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Investigating how p110α PI3K and ADAM10 contribute to pancreatic disease

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Jason Hall

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend

acceptance of this dissertation.

Howard Crawford, Ph.D.
Associate Professor, Department of Cancer Biology,
Mayo Clinic Cancer Center

Michael Frohman, M.D, Ph.D.
Professor and Chair, Department of Molecular and Cellular Pharmacology

Richard Z. Lin, M.D.
Professor, Department of Physiology and Biophysics

Feng-Qian Li, Ph.D.
Associate Professor, Department of Molecular and Cellular Pharmacology

Yupo Ma, M.D, Ph.D.
Professor, Department of Pathology

This dissertation is accepted by the Graduate School

Charles Taber
Dean of the Graduate School

Abstract of the Dissertation

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Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer-related deaths in the United States, with a 6% 5-year survival rate. Chronic pancreatitis (CP) is a debilitating disease that raises your risk for developing PDAC 13 fold. Since current therapies are not improving patient outcome, understanding the molecular mechanisms behind the formation and progression of these diseases are imperative for finding new molecularly targeted therapy. Kras is the earliest and most commonly mutated oncogene in PDAC. Pancreatic mouse models expressing oncogenic Kras spontaneously form pancreatic tumors. Since targeting Kras directly has been unsuccessful many have tried inhibiting other molecules in the cell-signaling pathway to block tumor formation. Previously, we have discovered that blocking molecules in the epidermal growth factor receptor (EGFR) pathway inhibits Kras activity and prevents tumor formation at an early stage of epithelial change. Acinar to ductal metaplasia (ADM) is an event commonly seen in CP, where acinar cells change structure to express a ductal phenotype. Normally, ADM

in CP progresses no further, but it is hypothesized that these metaplastic cells can become tumors if they express mutant Kras. My first study examines the catalytic subunit of phosphoinositide 3-kinase (PI3K), p110α, an enzyme that associates with Kras and is activated by receptor tyrosine kinases, such as EGFR. Here I show that knocking out or pharmacologically inhibiting p110α in mice prevents the development of CP. ADM and subsequent inflammatory responses in this disease does not occur when these mice are subjected to experimental pancreatitis. The actin-cytoskeleton rearrangement necessary for ADM is also blocked when p110α is ablated or inhibited, suggesting that p110α may have a role in actin remodeling. Secondly, I investigated A disintegrin and metalloproteinase (ADAM10), a protease known for causing the activation of Notch, whose signaling pathway has been shown to be downstream of EGFR. By ablating ADAM10 in a PDAC mouse model, we show tumor progression is altered leading to the development of large cystic lesions, decreased metastasis and a longer lifespan. In summary, my work demonstrates that p110α and ADAM10 may independently alter the course of pancreatic disease and may potentially be therapeutic targets.

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List of Abbreviations

ADAM A Disintegrin And Metalloproteinase
ADM Acinar-to-Ductal Metaplasia
AP Acute Pancreatitis
CCK Cholecystokinin
CP Chronic Pancreatitis
Cox Cyclooxygenase
CT Computer tomography
DMEM Dulbecco's Modified Eagle's medium
EGFR Epidermal Growth Factor Receptor
ERCP Endoscopic retrograde cholangiopancreatography
FBS Fetal Bovine Serum
GEF Guanine exchange factor
HBSS Hank's Buffered Saline Solution
NICD Notch Intra-Cellular Domain
IGFR Insulin growth factor receptor
IHC Immunohistochemistry
IP Intraperitoneal
Kuz Kuzbanian
MAPK Mitogen-activated protein kinase
MAML Mastermind
MEF Mouse embryonic fibroblasts
MCN Mucinus cystic neoplasms
MDL Metaplastic Ductal Lesion
MRCP Magnetic resonance cholangiopancreatography
PanIN Pancreatic Intraepithelial Neoplasia
PIK PI3K accessory domain
PI3K Phosphoinositide-3 kinase
PBS Phosphate Buffered Saline
PDAC Pancreatic Ductal Adenocarcinoma
PFA Paraformaldehyde
PTDIN Phosotidylinositol
RIP Regulated intra-membrane proteolysis
RTK Receptor Tyrosine Kinase
RegPr Regulatory binding domain
SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SVMP Snake venom metalloproteases
TBS Tris Buffered Saline
WT Wild type

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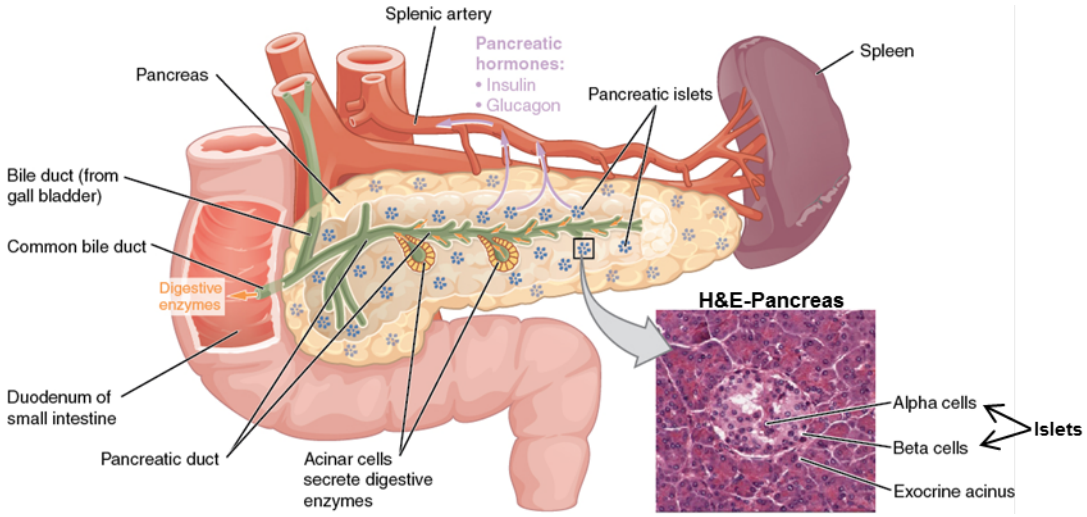
Chapter 1: Introduction to Pancreatic Disease and Associated Signaling Molecules

1-1 Introduction of Pancreatitis and Pancreatic Cancer

i. Anatomy and Physiology of the Pancreas

The pancreas is an organ in the gastrointestinal tract that facilitates digestion and regulates glucose metabolism[1]. Separate compartments of the pancreas, known as the exocrine and endocrine systems, regulate the two functions[1]. The endocrine compartment makes up about 1% of the pancreas and is composed of the Islets of Langerhans[1]. These structures are found throughout the pancreas and it generates the hormones (glucose, insulin, somatostatin) required for blood sugar regulation[1]. The other 99% of pancreas is composed of the exocrine compartment [1].The exocrine system is composed of acinar cells, that exist as berry-like clusters and generate digestive enzymes which are secreted into the lumen and flow into a branch like network of ducts that eventually converges into the main pancreatic duct[1]. Subsequently the enzymes flow into the small intestine to assist in the breakdown of food[1]. **Fig.1-1** shows an illustration and micrograph of the pancreas and its compartments. The portion of the pancreas that is attached to the spleen is referred to as the tail of the pancreas and the portion connected to the duodenum is the head[2].



Adapted from OpenStax College. (2013, June 19). The Endocrine Pancreas.

Figure 1-1 Pancreas anatomy

Illustration of the pancreas and its orientation with other internal organs (duodenum, spleen). Pancreatic compartments are labeled. Acinar cells and islets are depicted by hematoxylin and eosin stain (H&E).

ii. Pathology of Pancreatitis

Acute pancreatitis (AP) is the most common gastrointestinal disease that requires hospitalization in the United States, affecting 274,000 patients in 2012[3,4]. The number of hospitalizations for this illness has risen 30% since 2000[3,4]. Chronic Pancreatitis (CP), while not diagnosed as frequently still carries a severe prognosis, which is believed to stem from repeated bouts of AP[5]. AP is caused by the premature activation of the digestive enzymes, such as trypsin, leading to tissue auto-digestion (cell death) and a strong immune response[5]. The most prominent symptoms are abdominal and back pains[6]. Since patient symptoms are severe but general pains, the disease can only be properly diagnosed by computer

tomography (CT) and magnetic resonance cholangiopancreatography (MRCP)[6]. Imaging analysis shows edema, gas accumulation, and enlargement of pancreas[7]. CP is characterized by inflammation, fibrosis, and acinar cell loss[8]. Symptomatic pains are similar to acute pancreatitis, and patients may experience weight loss[8]. CT and MRCP are standard detections as well. Additionally, physicians look for the calcification and enlargement of the pancreas[5,8].

When searching for causes and risk factors for pancreatitis, researchers found a genetic component to be influential to AP and CP. Genes that regulate digestive enzyme production are mutated in a subset of pancreatitis patients. A mutation in the trypsinogen encoding gene, PSSR1 has been a well documented cause of familial pancreatitis[9]. While first manifesting as acute pancreatitis, people with familial mutations have an increased chance of developing chronic pancreatitis and a 50 fold chance of getting pancreatic cancer[10,11]. Though identifying genetic mutations in humans may give us insight into the cellular mechanisms that bring about this disease, environmental factors pose a greater risk. Obesity related gallstone formation remains the leading cause of acute pancreatitis, while chronic alcohol consumption is the greatest cause of chronic pancreatitis and second most common cause of acute attacks[3].

Treatment for AP and CP are limited, Endoscopic retrograde cholangiopancreatography (ERCP) therapy is available to those with gallstones but other sources of pancreatitis cannot be directly alleviated[8]. Through out the years, *in vivo* models of pancreatitis have been used to study the disease; currently new therapeutic targets are being examined in these models in hopes of finding new treatment options.

iii. Modeling Pancreatitis in mice

Various species of animals have been used to model pancreatitis throughout the years. Rodents are a favorable choice due to low maintenance costs, and their ability to produce a large sample size in a relatively short amount of time. In addition, mice can be genetically engineered, opening up a large field of research dedicated to studying how the loss or expression of certain genes can affect an organism. For these reasons, we conducted our studies using mouse models.

To model pancreatitis, mice were administered a series of intraperitoneal (IP) injections of caerulein, an analog of cholecystokinin (CCK). Both caerulein and CCK induce the secretion of digestive enzymes in the pancreas, a supramaximal dose results in the intracellular activation of digestive enzymes, which leads to auto-digestion of the pancreas and the recapitulation of pancreatitis[12,13]. **Figure 1-2** exhibits how the diseased pancreas appears compared to normal tissue. Acute pancreatitis is characterized by edema, vacuolization, acinar cell swelling, and focal areas of necrosis (**Figure 1-2)**. Chronic pancreatitis has stromal infiltration along with metaplastic ductal lesions and a depletion of acinar cells (**Figure 1-2)**. Depending on the dosage and length of treatment, the pathology of both acute and chronic pancreatitis can be observed and the molecular signals that cause acute bouts to become chronic can be elucidated[5].

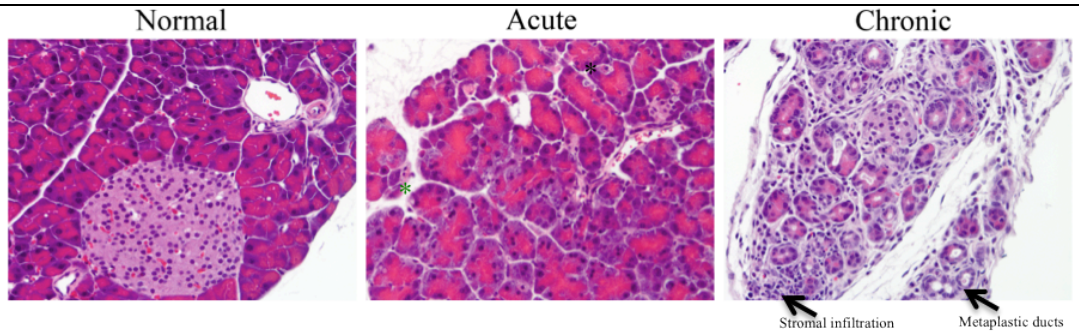


Figure 1-2 Histology of pancreatitis

H&E stain depicts acini, ducts, and islets, which compose a normal pancreas. The acini enlarge in acute pancreatitis (middle). Areas of discoloration indicate necrosis (*). Vacuolization, an indicator of auto-digestion is also visible (*). Chronic pancreatitis (right) is represented by the loss of acinar cells, accumulation of fibrotic tissue (arrow), and the appearance of metaplastic lesions (arrow).

iv. PDAC-Pathogenesis and Progression

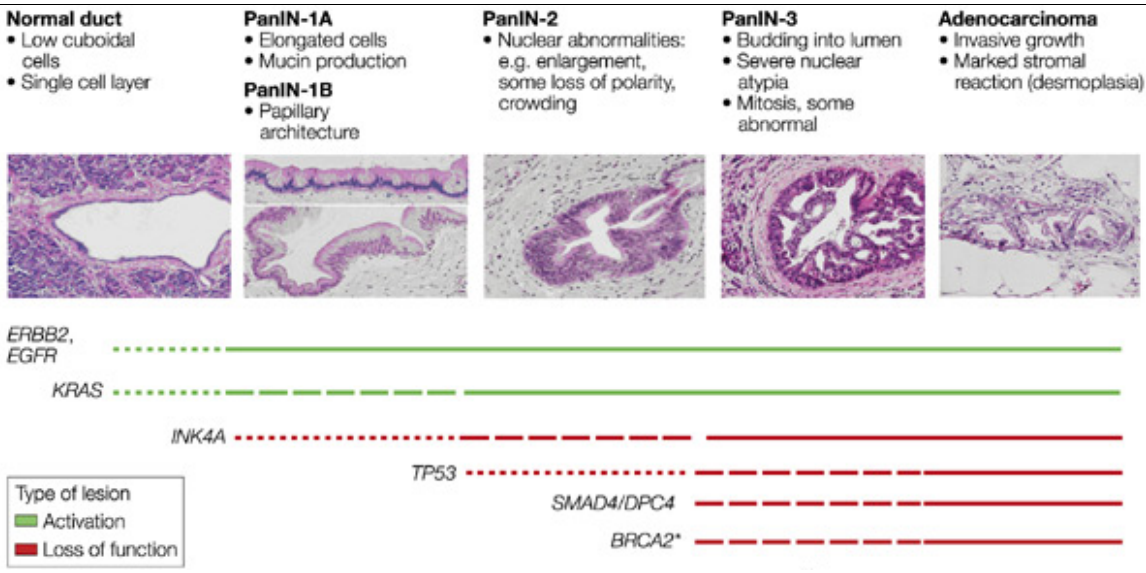
Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer-related death in the United States[14]. Chemotherapy and radiation therapy does little to effectively treat patients with PDAC, leaving surgery the only option. Sadly, even after surgery only 20% of the patients diagnosed with PDAC survive past five years[14]. Since current forms of treatment are not effective in ameliorating patient outcome, understanding the molecular mechanism behind the formation and progression of PDAC is imperative for discovering new targets for treatment.

The source of invasive carcinoma has been traced back to precursor tumor lesions known as pancreatic intra-epithelial neoplasia (Pan-IN)[15]. PanINs have been classified by a grading system, based on histological features[1,15]. PanIN-1 is a low-grade tumor structure

that resembles a pancreatic duct but is distinguished by elongated columnar cells with a thick mucinus layer **(Figure 1-3)**. PanIN-2 nuclei are no longer basally oriented (loss of polarity), and their cells are crowded and pseudostratified **(Figure 1-3).** PanIN-3 tumors have cells budding off into the lumen **(Figure 1-3).** Lastly, when the tumor breaks through the basement membrane it becomes truly invasive and is classified as PDAC **(Figure 1-3)**. PDAC development is described in the PanIN progression model, even the same genetic mutations are found at particular stages of diseases **(Figure 1-3).**

There are both activating mutation and loss of function mutations that shape PDAC progression[16]. KRAS is one of the earliest and most common oncogene mutated in PDAC; increased protein activity is found in early Pan-IN lesions and remains high in advanced tumors[17,18]. Kras is a small g-protein, responsible for activating the mitogen-activated protein kinase (MAPK) pathway, which induces cellular proliferation, survival, and invasion[19-21]. Kras functions as a GTPase; GTP bound Kras assumes a conformational state that allows for protein binding and activation of downstream signaling molecules[20]. Kras regulates its activity by hydrolyzing GTP to GDP; converting the active GTP bound protein into its GDP bound inactive form [22,23]. The KRAS mutations found in PanINs and PDAC patients results in Kras remaining in a constitutively active state [17,20]. The pro tumorigenic effects of Kras-MAPK signaling are initiated by the activation of growth factor recptors[23], concordantly epidermal growth factor receptor (EGFR) is highly expressed during pancreatic tumor formation and progression **(Figure 1-3).** The upregulation of EGFR and KRAS are critical for early stage tumor formation but it is the loss of tumor suppressor genes that push the PanINs into advanced stages[16,24].

The loss of tumor suppressor genes CDKN2A, TP53, and SMAD44 are observed in PDAC patients[16]. Loss of CDKN2A occurs in moderately advanced lesions **(Figure 1-3)**. CDKN2A encodes for Ink4A (p16), a tumor suppresser gene important for inducing cell cycle arrest[25]. The well studied, TP53 tumor suppressor gene is mutated in late stage tumors **(Figure 1-3)**. The genetic instability induced by a p53 mutation appears to promote the development of carcinoma[26,27]. SMAD4/DPC4 encodes Smad4, a necessary signaling molecule in TGF-β pathway[28]. This gene is lost very late in disease, and its absence is and indicator of poor survival outcome [1]**(Figure 1-3)**. Though not clearly understood, the gene may be important for regulating proliferation in PDAC and the loss of Smad4 may impact tumor-stromal signaling in a manner that may advance disease[1]. Although several other genes may be capable of regulating PDAC, *In vivo* studies in mice show that mutations of CDKN2A, TP53, SMAD4, and KRAS are sufficient to bring about the disease[29].



Adapted from Bardeesy N et al. Nature Reviews. 2012

Figure 1-3 Progression of Pancreatic Ductal Adenocarcinoma (PDAC)

Stages of PanIN progression leading to PDAC are depicted by H&E. Defining characteristics of PanIN-1,2,3 and adenocarcinoma are described above. Aberrant gene activations and loss of function mutations frequently seen throughout the development of PDAC are illustrated above. EGFR and KRAS are activated early in tumorigenesis and remain active during cancer progression. INK4A (P16) is deleted during the early PanIN stages and TP53 and SMAD4 loss occurs at the late stages of the disease

v. Modeling PDAC in mice

Currently, murine models of pancreatic cancer can be divided into 2 classifications: xenograft models and genetically engineered models. Xenograft models are mice that have been implanted with human cancer cells, either patient-derived or from an established cell line [29,30]. The cancer cells can be engrafted either subcutaneously or orthotopically. Though using human cells has the benefit of being representative of the cells you want to target, the tumor microenvironment is not representative unless you engraft cells directly into the pancreas[30]. Using patient-derived cancer cells, while more labor intensive and harder to obtain is a better option than using established cell lines[31]. Cell lines acquire mutations over time that makes the cells lose similarity to recently extracted patient tumors[31]. Also, patient derived cells include the tumor and stromal cells, which will better represent the microenvironment of the disease[30]. A drawback to this model is the need to use immune compromised mice for xenografts, since healthy mice reject cancer cells engraftment[31]. Genetic mouse models give investigators the benefit of studying pancreatic cancer *in situ*.

The mice will produce the tumor in the appropriate microenvironment with an intact immune system. It is critical to maintain the immune system since many studies show that the stromal-epithelial communication is especially important in pancreatic cancer[32]. Genetically modified organisms also give you the rare opportunity to study how cancer forms and becomes invasive[18]. Human cells are at many times too advanced to study progression, while it may take the mutation of one or two genes to establish PDAC in mice, human tumor cells could have at least half a dozen by the time we study them. The genetic mouse models allow us to know which mutations are necessary and which comes first.

To recapitulate PDAC in mouse models, we use the Lox-Stop-Lox $Kras^{G12D}$ (*LSL-KRAS^{G12D}*) knock-in mouse, which carries a loss of GTPase function mutation in the Kras oncogene [17,18]. The *LSL-KRAS^{G12D}* mouse model contains an allele with a $KRAS^{G12D}$ coding region that is targeted to the endogenous KRAS locus [33]. Expression of this gene is prevented by a transcriptional stop element that precedes it [33]. The stop element is flanked by lox-p sites, which can be excised upon Cre recombination [33]. To direct Cre-recombination we use a mouse model where pancreatic transcription factor -1a (Ptf1a) drives Cre expression (*Ptf1a-Cre*). Ptf1a is required for pancreatic development; expression of Ptf1a mRNA appears as early as E.9.5 and protein expression is seen at E.10.5 [33,34]. *The Ptf1a-Cre* mouse has Cre inserted into the Ptf1a locus, replacing the protein-coding region [35]. Only heterozygote mice avoid embryonic lethality because they still have one allele that encodes Ptf1a [35]. When the *LSL-Kras^{G12D}* knock-in mouse is crossed with the pancreatic specific Cre transgenic mouse (*Ptf1a-Cre*), the offspring generated have mutated Kras expressed wherever Ptf1a is. *The LSL-KRAS^{G12D}; Ptf1a-Cre* mice have pathology consistent with human PDAC **(Figure 1-4)**.

H&E and illustration show the morphological changes that the tumor cells undergo as cancer progresses from PanIN-1, 2, 3 to invasive carcinoma **(Figure 1-4)**.

Though it is possible to observe metastasis in the *LSL-KRAS^{G12D}; Ptf1a-Cre* mice, liver and lung metastases do not form until the mice are about one year old [36]. To address invasion and tumor formation at a secondary site, our lab introduces a p53 mutation (p53^{R172H}) to our mice to generate *LSL-KRAS^{G12D}; p53^{R172H};Ptf1a-Cre* mice [24]. These mice develop fast-progressing PDAC that are more suitable for invasion and metastases studies and serves as a model for targeted molecular therapy as well as standard chemotherapies.

Our lab also uses a tamoxifen inducible *Ptf1a-Cre* mouse strain (*Ptf1a$^{Cre\text{-}ERTM/+}$*)[37]. We use this mouse for studies where we want to induce Cre-recombination in adult mice. The *Ptf1a*$^{Cre\text{-}ERTM/+}$ mice express a fusion protein that is Cre-recombinase bound to an estrogen receptor[37,38]. The estrogen receptor confines Cre-recombinase to the cytoplasm until the estrogen analog, tamoxifen binds to the receptor and causes the fusion protein to enter the nucleus[38]. Once in the nucleus, Cre-recombinase is free to excise floxed genes. The benefit of the Ptf1a$^{Cre\text{-}ERTM/+}$ mouse model is that it gives the investigator temporal control of cre recombination[38]. This is beneficial when studying mice with *LSL-KRAS^{G12D} and p53^{R172H}* alleles because we may want to study PDAC progression after the mice are fully-grown. *Ptf1a$^{Cre\text{-}ERTM/+}$* mice are also usefully when studying ADM and early PanIN lesion. While Ptfla is expressed in pancreatic progenitor cells during development, the adult mouse only expresses Ptf1a in acinar cells[37]. Therefore, tumors from *LSL-KRAS^{G12D}; Ptf1a$^{Cre\text{-}ERTM/+}$* mice are of acinar origin. In order to assess the efficiency of Cre-recombination, the *Ptf1a$^{Cre\text{-}ERTM/+}$* mice carry a yellow fluorescent protien (YFP) transgene that is only expressed when Cre-recombinase excises the lox-stop-lox transcript that precedes it (*ROSA26$^{LSL\text{-}YFP}$*).

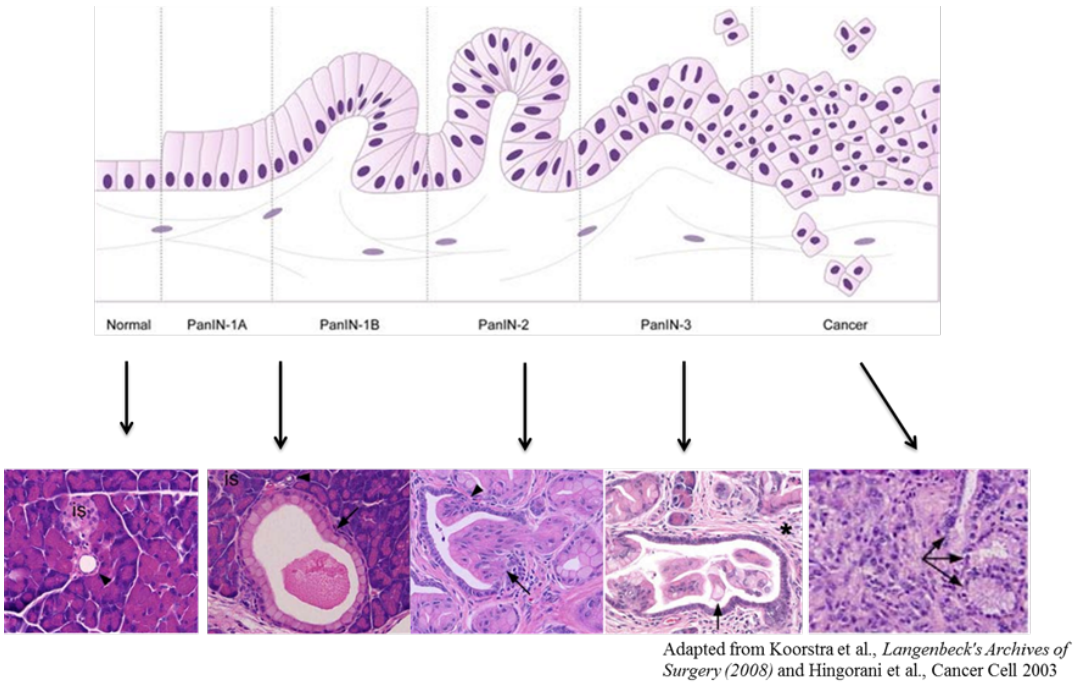


Figure 1-4 Tumor progression of the *LSL-KRAS^{G12D}; Ptf1a-Cre* mouse model

H&E shows PanIN progression is recapitulated in the *LSL-KRAS^{G12D}; Ptf1a-Cre* mouse model. PanIN-1 is represented by a large mucinus duct-like lesion. PanIN-2 shows a duct like tumor with nuclear crowding and a loss of polarity. Pan-IN 3describes clusters of cells budding off a tumor. Invasive carcinoma (cancer) is represented by Pan In lesions that have broken through the basement membrane and begun invading.

vi. Acinar to Ductal Metaplasia (ADM) – A link between CP and PDAC

In addition to studying tumorigenesis and tumor progression, the *LSL-KRAS^{G12D}; Ptf1a-Cre* mouse has been used to study the link between pancreatitis and cancer. Performing experimental pancreatitis in conjunction with the oncogenic Kras mutation increases the rate of tumor formation in our mouse model [36,39]. This data is consistent with epidemiological

studies, which show that patients with CP have a greater risk for developing PDAC[3]. Several studies show that the inflammatory microenvironment is pro-tumorigenic and that chronic pancreatitis constitutes a risk factor for PDAC[36]. The prolonged administration of cerulein causes inflammation along with the formation of metaplastic ductal lesion (MDL)[36]. Acinar to ductal metaplasia (ADM) is an event commonly seen in CP, where acinar cells change structure to express a ductal phenotype[40,41]. Normally, ADM in CP progresses no further, but it is hypothesized that these metaplastic cells can become tumors if they express mutated Kras[42]. MDLs are pro-inflammatory and express many molecular signals shown in early tumors[41,43]. It has been suggested that metaplastic lesions are precursors of PanINs, and studies show that blocking ADM prevent pancreatic tumorigenesis[36,44]. Furthermore, it has been shown that MDL lesions are capable of reverting back to acinar cells as part of the normal healing process but oncogenic Kras can reprogram these cells to initiate tumor formation[42,45]. Morris et al. shows that blocking acinar regeneration prevents Kras mediated tumor progression in caerulein treated mice[42].

Researchers also uncovered that genes that contribute to acinar and ductal identity can be instrumental in directing metaplastic conversion and tumor formation. The forced expression of acinar cell regulator Mist-1 can block ADM and PanIN formation, while its absence accelerates Kras mediated PanIN formation[46]. Conversely, ductal specific gene Sox-9 can accelerate tumor formation when over expressed in the *LSL-KRAS^{G12D}; Ptf1a-Cre* mouse model[45]. The loss of Sox-9 in this model does not prevent the formation of ADM but does stop tumorigenesis[45]. This finding suggests there are molecules in addition to Kras that can modulate the transition of metaplastic lesions to PanINs. We have dedicated much of our studies understanding what drives ADM and early PanIN formation, and have uncovered a

set of closely associated molecules that alters the fate of cerulein induced ADM and PDAC formation/progression.

1-2 Signaling Molecules to target against pancreatic disease

i. Pivotal Signaling Networks in Pancreatic Disease-

NFκB, EGFR-RAS-MAPK, and RAC

Understanding the cell signaling pathways involved in pancreatitis and PDAC lead to the elucidation of molecules necessary for the initiation and maintenance of these diseases. Pro-inflammatory genes in the pancreas modulate pancreatitis. Recently, NFκ-B signaling has been found necessary for the development of acute and chronic pancreatitis[47-49]. Over expression of the transcriptional subunit of NFκ-B, p65 worsened the damage caused by experimental pancreatitis[47]. Concordantly, genetic ablation of the inhibitory subunit, IκB yielded similar results[49]. Normally IκB remains bound to p65 preventing the nuclear translocation of the p65[47]. When IKK (inhibitor of IκB) gets activated by pro-inflammatory receptors it phosphorylates IκB which leads to its ubiquitin degradation
this frees p65, allowing NFκ-B to ensue[47]. This pathway has been established as a key regulator in pancreatitis and pancreatic tumorigenesis. In two independent studies, the NFK-B and EGFR pathway have been found to be essential for ADM[44,50]. Previous studies from our lab concluded that pancreatitis induced ADM is blocked by the loss/pharmacological inhibition of EGFR or the inhibition of downstream mediator, MEK[44]. When ADM is blocked, the inflammation associated with it resolves, suggesting metaplasia is pro-inflammatory[44]. Daniluk et al. (2012) shows that ablating IKK will also block ADM[50]. The loss of both of these genes can even prevent Kras mediated tumor formation [44,50]. The

importance of ADM in pancreatic disease is also demonstrated in Heid et.al (2011), where the loss of Rac1 reduced ADM and early tumorigenesis in a pancreatic cancer mouse model[51]. These finding, led us to further study molecules that control the ADM process, and test their therapeutic benefits in pancreatitis.

One of the most well studied molecule sin PDAC is Kras. Though many cell signaling pathways are altered in pancreatic cancer, the induction and constant expression of Kras is sufficient to drive tumorigenesis and tumor progression[52]. Targeting Kras has proven to be quite difficult since the inhibition or mutation of Kras causes a loss of function leading to constitutive signaling[20]. This monomeric G-protein is involved in a cell signaling cascade that results in the activation of MAP kinase proteins, Raf, MEK, and ERK which promote cell survival and proliferation [20,21]. Also, Kras can be activated by receptor tyrosine receptors, such as Epidermal growth factor receptor (EGFR)[44]. Blocking the functions of these upstream and downstream molecules has shown promise in preventing pancreatic tumors. Proteins that regulate EGFR-MAPK signaling or Rac mediated actin remodeling have been shown to block tumor formation by preventing the formation of the PanIN precusors, MDLs[44,51]. One such molecule, PI3K is known to contribute to EGFR, Kras and Rac signaling. My studies involve testing the role of PI3K in ADM and the PDAC risk factor, CP.

ii. PI3K- Introduction and mechanistic overview

Phosphoinositide 3 kinases (PI3K) are enzymes that phosphorylate the 3-position hydroxyl group on the inositol ring of their phosotidylinositol (PtdIns) lipid substrates[53]. This generates a second messenger molecule that serves as a docking site for specific signaling proteins[54]. PI3K signaling regulates a variety of cellular processes including, cell growth,

survival, and migration[55]. PI3Ks are separated into 3 classes based on structure and binding partners[56]. All isoforms have a 110kd catalytic subunit and a regulatory subunit. The most well understood and most clinically relevant class of PI3Ks in pancreatic disease are the class I proteins [55]**.**

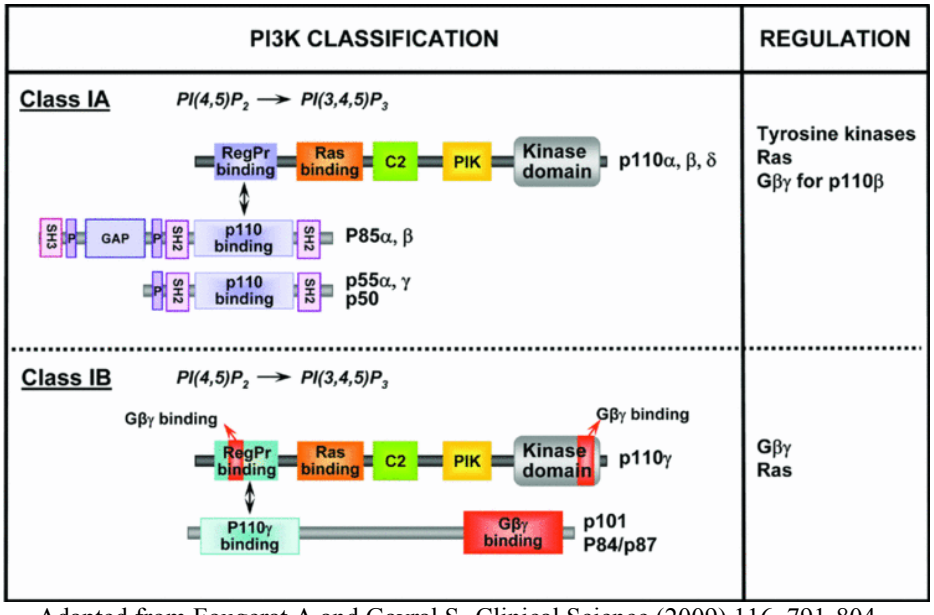
Class I subtypes are presents in all mammalian cell types and are divided into class IA (p110α, p110β and p110δ) and class IB (p110γ) **(Figure 1-5).** Class IA kinases have a p85 or p55 regulatory subunit **(Figure 1-5)**. Class IB kinases have a p101 or p87 subunit **(Figure 1-5)**. All 4 class I proteins can be bound to and regulated by Ras but p110α, p110β, and p110δ are also regulated by tyrosine kinases, while p110γ is selectively regulated by G-protein coupled receptors[56]. The regulatory subunits p85 and p55 contain an SH2 domain, which binds to phosphorylated tyrosines on receptor kinases[56]. p101 and p87 subunits found in Class IB proteins bind to Gβγ subunits released from G-protein coupled receptors[56]. p110β is a unique class 1 that can be activated by Gβγ subunits without binding to it[57].

The structural domains of these p110 subunits are highly conserved. There is a regulatory binding domain (RegPr) and Ras binding domain[56,58]. The C2 helical domain has the ability to bind to phospholipids and calcium, and the PI3K accessory domain (PIK) has an unknown function[59]. The catalytic domain is where PI3K converts PtdIns, $PI(4,5)P_2$ to $PI(3,4,5)P_3$[56].

There are many molecules that are upstream activators of PI3Ks. My studies look at the regulation of p110α, specifically. Insulin growth factor receptor (IGFR) and EGFR are receptor tyrosine kinases (RTK)s and well documented activators of p110α[60,61]. When the

RTK gets phosphorylated, the SH2 domain of p85 binds it, localizing p110α to the membrane so that it can generate $PI(3,4,5)P_3$ for proteins with PH domains[56].

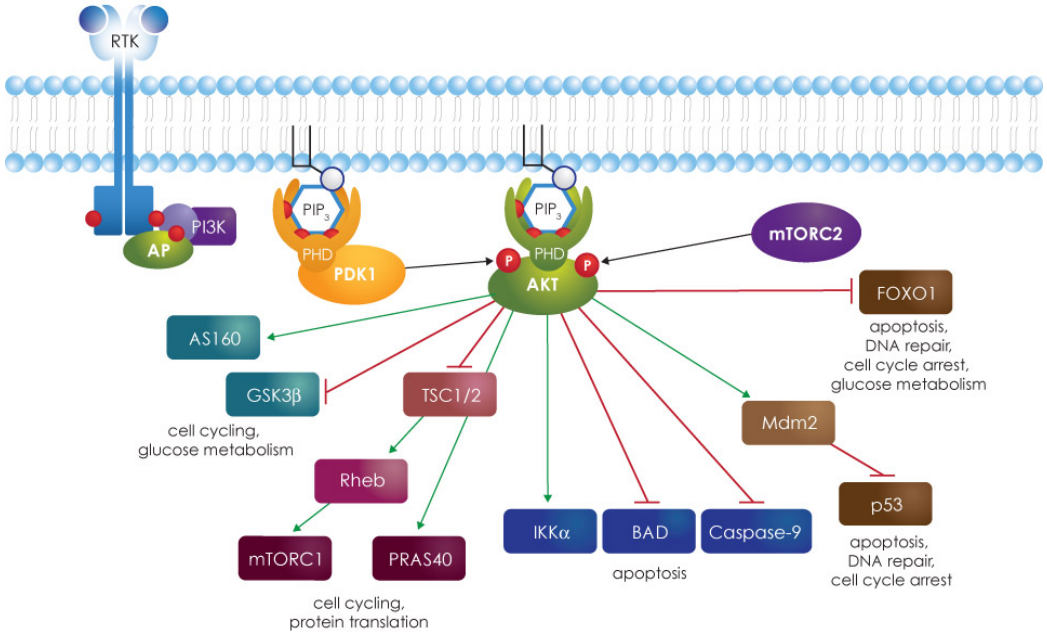
Two downstream targets of PI3K are AKT and PDK1[56]. They both require $PI(3,4,5)P_3$ to localize to the plasma membrane[56]. Once localized, PDK phosphorylates AKT at the threonine 308 (T308) site [56]. A second phosphorylation at the serine 473 (S473) by mTORC makes AKT fully active and capable of inducing many of the cellular processes that are associated with PI3K activation[56]. F**igure 1-6** illustrates the class I PI3K signaling network. Recent findings show that constitutively active expression of p110α accelerates tumor progression in the *LSL-KRAS^{G12D}; Ptf1a-Cre* mouse model[62]. The same study found that ablation of PDK stops Kras mediated tumorigenesis [62]. These findings encourage the importance of PI3K and PDK in pancreatic disease.



Adapted from Fougerat A and Gayral S, Clinical Science (2009) 116, 791-804

Figure 1-5 Structural Homology of Class I PI3K proteins

Class I PI3K proteins have a p110 catalytic subunit and a regulatory subunit; these kinases are further categorized into class 1A and class 1B. The figure illustrates that class IA (p110α, p110β and p110δ) have either a p85 or p55 regulatory subunit, while class IB (p110γ) proteins are partnered with P101 or p87. All Class 1 proteins have a regulatory binding domain, Ras binding domain, C2, PI3K accessory domain (PIK), and catalytic domain. Depending on the isoform, either Ras, tyrosine kinases, and G-protein coupled receptors subunits (Gβγ) can regulate PI3K activity. PI3K converts PtdIns, $PI(4,5)P_2$ to $PI(3,4,5)P_3$.



adapted from Brock T G, *The PI3K Pathway*: *Fast Forward for Cancer*, *2009*-03, *Cancer 2009.2134*

Figure 1-6 PI3K (class I) signaling network

Class I PI3Ks , such as p110α are recruited to the cell membrane my phosphorylated RTKs. PI3K converts PtdIns, $PI(4,5)P_2$ to $PI(3,4,5)P_3$ which will recruit PH domain containing proteins to the cell membrane. PDK and AKT are two PH domain containing proteins that bind to $PI(3,4,5)P_3$. Phosphorylation of AKT by PDK and mTORC leads several cellular responses.

iii. PI3K involvement with Ras and Rho Family GTPases

PI3K and Ras have a well-established relationship. Many studies have shown that Ras is required for PI3K activation[63,64]. Ras-PI3K studies in cell lines show that p110α and p110γ generate significantly higher levels of PIP3 when cells are transfected with of constitutively active Ras family members, including Kras[64]. This hypothesis has been demonstrated in a Kras mediated lung tumorigenesis model, where the Ras binding domain of p110α was mutated, preventing Ras-p110α interaction[58]. The loss of this interaction blocked tumor formation[58]. Other Rho family members that associate with PI3K are the actin modeling proteins Rac, Cdc42, and Rho. These GTPases are actually activated by PI3K[65,66]. The current model is that guanine exchange factors (GEFs) that load GTP onto Rac, Cdc42, and Rho requires $PI(3,4,5)P_3$ to be properly localized[65,67]. This PI3K mediated activation of GEFs in turn leads to GTPase activation[65]. PI3K has been shown to direct actin remodeling and promote chemotaxis, activating Rac, Cdc42, and Rho in the process[65,66]. The inhibition of any 3 of these GTPase is sufficient to block actin remodeling, even when PI3K is active which suggests that PI3K is an upstream effector[65,67]. Conversely, there have been groups

that have demonstrated that Rac can activate PI3K after binding to the p85 subunit of PI3K[67,68]. Yang et al. (2012) demonstrated that all 3 GTPases needed to be active for PI3K activation to occur in their model[65]. This response seems to be part of a positive feedback loop that requires PI3K initiation. How actin Rac, Cdc42, and Rho signal back to PI3K varies depending on the system. Nevertheless the data all corroborates that PI3K plays an important role in actin-cytoskeleton rearrangement, which is essential for the ADM process.

iv. PI3K- mouse models and relevance to pancreatitis

Studies interpreting the role of PI3K have been facilitated by the generation of isoform specific mutations and deletions. Complete knockouts of p110α and p110β are embryonic lethal but p110δ and p110γ knockouts are viable for adult studies[54]. Due to the prenatal death of p110α knockout mice at E.10.5, tissue specific knockout models have been utilized [54,69]. Constitutively active expression of p110α in mice was shown to have enlarged hearts while kinase dead p110α mice had decreased heart size [54]. This suggests that p110α can regulate cell size. p110δ and p110γ knockouts have defective lymphocytes[54]. p110γ knockouts have defective cardiac function as well which may suggests some overlap with p110α function[54]. Also, The full body ablation of p110γ can prevent damage caused by cerulein induced acute pancreatitis[70]. This phenotype could be a combined effect of both inflammatory and acinar cell processes being disrupted by the loss of p110γ. These data show that targeting Class I PI3K s may be a potential therapeutic for pancreatic disease.

v. Therapeutically targeting PI3K

Broad-spectrum inhibitors of Class I PI3Ks have been used for research and clinical development for almost 2 decades[71].Wortmanin, a fungal metabolite has been a long established inhibitor of PI3K[72]. The toxin is capable of blocking $PI(3,4,5)P_3$, but it can also disrupt other lipid signaling molecules such as Phospholipase D and Pleckstrin[72,73]. The first synthetic PI3K inhibitor was created shortly after and even though it was not as potent as Wortmannin, it was made freely available to the research community and gained popularity[74]. These compounds, though not as specific as inhibitors developed today pioneered PI3K research and laid the groundwork for better drug design[75]. The generation of isoform specific knockout mice gave dissimilar phenotypes demonstrating that the 4 class I molecules (p110α, p110β, p110γ, p110δ) have specific roles that cannot be compensated for by other class members[54]. These findings prompted the development of isoform specific PI3K inhibitors.

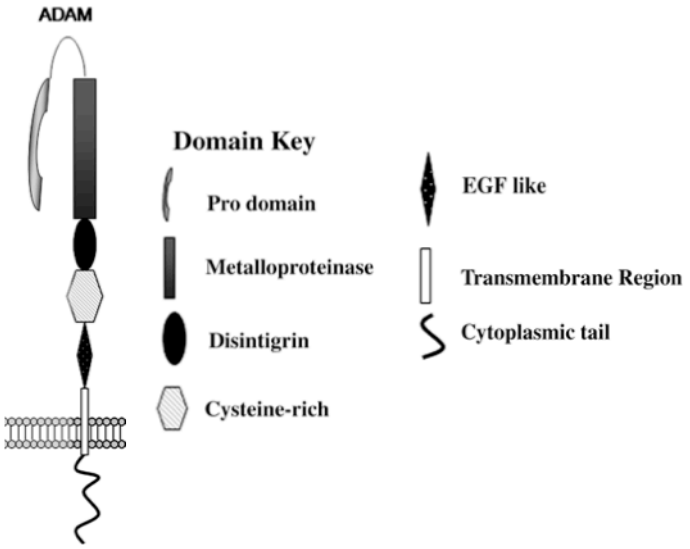
PIK-75 is a p110α selective kinase inhibitor, it has an IC 50 value of 5.8nM and it inhibits p110γ, p110β , and p110δ significantly less with IC_{50} values of 0.076 μM, 1.3 μM, and 0.51 μM respectively[76]. The inhibitor has been shown effective both *in vitro* and in mouse studies[77,78]. Studies using PIK-75 showed that loss of p110α can decrease the production of proinflammatory cytokines by inhibiting NF-κB signaling[77]. This resulted in an attenuation of experimental inflammation in the colon [77]. The effectiveness of PIK-75 in mouse models supports the use of this inhibitor for my studies. Our pharmacological study together with p110α knockout model experiments form a complimentary analysis of how the p110α subunit can affect pancreatic disease.

vi. ADAM 10- Introduction

A Disintegrin and Metalloproteinase 10 (ADAM 10) is a transmembrane protease that cleaves cell surface ligands and the ectodomain of cell-cell adhesion molecules[79]. ADAM 10 is also known to induce regulated intramembrane proteolysis (RIP)[80]. When the ectodomains of certain transmembrane proteins are cleaved, the event is followed by an intracellular cleavage, the second cleavage allows the intracellular domain to translocate to the nucleus and function as a transcription factor[80]. The types of proteins cleaved by ADAM 10 are diverse and each substrate can have a unique impact on cell regulation.

ADAM 10 belongs to a family of 33 ADAM proteins[81]. Its sequence and primary amino acid structure is highly conserved from flies to mammals[81]. ADAM 10 is composed of 6 protein domains and undergoes modification steps to become its fully mature form[82]. An illustration of ADAM 10 protein structure is shown in (**Figure 1-7).** On the N-terminal of ADAM 10 there is a pro-domain that keeps the catalytic domain inactive by occluding the active site[82]. The pro-domain also acts as a chaperone during protein folding at the endoplasmic reticulum[81,82]. Once folded, ADAM 10 is directed to the Golgi complex where pro-protein convertases cleave the pro-domain, allowing ADAM 10 to become catalytically active[82]. From the Golgi, ADAM 10 is sent to the plasma membrane, where it can perform its enzymatic function[82]. The metalloproteinase domain is where the proteolytic activity of ADAM 10 resides. The amino acid consensus sequence for the ADAM 10 active site is **HE**XG**H**NLGXX**H**D[79]. ADAMs require a zinc molecule (Zn^{2+}) to bind to the three histidine (**H**) residues, which assists the catalytic residue, glutamate (**E**) to break peptide bonds[83]. The disintegrin domain has the ability to bind to integrins. Snake venom metalloproteases (SVMP) are known for binding to platelet integrins which block platelet aggregation[79]. While

the purpose of the disintegrin domain in these related proteins are understood, how ADAMs may use this domain remains unclear[79]. There is a cysteine rich region in the disintegrin domain, which is believed to control substrate affinity and interaction[81]. Since many ADAMs are so similar in structure and function, small differences in this region may be an important distinguishing feature. The EGF-like domain, is a 30-40 amino acid residue which bears resemblance to the EGF ligand[81]. This domain is found in all ADAMs except for ADAM10 and 17[83]. Lastly, the cytoplamic tail has the ability to interact with other proteins[81]. Most ADAMs, including ADAM10, have a proline rich tail that can be an interaction domain for SH3 domain containing proteins[81]. The cytoplasmic tails of certain ADAMs can also undergo RIP[80]. ADAM 9 and 15 can actually cleave ADAM 10 leading to an intracellular cleavage of ADAM 10 by γ-secretase[80]. Though studying the cytoplasmic tail of ADAM 10 introduces an alternate way of understanding how this protein regulates cellular function, our research focuses on how the metalloproteinase domain regulates cell-signaling pathways.



Adapted from Edwards D, Molecular Aspects of Medicine (2008)

Figure 1-7 Protein domains of a Disintegrin and Metalloproteinases (ADAMs)

ADAM 10 contains a pro domain on the N-terminal, which masks the catalytic activity of the metalloproteinase domain. The pro-domain is shed by pro convertases prior to being localized to the plasma membrane. The metalloproteinase domain is responsible the cleavage of extracellular proteins. The active site of this domain consists of an amino acid motif containing 3 histidines and 1 glutamate, a zinc molecule (Zn^{2+}) is required for catalytic function. ADAM 10 does not have a EGF-like domain. The cytoplasmic tail is known to bind to other proteins and cleavage of this domain can induce nucleus translocation.

vii. ADAM 10 and Notch Signaling

Originally discovered for its role in neuronal development, the loss of ADAM 10 was found to be important for the process of lateral inhibition[84,85]. Drosophilae lacking the ADAM 10 homolog, Kuzbanian (Kuz) had progenitor cells that were unable to receive inhibitory signals from neighboring cells and they developed more sensory bristles compared to wild type flies[84]. The "multiple bristle" phenotype observed in flies lacking Kuz was also seen in flies lacking Notch[84]. A later study demonstrated that ADAM 10 is required for Notch activation during Drosophila wingspan development[86]. Studies prior to the discovery of ADAM 10 showed that Notch signaling pathway is important for neuronal development in flies[87]. Like ADAM 10, Notch controls cell fate through lateral inhibition. The role of Notch in neurogenesis extends to vertebrates as well[87]. Studies in mammalian cells discovered that Notch was universally important in maintaining the pluripotency of various cell type

lineages, such as the intestine[87]. The similar functions of ADAM 10 and Notch, together with the data from Sotillos et al. (1996) suggests that ADAM 10 may be regulating Notch signaling in many systems.

Notch and its ligands, Delta or Serrate are transmembrane proteins that bind to each other by cell-cell contact[87,88]. After Notch binds to its ligands its ectodomain is shed by an ADAM and undergoes RIP[87,88]. γ−secretase causes the cleavage of the intracellular domain of Notch (NICD)[87]. NICD functions as a transcription factor, interacting with the DNA binding protein, Rbpj-κ and its co-activators, Mastermind (MAML), and P300 [87,89]. Notable target genes transcribed by Notch are members of the Hes and Hey family and c-myc[87,89]. **Figure 1-8** illustrates the ADAM 10-Notch signaling pathway.

Many groups have performed studies to confirm if ADAM10 is the primary Notch sheddase[86,90-92]. ADAM 10 deficient mice were generated to understand how this protein may be important in vertebrates and at E.9.5 there were defects in development that resulted in lethality[90]. Embryos were smaller, had under developed hearts, and had neural deformities similar to Notch deficient mice[90]. *In situ hybridization* showed that Hes-5 expression in the neuro-epithelium was very disorganized compared to wild type and expression of Delta-like, which is normally low in wild type embryos was upregulated [90]. To avoid embryonic lethality, groups created conditional knockouts that were organ specific[93]. Jorrissen et al. (2010) found that ADAM 10 was important for brain development, and that the loss of ADAM 10 prevented the activation of Notch [93]. Though this evidence supports ADAM10 as the primary Notch sheddase, both ADAM 10 and ADAM 17 have been shown to cleave Notch[91,94].

One study compared the two ADAMs and concluded that the primary sheddase responsible may be context dependent[95]. Experiments using mammalian cells and mouse embryonic fibroblasts (MEFs) showed that ADAM 10 can cleave both endogenous and ectopically expressed Notch, but only when a ligand is present[95].

Interestingly, ADAM 17 cannot cleave endogenous Notch, it can only cleave ectopic Notch in a ligand independent manner[95]. The study proposes that ADAM17 does not normally associate with Notch and that ADAM17 can only cleave Notch when its cleavage site is exposed with out the presence of a ligand[95]. The aberrant manner that ADAM17 cleaves Notch reconciles previous data and explains why ADAM10 deficient mice phenocopied the same embryonic lethality found in Notch knockout mice, while the ADAM17 knockout mice experienced embryonic lethality during late pregnancy (E17.5) for defects in epithelia maturation as opposed to neuronal and vascular deficiencies[90,96]. These findings have led us to hypothesize that ADAM 10 can regulate Notch related cellular mechanisms in the pancreas.

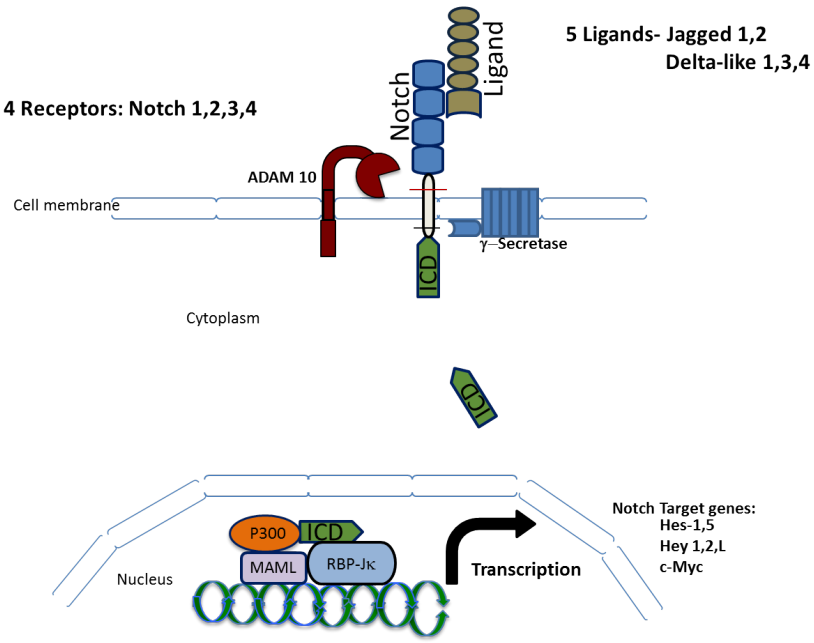


Figure 1-8 Diagram of ADAM 10 activating Notch signaling

The Notch receptor binds to ligands, Delta-like or Jagged. ADAM10 is a transmembrane protein that can cleave the Notch ectodomain after ligand binding. This is followed by and intracellular cellular cleavage by γ-secretase. The Notch intracellular domain (ICD) then translocates into the nucleus where it bind to Rbpj-κ and the transcriptional co-activators MamL and p300. This induces the transcription of Notch target genes, Hes, Hey and c-Myc

viii. ADAM 10 and Notch in the Pancreas

Both ADAM 10 and Notch are expressed during pancreatic development and can be found in adult tissue[97,98]. In the pancreas, Notch has been established as a positive regulator of progenitor cell maintenance and exocrine cell fate[98,99]. Over expressing Notch1 in cells expressing the pancreas specific promoter, PDX will block acinar and endocrine

differentiation[98]. Another study confirmed this finding by showing that Notch ICD or Hes-1 can block acinar differentiation by inhibiting the transcriptional activity of the pancreatic lineage promoter PTF1a[99]. Esni et al. demonstrates that Hes-1 and PTF1a are co-expressed in exocrine precursors cells but Hes-1 is lost in differentiated acinar cells. The loss of Notch signaling components yielded divergent results. An examination of the simultaneous loss of Notch 1 and 2 in pancreas showed that there were no developmental defects of note[100]. In contrast ablation of the Notch ICD DNA-binding partner, Rbpj-k resulted in a poorly differentiated pancreas and died a few days postpartum due to their inability to digest milk[100]. Hes-1 was reduced in all these models, confirming Notch signaling was downregulated[100]. These studies suggests that only an over expression of Notch may be important for pancreatic development but other studies in the field aimed to test how Notch can regulate adult tissue in the context of disease.

ix. ADAM 10 and Notch- Proposed Roles in Pancreatic Cancer

Both ADAM 10 and Notch are over expressed in both CP and PDA patients [101, 102]. Investigations in pancreatic cancer cell lines (Panc-1, Hpac, and Bx-PC3) show that down regulation of Notch 1 decreases cell growth and leads to apoptosis, demonstrating that Notch signaling is important in malignant cell signaling and that it may be a favorable therapeutic target[89].

All 4 Notch receptors are expressed in pancreatic cancer but currently there are only mouse models examining the roles of Notch 1 and Notch 2[103]. Notch 1 appears to function as a tumor suppressor, as studies have shown that the loss of Notch 1 in *LSL-KRAS^{G12D}; Ptf1a-Cre* mice enhances the progression of the disease [104]. However, this effect is seen without any changes in the expression of Notch target genes[104]. An different study shows that a

transgenic mouse expressing the Notch 1 ICD and mutant Kras has enhanced Notch activity as well as increased tumor burden[105]. These studies appear contradictory but may simply suggest that dysregulated Notch expression promotes tumor progression by causing downstream genes to be expressed at inappropriate levels. One other hypothesis is that losing Notch 1 is not sufficient to decrease the downstream signals and that other Notch receptors may be compensating in mice. Surprisingly, Notch 2 has its own distinct role in pancreatic cancer, contributing to tumor progression and increased malignancy[106]. *LSL-KRAS^{G12D}; Ptf1a-Cre* mice lacking Notch 2 produce large cystic lesions with characteristics of mucinus cystic neoplasms (MCN)s. This overt phenotype is accompanied by an improved survival rate[106]. While *LSL-KRAS^{G12D}; Ptf1a-Cre* mice live for about 15monts before succumbing to PDAC related death, the Notch 2 knockout mice can live almost a full 2 year lifespan[35,106]. These varying observations increases the importance of ADAM-10 and what possible outcome may arise from its deletion. We hypothesize that ADAM 10 promotes pancreatic disease by regulating Notch signaling

Chapter 2: PI3K subunit p110α mediates the development of chronic pancreatitis

2-1 Introduction

Pancreatitis is a gastrointestinal disease that is affecting more Americans each decade[3,11]. The disease can affect people in bouts of acute inflammation or as a chronic syndrome. Acute pancreatitis (AP) is the leading cause of hospitalization for patients with gastrointestinal diseases[4]. Chronic Pancreatitis (CP) is not as prevalent, but the damage caused by the disease can be irreversible[8]. AP is characterized by an upregulated production of digestive enzymes that become activated within the exocrine pancreas, this leads to tissue

autodigestion, necrosis and inflammation[5]. The leading causes for this disease are gallstone obstructions and chronic alcohol use[3]. CP is hypothesized to arise from consecutive acute attacks which leads to steady inflammation, permanent acinar cell loss, calcification of the pancreas, and an abundance of metaplastic ductal lesions (MDL)[11]. The greatest risk factors for CP are chronic alcohol use and smoking[3]. Several investigations have been conducted to study the etiology of the disease in order to find ways to prevent and manage pancreatitis. Understanding the cellular biology involved in the development of both AP and CP has lead to the discovery of potential therapeutic targets that can ameliorate the two diseases.

Phosphoinositide 3 kinase (PI3K) has been shown to promote AP and members of its signaling pathway have been shown to be necessary for the development of CP [44,70]. PI3K is an enzyme that can regulate many cellular processes such as, growth, motility, proliferation and survival[71]. It gets recruited to the plasma membrane by activated receptor tyrosine kinases (RTKs)[71]. Once PI3K is localized to the plasma membrane by RTKs they can act on their substrates, phosphoinositol (PtdIns) lipids. PI3K can coverts $PI(4,5)P_2$ to $PI(3,4,5)P_3$ leading to the activation of downstream signals[71]. $PI(3,4,5)P_3$ serves as a docking site for proteins with a pleckstrin homology (PH) domain. Binding to $PI(3,4,5)P_3$ is necessary for downstream molecules to be properly localized and potentiate signaling cascades[56]. PI3K dependent signals such as PDK regulate growth and proliferation while other molecules such as Rac GTPase, controls actin remodeling and motility[66].

Previous studies have found that the RTK epidermal growth factor receptor (EGFR) was necessary for acinar to ductal metaplasia (ADM)[44]. The genetic ablation of EGFR in mice showed that despite the administration of cerulein, MDLs were unable to form and the mice were protected from developing chronic pancreatitis[44]. Ablation of molecules

downstream of PI3K have also blocked ADM and protected mice from developing cerulein induced pancreatitis and Kras induced pancreatic tumorigenesis. Genetic knockouts of Rac or PDK have been shown to block ADM in two separate studies that phenocopy what was observed in the EGFR knockout model[51,62].

PI3K isoforms themselves have been implicated in alleviating inflammation and may protect against pancreatitis. Complete genetic knockouts of the class I PI3K isoform, p110γ are resistant to cerulein induced acute pancreatitis[70]. Since p110γ knockout mice have been shown to carry immunological defects, the phenotype observed may have been caused by either the malfunctioning immune response or the loss of p110γ in the pancreatic epithelium[54]. Pharmacological inhibition of another class I PI3K isoform, p110α also seemed to decrease the inflammatory response in an intestinal inflammation model[77]. The study showed a reduced release of cytokines due to decreased transcriptional activity of the proinflammatory molecule, NFκ-B. NFκ-B is yet another molecule that can regulate ADM, Ablating molecules that promote NFκ-B signaling in mice can stop acute pancreatitis and metaplastic conversion[50]. Taken together, these data supports our hypothesis that the p110α isoform of PI3K is necessary for the development of pancreatitis, and that targeting this molecule may be an effective treatment for those afflicted with this disease.

2-2 Materials and Methods

Mouse strains

PI3K;p110α$^{fl/fl}$,*Rac1*$^{fl/fl}$, and *Ptf1a*$^{Cre/+}$strains have been described previously and were genotyped accordingly[35,51,107,108]. Experiments were conducted in accordance with the Office

of Laboratory Animal Welfare and approved by the Institutional Animal Care and Use Committee at the Mayo Clinic and Stony Brook University.

Genotyping

Cre: Forward Primer Sequence 5'- 3': TCGCGATTATCTTCTATAT CTTCAG

Reverse Primer Sequence 5'-3': GCTCGACCAGTTTAGTTACC C

PI3K: P110α: Forward Primer Sequence 5'- 3': CTGAGCTATAGAACTTCGTAACG

Reverse Primer Sequence 5'-3': CTACACAGAGAAACCCTGTCTTG

Rac 1: Forward Primer Sequence 5'- 3': TCCGTGCAAAGTGGTATCCT

Reverse Primer Sequence 5'-3': TTCTTGACAGGAGGGGGACA

Induction of experimental pancreatitis

Control (saline injected) and experimental groups (cerulein injected) groups consisted of 8-12 week old wild type or knockout mice. To induce acute pancreatitis, mice were injected intraperitoneally (IP) with 7 hourly injections of 50 μg/kg cerulein (American Peptide Company) and allowed to recover for 1 hour. The staggered acute protocol consisted of 7 hourly injections of 50 μg/kg cerulein on day 1, no injections on day 2, and 7 hourly injections of 50 μg/kg cerulein on day three. This protocol was followed by 1 hour, 24hours, or 3 days recovery. Chronic pancreatitis was induced by I.P injections of 250 μg/kg cerulein, twice daily for seven days, followed by 1hr or 24 hours recovery. Modified chronic pancreatitis was induced by injection of 250 μg/kg cerulein, twice daily for three days, followed by 1 hour recovery.

Pharmacological inhibition of P110α

To inhibit p110α catalytic activity, mice were treated with a daily dose (50mg/kg) of PIK-75

(Selleckchem) via I.P injection. PIK-75 was administered to mice subjected to chronic pancreatitis. In our first protocol, daily I.P injections of PIK-75 were given in conjunction with the first cerulein injection for the duration of the chronic pancreatitis protocol. To test if PIK-75 can protect against chronic pancreatitis after the disease has been established, mice were first subjected to a chronic pancreatitis protocol followed subsequently by daily injections of PIK-75 and cerulein for 6 days. PIK-75 was resuspended in either 1.5% methycellulose or sterile corn oil.

Histology

Human samples of normal pancreas, and pancreatitis were procured from the Mayo Clinic and Vanderbilt University Medical Center with the approval of their Institutional Review Boards. Human tissue was fixed in 10% formalin and embedded in paraffin. Murine tissues were harvested and fixed overnight in 4% paraformaldehyde. Immunohistochemistry was completed by the Mayo Clinic Cancer Center Histology Core using an automated immunostainer (Dako Cytomation Immunostainer Plus) according to the company's protocols with minor modifications. IHC slides were counterstained with hematoxylin. IHC and hematoxylin and eosin (H&E) stained slides were photographed on an Olympus BX41 light microscope (Olympus, Tokyo, Japan). Aperio ScanScope XT slide scanner (Aperio Spectrum, Vista, CA) and image analysis software (ImageScope) were used for quantitative assessment. Quantitation represents the average of 5-7 10x/20x fields of view for 3-6 mice of each genotype, treatment protocol, or time point indicated.

Immunofluorescence

Pancreata were prepared for immunofluorescence post-extraction by 4% PFA fixation for 3-4 hours, followed by three 5 minute 0.1M PBS washes and an overnight incubation in 30%

sucrose. Pancreata were incubated in a 1:1 mixture of 30% sucrose in 0.1M PBS and OCT (Tissue-Tek) for 30 min, then embedded in OCT and frozen at -80°C. 7μm sections were cut and permeabilized with 0.1% Triton X-100 in 10mM PBS and blocked in 10mM 1xPBS, 5% normal donkey serum, 1% BSA for 60min at RT. Sections were then incubated with primary antibodies diluted in 10mM 1xPBS, 1% BSA, 0.1% Triton X-100 overnight at room temperature. Slides were then washed three times with 0.1% Triton X-100/PBS and incubated with Alexafluor488 and/or Alexafluor594-conjugated secondary antibody (Invitrogen, Carlsbad CA). Stained slides were washed three times, rinsed with deionized water and mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Images were acquired on a Zeiss 510LS Meta confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Western blot

Whole tissue lysates were harvested in ice-cold radioimmunoprecipitation assay buffer (RIPA) buffer supplemented with PhosStop phosphatase inhibitor and cOmplete EDTA-free protease inhibitor (Roche, Indianapolis, IN). 25μg of protein was run on a 10% SDS-gel and blotted to PVDF membrane for antibody incubation.

Antibodies

P110α (Cell signaling), F4/80 (AbD Serotec), Ly6-B.2 (AbD Serotec), CD3(abcam),CK19 (TROMA III, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa), CK19(abcam/epitomics), DBA lectin (Vector), NF-κB p65(Cell Signaling), Ki-67 (Abcam), Rhodamine Phalloidin (Invitrogen), ZO-1 (Invitogen), β-actin (sc4777, Santa Cruz), Cox-1 and Cox-2 (Santa Cruz)

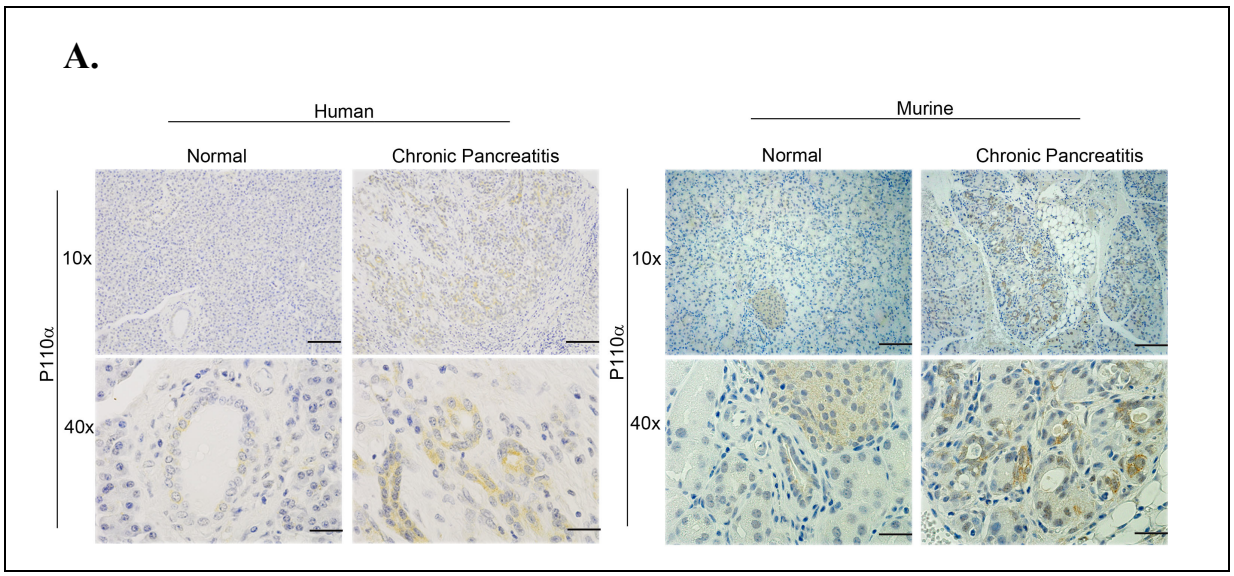
Serum Amylase Assay

Serum amylase levels were determined using Liquid Amylase Reagent (Pointe Scientific, Canton, MI). Serum of cerulein treated mice were diluted 1:100 before application. Amylase content was determined using a SpectraMax absorbance microplate reader (Molecular Devices, Sunny Valea, CA) according to manufacturer's instructions.

2-3 Results

i. The P110α subunit of PI3K is upregulated in the metaplastic lesions of chronic pancreatitis

Immunohistochemistry (IHC) was used to examine p110α expression in both human chronic pancreatitis samples and mice treated with cerulein **(Figure 2-1)**. The treatment leaves the pancreas in a state representative of human disease. p110α expression was found in islets and normal pancreatic ducts in control tissues, but was absent in healthy acinar cells. In contrast, both mouse and human pancreatitis tissue showed an upregulation of p110α in the metaplastic ducts **(Figure 2-1)**. Western blot confirmed an increased level of p110α in pancreatic lysates of mice with cerulein induced chronic pancreatitis **(Figure 2-1)**.



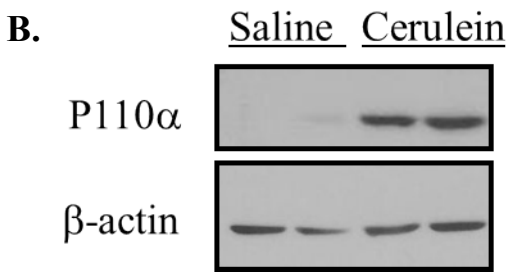


Figure 2-1. p110α is elevated in chronic pancreatitis

Human patient samples of healthy pancreata and chronic pancreatitis were tested for expression of p110α using immunohistochemistry (IHC). A) Murine samples of normal and cerulein induced pancreatitis tissue were also examined. IHC shows an increased expression of p110α in chronic pancreatitis tissue compared to normal in both human and mouse. B) Western blot of mouse tissue lysates yields equivalent results. Scale bars: 10x=120μm;

40x= 30 μm.

ii. Pancreas specific ablation of p110α blocks experimental pancreatitis

To determine if the p110α subunit of PI3K is involved in the epithelial and inflammatory changes that take place throughout the course of chronic pancreatitis, I administered cerulein to induce pancreatitis in p110α deficient mice. To generate these mice we crossed conditional p110α knockout mice ($p110\alpha^{fl/fl}$) with mice that carry a pancreas specific Cre recombinase ($Ptf1a^{cre/+}$). The $p110\alpha^{fl/fl};Ptf1a^{cre/+}$ mice, develop smaller pancreata compared to wild type (Data not shown), and histology shows that individual acinar cells were smaller **(Figure 2- 2)**. H&E shows that the acinar cell structure was phenotypically similar despite the difference in size. When mice are treated with cerulein, the

compound elicits an intracellular activation of digestive enzymes within the acinar cells[5], which damages the pancreas and leads to the recruitment of inflammatory cells. After serial I.P administrations of 250μg/kg of cerulein twice a day for 7 days, mice develop pathology representative of human chronic pancreatitis[5]. Western blot analysis on whole tissue lysates and IHC show decreased expression of p110α in the p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice compared to wild type **(Figure 2- 3)**. IHC also shows that p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice have a decreased immune response compared to controls **(Figure 2-4)**. Expressions of innate immune cells are represented by macrophages (F4/80) and neutrophils (Ly6.B). Adaptive inflammation is measured by the presence of T-cells ($CD3^+$).

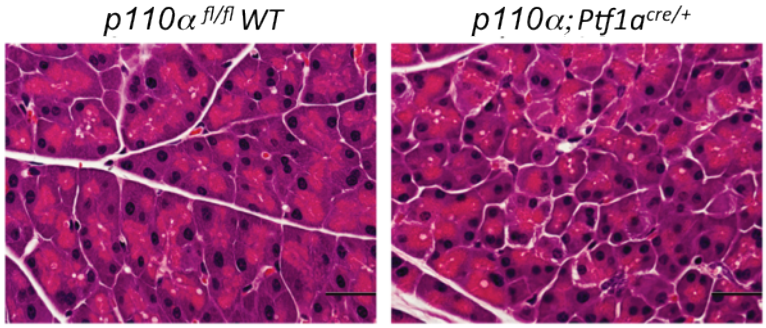


Figure 2-2. Ablation of p110α affects acinar cell size

p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice are born with a healthy but smaller pancreas, this due to having smaller acinar cells compared to control. Scale bars: 30μm (40x)

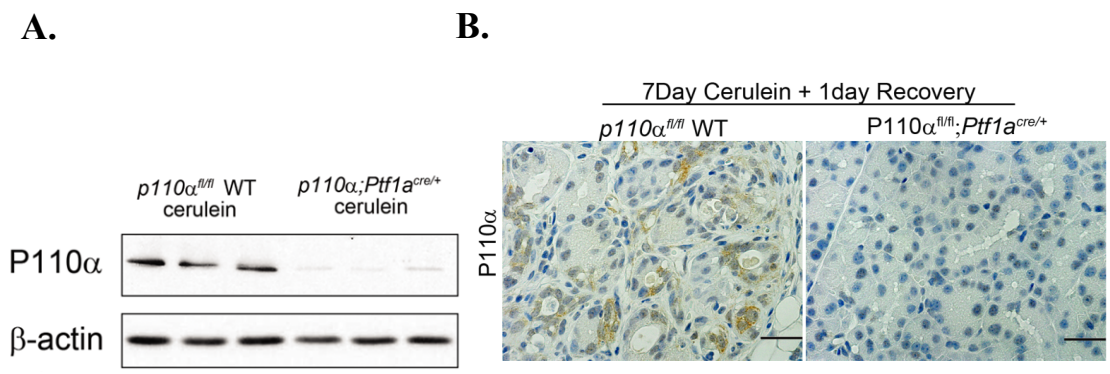


Figure 2-3. p110α expression is elevated in cerulein induced pancreatitis and not present in p110α$^{fl/fl}$; *Ptf1a*$^{cre/+}$ mice

A) Western blot of whole tissue lysates shows p110α expression in mice given chronic pancreatitis (2 daily injections of 250ug/kg for 7 days of cerulein). p110α knockout mice express significantly less p110α under the same treatment. B) IHC confirms that the p110α is primarily expressed in metaplastic ducts. Wild type mice show metaplastic lesions after 7 days. p110α $^{fl/fl}$*;Ptf1a*$^{cre/+}$ lack metaplastic ducts. Scale bars: 30μm (40x)

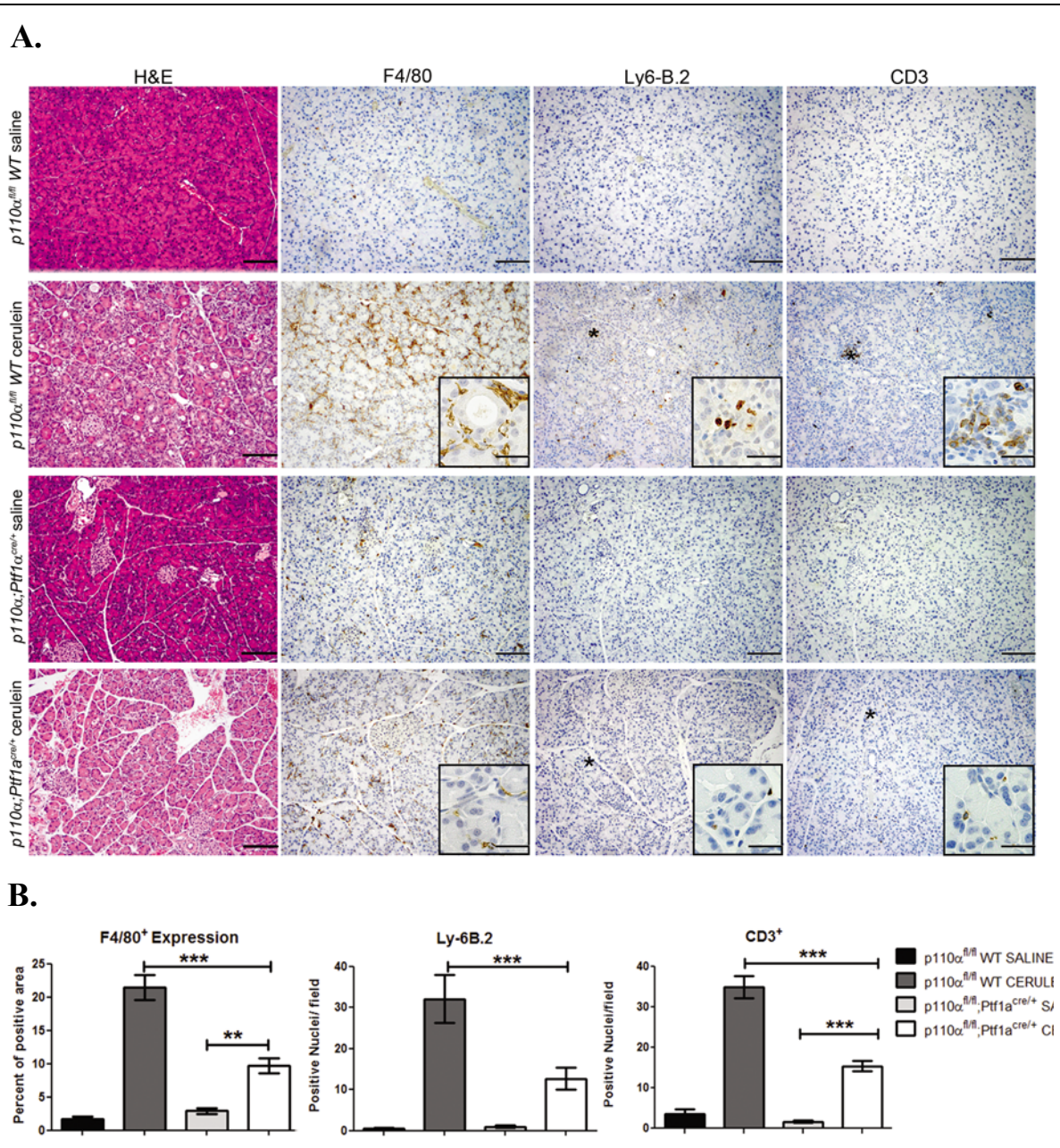


Figure 2-4. Ablation of p110α prevents cerulein induced chronic pancreatitis

2-3 month old p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice and wild type controls were treated with cerulein (2 daily doses for 7 days) to induce chronic pancreatitis. Mice were euthanized 1 day after final treatment. A) H&E depicts tissue morphology between cerulein and saline

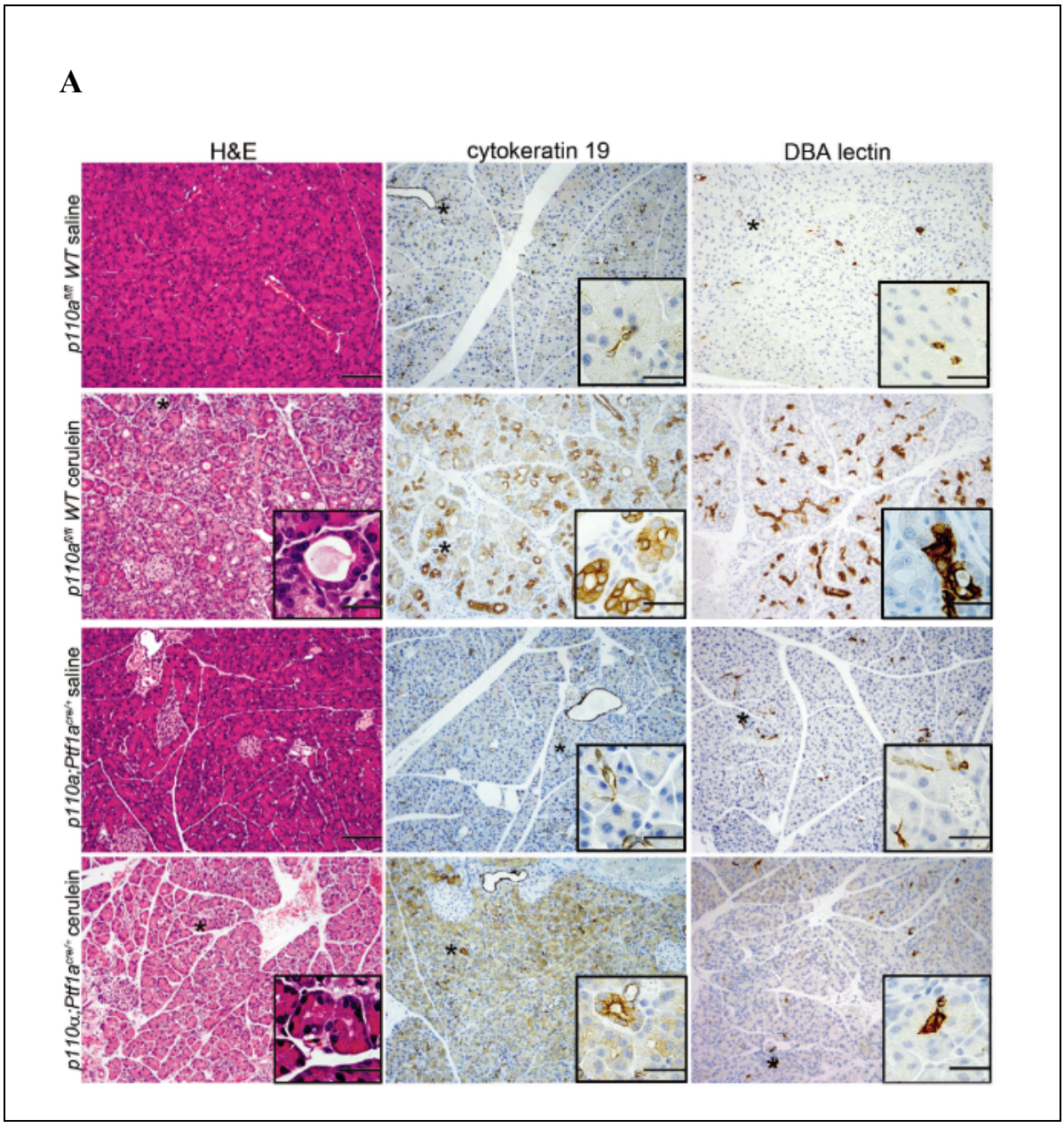
treated mice. Immunohistochemistry (IHC) shows immune infiltration increases after cerulein treatment compared to saline controls. p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice have decreased immune cell infiltration compared to wild type. The innate immune response is characterized by the presence of macrophages (F4/80) and neutrophils (Ly6.B). The adaptive immune response is assessed by counting T-cells ($CD3^+$). B) Cell count quantitation of theses immune markers reveals a significant difference between mice lacking p110α and wild type controls.

Scale bars: 120μm (10x); inserts: 30 μm (40x). Error bars, ± SDM ($^{***}P<0.001$, $^{**}P<0.01$).

iii. Loss of p110α impedes acinar to ductal metaplasia in chronic pancreatitis

Chronic pancreatitis is characterized by the presence of metaplastic ductal lesions (MDL) that arise from acinar cells[41]. Acinar to ductal metaplasia (ADM) requires EGFR and Rac1[44,51], whose respective pathways are both potentiated by PI3K[65,109]. PI3K has been hypothesized to be activated by the RTK, EGFR and once active PI3K can in turn activate Rac-GEFs[66]. To test if p110α is necessary for ADM, we administered cerulein to the p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice and quantitated the presence of ductal lesions using the ductal markers, cytokeratin 19 (CK19) and DBA lectin **(Figure 2-5)**. CK19 appears to be expressed earlier, in cells initiating metaplastic changes while DBA lectin appears in only a subset of metaplastic lesions, which may suggest DBA lectin only appears in more advanced ductal lesions. Both markers were expressed higher in cerulein treated controls compared to p110α

$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice **(Figure 2-5)**. The data supports the hypothesis that the loss of p110α blocks ADM, which protects the pancreas from chronic injury.



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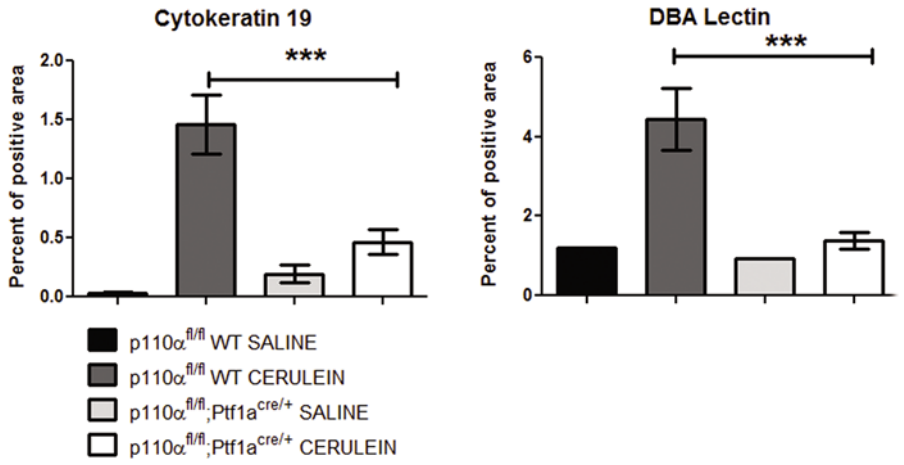


Figure 2-5. Ablation of p110α protects the pancreas from acinar-ductal metaplasia.

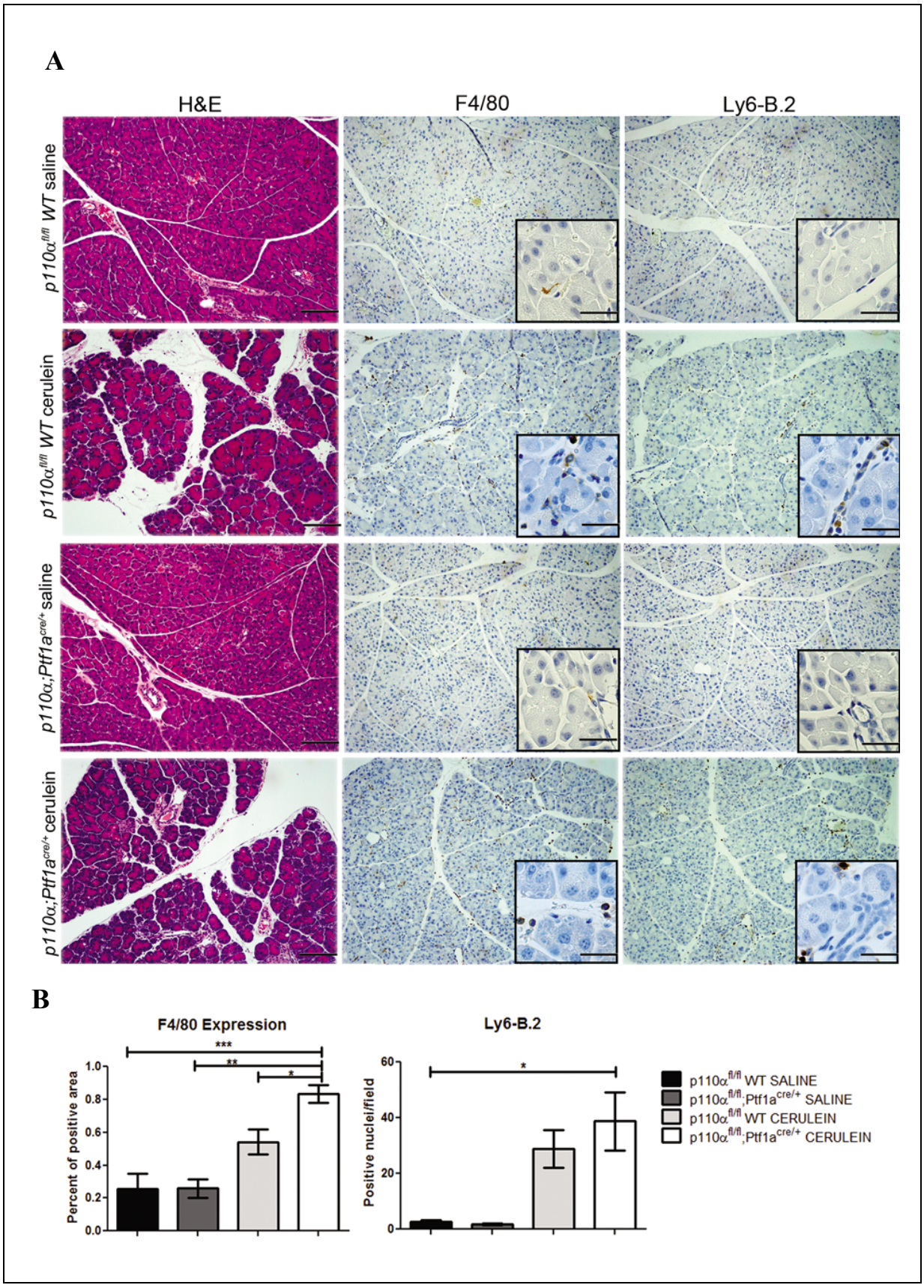
2-3 month old p110α$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice and wild type controls were treated with cerulein (2 daily doses for 7 days) to induce chronic pancreatitis. Mice were euthanized 1 day after final treatment. A) H&E stain shows metaplastic ducts are induced by i.p administration of cerulein in wild type mice, mice lacking p110α are resistant to metaplastic conversion. Immunohistochemistry (IHC) of ductal markers Cytokeratin 19 (CK19) and DBA lectin were used to quantify metaplastic lesions. B) IHC quantitation shows a significant difference in CK 19 and DBA lectin expression between cerulein treated wild type mice compared to p110α$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice and saline controls. Scale bars: 120μm (10x); inserts: 30 μm (40x).

Error bars, ± SDM (***P<0.001).

iv. p110α does not regulate the pre-metaplastic response induced by acute pancreatitis

Since our data show that early up-regulation of cytokeratin is blocked by the loss of p110α **(Figure 2-5)**, we investigated the initial pancreatic response to cerulein, which is representative of acute pancreatitis in humans [5]. Since the appearance of MDLs is unique to chronic pancreatitis, we hypothesized that ADM was the source of chronic inflammation and that acute pancreatitis would not be affected by the loss of p110α. Since the previous experiments (**Figure 2-4 and 2-5)** showed that P110α is blocking inflammation and ADM formation, we wanted to test if these effects were independent. Lupia et al. (2004) has shown that P110γ knockout mice are resistant to forming acute pancreatitis[70]. This led us to test if P110α was also involved in the pathology of acute pancreatitis and if the loss of P110α pancreatic tissue may be alleviating the disease in multiple ways.

To mimic acute pancreatitis in mice, they were given 7 hourly 50 mg/kg cerulein injections and were euthanized 1 hour after the last injection. Both wild type and p110α $^{fl/fl}$;$Ptf1a^{cre/+}$ mice show dilated acini and signs of edema by H&E **(Figure 2-6)**. Serum amylase, a marker of acute pancreatic damage was elevated equally **(Figure 2-6)**. The cerulein injections lead to an influx of macrophages and neutrophils, indicated by F4/80 and Ly6.B staining respectively **(Figure 2-6)**. Since the loss of p110α appears to have no therapeutic benefits to the early effects of cerulein induced pancreatitis, we performed different cerulein treatment protocols to evaluate disease progression, and test if p110α is only preventing pancreatitis because it impedes metaplastic conversion.


A
H&E
F4/80
Ly6-B.2
p110α fl/fl WT saline
p110α fl/fl WT cerulein
p110α;Ptf1a cre/+ saline
p110α;Ptf1a cre/+ cerulein
B
F4/80 Expression
Percent of positive area
0.0
0.2
0.4
0.6
0.8
1.0
***
**
*
Ly6-B.2
Positive nuclei/field
0
20
40
60
*
p110α fl/fl WT SALINE
p110α fl/fl;Ptf1a cre/+ SALINE
p110α fl/fl WT CERULEIN
p110α fl/fl;Ptf1a cre/+ CERULEIN

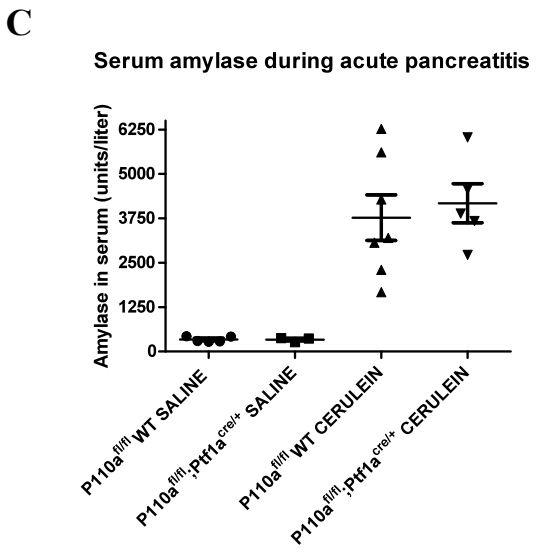


Figure 2-6. p110α is not necessary for early acinar cell response to cerulein

p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice and wild type controls were treated with levels of cerulein to induce acute pancreatitis (7 hourly doses at 50ug/kg). Mice were euthanized 1hr after last injection. A) H&E shows histological changes (dilated acinar cells, edema) apparent during acute pancreatitis. The loss of p110α does not alter the histological or physiological response seen in acute pancreatitis. Immunohistochemistry (IHC) of inflammatory markers; macrophages and neutrophils are characterized by F4/80 and Ly6B respectively (B). Quantitation of inflammatory response shows no significant difference between wild type and p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice when given an acute dose of cerulein . Acute pancreatitis is also identified by elevated levels of digestive enzyme secretion, which was measured by amylase units in blood serum (C). Scale bars: 120μm (10x); inserts: 30 μm (40x). Error bars, ± SDM (***P<0.001, **P<0.01,*P<0.05).

v. Metaplastic lesions are required for maintaining an inflammatory response

To assess the cell signaling response that occurs through the transition of acute pancreatitis and chronic pancreatitis, we conducted a modified cerulein treatment protocol, known as "staggered acute" [42,110] **(Figure 2-7)**. This protocol has been originally used to study pancreatitis damage and regeneration, but I hypothesized that using this protocol will allow us to assess the molecular signals upregulated in acinar cells before and after the ADM transition. The reason for this hypothesis is that the dosage of cerulein administered in this protocol elicits an acute response but metaplastic lesions form as well[42]. In this experiment, I showed that the pro-inflammatory NFκ-B subunit p65 is expressed in acinar cells before metaplastic changes **(Figure 2-7)**. In this model, p65 appears to be equally expressed 1 hr after the staggered acute cerulein treatments **(Figure 2-7)**. The 1 day acute protocol appears to upregulate nuclear NFκ-B in the centroacinar cells, whereas the staggered acute protocol induces p65 nuclear localization in acinar cells **(Figure 2-7)**. Despite seeing no significant difference between wild type and p110α$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice in acute pancreatitis, that data in **(Figure 2-7)** demonstrates that acinar cells express pro-inflammatory signals before becoming metaplastic.

Though cell autonomous inflammatory signals may subside after acute injury, they can be sustained in metaplastic ducts **(Figure 2-8).** Staggered acute treated wild type mice began to develop p65 positive metaplastic lesions 1 day after treatment while p110α$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice were resistant to forming metaplastic ducts and had less p65 positive acinar cells compared to the 1hr recovery time point **(Figure 2-7)**. The pancreas healed after 3 days and

neither p65 expression nor metaplastic ducts were present in wild type and p110α$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice **(Figure 2-8)**.

These observations lead us to test the hypothesis that p65 was consistently expressed in in the metaplastic lesions of chronic pancreatitis but not acinar cells. Wild type mice given cerulein twice a day for either 3 or 7 days (chronic treatment), displayed strong p65 staining in metaplastic ducts **(Figure 2-8)**. p110α$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice did not show any significant p65 expression **(Figure 2-8)**. In our models, p110α$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice do not maintain nuclear localization of the NFκ-B subunit p65 after acute injury subsides, and they lack the ability to form metaplastic lesions which would sustain pro-inflammatory signals. This data suggests that p110α's role in the pathology of pancreatitis lies in the regulation of pancreatic acinar to ductal metaplasia.

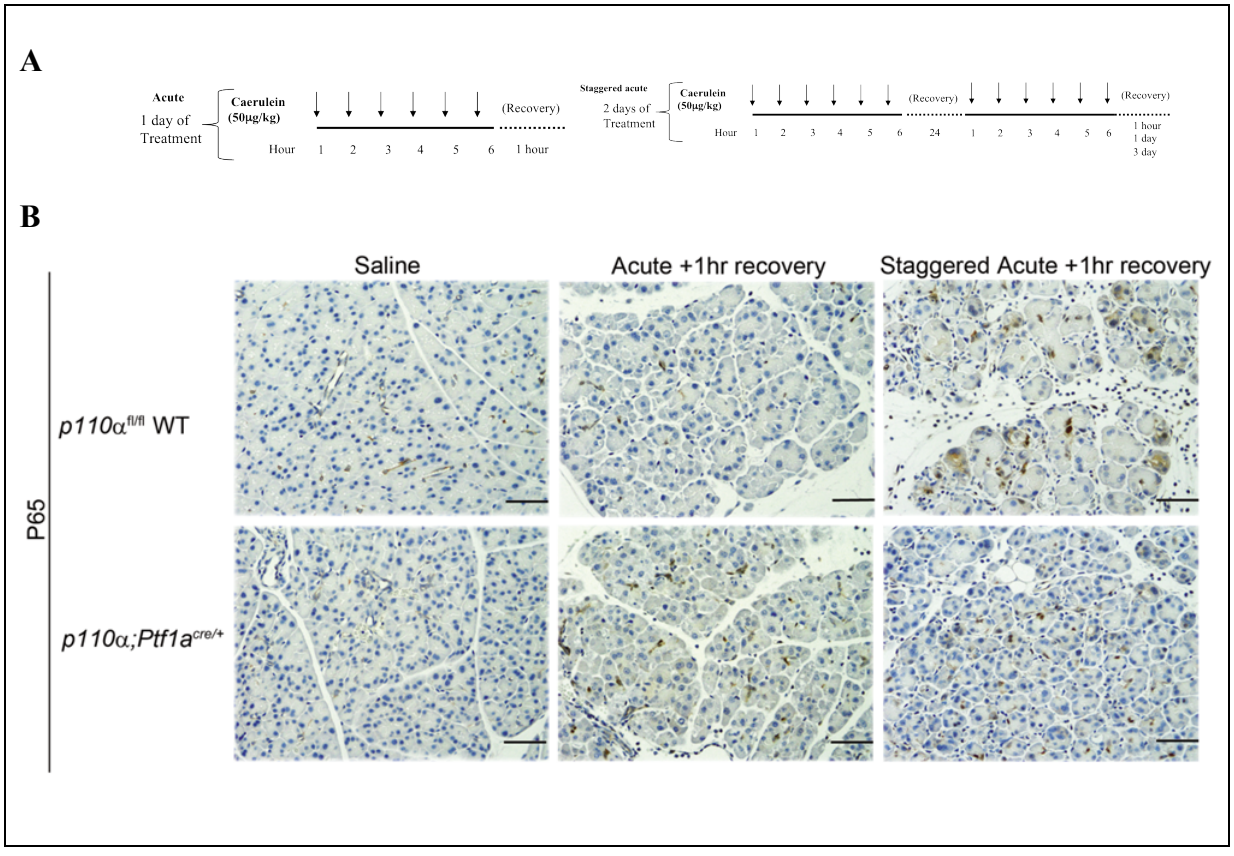
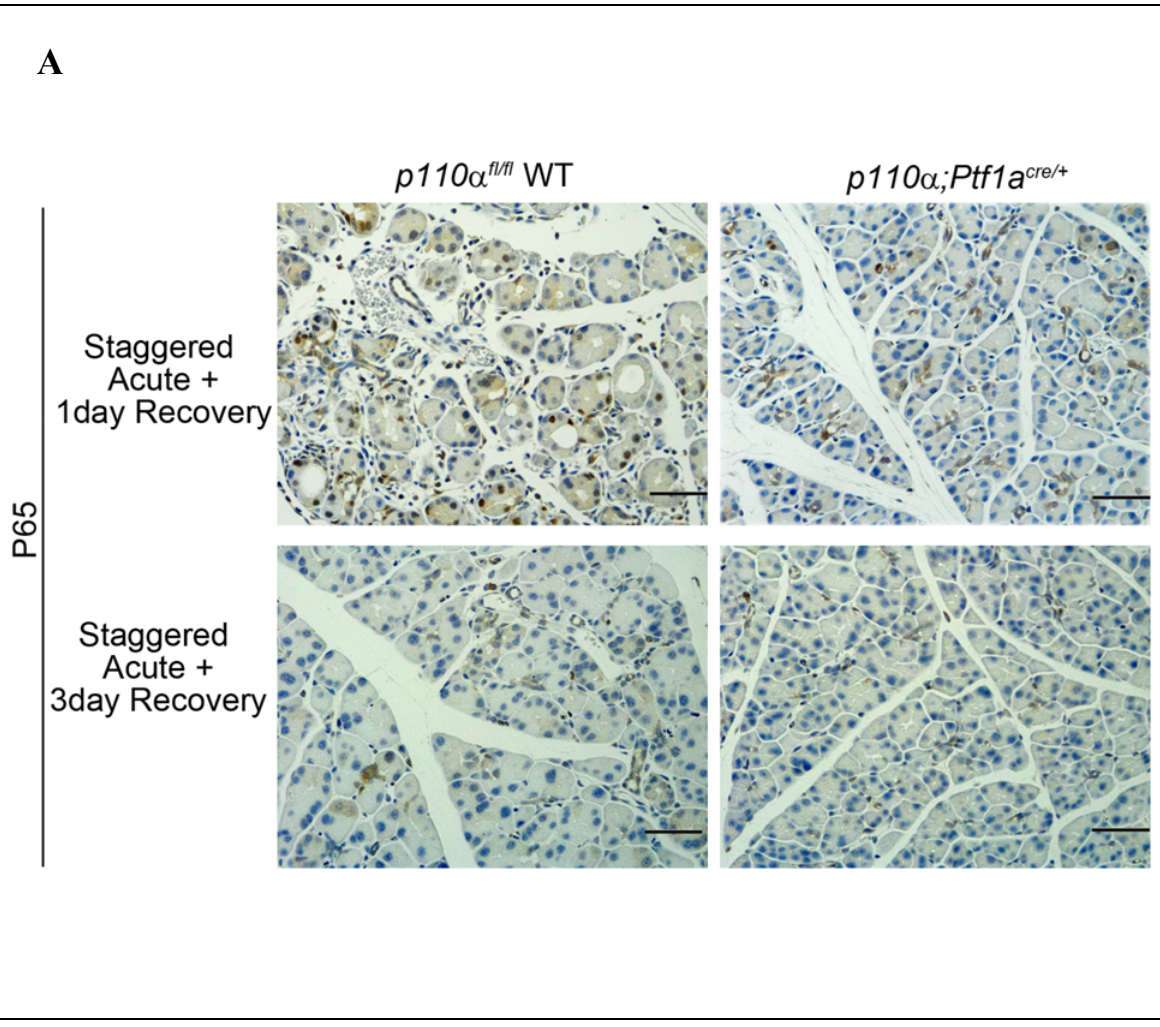


Figure 2-7. Acinar cells can initiate proinflammatory signals without p110α

A. Schematic of our acute pancreatitis protocol and the "staggered acute" protocol. Both protocols consists of a series of hourly injections of cerulein at a 50μg/kg concentration. B. p110α$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice and wild type mice were subjected to acute pancreatitis (7 hourly injections at 50μg/kg) or staggered acute treatments (2 series of acute injections with a day of rest in between). Mice were euthanized 1hr after treatment. Expression of NFκ-B subunit, p65 was assessed by IHC. An upregulation of p65 can be seen in both wild type and p110α knockout mice after both treatments. Scale bars: 60μm (20x).



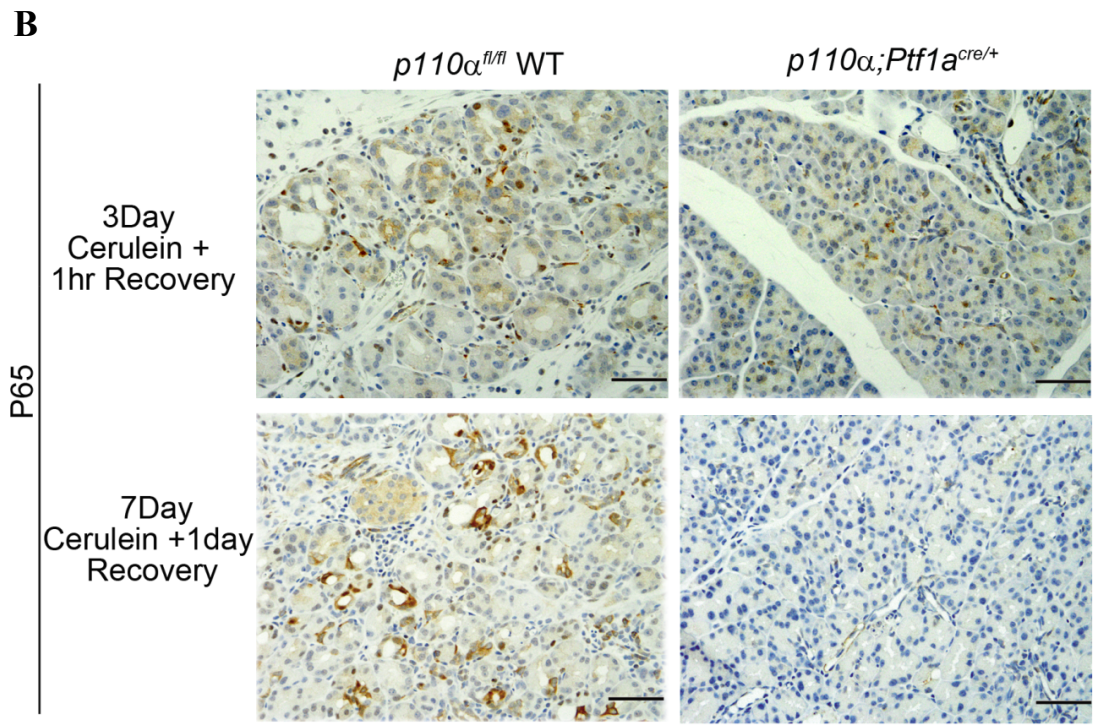


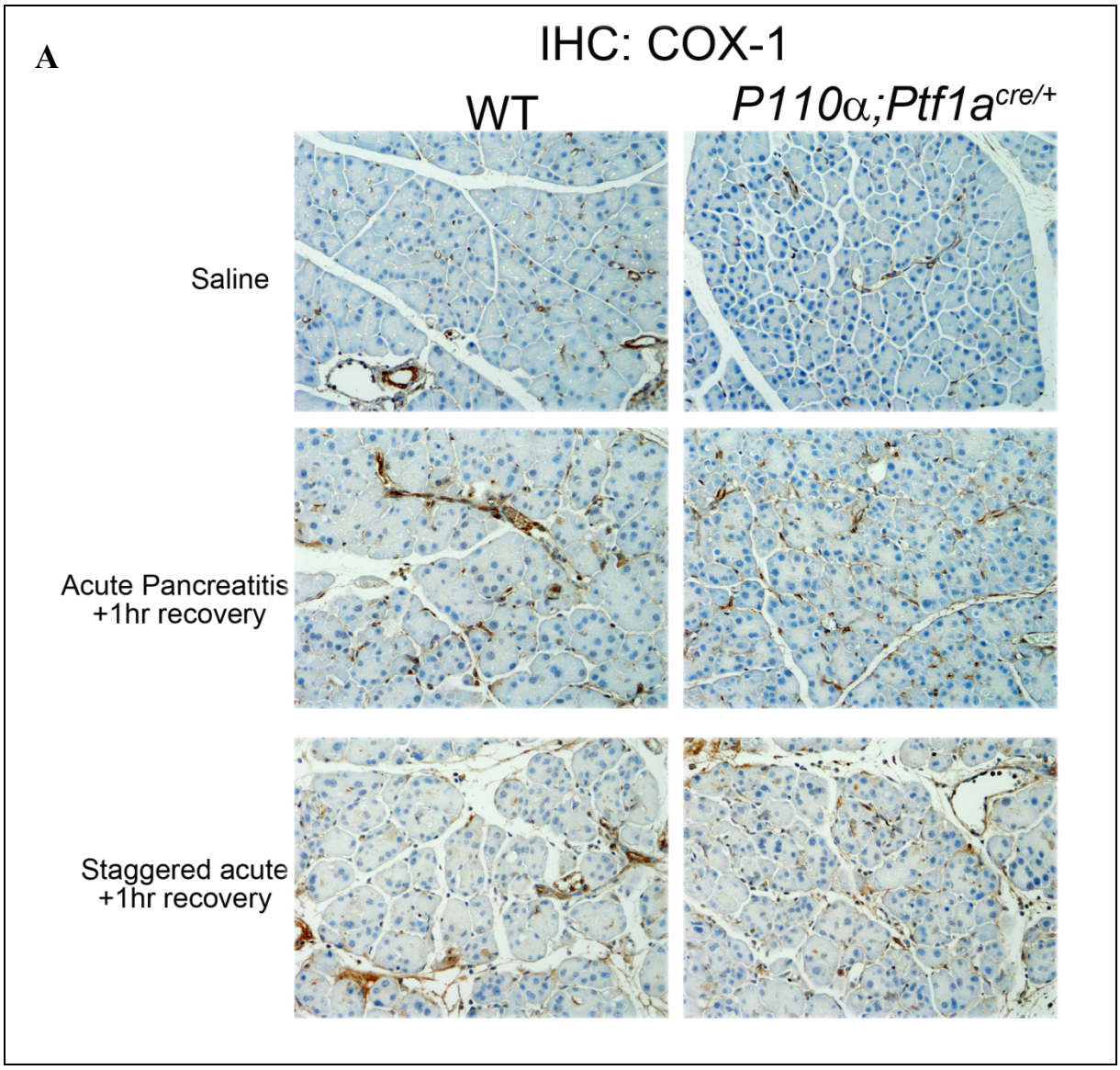
Figure 2-8. Metaplastic lesions sustain inflammatory signals

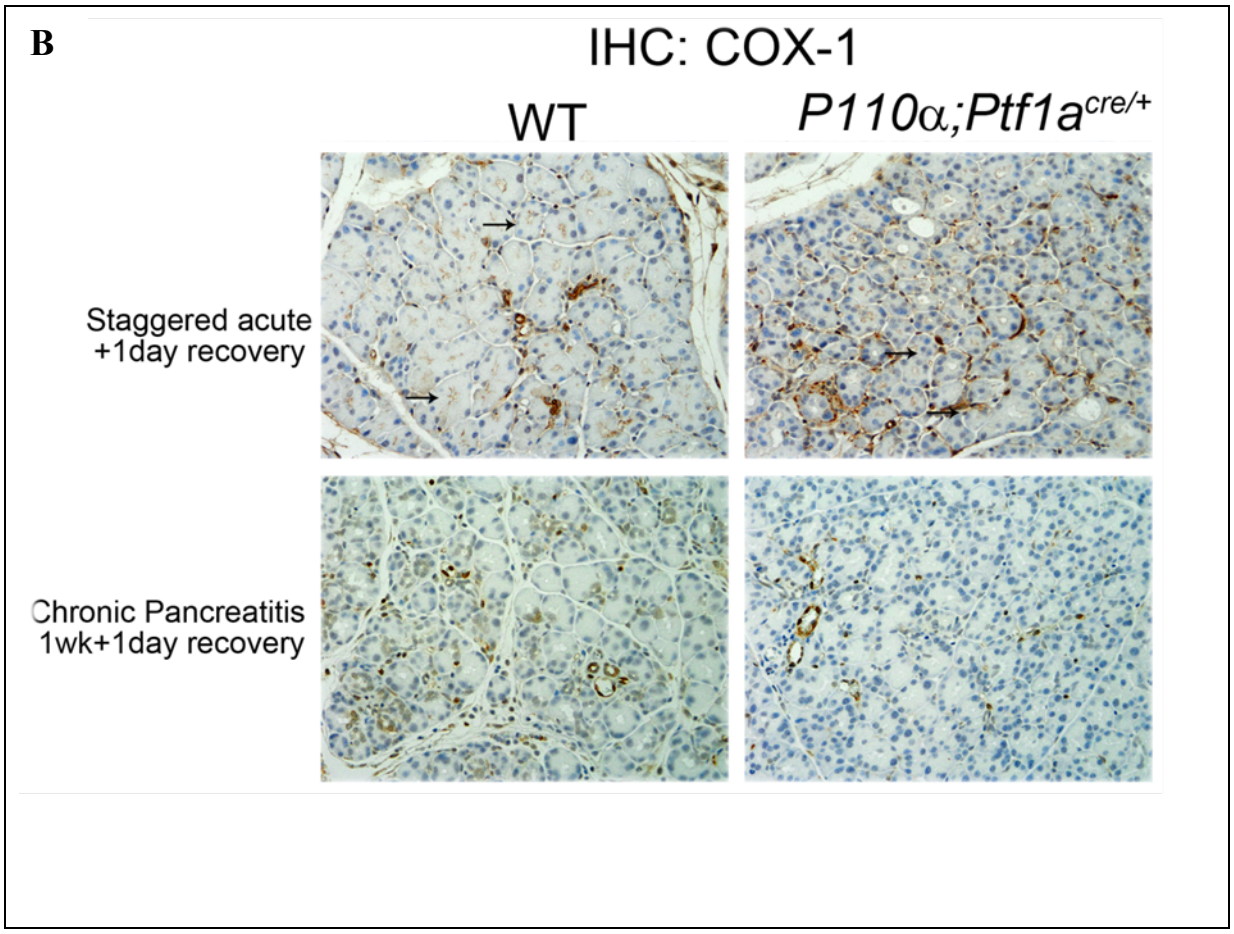
Wild type mice and p110α knockout mice underwent staggered acute treatments. Mice were euthanized 24hr or 3day after treatment and p65 expression was assessed by IHC. A) In wild type tissue, an upregulation of p65 can be seen in metaplasia and surrounding acinar cells after a 24hr recovery. p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice express p65 in the acinar cells but there are no metaplastic ducts. After 3 days recovery from the staggered acute treatment, the tissue in both mouse models healed and p65 expression disappeared. B) Chronically treated mice (3 day or 7 day) show that metaplastic lesions and acinar cells surrounding them express p65 at l hour recovery. After 1 day recovery only metaplastic ducts are p65 positive indicating that early p65 expression is transient in cells that do not

undergo ADM. p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice only express p65 after 1 hr recovery. There is no p65 expression or metaplastic ducts in p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice treated for 7 days and allowed 1 day recovery. Scale bars: 60μm.

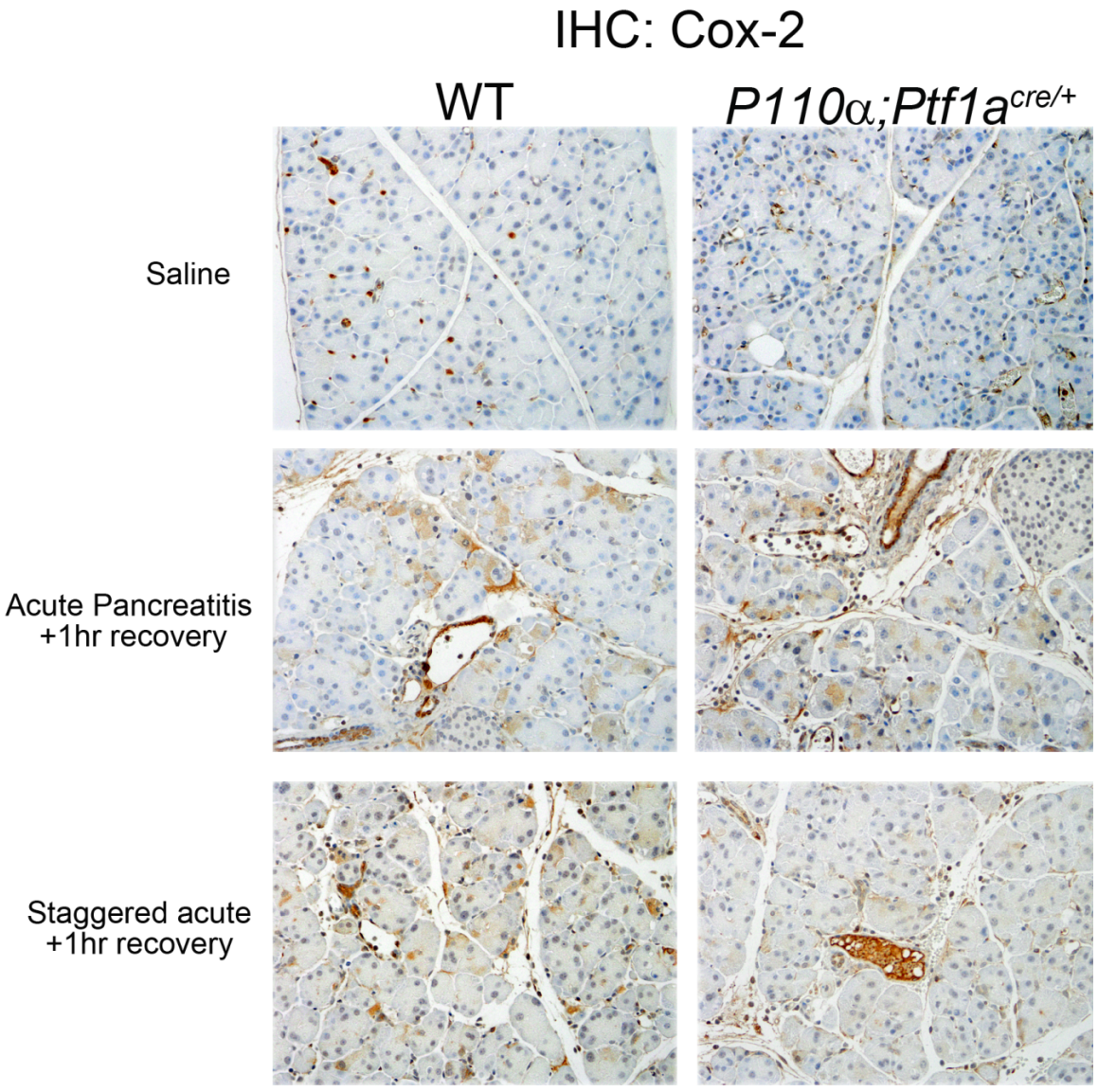
vi. Cox-1 and Cox-2 expression is upregulated in metaplastic ducts.

Pancreatic damage that leads to chronic inflammation is marked by the upregulation of pro inflammatory signals derived from the epithelium and stromal tissue. Along with NFκ-B, we looked at pro inflammatory molecule, Cyclooxygenase (Cox) 1 and 2. Cox-1 and Cox-2 are both able to able to breakdown the same downstream molecules but Cox-2 is known to be induced my many upstream activators, while Cox-1 is thought to be expressed more constitutively and in low levels[111]. Cox-2 is expressed in chronic pancreatitis, and arguably has a role in acute pancreatitis[112,113]. The role of Cox-1 in pancreatitis is still to be elucidated. By evaluating different cerulein protocols I tested to see when and where Cox-1 and Cox-2 was expressed by IHC. Cox-1 appears in mice that had 24 hrs to recover from a staggered acute treatment **(Figure 2-9)**. Cox-1 expression can be seen in acinar cells undergoing ADM **(Figure 2-9)**. Chronically treated mice have a well-defined expression of Cox-1 in metaplastic lesions and these lesions are absent in p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ **(Figure 2-9)**. Cox-2 is expressed in the acinar cells of mice treated with acute pancreatitis **(Figure 2-9)**. In chronic pancreatitis samples, Cox-2 is expressed strongly in ducts and lightly in areas of metaplasia. Ablation of p110α blocks the expression of Cox-1 and Cox-2 in the chronic model, most likely by impeding ADM. This data further supports the hypothesis that MDL are inflammatory mediators, that are regulated by p110α.


A
IHC: COX-1
WT
P110α;Ptf1a^cre/+
Saline
Acute Pancreatitis
+1hr recovery
Staggered acute
+1hr recovery


B
IHC: COX-1
WT
P110α;Ptf1a^cre/+
Staggered acute
+1day recovery
Chronic Pancreatitis
1wk+1day recovery

C


IHC: Cox-2
WT
P110α;Ptf1a cre/+
Saline
Acute Pancreatitis
+1hr recovery
Staggered acute
+1hr recovery

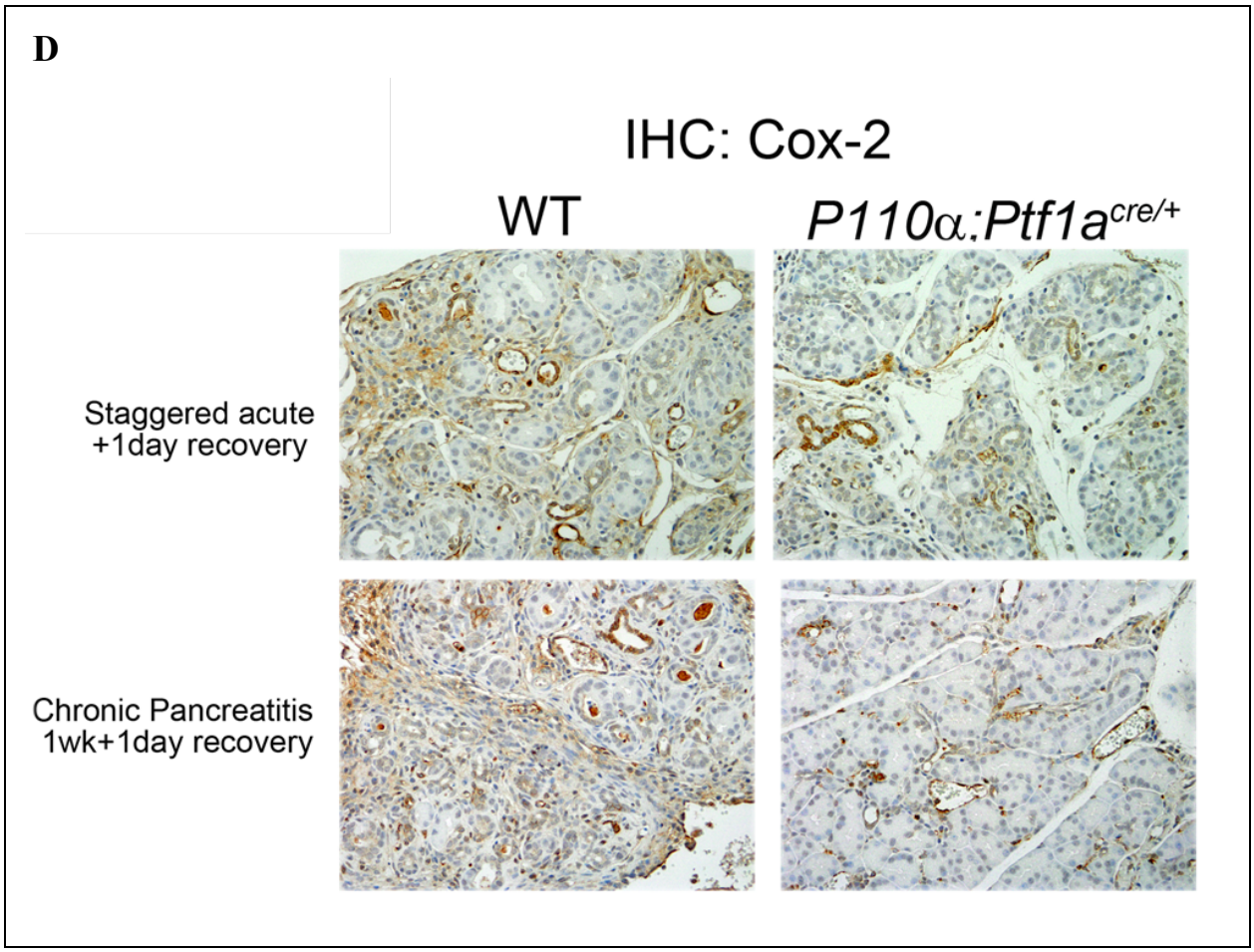


Figure 2-9. p110α can only impede pro-inflammatory signals expressed in metaplasia

The expression of pro-inflammatory signals, Cox-1 and 2 during different phases of pancreatitis were assessed by IHC. A&B) In wild type mice, Cox-1 is not expressed during early acute injury but it can be seen during early acinar cell dilation (staggered acute + 24hrs) and in metaplastic lesions (chronic pancreatitis). The p110α$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice express Cox-1 24 hrs after the staggered acute protocol but cannot be found in

chronically treated mice. Cox-1 is only sustained in metaplastic lesions, which are absent in the p110α knockout mice. C&D) Cox-2 is expressed in the early acute response. The p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice are unable to block the initial Cox-2 expression. In chronically treated mice, low expression of Cox-2 is observed in metaplasia and high expression is seen in ducts. The p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice treated with this protocol lack Cox-2 staining

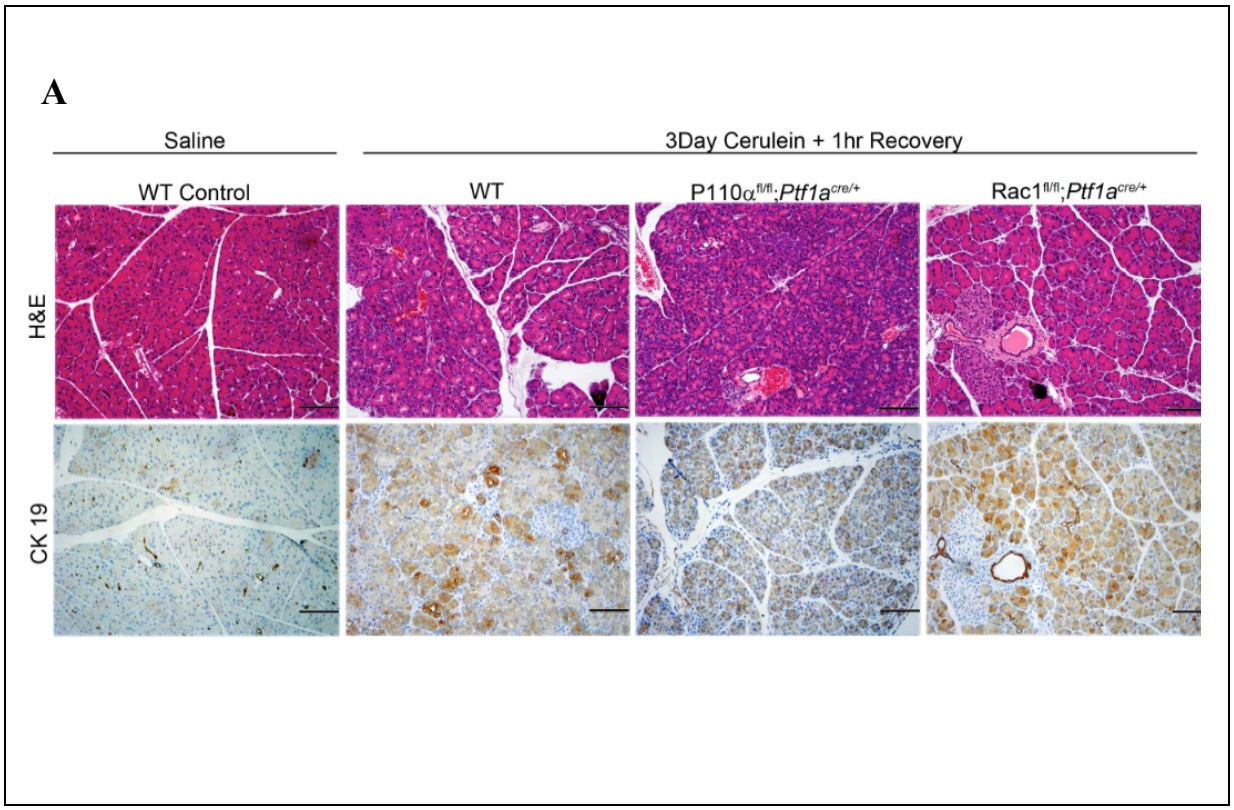
vii. Metaplastic conversion requires PI3K-Rac induced actin remodeling

After demonstrating that PI3K regulates MDL-induced inflammation, I set out to test how the loss of p110α is preventing ADM. To observe the development of MDLs at the onset of metaplasia, we employed a modified pancreatitis protocol (2 daily 250mg/kg injections of cerulein for 3 days and sacrificed the mice 1 hr after the final injection). Histology shows that the pancreas had different stages of disease, which include dilated acinar cells, and metaplasia **(Figure 2-10).** CK19 expression is up regulated in acinar cells subjected to pancreatitis, with strong staining in metaplastic lesion **(Figure 2-10).**

Given the previous findings that Rac1 is required for the formation of preneoplastic lesions[51], we tested if p110α is preventing ADM in a Rac1 dependent manner. To phenotypically assess PI3K-Rac signaling in the context of pancreatitis, we used pancreas specific Rac1 KO mice (Rac1 $^{fl/fl}$;*Ptf1a*$^{cre/+}$). When treated with cerulein for 3 days, both p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ and Rac1 $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice appear to lack well defined metaplasia and the lesions in these knockouts rarely progress beyond being dilated acinar cells with enlarged lumens **(Figure 2-10).** An interesting observation in this experiment shows that Rac1 $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice have up regulated CK19 despite the lack of metaplasia **(Figure 2-10).**

Since CK19 has been established as a ductal marker expressed in metaplasia[114], the up regulation of this marker without the actually metaplastic conversion led us to investigate the impeded acinar to ductal metaplasia (ADM) process.

Since Rac has been implicated in actin remodeling and PI3K can activate Rac through GEF localization[51,65], we hypothesized that p110α is manipulating actin-cytoskeleton rearrangment. Fluorescent staining shows that filamentous actin (phalloidin) is disrupted in cerulein treated wild type pancreata at this stage of disease **(Figure 2-10).** Conversely, Both p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ and Rac1 $^{fl/fl}$;*Ptf1a*$^{cre/+}$ pancreata are resistant to cerulein induced actin rearrangement **(Figure 2-10).** ZO-1 serves as an apical marker, to illustrate the actin remodeling that takes place upon cerulein administration **(Figure 2-10.)** Taken together these data suggests that the loss of p110α PI3k may protect against ADM formation by Rac mediated actin cytoskeleton rearrangement.



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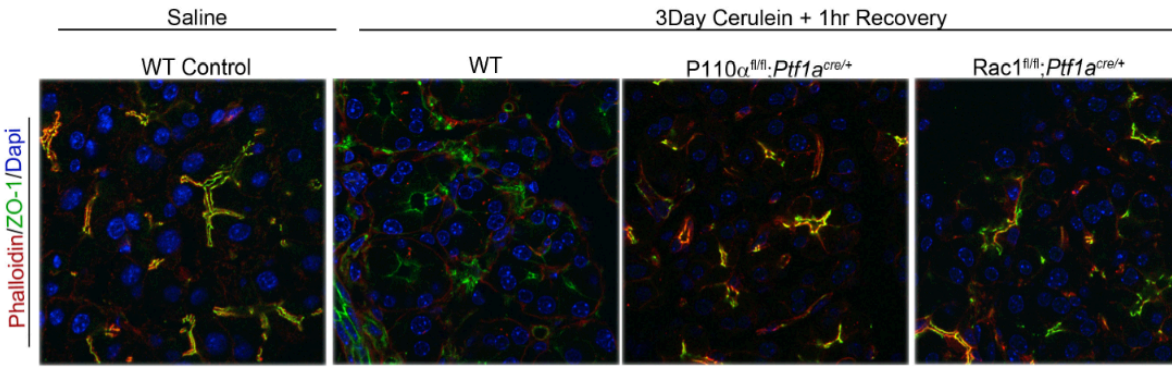


Figure 2-10. p110α signaling regulates Rac induced actin rearrangements

A modified cerulein treatment protocol was administered to observe early acinar cell changes during the development of chronic pancreatitis. A) H&E depicts dilated acini and lumens forming within acinar clusters in wild type mice treated with cerulein. p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ and Rac1 $^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice display dilated acini but have less acinar lumens compared to cerulein treated wild type tissue. B) Fluorescence staining shows actin rearrangement with phalloidin (red), a molecule that binds to filamentous actin. Apical, ZO-1 (green) is co-localized with phalloidin in control tissue but the actin spreads when the pancreas is treated with cerulein p110α $^{fl/fl}$;*Ptf1a*$^{cre/+}$ and Rac1$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice maintain the colocalization of ZO-1 and phalloidin, demonstrating that actin remodeling is impeded. Scale bars: 120μm (10x)

viii. Loss of Rac1 protects mice from developing metaplastic lesion and chronic pancreatitis

Early actin remodeling is blocked in both p110α $^{fl/fl}$*;Ptf1a*$^{cre/+}$ and Rac1$^{fl/fl}$*;Ptf1a*$^{cre/+}$ mice. Previously the loss of Rac1 was shown to block the formation ADM and PanINs when mice carry a pancreas specific oncogenic Kras^{G12D} mutation[51]. To demonstrate that this phenotype holds true in our cerulein induced chronic pancreatitis model, we injected Rac1 $^{fl/fl}$*;Ptf1a*$^{cre/+}$ mice with 250 μg/kg cerulein, twice daily for seven days. Rac1 deficient mice showed a significant decrease in inflammatory infiltration, as demonstrated by IHC for F4/80, Ly6.B, and CD3 **(Figure 2-11)**. Rac1 $^{fl/fl}$*;Ptf1a*$^{cre/+}$ mice also appear entirely resistant to forming metaplastic ducts, as characterized by CK19 expression **(Figure 2-11)**. These results are comparable to what we found in the P110a $^{fl/fl}$*;Ptf1a*$^{cre/+}$mice and demonstrates that Rac1 and p110α may function through the same pathway. Expression of CK19 and DBA lectin is higher in Rac1 $^{fl/fl}$*;Ptf1a*$^{cre/+}$ compared to untreated control, though the staining appears to be labeling dilated acinar cells and actual ducts rather than metaplasia **(Figure 2-11)**. This was not observed in the $^{fl/fl}$*;Ptf1a*$^{cre/+}$mice, and suggests that p110a may be necessary for the ADM process slightly earlier than Rac1.

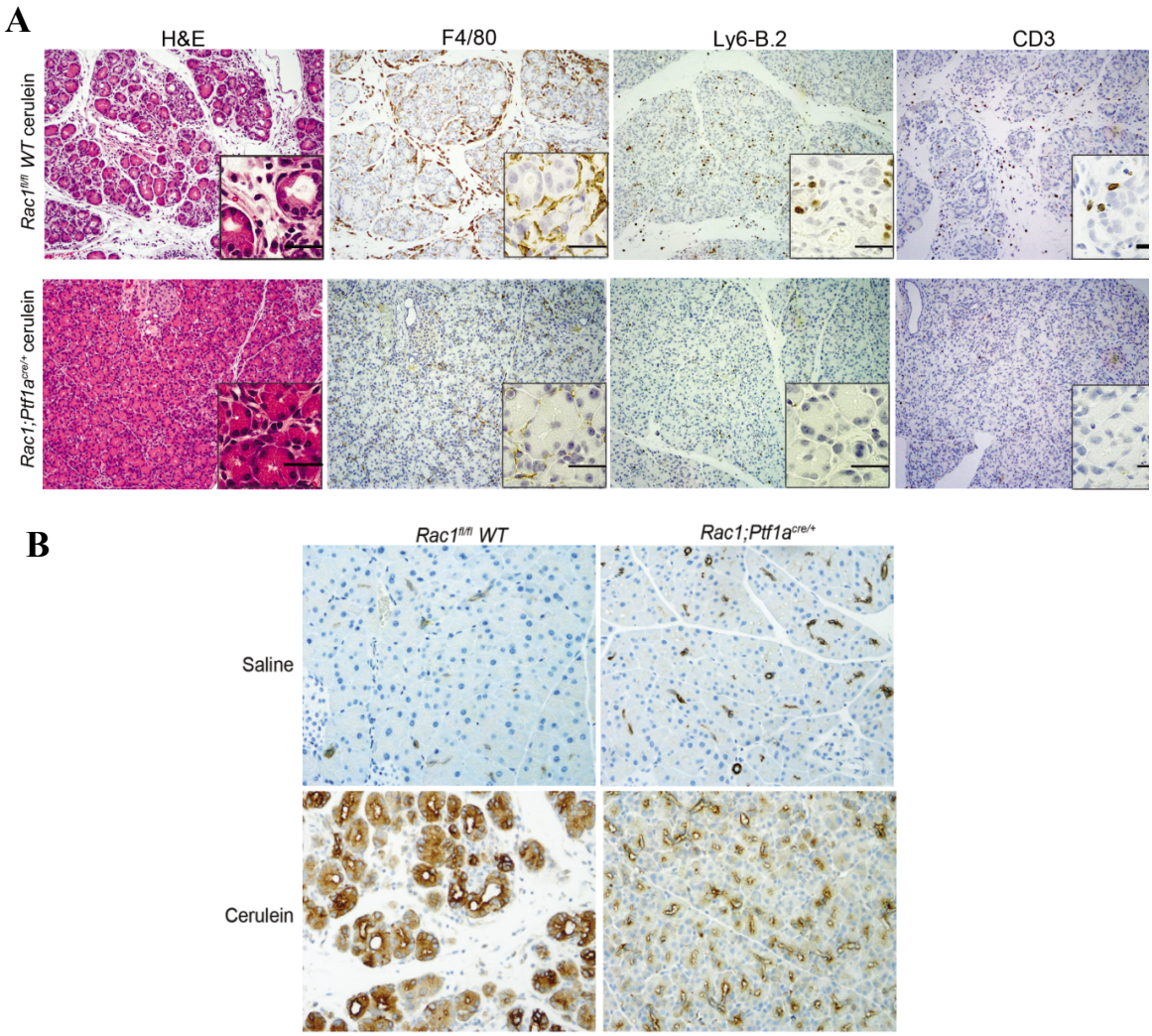


Figure 2-11. Loss of Rac1 protects mice from chronic pancreatitis and blocks ADM

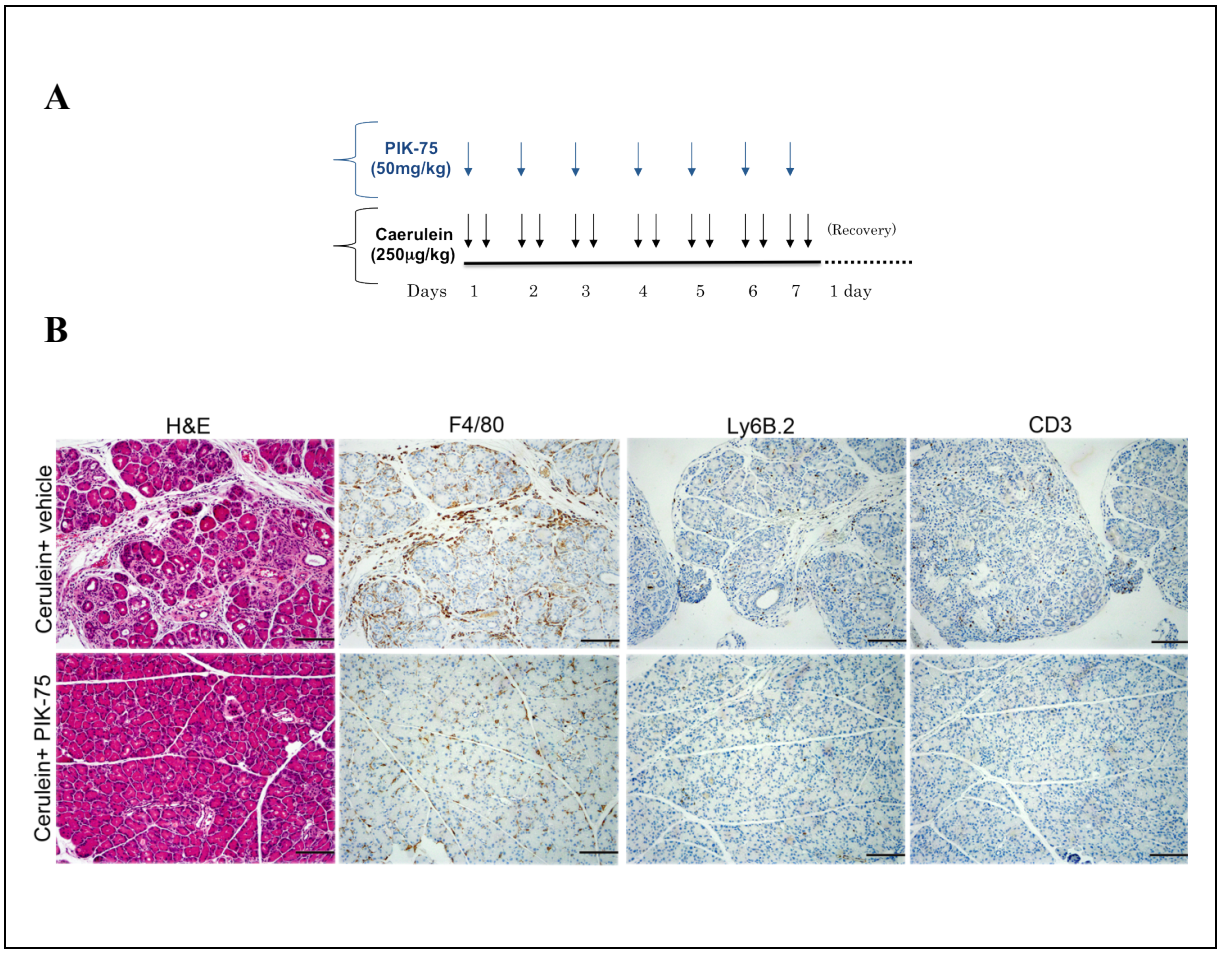
Ablation of Rac1 prevents cerulein mediated pancreatitis. A) IHC of F4/80 (macrophages), LY6B.2 (neutrophils), and CD3 (T-cells) shows that inflammatory cell infiltration is significantly lower in Rac1$^{fl/fl}$;*Ptf1a*$^{cre/+}$ mice compared to controls. B) There is higher CK19 expression in wild type cerulein treated mice compared to Rac1 $^{fl/fl}$;*Ptf1a*$^{cre/+}$. Expression of CK19 is higher in cerulein treated Rac1 $^{fl/fl}$;*Ptf1a*$^{cre/+}$ than saline control. Scale bars: 120μm (10x); inserts: 30μm (40x).

ix. Pharmacological inhibition of p110α protects against chronic pancreatitis as effectively as the genetic knockout

The results shown so far support the hypothesis that p110α is a good therapeutic target for CP. To test if pharmacological inhibition of p110α can serve as preventive therapy against CP, we treated wild type C57 Bl6J mice with the p110α inhibitor, PIK-75, together with cerulein. We administered PIK-75 at 50mg/kg, which fell within the manufacture's range of a specific and non-lethal daily dose. We performed the chronic pancreatitis protocol and administered a dose of PIK-75 each day immediately before the first injection. **Figure 2-12** shows that PIK-75 is able to block the development of chronic pancreatitis, leaving the tissue almost as healthy as control. IHC of the inflammatory markers F4/80, Ly6B.2, and CD3 verify that PIK-75 impeded cerulein induced inflammatory infiltration **(Figure 2-12)**. CK19 and DBA-Lectin staining demonstrates that there are fewer ductal lesions found in the PIK-75 treated group **(Figure 2-12)**. This data concludes that pharmacological inhibition of p110α phenotypically copies the genetic knockout model.

One hypothesis that we were unable to test using the $p110\alpha^{fl/fl};Ptf1a^{cre/+}$ mouse model, is if inhibiting p110α after a subject is afflicted with pancreatitis can have a therapeutic benefit. To address this question, we treated mice with PIK-75 after they already have pancreatitis. Mice recover from cerulein induced pancreatitis about 1 week after the last injection (Data not shown). To circumvent this issue, we established pancreatitis first (7 day chronic protocol) and then continued giving cerulein injections for 6 days in order to maintain the pancreatitis phenotype. By administering PIK-75 during those 6 days of

extended cerulein treatment, the tissue was protected from having significant metaplasia **(Figure 2-13)**. There was inflammation present since infiltration of macrophages, neutrophils, and T-cells were there prior to PIK-75 treatment and though metaplasia was gone, the inflammation did not completely resolve (Data not shown). Residual inflammation and fibrotic scarring is also seen in the tissue of wild type mice that was allowed to recover from cerulean induced pancreatitis. Together, this suggests that PIK 75 inhibits cerulein induced ADM signals and allows the tissue to heal despite a persistent insult.



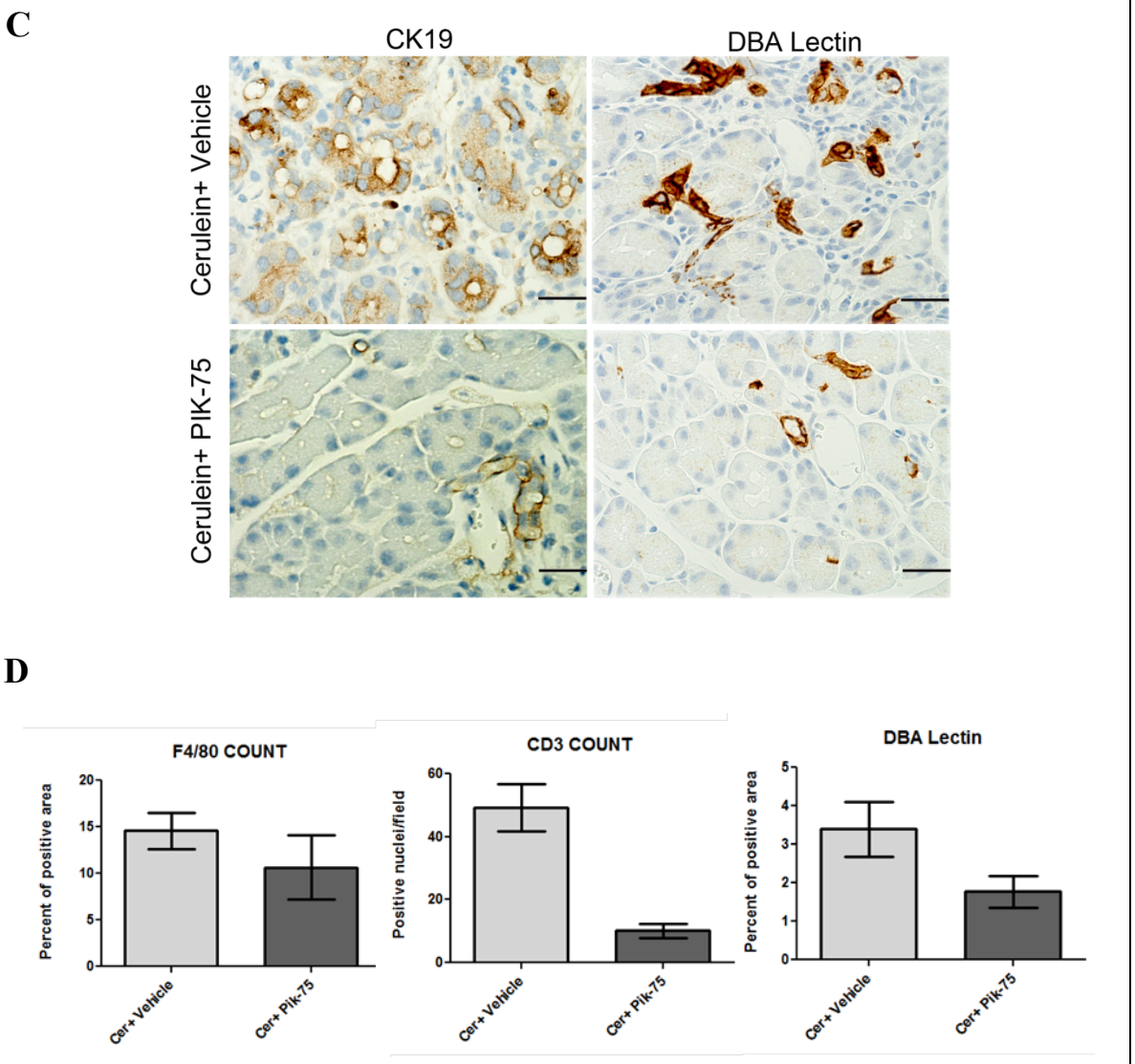


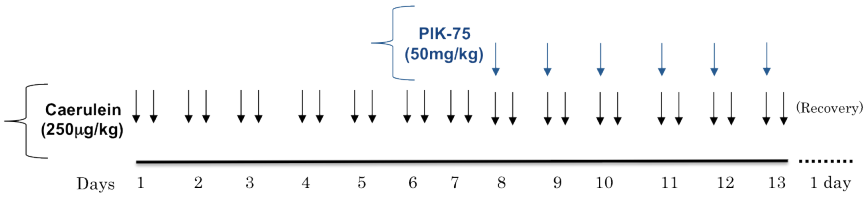
Figure 2-12. Pharmacological inhibition of the p110α subunit of PI3K protects against and ameliorates damage induced by chronic pancreatitis.

The administration of the p110α inhibitor, PIK-75 can block the effects of cerulein induced pancreatitis. A daily dose (50mg/kg) of PIK75 was administered via I.P during the duration of a chronic pancreatitis protocol (2 daily injection injection of cerulein for

7 days). The mice were given 1 day recovery after the last injection and were euthanized. A) Markers of inflammation: F4/80 (macrophages), LY6B.2 (neutrophils), and CD3 (T-cells) were more prevalent in wild type mice compared to PIK-75 treated mice. B) Ductal markers, CK 19 and DBA lectin had reduced expression in PIK 75 treated mice. C) Quantitation of innate immune marker (F4/80), adaptive immune response (CD3), and ductal marker DBA Lectin, shows decreased inflammation and metaplasia respectively. Scale bars: B) 120μm (10x)

C) 60μm (20x)

A



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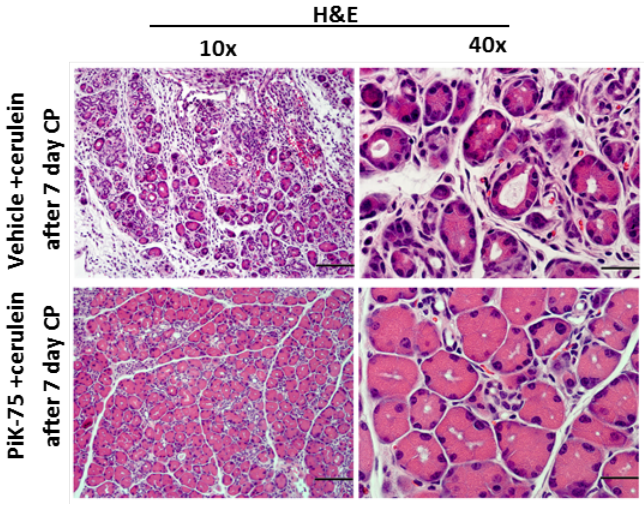

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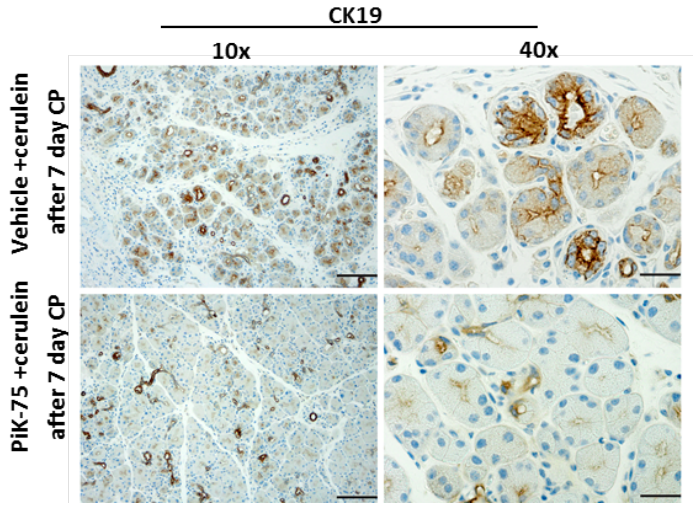
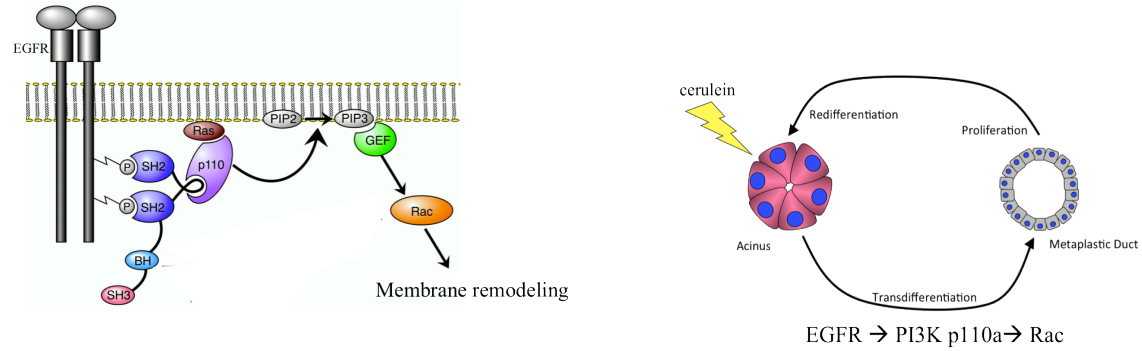


Figure 2-13. Inhibiting the catalytic activity of p110α blocks cerulein induced ADM allowing pre-existing pancreatitis to recover

A.) Chronic treatments of cerulein were administered to mice (twice a day for 1 week) which were followed by daily injections of PIK-75 and cerulein for 6 days. Mice were euthanized 24hrs after final injection. B&C.) H&E and CK-19 staining show that mice that were given cerulein for the 6 days after the chronic protocol, maintained a chronic pancreatitis phenotype. The combination of PIK-75 and cerulein blocked further pancreatic damage and allowed the pancreatic tissue to heal normally. Scale bars: 120μm (10x), 30μm (40x).



Adapted from K. Okkenhaug et al., *Science* (2001)

Figure 2-14 Model of PI3K-Rac signaling during cerulein induced ADM

A.) Mechanism by which p110α may induce Rac dependent cytoskeleton remodeling. Once EGFR is activated by its ligands, its phosphorylation allows for PI3K to bind and localize to the plasma membrane. At the membrane PI3K generates $PI(3,4,5)P_3$, providing a docking site for Rac GEFs. B.) This study proposes that PI3K and Rac are necessary for cerulein induced acinar to ductal metaplasia producing the metaplastic lesions that sustain chronic inflammation.

2-3 Discussion

Pancreatitis is a disease on the rise, due to better diagnostics we are aware that both chronic and acute pancreatitis affects a greater subset of the population than originally thought. Insight to how chronic pancreatitis develops can also help us understand how pancreatic cancer forms and perhaps discovering molecular targets that can improve the prognosis of patients with pancreatitis can also help those with PDAC.

The genetic ablation and pharmacological inhibition of p110α allowed us to study the role and therapeutic potential of p110α, the catalytic subunit of PI3K. By using the long standing cerulein induced pancreatitis model, we found that blocking p110α signaling can prevent the development of chronic pancreatitis because ADM cannot be sustained. Although it has already been shown that PI3K is expressed in chronic pancreatitis, we have demonstrated that p110α is an integral protein required for the maintenance of the disease.

Previous reports state that the p110γ catalytic subunit is important for developing acute pancreatitis[70]. p110γ knockout mice are protected from cerulein induced acute pancreatitis[70]. Since this model was a full body knockout, loss of this gene in the inflammatory and stellate cells could have been responsible for this phenotype. A second group tested this possibility and administered CCK to isolated the acinar cells of p110γ knockout mice[115]. They showed that NFκ-B signaling was diminished compared to wild type cells[115]. These data demonstrates that p110γ can direct acinar cells signaling and may suggest that p110γ and p110α have a complimentary role in pancreatitis since we did not find significant p110α expression in acinar cells before ADM. p110α is upregulated in metaplastic ducts and our findings conclude that this PI3k isoform is be important for the ADM transition and maintainence. Perhaps a model for pancreatitis can be proposed where different catalytic subunits of PI3K are expressed at different times and in different cell types.

The epithelial-inflammatory interaction is an important component of pancreatitis. Groups have targeted the inflammatory compartment to disrupt pancreatitis in rats and mice[77,111,116], while others have blocked cell autonomous signals[50]. In this study, we have

shown that the inflammatory response that follows the intracellular activation of digestive enzymes is transient and that metaplastic lesions are a cause for chronic inflammation. By blocking ADM, we can potentially stop chronic pancreatitis. As described in Daniluk et al. (2012), NFκ-B is necessary for the formation of chronic pancreatitis; by inhibiting PI3K signaling we prevent the formation of metaplastic ducts, which expresses sustained nuclear p65. Studies show that PIK75 is able to block NFκ-B signaling in monocyte cell lines, suggesting that this mechanism can occur in other systems. Without p110α metaplastic duct formation, expression of pro inflammatory markers Cox-1 and Cox-2 are also blocked. Though these molecules have not been shown to be necessary for driving chronic pancreatitis, they may still contribute to the inflammatory infiltration of the pancreas[117].

It is reassuring to observe that pharmacologically inhibiting p110α protects mice from cerulean induced pancreatitis just as well the genetic knockout models. PIK-75 and other p110α inhibitors have served in many cancer and immunology studies, and past studies have successfully treated mouse models with PIK-75. My current study adds to what is known about PI3K and may encourage clinical investigators to use p110α inhibitors to treat chronic patients or even to extend this treatment to those with ADM and early pancreatic tumor lesions.

2-4 Future Directions

The inhibition of EGFR and Rac1 were shown to be sufficient in preventing ADM *in vivo*[44,51]. PI3K may be functioning as an intermediate molecule, being activated by EGFR and then activating Rac **(Figure 2-14)**. This model may not be so simple, it is still to be determined which Rac-GEFs require PIP3 to be anchored to the plasma membrane, it is also

hypothesized that PI3K binds to Rac and its GEF in a complex[118]. This mechanism needs to be tested further in both cerulein induced pancreatitis tissue and in isolated pancreatic cells. How EGFR is important for PI3K and Rac may also be more than a scaffolding function especially since inhibition of MEK, downstream signal of EGFR but not PI3K or Rac can also prevent ADM[44]. How these three molecules interact and induce a positive feedback loop is worth investigating.

The PIK-75 treatment suggests that the loss of PI3K catalytic activity appears to be sufficient to prevent ADM. Both our data and that of Heid et al (2011) show that Rac induces cytoskeletol rearrangements[51]. Since the loss of p110α phenocopies these results, future experiments must test how PI3K is regulating actin remodeling and ADM. This may be dependent solely on the catalytic activity of p110α or protein interaction may be important. The implications of PI3K signaling to Rac1 are worth exploring further. Our lab has a kinase dead p110α mouse available to explore how p110α kinase activity may pancreatitis. This can be compared to the total p110α knockout model and or PIK-75 treatment. Colocalization studies of Rac and p110α can also be done if proper antibodies can be obtained. Lastly, we will also test if PIK-75 aids in the recovery process, by administering PIK-75 shortly after a cerulein treatment protocol. We hypothesize that we can evaluate if PI3K can revert MDL back to acinar cells if we administer PIK-75 and euthanize mice before the normal recovery process takes place.

In conclusion, there are still questions to be answered for how exactly p110α

Is exerting its function on pancreatic disease. The new insights on PI3K signaling and pancreatitis I have explored expand what we know about the field and may potentially benefit those afflicted with pancreatic disease.

Chapter 3: Ablation of A Disintegrin and Metalloproteinase 10 (ADAM10) disrupts typical PDAC progression

3-1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer related death in the U.S[14]. This disease has a poor prognosis due to late detection and rapid progression to metastasis. The search for viable drug targets to treat this disease has been an imperative aim for members of the field.

A Disintegrin and Metalloproteinase-10 (ADAM10) is a membrane bound metalloproteinase responsible for shedding bioactive molecules from the cell surface and initiating regulated intra-membrane proteolysis (RIP) of cell surface proteins such as Notch receptors[80]. Both ADAM10 and Notch are highly expressed in PDAC, with the latter being implicated in PDAC progression[101,103]. The 4 Notch receptors are all up regulated in human PDAC, with Notch 3 being the highest and Notch 1 and 2 still prominent[103].
Studies in pancreatic cancer cell lines have demonstrated that silencing Notch 1 decreases proliferation and increases cancer cell death [89]. Since past studies demonstrated that ADAM10 is the membrane bound protease that initiates Notch RIP, I hypothesize that the loss of ADAM 10 will decrease cancer cell viability [80,90,91,95].

Mouse models have been generated to understand the role of Notch in pancreatic cancer. The LSL-Kras$^{G12D/+}$;*Ptf1a*$^{cre/+}$ mouse model expresses all 4 Notches as well, with

Notch 1 and 2 having the highest protein expression[106]. The ablation of Notch 1 in LSL-Kras$^{G12D/+}$;*Ptf1a*$^{cre/+}$ mice increased the rate of tumor progression suggesting Notch 1 is a tumor suppressor[104]. However, another study showed that transgenic expression of the intracellular domain of Notch 1 (NICD) leads to faster tumor progression[105]. These mice also showed an upregulation of Notch target genes, members of the Hes and Hey family[105]. This study is consistent with research that shows that overexpression of (NICD) can promote tumor formation by inducing acinar to ductal transdifferentiation in isolated acinar cells[103]. The loss of Notch 2 has a unique phenotype. Pancreas specific Notch 2 knockout mice that express oncogenic Kras form large cystic lesions and have an increased lifespan.[106] The different Notch dependent phenotypes present in genetic mouse models demonstrates that the 4 Notch receptors may not have redundant roles. Thus, I set out to assess what would occur if we were to ablate the function all Notch proteins; to do this we generated conditional ADAM 10 knockout mice (ADAM10$^{fl/fl}$) and crossed them into the LSL-Kras$^{G12D/+}$;*Ptf1a*$^{cre/+}$ mouse model to generate an ADAM10$^{fl/fl}$; LSL Kras$^{G12D/+}$;*Ptf1a*$^{cre/+}$ mouse line. Our hypothesis is that if ADAM 10 is the primary protease for all 4 Notch receptors, the ablation of ADAM 10 would result in the decreased expression of Notch target genes, since the activity of all Notch proteins would be blocked. This could potentially result in decreased tumor formation if decreasing Notch target gene expression yields an opposite phenotype to the NICD transgenic mouse model[105]. Our studies aimed to verify if ADAM 10 is a promising target for treating PDAC.

3-2 Materials and Methods

Mouse strains

ADAM 10 $^{fl/fl}$ mice are described here, *Ptf1a$^{Cre/+}$*, LSL-Kras$^{G12D/+}$,LSL-Trp53$^{R172H/+}$; *ROSA26$^{LSL-YFP}$* strains have been described previously and were genotyped accordingly[18,35,119,120]. Experiments were conducted in accordance with the Office of Laboratory Animal Welfare and approved by the Institutional Animal Care and Use Committee at the Mayo Clinic and Stony Brook University.

Genotyping

ADAM10 Recombination

Forward Primer Sequence 5'- 3': CGTATCTCAAAACTACCCT CCC

Reverse Primer Sequence 5'-3': GTTGGACATAACTTTGGAT CTCC

Cre

Forward Primer Sequence 5'- 3': TCGCGATTATCTTCTATAT CTTCAG

Reverse Primer Sequence 5'-3': GCTCGACCAGTTTAGTTACC C

K-Ras

Forward Primer Sequence 5'- 3': CGCAGACTGTAGAGCAGC G

Reverse Primer Sequence 5'-3': CCATGGCTTGAGTAAGTCT GC

Histology

For methods see Chapter 2.

Quantitation

Aperio ScanScope XT slide scanner (Aperio Spectrum, Vista, CA) and image analysis software (ImageScope) were used for quantitative assessment. Quantitation represents the

average of 5-7 10x/20x fields of view for mice of each genotype. Number of mice quantitated for each experiment is stated in the figure legends.

Immuno fluorescence

For Methods see Chapter 2.

Cell culture

293FT cells (Life Technologies) and Mia PaCa2 cells (ATTC) were kept in Dulbecco's Modified Eagle's Medium (DMEM) with 5% FBS incubated at $37^{O}C$

Western blot

Pancreatic cancer cell lines were harvested in ice cold RIPA buffer supplemented with PhosStop phosphatase inhibitor and cOmplete EDTA-free protease inhibitor (Roche, Indianapolis, IN). 25µg of protein was run on a 8-12% SDS-gel and blotted to PVDF membrane for antibody incubation.

Antibodies

ADAM 10 (Millipore), Hes-1 (sc25392, Santa Cruz), Cyclin D (Epitomics), Estrogen receptor (Millipore), CK19 (TROMA III), GAPDH (cell signaling), Ki-67 (Abcam), β-actin (Santa Cruz).

Lentiviral production and infection

ViraPower™ Lentiviral Packaging Mix (Life Tecnologies) and ADAM 10 shRNA (Mission-Sigma) were transfected into 293FT cells using Lipofectamine 2000. Media containing virus was harvested 3 days after transfection and used to infect Mia PaCa2 cells. A stable ADAM10 knockdown cell line was established by puromycin selection.

Growth curve assay

To examine growth, cells were seeded in 12 well plates, 20,000 cells per well. The number of cells were counted every 24 hours for 3 day.

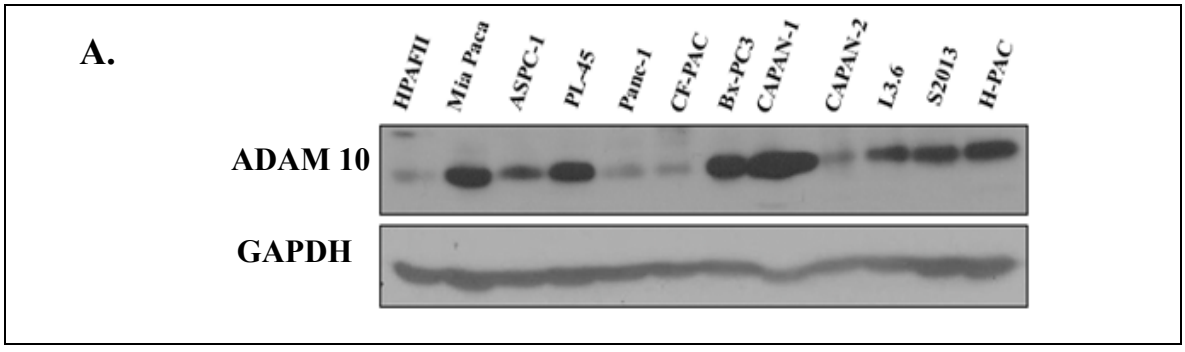
Colony formation (soft agar) assay

Mia PaCa2 cells were trypsinized and separated into a single cell suspension. 500 cells were embedded into a mixture of Sea Plaque Agarose (Lonza) and Dulbecco's Modified Eagle's medium (DMEM). Cells remained in Soft agar for 3 weeks before counting colonies.

3-3 Results

i. ADAM 10 is expressed in pancreatic cancer

To examine if ADAM 10 is expressed in pancreatic cancer, western blot analysis was performed on various pancreatic cancer cell lines **(Figure 3-1)**. ADAM 10 was highly expressed in 8 of the 12 cells lines tested. IHC showed that normal pancreatic tissue only expresses ADAM10 in the ducts while it is highly expressed in the tumors of PDAC tissue **(Figure 3-1)**. These data suggest that ADAM 10 may have a role in pancreatic cancer.



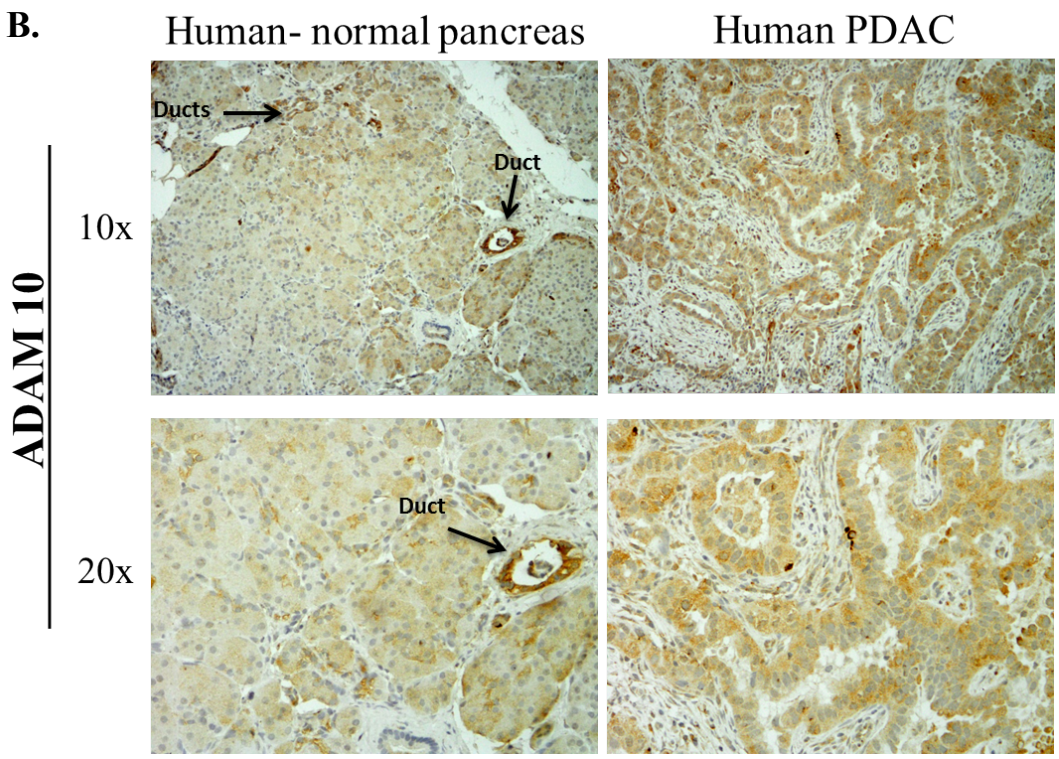


Figure 3-1. ADAM10 is expressed in pancreatic cancer

A) Western blot shows high expression of ADAM10 in several pancreatic cancer cell lines. B) IHC analysis reveals that ADAM 10 is normally expressed exclusively in the ducts. In contrast, PDAC specimens express high levels of ADAM 10 throughout tumor tissues.

ii. Silencing ADAM 10 decreases Notch signaling and proliferation of pancreatic cancer cells

To test if ADAM 10 acts as an oncogene or tumor suppresser in PDAC, ADAM 10 was knocked down in pancreatic cancer cell lines using shRNA. To generate ADAM 10 knockdown cells, lentiviral based ADAM 10 shRNA constructs (Mission-Sigma) were used to infect Mia-PaCa-2 cells. Stable cell lines were established by puromycin selection.

Western Blot analysis confirms ADAM10 knockdown, as wells as a down regulation of Hes-1, supporting the hypothesis that ADAM 10 is an essential Notch sheddase **(Figure 3-2)**.

To test if proliferation is affected by the lack of ADAM 10 in pancreatic cancer cells, a growth curve assay was performed on Mia-PaCa-2 cells infected with AD10shRNA. The 3-day growth curve shows that ADAM 10 knocked down cells proliferate slower than the non-target shRNA control **(Figure 3-2)**. The data demonstrates that ADAM 10 increases proliferation in cancer cell lines, which may contribute to the malignancy of the disease.

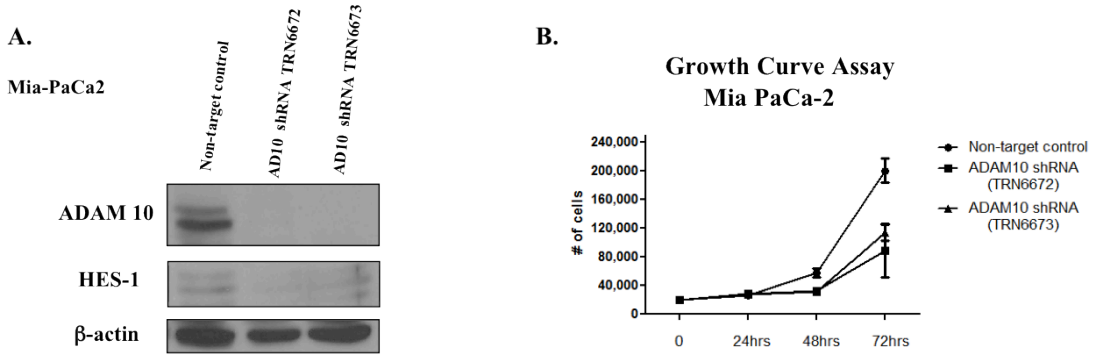


Figure 3-2. Loss of ADAM 10 in pancreatic cancer cell reduces Hes-1 expression and cell proliferation

A.) shRNA knockdown of ADAM 10 in the human pancreatic cancer cell line, Mia PaCa-2 yielded decreased HES-1 protein expression. B.) Growth curve for Mia PaCa-2 cells demonstrates that growth is inhibited with ADAM is knocked down.

iii. ADAM 10 promotes anchorage independent growth in pancreatic cancer cells

To test if ADAM 10 increases tumorigenicity, I set up a colony formation assay and then measured anchorage independent growth in ADAM 10 knockdown Mia-PaCa-2 cells

compared to non-target controls. The ability of single cells to form colonies in soft agar is a good indicator of a cell's ability to implant, proliferate and spread in xenograft mouse models. Orthotopic xenograft models of Mia-PaCa-2 cell lines have been established, and Mia-PaCa-2 cells that have been tail vein injected into mice have been shown to colonize multiple organs [121,122].

Furthermore, a colony formation assay assess if a cell line has a significant population of "tumor-initiating" cells, a cell type that retains stem cell characteristics that promote tumor progression [123]. Up-regulated Notch signaling has been shown to maintain cells in a stem-like state, which aids on tumor progression. I hypothesized that the loss of ADAM 10 would prevent colony formation by decreasing Notch signaling.

The results from this experiment showed that cells lacking ADAM 10 were unable to form as many colonies compared to control **(Figure 3-3).** Of the 500 cells seeded in each well, about 350 cells formed colonies in the non-target group, while ADAM 10 knockdown cells averaged about 50 colonies **(Figure 3-3).** These data suggests that cancer cells without ADAM 10 lack the ability to populate in an anchorage independent environment and may not form tumors in xenograft models.

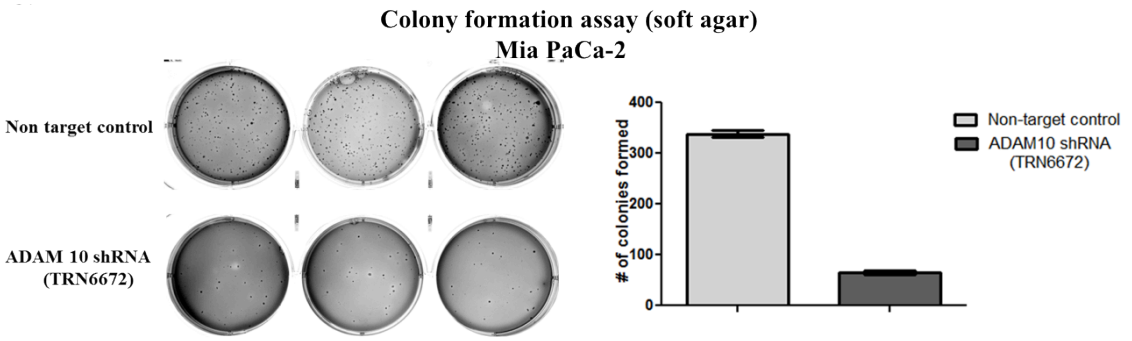


Figure 3-3. ADAM 10 promotes anchorage independent growth in pancreatic cancer cells

The pancreatic cancer cell line, Mia PaCa-2 was infected with lentiviral constructs of ADAM 10shRNA. A colony formation assay in soft agar tested if Mia PaCa-2 cells can still grow in an anchorage independent environment. The loss of ADAM 10 in Mia PaCa-2 cells prevented a significant number of colonies from forming.

iv. Generating a pancreas specific loss of ADAM 10 in the LSL-Kras$^{G12D/+}$;*Ptf1a*$^{cre/+}$ PDAC mouse model

Our data from Figure… has showed that ADAM 10 promotes cancer cell properties in pancreatic cancer cell lines. To test how the loss of ADAM 10 affects pancreatic tumor formation and progression *in vivo*, we generated a pancreas specific ADAM 10 knockout mouse model with an oncogenic Kras mutation (ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$;*Ptf1a*$^{cre/+}$) **(Figure 3-4)**. ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$;*Ptf1a*$^{cre/+}$ mice developed tumors at a rate similar to the LSL-Kras$^{G12D/+}$;*Ptf1a*$^{cre/+}$ control mice. IHC confirms a loss of ADAM 10 expression **(Figure 3-4).** Mice were aged and histology was analyzed in order to find a distinguishable difference between control mice and ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$;*Ptf1a*$^{cre/+}$ mice.

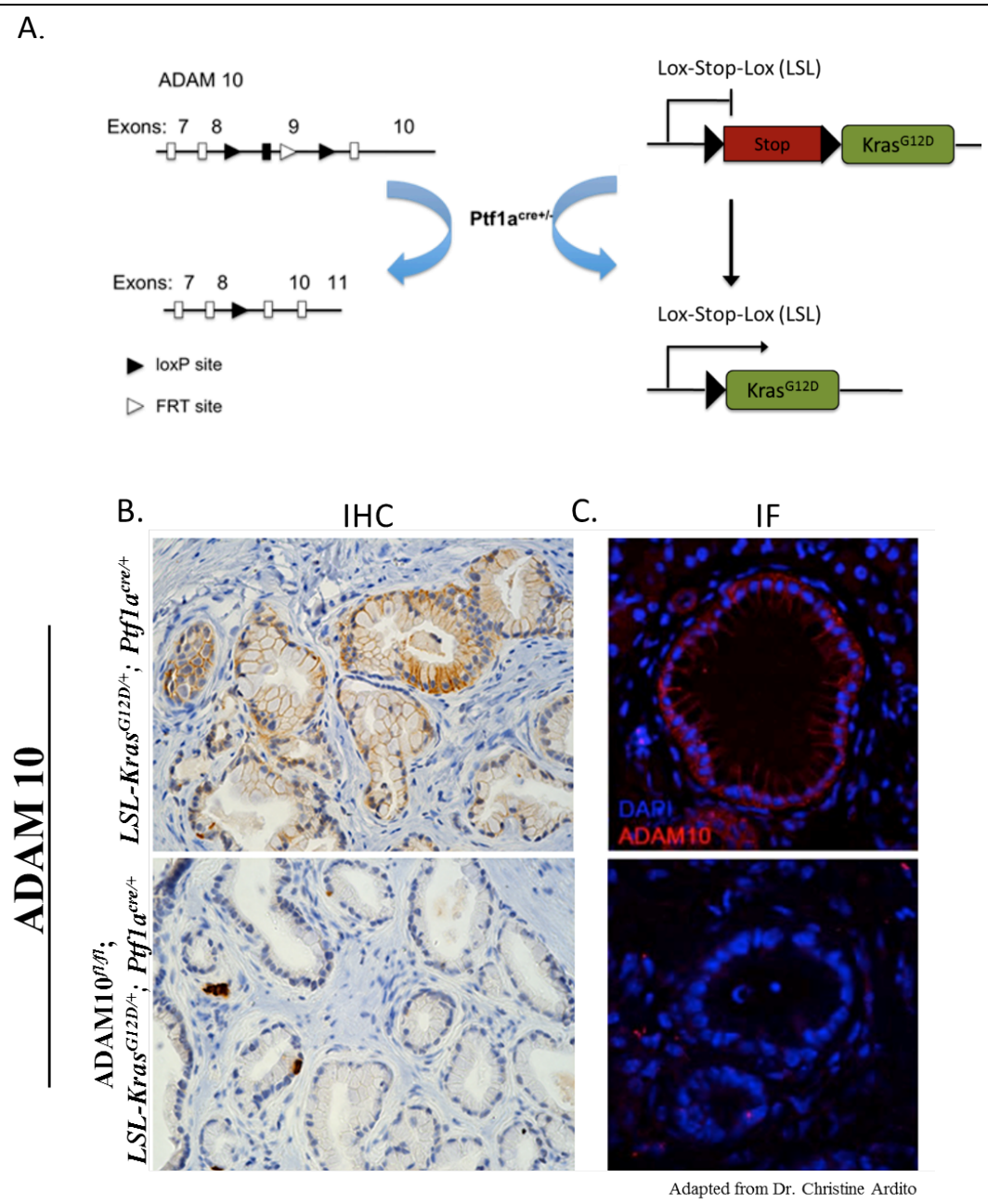


Figure 3-4. Pancreas-specific ablation of ADAM 10 in the LSL-Kras$^{G12D/+}$;*Ptf1a*$^{cre/+}$ mouse model

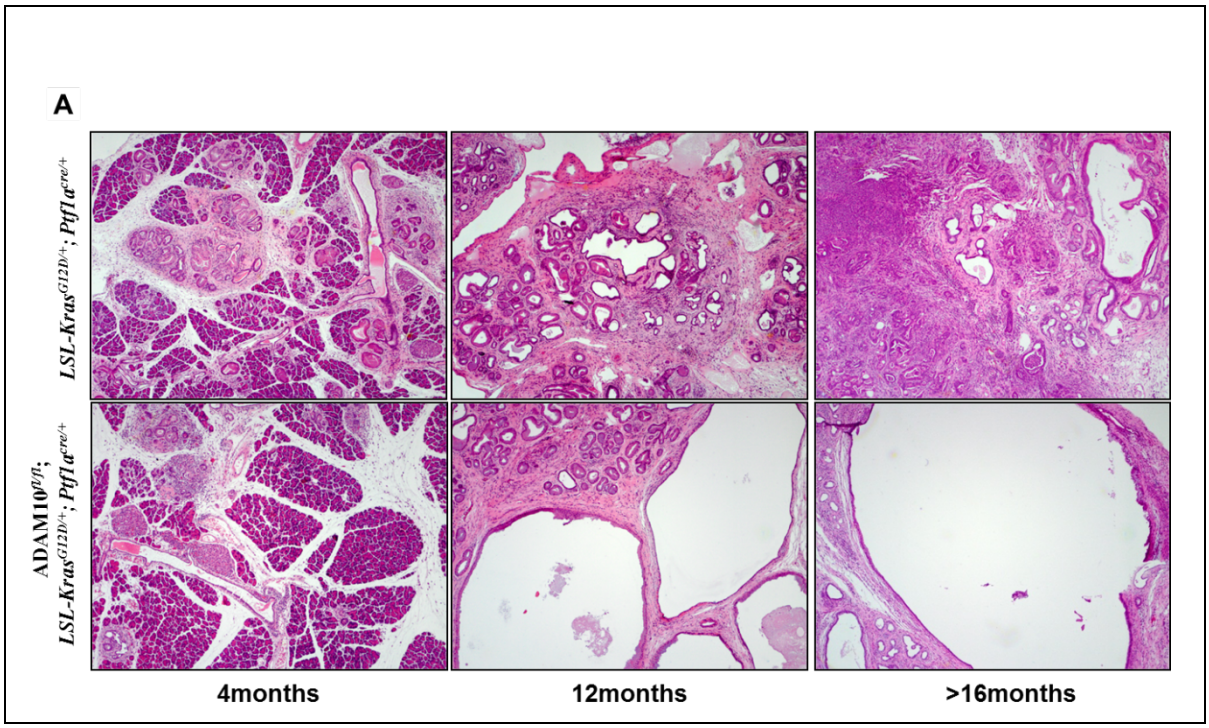
A) To ablate ADAM 10 zinc-binding domain encoding region (Exon 9) was flanked with loxP sites. If the sites are excised by cre-recombinase, the transcript will be out of

frame resulting in a nonsense transcript from that point on. LSL-Kras$^{G12D/+}$ mice and *Ptf1a*$^{Cre/+}$ mice were mated to our conditional ADAM10 knockout (ADAM10$^{fl/fl}$) mouse line. All mice were maintained in a C57Bl/6J background. When intercrossed, mice carrying both LSL-Kras$^{G12D/+}$ and *Ptf1a*$^{Cre/+}$ alleles (ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$) had simultaneous pancreas-wide expression of Kras^{G12D} and ablation of ADAM 10. B.) IHC and Immunofluorescence (IF) confirms the loss of ADAM 10 in the ADAM10$^{fl/fl}$; LSLKras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice compared to LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice.

v. Ablation of ADAM10 in LSL-Kras^{G12D}; *Ptf1a*$^{cre/+}$ mouse model leads to the formation of cystic lesions and improves survival

Despite observing no differences in early tumor formation, as the LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ and ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice aged it became clear that tumor progression was altered in the when ADAM was lost **(Figure 3-5)**. At 12 months of age, ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice had formed large cystic lesions rarely seen in LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice. The phenotype observed closely mimics Notch 2 knockout mice expressing mutant Kras [106]. We also examined mice that survived over 15 months, which was past the expected survival age for this mouse model[106]. ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice maintained large cystic lesions and PanIN 1 tumors while control mice had multiple stage PanIN lesions and progressed to invasive carcinoma **(Figure 3-5)**. In contrast to the advanced carcinoma found in Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice, ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice retain a high population of low-grade PanINs and healthy acinar

cells, despite having invasive tumors **(Figure 3-5)**. Cystic lesions in the ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice can be seen macroscopically and tend to form in the tail of the pancreas **(Figure 3-5)**. Survival is increased by approximately 40% in the ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice compared to LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice **(Figure 3-5)**. Consistent with the Notch-2 null phenotype[106], when ADAM 10 was ablated I observed and increased survival that supports ADAM 10 as both a Notch regulator and as a promising target for treating PDAC.



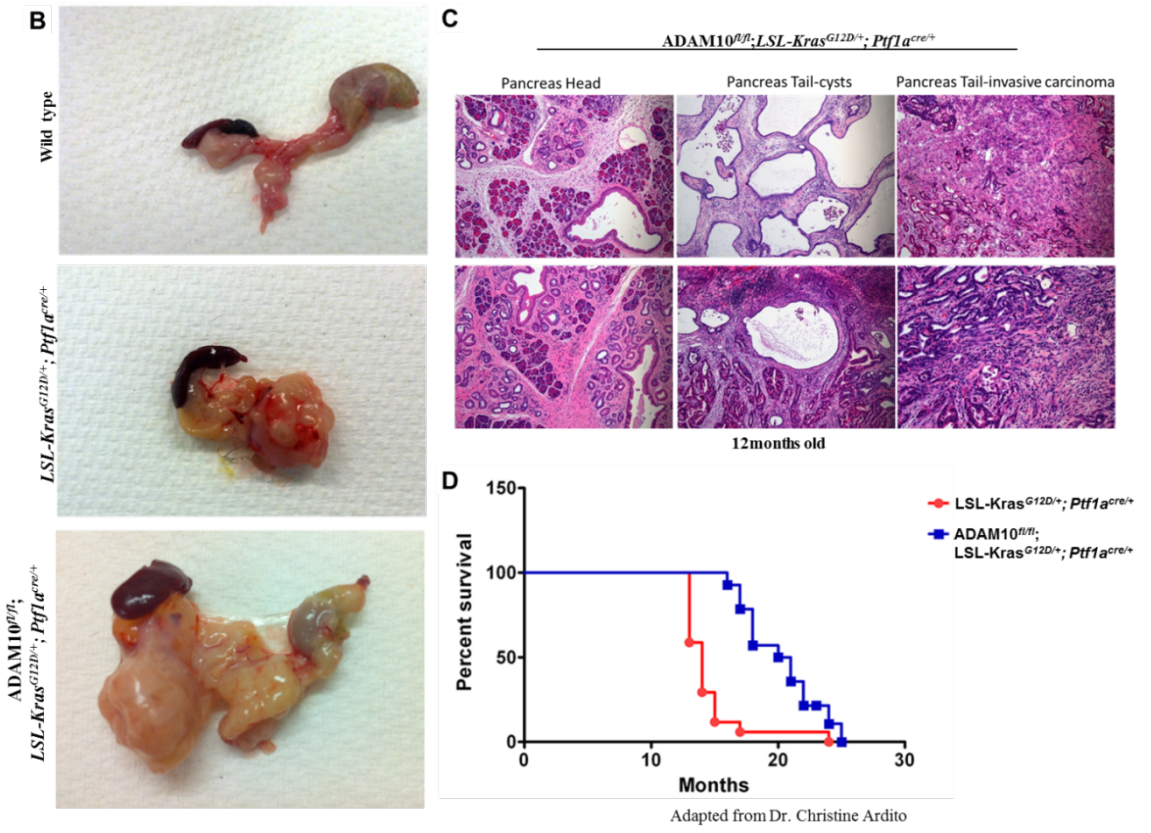


Figure 3-5. Ablation of ADAM10 leads to the developments of MCN-like lesions and improves survival

ADAM10 can only modestly deter tumor onset and early progression, in contrast tumor progression in older mice are noticeably affected by the loss of ADAM 10. A) At 12 months of age mice have large, cystic tumors that resemble mucinous cystic neoplasms (MCN).

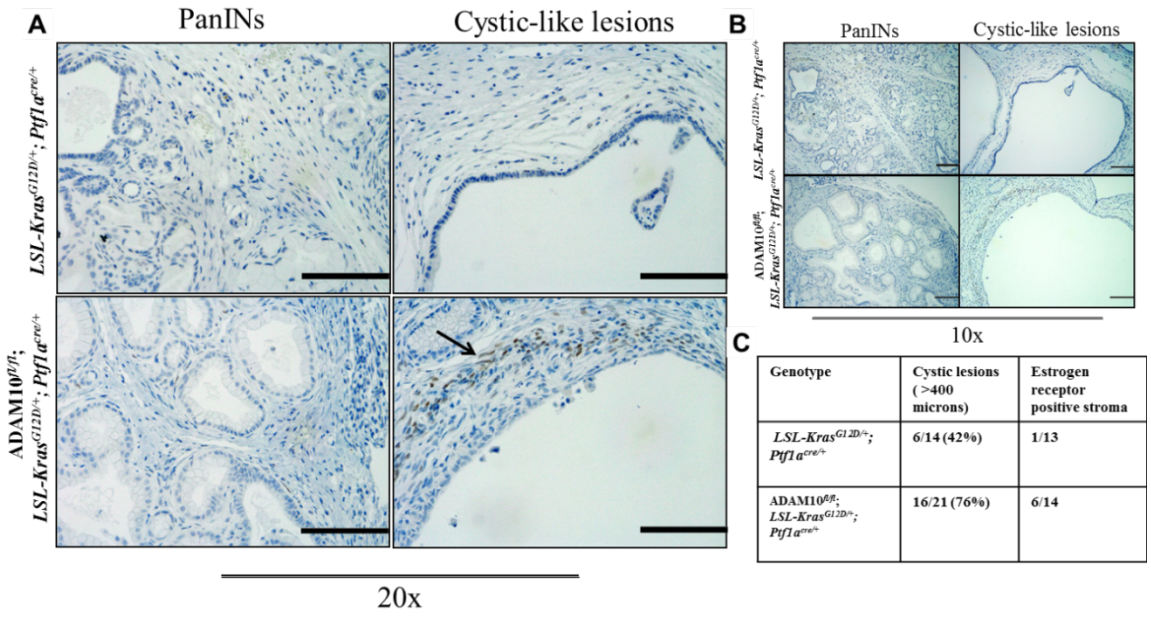
B) Gross morphology demonstrates the size of cysts compared to normal pancreas and tumors found in LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice. C) At greater than 1 year of age, pancreata of LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice are primarily composed of advanced PanINs and invasive cancer, while the ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$

pancreata frequently show areas of normal acinar cells low grade PanINs alongside very prominent cystic lesions and invasive cancer. D) Kaplan-Meier survival curve of LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ (red line) compared to ADAM10$^{fl/fl}$, LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice (blue line), demonstrated approximately 40% greater median lifespan in the mutant mice. Arrows=cysts; Asterisks*= tumors

vi. Cystic lesions in ADA10$^{fl/fl}$; *LSL-Kras*$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice are MCN-like as characterized by ovarian stoma

ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice have significantly more cystic lesions compared to controls **(Figure 3-6).** We performed histological analysis to characterize the cystic lesions in the ADAM10 deficient mice and compare them to lesions found in LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$model. From appearance, these large cysts resemble mucinous cystic neoplasia (MCN), one of a subset of enlarged cystic lesions found in the pancreas of human patients[124]. MCNs are large (> 3cm), mucinous cells that have a thick epithelial wall[124]. Despite their large appearance in relation to PanIN lesions, MCNs actually have a low prevalence for invasive carcinoma[125-127]. Pathologists identify MCN lesions by size, histological appearance and the presence of particular markers. Estrogen receptor is a marker found in the stroma of ovarian cysts and serves as a diagnostic of MCN in humans[128]. The Notch 2 null mouse has also been report to have large MCN-like lesions, confirmed by the presence of ovarian stroma surrounding the cysts. Using IHC, I tested the hypothesis that the cystic lesions found in the ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mouse model were representative of MCN lesion similar to those found in Notch 2 ablated LSL-Kras$^{G12D/+}$;

Ptf1a$^{cre/+}$mice. IHC shows ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice have estrogen receptor positive stroma **(Figure 3-6)**. LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice do not have ovarian stroma around their cysts, suggesting that they form distinct lesions. The phenotype observed in this study mimics the pancreas specific ablation of Notch 2, further supporting ADAM 10 as the upstream regulator of Notch 2 in the context of pancreatic cancer.



Genotype	Cystic lesions (>400 microns)	Estrogen receptor positive stroma
LSL-Kras$^{G12D/+}$; Ptf1a$^{cre/+}$	6/14 (42%)	1/13
ADAM10$^{fl/fl}$; *LSL-Kras$^{G12D/+}$; Ptf1a$^{cre/+}$*	16/21 (76%)	6/14

Figure 3-6. Ablation of ADAM 10 leads to MCN and other cystic neoplasms not often found in LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice

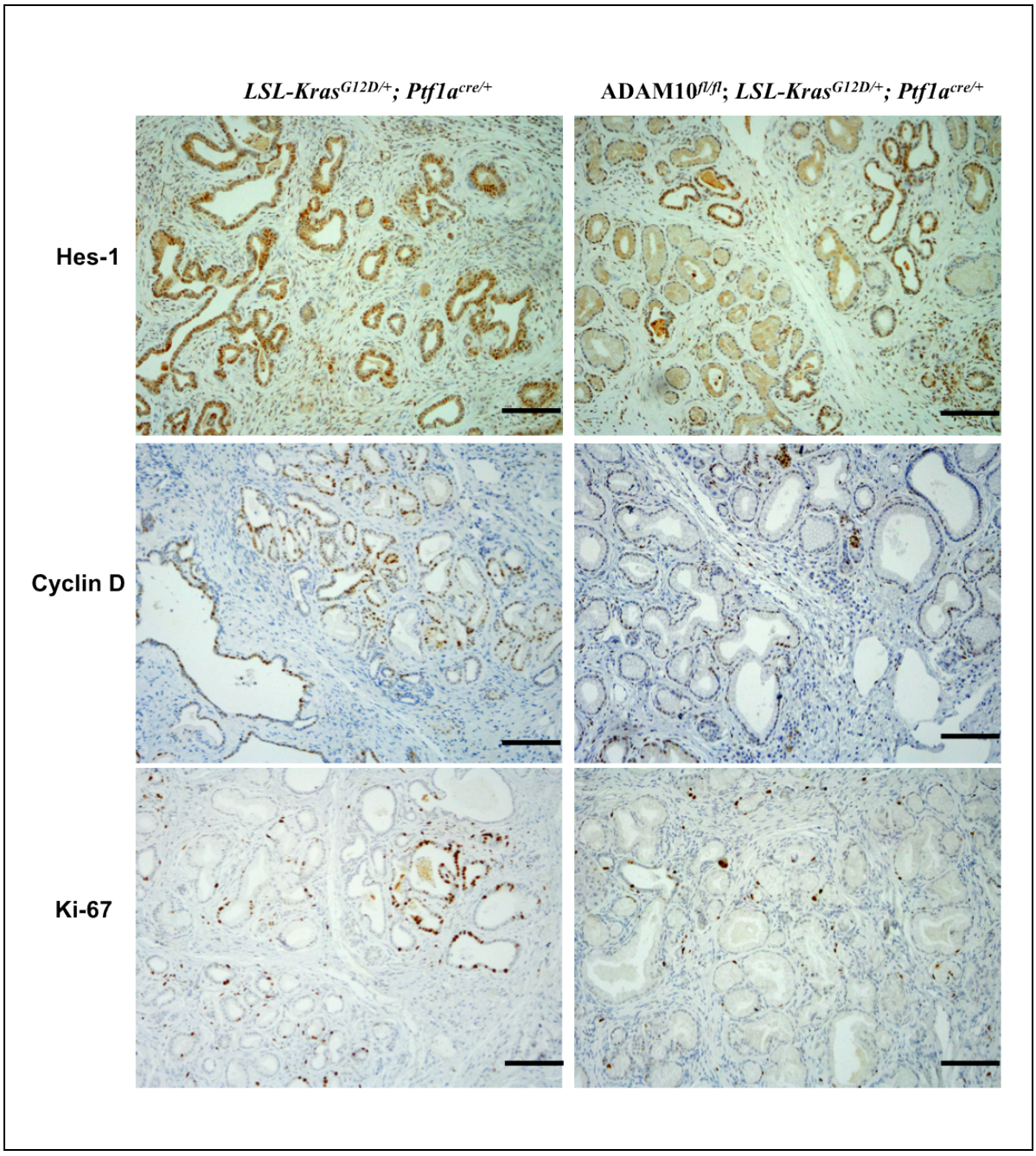
Qualitative assessment of ER positivity on comparable lesions between the LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ and ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice. 6/14 KC mice aged 1-2 years had cystic lesions, most where not macroscopic whereas 16/21 ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice of comparable ages had easily visible large cysts. Any lesion over 400mm diameter in width was considered a cyst-like lesion, and IHC for estrogen

receptor (ER) positive stroma was conducted on these tissues. Only 1/13 KC mice had cysts with ER positive stroma surrounding them compared to 6/14 ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$. Scale bars: 100mm. Arrows= ER positivity

vii. ADAM 10 promotes Notch and proliferative signals in the LSL-Kras$^{G12D/+}$;*Ptf1a*$^{cre/+}$ mouse model

To verify if the ADAM 10 dependent changes in tumor morphology is a result of aberrant Notch regulation, I performed IHC analysis on the expression of the Notch target gene, Hes-1. IHC shows that the PanINs and cystic lesions in ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice have decreased Hes-1 expression compared to LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice **(Figure 3-7).** These results parallel what was found in ADAM 10 knockdown Mia-PaCa 2 cells.

Although the amount of tumor burden seemed similar I observed more low grade tumors in ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice of an advanced age, and I hypothesized that the proliferative ability of tumors may positively regulated by ADAM 10. IHC shows that ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice aged for 1 year express Cyclin D1 and Ki-67 less frequently in PanIn lesions than the LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice **(Figure 3-7)**. The data demonstrates that ADAM 10 increases proliferation, which was similar to the results gathered *in vitro*. The down regulation of Notch and proliferative signals may be contributing to the longer lifespan of the ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice.


LSL-Kras$^{G12D/+}$; Ptf1a$^{cre/+}$
ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; Ptf1a$^{cre/+}$
Hes-1
Cyclin D
Ki-67

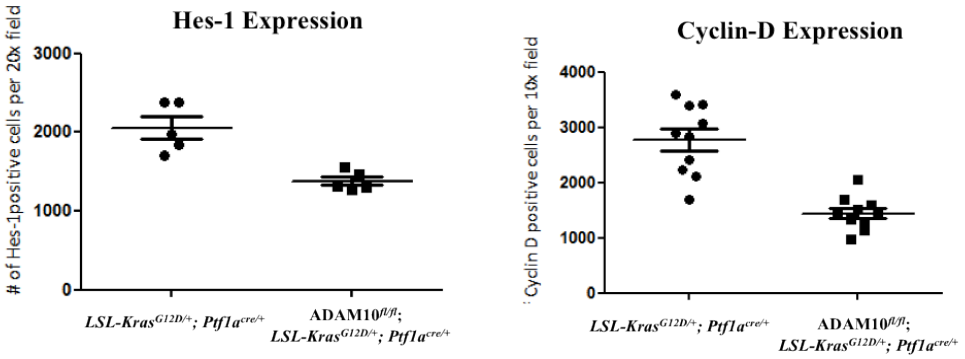


Figure 3-7. Ablation of ADAM 10 in LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice reduces the Hes-1 expression and proliferative signals

A.) The loss of ADAM 10 in our PDA mouse model has led to lower protein expression of the Notch target gene, Hes-1 throughout tumor lesions. IHC also shows that these mice have a decreased expression of Cell cycle marker, Cyclin D and proliferation marker, Ki-67. B.) Quantitation of Hes-1, Cyclin D, and Ki-67 positive nuclei confirm qualitative assessment. N=2 for each genotype.

viii. PDAC invasion is impeded by the loss of ADAM10

To investigate how the ablation of ADAM 10 is increasing the lifespan of ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice, we measured the amount of metastases in this model compared to Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice. The colony formation assay performed in Fig. 3-3 suggests that ADAM 10 aids in anchorage independent single cell growth and previous literature shows that Notch is necessary for cancer cells to have stem-like and tumor initiating ability, which is necessary for metastatic growth[129]. Given the data, I hypothesized that ablation of ADAM 10 down regulates Notch which in turn diminishes the ability of cancer cells to populate a secondary site. The results show that although both mouse models

have local invasive carcinoma, ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice were protected from forming metastatic lesions in the liver **(Figure 3-8)**. IHC of CK19, a pancreatic ductal marker highlights the metastatic formation in the liver **(Figure 3-8)**. It was unusual to observe local invasion without metastases, but this may demonstrate that ADAM 10-Notch signaling is essential in for secondary site colonization as opposed to tumor development.

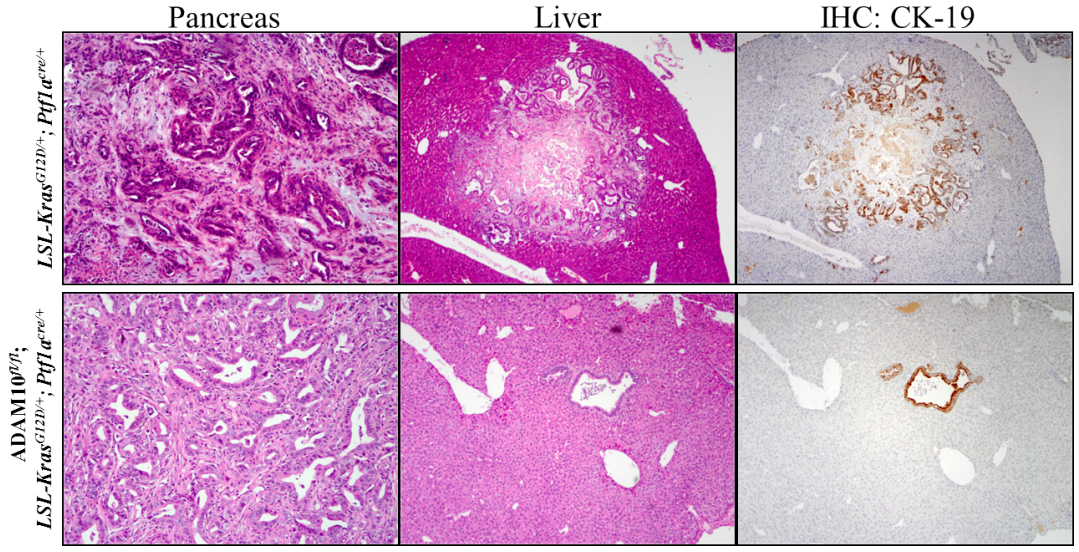


Figure 3-8. Despite local invasion ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice show limited metastases

Both LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ and ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice develop invasive carcinoma after reaching approximately one year of age. Liver metastases were found commonly in LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ and rarely in ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ (11/13 vs. 3/12). Metastases were also larger in the KC livers. Metastases were confirmed by expression of the pancreatic ductal marker, cytokeratin 19 (CK-19).

3-4 Discussion

This study has shown that ADAM 10 regulates pancreatic cancer. The genetic knock down of ADAM 10 in Mia-PaCa-2 cells demonstrates that Notch target gene, Hes-1 is down regulated and cell proliferation was inhibited. ADAM 10 knockdown Mia-PaCa-2 cells Also lost anchorage independence, which I hypothesize to be due to the loss of Notch signaling. The results from these *in vitro* experiments supported further investigations in an *in vivo* PDAC model.

The LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$mouse model has allowed us to investigate the development and progression of pancreatic tumors. By crossing this model with our conditional ADAM 10 knockout mouse strain, we gained insight on how the metalloproteinase may influence the fate of the disease. This data is consistent with previous literature that finds ADAM 10 as the primary sheddase for Notch[80,90,91,95]. Current literature is still in debate with how Notch regulation would affect tumor progression, the loss of ADAM 10 in the LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice appears to beneficial, and like the Notch 2 deficient mice, the mice have an improved life span, which is accompanied by large cystic tumors.

While the cystic phenotype may somehow be correlated with a longer lifespan, how this could happen is unclear. A study of 156 patients with MCN lesions was performed showing that 13.4% of patients had cysts accompanied by non-invasive PDAC and only 6% had invasive carcinoma along with their cystic lesion[125]. This suggests that the cellular processes that allow for MCN formation is not conducive to PDAC formation. The 5-year survival rate for patients with solely MCN lesions was 98%, while those with invasive carcinoma had a 50% 5-year survival rate[125]. This was a much better prognosis than the

average PDAC 5-year survival rate of 20%[130]. Further studies can be conducted to understand how the absence of Notch 2 and ADAM 10 allow for cyst formation.

Cell cycle progression and proliferation was reduced when ADAM 10 was ablated in LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice. Although it cannot be determined if blocking proliferation aided in extending the lives of the ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice, my study shows that inhibition of ADAM 10 has anti-proliferative effects in the context of pancreatic cancer.

The most likely reason the loss of ADAM 10 improves the life expectancy of PDAC mouse models is the lack of metastases. ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice are resistant to forming liver metastases. I hypothesize that ADAM 10 is required for cancer cells to colonize tumors in secondary sites. The results from the colony formation assay support this hypothesis. If pancreatic cancer cells infected with ADAM 10 shRNA are unable to form colonies in soft agar, then they have most likely lost their ability to colonize and grow *in vivo*. The reduced Hes expression in the ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$mice suggests that Notch signaling is decreased. Research has shown that Notch signaling regulates cell stemness and may promote tumor progression[123]. Previous studies have shown that down regulating Notch signaling using a gamma secretase inhibitor can prevent metastasis in both xenograft models and the *LSL-KRAS*^{G12D} *p53*^{R172H}*;Pdx-Cre* (KPC) PDAC model[131,132]. Although it seems that the effects of ADAM 10 are Notch related, evaluating how metastasis requires ADAM 10 need to be explored further.

Finally, although the data shows that ADAM 10 directs PDAC progression and metastasis, whether or not ADAM 10 is a suitable drug target is questionable. The data encourages therapeutic targeting of ADAM 10 will benefit PDAC patients but tests need to

be conducted to evaluate if cystic lesions immediately form in cancer patients when ADAM 10 is ablated, or if it cyst formation is something that occurs over a long stretch of time. The use of ADAM 10 inhibitors on PDAC mouse models should help answer this question. Taken together, my study adds to the relevance of Notch signaling in pancreatic cancer and demonstrates that the loss of ADAM 10 has multiple effects on PDAC progression.

3-4 Future Directions

To further understand how ADAM 10 exerts its effects on the ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mouse model, they are several *in vivo* and *in vitro* tests to be conducted. First it will be necessary to use a mouse model that will form metastases in a period of time shorter than a year. The addition of a mutant p53 allele in the Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mouse model causes invasive tumors at around 4 months. Unfortunately the mice expressing mutant p53 under the Ptf1a promoter form spinal tumors, causing paralysis and premature death. A mouse with a different pancreas specific promoter (Pdx) has been acquired to breed with the ADAM10$^{fl/fl}$. LSL-Kras$^{G12D/+}$; *p53*^{R172H} mice are a well-established fast progressing PDAC model. Metastasis can be assessed in this model, and tumors can actually be isolated and cultured.

Future studies can be conducted with the Mia-PaCa 2 ADAM 10 knockdown cell line. The cells can be collected and injected in xenograft models. By tail vein injecting cancer cells into the blood stream or directly adding cancer cells to an organ that is commonly metastasized, we can test if the growth of secondary site tumors requires ADAM 10.

Finally, cell biological studies can be conducted in either cell lines or murine tumors that can further explain which proteins are being activated by ADAM 10. Even though ADAM10$^{fl/fl}$; LSL-Kras$^{G12D/+}$; *Ptf1a*$^{cre/+}$ mice phenocopy the cystic lesions

of a Notch 2 knockout, ADAM 10 may be acting on all 4 notches. Isolating the Notch proteins regulated by ADAM 10 will be informative. Lastly, even though Notch signaling is down regulated by ADAM 10, AD10shRNA cells may have lost their ability too form anchorage independent colonies by Notch independent means. Two other ADAM 10 substrates E-cadherin and CD44 play a notable role in tumor invasion[133,134]. Creating knockdown of the 4 notch receptors as well as investigating other ADAM 10 substrates will determine if the phenotype observed in mouse models and cell lines are completely Notch dependent. A definitive experiment to test if the loss of ADAM 10 in cell lines is Notch dependent will be to reintroduce the Notch ICD in ADAM 10 knockdown cells. If introducing the downstream target of ADAM 10 restores anchorage independent growth, then we can conclude that my observations were due to Notch signaling being regulated by ADAM 10.

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