Mechanisms of KRAS-Mediated Pancreatic Tumor Formation and Progression: A Dissertation

Victoria A. Appleman

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MECHANISMS OF KRAS-MEDIATED PANCREATIC TUMOR FORMATION AND PROGRESSION

A Dissertation Presented

By

Victoria Ann Appleman

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MAY 31, 2012
MECHANISMS OF KRAS-MEDIATED PANCREATIC TUMOR INITIATION AND PROGRESSION

A Dissertation Presented By

Victoria Ann Appleman

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Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences
To My Grandfather
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Abstract

Pancreatic cancer is the 4th leading cause of cancer related death in the United States with a median survival time of less than 6 months. Pancreatic ductal adenocarcinoma (PDAC) accounts for greater than 85% of all pancreatic cancers, and is marked by early and frequent mutation of the KRAS oncogene, with activating KRAS mutations present in over 90% of PDAC. To date, though, targeting activated KRAS for cancer treatment has been very difficult, and targeted therapies are currently being sought for the downstream effectors of activated KRAS. Activation of KRAS stimulates multiple signaling pathways, including the MEK-ERK and PI3K-AKT signaling cascades, but the role of downstream effectors in pancreatic tumor initiation and progression remains unclear. I therefore used primary pancreatic ductal epithelial cells (PDECs), the putative cell of origin for PDAC, to determine the role of specific downstream signaling pathways in KRAS activated pancreatic tumor initiation. As one third of KRAS wild type PDACs harbor activating mutations in BRAF, and KRAS and BRAF mutations appear to be mutually exclusive, I also sought to determine the effect of activated BRAF (BRAFV600E) expression on PDECs and the signaling requirements downstream of BRAF.

I found that both KRASG12D and BRAFV600E expressing PDECs displayed increased proliferation relative to GFP expressing controls, as well as increased PDEC survival after challenge with apoptotic stimuli. This survival was found to depend on both the MEK-ERK and PI3K-AKT signaling cascades. Surprisingly, I found that this survival is also dependent on the IGF1R, and that activation of PI3K/AKT signaling
occurs downstream of MEK/ERK activation, and is dependent on signaling through the IGF1R. Consistent with this, I find increased IGF2 expression in KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> expressing PDECs, and show that ectopic expression of IGF2 rescues survival in PDECs with inhibited MEK, but not PI3K. Finally, I showed that the expression of KRAS<sup>G12D</sup> or BRAF<sup>V600E</sup> in PDECs lacking both the Ink4a/Arf and Trp53 tumor suppressors is sufficient for tumor formation following orthotopic transplant of PDECs, and that IGF1R knockdown impairs KRAS and BRAF-induced tumor formation in this model.

In addition to these findings within PDECs, I demonstrate that KRAS<sup>G12D</sup> or BRAF<sup>V600E</sup> expressing tumor cell lines differ in MEK-ERK and PI3K-AKT signaling from PDECs. In contrast to KRAS<sup>G12D</sup> or BRAF<sup>V600E</sup> expressing PDECs, activation of AKT at serine 473 in the KRAS<sup>G12D</sup> or BRAF<sup>V600E</sup> expressing tumor cell lines does not lie downstream of MEK, and only the inhibition of PI3K alone or both MEK and the IGF1R simultaneously results in loss of tumor cell line survival. However, inhibition of MEK, PI3K, or the IGF1R in KRAS<sup>G12D</sup> or BRAF<sup>V600E</sup> expressing tumor cell lines also resulted in decreased proliferation relative to DMSO treated cells, demonstrating that all three signaling cascades remain important for tumor cell growth and are therefore viable options for pancreatic cancer therapeutics.
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<th>Description</th>
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<tbody>
<tr>
<td>4EBP-1</td>
<td>4E Binding Protein 1</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>AG</td>
<td>AG1024 (IGF1R Inhibitor)</td>
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<tr>
<td>ALSV</td>
<td>Avian Leukosis-Sarcoma Virus</td>
</tr>
<tr>
<td>AMP</td>
<td>Amphiregulin</td>
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<tr>
<td>ARF</td>
<td>Alternate Reading Frame</td>
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<tr>
<td>BAX</td>
<td>BCL2-Associated X Protein</td>
</tr>
<tr>
<td>BRAF</td>
<td>v-Raf Murine Sarcoma Viral Oncogene Homolog B1</td>
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<tr>
<td>BRCA2</td>
<td>Breast Cancer 2 Susceptibility Protein</td>
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<tr>
<td>BTC</td>
<td>Betacellulin</td>
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<td>CDX2</td>
<td>Caudal Type Homeobox 2</td>
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<td>CHX</td>
<td>Cycloheximide</td>
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<tr>
<td>DPC4</td>
<td>Deleted in Pancreatic Carcinoma Target 4</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>EPI</td>
<td>Epigenin</td>
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<td>EPR</td>
<td>Epiregulin</td>
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<td>ER</td>
<td>Estrogen Receptor</td>
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<tr>
<td>ERB2</td>
<td>Erythroblastic Leukemia Viral Oncogene Homolog 2</td>
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<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinases</td>
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<tr>
<td>FGFR</td>
<td>Fibroblast Growth Factor Receptor</td>
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<tr>
<td>GAP</td>
<td>GTP-ase Activating Protein</td>
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<tr>
<td>GEF</td>
<td>Guanine Nucleotide Exchange Factor</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HBEGF</td>
<td>Heparin Binding Epidermal Growth Factor</td>
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<tr>
<td>HH</td>
<td>Hedgehog</td>
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<td>IGF1</td>
<td>Insulin-Like Growth Factor 1</td>
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<td>IGF1R</td>
<td>Insulin-Like Growth Factor 1 Receptor</td>
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<td>IGF2R</td>
<td>Insulin-Like Growth Factor 2 Receptor</td>
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<tr>
<td>IKBKE</td>
<td>Inhibitor of Nuclear Factor Kappa-B Subunit Epsilon</td>
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<td>INS2</td>
<td>Insulin 2</td>
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<tr>
<td>IPMN</td>
<td>Intraductal Papillary Mucinous Neoplasm</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin Receptor</td>
</tr>
<tr>
<td>IRS1</td>
<td>Insulin Receptor Substrate 1</td>
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<tr>
<td>IRS2</td>
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<td>K19</td>
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<td>KRAS</td>
<td>Kirsten Rat Sarcoma Viral Oncogene Homolog</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
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<td>--------------</td>
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<tr>
<td>LY</td>
<td>LY294002 (PI3K Inhibitor)</td>
</tr>
<tr>
<td>MCN</td>
<td>Mucinous Neoplasm</td>
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<tr>
<td>MDM2</td>
<td>Murine Double Minute 2</td>
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<tr>
<td>MEK</td>
<td>MAPK/ERK Kinases (MAP2Ks)</td>
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<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
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<tr>
<td>MUC2</td>
<td>Mucin 2</td>
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<tr>
<td>NFKB</td>
<td>Nuclear Factor Kappa-B</td>
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<tr>
<td>PanIN</td>
<td>Pancreatic Intraepithelial Neoplasm</td>
</tr>
<tr>
<td>PD</td>
<td>PD98059 (MEK Inhibitor)</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic Ductal Adenocarcinoma</td>
</tr>
<tr>
<td>PDEC</td>
<td>Pancreatic Ductal Epithelial Cells</td>
</tr>
<tr>
<td>PDX1</td>
<td>Pancreatic and Duodenal Homeobox 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-Kinase</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tension Homolog</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53- up-regulated Modulator of Apoptosis</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma</td>
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<tr>
<td>RCAS</td>
<td>Replication Competent ASLV Long Terminal Repeat with Splice Acceptor</td>
</tr>
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<td>RSV</td>
<td>Rous Sarcoma Virus</td>
</tr>
<tr>
<td>SHC</td>
<td>Src Homology 2 Domain-Containing</td>
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<tr>
<td>SMAD4</td>
<td>Mothers Against Decapentaplegic Homolog 4</td>
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<td>SMO</td>
<td>Smoothened</td>
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<tr>
<td>TGF-α</td>
<td>Transforming Growth Factor α</td>
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<tr>
<td>TGFβR2</td>
<td>Transforming Growth Factor -β Receptor 2</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous Sclerosis Complex</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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CHAPTER I: INTRODUCTION
Pancreatic cancer is the 4th leading cause of cancer related death within the US and has shown little improvement in its death rate in decades. In 2011 there were approximately 44,000 new cases of pancreatic cancer and approximately 38,000 deaths (Siegel, Ward et al. 2011). The median survival time of patients diagnosed with pancreatic cancer is less than 6 months, and the 5-year survival rate for pancreatic cancer is only 6% (Siegel, Ward et al. 2011). The bleak prognosis of pancreatic cancer is due in part to the late stage of the disease at the time of diagnosis, as frequently metastases are already present, and also to the resistance of pancreatic tumors to current chemotherapeutics. Surgery offers the only chance of a cure for pancreatic cancer, but is only an option for those patients diagnosed with localized disease. Of the small subset of patients who are able to undergo resection, the 5-year survival rate is still only 20%, although recent advances in adjuvant chemotherapy may improve the post-surgical outlook (Ahrendt and Pitt 2002). For these reasons, there is a critical need in pancreatic cancer research to discover better methods the early detection of pancreatic cancer, as well as more effective chemotherapeutics.

**Histological Progression of Pancreatic Ductal Adenocarcinoma**

Pancreatic Ductal Adenocarcinoma (PDAC) comprises the majority of pancreatic cancers, is thought to arise from pancreatic ductal epithelial cells, and has been shown to develop through a series of precursor lesions marked by characteristic morphological and genetic changes (Hruban, Goggins et al. 2000). These precursor lesions, known as Pancreatic intraepithelial neoplasia, or PanINs, are divided into three grades that progress
from least severe to most severe: PanIN-1A/1B, PanIN-2, and PanIN-3 (Hruban, Adsay et al. 2001). PDAC typically arises in the head of the pancreas, and invades surrounding tissues with common sites of metastasis including the liver and lungs (Hezel, Kimmelman et al. 2006). PDAC frequently displays desmoplasia, or a dense stroma of fibroblasts and inflammatory cells (Hezel, Kimmelman et al. 2006).

Typically, normal pancreatic ductal cells show cuboidal and low columnar epithelium absent of mucinous cytoplasm (Hruban, Adsay et al. 2001). PanIN-1A lesions display tall columnar cells, basally located nuclei, and supranuclear mucin, while PanIN-1B lesions have papillary, micropapillary, or basally pseudostratified architecture (Hruban, Adsay et al. 2001). PanIN-2 lesions are marked by nuclear abnormalities (such as loss of polarity or enlarged nuclei) and can be flat or papillary (Hruban, Adsay et al. 2001). PanIN-3 lesions show budding into the lumen, and are typically papillary or micropapillary, with loss of polarity and prominent nucleoli (Hruban, Adsay et al. 2001). PanIN-3 lesions represent carcinoma in situ, and progression from PanIN 3 to PDAC is characterized by invasion into the surrounding tissues (Hezel, Kimmelman et al. 2006).

Although the majority of PDAC appears to arise as the result of PanIN lesions, there are two other precursor lesions that have been characterized: mucinous neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN) (Hezel, Kimmelman et al. 2006). These and other cystic lesions contribute less than 1% of all pancreatic tumors, but they are increasingly common in tumors that are surgically resected, and comprise up to 15% of those lesions (Adsay 2007).
IPMNs are characterized by mucin production resulting in dilation of the pancreatic ducts. These lesions occur predominantly in the head of the pancreas, and are divided into main duct and branch duct classification (Tanaka, Chari et al. 2006). Overall, IPMNs comprise approximately 5% of all pancreatic neoplasms (Adsay 2003).

Genetically, IPMNs are more stable than PanIN lesions, with fewer KRAS, INK4A, and TRP53 mutations overall (Sessa, Solcia et al. 1994). Mutation of KRAS has been shown to correlate with the progression of IPMN lesions, however, and has been detected in up to 60% of IPMNs (Tada, Omata et al. 1991). In addition, approximately 30% of IPMNs have inactivation of LKB1, the gene that has been shown to be inactivated in Peutz-Jeghers syndrome, a disease associated with increased risk of pancreatic cancer (Sato, Rosty et al. 2001). Most IPMNs have also been found to frequently express MUC2 and CDX2, which have both been shown to have tumor suppressor activity (Adsay 2007). IPMNs can be classified into main duct and branch duct subtypes based on imaging or histological analysis (Furukawa, Takahashi et al. 1992). Although the majority IPMNs are non-invasive, main duct IPMNs have been found to be more likely to progress to invasive adenocarcinoma or colloid carcinoma, and therefore the classification of branch duct versus main duct can impact disease prognosis and treatment (Tanaka, Chari et al. 2006).

MCNs are characterized by an ovarian stroma and are lined with a mucin producing epithelium (Tanaka, Chari et al. 2006; Adsay 2007). The ovarian stroma is both ER and PR positive, and is considered a hallmark of MCNs (Izumo, Yamaguchi et al. 2003). These lesions are almost always found in the body and tail of the pancreas, and
occur predominantly in women (Zamboni, Scarpa et al. 1999; Reddy, Smyrk et al. 2004). Up to one third of all MCNs have been found to progress to invasive carcinoma, however they are typically less aggressive than other forms of invasive carcinoma in the pancreas (Thompson, Becker et al. 1999; Zamboni, Scarpa et al. 1999; Reddy, Smyrk et al. 2004). In contrast to most IPMNs, MCNs do not typically express MUC2 and CDX2, but often express MUC1 (Adsay 2007). In addition, 46% of MCNs have mutations in KRAS, and almost all malignant MCNs were found to have KRAS mutations, indicating a correlation between mutation of KRAS and disease progression (Jimenez, Warshaw et al. 1999).

**Genetic Progression of PDAC**

The progression from normal pancreas to PanINs and PDAC is marked by a well-established series of genetic alterations as illustrated in Figure 1.1 (Bardeesy and DePinho 2002). One of the earliest changes in the progression to PDAC is activating mutations in the KRAS2 oncogene, which have been detected in a small number of normal pancreas tissue, occur in approximately 30% of early PanIN lesions, and in over 95% of all cases of PDAC (Hezel, Kimmelman et al. 2006). KRAS mutations have also been detected in up to 30% of patients with chronic pancreatitis, a condition that has been shown to increase the risk of pancreatic cancer (Lohr, Maisonuneuve et al. 2000). Since activation of KRAS is both an early and prevalent event in the formation of pancreatic cancer, it is considered to have a critical role in the formation of pancreatic tumors. Overexpression of ERBB2 (HER2/NEU) and the EGFR also occur early in the formation of pancreatic tumors (Bardeesy and DePinho 2002). The EGFR is overexpressed in up to
60% of all pancreatic tumors, while ERBB2 is overexpressed in 70% of PDAC (Lemoine, Hughes, et al. 1992 and Hruban, Wilentz et al. 2000).

These initial genetic alterations are followed by the subsequent loss of the INK4A tumor suppressor, which occurs in over 85% of pancreatic tumors (Rozenblum, Schutte et al. 1997). As loss of INK4A is observed in approximately 30% of low-grade precursor lesions, this change is thought to occur only slightly later in the formation of PDAC than activation of KRAS, and increases in incidence to occur in nearly all cases of PDAC (Hruban, Wilentz et al. 2000).

Mutation of the TRP53 tumor suppressor has been identified in in over 50% of pancreatic tumors (Bardeesy and DePinho 2002). However, abnormal expression of p53 was only detected in 12% of high-grade precursor lesions, and was not detected at all in low-grade precursor lesions, indicating that the inactivation of TRP53 occurs late in the progression of PDAC (Hruban, Wilentz et al. 2000). Most often in PDAC, p53 is mutated within its DNA-binding domain (Hezel, Kimmelman et al. 2006).

As PDAC progresses, there is also eventual loss of the SMAD4 (DPC4) and BRCA2 tumor suppressor genes (Hruban, Wilentz et al. 2000). While the inactivation of SMAD4 occurs in up to 50% of all pancreatic tumors, it is not observed in low-grade precursor lesions, and is only observed in approximately 30% of high-grade precursor lesions, indicating that this is a later event in the formation of PDAC (Hruban, Wilentz et al. 2000). Similarly, inactivation of BRCA2 has been shown to occur in up to 10% of PDAC, but is not present in low-grade precursor lesions, also indicating that this is a late event in the formation of pancreatic tumors (Hruban, Wilentz et al. 2000).
Figure 1.1: Genetic Progression Model of Pancreatic Adenocarcinoma (Adapted from Bardeesy and DePinho 2002)
**Ink4a/Arf Tumor Suppressors**

*INK4A* and *ARF* are two tumor suppressors that are encoded in an overlapping region of the 9q21 locus (Sherr 2001). In PDAC, loss of INK4A has been found to occur in over 85% of all tumors through mutation, deletion, or promoter hypermethylation (Rozenblum, Schutte et al. 1997). Due to the shared gene locus, many pancreatic tumors are deficient for both INK4A and ARF, however point mutations in ARF are not detected in pancreatic tumors, indicating that INK4A is the primary target of inactivation in PDAC (Rozenblum, Schutte et al. 1997).

INK4A functions as a tumor suppressor by inhibiting the kinase activity of CDK4 and CDK6 (Serrano, Hannon et al. 1993). This inhibition is achieved by preventing CDK4 and CDK6 from interacting with Cyclin D, which is required for the kinase activity of CDK4 and CDK6 (Russo, Tong et al. 1998). This interference therefore causes hypophosphorylation of RB, and ultimately results in cell cycle arrest due to repression of E2F by RB (Dyson 1998).

*ARF* is encoded by an alternative first exon at this gene locus (exon 1β) but shares exons 2 and 3 with INK4A (Quelle, Zindy et al. 1995). As the name implies, *ARF* is encoded by an alternate reading frame from INK4A, and as a result, the two proteins share no amino acid homology. ARF has been found to function as a tumor suppressor by binding to MDM2 and inhibiting the ubiquitination of the p53 tumor suppressor (Kamijo, Weber et al. 1998; Pomerantz, Schreiber-Agus et al. 1998; Stott, Bates et al. 1998; Zhang, Xiong et al. 1998).
**Trp53 Tumor Suppressor**

p53 is a transcription factor that has been shown to function as a tumor suppressor through its transcriptional regulation of p21, MDM2, CYCLIN G, and BAX (Levine 1997). Mutations in p53 occur in over 50% of all human tumors (Hollstein, Rice et al. 1994). Most frequently, these mutations are missense mutations in a single allele of p53 that are followed by loss of the wild type allele (loss of heterozygozity). Consistent with this, p53 is mutated in over 50% of pancreatic tumors, and the majority of these mutations occur in the DNA binding domain (Rozenblum, Schutte et al. 1997).

p53 functions as a tetramer and is activated during cell stress, such as DNA damage, including double strand breaks, and hypoxia (Graeber, Osmanian et al. 1996; Guidos, Williams et al. 1996). Upon activation, p53 is able to affect multiple cellular processes through enhancing the transcription of many genes, including p21 and PUMA, which in turn function to induce cell cycle arrest, programmed cell death or senescence (Vousden and Prives 2009). In addition, p53 has also been found to cause cell cycle arrest and apoptosis through the transcriptional repression of other genes, such as BCL2 (Vousden and Prives 2009).

p53 has a short half-life and is regulated by the E3 ubiquitin ligase MDM2, which binds to p53 and targets it for ubiquitin mediated degradation (Marine, Francoz et al. 2006). Since MDM2 is itself transcriptionally regulated by p53, increased p53 expression results in a regulatory feedback loop involving increased expression of MDM2, and increased ubiquitination and degradation of p53 (Barak, Juven et al. 1993; Wu, Bayle et al. 1993). Because of its role in the regulation of p53, MDM2 has been found to be an
oncogene that is amplified in 7% of human tumors (Momand, Jung et al. 1998). As mentioned above, MDM2 also serves as a link between the p53 pathway and the ARF tumor suppressor, as ARF binds to MDM2 and stabilizes p53 (Kamijo, Weber et al. 1998; Pomerantz, Schreiber-Agus et al. 1998; Stott, Bates et al. 1998; Zhang, Xiong et al. 1998).

**SMAD4**

Of all human tumors, PDAC has the largest number of inactivating mutations in SMAD4, a tumor suppressor that has been shown to be involved in TGF-β signaling (Hahn, Schutte et al. 1996). Upon activation of receptor SMADs by TGF-β, SMAD4 is bound and translocates to the nucleus where it functions as a transcription factor (He, Dorn et al. 2006). SMAD4 is inactivated in over 50% of all pancreatic tumors, and this inactivation is due to either point mutation or, in approximately 30% of pancreatic tumors, deletion of the SMAD4 gene (Hahn, Schutte et al. 1996; Hansel, Kern et al. 2003). A recent study demonstrated that Smad4 expression is not needed for normal pancreas development, but that the loss of Smad4 results in an acceleration of KRAS-mediated pancreatic tumor formation, and shifted the tumors towards a progression through IPMNs rather than PanIN lesions (Bardeesy, Cheng et al. 2006). These findings demonstrate a role for SMAD4 loss in the progression of pancreatic tumors, as well as indicate a role for the loss of TGF-β signaling in PDAC.
TGF-β

TGF-β is able to function as both a tumor suppressor as well as an enhancer of cell growth (Zavadil and Bottinger 2005). While it has been shown that TGF-β and SMAD signaling can inhibit the growth of epithelial cells, increased TGF-β signaling can result in increased cell migration and EMT (Miyazono, ten Dijke et al. 2000; Zavadil and Bottinger 2005). In addition to the involvement of TGF-β signaling in PDAC that is implicated by the loss of SMAD4 in these tumors, mutations in the type II TGF-β receptor have also been identified in PDAC (Goggins, Shekher et al. 1998). A mouse model with pancreas specific loss of the TGF-β receptor 2 in conjunction with activation of KRAS demonstrated acceleration of tumor formation and a progression through PanIN lesions to PDAC, even without the loss of Ink4a or Trp53 (IJichi, Chytil et al. 2006). Although the complete mechanisms of how TGF-β and SMAD4 loss contributes to PDAC are still largely unknown, these findings further support the role of TGF-β signaling loss in the formation of pancreatic tumors.

IGF1R in Cancer

The IGF receptor family of receptor tyrosine kinases includes the IGF1R, IGF2R and the IR. The IR and IGF1R share 70% of their amino acid sequence, while the IGF2R does not, and instead acts as a negative regulator of IGF signaling due to its lack of intrinsic signaling (Riedemann and Macaulay 2006). The IGF1R is a heterotetramer with two alpha and two beta subunits, and can be activated by either IGF1 or IGF2 (Zha and Lackner 2010). Upon activation, the IGF1R is autophosphorylated and recruits the
docking proteins IRS1, IRS2, and SHC to the membrane (Baserga, Hongo et al. 1997; Pollak, Schernhammer et al. 2004). Through these docking proteins, the IGF1R is able to activate numerous signaling cascades, including PI3K/AKT and RAS/RAF/MEK/ERK (Pollak 2008).

In addition to the genetic lesions listed above for the progression of PDAC, it has also been demonstrated that both the IGF1R and IGF1 are aberrantly expressed in PDAC tumor cells, suggesting a possible role for IGF1R signaling in pancreatic tumorigenesis (Bergmann, Funatomi et al. 1995; Ouban, Muraca et al. 2003; Stoeltzing, Liu et al. 2003). In addition to increased expression of the IGF1R and IGF1 in pancreatic tumors, increased levels of *IRS1* mRNA and IRS1 protein have been detected in human pancreatic tumor tissue, further implicating IGF1R signaling in pancreatic tumorigenesis (Bergmann, Funatomi et al. 1996). In support of this, a recent study demonstrated that inhibition of the IGF1R decreased orthotopic tumor formation following transplant of human pancreatic cell lines (Moser, Schachtschneider et al. 2008).

**Current Pancreatic Cancer Therapeutics**

Despite the increase in knowledge about the histological and genetic progression of PDAC, little progress has been made in the development of effective chemotherapies to target pancreatic tumors. The current standard of care, Gemcitabine, is a nucleoside analog that has been shown to increase survival in patients when compared with the previous therapeutic option, 5-FU (Burris and Storniolo 1997). However, many
pancreatic tumors become resistant to treatment with gemcitabine, and it has done little as a chemotherapy agent to increase overall patient survival.

Several studies have attempted to combine gemcitabine with other therapeutics in an attempt to increase their efficacy. Examples of these include a combination with erlotinib, an inhibitor of the EGFR (Moore, Goldstein et al. 2007). A recent development in the treatment of PDAC is folfirinox, a four-drug combination therapy (Conroy, Desseigne et al. 2011). Unfortunately, while these combinations have fared better than the use of gemcitabine alone, they still have only showed marginal improvement in survival rates, or in the case of folfirinox, have an increased number of detrimental side effects, and therefore have done little to improve the outlook of PDAC.

Due to this ineffectiveness of DNA-analogs, either alone or in combination, and the dire need for more effective therapies, research has moved toward targeted therapy options. As mutational activation of KRAS2 is one of the most common changes detected in PDAC, much research has focused on a more intense study of activated KRAS, its consequences in PDAC, and potential therapeutics to target the effects of activated KRAS in tumor initiation and progression.

The RAS Family of Proteins

There are over 150 members of the human RAS superfamily of small GTPases, and members of this family are conserved across many species, including C. elegans and S. pombe (Colicelli 2004). These proteins function as monomeric G proteins, serving as GDP/GTP regulated molecular switches (Vetter and Wittinghofer 2001), and all Ras
superfamily members share a common and conserved G-box GDP/GTP binding domain (Wennerberg, Rossman et al. 2005). As GTPases, Ras proteins bind GDP and GTP with high affinity and are regulated by Guanine-nucleotide exchange factors (GEFs), which promote the exchange of Ras bound GDP for GTP, and GTPase activating proteins (GAPs), which promote GTP hydrolysis (Bernards and Settleman 2004). The binding of GTP to Ras proteins results in conformation changes in the Switch I (aa 30-38) and Switch II (aa 59-67) regions of the proteins, thus allowing the GTP bound Ras protein to bind effectors (Bishop and Hall 2000; Repasky, Chenette et al. 2004).

There are five subfamilies of the Ras superfamily, which have largely been determined through both structure and function analysis: Ras, Rho, Rab, Ran, and Arf. The three Ras oncogenes, HRAS, NRAS, and KRAS, are the founding members of the Ras subfamily. These three genes encode four Ras proteins: HRAS, NRAS, KRAS4A, and KRAS4B, with the two KRAS proteins resulting from alternative splicing.

The Ras proteins are initially synthesized in the cytosol on free polysomes, and then are post-translationally modified for targeting to the plasma membrane (Cox and Der 2002). This modification occurs due to the CAAX (Cysteine, Aliphatic, Aliphatic, Any Amino Acid) motif at the C terminus of RAS, which signals farnesyltransferase to farnesylate the cysteine (Reuther and Der 2000; Cox and Der 2002). These proteins are then trafficked to the endoplasmic reticulum, where cleavage of the AAX occurs, and the C terminus is methylated (Buday and Downward 2008). HRAS, NRAS, and KRAS4A are then transported to the Golgi and palmitoylated at the C terminus, and this modification results in the targeting of these proteins to the plasma membrane (Hancock,
Paterson et al. 1990). Instead of the palmitoylation, KRAS4B is targeted to the plasma membrane by the polybasic region at its C terminus (Hancock, Paterson et al. 1990). Recent evidence suggests that the three RAS isoforms may be targeted to different areas of the plasma membrane, and that this specific targeting could impact the ability of RAS to stimulate its downstream effectors.

The three isoforms of Ras (H, N, and K) are very closely related, and have been found to share 85% of their amino acid sequences (Downward 2003). This high degree of similarity between the proteins, as well as their shared downstream effectors, led to the belief that the three isoforms of RAS share the same functions. However, recent evidence is uncovering distinct roles and functions for each of the RAS isoforms. While mouse models have demonstrated that \textit{Hras} and \textit{Nras} are dispensable for normal mouse development, and can even be ablated in combination, loss of \textit{Kras} expression results in embryonic lethality in the developing mouse (Johnson, Greenbaum et al. 1997). In addition, one study has demonstrated differences in the ability of each RAS isoform to stimulate downstream effectors, with KRAS showing increased ability to bind and activate RAF, while HRAS was more capable of activating PI3K (Yan, Roy et al. 1998). These studies argue that the hypervariable region of RAS may in fact lead to differential functions of each isoform. Additional studies have recently shown that the localization of the RAS proteins may specify downstream effectors and signaling. In addition to the plasma membrane, signaling from RAS has been detected on endosomes, mitochondria, the Golgi apparatus, and the endoplasmic reticulum (Fehrenbacher, Bar-Sagi et al. 2009). While much remains unclear about the impact of RAS localization on its downstream
signaling, recent work has demonstrated that the ultimate effects of ERK activation may differ based upon the localization of its upstream activators (Harding, Tian et al. 2005). Therefore, it is possible that sub-cellular localization of the RAS proteins may provide an additional means of regulating the differential functions of the RAS proteins.

One of the most widely accepted mechanisms of RAS activation is by receptor tyrosine kinases (RTKs), such as the EGFR (Repasky, Chenette et al. 2004). In this model, EGFR is activated via ligand binding, is autophosphorylated, and binds GRB2, which is bound to the RAS-GEF SOS. This results in recruitment of SOS to the plasma membrane, bringing it in close proximity to RAS. This co-localization of SOS and RAS at the plasma membrane results in increased exchange of GDP for GTP bound to RAS, and increased activation of RAS (Downward 2003).

The Role of Activated RAS in Cancer

RAS proteins are among the most frequently mutated proteins in human cancer, with mutations present in approximately 30% of all human cancers (Cox and Der 2002). The most commonly mutated of the Ras proteins is KRAS, followed by NRAS, with HRAS mutations occurring in less than 1% of all tumors (Downward 2003). Mutations in Ras family members that disrupt the GTPase activity of these proteins, thus rendering them constitutively bound to GTP and activated, are commonly found in human malignancies, and hot spot mutations at codons 12, 13, and 61 are commonly observed in pancreatic cancer (Bos 1989).
Based upon the frequency of RAS mutations in human cancers, as well as evidence indicating that activation of RAS is key to the formation of many different tumor types, previous research has attempted to inhibit activated RAS through various mechanisms. One of the most well known attempts at inhibiting RAS was the generation of compounds intended to inhibit the targeting of RAS to the plasma membrane, as improperly targeted RAS proteins have been shown to be inactive (Downward 2003). As described above, the enzyme farnesyltransferase post-translationally modifies RAS proteins to target them to the plasma membrane. Based upon this, farnesyltransferase inhibitors (FTIs) were generated to prevent targeting of RAS to the plasma membrane, and thus prevent RAS from its ability to activate cytoplasmic signaling cascades. While effective against HRAS farnesylation and activity, further studies revealed that FTIs were ineffective at preventing KRAS and NRAS targeting to the plasma membrane (Lerner, Zhang et al. 1997). This is due to the fact that these two isoforms are also substrates for geranylgeranyltransferase, which adds a geranylgeranyl isoprenoid and therefore enables membrane targeting of the NRAS and KRAS proteins (Rowell, Kowalczyk et al. 1997; Whyte, Kirschmeier et al. 1997; Zhang, Burris et al. 1997).

In addition to attempts to prevent the targeting of RAS to the plasma membrane, inhibition of RAS activity has been attempted through targeting RAS protein expression, as well as through inhibiting upstream signaling pathways to counter RAS stimulated production of autocrine growth factors (Sibilia, Fleischmann et al. 2000; Dancey 2002). To date, however, none of these strategies have proven effective means of treating tumors
with RAS mutations, and many researchers have moved towards inhibiting the pathways activated downstream of the RAS proteins instead.

**RAS-Stimulated Signaling Pathways**

Stimulation of activated receptor tyrosine kinase results in the recruitment of RAS to the membrane, where it binds effector proteins resulting in the activation of downstream signaling cascades including, but not limited to, the RAF/MEK/ERK signaling cascade, the PI3K/AKT signaling cascade, the RAL/GDS pathway, and PLC-ε-induced calcium signaling (Shown in Figure 1.2, Adapted from Downward 2003). Through the activation of these various signaling cascades, activated RAS is able to impact multiple cellular processes that are critical to tumor progression, including proliferation and survival, as well as cell polarity and movement (Vigil, Cherfils et al. 2010).

The first of the RAS stimulated signaling pathways to be characterized was the RAF-MEK-ERK signaling cascade. The RAF family of proteins consists of three serine/threonine kinases: ARAF, BRAF, and CRAF (or RAF-1). These proteins bind to activated RAS at its RAF binding domain, and as a result are recruited to the plasma membrane and further phosphorylated. Once RAF kinase activity has been stimulated, RAF phosphorylates and activates MEK1 and MEK2, which in turn phosphorylate and activate the mitogen activated protein kinases (MAPK) ERK1 and ERK2. Once activated, ERK1 and ERK2 translocate to the nucleus and phosphorylate the Ets family of transcription factors and other targets (Downward 2003). As a result of the downstream
activation of cell cycle regulators such as CYCLIN D, the activation of the RAF-MEK-ERK signaling cascade has been associated with the increased cell proliferation (Pruitt and Der 2001). In support of this, the inhibition of MEK has been shown to result in the decreased proliferation of PDAC cell lines (Gysin, Lee et al. 2005).

The next of the RAS effector pathways to be characterized was the PI3K-AKT signaling cascade. Activation of PI3K-AKT by RAS was confirmed when the catalytic (p110) subunit of PI3K was shown to directly interact with and bind to RAS (Rodriguez-Viciana, Warne et al. 1994; Pacold, Suire et al. 2000). This results in the translocation of PI3K to the plasma membrane, where it phosphorylates PtdIns (4,5) to PtdIns (3,4,5). This results in the accumulation of AKT and PDK1 at the plasma membrane due to the binding of their pleckstrin-homology (PH) domains to PtdIns (3,4,5), and this close proximity results in the phosphorylation of AKT by PDK1 (Lawlor and Alessi 2001).

Once phosphorylated, AKT phosphorylates multiple targets, including TSC2, PRAS40, BAD, GSK3, and the Forkhead-related transcription factor 1 (FOXO1) (Brunet, Park et al. 2001). Phosphorylation of TSC2 or PRAS40 by AKT results in the inability of these proteins to inhibit mTORC1, leading to increased activation of mTORC1 and ultimately to increased phosphorylation of S6K and 4E-BP1, which results in mRNA translation and cell growth (Ma and Blenis 2009). Phosphorylation of pro-apoptotic BAD by AKT prevents its dimerization with Bcl-XL, which in turn enables Bcl-XL to inhibit apoptosis through the inhibition of cytochrome C (Datta, Dudek et al. 1997). Phosphorylation of GSK3 by AKT results in inhibition of this kinase, and this results in the accumulation of Cyclin D1 and increased cell proliferation (Diehl, Cheng et al. 1998).
The phosphorylation of FOXO1 results in its export from the nucleus, and thereby prevents the transcription of several pro-apoptotic genes, including *BIM* (Brunet, Bonni et al. 1999). Therefore, through these and other targets, activated AKT is able to impact cell growth, cell proliferation and cell survival, indicating a key role for this signaling cascade in cancer biology.

In addition to the many downstream targets of AKT that can contribute to tumor cell growth and survival, PDK1 has also been shown to phosphorylate and activates S6 kinase, further increasing the impact of this pathway on cell growth (Pullen, Dennis et al. 1998). Further supporting a role for this signaling cascade in pancreatic cancer is evidence of decreased expression of the PtdIns (3,4,5) phosphatase PTEN in human PDAC (Sakurada, Suzuki et al. 1997; Asano, Yao et al. 2004). Loss of PTEN leads to increased expression of PtdIns (3,4,5), and thus results in increased activation of PI3K (Di Cristofano and Pandolfi 2000).

Both RAF-MEK-ERK and PI3K-AKT signaling cascades can also be activated downstream of receptor tyrosine kinases, such as the IGF1R (Baserga, Hongo et al. 1997). In addition, many studies have also demonstrated extensive cross talk between the RAF-MEK-ERK and PI3K-AKT signaling cascades. One way such interactions occur is through the phosphorylation of TSC2 by ERK (Zoncu, Efeyan et al. 2011). As mentioned above, TSC2 is part of a complex that negatively regulates mTORC1, and its phosphorylation and inhibition by ERK results in increased activity of mTORC1 (Zoncu, Efeyan et al. 2011). ERK has also been shown to phosphorylate and activate RAPTOR, a member of the mTORC1 complex, thereby further increasing mTORC1 activity (Pearce,
Further demonstrating the complex interactions of these two signaling cascades, AKT has been found to negatively regulate RAF through phosphorylation of its N-terminal region, and it is hypothesized that this regulation may function to prevent cell cycle arrest due to high levels of MEK-ERK signaling (Cheung, Sharma et al. 2008).

It has also been shown that RAS activates the RAL GEFs RALGDS, RGL, RGL2/RIF, and RGL3 thereby leading to the increased activation of the Ras-family GTPases RAL A and RAL B. (Feng, Ouyang et al. 1996; Wolthuis and Bos 1999; Xu, Shi et al. 2007). In addition to these 4 RAL GEFs that are able to interact with RAS, RAP and R-RAS small GTP-ases have also been shown to interact with RAS, but it is not known if R-RAS and RAP proteins are able to activate the RAL proteins (Rodriguez-Viciana, Sabatier et al. 2004). Upon activation, RAL-GTP interacts with multiple downstream effectors, including proteins involved in cell proliferation and survival, endocytosis, and actin organization (Neel, Martin et al. 2011).

The two RAL isoforms, A and B, (named for being “Ras-like”) are encoded by two very similar genes, but have been shown to have distinct roles and functions in cancer cells (Neel, Martin et al. 2011). One such study demonstrated that RAL A is needed for the anchorage independent growth of tumor cell lines, while RAL B is required for survival of those same cell lines (Chien and White 2003). Although much of the prior research into RAS stimulated signaling cascades has focused on RAF-MEK-ERK and PI3K-AKT, these studies demonstrate that RAL A and RAL B may also be key regulations of cell proliferation and survival. In support of a role for this pathway in
pancreatic tumorigenesis, human pancreatic tumors show high levels of RAL A and RAL B as well as RALGEF and RGL2 (Lim, O'Hayer et al. 2006). In addition, shRNA mediated knockdown of RAL A in PDAC cell lines resulted in decreased anchorage independent growth \textit{in vitro} and decreased sub-cutaneous tumor formation \textit{in vivo}, while shRNA mediated knockdown of RAL B resulted in decreased invasion of PDAC cell lines, demonstrating a role for these proteins in pancreatic tumor progression (Lim, O'Hayer et al. 2006).

Activation of the PLC-$\varepsilon$ signaling cascades by RAS is less well understood, but recent studies have demonstrated stimulation of this pathway downstream of activated RAS, and indicate that this pathway may promote RAS induced activation of PKC and calcium mobilization (Downward 2003).

In addition to these pathways, numerous other downstream effectors of Ras have been identified, although the effects of their activation by Ras are not yet well characterized. These include AF6, NORE1, TIAM1, RIN1, RGS12, and IMP (Cox and Der 2010).
Figure 1.2:

**RAS Stimulated Signaling** (Adapted from Downward 2003)
Mouse Models of Pancreatic Cancer

In order to better study the genetic and morphological changes inherent to pancreatic cancer, numerous mouse models have been generated in an attempt to re-capitulate the formation of PDAC and most specifically the progression from normal pancreas to PanIN lesions to adenocarcinoma that is observed in the human disease. The earliest mouse models of pancreatic cancer made use of the observation that the Rat Elastase I promoter could direct gene expression specifically within acinar cells (Swift, Hammer et al. 1984; Ornitz, Palmiter et al. 1985; Ornitz, Hammer et al. 1987; Quaife, Pinkert et al. 1987). Using this, models were generated that targeted the expression of activated $Hras$ or SV40 T-antigen to the pancreas through coupling these genes with the Elastase promoter (Ornitz, Hammer et al. 1987; Quaife, Pinkert et al. 1987). These models resulted in acinar cell neoplasms, whereas Elastase driven expression of $c-myc$ resulted in a mixed acinar/ductal neoplasm (Sandgren, Quaife et al. 1991). These models fell short of truly re-capitulating human disease, though, due to their generation of tumors largely comprised of acinar cells.

In 2003, Lewis and colleagues developed a novel model of pancreatic cancer that utilized the RCAS-TVA mouse modeling system. This model targeted expression of the $PyMT$ and $c-Myc$ oncogenes to the pancreas using the Elastase promoter, and found that $PyMT$ induced acinar and ductal tumors, while $c-Myc$ resulted in exclusively endocrine tumors (Lewis, Klimstra et al. 2003). These findings revealed that different tumor types can arise from the same cell of origin, demonstrating the potential for pancreatic tumors to arise from common progenitors and that the type of tumor formed may depend upon
the genetic alterations that occur. This concept was later confirmed by Tyler Jacks and colleagues, who demonstrated the existence of pancreatic progenitor cells through the targeted expression of Cre in adult acinar cells, and showed that the fate of these cells is regulated at least in part by oncogenic stress and the expression of KRAS$^{G12D}$ (Gidekel Friedlander, Chu et al. 2009).

In order to more effectively generate relevant mouse models of pancreatic cancer, the biology of the pancreas and its development needed to be established. Lineage tracing experiments proved very useful in establishing the progression of pancreatic cell progenitors, and in providing lineage specific expression markers that could be used in mouse modeling. These studies identified PDX1 as an early marker of pancreas cells, and demonstrated that it is expressed in all pancreatic progenitor cells (Gu, Dubauskaite et al. 2002). Further research has since demonstrated that in the adult pancreas, expression of PDX1 is restricted to beta cells (Ashizawa, Brunicardi et al. 2004). Additional studies identified PTF1A as a second marker of pancreatic progenitor cells, and demonstrated that its expression is specific to the exocrine pancreas in the mature organ (Krapp, Knofler et al. 1996). With the increased understanding in pancreas development and the discovery of lineage specific promoters and markers, it became possible to generate more clinically relevant PDAC mouse models.

More recent models for PDAC generated through the pancreas-specific expression of an activated Kras allele result in the formation of PDAC and have confirmed the critical role of KRAS during pancreatic tumor initiation (Aguirre, Bardeesy et al. 2003; Guerra, Mijimolle et al. 2003; Hingorani, Petricoin et al. 2003;
Hingorani, Wang et al. 2005). The first of these models demonstrates that the expression of a conditional $Kras^{G12D}$ expressed from the endogenous $Kras$ promoter coupled with expression of a $Pdx1-Cre$ or $Ptf1-p48-Cre$ allele results in pancreas specific expression of $Kras^{G12D}$ and the formation of progressive PanIN lesions and occasional PDAC (Hingorani, Petricoin et al. 2003). These models served to confirm the PanIN progression model that was originally proposed for human PDAC based upon the observation of tumor samples (Hruban, Goggins et al. 2000).

As these models express activated $Kras$ from the endogenous $Kras$ promoter, these models provide a more relevant system to study the impact of activated KRAS expression in the pancreas, as $KRAS$ is activated but not overexpressed in human pancreatic tumors. Indeed, tumor progression in this model has been shown to be very similar to the progression of human pancreatic tumors (Hingorani, Petricoin et al. 2003). However, unlike the RCAS-TVA model, these models do not allow for the flexibility of using several different oncogenes or even combining oncogenes in the same mouse model without the need to generate a new transgenic mouse for each new oncogene. Unlike the RCAS-TVA model, the endogenous $Kras$ model also does not result in the activation of $Kras$ in only a small subset of pancreas cells, which is likely to occur in human tumor formation, but instead results in the widespread activation of $Kras$ in the pancreas.

Following the observation that pancreas specific activation of $Kras$ results in PanINs and PDAC in mice, several labs have gone on to combine $Kras$ activation with other genetic changes present in human PDAC and have observed an acceleration of
tumor development in mice. These models include a combination of *Ink4a/Arf* deficiency (Aguirre, Bardeesy et al. 2003), *p53* mutation (Hingorani, Wang et al. 2005), loss of the *TgfβR2* (Ijichi, Chytil et al. 2006), loss of *Smad4* (Bardeesy, Cheng et al. 2006), and *Pten* inactivation (Iwanaga, Yang et al. 2008) with pancreas specific expression of *Kras<sup>G12D</sup>.* It is important to note that mouse models with pancreas specific loss of *Ink4A/Arf* in the absence of *Kras* activation do not result in the formation of PanIN lesions or PDAC, demonstrating that the ability to initiate pancreatic tumors is unique to the expression of activated *Kras* (Aguirre, Bardeesy et al. 2003). Collectively, these models have confirmed the role of activated *Kras* in the initiation of PDAC, and have provided a tractable system to study the genetic events that cause PanIN lesions to progress to PDAC.

However, despite clearly showing the role for activated KRAS in the formation of pancreatic tumors, these models have been unable to address the mechanisms by which activated KRAS initiates pancreatic tumors. For this reason, an alternative model using pancreatic ductal epithelial cells, or PDECs, has proved useful in studying the earliest stages of pancreatic tumor initiation. PDECs are the putative cell of origin for PDAC, and unlike pancreatic tumor cell lines, they are non-transformed and will not form tumors without the added expression of an oncogene such as *KRAS<sup>G12D</sup>* (Hruban, Goggins et al. 2000; Morton, Mongeau et al. 2007). In addition, we and others have previously shown that PDECs embedded in matrigel after isolation will continue to form duct like structures, demonstrating that they retain the properties of ductal epithelial cells (Schreiber, Deramaudt et al. 2004; Morton, Mongeau et al. 2007). As a result, PDECs
provide an excellent model for studying the earliest events of KRAS-mediated pancreatic tumor initiation. Indeed, we have previously used this model to show that the expression of KRAS\(^{G12D}\) in PDECs results in increased proliferation, survival when challenged with apoptotic stimuli, and in the absence of Ink4a/Arf, the ability to form tumors following orthotopic transplant (Morton, Mongeau et al. 2007). These data show a clear role for the activation of Kras in the transformation of pancreatic epithelial cells, and underscore its importance as a critical player in the initiation of pancreatic tumorigenesis. Using a similar experimental approach, Lee and Bar-Sagi recently demonstrated a role for TWIST in bypassing oncogenic KRAS-induced cellular senescence, further demonstrating the ability of this model to elucidate the early changes in KRAS-mediated pancreatic tumorigenesis (Lee and Bar-Sagi 2010).

The Role of Ras-induced Signaling Pathways in Pancreatic Cancer

Many studies have been conducted assessing the role for activated RAS and its downstream signaling cascades in the formation and progression of pancreatic tumors. To address the question of which of the RAS stimulated signaling cascades are most important to pancreatic tumorigenesis, a series of RAS mutants have been used which have mutations in their effector loop that result in the preferential binding of a single downstream effector. These binding mutants were first characterized in HRAS using a yeast two-hybrid screen, which identified that the S35T mutant preferentially binds, RAF, while the C40Y mutant preferentially binds PI3K and the G37D mutant preferentially binds RALGDS (White, Nicolette et al. 1995). It has since been discovered that a
mutation at N38D will preferentially signal through the PLC-e signaling cascade, and collectively these mutations have become a vital tool in the study of RAS stimulated signaling.

Despite the availability of tools to assess signaling downstream of activated RAS, studies investigating the consequences of activated KRAS in pancreatic cancer have largely had conflicting results. One study, by Hamad et al in 2002 identified that the importance of a particular RAS effector is highly dependent on the cell context being studied (Hamad, Elconin et al. 2002). Through the expression of various HRAS effector binding mutants in human and murine cells, they determined that the signaling pathways required for anchorage independent growth can vary greatly from cell type to cell type. In murine fibroblasts, it was determined that the RAF-MEK-ERK, PI3K-AKT, and RALGDS signaling cascades all contribute to anchorage independent growth, with the greatest contribution coming from RAF-MEK-ERK signaling. However, in human fibroblasts, they determined that it was only the RALGDS pathway that contributes to anchorage independent growth of the cells, and that even the combination of RAS stimulated RAF-MEK-ERK and PI3K-AKT signaling in these cells was not sufficient for transformation. Yet when they continued this study by looking at the ability of human cells expressing the HRAS binding mutant that preferentially signals through RALGDS to form tumors following sub-cutaneous injection, they found that these cells are completely incapable of tumor formation (Lim and Counter 2005). In fact, tumor formation only results from the combination of signaling through both RALGDS and RAF-MEK-ERK, or RALGDS, RAF-MEK-ERK, and PI3K-AKT, and even those
combinations of the effector mutants are unable to re-capitulate tumor formation in HRAS$^{G12V}$ expressing cells.

Additional studies have since been conducted to investigate the role of RAL A and RAL B downstream of activated KRAS in human pancreatic cancer cell lines. These studies demonstrated that RAL A is required for anchorage independent growth of those cells, while RAL B is important for invasion in these cells (Lim, O'Hayer et al. 2006). However, these studies were still conducted in transformed cells, and as such fail to examine the role of RAL A and RAL B during KRAS-mediated pancreatic tumor initiation. More recent studies have looked at the effect of signaling downstream of activated KRAS in human pancreatic ductal cells, a cell culture model intended to be more relevant to the initiation of pancreatic tumors, but which still required genetic alterations and immortalization before any experiments were conducted (Campbell, Groehler et al. 2007). In contrast to the prior studies that implicated RAL as a key effector downstream of activated KRAS in the initiation of pancreatic tumors, this research found that RAF and PI3K were required for transformation and invasion of these cells (Campbell, Groehler et al. 2007).

Collectively, these studies demonstrate the conflicting findings that result from studying RAS-stimulated signaling in multiple cell contexts and underscore the need to study RAS-stimulated signaling in the most relevant cell context possible. In addition, as many of these previous studies involved the use of HRAS constructs and/or have been performed using transformed cells, there remains a need to understand the consequences of KRAS activation in the early stages of pancreatic tumor formation.
The Role of BRAF in Pancreatic Cancer

As discussed above, one of the pathways stimulated downstream of activated KRAS is the RAF-MEK-ERK signaling cascade, and a key family of proteins involved in this signaling is the RAF family of serine-threonine kinases. Previous studies have demonstrated that this signaling pathway is hyperactivated in approximately 30% of human cancers, indicating an important role for RAF-MEK-ERK signaling in tumorigenesis (Hoshino, Chatani et al. 1999). To underscore the role of RAF in cancer, BRAF is commonly mutated in multiple human malignancies including malignant melanoma, thyroid cancer, and ovarian cancer (Davies, Bignell et al. 2002). These mutations result in constitutive kinase activation of BRAF, often to a level significantly higher than that of wild type BRAF (Downward 2003). The most common mutation is V600E, which accounts for 90% of all BRAF mutations (Wan, Garnett et al. 2004). These findings point to a role for RAF, and more specifically BRAF, in the formation and progression of these and other tumor types.

However, BRAF gene mutations are almost always mutually exclusive with KRAS mutations, and thus given the high rate of KRAS mutations in PDAC, BRAF mutations are infrequently seen in this disease (Davies, Bignell et al. 2002). Previous work by Kern and colleagues has shown, though, that in the small subset of tumors that do not have activating mutations within the KRAS2 oncogene, 33% have activating mutations in BRAF (Calhoun, Jones et al. 2003). These findings raise the possibility that activating
BRAF mutations may functionally substitute for KRAS gene mutations during pancreatic tumor initiation.

Summary

It has been well established that activated KRAS has a key role in the formation of pancreatic tumors. Yet there is a lack of understanding of how KRAS-stimulated signaling contributes to the initiation and progression of the disease, as prior studies have produced contradicting evidence on the importance of the multiple signaling cascades downstream of activated KRAS in the formation and progression of pancreatic tumors. What these studies have demonstrated is that the effects of signaling through activated KRAS are highly context dependent, and that a complete understanding of KRAS-stimulated signaling requires research using the most relevant cell context possible.

Therefore, the goal of this work was to better address the question of the role of activated KRAS in pancreatic tumor initiation by investigating the roles of the RAF/MEK/ERK and PI3K/AKT signaling pathways in KRAS-mediated transformation of pancreatic epithelial cells, which are the putative cells of origin of PDAC. Since the activation of BRAF lies downstream of activated KRAS, and BRAF is activated in numerous human malignancies, this work also sought to investigate whether an activated BRAF molecule functionally substitutes for activated KRAS in pancreatic ductal epithelial cells.

The data provided in chapter 2 uncover an important role for the IGF1R in pancreatic tumor initiation and progression even in the context of KRAS or BRAF
activation, and show that MEK induced expression of IGF2 and subsequent activation of the IGF1R is necessary for pancreatic tumor formation. These studies also demonstrate key differences between signaling downstream of activated KRAS or BRAF in PDECs compared with transformed tumor cell lines, and as a result, identify potential key differences between KRAS and BRAF stimulated signaling during tumor initiation versus tumor progression, underscoring the need to study signaling downstream of KRAS and BRAF activation in the most relevant cell context possible.
Chapter II:

KRAS- and BRAF-Induced Pancreatic Tumor Formation Requires MEK-ERK Stimulated IGF1R Signaling
Figure Contribution

Jiu-Feng Cai provided the data in Figure 2.8, 2.9, 2.10 and Figure 2.25B.

Leanne Ahronian provided the data in Figure 2.11, 2.31.

Victoria Appleman provided the data for all other figures and tables in this chapter.
Introduction

Over 90% of all cases of PDAC have activating mutations in the KRAS2 oncogene, and activation of KRAS has been shown to be a very early event in the formation of pancreatic tumors (Hezel, Kimmelman et al. 2006). In addition, recent mouse models of pancreatic cancer have demonstrated an important role for Kras activation in the formation of pancreatic tumors by showing that the pancreas specific expression of activated Kras results in PanIN lesions and PDAC (Aguirre, Bardeesy et al. 2003; Guerra, Mijimolle et al. 2003; Hingorani, Petricoin et al. 2003; Hingorani, Wang et al. 2005). Targeting activated KRAS for cancer treatment has proved challenging, though, and recent work has sought to elucidate signaling downstream of activated KRAS and determine which of the KRAS stimulated signaling pathways are important for the formation and progression of pancreatic tumors. These studies have revealed that signaling downstream of activated KRAS is highly dependent on cell context, and that fully understanding the effects of activated KRAS in the initiation of pancreatic tumors requires the most relevant cell context possible (Lim and Counter 2005).

To address the question of what the roles of activated KRAS and its downstream effectors are in the initiation of pancreatic tumors, I sought to investigate the effects of activated KRAS expression in pancreatic ductal epithelial cells (PDECs), the putative cell of origin for PDAC (Hruban, Goggins et al. 2000). To further probe the role of the MEK/ERK signaling cascade, the best characterized of the KRAS downstream effectors, I also sought to determine the effect of expressing activated BRAF in PDECs. BRAF mutations are common in many types of human cancers, but are rare in pancreatic cancer,
due to the fact that BRAF and KRAS mutations are mutually exclusive (Davies, Bignell et al. 2002). However, 33% of all pancreatic tumors that do not have KRAS mutations have been shown to have BRAF mutations, illustrating the importance of MEK/ERK signaling in PDAC, and raising the question of whether BRAF mutations are able to functionally substitute for KRAS mutations (Calhoun, Jones et al. 2003).

To achieve expression of activated KRAS (KRAS$^{G12D}$) and activated BRAF (BRAF$^{V600E}$) in PDECs, I utilized the RCAS-TVA mouse modeling system. This system utilizes a subgroup-A avian leukosis-sarcoma virus (ALSV), a retrovirus that is normally only able to infect avian cells (Weiss, 1982). Mammalian cells can be rendered susceptible to infection with ALSVs through the expression of the viral receptor for ASLVs, TVA, on the cell surface (Bates, Young et al. 1993; Young, Bates et al. 1993). Mouse models with tissue and cell specific expression of the TVA receptor can be generated through the expression of TVA under the control of a tissue specific promoter. In these models, all dividing cells that express the TVA receptor are therefore susceptible to infection with ASLVs.

For these studies, the Rous Sarcoma Virus (RSV)- derived vector RCAS (replication competent ASLV long terminal repeat with splice acceptor) retroviruses were used for the expression of genes of interest (Hughes, Greenhouse et al. 1987; Greenhouse, Petropoulos et al. 1988; Petropoulos and Hughes 1991; Boerkoel, Federspiel et al. 1993). These vectors were generated by replacing the src gene with a multi-cloning site, which can accommodate up to 2.5kB of insert (Hughes, Greenhouse et al. 1987; Greenhouse, Petropoulos et al. 1988; Petropoulos and Hughes 1991; Boerkoel,
Federspiel et al. 1993) These viruses can be propagated in DF1 chicken fibroblasts, which provide a high titer, replication-competent viral stock (Himly, Foster et al. 1998; Schaefer-Klein, Givol et al. 1998). Following infection of these viruses into mammalian cells, the viral genome integrates into the host DNA, and the viral LTRs produce transcription of the provirus (Hughes, Greenhouse et al. 1987). The mRNA, expressing the gene of interest that has been inserted into the multi-cloning site, is then processed via an artificial splice acceptor (Hughes, Greenhouse et al. 1987). This system therefore allows the achievement of tissue or cell specific expression of genes of interest following RCAS virus infection of only those cells expressing the TVA receptor.

For these studies, I isolated PDECs from mice expressing the TVA receptor under the regulation of the Keratin-19 (K19) gene promoter and enhancer elements (Bader and Franke 1990; Hu and Gudas 1994; Grippo and Sandgren 2000; Orsulic 2002; Morton, Mongeau et al. 2007). It has been previously shown that the expression of TVA under the K19 promoter and enhancer elements results in the expression of TVA specifically within the duct epithelium (B. Lewis and H. Varmus, unpublished). Work within our lab has also demonstrated that PDECs embedded in matrigel after isolation will form duct like structures, demonstrating that they retain the properties of ductal epithelial cells (Schreiber, Deramaudt et al. 2004; Morton, Mongeau et al. 2007). In addition, our lab has previously demonstrated that PDECs isolated from mice expressing K19-TVA are susceptible to infection by ALV-A-based RCAS retroviruses (Morton, Mongeau et al. 2007). Therefore, these cells can be infected with RCAS-KRAS\textsuperscript{G12D} and RCAS-
BRAF$^{V600E}$, thereby providing a system to assess the effect of KRAS-stimulated signaling in pancreatic ductal epithelial cells.

Here, I demonstrate that both KRAS and BRAF stimulate the proliferation and survival of PDECs in culture, and that the induced survival is dependent on signaling through both the MEK/ERK and PI3K/AKT signaling pathways. In addition, I demonstrate that activation of AKT occurs in a manner dependent on MEK/ERK and the IGF1R, and that cells expressing activated KRAS and BRAF depend upon IGF2-stimulated IGF1R signaling for survival after exposure to apoptotic stimuli. Moreover, I show that KRAS$^{G12D}$- and BRAF$^{V600E}$-induced tumor formation in an orthotopic pancreatic tumor model is dependent on the IGF1R. Finally, I demonstrate that signaling in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing tumor cell lines is not the same as in PDECs. I show that while the tumor cell lines depend on PI3K, MEK, and the IGF1R for cell proliferation, they are less dependent than the PDECs on MEK and the IGF1R for their survival in response to an apoptotic stimulus. Collectively, these data provide new insights into the mechanisms underlying KRAS-mediated initiation of pancreatic tumorigenesis, as well as the differences between signaling downstream of KRAS$^{G12D}$ in pancreatic tumor initiation and tumor progression.
Results

Expressing KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} Within Pancreatic Ductal Epithelial Cells.

To investigate the effects of activated KRAS and BRAF on pancreatic ductal epithelial cells (PDECs), I isolated PDECs from transgenic mice expressing the avian leukemia virus subgroup A (ALV-A) receptor, TVA, under the control of the Keratin-19 (K19) gene promoter and enhancer elements (Bader and Franke 1990; Hu and Gudas 1994; Grippo and Sandgren 2000; Orsulic 2002; Morton, Mongeau et al. 2007). PDECs were also isolated from \textit{K19-tv-a} mice with pancreas specific deletion of the \textit{Ink4a/Arf}, and/or \textit{Trp53} tumor suppressor genes (Jonkers, Meuwissen et al. 2001; Krimpenfort, Quon et al. 2001; Morton, Mongeau et al. 2007). TVA-positive PDECs were infected with RCAS viruses encoding Flag epitope-tagged KRAS\textsuperscript{G12D}, BRAF\textsuperscript{V600E}, or GFP as a control (Morton, Mongeau et al. 2007). Infection of PDECs by RCAS-\textit{Kras}\textsuperscript{G12D} was confirmed by immunoblotting for the Flag epitope tag (Figure 2.1A). Importantly, quantitative RT-PCR (qRT-PCR) showed that the expression of the ectopic \textit{Kras}\textsuperscript{G12D} resulted in only a 3-fold increase in mRNA levels, indicating that any phenotypes observed from the expression of RCAS-\textit{Kras}\textsuperscript{G12D} are not likely to be non-specific effects due to overexpression of \textit{Kras} (Figure 2.1B). Elevated levels of BRAF in RCAS-\textit{Braf}\textsuperscript{V600E} infected cells relative to GFP infected cells were demonstrated by immunoblotting (Figure 2.2A). This increased expression was specific to BRAF, as increased levels of ARAF and CRAF were not observed (Figure 2.2B).
Figure 2.1

Infection of K19-TVA Expressing PDECs with RCAS-KRAS^{G12D} results in increased expression of Kras.

(A) Western blot confirming expression of ectopic Flag epitope-tagged KRAS^{G12D} in Ink4a/Arf, Trp53 null PDECs infected with RCAS-KRAS^{G12D}. β -actin is used as a loading control.

(B) Quantitative RT-PCR analysis of the expression of murine Kras in Ink4a/Arf, Trp53 null PDECs expressing RCAS-KRAS^{G12D} relative to Ink4a/Arf, Trp53 null PDECs expressing GFP. Results shown are from two individual primer sets targeting murine Kras with the expression level normalized to β-actin.
Figure 2.2

Infection of K19-TVA Expressing PDECs with RCAS-BRAF\textsuperscript{V600E} results in increased expression of BRAF.

(A) Western blot confirming elevated expression of BRAF in \textit{Ink4a/Arf}, \textit{Trp53} null PDECs infected with RCAS-BRAF\textsuperscript{V600E} relative to \textit{Ink4a/Arf}, \textit{Trp53} null PDECs infected with GFP. \(\beta\)-actin is used as a loading control. Values indicate the ratio of BRAF levels relative to \(\beta\)-actin levels as measured by densitometry and normalized such that GFP expressing PDECs have a ratio of 1.

(B) Western blot analysis showing similar levels of ARAF and CRAF expression in \textit{Ink4a/Arf}, \textit{Trp53} null PDECs infected with RCAS-BRAF\textsuperscript{V600E} or RCAS-GFP. \(\beta\)-actin is used as a loading control.
Expression of Kras$^{G12D}$ and BRAF$^{V600E}$ increases PDEC proliferation and survival

Following infection of PDECs with RCAS-KRAS$^{G12D}$, RCAS-BRAF$^{V600E}$, and RCAS-GFP, I next investigated the effect of activated KRAS and mutant BRAF had on the proliferation of PDECs. As previously shown within our lab, expression of activated KRAS in these cells resulted in increased proliferation over control cells, and this effect was similar in both tumor suppressor wild type as well as Ink4a/Arf, Trp53 null PDECs (Figure 2.3A and 2.4A) (Morton, Mongeau et al. 2007). Expression of mutant BRAF in PDECs also resulted in increased proliferation relative to control cells, but notably, this increase was not as great as in KRAS$^{G12D}$ expressing PDECs, both in tumor suppressor wild type and Ink4a/Arf, Trp53 null cells (Figure 2.3A and 2.4A). The ability of mutant BRAF to partially recapitulate the phenotype observed in KRAS$^{G12D}$ expressing PDECs indicates that increased proliferation downstream of activated KRAS is due in part to the RAF/MEK/ERK signaling pathway. However, BRAF$^{V600E}$’s inability to completely mimic the KRAS$^{G12D}$ phenotype may also suggest that the increased proliferation downstream of activated KRAS depends on other signaling cascades which are not activated downstream of mutant BRAF. It is possible, however, that the expression of BRAF$^{V600E}$ results in RAF/MEK/ERK signaling that either quantitatively or qualitatively differs from that downstream of KRAS$^{G12D}$ and this results in the differences seen in proliferation rates. In addition, I observed that PDECs expressing a wild type BRAF molecule had a similar level of proliferation as GFP expressing PDECs, indicating that the mutational status of BRAF is important for the increased proliferation observed in BRAF$^{V600E}$ expressing cells (Figure 2.5A and 2.6A).
I next sought to determine the effect of KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expression on PDEC survival when challenged with an apoptotic stimulus. For these assays, PDECs were treated with ultraviolet (UV) irradiation or cycloheximide, a cytotoxic agent that has been previously shown to cause apoptosis in PDECs and pancreatic cancer cells (Koehler and Drucker 2006; Morton, Mongeau et al. 2007). I found increased survival in both KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs relative to control cells when treated with either ultraviolet (UV) irradiation or cycloheximide (Figure 2.3B, 2.3C, 2.4B, and 2.4C). As observed in the proliferation studies, these effects were irrespective of tumor suppressor status, and similar results were obtained in wild type (Figure 2.3B and 2.3C) as well as \textit{Ink4a/Arf} (Figure 2.4C) and \textit{Ink4a/Arf, Trp53} null PDECs (Figure 2.4B). It should be noted, though, that apoptosis following treatment with UV irradiation depends upon wild type p53 in the cells. As a result, UV irradiation was only used for tumor suppressor wild type or \textit{Ink4a/Arf} null PDECs, and any survival assays involving \textit{Ink4a/Arf, Trp53} null PDECs were conducted using only cycloheximide as an apoptotic stimulus.

In addition, I observed increased survival relative to control cells in PDECs expressing wild type BRAF when treated with cycloheximide in both tumor suppressor wild type and \textit{Ink4a/Arf, Trp53} null cells (Figure 2.5B and 2.6B, respectively). These findings suggest that the expression of BRAF, both mutant and wild type, is able to functionally substitute for activated KRAS expression with regards to survival in PDECs, and therefore indicate that signaling downstream of the RAF/MEK/ERK pathway may be key to survival of pancreatic epithelial cells.
The expression of KRAS$^{G12D}$ and BRAF$^{V600E}$ in tumor suppressor wild type PDECs results in increased proliferation and survival when challenged with apoptotic stimuli.

(A) Cell numbers of tumor suppressor wild type PDECs showing increased proliferation at 15 days of KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs relative to GFP expressing control cells.

(B) Viability (as measured by trypan blue exclusion) of tumor suppressor wild type PDECs showing increased survival of KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing cells relative to GFP controls following treatment with 100µM cycloheximide. Values are normalized such that that viability of untreated cells is 1. *p < 0.01 for CHX treated KRAS$^{G12D}$ or BRAF$^{V600E}$ expressing PDECs compared with CHX treated GFP expressing controls.

(C) Viability (as measured by trypan blue exclusion) of tumor suppressor wild type PDECs showing increased survival of KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing cells relative to GFP controls following treatment with UV irradiation. Values are normalized such that that viability of untreated cells is 1. *p < 0.01 for UV treated KRAS$^{G12D}$ or BRAF$^{V600E}$ expressing PDECs compared with UV treated GFP expressing controls.
Figure 2.4
The expression of KRAS$^{G12D}$ and BRAF$^{V600E}$ in Ink4a/Arf, Trp53 and Ink4a/Arf null PDECs results in increased proliferation and survival when challenged with apoptotic stimuli.

(A) Cell numbers of Ink4a/Arf, Trp53 null PDECs showing increased proliferation at 15 days of KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs relative to GFP expressing control cells.

(B) Viability (as measured by trypan blue exclusion) of Ink4a/Arf, Trp53 null PDECs showing increased survival of KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing cells relative to GFP controls following treatment with 100µM cycloheximide. Values are normalized such that that viability of untreated cells is 1. *p < 0.01 for CHX treated KRAS$^{G12D}$ or BRAF$^{V600E}$ expressing PDECs compared with CHX treated GFP expressing controls.

(C) Viability (as measured by trypan blue exclusion) of Ink4a/Arf null PDECs showing increased survival of KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing cells relative to GFP controls following treatment with UV irradiation. Values are normalized such that that viability of untreated cells is 1. *p < 0.01 for UV treated KRAS$^{G12D}$ or BRAF$^{V600E}$ expressing PDECs compared with UV treated GFP expressing controls.
The expression of wild type BRAF in tumor suppressor wild type PDECs does not result in increased proliferation, but does result in increased survival when challenged with an apoptotic stimulus.

(A) Cell numbers of tumor suppressor wild type PDECs showing increased proliferation at 15 days of BRAF<sup>V600E</sup> expressing cells, but not wild type BRAF expressing cells, relative to GFP controls.

(B) Viability (as measured by trypan blue exclusion) of tumor suppressor wild type PDECs showing increased survival of BRAF<sup>V600E</sup> (V5 tagged) and wild type BRAF expressing cells relative to GFP controls following treatment with 100µM cycloheximide. Values are normalized such that that viability of untreated cells is 1.

*p < 0.01 for CHX treated WT BRAF or BRAF<sup>V600E</sup> expressing PDECs compared with CHX treated GFP expressing controls.
Figure 2.6

The expression of wild type BRAF in Ink4a/Arf, Trp53 null PDECs does not result in increased proliferation, but does result in increased survival when challenged with an apoptotic stimulus.

(A) Cell numbers of Ink4a/Arf, Trp53 null PDECs showing increased proliferation at 15 days of BRAF\(^{V600E}\) expressing cells, but not wild type BRAF expressing cells relative to GFP expressing control cells.

(B) Viability (as measured by trypan blue exclusion) of Ink4a/Arf, Trp53 null PDECs showing increased survival of BRAF\(^{V600E}\) (V5 tag) and wild type BRAF expressing cells relative to GFP expressing control cells following treatment with 100µM cycloheximide. Values are normalized such that that viability of untreated cells is 1.

*p < 0.05 for CHX treated WT BRAF or BRAF\(^{V600E}\) expressing PDECs compared with CHX treated GFP expressing controls
Expression of either KRAS$^{G12D}$ or BRAF$^{V600E}$ in Ink4a/Arf, Trp53 deficient PDECs results in tumor formation following orthotopic transplant

Having shown that BRAF$^{V600E}$ expression in PDECs results in increased proliferation and survival of those cells, I next sought to determine if the expression of BRAF$^{V600E}$ in PDECs was sufficient to induce tumor formation following orthotopic transplant. Previous studies within our lab have shown that the expression of KRAS$^{G12D}$ in Ink4a/Arf, Trp53 null PDECs is sufficient to induce tumor formation following orthotopic transplant (Morton, Mongeau et al. 2007), and in this study, I assessed if the same was true for BRAF$^{V600E}$ expressing PDECs null for Ink4a/Arf and Trp53. I found that implantation of KRAS$^{G12D}$- and BRAF$^{V600E}$-expressing PDECs resulted in efficient pancreatic tumor formation, whereas the implantation of GFP expressing cells did not efficiently result in tumor formation (Table 2.1). It should be noted that although in this cohort of mice, KRAS$^{G12D}$ expressing cells did form tumors sooner than BRAF$^{V600E}$ expressing cells (4 weeks versus 8 weeks) and tumors formed from KRAS$^{G12D}$ expressing cells were slightly larger than those from BRAF$^{V600E}$ expressing cells (average tumor volume of 1679 mm$^3$ versus 1197 mm$^3$), multiple orthotopic transplant experiments with KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs have demonstrated that this is not consistently the case.
Table 2.1

Tumor induction by $1 \times 10^6$ KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing Ink4a/Arf, Trp53 null PDECs following orthotopic transplant.

<table>
<thead>
<tr>
<th>Cells Implanted</th>
<th>Tumor Incidence</th>
<th>Time to Tumor Development (Weeks)</th>
<th>Average Tumor Volume (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>1/6</td>
<td>8</td>
<td>400mm$^3$ (±0)</td>
</tr>
<tr>
<td>KRAS$^{G12D}$</td>
<td>6/6</td>
<td>4</td>
<td>1679mm$^3$ (±607)</td>
</tr>
<tr>
<td>BRAF$^{V600E}$</td>
<td>5/6</td>
<td>8</td>
<td>1197mm$^3$ (±430)</td>
</tr>
</tbody>
</table>

Tumor Volume Calculated Using the Formula LxWxH
Subtle Differences Exist in the Tumors Formed from orthotopic transplant of KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} Expression PDECs

Next, I investigated whether the tumors formed following orthotopic transplant differed between mice transplanted with KRAS\textsuperscript{G12D} or BRAF\textsuperscript{V600E} expressing PDECs. Hematoxylin and eosin staining of tumor tissue showed that transplantation of both KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing cells primarily resulted in the formation of undifferentiated carcinomas, consistent with our previously published findings (Figure 2.7) (Morton, Mongeau et al. 2007). Surprisingly, tumors formed after the injection of BRAF\textsuperscript{V600E}-expressing PDECs additionally contained regions with features of skeletal, cartilaginous, or bone differentiation, traits consistent with mesenchymal differentiation (Figure 2.7C and 2.7D). Consistent with this, KRAS-induced tumors displayed cytokeratin 8 staining throughout the tumor, whereas BRAF-induced tumors displayed cytokeratin 8 staining only in regions displaying glandular differentiation (Figure 2.8). The mechanisms underlying this mesenchymal-like differentiation remain undetermined.

I evaluated proliferation within the various tumors via staining for Ki67, and observed that transplantation of both KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs resulted in tumors with similar levels of proliferation (2.9). Interestingly, tumors obtained from mice transplanted with KRAS\textsuperscript{G12D} expressing PDECs, though, frequently displayed regions of highly proliferative cells and other regions with very little proliferation (Figure 2.9A and 2.9B), while tumors from mice transplanted with BRAF\textsuperscript{V600E} expressing PDECs appeared to have a moderate level of proliferating cells dispersed throughout much of the tumor tissue (Figure 2.9C and 2.9D). Staining for the
pancreatic epithelial cell marker Pdx-1 showed Pdx-1 expression in regions of both KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing tumors, particularly within ductal cells (Figure 2.10A and 2.10C), as well as regions of both KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing tumors that were Pdx-1 negative (Figure 2.10B and 2.10D). These findings show that while a portion of the tumors retains expression of this epithelial cell marker, there are also cells present in the tumor that have either lost this epithelial marker during tumorigenesis, or are non-epithelial cells recruited to the tumor after initiation.
Figure 2.7

Tumors formed from orthotopic transplant of KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing Ink4a/Arf, Trp53 null PDECs have similar tumor histology.

Staining of tissue sections from orthotopic transplant tumors Hematoxylin and Eosin illustrating the similarities and differences between Kras$^{G12D}$ (A and B) and Brafi$^{V600E}$ (C and D) induced tumors. KRAS$^{G12D}$ induced tumors display undifferentiated histology, while BRAF$^{V600E}$ induced tumors display both regions of undifferentiated histology (C and D) as well as regions of glandular differentiation (C, white arrow) and bone differentiation (D, white arrow).
Figure 2.8

Tumors formed from orthotopic transplant of KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing Ink4a/Arf, Trp53 null PDECs show different patterns of Troma1 staining.

Staining of tissue sections from orthotopic transplant tumors with Troma1 (cytokeratin 8) illustrating the consistent cytokeratin 8 staining throughout Kras$^{G12D}$ induced tumors (A) in contrast to the presence of Troma1 positive cells only in the glandular regions of Braf$^{V600E}$ induced tumors (B).
Figure 2.9

Tumors formed from orthotopic transplant of KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing Ink4a/Arf, Trp53 null PDECs show similar overall levels of proliferation.

Staining of tissue sections from orthotopic transplant tumors with Ki67 illustrating the similarities in levels of proliferation in Kras\textsuperscript{G12D} (A and B) and Braf\textsuperscript{V600E} (C and D) induced tumors. However, KRAS\textsuperscript{G12D} induced tumors displayed regions of highly proliferative cells (A) and regions with low proliferation (B), while BRAF\textsuperscript{V600E} induced tumors display moderate levels of proliferation through the entire tumor tissue (C and D).
Figure 2.10

Tumors formed from orthotopic transplant of $\text{KRAS}^{G12D}$ and $\text{BRAF}^{V600E}$ expressing $\text{Ink4a/Arf, Trp53}$ null PDECs show different patterns of Pdx1 staining.

Staining of tissue sections from orthotopic transplant tumors with Pdx1 illustrating the similarity between $\text{Kras}^{G12D}$ (A and B) and $\text{Braf}^{V600E}$ (C and D) induced tumors. Both $\text{KRAS}^{G12D}$ and $\text{BRAF}^{V600E}$ induced tumors display regions with pdx1 positive cells (A and C) as well as regions that are pdx1 negative (B and D).
**KRAS^{G12D} and BRAF^{V600E} Expressing Tumor Cell Lines Have Differing Levels of Anchorage Independent Growth and Migration**

I additionally analyzed the transformation-associated phenotypes of cell lines derived from KRAS^{G12D} and BRAF^{V600E} expressing tumors. I found that while there was variation within each group, overall KRAS^{G12D} expressing cell lines had enhanced anchorage-independent growth capacity relative to tumor cell lines expressing BRAF^{V600E} (Figure 2.11A). In addition, KRAS^{G12D} expressing cell lines had enhanced migration activity relative to BRAF^{V600E} expressing cell lines (Figure 2.11B). Together, these data suggest that signaling pathways stimulated by activated KRAS, but not BRAF, contribute to the transformed phenotype in pancreatic cancer cells. These data also suggest that although activated BRAF is able to largely recapitulate the phenotype of activated KRAS in PDECs, there are subtle differences between the signaling downstream of these two oncogenes, and these differences may impact tumor progression.
**Figure 2.11**

KRAS$^{G12D}$ expressing tumor cell lines have enhanced migration and anchorage independent growth when compared with BRAF$^{V600E}$ expressing tumor cell lines.

**(A)** Average number of colonies formed in soft agar showing enhanced anchorage independent growth of three KRAS$^{G12D}$ expressing cell Ink4a/Arf, Trp53 null tumor cell lines compared with three BRAF$^{V600E}$ expressing Ink4a/Arf, Trp53 null tumor cell lines.

**(B)** Average number of migrating cells as assessed with BD Matrigel Migration Chambers showing enhanced migration of three KRAS$^{G12D}$ expressing cell Ink4a/Arf, Trp53 null tumor cell lines compared with three BRAF$^{V600E}$ expressing Ink4a/Arf, Trp53 null tumor cell lines.
Signaling downstream of MEK and PI3K is necessary for survival in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs

The PI3K/AKT signaling cascade is typically viewed as the survival-inducing pathway downstream of activated KRAS. As activated BRAF signals primarily through the MEK/ERK signaling cascade, it was surprising to observe similar levels of cell survival in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs after challenge with apoptotic stimuli. As a result, I next probed the signaling requirements of PDEC survival downstream of both activated KRAS and mutant BRAF, and investigated the effects of inhibiting either MEK or PI3K on survival in cells expressing KRAS$^{G12D}$ or BRAF$^{V600E}$. Surprisingly, I found that when PDECs were treated with the MEK inhibitor PD98059 or the PI3K inhibitor LY294002, survival in response to cycloheximide or UV irradiation in both KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs dropped to the level of GFP expressing cells (Figure 2.12A and 2.12B, respectively). These data indicate that both the MEK/ERK and the PI3K/AKT signaling cascades are necessary for the survival phenotype observed in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs. Similar results were found when PDECs were treated with rapamycin, an inhibitor of mTOR, as survival in both KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs dropped to that of GFP expressing cells (Figure 2.13), indicating that signaling through mTOR downstream of PI3K is needed for survival in these cells. When PDECs were treated simultaneously with both the MEK and PI3K inhibitors, survival in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs was once again decreased to the level of GFP expressing cells, but no additional decrease was seen with the combination of inhibitors, indicating that inhibition of either pathway...
resulted in the maximum impairment in survival measurable by this assay (Figure 2.14 and 2.15).

As a control to confirm that any observed loss of survival is not simply due to the addition of small chemical inhibitors, these survival assays also included PDECs that expressed Sonic Hedgehog (SHH). Our lab has previously demonstrated that SHH expressing PDECs have increased survival relative to GFP expressing cells, as well as increased activation of both RAF/MEK/ERK and PI3K/AKT signaling. (Morton, Mongeau et al. 2007). Despite activation of both of these pathways, SHH-induced survival is dependent on PI3K/AKT signaling, but not the MEK/ERK signaling cascade (Figure 2.12A and 2.12B) (Morton, Mongeau et al. 2007).

Our findings are consistent with previous studies implicating the PI3K/AKT pathway in survival downstream of activated KRAS, but differ from those studies by demonstrating that the RAF/MEK/ERK signaling cascade is also needed for survival in these cells. In addition, they present a novel finding demonstrating that activated BRAF depends on signaling through PI3K/AKT and does not rely solely on MEK/ERK signaling for its oncogenic effects.
Figure 2.12

Inhibition of MEK or PI3K results in decreased survival of KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> expressing PDECs when challenged with apoptotic stimuli.

(A) Viability (as measured by trypan blue exclusion) of Ink4a/Arf, Trp53 null PDECs expressing KRAS<sup>G12D</sup>, BRAF<sup>V600E</sup>, SHH, or GFP. For these assays, cells were first vehicle treated or treated with the MEK inhibitor PD98059 or the PI3K inhibitor LY2900042, and then one hour later either vehicle treated or treated with 100µM cycloheximide. Values are normalized such that viability of untreated cells is 1. *p < 0.05 for inhibitor treated plus CHX compared with CHX alone.

(B) Viability (as measured by trypan blue exclusion) of Ink4a/Arf null PDECs expressing KRAS<sup>G12D</sup>, BRAF<sup>V600E</sup>, SHH, or GFP. For these assays, cells were first vehicle treated or treated with the MEK inhibitor PD98059 or the PI3K inhibitor LY2900042, and then one hour later either left untreated or treated with ultraviolet (UV) irradiation. Values are normalized such that viability of untreated cells is 1. *p < 0.05 for inhibitor treated plus UV compared with UV alone.
Inhibition of mTOR results in decreased survival of KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs when challenged with an apoptotic stimulus.

(A) Viability (as measured by trypan blue exclusion) of Ink4a/Arf, Trp53 null PDECs expressing KRAS$^{G12D}$, BRAF$^{V600E}$, or GFP. For this assay, cells were first either vehicle treated, treated with the mTOR inhibitor rapamycin, or treated with the PI3K inhibitor LY294002, and then one hour later either vehicle treated or treated with 100µM cycloheximide. Values are normalized such that that viability of untreated cells is 1. *p < 0.05 for inhibitor treated plus CHX compared with CHX alone.
Figure 2.14

The combined inhibition of MEK and PI3K in KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs does not further decrease survival in response to cycloheximide when compared with either MEK or PI3K inhibition alone.

(A) Viability (as measured by trypan blue exclusion) of \textit{Ink4a/Arf, Trp53} null PDECs expressing KRAS\textsuperscript{G12D}, BRAF\textsuperscript{V600E}, or GFP. For these assays, cells were first vehicle treated or treated with the MEK inhibitor PD98059, the PI3K inhibitor LY2900042, or both PD98059 and LY294002, and then one hour later either vehicle treated or treated with 100µM cycloheximide. Values are normalized such that viability of untreated cells is 1.

*p < 0.05 for inhibitor treated plus CHX compared with CHX alone
The combined inhibition of MEK and PI3K in KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs does not further decrease survival in response to UV irradiation when compared with either MEK or PI3K inhibition alone.

(A) Viability (as measured by trypan blue exclusion) of Ink4a/Arf null PDECs expressing KRAS\textsuperscript{G12D}, BRAF\textsuperscript{V600E}, or GFP. For these assays, cells were first vehicle treated or treated with the MEK inhibitor PD98059, the PI3K inhibitor LY2900042, or both PD98059 and LY294002, and then one hour later either left untreated or treated with ultraviolet (UV) irradiation. Values are normalized such that viability of untreated cells is 1.

*p < 0.05 for inhibitor treated plus UV compared with UV alone
Expression of KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} results in activation of RAF/MEK/ERK and PI3K/AKT signaling cascades

The dependence of KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs on both the RAF/MEK/ERK and PI3K/AKT pathways for survival was unexpected, since BRAF\textsuperscript{V600E} has been previously shown to directly stimulate the MEK/ERK signaling cascade, but not the PI3K/AKT pathway. Because of this, I next sought to determine the activation status of these signaling pathways in KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs. PDECs were serum starved for 48 hours to eliminate pathway activation induced by exogenous growth factors, and protein lysates were generated from the serum-starved cells. Immunoblotting of these lysates showed increased ratios of phosphorylated AKT (pAKT; at ser473) to total AKT, and phosphorylated ERK1 and ERK2 (pERK; at Thr202/Tyr204) to total ERK, in KRAS\textsuperscript{G12D}- and BRAF\textsuperscript{V600E}-expressing PDECs relative to GFP expressing controls, indicating that both the MEK/ERK and PI3K/AKT signaling pathways are activated in these cells (Figure 2.16). As BRAF\textsuperscript{V600E} is not known to signal through AKT directly, these data suggest that BRAF\textsuperscript{V600E}, and potentially also KRAS\textsuperscript{G12D}, stimulates PI3K/AKT signaling in an indirect manner.

In addition, I found that KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs had increased levels of total AKT when compared with GFP expressing cells (Figure 2.17A). Immunoblotting for AKT1, AKT2, and AKT3 found that this increase in total AKT was due primarily to an increase in AKT1 in KRAS\textsuperscript{G12D} expressing PDECs, and an increase in AKT1 and AKT3 in BRAF\textsuperscript{V600E} expressing PDECs relative to GFP expressing PDECs (Figure 2.17A). KRAS\textsuperscript{G12D} expressing cells also displayed a slight decrease in both
AKT2 and AKT3 relative to GFP expressing PDECs (Figure 2.17A). Analysis of Akt family member gene expression via quantitative RT-PCR showed that KRAS$^{G12D}$ expressing PDECs had an increase in Akt3 message level relative to GFP expressing cells, and a decrease in Akt2 message level relative to GFP expressing cells, while Akt1 gene expression was unchanged relative to GFP expressing cells (Figure 2.17B). In contrast to KRAS$^{G12D}$ expressing PDECs, qRT-PCR analysis of BRAF$^{V600E}$ expressing PDECs showed increased levels of expression of all three Akt family members relative to GFP expressing PDECs (Figure 2.17B). These findings demonstrate that the changes in AKT family member protein expression in KRAS$^{G12D}$ and BRAF$^{V600E}$ do not coincide with the changes in gene expression, and indicate that the regulation of AKT protein translation and/or stability may differ downstream of activated KRAS versus activated BRAF.
**Figure 2.16**

KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDCs have increased pERK (1/2) and pAKT (ser473) expression when compared to control cells.

(A) Western blot analysis showing increased ERK (p42/44) and AKT (ser473) phosphorylation in serum starved KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing Ink4a/Arf, Trp53 null PDCs relative to GFP expressing controls. Values indicate the ratio of phosphorylated AKT relative to total AKT as measured by densitometry and normalized such that GFP expressing controls have a ratio of 1.
**Figure 2.17**

**KRAS**\(^{G12D}\) and **BRAF**\(^{V600E}\) expressing PDECs have increased total AKT expression and differential expression of AKT family members when compared to control cells.

(A) Western blot analysis of AKT isoform expression in Kras\(^{G12D}\), Braf\(^{V600E}\), and GFP expressing *Ink4a/Arf, Trp53* null PDECs showing increased expression of total AKT in Kras\(^{G12D}\) and Braf\(^{V600E}\) expressing cells relative to GFP expressing controls.

(B) Quantitative RT-PCR for murine *Akt1, Akt2*, and *Akt3* isoforms in Kras\(^{G12D}\) and Braf\(^{V600E}\) expressing *Ink4a/Arf, Trp53* null PDECs relative to GFP expressing *Ink4a/Arf, Trp53* null PDECs. \(\beta\)-actin is used as an endogenous control, and for all data, AKT isoform expression in GFP expressing cells is normalized to 1.
Activation of PI3K/AKT signaling in KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs
depends on signaling through the IGF1R

Since BRAF has not been previously shown to directly activate PI3K, I next sought to determine the mechanism by which BRAF\textsuperscript{V600E} stimulates signaling through the PI3K/AKT pathway in PDECs. I hypothesized that the activation of PI3K/AKT in these cells was downstream of the RAF/MEK/ERK signaling cascade, potentially through the activation of autocrine growth factor signaling. Previous studies have demonstrated increased expression of IGF1 in pancreatic tumors, indicating that the Insulin-Like Growth Factors and the IGF1R may play a role in pancreatic tumorigenesis (Bergmann, Funatomi et al. 1995). Based upon this, I hypothesized that the IGF1R could be activated downstream of RAF/MEK/ERK signaling. In support of this, a recent study demonstrated that melanoma cells expressing mutant NRAS or BRAF respond to treatment with an IGF1R inhibitor despite the activation of NRAS and BRAF (Yeh, Bohula et al. 2006). To further investigate this hypothesis, I next sought to determine if signaling through RAF/MEK/ERK induced the activation of the IGF1R in KRAS\textsuperscript{G12D} or BRAF\textsuperscript{V600E} expressing PDECs.

I first assessed the levels of IGF1R ligand expression in serum starved PDECs expressing KRAS\textsuperscript{G12D}, BRAF\textsuperscript{V600E}, or GFP by qRT-PCR. I found robustly increased levels of \textit{Igf2} in both KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing cells relative to GFP expressing cells, and a modest increase in \textit{Igf1} mRNA levels (Figure 2.18A). Interestingly, insulin mRNA levels were unaffected by the expression of activated KRAS and BRAF proteins. In contrast to the results for IGF1R ligands, I did not see a similar
increase in the expression level of any EGF family ligand in KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs by qRT-PCR (Figure 2.18B). For this study, the ligands included were Epidermal Like Growth Factor (\textit{Egf}), Amphiregulin (\textit{Amp}), Betacellulin (\textit{Btc}), Epigenin (\textit{Epi}), Transforming Growth Factor Alpha (\textit{TGF-\alpha}), Heparain Binding EGF (\textit{Hb-Egf}), and Epiregulin (\textit{Epr}). While it should be noted that the expression level of \textit{Egf} was increased in BRAF\textsuperscript{V600E} expressing PDECs, this was not true of KRAS\textsuperscript{G12D} expressing PDECs and the increased level observed was well below the increase observed in \textit{Igf2} expression in either cell type (Figure 2.18B). These data indicate that this increase is specific to \textit{Igf1} and \textit{Igf2} and supporting our hypothesis that KRas\textsuperscript{G12D} and BRAF\textsuperscript{V600E} activate the IGF1R downstream of RAF/MEK/ERK.
Figure 2.18

KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs greatly increased levels of IGF1R ligands and not EGFR ligands when compared with control cells.

(A) Quantitative RT-PCR demonstrating increased expression of $Igf2$ in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing $Ink4a/Arf$, $Trp53$ null PDECs relative to GFP expressing control cells. β-actin is used as an endogenous control, and for all data, ligand expression in GFP expressing cells is normalized to 1.

(B) Quantitative RT-PCR analyzing the expression of epidermal growth factor receptor (EGFR) ligands in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing $Ink4a/Arf$, $Trp53$ null PDECs relative to GFP expressing control cells. β-actin is used as an endogenous control, and for all data, ligand expression in GFP expressing cells is normalized to 1.
When I assessed IGF2 expression via western blot, I once again observed increased IGF2 levels in KRAS\(^{G12D}\) and BRAF\(^{V600E}\) expressing PDECs relative to GFP expressing PDECS (Figure 2.19A). Moreover, immunoblotting with specific antibodies against total and phosphorylated IGF1R in lysates from serum starved PDECs demonstrated increased total receptor levels as well as increased phosphorylation of the receptor in KRAS\(^{G12D}\) and BRAF\(^{V600E}\)-expressing cells (Figure 2.19B). Of note, the increase in total receptor levels was much higher in KRAS\(^{G12D}\) expressing PDECs than in BRAF\(^{V600E}\) expressing PDECs relative to control cells (Figure 2.19B).

Consistent with the hypothesis that \textit{Igf2} gene expression is stimulated downstream of the MEK/ERK cascade, treatment of KRAS- and BRAF-expressing PDECs with the MEK inhibitor PD98059, but not the PI3K inhibitor LY294002 impaired the elevation of \textit{Igf2}, but not \textit{Igf1} or \textit{Ins2}, mRNA levels in KRAS\(^{G12D}\) and BRAF\(^{V600E}\)-expressing PDECs (Figure 2.20A and 2.20B).
Figure 2.19

KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs increased levels of IGF2, IGF1R, and pIGF1R when compared with control cells.

(A) Western blot analysis showing increased IGF2 in serum starved KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing \textit{Ink4a}/\textit{Arf}, \textit{Trp53} null PDECs relative to GFP expressing control cells. \(\beta\)-actin is used as a loading control.

(B) Western blot analysis showing increased total and phosphorylated IGF1R in KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing \textit{Ink4a}/\textit{Arf}, \textit{Trp53} null PDECs relative to GFP expressing control cells. \(\beta\)-actin is used as a loading control.
**Figure 2.20**

**Inhibition of MEK reduces the levels of Igf2, but not Igf1 or Ins2 in KRAS\textsuperscript{G12D} expressing PDECs.**

(A) Quantitative RT-PCR demonstrating decreased expression of Igf2 in KRAS\textsuperscript{G12D} expressing Ink4a/Arf, Trp53 null PDECs treated with the MEK inhibitor PD98059 or the IGF1R inhibitor AG1024 relative to vehicle (DMSO) treated cells. β-actin is used as an endogenous control, and for all data, Igf2 expression in vehicle treated cells are normalized to 1.

(B) Quantitative RT-PCR of Igf1 and Ins2 in serum starved in KRAS\textsuperscript{G12D} expressing Ink4a/Arf, Trp53 null PDECs following treatment with either vehicle (DMSO), or the MEK inhibitor PD98059, the PI3K inhibitor LY294002, or the IGF1R inhibitor AG1024. β -actin is used as an endogenous control, and for all data, ligand expression in vehicle treated cells is normalized to 1.
To investigate whether the activation of AKT within \textit{KRAS}^{G12D} and \textit{BRAF}^{V600E} expressing cells depended upon signaling through MEK, and subsequently through the IGF1R, I inhibited MEK, PI3K, or IGF1R expression in \textit{KRAS}^{G12D} and \textit{BRAF}^{V600E} expressing PDECs using small molecule inhibitors, and determined the effect on activation of ERK 1/2 (p42/44) and AKT via immunoblotting. As expected, I found that in \textit{KRAS}^{G12D} and \textit{BRAF}^{V600E} expressing PDECs, pERK 1/2 is dramatically reduced in the presence of a MEK inhibitor. Surprisingly, I found that pERK 1/2 levels are also reduced in the presence of PI3K or IGF1R inhibitors in \textit{KRAS}^{G12D} and \textit{BRAF}^{V600E} expressing PDECs, indicating a potential feedback loop between the RAF/MEK/ERK and PI3K/AKT pathways within these cells (Figure 2.21A). In contrast, GFP expressing PDECs only showed a reduction in pERK 1/2 in the presence of the MEK inhibitor (Figure 2.21A). These results suggest that the potential feedback between the RAF/MEK/ERK and PI3K/AKT pathways is specific to signaling downstream of \textit{KRAS}^{G12D} and \textit{BRAF}^{V600E}.

Analysis of pAKT (ser473) levels following treatment with the various inhibitors demonstrated a decrease in pAKT (ser473) when \textit{KRAS}^{G12D}, \textit{BRAF}^{V600E}, and GFP cells were treated with the PI3K inhibitor, confirming efficient inhibition of this pathway downstream of PI3K inhibition (Figure 2.21A). In the \textit{BRAF}^{V600E} cells, though, there was also decreased pAKT (ser473) following treatment with the MEK and IGF1R inhibitors (Figure 2.21A). These data indicate that activation of PI3K/AKT signaling lies downstream of the MEK/ERK signaling cascade in \textit{BRAF}^{V600E} expressing PDECs. In contrast, I found that while treatment with the PI3K inhibitor robustly reduced pAKT
levels in KRAS\textsuperscript{G12D} -expressing PDECs, treatment with the MEK or IGF1R inhibitors had a more modest impact (Figure 2.21A). These data suggest that in addition to activation of PI3K/AKT downstream of MEK/ERK signaling, KRAS\textsuperscript{G12D} may also directly activate the PI3K/AKT pathway in PDECs as demonstrated previously in other cell types (Rodriguez-Viciana, Warne et al. 1994; Pacold, Suire et al. 2000).
Figure 2.21

The phosphorylation of AKT at ser473 in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs depends on signaling through MEK and the IGF1R.

(A) Western blot analysis of ERK (p42/44) and AKT (ser473) phosphorylation in serum starved in KRAS$^{G12D}$, BRAF$^{V600E}$, and GFP expressing Ink4a/Arf, Trp53 null PDECs following treatment with either vehicle (X), the MEK inhibitor PD98059, the PI3K inhibitor LY294002, or the IGF1R inhibitor AG1024.
To further assess the impact of the IGF1R on the MEK/ERK and PI3K/AKT signaling pathways, I utilized shRNA-mediated knockdown to reduce IGF1R levels and downstream signaling. Immunoblotting confirmed efficient knockdown of the IGF1R in PDECs infected with a lentivirus encoding an IGF1R-targeting shRNA relative to PDECs expressing a non-silencing control (Figure 2.22A). Of note, KRAS\(^{G12D}\) expressing PDECs treated with control shRNA no longer showed increased expression of the IGF1R when compared with BRAF\(^{V600E}\) expressing cells (Figure 2.22A). The cause of this difference in expression of the IGF1R was not determined, but it is possible that it was due to the puromycin selection of these cells, as it reduced IGF1R expression was consistently seen in KRAS\(^{G12D}\) cells treated with control shRNA and subsequently selected in puromycin.

Assessment of pERK and pAKT (ser473) levels by immunoblotting demonstrated that both the PI3K/AKT and MEK/ERK pathways were strongly inhibited in KRAS\(^{G12D}\)- and BRAF\(^{V600E}\)-expressing PDECs following IGF1R knockdown (Figure 2.22A). The effect of IGF1R knockdown was more robust than that observed upon AG1024 treatment. While the reasons for these differences are unclear, the data suggest that knockdown of IGF1R more completely inhibits downstream signaling than treatment with AG1024 at the concentration utilized for these studies (20\(\mu\)M). In addition, Yeh and colleagues demonstrated that decreases in pAKT and pERK following IGF1R inhibition in BRAF\(^{V600E}\) expressing melanoma cells required up to 4 days, indicating that the time of IGF1R inhibition, may impact the effect on the PI3K/AKT and MEK/ERK pathways (Yeh, Bohula et al. 2006). Nonetheless, these findings suggest that AKT activation
occurs downstream of the IGF1R. When coupled with our finding that IGF2 induction occurs downstream of MEK (Figure 2.20A), these data suggest that a MEK-IGF2-IGF1R signaling axis regulates AKT activation downstream of activated KRAS and BRAF in primary pancreatic ductal epithelial cells.
Knockdown of the IGF1R inhibits the phosphorylation of AKT at ser473 in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs

(A) Western blot analysis showing decreased ERK (p42/44) and AKT (ser473) phosphorylation in serum starved KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing Ink4a/Arf, Trp53 null PDECs with shRNA mediated knockdown of the IGF1R.
Inhibition of the IGF1R results in decreased survival in KRAS^{G12D} and BRAF^{V600E} expression PDECs

As the prior data has indicated that activation of the PI3K/AKT pathway lies downstream of the MEK/ERK signaling cascade and the IGF1R, and given the requirement for MEK/ERK and PI3K/AKT signaling for KRAS^{G12D}- and BRAF^{V600E}-induced survival, I next investigated the impact of IGF1R inhibition on this phenomenon. I found that both KRAS^{G12D} and BRAF^{V600E} expressing PDECs treated with an IGF1R inhibitor had decreased survival following challenge with both cycloheximide (Figure 2.23A) and UV irradiation (Figure 2.23B). Importantly, survival in SHH-expressing PDECs was not impacted by IGF1R inhibition, consistent with SHH-enhanced survival occurring in a MEK-independent manner, and in line with the hypothesis that IGF1R activation occurs downstream of MEK (Figure 2.23A, B).

If the hypothesis that IGF2 stimulates IGF1R-mediated signaling downstream of MEK was correct, then ectopic expression of IGF2 in KRAS^{G12D} and BRAF^{V600E}-expressing cells should rescue survival after apoptotic challenge in cells with inhibition of MEK but not PI3K. To test this hypothesis, I infected KRAS^{G12D} and BRAF^{V600E}-expressing PDECs with RCAS viruses encoding IGF2 or GFP as a control, and ascertained the effect of ectopic IGF2 expression on cell survival after apoptotic challenge in the presence of specific signaling pathway inhibitors. I found that ectopic IGF2 expression rescued KRAS^{G12D} - and BRAF^{V600E}-induced survival after challenge with cycloheximide in PDECs in which MEK was inhibited, but not in PDECs in which PI3K was inhibited (Figure 2.24A). Interestingly, IGF2 expression by itself was not
sufficient to promote the survival of PDECs after apoptotic challenge (Figure 2.24A), suggesting that other molecules and pathways regulated by oncogenic KRAS and BRAF are required to promote cell survival.
Inhibition of the IGF1R results in decreased survival of KRAS^{G12D} and BRAF^{V600E} expressing PDECs in response to apoptotic stimuli.

(A) Viability (as measured by trypan blue exclusion) of Ink4a/Arf, Trp53 null PDECs expressing KRAS^{G12D}, BRAF^{V600E}, SHH, or GFP. For these assays, cells were first vehicle treated or treated with the IGF1R inhibitor AG1024, and then one hour later either vehicle treated or treated with 100µM cycloheximide. Values are normalized such that viability of untreated cells is 1. *p < 0.05 for inhibitor treated plus CHX compared with CHX alone

(B) Viability (as measured by trypan blue exclusion) of Ink4a/Arf null PDECs expressing KRAS^{G12D}, BRAF^{V600E}, SHH, or GFP. For these assays, cells were first vehicle treated or treated with the IGF1R inhibitor AG1024, and then one hour later either left untreated or treated with ultraviolet (UV) irradiation. Values are normalized such that viability of untreated cells is 1. *p < 0.05 for inhibitor treated plus UV compared with UV alone
Expression of IGF2 rescues MEK inhibited survival in response to apoptotic stimuli in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs.

(A) Viability (as measured by trypan blue exclusion) of Ink4a/Arf, Trp53 null PDECs expressing KRAS$^{G12D}$, BRAF$^{V600E}$, or GFP which have been additionally been retrovirally transduced to express either GFP or IGF2. For these assays, cells were first vehicle treated or treated with the MEK inhibitor PD98059 or the PI3K inhibitor LY2900042, and then one hour later either vehicle treated or treated with 100µM cycloheximide. Values are normalized such that viability of untreated cells is 1.

*p < 0.05 for IGF2 expression plus inhibitor and CHX compared with inhibitor and CHX alone
**IGF1R is required for KRAS\(^{G_{12D}}\) - and BRAF\(^{V{600E}}\)-induced pancreatic tumorigenesis**

The data above indicated a critical role for IGF1R-mediated signaling in the survival of PDECs. To determine whether this effect contributes to KRAS\(^{G_{12D}}\) - and BRAF\(^{V{600E}}\)-induced transformation of pancreatic epithelial cells, I used targeting shRNAs to knock down IGF1R expression in PDECs expressing either KRAS\(^{G_{12D}}\) or BRAF\(^{V{600E}}\) and additionally null at the Ink4a/Arf and Trp53 tumor suppressor loci. Effective shRNA-mediated knockdown was confirmed by immunoblot (Figure 2.25A). Orthotopic implantation of 10\(^6\) PDECs resulted in efficient tumor formation in activated KRAS and BRAF-expressing PDECs, whereas tumor formation was robustly inhibited in mice implanted with cells that simultaneously expressed IGF1R shRNA (Table 1.2).

Interestingly, if mice implanted with cells expressing the IGF1R shRNA were allowed to remain on the study for a longer period of time, tumors eventually formed. Analysis of IGF1R expression in these tumors demonstrated equivalent IGF1R levels to tumors formed by cells expressing a non-silencing control (Figure 2.25B). Together, these data suggest that the IGF1R is required for KRAS\(^{G_{12D}}\) - and BRAF\(^{V{600E}}\)-induced pancreatic tumorigenesis.

We next investigated by immunostaining whether tumors generated through the pancreas specific expression of a KRAS\(^{G_{12D}}\) allele had increased levels of phosphorylated IGF1R. In support of the requirement for signaling through the IGF1R in KRAS-mediated pancreatic tumor initiation, tumor samples stained positive for phosphorylated IGF1R while normal pancreas tissues were negative for phosphorylated IGF1R staining (Figure 2.26A).
Table 2.2

Tumor induction by $1 \times 10^6 \text{KRAS}^{G12D}$ and BRAF$^{V600E}$ expressing Ink4a/Arf, Trp53 null PDECs following knockdown of IGF1R and subsequent orthotopic transplant.

<table>
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<tr>
<th>Cells Implanted</th>
<th>Tumor Incidence</th>
<th>Time to Tumor Development (Weeks)</th>
<th>Average Tumor Volume (SEM)</th>
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</thead>
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<tr>
<td>KRAS$^{G12D}$ Control</td>
<td>4/5</td>
<td>7</td>
<td>576mm$^3$ (±436)</td>
</tr>
<tr>
<td>KRAS$^{G12D}$ IGF1R shRNA</td>
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<td>7</td>
<td>N/A</td>
</tr>
<tr>
<td>BRAF$^{V600E}$ Control</td>
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<td>7</td>
<td>1211mm$^3$ (±239)</td>
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<tr>
<td>BRAF$^{V600E}$ IGF1R shRNA</td>
<td>5/6</td>
<td>7</td>
<td>72mm$^3$ (±20)</td>
</tr>
</tbody>
</table>

Tumor Volume Calculated Using the Formula LxWxH
Figure 2.25

Tumors formed following orthotopic transplant of KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs with IGF1R knockdown demonstrate restored levels of IGF1R.

(A) Western blot analysis showing decreased IGF1R expression in serum starved KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing Ink4a/Arf, Trp53 null PDECs infected with IGF1R shRNA. β-actin is used as a loading control.

(B) Western blot analysis showing IGF1R expression in tumor tissue following orthotopic transplant of BRAF$^{V600E}$ Ink4a/Arf, Trp53 null PDECs infected with either control shRNA or IGF1R shRNA. β-actin is used as a loading control.
Figure 2.26

KRAS$^{G12D}$ expressing pancreatic tumors exhibit strong expression of the IGF1R relative to normal pancreatic tissue.

(A) Immunostaining showing strong levels of phosphorylated IGF1R (Y1161) in KRAS$^{G12D}$ expressing pancreatic tumors relative to normal pancreas tissue.
Combined MEK and IGF1R inhibition impairs the survival of pancreatic cancer cells

Given that the IGF1R is required for PDEC survival after apoptotic challenge, and the requirement for IGF1R for KRASG12D- and BRAFV600E-induced pancreatic tumorigenesis, I investigated whether IGF1R is similarly required for the survival of pancreatic cancer cells. I first assessed the effect of IGF1R inhibition on the proliferation of pancreatic cancer cells derived from our orthotopic model. Consistent with our previously published data (Morton, Mongeau et al. 2007), I found that inhibition of MEK or PI3K strongly impaired the proliferation of a tumor cell line that harbors KRASG12D expression and deletion of Ink4a/Arf and Trp53 (Figure 2.27A). Similarly, I found that the inhibition of IGF1R also reduced proliferation in this cell line (Figure 2.27A).

I next determined the effect of MEK inhibition, PI3K inhibition, and IGF1R inhibition on the survival of pancreatic cancer cells. I found that inhibition of PI3K in these cells reduced survival after challenge with cycloheximide (Figure 2.28A). Interestingly, in contrast to the impact of MEK and IGF1R inhibition on survival in PDECs, I found that individual inhibition of these molecules did not significantly impact survival after apoptotic challenge in this cell line (Figure 2.28A). However, combined inhibition of MEK and IGF1R reduced survival to the levels seen with PI3K inhibition (Figure 2.28A). This finding is consistent with recent data from Engelman and colleagues who demonstrated an effect of combined MEK and IGF1R inhibition in colon cancer cells (Ebi, Corcoran et al. 2011).
To confirm that this phenomenon occurred following exposure to a clinically relevant compound, I treated the same tumor cell line with 50nM gemcitabine, a concentration that fails to elicit significant death in this cell line, in combination with inhibition of MEK, PI3K, and IGF1R. Consistent with our results following cycloheximide treatment, I found that PI3K inhibition, or combined inhibition of MEK and IGF1R (but not inhibition of MEK or IGF1R alone), sensitized cells to gemcitabine (Figure 2.28B). Similar results were obtained when IGF1R knockdown was combined with small molecule-mediated inhibition of MEK, demonstrating that these findings are not the consequence of toxicity induced by simultaneous exposure to the IGF1R and MEK chemical inhibitors (Figure 2.29B and 2.29C).

To exclude the possibility that these phenomena are specific to the cell line used, or particular to murine pancreatic cancer cells, I repeated the cycloheximide challenge experiment in the human Panc1 pancreatic cancer cell line, which expresses KRAS\textsuperscript{G12D}. Consistent with the results above, I found that combined inhibition of MEK and IGF1R increased sensitivity to cycloheximide-induced death, whereas inhibition of either molecule alone did not induce cell death (Figure 2.30A).

I next sought to determine if the decreased survival seen following the combined inhibition of MEK and the IGF1R in KRAS\textsuperscript{G12D} expressing cells was due to a decrease in the levels of pAKT at ser473. Western blot analysis of these cells confirms that both pERK 1/2 and pAKT at ser473 were effectively and specifically inhibited in the assays including the MEK and PI3K inhibitors, respectively (Figure 2.31A and 2.31B). In contrast, shRNA mediated knockdown of IGF1R or inhibition of the IGF1R did not affect
either pERK 1/2 or pAKT at ser473 (Figure 2.13D and 2.14D). Surprisingly, the combined inhibition of MEK and IGF1R, either by small molecule-mediated inhibition or shRNA-mediated knockdown, also did not impact the phosphorylation of AKT at ser473 (Figure 2.31A and 2.31B). These findings are interesting, as the combined inhibition of MEK and the IGF1R did lead to increased cell death, and all prior findings in our system have indicated that this corresponds with a decrease in the activation of AKT. Further analysis into the signaling changes that occur in KRAS$^{G12D}$ expressing tumor cells treated with both MEK and IGF1R inhibitors simultaneously is required to better understand the molecular consequences of this dual inhibitor treatment.
Figure 2.27

The inhibition of PI3K, MEK, or the IGF1R in KRAS$^{G12D}$ expressing tumor cell lines results in decreased cell proliferation.

(A) Cell numbers showing decreased proliferation at 72 hours of RCAS-KRAS$^{G12D}$ expressing, Ink4a/Arf, Trp53 null tumor cell lines treated with the MEK inhibitor PD98059, the PI3K inhibitor LY294002, or the IGF1R inhibitor AG1024 relative to vehicle treated cells.
Figure 2.28

The inhibition of PI3K or both MEK and the IGF1R in KRAS$^{G12D}$ expressing tumor cell lines results in decreased survival when challenged with apoptotic stimuli.

(A) Viability (as measured by trypan blue exclusion) of RCAS-KRAS$^{G12D}$ expressing, Ink4a/Arf, Trp53 null tumor cell lines. For this assay cells were treated with either vehicle, the MEK inhibitor PD98059, the PI3K inhibitor LY294002, the IGF1R inhibitor AG1024, PD98059 and LY294002, or PD98059+AG1024, and then either vehicle treated or treated with 100µM cycloheximide. Values are normalized such that that viability of vehicle treated cells is 1.

*p < 0.05 for inhibitor treated plus CHX compared with CHX alone

(B) Viability (as measured by trypan blue exclusion) of RCAS-KRAS$^{G12D}$ expressing, Ink4a/Arf, Trp53 null tumor cell lines. For this assay cells were treated with either vehicle, the MEK inhibitor PD98059, the PI3K inhibitor LY294002, the IGF1R inhibitor AG1024, PD98059 and LY294002, or PD98059+AG1024, and then either vehicle treated or treated with 50nM gemcitabine. Values are normalized such that that viability of vehicle treated cells is 1.

*p < 0.05 for inhibitor treated plus GEM compared with GEM alone
Figure 2.29

Knockdown of the IGF1R in KRAS\textsuperscript{G12D} expressing cells results in decreased cell growth, and combined knockdown of the IGF1R with MEK inhibition results in decreased cell survival when challenged with an apoptotic stimulus.

(A) Viability (as measured by trypan blue exclusion) of RCAS-KRAS\textsuperscript{G12D} expressing, \textit{Ink4a/Arf, Trp53} null tumor cell lines, following knockdown of IGF1R and treatment with vehicle, the MEK inhibitor PD98059, or the PI3K inhibitor LY294002, and then either vehicle treated or treated with 100\,\mu M cycloheximide. Values are normalized such that the viability of untreated cells is 1. *p < 0.05 for inhibitor treated plus CHX compared with CHX alone.

(B) Viability (as measured by trypan blue exclusion) of RCAS-KRAS\textsuperscript{G12D} expressing, \textit{Ink4a/Arf, Trp53} null tumor cell lines, following knockdown of IGF1R and treatment with vehicle, the MEK inhibitor PD98059, or the PI3K inhibitor LY294002, and then either vehicle treated or treated with 50\,nM gemcitabine. Values are normalized such that the viability of untreated cells is 1. *p < 0.05 for inhibitor treated plus GEM compared with GEM alone.
The inhibition of PI3K or both MEK and the IGF1R in a human KRAS$^{G12D}$ expressing tumor cell line results in decreased survival when challenged with an apoptotic stimulus.

(A) Viability (as measured by trypan blue exclusion) of Panc1 cells, a human tumor cell line. For this assay cells were treated with either vehicle, the MEK inhibitor PD98059, the PI3K inhibitor LY294002, the IGF1R inhibitor AG1024, PD98059 and LY294002, or PD98059+AG1024, and then either vehicle treated or treated with 100µM cycloheximide. Values are normalized such that that viability of vehicle treated cells is 1.

*p < 0.05 for inhibitor treated plus CHX compared with CHX alone
Figure 2.31

The inhibition of both MEK and the IGF1R in KRAS\textsuperscript{G12D} expressing tumor cell lines does not result in decreased phosphorylation of AKT at serine 473.

(A) Western blot analysis of ERK (p42/44) and AKT (ser473), phosphorylation in serum starved RCAS-KRAS\textsuperscript{G12D} expressing Ink4a/Arf, Trp53 null tumor cell lines following treatment with vehicle, the MEK inhibitor PD98059, the PI3K inhibitor LY294002, the IGF1R inhibitor AG1024, or both PD98059 and AG1024.

(B) Western blot analysis of ERK (p42/44) and AKT (ser473) phosphorylation in serum starved RCAS-KRAS\textsuperscript{G12D} expressing Ink4a/Arf, Trp53 null tumor cell lines following shRNA mediated knockdown of the IGF1R and treatment with vehicle, the MEK inhibitor PD98059, or the PI3K inhibitor LY294002.
Summary

These studies indicate that the expression of both activated KRAS and activated BRAF in PDECs results in increased proliferation, increased survival when challenged with apoptotic stimuli, and in cells lacking Ink4a/Arf and Trp53, the ability to form tumors following orthotopic transplant. These findings show that activated BRAF is able to functionally substitute for activated KRAS, and indicate a critical role for RAF/MEK/ERK signaling in KRAS-mediated pancreatic tumorigenesis. These findings further indicate that the increased survival in PDECs is dependent on signaling through MEK and PI3K, and consistent with this, that both the MEK/ERK and PI3K/AKT signaling cascades are activated downstream of KRAS$^{G12D}$ and BRAF$^{V600E}$. In addition, these studies uncover a novel autocrine signaling loop in which MEK/ERK signaling downstream of activated KRAS and activated BRAF stimulates increased expression of IGF2 and subsequent activation of the IGF1R (Illustrated in Figure 2.32). This signaling is necessary for the increased survival seen within KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing cells, and I find that the increased expression of IGF2 is sufficient to rescue that survival phenotype following MEK inhibition. Finally, I show that signaling through the IGF1R downstream of activated KRAS and activated BRAF is needed for tumor formation following orthotopic transplant, as shRNA mediated knockdown of the IGF1R inhibits tumor formation. These findings support a clear role for MEK/ERK stimulated IGF1R signaling in the initiation of pancreatic tumors, and identify a key role for receptor tyrosine kinase signaling even in the context of activated KRAS or BRAF.
In contrast to the role for MEK/ERK induced IGF1R activation in PDECs, I show that KRAS$^{G12D}$ expressing tumor cell lines do not depend on MEK or the IGF1R for survival, and only the inhibition of PI3K results in decreased survival of these cells. However, the combined inhibition of both MEK and the IGF1R does decrease cell survival, albeit in a manner that does not impact pAKT at ser473. These findings show a clear difference in signaling between KRAS mediated tumor initiation (as illustrated by the studies in PDECs) and KRAS mediated tumor progression (as illustrated by the studies in tumor cell lines). Lastly, I find that the inhibition of MEK, PI3K, and the IGF1R all result in decreased proliferation of the tumor cell lines, indicating that despite the differences in signaling of these cells, signaling through all three of these pathways is still important in these tumor cell lines and as such, all three are viable options for therapeutic targets.
Activated KRAS and BRAF induce increased expression of IGF2, resulting in the activation of the IGF1R and signaling through PI3K/AKT. (Adapted from Downward 2003)
Materials and Methods

Transgenic mice and animal care.

The keratin-19-tv-a, Ink4a/Arf$^{flox/lox}$, Trp53$^{flox/lox}$, and Ptf1a-cre strains have been previously described (Bader and Franke 1990; Hu and Gudas 1994; Grippo and Sandgren 2000; Jonkers, Meuwissen et al. 2001; Krimpenfort, Quon et al. 2001; Kawaguchi, Cooper et al. 2002; Orsulic 2002; Morton, Mongeau et al. 2007). Nude mice were purchased from Charles River Laboratories (Wilmington MA). All mice were housed in a specific pathogen-free facility with abundant food and water under guidelines approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Isolation, culture, and infection of mouse PDECs.

Isolation, culture, and infection of mouse PDECs were performed as previously described (Lewis, Chinnasamy et al. 2001; Schreiber, Deramaudt et al. 2004; Morton, Mongeau et al. 2007). Details are provided in the protocols below.

Isolation of PDECs: The pancreas of a K19-TVA expressing mouse was harvested into ~30ml G solution {HBSS (Gibco, Invitrogen, Carlsbad CA) plus 0.9g/L D-Glucose (Calbiochem, San Diego, CA) plus 1X Penicillin/Streptomycin (Gibco, Invitrogen, Carlsbad CA)} in a falcon tube on ice. In a sterile tissue culture hood, aspirate G solution and transfer the pancreas into a 50ml beaker. Add ~5ml G solution.
Cut up the pancreas with dissection scissors about 100 times (resulting in pieces ~1mm in size). Resuspend the cut tissue in ~50ml G solution and transfer to a fresh 50ml falcon tube. Allow tissue to settle and aspirate or decant G solution and any floating fatty tissue. Repeat the wash with G solution until all floating tissue has been removed. Resuspend tissue in 15ml of filter-sterilized collagenase V solution {1mg/ml of Collagenase V (Sigma, St Louis MO) in DMEM (Invitrogen, Carlsbad CA) plus 10% FCS (Atlanta Biologicals, Atlanta GA)} and transfer to 100ml bottle with stir bar. Digest tissue, with constant stirring, at 37°C for 20 minutes. Use a beaker set on a stirrer hot plate if you don’t have a submersible stirrer. Tissue should be free from large chunks at the end of digestion. If necessary, tissue can be digested up to 45 minutes to remove large chunks. Add 20ml G solution to stop reaction. Transfer the solution onto a 100µm nylon cell strainer over a waste container. Rinse the bottle with G solution if necessary to collect all remaining tissue. Invert the strainer over a fresh falcon tube and recover the undigested tissue by pipetting G solution onto the reverse side of the mesh and collecting washed off tissue/G solution in the falcon tube. If needed, top off the tissue solution to 50ml with G solution. Allow tissue to settle and pour off G solution to remove floating cells. (These are most likely acinar cells). Repeat. Add 50ml of G solution again and resuspend cells by inverting. Centrifuge cells at 1000rpm at 4°C, but stop the spin as soon as the centrifuge reaches speed and allow the centrifuge to decelerate using the minimum deceleration speed. Carefully decant or aspirate the supernatant. Repeat this wash step 3 times to remove all traces of collagenase. Add 2ml trypsin-EDTA (Invitrogen, Carlsbad CA) and incubate at room temperature for 5 minutes. Add 40mls DMEM plus 10% FCS
and invert to resuspend the cells. Centrifuge cells at 1000rpm 4°C and stop as soon as speed is reached (as in step 13). Remove the supernatant by decanting or by aspiration. Repeat this wash step 3 times. Resuspend the cells in 5ml PDEC medium and centrifuge at 1000rpm for 5 minutes at 4°C. (Reset the brake to the usual setting). Resuspend the tissue pellet in 12ml of PDEC medium (see description of media below) and plate 2ml onto each well of a 6-well plate. Culture in humidified incubator at 37°C, 5%CO₂. Note: Up to two pancreata can be isolated into one falcon tube at the start of the protocol. If this is done, the only change needed is to plate the cells onto two 6-well plates in step 20. Two days after isolation aspirate off medium and floating tissue. Wash gently with PBS. Replace PDEC medium and incubate until confluent (at least 5-7 days).

**Culturing of PDECs:** PDECs were cultured on collagen coated plates at 37 degrees Celsius with 5% CO₂. Cells were cultured in DMEM/F12 Medium (Gibco, Invitrogen, Carlsbad, CA) plus 5 mg/ml D-Glucose (Calbiochem, San Diego, CA), 0.1mg/mL Soybean Trypsin Inhibitor (Sigma, ), 5ml/L ITS+ (BD Biosciences, Billerica MA), 25ug/ml Bovine Pituitary Extract (BD Biosciences, Billerica MA), 20ng/ml EGF (BD Biosciences, Billerica MA), 5nM 3, 3, 5 tri-iodo-L-thyronine (Sigma, St Louis MO), 1uM dexamethasone (Sigma, St Louis MO), 1.22 mg/ml nicotinamide (Sigma, St Louis, MO), 5% Nu Serum (BD Biosciences, Billerica MA), and 1X Penicillin/Streptomycin (Gibco, Invitrogen, Carlsbad CA). To passage PDECs when confluent, media was removed and the collagen layer was pipetted into a filter-sterilized 1mg/ml collagenase V solution (Sigma, St Louis MO) and then incubated for 15-20 minutes at 37°C. Cells were
then pelleted by centrifugation at 1000rpm at 4°C for 5 minutes, and the supernatant removed by decanting or aspiration. 2ml trypsin-EDTA (Invitrogen, Carlsbad CA) were added to the cells, and the cells were incubated at room temperature for 5 minutes. Next, 40mls DMEM plus 10% FCS were added to the cells, and the cells were centrifuged at 1000rpm for 5 minutes at 4°C. Cells were washed twice more with DMEM plus 10% FCS and then resuspended into PDEC media and plated on collagen gel coated dishes.

**Infection of PDECs with RCAS Viruses:** Prior to infection, RCAS constructs were transfected into DF1 Chicken Fibroblasts using the Superfect Transfection Reagents (Qiagen, Valencia CA). Briefly, 5µg of plasmid was mixed with DMEM (Gibco, Invitrogen, Carlsbad CA) to a total volume of 150µL. To this mixture, 25µL of the Superfect reagent was added and the mixture was incubated at room temperature for 5 minutes. Following the incubation, 2mls of fresh DMEM plus 10% FCS plus 1% Penicillin/Streptomycin was added to the mixture, and the mixture was transferred onto DF1 cells. The DF1 cells plus transfection mixture were incubated for 2-3 hours at 37°C, following which fresh DMEM plus 10% FCS plus 1% Penicillin/Streptomycin was added and the DF1 cells were cultured as usual. At least one week after transfection of the RCAS constructs, the virus within the culture media from 3 confluent 10cm² dishes of DF1 cells was concentrated by ultracentrifugation at 27,000 rpms for 90minutes at 4°C. Following centrifugation, the supernatant was decanted, and the virus was resuspended through repeated pipetting of the remaining media. This virus mixture was then pipetted onto a single 10cm² dish of TVA expressing PDECs. For each virus, the infection
protocol was repeated so that the virus was added to PDECs a total of four times within 48 hours.

**Generation of RCAS Vectors**

The RCAS-GFP, RCAS-KRAS^{G12D}-IRES-GFP, RCAS-BRAF^{V600E} (V5 tag), and RCAS-BRAF (V5 tag) vectors have been described previously (Morton, Mongeau et al. 2007; Robinson, VanBroocklin et al. 2010). RCAS-Braf^{V600E} (myc tag) was generated by using PCR to add an XbaI restriction enzyme sequence (TCTAGA, 5’) and an EcoR1 restriction enzyme sequence (GAATTC, 3’) to a myc-tagged B-Raf^{V600E} cDNA. The resulting PCR product was cloned into the pCR-BluntII-TOPO vector (Invitrogen, Carlsbad CA). The clone was then sequence verified, digested with XbaI and EcoR1, and ligated into an XbaI/EcoR1 digested pYap6 vector. The cDNA fragment containing the myc-tagged B-Raf^{V600E} was then excised with PacI and PmeI and ligated into PacI/PmeI digested RCAS-X retroviral vector.

**ShRNA Mediated Knockdown of the IGF1R in PDECs**

For IGF1R knockdown, pGIPz shRNA targeting IGF1R (Open Biosystems, Huntsville AL, Clone ID V2LMM_188101), or a pGIPz empty vector control (Open Biosystems, Huntsville AL, Cat #RHS4349) was transfected into 293T cells using Effectene Transfection Reagents (Qiagen, Valencia CA). Briefly, 293T cells were plated onto a 15cm² dish, and the following day 2.5µg of the plasmid plus 1.55 µg of the pCMV del 8.2 plasmid and 1.0µg of the pMD.G plasmid were combined in EC Buffer to a total
volume of 370µL. To this, 40µL of enhancer reagent was added, and the mixture was incubated for 2-5 minutes at room temperature. Next, 125µL of Effectene reagent was added, and the mixture was incubated for 5-10 minutes at room temperature. Following the incubation, 5mls of DMEM plus 10% FCS plus 1% Penicillin/Streptomycin was added to the mixture, and the mixture was transferred onto the 293T cells. The cells plus transfection reagents were incubated overnight at 37°C and the next day fresh media was added to the cells. Supernatant from the transfected 293T cells was harvested at both 48 and 72 hours post transfection, concentrated by ultracentrifugation as described above for RCAS virus infection (Lewis, Chinnasamy et al. 2001), and the concentrated viral supernatant was added to a single 10cm² dish of PDECs. Infected PDECs were selected in media containing 2µg/ml puromycin for at least 4 days. For serum starved shRNA treated cells, puromycin was included in the media during serum starvation.

**PDEC Proliferation and Survival Assays**

All proliferation and survival assays were conducted as previously described (Morton, Mongeau et al. 2007). For proliferation assays, 1x10⁵ PDECs were plated per well in a 6 well plate on day zero. Fresh media was added to the cells every 5 days, and total cell numbers were counted on days 5, 10, and 15. For survival assays, 1x10⁶ PDECs were plated per well in a 6 well plate and incubated at 37°C overnight. The following day, the media was removed and fresh media was added. For cells treated with cycloheximide, the fresh media contained 100µM cycloheximide. For cells treated with
UV irradiation, following replacement of the media, the cells were treated with 40J/m² of ultraviolet irradiation. When inhibitors were used in the experiment, the inhibitors were added to the fresh media at the time it was changed, and cells were treated with either cycloheximide or UV irradiation one hour later. The MEK inhibitor PD980059 (Invitrogen, Carlsbad, CA) was used at a concentration of 25µM, the PI3 kinase inhibitor LY294002 (Invitrogen, Carlsbad, CA) was used at a concentration of 20µM, and the IGF1R inhibitor AG1024 (BD Biosciences, San Diego CA) was used at a concentration of 20µM. 24 hours after treatment with either cycloheximide or UV irradiation, live and dead cells were counted by trypan blue exclusion and percent survival was calculated.

**Culture and Treatment of Tumor Cell Lines**

All tumor cell lines were cultured in DMEM (Gibco, Invitrogen, Carlsbad CA) plus 10% FCS (Atlanta Biologicals, Atlanta GA) plus 1% Penicillin/Streptomycin (Gibco, Invitrogen, Carlsbad CA) in a humidified incubator at 37°C with 5% CO₂.

**Tumor Cell Line Proliferation and Survival Assays**

All proliferation and survival assays were conducted as previously described (Morton, Mongeau et al. 2007). For proliferation assays, 1x10⁵ cells were plated per well in a 6 well plate on day zero. Total cell numbers were counted after 24, 48, and 72 hours. For survival assays, 1x10⁶ cells were plated per well in a 6 well plate and incubated at 37°C overnight. The following day, the media was removed and fresh media was added.
For cells treated with cycloheximide, the fresh media contained 100µM cycloheximide.

For cells treated with Gemcitabine (Gemzar, Eli Lilly, Indianapolis IN), the fresh media contained 50nM Gemcitabine. When inhibitors were used in the experiment, the inhibitors were added to the fresh media at the time it was changed, and cells were treated with either cycloheximide or Gemcitabine one hour later. The MEK inhibitor PD980059 (Invitrogen, Carlsbad, CA) was used at a concentration of 25µM, the PI3 kinase inhibitor LY294002 (Invitrogen, Carlsbad, CA) was used at a concentration of 20µM, and the IGF1R inhibitor AG1024 (BD Biosciences, San Diego CA) was used at a concentration of 20µM. 24 hours after treatment with either cycloheximide or UV irradiation, live and dead cells were counted by trypan blue exclusion and percent survival was calculated.

**ShRNA Mediated Knockdown of the IGF1R in Tumor Cell Lines**

For IGF1R knockdown, pGIPz shRNA targeting IGF1R (Open Biosystems, Huntsville AL, Clone ID V2LMM_188101), or a pGIPz empty vector control (Open Biosystems, Huntsville AL, Cat #RHS4349) was transfected into 293T cells using Effectene Transfection Reagents (Qiagen, Valencia CA). Briefly, 5x10^5 293T cells were plated onto a 6-well plate, and the following day 0.5µg of the plasmid plus 0.33 µg of the pCMV del 8.2 plasmid and 0.2µg of the pMD.G plasmid were combined in EC Buffer to a total volume of 75µL. To this, 8µL of enhancer reagent was added, and the mixture was incubated for 2-5 minutes at room temperature. Next, 25µL of Effectene reagent was added, and the mixture was incubated for 5-10 minutes at room temperature. Following the incubation, 0.5mls of DMEM plus 10% FCS plus 1% Penicillin/Streptomycin was added to the mixture, and the mixture was transferred onto the 293T cells. The cells plus
transfection reagents were incubated overnight at 37°C and the next day fresh media was added to the cells. Supernatant from the transfected 293T cells was harvested at both 48 and 72 hours post transfection, and added to the tumor cell lines. Infected cells were in media containing 2µg/ml puromycin for at least 4 days.

**Isolation of Protein Lysates**

PDECs for lysates were isolated in a 1mg/mL collagenase V solution (Sigma, St Louis MO) incubated at 37°C for 10 minutes. Cells were then pelleted by centrifugation at 1000 rpm for 5 minutes at 4°C, and the cell pellet was incubated in lysis buffer on ice for 30 minutes. Lysis buffer contained: 10mM KPO4/EDTA pH 7.05, 5mM EGTA pH 7.2, 10mM MgCl2, 50mM b-glycerophosphate pH 7.2, 0.5% NP-40, 0.1% Brij-35, protease inhibitor mixture (Roche, San Francisco CA), 1mM DTT, 1mM Na3VaO4, and 1mM PMSF (Yang, Xia et al. 2011). Where noted, cells were serum starved for 48 hours prior to the generation of protein lysates.

Tumor cell lines for lysates washed once with PBS and then cells were scraped into 1mL of fresh PBS and transferred to an eppendorf tube. Cells were then pelleted by centrifugation at 1000 rpm for 5 minutes at 4°C, and the cell pellet was incubated in lysis buffer on ice for 30 minutes. Where noted, cells were serum starved for 48 hours prior to the generation of protein lysates.
Immunoblotting

Immunoblotting was performed as described previously (Lewis, Klimstra et al. 2003) using the following protocol. Protein lysates were subjected to SDS-PAGE and transferred to PVDF membranes (Amersham, Arlington Heights IL). PVDF membranes were then blocked in 1X TBS-T {1X TBS plus 0.1% Tween-20 (Sigma, St Louis MO)} plus 5% BSA for one hour at room temperature. PVDF membranes were then incubated with primary antibody diluted in 1X TBS-T plus 5% BSA at 4°C overnight, and the next day membranes were washed three times for ten minutes each wash in 1X TBS-T. Following washes, membranes were incubated with a horse radish peroxidase conjugated secondary antibody diluted 1:5000 in 1X TBS-T plus 5% BSA for one hour at room temperature, and then washed three times for twenty minutes each wash in 1X TBS-T. ECL (Amersham, Arlington Heights IL) or Supersignal West Pico (Pierce, Rockford, IL) chemoluminescence reagents were then used to visualize horse radish peroxidase signal on film. Primary antibody dilutions are listed in the following table.
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<td>Cat #sc-713</td>
</tr>
<tr>
<td>Rabbit anti-phospho IGF1R</td>
<td>1:500</td>
<td>Abcam, Cambridge, MA</td>
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**Quantitative RT-PCR.** RNA was extracted from serum starved PDECs using Trizol (Invitrogen, Carlsbad CA), purified with an RNeasy Mini Kit (Qiagen, Valencia CA), and treated with Turbo DNase (Ambion, Austin TX). cDNA was then generated using the Superscript III First Strand Synthesis System (Invitrogen, Carlsbad CA). For *Kras* expression analysis, 10ng of cDNA was mixed with TaqMan primers (Primer Set One: PN# Mm00517492_m1 and Primer Set Two PN# Mm01255197_m1) and TaqMan PCR Master Mix (Applied Biosystems, Foster City CA) using the Taqman standard protocol. For IGF- and EGF-family ligands, 10ng of cDNA was combined with SYBR Green Reaction Mix (Quanta Biosciences, Gaithersburg, MD), and 500nm of the appropriate primer pairs (IDT, Coralville, Iowa). Primer sequences can be found at the end of this methods section. PCR amplification was conducted using an ABI 7300 Real Time PCR system using Applied Biosystems standard conditions.
<table>
<thead>
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Orthotopic implantation of PDECs

For orthotopic transplant, 1x10⁶ PDECs were resuspended in 10µL of matrigel and injected into the pancreata of nude mice as previously described (Morton, Mongeau et al. 2007). Briefly, nude mice were anesthetized by IP injection of 100mg/kg ketamine (Henry Schein Inc., Melville NY) and 10mg/kg xylazine (Henry Schein Inc., Melville NY) diluted in sterile water. Once anesthetized, buprenorphine (Henry Schein Inc., Melville NY) was administered sub-cutaneously peri-operatively, and the surgical site was prepared through cleansing the abdomen of the mouse with betadine followed by isopropyl alcohol. A small incision was made in the abdomen and the pancreas was externalized, allowing for the injection of the PDEC and matrigel mixture using a 10µL Hamilton Syringe (Hamilton, Reno NV). Following injection, the matrigel was allowed to set undisturbed for approximately 30 seconds and then the pancreas was returned to the abdominal cavity. Lastly, Marcaine (Henry Schein Inc., Melville NY) was administered to the site of the wound, and the incision was closed with sutures on the body wall and wound clips in the skin. Following orthotopic transplant of PDECs, mice were monitored daily for tumor development.

Immunostaining

Immunostaining was performed as previously described (Lewis, Klimstra et al. 2003) using the following protocol: Deparaffinization of the slides was conducted using two five minute washes with Clear Rite 3 (Fisher Scientific, Pittsburgh PA) followed by three three minute washes with 100% ethanol, one three minute wash with 95% ethanol,
one three minute wash with 70% ethanol, one three minute wash with 50% ethanol, one three minute wash with 20% ethanol, and a one minute wash with distilled water. Next, slides were placed in a beaker with 7.5mL of Antigen Unmasking Solution (Vector Labs, Burlingame CA) mixed with 800mL of distilled water, and microwaved for 8 minutes on high power, followed by 15 minutes on low power. Slides were then cooled to room temperature for 40 minutes, and rinsed in purified water. Slides were then incubated for five to ten minutes in 3% Hydrogen Peroxide Solution, and then washed twice in TBS-T solution. Slides were then incubated with blocking solution at room temperature for one hour. Following blocking step, slides were washed twice in TBS-T solution. Slides were then incubated with primary antibody at 4°C overnight, and the next day slides were washed twice with TBS-T solution. Following washes, the slides were incubated with the secondary antibody at room temperature for thirty minutes, and then washed twice in TBS-T solution. Next, the slides were net incubated for thirty minutes at room temperature with the Vector ABC Reagent (Vector Labs, Burlingame CA), and then washed once with TBS-T solution. A NovaRed Peroxidase substrate (Vector Labs, Burlingame CA) was then added to the slides and slides were incubated five to ten minutes at room temperature until desired staining intensity had developed. Following this step, the slides were washed for five minutes in running water. Slides were then counterstained for five to ten seconds in Hematoxylin (Fisher Scientific, Pittsburgh PA) and then placed under running water until the water draining from the slides was clear. Slides were next placed into Bluing Solution (Fisher Scientific, Pittsburgh PA) for one minute, and then washed again in running water for five minutes. Lastly, slides were
dehydrated with three five minute washes in 95% ethanol, then two five minute washes in 100% ethanol, and lastly, three five minute washes in Clear Rite 3 (Fisher Scientific, Pittsburgh PA). Following this, slides were mounted with Permount Mounting Medium (Fisher Scientific, Pittsburgh PA) and clear coverslips and allowed to dry at room temperature.

Antibody dilutions for immunostaining were as follows: Rabbit anti-Ki-67 (1:1000, Novocastro, Cat #NCL-Ki67p), Rabbit anti-mouse Pdx-1 (1:5000, gift of Chris Wright), Rabbit anti-Keratin-8 (1:50, Developmental Studies Hybridoma Bank, University of Iowa). Rabbit anti-pIGF1R-Y1161 (1:100, Abcam, Cat #ab39398).

**Soft agar and migration assays**

Soft agar colony formation and transwell migration assays were performed as previously described (Lewis, Shim et al. 1997; Chen, Klimstra et al. 2007).

For soft agar colony formation, a hard agar layer was first prepared using a 1:1 mixture of 1.5% agarose and 2X DMEM (Gibco, Invitrogen, Carlsbad CA) and allowed to set overnight. The following day, 1x10^5 cells were resuspended in a soft agar layer comprised of 7% agarose and 2X DMEM, and this mixture was pipetted onto the hard agar layer. Once this layer had set, fresh media (1X DMEM plus 10% FCS plus 1% Penicillin/Streptomycin) was added to the plate, and plates were cultured at 37°C for 2-3 weeks. Following this incubation, the number of colonies per plate were counted.

For transwell migration assays, fresh media (DMEM plus 10% FCS plus 1% Penicillin/Streptomycin) was plated into the lower chamber of a BD Matrigel Migration
Chamber (BD Biosciences, Billerica MA). Next, 5x10⁴ cells were resuspended in serum free media (DMEM plus 1% Penicillin/Streptomycin) and added to the upper portion of the migration chamber. The migration chambers were then incubated at 37°C for 24 hours, and then fixed in methanol and the cells were stained with Giemsa (Fisher Scientific, Pittsburgh PA). The matrigel layer was then mounted onto a glass slide and the number of migrating cells were counted.
CHAPTER III:

DISCUSSION
Despite decades of research into pancreatic cancer progression and treatment, PDAC continues to have a dismal prognosis with few effective therapeutic options outside of surgical resection. Over 90% of all pancreatic tumors have activating mutations in the KRAS2 oncogene, and a clear role for activated KRAS in the formation of pancreatic tumors has been established through multiple mouse models of PDAC (Aguirre, Bardeesy et al. 2003; Guerra, Mijimolle et al. 2003; Hingorani, Petricoin et al. 2003; Hingorani, Wang et al. 2005; Hezel, Kimmelman et al. 2006). However, efforts to therapeutically target activated KRAS have thus far proved unsuccessful, and recent work has turned towards investigating the role of signaling downstream of activated KRAS in the search for new therapeutic targets. This recent research has highlighted the importance of taking cellular context into consideration when studying the consequences of signaling downstream of activated KRAS, as many of the studies used different cell types and resulted in conflicting reports regarding the significance of each of the KRAS activated signaling cascades on pancreatic tumorigenesis (Hamad, Elconin et al. 2002; Guerra, Mijimolle et al. 2003; Lim and Counter 2005; Campbell, Groehler et al. 2007).

To better address the question of which signaling pathways downstream of activated KRAS are important for pancreatic tumor initiation, I investigated the effects of activated KRAS and mutant BRAF expression in pancreatic ductal epithelial cells. As ductal epithelial cells are the putative targets of transformation in the initiation of pancreatic cancer, PDECs are a highly relevant model system to study the events of pancreatic tumor initiation (Hruban, Goggins et al. 2000).
Here, I determine that the expression of either KRAS\textsuperscript{G12D} or BRAF\textsuperscript{V600E} in PDECs results in increased proliferation relative to GFP expressing PDECs. These results were irrespective of tumor suppressor status, as the same results were observed in tumor suppressor wild type, \textit{Ink4a/Arf} null, and \textit{Ink4a/Arf, Trp53} null PDECs. These findings are consistent with the ability of BRAF\textsuperscript{V600E} to stimulate the MEK/ERK signaling cascade, and support the role of MEK/ERK signaling in cell proliferation downstream of activated KRAS in PDECs. In addition, I find that while both KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expression increases PDEC proliferation relative to GFP expressing cells, this increase is greater in KRAS\textsuperscript{G12D} expressing PDECs compared with BRAF\textsuperscript{V600E} expressing PDECs. As BRAF\textsuperscript{V600E} expression in PDECs does not fully mimic the expression of activated KRAS, these findings suggest that other signaling pathways stimulated by KRAS\textsuperscript{G12D} in addition to MEK/ERK are required for the KRAS-induced proliferation increase in PDECs. It is also possible that the signaling through MEK/ERK differs downstream of KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E}, and this signaling difference ultimately leads to the differential effect on proliferation that is observed.

In addition, I find that expression of either KRAS\textsuperscript{G12D} or BRAF\textsuperscript{V600E} in PDECs results in increased survival when cells are challenged with an apoptotic stimulus. Treatment of GFP expressing PDECs with either cycloheximide or UV irradiation results in increased cell death, and prior work within our lab has confirmed that this is due to apoptosis by demonstrating increased levels of cleaved caspase-3 in these cells (Morton, Mongeau et al. 2007). Here, I find that both KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs have increased survival when treated with either cycloheximide or UV
irradiation. As observed in cell proliferation studies, this increased survival is irrespective of tumor suppressor status, as the same results were observed in tumor suppressor wild type, *Ink4a/Arf* null, and *Trp53, Ink4a/Arf* null PDECs.

Prior studies assessing the role of signaling downstream of activated KRAS have typically associated signaling through RAF/MEK/ERK with increased proliferation, and signaling through PI3K/AKT with increased survival. Based upon this, I was surprised to find that the expression of activated BRAF was sufficient for increased survival in PDECs and next sought to determine if survival in these cells depended on signaling through either MEK or PI3K. I found that treatment of KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> expressing PDECs with either a MEK or a PI3K inhibitor resulted in decreased survival when challenged with an apoptotic stimuli. These findings indicate that signaling through both MEK and PI3K are required for KRAS- and BRAF-mediated survival in PDECs.

Having found that increased survival in KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> expressing PDECs depends on signaling through both MEK and PI3K, I next investigated whether activated BRAF was stimulating the PI3K/AKT signaling cascade in these cells. Immunoblotting for phosphorylated ERK 1/2 and phosphorylated AKT at ser473 confirmed that both the MEK/ERK and the PI3K/AKT signaling cascades were being activated downstream of KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> expression in PDECs. In addition, I found that the inhibition of MEK in BRAF<sup>V600E</sup> expressing PDECs resulted in decreased levels of both pERK 1/2 as well as pAKT at ser473, indicating that the activation of AKT in these cells is downstream of MEK/ERK signaling. While KRAS<sup>G12D</sup> expressing
PDECs did not show the same decrease in the levels of pAKT at ser473 following inhibition of MEK, this was likely due to the ability of activated KRAS to also directly stimulate PI3K/AKT signaling.

These data are significant as they support a connection between the signaling downstream of activated BRAF and the PI3K/AKT signaling cascade, as well as a dependence of mutant BRAF on signaling through PI3K for cell survival. Previous studies have clearly demonstrated mutant BRAF’s ability to signal through the MEK/ERK signaling cascade, but there is little evidence thus far to indicate the activation of PI3K/AKT downstream of BRAF (Cantwell-Dorris, O'Leary et al. 2011). In our studies, though, we see activation of both the RAF/MEK/ERK and PI3K/AKT signaling cascades downstream of KRAS^{G12D} and BRAF^{V600E} in PDECs, and we find that the activation of AKT lies downstream of the MEK/ERK signaling pathway in BRAF^{V600E} expressing PDECs.

Having found the activation of AKT downstream of MEK/ERK signaling in BRAF^{V600E} expressing PDECs, I next sought to determine the mechanism of AKT activation in these cells. I hypothesized that signaling through MEK/ERK resulted in the increased production of an autocrine growth factor, which would result in activation of growth factor receptor signaling, and subsequent activation of AKT. Since I observed increased levels of IGF2 at both the message and protein levels in KRAS^{G12D} and BRAF^{V600E} expressing PDECs, I hypothesized that signaling through the MEK/ERK pathway leads to increased IGF2 expression and results in activation of the IGF1R and subsequent activation of the PI3K/AKT signaling cascade.
Consistent with this hypothesis, there is increasing evidence for tumor cell dependence on growth factor receptor activation, even in the presence of activated KRAS mutations. One such study found that cells expressing activated RAS had increased expression of heparin-binding epidermal growth factor (HB-EGF), a ligand for the epidermal growth factor receptor (EGFR), and that these cells were sensitive to inhibiting HB-EGF with diphtheria toxin in spite of their mutant RAS expression (McCarthy, Samuels et al. 1995). Another recent study in immortalized human pancreatic ductal cells showed that the activation of KRAS and the loss of SMAD4 cooperated to induce the expression of EGFR, and that inhibition of EGFR in these cells resulted in decreased invasion even though the mutational status of KRAS was maintained (Zhao, Wang et al. 2010). An additional study demonstrated that melanoma cells had decreased survival following inhibition of the IGF1R, regardless of whether these cells expressed activated NRAS or BRAF (Yeh, Bohula et al. 2006). Lastly, a study recently published by Engelman and colleagues demonstrated that the IGF1R is the dominant activator of PI3K/AKT signaling in colorectal tumors, even in the context of KRAS mutations (Ebi, Corcoran et al. 2011).

In support of this hypothesis, I observe that inhibiton of MEK in KRAS\textsuperscript{G12D} expressing PDECs results in decreased levels of Igf2, indicating that increased expression of IGF2 is downstream of MEK/ERK signaling in these cells. In addition, I found increased expression of phosphorylated IGF1R in both KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs when compared with GFP expressing cells, supporting activation of the IGF1R in these cells. I also show that the activation of AKT at ser473 depends on
signaling through the IGF1R, since knockdown of the IGF1R results in loss of AKT phosphorylation at serine 473 in both KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs. Together, these data indicate that signaling through MEK/ERK downstream of activated KRAS- and BRAF- expression in PDECs results in increased levels of IGF2 and activation of the IGF1R, and this signaling through the IGF1R is required for activation of PI3K/AKT signaling.

I next confirmed the role of the signaling through IGF2 and the IGF1R in KRAS- and BRAF-mediated cell survival by showing that the ectopic expression of IGF2 was sufficient to rescue survival in the presence of a MEK inhibitor, but not a PI3K inhibitor. These results indicate that the requirement for signaling through the MEK/ERK signaling cascade downstream of activated KRAS or BRAF for cell survival is due to a requirement for increased expression of IGF2 downstream of this signaling cascade. In addition, I found that the inhibition of the IGF1R resulted in loss of survival in both KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs, indicating that signaling through the IGF1R is necessary for the activated KRAS and mutant BRAF induced survival phenotype.

Prior work within our lab has shown that the expression of KRAS\textsuperscript{G12D} in Ink4a/Arf, Trp53 null PDECs is sufficient for tumor formation following orthotopic transplant of these cells (Morton, Mongeau et al. 2007). Here, I show that the expression of BRAF\textsuperscript{V600E} in double null PDECs is also sufficient for tumor formation using an orthotopic model. I further show that shRNA-mediated knockdown of the IGF1R in either KRAS\textsuperscript{G12D} or BRAF\textsuperscript{V600E} expressing Ink4a/Arf, Trp53 null PDECs results in
decreased tumor formation, indicating that signaling through the IGF1R is needed for KRAS- and BRAF-mediated pancreatic tumor formation. These results are further supported by our findings that tumors formed from the pancreas specific expression of KRAS\textsuperscript{G12D} stain positive for phosphorylated IGF1R by immunostaining, underscoring a role for signaling through the IGF1R in KRAS-mediated pancreatic tumorigenesis.

An extension of these studies assessing the role of IGF1R signaling in KRAS-mediated pancreatic tumor formation could be conducted in the future using mouse modeling. If, as my research indicates, pancreatic tumor initiation following the activation of KRAS requires signaling through the IGF1R, then mouse models combining pancreas specific expression of KRAS\textsuperscript{G12D} in conjunction with pancreas specific ablation of the IGF1R should result in decreased tumor formation and increased tumor free survival when compared with littermate controls that are wild type for the IGF1R and expressing pancreas specific KRAS\textsuperscript{G12D}. These studies would not only confirm the role of the IGF1R in KRAS-mediated pancreatic tumor formation, but could provide new insight into the role of the IGF1R in tumor initiation through analysis of any precursor lesions (PanINs) or tumors that do form in the absence of IGF1R expression.

A potential signaling loop that remains to be characterized within our system is the ability of activated KRAS or BRAF to increase activation of mTORC1 via inhibition of the TSC1/TSC2 complex, which negatively regulates mTORC1 activity. Recent work has demonstrated that phosphorylation of TSC2 by pERK 1/2 results in inhibition of the ability of the TSC1/TSC2 protein complex to convert Rheb-GTP to Rheb-GDP (Ma, Chen et al. 2005). As Rheb-GTP is an activator of mTORC1, this results in increased
activation of mTORC1 (Shaw and Cantley 2006). mTORC1, which can additionally be activated downstream of AKT as well as through many other mechanisms, is involved in cell growth through its phosphorylation of S6K, stimulation of HIF-1α and inhibition of 4E-BP1 (Hara, Maruki et al. 2002; Hudson, Liu et al. 2002; Kim, Sarbassov et al. 2002). As our studies demonstrate that survival in both KRAS^{G12D} and BRAF^{V600E} expressing PDECs is sensitive to inhibition of mTORC1 by rapamycin, this complex is clearly important in signaling downstream of activated KRAS and BRAF. An intriguing question, therefore, is whether activation of mTORC1 downstream of KRAS^{G12D} and BRAF^{V600E} occurs only following the activation of AKT, or whether ERK is also contributing to this through the phosphorylation of TSC2.

To address this question, an investigation into the activation of mTORC1 downstream of KRAS and BRAF induced MEK/ERK signaling could be conducted by first determining if activation of mTORC1 occurs in PDECs in the absence of signaling through AKT. In addition, the impact of ERK 1/2 inhibition on the activation of mTORC1 and its downstream targets, such as S6K could be evaluated to assess how much ERK 1/2 contributes to the activation of mTORC1 downstream of KRAS^{G12D} and BRAF^{V600E} in PDECs.

BRAF expressing PDECs may also be increasing the activation of mTORC1 through the inhibition of LKB1. LKB1 has been shown to inhibit mTORC1 signaling by phosphorylating AMPKα, thereby activating AMPK (Hardie 2007). When activated, AMPK is able to phosphorylate TSC2 at Thr1227 and Ser1345 (Inoki, Zhu et al. 2003). Phosphorylation of TSC2 at this site results in the activation of the TSC1/TSC2 complex,
and increased conversion of Rheb-GTP to Rheb-GDP. This results in decreased
activation of mTORC1. A recent study has identified that in melanoma cells, BRAF<sup>V600E</sup>
is able to negatively regulate LKB1 by phosphorylating it at ser428 and ser325 and
preventing its binding to AMPK (Zheng, Jeong et al. 2009). Through inhibition of
LKB1, the expression of activated BRAF could therefore reduce the levels of activated
AMPK, and therefore impact the levels of activated TSC1/TSC2 in cells. These studies
identify a novel mechanism by which activated BRAF may increase mTORC1 activation
in tumor cells.

The potential for a similar signaling paradigm in activated BRAF expressing
PDECs is particularly interesting, since a role for LKB1 inhibition in pancreatic tumors
has already been established in several studies. Studies have demonstrated that patients
with Peutz-Jeghers syndrome, an autosomal dominantly inherited syndrome caused by
inactivating mutations in <i>LKB1</i>, have an increased risk of developing pancreatic cancer
(Hemminki, Avizienyte et al. 1998; Jenne, Reimann et al. 1998; Hearle, Schumacher et
al. 2006). Prior studies have also identified <i>LKB1</i> gene inactivation in IPMNs, and that
loss of <i>Lkb1</i> leads to benign cystadenomas (Sato, Rosty et al. 2001; Hezel, Gurumurthy et
al. 2008). Moreover, a recent study demonstrated that loss of <i>Lkb1</i> accelerated KRAS
induced pancreatic tumor formation in mice (Morton, Jamieson et al. 2010). In addition,
one study investigating genetic alterations in acinar cell carcinoma demonstrated that
while these tumors rarely have activating mutations in <i>KRAS</i> or loss of LKB1, there is at
least one case of acinar cell carcinoma in a patient with <i>LKB1</i> loss due to Peutz-Jeghers
syndrome, and this tumor responded to treatment with rapamycin (Hoorens, Lemoine et
al. 1993; Klumpen, Queiroz et al. 2011). These findings point to a potential role for loss of *LKB1* in the formation of pancreatic tumors, and highlight the possibility of effectively treating pancreatic tumors associated with LKB1 loss or inhibition with inhibitors of mTOR signaling. Therefore, it is important to better understand whether activated BRAF expression in PDECs and tumor cell lines is increasing mTOR signaling via inhibition of LKB1, as this could point to increased efficacy of mTOR inhibition in BRAF<sup>V600E</sup> expressing tumors.

Further support for the role of LKB1 and AMPK inhibition in pancreatic tumor initiation is found in studies investigating the impact of metformin on pancreatic tumors and tumor cell lines. Metformin is an anti-diabetes drug that has been shown to stimulate activation of AMPK in an LKB1 dependent manner (Shaw, Lamia et al. 2005). Recent studies have demonstrated that the use of metformin during the treatment of diabetes results in a decreased risk of cancer (Evans, Donnelly et al. 2005). More specifically, the use of metformin was shown to decrease the risk of pancreatic cancer in patients with Type II diabetes by 62% (Li, Yeung et al. 2009). A recent paper went on to demonstrate that treatment with metformin resulted in the inhibition of tumor growth in mice with sub-cutaneous injection of pancreatic cancer cell lines (Kisfalvi, Eibl et al. 2009). Collectively, these findings demonstrate a role for LKB1 and AMPK signaling in the inhibition of pancreatic tumors, and reveal an emerging role for metformin in the treatment of pancreatic tumors. However, because metformin functions through LKB1 dependent stimulation of AMPK, it is critical to understand if activated BRAF is
inhibiting LKB1 in pancreatic tumorigenesis, as this inhibition could prevent the efficacy of metformin in patients with BRAF mutations.

To investigate the potential role of LKB1 inhibition and mTORC1 activation in KRAS and BRAF expressing PDECs, the expression and localization of phosphorylated LKB1, as well as total LKB1, could be assessed within these cells. As LKB1 is phosphorylated both in its activated state as well as when inhibited, it is be important to determine which sites on LKB1 are phosphorylated downstream of activated KRAS and BRAF in PDECs. In addition, the level of phosphorylated AMPK expression could be ascertained downstream of KRAS$^{G12D}$ and BRAF$^{V600E}$ as further indication of whether this signaling pathway is being inhibited in these cells. Lastly, inhibition of ERK 1/2 could be used to determine if inhibition of LKB1 downstream of activated KRAS or BRAF in PDECs occurs due to phosphorylation of LKB1 by ERK as was seen in melanoma cells (Zheng, Jeong et al. 2009).

In these studies, I also identified a potential positive feedback loop between the RAF/MEK/ERK and PI3K/AKT signaling cascades, as inhibition of PI3K led to the decreased expression of phosphorylated ERK (1/2) in both KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs. Cross talk between these two signaling cascades has been identified previously in many studies of cancer cells, and so it is not surprising to see such an interaction, although the mechanisms of this interaction downstream of KRAS are still not clearly understood (Duckworth and Cantley 1997; Rubio and Wetzer 2000).

One potential mechanism for the decreased levels of phosphorylated ERK 1/2 is through a change in expression of the Sprouty genes. These proteins have been shown to
be inhibitors of MEK/ERK signaling downstream of receptor tyrosine kinases (Gross, Bassit et al. 2001; Yusoff, Lao et al. 2002). Although their mechanism of function remains largely to be determined, the Sprouty genes have been shown to function in multiple cell types as part of higher order complexes that inhibit ERK activation downstream of multiple RTKs, such as FGFR and EGFR (Mason, Morrison et al. 2006). Previous studies have demonstrated that melanomas expressing activated BRAF are able to maintain hyperactivated ERK 1/2 through resistance to regulation by one of the Sprouty genes, Spry2 (Tsavachidou, Coleman et al. 2004). Based upon these findings, one could speculate that it is possible that activated KRAS and BRAF expressing PDECs have developed a mechanism of avoiding regulation by the Sprouty genes in order to sustain hyperactivated ERK 1/2 levels. Following this logic, its possible to extend that speculation and hypothesize that these mechanisms potentially rely on signaling through PI3K or the IGF1R, and therefore the inhibition of either of these proteins results in an inability to avoid Sprouty regulation, and decreased levels of phosphorylated ERK 1/2 in these cells. Investigation into the expression of the Sprouty genes in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs treated with MEK, PI3K, and IGF1R inhibitors could begin to shed light on whether the Sprouty genes are involved in the feedback loop observed in these cells.

While I identified many similarities in KRAS and BRAF induced MEK/ERK signaling, it is possible that the ultimate downstream signaling, as well as the mechanisms of feedback, are not that same downstream of activated KRAS and activated BRAF. Indeed, the large disparity in KRAS mutations versus BRAF mutations in
pancreatic tumors would imply that these oncogenes are not fully redundant. A better understanding of how KRAS promotes pancreatic tumorigenesis and the signaling that is critical to KRAS mediated tumorigenesis therefore requires further exploration into how these signaling pathways may differ. One such difference lies potentially in the differential activation of MEK/ERK signaling and its downstream effects. Previous work within our lab has demonstrated that signaling through MEK/ERK can have different consequences depending upon how that signaling is initiated upstream. In these studies, we have shown that while both activated KRAS and SHH expression induces increased levels of pERK 1/2, only KRAS<sup>G12D</sup> expressing PDECs require signaling through MEK for survival when challenged with an apoptotic stimulus (Morton, Mongeau et al. 2007). An additional study has demonstrated that in melanoma cells, MEK inhibitors inhibit proliferation in those with BRAF mutations, while proliferation in those with RAS mutations are not effected by MEK inhibition (Solit, Garraway et al. 2006). In both of these cases, the MEK/ERK signaling cascade is being activated, but the impact of that activation is not the same. These findings indicate that the ultimate effect of MEK/ERK activation on tumorigenic phenotypes depends on the upstream stimulus, and it is possible that some of these differences in signaling lie in the downstream changes that occur after phosphorylation of ERK 1/2.

To further explore the downstream effects of increased pERK 1/2 in KRAS<sup>G12D</sup> and BRAF<sup>V600E</sup> expressing PDECs, and therefore potentially determine how signaling downstream of these two oncogenes may differ, gene expression analysis via microarray could be conducted. In addition, phosphoproteome analysis could be used to determine
which proteins are being phosphorylated downstream of activated KRAS and BRAF. These analyses could compare not only the signaling downstream of activated KRAS and BRAF in PDECs, but could also look at the effects of expressing a KRAS$^{G12D}$ binding mutant that preferentially signals through RAF/MEK/ERK in order to better focus on the effect of MEK/ERK signaling downstream of KRAS$^{G12D}$. Through assessing the differences in gene expression and protein phosphorylation downstream of MEK/ERK activation in KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing cells, one would potentially gain a better understanding of how activated KRAS and BRAF differ, and how these differences may impact pancreatic tumor treatment options.

It is also possible that the differential effects seen in ERK activation downstream of KRAS and BRAF may not be due entirely to the differential expression or activation of downstream targets. Many factors have been shown to regulate the effects of ERK activation, including the magnitude of ERK activation, the localization of ERK, and the expression of scaffold proteins that interact with ERK (Harding, Tian et al. 2005; Casar, Arozarena et al. 2009). These differences open up numerous avenues of study for further evaluating how MEK/ERK signaling may differ downstream of activated KRAS and BRAF, and as a result may provide insight into how to best treat pancreatic tumors.

One key difference that I already identified between KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs lies in the increased expression of the AKT family of proteins. Both KRAS$^{G12D}$ and BRAF$^{V600E}$ expressing PDECs showed increased levels of total AKT when compared with GFP expressing PDECs. However, the KRAS$^{G12D}$ expressing PDECs had increased expression of AKT1, while the BRAF$^{V600E}$ expressing PDECs were
found to have a large increase in AKT3, with only a slight increase in the levels of
AKT1. In addition, KRAS\textsuperscript{G12D} expressing PDECs had increased levels of Akt3 mRNA,
whereas BRAFV600E expressing PDECs had increased in mRNA levels of Akt1, Akt2,
and Akt3. These data indicate that KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expression in PDECs
results in changes in both AKT family member gene expression as well as protein levels,
and implicate a role for signaling downstream of activated KRAS and BRAF in the
regulation of the translation or post-translational modification of AKT.

The data regarding different AKT isoform expression downstream of activated
KRAS and activated BRAF are particularly interesting considering recent findings
regarding the roles of different AKT isoforms in tumorigenesis. In one recent study of
lung tumorigenesis, it was shown that the loss of AKT1 inhibited tumor formation in
cells expressing activated KRAS, indicating that the increased levels of AKT1
downstream of KRAS\textsuperscript{G12D} observed in PDECs may be important to Kras mediated
tumorigenesis (Hollander, Maier et al. 2011). AKT1 has also been shown to increase
IGF1R expression in human pancreatic cancer cell lines, further demonstrating its
potential role in pancreatic tumorigenesis (Tanno, Tanno et al. 2001). In additional
studies involving melanoma cells, it has been shown that increased expression of AKT3
can rescue survival loss following the knockdown of BRAF, while the inhibition of
AKT3 results in apoptosis, indicating that the increased expression of AKT3 that we
observe downstream of BRAF\textsuperscript{V600E} expression in PDECs may be aiding in the survival of
those cells (Madhunapantula and Robertson 2009; Shao and Aplin 2010). Expression
levels of AKT family members has also been linked to migration and invasion ability
within a tumor (Toker and Yoeli-Lerner 2006). In one study, it was demonstrated that in ovarian tumor cells, IGF1 induced increases in migration are mediated by mTORC2 activation of AKT1, not AKT2 (Kim, Yun et al. 2011). It is therefore possible that these differences in expression observed between the activated KRAS and the mutant BRAF expressing PDECs would result in different migration and invasion abilities, which were not addressed in PDECs in the present study.

To further investigate the role of AKT isoforms in pancreatic tumor initiation and progression, shRNA mediated knockdown of the individual isoforms could be conducted in both KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs. The impact of this knockdown on KRAS- and BRAF- induced tumor phenotypes such as proliferation, survival when challenged with an apoptotic stimulus, and the ability to form tumors following orthotopic transplant could then be assessed. If the differential expression of AKT isoforms seen within activated KRAS and activated BRAF expressing PDECs is important to KRAS- and BRAF-mediated tumorigenesis, as recent studies would indicate, then the knockdown of a single isoform of AKT should have different effects on KRAS\textsuperscript{G12D} expressing PDECs versus BRAF\textsuperscript{V600E} expressing PDECs. More specifically, I would expect that only the knockdown of AKT1 would decrease KRAS-mediated proliferation, survival, and tumor formation following orthotopic transplant, while only the knockdown of AKT3 would decrease these phenotypes in BRAF\textsuperscript{V600E} expressing PDECs. These studies would not only further characterize signaling downstream of activated KRAS and activated BRAF, but would also highlight a difference between
these two oncogenes and provide potential insight into how to better and more specifically target tumors expressing \( \text{KRAS}^{G12D} \) or \( \text{BRAF}^{V600E} \).

Likewise, similar studies using shRNA mediated knockdown of the AKT isoforms could be conducted to examine if the role of an individual isoform of AKT is important to activated KRAS or activated BRAF expressing tumor cell lines, thereby indicating if the expression of, and reliance on, the various isoforms is different between pancreatic tumor initiation and progression. Due to the increased ease of working with the tumor cell lines, these studies could include an assessment of not only proliferation and survival, but also address the role of the individual isoforms in KRAS and BRAF induced migration, invasion, and anchorage independent growth of pancreatic tumor cell lines.

If studies within PDECs and tumor cell lines indicate a role for specific AKT isoforms in KRAS- mediated pancreatic tumor formation, it is possible to then study this further through the use of mouse models with pancreas specific expression of \( \text{KRAS}^{G12D} \) in conjunction with ablation of individual AKT family members in the pancreas. If a specific AKT family member is required for KRAS- mediated tumor formation, then I would expect increased survival and decreased tumor formation in mice lacking expression of that AKT family member in the pancreas when compared with littermate controls. As with the tumor cell line and PDEC studies, these experiments would not only provide further insight into the role of the various AKT isoforms downstream of \( \text{KRAS}^{G12D} \), but could potentially result in more targeted therapies for pancreatic tumors.
through identifying the specific AKT isoforms required for KRAS- mediated pancreatic tumorigenesis.

An additional important question that remains to be addressed is how the IGF1R is signaling to the PI3K/AKT signaling cascade downstream of activated KRAS and BRAF. Upon activation, the IGF1R recruits IRS1 and IRS2 to the membrane, and this results in the activation of several downstream targets, including PI3K and RAS (Baserga, Hongo et al. 1997; Pollak, Schernhammer et al. 2004). A role for both IRS1 and IRS2 has been previously established in pancreatic cancer studies. One of these studies demonstrated that human pancreatic cancer cell lines have increased expression of both IRS1 and IRS1 expression (Bergmann, Funatomi et al. 1996). Other studies have demonstrated, though, that IRS2 is also overexpressed in pancreatic cancer cell lines, and that IRS2 but not IRS1 is involved in the regulation of the IGF1R in these cells (Kornmann, Maruyama et al. 1998; Kwon, Stephan et al. 2009). Determining which of the IRS proteins is needed for the signaling downstream of KRAS$^{G12D}$ and BRAF$^{V600E}$ to PI3K/AKT through knockdown studies of IRS1 and IRS2 in PDECs could prove useful in further elucidating exactly how MEK/ERK is signaling through the IGF1R to activate AKT.

Further studies could then explore the role of IRS1 and IRS2 in KRAS- mediated pancreatic tumor initiation through the use of mouse modeling. As described above for AKT family members and the IGF1R, pancreas specific ablation of IRS1 or IRS2 in conjunction with pancreas specific expression of KRAS$^{G12D}$ could be used to determine if IRS1 or IRS2 are required for KRAS- mediated pancreatic tumor formation. Together,
these in vitro and in vivo studies would help to further characterize signaling downstream of activated KRAS in pancreatic tumor initiation and progression.

In addition to elucidating how the IGF1R is signaling through PI3K/AKT in KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing cells, it is prudent to also investigate what role, if any, the insulin receptor is playing in KRAS-mediated tumor formation. IGF2 has been shown to have a comparable affinity for binding to the IR as for its binding to the IGF1R (De Meyts, Urso et al. 1995). As the insulin receptor has also been shown to stimulate signaling through PI3K/AKT, it is possible that IGF2 stimulated IR signaling is involved in KRAS and BRAF induced tumor formation (Buck, Gokhale et al. 2010). To investigate this, levels of phosphorylated IR in KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs should first be assessed. If there is increased phosphorylated IR in these cells compared with GFP expressing PDECs, then the impact of IR inhibition on KRAS- and BRAF- induced PDEC survival could be investigated to determine if signaling through the IR is required for this phenotype. Finally, knockdown of the IR in KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs followed by orthotopic transplant, or alternatively ablation of the IR in mice expressing pancreas specific KRAS\textsuperscript{G12D}, could be used to determine if signaling through the IR is required for KRAS- and BRAF- mediated tumor formation.

Finally, in these studies I found that although KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing tumor cell lines also display survival when challenged with cycloheximide, the signaling requirements for tumor cell line survival differ from what was observed in the PDECs. In KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing tumor cell lines, neither MEK inhibition nor IGF1R inhibition alone resulted in decreased survival when cells were
treated with cycloheximide. Instead, the survival phenotype in these cells decreases only
when either PI3K is inhibited, or MEK and the IGF1R are inhibited simultaneously. The
use of both the MEK and the IGF1R inhibitors simultaneously, however, was effective in
not only decreasing tumor cell survival in response to cycloheximide, but also caused
increased cell death when KRAS\textsuperscript{G12D} expressing tumor cell lines were treated with
gemcitabine. These findings demonstrate that the use of MEK and IGF1R inhibitors
simultaneously in combination with current gemcitabine therapies may be more effective
in treating pancreatic tumors than the use of gemcitabine alone.

In addition to the differences observed in cell survival, I find that the signaling
through ERK 1/2 and AKT differs between the KRAS\textsuperscript{G12D} expressing PDECs and tumor
cell lines, as the inhibition of MEK or the IGF1R in these cells does not impact pAKT at
ser473, and there does not appear to be any cross talk between the MEK and PI3K
signaling cascades as was observed in the PDECs. I further show that although the
simultaneous inhibition of MEK and the IGF1R results in decreased survival in
KRAS\textsuperscript{G12D} expressing tumor cells, immunoblotting of these cells still does not detect
decreased levels of pAKT at ser473. These findings show a stark contrast between the
signaling requirements in primary epithelial cells compared with transformed cells, and
illustrate the need to study signaling downstream of activated KRAS in the proper
context to fully understand the roles of specific signaling pathways in tumor initiation,
progression, and maintenance.

Despite these differences observed between PDECs and tumor cell lines, there is
still a clear role for both the IGF1R and MEK in tumor progression, as inhibition of either
results in decreased proliferation in KRAS^{G12D} expressing tumor cell lines. These data, in combination with the survival data in pancreatic tumor cell lines, argue that the combined inhibition of the IGF1R and MEK may prove to be an effective therapy for the treatment of pancreatic tumors, as studies have recently suggested for both colon carcinoma and melanoma (Villanueva, Vultur et al. 2010; Ebi, Corcoran et al. 2011).

In support of this, other recent studies have identified signaling between the IGF1R and G protein-coupled receptors in pancreatic cancer (Rozengurt, Sinnett-Smith et al. 2010). These studies propose that in the tumors studied, activation of the IGF1R lies downstream of PI3K/AKT activation, though, and not downstream of MEK/ERK signaling (Rozengurt, Sinnett-Smith et al. 2010). In combination with our own data, these studies raise the possibility that signaling through the IGF1R occurs downstream of the RAF/MEK/ERK signaling cascades during tumor initiation, but that during tumor progression, IGF1R is being activated instead by PI3K/AKT signaling. These studies add support to the idea that the IGF1R plays a critical role in pancreatic tumor progression and that the inhibition of the IGF1R could be an effective pancreatic tumor therapeutic. In fact, several clinical trials are currently underway to investigate the impact of IGF1R inhibition on pancreatic tumors and may soon shed light on if this is the case (Rozengurt, Sinnett-Smith et al. 2010).

A potential mechanism for the change in signaling between KRAS^{G12D} and BRAF^{V600E} expressing PDECs compared with tumor cell lines may involve the ability of FAK to compensate for the IGF1R. When phosphorylated, FAK has been shown to increase cell survival through PI3K/AKT signaling (Yamamoto, Sonoda et al. 2003). A
study in pancreatic cancer cell lines has recently demonstrated that FAK and the IGF1R physically interact in those cells, and that this interaction contributes to cell survival (Liu, Bloom et al. 2008). Moreover, Hochwald and colleagues go on to show that the inhibition of both FAK and the IGF1R is effective in decreasing pancreatic cancer cell survival, indicating that the combined inhibition of these two pathways may prove an effective chemotherapy option (Zheng, Golubovskaya et al. 2010). Understanding whether FAK is activated and able to interact with or compensate for IGF1R signaling downstream of activated KRAS or BRAF in PDECs or tumor cell lines could prove important in developing the most targeted therapeutics possible for pancreatic tumors.

An additional line of investigation that has not yet been pursued in this system involves the interaction between the IGF1R and the RON receptor. RON is a receptor tyrosine kinase that has been shown to be overexpressed in human pancreatic cancer, and to potentially contribute to resistance to treatment in pancreatic tumors (Thomas, Toney et al. 2007). A recent study has identified that the RON receptor is able to interact with the IGF1R in pancreatic tumor cell lines, and that this interaction is required for IGF1 induced migration of those cells (Jaquish, Yu et al. 2011). Based upon these findings, it could be interesting to assess what role, if any, the RON receptor is playing in KRAS\textsuperscript{G12D} and BRAF\textsuperscript{V600E} expressing PDECs and tumor cell lines. These experiments would be two fold and include the assessment of RON receptor activation in activated KRAS and BRAF expressing cells, as well an assessment of the impact of RON knockdown on proliferation and survival of these cells. It is possible that signaling from the IGF1R to the RON receptor may occur in the tumor cell lines and not the PDECs, and therefore at
least partially explain the differences seen between these two cell contexts. These findings could support an additional therapeutic option in which both the IGF1R and the RON receptor are inhibited.

Collectively, these studies support the importance of both the RAF/MEK/ERK and PI3K/AKT signaling cascades in KRAS-mediated tumor formation, and provide new evidence for the requirement of signaling through the IGF1R during KRAS-mediated pancreatic tumor initiation. These findings grant new understanding into the signaling downstream of activated KRAS in pancreatic tumor formation, and therefore provide new insight into effective therapeutic targets and options for combination drug therapies. These studies also indicate a requirement for signaling through receptor tyrosine kinases even in the context of activated KRAS and activated BRAF, uncovering a novel role for signaling through RTKs in KRAS mediated tumorigenesis and identifying RTK inhibitors as a viable option for pancreatic tumor therapeutics. In addition, these studies underscore the need to ensure that the system in which you are studying signaling downstream of Kras is as relevant as possible, as we demonstrate different signaling requirements for survival downstream of activated Kras in PDECs and transformed cells. This research indicates differences exist in the signaling cascades necessary for tumor formation versus those needed for tumor maintenance and further understanding of these differences in signaling requirements may prove key to effective tumor treatment when utilizing inhibitors targeting pathways downstream of activated KRAS.
APPENDIX A:

Investigation into the role of miRNAs in KRAS induced pancreatic tumor formation
Figure Contribution

Victoria Appleman conducted the work described in Appendix A and shown in Figure A.1

Brian Quattrochi is conducting all continuing and future experiments for this project.
**Introduction**

miRNAs are short, non-coding, single stranded segments of RNA that negatively regulate gene expression. miRNAs are initially transcribed in the nucleus as long precursors referred to as a pri-miRNAs. Following their transcription, pri-miRNAs are processed by Drosha and DGCR8 into pre-miRNAs, and are exported from the nucleus by Exportin 5. Following this, pre-miRNAs are processed by Dicer, which cleaves the hairpin structure and unwinds the miRNA duplex. The mature miRNA strand is then incorporated into the RISC complex, which binds to mRNA based upon sequence homology and results in either degradation of the mRNA or inhibition of mRNA translation (Garzon, Fabbri et al. 2006).

Many different human tumor samples, including those from pancreatic cancer, have abnormal expression of miRNAs when compared to normal tissue (Lee, Gusev et al. 2007). Based upon this, as well as their role in the regulation of gene expression, miRNAs have been implicated as having a role in tumorigenesis, and in fact, studies have shown that miRNAs can function as either tumor suppressors or oncogenes depending upon their target genes. Some of the most notable examples of miRNA involvement in cancer include the miR 17-92 cluster, which has been shown to accelerate tumor development in a B-cell lymphoma model, and the let-7 family of miRNAs, which have been found to regulate RAS proteins (He, Thomson et al. 2005; Johnson, Grosshans et al. 2005). In addition, a key study demonstrating the regulation of the miR 17-92 cluster by c-Myc showed that miRNA expression could be regulated by oncogenes, underscoring their role in cancer (O'Donnell, Wentzel et al. 2005).
While many prior studies have looked at the expression of miRNAs in human pancreatic tumors and pancreatic tumor cell lines, none to date have assessed the role of miRNAs following KRAS activation in the early stages of pancreatic tumor formation. Many of the studies profiling miRNA expression in pancreatic tumors have resulted in conflicting reports of expression changes in miRNA levels between normal tissue and tumor tissue (Bloomston, Frankel et al. 2007; Lee, Gusev et al. 2007; Szafranska, Davison et al. 2007). These differences could be due at least in part to the fact that the tumor samples being assessed are quite heterogeneous, and could include samples of stroma, acini, and inflammatory cells, in addition to ductal cells (Kent, Mullendore et al. 2009). Studies have also been conducted to determine the differences in miRNA expression within pancreatic tumor cell lines (Kent, Mullendore et al. 2009). However, these cell lines are often cultured for long periods of time, and may acquire significant genetic changes during this time that were not present within the tumor sample, making them a non-ideal comparison of human tumors. In addition, despite the emerging role of miRNAs in cancer and the findings of abnormal miRNA expression in pancreatic cancer, few studies have sought to address the cause or the effect of abnormal expression of miRNAs in pancreatic tumors. Therefore, I attempted to determine if activated KRAS was involved in the regulation of miRNAs during pancreatic tumor initiation through an investigation into the impact of activated KRAS on miRNA expression in PDECs, the putative cell of origin for PDAC (Hruban, Goggins et al. 2000). By demonstrating some of the earliest changes in miRNA expression following the expression of activated KRAS in PDECs, these studies can provide beneficial insight into those miRNAs that are critical
to pancreatic tumorigenesis, and potentially elucidate new targets for pancreatic tumor treatments.
Results

To investigate the role of miRNAs in KRAS mediated pancreatic tumorigenesis, PDECs null for Ink4a/Arf were isolated and infected with RCAS-GFP and RCAS-KRAS\textsuperscript{G12D}, and the differential expression of miRNAs between KRAS\textsuperscript{G12D} expressing and control cells was assessed via a microarray conducted by LC Sciences. Results of this microarray identified 49 miRNAs that had differential expression between the control cells and those expressing activated KRAS and a p value <0.01. 30 of these miRNAs were found to be overexpressed in KRAS\textsuperscript{G12D} expressing PDECs relative to GFP expressing PDECs, while the remaining 19 showed decreased expression. Of these, the differential expression of 14 miRNAs was subsequently validated via qRT-PCR, as shown in Figure A.1. Notably, several of the miRNAs found to be up regulated in KRAS\textsuperscript{G12D} expressing PDECs by our study have been identified as increased in human pancreatic tumors (Lee, Gusev et al. 2007).
**Discussion and Future Directions**

This study identified 49 miRNAs with differential expression between KRAS$^{G12D}$ and GFP expressing Ink4a/Arf null PDECs, thereby identifying miRNAs that are either potentially regulated by activated KRAS or are otherwise differentially expressed in the early stages of KRAS-mediated pancreatic tumorigenesis. In support of the role of these miRNAs in PDAC, several miRNAs identified have already been shown to have a role in the progression of other cancers, including miRNAs from the miR 17-92 cluster as well as miR 29b and miR 148b. In addition, several of these miRNAs have been identified as differentially expressed between human pancreatic tumors and normal pancreas, further implying a role for these miRNAs in pancreatic tumor formation and progression (Lee, Gusev et al. 2007). Having demonstrated differential regulation of miRNAs following the expression of activated KRAS, it is important to fully investigate whether these miRNAs are in fact regulated by activated KRAS and if so, how KRAS regulates miRNA expression. In addition, it is important to determine whether these miRNAs, either independently or collectively, are important to the formation or progression of pancreatic tumors.

To address the former of those questions, further studies should be conducted to examine the relationship between expression of activated KRAS in PDECs and changes in miRNA expression. A potential way in which to examine this would be to isolate PDECs from mice that express KRAS$^{G12D}$ under the expression of a tetracycline inducible promoter. Such a model would enable more precise control of when activated KRAS is being expressed in the cells, and would allow for the analysis of miRNA
expression changes at multiple time points after the expression of KRAS$^{G12D}$ to
determine how quickly miRNA expression changes, as well as if the expression of
different miRNAs change at different time points. In addition, such a system would
allow for the loss of KRAS$^{G12D}$ expression in PDECs, thus allowing for the assessment of
whether changes in miRNA expression are permanent following the expression of
KRAS$^{G12D}$ or if they depend upon the continued expression of activated KRAS.

Once it is confirmed that KRAS$^{G12D}$ is in fact regulating the expression of
miRNAs, further studies should be done to determine how this regulation occurs. One
possible way to address these questions would be to infect PDECs with RCAS constructs
that express KRAS$^{G12D}$ with an additional single site mutation that results in the
preferential activation of a single KRAS stimulated pathway (S35T stimulates RAF-MEK
ERK, C40Y stimulates PI3K-AKT, G37D stimulates RAL/GDS, and N38D stimulates
PLC-ε). miRNA expression levels could then be compared between these PDECs
expressing the additional site mutations, those expressing KRAS$^{G12D}$, and those
expressing GFP to determine if the stimulation of any single pathway results in similar
changes in miRNA expression to the expression of KRAS$^{G12D}$. If necessary, infection
with multiple RCAS constructs could be used to determine if a combination of pathways
downstream of activated KRAS are needed to alter the expression of miRNAs. In
addition, inhibitors to MEK or PI3K could be used to determine if inhibiting either of
these signaling cascades results in an inhibition of miRNA expression changes following
the expression of KRAS$^{G12D}$, further pinpointing the role of these signaling cascades in
KRAS regulated miRNA expression.
To address whether miRNAs are collectively required for the formation or progression of pancreatic tumors, a mouse model expressing pancreas specific Kras$^{G12D}$, in conjunction with loss of Dicer could be used. Previous studies have demonstrated that the pancreas specific expression of activated KRAS is sufficient for the formation of PDAC in mice (Aguirre, Bardeesy et al. 2003; Guerra, Mijimolle et al. 2003; Hingorani, Petricoin et al. 2003; Hingorani, Wang et al. 2005). If this KRAS induced tumor formation requires miRNAs, then the loss of Dicer in this mouse model would prevent the formation of PDAC and I would expect increased survival and decreased tumor formation in the Dicer null animals relative to their Dicer wild type littermate controls.

In addition, as the orthotopic transplant of PDECs null for Ink4a/Arf and expressing KRAS$^{G12D}$ has been shown to be sufficient for tumor formation in nude mice, a similar experiment could be done in which the processing of miRNAs is inhibited via knockdown of Drosha, DGCR8, or Dicer in PDECs lacking Ink4a/Arf and expressing KRAS$^{G12D}$. The cells could then be assessed for the impact of miRNA loss on KRAS$^{G12D}$ induced phenotypes, including increased proliferation, increased survival when challenged with apoptotic stimuli, and the ability to form tumors following orthotopic transplant. As in the above mouse model, if miRNAs are required for KRAS induced tumor formation, I would expect to see a decrease in tumor formation following the knockdown of miRNAs processing proteins.

To address whether individual miRNAs are required for pancreatic tumorigenesis, the same strategies as above could be applied, but instead of inhibiting global miRNA processing, mouse models could be generated with pancreas specific expression of
KRAS$^{G12D}$ and ablation of an individual miRNA. Likewise, PDECs could be generated that are null for Ink4a/Arf and express KRAS$^{G12D}$ in which the expression of a specific miRNA could then be inhibited or reduced. As above, these cells could then be assessed for impact on the KRAS$^{G12D}$ mediated phenotypes of increased proliferation, increased survival, and tumor formation following orthotopic transplant. In both of these experiments, I would expect that decreased expression of any miRNAs that are necessary for tumor formation would cause decreases tumor formation and increased survival relative to controls.

As mouse models and primary cells can be difficult and time consuming systems in which to address these questions, it is also possible to begin these investigations in tumor cell lines that express activated KRAS. Within these cells, it is possible to inhibit or overexpress individual miRNAs, or combinations of several miRNAs, and assess the impact on cell proliferation, survival, migration, and anchorage independent growth. In this manner, it is possible to first determine in the tumor cell lines which miRNAs, or combinations of miRNAs, may be most important to KRAS induced tumor phenotypes, and to then continue to study those particular miRNAs in primary cells and mouse models.

Collectively, these studies would better address the role of activated KRAS in the regulation of miRNA expression as well as which miRNAs are most important to KRAS induced pancreatic tumor formation and progression, and thus provide more insight into which miRNAs might serve as good therapeutic targets for the treatment of pancreatic tumors.
Figure A.1

KRAS$^{G12D}$ expression in Ink4a/Arf null PDECs results in differential miRNA expression levels when compared to control cells.

The expression of each individual miRNA as assessed by microarray and qRT-PCR showing differential miRNA expression between KRAS$^{G12D}$ expressing Ink4a/Arf null PDECs relative and GFP expressing control cells. miRNA expression levels are normalized such that the expression level of each miRNA in GFP expressing cells is 1.
Methods

Isolation, culture, and infection of mouse PDECs.

Isolation, culture, and infection of mouse PDECs were performed as previously described (Lewis, Chinnasamy et al. 2001; Schreiber, Deramaudt et al. 2004; Morton, Mongeau et al. 2007). Details are provided in the protocols below.

Isolation of PDECs: The pancreas of a K19-TVA expressing mouse was harvested into ~30ml G solution {HBSS (Gibco, Invitrogen, Carlsbad CA) plus 0.9g/L D-Glucose (Calbiochem, San Diego, CA) plus 1X Penicillin/Streptomycin (Gibco, Invitrogen, Carlsbad CA)} in a falcon tube on ice. In a sterile tissue culture hood, aspirate G solution and transfer the pancreas into a 50ml beaker. Add ~5ml G solution. Cut up the pancreas with dissection scissors about 100 times (resulting in pieces ~1mm in size). Resuspend the cut tissue in ~50ml G solution and transfer to a fresh 50ml falcon tube. Allow tissue to settle and aspirate or decant G solution and any floating fatty tissue. Repeat the wash with G solution until all floating tissue has been removed. Resuspend tissue in 15ml of filter-sterilized collagenase V solution {1mg/ml of Collagenase V (Sigma, St Louis MO) in DMEM (Invitrogen, Carlsbad CA) plus 10% FCS (Atlanta Biologicals, Atlanta GA)} and transfer to 100ml bottle with stir bar. Digest tissue, with constant stirring, at 37°C for 20 minutes. Use a beaker set on a stirrer hot plate if you don’t have a submersible stirrer. Tissue should be free from large chunks at the end of
digestion. If necessary, tissue can be digested up to 45 minutes to remove large chunks. Add 20ml G solution to stop reaction. Transfer the solution onto a 100µm nylon cell strainer over a waste container. Rinse the bottle with G solution if necessary to collect all remaining tissue. Invert the strainer over a fresh falcon tube and recover the undigested tissue by pipetting G solution onto the reverse side of the mesh and collecting washed off tissue/G solution in the falcon tube. If needed, top off the tissue solution to 50ml with G solution. Allow tissue to settle and pour off G solution to remove floating cells. (These are most likely acinar cells). Repeat. Add 50ml of G solution again and resuspend cells by inverting. Centrifuge cells at 1000rpm at 4°C, but stop the spin as soon as the centrifuge reaches speed and allow the centrifuge to decelerate using the minimum deceleration speed. Carefully decant or aspirate the supernatant. Repeat this wash step 3 times to remove all traces of collagenase. Add 2ml trypsin-EDTA (Invitrogen, Carlsbad CA) and incubate at room temperature for 5 minutes. Add 40mls DMEM plus 10% FCS and invert to resuspend the cells. Centrifuge cells at 1000rpm 4°C and stop as soon as speed is reached (as in step 13). Remove the supernatant by decanting or by aspiration. Repeat this wash step 3 times. Resuspend the cells in 5ml PDEC medium and centrifuge at 1000rpm for 5 minutes at 4°C. (Reset the brake to the usual setting). Resuspend the tissue pellet in 12ml of PDEC medium (see description of media below) and plate 2ml onto each well of a 6-well plate. Culture in humidified incubator at 37°C, 5%CO₂. Note: Up to two pancreata can be isolated into one falcon tube at the start of the protocol. If this is done, the only change needed is to plate the cells onto two 6-well plates in step 20.
Two days after isolation aspirate off medium and floating tissue. Wash gently with PBS. Replace PDEC medium and incubate until confluent (at least 5-7 days).

**Culturing of PDECs:** PDECs were cultured on collagen coated plates at 37 degrees Celsius with 5% CO₂. Cells were cultured in DMEM/F12 Medium (Gibco, Invitrogen, Carlsbad, CA) plus 5 mg/ml D-Glucose (Calbiochem, San Diego, CA), 0.1mg/mL Soybean Trypsin Inhibitor (Sigma, ), 5ml/L ITS+ (BD Biosciences, Billerica MA), 25ug/ml Bovine Pituitary Extract (BD Biosciences, Billerica MA), 20ng/ml EGF (BD Biosciences, Billerica MA), 1uM dexamethasone (Sigma, St Louis MO), 1.22 mg/ml nicotinamide (Sigma, St Louis, MO), 5% Nu Serum (BD Biosciences, Billerica MA), and 1X Penicillin/Streptomycin (Gibco, Invitrogen, Carlsbad CA). To passage PDECs when confluent, media was removed and the collagen layer was pipetted into a filter-sterilized 1mg/ml collagenase V solution (Sigma, St Louis MO) and then incubated for 15-20 minutes at 37°C. Cells were then pelleted by centrifugation at 1000rpm at 4°C for 5 minutes, and the supernatant removed by decanting or aspiration. 2ml trypsin-EDTA (Invitrogen, Carlsbad CA) were added to the cells, and the cells were incubated at room temperature for 5 minutes. Next, 40mls DMEM plus 10% FCS were added to the cells, and the cells were centrifuged at 1000rpm for 5 minutes at 4°C. Cells were washed twice more with DMEM plus 10% FCS and then resuspended into PDEC media and plated on collagen gel coated dishes.

**Infection of PDECs with RCAS Viruses:** Prior to infection, RCAS constructs were transfected into DF1 Chicken Fibroblasts using the Superfect Transfection Reagents
(Qiagen, Valencia CA). Briefly, 5µg of plasmid was mixed with DMEM (Gibco, Invitrogen, Carlsbad CA) to a total volume of 150µL. To this mixture, 25µL of the Superfect reagent was added and the mixture was incubated at room temperature for 5 minutes. Following the incubation, 2mls of fresh DMEM plus 10% FCS plus 1% Penicillin/Streptomycin was added to the mixture, and the mixture was transferred onto DF1 cells. The DF1 cells plus transfection mixture were incubated for 2-3 hours at 37°C, following which fresh DMEM plus 10% FCS plus 1% Penicillin/Streptomycin was added and the DF1 cells were cultured as usual. At least one week after transfection of the RCAS constructs, the virus within the culture media from 3 confluent 10cm² dishes of DF1 cells was concentrated by ultracentrifugation at 27,000 rpms for 90minutes at 4°C. Following centrifugation, concentrated virus was then pipetted onto a single 10cm² dish of TVA expressing PDECs. For each virus, the infection protocol was repeated so that the virus was added to PDECs a total of four times within 48 hours.

**RCAS Constructs**

The retroviral constructs RCAS-GFP and RCAS-KrasG12D-IRES-GFP have been previously described (Morton, Mongeau et al. 2007).

**Microarray.**

RNA was extracted from PDECs using Trizol (Invitrogen, Carlsbad CA) and purified with an RNeasy Mini Kit (Qiagen, Valencia CA). Microarray analysis of the total RNA was performed by LC Sciences (Houston, TX).
**Quantitative RT-PCR.**

RNA was extracted from PDECs using Trizol (Invitrogen, Carlsbad CA), and purified with an RNeasy Mini Kit (Qiagen, Valencia CA). miRNA expression analysis was conducted using TaqMan miRNA Assays and standard protocol (Applied Biosystems, Carlsbad CA). PCR amplification was conducted using an ABI 7300 Real Time PCR System.
APPENDIX B:

KRAS$^{G12D}$ induced proliferation and survival in PDECs requires the $Gli$ transcription factors
Figure Contribution

The experiments for Figure B.1 were conducted by Victoria Appleman as part of a collaboration with the laboratory of Junhao Mao. Wilfredo de Jesus-Monge conducted the experiment shown in Figure B.2. These data appear in the following manuscript as Supplementary Figure One, and Figure Three, respectively:

**Gli transcriptional activity is essential for Kras-induced pancreatic tumorigenesis and regulates IKBKE/NF-kB activity in the tumor epithelium**

Introduction

Hedgehog (Hh) signaling is not normally active in the adult pancreas, but is aberrantly activated in pancreatic tumors, indicating a role for Hh signaling in pancreatic tumorigenesis (Berman, Karhadkar et al. 2003; Thayer, di Magliano et al. 2003). GLI1, GLI2, and GLI3 are effectors of the Hh signaling pathway, and as such have been implicated as having a role in pancreatic cancer. Recent evidence has demonstrated that the expression of the Gli transcription factors is maintained in pancreatic tumor epithelium even when upstream Hh signaling is disrupted via Smo deletion, indicating that the Gli transcription factors may have a Hh independent role in pancreatic tumor formation and progression (Nolan-Stevaux, Lau et al. 2009). Analysis of human pancreatic tumors has also shown mutations in GLI1 and GLI3, further supporting an Hh independent role for Gli transcription in PDAC (Jones, Zhang et al. 2008). However, thus far the role of Gli transcription in KRAS mediated pancreatic tumorigenesis has remained unclear. These studies therefore sought to determine if Gli transcription was necessary for KRAS-induced pancreatic tumors, and how the Gli transcription factors might be contributing to pancreatic tumor initiation and progression through the use of Gli3T, a dominant negative construct that inhibits expression of Gli1 and Gli2.
Results

To investigate the role of Gli transcription factors in KRAS<sup>G12D</sup> induced pancreatic tumors, I assessed the impact of the expression of GLI3T on KRAS<sup>G12D</sup> induced proliferation and survival in PDECs. Ink4a/Arf, Trp53 null PDECs expressing an LSL-GLI3T allele were isolated and infected with either RCAS-GFP or RCAS-KRAS<sup>G12D</sup>. Both GFP and KRAS<sup>G12D</sup> expressing PDECs were then subsequently infected with either RCAS-GFP or RCAS-CRE, which generated four populations of PDECS: GFP, GLI3T, KRAS<sup>G12D</sup>, and KRAS<sup>G12D</sup> GLI3T expressing cells. The expression of GLI3T in combination with KRAS<sup>G12D</sup> in PDECs resulted in loss of survival when treated with cycloheximide, with the survival of these cells nearing that of control PDECs (Figure B.1). The expression of GLI3T in combination with KRAS<sup>G12D</sup> also resulted in decreased proliferation to the same level as GFP expressing PDECs (Figure B.2). Collectively, these findings indicate that the increased proliferation and survival of KRAS<sup>G12D</sup> expressing PDECs relative to controls depends on the Gli transcription factors.
Discussion and Future Directions

These results demonstrate that the proliferation and survival increases seen in KRAS$^{G12D}$ expressing PDECs depend upon the expression of the Gli transcription factors, and indicate a role for the Gli transcription factors in the formation of pancreatic tumors. Indeed, my collaborators went on to demonstrate that a mouse model with pancreas specific expression of GLI3T and activated KRAS resulted in decreased PanIN and tumor formation and increased tumor free survival when compared with pancreas specific expression of activated KRAS alone (Fig B.1). These findings further demonstrate the requirement of Gli transcription factors for activated KRAS induced formation and progression of pancreatic tumors.

An important question resulting from these findings is which targets of Gli transcription are needed for KRAS$^{G12D}$ induced tumor formation, and to address this, my collaborators have demonstrated that increased expression of Gli1 results in increased expression of Ikbke, a member of the NF-κB signaling cascade. They further demonstrate that shRNA mediated knockdown of Ikbke results in decreased colony formation and proliferation in vitro, and decreased tumor formation in vivo, demonstrating that the requirement for the Gli transcription factors in pancreatic tumor formation is at least partially due to a requirement for increased expression of Ikbke.

However, these studies have yet to identify if the expression of KRAS$^{G12D}$ is sufficient for the induction of Gli1 and Gli2 expression, and if so, the mechanism of that induction. To investigate this further, gene expression analysis could be done to compare the expression levels of the Gli transcription factors in PDECs expressing GFP compared
with those expressing KRAS^{G12D}. In addition, a tetracycline inducible construct of
KRAS^{G12D} could be used so that any changes in the expression levels of the *Gli*
transcription factors could be better correlated with expression of activated KRAS, and
the effect of removing the expression of activated KRAS on the levels of the *Gli*
transcription factors could be assessed. If these studies indicate that the expression of
KRAS^{G12D} results in increased expression of the *Gli* transcription factors, indicating that
activated KRAS stimulates the expression of *Gli*, further studies could be conducted to
determine which of the KRAS stimulated signaling pathways are responsible for this
induction of *Gli* and following this, continue to pinpoint a mechanism by which activated
KRAS induces *Gli* expression. Together, these studies would provide further insight into
the non-canonical regulation of *Gli* expression by activated KRAS.
Figure B.1

Inhibition of GLI results in decreased proliferation and survival of KRAS$^{G12D}$ expressing Ink4a/Arf null PDECs.

(A) Viability (as measured by trypan blue exclusion) of Ink4a/Arf, Trp53 null PDECs showing decreased survival in KRAS$^{G12D}$ plus GLI3T expressing PDECs relative to KRAS$^{G12D}$ expressing cells following treatment with 100 µM cycloheximide. Values are normalized such that the viability of untreated cells is 1.

(B) Cell numbers of Ink4a/Arf, Trp53 null PDECs showing decreased proliferation at 15 days of KRAS$^{G12D}$ plus GLI3T expressing PDECs relative to KRAS$^{G12D}$ expressing cells.
Figure B.2

Inhibition of the Gli transcription factors increases tumor free survival in mice expressing pancreas specific LSL-KRASG12D.

Kaplan-Meier plot of tumor free survival of Ptf1a-Cre;LSL-Kras$^{G12D};Trp53$flox/wt mice, with or without R26-Gli3T expression. Control mice were LSL-KrasG12D negative littermates.

p < 0.001 for comparison between R26-Gli3T-positive and –negative animals.
Methods

Transgenic Mice and Animal Care

As described in Rajurkar et al, offspring from the cross of LSL-KrasG12D;R26-Gli3T to Ptf1a-cre;Trp53flox/flox mice were followed longitudinally for tumor development for 270 days. (Rajurkar et al, In Press). All mice were housed in a specific pathogen-free facility with abundant food and water under guidelines approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Isolation, culture, and infection of mouse PDECs.

Isolation, culture, and infection of mouse PDECs were performed as previously described (Lewis, Chinnasamy et al. 2001; Schreiber, Deramaudt et al. 2004; Morton, Mongeau et al. 2007). Details are provided in the protocols below.

Isolation of PDECs: The pancreas of a K19-TVA expressing mouse was harvested into ~30ml G solution {HBSS (Gibco, Invitrogen, Carlsbad CA) plus 0.9g/L D-Glucose (Calbiochem, San Diego, CA) plus 1X Penicillin/Streptomycin (Gibco, Invitrogen, Carlsbad CA)} in a falcon tube on ice. In a sterile tissue culture hood, aspirate G solution and transfer the pancreas into a 50ml beaker. Add ~5ml G solution. Cut up the pancreas with dissection scissors about 100 times (resulting in pieces ~1mm in size). Resuspend the cut tissue in ~50ml G solution and transfer to a fresh 50ml falcon
tube. Allow tissue to settle and aspirate or decant G solution and any floating fatty tissue. Repeat the wash with G solution until all floating tissue has been removed. Resuspend tissue in 15ml of filter-sterilized collagenase V solution {1mg/ml of Collagenase V (Sigma, St Louis MO) in DMEM (Invitrogen, Carlsbad CA) plus 10% FCS (Atlanta Biologicals, Atlanta GA)} and transfer to 100ml bottle with stir bar. Digest tissue, with constant stirring, at 37°C for 20 minutes. Use a beaker set on a stirrer hot plate if you don’t have a submersible stirrer. Tissue should be free from large chunks at the end of digestion. If necessary, tissue can be digested up to 45 minutes to remove large chunks. Add 20ml G solution to stop reaction. Transfer the solution onto a 100µm nylon cell strainer over a waste container. Rinse the bottle with G solution if necessary to collect all remaining tissue. Invert the strainer over a fresh falcon tube and recover the undigested tissue by pipetting G solution onto the reverse side of the mesh and collecting washed off tissue/G solution in the falcon tube. If needed, top off the tissue solution to 50ml with G solution. Allow tissue to settle and pour off G solution to remove floating cells. (These are most likely acinar cells). Repeat. Add 50ml of G solution again and resuspend cells by inverting. Centrifuge cells at 1000rpm at 4°C, but stop the spin as soon as the centrifuge reaches speed and allow the centrifuge to decelerate using the minimum deceleration speed. Carefully decant or aspirate the supernatant. Repeat this wash step 3 times to remove all traces of collagenase. Add 2ml trypsin-EDTA (Invitrogen, Carlsbad CA) and incubate at room temperature for 5 minutes. Add 40mls DMEM plus 10% FCS and invert to resuspend the cells. Centrifuge cells at 1000rpm 4°C and stop as soon as speed is reached (as in step 13). Remove the supernatant by decanting or by aspiration.
Repeat this wash step 3 times. Resuspend the cells in 5ml PDEC medium and centrifuge at 1000rpm for 5 minutes at 4°C. (Reset the brake to the usual setting). Resuspend the tissue pellet in 12ml of PDEC medium (see description of media below) and plate 2ml onto each well of a 6-well plate. Culture in humidified incubator at 37°C, 5%CO₂. Note: Up to two pancreata can be isolated into one falcon tube at the start of the protocol. If this is done, the only change needed is to plate the cells onto two 6-well plates in step 20. Two days after isolation aspirate off medium and floating tissue. Wash gently with PBS. Replace PDEC medium and incubate until confluent (at least 5-7 days).

**Culturing of PDECs:** PDECs were cultured on collagen coated plates at 37 degrees Celsius with 5% CO₂. Cells were cultured in DMEM/F12 Medium (Gibco, Invitrogen, Carlsbad, CA) plus 5 mg/ml D-Glucose (Calbiochem, San Diego, CA), 0.1mg/mL Soybean Trypsin Inhibitor (Sigma, ), 5ml/L ITS+ (BD Biosciences, Billerica MA), 25ug/ml Bovine Pituitary Extract (BD Biosciences, Billerica MA), 20ng/ml EGF (BD Biosciences, Billerica MA), 5nM 3, 3, 5 tri-iodo-L-thyronine (Sigma, St Louis MO), 1uM dexamethasone (Sigma, St Louis MO), 1.22 mg/ml nicotinamide (Sigma, St Louis, MO), 5% Nu Serum (BD Biosciences, Billerica MA), and 1X Penicillin/Streptomycin (Gibco, Invitrogen, Carlsbad CA). To passage PDECs when confluent, media was removed and the collagen layer was pipetted into a filter-sterilized 1mg/ml collagenase V solution (Sigma, St Louis MO) and then incubated for 15-20 minutes at 37°C. Cells were then pelleted by centrifugation at 1000rpm at 4°C for 5 minutes, and the supernatant removed by decanting or aspiration. 2ml trypsin-EDTA (Invitrogen, Carlsbad CA) were
added to the cells, and the cells were incubated at room temperature for 5 minutes. Next, 40mls DMEM plus 10% FCS were added to the cells, and the cells were centrifuged at 1000rpm for 5 minutes at 4°C. Cells were washed twice more with DMEM plus 10% FCS and then resuspended into PDEC media and plated on collagen gel coated dishes.

**Infection of PDECs with RCAS Viruses:** Prior to infection, RCAS constructs were transfected into DF1 Chicken Fibroblasts using the Superfect Transfection Reagents (Qiagen, Valencia CA). Briefly, 5µg of plasmid was mixed with DMEM (Gibco, Invitrogen, Carlsbad CA) to a total volume of 150µL. To this mixture, 25µL of the Superfect reagent was added and the mixture was incubated at room temperature for 5 minutes. Following the incubation, 2mls of fresh DMEM plus 10% FCS plus 1% Penicillin/Streptomycin was added to the mixture, and the mixture was transferred onto DF1 cells. The DF1 cells plus transfection mixture were incubated for 2-3 hours at 37°C, following which fresh DMEM plus 10% FCS plus 1% Penicillin/Streptomycin was added and the DF1 cells were cultured as usual. At least one week after transfection of the RCAS constructs, the virus within the culture media from 3 confluent 10cm² dishes of DF1 cells was concentrated by ultracentrifugation at 27,000 rpms for 90minutes at 4°C. Following centrifugation, concentrated virus was then pipetted onto a single 10cm² dish of TVA expressing PDECs. For each virus, the infection protocol was repeated so that the virus was added to PDECs a total of four times within 48 hours.
**RCAS Constructs**

The retroviral constructs RCAS-GFP and RCAS-KrasG12D-IRES-GFP have been previously described (Morton, Mongeau et al. 2007). RCAS-CRE was a gift from Eric Holland (Memorial Sloan-Kettering Cancer Center, New York, NY). Isolated PDECs were infected with either RCAS-KrasG12D-IRES-GFP or RCAS-GFP, and subsequently infected with RCAS-CRE to induce Gli3T expression, or with RCAS-GFP as a control.

**PDEC Proliferation and Survival Assays**

All proliferation and survival assays were conducted as previously described (Morton, Mongeau et al. 2007). For proliferation assays, $1 \times 10^5$ PDECs were plated per well in a 6 well plate on day zero. Fresh media was added to the cells every 5 days, and total cell numbers were counted on days 5, 10, and 15. For survival assays, $1 \times 10^6$ PDECs were plated per well in a 6 well plate and incubated at 37°C overnight. The following day, the media was removed and fresh media was added. For cells treated with cycloheximide, the fresh media contained 100µM cycloheximide. 24 hours after treatment with cycloheximide, live and dead cells were counted by trypan blue exclusion and percent survival was calculated.
APPENDIX C:

Investigation into the genetic changes required for KRAS induced tumor formation
Figure Contribution

For the experiments described in Appendix C, Victoria Appleman generated the GFP, KRAS$^{G12D}$, and SHH expressing PDECs and isolated the RNA for the microarray studies. David Driscoll confirmed the data in Table C.1 and is conducting all ongoing and future studies for this project.
**Introduction**

Aberrant expression of *SHH* has been demonstrated in both PanIN lesions as well as in PDAC and is thought to be involved in the early stages of pancreatic tumor formation (Berman, Karhadkar et al. 2003; Thayer, di Magliano et al. 2003). However, prior work in our lab demonstrated that while expression of both SHH and KRAS\(^{G12D}\) in PDECs resulted in increased proliferation and survival, as well as activation of the MEK-ERK and PI3K-AKT signaling pathways, only the expression of KRAS\(^{G12D}\) in *Ink4a/Arf* null PDECs was sufficient for gross tumor formation following orthotopic transplant (Morton, Mongeau et al. 2007). Based upon these data, we hypothesized that although KRAS\(^{G12D}\) and SHH can stimulate the same signaling cascades, and both are involved in pancreatic tumorigenesis, activated KRAS is enacting changes in the cell that SHH is not, and these changes are necessary for pancreatic tumor initiation. This study therefore sought to identify which changes in gene expression might be necessary for KRAS induced tumor formation by assessing gene expression differences following the expression of activated KRAS versus the expression of SHH in PDECs.
Results

To investigate the gene expression changes following the expression of activated KRAS or SHH, PDECs null for Ink4a/Arf and Trp53 were isolated and infected with RCAS-GFP, RCAS-KRAS$^{G12D}$, and RCAS-SHH. Gene expression was then analyzed via microarray. Results of this microarray identified 693 genes that had greater than 1.5 fold increased expression in PDECs expressing KRAS$^{G12D}$ relative to control cells, but not in PDECs expressing SHH relative to control cells (p value < 0.01). Of these, the increased expression of 27 genes specifically in KRAS$^{G12D}$ and not SHH expressing PDECs relative to control has been confirmed via qRT-PCR, and is shown in Table C.1.
**Discussion and Future Directions**

The results of this study have identified many genes with increased expression following the expression of KRAS\(^{G12D}\), but not following expression of SHH, in PDECs. The differential expression of 27 of these genes has been verified by qRT-PCR, and a subset of those genes has been selected for future study. The next step to pursue those genes will be to determine which ones are potentially important for KRAS induced tumorigenesis by first looking at which genes are important for KRAS\(^{G12D}\) induced phenotypes in pancreatic tumor cell lines. This can be achieved through shRNA-mediated knockdown of an individual gene in KRAS\(^{G12D}\) expressing tumor cell lines, and an assessment of the impact of knockdown on proliferation, survival, migration, and anchorage independent growth of those cells. If a gene is important for KRAS mediated tumorigenesis, knockdown of that gene will likely result in decreases in some or all of those phenotypes.

Following this, those genes that are found to be needed for KRAS induced phenotypes in the tumor cell lines could be subsequently knocked down in KRAS\(^{G12D}\) expressing PDECs to assess impact in those cells. Again, any genes needed for KRAS mediated tumorigenesis will likely impact proliferation, survival, and tumor formation following orthotopic transplant of the PDECs. Ultimately, any genes found to impact KRAS\(^{G12D}\) induced phenotypes in the PDECs could be studied using a mouse model with pancreas specific expression of activated KRAS and ablation of the selected gene. In this model, if the gene is needed for KRAS induced tumor formation, those animals with
ablation of that gene will have decreased tumor formation and increased survival over their littermates that are not deficient in that gene’s expression.

Once a gene, or set of genes, has been confirmed in this manner to be important for KRAS mediated pancreatic tumor formation, further studies can examine how the expression of activated KRAS results in increased expression of those genes, and potentially which of the signaling pathways downstream of KRAS are required for the increased gene expression. Ultimately, these studies will identify novel genes that are critical to KRAS-mediated pancreatic tumor formation, as well as potentially demonstrate how activated KRAS is regulating the expression of those genes, thereby providing much needed further insight into the role of activated KRAS in the formation of pancreatic tumors and identifying potential new therapeutic targets for the treatment of pancreatic cancer.
Table C.1

Genes with greater than 1.5 fold increased expression in KRAS$^{G12D}$ expressing Ink4a/Arf, Trp53 null PDECs relative to GFP expressing control cells, but not in SHH expressing Ink4a/Arf, Trp53 null PDECs relative to GFP expressing control cells, as confirmed by qRT-PCR.

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Methods

**Isolation, culture, and infection of mouse PDECs.**

Isolation, culture, and infection of mouse PDECs were performed as previously described (Lewis, Chinnasamy et al. 2001; Schreiber, Deramaudt et al. 2004; Morton, Mongeau et al. 2007). Details are provided in the protocols below.

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Superfect reagent was added and the mixture was incubated at room temperature for 5 minutes. Following the incubation, 2mls of fresh DMEM plus 10% FCS plus 1% Penicillin/Streptomycin was added to the mixture, and the mixture was transferred onto DF1 cells. The DF1 cells plus transfection mixture were incubated for 2-3 hours at 37°C, following which fresh DMEM plus 10% FCS plus 1% Penicillin/Streptomycin was added and the DF1 cells were cultured as usual. At least one week after transfection of the RCAS constructs, the virus within the culture media from 3 confluent 10cm² dishes of DF1 cells was concentrated by ultracentrifugation at 27,000 rpms for 90 minutes at 4°C. Following centrifugation, concentrated virus was then pipetted onto a single 10cm² dish of TVA expressing PDECs. For each virus, the infection protocol was repeated so that the virus was added to PDECs a total of four times within 48 hours.

**RCAS Constructs**

The retroviral constructs RCAS-GFP and RCAS-KrasG12D-IRES-GFP have been previously described (Morton, Mongeau et al. 2007).

**Microarray.**

RNA was extracted from PDECs using Trizol (Invitrogen, Carlsbad CA) and purified with an RNeasy Mini Kit (Qiagen, Valencia CA). Microarray analysis of the total RNA was performed by MSKCC Genomics Core Laboratory (New York, NY).
**Quantitative RT-PCR.**

RNA was extracted from serum starved PDECs using Trizol (Invitrogen, Carlsbad CA), purified with an RNeasy Mini Kit (Qiagen, Valencia CA), and treated with Turbo DNase (Ambion, Austin TX). cDNA was then generated using the Superscript III First Strand Synthesis System (Invitrogen, Carlsbad CA). 50ng of cDNA was combined with SYBR Green Reaction Mix (Quanta Biosciences, Gaithersburg, MD), and 500nm of the appropriate primer pairs (IDT, Coralville, Iowa). PCR amplification was conducted using an ABI 7300 Real Time PCR system using Applied Biosystems standard conditions.


Lerner, E. C., T. T. Zhang, et al. (1997). "Inhibition of the prenylation of K-Ras, but not H- or N-Ras, is highly resistant to CAAX peptidomimetics and requires both a farnesyltransferase and a geranylgeranyltransferase I inhibitor in human tumor cell lines." Oncogene 15(11): 1283-1288.


cells with wild-type BRAF but not with the V599E mutant." Cancer Res 64(16): 5556-5559.


