage repair machinery to ensure that only those cells with no errors in the DNA sequence proliferate. If DNA damage is irreparable, programmed cell death is initiated to remove possible threats from the organism [29]. Further insights have unveiled proteins with tumour-suppressor abilities which repress cancer cell motility by inhibiting metastasis [30].

#### 1.2.2.1. TP53

Among the most significant tumour suppressors, tumour protein 53 (p53) was the first to be reported [31-32]. Codified by the TP53 gene, p53 is a transcription factor considered to be the master tumour-suppressor protein, which acts in response to diverse cellular stresses, regulating target genes involved in cell cycle arrest, apoptosis, senescence, DNA repair or changes in metabolism [33]. The tumour-suppressor activity of p53 entangles different mechanisms, like activation of DNA repair machinery when damage is detected, stopping the cell cycle at G1/S checkpoint to allow DNA to be repaired and induction of apoptosis if damage is beyond repair. Apart from DNA damage, activation of p53 is

induced by an extensive range of extracellular stimuli like osmotic shock, oxidative stress and oncogenic signals among others.

The classical model for p53 activation generally consists of three sequential activating steps: stress-induced stabilization mediated by phosphorylation, DNA binding and recruitment of the general transcriptional machinery.

P53 has a tight relationship with its direct inhibitor MDM2. The p53–MDM2 paradigm represents the best-studied relationship between a tumor suppressor gene which functions as a transcription factor and an oncogene, which functions primarily as an E3 protein ligase. In normal conditions, MDM2 couples with p53 preventing its translocation to the nucleus, whereas p53 induces transcription of MDM2. Important components that affect this auto-regulatory feedback loop include the tumor suppressor protein p14ARF (p19ARF in mice) [34]. The p14ARF protein binds the MDM2 protein, and inhibits the E3 activity of MDM2 [35], in addition to sequestering MDM2 into the nucleolus [36]. Consequently, p14ARF disrupts the negative feedback inhibition of p53 by the binding to MDM2 [37].

In unstressed conditions, low levels of p53 are continuously maintained due to the ubiquitin ligase activity of MDM2, which places post-translational ubiquitin modifications at lysine residues in p53 C-terminus. Ubiquitination of the p53-MDM2 complex is a target for degradation by the proteasome, thus maintaining low levels of p53. However, stress signal-induced p53 phosphorylation by ATM, ATR, and other kinases stabilizes p53 and promotes its binding to DNA. DNA-bound p53 then

recruits the transcriptional machinery to activate transcription of p53 target genes [38].

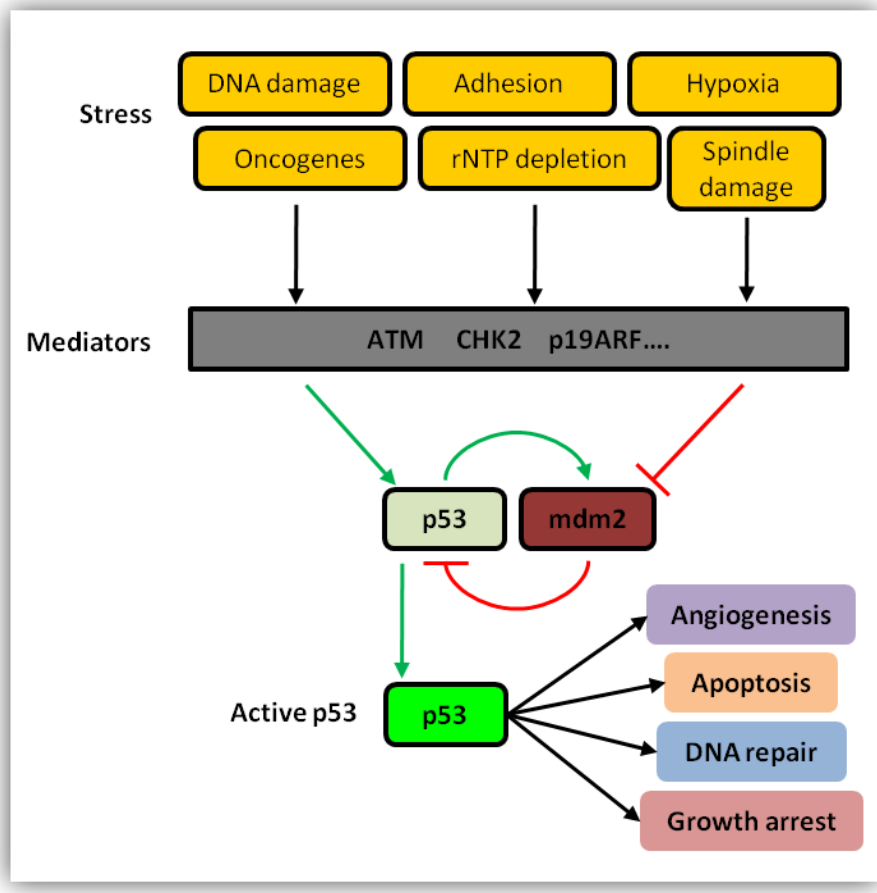



**Figure I.2. The p53 pathway.** Activation of the master regulator p53 is triggered by several stress stimuli through specific mediators. Induction of p53 and repression of mdm2 allows active p53 to act upon different mechanisms like angiogenesis, apoptosis, DNA repair or growth arrest. *Figure adapted from [39].*

The overwhelming list of reported p53 target genes encloses several ways of tumour suppression [33, 39]. Point mutations of TP53 gene affect its DNA binding domain region, thus inactivating DNA binding capacity of p53. As a result, tumour-suppressor mechanisms driven by p53 cannot be accomplished, favoring cancer progression of different

types of tumours [32]. Moreover, expression of mutant p53 has been recently been describe to be critical for TGFβ-induced metastasis [40].

### 1.2.2.2. CDKN2A

The cyclin-dependent kinase inhibitor 2A (CDKN2A), also known as the INK4a/ARF gene, encodes for two very important tumour-suppressor proteins: $p16^{INK4a}$ and $p14^{ARF}$ [41-42].

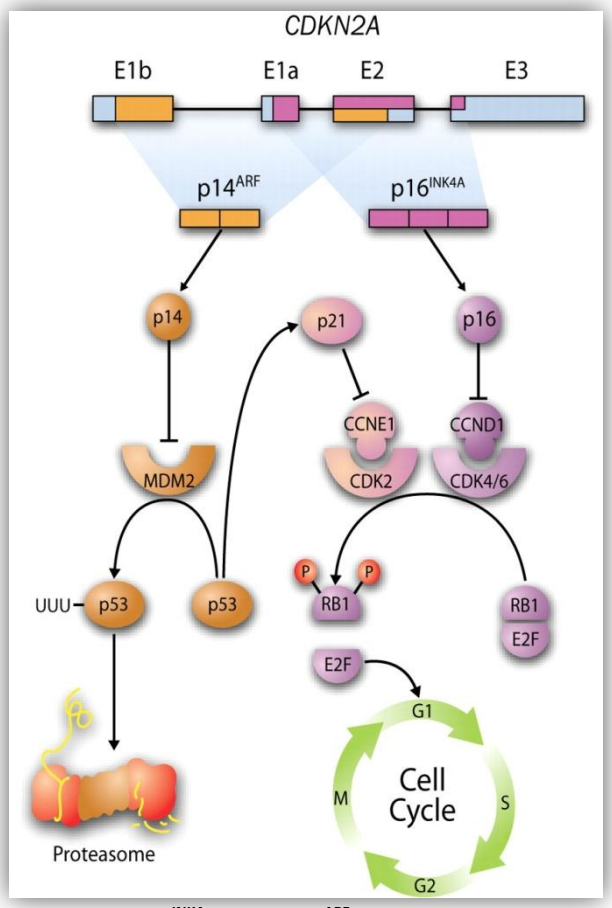



**Figure I.3: $p16^{INK4a}$ and $p14^{ARF}$ proteins**. The unusual structure of the CDKN2A locus codifies for 2 overlapping but very distinct translated proteins: $p16^{INK4a}$ and $p14^{ARF}$. Both proteins act as negative regulators of cell cycle progression and alteration in their expression is very common in a wide variety of cancers. *Figure apdated from [42].*

The $p16^{INK4a}$ protein has been reported to accumulate in several tissues as a function of advancing age [43-44]. Moreover, it is involved in senescence mechanisms [45] and acts as a strong inhibitor of Cdk4 [46], which is in charge of the phosphorylation of the Retinoblastoma (pRb) protein, which interacts with E2F, thus leading to cell cycle arrest [47-48]. The INK4A gene harbours a restricted pattern of expression during normal homeostasis and development [49] but INK4A transcription is increased in a stress-response manner to act as a tumour suppressor [50]. Mutations in the CDKN2A locus can disrupt both pRb and p53 pathways at the same time [51-52], which is a common feature in a wide range of human cancers [41].

## 1.3 Pathways involved in development and self-renewal

Recent studies regarding stem-cell machinery, cell fate decisions and embryogenesis have contributed to consolidate a broad knowledge about the signalling pathways involved in stem cell self-renewal and development. Due to their inferred importance in tissue homeostasis, alterations in these pathways have also been related to tumorigenesis.

### 1.3.1. Notch signalling pathway

The Notch signalling pathway is a highly conserved cascade in metazoans which participates in the development of multicellular organisms by maintaining the self-renewal potential of some tissues while inducing the differentiation of others [53]. Characterisation of the Notch pathway began with the analysis of the Notch gene in Drosophila, which encoded for a heterodimeric receptor with a large extracellular region, a single transmembrane domain and a small intracellular part.

Induction of the Notch pathway is accomplished by interaction of ligand proteins with the Notch extracellular domain. While in Drosophila there are only one Notch protein and two ligands – Delta (Dl) and Serrate (Ser), vertebrates possess four Notch proteins (NOTCH1-4) and five ligands, named Delta-like-1, -3 and -4 (DLL1, DLL3 and DLL4) and Jagged 1 and 2 (JAG1 and JAG2).

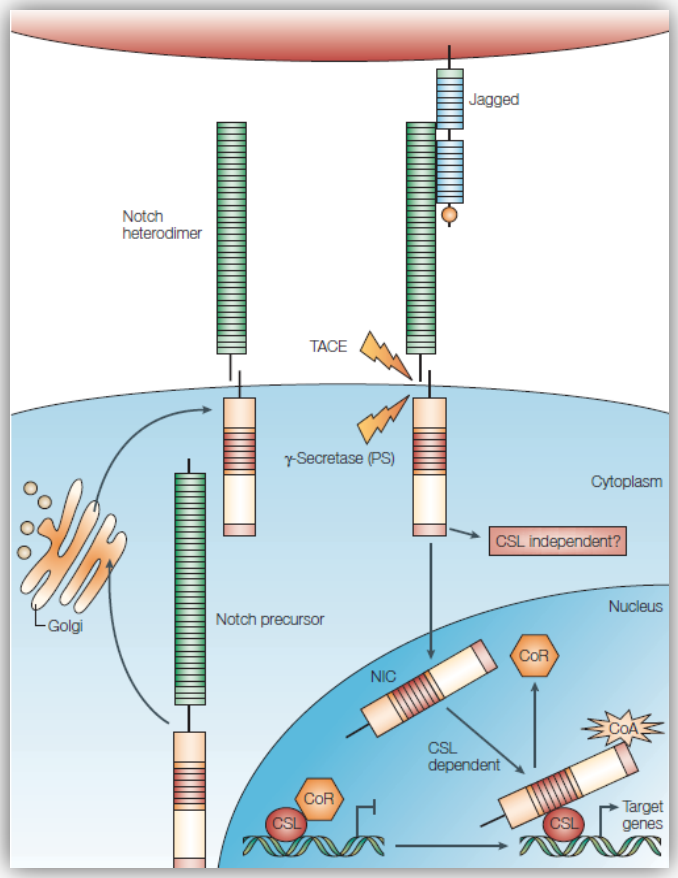



**Figure I.4: Notch signalling pathway.** Interaction of Notch receptors with their ligands (like Jagged) activates liberation of Notch intracellular domain (NIC), which enters the nucleus and binds to the transcripition factor CSL. Then, repressing machinery is displaced and co-activators (CoA) are recruited leading to transcriptional activation of target genes. *Figure adapted from [53].*

Interaction of Notch receptors with their ligands leads to a cascade of cleavages that detach the Notch intracellular domain. The liberated domain enters the cell nucleus and binds to transcription factor CSL, which recruits co-activators to induce transcriptional activation of downstream target genes.

Notch signalling affects several cellular processes, such as maintenance of stem cells, specification of cell fate, differentiation, proliferation and apoptosis. As a consequence, alterations in these functions can lead to cancer formation. Notch is in charge of maintaining the undifferentiated state in adult stem cells of different tissues, such as the vertebrate nervous system [54-55], the haematopoietic compartment [56] and the mammary gland [57]. Notch signalling also participates in binary cell-fate decisions to preserve the undifferentiated state of a group of cells while inducing differentiation in the other. Furthermore, DLL1-induced Notch signalling can initiate or enhance terminal differentiation [58].

Several members of the Notch pathway have been reported to be altered in different human diseases and types of cancer. The first oncogenic role of Notch to be reported was in T-cell acute lymphoblastic leukaemia (T-ALL). Later on, experiments performed in mouse models also involved Notch signalling in breast and salivary-gland cancer [59]. Interestingly, human breast cancers show increased levels of Notch proteins, but in this case Notch signalling is not inducing cancer formation but is necessary for tumorigenesis to take place [60].

### 1.3.2. Hedgehog signalling pathway

Another highly conserved pathway involved in embryonic development is the hedgehog signalling pathway [61-62]. The pathway is named after the Hedgehog gene (Hh) discovered in Drosophila, which is involved in

establishing the basis of the body plan. In mammals, three homologues for Hh have been characterised: Indian (Ihh), Desert (Dhh) and Sonic (Shh), being the latter the most studied of them.

Each mammalian hedgehog gene encodes transmembrane signalling ligands. When no ligand is present, hedgehog receptors Patched1 and Patched2 (Ptch) repress the hedgehog signalling molecule Smoothened (Smo) [63]. When hedgehog ligands bind to Ptch, Smo is freed from the inhibition, translocating Gli transcription factors to the cell nucleus, thus regulating hedgehog responsive genes such as cell cycle regulators Cyclin D1, p21 and Wnt proteins among others [64-65].

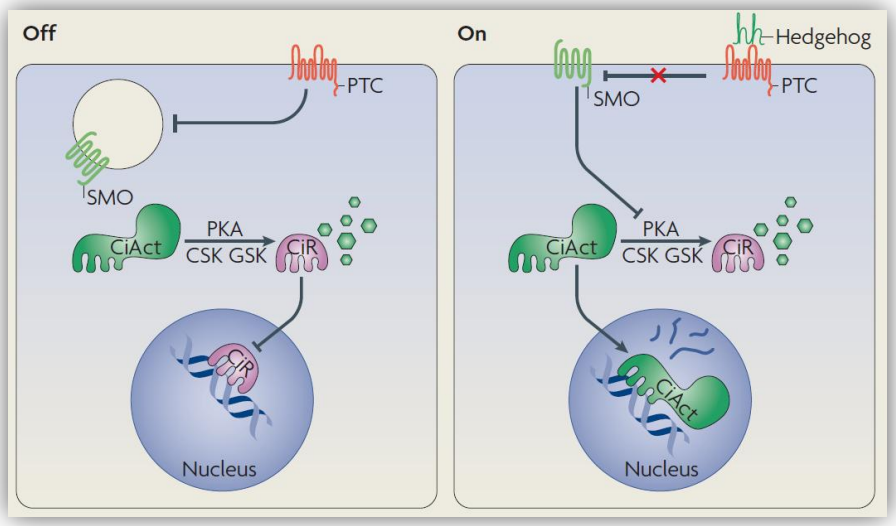



**Figure I.5: The Hedgehog signalling pathway.** Hedgehog (Hh) ligand binds to Patched (PTC), thus repressing the inhibition activity over Smoothened (SMO). As a consequence, activated Smoothened inhibits phosphorylation of GLI (Ci) proteins, thereby preventing degradation of the repressor form and allowing nuclear translocation of GLI transcriptional activators, thus inducing the expression of target genes. *Figure adapted from [66].*

Mammalian hedgehog signalling is involved in the maintenance of adult-cell populations [67-69] and is also controlling appropriate patterning of several epitheliums, such as lung, skin [70] and digestive tract [71].

Differentiation [72], survival [73] and proliferation of different cell populations are promoted by Sonic hedgehog in the ectoderm, mesoderm and endoderm-derived tissues [74]. This function is confirmed by analysing mice deficient in members of the hedgehog pathway, which show severe defects in different organs like skeleton, brain, muscle, gastrointestinal tract and lung [75-78].

Members of the hedgehog signalling pathway are found to be misexpressed in several human cancers, including small cell lung carcinomas, medulloblastomas, basal cell carcinomas, pancreatic adenocarcinoma [79-80], breast cancer [81], prostate cancer [82] and digestive tract tumours [71].

### 1.3.3. Wnt signalling pathway

Another pathway which has a central role in embryogenesis and adult tissue homeostasis is the Wnt/β-catenin signalling pathway [83]. It is a highly conserved pathway in which Wnt proteins activate a signalling cascade in the cell membrane that results in the modulation of β-catenin protein, which enters the nucleus to promote specific gene expression.

Although the effects of Wnt signalling depend on the ligand, the cell type and the organism, several members of the pathway are strikingly conserved throughout evolution. In the absence of Wnt proteins, the axin/GSK-3/APC proteolytic complex recruits and degrades free β-catenin in the cytosol. When Wnt proteins are bound to receptors of the Frizzled family, Dishevelled proteins become active and inhibit the axin/GSK-3/APC complex, thus changing the amount of β-catenin in the cytoplasm. As a consequence, a pool of β-catenin enters the nucleus, interacting with TCF/LEF family transcription factors to promote specific gene expression.

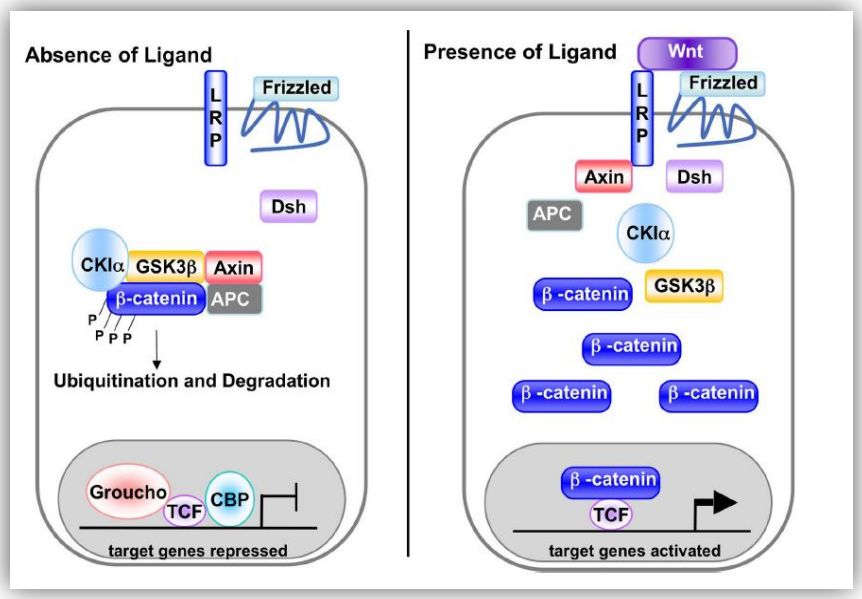



**Figure I.6: The Wnt signalling pathway.** In the absence of Wnt ligands, the axin/GSK-3/APC complex phosphorylates free β-catenin, which is targeted for degradation by the proteasome. In the presence of Wnt ligands, the Frizzled(Fz)/LRP coreceptor complex interacts with Axin, leading to the stabilization of β-catenin in the cytoplasm, which allows its nuclear translocation. In the nucleus, β-catenin displaces Groucho from Tcf/Lef to promote the transcription of Wnt target genes. *Figure adapted from [84].*

Members of the Wnt signalling pathway have been related to many human diseases, including cancers like colorectal cancer, hepatocellular tumours, pilomatricomas and melanomas among others [85-86].

Interestingly, all three signalling pathways here described – Notch, Hh and Wnt – are thought to crosstalk between each other. For instance, Notch acts as a tumour suppressor by inhibiting Hh and Wnt signalling pathways [53]. Moreover, Shh and Wnt are usually overexpressed in tumours, due to their requirement for tumour growth and they seem to be induced co-ordinately [87].

## 1.4 Epigenetics in cancer

With the constantly expanding knowledge in tumorigenesis, epigenetic machinery has found its place as a player in cancer formation and progression [88]. The term ‘‘epigenetics’’ was first mentioned by Conrad Hal Waddington in 1942, and originally meant to describe the process by which genotype gives rise to phenotype, for instance, through causal interactions among genes and their products [89]. The modern definition of “epigenetics” has more specific meaning, referring to heritable traits over cell division, and sometimes over generation, which do not involve changes to the underlying DNA sequence [90-91], but are related to the chromatin.

The chromatin is a complex system that includes DNA, RNA and proteins, by which the whole genome is stored inside the nuclei of every eukaryotic cell to create a compact but dynamic structure. Chromatin presents several levels of compaction based on consecutive folding of the DNA sequence around a basic repeat element, the nucleosome, which consists of DNA wrapped around a scaffold of histone proteins.

Epigenetic regulation has a very important role in initiating and modulating cellular differentiation and development, controlling the activation of proto-oncogenes and inactivation of tumour-suppressor genes, thus having a crucial role in cancer development and progression [92]. Inappropriate changes in chromatin structure due to epigenetic events can result in genomic instability, thus causing cellular transformation and malignant outgrowth. Besides the classical genetic changes found in cancer, like inactivation of tumour-suppressor genes, amplification of oncogenes and loss of heterozygosity or gene mutations in tumour associated genes, epigenetic machinery is also altered in

different types of tumour, which display altered DNA methylation, misregulation of histone modifications or aberrant expression of PcG proteins [93-98].

Two predominant mechanisms have been reported to be epigenetic: DNA methylation and histone modifications. These mechanisms collaborate with chromatin remodelling complexes, nuclear architecture and microRNAs to establish the chromatin structure of the genome and its transcriptional activity.

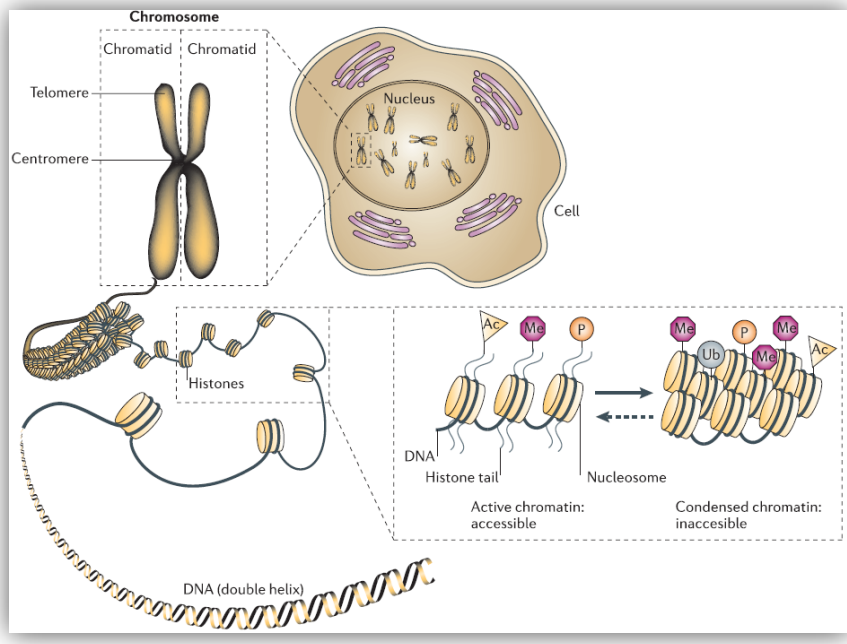



**Figure I.7: Chromatin compaction and histone modifications.** Storage of DNA presents several levels of compaction which culminates in the chromatin structure. Although chromatin culminates in very tight levels of compaction, the whole structure presents dynamic interactions with different layers of posttranscriptional modifiers of the N-terminal regions of histones, which extend from the nucleosome core. Proper organisation of the chromatin is crucial for transcription, replication, DNA repair and chromosome segregation. Specific point modifications in different aminoacids of histone tails alter chromatin conformation, thus allowing repression or activation. *Figure adapted from [99].*

### 1.4.1 DNA methylation

DNA methylation constitutes a stable epigenetic mark [100], being involved in processes such as X chromosome inactivation, imprinting, embryogenesis and silencing of repetitive DNA elements [101]. DNA methylation consists of covalent chemical modifications of the DNA sequence at cytosine residues of CpG sites. In mammals, DNA methyltransferases (DNMTs) are responsible for adding methyl groups to the cytosine residues at CpGs, converting cytosine to 5-methylcytosine and consequently silencing transcription of the region. CpG sites are clustered throughout the genome in regions referred to as CpG islands [102], which are usually found in the vicinity of mammalian gene promoters [103]. It has been reported that unmethylated CpG islands in promoter regions correlate with gene activation. On the other hand, methylation in CpGs at the gene promoters inhibits transcription.

Methylation of CpG sites allow anchoring of methyl-CpG binding domain (MBD) proteins to DNA [104]. Then, MDBs interact with a large group of histone deacetylases, histone methyltransferases and chromatin remodelling enzymes, rendering methylated DNA into a compacted chromatin state which is refractive to transcription [105].

### 1.4.2. Histone modifications

The histones are proteins that possess long tails which protrude from the nucleosome and are prone to suffer posttranslational modifications, which include acetylation, citrullination, methylation, phosphorylation, ADP-ribosylation, sumoylation and ubiquitination [92, 106-107]. These modifications in histone tails alter nucleosome spatial disposition, altering chromatin structure and eventually affecting DNA transcription. Histone modifications allow different patterns of gene expression,

changing from transcriptionally active to transcriptionally silent states, or vice versa.

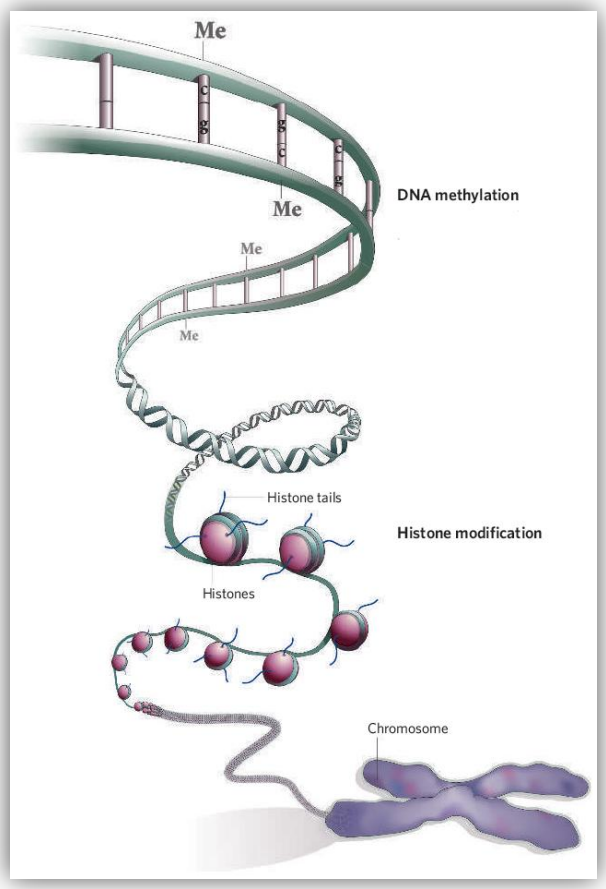



**Figure I.8: Classical epigenetic mechanisms.** DNA methylation and some histone modifications are the most studied epigenetic mechanisms, both acting as activators and repressors of gene transcription. *Figure adapted from [108].*

For instance, there are regions of chromatin exhibiting an open, permissive state for transcription which usually displays tri-methylation of histone H3 at lysine 4 and hyperacetylation of histones H3 and H4 [109]. On the other hand, transcriptionally repressed regions exhibit a compact chromatin structure that lacks H3/H4 acetylation and H3K4 methylation, but instead are enriched in di- and tri-methylations of H3K9

(H3K9me2/3), tri-methylation of H3K27 (H3K27me3), and tri-methylation of H4K20 (H4K20me3) [109-110]. These modifications have been hypothesized to work as an epigenetic mechanism directing specific and distinct modifications to the DNA sequence, but this hypothesis still has to be proven [111-112].

### 1.4.3. Polycomb group proteins

One important protein family involved in chromatin modification is the Polycomb group of proteins (PcG), which are epigenetic silencers that repress specific sets of genes by modulation of the chromatin structure [99, 113-114].

PcG proteins were initially identified in Drosophila as repressors of the HOX genes, which specify the anterior-posterior axis and segment identity during early embryonic development [115]. Preliminary characterisation showed that PcG complexes worked in combination with the Trithorax group of proteins (trxG) as members of a binary regulation switch of the same target genes, with antagonistic effects. Thus, while PcG proteins are related to repression, members of TrxG dictate an active state of transcription. Nevertheless, further insight expanded the list of target genes of PcG and the initial hypothesis was questioned by the fact that some genes remain functionally active even in the presence of PcG complexes [114].

PcG-mediated gene silencing mainly relies on two principal types of multimeric complexes called Polycomb repressive complex 1 (PRC1) and 2 (PRC2), which interact with the chromatin and modulate the transcriptional repression of specific regions of the genome [116]. PRCs were initially considered as static multimeric complexes. However,

further insights revealed the variability and dynamics of these complexes [117].

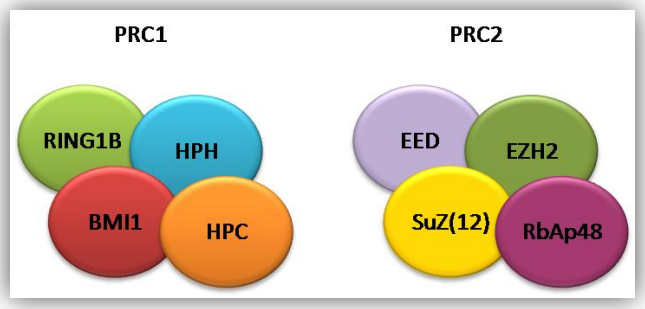



**Figure I.9: Core of Polycomb repressive complexes.** Figure adapted from [117].

The human PRC2 complex has a core of four members: Enhancer of Zeste (EZH2), Suppressor of Zeste 12 (SuZ12), Embryonic Ectoderm Development (EED) and RbAp48. EZH2 has a histone methyltransferase activity that catalyses the tri-methylation of lysine 27 at the histone H3 tail ($H3K27me^3$) [118]. Furthermore, EZH2 can also recruit DNA methyltransferases (DNMT), which are also involved in gene repression [119].

On the other hand, the PRC1 complex includes a more variable core of proteins, such as Human Polyhomeotic (HPH), Human Polycomb (HPC), Ring1B and Bmi1 polycomb ring finger oncogene (Bmi1). PRC1 is able to recognise the $H3K27me^3$ mark through the chromodomain of HPC [118] and triggers a transcriptional silencing state of the chromatin region by means of different mechanisms, like inhibition of transcriptional machinery, nucleosome compaction and ubiquitylation of lysine 199 of histone H2A tail (H2AK119) by the ligase activity of Ring1B [99, 120-122].

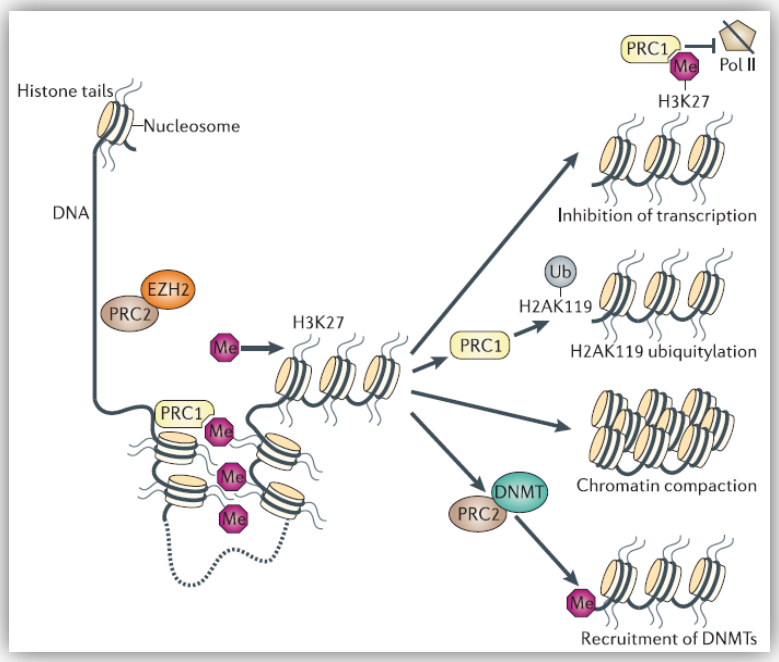



**Figure I.10: Silencing mechanisms by Polycomb group proteins.** PRC2 complex triggers the initiation of gene silencing by placing a mark with the trimethylation of H3K27. PRC1 recognises this mark and activates a wide range of mechanisms, directly inhibiting transcriptional machinery, PRC1-mediated ubiquitylation of H2AK119, chromatin compaction and recruitment of DNA methyltransferases (DNMTs) by PRC2. *Figure adapted from [99].*

PcG protein family has been highly conserved throughout evolution. Indeed, members of the PRC2 are consistently involved in developmental patterning. However, PRC1 components seem to have arisen in later evolution stages, as they are absent in nematodes and plants. Accordingly, knock-out mice for each core PRC2 components are embryonic lethal due to severe defects at the implantation and early post-implantation stages, while homozygous null mutant mice for PRC1 genes, except for Ring1B, survive to birth but display homeotic transformations and die perinatally [123].

These milder phenotypes of the PRC1 null mice could also be explained due to functional redundancy and compensation by the wide range of PRC1 genes that are found in vertebrates, with homologues exhibiting

partial divergence of tissue-specific expression patterns [124]. This divergence may affect the activity of PcG complexes, as *in vitro* experiments showed that while Bmi1 greatly stimulates the E3 ubiquitin ligase activity of Ring1B, Polycomb group ring finger protein 2 (Mel-18), a homologue that can substitute for Bmi1 in PRC1 complex assembly, does not fulfil this function [125]. Unlike Bmi1, which is overexpressed in various human tumors and generally accepted as a proto-oncogene, Mel-18 is shown to be either oncogenic or tumor suppressive, depending on the cancer system [126-127]. These findings may help to understand the prominent role of Bmi1 in tumorigenesis and at the same time find an explanation for the hypothetical tumor-suppressor properties of Mel18 [128].

Several PcG proteins have been reported to be involved in different cellular processes: cell cycle progression [129], cell memory and identity [130-131], stem cell maintenance [132] and neoplasia [133]. For instance, embryonic differentiation and development require the proper function of PcG proteins and of the DNA methylation machinery [134-135]. Interestingly, PRCs are not static complexes, but their protein members change depending on the cell type, tissue and stage [136].

Bmi1 was the first PcG protein reported to be involved in tumorigenesis, working as a proto-oncogene cooperating with CMYC to promote mouse B- and T-cell lymphomas by repressing the CDKN2A locus [137]. Later on, different reports showed that Bmi1 is involved in many types of human neoplasia, such as lung cancer [138], leukemia [139], brain tumour [140], prostate cancer [141] and breast cancer [142]. Bmi1 has also been reported to be involved in many other processes, like adult stem cell maintenance [143], axial skeletal development [144] and cell cycle

regulation [145], which may explain the prominent figure of Bmi-1 in tumorigenesis.

Even though Bmi1 was the first and still is the most frequently reported PcG protein to be involved in cancer development, other PcG members have been implicated in tumorigenesis [99]. For instance, EZH2 has been found to be upregulated in several human tumours, such as mantle cell lymphoma [146], prostate cancer [147-148], breast carcinoma [149] and bladder carcinoma [150] among others. Moreover, EZH2 expression has been hypothesised to be an indicative of metastasis and therefore a marker of low prognosis [151], despite this hypothesis needs further insight [152].

## 1.5 Epithelial cancers

Due to the heterogeneity of the different types of cancers, classifying the whole range of neoplasias can be approached from different levels – site of origin, histological characteristics or molecular features– and novel characterisation may obtain more specific treatments for each patient [153].

When tumours are classified following their site of origin, a wide range of cancers fits into a group called carcinomas, all coming from epithelial cells. Carcinomas, or malignant epithelial cancers, constitute the majority of human malignant neoplasias with higher incidence [154]. Interestingly, epithelial cancers seem to require more altered pathways for their progression than other tumours with higher motility [10, 155-156].

The International Classification of Diseases for Oncology (ICD-O) of the World Health Organisation (WHO) mainly distributes epithelial cancers in two subgroups [157-158]: cancers originating from the epithelium of the

skin, like basal cell carcinoma and melanoma, and those appearing in glands, like hepatocellular carcinoma, renal cell carcinoma and the family of adenocarcinomas. One of the human cancer with worse prognosis is the pancreatic adenocarcinoma, accounting for a 5-year survival rate of less than 5% since the diagnosis, while a total recovery is yet seldom achieved [159].

# 2. The pancreas

## 2.1 Physiology of the pancreas

The pancreas is an organ of the vertebrates, located near the stomach and the small intestine. Being the second largest gland involved in the digestive system, the pancreas displays both exocrine and endocrine functions. It is involved in regulating two major physiological processes: digestion and glucose metabolism [160].

At the histological level, the pancreas has two different types of parenchymal tissue. The microscopic appearance of the organ displays a series of cell-packed sphere-shaped structures called islets of Langerhans, surrounded by less dense and much larger acinar cells. The islets of Langerhans are responsible of the endocrine function while the acinar cells are in charge of the exocrine activity of the pancreas.

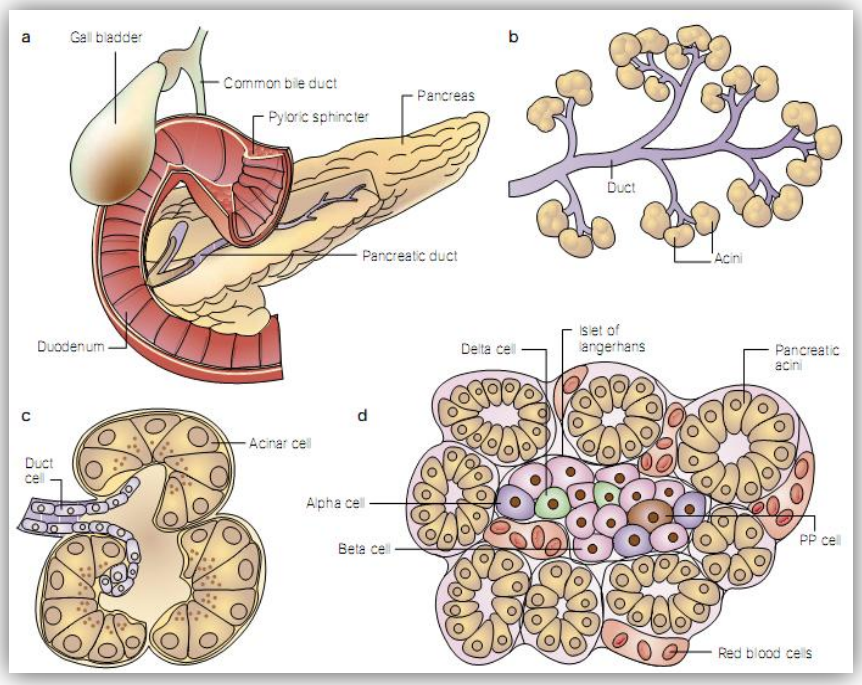



**Figure I.11: Anatomy of the pancreas.** The pancreas comprises an endocrine and an exocrine part. The function of the exocrine pancreas is to produce digestive enzymes, which are secreted by acinar cells and transported to the gut by the ductal system. The endocrine pancreas has four hormone-producing cell types: α-, β-, δ- and pancreatic polypeptide (PP) cells. α-cells and β-cells are in charge of glucose regulation, as they secrete glucagon and insulin, respectively. δ-cells secrete somatostatin and PP cells secrete pancreatic polypeptide. a | Gross anatomy of the pancreas. b | The exocrine pancreas. c | A single acinus. d | An Islet of Langerhans embedded in exocrine tissue. *Figure adapted from [161].*

### 2.1.1. Endocrine pancreas

The islets of Langerhans account for approximately 1-2% of total human pancreas, that is, around one million islets cells in healthy conditions. A

The islets comprise different cell types, each one of them secreting specific hormones to the bloodstream [162-165]. The α-cells account for approximately 30% of total islet cells and are in charge of producing glucagon, which is released in presence of hypoglycaemia to increase glucose levels. The β-cells represent the majority in the islets, accounting for around 60% of the total islet cells. They are responsible of insulin production and secretion, which activates glycogenesis in the context of

hyperglycaemia. The β-cells also produce amylin, which is also involved in glycemic control.

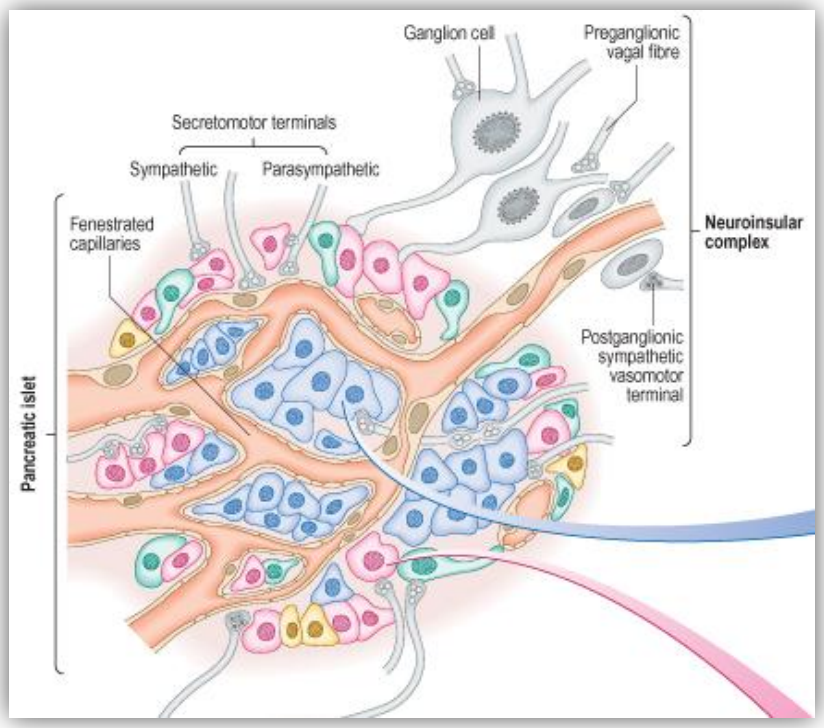



**Figure I.12: Endocrine pancreas.** The islets of Langerhans are complex spherical structures which contain several cell types which interact with the bloodstream through the capillaries. *Figure adapted from [166].*

Other less abundant cell types are found in the islets, such as δ, PP and ε-cells. The δ-cells constitute 3-10% of total islet cells and secrete somatostatin, which is responsible of suppressing the release of several gastrointestinal hormones.. The PP-cells (around 3-5% of total cells) secrete the pancreatic polypeptide (PP), whichregulates pancreatic and gastrointestinal secretions. Finally, the rare ε-cells account for less than 1% of total islet cells and produces ghrelin, a hormone which stimulates hunger [167].

Islets cells can influence each other through paracrine and autocrine communication. For instance, somatostatin inhibits both glucagon and insulin secretions at the paracrine level and also acinar and ductal secretions, whereas the pancreatic polypeptide acts in a paracrine manner inhibiting secretion of digestive enzymes from the acini.

### 2.1.2. Exocrine pancreas

The exocrine part of the pancreas comprises the bulk of the organ tissue. This fraction displays three main cell types: acinar, ductal and centroacinar cells. The acinar cells are arranged in grape-like structures at the smallest termini of the branching duct system, which covers the main area of the pancreatic tissue.

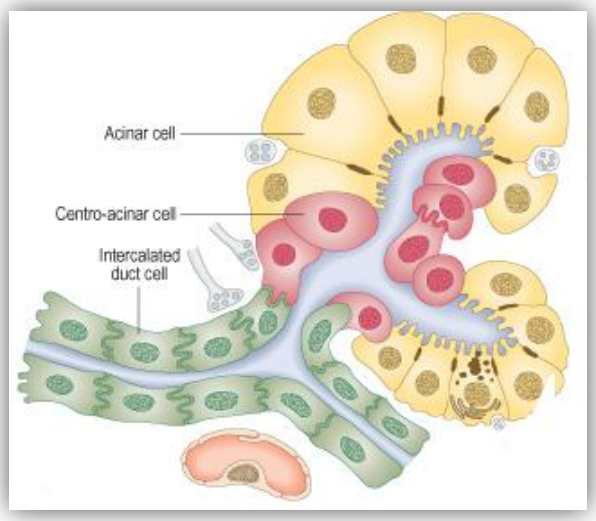



**Figure I.13: Exocrine pancreas.** Acinar cells constitute structures at the termini of the ducts. *Figure adapted from [166].*

Acinar cells possess large deposits of zymogen granules in the cytoplasm, which contain inactive forms of different digestive enzymes (trypsin, chymotrypsin, elastase, carboxypeptidase, lipase, amylase...). The acinar cells produce, store and secrete the digestive enzymes which are emptied to the ducts by exocytosis. Some proteases are the most

abundant enzymes in the pancreatic juice and in order to avoid self-digestion of the pancreatic tissue, acinar cells store inactive forms of these enzymes inside the zymogen granules of their cytoplasm. Then, they are secreted from the acini and emptied in the duodenal lumen.

The duct cells are organized in a network of increasing size culminating in main and accessory ducts. Their mission is to produce mucin and bicarbonate, which are added to the secreted digestive enzymes, thus obtaining the pancreatic juice. The enzyme mixture is then transported through the ducts and eventually emptied into the duodenum.

Finally, the centroacinar cells lie at the interface of the acinar structure and the ductal system, being continuous between both acinar and ductal lumens [168]. Both duct cells as well as centroacinar cells produce mucin and bicarbonate..

The exocrine secretion to the pancreatic duct is mediated by the hormone cholecystokinin (CCK), which is secreted by the duodenal and intestinal cells [169-170].. Once outside the pancreatic ducts, trypsinogen is activated by enteropeptidase, the latter which is produced by the duodenal mucous [171]. Trypsinogen is activated into trypsin, which activates the rest of inactive forms of proteases into carboxypeptidase, chymotrypsin, elastase and trypsin itself. Therefore, once the pancreatic juice is in the duodenum, the inactive enzymes are activated and ready to digest proteins, fat, carbohydrates and nucleic acids.

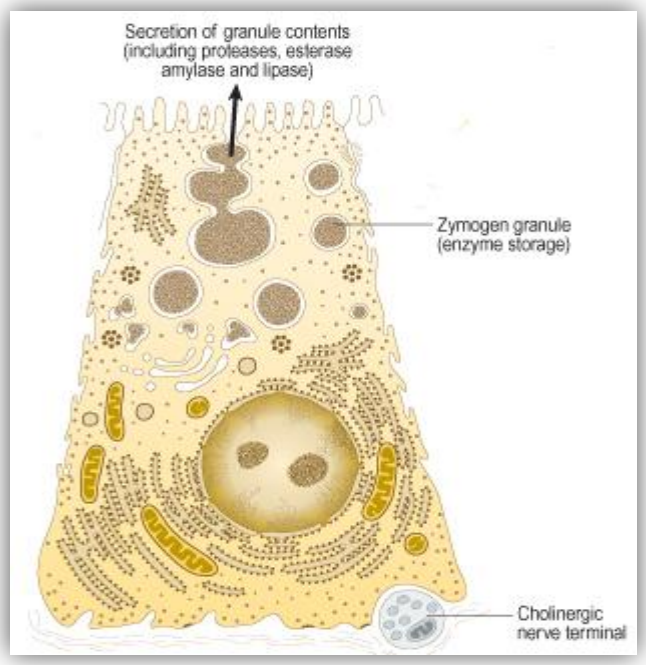



**Figure I.14: Secretion of pancreatic digestive enzymes from acinar cells.** Figure adapted from [166].

Pancreatic juice contains water, digestive enzymes (secreted by acinar cells) and electrolytes (secreted by ductal and centroacinar cells). The pancreatic juice is isotonic, and abundant in levels of $HCO_3^-$, $Na^+$, $K^+$, $Cl^-$ and scarce in $Ca^{2+}$ and $Mg^{2+}$. Pancreatic juice is alkaline due to high levels of bicarbonate [172], and not only transports the digestive enzymes to the intestinal tract but also neutralises the acid juices from the stomach. This neutralisation is very important because pancreatic enzymes need a neutral or slightly basic pH for their activity. Fat absorption is depending on the formation of micellae in the intestinal lumen, which needs a neutral or slightly basic pH as well. Finally, pancreatic juice protects the intestinal mucosa from ulcer formation by an excess of acid from the stomach.

| PANCREATIC DIGESTIVE ENZYMES | |
|---|---|
| Hydrolases | Nucleases |
| Amylase | Ribonucleases |
| | Deoxyribonucleases |
| Lipases | Proteases |
| Colipase | Chymotrypsinogen |
| Lipase | Trypsinogen |
| Prophospholipase A2 | Proelastase |
| Steapsin | Proprotease E |
| | Procarboxypeptidase A and B |

**Table I.1:** Digestive enzymes produced and secreted in acinar cells.

## 2.2 Pancreatogenesis

The first evidence of the pancreas during mouse development is a condensation of the mesenchyme of the endodermal gut tube in the duodenal primordium of the foregut. Two separate buds (dorsal and ventral) appear shortly after this condensation, around embryonic day 9.5 (E9.5) through evagination of the early gut endoderm into the mesenchyme [173-174].

Then, pancreatic buds undergo an increase in size and a spatial reorganization with the rotation of the stomach. A branching process initiates, sending epithelial protrusions into the mesenchyme, leading to a three dimensional structure with a tree-like epithelial network surrounding the mesenchyme. Meanwhile, the ventral bud is dramatically displaced towards the dorsal bud, becoming in contact with each other. This contact and the subsequent fusion of the pancreatic buds occur between E12 and E13. The dorsal bud will constitute the tail, the body and part of the head of the organ, while the ventral bud will become the rest of the head of pancreas.

Around E9 and E10, epithelial differentiation initiates and “early” endocrine cells can be found. Later on, the first acinar cells can be detected around E12 and E13.

During pancreatic formation, important signalling pathways are induced or silenced to promote differentiation of progenitor cells to an adult lineage, such as the case of the Hedgehog or the Notch pathway.

In this respect, several members like Sonic hedgehog (Shh) and Indian hedgehog (Ihh) induce growth, differentiation and function of many organs in embryogenesis and in adults [175]. However, increased Hedgehog signalling in the pancreas antagonises correct organogenesis [176-178], and inhibition of Hedgehog expression in the pre-pancreatic dorsal mesoderm induces the expression of transcription factor Pancreatic and duodenal homeobox 1 (PDX1), which is necessary for pancreas formation [179-181]. In addition, ectopic expression of Shh under the PDX1 promoter induces pancreatic mesoderm conversion to intestinal mesenchyme [178].

Another signalling pathway which has been reported to be involved in pancreatic formation is the Notch pathway. Indeed, analysis of pancreas development in mice genetically altered at several steps in the Notch signalling pathway showed that Notch signalling is crucial for the decision between the endocrine/exocrine progenitor fates in the developing pancreas [182]. Downregulation of several Notch pathway genes, like Hes1, Dll1 or RBPJ, leads to an increase of the transcription factor ngn3 which consequently triggers an increase in the formation of the endocrine compartment [183].

Moreover, recent discoveries have confirmed that Notch signalling is also involved in lateral specification of the pancreas and, similarly to other developing organ systems, in suppressing the differentiation and maintenance of the progenitors in an undifferentiated state [182-186].

## 2.3 Transcription factors involved in pancreatic development

Pancreatic development involves a complex combination of several signalling pathways and different transcription factors specifically expressed during time and in space. A subset of these transcription factors has a dual role: first, they are involved in differentiation and cell fate decision of the pancreatic progenitors during development. Later on, in the adult organ, they are related to functionality and the maintenance of the differentiated phenotype.

### 2.3.1. Pancreatic and duodenal homeobox 1 (Pdx1)

As mentioned above, one of the most important transcription factors in pancreatic development is Pdx1 which, in combination with Ptf1a, is crucial for proper pancreatogenesis and exocrine/endocrine progenitor differentiation [179-180, 187].

During mouse embryogenesis, Pdx1 is expressed by a subset of cells in the posterior foregut region of the definitive endoderm around E8.5-9, and it is present until around E12 [188]. Pdx1-expressing cells are multipotent progenitors which give rise to the developing pancreatic bud and subsequently to the exocrine, endocrine and ductal cell populations [181, 189]. Around E14-15, Pdx1 expression is localized in the endocrine tissue. Finally, Pdx1 is detected in the endocrine pancreas, mainly in β-cells [179] and to a lesser extent in other endocrine cell types [190-191].

Pdx1 has been found to be regulating the expression of many secretions from the endocrine pancreas, such as insulin and pancreatic polypeptide among others [192-194]. It has been also involved in the activation of the promoter of the elastase gene by means of constituting a trimer with Pbx1b and Meis2b which interacts with the Ptf1 complex [195-196]. Furthermore, interaction between Pdx1 and Pbx1 has been reported to be essential for pancreas development [197].

Homozygous Pdx1 knockout mice survive after birth but their lifespan is very short as they completely fail to develop an adult pancreas despite of developing pancreatic buds [179]. On the other hand, ectopic expression of Pdx1 expands the distribution of pancreatic progenitor cells in the dorsal domain of the embryo [198].

### 2.3.2. Pancreas specific transcription factor 1 alpha (Ptf1α)

In several organs, like the pancreas, a wide range of developmental and differentiation processes are regulated by the basic helix-loop-helix (bHLH) family of transcriptional regulatory proteins. bHLH proteins in mammals are classified in two groups, class B and class A. Class B bHLH transcription factors display a restricted pattern of expression, heterodimerising with class A bHLH molecules. Some of the class B members are muscle-specific factors like MyoD, NeuroD and the Pancreas specific transcription factor 1 alpha (Ptf1a), also known as p48 [199].

Ptf1a (henceforth p48) was initially identified as an exocrine specific transcription factor [200], constituting the PTF1 complex with two more molecules: E12/47, also called HEB, and Rbp-l [201].

However, this pancreatic specificity was questioned when p48 expression was also described in specific regions of the spinal cord and in the central nervous system (CNS) [202-203]. In addition, p48 expression was also found at early mouse embryonic stages (E9.5) in the developing exocrine and endocrine pancreas, rather than being exocrine restricted as previously described [187]. In the adult pancreas, p48 expression is restricted to acinar cells. Furthermore, overexpression of p48 in pancreatic acinar cell lines has an antiproliferative effect independent from the PTF1 complex [204].

Studies with knockout mice showed that the $p48^{-/-}$ null phenotype is postnatally lethal with associated below-average birth weight and complete failure of normal pancreatic development [200], while the ventral bud adopts an intestinal fate [187]. Moreover, p48 is a key regulator in cerebellar neurogenesis, as $p48^{-/-}$ null mice showed cerebellar agenesis at birth [205].

## 2.4 Pathologies of the pancreas

The main pancreatic pathologies reported in human patients have an effect on both exocrine and endocrine pancreas, like different congenital anomalies [206-208] or the formation of cysts [209]. The most common pathology in the endocrine pancreas is diabetes mellitus. However, our work is focused into pancreatic exocrine diseases, like pancreatitis and pancreatic cancer.

### 2.4.1. Acute pancreatitis

Acute pancreatitis is a sudden inflammation of the pancreas due to enzymatic necrosis, followed by an acute onset of abdominal pain. World deaths for acute pancreatitis are counted by 17,000 per year. The most

common causes of acute pancreatitis are pancreatic juice obstruction by gallstones, viral infections (paramyxovirus, Epstein-Barr virus), autoimmune diseases, excessive alcohol and drugs consumption, and duodenal ulcers.

As explained above, the exocrine pancreas produces a variety of enzymes that contribute to food digestion by breaking down food tissues. In acute pancreatitis, the main alteration is the auto-digestion of the tissue due to intrapancreatic activation of digestive enzymes such as the protease trypsinogen, and not in the intestinal lumen, thus leading to proteolysis, inflammation, destruction of pancreatic blood vessels, haemorrhage, tissue necrosis and pain.

Treatment for acute pancreatitis is based on pain relieving and blocking the pancreatic secretion until pancreas normalization is achieved.

### 2.4.2. Chronic pancreatitis

Chronic pancreatitis is a light or mild long-standing inflammation of the pancreas that alters its normal structure and functions, as the pancreatic parenchyma is progressively lost. Episodes of acute inflammation in a previously injured pancreas can also lead to a chronic pancreatitis, as well as chronic damage with persistent pain or absorption problems. At the histological level, chronic pancreatitis lesions show reduction of the acini, proliferation of ductal complexes and fibrosis with calcification.

Chronic pancreatitis shares many possible causes with acute pancreatitis, thus around 70% of adult patients with chronic pancreatitis presented chronic high alcohol consumption [210]. Gallstone-associated pancreatitis can become chronic after many acute inflammation and necrosis-fibrosis transition. Moreover, recent publications have unveiled

a hereditary chronic pancreatitis with an autosomal dominant pattern due to mutations in the serine protease inhibitor, Kazal type 1 (SPINK1), a pancreatic trypsin inhibitor [211]. However, several cases of chronic pancreatitis have an idiopathic origin.

Many studies have linked chronic pancreatitis with pancreatic cancer, pointing out a chronic, long-standing inflammation as a prelude of tumorigenesis [161, 212-214].

Palliative treatments for chronic pancreatitis include pain relieving and oral administration of digestive enzymes when the pancreas no longer secretes them.

### 2.4.3. Pancreatic cancer

Worldwide, over 200,000 people die annually of pancreatic cancer. The highest incidence and mortality rates of pancreatic cancer are found in developed countries. In the United States, pancreatic cancer is the 4$^{th}$ leading cause of cancer death, and in Europe it is the 6$^{th}$ [215]. Because of high fatality rates, pancreatic cancer incidence rates are almost equal to mortality rates. Patients with pancreatic cancer present a high mortality rate as the disease becomes clinically apparent during late stages, when it is refractory to conventional chemotherapy [216]. Therefore, there is a dismal prognosis of survival rate, while a total recovery is yet seldom achieved [159].

Several genetic and epidemiologic studies have been performed to understand the pathogenesis of the disease, and new insights are expanding the knowledge of pancreatic cancer. The incidence of the cancer increases in advanced age and environmental factors have an influence. The most common factors for pancreatic cancer are high

alcohol consumption, smoking habit [217], diets low in vegetables and fruits [218], chronic pancreatitis, inherited predisposition [219] and diabetes. Regarding familial pancreatic cancers, 10% of pancreatic cancers are inherited, but with a lower penetrance compared to other familial cancer syndromes [220].

The only efficient treatment at the moment is a partial pancreatoduodenectomy called the "Whipple procedure". Despite its aggressiveness, this treatment rarely achieves a complete recovery of the patient [221]. Chemotherapy and radiotherapy are an alternative to increase life expectancy. However, they are not as efficient as in other tumours at least in part due to the late diagnosis in advanced stages of tumorigenesis.

Pancreatic tumours are generally classified in two classes: endocrine and exocrine.

#### 2.4.3.1. Pancreatic endocrine tumours

Pancreatic endocrine tumours (PETs) are very rare, with an incidence of approximately 1:100,000 and they represent around 1–2% of all pancreatic neoplasms [222-223]. Regarding the cellular origin of PETs, while they were thought to be arising from the islets, new insights have suggested a pluripotent stem cell population in the ductal epithelium as their origin [223-224].

PETs are classified according to their secretion pattern, functional or non-functional. Functional PETs are associated with a clinical syndrome caused by inappropriate secretion of hormones. They were accounted for 70-80% of the diagnosed cases, but recent data has doubled the incidence of non-functional PETs [225-226]. The most common functional

PETs are insulinomas, followed by gastrinomas, glucagonomas, somatostatinomas, vasoactive intestinal polypeptide secreting tumours and other less frequent tumours [223, 227].

Non-functional PETs include lesions which have no relation with a specific hormonal syndrome because they do not present any secretion or because the actual secretion has no specific symptoms, like in the case of pancreatic polypeptide, ghrelin and neurotensin among others [227].

Despite both functional and non-functional PETs are very malignant, they are far less aggressive than their exocrine counterparts. Patients with PETs can achieve full recovery if the treatment is performed before apparition of metastasis [227]. Moreover, some PETs can appear outside the pancreas, like in the gastrointestinal tract, in the lungs or in neural tissue [227].

#### 2.4.3.2. Pancreatic exocrine tumours

The other group of pancreatic cancers includes pancreatic exocrine tumours, which account for around 95% of all cancers in the pancreas. Taking into account the histological classification of all types of malignant neoplastic lesions in the exocrine pancreas, 80% of the cases are classified as pancreatic ductal adenocarcinomas (PDACs), while the rest are ductal adenocarcinoma variants (like anaplastic carcinoma, adenosquamous carcinoma, mixed ductal-endocrine carcinoma…) and other malignancies which differ from the ductal phenotype, like mucinous cysticadenocarcinoma, intraductal papillary-mucinous invasive carcinoma and acinar cell carcinoma among others.

## 2.5 Pancreatic ductal adenocarcinoma (PDAC) and precursor lesions of PDAC

As mentioned above, pancreatic ductal adenocarcinoma (PDAC) is the most common type of malignant neoplasias of the pancreas, hence it is the most studied. Exhibiting aberrant ductal structures, three main types of non-invasive precursor lesions of PDAC have been reported and are being thoroughly studied to improve early diagnostic techniques and prognosis of the disease. These non-invasive lesions are pancreatic intraepithelial neoplasias (PanINs), mucinous cystic neoplasms (MCNs), and intraductal papillary mucinous neoplasms (IPMNs).

### 2.5.1. Pancreatic intraepithelial neoplasia (PanIN)

PanINs present microscopic noninvasive lesions which typically display columnar to cuboidal cells with mucin secretions, and cytologic and architectural atypia. Due to the variability of these lesions and the lack of agreement between researchers, characterising PanINs was very complex and chaotic in terms [228]. Therefore, a consensus was necessary to unify the histological criteria for grading PanINs [229-230].

PanINs can be separated in three grades according to the architectural and cytonuclear atypias: PanIN-1, PanIN-2, and PanIN-3. PanIN-1 is the lowest grade lesion, displaying flat epithelial lesions composed of tall columnar cells with abundant supranuclear mucin, with a minimal degree of atypias. This lesion is subclassified into PanIN-1A and PanIN-1B, according to the absence or the presence of epithelial micropapillary infoldings, respectively. PanIN-2 lesions are mostly papillary, with moderate atypias like loss of polarity, nuclear crowding, enlarged nuclei, pseudostratification, and hyperchromatism. Mitoses are not frequent. Finally, PanIN-3 lesions are also called “carcinoma in situ”, due to the

resemblance with carcinoma but with no invasive feature. Severe degree of atypias can be found, like papillary histology, cribriforming, luminal necrosis and atypical mitoses. The prevalence of PanIN lesions increases in advanced age. Indeed, low-grade stages are frequent in patients of 60 years of age while only a 3% show PanIN-3 lesions [231].

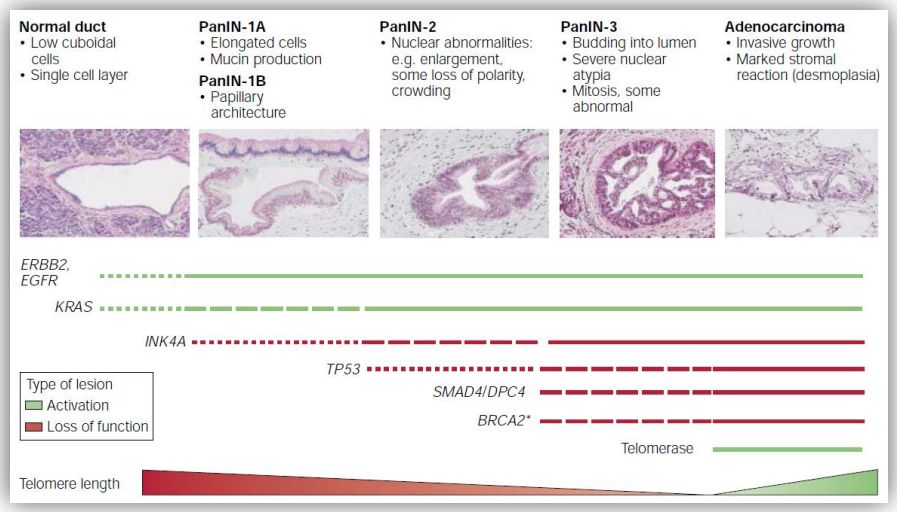



**Figure I.16: Pancreatic intraepithelial neoplasia (PanIN) progression.** PanIns preneoplastic lesions display progressive histological alterations which culminate into adenocarcinoma formation. A common pattern of gene alterations in different PDACs includes activation of oncogenes and a loss of function of many tumour-suppressor genes. *Figure adapted from [161, 229].*

There is solid evidence that PanINs are noninvasive precursor lesions of PDACs. Patients with previous pancreatic resection due to adenocarcinomas have been reported to display high-grade, noninvasive PanIN lesions which progressed to invasive cancers [232]. Furthermore, molecular analyses of PanINs have shown a correlation between the genetic alterations found in PanINs and in invasive pancreatic adenocarcinomas [233-234].

Furthermore, there is also a genetic progression in PanINs which fits with the histological grades [233, 235-236]. First of all, mutations in the KRAS oncogene have been reported in almost every case of PDAC, which

render KRAS constitutively active [237]. In PanIN lesions, mutations of KRAS are one of the earliest alterations. Moreover, there is a linear increase in the incidence of KRAS mutations in advancing PanIN stages [238-239]. In addition, loss of telomere integrity is also an early event in pancreatic cancer progression and in PanIN lesion. This event is related to chromosomal instability which leads to neoplasia [240-241]. Regarding PanIN-2 lesions, they display inactivating mutations in the INK4A/p16 gene, thus decreasing p16 expression. Mutations in this locus are found in 90% of the pancreatic cancers [242]. Advance-grade PanIN lesions display mutations in different tumour suppressor genes: TP53 [243], BRCA2 [244] and SMAD4/DPC4 [245].

### 2.5.2. Mucinous cystic neoplasm (MCN) and intraductal papillary mucinous neoplasm (IPMN)

The other two precursor lesions of PDAC, MCNs and IPMNs, are macroscopic lesions which present as cysts and sometimes as a dilation of the major pancreatic ducts. MCNs are cystic epithelial neoplasms with important secretion of mucin. They are usually found histologycally well defined and isolated with a surrounding ovarian-like stroma [246]. One third of MCNs have an invasive tubular component which has low prognosis compared to their non-invasive counterparts [247]. KRAS mutations can be detected at early stages of MCNs while inactivation of TP53 and SMAD4/DPC4 is usually detected only in invasive regions of MCNs [248-249].

Finally, IPMNs are the third type of precursor lesions, which are usually found in the main pancreatic duct. IPMNs are classified in two groups: main duct or branch duct, depending on their position in the pancreas. Branch-duct IPMNs are usually small, noninvasive lesions with better

prognosis than main-duct IPMNs, which are associated with invasion and malignancy [250-253]. IPMNs were first related to intestinal adenomas due to evident histological resemblances. Furthermore, recent analysis of different cases of IPMNs has unveiled a group of MUC-2 positive lesions with good prognosis and another group of MUC-1-expressing IPMNs and PanINs with associated PDACs and, thus, low prognosis [254-255].

Non-invasive IPMNs display a mutational pattern which significantly differs from the one of PanINs, probably due to differences of origin and histology. KRAS is mutated at early stages and this event increases during IPMN progression [256], like in PanINs. However, SMAD4/DPC4 gene is rarely inactivated in IPMNs and STK11/LKB1 is found to be mutated, which is the opposite in PanIN lesions [257-258]. Interestingly, the PI3KCA oncogene has been found to be mutated in IPMNs, which is a common alteration of colorectal neoplasia and would correlate with the intestinal-like phenotype of the lesion [259-260].

## 2.6 Genetic basis of PDAC

PDACs have a well-established mutational pattern which is consistent in the majority of analysed lesions. If we take into account the suggested precursor nature of PanINs, then the pattern should be considered as a progressive acquisition of mutations in tumour-suppressor genes, oncogenes, as well as telomere alterations [161].

One of the first genes to present alterations is the KRAS oncogene, which is mutated in almost every PDAC lesion [261]. Epidermal Growth-Factor (EGF) and the activated phosphatidylinositol 3-kinase (PI3K) pathway have been linked to KRAS in different cell lineages [262]. Consistently, EGF receptors (EGFR) and ligands (TGFα and EGF) are overexpressed in

pancreatic adenocarcinomas [263] and their knock-down inhibits cell growth in different pancreatic cell lines [264]. Moreover, EGF receptors are induced in low-grade PanINs, which might indicate EGF-family signalling to be active at early stages of neoplasia [265]. Expression of other oncogenes like cMyc is altered in around 25% of PDAC lesions [266].

On the other hand, PDACs show mutations in a wide range of tumour-suppressor genes. The p16/INK4A gene is the most commonly inactivated tumour-suppressor gene in the pancreas, accounting for around 90% of the cases [242]. Upon abnormal growth situation, p16/INK4A transcription is increased in a stress-response manner [50] to act as a tumour suppressor. Furthermore, p16/INK4A has been involved in the DNA damage response, thus its inhibition might favour PDAC resistance [267]. ARF is spared by sporadic mutations which target INK4A, thus suggesting that INK4A has a more important role than ARF in pancreatic cancer [268].

Another mutated tumour-suppressor in PDAC is TP53, which is found mutated in 50% of the cases [268].

Finally, the deleted in pancreatic carcinoma 4 gene (DPC4), also known as SMAD4, is a tumour-suppressor gene found mutated in 50% of PDACs [269]. The Smad4 protein has a crucial role in the TGF-β signalling pathway, which exerts inhibitory effects in cell growth. Loss of Smad4 in PDACs decreases TGF-β signalling, thus providing growth abnormalities [270].

## 2.7 Epigenetic mechanisms involved in pancreatic cancer

Recent studies have reported numerous epigenetic alterations in pancreatic cancers that, as describe for some genetic alterations can also be found in preneoplastic lesions such as PanINs and IPMNs [271]. It is the case of the DNA methylation machinery, which has been found to be altered in pancreatic cancers: expression of DNA methyltransferase 1 (DNMT1), which methylates daughter strands of newly replicated DNA to preserve parental methylation pattern [272], increases in advanced stages of the disease and may reflect the malignancy of PDAC [273].

The aberrant methylation of CpG islands has been frequently reported to occur in upstream regulatory regions of classical tumour-suppressor genes, thus impairing their normal transcription. Pancreatic cancers and precursor lesions (PanINs, IPMNs, MCNs) usually harbour abnormal hypermethylation in tumour-suppressor genes or in genes involved in important homeostatic pathways, like p16/INK4a [274], TSCL1 [275], BNIP3 [276] and preproenkephalin (ppENK) among others [277-278].

Members of the mucin family have been shown to undergo alterations of histone modifications in pancreatic cancer. Regulatory regions of the 5' of MUC1 have been reported to be enriched in H3K9 marks [279]. Moreover, MUC2 also shows enrichment in the 5' region, specifically in tri-methylated H3K4, acetylated H3K9 and acetylated H3K27 in pancreatic cancer cells [280].

Finally, analysis of the expression of Polycomb group genes in various human tissues – including the pancreas – at the RNA and protein levels unveiled an important variation in the expression among tissues and

even among specific cell types [136]. In these preliminary studies, Bmi1 and Ring1B expression were found to be expressed in both endocrine and exocrine compartments of the pancreas, while the signal was stronger in endocrine cells. No expression of PRC2 members was found.

## 2.8 Animal models for the study of exocrine pancreatic pathologies

There has been a strong interest to generate animal models that faithfully recapitulate the events occurring in human pancreatitis and in PDAC. A wide range of models have been created so far, involving induced expression of transforming growth factor α (TGFα) or overexpression of oncogenes CMYC and KRAS, among other strategies.

### 2.8.1. Caerulein-induced and duct-ligated-induced pancreatitis

Caerulein is a decapeptide similar in action and composition to cholecystokinin, stimulating gastric, biliary, and pancreatic secretion. It is used in paralytic ileus and as diagnostic aid in pancreatic malfunction. It is also used to induce pancreatitis in rodent models [281].

Another way of inducing experimental murine pancreatitis is by means of performing a ligation in pancreatic ducts next to the spleen, obstructing the exocrine fluid in the region [282]. Seven days later, exocrine metaplasia appears in the ligated part of the pancreas leading to transdifferentiation of the acinar cells to duct-like cells [283] while the unligated part remains intact. These metaplastic cells lose their acinar characteristics (decrease or loss of p48/Ptf1a and Mist1 expression) and start expressing genes related to development and cell growth, such as the embryonic transcription factor PDX1 and Notch receptors [284].

### 2.8.2. MT- TGFα and Ela-TGFα

Expression of epidermal growth factor receptor (EGFR) with its ligand transforming growth factor α (TGFα) has been linked with a decrease in the patient survival with pancreatic cancer [285].

Based on this observation, different TGFα transgenic mice were generated. One of these models uses the metallothionin-1 gene promoter to drive TGFα expression (MT-TGFα) [286-287]. This model displays high proliferation of fibroblasts and acinar cells in the pancreas, with focally altered acinar-to-ductal transdifferentiation. However, TGFα overexpression is not specifically directed to the pancreas, therefore this model was exceeded by more specific animal models for pancreatic diseases.

Another mouse model with induced TGFα using the elastase-1 promoter (Ela-TGFα) was developed, allowing specific induction in the acinar fraction of the pancreas [288-289]. About 25% of the animals display tumours at one year of age. Nevertheless, the spectrum of events in these lesions does not recapitulate those in human PDAC, as Ela-TGFα displayed abnormal ductal structures and fibrosis, but lacked many features found in human PDAC lesions.

### 2.8.3. Ela-myc

Half of human PDACs displays overexpression of oncogene CMYC both at the protein [290] and at RNA level [291]. Moreover, a recent study established that out of 31 pancreatic cancer cell lines, more than the half showed CMYC amplification [292].

In addition, the induction of pancreatic cancer in rats by chemical carcinogens, like azaserine, leaded to an increase in CMYC levels, thus

confirming the role of CMYC in human PDAC [293]. Moreover, a transgenic mouse was developed carrying CMYC under the elastase promoter (Ela-myc). These animals display pancreatic cancer lesions at an early age [294], and the resulting tumours are 50% acinar cell carcinomas while the other 50% are ductal or mixed ductal and acinar carcinomas.

### 2.8.4. $KRAS^{G12V}$

The first transgenic mouse able to recapitulate the whole spectrum of human PDAC events (PanINs and PDAC lesions) was a knock-in mouse which has a point mutation in the endogenous KRAS gene, which turns into an aminoacid substitution in the translated protein ($KRAS^{G12D}$), thus rendering KRAS constitutively active. Using pancreas-specific promoters of PDX1 and p48, $KRAS^{G12D}$ is specifically expressed in pancreatic lineages at E8.5 [295]. However, this mouse model does not provide information about the target cell in which KRAS mutation must take place for PDAC to develop.

Recently, a transgenic knock-in mouse was generated with the $KRAS^{G12V}$ oncogenic allele expressed specifically in acinar and centroacinar cells during development under the control of the elastase promoter [296]. This model recapitulates the full spectrum of PanINs and invasive PDAC found in humans and it can be ascertained that acinar and centroacinar cells can lead to ductal-like tumors.

Moreover, while oncogenic KRAS activation in embryonic stages was enough to induce the PanIN formation and PDAC progression, sole oncogenic KRAS induction in adult mice does not result in tumours. Interestingly, caerulein-induced chronic pancreatitis is needed to allow

tumorigenesis [296]. This correlates with the previously reported fact that chronic pancreatitis is a risk factor of PDAC [213].

## 2.9 Pancreatic cell plasticity

Many studies have reported the ability of pancreatic cell types to undergo metaplasia or transdifferentiation, thus converting into another cell type. *In vivo* and *in vitro* experiments have reported transdifferentiation between different pancreatic cell types and also into other tissue cell types [283]. This apparent pancreatic cell plasticity has opened an interesting research field with many implications, both clinical and therapeutical. Several *in vitro* models have been able to obtain a wide variety of metaplasias: acinar-to-ductal [284], acinar-to-islet [297-298], acinar-to-hepatocyte [299]. The studies regarding exocrine to endocrine transdifferentiation have meant a huge leap forward in pancreatic β-cell regeneration to improve the treatment on patients with diabetes mellitus [297]. However, a better understanding of the whole system must be achieved before transferring these models to the clinic.

*In vivo* models of transdifferentiation use the natural ability of the pancreas to spontaneously undergo acinoductal transdifferentiation upon tissue injury. As explained above, several experimental animal models with acinar-to-ductal metaplasia have been established, in which pancreatic lesion is induced, like caerulein-induced pancreatitis [296], obstruction or ligation of pancreatic ducts [300-301] and subtotal pancreatectomy [302]. Moreover, several transgenic mice with directed overexpression to the pancreas also show acinar-to-ductal transdifferentiation, like the cases of overexpressing transforming growth factor α (TGFα) [288-289], the oncogene CMYC [294] or the oncogene $KRAS^{G12V}$ [296].

Acinar-to-ductal metaplasia has been reported in human pancreatitis, in preneoplastic lesions of the pancreas and in PDACs. Exhaustive studies are being performed to understand the cellular origin of tumorigenesis in the pancreas, in order to tackle the disease early on its development [161, 212-213].

The identification of a population of “cancer stem cells” may pave the way to improve treatments against pancreatic cancer, targeting both tumour-initiating cells and differentiated cancer bulk population in order to achieve a cure instead of palliative treatments. In addition, it also may clarify the origin of the transdifferentiated population found in pancreatic lesions and as a consequence the cellular origin of pancreatic cancer. Recent evidences may suggest an origin from adult stem cells or progenitor cells which acquire self-renewing capacity due to acquisition of oncogenic mutations [295].

Until now, efforts to elucidate a stem-cell population in the pancreas have not yet succeeded. On the other hand, systematic analyses of different pancreatic adult cell types have been performed to characterise a population capable of initiating tumorigenesis. However, transgenic mice with directed expression of mutant KRAS oncogene to the pancreatic ducts show neither preneoplastic lesions nor neoplasia, suggesting that ductal cells are not capable of originating tumorigenesis [51].

At the moment, two cell types are considered to be as potential candidates. The first candidates are the centroacinar cells. Largely ignored during many years, interest in centroacinar cells re-emerged after findings that Notch signalling remains selectively active in these

cells [303]. The Notch signalling pathway has a key role in development stages to regulate cell differentiation, and in embryonic pancreas it is crucial for maintaining pancreatic progenitor cells in an undifferentiated state [182-183, 185-186]. The activated state of Notch in centroacinar cells, plus their strategic disposition in the pancreatic tissue, identify this pancreatic adult cell population as possible stem cell reservoir for the exocrine pancreas and therefore as a potential source for pancreatic cancer initiation.

On the other hand, acinar cells have been reported multiple times to acquire a precursor phenotype under pancreatic injury and regeneration. For instance, in animal models with caerulein-induced pancreatitis acinar cells repress differentiated exocrine characteristics and start expressing markers of undifferentiated pancreatic progenitors like Hes1 and PDX1 [304]. Moreover, strong evidence supporting the role of acinar and centroacinar cells in the origin of pancreatic cancer comes from the recent study above stated in which targeting a constitutively active $KRAS^{G12V}$ to elastase-positive acinar cells during development results in PanINs and PDAC formation.

During these last two years, several studies have been published regarding specific roles of members of the Polycomb family in pancreatic function. For instance, EZH2 was found to regulate the expression and regeneration of β-cells by means of repressing the INK4A locus. In aging islets, EZH2 expression was declined and p16 and p19 were upregulated. Interestingly, young mice with conditionally deleted EZH2 in β-cells had reduced β-cell proliferation and growth, hypoinsulinemia and mild diabetes. On the other hand, an induced lesion performed in the islets to diminish β-cell population was followed by an increase in EZH2

expression, by adaptive proliferation and by re-establishment of the β-cell mass [305].

Moreover, similar results were published with Bmi1 in β-cell regeneration and growth. Bmi1 was reported to be also regulating the INK4a/Arf locus in β-cells, and aging islets showed decreasing levels of Bmi1 which resulted in an increase of p16 and p19, leading to impairment in β-cell regeneration [306-307].

Finally, a recently published lineage-tracing study, using $BMI1^{Cre\text{-}ER/+}$; $Rosa26^{YFP/+}$ knock-out mice, reported that Bmi1 can allegedly work as a marker for a subpopulation of self-renewing acinar cells. This would indicate that self-renewal properties are not an exclusive feature of adult undifferentiated stem cells [308].

## OBJECTIVES

Epigenetic mechanisms are well established inducers of tumorigenesis. Their role in cancer formation and progression is incontrovertible. Moreover, several members of Polycomb group of proteins have been related to cancer formation and progression.

The main reason and hypothesis of this thesis project is that Polycomb members ought to have a role in formation, progression and invasiveness of pancreatic ductal adenocarcinoma. Therefore, in order to answer it this project has the following objectives:

**1. To characterise Polycomb expression pattern in normal mouse pancreas.**

**2. To study the role of Polycomb in pancreatic ductal adenocarcinoma by means of analysing possible modulations of its members in human pancreatic cancer and in PDAC mouse models *in vivo* and *in vitro*.**

**3. To elucidate the consequences of modulating Polycomb members in acinar differentiation and *in vitro* models.**

# 1. Immunohistochemistry

To perform immunohistochemical analysis of organ tissues, 5-µm sections were obtained from formalin-fixed, paraffin-embedded tissue blocks using a Leica microtome (RM2255). Sections were placed in microscope slides and deparaffined with immersion in histology grade xylene (3 buckets, 15 minutes each). Once paraffin is off the sample, excess of xylene is removed by hydrating samples in ethanol gradient battery (2 buckets of absolute ethanol, one at 96%, one at 70% and one at 50%, 5 minutes each). Sections are finally immersed in distilled water.

For the immunohistochemistry, horseradish peroxidase method (HRP) was used with the DAKOCYTOMATION Liquid DAB+ Substrate-Chromogen commercial system. The following protocol was followed:

- Antigen retrieval: citrate buffer solution at 10 mM (pH 7.3) was used at 120°C for 1 minute in an autoclave. Samples were allowed to gently cool down.
- Endogenous peroxidase inhibition: hydrogen peroxide solution ($H_2O_2$) at 4% in methanol was used for 10 minutes at RT and excess was washed with PBS, 3 times, 5 minutes each.
- Tissue is blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) solution, for 1 hour.
- Samples were incubated with the following primary antibodies for 12 hours, diluted with blocking solution:

| Marker | Antibody | Origin | Dilution |
|---|---|---|---|
| Bmi1 | Mouse | Millipore | 1:50 |
| Ring1B | Mouse | MBL | 1:50 |
| EED | Rabbit | Upstate | 1:100 |
| Amylase-2 | Rabbit | Sigma | 1:100 |

**Table M.1:** antibodies used in immunohistochemical assays.

- Sections were washed with blocking solution, 5 times, 5 minutes each.
- Envision system labelled polymer-HRP anti-mouse and anti-rabbit (DAKOCYTOMATION) were used as secondary antibodies. After washing, reactions were developed using 3,3-diaminobenzidine tetrahydrochloride plus (DAB+) as chromogenic substrate.
- For sequential horseradish peroxidase/alkaline phosphatase (HRP/AP) immunoenzymatic double staining analysis of Bmi1 and Amylase 2, primary anti-Bmi1 antibody was used first. Then, secondary HRP-conjugated antibody and after extensive washing, anti-Amylase 2 primary antibody was used.
- Finally, incubation with AP-conjugated secondary antibody was performed. HRP activity was developed as above, while AP detection was obtained by incubating with Envision doublestain system (K1395, DAKOCYTOMATION).
- Sections were counterstained with hematoxylin, dehydrated, and mounted. Two negative controls were routinely used, one where the primary antibody was substituted with antibody diluents and the other with an irrelevant monoclonal antibody.

Sections were visualized in a Leica DM6000 Digital microscope, and images were acquired using QWin software (Leica) at 20× objective magnification.

## 2. Cell line culture

The HEK293 Phoenix cell line was generated by transformation of cultures of normal human embryonic kidney cells with sheared adenovirus 5 DNA [309]. Two variants of this cell line were used in this project. First, the 293T cell line that contains, in addition, the simian virus 40 (SV40) Large T-antigen, thus allowing for episomal replication of transfected plasmids containing the SV40 origin of replication. Therefore, the cell line is widely used for amplification of transfected plasmids and extended temporal expression of the desired gene products. In this study, 293T cells were used to generate lentivirus codifying for cDNA of several PcG members.

Another HEK 293 variant we used in this project was the phoenix ecotropic 293 cells, which include constructs capable of producing gag-pol, and envelope protein for ecotropic viruses. Therefore, the phoenix 293 cells allowed us to generate retroviral vectors codifying for cDNA or iRNA of several PcG members.

On the other hand, we used pancreatic cell line 266-6 for infection / transfection experiments. The 266-6 cell line was derived from a young adult mouse with an induced tumour with an Elastase I/SV40 T-antigen fusion gene [310]. These cells partially retain a partially differentiated phenotype, expressing detectable levels of a number of digestive enzymes at the RNA level.

All three cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Foetal Bovine Serum (FBS), at 37°C in humid atmosphere of 5% $CO_2$ in air.

## 3. Retroviral generation and infection

Production of retroviruses was achieved by transfecting 293 Phoenix cells in 60 mm petri dishes (150326, NUNC) using FuGENE HD transfection reagent (04709705001, Roche Applied Science) in Opti-MEM I reduced-serum medium (11058-021, invitrogen), according to manufacturer's protocol. The protocol in use was the following:

- 293 Phoenix cells were plated at 50% of confluence 24 hours before transfection.
- For each condition, 500 µl of Opti-MEM medium were mixed with 25 µl of FuGENE HD reagent and incubated at RT for 5 minutes.
- The retroviral vectors used were the following: MSCV-hBMI-1-IRES-eGFP, pBabe-puromycin-Ring1B and pRetroSuper-puromycin-iGFP. 10 µg were added to the previous mix and left at RT for 15 minutes.
- The resulting solution was finally added to 5 ml of fresh DMEM+10% FBS medium, and then added to Phoenix 293 cells in culture, which were left at 37°C, 5% $CO_2$.
- 6 hours later, 5 ml of DMEM+10% FBS fresh medium were added.
- 24 hours later, medium was renewed. At the same time, 266-6 cell lines are plated at 50% of confluence.

- 24 hours later, medium supernatant was filtered through 0.45 μm filter to discard any cell debris and to obtain filtered retroviral particles. Then, medium from 266-6 cells was renewed with filtered medium with retroviruses and Polybrene reagent was added, which is a retroviral infection enhancer allowing an increase in infection rate up to 1000-fold. Cells were stored at 37$^{o}$C, 5% $CO_2$.
- Medium was renewed the following day with fresh DMEM+10% FBS with 4 μg/ml of puromycin antibiotic, in order to select the cells which had been infected.
- Once a selected population was achieved, which was in 5 days of selection, total RNA extraction was performed.

## 4. siRNA oligonucleotide transfection

266-6 cells were cultured in DMEM + 10% FBS medium. The following siRNA oligonucleotides (Dharmacon) were used: Bmi1 (GTATTGTCCTATTTGTGAT), GFP (GCTGACCCTGAAGTTCATC), both reported elsewhere [311]. Dharmafect 4 transfection reagent (T-2004-02, Dharmacon) was used like the following:

- Cells (1x10$^{6}$) were seeded in 60 mm culture dishes and kept in culture 16-24h at 37$^{o}$C, 5% $CO_2$.
- 2 μM of siRNA was mixed in 100 μl of Opti-MEM reduced-serum (invitrogen) and left for 5 minutes at RT. At the same time, 4 μl of Dharmafect 4 were added to 196 μl of Opti-MEM and left for 5 minutes at RT. Both mixes were pooled in one tube and left for 20 minutes at RT.

- Media was removed from cells and 2.5 ml of Opti-MEM were added (invitrogen). The siRNA-Dharmafect 4 mix was added to the cells dropwise and kept ON at 37$^{o}$C, 5% $CO_2$.
- Media was changed for Fresh DMEM+10% FBS and cells were cultured for 24 hours at 37$^{o}$C, 5% $CO_2$.
- Protein extraction and total RNA isolation were performed afterwards.

## 5. Total RNA isolation

RNA was obtained by using GenElute™ Mammalian Total RNA Miniprep Kit (RTN70, SIGMA-ALDRICH). The protocol in use was the one supplied by the manufacturer:

- Medium was thoroughly removed and cells were washed with PBS, twice.
- PBS was removed and 500 µl of Lysis Solution/2-Mercaptoethanol were added. Culture dish was rocked to completely cover the cells and rested for 1 minute at RT.
- Cell lysate was scraped and pipetted into a GenElute Filtration Column to remove cell debris and shear DNA. Column is centrifuged at maximum speed (12,000-16,000 x g) for 2 minutes. Column was discarded.
- 500 µl of 70% ethanol solution was added to the filtered lysate and mixed thoroughly. Solution is loaded into a GenElute Binding Column and centrifuged at maximum speed for 30 seconds.
- Column was retained and returned to a collection tube. 500 µl of Wash Solution 1 was added into the column and centrifuged at maximum speed for 30 seconds.

- Flow-through liquid was discarded and column was applied in a new collection tube. 500 µl of Wash Solution 2 were added to the column and it was centrifuged at maximum speed for 30 seconds.
- Flow-through was discarded and 500 µl of Wash Solution 2 were added to the column again and it was centrifuged at maximum speed for 2 minutes.
- Flow-through was again discarded and centrifuged 1 extra minute to eliminate residual ethanol.
- Column was inserted in a new collection tube and 50 µl of Elution Buffer were added and centrifuged at maximum speed for 1 minute.
- Eluted RNA was quantified with a Nanodrop spectrophotometer (Thermo-Scientific) at 260 nm and stored at -80°C.

## 6. Semiquantitative two-step RT-PCR

To perform semiquantitative RT-PCRs, commercial Transcriptor First Strand cDNA Synthesis Kit (04 896 866 001, Roche Applied Science) was used to obtain cDNA product from RNA extracts. The protocol in use was the following:

- Template-primer mix was prepared after quantifying RNA samples:

| Component | Volume | Final concentration |
|---|---|---|
| RNA + Elution buffer | 11 µl | 1 µg RNA |
| Random Hexamer Primers (600 pmol/µl) | 2 µl | 60 Mm |
| **Total Volume** | **13 µl** | |

**Table M.2:** RNA template-primer mix.

- Template-primer mix was denatured by heating for 10 minutes at 65°C in a T1 thermal block cycler (050-901, biometra) with a heated lid to minimize evaporation, in order to ensure denaturation of secondary structures. Solution was immediately cool on ice.
- The rest of the components were added to the template-primer mix tube in the following order:

| Component | Volume | Final concentration |
|---|---|---|
| Transcriptor Reverse Transcriptase Reaction Buffer (5x) | 4 µl | 8 mM $MgCl_2$ |
| Protector RNase Inhibitor (40 U/µl) | 0.5 µl | 20 U |
| Deoxynucleotide Mix (10 mM each) | 2 µl | 1 mM each |
| Transcriptor Reverse Transcriptase (20 U/µl) | 0.5 µl | 10 U |
| **Final Volume** | **20 µl** | |

**Table M.3:** Second mix of the RT assay.

- The RT reaction was performed in a thermal block cycler:
    - 10 minutes at 25°C.
    - 60 minutes at 50°C.
    - 5 minutes at 85°C.

Reaction was stopped by placing tubes on ice.

Standard semiquantitative PCR was performed using cDNA samples, which were previously normalised. The following mix was prepared:

| Component | Volume | Final concentration |
|---|---|---|
| BIOTAQ Buffer (10x) | 2.5 µl | 1x |
| $MgCl_2$ (50 mM) | 1 µl | 2 mM |
| Deoxynucleotide Mix (10 mM each) | 0.5 µl | 200 µM |
| Primer Forward (10 µM) | 1.5 µl | 0.6 µM |
| Primer Reverse (10 µM) | 1.5 µl | 0.6 µM |
| BIOTAQ polymerase (5U/µl) | 0.25 | 1.25 U |
| cDNA | Variable | 100-400 ng |
| $H_2O$ | Variable | |
| **Final Volume** | **25 µl** | |

**Table M.4:** Semiquantitative RT-PCR mix.

And the following PCR reaction was designed:

| PCR Step | Temperature | Time |
|---|---|---|
| 1. Initial denaturation | 94°C | 5-10 minutes |
| 2. Denaturation | 94°C | 30 seconds |
| 3. Annealing | 55-63°C (depending on Tm) | 30 seconds |
| 4. Extension | 68-72°C | 1-2 minutes |
| **Number of cycles (from 2. to 4.)** | **20-35 cycles (depending on abundance of transcript)** | |
| 5. Last extension | 68-72°C | 10 minutes |
| 6. End | 4°C | ∞ |

**Table M.5:** PCR conditions for semiquantitative RT-PCR.

The following pairs of oligonucleotides were used:

| Primer | Sequences | Length |
|---|---|---|
| HPRT | F 5'-GGCCAGACTTTGTTGGATTTG-3'<br>R 5'-TGCGCTCATCTTAGGCTTTGT-3' | 144 |
| Bmi1 | F 5'-CGTTACTTGGAGACCAGC-3'<br>R 5'-TGCAAGTTGGCCGAACTC-3' | 520 |
| Ring1B | F 5'-CATGAACAGATTACAGCGAGG-3'<br>R 5'-GGATAAGTGATCAACAGTGGC-3' | 325 |
| Amylase | F 5'- GGAGGACTGCTATTGTCCAC-3'<br>F 5'-CCAAGCAGAGTATGGAACTG-3' | 377 |
| P48 | F 5'-TGCAGTCCATCAACGACGC-3'<br>R 5'-GGACAGAGTTCTTCCAGTTC-3' | 1041 |

**Table M.6:** Primers used for semiquantitative RT-PCR experiments.

Products were visualized by ethidium bromide staining using agarose electrophoresis.

## 7. Quantitative Real-Time PCR

Real-Time PCR was performed using SYBR® Green PCR Master Mix (4312704, Applied Biosystems), working on a 96-well plate without cover. cDNA samples were obtained from RNA extracts of treated cells using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) and normalised to a specific concentration (25 ng/µl).

The following mixes were prepared:

| cDNA – SYBR Green mix | Volume | Concentration |
|---|---|---|
| cDNA | 2 µl | 5 ng/µl |
| SYBR® Green PCR Master Mix (2x) | 5 µl | 1x |

**Table M.7:** cDNA-SYBR Green mix.

| Primer – $H_2O$ mix | Volume | Concentration |
|---|---|---|
| Primer Forward (10 µM) | 0.3 µl | 300 nM |
| Primer Reverse (10 µM) | 0.3 µl | 300 nM |
| $H_2O$ | 2.4 µl | |

**Table M.8:** Primer-$H_2O$ mix.

Both mixes were combined in each well and the following PCR reaction was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems):

| PCR Step | Temperature | Time |
|---|---|---|
| 1. | 50°C | 2 minutes |
| 2. | 95°C | 10 seconds |
| 3. | 95°C | 15 seconds |
| 4. | 60°C | 1 minute |
| **Number of cycles (from 3. to 4.)** | **40 cycles** | |

**Table M.9:** PCR program for Real-Time PCR experiments.

The following pairs of oligonucleotides were used:

| Primer | Sequences | Length |
|---|---|---|
| HPRT | F 5'-TCATTATGCCGAGGATTTGGA-3'<br>R 5'-GCCTCCCATCTCCTTCATGAC-3' | 99 |
| GAPDH | F 5'-AGGCCGGTGCTGAGTATGTC-3'<br>F 5'-GGCGGAGATGATGACCCTTT-3' | 103 |
| Bmi1 | F 5'-AGTAAATAAAGAGAAGCCTAAGGAAGAG -3'<br>R 5'-TTCTCAAGTGCATCACAGTCATT-3' | 85 |
| Ring1B | F 5'-AGGCCGGTGCTGAGTATGTC-3'<br>F 5'-GGCGGAGATGATGACCCTTT-3' | 99 |
| EED | F 5'-CGGGAGACGAAAATGACGAT-3'<br>R 5'-CTTTTCCTTCCTGGTGCATTTG-3' | 99 |
| EZH2 | F 5'-GCTGACCATTGGGACAGTAAAAA-3'<br>R 5'-CCCAGCCTGCCACATCA-3' | 102 |
| P48 | F 5'-CCAGGCCCAGAAGGTTATCA-3'<br>R 5'-GGAAAGAGAGTGCCCTGCAA-3' | 99 |
| Amylase A2 | F 5'-GCCAAGGAATGTGAGCGATAC-3'<br>R 5'-AAGGTCTTGATGGGTTATGAACTACA-3' | 100 |
| Carboxypeptidase A1 | F 5'-GAGGCTGCTGGTTCTGAGTGT-3'<br>R 5'-TCTGCACCTGGGCTTCGT-3' | 107 |
| Chymotrypsinogen B | F 5'-CATCGTGTCCGAGGCTAAGTG-3'<br>R 5'-GAGTCACCCATGCAGGAAGAG-3' | 101 |
| Elastase | F 5'-ACCCTCATCCGAAGCAACTG-3' | 102 |

| | | |
|---|---|---|
| | R 5'-CGTCATTCTGGCTCAGGTTGT-3' | |
| PDX1 | F 5'-CGCGTCCAGCTCCCTTT-3'<br>R 5'-AGTACGGGTCCTCTTGTTTTCCT-3' | 113 |
| HES1 | F 5'-TACCCCAGCCAGTGTCAACA-3'<br>R 5'-TCTTGCCCTTCGCCTCTTC-3' | 99 |
| RBPJ | F 5'-CCAATTTCAGGCCACTCCAT-3'<br>R 5'-CGTGTACTCGGCCTTGTCTGT-3' | 99 |

**Table M.10:** Primer-$H_2O$ mix.

Data was analysed using SDS 2.3 and RQ Manager 1.2 software (Applied Biosystems).

## 8. Protein extraction

We treated the cells to obtain nuclear and total protein extracts. The following lysing buffers were used:

| Lysis Buffer A | Concentration |
|---|---|
| Sucrose | 0.25 M |
| HEPES pH 7.5 | 10 mM |
| $CaCl_2$ | 3 mM |
| NaCl | 10 mM |
| NP-40 | 0.25% |
| PMSF | 1 mM |
| DTT | 1 mM |

**Table M.11:** Lysis Buffer A composition.

| RIPA Buffer | Concentration |
|---|---|
| Sodium deoxycholate | 0.5 % |
| Tris-HCl pH 8.0 | 50 mM |
| NaCl | 150 mM |
| SDS | 0.1 % |
| NP-40 | 1.0 % |
| EDTA | 2 mM |
| PMSF | 2 mM |
| DTT | 1 mM |
| c0mplete cocktail | 1x |

**Table M.12:** RIPA buffer composition.

To obtain nuclear protein extracts, the following protocol was used:

- Medium was removed from cell dish and cells were washed with PBS, twice. 1 ml of PBS was added and cells were scraped, pipetted into an eppendorf tube on ice and centrifuged at 3,000 rpm for 5 minutes.
- Supernatant was discarded and pellet was resuspended with 1 ml of buffer A, and was incubated for 10 minutes on ice. Afterwards, cells were centrifuged at maximum speed for 1 minute at 4°C. This step was repeated twice.
- After three steps of washing cells with buffer A, a volume of RIPA buffer between 50-150 µl was added to the pellet of cells, and it was incubated for 30 minutes on ice. Finally, it was centrifuged at maximum speed for 15 minutes at 4°C. Supernatant was stored as it contained the nuclear protein fraction.
- To obtain total protein extracts, cells were washed with PBS, scraped and centrifuged at 3,000 rpm for 5 minutes as in the

above protocol. Supernatant was discarded and a volume of RIPA buffer between 50-150 µl was added to the pellet of cells and incubated for 30 minutes on ice. Cells were centrifuged at maximum speed for 15 minutes at 4°C.

Protein extracts were stored at -80°C.

## 9. Western blot

Protein extracts were quantified with RD DC Protein Assay Kit (500-0122, BIORAD), following provided protocol by the manufacturer. Absorbances of samples were read at 750 nm and quantified by setting up a standard gradient with BSA. The following buffers were used to perform western blot assay:

| Separating gel solution | Concentration |
|---|---|
| Tris-HCl pH 8.8 | 375 mM |
| Bis-acrylamide | 8 % |
| SDS | 0.1 % |
| APS | 0.1 % |
| TEMED | |

**Table M.13:** Separating gel solution composition.

| Stacking gel solution | Concentration |
|---|---|
| Tris-HCl pH 6.8 | 125 mM |
| Bis-acrylamide | 4 % |
| SDS | 0.1 % |
| APS | 0.1 % |
| TEMED | |

**Table M.14:** Stacking gel solution composition.

| Electrophoresis buffer | Concentration |
|---|---|
| Tris base | 25 mM |
| Glycine | 190 mM |
| SDS | 0.1 % |

**Table M.15:** Electrophoresis buffer composition.

| Transference buffer | Concentration |
|---|---|
| Tris pH 7.5 | 200 mM |
| Glycine | 1.5 M |
| Methanol | 20% |

**Table M.16:** Transference buffer composition.

| Blocking buffer | Concentration |
|---|---|
| Tween-20 | 0.1 % |
| Milk | 5 % |

**Table M.17:** Blocking buffer composition.

| Antibody dilution buffer | Concentration |
|---|---|
| Tween-20 | 0.1 % |
| BSA | 0.3 % |
| Sodium azide | 20% |

**Table M.18:** Antibody dilution buffer composition.

Proteins were normalized to a specific quantity and separated by vertical SDS-polyacrylamide gel electrophoresis using mini-protean III system (BioRad) at 100V for 2 hours at RT. Afterwards, proteins were transferred to Westran™ Polyvinylidine Fluoride (PVDF) Clear Signal membranes (10485287, Whatman). Blocking buffer was added to the membranes for 30 minutes to improve the sensitivity of the assay by reducing background interference and improving the signal to noise ratio.

After washing excess of blocking buffer, membranes were immunoblotted with the following primary antibodies ON at 4°C:

| Marker | Antibody | Origin | Dilution |
|---|---|---|---|
| **Lamin B1** | Rabbit | Abcam | 1:2000 |
| **Tubulin** | Mouse | SIGMA-ALDRICH | 1:10000 |
| **Bmi1** | Mouse | Millipore | 1:500 |
| **Ring1B** | Mouse | MBL | 1:500 |
| **Carboxypeptidase A1** | Rabbit | Biogenesis | 1:1000 |
| **Amylase** | Rabbit | SIGMA-ALDRICH | 1:1000 |
| **P48** | Rabbit | F.Real | 1:500 |

**Table M.19:** Antibodies used in western blotting assays. Anti-lamin B1 (E398L, abcam), anti-tubulin (SIGMA-ALDRICH), a-Bmi1 (05-637, Millipore), anti-Ring1b (D139-3, MBL), anti-Cpa1 (Biogenesis) and anti-amylase (SIGMA-ALDRICH).

The Ptf1/p48 rabbit antiserum was raised against a synthetic amino acid peptide (C-KSFDNIENEPPFEFVS) [312]. Primary antibodies were diluted in blocking buffer + 2.5% BSA

After washing excess of primary antibodies, membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit, K4003; anti-mouse, K4001, DakoCytomation) for 1 hour at RT.

Membranes were extensively washed and Pierce ECL Western Blotting Substrate (32106, Thermo-Scientific) was used to detect HRP signal.

## 10. Isolation of exocrine fraction of mouse pancreas

7-week old male CD1© mice (Crl:CD1, Charles River Laboratories), weighting between 33 and 37 g, were used to isolate the exocrine fraction of pancreas. The procedure was approved by the ethical committee of the institution and animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals.

To perform the isolation, a modified version of a previously described protocol was used [284]:

- Pancreas was retrieved from the mouse and to start disaggregation of the tissue, 2.5 ml of cool collagenase P (11 213 865 001, Roche Applied Science) at 1.33 mg/ml in Hank's Balanced Salt Solution (HBSS, 14065-04, invitrogen) were injected to the pancreatic tissue in different points. Tissue was

then immersed in 5 ml of chilled collagenase solution, cut and kept on ice to avoid any protease or RNAase activity.

- Tissue with collagenase was incubated in a shaking water bath at 37°C for 20 minutes and afterwards collagenase activity was stopped on ice and adding 5 ml of chilled HBSS + 5% FBS solution and left to sediment for 10 minutes.
- Supernatant was discarded and pellet was resuspended in 10 ml of HBSS + 5% FBS solution on ice and centrifuged at 1000 rpm for 90 seconds. This step was repeated twice.
- Supernatant was discarded and pellet was thoroughly resuspended with 5 ml of chilled HBSS + 5% FBS solution.
- Solution was filtered through a 500-µm polypropylene SpectraMesh© mesh (145613, SpectrumLabs) and filter was washed with 5 ml of HBSS + 5% FBS.
- 10 ml of filtered solution were filtered again through a 100-µm nylon SpectraMesh© mesh (145799, SpectrumLabs).
- Filtered solution was added to 20 ml of prewarmed HBSS + 30% FBS drop wise. Solution was centrifuged at 1000 rpm for 90 seconds and pellet of cells was resuspended in 10 ml of RPMI medium (12633-012, invitrogen) + 10% FBS + soy trypsin inhibitor + G418 in a non-treated petri dish to culture exocrine primary cultures in suspension.
- Cells were cultured for 5 days, changing media every 48 hours. Cells were harvested and performed RNA extracts.

## 11. Caerulein-induced pancreatitis

C57BL/6J male mice (Charles River Laboratories), weighting between 22 and 25 g, were used for the caerulein-induced pancreatitis experiments. To induce acute pancreatitis, the following protocol, previously reported [281], was used:

- Intraperitoneal injection of caerulein (C9026, SIGMA-ALDRICH) at a concentration of 50µg/kg was administered to the animal.
- Every hour, one injection was again performed until a total of seven.
- Pancreatic tissues were obtained at different time points and processed to obtain sections for immunostaining.

For chronic caerulein treatment, a modified version of a previously described protocol [296] was used:

- Single intraperitoneal injections of caerulein (0.1 ml of a 50 µg/ml) in saline solution were administered to one- month old mice, 5 days per week.
- Injections were repeated for four weeks.
- Animals were sacrificed 12 months later.

## 12. Duct ligation of the rat pancreas

Adult wistar rats were used in a previously reported protocol [284]:

- A silk thread was used to ligate the exocrine ducts draining the splenic part of the rat pancreas.
- After seven days of the ligation, pancreatic tissue was collected and processed to perform immunostaining analysis.

## 13. PDAC and chronic pancreatic injury-associated PDAC experimental models

The following mouse strains were used as previously described [296]:

- K-Ras$^{+/+}$; Elas-tTA; TetO-Cre.
- K-Ras$^{+/LSLG12Vgeo}$; Elas-tTA; TetO-Cre.

The following protocol was used:

- Doxycycline was depleted in the intake of mice, thus activating K-Ras$^{G12V}$ oncogene in cells expressing the Elastase gene, starting at E16.5.
- Pancretic tissue was obtained and processed for immunostaining analysis.

# 1. Expression of Polycomb group proteins in mouse pancreas

At the beginning of this thesis project, expression of Polycomb group proteins was barely characterised in the human pancreas and no articles describing PcG expression in the mouse pancreas had been published. Therefore, our first goal was to analyse the expression patterns of PRC1 and PRC2 members by using pancreatic tissue samples of wild-type mouse pancreas.

## 1.1. Analysing the expression of PcG members in normal mouse pancreas

Immunohistochemical analysis was performed on tissue sections of adult mouse pancreas using primary antibodies for PRC1 members Bmi1 and Ring1b, and PRC2 member EED to analyse their expression.

Expression of EED was localised in the cytoplasm of acinar cells, while no detectable expression could be found in the nuclei of ducts and endocrine cells **(Figure R.1A)**. Despite the unexpected cytoplasmic staining for a chromatin-interacting protein, EED has been recently reported to be recruited by the HIV-1 Nef protein to the plasma membrane [313]. Moreover, another member of the PRC2 complex, EZH2, has been found to be collaborating in actin polymerization and proliferation in the cytosol [314]. Nevertheless, the nature of EED cytoplasmic location in acinar cells was not further analysed.

Regarding the PRC1 complex, Bmi1 exhibited strong expression in the nuclei of endocrine cells **(Figure R.1A)**. In addition, expression of Bmi1 was found in ductal cells and in some scattered nuclei in the exocrine part of the pancreas.

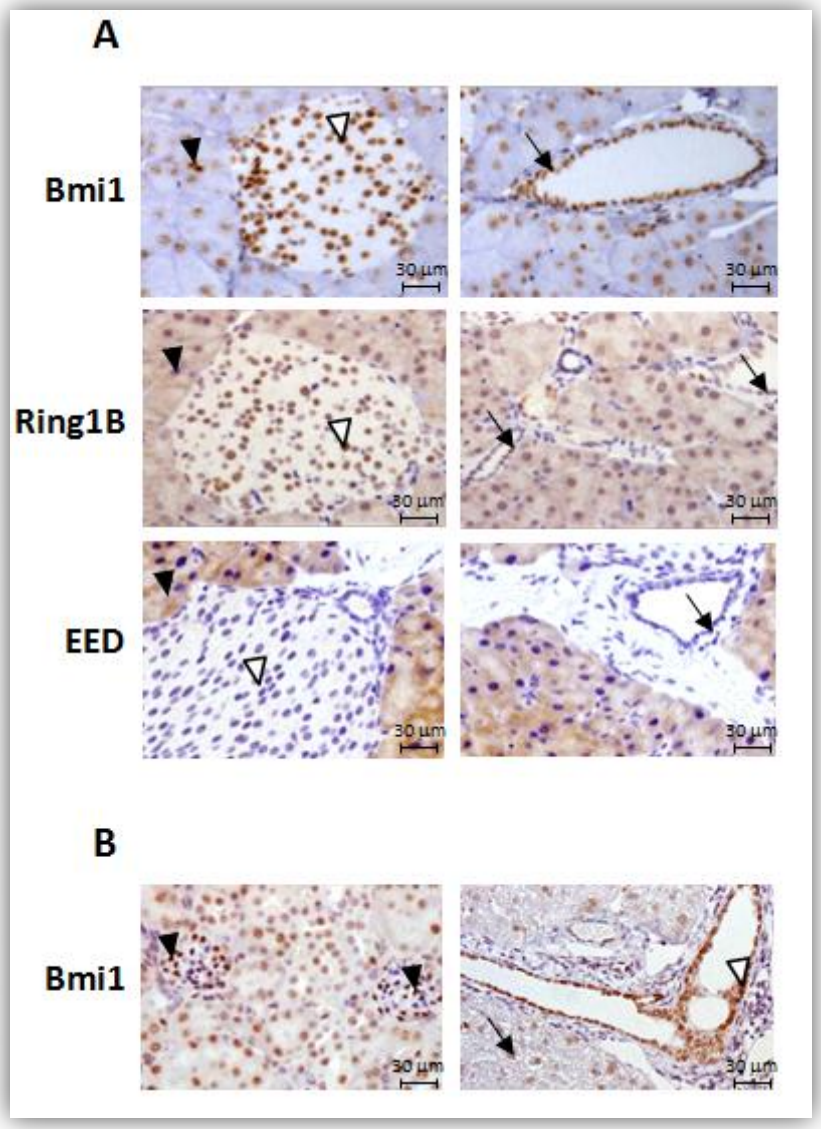



**Figure R.1: Immunohistochemical analysis of Bmi1, Ring1B and EED expression in normal mouse pancreas.** (A) Sections from adult mouse pancreas were labelled with Bmi1, Ring1B or EED antibodies. Specific nuclear signal of Bmi1 and Ring1B is found in the islet compartment (white arrowhead), in the ducts (arrow) and in some scattered cells within the exocrine fraction (arrowhead). EED expression is absent in endocrine cells (white arrowhead) and also in the ducts (arrow), while cytoplasmic signal is detected in the acini (arrowhead). (B) Sections of kidney (left panel) and liver (right panel) from normal mouse pancreas were used as positive controls. Nuclear specific signal of Bmi1 is found in all compartments of the kidney, especially in the glomerules (arrowheads). In liver sections, Bmi1 is highly expressed in the nuclei of hepatic duct cells (white arrowhead) and in some scattered hepatocytes (arrow).

The expression pattern of Ring1B was very similar to the one of Bmi1: it was found in nuclei of endocrine and ductal cells, and also in some scattered exocrine nuclei **(Figure R.1A)**. In addition, Ring1B showed signal in the cytoplasm of the acini which was identified as unspecific because samples incubated with a different monoclonal antibody against Ring1B [315] did not display any signal in the acinar cytoplasms.

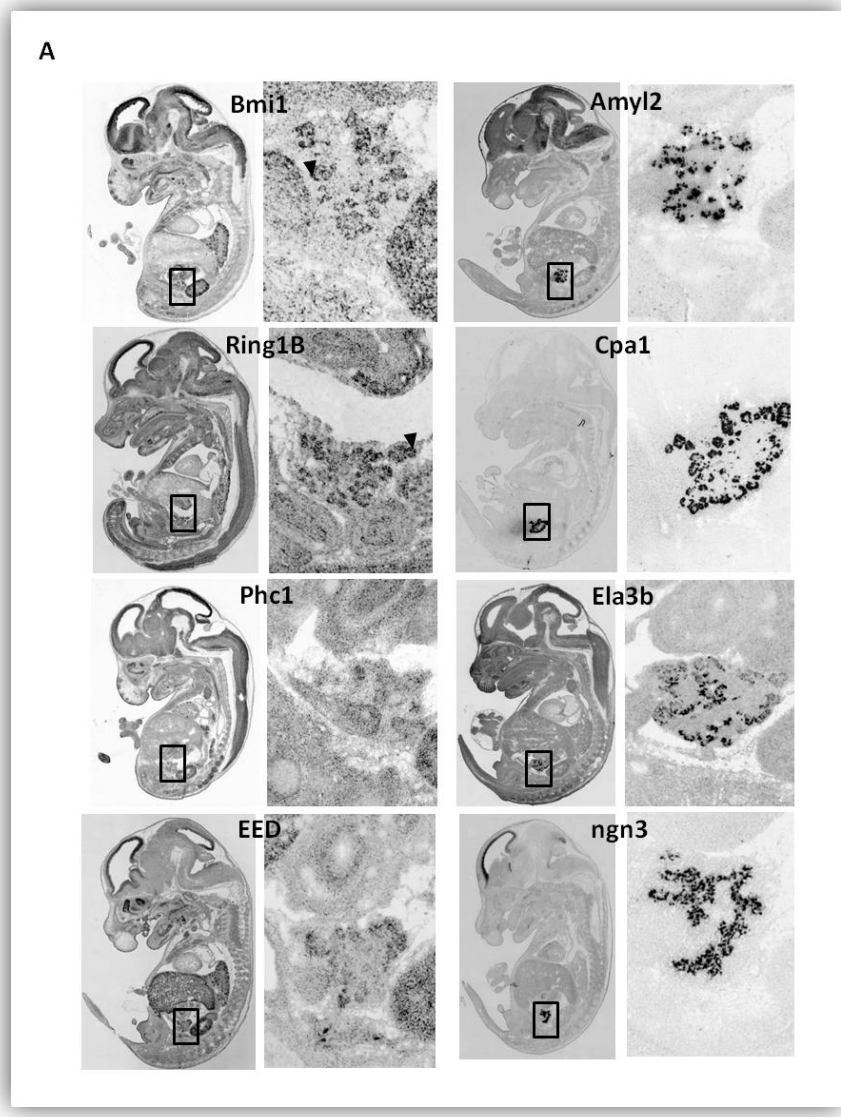
Kidney and liver sections from adult mouse were initially used as positive control for PRC1 expression (**Figure R.1B)**, as both tissues have been reported to express Bmi1 and also Ring1B [136]. All kidney cell types expressed Bmi1 in their nuclei, especially the glomerules. Liver sections showed nuclear staining of Bmi1 in the duct cells and in some scattered hepatocytes.

Bmi1 and Ring1B shared similar patterns of expression, which would be in agreement with reported studies that unveiled Ring1B to be interacting with Bmi1, thus having a synergistic effect on the Ring1B E3 ligase activity [316].

## 1.2. Analysing the expression of PcG members in the mouse embryo

After establishing the expression of PRC1 and PRC2 members in the pancreas of adult mice, we sought to determine their expression during embryonic development. We analysed the expression of different PcG members (Bmi1, Ring1B, Phc1 and EED) in E14.5 mouse embryos **(Figure R.2A)** by accessing to the online database Genepaint, which includes gene expression patters in mice determined by *in situ* hybridisation [317]. Expression of Bmi1 and Ring1B was localised at the distal tips of the pancreatic progenitor branch. Different pancreatic acinar markers

were checked, such as Elastase 3B (Ela3b), Amylase 2 (Amy2) and Carboxypeptidase A1 (Cpa1), and all of them were also expressed in the same region than Bmi1 and Ring1B **(Figure R.2A)**. In contrast, the trunk of the pancreatic branches was largely occupied by Neurogenin 3-positive endocrine cells. Moreover, Phc1 and EED were almost absent in the whole developing pancreas. On the other hand, Neurogenin 3 (Ngn3), which is an endocrine precursor marker, was expressed in the trunk of the branching epithelium.



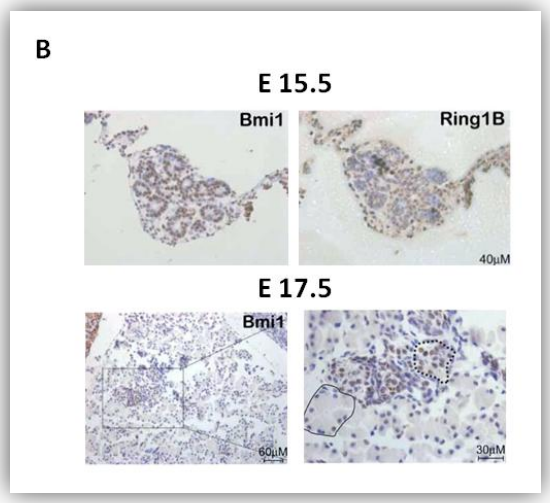



**Figure R.2: Expression of PcG in the mouse embryonic pancreas.** (A) Genepaint *in situ* hybridisation analysis of PcG components Bmi1, Ring1B, Phc1 and EED (left panels), exocrine markers Amylase 2, Elastase 3b and Carboxypeptidase A1, and endocrine transcription factor Neurogenin 3 (right panel). Insets on the right correspond to magnified views of expression in the sections. Bmi1 and Ring1B probes stain the distal tips (arrowheads), which are also labelled by exocrine markers Amylase 2 and Carboxypeptidase A1. In contrast, the trunk of the pancreatic branches is largely occupied by Neurogenin 3-positive endocrine cells. Moreover, Phc1 and EED are barely expressed. Images obtained from the public database http://www.genepaint.org. (B) Immunohistochemical analysis of Bmi1 and Ring1B in E15.5 (upper panels) and E17.5 (lower panels) mouse embryos. Bmi1 signal is detected in the nuclei of acinar cells at E 15.5, while Ring1B is barely expressed. E17.5 sections, expression of Bmi1 is absent in differentiated acinar cells but it is found in the nuclei of islet cells.

Due to the documented role of different PcG proteins in development, we sought to analyse Bmi1 and Ring1B expression during mouse pancreatic formation by immunohistochemistry **(Figure R.2B)**. Pancreatic sections of E15.5 mouse embryos showed Bmi1 signal in the nuclei of acinar cells. However, protein expression of Ring1B was undetectable during this stage. In pancreatic sections of E17.5 mouse embryos, when

there was an increase in endocrine cells which reorganise to form mature islets, Bmi1 expression was restricted to the nuclei of islet cells and in a few scattered exocrine cells.

Taking into account all these findings, we conclude that Bmi1 and Ring1B expression undergoes a modulation in exocrine and endocrine cells along pancreatic development and differentiation.

## 2. Expression of Polycomb group proteins in pancreatic exocrine cancer models

Characterising the expression of Bmi1 and Ring1B in the pancreas of wild-type mice showed specific signal of both proteins in the nuclei of islet cells and duct cells, while a subset of acinar cells displayed barely noticeable signal. In order to study their expression in the context of pancreatic ductal adenocarcinoma (PDAC), we used tissue samples from different mouse models of pancreatic cancer to accomplish this purpose.

### 2.1. MT-TGFα and Ela-myc

For our first approach in studying PcG expression in PDAC models, we used pancreatic sections of the available models at that time. We got access to samples from a transgenic mouse strain which bears constitutive activation of Transforming Growth Factor alpha by means of using the metallothionin-1 promoter gene (MT-TGFα). As TGFα expression is not restricted to the pancreatic tissue, this strain presents alterations in several tissues, like liver neoplasia, mammary epithelial hyperplasia and pancreatic metaplasia [286-287]. For this reason, this model has been widely used not only in the study of pancreatic cancer

[318], but also in the carcinogenesis of liver [319] and mammary gland [320-321].

The MT-TGFα mouse displays high proliferation of fibroblasts and acinar cells in the pancreas, with focalised areas of acinoductal metaplasia [318]. Immunohistochemical analysis of the expression of PRC1 and PRC2 members using pancreatic sections did not come into any conclusion, as no specific signal of Bmi1 could be found in any cell type and expression of EED was found in cytosol of duct cells **(Figure R.3A)**.

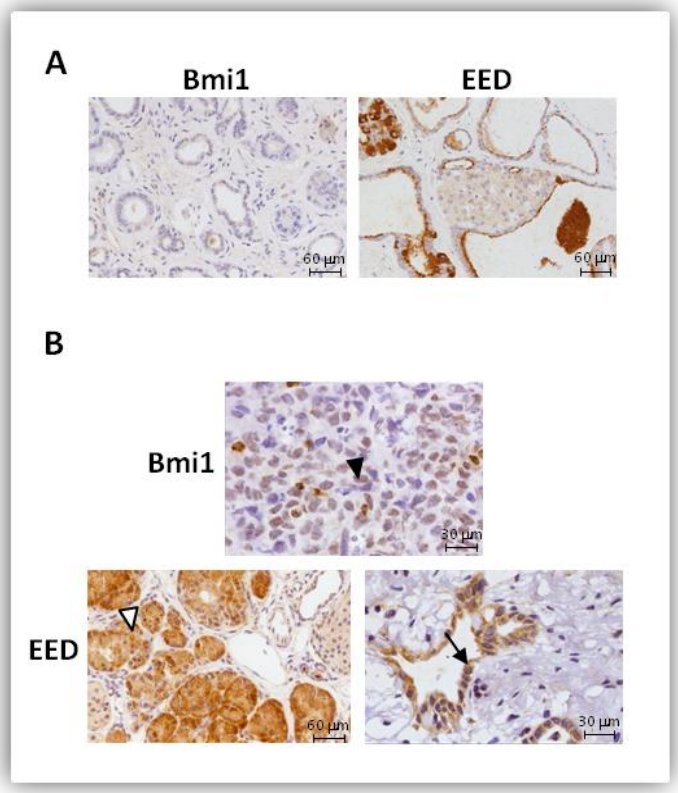



**Figure R.3: Immunohistochemical analysis of PcG members in MT-TGFα (A) and Ela-myc (B) PDAC mouse models.** (A) Expression of Bmi1 is absent in every cell type in pancreatic sections of MT-TGFα mice (left panel) while EED is expressed in ductal cells (right panel). (B) Regions of acinar cells display increased nuclear signal of Bmi1 (arrowhead). EED signal stains the cytoplasm of acinar cells (white arrowhead) and some neoplastic ducts (arrow).

In parallel, we got access to pancreatic samples of transgenic mice with constituve expression of oncogene cMyc driven under the Elastase promoter (Ela-myc), allowing specific expression in acinar cells [294]. Mice develop pancreatic cancer with 100% penetrance at an early age. 50% of tumours are acinar cell carcinomas; the remaining half are ductal adenocarcinomas or mixed ductal and acinar tumours [294].

Pancreas sections from different Ela-myc transgenic mice were used to study the expression of Bmi1. Several regions of acinar cells which displayed uncontrolled growth showed higher expression of Bmi1 than normal acini **(Figure R.3B)**. On the other hand, expression of EED could be found in ductal cells, but its specificity could not be assured.

## 2.2. $KRas^{G12V}$ mouse model

We had the opportunity to obtain pancreatic samples from a novel mouse model for PDAC: a conditional knock-in mouse strain with an inducible expression of endogenous $KRas^{G12V}$ activated by a Cre recombinase system under the Elastase promoter, thus specifically targeting $KRas^{G12V}$ expression to acinar and centroacinar cells [296]. $KRas^{G12V}$ mice display all the spectrum of pancreatic intraepithelial neoplasias (PanINs) and regions of acinoductal metaplasia, virtually recapitulating all the events of human PDAC.

Pancreatic sections of 12 month-old mice with induced $KRas^{G12V}$ at E16.5 were used to analyse the expression of the PRC1 members Bmi1 and Ring1B (samples kindly given by Carmen Guerra). Multiple desmoplastic PanIN lesions of different stages could be found in 80% of the analysed animals. Moreover, some mice also displayed at least one PDAC lesion [296].

An upregulation of Bmi1 expression was found in preneoplastic lesions, as Bmi1 was faintly expressed in acinar cells but showed moderate expression in PanINs and PDAC structures **(Figure R.4A)**. On the other hand, Ring1B was weakly expressed in PanINs but its expression was strengthened in PDAC lesions. This finding revealed a differential pattern in the kinetics of the expression of Bmi1 and Ring1B, which led us to the hypothesis of Bmi1 being involved in preneoplastic lesions, prior the formation of PDAC.

Therefore, we sought to analyse the expression of Bmi1 in the context of chronic pancreatitis, which has been reported to be a risk factor for pancreatic cancer [213, 220, 296, 322]. Pancreatic samples were extracted from mice with induced $KRas^{G12V}$ and with one-month caerulein treatment.

After a month of caerulein treatment, 5 days per week, administration was stopped. Then, when mice were 13 months old, pancreatic samples were retrieved, which displayed hyperplastic exocrine regions, metaplastic ducts, PanINs and PDAC formation. Expression of Bmi1 was found in hyperplastic acinar regions. Moreover, expression of Bmi1 was enhanced in late PanIN stages and PDAC lesions **(Figure R.4B)**. To test the nature of the Bmi1-positive cells, double staining analysis was performed with Bmi1 and the pancreatic acinar marker Amylase A2 (Amy2). Their signal exhibited an inverse correlation: normal acinar cells displayed their characteristic amylase signal while expression of Bmi1 was very weak **(Figure R.4C)**. On the other hand, hyperplastic acinar cells showed residual expression of amylase while expressing high levels of Bmi1.

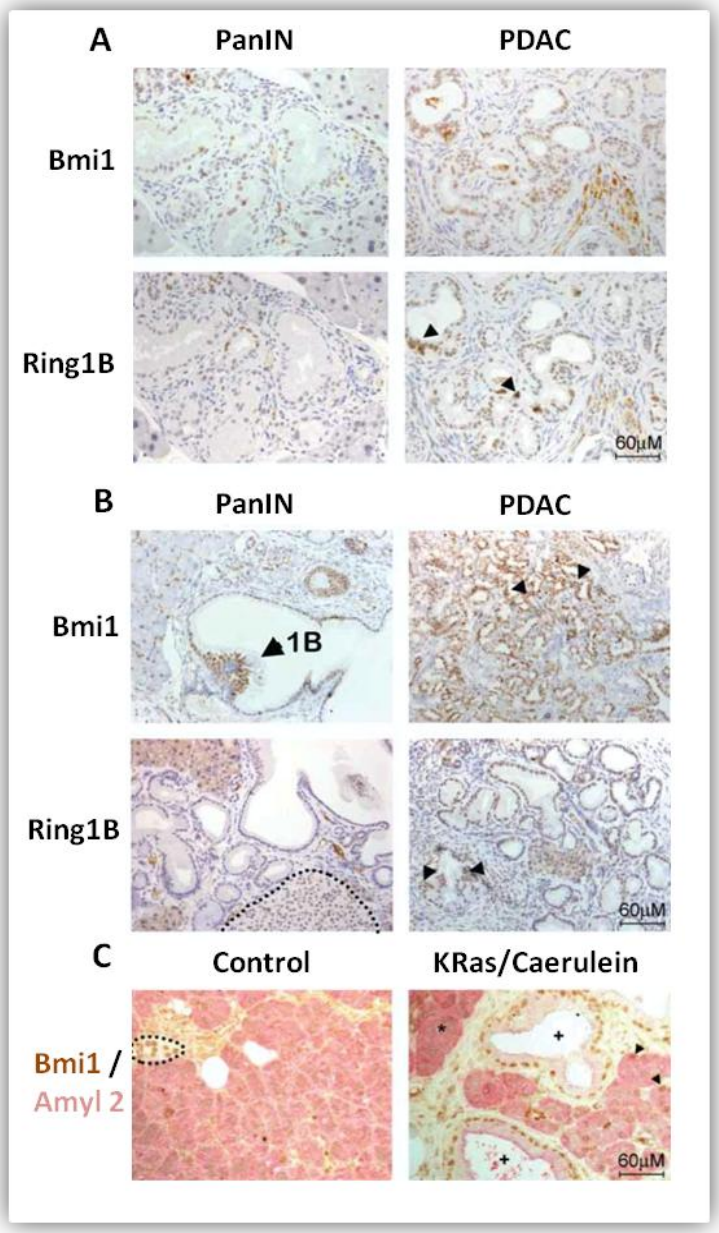



**Figure R.4: Immunohistochemical analysis of Bmi1 and Ring1B expression in murine experimental models for PDAC (A) and in chronic pancreatic injury-associated PDAC (B)**. (A) Bmi1 expression is increased in PanIN lesions (left panels) and in PDAC lesions (right panels) of K-Ras+/LSLG12Vgeo/Elas-tTA/TetO-Cre mice. On the other hand, Ring1B expression is induced later in PDAC lesions rather than in early preneoplastic lesions. (B) Pancreatic sections of K-Ras+/LSLG12Vgeo/ElastTA/TetO-Cre mice chronically treated with

caerulein show induction of Bmi1 expression in early PanIN lesions (left panel) which is maintained in PDAC lesions (right panel). Expression of Ring1B is again induced in PDAC lesions but not in low-grade PanINs.(C) Double staining analysis of Bmi1 (brown) and Amylase 2 (red) in normal pancreas and in the chronic pancreatic injury-associated PDAC model.

On the other hand, expression of Ring1B remained weak in early stages of preneoplastic lesions but it noticeably increased in late stages (PanIN 2 and 3) and in PDAC lesions.

In conclusion, experiments with the $KRas^{G12V}$ mouse model showed an increase of Bmi1 expression in the nucleus of acinar cells in early PanIN lesions. This increase of Bmi1 expression was sustained throughout all PanIN stages and was also found in PDAC lesions. In contrast, Ring1B expression was increased well after Bmi1, in late PanIN lesions and it was also found in PDAC.

We hypothesised about Bmi1 being involved in the formation of preneoplastic lesions and their progression to malignant PDAC. We focused our study on better understanding the pattern of expression of Bmi1 in this context.

## 2.3. Caerulein-induced pancreatitis models

After finding a specific increase of Bmi1 expression in preneoplastic lesions, our research focused on analysing the expression of Bmi1 in pancreatitis and preneoplastic lesions preceding PDAC. Acute and chronic pancreatitis mouse models were used to analyse the expression pattern of PRC1 members to understand the pancreatic landscape for tumour initiation.

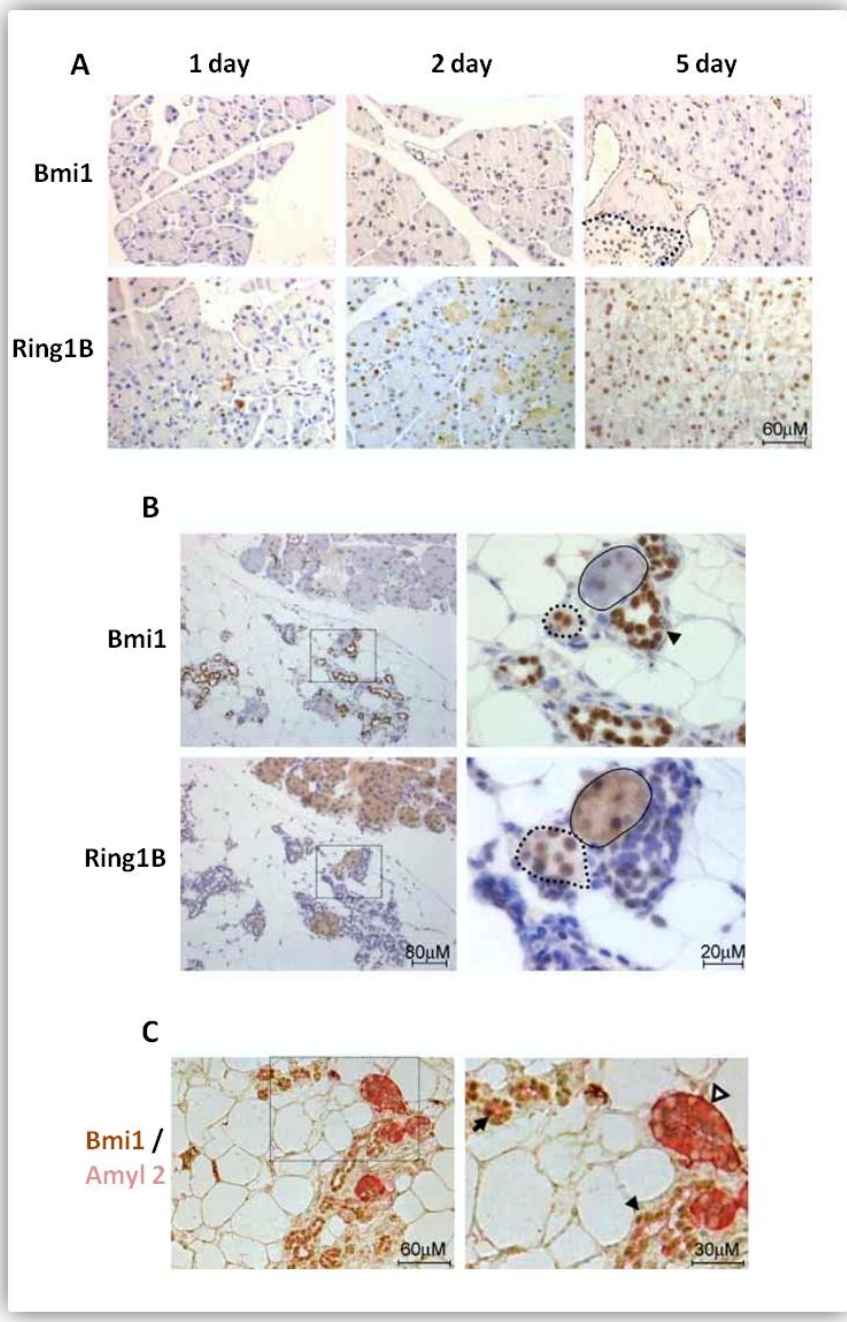



**Figure R.5: Analysis of Bmi1 and Ring1B expression in acutely or chronically caerulein-treated mouse.** (A) Immunohistochemical analysis of Bmi1 and Ring1B expression in pancreatic sections from mice with caerulein-induced acute pancreatitis. Pancreatic sections were obtained 1, 2, or 10 days after caerulein

administration. Both Bmi1 and Ring1B signals are induced at day 2 and this lasts for more than 10 days after treatment. (B) Immunohistochemical analysis of Bmi1 and Ring1B expression in pancreas from mice with caerulein-induced chronic pancreatitis. Insets on the right correspond to magnified views of expression in the sections. Bmi1 expression is increased in metaplastic acini while Ring1B is largely absent in them. (C) Double staining of Bmi1 (brown) and Amylase 2 (red) at two different magnifications of the same field. Normal acini display high levels of Amylase 2 while Bmi1 is barely detected (white arrowhead). Metaplastic ducts show strong Bmi1 expression while they have lost signal of Amylase 2 (arrowhead). Atrophic acinar cells partially retain Amylase 2 expression while showing strong signal of Bmi1 (arrow).

Regarding acute pancreatitis, a mouse model was used in which an acute pancreatic lesion was achieved by means of caerulein administration (samples kindly given by Xavier Molero) [281]. Mouse displayed pancreatic lesions soon after caerulein injection and both tissue histology and function were completely restored in about a week after. One day after caerulein treatment, subtle variations in the expression of Bmi1 and Ring1B were found **(Figure R.5A)**. However, signal of both proteins considerably increased in half of the acinar cell nuclei at day 2. Moreover, ten days after caerulein administration the majority of acinar cells still showed expression of Bmi1 and Ring1B in the nucleus.

About chronic pancreatitis, a mouse model was used in which daily caerulein administration induced the pancreatic lesion (kindly given by Carmen Guerra) [296]. One intraperitoneal low-dose injection of caerulein was administered each day to induce chronic pancreatic injury, five days a week. Treatment had one month of duration and the outcome was a mild injury to the pancreatic tissue, with panlobular lesions displaying atrophia in the acinar fraction, limited regions of inflammation and acinar-to-ductal transdifferentiation. In this chronic pancreatitis mouse model, Bmi1 was detected in the nuclei of atrophic acinar and was highly expressed in regions of acinoductal transdifferentiation

**(Figure R.5B)**. On the contrary, Ring1B could not be detected or was barely expressed in the nuclei of metaplastic acinar cell.

Double staining of Amy2 and Bmi1 fitted with previous experiments using the $KRas^{G12V}$ mouse model: normal acinar cells expressed strong levels of Amy2 while Bmi1 was weakly or barely detected, atrophic acini displayed high Bmi1 signal and residual Amy2 expression, and metaplastic duct showed no expression of Amy2 but strong signal of Bmi1 **(Figure R.5C)**.

Taking into account these findings, induction of Bmi1 in acinar cells is a response to pancreatitis and it is sustained after ending caerulein treatment.

# 3. Expression of Polycomb group proteins in acinar metaplasia models

## 3.1. Duct ligation in the rat pancreas

In order to further understand whether the expression of Bmi1 was a general feature upon pancreatic injury, a well established model of duct-ligated pancreatic injury in rats was used (kindly given by Ilse Rooman). Animals displayed generalised metaplastic acini in the ligated region, which would convert into duct-like structures [282], losing their acinar differentiated characteristics (decrease of transcription factor p48 and Mist1 expression) and expressing genes related to cell growth and development such as Pdx1 and Notch receptors [284].

In this model, Bmi1 was strongly induced in the nuclei of metaplastic acinar cells affected by duct ligation **(Figure R.6)**, while wild-type regions displayed weak expression of Bmi1.

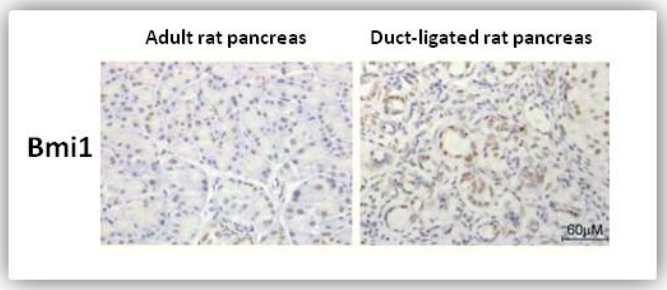



**Figure R.6: Bmi1 expression in acinoductal metaplasia in duct-ligated rat pancreas.** Immunohitochemical analysis displayed increase in Bmi1 expression in the nuclei of metaplastic acinar cells (right panel), in comparison to normal acinar regions (left panel).

## 3.2. Acinar-to-hepatocyte transdifferentiation in a pancreatic exocrine cell line

The first *in vitro* model of acinar metaplasia that we decided to use to study the effect of PcG modulation was treating the commercial pancreatic acinar cell line AR42J with glucocorticoid dexamethasone, as previously reported [299].

AR42J cells were originally derived from a rat pancreatic tumour and they bear a partially acinar differentiation although some neuroendocrine properties can also be found. The treatment of this cell line with dexamethasone triggered an acinar-to-hepatocyte transdifferentiation which resulted in a rapid change of the morphology in a subpopulation of cells which flattened onto the substratum **(Figure R.7)**. Downregulation of different specific acinar markers, such as amylase, was followed by a progressive induction of liver-specific markers glucose-6-phosphatase

and albumin since day 3 of treatment which culminated after two weeks [299].

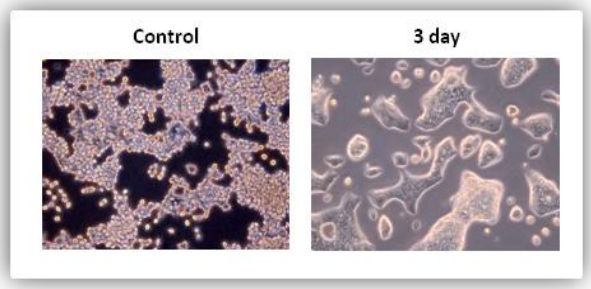



**Figure R.7: Dexamethasone treatment in AR42J cell line induces and acinar-to-hepatocyte transdifferentiation.** Treatment with dexamethasone in AR42J cell line triggers drastic changes in the phenotype in a few days of culture.

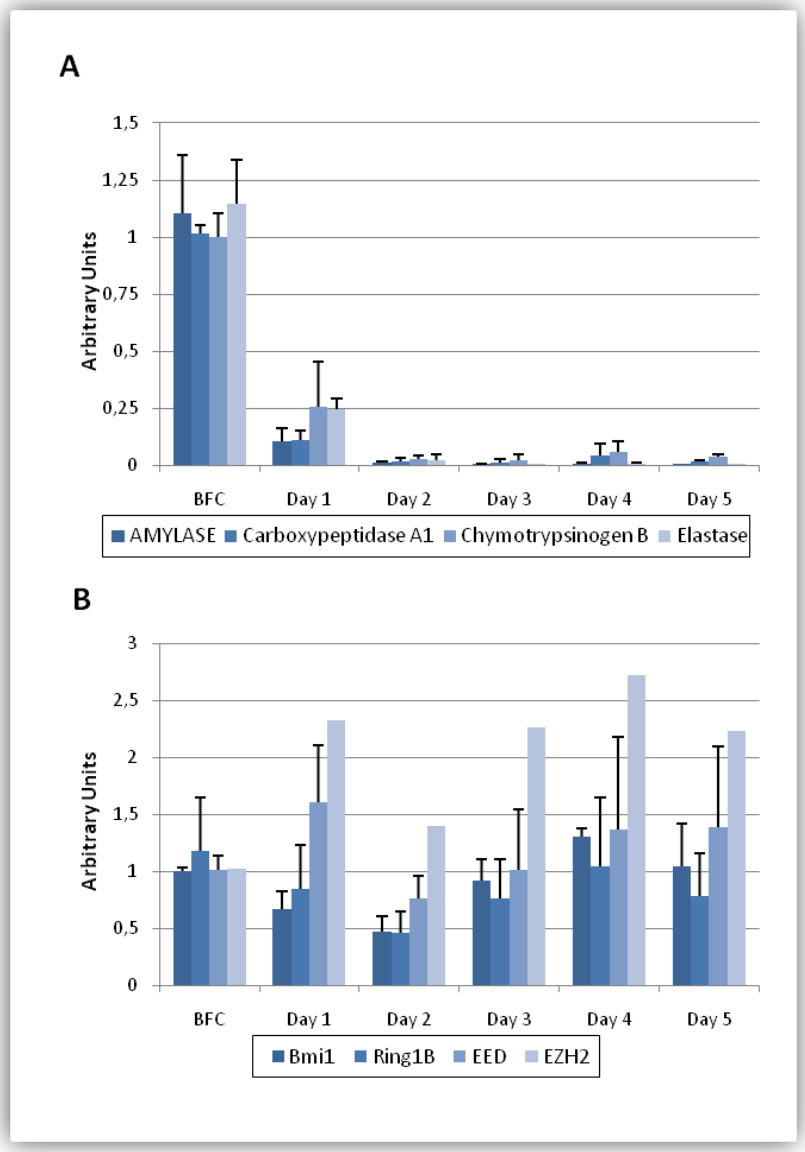
Nevertheless, we could not obtain conclusive results from this model because treatment with dexamethasone considerably inhibited cell growth, thus it was very difficult to obtain enough metaplastic cells to perform proper extracts. Moreover, while cells suffered an acinar metaplasia, a transdifferentiation to liver did not resemble the events in PDAC. Therefore, we focused on modulating the expression of PcG proteins in different *in vitro* models to obtain a better approach.

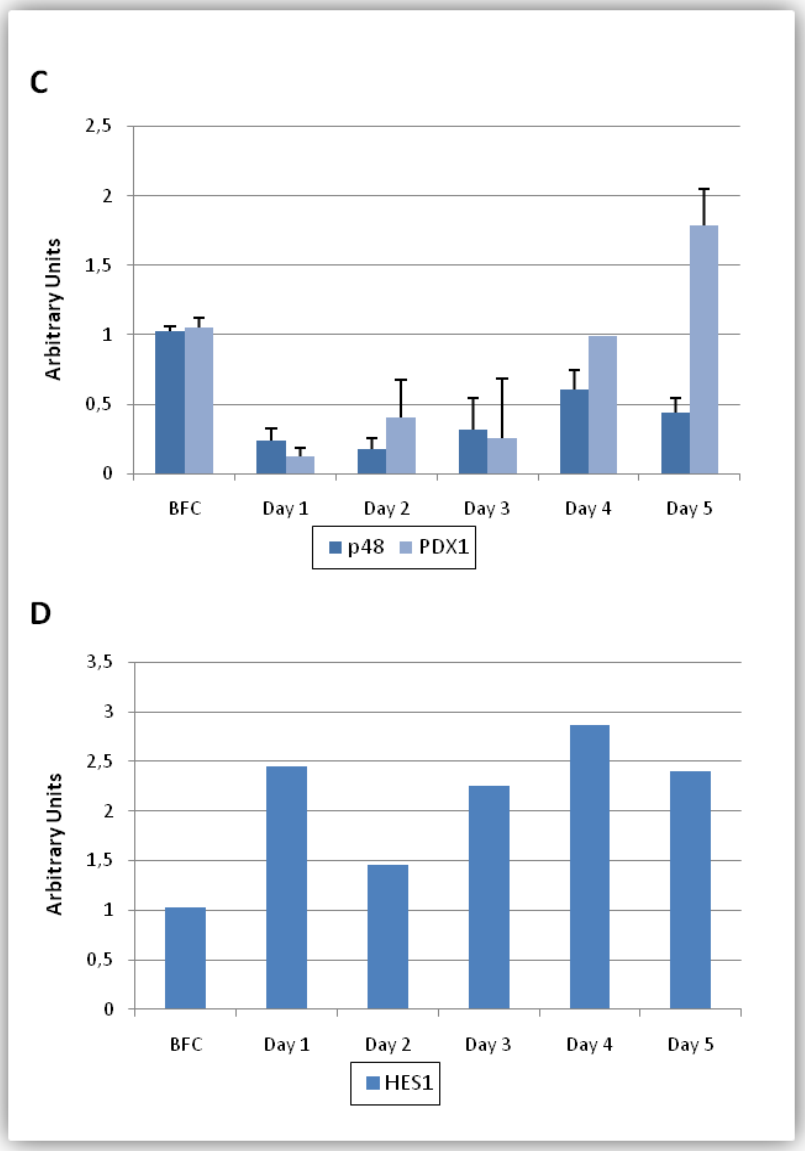
## 3.3. Primary culture of the exocrine fraction of mouse pancreas

We also used an *in vitro* model of exocrine cell metaplasia to study alterations in PcG expression during acinoductal transdifferentiation. This model is based on culturing the isolated exocrine fraction of the pancreas in suspension. During five days of culture, acinar cells spontaneously convert into duct-like cells, mimicking the duct ligation model to some extent [284].

As previously reported [284], RNA expression analysis of pancreatic acinar genes confirmed loss of the acinar differentiation program since

the first day of culture. Expression of amylase, carboxypeptiase A1, chymotrypsinogen B and elastase dramatically decreased since day 1 **(Figure R.8A)**. Two transcription factors involved in acinar differentiation, Pdx1 and p48, also decreased since day 1 **(Figure R.8C)**. Moreover, expression of Hes1, which is a protein downstream the Notch pathway, was induced **(Figure R.8D)**.




C
2,5
2
1,5
1
0,5
0
Arbitrary Units
BFC
Day 1
Day 2
Day 3
Day 4
Day 5
p48
PDX1
D
3,5
3
2,5
2
1,5
1
0,5
0
Arbitrary Units
BFC
Day 1
Day 2
Day 3
Day 4
Day 5
HES1

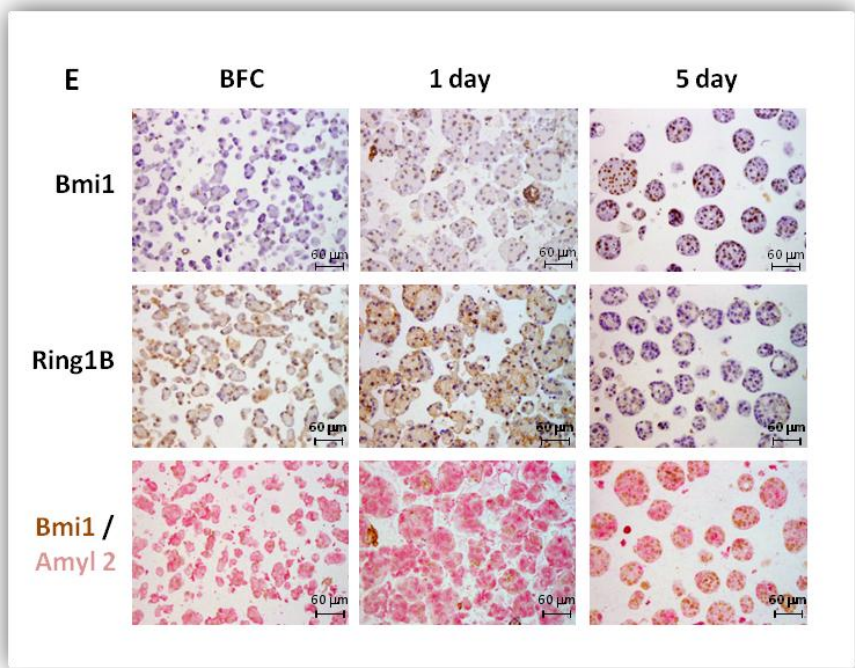



**Figure R.8: Expression analysis of PcG members in primary culture of the exocrine fraction of mouse pancreas.** (A) Analysis of pancreatic exocrine markers Amylase, Carboxypeptidase A1, Chymotrypsinogen B and Elastase show a pronounced decrease in all markers since day 1 of culture. (B) Expression of Bmi1, Ring1B and EED are slightly decreased while EZH2 expression is increased since day 1. (C) Transcription factors p48 and PDX1 decrease since day 1. (D) Hes1 is induced since day 1. (E) Immunohistochemical analysis in isolated acini shows increase in Bmi1 expression since day 1 of culture. On the other hand, Ring1B expression is not induced and Amylase2 signal is barely changed. BFC = Before culturing.

Regarding the analysis of the mRNA levels of PRC1 and PRC2 members, a faint impingement on Bmi1 and Ring1B levels could be detected since day 1, with a possible increase at day 3 **(Figure R.8B)**. On the other hand, PRC2 members EED and EZH2 were found to be slightly increased since day 1 **(Figure R.8B)**.

Nevertheless, statistic analysis of these findings could not be fulfilled during the 5 days of culture due to constraints of the model, since isolated exocrine cells cease to grow since day 1 of culture. Therefore, at day 4 the number of living cells was not enough to obtain conclusive results.

On the other hand, immunohistochemical analysis of Bmi1 expression in cultured acini showed a significant increase (samples kindly provided by Ilse Rooman). Before culturing, freshly isolated acini only showed 9% of Bmi1-positive cells. At day 1 of culture in suspension, 65% of metaplastic exocrine cells expressed Bmi1. At day 5, expression of Bmi1 could be found in virtually all cells in culture **(Figure R.8E)**. Moreover, there was also an increase in Ring1B expression. However, Amylase2 staining did not change over the 5 days of culture.

In conclusion, induction of Bmi1 may be related to the acinar-to-ductal transdifferentiation that undergo isolated acinar cells.

## 4. Expression of Polycomb group proteins in human PDAC

In order to correlate our findings from the different animal models with human PDAC lesions, Bmi1 and Ring1B expression was analysed in pancreatic tissue samples from different patients (n=35) who displayed a broad spectrum of prenoplastic and neoplastic lesions.

Consistent with the results obtained using pancreatitis and PDAC experimental models, a statistically significant upregulation of Bmi1 in regions of chronic pancreatitis versus histologically normal exocrine areas were observed ($1.5 \pm 0.4$ versus $0.7 \pm 0.4$; $p < 0.01$) **(Figure R.9A and R.9B)**. Moreover, aberrant ducts and tumour lesions showed higher increase of Bmi1 expression, regardless the level of dysplasia.

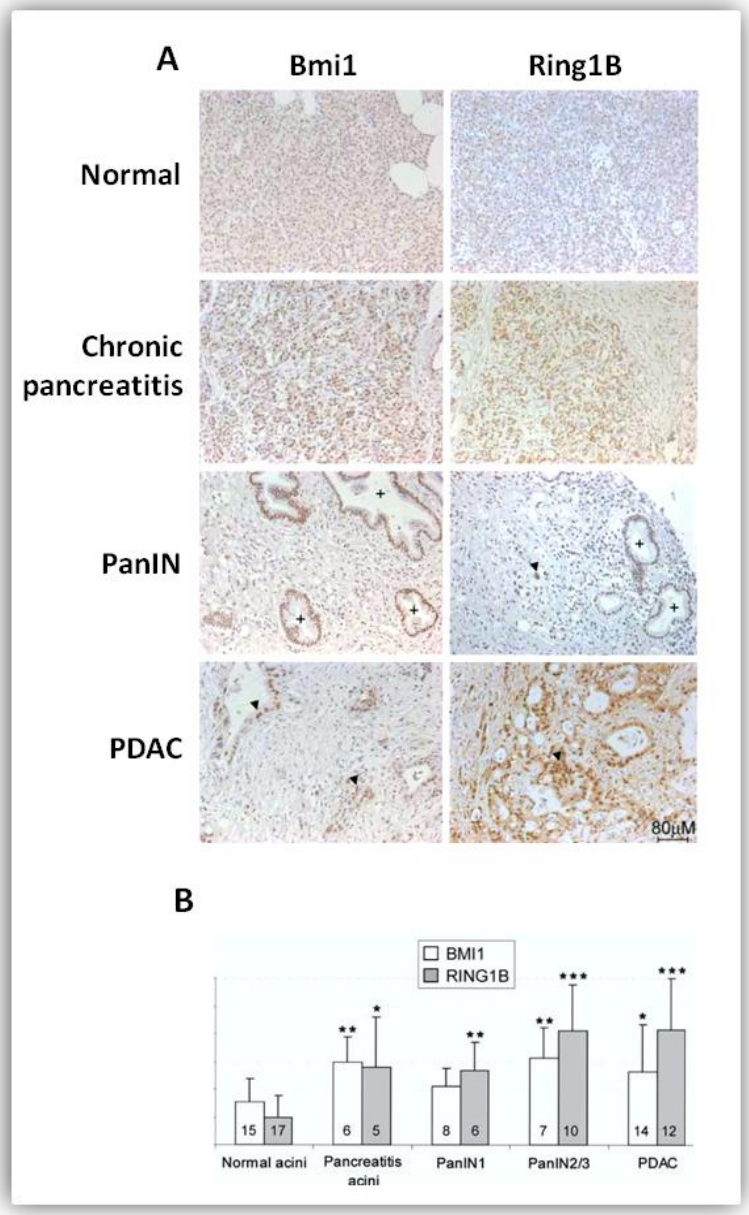



**Figure R.9. Immunohistochemical analysis of Bmi1 and Ring1B expression in human pancreas.** (A) Analysis of Bmi1 (left panels) and Ring1B (right panels) expression in normal, chronic pancreatitis, low-grade PanIN lesions and PDAC in human pancreatic sections. Bmi1 nuclear signal is increased in chronic pancreatitis, metaplastic ducts of PanIn lesions (+) and in tumour cells in PDAC (arrowhead). In contrast, Ring1B expression is later induced in high-grade PanINs and in PDAC lesions. (B) Quantification of Bmi1 and Ring1B expression analysis by immunohistochemistry in human pancreatic tissues. Numbers inside bars

indicate the amount of samples for each condition. The Mann-Whitney U-test was used to test the statistical significance of the results. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

Regarding Ring1B, its expression was slightly increased in pancreatitis lesions and in early stages of preneoplasia. However, late stages of PanINs and PDAC lesions displayed a higher induction of Ring1B (2.0 ± 0.8 in PanIN2/3 lesions and 2.1 ± 0.9 in PDAC lesions versus 0.5 ± 0.4 in normal pancreas; $p < 0.001$ for both situations) **(Figure R.9A and R.9B).**

After analysing the recollected data from human PDAC samples, a consistency was found between analysing different PDAC mouse models and analysing samples from human PDAC patients.

## 5. Bmi1 modulates the acinar differentiation program

After determining the pattern of expression of Bmi1 and Ring1B using the previous mentioned models, we sought to study if Bmi1 could be involved in the acinoductal metaplasia which happened during PDAC formation. Our first hypothesis was that Bmi1 would be acting upon the transcription factor Ptf1a/p48, which is regarded as the master regulator of the acinar differentiation programme [184, 187, 200-201, 204].

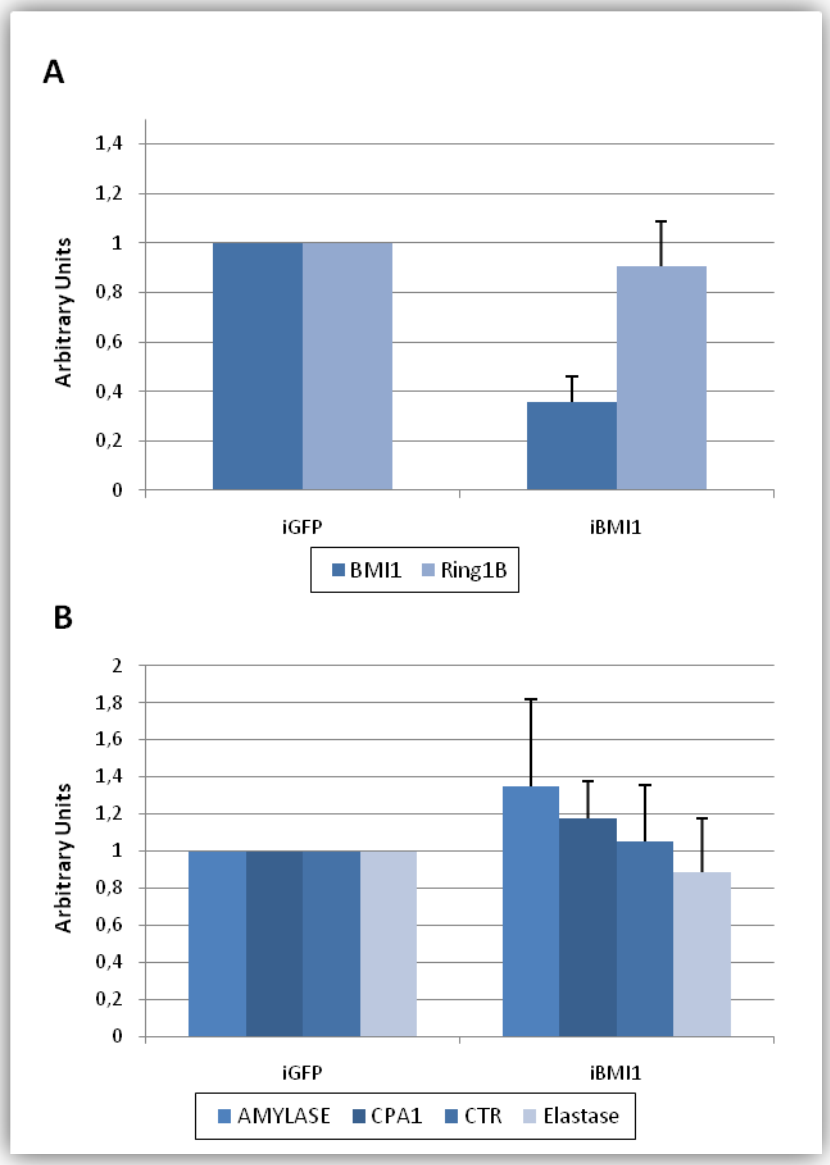
Pancreatitis and acinoductal metaplasia mouse models showed downregulation of several pancreatic markers and the transcription factor p48. On the contrary, protein expression of Bmi1 dramatically increased at the same time. To better understand the data obtained from *in vivo* models, *in vitro* models were used to analyse the effects of modulating Bmi1 expression on the expression of acinar cell differentiation markers.

## 5.1. Downregulation of Bmi1 in pancreatic acinar cell line

We mainly used two commercial acinar cell lines – AR42J and 266-6 – to study the effects of downregulating Bmi1. While we used both of them in parallel for the experiments, we decided to focus on the 266-6 cell line as transfection and infection yields were higher. The 266-6 cell line was reported to be obtained from a pancreatic tumour induced in a young adult mouse with an Elastase I/SV-40 T antigen fusion gene [310]. Partially retaining a differentiated phenotype, this cell line expresses detectable levels of transcription factor Ptf1/p48 and the exocrine digestive enzymes elastase, amylase and trypsin [323].

## 5.2. Effects of Bmi1 downregulation

A first approach was to downregulate Bmi1 by means of infecting 266-6 cells with retroviral vectors expressing Bmi1 siRNA. 293 Phoenix cells were transfected with plasmid vectors to generate these retroviral particles. Then, retroviruses were filtered and added to 266-6 cells, which were selected by antibiotic resistance. After total RNA isolation, quantitative Real-Time PCR was performed to analyse the expression of PcG members Bmi1 and Ring1B, the acinar markers amylase, carboxypeptidase A1, chymotrypsinogen B, elastase and the pancreatic transcription factors Pdx1 and p48 **(Figure R.10)**.


A
Arbitrary Units
1,4
1,2
1
0,8
0,6
0,4
0,2
0
iGFP
iBMI1
BMI1
Ring1B
B
Arbitrary Units
2
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
iGFP
iBMI1
AMYLASE
CPA1
CTR
Elastase

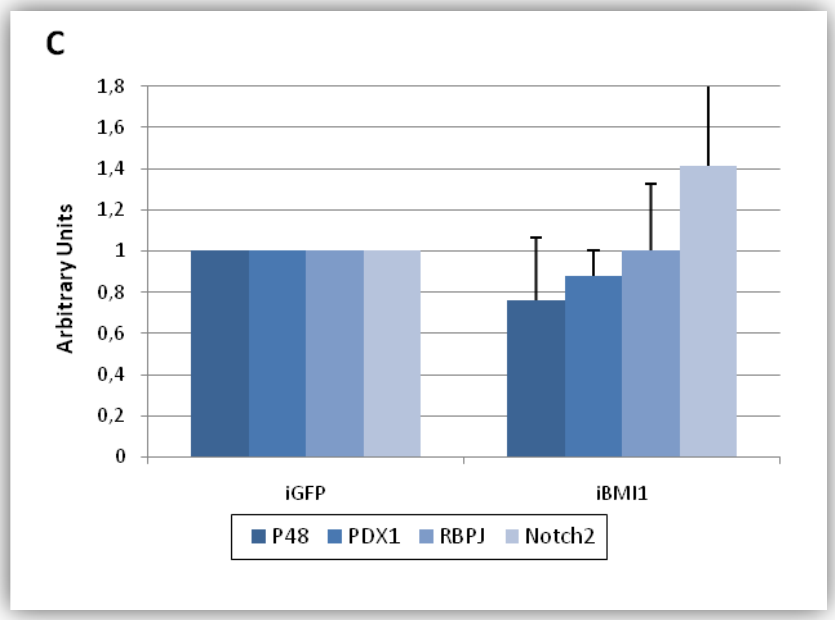


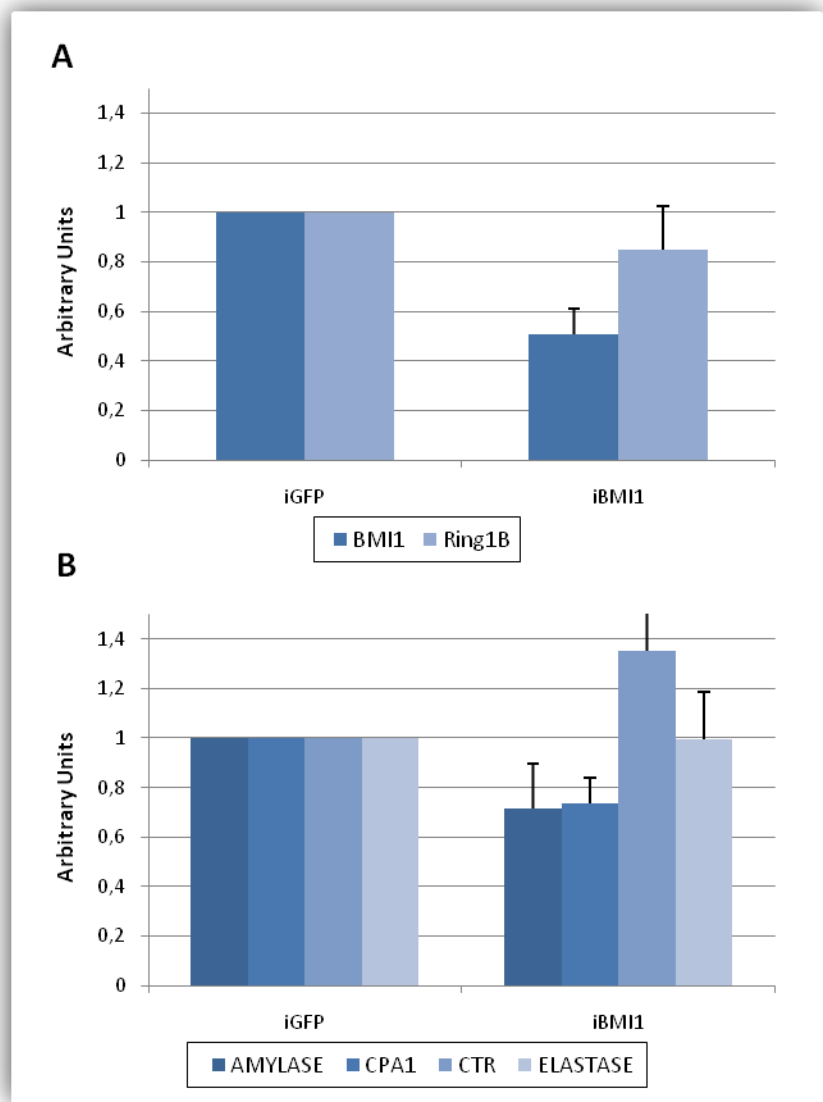

**Figure R.10: Effects of Bmi1 knockdown in the acinar differentiation programme using retroviral vectors in 266-6 acinar tumour cells, analysed by qPCR.** Levels of Bmi1 and Ring1B (A) were analysed, as well as Amylase 2, Carboxypeptidase A1, Chymotrypsinogen B and Elastase (B), and PDX1, Ptf1a/p48, RBPJ and Notch2 (C). iGFP = control infected with a lentivirus codifying for irrelevant iRNA.

Furthermore, the human homolog for the Drosophila Suppressor of Hairless, which is called RBPJ, was also analysed, as well as Notch 2 receptor. RBPJ was reported to be part of the PTF1 complex, directly interacting with p48 [201]. On the other hand, activation of the Notch signalling pathway is related to self-renewal potential in many tissues [53] and it is involved in pancreatic cell differentiation [182].

Upon retroviral infection of 266-6 with siRNA of Bmi1, mRNA levels of Bmi1 showed a 0.65-fold decrease compared to control **(Figure R.10A)**. Ring1B levels remained unchanged or slightly decreased **(Figure R.10A)**. On the contrary, amylase mRNA displayed a 1.35-fold increase. Levels of Carboxypeptidase A1, chymotrypsinogen B and elastase upon Bmi1

depletion barely changed **(Figure R.10B)**. Moreover, p48 and PDX1 showed a low decrease, while Notch2 expression was induced 1.4-fold **(Figure R.10C)**. RPBJ levels remain unchanged.

In parallel, we worked with a similar protocol using small interference siRNA oligonucleotides to induce Bmi1 knock-down, in order to assess the study of short-term effects due to depletion of Bmi1 [311].



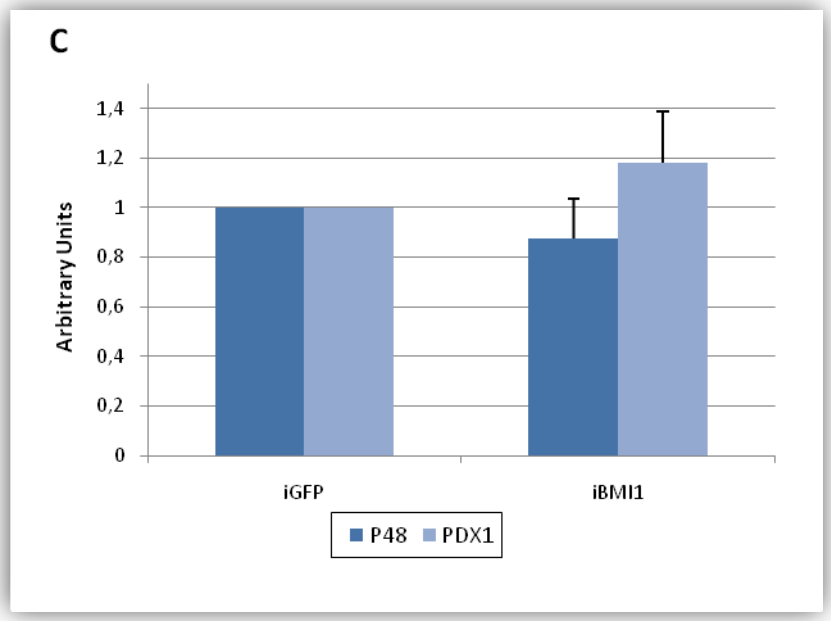



**Figure R.11. Effects of Bmi1 knockdown in the acinar differentiation program using siRNA transfection and analysed by qPCR.** mRNA levels of Bmi1 and Ring1B (A) were analysed, as well as Amylase 2, Carboxypeptidase A1, Chymotrypsinogen B and Elastase (B), and PDX1 and Ptf1a/p48 (C). iGFP = control transfected with irrelevant siRNA oligonucleotide.

Transfection of 266-6 cells with siRNA oligonucleotides against Bmi1 resulted in a 0.5-fold knock-down of RNA levels of Bmi1 **(Figure R.11A)**. While Ring1B remained unaltered **(Figure R.11A)**, amylase and carboxypeptidase showed a 0.25-fold decrease, chymotrypsinogen B displayed a 1.5-fold increase and elastase remained unchanged **(Figure R.11B)**. Moreover, levels of p48 experienced a slight decrease while PDX1 expression barely increased **(Figure R.11C)**.

Protein expression analysis was performed to analyse the effects of Bmi1 downregulation **(Figure R.12A and R.12B)**. Depletion of Bmi1 protein levels was achieved up to a 0.7 of decrease compared to control cells. Regarding acinar marker expression, Ptf1a/p48 displayed a modest

increase, while amylase was clearly induced with a 2.2-fold increase. Carboxypeptidase was also analysed and exhibited a 1.7-fold increase compared to control cells.

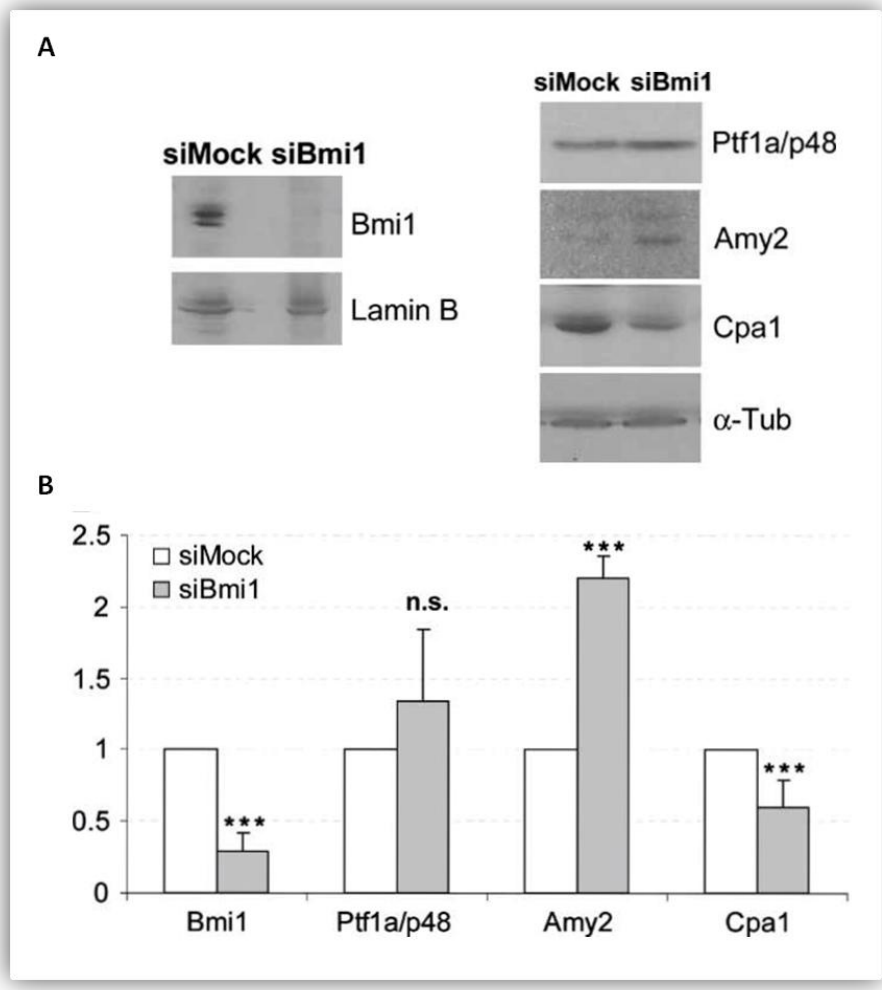



**Figure R.12. Effects of Bmi1 knockdown in the acinar differentiation program using siRNA transfection and analysed by Western Blot.** (A) Upon significant Bmi1 knock-down at the protein level (left panels), several pancreatic markers are altered (right panels). (B) Densitometric quantification of Bmi1, Ptf1a/p48, Amylase 2, and Carboxypeptidase A1 normalized to Lamin B or α-Tubulin. Data represent the average of at least three experiments and error bars correspond to the standard deviation. Statistics were performed by the Student's T-test. *** = $p < 0.001$; n.s. = non-significant difference ($p > 0.5$). siMock = control with transfected siRNA oligonucleotide.

In summary, downregulation of Bmi1 alters the expression pattern of the analysed exocrine pancreatic markers, modulating their expression levels and therefore hinting a possible modulation of acinar differentiation by Bmi1.

At the beginning of this thesis project, the expression profile of Polycomb group proteins in the pancreas was barely characterised. After taking into account published reports involving members of the PcG family in crucial mechanisms like cell cycle control [145], tumorigenesis [99, 126, 137, 141, 324] and stem cell maintenance [308, 325-326], our first approach was to conduct a proper analysis of PcG expression pattern in adult mouse pancreas. This was the first step to understand the role of the Polycomb family in the development, tumorigenesis and cell plasticity of the pancreas.

According to our immunohistochemical experiments in wild-type mice, expression of several PcG proteins is dependent on the pancreatic cell type and also on the developmental stage of the pancreas. Therefore, a general approach in analysing PcG expression considering the tissue as a whole could be misleading. Different analysis have been made using tissue microarrays which analysed expression of PcG members in the pancreas without getting into detail on cell types, obtaining questionable results [327].

Regarding our findings, at E14.5–15.5 Bmi1 expression is found in exocrine pancreatic progenitors, specifically at the tips of the branching epithelium, a distribution which is reminiscent of the stem cell/progenitor compartment at the tip of the acini [328]. This distribution is also consistent with recent insights in adult stem cell populations, which demonstrated that both mammary and intestinal stem cells are dependent on Bmi1 for self-renewal and maintenance of the differentiated pool of cells [326, 329]. At E17.5, when endocrine progenitor cells proliferate and begin to form mature cells, expression of Bmi1 is down-regulated in the majority of exocrine cells, becoming

localised in islet cell nuclei and only in a few scattered acinar cells. Finally, in the adult pancreas, expression of Bmi1 is restricted to ductal, islet and some scattered cells in the exocrine compartment.

These Bmi1+ isolated exocrine cells could be centroacinar cells, which are located at the termini of the duct network. These cells express Notch target gene Hes1 in the adult pancreas, the expression of which has been involved in keeping an undifferentiated state [168]. Centroacinar cells have been proposed as cells of origin for PanINs and PDAC [303, 330]. Evidence for this derives from pancreas-specific deletion of the *Pten* tumor suppressor, which causes expansion and transformation of Hes1-expressing centroacinar cells, without acinar-ductal reprogramming [331]. Although different models have shed light in understanding cell origin of PDAC, it has been difficult to reconcile their conclusions, or extrapolate to the human form of the disease. Not even the most recent mouse models for PDAC, like KRasG12D, can discriminate between acinar and centroacinar cells, as the Cre-loxP system is functional in both cell types [331]

Regarding Ring1B, its mRNA levels can be detected at E14.5 in the mouse pancreas but the protein could not be detected. This may suggest that the protein levels are below the detection threshold or some post-transcriptional regulation is happening. Recent findings revealed that translation of Ring1B protein needs a stable IRES in the 5'UTR region [332]. In the adult pancreas, Ring1B protein expression is restricted to the nuclei of islet, ductal and some acinar cells, sharing a similar distribution with Bmi1.

When planning to study members of the Polycomb family in pancreatic cancer, we chose to analyse Bmi1 and Ring1B expression in the exocrine pancreas because the majority of the human pancreatic tumours possess exocrine characteristics. Interestingly, our results in animal models of pancreatitis show an induction of Bmi1 expression which is related to tissue regeneration associated with acute and chronic pancreatic injury. Moreover, acinoductal metaplasia was found in Bmi1-positive chronic pancreatic lesions, which have been postulated to be preceding malignant tumorigenesis. These lesions may seem to display increased cell proliferation, which has been reported in association with repression of acinar cell differentiation by means of Ptf1a/p48 expression [204].

The role of Bmi1 in tumorigenesis is mainly considered to entail the escape from senescence or quiescence of preneoplastic cells by repressing the tumour-suppressor p16INK4A [138, 141, 333-334], which is almost universally inactivated by a variety of genetic mechanisms [41, 335]. Indeed, some PanINs arising in chronic pancreatitis may show loss of p16INK4 expression [336-337], suggesting that Bmi1 could play a role in this process.

Moreover, a very Interestingly, a very recent study allegedly reported to have found a Bmi1-positive pancreatic acinar cell subpopulation with stem-cell capabilities, maintaining tissue homeostasis [308]. However, these findings need to be further analysed, since the transgenic construct they used truncated the 3'UTR region of Bmi1, which may affect any hypothetical post-transcriptional regulation.

Furthermore, acinar cell proliferation may be induced by Bmi1-mediated repression of Ptf1a/p48, whose expression has been reported to have

growth inhibitory effects [204]. This would lead to hypothesize that Bmi1 overexpression in pancreatitis may be increasing the cell population sensitive to oncogenic transformation, by preventing terminal differentiation and decreasing cell cycle inhibition by p16INK4a and Ptf1a/p48. This hypothesis is consistent with the downregulation of Amy2 in Bmi1-positive cells.

Following this hypothesis, our experiments in Bmi1 knock-down *in vitro* using the 266-6 acinar cell line show modulation of a selection of pancreatic markers, hinting that downregulation of Bmi1 may be forcing differentiation of the acinar lineage, due to an increase in amylase and p48. However, expression of Cpa1 decreases, which has recently been reported to be expressed, along with Pdx1, Ptf1a/p48 and cMyc, by pancreatic multipotent progenitors at E9.5 during mouse development. Due to their high proliferation rate, these progenitor cells are located at the tips of the developmental branching of the pancreas, leaving differentiated progeny behind. The fact that Cpa1 is present in these multipotent cells questions the canonical views of pancreatic markers, as it is considered to be a marker for adult acinar cells.

Very recent findings have reported Bmi1 to be collaborating with HRAS to promote increased proliferation, invasion and resistance using *in vitro* models of breast cancer by altering several p16/INK4A-independent pathways [338], promoting an increased rate of spontaneous metastases from mammary fat pad xenografts including novel metastases to the brain [339]. Bmi1 could also be promoting pancreatic tumorigenesis in a similar way, as induction of KRAS is one of the first alterations in PDAC.

While induction of Bmi1 can be detected at early stages of preneoplastic lesions, Ring1B expression is increased in advanced PanIN stages. Despite the fact that Bmi1 cooperates with Ring1B in H2A ubiquitylation [340], taking into account our findings, both proteins seem to be regulated in an independent manner in pancreatitis and neoplastic lesions of the pancreas. Induction of Bmi1 expression is maintained throughout pancreatitis, preneoplastic lesions and PDAC. However, Ring1B expression becomes activated in advanced stages of tumorigenesis, hinting that Bmi1 and Ring1B could be cooperating in late PanINs and in PDAC.

One interesting hypothesis involves different barriers which PanINs need to overcome to progress into malignant PDAC [341]. Our results indicate that increase in Ring1B expression in late PanIN lesions and PDAC could be related to bypassing these hypothetical barriers to neoplasia, like senescent mechanisms induced by oncogenes [342].

In summary, our work suggests an important role for Polycomb in both early and late steps of pancreatic carcinogenesis. We conclude that these proteins may be crucial for inflammatory preneoplastic conditions. A better understanding of the mechanisms involved therein may provide clues for PDAC prevention in patients at risk, particularly in individuals with sporadic or hereditary pancreatitis.

1. In the normal mouse pancreas, Bmi1 and Ring1B are expressed at the protein level in the nuclei of endocrine cells, ductal cells and in some scattered cells of the exocrine compartment.

2. During mouse pancreatogenesis, mRNA levels of Bmi1 and Ring1B are localised at the distal tips of the pancreatic progenitor branch at E14.5. However, at E15.5, while Bmi1 protein expression is detected in the nuclei of acinar cells, Ring1B expression is undetectable. At E17.5, protein expression of Bmi1 is only found in the nuclei of islet cells and in a few scattered exocrine cells.

3. In pancreatic samples of $KRas^{G12V}$ mouse model for PDAC, protein expression of Bmi1 is upregulated since early PanIN stages and is maintained until PDAC lesions. On the contrary, expression of Ring1B is increased since late PanIN stages.

4. In mice with caerulein-induced acute pancreatitis, Bmi1 and Ring1B expression is increased in affected regions of the pancreas 2 days after treatment and this increase lasts up to 10 days. In chronic pancreatitis mouse models, Bmi1 is highly expressed in regions of acinar metaplasia, while Ring1B is not found.

5. Upon duct ligation in the rat pancreas, Bmi1 is increased in regions of acinar metaplasia.

6. In primary cultures of the exocrine fraction of mouse pancreas, while mRNA levels of Bmi1 and Ring1B barely change during the spontaneous acinoductal transdifferentiation of the cells, protein expression of Bmi1 highly increases after 1 day of culture and lasts until day 5. Ring1B expression is not induced during this time.

7. Human pancreatic samples from PDAC patients show a significant upregulation of Bmi1 protein expression in regions of chronic pancreatitis, aberrant ducts and tumour lesions. Ring1B is found to be induced in later PanIN stages and PDAC.

8. After knocking down expression of Bmi1 in an acinar tumour cell line, several pancreatic markers are altered thus the acinar differentiation program, suggesting a possible role of Bmi1 in the exocrine cell plasticity and commitment to an adult cell type.

**Martínez-Romero C, Rooman I, Skoudy A, Guerra C, Molero X, González A, Iglesias M, Lobato T, Bosch A, Barbacid M, Real FX, Hernández-Muñoz I.**
***The epigenetic regulators Bmi1 and Ring1B are differentially regulated in pancreatitis and pancreatic ductal adenocarcinoma.***
**J Pathol. 2009 Oct;219(2):205-13.**

## REFERENCES


1. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nature reviews 2008;**8**:755-768.
2. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;**414**:105-111.
3. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nature medicine 1997;**3**:730-737.
4. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;**445**:106-110.
5. Jordan CT. Cancer stem cell biology: from leukemia to solid tumors. Current opinion in cell biology 2004;**16**:708-712.
6. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 2003;**100**:3983-3988.
7. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V *et al.* Identification of pancreatic cancer stem cells. Cancer Res 2007;**67**:1030-1037.
8. Lang SH, Frame FM, Collins AT. Prostate cancer stem cells. The Journal of pathology 2009;**217**:299-306.
9. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nature reviews 2005;**5**:275-284; DOI nrc1590 [pii]
10.1038/nrc1590.
10. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nature medicine 2004;**10**:789-799.
11. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. Genes & development 2006;**20**:1218-1249; DOI 20/10/1218 [pii]
10.1101/gad.1415606.
12. Bar-Sagi D. A Ras by any other name. Molecular and cellular biology 2001;**21**:1441-1443; DOI 10.1128/MCB.21.5.1441-1443.2001.
13. Kranenburg O. The KRAS oncogene: past, present, and future. Biochimica et biophysica acta 2005;**1756**:81-82.
14. Gearhart J, Pashos EE, Prasad MK. Pluripotency redux--advances in stem-cell research. The New England journal of medicine 2007;**357**:1469-1472.
15. Lin CJ, Malina A, Pelletier J. c-Myc and eIF4F constitute a feedforward loop that regulates cell growth: implications for anticancer therapy. Cancer research 2009;**69**:7491-7494; DOI 0008-5472.CAN-09-0813 [pii]

10.1158/0008-5472.CAN-09-0813.
16. Matsumura I, Tanaka H, Kanakura Y. E2F1 and c-Myc in cell growth and death. Cell cycle (Georgetown, Tex 2003;**2**:333-338.
17. Wilson A, Murphy MJ, Oskarsson T, Kaloulis K, Bettess MD, Oser GM *et al.* c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes & development 2004;**18**:2747-2763; DOI 18/22/2747 [pii]
10.1101/gad.313104.
18. Nilsson JA, Cleveland JL. Myc pathways provoking cell suicide and cancer. Oncogene 2003;**22**:9007-9021.
19. Alarcon-Vargas D, Tansey WP, Ronai Z. Regulation of c-myc stability by selective stress conditions and by MEKK1 requires aa 127-189 of c-myc. Oncogene 2002;**21**:4384-4391.
20. Noguchi K, Kokubu A, Kitanaka C, Ichijo H, Kuchino Y. ASK1-signaling promotes c-Myc protein stability during apoptosis. Biochemical and biophysical research communications 2001;**281**:1313-1320.
21. Sears R, Leone G, DeGregori J, Nevins JR. Ras enhances Myc protein stability. Molecular cell 1999;**3**:169-179.
22. Stoneley M, Chappell SA, Jopling CL, Dickens M, MacFarlane M, Willis AE. c-Myc protein synthesis is initiated from the internal ribosome entry segment during apoptosis. Molecular and cellular biology 2000;**20**:1162-1169.
23. Ruggero D, Montanaro L, Ma L, Xu W, Londei P, Cordon-Cardo C *et al.* The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis. Nature medicine 2004;**10**:484-486.
24. Pelengaris S, Khan M, Evan G. c-MYC: more than just a matter of life and death. Nature reviews 2002;**2**:764-776.
25. Pelengaris S, Littlewood T, Khan M, Elia G, Evan G. Reversible activation of c-Myc in skin: induction of a complex neoplastic phenotype by a single oncogenic lesion. Molecular cell 1999;**3**:565-577.
26. Baudino TA, McKay C, Pendeville-Samain H, Nilsson JA, Maclean KH, White EL *et al.* c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. Genes & development 2002;**16**:2530-2543.
27. Schmidt EV. The role of c-myc in cellular growth control. Oncogene 1999;**18**:2988-2996.
28. Schmidt EV. The role of c-myc in regulation of translation initiation. Oncogene 2004;**23**:3217-3221.
29. Sherr CJ. Principles of tumor suppression. Cell 2004;**116**:235-246.

30. Hirohashi S, Kanai Y. Cell adhesion system and human cancer morphogenesis. Cancer science 2003;**94**:575-581.
31. Prives C, Hall PA. The p53 pathway. The Journal of pathology 1999;**187**:112-126.
32. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000;**408**:307-310.
33. Vousden KH, Prives C. Blinded by the Light: The Growing Complexity of p53. Cell 2009;**137**:413-431.
34. Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L *et al.* The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell 1998;**92**:713-723; DOI S0092-8674(00)81400-2 [pii].
35. Honda R, Yasuda H. Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. Embo J 1999;**18**:22-27; DOI 10.1093/emboj/18.1.22.
36. Tao W, Levine AJ. P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. Proc Natl Acad Sci U S A 1999;**96**:6937-6941.
37. Alarcon-Vargas D, Ronai Z. p53-Mdm2--the affair that never ends. Carcinogenesis 2002;**23**:541-547.
38. Kruse JP, Gu W. Modes of p53 regulation. Cell 2009;**137**:609-622; DOI S0092-8674(09)00511-X [pii]
10.1016/j.cell.2009.04.050.
39. Levine AJ, Hu W, Feng Z. The P53 pathway: what questions remain to be explored? Cell death and differentiation 2006;**13**:1027-1036.
40. Adorno M, Cordenonsi M, Montagner M, Dupont S, Wong C, Hann B *et al.* A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell 2009;**137**:87-98; DOI S0092-8674(09)00087-7 [pii]
10.1016/j.cell.2009.01.039.
41. Sharpless NE. INK4a/ARF: a multifunctional tumor suppressor locus. Mutation research 2005;**576**:22-38.
42. Sekulic A, Haluska P, Jr., Miller AJ, Genebriera De Lamo J, Ejadi S, Pulido JS *et al.* Malignant melanoma in the 21st century: the emerging molecular landscape. Mayo Clin Proc 2008;**83**:825-846.
43. Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L *et al.* Ink4a/Arf expression is a biomarker of aging. J Clin Invest 2004;**114**:1299-1307.
44. Nielsen GP, Stemmer-Rachamimov AO, Shaw J, Roy JE, Koh J, Louis DN. Immunohistochemical survey of p16INK4A expression

in normal human adult and infant tissues. Lab Invest 1999;**79**:1137-1143.

45. Campisi J. Cancer and ageing: rival demons? Nat Rev Cancer 2003;**3**:339-349.
46. Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: progress and puzzles. Curr Opin Genet Dev 2003;**13**:77-83.
47. Kato J, Matsushime H, Hiebert SW, Ewen ME, Sherr CJ. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev 1993;**7**:331-342.
48. Quelle DE, Cheng M, Ashmun RA, Sherr CJ. Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF. Proc Natl Acad Sci U S A 1997;**94**:669-673.
49. Sharpless NE, Bardeesy N, Lee KH, Carrasco D, Castrillon DH, Aguirre AJ *et al.* Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. Nature 2001;**413**:86-91.
50. Sherr CJ, DePinho RA. Cellular senescence: mitotic clock or culture shock? Cell 2000;**102**:407-410.
51. Bardeesy N, Aguirre AJ, Chu GC, Cheng KH, Lopez LV, Hezel AF *et al.* Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse. Proc Natl Acad Sci U S A 2006;**103**:5947-5952.
52. Ohtani N, Yamakoshi K, Takahashi A, Hara E. The p16INK4a-RB pathway: molecular link between cellular senescence and tumor suppression. J Med Invest 2004;**51**:146-153.
53. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nature reviews 2003;**3**:756-767.
54. Henrique D, Hirsinger E, Adam J, Le Roux I, Pourquie O, Ish-Horowicz D *et al.* Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. Curr Biol 1997;**7**:661-670.
55. Lewis J. Notch signalling and the control of cell fate choices in vertebrates. Seminars in cell & developmental biology 1998;**9**:583-589.
56. Varnum-Finney B, Purton LE, Yu M, Brashem-Stein C, Flowers D, Staats S *et al.* The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. Blood 1998;**91**:4084-4091.
57. Dontu G, Jackson KW, McNicholas E, Kawamura MJ, Abdallah WM, Wicha MS. Role of Notch signaling in cell-fate

determination of human mammary stem/progenitor cells. Breast Cancer Res 2004;**6**:R605-615.

58. Lowell S, Jones P, Le Roux I, Dunne J, Watt FM. Stimulation of human epidermal differentiation by delta-notch signalling at the boundaries of stem-cell clusters. Curr Biol 2000;**10**:491-500.
59. Gallahan D, Jhappan C, Robinson G, Hennighausen L, Sharp R, Kordon E *et al.* Expression of a truncated Int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis. Cancer research 1996;**56**:1775-1785.
60. Weijzen S, Rizzo P, Braid M, Vaishnav R, Jonkheer SM, Zlobin A *et al.* Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. Nature medicine 2002;**8**:979-986.
61. Ramalho-Santos M, Melton DA, McMahon AP. Hedgehog signals regulate multiple aspects of gastrointestinal development. Development (Cambridge, England) 2000;**127**:2763-2772.
62. Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes & development 2001;**15**:3059-3087.
63. Kalderon D. Transducing the hedgehog signal. Cell 2000;**103**:371-374.
64. Cohen MM, Jr. The hedgehog signaling network. American journal of medical genetics 2003;**123A**:5-28.
65. Gill PS, Rosenblum ND. Control of murine kidney development by sonic hedgehog and its GLI effectors. Cell cycle (Georgetown, Tex 2006;**5**:1426-1430.
66. Eaton S. Multiple roles for lipids in the Hedgehog signalling pathway. Nat Rev Mol Cell Biol 2008;**9**:437-445.
67. Ahn S, Joyner AL. In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature 2005;**437**:894-897.
68. Bhardwaj G, Murdoch B, Wu D, Baker DP, Williams KP, Chadwick K *et al.* Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. Nature immunology 2001;**2**:172-180.
69. Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW *et al.* Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. Cancer research 2006;**66**:6063-6071.
70. Ruiz i Altaba A, Stecca B, Sanchez P. Hedgehog--Gli signaling in brain tumors: stem cells and paradevelopmental programs in cancer. Cancer letters 2004;**204**:145-157.

71. Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K *et al.* Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature 2003;**425**:846-851.
72. Duprez D, Lapointe F, Edom-Vovard F, Kostakopoulou K, Robson L. Sonic hedgehog (SHH) specifies muscle pattern at tissue and cellular chick level, in the chick limb bud. Mechanisms of development 1999;**82**:151-163.
73. Miao N, Wang M, Ott JA, D'Alessandro JS, Woolf TM, Bumcrot DA *et al.* Sonic hedgehog promotes the survival of specific CNS neuron populations and protects these cells from toxic insult In vitro. J Neurosci 1997;**17**:5891-5899.
74. Duprez D, Fournier-Thibault C, Le Douarin N. Sonic Hedgehog induces proliferation of committed skeletal muscle cells in the chick limb. Development (Cambridge, England) 1998;**125**:495-505.
75. Morikawa Y, Maska E, Brody H, Cserjesi P. Sonic hedgehog signaling is required for sympathetic nervous system development. Neuroreport 2009;**20**:684-688.
76. Shah DK, Hager-Theodorides AL, Outram SV, Ross SE, Varas A, Crompton T. Reduced thymocyte development in sonic hedgehog knockout embryos. J Immunol 2004;**172**:2296-2306.
77. Washington Smoak I, Byrd NA, Abu-Issa R, Goddeeris MM, Anderson R, Morris J *et al.* Sonic hedgehog is required for cardiac outflow tract and neural crest cell development. Developmental biology 2005;**283**:357-372.
78. St-Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes & development 1999;**13**:2072-2086.
79. Kayed H, Kleeff J, Keleg S, Guo J, Ketterer K, Berberat PO *et al.* Indian hedgehog signaling pathway: expression and regulation in pancreatic cancer. International journal of cancer 2004;**110**:668-676.
80. Thayer SP, di Magliano MP, Heiser PW, Nielsen CM, Roberts DJ, Lauwers GY *et al.* Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature 2003;**425**:851-856.
81. Kasper M, Jaks V, Fiaschi M, Toftgard R. Hedgehog signalling in breast cancer. Carcinogenesis 2009;**30**:903-911.
82. Anton Aparicio LM, Garcia Campelo R, Cassinello Espinosa J, Valladares Ayerbes M, Reboredo Lopez M, Diaz Prado S *et al.*

Prostate cancer and Hedgehog signalling pathway. Clin Transl Oncol 2007;**9**:420-428.

83. Clevers H. Wnt/beta-catenin signaling in development and disease. Cell 2006;**127**:469-480.
84. Eisenmann DM. Wnt signaling. WormBook 2005:1-17; DOI 10.1895/wormbook.1.7.1.
85. Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet 2004;**5**:691-701.
86. Polakis P. Wnt signaling and cancer. Genes & development 2000;**14**:1837-1851.
87. Zhou BP, Hung MC. Wnt, hedgehog and snail: sister pathways that control by GSK-3beta and beta-Trcp in the regulation of metastasis. Cell cycle (Georgetown, Tex 2005;**4**:772-776.
88. Feinberg AP, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. Nat Rev Genet 2006;**7**:21-33.
89. Roloff TC, Nuber UA. Chromatin, epigenetics and stem cells. European journal of cell biology 2005;**84**:123-135.
90. Bird A. Perceptions of epigenetics. Nature 2007;**447**:396-398.
91. Delcuve GP, Rastegar M, Davie JR. Epigenetic control. Journal of cellular physiology 2009;**219**:243-250.
92. Lund AH, van Lohuizen M. Epigenetics and cancer. Genes Dev 2004;**18**:2315-2335.
93. Cadieux B, Ching TT, VandenBerg SR, Costello JF. Genome-wide hypomethylation in human glioblastomas associated with specific copy number alteration, methylenetetrahydrofolate reductase allele status, and increased proliferation. Cancer research 2006;**66**:8469-8476.
94. Enver T, Soneji S, Joshi C, Brown J, Iborra F, Orntoft T *et al.* Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. Human molecular genetics 2005;**14**:3129-3140.
95. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 2007;**8**:286-298.
96. Frigola J, Song J, Stirzaker C, Hinshelwood RA, Peinado MA, Clark SJ. Epigenetic remodeling in colorectal cancer results in coordinate gene suppression across an entire chromosome band. Nat Genet 2006;**38**:540-549.
97. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;**3**:415-428.

98. Squazzo SL, O'Geen H, Komashko VM, Krig SR, Jin VX, Jang SW *et al.* Suz12 binds to silenced regions of the genome in a cell-type-specific manner. Genome research 2006;**16**:890-900.
99. Sparmann A, van Lohuizen M. Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 2006;**6**:846-856.
100. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003;**33 Suppl**:245-254.
101. Li E. Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 2002;**3**:662-673.
102. Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A 2002;**99**:3740-3745.
103. Saxonov S, Berg P, Brutlag DL. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proc Natl Acad Sci U S A 2006;**103**:1412-1417.
104. Dhasarathy A, Wade PA. The MBD protein family-reading an epigenetic mark? Mutat Res 2008;**647**:39-43.
105. Bird AP, Wolffe AP. Methylation-induced repression--belts, braces, and chromatin. Cell 1999;**99**:451-454.
106. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z *et al.* High-resolution profiling of histone methylations in the human genome. Cell 2007;**129**:823-837.
107. Peterson CL, Laniel MA. Histones and histone modifications. Curr Biol 2004;**14**:R546-551.
108. Qiu J. Epigenetics: Unfinished symphony. Nature 2006;**441**:143-145.
109. Jenuwein T, Allis CD. Translating the histone code. Science 2001;**293**:1074-1080.
110. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J *et al.* Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 2007;**39**:232-236.
111. Suganuma T, Workman JL. Crosstalk among Histone Modifications. Cell 2008;**135**:604-607.
112. Turner BM. Defining an epigenetic code. Nat Cell Biol 2007;**9**:2-6.
113. Schwartz YB, Pirrotta V. Polycomb silencing mechanisms and the management of genomic programmes. Nat Rev Genet 2007;**8**:9-22.
114. Schwartz YB, Pirrotta V. Polycomb complexes and epigenetic states. Curr Opin Cell Biol 2008;**20**:266-273.

115. Lewis EB. A gene complex controlling segmentation in Drosophila. Nature 1978;**276**:565-570.
116. Marx J. Developmental biology. Combing over the Polycomb group proteins. Science 2005;**308**:624-626.
117. Otte AP, Kwaks TH. Gene repression by Polycomb group protein complexes: a distinct complex for every occasion? Curr Opin Genet Dev 2003;**13**:448-454.
118. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P *et al.* Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 2002;**298**:1039-1043.
119. Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C *et al.* The Polycomb group protein EZH2 directly controls DNA methylation. Nature 2006;**439**:871-874.
120. Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 2002;**111**:185-196.
121. de Napoles M, Mermoud JE, Wakao R, Tang YA, Endoh M, Appanah R *et al.* Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Developmental cell 2004;**7**:663-676.
122. Wang H, Wang L, Erdjument-Bromage H, Vidal M, Tempst P, Jones RS *et al.* Role of histone H2A ubiquitination in Polycomb silencing. Nature 2004;**431**:873-878.
123. Sauvageau M, Sauvageau G. Polycomb group genes: keeping stem cell activity in balance. PLoS Biol 2008;**6**:e113.
124. Gorfinkiel N, Fanti L, Melgar T, Garcia E, Pimpinelli S, Guerrero I *et al.* The Drosophila Polycomb group gene Sex combs extra encodes the ortholog of mammalian Ring1 proteins. Mechanisms of development 2004;**121**:449-462.
125. Cao R, Tsukada Y, Zhang Y. Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. Mol Cell 2005;**20**:845-854.
126. Leung C, Lingbeek M, Shakhova O, Liu J, Tanger E, Saremaslani P *et al.* Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas. Nature 2004;**428**:337-341.
127. Wiederschain D, Chen L, Johnson B, Bettano K, Jackson D, Taraszka J *et al.* Contribution of polycomb homologues Bmi-1 and Mel-18 to medulloblastoma pathogenesis. Molecular and cellular biology 2007;**27**:4968-4979; DOI MCB.02244-06 [pii]
10.1128/MCB.02244-06.

128. Kanno M, Hasegawa M, Ishida A, Isono K, Taniguchi M. mel-18, a Polycomb group-related mammalian gene, encodes a transcriptional negative regulator with tumor suppressive activity. Embo J 1995;**14**:5672-5678.
129. Martinez AM, Cavalli G. The role of polycomb group proteins in cell cycle regulation during development. Cell Cycle 2006;**5**:1189-1197.
130. Bantignies F, Cavalli G. Cellular memory and dynamic regulation of polycomb group proteins. Curr Opin Cell Biol 2006;**18**:275-283.
131. Ng RK, Gurdon JB. Epigenetic inheritance of cell differentiation status. Cell cycle (Georgetown, Tex 2008;**7**:1173-1177.
132. Ng JH, Heng JC, Loh YH, Ng HH. Transcriptional and epigenetic regulations of embryonic stem cells. Mutation research 2008;**647**:52-58.
133. Moss TJ, Wallrath LL. Connections between epigenetic gene silencing and human disease. Mutat Res 2007;**618**:163-174.
134. Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 1992;**69**:915-926.
135. O'Carroll D, Erhardt S, Pagani M, Barton SC, Surani MA, Jenuwein T. The polycomb-group gene Ezh2 is required for early mouse development. Mol Cell Biol 2001;**21**:4330-4336.
136. Gunster MJ, Raaphorst FM, Hamer KM, den Blaauwen JL, Fieret E, Meijer CJ *et al.* Differential expression of human Polycomb group proteins in various tissues and cell types. J Cell Biochem Suppl 2001;**Suppl 36**:129-143.
137. Jacobs JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. Genes Dev 1999;**13**:2678-2690.
138. Vonlanthen S, Heighway J, Altermatt HJ, Gugger M, Kappeler A, Borner MM *et al.* The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. British journal of cancer 2001;**84**:1372-1376.
139. Sawa M, Yamamoto K, Yokozawa T, Kiyoi H, Hishida A, Kajiguchi T *et al.* BMI-1 is highly expressed in M0-subtype acute myeloid leukemia. Int J Hematol 2005;**82**:42-47.
140. Dirks P. Bmi1 and cell of origin determinants of brain tumor phenotype. Cancer cell 2007;**12**:295-297.
141. Fan C, He L, Kapoor A, Gillis A, Rybak AP, Cutz JC *et al.* Bmi1 promotes prostate tumorigenesis via inhibiting p16(INK4A) and

p14(ARF) expression. Biochimica et biophysica acta 2008;**1782**:642-648.

142. Pietersen AM, Horlings HM, Hauptmann M, Langerod A, Ajouaou A, Cornelissen-Steijger P *et al.* EZH2 and BMI1 inversely correlate with prognosis and TP53 mutation in breast cancer. Breast Cancer Res 2008;**10**:R109.
143. Fasano CA, Phoenix TN, Kokovay E, Lowry N, Elkabetz Y, Dimos JT *et al.* Bmi-1 cooperates with Foxg1 to maintain neural stem cell self-renewal in the forebrain. Genes Dev 2009;**23**:561-574.
144. Courel M, Friesenhahn L, Lees JA. E2f6 and Bmi1 cooperate in axial skeletal development. Dev Dyn 2008;**237**:1232-1242.
145. Lee K, Adhikary G, Balasubramanian S, Gopalakrishnan R, McCormick T, Dimri GP *et al.* Expression of Bmi-1 in epidermis enhances cell survival by altering cell cycle regulatory protein expression and inhibiting apoptosis. The Journal of investigative dermatology 2008;**128**:9-17.
146. Visser HP, Gunster MJ, Kluin-Nelemans HC, Manders EM, Raaphorst FM, Meijer CJ *et al.* The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. Br J Haematol 2001;**112**:950-958.
147. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG *et al.* The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 2002;**419**:624-629.
148. Saramaki OR, Tammela TL, Martikainen PM, Vessella RL, Visakorpi T. The gene for polycomb group protein enhancer of zeste homolog 2 (EZH2) is amplified in late-stage prostate cancer. Genes, chromosomes & cancer 2006;**45**:639-645.
149. Raaphorst FM, Meijer CJ, Fieret E, Blokzijl T, Mommers E, Buerger H *et al.* Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. Neoplasia 2003;**5**:481-488.
150. Weikert S, Christoph F, Kollermann J, Muller M, Schrader M, Miller K *et al.* Expression levels of the EZH2 polycomb transcriptional repressor correlate with aggressiveness and invasive potential of bladder carcinomas. International journal of molecular medicine 2005;**16**:349-353.
151. Sellers WR, Loda M. The EZH2 polycomb transcriptional repressor--a marker or mover of metastatic prostate cancer? Cancer cell 2002;**2**:349-350.
152. Simon JA, Lange CA. Roles of the EZH2 histone methyltransferase in cancer epigenetics. Mutat Res 2008;**647**:21-29.

153. Swanton C, Caldas C. Molecular classification of solid tumours: towards pathway-driven therapeutics. British journal of cancer 2009;**100**:1517-1522.
154. ACS. *Cancer Facts & Figures 2009* edn). American Cancer Society: Atlanta, 2009.
155. Komarova NL, Sengupta A, Nowak MA. Mutation-selection networks of cancer initiation: tumor suppressor genes and chromosomal instability. Journal of theoretical biology 2003;**223**:433-450.
156. Nowell PC. Tumor progression: a brief historical perspective. Seminars in cancer biology 2002;**12**:261-266.
157. . *International Classification of Diseases for Oncology (ICD-O-3)* 2000. http://www.who.int/classifications/icd/adaptations/oncology/en/index.html [accessed 7th of August 2009].
158. . *International Classification of Diseases for Oncology (ICD-O-3)* 2009. http://en.wikipedia.org/wiki/International_Classification_of_Diseases_for_Oncology#.288010-8790.29_Epithelial [accessed 7th of August 2009].
159. Ghaneh P, Costello E, Neoptolemos JP. Biology and management of pancreatic cancer. Gut 2007;**56**:1134-1152.
160. Go VLW, DiMagno EP, Garner JD, Lebenthal E, Reber HA, Scheele GA. *The Pancreas: Biology, Pathobiology, and Disease* (Second edn). Raven Press Ltd: New York, 1993.
161. Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. Nat Rev Cancer 2002;**2**:897-909.
162. Brissova M, Fowler MJ, Nicholson WE, Chu A, Hirshberg B, Harlan DM *et al.* Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. J Histochem Cytochem 2005;**53**:1087-1097.
163. Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren PO, Caicedo A. The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proc Natl Acad Sci U S A 2006;**103**:2334-2339.
164. Sakuraba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S. Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. Diabetologia 2002;**45**:85-96.
165. Street CN, Lakey JR, Shapiro AM, Imes S, Rajotte RV, Ryan EA *et al.* Islet graft assessment in the Edmonton Protocol: implications

for predicting long-term clinical outcome. Diabetes 2004;**53**:3107-3114.

166. Standring S. *Gray's Anatomy: The Anatomical Basis of Clinical Practice* (39 edn). Churchill Livingstone, 2004.
167. Inui A, Asakawa A, Bowers CY, Mantovani G, Laviano A, Meguid MM *et al.* Ghrelin, appetite, and gastric motility: the emerging role of the stomach as an endocrine organ. Faseb J 2004;**18**:439-456.
168. Stanger BZ, Dor Y. Dissecting the cellular origins of pancreatic cancer. Cell cycle (Georgetown, Tex 2006;**5**:43-46.
169. Jena BP, Cho SJ, Jeremic A, Stromer MH, Abu-Hamdah R. Structure and composition of the fusion pore. Biophysical journal 2003;**84**:1337-1343.
170. Jeremic A, Kelly M, Cho SJ, Stromer MH, Jena BP. Reconstituted fusion pore. Biophysical journal 2003;**85**:2035-2043.
171. Mann NS, Mann SK. Enterokinase. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY 1994;**206**:114-118.
172. Feldman M, Friedman LS, Brandt LJ. *Feldman: Sleisenger & Fordtran's Gastrointestinal and Liver Disease* (8th edn). SAUNDERS ELSEVIER: Philadelphia, 2006.
173. Kallman F, Grobstein C. Fine Structure of Differentiating Mouse Pancreatic Exocrine Cells in Transfilter Culture. The Journal of cell biology 1964;**20**:399-413.
174. Pictet RL, Clark WR, Williams RH, Rutter WJ. An ultrastructural analysis of the developing embryonic pancreas. Developmental biology 1972;**29**:436-467.
175. Hammerschmidt M, Brook A, McMahon AP. The world according to hedgehog. Trends Genet 1997;**13**:14-21.
176. Kim SK, Melton DA. Pancreas development is promoted by cyclopamine, a hedgehog signaling inhibitor. Proc Natl Acad Sci U S A 1998;**95**:13036-13041.
177. Hebrok M, Kim SK, Melton DA. Notochord repression of endodermal Sonic hedgehog permits pancreas development. Genes Dev 1998;**12**:1705-1713.
178. Apelqvist A, Ahlgren U, Edlund H. Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. Curr Biol 1997;**7**:801-804.
179. Offield MF, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA *et al.* PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development 1996;**122**:983-995.

180. Jonsson J, Carlsson L, Edlund T, Edlund H. Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 1994;**371**:606-609.
181. Gu G, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 2002;**129**:2447-2457.
182. Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T *et al.* Notch signalling controls pancreatic cell differentiation. Nature 1999;**400**:877-881.
183. Jensen J, Pedersen EE, Galante P, Hald J, Heller RS, Ishibashi M *et al.* Control of endodermal endocrine development by Hes-1. Nat Genet 2000;**24**:36-44.
184. Esni F, Ghosh B, Biankin AV, Lin JW, Albert MA, Yu X *et al.* Notch inhibits Ptf1 function and acinar cell differentiation in developing mouse and zebrafish pancreas. Development 2004;**131**:4213-4224.
185. Hald J, Hjorth JP, German MS, Madsen OD, Serup P, Jensen J. Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development. Developmental biology 2003;**260**:426-437.
186. Murtaugh LC, Stanger BZ, Kwan KM, Melton DA. Notch signaling controls multiple steps of pancreatic differentiation. Proc Natl Acad Sci U S A 2003;**100**:14920-14925.
187. Kawaguchi Y, Cooper B, Gannon M, Ray M, MacDonald RJ, Wright CV. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 2002;**32**:128-134.
188. Stanger BZ, Tanaka AJ, Melton DA. Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver. Nature 2007;**445**:886-891.
189. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG *et al.* Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nature biotechnology 2006;**24**:1392-1401.
190. Miller CP, McGehee RE, Jr., Habener JF. IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene. Embo J 1994;**13**:1145-1156.
191. Ohlsson H, Karlsson K, Edlund T. IPF1, a homeodomain-containing transactivator of the insulin gene. Embo J 1993;**12**:4251-4259.

192. Babu DA, Chakrabarti SK, Garmey JC, Mirmira RG. Pdx1 and BETA2/NeuroD1 participate in a transcriptional complex that mediates short-range DNA looping at the insulin gene. J Biol Chem 2008;**283**:8164-8172.
193. Peers B, Leonard J, Sharma S, Teitelman G, Montminy MR. Insulin expression in pancreatic islet cells relies on cooperative interactions between the helix loop helix factor E47 and the homeobox factor STF-1. Molecular endocrinology (Baltimore, Md 1994;**8**:1798-1806.
194. Serup P, Jensen J, Andersen FG, Jorgensen MC, Blume N, Holst JJ *et al.* Induction of insulin and islet amyloid polypeptide production in pancreatic islet glucagonoma cells by insulin promoter factor 1. Proc Natl Acad Sci U S A 1996;**93**:9015-9020.
195. Liu Y, MacDonald RJ, Swift GH. DNA binding and transcriptional activation by a PDX1.PBX1b.MEIS2b trimer and cooperation with a pancreas-specific basic helix-loop-helix complex. J Biol Chem 2001;**276**:17985-17993.
196. Swift GH, Rose SD, MacDonald RJ. An element of the elastase I enhancer is an overlapping bipartite binding site activated by a heteromeric factor. J Biol Chem 1994;**269**:12809-12815.
197. Dutta S, Gannon M, Peers B, Wright C, Bonner-Weir S, Montminy M. PDX:PBX complexes are required for normal proliferation of pancreatic cells during development. Proc Natl Acad Sci U S A 2001;**98**:1065-1070.
198. Grapin-Botton A, Majithia AR, Melton DA. Key events of pancreas formation are triggered in gut endoderm by ectopic expression of pancreatic regulatory genes. Genes Dev 2001;**15**:444-454.
199. Massari ME, Murre C. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Molecular and cellular biology 2000;**20**:429-440.
200. Krapp A, Knofler M, Frutiger S, Hughes GJ, Hagenbuchle O, Wellauer PK. The p48 DNA-binding subunit of transcription factor PTF1 is a new exocrine pancreas-specific basic helix-loop-helix protein. Embo J 1996;**15**:4317-4329.
201. Beres TM, Masui T, Swift GH, Shi L, Henke RM, MacDonald RJ. PTF1 is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or its paralogue, RBP-L. Mol Cell Biol 2006;**26**:117-130.
202. Glasgow SM, Henke RM, Macdonald RJ, Wright CV, Johnson JE. Ptf1a determines GABAergic over glutamatergic neuronal cell

fate in the spinal cord dorsal horn. Development 2005;**132**:5461-5469.
203. Hoshino M, Nakamura S, Mori K, Kawauchi T, Terao M, Nishimura YV *et al.* Ptf1a, a bHLH transcriptional gene, defines GABAergic neuronal fates in cerebellum. Neuron 2005;**47**:201-213.
204. Rodolosse A, Chalaux E, Adell T, Hagege H, Skoudy A, Real FX. PTF1alpha/p48 transcription factor couples proliferation and differentiation in the exocrine pancreas [corrected]. Gastroenterology 2004;**127**:937-949.
205. Sellick GS, Barker KT, Stolte-Dijkstra I, Fleischmann C, Coleman RJ, Garrett C *et al.* Mutations in PTF1A cause pancreatic and cerebellar agenesis. Nat Genet 2004;**36**:1301-1305.
206. Goossens A, Gepts W, Saudubray JM, Bonnefont JP, Nihoul F, Heitz PU *et al.* Diffuse and focal nesidioblastosis. A clinicopathological study of 24 patients with persistent neonatal hyperinsulinemic hypoglycemia. Am J Surg Pathol 1989;**13**:766-775.
207. Montgomery EA, Popek EJ. Intussusception, adenovirus, and children: a brief reaffirmation. Human pathology 1994;**25**:169-174.
208. Mourra N, Tiret E, Caplin S, Gendre JP, Parc R, Flejou JF. Involvement of Meckel diverticulum in Crohn disease associated with pancreatic heterotopia. Archives of pathology & laboratory medicine 2003;**127**:E99-E100.
209. Xie XY, Strauch E, Sun CC. Choledochal cysts and multilocular cysts of the pancreas. Human pathology 2003;**34**:99-101.
210. Nair RJ, Lawler L, Miller MR. Chronic pancreatitis. American family physician 2007;**76**:1679-1688.
211. Witt H, Luck W, Hennies HC, Classen M, Kage A, Lass U *et al.* Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. Nat Genet 2000;**25**:213-216.
212. Bansal P, Sonnenberg A. Pancreatitis is a risk factor for pancreatic cancer. Gastroenterology 1995;**109**:247-251.
213. Lowenfels AB, Maisonneuve P, DiMagno EP, Elitsur Y, Gates LK, Jr., Perrault J *et al.* Hereditary pancreatitis and the risk of pancreatic cancer. International Hereditary Pancreatitis Study Group. J Natl Cancer Inst 1997;**89**:442-446.
214. Whitcomb DC, Lowe ME. Human pancreatic digestive enzymes. Dig Dis Sci 2007;**52**:1-17.
215. Michaud DS. Epidemiology of pancreatic cancer. Minerva Chir 2004;**59**:99-111.

216. Magee CJ, Ghaneh P, Neoptolemos JP. Surgical and medical therapy for pancreatic carcinoma. Best practice & research 2002;**16**:435-455.
217. Iodice S, Gandini S, Maisonneuve P, Lowenfels AB. Tobacco and the risk of pancreatic cancer: a review and meta-analysis. Langenbeck's archives of surgery / Deutsche Gesellschaft fur Chirurgie 2008;**393**:535-545.
218. Chan JM, Wang F, Holly EA. Vegetable and fruit intake and pancreatic cancer in a population-based case-control study in the San Francisco bay area. Cancer Epidemiol Biomarkers Prev 2005;**14**:2093-2097.
219. Efthimiou E, Crnogorac-Jurcevic T, Lemoine NR, Brentnall TA. Inherited predisposition to pancreatic cancer. Gut 2001;**48**:143-147.
220. Lynch HT, Smyrk T, Kern SE, Hruban RH, Lightdale CJ, Lemon SJ *et al.* Familial pancreatic cancer: a review. Seminars in oncology 1996;**23**:251-275.
221. Warshaw AL, Fernandez-del Castillo C. Pancreatic carcinoma. N Engl J Med 1992;**326**:455-465.
222. Eriksson B, Oberg K. Neuroendocrine tumours of the pancreas. The British journal of surgery 2000;**87**:129-131.
223. Oberg K, Eriksson B. Endocrine tumours of the pancreas. Best practice & research 2005;**19**:753-781.
224. Pour PM, Schmied B. The link between exocrine pancreatic cancer and the endocrine pancreas. Int J Pancreatol 1999;**25**:77-87.
225. Ito T, Tanaka M, Sasano H, Osamura YR, Sasaki I, Kimura W *et al.* Preliminary results of a Japanese nationwide survey of neuroendocrine gastrointestinal tumors. Journal of gastroenterology 2007;**42**:497-500.
226. Panzuto F, Nasoni S, Falconi M, Corleto VD, Capurso G, Cassetta S *et al.* Prognostic factors and survival in endocrine tumor patients: comparison between gastrointestinal and pancreatic localization. Endocrine-related cancer 2005;**12**:1083-1092.
227. Metz DC, Jensen RT. Gastrointestinal neuroendocrine tumors: pancreatic endocrine tumors. Gastroenterology 2008;**135**:1469-1492.
228. Takaori K, Hruban RH, Maitra A, Tanigawa N. Pancreatic intraepithelial neoplasia. Pancreas 2004;**28**:257-262.
229. Hruban RH, Adsay NV, Albores-Saavedra J, Compton C, Garrett ES, Goodman SN *et al.* Pancreatic intraepithelial neoplasia: a new

nomenclature and classification system for pancreatic duct lesions. Am J Surg Pathol 2001;**25**:579-586.

230. Hruban RH, Takaori K, Klimstra DS, Adsay NV, Albores-Saavedra J, Biankin AV *et al.* An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms. Am J Surg Pathol 2004;**28**:977-987.
231. Kern SE, Hruban RH, Hidalgo M, Yeo CJ. An introduction to pancreatic adenocarcinoma genetics, pathology and therapy. Cancer biology & therapy 2002;**1**:607-613.
232. Doi R, Ikeda H, Kobayashi H, Kogire M, Imamura M. Carcinoma in the remnant pancreas after distal pancreatectomy for carcinoma. Eur J Surg Suppl 2003:62-65.
233. Maitra A, Fukushima N, Takaori K, Hruban RH. Precursors to invasive pancreatic cancer. Advances in anatomic pathology 2005;**12**:81-91.
234. Maitra A, Kern SE, Hruban RH. Molecular pathogenesis of pancreatic cancer. Best practice & research 2006;**20**:211-226.
235. Luttges J, Kloppel G. Precancerous conditions of pancreatic carcinoma. J Hepatobiliary Pancreat Surg 2000;**7**:568-574.
236. Maitra A, Adsay NV, Argani P, Iacobuzio-Donahue C, De Marzo A, Cameron JL *et al.* Multicomponent analysis of the pancreatic adenocarcinoma progression model using a pancreatic intraepithelial neoplasia tissue microarray. Mod Pathol 2003;**16**:902-912.
237. Deramaudt T, Rustgi AK. Mutant KRAS in the initiation of pancreatic cancer. Biochim Biophys Acta 2005;**1756**:97-101.
238. Moskaluk CA, Hruban RH, Kern SE. p16 and K-ras gene mutations in the intraductal precursors of human pancreatic adenocarcinoma. Cancer Res 1997;**57**:2140-2143.
239. Yamano M, Fujii H, Takagaki T, Kadowaki N, Watanabe H, Shirai T. Genetic progression and divergence in pancreatic carcinoma. Am J Pathol 2000;**156**:2123-2133.
240. Meeker AK, De Marzo AM. Recent advances in telomere biology: implications for human cancer. Current opinion in oncology 2004;**16**:32-38.
241. van Heek NT, Meeker AK, Kern SE, Yeo CJ, Lillemoe KD, Cameron JL *et al.* Telomere shortening is nearly universal in pancreatic intraepithelial neoplasia. Am J Pathol 2002;**161**:1541-1547.
242. Schutte M, Hruban RH, Geradts J, Maynard R, Hilgers W, Rabindran SK *et al.* Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. Cancer Res 1997;**57**:3126-3130.

243. Luttges J, Galehdari H, Brocker V, Schwarte-Waldhoff I, Henne-Bruns D, Kloppel G *et al.* Allelic loss is often the first hit in the biallelic inactivation of the p53 and DPC4 genes during pancreatic carcinogenesis. Am J Pathol 2001;**158**:1677-1683.
244. Goggins M, Hruban RH, Kern SE. BRCA2 is inactivated late in the development of pancreatic intraepithelial neoplasia: evidence and implications. Am J Pathol 2000;**156**:1767-1771.
245. Wilentz RE, Iacobuzio-Donahue CA, Argani P, McCarthy DM, Parsons JL, Yeo CJ *et al.* Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. Cancer Res 2000;**60**:2002-2006.
246. Zamboni G, Scarpa A, Bogina G, Iacono C, Bassi C, Talamini G *et al.* Mucinous cystic tumors of the pancreas: clinicopathological features, prognosis, and relationship to other mucinous cystic tumors. Am J Surg Pathol 1999;**23**:410-422.
247. Wilentz RE, Albores-Saavedra J, Zahurak M, Talamini MA, Yeo CJ, Cameron JL *et al.* Pathologic examination accurately predicts prognosis in mucinous cystic neoplasms of the pancreas. Am J Surg Pathol 1999;**23**:1320-1327.
248. Iacobuzio-Donahue CA, Wilentz RE, Argani P, Yeo CJ, Cameron JL, Kern SE *et al.* Dpc4 protein in mucinous cystic neoplasms of the pancreas: frequent loss of expression in invasive carcinomas suggests a role in genetic progression. Am J Surg Pathol 2000;**24**:1544-1548.
249. Jimenez RE, Warshaw AL, Z'Graggen K, Hartwig W, Taylor DZ, Compton CC *et al.* Sequential accumulation of K-ras mutations and p53 overexpression in the progression of pancreatic mucinous cystic neoplasms to malignancy. Annals of surgery 1999;**230**:501-509; discussion 509-511.
250. Kobari M, Egawa S, Shibuya K, Shimamura H, Sunamura M, Takeda K *et al.* Intraductal papillary mucinous tumors of the pancreas comprise 2 clinical subtypes: differences in clinical characteristics and surgical management. Arch Surg 1999;**134**:1131-1136.
251. Sugiyama M, Izumisato Y, Abe N, Masaki T, Mori T, Atomi Y. Predictive factors for malignancy in intraductal papillary-mucinous tumours of the pancreas. The British journal of surgery 2003;**90**:1244-1249.
252. Tanaka M, Kobayashi K, Mizumoto K, Yamaguchi K. Clinical aspects of intraductal papillary mucinous neoplasm of the pancreas. Journal of gastroenterology 2005;**40**:669-675.

253. Terris B, Ponsot P, Paye F, Hammel P, Sauvanet A, Molas G *et al.* Intraductal papillary mucinous tumors of the pancreas confined to secondary ducts show less aggressive pathologic features as compared with those involving the main pancreatic duct. Am J Surg Pathol 2000;**24**:1372-1377.
254. Adsay NV, Merati K, Andea A, Sarkar F, Hruban RH, Wilentz RE *et al.* The dichotomy in the preinvasive neoplasia to invasive carcinoma sequence in the pancreas: differential expression of MUC1 and MUC2 supports the existence of two separate pathways of carcinogenesis. Mod Pathol 2002;**15**:1087-1095.
255. Adsay NV, Merati K, Basturk O, Iacobuzio-Donahue C, Levi E, Cheng JD *et al.* Pathologically and biologically distinct types of epithelium in intraductal papillary mucinous neoplasms: delineation of an "intestinal" pathway of carcinogenesis in the pancreas. Am J Surg Pathol 2004;**28**:839-848.
256. Yoshizawa K, Nagai H, Sakurai S, Hironaka M, Morinaga S, Saitoh K *et al.* Clonality and K-ras mutation analyses of epithelia in intraductal papillary mucinous tumor and mucinous cystic tumor of the pancreas. Virchows Arch 2002;**441**:437-443.
257. Iacobuzio-Donahue CA, Klimstra DS, Adsay NV, Wilentz RE, Argani P, Sohn TA *et al.* Dpc-4 protein is expressed in virtually all human intraductal papillary mucinous neoplasms of the pancreas: comparison with conventional ductal adenocarcinomas. Am J Pathol 2000;**157**:755-761.
258. Sahin F, Maitra A, Argani P, Sato N, Maehara N, Montgomery E *et al.* Loss of Stk11/Lkb1 expression in pancreatic and biliary neoplasms. Mod Pathol 2003;**16**:686-691.
259. Schonleben F, Qiu W, Ciau NT, Ho DJ, Li X, Allendorf JD *et al.* PIK3CA mutations in intraductal papillary mucinous neoplasm/carcinoma of the pancreas. Clin Cancer Res 2006;**12**:3851-3855.
260. Singh M, Maitra A. Precursor lesions of pancreatic cancer: molecular pathology and clinical implications. Pancreatology 2007;**7**:9-19.
261. Caldas C, Kern SE. K-ras mutation and pancreatic adenocarcinoma. Int J Pancreatol 1995;**18**:1-6.
262. Sibilia M, Fleischmann A, Behrens A, Stingl L, Carroll J, Watt FM *et al.* The EGF receptor provides an essential survival signal for SOS-dependent skin tumor development. Cell 2000;**102**:211-220.
263. Korc M, Chandrasekar B, Yamanaka Y, Friess H, Buchier M, Beger HG. Overexpression of the epidermal growth factor receptor in human pancreatic cancer is associated with concomitant

increases in the levels of epidermal growth factor and transforming growth factor alpha. J Clin Invest 1992;**90**:1352-1360.

264. Watanabe M, Nobuta A, Tanaka J, Asaka M. An effect of K-ras gene mutation on epidermal growth factor receptor signal transduction in PANC-1 pancreatic carcinoma cells. International journal of cancer 1996;**67**:264-268.
265. Day JD, Digiuseppe JA, Yeo C, Lai-Goldman M, Anderson SM, Goodman SN *et al.* Immunohistochemical evaluation of HER-2/neu expression in pancreatic adenocarcinoma and pancreatic intraepithelial neoplasms. Human pathology 1996;**27**:119-124.
266. Nowak NJ, Gaile D, Conroy JM, McQuaid D, Cowell J, Carter R *et al.* Genome-wide aberrations in pancreatic adenocarcinoma. Cancer Genet Cytogenet 2005;**161**:36-50.
267. Shapiro GI, Edwards CD, Rollins BJ. The physiology of p16(INK4A)-mediated G1 proliferative arrest. Cell biochemistry and biophysics 2000;**33**:189-197.
268. Rozenblum E, Schutte M, Goggins M, Hahn SA, Panzer S, Zahurak M *et al.* Tumor-suppressive pathways in pancreatic carcinoma. Cancer Res 1997;**57**:1731-1734.
269. Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E *et al.* DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 1996;**271**:350-353.
270. Siegel PM, Massague J. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat Rev Cancer 2003;**3**:807-821.
271. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. Science 2001;**293**:1068-1070.
272. Robert MF, Morin S, Beaulieu N, Gauthier F, Chute IC, Barsalou A *et al.* DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. Nat Genet 2003;**33**:61-65.
273. Wang W, Gao J, Man XH, Li ZS, Gong YF. Significance of DNA methyltransferase-1 and histone deacetylase-1 in pancreatic cancer. Oncology reports 2009;**21**:1439-1447.
274. Klump B, Hsieh CJ, Nehls O, Dette S, Holzmann K, Kiesslich R *et al.* Methylation status of p14ARF and p16INK4a as detected in pancreatic secretions. British journal of cancer 2003;**88**:217-222.
275. Jansen M, Fukushima N, Rosty C, Walter K, Altink R, Heek TV *et al.* Aberrant methylation of the 5' CpG island of TSLC1 is common in pancreatic ductal adenocarcinoma and is first manifest in high-grade PanINs. Cancer biology & therapy 2002;**1**:293-296.

276. Okami J, Simeone DM, Logsdon CD. Silencing of the hypoxia-inducible cell death protein BNIP3 in pancreatic cancer. Cancer research 2004;**64**:5338-5346.
277. Sato N, Goggins M. The role of epigenetic alterations in pancreatic cancer. Journal of hepato-biliary-pancreatic surgery 2006;**13**:286-295.
278. Nakayama S, Semba S, Maeda N, Matsushita M, Kuroda Y, Yokozaki H. Hypermethylation-mediated reduction of WWOX expression in intraductal papillary mucinous neoplasms of the pancreas. British journal of cancer 2009;**100**:1438-1443.
279. Yamada N, Nishida Y, Tsutsumida H, Hamada T, Goto M, Higashi M *et al.* MUC1 expression is regulated by DNA methylation and histone H3 lysine 9 modification in cancer cells. Cancer Res 2008;**68**:2708-2716.
280. Yamada N, Hamada T, Goto M, Tsutsumida H, Higashi M, Nomoto M *et al.* MUC2 expression is regulated by histone H3 modification and DNA methylation in pancreatic cancer. International journal of cancer 2006;**119**:1850-1857.
281. Niederau C, Ferrell LD, Grendell JH. Caerulein-induced acute necrotizing pancreatitis in mice: protective effects of proglumide, benzotript, and secretin. Gastroenterology 1985;**88**:1192-1204.
282. Wang RN, Kloppel G, Bouwens L. Duct- to islet-cell differentiation and islet growth in the pancreas of duct-ligated adult rats. Diabetologia 1995;**38**:1405-1411.
283. Lardon J, Bouwens L. Metaplasia in the pancreas. Differentiation 2005;**73**:278-286.
284. Rooman I, De Medts N, Baeyens L, Lardon J, De Breuck S, Heimberg H *et al.* Expression of the Notch signaling pathway and effect on exocrine cell proliferation in adult rat pancreas. Am J Pathol 2006;**169**:1206-1214.
285. Kleeff J, Friess H, Berberat PO, Martignoni ME, Z'Graggen K, Buchler MW. Pancreatic cancer--new aspects of molecular biology research. Swiss surgery = Schweizer Chirurgie = Chirurgie suisse = Chirurgia svizzera 2000;**6**:231-234.
286. Jhappan C, Stahle C, Harkins RN, Fausto N, Smith GH, Merlino GT. TGF alpha overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell 1990;**61**:1137-1146.
287. Sandgren EP, Luetteke NC, Palmiter RD, Brinster RL, Lee DC. Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. Cell 1990;**61**:1121-1135.

288. Greten FR, Wagner M, Weber CK, Zechner U, Adler G, Schmid RM. TGF alpha transgenic mice. A model of pancreatic cancer development. Pancreatology 2001;**1**:363-368.
289. Schmid RM, Kloppel G, Adler G, Wagner M. Acinar-ductal-carcinoma sequence in transforming growth factor-alpha transgenic mice. Annals of the New York Academy of Sciences 1999;**880**:219-230.
290. Schleger C, Verbeke C, Hildenbrand R, Zentgraf H, Bleyl U. c-MYC activation in primary and metastatic ductal adenocarcinoma of the pancreas: incidence, mechanisms, and clinical significance. Mod Pathol 2002;**15**:462-469.
291. Han H, Bearss DJ, Browne LW, Calaluce R, Nagle RB, Von Hoff DD. Identification of differentially expressed genes in pancreatic cancer cells using cDNA microarray. Cancer Res 2002;**62**:2890-2896.
292. Mahlamaki EH, Barlund M, Tanner M, Gorunova L, Hoglund M, Karhu R *et al.* Frequent amplification of 8q24, 11q, 17q, and 20q-specific genes in pancreatic cancer. Genes, chromosomes & cancer 2002;**35**:353-358.
293. Silverman JA, Kuhlmann ET, Zurlo J, Yager JD, Longnecker DS. Expression of c-myc, c-raf-1, and c-Ki-ras in azaserine-induced pancreatic carcinomas and growing pancreas in rats. Molecular carcinogenesis 1990;**3**:379-386.
294. Sandgren EP, Quaife CJ, Paulovich AG, Palmiter RD, Brinster RL. Pancreatic tumor pathogenesis reflects the causative genetic lesion. Proc Natl Acad Sci U S A 1991;**88**:93-97.
295. Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA *et al.* Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 2003;**4**:437-450.
296. Guerra C, Schuhmacher AJ, Canamero M, Grippo PJ, Verdaguer L, Perez-Gallego L *et al.* Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. Cancer Cell 2007;**11**:291-302.
297. Baeyens L, Bouwens L. Can beta-cells be derived from exocrine pancreas? Diabetes, obesity & metabolism 2008;**10 Suppl 4**:170-178.
298. Baeyens L, De Breuck S, Lardon J, Mfopou JK, Rooman I, Bouwens L. In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells. Diabetologia 2005;**48**:49-57.

299. Shen CN, Slack JM, Tosh D. Molecular basis of transdifferentiation of pancreas to liver. Nat Cell Biol 2000;**2**:879-887.
300. Rooman I, Lardon J, Bouwens L. Gastrin stimulates beta-cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreas tissue. Diabetes 2002;**51**:686-690.
301. Rooman I, Lardon J, Flamez D, Schuit F, Bouwens L. Mitogenic effect of gastrin and expression of gastrin receptors in duct-like cells of rat pancreas. Gastroenterology 2001;**121**:940-949.
302. Plachot C, Portha B. Impaired pancreatic duct-cell growth in focal areas of regeneration after partial pancreatectomy in the adult Goto-Kakizaki rat, a spontaneous model of non-insulin dependent diabetes mellitus. The Histochemical journal 2001;**33**:141-147.
303. Miyamoto Y, Maitra A, Ghosh B, Zechner U, Argani P, Iacobuzio-Donahue CA *et al.* Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. Cancer Cell 2003;**3**:565-576.
304. Grippo PJ, Nowlin PS, Demeure MJ, Longnecker DS, Sandgren EP. Preinvasive pancreatic neoplasia of ductal phenotype induced by acinar cell targeting of mutant Kras in transgenic mice. Cancer Res 2003;**63**:2016-2019.
305. Chen H, Gu X, Su IH, Bottino R, Contreras JL, Tarakhovsky A *et al.* Polycomb protein Ezh2 regulates pancreatic beta-cell Ink4a/Arf expression and regeneration in diabetes mellitus. Genes Dev 2009;**23**:975-985.
306. Dhawan S, Tschen SI, Bhushan A. Bmi-1 regulates the Ink4a/Arf locus to control pancreatic beta-cell proliferation. Genes Dev 2009;**23**:906-911.
307. Tschen SI, Dhawan S, Gurlo T, Bhushan A. Age-dependent decline in beta-cell proliferation restricts the capacity of beta-cell regeneration in mice. Diabetes 2009;**58**:1312-1320.
308. Sangiorgi E, Capecchi MR. Bmi1 lineage tracing identifies a self-renewing pancreatic acinar cell subpopulation capable of maintaining pancreatic organ homeostasis. Proc Natl Acad Sci U S A 2009;**106**:7101-7106.
309. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. The Journal of general virology 1977;**36**:59-74.
310. Ornitz DM, Palmiter RD, Messing A, Hammer RE, Pinkert CA, Brinster RL. Elastase I promoter directs expression of human

growth hormone and SV40 T antigen genes to pancreatic acinar cells in transgenic mice. Cold Spring Harb Symp Quant Biol 1985;**50**:399-409.

311. Hernandez-Munoz I, Lund AH, van der Stoop P, Boutsma E, Muijrers I, Verhoeven E *et al.* Stable X chromosome inactivation involves the PRC1 Polycomb complex and requires histone MACROH2A1 and the CULLIN3/SPOP ubiquitin E3 ligase. Proc Natl Acad Sci U S A 2005;**102**:7635-7640.
312. Gesina E, Blondeau B, Milet A, Le Nin I, Duchene B, Czernichow P *et al.* Glucocorticoid signalling affects pancreatic development through both direct and indirect effects. Diabetologia 2006;**49**:2939-2947.
313. Witte V, Laffert B, Rosorius O, Lischka P, Blume K, Galler G *et al.* HIV-1 Nef mimics an integrin receptor signal that recruits the polycomb group protein Eed to the plasma membrane. Mol Cell 2004;**13**:179-190.
314. Su IH, Dobenecker MW, Dickinson E, Oser M, Basavaraj A, Marqueron R *et al.* Polycomb group protein ezh2 controls actin polymerization and cell signaling. Cell 2005;**121**:425-436.
315. Atsuta T, Fujimura S, Moriya H, Vidal M, Akasaka T, Koseki H. Production of monoclonal antibodies against mammalian Ring1B proteins. Hybridoma 2001;**20**:43-46.
316. Hruban RH, Adsay NV, Albores-Saavedra J, Anver MR, Biankin AV, Boivin GP *et al.* Pathology of genetically engineered mouse models of pancreatic exocrine cancer: consensus report and recommendations. Cancer research 2006;**66**:95-106.
317. . *genepaint.org* 2009. http://www.genepaint.org/.
318. Liao DJ, Wang Y, Wu J, Adsay NV, Grignon D, Khanani F *et al.* Characterization of pancreatic lesions from MT-tgfalpha, Ela-myc and MT-tgfalpha/Ela-myc single and double transgenic mice. J Carcinog 2006;**5**:19.
319. Thorgeirsson SS, Factor VM, Snyderwine EG. Transgenic mouse models in carcinogenesis research and testing. Toxicology letters 2000;**112-113**:553-555.
320. Liao DJ, Dickson RB. c-Myc in breast cancer. Endocrine-related cancer 2000;**7**:143-164.
321. Liao DJ, Natarajan G, Deming SL, Jamerson MH, Johnson M, Chepko G *et al.* Cell cycle basis for the onset and progression of c-Myc-induced, TGFalpha-enhanced mouse mammary gland carcinogenesis. Oncogene 2000;**19**:1307-1317.

322. Lynch HT, Brand RE, Lynch JF, Fusaro RM, Kern SE. Hereditary factors in pancreatic cancer. J Hepatobiliary Pancreat Surg 2002;**9**:12-31.
323. Swift GH, Kruse F, MacDonald RJ, Hammer RE. Differential requirements for cell-specific elastase I enhancer domains in transfected cells and transgenic mice. Genes Dev 1989;**3**:687-696.
324. Kim JH, Yoon SY, Kim CN, Joo JH, Moon SK, Choe IS *et al.* The Bmi-1 oncoprotein is overexpressed in human colorectal cancer and correlates with the reduced p16INK4a/p14ARF proteins. Cancer letters 2004;**203**:217-224.
325. Takihara Y. Role of Polycomb-group genes in sustaining activities of normal and malignant stem cells. Int J Hematol 2008;**87**:25-34.
326. Sangiorgi E, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. Nat Genet 2008;**40**:915-920; DOI ng.165 [pii]
10.1038/ng.165.
327. Sanchez-Beato M, Sanchez E, Gonzalez-Carrero J, Morente M, Diez A, Sanchez-Verde L *et al.* Variability in the expression of polycomb proteins in different normal and tumoral tissues. A pilot study using tissue microarrays. Mod Pathol 2006;**19**:684-694; DOI 3800577 [pii]
10.1038/modpathol.3800577.
328. Zhou Q, Law AC, Rajagopal J, Anderson WJ, Gray PA, Melton DA. A multipotent progenitor domain guides pancreatic organogenesis. Developmental cell 2007;**13**:103-114.
329. Pietersen AM, Evers B, Prasad AA, Tanger E, Cornelissen-Steijger P, Jonkers J *et al.* Bmi1 regulates stem cells and proliferation and differentiation of committed cells in mammary epithelium. Curr Biol 2008;**18**:1094-1099; DOI S0960-9822(08)00874-9 [pii]
10.1016/j.cub.2008.06.070.
330. Maitra A, Hruban RH. Pancreatic cancer. Annual review of pathology 2008;**3**:157-188.
331. Stanger BZ, Stiles B, Lauwers GY, Bardeesy N, Mendoza M, Wang Y *et al.* Pten constrains centroacinar cell expansion and malignant transformation in the pancreas. Cancer Cell 2005;**8**:185-195; DOI S1535-6108(05)00236-9 [pii]
10.1016/j.ccr.2005.07.015.
332. Boutsma E, Noback S, van Lohuizen M. The Polycomb protein and E3 ubiquitin ligase Ring1B harbors an IRES in its highly conserved 5' UTR. PLoS ONE 2008;**3**:e2322.
333. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell

proliferation and senescence through the ink4a locus. Nature 1999;**397**:164-168.

334. Bracken AP, Kleine-Kohlbrecher D, Dietrich N, Pasini D, Gargiulo G, Beekman C *et al.* The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes & development 2007;**21**:525-530.
335. Sherr CJ. The INK4a/ARF network in tumour suppression. Nature reviews 2001;**2**:731-737.
336. Fukushima N, Sato N, Ueki T, Rosty C, Walter KM, Wilentz RE *et al.* Aberrant methylation of preproenkephalin and p16 genes in pancreatic intraepithelial neoplasia and pancreatic ductal adenocarcinoma. Am J Pathol 2002;**160**:1573-1581.
337. Rosty C, Geradts J, Sato N, Wilentz RE, Roberts H, Sohn T *et al.* p16 Inactivation in pancreatic intraepithelial neoplasias (PanINs) arising in patients with chronic pancreatitis. Am J Surg Pathol 2003;**27**:1495-1501.
338. Datta S, Hoenerhoff MJ, Bommi P, Sainger R, Guo WJ, Dimri M *et al.* Bmi-1 cooperates with H-Ras to transform human mammary epithelial cells via dysregulation of multiple growth-regulatory pathways. Cancer research 2007;**67**:10286-10295; DOI 67/21/10286 [pii]

10.1158/0008-5472.CAN-07-1636.

339. Hoenerhoff MJ, Chu I, Barkan D, Liu ZY, Datta S, Dimri GP *et al.* BMI1 cooperates with H-RAS to induce an aggressive breast cancer phenotype with brain metastases. Oncogene 2009;**28**:3022-3032; DOI onc2009165 [pii]

10.1038/onc.2009.165.

340. Wei J, Zhai L, Xu J, Wang H. Role of Bmi1 in H2A ubiquitylation and Hox gene silencing. J Biol Chem 2006;**281**:22537-22544; DOI M600826200 [pii]

10.1074/jbc.M600826200.

341. Real FX, Cibrian-Uhalte E, Martinelli P. Pancreatic cancer development and progression: remodeling the model. Gastroenterology 2008;**135**:724-728; DOI S0016-5085(08)01419-4 [pii]

10.1053/j.gastro.2008.07.033.

342. Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M *et al.* Tumour biology: senescence in premalignant tumours. Nature 2005;**436**:642; DOI 436642a [pii]

10.1038/436642a.

The printing of this thesis was financially assisted by Fundació IMIM.