

Investigation of the Role of Wild-type Ras Isoforms in KRAS driven Cancers

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor  
of Philosophy in the University Program in  
Genetics and Genomics in the Graduate School  
of Duke University

2015

ABSTRACT

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## Abstract

The RAS family is a group of small GTPases that can become constitutively activated by point mutations that are found in about 30% of all cancer patients. There are three well-characterized RAS family members: *HRAS*, *NRAS*, and *KRAS*, the latter of which is alternatively spliced at the C-terminus into *KRAS4A* and *KRAS4B*. The RAS proteins are all nearly identical at their N-termini and core effector binding domains, but have divergent C-terminal membrane-binding regions that impart different subcellular localization and subtle differences in signaling. Although the role of constitutively activated oncogenic RAS has been well established to play a role in cancer, recent work has suggested that wild-type RAS signaling may also be important in tumorigenesis. Wild-type RAS proteins have been shown to be activated in the presence of oncogenic *KRAS*. However, the consequences of this activation are context-dependent, as signaling through the wild-type RAS proteins has been shown to both suppress neoplastic growth and promote tumorigenesis under different circumstances.

I sought to investigate the role of the wild-type RAS proteins in two clinically – relevant models of cancer: pancreatic, the type of cancer most frequently associated with *KRAS* mutations, and lung cancer, the cancer in which *KRAS* mutations affect the highest number of patients. First, I tested whether a loss of wild type Hras altered tumorigenesis in a mouse model of pancreatic cancer driven by oncogenic Kras. *Hras* homozygous null mice (*Hras*<sup>-/-</sup>) exhibited more precancerous lesions of the pancreas as well as more off-target skin papillomas compared to their wild type counterparts,

indicating that Hras suppresses early Kras-driven pancreatic tumorigenesis. Loss of *Hras* also reduced the survival of mice engineered to develop aggressive pancreatic cancer by the additional disruption of one allele of the tumor suppressor p53 (*Trp53*<sup>R172H/+</sup>). However, this survival advantage was lost when both alleles of *Trp53* were mutated, suggesting that wild-type HRas inhibits tumorigenesis in a p53-dependant manner.

Next, I investigated the role that wild-type Hras and Nras play in a chemical carcinogen-induced model of lung cancer. In mice treated with urethane, a carcinogen that induces Kras-mutation positive lung lesions, *Hras*<sup>-/-</sup> mice once again developed more tumors than wild-type mice. Interestingly, however, this effect was not observed in mice lacking wild-type *Nras*. Mice lacking both *Hras* and *Nras* alleles developed approximately the same number of tumors as *Hras*<sup>-/-</sup> mice, thus the additional loss of *Nras* does not appear to enhance the tumor-promoting effects of loss of Hras. In summary, signaling through wild-type Hras, but not Nras, suppresses tumorigenesis in a carcinogen-induced model of lung cancer.

The tumor-suppressive effects of wild-type Ras signaling were traced to the earliest stages of pancreatic tumorigenesis, suggesting that wild-type Ras signaling may suppress tumorigenesis as early as the time of initiation. These findings suggest that differences in expression of the wild-type Ras isoforms could potentially play a role in an individual's predisposition for developing cancer upon oncogenic insult.

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# 1. Introduction

## 1.1 Overview

The RAS family of small GTPases act as molecular switches, alternating between an inactive, GDP-bound state, and an active-GTP bound state. Oncogenic mutations that render these proteins constitutively active are found in about 30% of all cancer cases, resulting in constitutive signaling through downstream pathways involved in cellular proliferation and survival. There are three genes in the RAS family: *HRAS*, *NRAS*, *KRAS*, which is alternatively spliced into two isoforms, *KRAS4A* and *KRAS4B*. The effects of constitutive activation of RAS proteins have been well studied in cancer, but the roles that the remaining wild-type proteins play in tumorigenesis are not yet well-understood. It has been shown that wild-type RAS proteins are activated in the presence of oncogenic *KRAS*, but the effects of this activation have seemed to have divergent consequences in different contexts and tissues. For this reason, I sought to determine the role that activation of wild-type RAS proteins played in mouse models of pancreatic and lung cancer, two diseases in which mutant *KRAS* has been identified as a driver oncogene. In this introduction, I will first discuss RAS signaling and how RAS mutations contribute to tumorigenesis. I will then describe the different RAS family members. I will also delineate the ways in which wild-type RAS proteins are activated in the presence of mutant RAS, and what was known regarding the consequences of this activation. Finally, I will discuss the significance of understanding the roles that both wild-type and oncogenic RAS proteins play in tumorigenesis, and the two cancers, pancreatic and lung, in which I chose to study these roles.

## **1.2 *Ras family signaling***

### **1.2.1 RAS proteins act as molecular switches**

The RAS family of proteins are a group of 21 kilodalton small GTPases that cycle between inactive, GDP-bound, and active, GTP-bound states (Karnoub and Weinberg 2008). RAS proteins are typically located along the inner surface of the plasma membrane, where they are activated downstream of activated growth factors that recruit guanine nucleotide exchange factors (GEFs). When bound to RAS, these GEFs promote the exchange of GDP for GTP (Takai, Sasaki et al. 2001). GTP-bound (active) RAS proteins undergo a conformational change, enabling them to interact with a number of downstream effectors broadly involved in cellular growth, proliferation, and survival (Karnoub and Weinberg 2008). The signal is then terminated by the binding of GTPase-activating proteins (GAPs), which enhance the hydrolysis of GTP, reverting RAS proteins back to their inactive, GDP-bound states (Bos, Rehmann et al. 2007). As such, RAS proteins act as molecular switches, transducing extracellular signals to downstream intracellular effects when GTP-bound, but shutting off these signaling pathways when they are GDP-bound.

### **1.2.2 Downstream signaling pathways of RAS proteins**

When GTP-bound, the RAS family of proteins activate a number of downstream pathways that are broadly involved in cellular growth, proliferation, and survival. The three best characterized RAS effector pathways are the MAPK, PI3K, and RalGEF pathways, which are described here.

The MAPK pathway is initiated when activated RAS binds to the RAF serine/threonine kinases (consisting of c-RAF1, A-RAF, and B-RAF), thereby recruiting them to the plasma membrane. In turn, RAF proteins phosphorylate MEK1 and MEK2 at multiple serine residues, which then activate ERK1 and ERK2 by phosphorylation at threonine and tyrosine residues (Friday and Adjei 2008). The ERK proteins have a multitude of downstream targets, and activate numerous transcription factors, including important regulators of the cell cycle, mitosis, and apoptosis (McCubrey, Steelman et al. 2007). Thus, aberrant activation of this pathway can have a significant impact on cellular proliferation and survival.

Another downstream pathway stimulated by activated RAS is the PI3K pathway. This pathway is activated when RAS binds to the p110 catalytic subunit of PI3K, converting phosphatidylinositol (4,5)-bisphosphate (PIP<sub>2</sub>) into phosphatidylinositol (3,4,5)-triphosphate (PIP<sub>3</sub>). PIP<sub>3</sub> can then bind to the pleckstrin homology (PH) domain of AKT/PKB, activating its kinase activity (Castellano and Downward 2011). Activation of AKT proteins promotes cellular growth and survival in a number of ways. First, it can inhibit pro-apoptotic proteins from the Bcl2 family (Engelman, Luo et al. 2006). Activation of AKT also negatively regulates the activity of NF-κB, resulting in increased transcription of prosurvival genes (Duronio 2008). Furthermore, the AKT kinases phosphorylate Mdm2, antagonizing signaling through the p53 tumor suppressor pathway (Duronio 2008). Activated AKT kinases also negatively regulate Forkhead transcription factors, reducing the production of proteins involved in cell death (Castellano and Downward 2011). The mammalian target of rapamycin (mTOR), is also

activated downstream of AKT kinases, promoting cell growth (Sarbasov, Guertin et al. 2005). Other downstream effectors of the AKT pathway include endothelial nitric oxide synthase (eNOS), important for angiogenesis (Fulton, Gratton et al. 1999). Thus, signaling through AKT has also been shown to be involved in cell growth and survival through a number mechanisms (reviewed in Franke 2008).

The third well-characterized downstream pathway activated by RAS is the RalGEF pathway. Binding of activated RAS to members of the RalGEF family promotes their translocation to the plasma membrane (Ferro and Trabalzini 2010). There, RalGEFs serve as guanine nucleotide exchange factors (GEFs) for the Ras-like small GTPases RalA and RalB. Activated Ral proteins have a number of downstream targets, including Sec5, Exo84, Filamin, ZONAB, and RalBP1 (Cantor, Urano et al. 1995, Ohta, Suzuki et al. 1999, Moskalenko, Henry et al. 2002, Frankel, Aronheim et al. 2005, Kashatus, Lim et al. 2011). These signaling pathways are involved in cellular processes such as endocytosis, exocytosis, cytoskeletal organization, mitochondrial dynamics, and gene expression, and their activation has overall been shown to have pro-proliferative and pro-survival effects (reviewed in Kashatus, 2011).

Overall, signaling through each of these three primary RAS effector pathways has mitogenic, anti-apoptotic, and pro-survival effects. Thus, activation of these pathways is important during normal cell division and differentiation, but when uncontrolled can have devastating consequences. Gain-of-function mutations in RAS genes causing constitutive signaling through these pathways have been shown to contribute to excessive

proliferation and tumor development in a number of cancer types (reviewed in (Schubbert, Shannon et al. 2007)).

### **1.2.3 RAS family and cancer**

Somatic mutations in one of the *RAS* genes are one of the most common events in human cancer, found in approximately 30% of all cases (COSMIC Database, 2014).

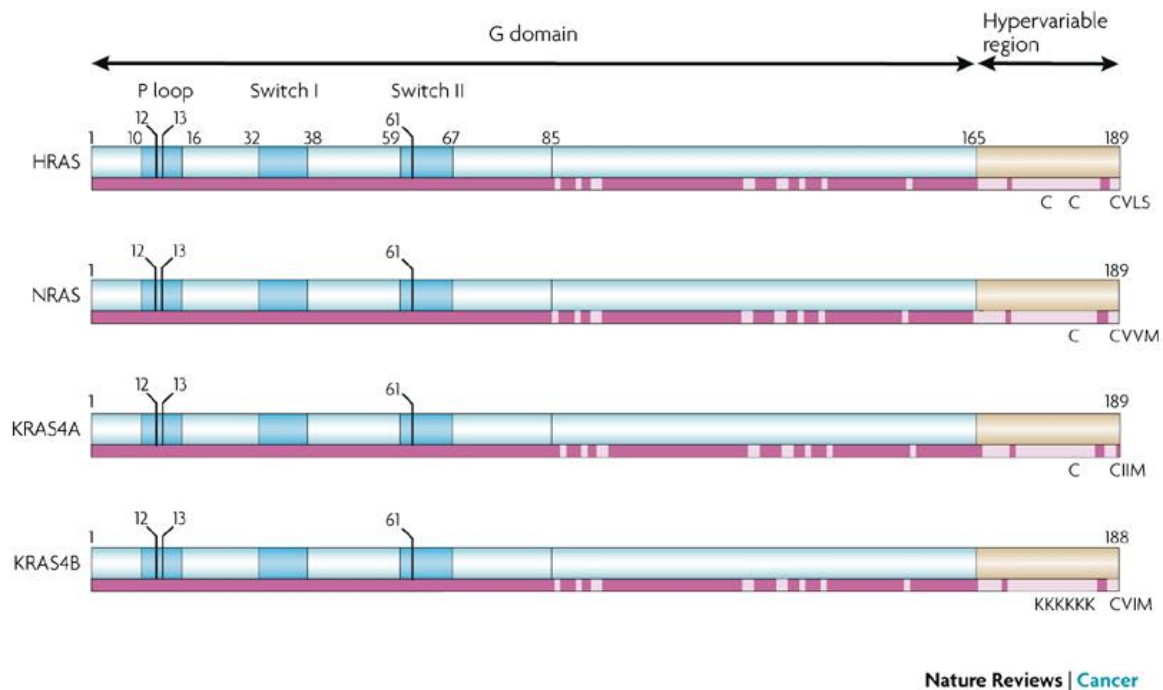
These oncogenic mutations are most frequently point mutations found at very specific locations in the gene, namely at codons 12, 13, or 61 (Bos 1989). These mutations either impair the intrinsic GTPase activity of RAS proteins, or make them insensitive to GAPs, rendering them constitutively GTP-bound, and resulting in chronic activation of the aforementioned downstream signaling pathways (Scheffzek, Ahmadian et al. 1997). Unchecked activation of these pro-proliferation, pro-survival pathways contributes to tumor development and growth (Schubbert, Shannon et al. 2007).

Specifically, mutations at glycine-12 and glycine-13, result in substitutions of amino acids with side chains in place of glycine in the phosphate-binding loop (P-loop) of RAS, sterically inhibiting the binding of GAPs, and thus making these molecules insensitive to GTP-hydrolysis by GAPs (Scheffzek, Ahmadian et al. 1997). Mutations at codon 61 instead inactivate the catalytic amino acid required to hydrolyze GTP (Krengel, Schlichting et al. 1990). There are also a host of other *RAS* mutations found at much lower frequencies in cancer that similarly render RAS constitutively active, but typically at much lower levels (Prior, Lewis et al. 2012). Collectively, gain-of-function mutations in RAS render the protein constitutively active by a variety of means.

## **1.3 Different RAS isoforms**

### **1.3.1 Four highly homologous RAS family members**

The RAS family is comprised of three genes: *KRAS*, *HRAS*, and *NRAS* (Bos 1989). *KRAS* is further divided into two isoforms, *KRAS4A*, and *KRAS4B*, which are the result of alternatively spliced terminal exons 4A and 4B (McGrath, Capon et al. 1983). All of the encoded proteins are highly homologous, sharing approximately 85% sequence identity (Schubbert, Shannon et al. 2007). The first 85 amino acids, coding for the domains where these proteins bind to regulatory molecules as well as downstream-effectors, are completely identical between all four isoforms (Schubbert, Shannon et al. 2007). Thus signaling in these proteins is regulated in a similar manner by GEFs and GAPs, and they are all able to engage the same downstream pathways (Downward 2003). The proteins are also nearly identical in codons 86-164, where only fifteen amino acid residues show variation between isoforms (Schubbert, Shannon et al. 2007). Where the RAS proteins do differ are at the carboxy-termini (C-termini), from amino acids 165-188 or 189, the regions responsible for the localization of these proteins to the inner surface of the plasma membrane within the cell (Schubbert, Shannon et al. 2007). The amino acid sequences of the four RAS proteins are depicted in **Figure 1**. Due to their nearly identical structures, signaling differences observed between the RAS family members are typically ascribed to different subcellular localization imparted by the “hypervariable” C-termini (Hancock 2003).



**Figure 1: Four highly homologous RAS isoforms**

HRAS, NRAS, KRAS4A, and KRAS4B are highly homologous, with 100% shared amino acid sequence identity in the first 85 amino acids and approximately 85-90% identity from amino acids 86-164, but differing at the C-termini from amino acids 165-188 or 189. The box at the bottom of each isoform demonstrates the degree of homology, with conserved residues shown in magenta, and variable residues shown in pink (from Schubbert and Shannon, 2007- used with permission from the publisher).

### 1.3.2 RAS isoforms have different post-translational modifications at the C-termini

As described, the primary differences between the four Ras isoforms lies within the C-terminus, known as the hypervariable region (HVR) of these proteins. This region is the site of many post-translational modifications to RAS proteins. More specifically, all four of these proteins have a terminal CAAX motif, in which C is cysteine, A is an aliphatic amino acid, and X is any amino acid (Cox, Der et al. 2015). This hydrophobic CAAX motif allows these proteins to be prenylated, enabling them to associate with the plasma membrane (Cox, Der et al. 2015). Additional features foster the anchoring of

these proteins to the membrane. The addition of two palmitic acids just after the CAAX motif in HRAS, NRAS, and KRAS4A provides a second hydrophobic signal that can give additional support for localizing these proteins at the membrane (Schubbert, Shannon et al. 2007). KRAS4B, however, contains instead a stretch of positively-charged polybasic lysine residues (polybasic region or PBR) that act as a second signal for anchoring this protein to the membrane (Schubbert, Shannon et al. 2007). Recent work has also shown that although KRAS4A is palmitoylated like HRAS and NRAS, it has two additional smaller polybasic residues similar to the one found in KRAS4B that allow it to be localized to the membrane in the absence of palmitoylation, suggesting a unique hybrid membrane-targeting mechanism for this protein (Tsai, Lopes et al. 2015). Thus, although the RAS family of proteins are highly identical, subtle differences in post-translational modification, and thus membrane localization account, at least in part, for differences in signaling observed between these proteins.

### **1.3.3 Differences in signaling between the RAS isoforms**

Although each of the RAS isoforms has the ability to bind to the same downstream effectors, subtle differences in downstream signaling between these proteins have been observed. For example, KRAS has been identified as a more potent activator of RAF1 in comparison to HRAS, while HRAS appears to activate PI3K more efficiently than KRAS (Yan, Roy et al. 1998, Voice, Klemke et al. 1999). Furthermore, KRAS also recruits RAF1 to the plasma membrane more effectively in comparison to HRAS, although HRAS could be engineered to recruit similar levels of RAF1 by replacing the hypervariable region of the HRAS protein with a shortened form that more closely

resembled that of KRAS, providing evidence that the differences in membrane localization between these isoforms (as described in Section 1.3.2) is most likely responsible for these differences in signaling (Yan, Roy et al. 1998).

The hypervariable C-termini of the RAS isoforms most likely target these proteins to different microdomains of cellular membranes, where it is possible that different effectors may be more or less available. Electron microscopy has revealed that the plasma membrane is made of various components or microdomains, containing cholesterol-dependent lipid rafts as well as cholesterol-independent, disordered regions (Prior, Muncke et al. 2003). KRAS has been shown to localize primarily to the disordered, non-raft regions, while HRAS appears to be evenly distributed among lipid-rafts and disorderly microdomains, although upon activation of this protein, GTP-bound HRAS shifts to higher levels of localization in the disordered, non-raft regions (Hancock 2003). Thus, these different affinities for different locations within the plasma membrane may contribute to differences in signaling that have been observed between the RAS isoforms.

The RAS proteins also signal at endomembranes within the cell, and compartmentalized signaling due to differences in localization within these membranes may also play a role in the observed differences between the RAS isoforms (Chiu, Bivona et al. 2002). For example, HRAS and NRAS are able to localize on the Golgi membrane, while KRAS4B cannot (Ahearn, Haigis et al. 2012). Palmitoylation of HRAS and NRAS on the Golgi apparatus is important for transporting these proteins to the plasma membranes on vesicles, where they can activate different effectors. This

modification is reversible, and after a period of time, HRAS and NRAS are depalmitoylated and released back into the cytosol, where they can again be repalmitoylated at the Golgi membrane and the cycle continues (Ahearn, Haigis et al. 2012). This trafficking between membranes has been suggested as a mechanism by which RAS proteins modulate signaling, as different effector pathways are activated to different degrees at different membranes (Lynch, Snitkin et al. 2015). Furthermore, recent evidence suggests that even within the endomembranes, HRAS and NRAS have distinct means for localization and activation of downstream signaling pathways. Use of confocal live-cell imaging has shown that within Golgi stacks, the doubly-palmitoylated HRAS is distributed throughout the membrane, while the mono-palmitoylated NRAS is localized primarily to the cis Golgi (Lynch, Snitkin et al. 2015). Thus, the variation in signaling observed between different RAS isoforms may result from both their distinct affinities for different membranes, as well as divergent localization within these membranes.

#### **1.3.4 Differences in protein expression between RAS isoforms**

In addition to signaling differences between the RAS isoforms, there are also differences in levels of protein expression between the different isoforms. For example, when oncogenic HRAS was ectopically expressed in a number of different human cell lines, it consistently produced more than twenty times the amount of protein than ectopically expressed oncogenic KRAS, as a result of differences in the rates of translation of the two transcripts (Lampson, Pershing et al. 2013). More specifically, even though these proteins share approximately 85% amino acid sequence identity,

synonymous differences in the coding sequences for these genes has significant effects on their expression, namely KRAS is enriched in rare codons that we know to impede translation (Lampson, Pershing et al. 2013). Converting rare to common codons increases KRAS expression to mirror that of HRAS in vivo and in vitro (Lampson, Pershing et al. 2013, Pershing, Lampson et al. 2015). Therefore, in addition to differences in membrane-targeting mechanisms, differences in translation between these isoforms may be another form of regulation.

### **1.3.5 Differences in RAS isoforms at the organismal level**

Further evidence for overlapping, yet distinct roles for the RAS family members comes from studies using knockout mice. Knockout mouse models have shown that mice lacking *Hras*, *Nras*, or both *Hras* and *Nras* develop normally with no apparent phenotypic abnormalities (Umanoff, Edelman et al. 1995, Esteban, Vicario-Abejon et al. 2001). Likewise, *Kras4A* is dispensable for development, at least in the presence of *Kras4B* (Plowman, Williamson et al. 2003). *Kras4B*, however, has been shown as essential for development, as mice lacking this protein died between embryonic day 12.5 and birth, indicating that signaling through this protein could not be compensated for by *Hras* or *Nras* (Koera, Nakamura et al. 1997). Interestingly, however, when a knock-in allele encoding *Hras* protein from the *Kras* locus was engineered, mice lacking *Kras* developed normally, further supporting the idea that these isoforms can activate redundant downstream signaling pathways during embryonic development. (Potenza, Vecchione et al. 2005). Adult mice lacking *Kras*, however, did eventually develop cardiomyopathy, demonstrating a unique role for this protein in cardiovascular

homeostasis (Potenza, Vecchione et al. 2005). Thus, knockout mouse models provide evidence for both overlapping and diverse roles for the RAS family members.

### **1.3.6 Differences in RAS isoforms in cancer**

As previously discussed, oncogenic mutations in the *RAS* genes are common events in human cancer, being present in about 30% of all cases (Fernandez-Medarde and Santos 2011). Interestingly, however, although similar mutations (usually at codons 12, 13, and 61) are observed in all of the *RAS* family members, these mutations are not observed in the same tissue types or at the same frequencies. *KRAS* mutations are found most frequently in pancreatic, colorectal, and lung tumors, and *NRAS* mutations are more often associated with hematopoietic malignancies and melanoma (Bos 1989, Karnoub and Weinberg 2008, Pylayeva-Gupta, Grabocka et al. 2011). *HRAS* mutations are rare, but more frequently found in head, neck, and bladder cancers (Karnoub and Weinberg 2008, Pylayeva-Gupta, Grabocka et al. 2011). These differences in driver mutation specificity in separate tissue types suggest divergent roles for these isoforms within these tissues.

In addition to tissue specificity, the frequency at which the three genes are found mutated in cancer also varies. The majority of oncogenic mutations found in human cancer typically occur in *KRAS*, accounting for nearly 90% of total *RAS* mutations. Approximately 8% of *RAS* mutations are detected in *NRAS*, while *HRAS* mutations are much less common, accounting for only about 2% of the total number of mutations (Fernandez-Medarde and Santos 2011). These differences also suggest some variation in the signaling output of these mutations in different tissue types.

Finally, the distribution of the types of mutations is also different between the *RAS* family members. While most mutations in the *RAS* genes occur at codons 12, 13, or 61 (as described in Section 1.1.3), different isoforms exhibit different mutational spectra. In *KRAS*, nearly all of the mutations observed are substitutions for glycine 12 or 13 (86% and 13% respectively), while only 1% of *KRAS* mutations are at glutamine 61 (Forbes, Bindal et al. 2011). In contrast, a higher proportion of mutations in *NRAS* are found at codon 61, with about 63% of all *NRAS* mutations resulting in substitutions for glutamine at this codon. Glycine 12 or 13 substitutions occur less frequently in *NRAS*, at rates of approximately 24 % and 13 %, respectively (Fernandez-Medarde and Santos 2011, Forbes, Bindal et al. 2011). *HRAS* mutations occur at yet different frequencies, with about 54% occurring a glycine 12, approximately 35% at glutamine 61, and 11% at glycine 13 (Fernandez-Medarde and Santos 2011, Forbes, Bindal et al. 2011) . Thus, these differences in the mutational spectra of the three *RAS* family members further argues that these genes are not identical in function.

## ***1.4 The Role of wild-type RAS in cancer***

### **1.4.1 Wild-type RAS proteins are activated in the presence of oncogenic RAS**

Constitutively-activated oncogenic *RAS* proteins have been well-established as driver mutations in a number of different cancer types, as previously described in sections 1.2.3 and 1.3.6. Interestingly, the remaining wild-type *RAS* proteins can also be activated in the presence of oncogenic *RAS* signaling, although the consequences of this

activation are not yet well understood. Wild-type RAS proteins were first considered as possible players in tumorigenesis when it was reported that localization of a RasGAP that specifically inhibited the activity of the endogenous wild-type form of HRAS could suppress the transforming activity of oncogenic HRAS in fibroblasts (Huang, Marshall et al. 1993). Indeed it has been determined that the wild-type RAS family members can be activated downstream of oncogenic signaling through several mechanisms (Lim, Ancrile et al. 2008, Jeng, Taylor et al. 2012, Young, Lou et al. 2013).

First, it has been shown that activation of AKT downstream of oncogenic KRAS signaling results in the phosphorylation of one of its substrates, endothelial nitric oxide synthase (eNOS), which in turn enhances nitrosylation and activation of wild-type HRAS and NRAS (Lim, Ancrile et al. 2008, Huang, Carney et al. 2014, Huang and Counter 2015, Huynh and Campbell 2015). Another proposed mechanism for activation of the wild-type RAS proteins is through the GEF Son-of-sevenless (SOS), which has an allosteric binding site for activated RAS. This allosteric binding site has been shown to preferentially bind to mutant KRAS, enhancing cross-activation of wild-type HRAS and NRAS at a second binding site (Jeng, Taylor et al. 2012). Finally, a third mechanism for activation of wild-type RAS proteins is through activation downstream of growth factors, as wild-type RAS proteins have been shown to be susceptible to this activation, even in the presence of an oncogenic RAS mutation (Young, Lou et al. 2013).

While wild-type proteins are activated in the presence of oncogenic RAS, the consequences of this activation remain ambiguous. Divergent effects resulting from wild-type RAS signaling have been reported. As discussed next, in some contexts they

appear to antagonize or suppress oncogene-driven tumorigenesis, while in other examples the wild-type RAS proteins seem to cooperate with the oncogene to promote tumorigenesis.

#### **1.4.2 Wild-Type RAS proteins can suppress tumorigenesis**

Although one might suspect that signaling through the wild-type RAS proteins would further serve to enhance the activity of the mutant protein since they are capable of activating the same effectors, quite interestingly it has been reported that there are consequences in which wild-type RAS activity appears to suppress tumorigenesis in the presence of oncogenic RAS. First, mice lacking one allele of wild-type *Kras* (+/-) are more susceptible to chemical carcinogen-induced lung tumors than wild-type *Kras* mice (+/+). Even though these chemicals, urethane and MNU, induce oncogenic mutations in *Kras*, losing one-allele produced significantly more tumors, suggesting that wild-type *Kras* could act to suppress oncogenic *Kras*-driven lung tumorigenesis (Zhang, Wang et al. 2001). Furthermore, it has also been observed that loss-of- heterozygosity of the wild-type allele occurs in KRAS-driven lung cancer in both mice (Hegi, Devereux et al. 1994, Zhang, Wang et al. 2001), and humans (Li, Zhang et al. 2003), suggesting a tumor-suppressive role for this corresponding wild-type allele, as its loss appears to provide a growth advantage to tumorigenic cells. Likewise, in a mouse model of thymic lymphoma, wild-type *Nras* appeared to suppress the formation of oncogenic-*Nras*-driven malignancies (Diaz, Ahn et al. 2002). More recently, it has been shown that additional wild-type RAS family members can also antagonize tumorigenic effects of the oncogene, as mice lacking *Hras* or *Nras* developed higher numbers of carcinogen-induced, *Kras*-

mutation-positive lung tumors (To, Rosario et al. 2013). Thus, it appears that wild-type RAS proteins can inhibit tumor growth, at least during the early stages of tumorigenesis that are modelled in these mice.

### **1.4.3 Wild-Type RAS proteins can enhance tumorigenesis**

The effects that wild-type RAS proteins have on tumorigenesis appear to be quite context-dependent. In contrast to the examples discussed above, there are also cases in which wild-type RAS signaling appears to promote tumor growth. First, inhibiting wild-type HRAS signaling in HRAS-transformed human NIH3T3 fibroblasts reduced their transforming properties in culture (Huang, Marshall et al. 1993). Other *in vitro* experiments suggested that wild-type NRAS is required for signaling through the MAPK pathway in fibroblasts transformed by oncogenic HRAS, and that inhibiting this pathway reduced the proliferative capacity of these cells (Hamilton and Wolfman 1998). *In vivo*, xenografts of KRAS-driven human pancreatic cancer cell lines grown in mice showed reduced tumor growth when wild-type HRAS or NRAS was knocked down, suggesting a pro-tumorigenic role for the wild-type proteins (Lim, Ancrile et al. 2008). KRAS-driven human pancreatic cancer cells lines also show reduced viability and proliferation *in vitro* when wild-type HRAS or NRAS was knocked down (Grabocka, Pylayeva-Gupta et al. 2014). In addition, inhibiting the activation of wild type HRAS and NRAS by the Ras GEF SOS (Jeng, Taylor et al. 2012) or EGF (Young, Lou et al. 2013) reduced the viability and transforming properties of KRAS-driven human pancreatic cancer cell lines. The wild-type RAS proteins also appear to enhance tumorigenesis in a model of HRAS-driven cancer: mice lacking wild-type Hras or Nras developed fewer DMBA/TPA-

induced skin papilloma, in which *Hras* mutations at codon 61 are the driving oncogene (To, Rosario et al. 2013). Thus, there are a number of contexts and tissue types in which wild-type RAS signaling can promote tumorigenesis, especially in more-advanced disease models.

#### **1.4.4 The importance of investigating the role of wild-type RAS proteins in clinically relevant models of cancer associated with RAS oncogenes**

As described in sections 1.4.2 and 1.4.3, the role that wild-type RAS signaling plays in oncogenic RAS-driven tumorigenesis is variable. Divergent results in different models and contexts suggest that the wild-type RAS proteins may confer varying properties to tumorigenic cells, and that a number of factors such as mutational status, tissue type, stage of tumorigenesis, and other signaling pathways may all influence the consequences of signaling through the wild-type RAS proteins. As activation of the wild-type RAS proteins is closely linked to activation of oncogenic proteins, I propose that elucidating the roles that wild-type RAS proteins play in tumorigenesis is critical to understanding how to best treat RAS-driven cancers, which affect 30% of all human cancer patients. Thus, I sought to investigate the consequences of loss of wild-type RAS signaling in pancreatic cancer, the cancer that is most frequently associated with *KRAS* mutations, and lung cancer, in which *KRAS* mutations affect the highest number of patients. I wanted to test the effects of loss of these proteins when RAS was spontaneously activated within the target organs with surrounding stroma that would recapitulate human disease as closely as possible. I will discuss these types of cancers and the models that we used in the proceeding sections.

## **1.5 Pancreatic cancer**

### **1.5.1 Critical need for improvements in the treatment of pancreatic cancer**

It is estimated that approximately 1.5% of Americans will develop pancreatic cancer, primarily pancreatic ductal adenocarcinoma (PDAC) (Cox, Der et al. 2015). Despite significant efforts to find appropriate treatments, the five-year survival rate for pancreatic cancer is still only 6.7%, with most patients succumbing to the disease within a year of diagnosis (SEER Database, 2014). Because the symptoms of pancreatic cancer often do not present until late stages of disease, only 15-20% of all patients have localized tumors that can be resected at the time of diagnosis, and of those, 80% relapse and die of metastatic disease within five years (Li, Xie et al. 2004). Pancreatic cancer responds poorly to radiation, and to date, little success has been met in targeting the KRAS pathway, the primary genetic driver of this disease, as discussed in the following section. The current standard-of-care chemotherapeutic agent, gemcitabine, extends survival for only a few months (Burris, Moore et al. 1997). Thus, there is a critical need to further elucidate the signaling pathways that contribute to this extremely devastating health problem.

### **1.5.2 KRAS is activated in nearly all cases of pancreatic cancer**

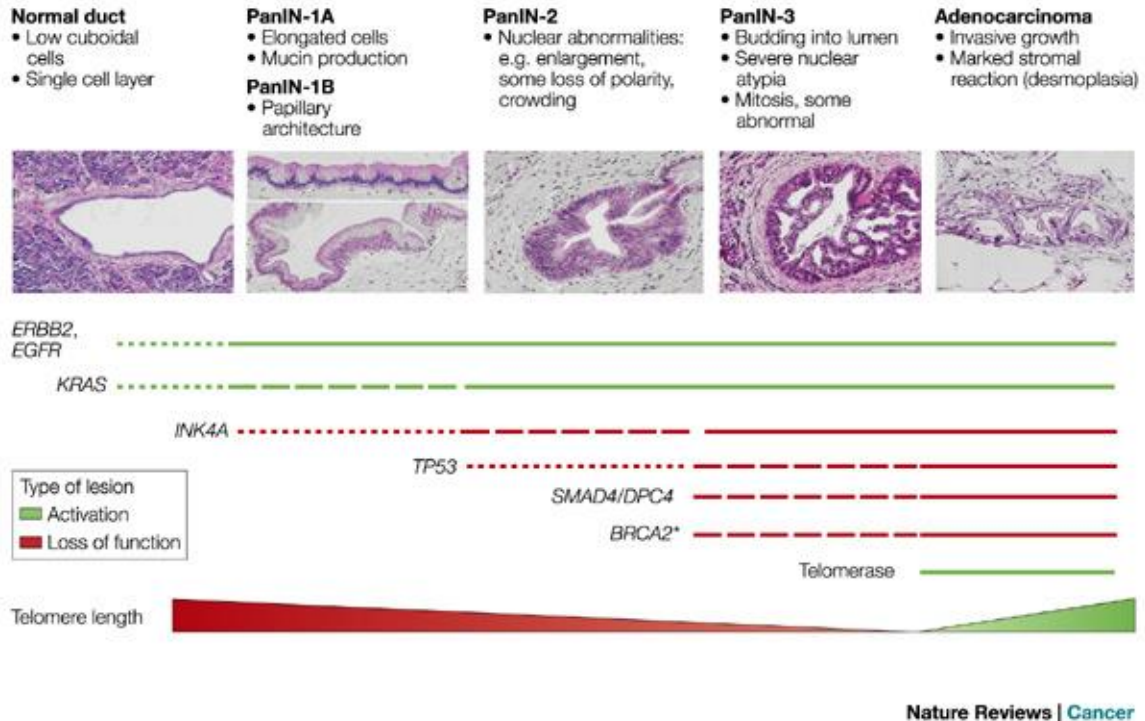
Activating, oncogenic mutations in *KRAS* are the most common genetic alterations found in pancreatic cancer, being present in over 90% of cases (COSMIC Database, 2014). Even when *KRAS* itself is not mutated, this pathway is found to be active in virtually all pancreatic cancers (Eser, Schnieke et al. 2014). Nearly all

(approximately 98%) of these mutations are at glycine-12, but glycine-13 and glutamine-61 mutations are also occasionally found in pancreatic cancer (Pylayeva-Gupta, Grabocka et al. 2011). These mutations, all of which result in constitutively active, GTP-bound KRAS, are considered to be the driver mutations of pancreatic carcinoma, as they are found even in the earliest stages of this disease (Eser, Schnieke et al. 2014). When mice were engineered to express a *Kras*<sup>G12D</sup> mutation in their pancreatic cells, they developed early-stage lesions that progressed to pancreatic adenocarcinoma over time, providing further evidence that mutations in *Kras* can drive pancreatic tumorigenesis (Hingorani, Petricoin et al. 2003).

### **1.5.3 Pancreatic cancer develops through a series of stages**

Pancreatic Cancer develops through a series of stages, as shown in **Figure 2**. Oncogenic *KRAS* mutations are found in the earliest pre-cancerous lesions, known as Pancreatic Intraepithelial Neoplasia, or PanIN, which are precursors that eventually proliferate and progress to pancreatic ductal adenocarcinoma (PDAC) (Bardeesy and DePinho 2002). Most often, additional mutations are accumulated as these lesions progress to later stages, particularly in tumor suppressor genes, such as *CDKN2A*, *TP53*, and *SMAD4* (Bardeesy and DePinho 2002). The cell-of-origin for these lesions remains unclear. While it was typically thought that these lesions arose from ductal cells in the pancreas, given their ductal-like characteristics, evidence has suggested that the more abundant cell type in the pancreas, acinar cells, could also form PanIN lesions (Guerra, Schuhmacher et al. 2007, De La, Emerson et al. 2008, Gidekel Friedlander, Chu et al. 2009, Hill, Calvopina et al. 2010, Shi, DiRenzo et al. 2013). It has been shown that

acinar cells expressing oncogenic KRAS can transdifferentiate into a ductal cell phenotype (Shi, DiRenzo et al. 2013), and that mice engineered with a *Kras*<sup>G12D</sup> mutation targeted to the acinar cells of the pancreas developed a full spectrum of PanIN lesions (Habbe, Shi et al. 2008). Thus, an intermediate cell type known as acinar-to-ductal metaplasia (ADM), commonly found amongst PanIN lesions (Bardeesy and DePinho 2002), may instead be the earliest pathology following an oncogenic mutation in *KRAS* in the pancreas, although this remains unclear (Caldwell, DeNicola et al. 2012).



**Figure 2: Progressive stages of pancreatic adenocarcinoma**

Pancreatic adenocarcinoma develops through a series of progressive stages, beginning with pre-cancerous neoplastic lesions known as Pancreatic Epithelial Neoplasia (PanIN). These lesions sequentially develop additional changes in cellular architecture as they acquire additional genetic mutations and progress toward pancreatic ductal adenocarcinoma (PDAC). (From Bardeesy and dePinho, 2002- used with permission from the publisher).

## ***1.6 Investigating the role of wild-type RAS signaling in pancreatic cancer***

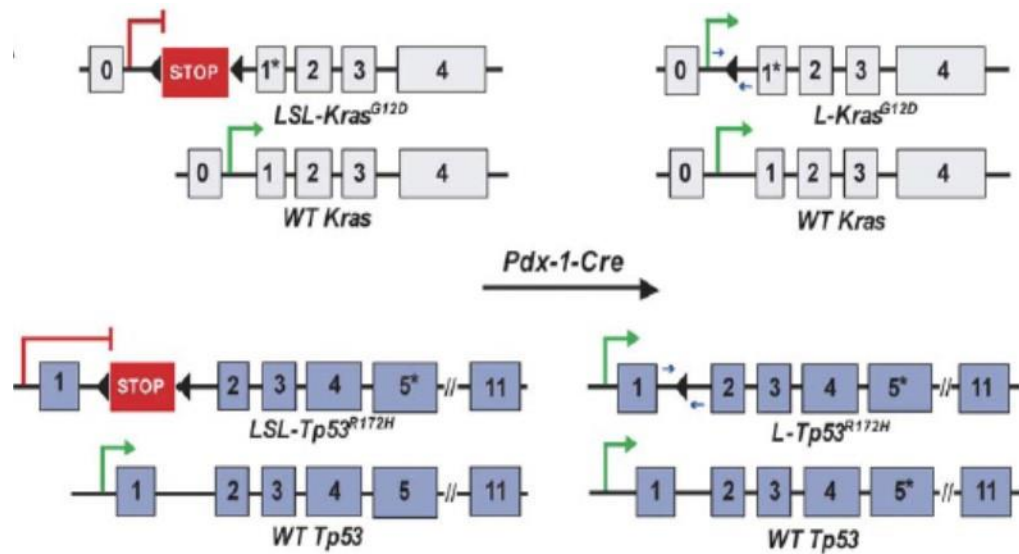
### **1.6.1 Pancreatic cancer is the type of cancer most frequently associated with RAS mutations**

As discussed in section 1.4, understanding the role that wild-type RAS proteins play in oncogenic KRAS-driven cancers is vital to elucidating the signaling involved and identifying treatment strategies for these diseases. Because pancreatic cancer is the type of cancer most frequently associated with *RAS* mutations (Bos 1989, Eser, Schnieke et al. 2014), and improved treatments for this disease are desperately needed (see section 1.5.1), I sought to determine the role that wild-type RAS proteins played in a clinically relevant model of pancreatic adenocarcinoma.

### **1.6.2. Mouse models of pancreatic adenocarcinoma closely recapitulate human disease**

In a xenograft model of KRAS- transformed human pancreatic cancer cell lines, shRNA-mediated knockdown of wild-type HRAS , and to a lesser degree, NRAS, reduced tumor growth, suggesting that signaling through these wild-type isoforms was required for KRAS-driven tumorigenesis (Lim, Ancrile et al. 2008). Because these human pancreatic cancer cell lines were derived from late-stages of this disease, and xenograft models do not closely re-capitulate the growth of the cancer cells within the target organ and lack the typical stomal cells and microenvironment of this cancer, I tested the effects of wild-type RAS signaling in a model that would more closely resemble human pancreatic cancer.

To achieve this goal, I utilized a genetically engineered mouse (GEM) model of pancreatic cancer whereby an inducible oncogenic *Kras*<sup>G12D</sup> knock-in allele was activated in the developing pancreas (Hingorani, Petricoin et al. 2003). Specifically, mice with an allele containing oncogenic *Kras*<sup>G12D</sup> downstream of a Lox-Stop-Lox (LSL) sequence, in which a stop cassette is flanked by LoxP sites, were bred with mice containing a *Pdx-1-Cre* driver. Upon Cre-mediated recombination, the LSL cassette is excised, allowing expression of the *Kras*<sup>G12D</sup> allele in pancreatic progenitor cells (as shown in **Figure 3**). These mice, known as KC mice, develop the full-spectrum of pancreatic lesions (PanIN1-3) with histological characteristics similar to those found in humans, and their neoplasia progress over time such that a small percentage progress to ductal adenocarcinoma (Hingorani, Petricoin et al. 2003). While this GEM models the early stages of pancreatic cancer, later stages of this disease can also be studied by additionally recombining *Trp53*<sup>LSL-R172H</sup> alleles, which encode an inducible inactivating mutation in the *Trp53* tumor suppressor allele, a mutation that is also frequently found in human pancreatic cancer patients. These mice, known as KPC mice, develop PanIN lesions that progress to metastatic pancreatic ductal adenocarcinoma (PDAC) with symptoms such as cachexia and abdominal distention that closely mimic the later stages of human disease (Hingorani, Wang et al. 2005). Thus, use of these two models allowed me to study the effects of wild-type RAS signaling at both early and late stages of tumor progression.



**Figure 3: Genetically engineered mouse model of pancreatic cancer**

Recombination driven by *Pdx-1-Cre* excises the Stop cassette flanked by LoxP sites, expressing *Kras*<sup>G12D</sup> and *Trp53*<sup>R172H</sup> alleles in pancreatic cells during development (from Hingorani, Wang et al. 2005- used with permission from the publisher).

### 1.6.3 Investigating the role of wild-type HRAS in a model of pancreatic cancer

Because wild-type HRAS had the greatest effects on tumor growth in the aforementioned xenograft model (Lim, Ancrile et al. 2008), and because *Hras* is the only one of the three Ras alleles that is not embryonic lethal when knocked out in the KC background (described in section 1.6.2) which encodes only one functional *Kras* allele, I chose to investigate the effects of wild-type HRAS on KRAS-driven pancreatic tumorigenesis. I bred KC and KPC mice into an *Hras*-knockout background to investigate signaling at both early and late stages of disease. Mice were a kind gift from Eugenio Santos (Esteban, Vicario-Abejon et al. 2001). I describe and discuss my work

comparing tumor development at various stages in wild-type versus *Hras*-knockout littermates in these clinically relevant models in Chapter 2.

## **1.7 Lung cancer**

### **1.7.1 Need for improvements in treatment of lung cancer**

Approximately 6.6% of the population in the United States will develop lung cancer during their lifetime. Although improvements in the detection and treatment of lung cancer have been made in recent years, only about 17.5% of patients survive longer than five years past the time of their diagnosis (SEER database, 2014). Lung cancer is responsible for the most deaths from metastatic cancer, killing over 158,000 Americans annually (SEER database, 2014). Thus, there is a need to find novel means of detecting this disease earlier and to develop new therapies. More insight into the molecular biology and signaling of this disease is critical to improving care for these patients.

### **1.7.2 KRAS mutations are found in many cases of non-small cell lung carcinoma**

Non-small-cell lung carcinoma (NSCLC) is the most prevalent type of lung cancer, and *KRAS* is the driving oncogene for about 20-30% of these cases (Aviel-Ronen, Blackhall et al. 2006). As in other types of *KRAS*-driven cancers, most of these mutations result in an amino acid substitution at exons 12, 13, or 61 (Karachaliou, Mayo et al. 2013). Evidence for *KRAS* acting as the driver-mutation in lung cancer first came from studies done in *KRAS*-mutation positive cancer cell lines, where inhibition of *KRAS* expression by knockdown with anti-sense RNA reduced *in vitro* growth and proliferation

(Mukhopadhyay, Tainsky et al. 1991, Alemany, Ruan et al. 1996). Later, mice engineered with an allele containing oncogenic *Kras* that could be spontaneously activated were shown to be highly predisposed to the development of lung cancer (Johnson, Mercer et al. 2001), further evidence that mutant *KRAS* could initiate lung tumorigenesis. As *KRAS* is the driving oncogene in a significant number of lung cancer cases, having a better understanding of this signaling pathway and its interactions with other molecular pathways could help to identify better therapeutic targets for this disease.

## **1.8 Role of wild-type RAS signaling in lung cancer**

### **1.8.1 Lung cancer is the RAS-associated cancer that occurs at the highest incidence**

As understanding the role that wild-type RAS signaling plays in tumorigenesis may be important for treating RAS-induced cancers, it is important to investigate the role of these proteins in lung cancer, as *KRAS* mutations affect the largest number of patients with this disease. Although only 20-30% of patients with lung cancer have an oncogenic mutation in *KRAS*, lung cancer is the second most common type of cancer, and the most deadly, thus affecting a higher number of patients than pancreatic cancer (Pylayeva-Gupta, Grabocka et al. 2011). For this reason, I extended my studies of wild-type RAS signaling to lung cancer.

## **1.8.2 Advantages of the urethane model for investigating the role of wild-type HRAS and NRAS in lung cancer**

The urethane model of lung cancer allowed me to not only investigate the role of wild-type RAS signaling in a second tissue type (the one in which RAS mutations affect the greatest number of people), but also to investigate the effects of loss of multiple wild-type alleles, the effects of wild-type RAS in the presence of a second *Kras* mutation, and the effects of wild-type HRAS following spontaneous *RAS* mutation in adult mice.

These advantages are described in more detail below.

### **1.8.2.1 Investigation of the effects of loss of wild-type Hras and Nras, individually and in combination**

Unlike in the pancreatic model, in which *Hras* was the only RAS family member that could be knocked out in the presence of the oncogenic *Kras*<sup>*LSL-G12D*</sup> allele to get viable pups, I was able to investigate the role of loss of both wild-type Hras and Nras in the urethane model, both individually and in combination. I wanted to assess the role of both of these proteins in Kras-driven tumorigenesis, as both were previously shown to be important in RAS-driven tumorigenesis in xenografts with human pancreatic cancer cell lines (Lim, Ancrile et al. 2008). In addition, I wanted to investigate whether the role of dosage of the RAS family members was important, and thus bred mice that were heterozygous knockouts for both *Nras* and *Hras* together to get littermates with different combinations of the wild-type and knockout *Hras* and *Nras* alleles. I then could compare the effects of loss of one allele versus two, as well as different combinations of loss of both family members on lung tumorigenesis.

### **1.8.2.2 Investigation of the role of wild-type Ras proteins in a model that predominantly exhibits mutations at Q61**

Most mutations that form in urethane-treated mice exhibit amino acid substitutions at Q61 (Cazorla, Hernandez et al. 1998). Thus, I also selected the urethane model to investigate the role of wild-type RAS proteins in the presence of a second *Kras* mutation, since the previous analysis of the role of wild-type Ras in pancreatic cancer was driven by a G12D mutation. Different *RAS* mutations have been shown to exhibit slightly different signaling profiles and transforming abilities (Miller and Miller 2011), and thus I also wanted to assess the role of the wild-type Ras proteins in combination with different oncogenic *Kras* mutations.

### **1.8.2.3 Investigation of the role of wild-type Ras proteins in a spontaneous model of Kras-driven tumorigenesis in adult mice**

While the pancreatic models of adenocarcinoma allowed me to investigate the role of wild-type Hras signaling at different stages of tumorigenesis, I also wanted to determine the role of the wild-type Ras proteins in a chemical-carcinogen induced model. The transgenic mouse models closely recapitulate human disease, but one disadvantage is that oncogenic *Kras* is turned on in embryonic tissues, rather than differentiated adult tissues, where most somatic mutations occur in human patients. Signaling through the RAS proteins may have different effects at different stages of tissue development and differentiation, as it has been shown that the expression levels of these proteins changes over time (Muller, Slamon et al. 1982, Leon, Guerrero et al. 1987). Thus, a spontaneous,

carcinogen-driven model of tumorigenesis allowed me to investigate the role of the wild-type Ras proteins in *Kras*-driven tumorigenesis in fully developed mice.

### **1.8.3 Investigating the role of wild-type RAS proteins urethane-induced lung cancer**

As described above, the urethane model of lung cancer provided several advantages for determining the role of wild-type RAS proteins in *KRAS*-driven tumorigenesis. I used this model to compare the effects of loss of *Hras* and *Nras*, alone, and in combination, on *Kras*-driven tumor formation, tumor burden, and molecular tumor characteristics. These experiments are summarized in Chapter 3. I was also able to micro-dissect the tumors from these mice for sequencing of the *Kras* allele in order to determine the mutational spectrum found in these lesions. Future experiments in this regard are discussed in Chapter 5.

## **1.9 Summary**

Oncogenic mutations in *RAS* genes render the encoded proteins constitutively activated, and are common driver mutations in a variety of cancers. Wild-type RAS proteins have also been shown to be activated downstream of oncogenic RAS signaling, but the consequences of this activation is not well understood. While in some contexts, activation of the wild-type RAS proteins seems to promote tumorigenesis, in others, this activation appears to suppress the growth of tumor cells. In this thesis, I will investigate the role that wild-type RAS proteins play in clinically relevant mouse models of both pancreatic and lung cancer. In Chapter 2, I show that loss of wild-type *Hras* promoted

the early stages of pancreatic tumorigenesis, possibly even at the time of initiation of oncogenic Kras signaling, suggesting a tumor suppressive role for this protein in early stages of cancer. In Chapter 3, I show that loss of wild-type Hras also enhances tumorigenesis in a carcinogen-induced model of lung cancer, but loss of wild-type Nras has no effect on tumor number. Thus, the tumor suppressive effects of wild-type RAS signaling appear to occur at the earliest stages of tumorigenesis, and appear to be isoform-specific, as wild-type Nras status did not affect tumor formation. In Chapter 4, through mechanistic studies, I provide further evidence that the effects of wild-type RAS proteins occur at the earliest stages of tumorigenesis, as no differences in proliferation were observed *in vitro* in later stage cell lines or in xenograft growth of mouse PDAC cell lines. Furthermore, there were no apparent differences in the amplitudes of signaling through the canonical downstream pathways, at least in tumor cell lines. Collectively, these data suggest a growth-suppressive role for wild-type HRAS in early KRAS-driven tumorigenesis. This knowledge is important for not only understanding the biology and signaling interactions between the RAS isoforms, but for determining how to best treat cancers driven by oncogenic RAS mutations, as the RAS isoforms are molecularly similar, and drugs designed to inhibit one protein would most likely have effects on the others. Moreover, these data also suggest that variations in wild-type RAS signaling between individuals could possibly predispose some to the development of cancer in the presence of an oncogenic mutation.

## **2. Wild-type Hras suppresses the earliest stages of tumorigenesis in a genetically engineered mouse model of pancreatic cancer**

NOTE: Chapter 2 was modified from a manuscript (of the same title) published in PLoS One 2015 Oct 9;10(10):e0140253. The authors were Jamie D. Weyandt, Benjamin L. Lampson, Sherry Tang, Matthew Mastrodomenico, Diana M. Cardona, and Christopher M. Counter. Benjamin Lampson provided assistance in early experimental planning and breeding of mice. Sherry Tang, Matthew Mastrodomenico, and Diana Cardona graded the PanIN lesions from H&E stained sections from pancreases that I isolated from the mice. I performed all other experiments reported in this study.

### **2.1 Introduction**

It is estimated that approximately 1.5% of Americans will develop pancreatic cancer, primarily pancreatic ductal adenocarcinoma (PDAC). The vast majority of these individuals will succumb to this disease, with a five-year survival rate of only 6.7% (SEER Database, 2014). Thus, it is critical to elucidate the signaling pathways underlying this incredibly deadly cancer. Activating mutations in *KRAS* are the most common genetic alteration found in human PDAC, being present in over 90% of all cases. In fact, the Ras pathway is argued to be engaged in essentially all PDAC (Jones, Zhang et al. 2008).

*KRAS*, and the other Ras family members *NRAS* and *HRAS*, encode highly related small GTPases. Normally, stimulation of guanine nucleotide exchange factors (GEFs)

promotes the conversion of Ras from an inactive, GDP-bound state to an active, GTP-bound state. Ras-GTP binds to and activates effector proteins that engage the MAPK, PI3K, RalGEF, and other signaling pathways involved in cellular growth and survival. GTPase-activating proteins (GAPs) then enhance hydrolysis of GTP and revert Ras back to an inactive state (Pylayeva-Gupta, Grabocka et al. 2011).

The somatic mutations detected in the *KRAS* gene inactivate the endogenous or GAP-stimulated GTPase activity of the encoded protein, resulting in constitutively GTP-bound and activated KRAS (Pylayeva-Gupta, Grabocka et al. 2011). In addition to being commonly detected in PDAC (Jones, Zhang et al. 2008), these mutations are also found in the earliest stages of this disease (Eser, Schnieke et al. 2014) and, when introduced into the murine *Kras* gene, induce early stage pancreatic cancer that can progress to frank PDAC (Hingorani, Petricoin et al. 2003). As such, oncogenic mutations in *KRAS* are thought to be the driver mutations in PDAC.

Although the involvement of the oncogenic mutant form of KRAS in pancreatic cancer is well described, the remaining wild-type Ras isoforms may also be activated. The first hints in this regard were the findings that targeting the negative regulator RasGAP to the plasma membrane (Huang, Marshall et al. 1993) or reducing wild-type Nras expression (Hamilton and Wolfman 1998) dampened oncogenic Hras signaling. Indeed, wild-type Nras and Hras have been found to be activated downstream of oncogenic Kras in a manner dependent upon S-nitrosylation (Lim, Ancrile et al. 2008) or expression of the GEF son-of-sevenless (SOS) (Jeng, Taylor et al. 2012). Moreover,

wild-type Ras proteins are still susceptible to activation by growth factors, even in the presence of oncogenic Kras (Young, Lou et al. 2013).

Wild-type Ras proteins can have divergent effects depending on the setting. On one hand, they can inhibit tumorigenesis. Specifically, oncogenic Ras can induce a senescent growth arrest when expressed in normal cells (Collado and Serrano 2010) and inhibit tumor formation (Sarkisian, Keister et al. 2007), suggesting a tumor suppressive role for the wild-type Ras proteins. In agreement, carcinogen-induced oncogenic mutations in *Kras* or *Hras* in lung and skin tumors of mice are often accompanied by loss of the reciprocal wild-type allele (Balmain, Brown et al. 1990, Hegi, Devereux et al. 1994, Zhang, Wang et al. 2001). Similarly, loss of heterozygosity of the oncogenic *RAS* gene has been reported in human cancers (Li, Zhang et al. 2003). Moreover, mice lacking one allele of wild-type *Kras* (Zhang, Wang et al. 2001), or both alleles of either *Hras* or *Nras*, develop more Kras mutation-positive lung tumors (To, Rosario et al. 2013). A tumor-suppressive role for wild-type *Nras* was also observed in a mouse model of thymic lymphomas driven by oncogenic *Nras* (Diaz, Ahn et al. 2002). Thus, wild-type Ras proteins can be tumor suppressive, especially in settings of early tumorigenesis.

Wild-type Ras proteins can also foster tumorigenesis. Specifically, mice lacking one or both alleles of wild-type *Hras* or *Nras* develop fewer carcinogen-induced *Hras* mutation-positive skin tumors (To, Rosario et al. 2013). Knocking down the expression of wild-type Ras proteins (Lim, Ancrile et al. 2008, Grabocka, Pylayeva-Gupta et al. 2014), or reducing their activation by eNOS (Lim, Ancrile et al. 2008) or SOS (Jeng, Taylor et al. 2012), inhibits cell viability, transformation, and/or tumorigenic growth of

*KRAS* mutation-positive human cancer cell lines. Conversely, stimulation of wild-type Ras proteins by EGF promotes proliferation of such cells (Young, Lou et al. 2013). Thus, particularly in models of more advanced disease, wild-type Ras proteins can enhance tumorigenesis.

Given that wild-type Ras proteins can be activated downstream of oncogenic *Kras*, yet give rise to opposite effects on tumorigenesis depending on the context, it is important to ascertain their role in the cancer most frequently associated with *KRAS* mutations, namely pancreatic. Thus, we assessed the consequence of disrupting the endogenous, wild-type *Hras* gene on the development of oncogenic *Kras*-driven tumorigenesis in the pancreas of mice. We report that loss of wild-type *Hras* promotes tumorigenesis in this model, suggesting a tumor suppressive role for wild-type *Hras* at the early stages of pancreatic cancer.

## **2.2 Materials and Methods**

### **2.2.1 Mouse pancreatic cancer models**

*Kras*<sup>LSL-G12D/+</sup>, *Trp53*<sup>LSL-R172/+</sup>, and *Pdx-1-Cre*<sup>tg/+</sup> mice were obtained from Jackson Labs and as a kind gift from David Kirsch. *Hras*<sup>-/-</sup> mice were a kind gift from the NCI and Eugenio Santos. *Kras*<sup>LSL-G12D/+</sup>;*Hras*<sup>+/-</sup> and *Pdx-1-Cre*<sup>tg/+</sup>;*Hras*<sup>+/-</sup> mice were bred to generate *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> KC (*LSL-Kras*<sup>G12D/+</sup>;*Pdx-1-Cre*<sup>tg/+</sup>) littermates. *Kras*<sup>LSL-G12D/+</sup>;*Trp53*<sup>LSL-R172/+</sup>;*Hras*<sup>+/+</sup> and *Pdx-1-Cre*<sup>tg/+</sup>;*Hras*<sup>+/+</sup> mice were bred to generate *Hras*<sup>+/+</sup> KPC (*LSL-Kras*<sup>G12D/+</sup>;*Trp53*<sup>LSL-R172/+</sup>;*Pdx-1-Cre*<sup>tg/+</sup>) mice. *Kras*<sup>LSL-G12D/+</sup>;*Trp53*<sup>LSL-R172/+</sup>;*Hras*<sup>-/-</sup> and *Pdx-1-Cre*<sup>tg/+</sup>;*Hras*<sup>-/-</sup> mice were bred to generate *Hras*<sup>-/-</sup>

KPC mice.  $Kras^{LSL-G12D/+};Trp53^{LSL-R172/+};Hras^{+/+}$  and  $Pdx-1-Cre^{tg/+};Trp53^{LSL-R172/+};Hras^{+/+}$  mice were bred to generate  $Hras^{+/+}$  KPPC ( $LSL-Kras^{G12D/+};Trp53^{LSL-R172/+};Hras^{+/+};Pdx-1-Cre^{tg/+}$ ) mice.  $Kras^{LSL-G12D/+};Trp53^{LSL-R172/+};Hras^{-/-}$  and  $Pdx-1-Cre^{tg/+};Trp53^{LSL-R172/+};Hras^{-/-}$  mice were bred to generate  $Hras^{-/-}$  KPPC mice. The described mice were monitored for health and weight three times per week and were euthanized at either the indicated fixed time points or upon reaching a moribundity endpoint. Moribundity endpoints were defined as weight loss exceeding 15% of total body weight, indications of abdominal ascites or swelling, or signs of pain or distress. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. A protocol for this study was specifically approved by the Institutional Animal Care and Use Committee at Duke University (protocol A279-13-11).

## 2.2.2 Mouse PDAC cell lines

Pancreatic tumor tissue from three  $Hras^{+/+}$  and three  $Hras^{-/-}$  KPC mice was minced in collagenase V (Sigma-Aldrich) for 30 minutes at 37°C, and resultant cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) + 10% FBS for at least 4 passages.

## 2.2.3 Quantification of normal pancreatic acinar area.

The percentage of normal acinar area was quantified at 8 and 36 week time-points in KC mice in a blinded study of 4-5 randomly selected high-power fields of H & E-stained pancreatic section per mouse. The amount of acinar area per section was

expressed as a percentage of the total area of tissue in the field, determined using Adobe Photoshop freehand selection tool.

#### **2.2.4 Grading of pancreatic lesions**

H&E stained sections were blindly reviewed by pathologists (S. Tang, M. Mastrodomenico, and D. Cardona) at 4, 8, and 36 week time points. Each lobule of the pancreas from each section was examined, and the highest grade lesion (ADM or PanIN) from each lobule was identified, and the percent of lobules with the highest grade of each type of lesion was determined based upon the total number of lobules counted.

#### **2.2.5 ADM immunofluorescent staining**

Pancreatic specimens from KC mice were snap frozen in liquid nitrogen and 8  $\mu$ m-thick sections were cut using a cryotome. The samples were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton-X-100. Samples were blocked in normal donkey serum then incubated with 1:100 dilution of the primary antibodies Goat Anti-Amylase C-20 and sc-12821 Rabbit Anti-CK-19 H-60 (Santa Cruz Biotechnology Inc.) overnight at 4<sup>0</sup>C. Samples were then incubated with the secondary antibodies Alexa fluor 488 Donkey anti-rabbit IgG (Invitrogen A-21206) or Alexa-fluor 594 Donkey anti-goat IgG (Invitrogen A-11058) at a 1:500 dilution for 1 hour at room temperature, then mounted with ProLong Gold antifade reagent with DAPI (Life Technologies P36931). Total number of lesions with cells co-staining for amylase and CK-19 were quantified per high power field in merged images from a Zeiss Axio Imager widefield fluorescence microscope in a blinded fashion.

### **2.2.6 Senescence-associated $\beta$ -galactosidase staining**

Snap-frozen pancreatic samples were stained as described by Debacq-Chainiaux et al (Debacq-Chainiaux, Erusalimsky et al. 2009) and 5 randomly selected, high-power, images from an Olympus Vanox X microscope were quantified using Image J software in a blinded fashion. Color thresholding was used to determine the total amount of staining per high power field, and the freehand selection tool was used to select only the lesions, where color thresholding was applied to calculate the percentage of positive staining area from only the lesions.

### **2.2.7 Ki67, CC3 and p16 immunohistochemistry**

Heat-induced antigen retrieval was performed on formalin-fixed, paraffin-embedded sections, followed by staining with an anti-Ki67 primary antibody at a 1:50 dilution (Dako M7249), an anti-CC3 primary antibody at a 1:100 dilution (Cell Signaling, D175), or anti-p16 primary antibody (BD Biosciences, 551153) at a 1:100 dilution. Peroxidase-based detection was performed using Vectastain Elite ABC Kit (Vector labs). The total number of Ki67-positive staining cells per field in each of 5 randomly selected, high-power fields was counted in a blinded fashion, as well as the number of lesions with at least one positive-staining cell and quantified as a percentage of the total number of lesions. For CC3 and p16, 5 randomly selected, high-power fields were quantified in a blinded fashion using Image J software. Color thresholding was used to determine the total amount of CC3 and p16 staining per high-power field, and the freehand selection tool was used to select only the lesions, where color thresholding was applied to calculate the percentage of positive staining area from only the lesions.

### 2.2.8 Hras-GTP analysis

Early passage (within 5 passages from adaption to culture) *Hras*<sup>-/-</sup> KPC cell lines were stably infected with retroviruses derived from pBabePuro (vector control) or pBabePuroFLAG-Hras encoding wild-type mouse *Hras* cDNA, and selected with puromycin, as previously described (Lim, Ancrile et al. 2008). Stable lines were then tested within 2 passages for Hras GTP levels by affinity capture with the RBD of Raf followed by immunoblot for HRAS with an  $\alpha$ -HRAS antibody (Santa Cruz, sc520), as previously described (de Rooij and Bos 1997).

### 2.2.9 PCR of *Kras* alleles

DNA was isolated from pancreatic tissue, facial papillomas, or vulvar tumors and amplified by PCR to detect the wild-type and recombined *Kras* alleles as previously described (Hingorani, Petricoin et al. 2003).

### 2.2.10 Statistics

Statistical analyses were performed using GraphPad Prism v5 (GraphPad Software). A 2-sided, unpaired t-test was used to compare the amount of normal acinar tissue remaining in the pancreata in *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> KC and KPPC mice, and to compare levels of Ki67, p16, ADM, SA- $\beta$ -gal, and CC3 staining in each cohort. A 2-sided unpaired t-test was also used to compare the levels of each type of graded lesions at the specified time-points and the number of facial papillomas between cohorts. These results were then confirmed by Mann-Whitney test using the same software. Kaplan-Meier survival curves were generated for each of the KPC and KPPC cohorts, as well as

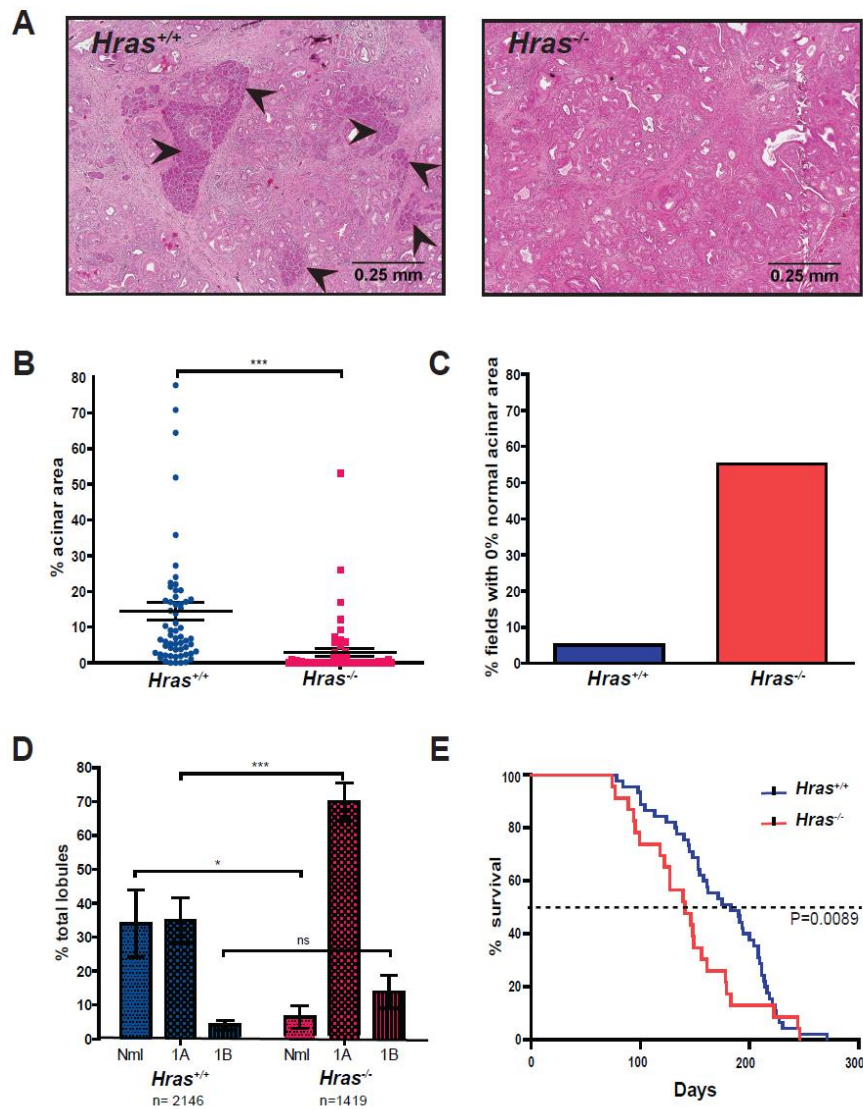
for the development of facial and vulvar papillomas over time in the KPC cohort, and P-values were calculated using the log-rank (Mantel-Cox) test.

## 2.3 Results

### 2.3.1 Increased number of PanIN lesions in the absence of wild-type Hras

To assess the role of wild-type Ras proteins on oncogenic *Kras*-driven pancreatic tumorigenesis *in vivo*, mice were generated with either homozygous null *Hras*<sup>-/-</sup> (Esteban, Vicario-Abejon et al. 2001) or wild-type *Hras*<sup>+/+</sup> alleles in a *Kras*<sup>LSL-G12D/+</sup>; *Pdx-1-Cre*<sup>tg/+</sup> (KC) background (Hingorani, Petricoin et al. 2003). We chose Hras, as it is activated downstream of oncogenic Kras in PDAC cell lines (Lim, Ancrile et al. 2008, Jeng, Taylor et al. 2012, Young, Lou et al. 2013), and of the three *Ras* genes, is the only one that is not embryonic lethal in a background like that of KC mice which encodes only one functional *Kras* allele (Esteban, Vicario-Abejon et al. 2001). We chose the KC model because the activation of an endogenous oncogenic (G12D) *Kras* in the developing pancreas of these mice results in the development of pre-invasive pancreatic intraepithelial neoplasias (PanINs) that progress at a low frequency to PDAC through a series of histological changes that resemble those seen in human patients (Hingorani, Petricoin et al. 2003). Cohorts of 12 *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> KC mice were generated and then euthanized at 36 weeks of age, a time when a spectrum of PanIN lesions are easily detected (Hingorani, Petricoin et al. 2003). H & E staining of pancreatic sections revealed a frank loss of normal tissue and expansion of PanIN lesions in the *Hras*<sup>-/-</sup> KC

mice (**Figure 4A**). Quantification of the amount of normal acinar tissue revealed that *Hras*<sup>+/+</sup> KC mice had an average of 14.5% normal tissue/field, whereas *Hras*<sup>-/-</sup> KC mice had 2.9%, a significant decrease of nearly 5-fold in *Hras*<sup>-/-</sup> KC mice (**Figure 4B**). This effect was more evident when the percentage of sections lacking normal acinar tissue was quantified. 5% of sections in *Hras*<sup>+/+</sup> KC mice lacked normal acinar tissue whereas this value was 55.1% in *Hras*<sup>-/-</sup> KC mice, an increase of over 11-fold in *Hras*<sup>-/-</sup> KC mice (**Figure 4C**). Pathological grading of 2146 pancreatic lobes from *Hras*<sup>+/+</sup> KC mice and 1419 lobes from *Hras*<sup>-/-</sup> KC mice revealed 27.4% fewer lobes with no lesions and 30% more lobes with the highest grade being PanIN 1A in *Hras*<sup>-/-</sup> KC mice (**Figure 4D**). Thus, wild-type *Hras* suppresses the tumorigenic activity of oncogenic *Kras* during pancreatic tumorigenesis.



**Figure 4 Mice lacking wild-type *Hras* develop more PanIN lesions and have reduced survival in oncogenic *Kras*-driven models of pancreatic cancer**

(A) Representative H & E stained sections (arrowheads: normal acinar cells), (B) quantification of % total normal acinar area remaining per field (at 4x magnification, 4–5 fields from 12 mice, bar: mean  $\pm$  S.E.M.), (C) % of fields (at 4x magnification) with no normal acinar tissue (based on the data from B), and (D) % of lobules with the indicated highest grade lesion (Nml: normal, 1A: PanIN-1A, 1B: PanIN-1B, bar: mean  $\pm$  S.E.M.) of pancreata isolated from *Hras*<sup>+/+</sup> versus *Hras*<sup>-/-</sup> KPC mice at 9 months of age. (E) Kaplan-Meier curve of *Hras*<sup>+/+</sup> (n = 45) versus *Hras*<sup>-/-</sup> (n = 23) KPC mice. ns: not significant. \*P<0.05. \*\*\*P<0.0001.

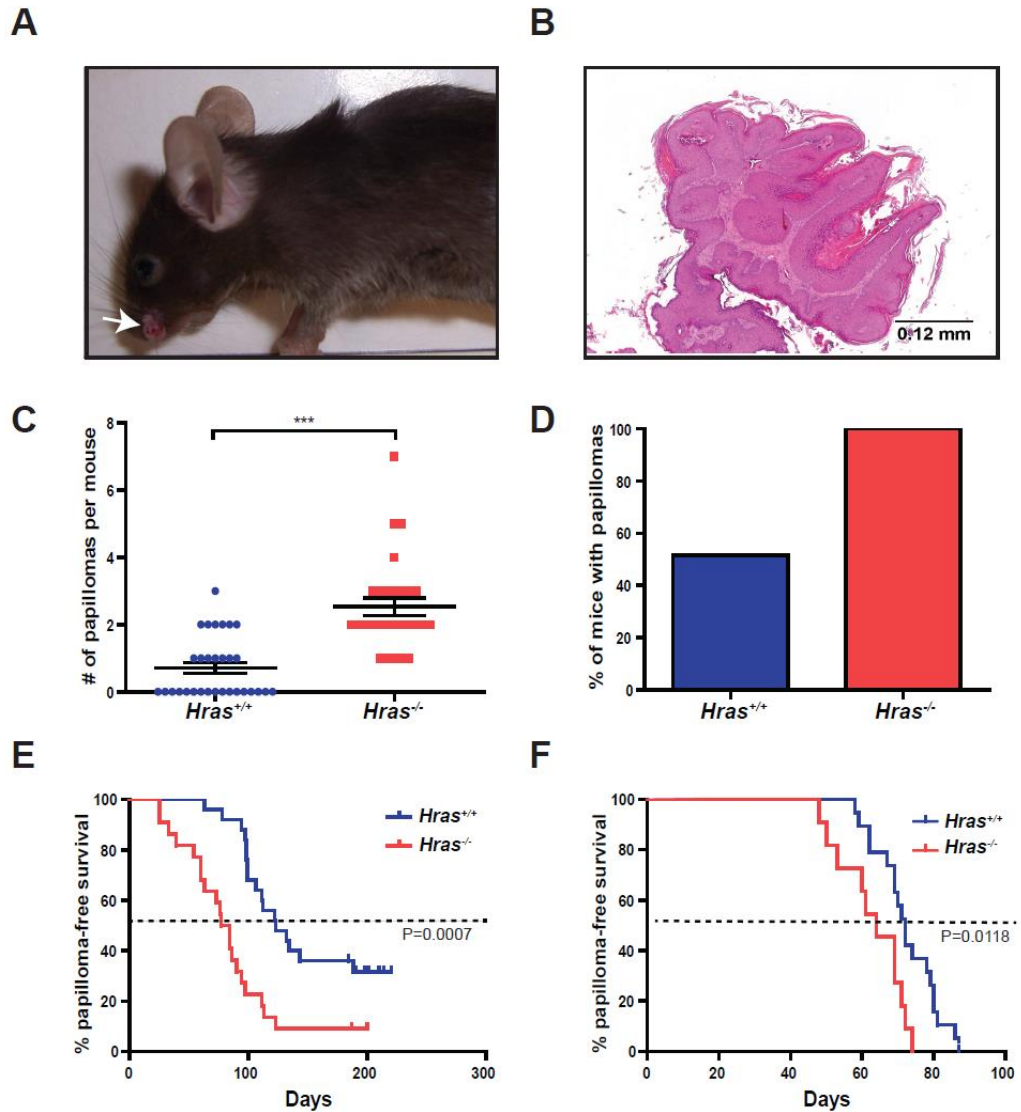
### 2.3.2 Decreased survival in the absence of wild-type Hras

Admittedly, the above approach did not determine the effect of *Hras* loss on the commonly diagnosed stage of PDAC (Vincent, Herman et al. 2011) or on survival, the most clinically relevant endpoint. To overcome these shortcomings, we generated *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> mice in a *Kras*<sup>LSL-G12D/+</sup>; *Trp53*<sup>LSL-R172H/+</sup>; *Pdx-1-Cre*<sup>tg/+</sup> (KPC) setting (Hingorani, Wang et al. 2005). These mice are based on the KC genetic background, but contain an additional inducible dominant-negative *Trp53*<sup>R172H</sup> allele, which promotes PDAC that pathologically resembles the human disease (Hingorani, Wang et al. 2005). Cohorts of 45 *Hras*<sup>+/+</sup> and 23 *Hras*<sup>-/-</sup> KPC mice were generated and monitored regularly. Mice were euthanized upon reaching humane moribundity endpoints to determine their lifespan. This analysis revealed that the median survival in *Hras*<sup>+/+</sup> KPC mice was 183 days, but 141 days in *Hras*<sup>-/-</sup> KPC mice, a significant reduction of nearly a quarter in *Hras*<sup>-/-</sup> KPC mice (**Figure 4E**). Thus, loss of wild-type *Hras* not only potentiates early oncogenic *Kras*-driven pancreatic tumorigenesis, but is also associated with a reduction in lifespan in mice developing PDAC.

### 2.3.3 Increased number of skin papillomas in the absence of wild-type Hras

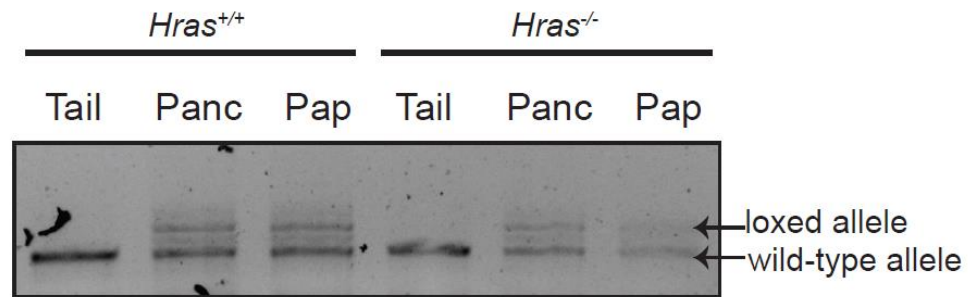
In addition to PanIN lesions, KC mice are prone to develop skin papillomas in the face and vulvar tissues due to Cre expression by *Pdx-1* in these tissues (Hingorani, Petricoin et al. 2003, Gades, Ohash et al. 2008, Mazur, Gruner et al. 2010, Lampson, Kendall et al. 2012). The development of papillomas in the same KC mice provides a well-controlled system to assess the role of wild-type *Hras* in a second independent tissue

type (Lampson, Kendall et al. 2012). To this end, we monitored the number of facial papillomas developing in cohorts of 24 *Hras*<sup>+/+</sup> and 27 *Hras*<sup>-/-</sup> KC mice. Visual inspection revealed an obvious increase in the number of facial lesions (**Figure 5A**) of papilloma pathology (**Figure 5B**) in *Hras*<sup>-/-</sup> KC mice. Quantification revealed that there was an average of 0.7 facial papillomas/mouse in the *Hras*<sup>+/+</sup> KC cohort, but 2.5 in the *Hras*<sup>-/-</sup> KC cohort, a significant increase of nearly 4-fold in *Hras*<sup>-/-</sup> KC mice (**Figure 5C**). Furthermore, while only about half the *Hras*<sup>+/+</sup> KC mice developed a papilloma by the study endpoint, all the *Hras*<sup>-/-</sup> KC mice developed at least one papilloma (**Figure 5D**). PCR of DNA from a subset of these papillomas revealed the expected recombination of the *LSL-Kras*<sup>G12D</sup> allele (**Figure 6**). Perhaps most telling, the average time when these lesions first appeared in *Hras*<sup>+/+</sup> KPC mice was 123 days, but 80.5 days in *Hras*<sup>-/-</sup> KPC mice, a significant decrease of 42.5 days in *Hras*<sup>-/-</sup> KPC mice (**Figure 5D**). Similarly, vulvar papillomas also appeared, on average, 8 days earlier in *Hras*<sup>-/-</sup> KPC mice (**Figure 5E**). Taken together, these results suggest that loss of Hras promotes oncogenic Kras-driven skin tumorigenesis, potentially at a very early stage such as initiation.



**Figure 5: Mice lacking wild-type *Hras* develop more oncogenic *Kras*-driven skin tumors**

(A) Representative photograph (white arrow) and (B) H & E stained section of facial papillomas that develop in KC mice. (C) Number of facial papillomas per *Hras*<sup>+/+</sup> (n = 31) versus *Hras*<sup>-/-</sup> (n = 28) KC mice (bar: mean ± S.E.M.). (D) % of total *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> KC mice (from C) that developed facial papillomas. (E) % facial papilloma-free survival of *Hras*<sup>+/+</sup> (n = 45) versus *Hras*<sup>-/-</sup> (n = 23) KPC mice. (F) % vulvar papilloma-free survival of *Hras*<sup>+/+</sup> (n = 19) versus *Hras*<sup>-/-</sup> (n = 11) female KPC mice. \*\*\*P<0.0001.



**Figure 6: The *LSL-Kras<sup>G12D</sup>* allele is recombined in the pancreas and skin papillomas of KC mice**

Representative PCR amplification using primers specific for the wild-type *Kras* allele or the *LSL-Kras<sup>G12D</sup>* allele following Cre-excision (loxed allele) shows successful recombination in the pancreata (panc) and facial papillomas (pap), but not the negative control tails. DNA was isolated from the tissues of 9-month old *Hras<sup>+/+</sup>* and *Hras<sup>-/-</sup>* mice.

#### 2.3.4 Loss of wild-type *Hras* affects early pancreatic tumorigenesis

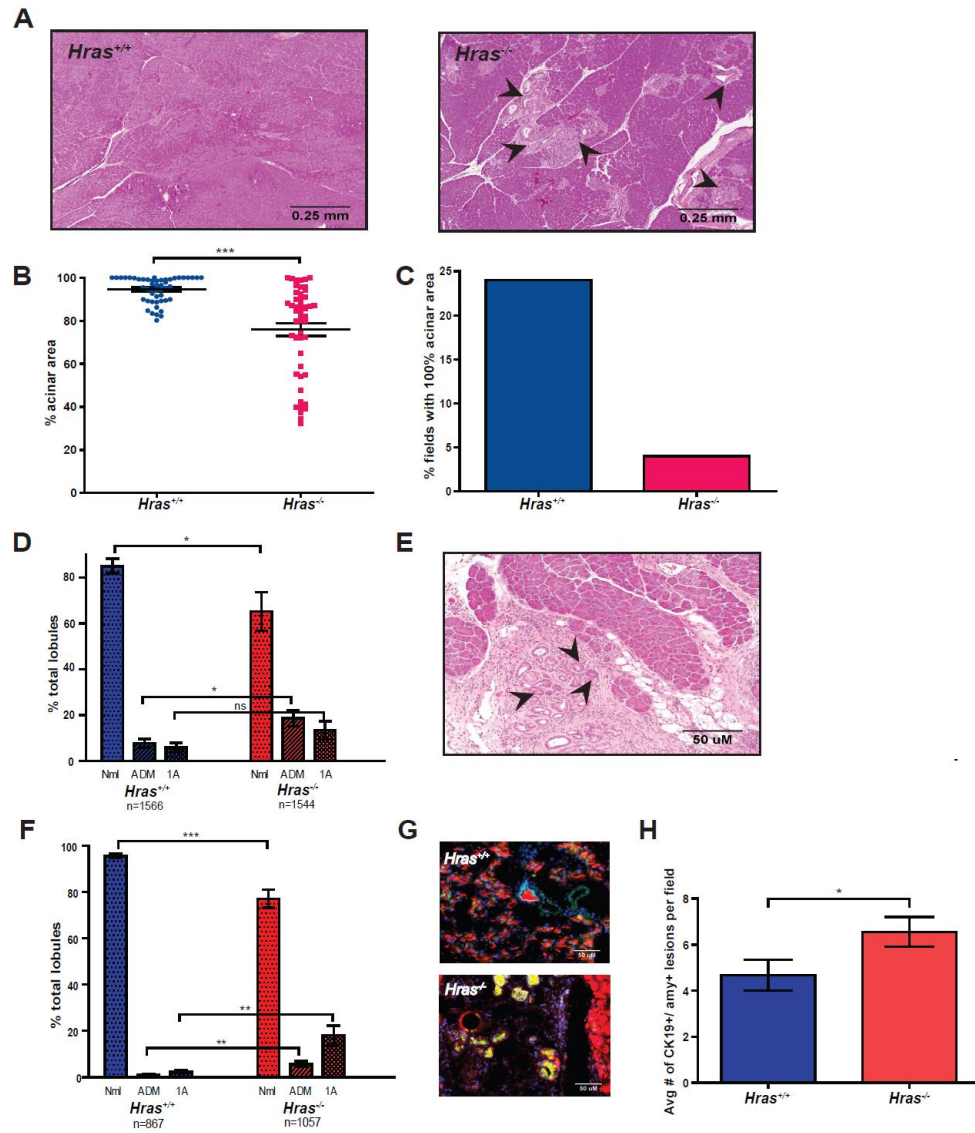
The above observations point towards the *Hras* null background affecting an early stage of tumorigenesis, such as initiation. To assess whether this may explain the observed increase in pancreatic lesions in KC mice and reduced lifespan in KPC mice upon loss of wild-type *Hras*, pancreatic tumorigenesis was analyzed at 8 weeks of age, when KC mice typically exhibit fewer and lower grade lesions (Hingorani, Petricoin et al. 2003). H & E staining of pancreatic sections again revealed a reduction in normal tissue and expansion of PanIN lesions in the *Hras<sup>-/-</sup>* KC mice (**Figure 7A**). Quantification of the amount of normal acinar tissue revealed that *Hras<sup>+/+</sup>* KC mice had an average of 94.6% normal tissue/field, whereas *Hras<sup>-/-</sup>* KC mice had 75.9%, a significant reduction by nearly 20% in *Hras<sup>-/-</sup>* KC mice (**Figure 7B**). This effect was particularly evident when the analysis was repeated by quantitating the number of sections with all the normal

acinar tissue remaining. 24% of the sections in *Hras*<sup>+/+</sup> KC mice had all normal acinar tissue, compared to 4% in *Hras*<sup>-/-</sup> KC mice, a reduction of 6-fold in *Hras*<sup>-/-</sup> KC mice (**Figure 7C**). Pathological grading of 1566 pancreatic lobes from *Hras*<sup>+/+</sup> KC mice and 1544 pancreatic lobes from *Hras*<sup>-/-</sup> KC mice revealed 18.7% more lobes with no lesions and a trend towards more PanIN 1A lesions in *Hras*<sup>-/-</sup> KC mice (**Figure 7D**).

Interestingly, pathological grading also revealed a significant increase of over 2-fold in the number of lobules with acinar-to-ductal metaplasia (ADM) as the highest grade lesion in *Hras*<sup>-/-</sup> KC mice (**Figure 7D**). ADM (*e.g.* **Figure 7E**) is one of the earliest changes in the pancreas of PDAC mouse models, and has been suggested to be a possible precursor to PanIN lesions (Hill, Calvopina et al. 2010, Shi, DiRenzo et al. 2013).

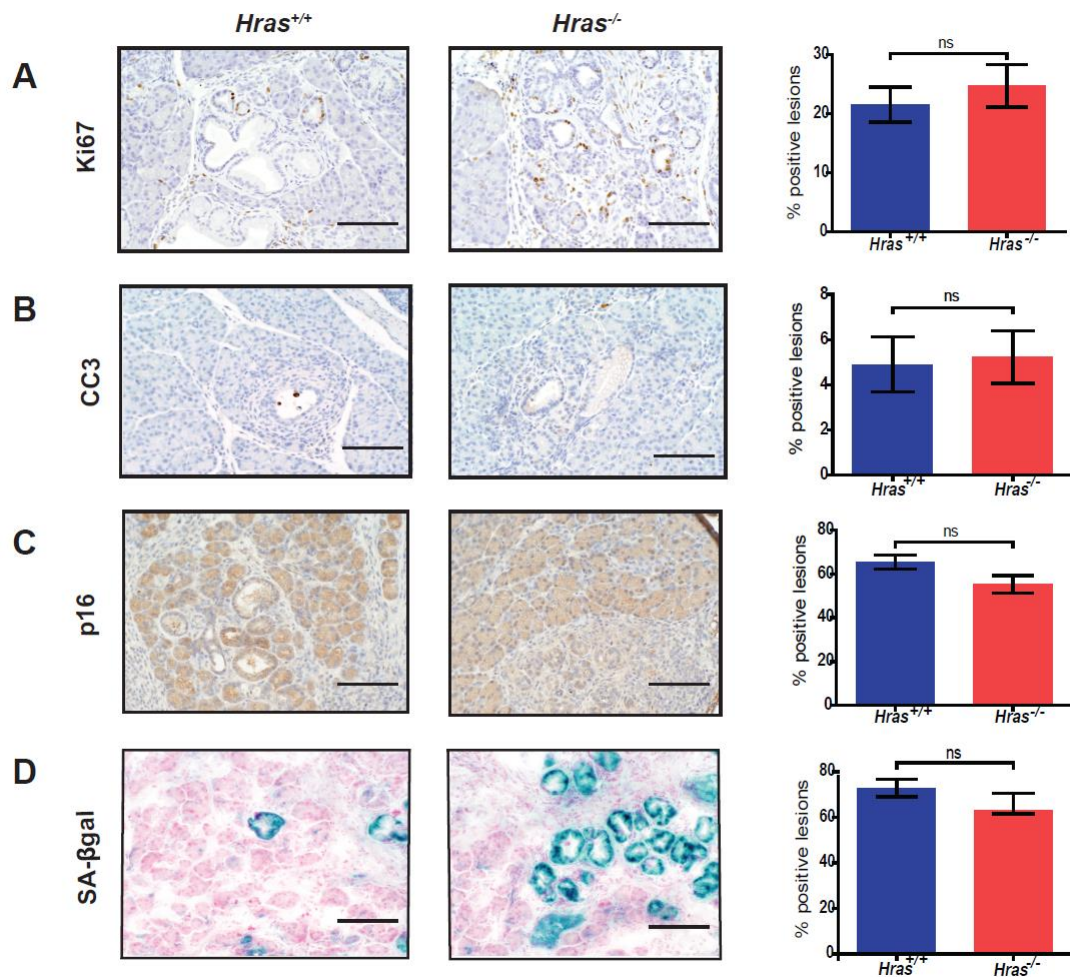
Given the increase in the early grade lesions at 8 weeks in *Hras*<sup>-/-</sup> KC mice, we explored the impact of the *Hras* null genotype at 4 weeks of age when typically there is little or no evidence of pancreatic lesions in KC mice. Pathological grading of 867 lobes from *Hras*<sup>+/+</sup> KC mice revealed, as expected, nearly all the lobes (95.6%) lacked any lesion (**Figure 7F**). In contrast, there was a significant increase of nearly 5-fold in the number of lobes with highest grade of ADM lesions and nearly 7-fold in the number of lobes with PanIN 1A lesions in the *Hras*<sup>-/-</sup> KC mice (**Figure 7F**). We also independently and directly validated the increase in ADM lesions in the *Hras*<sup>-/-</sup> KC mice by quantitating the number of cells co-staining for immunofluorescent markers of acinar (amylase) and ductal (CK-19) cells (**Figure 7G**). Quantification revealed an average of 4.7 amylase/CK-19 co-staining pancreatic lesions/field in *Hras*<sup>+/+</sup> KC mice, but 6.6 in *Hras*<sup>-/-</sup> KC mice, a significant increase in *Hras*<sup>-/-</sup> KC mice (**Figure 7H**). There were no

differences in the amount of Ki67 (a marker of cellular proliferation), CC3 (a marker of apoptosis), SA- $\beta$ -gal or p16 (markers of senescence) positive-staining lesions between *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> KC mice (**Figure 8A-D**), suggesting that the difference imparted by the loss of Hras had already occurred by the time lesions were detected. Taken together, these data suggest that Hras suppresses early pancreatic tumorigenesis, perhaps at the stage of initiation.



**Figure 7: Mice lacking wild-type *Hras* develop more ADM and PanIN lesions at early time points**

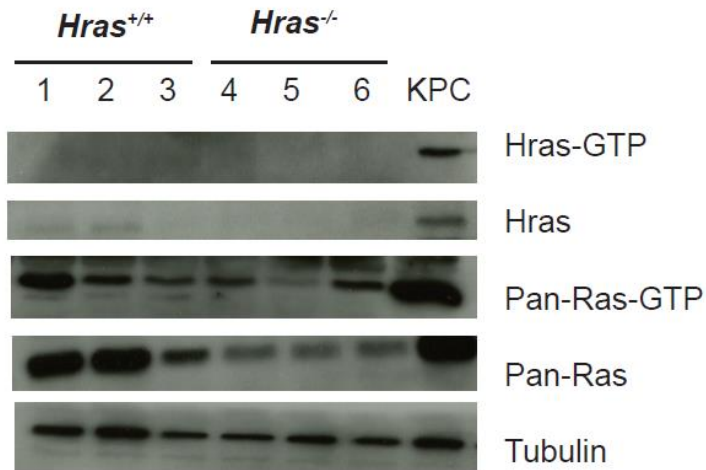
(A) H&E stained section (arrowhead: PanIN), (B) quant. of % acinar area remaining per field (at 4x, 5 fields \*10 mice), (C) % fields with all acinar tissue (from B), and (D) % lobules with the indicated highest grade lesion (Nml: normal, ADM: acinar-to-ductal metaplasia, 1A: PanIN-1A) of pancreata isolated from *Hras*<sup>+/+</sup> vs *Hras*<sup>-/-</sup> KC mice at 8 wks of age. (E) Rep. H&E stained section of a pancreas from an 8-wk old KC mouse (arrowhead: ADM). (F) % lobules with indicated highest grade lesion of pancreata isolated from *Hras*<sup>+/+</sup> versus *Hras*<sup>-/-</sup> KC mice at 4 wks of age. (G) Rep. pancreatic section from a 4-wk old *Hras*<sup>+/+</sup> versus *Hras*<sup>-/-</sup> KC mouse immunostained for cells (DAPI, blue) and markers of acinar (amylase, red) and ductal (CK-19, green) cells to highlight ADM lesions (co-staining, yellow). (H) # amylase<sup>+</sup>/CK-19<sup>+</sup> positive (ADM) cells per field (at 4x, 5 fields \*10 mice) from pancreata isolated from *Hras*<sup>+/+</sup> versus *Hras*<sup>-/-</sup> KC mice at 4 wks of age. (bar: mean ± S.E.M.) \*P<0.05. \*\*\*P<0.0001.



**Figure 8: Immunohistochemical analysis of lesions from 8-week old KC mice** Representative stained sections and quantification of % positive-staining lesions for (A) Ki67 as a marker for proliferation, (B) CC3 as a marker for apoptosis, (C) p16 and, (D) SA-βgal as markers for senescence. For each, the % of positive-staining lesions was quantified in 10 random high-power fields from 5 mice of each cohort. (bar: mean ± S.E.M.). ns = not significant. Line = 50 μM.

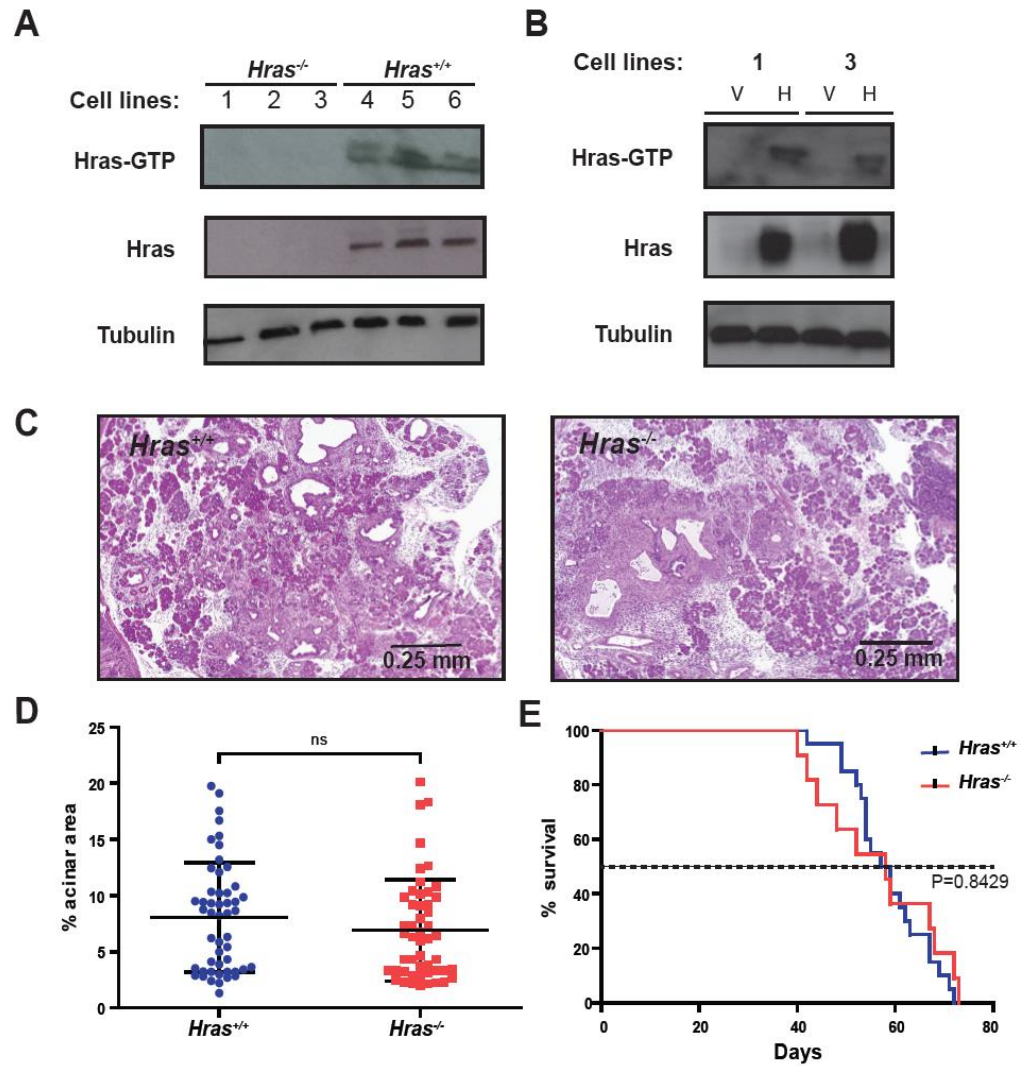
### 2.3.5 Wild-type Hras is activated in PDAC

Wild-type Ras can be activated in the presence of a mutant oncogenic allele in cancer cell lines (Lim, Ancrile et al. 2008, Jeng, Taylor et al. 2012, Young, Lou et al. 2013, Grabocka, Pylayeva-Gupta et al. 2014). In pancreatic tissue at 8 weeks of age, when there are few PanIN lesions in *Hras*<sup>+/+</sup> KC mice (**Figure 7A, B**), Hras was not detected at appreciable levels (**Figure 9**). To assess whether the loss of Hras reduced the pool of active GTP-bound Ras in PDAC, we first measured whether endogenous Hras was activated in PDAC tumor cells derived from KPC mice. To this end, PDAC cell lines were established from three different *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> KPC mice. GTP-bound Hras was then affinity captured with Raf-RBD followed by immunoblotting with an anti-Hras antibody to detect the protein. In all three *Hras*<sup>+/+</sup> KPC cell lines, Hras-GTP was recovered. In contrast, this pool of active Hras was completely absent in the PDAC tumor cells derived from the three *Hras*<sup>-/-</sup> KPC mice (**Figure 10A**). Moreover, this pool of active Ras was restored upon re-expressing wild-type Hras in two *Hras*<sup>-/-</sup> KPC cell lines at early passage (**Figure 10B**). Thus, loss of Hras removes a pool of active Ras from pancreatic tumor cells.



**Figure 9: Ras-GTP levels in 8-week old KC mice**

Lysates were prepared from flash frozen pancreatic tissue from 3 *Hras*<sup>+/+</sup> (1-3) and 3 *Hras*<sup>-/-</sup> (4-6) KC mice. Immunoblot analysis was performed for Pan-Ras, Pan-Ras-GTP, Hras, and Hras-GTP levels, with an *Hras*<sup>+/+</sup> KPC cell line used as a control for Ras-activation. The overall levels of Pan-Ras were lower in the *Hras*<sup>-/-</sup> mice, and 1 of 3 lysates appeared to have lower levels of Pan-Ras activation in comparison to the wild-type cohort. However, wild-type Hras-GTP could not be detected at this early time point. Hras input levels were also very low in this early pancreatic tissue, as Hras could only be detected at low levels in 2 of the 3 *Hras*<sup>+/+</sup> lysates.



**Figure 10: Wild-type Hras is activated in KPC cell lines and *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> KPPC mice exhibit similar tumor burden and lifespan**

Immunoblot analysis of Hras and Hras-GTP in PDAC cell lines derived from (A) *Hras*<sup>-/-</sup> (cell lines 1–3) and *Hras*<sup>+/+</sup> (cell lines 4–6) KPC mice or (B) PDAC cell lines 1 and 3 derived from *Hras*<sup>-/-</sup> KPC mice stably infected with a retrovirus encoding no transgene (vector, V) or wild-type Hras (H). Tubulin serves as a loading control. (C) Representative H&E stained sections from pancreata of *Hras*<sup>+/+</sup> versus *Hras*<sup>-/-</sup> KPPC mice at 14 days of age. (4x magnification) (D) Quantification of the % normal acinar area remaining per field (at 4x magnification, 5 fields from 10 mice) from *Hras*<sup>+/+</sup> versus *Hras*<sup>-/-</sup> KPPC mice (bar: mean ± S.E.M). (E) Kaplan-Meier survival curve of *Hras*<sup>+/+</sup> (n = 20) versus *Hras*<sup>-/-</sup> (n = 11) KPPC mice. ns: not significant.

### 2.3.6 Loss of wild-type Hras has no overt effect on pancreatic tumorigenesis in a homozygous mutant p53 background

To assess the effect on pancreatic tumorigenesis upon losing Hras in a homozygous *Trp53<sup>R172H</sup>* background, which has been reported to suppress a loss of pancreatic cells when the *LSL-Kras<sup>G12D</sup>* allele is activated (Morton, Timpson et al. 2010), cohorts of 10 *Hras<sup>+/+</sup>* versus *Hras<sup>-/-</sup>* mice in a *Kras<sup>LSL-G12D/+</sup>;Trp53<sup>LSL-R172H/LSL-R172H</sup>;Pdx-1-Cre<sup>tg/+</sup>* (KPPC) background were euthanized at a fixed time point of 14 days of age. The amount of normal acinar tissue remaining in the pancreas was then assessed (**Figure 10C**). Quantification of H & E stained pancreatic sections revealed no significant difference in the amount of normal tissue remaining between the two cohorts (**Figure 10D**). Cohorts of 20 *Hras<sup>+/+</sup>* and 11 *Hras<sup>-/-</sup>* KPPC mice were also allowed to age until moribundity endpoints, revealing a nearly identical median survival of 50 and 47.5 days, respectively (**Figure 10E**). With the caveat that the KPPC background may be so aggressive as to mask any effect on early tumorigenesis caused by the loss of *Hras*, these data are consistent with a model whereby a homozygous mutant p53 background can negate the tumor-suppressive effects of wild-type *Hras* on both pancreatic tumorigenesis and survival.

## 2.4 Discussion

We report that a homozygous null *Hras* background led to more and higher grade pre-invasive PanIN lesions in the KC mouse model of early pancreatic cancer. In agreement, reducing the levels of wild-type Ras signaling also promotes tumorigenesis in

other models of pre-invasive cancer (Balmain, Brown et al. 1990, Zhang, Wang et al. 2001, Diaz, Ahn et al. 2002, To, Rosario et al. 2013). Multiple lines of evidence point towards these phenotypes being a manifestation of the loss of Hras at a very early stage of pancreatic tumorigenesis. First, the percentages of proliferation, apoptosis, and senescence marker-positive PanIN lesions was similar between *Hras*<sup>-/-</sup> and *Hras*<sup>+/+</sup> KC mice at an early time point, suggestive of a difference prior to the detection of these lesions. Second, skin papillomas were detected earlier and at a higher frequency in the *Hras*<sup>-/-</sup> background, consistent with an effect on tumor initiation. In agreement, there were more ADM and PanIN1A lesions in *Hras*<sup>-/-</sup> KC mice at early time points, both of which have been argued to reflect an initiating event (Reichert and Rustgi 2011). We attempted to assess if this effect occurred at an even earlier time point by assaying for recombination of LSL-EGFP as a surrogate marker for cells with an activated *Kras*<sup>G12D</sup> allele, but mosaic staining for GFP precluded any meaningful analysis (**Figure 11**). Collectively, these results suggest that the loss of Hras enhances the earliest stages of pancreatic tumorigenesis, perhaps even initiation. Taking this concept one step further, perhaps epigenetic differences in levels of Ras proteins may influence whether an oncogenic mutation in *KRAS* leads to pancreatic tumorigenesis. As KPC mice had a reduced lifespan in the *Hras*<sup>-/-</sup> background, such early effects of wild-type Ras proteins may have lasting repercussions.

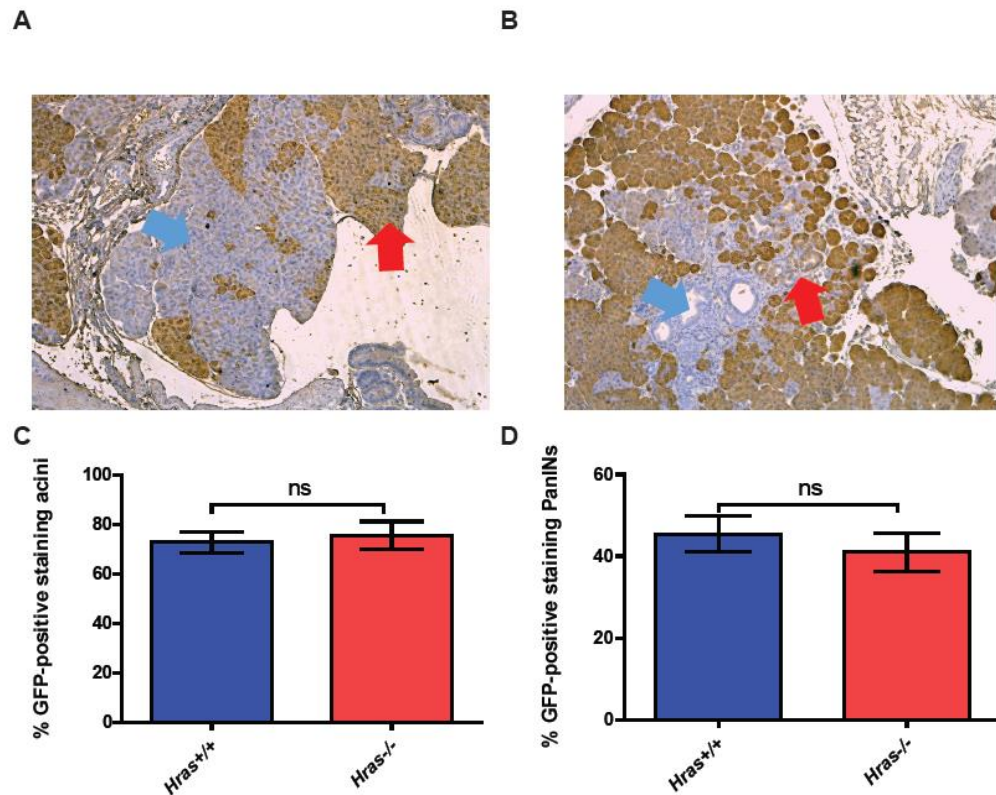
While it remains to be determined how wild-type Hras suppresses early pancreatic tumorigenesis, a number of mechanisms are possible. One possibility is that the loss of Hras may reduce the pool of active Ras to a level below that which triggers a growth

arrest in response to activation of oncogenic *Kras*, increasing the chances that an oncogenic mutation in *Kras* will initiate tumorigenesis. More specifically, normal cells can be sensitive to high levels of Ras signaling, resulting in senescence rather than proliferation, which can inhibit tumor formation (Serrano, Lin et al. 1997, Collado, Gil et al. 2005). In this regard, pancreatic cells expressing oncogenic *Kras* have been reported to be selectively lost at two to six weeks of age, apparently a consequence of high *Kras* signaling (Morton, Timpson et al. 2010). As such, changes that reduce this signaling may promote tumor initiation. In this regard, we demonstrated that loss of *Hras* reduced the pool of active Ras in pancreatic cancer cells. Bi-allelic loss of wild-type p53 has been shown to rescue the early loss of pancreatic cells upon activation of oncogenic *Kras* (Morton, Timpson et al. 2010). In agreement, both tumor burden and lifespan were similar between the *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> genotypes in a homozygous mutant p53 background of pancreatic cancer. Admittedly, there was no difference in  $\beta$ -galactosidase staining, a common marker for senescence, between *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> *KC* mice at 8-weeks of age, and endogenous *Hras* was not detected by immunoblot in the pancreatic tissue at this time point. A number of other mechanisms are most certainly possible. For example, loss of *Hras* may reduce apoptosis or increase proliferation. However, as with the case of senescence markers, there was no difference in Ki67, cleaved caspase 3, or TUNEL staining between *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> *KC* mice at 8-weeks of age, arguing either against this model or pointing towards an effect at an earlier time point. Alternatively, rather than the loss of *Hras* reducing the amplitude of Ras signaling, it is also possible that this loss changes the spectrum of this signaling. More specifically, Ras isoforms

exhibit differences in their subcellular localizations, which may result in engagement of effector proteins in different locations within the cells (reviewed in Hancock, 2003). Loss of Hras may thus reduce the diversity of this signaling. It has also been suggested that wild-type Ras proteins may sequester effectors from the oncogenic protein, thereby reducing the amplitude or altering the signaling output, which may manifest as increased tumorigenesis when wild-type Hras is deleted (Pylayeva-Gupta, Grabocka et al. 2011) . The loss of Hras during development may lead to an increase in the type of cells that ultimately give rise to pancreatic cancer, due to increased proliferation, decreased apoptosis or senescence, or altered differentiation. Finally, it is even possible that the loss of Hras in the stroma underlies the increase in pancreatic lesions observed in *Hras*<sup>-/-</sup> *KC* mice.

We fully recognize that the tumor-suppressive effect of wild-type Hras may very well be context dependent. Indeed, loss of *Nras* has been shown to promote oncogenic *Kras*-driven lung tumorigenesis, yet inhibit oncogenic Hras-driven skin tumorigenesis (To, Rosario et al. 2013). Similarly, two oncogenic alleles of *Nras* were found to be more tumorigenic than one allele in a mouse model of CMML and JMML (Xu, Haigis et al. 2013). The negative impact of wild-type Ras proteins on tumorigenesis may also be negated during tumor progression, for example, when cells become resistant to higher levels of Ras signaling. Wild-type Ras proteins may even promote more malignant stages of tumorigenesis, as shRNA-mediated knock down of wild-type Ras proteins has been shown to inhibit the tumor growth of *KRAS* mutation-positive cancer cell lines isolated from late stage disease (Lim, Ancrile et al. 2008, Jeng, Taylor et al. 2012,

Young, Lou et al. 2013, Grabocka, Pylayeva-Gupta et al. 2014). Nevertheless, we find that *Hras* is clearly tumor suppressive in pancreatic cancer, at least at early stages of this disease, which could have important ramifications on the susceptibility of developing pancreatic cancer upon oncogenic insult.



**Figure 11: Immunostaining for GFP in KC mice**

5 *Hras*<sup>+/+</sup> and 5 *Hras*<sup>-/-</sup> KC mice containing *LSL-EGFP* were stained for GFP. (A), Acinar tissue showed mosaic staining as indicated by the arrows. The blue arrow shows an area of negative-staining for GFP, while the red arrow shows an area positive for GFP-staining. (B), An example of PanINs staining negative are shown by the blue arrow, while a positive area of PanINs is indicated by the red arrow. (C), Quantification of GFP-positive staining acinar cells (n=25 for each cohort, 5 fields X 5 mice). (D), Quantification of percentage of GFP-positive staining PanINs (n=25 for each cohort, 5 fields X 5 mice).

### **3.0 Wild type Hras, but not Nras, suppresses carcinogen-induced lung cancer in mice.**

This work was done with the assistance of pathologists John Carney, Elizabeth Pavlisko, and Diana Cardona. Drs. Carney and Pavlisko quantified and graded the H & E stained lung lesions. Immunohistochemical staining of lung sections was performed by the Duke Research Pathology Laboratory. I performed all mouse breeding and genotyping, assessment, preparation and H&E staining of slides, presentation of data, and statistical analyses.

#### **3.1 Introduction**

Approximately 30% of all individuals diagnosed with cancer harbor an activating mutation in one of the *RAS* genes (COSMIC database, 2014). This family of genes, composed of three members, *HRAS*, *NRAS*, and *KRAS*, encode highly similar proteins that function as molecular switches, alternating between an inactive, GDP-bound form, and an active-GTP bound form (Pylayeva-Gupta, Grabocka et al. 2011). Regulation of these proteins is carried out by guanine nucleotide exchange factors (GEFs), which promote the exchange of GDP for GTP, activating the protein, and GTPase activating proteins, or GAPs, which enhance the hydrolysis of GTP, reverting these proteins back to their inactive, GDP-bound states (Takai, Sasaki et al. 2001). Oncogenic mutations in these genes, usually found at codons 12, 13, or 61, inactivate the inherent or GAP-stimulated GTPase activity of the enzyme, thereby keeping RAS in a constitutively activated, GTP-bound state. In this state, chronic activation of downstream signaling

pathways, including MAPK, PI3K, and RalGEF, results in uncontrolled cellular proliferation, driving tumor formation and growth (Karnoub and Weinberg 2008). Of the RAS family members, mutations in *KRAS* are most common, and drive tumorigenesis in a number of tissue types, especially pancreatic, colorectal, and lung cancers (Miller and Miller 2011).

Although mutant RAS proteins have been long-studied as drivers of tumorigenesis in a number of different tissue types, it has been more recently shown that the remaining wild-type family members are also activated in the presence of the oncogenic proteins (Lim, Ancrile et al. 2008, Jeng, Taylor et al. 2012, Young, Lou et al. 2013, Huang and Counter 2015, Weyandt, Lampson et al. 2015). The consequences of this activation, however, are complex. In some cases, particularly in models of early stage cancer, the wild-type isoforms inhibit oncogenic RAS-driven tumorigenesis (Zhang, Wang et al. 2001, Diaz, Ahn et al. 2002, To, Rosario et al. 2013, Weyandt, Lampson et al. 2015). In contrast, in *KRAS*-mutation-positive cancer cell lines, wild-type RAS signaling instead promotes mutant RAS-driven proliferation and tumorigenesis (Lim, Ancrile et al. 2008, Jeng, Taylor et al. 2012, To, Rosario et al. 2013, Young, Lou et al. 2013, Grabocka, Pylayeva-Gupta et al. 2014, Huang and Counter 2015). These seemingly confounding results suggest that wild-type RAS proteins may have both tissue and context-dependent effects in oncogenic RAS-driven cancers.

With regards to lung cancer, loss of both alleles of *Hras* or *Nras*, or loss of the remaining wild-type *Kras* allele, significantly increased the number of oncogenic *Kras*-driven lung tumors in mice exposed to the carcinogen urethane (You, Candrian et al.

1989, Zhang, Wang et al. 2001, To, Rosario et al. 2013). These findings suggest that the loss of wild-type Ras proteins may have an additive effect in enhancing lung tumorigenesis. To test this possibility, I generated littermates with different allele combinations of *Nras* and/or *Hras* null alleles. I then used chemical carcinogenesis to assess the effects of the loss of these different alleles on tumor formation and growth.

## **3.2 Materials and Methods**

### **3.2.1 Generation of mice and genotyping**

*Hras*<sup>-/-</sup> and *Nras*<sup>-/-</sup> mice were obtained from the NCI and Eugenio Santos. *Hras*<sup>+/-</sup>; *Nras*<sup>+/-</sup> mice on a mixed 129/BL6 background were bred to obtain littermates with various combination of native and null *Hras* and *Nras* alleles. Genomic DNA isolated from a total of 268 pups were genotyped for *Hras* and *Nras* wild-type and null alleles as previously described (Esteban, Vicario-Abejon et al. 2001).

### **3.2.2 Urethane Carcinogenesis**

7-9 week-old mice (9-14 mice from each cohort) were injected intraperitoneally with urethane at 1mg/per g body weight as previously described (Zhang, Wang et al. 2001), and regularly monitored for moribundity endpoints of greater than 15% weight gain or signs of pain and distress according to the Duke University Institutional Animal Care and Use Guidelines until they were euthanized at 11-months post-injection.

### **3.2.3 Quantification of Lesions**

Mice were euthanized 11-months after administration of urethane, the lungs were removed, examined, and tumors were measured and counted with the investigator blinded to the genotype. The left lung from each mouse was formalin-fixed and paraffin-embedded for pathological analyses. The paraffin-embedded lungs were sectioned every 200 microns and each 8 micron slice was H&E stained. Pathologists John Carney and Elizabeth Pavlisko graded and counted the lesions in each section from 9-14 mice per cohort in a blinded fashion.

### **3.2.4 Immunohistochemistry**

8  $\mu\text{m}$ -thick sections cut from the left lungs of urethane-treated mice were subjected to epitope retrieval and stained with, anti-Ki67 (Thermo Scientific RM9106), anti-CC3 (Cell Signaling, D175), anti-P(Thr 202/Tyr 204)-Erk1/2 (Cell Signaling 4376), and anti-P(Ser473)-Akt (Cell Signaling 4060) antibodies followed by peroxidase-based detection with Vectastain Elite ABC Kits (Vector Labs) and counterstained with haematoxylin. Photographs were taken of 6-10 (10X) random fields in a blinded fashion. The tumor areas were determined from these photographs using the freehand selection tool in Image J ([imagej.nih.gov](http://imagej.nih.gov)). Color thresholding was applied to determine positive-staining areas, using the same parameters for each tumor image. Areas staining positive by these parameters were selected and the positive-staining area in pixels recorded. The percentage of positive-staining area was calculated by dividing the positive-staining area of the tumor in pixels by the total area of tumors in pixels.

### 3.2.5 Statistics

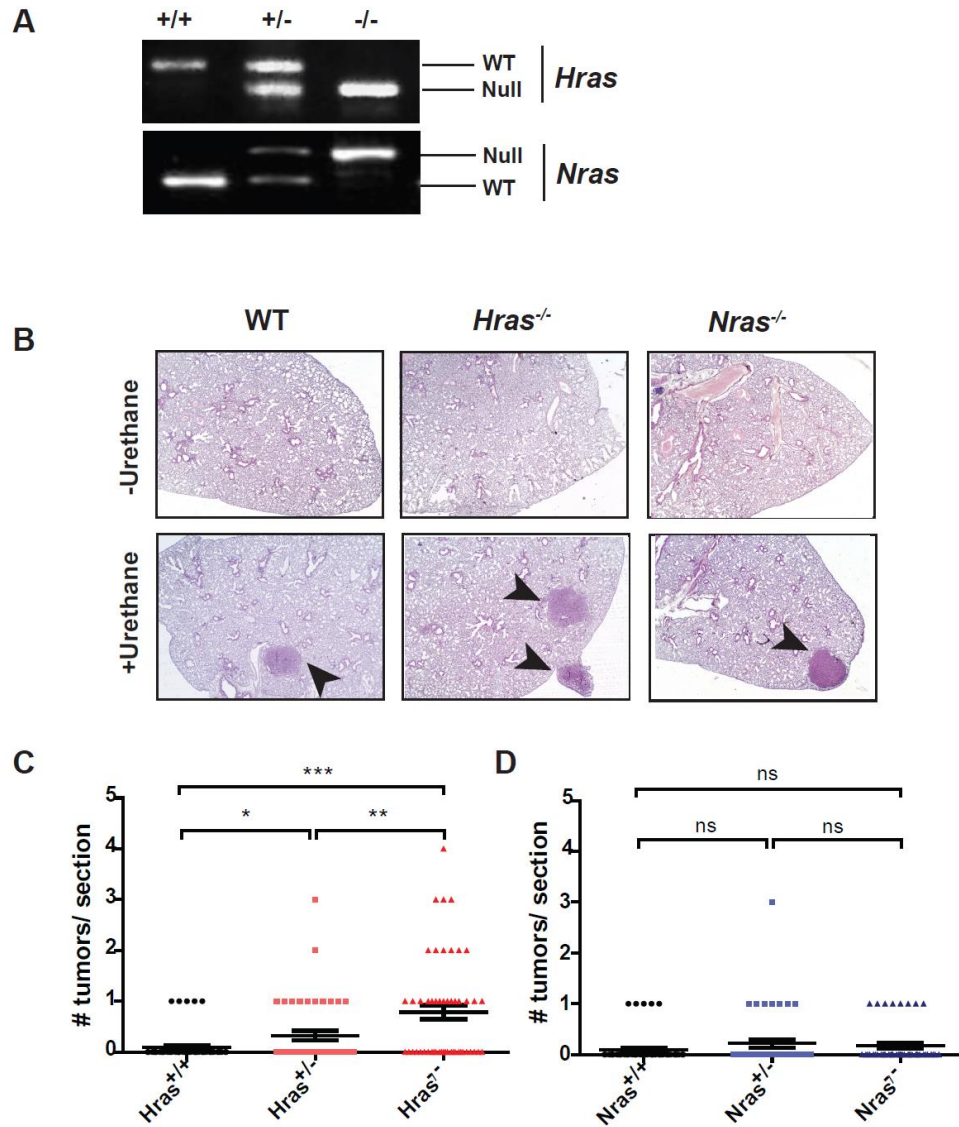
Statistical Analyses were performed using GraphPad Prism v5 (GraphPad Software). A 2-sided, unpaired *t*-test was used to compare the number of lesions and levels of IHC staining between cohorts, and P-values were calculated using the log-rank (Mantel-Cox) test.

## 3.3 Results

### 3.3.1 Loss of wild-type Hras, but not Nras, promotes the formation of urethane-induced lung tumors

To compare the roles of the wild-type Hras and Nras isoforms on lung tumorigenesis, I interbred *Hras*<sup>+/-</sup>;*Nras*<sup>+/-</sup> mice on a mixed 129/B6 background, generating littermates with the following genotypes: *Hras*<sup>+/+</sup>;*Nras*<sup>+/+</sup>, *Hras*<sup>+/-</sup>;*Nras*<sup>+/+</sup>, *Hras*<sup>+/+</sup>;*Nras*<sup>+/-</sup>, *Hras*<sup>+/-</sup>;*Nras*<sup>+/-</sup>, *Hras*<sup>-/-</sup>;*Nras*<sup>+/+</sup>, *Hras*<sup>+/+</sup>;*Nras*<sup>-/-</sup>, *Hras*<sup>+/-</sup>;*Nras*<sup>-/-</sup>, *Hras*<sup>-/-</sup>;*Nras*<sup>+/-</sup>, and *Hras*<sup>-/-</sup>;*Nras*<sup>-/-</sup>. PCR was used to identify genotypes (**Figure 12A**) of 268 pups, which revealed the expected distribution of genotypes (**Table 1**). 9-14 mice of each genotype were then treated with the chemical carcinogen urethane at 7-9 weeks of age to induce *Kras*-mutation-positive lung lesions. These mice were then euthanized 11 months later, and tumor number and size determined. Consistent with previous studies (To, Rosario et al. 2013), microscopic analysis of the left lungs from these mice revealed that mice with one null allele of *Hras* developed roughly three times more tumors ( $p=0.0214$ , **Figure 12B, C**). This effect was further exasperated by loss of the second wild-type allele, as *Hras*<sup>-/-</sup> mice developed on average, more than twice as many tumors

as *Hras*<sup>+/-</sup> mice (p=0.0021 **Figure 12B, C**). In contrast, however, there was no statistically significant difference in the number of tumors in mice having either one (p=0.1738) or two (p=0.2437) null *Nras* alleles (**Figure 12B, D**). We conclude that in this mixed genetic background, loss of *Hras*, but not *Nras* results in an increase in tumor burden upon exposure to the carcinogen urethane.



**Figure 12: Loss of wild-type Hras, but not Nras, promotes formation of urethane induced lung tumors**

(A) PCR-genotyping for *Hras* (top panel: wild-type (WT) band =434 bp; Null band = 336 bp), and *Nras* (lower panel: wild-type (WT) band = 146 bp; Null band =315 bp). (B) Representative H&E-stained lung tissue (magnification =2X) from wild-type (WT), *Hras*<sup>-/-</sup>, and *Nras*<sup>-/-</sup> mice (top) untreated and (bottom) 11-months after urethane administration. Arrowheads: lung lesions. (C and D) Number of lesions counted per H&E stained section from left lungs of mice 11-months post-urethane treatment in mice with the indicated combinations of wild-type (+) and null (-) alleles of (C) *Hras* and (D) *Nras*. (bar: mean ± S.E.M.) ns: not significant. \*P<0.05. \*\* P<0.001 \*\*\*P<0.0001. n=4-6 sections from 9-14 mice per cohort.

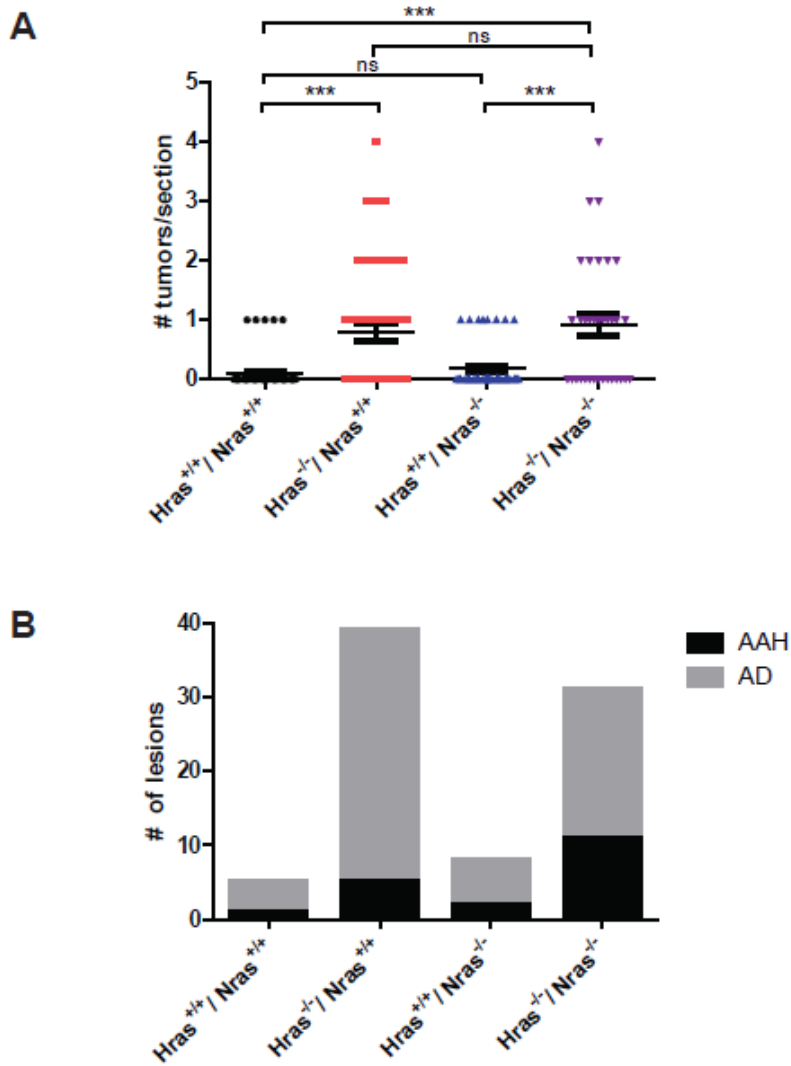
**Table 1: Distribution of Genotypes Obtained from Crossing *Hras*<sup>+/-</sup>;*Nras*<sup>+/-</sup> mice** (total # of mice generated = 268)

<b>Genotype</b>	<b>Expected Ratio</b> % (#)	<b>Observed Ratio</b> % (#)
<i>Hras</i> <sup>+/+</sup> ; <i>Nras</i> <sup>+/+</sup>	6.25 (16.75)	7.46 (20)
<i>Hras</i> <sup>+/-</sup> ; <i>Nras</i> <sup>+/+</sup>	12.5 (33.5)	13.06 (35)
<i>Hras</i> <sup>+/+</sup> ; <i>Nras</i> <sup>+/-</sup>	12.5 (33.5)	12.31 (33)
<i>Hras</i> <sup>+/-</sup> ; <i>Nras</i> <sup>+/-</sup>	25.0 (67)	24.25 (65)
<i>Hras</i> <sup>-/-</sup> ; <i>Nras</i> <sup>+/+</sup>	6.25 (16.75)	5.22 (14)
<i>Hras</i> <sup>+/+</sup> ; <i>Nras</i> <sup>-/-</sup>	6.25 (16.75)	7.46 (20)
<i>Hras</i> <sup>-/-</sup> ; <i>Nras</i> <sup>+/-</sup>	12.5 (33.5)	12.69 (34)
<i>Hras</i> <sup>+/-</sup> ; <i>Nras</i> <sup>-/-</sup>	12.5 (33.5)	14.18 (38)
<i>Hras</i> <sup>-/-</sup> ; <i>Nras</i> <sup>-/-</sup>	6.25 (16.75)	3.36 (9)

### 3.3.2 Concomitant loss of wild-type Hras and Nras does not further effect the formation of urethane-induced tumors

To explore the influence of losing Nras expression on the increase in lung lesions induced by loss of Hras, I also compared the number of lung lesions in the aforementioned *Hras*<sup>+/+</sup>;*Nras*<sup>+/+</sup>; *Hras*<sup>-/-</sup>;*Nras*<sup>+/+</sup>; and *Hras*<sup>+/+</sup>;*Nras*<sup>-/-</sup> mice to *Hras*<sup>-/-</sup>;*Nras*<sup>-/-</sup> double homozygous knockout mice. These latter mice developed approximately the same number of tumors as *Hras*<sup>-/-</sup>; *Nras*<sup>+/+</sup> mice (p=0.5762) (**Figure 13A**). The tumors from these four genotypes were graded independently in a blinded fashion by two pathologists. All but one of the lesions were graded as either atypical adenomatous hyperplasia (AAH) or adenoma (AD), both of which are early stages of lung tumorigenesis. One mouse (*Hras*<sup>-/-</sup>) had a lesion graded as adenocarcinoma (not

shown in figure). Comparing tumors from all four genotypes revealed no significant difference in the grade of lesions between the different genotypes (**Figure 13B**). Based on these observations, I conclude that loss of Hras increases early-stage lung tumorigenesis induced by the carcinogen urethane in the tested mixed strain background, and moreover, loss of Nras does not influence that effect.

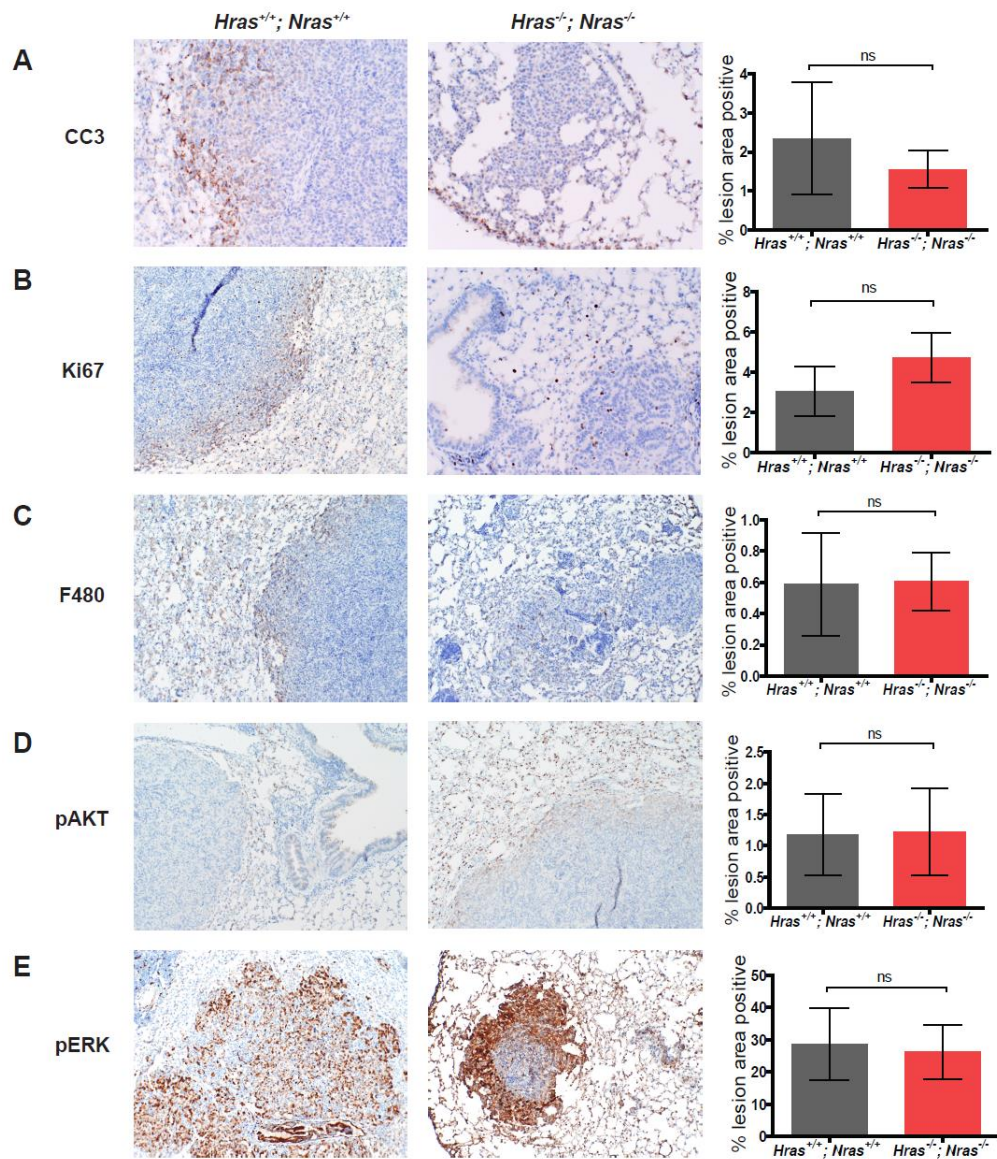


**Figure 13: Effects of concomitant loss of wild-type Hras and Nras on urethane-induced tumorigenesis**

(A.) Number of lesions counted per H&E-stained section of lung tissue from urethane-treated mice of the indicated combinations of wild-type (+) and null (-) alleles for *Hras* and *Nras*. (B.) Quantification of graded lesions from H&E-stained lung sections, AAH=atypical adenomatous hyperplasia, AD = adenoma. (bar: mean  $\pm$  S.E.M.) ns: not significant. \*\*\*P<0.0001. n=4-6 sections from 9-14 mice per cohort.

### 3.3.3 Loss of wild-type Hras and Nras does not affect levels of proliferation, apoptosis, or downstream-signaling within urethane-induced tumors.

To investigate whether the increased number of urethane-induced lung tumors in the absence of wild-type Hras was due to differences in downstream signaling, I assessed immunohistochemical staining of lesions from *Hras*<sup>+/+</sup>;*Nras*<sup>+/+</sup> and *Hras*<sup>-/-</sup>;*Nras*<sup>-/-</sup> mice for markers of apoptosis, proliferation, and activation of downstream pathways. The percentage of positively staining tumor-area was determined for 6-10 lesions from each cohort. No significant differences in immunohistochemical staining using antibodies for markers for apoptosis (Cleaved Caspase 3, **Figure 14A**), proliferation (Ki67, **Figure 14B**), or inflammation, (F480, **Figure 14C**) were observed between these two cohorts. Furthermore, the average levels of pAKT (**Figure 14D**) and pERK (**Figure 14E**) immuno-staining within the lesions were also similar between these two cohorts. Thus, at the stage tested, signaling through the downstream effector pathways of Ras is not significantly different in lesions lacking wild-type Hras and Nras by immunohistochemical analysis.

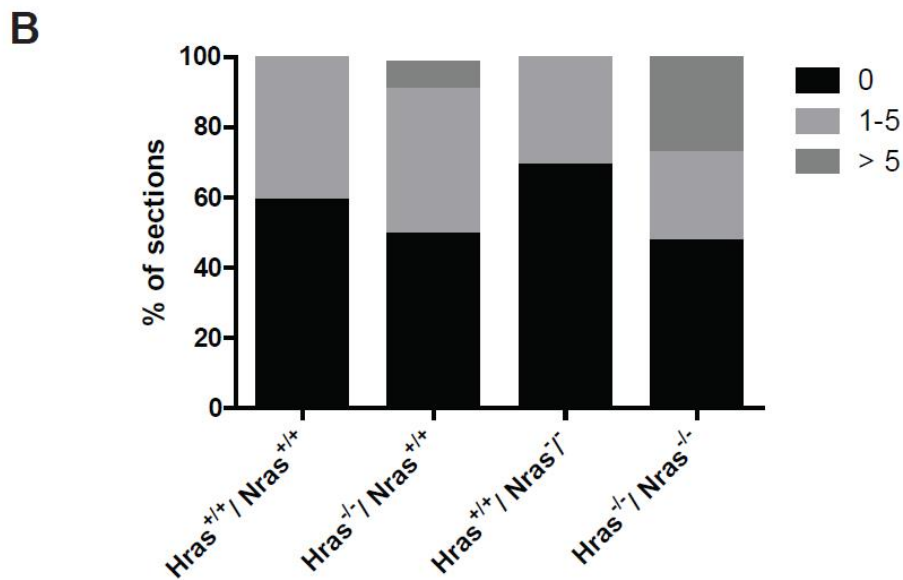
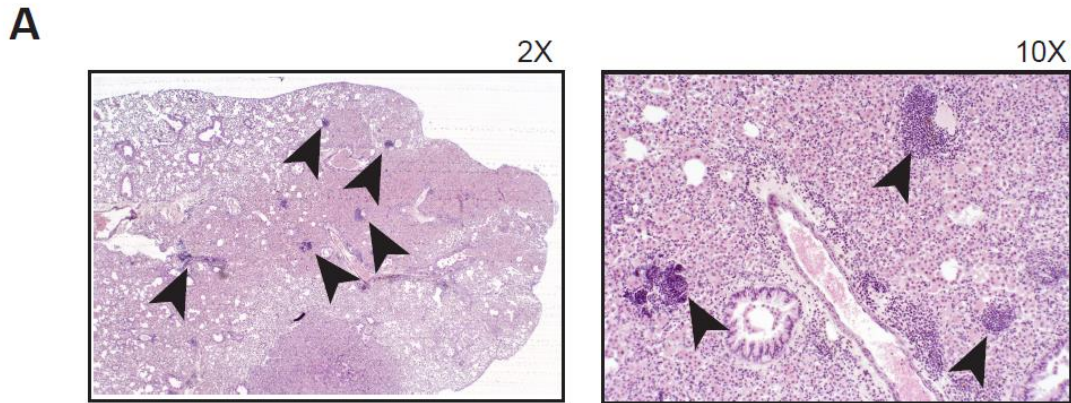


**Figure 14: Immunohistochemical staining in urethane-induced lung lesions.**

Immunohistochemical staining was performed for makers of (A), apoptosis (CC3: cleaved caspase 3), (B), proliferation (Ki67), (C), inflammation (F480 macrophage marker), (D) PI3K pathway signaling (pAKT), and (E) MAPK pathway signaling (pERK). For each antibody, the percentage of positively-staining tumor area was calculated for 6-10 lesions from 3-5 mice with the indicated combinations of wild-type (+) and null (-) alleles of *Hras* and *Nras*. (bar: mean  $\pm$  S.E.M.) ns: not significant.

**3.3.4 Loss of wild-type *Hras* and *Nras* in combination increased the number of lymphoid aggregates in the lungs of urethane-treated mice**

Pathological examination of lung sections from mice revealed an aggregation of lymphoid cells in urethane-treated, (**Figure 15A**), but not untreated (see **Figure 12B**) mice. To assess whether disruption of either or both *Hras* and *Nras* alleles influenced this phenotype, two pathologists determined the number of lymphoid aggregates in each section of lungs isolated from the four genotypes *Hras*<sup>+/+</sup>;*Nras*<sup>+/+</sup>, *Hras*<sup>-/-</sup>;*Nras*<sup>+/+</sup>, *Hras*<sup>+/+</sup>;*Nras*<sup>-/-</sup>, and *Hras*<sup>-/-</sup>;*Nras*<sup>-/-</sup>. Binning this data into groups of no aggregates, 1-5 aggregates, or greater than 5 aggregates revealed that the number of lymphoid cell clusters were higher in *Hras*<sup>-/-</sup>;*Nras*<sup>+/+</sup> mice, and even more prevalent in *Hras*<sup>-/-</sup>;*Nras*<sup>-/-</sup> mice (**Figure 15B**). Thus, loss of wild-type Hras enhances the lymphoid response in the lungs to the chemical carcinogen urethane, and this response may be further augmented by the additional loss of wild-type Nras.



**Figure 15: Loss of wild-type Hras alone or in combination with loss of wild-type Nras promotes lymphoid infiltration in the lungs of urethane-treated mice.**

Lymphoid aggregates were observed in the lungs of mice treated with urethane. (A), Representative photograph at 2X (right), and 10X (left) of H&E stained lung tissue containing lymphoid aggregates, indicated by arrowheads. (B), Quantification of the percentage of sections from mice with the indicated wild-type (+) or null (-) alleles with lymphoid aggregates binned as no aggregates, 1-5 aggregates, or >5 aggregates. n=4-6 sections from 9-14 mice per cohort.

### 3.4 Discussion

Loss of wild-type Hras enhances carcinogen-induced lung tumorigenesis in mice. These data are consistent with the effects of the *Hras* null allele in other models of early lung and pancreatic tumorigenesis (To, Rosario et al. 2013, Weyandt, Lampson et al. 2015). Interestingly, however, this effect was not observed in mice lacking wild-type Nras. Loss of Nras had previously been shown to result in a greater urethane-induced tumor burden, but the mice used in that study were from a pure 129 background (To, Rosario et al. 2013). Strain backgrounds greatly influence the susceptibility of mice to urethane-induced tumorigenesis (Manenti, Trincucci et al. 2008). Indeed, in the tested background, the number of tumors observed was less than that found in the 129 background, which may mask a more subtle role of *Nras*<sup>-/-</sup> alleles in tumorigenesis. Nonetheless, in the tested background, the loss of wild-type Hras and wild-type Nras differentially affect oncogenic Kras-driven tumorigenesis.

Loss of both Hras and Nras led to significantly more tumors in comparison to the wild-type cohort or the Nras-knockout cohort, however, the number of tumors was not significantly different from those detected in *Hras*<sup>-/-</sup> mice. These results suggest that the increase in tumorigenicity in the double knockout mice was due to loss of the *Hras* allele, and that loss of *Nras* did not change the tumor-promoting effect of losing wild-type Hras.

Loss of Hras and Nras did not appear to significantly alter the levels of signaling through the canonical pathways activated downstream of Ras. Levels of pAKT and pERK were similar in the lesions from wild-type and *Hras*<sup>-/-</sup>;*Nras*<sup>-/-</sup> mice as assessed by immunohistochemical staining. Furthermore, these lesions also showed similar staining

patterns for markers of proliferation, apoptosis, and an inflammatory marker that stains macrophages. Collectively, these data suggest that the effects of loss of wild-type Hras on tumorigenesis could be the result of stromal cell influences, rather than differences in signaling within the tumor cells themselves. Alternatively, the effects on signaling and tumor markers may be below the levels of detection by immunohistochemical staining.

The idea that signaling through a wild-type Ras isoform could suppress tumorigenesis in lung cancer is supported by the fact that the wild-type allele of *KRAS* is often lost in both human lung cancer patients and in mouse models of lung cancer (Hegi, Devereux et al. 1994, Zhang, Wang et al. 2001). While such findings could reflect a gene conversion event, they may also point toward the loss of wild-type Kras promoting tumorigenesis. As such, wild-type RAS proteins may influence the susceptibility to lung tumorigenesis following mutagenic insult.

Interestingly, I observed a high rate of lymphocyte infiltration in urethane-treated mice lacking both wild-type Hras and Nras. Aggregates of lymphoid tissue in the lung are most often in response to infection, but occasionally are seen in cases of non-small-cell-lung-carcinoma (NSCLC) and have been suggested in at least one study to be prognostic of a more favorable outcome (Brambilla E 2012). Although the consequences of this immune response were not investigated, previous studies linking wild-type Hras and Nras isoforms with immune function suggest that loss of signaling through these pathways could also alter the inflammatory response to tumorigenesis. An investigation of genomic expression of transcriptional profiles in fibroblast cell lines in which each of the RAS isoforms had been knocked down individually showed that wild-

type NRAS significantly upregulated a number of pathways that were related to immune response (Castellano, De Las Rivas et al. 2007). In addition, previous studies showed that *Hras*<sup>-/-</sup>;*Nras*<sup>-/-</sup> mice are unable to mount an immune response to parasitic infection, due to hampered Th1 immunity (Iborra, Soto et al. 2011). Thus, further investigation into the role that immune response plays in this model of tumorigenesis has potential to shed some light on the different activities of the different wild-type Ras isoforms.

Understanding the molecular interactions between the wild-type and mutant RAS isoforms is critical not only for elucidating the biology of the RAS signaling pathway, but for determining whether differences in signaling in these various isoforms may play a role in whether an individual is pre-disposed to RAS-driven tumorigenesis. My findings suggest that levels of wild-type HRAS signaling could play a role in enhancing or suppressing KRAS-driven lung tumorigenesis.

## **4. Investigation of possible mechanisms for suppression of early tumorigenesis by wild-type Hras.**

### **4.1 Introduction**

I have shown that wild-type Hras suppresses the growth of oncogenic Kras driven tumors in mouse models of early pancreatic and lung tumorigenesis (described in Chapters 2 and 3). Loss of wild-type *Hras* in mice led to increased Kras-driven tumor loads in three different tissue types: pancreas (Chapter 2), skin (Chapter 2), and lungs (Chapter 3). The fact that I was able to detect this effect in different tissue types suggests that loss of wild-type Hras alters signaling through pathways critical for tumor suppression following an oncogenic mutation in *Kras*. In Chapter 2, I demonstrated that loss of normal Trp53 signaling suppressed the effects of the loss of wild-type Hras in a model of pancreatic cancer, indicating that signaling through the p53 tumor suppressor protein may be responsible for the observed differences in tumor growth in *Hras*<sup>-/-</sup> mice. To further investigate the mechanisms by which wild-type Hras suppresses early tumorigenesis, I performed a variety of experiments geared toward detecting differences in signaling in the presence and absence of wild-type Hras, as well as changes in proliferation or cell survival following loss of wild-type Hras. These experiments are described here.

## **4.2 Materials and Methods**

### **4.2.1 Mouse PDAC cell lines**

Pancreatic tumor tissues from *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> KPC mice were minced in collagenase V (Sigma-Aldrich) for 30 minutes at 37°C, and resultant cells were cultured in Dulbecco's Modified Eagle's Media (DMEM) + 10% FBS for at least 4 passages.

### **4.2.2 Ras-GTP analysis**

Ras GTP levels were detected by affinity capture with the RBD of Raf followed by immunoblot for HRAS with an  $\alpha$ -HRAS (Santa Cruz, sc520),  $\alpha$ -KRAS (Santa Cruz, sc30),  $\alpha$ -NRAS (Santa Cruz, sc31) or  $\alpha$ -Pan-Ras (Santa-Cruz, sc32) antibody, as previously described (de Rooij and Bos 1997).

### **4.2.3 Protein analyses**

Lysates were obtained from flash frozen pancreatic tissue or from pelleted cell lines and immunoblotted for p44/42 MAPK (Erk1/2) (Cell Signaling), phosphoERK (Thr202/204) (Erk1/2) (Cell Signaling), AKT (Cell Signaling), phosphoAKT (Ser/Thr) (Cell Signaling), or tubulin (Sigma-Aldrich).

### **4.2.4 PCR of *Kras* and *Trp53* alleles**

DNA was isolated from flash frozen pancreatic tissue and amplified by PCR to detect the wild-type and recombined *Kras* and *Trp53* alleles as previously described (Hingorani, Petricoin et al. 2003, Hingorani, Wang et al. 2005).

#### 4.2.5 Colony formation assays

PDAC cell lines from KPC mice were plated at low densities of 25, 50, 100, 500, and 1000 cells per well in triplicate in 6-well plates. Colony formation was assessed at intervals of 1, 3, 5, and 7 days after plating. The colonies were stained with 1% crystal violet solution and counted for each interval.

#### 4.2.6 Growth in IMR-90 cell lines

IMR-90 cell lines obtained from the Duke Cell Culture Facility (population doubling 36-40), were infected with pBabeHygro retroviral vectors encoding either wild-type Hras or empty vector. Following selection with hygromycin, the cell lines were then infected with pBabePuro retroviral vectors encoding either Kras<sup>G12D</sup> or empty vector and selected with puromycin. 3 days after selection with puromycin was started, cells were counted and plated at 4,000 cells per well of a 24-well plate in quadruplicate. Selective media was changed every other day and cells were maintained for intervals of 1, 3, 8, 12, 15, and 18 days. At the indicated timepoints, cells were fixed in 10% formalin and stained with 1% crystal violet. Upon completion of the experiments, the crystal violet was extracted with 1 mL of acetic acid in each well, and quantified by absorbance at 590 nm. Values at all timepoints were normalized to the mean for each cell line at Day 0.

#### 4.2.7 3D-culture of pancreatic acinar cells

5 *Hras*<sup>+/+</sup> and 5 *Hras*<sup>-/-</sup> mice encoding a *LSL-Kras*<sup>G12D</sup> allele were euthanized at 4 weeks of age and acinar cells were isolated and treated with Ad-Cre to activate oncogenic *Kras*. The cells were then plated in collagen 3D culture in quadruplicate, as previously

described (Garcia, Grasso et al. 2014), and assessed for conversion to ductal structures after 5 days. The percentage of ductal structures was divided by the total number of cell clusters to determine the percentage of cells that had converted.

#### **4.2.8 Allograft assays**

PDAC cell lines from KPC mice were cultured and 10,000 cells from each line were suspended in 100  $\mu$ l of Matrigel (BD Biosciences) and were injected subcutaneously into the flanks of KPC mice. The resultant tumors were measured 3X per week. Mice were euthanized once any of the tumors reached a size of 1.0 cm<sup>3</sup>, after which tumors were removed from all mice and their volume determined.

#### **4.2.9 Knockdown of wild-type Hras in KPC lines**

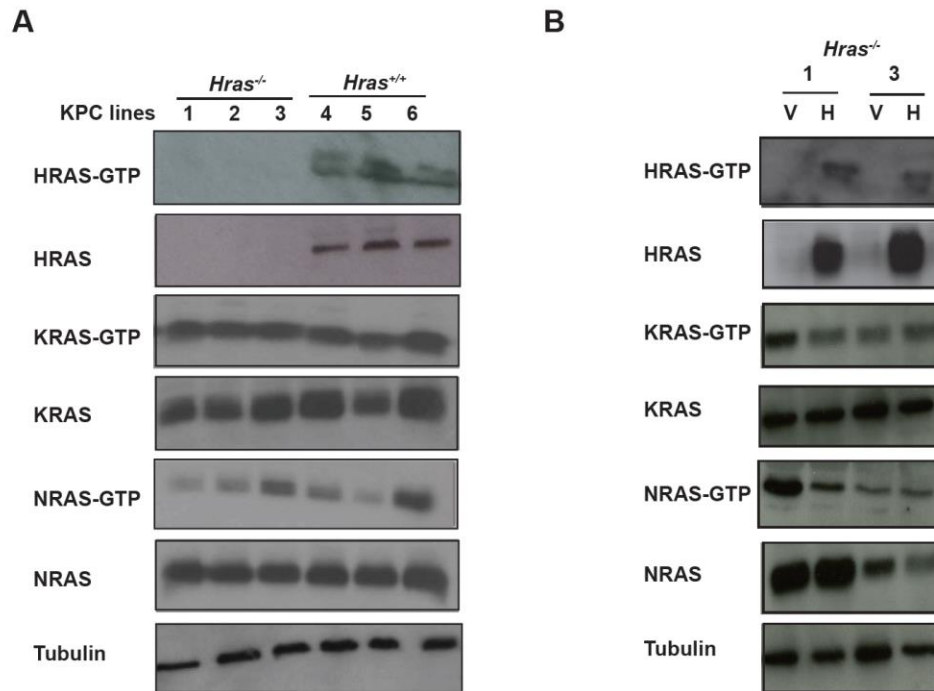
*Hras* shRNA (5'-GGCAAGAGTGCGCTGACCATC) sequence was cloned into pSUPER-PURO-RETRO (Oligoengine). Selection was performed in media containing puromycin, and confirmation of knockdown was assayed by immunoblot for Hras (described above in section 4.2.2).

### **4.3 Results**

#### **4.3.1 Loss of wild-type Hras does not alter the expression or activation of the other Ras proteins**

As the three members of the Ras family are closely related and show redundant signaling in mice (Esteban, Vicario-Abejon et al. 2001), I wanted to determine whether the loss of Hras could result in altered expression of Nras or Kras. I hypothesized that it was possible that these isoforms may be upregulated in compensation for loss of wild-

type Hras, and if so, that higher levels of signaling through the mutant Kras protein in the absence of wild-type Hras could be responsible for increased oncogenic Kras-driven tumorigenicity in the *Hras<sup>-/-</sup>* mice. To investigate this hypothesis, I assessed the levels of total and GTP-bound (active) Ras isoforms in lysates derived from KPC cell lines from wild-type and *Hras<sup>-/-</sup>* mice, and no consistent differences were observed in the levels of protein expression between these two genotypes by immunoblot analysis (**Figure 16A**). Because the levels of the different RAS proteins appeared to be variable between cell lines, I also compared the levels in two independent *Hras<sup>-/-</sup>* cell lines in which Hras protein was restored by a retroviral expression vector encoding murine wild-type Hras. Once again, I did not see a change in the expression or activation of the other Ras isoforms in the presence of wild-type Hras (**Figure 16B**). Thus, the loss of wild-type Hras does not appear to alter protein levels or activation of the other Ras family members, at least as assessed by immunoblot.



**Figure 16: Loss of wild-type Hras does not alter expression or activation of other Ras Isoforms**

**(A)** Lysates prepared from 3 *Hras*<sup>-/-</sup> (1-3) and 3 *Hras*<sup>+/+</sup> (4-6) KPC cell lines were immunoblotted for each of the Ras isoforms. Activated Ras levels were determined by affinity capture with Raf-BD, followed by immunoblotting for the Ras isoforms. Tubulin served as loading control. **(B)** Wild-type Hras was added back to *Hras*<sup>-/-</sup> cell lines 1 and 3 by a retroviral expression vector encoding wild-type Hras (V=empty vector control, H=vector encoding Hras). Immunoblotting was performed for each of the Ras Isoforms following affinity capture with Raf-BD. Tubulin served as loading control.

#### 4.3.2 Loss of wild-type Hras does not overtly alter specific downstream signaling pathways in pancreatic cancer cell lines

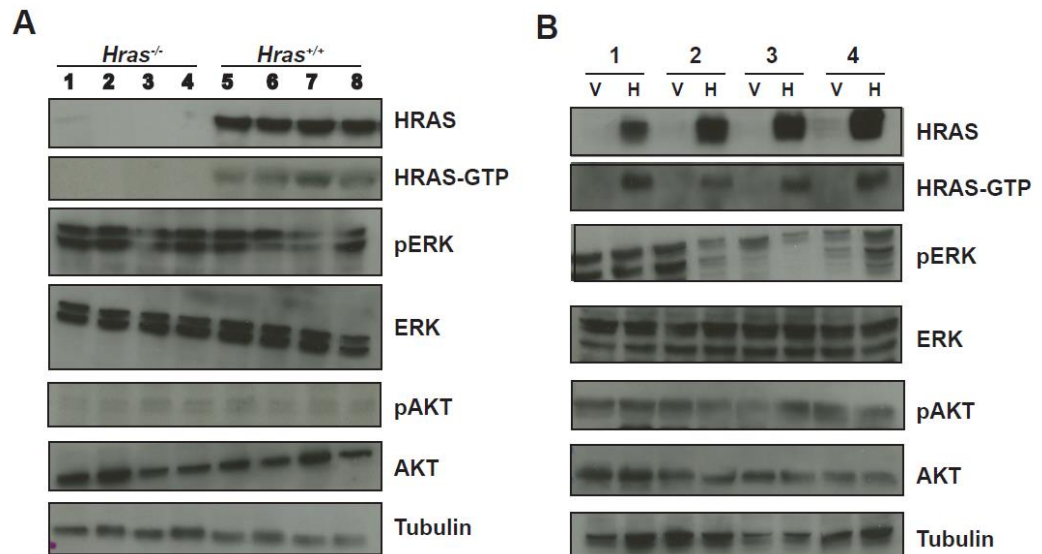
Because the Ras proteins can activate the same downstream signaling pathways (Pylayeva-Gupta, Grabocka et al. 2011), I wanted to determine whether loss of wild-type Hras, which has been previously shown to reduce total GTP-loaded Ras (see **Figure 9**),

altered signaling through downstream effector pathways. Changes in signaling through specific effector pathways could provide clues for identifying the mechanism behind the higher tumor loads in *Hras*-deficient mice. For example, signaling through the MAPK pathway is essential for Ras-induced senescence, a growth arrest in early tumorigenic cells that suppresses tumor growth through p53 and p16-mediated pathways (Serrano, Lin et al. 1997, Lin, Barradas et al. 1998, Sun, Yoshizuka et al. 2007). Thus, higher levels of signaling through the MAPK pathway (measured by ERK phosphorylation) in the presence of wild-type *Hras* would suggest a potential role for this pathway in tumor suppression.

To investigate the amplitude of signaling through the MAPK and PI3K pathways, levels of total and phosphorylated ERK and AKT were compared in pancreatic cancer cell lines from *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> mice. Signaling through these downstream pathways appeared to be cell line-dependent, as variable levels of pAKT and pERK were found in cell lines from mice of both genotypes (**Figure 17A**).

In order to test the levels of signaling with and without *Hras* in the same cell lines, I immunoblotted for these proteins in four independent *Hras*<sup>-/-</sup> KPC cell lines infected with either an empty retroviral vector or a vector encoding wild-type *Hras*. Once, again, changes in the levels of pAKT and pERK upon expression of wild-type *Hras* appeared to vary between cell lines: levels of pERK stayed approximately the same in one cell line (#1), went down in two cells lines (#2, 3), and went up in one cell line (#4) when wild-type *Hras* was ectopically expressed (**Figure 17B**).

Collectively, these findings suggest that the spectrum of signaling through downstream pathways is not altered in the presence or absence of wild-type Hras, at least by this approach. These experiments were performed in late-stage adenocarcinoma cell lines, so it is possible that there may be subtle differences in the amplitude of signaling at earlier timepoints that could have an effect on cells in earlier stages of growth. Nevertheless, I did not detect reproducible differences in effector signaling between matched cell lines with and without Hras expression.

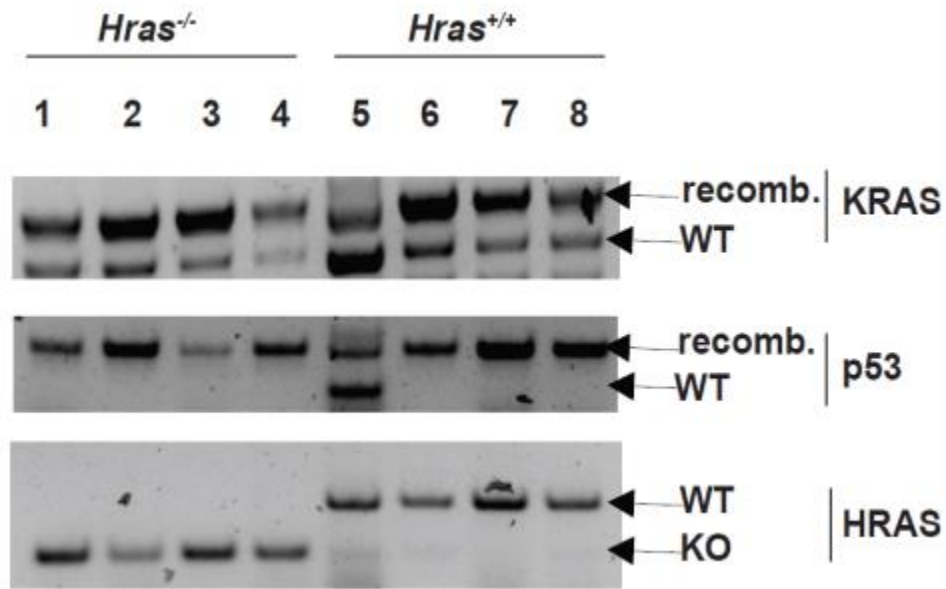


**Figure 17: Loss of wild-type Hras does not overtly alter specific downstream signaling pathways in pancreatic cancer cell lines**

(A.) Immunoblot of lysates derived from four *Hras*<sup>-/-</sup> (1-4) and four *Hras*<sup>+/+</sup> (5-8) KPC cell lines. (B.) Immunoblot from four *Hras*<sup>-/-</sup> cell lines (1-4) infected with either empty retroviral (V) or a vector encoding wild-type murine Hras (H).

### 4.3.3 Loss of wild-type Hras does not affect loss of heterozygosity in the *Kras* and *Trp53* alleles

Loss-of-heterozygosity of the wild-type alleles for *KRAS* and *p53* have been observed in both human cancer (Li, Zhang et al. 2003), as well as mouse models of *Kras*-driven-tumorigenesis (Hingorani, Wang et al. 2005). Thus, another possible explanation for higher levels of *Kras*-driven tumorigenesis in *Hras*<sup>-/-</sup> mice is that loss of the wild-type *Kras* allele, which can suppress proliferation (Zhang, Wang et al. 2001), or loss of the wild-type *Trp53*-tumor suppressor allele, are enhanced in the absence of wild-type Hras. For this reason, I assessed loss-of-heterozygosity of both the *Kras* and *Trp53* alleles in genomic DNA isolated from pancreatic cancer cell lines derived from *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> KPC mice. Consistent with the original description of this model (Hingorani, Wang et al. 2005), I found that the wild-type *Kras* allele was indeed retained in all of cell lines, regardless of the status of the *Hras* gene (**Figure 18**). Again, in agreement with the aforementioned published study, I also found that the wild-type *Trp53* allele was similarly lost in all but one cell line (#5) (**Figure 18**). Therefore, the presence or absence of the wild-type *Hras* does not appear to affect the loss of heterozygosity of the wild-type *Kras* or *Trp53* alleles



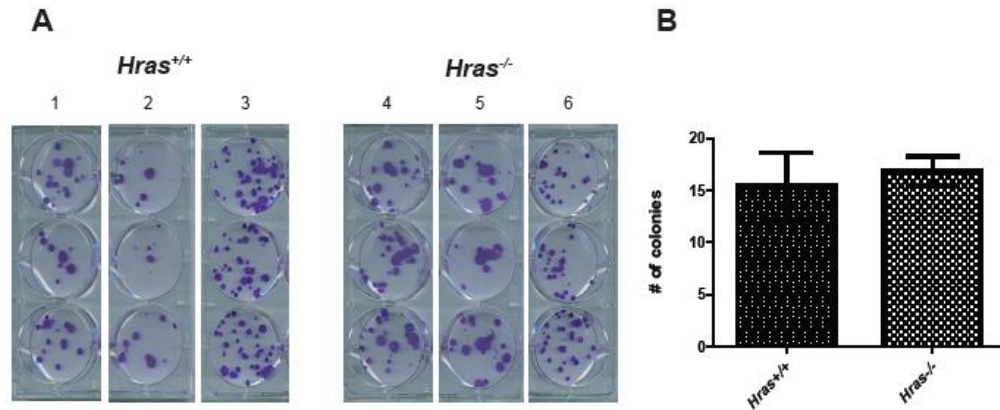
**Figure 18: Loss of wild-type Hras does not affect loss of heterozygosity in the wild-type *Kras* and *Trp53* alleles.**

PCR of genomic DNA to detect recombined (recomb.) and wild-type (WT) *Kras* and *Trp53* alleles, as well as the wild-type and knockout *Hras* alleles in PDAC cell lines from *Hras*<sup>-/-</sup> (1-4) and *Hras*<sup>+/+</sup> (5-8) KPC mice. WT: wild-type, KO: knockout (*Hras*<sup>-/-</sup>)

#### 4.3.4 Wild-type Hras does not significantly alter proliferation of KPC or IMR-90 cell lines *in vitro*

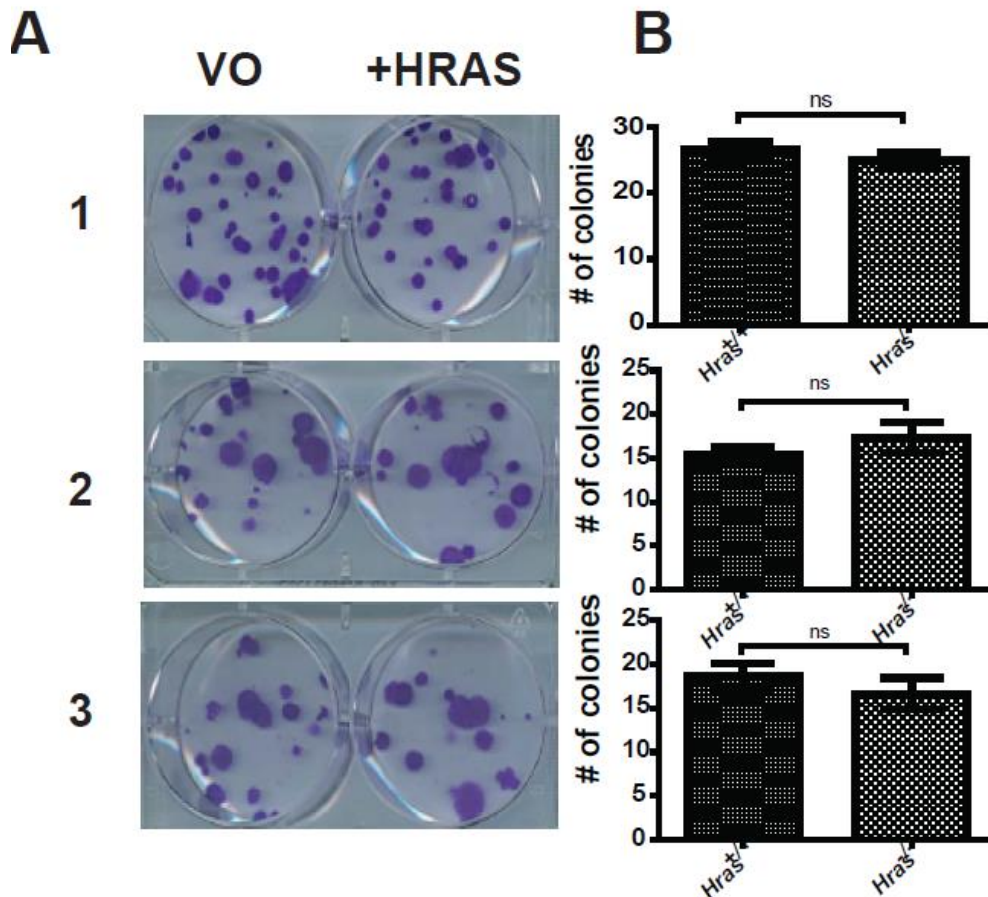
Because I previously showed that wild-type Hras was activated in pancreatic cancer cell lines from KPC mice, I wanted to determine whether this activation enhanced cellular proliferation in an *in vitro* model. I thus assessed colony formation using three independent cell lines from both *Hras*<sup>-/-</sup> and *Hras*<sup>+/+</sup> mice. I performed crystal violet staining, and observed no significant differences in the average number of colonies formed (**Figure 19A and B**). Similarly, no differences in colony formation were observed when wild-type Hras was added back to three *Hras*<sup>-/-</sup> cell lines by infection with

a retroviral vector encoding the wild-type Hras protein (**Figure 20**). Thus, the presence of wild-type Hras does not appear to overtly alter the proliferation of advanced adenocarcinoma cell lines.



**Figure 19: Colony formation in PDAC cell lines from KPC mice.**

Three *Hras*<sup>+/+</sup> (1-3) and three *Hras*<sup>-/-</sup> (4-6) KPC PDAC cell lines were plated at the low density of 50 cells/ well in triplicate (e.g. n=9 for each cohort), and after 5 days cells were stained with crystal violet to visualize colonies, (**A**), which are shown in a representative photograph, and (**B**), quantified and plotted. Bar: mean  $\pm$  S.E.M.



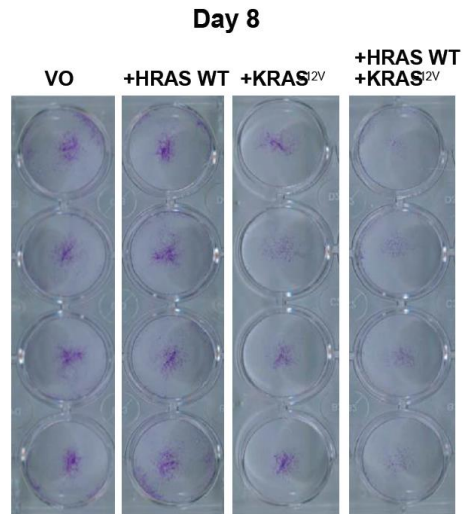
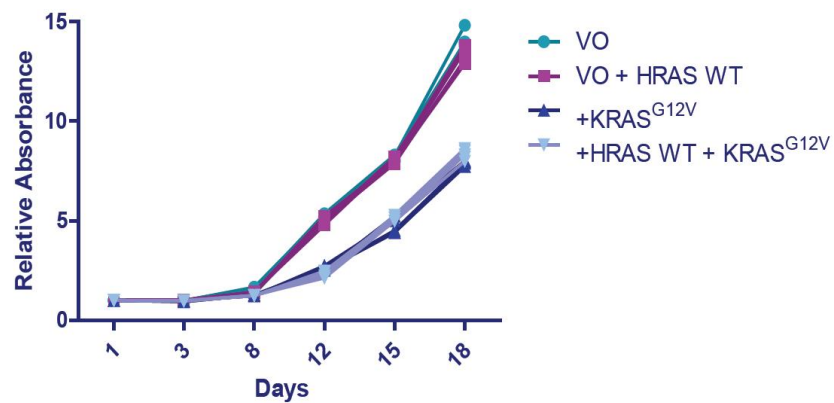
**Figure 20: Colony formation in *Hras*<sup>-/-</sup> KPC cell lines with rescued *Hras* expression**

Colony formation was assayed in three *Hras*<sup>-/-</sup> KPC cell lines (1-3) infected with either empty vector (VO: vector only) or a retroviral vector encoding wild-type *Hras* (+HRAS). Cell lines were plated at the low density of 50 cells/ well in triplicate, and after 5 days cells were stained with crystal violet to visualize colonies. (A) Representative photograph and (B) the number of colonies for each cell line quantified and plotted. Bar: mean ± S.E.M.

Next, because we have shown in our mouse models that wild-type *Hras* appears to suppress tumorigenesis at a very early stage, I also wanted to assess the effects of wild-type *Hras* in non-transformed cell lines. Therefore, I infected IMR-90 cells with retroviral vectors that were either empty or encoded wild-type HRAS, followed by empty

vector or oncogenic KRAS. I used crystal violet staining to measure the proliferation of these cells. High levels of Ras signaling can induce a cell growth arrest (Sarkisian, Keister et al. 2007), and in agreement, we observed lower levels of proliferation in the presence of oncogenic KRAS. However, the addition of wild-type Hras did not appear to significantly alter this cell growth arrest. Specifically, cells in which wild-type Hras was expressed trended toward having lower levels of proliferation, but repeated assays did not reach significance (**Figure 21**).

Based on these experiments, I was not able to elucidate the mechanism by which wild-type Hras reduces urethane-induced lung tumorigenesis. As discussed in Chapter 2 and 3 (Figures 8 and 14), I also did not see a difference in the number of Ki67-positive lesions in the KC pancreatic cancer model (**Figure 8**) or in the percentage of Ki67-positive staining lung tumors in the urethane model (**Figure 14**). Thus, although mice lacking wild-type Hras develop more Kras-driven lesions, collectively these data indicate that wild-type Hras has no overt effects on cellular proliferation in cells expressing oncogenic Kras.

**A****B**

**Figure 21: The presence of absence of wild-type Hras does not enhance the oncogenic Kras-driven suppression of growth of IMR-90 cells.**

IMR-90 cells were infected with empty vector (VO: vector only) or wild-type HRAS (HRAS-WT) followed by empty vector or KRAS<sup>G12V</sup>. Cells infected with oncogenic KRAS showed an arrest in proliferation, but the addition of wild-type HRAS did not appear to significantly alter this growth arrest. (A) Representative photograph of cell lines stained with Crystal Violet at Day 8 post-infection with oncogenic Kras, and (B) plot of the relative absorbance of these cell lines at the indicated timepoints.

#### 4.3.5 No differences in allograft tumor size when Hras was knocked down in KPC cell lines

Because knockdown of wild-type HRAS in human pancreatic cancer cell lines reduced tumor growth when these cell lines were injected into the flanks of mice (Lim, Ancrile et al. 2008), I wanted to determine whether the higher levels of tumorigenesis observed in *Hras*<sup>-/-</sup> mice may be due to differences in human versus mouse Ras signaling. I have shown that the presence of wild-type Hras appears to suppress tumorigenesis in early stage pancreatic and lung cancer in mouse models (Chapters 2 and 3), but these data seem to contradict the xenograft data using human pancreatic cancer cell lines, where wild-type Hras was shown to promote tumor growth (Lim, Ancrile et al. 2008). Therefore, I wanted to extend these experiments by knocking down wild-type Hras in mouse pancreatic cell lines to determine the effects of wild-type Hras on allograft tumor growth. When wild-type Hras was knocked down in a mouse PDAC cell line (**Figure 22A**) no significant differences in the size of tumor growth were observed in comparison to cells infected with a scramble control (**Figure 22B**), with the caveat that knockdown of Hras was not complete. These data suggest that the presence of wild-type Hras has no effect on the growth of later stage tumor cell lines in mice, and furthermore, that there may be some differences in the role of HRAS signaling between human and mouse cell lines, as in human pancreatic cancer cells, loss of wild-type HRAS reduced tumor growth.

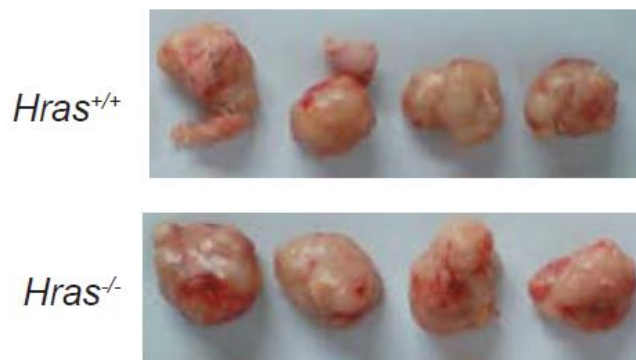


**Figure 22: Knockdown of wild-type Hras in KPC cell line**

A PDAC cell line from a KPC mouse was infected with either a pSuperRetroPuro vector encoding a Scramble control (+Scramble) or short-hairpin sequence knocking down wild-type Hras (+Hras shRNA). 10,000 cells from each were then injected into the flanks of wild-type mice from similar backgrounds as the KPC mice. (A) Knockdown of wild-type Hras was confirmed by immunoblot, and (B) final tumor growth was measured. No significant differences in tumor sizes were observed.

#### 4.3.6 No differences in tumor growth of KPC cell lines injected into *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> mice

As similar growth has been shown *in vitro* and *in vivo* in advanced stage Kras-driven mouse pancreatic cancer cell lines in the presence and absence of wild-type Hras, I wanted to determine whether the loss of wild-type Hras in the tumor environment, rather than in the tumor cells themselves, may effect growth. Therefore, I injected the same pancreatic cancer cell lines from KPC mice into the flanks of both *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> mice of similar backgrounds. No significant differences in tumor size were observed between the *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> mice (Figure 23), suggesting that loss of Hras in the stromal component and/or immune cells does not play a role in differences observed in tumorigenesis between these cohorts. However, because these cells are injected subcutaneously, rather than into the target organ, the stromal microenvironment is not well-recapitulated, and thus further studies are needed to determine whether loss of Hras within the tumor microenvironment within the target organ plays a role.



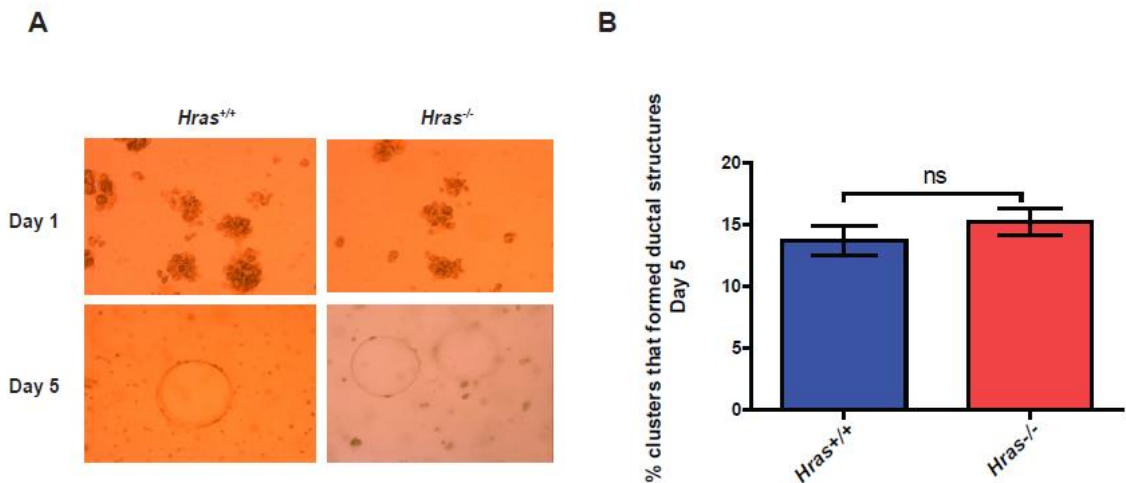
**Figure 23: Growth of KPC cell lines in  $Hras^{+/+}$  and  $Hras^{-/-}$  mice**

10,000 PDAC cells derived from KPC mice were injected into the flanks of  $Hras^{+/+}$  and  $Hras^{-/-}$  littermates. Growth of the same cell line was compared between  $Hras^{+/+}$  and  $Hras^{-/-}$  mice, and no significant differences in allograft tumor size or volume were observed.

#### **4.3.7 Loss of wild-type Hras does not alter the conversion of acinar to ductal cells *in vitro***

Since I observed a higher number of cells co-staining for markers of ADM in a mouse model of pancreatic cancer (**Figure 7G**), I wanted to determine whether there was a difference in the conversion of acinar-to-ductal cells *in vitro*. I compared the percentage of acinar cells that converted to ductal cells in 3D culture from  $Hras^{+/+}$  and  $Hras^{-/-}$  mice with a LSL- $Kras^{G12D}$  allele following activation of  $Kras^{G12D}$  by Ad-Cre, as previously described (Shi, DiRenzo et al. 2013, Garcia, Grasso et al. 2014). However, there was no significant difference in the percentage of ADM events that occurred *in vitro* between these two cohorts ( $P=0.5622$ ) (**Figure 24**). However, as it has been reported that over 90% of  $Kras^{G12D}$ -expressing acinar cells differentiate to form ductal cysts in this type of 3D-culture (Shi *et al*, Oncogene, 2013), it may be difficult to show an increase in ADM *in vitro*. Nevertheless,  $Hras^{-/-}$  mice have more lesions identified as

ADM histology by two independent pathologists, and have shown higher numbers of lesions co-staining for immunofluorescent markers of both acinar and ductal cells.



**Figure 24: 3D culture of pancreatic acinar cells**

5 *Hras*<sup>+/+</sup> and 5 *Hras*<sup>-/-</sup> mice expressing *LSL-Kras*<sup>G12D</sup> were euthanized at 4 weeks of age and acinar cells were isolated and treated with Ad-Cre to activate oncogenic *Kras*. The cells were then plated in collagen 3D culture in quadruplicate. **(A)** Representative photographs at Day 1 and Day 5 after plating, and **(B)** quantification of the percentage of acinar clusters which converted to ductal structures *in vitro* after activation of *Kras*<sup>G12D</sup>. Bar: mean  $\pm$  S.E.M

#### 4.4 Summary and Discussion

There appear to be no significant differences in the levels of signaling through the different Ras isoforms or through downstream signaling pathways in the presence or absence of wild-type Hras, at least at the stages that I was able to study. As the previously observed tumor-suppressive effects of wild-type Hras were not observed in later stage cell lines, it appears that the loss of Hras affects tumorigenesis at a very early

timepoint, possibly at the stage of initiation. Collectively, loss of Hras in later stage cell lines did not appear to affect levels of proliferation or signaling, supporting the hypothesis that the effects of wild-type Ras proteins on tumorigenesis affect very early stages of tumorigenesis.

Allograft studies revealed no differences in tumor growth in mouse cell lines when Hras expression was knocked down, suggesting that the effects of wild-type Hras are different not only at different stages of tumorigenesis, but potentially also between mouse and human cell lines.

Allograft studies comparing the growth of mouse pancreatic cancer cell lines in *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> mice revealed no significant effects of loss of wild-type Hras in the stromal component on tumor growth, at least in subcutaneous tumors. As allograft studies do not recapitulate the same stromal microenvironment found within the organ, more specific studies of loss of wild-type Hras within the target organ are needed to definitively rule-out stromal cells as contributing to differences in tumor growth.

Collectively, these mechanistic studies show that the loss of wild-type Hras promotes tumorigenesis at an extremely early timepoint after activation of oncogenic Ras, and that differences in the amplitude and diversity of signaling do not appear to be responsible for this phenotype, although it is possible that subtle differences at an early stage not investigated could contribute to the tumorigenic phenotype in *Hras*<sup>-/-</sup> mice.

## 5. Future Directions and Discussion

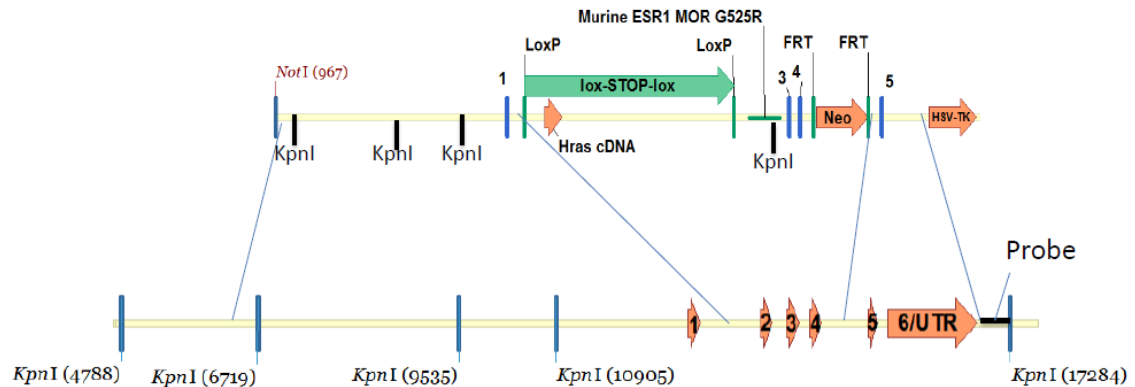
### 5.1 Future Directions

#### 5.1.1 Investigating the effects of wild-type RAS Signaling in early vs. late tumorigenesis.

Although signaling through the wild-type RAS proteins seems to exhibit divergent effects depending upon the context, early models of tumorigenesis appear to demonstrate a tumor-suppressive role for wild-type RAS, while experiments in more advanced stages of disease indicate that wild-type RAS can be either tumor-promoting or have no effect on tumor growth and proliferation. I have shown a clear role for wild-type Hras in suppressing early lung and pancreatic cancer in mouse models, tracing pancreatic lesions back to the earliest stages. Thus wild-type Hras may suppress tumorigenic growth at the time of initiation of oncogenic Kras.

To further investigate the role of wild-type Hras during different stages of cancer, we have designed a mouse with a tissue-specific, inducible *Hras* allele. With the help of Gary Kucera from the Duke Cancer Institute Transgenic Facility, we knocked into the *Hras* allele a loxP site, followed by *Hras* cDNA, a STOP sequence, and another loxP site, then a cDNA encoding ER-*Hras* fusion protein. (**Figure 25**). Thus, normally the *Hras* cDNA will be expressed in cells, but in the presence of Cre-recombinase the tamoxifen-inducible ERHras protein would be expressed. This allele will allow us to not only assess the role of wild-type Hras signaling specifically within the target tissue, but will also allow us to temporally control expression of Hras to determine whether the effects are

different at early and later timepoints during tumorigenesis when combined with mutant Kras.



**Figure 25: ERHras knock-in targeting strategy**

ERHras knock-in allele designed so that expression of wild-type Hras is under the control of the murine estrogen receptor, making it tamoxifen-inducible. Upon Cre-excision of the Lox-STOP-Lox cassette, wild-type Hras expression can be induced by the administration of tamoxifen (Figure courtesy of Gary Kucera, Duke Transgenic Facility).

### 5.1.2 Investigating the immune response as a potential contributor to tumor suppression by wild-type HRAS

The body's innate immune response can also play a role in tumorigenesis.

Tumor cells are recognized as foreign, and can be recognized and attacked by circulating immune cells. Thus these defenses must be overcome for a tumor cell to survive. For this reason the immune system can play a role in suppressing tumorigenesis.

Interestingly, however, the attraction of chemokines as part of this defense can also serve to promote tumorigenesis as well, as some of these factors are required for tumor cell growth (Gajewski, Schreiber et al. 2013). Therefore, differences in immune response

can also play a role in determining whether a tumor will form, and in promoting growth of those cells once the tumor is initiated.

Thus, another possible mechanism for the observed differences in tumor growth between wild-type and *Hras*<sup>-/-</sup> mice could be the immune response. Loss of wild-type Hras has previously been implicated for playing a role in Th-1 T cell response to parasitic infections in mice (Iborra, Soto et al. 2011), and we have shown elevated levels of lymphoid aggregates in the urethane-treated lung tissues of mice lacking both wild-type Hras and Nras (See section 3.3.4). Therefore, in future experiments, we plan to investigate whether the differences in tumor number observed in the absence of wild-type Hras may be due in part to differences in immunity.

Because the animals that we have previously used for assessment of the role of wild-type Hras in Kras-driven cancers were whole-animal knockouts, the above mentioned- ERHras allele will also allow us to specifically examine the role that wild-type Hras plays specifically within cells of the target organ. These animals will express wild-type Hras only within the targeted tissue type when treated with tamoxifen, and thus if the immune response were responsible for the previously observed differences in tumor number, these differences should no longer be apparent using this model in cells expressing *Hras* versus *Hras*<sup>-/-</sup>.

### 5.1.3 Investigating GEF sequestration as a possible mechanism for tumor suppression by wild-type HRAS

We have found no consistent differences in the levels of signaling through downstream effector pathways when wild-type HRAS is activated in the presence of oncogenic KRAS (sections 4.3.1 and 4.3.2). Furthermore, there does not appear to be a difference in the levels of proliferation, senescence, or apoptosis in tumors isolated from *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> mice (see **Figures 8 and 14.**) Therefore, it does not appear that overt changes in Ras signaling amplitude or diversity are responsible for the higher number of Kras-driven tumors in mice lacking wild-type Hras. Another possible explanation for this difference could be that the wild-type RAS proteins sequester GEFs away from mutant *KRAS*, resulting in overall lower-levels of activation of the mutant protein in cells with wild-type HRAS, possibly at subtle levels of which differences are not detected by immunoblot.

To test this hypothesis in the future, we could design a mouse with an *HRAS*<sup>N17</sup> mutant allele. *RAS*<sup>N17</sup> is a dominant-negative RAS mutant that binds GDP with preferential affinity over GTP, allowing *RAS*<sup>N17</sup> to inhibit endogenous RAS activation by sequestering RasGEFs (Stewart and Guan 2000). While this mutant has an affinity for GEFs, it does not bind to effector molecules, and thus wild-type HRAS would be “activated”, but would not be able to initiate downstream signaling. In this way, we could dissect whether it was the signaling or the sequestration of GEFs that contributes to the tumor-suppressive actions of wild-type Hras when compared to *Hras*<sup>-/-</sup> mice.

#### **5.1.4 Investigating the effects of wild-type Hras on the cell of origin in Kras-driven cancers**

I have shown that wild-type Hras suppresses Kras-driven tumorigenesis at the very earliest stages of pancreatic cancer; specifically mice lacking wild-type Hras get more ADM lesions (Chapter 2). Although these data suggest a very early-stage effect for wild-type Hras, we do not know the cell of origin in pancreatic cancer (Seton-Rogers 2013). One explanation for the difference observed could be that the presence of wild-type Hras results in a higher number of the particular cell type that is susceptible to oncogenic KRAS mutations. Although this cell type is not yet well-defined in pancreatic cancer, recent work has identified the cell-of-origin for lung cancer as alveolar type II cells, which can be identified by immunohistochemical staining (Xu, Huang et al. 2014).

To determine whether wild-type Hras affects the number of alveolar type II cells, the cells of origin for non-small cell-lung carcinoma immunohistochemical staining for makers of these cell types could be performed. These cells stain positive for both Sox2 and Notch 1 (Xu, Huang et al. 2014). By assessing the number of cells staining positive for these markers in lung sections from *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> mice, it would be possible to determine whether loss of wild-type Hras influences the development and retention of this cell type. Such a study would allow us to determine whether alterations in the number of a precursor cell type are responsible for the higher numbers of lung tumors in Hras-knockout mice.

### 5.1.5 Investigating the potential for reprogramming of cells following loss of wild-type Hras

As discussed in the previous section, the cell of origin in pancreatic cancer remains unknown. However, the significant effects of loss of wild-type Hras suggest that one explanation for the higher numbers of tumors in mice lacking this protein could be that loss of Hras results in the reprogramming of cell types in the pancreas to those more susceptible to oncogenic insult. Pancreatic cells, in particular, may be especially susceptible to reprogramming, as loss of a single gene (*Fbw7*), has been shown to render adult pancreatic ductal cells multipotent, such that they can differentiate into exocrine or endocrine cell types (Sancho, Gruber et al. 2014). To investigate the possibility that loss of wild-type Hras reprograms cells in the adult pancreas, we could also utilize our new inducible ERHras mouse, as tamoxifen-treatment could be discontinued in adult cells to investigate the effects of loss of wild-type Hras on different cell types in the pancreas. By comparing immunohistochemical markers for the various endocrine and exocrine cell types between cells in which Hras is knocked out and wild-type cells, we could determine whether loss of wild-type Hras affects the ratio of these cell types, and thus might alter the number of cells susceptible to tumor growth following an oncogenic mutation in *Kras*.

### 5.1.6 Investigating other genes involved in proliferation or tumor suppression

Another possible explanation for the tumor suppressive effects of wild-type *Hras* could be that loss of wild-type *Hras* alters the expression of other genes involved in tumor proliferation or suppression. For example, loss of *Notch1* and *Icmt* have also been identified as functioning as tumor suppressors in a mouse model of PDAC (Hanlon, Avila et al. 2010, Court, Amoyel et al. 2013). If loss of wild-type *Hras* resulted in lower levels of expression of these or other tumor suppressor genes, this could help to explain the higher numbers of tumors in the *Hras*<sup>-/-</sup> mice. Likewise, if loss of wild-type *Hras* increased the expression of tumor-promoting genes, the higher levels of expression of these genes could help to explain the more severe tumor-phenotype in *Hras*<sup>-/-</sup> mice. For example, YAP and TAZ have also been found to be upregulated in PanINs and PDAC in mice and human pancreatic tumor samples (Morvaridi, Dhall et al. 2015). These genes function as transcriptional cofactors for a host of other genes involved in cell growth, proliferation, and mobility (Zhao, Li et al. 2010). Thus, alteration in the expression levels of these or other genes involved in tumorigenesis could promote tumorigenesis in the absence of wild-type *Hras*.

In order to investigate the effect of loss of wild-type *Hras* on the expression of other genes involved in promoting or suppressing tumorigenesis, in the future gene expression array analyses or RNASeq on cDNA isolated from PDAC samples from *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> mice could be performed to identify any significant differences in expression of other genes that may play a role in cancer growth. Candidate genes could

also be investigated by comparing differences in gene expression by RT-PCR or immunohistochemical staining in tissues from *Hras*<sup>+/+</sup> and *Hras*<sup>-/-</sup> mice.

### 5.1.7 Summary of Future Experiments

I have identified wild-type Hras as playing a role in suppressing tumorigenesis at a very early timepoint in Kras-driven cancers. The mechanism behind this phenotype appears to be dependent upon normal p53 signaling, and consistent with this, in experiments performed using advanced-stage cell lines which have lost normal p53-signaling, wild-type Hras does not seem to exhibit these tumor-suppressive characteristics.

Future experiments will use novel mouse models to temporally and spatially dissect the roles of wild-type Hras during different timepoints, cell types, and stages of tumorigenesis, as well as to determine whether it is altered signaling or the sequestering of GEFs by the wild-type proteins that contributes to differences in tumor formation. Furthermore, we could also use these mouse models to distinguish whether losing Hras in the tumor cells or the stroma or both contributes to the more tumorigenic phenotype. We could also assess whether the presence of wild-type Hras affects the cell types that are sensitive to mutant *Kras* and go on to form tumors, or whether the loss of wild-type Hras can reprogram cells in the adult organs, making them more susceptible to growth following Ras mutation. Finally, we could investigate the effects of loss of wild-type Hras on the expression of other proteins that may be involved in tumorigenesis or tumor suppression.

## **5.2 Significance**

### **5.2.1 Wild-type HRAS expression may influence susceptibility to cancer**

We have shown that loss of wild-type Hras promotes tumorigenesis in mouse models of pancreatic cancer, lung cancer, and skin cancer. Mutations in one of the RAS proteins are found in about 30% of all cancer patients, and thus millions are affected by these mutations each year. Our finding that wild-type HRAS actually suppresses oncogenic KRAS-driven tumorigenesis suggests that subtle differences in the expression levels of wild-type HRAS could influence an individual's susceptibility to developing a tumor following an oncogenic mutation in *KRAS*. These findings are not only important for understanding the nuances of RAS-driven tumorigenesis, but also could play a role in early monitoring or detection. Pancreatic cancer, in particular, is very difficult to detect in its earliest stages. A way to assess whether an individual may be predisposed for developing this disease could be a potentially helpful tool for monitoring.

### **5.2.2. Wild-type RAS signaling may be an important consideration when designing therapeutics**

To date, efforts made toward targeting the mutant RAS proteins have been largely ineffective. The class of drugs once thought to be the most promising for targeting RAS membrane-targeting, farnesyl-transferase inhibitors (FTIs) failed in clinical trials largely in part due to the differences in the post-translational processing of the different RAS proteins. As discussed in Chapter 1, both NRAS and KRAS, the most commonly mutated form of RAS, undergo additional modifications that make them resistant to this type of targeting (Downward 2003, Cox, Der et al. 2015). HRAS, however, is best

targeted by the class of drugs. For this reason, it is important to consider the potential roles that wild-type RAS proteins play in oncogenic-KRAS-driven tumorigenesis. Our data would suggest that treating a patient with an inhibitor that would target multiple RAS isoforms early in tumorigenesis could actually promote proliferation at early timepoints, since wild-type HRAS actually suppressed tumor growth during this time. Understanding the subtle differences in signaling between the RAS family of proteins is important to understanding how to best treat this disease, as it appears that even the very earliest effects have lasting consequences in our model systems.

### **5.3 Summary**

I find a significant role for wild-type Hras in suppressing the early stages of pancreatic, lung, and skin tumorigenesis driven by oncogenic Kras. These effects, traced to the earliest stages of disease, have lasting impacts, as loss of wild-type *Hras* significantly reduced survival in a mouse model of pancreatic adenocarcinoma. I also show that the tumor suppressive activity of wild-type Hras is dependent on normal p53 signaling, and that these effects are ablated in a homozygous mutant-p53 background.

I also show, for the first time, a comparison of the effects of the loss of multiple wild-type RAS proteins side-by-side in an *in vivo* tumor model using littermate controls. In the urethane-induced model of lung cancer, the loss of wild-type Hras significantly enhanced tumorigenesis, but loss of wild-type Nras had no apparent effects on tumor number in these mice. In addition, concomitant loss of both of these proteins resulted in

an increased tumor load at a level nearly identical to that of loss of wild-type Hras alone, and thus the absence of wild-type Nras does not appear to effect the consequences of loss of wild-type Hras.

I show that wild-type Hras is activated in the presence of oncogenic Kras, but that there does not seem to be consistent alteration in the signaling through downstream effectors. Furthermore, there were no significant differences in markers for cellular proliferation, apoptosis, or senescence in tissues from mice lacking wild-type Hras. The tumor suppressive effects of wild-type Hras therefore seem to be an extremely early event, possibly at the time of initiation of the oncogenic mutation.

These findings are important for understanding the interactions between the different RAS proteins, as it is critical to understand the nuances of this signaling for developing potential therapies against RAS-driven cancers. Furthermore, these findings suggest that subtle differences in the levels of wild-type HRAS protein could play a role in determining whether an oncogenic RAS mutation eventually results in formation of a tumor. Thus, these findings could play a critical role in determining an individual's susceptibility to RAS-driven cancers

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## Biography

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