

THE IMPACT OF MTORC2 SIGNALING ON THE INITIATION AND
PROGRESSION OF KRAS-DRIVEN PANCREATIC NEOPLASIAS

A Dissertation Presented

By

DAVID REISS DRISCOLL

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DAVID REISS DRISCOLL

The signatures of the Dissertation Defense Committee signify completion and approval as to style and content of the Dissertation

Brian C. Lewis, Ph.D., Thesis Advisor

Junhao Mao Ph.D., Member of Committee

Craig Ceol, Ph.D., Member of Committee

Leslie Shaw, Ph.D., Member of Committee

Katerina Politi, Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Lucio Castilla, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school.

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program
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Dedication

I dedicate this work to my loving wife Marci Driscoll who has supported my research vocation for over a decade.

Marci, you have suffered each setback with me and delighted in with each success. You are my soul mate and the most amazing person I have ever known. This work would not have been possible without your love and support.

Thank You. I Love You.

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To Tyke Crowley, Charles Hillman, Marius Mößmer, Dorothy Driscoll, Joe Smith Sr. and Joanne Neely, I’m sorry this took me so long. I wish you could have been here.

Abstract

Pancreatic ductal adenocarcinoma (PDAC), the most common form of pancreatic cancer, develops through progression of premalignant pancreatic intraepithelial neoplasias (PanINs). In mouse-models, KRAS-activation in acinar cells induced an acinar-to-ductal metaplasia (ADM), and mutation of the *Kras* oncogene is believed to initiate PanIN formation. ADM is also promoted by pancreatic injury, which cooperates with activated KRAS to stimulate PanIN and PDAC formation from metaplastic ducts.

Our lab, and others, have shown that the downstream PI3K/AKT pathway is important for KRAS-mediated proliferation and survival *in vitro* and *in vivo*. Prior studies have demonstrated that full activation of AKT requires both PDK1-mediated phosphorylation of AKT^{T308} and mTOR complex 2 (mTORC2)-mediated phosphorylation of AKT^{S473}. Given the importance of the PI3K/AKT signaling axis, I hypothesized that mTORC2 is required for KRAS-driven pancreatic tumorigenesis and investigated this relationship in mice by combining pancreas-specific expression of an activated KRAS^{G12D} molecule with deletion of the essential mTORC2 subunit RICTOR.

In the context of activated KRAS, *Rictor-null* pancreata developed fewer PanIN lesions; these lesions lacked mTORC2 signaling and their proliferation and progression were impaired. Higher levels of nuclear cyclin dependent kinase inhibitors (CDKIs) were maintained in *Rictor-null* lesions, and nuclear BMI1, a known regulator of the CDKI *Cdkn2a*, inversely correlated with their expression.

Rictor was not required for KRAS-driven ADM following acute pancreatitis, however the inverse correlation between CDKIs and BMI1 was maintained in this system. Treatment of *PDX-Cre;KRAS^{G12D/+};Trp53^{R172H/+}* mice with an mTORC1/2 inhibitor delayed tumor formation, and prolonged the survival of mice with late stage PDAC. Knockdown of *Rictor* in established PDAC cell lines impaired proliferation and anchorage independent growth supporting a role for mTORC2 in fully transformed cells.

These data suggest that mTORC2 cooperates with activated KRAS in the initiation and progression of PanIN lesions and is required for the transformation and maintenance of PDAC. My work illustrates phenotypic differences between pancreatic loss of *Rictor* and *PDK1* in the context of KRAS, broadens our understanding of this signaling node and suggests that mTORC2 may potentially be a viable target for PDAC therapies.

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List of Abbreviations

4EBP1	eif4E binding protein 1
5FU	5 fluoro-uracil
ADAM17	ADAM metalloproteinase domain 17
ADM	Acinar-to-ductal metaplasia
AGC	Protein kinase A, G, and C families
AKT	Protein kinase B
AML	Acute myeloid leukemia
AMPK	5' adenosine monophosphate-activated protein kinase
AP-1	Activator protein 1
ARF	Alternative Reading Frame
ATG13	Autophagy related 13
BIRC2	Baculoviral IAP repeat-containing protein 2
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
BMP	Bone morphogenic protein
BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
Ca ²⁺	Calcium ions
CDK4	Cyclin dependent kinase 4
CDK6	Cyclin dependent kinase 6
CDKI	Cyclin dependent kinase inhibitor
CDKN2A	Cyclin dependent kinase inhibitor 2A
CDKN2B	Cyclin dependent kinase inhibitor 2B
CRE	Causes Recombination
CT	Computed tomography
DAG	Diacylglycerol
DAPI	4',6-Diamidino-2-phenylindole
DEPTOR	DEP domain-containing mTOR-interacting protein
DNA	Deoxyribonucleic acid
E2F	E2F transcription factor
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF4E	Eukaryotic translation initiation factor 4E
EMT	Epithelial–mesenchymal transition
EPCAM	Epithelial cell adhesion molecule
ER	Endoplasmic reticulum
ERB-B2	Erb-b2 receptor tyrosine kinase 2
ERK	Extracellular-signal-regulated kinase
<i>et al.</i>	<i>Et alii</i>

FDA	Food and Drug Administration
FFPE	Formalin fixed, paraffin embedded
FKBP12	FK506-binding protein
flp	flippase
FoxO	Forkhead transcription factor subclass O
FSF	FRT-stop cassette- FRT
GAP	GTPase activating proteins
gDNA	Genomic DNA
GDP	Guanosine diphosphate
GEF	Guanine exchange factor
GFP	Green Fluorescent Protein
GLI2	GLI family zinc finger 2
GLUT4	Glucose transporter 4
GNAS	Guanine nucleotide binding protein, alpha stimulating activity polypeptide 1
GRB10	Growth factor receptor bound 10
GRB2	Growth factor receptor-bound protein 2
GSK3B	Glycogen synthase kinase 3 beta
GTP	Guanosine triphosphate
H&E	Hematoxylin and eosin
HRAS	Harvey rat sarcoma viral oncogene homolog
ID3	Inhibitor Of DNA Binding 3
IF	Immunofluorescence
IGF1	Insulin growth factor 1
IGF1R	Insulin growth factor receptor
IHC	Immunohistochemistry
IL-6	Interleukin 6
INK4A	Inhibitor of CDK4-a
IP3	1,4,5 triphosphate
IPMN	Intraductal papillary mucinous neoplasm
KC	<i>PTF1a</i> ^{Cre/+} ; <i>LSL-KRAS</i> ^{G12D/+}
KRAS	Kirsten rat sarcoma viral oncogene homolog
LKB1	Serine-threonine-protein kinase 11/ liver kinase B1
LSL	Lox-stop cassette- Lox
Mb	Mega-base pair
MCN	Mucinous cystic neoplasm
MDM2	Mouse Double Minute 2
MEK	Mitogen-activated protein kinase kinase
mLST8	Mammalian lethal with SEC13 protein 8
MMP	Matrix Metalloproteinase family
MRI	Magnetic resonance imaging
mRNA	Messenger RNA

mSIN1	Mammalian stress-activated map kinase-interacting protein 1
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
MYC	C-Myc
MYCN	N-Myc
MYO1C	Myosin 1c
n	Number of samples
NDRG	N-myc downstream regulated gene
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NF1	Neurofibromin 1
NRAS	Neuroblastoma ras oncogene
NSCLC	Non-small cell lung cancer
p-	Phosphorylated
p16 ^{INK4A}	Cyclin-dependent kinase inhibitor 2A
p21 ^{Cip}	Cyclin-dependent kinase inhibitor 1
p27 ^{Kip1}	Cyclin-dependent kinase inhibitor 1B
PALB2	Partner and localizer of BRCA2
PanIN	Pancreatic intraepithelial neoplasia
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PDEC	Pancreatic ductal epithelial cells
PDK1	3-phosphoinositide-dependent protein kinase 1
PDX1	Pancreatic and duodenal homeobox 1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP ₃	Phosphatidylinositol3,4,5-triphosphate
PKC	Protein kinase C
PKCA	α subunit of class 1 PI3K complex
PLC	Phospholipase C
PRAS40	40-kDa pro-rich AKT-substrate
PROTOR	Protein observed with RICTOR
PRSS1	Cationic trypsinogen gene
PtdIns(3,4,5)P3	Phosphatidylinositol (3,4,5)-trisphosphate
PTEN	Phosphatase and tensing homolog
PTF1A	Pancreas transcription factor 1 subunit alpha
Q-PCR	Quantitative-polymerase chain reaction
RAC1	Ras-Related C3 Botulinum Toxin Substrate 1
RAF	Rapidly Accelerated Fibrosarcoma proto-oncogene
RAL	Ras-like
RALBP1	RAS-like binding protein 1

RALGDS	Ral Guanine Nucleotide Dissociation Stimulator
RAPTOR	Regulatory-associated protein of mTOR
RAS	Rat sarcoma viral oncogene family of proteins
RB	Retinoblastoma 1
RCAS	Replication-competent avian sarcoma-leukosis virus long-terminal repeat with splice acceptor
RCE1	Ras-converting enzyme 1
RHEB	Ras homologue enriched in brain
RHOGLI2	Rho-GDP dissociation inhibitor 2
RICTOR	Rapamycin-insensitive companion of mTOR
Rictor ^{+/+}	Homozygous wild-type Rictor alleles
Rictor ^{Δ/Δ}	Homozygous floxed Rictor alleles
RNA	Ribonucleic acid
RNF43	Ring finger 43
RTK	Receptor tyrosine kinase
S6K	S6 kinase
SREBP	Sterol regulatory element-binding protein
SGK	Serum and glucocorticoid-regulated kinase
SHH	Sonic Hedgehog
shRNA	Short hairpin RNA
SMAD4	Deleted in Pancreatic Carcinoma 4
SOS	Son of Sevenless
SPARC	Secreted protein acidic and rich in cysteine
SPINK1	Serine protease inhibitor gene
STAT3	Signal transducer and activator of transcription 3
TBS	Tris-buffered saline
TBX3	T-Box Protein 3
Tet-O	Tetracycline operator sequence
TGF-β	Transforming growth factor-β
TGF-β	Transforming growth factor
TGFBR1	Transforming growth factor-β receptor 1
TGFBR2	Transforming growth factor-β receptor 2
TIAM1	T lymphoma invasion and metastasis 1
TSC1	Tuberous sclerosis complex 1
TSC2	Tuberous sclerosis complex 2
ULK1	Unc-51 like autophagy activating kinase 1
WNT	Wingless-Type MMTV Integration Site Family
YAP	Yes-associated protein
ZAK	Zipper containing kinase AZK

Chapter I:
Introduction

A diagnosis of pancreatic cancer carries one of the worst prognoses in modern medicine. It is currently the fourth leading cause of cancer related death in the United States and although survival rates for various other malignancies have dramatically improved in recent decades, progress in the treatment of pancreatic cancer has been tepid at best (Siegel, Miller et al. 2015). Early symptoms of pancreatic cancer are rare and reliable screening methods for identification of early stage disease are not yet available. At the time of diagnosis most patients present with distant metastasis and have a dismal 2% 5-year survival rate (Siegel, Miller et al. 2015). Only 9% of patients are diagnosed with localized disease. These patients are eligible for potentially curative surgery, and while their 5-year survival increases to 25% (Siegel, Miller et al. 2015), it is clear from the frequent recurrences of this malignancy that even “localized” disease has often disseminated. Primary and metastatic pancreatic cancers are often resistant to current chemotherapeutics and even resected local disease frequently recurs despite supplementary chemotherapeutic treatment. A better understanding of this disease will allow for the development of more effective therapies that are desperately needed to improve patient outcomes and survival.

Overview of Pancreatic Endocrine and Exocrine Function

The pancreas is comprised of both endocrine and exocrine tissues. The endocrine component of the pancreas includes α , β , δ , ϵ and PP cells which produce glucagon, insulin, somatostatin, ghrelin and pancreatic-polypeptide

respectively (Jain and Lammert 2009). Pancreatic endocrine cells reside in the islets of Langerhans, spherical structures comprised predominantly of β -cells (28% to 75%), α -cells (10% to 65%), and δ -cells (1.2% to 22%) (Figure 1.1). In humans the organization of cells is heterogeneous, while mice display a more core-mantle structure with centralized β -cells and α , and δ cells localized to the periphery. (Brissova, Fowler et al. 2005, Jain and Lammert 2009).

Islets account for only 2% of the human pancreas; the majority of the organ is comprised of acinar tissue responsible for the secretion of digestive enzymes, including trypsinogen, chymotrypsinogen, α -amylase, and pancreatic lipase (Maclean and Ogilvie 1955, Leung and Ip 2006). The architecture of the pancreatic exocrine system has been described as a cluster of grapes on the vine (Figure 1.1). Groups of acinar cells form grape like acini around centroacinar cells at the terminal ends of intercalated ducts. Apically located zymogen granules in acinar cells secrete inactive digestive enzymes into the ductal lumina, which empty into progressively larger intralobular, small interlobular and large interlobular (main) ducts (Reichert and Rustgi 2011). The large interlobular duct junctions with the common bile duct shortly before emptying into the duodenum. In the small intestine enteropeptidase initiates the cleavage of trypsinogen resulting in active trypsin protease. This active enzyme then cleaves and activates other trypsinogen and chymotrypsinogen molecules (Rinderknecht 1986, Hofbauer, Saluja et al. 1998). Secretion of digestive enzymes by acinar cells is stimulated by cholecystokinin, a hormone produced by duodenal

enteroendocrine I-cells in response to the amino acid phenylalanine (Mangel, Prpic et al. 1995).

Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinomas (PDACs) are the most common and deadly pancreatic tumors. They comprise over 85% of pancreatic malignancies while cystic neoplasms, acinar carcinomas, and islet cell endocrine tumors are less frequent (Bardeesy and DePinho 2002). Symptoms of PDAC are non specific including lower back pain, weight loss, fatigue, and, in instances where the tumor compresses the bile duct, jaundice (Livstone E.P. 2014). The central abdominal position of the pancreas, dorsal to the liver and ventral to the kidneys, likely contributes to this lack of specific symptoms; diagnosis is difficult and usually requires expensive and invasive techniques such as contrast-enhanced computed tomography (CT) scan and endoscopic ultrasound with needle biopsy. At the time of diagnosis over half of patients with PDAC have distant metastases and only 9% have localized disease; the majority of pancreatic tumors detected at an early stage are identified during imaging for unrelated pathologies (Siegel, Miller et al. 2015). Patients with regional or distant metastases have 10% and 2% 5-year survival rates respectively. Individuals diagnosed with localized disease are eligible for potentially curative surgery and their 5-year survival increases to 25%(Siegel, Miller et al. 2015). Although the lifetime risk for developing PDAC is approximately 1.5% (SEER, 2015), only 1 in 8000

individuals will be diagnosed in a given year (Siegel, Miller et al. 2015). The low incidence in the general population, coupled with the expensive and invasive nature of tests used to confirm the diagnosis, demands that any widely used screening technology be highly sensitive with a very low false positive rate. For individuals known to be at high risk for developing PDAC, the carbohydrate antigen 19-9 is an FDA approved blood biomarker for the disease. However, the specificity (87%), and sensitivity (70%) of these tests are too low to be of value in screening the general population (Becker, Hernandez et al. 2014). The advent of newer methods in which circulating tumor cells or circulating tumor exosomes are isolated from blood samples via EPCAM or Glypican-1 epitopes may achieve the high specificity and sensitivity needed for screening of lower risk populations, but these technologies require further development (Yu, Ting et al. 2012, Melo, Luecke et al. 2015).

Environmental Risk Factors for PDAC

Epidemiological studies have identified various risk factors that increase the risk of developing PDAC. Chronic pancreatitis and tobacco use are the most common of these environmental risks, with 13-27 and 3.2-4.8 fold increases respectively (Talamini, Bassi et al. 1999, Malka, Hammel et al. 2002). The increased risk of PDAC from obesity and diabetes are smaller but still significant (Lowenfels and Maisonneuve 2006). Excessive alcohol consumption can cause

pancreatitis and increases the risk of PDAC in that context (Talamini, Bassi et al. 1999).

Pancreatitis is an inflammatory response in the pancreas that can be either acute or chronic. The inflammation is associated with upper abdominal pain, which is more severe in acute cases. Acute pancreatitis is, by definition, temporary; the inflammatory response resolves and the pancreatic composition returns to normal. Acute acinar cell injury initiates a pro-inflammatory response, mediated primarily by lymphocytes and macrophages; this response also activates pancreatic stellate cells. Following an initial phase of inflammation, anti-inflammatory cytokines such as interleukin-10 and transforming growth factor β are produced, promoting stellate cell production of extracellular matrix proteins. Barring further injury or genetic predisposition, acinar tissue will recover to a normal state; however, repeated pancreatic injury and recurrent acute pancreatitis can maintain the fibrotic response resulting in a chronic pancreatitis phenotype (Whitcomb 2003). Chronic pancreatitis results in fibrosis, which permanently alters the makeup of the organ and may result in both exocrine and endocrine pancreatic insufficiency (Freedman, S.D. 2012). While patients with acute pancreatitis and chronic pancreatitis both had elevated levels of the cytokine interleukin-6 (IL-6), interleukin-12 levels were elevated only in the blood of chronic pancreatitis patients. Furthermore, while T-cell populations in acute pancreatitis patients remained mixed, T-helper 1 cells were the predominant subtype in patients with chronic pancreatitis (Bhatnagar, Wig et al. 2003).

Any event preventing the efficient drainage of pancreatic ducts, such as inflammation-induced pressure on these ducts, presents a risk for premature activation of digestive enzymes. Activation of these enzymes within the pancreas evokes a positive feedback loop, cleaving and activating additional digestive enzymes, which induce necrotic death of acinar cells and subsequent inflammatory responses (Willemer, Kloppel et al. 1989). Models of pancreatitis induced by the cholecystokinin analogue caerulein, showed independent activation of both trypsinogen and the inflammatory NF- κ B pathway; both contributed to necrotic cell death and induction of acute pancreatitis (Han, Ji et al. 2001, Dawra, Sah et al. 2011). While environmental factors such as chronic pancreatitis or smoking increase the risk of developing PDAC, many cases develop in the absence of these factors. Although pancreatitis is known to increase the risk for developing pancreatic cancer, and accelerates the formation of pancreatic cancers in mouse models, the effect of pancreatitis on pancreatic cancer outcomes in humans has not been studied. It is possible that increased surveillance of this higher risk population could be used to detect disease at an earlier stage and thus improve survival.

Hereditary Risk Factors for PDAC

Roughly 10% of patients who develop PDAC have a genetic predisposition to the disease (Sheridan and Downward 2013). According to the

National Familial Pancreas Tumor Registry at Johns Hopkins University

individuals with family histories that include two or more first degree relatives diagnosed with pancreatic cancer, have a 6.8-fold increased risk of developing the disease themselves (nfptr.org, 2015). Germ-line mutations and genetic syndromes have been identified in roughly 20% of these families (Whitcomb, Shelton et al. 2015). Individuals with Peutz-Jeghers syndrome develop multiple hamartomas in their gastrointestinal tracts and present with hyper-pigmentation of the lips and hands (Wolfgang, Herman et al. 2013). This autosomal dominant disorder is most often caused by mutations in the serine-threonine-protein kinase 11/ liver kinase B1 (*LKB1*) gene, a master regulator of the AMPK serine threonine kinase family, and these individuals have a 132-fold increased risk of developing PDAC (Hezel and Bardeesy 2008, Wolfgang, Herman et al. 2013). Individuals with familial atypical multiple mole melanoma syndrome caused by germ line mutations in the *CDKN2A* tumor suppressor gene have a 38-fold increased risk of developing PDAC in addition to a dramatically elevated risk of melanoma (Wolfgang, Herman et al. 2013). Individuals with hereditary pancreatitis caused by mutations in either the cationic trypsinogen gene (*PRSS1*) or the serine protease inhibitor gene (*SPINK1*) have a 58-fold increased risk of PDAC (Wolfgang, Herman et al. 2013). Additionally, germ-line mutations in the *BRCA2*, *PALB2*, and *BRCA1* genes also increase the risk of PDAC, although mutations in these DNA damage repair genes are less penetrant than the previously discussed syndromes. (Downward 2009, Castellano and Downward

2010, Sheridan and Downward 2013). As is often the case, analysis of hereditary mutations that predispose individuals to cancer has helped inform the field with regards to sporadic malignancies. This is highlighted by the frequent deletions and mutations of the *CDKN2A* locus, and less frequent but clinically relevant alterations of *BRCA2* (7%) and *LKB1*(5%) in sporadic PDAC (Morran, Wu et al. 2014, Waddell, Pajic et al. 2015).

Current Treatments for PDAC

Surgical resection is currently the only curative option for PDAC, though the surgery, usually a Whipple procedure, carries great risk and is available only to the minority of patients diagnosed with localized disease. All patients, whether surgical candidates or not, are offered cytotoxic chemotherapy. The pre-operative, neo-adjuvant, and post-operative, adjuvant, use of chemotherapeutics significantly improves outcomes for patients undergoing surgical resection of PDAC, although the disease will recur in the majority of these patients (Hattangadi, Hong et al. 2009). In the absence of surgery, chemotherapy provides a palliative benefit and a small but significant increase in survival. Prior to 1997 the standard of care was 5 fluoro-uracil (5FU). The 2' difluorinated deoxycytidine analog gemcitabine provided a minimal 1.2-month increase in survival relative to 5FU; however, the drug was well tolerated and 24% of patients reported a clinical benefit, mainly reduced pain, whereas only 5% of patients on 5FU reported a benefit (Burris, Moore et al. 1997). Gemcitabine

became the standard of care for treatment of PDAC in the United States and is still widely used in combination therapies. Recently, FOLFIRINOX, a four drug cocktail consisting of oxaliplatin, irinotecan, leucovorin, and fluorouracil showed a median survival of 11.1 months compared to 6.8 months for patients receiving gemcitabine (Conroy, Desseigne et al. 2011). This drug combination, while the most effective to date, is highly toxic and is not well tolerated by many patients (Thota, Pauff et al. 2014). Combined therapy with gemcitabine and nanoparticle albumin-bound (nab)-paclitaxel, a less toxic therapy, was approved for first line treatment of PDAC in 2013 (Von Hoff, Ervin et al. 2013). This combination therapy provided a median survival roughly 2-months better than gemcitabine and due to favorable patient tolerance is becoming the preferred treatment (Von Hoff, Ervin et al. 2013). While these advances in treatment are significant, they are far from a cure and represent a relatively modest improvement in PDAC treatment relative to the progress that has been made in treating other cancers over the last two decades. The recurrence of resected disease and emergence of metastatic lesions in spite of these therapies suggests that these metastatic cells possess an intrinsic resistance to cytotoxic therapies. Identifying and targeting signaling pathways essential to the survival of PDAC may provide more effective therapeutic options.

Diagnosis and Staging of PDAC

Pancreatic cancer is most often visualized and diagnosed by contrast-enhanced CT scan, magnetic resonance imaging (MRI), or endoscopic ultrasound. While CT and MRI allow the tumor to be visualized and staged in the context of surrounding tissue, endoscopic ultrasound provides the opportunity to biopsy the mass, providing tissue for a definitive pathological diagnosis (Hidalgo 2010, Wolfgang, Herman et al. 2013). PDAC are staged from I-to-IV according to the tumor–node–metastasis classification set forth by the American Joint Committee on Cancer, which attempts to classify these tumors based on resectability (Edge, Byrd et al. 2010). Stage-Ia and stage-Ib are restricted to the pancreas with no involvement of the lymph nodes or metastatic disease. Stage-IIa tumors have extended beyond the pancreas, but neither the celiac axis nor the superior mesenteric artery are involved. Stage-IIb includes any tumor without involvement of the celiac axis or the superior mesenteric artery, but with nodal involvement. Stage-I and stage-II tumors are defined as candidates for surgical resection (Wolfgang, Herman et al. 2013). Stage-III tumors have involvement of either the celiac axis or the superior mesenteric artery and while some are classified as borderline resectable, others are locally advanced and are no longer surgical candidates. Any tumor which has metastasized from the pancreas is defined as stage-IV, for which surgery is contraindicated (Wolfgang, Herman et al. 2013)(AJCC, 2009).

The Role of Stromal Tissue in PDAC

Stromal tissue often makes up the majority of a PDAC tumor's mass, and is comprised of proliferating fibroblast, pancreatic stellate cells, endothelial, and immune cells (Feig, Gopinathan et al. 2012). Pancreatic stellate cells proliferate and deposit a dense extracellular matrix (ECM) of fibronectin, and type I and III collagen in response to signals from PDAC (Bachem, Schunemann et al. 2005). Growth factors and cytokines secreted by immune cells, endothelial cells, and tumor cells are held in close proximity to the tumor by the ECM, increasing proliferation of both tumor cells and pancreatic stellate cells (Mews, Phillips et al. 2002, Korc 2007, Feig, Gopinathan et al. 2012). Secretions of pancreatic stellate cells have been shown to increase proliferation and migration as well as pro-oncogenic AKT and ERK signaling in PDAC cell lines (Hwang, Moore et al. 2008, Vonlaufen, Joshi et al. 2008). These findings were supported *in vivo* by an orthotopic model in which co-injection of PDAC and pancreatic stellate increased tumor progression and metastasis (Hwang, Moore et al. 2008).

The role of the overall tumor micro-environment is quite nuanced. In addition to proliferative signals, pancreatic stellate cells produce collagen and express α -smooth muscle actin (Apte, Park et al. 2004). While high α -smooth muscle actin levels correlate with a poor patient prognosis, tumors with high collagen levels carry a better prognosis (Erkan, Michalski et al. 2008). The importance of stromal tissue in treating PDAC was firmly established in 2009 when Olive et al. identified a shielding effect of tumor associated pancreatic

stromal tissue in a mouse-model of PDAC, and showed that small molecule delivery to tumor cells could be dramatically improved through depletion of this tissue using a Sonic Hedgehog (SHH) inhibitor (Olive, Jacobetz et al. 2009). However, subsequent models using genetic or small molecule disruption of the SHH pathway to decrease the stromal component of PDAC resulted in accelerated growth of precursor lesions and resulted in more aggressive undifferentiated tumors with increased vasculature and metastatic potential (Lee, Perera et al. 2014, Rhim, Oberstein et al. 2014). A popular notion explaining these seemingly contradictory roles of the stromal response is that the fibrotic deposition of ECM provides a nourishing and protective environment for neoplastic cells while at the same time inhibiting angiogenesis and the metastatic potential of these tumors. While inhibition of the SHH pathway in mouse-models accelerated tumor growth, the success of combined nab-paclitaxel-gemcitabine therapy in humans is attributed to secreted protein acidic and rich in cysteine (SPARC) sequestration of paclitaxel in stromal fibroblasts; this results in gradual disruption of the ECM and improved delivery of gemcitabine to the tumor cells (Rhim, Oberstein et al. 2014, Thota, Pauff et al. 2014). This suggests that cautious targeting of the stromal response in PDAC through additional mechanisms may provide therapeutic benefits.

Precursor Lesions Giving Rise to PDAC

PDAC is believed to arise from one of three non-metastatic neoplastic lesions, pancreatic intraepithelial neoplasias (PanINs), mucinous cystic neoplasms (MCNs), and intraductal papillary mucinous neoplasms (IPMNs) (Gomez, Lee et al. 2001, Hezel, Kimmelman et al. 2006, Morris, Wang et al. 2010). The best studied and most common precursor lesions, PanINs, arise in the smaller caliber ducts (Hruban, Adsay et al. 2001, Morris, Wang et al. 2010). These lesions are graded histologically from PanIN-1a to PanIN-3 with increasing levels of dysplasia. While normal pancreatic ducts are characterized by low cuboidal cells, PanIN-1a lesions are hyperplastic and contain epithelial cells with a high columnar architecture, abundant supranuclear mucin, and basally located nuclei lacking atypia (Hruban, Adsay et al. 2001). PanIN-1b lesions have similar cellular attributes, and are differentiated by the formation of hyperplastic papillary structures. Lesions that have developed low to moderate levels of nuclear atypia are classified as PanIN-2; lesions of this grade tend to have papillary architecture, although this is not required. PanIN-3 lesions are classified as carcinoma *in situ* with advanced nuclear atypia and luminal budding of papillary structures (Hruban, Adsay et al. 2001). Once these neoplastic cells cross the basement membrane they become invasive carcinoma (Hanahan and Weinberg 2011). Stromal reactivity increases with the progression of PanIN lesions. This is likely a response to the increased dysplasia of the epithelial PanIN cells, but this stromal response may also promote PanIN progression and recent work

shows IL-6, secreted by stromal macrophages, to be involved in this process (Lesina, Kurkowski et al. 2011).

Surveys of human pancreata show an increased incidence of PanIN lesions with age and suggest that sporadic PanIN1a lesions are common in adults over age fifty. These studies also demonstrated an increased prevalence of PanIN lesions associated with chronic pancreatitis (60%) and in non-tumor pancreas tissue from PDAC patients (82%) relative to normal pancreas (16%) (Strobel, Dor et al. 2007). These findings reveal that early PanINs are not rare in the general population and suggest that only a small minority of these lesions will progress.

While MCNs and IPMNs are bona fide precursor lesions (Li, Hu et al. 2014), they take longer than PanINs to become dysplastic. Due to their larger size they can be identified by imaging, monitored, and removed prior to malignancy (Wolfgang, Herman et al. 2013). IPMN lesions are characterized by papillary growth in the main or branch ducts. Notably, lesions in the branch ducts are more likely to become neoplastic and require intervention (Tanaka, Chari et al. 2006). Alternatively MCNs tend to occur in small ducts at the tail of the pancreas; they are characterized by the involvement of ovarian-like stroma and are most common in women of child-bearing age (Tanaka, Chari et al. 2006). While pancreatic cysts are common, only 3.4% of the population harbors cystic neoplastic precursor lesions (Kimura, Nagai et al. 1995)

Genetics of PDAC

The KRAS Oncogene

Among all the genetic changes seen in PDAC, mutations in the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene are the most frequent with activating mutations being present in greater than 95% of tumors (Kanda, Matthaei et al. 2012, Waddell, Pajic et al. 2015). Activating mutations in *KRAS* are also the earliest and most frequent mutation seen in PanIN lesions. They are found in over 90% of micro-dissected human PanIN-1a lesions and in greater than 95% of PanIN3 lesions (Kanda, Matthaei et al. 2012, Waddell, Pajic et al. 2015). MCNs and IPMNs harbor fewer *KRAS* mutations than PanIN lesions, 33% and 81% respectively (Wu, Matthaei et al. 2011). Given the prevalence of activating *KRAS* mutations in PDAC, studies involving this gene play a central role in pancreatic cancer research.

KRAS is a member of the RAS family of small GTPase proteins, which also include *NRAS* and *HRAS*. All three family members are highly homologous in their first 168 residues, which contain the switch domains, GTP binding domains, and effector binding domains (Prior, Lewis et al. 2012). They also contain a short C-terminal variable domain by which these proteins are localized to various membranes within the cell (Wright and Philips 2006). These proteins are signaling molecules and their constitutive activation by point mutations is frequently observed in human cancers (Prior, Lewis et al. 2012). RAS proteins

with these activating mutations were shown in 1982 to have the potential to transform mammalian cells (Der, Krontiris et al. 1982, Parada, Tabin et al. 1982).

Under normal circumstances KRAS is maintained in a GDP bound state and signal transduction is absent. A guanine exchange factor (GEF) facilitates the removal of GDP from an inactive RAS protein, and the subsequent binding of a GTP molecule in its place. RAS binding of GTP induces a conformational change in the Switch1 (amino acids 30–38) and Switch2 (amino acids 59–67) domains of RAS (Jancik, Drabek et al. 2010). In its GTP bound form RAS is a signal transducer and activates downstream signaling pathways including but not limited to the RAF/MEK/ERK, PI3K/PDK1/AKT, RALGDS/RAL, RAC, and PLC ϵ pathways (Gupta, Ramjaun et al. 2007, Young, Lyons et al. 2009). The intrinsic GTPase activity of RAS proteins is enhanced by GTPase activating proteins (GAPs) which catalyze GTP hydrolysis by up to 10^5 fold, thus inhibiting RAS mediated signaling (Gideon, John et al. 1992). The neurofibromin 1 (NF1) tumor suppressor protein acts as a GAP and its loss has been shown to increase KRAS signaling (Cichowski and Jacks 2001).

Point mutations that prevent the hydrolysis of GTP by RAS, thus constitutively activating RAS signal transduction, are present in 17- 30% of all human tumors (Prior, Lewis et al. 2012). While RAS family members are largely homologous, they are not equivalent. Despite expression of all three RAS isoforms in most tissues, preferential mutation of individual isoforms is observed in a tumor specific manner. *NRAS* mutations are prevalent in skin (16-29%)

malignancies and acute myeloid leukemia (AML)(10-15%) while *KRAS* mutations are less common in AML (5%) but frequent in lung (17%), intestinal (20-34%), biliary tract (31%) and pancreatic (80-95%) malignancies. *HRAS* is most commonly mutated in salivary gland and urinary tract tumors (Ball, Yohn et al. 1994, Renneville, Roumier et al. 2008, Forbes, Bindal et al. 2011, Prior, Lewis et al. 2012, Waddell, Pajic et al. 2015). Ninety-nine percent of RAS activating mutations affect 3 codons, Gly-12, Gly-13, and Gln-61 (Prior, Lewis et al. 2012). Gln-61 is required to stabilize a water molecule used in GTP hydrolysis while Gly-12 and Gly-13 orient the Gln-61 residue and form Vander Waals interactions between the RAS-GTP loop and a GAP (Gideon, John et al. 1992, Scheffzek, Ahmadian et al. 1997, Jancik, Drabek et al. 2010, Pylayeva-Gupta, Grabocka et al. 2011). All three mutations impair the ability of RAS to hydrolyze GTP, locking RAS into its active conformation and activating its effectors (Malumbres and Barbacid 2003). For reasons yet unknown, the vast majority of *KRAS* mutations in PDAC affect the Gly-12 residue (Prior, Lewis et al. 2012).

Membrane localization of RAS plays an important role in signal transduction (Song, Hu et al. 2001). This localization is mediated by posttranslational modifications to their variable c-terminal regions (Hancock, Paterson et al. 1990, Jackson, Cochrane et al. 1990). All RAS family members contain a c-terminal CAAX motif (C is cysteine, A is any aliphatic residue, and X is any residue). Prenylation (farnesylation or geranylgeranylation) of the cysteine residue by farnesyltransferase or geranylgeranyltransferase provides a

hydrophobic anchor allowing the protein to associate with the lipid bi-layer of the endoplasmic reticulum (ER) where the Ras-converting enzyme 1 (RCE1) cleaves the AAX residues (Wright and Philips 2006). Unlike *HRAS* and *NRAS*, *KRAS* encodes 2 isoforms, *KRAS4A* and *KRAS4B*. Alternative splicing of exon 4 produces distinct variable c-terminal regions and changes the mechanisms by which these splice isoforms localize to the membrane (Tsai, Lopes et al. 2015). *NRAS*, *HRAS*, and *KRAS4A* have additional cysteine residues in their C-terminal region, which allow for palmitoylation in the ER or Golgi and subsequent plasma membrane localization (Hancock, Magee et al. 1989, Laude and Prior 2008). *KRAS4B* lacks a palmitoylation site and is localized to the plasma membrane via a lysine rich polybasic region (Hancock, Paterson et al. 1990). Notably, *KRAS4A* also contains two smaller polybasic regions and is capable of membrane localization independent of palmitoylation (Tsai, Lopes et al. 2015). These posttranslational modifications and localizations play an important role in oncogenic RAS signaling.

Loss of Tumor Suppressors in PDAC

The *CDKN2A* locus encodes the Inhibitor of CDK4-a ($p16^{\text{INK4A}}$) gene, a member of the INK4 family of CDKIs which also includes $p15^{\text{INK4B}}$, $p18^{\text{INK4C}}$, and $p19^{\text{INK4D}}$ (Roussel 1999). INK4 proteins inhibit cell cycle progression by preventing cyclin dependent kinase 4 (CDK4) and cyclin dependent kinase 6 (CDK6) from binding Cyclin D, thus maintaining active retinoblastoma (RB) and

inhibiting the activity of E2F transcription factors (Russo, Tong 1998).

Homozygous deletion of the *CDKN2A* locus is frequent in PDACs (Schutte, Hruban et al. 1997). Tumors lacking homozygous deletions often carry intragenic mutations, resulting in complete functional loss of p16^{INK4A} in 85% of cases. Epigenetic silencing is also common and overall, as many as 98% of PDACs may lack p16^{INK4A} expression. (Rozenblum, Schutte et al. 1997, Schutte, Hruban et al. 1997, Waddell, Pajic et al. 2015). The loss of *CDKN2A* has also been identified in PanIN precursor lesions. Deletions in this gene have been reported in 11.5% of PanIN1/2 lesions (Kanda, Matthaei et al. 2012), and expression of this tumor suppressor was lacking in 30% of PanIN-1a, 26% of PanIN-1b, 55% of PanIN-2 and 71% of PanIN3 as measured by immunostaining (Wilentz, Geradts et al. 1998).

In addition to coding for p16^{INK4A}, the *CDKN2A* locus also encodes the Alternative Reading Frame (p14^{ARF}, p19^{ARF} in rodents) protein (Serrano, Hannon et al. 1993). Their transcriptional start sites are separated by 13 kilobases, and although exons 2 and 3 overlap, p16^{INK4A} and p14^{ARF} translations occur in different reading frames and yield entirely unique proteins (Quelle, Zindy et al. 1995, Stone, Jiang et al. 1995). p14^{ARF} is a suppressor of the MDM2 protein and thus it functions to activate the tumor suppressor TP53 (Weber, Taylor et al. 1999). While deletions of these overlapping transcripts are frequent in PDAC, inactivating point mutations disrupting only the p16^{INK4A} encoding transcript have also been identified. In contrast mutations specifically targeting the p14^{ARF}

protein product have not been identified (Rozenblum, Schutte et al. 1997, Schutte, Hruban et al. 1997). This suggests that p16^{INK4A} plays the more important role in PDAC. Despite the lack of p14^{ARF} specific mutations, mouse-models show cooperation between deficiencies in p16^{INK4A} and TRP53 tumor suppressor pathways, indicating that deletion of p14^{ARF} benefits PDAC (Bardeesy, Aguirre et al. 2006).

The *CDKN2A* and *CDKN2B* loci encoding p16^{Ink4A} and P15^{Ink4B} respectively are separated by only 7,870 base pairs and are co-deleted in 29% of PDACs. However, lack of inactivating point mutations in *CDKN2B* suggest a proximal rational for these deletions rather than a tumor suppressive role for P15^{INK4B} in PDAC (Cerami, Gao et al. 2012, Gao, Aksoy et al. 2013, Waddell, Pajic et al. 2015).

Inactivation of a second major tumor suppressor gene, *TP53* (*Trp53* in mice), was observed in 50-75% of PDACs (Scarpa, Capelli et al. 1993, Rozenblum, Schutte et al. 1997). Loss of this tumor suppressor gene appears to occur later in the PanIN to PDAC progression as deletions were not observed in PanIN-1 or PanIN-2 lesions, and were found in only 12% of PanIN-3 lesions (DiGiuseppe, Hruban et al. 1994). Regulation of cell cycle arrest or apoptosis in response to DNA damage, hypoxia or oncogenic signaling, is coordinated by the TP53 transcription factor (Giaccia and Kastan 1998, Hu, Feng et al. 2012). TP53 protein function and levels are negatively regulated by the E3 ubiquitin ligase MDM2 which binds to and ubiquitinates TP53 resulting in its nuclear export and

proteasomal degradation (Roth, Dobbstein et al. 1998). DNA damage and ribosomal stress activate TP53 by inhibition of MDM2 (Hu, Feng et al. 2012). The p14^{ARF} protein directly inhibits MDM2 by nucleolar sequestration and inhibition of ubiquitin ligase activity, thus activating TP53 (DeGregori, Leone et al. 1997). In rat fibroblasts, overexpression of the RB pathway targets E2F1 or E2F2 induced expression of p19^{ARF}, but not p16^{INK4A} (DeGregori, Leone et al. 1997). The *CDKN1A* gene codes the cyclin dependent kinase inhibitor p21^{CIP}. Like p16^{INK4A}, p21^{CIP1} inhibits the function of E2F transcription factors through the RB pathway, and is a transcriptional target of the TP53 transcription factor (el-Deiry, Harper et al. 1994). This highlights the interconnected nature of the RB and TP53 pathways; the tumor suppressor pathways most commonly impaired in PDAC (Cerami, Gao et al. 2012, Gao, Aksoy et al. 2013).

TGF- β Signaling in PDAC

Disruption of the TGF- β signaling pathway is also common in PDAC. While TGF- β receptor 1 and 2 (TGFR1 and TGFR2) are inactivated through deletion or mutation in only 1-7% and 4-7% of PDACs respectively (Cerami, Gao et al. 2012, Gao, Aksoy et al. 2013), the downstream SMAD4 protein, also known as Deleted in Pancreatic Carcinoma 4, is frequently lost (Hahn, Schutte et al. 1996, Goggins, Shekher et al. 1998). Biallelic deletion of *SMAD4* is seen in 30% of PDAC and an additional 25% of tumors loose expression due to intragenic mutations (Hahn, Schutte et al. 1996). *Smad4* loss occurs later in PanIN

progression with expression being retained in PanIN-1/2 lesions but lost in 30% of PanIN-3 lesions (Wilentz, Iacobuzio-Donahue et al. 2000).

TGF- β is a well-studied inhibitory cytokine family involved in signaling of multiple cell types with tissue specific effects. Signaling through the TGF- β pathway is broadly grouped into TGF- β signaling and bone morphogenic protein (BMP) signaling. Upon binding to ligands such as TGF β 1, TGF β 2, TGF β 3, BMP2, BMP4 or BMP7, TGF- β type-1, and 2 receptors form a complex in which type-2 receptors phosphorylate type-1 receptors. The heterodimeric composition of these receptor complexes provides ligand specificity, and directs downstream signaling (Shi and Massague 2003). Phosphorylated type-1 receptors signaling through the TGF- β arm of this pathway phosphorylate SMAD2 and SMAD3, while type-1 receptors signaling through the BMP arm phosphorylate SMAD1, SMAD5, and SMAD8 (Shi and Massague 2003). These receptor-regulated SMAD proteins (R-SMAD) are phosphorylated on their C terminal SXS motif causing the release of cytosol-sequestering cofactors, and the formation of heterodimers or heterotetramers with the SMAD4 protein (Siegel and Massague 2003). These R-SMAD-SMAD4 complexes are shuttled to the nucleus, where together with other DNA binding cofactors, they selectively bind and regulate hundreds of genes including *CDKN2A* and *CDKN2B* (Shi and Massague 2003, Siegel and Massague 2003). Therefore, loss of SMAD4 inhibits TGF- β signaling through both the TGF- β and BMP pathways (Bierie and Moses 2006).

Paradoxically, TGF- β ligands are overexpressed in numerous tumor types including PDAC (Friess, Yamanaka et al. 1993, Bieri and Moses 2006, Bieri and Moses 2006). While loss of SMAD4, and thus TGF- β signaling, correlates with poor surgical outcomes and increased metastasis (Blackford, Serrano et al. 2009, Iacobuzio-Donahue, Fu et al. 2009), expression of TGF- β ligands also correlates with decreased survival (Friess, Yamanaka et al. 1993). These contradictory results support a dual role for TGF- β signaling in PDAC. TGF- β signals in epithelial tumor cells inhibit the initial formation and early progression of neoplasia, while in established disease these same signals shape the stromal reaction into an environment favorable for tumor growth and dissemination (Bieri and Moses 2006).

Genetics of IPMNs and MCNs

In addition to PanIN lesions, PDAC may also arise from IPMN and MCN precursor lesions. The early genetic profiles of IPMN and MCN lesions differ from those of PanINs, and, consistent with the clonal nature of cancer, these genetics are carried into the subset of PDACs arising from these lesions (Kanda, Matthaei et al. 2012). Guanine nucleotide binding protein, alpha stimulating activity polypeptide 1 (*GNAS*) encodes the G_s-alpha subunit of heterotrimeric G-proteins, and activating mutations in codon 201 result in constitutive activation of the cAMP pathway. These *GNAS* mutations are seen in roughly 11% of PanIN

lesions but are much more common in IPMNs (66%); they are absent in MCN lesions (Wu, Matthaei et al. 2011). Coincident mutations of *GNAS* and *KRAS* were frequent in IPMN with 51% of these lesions harboring both *KRAS* and *GNAS* mutations; 96% of IPMNs contained activating mutations in at least one of these genes (Wu, Jiao et al. 2011, Wu, Matthaei et al. 2011). Roughly 33% of MCN lesions had activating mutations in *KRAS*, but no *GNAS* mutations were identified (Wu, Jiao et al. 2011, Hosoda, Sasaki et al. 2015)

The ubiquitin E3 ligase ring finger 43 (*RNF43*) targets the WNT ligand receptor frizzled, and its inactivation has been shown to induce a WNT-dependent phenotype in PDAC cell lines (Jiang, Hao et al. 2013). Activation of the WNT/ β -catenin pathway is common in MCNs (Sano, Driscoll et al. 2014), and genetic inactivation or deletion of the *RNF43* gene is common in both IPMNs (75%) and MCNs(50%) (Wu, Jiao et al. 2011). This gene was mutated in only 7% of PanIN lesions (Forbes, Bindal et al. 2011, Wu, Jiao et al. 2011). Slow growing IMPN and MCN lesions, if identified, have the potential to be observed and resected prior to malignant progression. Therefore, understanding the genetics of these lesions and how they relate to progression could provide invaluable information regarding the necessity of high-risk surgery.

Other Pathways Altered in PDAC

The prevalence of *KRAS* mutations and *CDKN2A*, *TP53* and *SMAD4* loss

in PDAC can lead to the false impression that PDAC is genetically homogeneous. Recent sequencing efforts have identified hundreds of mutations, amplifications and deletions, which provide a layer of variability to the disease. Although alterations in any one gene are rare, grouping the proteins affected into broad functional categories highlights a number of pathways known to play a role in PDAC (Jones, Zhang et al. 2008, Biankin, Waddell et al. 2012). An example of this is seen in the frequent inactivation of the SWI/SNF chromatin-remodeling complex, which antagonizes polycomb group complex inhibition of p16^{INK4A} expression (Kia, Gorski et al. 2008, Wilson, Wang et al. 2010). Although genetic inactivation of any one component is infrequent, loss of at least one subunit, and thus SWI/SNF complex inactivation, was seen in roughly 33% of PDAC (Shain, Giacomini et al. 2012). Further studies identified aberrations in the Robo2/Slit2 axon guidance pathway, and found that decreased ROBO2 expression correlated with worse patient outcomes (Biankin, Waddell et al. 2012).

While it is clear that alterations in only 4 genes, *KRAS*, *CDKN2A*, *TP53* and *SMAD4*, constitute the core of genetic changes in PDAC, pharmacological exploitation of these changes has thus far been unsuccessful. The newly identified layer of genetic diversity presents an opportunity for highly personalized therapies. As a proof of principal, Waddell et al recently published a study showing a robust response to platinum based therapies in 4 of 5 of patients with *BRCA1*, *BRCA2*, or *PALB2* deficient PDACs (Waddell, Pajic et al. 2015). While previous drug trials often had a few patients with robust responses,

we may now be able to mine such studies for clues that will help to replicate these “anomalies” in tumors with similar genetic profiles.

Attempts to Pharmacologically Inhibit KRAS

In 1990, the cysteine residue on which farnesylation occurs was shown to be required for the transformative abilities of mutant KRAS (Jackson, Cochrane et al. 1990). Given the interest in RAS as a therapeutic target, a race to develop inhibitors of farnesyltransferase ensued. While preclinical reports of farnesyltransferase inhibitors were extraordinary, essentially curing Mouse Mammary Tumor Virus-HRAS induced breast and salivary gland tumors, clinical trials were a disappointment (Kohl, Omer et al. 1995, Berndt, Hamilton et al. 2011). Farnesyltransferase inhibitors provided no survival benefit to patients with pancreatic cancer, colorectal cancer or acute myeloid leukemia (Rao, Cunningham et al. 2004, Van Cutsem, van de Velde et al. 2004, Harousseau, Martinelli et al. 2009). Subsequent studies showed that while HRAS relies exclusively on farnesyltransferase, geranylgeranylation can act as a substitute in KRAS and NRAS (Lerner, Zhang et al. 1997, Rowell, Kowalczyk et al. 1997, Whyte, Kirschmeier et al. 1997, Sun, Qian et al. 1998).

Despite the failure of farnesyltransferase inhibitors to provide significant benefits in the clinic, some individuals did respond positively. Neither the clinical responses nor the preclinical successes correlated with KRAS status implying

that these diseases may be “addicted” to a different protein or proteins that depend exclusively on farnesylation (End, Smets et al. 2001, Kurzrock, Kantarjian et al. 2003). One such protein that merits mention is RAS homologue enriched in brain (RHEB). A small GTPase, RHEB acts downstream of AKT to activate mTOR complex1 (mTORC1), and shows elevated expression in some cancers. Farnesyltransferase inhibition resulted in decreased mTORC1 activity and increased apoptosis in breast and ovarian cancer cell lines. These phenotypes were reversed upon expression of a mutant RHEB designed to be geranylgeranylated (Basso, Mirza et al. 2005).

Additional drugs designed to inhibit geranylgeranyltransferase have since been developed. One has undergone phase 1 clinical trials yielding stable disease in 2 of 9 patients with refractory colon cancer (Berndt, Hamilton et al. 2011, Holstein and Hohl 2012). Both farnesyltransferase and geranylgeranyltransferase1 inhibitors are highly toxic due to the wide range of proteins modified by these enzymes (Lobell, Omer et al. 2001, Cox and Der 2002). Fears of synergistic toxicity from combination of these inhibitors have shifted current enthusiasm towards understanding the downstream effectors of activated KRAS. These downstream effectors contain a plethora of kinase rich signaling pathways with vast potential for specific inhibition by small molecules. However, they are also riddled with feedback loops and cross talk, making a thorough understanding of these pathways essential before targeting them in humans.

KRAS-driven Signaling Cascades

The activation of KRAS results in constitutive signaling through its downstream effectors and should make activating mutations in these effectors redundant. The low prevalence of activating mutations in the RAF and PI3K effector pathways, such as BRAF^{V600} and PI3KCA^{H1047} respectively, suggests that they are indeed redundant and that PDAC benefits from activation of multiple KRAS effector pathways (Cerami, Gao et al. 2012, Gao, Aksoy et al. 2013). Although KRAS^{G12D} causes constitutive activation of multiple pathways (Figure 1.1), the RAF/MEK/ERK, and PI3K/AKT pathways are the best studied and arguably the most important in the context of PDAC (Lim, Cho et al. 2005, Feldmann, Mishra et al. 2010, Eser, Schnieke et al. 2014).

BRAF Signaling in PDAC

BRAF is a member of the RAF family of serine/threonine kinases, which also include ARAF, and CRAF. RAF proteins contain an N-terminal auto-inhibitory domain, which obscures the catalytic domain of RAF. This N-terminal domain binds to activated RAS, altering the conformation of RAF to make its catalytic site accessible (Tran, Wu et al. 2005). RAS bound BRAF is localized to the plasma membrane where it undergoes additional phosphorylation events and forms a dimer, primarily with CRAF (Weber, Slusky et al. 2001). Activated BRAF protein phosphorylates and activates MEK1 and MEK2, which subsequently phosphorylate and activate ERK1 and ERK2 (Figure 1.1). The

activated ERK proteins translocate to the nucleus where they lead to activation of the AP1 transcription factor and subsequent expression of D-type cyclins (Pruitt and Der 2001, Downward 2003). In pancreatic ductal cells BRAF-MEK-ERK signaling imparts proliferative and pro-survival cellular responses. These responses are dependent on IGF1R and PI3K signaling (Appleman, Ahronian et al. 2012). While constitutively active KRAS activates the RAF-MEK-ERK signaling cascade in the majority of PDACs, BRAF^{V600} mutations activate this pathway in 9 to 37% of tumors without mutant KRAS (Calhoun, Jones et al. 2003, Cerami, Gao et al. 2012, Gao, Aksoy et al. 2013).

PI3K Signaling in PDAC

The PI3K signaling pathway also plays a crucial role in human PDAC. PI3Ks are lipid kinase heterodimers comprised of catalytic and regulatory subunits. Class1 PI3Ks have been implicated in oncogenic transformation (Yuan and Cantley 2008). These dimers are composed of a p110 α , p110 β , or p110 δ catalytic subunit, which bind to a p85 regulatory subunit, or a p110 γ catalytic subunit which binds to either a p110 or a p87 regulatory subunit (Castellano and Downward 2011). These heterodimers are activated via receptor tyrosine kinases (RTKs) at the plasma membrane (Castellano and Downward 2011). Src homology 2 domains on the p85 subunit can interact with the phosphorylated kinase directly, via a GRB2-GAB adaptor complex (McGlade, Ellis et al. 1992) or via insulin-receptor substrate 1 or 2 (IRS1/2) (Sun, Rothenberg et al. 1991, Araki,

Lipes et al. 1994). Alternatively, RTK bound GRB2 can activate SOS, a GEF responsible for RAS activation (Figure 1.1) (Boriack-Sjodin, Margarit et al. 1998).

Active PI3K converts phosphatidylinositol 4,5- bisphosphate (PIP₂) to phosphatidylinositol3,4,5-triphosphate (PIP₃), which binds pleckstrin homology (PH) domains to localize proteins such as AKT and 3-phosphoinositide-dependent kinase 1 (PDK1) to the plasma membrane, a crucial step in their activation (Figure 1.1)(Pearce, Komander et al. 2010, Castellano and Downward 2011). The phosphatase and tensing homolog (PTEN) acts contra to PI3K by dephosphorylating PIP₃, and is widely described as a tumor suppressor. Low PTEN levels in PDAC patient samples correlates with poor outcomes(Morran, Wu et al. 2014), and murine models of PTEN-deficient KRAS-driven PDAC respond to the mTOR inhibitor rapamycin (Morran, Wu et al. 2014).

Additional RAS effector pathways

Constitutively activated RAS signaling activates a number of downstream pathways in addition to the RAF/MEK/ERK and PI3K/AKT. The Ral, Tiam1/RAC, and PLC_ε pathways are downstream effector pathways of RAS that have been implicated in RAS-mediated tumorigenesis (Figure 1.1). In humans the Ras-like (Ral) family of GTPases are highly conserved from *Caenorhabditis elegans* to *Homo sapiens* (Neel, Martin et al. 2011). To enable efficient signal transduction,

these GTPases require GEF mediated replacement of RAL bound GDP with GTP. Four of the six RAL specific GEFs , RALGDS and RalGDS-like 1,2, and 3, contain highly conserved C-terminal RAS binding domains (Neel, Martin et al. 2011). Elevated levels of GTP bound RALA and RALB were observed in human PDAC; isoform specific knockdown with shRNA implicated the former in tumor growth and the latter in metastasis (Lim, O'Hayer et al. 2006). Like RAS, RAL proteins influence multiple downstream pathways including RAC, CDC42 and NF κ B (Figure 1.1)(Neel, Martin et al. 2011). RAL binding protein 1 (RALBP1) interacts with GTP bound RAL via a RAL binding domain to regulate RALBP1 cellular localization (Matsubara, Hinoi et al. 1997). RALBP1 acts as a GAP for the Rho family members RAC and Cdc42. By catalyzing the GTPase activity of these family members RALBP1, mediates cytoskeletal organization; by regulating RALBP1 localization, RAL regulates the formation of RAC1 mediated membrane ruffling and CDC42 mediated filopodia formation (Neel, Martin et al. 2011). RAL activity can also influence cell cycle progression by inducing transcription of Cyclin D1 in an NF- κ B dependent, RALBP1 independent manner (Matsubara, Hinoi et al. 1997).

Ras activity can also regulate RAC1 activation through the T lymphoma invasion and metastasis 1 (TIAM1) protein (Figure 1.1). TIAM1 is a RAC specific GEF, which associates with GTP bound RAS via a RAS binding domain and increases RAC1-GTP levels (Lambert, Lambert et al. 2002). RAC1 activation by TIAM1 is independent of PI3K activity, and is constitutively activated by mutant

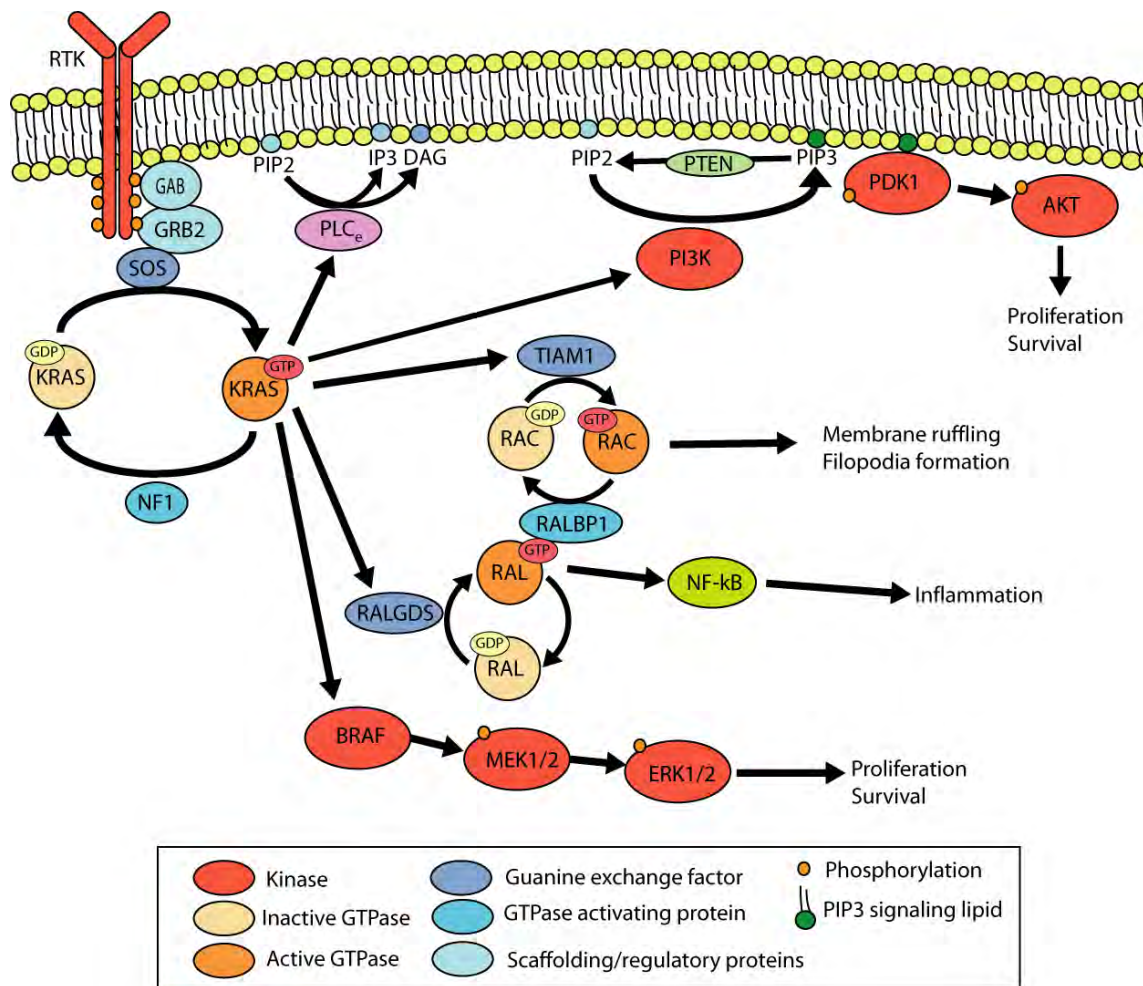
RAS (Matsubara, Hinoi et al. 1997, Nimnual, Yatsula et al. 1998, Scita, Nordstrom et al. 1999, Lambert, Lambert et al. 2002).

Direct binding of the C-terminal region of RAC1 with mTOR has led to the suggestion that localization of mTORC1 and mTORC2 are mediated by RAC1 (Saci, Cantley et al. 2011). The significance of these findings is supported by the co-localization of both mTORC1 and mTORC2 components with RAC1 at the plasma membrane, the failure of both complexes to properly localize in the absence of RAC1, and the down regulation of both mTORC1 and mTORC2 signaling in the absence of RAC1 (Saci, Cantley et al. 2011). Notably, these effects appeared to be independent of the GDP/GTP binding status of RAC1 (Saci, Cantley et al. 2011).

Members of the phospholipase C (PLC) protein family act to hydrolyze phosphatidylinositol 4,5 bisphosphate to produce two second-messenger molecules, 1,4,5 triphosphate (IP3) and diacylglycerol (DAG) in response to RAS activation (Figure 1.1)(Rhee and Bae 1997). The presence of IP3 results in the cytoplasmic release of Ca^{2+} , and DAG is involved in the activation of PKC proteins (Reuther and Der 2000). The PLC_ϵ member of this family contains two RAS-association domains, which, upon expression of activated HRAS, bound to the HRAS and increased PLC_ϵ activity (Kelley, Reks et al. 2001). This activation appears to be the result of active RAS localizing PLC_ϵ to the plasma membrane (Song, Hu et al. 2001).

Figure 1.1 Schematic representation of oncogenic KRAS signaling.

Active receptor tyrosine kinases bind GRB2, which binds and activates SOS. As a guanine exchange factor (GEF), SOS facilitates the removal of GDP and binding of a GTP molecule to KRAS, thus activating KRAS. GAP proteins, such as NF1, catalyze hydrolysis of GTP by KRAS; activating mutations in *KRAS* impair GTP hydrolysis constitutively activating the protein. Active KRAS signals through multiple tumorigenic pathways including the PI3K-AKT and RAF-MEK-ERK pathways which promote proliferation and survival. KRAS also activates the TIAM1 and RALGDS GEFs resulting in RAC and RAL activation respectively and promote cell motility and inflammation. Active RAL binds and localizes RALBP1 a GAP that regulates RAC activity. KRAS also activates PLC ϵ which hydrolyzes PIP₂ to form IP₃ and DAG lipids. These lipids promote cytoplasmic release of Ca²⁺ and activation of PKC proteins respectively.



Receptor Tyrosine Kinase Signaling in PDAC

Upon ligand interaction with their extracellular regions, the receptor tyrosine kinase (RTK) class of trans-membrane signaling molecules homo or heterodimerize resulting in conformational changes, post-translational modifications, and kinase activity (Lemmon and Schlessinger 2010). The epidermal growth factor receptor (EGFR) and insulin growth factor receptor (IGF1R) family members are among these proteins and are known to be involved in PDAC development. High levels of EGFR expression are ubiquitous in PDAC, and expression of its family member and occasional heterodimeric partner, ERBB2, was elevated in 68% of moderately differentiated PDACs (Korc, Chandrasekar et al. 1992). A canonical view of these RTKs places them upstream of KRAS, with GRB2 bound RTKs activating SOS, a RAS activating guanine exchange factor (Figure 1.1). In this simplistic model, constitutive KRAS activation would make these signals irrelevant. The mutual exclusivity of EGFR and KRAS mutations in non-small cell lung cancers (NSCLCs), the contraindication of mutant KRAS to EGFR targeted therapies in NSCLCs, and genetic mouse-models of this disease support this pathway model (Shigematsu, Takahashi et al. 2005, Amado, Wolf et al. 2008, Karapetis, Khambata-Ford et al. 2008). In PDAC however, KRAS mutations are found in concert with EGFR overexpression (Korc, Chandrasekar et al. 1992), amplifications (2%) (Biankin, Waddell et al. 2012), and activating mutations (4%) (Kwak, Jankowski et al. 2006, Cerami, Gao et al. 2012, Gao, Aksoy et al. 2013). Mouse-models also

show that EGFR signaling is essential for KRAS-driven PDAC initiation, contrary to the canonical view from lung cancer (Navas, Hernandez-Porras et al. 2012). Indeed, erlotinib combined with gemcitabine provided a small but significant increase in survival for patients suffering from PDAC and is the first molecular targeted therapy to do so (Moore, Goldstein et al. 2007). Furthermore, work by Appleman et al. showed signaling through the RAF-MEK-ERK pathway activated IGF1R signaling which was required for subsequent activation of the PI3K pathway (Appleman, Ahronian et al. 2012). These studies have led to a much greater appreciation for the non-linear and complex roles RTK signaling plays in PDAC.

The mTOR Kinase Complexes

The mammalian target of rapamycin (mTOR) kinase acts both upstream and downstream of the PI3K-AKT signaling pathway. It regulates growth, proliferation and survival in response to nutrient availability and growth factor signaling (Sengupta, Peterson et al. 2010, Zoncu, Efeyan et al. 2011). The mTOR kinase protein is the catalytic subunit in two distinct complexes. Both mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2) contain the DEP domain-containing mTOR-interacting protein (DEPTOR) and mammalian lethal with SEC13 protein 8 (mLST8 also known as GBL) in addition to the mTOR kinase (Figure 1.2). The Regulatory-associated protein of mTOR (RAPTOR)

and 40-kDa pro-rich AKT-substrate (PRAS40) are unique to mTORC1, while the rapamycin-insensitive companion of mTOR (RICTOR), the protein observed with RICTOR (PROTOR), and the mammalian stress-activated map kinase-interacting protein 1 (mSIN1 also known as MAPKAP1) are all specific to mTORC2 (Figure 1.2) (Loewith, Jacinto et al. 2002, Jacinto, Loewith et al. 2004, Sarbassov, Ali et al. 2004, Frias, Thoreen et al. 2006, Jacinto, Facchinetti et al. 2006, Yang, Inoki et al. 2006, Pearce, Huang et al. 2007, Thedieck, Polak et al. 2007, Woo, Kim et al. 2007, Peterson, Laplante et al. 2009). mTOR kinase phosphorylates members of the AGC kinase family, including AKT, SGK and S6K, at a hydrophobic motif (Figure 1.2) (F-X-X-F/Y-S/T-F/Y) adjacent to the kinase domain (Huang, Wu et al. 2009). Growth of mice lacking mTORC1 is arrested at embryonic day 5.5 and while some embryos are able to implant, they cannot proliferate and die at or shortly after embryonic day 6.5 (Gangloff, Mueller et al. 2004, Guertin, Stevens et al. 2006). In the absence of mTORC2 signaling mice die by embryonic day 10.5 (Guertin, Stevens et al. 2006, Shiota, Woo et al. 2006).

mTOR Complex 1 Signaling

Signaling through mTORC1 provides a single hub through which cells can respond to growth signals, nutrient, and oxygen levels to influence cellular responses including protein synthesis, lipid metabolism, and autophagy (Hung,

Garcia-Haro et al. 2012). Regulation of protein synthesis is largely accomplished by mTORC1 phosphorylation of S6K and 4EBP1 proteins. Phosphorylation of the hydrophobic motif of S6K at the threonine 389 residue results in activation of numerous proteins responsible for mRNA maturation and protein translation (Ma and Blenis 2009). In addition mTORC1 phosphorylates the eukaryotic translation initiation factor 4E (eIF4E) inhibitory protein, eIF4E binding protein 1 (4EBP1) at multiple sites, decreasing the affinity of these two proteins, and freeing the eIF4E protein to initiate cap-dependent translation (Figure 1.2)(Ma and Blenis 2009). The sterol regulatory element-binding protein (SREBP) family of transcription factors control expression of multiple fatty acid and cholesterol biogenesis genes (Horton, Goldstein et al. 2002). Activity of these proteins is inhibited by nuclear LIPIN 1, and phosphorylation of LIPIN 1 by mTORC restricts its access to the nucleus, allowing SREBP1 mediated expression of fatty acid and cholesterol biosynthetic programs (Figure 1.2)(Peterson, Sengupta et al. 2011). Inhibition of autophagy by mTORC 1 is achieved by direct phosphorylation of the unc-51 like autophagy activating kinase 1 (ULK1) and autophagy related 13 (ATG13) members of the ULK complex (Figure 1.2). In a nutrient sufficient setting, mTORC1 phosphorylation of these proteins inhibits the induction of autophagy (Shang, Chen et al. 2011). Through these pathways mTORC1 allows cells to regulate their biosynthetic processes, building when materials are plentiful, and recycling through autophagy when resources are scarce.

mTORC1 activity is modulated by intracellular energy levels and amino

acid availability. In a glucose deficient environment, adenosine triphosphate (ATP) levels decrease and adenosine monophosphate (AMP) levels increase. Increased AMP activates AMPKinase, which directly phosphorylates and activates tuberous sclerosis complex 2 (TSC2) (Inoki, Li et al. 2002). TSC2 and its binding partner tuberous sclerosis complex 1 (TSC1) inhibits the small GTPase Ras Homolog Enriched In Brain (RHEB) by acting as a GAP protein to promote hydrolysis of RHEB-bound GTP (Manning and Cantley 2007). Active RHEB is a potent activator of mTORC1, and TSC1/2 deactivation of RHEB results in mTORC1 inhibition (Figure 1.2)(Inoki, Li et al. 2003). The importance of this regulatory mechanism is underscored by apoptosis of TSC2^{-/-} cells upon glucose starvation (Inoki, Zhu et al. 2003). TSC2 deletion uncouples cell growth from resource availability placing cells in crisis. While some details remain unresolved, the depletion of ATP likely leads to increased AMPK mediated TP53 stabilization. In addition, hyper-activation of mTORC1 and its subsequent activation and stabilization of S6K and GRB10 respectively, results in the inhibition of IRS1 and mTORC2, decreasing PI3K/AKT survival signals and promoting cell death (Hsu, Kang et al. 2011, Zoncu, Efeyan et al. 2011, Hung, Garcia-Haro et al. 2012) AMP kinase can also phosphorylate the mTORC1 subunit RAPTOR; this phosphorylation of RAPTOR inhibits mTORC1 activity (Gwinn, Shackelford et al. 2008).

mTORC1 activity is also responsive to amino acid availability. Although cells require RHEB for amino acid induced mTORC1 activation, TSC2-deficient

cells remain sensitive to amino acid depletion, indicating that the mTORC1 response to amino acid levels is TSC1/2 independent (Smith, Finn et al. 2005). In the presence of lysosomal amino acids, RAG GTPase, tethered to the lysosome by RAGULATOR, binds to and recruits mTORC1 to the lysosome (Kim, Goraksha-Hicks et al. 2008, Sancak, Peterson et al. 2008, Sancak, Bar-Peled et al. 2010). This is presumed to bring mTORC1 into proximity with RHEB proteins residing on the lysosome to allow for mTORC1 activation (Hung, Garcia-Haro et al. 2012)

mTOR Complex 2 Signaling

mTORC2 was identified well after mTORC1 and comparatively little is known about this complex. *In vitro* stimulation with insulin or insulin-like growth factor 1 increases mTORC2 kinase activity, and this activity has been shown to induce hydrophobic motif phosphorylation of AKT at the serine 473 residue, and SGK at the serine 422 residue as well as some members of the PKC family, including PKC α (Figure 1.2)(Sarbasov, Ali et al. 2004, Sarbasov, Guertin et al. 2005, Frias, Thoreen et al. 2006, Jacinto, Facchinetti et al. 2006, Garcia-Martinez and Alessi 2008) In addition, mTORC2-dependent phosphorylation of turn motifs has also been identified in AKT and PKC family members (Figure 1.2) (Facchinetti, Ouyang et al. 2008, Ikenoue, Inoki et al. 2008).

Cellular location of mTORC2 likely modulates its activity. It has been reported that prostate epithelial cells lacking PTEN have increased localization of RICTOR to the plasma membrane (Guertin, Stevens et al. 2009). However, fractionation of cell lines derived from lung carcinoma and melanoma identified the endoplasmic reticulum as the primary site of the mTORC2 components RICTOR, mSIN1 and mTOR. While mTORC2 activity in the plasma membrane fraction required insulin-like growth factor stimulation, mTORC2 activity the ER and cytoplasm was not altered by this growth factor (Boulbes, Shaiken et al. 2011). Further studies have shown that mTORC2 localizes to the mitochondria-associated sub-compartment of the endoplasmic reticulum in a growth factor dependent manner and mediates mitochondrial integrity via AKT activity (Betz, Stracka et al. 2013). The discovery of a pleckstrin homology like domain in mSIN suggested a possible mechanism for membrane localization via $\text{PtdIns}(3,4,5)\text{P}_3$ (Schroder, Buck et al. 2007). Recent findings showed that the PH domain of mSIN inhibits mTOR catalytic activity in mTORC2. This inhibition is removed upon PIP_3 - PH domain interaction (Liu, Gan et al. 2015). An additional level of regulation may be afforded by S6K phosphorylation of RICTOR, which creates a 14-3-3 binding site, and appears to regulate AKT^{S473} phosphorylation but not phosphorylation of SGK or $\text{PKC}\alpha$ (Dibble, Asara et al. 2009).

The TSC1/2 complex responsible for inhibiting mTORC1 may also promote mTORC2 activity independent of mTORC1 inhibitory feedback (Huang and Manning 2008). These findings are supported by a decrease in mTORC2

signaling in the absence of TSC1/2 that is not reversed by mTORC1 inhibition (Huang and Manning 2008). Furthermore, altering RHEB expression levels caused no change in mTORC2 activity levels (Yang, Inoki et al. 2006, Huang and Manning 2008, Sancak, Peterson et al. 2008).

Transcriptional regulation of mTORC2 subunits is also likely to regulate mTORC2 levels, and thus its activity. The AKT target FOXO1 can induce expression of *Rictor* mRNA and increase its protein levels (Chen, Jeon et al. 2010). Regulation of RICTOR and mSIN1 through expression and post-translational modification likely provide a series of regulatory mechanisms by which mTORC2 activity is regulated independently of its mTOR subunit. While an understanding of mTORC2 regulation is still emerging, it is clear that activity of this complex plays a role in activating the AKT, SGK and PKC signaling pathways.

Activation and Effects of the AKT Kinase Family

The AKT signaling pathway is one of the most studied pathways in cancer. The three AKT kinase family members, AKT1, AKT2 and AKT3, are involved in regulating numerous hallmarks of cancer, including proliferation, survival, and metabolism (Gonzalez and McGraw 2009). The three isoforms are expressed from distinct genetic loci but are highly homologous (Hanada, Feng et al. 2004). Despite this homology, whole animal knockout of individual isoforms in mice has

shown that these proteins are not fully redundant. *Akt1* knockout mice are smaller, *Akt2* knockout mice develop insulin resistance, and *Akt3* knockout mice have defects in brain development (Chen, Xu et al. 2001, Cho, Mu et al. 2001, Cho, Thorvaldsen et al. 2001, Garofalo, Orena et al. 2003, Tschopp, Yang et al. 2005). AKT1 over-expression failed to rescue insulin dependent glucose transport in the absence of AKT2 supporting non-redundant roles for each isozyme (Bae, Cho et al. 2003). However, membrane targeted versions of AKT1 were able to rescue this phenotype (Gonzalez and McGraw 2009) supporting a role for subcellular localization of AKT isoforms in their specificity.

Multiple post-translational modifications are involved in the activation of AKT. Phosphorylation of the turn motif at AKT^{T450} occurs in an mTORC2-dependent manner, during or soon after translation, and is believed to stabilize phosphorylation of the hydrophobic motif (Facchinetti, Ouyang et al. 2008, Ikenoue, Inoki et al. 2008). In the absence of this phosphorylation, the HSP90 protein acts as a chaperone to prevent ubiquitin-mediated degradation of the AKT protein allowing for the maintenance of AKT protein levels and a basal level of signaling even in the absence of mTORC2 (Facchinetti, Ouyang et al. 2008). AKT is recruited to the membrane by interaction of its pleckstrin homology (PH) domain with PIP₃ (Calleja, Alcor et al. 2007). This interaction also induces a conformational change in AKT allowing phosphorylation of the activation motif, at the threonine 308 residue, by PDK1 (Alessi, Deak et al. 1997). This level of phosphorylation activates AKT signaling (Guertin, Stevens et al. 2006, Jacinto,

Facchinetti et al. 2006, Shiota, Woo et al. 2006), however, full activation requires phosphorylation of the hydrophobic motif at AKT^{S473} by mTORC2 (Sarbasov, Guertin et al. 2005, Jacinto, Facchinetti et al. 2006).

AKT activity results in cell survival, cell growth, proliferative, and metabolic phenotypes. AKT mediated phosphorylation of R-X-R-X-X-S/T-B motifs, where B is a bulky hydrophobic residue and X is any residue, is context specific and can moderate the activity of numerous proteins (Alessi, Caudwell et al. 1996).

Phosphorylation of pro-apoptotic BCL-2 family members such as BAD on S136 results in a 14-3-3 protein binding site that removes the inhibitory BAD protein from pro-survival BCL-2 family members (Figure 1.2)(Datta, Katsov et al. 2000).

Nuclear activity of AKT can also regulate cell survival through phosphorylation of FOXO family members such as FOXO1 at T24 and S256 (Figure 1.2).

Phosphorylation of these sites creates a 14-3-3 protein binding site, and such binding results in nuclear export of these proteins (Tran, Brunet et al. 2003). The FOXO family of transcription factors induces expression of pro-apoptotic genes such as Fas ligands and BIM (Brunet, Bonni et al. 1999, Dijkers, Birkenkamp et al. 2002). MDM2 mediated TP53 degradation is also regulated by AKT; MDM2 is localized to the nucleus upon phosphorylation of residues S166 and S186 by AKT (Mayo and Donner 2001, Zhou, Liao et al. 2001). Inhibitory AKT phosphorylation of Glycogen synthase kinase-3 beta (GSK3 β) impacts numerous downstream targets including its activation of pro-apoptotic c-Jun N-terminal kinase (JNK) signaling and inhibition of the MCL-1, a pro survival BCL-2 family

member (Maurer, Charvet et al. 2006, Marchand, Tremblay et al. 2012). AKT can also negatively regulate this pathway by inhibition of apoptosis signal-regulating kinase 1 (ASK1) upstream of JNK (Kim, Khursigara et al. 2001).

In addition to restraining apoptotic signaling, AKT can regulate cell cycle arrest. AKT has been shown to phosphorylate human p21^{CIP1} and p27^{KIP1} at T145 and T157 respectively. In both cases these modifications result in protein localization to the cytoplasm and subsequent loss of their nuclear functions as cell cycle inhibitors (Zhou, Liao et al. 2001, Sekimoto, Fukumoto et al. 2004). The T157 residue of p27^{KIP1} is not conserved in mice, however the regulation of p27^{KIP1} by AKT is maintained at a transcriptional level by the inhibition of FOXO transcription factors by AKT (Medema, Kops et al. 2000). Expression of p21^{CIP1} may also be regulated by AKT driven inactivation of the TP53 transcription factor via MDM2 (Mayo and Donner 2001, Zhou, Liao et al. 2001).

AKT plays multiple roles in the regulation of metabolism. As described above, the TSC1/TSC2 heterodimer inhibits the potent mTORC1 activating protein RHEB. AKT phosphorylates TSC2 at multiple sites, of which S939 and T1462 are evolutionarily conserved through drosophila, and inactivates the protein resulting in mTORC1 activation (Figure 1.2) (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). A second mechanism for AKT-dependent mTORC1 activation is through its inhibition of proline-rich Akt substrate of 40 kDa (PRAS40). This protein binds to and negatively regulates mTORC1, but upon

AKT phosphorylation at T246, 14-3-3 proteins bind PRAS40, dissociating it from mTORC1 and targeting it for degradation (Kovacina, Park et al. 2003, Sancak, Thoreen et al. 2007, Vander Haar, Lee et al. 2007). These parallel pathways allow AKT to enhance anabolic processes such as protein synthesis and cap dependent mRNA translation through mTORC1 activity.

Stimulation of glucose uptake in response to insulin is also mediated by AKT. Upon insulin stimulation, AKT, primarily AKT2, associates with glucose transporter 4 (GLUT4) containing vesicles and stimulates the translocation of GLUT4 to the plasma membrane (Kohn, Summers et al. 1996, Calera, Martinez et al. 1998). Furthermore, insulin stimulated AKT activity can, through mTORC1, induce translational expression of Glucose transporter 1 (Taha, Liu et al. 1999). In the context of PDAC, KRAS-activation has been shown to enhance glucose uptake, glycolysis and ribosome biogenesis; this metabolic shift is essential for tumor survival (Ying, Kimmelman et al. 2012). Despite the multiple mechanisms through which AKT impacts cell metabolism, small molecule inhibition of PI3K/AKT did not greatly affect KRAS-driven metabolic reprogramming in PDAC (Ying, Kimmelman et al. 2012).

The extent to which differential AKT phosphorylation regulates target specificity has not been fully defined. While phosphorylation of some AKT targets, such as FoxO1^{T24} and FoxO3a^{T32}, appears to require AKT^{S473}, other pathways, such as mTORC1 activation, do not (Guertin, Stevens et al. 2006,

Jacinto, Facchinetti et al. 2006). The potential for differential signaling based on AKT phosphorylation status suggests that blocking AKT^{S473} phosphorylation may inhibit a subset of AKT effectors, while maintaining signaling through other, potentially beneficial pathways.

Activation and Effects of the SGK Kinase Family

The serum and glucocorticoid-regulated kinase (SGK) family is also activated by mTORC2. It contains three family members; SGK1, and SGK3, which are expressed in all tissues, and SGK2, which is expressed specifically in the pancreas, liver, and kidneys (Kobayashi, Deak et al. 1999). These proteins are involved in the cellular stress response and in parallel to AKT regulation of glucose transport, SGK1 regulates sodium, calcium, potassium and chlorine channels (Lang and Shumilina 2013). Most notably, *Sgk1*^{-/-} mice are unable to stem the efflux of Na⁺ upon dietary restriction of Sodium Chloride (Wulff, Vallon et al. 2002). Unlike AKT proteins, this family of proteins lacks PH domains and requires mTORC2 phosphorylation of the hydrophobic motif at SGK^{S422} as a primer for activation (Figure 1.2)(Garcia-Martinez and Alessi 2008). This phosphorylated residue of SGK interacts with the PDK1-interacting fragment (PIF) pocket of PDK1, stimulating phosphorylation of the SGK activation motif by PDK1 and thus activating SGK (Pearce, Komander et al. 2010).

The consensus target sequence for phosphorylation by SGK proteins is R-X-R-X-X-S/T, a motif shared by the AKT family (Kobayashi, Deak et al. 1999). Multiple proteins, such as GSK3 β and p27^{KIP1}, are phosphorylated by AKT and SGK at the same residues (Kobayashi and Cohen 1999, Hong, Larrea et al. 2008). Other proteins such as FOXO3a are selectively phosphorylated by AKT and SGK at independent residues, although low levels of cross reactivity between target residues were observed in the presence of constitutively active AKT or SGK proteins (Brunet, Park et al. 2001).

The N-myc downstream regulated gene (NDRG) family contains 4 proteins, two of which, NDRG1 and NDRG2, have been identified as targets for SGK1 (Murray, Campbell et al. 2004). Under physiological conditions, these proteins are phosphorylated at multiple sites in an SGK1 specific manner (Figure 1.2) (Murray, Campbell et al. 2004). NDRG1 expression is down regulated by MYC and MYCN oncoproteins and is upregulated by numerous stresses including hypoxia and oxidative stress (Said, Stein et al. 2009, Cheng, Xie et al. 2011). Although often referred to as a metastasis suppressor, the effect of NDRG1 expression in cancer appears to vary based on malignancy type. In PDAC, NDRG1 levels appear to correlate with improved survival, possibly due to reduced metastatic and angiogenic potential (Maruyama, Ono et al. 2006). However, in a recent study looking at glioblastoma, NDRG1 expression was shown to provide resistance to alkylating chemotherapy in an MGMT-dependent manner (Weiler, Blaes et al. 2014). SGK1 phosphorylation of NDRG1 at T328,

S330, and T346 primes this protein for phosphorylation by GSK3 β at S342, S352 and S362 (Murray, Campbell et al. 2004). The effect of NDRG1 phosphorylation is unclear, but phosphorylation of the previously mentioned residues is often used as a measure of SGK1 activity.

Activation and Effects of the PKC Kinase Family

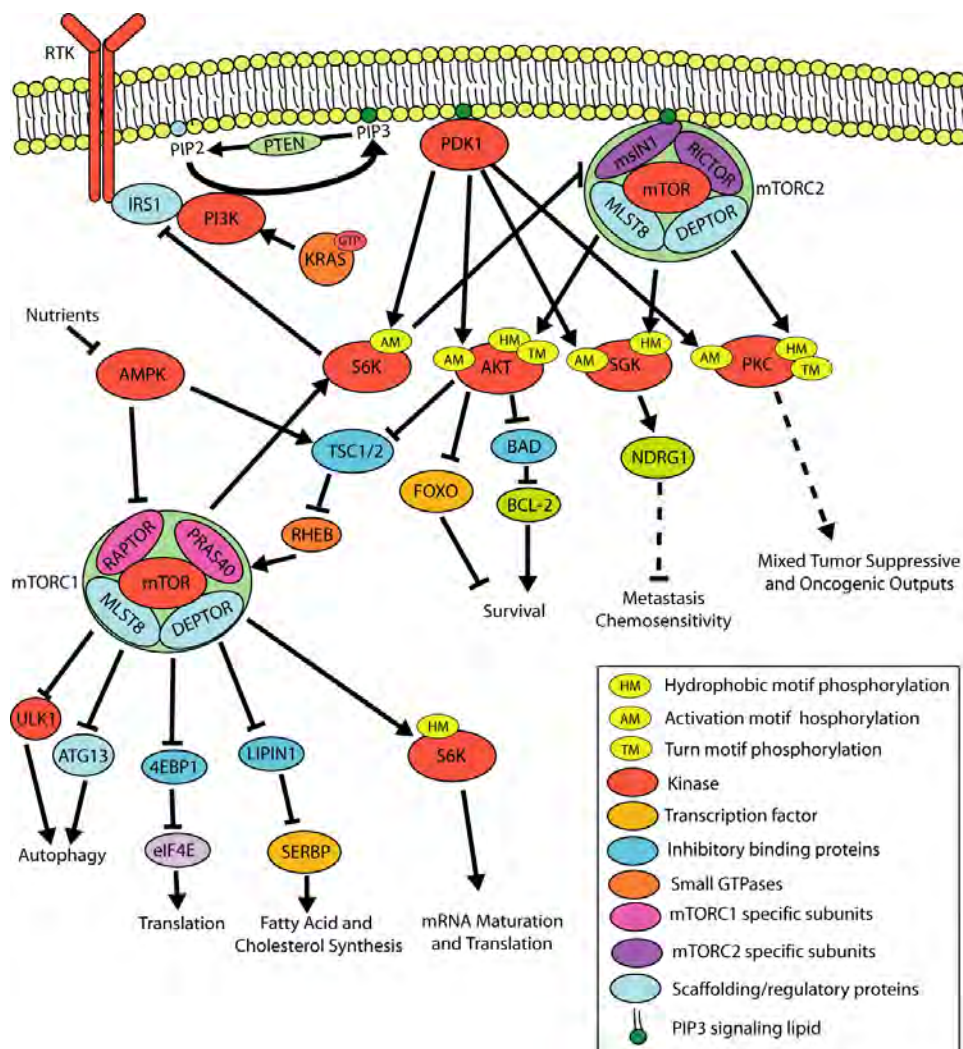
Some members of the protein kinase C (PKC) family are also activated by mTORC2 kinase activity. The PKC family contains three sub categories; conventional PKCs include PKC α , PKC β and PKC γ , novel PKCs include PKC δ , PKC ϵ , PKC η and PKC θ , and atypical PKCs include PKC ζ and PKC λ (Pearce, Komander et al. 2010). The activation of PKCs requires phosphorylation events in the activation domain, the hydrophobic motif, and the turn motif. These steps prime the protein for activation which is achieved by binding of the auto-inhibitory N-terminal domains to diacylglycerol and calcium, thus exposing the activation domain (Newton 2003). Although phosphorylation of the hydrophobic and turn motifs was originally classified as autophosphorylation, mTORC2 is now known to be responsible for phosphorylation of the PKC α hydrophobic motif (S657), and turn motif (T638) (Figure 1.2) (Newton 2003, Sarbassov, Ali et al. 2004, Guertin, Stevens et al. 2006, Ikenoue, Inoki et al. 2008). These sites may be phosphorylated by mTORC2 in additional PKC family members, however, phosphorylation of PKC ϵ and PKC η hydrophobic motifs were insensitive to

mTORC2 depletion suggesting that alternative methods exist for activation of these sites in other PKC family members(Sarbassov, Ali et al. 2004).

Despite extensive study, the role of PKC isozymes in cancer remains unclear, as numerous studies have produced conflicting results. This is not unexpected in a large and diverse family of kinases, however disagreement exists regarding the oncogenic function of individual isozymes even within the same tumor and cell types. For instance, PKC α activity was shown to both enhance and suppress cell line proliferation in colon cancer cell lines (Walsh, Woo et al. 2004, Gwak, Jung et al. 2009, Wu, Zhou et al. 2013). Likewise, PKC ζ loss both increased and decreased growth of colorectal tumors (Luna-Ulloa, Hernandez-Maqueda et al. 2011, Ma, Tao et al. 2013). PKC δ overexpression in a pancreatic cancer cell line promoted tumor growth and metastasis upon subcutaneous injection of nude mice (Mauro, Grossoni et al. 2010). The prevailing view that PKC proteins have a net oncogenic effect has been revised based on two recent studies. The only phase III trial of a PKC inhibitor showed decreased survival in NSCLC patients (Mackay and Twelves 2007). In addition, it was recently determined that the majority of mutations in human PKC isozymes resulted in suppression rather than activation of PKC activity. In fact, some mutations were dominant negative while others had no functional impact, but no mutations tested increased PKC activity when tested in their respective cell types (Antal, Hudson et al. 2015). These findings suggest that the actual role of PKC proteins is tumor suppressive rather than oncogenic.

Figure 1.2: Signaling through mTORC1 and mTORC2 in cancer.

Activation of PI3K by KRAS or IRS mediated receptor tyrosine kinase interaction results in PIP2 phosphorylation and produces a PIP3 signaling lipid. Binding of PH domains by PIP3 localizes both PDK1 and mTORC2, via its mSIN1 subunit, to the plasma membrane. PDK1 phosphorylates the activation motif of S6K, AKT, SGK and some PKC family members. mTORC2 phosphorylates the hydrophobic motif of SGK and the hydrophobic and turn motifs of AKT and some PKC family members. mTORC1 is activated downstream of mTORC2 in response to inhibitory phosphorylation of TSC2 by AKT. Low cellular ATP levels result in elevated AMPK which impairs mTORC1 by activating TSC2 and phosphorylating the mTORC1 subunit RAPTOR. Active mTORC1 phosphorylates and activates S6K, which phosphorylates and inhibits mSIN1, RICTOR and IRS, providing a negative feedback loop.



Mouse-models of KRAS Induced PDAC

The current standard in the field of KRAS initiated PDAC mouse-models was first described by Hingorani et al in 2003. In this study they used mice with one wild-type allele of *Kras* and one mutant allele preceded by a Lox-P flanked stop cassette. Placing a CRE recombinase under either the *Pdx1* or the *Ptf1a*-promoter resulted in excision of the stop cassette in pancreatic progenitor cells beginning at embryonic day 8.5 or 9.5 respectively, and the subsequent expression of the mutant *Kras* allele from its endogenous locus (Offield, Jetton et al. 1996, Kawaguchi, Cooper et al. 2002, Hingorani, Petricoin Iii et al. 2003). Beginning as early as two weeks of age, these mice develop PanIN lesions that mirror human pathology. These lesions develop into invasive and metastatic PDAC over a long latency with about half of these mice developing tumors by 400 days of age (Tuveson and Hingorani 2005). Lineage tracing versions of this model showed that PanIN lesions can arise from acinar cells through a process known as acinar-to-ductal metaplasia (ADM)(Habbe, Shi et al. 2008). During this process acinar cells dedifferentiate, altering their transcriptional program to a more embryonic state (Fendrich, Esni et al. 2008). These primitive pancreatic progenitor cells can repopulate exocrine cells in the pancreas, but in the context of mutant KRAS they develop into neoplastic duct like lesions (Habbe, Shi et al. 2008). Given the success of Hingorani's model, it was quickly adopted as the field standard. Commonly referred to as *LSL-Kras* or "*KC*" mice, they have been

combined with many additional genetic models to enhance our understanding of PDAC.

Given the prevalence of *CDKN2A*, *TP53* and *SMAD4* loss in human PDAC, *KC* mouse-models deficient in the murine equivalent of these genes were developed to better understand their roles in the disease. Additional deletion of the *Cdkn2a* locus in the *KC* mouse-model resulted in formation of PanIN lesions by 3 weeks of age, and poorly differentiated PDAC by 6 weeks of age. All mice succumbed to disease between 7 and 11 weeks and distant metastases were detected in 21% of mice (Aguirre, Bardeesy et al. 2003). Further efforts to tease apart the different roles of p16^{INK4A} and P14^{ARF} contributions to PDAC found that *KC* mice with germ line loss of the p16^{INK4A} *Cdkn2a* product that retained wild-type P19^{ARF}, developed ductal adenocarcinoma with sarcomatoid differentiation. These tumors developed with an average latency of 18.3 weeks and 33% of these tumors were metastatic (Bardeesy, Aguirre et al. 2006). By comparison, *KC* mice developed tumors of similar histology but the latency was 57 weeks and 67% of animals developed metastatic disease. These studies also showed that mice lacking a single allele of *Cdkn2a* produced tumors with adenocarcinoma and sarcomatoid histology in which the remaining copy of *Cdkn2a* is lost, reducing survival of these mice to 34.2 weeks (Bardeesy, Aguirre et al. 2006). Additionally, tumor lysates and cell lines from tumors arising in tumor suppressor wild-type *KC* mice lacked expression of both p16^{INK4A} and P19^{ARF} (Bardeesy, Aguirre et al. 2006). These findings suggested that KRAS-activation was not

sufficient for PDAC formation and that additional loss of tumor suppressors was required.

Conditional loss of *Trp53* in *KC* mouse-models accelerated disease progression to 6.2 weeks (Bardeesy, Aguirre et al. 2006). Loss of a single *Trp53* allele resulted in adenocarcinoma at a mean age of 21.8 weeks, and these tumors were found to have inactivated the second copy of *Trp53*, mirroring the loss of wild-type alleles seen in the *KC-Cdkn2a* model (Bardeesy, Aguirre et al. 2006). A second study using the *Trp53*^{R172H} allele, which has been shown to inactivate wild-type TRP53 as well as display oncogenic gain of functions (de Vries, Flores et al. 2002), also showed acceleration of PDAC with mice succumbing to the disease around 5 months of age (Hingorani, Wang et al. 2005). Although mice with bi-allelic deletion of *Trp53* rapidly developed malignant disease, they did not develop metastatic disease and showed few sites of micro-metastasis (Bardeesy, Aguirre et al. 2006). Mice with single copy loss of *Trp53* had a metastasis rate of 33%, and 74% of *KC* mice with germ line *Trp53*^{R172H} mutations presented with metastatic disease upon necropsy, suggesting that the increased latency of these models may afford the opportunity for metastatic disease (Hingorani, Wang et al. 2005, Bardeesy, Aguirre et al. 2006).

Tumors arising in pancreata with heterozygous loss of the *Cdkn2a* p16^{INK4A} transcript and *Trp53* frequently developed complete genetic loss of *Trp53*, and decreased or lost their expression of p16^{INK4A}, suggesting that both the p16^{INK4A} and TP53 pathways play important roles in the development of

KRAS-driven PDAC (Bardeesy, Aguirre et al. 2006). In *KC* mice with homozygous deletion of *Trp53*, the subsequent heterozygous or homozygous loss of p16^{INK4A} coding *Cdkn2a*, altered the dominant histology from adenocarcinoma, defined as neoplastic ductal epithelial cells in a dense stromal deposition, to an anaplastic histology, defined by ductal differentiation with detached tumor cells of varying sizes, however the latency of tumor formation was not altered (Bardeesy, Aguirre et al. 2006). This suggests a limitation of the *KC-Trp53^{f/f}* model in that further acceleration of tumor growth has proven difficult.

Pancreas specific *Smad4* knockout in *KC* mice also accelerated PDAC formation (Bardeesy, Cheng et al. 2006, Izeradjene, Combs et al. 2007, Kojima, Vickers et al. 2007). As with other tumor suppressor deficient backgrounds, these mice developed rapidly-progressing PanIN lesions (Bardeesy, Cheng et al. 2006, Izeradjene, Combs et al. 2007, Kojima, Vickers et al. 2007). They also formed IPMN lesions as identified by positive staining for MUC1, MUC4, and MUC5AC indicating that these lesions are similar to “gastric” type human IPMNs (Bardeesy, Cheng et al. 2006, Kojima, Vickers et al. 2007) and MCN lesions identified by expanded epithelial cysts surrounded by ovarian-like stroma (Izeradjene, Combs et al. 2007). Despite accelerated tumor formation, the invariable loss of either TRP53 or p16^{INK4A} expression in tumors derived from a *KC-Smad4* deficient background suggests that SMAD4 loss is not a sufficient second hit for KRAS-driven tumorigenesis (Izeradjene, Combs et al. 2007).

Models with conditional deletion of *Smad4* and *Cdkn2a* support this notion by further accelerating KRAS-driven tumor development (Bardeesy, Cheng et al. 2006). Further mouse studies in which TGF- β receptor 2, an upstream component of the TGF- β signaling pathway, was knocked out conditionally in *KC* mice, showed dramatic acceleration of PDAC. The majority of these mice succumbed to the disease between 7 and 10 weeks and mice that lived beyond 23 weeks developed metastatic lesions in the liver (Ijichi, Chytil et al. 2006). These findings not only support an important role for TGF- β signaling, but also fit a more general inverse correlation between the aggressiveness of a primary tumor and the development of metastatic disease in mouse-models. This highlights a limitation of current mouse-models, that the burden of an aggressive primary tumor is often lethal to a mouse, precluding the development of metastatic disease so commonly seen in human PDAC.

Activation of KRAS in the *KC* models occurs during embryogenesis, but in humans, the average age of PDAC onset is 71 years old, and it is presumed to arise from somatic mutations occurring in adult cells (Whitcomb, 2015). To address this discrepancy, mouse-models with *Elas-tTA/tetO-Cre* (Guerra, Schuhmacher et al. 2007) have been combined with a conditional LSL-KRAS^{G12V} strain allowing doxycycline inducible acinar (and centroacinar) specific expression of activated KRAS from the endogenous locus. While these mice develop PanINs when KRAS is activated during embryogenesis, delayed KRAS-activation decreases the efficiency of PanIN formation and progression, a finding

confirmed by a Pdx1-Cre^{ERT} inducible model (Guerra, Schuhmacher et al. 2007, Gidekel Friedlander, Chu et al. 2009). Delaying KRAS-activation until postnatal day 60 completely prevented the formation of PanIN lesions in the model described by Guerra et al., while KRAS-activation at postnatal day 56 dramatically reduced the number of PanIN lesions and prevented their progression beyond PanIN-1 in the model used by Gidekel et al. (Guerra, Schuhmacher et al. 2007, Gidekel Friedlander, Chu et al. 2009). Chronic pancreatitis induced by the cholecystokinin analogue caerulein was sufficient to induce ADM, PanIN lesions and PDAC in adult mice with activated KRAS (Guerra, Schuhmacher et al. 2007).

Induction of pancreatitis in with the cholecystokinin analogue caerulein also induces rapid and widespread PanIN formation in *KC* models with embryonic activation of KRAS (Lerch and Gorelick 2013). In the context of a KRAS wild-type pancreas, acute pancreatitis results in acinar cell dedifferentiation followed by recovery of the acinar tissue; however, in the context of activated KRAS caerulein induced acute pancreatitis causes ADM and PanIN lesions following KRAS activation in adult mice (Lerch and Gorelick 2013).

Models of pancreatitis induced by caerulein, show independent activation of trypsinogen and the inflammatory NF- κ B pathway, and demonstrate that both contribute to necrotic cell death and induction of acute pancreatitis (Han, Ji et al. 2001, Dawra, Sah et al. 2011). Similar models using adult onset KRAS-activation found that the inflammatory NF- κ B pathway played an important role in KRAS-

driven ADM and PanIN formation, and maintenance of elevated KRAS activity (Daniluk, Liu et al. 2012). The importance of trans-signaling between myeloid cells and PDAC cells through the IL6-signal transducer and activator of transcription 3 (STAT3) pathway was determined in the conditional *KC* model and further supports a vital role for inflammation in PanIN progression and PDAC development (Lesina, Kurkowski et al. 2011). Furthermore, work by Bielas *et al.* shows mutation rates are increased roughly 4 fold in inflammatory microenvironments relative to normal tissue (Bielas, Loeb et al. 2006). Mutational analysis of *TP53* in human samples of chronic pancreatitis identified an increase in polymorphisms, both silent and deleterious, providing additional evidence that chronic pancreatitis can promote genetic instability (Gansauge, Schmid et al. 1998). Therefore, chronic pancreatitis not only provides pro-tumor signals, but it increases the rate of mutation within pancreatic cells, increasing the risk for the acquisition of oncogenic mutations.

The requirement for sustained KRAS signaling in PDAC was a long-standing assumption that has now been confirmed *in vivo*. By using a *Kras*^{G12D} transgene under a *Tet-O*-promoter, and *Ptf1a* driven CRE mediated activation of a reverse tetracycline trans-activator, Collins et al. developed a mouse in which pancreas specific expression of KRAS^{G12D} could be turned on by administration of doxycycline and expression of KRAS^{G12D} could be ablated by removal of the drug. Concurrent with KRAS^{G12D} expression pancreatitis was induced with caerulein. They showed that when oncogenic KRAS signal was eliminated 3-

weeks after pancreatitis, PanIN lesions reverted to acinar cells. However, removal of mutant KRAS expression following 5 weeks of its expression induced apoptosis (Collins, Bednar et al. 2012). In addition, mono-allelic and bi-allelic deletion of *Trp53* in tetracycline inducible KRAS models produced PDAC that regressed dramatically upon KRAS ablation (Collins, Bednar et al. 2012, Ying, Kimmelman et al. 2012). These experiments show that KRAS-activation is responsible not only for initiation of neoplastic lesions, but is required for maintenance of both PanINs and PDAC. Subsequent research by Collins et al. showed that KRAS is also required for the maintenance of metastatic lesions (Collins, Brisset et al. 2012).

Mouse-models of PDAC have recently begun to illuminate downstream pathways required for KRAS-driven initiation and progression of PDAC. By activating and knocking out critical components of KRAS signaling pathways we are not only gleaning a better understanding of this disease, but we are identifying pathways of potential therapeutic value in this disease. Two recent studies by Collisson and Eser, while contradictory in some respects, highlight the power of this approach. In 2011 Collisson found that conditional activation of $BRAF^{V600E}$ in 2 week postnatal pancreata resulted in PanIN development, but these lesions did not progress to PDAC. When combined with mutant *Trp53*, $Braf^{V600E}$ mice developed PDAC at about 6 months of age (Collisson, Sadanandam et al. 2011). Eser et al. did not directly address BRAF in their work, however they used a knockout of the related *CRAF* gene and showed its

requirement for non-small cell lung cancer but not PDAC initiation (Eser, Reiff et al. 2013). Treatment with the MEK1/2 inhibitor, PD325901, stabilized disease in a *KRAS* mutant, *Cdkn2a*^{fl/+} orthotopic injection model of PDAC for two weeks (Collisson, Trejo et al. 2012). These studies also investigated the role of the PI3K pathway. Eser showed that activating mutations in the p110 α subunit of PI3K were sufficient to induce acinar-to-ductal metaplasia, PanIN lesions and PDAC in a wild-type *KRAS* background (Eser, Reiff et al. 2013). Deletion of the p110 α subunit of PI3K, but not the p110 β subunit, was subsequently shown to block *KRAS*-driven PanIN formation even in the context of caerulein-induced pancreatitis (Baer, Cintas et al. 2014). The prominence of this pathway in PDAC was further supported by data showing that PDK1 is required for *KRAS*-driven PDAC in mice (Eser, Reiff et al. 2013). Conflicting results published by Collisson *et al.* showed the same p110 alpha mutation to be insufficient for PanIN or PDAC formation (Collisson, Trejo et al. 2012). This discrepancy may be explained by Collisson's use of a tamoxifen inducible PDX1-Cre^{ER} system and activation PI3K at postnatal day 14 while Eser used a Ptf1a-Cre system in which Cre was activated in pancreatic precursor cells at embryonic day 9.5. Temporal differences in CRE activation as well as changes in the cell types expressing CRE may account for this difference. Collinsson *et al.* did not address the requirement of p110 alpha in *KRAS*-driven PDAC (Collisson, Trejo et al. 2012).

The use of small molecule inhibitors of PI3K and MEK inhibitors in mouse-models of PDAC has provided further insight into the therapeutic potential of

targeting these pathways. Treatment with the pan class I PI3K inhibitor GDC-0941 stabilized disease in *KC-Trp53^{R172H/fi}* mice during the two weeks of treatment, a finding that was not maintained in non-small cell lung cancer (Eser, Reiff et al. 2013). These findings were supported by data from an additional study, which showed a mild increase in survival of *KC-Trp53^{fi/+}* mice treated with the BMK120 PI3K inhibitor (Alagesan, Contino et al. 2015). This study found that dual inhibition of PI3K and MEK significantly delayed the onset of PDAC relative to gemcitabine treatment alone (Alagesan, Contino et al. 2015). A similar set of experiments were done in *KC-Cdkn2a^{fi/fi}* mice, and although dual inhibition of PI3K and MEK significantly increased survival relative to vehicle treated animals, the survival benefit was not significant when the dual inhibition was combined with gemcitabine and compared with a gemcitabine treated control arm (Junttila, Devasthali et al. 2015). The difference in responses observed in these studies suggests that the tumor suppressor background of individual tumors may dictate their responses to targeted therapies.

The role of the EGFR in the development of PDAC has recently gained attention. As previously mentioned, conditional mouse-models of KRAS-driven PDAC lacking EGFR or ADAM17, a sheddase which activates EGFR ligands, failed to form tumors or PanIN lesions, while a similar model of NSCLC and intestinal carcinomas were unaffected by the status of EGFR (Ardito, Gruner et al. 2012, Navas, Hernandez-Porras et al. 2012). *Trp53* deletion allowed the development of EGFR-deficient KRAS-driven PanINs and PDAC, while loss of

Cdkn2a did not (Navas, Hernandez-Porras et al. 2012). These data provide further support for a differential role of these tumor suppressors in the regulation of oncogenic signaling in PDAC. These findings are also consistent with studies showing over expression of EGFR ligands can cause *in vivo* and *in vitro* ADM and acceleration of KRAS-driven PDAC by the EGFR ligand TGF α (Wagner, Greten et al. 2001, Means, Meszoely et al. 2005, Siveke, Einwachter et al. 2007).

A Role for mTORC2 in the Development of PDAC

Given the importance of the PI3K and AKT signaling pathways in various cancers the mTOR inhibitor rapamycin was tested as a targeted therapy and tumors with PTEN loss were found to have an increased sensitivity to the drug (Morran, Wu et al. 2014). While rapamycin treatment showed promising results in PDAC model systems with enhanced PI3K-AKT activity (Sun, Rosenberg et al. 2005, Morran, Wu et al. 2014), phase II human trials of two rapamycin analogues failed to provide clinical benefit to PDAC patients and were terminated early (Javle, Shroff et al. 2010). One potential explanation for this outcome is a loss of negative feedback loops associated with the mTORC1 targets S6K and growth factor receptor bound 10 (GRB10) (Yu, Yoon et al. 2011, Soares, Ni et al. 2013). Upon mTORC1 down-regulation, S6K mediated IRS1 inhibition is removed resulting in amplification of Pi3K-AKT signaling (Sun, Rosenberg et al. 2005, O'Reilly, Rojo et al. 2006). Inhibition of mTORC1 impairs phosphorylation of

GRB10, destabilizes this protein, and subsequently increases PI3K and ERK signaling (Yu, Yoon et al. 2011). Loss of the inhibitory phosphorylation of the essential mTORC2 component mSIN by either S6K or GRB10 can further amplify PI3K-AKT signaling upon mTORC1 inhibition (Liu, Gan et al. 2013).

A recent mouse model of EGFR driven glioblastoma placed mTORC2 activation downstream of the tyrosine kinase receptor and found that its activation was PTEN-dependent (Tanaka, Babic et al. 2011). Prior to this, mTORC2 signaling was demonstrated to have an essential role in the formation of PTEN mutant murine prostate cancers (Guertin, Stevens et al. 2009). Both the EGF and IGF1 receptors play important roles in PDAC, and are upstream of mTORC2 signaling, suggesting that mTORC2 may play a role in the pathology of this disease. Both tumor types in which mTORC2 has been shown to play an essential role have enhanced PI3K signaling, a pathway that is also essential for PDAC (Liu, Gan et al. 2015). To date, a role for mTORC2 in KRAS-driven tumors has not been identified. This body of work uses multiple *in vivo* and *in vitro* models to identify and characterize an essential role for mTORC2 signaling in the initiation, progression, and maintenance of KRAS-driven PDAC.

Although the PI3K-AKT-mTOR signaling pathway is altered in roughly 1/3 of all human cancers (Shaw and Cantley 2006, Akinleye, Avvaru et al. 2013), the importance of mTORC2 signaling in oncogenesis is only now coming to light. With the advent of whole genome sequencing and copy number variant analysis,

recent studies have identified amplifications of mTORC2 subunits. When combined, amplifications in *RICTOR*, *SIN1*, *DEPTOR*, and *mTOR* genes were observed in 28% of human pancreatic cancers (Witkiewicz, McMillan et al. 2015). Furthermore, these genes were amplified in 38% of human prostate adenocarcinomas (Kumar, Coleman et al. 2016), 23% of invasive lobular breast carcinomas (Ciriello, Gatz et al. 2015), 14% of lung adenocarcinomas (Cancer Genome Atlas Research 2014), 23% of esophageal carcinomas, and 39% of ovarian serous cystadenocarcinoma (These results are based upon data generated by the TCGA Research Network: <http://cancergenome.nih.gov/>). The frequent amplification of mTORC2 subunits in multiple human tumor types suggests that mTORC2 signaling plays an important role in oncogenic PI3K-AKT signaling. Although the mTORC1 specific component Raptor is amplified in 8% of cases it is also deleted in 4% of human PDACs. Furthermore the mTORC1 inhibitor TSC2 is amplified in 14% of cases (Witkiewicz, McMillan et al. 2015), suggesting that reduction of mTORC1 activity may, in some contexts benefit these tumors. This is consistent with the failure of Rapamycin analogues to improve survival in clinical trials of PDAC.

Recently, a keen interest in developing both isoform specific and pan-PI3K inhibitors as therapeutics agents has led to the production and clinical testing of many such compounds. The clinical benefit of this class of drugs has been less than ideal, and although many compounds have been shown to effectively inhibit PI3K signaling, the few responses recorded have been limited to temporary

stabilization of disease rather than regression (Khan, Yap et al. 2013). Clinical attempts to combine various PI3K inhibitors with other kinases inhibitors, such as AKT, MEK and mTOR inhibitors, are ongoing. Although currently available mTOR kinase inhibitors target both mTORC1 and 2, no mTORC2 specific inhibitors currently exist. My work demonstrates an important role for mTORC2 signaling in the initiation of progression of PDACs, and supports the possibility that specific inhibition of mTORC2 may be a viable therapeutic strategy by which to modulate the PI3K-AKT-mTOR signaling pathway.

Chapter II:
**mTORC2 signaling is required for pancreatic cancer development and
progression**

Preface

This chapter is a manuscript currently under review for publication. It represents a collaborative effort by a number of individuals whose contributions are listed below. With the following exceptions I have produced and analyzed the data contained within Figures 1 through 9 and Figure 11: Makoto Sano performed the histopathological analyses presented in Figures 2B, 2D and 8B. Jennifer P Morton performed the immunohistochemical staining for pS6 presented in the bottom row of Figure 2.3. Appendix A contains additional data not included in this manuscript and directly related to the role of mTORC2 in KRAS-driven pancreatic ductal adenocarcinoma. Appendix A also contains an expanded materials and methods section for the benefit individuals wishing to expand on my research.

The following authors contributed to this manuscript:

David R Driscoll, Saadia A Karim, Makoto Sano, David M Gay, Wright Jacob, Yusuke Mizukami, Aarthi Gopinathan, Duncan I Jodrell, TR Jeffry Evans, Nabeel Bardeesy, Michael N Hall, Brian J. Quattrochi, David S. Klimstra, Simon T Barry, Owen J Sansom, Brian C Lewis, and Jennifer P Morton.

Abstract

Pancreatic cancer is a devastating disease with limited therapeutic options. Therefore, new strategies are urgently required. mTOR signaling controls several critical cellular functions and is deregulated in many cancers including pancreatic cancer. To date, most effort has focused on inhibiting the mTORC1 complex, however clinical trials of mTORC1 inhibitors in pancreatic cancer have failed, raising questions about this therapeutic approach. Recent publications have confirmed that PI3K and PDK1 are essential for PDAC initiation, but the role of mTORC2 signaling, which converges with PDK1 signaling, and is required for full activation of multiple downstream kinases, has not been studied. We now use a genetic approach to delete the obligate mTORC2 subunit *Rictor* and identify the critical times during tumorigenesis where mTORC2 signaling is important. We find that *Rictor* deletion results in profoundly delayed tumorigenesis. Whereas we previously showed that most pancreatic tumors were insensitive to rapamycin, we demonstrate that treatment with a dual mTORC1/2 inhibitor strongly suppresses tumorigenesis, while in late-stage tumor-bearing mice, combined mTORC1/2 and PI3K inhibition significantly increases survival. Thus, targeting mTOR may be a potential therapeutic strategy in pancreatic cancer.

Statement of Significance

Novel therapeutic strategies are urgently required in pancreatic cancer. We now identify a key role for mTORC2 signaling in pancreatic cancer and demonstrate a novel potential therapeutic strategy for use in this disease.

Introduction

Pancreatic Ductal Adenocarcinoma (PDAC) accounts for 85% of all cases of pancreatic cancer and is a highly aggressive form of the disease (Ryan, Hong et al. 2014). Indeed, PDAC is predicted to become the second leading cause of cancer death in the US by 2030 (Rahib, Smith et al. 2014). At diagnosis, the disease is characterized by invasion into adjacent anatomic structures and distant metastases, features that render > 80% of patients ineligible for tumor resection (Ryan, Hong et al. 2014). Even the few patients eligible for potentially curative resection inevitably develop recurrent or metastatic disease (Ryan, Hong et al. 2014). Indeed recent work has demonstrated that cells in PDAC precursor lesions migrate and seed distant sites prior to the presence of overt carcinoma (Rhim, Oberstein et al. 2014). Moreover, most systemic therapies are largely ineffective, and as a result, PDAC is almost universally lethal, with a 5-year survival rate of ~7% (Siegel, Miller et al. 2015). Up until the recent adoption of combination chemotherapy regimens - FOLFIRINOX (5-FU, Leucovorin, Irinotecan and Oxaliplatin) and gemcitabine combined with albumin-bound paclitaxel - gemcitabine monotherapy had been the standard of care for PDAC patients since 1997 (Burris, Moore et al. 1997, Conroy, Desseigne et al. 2011, Goldstein, El-Maraghi et al. 2015). Yet, median survival with FOLFIRINOX or gemcitabine combined with albumin-bound paclitaxel is 11 months and 9 months

respectively. Thus, PDAC presents a significant therapeutic challenge and novel, effective therapies are required.

The genetic drivers of pancreatic cancer development and progression have been well studied. Activating mutations in *KRAS* occur in around 90% of cases and are thought to be the initiating event driving the formation of precursor lesions termed pancreatic intraepithelial neoplasia (PanINs) (Almoguera, Shibata et al. 1988). Accumulation of further mutations in a number of tumor suppressor genes, including *CDKN2A*, *TP53*, *DPC4* and *BRCA2* occurs throughout tumor development and progression (Hruban, Wilentz et al. 2000). Moreover, recent wide-scale genomic studies have revealed that, in fact, 100s of genetic aberrations exist in this pathology (Biankin, Waddell et al. 2012). In addition, pancreatic tumors are histologically complex, and interactions between the tumor cells and the microenvironment also contribute to tumor progression. Indeed, the extensive desmoplastic stroma that characterizes PDAC - consisting of pancreatic stellate cells, fibroblasts and immune cells and a dense collagenous matrix - may also protect tumor cells from a number of stresses, including chemotherapy, although this is somewhat controversial (Olive, Jacobetz et al. 2009, Lee, Perera et al. 2014, Ozdemir, Pentcheva-Hoang et al. 2014, Rhim, Oberstein et al. 2014). Therefore, a greater understanding of the signals that drive tumor progression is required to develop more effective drug therapies.

Activated KRAS drives a complex signaling network, involving multiple downstream effectors, including the MEK/ERK, PI3K/AKT and RAL pathways (Baines, Xu et al. 2011). The PI3K pathway regulates a number of processes integral to cancer development and progression including cell growth, cell cycle entry, cell survival and metabolism (Cully, You et al. 2006). Indeed, PI3K signaling has been shown to be a key mediator of the effects of oncogenic KRAS in the pancreas (Eser, Reiff et al. 2013). PI3K inhibitors have been developed for clinical trials, however, the pathway interacts with many other signals and there are several feedback loops making it difficult to predict the effects of suppressing the pathway (O'Reilly, Rojo et al. 2006, Rodon, Dienstmann et al. 2013).

One of the major downstream effectors of PI3K signaling is mTOR, a serine/threonine kinase that acts as part of two distinct complexes, mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) (Huang and Fingar 2014). The mTORC1 complex, of which Raptor is an essential subunit, is responsible for several well-studied functions of mTOR, including sensing nutrient stress and regulating translation through interaction with p70S6K and 4E-BP1 (Huang and Fingar 2014). p70S6K can also phosphorylate IRS1 on an inhibitory serine residue, thus causing a negative feedback loop that inhibits insulin and IGF signaling to PI3K and AKT (Zhang, Gao et al. 2008). In addition, inhibition of mTORC1 can lead to feedback activation of MEK/ERK signaling (Carracedo, Ma et al. 2008).

The mTORC2 complex, which includes the essential subunit RICTOR, regulates cell growth, metabolism and survival, as well as organization of the actin cytoskeleton, through activation of AKT (at Ser⁴⁷³), SGK and PKC (Guertin, Stevens et al. 2006, Garcia-Martinez and Alessi 2008). We previously showed that high levels of phospho-AKT^{S473} in human PDAC were significantly associated with poor survival (Kennedy, Morton et al. 2011). More recently, *RICTOR* amplifications have been described in human lung cancers and melanomas (Cheng, Zou et al. 2015, Laugier, Finet-Benyair et al. 2015). However, since the tumor promoting properties of mTORC2 signaling have only recently been identified (Guertin, Stevens et al. 2009, Nardella, Carracedo et al. 2009, Roulin, Cerantola et al. 2010, Gupta, Hau et al. 2013), a full understanding of the mechanisms underlying this effect is still developing.

Initial strategies to inhibit mTOR function focused on mTORC1 and the development of analogs of the potent mTORC1 inhibitor rapamycin (Nelson, Altman et al. 2013). However, clinical trials using these compounds have proved disappointing, with the exception of a few rare responders (Klumpen, Queiroz et al. 2011). More recently, small molecule inhibitors that block mTOR kinase activity, and therefore impair both mTORC1 and mTORC2 have been developed. Not only do these inhibitors target both arms of mTOR signaling, they also target 4E-BP1 phosphorylation, and thus translational initiation, more efficiently than

rapalogs, which primarily inhibit S6 kinase (Guertin and Sabatini 2009). We recently showed in a PDAC genetically engineered mouse-model (GEMM) that mice bearing pancreas-specific mutations in *Kras* and *Pten*, a negative regulator of PI3K-mTOR signaling, were exquisitely sensitive to rapamycin whereas mice wild-type for *Pten*, but expressing mutant *Trp53*, were resistant (Morran, Wu et al. 2014). These findings suggest that mTORC2 signaling may play a critical role downstream of commonly occurring PDAC gene mutations and that inhibition of this complex may enhance therapeutic efficacy in PDAC.

Global deletion of *Rictor* or *Raptor* in the mouse embryo results in embryonic lethality (Guertin, Stevens et al. 2006, Shiota, Woo et al. 2006), however, conditional models of *Rictor* and *Raptor* deletion are now available, and these allow interrogation of tissue-specific roles of mTORC1 and 2 (Shiota, Woo et al. 2006, Bentzinger, Romanino et al. 2008). We have now utilized pancreas-specific deletion of *Rictor* to discern the requirement for mTORC2 signaling during pancreatic tumorigenesis. We show that genetic inactivation of mTORC2 significantly blocks precursor PanIN development, impairs progression to invasive carcinoma, and prolongs survival in a PDAC GEMM. Moreover, pharmacologic inhibition of mTOR function extends survival, even in mice with late stage disease. Our findings demonstrate an important role for mTORC2 signaling during pancreatic tumorigenesis and support the potential use of mTOR inhibitors as part of therapeutic strategies in PDAC.

Results

Loss of Rictor does not impair pancreas differentiation and function

To determine the impact of mTORC2 inactivation on pancreas development, we generated mice lacking the essential mTORC2 subunit Rictor, specifically within the pancreas, by crossing mice bearing *Ptf1a-cre* and *Rictor^{fllox}* alleles (Kawaguchi, Cooper et al. 2002, Shiota, Woo et al. 2006) (Figure 2.1A). The absence of Rictor protein was confirmed by immunoblotting of protein lysates isolated from 2-month old pancreata (Figure 2.1B). Mice lacking pancreatic *Rictor* were viable and showed no difference in body weight relative to their wild-type littermates (Figure 2.1C). However, *Rictor*-null pancreata weighed significantly less than *Rictor* intact pancreata (Figure 2.1D). Measurement of cell size (Figure 2.1E) and quantification of the total amount of genomic DNA as a surrogate measurement for cell number (Figure 2.1F), demonstrated that the reduced pancreas size likely results from decreased cell numbers. Importantly, *Rictor* deletion did not impair pancreas differentiation as evidenced by histologic appearance as well as the relative abundance of acinar, α - and β -cells as determined by immunostaining for the markers amylase, glucagon and insulin, respectively (Figure 2.2). In combination with the normal weight gain displayed by mice with *Rictor* deleted pancreata, these data demonstrate that mTORC2 activity is not required for pancreas differentiation and function.

Figure 2.1: Pancreatic Loss of *Rictor* does not impair fitness

(A) Schematic showing the pancreas specific deletion of *Rictor*. (B) Western blot showing Rictor levels in pancreas tissue lysate from 2-month old mice. α -tubulin is shown as a loading control. (C) Total mouse weight of 2-month old males of the indicated genotypes. (D) Weight of pancreata isolated from 2-month old males of the indicated genotypes. (E) Average cell area in pancreatic cross section of 2-month old males. (F) Total genomic DNA per pancreas, as an estimation of relative cell number, in 2-month old males (n=4 mice per cohort for C-F). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by student's t-test.

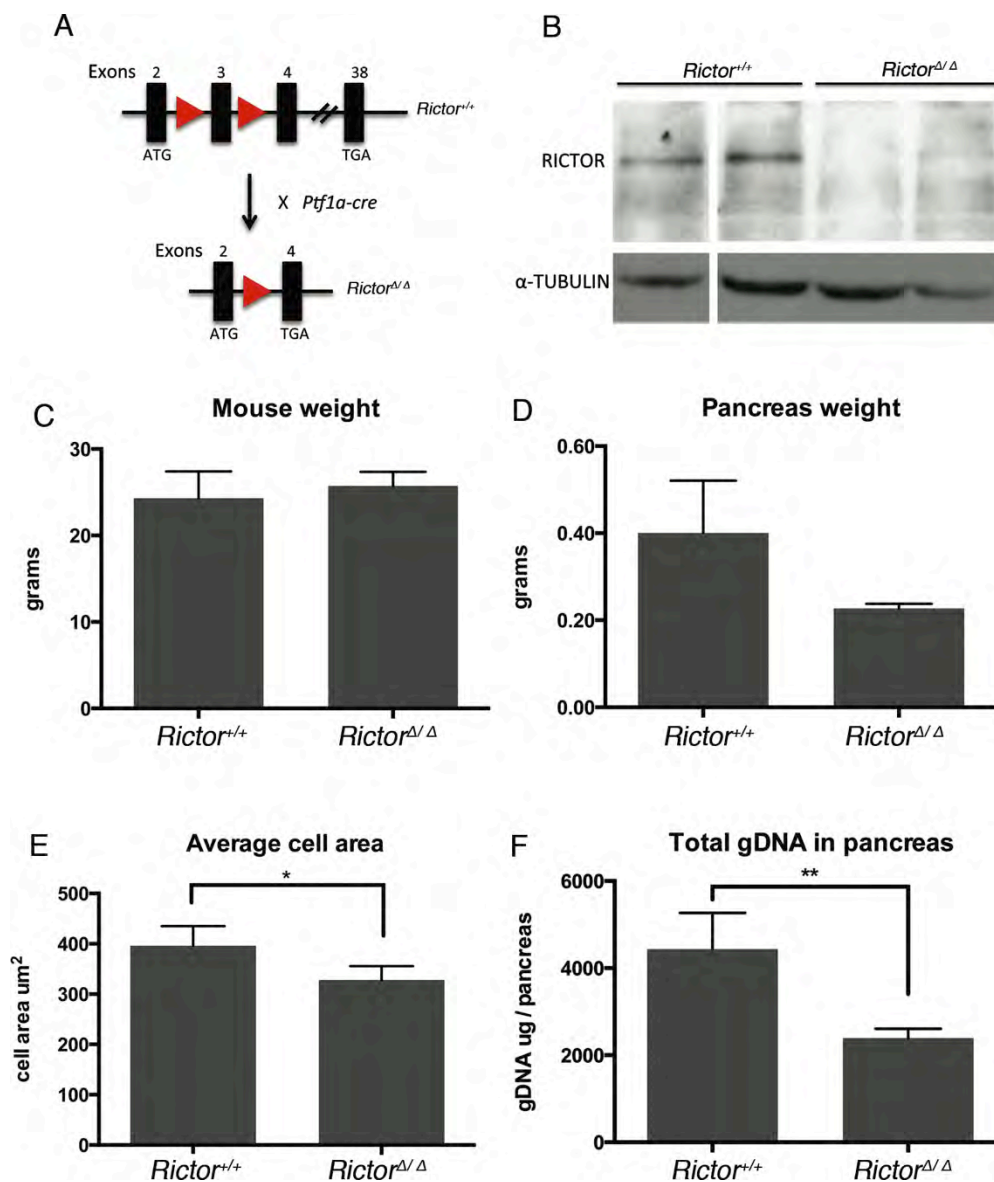
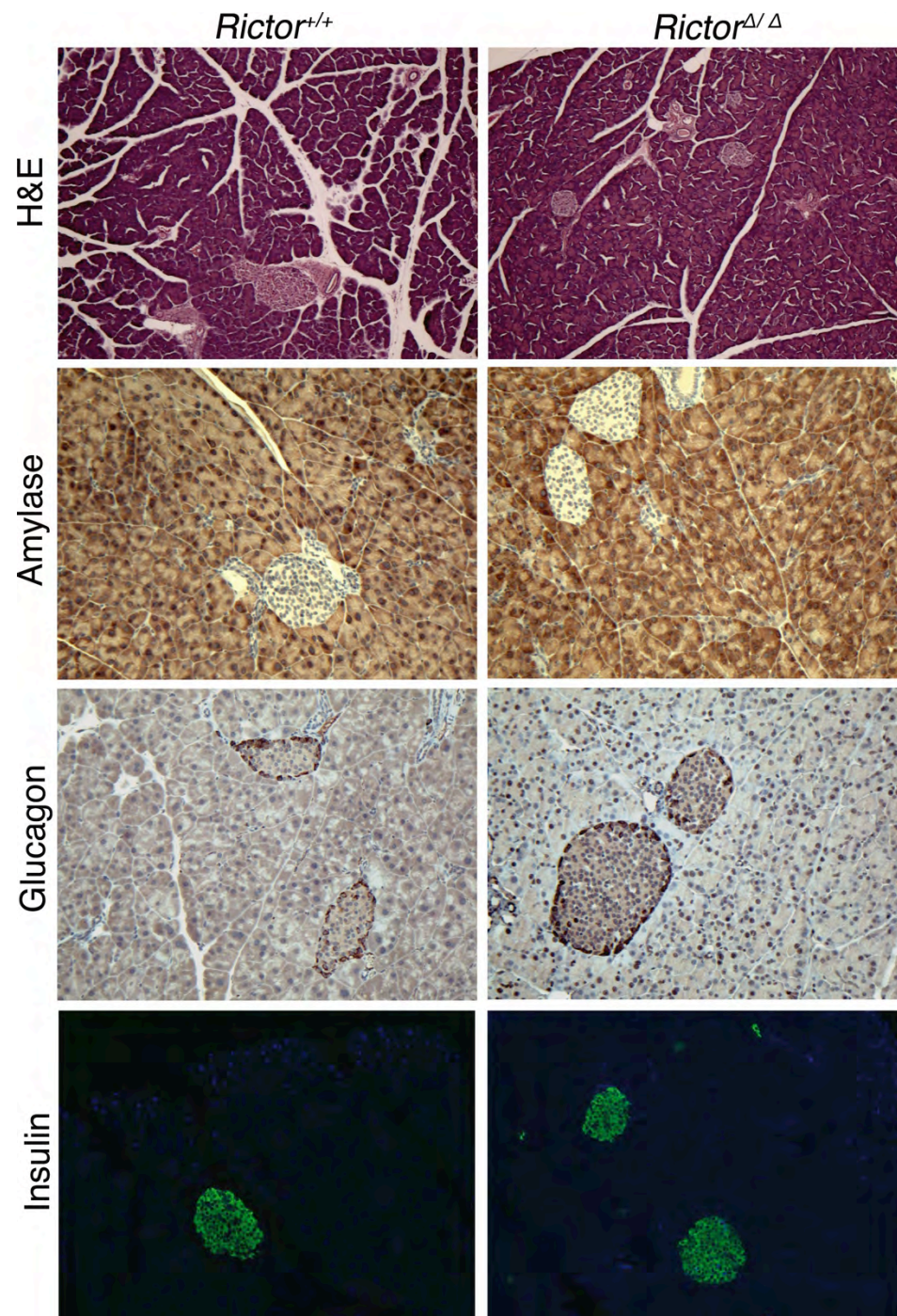


Figure 2.2: Loss of *Rictor* does not prevent formation or function of the pancreas

H&E staining (top row), IHC for amylase (2nd row) and glucagon (3rd row), and IF for insulin (bottom row) in 2-month old males.



mTORC2 deficiency impairs development and progression of KRAS^{G12D} driven PanIN lesions

Both 3-phosphoinositide-dependent protein kinase 1 (PDK1) and mTORC2 are required for full activation of AKT and SGK family members. PDK1 is essential for the development of KRAS-driven PanIN lesions and PDAC (Eser, Reiff et al. 2013). We therefore hypothesized that mTORC2 is similarly required for KRAS-driven tumorigenesis. The well-established *Ptf1a*^{Cre/+}; *LSL-Kras*^{G12D/+} mouse-model develops PanIN lesions that mirror the human lesions and can progress to PDAC after a long latency (Hingorani, Petricoin et al. 2003). To investigate the role of mTORC2 signaling in this model, *Ptf1a*^{Cre/+}; *LSL-KRAS*^{G12D/+}; *Rictor*^{+/+} (KC) and *Ptf1a*^{Cre/+}; *LSL-KRAS*^{G12D/+}; *Rictor*^{fl/fl} (*KC-Rictor*^{Δ/Δ}) siblings were produced and their pancreata analyzed at 4 or 8-months of age. Consistent with published studies, significant replacement of acinar cells by PanIN lesions containing abundant reactive stroma was evident in KC mice at 4-months of age (Figure 2.3A, B). In contrast, *KC-Rictor*^{Δ/Δ} pancreata displayed fewer PanIN lesions and significant retention of acinar tissue (Figure 2.3A, B). Moreover, while *KC* pancreata displayed PanIN1B and PanIN2 lesions, precursors lesions observed in *KC-Rictor*^{Δ/Δ} pancreata were restricted to PanIN1A. Quadchrome staining, which allows the simultaneous visualization of apical mucins produced by PanIN cells and the collagen deposited by the reactive stromal cells, highlighted the differences between pancreata of the two genotypes (Figure 2.3A). *KC-Rictor*^{Δ/+} pancreata observed by H+E staining at 4

and 8-months of age were phenotypically similar to *KC* organs of the same age (Data not shown).

Acinar replacement in *KC* mice was nearly complete by 8-months and most PanIN lesions had progressed to PanIN2 (Figure 2.4A, B). This KRAS-driven phenotype is significantly impaired in *KC-Rictor^{Δ/Δ}* pancreata, with retention of acinar tissue, fewer PanIN lesions and reduced reactive stroma (Figure 2.4A, B). Moreover, the PanIN lesions present failed to progress beyond PanIN1 (Figure 2.4B). Together, these data indicate that *Rictor* deletion impairs the development of KRAS-driven PanIN lesions and blocks their progression to higher-grade lesions.

Figure 2.3: Loss of *Rictor* impairs the development of *Kras*^{G12D} driven PanIN lesions

Histological evaluation of PanIN lesions in pancreata from 4-month old *KC* and *KC-Rictor*^{Δ/Δ} mice. (A) Representative images from hematoxylin and eosin (H&E) stained tissues (25x magnification, top panels; 100x magnification, middle panels), and quadchrome stained tissues (simultaneous Alcian Blue and Sirius Red staining; 50x magnification, bottom panels) are shown. The quadchrome stain marks collagen red, mucin blue, cytoplasm yellow-brown, and nucleic acids black. (B) Quantification of normal and neoplastic cell types as a percentage of total tissue area in 4-month old *KC* and *KC-Rictor*^{Δ/Δ} pancreata. p values as determined by student's t test: *p<0.05, **p<0.01, ***p < 0.001.

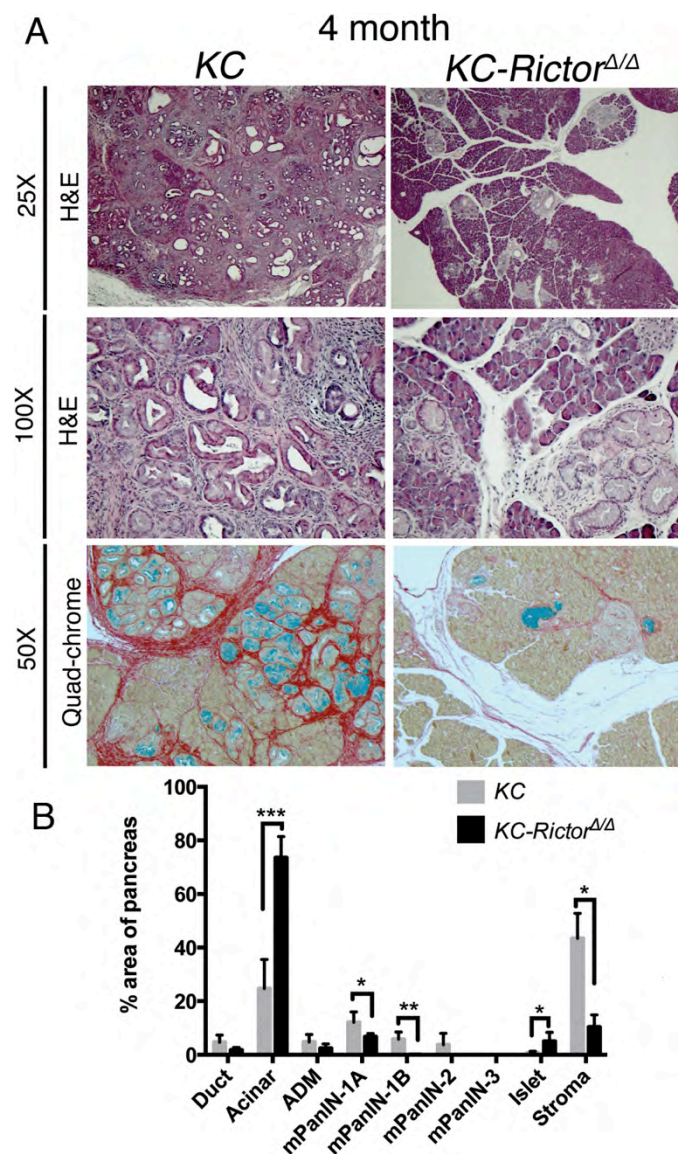
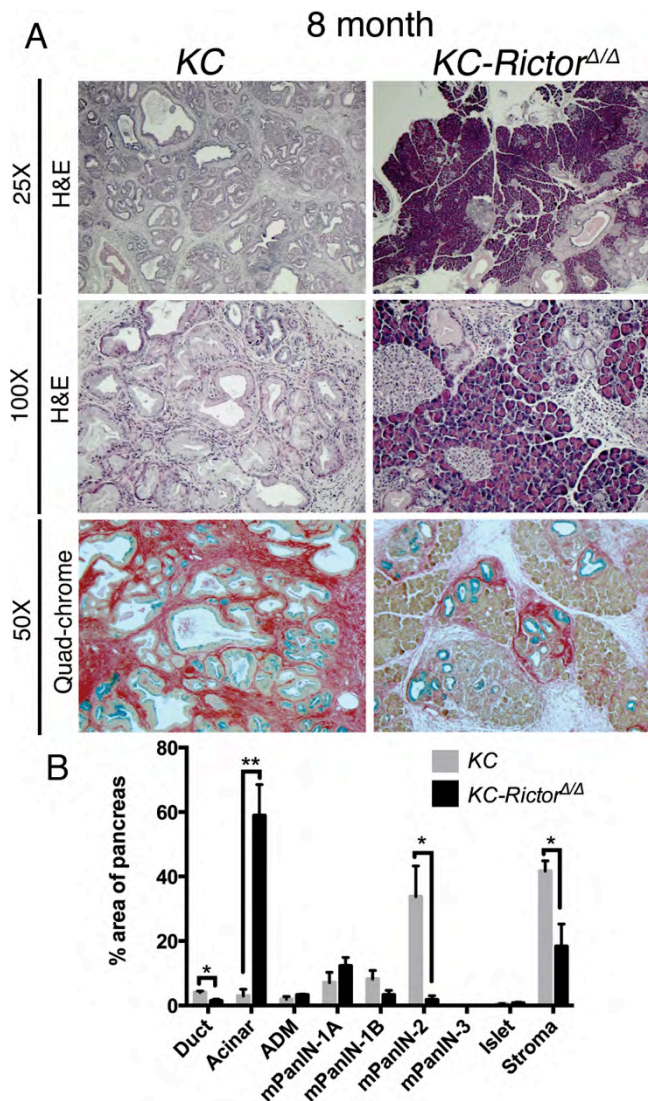


Figure 2.4: Loss of *Rictor* impairs the progression of *Kras*^{G12D} driven PanIN lesions

Histological evaluation of PanIN lesions in pancreata from 8-month old *KC* and *KC-Rictor*^{Δ/Δ} mice. (A) Representative images from hematoxylin and eosin (H&E) stained tissues (25x magnification, top panels; 100x magnification, middle panels), and quadchrome stained tissues (simultaneous Alcian Blue and Sirius Red staining; 50x magnification, bottom panels) are shown. The quadchrome stain marks collagen red, mucin blue, cytoplasm yellow-brown, and nucleic acids black. (B) Quantification of normal and neoplastic cell types as a percentage of total tissue area in 8-month old *KC* and *KC-Rictor*^{Δ/Δ} pancreata. p values as determined by student's t test: *p<0.05, **p<0.01.

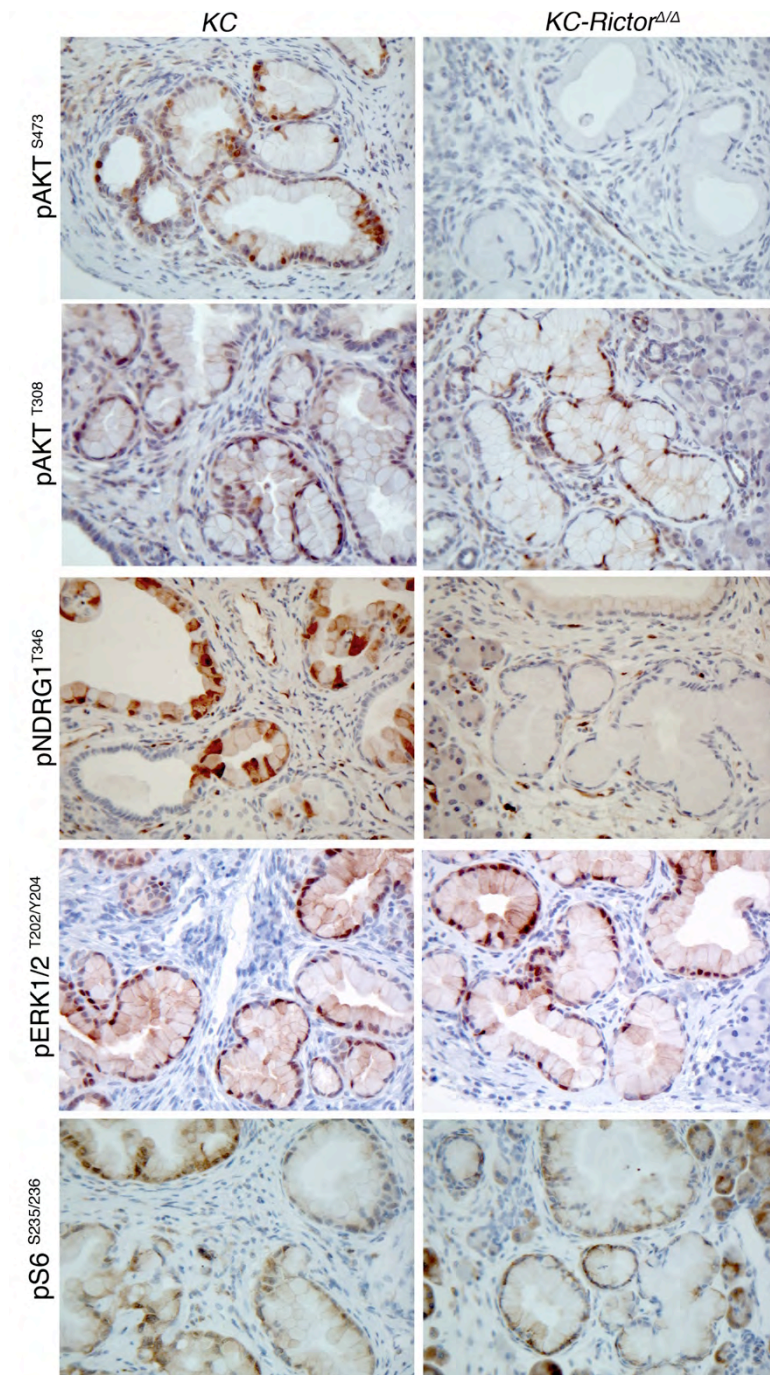


Rictor-null PanIN lesions display impaired mTORC2 signaling

To determine the status of mTORC2 signaling in PanIN lesions, pancreata from 8-month old mice were stained for mTORC2 targets. Relative to PanINs present in *KC* mice, *KC-Rictor^{Δ/Δ}* PanIN lesions displayed significantly reduced mTORC2 signaling, as measured by phospho-AKT^{S473} and phospho-NDRG1^{T346} (Figure 2.5). The reduced activity was specific to mTORC2 as PDK1-dependent phosphorylation of AKT on Thr308 was unaffected, as was phosphorylation of the mTORC1 downstream target phospho-S6^{S235/236}, and the MAP kinase signaling components ERK1/2 (Figure 2.5). Thus, the PanIN lesions present in *KC-Rictor^{Δ/Δ}* pancreata have not escaped *Rictor* gene deletion and develop in the absence of active mTORC2 signaling.

Figure 2.5: mTORC2, but not mTORC1, is functionally absent in *KC-Rictor^{Δ/Δ}* PanIN lesions

Immunostaining for the phosphorylated forms of AKT, NDRG1, ERK and S6 at the indicated epitopes, in pancreata harvested from 8-month old *KC* and *KC-Rictor^{Δ/Δ}* mice (200x magnification).



Loss of mTORC2 signaling induces proliferative arrest

The lack of compensatory activation in the PDK1, mTORC1, and MEK signaling pathways and the limited progression of *KC-Rictor*^{Δ/Δ} PanIN lesions indicated fundamental phenotypic differences between *KC* and *KC-Rictor*^{Δ/Δ} PanIN lesions. We therefore compared the proliferation and apoptosis rates in PanIN lesions from the two genotypes. Since comparison of different grade PanINs might confound the findings, analysis was restricted to PanIN1 lesions. We observed that the percentage of Ki67-positive nuclei in *KC-Rictor*^{Δ/Δ} PanIN1 lesions was significantly lower than PanIN1 lesions in *KC* mice at both 4 and 8-months (Figure 2.6A, B). Apoptosis rates, as assessed by cleaved caspase 3 staining, were very low in lesions found in both genotypes and not different between the groups (Figure 2.6C, D). Together, these data suggest that mTORC2 inactivation impairs cell cycle progression in early PanIN lesions.

The cyclin dependent kinase (CDK) inhibitor p16^{INK4A} has been proposed as a barrier to KRAS-driven PDAC development (Aguirre, Bardeesy et al. 2003). We therefore assessed p16^{INK4A} expression in *KC* and *KC-Rictor*^{Δ/Δ} PanIN lesions by immunostaining. We found that the number of cells with nuclear localized p16^{INK4A} was significantly increased in *KC-Rictor*^{Δ/Δ} PanIN lesions, relative to *KC* PanINs, at both the 4 and 8-month time points (Figure 2.7A, Figure 2.8A). We have shown that the levels of the CDK inhibitor p21^{CIP1} correlate with survival following tumor resection and that heterozygous *Cdkn1a* deletion accelerates

PanIN development and progression to PDAC (Morton, Jamieson et al. 2010). We therefore evaluated expression of p21^{CIP1} and another CDK inhibitor p27^{KIP1} in PanIN lesions observed in *KC* and *KC-Rictor*^{Δ/Δ} mice. The number of p21^{CIP1} and p27^{KIP1}-positive nuclei was similar between groups at the 4-month time point (Figure 2.7B, C; Figure 2.8B, C). However, we observed that while the number of positive nuclei decreased in PanIN lesions in 8-month old *KC* mice, they remained elevated in *KC-Rictor*^{Δ/Δ} PanIN lesions (Figure 2.7B, C; Figure 2.8B, C). Thus, *KC-Rictor*^{Δ/Δ} PanIN lesions display coordinate elevation of multiple CDK inhibitors.

The concomitant elevation in p16^{INK4A}, p21^{CIP1}, and p27^{KIP1} levels suggested the possibility that their expression may be regulated by a common factor. The polycomb repressor group complex 1 protein BMI1 is an established negative regulator of *CDKN2A* expression and has been implicated in *CDKN1A* and *CDKN1B* down-regulation (Jacobs, Kieboom et al. 1999, Zheng, Wang et al. 2014). Moreover, BMI1 is required for KRAS-driven PanIN formation and progression (Bednar, Schofield et al. 2015). We therefore evaluated BMI1 expression in *KC* and *KC-Rictor*^{Δ/Δ} PanIN1 lesions by immunostaining. We found that the percentage of cells with nuclear BMI1 staining was decreased in *KC-Rictor*^{Δ/Δ} PanIN lesions relative to *KC* controls at both the 4-month and 8-month time points (Figure 2.7D, Figure 2.8D).

Figure 2.6: *Rictor* deletion decreases proliferation in PanIN1 lesions

Immunostaining for Ki67 in the pancreata of (A) 4-month old mice and (B) 8-month old mice of the indicated genotypes. Quantification is shown in the panels on the right. Immunostaining for cleaved caspase 3 in the pancreata of (C) 4-month old mice and (D) 8-month old mice of the indicated genotypes. Quantification is shown in the panels on the right. N=4 for each group. * $p < 0.05$, ** $p < 0.01$ by student's t-test.

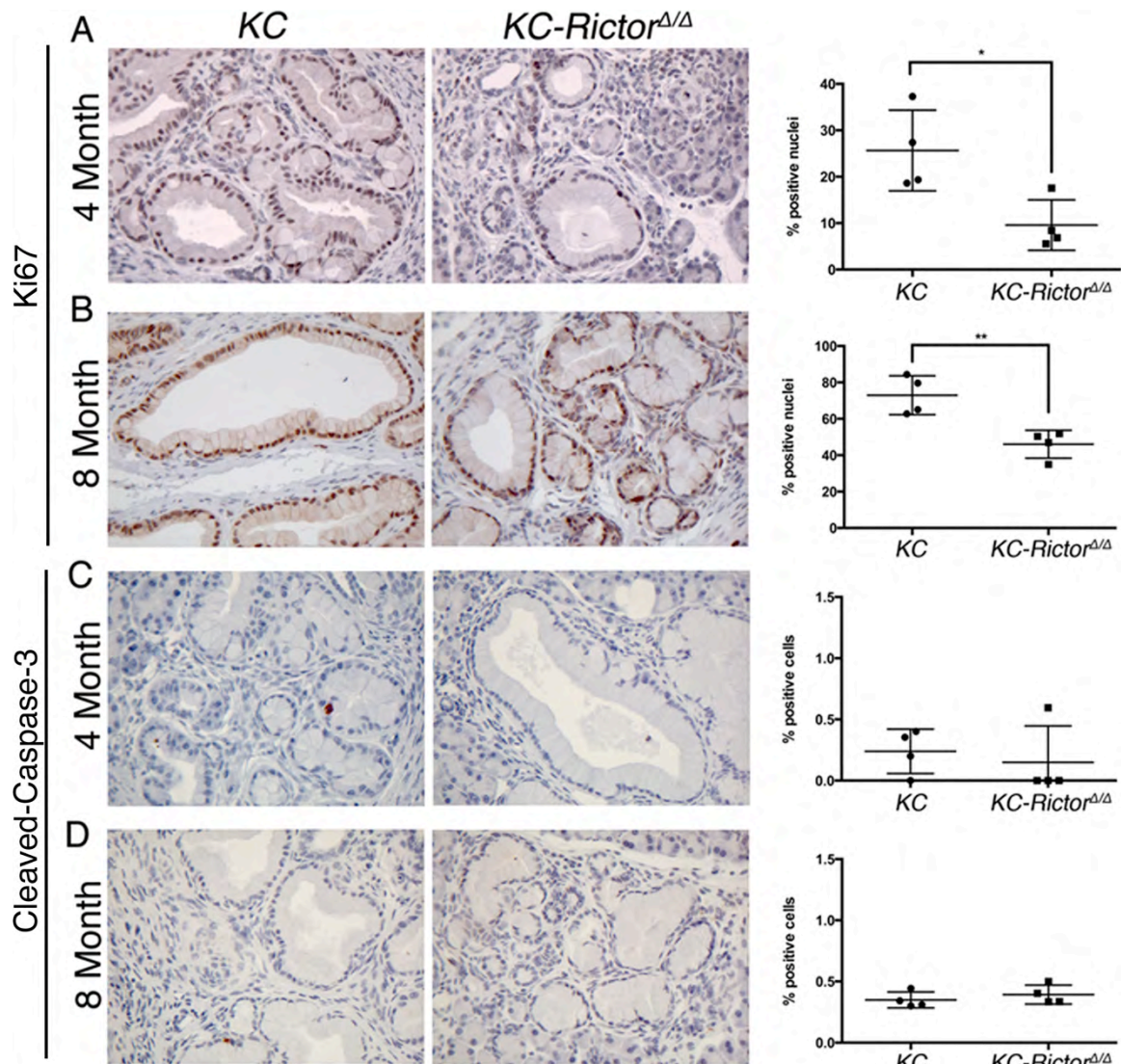


Figure 2.7 *Rictor*-deficient PanIN lesions display increased nuclear expression of cyclin-dependent kinase inhibitors and decreased nuclear expression of BMI1

(A-D) Quantification of positive nuclei in PanIN1 lesions identified in pancreata harvested from 4- (left) and 8- (right) month old *KC* and *KC-Rictor*^{Δ/Δ} mice and stained for (A) p16^{INK4A}, (B) p21^{CIP1}, (C) p27^{KIP1} and (D) BMI1. *p<0.05, **p<0.01, ***p < 0.001 as determined by student's t-test.

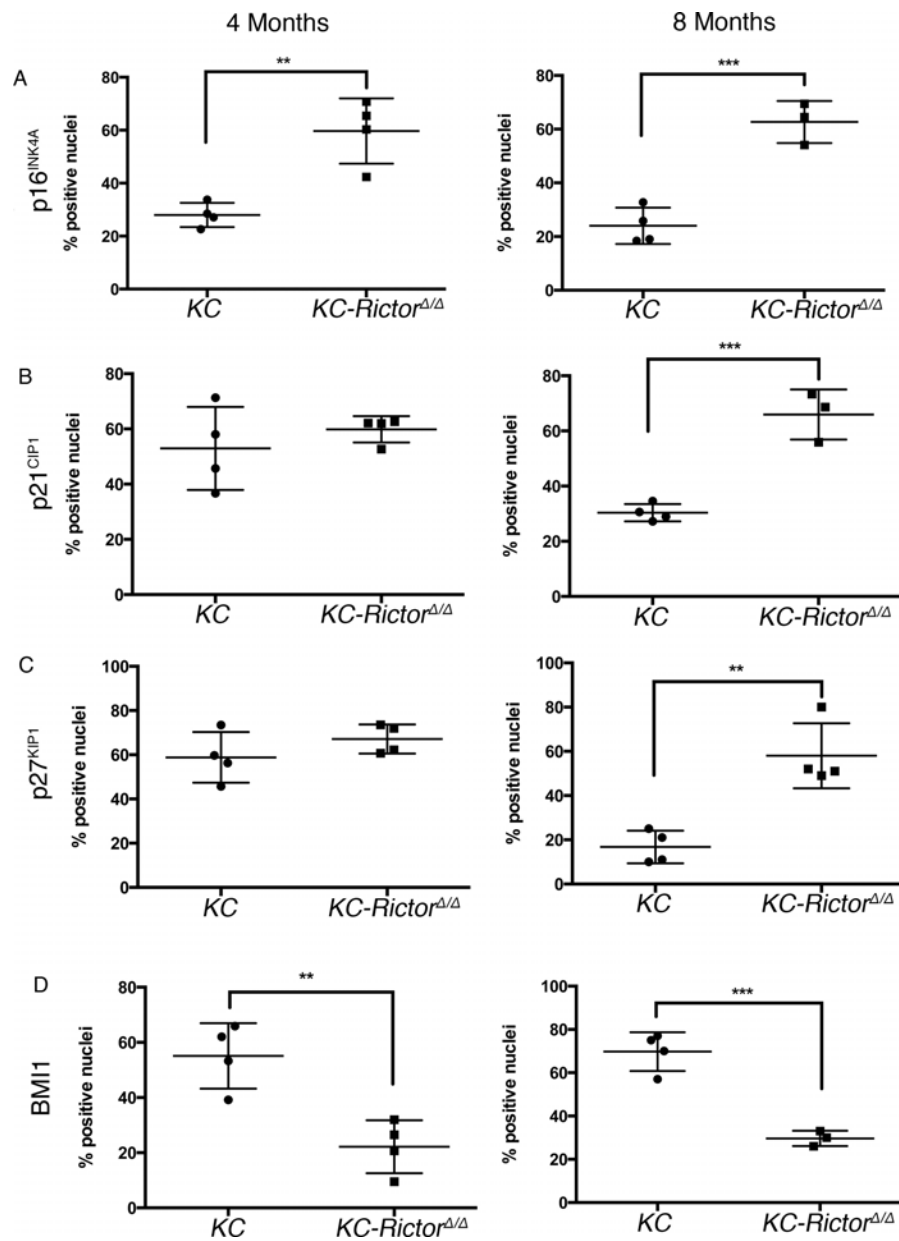
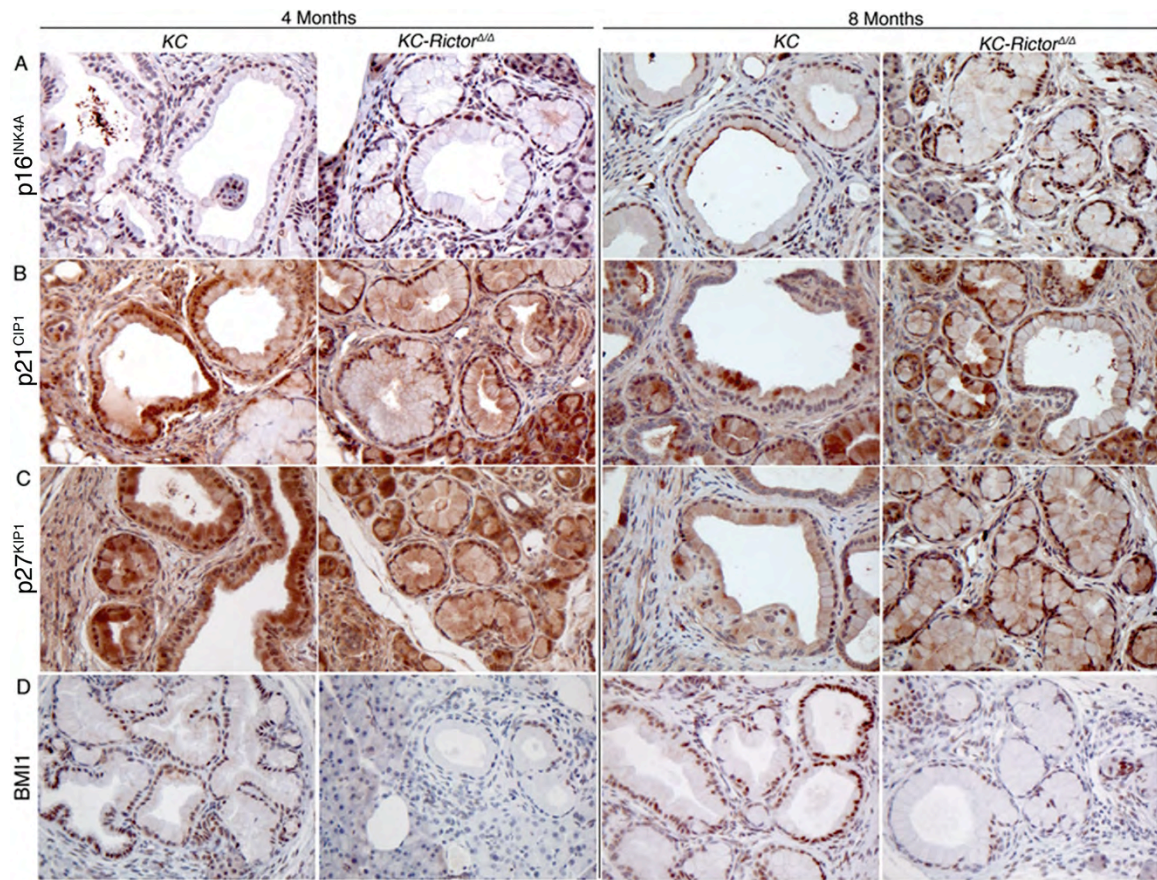


Figure 2.8: *Rictor* deletion alters CDKI and BMI1 expression and localization

(A-D) Immunostaining for the CDK inhibitors p16^{INK4A}, p21^{CIP1} and p27^{KIP1} and BMI1 in the pancreata of 4- and 8-month old *KC* and *KC-Rictor*^{Δ/Δ} mice (200x magnification).



We next sought to model the effect of mTORC2 inhibition in PanIN cells in an *in vitro* system. We performed shRNA-mediated knockdown of Rictor in two primary PanIN cell lines, AH2375 and RP2294 established from *KC* mice with early PanIN lesions (Corcoran, Contino et al. 2011). Rictor knockdown significantly reduced proliferation in both cell lines and enhanced the expression of the senescence-associated beta galactosidase (Figure 2.9A, B; Figure 2.10A). However, in contrast to our observations *in vivo*, we did not observe any changes in the expression of the CDK inhibitors p16^{INK4A}, p21^{CIP1} or p27^{KIP1}, nor did we observe changes in BMI1 expression (Figure 2.10B, C). Nonetheless, our data suggest that Rictor knockdown impairs PanIN development and progression at least in part by robustly blocking cell proliferation.

Figure 2.9 *Rictor*-knockdown impairs proliferation and induces senescence in cultured PanIN cells

(A) Doubling time of a PanIN derived cell line 4-6 days following shRNA-mediated *Rictor* knockdown. (B) Senescence associated β -galactosidase positivity in a PanIN derived cell line 4 days following shRNA-mediated *Rictor* knockdown. P values in (A) determined by counter variance analysis of inverse slopes. P values in (B) determined by student's t-test. ** $p < 0.01$, *** $p < 0.001$.

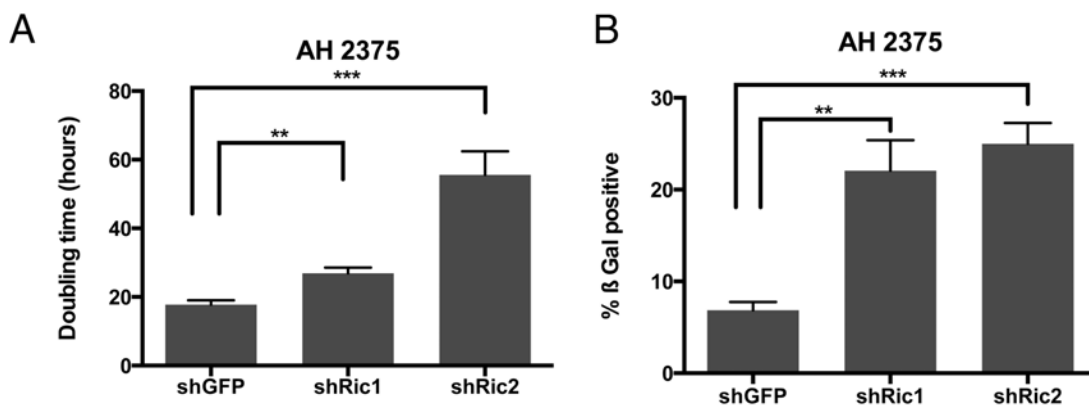
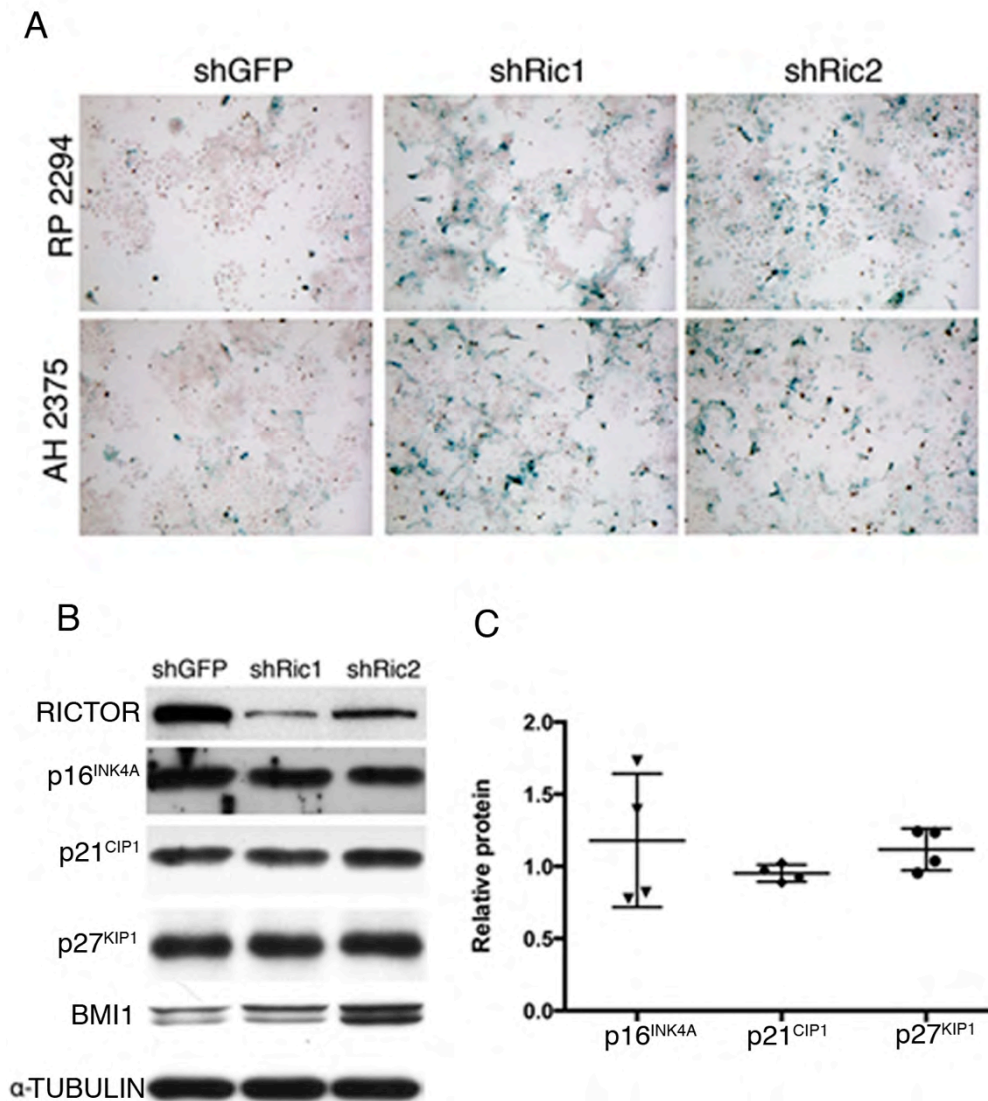


Figure 2.10: *Rictor* knockdown induces senescence but does not alter CDKI or BMI1 levels in cultured PanINs

(A) Staining for senescence associated β -galactosidase activity in the PanIN derived cell lines RP 2294 and AH 2375 4 days following *Rictor* knockdown. (B) Western blot for RICTOR, indicated CDKIs, and BMI1 in PanIN derived cell line AH 2375 6 days post *Rictor* knockdown. (C) Quantification of CDKI levels in western blots for the proteins shown in (B) (n=4 for each antibody).



mTORC2 loss impairs PanIN progression following pancreas injury

Pancreatitis is a major risk factor for the development of PDAC and can be modeled in mice using caerulein-induced pancreatic injury (Lerch and Gorelick 2013). To determine whether *Rictor* deletion impacted the response to caerulein-induced injury we performed serial caerulein injections in 6-week old wild-type and *Rictor*^{Δ/Δ} mice. Wild-type and *Rictor*^{Δ/Δ} mice displayed similar responses with stereotypical histologic changes - including acinar-to-ductal metaplasia (ADM) and increased separation of acinar lobules - evident 2 days following injection (Figure 2.11) (Morris, Cano et al. 2010). As expected, injury was fully resolved by 21 days post-injection (Figure 2.11). The effect of caerulein insult was not investigated in *Rictor* heterozygous animals.

The expression of activated Kras enhanced the response to caerulein injection, with extensive ADM visible 2 days following caerulein treatment (Figure 2.12). However, *Rictor* deletion blunted ADM development (Figure 2.12). Moreover, while *KC* mice evaluated 21 days following caerulein injection displayed complete replacement of the pancreas parenchyma with PanIN lesions, many of which had progressed to PanIN1B and PanIN2, *KC-Rictor*^{Δ/Δ} pancreata displayed significant retention of normal acinar tissue, more areas with ADM and significantly fewer PanIN1B and PanIN2 lesions and associated stroma (Figure 2.12; Figure 2.13).

Evaluation of mTORC2 signaling via immunostaining for p-AKT^{S473} demonstrated that mTORC2 activity was impaired in *KC-Rictor*^{Δ/Δ} lesions as compared to *KC* lesions (Figure 2.14). By contrast, p-AKT^{T308} staining was equivalent between both genotypes (Figure 2.14). Intriguingly, we observed that p-ERK staining was elevated in *KC* lesions relative to *KC-Rictor*^{Δ/Δ} lesions, suggesting that mTORC2 signaling is required for activation of the MEK/ERK signaling cascade following caerulein injury-mediated PanIN progression (Figure 2.14). To ascertain whether similar mechanisms mediate inhibition of PanIN progression in the caerulein injury model, we assessed the presence of nuclear localization of the CDK inhibitors p16^{INK4A}, p21^{CIP1} and p27^{KIP1}. We found that *KC-Rictor*^{Δ/Δ} lesions had elevated levels of these markers (Figure 2.15A-C; Figure 2.16A-C). Moreover, the CDK inhibitor levels inversely correlated with nuclear BMI1 localization (Figure 2.15D; Figure 2.16D). Together, these data suggest that the absence of mTORC2 signaling impairs the progression of PanIN lesions following pancreatic injury.

Figure 2.11: *Rictor* deletion does not affect response to caerulein-induced pancreatic injury

H&E staining of pancreata harvested from *Rictor*^{+/+} or *Rictor*^{Δ/Δ} mice 2 or 21 days after injection with caerulein or PBS as a control. Note acinar-to-ductal metaplasia (ADM) and increased separation of acinar lobules evident 2 days post-injection of caerulein (middle panels) and fully resolved by 21 days post-injection (lower panels). Arrows denote islets and arrowheads denote pancreatic ducts.

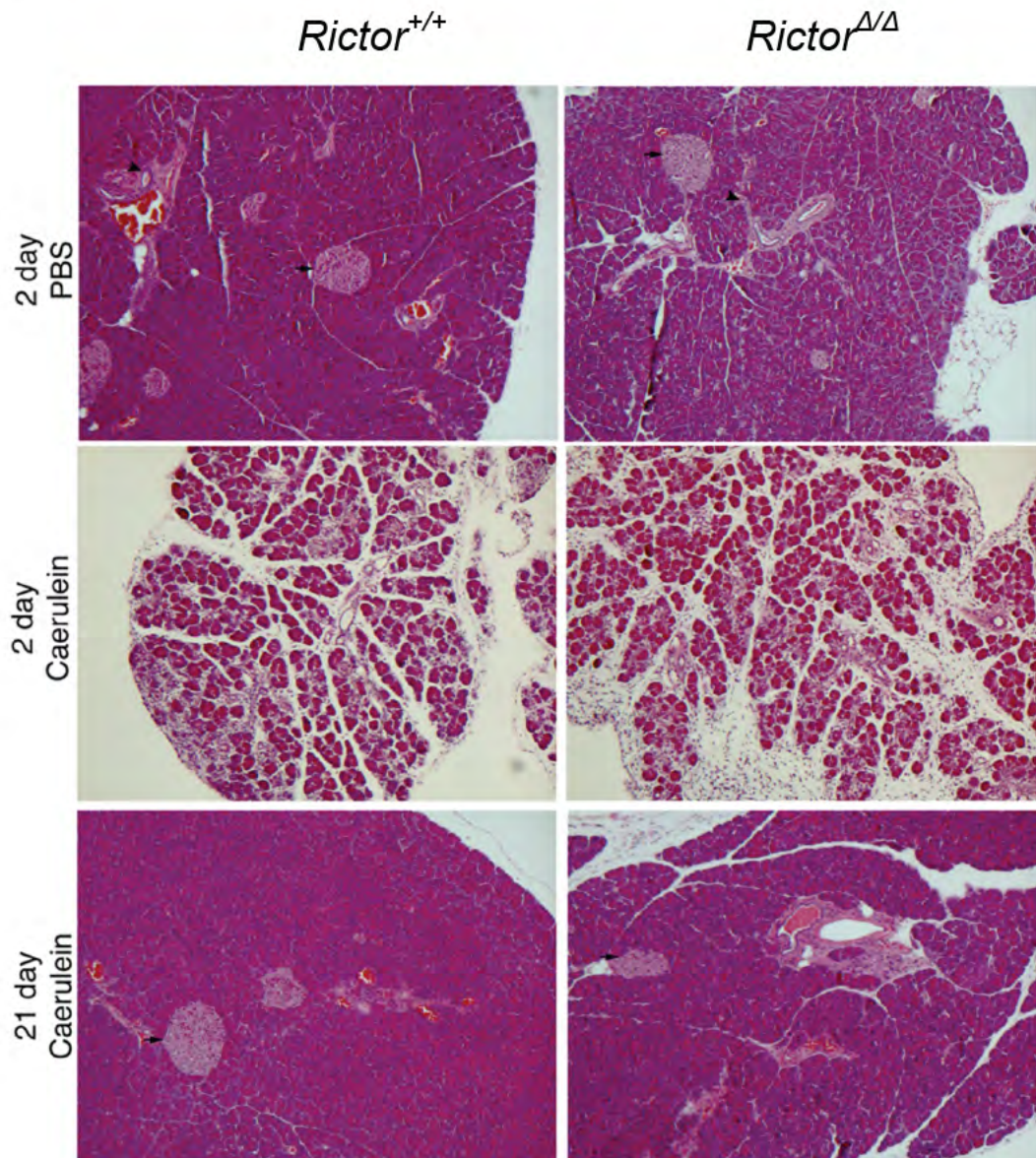


Figure 2.12: *Rictor* deletion impairs the formation of pancreatitis-induced PanIN lesions

H&E and quadchrome staining of pancreata from *KC* and *KC-Rictor^{Δ/Δ}* mice harvested 2 or 21 days following injection of caerulein, or PBS as a control (100x magnification).

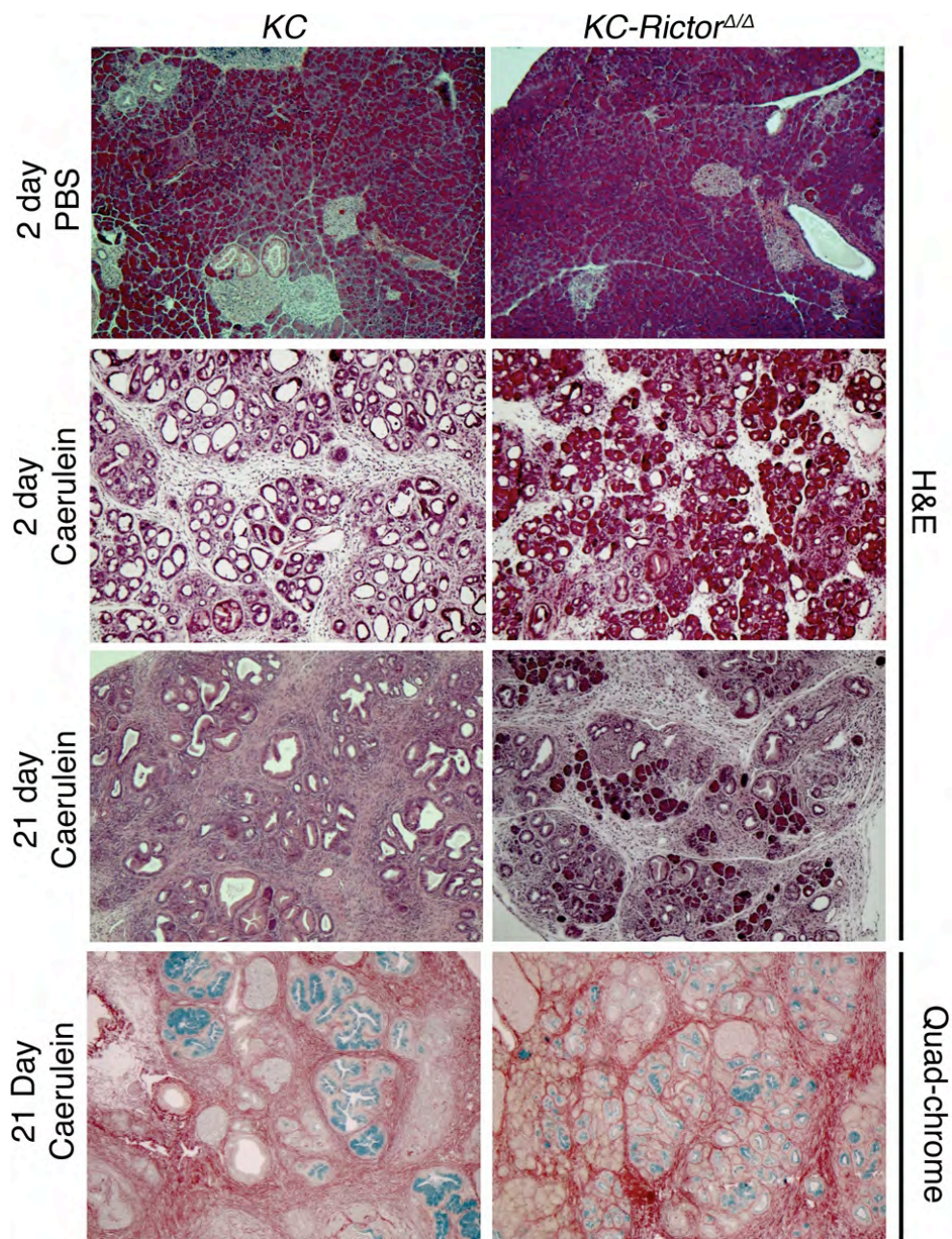


Figure 2.13: *Rictor* deletion impairs the progression of pancreatitis-induced PanIN lesions

Quantification of normal and neoplastic cell types as a percentage of total tissue area in pancreata harvested from *KC* and *KC-Rictor Δ/Δ* mice 21 days post caerulein injection (n=5-6 mice per cohort). p values as determined by student's t-test: *p<0.05, **p<0.01, ***p < 0.001.

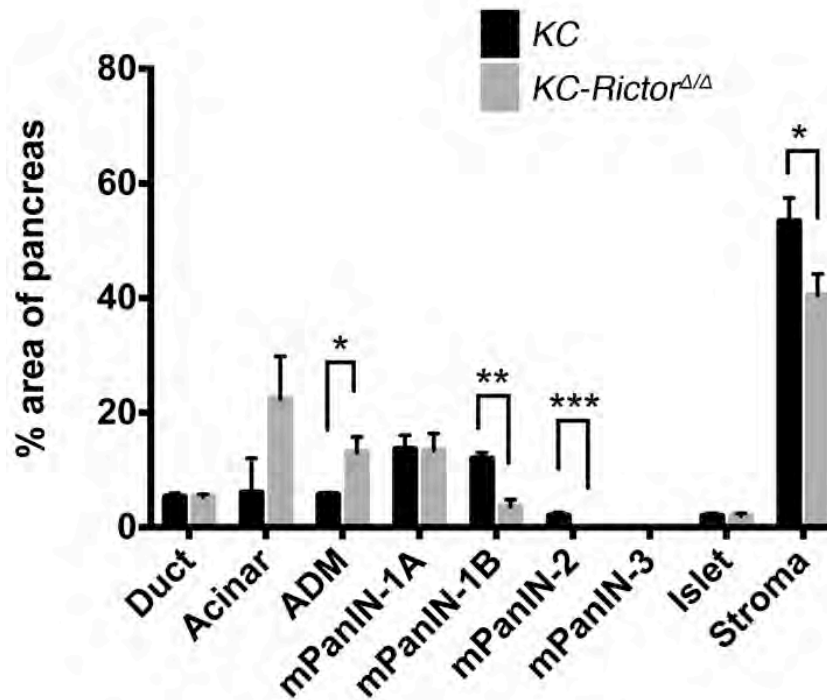


Figure 2.14: mTORC2 is functionally absent in pancreatitis-induced *KC-Rictor*^{Δ/Δ} PanIN lesions

IHC for pAKT^{S473}, pAKT^{T308}, and pERK1/2^{T202/Y204} in pancreata harvested from *KC* and *KC-Rictor*^{Δ/Δ} mice 21 days post caerulein injection (200x magnification).

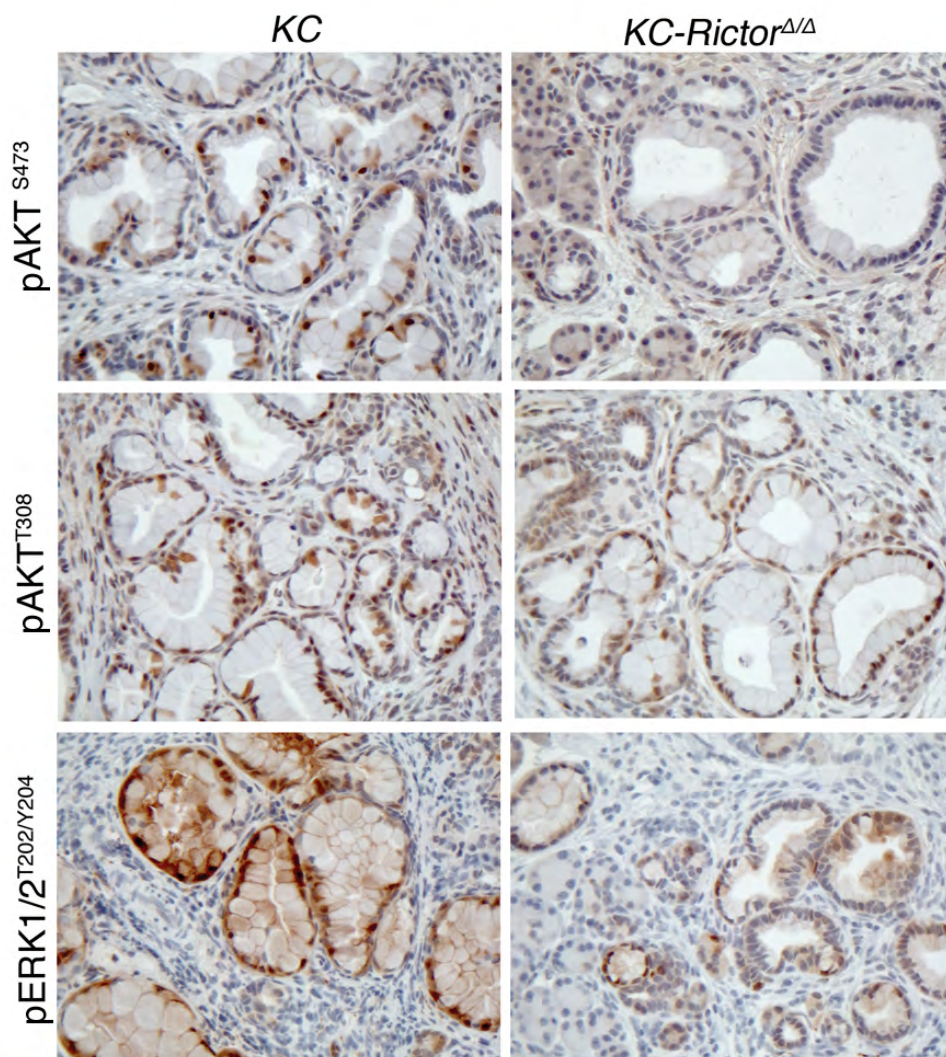


Figure 2.15: *Rictor*-deficiency increases the frequency of nuclear cyclin-dependent kinase inhibitor expression in pancreatitis induced PanIN lesions

Quantification of positive nuclei in PanIN1 lesions identified in pancreata harvested from *KC* and *KC-Rictor Δ/Δ* mice 21 days post caerulein injection. (A) p16^{INK4A}, (B) p21^{Cip}, (C) p27^{KIP1} and (D) BMI1. P values as determined by student's t-test: *p<0.05, **p<0.01, ***p < 0.001.

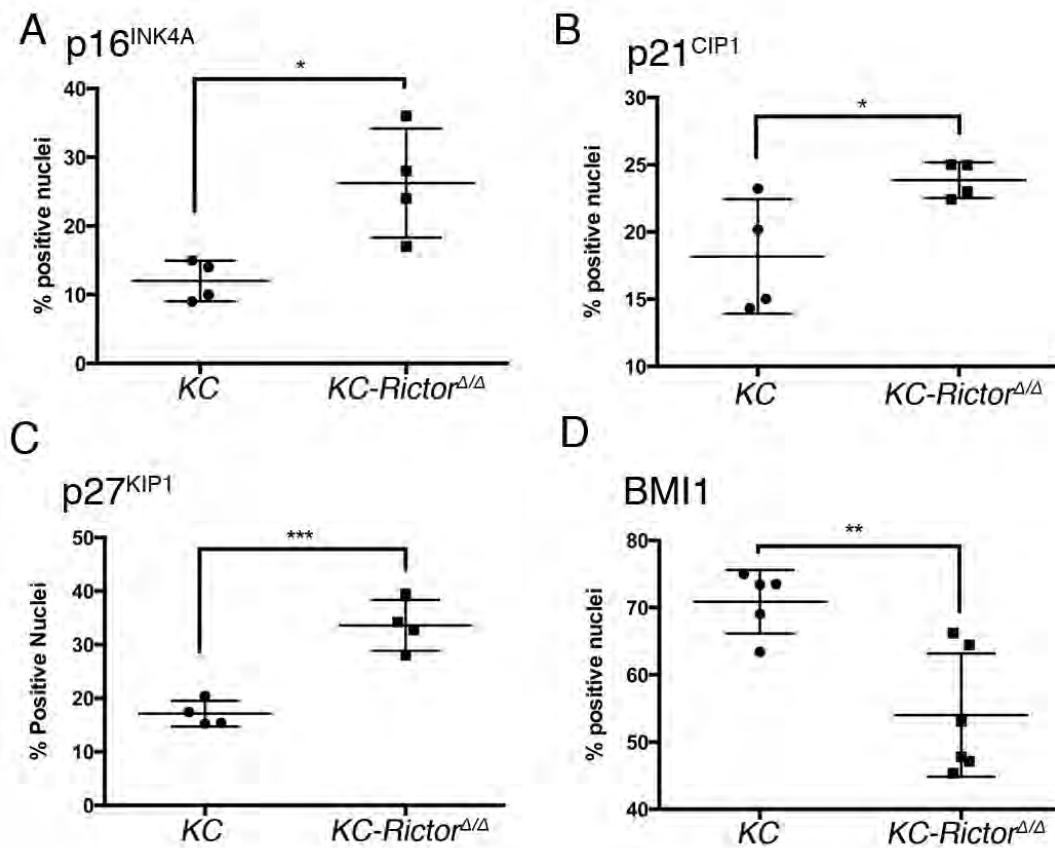
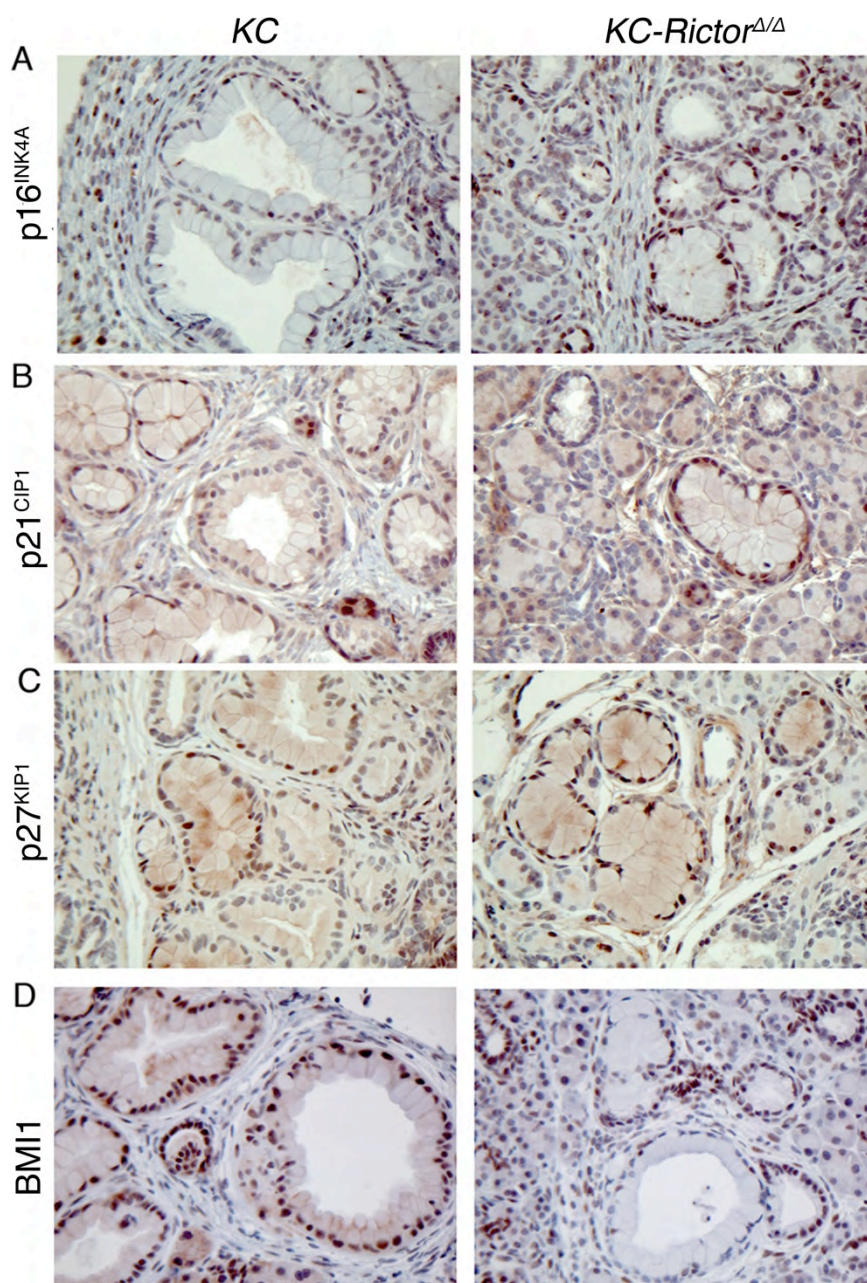


Figure 2.16: Absence of mTORC2 activity increases the nuclear expression of CDKIs in PanIN1 lesions driven by $KRAS^{G12D}$ and caerulein-induced pancreas injury

(A-D) Immunostaining for the CDK inhibitors $p16^{INK4A}$, $p21^{CIP1}$ and $p27^{KIP1}$ and BMI1 in the pancreata of *KC* and *KC-Rictor Δ/Δ* mice harvested 21 days following caerulein injection (200x magnification).



Rictor deletion delays tumor formation in KPC mice

Our data above demonstrated that *Rictor* gene deletion severely impairs PanIN development and progression. To determine whether *Rictor* deletion inhibits the progression from PanIN to PDAC in an aggressive model of pancreatic tumorigenesis, we crossed *Rictor^{fl/fl}* mice with *KPC* (*Pdx1-Cre*, *LSL-Kras^{G12D/+}*, *LSL-Trp53^{R172H/+}*) mice, to generate *KPC-Rictor^{Δ/Δ}* mice. We found that deletion of *Rictor* dramatically delayed tumor formation in the *KPC* model, with median survival almost doubled in *KPC-Rictor^{Δ/Δ}* mice (319 days) compared with *KPC-Rictor^{+/+}* mice (182 days) (Figure 2.17A, B). Further, 8 out of 47 *KPC-Rictor^{Δ/Δ}* mice were found to be tumor-free at over 200 days old and were sacrificed due to other pathologies. Deletion of one copy of *Rictor* was insufficient to affect tumor development, with median survival unchanged between *KPC-Rictor^{+/+}* and *KPC-Rictor^{Δ/+}* mice (Figure 2.17A, B). Interestingly, we did not observe any apparent histological or pathological differences in the tumors that eventually developed in the *KPC-Rictor^{Δ/Δ}* mice compared with those in *KPC-Rictor^{+/+}* mice. Gross metastatic disease was observed in 50% of *KPC-Rictor^{+/+}* animals and 25% of *KPC-Rictor^{Δ/Δ}* animals at the time of death (p-value = 0.1053)(data not shown). Using RNAscope® we confirmed the absence of *Rictor* RNA demonstrating that the tumors were not derived from cells that failed to delete the *Rictor* locus (Wang, Flanagan et al. 2012) (Figure 2.17C). These findings are supported by *in vitro* observations in murine PDAC cell lines in which *Rictor* knockdown impairs cell

proliferation (Figure 2.18A-B, D-E), and reduces anchorage independent growth (Figure 2.18C, F).

We next examined the effects of *Rictor* deletion on mTOR targets by immunohistochemistry. *Rictor* deletion had no effect on phosphorylation of the mTORC1 signaling targets, S6 and 4E-BP1, (Figure 2.17D, left panels) but as expected, completely abrogated phosphorylation of the mTORC2 downstream specific targets pAKT^{S473} and pNDRG1 (Figure 2.17D, right panels). Thus, we conclude that mTORC2 signaling plays a significant role in pancreatic tumorigenesis and that targeting mTORC2 could have therapeutic impact in this disease.

Figure 2.17: Rictor deletion delays tumor formation in a mouse-model of pancreatic cancer

(A) Kaplan-Meier survival analysis showing pancreatic tumor-free survival in *Pdx1-Cre;Kras^{G12D/+};Trp53^{R172H/+}* (KPC) mice, *Pdx1-Cre;Kras^{G12D/+};Trp53^{R172H/+};Rictor^{Δ/+}* (KPC-Rictor^{Δ/+} mice), and *Pdx1-Cre;Kras^{G12D/+};Trp53^{R172H/+};Rictor^{Δ/Δ}* mice (KPC-Rictor^{Δ/Δ}). Censor ticks indicate mice sacrificed due to extra-pancreatic pathologies. (B) Table showing number of mice and median survival per cohort. P values calculated by Log Rank test. (C) RNAscope® detecting Rictor showing absence of Rictor in tumors from *KPC-Rictor^{fl/fl}* mice (right) compared with *KPC* mice (left). (D) IHC for downstream mTOR signaling pathway components in *KPC* and *KPC-Rictor^{Δ/Δ}* tumors as indicated.

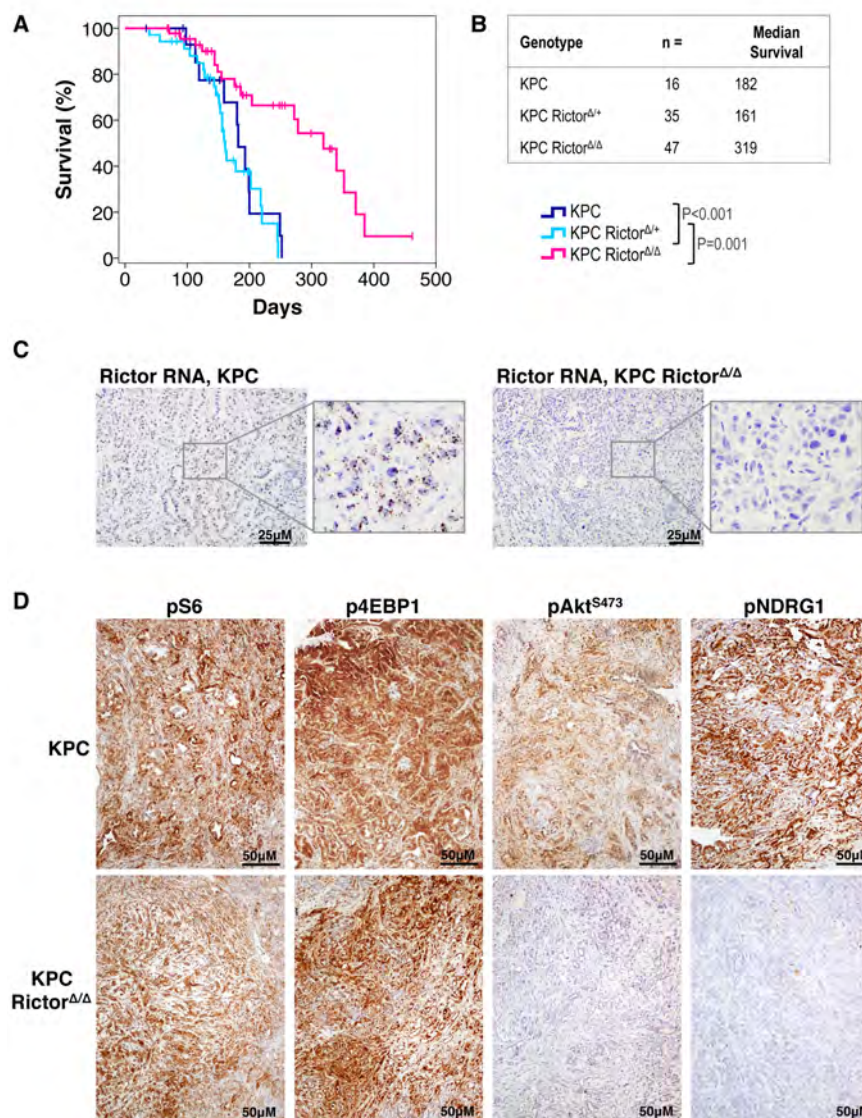
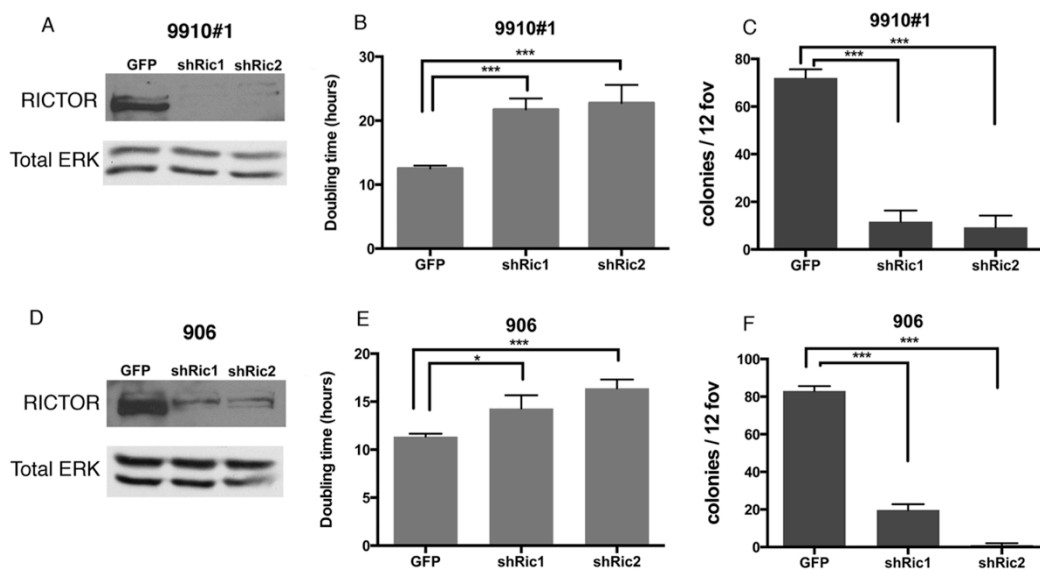


Figure 2.18: Rictor knockdown impairs the transformed phenotype in murine PDAC cells

(A, D) Western blot confirming efficient Rictor knockdown in the 9910#1 (A) and 906 (D) murine PDAC cell lines. Total ERK protein levels are used as a loading control. (B, E) Bar graphs showing the impact of Rictor knockdown on PDAC cell proliferation. (C, F) Bar graphs showing the impact of Rictor knockdown on anchorage independent growth as measured by soft agar colony formation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by student's t- test.



Pharmacological inhibition of mTORC1/2 delays tumor formation in KPC mice

To test this hypothesis we used AZD2014, a dual mTORC1/2 inhibitor recently investigated in a phase I clinical trial (Basu, Dean et al. 2015, Guichard, Curwen et al. 2015). We previously showed that *KPC* mice were resistant to treatment with rapamycin, and therefore not dependent on mTORC1 signaling to S6 kinase (Morran, Wu et al. 2014), however, AZD2014 allowed us to evaluate the impact of inhibiting both mTOR signaling complexes using a clinically relevant inhibitor. We first tested AZD2014 *in vitro* using *KPC* tumor cell lines and showed that signaling downstream of both mTORC1 and mTORC2, assessed by immunoblotting for pAKT^{S473}, pS6 and p4E-BP1, was effectively inhibited (Figure 19A). We next treated cohorts of *KPC* mice from 10 weeks of age with gemcitabine, AZD2014 or AZD2014 in combination with gemcitabine. This timepoint was selected in order to mimic surgically resectable disease, and to exclude tumor-promoting effects that may not be evident when mice with late-stage disease are treated (Rhim, Oberstein et al. 2014). In line with previous studies, gemcitabine treatment offered negligible benefit compared with untreated controls (Figure 2.20A, B). In contrast, AZD2014 treatment significantly prolonged survival, although not to the same extent as *Rictor* deletion. Combination of AZD2014 and gemcitabine further prolonged survival, and mice treated with this combination had a median survival of 280 days compared with 147 days for those treated with gemcitabine alone (Figure 2.20A, B). These data

were supported by our demonstration that AZD2014 and gemcitabine act synergistically to induce death of pancreatic tumor cells *in vitro* (Figure 2.19B). When we assessed inhibition of mTOR targets by IHC we found that phosphorylation of both mTORC1 and mTORC2 targets was suppressed, although mTORC2 signaling was more efficiently inhibited (Figure 2.20C). Taken together, our data show that dual mTORC1/2 inhibition can effectively block signaling downstream of mTOR, and may hold promise as a novel therapeutic strategy in pancreatic cancer.

Figure 2.19: AZD2014 treatment blocks signaling downstream of mTORC1 and mTORC2

(A) Immunoblots showing levels of pAkt, total Akt, pS6, total S6, p4E-BP1, total 4E-BP1 and β -actin in 3 KPC cell lines, untreated, or treated with either vehicle, or 50nM AZD2014. (B) Cytotoxicity assay in K8484 KPC cells (top panel) and MIA PaCa-2 human pancreatic cancer cells treated with gemcitabine (x-axis) and AZD2014 (y-axis) for 96 hours. Combeneft software was used to analyze the experimental data (left, percentage growth inhibition compared with control), generate predicted inhibition data (middle), and compare for each combination (right). The greater the difference, the more synergistic the combination (shown in cyan and blue).

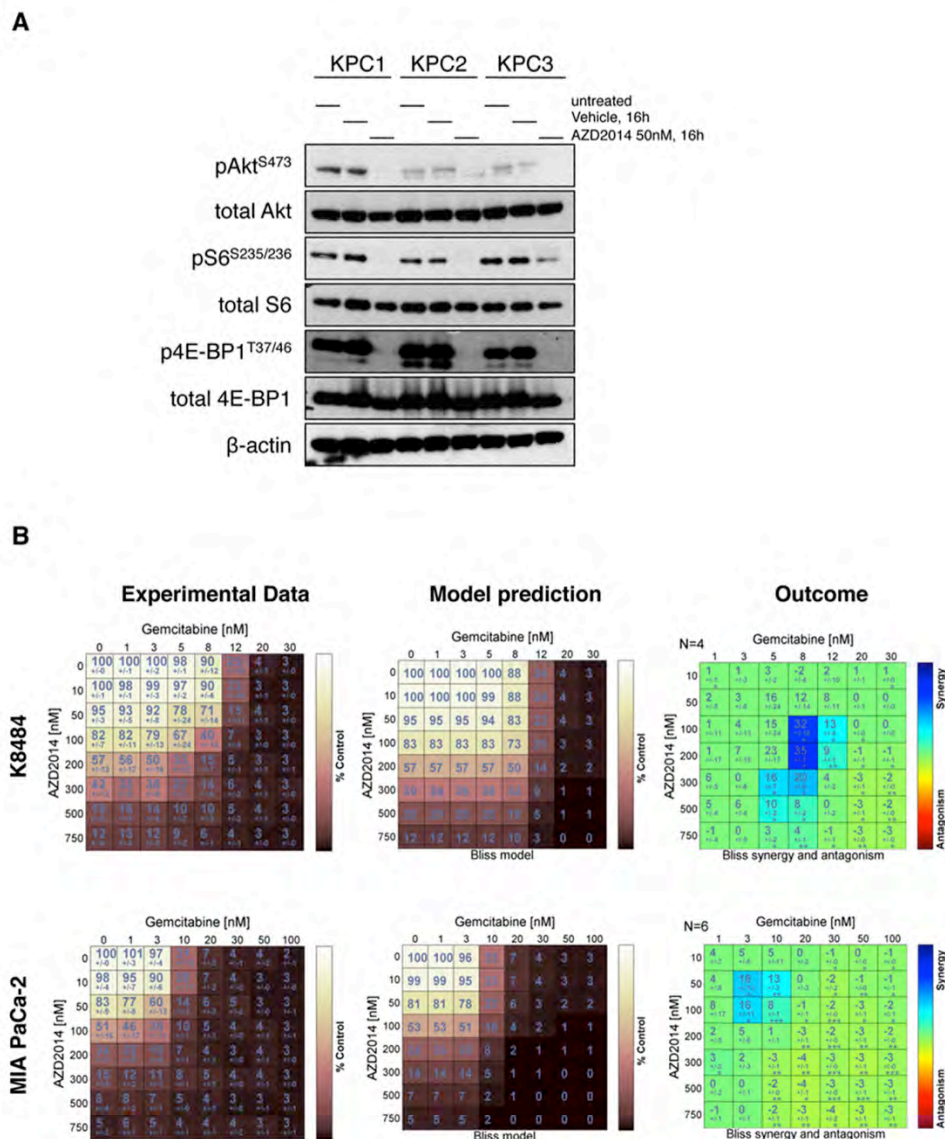
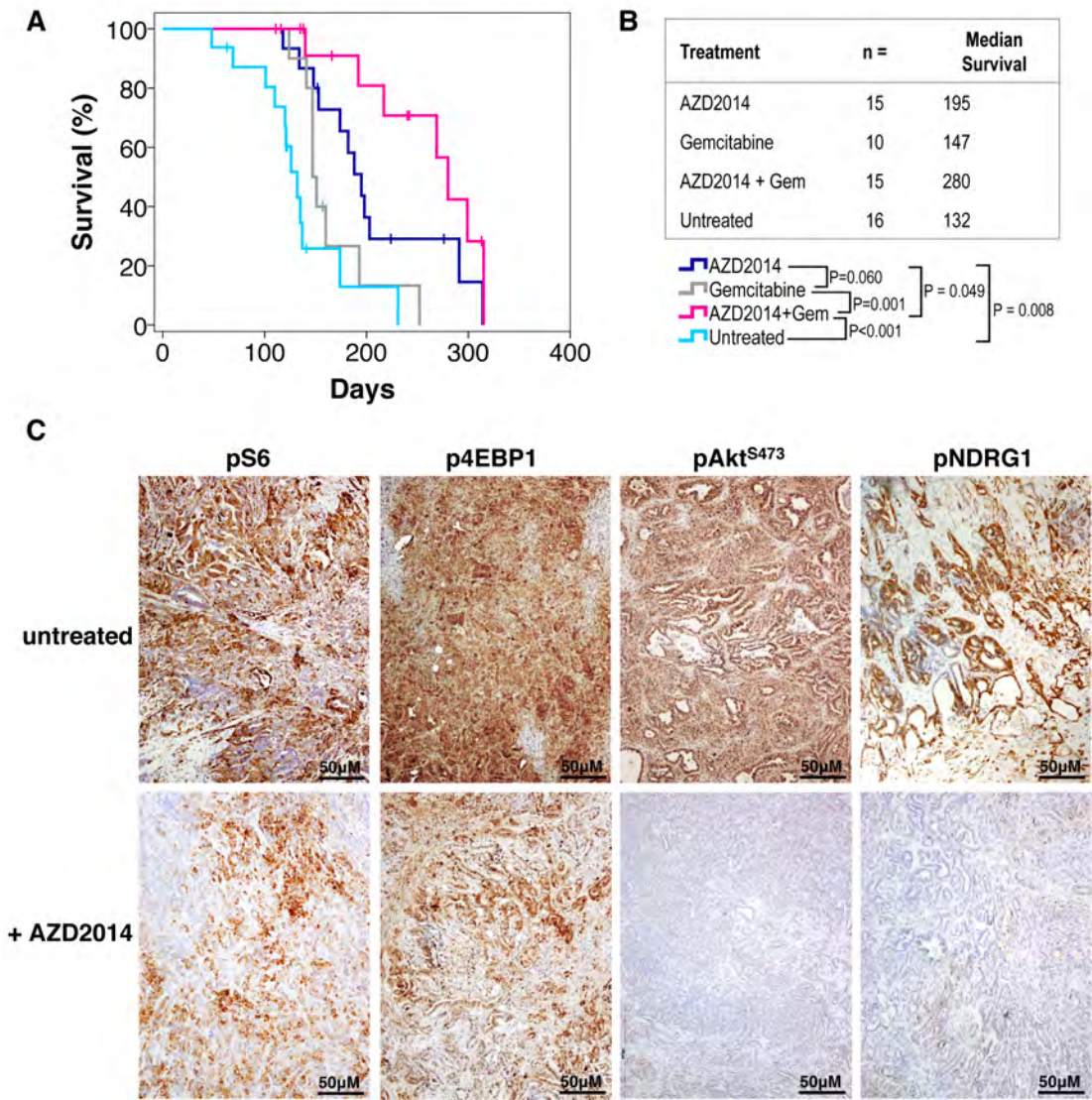


Figure 2.20: Pharmacological inhibition of mTORC1/2 delays tumor formation in *KPC* mice

(A) Kaplan-Meier survival analysis showing pancreatic tumor-free survival in untreated *KPC* mice, and *KPC* mice treated from 10 weeks of age with the dual mTORC1/2 inhibitor (AZD2014), Gemcitabine, or AZD2014 in combination with Gemcitabine. Censor ticks indicate mice sacrificed due to extra-pancreatic pathologies. (B) Table showing number of mice and median survival per cohort. P values calculated by Log Rank test. (C) IHC for downstream mTOR signaling pathway components in tumors from AZD2014 treated and untreated *KPC* mice as indicated.

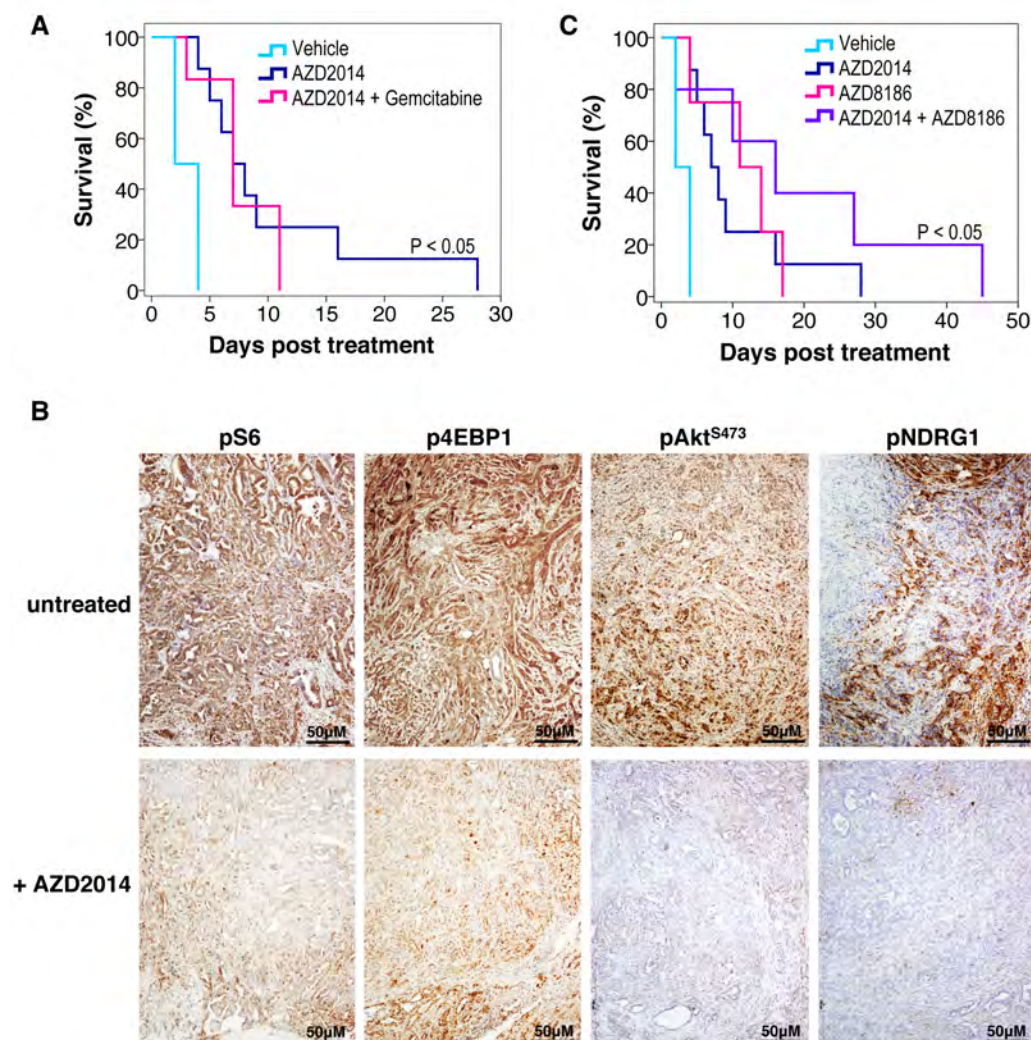


Inhibition of mTORC1/2 prolongs survival in late stage tumors

To determine the efficacy of mTOR inhibition as a therapeutic strategy, we tested the effects of AZD2014 treatment in mice with late stage tumors. We monitored *KPC* mice until they exhibited clinical signs of PDAC, including swollen abdomen, loss of body conditioning resembling cachexia, hunching, immobility and the presence of a palpable tumor. At this stage mice were randomized onto treatment with vehicle, gemcitabine, AZD2014, or AZD2014 combined with gemcitabine. We found that survival from the commencement of treatment was significantly prolonged by AZD2014 treatment in these mice with late-stage tumors (Figure 2.21A). Interestingly, combination of AZD2014 with gemcitabine did not further extend survival (Figure 2.21A). When we assessed inhibition of mTOR targets by IHC we found that phosphorylation of downstream targets of both mTORC1 (S6, 4E-BP1) and mTORC2 (AKT, NDRG1) was suppressed (Figure 2.21B), although again, mTORC2 signaling was more efficiently suppressed. Although we saw an impact in a very aggressive late stage model, mice still rapidly progressed to end stage disease. Consistent with this, in this late setting *in vivo* we did not observe induction of CDK inhibitors that we observed earlier, nor did we observe changes in other potential targets of RICTOR such as c-MYC (Guo, Zhou et al. 2012, Kuo, Huang et al. 2015), showing that as tumors progress *in vivo* they become less susceptible to mTORC2 inhibition as a single agent.

Figure 2.21: Pharmacological inhibition of mTORC1/2 prolongs survival in mice with late stage tumors

(A) Kaplan-Meier survival analysis showing survival in *KPC* mice treated from the onset of clinical symptoms with vehicle (cyan line, $n = 4$), AZD2014 (blue line, $n = 8$), or AZD2014 in combination with Gemcitabine (pink line, $n = 6$). (B) IHC for downstream mTOR signaling pathway components in treated and untreated *KPC* tumors as indicated. (C) Kaplan-Meier survival analysis showing survival in *KPC* mice treated from the onset of clinical symptoms with vehicle (cyan line, $n = 4$), AZD2014 (blue line, $n = 8$), PI3K inhibitor (AZD8186, pink line, $n = 4$), or AZD2014 in combination with AZD8186 (purple line, $n = 5$). P values calculated by Log Rank test.

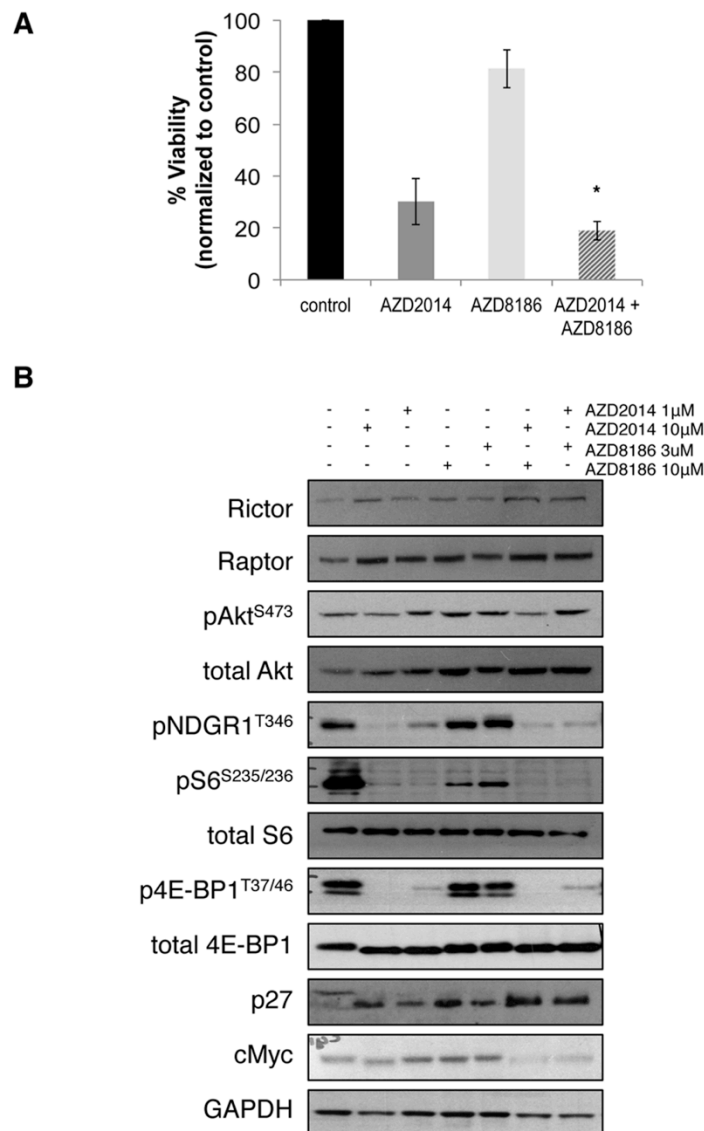


Inhibition of PI3K co-operates with mTORC1/2 inhibition to prolong survival in late-stage tumor-bearing mice

Recent studies have suggested that cancer cells can escape the requirement for mTOR through uptake of nutrients via other means (Palm, Park et al. 2015). In addition, mTORC1 inhibition results in feedback activation of the PI3K/AKT axis (O'Reilly, Rojo et al. 2006). Therefore, we tested whether inhibiting PI3K signaling could enhance the chemotherapeutic effects of AZD2014. Previous elegant studies have shown deletion of PI3K α blocked tumor formation but not established tumor growth (Eser, Reiff et al. 2013), consistent with our findings with *Rictor* gene deletion. Moreover *Rac1* deletion strongly suppresses pancreatic carcinogenesis and this was suggested to be downstream of PI3K (Heid, Lubeseder-Martellato et al. 2011, Wu, Carpenter et al. 2014). Given *Rac1* can also modify macropinocytosis (West, Prescott et al. 2000), inhibiting PI3K may also suppress some of the mechanisms of nutrient uptake when mTOR is depleted. Since PI3K β is the primary isoform that links PI3K signaling to RAC1 (Fritsch, de Krijger et al. 2013, Yuzugullu, Baitsch et al. 2015), we combined AZD2014 with a PI3K β kinase inhibitor, AZD8186 (Hancox, Cosulich et al. 2015). We first investigated whether AZD2014 and AZD8186 caused an increased loss of cell viability in *KPC* tumor cells *in vitro* (Figure 2.22A). AZD2014 proved potent in reducing cell viability (as observed previously with *Rictor* knockdown) and combined treatment with AZD8186 (which alone had little effect on cell viability) further reduced cell viability (Figure 2.22A).

Interestingly, in this scenario we were able to detect diminished Myc expression and p27 upregulation by western in the dual treated cell lines (Figure 2.22B).

Therefore, we decided to investigate the effects of inhibiting mTORC1/2 along with PI3K in mice with late stage tumors. As previously, we monitored *KPC* mice until they exhibited symptoms of PDAC and a palpable tumor, and then treated with AZD2014 and/or AZD8186. We found that survival was extended by AZD2014 and AZD8186 co-treatment relative to treatment with either compound alone (Figure 2.21C). Taken together, our data show that Rictor-mediated signaling plays a crucial role in pancreatic cancer development and progression. Moreover, mTORC2 inhibition, particularly as part of a combinatorial approach, potentially represents a new therapeutic strategy for this disease.



Discussion

Pancreatic cancer is an almost universally lethal disease, and better understanding of the signaling pathways involved in its development and progression is required to develop improved therapeutic options for patients. Cytotoxic chemotherapy has limited efficacy in this malignancy, therefore the development of targeted therapeutic strategies is an attractive approach. Mutation of *KRAS* is almost universally the driving force in pancreatic tumor initiation and progression. Indeed, inducible mouse-models have demonstrated that pancreatic tumors retain dependence on activated *KRAS* signaling (Collins, Bednar et al. 2012, Ying, Kimmelman et al. 2012). Past attempts to directly inhibit *KRAS* have failed, however inhibition of key signaling pathways downstream of *KRAS*, particularly as part of combination inhibition strategies may hold promise. Prior elegant studies have shown PI3K signaling to be a key mediator of oncogenic *KRAS* in the pancreas (Eser, Reiff et al. 2013). mTOR is one of the major effectors downstream of PI3K signaling, however the involvement of mTOR signaling in pancreatic tumorigenesis was relatively poorly understood until now. Clinical trials aimed at inhibiting mTORC1 have failed, and given the high cost of trials and the high attrition rate for most novel putative therapeutic agents, a far greater understanding of how signaling through the mTOR-containing signaling complexes mTORC1 and mTORC2 contributes to pancreatic

tumor development and progression is required to develop more effective therapeutic strategies for targeting mTOR.

Here we demonstrate that genetic deletion of the required mTORC2 complex component, *Rictor*, strongly suppresses tumor development and progression in mouse-models of pancreatic cancer. This anti-tumorigenic effect of *Rictor* deletion was associated with increased expression of cyclin dependent kinase inhibitors, which may be a result of a G1 arrest, reduced proliferation, and features of senescence in cells *in vitro*. Our findings are in line with recent studies, which have demonstrated a requirement for mTORC2 signaling in tumor progression in both prostate cancer (Guertin, Stevens et al. 2009) and melanoma (Damsky, Micevic et al. 2015). Importantly, while loss of *Rictor* significantly prolonged tumor latency in *KPC* mice, tumors were ultimately able to progress, and the anti-proliferative signals observed in pre-neoplastic lesions were lost in late-stage tumors, perhaps due to loss of TRP53. Indeed, the presence of wild-type TRP53 has previously been shown to augment the anti-proliferative effects of mTOR inhibition (Herzog, Bian et al. 2013). Together, these findings suggested that mTORC2 signaling plays an important role during pancreatic tumorigenesis. However, it remains possible that mTORC2-independent functions of Rictor contribute to the observed phenotypes. Additional studies utilizing null alleles for other mTORC2 components would address this issue.

Given our prior finding that pharmacologic inhibition of mTORC1 with rapamycin analogs failed to demonstrate any therapeutic benefit in the 'gold-standard' *KPC* mouse-model (Morran, Wu et al. 2014), it was important to determine whether simultaneous inhibition of both mTOR signaling complexes is an effective strategy. Our data using the mTOR kinase inhibitor AZD2014 demonstrate that blockade of mTOR signaling impairs the progression to end stage disease. Moreover, mTOR inhibition enhanced the benefit of gemcitabine in mice with established disease. These findings indicate that targeting mTOR may represent a potential treatment strategy in PDAC. The mechanism for this cooperation is unclear thus far, however, inhibition of mTOR signaling has been shown to reduce expression of the low density lipoprotein receptor (LDLR) (John, Paraiso et al. 2012), and LDLR depletion in *KPC* mice can increase the efficacy of gemcitabine *in vivo* (Guillaumond, Bidaut et al. 2015).

The dual mTORC1/2 inhibitor, AZD2014, has a number of benefits. mTORC2 signaling is almost completely abrogated in tumors from treated mice, similar to the effects we observed when *Rictor* was deleted. Further, it was recently reported that mTORC1 inhibition alone can actually enhance proliferation of activated KRAS-expressing cells in the setting of amino acid deprivation, by increasing the efficiency of utilization of extracellular proteins (Palm, Park et al. 2015). Considering that PDAC is characterized by a dense extracellular matrix that may provide not only physical, but nutritional support for pancreatic tumor cells, it is perhaps not surprising that inhibition of both mTORC1

and mTORC2 enhances survival whereas inhibiting mTORC1 alone is not efficacious. It should also be noted that in all *in vitro* systems we found that AZD2014 or Rictor knockdown had profound effects on soft agar growth and tumor cell proliferation suggesting that the tumor microenvironment might also be the source of support for the late stage aggressive tumors.

Molecularly targeted therapeutics often fail to display expected anti-tumor effects due to drug induced stimulation of feedback mechanisms. Indeed, mTOR inhibition has been shown to promote feedback activation of PI3K (O'Reilly, Rojo et al. 2006). In turn, PI3K activation can specifically activate mTORC2, through release of SIN1 mediated suppression of mTOR by PIP₃ binding (Liu, Gan et al. 2015). Therefore, our finding that combined mTOR and PI3K β inhibition prolonged survival in mice with end stage disease provides further support for the evaluation of mTOR kinase inhibitors in PDAC where the majority of patients present with disseminated disease.

The pathways downstream of mTORC2 signaling may also represent potential therapeutic targets. For example, in addition to AKT, mTORC2 signaling also regulates SGK1 (Garcia-Martinez and Alessi 2008) and PKC α (Sarbasov, Ali et al. 2004). SGK is involved primarily in regulating sodium transport, however, increased expression has been observed in several cancer types (Tessier and Woodgett 2006), while several cancer cell lines bearing PIK3CA mutations have been shown to be dependent on SGK (Vasudevan, Barbie et al. 2009). mTORC2

can also regulate PKC α and RAC (Jacinto, Loewith et al. 2004, Sarbassov, Ali et al. 2004, He, Li et al. 2013), both of which potentially influence tumorigenesis by affecting the spatial control of cell growth. In fact, RAC1 is reported to play an important role in KRAS-driven pancreatic tumorigenesis (Heid, Lubeseder-Martellato et al. 2011, Wu, Carpenter et al. 2014). MYC is also a reported target of mTORC2 (Guo, Zhou et al. 2012, Kuo, Huang et al. 2015), and is regulated at the level of translation by mTORC1 (Gera, Mellinghoff et al. 2004). We recently demonstrated that pancreatic tumorigenesis is significantly delayed by Myc deficiency (Walz, Lorenzin et al. 2014). Defining the relative contributions of these downstream molecules will be required to determine the molecular mechanisms underlying the importance of mTORC2 signaling that we have described here. In addition, recent work from Yaffee and colleagues demonstrated that the temporal sequence of drug delivery can dramatically alter cellular response (Lee, Kim et al. 2012). Therefore, it will be vital to determine the optimal sequence and timing of delivery of mTOR inhibitors (and other targeted drugs) when used in combination with each other or cytotoxic drugs such as gemcitabine.

Methods

Animal Experiments

Mice were maintained on a mixed background and given access to standard diet and water *ad libitum*. *Ptf1a-cre*, *Pdx1-Cre*, *LSL-Kras^{G12D}*, *Trp53^{R172H}* mice and *Rictor^{fl/fl}* mice have been described previously (Kawaguchi, Cooper et al. 2002, Hingorani, Wang et al. 2005, Shiota, Woo et al. 2006, Bentzinger, Romanino et al. 2008). *Rictor^{fl/+}* mice were intercrossed to generate *Rictor^{Δ/Δ}* and *Rictor^{+/+}* siblings for comparison in PanIN development and caerulein injection studies. In tumor development studies, mice were monitored for symptoms such as abdominal distension, cachexia, jaundice, hunching, reduced mobility, staring coat and diarrhea. For late stage treatment studies pancreatic malignancy was confirmed by abdominal palpation. Mice were euthanized when disease progressed and post mortem tissue was harvested for histological analysis. Survival between cohorts was compared by Kaplan-Meier and Log Rank statistical analysis. Animal experiments were approved by the University of Massachusetts Medical School institutional Animal Care and Use Committee or performed under Home Office regulations and under license approved by the local ethics committee.

Caerulein injections

Caerulein (Sigma C9026, 50µg/kg) was injected intraperitoneally into 5-7-week old mice as previously described (Lugea, Nan et al. 2006). Injection of vehicle (0.05M Ammonium hydroxide in PBS) was used as a control. Mice were sacrificed at 2 or 21 days following the final injection series.

In vivo treatment experiments

For drug treatments, mice were randomly assigned to cohorts. Treatments were administered as follows: AZD2014 (AstraZeneca), in 20% w/v Captisol in H₂O, at 15mg/kg daily by oral gavage daily; Gemcitabine (LC labs), in PBS, at 100mg/kg by intraperitoneal injection twice weekly; and AZD8186 (AstraZeneca), in 0.5% HPMC in H₂O, at 50mg/kg by oral gavage twice daily.

Histology

Following necropsy pancreata were formalin fixed overnight, embedded in paraffin, and 4- or 5-micron sections were mounted on positively charged slides. H&E staining was done according to field standard. The quadchrome consists of a hybrid protocol derived from Sirius Red staining for collagen and Alcian Blue staining for mucin. Briefly, slides were rehydrated through a graded alcohol series to distilled water. They were then incubated sequentially in Weigert's Hematoxylin for one hour, Sirius Red for one hour, and Alcian Blue for 30 minutes, with rapid washes in acidified water (15 dips in 0.5% acetic acid) after

each incubation. In this stain, collagen appears bright red, mucin is blue, nuclei are black, and cytoplasm is a weak yellow.

A licensed pathologist who was blinded to tissue genotypes performed quantification of tissue area. Four images were quantified per section representing 1) the area of greatest neoplastic progression, 2) the area of lowest neoplastic progression, and 3,4) areas of the pancreas that were consistent with the average progression for that tissue section. Acini, ducts, PanINs, ADM lesions, and stromal tissue not including blood vessels were manually outlined using ImageJ software and are graphed as the percentage area of all areas quantified.

Estimation of cell number and cell size in $Rictor^{+/+}$ and $Rictor^{\Delta/\Delta}$ pancreata. Total genomic DNA was isolated using standard phenol-chloroform extraction from a tissue fragment resected from the tail of the pancreas. DNA concentration was determined by dividing the quantity of DNA retrieved, by the mass of the tissue fragment. The amount of total genomic DNA was extrapolated by multiplying the DNA concentration by the mass of the entire pancreas. Calculations assume that each cell contains an equivalent amount of genomic DNA.

For estimation of cell size, 5mm thick tissue sections were stained with DAPI and the number of DAPI-positive cells in 5-8 fields (100x magnification) counted in

ImageJ. Tissue area was calculated in ImageJ. Cell size was estimated by dividing the number of DAPI-positive nuclei in the tissue area.

Immunohistochemistry

4- or 5µm formalin-fixed paraffin-embedded (FFPE) sections were deparaffinized and rehydrated by passage through Xylene and a series of graded alcohols. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide, and antigen retrieval was performed using citrate buffer, pH6. Sections were blocked in 5% serum, and then incubated with primary antibody. Sections were incubated in secondary antibody (Vectastain ABC system) and staining visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) or NovaRed. A list of antibodies is provided in Table 2.1.

For insulin staining, a FITC-conjugated donkey anti-guinea pig secondary antibody (Jackson Immuno 706-095-148, 1:500) was used in lieu of a biotinylated antibody. Samples were mounted with DAPI containing hard-set (Vector) and observed with a Nikon Eclipse E400 microscope.

RNAscope

In situ detection of Rictor transcripts in FFPE mouse PDAC samples was performed using a Rictor specific RNAscope® assay (Wang, Flanagan et al.

2012) (Advanced Cell Diagnostics, USA) according to the manufacturers protocol and using the Rictor probe diluted 1:5.

Cell culture experiments

Primary PanIN lines (AH 2375, RP 2294) were grown on Laminin coated plates as previously described (Schreiber, Deramaudt et al. 2004). Cells were infected twice at 5 hour intervals with lentiviruses encoding Rictor-targeting shRNAs or GFP-targeting shRNAs as a control. shRNA sequences are provided in Table 2.2. Cells were plated at a density of 2.7×10^4 cells/well in 6 well plates. For the generation of protein lysates, cells were plated at a density of 1.3×10^5 cells/10cm dish.

Proliferation assay in PanIN cell lines: Viable cells were counted using trypan blue exclusion on days 4, 5 and 6 post-infection. \log_2 of cell numbers/well were plotted against time and doubling time was derived from the inverse slope. Significant differences in doubling time were determined by analysis of covariance using Prism Graphpad.

Senescence associated β -galactosidase staining of PanIN cell lines: Senescence Associated β -Galactosidase activity was assessed 4 days post lentiviral infection. Cells were fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 10 min at RT, stained (40mM Citric acid 0.1M/sodium phosphate 0.2M buffer

pH 6.0, 5mM potassium ferrocynide, 5mM potassium ferricynide, 150mM sodium chloride, 2mM magnesium chloride and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside 1mg/ml in water) for 16 hours at 37°C and counterstained with nuclear fast red (Sigma).

Proliferation assay in PDAC cell lines: Generation of murine pancreatic cancer cell lines from *KPC* mice has been described previously (Morran, Wu et al. 2014). Testing was performed by an in-house service to confirm that cells were mycoplasma-free. Cells were maintained at 37°C and 5% CO₂ in Dulbecco's modified eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (PAA), 2mM L-Glutamine (Invitrogen), and 1% Penicillin/Streptomycin (Invitrogen). Cells were treated with AZD2014 +/- AZD8186. Cell viability was measured by absorbance using the Cell Titer Blue assay (Promega). Fluorescence was measured using an Envision plate reader (Perkin Elmer).

Synergy assay in PDAC cell lines: Primary *KPC* tumor cell line K8484 and human pancreatic cancer cell line MiaPaca2 were treated with AZD2014 +/- Gemcitabine for 96 hours. Cell viability was determined by measuring the total protein content using the sulforhodamine B assay. Combenefit software was used to analyze the data for potential synergy (<http://www.cruk.cam.ac.uk/research-groups/jodrell-group/combenefit>).

Western Blotting

Cell lysates were prepared and Western blot analysis was performed using standard protocols. Antibodies used are listed in Table 2.3.

Table 2.1: Antibodies for immunohistochemistry

Protein	Conc.	Supplier
pS6	1:800	Cell Signaling Technology
p4E-BP1	1:500	Cell Signaling Technology
pAkt	1:25	Cell Signaling Technology
pNDRG1	1:800	Cell Signaling Technology
Ki67	1:200	Thermo Scientific
Cleaved caspase 3	1:50	Cell Signaling Technology
p21	1:500	Santa Cruz

Table 2.2: shRNA sequences

Target	Sequence
Rictor shRNA1	GCGGTTTCATACAAGAGTTATT
Rictor shRNA2	GCCAGTAAGATGGGAATCATT
GFP	TACAACAGCCACAACGTCTAT

Table 2.3: Antibodies for western blotting

Protein	Conc.	Supplier
S6	1:1,000	Cell Signaling Technology
pS6	1:1,000	Cell Signaling Technology
4E-BP1	1:1,000	Cell Signaling Technology
p4E-BP1	1:1,000	Cell Signaling Technology
Akt	1:1,000	Cell Signaling Technology
pAkt	1:1,000	Cell Signaling Technology
pNDRG1	1:1,000	Cell Signaling Technology
Rictor	1:1,000	Cell Signaling Technology
Raptor	1:1,000	Cell Signaling Technology
cMyc	1:1,000	Cell Signaling Technology
p27	1:1,000	Cell Signaling Technology
GAPDH	1:10,000	Cell Signaling Technology

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Chapter III:

Discussion

A Role for mTORC2 in Pancreatic Neoplasia

A critical role for mTORC2 signaling has been identified in genetically engineered mouse-models of prostate cancer driven by PTEN deficiency (Guertin, Stevens et al. 2009), and orthotropic models of glioblastoma driven by mutant EGFR (Tanaka, Babic et al. 2011). My study is the first to identify a requirement for mTORC2 in a genetically engineered mouse-model of a KRAS-driven tumor. Given the prevalence of KRAS mutations in human cancers, and the difficulty in treating KRAS-driven tumors (Ostrem, Peters et al. 2013), identification of a requirement for mTORC2 in KRAS-driven initiation and progression of pancreatic neoplasias expands our understanding the pathways by which KRAS induces oncogenic transformation.

Pancreatic knockout of *Rictor* in the context of activated KRAS resulted in a dramatic reduction, but not complete prevention, of PanIN lesions (Figure 2.2). mTORC2 activity was absent in the PanIN lesions that did form in *KC-Rictor^{Δ/Δ}* pancreata (Figures 2.3, A.1); proliferation and progression were reduced in these low-grade lesions relative to their *KC* counterparts (Figures 2.2, 2.4). These PanIN1 lesions also contained a higher percentage of cells positive for nuclear CDKIs in the absence of mTORC2, and a lower percentage of cells positive for nuclear BMI1 (Figures 2.5, 2.6). The increased presence of nuclear p16^{INK4A}, p21^{Cip}, and p27^{KIP1}, in PanIN lesions lacking mTORC2 signaling suggests that KRAS is down regulating these CDKIs in an mTORC2-dependent manner. The

decreased levels of BMI1 in *KC-Rictor*^{Δ/Δ} lesions supports the possibility that this polycomb group protein may be involved in the down regulation of some or all of these CDKIs. While only the inverse correlation between p16^{INK4A} and BMI1 was close to significant when calculated in pancreata harvested at 4-months of age (Table A.1), by 8-months of age, strong and significant inverse correlations were observed between BMI1 and all three CDKIs (p16^{INK4A}, p21^{CIP}, and p27^{KIP1}) (Table A.1). *In vitro* models were unable to recapitulate the *in vivo* decrease in BMI1 and increase in CDKIs observed in the *KC-Rictor*^{Δ/Δ} PanINs relative to their *KC* counterparts. However, the proliferative deficit observed in *KC-Rictor*^{Δ/Δ} PanINs was observed upon knockdown of *Rictor* in PanIN derived cell lines as well as in cell lines derived from established KRAS-driven murine PDAC, suggesting a robust role for mTORC2 in the maintenance of the KRAS^{G12D} mediated proliferative phenotype.

Of all the questions raised by the findings in this work, perhaps the most obvious is whether inhibition of mTORC2 in established PDAC provides a therapeutic benefit. Dual inhibition of mTORC1 and mTORC2 in *KPC* mice (Figure 2.13) and knockdown of *Rictor* in PDAC cell lines (Figures 2.11, A.9), suggest that mTORC2 specific inhibition could provide a therapeutic benefit in established PDACs. Although mTORC2 specific inhibitors are not available, the genetic tools to ask such a question have already been developed. The use of flippase (FLP) based recombination systems, which employ FLP mediated recombination of flippase recognition target (FRT) sites in a manner analogous to

Cre mediated recombination of flox sites, would allow loss of *Rictor* and activation of KRAS to be accomplished by distinct mechanisms. Similar to the LSL-cassettes used in LSL- *KRAS*^{G12D/+} animals, FRT-Stop-FRT (FSF) cassettes prevent read through of the genes they control until the stop cassette is excised by FLP. Combining *Pdx1-Flp;FSF-KRAS*^{G12D/+} and *FSF-Rosa26*^{CAG-CreERT2/+} alleles published by the Saur group in Germany (Schonhuber, Seidler et al. 2014) with the *Rictor*^{Δ/Δ} model (Shiota, Woo et al. 2006) will produce an animal in which flippase expressed under the pancreas specific *Pdx1*-promoter will excise the FSF-cassettes resulting in endogenous levels of activated KRAS beginning at embryonic day 8.5. Flippase will also remove the stop-cassette preceding the *Rosa26*^{CAG-CreERT2/+} allele in pancreatic tissue. Because delivery of tamoxifen is required for CRE expression by this activated allele, *Rictor* knockout can be delayed until animals present with specific stages of PanINs or PDACs. Inhibition of mTORC1 can increase AKT signaling by removing feedback inhibition of IRS1 and mSIN by the mTORC1 target S6K (Harrington, Findlay et al. 2004, Liu, Gan et al. 2013), and has been shown to increase proliferation in certain contexts (Palm, Park et al. 2015). These animals, and similar models with floxed *mTOR* or *Raptor* alleles (Risson, Mazelin et al. 2009, Sengupta, Peterson et al. 2010), would allow the delayed genetic ablation of mTORC1, mTORC2 or both mTORC1 and 2 in the context of established KRAS-driven lesions. Addition of the *Rosa*^{mT/mG} allele to this cross would allow confirmation of *Rictor* deletion via GFP and these lesions could be compared to mTOMATO expressing *Rictor*^{+/+}

lesions; titration of the tamoxifen pulse could allow visualization of both cell types within the same pancreas. These mouse models could provide important *in vivo* insight into potential benefits of an mTORC2 specific inhibitor relative to mTORC1, mTORC1/2 or PI3K inhibitors in established PDACs. One potential benefit for mTORC2 specific inhibition, compared to either PI3K or mTOR kinase inhibition, is a reduced number of targets. If mTORC2 specific loss can arrest established KRAS driven PDACs, it would suggest that mTORC2 specific inhibitors could provide efficacy by impeding a restricted subset of PI3K effectors. Although not always the case, reducing the incidental targets of molecular therapies should reduce adverse toxicities.

mTORC2 Signaling in Acinar-To-Ductal Metaplasia

Activating KRAS mutations in acinar cells have been shown to induce acinar-to-ductal metaplasia (ADM) resulting in duct like lesions capable of forming PanIN lesions (Habbe, Shi et al. 2008), and it is largely accepted that the majority of PanIN lesions observed in the *KC* model originate from acinar tissue that has undergone ADM. The presence of low numbers of PanIN lesions in the *KC-Rictor^{Δ/Δ}* pancreata observed at 4 and 8-months led to my hypothesis that in the absence of mTORC2 signaling, these lesions originate from pancreatic ductal tissue but not from trans-differentiated acinar tissue. Recent data demonstrating that the P110 α subunit of PI3K is required for KRAS-driven ADM in a caerulein induced model of pancreatic injury (Baer, Cintas et al. 2014) suggest that mTORC2 signaling may also be involved in ADM. Using a model of caerulein

induced pancreatic injury known to initiate KRAS-driven ADM (Carriere, Young et al. 2009), I found that mTORC2 was not required for ADM (Figures 2.7, 2.8).

The presence of PanIN1A lesions in both *KC* and *KC-Rictor^{Δ/Δ}* mice 21 days post caerulein injection was equivalent, and a larger percentage of cells were identified in the ADM transitional state in *KC-Rictor^{Δ/Δ}* mice than in *KC* mice (Figure 2.8). One explanation for this finding is that mTORC2 deficiency does not prevent ADM but impairs PanIN lesion progression at all stages including potentiated acinar cells and cells in an ADM transitional state. In support of this idea, the majority of exocrine cells in *KC-Rictor^{Δ/Δ}* pancreata were observed in an acinar, ADM or PanIN1a morphology at 21 days post caerulein insult, while exocrine cells in *KC* pancreata were primarily observed in PanIN1b or PanIN2 morphologies (Figure 2.8).

The formation of duct like structures 2 days following pancreatic injury (Figures 2.8, A.6) further supports the conclusion that caerulein can induce ADM in KRAS potentiated acinar cells even in the absence of mTORC2. The number of duct like structures, and the speed with which they formed, preclude their origination exclusively from preexisting ducts, and the most logical conclusion is that a significant number of these duct like lesions originate from acinar tissue that has undergone ADM. This assertion is further supported by the maintenance of a significant portion of *KC-Rictor^{Δ/Δ}* pancreata in an ADM transitional state 21 days following caerulein insult. While mTORC2 does not appear to be required for acinar tissue to transition to a ductal phenotype

following caerulein insult, the smaller nature and lower concentration of duct like structures observed in *KC-Rictor^{Δ/Δ}* pancreata relative to *KC* pancreata (Figure 2.8) does support a role for mTORC2 during KRAS-driven progression of acinar cells through ADM and into neoplastic ducts. These experiments show that KRAS-driven ADM can, in the context of caerulein injury, proceed to the formation of PanIN lesions even in the absence of mTORC2; however, they do not necessarily show that this is what occurred in the 4 and 8-month cohorts. In the absence of caerulein injury, the possibility that *KC-Rictor^{Δ/Δ}* PanINs are originating from pancreatic ducts remains. To assess the potential of *KRAS^{G12D}-Rictor^{Δ/Δ}* acinar tissue to trans-differentiate and form PanIN lesions, a lineage-tracing approach using inducible-conditional alleles could be employed. The use of *Mist1-Cre^{ERT2/+}* and *Ela-CreERT2^{Tg/+}* mouse strains combined with the *LSL-KRAS^{G12D}* both result in PanIN formation following acinar and acinar/centroacinar specific expression of Cre respectively, at 6 weeks of age (Habbe, Shi et al. 2008). Adding the *Rictor^{Δ/Δ}* allele to these mice would allow me to ask if PanINs arise from acinar or centroacinar cells in the absence of mTORC2. The converse experiment, to confirm the ability of *Rictor*-deficient ductal cells to form PanINs, could be achieved using a duct specific *CK19-CreERT* allele (Means, Xu et al. 2008) combined with *LSL-KRAS^{G12D}* and *Rictor^{Δ/Δ}*. Although the *CK19* driven CRE is expressed in multiple epithelial cell types, *LSL-Kras^{G12D}* activated by this allele has been shown to induce PanIN lesions with a pancreatic ductal origin (Ray, Bell et al. 2011). Unless combining *Rictor*-loss with KRAS-activation in

these other cell types results in a lethal or otherwise confounding phenotype, the ability of KRAS expressing ductal cells to induce PanINs should be apparent within 6 months of induction with tamoxifen. The added utility of tamoxifen inducibility in these systems would provide temporal control, allowing initiation of KRAS-activation and mTORC2 deletion at a similar developmental age across these three models. Although the PanIN forming potential of the pancreas decreases with delayed KRAS-activation, and these models would initiate KRAS-activation and *Rictor* deletion later than the *Ptf1a*^{Cre} allele used in my studies, KRAS has been shown to induce sufficient PanIN lesions to test this hypothesis when initiated at postnatal day 14 (Gidekel Friedlander, Chu et al. 2009). Despite the acceptance that PDAC can arise from acinar or ductal tissue in pertinent mouse models, conclusive evidence for either of these cellular origins in human PDAC is not yet available.

An alternative interpretation of results obtained from induction of acute pancreatitis that cannot be discounted is that caerulein-induced inflammation provides a partial *in vivo* rescue of the *KC-Rictor*^{Δ/Δ} phenotype. My observation of rapid formation of duct like lesions which progress to PanINs in the absence mTORC2 signaling, may result from caerulein mediated activation of a downstream mTORC2 target essential for KRAS-driven ADM. Intra-pancreatic trypsinogen activation and NF-κB activity act in parallel pathways to induce the pancreatic injury and inflammation associated with caerulein induced acute pancreatitis (Han, Ji et al. 2001, Dawra, Sah et al. 2011, Sah, Garg et al. 2012).

It is possible that mTORC2 is involved in the activation of one or both of these pathways during the initiation and progression of KRAS-driven PanIN lesions. NF- κ B activity has been placed downstream of both mTORC2 and AKT and is a likely candidate (Tanaka, Babic et al. 2011, Sah, Garg et al. 2012). Staining for nuclear localization of the REL-A subunit of NF- κ B would provide insight into this possibility. If caerulein injection is inducing a partial rescue by activating an NF- κ B mediated inflammatory response, I would predict that nuclear REL-A would be decreased in the pancreata of 4 and 8-month *KC-Rictor^{Δ/Δ}* animals relative to their *KC* counterparts, while nuclear REL-A would be observed in the pancreata of both *KC* and *KC-Rictor^{Δ/Δ}* pancreata following caerulein insult.

It is also possible that active trypsin may act downstream of mTORC2. The original identification of PKC enzymes identified them as protease-activated protein kinases (Takai, Kishimoto et al. 1977), and proteolytic activation by trypsin was shown to result in PKC enzymes whose activity was independent of Ca^{2+} and DAG (Kishimoto, Kajikawa et al. 1983). Staining for active trypsin as well as active PKC isozymes in both caerulein treated and untreated samples could provide some insight into the potential roles that trypsin mediated PKC activation may play in bypassing mTORC2 signaling in the development of PanIN lesions.

Trypsin can initiate signaling through the PAR-2 receptor (Dery, Corvera et al. 1998) which, along with PKC ϵ , has been shown to activate ERK in prostate stromal cell (Myatt and Hill 2005). Although my staining for ERK following

caerulein injection identifies decreased p-ERK in *KC-Rictor^{Δ/Δ}* PanIN lesions 21 days post caerulein injection (Figure 2.8), it is possible that ERK signaling was temporarily elevated immediately following insult. This possibility could be easily addressed by staining for p-ERK in the samples harvested 2 days following administration of caerulein. The use of an L-arginine driven model of pancreatitis, which has been shown to decrease rather than increase pancreatic secretions (Lechin and van der Dijs 2008), could be employed to avoid any trypsin mediated signaling through PKC or ERK.

While PI3K and PDK1 were required for the plasticity of acinar cells, preventing the subsequent formation of PanIN and PDAC (Eser, Reiff et al. 2013, Baer, Cintas et al. 2014), loss of mTORC2 appears to impair the progression of acinar cells through ADM and during their subsequent evolution through the PanIN grades. Although it could be argued that the mTORC2 deficient phenotype is a less robust version of that induced by either the PI3K or PDK, the cooperation observed when treating established *KPC* tumors with the dual mTORC1/2 inhibitor AZD2014 and the PI3K inhibitor AZD8186 (Figure 2.14), suggests that the roles of these pathways are distinct and can be combined for clinical benefit.

The maintenance of KRAS induced neoplasticity may require mTORC2

Regression from PanIN lesions back to acinar tissue has been shown in caerulein models upon ablation of mutant KRAS expression (Collins, Bednar et al. 2012). Notably, phosphorylation of ERK is dramatically decreased during this regression, and treatment of mice expressing pancreatic KRAS^{G12D} with a small molecule MEK inhibitor is sufficient to induce a PanIN to acinar regression (Collins, Yan et al. 2014). My observation of reduced p-ERK staining in *KC-Rictor^{Δ/Δ}* mice 21 days post caerulein treatment (Figure 2.8) make it worth considering the possibility that progression of *KC-Rictor^{Δ/Δ}* PanIN lesions may not be linear and that higher grade lesions may regress and even revert back to acinar tissue. Observations of intermediary time points following caerulein insult would provide insights regarding this possibility, but to confirm reversion of ductal cells into acinar cells, a lineage tracing approach which combines the use of a tamoxifen inducible Cre-loxP and flippase-FRT recombination technologies could be used. Crossing existing mouse strains to produce *CK19^{CreERT}; Rictor^{Δ/Δ}; Rosa26^{mT/mG}; Pdx1-Flp; FSF-KRAS^{G12D/+}* mice (Shiota, Woo et al. 2006, Muzumdar, Tasic et al. 2007, Means, Xu et al. 2008, Schonhuber, Seidler et al. 2014) would afford temporal control of Cre expression under the duct specific cytokeratin-19-promoter. Expression of flippase under the *Pdx1*-promoter would result in KRAS-activation beginning at embryonic day 8.5 (Offield, Jetton et al. 1996). Subsequent activation of Cre by intraperitoneal tamoxifen injection could be administered after PanIN lesions are known to have developed in the FSF-

KRAS model both in the context of caerulein induced acute pancreatitis, and in its absence. Cre activation would excise *Rictor* exon3 and ablate mTORC2-expression in ducts and PanIN lesions, while also deleting the Tomato marker in the lineage tracing *Rosa26^{mT/mG}* allele and expressing a membrane targeted GFP-reporter in its place. The presence of both positive and negative reporter genes is important given that cytokeratin-19 is expressed in multiple epithelial cell types. It is possible that titration of tamoxifen may be able to reduce the impact of non-pancreatic *Rictor* loss. The development of interfering phenotypes would require the design of new mouse lineages, specifically, the development of a *flp-stop-flp-CK19^{CreERT}*, would restrict CRE expression to epithelium derived from the pancreas. This model could also be made more equivalent to my current model by developing and using a *Rictor^{flp/flp}* conditional mouse to produce a *FSF-CK19^{CreERT}; Rictor^{flp/flp}; Rosa26^{mT/mG}; Pdx1-Flp; FSF-KRAS^{G12D/+}* mouse-model. In this model tamoxifen mediated expression of Cre could be restricted to adults and coordinated with caerulein insult, and as a result of *Rosa26^{mT/mG}*, acinar cells which had reverted from a ductal state would express GFP. These mouse-models could test the hypothesis that the ability of KRAS^{G12D} to “lock” pancreatic cells in an epithelial state requires *Rictor*.

KRAS Modulation of the Microenvironment Through mTORC2

The stromal component of PDAC plays a vital role in the development of these tumors. In established PDAC, stromal tissue can represent the majority of a tumor mass, and secretions from pancreatic stellate cells have been shown to potentiate neoplastic lesions (Hwang, Moore et al. 2008, Feig, Gopinathan et al. 2012). In *KC-Rictor^{Δ/Δ}* pancreata observed at 4 or 8-months of age, or 21 days post caerulein insult, quadchrome staining showed a dramatic reduction in collagen deposition by pancreatic stellate cells relative to *KC* pancreata (Figures 2.2, 2.8). Histopathological analysis confirmed that stromal tissue constituted a significantly smaller proportion of *KC-Rictor^{Δ/Δ}* pancreata than their *KC* counterparts. This supports the possibility that mTORC2 activity contributes to KRAS mediated effects on the neoplastic microenvironment. Although the administration of caerulein was not sustained, the fibrotic response in both *KC* and *KC-Rictor^{Δ/Δ}* animals was indicative of chronic pancreatitis. A cohort of these animals was allowed to progress to assess the role that mTORC2 plays on KRAS driven PDAC formation in the context of chronic pancreatitis. Experiments to address this question are ongoing but have been confounded by the presence of comorbidities such as pancreatic insufficiency.

Following caerulein insult, *KC-Rictor^{Δ/Δ}* pancreata were not subject to the rapid increase in weight and size observed in their *KC* counterparts (Figure A.5). The rapid onset of this phenotype in *KC* mice precludes a proliferative cause and

is consistent with inflammation-associated edema which has been described in similar models (Grady, Saluja et al. 1996). Immunohistochemical staining for B220, CD3, and F480 to identify B-cells, T-cells and macrophages respectively, within the stromal components of pancreata harvested 2 and 21 days post caerulein injection, may provide insight into differential immune responses in *KC-Rictor^{Δ/Δ}* and *KC* pancreata.

The linear view of PanINs recruiting and activating more stromal tissue as they progress has been revised based on work showing that signaling from stromal cells promote PanIN progression. Specifically, IL-6, presumably secreted by stromal macrophages, was shown to be involved in this process (Lesina, Kurkowski et al. 2011). IL-6 was shown to be a driver of STAT3 activity in KRAS-driven PDAC, and while STAT3 activation, achieved by conditional knockout of the STAT3 inhibitor *Socs3*, has not been shown to induce PDAC alone, it does cooperate with activated KRAS to dramatically accelerate disease in the *KC* model (Lesina, Kurkowski et al. 2011). Furthermore, pancreas specific deletion of STAT3 in PDAC mouse-models yielded phenotypes similar to the *KC-Rictor^{Δ/Δ}* model, decreasing, but not eliminating, the formation of PanIN lesions (Corcoran, Contino et al. 2011, Lesina, Kurkowski et al. 2011).

The NF-κB transcription factor has been shown to regulate inflammatory cytokines, including IL-6 in multiple cell types (Miyazawa, Mori et al. 1998, Xiao, Hodge et al. 2004), and studies showing activation of NF-κB downstream of

KRAS and mTORC2 suggest a mechanism by which these proteins may influence the inflammatory response which surrounds PanIN lesions and PDACs (Tanaka, Babic et al. 2011, Daniluk, Liu et al. 2012). *In vivo* analysis of inflammatory response in the ischemic hind limb adductor muscle also showed that dual mTORC1/2 inhibition, but not low dose rapamycin, reduced IL-6 expression, further suggesting a connection between mTORC2 and IL-6 expression (Fan, Cheng et al. 2013). In MEFs and dendritic cells mTORC2 repression of FOXO1 has been proposed as a negative regulator of numerous cytokines including IL-6 (Brown, Wang et al. 2011). This suggests that any regulation of IL-6 by mTORC2 may be context dependent.

The robust co-localization of IL-6 with cells expressing the macrophage marker CD68 suggests, that in the context of normal acinar tissue, macrophages are the major providers of IL-6 (Lesina, Kurkowski et al. 2011). However, activation of KRAS has previously been shown to induce IL-6 secretion from multiple cell types, and knockdown of IL-6 in established human PDAC lines impaired their growth in an orthotopic mouse-model (Ancrile, Lim et al. 2007). Importantly, the *SCID/beige* mice used for these orthotopic transplants maintain normal populations of macrophages despite being deficient in T and B cell lineages (Xing, Zganiacz et al. 2001). Together, these data suggest that macrophage derived IL-6 may play a role in the initiation of PanIN lesions, but that in established lesions, KRAS may drive secretion of IL-6 from within exocrine cells to create an autocrine signaling loop in established lesions. Combined

amplification of the loci expressing the IL-6 receptor (IL6R) and kinase STAT3 in over 20% of micro-dissected samples of PDAC support an important role for this signaling axis in the development and maintenance of these tumors (Witkiewicz, McMillan et al. 2015). In addition to Immunohistochemical staining for the NF- κ B subunit REL-A and immune markers for B cells, T cells and macrophages, staining for the IL-6 cytokine and its downstream target p-STAT3^{Y705} will help to provide further insight into PanIN formation and progression both in the absence of caerulein injury, and in the context of a caerulein induced pancreatitis model. If mTORC2 promotes IL-6 and STAT3 signaling, pancreas specific knockout of the STAT3 inhibitor SOCS3 should provide, at least in part, a rescue of PanIN formation in *KC-Rictor^{Δ/Δ}* pancreata (Lesina, Kurkowski et al. 2011). Should SOCS3 rescue mTORC2 deficiency, parsing the autocrine or paracrine nature of mTORC2 mediated IL-6 effect by developing a flippase mediated conditional *IL-6* knockout allele and combining it with pancreas specific or macrophage specific FLP expression would be an interesting and worthwhile endeavor.

Given the importance the tumor microenvironment plays in the establishment and support of PDAC, understanding the mechanisms employed by KRAS to shape the stromal response is an active area of research. If inhibition of mTORC2 proves to be a method by which active KRAS can be decoupled from the stromal response, targeting that leg of KRAS signaling may provide a significant clinical benefit.

While a decoupling of KRAS activation from the stromal response is desirable, in a clinical setting, the inhibition of mTORC2 by small molecule inhibitors would not be restricted to pancreatic cells. As stromal tissue makes up a significant portion of pancreatic tumors, mTORC2 inhibition would also affect these cells, and understanding the effect that such inhibition would have on the stromal element of the tumor is important. Although our studies don't allow for direct analysis of mTORC2 specific loss in the stroma, the pancreas cell specific loss of mTORC2 signaling in *KPC-Rictor^{Δ/Δ}* tumors and the global inhibition of mTORC2 with the dual mTORC1/2 inhibitor AZD2014 provide models which could provide insight into the effects of mTORC2 inhibition on the stromal tissue. To gain the clearest picture possible from these models the differences between *KPC* and *KPC-Rictor^{Δ/Δ}* stroma should be compared to the differences between stroma in control treated and AZD2014 treated *KPC* tumors. Any distinctions identified may be the result of mTORC2 specific inhibition in the stromal tissue, but could also be the result of mTORC1 inhibition or combined mTORC1/2 inhibition. Furthermore, differences identified could be the result of complete mTORC2 loss in the genetic model and less than complete loss in response to the drug. Regardless, information from such an analysis could inform further questions and experiments into the role that mTORC2 plays within the stromal element of pancreatic tumors.

Bypass of KRAS Induced Senescence Involves mTORC2

My studies show that a greater percentage of PanIN1 cells express nuclear CDKIs when mTORC2 signaling is absent. In pancreata harvested at 4-months, only p16^{INK4A} was elevated in *KC-Rictor^{Δ/Δ}* relative to *KC*, but at 8-months of age the percentage of p21^{CIP1} and p27^{KIP1} positive nuclei in *KC* PanIN1 lesions decreased, while they remained elevated in *KC-Rictor^{Δ/Δ}* lesions (Figures 2.5, 2.6). These findings are supported by Pearson's coefficients, which showed that only the inverse correlation of nuclear p16^{INK4A} with nuclear BMI1 was close to being statistically significant at 4-months of age, while at 8-months of age, the nuclear frequency of all three CDKIs (p16^{INK4A}, p21^{CIP1} or p27^{KIP1}) showed strong and statistically significant inverse correlations with nuclear BMI1 (Figure A.1). This combined with the impaired progression of *KC-Rictor^{Δ/Δ}* PanIN lesions (Figure 2.2), suggests that mTORC2 is required for the inactivation of CDKIs in KRAS mutant PanIN cells. Previous studies have determined that PDAC formation is accelerated when *KC* mouse-models are combined with conditional knockout of *Cdkn2a*, *Cdkn1a* or *Cdkn1b* (Bardeesy, Aguirre et al. 2006, Morton, Jamieson et al. 2010, Diersch, Wenzel et al. 2013). The ubiquitous loss of p16^{INK4A} expression in human PDACs suggests that this protein functions as a potent suppressor of KRAS-driven oncogenesis (Schutte, Hruban et al. 1997). Furthermore, low p21^{CIP1} levels correlate with poor prognosis following surgical resection (Morton, Jamieson et al. 2010), and p27^{KIP1} loss correlates with more advanced tumor grade and clinical stage in human PDAC (Hu, Watanabe et al.

2000). These studies strongly suggest a role for these CDKIs in restricting PDAC, and that the function of these cell cycle inhibitors must be eliminated to allow KRAS-driven oncogenesis.

Delayed activation of KRAS has been reported to impair the formation of PanIN lesions (Guerra, Schuhmacher et al. 2007, Gidekel Friedlander, Chu et al. 2009). The reduced potential for KRAS-driven transformation in adult pancreatic murine tissue suggests that actively cycling cells, the numbers of which decrease as the pancreas matures (Collado, Gil et al. 2005), are more susceptible to KRAS induced transformation. Deletion of *Cdkn2a* in a *Pdx1-CreERTM;LSL-KRAS^{G12D}* model induced at postnatal day 28, accelerated the formation of PanINs relative to *Pdx1-CreERTM;LSL-KRAS^{G12D}* mice induced at a similar age (Gidekel Friedlander, Chu et al. 2009), suggesting that *Cdkn2a* expression in adult pancreatic tissue may contribute to this resistance.

Ectopic expression of RAS isoforms can induce expression of CDKIs and often results in oncogene-induced senescence (Serrano, Lin et al. 1997). However, senescence associated β -galactosidase activity in pancreatic ducts occurring in response to caerulein insult was absent in the context of endogenous KRAS^{G12D} as was p16^{INK4A} expression (Lee and Bar-Sagi 2010). Furthermore, when activated KRAS was expressed at endogenous levels *in vitro*, p16^{INK4A} expression was reduced rather than elevated (Lee and Bar-Sagi 2010). *In vivo*, KRAS-driven PanIN lesions identified at 6 weeks of age in *KC* and *KC-LSL-Trp53^{R172H}* animals were positive for senescence associated β -

galactosidase activity, while PanIN lesions from *KC-Cdkn2a*^{-/+} animals were negative (Morton, Timpson et al. 2010), suggesting that the proteins encoded by *Cdkn2a* are required for KRAS induced senescence. Understanding how this tumor-suppressor is inactivated by KRAS is important, as a significant number of human PDACs maintain intact *CDKN2A* alleles, but do not express p16^{INK4A} (Schutte, Hruban et al. 1997).

p21^{CIP1} or p27^{KIP1} are both reported to be regulated by the mTORC2 target AKT. Direct phosphorylation of these CDKIs by AKT has been shown to localize them to the cytoplasm thus preventing their nuclear CDKI functions (Zhou, Liao et al. 2001, Sekimoto, Fukumoto et al. 2004). Additionally, transcriptional regulation of *Cdkn1a* is achieved by AKT mediated down regulation of TRP53 via MDM2 activation (Mayo and Donner 2001, Zhou, Liao et al. 2001), and transcription of *Cdkn1b* is repressed by AKT mediated inhibition of FOXO transcription factors (Medema, Kops et al. 2000). Given that p-AKT⁴⁷³ was absent in *KC-Rictor*^{Δ/Δ} PanINs, while p-AKT^{T308} remained, it is possible that AKT mediated regulation of transcription and post-translational modification of p21^{CIP1} and p27^{KIP1} require a fully activated p-AKT⁴⁷³ protein. This would be consistent with findings in mouse embryonic fibroblasts, which showed that mTORC2 activity was required for FOXO3a phosphorylation (Guertin, Stevens et al. 2006).

While my study has identified elevated frequencies of PanIN1 cells with nuclear CDKIs in the absence of mTORC2, and has done so in multiple KRAS-driven PanIN models, these findings are still correlative. Immunostaining for

nuclear TRP53 and FOXO transcription factors in *KC-Rictor^{Δ/Δ}* PanIN lesions could provide some insight into a mechanism for the increased nuclear expression of their transcriptional targets; however, these findings would also be correlative. Ultimately, combining the *KC-Rictor^{Δ/Δ}* model with conditional alleles for *Cdkn2a*, *Cdkn1a* and *Cdkn1b* would allow an investigation into which, if any, of these CDKIs are required for the inhibition of PanIN formation and progression seen in the *KC-Rictor^{Δ/Δ}* animals.

BMI1 is a Potential Mechanism by which mTORC2 Fosters Cell Cycle Progression

Upon identification of CDK inhibitor activity in mTORC2-deficient PanIN lesions I became interested in understanding how these inhibitors may be regulated. My inquiry into the status of BMI1 in the absence of mTORC2 signaling stemmed from its well-established role as an inhibitor of *Cdkn2a* expression (Jacobs, Kieboom et al. 1999). A member of the polycomb repressor group, BMI1 is required for the maintenance of hematopoietic and neuronal stem cells (Molofsky, Pardal et al. 2003, Park, Qian et al. 2003), as well as regeneration of the exocrine pancreas following caerulein insult (Fukuda, Morris et al. 2012). A recent study in which BMI1 was knocked out in the *KC* mouse-model (*KC-BMI1^{-/-}*) showed a phenotype similar to knockout of the PI3K pathway; PanIN formation was almost entirely prevented upon homozygous deletion of BMI1 (Bednar, Schofield et al. 2015). I observed a strong inverse correlation

between nuclear BMI1 expression and nuclear p16^{INK4A} expression *in vivo* (Figures 2.5, 2.6, 2.8, 2.9 and tables A.1, A.2), and while this finding is correlative, it fits with and provides context for the emerging role of BMI1 in KRAS-driven PanIN progression. Although the majority of human PDACs have genetic loss of p16^{INK4A}, many retain an intact *CDKN2A* locus but repress p16^{INK4A} expression. The mechanism by which p16^{INK4A} is repressed in KRAS-driven PDACs has not been confirmed *in vivo*, but both BMI1 and TWIST dependent mechanisms have been proposed. While my study did not investigate TWIST, it provides correlative *in vivo* support for a KRAS-driven, mTORC2-dependent, silencing of p16^{INK4A} by BMI1 (Figures 2.5, 2.6, 2.8, 2.9).

It is notable that in *KC-BMI1*^{-/-} pancreata, PanIN formation was not rescued by the additional loss of p16^{INK4A}, suggesting that BMI1 affects additional mechanisms involved in PanIN formation (Bednar, Schofield et al. 2015). A role for BMI1 in the regulation of *CDKN1A* and *CDKN1B* in cultured NSCLC cells suggests that BMI1 may act as a regulator of multiple CDKs in RAS driven tumors (Zheng, Wang et al. 2014). Significant inverse correlations were observed between nuclear frequencies of BMI1 and p27^{KIP1} at both 8-months of age and 21 days post pancreatic injury. A significant inverse correlation between BMI1 and p21^{CIP1} was only observed in the 8-month cohort (Table A.1). The lack of inverse correlations observed between BMI1 and p21^{CIP1} or p27^{KIP1} in 4-month cohorts was caused by the maintenance of nuclear CDKs in *KC* PanIN1s rather than a decrease in the frequency of nuclear CDK expression in *KC-Rictor*^{Δ/Δ}

PanIN1s. It therefore remains possible that mTORC2-dependent activation of BMI1 plays a role in KRAS-driven down regulation of multiple CDK inhibitors, but that frequencies of nuclear p27^{KIP1} and p21^{CIP1} positive PanIN1 cells are maintained for a longer period of time than p16^{INK4A} following KRAS-activation. My data do not preclude BMI1 independent mechanisms of mTORC2-dependent regulation of any of these CDKIs.

Further studies need to be done to elaborate the connection between mTORC2 and BMI1 as well as provide a better understanding of BMI1 regulation of CDK inhibitors. A series of co-immunofluorescence staining experiments in the current cohort of *KC* and *KC-Rictor^{Δ/Δ}* mice could provide insight into how individual CDKIs respond to increased BMI1 on a cell by cell basis. This method could also be used to identify expression patterns of BMI1 in relation to CDKIs. Mutual exclusion between BMI1 and any of the CDKIs would strongly support BMI1 regulation of that CDKI.

To directly address the role of BMI1 in KRAS-driven PanIN formation and its relationship to mTORC2, I propose the integration of a *Rosa26-LSL-Bmi1* allele into the *KC-Rictor^{Δ/Δ}* model. The conditional overexpression of BMI1 by a CAGs-promoter at the *Rosa26* locus would allow overexpression of BMI1 upon pancreas specific expression of CRE recombinase (Westerman, Blom et al. 2012). If BMI1 is a critical component of KRAS-mTORC2 driven PanIN formation, the overexpression of BMI1 in *KC-Rictor^{Δ/Δ}* pancreata should rescue

PanIN formation and decrease CDKI expression in these PanINs. As BMI1 has been shown to cause glioblastoma in the context of RB deficiency (Westerman, Blom et al. 2012), it would be important to control for a potential cooperative effect of BMI1 over-expression and KRAS-activation by comparing rates of PanIN formation in *KC* and *KC-LSL-Bmi1* models to avoid misinterpreting a cooperative effect as a rescue.

Although, the specific manner in which BMI1 might be regulated by mTORC2 or KRAS was not addressed in my studies, previous work in breast cancer has shown BMI1 levels to be regulated by the Gli2 transcription factor (Liu, Dontu et al. 2006). In a gene expression array described in Appendix C, Gli2 expression levels were found to be elevated in primary ductal epithelial cells upon infection with activated KRAS. The Sonic Hedgehog signaling pathway culminates with nuclear translocation of GLI transcription factors (Pasca di Magliano and Hebrok 2003) and is known to cooperate with KRAS throughout PDAC initiation and development (Morton, Mongeau et al. 2007). Ablation of GLI1 and GLI2 activity via expression of a dominant negative *Gli3T* allele has been shown to prevent PanIN formation in the *KC* model (Rajurkar, De Jesus-Monge et al. 2012). To confirm *in vivo* regulation of BMI1 by Gli2 downstream of mTORC2 signaling, the *KC-Rictor^{Δ/Δ}* model could be combined with the conditional *R26^{ΔNGli2/+}* knockin allele. Upon CRE mediated recombination, *R26^{ΔNGli2/+}* expresses a constitutively active N-terminally truncated form of GLI2 (Joeng and Long 2009). Rescue of a robust PanIN phenotype with increased

nuclear BMI1 in *KC-Rictor*^{Δ/Δ}; R26^{ΔNGli2/+} pancreata would provide support for GLI2 regulation of BMI1 during PanIN progression. I find this model preferable to an alternative conditional model, which expresses active Gli2 constitutively under a CAG-promoter and rapidly produced undifferentiated carcinoma of the pancreas, likely due to the robust synthetic promoter (Pasca di Magliano, Sekine et al. 2006). The less robust expression in the R26^{ΔNGli2/+} model should provide a larger window in which to observe rescue of a PanIN phenotype. Further experiments using a conditional BMI1^{fl/fl} animals combined with the *Ptf1a*^{Cre/+};R26^{ΔNGli2/+} mice could investigate the *in vivo* connection of BMI1 downstream of Gli2 (Bednar, Schofield et al. 2015).

An additional manner in which BMI1 may be regulated by mTORC2 is through post-translational modification. Post-translational modification of BMI1 by AKT has been observed in prostate cancer cell lines and in mouse embryonic fibroblasts (Liu, Liu et al. 2012, Nacerddine, Beaudry et al. 2012). These two studies reached differing conclusions as to the physiological results of BMI1 phosphorylation by AKT, with prostate cancers showing increased ubiquitination of H2A and MEFs showing decreased ubiquitination in response to BMI1 phosphorylation. These contradicting findings may be explained by differences in cell types and tumor suppressor backgrounds. It is also important to recognize that in the experiments done in MEFs, Liu et al. utilized BMI1^{S316A} and BMI1^{S316D} alleles in which the canonical site of AKT phosphorylation was altered. In Prostate cancer cell lines, Nacerddine *et al.* identified a more complex pattern of

BMI1 regulation in which three serine residues, which lack the canonical AKT target motif, appeared to regulate an AKT-dependent pro-tumorigenic role of BMI1 (Nacerddine, Beaudry et al. 2012).

In vivo analysis of PanIN1 lesions showed decreased proliferation, decreased nuclear BMI1, and elevated nuclear CDKI, in the absence of mTORC2 signaling (Figures 2.3, 2.4, 2.5, 2.6). While these *in vivo* findings are powerful, they are also correlative. To establish a direct relationship between BMI1, CDKIs and proliferative defects in response to mTORC2 loss, I attempted to replicate loss of mTORC2 in PanIN lesions in a tissue culture model. Substantial care was taken to select a cell culture system that represented the PanIN lesions in which I observed differences in CDKI expression. With this in mind I investigated *Rictor* knockdown in the context of primary cell lines derived from KRAS mutant PanIN lesions provided by Dr. Nabeel Bardeesy. These cells should, in theory, have relatively few mutations and signaling should be similar to early PanIN lesions *in vivo*. Although *Rictor* knockdown reproduced the decrease in proliferative rate, and increased senescence in these cells, CDKI levels and nuclear localization were unchanged, suggesting a fundamental difference between mTORC2 signaling in PanIN lesions grown in tissue culture verses *in vivo*. One possibility explaining the lack of corresponding phenotype is that primary PanIN cells grown in culture are under stress from non-physiological conditions, and, as a result, baseline levels of CDKI activity are elevated. Inhibition of mTORC2 may be unable to further activate CDKIs in these cells, but

by impairing proliferative signals from growth factors in the culture media, the balance of cell cycle progression verses inhibition is altered and these cells enter a senescent state. Alternatively, if the role of mTORC2 in regulating CDKIs were primarily directed at their degradation, *Rictor* knockdown would be unable to elevate the levels of CDKIs above the baseline observed in tissue culture. If basal levels of CDKIs in these PanINs are low, providing an additional cellular stress, such as serum starvation or cycloheximide, may be required to establish a context in which to observe differential CDKI activity.

The fact that the BMI1 and CDKI signaling observed in multiple *in vivo* settings was unable to be faithfully reproduced in a tissue culture model does not negate the findings in mice, but, thus far, it has precluded a robust biochemical analysis of how BMI1 and CDKIs are regulated by mTORC2. Initial observations in PDAC cell lines showed no difference in CDKI levels and lead to the selection of the PanIN derived cell lines. While it is possible that epithelial pancreatic ductal cells cultured in the continued presence of super-physiological glucose and serum levels may be an inappropriate model in which to observe the subtle effects of a growth factor mediated signaling pathway involved in metabolism, if a KRAS-driven *in vitro* system, in which mTORC2 loss consistently results in decreased BMI1 and increased CDKI nuclear expression, can be developed, shRNA mediated knockdown of p16^{INK4A}, p21^{Cip}, and p27^{KIP1} should be employed to determine if individual or combined loss of CDKIs can rescue the proliferative phenotype associated with mTORC2 loss. Furthermore, ectopic

expression of BMI1 following loss of mTORC2 could also be tested in such a system. If CDKIs in KRAS mutant cells are down regulated in an mTORC2, BMI1-dependent manner, it is possible that targeting this pathway may provide a mechanism by which certain CDKIs, silenced but not lost in various subsets of human PDACs, could be reactivated to provide clinical benefit. A more in depth understanding of the *in vivo* effects of mTORC2 signaling in the context of KRAS^{G12D} mutant PDAC with various tumor suppressor profiles is required to see if such an approach is feasible or wise.

Differentiating between PI3K/PDK1 and mTORC2 Signaling

The data presented in this thesis describe a phenotype in which the formation and progression of KRAS-driven PanIN lesions is inhibited by the loss of mTORC2. Perturbation of PI3K signaling through knockout of either PDK1 or the PI3K subunit P110 α in *KC* mouse-models of PDAC, resulted in the near complete ablation of ADM and PanIN lesions (Eser, Reiff et al. 2013, Baer, Cintas et al. 2014). While the PI3K knockout phenotype was dose dependent (Baer, Cintas et al. 2014), mono-allelic loss of *Rictor* in *KC* pancreata showed no discernable phenotype. Direct quantifications of PanIN formation in *KC-Pdk1^{fl/+}* heterozygotes versus *KC* animals were not reported, however survival of *KC-Pdk1^{fl/+}* animals was not significantly different than *KC* animals (Eser, Reiff et al. 2013). Bi-allelic *Rictor* loss dramatically decreased PanIN lesions and their progression, but not to the same extent as PI3K α or PDK1 ablation (Eser, Reiff et

al. 2013, Baer, Cintas et al. 2014). The absolute requirement for the PI3K-PDK1 pathway in PanIN initiation contrasts with the important, but non-essential, role played by mTORC2 signaling. PDK1 and mTORC2 co-phosphorylate proteins in the AKT and SGK families, PDK1 phosphorylates the activation domain, while mTORC2 targets the hydrophobic motif. For SGK family members, phosphorylation at the hydrophobic motif primes them for activation by PDK1 and is required for their activity. AKT family members contain PH domains, which localize them to the plasma-membrane, allowing partial activation by PDK1, but mTORC2 phosphorylation of the hydrophobic motif is required for full AKT activity (Guertin, Stevens et al. 2006, Pearce, Komander et al. 2010). The formation of some KRAS-driven PanIN lesions in the absence of mTORC2 signaling suggested that partial AKT signaling may be sufficient for a subset of cells to progress to PanIN lesions. The presence of these lesions afforded the opportunity to characterize these lesions and determine that their proliferative potential was reduced in the absence of mTORC2.

Following the development of focal PDAC, treatment of *Pdx1-Cre; LSL-Kras^{G12D/+}; Trp53^{fl/+}* mice with a PI3K inhibitor failed to significantly improve survival relative to mice treated with gemcitabine (Alagesan, Contino et al. 2015). In a similar study, *Pdx1-Cre; Kras^{G12D}; Cdkn2a^{fl/fl}* mice treated with a combination of PI3K and gemcitabine did not demonstrate a significant increase in survival relative to animals treated with gemcitabine mono-therapy (Junttila, Devasthali et al. 2015).

One of the major downstream consequences of PI3K signaling is the activation of mTORC1 signaling (Guertin and Sabatini 2007). Rapamycin provides acute inhibition of mTORC1, although long term exposure has been shown to inhibit mTORC2 activity in some cellular contexts (Sarbasov, Ali et al. 2006). Rapamycin treatment provided no survival benefit to *Pdx1-Cre; LSL-KrasG12D; LSL-Trp53^{R172H/+}* mice (Morran, Wu et al. 2014), and was shown to increase proliferation in the nutrient deprived interiors of tumors derived from this model (Palm, Park et al. 2015). Our findings that the dual mTORC1/2 inhibitor AZD2014 improved survival in *Pdx1-Cre; LSL-KrasG12D; LSL-Trp53^{R172H/+}* animals (Figure 2.13) and that this drug provides a synergistic effect with gemcitabine (Figures 2.12, 2.13), while inhibition of mTORC1 provided no benefit (Morran, Wu et al. 2014), suggests that mTORC2 inhibition plays an important role in this response.

The failure of mTORC1 specific inhibitors to induce cell death in cancer cells is often attributed to the inactivation of negative feedback loops and subsequent hyper-activation of AKT; in PDAC cell lines, acute inhibition of mTORC1 removed the feedback inhibition of S6K on IRS and mTORC2 (Soares, Ni et al. 2013). Recently, it has also been shown that in nutrient deprived settings, inhibition of mTORC1 increases the efficiency of lysosomal degradation of extracellular proteins, providing a source of amino acids to these cells (Palm, Park et al. 2015). The use of mTOR kinase active site inhibitors, which inhibit both mTORC1 and mTORC2, avoided hyper-activation of AKT in human PDAC

cell lines; however, levels of p-ERK increased. Furthermore, specific inhibition of mTORC2 also dramatically increased ERK activity in human PDAC cell lines (Soares, Ni et al. 2013, Soares, Ming et al. 2015), a result that contrasts with both *in vitro* and *in vivo* results from my studies showing no compensatory activation of ERK in the absence of *Rictor* (Figure 2.3, 2.8). Our data showing a cooperative effect of mTORC1/2 and PI3K β inhibition on the treatment of established late stage tumors arising in *Pdx1-Cre; LSL-KrasG12D; LSL-Trp53^{R172H/+}* animals (Figure 2.14) and *in vitro* (Figure 2.15) suggest that combining dual mTORC1/2 inhibition with inhibition of appropriate isoforms of PI3K may, in some highly relevant contexts, provide therapeutic benefits.

Compensatory ERK Signaling and Its Confounding Role in PDAC

Treatment

Both the RAF-MEK-ERK and PI3K-PDK1-AKT-mTORC1 pathways have been validated as KRAS effector pathways important for the initiation and progression of PDAC (Collisson, Trejo et al. 2012, Eser, Reiff et al. 2013). AKT has been shown to negatively regulate the RAF-MEK-ERK pathway by phosphorylating B-RAF at multiple sites within its amino-terminal regulatory domain (Zimmermann and Moelling 1999, Guan, Figueroa et al. 2000). Although 1 of 2 human PDAC cell lines showed elevated p-ERK levels upon treatment with a PI3K inhibitor (Alagesan, Contino et al. 2015), significant increases in p-ERK levels were not observed following treatment of advanced PDAC in GEMM

models with PI3K inhibitors (Alagesan, Contino et al. 2015, Junttila, Devasthali et al. 2015). Upon combined treatment with PI3K and MEK inhibitors, survival of *Pdx1-Cre;LSL-KrasG12D; Trp53^{fl/+}* and *Pdx1-Cre;KrasG12D;Cdkn2a^{fl/fl}* mice was extended relative to vehicle treated mice; however, significant increases in survival relative to gemcitabine treated animals were observed only in the *Pdx1-Cre; LSL-KrasG12D; Trp53^{fl/+}* mice (Alagesan, Contino et al. 2015, Junttila, Devasthali et al. 2015). This raises an intriguing question as to whether p16^{INK4A} may be required for the response to dual PI3K-ERK inhibition.

In *KC-Rictor^{Δ/Δ}* PanIN lesions observed at 4 and 8-months of age, ERK activity was not changed relative to their *KC* counterparts (Figures 2.2, A.1), suggesting that differential ERK signaling was not involved in the impaired progression phenotype. However, the difference in the average PanIN grades between *KC* and *KC-Rictor^{Δ/Δ}* mice presents the possibility that the apparent equivalence of p-ERK staining resulted from the comparison of RAS driven p-ERK in late stage lesions of *KC* mice with compensatory p-ERK seen in lower grade PanINs of *KC-Rictor^{Δ/Δ}* mice. My observations of low grade PanINs do not support this; however, a dual analysis of p-ERK staining intensity and PanIN grade, conducted by a certified pathologist, would provide a quantitative analysis with a higher resolution of disease state, and might identify differential p-ERK levels between PanIN1a, and PanIN1b, or PanIN1b and PanIN2, too subtle to be recognized without extensive pathological training.

These findings of unchanged p-ERK levels in 4 and 8-month old *KC-Rictor^{Δ/Δ}* animals are consistent with data from *KC* mice haploinsufficient for P110α as well as *KC-Trp53^{-/-}* and *KC-Cdkn2a^{-/-}* mice treated with a small molecule inhibitor of PI3K (Baer, Cintas et al. 2014, Alagesan, Contino et al. 2015, Junttila, Devasthali et al. 2015). These *in vivo* findings are also supported by my own *in vitro* data, in which any increases in p-ERK levels were minor following shRNA mediated *Rictor* knockdown (Figure A.8). Results published by Soares et al, in which dual mTOR/PI3K inhibition or *Rictor* knockdown in two human PDAC lines resulted in robust elevation of p-ERK levels, differ from my *in vitro* findings (Soares, Ming et al. 2015). This discrepancy may be explained as a difference between mouse and human samples or as an artifact of extended time in culture of the human PDAC lines. Differences in the experimental time line might also contribute to these divergent findings. Cell lysates from siRNA mediated knockdown of *Rictor* in human PDAC cell lines were harvested 48 hours earlier than from my shRNA mediated knockdown in murine PDAC; the robust ERK1/2 phosphorylation induced by dual inhibition of mTOR and PI3K was measured 2 hours after treatment (Soares, Ming et al. 2015). While *Rictor* knockdown was maintained in my cells, it is possible that ERK activation in response to mTORC2 inhibition is transient, not sustained.

In the context of caerulein treatment, *KC-Rictor^{Δ/Δ}* pancreata showed decreased levels of p-ERK. This was an unexpected finding that warrants discussion and further investigation. It is unclear from my data whether or not

phosphorylation of ERK in *KC-Rictor*^{Δ/Δ} pancreata occurs in response to caerulein induced acute pancreatitis. In *KC* and *KC-P110α* deficient animals ERK phosphorylation was shown to occur rapidly in response to caerulein insult, and while p-ERK levels were maintained over time in *KC* pancreata, they decreased in *KC-P110α*^{-/+} and *KC-P110α*^{-/-} pancreata (Baer, Cintas et al. 2014). This supports the possibility that ERK may initially be activated in *KC-Rictor*^{Δ/Δ} following caerulein insult, but that mTORC2, like PI3K, is required for it's sustained activity. It is also possible that in the absence of mTORC2, ERK is not activated in response to caerulein. Observation of p-ERK levels in *KC-Rictor*^{Δ/Δ} pancreata at 1, 2, and 5 days following caerulein injection should provide insight into the role of mTORC2 in the activation and maintenance of ERK signaling in response to acute pancreatitis. If *Rictor* proves to be required for early activation of ERK in response to acute pancreatitis, additional observations of ERK levels in KRAS wild-type pancreata, both with and without *Rictor*, could be used to investigate whether mTORC2-dependent ERK activation in response to caerulein insult is independent of constitutive KRAS signaling. Furthermore, the delayed progression of caerulein induced *KC-Rictor*^{Δ/Δ} PanINs permits the possibility that in the context of pancreatitis, mTORC2 mediated ERK signaling is required for the progression of PanINs.

Given the importance of the RAF-MEK-ERK pathway in the development of PDAC (Collisson, Trejo et al. 2012), understanding contexts in which targeted inhibition of pathways could enhance signaling through RAF-MEK-ERK is vital.

Given the possibility that mTORC2 inhibition may result in elevated signaling through this pathway, observing p-ERK levels in the animals treated with AZD2014 would provide valuable insight into possible compensatory activation in this model. Identifying common mechanisms of compensation and developing therapeutics directed at those pathways has proven effective in the treatment of NSLCs refractor to EGFR tyrosine kinase inhibitors (Janne, Yang et al. 2015). Predicting and targeting the pathways by which PDAC might evade targeted therapies may inform the selection of therapeutics and extend patient survival.

Identifying Phenotypically Relevant Targets of mTORC2

Both PDK1 and mTORC2 act in concert to phosphorylate targets known to play important roles in PDAC signaling. Understanding how these kinases function to promote tumorigenesis may provide insights into molecular vulnerabilities of this disease. My data have shown that mTORC2 activity plays a role in the formation of PanIN lesions, as well as shaping the microenvironment in which these lesions develop into PDAC. Conditional mouse-models provide excellent systems in which to investigate the loss of specific downstream components of this signaling complex in the context of a dynamic mammalian system. To further investigate the roles of PDK1 and mTORC2 downstream targets, and their contributions to PanIN and PDAC development, the *KC* mouse-model could be combined with conditional knockouts of AKT1, AKT2 and SGK1

(Bell, Horita et al. 1984, Fejes-Toth, Frindt et al. 2008, Wan, Easton et al. 2012). Bridget Shafit-Zagardo at Albert Einstein College of Medicine has proposed a conditional allele for AKT3, but such an allele has not yet been published (Shafit-Zagardo 2014). No conditional alleles have been published for SGK2 or SGK3. Ideally such mice would be developed to enable a full understanding of how the members of these two families contribute to PanIN formation and progression. Although it is unlikely that pancreatic loss of AKT or SGK isoforms would have dramatic developmental phenotypes, given the lack of such phenotypes in the absence of mTORC2, PDK1 or PI3Ka (Eser, Reiff et al. 2013, Baer, Cintas et al. 2014), temporal control afforded by inducible CRE systems would allow both the visualization of such phenotypes and the potential to circumvent them.

Although the development and use of mouse-models to address the questions proposed would be ideal, limited time, and money as well as the ethical responsibilities that accompany animal research, make the use of cell culture models more realistic approach to try and identify relevant targets to be further studied *in vivo*. I utilized cell lines derived from established PDAC to assess the loss mTORC2 signaling in a model of late stage disease. Increased doubling time upon shRNA mediated *Rictor* knockdown (Figures 2.11, A.9) showed that the reduced proliferation observed in early PanIN lesions could also be obtained in established PDACs deficient for *Trp53* or *Cdkn2a* by inhibiting mTORC2. Furthermore, the reduction of anchorage independent growth in these

cell lines (Figures 2.11, A.9) suggested that RAS driven tumorigenicity, not just proliferation, was affected by *Rictor* knockdown.

While p-AKT⁴⁷³ and p-NDRG1^{T346}, measures of mTORC2 activity, were generally decreased upon *Rictor* knockdown (Figure A.8), their decrease was moderate suggesting that both AKT^{S473} and SGK^{S422} may be phosphorylated by additional players in the context of mTORC2 knockdown. These findings contrast my *in vivo* observations, that phosphorylation of AKT⁴⁷³ and NDRG1^{T346} were all but eliminated in KC-Rictor^{Δ/Δ} PanIN lesions at 4 and 8-months of age (Figures 2.3, A.1). Furthermore, tumors that arose in *KPC* mice treated with AZD2014 maintained a reduction in mTORC2 signaling (Figure 2.13) suggesting that any phosphorylation of AKT^{S473} and SGK^{S422} by alternative mechanisms may be context specific. mTORC1 signaling, as assessed by p-S6K^{T389}, was moderately elevated following *Rictor* knockdown and a modest increase (1.0 - 1.8 fold) in p-ERK was also observed (Figure A.8). The increase in p-S6K is consistent with previously reported data (Soares, Ming et al. 2015), but given the quality of these blots, probing with the p-S6K target p-S6 to confirm these findings would be prudent. The mild elevation of p-ERK, which I observed in response to *Rictor* knockdown, is much less robust than observations in human PDAC cell lines (Soares, Ming et al. 2015).

Although PDK1 and mTORC2 share many targets, they target different sites and have varying effects on the activity of such targets. Understanding the signaling changes effected by mTORC2 inhibition and how they differ from those

resulting from PDK1 inhibition may provide valuable insight into efforts to inhibit this pathway. Rather than probing for all known proteins effected by mTORC2 and PI3K, a more global *in vitro* screening approach, such as a dot blot or reverse phase protein array, in which the signaling responses of KRAS-driven PDAC lines to mTORC2 or PDK1 loss are compared, should provide valuable insight into the differences between these two collaborators.

In attempting to determine which downstream mTORC2 pathway is required for the anchorage independence phenotype observed upon *Rictor* knockdown, I utilized constitutively active SGK1 and myristoylated-AKT1. Elevated SGK1 in Breast cancer has been shown to increase resistance to AKT inhibitors (Sommer, Dry et al. 2013), while myristoylated-AKT1 is strongly activated and has been shown to gain some functions of the AKT2 isoform (Kohn, Summers et al. 1996). The activated SGK1^{S422D} mutant increased the phosphorylation of its target NDRG1; however, it did not rescue the *Rictor* knockdown phenotype and mTORC2 retained its influence on SGK1 activity (Figure A.10). Of note, multiple NDRG1 bands recognized by both the p-NDRG1 and total NDRG1 antibodies are the result of a recently identified proteolytic cleavage event and not phosphorylation or glycosylation (Ghalayini, Dong et al. 2013).

The ectopic expression of myristoylated-AKT1 provided a robust expression of phosphorylated AKT^{S473}, which was insensitive to mTORC2

inhibition. While mTORC2 is the primary mechanism by which AKT^{S473} is phosphorylated (Guertin, Stevens et al. 2006), other kinases, including IKBKE, can phosphorylate this site in the absence of mTORC2 activity (Rajurkar et al. 2016 under review). Localization of myristoylated-AKT1 to the membrane appears to increase phosphorylation of AKT^{S473} through mTORC2 independent mechanisms, as expression of myristoylated-AKT1 rendered the endogenous AKT^{S473} insensitive to mTORC2 inhibition (Figure A.10).

In addition to the anticipated increase of AKT^{S473} levels, myristoylated-AKT1 increased NDRG1 phosphorylation in an mTORC2 independent fashion (Figure A.10). AKT and SGK recognize nearly identical consensus sequences as targets for phosphorylation (Alessi, Caudwell et al. 1996, Kobayashi, Deak et al. 1999), and subtle differences in binding site preference, as well as localization of kinase-targets, play roles in the specificity of SGK and AKT isoforms (Tran, Brunet et al. 2003). It is therefore reasonable to conjecture that expression of myristoylated-AKT1 may target proteins and phosphorylation sites that fall outside its normal regulatory sphere. A similar expansion of AKT1 targets was demonstrated by the ability of membrane bound AKT1 to localize Glut4 to the membrane, a function normally specific to AKT2 (Kohn, Summers et al. 1996, Calera, Martinez et al. 1998). This is likely the cause of the increased mTORC2 insensitive NDRG1 phosphorylation seen in response to myristoylated-AKT1 expression (Figure A.10). It is possible that increased AKT activity in PanIN lesions and PDAC might also result in phosphorylation of a broadened spectrum

of targets. The inability of myristoylated-AKT1 to rescue anchorage independence suggests that AKT alone is not responsible for this phenotype.

PDAC cells lines infected with SGK1^{S422D} demonstrated elevated SGK1 activity as measured by p-NDRG1^{T346}, and while these p-NDRG1 levels were decreased upon *Rictor* knockdown, they decreased only to the levels of the empty vector controls, suggesting that the SGK1 activity was roughly equivalent to the unperturbed empty vector control (Figure A.10). Thus, constitutive activation of SGK1 does not, by itself, rescue *Rictor* knockdown, and SGK1 alone is not responsible for the anchorage independent growth phenotype.

The phosphorylation of NDRG1 in response to expression of myristoylated-AKT1 suggests that this protein activated at least some of the downstream targets of SGK1. This further supports a failure of SGK1 to rescue the *Rictor* knockdown phenotype, and suggests that combining constitutively active SGK1 and AKT1 would not rescue the anchorage independent growth phenotype perpetuated by *Rictor* knockdown.

While rescue experiments conducted with additional isoforms of AKT and SGK might result in the identification of a critical downstream target, identification of additional targets through an unbiased approach may provide clearer direction to future investigations. A temporal analysis of signaling following *Rictor* knockdown would provide a useful understanding of the compensatory signaling responses in PDAC, and help to explain how these cells arrive at a new, less tumorigenic homeostasis. It would be wise to consider broadening such an

analysis beyond well described mTORC2 targets and feedback loops, employing approaches such as multiplexed reverse phase protein arrays, to identify novel targets and pathways in which they are altered.

A Role for mTORC2 in Pancreas Size

Given the importance of AKT signaling on proliferation (Testa and Tsichlis 2005), and regulation of cell size (Fingar, Salama et al. 2002), it was not surprising to find that both the number and size of cells was reduced in *Rictor*^{Δ/Δ} pancreata (Figure 2.1). While a portion of the decreased organ mass was a result of smaller cells, I found a reduction in overall cell number to be primarily responsible for this phenotype. This contrasts findings in whole body hypomorphic PDK1 mice, in which the sizes of the kidney, pancreas, spleen and adrenal gland were reduced. The decrease in the size of the adrenal gland was determined to be the result of decreased cell size; however, the pancreata were not analyzed regarding cell size and number (Lawlor, Mora et al. 2002). My findings suggest that mTORC2 signaling may regulate cellular proliferation during pancreatic development. Published data showing that beta-cell specific knockout of *Rictor* decreases their proliferation also supports my findings (Gu, Lindner et al. 2011). Although opposing studies observing conditional knockout of PDK1 in pancreatic development have not been published, they could help to identify disparities between PDK1 and mTORC2 signaling and may identify *in vivo*

differences between mTORC2 and PI3K-PDK1 signaling during pancreatic development.

PTF1a-CRE is expressed at some point during the development of all acinar and most ductal cells as well as 95% of β -cells and 75% of α -cells. Given the importance of exocrine and endocrine function in digestion and glucose homeostasis respectively, disruption of either of these pancreatic roles could produce phenotypes including development of diabetes or decreased mouse weight. β -cell specific knockout of *Rictor* did not alter the size of these cells or the weight of mice; however, a decrease in β -cell mass and subsequent decrease in pancreatic insulin content were observed (Gu, Lindner et al. 2011). The finding that β -cell content was decreased while β -cell size was maintained (Gu, Lindner et al. 2011) supports my conclusion that the differences in pancreas size in *Rictor* knockout pancreata are primarily driven by decreased cell numbers. Furthermore, this study supports my finding that *Rictor-deficient* pancreata did not affect the overall fitness of the mice and adds credence to my assertion that pancreatic function was normal, or at least sufficient for the needs of mouse development despite the loss of *Rictor* during organogenesis (Figure 2.1).

With regards to pancreas specific *Rictor* deletion, the intent of my analysis was to identify systemic defects that might preclude subsequent development of this model to observe the role of mTORC2 in PDAC. My data provided a snapshot of pancreatic composition roughly 2.5 months following *Rictor* loss at

embryonic day 9.5, and, while sufficient to answer my question, it was not a comprehensive analysis of pancreatic development and function, and would be insufficient to identify subtle changes in total islet numbers or sizes. Additionally, my findings were obtained at 2-months of age and do not preclude the possibility that pre-natal or juvenile *Rictor*^{Δ/Δ} mice may have pancreatic deficiencies that resolve by this point. These data do, however, show the viability and generally equivalent fitness of *Rictor*^{Δ/Δ} mice. The development of a functional, albeit smaller, pancreas suggests that the phenotypes observed in the context of activating KRAS mutations were not a result of a developmental defect.

The Role of mTORC2 Signaling in Metastasis Remains Unclear

Patients diagnosed with PDAC usually succumb to metastatic disease rather than the primary tumor; however, due to their aggressive nature, the primary tumors in *KPC* mice are usually responsible for the demise of the animals. The *KPC* animals used in our study contained a gain of function *p53*^{R172H} allele and, consistent with previous studies, gross metastatic lesions were observed in roughly 50% of these animals. In *KPC-Rictor*^{Δ/Δ} animals, gross metastatic disease was observed in roughly 25% of these animals at the time of death, although this difference was not statistically significant. Histological analysis of these samples for micro-metastatic lesions has not yet been conducted. The increased lifespan of the *KPC-Rictor*^{Δ/Δ} animals relative to the

KPC animals presents increased time for the development of metastatic disease and the presence of metastatic lesions in the long-lived *KPC-Rictor^{Δ/Δ}* animals may suggest that mTORC2 deficient metastatic lesions have reduced proliferative capacity. An analysis of micro-metastatic frequency would be required to determine if fewer lesions occur or if these lesions are simply growing more slowly in the absence of Rictor.

A role for mTORC2 has been implicated in metastatic disease in both bladder cancer and colorectal cancer. Phosphorylation of the mTORC2 target AKT^{S473} was observed in invasive bladder carcinoma cell lines and the migratory ability of these cells, as measured by wound healing assay, was impaired by *Rictor* knockdown (Gupta, Hau et al. 2013). Both mTOR and RICTOR were found to be elevated in late stage colorectal cancers and metastatic lesions relative to normal colon epithelium. Furthermore, trans-well migration was impaired by siRNA mediated knockdown of *RICTOR*, and intravenous injection of *RICTOR* deficient colorectal cancer cell lines into athymic nude mice prevented these cells from establishing lesions in the lungs, bones, and lymph nodes of these mice (Gulhati, Bowen et al. 2011). Notably, the role for mTORC2 in metastatic bladder and colorectal cancers has been linked to RAC1, and while RAC1 has been shown to regulate the localization of both mTORC1 and 2 (Saci, Cantley et al. 2011), it has not been shown to be a target of either mTOR complex. RICTOR has, however, been shown to regulate RAC1 in an mTORC2 independent manner.

mTORC2 Independent Roles of RICTOR

Throughout this thesis I have equated the expression of *Rictor* with mTORC2 activity, and while it is true that RICTOR is required for the formation of mTORC2, other mTORC2 independent functions of RICTOR also exist. Specifically, RICTOR, but not the mTORC2 specific cofactor mSIN1, has been identified as a regulator of cell migration. Knockout of RICTOR in MEFs reduced cell migration as well as GTP bound levels of the Rho family members CDC42 and RAC1 (Agarwal, Chen et al. 2013). CDC42 and RAC1 are known to be involved in the dynamic assembly of actin filaments, an important function during cell migration. RICTOR inhibits Rho-GDP dissociation inhibitor 2 (RHOGDI2), which interacts with RAC1 and CDC42 to prevent GTP binding in these proteins. In the absence of *Rictor*, knockdown of RHOGDI2 rescued the migratory ability of MEFs (Agarwal, Chen et al. 2013) confirming this relationship. RICTOR has also been identified in a complex with the actin-based molecular motor myosin 1c (MYO1C). This complex is reported to regulate the actin filament regulatory protein paxillin by phosphorylation at tyrosine 118. Knockdown of either *Rictor* or *Myo1c* impaired this phosphorylation and the formation of membrane ruffles, implying a role for the RICTOR-MYO1C complex in cortical actin remodeling (Hagan, Lin et al. 2008).

Confirmation that the phenotypes observed in my research are specific to mTORC2 and not an mTOR independent function of RICTOR would be achieved by conducting parallel experiments with a conditional mSIN1 knockout allele *in vivo* or mSIN1 knockdown *in vitro*. A rescue of the *Rictor* knockdown phenotype with subsequent knockdown of RHOGDI2 would also support an mTORC2 independent role for RICTOR. It is notable, however, that the mTORC2 independent functions of RICTOR have centered around cell migration, while neither my *in vitro* or *in vivo* data recognized a migratory phenotype. Furthermore, the increased survival in *KPC* mice treated with mTORC1/2 inhibitors support an mTORC2-dependent rather than independent role for RICTOR in the support of KRAS-driven PDAC.

Translational Implications for Understanding mTORC2 Signaling in PDAC

For decades the treatment of pancreatic cancer remained stagnant. While the sequencing of the human genome and the advent of targeted therapies held great promise for the introduction of personalized therapies, the cures that were envisioned have been slow to materialize for pancreatic cancer. In recent years pancreatic cancer oncology has seen some movement; the addition of nanoparticle bound albumin–paclitaxel to gemcitabine, and the introduction of the more effective, albeit more toxic, FOLFIRINOX regimen, have greatly improved the treatment options for this disease. Although these drugs are cytotoxic at their

core and are not molecularly targeted therapeutics in the classical sense, the use of albumin bound–paclitaxel, which is likely sequestered by SPARC in pancreatic fibroblasts and successfully diminishes pancreatic stromal content, signals a movement towards smarter drug development (Al-Batran, Geissler et al. 2014). Furthermore, erlotinib, which targets the EGF-receptor, combined with gemcitabine, provides a robust clinical benefit (10.5 months) in a subset of patients, who present with a rash upon treatment (Moore, Goldstein et al. 2007). These therapies prove that smarter drug design and molecularly targeted therapeutics have the potential to dramatically improve outcomes for individuals diagnosed with PDAC. With significant efforts to target members of the PI3K/PDK1 pathway ongoing, an understanding of how mTORC2 regulates its downstream targets may provide great insight into the design of future therapeutics targeting these pathways. While the preventative *in vivo* effects of *Rictor* ablation are less robust than PI3K/PDK1 knockout, it is possible that this difference may allow for differential regulation of oncogenic signals, preventing the loss of anti-proliferative negative feedback signals. Furthermore, an understanding of the role played by CDKs in the response to mTORC2 and PI3K inhibition will be critical to predicting patient response to such targeted therapies.

My data suggest that specific targeting of mTORC2 may constitute a rational drug target that, in combination with other targeted therapies, including but not limited to mTORC1 and PI3K β inhibitors, would provide increased therapeutic options to PDAC patients. Further understanding of the mechanism

by which mTORC2 signaling enables KRAS-driven PanIN formation and tumor maintenance, combined with proper genetic screening of tumors, will enable treatment design that is specific to an individual's disease, and allow more accurate predictions of drug response.

Appendix A:

Additional Data Regarding the Impact of mTORC2 Signaling on the Initiation and Progression of KRAS-Driven Pancreatic Neoplasias

Preface

This appendix contains data that are relevant to a more complete understanding of the role of mTORC2 and its signaling in the context of KRAS-driven PDAC. These data are meant to complement and provide further insight into the findings presented in Chapter II, and have been discussed in Chapter III.

All data in this appendix were produced and analyzed by David Driscoll.

Results

***Rictor-null* PanIN Lesions Display Impaired mTORC2 Activity**

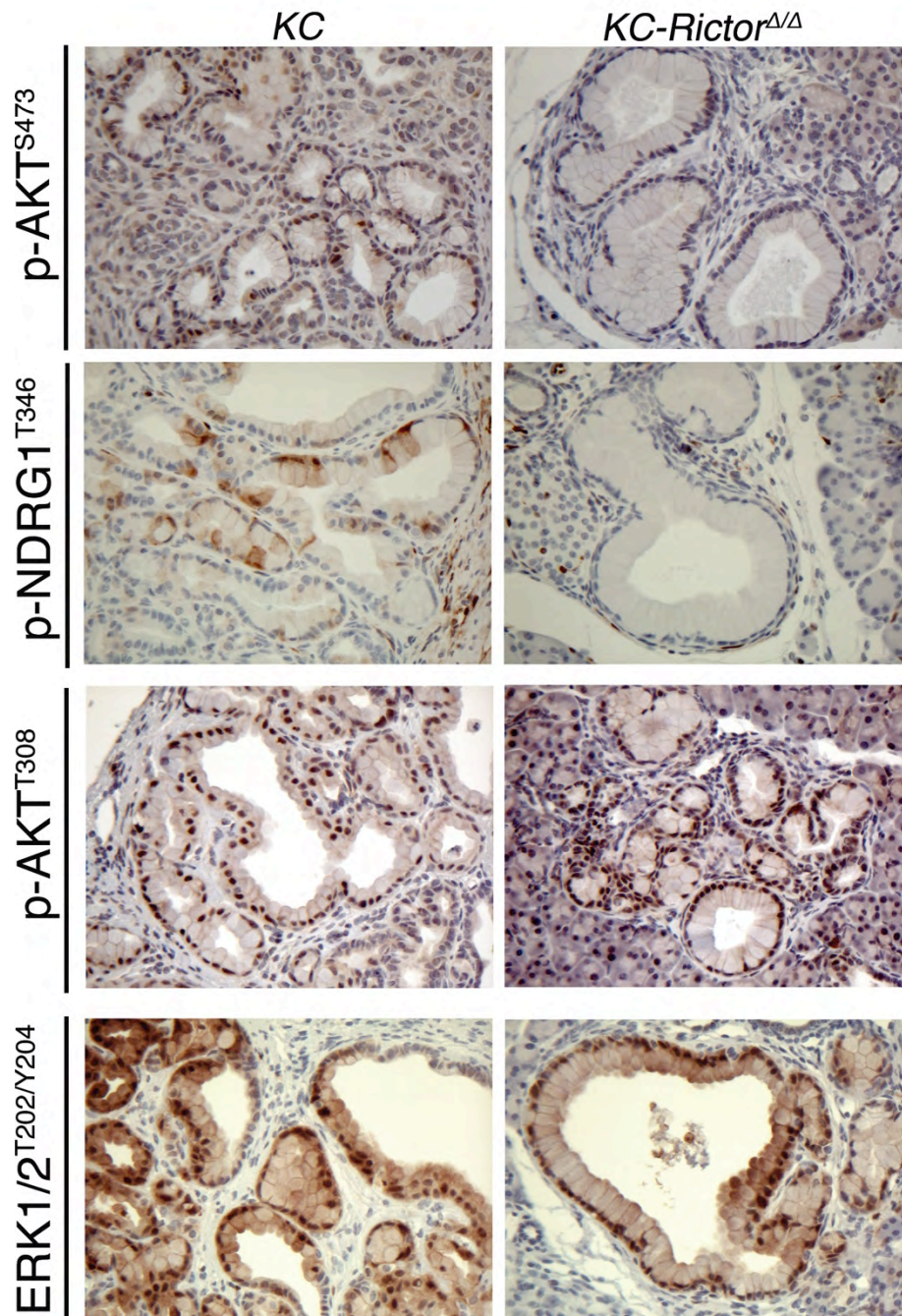
Although the inhibition of PanIN formation in *KC-Rictor^{Δ/Δ}* mice was dramatic, it was incomplete. I therefore hypothesized that PanIN lesions in these mice arose from cells in which KRAS was activated, but at least 1 *Rictor* allele escaped recombination. To determine the status of mTORC2 signaling in these lesions, pancreata from 4 and 8-month old mice (Figures 2.3 and A.1) were stained for targets of mTORC2 activity. Relative to their *KC* counterparts, *KC-Rictor^{Δ/Δ}* PanINs had significantly reduced mTORC2 signaling, as measured by p-AKT^{S473} and SGK1 activity measured by p-NDRG1^{T346} (Figures 2.3 and A.1). Reduced mTORC2 signaling is consistent in *KC-Rictor^{Δ/Δ}* PanINs observed at 4 and 8-month of age, thereby disproving my hypothesis and showing that early PanIN lesions were indeed formed and maintained in the absence of mTORC2 signaling.

Pharmacological attempts to inhibit mTOR activity have identified inhibitory feedback mechanisms which, when impaired, can negate the therapeutic benefit of mTOR inhibition. In PDAC cell lines, inhibition of mTORC1 via rapamycin, or inhibition of both mTORC1 and mTORC2 via mTOR active site inhibitors, resulted in increased AKT or ERK phosphorylation respectively (Soares, Ni et al. 2013). To better understand the effects of mTORC2 specific loss on oncogenic signaling in PanINs, these lesions were further characterized for PDK1 activity as measured by p-AKT^{T308}, and RAF/MEK/ERK activity as

measured by p-ERK1/2^{T202/T204}. No difference was observed in the levels of p-AKT^{T308} or p-ERK1/2^{T202/T204} in PanINs from 4 or 8-month old mice (Figures 2.3 and A.1). These data suggested that the effects of mTORC2 loss did not alter signaling of the MEK pathway or mTORC2 independent signaling of PDK1.

Figure A.1: PanIN lesions form in the absence of mTORC2 signaling.

IHC staining for the mTORC2 target p-AKT^{S473}, the SGK1 target p-NDRG1^{T346}, the PDK1 target p-AKT^{T308}, and the MEK target p-ERK1/2^{T202/Y204} at 4-months of age. 200X magnification.



mTORC2 does not regulate CDKI nuclear localization through TGF- β signaling

To determine the mechanism by which mTORC2 may regulate CDKI activity, I looked for alterations in pathways known to regulate multiple CDKI proteins. Signaling through the TGF- β pathway has been shown to increase expression of the p16^{INK4A}, p21^{CIP1} and p27^{KIP1} CDKIs (Reynisdottir, Polyak et al. 1995). Given the differences in stromal reaction between *KC* and *KC-Rictor* ^{Δ/Δ} pancreata, as well as the importance of TGF- β signaling in the tumor microenvironment and PDAC progression, I hypothesized that differential TGF- β signaling may be present between *KC* and *KC-Rictor* ^{Δ/Δ} PanIN lesions. However, immunostaining of p-Smad3 showed no difference in levels of canonical TGF- β pathway activity at 4 or 8-months of age (Figure A.2).

A second protein with the potential to coordinately regulate these CDKIs was the proto-oncogene B lymphoma Mo-MLV insertion region 1 homolog (BMI1). The inverse correlations between BMI1 and the CDKIs reported in Chapter II (Figures 2.5 and 2.6) were quantified using Pearson's product-moment coefficients (Pearson *r*). A Pearson *r* was calculated between BMI1 and each of the CDKIs for both 4 and 8-month sample sets. At 4-months of age, the inverse correlation between BMI1 and p16^{INK4A} was moderately strong with a P value of 0.0576, while the correlations between BMI1 and p21^{CIP1} and p27^{KIP1} were weak and not significant (Table A.1). At 8-months of age strong inverse correlations

were observed between BMI1 and p16^{INK4A}, p21^{CIP}, and p27^{KIP1}; these correlations were highly significant with P values of 0.0032, 0.0099 and 0.0068 respectively (Table A.1).

Figure A.2: TGF- β signaling, as measured by nuclear p-Smad3, was unaffected by *Rictor* loss in PanIN1 lesions.

IHC staining for p-Smad3 in *KC* and *KC-Rictor* $^{\Delta/\Delta}$ PanIN lesions from 4 (A) and 8-month (B) old mice. (C) Quantification of nuclear p-Smad3 staining in PanIN1 lesions of 4-month old mice (n=4). (D) Quantification of nuclear staining in PanIN1 lesions of 8-month old mice (n=3-4). P value: No significant differences observed. Arrows and arrowheads indicate representative positive and negative nuclei respectively.

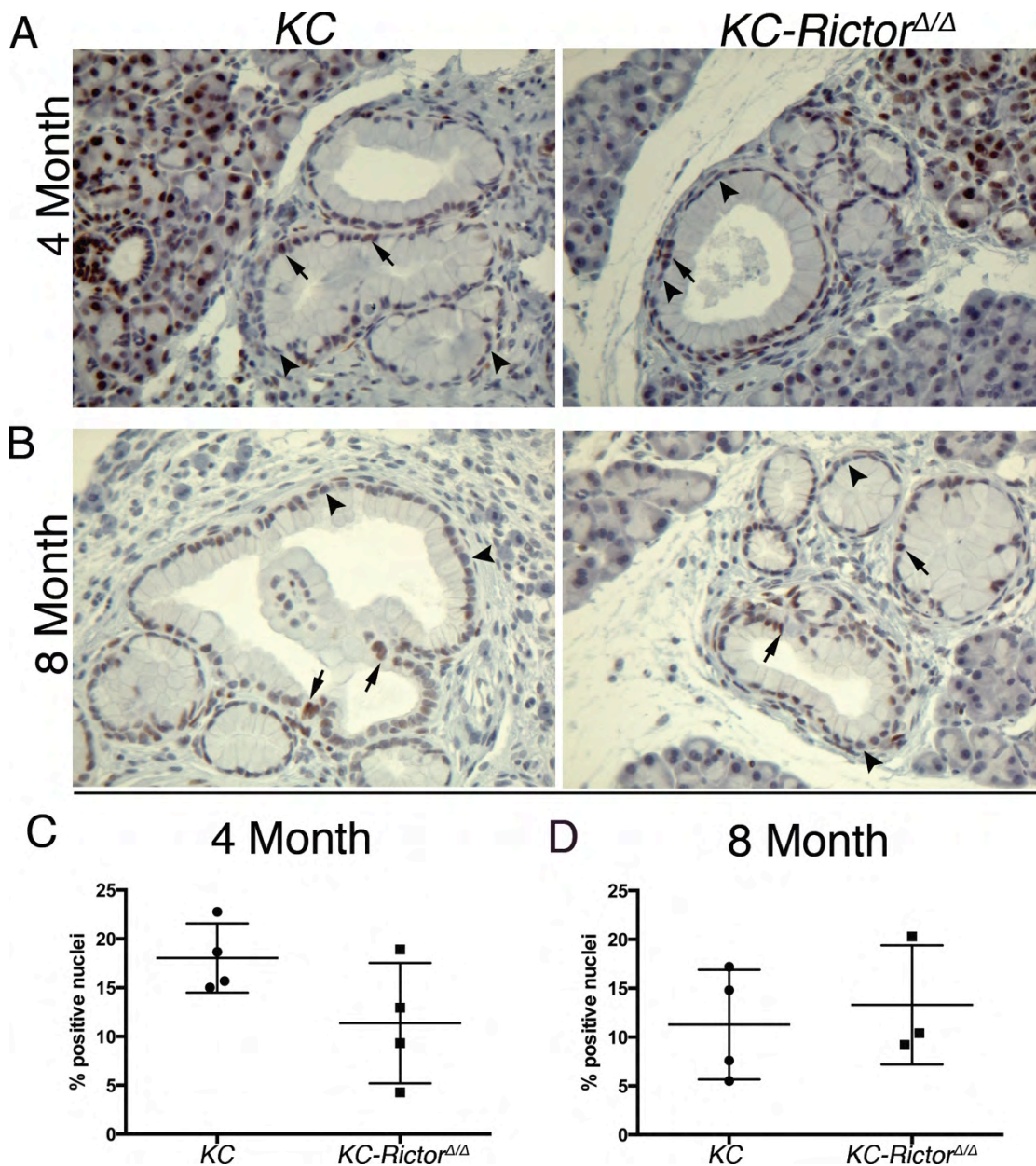


Table A.1 Quantification of Inverse correlation between BMI1 and CDKIs

Pearson's product moment coefficients were calculated between BMI1 and each individual CDKI (p16^{INK4A}, p21^{CIP}, and p27^{KIP1}) by comparing the percentages of positive nuclei within PanIN1 lesions for each sample harvested at either 4 or 8-months of age. Negative Pearson r values represent an inverse correlation and the correlation strengthens as the absolute value of r approaches 1.

		Pearson r	P value
4-month	p16 ^{INK4A} vs. BMI1	-0.6913252	0.05755543
	p21 ^{CIP} vs. BMI1	-0.3072842	0.4590833
	p27 ^{KIP1} vs. BMI1	-0.2802371	0.501417
8-month	p16 ^{INK4A} vs. BMI1	-0.9211026	0.003217379
	p21 ^{CIP} vs. BMI1	-0.8751158	0.009886242
	p27 ^{KIP1} vs. BMI1	-0.8928242	0.006812295

Impaired Proliferation and Induction of Senescence Following *Rictor* Knockdown in PanIN Derived Cell Lines

While staining of paraffin embedded tissues provides valuable insights regarding the *in vivo* consequences of *Rictor* knockout in the context of KRAS, they are observations of a static point in time. Cell lines provide an *in vitro* system, which, while artificial, can be manipulated and subjected to biochemical analysis. To assess mTORC2 signaling in a comparable *in vitro* model, I employed an shRNA mediated knockdown of *Rictor* in primary cell lines derived from KRAS-driven murine PanIN lesions. Efficient knockdown of *Rictor* in two such cell lines, RP 2294 and AH 2375, was confirmed by western blot in two cell lines generously provided by Dr. Nabeel Bardeesy (Corcoran, Contino et al. 2011) (Figure A.3 A,B) Given the proliferative phenotype and induction of CDKIs observed *in vivo*, I hypothesized that *Rictor* knockdown would impair proliferation and induce senescence *in vitro*. Consistent with this hypothesis, *Rictor* knockdown decreased proliferation, as measured by increased doubling time (Figures 2.5E and A.3C) and increased expression of senescence associated beta-galactosidase (Figures 2.5F, 2.6E and A.3D). Consistent with the lack of CDKI or BMI1 protein level changes observed following *Rictor* knockdown in AH2375 cells (Figure 2.6F,G), quantification of nuclear p21^{CIP1} and p27^{KIP1} by immunostaining showed no difference in the frequency of nuclear localization of these proteins in either the AH 2375 or RP 2294 PanIN cell lines following *Rictor* knockdown (Figure A.4). Notably p16^{INK4A} protein was not detected in the RP

2294 cell line, precluding its necessity for the senescence phenotype. While senescence was induced upon mTORC2 depletion, the absence of differential CDKI or BMI1 levels highlights a disparity between tissue culture and *in vivo* PanIN models. Therefore, reduction of RICTOR *in vitro* decreased proliferation and increased senescence in PanIN derived cell lines, but these *in vitro* phenotypes appear to be independent of nuclear expression of BMI1 or the observed CDKIs.

Figure A.3: *Rictor* knockdown impairs proliferation and induces senescence in PanIN derived cell lines.

(A,B) Western blots for RICTOR in of RP 2294 and AH 2375 cells 6 days post shRNA mediated knockdown of *Rictor* or a GFP control. α -tubulin was used as a loading control. (C) Doubling time of RP 2294 cells between 5 and 7 days post *Rictor* knockdown. (D) Quantification of beta-galactosidase positive RP 2294 cells. P value: * <0.05 , ** <0.01 , *** <0.001 .

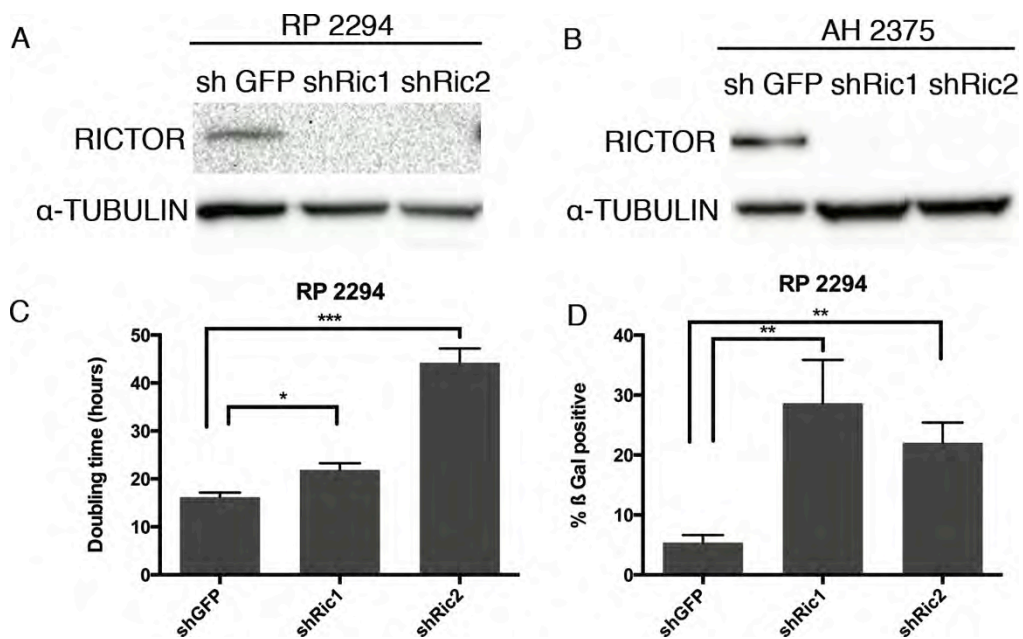
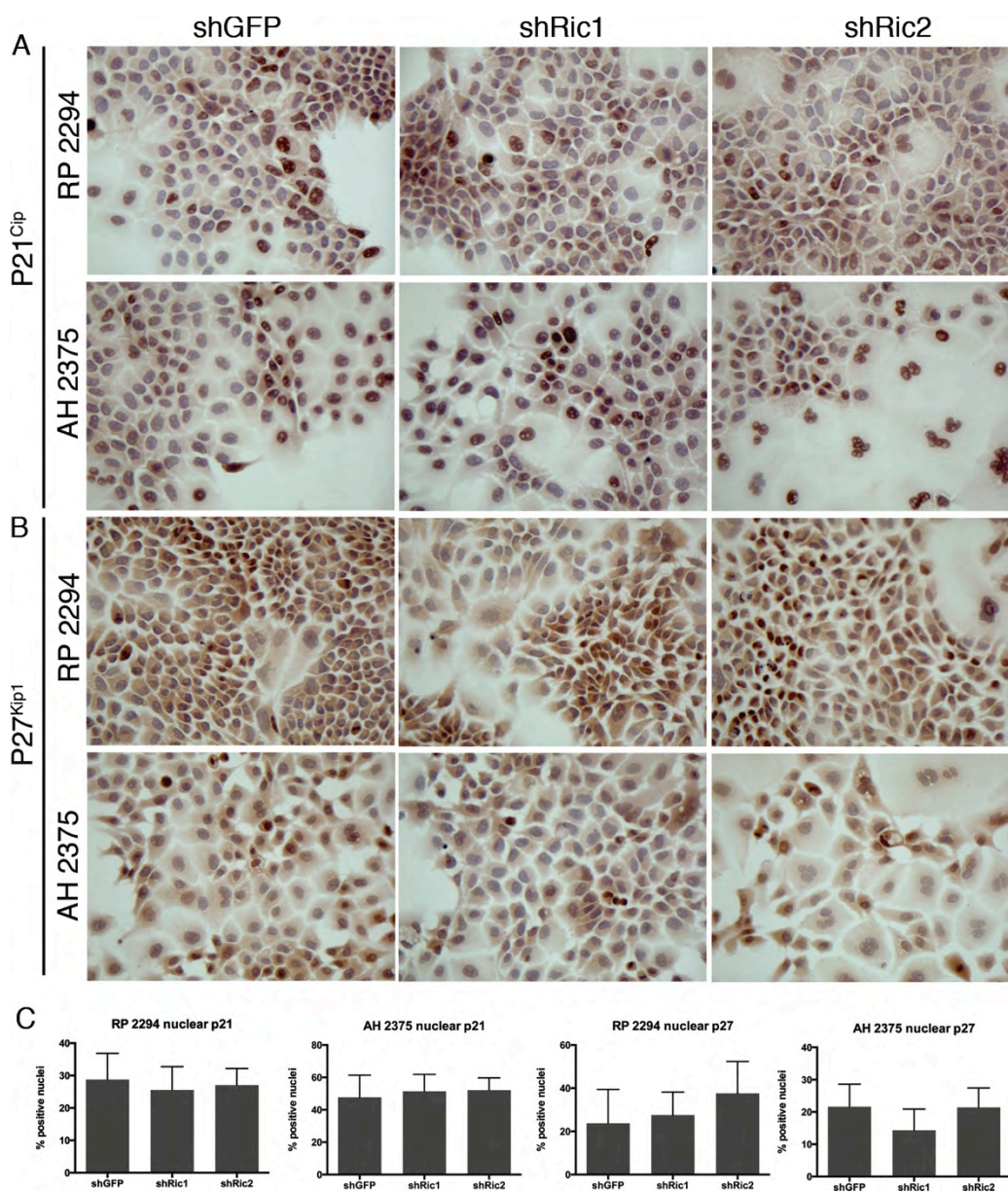


Figure A.4: Nuclear localization of CDKIs is unaffected by *Rictor* knockdown in cultured PanIN cells.

Immunocytochemical staining for p21^{CIP} (A) and p27^{KIP1} (B) in cultured PanIN cells from Pdx1-Cre;LSL-KRAS^{G12D} pancreata following shRNA mediated knockdown of *Rictor*. Quantification of nuclear p21^{CIP} and p27^{KIP1} in the indicated cell lines (C). No significant differences were observed.



mTORC2 Loss Impairs KRAS Mediated Responses to Pancreas Injury

Pancreatitis is a major risk factor for the development of PDAC and can be modeled in mice using caerulein induced pancreatic injury (Niederau, Ferrell et al. 1985, Malka, Hammel et al. 2002). Upon caerulein injury, acinar cells dedifferentiate, and, in the context of mutant KRAS these cells undergo acinar-to ductal-metaplasia (ADM) culminating in the formation of neoplastic ducts (Carriere, Young et al. 2009). ADM is a well-observed phenomenon in the *KC* model and, in some adult onset models of activated KRAS, ADM, driven by an inflammatory response, is required for initiation of PDAC (Guerra, Schuhmacher et al. 2007). Given the limited number of PanIN lesions in *KC-Rictor^{Δ/Δ}* pancreata, I hypothesized that mTORC2 signaling may be required for ADM, thus restricting the cells of origin for PanINs formed in *KC-Rictor^{Δ/Δ}* pancreata to ductal cells, and preventing their formation from acinar cells. The pancreatic-caerulein response was used to assess the requirement for mTORC2 in acinar cell trans-differentiation within the contexts of both wild-type and activated KRAS. To induce acute pancreatitis, mice were subjected to 2 sets of 7 hourly caerulein injections separated by a days rest.

Gross observation of *KC* pancreata 2 days post caerulein injection revealed enlarged organs with dissociated acinar clusters. On average, the ratio of pancreas weight to mouse weight in caerulein treated *KC* mice was elevated relative to PBS injected control mice when measured 2 days post injections (P

value = 0.0688) (Figure 2.6). Only 1 in 4 *KC-Rictor^{Δ/Δ}* pancreata showed a similar increase when observed 2 days post injections (Figure A.5). By 21 days, the average pancreas to body mass ratio for both *KC* and *KC-Rictor^{Δ/Δ}* caerulein injected cohorts were comparable to their respective controls measured 2 days post PBS injection (Figure A.5). At 21 days post injection, the caerulein treated pancreata of both phenotypes were fibrotic, as observed by necropsy.

Histopathological analysis and quadchrome staining of the pancreata 21 days post caerulein insult confirmed that the stromal composition was reduced in the *KC-Rictor^{Δ/Δ}* pancreata (Figure 2.8 A,B). In both genotypes, the deposition of collagen by pancreatic stellate cells, as measured by quadchrome staining 2 days post caerulein insult, was minimal (Figure A.6) and did not correlate with the rapid increase of pancreatic weight observed in *KC* pancreata 2 days post caerulein insult (Figure A.5). Interstitial pancreatic edema is a well-established response to caerulein injury (Grady, Saluja et al. 1996), and the rapid increase in organ mass over a short period of time and its subsequent resolution, suggests that this phenotype is the result of pancreatic edema. These data suggest that mTORC2 plays a role in regulating the response of KRAS mutant pancreata to injury.

Figure A.5: The rapid initial increase in KC pancreas mass following caerulein injection requires *Rictor*.

Following intraperitoneal injection of caerulein (50 µg/kg) or sterile phosphate buffered saline, administered in 2 sets of 7 hourly injections spaced 48 hours apart, the ratio of pancreas mass to mouse mass was determined at the time of harvest.

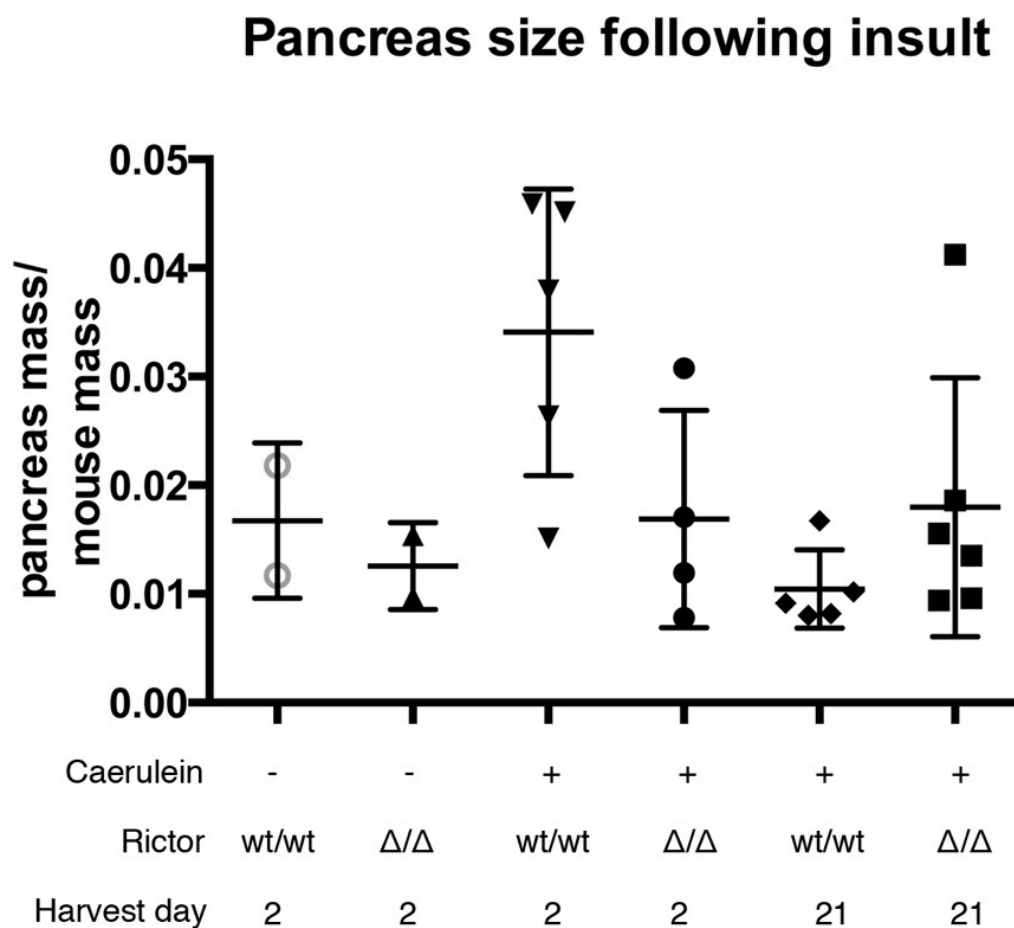
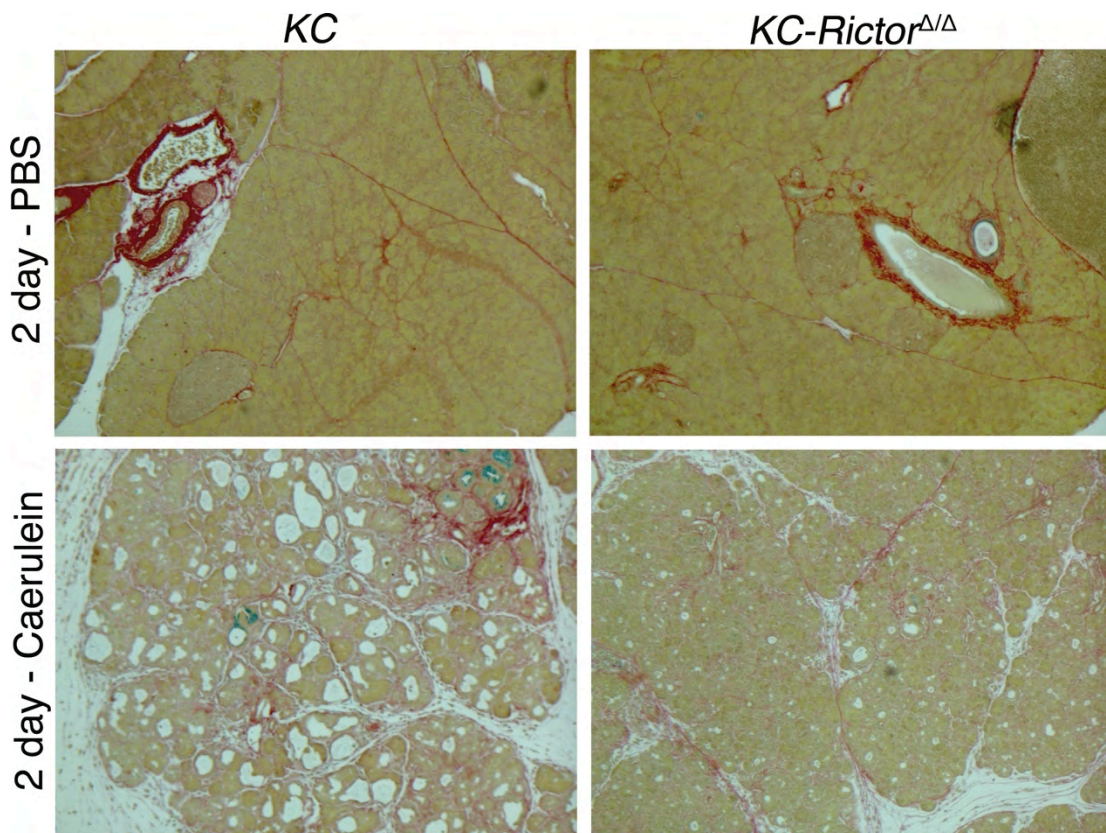


Figure A.6 Collagen deposition is unaffected by *Rictor* status directly following caerulein insult.

KC and *KC-Rictor*^{Δ/Δ} mice were subjected to intraperitoneal injection of caerulein (50 μg/kg) or sterile phosphate buffered saline, administered in 2 sets of 7 hourly injections spaced 48 hours apart. Quadchrome stains of pancreata from mice sacrificed 2 days after the second set PBS injections (top row), or 2 days after the second set of caerulein injections (bottom row). Red is collagen, blue is mucin, yellow is cytoplasm and grey is nuclear staining. All images taken at 50x magnification.

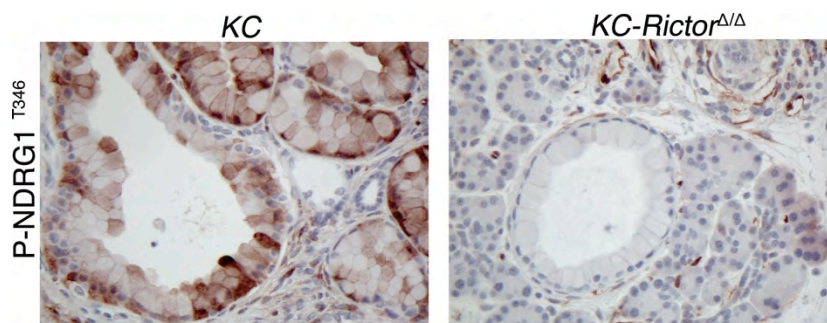


mTORC2 Loss Reduces SGK Signaling Following Pancreatic Injury

Signaling through the SGK pathway, as measured by immunostaining for p-NDRG1^{T346}, was reduced in *KC-Rictor*^{Δ/Δ} PanINs relative to their *KC* counterparts in pancreata harvested 21 days post caerulein injection (Figure A.7). These data complement findings presented in Figure 2.8C showing reduced p-AKT^{S473} levels in *KC-Rictor*^{Δ/Δ} following pancreatic insult. Together these data provide evidence that both these downstream readouts of mTORC2 activity are decreased in the absence of *Rictor* following pancreatic insult by caerulein, and are consistent with observations in uninjured 4 and 8-month old pancreata.

Figure A.7: mTORC2 signaling through SGK1 is impaired in *KC-Rictor*^{Δ/Δ} PanIN lesions 21 days post caerulein insult.

Pancreata harvested from *KC* and *KC-Rictor*^{Δ/Δ} mice 21 days post caerulein injection were stained by IHC for p-NDRG1^{T346}. Images taken at 200x magnification.



Nuclear BMI1 Ratios Inversely Correlated With Nuclear CDKIs in Caerulein Provoked *KC* and *KC-Rictor^{Δ/Δ}* PanIN1s

Given the accelerated context of PanIN development in my caerulein injected model, I sought to determine the role of mTORC2 loss on cell cycle progression in KRAS-driven PanINs following caerulein insult. Characterization of CDKI activity in these lesions was accomplished by immunohistochemical staining and quantification of positive nuclei within PanIN1 lesions and was described in Chapter II (Figures 2.8 and 2.9). The inverse correlations observed between BMI1 and the CDKIs were quantified by deriving Pearson's product-moment coefficients for nuclear BMI1 relative to each CDKI (p16^{INK4A}, p21^{CIP1} and p27^{KIP1}) in PanIN1 lesions from samples harvested 21 days following the second caerulein injection set (Table A.2). Both p16^{INK4A} and p27^{KIP1} showed strong, statistically significant, inverse correlations with BMI1, while the inverse relationship between nuclear p21^{CIP1} and BMI1 was weaker and not significant. These data provide correlative evidence suggesting that BMI1 is a potential mechanism by which KRAS may regulate these CDKIs in PanIN lesions.

Table A.2 Quantification of inverse correlation between BMI1 and CDKIs following caerulein insult.

Pearson's product moment coefficients were calculated between BMI1 and each individual CDKI (p16^{INK4A}, p21^{CIP}, and p27^{KIP1}) by comparing the percentages of positive nuclei within PanIN1 lesions for each sample harvested 21 days following the second set of caerulein injections. Negative Pearson r values represent an inverse correlation and the correlation strengthens as the absolute value of r approaches 1.

		Pearson r	P value
21 Days Post Caerulein Injection	p16 ^{INK4A} vs. BMI1	-0.8288	0.011
	p21 ^{CIP} vs. BMI1	-0.4259	0.2927
	p27 ^{KIP1} vs. BMI1	-0.8444	0.0083

KRAS-driven PDAC Cell Lines Require mTORC2 for Maintenance of Tumorigenic Properties

Having established a role for mTORC2 in the development and progression of PanIN lesions, I sought to provide insight into the worth of mTORC2 specific inhibition as a therapeutic intervention in established PDAC by assessing its role in tumor maintenance. I hypothesized that disruption of mTORC2 activity would impair the proliferative rates and anchorage independent growth abilities of PDAC cell lines. To determine the necessity for mTORC2 signaling in established PDAC, cell lines derived from LSL-KRAS driven tumors, designed to be deficient in *Trp53*, were infected with 2 independent shRNAs targeting the essential *Rictor* component of mTORC2. Puromycin selection began 30 hours post infection and cells remained in selection for the duration of these assays. Whole cell lysates were harvested, following 24 hour serum starvation and 15 minute re-stimulation, and showed robust knockdown of RICTOR. Reductions of the mTORC2 target p-AKT^{S473}, and SGK signaling through p-NDRG1^{T346} were consistent between both shRNAs in the *Cdkn2a*; *Smad4*-deficient 906 cell line (Figure A.8). Decreased p-AKT^{S473} was observed following knockdown of *Rictor* with either shRNAs in the *Trp53*-deficient 9910#1 cell line, however only one of the two Rictor targeting shRNAs reduced levels of p-NDRG1^{T346} (Figure A.8). The mTORC1 target p-S6K showed a 1.3 to 4.5 fold increase, with shRic 2 providing a greater increase in phosphorylation of S6K (Figure A.8). The MEK1/2 target p-ERK showed little change in the 9910 cells

and a moderate increase in the 906 cells following RICTOR knockdown (Figure A.8).

Anchorage independent growth, as measured by the formation of colonies in soft agar, and proliferation rate, inversely measured by doubling time, were significantly reduced upon *Rictor* knockdown in the KRAS-driven, *Trp53*-deficient, 6823#3 and 9415#1 (Figure A.9), consistent with the findings for 906 and 9910#1 cells presented in Figure 2.11.

Figure A.8: Knockdown of *Rictor* in PDAC cell lines reduced phosphorylation of mTORC2 targets but not mTORC1 or MEK targets.

Cell lines derived from KRAS-driven mouse PDAC were infected with lentiviral shRNAs targeting *Rictor*. At 4 days post infection cells were serum starved for 24 hours. Following starvation, cells were fed with 10% serum for 15 minutes and harvested for whole cell lysates. In addition to activated KRAS, 9910#1 cells are *Trp53*-deficient, and 906 cells lack *Smad4* and *Cdkn2a*. Numbers below the phosphorylated protein bands represent the quantification of band intensities for phosphorylated proteins normalized first to the corresponding total protein level and then to the normalized value for the shGFP control.

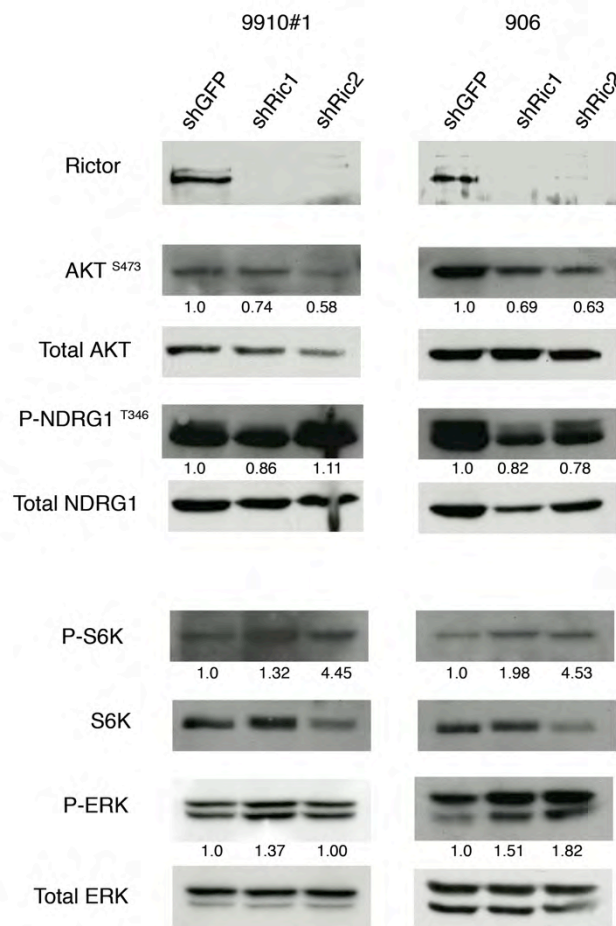
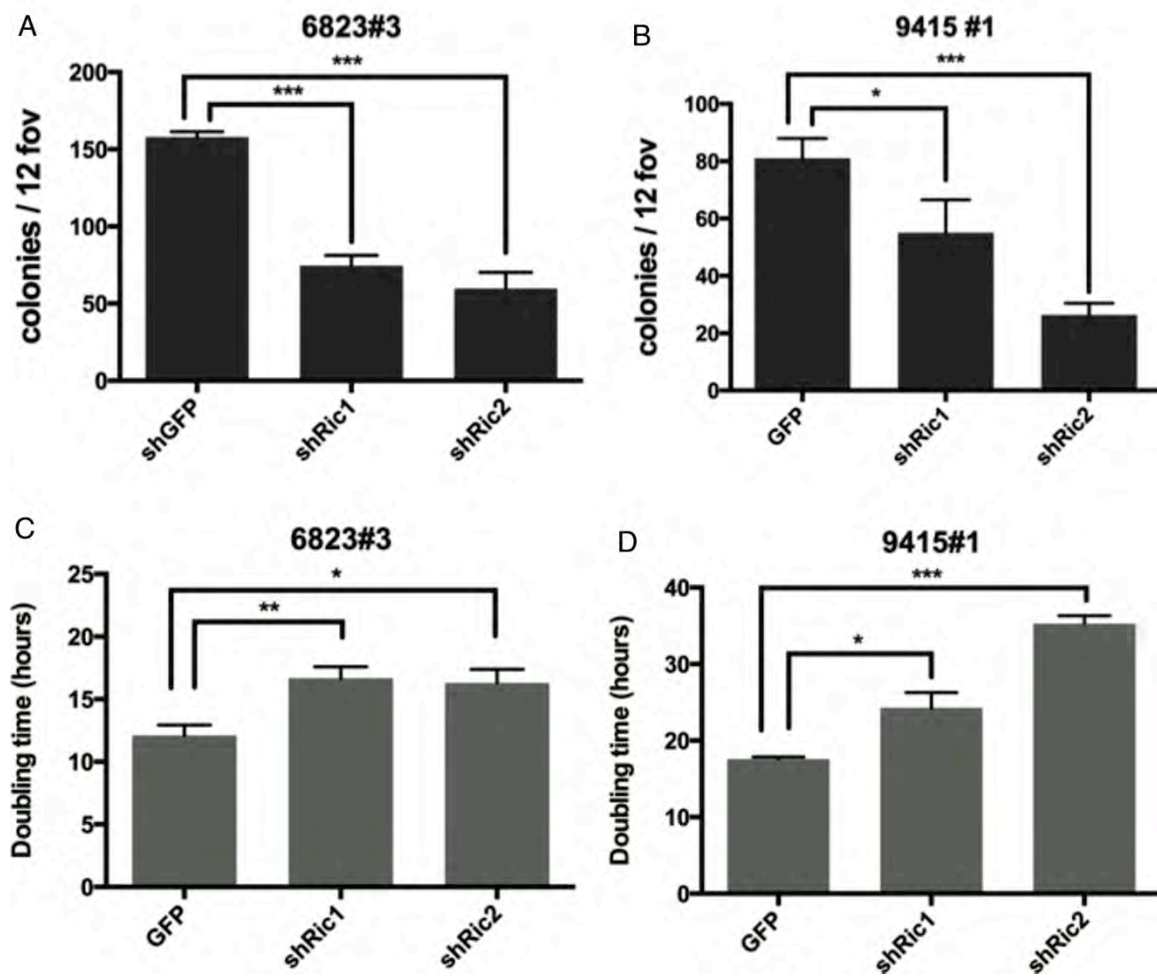


Figure A.9 Anchorage independent growth and proliferation of KRAS-driven PDAC cell lines is impaired by *Rictor* knockdown.

Following shRNA mediated Rictor knockdown and puromycin selection in 6823#3, and 9415#1 mouse PDAC cell lines, (A,B) colony formation in soft agar was measured by seeding 1×10^5 cells in 0.4% agar and allowed to grow for 2-3 weeks. Assays were plated in triplicate and 12 fields of view were counted for each plate. P values were derived via Student's T-test. (C,D) To measure proliferative rates cells were seeded at 1×10^4 cells/well. Counts were taken at 24 hour intervals for 72 hours. Proliferative rate was plotted on a \log_2 scale and doubling time was determined as the inverse slope of this line. P values were derived from the inverse slopes of doubling times using analysis of counter variance. Error bars represent 95% confidence intervals.

P values: * <0.05 , ** <0.01 , *** <0.001 .



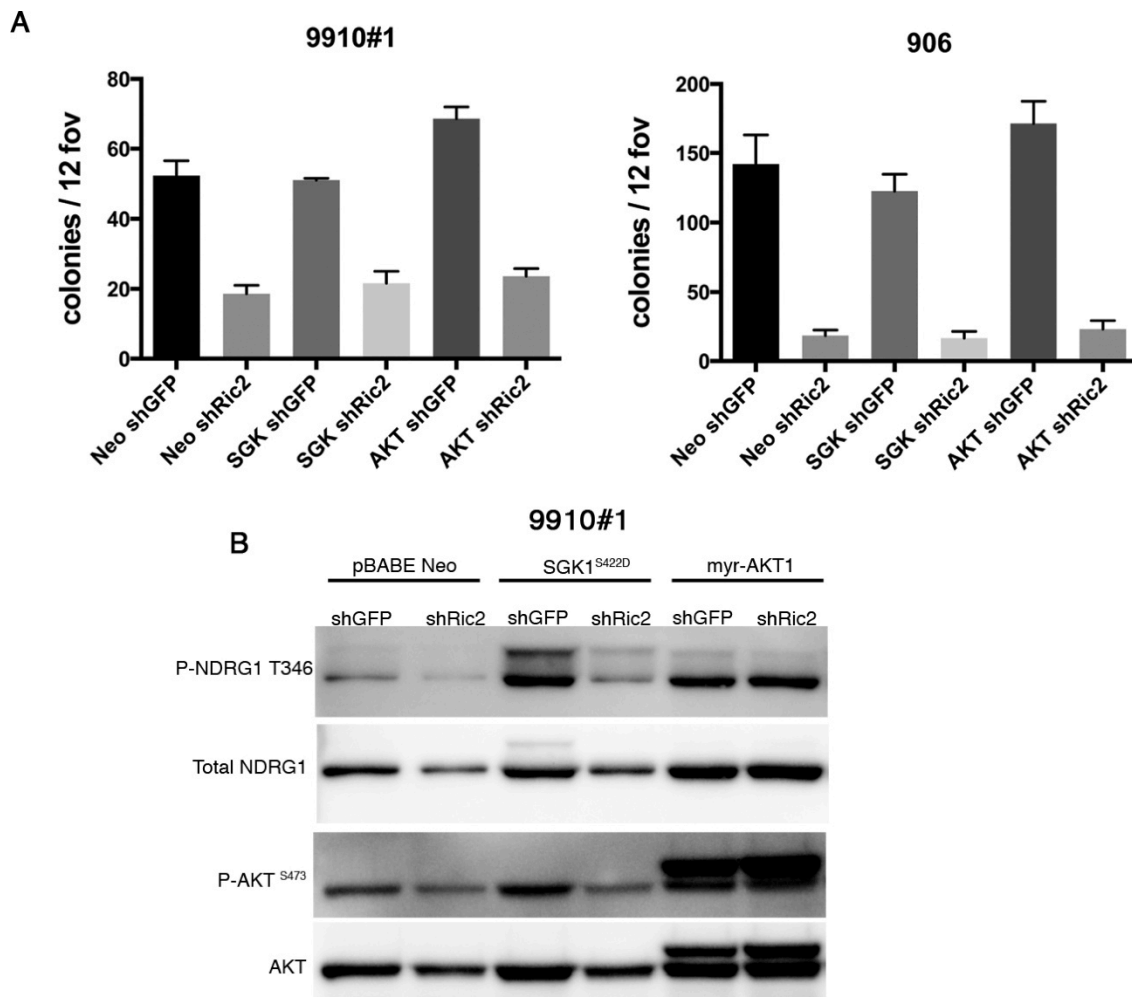
I hypothesized that constitutive activation of either AKT or SGK signaling would replace the oncogenic activity lost by mTORC2 ablation and rescue the anchorage independent growth deficit induced by *Rictor* knockdown. To assess the roles of these downstream mTORC2 targets, constitutively active SGK1^{S422D} and myristoylated AKT1 were ectopically expressed using a pBABE-neomycin retroviral system. Cells were maintained in selection for 4 days prior to lentiviral knockdown of *Rictor* after which cells were maintained in dual puromycin and neomycin selection for the remainder of these assays. Consistent with our previous findings, knockdown of *Rictor* decreased soft agar colony formation in pBABE-neomycin infected cell lines. Decreased anchorage independent growth mediated by *Rictor* knockdown was unaffected by ectopic expression of either SGK1 or AKT in either *Trp53* (9910#1) or *Cdkn2a/Smad4* (906) deficient cell lines (Figure A.10A).

Whole cell lysates harvested 5 days post infection, following 24 hours of serum starvation and 15 minute re-stimulation, were probed for levels of phosphorylated p-NDRG1^{T346} and p-AKT^{S473} via western blot as measures of mTORC2 pathway activity. Knockdown of *Rictor* decreased p-NDRG1^{T346} and p-AKT^{S473} levels in cells lacking ectopic expression of SGK1 or AKT1 (Figure A.10B). Phosphorylation of NDRG1^{T346} was increased by ectopic expression of SGK1^{S422D}, and this activity was reduced upon mTORC2 depletion. Surprisingly, myristoylated AKT1 also increased NDRG1 activity in an mTORC2

independent manner. Phosphorylated AKT⁴⁷³ levels were not altered by ectopic expression of SGK1^{S422D} and remained sensitive to *Rictor* knockdown (Figure A.10B). Expression of myristoylated AKT1 at levels similar to endogenous AKT resulted in an mTORC2 insensitive elevation of p-AKT⁴⁷³ levels. Moreover, in the context of myristoylated AKT, the faster migrating endogenous AKT⁴⁷³ was also insensitive to *Rictor* knockdown (Figure A.10B). Taken together these data suggest that the anchorage independent growth phenotype seen in PDAC cells is independent of AKT1. However, given that the constitutively active SGK1^{S442} was still susceptible to mTORC2 depletion, SGK1 mediated regulation of this phenotype cannot be completely ruled out.

Figure A.10: Following *Rictor* knockdown, neither AKT1 nor SGK1 rescued transformative ability of *KPC* cells.

KRAS^{G12D} driven mouse PDAC cell lines, 9910#1, and 906 were infected with either myristoylated-AKT1 or constitutively active SGK1^{S422A}. Cells were then selected with neomycin and infected with lentiviral shRNAs targeting *Rictor*. (A) Following puromycin selection 1x10⁵ cells/plate were seeded in 0.4% agar. Colonies were maintained in the presence of puromycin but not neomycin, and were counted between 2 and 3 weeks. (B) Protein levels of phosphorylated and total AKT and NDRG1 were probed by western blot.



Expanded Materials and Methods

Mouse strains

All procedures and care were provided under protocols approved by the University of Massachusetts Institutional Animal Care and Use Committee. The *Ptf1a*^{Cre} allele was developed and previously described by Kawaguchi et al. (Kawaguchi, Cooper et al. 2002); these mice were a gift from Chris Wright. The LSL-KRAS^{G12D} allele was developed and previously described by Jackson et al. (Jackson, Willis et al. 2001) and was obtained from Jackson laboratories. The conditional *Rictor* allele has also been previously described (Shiota, Woo et al. 2006), and was a gift from David Guertin. *Ptf1a*^{Cre/+};*LSL-KRAS*^{G12D/+};*Rictor*^{+/+} (KC) and *Ptf1a*^{Cre/+};*LSL-KRAS*^{G12D/+};*Rictor*^{Ex3cond/Ex3cond} (KC-*Rictor*^{Δ/Δ}) siblings were euthanized for analysis at 4 or 8-months of age. F2 of the *Rictor* heterozygous crosses provided *Ptf1a*^{Cre/+} (*Rictor*^{+/+}) and *Ptf1a*^{Cre/+};*Rictor*^{Ex3cond/Ex3cond} (*Rictor*^{Δ/Δ}) animals for the characterization of pancreatic *Rictor* knockout and caerulein injections, as well as KC and KC-*Rictor*^{Δ/Δ} animals for caerulein injections. *Rictor*^{+/+} and *Rictor*^{Δ/Δ} animals were euthanized at 2-months of age to assess the role of *Rictor* in pancreatic development.

Caerulein injections

Caerulein (Sigma C9026, 50 µg/kg) was delivered intraperitoneally into 5 - 7 week old mice in a series of two sets of 7 hourly injections separated by 42 hours. Injection of vehicle (0.05M Ammonium hydroxide in PBS) was used as a control. Mice were sacrificed at 2 or 21 days following the final injection series.

Histology

Following necropsy, pancreata were formalin fixed overnight, embedded in paraffin (FFPE), and 5-micron sections were mounted on positively charged slides. H&E staining was done according to field standard. For quadchrome staining, previously described staining methods for picro-sirius red staining for collagen (Junqueira, Bignolas et al. 1979, Whittaker, Kloner et al. 1994), and alcian blue staining for mucin (Steedman 1950) were adapted to allow co-staining of these components. Rehydrated slides were stained with fresh Wiegerts' hematoxylin (Sigma HT-1079) for 1 hour, alcian blue (2.5g alcian blue 8GX in 250ml 3% glacial acetic acid, pH 2.5) for 30 minutes, and sirius red staining (0.5g of Sirius Red (Sigma 365548) in 500ml of saturated picric acid (Sigma P6744)) for 1 hour. Each stain was followed by a brief wash in acid water (3% glacial acetic acid). Samples were rapidly dehydrated in ethanol and Xylene prior to mounting with PermountTM mounting media. In this stain, collagen appears bright red, mucin is a bright blue, nuclei are grey to black, and cytoplasm is a weak yellow.

A blinded histopathological analysis was conducted on H&E stained slides by Dr. Makoto Sano, a licensed pathologist. Tissue type composition was determined for 4 fields of view per sample; fields with the greatest and least neoplastic progression as well as two fields that were consistent with the average progression seen on that tissue section were quantified.

Immunohistochemistry

Five-micron sections from FFPE samples were deparaffinized and rehydrated by passage through Clear-Rite 3TM (Thermo Scientific, 6901) and a graded series of ethanols, 100% to 20%. Antigen exposure was accomplished using Antigen Unmasking Solution (Vector H-3300) and heating the samples to a gentle boil via microwave. This was followed by inactivation of endogenous peroxidases with 3% hydrogen peroxide, and overnight blocking with 3% normal goat serum or Mouse on Mouse blocking reagent (Vector labs M.O.M kit BMK-2202). Samples were incubated with primary antibody diluted into blocking solution as listed in Table 2.2 for 1 hour at room temperature. Subsequent incubation with biotinylated secondary antibodies for 1 hour at room temperature was followed by Avidin/biotinylated enzyme complex (Elite ABC, Vector labs PK-6101) incubation for 30min at room temperature and development using Nova-Red (Vector Labs SK4800). Specimens were observed using a Leica DM LB2 microscope.

For insulin staining, a FITC-conjugated donkey anti-guinea secondary (Jackson Immuno 706-095-148, 1:500) was used in lieu of a biotinilated antibody. Samples were mounted with dapi containing hard-set (Vector H-1500) and observed with a Nikon Eclipse E400 microscope.

Estimation of relative cell number and size

Total gDNA was isolated from a tissue fragment resected from the tail of the pancreas by standard phenol-chloroform extraction. The gDNA concentration of the tissue was determined by dividing the mass of the gDNA isolated by the weight of the fragment. The amount of total genomic DNA was extrapolated by multiplying the gDNA tissue concentration by the mass of the pancreas. These calculations assume equivalent amounts of gDNA in all cells.

To estimate cell size, 5 μ m thick sections from formalin fixed, paraffin embedded tissues were deparaffinized and stained with dapi (1:2000 in PBS) for 5 minutes. Followed by 2 washes in PBS. The number of dapi positive nuclei in 5-8 fields of view per sample was counted using ImageJ. The area of pancreatic tissue, measured in pixels, was determined via ImageJ, and an image of a hemocytometer was used to determine that 400 μ m² is equal to 450pixels and derive a conversion factor of 0.8888889 μ m²/pixel. The average number of pixels/cell was calculated and multiplied by this conversion factor to convert to μ m²/cell.

PanIN Cell culture experiments

Cell lines

Primary PanIN lines (AH 2375, RP 2294), a gift from Dr. Nabeel Bardeesly (Corcoran, Contino et al. 2011), were grown on laminin coated plates in media described by Schreiber et al in 2004 (Schreiber, Deramaudt et al. 2004) for culture of pancreatic ductal cells. Lentivirus was produced by transfection of HEK 293T cells (5×10^5 cells in a 9.5 cm^2 area) with pCMV delta 8.9 packaging plasmid ($0.3 \mu\text{g}$), pMDG envelope plasmids ($0.2 \mu\text{g}$) and a pLKO plasmid ($0.5 \mu\text{g}$) targeting either *Rictor* or GFP (Table 2.2). PanIN cell were subject to 2 lentiviral infections at 5-hour intervals. Cells were plated at concentrations of 2.7×10^4 cells/well in 6 well plates for proliferation assays, and β -galactosidase assays, and 1.3×10^5 cells/10cm dish for cell lysates. All cells were maintained in $3 \mu\text{g/ml}$ puromycin selection beginning 30 hours after the initial infection.

Proliferation assays

Viable cells were counted using trypan blue exclusion on days 4, 5 and 6 post-infection. Log_2 of cell/well numbers were plotted against time and doubling time was derived from the inverse slope. Significant differences in slopes, and thus doubling time, were determined by analysis of covariance using Prism Graphpad.

Senescence associated β -galactosidase staining

Senescence Associated β -Galactosidase activity was assessed 4 days post lentiviral infection. Cells were gently fixed, stained (40 mM Citric acid 0.1M/sodium phosphate 0.2M buffer pH 6.0, 5mM potassium ferrocynide, 5 mM potassium ferricynide, 150mM sodium chloride, 2mM magnesium chloride and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside 1mg/ml in water) for 16 hours at 37°C and counterstained with nuclear fast red (sigma N3020).

Western blots

Cell pellets for whole cell lysates were obtained from sub-confluent plates 6 days post infection. Cell pellets were lysed in fresh phospho-lysis buffer (10mM EDTA, 5mM EGTA, 10mM MgCl₂, β -glycerophosphate 50mM, nonidet P-40 0.5%, brij-35 0.1%, dithiothreitol 1mM, sodium orthovanadate 1mM, phenylmethanesulfonyl fluoride 1mM, CompleteTM Protease inhibitor cocktail 1 tablet/20ml recipe from Kelliher lab).

Protein lysates (15ug) were subject to SDS-Page in acrylamide gels and transferred onto polyvinylidene difluoride transfer membranes (Amersham Hybond, pore size 0.45 μ m, 45004110). Membranes were blocked in 5% milk in TBS+ 0.1% Tween 20 (TBST) for 1.5 hours at room temperature followed by overnight 4°C incubation in primary antibodies as listed (Table 2.1). Blots washed in TBST were incubated for 1 hour with peroxidase conjugated

secondary antibody, diluted 1:5000 and developed using Westernbright (Advansta, K-12045).

PDAC cell culture experiments

Cell lines

906, 9910#1, 9415#1, 6823#3 were cultured in Dulbecco's Modified Eagle Medium, 4.5 g/L D-Glucose, + L-Glutamine (Gibco 11965-092), with 10% Fetal Bovine Serum (FBS) and 1% penicillin streptomycin (Gibco 15140-122). PDAC cell lines were infected with a single round of lentivirus as described for PanIN cell lines. Cells were split and placed in selection (3ug/ul puromycin) 30 hours post infection and remained in selection for the duration of the experiments. All assays were plated 4 days post-infection.

Infection with retrovirus for constitutively active SGK1 or myristoylated AKT1 were preformed as described for PanIN cell lines, 6 days prior to lentiviral knockdown. Retroviruses were produced using the Effectene transfection reagent kit with 0.5 µg of SGK1^{S422D}-pBABE-neo, myristoylated AKT1-pBABE-neo, or empty vector pBABE-neo and 0.5 µg of ψ-eco, in 5x10⁵ phoenix cells plated on 6 well plates. 48 hours post transfection, viral supernatant (2 ml from each well) was filtered through a 0.45 micron syringe filter and 1.5 ml of filtered viral supernatant was combined with 15 µg of polybrene, added to the cells, and spun at 1800 RPM at room temperature for 45 minutes. The cells were then incubated for 4.25 hours at 37°C after which fresh DMEM with 10% FCS and 1%

antibiotics was added. Cells were subjected to 4 days of neomycin selection (1mg/ml G418 reagent) and then subjected to lentiviral knockdown as described above.

Anchorage independent growth assays

Soft agar assays were seeded at 1×10^5 cells/10cm dish in 3 ml of media with 0.4% agarose and 3ug/ml puromycin poured over solidified media with 0.7% agarose. Once solidified the cell suspension was covered with 7ml of complete media with 3ug/ml puromycin. Colonies were counted 2-3 weeks post plating using size exclusion criteria.

Proliferation assays

Proliferation assays were seeded in quadruplet at 1×10^4 cells/well in 24 well plates. Using trypan blue exclusion viable cells were counted at 24, 48 and 72 hours via hemocytometer. Doubling time and significance were determined as described above.

Western blots

Cell pellets for whole cell lysates were obtained from sub-confluent plates 5 days post infection following 24 hours of serum starvation and a 15 minute retreatment with media containing 10% FBS. Cell pellets were lysed in fresh phospho-lysis buffer (10mM EDTA, 5mM EGTA, 10mM MgCl_2 , β -

glycerophosphate 50mM, nonidet P-40 0.5%, brij-35 0.1%, dithiothreitol 1mM, sodium orthovanadate 1mM, phenylmethanesulfonyl fluoride 1mM, Complete™ Protease inhibitor cocktail 1 tablet/20ml recipe from Kelliher lab).

Protein lysates (15ug) were subject to SDS-Page in acrylamide gels and transferred onto polyvinylidene difluoride transfer membranes (Amersham Hybond, pore size 0.45 µm, 45004110). Membranes were blocked in 5% milk or bovine serum albumin (BSA) in TBS+ 0.1% Tween 20 (TBST) for 1.5 hours at room temperature followed by overnight 4°C incubation in primary antibodies as listed (Table 2.1). Blots washed in TBST were incubated for 1 hour with peroxidase conjugated secondary antibody diluted 1:5000 and developed using Westernbright (Advansta, K-12045).

Generation of Plasmids

pBABE-neo was a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg (Addgene plasmid # 1767) (Morgenstern and Land 1990). pBabe-Neo-Myr-Flag-AKT1 was a gift from William Hahn (Addgene plasmid # 15266) (Boehm, Zhao et al. 2007). pBABE-Puro-SGK1^{S422D}Δ60N was a gift from David Guertin and the SGK1^{S422D}Δ60N insert was cloned into the pBABE-neo backbone using the BamHI-HF and Sall-HF restriction enzymes from New England Biolabs.

Location of Reagents

Plasmids are located in the west – 20°C freezer located in 570P1 as are the all CST antibodies. All other antibodies are located in the 4°C glass front refrigerator located in 570P1. shRic1 was a gift from David Guertin. Ric2 was a gift from David Sabatini (Addgene plasmid # 21341)(Thoreen, Kang et al. 2009).

Table A3: Antibodies, dilutions and blocking medium used in IHC, IF and western blots

Target	Host	Company	IHC dilution	WB dilution	WB blocking
Amylase	rabbit	Abcam 21156	1:500		
Glucagon	rabbit	Dako A0565	1:50		
Insulin	guinea pig	Dako A0564	1:150		
pAKT ^{S473}	rabbit	CST 4060	1:400	1:2000	5% BSA
pAKT ^{T308}	rabbit	CST 4056	1:200		
Total AKT	rabbit	CST 9272		1:1000	5% BSA
pNDRG1 ^{T436}	rabbit	CST 5482	1:500	1:1000	5% BSA
Total NDRG1	rabbit	CST 9408		1:1000	5% BSA
pERK1/2 ^{T202/Y204}	rabbit	CST 9101	1:400	1:2000	5% BSA
KI67	rabbit	Abcam 66155	1:500		
Cleaved Caspase3	Rabbit	CST 9664	1:800		
p16 ^{INK4A}	mouse	Santa Cruz 1661	1:250		
p16 ^{INK4A}	rabbit	SC 1207		1:1000	5% milk
p21 ^{CIP1}	mouse	Santa Cruz 6246	1:250	1:1000	5% milk
p27 ^{KIP1}	mouse	Santa Cruz 1641	1:250	1:1000	5% milk
BMI1	rabbit	CST 5856	1:300		
BMI1	rabbit	Abcam 3829		1:2000	5% milk
RICTOR	rabbit	CST 1224		1:1000	5% milk
Alpha tubulin	mouse	DSHB 12G10		1:1000	5% milk

Table A.4 shRNA sequences targeting *Rictor* delivered in pLKO lentiviral constructs

Name	Target	Vector	Stem Sequence
shRIC1	<i>Rictor</i>	pLKO.1	5'-GCGGTTCATACAAGAGTTATT-3'
shRIC2	<i>Rictor</i>	pLKO.1	5'-GCCAGTAAGATGGGAATCATT-3'
shGFP	GFP	pLKO.1	5'-TACAACAGCCACAACGTCTAT-3'

Appendix B:

Pancreas Specific Loss of mTORC1 Impairs Pancreas Development

Introduction

mTOR kinase was originally discovered as the target of the drug rapamycin, a natural product isolated from *Streptomyces hygroscopicus* collected in soil samples from Easter Island (Vezina, Kudelski et al. 1975). Rapamycin works by simultaneously binding hydrophobic pockets in the mTOR protein and the FK506-binding protein (FKBP12) (Choi, Chen et al. 1996), thus creating a protein dimer whereby FKBP12 is inappropriately bound to the FRB domain of mTOR by rapamycin. mTOR kinase is known to be present in two functionally distinct complexes, mTORC1 and mTORC2. Rapamycin has been shown to be a specific inhibitor of mTORC1 (Thoreen, Kang et al. 2009, Kang, Pacold et al. 2013). While mTORC2 was originally believed to be insensitive to rapamycin, chronic treatment with rapamycin has been shown to inhibit mTORC2 activity levels in some cell types (Zeng, Sarbassov et al. 2007).

mTORC1 is a master regulator of cellular metabolic processes and is responsible for regulation of protein biosynthesis, ribosome biogenesis and autophagy in response to nutrient levels in the cell (Gibbons, Abraham et al. 2009). Interestingly, while mTORC1-dependent phosphorylation of S6K requires the FRB domain and is sensitive to rapamycin, phosphorylation of 4E-BP1 and ULK1 by mTORC1 are insensitive to rapamycin (Thoreen, Kang et al. 2009, Kang, Pacold et al. 2013). While the precise mechanism by which rapamycin provides its inhibition is still being worked out, it appears that the mTOR -

FKBP12 - rapamycin complex prevents the recruitment and positioning of some but not all substrates resulting in a selective inhibition for some mTORC1 targets (Shimobayashi and Hall 2014).

Although originally used as an anti-fungal and immune suppressant, rapamycin has since been investigated for its potential uses as an anti cancer therapy (Zaytseva, Valentino et al. 2012). The ability of rapamycin to decrease proliferation as well as its ability to specifically target one of the major downstream effectors of the PI3K-AKT signaling pathway made rapamycin a promising cancer therapeutic (Chiang and Abraham 2007). Encouraging results were observed in phase II trials with the rapalog everolimus with objective response rates of 47%, 30% and 12%, in Hodgkin lymphoma, non-Hodgkin's lymphoma and breast cancer respectively and phase II/III trials with the rapalog temsirolimus produced objective responses in 4 to 14% and 22% of endometrial cancers and mantle-cell lymphomas, respectively (Populo, Lopes et al. 2012). Temsirolimus is a more soluble analog of rapamycin that is processed into the natural form of the drug, and it has proven to be efficacious in treating renal cell carcinomas, a malignancy which is notoriously resistant to therapies and has been approved for treatment of this disease (Hudes, Berkenblit et al. 2009)

It has been proposed that tumors derived from cell types in which rapamycin also inhibits mTORC2 functions may be more sensitive to rapalogs, while in cell types with rapamycin insensitive mTORC2, release of negative feedback loops may enhance AKT signaling (Soares, Ni et al. 2013). It has also

been suggested that tumors with activated PI3K signaling may be “addicted” to mTORC1 activity and be sensitive to rapamycin and its analogs (Meric-Bernstam, Akcakanat et al. 2012). The PI3K-AKT signaling cascade is known to be involved in numerous human malignancies and correlates with reduced survival in multiple tumor types (Castellano and Downward 2011). Although 31% of renal cell carcinomas have decreased PTEN expression (Shin Lee, Seok Kim et al. 2003), the baseline levels of PTEN in renal cell carcinomas did not correlate with efficacy of Temsirolimus (Figlin, de Souza et al. 2009).

Human pancreatic tumors with low levels of the PI3K inhibitor PTEN have a poorer prognosis than those with high PTEN (Morran, Wu et al. 2014). Mouse-models show that *KC* driven PDACs deficient for PTEN are more sensitive to rapamycin treatment. *Pdx1-Cre; Kras^{G12D/+}; Pten^{flox/+}* animals responded better to rapamycin than the standard of care gemcitabine; however, tumors in *Pdx1-Cre; Kras^{G12D/+}; LSL-Trp53^{R172H}* animals were insensitive to rapamycin (Morran, Wu et al. 2014).

The tumor suppressor LKB1 is mutated in Peutz-Jeghers syndrome, which carries an increased risk of developing PDAC (Hezel and Bardeesy 2008, Wolfgang, Herman et al. 2013). In the context of low ATP levels, LKB1 has been shown to regulate mTOR1 activity in an AMPK, TSC1/2 dependent manner (Shaw, Bardeesy et al. 2004). Pancreas specific homozygous deletion of *Lkb1* was shown to be sufficient for the development of pancreatic cancer, and

haploinsufficiency in *Lkb1* cooperated with KRAS^{G12D} to accelerate PDAC formation (Morton, Jamieson et al. 2010).

Loss of mTOR kinase function due to ablation in the germ line results in embryonic lethality between day 5.5 and 6.5 (Gangloff, Mueller et al. 2004, Murakami, Ichisaka et al. 2004). mTOR-deficient embryos implanted but, being unable to proliferate or differentiate, they subsequently died (Gangloff, Mueller et al. 2004, Guertin, Stevens et al. 2006). Germ line loss of mTORC1 by ablation of the obligate subunit RAPTOR resulted in a similar phenotype (Guertin, Stevens et al. 2006); while animals with germ line loss of the mTORC2 obligate subunit RICTOR die around embryonic day 10.5 (Guertin, Stevens et al. 2006, Shiota, Woo et al. 2006).

Given the connection between efficacy of rapamycin on a subset of PDAC tumors as well as the cell type specific effect of rapamycin on mTORC2 activity, I became interested in the specific roles of mTORC1 and mTORC2 in the development of PDAC. Due to the lethality of germ line loss of mTORC1 and mTORC2, conditional knockout alleles for *Raptor* and *Rictor* were developed to aid in the observation of the role these compounds play in specific tissues (Shiota, Woo et al. 2006, Sengupta, Peterson et al. 2010). To address the differential role of these mTOR complexes during the development of PDAC, I combined these conditional models with the *Ptf1a*^{Cre/+};LSL-KRAS^{G12D/+} mouse-model (Hingorani, Petricoin Iii et al. 2003). While the role of mTORC2 in KRAS-driven PDAC is the central theme of this dissertation, the role of mTORC1 was

also of particular interest due to the cooperative effect of reduced LKB1 activity on KRAS-driven PDAC, and the effectiveness of rapamycin on KRAS-driven mouse-models deficient for PTEN. The incomplete inhibition of mTORC1 activity by rapamycin leaves the possibility that fully inhibiting mTORC1 activity might still be effective in treating PDAC. My ability to investigate the role of mTORC1 in the context of KRAS-driven PDAC was hampered by impaired pancreatic development and incomplete *Raptor* loss. The phenotype described in this appendix suggests that mTORC1 is required during the development of the pancreas.

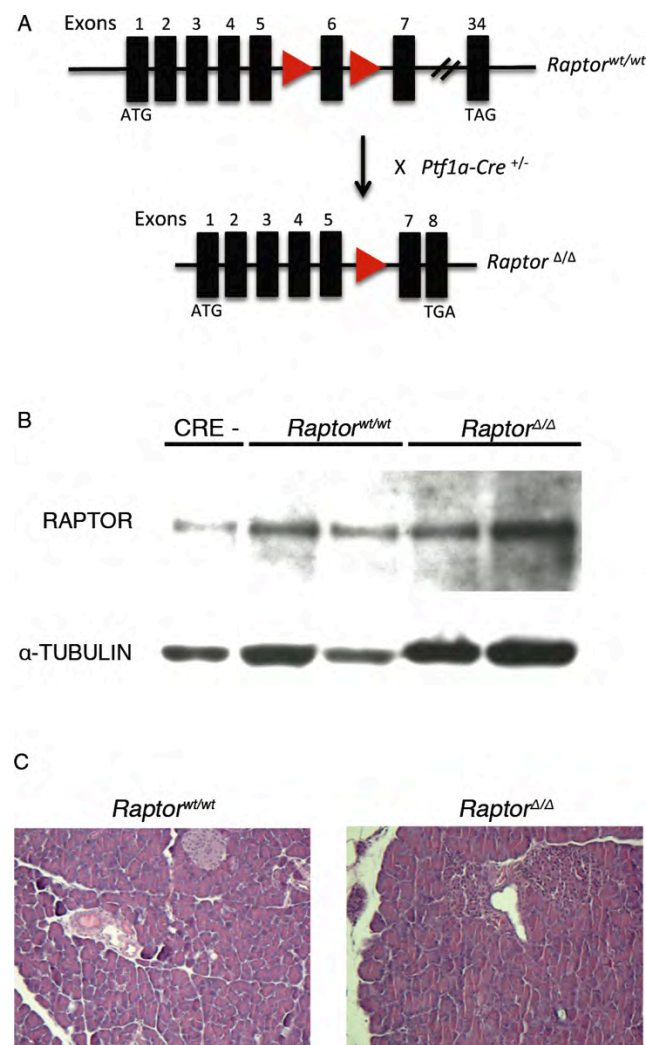
Results

Pancreata in *Raptor* Conditional Knockout Mice Retain RAPTOR

mTORC1 plays a central role in regulating protein and ribosomal biogenesis in response to cellular levels of nutrients, oxygen, and amino acids. Given the importance of mTORC1 in regulating both cell growth and autophagy, I hypothesized that mTORC1 may be required for pancreatic development. To address the specific requirement of mTORC1 in the development of the pancreas, I utilized a conditional knockout of the *Raptor* gene, which is an essential component of mTORC1. Pancreas specific knockout of the *Raptor* gene was achieved by crossing mice with LoxP sites flanking exon 6 of *Raptor* (Sengupta, Peterson et al. 2010), with the pancreas specific *Ptf1a*^{Cre} allele (Kawaguchi, Cooper et al. 2002). Upon expression of CRE at embryonic day 9.5, Exon 6 is excised resulting in a frame-shift and subsequent early termination in exon 8 (Figure B.1 A). To confirm pancreatic knockout of the *Raptor* allele, whole tissue lysate was prepared from *Ptf1a*^{Cre/+};*Raptor*^{+/+} (*Raptor*^{+/+}) and *Ptf1a*^{Cre/+};*Raptor*^{flox/flox} (*Raptor*^{Δ/Δ}) pancreata. Levels of RAPTOR protein were unchanged in these organs (Figure B.1B). Although the histology of the pancreatic tissue in *Raptor*^{Δ/Δ} mice was similar to *Raptor*^{+/+} pancreata, containing both endocrine and exocrine components (Figure B.1 C), *Raptor*^{Δ/Δ} pancreata were significantly smaller than their *Raptor*^{+/+} counterparts.

Figure B.1 *Raptor*^{Δ/Δ} mice form pancreata that retain RAPTOR expression.

- Schematic describing the conditional *Raptor* knockout allele and the frameshift and truncation resulting from exon6 excision by *Ptf1a* driven CRE recombinase.
- Western blots with pancreatic tissue lysates probed for RAPTOR or α-TUBULIN as a loading control.
- H&E staining of formalin fixed and paraffin embedded pancreatic tissue from 2-month old mice.



***Raptor*^{ΔΔ} animals and their pancreata were smaller than *Raptor*^{+/+} animals**

Raptor^{ΔΔ} pups were frequently runty and were noticeably smaller when weaned at 21 days of age. The body weight of males sacrificed at 2, 4, and 8-months of age was significantly lower in *Raptor*^{ΔΔ} mice at all time points (Figure B.2 A). The smaller mouse size correlated with lower pancreatic weight in *Raptor*^{ΔΔ} mice relative to *Raptor*^{+/+} mice of the same age (Figure B.2B). To determine if the decreased pancreas mass was simply a function of smaller mice, the mass of the pancreas relative to body mass was calculated for each mouse. This showed that *Raptor*^{ΔΔ} pancreata were indeed smaller than *Raptor*^{+/+} even when corrected for differences in body size (Figure B.2C). The pancreas to body mass ratio did not change significantly as mice aged (Figure B.2C).

***Raptor*-deficient pancreata are smaller in both sexes due to reduced cell numbers**

At 2-months of age the average body mass of female *Raptor*^{ΔΔ} mice was lower than *Raptor*^{+/+} mice, however, unlike males, this difference was not significant (Figure B.3A). Enrolling more female mice into this study may provide significance. Both the pancreatic weight and mass of the pancreas relative to body mass were significantly lower in *Raptor*^{ΔΔ} females at 2-months of age. While the weights of the female pancreata were generally lower than their male counterparts, the pancreas to body mass ratios were similar between the sexes

(Figure B.3 B-C). The decreased pancreatic proportions suggest that the decreased pancreas size was not simply a function of smaller animal size. Given the expression of *Raptor* in the residual pancreas and the similar histological appearance of the acinar tissue, I hypothesized that the difference in size was a result of decreased cell number in the pancreas. To test this, genomic DNA from a pancreatic fragment of known mass was isolated and quantified. The total amount of genomic DNA was extrapolated using the total mass of the pancreas. These results supported my hypothesis, showing that the total amount of genetic material in *Raptor*^{Δ/Δ} pancreata of both males and females was significantly less than in their *Raptor*^{+/+} counterparts (Figure B.3).

Figure B.2: Decreased animal weight and pancreatic mass in *Raptor*^{Δ/Δ} animals.

- A. Whole body weight of males sacrificed at 2, 4 and 8-months of age.
 B. Weight of pancreata from males sacrificed at 2, 4, and 8-months of age.
 C. Percentage of mouse weight comprised of pancreas mass in males sacrificed at 2, 4, and 8-months of age.
 P. values : **<0.01, ***<0.001. (n=4-10)

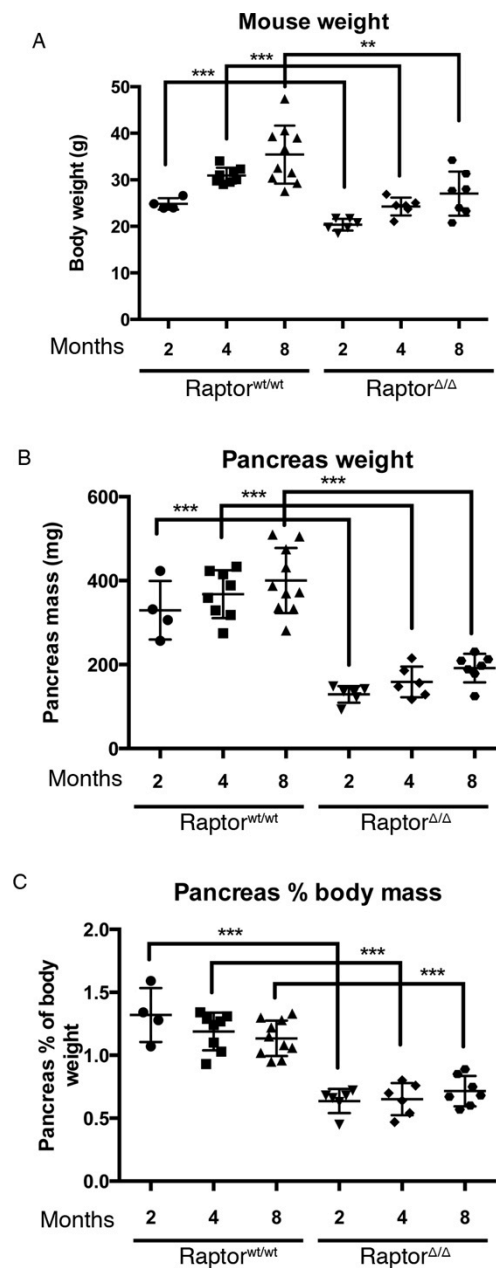
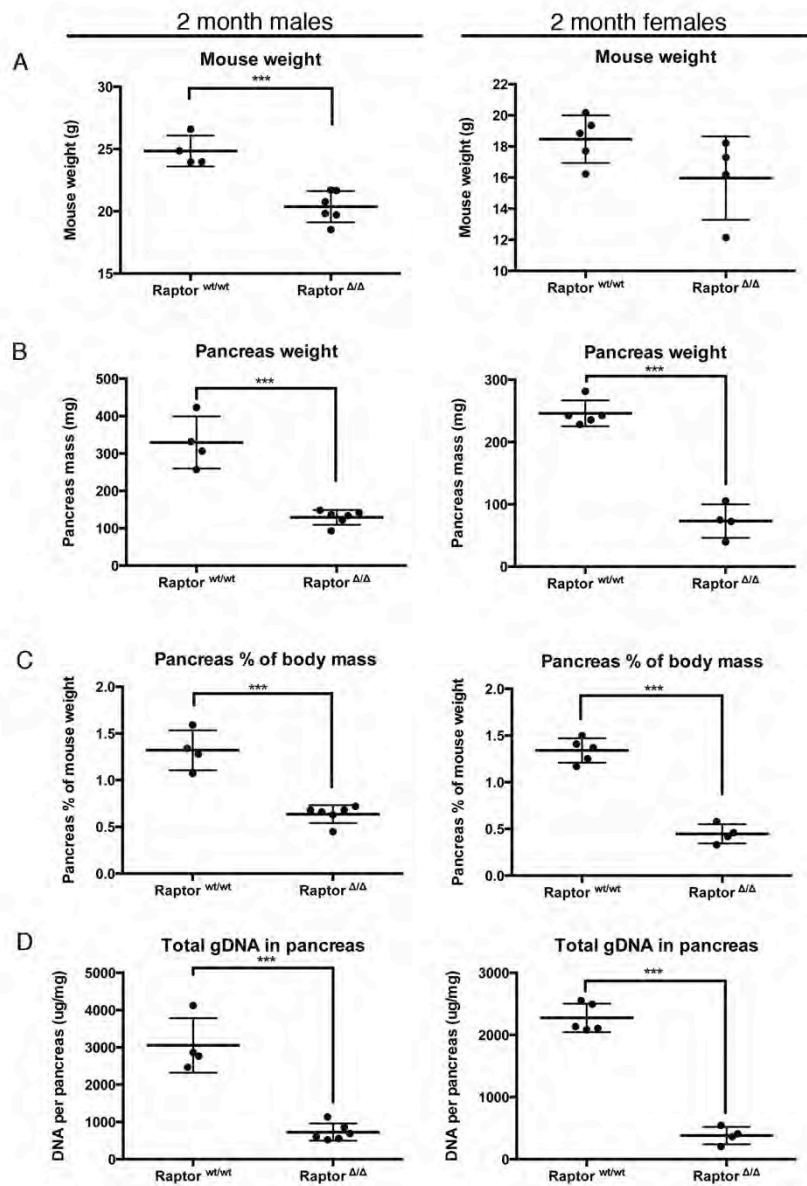


Figure B.3: Smaller pancreata in *Raptor*^{Δ/Δ} animals have fewer cells.

- A. Whole body weight of males and females sacrificed at 2-months of age.
 B. Weight of pancreata from males and females sacrificed at 2-months of age.
 C. Percentage of mouse weight comprised of pancreas mass in males and females sacrificed at 2-months of age.
 P values : ***<0.001. (n=4-6)



Size, proliferation, and apoptosis were similar in acinar cells of *Raptor*^{ΔΔ} animals

To characterize the pancreas *Raptor*^{ΔΔ} mice, I looked specifically at acinar cells as they comprised the majority of the pancreatic tissue and as such would play the greatest role in organ size. Furthermore, *Ptf1a* driven CRE expression should be maintained in acinar tissue and I hypothesized that delayed activation in a subset of these cells might reduce cell size or induce apoptosis. The relative size of acinar cells was similar between the genotypes in 2-month old animals, and no significant difference was seen in proliferation rates, as measured by KI67 staining, or apoptotic rates, as measured by cleaved-Caspase 3 staining (Figure B.4). These findings suggest that the acinar cells comprising the pancreata of *Raptor*^{ΔΔ} mice are of normal size and that the decreased organ mass is due to reduced cell numbers. The statistical power of these findings is low, as only 2 animals per cohort were analyzed.

***Raptor* knockdown impairs proliferation and anchorage independent growth in a PDAC cell line**

Although the maintenance of RAPTOR in the residual pancreas of *Raptor*^{ΔΔ} mice precludes the use of this model for observations of mTORC1 in the traditional *KC* mouse-model, shRNA mediated knockdown of *Raptor* in KRAS-driven tumor cell lines decreased protein levels of RAPTOR (Figure B.5 A) allowing for the observation of mTORC1 inhibition *in vitro*. Anchorage

independent growth in this KRAS-driven, *Trp53*-deficient cell line was significantly decreased by two independent shRNAs (Figure B.5 B). Proliferation rate was approximated by measuring the formazan produced by viable cells upon reduction of MTS tetrazolium. The doubling time, derived from the inverse slope of the natural log of the absorbance readings, trended towards significance, with one shRNA providing a significant increase in doubling time and the other near significance (Figure B.5 C).

Figure B.4 Cell size, proliferation, and apoptosis rates of acinar cells are unchanged in pancreata of *Raptor*^{Δ/Δ} mice.

- A. Relative area of acinar cells from 2-month old mice. (n=2)
- B. Percentage of KI67 positive acinar nuclei in 2-month old mice, stained by IHC. (n=2)
- C. Percentage of cleaved-Caspase 3 positive acinar nuclei in 2-month old mice, stained by IHC. (n=2)

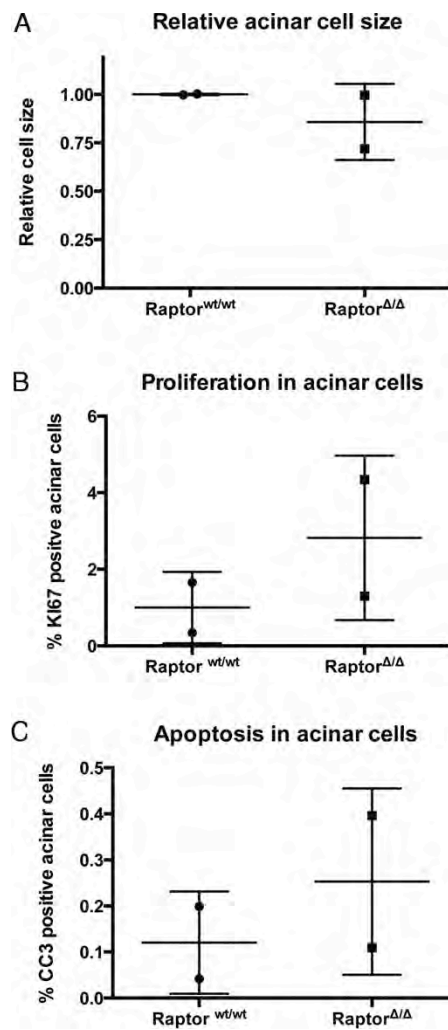
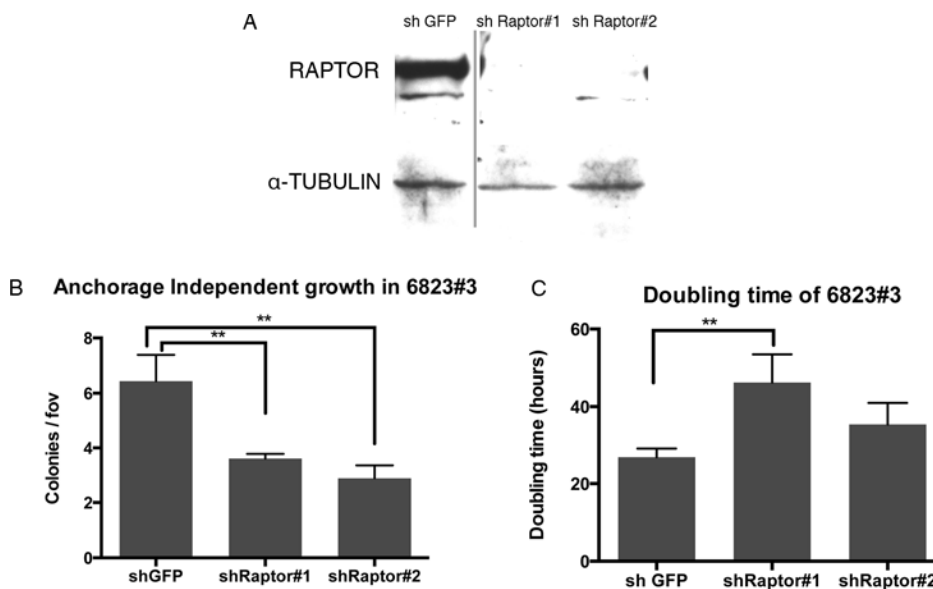


Figure B.5 Anchorage independent growth and proliferation are impaired by *Raptor* loss *in vitro*.

- A. Western blot with whole cell lysates of 6823#3 cells harvested 5 days post *Raptor* knockdown. Membranes were probed for RAPTOR or α -TUBULIN as a loading control. The vertical line indicates the movement of the GFP lanes from the same blot image to provide presentation consistency.
- B. Anchorage independent growth of 6823#3 cells following *Raptor* knockdown was measured by colony formation in 0.4% agar over 2 weeks. Assay was plated in triplicate and 12 fields of view per plate were counted. P.Values **<0.01
- C. Doubling time of cells as determined by metabolism of MTS tetrazolium to formazan by viable cells. Formazan levels were assessed by absorbance at 490nm 24, 48, 72, and 96 hours after seeding of 1×10^3 cells/well. Doubling time was determined as the inverse slope of the \log_2 of the absorbance values. P.Values were derived from the inverse slopes of doubling times using analysis of covariance of a line. Error bars represent 95% confidence intervals.



Discussion

Reduced pancreatic function in *Raptor*^{ΔΔ} animals

The pancreata obtained from two month old *Raptor*^{ΔΔ} animals retained expression of RAPTOR protein. This suggests that a subset of tissue has avoided CRE expression or CRE mediated excision of *Raptor* exon 6 at one or both alleles and that this subset has expanded to constitute a pancreas functional enough for these mice to survive. The decreased pancreas to body-mass ratio in these animals suggests that they are smaller due to the reduced pancreatic function and that reduced pancreas size is not a consequence of reduced animal size. Interestingly, pancreas size did not increase substantially relative to body mass as mice aged from 2 to 4 to 8-months, suggesting that the reduced pancreas size measured at 2-months of age meets the basic needs of the adult mouse; these pancreata either lack the capacity or the physiological impulse to regenerate a full sized pancreas. Measurements of relative proliferative and apoptotic rates in acinar tissue of 2-month old *Raptor*^{ΔΔ} males showed possible increases in both relative to their wild-type counterparts; however, due to the low number of samples tested these findings have little statistical value. If increases in proliferation and apoptosis in this tissue are substantiated it might suggest a cycle in which acinar tissues lacking CRE expression proliferate to maintain the pancreas while activation of CRE and subsequent loss of *Raptor* occurring in the adult acinar tissue results in

apoptosis. This cycle could prevent the expansion of the pancreas beyond the observed size.

Origins of the RAPTOR positive acinar tissue in *Raptor*^{ΔΔ} pancreata

The reduced size of *Raptor*^{ΔΔ} pancreata and the expression of RAPTOR therein suggested that mTORC1 was required for pancreatic development. The question posed by the expression of *Raptor* in the *Raptor*^{ΔΔ} pancreata is how it has avoided recombination. Possible mechanisms for the maintenance of *Raptor* include, silencing of the CRE expressing *Ptf1a* allele either through loss of *Ptf1a* expression or by failure to produce CRE recombinase following its transcription under the *Ptf1a*-promoter. Alternatively, changes to one of the floxed *Raptor* alleles might prevent CRE mediated recombination of exon 6. Mice lacking PTF1A expression failed to form pancreatic exocrine tissue and died within 2 days of birth (Krapp, Knofler et al. 1998). The possibility that acinar tissue is being reconstituted from a lineage that has not expressed PTF1A is unlikely as continued expression of this protein is required for the maintenance of acinar tissue and loss of PTF1A promoted ADM (Krah, De La et al. 2015). To address the question as to how these cells have avoided CRE recombination, these tissues could be stained for PTF1A expression, which should be present in acinar tissue, CRE expression, which, under the control of the *Ptf1a*-promoter, should also be expressed in the acinar tissue, and p-S6, to confirm the lack of functional mTORC1 in acinar tissues. From the results of this staining I could

ascertain if *Ptf1a* gene expression is silenced in these cells, if CRE expression is somehow impaired, or if recombination of *Raptor* is somehow blocked.

It is possible that mTORC1-deficient pancreata develop some level of exocrine tissue, which is sufficient to allow survival of these animals, and that in the absence of mTORC1 this exocrine tissue eventually dies or fails to proliferate. The pancreas is subsequently populated by trans-differentiation of cells, a small number of endocrine cells develop in the absence of PTF1A (Krapp, Knofler et al. 1998), which have retained *Raptor*. This possibility does not explain how acinar tissue is avoiding expression of *Ptf1a* driven CRE.

Ptf1a-null embryos lack pancreatic exocrine structures at embryonic day 18 (Krapp, Knofler et al. 1998). Morphological observations of *Raptor*^{Δ/Δ} pancreata at embryonic day 18 and in newborns would help to answer whether or not the pancreas is forming in the absence of mTORC1. If exocrine tissue in these embryos and newborns exists, staining for markers of mTORC1 activity, apoptosis, proliferation and autophagy should provide some insight into how these cells are responding to mTORC1 deficiency.

mTORC1 loss in the context of KRAS^{G12D}

The data presented suggest that loss of mTORC1 in KRAS-driven PDAC may have an effect on proliferation and tumorigenicity, however my ability to test this *in vivo* was obscured by a developmental phenotype. Mouse crosses which resulted in *KC-Raptor*^{Δ/Δ} animals were conducted and the pancreata of their

progeny displayed lesions consistent with *KC* pancreata. While the weight of these *KC-Raptor*^{Δ/Δ} pancreata was lower than their *KC* counterparts (data not shown), consistent with these pancreata originating from smaller organs, the possibility that mTORC1 deficiency is tolerated in the context of activated KRAS during pancreatic development has not been fully excluded. Due to the presence of extensive stromal infiltration, these tissues were not probed for RAPTOR via western blot, but immunostaining for p-S6 and p-4EBP1 could confirm the presence of RAPTOR in these pancreata.

Delaying mTORC1 loss until adulthood

While the data presented suggest that mTORC1 is required during pancreatic development, a more pertinent question with regard to pancreatic cancer is how the adult pancreas responds to loss of mTORC1 and how the adult pancreas responds to such a loss in the context of activated KRAS. To address this question a pancreas specific inducible Cre allele is being utilized,

Ptf1a^{CreERTM/+}; *Raptor*^{flox/flox} animals (Sengupta, Peterson et al. 2010, Kopinke, Brailsford et al. 2012) are being produced. By adding tamoxifen at 6 weeks of age, the requirement of mTORC1 for the maintenance of exocrine tissue in the adult pancreas can be observed. Furthermore, I will be able to assess the requirement of mTORC1 in KRAS-driven PanIN formation. Combining Flippase and Cre recombinase systems by generating *Pdx1-Flp*; *FSF-Kras*^{G12D/+}; *FSF-Rosa26*^{CreERTM/+}; *Raptor*^{flox/flox} animals (Sengupta, Peterson et al. 2010,

Schonhuber, Seidler et al. 2014) would cause pancreas specific activation of KRAS^{G12D} while allowing pancreas specific tamoxifen inducible loss of Raptor which could be delayed to observe the effects of mTORC1 loss during various stages of PanIN and PDAC. This would provide insight into the effectiveness of mTOR inhibitors and, combined with data from the similar mTORC2 model, may suggest benefits to targeting mTOR complexes independently as opposed to the combined or semi-combined inhibition observed in rapalogues and mTOR active site inhibitors.

Development of neurological phenotypes

One empirical observation in *Raptor*^{Δ/Δ} animals was an increased rate of hydrocephaly. Although determination of the pancreatic lineage is the first and best known function of PTF1A, this protein is also expressed in a neuroepithelial region of the embryonic hindbrain tail (Yamada, Terao et al. 2007), and during the development of GABAergic neurons in both the cerebellum and dorsal spinal cord (Glasgow, Henke et al. 2005). This phenotype was not investigated further, however it is plausible that hydrocephaly resulted from defects in the hindbrain or cerebellum due to the loss of *Raptor* and thus mTORC1 during their development.

Materials and Methods

Mouse Strains

All procedures and care were provided under protocols approved by the University of Massachusetts Institutional Animal Care and Use Committee. *Ptf1a*^{Cre/+} animals have been previously described and were obtained from Chris Wright (Kawaguchi, Cooper et al. 2002). Conditional *Raptor*^{fl/fl} alleles have also been previously described (Sengupta, Peterson et al. 2010), and were a gift from David Guertin. *Ptf1a*^{+/+}; *Raptor*^{fl/fl} (*Raptor*^{+/+}) and *Ptf1a*^{Cre/+}; *Raptor*^{fl/fl} (*KC-Raptor*^{Δ/Δ}) siblings were euthanized for analysis at 2-months of age.

Characterization of mouse and pancreas mass

The weight of each animal was measured immediately following sacrifice, after which the animals were perfused with 15-20 ml of phosphate buffered saline. The pancreas was harvested and weighed and the pancreatic percentage of body mass was calculated as the ((pancreas weight / mouse weight) x 100).

Estimation of relative cell number by DNA content

Total gDNA was isolated from a tissue fragment resected from the tail of the pancreas by standard phenol-chloroform extraction. The gDNA concentration of the tissue was determined by dividing the mass of the gDNA isolated by the weight of the fragment. The amount of total genomic DNA was

extrapolated by multiplying the gDNA tissue concentration by the mass of the pancreas. These calculations assume equivalent amounts of gDNA in all cells.

Histology

Formalin fixed, paraffin embedded pancreata from *Raptor*^{+/+} and *Raptor*^{Δ/Δ} animals were sectioned at 5 microns and mounted on positively charged slides. These sections were deparaffinized and rehydrated by passage through Clear-Rite 3™ (Thermo Scientific, 6901) and a graded series of ethanols, 100% to 20% followed by water. Samples were then stained for H&E according to the field standard.

Two samples each of *Raptor*^{+/+} and *Raptor*^{Δ/Δ} were immunostained for KI67 and cleaved caspase 3. Antigen exposure was accomplished using Antigen Unmasking Solution (Vector H-3300) and heating the samples to a gentle boil via microwave. This was followed by inactivation of endogenous peroxidases with 3% hydrogen peroxide, and overnight blocking with 3% normal goat serum or Mouse on Mouse blocking reagent (Vector labs M.O.M kit BMK-2202). Samples were incubated with primary antibody against KI67 (Abcam 66155) diluted 1:500 or CC3 (CST 9664) diluted 1:800 in blocking solution for 1 hour at room temperature. Subsequent incubation with biotinylated rabbit secondary antibodies (1:300) for 1 hour at room temperature was followed by Avidin/biotinylated enzyme complex (Elite ABC, Vector labs PK-6101) incubation for 30min at room temperature and development using Nova-Red (Vector Labs

SK4800). Specimens were counterstained in hematoxylin, dehydrated through graded ethanol to xylenes, fixed with Permount, and observed using a Leica DM LB2 microscope. Positive KI63 and CC3 cells were quantified in five 100x fields of view per sample with the exception of one *Raptor*^{Δ/Δ} sample for which only 2 fields of view were countable. The number of positive cells was compared to the total number of nuclei stained by hematoxylin.

Estimation of cell size

The average area of an acinar cell was calculated using the total number of cells counted in the KI67 quantifications, calculating the total area of acinar tissue in these images, measured in pixels using ImageJ, dividing the number of cells by the tissue area, and normalizing this ratio to the average cells/pixel in *Raptor*^{+/+} tissue.

Western blots

Whole tissue lysates were prepared from flash frozen tissue from the body of the pancreas, pulverized by mortar and pestle on dry ice. Cell pellets for whole cell lysates were obtained from 6823#3 cells 5 days post *Raptor* knockdown. Both powdered whole tissue and cell pellets were lysed in fresh phospho-lysis buffer (10mM EDTA, 5mM EGTA, 10mM MgCl₂, β-glycerophosphate 50mM, nonidet P-40 0.5%, brij-35 0.1%, dithiothreitol 1mM, sodium orthovanadate 1mM, phenylmethanesulfonyl fluoride 1mM, Complete™

Protease inhibitor cocktail 1 tablet/20ml recipe from Kelliher lab) for 2 hours at 4°C. Cell debris was cleared by centrifugation and the protein lysates were stored at -20°C.

Protein lysates (15µg) were subject to SDS-Page in acrylamide gels and transferred onto polyvinylidene difluoride transfer membranes (Amersham Hybond, pore size 0.45 µm, 45004110). Membranes were blocked in 5% milk in TBS+ 0.1% Tween 20 (TBST) for 1.5 hours at room temperature followed by overnight 4°C incubation with RAPTOR (Abcam ab40768) α-TUBULIN (DSHB 12G10) primary antibodies diluted 1:1000. Blots were triple washed in TBST, incubated for 1 hour with peroxidase conjugated rabbit or mouse secondary antibody respectively; both were diluted 1:5000. Membranes were then triple washed again and developed using Westernbright (Advansta, K-12045).

Lentiviral production and infection

Lentivirus was produced by transfection of HEK 293T cells (5×10^5 cells in a 9.5cm² area) with pCMV delta 8.9 packaging plasmid (0.3µg), pMDG envelope plasmids (0.2µg) and a pLKO plasmid (0.5µg) targeting *Raptor* or GFP (Table A.1). 6823#3 cells were subject to a single round of lentiviral infection which included a 45 minute room temperature spin at 1800 RPM followed by a 4 hour and 15 minute incubation at 37°C. Cells were placed in 3µg/ml puromycin selection 48 hours after the infection. Assays were set up and cell lysates were obtained 5 days post infection.

Anchorage independent growth assay

6823#3 cells were seeded at 1×10^5 cells/10cm dish in 3 ml of media with 0.4% agarose and 3 μ g/ml puromycin poured over solidified media with 0.7% agarose. Once solidified the cell suspension was covered with 7ml of complete media with 3 μ g/ml puromycin. Colonies were counted 2-3 weeks post plating using size exclusion criteria.

Proliferation as measured by MTS assay

In 6823#3 cells with *Raptor* knockdown, the ability of cells to metabolize MTS tetrazolium was used as to estimate the relative number of viable cells. Cells maintained in 3 μ g/ml puromycin selection were seeded in triplicate at 1×10^3 on a 96 well plate. At 24, 48, 72, and 96 hours media was replaced with complete media containing 16.7% MTS reagent (Abcam ab197010). The cells were incubated at 37°C for 30 minutes after which formazan produced by reduction of MTS tetrazolium was measured by absorbance at 490 nm. Absorbance of the MTS-media in the absence of cells was subtracted from the readings and the average OD 490 was plotted for *Raptor* knockdown verses GFP knockdown in 6823#3 cells.

Table B.1 shRNA sequences targeting *Raptor* delivered in pLKO lentiviral constructs

Name	Target	Vector	Stem Sequence
shRaptor1	<i>Raptor</i>	pLKO.1	5'-CCTCATCGTCAAGTCCTTCAAC-3'
shRaptor2	<i>Raptor</i>	pLKO.1	5'-GCCCCGAGTCTGTGAATGTAATC-3'
shGFP	GFP	pLKO.1	5'-TACAACAGCCACAACGTCTAT-3'

Raptor targeting shRNAs were a gift from David Guertin.

These plasmids are stored in the western -20 freezer in 570-P.

Appendix C:

KRAS^{G12D} Specific Gene Expression Changes in Primary Pancreatic Ductal Epithelial Cells

Introduction:

SHH is a ligand in the hedgehog family, which binds to the patched 1 (PTCH1) receptor, removing PTCH1 inhibition of the smoothened (SMO) transmembrane protein and subsequently activating GLI signaling (Hooper and Scott 2005). Although SHH is absent in the developing and mature pancreas (Kim and Hebrok 2001), its presence is observed in PanIN lesions and its expression increases as these lesions progress to PDAC (Berman, Karhadkar et al. 2003, Thayer, di Magliano et al. 2003). Previous work in our lab showed that when primary ductal epithelial cells (PDECs) lacking *Cdkn2a*, *Trp53*, or both were infected with KRAS^{G12D}, or SHH, the KRAS^{G12D} infected PDEC cells were capable of producing pancreatic tumors upon orthotopic injection into the pancreata of nude mice, while PDEC cells infected with SHH did not form tumors (Morton, Mongeau et al. 2007). Although infection with either SHH or KRAS^{G12D} increased the p-ERK and p-AKT levels in PDECs, the PDECs expressing SHH produced nothing more than microscopic lesions over a 4-month period (Morton, Mongeau et al. 2007).

Increased outputs of the ERK and AKT signaling pathways are hallmarks of KRAS-driven tumorigenesis, yet activation of these pathways was not sufficient for SHH infected PDEC to form tumors, even in the context of tumor suppressor deficiency (Morton, Mongeau et al. 2007). This provided an opportunity to identify the differences between the changes effected by activated KRAS and SHH. I hypothesized that differences in the gene expression profiles

of PDECs stimulated by KRAS and SHH would include genes required for KRAS-driven tumorigenesis. Identification of genes and pathways required for the transformative ability of RAS might provide novel sets of targets for therapeutic intervention.

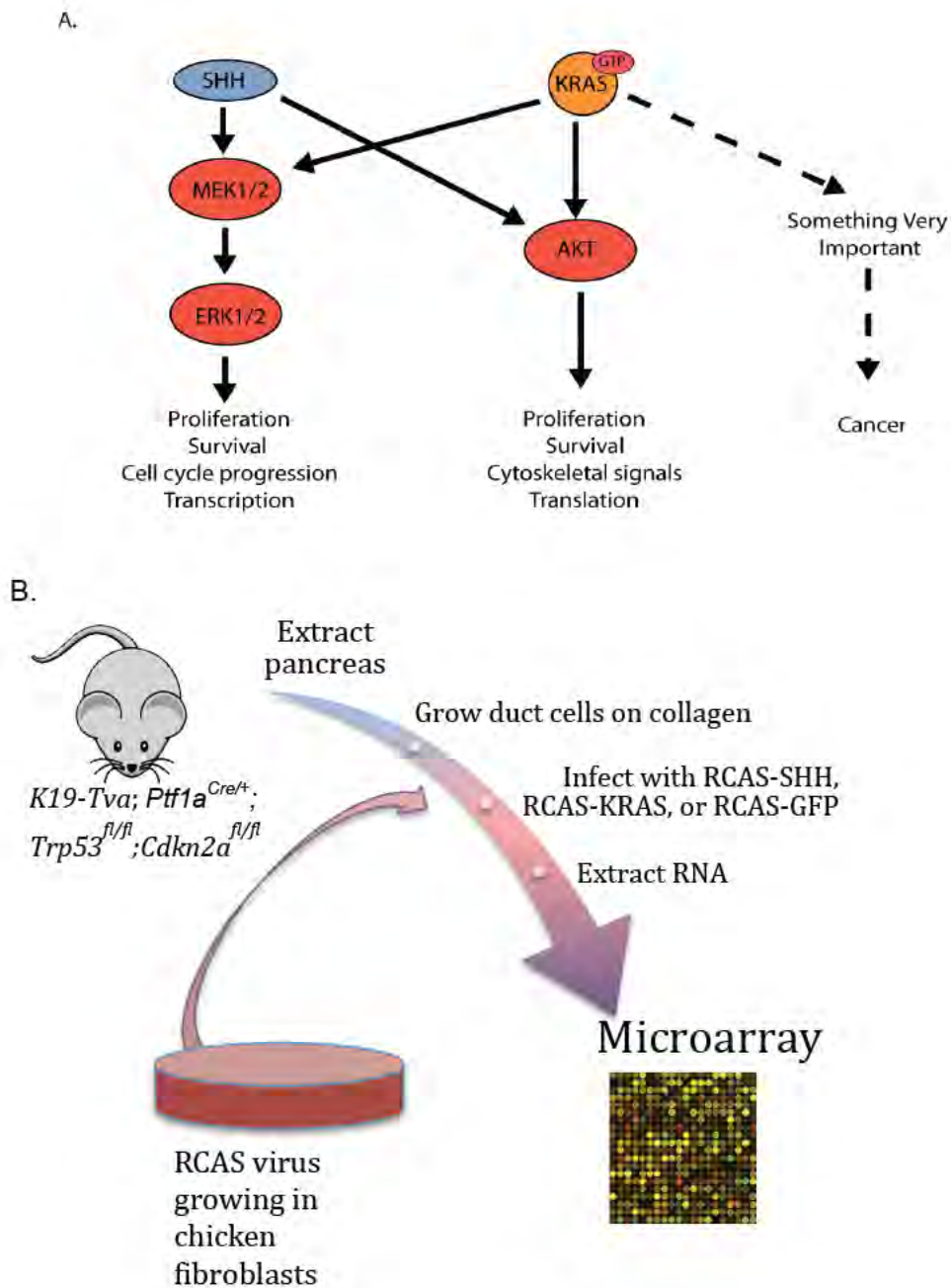
Results

Identification of genes differentially regulated by KRAS^{G12D} and SHH

Both KRAS^{G12D} and SHH increased p-AKT and p-ERK levels in PDEC, yet only KRAS^{G12D} was capable of imparting tumor forming potential to PDECs (Figure C.1A). To identify differential expression of genes resulting from KRAS^{G12D} and SHH expression in PDECs, I obtained gene expression profiles using an affymetrix microarray. PDEC cells expressing the avian retroviral receptor TVA under the cytokeratin-19-promoter, and lacking both *Cdkn2a* and *Trp53* as a result of *Ptf1a*-driven CRE expression during pancreatic development, were infected with retroviral RCAS viruses encoding KRAS^{G12D}, SHH, or GFP (Figure C.1). Three independent infections were conducted at passage 5, passage 8, and passage 9. Following each infection mRNA was isolated and stored at -80°C. Infections and RNA isolations were done by Victoria Appleman. The mRNA was processed by the Genomics Core Lab at Memorial Sloan Kettering Cancer Center and run on an MOE430 2.0 Affymetrix Expression Array to probe for mRNA levels of known and estimated genes (Figure C.1B). This Array contained 45000 probe sets for 39000 transcripts of 34000 genes. The density of the array was 1.3 probes per gene.

Figure C.1 Rational and method behind gene expression profiling.

(A) Both SHH and activated KRAS induce ERK and AKT activity however only KRAS activation potentiates PDEC cells to develop into PDAC *in vivo*. (B) Gene-expression analysis from *CK19-TVA;Ptf1a^{Cre/+};Trp53^{fl/fl};Cdkn2a^{fl/fl}* primary ductal cells infected with RCAS-SHH, -KRAS^{G12D}, or -GFP.



The changes in gene expression of KRAS^{G12D} relative to GFP infected PDECs were compared to changes in gene expression of SHH relative to GFP infected PDECs. The bioinformatics analysis was conducted by Julie Zhu and Jianhong Ou. Triplicate samples were compared as blocks and a threshold of $\log_2(1.5)$ fold change with an unadjusted P. value of 0.01 was used to identify genes for which the changes induced by KRAS^{G12D} relative to GFP infection were significantly different than the changes induced by SHH relative to GFP infection. By these criteria, expression of 693 genes were increased by KRAS and expression of 788 genes were decreased; these genes included both validated and predicted genes. Multiple probes were present for many of these genes, but only sterile alpha motif and leucine zipper containing kinase AZK (ZAK) had probes identified in both data sets.

To select targets for validation, these data sets were curated using a combination of gene ontology, and a review of published literature. Gene ontology analysis was conducted using the functional annotation clustering tool in the DAVID bioinformatics resource (Figure C.2) (Huang da, Sherman et al. 2009, Huang da, Sherman et al. 2009). Using the pathways flagged by the gene ontology analysis as a guide, a review of published literature was used to select 29 gene targets known to be involved in or related to pathways involved in cancer, that were up regulated in KRAS infected cells relative to their SHH infected counterparts. These genes were validated by qPCR (Figure C.3) and included secreted factors, genes involved in epithelial to mesenchymal transition,

growth factor receptors, Wnt family ligands, and Fmo gene family responsible for metabolism of xenobiotics (Lawton, Cashman et al. 1994). With the exception of *Mmp20*, elevated expression of mRNA was confirmed for all genes of interest in passage 5 (data not shown) and passage 8 KRAS infected cells (Figure C.3).

To further investigate the roles these proteins were playing in KRAS-driven PDAC, shRNA mediated knockdown of selected targets was conducted in cell lines derived from KRAS-driven murine pancreatic tumors. Based on the notion that genes involved in transcriptional regulation may be drivers of the observed differential gene expression, Gli2, Id3, Tbx3, and Yap1 were selected for knockdown. Gli2 and Yap1 are transcription factors while ID3 and TBX3 are transcriptional repressors. All four of these genes have been implicated in cancer development and metastasis, and had multiple shRNAs available from the UMass shRNA core (Narita, So et al. 2008, Yarosh, Barrientos et al. 2008, Hall, Wang et al. 2010, Liu, Xu et al. 2010, Shuno, Tsuno et al. 2010, Javelaud, Alexaki et al. 2011, Mowla, Pinnock et al. 2011). Following infection of 170#3 cells, derived from a tumor resulting from orthotopic transplant of KRAS infected *Trp53^{-/-}*, *Cdkn2a^{-/-}* PDECs into the pancreas of athymic nude mice, with lentiviral constructs targeting these four genes, consistent knockdown of Yap1 was achieved with multiple independent shRNAs (Figure C.4-A). The shYapB and shYapE constructs were the most effective and were used for phenotypic assays. Knockdowns of Gli2, Id3, and Tbx3 were unsuccessful despite testing with all

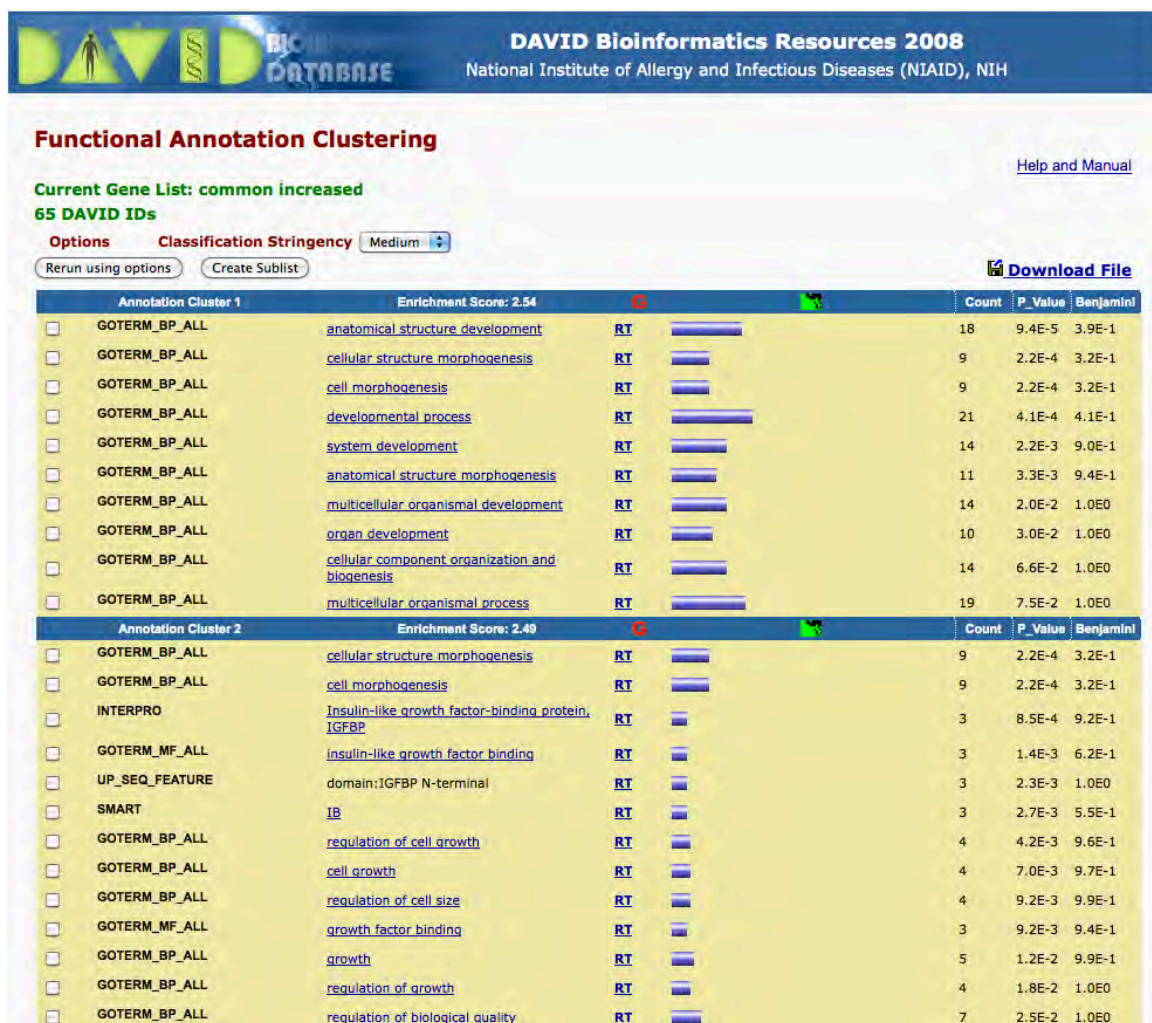
available shRNAs designed to target these genes in the UMass shRNA library (data not shown).

Knockdown of YAP in the 170#3 cell line was confirmed by western blot (Figure C.4-A). To characterize relevant phenotypic changes following loss of YAP, proliferation, migration, and soft agar colony formation were measured. YAP knockdown significantly decreased anchorage independent growth as measured by soft agar colony formation (Figure C.4-B). However, Yap knockdown did not provide consistent changes to migration or proliferation rates as measured by trans-well migration towards a chemo attractant and MTS assay respectively (Figure C.4-C-D).

Figure C.2 Gene ontology analysis of microarray data sets

- Functional annotation clustering of transcripts significantly increased in KRAS infected PDECs relative to SHH infected PDECs.
- Functional annotation clustering of transcripts significantly decreased in KRAS infected PDECs relative to SHH infected PDECs.

A.



A. (Continued)

Annotation Cluster 3		Enrichment Score: 1.94		Count	P_Value	Benjamini
<input type="checkbox"/>	INTERPRO	von Willebrand factor, type C	RT	4	1.6E-4	6.1E-1
<input type="checkbox"/>	SP_PIR_KEYWORDS	heparin-binding	RT	4	3.6E-4	2.7E-1
<input type="checkbox"/>	SMART	VWC	RT	4	5.2E-4	2.7E-1
<input type="checkbox"/>	GOTERM_MF_ALL	heparin binding	RT	4	6.1E-4	8.1E-1
<input type="checkbox"/>	GOTERM_MF_ALL	carbohydrate binding	RT	6	1.2E-3	8.1E-1
<input type="checkbox"/>	GOTERM_MF_ALL	glycosaminoglycan binding	RT	4	1.3E-3	7.0E-1
<input type="checkbox"/>	UP_SEQ_FEATURE	domain:VWFC	RT	3	1.6E-3	1.0E0
<input type="checkbox"/>	GOTERM_MF_ALL	polysaccharide binding	RT	4	1.7E-3	6.1E-1
<input type="checkbox"/>	GOTERM_MF_ALL	pattern binding	RT	4	2.2E-3	6.4E-1
<input type="checkbox"/>	SP_PIR_KEYWORDS	signal	RT	15	1.1E-2	9.1E-1
<input type="checkbox"/>	GOTERM_CC_ALL	extracellular region	RT	13	3.5E-2	9.9E-1
<input type="checkbox"/>	UP_SEQ_FEATURE	signal peptide	RT	15	4.7E-2	1.0E0
<input type="checkbox"/>	SP_PIR_KEYWORDS	glycoprotein	RT	14	8.2E-2	1.0E0
<input type="checkbox"/>	UP_SEQ_FEATURE	disulfide bond	RT	12	1.1E-1	1.0E0
<input type="checkbox"/>	UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNac...)	RT	14	1.8E-1	1.0E0
<input type="checkbox"/>	GOTERM_CC_ALL	extracellular space	RT	9	2.4E-1	1.0E0
<input type="checkbox"/>	GOTERM_CC_ALL	extracellular region part	RT	9	3.0E-1	1.0E0
<input type="checkbox"/>	SP_PIR_KEYWORDS	Secreted	RT	5	4.6E-1	1.0E0
<input type="checkbox"/>	UP_SEQ_FEATURE	transmembrane region	RT	8	9.2E-1	1.0E0
Annotation Cluster 4		Enrichment Score: 1.44		Count	P_Value	Benjamini
<input type="checkbox"/>	GOTERM_BP_ALL	biological adhesion	RT	8	4.9E-3	9.4E-1
<input type="checkbox"/>	GOTERM_BP_ALL	cell adhesion	RT	8	4.9E-3	9.4E-1
<input type="checkbox"/>	INTERPRO	Concanavalin A-like lectin/glucanase, subgroup	RT	3	1.6E-2	1.0E0
<input type="checkbox"/>	SP_PIR_KEYWORDS	egf-like domain	RT	4	2.4E-2	9.5E-1
<input type="checkbox"/>	INTERPRO	EGF-like calcium-binding	RT	3	2.4E-2	1.0E0
<input type="checkbox"/>	GOTERM_MF_ALL	calcium ion binding	RT	7	3.0E-2	1.0E0
<input type="checkbox"/>	INTERPRO	EGF-like region	RT	4	3.2E-2	1.0E0
<input type="checkbox"/>	SMART	EGF_CA	RT	3	5.5E-2	1.0E0
<input type="checkbox"/>	INTERPRO	EGF-like, type 3	RT	3	9.3E-2	1.0E0
<input type="checkbox"/>	INTERPRO	EGF	RT	3	9.4E-2	1.0E0
<input type="checkbox"/>	SMART	EGF	RT	3	1.9E-1	1.0E0
<input type="checkbox"/>	SP_PIR_KEYWORDS	calcium	RT	4	2.9E-1	1.0E0

B.

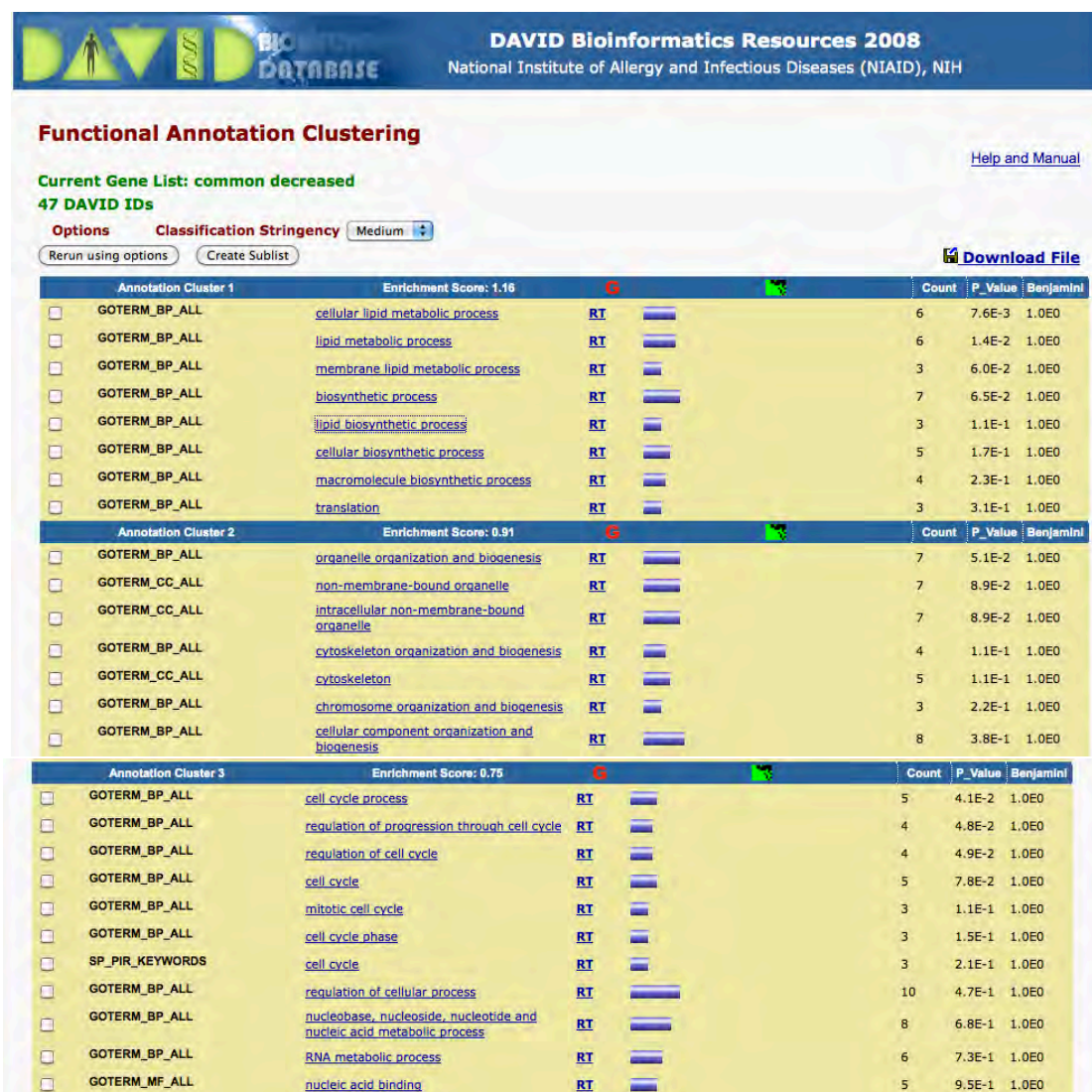


Figure C.3 Q-PCR validation of selected microarray targets

Validation of selected Array targets by qPCR. Array data obtained by block factor analysis of three independent infections. Displayed qPCRs were run with mRNA from the passage 8 infection set. PDECS infected with KRAS^{G12D} are indicated in red and pink while PDECS infected with SHH are indicated in dark and light blue.

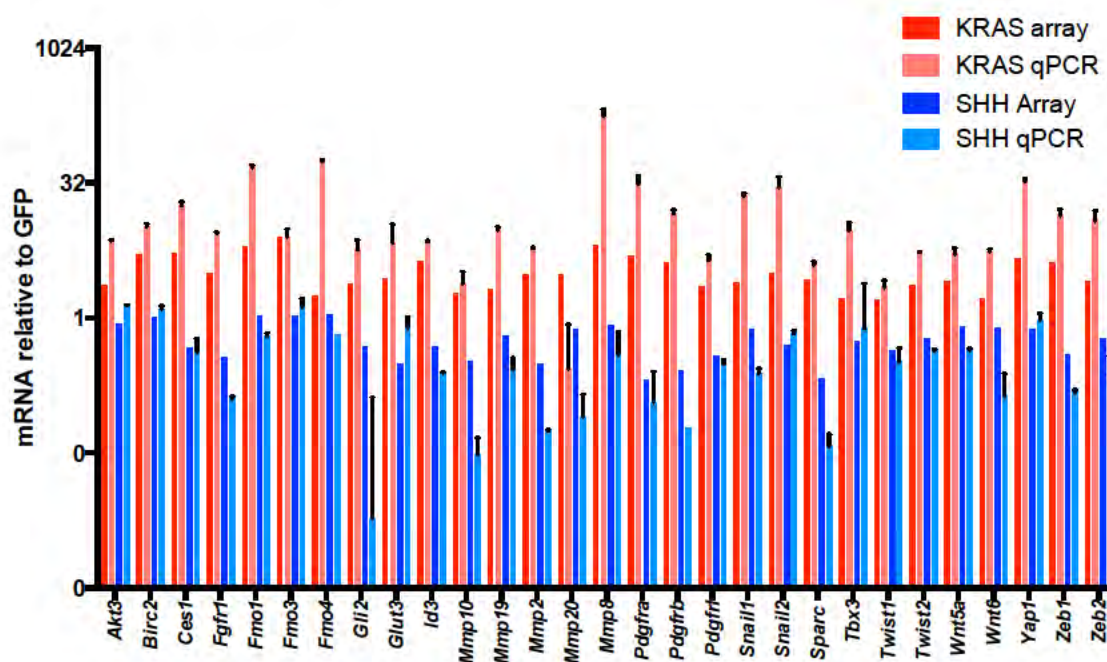
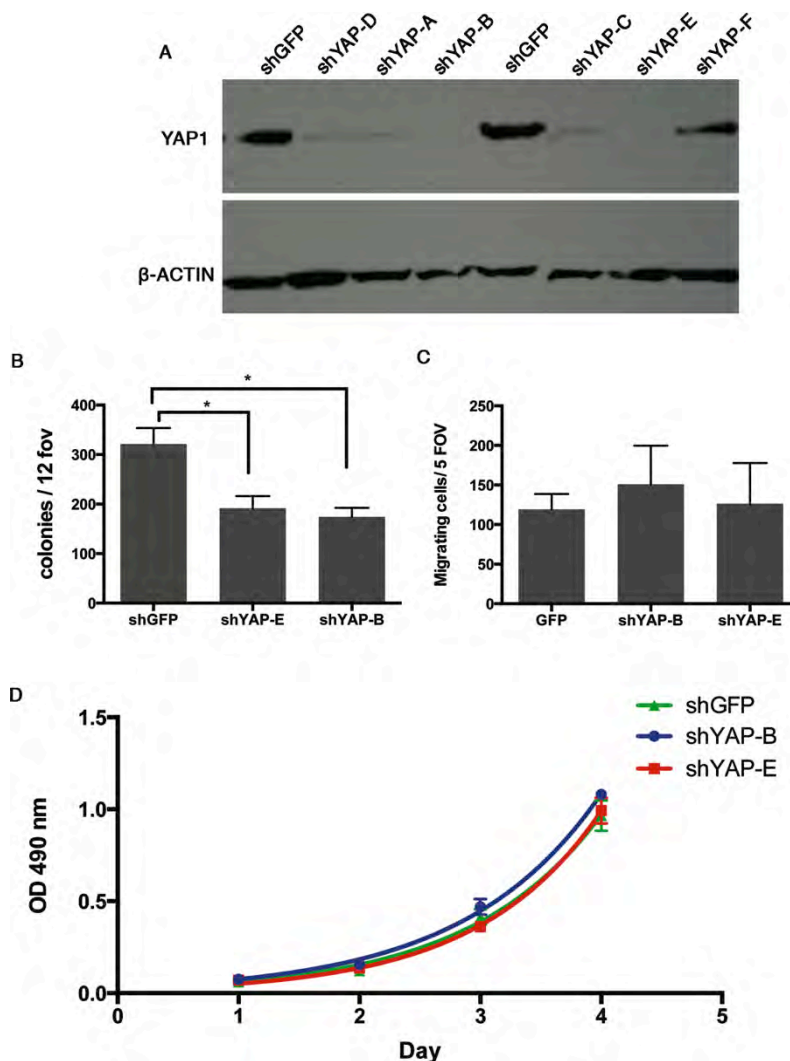


Figure C.4 Knockdown of *Yap* in an orthotropic transplant tumor cell line.

- A. Protein levels of YAP and B-ACTIN following shRNA mediated knockdown of YAP with 6 independent shRNA constructs
- B. Anchorage independent growth as measured colony formation in soft agar. Cells were plated at 1×10^5 cells/ dish 14 days prior to counting colonies using size exclusion criteria.
- C. Migration potential following Yap knockdown was measured as successful migration towards a serum gradient through 8micron pores.
- D. The ability of cells to metabolize MTS was assessed by formazan production, measured by absorbance at 490 nm. Metabolic rates were used to compare the relative numbers of cells with active metabolism over a 4-day period.



Knockdown of *Yap* in KRAS^{G12D}; *Trp53*^{-/-}; *Cdkn2a*^{-/-} PDECs

To assess the contributions of increased *Yap* expression to the tumorigenic role played by KRAS, I sought to determine if YAP knockdown could impair KRAS-driven phenotypes in PDECs. Following retroviral infection with RCAS-KRAS^{G12D}, qPCR confirmed an increase in YAP mRNA levels, but the magnitude of this change was less than seen in the microarray (Figure C.5-A). These KRAS expressing PDECs were then subject to shRNA mediated knockdown of *Yap* via lentiviral infection. Knockdown was confirmed at the protein level by western blot (Figure C.5-B), and the mRNA level by qPCR (Figure C.5-C). Knockdown of *Yap* in KRAS infected PDECs did not have a significant impact on proliferation of these cells (Figure C.5-D).

To more closely model the system from which the rationale for the gene expression profiling was conceived, I wanted to test if YAP-deficient KRAS^{G12D} PDECs were capable of tumor formation *in vivo*. I injected KRAS^{G12D} expressing *Cdkn2a*^{-/-}, *Trp53*^{-/-} PDECs cells, expressing shRNAs targeting YAP or GFP, subcutaneously into athymic nude mice. These mice were sacrificed 32 days following injection and the tumors were excised (Figure C.6A). Tumors derived from PDECs with either *Yap* targeting shRNA did not have significantly reduced tumor volumes relative to tumors formed with PDECs expressing shRNAs targeting GFP (Figure C.6B). Tumor cell lines were successfully grown from 10 of the subcutaneous tumors. mRNA and protein lysates were derived from these cell lines and YAP mRNA and protein levels were measured by qPCR and

western blot respectively (Figure C.6C-D). Yap knockdown was not maintained in these cell lines, and neither mRNA nor protein levels of YAP correlated with tumor size (Figure C.6).

Figure C.5 Knockdown of *Yap* in PDEC did not significantly decrease proliferation.

- Relative quantification of *Yap* mRNA as measured by qPCR following independent infections of *Trp53*^{-/-}, *Cdkn2a*^{-/-} PDECs with RCAS-GFP or RCAS-KRAS^{G12D}.
- Western blot for Yap1 and β -Actin protein levels following shRNA mediated knockdown of *Yap* with 2 independent hairpin sequences.
- Relative quantification of *Yap1* mRNA in KRAS^{G12D} infected PDECs following shRNA mediated knockdown.
- Doubling time of KRAS^{G12D} infected PDECs was not significantly altered following Knockdown of *Yap*. Analysis of covariance of a line determined that the slopes derived from 4 independent cell counts at days 5, 10 and 15, plotted on a log₂ scale were not significantly different.

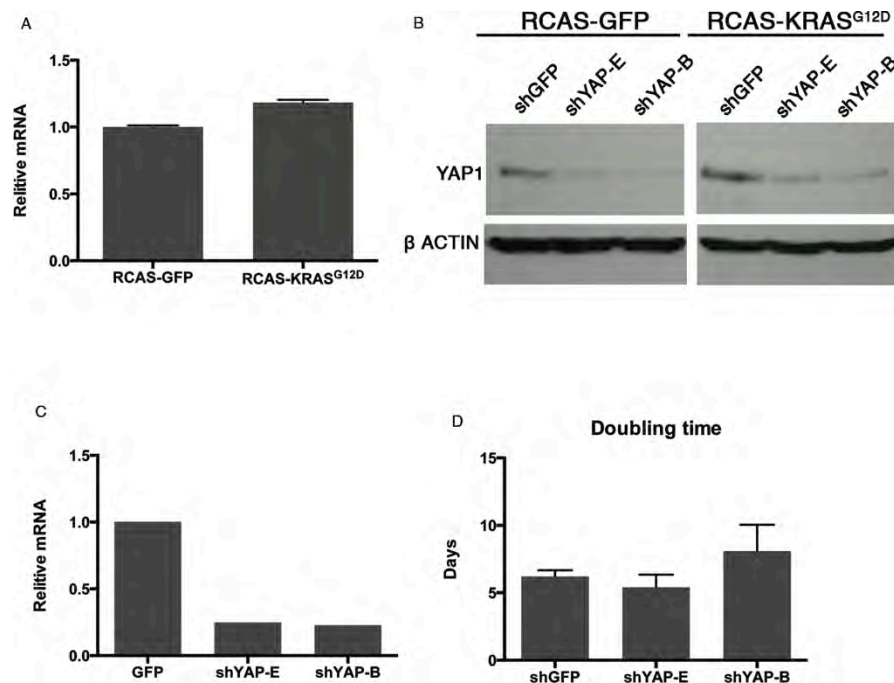
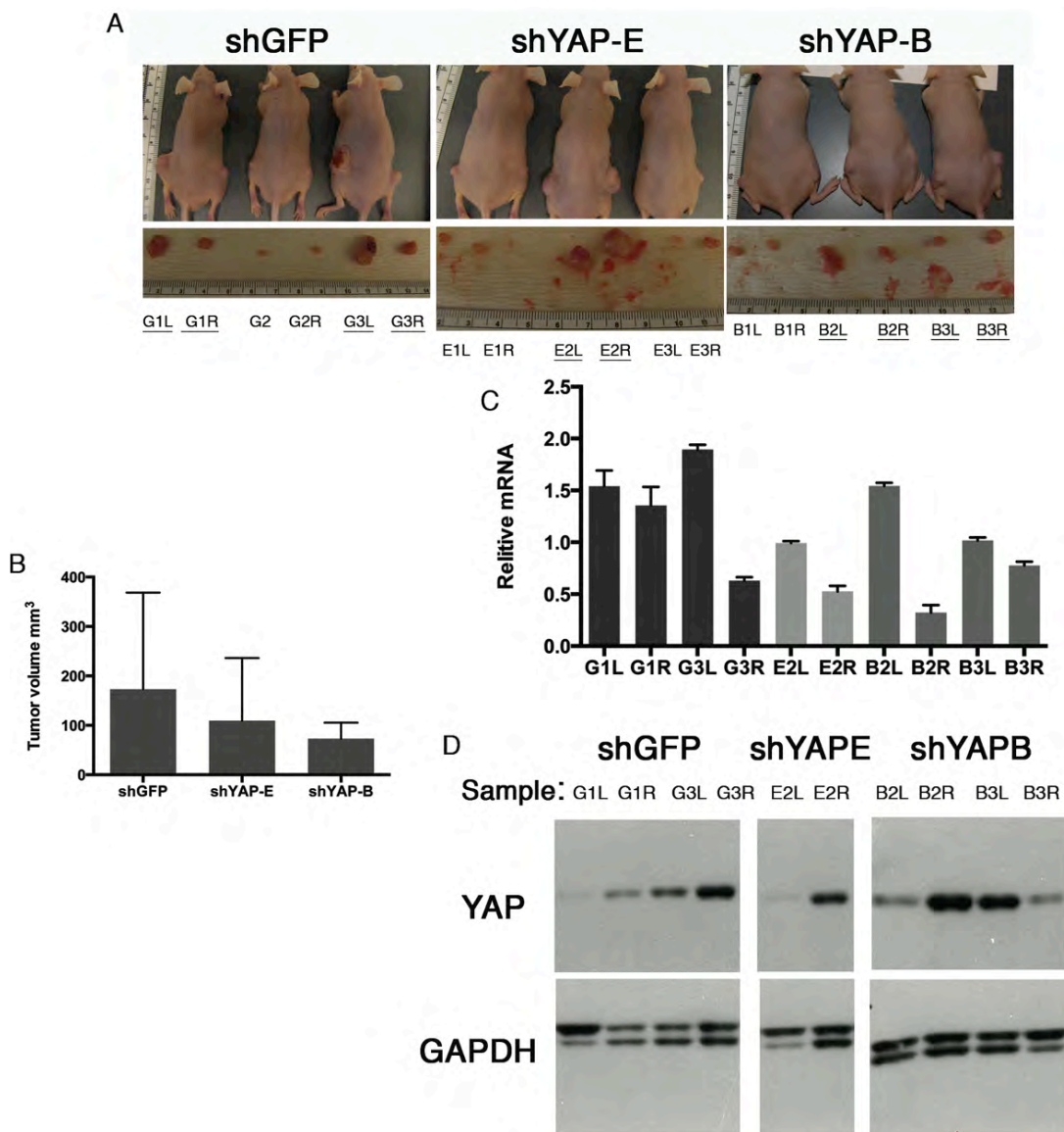


Figure C.6 Subcutaneous injection of KRAS infected YAP-deficient PDEC.

- A. Injection of 1×10^6 RCAS-KRAS^{G12D} infected PDEC with lentiviral knockdown of GFP or Yap. Mice were sacrificed at 32 days.
- B. Average volume of subcutaneous tumors 32 days post injection. (Volume = $1/2$ (height x width x length)). No significant difference by T-Test was observed in mice injected PDECs containing shRNAs targeting YAP.
- C. *Yap* levels measured by mRNA in select tumors isolated from subcutaneous tumors.
- D. YAP protein levels in select subcutaneous tumors measured by Western blot.

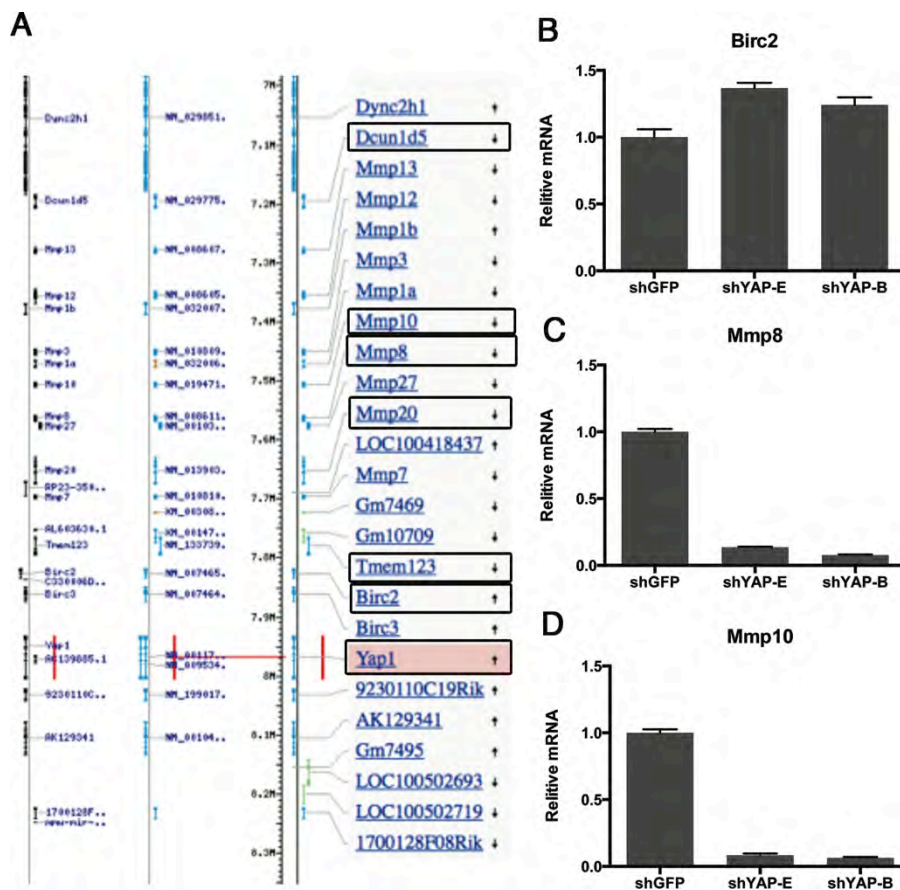


Possible coordinate regulation of Chromosome 11q22

The locus that encodes the YAP protein is located on chromosome 11 in the q22 region. The results of my microarray found that 7 genes in a 1Mb region of this chromosome were upregulated in KRAS^{G12D} versus SHH infected PDECs (Figure C.7 A). Because YAP is a transcription factor, I hypothesized that increased YAP in response to KRAS^{G12D} may be responsible for the upregulation of these additional proteins. To begin to assess this possibility, I observed mRNA levels of *Birc2*, *Mmp8* and *Mmp10* following knockdown of Yap in PDEC cells. While *Birc2* levels were not significantly altered, both *Mmp10* and *Mmp8* were decreased following knockdown of Yap (Figure C.7 B-D), supporting the possibility that the YAP transcription factor may be regulating expression of multiple genes in this region.

Figure C.7 Close proximity of KRAS up-regulated genes to *Yap* on chromosome 11q22 (Ch 9a1 in *mus musculus*).

- A. A screenshot from NCBI map viewer tool showing a 1 mb region in which 7 genes were identified by microarray as upregulated in KRAS vs SHH infected PDECs. Genes present in microarray are boxed.
- B. Q-PCR measuring *Birc2* levels following shRNA knockdown in KRAS^{G12D} Infected *Trp53*^{-/-}, *Cdkn2a*^{-/-} PDECs.
- C. Q-PCR measuring *Mmp8* levels following shRNA knockdown in KRAS^{G12D} Infected *Trp53*^{-/-}, *Cdkn2a*^{-/-} PDECs.
- D. Q-PCR measuring *Mmp10* levels following shRNA knockdown in KRAS^{G12D} Infected *Trp53*^{-/-}, *Cdkn2a*^{-/-} PDECs.



Discussion

Identification of functional groups upregulated by KRAS

The question as to which downstream effectors of activated KRAS are required for its tumorigenic potential remains an active area of research, and the answers to this question will provide valuable information in the fight against KRAS-driven tumors. By using ectopic expression of SHH in the pancreas as a gate to screen out changes specific to the ERK and AKT pathways, which have been shown to play major roles in PDAC initiation and progression, but were not sufficient to drive PDAC in our pancreatic orthotopic transplant mouse-model, I acquired a novel data set which I believe has identified important downstream pathways enhanced by activated KRAS in PDECs.

Independent studies have identified pathways flagged by this microarray as vital to PDAC. The formation of PDACs has been shown to increased anabolic metabolism, and loss of KRAS resulted in decreased expression of the *Glut1* glucose transporter(Ying, Kimmelman et al. 2012). My array identified upregulation of the neuronal glucose transporter GLUT3 in PDECs expressing activated KRAS, suggesting that upregulation of additional glucose transporters may result for KRAS-activation. The identification of *Pdgfra*, *Pdgfrb* *Pdgfrl*, *Fgfr1* suggest that KRAS^{G12D} increases transcription of numerous growth factor receptors resulting in increased sensitivity of PDAC cells to extracellular signals. My array also identified multiple genes involved in epithelial the mesenchymal transition (EMT). The identification of *Snai1*, *Snail2*, *Twist1*, *Twist2*, *Zeb1* and

Zeb2 strongly suggests a role for KRAS in the activation of an EMT transcriptional program. Genetic loss of YAP in the pancreas has since been shown to prevent KRAS-driven tumor initiation (Zhang, Nandakumar et al. 2014, Yang, Zhang et al. 2015) and YAP has been shown to regulate EMT in PDAC (Shao, Xue et al. 2014). Furthermore, upon withdrawal of activated KRAS PDAC tumors regress, but YAP activity can drive recurrent tumors (Kapoor, Yao et al. 2014). Taken together, these studies providing a positive validation for this data set.

While KRAS is not a transcription factor, it is known to activate transcription factors through multiple downstream effectors including ERK and AKT. The prevalence of transcription factors upregulated in my data sets suggest that KRAS activates a transcriptional cascade whereby KRAS mediated regulation of specific proteins results in enhanced transcription of a transcription factor which in turn, upregulates other transcription factors. This possibility is supported by previous findings that Twist1 expression is increased upon KRAS-activation in PDEC (Lee and Bar-Sagi 2010), however the manner by which KRAS-activation increases Twist1 expression has not been identified and it may be secondary or tertiary to KRAS-activation. I did investigate the levels of the Twist, Snail, and Zeb genes in response to YAP knockdown to inquire if YAP may be regulating some or all of these EMT regulating transcription factors, but I found the levels of these genes to be unchanged (data not shown). Interestingly, regulation of Glut3 by Zeb2 has been shown in in non-small cell lung cancers

(Masin, Vazquez et al. 2014) suggesting that transcription factors in my screen may be directly responsible for the regulation of other transcripts identified in the screen. The concept of transcription factors regulating a series of targets, some of which may be other transcription factors, is not novel. In the context of my microarray results, however, it is important to identify which transcription factors are the primary players resulting from KRAS-activation and which are secondary or tertiary players regulated by the primary transcript factors. Additional bioinformatics analysis of my data sets, such as mapping transcription factors to their known targets, may provide a more substantial understanding of the gene regulation which results from activation of KRAS and should allow for the changes in signaling to be mapped back to a few transcription factors. As secondary and tertiary transcription factors may be altered more robustly than the primary transcription factors, it is possible that increases in some primary transcription factors may fall below the current threshold and would need to be confirmed in the original microarray data set. Understanding how the identified transcription factors are regulated by KRAS will provide insight into pathways required for KRAS mediated transformation of pancreatic ductal cells, and could direct future therapeutic efforts.

Coordinate regulation possible

The identification of 7 genes in a 1 Mb region that are up regulated in response to activated KRAS but not SHH raises the possibility of coordinate

regulation. An analysis of 145 human PDACs from The Cancer Genome Atlas (<http://cancergenome.nih.gov/>) did not identify any amplifications of this chromosomal region in human disease, suggesting that increased expression is occurring as a result of transcription rather than amplification. There are multiple possibilities that could lead to coordinate regulation of gene expression including binding of strong promoting factors in this region, and alterations to the tertiary structure of the DNA that facilitates transcription. Analysis of histone densities throughout this region and looking for repetitive sequences to which promoters are known to bind, may provide some insight into coordinate regulation of this region. A more thorough analysis could be achieved using a 5C analysis to map the cis- and trans- interactions and identify changes in the higher order chromosome structure associated with this region. The use of primary ductal epithelial cells may provide an ideal system in which to investigate KRAS^{G12D} mediated changes in chromosome structure of untransformed pancreatic ductal cells.

The multiple gene targets upregulated on chromosome 11q22 were identified in the normal course of investigating the attributes of YAP. It is possible that other regions of the genome also contain clusters of genes regulated by KRAS-activation. The gene expression profiles obtained in this study provide a ready source to further investigate this possibility by simply mapping these genes on their chromosomes.

Materials and Methods

Microarray and Q-PCR validation.

RNA was isolated from *Keratin-19-tv-a*; *Ptf1a*^{Cre/+}; *Trp53*^{fl/fl}; *Cdkn2a*^{fl/fl} PDECs infected with RCAS-KRAS, RCAS-SHH or RCAS-GFP at passage 5, passage 8 or passage 9. These PDEC isolations, RCAS infections and RNA isolations were conducted by Victoria Appleman. RNA was stored at -80°C. RNA aliquots with concentrations ranging from 0.08 µg/ul to 2.07 µg/µl (median of 0.42 µg/µl) were sent to the Memorial Sloan Kettering Cancer Center Genomics Core Laboratory where the samples were labeled and hybridized to a MOE430 2.0 Affymetrix microarray. Julie Zhu and Jianhong Ou analyzed data from these microarrays. The three independent infections with each RCAS virus were compared as blocks and a threshold of log₂(1.5) fold change with an unadjusted P. value of 0.01 was used to identify targets up and down-regulated in KRAS infected PDEC verses SHH infected PDEC.

Twenty-nine targets from the upregulated in KRAS verses SHH gene set were selected and q-PCR primers were designed using the promega primer design tool Plexor (<http://www.promega.com>). These primer sets were functionally validated for q-PCR by confirming the generation of a linear standard curve for each set. Primer sets are listed in Table B.1. Validations of selected targets were done using mRNA from the passage 5 and passage 8 infection sets. Superscript III First Strand Synthesis System for RT-PCR (ThermoFisher Scientific 18080-51) was used to produce cDNA from the RNA templates with

OligoDTs as the primers. 200ng of cDNA was combined with sybr green (Quanta Biosciences) and appropriate primer pairs at a final concentration of 0.5 μ M. Simultaneous PCR amplification and signal detection was accomplished on an ABI 7300 Real Time PCR system. Amplification of β -*Actin* was used as an endogenous control.

Mouse-models and animal care

The *keratin-19-tv-a*; *Ptf1a*^{Cre/+}; *Trp53*^{fl/fl}; *Cdkn2a*^{fl/fl} have been described previously (Morton, Mongeau et al. 2007). Athymic nude mice used for subcutaneous orthotropic transplants were purchased from Charles River Laboratories. All animals were housed in pathogen free environments and food and water were provided *ad libitum*. Care and euthanasia of these animals was conducted under the guidelines approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Culture and Infection of Pancreatic Ductal Epithelial Cells (PDECs)

Isolation and culture of PDEC were accomplished as previously described (Schreiber, Deramautd et al. 2004). Of note, the PDECs for these experiments were cultured on a layer of type-I collagen and not laminin, which has subsequently been used in the lab.

RCAS-Kras, RCAS-Shh, and RCAS-GFP vectors have been previously described (Morton, Mongeau et al. 2007). Virus was produced by transfecting 5

µg of RCAS plasmid into DF1 chicken fibroblasts using Superfect Transfection reagent (Quiagen 301305). DF1 cells were cultured in Dulbecco's Modified Eagle Medium, 4.5 g/L D-Glucose, + L-Glutamine (Gibco 11965-092), with 10% Fetal Bovine Serum (FBS) and 1% penicillin streptomycin (Gibco 15140-122). The virus produced is replication competent in avian cells and transfected DF1 cells were maintained for at least 1 week prior to viral harvest to allow the viral titer to reach its maximum sustainable levels. These virus producing DF1 cells were never cultured for more than 4 weeks in an attempt to prevent propagation of advantageous replicative errors in the RCAS virus or the gene it is designed to express.

Following expansion of transfected virus producing DF1 cells into three 10cm dishes, 30 ml of viral supernatant was collected from these confluent plates (10ml each), filtered through 0.45 micron syringe filters, concentrated by centrifugation at 27,000 RPMs for 90 minutes at 4°C, resuspended in 100µl of DMEM media, and added to a single 10 cm diameter dish of *K19-tv-a; Ptf1a^{Cre/+}; Trp53^{fl/fl}; Cdkn2a^{fl/fl}* PDECs. Fresh DMEM was added to the virus producing DF1 cells and subsequent infections were conducted for a total of 4 infections in a 48 hour window. DF1 cells were incubated at 39°C while PDEC were incubated at 37°C. Both cell lines were maintained at 5% CO₂ in humidified incubators.

Lentivirus was produced by transfection of HEK 293T cells (5x10⁵ cells in a 9.5cm² area) with pCMV delta 8.9 packaging plasmid (0.3µg), pMDG envelope plasmids (0.2µg) and a pLKO plasmid (0.5µg) targeting *Yap* or GFP (Table B.2).

PDEC cells previously infected with RCAS-KRAS or RCAS-GFP were subject to a single round of lentiviral infection, which included a 45 minute spin at 1800 RPM and a 4 hour and 15 minute incubation at 37°C. These cells were placed into 3µg/ml puromycin selection 48 hours following lentiviral infection.

Proliferation in PDEC cells

Ten days following lentiviral infection, and 8 days following selection, 1×10^5 cells/well were plated on 6-well plates. Cells were plated in duplicate on three 6-well plates. Viable cells were counted using trypan blue exclusion on days 5, 10 and 15 post-infection. \log_2 of cell/well numbers were plotted against time and doubling time was derived from the inverse slope. The significance of the difference in these slopes, and thus doubling time, were determined by analysis of covariance using Prism Graphpad.

Processing of PDECs for orthotropic injection

To remove the collagen layer on which PDECs were grown, 1mg/ml collagenase V (Sigma C9263) was used to dissolve it. Cells were then treated with 0.25% trypsin-EDTA (TCF-Invitrogen 25200056) for 10 minutes at room temperature and washed three times in DMEM. Cells were pelleted by centrifugation at 1000RPM for 5 min at 4°C between each step.

PDEC cells with *Yap* knockdown used for subcutaneous orthotropic transplant were resuspended in 10 ml of DMEM following the third wash. Cells

were counted in duplicate with a hemocytometer to ensure accuracy, and 8×10^6 cells were pelleted and resuspended in 800 μ l of sterile PBS at 4°C. Athymic nude mice were injected subcutaneously with 100 μ l of PBS containing 1×10^6 cells their left and right flanks. A total of six injections in three mice were completed for each PDEC variant. Tumors harvested 32 days after subcutaneous injections were measured and the volumes of these tumors were calculated as $1/2$ the (height x width x length).

Culture of cell lines from subcutaneous orthotropic transplants

Tumors harvested following subcutaneous injections of KRAS^{G12D} PDEC cells were cultured in Dulbecco's Modified Eagle Medium, 4.5 g/L D-Glucose, + L-Glutamine (Gibco 11965-092), with 10% Fetal Bovine Serum (FBS) and 1% penicillin streptomycin (Gibco 15140-122). Ten of the 16 cell lines grew out and these cells were scraped into PBS, pelleted and used to isolate mRNA or make whole cell lysates. RNA isolation and whole cell lysate preparation is described below.

Confirmation of target gene knockdown

The ability of shRNAs to knockdown their target genes was assessed in a cell line derived from orthotropic pancreatic transplant of KRAS^{G12D} infected PDECs (170#3). Production of lentivirus was produced by transfection of HEK 293T cells (5×10^5 cells in a 9.5cm² area) with pCMV delta 8.9 packaging plasmid

(0.3µg), pMDG envelope plasmids (0.2µg) and a pLKO or pGIPZ plasmid (0.5µg) targeting *Yap*, *Gli2*, *TBX3*, *ID3* or GFP (Table B.1). 170#3 cells were subject to a single round of lentiviral infection, which consisted of a 45 minute at 1800 RPM and a 4 hour and 15 minute incubation at 37°C. Infection was confirmed by maintaining cells in 3µg/ml puromycin selection beginning 48 hours following lentiviral infection. Cells were used to set up phenotypic assays and harvested for protein lysates 10 days following infection.

RNA Isolation

RNA was extracted from PDECs using TRIzol reagent (Thermo Fisher Scientific 15596026). TRIzol (1ml) was added to the washed cell pellets and incubated at room temperature for 5 minutes. Chloroform (0.2ml) was added, tubes were vortexed, incubated for 3' at room temperature and centrifuged for 10 minutes at 13000 RPM. The aqueous phase was transferred, RNA was precipitated with 0.5ml of isopropanol and resuspended in DEPC treated water. Production of cDNA and subsequent qPCRs are described above.

Western blots

Cell pellets for whole cell lysates were obtained from 170#3 cells 10 days post Yap knockdown and from subcutaneous tumor cell lines 18 days they were isolated in culture. Cell pellets were lysed in fresh phospho-lysis buffer (10mM EDTA, 5mM EGTA, 10mM MgCl₂, β-glycerophosphate 50mM, nonidet P-40

0.5%, brij-35 0.1%, dithiothreitol 1mM, sodium orthovanadate 1mM, phenylmethanesulfonyl fluoride 1mM, CompleteTM Protease inhibitor cocktail 1 tablet/20ml recipe from Kelliher lab).

Protein lysates (15ug) were subject to SDS-Page in acrylamide gels and transferred onto polyvinylidene difluoride transfer membranes (Amersham Hybond, pore size 0.45 μ m, 45004110). Membranes were blocked in 5% milk in TBS+ 0.1% Tween 20 (TBST) for 1.5 hours at room temperature followed by overnight 4°C incubation in YAP1 (Cell signaling technology 4912), β -ACTIN (Santa Cruz 1615R) or GAPDH (Cell signaling technology 2118) primary antibodies diluted 1:1000. Blots washed in TBST were incubated for 1 hour with peroxidase conjugated rabbit secondary antibody diluted 1:5000 and developed using ECL (Fisher 45-000-885).

MTS assay

In 170#3 cells with *Yap* knockdown, the ability of cells to metabolize MTS tetrazolium was used as to estimate the relative number of viable cells. Cells maintained in 3ug/ml puromycin selection were seeded in triplicate at 1×10^3 on a 96 well plate. At 24, 48, 72, and 96 hours media was replaced with complete media containing 16.7% MTS reagent (Abcam ab197010). The cells were incubated at 37°C for 30 minutes after which formazan produced by reduction of MTS tetrazolium was measured by absorbance at 490 nm. Absorbance of the MTS-media in the absence of cells was subtracted from the readings and the

average OD 490 was plotted for *Yap* knockdown and GFP knockdown in 170#3 cells verses time.

Anchorage independent growth assays

Soft agar assays were seeded at 1×10^5 cells/10cm dish in 3 ml of media with 0.4% agarose and 3ug/ml puromycin poured over solidified media with 0.7% agarose. Once solidified the cell suspension was covered with 7ml of complete media with 3ug/ml puromycin. Colonies were counted 2-3 weeks post plating using size exclusion criteria.

Table C.1 Validated qPCR primers for murine target genes

	Primer F		Primer R
PdgfrbF1	GCCATGGTCGTTCA CACTCAC	PdgfrbR1	CTCCATCCTGCATATC CCCAC
PdgfrbF3	ATGATCTCATAGATC TCGTCGGAGG	PdgfrbR3	TTACCCAGAG CTGCCCATGA AC
PdgfraF1	AGTGTCATTATTACA TTTCTTAATATGCTT GCAGA	PdgfraR1	ACCCTAGTTCCTGCAT CCATTTT
PdgfraF2	CGGTGGACACAATTT TTCGAAGC	PdgfraR2	GGGTCTGACTTTGCTG GATCTATTG
PdgfrlF1	TTGAGTCAGTGGAGT CCCTGT	PdgfrlR1	GGAGCAGGGAGAAAA CAGGATCAA
PdgfrlF3	CGGAGATTCTGAGC TGTGCATA	PdgfrlR3	TGGGACACACCACAAG AATCTCC
Fmo1F2	GGACCTGAACAACC CATCGAG	Fmo1R2	CGCATCTGCCAAAACC AACTC
Fmo1F4	TGGATGTTTATACTG TCGGCTGTGG	Fmo1R4	TAACTAACCCACATCT GCCCCTG
Fmo3F1	CCTTGGCAAATGAAG TGATGTATTCT	Fmo3R1	CCAAAGAGATGATGTG TTTTCCAGAC
Fmo3F3	AACTCCTTTACATTA GGCTTGATGGT	Fmo3R3	TGCCTTTAAACAGAAC ACTCAGGAAAG
Fmo4F2	GGGGTAATCTTCTCG GAATGGGAA	Fmo4R2	GTTTGCTGACACTTCC GAGGAT
Fmo4F4	CGTGAAGGCAGAAC TCGTACAAG	Fmo4R4	TCAGCACTAGAACTGG CACCTG
SparcF1	TGTTGTCATTGCTGC ATACCTTCTC	SparcR1	TGTGAGCTGGACGAGA GCAACA
SparcF3	ATACCTTCTCAAAC CGCCAATGG	SparcR3	TTGCAAACATGGCAAG GTGTGTGA
Glut3	CTTCTAACCGCTCTT	Glut3	ACGATCGGCTCTTTCC

	CCAAAGTG		AGTTTG
Glut3	CAGGTCCAATCTCAA AGAAGGCTAC	Glut3	CATGACGATTTGCTG TACTAAAGG
Ces1F1	ATTAGGGTTCCCATT CCGAGCAAA	Ces1R1	CCGTTTTTAAAAGAGG GTGCTTCAGA
Ces1F4	GTAGGGGACAGTGT TGAAACTCTT	Ces1R4	GAGAGCTATCCCTTCC TCCCTAC
Mmp10F1	GGAAGTTCTGCATTT CTTGAATTTTTTTGA CA	Mmp10R 1	GCTCAGCAATACCTAG AAAAATACTACAAC
Mmp10F2	GGTACTGAAGCCAC CAACATCAG	Mmp10R 2	GTTGTCAAAAAAATTCA AGAAATGCAGAAGT
Mmp19F3	TTTGGCATGGGACC AGGTTTT	Mmp19R 3	CCAGAGACAAGAGATG AGGAGGAAGA
Mmp19F4	AAACAGGAACACCTT TTGATTAACAGG	Mmp19R 4	ATGTGGATTTCAAGAT GTCTCCTGG
Mmp2F2	TGGTCAGTGGCTTG GGGTATC	Mmp2R2	AGAAAAGATTGACGCT GTGTATGAGG
Mmp8F3	CCCTGTAAGCATAGT TTGGGTACA	Mmp8R3	TCTTTGGGACTCTCTC ACTCCAC
Mmp20F1	GGGTTCTGGTACTTA TAAGTTGGGTACA	Mmp20R 1	GACTATGGGAACGAAT GGGTTCAAT
Mmp20F3	G TTCAGAGGGACAG CTGTACTCCA	Mmp20R 3	ACAGAATCTCTAAGTA CACACCCTCC
Zeb1F1	GGGACTACATTTACA ACTGGTTCTGAG	Zeb1R1	CCCTTGAAGTTGTCTT GTGCAAAAAAG
Zeb2F2	CCAAGTTGTTCTTA ACTGAGTAATGG	Zeb2R2	AATGGCCGAATGAGAA ACAATATCAAG
Snail1F3	CTGCTGGAAGGTGA ACTCCAC	Snail1R3	TGCCGCGCTCCTTCCT GGTCA
Snail1F4	TGGTCGTAGGGCTG CTGGAA	Snail1R4	CGGAAGCCCAACTATA GCGAG

Snail2F3	GCCCAGAGAACGTA GAATAGGTCTT	Snail2R3	G TTCAGAAAGTCCCAT TAGTGACGAAG
Snail2F4	GAGAAGGTTTTGGA GCAGTTTTTGC	Snail2R4	CAGATCAAACCTGAG GGCACATC
Twist1F3	CCATCTTGGAGTCCA GCTCG	Twist1R3	GCTGAGCAAGATTGAG ACCCTC
Twist1F4	TGCAGCTCCTCGTAC GACTG	Twist1R4	CAAGAAATCTGCGGGC GGAG
Twist2F1	GCTGGTCATCTTATT GTCCATCTCG	Twist2R1	AGCAAGATCCAGACGC TCAAG
Twist2F2	CTTCGCTCGATTTCT TGCTGTAG	Twist2R2	CCAGTGAGGAAGAGCT GGAGAG
Fgfr1F2	CTTGAAAAGTTCCTC CACAGGCACA	Fgfr1R2	TGTGTGGTCTTTTGA GTGCTCT
Tbx3F1	GCCTCTCCTCAAACA CTCTCATG	Tbx3R1	GGGTTTTTCGAGACACT GGCAA
Tbx3F2	TCGCCTTCCTGACTT CGTGATGA	Tbx3R2	GGAACCCGAAGAAGA CGTAGAAG
Yap1F1	CATGTTGTTGTCTGA TCGTTGTGATT	Yap1R1	TTGAGATCCCTGATGA TGTACCACT
Yap1F3	CCATTTTCATCCACAC TGTTGAGG	Yap1R3	GTGGCACCTATCACTC TCGAGA
Birc2F1	GGGATCCATCCTTGA TGAGAAGTCT	Birc2R1	AGATTTGCACATTCGT CACCTCT
Birc2F4	CAAGAACTCACACCT TGGAACC	Birc2R4	GTCAAGTGCTTTTGTT GTGATGGT
Akt3F2	AGGCTTAAAAGGAG GTACAAGCTT	Akt3R2	GGCCAGATGATGCAAA AGAAATCATG
Akt3F3	GCTTAAAAGGAGGTA CAAGCTTTTTGTC	Akt3R3	TGGAGGGCCAGATGAT GCAAAA
Id3F1	CTATGACACGCTGCA GGATTTC	Id3R1	CTCTTGGACGACATGA ACCACTG
Id3F2	CCACCTGAAGGTCG	Id3R2	GGGAAGTGGTGCCGG

	AGGATGT		GAGTC
Gli2F2	TCTTCACATGCTTGC GGAGTG	Gli2R2	ACTCACTCCAATGAGA AACCCTACA
Gli2F3	GCTGGCTTCTGTTG GACCACA	Gli2R3	GCAGTGGAATGAGGT GAGTTCT
Wnt5aF1	GCGCTCTCATAGGA ACCCTTAG	Wnt5aR1	CTGCGGAGACAACATC GACTATG
Wnt5aF2	ATTTGCACACGAACT GATCCACA	Wnt5aR2	GACCAGTTTAAGACAG TGCAGACC
Wnt6F1	GAAAGCTGTCTCTCG GATGTCCTG	Wnt6R1	GGGGTTCGAGAATGTC AGTTCCA
Wnt6F2	GTCTTGACTTCTCAT CCCCGAAG	Wnt6R2	TTCTGGGCACTCCTGG ACCTC

Table C.2 shRNA clones used for knockdown of target genes.

shRNA clones used to attempt knockdown in murine cell lines. Only constructs targeting Yap were effective and the clones used in phenotypic assays and orthotopic transplant are labeled shYAP-B and shYAP-E.

Gene	Vector	Clone ID
TBX3	pLKO.1	TRCN00000 05079
TBX3	pLKO.1	TRCN00000 95873
TBX3	pLKO.1	TRCN00000 95869
TBX3	pLKO.1	TRCN00000 95870
TBX3	pLKO.1	TRCN00000 95871
TBX3	pLKO.1	TRCN00000 95872
YAP1	pLKO.1	TRCN00000 95864
YAP1 (shYAP-B)	pLKO.1	TRCN00000 95864
YAP1	pLKO.1	TRCN00000 95865
YAP1	pLKO.1	TRCN00000 095866
YAP1 (shYAP-E)	pLKO.1	TRCN00000 095867
YAP1	pLKO.1	TRCN00000 95868
Id3	pLKO.1	TRCN00000 71438
Id3	pLKO.1	TRCN00000 71439
Id3	pLKO.1	TRCN00000 71440
Id3	pLKO.1	TRCN00000 71441

Gli2	pGIPZ	V2LMM_111 857
Gli2	pGIPZ	V3LMM_441 632
Gli2	pGIPZ	V3LMM_441 635
Gli2	pGIPZ	V3LMM_441 631
TBX3	pGIPZ	V3LMM_494 986
TBX3	pGIPZ	V3LMM_520 617
TBX3	pGIPZ	V2LMM_831 63
TBX3	pGIPZ	V2LMM_190 516

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