Using Mouse Models to Investigate the Initiation and Progression of Pancreatic Cancer

by

Heather K. Schofield

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Cellular and Molecular Biology)
in the University of Michigan
2017

Doctoral Committee:

Associate Professor Marina Pasca di Magliano, Chair
Assistant Professor Benjamin L. Allen
Professor Howard C. Crawford
Professor Andrzej A. Dlugosz
Professor Celina G. Kleer

schofihe@umich.edu

ORCID 0000-0002-1613-1940

For Sharan, who inspires and motivates everything I do

Acknowledgements

I owe a great deal of gratitude to the many people who have nurtured my research career over the years. First among these is my mentor, Marina Pasca di Magliano. Your abilities as a scientist and research mentor inspire me and have played a huge role in guiding me through grad school. Your constant support of my interests, both in and out of lab, and your example of work-life balance, continues to convince me that I can reach my career and life goals.

I would also like to thank members of the Pasca di Magliano lab, both past and present, including Meredith Collins, Esha Mathew, Yaqing Zhang and many others. I always looked forward to coming in to lab, and your patient help, especially during my early years of grad school, enhanced my research projects and my life during this experience. To Esther Kim and Annachiara del Vecchio, thank you for your endless help. You are both so competent, smart and thoughtful. Your time in lab makes my work easier, and I always appreciate that.

To the members of the “Pantera” research group, I am always thankful for your presence and support. Having a larger group of labs to share ideas, reagents and techniques makes my work here easier and more enjoyable, and it has been fun to have a team to be a part of. I would like to particularly thank Chris Halbrook for all he has done to help me. Chris, I ask a lot from you and you always enthusiastically help. Your excitement about pancreatic cancer research and scientific discovery has opened

new directions in my research projects, and you seem to never tire of me depending on your opinions.

I would like to thank my thesis committee: Ben Allen, Howard Crawford, Anj Dlugosz and Celina Kleer for their comments and guidance. In particular I would like to thank Howard and his lab for advice and reagents, and I would like to thank Costas Lyssiotis and his lab for the same. My experience as a Michigan graduate student has been full of wonderful people and resources, and for that I am grateful.

My friends at Michigan have been an incredible support system going through grad school and the MSTP. To David Giles and Julia Wu, I am so happy that some of my best friends at Michigan are in the same MSTP cohort as myself. This whole process feels a little easier when I have supportive and understanding friends by my side. I'd also like to thank my good friends Cher Zhao, Markus Nemitz, Brittany Allen and Steve Allen. I've had so many fun times with you all, it feels like we have family here in Michigan.

To my parents and brothers, thank you for your endless support, even when I moved halfway across the country from you guys to attend a nine-year long graduate program – I know you all thought I was crazy. I'm so happy that you still visit me here and have all adopted Michigan sports fandom. To my mom especially, thank you for the phone calls listening me talk to what happened to me in lab, I know you probably didn't understand everything, but you were always willing to listen.

And to Sharan, it feels like making it through grad school would have been impossible without you. Thank you for always being willing to talk about experiments and results with me, I treasure the fact that we both have such an interest in science. I

am so lucky to have you (and Brady!) in my life, and I look forward to sharing our lives and careers together.

Table of Contents

List of Figures

Abstract

Pancreatic cancer is one of the deadliest human malignancies, and is expected to become the second leading cause of cancer related death in the United States by the year 2020. Standard chemotherapy approaches have proven ineffective in the treatment of pancreatic cancer, highlighting the need to understand the basic biology of the disease in order to identify future therapeutic targets. In humans, pancreatic tumors almost universally display activating mutations in the oncogene Kras, which are considered to be the initiating factor in carcinogenesis. Pancreatic cancer is also associated with later mutations in tumor suppressors, most commonly point mutations in the gene p53. This information from human tumors can be used to model pancreatic cancer in mice, where expression of oncogenic Kras recapitulates the precursor lesions seen in humans. Addition of tumor suppressor mutations leads to mice that reliably mimic all the stages of human disease. The overarching goal of this work is to use existing mouse models, as well as develop new ones, in order to further our understanding of the initiation and progression of pancreatic cancer.

In the first part of this dissertation, I focus on the initiation of pancreatic cancer and the role that the epigenetic regulator Bmi1 plays in this process. Using a mouse model of early pancreatic carcinogenesis, I show that pancreatic expression of Bmi1 is required for the development of precancerous lesions, and therefore the initiation of pancreatic cancer. I showed that Bmi1 knockdown in pancreatic cancer cell lines

increased levels of reactive oxygen species (ROS), indicating that the requirement of Bmi1 expression during pancreatic cancer initiation may be due to its control of cellular ROS levels. Next, I explore the mechanism for Bmi1 requirement in pancreatic cancer initiation. I find that when Bmi1 is knocked down in pancreatic cancer cells, HIF1a is also down, suggesting that Bmi1 may be regulating HIF1a. *In vivo*, pancreatic HIF1a stabilization recovers the lack of precancerous lesion phenotype seen in animals lacking Bmi1 expression, indicating that the reason for Bmi1 requirement in pancreatic cancer initiation is through its regulation of HIF1a levels.

In the next sections of this dissertation work, I develop new mouse models that will help in the understanding of the basic biology of pancreatic cancer. First, I analyze HIF2a stabilization in the murine pancreas. Pancreatic HIF2a stabilization results in a phenotype that resembles human chronic pancreatitis, including inflammatory infiltrates and extensive fibrosis. In the context of oncogenic Kras expression, HIF2a stabilization leads to the development of large cystic lesions that resemble human mucinous cystic neoplasm (MCN), a less common precancerous lesion of pancreatic cancer. This work provides new mouse models of chronic pancreatitis and MCN, which can be of use in the future to study these conditions. Next, I create a new mouse model to study the role of tumor suppressor mutations in pancreatic cancer. Previously, only the most common point mutation in the tumor suppressor p53 has been modeled in mice, however in human pancreatic tumors a spectrum of p53 mutations is observed. In this work I create a new model that uses the second most common point mutation in p53, as well as models the sequence of events that occur in human tumors. I use these mice to

determine that mutant p53 expression promotes formation of precancerous lesions, but it is not necessary for the growth of established tumors.

Taken together, this dissertation work utilizes both existing and newly developed mouse models in order to provide new insights into the initiation and progression of pancreatic cancer. Overall, the use of mouse models provides an important scientific basis for experimentation that will eventually lead to new therapeutic options for pancreatic cancer, a truly devastating disease.

Chapter One

Introduction

Pancreatic cancer overview

Pancreatic cancer is one of the deadliest malignancies, with a five-year-survival rate of just 8% (SEER Database – www.seer.cancer.gov). It is expected to become the second leading cause of cancer-related death in the United States by the year 2020 (Rahib, Smith et al. 2014). Few specific symptoms of pancreatic cancer exist, and therefore most patients present with metastatic and unresectable lesions (Gillen, Schuster et al. 2010, Teague, Lim et al. 2015). Unfortunately, even those patients that undergo potentially curative resection have a median survival of less than two years (Katz, Wang et al. 2009). Standard chemotherapy approaches have proven unsuccessful in the treatment of pancreatic cancer (Hidalgo 2010), highlighting the need to understand the underlying biology of the disease in order to identify potential new therapeutic options.

Pancreatic cancer pathology

Pancreatic ductal adenocarcinoma, or PDAC, the most common pancreatic malignancy, arises from the exocrine pancreas and makes up the vast majority of pancreatic malignancies. The pancreas epithelium includes an exocrine and an endocrine component. The exocrine pancreas is comprised of acinar, centroacinar and

ductal cells. Acinar cells synthesize and secrete inactive precursors of digestive enzymes and ductal cells serve as a conduit for the passage of these enzymes into the intestine. The cell of origin of human PDAC is not confirmed, but animal studies suggest that PDAC arises primarily from acinar cells (Means, Meszoely et al. 2005, Morris, Cano et al. 2010, Kopp, von Figura et al. 2012), although other cells of origin have been identified (Gidekel Friedlander, Chu et al. 2009, Bailey, Hendley et al. 2016). PDAC proceeds through a series of well-defined histological stages, beginning with precursor lesions, termed pancreatic intraepithelial neoplasia (PanIN) and eventually progressing to frank carcinoma (Hruban, Goggins et al. 2000). PanINs are separated into low grade and high-grade lesions, a classification that corresponds with increased levels of celluar atypia (Hruban, Goggins et al. 2000). While PanINs are thought to be the most common precursor lesion to PDAC, other types of lesions do progress to carcinoma, although the underlying biology of those lesions is less well understood. These other types of precursors include intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN) (Hruban, Maitra et al. 2007, Yonezawa, Higashi et al. 2008).

Genetics of pancreatic cancer

The histologic changes observed during the progression of pancreatic cancer are accompanied by genetic changes (Amundadottir, Kraft et al. 2009, Petersen, Amundadottir et al. 2010, Wu, Miao et al. 2011). The most common of these are activating mutations in the oncogene Kras, which are found in over 90% of human tumors (Smit, Boot et al. 1988, Pellegata, Sessa et al. 1994, Moskaluk, Hruban et al. 1997, Jones, Zhang et al. 2008). Additionally, mutations in tumor suppressors, such as

p53, Ink4a/ARF, and BRCA2 are frequently observed (DiGiuseppe, Hruban et al. 1994, Wilentz, Geradts et al. 1998, Goggins, Hruban et al. 2000, Wilentz, Iacobuzio-Donahue et al. 2000). Kras is a small protein GTPase and a component of many cell signal transduction pathways (Khosravi-Far and Der 1994, Pylayeva-Gupta, Grabocka et al. 2011). In normal cells, Kras activation is brief, and the protein predominantly exists in the inactive GDP bound form. Kras mutations, most commonly the G12D mutation, result in a Kras protein that favors the active GTP-bound state (Hezel, Kimmelman et al. 2006). Therefore, mutant Kras can activate downstream effector pathways, including both MAP kinase and AKT signaling (Hezel, Kimmelman et al. 2006). It is the inappropriate activation of these cellular processes downstream of Kras that are thought to be the driving force behind the initiation of pancreatic cancer.

While Kras activation is often the first mutation, progression of pancreatic cancer is associated with later mutations in tumor suppressor genes, including p53, Ink4a/ARF and BRCA2 (Moskaluk, Hruban et al. 1997, Wilentz, Geradts et al. 1998, Goggins, Hruban et al. 2000, Wilentz, Iacobuzio-Donahue et al. 2000, Jones, Zhang et al. 2008). Mutations in the tumor suppressor gene p53 are common, being present in up to 75% of human pancreatic tumors (Bailey, Chang et al. 2016). TP53 is a protein that plays a role in normal cells by protecting organs from carcinogenesis, being activated in response to cell stress in order to direct cells toward either DNA repair or apoptosis (Vousden and Lu 2002). Mutations in p53 are found commonly in all human cancers (Hollstein, Sidransky et al. 1991, Muller and Vousden 2013). These mutations disrupt the normal protective functions of wild type p53, leading to inappropriate cell growth and survival (Oren and Rotter 2010, Muller and Vousden 2014). Missense p53 mutations are the

most common type of alteration, with a spectrum of missense mutations observed in human pancreatic tumors (Jones, Zhang et al. 2008). Importantly, p53 missense mutations in human cancer fall into two main categories – those that contain changes in the amino acids that normally directly contact DNA (contact mutants, typified by the R273H mutation) and those that more generally disrupt the structure of the p53 protein (structural mutations, like R175H) (Freed-Pastor and Prives 2012). Both contact mutants and structural mutants are prominent in human pancreatic cancer (Bailey, Chang et al. 2016). In general, TP53 missense mutants act as a dominant negative on the wild type, suppressing its function (de Vries, Flores et al. 2002, Willis, Jung et al. 2004). Additionally, certain p53 mutants can have gain of function roles, activating downstream targets beyond those typically activated by the wild type version (Oren and Rotter 2010). Tp53 gain of function can be achieved through interaction with different groups of binding partners or transcriptional activation of unique downstream target genes. Many of these binding proteins and target genes were identified in the context of particular p53 mutations, indicating that different p53 mutations result in unique downstream effects (Freed-Pastor and Prives 2012).

Pancreatic cancer subtypes

Several research groups have further classified genetic alterations in PDAC using human tumor data, leading to the definition of several subtypes of pancreatic cancer (Collisson, Sadanandam et al. 2011, Moffitt, Marayati et al. 2015, Waddell, Pajic et al. 2015, Bailey, Chang et al. 2016, Makohon-Moore, Zhang et al. 2017). Each of these groups uses different criteria, and so define different numbers of subtypes.

However, each group uses the common method of a genetic signature in the tumors to define each of the subtypes. Importantly, differences in underlying tumor biology and responses to therapy are still poorly defined. Given the advent of personalized medicine techniques, understanding the differences that arise in human pancreatic cancer with different mutational profiles will be important in the development of future treatments. However, without understanding the underlying biology of pancreatic cancer, the ease of development and testing of potential therapeutics is limited. Therefore, the development of proper mouse models of disease is paramount for the advancement of future treatment options.

Modeling pancreatic cancer in mice

$Kras^{G12D}$ in pancreatic cancer initiation

Mouse models of pancreatic cancer have been an essential tool in the study of the initiation and progression of PDAC. The well-defined genetics of human pancreatic tumors have provided the basis for modeling the disease in mice. Mouse models of pancreatic cancer that dependably recapitulate the human disease rely on pancreas-specific expression of oncogenic Kras, most commonly the $Kras^{G12D}$ allele. The most widely used of the models expressing oncogenic Kras, termed KC, uses a pancreas-specific cre recombinase (either $Ptf1a^{Cre/+}$ or Pdx1-Cre) to drive expression of an LSL-$Kras^{G12D}$ cassette (Aguirre, Bardeesy et al. 2003, Hingorani, Petricoin et al. 2003). This results in animals that express $Kras^{G12D}$ specifically in the pancreas, starting at embryonic stages. Despite $Kras^{G12D}$ expression during embryogenesis, KC animals are born with a normal pancreas and PanINs develop over time. PanIN formation and

progression in KC mice can be accelerated by the induction of pancreatitis (Guerra, Schuhmacher et al. 2007, Carriere, Young et al. 2009, Morris, Cano et al. 2010). Pancreatitis is induced in animals by administration of the cholecystokinin orthologue caerulein, which results in acinar cell necrosis and a fibroinflammatory response. In KC animals, caerulein-induced damage synergizes with the expression of oncogenic Kras, driving the rapid development of PanIN formation. Consequently, caerulein treated KC mice will typically have pancreata in which normal tissue is entirely replaced by PanIN lesions three weeks after pancreatitis (Guerra, Schuhmacher et al. 2007, Morris, Cano et al. 2010). KC animals, with or without pancreatitis treatment, reliably develop PanINs over time, however progression to frank carcinoma is infrequent.

$Kras^{G12D}$ in pancreatic cancer maintenance

While KC mice are useful to study the initiation of PanINs in mice, direct investigation of the mechanism of oncogenic Kras action in the pancreas is difficult due to the fact that KC mice permanently express oncogenic Kras starting from embryonic stages. iKras* mice express the $Kras^{G12D}$ mutation in an inducible and reversible manner, and are thus suitable to study the role of this oncogene in tumor maintenance (Collins, Bednar et al. 2012). iKras mice are generated using a pancreas-specific Cre recombinase ($Ptf1a^{Cre/+}$ or Pdx1-Cre) that drives excision of a stop cassette from the Rosa26-LSL-rtTa allele, thus activating the expression of rtTa. rtTa, a bacterial transcription factor, is active in presence of doxycycline (dox) and inactive in absence of the antibiotic (Belteki, Haigh et al. 2005). The third transgene in the iKras* model is TetO- $Kras^{G12D}$. Thus, in the presence of dox, mutant Kras expression is induced at will.

In contrast, removal of dox reverses Kras expression. Similar to KC animals, the induction of $Kras^{G12D}$ expression (by dox administration) during embryonic stages results in the extensive formation of PanINs in iKras animals. $Kras^{G12D}$ expression in iKras animals can be induced in adult mice, more closely mimicking the mutation timing observed in human pancreatic tumors. When $Kras^{G12D}$ is activated in adult animals using dox, there is a long latency to PanIN development, and induction of pancreatitis can accelerate and increase the penetrance of these lesions (Collins, Bednar et al. 2012). Once dox is removed from iKras mice, turning off expression of $Kras^{G12D}$, PanINs regress and the pancreas is replaced by normal appearing acinar cells after two weeks. While the development of KC animals showed that $Kras^{G12D}$ expression is sufficient to promote PanIN formation, iKras mice demonstrated that continued $Kras^{G12D}$ expression is sufficient for PanIN initiation and necessary for their maintenance.

Using $Kras^{G12D}$ mouse models to research pancreatic cancer initiation

Oncogenic Kras expression is frequently combined with pancreas-specific deletion of a gene of interest in order to identify the importance of that gene during PanIN formation (Zhang, Morris et al. 2013, Wu, Carpenter et al. 2014, Bednar, Schofield et al. 2015). For example, KC mice with pancreas specific deletion of the Polycomb group protein Bmi1 do not develop PanINs, demonstrating the necessity for Bmi1 expression for pancreatic cancer initiation (Bednar, Schofield et al. 2015). These types of studies have allowed numerous scientific insights into the biology of the early stages of pancreatic cancer. Depending on the gene of interest, a diverse array of cancer related processes can be explored using mice that express oncogenic Kras,

including the roles of hypoxia (Criscimanna, Duan et al. 2013, Lee, Spata et al. 2016), epigenetics (Mallen-St Clair, Soydaner-Azeloglu et al. 2012, Bednar, Schofield et al. 2015) and many other mechanistic aspects of disease development.

Tumor suppressor mutations in pancreatic cancer mouse models

While the KC and iKras mice have been vital in establishing the role of oncogenic $Kras^{G12D}$ mutation in precancerous lesion initiation and maintenance, neither of these models reliably progress to frank carcinoma. Similar to the genetic changes seen in human tumors, the addition of a tumor suppressor mutation to the KC or iKras animals leads to the formation of pancreatic cancer. Several tumor suppressor mutations have been used in the mice, guided by the spectrum of mutations seen in human cancer, including p53, Ink4a/ARF and BRCA2 (Aguirre, Bardeesy et al. 2003, Hingorani, Wang et al. 2005, Skoulidis, Cassidy et al. 2010). In the most extensively studied of these models, Kras mutation is combined with alterations in the tumor suppressor p53, termed KPC (Hingorani, Wang et al. 2005). KPC animals reliably develop pancreatic tumors with invasive cancer, similar to what is seen in human patients, and so are commonly used to explore the biology of the disease as well as in drug treatment experiments. Multiple versions of the KPC mouse model exist, using different alterations in p53. Some KPC animals express a floxed version of the p53 gene (KP(fl)C), resulting in a null allele of p53 in the pancreas, along with oncogenic Kras (Morton, Timpson et al. 2010). These mice develop pancreatic tumors quickly, within 4-5 months of life, but show few metastatic lesions, due to the rate at which they succumb to the disease. KP(fl)C animals are frequently used in drug studies given that they quickly and reliably

develop pancreatic tumors. However, null mutations are rarely seen in human pancreatic tumors, potentially limiting the applicability of the KP(fl)C system to human tumor biology.

Point mutations in p53 are more common in human pancreatic cancer than null alleles (Bailey, Chang et al. 2016). Additionally, these p53 point mutants can have gain-of-function effects (Olive, Tuveson et al. 2004, Oren and Rotter 2010), making it important to study these mutations instead of only null alleles of p53. The most common p53 point mutant in human pancreatic tumors is R175H, which corresponds to R172H in mice (Olivier, Eeles et al. 2002). KPC mice that express oncogenic Kras along with $p53^{R172H}$ reliably develop pancreatic tumors with metastatic lesions particularly in the liver.

KPC animals are a vital tool for studying the cellular processes involved in the later stages of cancer development. For example, epithelial-to-mesenchymal transition (EMT) and metastasis occur late in cancer progression (Kalluri and Weinberg 2009), and given that most human pancreatic cancer patients have metastatic lesions at the time of diagnosis, understanding the process of metastasis is vital for cancer treatment. KPC animals reliably develop metastases in a similar pattern to human tumors, and so serve as a tool for studying this step in cancer progression. In fact, cell lines made from KPC mice were used to show that mutant p53 helps drive metastasis of pancreatic cancer through expression of PDGFRβ (Weissmueller, Manchado et al. 2014). In addition to their utility for EMT and metastasis studies, KPC animals are useful in designing experiments to test new therapeutic options for pancreatic cancer. KPC animals reliably develop metastatic tumors similar to those seen in humans, over a

relatively short time frame. Therefore, treatment studies can easily be designed that may help mirror what reaction to the drugs would be seen in human tumors.

Overall, the development and use of KC, iKras and KPC mice has been instrumental in beginning to understand and treat pancreatic cancer in humans (Westphalen and Olive 2012, Gopinathan, Morton et al. 2015). These mice recapitulate genetic lesions seen in human pancreatic tumors and serve as a tool to probe their underlying biology.

Emerging mouse models in pancreatic cancer

Using multiple recombinase systems to target separate cellular compartments

There are intrinsic shortcomings in using embryonic transcription factor driven cre-mediated recombination to study carcinogenesis. New mouse model technologies help circumvent these shortcomings and expand the scope of animal model research in pancreatic cancer. One such example is a mouse model that expresses oncogenic Kras driven by flp recombinase instead of the more widely used cre recombinase. In these animals, termed KF, transgenic flp recombinase under the control of the *Pdx1* promoter drives excision of the stop cassette from an FSF-Kras^{G12D} allele, resulting in pancreas specific expression of Kras^{G12D}. Importantly, cre recombinase can be used for other functions in the model. For example, mast cells are present in the pancreas during PanIN initiation, but their function there is unknown. KF animals were combined with a cre-driven system to delete the entire mast cell lineage. Histologic analysis at 9 months of age showed similar PanIN burden in KF animals with or without mast cells, indicating that mast cell presence is dispensable for pancreatic cancer initiation and progression

(Schonhuber, Seidler et al. 2014). KF mice can be used in this manner to investigate the functional role of genes during pancreatic cancer development in non-epithelial compartments.

Methods to temporally regulate the expression of multiple transgenes

In addition to targeting separate compartments, KF animals have the advantage of allowing cre-mediated gene editing after tumor formation by the use of a CreER system. In this system, cre recombinase is only active in the presence of tamoxifen treatment, which can allow deletion or expression of floxed genes after PanINs have already been formed in KF mice. Thus, cre-mediated gene ablation can occur in a temporal fashion instead of simultaneously with $Kras^{G12D}$ activation, as required in KC animals. This allows study of the role of genes at later time points during pancreatic cancer development, which is likely to be more analogous to human tumor biology. As an example of this system, Pdpk1 expression was known to be required for PanIN initiation (Eser, Reiff et al. 2013), however its role in later tumor stages remained unknown. To answer this question, KF mice were allowed to develop PanINs, and then the CreER system was used to delete Pdpk1 specifically in the pancreas, including in these established lesions. At 9 months of age, animals with Pdpk1 deletion lacked PanINs, while KF controls had pancreata almost entirely replaced by lesions, demonstrating that continued Pdpk1 expression is required after PanIN initiation. Treatment of human pancreatic cancer begins after tumor establishment, making these studies more analogous to human pancreatic caner treatment than genetic deletion in traditional mouse models (KC or KPC) (Schonhuber, Seidler et al. 2014).

Tumor suppressors in multiple recombinase systems

KF animals can be combined with a null allele of p53, creating KPF mice (Lee, Moding et al. 2012, Schonhuber, Seidler et al. 2014). Importantly, KPF mice have cre recombinase available for use in other capacities than expression of $Kras^{G12D}$ and p53 null. This confers similar advantages to KF mice, such as analysis of multiple cell compartments or temporal regulation, but in later tumor stages. This allows study of aspects of carcinogenesis that are unique to later tumor stages, such as EMT and metastasis. Given the complexity of pancreatic cancer biology, this flexibility in mouse models is an exciting new development that will allow many new types of experiments that were impossible with the use of only cre recombinase.

CRISPR/Cas9 in mouse models of pancreatic cancer

Other biological technologies are becoming useful in pancreatic cancer mouse model research (Sanchez-Rivera and Jacks 2015). CRISPR/Cas9 is a system that creates specific, permanent changes in cellular DNA (Doudna and Charpentier 2014). The CRISPR/Cas9 system can make changes in any region of DNA, which can be designed to produce many types of effects, including deletions, mutations, or overexpression. There are several ways that the CRISPR/Cas9 system can be useful in pancreatic cancer research. First, the DNA of mouse embryos can be modified by this technology (Wang, Yang et al. 2013, Yang, Wang et al. 2013). Wild type embryos can be engineered to express both oncogenic Kras and mutant p53, creating KPC animals. Given that these can be created from wild type embryos, large numbers of KPC mice

can be created at once without using a traditional breeding system. This allows the set up of large experiments using many mice at once that would be difficult to perform with typical mouse breeding. Using larger numbers of animals in research studies improves the reliability of research findings.

Given that CRISPR/Cas9 can edit any cellular DNA, pancreatic cancer mouse models can also be developed from adult wild type mice (Chiou, Winters et al. 2015, Mazur, Herner et al. 2015, Maresch, Mueller et al. 2016). Mutations that lead to or modify pancreatic cancer can be engineered into the adult pancreas through a delivery method in order to initiate the development of pancreatic cancer. One delivery method is injection directly into the pancreas, with subsequent development of a tumor at that site. Again, this method may be superior to traditional breeding schemes because it allows the use of wild type mice, making it easier to obtain the correct animals. Additionally, traditional mouse models (KC and KPC) induce mutations into the embryonic pancreas, a situation that is not analogous to a human tumor. Tumor initiation in the adult murine pancreas may be more similar to human tumor biology, and the study of how a tumor develops in an adult mouse may be a more close parallel.

Third, CRISPR/Cas9 is used to create specific, desired mutations and deletions. So far, the number of mutations created in typical pancreatic cancer mouse model research is quite small compared to the breadth of mutations observed in human pancreatic tumors (Bailey, Chang et al. 2016). Given that CRISPR/Cas9 is a fast way to induce mutation in mice, it could be used to recapitulate the spectrum of mutations seen in humans. Given the rise of personalized medicine, it is possible that CRISPR/Cas9 could eventually be used to develop mouse models of the tumors of specific human

patients, according to their mutation profile. These mouse models could then be used to determine which treatment options were most appropriate for that specific tumor, and to eventually be used in that patient. There is a vast array of combinations of mutations that are seen in human pancreatic tumors, and the development of CRISPR/Cas9 raises the possibility that in the near future many more of these types of tumors could be studied and potentially treated more specifically than has been previously possible.

Dissertation Overview

Bmi1 and HIF1α in pancreatic cancer

In this dissertation, I use existing mouse models as well as develop new tools in order to further understand the mechanisms the underlie the initiation and progression of pancreatic cancer. In Chapter Two, which has been published (Bednar, Schofield et al. 2015), I explore the role of the epigenetic regulator and known oncogene Bmi1 in the initiation of pancreatic cancer. I generated KC mice with pancreas specific deletion of Bmi1. These mice fail to develop PanINs, indicating an absolute requirement for Bmi1 in pancreatic cancer initiation. I endeavored to investigate the mechanism underlying the requirement for Bmi1. For this purpose, I followed a candidate approach. The requirement for Bmi1 expression in the initiation of pancreatic cancer was not due to the known ability of Bmi1 to regulate the tumor suppressors Ink4a and ARF. I did determine that Bmi1 is required for the regulation of reactive oxygen species (ROS) levels in pancreatic cancer cells. Notably, regulation of ROS accumulation is a prerequisite for PanIN formation (DeNicola, Karreth et al. 2011). Confusingly, however, none of the “classic” regulators of intracellular ROS accumulation, namely Nrf2 and Keap1, were

affected by Bmi1 inactivation. In Chapter Three, I deepened my investigation into the interplay between Bmi1, ROS accumulation and pancreatic carcinogenesis. I found that Bmi1 expression regulates levels of HIF1α and HIF2α in pancreatic cancer cells. Although their best-known function is the regulation of response to hypoxia, HIF factors are also important to modulate ROS accumulation (Galanis, Pappa et al. 2008). I thus tested whether expression of an oxygen stable form of HIF1α in the pancreas could rescue Kras-driven carcinogenesis in absence of Bmi1. KC mice lacking Bmi1 do not develop PanINs, but the additional expression of HIF1α in these animals recovers PanIN formation. This recovery of lesion formation suggests that Bmi1 regulation of HIF1α in the precancerous pancreas may be the reason for the requirement for Bmi1 expression during pancreatic cancer initiation.

HIF2α stabilization as a mouse model for chronic pancreatitis and MCN

In Chapter Four, I focus on the role of HIF2α in both the normal and neoplastic pancreas. I find that expression of an oxygen stable form of HIF2α specifically in the pancreas causes a phenotype that resembles human chronic pancreatitis. Mice expressing HIF2α display pancreata with increased fibrosis, inflammation and ductal structures. There are very few mouse models of chronic pancreatitis that closely resemble the human disease, and so the finding that pancreata with HIF2α stabilization recreate this phenotype could lead to its use as a tool to study pancreatitis. Given that chronic pancreatitis is a risk factor for development of pancreatic cancer, I also studied pancreatic HIF2α stabilization in the context of mutant Kras expression. Mice with $Kras^{G12D}$ and HIF2α expression do not develop PanINs, but instead display a phenotype

resembling human mucinous cystic neoplasm (MCN). MCN is a pancreatic cystic lesion that, while less common than PanINs, also serves as a precursor lesion to pancreatic cancer. MCN biology is much less well understood than that of PanINs, and so mice that model MCN are important in studying this type of pancreatic lesion. Overall, in Chapter Four I show that HIF2α stabilization in the pancreas causes chronic pancreatitis and leads to MCN in the context of mutant Kras expression. Both of these conditions serve as new mouse models for human diseases that are currently lacking viable research tools.

Creating a mouse model of mutant p53^{R270H} expression in pancreatic cancer

In Chapter Five, I use what we know about human pancreatic cancer genetics to create a new model of the disease. While KPC mice recapitulate the stages of human pancreatic cancer, they only employ one specific p53 mutation. However, in humans a spectrum of p53 mutations is observed. Here I created a model, termed KCip53, which expresses a p53 mutation that is common in human pancreatic cancer but has not been studied in mice. Additionally, in KCip53 animals I can turn on and off p53 mutation at will, allowing study specifically of mutant p53 action in pancreatic cancer initiation and progression. Using these mice, I find differential roles for mutant p53 during carcinogenesis, with mutant p53 expression promoting pancreatic cancer initiation but being dispensable for established tumor growth.

Finally, in Chapter Six I discuss further questions and directions that are raised by this work. I will discuss how this work fits into the greater field of pancreatic cancer mouse modeling and research, and what further questions this dissertation work raises.

This work will create opportunities to help move the understanding of pancreatic cancer forward, which will be highlighted in the final chapter.

References

Aguirre, A. J., N. Bardeesy, M. Sinha, L. Lopez, D. A. Tuveson, J. Horner, M. S. Redston and R. A. DePinho (2003). "Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma." Genes Dev **17**(24): 3112-3126.

Amundadottir, L., P. Kraft, R. Z. Stolzenberg-Solomon, C. S. Fuchs, G. M. Petersen, A. A. Arslan, H. B. Bueno-de-Mesquita, M. Gross, K. Helzlsouer, E. J. Jacobs, A. LaCroix, W. Zheng, D. Albanes, W. Bamlet, C. D. Berg, F. Berrino, S. Bingham, J. E. Buring, P. M. Bracci, F. Canzian, F. Clavel-Chapelon, S. Clipp, M. Cotterchio, M. de Andrade, E. J. Duell, J. W. Fox, Jr., S. Gallinger, J. M. Gaziano, E. L. Giovannucci, M. Goggins, C. A. Gonzalez, G. Hallmans, S. E. Hankinson, M. Hassan, E. A. Holly, D. J. Hunter, A. Hutchinson, R. Jackson, K. B. Jacobs, M. Jenab, R. Kaaks, A. P. Klein, C. Kooperberg, R. C. Kurtz, D. Li, S. M. Lynch, M. Mandelson, R. R. McWilliams, J. B. Mendelsohn, D. S. Michaud, S. H. Olson, K. Overvad, A. V. Patel, P. H. Peeters, A. Rajkovic, E. Riboli, H. A. Risch, X. O. Shu, G. Thomas, G. S. Tobias, D. Trichopoulos, S. K. Van Den Eeden, J. Virtamo, J. Wactawski-Wende, B. M. Wolpin, H. Yu, K. Yu, A. Zeleniuch-Jacquotte, S. J. Chanock, P. Hartge and R. N. Hoover (2009). "Genome-wide association study identifies variants in the ABO locus associated with susceptibility to pancreatic cancer." Nat Genet **41**(9): 986-990.

Bailey, J. M., A. M. Hendley, K. J. Lafaro, M. A. Pruski, N. C. Jones, J. Alsina, M. Younes, A. Maitra, F. McAllister, C. A. Iacobuzio-Donahue and S. D. Leach (2016). "p53 mutations cooperate with oncogenic Kras to promote adenocarcinoma from pancreatic ductal cells." Oncogene **35**(32): 4282-4288.

Bailey, P., D. K. Chang, K. Nones, A. L. Johns, A. M. Patch, M. C. Gingras, D. K. Miller, A. N. Christ, T. J. Bruxner, M. C. Quinn, C. Nourse, L. C. Murtaugh, I. Harliwong, S. Idrisoglu, S. Manning, E. Nourbakhsh, S. Wani, L. Fink, O. Holmes, V. Chin, M. J. Anderson, S. Kazakoff, C. Leonard, F. Newell, N. Waddell, S. Wood, Q. Xu, P. J. Wilson, N. Cloonan, K. S. Kassahn, D. Taylor, K. Quek, A. Robertson, L. Pantano, L. Mincarelli, L. N. Sanchez, L. Evers, J. Wu, M. Pinese, M. J. Cowley, M. D. Jones, E. K. Colvin, A. M. Nagrial, E. S. Humphrey, L. A. Chantrill, A. Mawson, J. Humphris, A. Chou, M. Pajic, C. J. Scarlett, A. V. Pinho, M. Giry-Laterriere, I. Rooman, J. S. Samra, J. G. Kench, J. A. Lovell, N. D. Merrett, C. W. Toon, K. Epari, N. Q. Nguyen, A. Barbour, N. Zeps, K. Moran-Jones, N. B. Jamieson, J. S. Graham, F. Duthie, K. Oien, J. Hair, R. Grutzmann, A. Maitra, C. A. Iacobuzio-Donahue, C. L. Wolfgang, R. A. Morgan, R. T. Lawlor, V. Corbo, C. Bassi, B. Rusev, P. Capelli, R. Salvia, G. Tortora, D. Mukhopadhyay, G. M. Petersen, I. Australian Pancreatic Cancer Genome, D. M. Munzy, W. E. Fisher, S. A. Karim, J. R. Eshleman, R. H. Hruban, C. Pilarsky, J. P. Morton, O. J. Sansom, A. Scarpa, E. A. Musgrove, U. M. Bailey, O. Hofmann, R. L. Sutherland, D. A. Wheeler, A. J. Gill, R. A. Gibbs, J. V. Pearson, N. Waddell, A. V. Biankin and S. M. Grimmond (2016). "Genomic analyses identify molecular subtypes of pancreatic cancer." Nature **531**(7592): 47-52.

Bednar, F., H. K. Schofield, M. A. Collins, W. Yan, Y. Zhang, N. Shyam, J. A. Eberle, L. L. Almada, K. P. Olive, N. Bardeesy, M. E. Fernandez-Zapico, D. Nakada, D. M. Simeone, S. J. Morrison and M. Pasca di Magliano (2015). "Bmi1 is required for the initiation of pancreatic cancer through an Ink4a-independent mechanism." Carcinogenesis **36**(7): 730-738.

Belteki, G., J. Haigh, N. Kabacs, K. Haigh, K. Sison, F. Costantini, J. Whitsett, S. E. Quaggin and A. Nagy (2005). "Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction." Nucleic Acids Res **33**(5): e51.

Carriere, C., A. L. Young, J. R. Gunn, D. S. Longnecker and M. Korc (2009). "Acute pancreatitis markedly accelerates pancreatic cancer progression in mice expressing oncogenic Kras." Biochem Biophys Res Commun **382**(3): 561-565.

Chiou, S. H., I. P. Winters, J. Wang, S. Naranjo, C. Dudgeon, F. B. Tamburini, J. J. Brady, D. Yang, B. M. Gruner, C. H. Chuang, D. R. Caswell, H. Zeng, P. Chu, G. E. Kim, D. R. Carpizo, S. K. Kim and M. M. Winslow (2015). "Pancreatic cancer modeling using retrograde viral vector delivery and in vivo CRISPR/Cas9-mediated somatic genome editing." Genes Dev **29**(14): 1576-1585.

Collins, M. A., F. Bednar, Y. Zhang, J. C. Brisset, S. Galban, C. J. Galban, S. Rakshit, K. S. Flannagan, N. V. Adsay and M. Pasca di Magliano (2012). "Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice." J Clin Invest **122**(2): 639-653.

Collisson, E. A., A. Sadanandam, P. Olson, W. J. Gibb, M. Truitt, S. Gu, J. Cooc, J. Weinkle, G. E. Kim, L. Jakkula, H. S. Feiler, A. H. Ko, A. B. Olshen, K. L. Danenberg, M.

A. Tempero, P. T. Spellman, D. Hanahan and J. W. Gray (2011). "Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy." Nat Med **17**(4): 500-503.

Criscimanna, A., L. J. Duan, J. A. Rhodes, V. Fendrich, E. Wickline, D. J. Hartman, S. P. Monga, M. T. Lotze, G. K. Gittes, G. H. Fong and F. Esni (2013). "PanIN-specific regulation of Wnt signaling by HIF2alpha during early pancreatic tumorigenesis." Cancer Res **73**(15): 4781-4790.

de Vries, A., E. R. Flores, B. Miranda, H. M. Hsieh, C. T. van Oostrom, J. Sage and T. Jacks (2002). "Targeted point mutations of p53 lead to dominant-negative inhibition of wild-type p53 function." Proc Natl Acad Sci U S A **99**(5): 2948-2953.

DeNicola, G. M., F. A. Karreth, T. J. Humpton, A. Gopinathan, C. Wei, K. Frese, D. Mangal, K. H. Yu, C. J. Yeo, E. S. Calhoun, F. Scrimieri, J. M. Winter, R. H. Hruban, C. Iacobuzio-Donahue, S. E. Kern, I. A. Blair and D. A. Tuveson (2011). "Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis." Nature **475**(7354): 106-109.

DiGiuseppe, J. A., R. H. Hruban, S. N. Goodman, M. Polak, F. M. van den Berg, D. C. Allison, J. L. Cameron and G. J. Offerhaus (1994). "Overexpression of p53 protein in adenocarcinoma of the pancreas." Am J Clin Pathol **101**(6): 684-688.

Doudna, J. A. and E. Charpentier (2014). "Genome editing. The new frontier of genome engineering with CRISPR-Cas9." Science **346**(6213): 1258096.

Eser, S., N. Reiff, M. Messer, B. Seidler, K. Gottschalk, M. Dobler, M. Hieber, A. Arbeiter, S. Klein, B. Kong, C. W. Michalski, A. M. Schlitter, I. Esposito, A. J. Kind, L. Rad, A. E. Schnieke, M. Baccarini, D. R. Alessi, R. Rad, R. M. Schmid, G. Schneider

and D. Saur (2013). "Selective requirement of PI3K/PDK1 signaling for Kras oncogene-driven pancreatic cell plasticity and cancer." Cancer Cell **23**(3): 406-420.

Freed-Pastor, W. A. and C. Prives (2012). "Mutant p53: one name, many proteins." Genes Dev **26**(12): 1268-1286.

Galanis, A., A. Pappa, A. Giannakakis, E. Lanitis, D. Dangaj and R. Sandaltzopoulos (2008). "Reactive oxygen species and HIF-1 signalling in cancer." Cancer Lett **266**(1): 12-20.

Gidekel Friedlander, S. Y., G. C. Chu, E. L. Snyder, N. Girnius, G. Dibelius, D. Crowley, E. Vasile, R. A. DePinho and T. Jacks (2009). "Context-dependent transformation of adult pancreatic cells by oncogenic K-Ras." Cancer Cell **16**(5): 379-389.

Gillen, S., T. Schuster, C. Meyer Zum Buschenfelde, H. Friess and J. Kleeff (2010). "Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages." PLoS Med **7**(4): e1000267.

Goggins, M., R. H. Hruban and S. E. Kern (2000). "BRCA2 is inactivated late in the development of pancreatic intraepithelial neoplasia: evidence and implications." Am J Pathol **156**(5): 1767-1771.

Gopinathan, A., J. P. Morton, D. I. Jodrell and O. J. Sansom (2015). "GEMMs as preclinical models for testing pancreatic cancer therapies." Dis Model Mech **8**(10): 1185-1200.

Guerra, C., A. J. Schuhmacher, M. Canamero, P. J. Grippo, L. Verdaguer, L. Perez-Gallego, P. Dubus, E. P. Sandgren and M. Barbacid (2007). "Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice." Cancer Cell **11**(3): 291-302.

Hezel, A. F., A. C. Kimmelman, B. Z. Stanger, N. Bardeesy and R. A. Depinho (2006). "Genetics and biology of pancreatic ductal adenocarcinoma." Genes Dev **20**(10): 1218-1249.

Hidalgo, M. (2010). "Pancreatic cancer." N Engl J Med **362**(17): 1605-1617.

Hingorani, S. R., E. F. Petricoin, A. Maitra, V. Rajapakse, C. King, M. A. Jacobetz, S. Ross, T. P. Conrads, T. D. Veenstra, B. A. Hitt, Y. Kawaguchi, D. Johann, L. A. Liotta, H. C. Crawford, M. E. Putt, T. Jacks, C. V. Wright, R. H. Hruban, A. M. Lowy and D. A. Tuveson (2003). "Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse." Cancer Cell **4**(6): 437-450.

Hingorani, S. R., L. Wang, A. S. Multani, C. Combs, T. B. Deramaudt, R. H. Hruban, A. K. Rustgi, S. Chang and D. A. Tuveson (2005). "Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice." Cancer Cell **7**(5): 469-483.

Hollstein, M., D. Sidransky, B. Vogelstein and C. C. Harris (1991). "p53 mutations in human cancers." Science **253**(5015): 49-53.

Hruban, R. H., M. Goggins, J. Parsons and S. E. Kern (2000). "Progression model for pancreatic cancer." Clin Cancer Res **6**(8): 2969-2972.

Hruban, R. H., A. Maitra, S. E. Kern and M. Goggins (2007). "Precursors to pancreatic cancer." Gastroenterol Clin North Am **36**(4): 831-849, vi.

Jones, S., X. Zhang, D. W. Parsons, J. C. Lin, R. J. Leary, P. Angenendt, P. Mankoo, H. Carter, H. Kamiyama, A. Jimeno, S. M. Hong, B. Fu, M. T. Lin, E. S. Calhoun, M. Kamiyama, K. Walter, T. Nikolskaya, Y. Nikolsky, J. Hartigan, D. R. Smith, M. Hidalgo, S. D. Leach, A. P. Klein, E. M. Jaffee, M. Goggins, A. Maitra, C. Iacobuzio-Donahue, J.

R. Eshleman, S. E. Kern, R. H. Hruban, R. Karchin, N. Papadopoulos, G. Parmigiani, B. Vogelstein, V. E. Velculescu and K. W. Kinzler (2008). "Core signaling pathways in human pancreatic cancers revealed by global genomic analyses." Science **321**(5897): 1801-1806.

Kalluri, R. and R. A. Weinberg (2009). "The basics of epithelial-mesenchymal transition." J Clin Invest **119**(6): 1420-1428.

Katz, M. H., H. Wang, J. B. Fleming, C. C. Sun, R. F. Hwang, R. A. Wolff, G. Varadhachary, J. L. Abbruzzese, C. H. Crane, S. Krishnan, J. N. Vauthey, E. K. Abdalla, J. E. Lee, P. W. Pisters and D. B. Evans (2009). "Long-term survival after multidisciplinary management of resected pancreatic adenocarcinoma." Ann Surg Oncol **16**(4): 836-847.

Khosravi-Far, R. and C. J. Der (1994). "The Ras signal transduction pathway." Cancer Metastasis Rev **13**(1): 67-89.

Kopp, J. L., G. von Figura, E. Mayes, F. F. Liu, C. L. Dubois, J. P. t. Morris, F. C. Pan, H. Akiyama, C. V. Wright, K. Jensen, M. Hebrok and M. Sander (2012). "Identification of Sox9-dependent acinar-to-ductal reprogramming as the principal mechanism for initiation of pancreatic ductal adenocarcinoma." Cancer Cell **22**(6): 737-750.

Lee, C. L., E. J. Moding, X. Huang, Y. Li, L. Z. Woodlief, R. C. Rodrigues, Y. Ma and D. G. Kirsch (2012). "Generation of primary tumors with Flp recombinase in FRT-flanked p53 mice." Dis Model Mech **5**(3): 397-402.

Lee, K. E., M. Spata, L. J. Bayne, E. L. Buza, A. C. Durham, D. Allman, R. H. Vonderheide and M. C. Simon (2016). "Hif1a Deletion Reveals Pro-Neoplastic Function of B Cells in Pancreatic Neoplasia." Cancer Discov **6**(3): 256-269.

Makohon-Moore, A. P., M. Zhang, J. G. Reiter, I. Bozic, B. Allen, D. Kundu, K. Chatterjee, F. Wong, Y. Jiao, Z. A. Kohutek, J. Hong, M. Attiyeh, B. Javier, L. D. Wood, R. H. Hruban, M. A. Nowak, N. Papadopoulos, K. W. Kinzler, B. Vogelstein and C. A. Iacobuzio-Donahue (2017). "Limited heterogeneity of known driver gene mutations among the metastases of individual patients with pancreatic cancer." Nat Genet **49**(3): 358-366.

Mallen-St Clair, J., R. Soydaner-Azeloglu, K. E. Lee, L. Taylor, A. Livanos, Y. Pylayeva-Gupta, G. Miller, R. Margueron, D. Reinberg and D. Bar-Sagi (2012). "EZH2 couples pancreatic regeneration to neoplastic progression." Genes Dev **26**(5): 439-444.

Maresch, R., S. Mueller, C. Veltkamp, R. Ollinger, M. Friedrich, I. Heid, K. Steiger, J. Weber, T. Engleitner, M. Barenboim, S. Klein, S. Louzada, R. Banerjee, A. Strong, T. Stauber, N. Gross, U. Geumann, S. Lange, M. Ringelhan, I. Varela, K. Unger, F. Yang, R. M. Schmid, G. S. Vassiliou, R. Braren, G. Schneider, M. Heikenwalder, A. Bradley, D. Saur and R. Rad (2016). "Multiplexed pancreatic genome engineering and cancer induction by transfection-based CRISPR/Cas9 delivery in mice." Nat Commun **7**: 10770.

Mazur, P. K., A. Herner, S. S. Mello, M. Wirth, S. Hausmann, F. J. Sanchez-Rivera, S. M. Lofgren, T. Kuschma, S. A. Hahn, D. Vangala, M. Trajkovic-Arsic, A. Gupta, I. Heid, P. B. Noel, R. Braren, M. Erkan, J. Kleeff, B. Sipos, L. C. Sayles, M. Heikenwalder, E. Hessmann, V. Ellenrieder, I. Esposito, T. Jacks, J. E. Bradner, P. Khatri, E. A. Sweet-Cordero, L. D. Attardi, R. M. Schmid, G. Schneider, J. Sage and J. T. Siveke (2015). "Combined inhibition of BET family proteins and histone deacetylases as a potential epigenetics-based therapy for pancreatic ductal adenocarcinoma." Nat Med **21**(10): 1163-1171.

Means, A. L., I. M. Meszoely, K. Suzuki, Y. Miyamoto, A. K. Rustgi, R. J. Coffey, Jr., C. V. Wright, D. A. Stoffers and S. D. Leach (2005). "Pancreatic epithelial plasticity mediated by acinar cell transdifferentiation and generation of nestin-positive intermediates." Development **132**(16): 3767-3776.

Moffitt, R. A., R. Marayati, E. L. Flate, K. E. Volmar, S. G. Loeza, K. A. Hoadley, N. U. Rashid, L. A. Williams, S. C. Eaton, A. H. Chung, J. K. Smyla, J. M. Anderson, H. J. Kim, D. J. Bentrem, M. S. Talamonti, C. A. Iacobuzio-Donahue, M. A. Hollingsworth and J. J. Yeh (2015). "Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma." Nat Genet **47**(10): 1168-1178.

Morris, J. P. t., D. A. Cano, S. Sekine, S. C. Wang and M. Hebrok (2010). "Beta-catenin blocks Kras-dependent reprogramming of acini into pancreatic cancer precursor lesions in mice." J Clin Invest **120**(2): 508-520.

Morton, J. P., P. Timpson, S. A. Karim, R. A. Ridgway, D. Athineos, B. Doyle, N. B. Jamieson, K. A. Oien, A. M. Lowy, V. G. Brunton, M. C. Frame, T. R. Evans and O. J. Sansom (2010). "Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer." Proc Natl Acad Sci U S A **107**(1): 246-251.

Moskaluk, C. A., R. H. Hruban and S. E. Kern (1997). "p16 and K-ras gene mutations in the intraductal precursors of human pancreatic adenocarcinoma." Cancer Res **57**(11): 2140-2143.

Muller, P. A. and K. H. Vousden (2013). "p53 mutations in cancer." Nat Cell Biol **15**(1): 2-8.

Muller, P. A. and K. H. Vousden (2014). "Mutant p53 in cancer: new functions and therapeutic opportunities." Cancer Cell **25**(3): 304-317.

Olive, K. P., D. A. Tuveson, Z. C. Ruhe, B. Yin, N. A. Willis, R. T. Bronson, D. Crowley and T. Jacks (2004). "Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome." Cell **119**(6): 847-860.

Olivier, M., R. Eeles, M. Hollstein, M. A. Khan, C. C. Harris and P. Hainaut (2002). "The IARC TP53 database: new online mutation analysis and recommendations to users." Hum Mutat **19**(6): 607-614.

Oren, M. and V. Rotter (2010). "Mutant p53 gain-of-function in cancer." Cold Spring Harb Perspect Biol **2**(2): a001107.

Pellegata, N. S., F. Sessa, B. Renault, M. Bonato, B. E. Leone, E. Solcia and G. N. Ranzani (1994). "K-ras and p53 gene mutations in pancreatic cancer: ductal and nonductal tumors progress through different genetic lesions." Cancer Res **54**(6): 1556-1560.

Petersen, G. M., L. Amundadottir, C. S. Fuchs, P. Kraft, R. Z. Stolzenberg-Solomon, K. B. Jacobs, A. A. Arslan, H. B. Bueno-de-Mesquita, S. Gallinger, M. Gross, K. Helzlsouer, E. A. Holly, E. J. Jacobs, A. P. Klein, A. LaCroix, D. Li, M. T. Mandelson, S. H. Olson, H. A. Risch, W. Zheng, D. Albanes, W. R. Bamlet, C. D. Berg, M. C. Boutron-Ruault, J. E. Buring, P. M. Bracci, F. Canzian, S. Clipp, M. Cotterchio, M. de Andrade, E. J. Duell, J. M. Gaziano, E. L. Giovannucci, M. Goggins, G. Hallmans, S. E. Hankinson, M. Hassan, B. Howard, D. J. Hunter, A. Hutchinson, M. Jenab, R. Kaaks, C. Kooperberg, V. Krogh, R. C. Kurtz, S. M. Lynch, R. R. McWilliams, J. B. Mendelsohn, D. S. Michaud, H. Parikh, A. V. Patel, P. H. Peeters, A. Rajkovic, E. Riboli, L. Rodriguez, D. Seminara, X. O. Shu, G. Thomas, A. Tjonneland, G. S. Tobias, D. Trichopoulos, S. K. Van Den Eeden, J. Virtamo, J. Wactawski-Wende, Z. Wang, B. M. Wolpin, H. Yu, K.

Yu, A. Zeleniuch-Jacquotte, J. F. Fraumeni, Jr., R. N. Hoover, P. Hartge and S. J. Chanock (2010). "A genome-wide association study identifies pancreatic cancer susceptibility loci on chromosomes 13q22.1, 1q32.1 and 5p15.33." Nat Genet **42**(3): 224-228.

Pylayeva-Gupta, Y., E. Grabocka and D. Bar-Sagi (2011). "RAS oncogenes: weaving a tumorigenic web." Nat Rev Cancer **11**(11): 761-774.

Rahib, L., B. D. Smith, R. Aizenberg, A. B. Rosenzweig, J. M. Fleshman and L. M. Matrisian (2014). "Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States." Cancer Res **74**(11): 2913-2921.

Sanchez-Rivera, F. J. and T. Jacks (2015). "Applications of the CRISPR-Cas9 system in cancer biology." Nat Rev Cancer **15**(7): 387-395.

Schonhuber, N., B. Seidler, K. Schuck, C. Veltkamp, C. Schachtler, M. Zukowska, S. Eser, T. B. Feyerabend, M. C. Paul, P. Eser, S. Klein, A. M. Lowy, R. Banerjee, F. Yang, C. L. Lee, E. J. Moding, D. G. Kirsch, A. Scheideler, D. R. Alessi, I. Varela, A. Bradley, A. Kind, A. E. Schnieke, H. R. Rodewald, R. Rad, R. M. Schmid, G. Schneider and D. Saur (2014). "A next-generation dual-recombinase system for time- and host-specific targeting of pancreatic cancer." Nat Med **20**(11): 1340-1347.

Skoulidis, F., L. D. Cassidy, V. Pisupati, J. G. Jonasson, H. Bjarnason, J. E. Eyfjord, F. A. Karreth, M. Lim, L. M. Barber, S. A. Clatworthy, S. E. Davies, K. P. Olive, D. A. Tuveson and A. R. Venkitaraman (2010). "Germline Brca2 heterozygosity promotes Kras(G12D) -driven carcinogenesis in a murine model of familial pancreatic cancer." Cancer Cell **18**(5): 499-509.

Smit, V. T., A. J. Boot, A. M. Smits, G. J. Fleuren, C. J. Cornelisse and J. L. Bos (1988). "KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas." Nucleic Acids Res **16**(16): 7773-7782.

Teague, A., K. H. Lim and A. Wang-Gillam (2015). "Advanced pancreatic adenocarcinoma: a review of current treatment strategies and developing therapies." Ther Adv Med Oncol **7**(2): 68-84.

Vousden, K. H. and X. Lu (2002). "Live or let die: the cell's response to p53." Nat Rev Cancer **2**(8): 594-604.

Waddell, N., M. Pajic, A. M. Patch, D. K. Chang, K. S. Kassahn, P. Bailey, A. L. Johns, D. Miller, K. Nones, K. Quek, M. C. Quinn, A. J. Robertson, M. Z. Fadlullah, T. J. Bruxner, A. N. Christ, I. Harliwong, S. Idrisoglu, S. Manning, C. Nourse, E. Nourbakhsh, S. Wani, P. J. Wilson, E. Markham, N. Cloonan, M. J. Anderson, J. L. Fink, O. Holmes, S. H. Kazakoff, C. Leonard, F. Newell, B. Poudel, S. Song, D. Taylor, N. Waddell, S. Wood, Q. Xu, J. Wu, M. Pinese, M. J. Cowley, H. C. Lee, M. D. Jones, A. M. Nagrial, J. Humphris, L. A. Chantrill, V. Chin, A. M. Steinmann, A. Mawson, E. S. Humphrey, E. K. Colvin, A. Chou, C. J. Scarlett, A. V. Pinho, M. Giry-Laterriere, I. Rooman, J. S. Samra, J. G. Kench, J. A. Pettitt, N. D. Merrett, C. Toon, K. Epari, N. Q. Nguyen, A. Barbour, N. Zeps, N. B. Jamieson, J. S. Graham, S. P. Niclou, R. Bjerkvig, R. Grutzmann, D. Aust, R. H. Hruban, A. Maitra, C. A. Iacobuzio-Donahue, C. L. Wolfgang, R. A. Morgan, R. T. Lawlor, V. Corbo, C. Bassi, M. Falconi, G. Zamboni, G. Tortora, M. A. Tempero, I. Australian Pancreatic Cancer Genome, A. J. Gill, J. R. Eshleman, C. Pilarsky, A. Scarpa, E. A. Musgrove, J. V. Pearson, A. V. Biankin and S. M. Grimmond (2015).

"Whole genomes redefine the mutational landscape of pancreatic cancer." Nature **518**(7540): 495-501.

Wang, H., H. Yang, C. S. Shivalila, M. M. Dawlaty, A. W. Cheng, F. Zhang and R. Jaenisch (2013). "One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering." Cell **153**(4): 910-918.

Weissmueller, S., E. Manchado, M. Saborowski, J. P. t. Morris, E. Wagenblast, C. A. Davis, S. H. Moon, N. T. Pfister, D. F. Tschaharganeh, T. Kitzing, D. Aust, E. K. Markert, J. Wu, S. M. Grimmond, C. Pilarsky, C. Prives, A. V. Biankin and S. W. Lowe (2014). "Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor beta signaling." Cell **157**(2): 382-394.

Westphalen, C. B. and K. P. Olive (2012). "Genetically engineered mouse models of pancreatic cancer." Cancer J **18**(6): 502-510.

Wilentz, R. E., J. Geradts, R. Maynard, G. J. Offerhaus, M. Kang, M. Goggins, C. J. Yeo, S. E. Kern and R. H. Hruban (1998). "Inactivation of the p16 (INK4A) tumor-suppressor gene in pancreatic duct lesions: loss of intranuclear expression." Cancer Res **58**(20): 4740-4744.

Wilentz, R. E., C. A. Iacobuzio-Donahue, P. Argani, D. M. McCarthy, J. L. Parsons, C. J. Yeo, S. E. Kern and R. H. Hruban (2000). "Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression." Cancer Res **60**(7): 2002-2006.

Willis, A., E. J. Jung, T. Wakefield and X. Chen (2004). "Mutant p53 exerts a dominant negative effect by preventing wild-type p53 from binding to the promoter of its target genes." Oncogene **23**(13): 2330-2338.

Wu, C., X. Miao, L. Huang, X. Che, G. Jiang, D. Yu, X. Yang, G. Cao, Z. Hu, Y. Zhou, C. Zuo, C. Wang, X. Zhang, Y. Zhou, X. Yu, W. Dai, Z. Li, H. Shen, L. Liu, Y. Chen, S. Zhang, X. Wang, K. Zhai, J. Chang, Y. Liu, M. Sun, W. Cao, J. Gao, Y. Ma, X. Zheng, S. T. Cheung, Y. Jia, J. Xu, W. Tan, P. Zhao, T. Wu, C. Wang and D. Lin (2011). "Genome-wide association study identifies five loci associated with susceptibility to pancreatic cancer in Chinese populations." Nat Genet **44**(1): 62-66.

Wu, C. Y., E. S. Carpenter, K. K. Takeuchi, C. J. Halbrook, L. V. Peverley, H. Bien, J. C. Hall, K. E. DelGiorno, D. Pal, Y. Song, C. Shi, R. Z. Lin and H. C. Crawford (2014). "PI3K regulation of RAC1 is required for KRAS-induced pancreatic tumorigenesis in mice." Gastroenterology **147**(6): 1405-1416 e1407.

Yang, H., H. Wang, C. S. Shivalila, A. W. Cheng, L. Shi and R. Jaenisch (2013). "One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering." Cell **154**(6): 1370-1379.

Yonezawa, S., M. Higashi, N. Yamada and M. Goto (2008). "Precursor lesions of pancreatic cancer." Gut Liver **2**(3): 137-154.

Zhang, Y., J. P. t. Morris, W. Yan, H. K. Schofield, A. Gurney, D. M. Simeone, S. E. Millar, T. Hoey, M. Hebrok and M. Pasca di Magliano (2013). "Canonical wnt signaling is required for pancreatic carcinogenesis." Cancer Res **73**(15): 4909-4922.

Chapter Two

Bmi1 is required for the initiation of pancreatic cancer through an Ink4a-independent mechanism

Abstract

Epigenetic dysregulation is involved in the initiation and progression of many epithelial cancers. BMI1, a component of the Polycomb protein family, plays a key role in these processes by controlling the histone ubiquitination and long-term repression of multiple genomic loci. BMI1 has previously been implicated in pancreatic homeostasis and the function of pancreatic cancer stem cells. However, no work has yet addressed its role in the early stages of pancreatic cancer development. Here, we show that BMI1 is required for the initiation of murine pancreatic neoplasia using a novel conditional knockout of *Bmi1* in combination with a $Kras^{G12D}$-driven pancreatic cancer mouse model. We also demonstrate that the requirement for Bmi1 in pancreatic carcinogenesis is independent of the *Ink4a/Arf* locus and at least partially mediated by dysregulation of reactive oxygen species (ROS). Our data provide new evidence of the importance of this epigenetic regulator in the genesis of pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDA) is one of the most lethal malignancies, with a 5-year survival rate of 6%. The National Cancer Institute estimated that 45,220 patients would be diagnosed with the disease in 2013 in the United States and 38,460 would die from it (SEER database). Although systemic chemotherapeutic options exist, these have limited efficacy in pancreatic cancer. The development of high-fidelity genetically engineered mouse models of PDA that recapitulate the developmental and pathologic characteristics of the human disease has led to remarkable insights into the biology of pancreatic cancer over the last decade (Aguirre, Bardeesy et al. 2003, Hingorani, Petricoin et al. 2003, Hingorani, Wang et al. 2005, Guerra, Schuhmacher et al. 2007). Complementary systems biology and genomic approaches using human samples have begun to shed some light on the mutational complement of human PDA and the pathways potentially involved in pancreatic tumorigenesis (Jones, Zhang et al. 2008). Together with genetic mutations, epigenetic dysregulation has also been implicated in the pathogenesis of multiple hematopoietic and epithelial cancers (Berdasco and Esteller 2010). However, less is known about the contribution of epigenetic regulators such as the Polycomb repressive complexes (PRCs) in pancreatic cancer initiation and progression.

B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) belongs to the Polycomb group (PcG) of proteins that comprise the Polycomb repressive complex 1 (PRC1) (Schuettengruber, Chourrout et al. 2007) and was originally identified as a cooperating oncogene with c-Myc in the Em-myc transgenic mouse model of B-cell lymphoma (van Lohuizen, Verbeek et al. 1991). Bmi1 regulates murine embryonic fibroblast proliferation and senescence by suppressing the expression of the tumor

suppressor genes *p16*^{Ink4a} and *p19*^{Arf,} which are both encoded by the Ink4a locus (Jacobs, Kieboom et al. 1999). *Bmi1*$^{-/-}$ mice have severe neurologic and hematopoietic developmental defects (van der Lugt, Domen et al. 1994), which are at least partially reversed when the BMI1-deficient mice are bred onto an *Ink4a/Arf* null background (Jacobs, Kieboom et al. 1999). Derepression of *p16*^{Ink4a} and *p19*^{Arf} each individually contribute to the phenotypes observed in Bmi-1 deficient mice (Bruggeman, Valk-Lingbeek et al. 2005, Molofsky, He et al. 2005). More recently, BMI1 has been implicated in the regulation of reactive oxygen species (ROS) accumulation. Deletion of *Bmi1* induces the upregulation of several genes involved in redox homeostasis leading to increased ROS generation, DNA oxidative damage, and activation of the DNA damage response pathways (Liu, Cao et al. 2009). Deletion of *Chk2,* a mediator of the DNA damage response, in BMI1-deficient mice leads to increased thymocyte survival and differentiation, improved hematopoietic progenitor function, partial rescue of cerebellar development, and overall increased survival (Liu, Cao et al. 2009). Notably, all of these effects appear to be independent of the upregulation of *Ink4a/Arf*.

BMI1 plays a key role in pancreatic biology through the regulation of both pancreatic β cell proliferation and acinar regeneration following injury. *Bmi1* ^{null} mice demonstrate impaired glucose tolerance and decreased β cell mass due to the increased expression of the *Ink4a/Arf* locus (Dhawan, Tschen et al. 2009). In addition, *Bmi1*-expressing cells are found in murine pancreatic islets and acini, where BMI1 is required for proper regeneration after pancreatitis or toxin-mediated cellular ablation (Dhawan, Tschen et al. 2009, Sangiorgi and Capecchi 2009, Fukuda, Morris et al. 2012). Importantly, BMI1 is highly expressed in human and murine PDA compared to

normal pancreatic tissues (Tateishi, Ohta et al. 2006, Martinez-Romero, Rooman et al. 2009, Song, Tao et al. 2010, Proctor, Waghray et al. 2013) and its overexpression in tumors correlates with poorer prognosis in pancreatic cancer patients (Song, Tao et al. 2010). Recent work has also implicated Bmi1 in the maintenance of the cancer stem cell compartment in human PDA (Proctor, Waghray et al. 2013). Despite these results, it is still unknown if BMI1 plays a role in the initiation of pancreatic cancer.

Here, we utilize the *Pdx1-Cre;Kras*$^{LSL\text{-}G12D}$ (KC) murine model of pancreatic cancer (Hingorani, Petricoin et al. 2003) in combination with pancreas-specific inactivation of *Bmi1* (*Bmi1*$^{fl/fl}$) to generate *Pdx1-Cre;Kras*$^{LSL\text{-}G12D/+}$*;Bmi1*$^{fl/fl}$ (KC;Bmi1$^{fl/fl}$) mice. We demonstrate that BMI1 is required for murine pancreatic cancer initiation. This process is *Ink4a/Arf*-independent, as the lack of carcinogenesis is not rescued in KC;*Bmi1*$^{fl/fl}$*;Ink4a*$^{-/-}$ mice, which lack the *Ink4a/Arf* locus. We also show that inhibition of *Bmi1* in primary mouse pancreatic cancer cells leads to the upregulation of ROS. Our data suggest that BMI1 regulates the protection from excess ROS in pancreatic cells undergoing neoplastic transformation, which is required for their survival and subsequent pancreatic neoplasia development (DeNicola, Karreth et al. 2011).

Materials and Methods

Mice Mice were housed in the specific pathogen free facilities of the University of Michigan Comprehensive Cancer Center. This study was approved by the University of Michigan University Committee on Use and Care of Animals (UCUCA). The Pdx1-Cre, p48-Cre, Kras$^{LSL\text{-}G12D}$, Trp53$^{R172H/+}$, Ink4a$^{-/-}$, R26^{lacZ}, and Bmi1$^{CreER/+}$ strains have been previously described (Hingorani, Petricoin et al. 2003, Hingorani, Wang et al. 2005,

Bardeesy, Aguirre et al. 2006, Sangiorgi and Capecchi 2009). Bmi1$^{fl/fl}$ mice were developed in the Morrison lab at the University of Michigan (Mich, Signer et al. 2014). All mice were genotyped by PCR analysis. Caerulein and tamoxifen were administered as previously described (Sangiorgi and Capecchi 2009, Collins, Bednar et al. 2012). Caerulein was intraperitoneally injected at a dose of 75ug/kg hourly for 8 hours, two days in a row, for a total of 16 injections. Tamoxifen was administered by intraperitoneal injection at a dose of 9mg per 40g body weight, in 3 to 6 week-old mice.

β-galactosidase Staining We stained cryosections of mouse pancreas or intestine for β-galactosidase activity as previously described(Collins, Bednar et al. 2012).

Immunohistochemistry Histology and immunohistochemistry were performed as previously described (Collins, Bednar et al. 2012). Images were acquired with an Olympus BX-51 microscope, Olympus DP71 digital camera, and DP Controller software. For histopathological analysis, a minimum of 50 acinar or ductal clusters were scored from at least 3 independent animals per experimental condition. Five non-overlapping, high power images were selected from each slide, and each cluster was classified based on the classification consensus (Hruban, Rustgi et al. 2006). A list of antibodies used is included in **Table 3.1**.

Western Blotting Western blots were performed as previously described (Collins, Bednar et al. 2012). Antibodies used for Western blotting are described in Supplementary Table 1.

Quantitative RT-PCR RNA extraction, cDNA preparation, and quantative PCR was performed as previously described (Collins, Bednar et al. 2012). RNA was isolated using RNeasy protect (QIAGEN) according to the manufacturer's instructions. Reverse transcription reactions were conducted using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantative PCR was performed using 1x SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences used were: *Bmi1* F- 5' ATGGCCGCTTGGCTCGCATT 3', R- 5' GATAAAAGATCCCGGAAAGAGCGGC 3'; *Ezh2* - 5' CCCTTCCATGCAACACCCAACACA 3', R- 5' ACGCTCAGCAGTAAGAGCAGCA 3'; *p16* F- 5' TTTCGCCCAACGCCCCGAAC 3', R- 5' CACCGGGCGGGAGAAGGTAGT 3'; *p19* F- 5' CACCGGAATCCTGGACCAG 3', R- 5' GCAGTTCGAATCTGCACCGT 3'; *Chk2* F- 5' TGACAGTGCTTCCTGTTCACA 3', R- 5' GAGCTGGACGAACCCTGATA 3'; *Nrf2* F- 5' CTCGCTGGAAAAAGAAGTG 3', R- 5' CCGTCCAGGAGTTCAGAGG 3'; *ATM* F-5' GATCTGCTCATTTGCTGCCG 3', R-5' GTGTGGTGGCTGATACATTTGAT 3'; *BRCA1* F-5' CGAATCTGAGTCCCCTAAAGAGC 3', R- 5' AAGCAACTTGACCTTGGGGTAC 3'. Gapdh or cyclophilin was used as the housekeeping gene expression control. These sequences were: *Gapdh* F- 5' TTGATGGCAACAATCTCCAC 3', R- 5' CGTCCCGTAGACAAAATGGT 3' and *Cyclophilin* F- 5' TCACAGAATTATTCCAGGATTCATG 3', R- 5' TGCCGCCAGTGCCATT 3'.

Transfection and ROS levels KC; Ink4a$^{-/-}$ cells (line 35) were generated in the Bardeesy lab from a KC;Ink4a$^{-/-}$ (as confirmed by PCR) mouse tumor. KPC cells (line 8041) were isolated in the Pasca di Magliano lab from a p48Cre;LSLKras^{G12D};p53^{R172H} (as confirmed by PCR) mouse tumor in 2012. For authentication in Fall 2014,

genotyping was performed via PCR on DNA isolated from cells growing in culture to confirm Cre transgene expression in both cell lines **(Figure 2.1).** Cells were transfected using Lipofectamine according to the manufacturer's instructions. Control or Bmi1 knockdown siRNAs were purchased from Dharmacon. Cells were subjected to siRNA treatment for 48 hours. Hydrogen peroxide was added directly to the media at a concentration of 500uM for 2 hours for ROS analysis. ROS levels were measured using the CellROX Green reagent (Life Technologies). After exposure to CellROX Green, five high power, non overlapping images were taken from each slide. Fluorescence levels were then measured and each group was normalized to the level of the cells without H_2O_2 exposed to scrambled control siRNA.

Chromatin Immunoprecipitation cells were transfected using a siRNA targeting Bmi1 or control siRNA, 72 hours post-transfection cells were fixed using formaldehyde, lysed and processed for ChIP. ChIP was preformed as previously described. (Mathew, Collins et al. 2014)

Results

BMI1$^+$ cells in the mouse pancreas can serve as the cell of origin for pancreatic cancer.

Our first goal was to determine whether BMI1 expressing cells within the mouse pancreas could give rise to PanIN lesions. Initially, we set out to identify BMI1 expression in normal pancreatic tissue. For this purpose, we crossed *Bmi1-IRES-CreER* mice, which express a tamoxifen inducible Cre knocked in to the Bmi1 locus, (Sangiorgi

and Capecchi 2008) with *Rosa26*^{lacZ} mice, expressing beta galactosidase from the ubiquitous *Rosa26* locus only in cells that are also expressing the Cre (**Figure 2.2A)** (Soriano 1999). Double transgenic mice (*Bmi1*$^{CreER/+}$*; R26*^{lacZ}) were orally gavaged at 3-6 weeks of age with tamoxifen for three consecutive days to induce Cre-mediated recombination. We harvested the pancreata and duodenum from all mice 1 week following tamoxifen administration (scheme in **Figure 2.2B, n=3**). The vast majority of *LacZ*$^{+}$ cells in the pancreas were single acinar cells, as previously described (Sangiorgi and Capecchi 2009) (**Figure 2.2C**). We also observed isolated *LacZ*$^{+}$ cells in the pancreatic islets, consistent with the previously described role of BMI1 in β cell homeostasis (Dhawan, Tschen et al. 2009) (data not shown). In the duodenum, *LacZ*$^{+}$ cells lined the mucosal epithelium extending from the crypts to the tips of the villi presumably originating from BMI1^{+} crypt stem cells, as previously described (**Figure 2.2C**) (Sangiorgi and Capecchi 2008). Thus, BMI1 is expressed in a subset of pancreatic exocrine and endocrine cells.

To determine whether BMI1^{+} cells in the pancreas can serve as cells of origin for pancreatic cancer, we generated *Bmi1*$^{CreER/+}$*;Kras*$^{LSL\text{-}G12D}$ mice, where oncogenic Kras^{G12D} expression can be induced specifically in *Bmi1*-expressing cells upon Cre activation (**Figure 2.2D**). We treated mice at 3-6 weeks of age with tamoxifen as before. One week later, we induced acute pancreatitis with intraperitoneal injections of the cholecystokinin analog caerulein, as previously described (Collins, Bednar et al. 2012). The pancreata were harvested 3 weeks after the induction of pancreatitis (scheme in **Figure 2.2E, n=4**). As expected, control mice had undergone tissue repair within this time frame with full recovery of normal pancreatic histology (data not shown). In

contrast, in *Bmi1*$^{CreER/+}$;*Kras*$^{LSL-G12D}$ mice, we observed low-grade PanIN lesions, positive for mucin accumulation as identified by PAS staining, in a sporadic manner through the tissue, consistent with mosaic induction of Cre activation. The lesions were also positive for phosphorylated ERK (pERK) and were surrounded by fibro-inflammatory stroma (**Figure 2.2F**). Therefore, our data indicates that PanIN lesions can arise from BMI1^{+} cells within the adult mouse pancreas.

Bmi1 is required for pancreatic carcinogenesis.

To address the requirement for Bmi1 in murine pancreatic neoplasia we crossed the *Pdx1-Cre;Kras*$^{LSL-G12D/+}$ (KC) mouse model of pancreatic cancer (Hingorani, Petricoin et al. 2003) with a novel conditional knockout of Bmi1 (*Bmi1*$^{fl/fl}$) (Mich, Signer et al. 2014) (**Figure 2.3A**) to generate *Pdx1-Cre;Kras*$^{LSL-G12D/+}$;*Bmi1*$^{fl/fl}$ (KC; *Bmi1*$^{fl/fl}$) mice. Recombination of the *Bmi1* locus in the pancreas was verified by PCR of pancreatic genomic DNA isolated from *Pdx1-Cre;Bmi1*$^{+/+}$, *Pdx1-Cre;Bmi1*$^{fl/+}$, and *Pdx1-Cre;Bmi1*$^{fl/fl}$ mice (**Figure 2.4**). Additionally, Bmi1 was expressed in PanINs in KC pancreata, but not in the pancreas of KC*;Bmi1*$^{fl/fl}$ mice (**Figure 2.3B**). Bmi1 expression was similarly observed in PanINs in Pdx1Cre;Kras$^{LSLG12D/+}$;p53$^{R172H/+}$ (KPC) mice, a model that combines Kras and p53 mutations and develops PanINs at an earlier age than KC mice (**Figure 2.5**) (Hingorani, Wang et al. 2005). To investigate PanIN formation, we analyzed KC*;Bmi1*$^{fl/fl}$ mice along with KC and KC;Bmi$^{fl/+}$ littermates at 12 and 20 weeks after birth (n=4-8mice/genotype/timepoint). KC mice developed PanIN lesions with the expected progression: rare lesions were present at 12 weeks, but more abundant lesions were observed at 20 weeks (**Figure 2.3C**). KC;*Bmi1*$^{fl/+}$ mice had

similar PanIN development to KC animals, with a comparable number and grade of the lesions (**Figure 2.3D**). The vast majority of the lesions were classified as PanIN1A/1B and PanIN2 (**Figure 2.3F**) that presented with characteristic high proliferation index as measured by $Ki67^{+}$ immunostaining (**Figures 2.6A and 2.6B**) and intracellular accumulation of mucin (**Figure 2.3G, 2.6D and 2.6E**). Strikingly, PanIN formation was almost completely abrogated in KC;$Bmi1^{fl/fl}$ mice (**Figure 2.3E, 2.3F and Figures 2.6C and 2.6F**), with a single PanIN1A observed in a single animal. These results implicate BMI1 as a key factor in the initiation of murine pancreatic neoplasia.

The induction of acute pancreatitis synergizes with expression of oncogenic Kras to drive PanIN formation (Morris, Cano et al. 2010, Carriere, Young et al. 2011, Guerra, Collado et al. 2011). In the next series of experiments, we investigated whether Bmi1 expression was required in pancreatitis-induced carcinogenesis. For this purpose, we induced acute pancreatitis with the cholecystokinin analog caerulein, as previously described (Morris, Cano et al. 2010), starting three to four weeks after birth, and collected the pancreatic tissues at time points ranging from 24 hours to 3 weeks later (n=3-10mice/genotype/timepoint, (scheme in **Figure 2.7A**). Wild type pancreata demonstrated acinar damage and acinar-ductal metaplasia, accompanied by transient upregulation of the MAPK signaling pathway 24 hours after pancreatitis (**Figure 2.7B**), and exhibited complete tissue recovery 3 weeks later (**Figure 2.7F**). Analysis of KC, KC;$Bmi1^{fl/+}$ and KC;$Bmi1^{fl/fl}$ mice 24 hours after pancreatitis induction (**Figures 2.7C-E**) revealed increased acinar damage and elevated p-ERK1/2 staining, consistent with previous observations in mice expressing oncogenic Kras (Collins, Bednar et al. 2012). The prevalence of inflammatory cell infiltration and of acinar damage, and the induction

of phosphorylated-ERK1/2 in the acinar cells did not differ based on the Bmi1 status of the tissues. Thus, epithelial Bmi1 expression did not affect the early response to caerulein-induced pancreatitis.

Three weeks after pancreatitis, in both KC and KC;*Bmi1*$^{fl/+}$ mice, the pancreas parenchyma was largely replaced by low-grade PanIN lesions surrounded by desmoplastic stroma (**Figures 2.7G, 2.7H, and 2.7J**). In contrast, KC;Bmi1$^{fl/fl}$ pancreata presented with almost completely normal acinar and ductal architecture (**Figures 2.7I and 2.7J**). Rarely, acinar-ductal metaplasia was observed in isolated areas of a subset of the KC;Bmi1$^{fl/fl}$ mice, but frank PanINs were generally absent. These areas of acinar-ductal metaplasia in KC;Bmi1$^{fl/fl}$ mice stained positive for Bmi1, indicating failure to recombine both alleles of Bmi1 rather than an ability to circumvent the requirement of Bmi1 in PanIN initiation (**Figure 2.8**). p-ERK1/2 levels were elevated both within the lesions and in the surrounding stroma of KC and KC;*Bmi1*$^{fl/+}$ pancreata, as well as in the rare areas of ADM in KC;Bmi1$^{fl/fl}$ pancreata (**Figures 2.7G-I**). Thus, while the inflammatory response and the pancreatitis-induced tissue damage was not dependent on epithelial BMI1 expression, the subsequent development of PanINs required at least one wild type *Bmi1* allele.

BMI1 controls pancreatic neoplasia independently of *Ink4a/ARF*.

BMI1 has been shown to repress the *Ink4a/Arf* locus, which encodes for the cell cycle regulators p16^{INK4A} and p19^{ARF} (Jacobs, Kieboom et al. 1999). Inactivation of p16 expression is essential to bypass oncogenic Kras-induced senescence and thus for the onset of carcinogenesis (Lee and Bar-Sagi 2010, Guerra, Collado et al. 2011). Thus, we

considered the hypothesis that Bmi1 was required to suppress the *Ink4a* locus therefore allowing the onset of pancreatic neoplasia. We used two complementary approaches to address this hypothesis.

First, we utilized primary low passage mouse pancreatic tumor cell lines derived from *p48/Ptfa-1*$^{Cre/+}$*;Kras*$^{LSL\text{-}G12D/+}$*;Trp53*$^{R172H/+}$ (KPC mice) (Hingorani, Wang et al. 2005) and *Pdx1*$^{Cre/+}$*;Kras*$^{LSL\text{-}G12D/+}$*;Ink4a*$^{-/-}$, which lack expression of both Ink4a and ARF, (KC;*Ink4a*$^{-/-}$) (Aguirre, Bardeesy et al. 2003) to determine whether inhibition of *Bmi1* expression led to derepression of the *Ink4a* locus. Multiple independent cell lines were used for each genotype (KPC: 8041, 8206, 65671; KC; *Ink4a*$^{-/-}$*: 35, 45).* We transfected two distinct *Bmi1*-specific siRNAs individually or in combination into the cell lines to inhibit *Bmi1* expression. The siRNA treatment resulted in a >60-80% knockdown of *Bmi1* expression across all of the cell lines, as determined by both qPCR (**Figure 2.9A**) and Western Blot (**Figure 2.9B,** full blot seen in **Figure 2.10**). In comparison, the expression of *Ezh2*, a component of the Polycomb repressor complex 2, was not affected (**Figure 2.9C**). We then analyzed the expression of both *p16*^{Ink4a} and *p19*^{Arf} in the presence and absence of BMI1. Despite robust *Bmi1* knockdown, no significant changes in *p16/p19* expression were noted in the KPC tumor-derived cell lines (**Figures 2.9D and 2.9E**). As expected, the cell lines derived from the KC;*Ink4a*$^{-/-}$ mice did not demonstrate any *p16/p19* expression regardless of the presence or absence of Bmi1 (**Figure 2.9D and 2.9E**). These results suggest that Bmi1 may exert its role in pancreatic neoplasia independently of its regulation of the Ink4a locus.

Second, to determine whether the requirement for Bmi1 during the onset of pancreatic carcinogenesis was mediated by its ability to repress the Ink4a locus, we

generated KC; Bmi1$^{fl/fl}$;*Ink4a*$^{-/-}$ mice. The *KC;Ink4a*$^{-/-}$model of pancreatic cancer develops advanced neoplastic lesions rapidly even in the absence of pancreatitis (Aguirre, Bardeesy et al. 2003, Bardeesy, Aguirre et al. 2006). KC, KC;Ink4a$^{-/-}$, KC;Bmi1$^{fl/fl}$, and KC;Bmi1$^{fl/fl}$;*Ink4a*$^{-/-}$, mice were treated with caerulein to induce pancreatitis, and the pancreata were harvested 3 weeks later (n=3-5 mice/genotype/timepoint). As expected, KC and KC;Ink4a$^{-/-}$ mice had extensive PanIN formation at this time point; however, neither KC;Bmi1$^{fl/fl}$ nor KC;Bmi1$^{fl/fl}$;*Ink4a*$^{-/-}$ mice presented with lesions (**Figure 2.9F**). Since *Bmi1* inactivation abrogated PanIN formation even in Ink4a null animals, this indicates that the requirement for BMI1 during the onset of pancreatic carcinogenesis is independent of its regulation of the *Ink4a* locus.

BMI1 deficiency impairs the reactive oxygen species detoxification program in pancreatic tumor cells.

BMI1 regulates the detoxification of reactive oxygen species (ROS) generated in the mitochondria and the subsequent induction of the DNA damage response (DDR) pathway in hematopoietic stem cells and thymocytes (Liu, Cao et al. 2009). ROS detoxification is an essential step during the onset of pancreatic cancer (DeNicola, Karreth et al. 2011). To determine whether Bmi1 was required for ROS regulation in pancreatic cancer cells, we measured ROS levels in mouse primary pancreatic cancer cells upon siRNA-mediated *Bmi1* inactivation. We measured the baseline ROS levels and those induced by hydrogen peroxide exposure in control and *Bmi1*-knockdown cells. As before, *Bmi1* expression was inhibited with high efficiency (**Figure 2.11A**). At

baseline, before any hydrogen peroxide exposure, the control cells demonstrated a trend toward lower levels of ROS compared to cells with *Bmi1* knockdown (**Figure 2.11B**). Once the cells were exposed to 500mM H_2O_2, there was a significant increase in the production of intracellular ROS within 2 hours of the exposure (**Figures 2.11B, 2.11C, and 2.11D**). Under these conditions, the *Bmi1* knockdown cells accumulated a greater level of intracellular ROS compared to cells transfected with control siRNA. These results provide the first evidence that BMI1 is required for the regulation of ROS generation in pancreatic tumor cells.

Oxidative stress can induce the DNA damage response (DDR) pathway in cells to protect the integrity of the genome. Chk2, a DDR pathway component, is activated in the thymocytes of Bmi1 knockout mice (Liu, Cao et al. 2009). Furthermore, deletion of *Chk2* rescued the defect in survival and body size in Bmi1 knockout mice. Thus, at least during normal organ maintenance, a key function of Bmi1 is to repress expression of Chk2. To determine the effect of Bmi1 loss on Chk2 expression in our system, we measured expression levels of *Chk2*, with or without the induction of ROS. *Bmi1* knockdown did not appreciably increase *Chk2* expression in Bmi1 knockdown cells compared to control, either without or with exposure to ROS as tested by Western Blot (**Figure 2.10)** or qPCR (**Figure 2.10**). However, the knockdown of Bmi1 significantly reduced the levels of H2AK119Ub at the Chk2 locus. This shows that at the epigenetic level we are seeing the changes we would expect as a result of Bmi1 knock down **(Figures 2.10C-2.10F)**. Similarly, we did not observe expression changes of the antioxidant enzyme Nrf2, *in vitro* or *in vivo* (**Figure 2.12A and 2.12B**), or its binding partner Keap1 (**Figure 2.12C**). Finally, we did not observe significant changes in other

proteins involved in the DNA damage response, including ATM and BRCA1 (**Figures 2.12D and 2.12E**). Therefore, *Bmi1* loss induced ROS generation but did not cause a subsequent induction of the DNA damage response pathway.

Discussion

Our work is the first study addressing the role of Bmi1 during pancreatic carcinogenesis in the context of an intact microenvironment. Our results provide the first direct evidence that BMI1 is required for pancreatic cancer initiation.

We used genetically engineered mouse models of pancreatic cancer combined with tissue-specific inactivation of *Bmi1* to study the role of BMI1 in the initiation and progression of pancreatic neoplasia. When *Bmi1* was inactivated in the pancreas, neoplastic transformation did not occur in either the KC;Bmi1$^{fl/fl}$ or the KC;Bmi1$^{fl/fl}$;*Ink4a*$^{-/-}$ mice. Thus, BMI1 is required for the establishment and survival of KRAS-driven neoplastic cells and this process is independent of Bmi1 control of the *Ink4a* locus. Bmi1 requirement in the initiation of neoplasia recapitulates previous observations in a KRAS-driven mouse model of lung cancer (Dovey, Zacharek et al. 2008). However, in that case, the inhibition of lung neoplastic transformation was dependent on the upregulation of *p19^{Arf}* from the *Ink4a/ARF* locus. In contrast, we find that the tumor-promoting role of BMI1 in pancreatic cancer is independent of the status of the *Ink4a* locus. Bmi1 control of cancer initiation independent of Ink4a expression has been previously reported in an orthotopic transplantation model of glioma (Bruggeman, Hulsman et al. 2007). Therefore, BMI1 may regulate tumorigenesis differently depending on the tissue context, indicating the need for tissue specific studies.

PRCs play a key role in the regulation of multiple cancers (Sparmann and van Lohuizen 2006). One of the other most studied components is EZH2, a member of PRC2 that also plays an integral role in gene silencing (Cao, Wang et al. 2002). BMI1 and EZH2 are classically thought to cooperate in gene silencing. However, our results following *Bmi1* deletion are in stark contrast with the analysis of *p48-Cre, Kras*$^{LSL\text{-}G12D/+}$ mice where *Ezh2* is genetically inactivated (Mallen-St Clair, Soydaner-Azeloglu et al. 2012). In the KC;*Ezh2*^{DSET} mice, loss of *Ezh2* led to a rapid onset of PanINs and early mortality by 12-16 weeks. In this model EZH2 acted at least partially through the *Ink4a* locus, which led to the inability of the acinar compartment to recover from transient injury. This effect was compounded by an increased inflammatory infiltrate resulting in additional injury and early fibrosis and neoplasia (Mallen-St Clair, Soydaner-Azeloglu et al. 2012). In contrast, our *Bmi1* knockout mice demonstrated complete abrogation of pancreatic neoplasia under both caerulein-induced pancreatitis and quiescent conditions. These results highlight the complex roles of the different PRCs in pancreatic neoplasia and the need for further exploration of the epigenetic regulation mechanisms controlling pancreatic transformation.

BMI1 plays a role in additional cellular processes, including the dysregulation of ROS generation (Liu, Cao et al. 2009). Our experiments revealed increased ROS generation in pancreatic cancer cells when *Bmi1* expression was inhibited. However, ROS accumulation did not correlate with upregulation of CHK2 or other components of the DNA damage response pathway, suggesting that lack of BMI1 leads to significant oxidative stress without upregulation of the DNA damage response pathway during the early stages of pancreatic neoplasia. Here, BMI1 upregulation observed in early PanINs

may represent a protective response of transformed cells to the KRAS-driven oxidative stress. Interestingly, activation of a ROS detoxification program has been recently shown to be an essential step during the onset of pancreatic carcinogenesis (DeNicola, Karreth et al. 2011), and inactivation of the key ROS detoxification component Nrf2 was sufficient to inhibit carcinogenesis. However, given the complexity of the epigenetic regulation in pancreatic cancer, it is likely that Bmi1 exerts its role in carcinogenesis though regulation of multiple pathways, warranting further investigation in the future. Importantly, recent pre-clinical testing of a Bmi1 inhibitor slowed tumor growth in a colon cancer xenograft model, revealing Bmi1's potential as a therapeutic target (Kreso, van Galen et al. 2014). Together with previous work indicating a role for Bmi1 in the growth of human pancreatic cancer cells in an orthotopic transplantation model in immunocompromised mice (Proctor, Waghray et al. 2013), our observations provide rationale for Bmi1 inhibition as a potential therapeutic target for pancreatic cancer.

Acknowledgements

FB was supported by the American College of Surgeons Resident Research Scholarship and by NIH T32 HD007505. HKS is supported by a University of Michigan Program in Cellular and Molecular Biology training grant (NIH T32 GM07315). MAC was supported by a University of Michigan Program in Cellular and Molecular Biology training grant (NIH T32 GM07315), a University of Michigan Center for Organogenesis Training Grant (5-T32-HD007515). Research in the Pasca di Magliano lab is supported by NCI-1R01CA151588-01, by ACS RSG-14-173-01-CSM and by the Elsa Pardee Foundation. This project was supported by a National Pancreas Foundation pilot grant.

LLA and MEF-Z are supported by Mayo Clinic Center for Cell Signaling in Gastroenterology Grant DK84567.

References

Aguirre, A. J., N. Bardeesy, M. Sinha, L. Lopez, D. A. Tuveson, J. Horner, M. S. Redston and R. A. DePinho (2003). "Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma." Genes Dev **17**(24): 3112-3126.

Bardeesy, N., A. J. Aguirre, G. C. Chu, K. H. Cheng, L. V. Lopez, A. F. Hezel, B. Feng, C. Brennan, R. Weissleder, U. Mahmood, D. Hanahan, M. S. Redston, L. Chin and R. A. Depinho (2006). "Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse." Proc Natl Acad Sci U S A **103**(15): 5947-5952.

Berdasco, M. and M. Esteller (2010). "Aberrant epigenetic landscape in cancer: how cellular identity goes awry." Dev Cell **19**(5): 698-711.

Bruggeman, S. W., D. Hulsman, E. Tanger, T. Buckle, M. Blom, J. Zevenhoven, O. van Tellingen and M. van Lohuizen (2007). "Bmi1 controls tumor development in an Ink4a/Arf-independent manner in a mouse model for glioma." Cancer Cell **12**(4): 328-341.

Bruggeman, S. W., M. E. Valk-Lingbeek, P. P. van der Stoop, J. J. Jacobs, K. Kieboom, E. Tanger, D. Hulsman, C. Leung, Y. Arsenijevic, S. Marino and M. van Lohuizen (2005). "Ink4a and Arf differentially affect cell proliferation and neural stem cell self-renewal in Bmi1-deficient mice." Genes Dev **19**(12): 1438-1443.

Cao, R., L. Wang, H. Wang, L. Xia, H. Erdjument-Bromage, P. Tempst, R. S. Jones and Y. Zhang (2002). "Role of histone H3 lysine 27 methylation in Polycomb-group silencing." Science **298**(5595): 1039-1043.

Carriere, C., A. L. Young, J. R. Gunn, D. S. Longnecker and M. Korc (2011). "Acute pancreatitis accelerates initiation and progression to pancreatic cancer in mice expressing oncogenic Kras in the nestin cell lineage." PLoS One **6**(11): e27725.

Collins, M. A., F. Bednar, Y. Zhang, J. C. Brisset, S. Galban, C. J. Galban, S. Rakshit, K. S. Flannagan, N. V. Adsay and M. Pasca di Magliano (2012). "Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice." J Clin Invest **122**(2): 639-653.

DeNicola, G. M., F. A. Karreth, T. J. Humpton, A. Gopinathan, C. Wei, K. Frese, D. Mangal, K. H. Yu, C. J. Yeo, E. S. Calhoun, F. Scrimieri, J. M. Winter, R. H. Hruban, C. Iacobuzio-Donahue, S. E. Kern, I. A. Blair and D. A. Tuveson (2011). "Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis." Nature **475**(7354): 106-109.

Dhawan, S., S. I. Tschen and A. Bhushan (2009). "Bmi-1 regulates the Ink4a/Arf locus to control pancreatic beta-cell proliferation." Genes Dev **23**(8): 906-911.

Dovey, J. S., S. J. Zacharek, C. F. Kim and J. A. Lees (2008). "Bmi1 is critical for lung tumorigenesis and bronchioalveolar stem cell expansion." Proc Natl Acad Sci U S A **105**(33): 11857-11862.

Fukuda, A., J. P. t. Morris and M. Hebrok (2012). "Bmi1 is required for regeneration of the exocrine pancreas in mice." Gastroenterology **143**(3): 821-831 e821-822.

Guerra, C., M. Collado, C. Navas, A. J. Schuhmacher, I. Hernandez-Porras, M. Canamero, M. Rodriguez-Justo, M. Serrano and M. Barbacid (2011). "Pancreatitis-induced inflammation contributes to pancreatic cancer by inhibiting oncogene-induced senescence." Cancer Cell **19**(6): 728-739.

Guerra, C., A. J. Schuhmacher, M. Canamero, P. J. Grippo, L. Verdaguer, L. Perez-Gallego, P. Dubus, E. P. Sandgren and M. Barbacid (2007). "Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice." Cancer Cell **11**(3): 291-302.

Hingorani, S. R., E. F. Petricoin, A. Maitra, V. Rajapakse, C. King, M. A. Jacobetz, S. Ross, T. P. Conrads, T. D. Veenstra, B. A. Hitt, Y. Kawaguchi, D. Johann, L. A. Liotta, H. C. Crawford, M. E. Putt, T. Jacks, C. V. Wright, R. H. Hruban, A. M. Lowy and D. A. Tuveson (2003). "Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse." Cancer Cell **4**(6): 437-450.

Hingorani, S. R., L. Wang, A. S. Multani, C. Combs, T. B. Deramaudt, R. H. Hruban, A. K. Rustgi, S. Chang and D. A. Tuveson (2005). "Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice." Cancer Cell **7**(5): 469-483.

Hruban, R. H., A. K. Rustgi, T. A. Brentnall, M. A. Tempero, C. V. Wright and D. A. Tuveson (2006). "Pancreatic cancer in mice and man: the Penn Workshop 2004." Cancer Res **66**(1): 14-17.

Jacobs, J. J., K. Kieboom, S. Marino, R. A. DePinho and M. van Lohuizen (1999). "The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus." Nature **397**(6715): 164-168.

Jones, S., X. Zhang, D. W. Parsons, J. C. Lin, R. J. Leary, P. Angenendt, P. Mankoo, H. Carter, H. Kamiyama, A. Jimeno, S. M. Hong, B. Fu, M. T. Lin, E. S. Calhoun, M. Kamiyama, K. Walter, T. Nikolskaya, Y. Nikolsky, J. Hartigan, D. R. Smith, M. Hidalgo, S. D. Leach, A. P. Klein, E. M. Jaffee, M. Goggins, A. Maitra, C. Iacobuzio-Donahue, J. R. Eshleman, S. E. Kern, R. H. Hruban, R. Karchin, N. Papadopoulos, G. Parmigiani, B. Vogelstein, V. E. Velculescu and K. W. Kinzler (2008). "Core signaling pathways in human pancreatic cancers revealed by global genomic analyses." Science **321**(5897): 1801-1806.

Kreso, A., P. van Galen, N. M. Pedley, E. Lima-Fernandes, C. Frelin, T. Davis, L. Cao, R. Baiazitov, W. Du, N. Sydorenko, Y. C. Moon, L. Gibson, Y. Wang, C. Leung, N. N. Iscove, C. H. Arrowsmith, E. Szentgyorgyi, S. Gallinger, J. E. Dick and C. A. O'Brien (2014). "Self-renewal as a therapeutic target in human colorectal cancer." Nat Med **20**(1): 29-36.

Lee, K. E. and D. Bar-Sagi (2010). "Oncogenic KRas suppresses inflammation-associated senescence of pancreatic ductal cells." Cancer Cell **18**(5): 448-458.

Liu, J., L. Cao, J. Chen, S. Song, I. H. Lee, C. Quijano, H. Liu, K. Keyvanfar, H. Chen, L. Y. Cao, B. H. Ahn, N. G. Kumar, Rovira, II, X. L. Xu, M. van Lohuizen, N. Motoyama, C. X. Deng and T. Finkel (2009). "Bmi1 regulates mitochondrial function and the DNA damage response pathway." Nature **459**(7245): 387-392.

Mallen-St Clair, J., R. Soydaner-Azeloglu, K. E. Lee, L. Taylor, A. Livanos, Y. Pylayeva-Gupta, G. Miller, R. Margueron, D. Reinberg and D. Bar-Sagi (2012). "EZH2 couples pancreatic regeneration to neoplastic progression." Genes Dev **26**(5): 439-444.

Martinez-Romero, C., I. Rooman, A. Skoudy, C. Guerra, X. Molero, A. Gonzalez, M. Iglesias, T. Lobato, A. Bosch, M. Barbacid, F. X. Real and I. Hernandez-Munoz (2009). "The epigenetic regulators Bmi1 and Ring1B are differentially regulated in pancreatitis and pancreatic ductal adenocarcinoma." J Pathol **219**(2): 205-213.

Mathew, E., M. A. Collins, M. G. Fernandez-Barrena, A. M. Holtz, W. Yan, J. O. Hogan, Z. Tata, B. L. Allen, M. E. Fernandez-Zapico and M. P. di Magliano (2014). "The transcription factor GLI1 modulates the inflammatory response during pancreatic tissue remodeling." J Biol Chem **289**(40): 27727-27743.

Mich, J. K., R. A. Signer, D. Nakada, A. Pineda, R. J. Burgess, T. Y. Vue, J. E. Johnson and S. J. Morrison (2014). "Prospective identification of functionally distinct stem cells and neurosphere-initiating cells in adult mouse forebrain." Elife **3**: e02669.

Molofsky, A. V., S. He, M. Bydon, S. J. Morrison and R. Pardal (2005). "Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways." Genes Dev **19**(12): 1432-1437.

Morris, J. P. t., D. A. Cano, S. Sekine, S. C. Wang and M. Hebrok (2010). "Beta-catenin blocks Kras-dependent reprogramming of acini into pancreatic cancer precursor lesions in mice." J Clin Invest **120**(2): 508-520.

Proctor, E., M. Waghray, C. J. Lee, D. G. Heidt, M. Yalamanchili, C. Li, F. Bednar and D. M. Simeone (2013). "Bmi1 enhances tumorigenicity and cancer stem cell function in pancreatic adenocarcinoma." PLoS One **8**(2): e55820.

Sangiorgi, E. and M. R. Capecchi (2008). "Bmi1 is expressed in vivo in intestinal stem cells." Nat Genet **40**(7): 915-920.

Sangiorgi, E. and M. R. Capecchi (2009). "Bmi1 lineage tracing identifies a self-renewing pancreatic acinar cell subpopulation capable of maintaining pancreatic organ homeostasis." Proc Natl Acad Sci U S A **106**(17): 7101-7106.

Schuettengruber, B., D. Chourrout, M. Vervoort, B. Leblanc and G. Cavalli (2007). "Genome regulation by polycomb and trithorax proteins." Cell **128**(4): 735-745.

Song, W., K. Tao, H. Li, C. Jin, Z. Song, J. Li, H. Shi, X. Li, Z. Dang and K. Dou (2010). "Bmi-1 is related to proliferation, survival and poor prognosis in pancreatic cancer." Cancer Sci **101**(7): 1754-1760.

Soriano, P. (1999). "Generalized lacZ expression with the ROSA26 Cre reporter strain." Nat Genet **21**(1): 70-71.

Sparmann, A. and M. van Lohuizen (2006). "Polycomb silencers control cell fate, development and cancer." Nat Rev Cancer **6**(11): 846-856.

Tateishi, K., M. Ohta, F. Kanai, B. Guleng, Y. Tanaka, Y. Asaoka, M. Tada, M. Seto, A. Jazag, L. Lianjie, M. Okamoto, H. Isayama, M. Tada, H. Yoshida, T. Kawabe and M. Omata (2006). "Dysregulated expression of stem cell factor Bmi1 in precancerous lesions of the gastrointestinal tract." Clin Cancer Res **12**(23): 6960-6966.

van der Lugt, N. M., J. Domen, K. Linders, M. van Roon, E. Robanus-Maandag, H. te Riele, M. van der Valk, J. Deschamps, M. Sofroniew, M. van Lohuizen and et al. (1994). "Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene." Genes Dev **8**(7): 757-769.

van Lohuizen, M., S. Verbeek, B. Scheijen, E. Wientjens, H. van der Gulden and A. Berns (1991). "Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging." Cell **65**(5): 737-752.

Cre

KPC-8041 | KC; Ink4a$^{-/-}$-35 | Neg

Figure 2.1: Pancreatic cancer cell line validation
PCR of DNA isolated from KPC – 8041 and KC; Ink4a$^{-/-}$ - 35 cell lines, confirming transgene expression

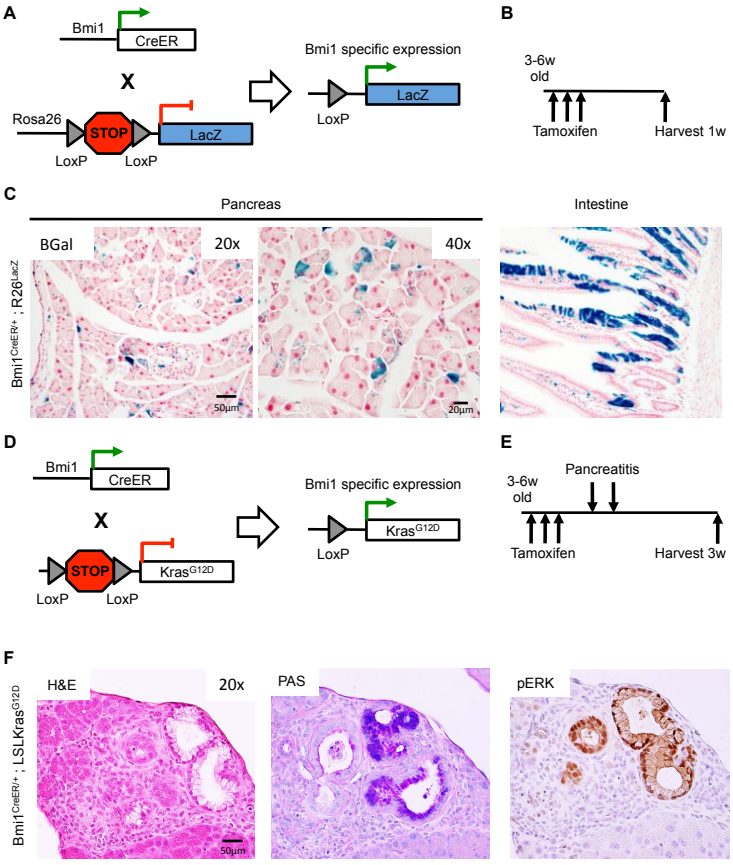


Figure 2.2: Bmi1 expressing cells can serve as a cell of origin for pancreatic cancer
(A) Genetic make up of *Bmi1-IRES-CreER;R26^{lacZ}* mice. **(B)** Experimental design. **(C)** BMI1+ cells identified by β-Gal staining for the LacZ reporter. **(D)** Genetic makeup of *Bmi1-IRES-CreER;Kras$^{LSL-G12D}$* mice. **(E)** Experimental design. **(F)** HE, PAS staining, and pERK immunohistochemistry of *Bmi1-IRES-CreER;Kras$^{LSL-G12D}$* tissues 3w following pancreatitis.

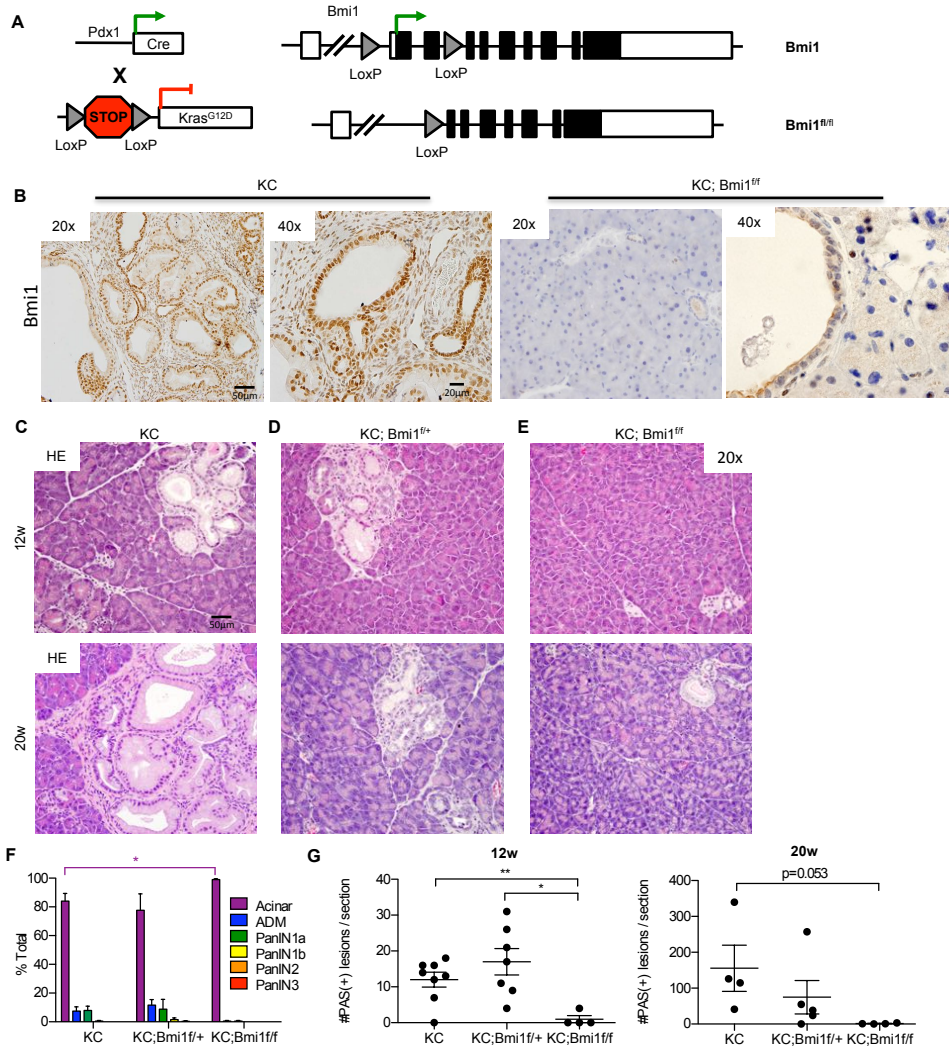


Figure 2.3: Bmi1 is required for PanIN formation
(A) Genetic make up of *Pdx1-Cre;Kras$^{LSL-G12D/+}$;Bmi1$^{fl/fl}$* (KC; *Bmi1$^{fl/fl}$*) mice. **(B)** IHC staining for Bmi1 in KC and KC; *Bmi1$^{fl/fl}$* mice. HE staining for **(C)** KC, **(D)** KC;Bmi1$^{fl/+}$, and **(E)** KC;Bmi1$^{fl/fl}$ mice at 12 and 20 weeks. Histopathological analysis (2-way ANOVA: *p<0.05**) (F)** and quantification of the number of PAS+ lesions (student's t-test: **p<0.01, *p<0.05) **(G)** of KC, KC;Bmi1$^{fl/+}$, and KC;Bmi1$^{fl/fl}$ mice at 12 and 20 weeks (n=3-6mice/genotype/timepoint).

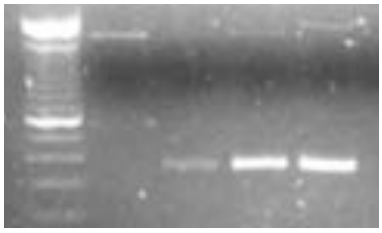


Figure 2.4: Successful recombination of the Bmi1 locus in the murine pancreas. PCR of pancreatic genomic DNA isolated from isolated from *Pdx1-Cre; Bmi1*$^{+/+}$, *Pdx1-Cre; Bmi1*$^{fl/+}$, and *Pdx1-Cre; Bmi1*$^{fl/fl}$ mice.

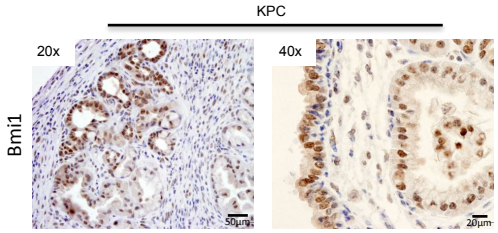


Figure 2.5: Bmi1 is expressed specifically in PanINs in KPC mice.
Immunohistochemistry for Bmi1 expression in Pdx1-Cre; LSLKrasG12D; p53R172H pancreata.

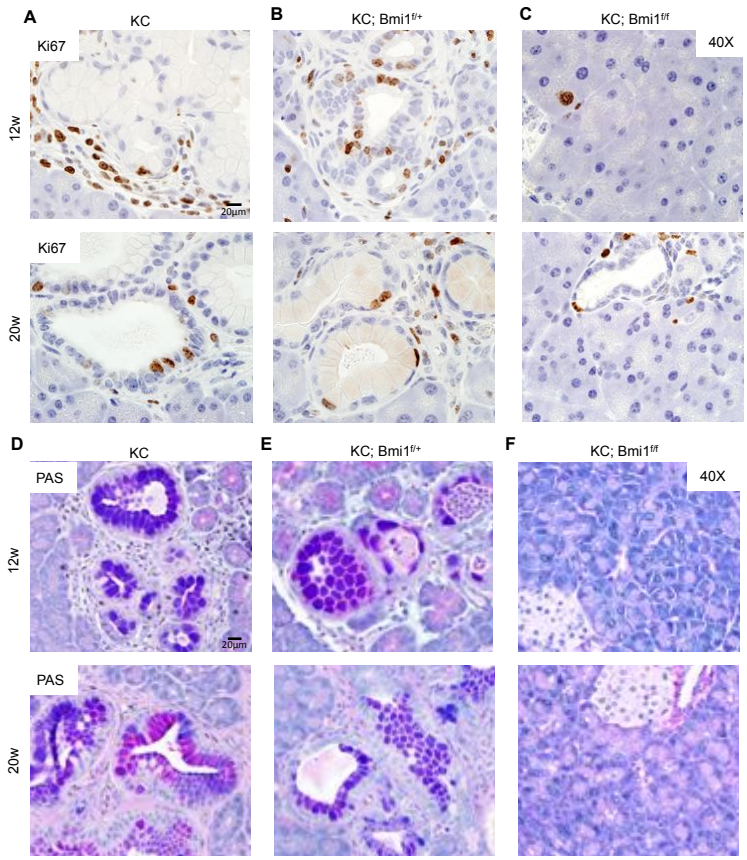


Figure 2.6: Bmi1 null pancreata do not develop PanINs.
IHC staining for Ki67 in (**A**) KC, (**B**) KC; Bmi1$^{fl/+}$, and (**C**) KC; Bmi1$^{fl/fl}$ pancreata at 12 and 20 weeks. PAS staining in (**D**) KC, (**E**) KC; Bmi1$^{fl/+}$, and (**F**) KC; Bmi1$^{fl/fl}$ pancreata at 12 and 20 weeks.

6

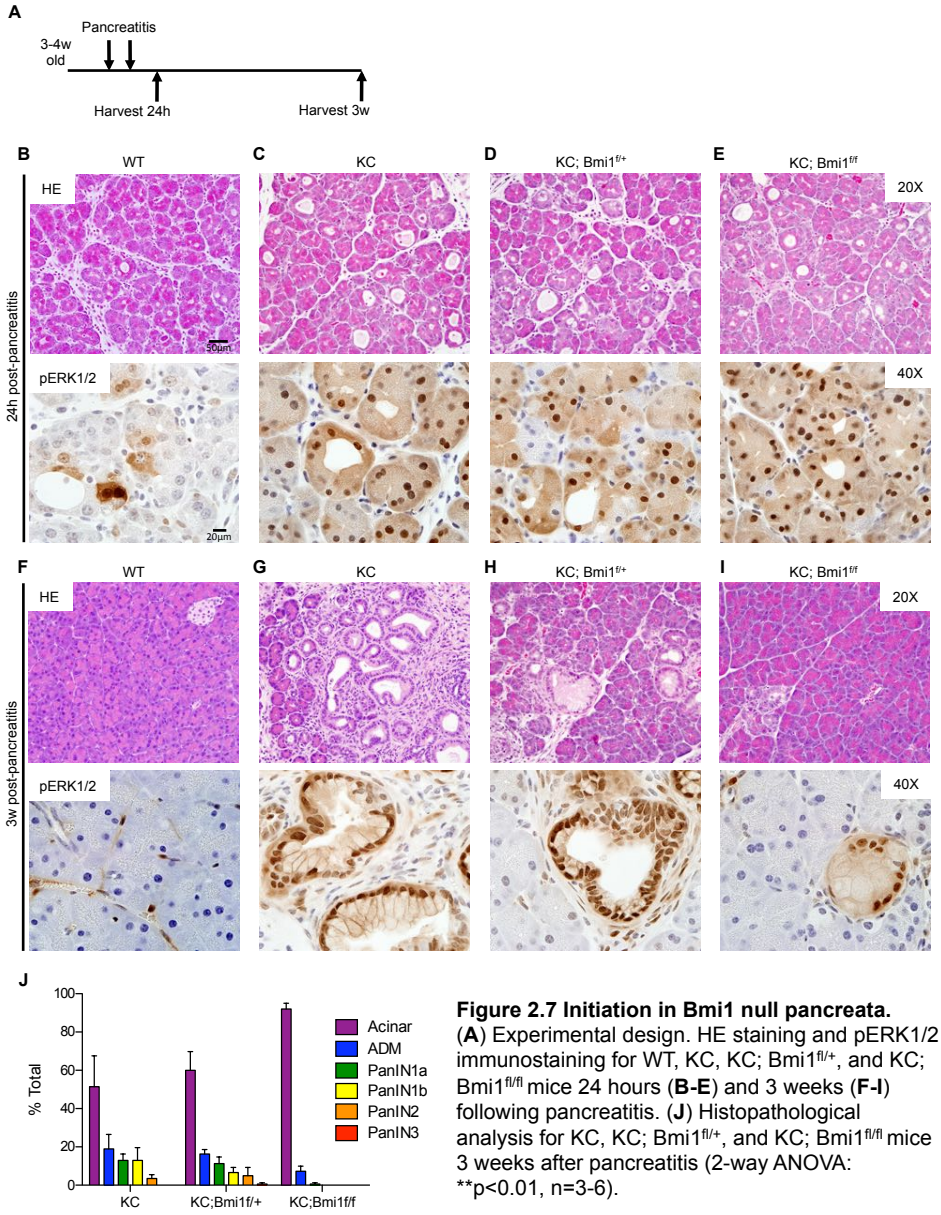


Figure 2.7 Initiation in Bmi1 null pancreata. (**A**) Experimental design. HE staining and pERK1/2 immunostaining for WT, KC, KC; Bmi1$^{fl/+}$, and KC; Bmi1$^{fl/fl}$ mice 24 hours (**B-E**) and 3 weeks (**F-I**) following pancreatitis. (**J**) Histopathological analysis for KC, KC; Bmi1$^{fl/+}$, and KC; Bmi1$^{fl/fl}$ mice 3 weeks after pancreatitis (2-way ANOVA: **p<0.01, n=3-6).

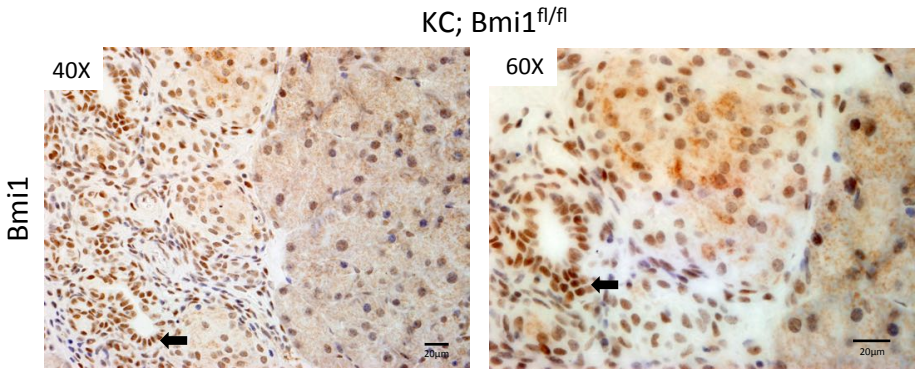


Figure 2.8: Bmi1 is expressed in areas of ADM in KC; Bmi1$^{fl/fl}$ mice
IHC staining for Bmi1 in KC; Bmi1$^{fl/fl}$ mice 3 weeks post pancreatitis. Arrows point to areas of Bmi1-positive ductal cells in areas of acinar ductal metaplasia.

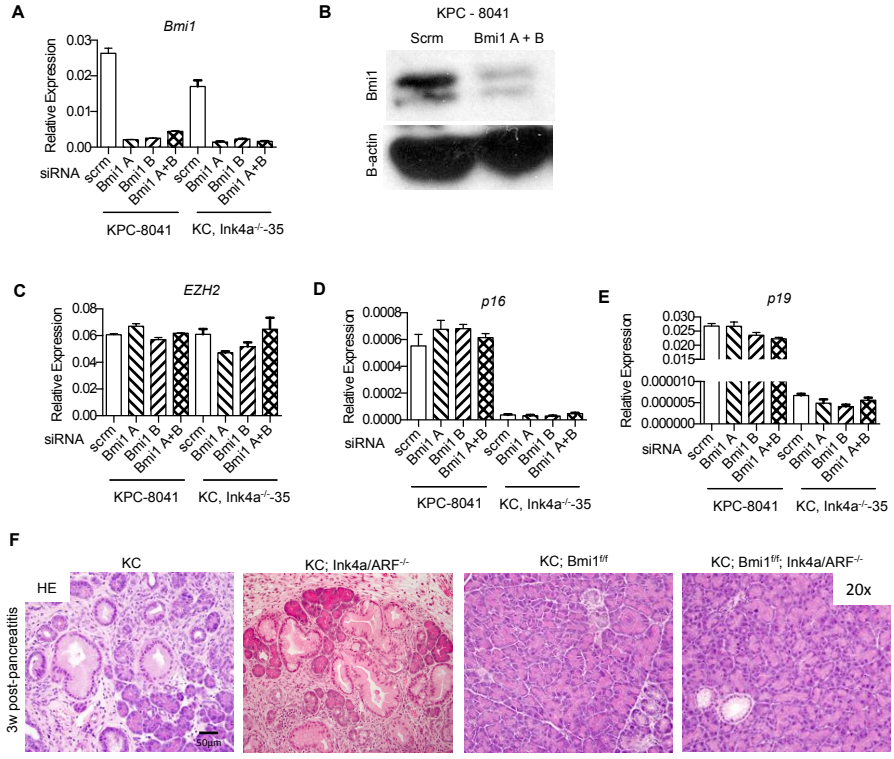


Figure 2.9: Bmi1-regulated pancreatic carcinogenesis is independent of Ink4a/ARF expression

RT-qPCR **(A)** and Western Blot **(B)** for Bmi1. RT-qPCR for **(C)** EZH2**, (D)** p16, and **(E)** p19. **(F)** HE staining for KC, KC;Ink4a/ARF$^{-/-}$, KC;Bmi1$^{fl/fl}$, and KC;Bmi1$^{fl/fl}$; Ink4a/ARF$^{-/-}$ pancreata.

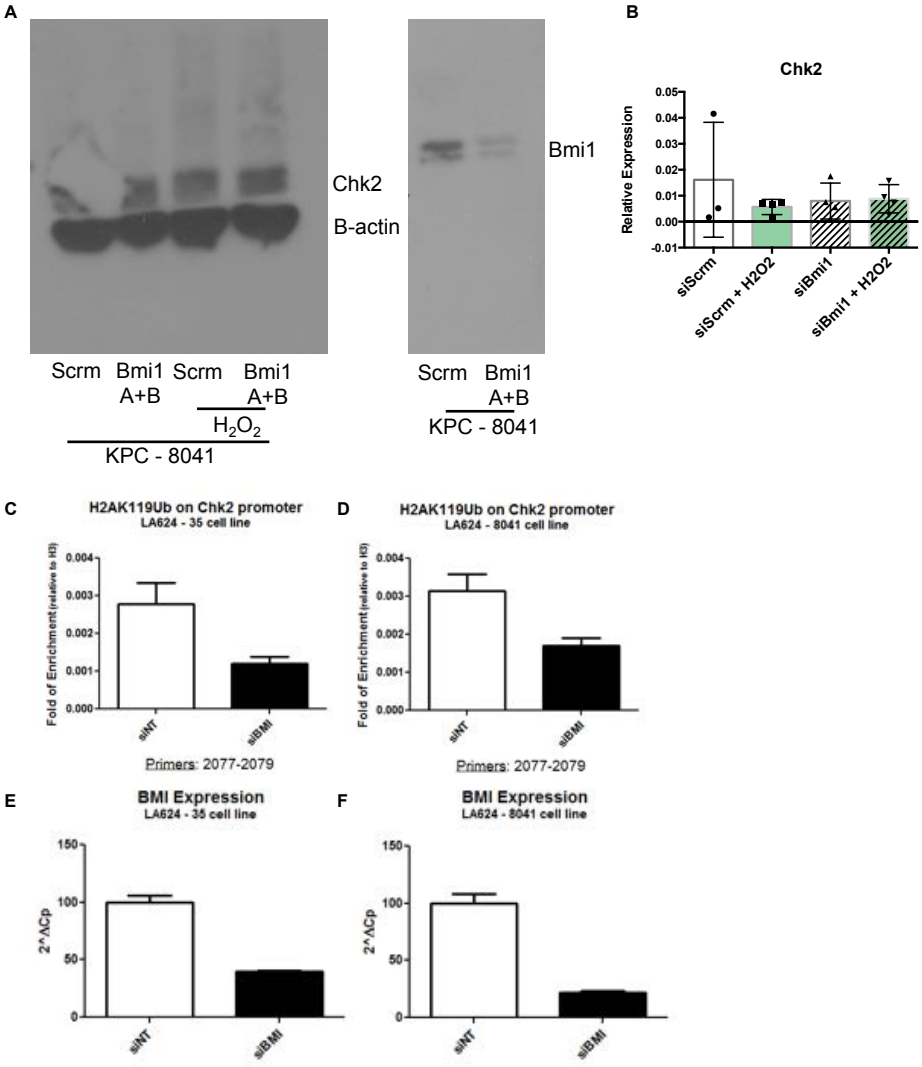


Figure 2.10: Chk2 expression is unchanged upon Bmi1 knock down
(A) Western Blot for Chk2, B-actin, and Bmi1 and **(B)** RT-PCR analysis for Chk2 in KPC – 8041 cells, in the presence of scrambled siRNA or Bmi1 knock down siRNA. Chromatin immunoprecipitation for H2AK119Ub on the Chk2 promoter in **(C)** 35 and **(D)** 8041 cells. RT-PCR confirming Bmi1 knock down in **(E)** 35 and **(F)** 8041 cells.

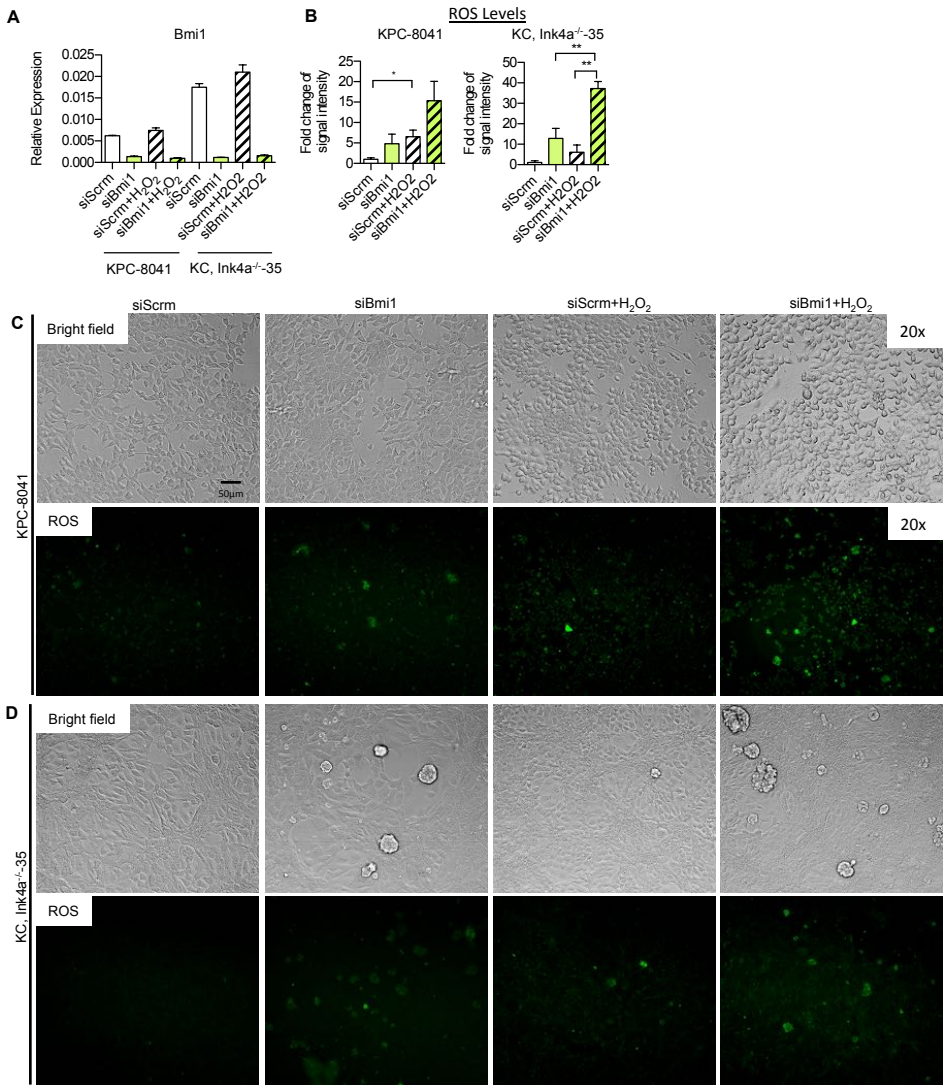


Figure 2.11: Activation of ROS detoxification is regulated by Bmi1
(A) RT-qPCR analysis for Bmi1 expression. **(B)** Quantification of ROS intensity for KPC – 8041 cells and KC; Ink4a$^{-/-}$ - 35 cells. Brightfield and ROS images for **(C)** KPC - 8041 cells and **(D)** KC; Ink4a$^{-/-}$ - 35 cells following siRNA and H_2O_2 treatments.

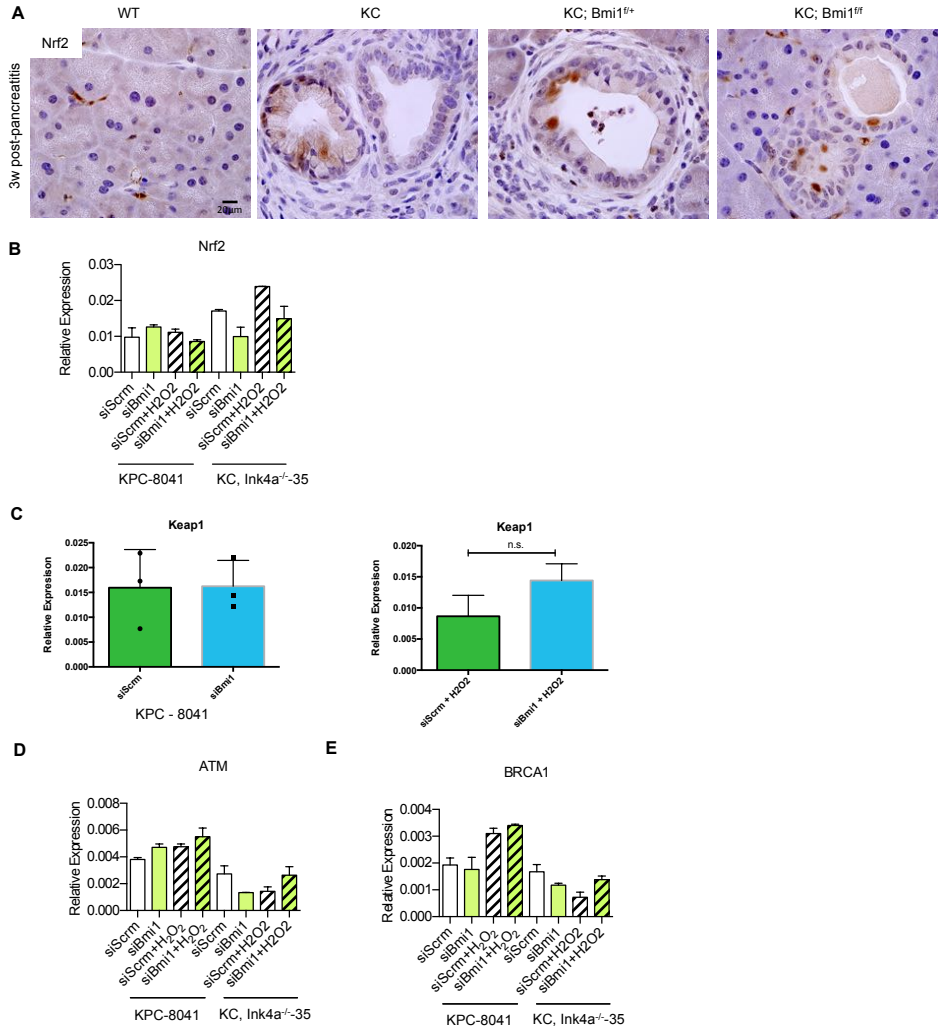


Figure 2.12: Significant changes are not observed in other ROS detoxification or DNA damage repair enzymes in the absence of Bmi1 expression.
IHC staining for Nrf2 in WT, KC, KC; Bmi1$^{fl/+}$, and KC; Bmi1$^{fl/fl}$ pancreata at 3 weeks post-pancreatitis (**A**). RT-qPCR analysis for Nrf2 (**B**), Keap1 (**C**), ATM (**D**), and BRCA1 (**E**) in KPC – 8041 cells and KC; Ink4a$^{-/-}$ - 35 cells following siRNA and H_2O_2 treatments. 12

Chapter Three

The Polycomb Group 1 component Bmi1 is required for HIF1α pathway activation, a limiting step for pancreatic carcinogenesis

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest human malignancies, and is predicted to become the second leading cause of cancer-related death by the year 2020 (Rahib, Smith et al. 2014). Notwithstanding current therapeutic options, the five-year-survival rate is a dismal 9% (Rahib, Smith et al. 2014). Future options for targeted therapy depend on improving our understanding of the biology of this disease.

Activating mutations in the oncogene Kras are observed in over 90% of human pancreatic cancers (Jones, Zhang et al. 2008, Waddell, Pajic et al. 2015, Bailey, Chang et al. 2016) and in the precancerous lesion of pancreatic cancer (Kanda, Matthaei et al. 2012), pancreatic intraepithelial neoplasia (PanIN). Mice that express mutant Kras in the pancreas (Pdx1-Cre; LSLKras^{G12D}, termed KC) develop PanINs that resemble the progression of the human disease (Hingorani, Petricoin et al. 2003). Bmi1 is a key component of the Polycomb Repressive Complex 1, a group of proteins that modulates gene transcription by placing repressive marks on chromatin. Bmi1 is upregulated in many types of cancer, including pancreatic, and often a high level of Bmi1 expression correlates with a worse prognosis (Song, Tao et al. 2010, Guo, Xu et al. 2014). Bmi1 is

required for the initiation and/or progression of several cancer types using mouse models, including lung and medulloblastoma, even in the presence of oncogene expression (Dovey, Zacharek et al. 2008, Michael, Westerman et al. 2008, Becker, Korn et al. 2009, Fan, Xu et al. 2012, Maynard, Ferretti et al. 2014).

In vitro, expression of Bmi1 in human pancreatic cancer cell lines that do not normally express Bmi1 enhanced invasiveness, migration, and markers of EMT. Conversely, Bmi1 knockdown in normally Bmi1-expressing human pancreatic cancer cell lines reduced these same properties (Proctor, Waghray et al. 2013). We previously showed that deletion of Bmi1 in the presence of oncogenic Kras is sufficient to block the onset of pancreatic carcinogenesis (Bednar, Schofield et al. 2015). The mechanism of Bmi1 requirement in some cancer types is through its repression of the expression of cell cycle regulators Ink4a and ARF (Dovey, Zacharek et al. 2008), which allows subsequent escape from senescence and continuation of cell proliferation. In PDAC and other cancer types that that are not regulated in this manner the mechanism of Bmi1 involvement remains unknown (Bruggeman, Hulsman et al. 2007, Douglas, Hsu et al. 2008).

In cancer types where Bmi1 was not acting to regulate cancer progression through Ink4a and ARF expression, a common characteristic was that lack of Bmi1 reduced proliferation and tumorigenicity of the cancer cells (Bruggeman, Hulsman et al. 2007, Douglas, Hsu et al. 2008). A feature of pancreatic cancer known to promote tumorigenicity is a hypoxic environment (Duffy, Eibl et al. 2003, Buchler, Reber et al. 2004). Human pancreatic tumors are extensively hypoxic (Koong, Mehta et al. 2000), a feature that contributes to the propensity to metastasize (Duffy, Eibl et al. 2003, Buchler,

Reber et al. 2004) and inhibition of hypoxia signaling for the treatment of pancreatic cancer is an active area of investigation (Onnis, Rapisarda et al. 2009, Erkan, Kurtoglu et al. 2016). Hypoxia signaling is regulated via the HIF pathway. There are three HIF family members, HIF1α, HIF2α, and HIF3α. HIF1α is ubiquitously expressed (Semenza 2007, Triner and Shah 2016). During states of normal oxygen levels, HIF proteins are bound with cellular oxygen, which allows their recognition and tagging for degradation in the proteasome by the protein VHL. When cellular oxygen levels decrease, lower oxygen binding allows HIF protein stabilization via binding with their β-subunit. This complex is then translocated to the nucleus and induces expression of downstream targets (Semenza 2012). These downstream targets include genes that control angiogenesis, glycolysis, and proliferation(Hu, Wang et al. 2003).

In the pancreas, a high level of HIF1α expression in human tumors correlates to faster tumor progression and lower levels of survival (Hoffmann, Mori et al. 2008). Deletion of HIF1α in the pancreas in animal models of pancreatic cancer promotes tumor initiation, an effect that is mediated, at least in part, through increasing tumor B-cell accumulation (Lee, Spata et al. 2016). This indicates that hypoxia signaling, specifically through HIF1α, may in fact act to restrain tumor growth at least partially through controlling the tumor microenvironment. Despite this result, the factors that regulate HIF1α expression in pancreatic cancer remain unknown.

Previous studies showed that Bmi1 is required for the initiation of pancreatic cancer in animal models, and that Bmi1 levels control tumorgenicity of human cell lines (Proctor, Waghray et al. 2013, Bednar, Schofield et al. 2015). Despite this, the mechanism of Bmi1 action in pancreatic cancer remained unknown. Here we

demonstrate that HIF1α stabilization recovers the lack of PanIN phenotype observed in animals lacking Bmi1, indicating that the reason for Bmi1 requirement in pancreatic cancer initiation is due to its control of HIF1α expression. This understanding of the interplay between Bmi1 and HIF1α during pancreatic cancer will be increasingly important as inhibitors for both proteins are actively being developed for cancer therapy (Masoud and Li 2015, Mayr, Wagner et al. 2016, Yin, Zhang et al. 2016).

Results

HIF1α expression is regulated by Bmi1 in pancreatic cancer cells

Bmi1 is required for the initiation of pancreatic cancer, however the mechanism for Bmi1 involvement in this process remains unknown (Bednar, Schofield et al. 2015) We used siRNA to knock down Bmi1 expression in mouse pancreatic cancer cell lines. Analysis of expression levels by qPCR revealed that in pancreatic cancer cells with Bmi1 knockdown, levels of HIF1α were significantly decreased (Figure 3.1A). This finding indicates that mechanism for Bmi1 action in pancreatic cancer progression may be via control of HIF1α expression.

The HIF signaling pathway is inactive in cells with normal oxygen levels, and becomes activated in response to hypoxia, initiating the transcription of downstream target genes. In order to test whether the disruption of Bmi1 expression in pancreatic cancer cells caused functional differences in HIF pathway activation, we grew cells in hypoxia to induce HIF pathway activation. Cells were treated for 24 hours with siRNA for Bmi1 knockdown or control, and then grown overnight in low oxygen conditions. Gene expression analysis for levels of HIF pathway target expression revealed that

expression of HIF targets was lower in cells with Bmi1 knockdown than control cells (Figure 3.1B). This indicates that Bmi1 knockdown in pancreatic cancer cells causes a functional downregulation of the HIF pathway.

Pancreata lacking Bmi1 and with stabilized HIF1α fail to recover from pancreatitis

Given the functional role for Bmi1 control of the HIF pathway *in vitro*, we next explored whether this also applied *in vivo.* We used mice that express a pancreas specific cre recombinase, Pdx1-Cre, in order to look directly at the role of Bmi1 and HIF1α in the murine pancreas. Next, to mirror the Bmi1 knockdown used in cells, we used animals that lack Bmi1 expression ($Bmi1^{fl/fl}$) upon cre recombination (Mich, Signer et al. 2014, Bednar, Schofield et al. 2015). To evaluate whether re-expressing HIF1α would recover the phenotypes seen by Bmi1 knockdown, we used a mouse that expresses an oxygen stable form of HIF1α ($R26\text{-}^{LSL\text{-}HIF1\alpha/+}$) cre recombination(Kim, Safran et al. 2006) (Figure 3.2A). First, we evaluated the histology of normal pancreata without treatment. Animals expressing the oxygen stable form of HIF1α, of the genotype Pdx1Cre; $R26^{LSL\text{-}HIF1\alpha/+}$, appeared histologically similar to control pancreata at 3 months of age (Figure 3.2B), indicating that HIF1α expression does not noticeably disrupt the normal pancreas. Similarly, animals lacking Bmi1 expression (Pdx1-Cre; $Bmi1^{fl/fl}$) had no noticeable histological differences when compared to control. Last, Pdx1-Cre; $Bmi1^{fl/fl}$; $HIF1\alpha^{-LSL/+}$ mice also looked similar to control (Figure 3.2B). Therefore, HIF1α stabilization, lack of Bmi1 expression, or both simultaneously, had no affect on the normal murine pancreas.

Bmi1 controls proliferation in pancreatic cancer cells (Proctor, Waghray et al. 2013) and in pancreatic islets (Dhawan, Tschen et al. 2009). Therefore we investigated the role of Bmi1 and HIF1a in another proliferative process of the pancreas, the recovery from pancreatitis. Animals were administered caerulein in order to induce pancreatitis, and tissue was harvested one week later (Figure 3.2C). After one week, control animals are expected to display full pancreatic recovery from the injuries produced by pancreatitis (Morris, Cano et al. 2010). One week after pancreatitis, animals lacking Bmi1 expression had similar histology to control mice, suggesting that Bmi1 expression is not required for the recovery from pancreatitis. Similarly, animals expressing HIF1α also fully recovered from pancreatitis, indicating that HIF1α stabilization during normoxia does not disrupt pancreatic recovery after pancreatitis. Animals lacking Bmi1 expression, but with HIF1α stabilization, however did not display normal histology one week after pancreatic insult. These animals showed extensive fibrosis and inflammatory infiltrates, along with few remaining acinar cells and small areas of acinar-ductal metaplasia (Figure 3.2D). Therefore in the pancreas, either lack of Bmi1 expression or stabilization of HIF1α expression during normoxia does not prevent pancreatic recovery post-pancreatitis. However, Pdx1-Cre; $Bmi1^{fl/fl}$; HIF1α-LSL/+ animals, without Bmi1 expression and with HIF1α stabilized, do not recover from pancreatic injury, indicating that Bmi1 and HIF1α may play a dual functional role in the reaction to pancreatic stress.

HIF1α expression recovers the lack of PanINs seen in mice lacking Bmi1 in a mouse model of pancreatic cancer

Our group previously showed that Bmi1 expression is required for the initiation of pancreatic cancer (Bednar, Schofield et al. 2015), but the mechanism for Bmi1 control of this process remained unknown. Given that Bmi1 knockdown led to reduced levels of HIF pathway activation in pancreatic cancer cells, we sought to determine whether Bmi1 control of HIF1α expression was the reason for Bmi1 requirement in the initiation of pancreatic cancer. Mice that express oncogenic Kras specifically in the pancreas, of the genotype Pdx1-Cre; LSLKras^{G12D} reliably model the early stages of pancreatic cancer. This animal model is termed KC, and KC mice develop PanIN lesions that resemble those seen in human pancreatic cancer. Here we used KC animals to investigate the interaction between Bmi1 and HIF1α expression during the initiation of pancreatic cancer. KC animals were combined with the Bmi1 floxed allele and HIF1α stabilized allele (Figure 3.3A), allowing evaluation of Bmi1 deletion and HIF1α stabilization in the context of oncogenic Kras expression.

At 12 weeks of age, KC mice developed sporadic PanINs, as expected. These PanINs expressed pERK, indicating MAPK pathway activation, and mucins, both features that are characteristic for pancreatic precancerous lesions (Figure 3.3B). As previously described, KC;Bmi1$^{fl/fl}$ animals do not develop PanIN lesions, highlighting the requirement for Bmi1 in the initiation of pancreatic cancer (Figure 3.3C). Pancreata in these animals appear histologically normal and do not display pERK or PAS positivity, due to lack of pancreatic precancerous lesions (Figure 3.3C). Occasional PanIN lesions seen in KC;Bmi1$^{fl/fl}$ animals continue to express Bmi1 and therefore presumably develop due to failure of recombination of the Bmi1 floxed allele, as previously

described(Bednar, Schofield et al. 2015). KC;HIF1α-LSL/+ animals, expressing both oncogenic Kras and stabilized HIF1α also develop PanINs, indicating that HIF1α stabilization does not disrupt PanIN formation driven by oncogenic Kras. These lesions are positive for pERK and mucin expression (by PAS) (Figure 3.3D). KC; $Bmi1^{fl/fl}$; HIF1α-LSL/+ mice at 12 weeks of age develop PanINs similar to KC animals. These lesions display pERK and PAS positivity, helping to confirm their identity as PanINs (Figure 3.3E). Therefore, HIF1α stabilization recovers the lack of PanIN phenotype observed in the KC;$Bmi1^{fl/fl}$ animals that lack Bmi1 expression. This indicates that the reason for the requirement for Bmi1 expression during the initiation of pancreatic precancerous lesions may be due to Bmi1 control of HIF pathway activation. Taken together with the reaction to pancreatitis, this data indicates that the combination of lack of pancreatic Bmi1 expression and stabilization of HIF1α renders the pancreas unable to respond to cellular insults, including pancreatitis and Kras activation.

Bmi1 and HIF1α control metabolic enzyme levels in the normal and precancerous pancreas

Given that Bmi1 and HIF1α collaborate in the murine pancreas in the reaction to cellular stresses, we questioned which processes were controlled by this interaction. HIF pathway stimulation is known to activate many downstream target genes, including those involved in angiogenesis, growth and metabolism (Triner and Shah 2016). Both the reaction to pancreatitis and the initiation of pancreatic cancer are highly metabolic processes (Halbrook and Lyssiotis 2017), therefore we investigated whether Bmi1 and

HIF1α control metabolic changes during these pancreatic states. First, we wished to define the changes in levels of expression of metabolic enzymes during the reaction to pancreatitis. In order to do this we induced pancreatitis, either in normal pancreata or in the context of oncogenic Kras, and harvested pancreata after one day, two days or one week (Figure 3.4A).

In the normal pancreas, there are low levels of the metabolic enzyme Pdk1 in untreated animals. These levels increase dramatically one day after the administration to pancreatitis, and return to normal after two days, corresponding with the histologic injury and recovery timeline in mice (Figure 3.4B). This indicates that metabolic enzymes change levels and may play a role during the reaction to pancreatitis in mice. To investigate this in the context of Bmi1 and HIF1α expression, we analyzed Pdk1 expression in our genetically engineered mice. Mice were analyzed one week after pancreatitis, where those lacking Bmi1 expression (Pdx1-Cre; $Bmi1^{fl/fl}$) displayed significantly lower levels of Pdk1 expression compared to control animals. However in animals additionally having stabilized HIF1α, Pdx1-Cre; $Bmi1^{fl/fl}$; HIF1α-LSL/+, Pdk1 expression was recovered to the level seen in control animals (Figure 3.4C). In animals lacking Bmi1 expression, lack of Bmi1 may lead to inability of the HIF pathway to activate downstream target genes, such as Pdk1. The additional expression of HIF1α in this context allows recovery of HIF pathway activation.

We performed the same experiments in the context of oncogenic Kras activation. 12-week-old KC animals were subjected to pancreatitis and tissues were harvested after one day, two days or one week (Figure 3.4A). Untreated KC animals expressed a similar level of Pdk1 to untreated control mice (Figure 3.4B and Figure 3.4D). Similar to

control animals, in KC mice levels of Pdk1 rose one day after pancreatitis and fell back to untreated levels by two days after pancreatitis, and persisted at that untreated level one week after pancreatitis (Figure 3.4D), indicating that Pdk1 levels also respond to pancreatitis in the context of the induction of oncogenic Kras. In order to discern the pattern of Pdk1 expression in terms of Bmi1 and HIF1α, we analyzed Pdk1 levels in 12-week-old pancreata. KC;Bmi1$^{fl/fl}$ mice displayed very low levels of Pdk1 compared to control KC animals. These Pdk1 levels were recovered in KC;Bmi1$^{fl/fl}$; HIF1α-LSL/+ animals, indicating that lack of Bmi1 expression is inhibiting HIF pathway activation of Pdk1 (Figure 3.4E). This data indicates that Bmi1 expression may play a protective role in the pancreas by inhibiting HIF pathway activation of metabolic enzymes, such a Pdk1, in reaction to cell stress. When the HIF pathway is additionally activated, the levels of Pdk1 recover, which corresponds histologically to lack of pancreatic recovery from pancreatitis and the development of PanIN lesions in the context of oncogenic Kras expression.

Differential roles for Bmi1 expression in the endocrine and exocrine pancreas

Mice with full body knockout of Bmi1 have been previously shown to have defects in the endocrine pancreas (Dhawan, Tschen et al. 2009). Given that our data suggests that the requirement for Bmi1 expression during the initiation of pancreatic cancer is through Bmi1 regulation of HIF1α expression, we also investigated this relationship in the endocrine pancreas. To test endocrine pancreas function, we subjected animals to glucose tolerance testing. In response to glucose injection, control

animals showed a spike in blood glucose levels after 15 minutes, which slowly returned to baseline levels after two hours. In contrast, animals lacking Bmi1 expression showed impaired glucose tolerance, showing a similar spike in blood glucose in response to glucose injection. However, this spike lasted longer than in control animals and Pdx-Cre;Bmi1$^{fl/fl}$ animals retained the highest levels of blood glucose for 30 minutes, but did return to baseline within two hours (Figure 3.5A). Similar to previous observations, this indicates that lack of Bmi1 expression in the pancreas leads to endocrine pancreas dysfunction. HIF1α expression seemed to rescue this defect, with Pdx1-Cre;Bmi1$^{fl/fl}$;HIF1α-$^{LSL/+}$ animals displaying a similar glucose response to control mice (Figure 3.5A). A similar pattern was observed in the context of oncogenic Kras, where KC mice that were injected with glucose showed a rapid spike in blood glucose levels that then returned to baseline by two hours after injection (Figure 3.5B). KC;Bmi1$^{fl/fl}$ mice also displayed a rapid spike in blood glucose in response to glucose injection, and this maximum level of blood glucose lasted longer than observed in control, before also returning to baseline within two hours (Figure 3.5B). KC;Bmi1$^{fl/fl}$;HIF1α-LSL/+ mice showed a similar response to glucose injection as KC (Figure 3.5B), indicating that Bmi1 control of HIF1α in the pancreas may contribute to its role in endocrine function.

To investigate the role of Bmi1 in the endocrine pancreas at a cellular level, we used Min6 cells, a mouse insulinoma cell line that is composed of β-cells and has endocrine function (Ishihara, Asano et al. 1993). Min6 cells with Bmi1 knockdown showed lower levels of HIF1α expression than controls, consistent with the pattern seen in pancreatic cancer cell lines (Figure 3.5C). However measurement of ROS levels by DCFDA analysis showed no difference in ROS accumulation in Min6 cells with or

without Bmi1 knockdown (Figure 3.5D), in contrast to previous observations that Bmi1 knockdown results in higher ROS levels in pancreatic cancer cell lines (Bednar, Schofield et al. 2015). This suggests that Bmi1 may be important for the control of different cellular processes in the endocrine and the exocrine pancreas. Other work has shown that an important role for Bmi1 in many systems is inhibition of the cell cycle regulators Ink4a and ARF (Jacobs, Kieboom et al. 1999, Bruggeman, Valk-Lingbeek et al. 2005, Molofsky, He et al. 2005, Dhawan, Tschen et al. 2009). Our previous studies indicated that the reason for the requirement of Bmi1 expression during the initiation of pancreatic cancer was not through control of Ink4a and ARF, suggesting that this mechanism is not predominant in the exocrine pancreas (Bednar, Schofield et al. 2015). In Min6 cells, gene expression analysis showed that levels of p16 and p19, the genes encoding Ink4a and ARF, were increased in response to Bmi1 knockdown (Figure 3.5E). This indicates that Bmi1 may play differential roles in the pancreas, controlling pancreatic cancer initiation through the HIF pathway in the exocrine pancreas and inhibiting the cell cycle regulators Ink4a and ARF in the endocrine pancreas.

Discussion

Our previous work showed that Bmi1 expression is required for the initiation of pancreatic cancer, using a mouse model of the disease with an intact microenvironment (Bednar, Schofield et al. 2015). However, which cellular processes Bmi1 controls in this context, and therefore the reason for its requirement in pancreatic cancer initiation, remained unknown. In this current work, we show that the mechanism for Bmi1 requirement in the initiation of pancreatic cancer is through its control of HIF pathway

activation, providing new insights into the development of the early stages of the disease.

In vivo we explored the interaction between Bmi1 and HIF1α expression using animals that express an oxygen stable form of HIF1α specifically in the pancreas (Kim, Safran et al. 2006). During the recovery from pancreatitis, animals that either lack Bmi1 expression or express stabilized HIF1α recover from their injury in a similar time frame to control pancreata. However, mice that both lack Bmi1 and express stabilized HIF1α do not recover from pancreatitis, displaying extensive fibrosis and inflammation one week after injury. During the initiation of pancreatic cancer, using an oncogenic Kras driven mouse model, animals that express $Kras^{G12D}$ but lack Bmi1 do not develop PanINs. Additional HIF1α stabilization however recovers this lack of PanIN phenotype, allowing pancreatic cancer initiation. These data together indicate that Bmi1 plays in important protective role when the pancreas is stressed via its control of HIF1α activation. When Bmi1 is inactivated and HIF1α is stabilized, the pancreas is not able to recover from insults, which include pancreatitis or oncogenic Kras expression. This suggests that Bmi1 control of HIF pathway levels is crucial to maintain a healthy pancreas and to prevent disease progression.

The recovery from pancreatitis and the initiation of pancreatic cancer are both processes that involve many changes in tissue metabolism in order to proceed. Here we show that HIF pathway activation, via Bmi1 expression, may be crucial to control the metabolic state of the pancreas in these contexts. New research has revealed many insights into metabolic changes in pancreatic cancer cells, and so it will be crucial going forward to understand the pathways that control these changes. Here we show that

HIF1α expression is crucial for the initiation of pancreatic cancer, and that this may be through its control of downstream metabolic changes. Given that HIF1α inhibitors are actively being developed for cancer therapy, it is important to elucidate precisely how HIF expression controls cancer development in order to fully understand these future therapies.

Investigation of the endocrine pancreas revealed different functions for Bmi1 in the exocrine and endocrine compartments of the pancreas. Knockdown of Bmi1 did not result in increased ROS in β-cells, but it did lead to higher levels of expression of p16 and p19, similar to previous *in vivo* studies (Dhawan, Tschen et al. 2009). This indicates that Bmi1 may have separate pancreatic functions, controlling the cell cycle through p16 and p19 expression in the endocrine pancreas and regulating the levels of HIF pathway activation in the exocrine pancreas.

Here we show that the reason for the requirement of Bmi1 expression during the initiation of pancreatic cancer is due to Bmi1 regulation of HIF pathway activation, specifically HIF1α. Understanding the mechanisms of HIF pathway activation in pancreatic cancer will be increasingly important as HIF1α inhibitors are currently being developed for cancer therapy (Onnis, Rapisarda et al. 2009, Burroughs, Kaluz et al. 2013, Wigerup, Pahlman et al. 2016). Overall, this work will provide a deeper understanding of the mechanisms behind the initiation of pancreatic cancer, which will help inform future therapeutic options.

Methods

Mice

Mice were housed in the specific pathogen free facilities at the University of Michigan Comprehensive Cancer Center. This study was approved by the University of Michigan University Committee on Use and Care of Animals (UCUCA) guidelines. Pdx1-Cre, Bmi1 floxed, R26-$^{LSLHIF1\alpha}$, and LSLKras^{G12D} animals have been previously described (Hingorani, Petricoin et al. 2003, Kim, Safran et al. 2006, Mich, Signer et al. 2014). To induce pancreatitis, mice were subjected to two consecutive days of 8 hourly intraperitoneal injections of caerulein at a concentration of 75ug/kg, as previously described (Collins, Bednar et al. 2012).

Histology and Immunohistochemistry

All histology and immunohistochemistry studies were performed as previously described (Collins, Bednar et al. 2012). Embedding and sectioning was performed by the University of Michigan Cancer Center Histopathology Core. All antibodies used are detailed in Supplemental Materials and Methods.

Cell Culture

Mouse pancreatic cancer cell lines were maintained as previously described (Bednar, Schofield et al. 2015). Bmi1 knockdown was performed using siRNA. Cells were transfected using Lipofectamine according to the manufacturer's instructions. Control or Bmi1 specific siRNA (Dharmacon) was applied to the cells for Bmi1 knockdown. Min6 mouse insulinoma cells were grown in culture as previously described, using DMEM

growth media containing FBS, penicillin/streptomycin, sodium pyruvate, and β-mercaptoethanol(Soleimanpour, Ferrari et al. 2015).

ROS Detection Assay

Hydrogen peroxide was added directly to the cell media at a concentration of 500uM for a time period of two hours preceding ROS analysis. ROS levels were measured by DCFDA assay according to the manufacturer's instructions.

qRT-PCR

RNA isolation, rt-PCR, and qRT-PCR were performed as previously described (Collins, Bednar et al. 2012). *Cyclophilin A* was used as a control housekeeping gene for normalization. All primer sequences used are included in Supplemental Materials and Methods. Statistical significance for qRT-PCR was determined by unpaired two-tailed t-test, with the threshold for significance set at $p<0.05$.

Glucose Tolerance Test

Glucose tolerance testing was performed as previously described (Flak, Patterson et al. 2014). Prior to testing, animals were fasted for four hours during the light cycle. Initial blood glucose levels were measured using tail blood sample. Animals were next administered glucose by intraperitoneal injection at a dose of 2g glucose per kg body weight. Tail blood samples were used for glucose measurement at 15, 30, 60, 90, and 120 minutes post-glucose injection. Blood glucose was measured using Accu-Chek Aviva diabetes monitoring kit and Accu-Chek Aviva Plus testing strips.

References

Bailey, P., D. K. Chang, K. Nones, A. L. Johns, A. M. Patch, M. C. Gingras, D. K. Miller, A. N. Christ, T. J. Bruxner, M. C. Quinn, C. Nourse, L. C. Murtaugh, I. Harliwong, S. Idrisoglu, S. Manning, E. Nourbakhsh, S. Wani, L. Fink, O. Holmes, V. Chin, M. J. Anderson, S. Kazakoff, C. Leonard, F. Newell, N. Waddell, S. Wood, Q. Xu, P. J. Wilson, N. Cloonan, K. S. Kassahn, D. Taylor, K. Quek, A. Robertson, L. Pantano, L. Mincarelli, L. N. Sanchez, L. Evers, J. Wu, M. Pinese, M. J. Cowley, M. D. Jones, E. K. Colvin, A. M. Nagrial, E. S. Humphrey, L. A. Chantrill, A. Mawson, J. Humphris, A. Chou, M. Pajic, C. J. Scarlett, A. V. Pinho, M. Giry-Laterriere, I. Rooman, J. S. Samra, J. G. Kench, J. A. Lovell, N. D. Merrett, C. W. Toon, K. Epari, N. Q. Nguyen, A. Barbour, N. Zeps, K. Moran-Jones, N. B. Jamieson, J. S. Graham, F. Duthie, K. Oien, J. Hair, R. Grutzmann, A. Maitra, C. A. Iacobuzio-Donahue, C. L. Wolfgang, R. A. Morgan, R. T. Lawlor, V. Corbo, C. Bassi, B. Rusev, P. Capelli, R. Salvia, G. Tortora, D. Mukhopadhyay, G. M. Petersen, I. Australian Pancreatic Cancer Genome, D. M. Munzy, W. E. Fisher, S. A. Karim, J. R. Eshleman, R. H. Hruban, C. Pilarsky, J. P. Morton, O. J. Sansom, A. Scarpa, E. A. Musgrove, U. M. Bailey, O. Hofmann, R. L. Sutherland, D. A. Wheeler, A. J. Gill, R. A. Gibbs, J. V. Pearson, N. Waddell, A. V. Biankin and S. M. Grimmond (2016). "Genomic analyses identify molecular subtypes of pancreatic cancer." Nature **531**(7592): 47-52.

Becker, M., C. Korn, A. R. Sienerth, R. Voswinckel, K. Luetkenhaus, F. Ceteci and U. R. Rapp (2009). "Polycomb group protein Bmi1 is required for growth of RAF driven non-small-cell lung cancer." PLoS One **4**(1): e4230.

Bednar, F., H. K. Schofield, M. A. Collins, W. Yan, Y. Zhang, N. Shyam, J. A. Eberle, L. L. Almada, K. P. Olive, N. Bardeesy, M. E. Fernandez-Zapico, D. Nakada, D. M. Simeone, S. J. Morrison and M. Pasca di Magliano (2015). "Bmi1 is required for the initiation of pancreatic cancer through an Ink4a-independent mechanism." Carcinogenesis **36**(7): 730-738.

Bruggeman, S. W., D. Hulsman, E. Tanger, T. Buckle, M. Blom, J. Zevenhoven, O. van Tellingen and M. van Lohuizen (2007). "Bmi1 controls tumor development in an Ink4a/Arf-independent manner in a mouse model for glioma." Cancer Cell **12**(4): 328-341.

Bruggeman, S. W., M. E. Valk-Lingbeek, P. P. van der Stoop, J. J. Jacobs, K. Kieboom, E. Tanger, D. Hulsman, C. Leung, Y. Arsenijevic, S. Marino and M. van Lohuizen (2005). "Ink4a and Arf differentially affect cell proliferation and neural stem cell self-renewal in Bmi1-deficient mice." Genes Dev **19**(12): 1438-1443.

Buchler, P., H. A. Reber, R. S. Lavey, J. Tomlinson, M. W. Buchler, H. Friess and O. J. Hines (2004). "Tumor hypoxia correlates with metastatic tumor growth of pancreatic cancer in an orthotopic murine model." J Surg Res **120**(2): 295-303.

Burroughs, S. K., S. Kaluz, D. Wang, K. Wang, E. G. Van Meir and B. Wang (2013). "Hypoxia inducible factor pathway inhibitors as anticancer therapeutics." Future Med Chem **5**(5): 553-572.

Collins, M. A., F. Bednar, Y. Zhang, J. C. Brisset, S. Galban, C. J. Galban, S. Rakshit, K. S. Flannagan, N. V. Adsay and M. Pasca di Magliano (2012). "Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice." J Clin Invest **122**(2): 639-653.

Dhawan, S., S. I. Tschen and A. Bhushan (2009). "Bmi-1 regulates the Ink4a/Arf locus to control pancreatic beta-cell proliferation." Genes Dev **23**(8): 906-911.

Douglas, D., J. H. Hsu, L. Hung, A. Cooper, D. Abdueva, J. van Doorninck, G. Peng, H. Shimada, T. J. Triche and E. R. Lawlor (2008). "BMI-1 promotes ewing sarcoma tumorigenicity independent of CDKN2A repression." Cancer Res **68**(16): 6507-6515.

Dovey, J. S., S. J. Zacharek, C. F. Kim and J. A. Lees (2008). "Bmi1 is critical for lung tumorigenesis and bronchioalveolar stem cell expansion." Proc Natl Acad Sci U S A **105**(33): 11857-11862.

Duffy, J. P., G. Eibl, H. A. Reber and O. J. Hines (2003). "Influence of hypoxia and neoangiogenesis on the growth of pancreatic cancer." Mol Cancer **2**: 12.

Erkan, M., M. Kurtoglu and J. Kleeff (2016). "The role of hypoxia in pancreatic cancer: a potential therapeutic target?" Expert Rev Gastroenterol Hepatol **10**(3): 301-316.

Fan, L., C. Xu, C. Wang, J. Tao, C. Ho, L. Jiang, B. Gui, S. Huang, M. Evert, D. F. Calvisi and X. Chen (2012). "Bmi1 is required for hepatic progenitor cell expansion and liver tumor development." PLoS One **7**(9): e46472.

Flak, J. N., C. M. Patterson, A. S. Garfield, G. D'Agostino, P. B. Goforth, A. K. Sutton, P. A. Malec, J. T. Wong, M. Germani, J. C. Jones, M. Rajala, L. Satin, C. J. Rhodes, D. P. Olson, R. T. Kennedy, L. K. Heisler and M. G. Myers, Jr. (2014). "Leptin-inhibited PBN neurons enhance responses to hypoglycemia in negative energy balance." Nat Neurosci **17**(12): 1744-1750.

Guo, S., X. Xu, Y. Tang, C. Zhang, J. Li, Y. Ouyang, J. Ju, P. Bie and H. Wang (2014). "miR-15a inhibits cell proliferation and epithelial to mesenchymal transition in pancreatic

ductal adenocarcinoma by down-regulating Bmi-1 expression." Cancer Lett **344**(1): 40-46.

Halbrook, C. J. and C. A. Lyssiotis (2017). "Employing Metabolism to Improve the Diagnosis and Treatment of Pancreatic Cancer." Cancer Cell **31**(1): 5-19.

Hingorani, S. R., E. F. Petricoin, A. Maitra, V. Rajapakse, C. King, M. A. Jacobetz, S. Ross, T. P. Conrads, T. D. Veenstra, B. A. Hitt, Y. Kawaguchi, D. Johann, L. A. Liotta, H. C. Crawford, M. E. Putt, T. Jacks, C. V. Wright, R. H. Hruban, A. M. Lowy and D. A. Tuveson (2003). "Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse." Cancer Cell **4**(6): 437-450.

Hoffmann, A. C., R. Mori, D. Vallbohmer, J. Brabender, E. Klein, U. Drebber, S. E. Baldus, J. Cooc, M. Azuma, R. Metzger, A. H. Hoelscher, K. D. Danenberg, K. L. Prenzel and P. V. Danenberg (2008). "High expression of HIF1a is a predictor of clinical outcome in patients with pancreatic ductal adenocarcinomas and correlated to PDGFA, VEGF, and bFGF." Neoplasia **10**(7): 674-679.

Hu, C. J., L. Y. Wang, L. A. Chodosh, B. Keith and M. C. Simon (2003). "Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation." Mol Cell Biol **23**(24): 9361-9374.

Ishihara, H., T. Asano, K. Tsukuda, H. Katagiri, K. Inukai, M. Anai, M. Kikuchi, Y. Yazaki, J. I. Miyazaki and Y. Oka (1993). "Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets." Diabetologia **36**(11): 1139-1145.

Jacobs, J. J., K. Kieboom, S. Marino, R. A. DePinho and M. van Lohuizen (1999). "The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus." Nature **397**(6715): 164-168.

Jones, S., X. Zhang, D. W. Parsons, J. C. Lin, R. J. Leary, P. Angenendt, P. Mankoo, H. Carter, H. Kamiyama, A. Jimeno, S. M. Hong, B. Fu, M. T. Lin, E. S. Calhoun, M. Kamiyama, K. Walter, T. Nikolskaya, Y. Nikolsky, J. Hartigan, D. R. Smith, M. Hidalgo, S. D. Leach, A. P. Klein, E. M. Jaffee, M. Goggins, A. Maitra, C. Iacobuzio-Donahue, J. R. Eshleman, S. E. Kern, R. H. Hruban, R. Karchin, N. Papadopoulos, G. Parmigiani, B. Vogelstein, V. E. Velculescu and K. W. Kinzler (2008). "Core signaling pathways in human pancreatic cancers revealed by global genomic analyses." Science **321**(5897): 1801-1806.

Kanda, M., H. Matthaei, J. Wu, S. M. Hong, J. Yu, M. Borges, R. H. Hruban, A. Maitra, K. Kinzler, B. Vogelstein and M. Goggins (2012). "Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia." Gastroenterology **142**(4): 730-733 e739.

Kim, W. Y., M. Safran, M. R. Buckley, B. L. Ebert, J. Glickman, M. Bosenberg, M. Regan and W. G. Kaelin, Jr. (2006). "Failure to prolyl hydroxylate hypoxia-inducible factor alpha phenocopies VHL inactivation in vivo." EMBO J **25**(19): 4650-4662.

Koong, A. C., V. K. Mehta, Q. T. Le, G. A. Fisher, D. J. Terris, J. M. Brown, A. J. Bastidas and M. Vierra (2000). "Pancreatic tumors show high levels of hypoxia." Int J Radiat Oncol Biol Phys **48**(4): 919-922.

Lee, K. E., M. Spata, L. J. Bayne, E. L. Buza, A. C. Durham, D. Allman, R. H. Vonderheide and M. C. Simon (2016). "Hif1a Deletion Reveals Pro-Neoplastic Function of B Cells in Pancreatic Neoplasia." Cancer Discov **6**(3): 256-269.

Masoud, G. N. and W. Li (2015). "HIF-1alpha pathway: role, regulation and intervention for cancer therapy." Acta Pharm Sin B **5**(5): 378-389.

Maynard, M. A., R. Ferretti, K. I. Hilgendorf, C. Perret, P. Whyte and J. A. Lees (2014). "Bmi1 is required for tumorigenesis in a mouse model of intestinal cancer." Oncogene **33**(28): 3742-3747.

Mayr, C., A. Wagner, M. Loeffelberger, D. Bruckner, M. Jakab, F. Berr, P. Di Fazio, M. Ocker, D. Neureiter, M. Pichler and T. Kiesslich (2016). "The BMI1 inhibitor PTC-209 is a potential compound to halt cellular growth in biliary tract cancer cells." Oncotarget **7**(1): 745-758.

Mich, J. K., R. A. Signer, D. Nakada, A. Pineda, R. J. Burgess, T. Y. Vue, J. E. Johnson and S. J. Morrison (2014). "Prospective identification of functionally distinct stem cells and neurosphere-initiating cells in adult mouse forebrain." Elife **3**: e02669.

Michael, L. E., B. A. Westerman, A. N. Ermilov, A. Wang, J. Ferris, J. Liu, M. Blom, D. W. Ellison, M. van Lohuizen and A. A. Dlugosz (2008). "Bmi1 is required for Hedgehog pathway-driven medulloblastoma expansion." Neoplasia **10**(12): 1343-1349, 1345p following 1349.

Molofsky, A. V., S. He, M. Bydon, S. J. Morrison and R. Pardal (2005). "Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways." Genes Dev **19**(12): 1432-1437.

Morris, J. P. t., D. A. Cano, S. Sekine, S. C. Wang and M. Hebrok (2010). "Beta-catenin blocks Kras-dependent reprogramming of acini into pancreatic cancer precursor lesions in mice." J Clin Invest **120**(2): 508-520.

Onnis, B., A. Rapisarda and G. Melillo (2009). "Development of HIF-1 inhibitors for cancer therapy." J Cell Mol Med **13**(9A): 2780-2786.

Proctor, E., M. Waghray, C. J. Lee, D. G. Heidt, M. Yalamanchili, C. Li, F. Bednar and D. M. Simeone (2013). "Bmi1 enhances tumorigenicity and cancer stem cell function in pancreatic adenocarcinoma." PLoS One **8**(2): e55820.

Rahib, L., B. D. Smith, R. Aizenberg, A. B. Rosenzweig, J. M. Fleshman and L. M. Matrisian (2014). "Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States." Cancer Res **74**(11): 2913-2921.

Semenza, G. L. (2007). "Hypoxia-inducible factor 1 (HIF-1) pathway." Sci STKE **2007**(407): cm8.

Semenza, G. L. (2012). "Hypoxia-inducible factors in physiology and medicine." Cell **148**(3): 399-408.

Soleimanpour, S. A., A. M. Ferrari, J. C. Raum, D. N. Groff, J. Yang, B. A. Kaufman and D. A. Stoffers (2015). "Diabetes Susceptibility Genes Pdx1 and Clec16a Function in a Pathway Regulating Mitophagy in beta-Cells." Diabetes **64**(10): 3475-3484.

Song, W., K. Tao, H. Li, C. Jin, Z. Song, J. Li, H. Shi, X. Li, Z. Dang and K. Dou (2010). "Bmi-1 is related to proliferation, survival and poor prognosis in pancreatic cancer." Cancer Sci **101**(7): 1754-1760.

Triner, D. and Y. M. Shah (2016). "Hypoxia-inducible factors: a central link between inflammation and cancer." J Clin Invest **126**(10): 3689-3698.

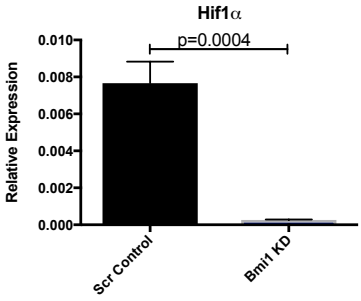
Waddell, N., M. Pajic, A. M. Patch, D. K. Chang, K. S. Kassahn, P. Bailey, A. L. Johns, D. Miller, K. Nones, K. Quek, M. C. Quinn, A. J. Robertson, M. Z. Fadlullah, T. J. Bruxner, A. N. Christ, I. Harliwong, S. Idrisoglu, S. Manning, C. Nourse, E. Nourbakhsh, S. Wani, P. J. Wilson, E. Markham, N. Cloonan, M. J. Anderson, J. L. Fink, O. Holmes, S. H. Kazakoff, C. Leonard, F. Newell, B. Poudel, S. Song, D. Taylor, N. Waddell, S. Wood, Q. Xu, J. Wu, M. Pinese, M. J. Cowley, H. C. Lee, M. D. Jones, A. M. Nagrial, J. Humphris, L. A. Chantrill, V. Chin, A. M. Steinmann, A. Mawson, E. S. Humphrey, E. K. Colvin, A. Chou, C. J. Scarlett, A. V. Pinho, M. Giry-Laterriere, I. Rooman, J. S. Samra, J. G. Kench, J. A. Pettitt, N. D. Merrett, C. Toon, K. Epari, N. Q. Nguyen, A. Barbour, N. Zeps, N. B. Jamieson, J. S. Graham, S. P. Niclou, R. Bjerkvig, R. Grutzmann, D. Aust, R. H. Hruban, A. Maitra, C. A. Iacobuzio-Donahue, C. L. Wolfgang, R. A. Morgan, R. T. Lawlor, V. Corbo, C. Bassi, M. Falconi, G. Zamboni, G. Tortora, M. A. Tempero, I. Australian Pancreatic Cancer Genome, A. J. Gill, J. R. Eshleman, C. Pilarsky, A. Scarpa, E. A. Musgrove, J. V. Pearson, A. V. Biankin and S. M. Grimmond (2015). "Whole genomes redefine the mutational landscape of pancreatic cancer." Nature **518**(7540): 495-501.

Wigerup, C., S. Pahlman and D. Bexell (2016). "Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer." Pharmacol Ther **164**: 152-169.

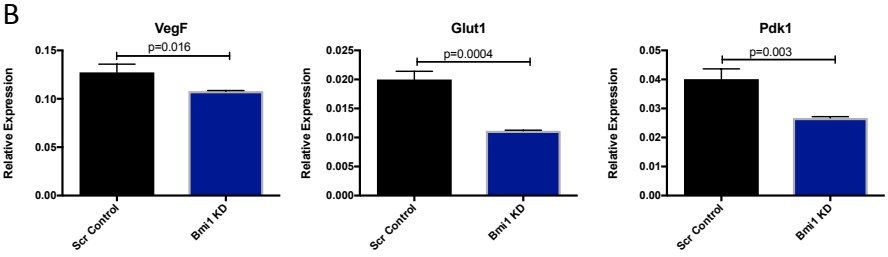
Yin, T., Z. Zhang, B. Cao, Q. Duan, P. Shi, H. Zhao, S. N. Camara, Q. Shen and C. Wang (2016). "Bmi1 inhibition enhances the sensitivity of pancreatic cancer cells to gemcitabine." Oncotarget **7**(24): 37192-37204.

Figure 3.1 – HIF1α expression is regulated by Bmi1 in pancreatic cancer cells

A

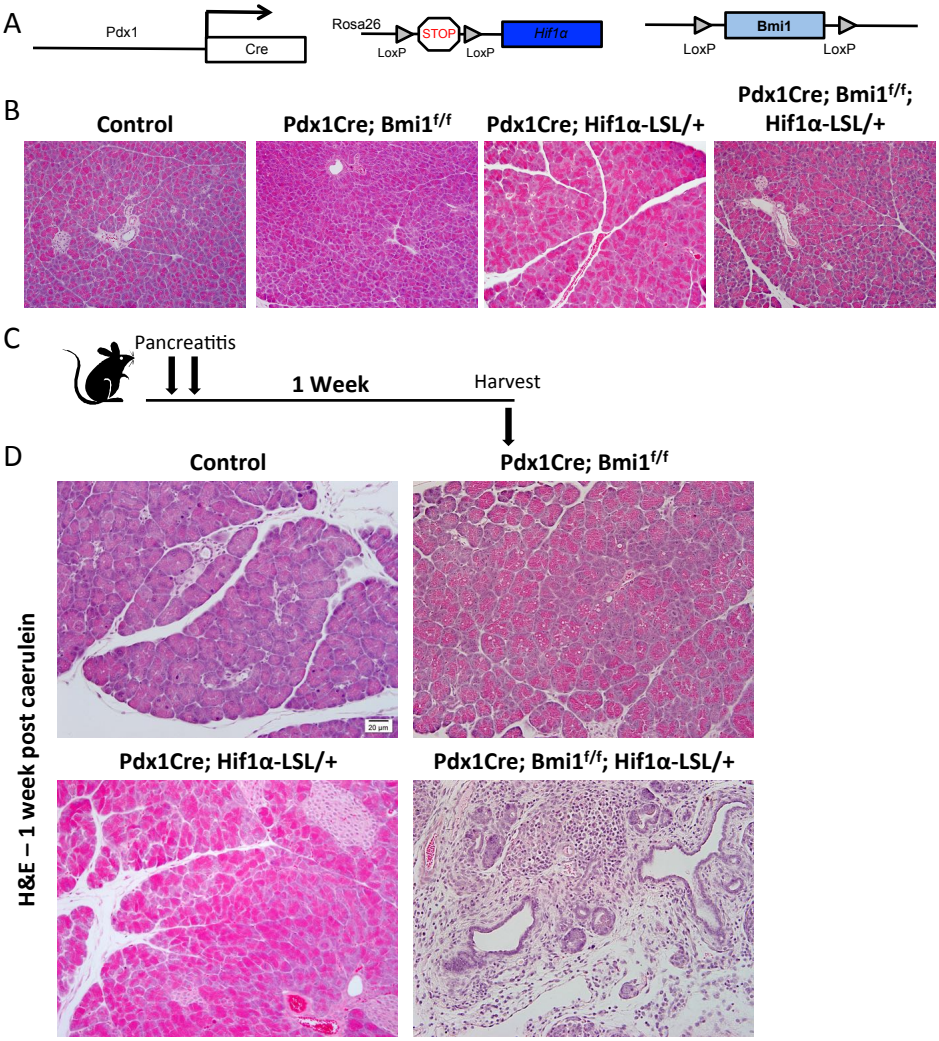


B



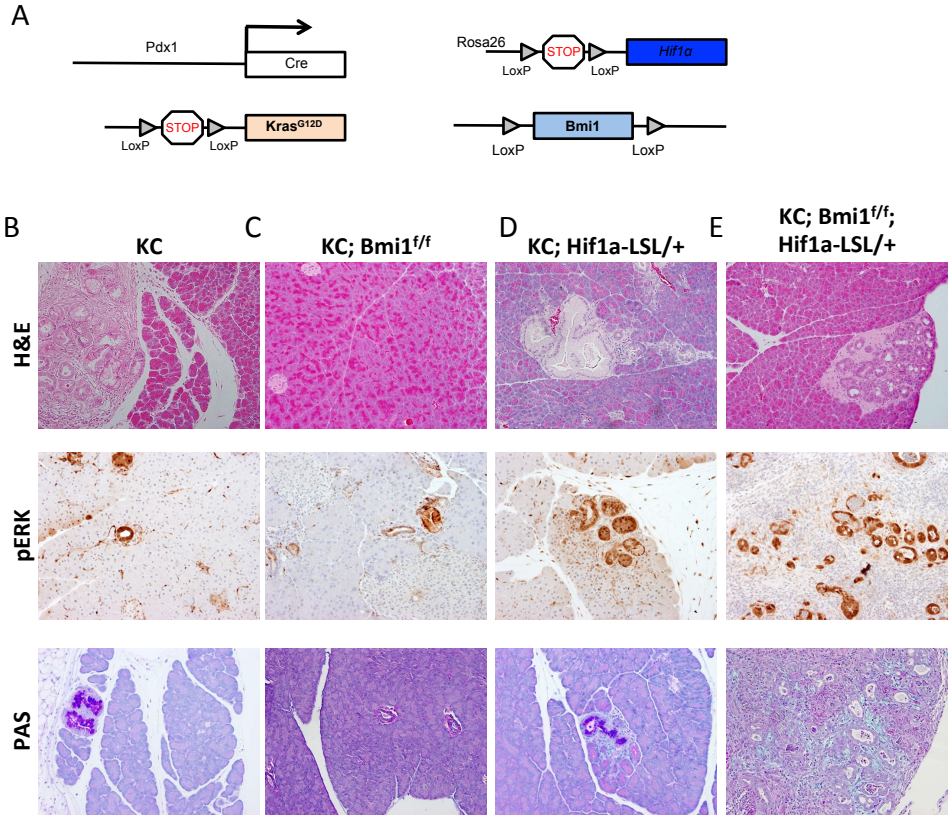
(A) qPCR analysis of KPC cells with Bmi1 knockdown siRNA or scrambled control. (B) qPCR analysis of KPC cells grown overnight in hypoxia with or without Bmi1 knockdown siRNA.

Figure 3.2 – Pancreata lacking Bmi1 and with stabilized HIF1α fail to recover from pancreatitis



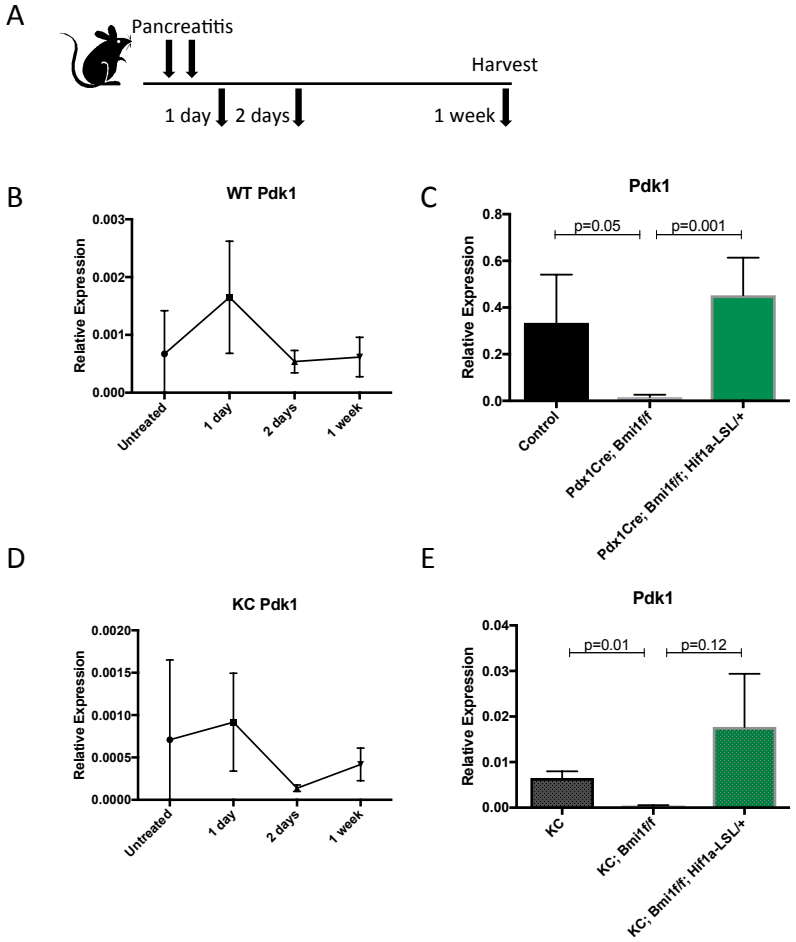
(A) Mouse scheme for transgenic animals. (B) H&E analysis of non-treated pancreata. (C) Scheme for pancreatitis treatment and harvesting. (D) H&E analysis of pancreata one week after pancreatitis treatment.

Figure 3.3 – HIF1α expression recovers the lack of PanINs observed in the absence of Bmi1



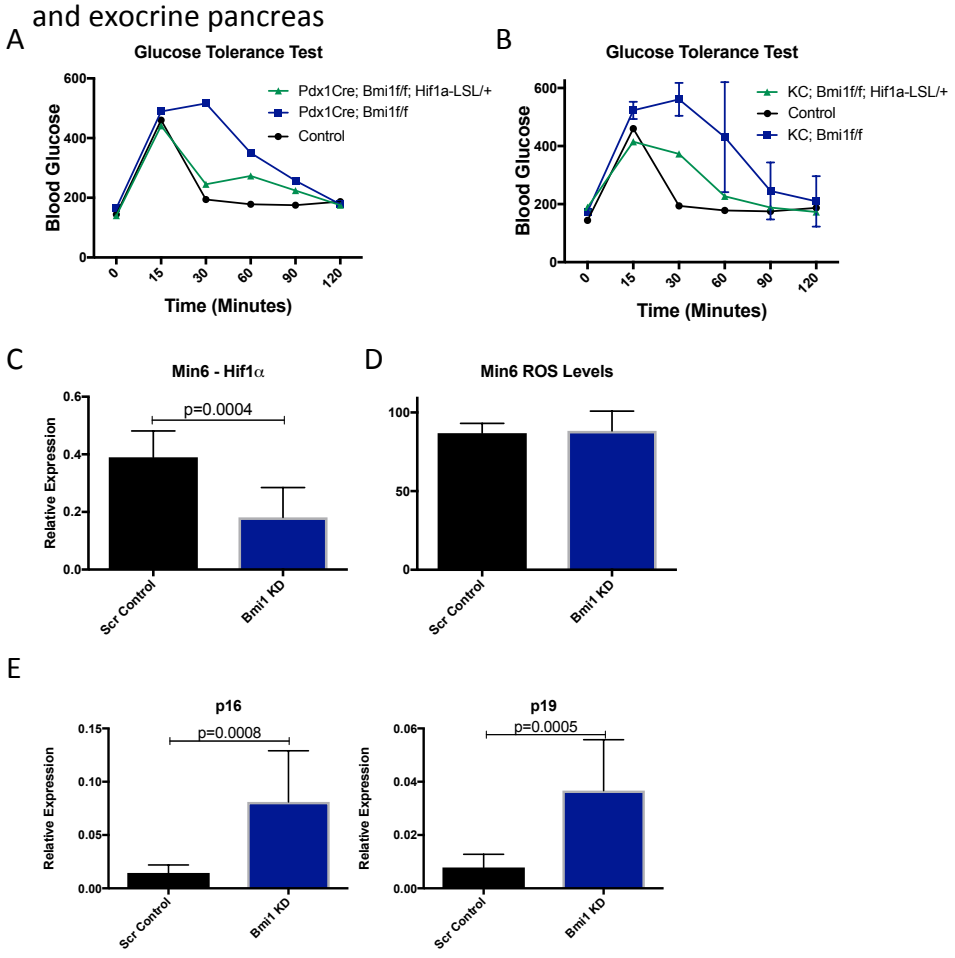
(A) Mouse scheme for transgenic animals. Histologic analysis, including H&E, pERK immunohistochemistry, and PAS staining in 12 week old (B) KC, (C) KC;Bmi1$^{f/f}$, (D) KC;HIF1α-LSL/+ and (D) KC;Bmi1$^{f/f}$;HIf1α-LSL/+ animals.

Figure 3.4 – Bmi1 and HIF1α control metabolic enzyme levels in the normal and precancerous pancreas



(A) Mouse scheme for pancreatitis treatment and harvesting. (B) qPCR analysis of mRNA levels in wild type pancreata treated with pancreatitis. (C) qPCR analysis of pancreata from transgenic animals one week after pancreatitis. (D) qPCR analysis of mRNA levels in KC pancreata treated with pancreatitis. (E)) qPCR analysis of pancreata from transgenic animals at 12 weeks of age.

Figure 3.5 – Differential roles for Bmi1 expression in the endocrine and exocrine pancreas



Glucose tolerance testing in (A) transgenic and (B) Kras expressing animals. (C) qPCR analysis of mRNA levels in Min6 cells with or without Bmi1 knockdown using siRNA. (D) ROS levels in Min6 cells with Bmi1 targeting siRNA or scrambled control, as measured by DCFDA. qPCR analysis of mRNA levels in Min6 cells for (E) p16 and (F) p19.

Chapter Four

Pancreatic HIF2α stabilization leads to chronic pancreatitis and predisposes to mucinous cystic neoplasm

Abstract

Tissue hypoxia controls cell differentiation in the embryonic pancreas, and promotes tumor growth in pancreatic cancer. The cellular response to hypoxia is controlled by the HIF proteins, including HIF2α. Here we show that HIF2α is not expressed in the normal human pancreas, however it is upregulated in human chronic pancreatitis. Stabilization of HIF2α in mouse pancreata leads to the development of chronic pancreatitis. In the presence of oncogenic Kras, HIF2α stabilization drives the formation of cysts resembling mucinous cystic neoplasm in human. Overall, we demonstrate the importance of HIF2α expression in the development of human and murine pancreatic disease.

Introduction

During pancreas development, oxygen levels are primarily ascribed to positive control of β-cell differentiation (Heinis, Simon et al. 2010). Pancreatic cancer, the third leading cause of cancer related death (Rahib, Smith et al. 2014), is extensively hypoxic, with low levels of oxygen throughout tumors (Buchler, Reber et al. 2003). Hypoxia has

been shown to promote pancreatic tumor growth, invasion, and metastasis (Shi, Abbruzzese et al. 1999, Duffy, Eibl et al. 2003). At the cellular level, the response and adaptation to hypoxia is controlled by hypoxia inducible factors, or HIFs. In vertebrates, the HIF family contains three isoforms, HIF1α, HIF2α, and HIF3α. The HIF proteins are transcription factors, activating genes containing a Hypoxia Response Element in response to low levels of cellular oxygen (Semenza and Wang 1992, Wang and Semenza 1993). In normal oxygen conditions, HIF proteins are hydroxylated post-translationally, allowing association with the von Hippel-Lindau tumor suppressor and tagging for proteosomal degradation (Triner and Shah 2016).

Hypoxia induces HIF1α and HIF2α expression in the pancreas (Wiesener, Jurgensen et al. 2003). HIF1α expression is induced during the development of pancreatic cancer, and its deletion promotes pancreatic tumorigenesis in a Kras driven model of pancreatic cancer (Lee, Spata et al. 2016). HIF2α expression is required for the development of the pancreas, and a lack of HIF2α expression in developing mice leads to smaller pancreata and decreased branching (Chen, Houshmand et al. 2010). In the presence of oncogenic Kras, HIF2α inactivation inhibited progression of precancerous lesions (Criscimanna, Duan et al. 2013).

The stress induced by the development of tumors can lead to the activation of multiple cellular response pathways in an attempt to restore homeostasis. In fact, while the hypoxia that exists in tumors does activate the HIF pathway, it can also activate other cell reactions, including the unfolded protein response. Activation of the unfolded protein response, via endoplasmic reticulum (ER) stress, and HIF signaling can collaborate in order to control the cellular response to tumorigenesis (Bi, Naczki et al.

2005, Wouters and Koritzinsky 2008, Hess, Humphrey et al. 2011, Pereira, Frudd et al. 2014). Given that pancreatic tumors are extensively hypoxic, it is vitally important to understand the role that the cellular response to hypoxia plays in the development and the progression of this disease.

Here we explored the effects of HIF2α stabilization in the pancreas, both in the context of pancreatitis and the initiation of pancreatic cancer.

Results and Discussion

HIF2α protein expression is not detectable in the normal human pancreas. However, we observed abundant HIF2α expression in human samples of chronic pancreatitis (Figure 4.1A). Since a functional role for HIF2α had not been described in this disease, we generated Pdx1-Cre;R26-$^{LSLHIF2\alpha\ /+}$ mice. In these animals, an oxygen-stable form of HIF2α(Kim, Safran et al. 2006) is expressed in the pancreas upon Cre recombination (Figure 4.1B). We observed that pancreata with stabilized HIF2α were smaller than in their littermate controls (Figure 4.1C, shown at 9 weeks of age). However, there was no difference in total body weight between the controls and HIF2α stabilized mice at all ages analyzed. From 7 weeks to 1 year of age HIF2α stabilized animals displayed similar total body weight to age-matched littermate control animals (Figure 4.1D).

At two weeks of age (n=2), animals expressing HIF2α had atrophic pancreatic parenchyma resembling end stage chronic pancreatitis (Figure 4.1E). By 4 weeks of age, shortly after weaning, HIF2α stabilized animals had developed further signs of chronic pancreatitis (Figure 4.1F). These observed changes progressed over time, with

few residual acini and significant inflammatory infiltrates observed at 9 weeks of age (n=5) (Figure 4.1G). In older mice, (n=4 analyzed at one year of age), pancreata were mostly replaced by adipose tissue with small remnant clusters of acinar cells, dilated ducts, and intermixed inflammatory infiltration (Figure 4.1H). These changes were quantified in 2-week-old animals using qPCR analysis for gene expression levels of pancreatic cell markers. By 2 weeks of age, HIF2α stabilized animals were not expressing amylase, indicating a loss of acinar cell function. At the same age, animals still expressed similar levels of CK19 compared to littermate controls, indicating that their ducts were still functional. Lastly, smooth muscle actin expression was increased in HIF2α stabilized mice, indicating an increase in fibroblast activity (Figure 4.1I). Thus, HIF2α overexpression in the pancreas resulted in a smaller pancreas with severe atrophy and chronic pancreatitis. Importantly, no mouse models of persistent chronic pancreatitis currently exist, making HIF2α stabilized mice a potentially useful tool for further understanding this condition.

HIF2α stabilization in experimental animals was confirmed by western blot. HIF2α expression was observed in those animals with HIF2α stabilized in the pancreas, and was not seen in control littermates (Figure 4.2A). Further, IHC analysis showed increased levels of HIF2α in the HIF2α stabilized pancreata, reflecting histologically that HIF2α is indeed stabilized in these mice (Figure 4.2B). Gene expression analysis by qPCR for HIF target genes revealed upregulation of HIF targets, including Pdk1 (Kim, Tchernyshyov et al. 2006), indicating functional upregulation of the HIF signaling pathway in HIF2α stabilized animals (Figure 4.2C). Given that there are two prevalent HIF proteins, HIF1α and HIF2α, we probed HIF1α protein expression in the same

human chronic pancreatitis samples in which we observed HIF2α upregulation. In human chronic pancreatitis HIF1α expression was not observed by western blot, indicating that HIF2α is upregulated independent of HIF1α in this disease (Figure 4.2D).

To further define the importance of HIF2α expression in the development of chronic pancreatitis, we developed further animals modeling HIF2α stabilization. First, we used a second pancreas specific cre recombinase, this time Ptf1a-Cre, to drive the same oxygen stable form of HIF2α, R26-$^{HIF2\alpha\ -LSL/+}$ (Figure 4.3A). Similar to our previous observations, Ptf1a-Cre;R26-$^{HIF2\alpha\ -LSL/+}$ animals displayed inflammatory infiltration and pancreatic atrophy resembling chronic pancreatitis (Figure 4.3B). The phenotype developed more slowly, with small pocket of inflammation at 1 month, progressing to full pancreatic involvement by 7 months of age (Figure 4.3B). Differences in the speed of phenotype progression may be due to differences between the two different cre recombinase alleles. We next sought to determine whether the observed chronic pancreatitis phenotype is specific to the HIF pathway by stabilizing HIF2α using a different method. We used Ptf1a-Cre along with expression of a floxed allele of VHL (Haase, Glickman et al. 2001) (Figure 4.3C). In normoxia, VHL acts to inhibit HIF function by tagging HIF proteins for degredation in the proteasome. Therefore, in Ptf1a-Cre;VHL$^{fl/fl}$ animals, absence of VHL expression leads to HIF stabilization specifically in the mouse pancreas. Histologic analysis of Ptf1a-Cre;VHL$^{fl/fl}$ mice revealed a similar chronic pancreatitis phenotype that progressed over time, observable from as early as 3 months of age (Figure 4.3D). Taken together, these data show that pancreatic stabilization of HIF2α, through a variety of methods, leads to the development of a phenotype resembling human chronic pancreatitis.

To determine whether HIF2α expression adversely affected embryonic development, we analyzed pancreata from very young animals. At one day of age, we observed no major differences in histology between HIF2α stabilized animals and their control littermates (n=2) (Figure 4.4A). Both control and HIF2α stabilized animals showed high levels of proliferation, as evidenced by positive staining for Ki67. Quantification of Ki67 positivity showed no difference in levels of proliferation between HIF2α stabilized animals and their control littermates (Figure 4.4B). One-day-old animals with HIF2α stabilization and their control littermates both showed low levels of cell death, as indicated by cleaved caspase 3 staining and quantified by cleaved caspase 3 positivity (Figure 4.4C). HIF2α stabilization was confirmed by IHC, where both control and HIF2α stabilized animals showed positive HIF2α expression in the developing islets, as expected, and only HIF2α stabilized pancreata displayed positive HIF2α staining throughout the pancreas (Figure 4.4D). The similarities in histology as well as cell proliferation and death in control and HIF2α stabilized animals at one day of age indicate that the HIF2α stabilization did not cause developmental defects.

We next further analyzed the phenotype of the development of chronic pancreatitis in young animals. By two weeks of age, we observed a drastic reduction in the numbers of acini, chronic inflammation, and foci of acinar-to-ductal metaplasia. At this age, the pancreas is undergoing active proliferation indicated by a high percentage of Ki67 positive cells, and, although the acinar cell population was reduced, proliferation in this compartment was still elevated as a result of HIF2α stabilization (Figure 4.5A). This was quantified by counting Ki67 positivity in both cohorts, showing an increase in proliferation in the HIF2α stabilized animals compared to control littermates (Figure

4.5A). Additionally, HIF2α expressing pancreata had increased apoptosis indicated by cleaved caspase 3 expression in epithelial cells, as seen by IHC and quantified (Figure 4.5B). Thus, in two-week old animals the chronic pancreatitis phenotype is actively developing, with evident loss of acini, cell death, and inflammatory cell infiltration. We next analyzed animals shortly after weaning, at 4 weeks of age. At this timepoint we observed a continuation of the phenotype that was developing at two weeks of age. Proliferation was increased in HIF2α stabilized animals as compared to control littermates, shown by staining for Ki67 and subsequent quantification (Figure 4.5C). Similarly, cell death, as measured by cleaved caspase 3, was elevated in HIF2α animals compared to controls (Figure 4.5D). Therefore, pancreatic HIF2α stabilization leads to chronic pancreatitis that actively progresses in developing mice, leading to increases in proliferation and cell death at both two weeks and four weeks of age.

We next sought to further define the identity of the remaining cells in the pancreata of HIF2α stabilized animals. Immunohistochemical analysis in nine-week-old HIF2α stabilized pancreata revealed individual acinar, ductal, and endocrine cells. However, Mist1 expression was reduced in acinar cells compared with control (Figure 4.6A). This was quantified by analyzing numbers of Mist1 positive cells, with control animals displaying much higher Mist1 positivity compared to HIF2α stabilized littermates (Figure 4.6A). As for ducts, we observed more Sox9-expressing tubules, indicating increased acinar-to-ductal metaplasia, and as quantified by analyzing Sox9 positivity (Figure 4.6B). Chromogranin A positivity in HIF2α stabilized animals highlighted the fact that islets remain in HIF2α stabilized pancreata (Figure 4.6C). qPCR analysis revealed higher levels of expression of the genes Bip and Chop in HIF2α stabilized animals,

indicating increases in the unfolded protein response due to endoplasmic reticulum (ER) stress(Hess, Humphrey et al. 2011) (Figure 4.6D). Previous studies have shown an association between ER stress and chronic pancreatitis(Sah, Garg et al. 2014). Therefore, HIF2α stabilized animals show higher levels of ductal markers and ER stress compared to control littermates, both consistent with chronic pancreatitis.

To further define the chronic pancreatitis phenotype in HIF2α stabilized mice, we analyzed animals for characteristic features of pancreatitis. Immunostaining for CD45 confirmed the presence of abundant inflammatory infiltrates in HIF2α stabilized pancreata, both by histology and quantification (Figure 4.7A). Gomori trichrome staining revealed increased levels of fibrosis (Figure 4.7B). Additionally, HIF2α stabilized pancreata expressed higher levels of genes that are associated with the fibrosis present in chronic pancreatitis, including TGFβ and MMP9 (Venkateshwari, Sri Manjari et al. 2011) (Branton and Kopp 1999) (Figure 4.7C). Overall, HIF2α stabilized animals display characteristic features of chronic pancreatitis, including immune infiltration, increased fibrosis and upregulation of expression of cytokines and fibrosis-associated genes. These features make HIF2α stabilized animals an exciting new experimental model of chronic pancreatitis.

Given the role of hypoxia in β-cell development, we analyzed the endocrine islets in nine-week-old HIF2α-stabilized mice. The islets in HIF2α stabilized pancreata were morphologically normal, and β-cells stained for insulin (Figure 4.8A). To measure islet function, we subjected nine-week-old animals to glucose tolerance testing. HIF2α stabilized animals had impaired glucose tolerance, with a sharper initial rise and sustained elevation of blood glucose levels compared to controls (n=5 animals per

genotype) (Figure 4.8B). To test for insulin secretion, we measured blood insulin levels in animals subjected to a glucose challenge. HIF2α stabilized animals showed no rise in blood insulin levels after glucose challenge (Figure 4.8C), indicating β-cell dysfunction (n=3 animals per genotype). This finding is consistent with the development of glucose intolerance in human chronic pancreatitis patients, termed Type 3c diabetes. (Ewald and Hardt 2013, Makuc 2016)

Chronic pancreatitis is a risk factor for the development of pancreatic cancer (Raimondi, Lowenfels et al. 2010). Additionally, animals lacking HIF2α expression in a mouse model of pancreatic cancer develop lesions that fail to progress to cancer, suggesting a role for HIF2α in pancreatic cancer development (Criscimanna, Duan et al. 2013). Therefore, we evaluated HIF2α stabilization in the context of pancreatic cancer. Mice that express oncogenic Kras in the pancreas, such as Pdx1-Cre; LSL-$Kras^{G12D}$ (KC) recapitulate the formation of pancreatic intraepithelial neoplasia (PanIN), a precursor lesion to pancreatic cancer (Hingorani, Petricoin et al. 2003). KC mice were crossed into mice with stably-expressed HIF2α, generating KC;HIF2α animals (Figure 4.9A). KC mice have sporadic PanINs within a largely normal pancreas at 9-12 weeks. Age-matched KC;HIF2α animals developed large cystic lesions with full penetrance (Figure 4.9B, arrows) (n=7 mice). Histologically, at nine to ten weeks of age, KC;HIF2α mice presented with a fibrotic pancreas and large cysts, resembling human mucinous cystic neoplasm (Figure 4.9C). To further define the KC;HIF2α phenotype, we developed multiple models of pancreatic HIF2α stabilization in the context of oncogenic Kras expression and analyzed their histology over time. We used another pancreas specific cre, Ptf1a-Cre, to express $Kras^{G12D}$. HIF2α stabilization was achieved either by

expressing an oxygen stable form of HIF2α or by deletion of VHL expression. KC animals develop PanINs, with their prevalence increasing over time from sporadic at one month of age to almost prevalent in the pancreas by 9 months (Figure 4.9D). Conversely, KC;HIF2α developed cystic lesions resembling human MCN. These cysts were small at one month of age and increased in size over time, up to 9 months (Figure 4.9E). Similar MCN histology was observed in KC;$VHL^{fl/fl}$ animals, with small, sporadic cystic lesions present at one month of age (Figure 4.9F). By 9 months pancreata in KC;$VHL^{fl/fl}$ mice were replaced by large cystic lesions similar to human MCN, indicating that the development of MCN in the context of oncogenic Kras is specific to the HIF pathway.

Similar to human histology, in KC;HIF2α mice the cystic lesions were lined by flat cuboidal epithelial cells with no papillary architecture and surrounded by stroma. Pdx1-Cre;Hif2a-stabilized mice expressed further histologic features characteristic of MCN, as described previously (Izeradjene, Combs et al. 2007). These include apical expression of mucin in the cystic epithelial cells, shown by PAS staining (Figure 4.10A). Additionally, human MCN displays a characteristic ovarian-type stroma, as evidenced by positive staining for estrogen receptor (ER) in the stromal cells, which is also found in KC;HIF2α animals (Figure 4.10B). Other histologic features characteristic of human MCN seen in the KC;HIF2α mice include CK19 expression in the cystic epithelial cells and not the stromal cells (Figure 4.10C) and expression of mesenchymal markers, including vimentin, in the stroma and not the epithelium (Figure 4.10D). Analysis of mucin expression in the animals showed expression of Muc1 in both KC and KC;HIF2α animals, as expected in both PanIN and MCN type lesions (Lau, Weiss et al. 2004)

(Figure 4.10E). Additionally, both the PanINs in KC animals and the cystic lesions in KC;HIF2α mice were positive for Muc5ac expression (Figure 4.10F). This pattern of Muc1 and Muc5ac expression has been previously described in MCNs, consistent with the pattern seen in KC;HIF2α animals (Matthaei, Schulick et al. 2011). Gene expression analysis of pancreatic cell type markers revealed decreased levels of amylase expression in KC;HIF2a mice compared to KC, as well as increases in both CK19 and SMA expression (Figure 4.10G). Overall, stabilization of HIF2α in the presence of oncogenic Kras recapitulated formation of human mucinous cystic neoplasm, a precursor lesion of pancreatic cancer.

MCN is associated with de-regulated Wnt signaling (Sano, Driscoll et al. 2014) and was modeled in mice by constitutively activating Wnt signaling (Sano, Driscoll et al. 2014). Additionally, HIF2α modulates Wnt expression during development of PanINs in the KC mouse model (Criscimanna, Duan et al. 2013). Accordingly, our analysis showed increased levels of Wnt target genes (Lef1, MYC and Axin) in KC;HIF2α animals compared to KC at nine weeks of age (Figure 4.11A). However given the significant remodeling of the KC;HIF2α pancreata, it is difficult to discern whether these changes in gene expression were caused by, or the cause of the MCN phenotype. Therefore, we analyzed expression of Wnt target genes histologically by IHC. Increased levels of Lef1 expression were observed in the stroma of MCN of KC;HIF2α animals (Figure 4.11B). Importantly, very few mouse models of MCN have been described. Given that human MCN is a much less well-understood precursor lesion than PanINs, it is vital to develop additional mouse models to probe MCN biology and understand its formation and progression.

Here we show that HIF2α expression increases in human pancreata with chronic pancreatitis. The expression of an oxygen stable form of HIF2α in the mouse pancreas results in chronic pancreatitis and atrophy, with loss of acini and increased chronic inflammation in the lobule. Additionally, stabilization of HIF2α along with oncogenic Kras expression recapitulates human MCN. Thus, we describe two new models of human disease, and provide new insight into the role of hypoxia signaling, specifically through HIF2α, in the pancreas.

Methods

Mice

Mice were housed in the specific pathogen free facility at the University of Michigan Comprehensive Cancer Center. This study was approved by the University of Michigan Committee on Use and Care of Animals (UCUCA). Pdx1-Cre, Ptf1a-Cre, LSL-$Kras^{G12D}$, R26-$^{LSLHif2a/+}$ and VHL floxed mice have been described previously (Haase, Glickman et al. 2001, Hingorani, Petricoin et al. 2003, Kim, Safran et al. 2006).

Glucose tolerance testing

Glucose tolerance testing was performed as previously described (Flak, Patterson et al. 2014). Prior to testing, animals were fasted for four hours during the light cycle. Initial blood glucose levels were measured using tail blood samples. Then animals were administered glucose at a dose of 2g glucose per kg body weight by intraperitoneal injection. Tail blood samples were then measured for blood glucose levels at 15, 30, 60,

90, and 120 minutes post-glucose injection. Blood glucose was measured using Accu-Chek Aviva diabetes monitoring kit and Accu-Chek Aviva Plus testing strips.

Glucose Stimulated Insulin Secretion

Overnight fasted mice were anesthetized by injecting intraperitoneally with Avertin. Anesthetized mice were then injected intraperitoneally with glucose at 3g/kg body weight and blood was collected retroorbitally at 0, 2, 7, 15 and 30 minutes. Serum was separated by centrifuging the blood at 8,000 rpm for 8 min at 4C. Serum insulin was measured using Ultrasensitive mouse insulin ELISA kit (CrystalChem, Downers Grove, IL), following manufacturers recommendation.

Immunohistochemistry and Immunofluorescence

Histology and immunohistochemistry studies, as well as PAS and Gomori Trichrome staining, were performed as previously described (Collins, Bednar et al. 2012). To prepare for staining, tissue was collected and fixed overnight in 10% neutral buffered formalin. Tissue was then embedded in paraffin and sectioned. The University of Michigan Cancer Center Histopathology Core performed embedding and sectioning. Sections were imaged using an Olympus BX-51 microscope, Olympus DP71 digital camera, and CellSens Standard software. Primary antibodies used are included in Supplemental Table 1.

qRT-PCR

Tissue for RNA extraction was collected in lysis buffer (Ambion) and RNA was isolated using the PureLink RNA Mini Kit (Ambion). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers were optimized for amplification conditions of: 95C for 10 minutes, then 40 cycles of 95C for 15 seconds and 60C for 1 minute. Melt curve analysis was performed for all samples. *Cyclophilin A* was used as the control housekeeping gene for normalization. Primer sequences for genes analyzed are: Axin F – GCCAATGGCCAAGTGTCTCT, Axin R – GCGTCATCTCCTTGGGCA, Lef1 F – AGTGCAGCTATCAACCAGATCCT, Lef1 – R TTTCCGTGCTAGTTCATAGTATTTGG, MYC F – TGAGCCCCTAGTGCTGCAT, MYC R – AGCCCGACTCCGACCTCTT, TGFB3 F – CAGGCCAGGGTAGTCAGAG, TGFB3 R – ATTTCCAGCCTAGATCCTGCC, MMP9 F – CTGGACAGCCAGACACTAAAG, MMP9 R – CTCGCGGCAAGTCTTCAGAG.

Western Blot

Tissue for protein extraction was collected in RIPA buffer with protease inhibitor. Equal amounts of protein were added per lane, run by electrophoresis in SDS-PAGE gels, and transferred to PVDF membranes. Each membrane was blocked with 5% milk. Primary antibody incubation was performed overnight at 4C. Secondary antibody incubation was performed for two hours at room temperature. Protein bands were detected using Western Lightning Plus Enhanced Chemiluminescence (PerkinElmer, NEL103001EA) and film.

Statistics

Statistical significance for qRT-PCR results was determined by unpaired two-tailed t test, with the threshold for statistical significance determined to be $p<0.05$.

Study Approval

The University of Michigan Institutional Review Board (IRB) approved all human studies. Informed consent was received from all human patients prior to inclusion in the studies detailed in this work. All animal studies were approved by the University of Michigan Committee on Use and Care of Animals (UCUCA).

References

Bi, M., C. Naczki, M. Koritzinsky, D. Fels, J. Blais, N. Hu, H. Harding, I. Novoa, M. Varia, J. Raleigh, D. Scheuner, R. J. Kaufman, J. Bell, D. Ron, B. G. Wouters and C. Koumenis (2005). "ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth." EMBO J **24**(19): 3470-3481.

Branton, M. H. and J. B. Kopp (1999). "TGF-beta and fibrosis." Microbes Infect **1**(15): 1349-1365.

Buchler, P., H. A. Reber, M. Buchler, S. Shrinkante, M. W. Buchler, H. Friess, G. L. Semenza and O. J. Hines (2003). "Hypoxia-inducible factor 1 regulates vascular endothelial growth factor expression in human pancreatic cancer." Pancreas **26**(1): 56-64.

Chen, H., G. Houshmand, S. Mishra, G. H. Fong, G. K. Gittes and F. Esni (2010). "Impaired pancreatic development in Hif2-alpha deficient mice." Biochem Biophys Res Commun **399**(3): 440-445.

Collins, M. A., F. Bednar, Y. Zhang, J. C. Brisset, S. Galban, C. J. Galban, S. Rakshit, K. S. Flannagan, N. V. Adsay and M. Pasca di Magliano (2012). "Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice." J Clin Invest **122**(2): 639-653.

Criscimanna, A., L. J. Duan, J. A. Rhodes, V. Fendrich, E. Wickline, D. J. Hartman, S. P. Monga, M. T. Lotze, G. K. Gittes, G. H. Fong and F. Esni (2013). "PanIN-specific regulation of Wnt signaling by HIF2alpha during early pancreatic tumorigenesis." Cancer Res **73**(15): 4781-4790.

Duffy, J. P., G. Eibl, H. A. Reber and O. J. Hines (2003). "Influence of hypoxia and neoangiogenesis on the growth of pancreatic cancer." Mol Cancer **2**: 12.

Ewald, N. and P. D. Hardt (2013). "Diagnosis and treatment of diabetes mellitus in chronic pancreatitis." World J Gastroenterol **19**(42): 7276-7281.

Flak, J. N., C. M. Patterson, A. S. Garfield, G. D'Agostino, P. B. Goforth, A. K. Sutton, P. A. Malec, J. M. Wong, M. Germani, J. C. Jones, M. Rajala, L. Satin, C. J. Rhodes, D. P. Olson, R. T. Kennedy, L. K. Heisler and M. G. Myers, Jr. (2014). "Leptin-inhibited PBN neurons enhance responses to hypoglycemia in negative energy balance." Nat Neurosci **17**(12): 1744-1750.

Haase, V. H., J. N. Glickman, M. Socolovsky and R. Jaenisch (2001). "Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor." Proc Natl Acad Sci U S A **98**(4): 1583-1588.

Heinis, M., M. T. Simon, K. Ilc, N. M. Mazure, J. Pouyssegur, R. Scharfmann and B. Duvillie (2010). "Oxygen tension regulates pancreatic beta-cell differentiation through hypoxia-inducible factor 1alpha." Diabetes **59**(3): 662-669.

Hess, D. A., S. E. Humphrey, J. Ishibashi, B. Damsz, A. H. Lee, L. H. Glimcher and S. F. Konieczny (2011). "Extensive pancreas regeneration following acinar-specific disruption of Xbp1 in mice." Gastroenterology **141**(4): 1463-1472.

Hingorani, S. R., E. F. Petricoin, A. Maitra, V. Rajapakse, C. King, M. A. Jacobetz, S. Ross, T. P. Conrads, T. D. Veenstra, B. A. Hitt, Y. Kawaguchi, D. Johann, L. A. Liotta, H. C. Crawford, M. E. Putt, T. Jacks, C. V. Wright, R. H. Hruban, A. M. Lowy and D. A. Tuveson (2003). "Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse." Cancer Cell **4**(6): 437-450.

Izeradjene, K., C. Combs, M. Best, A. Gopinathan, A. Wagner, W. M. Grady, C. X. Deng, R. H. Hruban, N. V. Adsay, D. A. Tuveson and S. R. Hingorani (2007). "Kras(G12D) and Smad4/Dpc4 haploinsufficiency cooperate to induce mucinous cystic neoplasms and invasive adenocarcinoma of the pancreas." Cancer Cell **11**(3): 229-243.

Kim, J. W., I. Tchernyshyov, G. L. Semenza and C. V. Dang (2006). "HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia." Cell Metab **3**(3): 177-185.

Kim, W. Y., M. Safran, M. R. Buckley, B. L. Ebert, J. Glickman, M. Bosenberg, M. Regan and W. G. Kaelin, Jr. (2006). "Failure to prolyl hydroxylate hypoxia-inducible factor alpha phenocopies VHL inactivation in vivo." EMBO J **25**(19): 4650-4662.

Lau, S. K., L. M. Weiss and P. G. Chu (2004). "Differential expression of MUC1, MUC2, and MUC5AC in carcinomas of various sites: an immunohistochemical study." Am J Clin Pathol **122**(1): 61-69.

Lee, K. E., M. Spata, L. J. Bayne, E. L. Buza, A. C. Durham, D. Allman, R. H. Vonderheide and M. C. Simon (2016). "Hif1a Deletion Reveals Pro-Neoplastic Function of B Cells in Pancreatic Neoplasia." Cancer Discov **6**(3): 256-269.

Makuc, J. (2016). "Management of pancreatogenic diabetes: challenges and solutions." Diabetes Metab Syndr Obes **9**: 311-315.

Matthaei, H., R. D. Schulick, R. H. Hruban and A. Maitra (2011). "Cystic precursors to invasive pancreatic cancer." Nat Rev Gastroenterol Hepatol **8**(3): 141-150.

Pereira, E. R., K. Frudd, W. Awad and L. M. Hendershot (2014). "Endoplasmic reticulum (ER) stress and hypoxia response pathways interact to potentiate hypoxia-inducible factor 1 (HIF-1) transcriptional activity on targets like vascular endothelial growth factor (VEGF)." J Biol Chem **289**(6): 3352-3364.

Rahib, L., B. D. Smith, R. Aizenberg, A. B. Rosenzweig, J. M. Fleshman and L. M. Matrisian (2014). "Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States." Cancer Res **74**(11): 2913-2921.

Raimondi, S., A. B. Lowenfels, A. M. Morselli-Labate, P. Maisonneuve and R. Pezzilli (2010). "Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection." Best Pract Res Clin Gastroenterol **24**(3): 349-358.

Sah, R. P., S. K. Garg, A. K. Dixit, V. Dudeja, R. K. Dawra and A. K. Saluja (2014). "Endoplasmic reticulum stress is chronically activated in chronic pancreatitis." J Biol Chem **289**(40): 27551-27561.

Sano, M., D. R. Driscoll, W. E. De Jesus-Monge, D. S. Klimstra and B. C. Lewis (2014). "Activated wnt signaling in stroma contributes to development of pancreatic mucinous cystic neoplasms." Gastroenterology **146**(1): 257-267.

Semenza, G. L. and G. L. Wang (1992). "A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation." Mol Cell Biol **12**(12): 5447-5454.

Shi, Q., J. L. Abbruzzese, S. Huang, I. J. Fidler, Q. Xiong and K. Xie (1999). "Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic." Clin Cancer Res **5**(11): 3711-3721.

Triner, D. and Y. M. Shah (2016). "Hypoxia-inducible factors: a central link between inflammation and cancer." J Clin Invest **126**(10): 3689-3698.

Venkateshwari, A., K. Sri Manjari, D. Krishnaveni, P. Nallari, A. Vidyasagar and A. Jyothy (2011). "Role of Plasma MMP 9 levels in the Pathogenesis of Chronic Pancreatitis." Indian J Clin Biochem **26**(2): 136-139.

Wang, G. L. and G. L. Semenza (1993). "Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia." J Biol Chem **268**(29): 21513-21518.

Wiesener, M. S., J. S. Jurgensen, C. Rosenberger, C. K. Scholze, J. H. Horstrup, C. Warnecke, S. Mandriota, I. Bechmann, U. A. Frei, C. W. Pugh, P. J. Ratcliffe, S. Bachmann, P. H. Maxwell and K. U. Eckardt (2003). "Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs." FASEB J **17**(2): 271-273.

Wouters, B. G. and M. Koritzinsky (2008). "Hypoxia signalling through mTOR and the unfolded protein response in cancer." Nat Rev Cancer **8**(11): 851-864.

Acknowledgements

We would like to thank Howard Crawford (University of Michigan) for use of his lab equipment and helpful scientific discussion. HKS is supported by National Institutes of Health (NIH) grants T32 GM007315-38 and T32 DK094775-04. MJP was supported by a University of Michigan Postdoctoral Translational Scholars Program Award (UL1TR000433) and SKR was supported by K99/R00 award (DK110537). This project was supported by R01CA151588, by Funding from the Hirshberg Foundation for Cancer Research (MPdM) and by The Cancer Center Core Grant P30CA46592; R01 CA148828494 (YMS); R01 DK47918 and the Department of Veterans Affairs (MBO); and NIH grant P30 DK34933 to the University of Michigan.

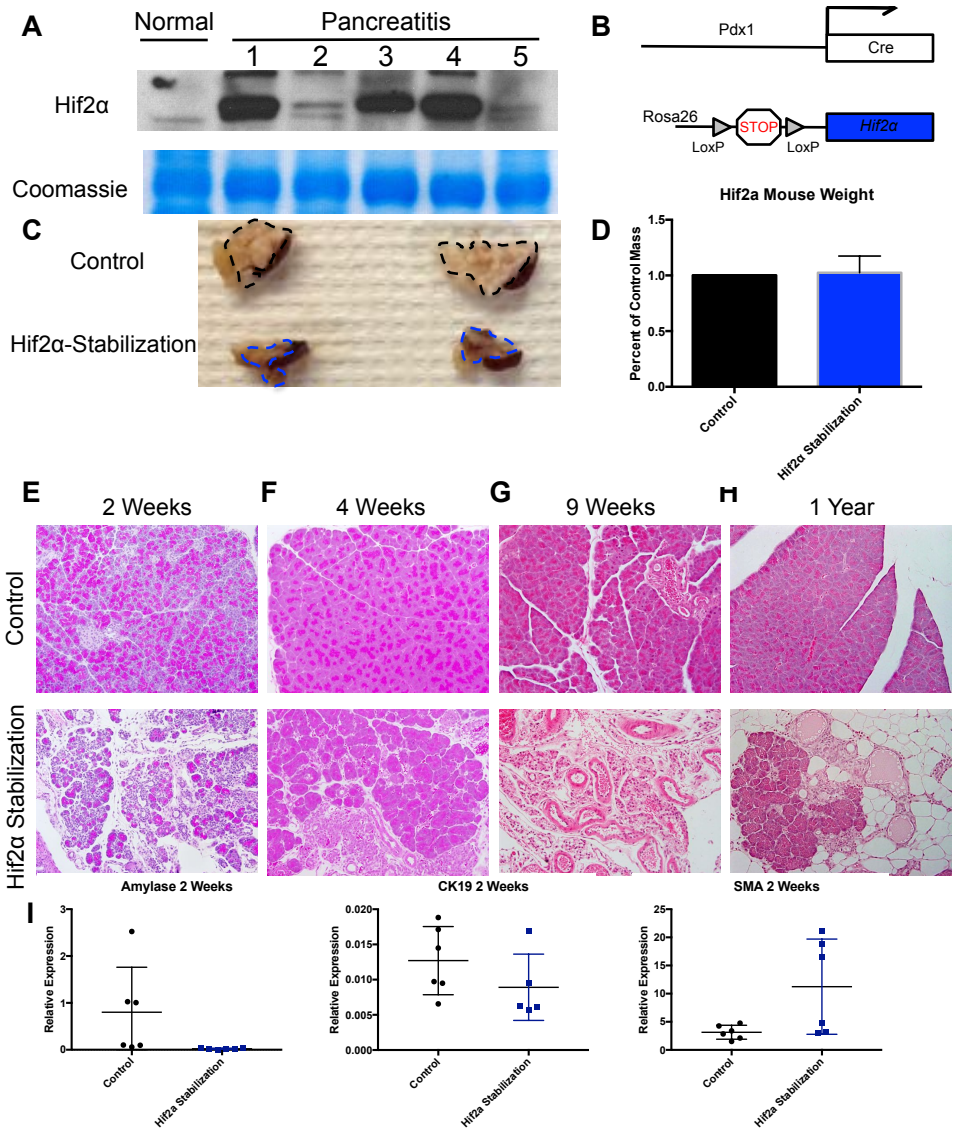


Figure 4.1 – HIF2α expression is associated with chronic pancreatitis in human and mouse
(A) Western blot analysis of HIF2α expression in lysates from human normal and chronic pancreatitis pancreata. (B) Transgenic mouse scheme. (C) Gross morphology of control and HIF2α stabilized pancreata. (D) Average mouse weight of animals with HIF2α stabilization as compared to controls. H&E evaluation of (E) 2 week, (F) 4 week, (G) 9 week and (H) 1 year old pancreata. (I) qPCR analysis of Amylase, CK19 and SMA at 2 weeks of age.

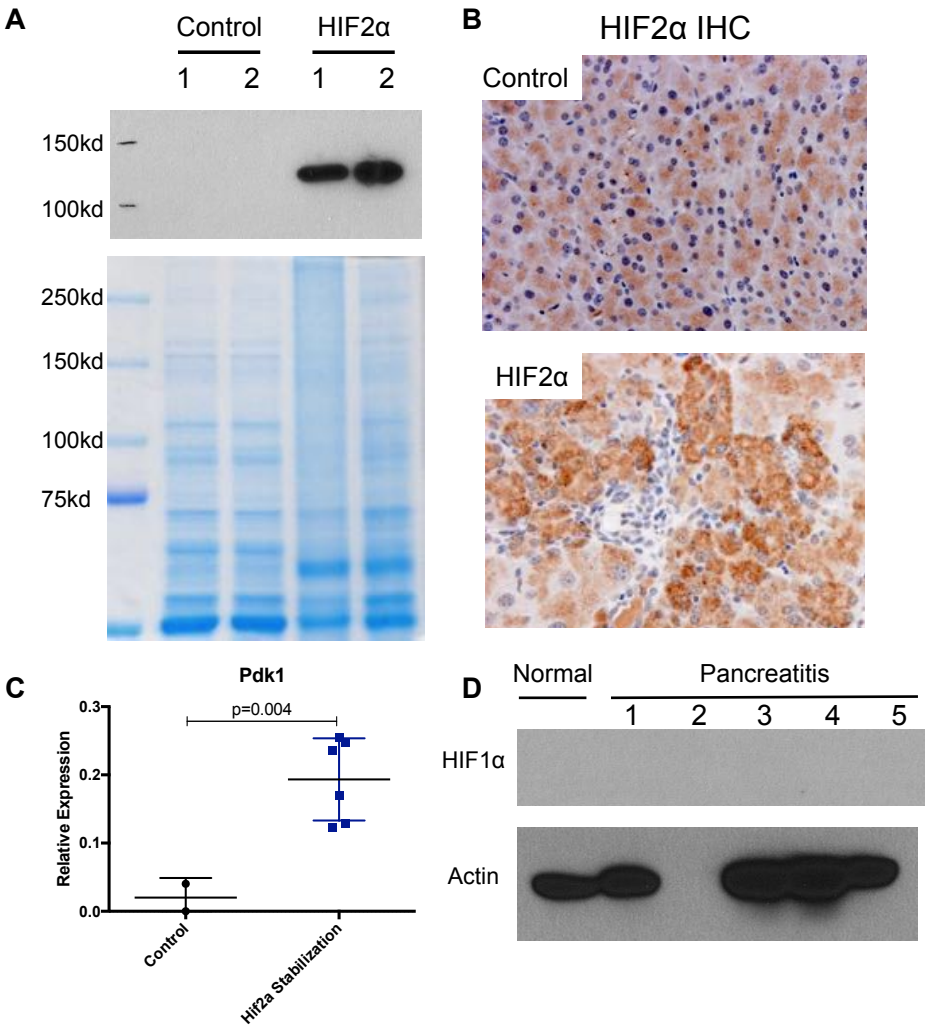


Figure 4.2– Validation of HIF2α stabilization in mouse pancreata
(A) Western blot for HIF2α in lysates from control and HIF2α stabilized pancreata, with commassie stain for loading control. (B) Immunohistochemistry for HIF2α in control and HIF2α stabilized pancreata. (C) qPCR analysis for levels of HIF target Pdk1. (D) Western blot for HIF1a levels in human normal and chronic pancreatitis samples.

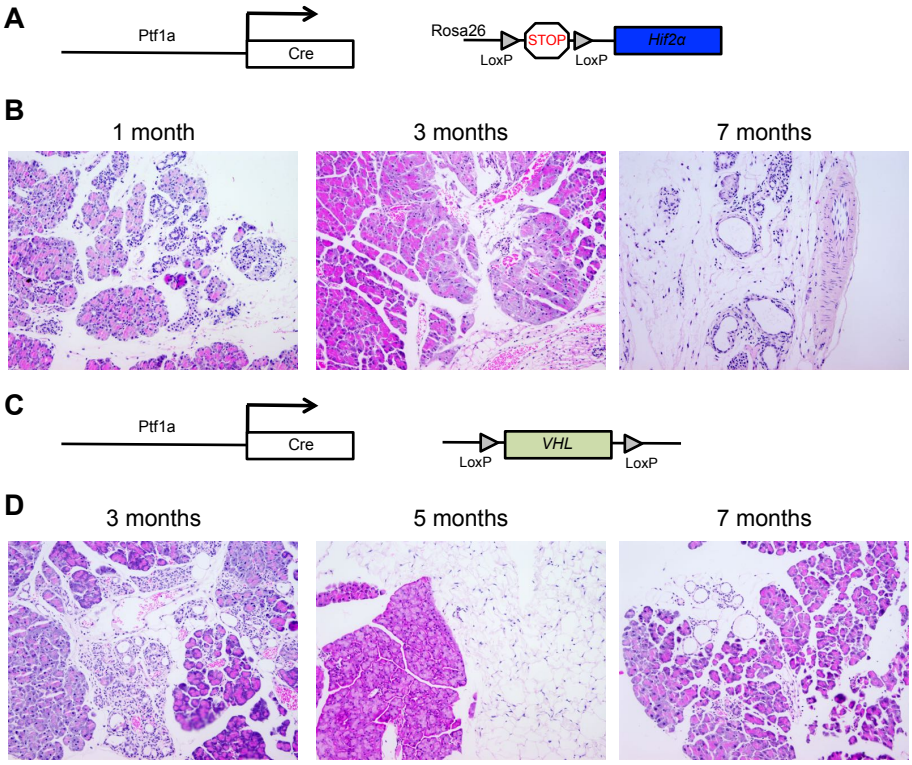


Figure 4.3 –HIF2α stabilization leads to chronic pancreatitis in multiple mouse models
(A) Transgenic mouse scheme for HIF2a stabilization. (B) H&E evaluation of HIF2a stabilized pancreata at 1 month, 3 months and 7 months. (C) Transgenic mouse scheme for HIF2a stabilization via pancreatic VHL deletion. (D) H&E evaluation of pancreata lacking VHL expression at 3 months, 5 months and 7 months.

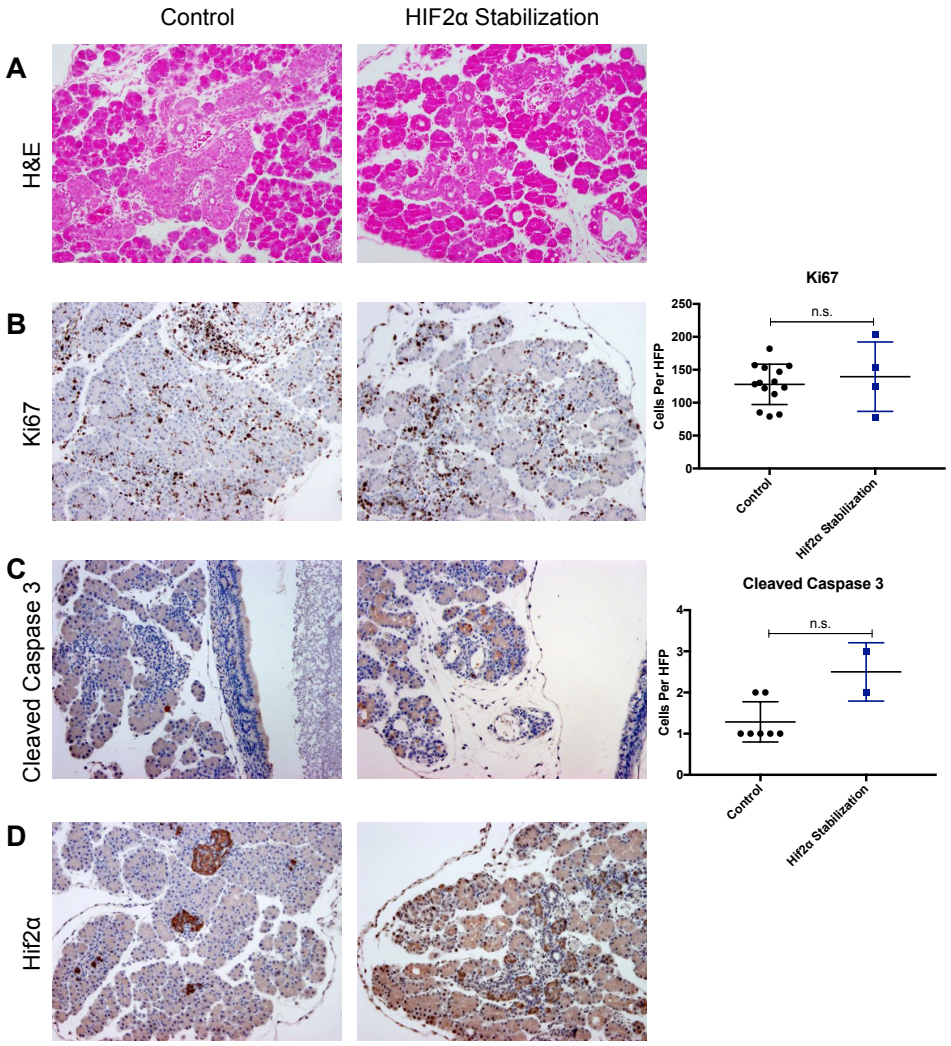


Figure 4.4– Pancreas histology in 1-day old HIF2α pancreata
(A) H&E analysis for control and HIF2a stabilized pancreata at one day. Immunohistochemistry and quantification for (B) Ki67 and (C) Cleaved Caspase 3 in 1-day-old animals. (D) Immunohistochemistry for HIF2a.

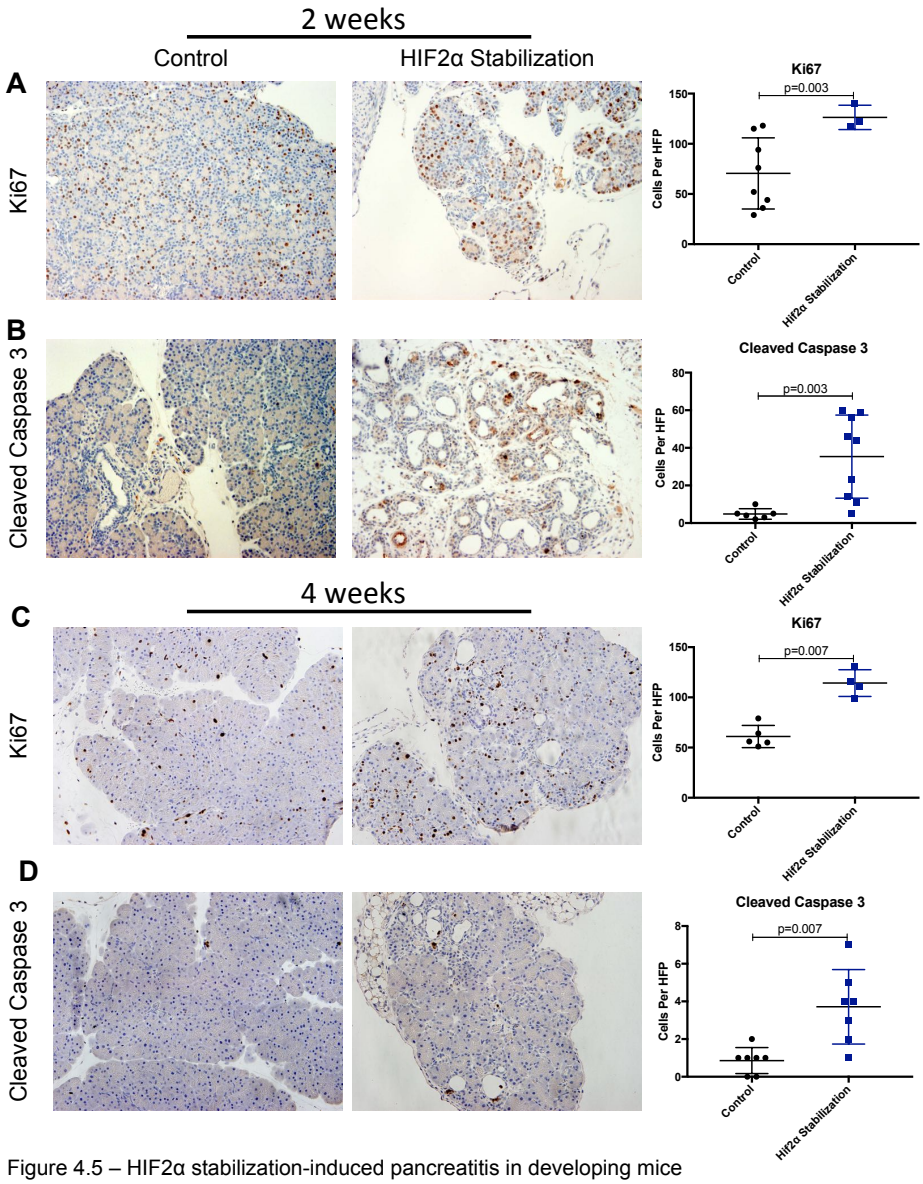


Figure 4.5 – HIF2α stabilization-induced pancreatitis in developing mice
Immunohistochemistry and quantification for (A) Ki67 and (B) Cleaved Caspase 3 in 2-week-old animals. Immunohistochemistry and quantification for (C) Ki67 and (D) Cleaved Caspase 3 in 4-week-old animals.

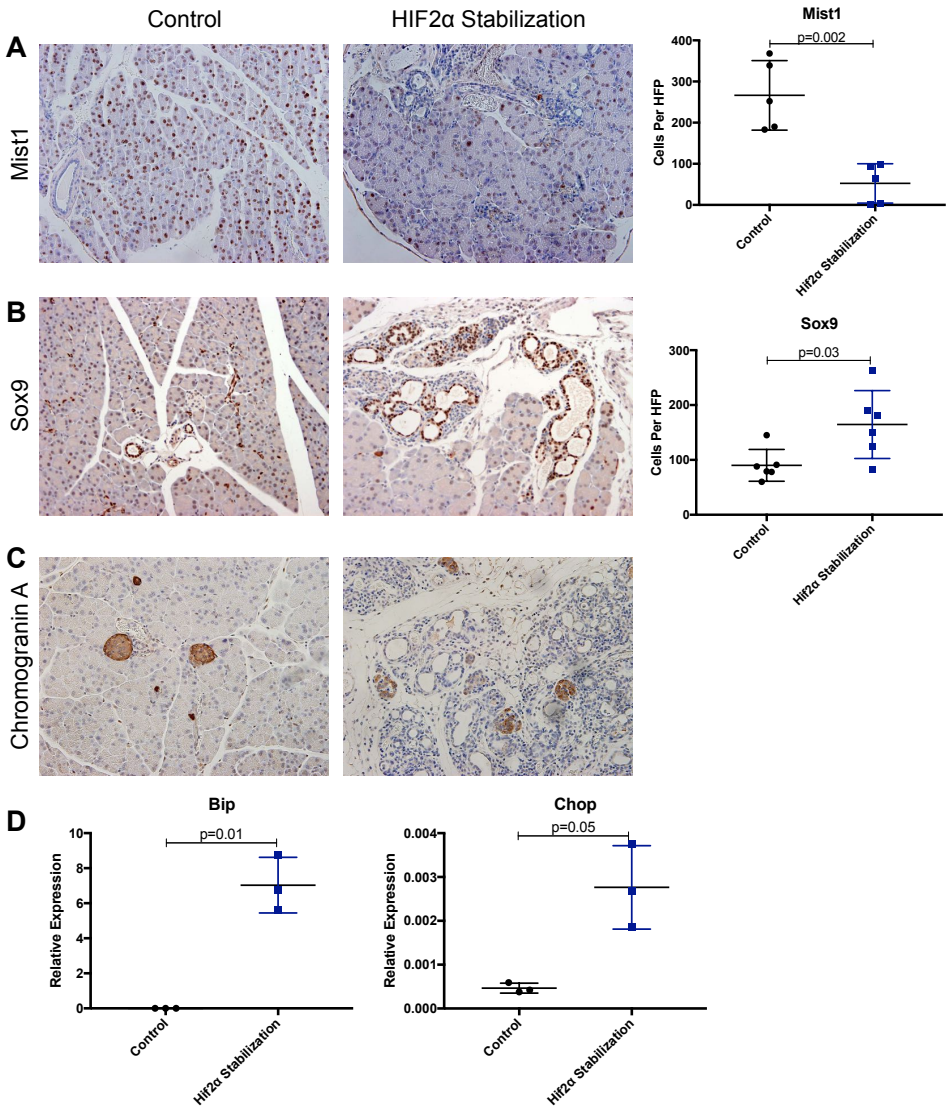


Figure 4.6 – Immunostaining for lineage markers in 9-week-old HIF2α pancreata Immunohistochemistry and quantification for (A) Mist1 and (B) Sox9 in 9-week-old animals. (C) Immunohistochemistry for Chromogranin A in 9-week-old animals. (D) qPCR analysis for ER stress markers Bip and Chop in 9-week-old animals.

Figure 7 – Chronic pancreatitis 9-week-old in HIF2α mice

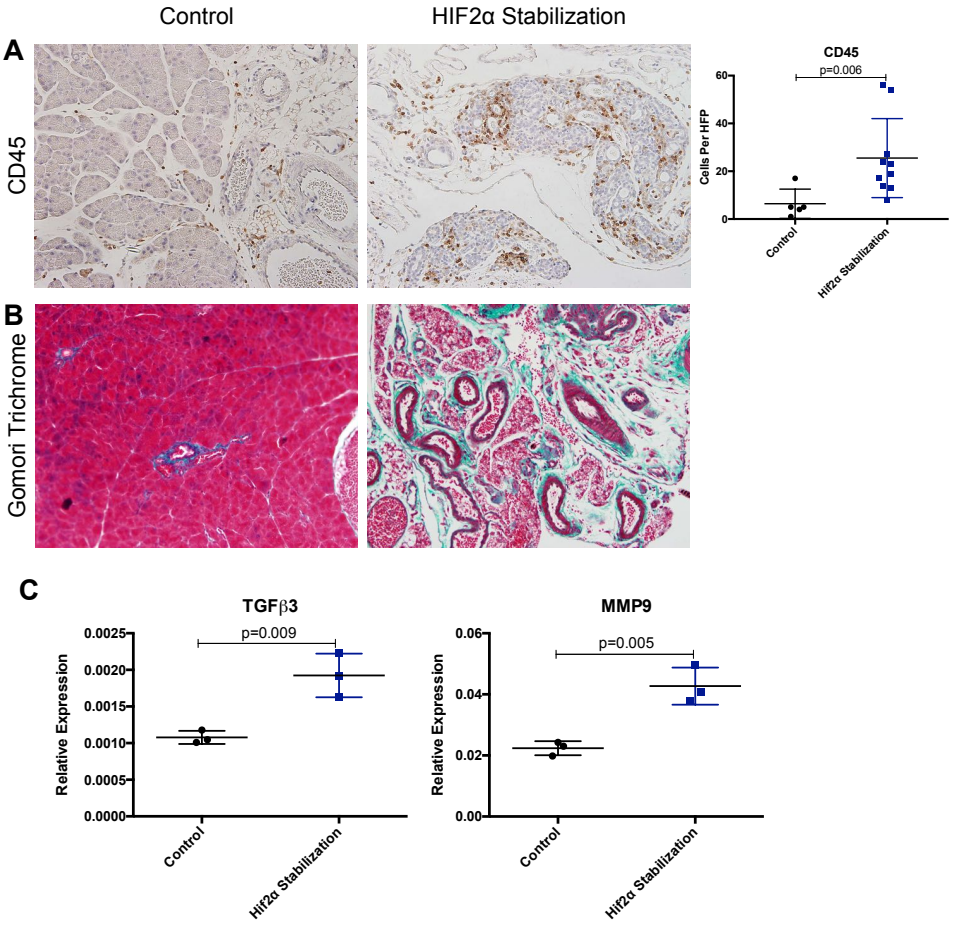


Figure 4.7 – Chronic pancreatitis 9-week-old in HIF2α mice
(A) Immunohistochemistry and quantification for CD45 in 9-week-old mice. (B) Gomori trichrome staining in 9-week-old animals. (C) qRT-PCR analysis of control and HIF2α stabilized pancreata.

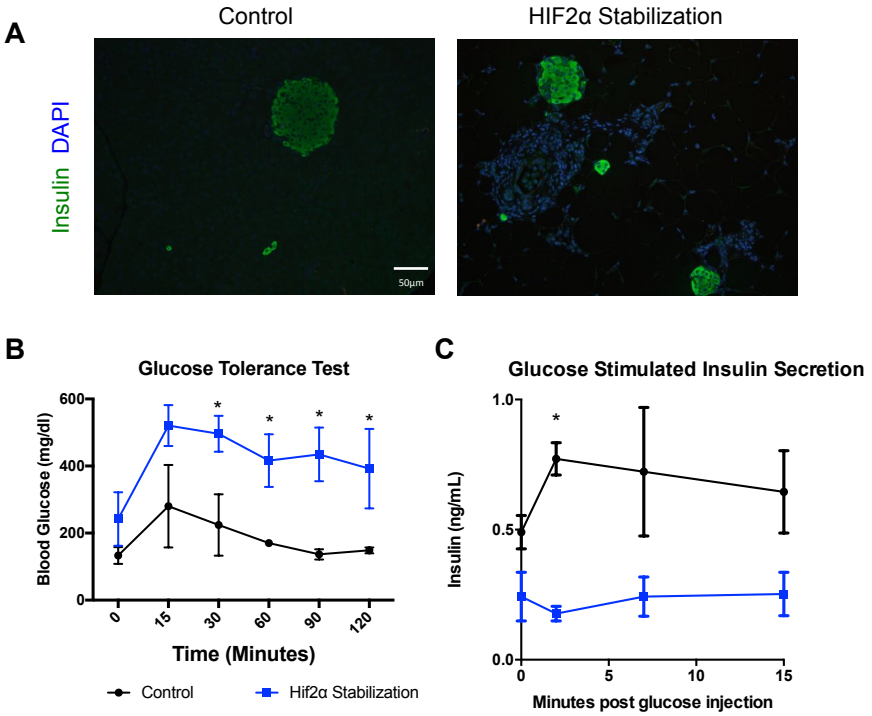


Figure 4.8 – HIF2α stabilization causes endocrine pancreas dysfunction
(A) Immunofluorescence for Insulin and DAPI in 9-week-old animals. (B) Glucose tolerance test and (C) glucose stimulated insulin secretion in 9-week-old animals.

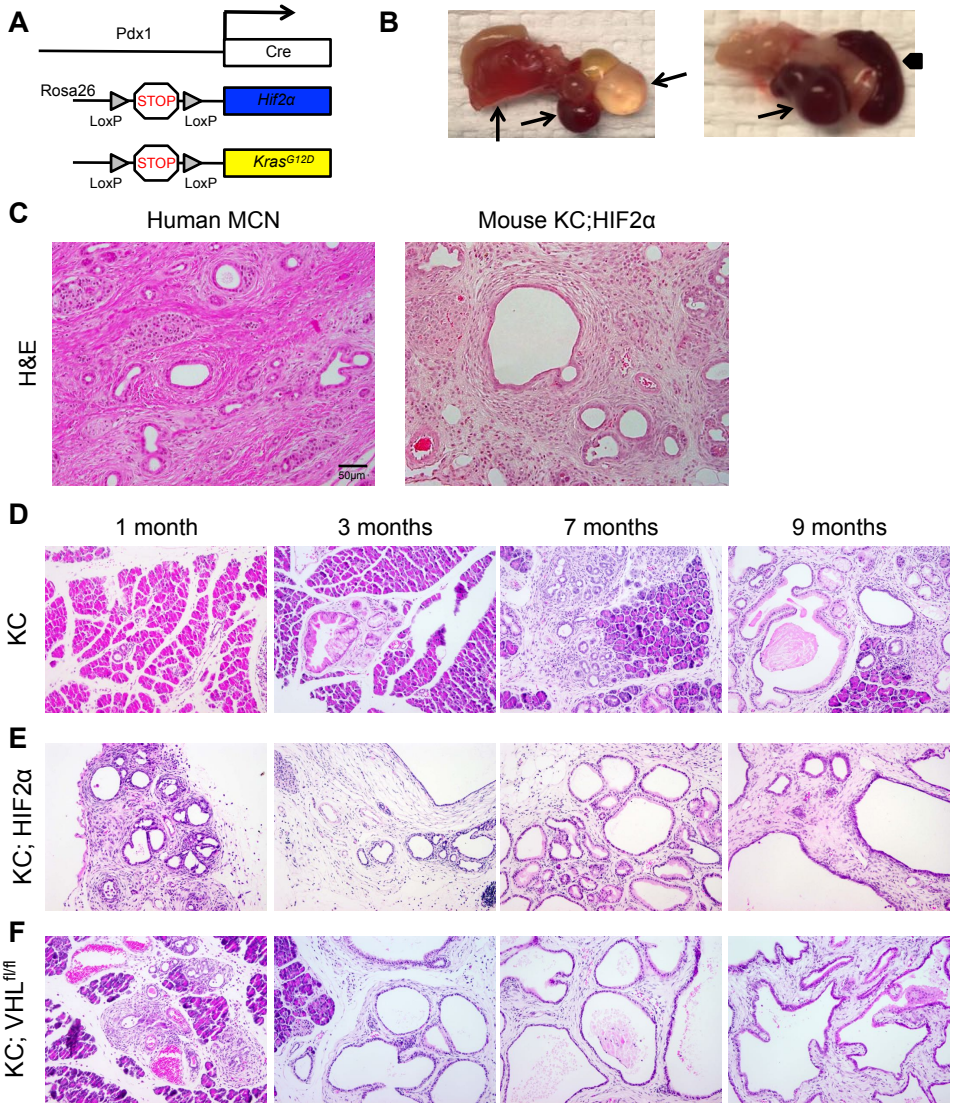


Figure 4.9 – HIF2α stabilization during pancreatic cancer initiation mimics Mucinous Cystic Neoplasms

(A) Transgenic mouse scheme. (B). Gross morphology of two separate KC;HIF2α animals at 9 weeks of age. Arrows indicate cysts, arrowhead indicates spleen. (C) H&E evaluation of human MCN and KC; HIF2α pancreata. H&E evaluation of (D) KC, (E) KC;HIF2a and (F) KC;VHL$^{fl/fl}$ animals at 1 month, 3 months, 7 months and 9 months of age.

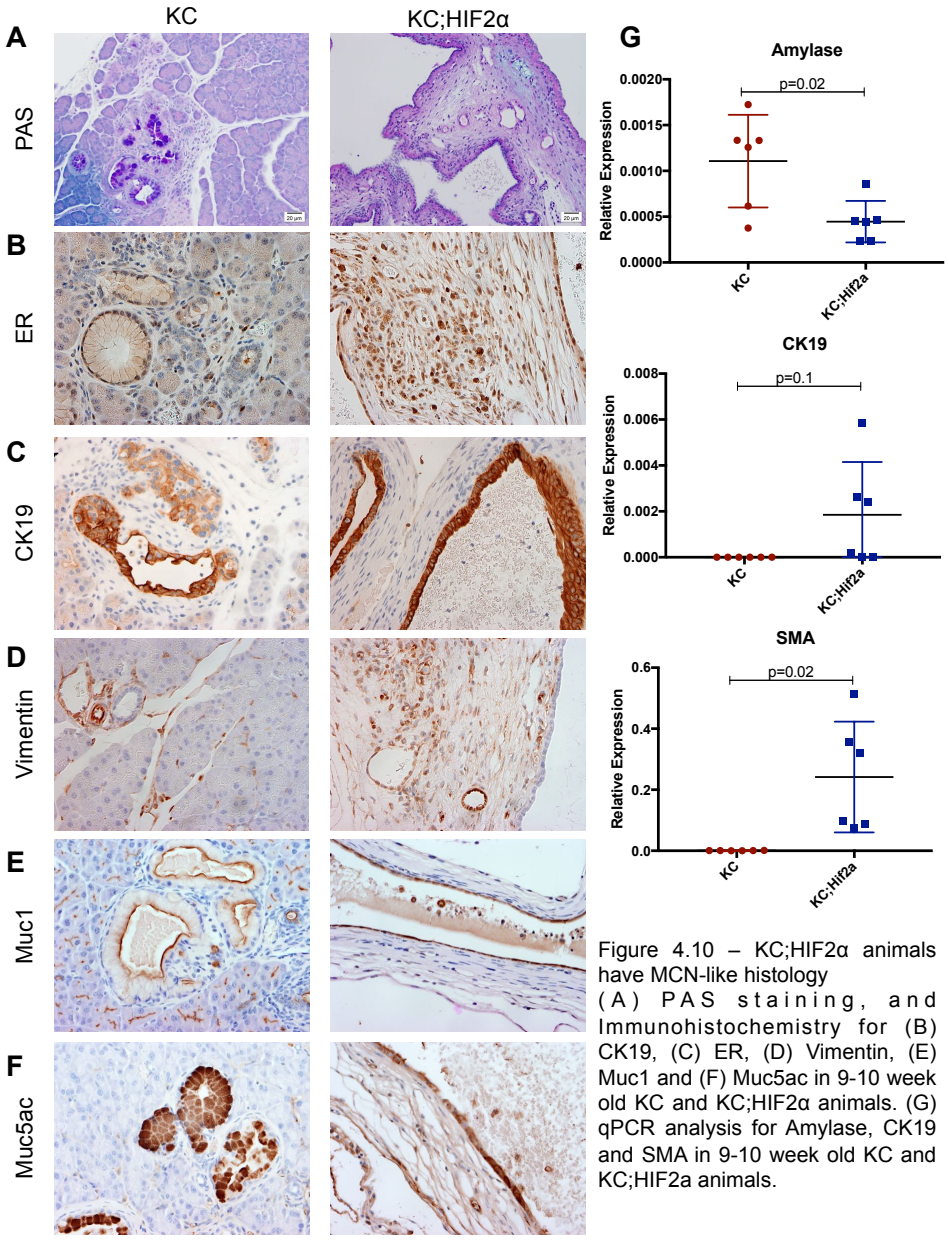


Figure 4.10 – KC;HIF2α animals have MCN-like histology (A) PAS staining, and Immunohistochemistry for (B) CK19, (C) ER, (D) Vimentin, (E) Muc1 and (F) Muc5ac in 9-10 week old KC and KC;HIF2α animals. (G) qPCR analysis for Amylase, CK19 and SMA in 9-10 week old KC and KC;HIF2a animals.

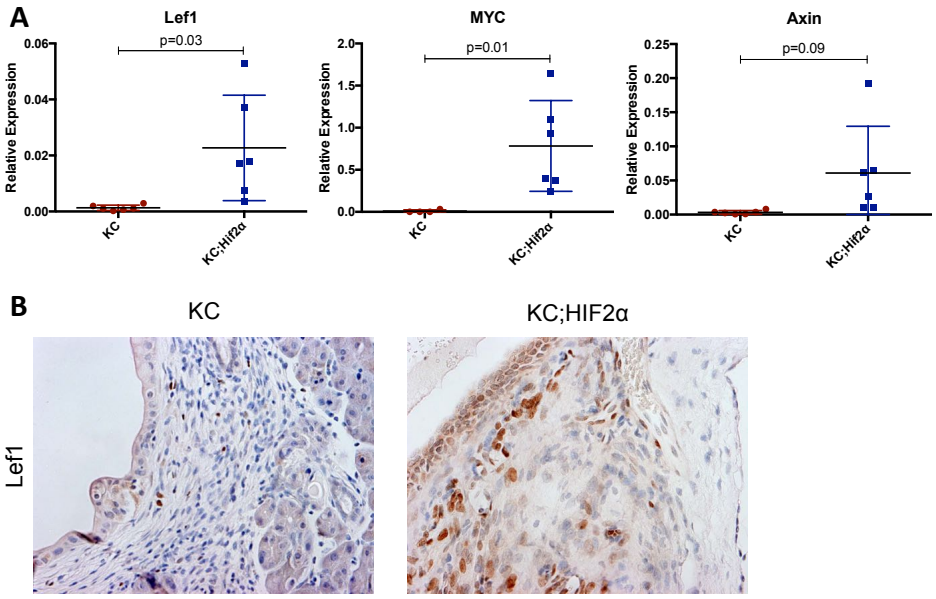


Figure 4.11 – Wnt pathway is upregulated in KC;HIF2a animals
(A) qPCR for Wnt pathway targets in KC and KC;HIF2a animals at 9-10 weeks of age. (B) Immunohistochemistry for Lef1 in KC and KC;HIF2a animals.

Chapter Five

Differential Roles of Mutant p53^{R270H} in Cancer Development Versus Established Growth in Pancreatic Cancer

Abstract

Pancreatic cancer is characterized by nearly universal activating mutations in *KRAS*. Among other somatic mutations, *TP53* is mutated in over 75% of human pancreatic tumors. Genetically engineered mice have proven instrumental in studies of the contribution of individual genes in carcinogenesis. However, current models recapitulate just a subset of the mutations observed in human tumors. The most common mutation in *TP53* in pancreatic cancer is the amino acid substitution R175H. However, the most commonly mutated *TP53* codon is R273, with R273H being the most prevalent substitution. Here we generated a new mouse model that combines expression of *Trp53*^{R270H} (mouse ortholog to human R273H) and oncogenic Kras^{G12D}. *Trp53*^{R270H} expression is regulated by doxycycline administration, so is inducible and reversible. Using this model, we recapitulated sequential Kras and p53 mutations in pancreatic carcinogenesis. Further, with the reversibility of mutant p53 expression, we restored wild type p53 at different stages of carcinogenesis. Continuous mutant p53 was required for progression of precancerous lesions. Mutant p53 was however not required for maintenance of tumor growth, but was critical in the invasive properties of

cancer cells. Mutant p53 expression contributed to pancreatic cancer via reprogramming of cellular metabolism and induction of signaling pathways that regulate proliferation.

Introduction

Pancreatic cancer is a devastating disease, and is the third leading cause of cancer-related death in the United States (Rahib, Smith et al. 2014). Effective therapies are currently lacking and the five-year-survival rate is less than 10%. Activating (oncogenic) mutations in *KRAS* are present in over 90% of human pancreatic cancers (Kanda, Matthaei et al. 2012). *KRAS* mutations are present with high frequency in pancreatic intraepithelial neoplasias (PanINs), precursor lesions to pancreatic cancer, suggesting an initiating role for mutant KRAS. Mutations in the *TP53* and *INK4A* tumor suppressor genes are common at later stages of pancreatic cancer development (Hruban, Goggins et al. 2000), with *TP53* mutations present in up to 75% of pancreatic cancers (Jones, Zhang et al. 2008).

Cancer-associated missense mutations in the TP53 protein have pleiotropic contributions in tumorigenesis, including abrogation of the *wild type* p53 protein's ability regulate cell cycle checkpoints, apoptosis, and inhibition of angiogenesis. At the same time, these missense-mutant P53 proteins can behave as gain-of-function mutants (for review see (Oren and Rotter 2010)). In pancreatic cancer, missense mutations are most common, although null alleles are occasionally observed (Jones, Zhang et al. 2008, Bailey, Chang et al. 2016).

Mice expressing a conditionally activated, oncogenic $Kras^{G12D}$ protein in pancreatic epithelial cells (Ptf1a-Cre;LSL-$Kras^{G12D}$, known as KC mice) develop PanINs, with features akin to those seen in humans, with incomplete penetrance and long latency to malignancy (Hingorani, Petricoin et al. 2003). Combining mutant $Kras^{G12D}$ expression with $p53^{R172H}$ mutant protein expression in the mouse pancreas- in the model known as KPC - results in the development of metastatic pancreatic cancer (Hingorani, Wang et al. 2005). While KPC mice have been essential to establish the role of mutant p53 in cancer progression, they only represent one of a range of different TP53 mutations seen in human pancreatic cancer. Furthermore, in KPC mice, the mutations in *Kras* and Trp53 are introduced concurrently. In contrast, in human pancreatic cancer, activation of Kras is an initiating event present at high frequency in PanINs (Kanda, Matthaei et al. 2012), while *TP53* mutations occur later during disease progression. When a *Trp53* null allele was compared to the $Trp53^{R172H}$ missense allele in the mouse pancreas model, the mice carrying the $Trp53^{R172H}$ allele had both longer disease latency and increased metastatic burden (Morton, Timpson et al. 2010, Weissmueller, Manchado et al. 2014). Similarly, different p53 mutant proteins have differing contributions to tumorigenesis in mouse lung cancer models (Jackson, Olive et al. 2005). Human pancreatic cancer samples contain a spectrum of TP53 mutations. While complete loss of function is rare, mutations occur in several codons, with potentially variable effects on p53 protein properties (Jones, Zhang et al. 2008). Of the point mutations detected in human pancreatic cancer, only $Tp53^{R172H}$ has been recapitulated in a mouse model. Here, we set out to study the functional contributions of $Tp53^{R270H}$, the ortholog to human R273H, in the context of mutant Kras. Further, by

using an inducible and reversible approach to express mutant Tp53, we model the sequence of mutations of the human disease, and explore the role of mutant p53 in the maintenance of PanINs and cancer.

With the advent of precision oncology approaches and the identification of different subsets of pancreatic cancer (Collisson, Sadanandam et al. 2011, Moffitt, Marayati et al. 2015, Waddell, Pajic et al. 2015, Bailey, Chang et al. 2016, Makohon-Moore, Zhang et al. 2017), mouse models recapitulating different human cancer genotypes are likely to be of increasing relevance.

Results

Mutant $p53^{R270H}$ synergizes with oncogenic Kras to promote pancreatic cancer progression

We assessed the nature of TP53 mutations in human pancreatic cancer using publically available data in the Catalog of Somatic Mutations in Cancer (COSMIC) database (http://cancer.sanger.ac.uk/cosmic). Similar to many other human malignancies, the majority of *TP53* mutations in pancreatic cancer are missense substitutions in the DNA binding domain of the p53 protein (Jones, Zhang et al. 2008). The most common Tp53 missense mutation in this data set was $p53^{R175H}$, which corresponds to mouse $p53^{R172H}$. The next most commonly mutated codon in human p53 in all cancer types and the most commonly mutated codon in pancreatic cancer was codon 273. Substitution of histidine for arginine (R273H) is the most common

substitution at that position. The corresponding change in the mouse gene is R270H (Figure 5.1A).

To explore the role of $p53^{R270H}$ in pancreatic cancer, we designed a construct to express this mutant, as well as the fluorescent reporter dsRed, under the control of a Tetracycline Responsive Element (TRE). We initially verified the functionality of the $TREp53^{R270H}$ cassette in tissue culture. HEK-293 cells were transfected with individual plasmids encoding for rtTa and the $TREp53^{R270H}$ construct. Accumulation of p53 protein was observed by immunoblot in doxycycline (dox) treated cells (Figure 5.2A). We then generated transgenic mice carrying the $TREp53^{R270H}$ expression construct. Transgenic $TREp53^{R270H}$ animals were crossed with the Krt5-tTa mouse strain, which expresses tTa in the skin, to establish *in vivo* regulation of the transgene. Both p53 and dsRed expression were observed by IHC in the Krt5-tTa; $TREp53^{R270H}$ animals and not in control littermates (Figure 5.2B). Therefore, the $TREp53^{R270H}$ construct allows for mutant p53 expression in an inducible manner *in vitro* and *in vivo.*

To assess the effects of expressing mutant p53 in the context of mutant Kras pancreata we generated Ptf1a-Cre;LSL-$Kras^{G12D}$;$TREp53^{R270H}$;$R26^{rtTa/rtTa}$ mice, hereby designated as KCip53 mice. The Ptf1a-Cre allele expresses the Cre recombinase in the pancreatic epithelium, thus leading to expression of both $Kras^{G12D}$ and the reverse tetracycline transactivator (rtTa). Administration of dox to the mice induces rtTa-mediated transcriptional activation of $p53^{R270H}$. A dsRed reporter is expressed at the same time as mutant p53, allowing for a surrogate marker of dox-inducible gene expression (Figure 5.1B). KCip53 animals were placed on dox water or chow at approximately four weeks of age, and the animals were maintained on continuous dox

administration. KC littermates were similarly kept on dox for the duration of the experiments, to control for other potential effects of the antibiotic. Starting at 8 months of age, KCip53 animals developed large pancreatic tumors with metastases to the liver and lung (Figure 5.1C). Accumulation of p53 protein in the tumors was confirmed by IHC analysis comparing PanIN lesions in KCip53 mice to lesions in KC mice (Figure 5.1D). In KC mouse lesions, p53 protein accumulation was rare. In KCip53 lesions we observed increased accumulation of p53, although intriguingly only in a subset of cells, similar to the Krt5-tTa; TREp53^{R270H} animals (Figure 5.1D and Figure 5.2B). To determine whether the expression of mutant p53 in this model affected tumor progression we aged the animals until they reached humane endpoints. KCip53 mice had a median survival of 275 days, compared to 395 days in KC mice (Figure 5.1E, n=60 mice for KCip53, 24 for KC). At necropsy, we determined that KCip53 mice had a significant metastatic burden, with a prevalence of liver and lung metastases. Thus, expression of p53^{R270H} accelerates tumorigenesis of KC mice and leads to the development of metastatic disease.

The TREp53^{R270H} allele in the KCip53 animals is a transgene, and therefore the animals still retain two wild type alleles of *Trp53*, potentially mitigating some of the effects of mutant p53^{R270H} expression. To mimic the human scenario, where one allele of *TP53* is mutated and only one wild type allele is present, we generated animals with the genotype Pdx1-Cre; LSL-Kras^{G12D}; LSL-Trp53^{R270H}, called KP^{R270H}C here (Figure 5.3A). In brief, the sequence encoding for P53^{R270H} was inserted in the p53 locus, preceded by a floxed stop cassette. Thus, the mutant p53 was expressed upon Cre recombination in the pancreas at the same time as mutant Kras, in a model similar to

the KPC model (Hingorani, Wang et al. 2005). $KP^{R270H}C$ mice, similar to KCiP53 animals, have shortened lifespan compared to KC littermates (Figure 5.3B, n=43 $KP^{R270H}C$ and 39 KC). Histology from mice at necropsy revealed extensive tumor burden in the $KP^{R270H}C$ animals, with metastatic lesions in the liver and lungs (Figure 5.3C). Thus, analysis of this model corroborates the phenotype of the KCip53 mouse.

Mutant $p53^{R270H}$ promotes formation, progression and maintenance of PanINs

Given that mice expressing mutant $p53^{R270H}$ developed cancer earlier than KC littermates, we sought to determine whether this effect was associated with earlier PanIN development. KCip53 and KC littermates were placed on dox at four weeks of age, activating expression of mutant $p53^{R270H}$ protein. At 10 weeks of age, pancreata were harvested and subjected to histopathological analysis (Figure 5.4A). KCip53 pancreata had more PanINs, and less normal tissue than pancreata from KC littermates (Figure 5.4B and quantification in Figure 5.4C, n=3-6 mice per group). The lesions displayed characteristic PanIN features, including intracellular mucin accumulation (as indicated by PAS staining) and elevated pERK, indicating elevated MAPK signaling (Figure 5.4B). Additionally, KCip53 animals had more CD45+ immune cells (Figure 5.5A) than KC controls. STAT3 activation was previously identified as activated downstream of $p53^{R172H}$ in pancreatic cancer cells and a contributing factor in their growth(Wormann, Song et al. 2016). However, immunostaining for the active, phosphorylated form of STAT3 (pSTAT3), revealed no difference in expression of

pSTAT3 between KC and KCip53 PanINs, suggesting that a different mechanism might be at play during PanIN formation (Figure 5.5B).

KCip53 mice carry two alleles of wild type *Trp53*, in addition to the mutant transgene. Thus, inactivation of mutant expression mimics restoration of wild type function. We pursued studies to determine whether mutant $p53^{R270H}$ expression regulated the progression and maintenance of PanINs. Mice were placed on dox at four weeks of age for 6 weeks. At that point, the animals were randomized in cohorts that either stayed on dox or were removed from dox. Pancreata from both groups were harvested at 2 days, one week or 3 weeks later (Figure 5.6A, n=3 or more mice per group). Inactivation of mutant $p53^{R270H}$ in KCip53 mice effectively re-establishes expression of wild type p53, with two copies of wild type p53 present in these animals. The level of p53 protein accumulation was assessed by Western blot of whole pancreas lysates. In KC pancreata, p53 protein was undetectable. In KCip53 mice on dox, we observed p53 accumulation, which decreased upon dox removal (Figure 5.6B). We then compared pancreas histology across the different groups. KCip53 mice that were continuously maintained on dox had extensive PanINs and limited acinar clusters. In contrast, pancreata from mice that were taken off dox for 3 weeks after the initial 6-week dosing period had limited, low grade lesions and large areas of normal acinar clusters (Figure 5.6C). Within lesions, PAS and pERK1/2 staining were present in both on and off dox pancreata, but fewer positive lesions were observed in the animals taken off dox (Figure 5.6C). These data are consistent with continuous expression of mutant $p53^{R270H}$ being required for maintenance of PanIN lesions and their continued progression.

Given the dramatic differences seen at the 3-week time point after inactivation of mutant p53, we assessed the effects at one week or two days after removing dox and the associated mutant p53 expression. No major histological changes were apparent at these earlier time points (Figure 5.7). We performed qPCR for selected p53-regulated target genes in samples from both cohorts over time. Two days following inactivation of mutant p53, KCip53 animals had a higher ratio of pro-apoptotic to anti-apoptotic factors, compared to KCip53 animals that remained on dox (Figure 5.6D). Furthermore, animals that had been off dox for one week had higher levels of wild type p53-regulated target gene expression, including for *p21*, *Thrombospondin1* and *E-Cadherin*, compared to KCip53 animals maintained on dox (Figure 5.6E). These data suggest that once taken off dox, wild type p53 function is restored, potentially explaining the reduced PanIN progression observed over time.

Expression of mutant p53^{R270H} is not required for growth of invasive cancer

We next sought to determine whether continued expression of mutant p53^{R270H} was required for survival and growth of pancreatic cancer cells. As described above, KCip53 mice maintained on dox treatment develop invasive, metastatic tumors with variable latency (Figure 5.1C and 5.1E). The disease burden in individual animals is heterogeneous, complicating tumor burden analysis at later ages. To study a cohort of mice carrying genetically identical tumors, where we could modulate mutant p53 expression, we established several primary cell lines from cancer-bearing KCip53 mice (Figure 5.8A). To establish these cell lines, we generated single cell suspensions from

the original primary tumors and sorted the cells by flow cytometry for expression of dsRed, as a surrogate marker for cells expressing mutant $p53^{R270H}$ (Figure 5.9A). The cell lines were then propagated in culture in presence of dox (Figure 5.8B). We performed qPCR to study expression of selected p53-regulated target genes, with the goal of determining how changes in expression of mutant p53 affected p53 transcriptional activity in the cells. In cells treated with dox, we saw a reduction in the expression of the wild type p53 target genes *p21* and *Thrombospondin*. Conversely, *vimentin*, a marker of epithelial-mesenchymal transition linked to mutant p53 (Dong, Karaayvaz et al. 2013) was upregulated when dox was removed to abrogate $p53^{R270H}$ expression (Figure 5.9B).

We injected KCip53 cells subcutaneously into immunocompromised (NSG) mice and tracked tumor growth over time by caliper measurement. For each cell line, we included three conditions: i) animals never given dox (no mutant $p53^{R270H}$ expression – termed “No dox”); ii) animals always given dox (continued mutant $p53^{R270H}$ expression, “Plus dox”); and iii) animals started on dox and then removed once tumors had become palpable (termed “Off dox”) (Figure 5.8C, n=8 or more tumors per group, experiment performed at least twice per cell line). No differences in tumor growth were observed among these three groups for the KCip53-1 and KCip53-2 cell lines (Figure 5.8D and 5.8F), and there was no difference in final tumor volume or weight among the groups (Figure 5.8E and 5.8G). We did not observe differences in the prevalence of epithelial cells within the tumors, as measured by expression of CK19, nor in the levels of proliferation (Ki67) or apoptosis (cleaved caspase 3) (Supplemental Figure 5.10A,B,C). To verify the modulation of $Trp53^{R270H}$ gene expression following dox removal, we

performed immunostaining for dsRed. We did not observe any dsRed staining in the tumors from the no dox cohort. In the plus dox cohort, we observed heterogeneous accumulation of dsRed, similar to our observations in PanINs. Fewer dsRed-expressing cells were observed in tumors from the cohort removed from dox (off dox). Hence, dox removal reduced the number of cells expressing the transgene, although a few cells appeared to have escaped regulation and continued to express the transgene in the absence of dox (Figure 5.10D). Quantification of dsRed expression revealed a significantly higher number of cells per high power field being seen in the dox-treated cohort (Figure 5.10D).

Interestingly, comparison of the histology of the tumors from the three groups showed differences in the epithelial cell morphology. We observed increased muscle invasion in the plus dox group (Figure 5.11A, arrows indicate muscle invasion). Immunostaining for CK19 staining highlighted smaller cells with sarcomatoid features in the same group (Supplemental Figure 5.10A). Quantification of the phenotype revealed muscle invasion in 39.3% of tumors in mice that were never on dox, and 69.7% percent of tumors always on dox, suggesting that mutant $p53^{R270H}$ expression promotes tumor invasion into the muscle layer (Figure 5.11B). We then measured the expression of genes associated with epithelial mesenchymal transition (EMT), namely *Zeb1*, *Vimentin*, and *Twist* by qRT-PCR. While the expression of these genes was generally higher in tumors that maintained expression of mutant $p53^{R270H}$, there was not statistical significance (Figure 5.11C). Since the interpretation of the *in vivo* data is confounded by differences in the microenvironment, we used an *in vitro* system for functional studies. For this purpose, we performed scratch assays using KCip53 cell lines. After growing

the cells in the presence or absence of dox, we scratched the plate and monitored time to scratch closure. In both KCip53-1 and KCip53-2 lines, cells grown with dox had shorter time to scratch closure, consistent with an increased migration potential (Figure 5.11D). In previous studies, the ability of mutant $p53^{R172H}$ to promote invasion and metastasis of pancreatic cancer cells carrying this specific mutation was mediated by mutant-p53 mediated expression of *PDGFRβ* (Weissmueller, Manchado et al. 2014). However, we did not observe any difference in PDGFRβ levels by IHC in subcutaneous tumors grown from KCip53-2 cells (Figure 5.12A). Additionally, qRT-PCR for *PDGFRβ* in KCip53 pancreata 3 weeks off dox was actually slightly higher than animals on dox (Figure 5.12B). Together, these data indicate that $p53^{R270H}$ likely controls invasive behavior through a different mechanism than $p53^{R172H}$.

Given the changes in *in vitro* migration, and the increased muscle invasion observed in subcutaneous models, we implanted KCip53-1 cells orthotopically into the pancreata of immunocompromised mice to determine whether mutant $p53^{R270H}$ expression conferred increased metastatic potential, as previously described for $p53^{R172H}$(Morton, Timpson et al. 2010, Weissmueller, Manchado et al. 2014). As in the subcutaneous experiment, we divided the animals in 3 cohorts (no dox, plus dox, and off dox). We then measured tumor growth over time by magnetic resonance imaging (MRI), starting two weeks after cell implantation and continuing for three weeks, when the tumors were harvested (Figure 5.13A, n=3-4 animals per group). Similar to the subcutaneous experiment, there was no difference in tumor growth rate, final tumor volume, or metastatic spread between the “no dox” and “plus dox” groups. Tumors in the cohort taken off dox were slightly smaller (Figure 5.13B-F). Histology of the tumors

across cohorts were similar. We concluded that mutant $p53^{R270H}$ can confer increased migration potential to cells in certain contexts, and that it is dispensable for the growth of invasive tumors.

Transcriptional profile of genes activated downstream of $p53^{R270H}$ reveals alterations in cellular metabolism

To understand the global effects of expressing $p53^{R270H}$ we performed RNA sequencing. We subcutaneously injected KCip53-1 cells in NSG mice and divided the injected mice into three experimental groups, as described above: No dox, Plus dox and Off dox (Figure 5.14A). RNA sequencing analysis revealed marked differences in gene expression profiles among the three treatment groups (n=4 mice/group), with the most significant gene expression signature differences between the group that had no mutant $p53^{R270H}$ expression and the group of tumors always expressing mutant $p53^{R270H}$ (Figure 5.14B). Intriguingly, the gene profile established by $p53^{R270H}$ expression did not reverse upon inactivation of the mutant (Figure 5.14B and Table 5.1).

To analyze the data, we performed pathway analysis and assessed groups of genes changed between the “No dox” and “plus dox” groups. We also observed changes in cell signaling, cell fate, extracellular matrix modeling, and cell motility (Figure 5.15, Table 5.2). Mutant $p53^{R270H}$ expression also reduced apoptotic pathways, while conversely up-regulating signaling pathways that might reflect the increased growth potential of these cells (growth factor activity, regulation of the ERK1/2 cascade and regulation of the insulin receptor signaling pathway). Further, mutant $p53^{R270H}$

expression correlated with increased ECM remodeling pathways, cell mobility pathways, and activation of Rho pathway activities which control cytoskeletal dynamics, all linked with cell invasion and migration. Among the pathways altered by mutant p53 expression, we identified several which suggested changes in metabolism (Figure 5.14C), including regulation of the metabolism of amino acids, carbon sources, fatty acids, and autophagy.

To functionally investigate whether $p53^{R270H}$ regulates PDA metabolism, we analyzed intracellular metabolites from primary KCip53 cells with or without doxycycline treatment by targeted liquid chromatography-mass spectrometry (LC-MS)-based metabolomics (Scheme in Figure 5.16A, Figure 5.17A, Table 5.3). As the pathway analysis suggested, we observed profound changes across the metabolome. Among these, and consistent with the pathway analysis, we found that intracellular branched chain amino acid (BCAA) levels were elevated (Figure 5.14E), which could reflect lower catabolism. BCAAs can be used as a carbon source to fuel the tricarboxylic acid (TCA) cycle in the mitochondria. Indeed, we also observed lower levels of several metabolites in the TCA cycle (Figure 5.14F, Figure 5.17B), which could again reflect lower mitochondrial activity. Thus, to assess bioenergetic activity directly, we used the Seahorse instrument to measure changes in mitochondrial metabolism and glycolysis, as read out by oxygen consumption rate (OCR) and extracellular acidification (ECAR), respectively (Figure 5.16C). This was performed for primary KCip53 cell lines and a primary cell line isolated from a mouse PDX-Cre;$Kras^{LSL-G12D}$;$p53^{R172H}$ (KPC) tumor. The latter do not depend on DOX for their mutant p53 expression and served as control. We observed a decrease in the levels of OCR in the KCip53 cell lines, but not in the KPC

line, upon doxycycline treatment (Figure 5.14D, Figure 5.16B). These functional data provide direct evidence that mutant p53 expression impairs mitochondrial activity, which may result from decreased BCAA metabolism.

Mutant p53^{R270H} in pancreatic cancer treatment

Upwards of 90% of pancreatic cancers harbor oncogenic Kras mutations, but no targeting agents are currently available (for review see (Cox, Fesik et al. 2014)). Attempts to target downstream effector pathways activated by oncogenic Kras, such as PI3K/AKT or MAPK signaling (Cox, Fesik et al. 2014) – both important in pancreatic carcinogenesis (Ardito, Gruner et al. 2012, Collisson, Trejo et al. 2012, Navas, Hernandez-Porras et al. 2012, Eser, Reiff et al. 2013, Collins, Yan et al. 2014)- have similarly been unsuccessful (Infante, Somer et al. 2014). To determine whether inactivation of *Trp53^{R270H}* expression, sensitized pancreatic cancer cells to MAPK or AKT inhibition, we designed a set of experiments. We injected NSG mice subcutaneously with KCip53-1 cells. Initially, all the mice were kept on dox to express mutant p53. In our *first* experiment we tested for MEK sensitivity. Once the tumors were palpable (approximately two weeks after injection), we subdivided the mice in four groups: i) On dox with vehicle; ii) on dox with MEK inhibitor (PD325901, 5mg/kg administered daily by oral gavage); iii) Off dox with vehicle and iv) Off dox with MEK inhibitor (Figure 5.18A, n=5 mice per group, two tumors implanted per mouse). We measured tumor volume over time by MRI. Similar to previous observations (Collins, Yan et al. 2014, Zhang, Velez-Delgado et al. 2017), MEK inhibition slowed, but did not

reverse tumor growth; whether or not mutant *Trp53*^{R270H} was expressed. Abrogation of mutant p53 expression slightly decreased tumor growth, while MEK inhibition cooperated with abrogation of mutant p53^{R270H} to decrease tumor growth (Figure 5.18B). By immunostaining and western blot we observed decreased phospho-ERK1/2 (indicating MAPK activity) in the MEK inhibitor treated samples and expression of dsRed (as readout for mutant p53 expression) in samples from mice on dox (Figure 5.19). We did not observe changes in proliferation (measured by Ki67) or apoptosis (by cleaved caspase 3 staining) in any of the groups. The MEK inhibited and off dox group displayed higher levels of fibrosis, reflecting the slowing of cell growth in these tumors (Figure 5.19). Histological characterization of the tumors showed changes in cellular architecture, with more ductal structures and less sarcomatoid appearance in the tumors from MEK inhibited groups (Figure 5.18C).

Following the MEK inhibition studies, we pursued efforts to study combined inhibition of MEK and PI3 kinase (PI3K), an upstream regulator of AKT, in mice harboring the KCip53 tumors. We injected NSG mice subcutaneously with KCip53-1 cells and animals remained on dox, with mutant p53^{R270H} expression, throughout the experiment. Once the tumors were palpable, we started drug treatment in all four groups: vehicle, MEK inhibition, PI3K inhibition and dual MEK and PI3K inhibition (Figure 5.18D, n=4 animals per group, two tumors implanted per animal). The MEK inhibitor PD325901 was administered orally, once daily at a dose of 5mg/kg. The PI3K inhibitor ZSTK-474 was administered orally once daily at a dose of 100mg/kg. Tumor growth was assessed using MRI for exact volume measurements. As expected, tumors in the control group grew the fastest, while single MEK or PI3K inhibition had a modest

inhibitory effect on growth, which was statistically significant only for PI3K inhibition. The combination of MEK and PI3K inhibition potently inhibited tumor growth, with no increase in tumor volume seen in 4 out of 8 tumors although no evident regression of tumors was observed (Figure 5.18E). We confirmed MEK and PI3K inhibition in the respective treatment groups by Western blot; observing downregulation of pERK1/2 in the MEK inhibitor treated groups and reduced pAKT in the PI3K inhibitor groups (Figure 5.20). Interestingly, while vehicle treated tumors presented with sarcomatoid histology, consistent with the histology of this cell line, inhibition of either MEK or PI3K led to an increase in ductal structures, surrounded by fibrotic stroma (Figure 5.18F). In the dual MEK/PI3K inhibitor treated samples we observed ductal structures surrounded by stroma with lower cellularity than any of the other groups. Proliferation, measured as Ki67 positive nuclei, was lowest in the dual inhibitor-treated tumors. In contrast, we did not observe changes in apoptosis (cleaved caspase 3 expression) in any of the groups, although it is possible that we missed an earlier wave of cell death (Figure 5.20). Thus restoration of wild type p53 function at least partially sensitizes pancreatic tumor cells to MEK inhibition; however, expression of the specific $Trp53^{R270H}$ mutant did not change response to combined MEK and PI3K inhibition, compared to other pancreatic cancer cell lines. These novel KCip53 cell lines constitute a platform for testing the effects of wild type p53 restoration in combination with targeted inhibitors.

Discussion

Mutations in TP53 are detected in about 70% of human pancreatic cancers (Jones, Zhang et al. 2008, Waddell, Pajic et al. 2015, Bailey, Chang et al. 2016) making

it the second most common mutated gene in this disease. While Kras is mutated in low-grade PanINs, consistent with its requirement for tumor initiation (Kanda, Matthaei et al. 2012), p53 mutations accumulate at later stages of disease, and encompass a wide spectrum of mutations. In this study, we re-analyzed public sequencing databases (COSMIC) to determine the prevalence of individual TP53 mutations in human pancreatic cancer. The codon encoding for amino acid 273 of the human protein was the most frequent mutation site; at this site, the most common mutation was R273H. Interestingly, this mutation is considered a p53 “hotspot” mutation, and is one of the most common mutations in human tumors of all types (Olivier, Hollstein et al. 2010). This mutation has, however, not been modeled in pancreatic cancer mouse models.

In human tumors, the majority of TP53 mutations in pancreas cancer lead to the expression of a missense mutant p53 protein that can still oligomerize with wild type p53 but which fails to function as a sequence-specific DNA transcription factor (Hollstein, Sidransky et al. 1991, Hruban, Goggins et al. 2000, Jones, Zhang et al. 2008). Consistent with the human data, expression of mutant Kras in the pancreas is sufficient to initiate carcinogenesis while *Trp53* mutations have little effect on their own, but promote carcinogenesis in presence of oncogenic Kras (Hingorani, Petricoin et al. 2003, Hingorani, Wang et al. 2005). Of the spectrum of human mutations, two types have been studied in mouse models: p53 deletion and a substitution of histidine for arginine at codon 172 of the mouse protein ($p53^{R172H}$), ortholog to R175H in the human p53 protein. Deletion of one or both alleles of p53, in the presence of oncogenic Kras, leads to invasive pancreatic tumors with short latency and high penetrance (Bardeesy, Aguirre et al. 2006). In the commonly used KPC model, $p53^{R172H}$ is expressed alongside

oncogenic Kras; this model has slightly longer latency to invasive disease and, at least in some reports, increased metastatic potential (Hingorani, Wang et al. 2005).

Here, we set out to model the subset of pancreatic cancers expressing *Trp*^{R270H}, the mouse ortholog to human *TP53*^{R273H}. The KCip53 mouse combines expression of oncogenic Kras with *Trp*^{R270H,} and it is designed to have two unique features: 1) *Trp*^{R270H} expression in this model is inducible, thus allowing sequential activation of mutant genes; 2) *Trp*^{R270H} expression is reversible, thus inactivation of its expression and, consequently, restoration of wild type function, can be regulated at will. Similar to p53^{R172H}, p53^{R270H} synergizes with oncogenic Kras to promote pancreatic carcinogenesis. However, a comparison between KPC mice, expressing p53^{R172H} and the KCip53 model revealed slowed progression to malignancy in the latter. Different explanations are possible for this finding, including the later time of activation of p53 (in adult mice rather than in embryogenesis), as well as the fact that the KCip53 model retains two copies of the *wild type* allele. Interestingly, KCip53 mice develop a highly metastatic tumor, which is consistent with a role for p53 in inducing epithelial mesenchymal transition (EMT), one of the mechanisms of metastatic disease spread. Further, tumors expressing mutant p53 showed increased intramuscular invasion, in the subcutaneous setting, reflecting our observation in vitro, where mutant p53 promotes cellular migration.

A unique feature of the KCip53 model is the reversible nature of mutant p53 expression, which allows us to restore wild type function at will, as well as to understand mutant-specific phenotypes. In PanIN lesions, expression of the mutant was continuously required for progression, and inactivation of mutant p53 led to an increase

of wild type p53 target genes, such as p21, that inhibit growth. The mutant protein also facilitated survival of epithelial cells exposed to Kras-driven oncogenic stress, and restoration of wild type function increased the ratio of pro-apoptotic to anti-apoptotic factors. Accordingly, restoration of wild type p53 in liver and lung tumors limits cancer progression by inducing apoptosis (Ventura, Kirsch et al. 2007, Xue, Zender et al. 2007, Feldser, Kostova et al. 2010). In those studies, wild type p53 expression was restored to autochotonous tumors that had developed in the absence of p53 expression in mutant *Ras* driven liver or lung tumor models. In each of these models, wild type p53 restoration led to tumor regression. For this reason, restoration of wild type p53 function has been hypothesized as a potential therapeutic approach, an attractive prospect given that p53 is lost or mutated in the vast majority of human malignancies (Bykov, Issaeva et al. 2002)[13,32,53-55.] Each of the p53 restoration studies relied on models that had lost p53 function. Given that p53 mutation, instead of loss of function, is common in human cancer development, the KCip53 animals provide a unique system to test questions of wild type p53 restoration in tumor therapy.

Our PanIN studies supported the idea that wild type p53 restoration limits tumor progression. Therefore, we extended our studies to invasive tumors. Surprisingly, inactivation of mutant p53 did not reduce tumor growth in a transplantation system. To understand this finding, we generated RNA from transplanted tumors harvested prior and after inactivation of mutant p53 and performed RNAseq to establish a global gene expression signature downstream of mutant $p53^{R270H}$ expression. We found that expression of $p53^{R270H}$ drastically changed global gene expression in pancreatic tumors, compared to tumors that had not expressed mutant $p53^{R270H}$ at all. However,

inactivation of $p53^{R270H}$ in established tumors failed to restore the baseline gene expression pattern. In other words, expression of mutant p53 altered the transcriptome of the tumor cells, but restoration of wild type function failed to restore transcription to the baseline pattern. This finding might explain the lack of dependency on mutant p53 of the KCip53 cell lines. Pathway analysis illuminated many processes that are perturbed by expression of mutant $p53^{R270H}$, many of which have been previously identified as important in pancreatic cancer. Among these, we found changes in the MAPK and Rho family signaling pathways, both critical for pancreatic tumorigenesis (Heid, Lubeseder-Martellato et al. 2011, Collisson, Trejo et al. 2012, Baer, Cintas et al. 2014, Wu, Carpenter et al. 2014). Mutant $p53^{R172H}$ expression in pancreatic cancer cells induces metastasis, an effect that was attributed to activation of PDGFRβ (Weissmueller, Manchado et al. 2014). Analysis of the RNAseq data revealed induction of PDGFRβ upon expression of $p53^{R270H}$, albeit to a modest extent. IHC staining for PDGFRβ expression in subcutaneous tumor tissue revealed high levels of PDGFRβ expression even in “No dox” tumors, which may explain the only modest increase in the “Plus dox” condition. Other mechanisms of invasion, including activation of Rho family members, might be at play in our model.

In addition to the changes in signaling pathways, we also observed changes in the cellular metabolism downstream of mutant p53 in pancreatic cancer cells. The complex role of p53 in the regulation of cellular metabolism has been described before (Berkers, Maddocks et al. 2013), but it has not been studied extensively in the context of pancreatic cancer. An interesting observation, based on pancreatic cancer patient derived xenografts, is that lack of p53 function sensitizes tumors to inhibition of lactate

dehydrogenase (Rajeshkumar, Dutta et al. 2015). It has also been suggested that p53 function can play a role in the regulation of autophagy in pancreatic tumorigenesis, however this result appears to be context dependent on the manner of p53 loss of function (Rosenfeldt, O'Prey et al. 2013, Yang, Rajeshkumar et al. 2014). In our gene expression analysis, we did identify a connection between the expression of $p53^{R270H}$ and regulation of glycolysis and autophagy pathways, and our metabolic profiling indicates that there is indeed a regulation of central metabolic pathways by mutant p53. The drop in mitochondrial activity could suggest that p53 mutations play a role in the metabolic wiring of PDA cells and their respective glycolytic versus lipogeneic behavior, which has been previously suggested to be controlled by an epithelial vs. mesenchymal cell fate (Daemen, Peterson et al. 2015).

Interestingly, one of the most significant metabolic pathways identified through pathway analysis, and confirmed by measuring metabolite abundance, was the downregulation of BCAA metabolism upon expression of $p53^{R270H}$. Recently Mayers et al. demonstrated that the incorporation of BCAA-derived carbon into the TCA cycle is decreased in pancreatic tumors harboring a Kras mutation and p53 LOH, relative to corresponding normal pancreas (Mayers, Torrence et al. 2016). Similarly, our data show that the BCAA degradation pathway is downregulated upon expression of $p53^{R270H}$, where p53 mutation acts similarly to p53 loss. Mayers, et al. suggest that decreased mitochondrial BCAA metabolism occurs due to decreased uptake and thus decreased intracellular levels. It will be important to determine the role of mutant p53 in nutrient acquisition and if this accounts for the decreased BCAA metabolism observed in our experiments.

We, and others, have previously shown that pancreatic cancer requires continuous oncogenic Kras activity (Collins, Bednar et al. 2012, Collins, Brisset et al. 2012, Ying, Kimmelman et al. 2012). Two downstream effector pathways of Kras, MAPK and PI3K/AKT, are required for formation of pancreatic cancer and, at least at early stages, its progression (Ardito, Gruner et al. 2012, Hofmann, Weiss et al. 2012, Navas, Hernandez-Porras et al. 2012, Zhong, Sanchez et al. 2013, Collins, Yan et al. 2014, Watson, Anderson et al. 2014). Inactivation of oncogenic Kras in invasive cancer carrying p53 mutations leads to tumor regression, but not to eradication of tumor cells, and in fact relapse over time is common (Kapoor, Yao et al. 2014, Viale, Pettazzoni et al. 2014, Genovese, Carugo et al. 2017). Further, inhibition of MAPK (via MEK inhibitors) or AKT (using inhibitors of PI3K) or even a combination of both has shown little efficacy in pancreatic cancer patients (Hofmann, Weiss et al. 2012, Infante, Somer et al. 2014). Given the strong anti-apoptotic function ascribed to mutant p53 (Haupt, Berger et al. 2003), we hypothesized that its presence might constitute a mechanism allowing pancreatic cancer cells to bypass MEK inhibition. Further, a new generation of conformational drugs has been developed to restore the wild type function of mutant p53 (Bykov, Issaeva et al. 2002, Feldser, Kostova et al. 2010, Bykov and Wiman 2014, Liu, Read et al. 2015, Mohell, Alfredsson et al. 2015, Fransson, Glaessgen et al. 2016). Conceivably, combined restoration of wild type p53 could be combined with MEK inhibition in pancreatic cancer, a concept that has not been tested before. As a proof of principle approach for this concept, we treated mice bearing implanted KCip53 cells the PD325901 MEK inhibitor and/or the ZSTK-474 PI3K inhibitor. MEK inhibition alone had a modest impact on tumor growth in the presence of mutant $p53^{R270H}$ expression.

However the combination of MEK inhibition with inactivation of mutant $p53^{R270H}$ significantly delayed tumor growth, thus restoration of wild type p53 may be explored as an component in combinatorial therapeutic strategies to target pancreatic cancer. In a separate set of experiments, we explored whether cells expressing $p53^{R270H}$ have a unique sensitivity to combined MEK/PI3K inhibition. While the combination of both inhibitors slowed tumor growth compared to each inhibitor alone, the effect was comparable to previous studies and failed to highlight a unique susceptibility in cells carrying this specific p53 mutation.

In summary, KCip53 mice represent a new mouse model of pancreatic cancer that recapitulates the subset of human tumors that express the R275H mutation of p53. Further, this mouse allows us to model the sequential introduction of mutations in the pancreas, and, thanks to the reversible nature of mutant p53 expression, to determine the role of this tumor suppressor past the initiation stages of carcinogenesis. As new personalized medicine approaches are developed, this model may be useful to predict clinical responses of patients carrying this specific gene mutation.

Methods

Mice

Mice were housed in the specific pathogen free facilities at the University of Michigan Comprehensive Cancer Center. This study was approved by the University of Michigan University Committee on Use and Care of Animals (UCUCA) guidelines. Ptf1a-Cre, Pdx1-Cre, LSL-$Kras^{G12D}$, LSL-$Tp53^{R270H}$ and R26-rtTa animals have been previously

described (Hingorani, Petricoin et al. 2003, Olive, Tuveson et al. 2004, Hingorani, Wang et al. 2005, Collins, Bednar et al. 2012), and TRE-p53^{R270H} animals were generated at the University of Michigan (Eric Fearon). Doxycycline was administered in the drinking water (0.2g/L in a 5% sucrose solution) or the chow (1g/kg) and replaced every 3-4 days. Immunocompromised (NSG) animals were used for all subcutaneous or orthotopic tumor growth experiments. For subcutaneous tumors, 500,000 cells were injected in a 200uL solution of 50% Matrigel and 50% RPMI media. Each mouse was implanted subcutaneously with two tumors, and tumor growth was recorded at least 3X weekly until tumors reached a 1.5cm diameter. For orthotopic tumors, mice were anesthetized using vaporized isoflurane. 500,000 cells were injected in a 50uL solution of 50% Matrigel and 50% RPMI media directly into the pancreas. The incision was closed using absorbable sutures and clips. Post-surgical animals were monitored daily for ten days, and then at least 3X weekly for the duration of the experiment. For drug treatments, animals were treated with PD325901 at a dose of 5mg/kg, ZSTK-474 at a dose of 100mg/kg, or vehicle. Combination treatment was given using the same doses for single drug treatment. All treatments were given by oral gavage once daily.

Immunohistochemistry

Histology and immunohistochemistry studies were performed as previously described (Collins, Bednar et al. 2012). Primary antibodies used are detailed in Supplemental Materials and Methods.

qRT-PCR

RNA isolation, rt-PCR, and qRT-PCR were performed as previously described (Collins, Bednar et al. 2012). *Cyclophilin A* was used as the control housekeeping gene for normalization. Primers used are included in Supplemental Materials and Methods.

Histopathological Analysis

Histopathological analysis was performed using H&E stained sections, with the pathologist being blinded to each animal's genotype. At least 3 independent animals were analyzed from each group, with a minimum of 50 acinar or ductal clusters being counted from each animal. Five representative, non-overlapping, high-power images were analyzed from each slide, with one slide being analyzed per animal. Each cluster was classified as acinar, PanIN1A, 1B, 2, or 3, based on the classification consensus.

Western Blot

Protein isolation and Western blot were performed as previously described (Collins, Bednar et al. 2012). Antibodies used are detailed in Supplemental Materials and Methods.

Cell Culture

KCip53 cell lines were derived from mice of the genotype Ptf1a-Cre; LSL-Kras^{G12D}; TRE-p53^{R270H}; R26-$^{rtTa/rtTa}$. Pancreata or tumors from these animals were minced with scissors and digested in 1mg/mL collagenase. Tumor cells were then sorted from these cultures using Flourescence Activated Cell Sorting, sorting for the expression of dsRed. All cells were cultured in RPMI supplemented with 10% FBS and 1%

penicillin/streptomycin (Gibco). Doxycycline was administered to cells at a concentration of 2ug/mL.

Scratch Assay

Cells were plated in a 6 well plate and grown until confluent. Scratches were created with a pipet tip. Scratches were then measured in the same location at each timepoint. Distance of scratch closure was measured using Image J.

MRI

Mice were anesthetized with 1%–2% isoflurane/air, and body temperature was maintained using a Multistation Temperature Control Unit (Minerve Equipment Veterinaire.) MRI scanning was performed using a 3T Translational, Cryogen-free, preclinical MRI (MR Solutions; MRS 3000 series) with a quadrature mouse body volume coil. Mice were placed supine in the mouse bed. To reduce respiratory motion, surgical tape was used to secure the mice below the thoracic cavity on the bed. T2-weighted images were acquired using a fast spin echo multi-slice sequence with the following parameters: repetition time (TR)/echo time (TE) = 4,500/34 ms, 8 echo trains, 4 averages, Field of View (FOV) = 35 x 35 mm^2, Matrix size = 128 x 128, slice thickness = 2 mm, number of slices = 30, and no gap. Using in-house software, the tumor boundary was manually defined on each slice and then integrated across slices to measure the volume.

RNA Sequencing

Tissue from subcutaneous tumors was collected in lysis buffer for RNA extraction. RNA was isolated using the Qiagen AllPrep DNA/RNA/miRNA Universal kit according to the manufacturer's instructions. PolyA+, non strand specific libraries were prepared by the University of Michigan Sequencing Core, and all samples were sequenced on an Illumina HiSeq 4000.

RNA-seq data analysis

50 bp, single-end reads were mapped to the mouse reference genome (mm9) using TopHat v 1.4.1 (Trapnell, Pachter et al. 2009). The NCBI RefSeq transcript isoform annotation was condensed to an unstranded, gene-level annotation and quantification performed over unambiguous exonic spans per gene. Gene expression was calculated as RPKM (using exonic, base-wise coverage, normalized by summed lengths of exons and total mapped read count). Differential gene expression was performed using DESeq2 v1.12.4 (Love, Huber et al. 2014). Differentially expressed genes were defined as being expressed more than 0.25 RPKM (mean value across all samples in a given comparison), greater than 250 bp in length, changing more than 1.5 fold, and having an adjusted p-value from DESeq2 of less than 0.1. For cluster analysis and heatmap generation, gene expression values were $\log_{10}$-transformed and z-score standardized across samples. Hierarchical clustering was performed using Euclidean distance and complete linkage.

Metabolic Flux Assay

To estimate the rate of glycolysis and mitochondrial respiration Seahorse Metabolic Flux Analyzer e96 XF instrument (Agilent) was used according to the manufacturer's manual. 20 000 cells/well of KPC, KCip53-1 or KCip53-2 cells were seeded in the respective culture media the day prior to the assay. The next day media was exchanged to the Seahorse assay media, containing 25 mM glucose, adjusted to 7.4 pH. Cell plate was allowed to equilibrate for 1 hr in non-CO2 37 C incubator, following 3 sequential measurements for the basal respiration, mitostress assay was performed by injections of 1 μM Oligomycin, 1μM FCCP, 0.5 μM rotenone/0.5 μM Antimycin A. All the chemicals were obtained from Sigma. The cell number adjustment was performed using CyQuant NF (Thermo) after the assay. The data is presented as the mean and SD.

Label-free targeted metabolomics and data analysis

PDAC cells were plated in triplicate and treated with doxycycline containing medium or normal RPMI with 5% FBS for 3 days. The medium was removed and the cell lysate harvested with ice cold 80% MeOH. The soluble metabolite fractions were cleared by centrifugation, dried via speedvac (Thermo Fisher), then resuspended in 50:50 MeOH:H2O mixture for LC–MS analysis. We performed label-free targeted metabolomics using in-house liquid chromatography-mass spectrometry (LC-MS) to measure over 200 metabolites. The bioinformatic data analysis was done using R/Bioconductor. Further details are provided in Supplemental Materials and Methods.

Acknowledgements

The authors thank Kevin Heist for his contribution to MRI measurements. We apologize to the authors of any previous work we were not able to cite due to space constraints. This project was supported by the following funding sources: HKS is supported by National Institutes of Health (NIH) grants T32 GM007315-38 and T32 DK094775-04. This project was supported by R01CA151588, by Funding from the Hirshberg Foundation for Cancer Research (MPdM) and by The Cancer Center Core Grant P30CA46592. CAL is supported by an AACR/Pancreatic Cancer Action Network Pathway to Leadership Award (13-70-25-LYSS). JPM is supported by Cancer Research UK grants C596/A18076 and C596/A17196

References

Ardito, C. M., B. M. Gruner, K. K. Takeuchi, C. Lubeseder-Martellato, N. Teichmann, P. K. Mazur, K. E. Delgiorno, E. S. Carpenter, C. J. Halbrook, J. C. Hall, D. Pal, T. Briel, A. Herner, M. Trajkovic-Arsic, B. Sipos, G. Y. Liou, P. Storz, N. R. Murray, D. W. Threadgill, M. Sibilia, M. K. Washington, C. L. Wilson, R. M. Schmid, E. W. Raines, H. C. Crawford and J. T. Siveke (2012). "EGF receptor is required for KRAS-induced pancreatic tumorigenesis." Cancer Cell **22**(3): 304-317.

Baer, R., C. Cintas, M. Dufresne, S. Cassant-Sourdy, N. Schonhuber, L. Planque, H. Lulka, B. Couderc, C. Bousquet, B. Garmy-Susini, B. Vanhaesebroeck, S. Pyronnet, D. Saur and J. Guillermet-Guibert (2014). "Pancreatic cell plasticity and cancer initiation induced by oncogenic Kras is completely dependent on wild-type PI 3-kinase p110alpha." Genes Dev **28**(23): 2621-2635.

Bailey, P., D. K. Chang, K. Nones, A. L. Johns, A. M. Patch, M. C. Gingras, D. K. Miller, A. N. Christ, T. J. Bruxner, M. C. Quinn, C. Nourse, L. C. Murtaugh, I. Harliwong, S. Idrisoglu, S. Manning, E. Nourbakhsh, S. Wani, L. Fink, O. Holmes, V. Chin, M. J. Anderson, S. Kazakoff, C. Leonard, F. Newell, N. Waddell, S. Wood, Q. Xu, P. J. Wilson, N. Cloonan, K. S. Kassahn, D. Taylor, K. Quek, A. Robertson, L. Pantano, L. Mincarelli, L. N. Sanchez, L. Evers, J. Wu, M. Pinese, M. J. Cowley, M. D. Jones, E. K. Colvin, A. M. Nagrial, E. S. Humphrey, L. A. Chantrill, A. Mawson, J. Humphris, A. Chou, M. Pajic, C. J. Scarlett, A. V. Pinho, M. Giry-Laterriere, I. Rooman, J. S. Samra, J. G. Kench, J. A. Lovell, N. D. Merrett, C. W. Toon, K. Epari, N. Q. Nguyen, A. Barbour, N. Zeps, K. Moran-Jones, N. B. Jamieson, J. S. Graham, F. Duthie, K. Oien, J. Hair, R. Grutzmann, A. Maitra, C. A. Iacobuzio-Donahue, C. L. Wolfgang, R. A. Morgan, R. T. Lawlor, V. Corbo, C. Bassi, B. Rusev, P. Capelli, R. Salvia, G. Tortora, D. Mukhopadhyay, G. M. Petersen, I. Australian Pancreatic Cancer Genome, D. M. Munzy, W. E. Fisher, S. A. Karim, J. R. Eshleman, R. H. Hruban, C. Pilarsky, J. P. Morton, O. J. Sansom, A. Scarpa, E. A. Musgrove, U. M. Bailey, O. Hofmann, R. L. Sutherland, D. A. Wheeler, A. J. Gill, R. A. Gibbs, J. V. Pearson, N. Waddell, A. V. Biankin and S. M. Grimmond (2016). "Genomic analyses identify molecular subtypes of pancreatic cancer." Nature **531**(7592): 47-52.

Bardeesy, N., A. J. Aguirre, G. C. Chu, K. H. Cheng, L. V. Lopez, A. F. Hezel, B. Feng, C. Brennan, R. Weissleder, U. Mahmood, D. Hanahan, M. S. Redston, L. Chin and R. A. Depinho (2006). "Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse." Proc Natl Acad Sci U S A **103**(15): 5947-5952.

Berkers, C. R., O. D. Maddocks, E. C. Cheung, I. Mor and K. H. Vousden (2013). "Metabolic regulation by p53 family members." Cell Metab **18**(5): 617-633.

Bykov, V. J., N. Issaeva, A. Shilov, M. Hultcrantz, E. Pugacheva, P. Chumakov, J. Bergman, K. G. Wiman and G. Selivanova (2002). "Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound." Nat Med **8**(3): 282-288.

Bykov, V. J. and K. G. Wiman (2014). "Mutant p53 reactivation by small molecules makes its way to the clinic." FEBS Lett **588**(16): 2622-2627.

Collins, M. A., F. Bednar, Y. Zhang, J. C. Brisset, S. Galban, C. J. Galban, S. Rakshit, K. S. Flannagan, N. V. Adsay and M. Pasca di Magliano (2012). "Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice." J Clin Invest **122**(2): 639-653.

Collins, M. A., J. C. Brisset, Y. Zhang, F. Bednar, J. Pierre, K. A. Heist, C. J. Galban, S. Galban and M. P. di Magliano (2012). "Metastatic pancreatic cancer is dependent on oncogenic Kras in mice." PLoS One **7**(12): e49707.

Collins, M. A., W. Yan, J. S. Sebolt-Leopold and M. Pasca di Magliano (2014). "MAPK signaling is required for dedifferentiation of acinar cells and development of pancreatic intraepithelial neoplasia in mice." Gastroenterology **146**(3): 822-834 e827.

Collisson, E. A., A. Sadanandam, P. Olson, W. J. Gibb, M. Truitt, S. Gu, J. Cooc, J. Weinkle, G. E. Kim, L. Jakkula, H. S. Feiler, A. H. Ko, A. B. Olshen, K. L. Danenberg, M. A. Tempero, P. T. Spellman, D. Hanahan and J. W. Gray (2011). "Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy." Nat Med **17**(4): 500-503.

Collisson, E. A., C. L. Trejo, J. M. Silva, S. Gu, J. E. Korkola, L. M. Heiser, R. P. Charles, B. A. Rabinovich, B. Hann, D. Dankort, P. T. Spellman, W. A. Phillips, J. W. Gray and M. McMahon (2012). "A central role for RAF-->MEK-->ERK signaling in the genesis of pancreatic ductal adenocarcinoma." Cancer Discov **2**(8): 685-693.

Cox, A. D., S. W. Fesik, A. C. Kimmelman, J. Luo and C. J. Der (2014). "Drugging the undruggable RAS: Mission possible?" Nat Rev Drug Discov **13**(11): 828-851.

Daemen, A., D. Peterson, N. Sahu, R. McCord, X. Du, B. Liu, K. Kowanetz, R. Hong, J. Moffat, M. Gao, A. Boudreau, R. Mroue, L. Corson, T. O'Brien, J. Qing, D. Sampath, M. Merchant, R. Yauch, G. Manning, J. Settleman, G. Hatzivassiliou and M. Evangelista (2015). "Metabolite profiling stratifies pancreatic ductal adenocarcinomas into subtypes with distinct sensitivities to metabolic inhibitors." Proc Natl Acad Sci U S A **112**(32): E4410-4417.

Dong, P., M. Karaayvaz, N. Jia, M. Kaneuchi, J. Hamada, H. Watari, S. Sudo, J. Ju and N. Sakuragi (2013). "Mutant p53 gain-of-function induces epithelial-mesenchymal transition through modulation of the miR-130b-ZEB1 axis." Oncogene **32**(27): 3286-3295.

Eser, S., N. Reiff, M. Messer, B. Seidler, K. Gottschalk, M. Dobler, M. Hieber, A. Arbeiter, S. Klein, B. Kong, C. W. Michalski, A. M. Schlitter, I. Esposito, A. J. Kind, L. Rad, A. E. Schnieke, M. Baccarini, D. R. Alessi, R. Rad, R. M. Schmid, G. Schneider and D. Saur (2013). "Selective requirement of PI3K/PDK1 signaling for Kras oncogene-driven pancreatic cell plasticity and cancer." Cancer Cell **23**(3): 406-420.

Feldser, D. M., K. K. Kostova, M. M. Winslow, S. E. Taylor, C. Cashman, C. A. Whittaker, F. J. Sanchez-Rivera, R. Resnick, R. Bronson, M. T. Hemann and T. Jacks

(2010). "Stage-specific sensitivity to p53 restoration during lung cancer progression." Nature **468**(7323): 572-575.

Fransson, A., D. Glaessgen, J. Alfredsson, K. G. Wiman, S. Bajalica-Lagercrantz and N. Mohell (2016). "Strong synergy with APR-246 and DNA-damaging drugs in primary cancer cells from patients with TP53 mutant High-Grade Serous ovarian cancer." J Ovarian Res **9**(1): 27.

Genovese, G., A. Carugo, J. Tepper, F. S. Robinson, L. Li, M. Svelto, L. Nezi, D. Corti, R. Minelli, P. Pettazzoni, T. Gutschner, C. C. Wu, S. Seth, K. C. Akdemir, E. Leo, S. Amin, M. D. Molin, H. Ying, L. N. Kwong, S. Colla, K. Takahashi, P. Ghosh, V. Giuliani, F. Muller, P. Dey, S. Jiang, J. Garvey, C. G. Liu, J. Zhang, T. P. Heffernan, C. Toniatti, J. B. Fleming, M. G. Goggins, L. D. Wood, A. Sgambato, A. Agaimy, A. Maitra, C. W. Roberts, H. Wang, A. Viale, R. A. DePinho, G. F. Draetta and L. Chin (2017). "Synthetic vulnerabilities of mesenchymal subpopulations in pancreatic cancer." Nature **542**(7641): 362-366.

Haupt, S., M. Berger, Z. Goldberg and Y. Haupt (2003). "Apoptosis - the p53 network." J Cell Sci **116**(Pt 20): 4077-4085.

Heid, I., C. Lubeseder-Martellato, B. Sipos, P. K. Mazur, M. Lesina, R. M. Schmid and J. T. Siveke (2011). "Early requirement of Rac1 in a mouse model of pancreatic cancer." Gastroenterology **141**(2): 719-730, 730 e711-717.

Hingorani, S. R., E. F. Petricoin, A. Maitra, V. Rajapakse, C. King, M. A. Jacobetz, S. Ross, T. P. Conrads, T. D. Veenstra, B. A. Hitt, Y. Kawaguchi, D. Johann, L. A. Liotta, H. C. Crawford, M. E. Putt, T. Jacks, C. V. Wright, R. H. Hruban, A. M. Lowy and D. A.

Tuveson (2003). "Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse." Cancer Cell **4**(6): 437-450.

Hingorani, S. R., L. Wang, A. S. Multani, C. Combs, T. B. Deramaudt, R. H. Hruban, A. K. Rustgi, S. Chang and D. A. Tuveson (2005). "Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice." Cancer Cell **7**(5): 469-483.

Hofmann, I., A. Weiss, G. Elain, M. Schwaederle, D. Sterker, V. Romanet, T. Schmelzle, A. Lai, S. M. Brachmann, M. Bentires-Alj, T. M. Roberts, W. R. Sellers, F. Hofmann and S. M. Maira (2012). "K-RAS mutant pancreatic tumors show higher sensitivity to MEK than to PI3K inhibition in vivo." PLoS One **7**(8): e44146.

Hollstein, M., D. Sidransky, B. Vogelstein and C. C. Harris (1991). "p53 mutations in human cancers." Science **253**(5015): 49-53.

Hruban, R. H., M. Goggins, J. Parsons and S. E. Kern (2000). "Progression model for pancreatic cancer." Clin Cancer Res **6**(8): 2969-2972.

Infante, J. R., B. G. Somer, J. O. Park, C. P. Li, M. E. Scheulen, S. M. Kasubhai, D. Y. Oh, Y. Liu, S. Redhu, K. Steplewski and N. Le (2014). "A randomised, double-blind, placebo-controlled trial of trametinib, an oral MEK inhibitor, in combination with gemcitabine for patients with untreated metastatic adenocarcinoma of the pancreas." Eur J Cancer **50**(12): 2072-2081.

Jackson, E. L., K. P. Olive, D. A. Tuveson, R. Bronson, D. Crowley, M. Brown and T. Jacks (2005). "The differential effects of mutant p53 alleles on advanced murine lung cancer." Cancer Res **65**(22): 10280-10288.

Jones, S., X. Zhang, D. W. Parsons, J. C. Lin, R. J. Leary, P. Angenendt, P. Mankoo, H. Carter, H. Kamiyama, A. Jimeno, S. M. Hong, B. Fu, M. T. Lin, E. S. Calhoun, M. Kamiyama, K. Walter, T. Nikolskaya, Y. Nikolsky, J. Hartigan, D. R. Smith, M. Hidalgo, S. D. Leach, A. P. Klein, E. M. Jaffee, M. Goggins, A. Maitra, C. Iacobuzio-Donahue, J. R. Eshleman, S. E. Kern, R. H. Hruban, R. Karchin, N. Papadopoulos, G. Parmigiani, B. Vogelstein, V. E. Velculescu and K. W. Kinzler (2008). "Core signaling pathways in human pancreatic cancers revealed by global genomic analyses." Science **321**(5897): 1801-1806.

Kanda, M., H. Matthaei, J. Wu, S. M. Hong, J. Yu, M. Borges, R. H. Hruban, A. Maitra, K. Kinzler, B. Vogelstein and M. Goggins (2012). "Presence of somatic mutations in most early-stage pancreatic intraepithelial neoplasia." Gastroenterology **142**(4): 730-733 e739.

Kapoor, A., W. Yao, H. Ying, S. Hua, A. Liewen, Q. Wang, Y. Zhong, C. J. Wu, A. Sadanandam, B. Hu, Q. Chang, G. C. Chu, R. Al-Khalil, S. Jiang, H. Xia, E. Fletcher-Sananikone, C. Lim, G. I. Horwitz, A. Viale, P. Pettazzoni, N. Sanchez, H. Wang, A. Protopopov, J. Zhang, T. Heffernan, R. L. Johnson, L. Chin, Y. A. Wang, G. Draetta and R. A. DePinho (2014). "Yap1 activation enables bypass of oncogenic Kras addiction in pancreatic cancer." Cell **158**(1): 185-197.

Liu, D. S., M. Read, C. Cullinane, W. J. Azar, C. M. Fennell, K. G. Montgomery, S. Haupt, Y. Haupt, K. G. Wiman, C. P. Duong, N. J. Clemons and W. A. Phillips (2015). "APR-246 potently inhibits tumour growth and overcomes chemoresistance in preclinical models of oesophageal adenocarcinoma." Gut **64**(10): 1506-1516.

Love, M. I., W. Huber and S. Anders (2014). "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2." Genome Biol **15**(12): 550.

Makohon-Moore, A. P., M. Zhang, J. G. Reiter, I. Bozic, B. Allen, D. Kundu, K. Chatterjee, F. Wong, Y. Jiao, Z. A. Kohutek, J. Hong, M. Attiyeh, B. Javier, L. D. Wood, R. H. Hruban, M. A. Nowak, N. Papadopoulos, K. W. Kinzler, B. Vogelstein and C. A. Iacobuzio-Donahue (2017). "Limited heterogeneity of known driver gene mutations among the metastases of individual patients with pancreatic cancer." Nat Genet **49**(3): 358-366.

Mayers, J. R., M. E. Torrence, L. V. Danai, T. Papagiannakopoulos, S. M. Davidson, M. R. Bauer, A. N. Lau, B. W. Ji, P. D. Dixit, A. M. Hosios, A. Muir, C. R. Chin, E. Freinkman, T. Jacks, B. M. Wolpin, D. Vitkup and M. G. Vander Heiden (2016). "Tissue of origin dictates branched-chain amino acid metabolism in mutant Kras-driven cancers." Science **353**(6304): 1161-1165.

Moffitt, R. A., R. Marayati, E. L. Flate, K. E. Volmar, S. G. Loeza, K. A. Hoadley, N. U. Rashid, L. A. Williams, S. C. Eaton, A. H. Chung, J. K. Smyla, J. M. Anderson, H. J. Kim, D. J. Bentrem, M. S. Talamonti, C. A. Iacobuzio-Donahue, M. A. Hollingsworth and J. J. Yeh (2015). "Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma." Nat Genet **47**(10): 1168-1178.

Mohell, N., J. Alfredsson, A. Fransson, M. Uustalu, S. Bystrom, J. Gullbo, A. Hallberg, V. J. Bykov, U. Bjorklund and K. G. Wiman (2015). "APR-246 overcomes resistance to cisplatin and doxorubicin in ovarian cancer cells." Cell Death Dis **6**: e1794.

Morton, J. P., P. Timpson, S. A. Karim, R. A. Ridgway, D. Athineos, B. Doyle, N. B. Jamieson, K. A. Oien, A. M. Lowy, V. G. Brunton, M. C. Frame, T. R. Evans and O. J.

Sansom (2010). "Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer." Proc Natl Acad Sci U S A **107**(1): 246-251.

Navas, C., I. Hernandez-Porras, A. J. Schuhmacher, M. Sibilia, C. Guerra and M. Barbacid (2012). "EGF receptor signaling is essential for k-ras oncogene-driven pancreatic ductal adenocarcinoma." Cancer Cell **22**(3): 318-330.

Olive, K. P., D. A. Tuveson, Z. C. Ruhe, B. Yin, N. A. Willis, R. T. Bronson, D. Crowley and T. Jacks (2004). "Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome." Cell **119**(6): 847-860.

Olivier, M., M. Hollstein and P. Hainaut (2010). "TP53 mutations in human cancers: origins, consequences, and clinical use." Cold Spring Harb Perspect Biol **2**(1): a001008.

Oren, M. and V. Rotter (2010). "Mutant p53 gain-of-function in cancer." Cold Spring Harb Perspect Biol **2**(2): a001107.

Rahib, L., B. D. Smith, R. Aizenberg, A. B. Rosenzweig, J. M. Fleshman and L. M. Matrisian (2014). "Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States." Cancer Res **74**(11): 2913-2921.

Rajeshkumar, N. V., P. Dutta, S. Yabuuchi, R. F. de Wilde, G. V. Martinez, A. Le, J. J. Kamphorst, J. D. Rabinowitz, S. K. Jain, M. Hidalgo, C. V. Dang, R. J. Gillies and A. Maitra (2015). "Therapeutic Targeting of the Warburg Effect in Pancreatic Cancer Relies on an Absence of p53 Function." Cancer Res **75**(16): 3355-3364.

Rosenfeldt, M. T., J. O'Prey, J. P. Morton, C. Nixon, G. MacKay, A. Mrowinska, A. Au, T. S. Rai, L. Zheng, R. Ridgway, P. D. Adams, K. I. Anderson, E. Gottlieb, O. J. Sansom

and K. M. Ryan (2013). "p53 status determines the role of autophagy in pancreatic tumour development." Nature **504**(7479): 296-300.

Trapnell, C., L. Pachter and S. L. Salzberg (2009). "TopHat: discovering splice junctions with RNA-Seq." Bioinformatics **25**(9): 1105-1111.

Ventura, A., D. G. Kirsch, M. E. McLaughlin, D. A. Tuveson, J. Grimm, L. Lintault, J. Newman, E. E. Reczek, R. Weissleder and T. Jacks (2007). "Restoration of p53 function leads to tumour regression in vivo." Nature **445**(7128): 661-665.

Viale, A., P. Pettazzoni, C. A. Lyssiotis, H. Ying, N. Sanchez, M. Marchesini, A. Carugo, T. Green, S. Seth, V. Giuliani, M. Kost-Alimova, F. Muller, S. Colla, L. Nezi, G. Genovese, A. K. Deem, A. Kapoor, W. Yao, E. Brunetto, Y. Kang, M. Yuan, J. M. Asara, Y. A. Wang, T. P. Heffernan, A. C. Kimmelman, H. Wang, J. B. Fleming, L. C. Cantley, R. A. DePinho and G. F. Draetta (2014). "Oncogene ablation-resistant pancreatic cancer cells depend on mitochondrial function." Nature **514**(7524): 628-632.

Waddell, N., M. Pajic, A. M. Patch, D. K. Chang, K. S. Kassahn, P. Bailey, A. L. Johns, D. Miller, K. Nones, K. Quek, M. C. Quinn, A. J. Robertson, M. Z. Fadlullah, T. J. Bruxner, A. N. Christ, I. Harliwong, S. Idrisoglu, S. Manning, C. Nourse, E. Nourbakhsh, S. Wani, P. J. Wilson, E. Markham, N. Cloonan, M. J. Anderson, J. L. Fink, O. Holmes, S. H. Kazakoff, C. Leonard, F. Newell, B. Poudel, S. Song, D. Taylor, N. Waddell, S. Wood, Q. Xu, J. Wu, M. Pinese, M. J. Cowley, H. C. Lee, M. D. Jones, A. M. Nagrial, J. Humphris, L. A. Chantrill, V. Chin, A. M. Steinmann, A. Mawson, E. S. Humphrey, E. K. Colvin, A. Chou, C. J. Scarlett, A. V. Pinho, M. Giry-Laterriere, I. Rooman, J. S. Samra, J. G. Kench, J. A. Pettitt, N. D. Merrett, C. Toon, K. Epari, N. Q. Nguyen, A. Barbour, N. Zeps, N. B. Jamieson, J. S. Graham, S. P. Niclou, R. Bjerkvig, R. Grutzmann, D. Aust,

R. H. Hruban, A. Maitra, C. A. Iacobuzio-Donahue, C. L. Wolfgang, R. A. Morgan, R. T. Lawlor, V. Corbo, C. Bassi, M. Falconi, G. Zamboni, G. Tortora, M. A. Tempero, I. Australian Pancreatic Cancer Genome, A. J. Gill, J. R. Eshleman, C. Pilarsky, A. Scarpa, E. A. Musgrove, J. V. Pearson, A. V. Biankin and S. M. Grimmond (2015). "Whole genomes redefine the mutational landscape of pancreatic cancer." Nature **518**(7540): 495-501.

Watson, A. L., L. K. Anderson, A. D. Greeley, V. W. Keng, E. P. Rahrmann, A. L. Halfond, N. M. Powell, M. H. Collins, T. Rizvi, C. L. Moertel, N. Ratner and D. A. Largaespada (2014). "Co-targeting the MAPK and PI3K/AKT/mTOR pathways in two genetically engineered mouse models of schwann cell tumors reduces tumor grade and multiplicity." Oncotarget **5**(6): 1502-1514.

Weissmueller, S., E. Manchado, M. Saborowski, J. P. t. Morris, E. Wagenblast, C. A. Davis, S. H. Moon, N. T. Pfister, D. F. Tschaharganeh, T. Kitzing, D. Aust, E. K. Markert, J. Wu, S. M. Grimmond, C. Pilarsky, C. Prives, A. V. Biankin and S. W. Lowe (2014). "Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor beta signaling." Cell **157**(2): 382-394.

Wormann, S. M., L. Song, J. Ai, K. N. Diakopoulos, M. U. Kurkowski, K. Gorgulu, D. Ruess, A. Campbell, C. Doglioni, D. Jodrell, A. Neesse, I. E. Demir, A. P. Karpathaki, M. Barenboim, T. Hagemann, S. Rose-John, O. Sansom, R. M. Schmid, M. P. Protti, M. Lesina and H. Algul (2016). "Loss of P53 Function Activates JAK2-STAT3 Signaling to Promote Pancreatic Tumor Growth, Stroma Modification, and Gemcitabine Resistance in Mice and Is Associated With Patient Survival." Gastroenterology **151**(1): 180-193 e112.

Wu, C. Y., E. S. Carpenter, K. K. Takeuchi, C. J. Halbrook, L. V. Peverley, H. Bien, J. C. Hall, K. E. DelGiorno, D. Pal, Y. Song, C. Shi, R. Z. Lin and H. C. Crawford (2014). "PI3K regulation of RAC1 is required for KRAS-induced pancreatic tumorigenesis in mice." Gastroenterology **147**(6): 1405-1416 e1407.

Xue, W., L. Zender, C. Miething, R. A. Dickins, E. Hernando, V. Krizhanovsky, C. Cordon-Cardo and S. W. Lowe (2007). "Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas." Nature **445**(7128): 656-660.

Yang, A., N. V. Rajeshkumar, X. Wang, S. Yabuuchi, B. M. Alexander, G. C. Chu, D. D. Von Hoff, A. Maitra and A. C. Kimmelman (2014). "Autophagy is critical for pancreatic tumor growth and progression in tumors with p53 alterations." Cancer Discov **4**(8): 905-913.

Ying, H., A. C. Kimmelman, C. A. Lyssiotis, S. Hua, G. C. Chu, E. Fletcher-Sananikone, J. W. Locasale, J. Son, H. Zhang, J. L. Coloff, H. Yan, W. Wang, S. Chen, A. Viale, H. Zheng, J. H. Paik, C. Lim, A. R. Guimaraes, E. S. Martin, J. Chang, A. F. Hezel, S. R. Perry, J. Hu, B. Gan, Y. Xiao, J. M. Asara, R. Weissleder, Y. A. Wang, L. Chin, L. C. Cantley and R. A. DePinho (2012). "Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism." Cell **149**(3): 656-670.

Zhang, Y., A. Velez-Delgado, E. Mathew, D. Li, F. M. Mendez, K. Flannagan, A. D. Rhim, D. M. Simeone, G. L. Beatty and M. Pasca di Magliano (2017). "Myeloid cells are required for PD-1/PD-L1 checkpoint activation and the establishment of an immunosuppressive environment in pancreatic cancer." Gut **66**(1): 124-136.

Zhong, H., C. Sanchez, D. Spitzer, S. Plambeck-Suess, J. Gibbs, W. G. Hawkins, D. Denardo, F. Gao, R. A. Pufahl, A. C. Lockhart, M. Xu, D. Linehan, J. Weber and A.

Wang-Gillam (2013). "Synergistic effects of concurrent blockade of PI3K and MEK pathways in pancreatic cancer preclinical models." PLoS One **8**(10): e77243.

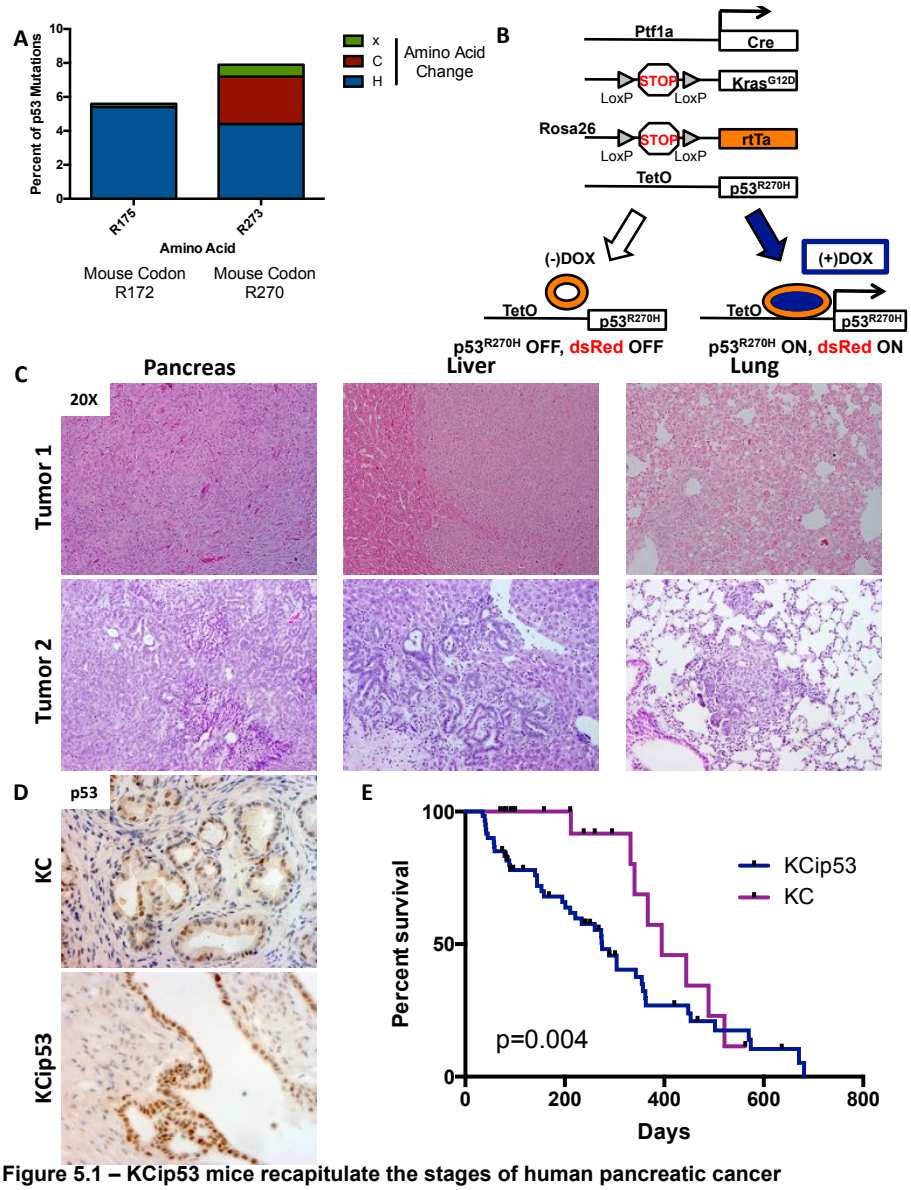


Figure 5.1 – KCip53 mice recapitulate the stages of human pancreatic cancer
(A) Percent of human pancreatic tumor samples with p53R175 or R273 mutations, from the COSMIC database. (B) Scheme of Ptf1a-Cre; LSLKrasG12D; TREp53R270H; R26rtTa/rtTa animals, termed KCip53 here. (C) H&E from pancreas, liver, and lung from two separate KCip53 animals with tumors. (D) IHC for p53 in KC and KCip53 tissue. (E) Survival curve for KC and KCip53 animals.

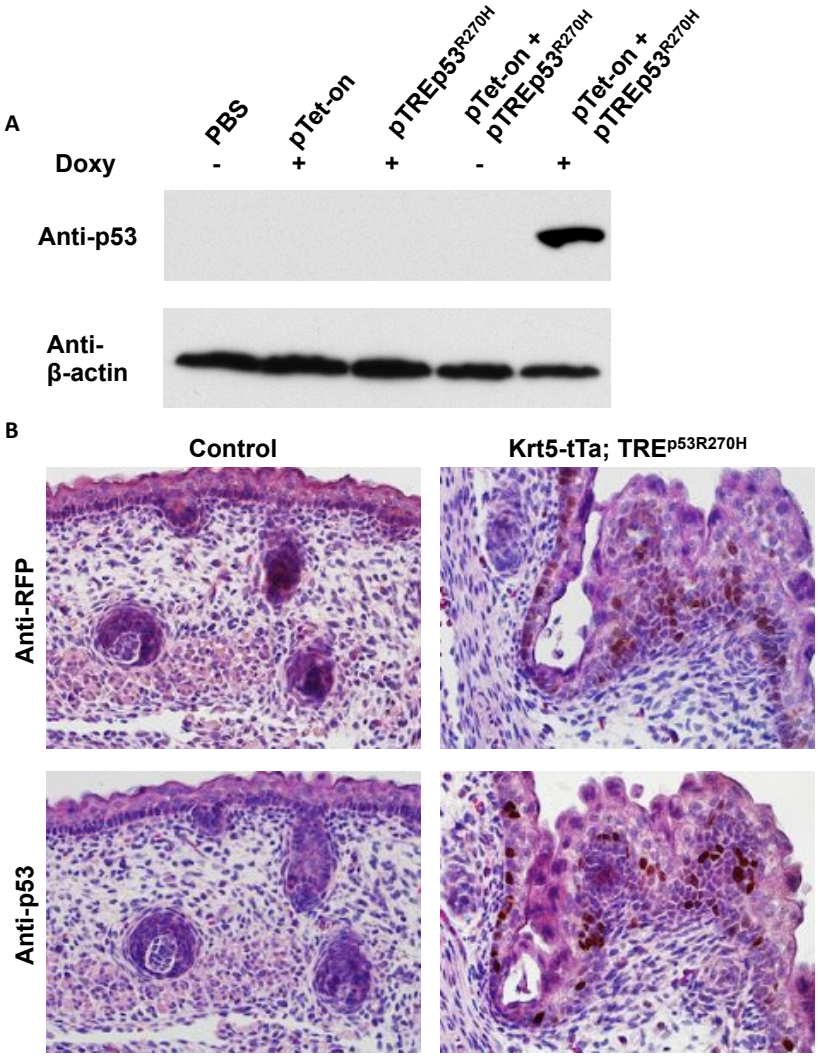


Figure 5.2 – TREp53^{R270H} Validation *in vitro* and *in vivo*
(A) Western Blot for p53 to confirm p53^{R270H} expression upon dox administration. (B) IHC for RFP (dsRed) and p53 in Control and Krt5-tTa; TRE^{p53R270H} animal epithelium to confirm p53^{R270H} expression.

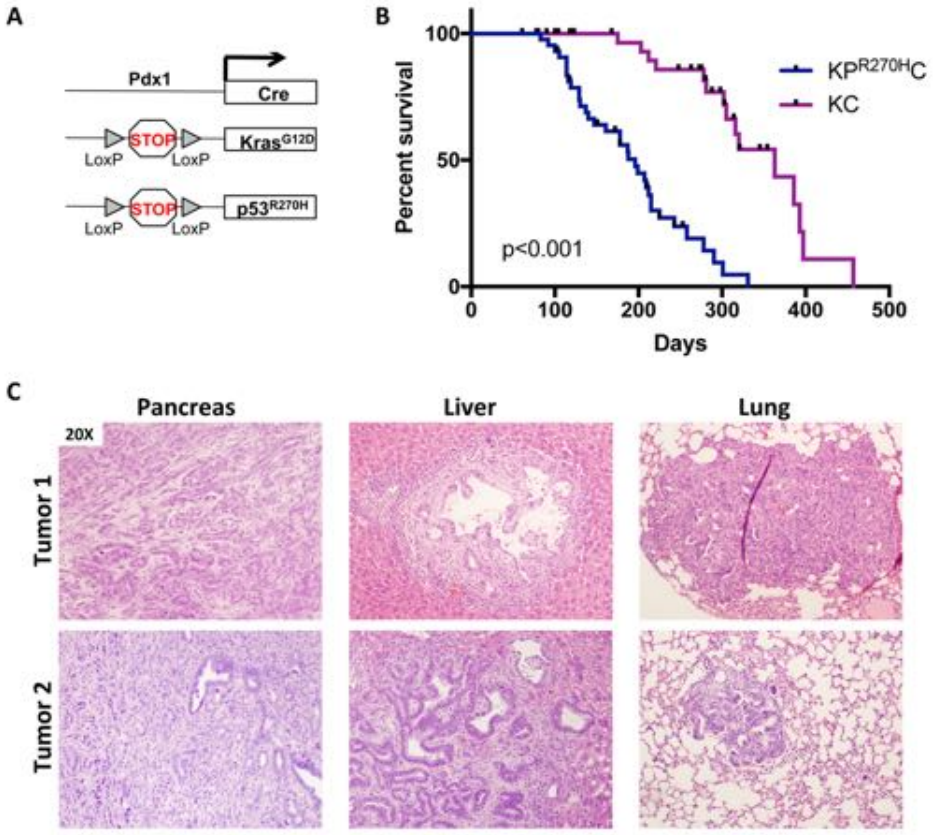


Figure 5.3 – KP^{R270H}C animals develop metastatic pancreatic tumors
(A) Scheme of Pdx1-Cre; LSLKras^{G12D}; Tp53$^{R270H/+}$ animals, termed KP^{R270H}C here (B) Survival curve for KC and KP^{R270H}C animals. (C) H&E from pancreas, liver and lung from two separate KP^{R270H}C animals with tumors.

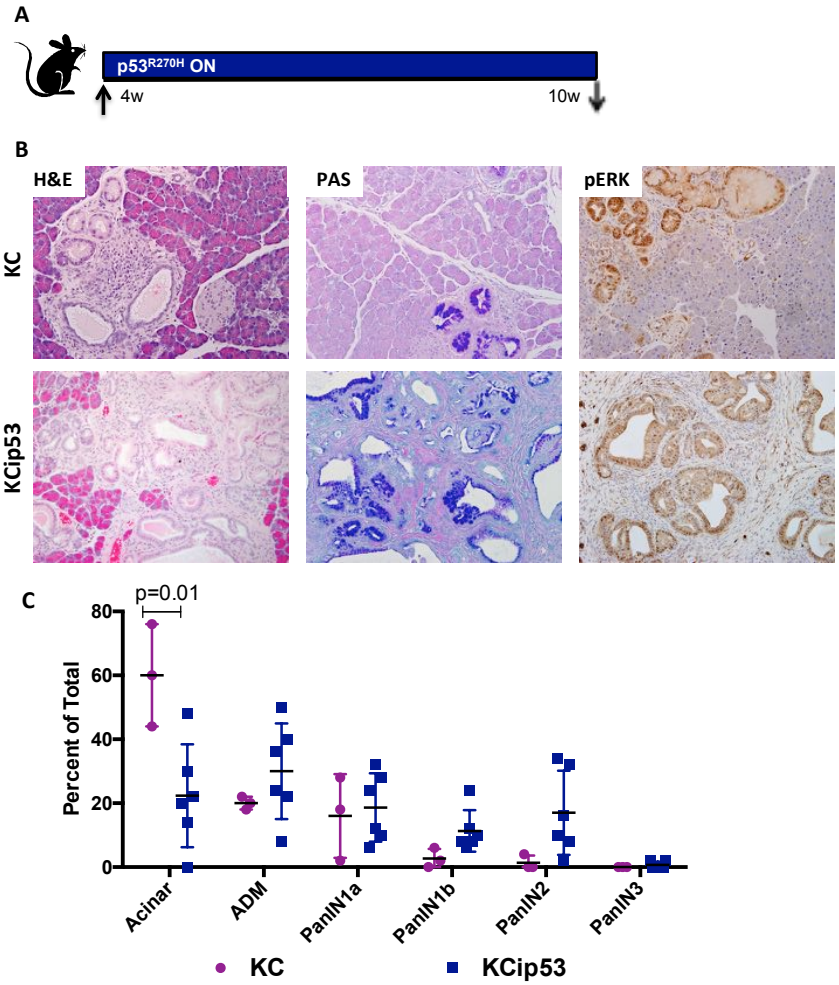


Figure 5.4 – Mutant p53^{R270H} expression promotes PanIN formation
(A) Scheme for mouse treatment for early timepoint analysis. (B) H&E, PAS and IHC for pERK in KC and KCip53 animals at ten weeks of age. (C) Quantification of pancreatic pathology in KC and KCip53 animals.

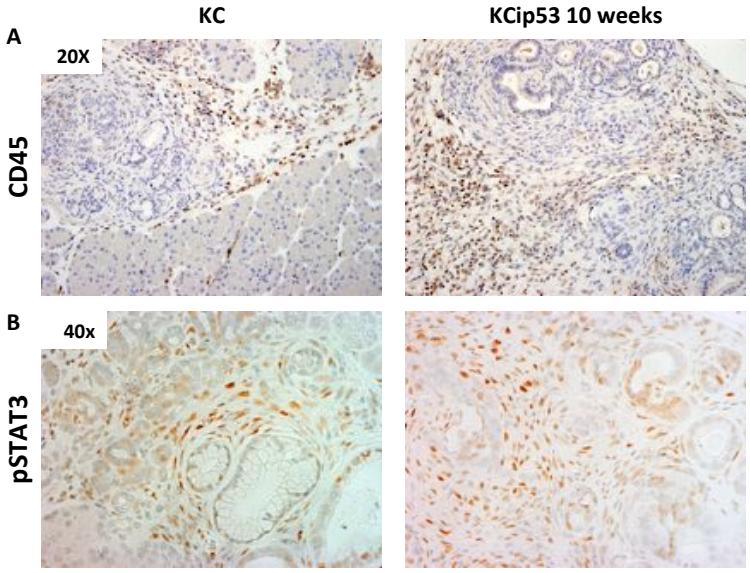


Figure 5.5– KCip53 histology at ten weeks of age
IHC for (A) CD45 and (B) pSTAT3 in 10-week-old KC or KCip53 animals on dox.

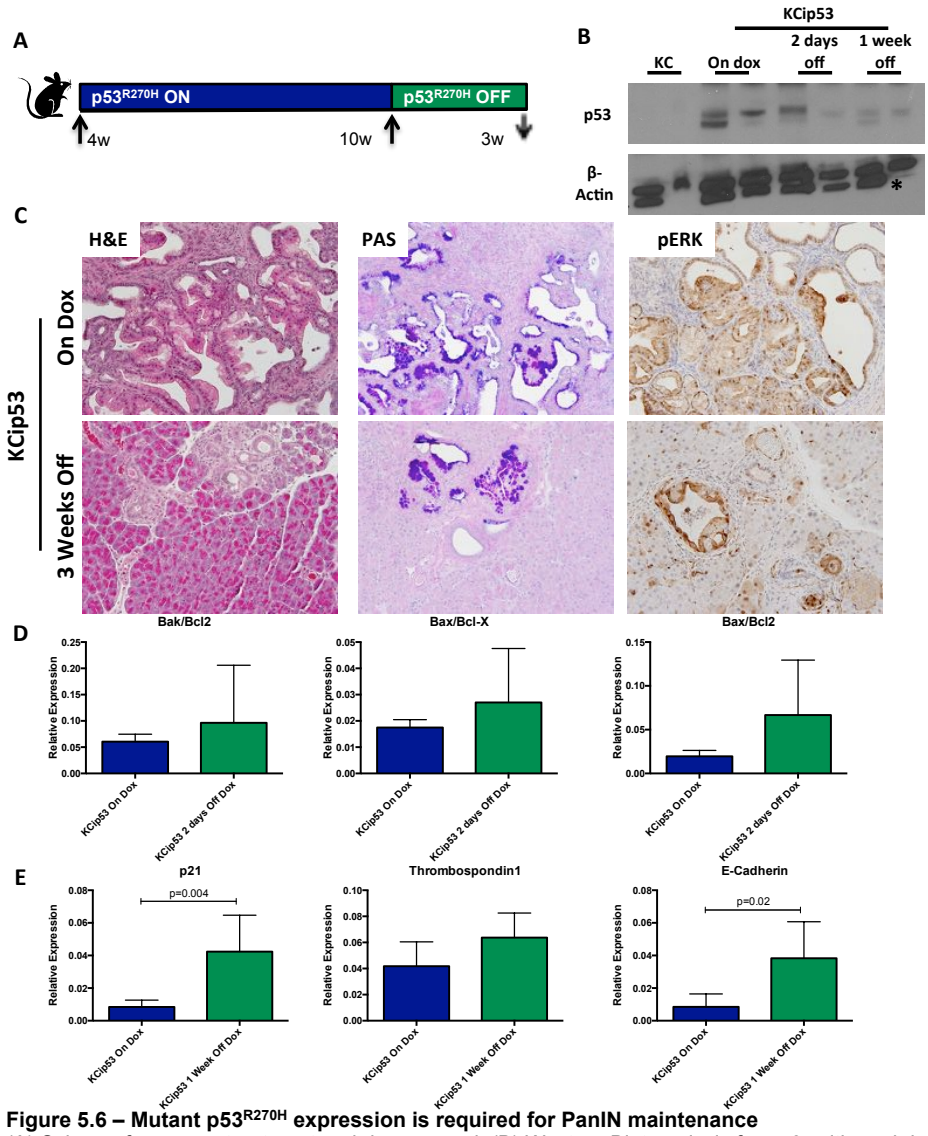


Figure 5.6 – Mutant p53^{R270H} expression is required for PanIN maintenance
(A) Scheme for mouse treatment and dox removal. (B) Western Blot analysis for p53, with each lane representing the lysate from an individual animal. * Indicates a non-specific band seen in the B-Actin blot. (C) H&E, PAS and IHC for pERK in KCip53 animals either on dox or 3 weeks off dox. (D) qRT-PCR analysis of pro- and anti-apoptotic factors in KCip53 on dox and KCip53 2 days off dox animals. (E) qRT-PCR analysis of targets of wild type p53 in KCip53 on dox and KCip53 1 week off dox animals.

A

B

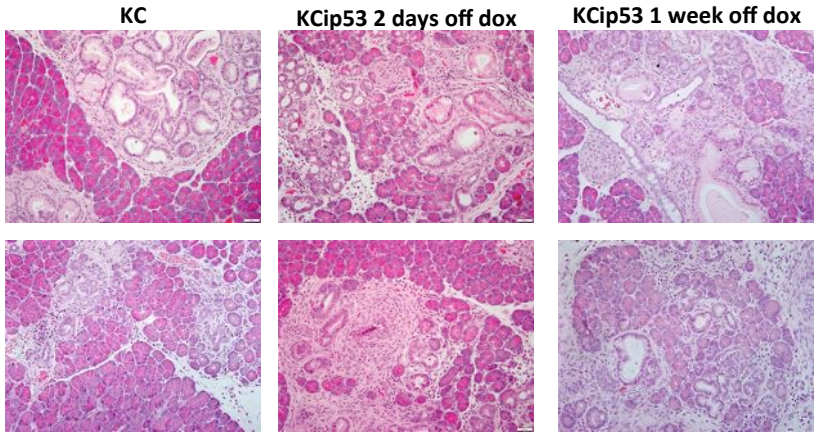


Figure 5.7 – KCip53 histology at two days and one week off dox
(A) Scheme for dox treatment in KCip53 animals. (B) H&E analysis of KC and KCip53 animals taken off dox.

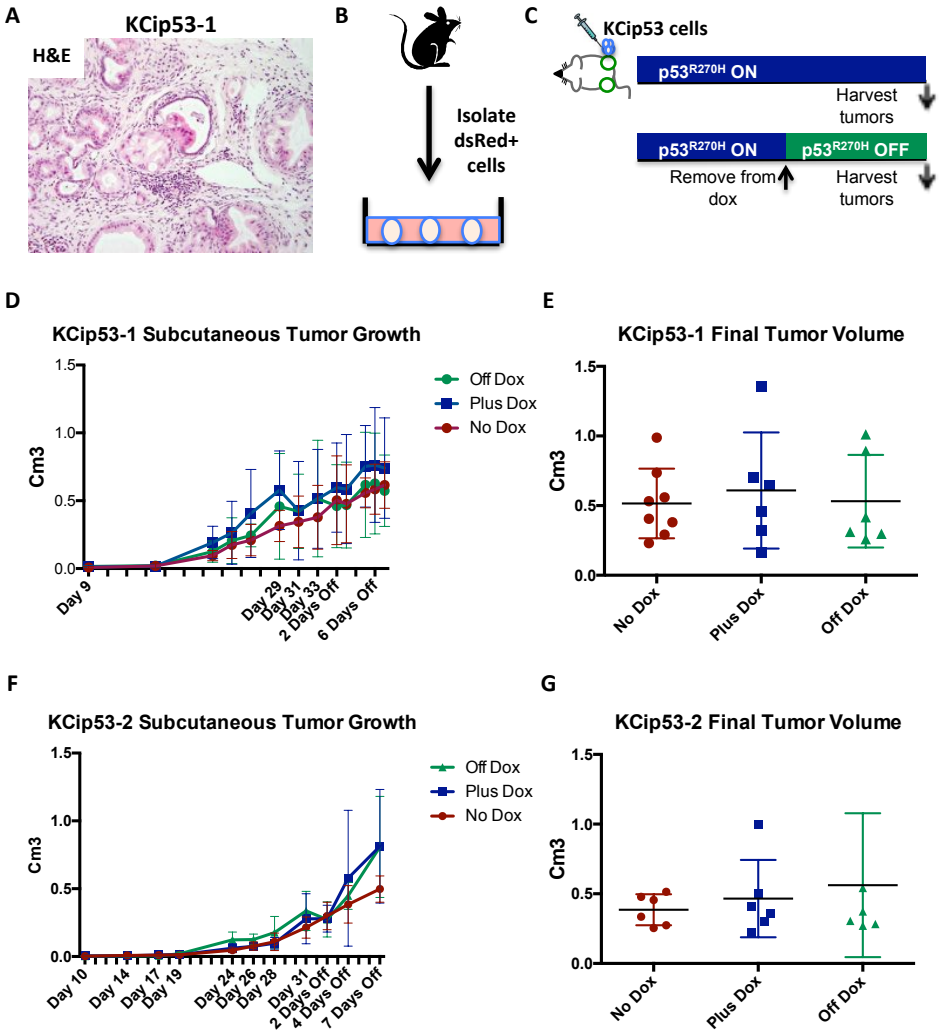


Figure 5.8 – Tumor growth is not affected by mutant p53^{R270H} expression
(A) H&E of original tumor from the animal from which cell line KCip53-1 was generated. (B) Scheme of cell line generation from KCip53 animals. (C) Scheme for subcutaneous tumor growth assay. (D) Subcutaneous tumor growth curve and (E) final tumor volume for KCip53-1 cell line. (F) Subcutaneous tumor growth curve and (G) final tumor volume for KCip53-2 cell line.

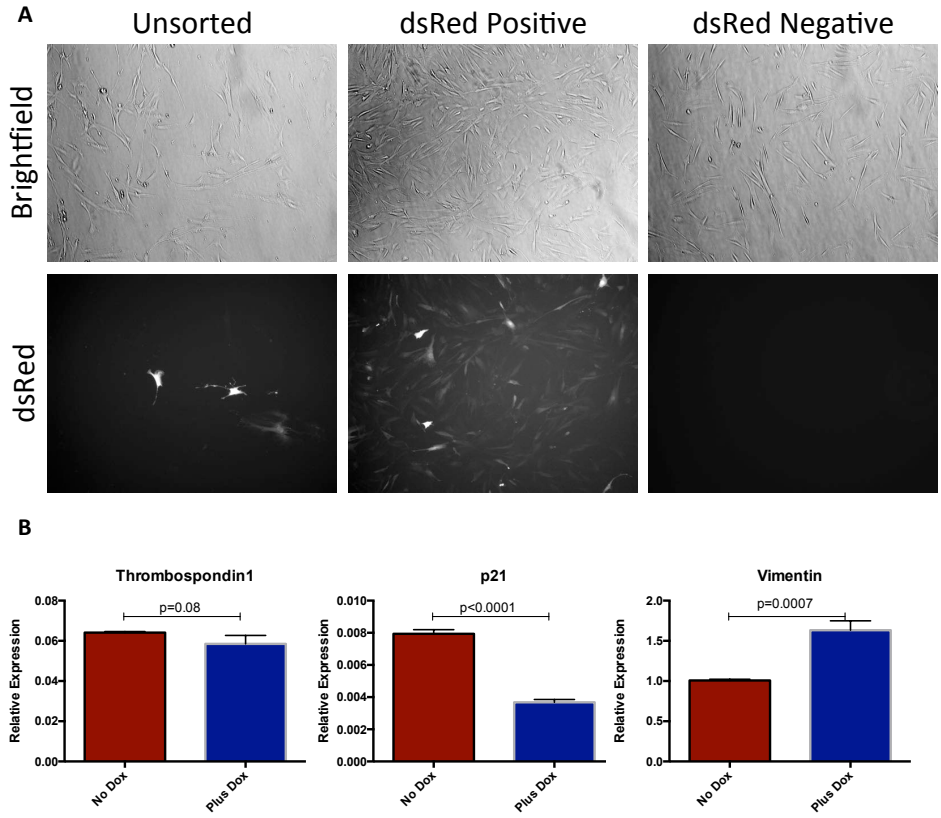


Figure 5.9 – KCip53-1 Cell line validation by dsRed and p53 gene target expression
(A) Brightfield and dsRed fluorescence images of KCip53-1 cells before and after cell sorting for dsRed expression. (B) qRT-PCR analysis for p53 targets in KCip53-1 cells grown with or without dox.

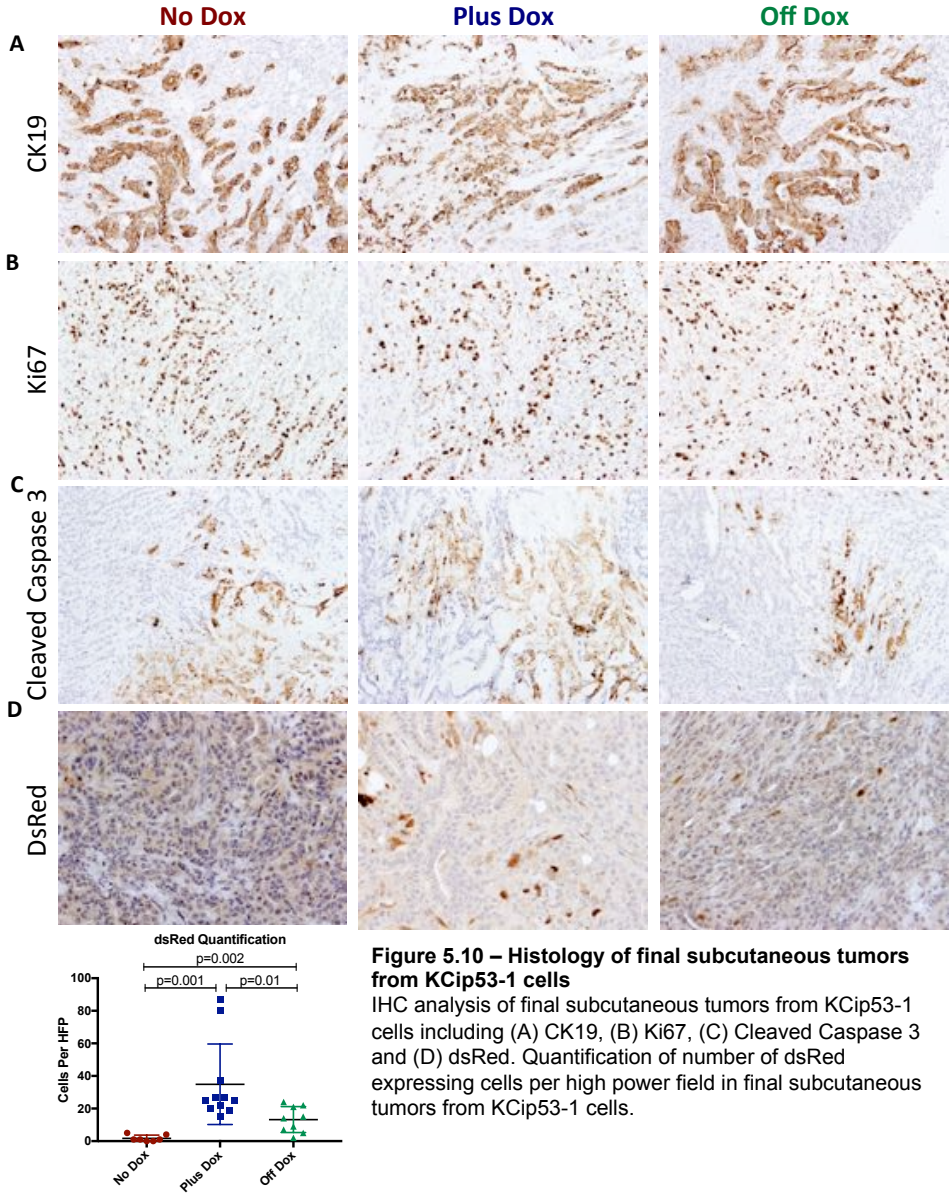


Figure 5.10 – Histology of final subcutaneous tumors from KCip53-1 cells
IHC analysis of final subcutaneous tumors from KCip53-1 cells including (A) CK19, (B) Ki67, (C) Cleaved Caspase 3 and (D) dsRed. Quantification of number of dsRed expressing cells per high power field in final subcutaneous tumors from KCip53-1 cells.

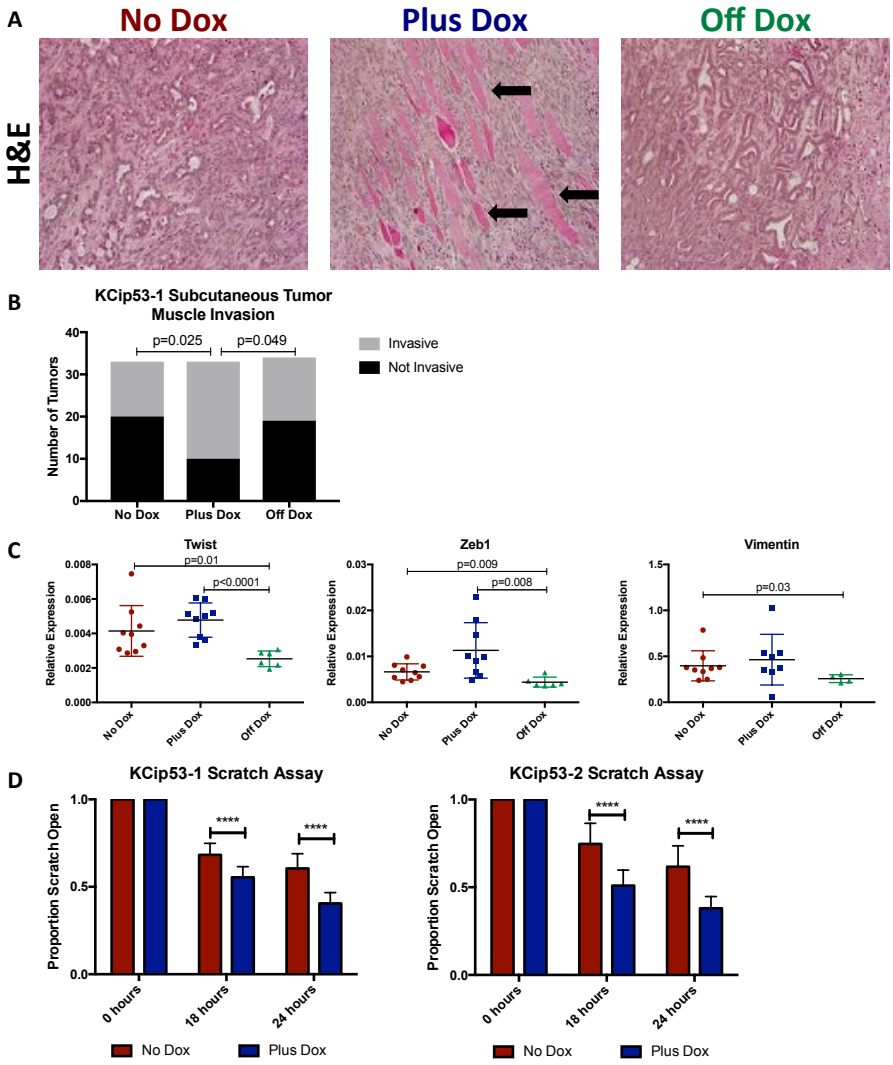


Figure 5.11 – KCip53 Cells on dox display more invasive and migratory characteristics than those not on dox

(A) H&E analysis of final subcutaneous tumors grown from KCip53-1 cells. Arrows indicate some muscle fibers within tumor. (B) Quantification of percentage of final tumors that have invasion into muscle by H&E. n=33 or 34 tumors per group. (C) qRT-PCR analysis of EMT associated gene expression in final subcutaneous tumors grown from KCip53-1 cells. (D) Scratch assay in KCip53-1 and KCip53-2 cell lines, shown as percentage original of scratch closed at specified timepoints.

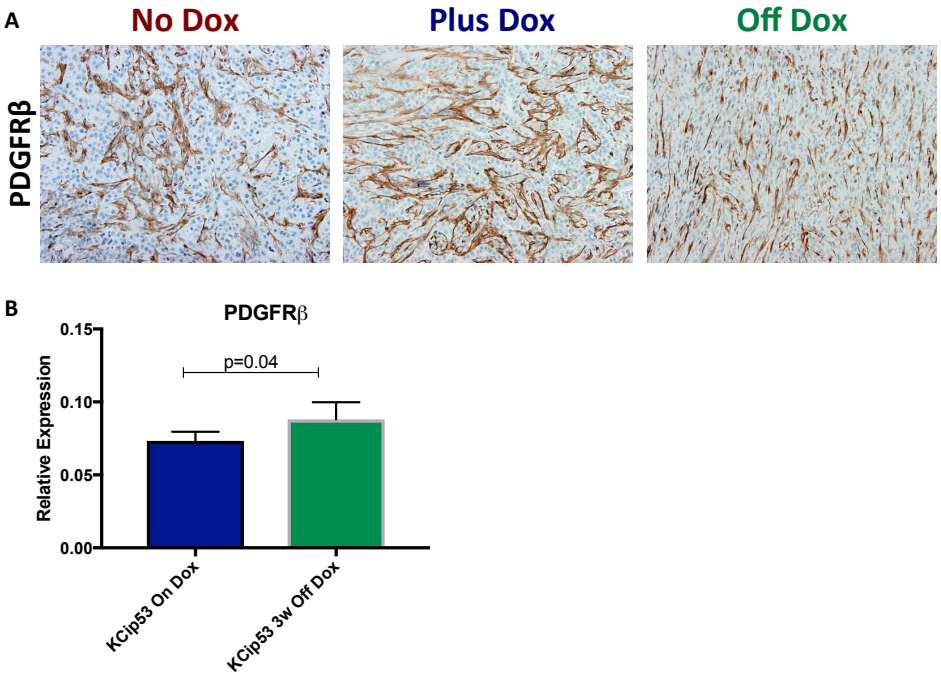


Figure 5.12 – KCip53 Cells on dox do not display increased levels of PDGFRb compared to those not on dox
(A) IHC analysis for PDGFRβ of final subcutaneous tumors grown from KCip53-2 cells. Arrows indicate some muscle fibers within tumor. (B) qRT-PCR analysis of PDGFRβ levels in KCip53 animals on dox compared to those removed from dox for 3 weeks.

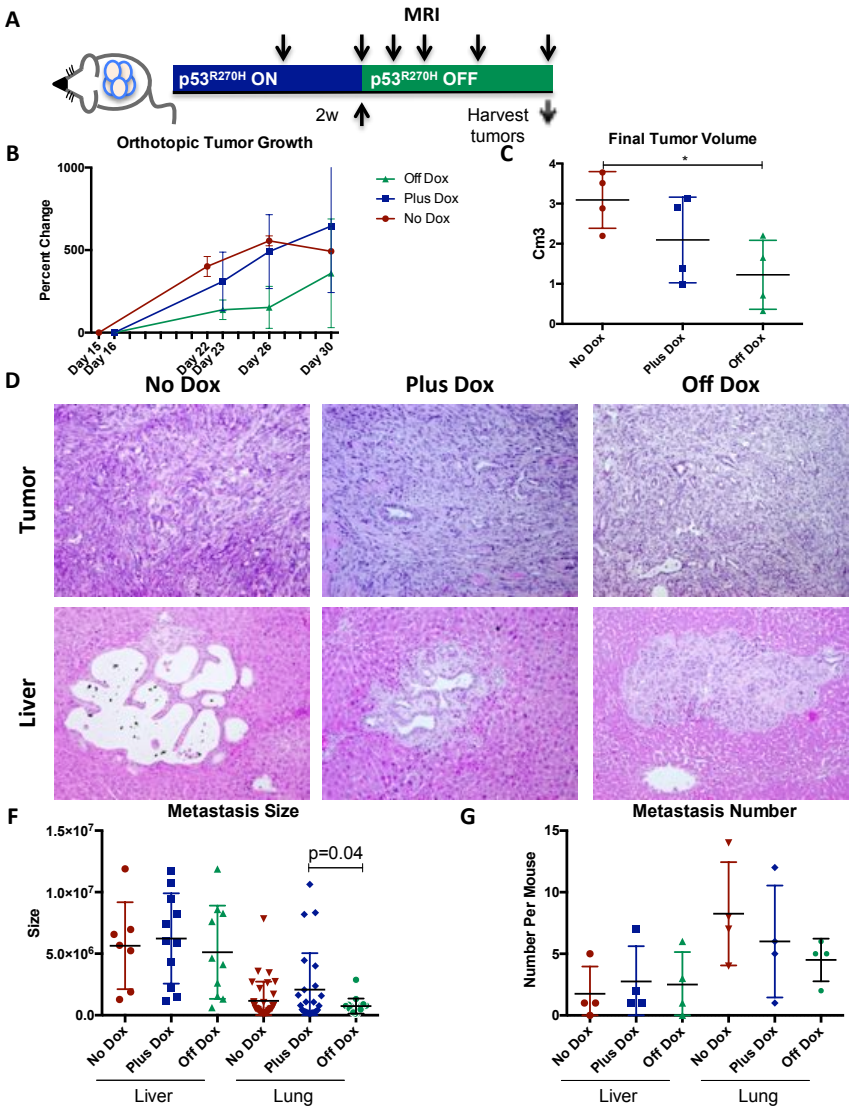


Figure 5.13 - Assessing the effect of mutant p53^{R270H} expression on orthotopic tumor growth

(A) Scheme for orthotopic tumor growth and MRI measurement using KCip53-1 cell lines. (B) Orthotopic tumor growth, measured by MRI. (C) Final tumor volume. (D) H&E for final orthotopic tumors and representative liver metastases. (E) Quantification of average metastasis size. (F) Quantification of average number of metastases.

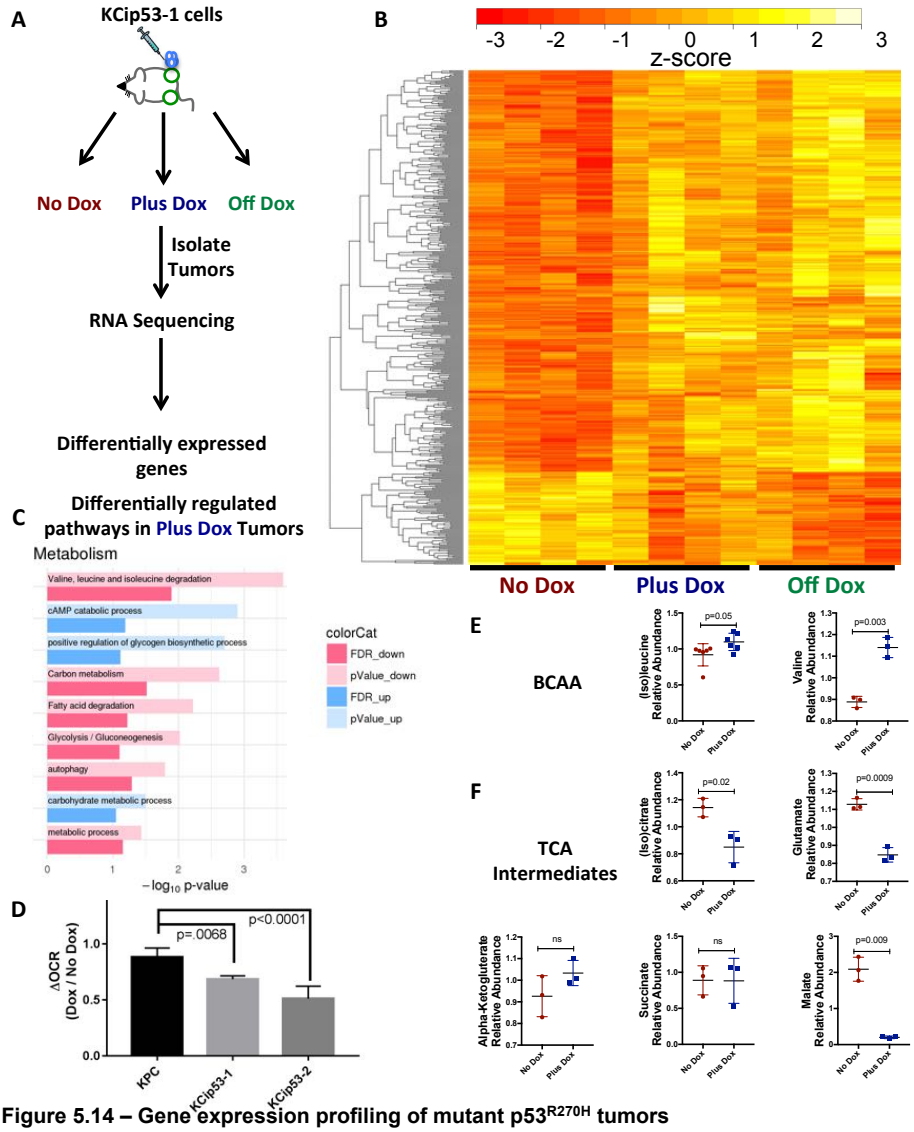


Figure 5.14 – Gene expression profiling of mutant p53^{R270H} tumors
(A) Scheme for subcutaneous tumor growth and RNA collection for RNA sequencing. (B) Heat map showing differential gene expression in tumor groups. (C) Pathway analysis of gene expression differences in “No dox” and “Plus dox” conditions. (D) Change is OCR levels as measured by mitochondrial stress test in KPC, KCip53-1 and KCip53-2 cells with or without dox. Difference of levels of (E) branched chain amino acids and (F) TCA intermediates in KCip53-1 cells grown with or without dox.

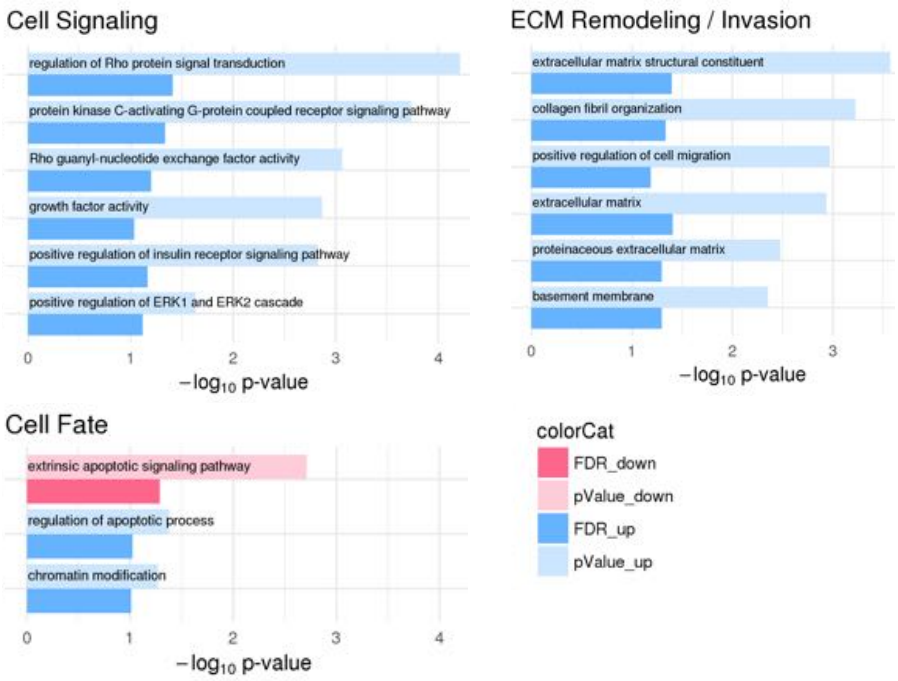


Figure 5.15 – Further Differentially Regulated Pathways in Plus Dox Tumors
Pathway analysis in “No dox” compared to “Plus dox” tumors.

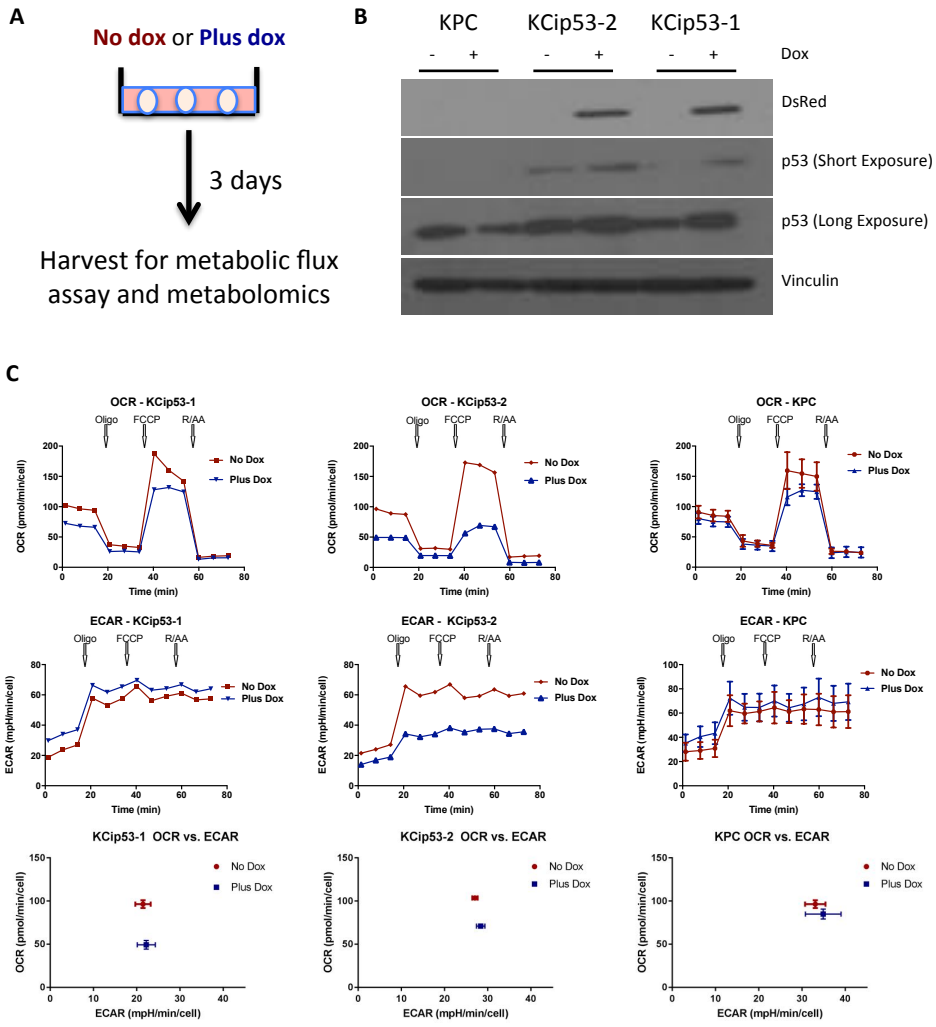


Figure 5.16 – Changes in OCR but not ECAR in KCip53 cell lines with p53^{R270H} expression
(A) Scheme for cell growth for metabolic flux assay (B) Western blot analysis of KPC, KCip53-1, and KCip53-2 cell lines for dsRed expression and p53 expression. (B) Traces showing change in OCR and ECAR during a mitochondrial stress test in KCip53-1, KCip53-2, and KPC cells grown with or without dox.

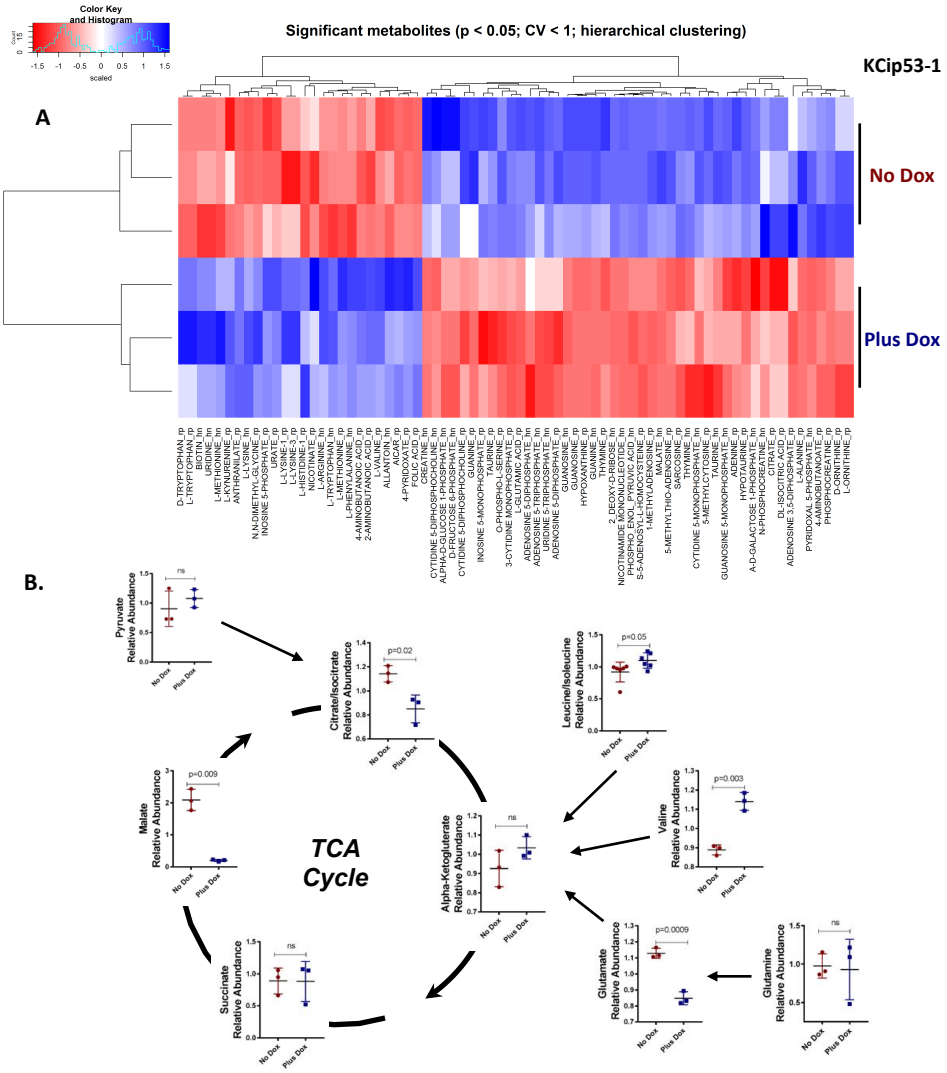


Figure 5.17 – Metabolomics Analysis of KCip53 cells
A. Heatmap of the significant metabolites changed in KCip53-1 cells under expression on $p53^{R270H}$ (Plus dox) or without (No Dox). **B.** Summary of TCA intermediates and substrates.

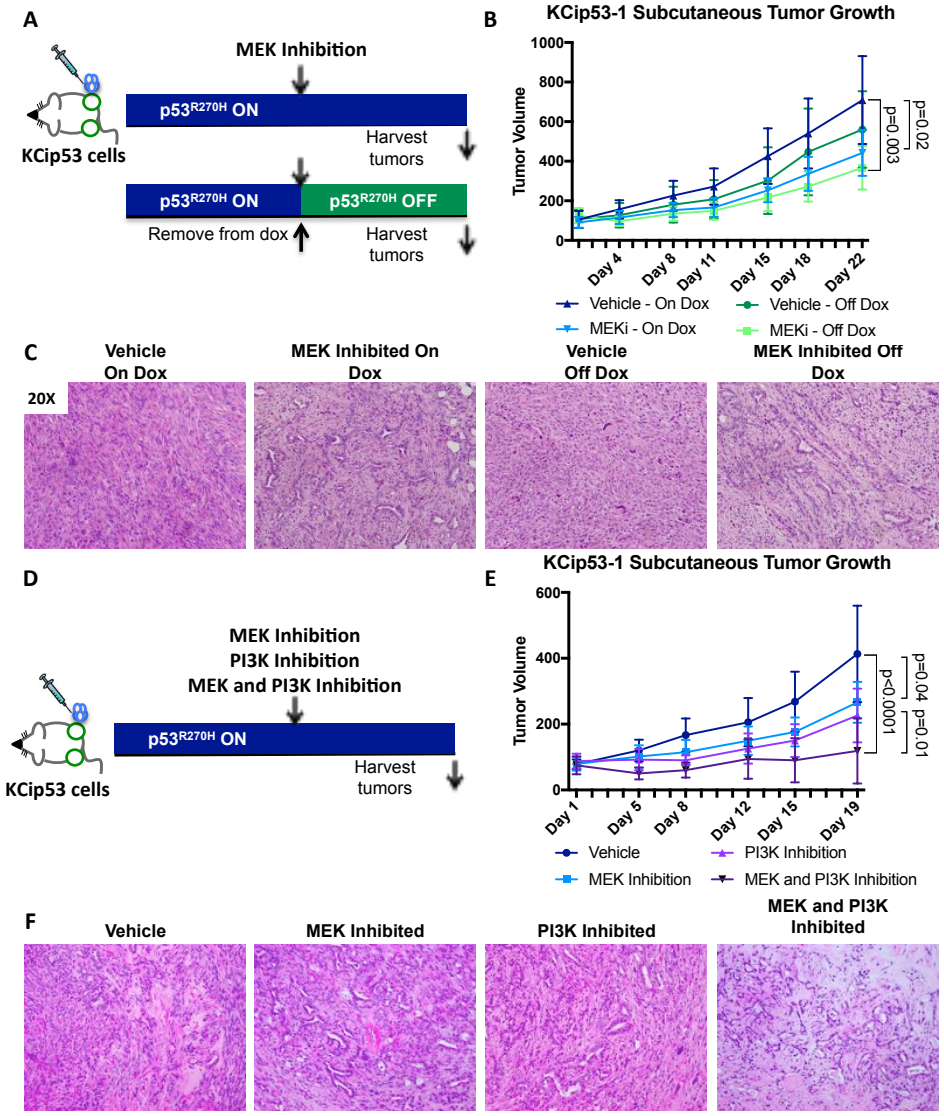


Figure 5.18 – Combination of mutant p53^{R270H} inactivation and MEK inhibition results in slower tumor growth

(A) Scheme for subcutaneous tumor growth and drug treatment. (B) Subcutaneous tumor growth curve for treatment groups using KCip53-1 cells. (C) H&E analysis of final tumors. (D) Scheme for subcutaneous tumor growth and drug treatment. (E) Subcutaneous tumor growth curve for treatment groups using KCip53-1 cells. (F) H&E analysis of final tumors.

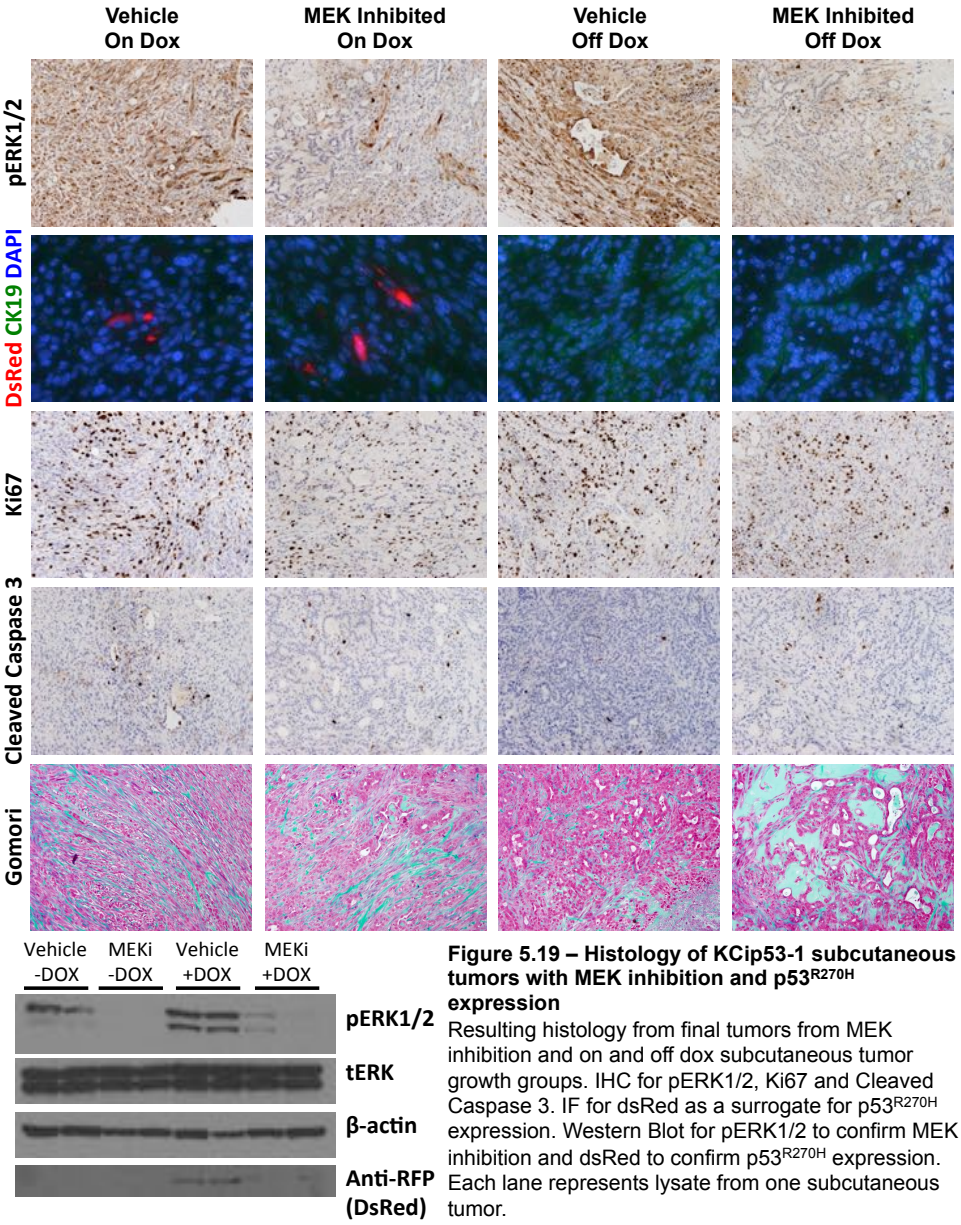
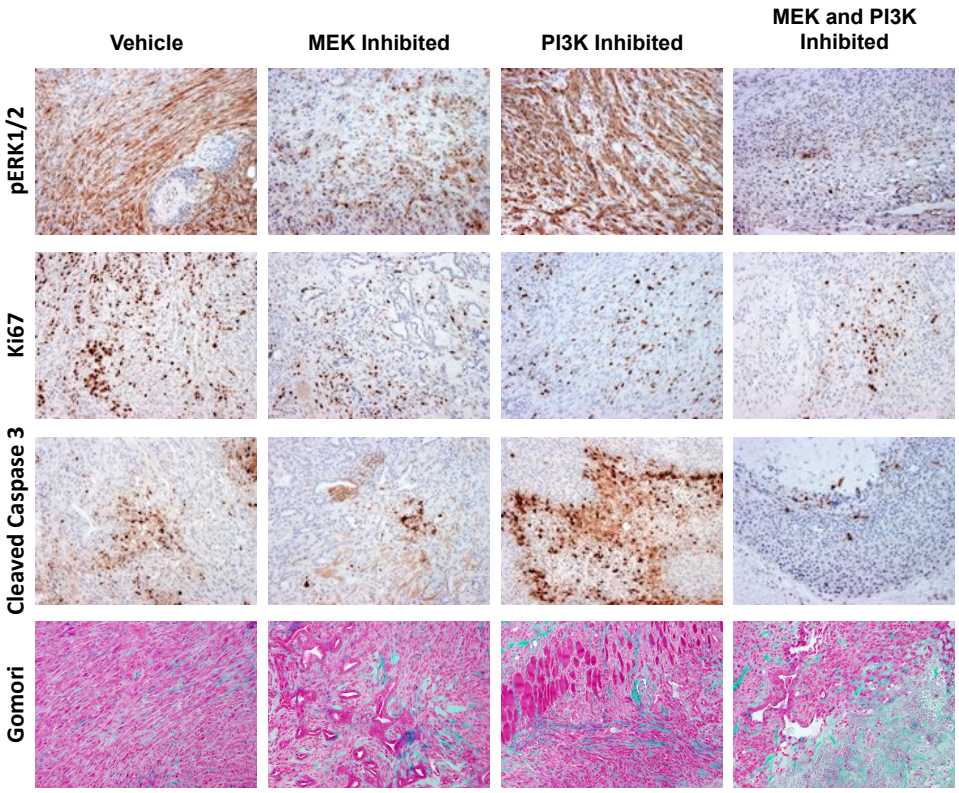


Figure 5.19 – Histology of KCip53-1 subcutaneous tumors with MEK inhibition and p53^{R270H} expression

Resulting histology from final tumors from MEK inhibition and on and off dox subcutaneous tumor growth groups. IHC for pERK1/2, Ki67 and Cleaved Caspase 3. IF for dsRed as a surrogate for p53^{R270H} expression. Western Blot for pERK1/2 to confirm MEK inhibition and dsRed to confirm p53^{R270H} expression. Each lane represents lysate from one subcutaneous tumor.



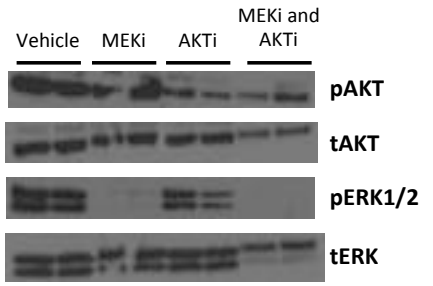


Figure 5.20 – Histology of KCip53-1 subcutaneous tumors with MEK and/or PI3K inhibition

Resulting histology from final tumors from MEK and/or PI3K inhibition in subcutaneous tumor growth groups. IHC for pERK1/2, Ki67 and Cleaved Caspase 3. Western Blot for pAKT and total AKT to confirm PI3K inhibition and pERK1/2 to confirm MEK inhibition.

Chapter Six

Discussion and Future Directions

Pancreatic cancer is a devastating disease, with a five-year-survival rate of just 9%(Rahib, Smith et al. 2014). It is crucial to understand the biology of how pancreatic cancer develops and progresses in order to identify effective options for future therapy. Sequencing information from human tumor samples has provided a picture of the genetic changes found in human pancreatic cancer. Using this data, introducing these changes into the pancreata of mice creates models that reliably recapitulate the human disease(Hingorani, Petricoin et al. 2003, Hingorani, Wang et al. 2005, Collins, Bednar et al. 2012, Collins, Brisset et al. 2012). Using mouse models of pancreatic cancer, studies can be performed that illuminate the genetic and environmental changes that lead of pancreatic cancer progression in ways that could not be achieved in humans, providing a more clear picture of the biology of the disease. My thesis work focused on using existing mouse models to explore the factors that contribute to the initiation of pancreatic cancer, as well as creating new mouse models for use as tools to discover new aspects of pancreatic cancer biology.

Defining the role of Bmi1 and HIF1a expression in pancreatic cancer initiation

Future Experiments

In Chapter Two I showed that expression of Bmi1 is required for the formation of pancreatic precancerous lesions using an oncogenic Kras driven mouse model of pan

creatic cancer. Using mouse pancreatic cancer cell lines, I showed that knockdown of Bmi1 in these cells led to increased levels of ROS, indicating that the requirement for Bmi1 expression during pancreatic cancer initiation may be due to its role in regulating ROS accumulation. In Chapter Three I explored the mechanism of Bmi1 requirement in the process of pancreatic cancer initiation. I observed that in pancreatic cancer cell lines with Bmi1 knockdown, levels of HIF1α were also reduced. Further, re-expression of HIF1α in animals lacking Bmi1 led to the formation of PanINs, indicating that Bmi1 control of HIF1α levels may be the reason for Bmi1 requirement during pancreatic cancer initiation.

The studies in Chapters Two and Three helped define the role of Bmi1 and its control of HIF1α during pancreatic carcinogenesis, however detailed studies of the mechanism of Bmi1 action were limited by the reagents used to perform the experiments. Mechanistic studies were limited by the fact that Bmi1 knockdown in mouse pancreatic cancer cell lines was performed using siRNA, a temporary method with knockdown that only lasts for a couple of days. Given this, it was difficult to assess the true role of Bmi1 expression in cells. For future experiments, the availability of newer reagents will help to change this, and provide further insights into Bmi1 function in pancreatic cancer.

Using the CRISPR/Cas9 system, we have created mouse pancreatic cancer cell lines that have permanent deletion of Bmi1 at the genetic level (Figure 6.1A). This allows direct comparison of pancreatic cancer cells that have Bmi1 expressed to those that do not, and the ability to perform cellular assays to determine Bmi1 function in this context. Using these cells, we see that permanent lack of Bmi1 leads to lower levels of

HIF1α expression, mirroring what is seen using siRNA and suggesting that the CRISPR/Cas9 knockout of Bmi1 recapitulates what we see in other experiments (Figure 6.1B). Similarly, when grown in hypoxia, pancreatic cancer cells lacking Bmi1 show lower levels of expression of HIF1α target genes, suggesting a functional decrease in HIF pathway output (Figure 6.1C). Further experiments show that Bmi1 deletion leads to lower levels of proliferation compared to control pancreatic cancer cells (Figure 6.2). Additionally, when injected subcutaneously into syngeneic mice, pancreatic cancer cells with Bmi1 knockdown grow more slowly than those with Bmi1 expressed (Figure 6.3A-C). Future experiments will test whether re-expression of HIF1α in these cell lines lacking Bmi1 rescues these observed phenotypes.

The creation of murine pancreatic cancer cell lines that permanently lack Bmi1 expression creates the opportunity to explore some of the open questions regarding the role of Bmi1 and HIF1α during pancreatic cancer initiation. Namely, given the changes observed in metabolic enzymes using mouse models, these cell lines will be used to further define metabolic changes. Metabolomics analysis comparing pancreatic cancer cell lines with and without Bmi1 expression will give a more comprehensive view of the difference in metabolite levels caused by Bmi1 knockout. Reintroduction of changed metabolites and analysis of whether this results in rescue of observed phenotypes, such as cell proliferation, may help elucidate specifically what changes at the cellular level are the cause for Bmi1 requirement in pancreatic cancer initiation.

Open Questions

Some remaining questions pertaining to the role of Bmi1 and HIF1α in the initiation of pancreatic cancer are not as easily answered using CRISPR/Cas9 knockout of Bmi1. Recently, it has been shown that HIF1α expression in pancreatic cancer has an impact on immune cell populations(Lee, Spata et al. 2016). This prompts the question of whether lack of Bmi1 expression, and its subsequent effects on HIF1α, changes the immune microenvironment of the precancerous pancreas. Flow cytometry analysis of the pancreata of these animals may reveal differences in immune cell populations, which are critical to pancreatic cancer development(Neesse, Algul et al. 2015). While our current data suggests that Bmi1 and HIF1α interact to control the metabolic state of the pancreatic cancer cells, it is possible that this interaction affects multiple aspects of carcinogenesis.

Lastly, it remains unknown whether the Bmi1/HIF1α collaboration gives us any valuable insight into pancreatic cancer treatment. Given that Bmi1 is required for pancreatic cancer initiation, it is possible that Bmi1 inhibitors may slow pancreatic cancer growth. A commercially available Bmi1 inhibitor has been used previously(Mayr, Wagner et al. 2016), but in our experiments did not specifically inhibit Bmi1. The development of Bmi1 inhibitors is ongoing in other contexts, and when they are available could be used to test whether Bmi1 inhibition delays pancreatic cancer growth. The effect of inhibiting Bmi1 in established tumors, as would be the case in human pancreatic cancer treatment, could be assessed using the newer KPF mouse model(Schonhuber, Seidler et al. 2014). KPF mice express oncogenic Kras and mutant p53, both driven by flp recombinase. These would be combined with a temporally activatable CreERT allele and Bmi1 floxed. When the KPF mice developed tumors, Cre

could be activated, deleting Bmi1 expression. Assessment of the resulting tumors over time would show the outcome of Bmi1 deletion in established tumors, and provide a basis for future drug treatment in human pancreatic cancer.

Overall, Bmi1 seems to be required in pancreatic cancer through its control of HIF1α expression. Newer techniques will provide ways to determine the exact cellular mechanisms of Bmi1 and HIF1α action in pancreatic cancer. Additionally, this work raises the question of whether Bmi1 inhibition would be a valid therapeutic option in human pancreatic cancer, although some experiments remain in order to move forward with this strategy.

HIF2a stabilization as a mouse model of pancreatic disease

A new model of chronic pancreatitis and MCN

In Chapter Four of this work, I detail the effects of pancreatic HIF2α stabilization on the murine pancreas. I show that stabilization of HIF2α in the pancreas results in a phenotype that mimics human chronic pancreatitis, including inflammatory infiltrates and extensive fibrosis. With the additional expression of oncogenic Kras, HIF2α stabilization results in the development of mucinous cystic neoplasm (MCN), a less common precursor lesion of human pancreatic cancer. These observations are very important because they detail new mouse models that provide the opportunity to further explore these human conditions. Chronic pancreatitis is a common human condition with many causes, including alcohol use, autoimmune conditions and tumors(Etemad and Whitcomb 2001). Chronic pancreatitis is characterized by irreversible damage to the pancreas, distinguishing it from acute pancreatitis, a temporary inflammatory injury from

which the pancreas recovers to normal(Banks, Freeman et al. 2006). There are many experimental procedures that mimic chronic pancreatitis, including repeated injections of caerulein or pancreatic ductal ligation(Lerch and Gorelick 2013). However, at the end of each of these procedures the murine pancreas eventually recovers from the insult, in contract to human chronic pancreatitis. Importantly, HIF2α stabilized animals are the first model of chronic pancreatitis where the pancreas does not eventually recover to normal histology, more closely resembling the human condition than other existing models. This provides a new tool to use to study the biology of chronic pancreatitis and understand the etiology of and potential treatments for this disease.

In the context of oncogenic Kras, HIF2α stabilized animals develop MCN, a cystic lesion of the pancreas. In humans these lesions can be benign or malignant, complicating their prognosis and treatment(Tanaka, Chari et al. 2006, Testini, Gurrado et al. 2010). Given this variability in MCN characteristics, it is important to develop models of this condition in order to better understand its development and progression. Very few other mouse models of MCN have been described(Izeradjene, Combs et al. 2007, Sano, Driscoll et al. 2014), highlighting the importance of finding new ways to model this lesion. KC;HIF2α animals are a new addition to this group, providing further insight into the mechanism of MCN development.

Open Questions

Given that HIF2α stabilization in the murine pancreas results in the development of chronic pancreatitis or MCN (in the context of oncogenic Kras), the question remains of whether this role is recapitulated in the human pancreas. Further experiments in

human systems or with existing human data can illuminate whether HIF2α expression has a causative or prognostic role in human chronic pancreatitis or MCN. In Chapter Four I showed that in lysates from human chronic pancreatitis samples there is upregulation of HIF2α compared to the normal human pancreas. This relationship however should be explored further. Rare human cases of gain-of-function HIF2α mutations have been described(Percy, Furlow et al. 2008, Zhuang, Yang et al. 2012), however in these cases no evaluation of the pancreas was performed. It is possible that these patients have mild chronic pancreatitis or a condition that develops later in life, and histologic analysis of pancreatic biopsies would illuminate whether HIF2α expression in humans leads to chronic pancreatitis. Conversely, chronic pancreatitis is of idiopathic origin in up to 25% of human cases(Etemad and Whitcomb 2001). Given the fact that HIF2α stabilization causes chronic pancreatitis in mice, it is possible that a portion of these chronic pancreatitis cases with unknown origin arise from HIF2α stabilizing mutations. HIF2α inhibitors are an area of active development(Wallace, Rizzi et al. 2016), and so if HIF2α blockade could alleviate some cases of chronic pancreatitis, this could help a portion of patients.

This work also raises the question of the role of HIF2α in human MCN. Given that KC;HIF2α animals develop MCN, it is possible that HIF2α stabilization in the human pancreas is a cause of MCN. A first experiment to help determine this relationship would be to examine HIF2α expression in human MCN samples. HIF2α expression in MCN, but not normal pancreas or other pancreatic lesions, would suggest that HIF2α expression plays a role in human MCN development. Another question would be whether expression of HIF2α in MCN plays a prognostic role in the disease. MCN is a

condition that can be benign or malignant, however the differences between these two types are not well understood and therefore treatment decisions can be difficult(Jana, Shroff et al. 2015). Evaluation of whether HIF2α expression is a marker for malignancy could help differentiate between benign and malignant MCN and help choose more appropriate treatment options.

Overall, mice with pancreatic stabilization of HIF2α provide an important new animal model of two human pancreatic diseases, chronic pancreatitis and mucinous cystic neoplasm. Previous to the development of these mice there were no animal systems that accurately modeled chronic pancreatitis, and so HIF2α stabilized mice will provide an important tool for studying this condition. Similarly, there have been very few models of MCN described previously, so KC;HIF2α stabilized mice help understand this disease and provide more mechanistic insight into its development. Beyond the role of HIF2α stabilization to create animal models, it will next be important to determine whether HIF2α has a functional role in human pancreatic disease. Evaluation of humans with HIF2α stabilizing mutations will illuminate whether HIF2α expression is a causative factor in chronic pancreatitis development. Additional evaluation of HIF2α expression in human MCN samples may provide a biomarker for MCN development or progression.

Modeling common p53 point mutations and their role in pancreatic cancer initiation and progression

Future Experiments

In Chapter Five I developed a new mouse system to model the effects of expression of mutant $p53^{R270H}$, a mutation commonly found in human pancreatic cancer

that has not been explored experimentally in animals. Importantly, this model, termed KCip53, permitted for expression of mutant $p53^{R270H}$ in an inducible and reversible manner, allowing for specific studies into the role of $p53^{R270H}$ in pancreatic cancer initiation and progression. We found that expression of $p53^{R270H}$, in the context of mutant Kras, leads to the formation of metastatic pancreatic tumors. Using KCip53 animals we found that expression of $p53^{R270H}$ promotes formation of PanINs and its continued expression is required for PanIN maintenance. In later tumor stages, use of KCip53 cell lines suggested that mutant $p53^{R270H}$ expression does not have an effect on tumor growth or metastasis. Further profiling of gene expression signature and levels of metabolites revealed differences in cells with or without $p53^{R270H}$ expression at the cellular level.

Although the use of KCip53 mice has revealed many aspects of mutant $p53^{R270H}$ function in pancreatic cancer, questions still remain. One major subject for further exploration is the mechanism of $p53^{R270H}$ action in pancreatic cancer initiation and progression. Global gene expression analysis using RNA sequencing revealed differences in many cellular pathways between subcutaneous tumors grown with or without mutant $p53^{R270H}$ expression. However which of these changes causes the functional difference in tumor characteristics is still unknown. One candidate is changes in metabolism due to mutant $p53^{R270H}$ expression. Metabolomics analysis in cell lines grown with or without $p53^{R270H}$ expression revealed different levels of metabolites between the two conditions, including those involved in the TCA cycle. It remains to be determined whether these changes are the cause for the role of mutant $p53^{R270H}$ in the progression of pancreatic cancer. Future experiments may help determine this

relationship. Given that metabolomics analysis was performed in cell lines, it will next be important to determine whether the same changes occur in tumors. Additionally, re-addition of changed metabolites to the media of tumor cells and downstream phenotypic analysis can help determine whether controlling changes in metabolism is a major role for $p53^{R270H}$ in pancreatic cancer.

Another remaining question is the surprising finding that $p53^{R270H}$ does not promote tumor growth or metastasis. In other cancer systems expression of $p53^{R270H}$ promotes cancer progression(Olive, Tuveson et al. 2004, Jackson, Olive et al. 2005) and in pancreatic cancer p53 mutation or deletion promotes tumor growth and metastasis(Hingorani, Wang et al. 2005, Morton, Timpson et al. 2010). It is possible that $p53^{R270H}$ does not have a role in later tumor stages, or it is possible that there are limitations to our current experimental system. It is well known that in pancreatic cancer interactions between the tumor cells and the immune environment play an important role in tumor progression(Olive, Tuveson et al. 2004, Jackson, Olive et al. 2005). Given that KCip53 mice were developed on a mixed background, the subcutaneous and orthotopic tumor growth experiments were performed in immunocompromised mice, removing potential interactions between the tumor cells and immune cells. KCip53 animals should be backcrossed onto a pure mouse background and new cell lines developed so that subcutaneous tumor growth experiments can be performed in immunocompetent, syngeneic animals. However, this is a very time consuming process, and so alternative methods to access the interaction between the immune environment and mutant $p53^{R270H}$ expression should be considered. Preliminary analysis of gene expression in KCip53 pancreata suggests changes in macrophage differentiation status

and CD8 T-Cell activation in animals with mutant $p53^{R270H}$ turned off, indicating that $p53^{R270H}$ expression may induce changes in the immune microenvironment of pancreatic cancer (Figure 6.4A,B). Further experiments, including flow cytometry analysis of immune cell populations in KCip53 pancreata both with and without $p53^{R270H}$ expression, may help reveal a role for $p53^{R270H}$ in pancreatic cancer progression.

Open Questions

More generally, KCip53 mice are a model for a common p53 mutation found in human pancreatic cancer that has not previously been explored experimentally. Almost all experimental models in pancreatic cancer have relied on a very small subset of mutations, which is not consistent with the reality of human pancreatic cancer. Given that several recent studies have identified subtypes of pancreatic cancer that correspond to different genetic signatures(Collisson, Sadanandam et al. 2011, Moffitt, Marayati et al. 2015, Waddell, Pajic et al. 2015, Bailey, Chang et al. 2016), it is possible that different tumor types have unique responses to therapy. In the future it will become important to develop methods to model different combinations of human mutations in order to elucidate these differences. New developments in Crispr/Cas9 technology should make this process much faster and easier than previous methods, allowing creation of many models that recapitulate the wide spectrum of mutations found in human pancreatic tumors.

Summary

Overall, this work explored aspects of the initiation and progression of pancreatic cancer using both existing and newly developed mouse models of the disease. In Chapters Two and Three I found that Bmi1 expression is required for pancreatic cancer initiation, and that this is likely due to Bmi1 control of HIF1α expression. In Chapter Four I found that pancreatic HIF2α stabilization leads to chronic pancreatitis and MCN in the context of oncogenic Kras, providing valuable new models of less well understood pancreatic conditions. In Chapter Five I developed a mouse model of mutant $p53^{R270H}$ in pancreatic cancer, a mutation found commonly in humans but not previously modeled in animals. Taken together, this work demonstrates the wide array of possible uses of mouse models in pancreatic cancer research, as well as provides new functional insights into the mechanisms of pancreatic cancer initiation and progression. The use of mouse models in research is a critical tool to understand the biology of human pancreatic cancer, and hopefully provides the basis for the development of future effective therapeutic options.

References

Bailey, P., D. K. Chang, K. Nones, A. L. Johns, A. M. Patch, M. C. Gingras, D. K. Miller, A. N. Christ, T. J. Bruxner, M. C. Quinn, C. Nourse, L. C. Murtaugh, I. Harliwong, S. Idrisoglu, S. Manning, E. Nourbakhsh, S. Wani, L. Fink, O. Holmes, V. Chin, M. J. Anderson, S. Kazakoff, C. Leonard, F. Newell, N. Waddell, S. Wood, Q. Xu, P. J. Wilson, N. Cloonan, K. S. Kassahn, D. Taylor, K. Quek, A. Robertson, L. Pantano, L. Mincarelli, L. N. Sanchez, L. Evers, J. Wu, M. Pinese, M. J. Cowley, M. D. Jones, E. K.

Colvin, A. M. Nagrial, E. S. Humphrey, L. A. Chantrill, A. Mawson, J. Humphris, A. Chou, M. Pajic, C. J. Scarlett, A. V. Pinho, M. Giry-Laterriere, I. Rooman, J. S. Samra, J. G. Kench, J. A. Lovell, N. D. Merrett, C. W. Toon, K. Epari, N. Q. Nguyen, A. Barbour, N. Zeps, K. Moran-Jones, N. B. Jamieson, J. S. Graham, F. Duthie, K. Oien, J. Hair, R. Grutzmann, A. Maitra, C. A. Iacobuzio-Donahue, C. L. Wolfgang, R. A. Morgan, R. T. Lawlor, V. Corbo, C. Bassi, B. Rusev, P. Capelli, R. Salvia, G. Tortora, D. Mukhopadhyay, G. M. Petersen, I. Australian Pancreatic Cancer Genome, D. M. Munzy, W. E. Fisher, S. A. Karim, J. R. Eshleman, R. H. Hruban, C. Pilarsky, J. P. Morton, O. J. Sansom, A. Scarpa, E. A. Musgrove, U. M. Bailey, O. Hofmann, R. L. Sutherland, D. A. Wheeler, A. J. Gill, R. A. Gibbs, J. V. Pearson, N. Waddell, A. V. Biankin and S. M. Grimmond (2016). "Genomic analyses identify molecular subtypes of pancreatic cancer." Nature **531**(7592): 47-52.

Banks, P. A., M. L. Freeman and G. Practice Parameters Committee of the American College of (2006). "Practice guidelines in acute pancreatitis." Am J Gastroenterol **101**(10): 2379-2400.

Collins, M. A., F. Bednar, Y. Zhang, J. C. Brisset, S. Galban, C. J. Galban, S. Rakshit, K. S. Flannagan, N. V. Adsay and M. Pasca di Magliano (2012). "Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice." J Clin Invest **122**(2): 639-653.

Collins, M. A., J. C. Brisset, Y. Zhang, F. Bednar, J. Pierre, K. A. Heist, C. J. Galban, S. Galban and M. P. di Magliano (2012). "Metastatic pancreatic cancer is dependent on oncogenic Kras in mice." PLoS One **7**(12): e49707.

Collisson, E. A., A. Sadanandam, P. Olson, W. J. Gibb, M. Truitt, S. Gu, J. Cooc, J. Weinkle, G. E. Kim, L. Jakkula, H. S. Feiler, A. H. Ko, A. B. Olshen, K. L. Danenberg, M. A. Tempero, P. T. Spellman, D. Hanahan and J. W. Gray (2011). "Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy." Nat Med **17**(4): 500-503.

Etemad, B. and D. C. Whitcomb (2001). "Chronic pancreatitis: diagnosis, classification, and new genetic developments." Gastroenterology **120**(3): 682-707.

Hingorani, S. R., E. F. Petricoin, A. Maitra, V. Rajapakse, C. King, M. A. Jacobetz, S. Ross, T. P. Conrads, T. D. Veenstra, B. A. Hitt, Y. Kawaguchi, D. Johann, L. A. Liotta, H. C. Crawford, M. E. Putt, T. Jacks, C. V. Wright, R. H. Hruban, A. M. Lowy and D. A. Tuveson (2003). "Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse." Cancer Cell **4**(6): 437-450.

Hingorani, S. R., L. Wang, A. S. Multani, C. Combs, T. B. Deramaudt, R. H. Hruban, A. K. Rustgi, S. Chang and D. A. Tuveson (2005). "Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice." Cancer Cell **7**(5): 469-483.

Izeradjene, K., C. Combs, M. Best, A. Gopinathan, A. Wagner, W. M. Grady, C. X. Deng, R. H. Hruban, N. V. Adsay, D. A. Tuveson and S. R. Hingorani (2007). "Kras(G12D) and Smad4/Dpc4 haploinsufficiency cooperate to induce mucinous cystic neoplasms and invasive adenocarcinoma of the pancreas." Cancer Cell **11**(3): 229-243.

Jackson, E. L., K. P. Olive, D. A. Tuveson, R. Bronson, D. Crowley, M. Brown and T. Jacks (2005). "The differential effects of mutant p53 alleles on advanced murine lung cancer." Cancer Res **65**(22): 10280-10288.

Jana, T., J. Shroff and M. S. Bhutani (2015). "Pancreatic cystic neoplasms: Review of current knowledge, diagnostic challenges, and management options." J Carcinog **14**: 3.

Lee, K. E., M. Spata, L. J. Bayne, E. L. Buza, A. C. Durham, D. Allman, R. H. Vonderheide and M. C. Simon (2016). "Hif1a Deletion Reveals Pro-Neoplastic Function of B Cells in Pancreatic Neoplasia." Cancer Discov **6**(3): 256-269.

Lerch, M. M. and F. S. Gorelick (2013). "Models of acute and chronic pancreatitis." Gastroenterology **144**(6): 1180-1193.

Mayr, C., A. Wagner, M. Loeffelberger, D. Bruckner, M. Jakab, F. Berr, P. Di Fazio, M. Ocker, D. Neureiter, M. Pichler and T. Kiesslich (2016). "The BMI1 inhibitor PTC-209 is a potential compound to halt cellular growth in biliary tract cancer cells." Oncotarget **7**(1): 745-758.

Moffitt, R. A., R. Marayati, E. L. Flate, K. E. Volmar, S. G. Loeza, K. A. Hoadley, N. U. Rashid, L. A. Williams, S. C. Eaton, A. H. Chung, J. K. Smyla, J. M. Anderson, H. J. Kim, D. J. Bentrem, M. S. Talamonti, C. A. Iacobuzio-Donahue, M. A. Hollingsworth and J. J. Yeh (2015). "Virtual microdissection identifies distinct tumor- and stroma-specific subtypes of pancreatic ductal adenocarcinoma." Nat Genet **47**(10): 1168-1178.

Morton, J. P., P. Timpson, S. A. Karim, R. A. Ridgway, D. Athineos, B. Doyle, N. B. Jamieson, K. A. Oien, A. M. Lowy, V. G. Brunton, M. C. Frame, T. R. Evans and O. J. Sansom (2010). "Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer." Proc Natl Acad Sci U S A **107**(1): 246-251.

Neesse, A., H. Algul, D. A. Tuveson and T. M. Gress (2015). "Stromal biology and therapy in pancreatic cancer: a changing paradigm." Gut **64**(9): 1476-1484.

Olive, K. P., D. A. Tuveson, Z. C. Ruhe, B. Yin, N. A. Willis, R. T. Bronson, D. Crowley and T. Jacks (2004). "Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome." Cell **119**(6): 847-860.

Percy, M. J., P. W. Furlow, G. S. Lucas, X. Li, T. R. Lappin, M. F. McMullin and F. S. Lee (2008). "A gain-of-function mutation in the HIF2A gene in familial erythrocytosis." N Engl J Med **358**(2): 162-168.

Rahib, L., B. D. Smith, R. Aizenberg, A. B. Rosenzweig, J. M. Fleshman and L. M. Matrisian (2014). "Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States." Cancer Res **74**(11): 2913-2921.

Sano, M., D. R. Driscoll, W. E. De Jesus-Monge, D. S. Klimstra and B. C. Lewis (2014). "Activated wnt signaling in stroma contributes to development of pancreatic mucinous cystic neoplasms." Gastroenterology **146**(1): 257-267.

Schonhuber, N., B. Seidler, K. Schuck, C. Veltkamp, C. Schachtler, M. Zukowska, S. Eser, T. B. Feyerabend, M. C. Paul, P. Eser, S. Klein, A. M. Lowy, R. Banerjee, F. Yang, C. L. Lee, E. J. Moding, D. G. Kirsch, A. Scheideler, D. R. Alessi, I. Varela, A. Bradley, A. Kind, A. E. Schnieke, H. R. Rodewald, R. Rad, R. M. Schmid, G. Schneider and D. Saur (2014). "A next-generation dual-recombinase system for time- and host-specific targeting of pancreatic cancer." Nat Med **20**(11): 1340-1347.

Tanaka, M., S. Chari, V. Adsay, C. Fernandez-del Castillo, M. Falconi, M. Shimizu, K. Yamaguchi, K. Yamao, S. Matsuno and P. International Association of (2006). "International consensus guidelines for management of intraductal papillary mucinous

neoplasms and mucinous cystic neoplasms of the pancreas." Pancreatology **6**(1-2): 17-32.

Testini, M., A. Gurrado, G. Lissidini, P. Venezia, L. Greco and G. Piccinni (2010). "Management of mucinous cystic neoplasms of the pancreas." World J Gastroenterol **16**(45): 5682-5692.

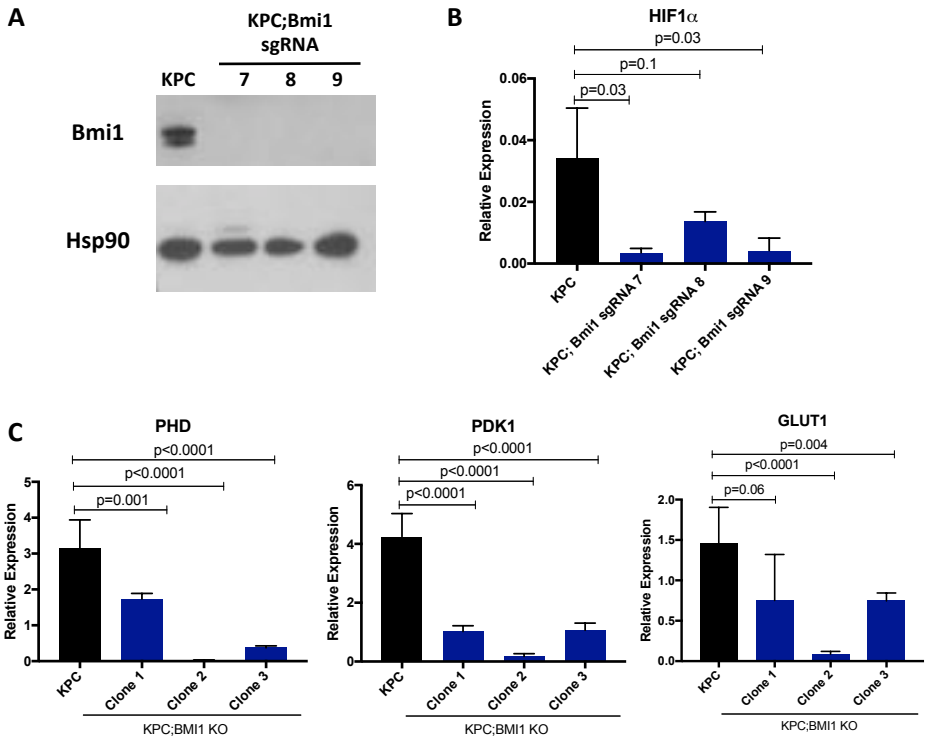
Waddell, N., M. Pajic, A. M. Patch, D. K. Chang, K. S. Kassahn, P. Bailey, A. L. Johns, D. Miller, K. Nones, K. Quek, M. C. Quinn, A. J. Robertson, M. Z. Fadlullah, T. J. Bruxner, A. N. Christ, I. Harliwong, S. Idrisoglu, S. Manning, C. Nourse, E. Nourbakhsh, S. Wani, P. J. Wilson, E. Markham, N. Cloonan, M. J. Anderson, J. L. Fink, O. Holmes, S. H. Kazakoff, C. Leonard, F. Newell, B. Poudel, S. Song, D. Taylor, N. Waddell, S. Wood, Q. Xu, J. Wu, M. Pinese, M. J. Cowley, H. C. Lee, M. D. Jones, A. M. Nagrial, J. Humphris, L. A. Chantrill, V. Chin, A. M. Steinmann, A. Mawson, E. S. Humphrey, E. K. Colvin, A. Chou, C. J. Scarlett, A. V. Pinho, M. Giry-Laterriere, I. Rooman, J. S. Samra, J. G. Kench, J. A. Pettitt, N. D. Merrett, C. Toon, K. Epari, N. Q. Nguyen, A. Barbour, N. Zeps, N. B. Jamieson, J. S. Graham, S. P. Niclou, R. Bjerkvig, R. Grutzmann, D. Aust, R. H. Hruban, A. Maitra, C. A. Iacobuzio-Donahue, C. L. Wolfgang, R. A. Morgan, R. T. Lawlor, V. Corbo, C. Bassi, M. Falconi, G. Zamboni, G. Tortora, M. A. Tempero, I. Australian Pancreatic Cancer Genome, A. J. Gill, J. R. Eshleman, C. Pilarsky, A. Scarpa, E. A. Musgrove, J. V. Pearson, A. V. Biankin and S. M. Grimmond (2015). "Whole genomes redefine the mutational landscape of pancreatic cancer." Nature **518**(7540): 495-501.

Wallace, E. M., J. P. Rizzi, G. Han, P. M. Wehn, Z. Cao, X. Du, T. Cheng, R. M. Czerwinski, D. D. Dixon, B. S. Goggin, J. A. Grina, M. M. Halfmann, M. A. Maddie, S. R.

Olive, S. T. Schlachter, H. Tan, B. Wang, K. Wang, S. Xie, R. Xu, H. Yang and J. A. Josey (2016). "A Small-Molecule Antagonist of HIF2alpha Is Efficacious in Preclinical Models of Renal Cell Carcinoma." Cancer Res **76**(18): 5491-5500.

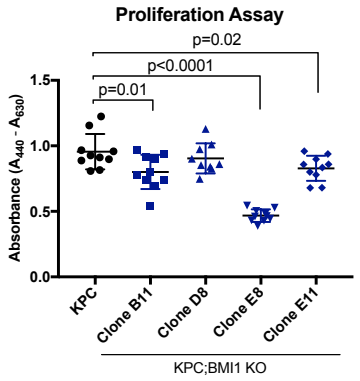
Zhuang, Z., C. Yang, F. Lorenzo, M. Merino, T. Fojo, E. Kebebew, V. Popovic, C. A. Stratakis, J. T. Prchal and K. Pacak (2012). "Somatic HIF2A gain-of-function mutations in paraganglioma with polycythemia." N Engl J Med **367**(10): 922-930.

Figure 6.1 – Bmi1 knockout with Crispr/Cas9 recapitulates phenotype seen with Bmi1 siRNA



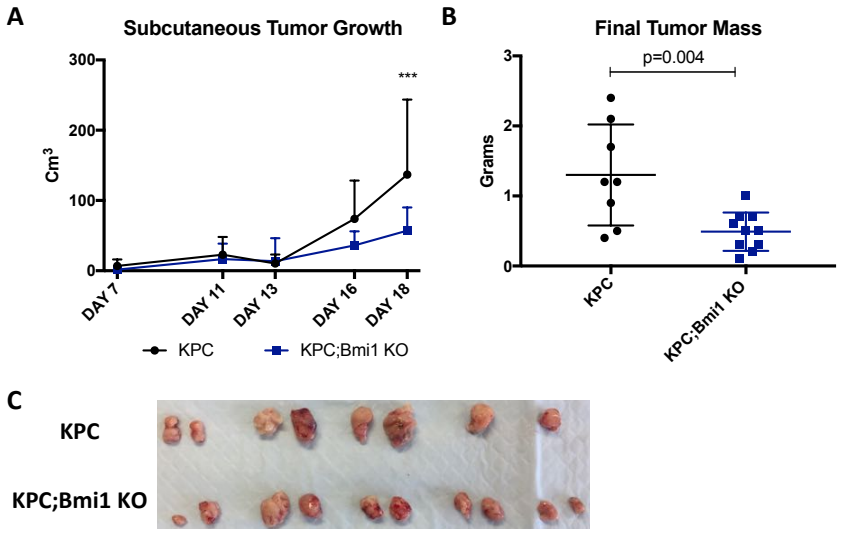
(A) Western blot in KPC and KPC;Bmi1 sgRNA cells for Bmi1. (B) qPCR analysis of mRNA levels in KPC and KPC;Bmi1 sgRNA cells. (C) qPCR analysis for HIF target genes in KPC and KPC;Bmi1 KO clonal cells grown for 24 hours in hypoxia.

Figure 6.2 – Bmi1 expression is required for proliferation in pancreatic cancer cell lines



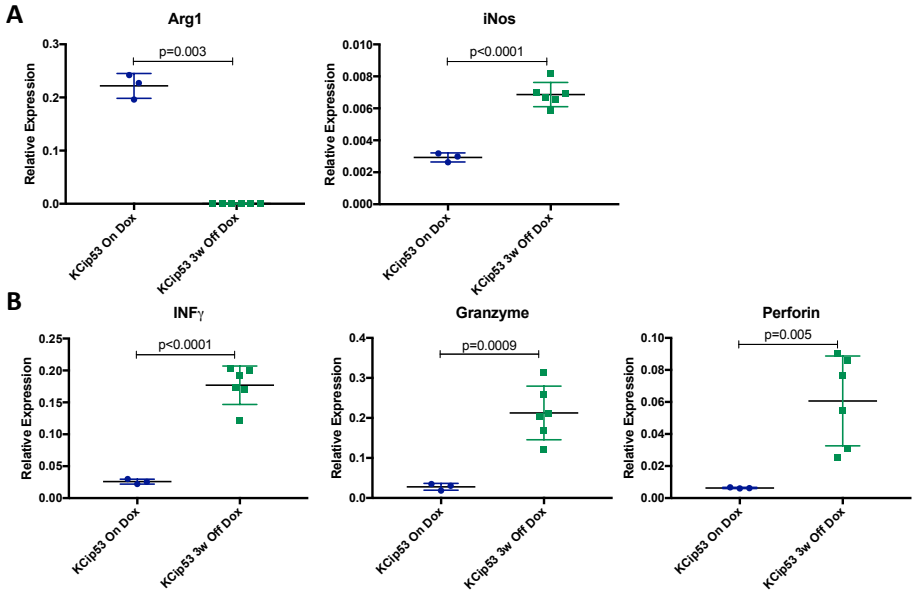
Proliferation assay in KPC and KPC;Bmi1 KO clonal cells as measured by WST-1 reagent.

Figure 6.3 – Lack of Bmi1 expression results in slower tumor growth in pancreatic cancer cells



(A) Subcutaneous tumor growth rate in KPC and KPC;Bmi1 KO cells. (B) Final tumor mass in KPC and KPC;Bmi1 KO cells. (C) Final gross subcutaneous tumors.

Figure 6.4 – Mutant p53^{R270H} expression results in changes in macrophage differentiation markers and CD8 T-Cell activation



qPCR analysis of (A) macrophage differentation markers and (B) markers of CD8 T-cell activations in pancreata from KCip53 animals on dox or off dox for three weeks.