Tissue-specific interactions between oncogenic K-ras and the p19^{Arf}-p53 pathway determine susceptibility to transformation

by

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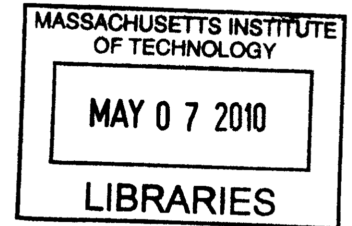
B.S. Molecular and Cellular Biology
Vanderbilt University, 2003



SUBMITTED TO THE DEPARTMENT OF BIOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTORATE OF PHILOSOPHY

AT THE

ARCHIVES

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

APRIL 2010
[June 2010]

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Tissue-specific interactions between oncogenic K-ras and the p19^{Arf}-p53 pathway determine susceptibility to transformation

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Submitted to the Department of Biology on April 30, 2010 in partial fulfillment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Tumor development is a multi-step process driven by the collective action of gain-of-function mutations in oncogenes and loss-of-function alterations in tumor suppressor genes. The particular spectrum of mutations in a given cancer is rarely the result of random chance but instead derives from the intimate connections between proliferative networks and those suppressing growth and transformation. Specifically, hyper-active oncogenes directly engage tumor suppressor programs, such that cells harboring oncogenic lesions frequently must acquire secondary mutations that disable these anti-proliferative responses before progressing to overt transformation. This tight coupling represents a critical checkpoint protecting against tumor formation. Whether different cell types exhibit variability in the extent and/or timing of this oncogene-induced tumor suppression is largely unknown.

The ability of oncogenic Ras to induce the tumor suppressive p19^{Arf}-p53 pathway and cause irreversible cell cycle arrest typifies this phenomenon. Using this-well established interaction as model, we investigated the cell-type specificity of oncogene-induced tumor suppression. By combining *K-ras^{LA2}* mice with a reporter for p19^{Arf} expression (*Arf^{GFP}*), we identify a tissue-specific, onocogenic K-ras-dependent expression pattern of p19^{Arf} in lung tumors and sarcomas that correlates with each tissue's genetic requirements for tumorigenesis. Lung tumors, which can arise in the presence of p19^{Arf} and show modest increases in tumor progression in its absence, exhibit very minimal p19^{Arf} induction. Conversely, sarcomas, which depend on p19^{Arf}-p53 mutation for tumor formation, display robust p19^{Arf} up-regulation. While previous studies proposed oncogene levels as the main determinant of p19^{Arf} induction, we find equivalent signaling levels and instead highlight tissue-specific differences in the epigenetic regulation of *Ink4a/Arf*. Using *in vivo* RNAi, we implicate Polycomb group (PcG) protein-mediated repression in lung tumors and SWI/SNF-dependent activation in sarcomas as being critically important for each tissue's unique expression pattern of p19^{Arf}.

During normal tumor progression, mutations in oncogenes and tumor suppressors occur in a sequential fashion, although whether unique orders of mutations dictate distinct phenotypes is unknown. The requirement for complete p53 pathway abrogation during oncogenic K-ras-dependent sarcomagenesis suggested that tumor development in the muscle critically depends on early p53 mutation. To test this we generated a Flp-inducible allele of K-ras^{G12D} (*K-ras$^{FSF-G12D}$*) that when combined with established reagents for Cre-dependent p53 deletion permits the separate regulation of K-ras activation and p53 loss. Strikingly, although simultaneous mutation results in robust tumor formation, delaying p53 deletion relative to oncogenic K-ras expression

significantly diminishes tumor penetrance. This indicates that the tumorigenic capacity of K-ras^{G12D} mutant muscle cells is rapidly and severely compromised by a strong p53-dependent response, which is entirely different from the mode of action of p53 during lung tumorigenesis. Further genetic analysis implicates the p53 target gene p21 in this suppression, implying that p53 irreversibly constrains sarcoma development through cell cycle arrest mechanisms.

Together, these results highlight tissue-specific variability in the relationship of oncogenic K-ras and the p53 pathway. Robust pathway up-regulation, as seen in muscle cells, affords potent inhibition of tumor initiation, while modest induction, such as in lung cells, permits tumor development and only hinders more advanced stages of progression. These differences might help explain the spectrum of tumors associated with *K-Ras* mutations as well as the overall frequency of difference cancer types.

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N. Young and T. Jacks. A new system for sequential mutagenesis reveals the importance of early p53 mutation in K-ras^{G12D}-driven sarcomagenesis. In preparation.

D.G. Kirsch, D.M. Dinulescu, J.B. Miller, J. Grimm, P.M. Santiago, **N.P. Young**, G.P., Nielsen, B.J. Quade, C.J. Chaber, C.P. Schultz, O Takeuchi, R.T. Bronson, D. Crowley, S.J. Korsmeyer, S.S. Yoon, F.J. Hornicek, R. Weissleder, and T. Jacks. A spatially and temporally restricted mouse model of soft tissue sarcoma. *Nature Medicine*. 2007 August; 13(8): 992-7.

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N.P. Young and T Jacks. "Tissue-specific p19Arf-p53 up-regulation dictates the response to oncogenic K-ras". **Poster**. Cold Spring Harbor Conference: Mechanisms and Models of Cancer. Cold Spring Harbor, NY. 2008.

N.P. Young and T. Jacks. "Novel Tools for Analysis of Stepwise Mutational Events in Mouse Models of Human Cancer". **Poster**. Mouse Models of Human Cancer Consortium Steering Committee. Colorado Springs, CO. 2008.

"Tissue-specific p19Arf-p53 up-regulation dictates the response to oncogenic K-ras." **Talk**. Colrain Meeting. 2007.

N.P. Young and T. Jacks. "Novel Tools for Analysis of Stepwise Mutational Events in Mouse Models of Human Cancer". **Poster**. Beatson International Cancer Conference: 24 years of ras and human cancer. Glasgow, Scotland. 2006.

ACKNOWLEDGEMENTS

These past 7 years at MIT have been filled with so many varied experiences, all of which have left their own indelible marks. At times full of despair and frustration, only to be countered by moments of irrepressible excitement and elation that accompanies newfound knowledge, this veritable rollercoaster ride has not been experienced in isolation. The personal and professional relationships I have cultivated during this time not only greatly contributed to my development as a young scientist, but also provided constant enjoyment and support that truly enriched my life in Boston and will continue to do so in the future. In particular I would like to acknowledge…

…Tyler, for giving me the opportunity to be a part of his lab. He takes special care to generate a lab environment full of intelligent, thoughtful, and personable scientists, and his efforts pay off year in and year out. Furthermore, despite his overwhelming schedule, he always somehow makes time if serious situations arise, for among his many responsibilities throughout the Institute he truly cares for the welfare of lab-mates above all else. Most importantly, in his ability to be such dedicated family man while having so many professional duties, he serves as a role model for how to keep perspective and lead a balanced life.

…Jackie Lees and Michael Hemann, for sitting on my thesis committee from the beginning and providing me with helpful advice and guidance over the years. Despite my pessimistic tendencies, especially during the middle years, they continuously supported and encouraged me to see it through. Their willingness to meet and discuss my projects outside of formal committee meetings was truly appreciated. I would also like to thank Bob Weinberg and Karen Cichowski for reading my thesis and participating in my final meeting. Special thanks to Bob for being a critical member of the audience during all of my floor meetings, reminding me that one should be prepared to defend their scientific claims.

…Michel DuPage, for being my partner in crime for so many years in the Jacks lab. Our shared sense of humor, taste in music, and interest in certain extracurricular activities provided me with some of my best memories from graduate school. Just as important, his supreme intellect constantly challenged me to think more critically and creatively, and I am a much better scientist as a result.

…Keara Lane and Ann Cheung, for being exceptional grad student role models to me in the lab from day one. The challenging, independent research projects they assumed as young graduate students encouraged me to do the same, for better or worse. Aside from expert scientific advice over the years, their willingness to have the occasional drink or two contributed greatly to my sanity. A special thanks to Keara for being my go-to person when things weren't going so well. Misery loves company.

….Judy Teixeira and Anne Deconinck, who work tirelessly, often behind the scenes, to keep the Jacks lab afloat. They both contribute so much to making the Jacks lab a place not only where fantastic science is done where much fun is had in the process.

…past Jacks lab members, for your guidance during my formative years. Especially to Kevin Haigis, whose extreme sarcasm is matched only by his consummate skills as a scientist. Additional thanks to Carla, Andrea, and David K. for their scientific acuity, optimism, and helpful advice and encouragement during their time in the lab and beyond.

…current Jacks Lab members, who continue to make the Jacks lab a great place to live and work. A special thanks to Irene, who has done a great job as the new social chair of the lab, making sure that working hard is accompanied by playing hard.

…classmates and friends outside of MIT, with a special thanks to the "Rat Pack", who made the first year exceptionally fun (if hard to remember), and H. Dave (and Heather) for all of our fun at DMB concerts and on camping trips.

…my friends back home in Rochester, especially Marc, Mary K, and Shelly. You never quite understood what was taking me so long, but remained supportive throughout the process.

…the 5th floor, entire Cancer Center, floor meeting labs, and Department of Biology, for making MIT such a unique place to do science. Being surrounded by so many smart people who are so dedicated to the pursuit of knowledge continues to inspire me every day.

…my parents and other family members, who have unwaveringly supported me in everything I've ever pursued. Over the years they have always provided encouragement while never putting pressure on me, which has been a large part of my success.

…my son, Nate Jr., for giving me a huge smile every evening I walk through the door, for correcting some sleeping habits that could have wreaked havoc on this thesis, and for reminding me that regardless of how badly an experiment fails, he still needs someone to change him , feed him , and play with him, so I better get over it. This shift in perspective has been one of my most important lessons learned, and I look forward to many more he will be sure to teach me over the years.

…my wife, Amanda, for everything. From the first day of graduate school orientation through the dark days of the middle years and continuing on to the writing of this thesis and everything in between, she has been there for me, and I am not sure I would have been able to do it without her unceasing friendship, sense of humor, intelligence, and love. Despite not always being the most pleasant person to be around, she continued to stand by me, and for that I am forever grateful. Among her countless traits that I would be wise to emulate, her ever-present optimism has truly enriched my life experience above all else. Professionally, she has also been a tremendous colleague, and her amazing abilities as scientist are surpassed only by the job she has done being an exceptional mother to our son. I dedicate this thesis to you.

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CHAPTER 1: INTRODUCTION

Cancer is a genetic disease in which normal tissue homeostasis is compromised due to the acquisition of various traits by effected cells, including excessive proliferation, immortalization, and resistance to apoptosis (Hanahan and Weinberg 2000). These traits are largely obtained through the sequential accumulation of two broad classes of mutations: activating, gain-of-function alterations in pro-proliferative oncogenes, and inactivating, loss-of-function disruptions in growth inhibitory tumor suppressor genes. The co-occurrence of these types of mutations underscores an intimate association between networks that antagonistically regulate cellular proliferation and survival (Lowe et al. 2004). This thesis is largely devoted to such a relationship between oncogenic K-ras and the p19Arf-p53 tumor suppressor pathway, uncovering important tissue specificity in the nature of this connection. Using a variety of mouse genetics tools, including a novel system for sequential mutagenesis, this thesis describes how such variability can directly affect the ability of different cell types to be transformed by particular mutations.

A. The p19^{Arf}-p53 tumor suppressor pathway

To protect against aberrant cellular expansion, normal cells employ a variety of mechanisms that potently inhibit proliferation (Hanahan and Weinberg 2000; Sherr 2004). The precise regulation and functioning of these checkpoints ensures controlled growth only in the appropriate settings. Accordingly, these anti-growth pathways respond to a wide variety of cellular stresses indicative of abnormal environmental conditions and execute downstream effector functions such as cell cycle arrest or cell death if properly engaged. Many of these pathways comprise canonical tumor suppressor pathways, and not surprisingly, their functional inactivation represents one of the critical steps for tumor initiation and progression. The

following section introduces two components of one of the most important of these pathways, the p19^{Arf}-p53 tumor suppressor axis, which plays a central role in Chapters 2 and 3.

i. p19^{Arf}

a. Discovery

The story of p19^{Arf} begins with the initial discovery and characterization of the *CDKN2* locus. This genomic region was originally shown to encode two small proteins, p16^{Ink4a} and p15^{Ink4b}, both novel binding partners and inhibitors of cyclin-dependent kinase 4 (CDK4), a critical component of the cell cycle machinery that inactivates the Rb tumor suppressor and helps drive the G1/S transition (Serrano et al. 1993; Hannon and Beach 1994; Weinberg 1995). As normal cell cycle progression was known to require CDK-dependent phosphorylation of Rb to promote E2F-dependent transcriptional activation of key proliferative genes, the CDK inhibitory functions of these newly discovered proteins implicated this locus as an upstream activator of Rb, ultimately functioning to inhibit the cell cycle (Weinberg 1995). Interestingly, p16^{Ink4a} was initially thought to have two transcripts that differed in their first exon (Duro et al. 1995; Mao et al. 1995; Stone et al. 1995). Upon closer inspection it was determined that despite splicing into the same second exon the resulting mRNAs would be in different reading frames and thus encode two distinct polypeptides. While exon E1α usage resulted in p16^{Ink4a}, this new 19-kilodalton (kd) protein initiating from the upstream exon E1β was named p19^{Arf} (alternate reading frame) (p14^{Arf} in humans) (Quelle et al. 1995).

Given that Rb was an established tumor suppressor, the functions ascribed to p16^{Ink4a} and p15^{Ink4b} immediately suggested a role for this genomic region in tumor suppression as well. Mutational analyses supported this idea, as a variety of tumor types, including melanoma,

pancreatic cancer, and non-small cell lung cancer harbored mutations in this locus (Hussussian et al. 1994; Nobori et al. 1994; Goldstein et al. 1995; Okamoto et al. 1995; Walker et al. 1995). Many different types of mutations were found, including large-scale deletions, point mutations, and promoter hypermethylation (Hussussian et al. 1994; Cairns et al. 1995; Herman et al. 1995). In light of the physical overlap of *Ink4a* and *Arf*, the distribution of some of these mutations potentially implicated $p19^{Arf}$ as a critical tumor suppressor as well. In addition, initial experiments in NIH 3T3 fibroblasts demonstrated $p19^{Arf}$'s ability to cause cell cycle arrest (Kamijo et al. 1997). Together, these data supported a model in which *CDKN2* (*CDKN2A* or *Ink4a/Arf* for the region encompassing only $p19^{Arf}$ and $p16^{Ink4a}$) encoded genetically linked but structurally distinct tumor suppressor genes.

b. Functional analyses

p53-dependent functions

The G1 cell cycle arrest executed by $p19^{Arf}$ overexpression suggested that this newest member of *CDKN2* could also be a CDK inhibitor. However, unlike $p16^{Ink4a}$ and $p15^{Ink4b}$, immunoprecipitates of a variety of cyclins and CDKs did not contain $p19^{Arf}$, and *in vitro* CDK assays were unaffected by its presence (Kamijo et al. 1997). Moreover, the protein was predominantly localized to the nucleolus, unlike known CDK inhibitors. These results argued for a novel mechanism for $p19^{Arf}$ function independent of the regulation of cyclin/CDKs.

In the search for molecular determinants of $p19^{Arf}$ function, a number of observations implicated the well-known tumor suppressor p53. First, the ability of $p19^{Arf}$ to arrest rodent cells was abrogated in the absence of functional p53 (Kamijo et al. 1997). This p53-dependence was also noted in standard transformation assays in which $p19^{Arf}$'s transformation-suppressing

functions were lost in the presence of p53-neutralizing agents (Pomerantz et al. 1998). Together with studies showing a mutually exclusive pattern of mutation in either *Arf* or *p53* during spontaneous immortalization of primary mouse cells, these data suggested that p19^{Arf} acted upstream of p53 in a linear pathway (Kamijo et al. 1997).

Subsequent biochemical analysis confirmed this genetic model when it was discovered that p19^{Arf} physically interacted with Mdm2, a known inhibitor of p53 (Pomerantz et al. 1998; Zhang et al. 1998). Previous work had shown that through its activity as an E3 ubiquitin ligase, Mdm2 was able to bind to p53 and promote the proteosomal degradation of this tumor suppressor (Haupt et al. 1997; Honda et al. 1997; Kubbutat et al. 1997). However, binding of p19^{Arf} to Mdm2 resulted in the degradation of Mdm2 itself, thus freeing p53 from this negative regulation. In addition to its effect on Mdm2 protein stability, p19^{Arf} could also sequester Mdm2 in the nucleolus, preventing further interaction with p53 (Weber et al. 1999). These data led to a model attributing p19^{Arf}'s growth suppressive function to its ability to indirectly increase p53 protein levels, which once stabilized can initiate one a variety growth-inhibitory transcriptional programs. Details of these effector pathways downstream of p53 will be discussed further in Section A ii b.

Together with studies of p16^{Ink4a}, these results implicated the *Ink4/Arf* locus as a critical regulatory node in two of the most important tumor suppressor pathways, p53 and Rb (Figure 1). p16^{Ink4a}, through its inhibition of cyclinD/CDK4 complexes, maintains Rb in a hypophosphorylated state and thus competent to repress target genes required for normal cell cycle progression. In parallel, p19^{Arf} potently up-regulates p53, leading to the induction of various cytostatic or cytotoxic gene expression programs.

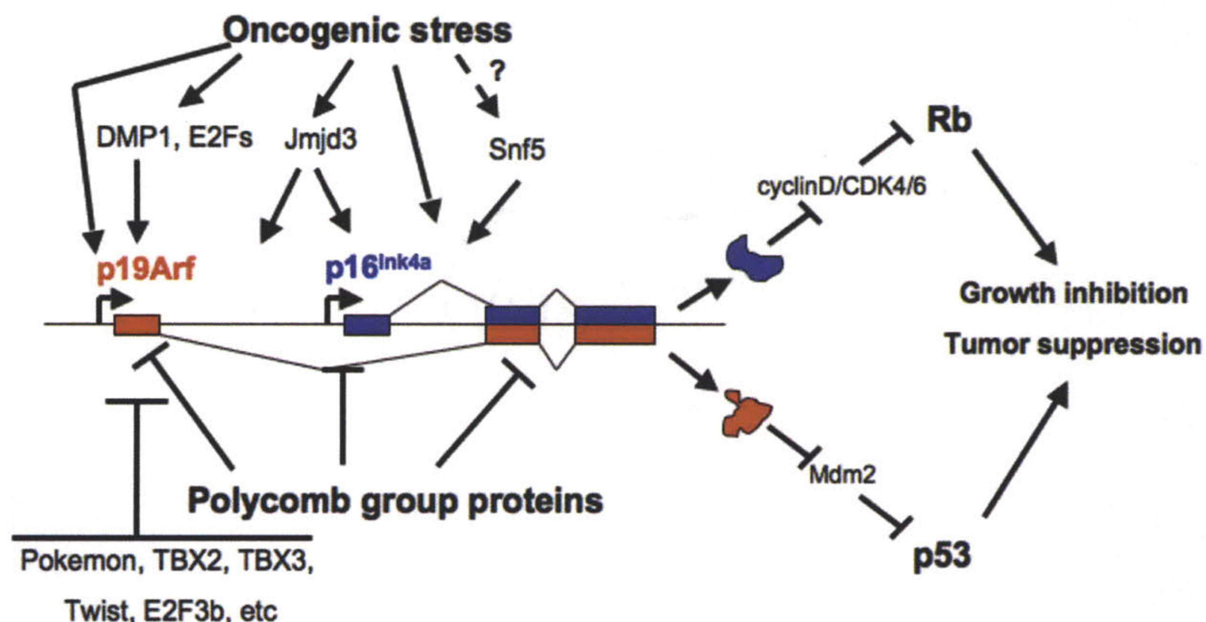
p53-independent functions

In addition to the large body of work showing that $p19^{Arf}$ acts in a p53-dependent manner, there is growing evidence that it can also have growth inhibitory activity in the absence of p53. The first insight into potential p53-independent functions came from experiments demonstrating that exogenous expression of $p19^{Arf}$ in *Trp53*$^{-/-}$; *Mdm2*$^{-/-}$ MEFs caused a significant G1 cell cycle arrest (Weber et al. 2000). Interestingly, Mdm2 deletion was required for this effect, suggesting that it normally suppresses this function of $p19^{Arf}$. Additional biochemical studies have generated a long list of binding partners of $p19^{Arf}$, many of which could be involved in these p53-independent functions (reviewed in (Sherr 2006). For example, $p19^{Arf}$ interacts with and potentially sequesters nucleophosmin (NPM), a gene important for the increased ribosomal RNA synthesis required in proliferating cells (Itahana et al. 2003). Furthermore, $p19^{Arf}$ has been shown to bind to and inhibit transactivation capabilities of several transcription factors important in cell cycle progression, such as E2F1 and c-myc (Martelli et al. 2001; Qi et al. 2004). Finally, a smaller form of $p19^{Arf}$ derived from an internal start codon, named smArf, preferentially localizes to mitochondria and regulates autophagy (Reef et al. 2006). Clearly, despite the strong connections between $p19^{Arf}$ and the p53 pathway, $p19^{Arf}$ also possesses additional functions apart from regulating p53. Defining the contexts in which these newly appreciated roles are important is an area of active investigation.

c. Regulation

Transcriptional repression

Expression from the entire *CDKN2A* locus is not readily detectable in most mouse tissues or early passage mouse embryonic fibroblasts (MEFs). Studies involving the Polycomb group



<u>Figure 1:</u> The *Ink4a/Arf* locus is an important regulatory node in the p53 and Rb tumor suppressor pathways.
The overlapping genomic arrangement of $p19^{Arf}$ (red) and $p16^{Ink4a}$ (blue) coding regions is shown, as are the major upstream regulatory signals mediating both transcriptional induction and repression of the locus. See text for details of this regulation. Once induced, this locus can indirectly activate the Rb tumor suppressor through $p16^{Ink4a}$-mediated inhibition of cyclin/CDK complexes. In addition, $p19^{Arf}$ up-regulation can induce the p53 tumor suppressor pathway by interfering with Mdm2's ability to destabilize p53.

(PcG) protein Bmi-1 provided the first evidence that this lack of expression was largely due to PcG-mediated transcriptional repression of *Ink4a/Arf*, by showing that *Bmi-1*$^{-/-}$ MEFs and tissues displayed elevated levels of p19^{Arf} and p16^{Ink4a} (Jacobs et al. 1999). PcG proteins are found in one of two main Polycomb repressive complexes (PRC). PRC2 contains a histone methyltransferase named Ezh2, that along with binding partners including Eed and Su(z)12, methylates lysine 27 on histone H3 (H3K27me3). This mark serves as a docking site for another complex, PRC1 (including Bmi-1), which binds H3K27me3 and mediates gene repression. The exact mechanism of this repression is still under investigation but could involve the physical blockade of transcription factor binding by the generation of a compact chromatin environment that limits the accessibility of DNA binding sites (reviewed in Schuettengruber et al. 2007)).

Since the initial experiments with Bmi-1, numerous studies have reported H3K27me3 as well as both PRC2 and PRC1 complexes binding throughout *Ink4a/Arf* in a variety of cell types *in vitro* and *in vivo* (Bracken et al. 2007; Dietrich et al. 2007; Miyazaki et al. 2008; Chen et al. 2009; Dhawan et al. 2009). Along with Bmi-1, the PRC1 components CBX7 and CBX8 have been shown to directly bind the locus (Maertens et al. 2009), and genetic studies have validated a functional role for many PcG proteins in repressing *Ink4a/Arf* (Gil et al. 2004; Chen et al. 2009). In fact, several defects resulting from genetic disruption of particular PcG members are rescued by simultaneously deleting *Ink4a/Arf*, suggesting that one of the main physiological roles of PcG is to prevent aberrant expression from this locus (Jacobs et al. 1999; Molofsky et al. 2005). Interestingly, the cell-type specificity of this mode of repression seems to vary across tissues. In the hematopoietic and central nervous systems, this regulation appears to be especially important in stem cells for the maintenance of self-renewal capabilities (Molofsky et al. 2003; Park et al.

2003). In other settings PcG controls *Ink4a/Arf* expression throughout entire tissues (Dovey et al. 2008; Dhawan et al. 2009).

Aside from the coordinated, *Ink4a/Arf*-wide regulation by PcG proteins, a number of other transcriptional repressors specific for $p19^{Arf}$ exist. Zbtb7, a transcription factor with diverse roles in cellular differentiation, was shown to inhibit $p19^{Arf}$ transcription in MEFs by directly binding its promoter (Maeda et al. 2005). Similar observations have been made with the repressive E2F family member E2F3b (Aslanian et al. 2004). Additional regulators have been identified through functional cDNA screens attempting to bypass the strong growth inhibitory functions of the $p19^{Arf}$-p53 pathway *in vitro*. Multiple transcription factors, including two members of the T-box family, TBX2 and TBX3, as well as bHLH-containing Twist and Twist2, were isolated in this manner and subsequently shown to repress $p19^{Arf}$ transcription (Maestro et al. 1999; Jacobs et al. 2000). The functional relevance of many of these non-PcG regulators in controlling $p19^{Arf}$ expression *in vivo* remains to be determined.

Transcriptional Activation

While levels of $p19^{Arf}$ are low in most settings, its expression can be induced under several conditions. Prolonged passaging of primary cells *in vitro* leads to up-regulation of $p19^{Arf}$ (and/or $p16^{Ink4a}$, depending on the cell type), which activates p53 and causes irreversible growth arrest, a process known as replicative senescence (Sherr and DePinho 2000). Accordingly, functional inactivation of the $p19^{Arf}$-p53 pathway is the main route to immortalization of MEFs (Kamijo et al. 1997). The factors responsible for this activation are unknown, although hypoxia and/or sustained growth factor signaling may play a role (Parrinello et al. 2003).

The predominant stimulus eliciting p19^{Arf} up-regulation both *in vitro* and *in vivo* is expression of oncogenes such as, E1A, Ras, and c-myc (de Stanchina et al. 1998; Palmero et al. 1998; Zindy et al. 1998). Loss of tumor suppressor genes such as PTEN and Rb family members can also promote p19^{Arf} activation (Dannenberg et al. 2000; Chen et al. 2005). The common link between these alterations is the excessive nature of downstream proliferative signals that denote abnormal mitogenic pathway flux. This hyperactivity ultimately turns on transcriptional regulators that directly engage the p19^{Arf} promoter, and at times, the entire *Ink4a/Arf* locus (reviewed in Gil and Peters 2006).

Several studies have implicated specific transcription factors with particular oncogenic insults. For example, increased abundance of activating E2Fs (E2F1-3a) underlies a number of oncogenic states, and their ability to directly bind and activate the p19^{Arf} promoter suggests a central role in p19^{Arf} induction (Bates et al. 1998; Aslanian et al. 2004; Iaquinta et al. 2005). However, redundancy of family members has complicated functional analyses (Palmero et al. 2002). In contrast, genetic studies with the transcriptional activator Dmp1 have convincingly demonstrated its importance in relaying signals from both c-myc and Ras to p19^{Arf} under certain conditions, including myc-induced lymphomas (Inoue et al. 2000; Inoue et al. 2001). Other factors have been shown to have stimulatory effects in distinct settings, such as c-Jun, β-catenin, Runx1 and FOXO3 (Gil and Peters 2006). p19^{Arf}'s role as a detector of hyperproliferative stimuli has led to the concept that its main function is to serve as a checkpoint, limiting the outgrowth of cells harboring oncogenic insults.

Given the repressive chromatin state of *Ink4a/Arf* under non-stressed conditions, the activation of this locus must involve chromatin-remodeling activities. Genetic screens performed in *D. melanogaster* identified SWI/SNF chromatin-remodeling complexes as critical for the

activation of PcG-regulated genes during development (Tamkun et al. 1992; Gebuhr et al. 2000). This class of ATPase –dependent chromatin modifiers alters nucleosome occupancy around specific genomic regions, which exposes distinct stretches of DNA and allows for transcription factor binding (Gebuhr et al. 2000). Interestingly, recent studies have demonstrated a role for the SWI/SNF member Snf5 during *Ink4a/Arf* activation, suggesting that nucleosomal remodeling is an important component of this process (Kia et al. 2008). Moreover, the H3K27me3 demethylase Jmjd3, which effectively erases PcG histone marks, has been shown to be required for maximal induction of $p19^{Arf}$ and $p16^{Ink4a}$ in response to oncogenic H-ras (Agger et al. 2009; Barradas et al. 2009). Finally, MLL1, a histone methyltransferase responsible for the generation of transcriptionally active chromatin configurations, has also been implicated in directly modifying the *Ink4a/Arf* locus and regulating its expression during oncogenic stress (Kotake et al. 2009). Clearly, alteration of the local chromatin state represents a critical component to $p19^{Arf}$ activation.

Post-translational control

Although most well studied at the transcriptional level, $p19^{Arf}$ regulation also occurs post-translationally. One of the most abundant binding partners of $p19^{Arf}$ is the nucleolar-localized protein NPM (Sherr 2006). *NPM*$^{-/-}$ MEFs display a dramatic reduction in the half-life of $p19^{Arf}$, suggesting that NPM regulates $p19^{Arf}$ protein stability (Colombo et al. 2005). This most likely results from NPM's ability to block a unique N-terminal polyubiquitination of $p19^{Arf}$, which normally leads to its proteosomal-mediated degradation (Kuo et al. 2004; Colombo et al. 2005). Notably, removing this regulation significantly hindered $p19^{Arf}$'s ability to inhibit Ras and myc-dependent transformation, as *NPM*$^{-/-}$; *Trp53*$^{-/-}$ MEFs were more susceptible to colony formation

than *Trp53*$^{-/-}$ MEFs (Colombo et al. 2005). While NPM is mutated in human cancers, it is unclear if this p19^{Arf}-associated function is involved in its tumor suppressor activity.

d. Mouse models

The first mouse model involving p19^{Arf} was a knockout of exons 2 and 3 in *Ink4a/Arf*, which removed the function of both p16^{Ink4a} and p19^{Arf}. These mice were highly susceptible to tumorigenesis, with homozygous mutants (*Ink4a/Arf*$^{-/-}$) presenting with both spontaneous and carcinogen-induced lymphomas and sarcomas (Serrano et al. 1996). In addition, knockout MEFs were immortalized and readily transformed by oncogenic H-ras, directly implicating the endogenous locus in cell cycle control and transformation. While these initial studies functionally validated this genomic region as a tumor suppressor locus, it didn't distinguish between p19^{Arf}, p16^{Ink4a}, or both as the critical player(s). Soon thereafter, exon 1β was targeted, thus creating a knockout of p19^{Arf} specifically. Interestingly, *Arf*$^{-/-}$ mice and cells closely phenocopied their *Ink4a/Arf*$^{-/-}$ counterparts, especially in *in vitro* assays, suggesting that p19^{Arf} alone was the predominant tumor suppressor, at least in mice (Kamijo et al. 1997). However, analysis of *Ink4a*$^{-/-}$ mice indicated tumor suppressor activity for p16^{Ink4a} alone, and careful studies of *Arf*$^{-/-}$, *Ink4a*$^{-/-}$, and *Ink4a/Arf*$^{-/-}$ on the same genetic background revealed that *in vivo*, both p16^{Ink4a} and p19^{Arf} exhibit tumor suppressor functions in mice (Sharpless et al. 2004).

Subsequent analyses with p19^{Arf} knockout animals have provided *in vivo* validation for many of its characteristics described in previous sections. For example, introducing *Arf*$^{-/-}$ onto a *Trp53*$^{-/-}$ background fails to increase tumor predisposition, underscoring a largely p53-dependent role for p19^{Arf} in tumor suppression (Weber et al. 2000). However, differential effects of *Arf* or *p53* mutations have been reported in some contexts, suggesting that p19^{Arf} can act in a p53-

independent manner *in vivo* (Tsai et al. 2002; Ha et al. 2007). Most importantly, the exacerbation of tumor phenotypes upon inclusion of $Arf^{-/-}$ in a variety of tumor-prone models has conclusively demonstrated its role as a checkpoint limiting the outgrowth of tumorigenic clones *in vivo* in diverse settings (Eischen et al. 1999; Kelly-Spratt 2004; Williams et al. 2006). To complement these loss-of-function approaches, transgenic mice containing an extra copy of the entire *CDKN2* locus have been described. These mice are more tumor resistant than their wild-type counterparts when treated with carcinogens, suggesting that members of this locus act as dose-dependent tumor suppressors (Matheu et al. 2004).

In light of the difficulties in detecting $p19^{Arf}$ expression *in vivo*, the Sherr group recently constructed a reporter allele, Arf^{GFP} in which GFP was placed in-frame into exon 1β, thus knocking out $p19^{Arf}$ and putting GFP under control of the endogenous $p19^{Arf}$ promoter (Zindy et al. 2003). In the large majority of cells within these mice, GFP is undetectable. However, during spontaneous or c-myc-induced tumorigenesis, GFP expression is induced in the tumor cells, supporting the notion that the $p19^{Arf}$ promoter is a sensor of hyperproliferative signals. To achieve more sensitivity of detection, as well as possibly identify transient expression of $p19^{Arf}$, the same group created a Cre knock-in allele, Arf^{Cre}, which also deleted exon 1β (Gromley et al. 2009). By crossing in a Cre-dependent YFP reporter, they were able to permanently mark cells that expressed $p19^{Arf}$, owing to the genomic recombination event mediated by Cre. Using this system, they documented $p19^{Arf}$ expression in spermatogonia and the mural components of hyaloid vascular system in the eye (HVS). HVS-specific expression of $p19^{Arf}$, which normally leads to the involution of the HVS and proper eye function, is one of the only non-tumor suppressive functions ascribed to $p19^{Arf}$ (McKeller et al. 2002). In addition to being a very

sensitive reporter, use of this strain will allow for other Cre-controlled genetic events to be targeted specifically to p19^{Arf}-expressing cells in the future.

e. mouse vs. human differences in Ink4a/Arf tumor suppressive functions

The initial genetic analyses of *Ink4a/Arf*$^{-/-}$ mice and cells implicating p19^{Arf} as the more critical tumor suppressor compared to p16^{Ink4a} was surprising given the mutational data from humans. A number of alterations from human tumors, including promoter hypermethylation and exon 1α-specific point mutations, selectively affect p16^{Ink4a} while sparing p14^{Arf} (Holland et al. 1995; Walker et al. 1995). Furthermore, functional studies in human cell lines have questioned the role of p14^{Arf} in mediating p53-dependent effects downstream of oncogenic insults (Voorhoeve and Agami 2003). The reasons for these discrepancies are currently unknown, but could relate to the greater importance of other upstream activating signals for p53 in some human cells (further discussed in Section A ii c below). While these observations led some to speculate that Ink4a/Arf function varies across species, it could also be explained by intraspecies cell-type specificity, as the relative roles of p19^{Arf} versus p16^{Ink4a} vary among different cell types within the mouse as well. For example, while MEFs and pre-B cells require only p19^{Arf} loss for immortalization, macrophages must disable p16^{Ink4a} as well (Randle et al. 2001). Moreover, *Arf*$^{/-}$ and *Ink4a*$^{/-}$ animals have unique tumor spectra, suggesting that different cells types are more susceptible to removal of a particular gene (Sharpless et al. 2004). Finally, p14^{Arf}-specific mutations have been documented in human tumors, indicating that some human cells rely on this component of *Ink4a/Arf* for tumor suppression (Gazzeri et al. 1998).

ii. p53

a. Discovery

p53 was originally identified as a cellular protein interacting with the large T antigen in cells transformed by SV-40 T-Ag (Lane and Crawford 1979; Linzer and Levine 1979). Initial functional experiments suggested an active role for p53 in transformation, as it was shown that expression of its cDNA could cooperate with H-ras in cellular transformation (Eliyahu et al. 1984; Parada et al. 1984). Only a few years later was it discovered that the original cDNAs used in these experiments actually contained point mutations (Hinds et al. 1989). In contrast to these earlier studies, introduction of wild-type p53 into rodent cells suppressed transformation, suggesting its normal function was to constrain tumorigenesis (Finlay et al. 1989).

Subsequent mutational analyses uncovered mutations in the *Trp53* gene in the cancer-predisposing Li-Fraumeni syndrome (Malkin et al. 1990; Srivastava et al. 1990). Further profiling of a variety of spontaneous tumors indicated an array of somatic mutations in *Trp53*, firmly establishing its role as a tumor suppressor (Hollstein et al. 1991; Greenblatt et al. 1994; Vogelstein et al. 2000). It is estimated that more than half of all human tumors mutate p53, with perhaps all maligancies inactivating its associated pathways at some level, making it one of the most important tumor suppressors in all of cancer biology.

b. Functional analysis

Initial studies with wild-type p53 demonstrated its ability to suppress transformation by oncogenic Ras, suggesting a role in growth inhibitory pathways. Insights into the manner in which p53 exerted this function came from multiple lines of evidence indicating that p53 was a transcription factor. First, its N-terminus was shown to contain transactivation capabilities in Gal4 fusion studies (Fields and Jang 1990; Raycroft et al. 1990). Secondly, a consensus DNA

binding site was revealed and subsequently shown to be in an internal segment of the protein (el-Deiry et al. 1992; Bargonetti et al. 1993; Pavletich et al. 1993; Wang et al. 1993). Other studies indicated that p53 formed a homotetramer to perform these functions (Sturzbecher et al. 1992; Hainaut et al. 1994). Importantly, cancer associated mutations abrogated some of these functions (Kern et al. 1992; Bargonetti et al. 1993). Together, these results suggested that p53 engaged transcriptional programs to inhibit transformation. As described below, additional work further refined this model and illustrated a general role for p53 in mediating cell cycle arrest or cell death downstream of various stresses (Fig 2).

Cell cycle arrest

One of the earliest documented effects of p53 expression was the induction of G1 cell cycle arrest. This was seen both in the context of a cellular response to irradiation (IR), as well as after exogenous expression of p53 (Kastan et al. 1991; Kuerbitz et al. 1992; Agarwal et al. 1995). The defective IR-induced arrest in *Trp53*$^{-/-}$ MEFs conclusively demonstrated a functional role for endogenous p53 in this process (Kastan et al. 1992). Given p53's suspected activity as a transcription factor, a number of groups performed gene expression studies to identify p53-responsive genes that potentially mediated these effects. Using such a strategy, p21$^{WAF/CIP}$ (p21), a potent inhibitor of G1 cyclin/CDK complexes, was identified (el-Deiry et al. 1993). Indeed, *p21*$^{-/-}$ MEFs displayed defects similar to *Trp53*$^{-/-}$ cells following IR treatment, suggesting a functional link between p53 and p21 in mediating cell cycle arrest (Brugarolas et al. 1995; Deng et al. 1995). However, in other settings this connection is not as clear, indicating the presence of additional p53-dependent factors. p53 can also induce a G2/M arrest, which is mediated by its transcriptional activation of factors such as 14-3-3σ, cdc25c, and Gadd45, all of which impinge

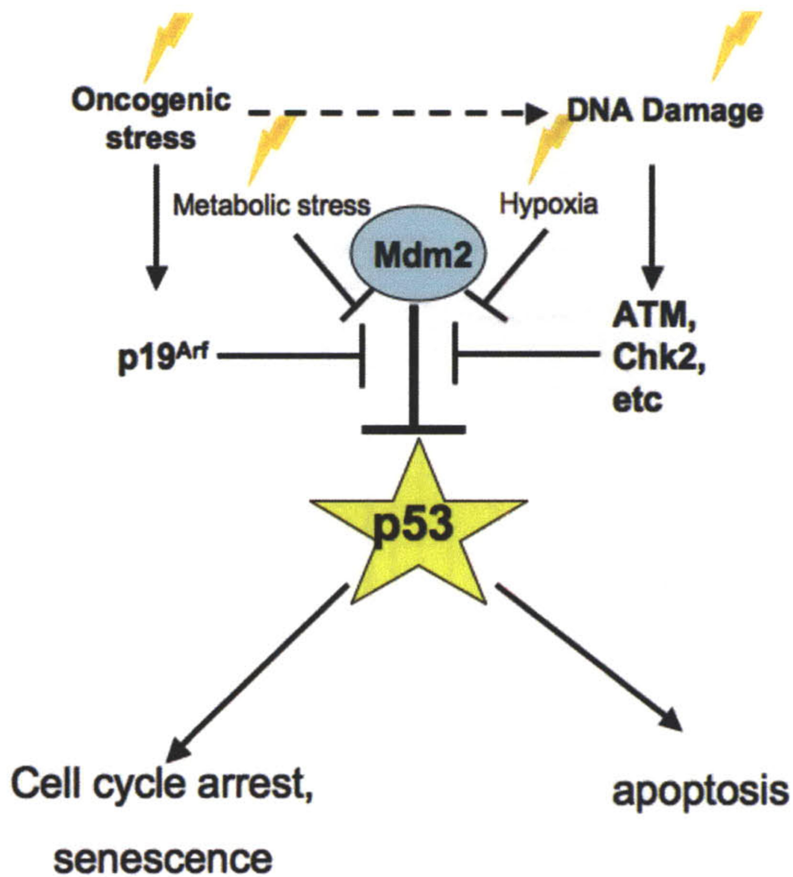


Figure 2: p53 responds to multiple stress-associated signals by inducing diverse gene expression programs.

Under normal conditions, Mdm2 negatively regulates the stability and activity of the p53 protein. Many stresses (lightning bolts) disrupt this interaction, which leads to enhanced p53 levels and function. As a result, this master transcriptional regulator is able to induce its cytostatic or cytotoxic gene expression programs. The extensive cross-talk between stresses is just beginning to be appreciated, such as the ability of oncogenic stress to directly activate both $p19^{Arf}$ and a DNA damage response, both of which might be the critical for p53 induction in distinct settings.

on the cyclinB/CDK1 complex important in the transition into mitosis (reviewed in Taylor and Stark 2001)). By arresting cells after various stresses such as DNA damage, these checkpoint functions of p53 ensure that cells respond to these insults before continuing through the cell cycle, thus limiting the propagation of potential damage. Accordingly, a prominent feature of *Trp53*$^{-/-}$ cells is the accumulation of various kinds of DNA alterations (Harvey et al. 1993; Tarapore and Fukasawa 2002).

Senescence

In some instances, the arrest executed by p53 falls into a unique category called senescence. Numerous stimuli, such as continued passage *in vitro*, dysfunctional telomeres, DNA damaging agents, and oncogene expression can bring about this response (reviewed in Collado and Serrano 2006). Of the defining characteristics, such as an enlarged, flattened cell morphology and a poorly understood senescence associated β-galactosidase (SA-βgal) activity (Dimri et al. 1995), the most functionally relevant feature is the presumed irreversibility of the arrest. While the role of p53 in senescence has been validated from studies with *Trp53*$^{-/-}$ cells and mice, the factors controlling induction of senescence versus a more canonical cell cycle arrest are not well defined. While p21 levels increase in both settings, recent work has highlighted cytokines and extracellular matrix-degrading enzymes as potential senescence-specific p53 target genes. These factors are important for innate immune cell recognition and clearance of senescing cells, a phenomenon seen in multiple models *in vivo* (Xue et al. 2007; Krizhanovsky et al. 2008). It is possible that the p16^{Ink4a}-Rb pathway plays a critical role in selecting the senescence program. p16^{Ink4a} is frequently up-regulated during senescence and might contribute to Rb's senescence-specific function of stable heterochromatin formation in the vicinity of cell cycle genes (Narita et al. 2003). Senescence is discussed further in Part C of the introduction.

Apoptosis

As p53 was investigated in more cell types, it was soon discovered that it had functions apart from cell-cycle arrest. Interestingly, IR treatment of thymocytes elicited a strong p53-dependent cell death response, as opposed to the cell cycle arrest seen in MEFs under the same conditions (Lowe et al. 1993b). Other stimuli, such as chemotherapeutic agents and particular oncogenic insults, were shown to cause p53-dependent apoptosis as well (Lowe and Ruley 1993; Lowe et al. 1993a). Once again, mechanistic insight was provided by studies identifying known apoptosis regulators as p53-responsive genes, such as members of the mitochondrial death pathway like Bax and Puma, as well as some mediators of the extrinsic death pathway such as Fas (Miyashita and Reed 1995; Owen-Schaub et al. 1995; Nakano and Vousden 2001).

Apparently, under certain conditions and in particular cell types, p53 functions to eliminate cells rather than simply arresting them. Given the drastic differences in potential fates downstream of activating p53, a critical question concerns how the decision between arrest and apoptosis is made. Intriguingly, early studies demonstrated that the p53-dependent, IR-mediated G1 arrest in MEFs could be shifted to apoptosis in the presence of the E1A oncogene, suggesting some plasticity in p53 function (Lowe et al. 1993a). An emerging model implicates specific post-translational modifications of p53, such as acetylation and phosphorylation, in the choice of p53 effector functions (Oda et al. 2000; Tang et al. 2006). Such modifications are hypothesized to affect co-factor binding and potential target gene selection (Murray-Zmijewski et al. 2008). Perhaps in certain cell types or following distinct types of stress, particular modifying enzymes are sufficiently expressed such that p53 activation is accompanied by specific modifications, leading to stereotypic downstream function. An entirely separate possibility is that cell-type specific chromatin states surrounding potential target genes affect the accessibility of p53 to

these loci. Finally, the presence of other factors (ie- excess survival signals) could bias a cell towards one fate or the other given a particular gene expression program.

c. Regulation

Under normal conditions, p53 levels are extremely low. This is largely due to a variety of processes imparting a short half-life on the protein, the most well-established being polyubiquitination and subsequent proteosomal-mediated degradation. The E3 ubiquitin ligase Mdm2 is the predominant enzyme controlling this mode of regulation of p53 (Haupt et al. 1997; Honda et al. 1997; Kubbutat et al. 1997). In addition to affecting protein levels, Mdm2 also inhibits the transactivation capabilities of p53 (Momand et al. 1992). Supporting its critical function in p53 regulation, *Mdm2*$^{-/-}$ animals normally die early in embryogenesis but can be rescued on a *Trp53*$^{-/-}$ background (Jones et al. 1995; Montes de Oca Luna et al. 1995). Conversely, high levels of Mdm2 have been observed in cancers, where its overexpression is sufficient to functionally inactivate the p53 pathway and thus obviates the need for p53 mutations (Leach et al. 1993).

In response to numerous cellular stresses, such as DNA damage, hyperproliferative signaling, hypoxia, and nucleotide depletion, p53 protein levels increase rapidly while its mRNA remains relatively constant (Vousden and Lu 2002). Most stimuli mediate this up-regulation by interfering with Mdm2's ability to bind to and regulate p53. Interestingly, Mdm2 is itself a p53 target gene, which creates a negative feedback loop to control p53 levels (Wu et al. 1993). While the list of potential upstream activating signals continues to grow, below I review two of the most well-known examples of such stresses regulating p53 abundance and activity.

DNA damage

The first known treatment to induce p53 protein levels was UV irradiation (Maltzman and Czyzyk 1984). Subsequent studies using genotoxic agents showed similar effects (Lowe et al. 1993a). The DNA damage generated by these various treatments is thought to underlie their ability to up-regulate p53. Through different mechanisms, distinct forms of damage activate specific DNA damage responses (DDR) made up of kinase signaling cascades, some of which eventually impinge on the p53 protein (reviewed in (Kastan and Bartek 2004). For example, IR treatment creates double stranded breaks in DNA that activate a signaling pathway consisting of the serine/threonine kinases ATM and Chk2. Both of these kinases can directly phosphorylate p53 on particular residues in its N-terminus, such as S20 for Chk2, and this disrupts binding of Mdm2 to p53 (Canman et al. 1998; Hirao et al. 2000). Additional DDR-dependent phosphorylations and other post-translational modifications, including acetylation and methylation, have been reported and can have diverse effects on p53 abundance and transactivation functions (Huang et al. 2006; Tang et al. 2006). While varying according to the specific form of DNA damage, the totality of these events results in increased p53 levels and activity and allows p53 to orchestrate downstream gene expression programs.

Excessive mitogenic signaling

Another important route to p53 stabilization and increased activity is the expression of oncogenes such as c-myc, E1A, and Ras (Lowe and Ruley 1993; Hermeking and Eick 1994; Wagner et al. 1994; Serrano et al. 1997). As described in Section A i c., many of these same genes activate $p19^{Arf}$ owing to the excessive nature of their downstream hyperproliferative signals. Following its induction, $p19^{Arf}$ directly binds and inhibits Mdm2, resulting in p53 up-regulation (see Section A.i.b.). Studies showing dampened p53 responses to a variety of oncogenes in *Arf*$^{-/-}$ cells and tissues support a critical role for $p19^{Arf}$ in oncogene-dependent p53

induction (de Stanchina et al. 1998; Palmero et al. 1998; Eischen et al. 1999; Schmitt et al. 1999). In some contexts, oncogenic signaling may also affect p53 through post-translational modifications. For example, oncogenic Ras was shown to promote the relocalization and subsequent p300-dependent acetylation of p53 through the up-regulation of the PML, a tumor suppressor gene (Ferbeyre et al. 2000; Pearson et al. 2000). Moreover, oncogenic Ras-induced p38 MAPK pathway activation can lead to direct phosphorylation of p53 at S37, which is necessary for its transactivation function (Sun et al. 2007).

To add additional complexity, recent work has shown that oncogene-induced p53 activation is largely due to a DDR caused by unregulated cell proliferation. In these studies oncogene expression was shown to generate a significant DDR, and removing core components of the DDR such as ATM or Chk2 blocked oncogene-induced p53 up-regulation (Bartkova et al. 2006; Di Micco et al. 2006). A potential reconciliation of the conflicting evidence that both $p19^{Arf}$-dependent (hyperproliferative stimuli) and DDR-dependent factors up-regulate p53 following oncogenic insults might be found in mouse versus human differences (Efeyan et al. 2009). Studies implicating $p19^{Arf}$ were largely from mice, where $p19^{Arf}$ may play a more predominant role, and those implicating the DDR were mostly performed in human cells, which might have unique circuitry connecting oncogenes to p53 (Voorhoeve and Agami 2003). Alternatively, the DDR and $p19^{Arf}$ ($p14^{Arf}$) could be important in both settings through partially overlapping pathways, as there is limited evidence of bidirectional cross-talk between DNA damage and Arf regulation (Khan et al. 2000; Eymin et al. 2006).

d. Mouse models

p53 was the first tumor suppressor gene to be targeted for inactivation in the mouse. While development appeared to be relatively normal in *Trp53*$^{-/-}$ mice, they were extremely tumor-prone and succumbed mostly to T-cell lymphomas and soft-tissue sarcomas within a few months of life (Donehower et al. 1992; Jacks et al. 1994). Cooperation with numerous other models of cancer, both genetically engineered and carcinogen-induced, highlighted a broad tumor suppressive role for p53 beyond the tissues affected in *Trp53*$^{-/-}$ mice. Generation of an allele affording Cre-regulated p53 deletion, *Trp53*^{flox}, allowed for cell-type specific knockouts and was instrumental in expanding the tissues in which p53 was studied (reviewed in (Donehower and Lozano 2009).

Given the diverse functions ascribed to p53 from cell culture studies, a critical question became which of these roles were important for its tumor suppression activities *in vivo*. Introducing *Trp53*$^{-/-}$ into a mouse model of T-antigen-driven brain tumors suppressed apoptosis and accelerated tumor progression, implicating p53's promotion of cell death as a critical tumor suppressive mechanism (Symonds et al. 1994). On the other hand, a point mutant allele of p53 incapable of inducing apoptosis but competent for cell cycle arrest, *Trp53*^{515C}, still retained significant tumor suppressor activities, suggesting that cell cycle arrest is also important in particular contexts (Liu et al. 2004). As with *in vitro* studies, the choice between effector functions appears to be cell-type specific. However, it has been recently shown that incipient lymphomas, which normally undergo p53-dependent apoptosis, can undergo senescence if cell death programs are inhibited, suggesting that there is some plasticity in this decision (Post et al. ; Feldser and Greider 2007).

Deciphering which of the many potential upstream signaling events are necessary for p53-dependent tumor suppression has also been addressed in mouse models. In some settings,

p53 function appears to be p19^{Arf}-independent (Tolbert et al. 2002), while other studies strongly implicate this pathway. For example, Evans and colleagues constructed a knock-in allele of p53 expressing a tamoxifen-regulated version of the p53 protein (p53^{ER}), allowing them switch p53 function on and off (Christophorou et al. 2005). By adjusting the time at which p53 was active following IR treatment, they were able to separate p53-dependent lymphoma suppression from the DDR associated with IR. Moreover, this suppression was abolished in the absence of p19^{Arf} , suggesting that oncogenic signals were the critical inducer in this setting (Christophorou et al. 2006). Further work with this system, along with two others that allowed for p53 reactivation in established tumors, demonstrated that p53 deficiency was required for tumor maintenance and also provided additional evidence for cell-type specific modes of p53-dependent tumor suppression, including apoptosis for lymphomas and senescence in sarcomas (Martins et al. 2006; Ventura et al. 2007; Xue et al. 2007).

While most of these studies dissected tumor-associated p53 properties with complete gene inactivation, they did not faithfully recapitulate the situation in human tumors. Most mutations found in human cancer are point mutations in so-called hotspot regions that interfere with normal function but maintain or even increase expression of p53 (reviewed in Petitjean et al. 2007). Based on *in vitro* studies it has been proposed that these point mutant versions have dominant negative or gain-of-function attributes, including the ability to bind and inhibit related family members p63 and p73 (Strano et al. 2000; Gaiddon et al. 2001). Therefore, several groups generated mice containing these mutations and compared them to *Trp53*$^{-/-}$ strains (Lang et al. 2004; Olive et al. 2004). While overall survival was not affected, there was a shift in tumor spectrum to more epithelial cancers in one study, potentially implicating novel functions for these mutated versions of p53 in certain cell types (Olive et al. 2004).

B. The Ras oncogenes

Proper organismal growth and development relies on substantial cellular proliferation. Normally, this expansion requires extracelluar signals to be received and propagated through intracellular pathways, ultimately leading to activation of the cell cycle machinery (Hanahan and Weinberg 2000). Such a tightly controlled system ensures that cells divide only under the right conditions. Tumors invariably overcome this mode of regulation, leading to the excessive proliferation often associated with cancer cells. One of the most common ways cancers acquire this trait is through mutations in mitogenic signaling pathways, which result in the uncoupling of cell cycle progression from proper environmental cues. Activating mutations in the Ras family of proto-oncogenes are prime examples of this type of alteration and represent one of the most common genomic events in cancer. This section introduces Ras biology, as the tumor models used throughout this thesis are driven by oncogenic K-ras.

a. Discovery

The *Ras* genes were first described as transforming segments within the genomes of the Harvey and Kirsten rat sarcoma viruses (Chien et al. 1979; DeFeo et al. 1981; Ellis et al. 1981). Interestingly, their relationship to human cancer was revealed from an entirely separate line of studies involving the transformation of NIH 3T3 cells with transfected human DNA from chemically treated cells (Shih et al. 1979a). Using the unique patterns of human DNA-derived Alu sequences within transformed clones, it was determined that defined genetic elements from the transferred human DNA were causing the transformation (Perucho et al. 1981; Shih et al. 1981). Soon thereafter these specific genetic regions were cloned from a variety of cancer cells (Goldfarb et al. 1982; Pulciani et al. 1982; Shih and Weinberg 1982) and subsequently shown to be homologous to the previously identified viral *Ras* genes (Chang et al. 1982; Parada et al.

1982). A few years after the identification of cellular *H-ras* and *K-ras2* (hereafter referred to as *K-ras*), *N-ras* was discovered (Shimizu et al. 1983). Subsequent work indicated that in most cases there was only a single missense mutation that distinguished the wild-type and oncogenic versions (Reddy et al. 1982; Tabin et al. 1982; Taparowsky et al. 1982; Capon et al. 1983). Further sequencing efforts have found these mutations in a variety of cancers, with *K-ras* being the most frequent target, especially in tumors from the lung, colon and pancreas (Bos 1989). As such, *Ras* genes represent some of the most frequent mutational targets in cancer.

b. Regulation

Initial characterization of the Ras proteins offered several insights into their normal cellular function and modes of regulation. Early studies indicated they were 21 kd membrane-localized proteins that bound guanine nucleotides and contained GTPase activity (Shih et al. 1979b; Shih et al. 1980; Willingham et al. 1980; Gibbs et al. 1984; Sweet et al. 1984). These features were reminiscent of small G-proteins (Hurley et al. 1984), a class of proteins known to be involved in transduction of signals across the plasma membrane and regulated by their binding of either GTP (active state) or GDP (inactive state). The finding that EGF induced GTP binding indicated that Ras activity was regulated by mitogenic signaling (Kamata and Feramisco 1984). A large number of genetic and biochemical studies identified additional factors linking growth factor receptor signaling to Ras-GTP binding, culminating in a model in which activated growth factor receptors bind adaptor proteins (such as GRB2) which then recruit guanine nucleotide exchange factors (GEFs, such as SOS) to the plasma membrane, bringing them in close proximity to Ras. These GEFs interact with Ras and stimulate its release of GDP, allowing for binding of the more abundant GTP, resulting in Ras activation (reviewed in (McCormick 1993). The subsequent hydrolysis of GTP to GDP, which inactivates Ras, is greatly stimulated

by additional cellular factors called GTPase-activating proteins (GAPs) such as p210GAP and the tumor suppressor *NF1* (Trahey and McCormick 1987; Gibbs et al. 1988; Ballester et al. 1990; Cawthon et al. 1990; Martin et al. 1990). Thus, Ras activity is regulated by a GTP-GDP cycle. Consequently, most oncogenic point mutations in *Ras* genes, comprising those in codons 12, 13, and 61, disrupt this mode of regulation, either by reducing its intrinsic GTPase activity or by inhibiting interactions with GAPs (Gibbs et al. 1984; Sweet et al. 1984; Der et al. 1986; Trahey and McCormick 1987). This results in its constitutive binding to GTP, leading to activation even in the absence of appropriate upstream signals.

c. Ras signaling and effector mechanisms

The connection between EGF stimulation and Ras-GTP binding and suggested a role for Ras proteins in mitogenic signaling. A functional link was soon demonstrated by studies in which inhibition of Ras through microinjected antibodies blocked serum-induced growth of NIH 3T3 cells (Mulcahy et al. 1985). Work in a number of other systems went on to document roles for Ras in other processes such as differentiation and response to cytokines (Samid et al. 1985a; Samid et al. 1985b; Hagag et al. 1986). Together, these studies indicated that Ras proteins were critical for transducing signals from the extracellular environment to intracellular networks in order to generate appropriate responses (Figure 3). Consequently, an important question became how Ras executed these diverse downstream functions. Over the years a number of groups have identified binding partners of Ras that specifically interact with active Ras-GTP,

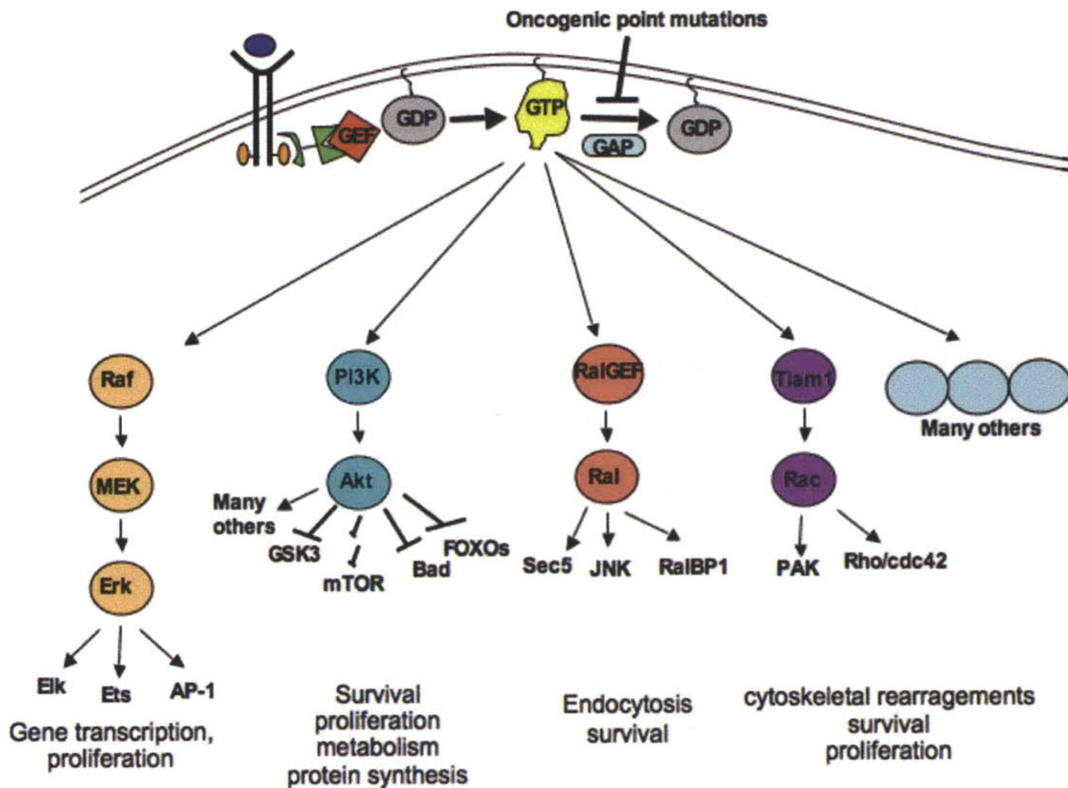


Figure 3: Overview of Ras signaling.
Ras activity is normally controlled by a GTP-GDP cycle. In wild-type cells, receptor binding to an extracellular growth factor (purple oval) leads to phosphorylation of cytoplasmic residues on the receptor (orange ovals), which serve as docking sites for adaptor proteins like Grb2 (green structure). Through separate domains these adaptor proteins also bind guanine nucleotide exchange factors (GEF-red square) such as SOS, which brings them in close proximity to membrane localized Ras (grey circle). GEFs stimulate the release of GDP from Ras, allowing the more abundant GTP to bind and activate Ras (yellow). This creates a confirmation change in Ras, giving it the ability to interact with a number of proteins that serve as upstream signaling nodes in an array of intracellular pathways, a few of which are shown. The activation of these pathways mediates the large number of output functions ascribed to Ras, such as proliferation, survival, and cytoskeletal rearrangements. Normally, Ras activity is terminated by GTPase activating proteins (GAPs-light blue oval) that bind Ras-GTP and enhance its intrinsic GTPase activity. Oncogenic point mutations disrupt this interaction, thus keeping Ras in its active, GTP bound state and able to constitutively signal to downstream pathways.

implicating them as potential Ras effectors. Below is an overview of some of the most well-established examples.

MAPK

The first Ras effectors to be identified were Raf family members, which were themselves previously identified oncogenes (Vojtek et al. 1993; Warne et al. 1993; Zhang et al. 1993). This interaction localizes Raf to the plasma membrane and initiates the mitogen activated protein kinase (MAPK) signaling cascade in which Raf phosphorylates MEK family members (MEK1/2), which then phosphorylate and activate ERK1/2 kinases (Hagemann and Blank 2001). While targets of the ERKs vary widely among cell types, canonical examples include the Ets family of transcription factors and members of the AP-1 complex (reviewed in Yordy and Muise-Helmericks 2000). These factors subsequently induce a number of genes involved in the cell cycle, such as cyclin D1, providing a direct link between Ras signaling and cell cycle progression (Hitomi and Stacey 1999). Additional MAPK subfamilies, including the c-Jun N-terminal kinase (JNK) and p38 pathways (reviewed in Wagner and Nebreda 2009), also signal downstream of Ras. Generally associated with stress responses, these kinase cascades have unique and overlapping targets with the Raf-MAPK pathway, indicating that Ras can direct distinct outputs depending on which MAPK pathways it activates.

PI3K

Another important Ras effector arm is the class I phosphoinositide 3-kinase (PI3K) pathway, first shown by the interaction between Ras-GTP and the p110 catalytic subunit of PI3K (Rodriguez-Viciana et al. 1994). Upon binding to Ras this lipid kinase localizes to the plasma membrane, allowing it to phosphorylate position 3 on the inositol ring, which results in increased phosphatidylinostiol 3,4,5 triphosphate (PIP_3) on the intracellular surface of the cell membrane

(Vivanco and Sawyers 2002). PIP_3 serves as a docking site for the serine/threonine kinase Akt, whose membrane localization leads to its activation and subsequent ability to phosphorylate numerous proteins (Franke et al. 1997). Through its phosphorylation and inactivation of the pro-apoptotic protein Bad, Akt can promote cell survival (Datta et al. 1997), while substrates such as glycogen synthase kinase 3 (GSK3) and the Forkhead family of transcription factors (FOXOs) link this kinase to cell cycle progression (Medema et al. 2000; Hill and Hemmings 2002). In addition, Akt can activate the mTOR pathway, which has pleiotropic effects on proliferation, cell size, metabolism, and protein translation (reviewed in Shaw and Cantley 2006). Clearly, by activating the PI3K/Akt pathway, Ras is able to generate diverse signaling outputs.

Other pathways

A number of other Ras-GTP interacting partners have been described over the years. For example, RalGDS and RalGRL, two GEFs for RalA and RalB small GTPases, bind activated Ras, implicating this class of proteins as Ras effectors (Kikuchi et al. 1994; Spaargaren and Bischoff 1994). Most well known for their role in vesicle trafficking and receptor recycling, Ral proteins (specifically RalB) have recently been shown to have important roles in cell survival through their activation of the IKK-related kinase TBK1 (Bodemann and White 2008).

An additional group of Ras effectors comprise the Rho family of small GTPases. Initially thought to be only indirectly affected by Ras signaling, an interaction between Ras-GTP and Tiam1, a GEF for Rac GTPases, suggested a more direct relationship (Lambert et al. 2002). Rho-GTPases play important roles in cytoskeletal reorganization and can mediate the formation of stress fibers, lamellipodia, and membrane ruffling, depending on the context (Ridley and Hall 1992; Ridley et al. 1992). Moreover, by activating other pathways such as NFκB, this family can also impact cell proliferation and survival (Joyce et al. 1999). As research continues, an

increasing number of binding partners for Ras are found, and their varied functions implicate Ras signaling in more and more biological processes.

Identifying effectors important in tumorigenesis

Given that Ras has a direct role in cellular transformation, a long-standing question has been which of its downstream signaling pathways are critical for its tumorigenic functions. Early studies overexpressing dominant negative or constitutively active cDNAs showed that members of the Raf-MAPK pathway were necessary and sufficient for Ras-induced transformation of NIH 3T3 cells (Schaap et al. 1993; Cowley et al. 1994). Dominant negative versions of Rho family members proteins similarly blocked Ras-dependent transformation (Qiu et al. 1995a; Qiu et al. 1995b). A set of very informative studies relied on a collection of Ras mutants that differed in their ability to bind various effectors owing to effector-binding domain point mutations (White et al. 1995). For example, an oncogenic H-ras mutant unable to bind Raf proteins (G12V, E37G) lacked transformation capabilities, further demonstrating the importance of this pathway. Subsequently, the Downward group generated additional mutants that bound only Raf, only PI3K, or only RalGDS and showed that all failed to individually transform NIH 3T3 cells but had partial activity when used in various combinations. These results, along with others indicating that constitutively active effector proteins (ie- Raf-CAAX or p110-CAAX, which are targeted to the membrane) could only transform cells when expressed together, suggested that multiple effector pathways were necessary to completely recapitulate Ras function (Rodriguez-Viciana et al. 1997). More recently, multiple groups have interrogated Ras effectors using shRNA-mediated knockdown of pathway components. A benefit of this approach is that one can implicate specific family members, as has been done with b-Raf/c-Raf and RalA/RalB in different tumor cell lines (Dumaz et al. 2006; Lim et al. 2006). Additionally, RNAi systems can

be readily applied to large-scale screens. Several of these types of screens have been carried out and have revealed important pathway dependencies of sensitivities of Ras-mutant cells (Barbie et al. 2009; Luo et al. 2009; Scholl et al. 2009).

Both cell-type and species dependent effects have been observed in Ras effector studies. While MEFs require only Raf to phenocopy Ras-transforming functions, human fibroblasts depend on both Raf and RalGEF activities. Among human cells, further differences have been noted between fibroblasts and epithelial cell types such as kidney cells, which require PI3K and RalGEF, and mammory cells, which rely on Raf, PI3K, and RalGEF pathways (Rangarajan et al. 2004). Although the reasons for these discrepancies are unknown, they highlight the complexity of Ras signaling and underscore the importance of considering cell type when studying Ras effector pathways.

d. Ras family members

The Ras family consists of the closely related *H-ras, N-ras,* and *K-ras (4A* or *4B*-determined by alternative splicing to its last exon (Capon et al. 1983; McGrath et al. 1983)) genes. While often referred to collectively, important differences exist between these family members. Interestingly, *H-ras*$^{-/-}$ and *N-ras*$^{-/-}$ mice are viable while *K-ras*$^{-/-}$ embryos die *in utero* (Umanoff et al. 1995; Johnson et al. 1997). In addition, different tumor types specifically harbor mutations in a particular family members (Karnoub and Weinberg 2008). Unique expression patterns have been proposed as one possible explanation for these differences, but other factors may play a role as well. While a majority of their primary amino acid sequence is very similar, there are substantial differences in the C-terminal portion of these proteins, which allows for different modifications and thus might lead to distinct subcellular localizations (Apolloni et al.

2000). Interestingly, when studied side-by-side in the same overexpression systems, the different family members have shown unique effects on transformation (Voice et al. 1999; Quinlan et al. 2008). Therefore, it is most likely that distinct biological properties of the Ras proteins also influence the tumor spectrums associated with their mutations.

e. Mouse models

Even before *Ras* genes were targeted in animal studies, oncogenic point mutations in *H-ras* were identified in benign papillomas from mice treated with chemical carcinogens (Balmain et al. 1984). A more causal role in tumor initiation was provided by transgenic mice expressing oncogenic H-ras driven by the MMTV promoter, which formed mammory hyperplasias (Sinn et al. 1987). Subsequently, several transgenics have been made in a variety of tissues, highlighting the potency of Ras oncogenes in initiating tumors.

More recently, sophisticated mouse models have illustrated important concepts in Ras tumor biology. For example, using reversible systems in which oncogenic Ras could be shut off after tumors developed, several groups showed that tumor maintenance required the continued expression of oncogenic Ras (Chin et al. 1999; Fisher et al. 2001). In addition, mice harboring a "latent" allele of K-ras^{G12D} (*K-ras*^{LA}), which presumably can spontaneously activate endogenous K-ras^{G12D} in any cell type following an intrachromosomal recombination event that removes an engineered exon duplication, showed a very limited tumor spectrum, with 100 percent of mice presenting with lung tumors (Johnson et al. 2001). While this could be due to cell type variability in recombination efficiency, these results might also suggest that only certain cell are responsive to endogenous oncogenic K-ras signaling (See Chapter 2). Finally, Cre-inducible alleles of endogenous K-ras^{G12D}, such as *K-ras*$^{LSL\text{-}G12D}$, have permitted the targeting of endogenous

oncogenic K-ras to specific cell types, as well as highlighted differences between endogenous and over-expressed oncogenic Ras (Tuveson et al. 2004) (see Section C of the Introduction).

Many of these models have been used to investigate the importance of different effector pathways in Ras-mediated tumor development. In one study, deletion of Rac1 strongly inhibited K-ras^{G12D}-induced lung tumor formation, while another demonstrated a critical role for RalGDS in H-Ras-driven skin tumor initiation (Gonzalez-Garcia et al. 2005; Kissil et al. 2007). Furthermore, disrupting the ability of p110α to interact with Ras-GTP dramatically blocked lung tumor formation in *K-ras^{LA}* animals (Gupta et al. 2007). The use of specific pathway inhibitors in tumor-bearing animals has allowed for studies on established tumors as well. For example, combined administration of drugs inhibiting MAPK and PI3K pathways led to regression in K-ras^{G12D}-initated lung tumors (Engelman et al. 2008). The ability to perform these types of pathway analyses in mouse models that closely recapitulate human cancers will hopefully inform therapeutic strategies in the future.

C. Oncogene-induced tumor suppression

Tumorigenesis is driven by multiple mutations, including those that activate growth-promoting oncogenes, such as Ras (Section B) and others that disable anti-proliferative tumor suppressors such as p19^{Arf} or p53 (Section A). This mutational spectrum endows cancer cells with the ability to expand uncontrollably and progress towards malignancy. The co-occurrence of these different classes of mutations is not merely a coincidence but instead underscores an intimate connection between pathways regulating cell proliferation and those with growth inhibitory properties (Lowe et al. 2004). Surveillance systems that are intertwined with

proliferative networks sense and respond to inappropriate signals by impeding growth or executing cell death. Paradoxically, this means that oncogenic mutations directly engage the tumor suppressor pathways that constrain cancer progression. Therefore, the full potential of oncogenic lesions is unleashed only after inactivation of these anti-growth programs (Figure 4). Such tight coupling of oncogenes and tumor suppressors represents a critical checkpoint guarding against tumor formation. This section highlights the main types of oncogene-induced tumor suppression and ends with a discussion of potential causes of variability in these responses.

a. Early insights - oncogene cooperativity for transformation of primary cells

The cellular *Ras* genes were functionally cloned by their ability to transform immortalized NIH 3T3 cells (Perucho et al. 1981; Shih et al. 1981). This initially suggested that oncogenic Ras was sufficient for transformation. However, when similar experiments were conducted in primary rodent fibroblasts, Ras lacked transforming capabilities. Only introduction of additional oncogenes, such as E1A or c-myc, elicited overt transformation (Land et al. 1983; Newbold and Overell 1983; Ruley 1983). This provided the first evidence that multiple oncogenic events were necessary for tumor formation. Subsequent work expanded the list of secondary events that cooperated with Ras in transformation, including deletion of *Trp53* or *Ink4a/Arf* (Tanaka et al. 1994; Serrano et al. 1996). As many of these cooperating events were known to immortalize cells, preventing the eventual growth arrest, or replicative senescence, observed after prolonged passage in tissue culture, it was suggested that oncogenic Ras could transform cells only after they underwent immortalization, further refining the model of multistep tumorigenesis (Ruley 1990). These *in vitro* studies nicely aligned with mutational data

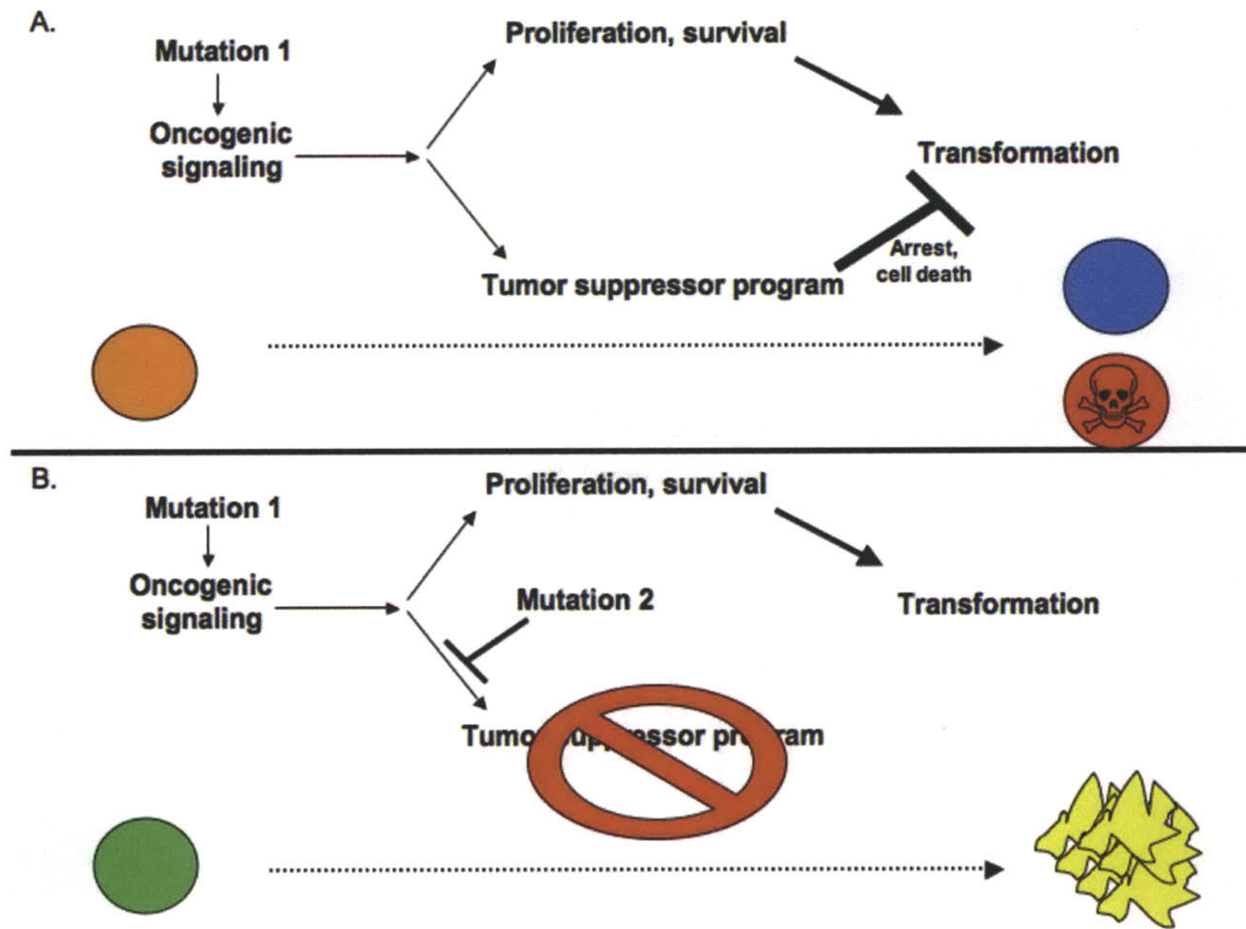


Figure 4: Oncogene-induced tumor suppression.
A. The activation of oncogenes not only induces pathways that promote proliferation and survival but also directly engages tumor suppressor programs that inhibit these processes. Because of this intrinsic coupling of oncogenes and tumor suppressors, cells that acquire oncogenic mutations (orange circle) usually undergo either irreversible cell cycle arrest (blue circle) or cell death (red circle), depending on the cell type and/or oncogenic stimulus. This effectively inhibits transformation. **B.** If the same oncogenic insult occurs in a cell that has disabled these tumor suppressor programs (green circle), perhaps through a secondary mutation, the oncogenic pathways controlling proliferation and enhanced survival can promote transformation (yellow shapes). This phenomenon helps to rationalize the multi-step nature of tumorigenesis, as additional mutations are required to unleash the full potential of oncogenes.

from human cancers which indicated that established tumors harbored multiple mutations (Kinzler and Vogelstein 1996).

b. Oncogene-induced senescence

While the general principle of oncogene cooperativity had been appreciated for many years, a mechanistic explanation was still lacking. To gain insight into why Ras required additional events to transform primary cells, Serrano and colleagues investigated the response of primary mouse and human fibroblasts to sustained expression of oncogenic H-ras. Interestingly, H-ras^{G12V}-expressing cells rapidly up-regulated the tumor suppressors p16^{Ink4a} and p53, underwent a robust and irreversible G1 cell cycle arrest, and presented with additional characteristics of the well-established phenomenom of replicative senescence (Serrano et al. 1997). Cooperating events already known to combine with Ras in transformation, such as dominant negative p53 and E1A expression, completely inhibited the appearance of these senescence markers, suggesting that their true function in oncogene cooperativity might be to subvert this senescent phenotype. This newly identified program of premature senescence, named oncogene-induced senescence (OIS), was hypothesized to be a failsafe mechanism preventing the outgrowth of incipient tumor cells harboring oncogenic mutations.

Numerous studies have since implicated the p19^{Arf}-p53 and p16^{Ink4a}-Rb tumor suppressor pathways as the main mediators of OIS, with the relative importance of each being dependent on the species and cell type (reviewed in Dimri 2005). One of the main mechanisms linking oncogenes to senescence induction is the excessive mitogenic signaling associated with activated oncogenes, which then transcriptionally engages the *Ink4a/Arf* locus (see Section A i c), resulting in p53 and Rb pathway activation (Collado and Serrano 2010). In the case of oncogenic Ras,

hyper-active Raf-MEK-ERK signaling has been shown to be necessary and sufficient for $p19^{Arf}$ and $p16^{Ink4a}$ up-regulation (Lin et al. 1998; Zhu et al. 1998; Sreeramaneni et al. 2005). Interestingly, activation of p38MAPK, either by Raf or reactive oxygen species (ROS), might be critical for this effect in certain contexts (McCubrey et al. 2006). High levels of activating E2Fs associated with many oncogenic insults could also play a role, as overexpression of E2Fs can activate Ink4a/Arf and induce senescence on their own (Dimri et al. 2000; Lazzerini Denchi et al. 2005). In contrast to models invoking increased flux through pathways, others have highlighted the importance of negative feedback mechanisms in controlling induction of p53 and Rb during OIS. In one study, simply inhibiting the PI3K pathway generated a senescent phenotype, although the role of *Ink4a/Arf* was not addressed (Courtois-Cox et al. 2006). As discussed earlier, oncogenes can also activate p53 through a DDR, and several groups have demonstrated the requirement for these pathways in OIS (Bartkova et al. 2006; Di Micco et al. 2006).

Moving downstream of p53/Rb activation, little is known about the mechanism of OIS, although p21 might be involved in some contexts (Brown et al. 1997). Gene expression studies recently identified a number of markers expressed specifically in cells undergoing OIS, including Dec1 and DcR2, although their functional role is unclear (Collado et al. 2005). Interestingly, unique chromatin patterns called senescence associated heterochromatic foci (SAHF) have been observed specifically in senescent cells compared to those undergoing more traditional cell cycle arrest. These condensed chromatin structures, which are dependent on $p16^{Ink4a}$-Rb activity, encompass genes important in cell-cycle progression and could be critical in directing the irreversible cell cycle arrest associated with senescence (Narita et al. 2003).

Despite the wealth of *in vitro* data, evidence for OIS as a tumor suppressive mechanism *in vivo* remained elusive for some time. However, a recent spate of papers, using the newly

identified markers, reported widespread OIS in a number of mouse tumor models including those of the lung, prostate, hematopoietic, mammory, thyroid, and skin (Braig et al. 2005; Chen et al. 2005; Collado et al. 2005; Sarkisian et al. 2007; Shamma et al. 2009). There is also mounting evidence for senescence in human tumors (Collado and Serrano 2010). Importantly, many of these studies correlated more advanced tumor stages with a loss of OIS markers. Moreover, in several mouse models, directly disabling the senescence program resulted in enhanced tumor progression, providing causal relationship between senescence and tumor suppression. Most notably, deletion of suv39h1, a histone methyltransferase that physically and functionally interacts with Rb, was shown to have such an effect, providing *in vivo* evidence that chromatin modifications mediate the senescent program (Braig et al. 2005; Shamma et al. 2009). In many of these studies, senescent cells were found in small, pre-malignant lesions, suggesting that oncogene expression allowed for some cellular expansion but eventually engaged the senescence program, which limited subsequent tumor growth. Given that senescence is potentially irreversible yet many tumors ultimately progress to more advanced stages, an intriguing question concerns the origins of these malignant clones. One possibility is that they derive from cells that undergo senescence but somehow eventually bypass this checkpoint, possibly through secondary mutations. Alternatively, they could represent cells that never fully engaged the program and therefore might still be susceptible to senescence. As intact senescence programs might represent an important chemotherapeutic target in established tumors (Schmitt et al. 2002b), this question needs to be addressed.

c. Oncogene-induced apoptosis

Studying additional oncogenes in primary cells demonstrated a paradoxical role for some in mediating apoptosis in certain contexts. For example, under a variety of conditions the viral oncogene E1A elicited a robust apoptotic response in rat embryonic fibroblasts (Lowe and Ruley 1993). Likewise, ectopic expression of c-myc in primary cells deprived of nutrients or arrested by other means resulted in widespread cell death (Evan et al. 1992). Given the established role for p53 in mediating apoptosis, a connection was quickly made between the aberrant expression of these oncogenes and p53 up-regulation, and p53 deletion was shown to severely hinder this oncogene-induced apoptosis (OIA) (Hermeking and Eick 1994; Wagner et al. 1994). Further genetic dissection of the p53 pathway in cells undergoing OIA implicated $p19^{Arf}$ as a critical upstream regulator of p53 in many settings (de Stanchina et al. 1998; Eischen et al. 1999). As with OIS, inappropriate mitogenic signaling downstream of oncogene expression is thought to underlie $p19^{Arf}$ transcriptional activation. For both E1A and c-myc, their ability to directly or indirectly activate E2F transcription factors has been proposed as a mechanism to explain $p19^{Arf}$ induction (Lowe et al. 2004), although redundancy in the E2F family has made it difficult to definitively prove this model. Whatever the mechanism of p53 induction, its subsequent stabilization results in the transcriptional activation of a number of pro-apoptotic genes (see Section A ii b). Additionally, c-myc and E1A can directly impinge on apoptotic cascades, further connecting these oncogenes with apoptosis (Klefstrom et al. 2002; Perez and White 2003). The relative importance of p53-dependent and independent routes to apoptosis most likely varies between different cell types.

The dual functions of oncogenes such as c-myc in cell cycle progression as well as apoptosis induction led to the speculation that the oncogenicity of such genes would be uncovered only when cell death was inhibited. Indeed, in numerous *in vivo* models driven by

exogenous c-myc expression, including those directing pre-B cell or pancreatic β-cell-specific expression, massive apoptosis has been documented, correlating with a relatively weak tumor phenotype (Eischen et al. 1999; Pelengaris et al. 2002). However, disabling the cell death machinery suppressed this high apoptotic index and led to extensive c-myc driven tumorigenesis. This has been accomplished both through inactivation of pro-apoptotic genes such as BH3-only killer protein Bim, as well as with overexpression of cell death inhibitors such as Bc1-2 or Bcl-xl (Pelengaris et al. 2002; Schmitt et al. 2002a; Egle et al. 2004). These results strongly implicate OIA as a critical tumor suppressor mechanism downstream of c-myc overexpression.

d. Determinants of OIS and OIA

The tight coupling of oncogenic signaling to tumor suppressor pathways makes it difficult to envision oncogenic mutations as drivers of tumor initiation, instead placing their mutation later in disease progression. This contrasts with studies implicating some oncogenic lesions as initiating, or at least early events (Moskaluk et al. 1997; Shet et al. 2002; Kemp 2005). Given the model that hyperactive signaling underlies engagement of growth-inhibitory checkpoints, it is conceivable that intermediate levels of signaling, closer to those seen within the normal functioning range of mitogenic signaling pathways, could drive proliferation while minimally inducing tumor suppressors. Support for such a model has been documented in a number of experimental settings, as described below.

Most early studies of oncogenic Ras function relied on overexpression. To investigate the most frequently mutated Ras family member under more physiological settings, a Cre-inducible allele of endogenous K-ras^{G12D} was generated (*K-ras*$^{LSL-G12D}$) (Tuveson et al. 2004). Initial characterization of MEFs expressing endogenous K-ras^{G12D} (*K-ras*$^{Lox-G12D}$ MEFs) revealed

dramatic differences compared to cells with ectopic oncogenic Ras expression. In contrast to the traditional OIS response, *K-ras*$^{Lox\text{-}G12D}$ MEFs appeared immortalized and even partially transformed. This phenotypic difference was attributed to relatively low p19^{Arf}-p53 pathway activation, possibly due to attenuated signaling through Ras effector pathways seen with endogenous K-ras^{G12D}. Furthermore, directing physiological K-ras^{G12D} expression to multiple tissues resulted in hyperproliferation without any signs of senescence. Together, these data suggested that oncogene-induced tumor suppression relied on oncogene overexpression. In contrast to these results, work with another knock-in model of oncogenic K-ras (*K-ras*$^{LSL\text{-}G12V\text{-}IRES_{\beta}Geo}$) reported senescence in pre-malignant lesions of the lung and pancreas (Collado et al. 2005). Importantly, inserting the βGeo into the 3' UTR of *K-ras* could increase K-ras expression levels in this model, providing a possible explanation for phenotypic differences between these alleles.

To more directly test the relationship between oncogenic Ras expression levels and functional outputs *in vivo*, Chodosh and colleagues generated a doxycycline-regulated allele of H-ras^{G12V} (Sarkisian et al. 2007). With this system, they could carefully manipulate H-ras levels and assess downstream consequences. While moderate induction of H-ras^{G12V} elicited modest proliferation and no Ink4a/Arf or p53 expression, high levels of the oncogene led to a burst of cell cycle entry followed by robust tumor suppressor induction and cellular senescence. Deleting *Ink4a/Arf* or *Trp53* blunted the senescence response and permitted tumor formation.

Similar observations have been reported for c-myc. MycER^{T2}, a fusion protein of c-myc and a fragment of the estrogen receptor (ER) that affords tamoxifen-regulated control of c-myc activity, was targeted to the *R26* locus (Murphy et al. 2008). Despite only modest expression, activation of mycER^{T2} was able to induce proliferation in a number of tissues. Interestingly, this

was not accompanied by significant $p19^{Arf}$ induction or cell death, which required c-myc over-expression via a different transgene. The ability of $mycER^{T2}$ to cooperate with endogenous K-ras^{G12D} in lung tumor progression suggested that this low level of c-myc activity, which escaped $p19^{Arf}$-mediated surveillance, could still contribute to tumorigenesis.

Together, these studies indicate that the dual nature of oncogenic signaling can be uncoupled by adjusting expression levels. Moderate oncogene expression, perhaps corresponding to more physiological levels associated with normal cellular functions, allows for proliferative responses without appreciable tumor suppressor induction. In contrast, inducing higher levels of oncogenes engages anti-proliferative networks that constrain growth and tumorigenesis. An attractive model to explain these observations is that early in tumor development, initiating oncogenic lesions lie below the detection of tumor suppressor programs, allowing for cell proliferation. As these lesions advance, increased signaling, perhaps through gene amplification or loss of negative regulators, could potentially induce checkpoints. This would limit tumor progression but also provide selective pressure for clones in which these anti-tumor pathways have been inactivated. Once these secondary mutations occur, high oncogene levels can drive cancer cells towards full malignancy.

Aside from oncogene expression levels, whether there are additional factors that might control the decision to proliferate or induce tumor suppressors downstream of oncogenic insults are unknown. As mentioned in Section A I c, cell types differ in their extent of PcG repression of *Ink4a*/Arf, and this could set different thresholds for gene activation. A few studies have suggested that such differences between hematopoietic progenitors and more differentiated T and B cells might affect their tumorigenic potential. Specifically, while expression of the Bcr-Abl or Notch-IC oncogenes in bone-marrow derived (more progenitor-like) cells did not require *Arf*

deletion, equivalent tumor formation when using or pre-B or pre-T-cell (more differentiated) was only seen in *Arf*$^{-/-}$ backgrounds. While differential PcG-repression of p19^{Arf} was proposed as an explanation, this was not tested (Williams et al. 2006; Volanakis et al. 2009). In Chapter 2, I describe a functionally relevant, tissue-specific p19^{Arf} expression pattern in the context of similar, endogenous K-ras^{G12D} levels. Furthermore, I implicate distinct chromatin remodeling activities in the control of this response, providing additional support that inherent differences in p19^{Arf} transcriptional regulation among cell types can affect oncogene-induced tumor suppression.

D. Genetic tools for modeling cancer in the mouse

Cancer is a highly complex disease that arises within intact organisms and therefore has multiple interactions with both local and systemic factors that significantly affect tumor initiation, progression, and treatment responses. While tissue culture studies have undoubtedly contributed to our understanding of tumorigenesis, they do not recapitulate an endogenous *in vivo* environment and as such limit our ability to interrogate certain aspects of the disease. The advent of genetic engineering in the mouse has generated experimental platforms allowing for the modeling and careful study of cancer within a more physiological context. From validating causal and cooperative roles for various oncogenes and tumor suppressors in tumor initiation and maintenance in a variety of tissues, to providing controlled systems for mechanistic and therapeutic studies, as well as highlighting the importance of tumor cell-microenvironment interactions, these models have been and continue to be highly informative. In this section I give an overview of the technology that has made this possible and end with a discussion of sequential mutagenesis (Figure 5).

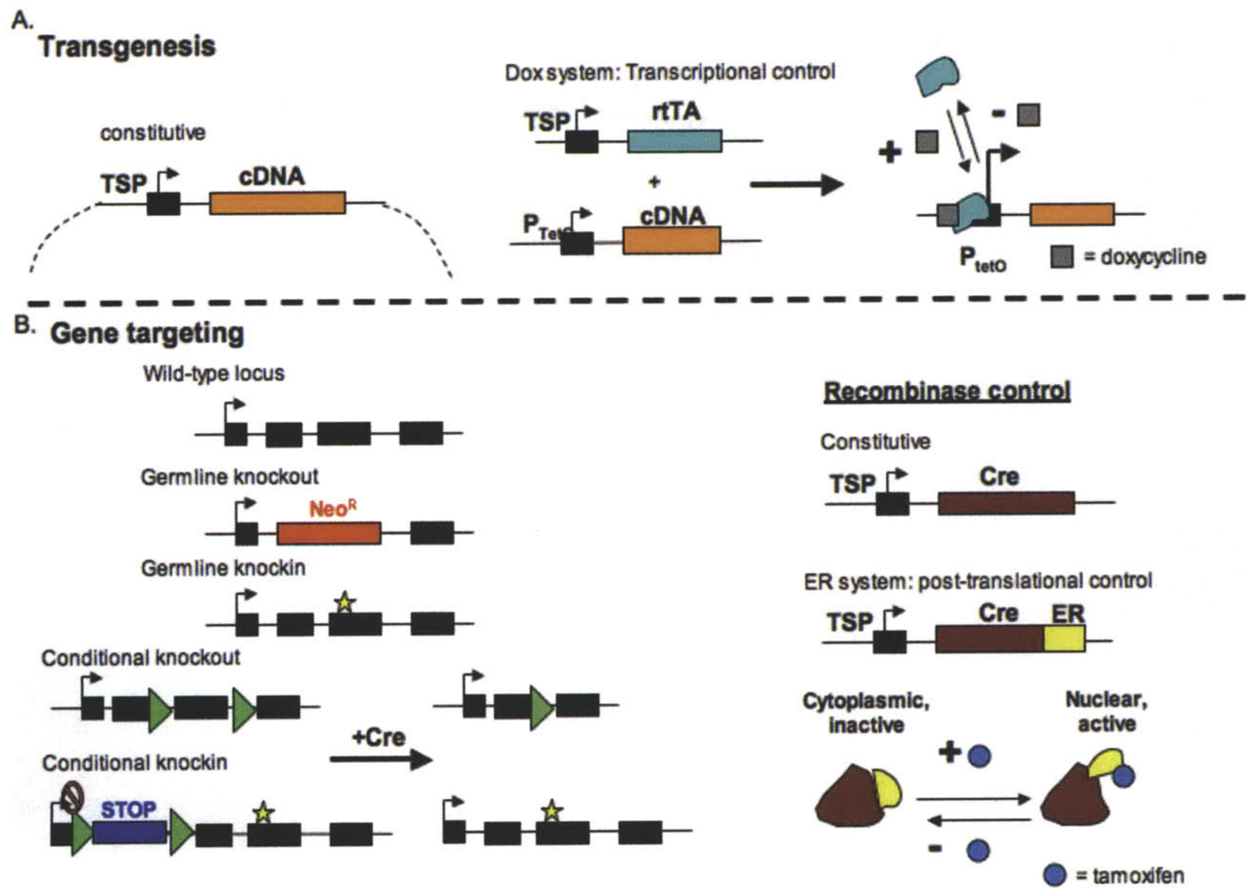


Figure 5: Overview of common reagents used in the mouse models of cancer
A. Transgenics and **B.** Gene targeting strategies. Both constitutive and inducible systems are shown for each. See text for details. TSP = tissue specific promoter. Neo^R = neomycin resistance cassette, which is an example of a heterologous DNA segment one can use to replace endogenous sequences when constructing a knockout. Star = point mutation. Green triangles, = LoxP sites. STOP = transcriptional STOP cassette.

a. Transgenics

Pioneering work in the late 1970s and early 80s demonstrated that foreign plasmid DNA could be introduced into mouse oocytes which could then be used to generate intact animals that carried the DNA in their germline (reviewed in (Jaenisch 1988). In an early tumor-related study, mice harboring SV40 T-antigen DNA were shown to efficiently generate brain tumors, validating the technique (Brinster et al. 1984). In subsequent work, specific regulatory sequences were placed upstream of the gene of interest to more carefully control gene expression. Using the mouse mammory tumor virus (MMTV) LTR as a promoter, c-myc and v-H-ras transgenes were generated and shown to cooperate with each other in mammory tumorigenesis, validating the oncogene cooperativity observed *in vitro* (Sinn et al. 1987). Since these initial studies numerous transgenic mice have been constructed, most of which use cell-type specific promoters to drive expression of various oncogenes in distinct cellular compartments (Frese and Tuveson 2007). Such a strategy can also be applied to the functional inactivation of multiple family members through the use of dominant negative constructs (Pikarsky et al. 2004).

While very useful, transgenics have multiple drawbacks, most notable being the difficulty in directing proper expression patterns and levels. Tissue-specific expression relies on the careful selection and construction of regulatory elements, usually taken from proximal promoters. However, important regulatory sequences are often located far away from the transcription start site and therefore might be difficult to identify and include in the transgene. In addition, the random insertion of the introduced DNA could place it in local chromatin environments that inhibit or inappropriately activate expression, so careful screening of founders must be done. Finally, because the genes are not under control of their endogenous loci, selected transgenic

founders can drive supraphysiological expression levels, which depending on the study, could lead to experimental artifacts.

b. Gene targeting

A major breakthrough in mouse modeling came with technological advancements allowing for targeting of specific loci in the mouse genome. Techniques were developed in embryonic stem (ES cells) in which homologous recombination events of introduced DNA could be preferentially selected over random insertion (Mansour et al. 1988). Correctly targeted clones could ultimately be propagated to the germline of mice. Using this technique, endogenous genes could now be manipulated, allowing for reverse genetic analysis in mice. Typically, gene function is removed in so-called "knock-outs" by replacing segments of their coding region with foreign DNA. Many tumor suppressor genes, such as p53, Rb and PTEN, have been validated as such in knockout models in which animals deficient in these genes are tumor prone (Jacks et al. 1992; Jacks et al. 1994; Di Cristofano et al. 1998). Recently, more sophisticated alleles of different genes have been constructed. For example, *in vivo* structure-function analyses of proteins can be performed if specific residues or regions of proteins, such as phosphoacceptor sites or binding domains, are mutated and associated with particular phenotypic consequences (MacPherson et al. 2004; Gupta et al. 2007). In addition, targeting genes to loci with desired expression patterns, such as the ubiquitously expressed *R26* locus, can give more control compared to traditional transgenic techniques. A similar strategy can also be used to place reporter genes within specific regulatory segments, creating accurate reporter strains (Zindy et al. 2003).

c. Spatiotemporal control

Gene targeting has been instrumental in demonstrating many important functions of cancer-relevant genes. Despite these advances, the inability to control the time and place of the manipulations proved to be a hindrance in some cases. For example, both *Rb*$^{-/-}$ and *PTEN*$^{-/-}$ animals died during embryogenesis, precluding the study of tumor formation. While heterozygous animals eventually succumbed to cancer usually due to loss of heterozygosity (LOH), the long latency and lack of control over where LOH took place limited the scope of these studies. To circumvent these issues, site-specific recombinase (SSR) systems, most notably Cre-LoxP from bacteriophage P1, have been introduced into mouse models (Orban et al. 1992). In this system, the Cre enzyme catalyzes the recombination between two specific DNA sequences, called LoxP sites (Kilby et al. 1993). If two LoxP sites are in the same orientation and flank a segment of DNA, Cre expression will remove the intervening DNA. In this way, removal of particular regions of DNA can be regulated by controlling Cre expression. For example, by flanking a critical segment of Rb with two LoxP sites , one can delete this gene only in cells expressing Cre, leaving other cells unaffected. Such a strategy has been used to inactivate a variety of tumor suppressor genes in distinct tissues by combining floxed alleles with cell-type specific Cre transgenes, knock-ins, or virally-administered Cre (reviewed in (Frese and Tuveson 2007). In addition to these loss-of-function approaches, Cre-LoxP can also be utilized to activate genes. In this strategy, a Lox-STOP-Lox (LSL) cassette, which is a LoxP-flanked stretch of polyA sequences that prevent transcriptional read-through, is targeted to a gene to block its transcription. Introduction of Cre removes the STOP cassette, allowing the targeted gene to be expressed. LSL has been used to activate a number of oncogenes, such as K-ras (*K-ras*$^{LSL\text{-}G12D}$) (Tuveson et al. 2004). Additional SSR systems, such as Flp-Frt, have also been used to control gene expression in the mouse (Vooijs et al. 1998; Awatramani et al. 2001). In Chapter 3 I show

how combining multiple SSRs into one mouse model permits distinct regulation of two different genetic events.

While both transgenic and SSR/gene targeting systems afford cell-type specific gene manipulation, the strategies discussed above do not allow for temporal control. This additional layer of regulation could be important if one is interested in the kinetics or developmental stage-specificity of responses to gene activation/inactivation events. Such control has been introduced into SSR technology through the fusion of a modified ligand-binding domain of the estrogen receptor (ER) to Cre, creating CreER proteins (Indra et al. 1999). Under normal conditions, CreER is retained in the cytoplasm owing to heat shock protein-mediated ER regulation. Upon addition of the estrogen analog tamoxifen, this regulation is relieved and CreER translocates into the nucleus to mediate recombination. Showing the utility of CreER systems, a recent study activated oncogenic K-ras in the pancreas of mice of different ages and documented a decrease in tumorigenic potential with age, possibly reflecting a decline in stem cell number or function (Gidekel Friedlander et al. 2009). Interestingly, other proteins relying on nuclear translocation for proper function, such as p53 and c-myc, have been tightly controlled using ER technology as well (see A ii d and C d).

Transgene expression can also be temporally controlled using the tetracycline (tet) system. This transcription-based system has two parts: the gene of interest (such as an oncogene) placed under the control of a promoter containing regulatory sequences of the tet operator (tetO), and a tet-responsive transactivator (tTA), encoding a fusion protein of the VP16 transcriptional co-activator with the tet-repressor protein that binds tetO sequences in a doxycycline (dox - an analog of tet) - dependent manner (Gossen and Bujard 1992; Kistner et al. 1996). Depending on which of two tTAs are used (tTA or rtTA), addition of dox either represses or activates

transcription of the tetO-controlled gene, allowing one to carefully manipulate gene expression in a reversible manner (reviewed in (Branda and Dymecki 2004). Putting Cre under the control of tetO is another way to temporally regulate this recombinase. Highlighting the benefits of the tet system, multiple groups have de-activated expression of initiating oncogenic lesions and shown that tumor maintenance requires their continued expression (Chin et al. 1999; Fisher et al. 2001). These types of experiments provide insight into potential therapeutic targets.

d. RNAi platforms

The discovery of RNA interference (RNAi) has revolutionized mammalian genetics. This endogenous gene regulatory system can be harnessed to silence any gene by introducing small, double-stranded RNA that is complementary to a target gene of interest. If properly designed, this RNA species is incorporated into the normal cellular machinery and ultimately directs target gene repression (Sandy et al. 2005). This technology has rapidly accelerated the pace of loss-of-function studies in mammalian systems, obviating the need to use gene targeting in many cases. While primarily used in cell culture studies, RNAi technology has recently transitioned into *in vivo* settings as well. Initial studies introduced short hairpin RNA (shRNA)-containing lentiviruses into mouse ES cells and subsequently generated mice that displayed partial gene knockdown (Rubinson et al. 2003). An improved version of this vector produced an observable diabetes-associated phenotype *in vivo* (Kissler et al. 2006).

In regards to *in vivo* cancer biology, RNAi has been most widely used in systems involving *ex vivo* manipulation of cells followed by their re-introduction into endogenous environments. The Eμ-myc lymphoma model has been extremely successful in substituting traditional gene targeting strategies with shRNA modalities. In this system, hematopoetic stem-

and progenitor cells are isolated from fetal livers, infected with shRNA-containing viruses and re-introduced into recipient mice. Knockdown of known tumor suppressors has been shown to promote lymphomagenesis, validating this approach (Hemann et al. 2003; Hemann et al. 2004). The potential to generate pools of shRNA libraries targeting thousands of genes creates the possibility for *in vivo* screens, including those for tumor suppressors as well as drivers of oncogenesis. To date, both positive and negative selection screens in the lymphoma model have reported novel hits in both classes of genes (Bric et al. 2009; Meacham et al. 2009). For an additional layer of control, multiple groups have incorporated spatiotemporal components into these viral-based RNAi systems. These include a Cre-controlled lentiviral system, as well as versions of tet-regulated shRNAs (Ventura et al. 2004; Dickins et al. 2005)

To further the potential uses of *in vivo* RNAi beyond infection and transplantation systems, traditional mouse genetics techniques have merged with shRNA platforms. For example, tet-regulated shRNA expression systems have been made into transgenics. In one report, crossing in different tTA/rtTA systems with a tetO-shp53 transgene afforded reversible and tissue-specific p53 knockdown (Dickins et al. 2007). Although reliant on finding extremely efficient shRNAs, such a system represents a fast and powerful approach to loss-of-function mouse genetics that will hopefully make *in vivo* studies more efficient in the future.

e. Sequential mutagenesis

Tumorigenesis is a multi-step process driven by numerous mutations in oncogenes and tumor suppressor genes that occur in a sequential manner (Hanahan and Weinberg 2000; Vogelstein and Kinzler 2004). However, the large majority of mouse models incorporating multiple mutations rely on strategies that require simultaneous genetic alterations, or at best,

control of only one mutation. As a result, one cannot accurately model stepwise mutations inherent in tumorigenesis or genetically dissect the relevance of these events in various stages of cancer.

The sequential nature of accumulated mutations during tumor formation is most well established in colorectal cancer. Early studies implicated *K-ras* and *APC* as frequent mutational targets, and genotype-phenotype correlations linked specific disease progression states with distinct combinations of mutations (Jen et al. 1994). Alterations of other genes, such as p53, have been linked to more advanced stages of colon cancer (Baker et al. 1990). Interestingly, the order of these mutations has been suggested to be a critical determinant of potential malignant progression. For example, from an analysis of human colon cancer samples, it appeared that lesions with an initiating mutation in *APC* were more likely to progress to a malignant state than their counterparts with initiating *K-ras* mutations. Interestingly, these early *K-ras* alterations seemed to steer cells down an alternate, less malignant state (Jen et al. 1994). Similar observations have been made in mouse studies. In a model of pancreatic cancer involving oncogenic K-ras activation and deletion of both p53 and Smad4, the timing of Smad4 loss relative to the other mutations was suggested to affect overall invasiveness of the end-stage disease, although this was not definitively shown (Izeradjene et al. 2007). Having temporal control of multiple mutations in mouse models would provide the opportunity to directly test these hypotheses concerning the importance of mutational order.

In addition to creating more accurate models of human cancer, sequential mutagenesis would also allow for more advanced mechanistic studies. By separating tumor-initiating mutations from other genetic perturbations, one could study the effects of gene inhibition or activation at distinct stages of tumor progression. While a number of groups have used genetics

to document robust inhibition of tumorigenesis in a variety of models, most studies have been focused on effects in tumor initiation (Gonzalez-Garcia et al. 2005; Gupta et al. 2007; Kissil et al. 2007). This experimental set-up not only limits tumor material available for further mechanistic analysis, it also precludes an assessment of the relevant gene in tumor maintenance, which is more important when considering it as a potential therapeutic target.

In Chapter 3 I describe the generation and characterization of a Flp-inducible allele of oncogenic K-ras that can be combined with Cre-LoxP reagents to perform sequential mutagenesis.

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CHAPTER 2:

Tissue-specific p19^{Arf} regulation dictates the response to oncogenic K-ras

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This chapter is an extended version of a manuscript in review at *PNAS*.

Nathan Young performed all experiments in the laboratory of Tyler Jacks.

ABSTRACT

The ability of oncogenes to engage tumor suppressor pathways represents a key checkpoint limiting the outgrowth of incipient tumor cells. For example, in a number of settings oncogenic Ras strongly activates the *Ink4a/Arf* locus, resulting in cell cycle arrest or senescence The capacity of different cell types to execute tumor suppressor programs following expression of endogenous K-ras^{G12D} has not been examined. Using compound mutant mice containing the *Arf*^{GFP} reporter and the spontaneously activating *K-ras*^{LA2} allele, we have uncovered dramatic tissue-specificity of K-ras^{G12D}-dependent p19^{Arf} up-regulation. Lung tumors, which can arise in the presence of functional p19^{Arf}, rarely display p19^{Arf} induction. In contrast, sarcomas always show robust activation, which correlates with genetic evidence suggesting that loss of the p19^{Arf}-p53 pathway is a requisite event for sarcomagenesis. Using constitutive and inducible RNAi systems *in vivo,* we highlight cell-type specific chromatin regulation of *Ink4a/Arf* as a critical determinant of cellular responses to oncogenic K-ras. Polycomb-group complexes repress the locus in lung tumors while the SWI/SNF family member Snf5 acts as an important mediator of p19^{Arf} induction in sarcomas. This variation in tumor suppressor induction might explain the inherent differences between tissues in their sensitivity to Ras-mediated transformation.

INTRODUCTION

An emerging paradigm in cancer biology is the duality of oncogenic signaling. Through their ability to activate a number of pro-growth and pro-survival pathways, oncogenes potently promote tumor initiation and progression. However, oncogenes also can engage tumor suppressor pathways, which results in a permanent cell cycle arrest (termed senescence) and/or cell death. These findings have led to the concepts of oncogene-induced senescence (OIS) and oncogene-induced apoptosis (OIA) as two crucial tumor suppressor checkpoints functioning to restrain tumor growth (Lowe et al. 2004; Collado et al. 2005; Collado and Serrano 2006; Mooi and Peeper 2006).

Two of the main mediators of these anti-oncogenic programs are encoded by the *Ink4a/Arf* locus, which contains two overlapping but structurally distinct tumor suppressors: $p19^{Arf}$ ($p14^{Arf}$ in humans) and $p16^{Ink4a}$. $p19^{Arf}$ is a nucleolar protein whose most well-established role is to indirectly activate the p53 transcription factor by interfering with the function of its inhibitor Mdm2. $p16^{Ink4a}$ functions as a cyclin-dependent kinase inhibitor that promotes cell cycle arrest by binding cyclin D/CDK4/6 complexes and preventing RB phosphorylation (Sherr and McCormick 2002; Lowe and Sherr 2003). Underscoring the importance of this locus in tumor suppression, knockout studies in mice have revealed increased tumor predisposition, while human data implicates both genes as frequent mutational targets in various malignancies (Serrano et al. 1996; Kamijo et al. 1997; Ruas and Peters 1998; Sharpless 2005). In response to a variety of hyper-proliferative stimuli, including oncogene activation, these genes are coordinately up-regulated (Gil and Peters 2006). For example, enhanced signaling downstream of oncogenic Ras has been shown to strongly induce $p19^{Arf}$ and $p16^{Ink4a}$ through a variety of transcription factors, such as cJun and DMP1 (Inoue et al. 2000; Ameyar-Zazoua et al. 2005). As

a result, both p53 and Rb pathways are activated, and cells usually undergo OIS by executing an irreversible cell cycle arrest (Serrano et al. 1997; Collado et al. 2005). Therefore, *Ink4a/Arf* up-regulation serves as a critical node linking upstream signals to downstream effector pathways in many types of oncogene-induced tumor suppression.

Whether or not various cell types in distinct tissues have different inherent abilities to engage these programs downstream of oncogenic insults remains largely unknown. Any such variability could have a profound influence on tumor susceptibility in different tissues and cell types, with cells responding either by proliferating as a result of low tumor suppressor induction or robustly up-regulating checkpoints and halting tumor initiation. We have explored this by question utilizing the "latent" K-ras^{G12D} (*K-ras*^{LA2}) mouse model (Johnson et al. 2001). In this model, an oncogenic allele of K-ras was engineered such that a duplication of exon 1 prevents its expression. Following a spontaneous recombination event, which in theory can occur in any cell type, the duplication is resolved and K-ras^{G12D} is expressed at endogenous levels. Despite the presumed random nature of oncogenic K-ras expression, these mice predominantly succumb to lung tumors; a subset also develops thymic lymphomas (Johnson et al. 2001). A potential explanation for this strong lung tumor phenotype is that certain lung cells are sensitive to oncogenic K-ras-mediated transformation because they fail to effectively induce p19^{Arf} and p16^{Ink4a} and, therefore, hyper-proliferate. In contrast, numerous other cell types in these animals could strongly activate *Ink4a/Arf* following K-ras^{G12D} expression, which would block tumor formation.

Previous studies have shown that oncogene levels can determine whether a cell undergoes proliferation or cell cycle arrest/death. For example, novel alleles of oncogenic K-ras allowing for Cre-dependent expression of endogenous K-ras^{G12D} (one being *K-ras*$^{LSL\text{-}G12D}$)

immortalized primary mouse embryonic fibroblasts, as opposed to inducing OIS, as was seen with retroviral-mediated over-expression of K-ras^{G12D} (Tuveson et al. 2004). This concept has also been validated *in vivo* (Sarkisian et al. 2007; Murphy et al. 2008). For example, Sarkisian et al. have used doxycycline-inducible K-ras to alter oncogene levels and shift the response from hyper-proliferation (low K-ras) to growth suppression (high K-ras). These data provide support for a model of oncogene levels dictating OIS or OIA versus proliferation in a given cell type, and could also explain any different responses across tissues if oncogene levels varied accordingly.

An alternative explanation for variable *Ink4a/Arf* induction in response to oncogenic signaling is underlying cell type-specific locus regulation that sets different thresholds for gene activation. A growing number of positive and negative regulators of p19^{Arf} and p16^{Ink4a} have roles in regulating chromatin configuration, thus allowing them to confer upon the locus a particular chromatin confirmation and affect the accessibility of other transcription factors following oncogene activation. Polycomb-group (PcG) proteins repress the *Ink4a/Arf* locus in a number of cell types through direct binding of the histone methyltransferase complex PRC2 (including Ezh2 and Suz12) and the repressive PRC1 complex (including Bmi-1 and CBX8) (Bracken et al. 2007; Miyazaki et al. 2008; Chen et al. 2009; Dhawan et al. 2009). This mode of regulation seems to be especially important in multiple types of stem cells compared to their differentiated progeny (Valk-Lingbeek et al. 2004). Distinct chromatin patterns within certain lineages have been proposed to explain the inherent differences in transformation capabilities of progenitor-like hematopoietic cells versus B and T cells (Williams and Sherr 2007; Volanakis et al. 2009). Presumably, the loss of repressive chromatin domains within differentiated cells allows for greater ease of *Ink4a/Arf* induction when positive regulators are recruited following oncogene expression, although this has not been directly tested. In other systems, it is

conceivable that across cell types with similar chromatin-bound PcG complexes, oncogene activation could result in cell type-specific chromatin remodeling and *Ink4a/Arf* expression. For example, the proper up-regulation of genes normally repressed by PcG involves additional chromatin modifiers, including the SWI/SNF family of nucleosome remodelers (Tamkun et al. 1992; Gebuhr et al. 2000). This complex might have tissue-specific functions that allow it to exert influence over PcG regulation only in some settings. Overall, the balance between classes of chromatin regulators could have a large influence on the ability of cells to engage the *Ink4a/Arf* locus in response to oncogenic signaling.

In order to explore potential tissue-specific interactions involving oncogenic K-ras and the $p19^{Arf}$ tumor suppressor pathway, we have examined the effects on tumor predisposition in compound mutant mice. Specifically, using the $K\text{-}ras^{LA2}$ model, absence of $p19^{Arf}$ function promoted sarcoma development while only modestly affecting lung tumor progression. By utilizing a reporter for $p19^{Arf}$ transcriptional activity, we were able to show a striking tumor-type specific expression pattern from this locus in lung tumors and sarcomas. Importantly, this expression pattern correlated with the different genetic requirements for transformation in lung versus muscle cells. While oncogene levels and signaling outputs appeared similar between the tumor types, we identified and functionally validated direct chromatin regulators of *Ink4a/Arf* in both lung tumors and sarcomas, suggesting that tissue-specific chromatin remodeling controls the engagement of this tumor suppressor locus. Cell-type specificity in the degree of tumor suppressor induction in response to oncogenic K-ras provides an explanation for the different inherent sensitivities of various tissues to K-ras mediated transformation.

RESULTS

Lung tumors expressing endogenous levels of K-ras^{G12D} weakly induce Ink4a/Arf

To determine if K-ras^{G12D} expressed at endogenous levels can effectively engage tumor suppressor checkpoints in different tissues *in vivo*, we began by monitoring the expression of both 19^{Arf} and p16^{Ink4a} in lung tumors isolated from aged *K-ras^{LA2}* mice. As shown in Figure 1A, neither gene product was robustly up-regulated at the protein level, with p19^{Arf} detectable in only one of seven tumors examined. As a positive control for our ability to detect induction of these proteins, we generated *K-ras$^{LSL-G12D}$; Trp53$^{flox/flox}$* tumors (which also have endogenous levels of K-ras^{G12D}) as it is known that loss of p53 leads to up-regulation of both p19^{Arf} and p16^{Ink4a} (Stott et al. 1998). Indeed, in the context of p53 deficiency, both proteins were significantly induced (Fig 1B). These data suggest that the *K-ras^{LA2}* lung tumors in Fig 1A maintain functional p53. To achieve more sensitivity of detection, we also measured mRNA levels by qRT-PCR. Across a panel of tumors, p16^{Ink4a} was only slightly induced, and p19^{Arf} levels varied from very low to moderate (Fig 1C). Importantly, all tumors showed some induction from *Ink4a/Arf*, demonstrating that this locus is neither silenced nor deleted tumor lung tumor progression. Additionally, while several tumors appeared to significantly up-regulate p19^{Arf}, it should be noted that none displayed the high levels seen in endogenous K-ras^{G12V}-driven lung tumors reported by Serrano and colleagues (Guerra et al. 2003), further highlighting differences between this allele and those from our lab (Tuveson et al. 2004). Finally, we also utilized an *Arf^{GFP}* reporter mouse, in which GFP has been knocked into the endogenous p19^{Arf} locus such that p19^{Arf} function is abolished and GFP is under the control of the endogenous promoter (Zindy et al. 2003). By crossing this mouse with the *K-ras^{LA2}* strain, we hoped to monitor p19^{Arf} promoter activity independently of any selective pressure to keep this tumor suppressor locus inactive

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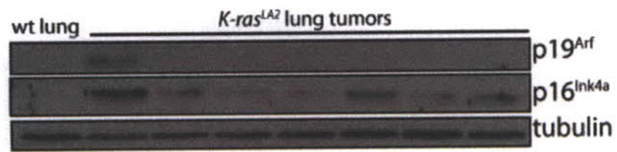

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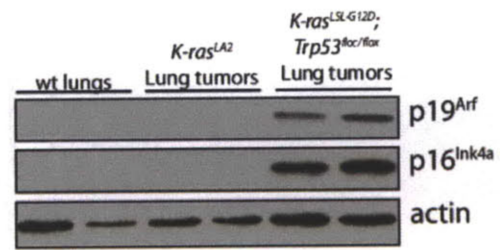

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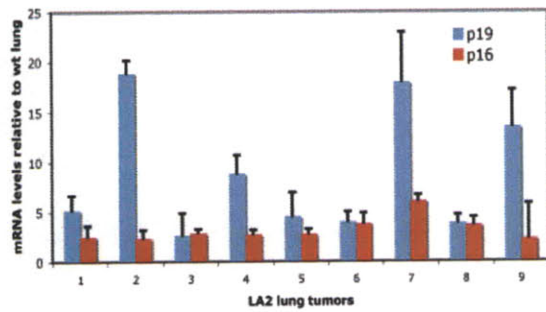

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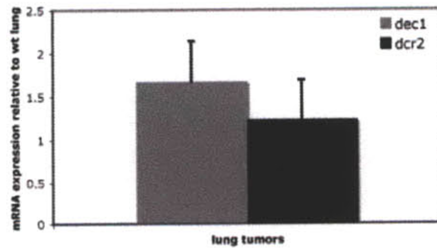

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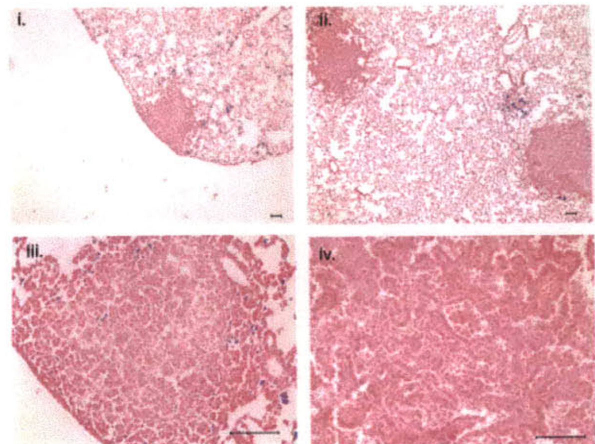

Figure 1: Characterization of p19^{Arf} and p16^{Ink4a} expression and activity in lung tumor models driven by endogenous K-ras^{G12D}.

(A. and **B.)** Western blot analysis of p19^{Arf} and p16^{Ink4a} in wild-type lungs and lung tumors. All tumors are from 5-8 month old *K-ras*^{LA2} mice except for those from *K-ras*$^{LSL\text{-}G12D}$; *Trp53*$^{flox/flox}$ mice, which were harvested 16 weeks post adeno-Cre delivery into the lungs. **(C.)** qRT-PCR of p19^{Arf} and p16^{Ink4a} mRNA levels in *K-ras*^{LA2} tumors similar to those in **A.** β-actin was used as a standard and relative expression was calculated by normalized to wt lungs (N = 2).
(D.) GFP western blot on lungs and lung tumors taken from *K-ras*^{LA2}; *Arf*^{GFP} compound mutant mice. **(E.)** qRT-PCR analysis of the senescence-associated genes Dec1 and DcR2 in lung tumors. Relative expression was calculated as in **C. (F.)** Senescence-associated β-galactosidase (SA-βgal) assay on frozen sections of *K-ras*^{LA2}; *Arf*$^{GFP/+}$ mice. Panels **i.** and **ii.** are 4X and **iii.** and **iv.** are 20X magnification. Scale bars represent 200 μm. The lack of SA-βgal activity, as shown in these images, is representative of results seen with a number of lung tumors (>50). The tuft of blue cells in the upper left panel and those scattered in the bottom right are most likely macrophages. For qRT-PCR, error bars indicate standard deviation.

during K-ras^{G12D}-driven tumor progression. Analyzing GFP levels in lung tumors from compound mutant mice revealed variability in the degree of induction, with a majority of tumors showing little signal (Fig 1D). Taken together, these data suggest that *K-ras*^{LA2} lung tumors only moderately activate the p19^{Arf}-p16^{Ink4a} locus, if at all.

One of the main functional outputs of p19^{Arf} and p16^{Ink4a} expression is senescence (Collado and Serrano 2006). To examine whether the low levels of induction discussed above could still be engaging this program, we tested for well-established markers of senescence. As shown in Figure 1E, qRT-PCR analysis revealed only a slight increase in expression of Dec1 and DcR2 in *K-ras*^{LA2} lung tumors over normal lung tissue. In addition, we never detected senescence-associated beta-galactosidase activity (SA-βgal) in tumors, irrespective of their size or grade (Fig 1F and data not shown). These data indicate that lung tumors from the *K-ras*^{LA2} model fail to undergo senescence, perhaps because the two main upstream activators, p19^{Arf} and p16^{Ink4a}, are only weakly induced.

p19^{Arf}-deficiency mildly affects lung tumor progression

The low levels of p19^{Arf} and p16^{Ink4a} induction during the course of lung tumor progression predict that deletion of these genes will have a minimal effect on tumorigenesis. To test this, we made use of the fact that the *Arf*^{GFP} allele eliminates p19^{Arf} function. We generated cohorts of *K-ras*^{LA2} animals that were either *Arf*$^{GFP/+}$ or *Arf*$^{GFP/GFP}$ and compared tumors at two different timepoints. At an early timepoint (6 weeks of age) the two genotypes were indistinguishable from one another in terms of tumor number, average tumor size, and histopathological grade (Fig 2A-C). By 12 weeks of age, while tumor number tumor did not change among genotypes, *Arf*$^{GFP/GFP}$ mice tended to have slightly larger tumors, although this

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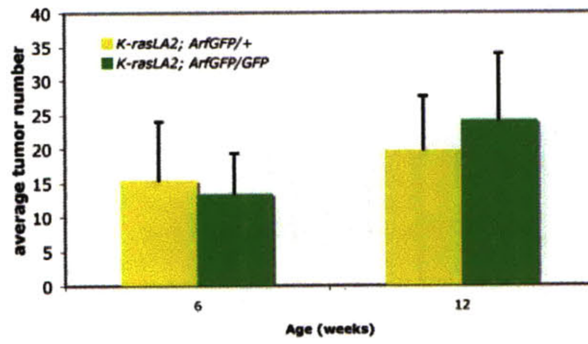

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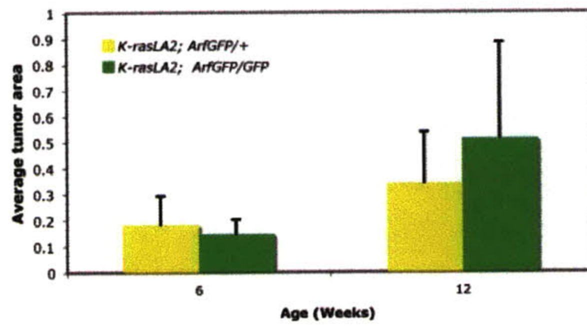

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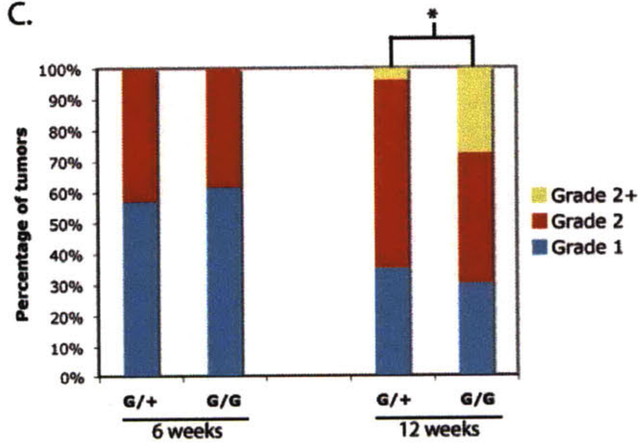

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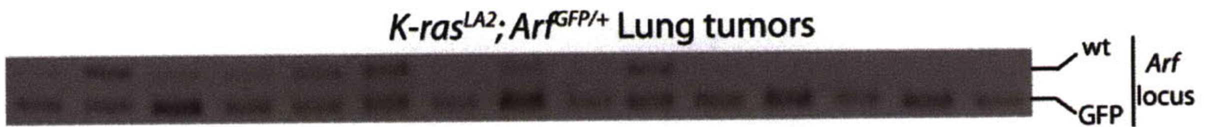


Figure 2: Effects of p19^{Arf} deficiency on *K-ras^{LA2}* lung tumors.

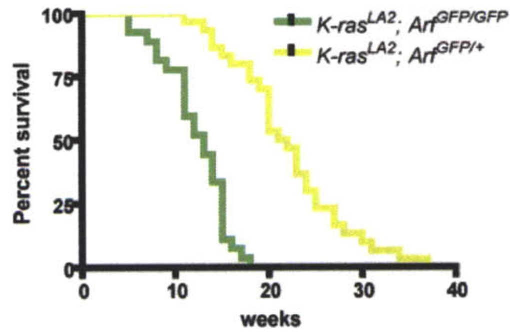
K-ras^{LA2}; Arf$^{GFP/+}$ (yellow bars, **A** and **B**) and *K-ras^{LA2}; Arf$^{GFP/GFP}$* (green bars, **A** and **B**) littermates were aged either 6 or 12 weeks, at which point their lungs were processed for histological examination. One representative section per mouse was analyzed to calculate average tumor number (**A**), average tumor size (**B**), and each individual tumor's histopathological grade (**C**). For (**C**), G/+ = *K-ras^{LA2}; Arf$^{GFP/+}$* and G/G = *Arf$^{GFP/GFP}$*. For **A** and **B**, $P > .05$ for comparison between genotypes within one age group (Students T-test). For **C**., * = $P < .001$ (Fisher's Exact Test). N = 7 (*Arf$^{GFP/+}$* and *Arf$^{GFP/GFP}$* at 6 weeks). N = 12 (*Arf$^{GFP/+}$*) and N = 10 (*Arf$^{GFP/GFP}$*) at 12 weeks. Error bars indicate standard deviation. (**D.**) PCR analysis of the *Arf* locus on LCM-captured genomic DNA from a panel of tumors from 7 month old *K-ras^{LA2}; Arf$^{GFP/+}$* animals.

was not statistically significant due to high variability (Fig 2A, B). Moreover, a significant subset of tumors in *Arf*$^{GFP/GFP}$ animals presented with a more advanced histopathological grade compared to their *Arf*$^{GFP/+}$ counterparts (Fig 2C). Finally, genomic PCR analysis of advanced lesions from aged *K-ras*^{LA2}; *Arf*$^{GFP+}$ animals indicated that several tumors had undergone loss-of-heterozygosity (LOH) of the wild-type *Arf* allele during tumor progression (Fig 2D). Together, these studies suggest that p19^{Arf} acts to impair the progression of *K-ras*^{LA2} lung tumors, as opposed to blocking the earliest stages of tumor initiation. This is consistent with the low level of p19^{Arf} induction observed in most of the tumors.

Expansion of the tumor spectrum in K-ras^{LA2}*; Arf*$^{GFP/GFP}$ *mice*

Despite the modest effect on lung tumor development in compound mutant mice, a striking difference in median survival was noted between *K-ras*^{LA2}; *Arf*$^{GFP/+}$ and *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ littermates (Fig 3A). Upon close examination of aged *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ mice, it became evident that a number of animals had grossly detectable masses before death. Through histological examination, we determined that these masses represented two broad types of novel tumors. About 35% of mice had muscle-derived sarcomas that contained mostly spindle-shaped cells, with varying degrees of differentiation. A quarter of compound mutant mice displayed enlarged spleens and livers that contained large cells with irregular nuclei and abundant cytoplasm, features reminiscent of histiocytic sarcomas (Fig 3B, C). Given that p19^{Arf} knockout mice alone are susceptible to muscle-derived sarcomas (Kamijo et al. 1999), it was important to determine if this novel tumor spectrum in *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ animals was due to cooperation between oncogenic K-ras and p19^{Arf} loss. Evidence for genetic cooperation includes the shorter latency for sarcoma formation in *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ versus p19^{Arf} knockout mice

A.



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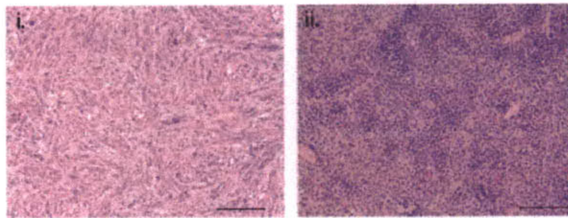

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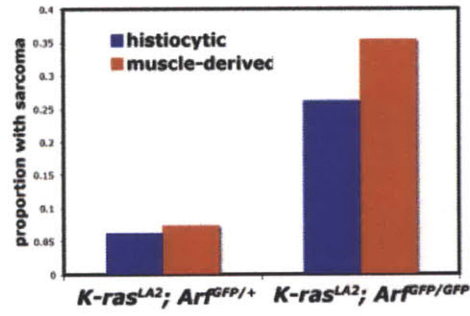

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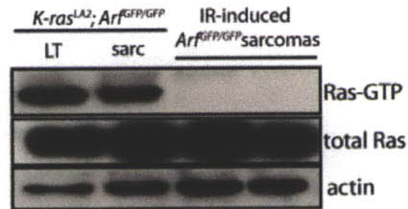


<u>Figure 3:</u> Expanded tumor spectrum in *K-ras*^{LA2}*; Arf*$^{GFP/GFP}$ mice.

(A.) Kaplan-Meier plot showing the differential survival of *K-ras*^{LA2}*; Arf*$^{GFP/+}$ (yellow line) and *K-ras*^{LA2}*; Arf*$^{GFP/GFP}$ (green line) mice. (P <.0001) **(B.)** Representative hematoxylin and eosin (HE) pictures of novel tumors in *K-ras*^{LA2}*; Arf*$^{GFP/GFP}$ mice. **i.** muscle-derived sarcomas. **ii.** histiocytic sarcomas. Scale bar is 200 µm.**(C.)** Percentage of *K-ras*^{LA2}*; Arf*$^{GFP/+}$ and *K-ras*^{LA2}*; Arf*$^{GFP/GFP}$ animals presenting with macroscopic muscle-derived sarcomas (red bars) or histiocytic sarcomas after 7 weeks of age. **(D.)** Ras-GTP assay demonstrating activated Ras in muscle-derived sarcomas from *K-ras*^{LA2}*; Arf*$^{GFP/GFP}$ compound mutant mice. For a negative control, tumors induced by IR irradiation of *Arf*$^{GFP/GFP}$ mice do not show activated Ras.

(Kamijo et al. 1999) as well as the fact that we could readily detect hyper-activated Ras in lysates from *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ tumors compared with control *Arf*$^{GFP/GFP}$ sarcomas (Fig 3D). One interpretation of the expanded tumor spectrum in *K-ras*^{LA2} mice upon deleting p19^{Arf} is that there are cells in *K-ras*^{LA2} animals that express oncogenic K-ras but fail to form tumors due to the strong induction of the p19^{Arf}-p53 pathway.

Variation in the degree of Ink4a/Arf activation across tumor types in K-ras^{LA2}*; Arf*$^{GFP/GFP}$ *mice*

We hypothesized that cells that give rise to tumors in *K-ras*^{LA2} mice only when *Arf* is deleted should have high levels of p19^{Arf} expression upon K-ras^{G12D} expression. Given that all the tumors arose in the presence of the *Arf*^{GFP} reporter, we compared GFP induction across the various tumor types that form in *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ mice. This analysis revealed dramatic differences in reporter induction (Fig 4A). As the expression pattern was most striking and reproducible between lung tumors and muscle-derived sarcomas, we focused on these two tumor types for further molecular analysis (Fig 4B). To extend these findings beyond the GFP reporter, we assessed levels of endogenous p19^{Arf} protein in lung tumors and sarcomas from another mouse model in which we were able to control the timing and location of oncogenic K-ras activation. In the *K-ras*$^{LSL\text{-}G12D}$; *Trp53*$^{flox/flox}$ mouse model lung tumors and sarcomas can be induced through administration of recombinant adenovirus expressing Cre recombinase (Ad-Cre) either to the lung or the muscle (Jackson et al. 2001; Kirsch et al. 2007). As shown in Figure 4C, even though both tumor types developed in the absence of p53 and so might have been expected to both up-regulate p19^{Arf}, steady-state levels of the protein were much higher in sarcomas than lung tumors. Additionally, the expression pattern of p16^{Ink4a}, which shares its locus with p19^{Arf} and is often co-regulated, was identical to that of GFP, while an unrelated tumor suppressor,

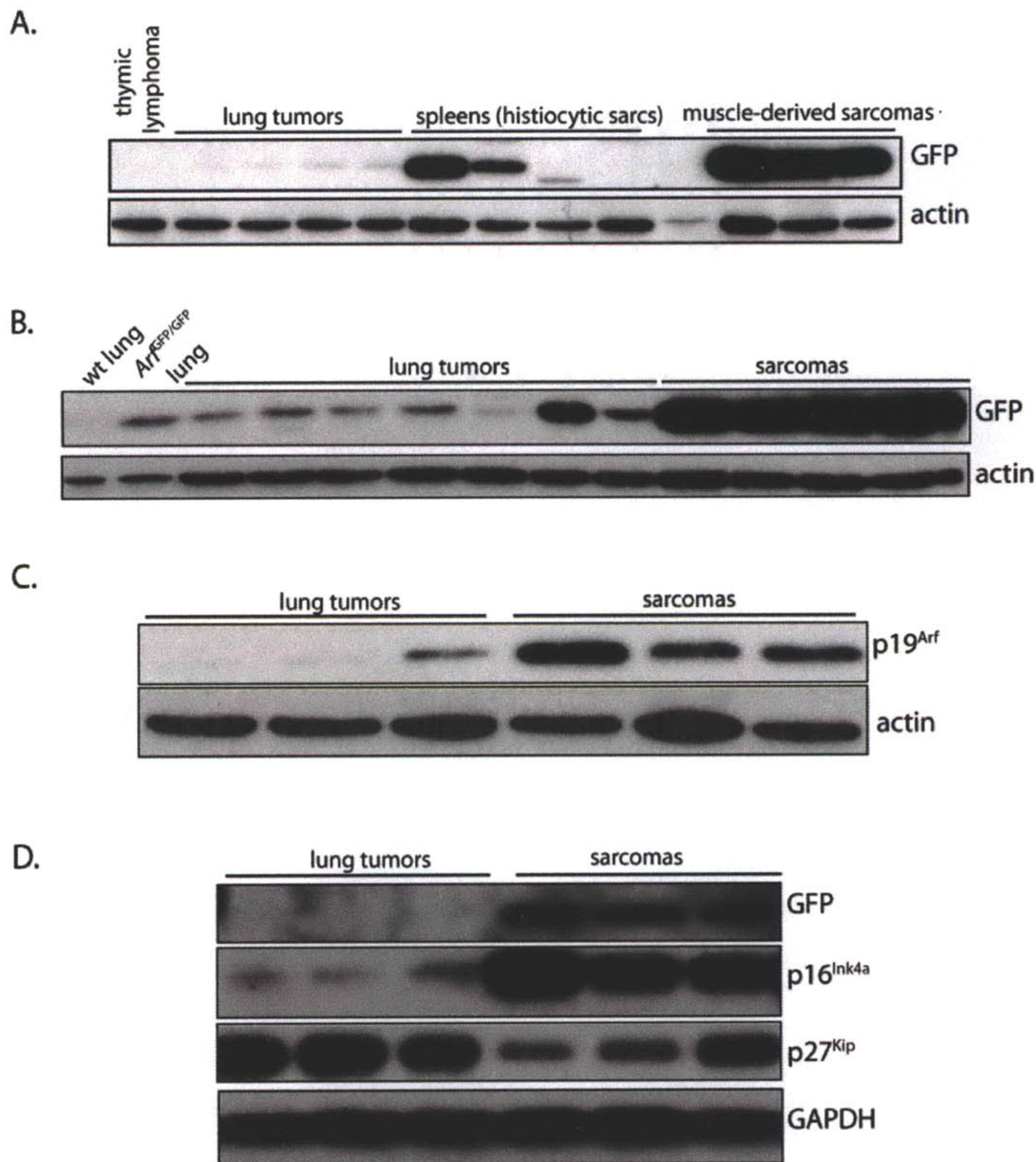


Figure 4: **Differential p19^{Arf} expression in K-ras^{G12D}-induced lung tumors and sarcomas.**

(**A.**) Western blot analysis of GFP from multiple tumor types originating in *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ animals, including thymic lymphomas, lung tumors, histiocytic sarcomas, and muscle-derived sarcomas. (**B.**) GFP western blots on lung tumors and muscle-derived sarcomas from *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ mice. (**C.**) Comparison of endogenous p19^{Arf} protein levels in lung tumors and sarcomas generated from *K-ras*$^{LSL-G12D}$; *Trp53*$^{flox/flox}$ mice infected with Ad-Cre either intratracheally (for lung tumors) or intramuscularly (for sarcoma formation). (**D.**) Western blots of GFP as well as additional tumor suppressor genes, p16^{Ink4a} and p27^{Kip1}, from lung tumors and sarcomas taken from *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ mice. The pattern of tumor suppressor induction in lung tumors and sarcomas appears to be specific to *Ink4a/Arf*, as p27, an unrelated tumor suppressor, shows the opposite correlation.

$p27^{Kip1}$, showed the opposite pattern (Fig 4D). In summary, although both tumors are driven by the same oncogene, sarcomas induce *Ink4a/Arf* expression much more strongly than lung tumors.

Different levels of $p19^{Arf}$ activation lead to differences in the nature of tumor suppression

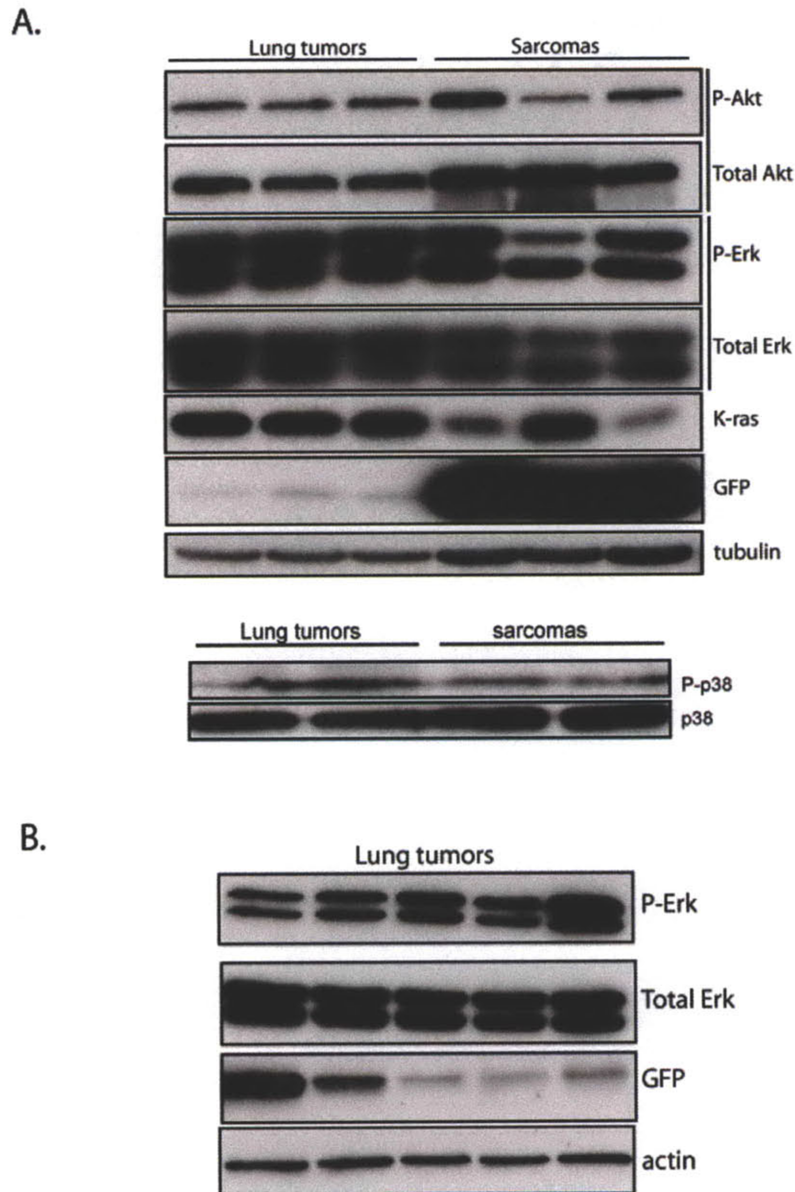
Having established a differential expression pattern of $p19^{Arf}$ and $p16^{Ink4a}$ between lung tumors and sarcomas, we wished to determine if this had any functional significance. As the activation of the *Ink4a/Arf* locus engages two potent tumor suppressor pathways, p53 and Rb, it is conceivable that differences in the magnitude of induction could significantly affect the ability of cells harboring oncogenic mutations to form early lesions. As shown in Figures 1 and 2, lung tumors in *K-ras*^{LA2} mice display very modest levels of $p19^{Arf}$and $p16^{Ink4a}$, and this correlated with the ability of oncogenic K-ras alone to promote tumor formation in the lung. Conversely, in K-ras^{G12D}-mutant sarcomas, the *Ink4a/Arf* locus was robustly activated (Fig 4), leading us to hypothesize that in those target cells the tumorigenic process is inhibited at a much earlier stage, perhaps even at initiation. To test this, we again utilized the sarcoma model based on Ad-Cre delivery into the limbs of *K-ras*$^{LSL-G12D}$; *Trp53*$^{flox/flox}$ mice. We have previously shown that sarcomas formed in this model only when both alleles of p53 were inactivated (Kirsch et al. 2007). Substituting homozygous *Arf*^{GFP} alleles for *Trp53*^{flox} also supported sarcoma formation (data not shown). When leg muscles of both *Trp53*$^{flox/+}$ and *Arf*$^{GFP/+}$ (both *K-ras*$^{LSL-G12D}$) animals were subjected to systematic histological examination 4-7 months post Cre delivery there was no sign of any preneoplastic lesions in the region of Ad-Cre delivery (data not shown). This suggests that K-ras^{G12D} mutant cells are completely blocked at a very early stage of tumor initiation when even one copy of $p19^{Arf}$ or p53 is present, most likely because of rapid and robust

pathway activation. Therefore, we conclude that the expression pattern of p19^{Arf} between lung tumors and sarcomas leads to dramatically different functional outputs of the p19^{Arf}-p53 pathway in cells expressing oncogenic K-ras in the two tissues.

Neither oncogenic signaling levels nor canonical transcriptional activators explain tumor-specific p19^{Arf} and p16^{Ink4a} induction

We next sought to understand the mechanistic basis of the tissue-specific expression pattern of *Ink4a/Arf*. The regulation of p19^{Arf} and p16^{Ink4a} expression is largely controlled by the intensity of signaling pathways downstream of oncogenic K-ras, which are themselves directly regulated by overall oncogene levels (Lowe and Sherr 2003; Tuveson et al. 2004; Gil and Peters 2006). Our initial hypothesis was that sarcomas have increased oncogenic signaling compared to lung tumors, which drives higher tumor suppressor induction. Therefore, we analyzed steady-state levels of K-ras and some of the relevant downstream signaling outputs, including the Erk-MAPK, PI3K, and p38MAPK pathways, between lung tumors and sarcomas from *K-ras^{LA2}; Arf$^{GFP/GFP}$* mice. This analysis did not reveal any correlations between oncogene or signaling pathway levels and GFP induction across the two tumor types (Fig 5A). Furthermore, similar analyses across a panel of lung tumors with differing GFP levels failed to link increased GFP with elevated signaling (Fig 5B).

As the *Ink4a/Arf* locus is more proximately controlled at the level of transcriptional induction (Gil and Peters 2006), we next measured the levels of known transcriptional activators including E2F1, E2F3, cJun, and DMP-1 across the tumor types. Interestingly, all of these genes were more highly expressed in sarcomas compared to lung tumors, potentially implicating them in p19^{Arf} and p16^{Ink4a} regulation (Fig 6).



<u>Figure 5:</u> Levels of K-ras and downstream signaling pathways in lung tumors and sarcomas from *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ mice do not correlate with GFP expression.

(**A.**) Western blot analysis of oncogenic signaling in lung tumors and sarcomas from *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ mice. Erk-MAPK (P-Erk) PI3K (P-Akt), and p38MAPK signaling pathways were analyzed, as was total K-ras levels. (**B.**) Erk-MAPK signaling was assessed in a panel of lung tumors showing differential GFP expression.

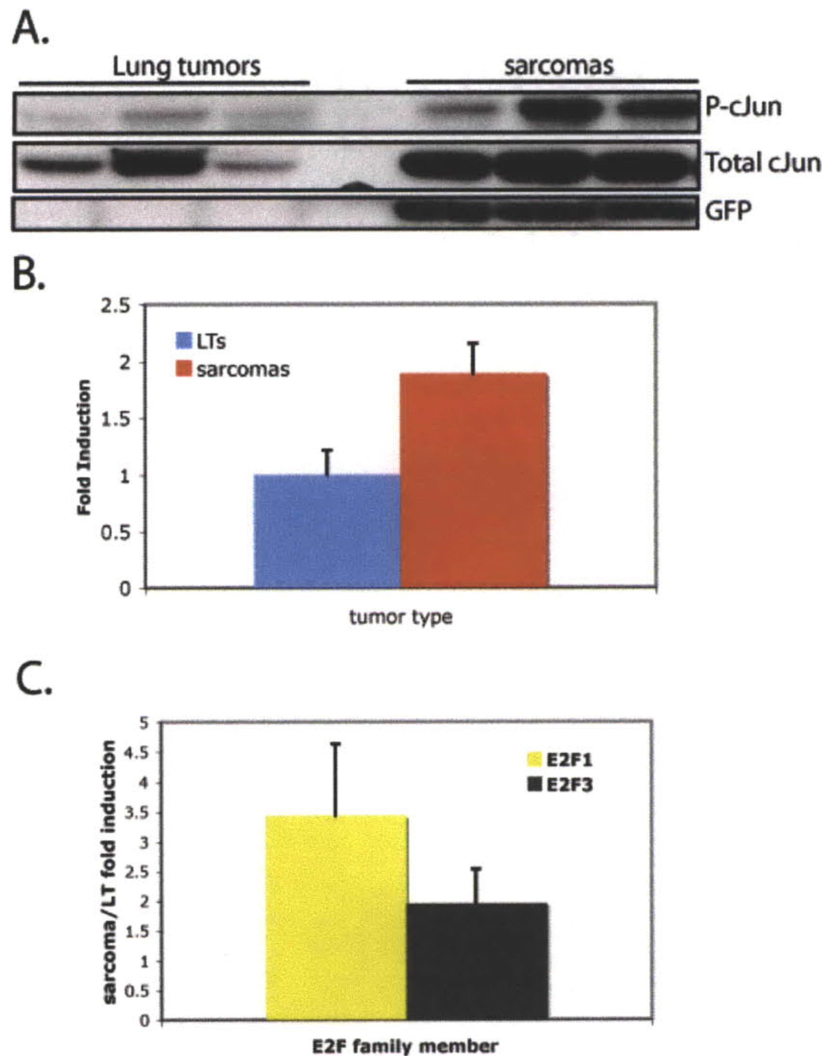


Figure 6: A number of putative activators of *Ink4a/Arf* are up-regulated in sarcomas compared to lung tumors in *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ mice.

Immunoblot (**A.**) and qRT-PCR (**B. and C.**) analysis of activators of p19^{Arf} and/or p16^{Ink4a} in lung tumors and sarcomas from *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ tumors. cJun (**A**), DMP-1 (**B**), and E2F1 and E2F3 (**C**) all show up-regulation in sarcomas compared to lung tumors. For qRT-PCR analyses, N = 3 for each tumor type, and $P < .05$ for gene expression differences between lung tumors and sarcomas (Student's T-test). Error bars indicate standard deviation.

To investigate if any of these genes contribute to the positive regulation of *Ink4a/Arf* in sarcoma development, we devised a functional assay using lentivirus-mediated RNAi in the muscle (Fig 7A and B). It was necessary to perform these experiments on a *Rag2*$^{-/-}$ background to achieve adequate tumor initiation following lentiviral infection. Infection of *K-ras*$^{LSL-G12D}$; *Arf*$^{GFP/+}$; *Rag2*$^{-/-}$ mice with lentiviruses containing just Cre failed to induce tumor formation, presumably due to the remaining functional allele of p19^{Arf}. However, lentiviruses that contained both Cre and a shRNA targeting both p19 and p16 were able to efficiently generate sarcomas (Fig 7C and D). To assess knockdown efficiency of a panel of shRNAs targeting p19^{Arf} activators, we infected *K-ras*$^{LSL-G12D}$; *Arf*$^{GFP/GFP}$; *Rag2*$^{-/-}$ mice. While a number of shRNAs appeared to silence their target genes in the resulting sarcomas (including those targeting E2F1, E2F3, and DMP-1; data not shown), infection of *K-ras*$^{LSL-G12D}$; *Arf*$^{GFP/+}$; *Rag2*$^{-/-}$ animals with these viruses failed to induce sarcomas (data not shown). While we cannot rule out the possibility of functional redundancy of multiple activators or insufficient knockdown, these results may indicate that these genes do not play a significant role in K-ras^{G12D}-dependent p19^{Arf} up-regulation in the muscle.

Tumor type-specific Polycomb-group (PcG) occupancy at Ink4a/Arf

Chromatin structure has a major influence on the ability of upstream signals to execute transcriptional programs at target loci. Therefore, we decided to monitor chromatin regulators as well as the chromatin composition of the *Ink4a/Arf* locus in lung tumors and sarcomas. Given their established role in *Ink4a/Arf* chromatin regulation in a number of settings, we began by investigating PcG proteins (Bracken et al. 2007; Miyazaki et al. 2008; Chen et al. 2009; Dhawan et al. 2009). Chromatin immunoprecipitation (ChIP) experiments revealed a striking difference

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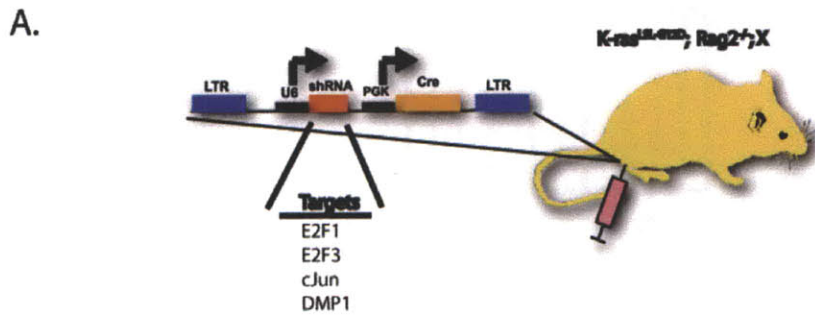

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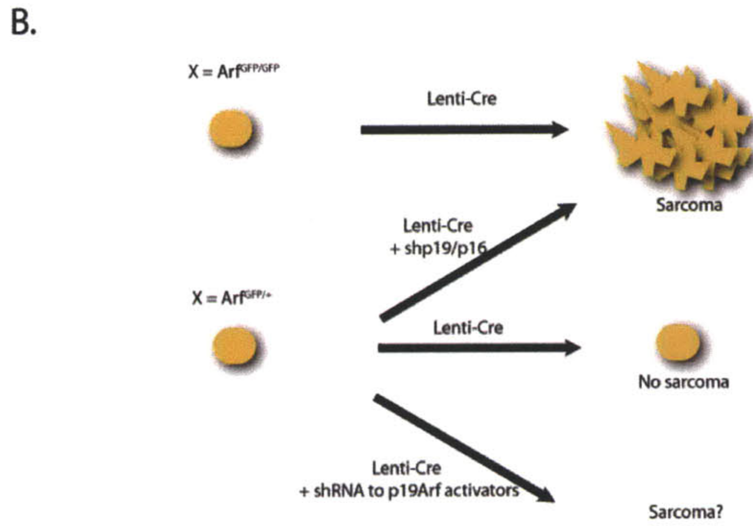

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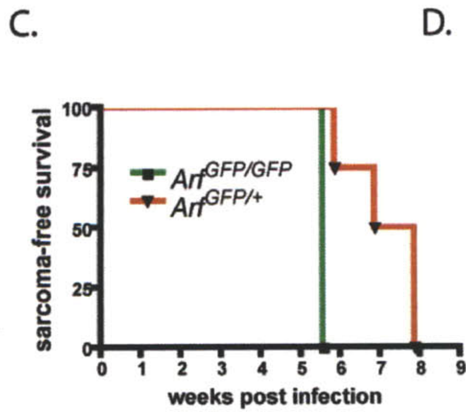

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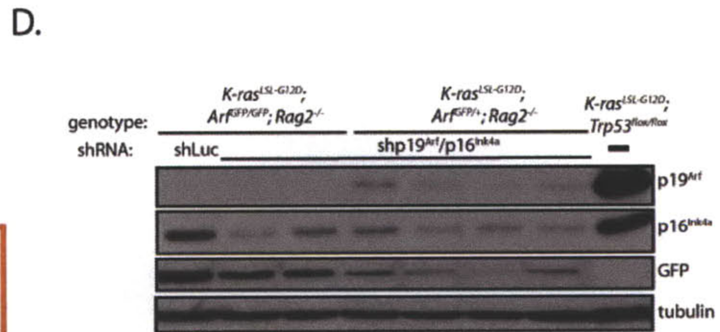

Figure 7: Use of bifunctional lentiviruses to identify functionally significant activators of $p19^{Arf}$ during sarcomagenesis.

(A.) A bifunctional lentivirus expressing Cre and a shRNA is used to infect the muscles of *K-ras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$ mice that are either heterozygous (*Arf*$^{GFP/+}$) or homozygous null (*Arf*$^{GFP/GFP}$) for $p19^{Arf}$. **(B.)** Sarcoma formation is completely inhibited by the presence of an activated, functional copy of $p19^{Arf}$. Therefore, sarcomas form only when infecting *Arf*$^{GFP/GFP}$ animals with Cre alone or infecting *Arf*$^{GFP/+}$ mice with a shRNA to $p19^{Arf}$. We hoped identify the critical activator(s) of $p19^{Arf}$ by using the system in **A** to knock down putative regulators in *Arf*$^{GFP/+}$ mice and screening for sarcoma induction. **(C.)** Kaplan-Meier graph of sarcoma-free survival of *K-ras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$ mice that are either heterozygous (*Arf*$^{GFP/+}$-red bar) or homozygous null (*Arf*$^{GFP/GFP}$-green bar) for $p19^{Arf}$. Both groups of mice were infected intramuscularly with lentiviruses containing Cre and a shRNA to a shared exon of $p19^{Arf}$ and $p16^{Ink4a}$. **(D.)** Western blot analysis of tumors from animals in **C** as well as a negative control for $p19^{Arf}$ (*K-ras*^{G12D}; *Arf*$^{GFP/GFP}$; *Rag2*$^{-/-}$ sarcoma; left-most lane) and a positive control for $p19^{Arf}$ (*K-ras*^{G12D}; *Trp53*$^{flox/flox}$ sarcoma; right-most lane). The knockdown tumors show reduced levels of $p19^{Arf}$ and $p16^{Ink4a}$.

in PcG occupancy at *Ink4a/Arf* between lung tumors and sarcomas from *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ animals. Lung tumors consistently showed an enrichment of the PcG-associated histone mark, H3K27me3, at the promoters of both p19^{Arf} and p16^{Ink4a} compared with sarcomas (Fig 8A and B). In addition, the binding of the PRC1 component Bmi-1 was much more robust in lung tumors (Fig 8A and B). We next assessed if tumor type-specific expression of PcG components could explain these differences in *Ink4a/Arf* occupancy. However, neither Bmi-1 nor the H3K27 methyltransferase Ezh2 displayed significantly different mRNA levels among the tumor types (Fig 8C). Furthermore, while the H3K27 de-methylase Jmjd3 was differentially expressed, it showed the opposite correlation than one would expect, with higher abundance in lung tumors compared to sarcomas (Fig 8D). Despite these inclusive expression data, the ChIP analysis suggests that there is lung tumor-specific PcG-mediated gene silencing of *Ink4a/Arf*.

The abundant PcG binding across *Ink4a/Arf* in established lung tumors was surprising given that these cells harbor significant oncogenic stress, which should remove this form of chromatin regulation. Recent studies have implicated Jmjd3 in the eviction of PcG from *Ink4a/Arf* downstream of oncogenic Ras (Agger et al. 2009; Barradas et al. 2009), so we wondered if the continued PcG occupancy could be explained by insufficient Jmjd3 induction following K-ras^{G12D} expression in lung cells. Indeed, an assessment of Jmjd3 mRNA abundance revealed equivalent levels between normal lung tissue and lung tumors (Fig 9A). In addition, ChIP analysis indicated a very similar enrichment of H3K27me3 at *Ink4a/Arf* in normal and tumor tissue from the lung (Fig 9B). Together, these data might suggest that significant PcG binding of *Ink4a/Arf* in lung tumors results from the inability of oncogenic signaling to induce programs that normally remove PcG under stress conditions.

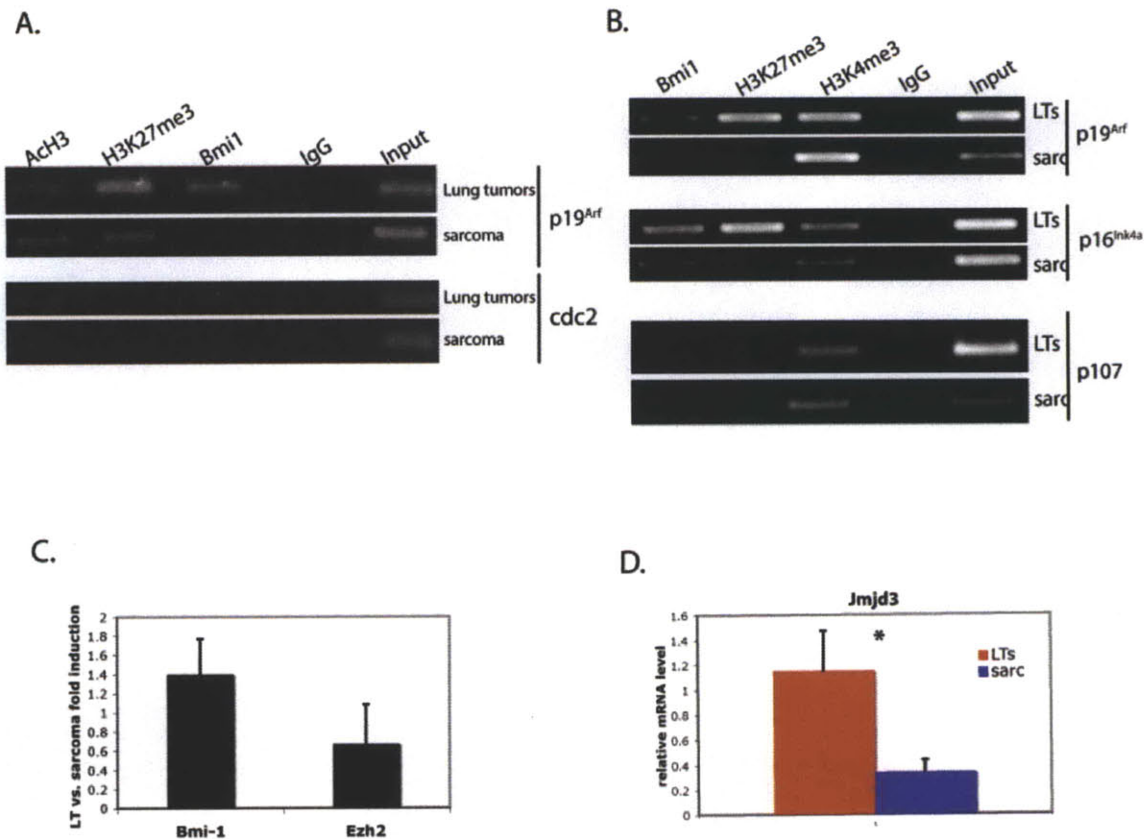

Figure 8: Differential PcG occupancy at *Ink4a/Arf* in lung tumors and sarcomas

(**A.** and **B.**) Representative ChIP analyses of PcG markers in lung tumors and sarcomas from *K-ras^{LA2}; Arf$^{GFP/GFP}$* mice. AcH3 = acetylated histone H3; H3-K4 = trimethylated histone lysine 4 of histone H3, both marks of transcriptionally active promoters. H3K27me3 = tri-methylated lysine 27 on histone H3, the PcG-associated chromatin mark. IgG corresponds to a control IP with rabbit IgG. Immunoprecipitated DNA was amplified with primers specific to the promoters of p19^{Arf}, p16^{Ink4a}, cdc2, and/or p107. (**C** and **D.**) qRT-PCR of selected PRC2 (Ezh2) and PRC1 (Bmi-1) components (**C**) as well as Jmjd3 (**D**) from *K-ras^{LA2}; Arf$^{GFP/GFP}$* derived tumors. The Y-axis denotes fold induction of lung tumors vs. sarcomas. N = 3 for each tumor type. $P > .05$ for both genes in **C**, while $P < .05$ in **D** (Student's T-test). Error bars indicate standard deviation.

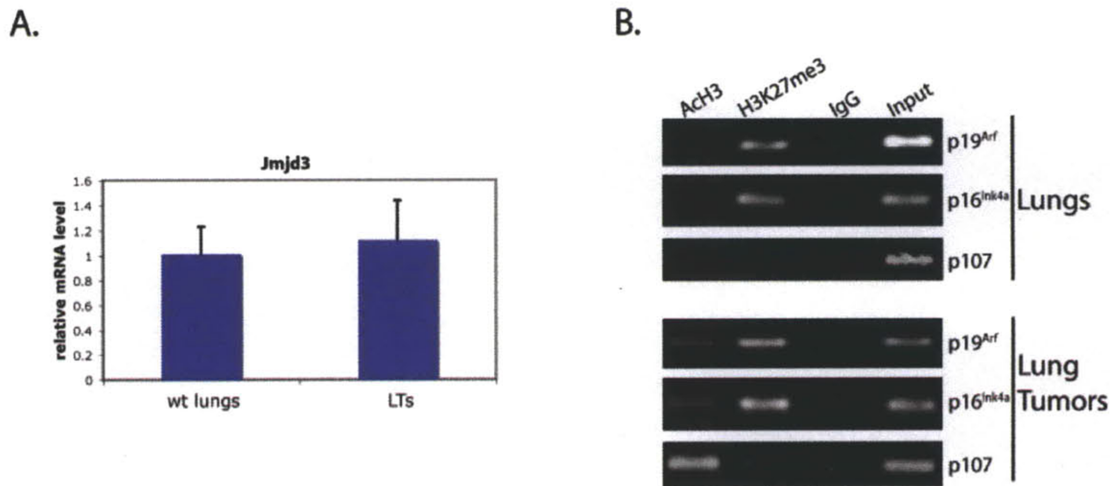

Figure 9: **Similar Jmjd3 levels and PcG enrichment in normal lung and lung tumors.**

(**A.**) qRT-PCR of Jmjd3 from *Arf*$^{GFP/GFP}$ lungs (N = 2) and *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ lung tumors (N = 3). (**B.**) ChIP on *Arf*$^{GFP/GFP}$ lungs ("Lungs") and *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ lung tumors ("Lung tumors". Labels are the same as in Figure 8. Except for the slight increase in AcH3 enrichment in the tumors, normal lung tissue looks very similar to lung tumors in terms of PcG occupancy at *Ink4a/Arf*. The increase in AcH3 at p107 is a mark of actively dividing cells, and is therefore seen in the tumors only.

Bmi-1 actively represses Ink4a/Arf in established lung tumors

To address if the observed histone methylation pattern and binding of Bmi-1 actively repressed $p19^{Arf}$ in established lung tumors, we combined the Arf^{GFP} reporter with a novel system for inducible RNAi *in vivo*. We crossed mice carrying a Flp recombinase-inducible K-ras^{G12D} allele ($K\text{-}ras^{FSF\text{-}G12D}$; see Chapter 3) with those carrying a widely expressed tamoxifen-inducible allele of Cre, $R26^{CreER\text{-}T2}$ (Ventura et al. 2007). To initiate tumors, bifunctional lentiviruses containing Flpo (Raymond and Soriano 2007) were administered intratracheally into these mice. These viruses also contained a Cre-inducible shRNA cassette for expression of shRNAs to luciferase or Bmi-1. 12-16 weeks after tumor initiation, shRNAs were induced by activating CreER via injection with tamoxifen. One week after tamoxifen treatment, lung tumors were harvested and analyzed for Bmi-1 knockdown and GFP expression (see scheme in Fig 10A). As shown in Figure 10B, this system achieved reliable knockdown of Bmi-1, to levels about one-third observed in control hairpin tumors. Importantly, Bmi-1 knockdown tumors displayed increased GFP levels compared to controls (Fig 10C), and $p16^{Ink4a}$ was up-regulated as well (Fig 10D). These data are consistent with a model in which PcG proteins function to maintain a closed chromatin state of *Ink4a/Arf* in lung tumors, leading to partial repression of $p19^{Arf}$ and $p16^{Ink4a}$.

The SWI/SNF family member Snf5 links K-ras^{G12D} activation to p19^{Arf} induction in sarcomas

The presence of the PcG histone mark in sarcomas suggested that this chromatin remodeling complex had some residual function at *Ink4a/Arf*, even in the context of the robust transcriptional activation taking place. As PcG is known to repress this locus in a number of wild-type tissues, it was possible that the observed pattern reflected the activity of PcG in the

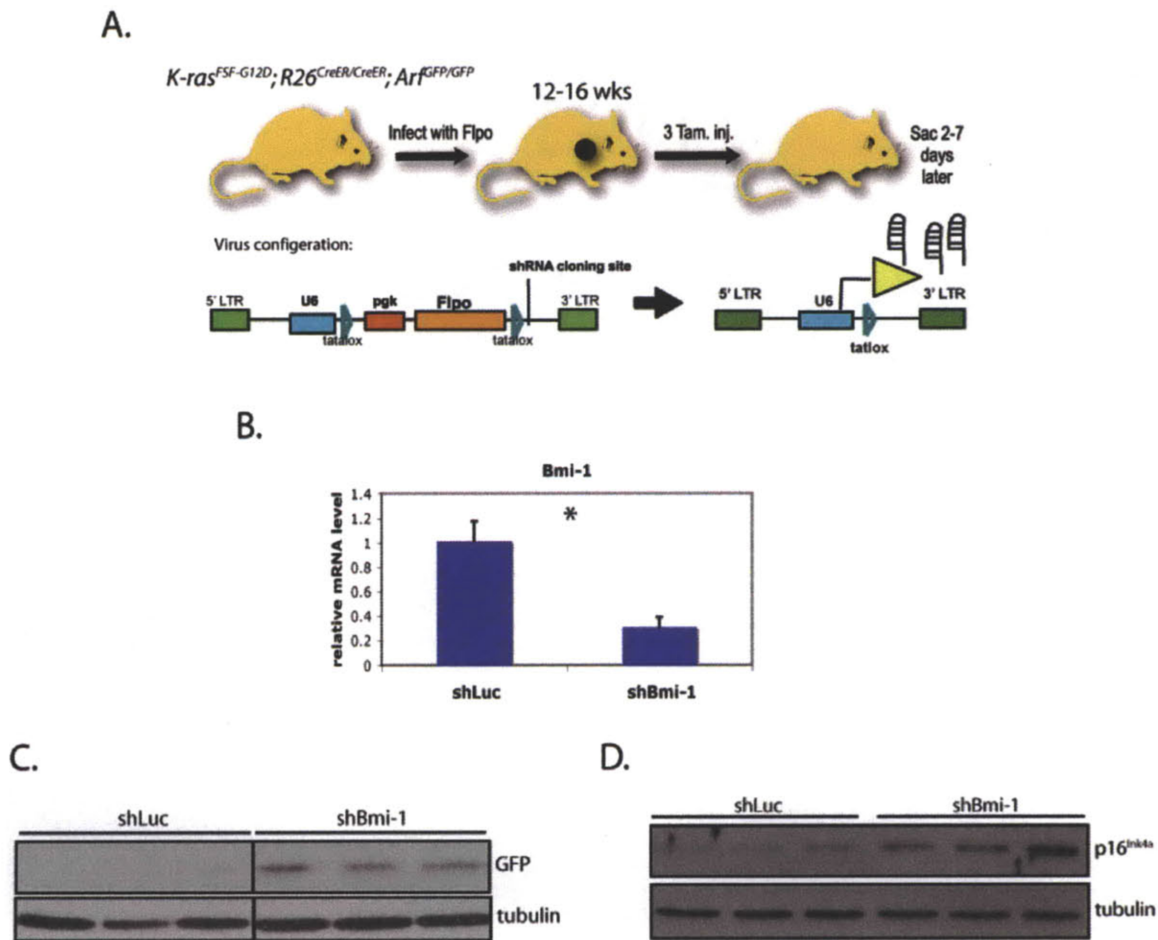

Figure 10: PcG proteins repress the *Ink4a/Arf* locus in established lung tumors.

(**A.**) Schematic of inducible RNAi strategy. See text for details. **(B.)** qRT-PCR of Bmi-1 in tumors in which Luc (shLuc) (N = 6) or Bmi-1 (shBmi-1) (N = 6) shRNAs had been induced. *, $p< .0001$ (Student's T-test) Error bars indicate standard deviation **(C.)** Western blot of GFP in shLuc and shBmi1 tumors. The two sides of the image (separated by a vertical line) are from the same exposure of the same blot, but the samples were not adjacent to each other. (**D.**) $p16^{Ink4a}$ western blot in additional tumors from inducible knockdown experiments.

cell-of-origin of the sarcomas. We hypothesized that following K-ras^{G12D} expression, the locus might get remodeled such that PcG is mostly evicted and replaced by a distinct chromatin structure more conducive to gene activation. Studies in other model systems have implicated the SWI/SNF chromatin remodeling complex as a critical player in this sort of antagonism of PcG-controlled gene repression (Tamkun et al. 1992; Gebuhr et al. 2000). Intriguingly, one member of SWI/SNF, Snf5, has recently been shown to directly bind and activate the *Ink4a/Arf* locus (Kia et al. 2008). Therefore, we investigated the requirement for Snf5 in the activation of p19^{Arf} in K-ras^{G12D}-driven sarcomas. Importantly, Snf5 was detectable by ChIP at *Ink4a/Arf* in multiple *K-ras$^{LSL-G12D}$; Arf$^{GFP/GFP}$* cell lines derived from sarcomas (Fig 11A). Furthermore, acute knockdown of Snf5 in these cell lines resulted in diminished GFP levels, suggesting a functional role for this chromatin modifier in p19^{Arf} regulation (Fig 11B).

To test the significance of these findings *in vivo*, we utilized lentiviral RNAi (Fig 7A). Strikingly, Snf5 knockdown in *K-ras$^{LSL-G12D}$; Arf$^{GFP/+}$; Rag2$^{-/-}$* animals led to reproducible sarcoma formation with stable repression of Snf5 levels in the resulting tumors (Fig 11C, E). Histological analysis revealed very similar histopathologies between Snf5 knockdown tumors and those originating from control infections of *K-ras$^{LSL-G12D}$; Arf$^{GFP/GFP}$; Rag2$^{-/-}$* animals. Both groups had tumors with areas of both large epithelioid cells as well as more spindle-shaped cells (Fig 11D). Importantly, genomic PCR analysis showed retention of the wild-type allele of *Arf*, indicating there was no selective pressure to further inactivate the p19^{Arf}-p53 pathway during tumor progression (Fig 11E). Together, these *in vitro* and *in vivo* studies suggest that Snf5 functions as a critical link between oncogenic K-ras expression and induction of the tumor suppressor pathway controlled by p19^{Arf}.

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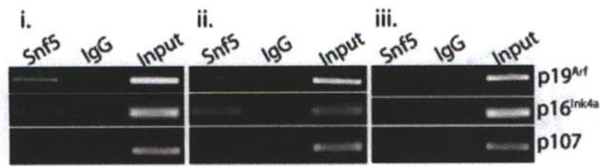

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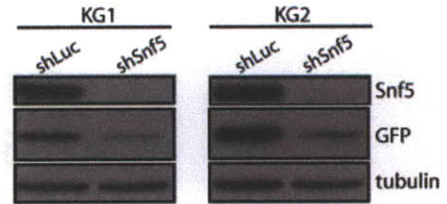

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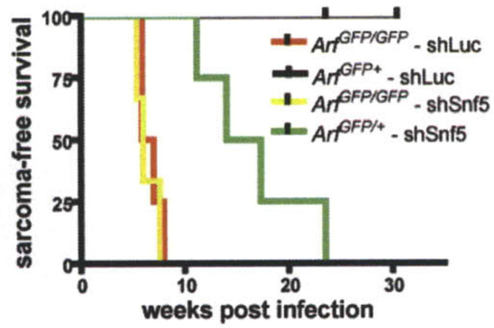

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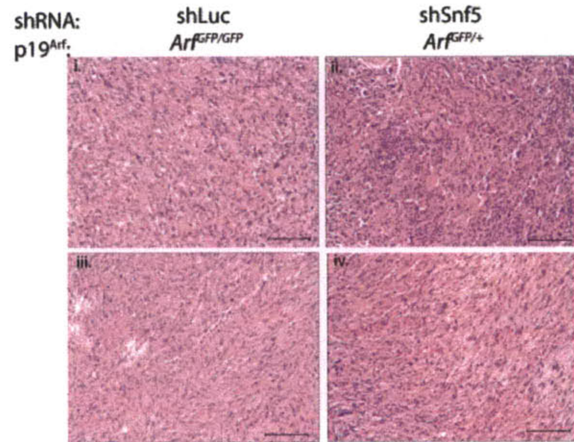

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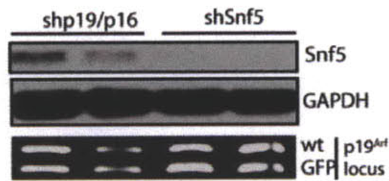


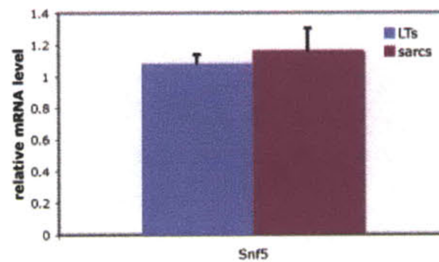
Figure 11: The chromatin remodeler Snf5 contributes to the activation of $p19^{Arf}$ following oncogenic K-ras induction in the muscle.

(A.) Representative ChIP analysis of Snf5 on sarcoma-derived cell lines. **i.** and **ii.** Cell lines derived from *K-ras*$^{LSL\text{-}G12D}$; *Arf*$^{GFP/GFP}$ sarcomas induced with Ade-Cre infection. **iii.** A cell line from a *K-ras*$^{LSL\text{-}G12D}$; *Arf*$^{GFP/GFP}$; *Rag2*$^{-/-}$ animal infected with a Cre-shSnf5 lentivirus. Snf5 enrichment at *Ink4a/Arf* is absent in the knockdown cell lines. The p107 locus serves as a negative control for Snf5 binding. **(B.)** Immunoblot analysis following *in vitro* knockdown of Snf5 in cell lines **i.** and **ii.** from **A.** **(C.)** Kaplan-Meier graph of sarcoma-free survival for a cohort of *K-ras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/+}$(black and green lines) or *Arf*$^{GFP/GFP}$ (red and yellow lines) mice infected intramuscularly with Cre-shLuc (red and black lines) or Cre-shSnf5 (yellow and green lines) lentiviruses. **(D.)** HE pictures of sarcomas from lenti-Cre; shLuc infected *K-ras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/GFP}$ (**i.** and **iii**) or lenti-Cre; shSnf5 infected *K-ras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/+}$ (**ii.** and **iv**) animals. Panels **i.** and **ii.** show regions containing, circular epithelioid cells while **iii.** and **iv.** are from areas comprised of more spindle-shaped cells. Scale bar is 200 μm. **(E.)** Western blot and genomic PCR analysis on a panel of sarcomas from *K-ras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/+}$ animals derived from lentiviruses expressing Cre and hairpins to Snf5 or $p19^{Arf}/p16^{Ink4a}$.

Snf5 expression is similar between lung tumors and sarcomas

The important functional role of Snf5 in K-ras^{G12D}-dependent p19^{Arf} induction in the muscle prompted us to investigate whether differential expression of this chromatin remodeler could explain the tissue-specific levels of p19^{Arf} in lung tumors and sarcomas. To this end, we assessed Snf5 levels at both the mRNA and protein levels in a panel of *K-ras*^{LA2}*; Arf*$^{GFP/GFP}$ lung tumors and sarcomas. Interestingly, both analyses revealed a very similar expression pattern between the tumor types (Fig 12), arguing against tumor type-specific Snf5 levels as the mechanism behind differential p19^{Arf} expression.

A.



B.

Figure 12: **Similar Snf5 expression in lung tumors and sarcomas**

qRT-PCR (**A.**) and western blot (**B.**) analyses of Snf5 in lung tumors and sarcomas from *K-ras*^{LA2}; *Arf*$^{GFP/GFP}$ mice. Snf5 expression is equivalent at both the mRNA and protein level. N = 3 for each tumor type, and error bars indicate standard deviation in **A.**

DISCUSSION

The ability of oncogenic signaling to directly engage tumor suppressor pathways prevents the outgrowth of incipient tumor cells harboring initiating oncogenic mutations. Any variability in the nature or strength of any such tumor suppressor induction could have a profound influence on the potential tumorigenicity of mutated cells. While a number of earlier studies have shown that oncogene levels play a critical role in determining whether cells become transformed or arrested, they have generally relied on experimentally manipulating the oncogene expression in a given cell type. In the present study, we identified naturally occurring variability in the degree of $p19^{Arf}$ and $p16^{Ink4a}$ induction following endogenous expression of K-ras^{G12D} in different cell types. Lung tumors display relatively low levels of these tumor suppressors, and this correlates with the ability of these cells to start the tumorigenic process with K-ras mutation alone. Introducing a $p19^{Arf}$ mutant allele into these models moderately affects the tumor phenotype, and the loss of this pathway is still most likely a requisite event for development of advanced tumors (Jackson et al. 2005). In contrast, sarcomas originating from K-ras^{G12D}-mutant cells robustly up-regulate the *Ink4a/Arf* locus. This strong induction effectively blocks the transformation of K-ras^{G12D}-expressing muscle cells, such that the $p19^{Arf}$-p53 pathway must be completely abolished for tumor initiation to occur at all. Thus, it appears that resistance to oncogenic K-ras directly correlates with the strength of tumor suppressor induction.

As discussed above, one of the major determinants of oncogene-induced tumor suppression is thought to be the relative expression level of the oncogene. This does not appear to be the explanation for the cell-type specific responses described here. A comparative analysis of K-ras expression as well as downstream signaling pathways failed to identify significant differences between established lung tumors and sarcomas. Instead, we found that dynamic

chromatin regulation plays an important role in tissue-specific *Ink4a/Arf* regulation. For example, we provide evidence for active repression of the locus in established lung tumors mediated by PcG. ChIP analysis indicated a strong local enrichment of PcG proteins and histone marks in lung tumors compared with sarcomas. The significant PcG occupancy in lung tumors could be due to the fact that Jmjd3, a H3K27me3 demethylase implicated in Ras-induced *Ink4a/Arf* activation, is not elevated in lung tumors compared to normal lung tissue. Importantly, knockdown of Bmi-1 in established lung tumors using inducible RNAi *in vivo* confirmed PcG-mediated gene repression. This active silencing of the *Ink4a/Arf* locus most likely contributes to the observed low levels of $p19^{Arf}$ and $p16^{Ink4a}$ and relative susceptibility of lung cells to transformation by oncogenic K-ras. These results are in agreement with a previous study that investigated lung tumor formation in Bmi-1-/- mice (Dovey et al. 2008), although we also demonstrate a role for PcG in established lung tumors.

The observation that sarcomas maintained some PcG-associated marks at *Ink4a/Arf* suggested that perhaps the initiating cell did repress the locus, but then further remodeled the local chromatin to allow for robust transcription in response to K-ras^{G12D}. As SWI/SNF remodeling complexes have been shown to be important for induction of PcG-regulated genes, we examined this class of genes as a possible link between oncogenic K-ras activation and *Ink4a/Arf* induction. Knockdown of Snf5 led to efficient sarcoma formation in a background normally completely resistant to tumor formation owing to robust $p19^{Arf}$-p53 pathway activation. Importantly, additional studies *in vitro* involving ChIP and acute knockdown suggested that Snf5 directly binds to and activates the *Ink4a/Arf* locus. Thus, in the muscle SWI/SNF complexes play a critical role in oncogene-induced tumor suppression.

One current model to explain tissue-specific $p19^{Arf}/p16^{Ink4a}$ induction presumes that under normal conditions PcG represses *Ink4a/Arf* in the cell-of-origin of both lung tumors and sarcomas. However, following oncogene activation only lung tumors retain functional PcG repression. One possibility to account for this tissue-specific difference is that Snf5's main role in sarcomagenesis is to evict PcG from *Ink4a/Arf* in sarcomas, as it does in malignant rhabdoid tumors (Kia et al. 2008). It would appear that a similar effect does not occur in the cell-of-origin of lung tumors. While the expression level of Snf5 is similar between the two tumor types, it is still unclear if tissue-specific activity or localization of SWI/SNF complexes could be the explanation for this difference. Another factor mediating PcG loss from chromatin is enhanced p38 MAPK activity (Voncken et al. 2005; Wong et al. 2009). However, we have been unable to observe differences in the activation status of this pathway between the two tumors types.

An alternative model for the different levels of PcG regulation in the established tumors is that they represent the relative amount or activity state in the respective cells-of-origin. Having a lower degree of PcG-bound chromatin initially could reduce the requirements for gene activation in the muscle following oncogenic K-ras induction. Such a scenario has been suggested to explain the cell-type specific requirements for $p19^{Arf}$ loss in the transformation of progenitor versus more differentiated B and T-cells in the hematopoietic system, where PcG is thought to repress *Ink4a/Arf* mainly in stem cells (Williams and Sherr 2008). The precise identity of the respective cell-of-origins for lung tumors and sarcomas is currently unknown, thus precluding a meaningful analysis of PcG recruitment pre-K-ras activation.

Cell-type specificity in the activation threshold for the *Ink4a/Arf* locus might relate to the basal proliferative rate of the tissues in question. The lung epithelium constantly receives damage from irritants in the environment, thus requiring significant repair in the form of cellular

regeneration. To allow for this constant potential for repair, perhaps certain cells in the lung stably silence $p19^{Arf}$ and $p16^{Ink4a}$ via PcG. In the context of oncogenic K-ras expression, the low level of expression at the locus allows cells to proliferate and form tumors. In contrast, muscles require minimal proliferative capacity and, therefore, the *Ink4a/Arf* locus is kept in a chromatin state that allows for easier engagement of the $p19^{Arf}$ and $p16^{Ink4a}$ tumor suppressor pathways. If these cells acquire an oncogenic Ras mutation, they robustly induce $p19^{Arf}$ and $p16^{Ink4a}$ and effectively block tumor formation.

Lung tumors, as well as a variety of other epithelial cancers that originate from cells with a relatively high turn-over rate, are much more common than soft-tissue sarcomas. The inherent differences in oncogene-induced tumor suppression across cell types could be the mechanistic basis for these observations. In addition, the fact that early lesions in some tissues have key tumor suppressor pathways intact might have profound clinical implications if such anti-growth and pro-cell death pathways could be mobilized therapeutically.

MATERIALS AND METHODS

Mice. *Arf*^{GFP} mice were kindly provided by C. Sherr (St. Jude Children's Hospital, Memphis, TN), *Trp53*^{flox} were provided by A. Berns (The Netherlands Cancer Institute, Amsterdam, the Netherlands), and *Rag2*$^{-}$ mice were purchased from The Jackson Laboratory. *K-ras*^{LA2}, *K-ras*$^{LSL-G12D}$, *R26*^{CreER}, and *K-ras*$^{FSF-G12D}$ mice were generated in our laboratory. All animals were maintained on a mixed background comprising 129S4/SvJae and C57BL/6 strains. Lung tumors in *K-ras*$^{LSL-G12D}$ and *K-ras*$^{FSF-G12D}$ were induced by intratracheal instillation of either Ad-Cre or Lenti-Flpo as previously described (DuPage et al. 2009). To induce sarcomas mice were infected intramuscularly with either Ad-Cre or Lenti-Cre as shown previously (Kirsch et al. 2007). Tamoxifen (Sigma) was dissolved in corn-oil at 15 mg/ml and injected intraperitoneally every other day for 5 days. For lung tumor studies, *K-ras*^{LA2}*; Arf*^{GFP} compound mutant mice were sacrificed at 6 or 12 weeks of age, and their lungs were processed as previously described (Jackson et al. 2001). Bioquant Image Analysis was used to quantitate tumor burden. Aged cohorts of *K-ras*^{LA2}*; Arf*^{GFP} mice as well as those used for sarcoma generation were monitored for visible masses or until they became moribund. Masses were processed similarly to the lungs. Animal studies were approved by Massachusetts Institute of Technology's (MIT) Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals, and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory Animals (institutional animal welfare assurance number, A-3125-01).

Senescence-associated β-galactosidase assay. At necropsy, lungs were inflated with 70% OCT (in PBS), then allowed to equilibrate in 100% OCT on ice for 15 minutes. Individual lobes were placed in cassettes and frozen in OCT. 10-15 μm sections were cut, and the resulting slides were fixed in .5% glutaraldehyde for 15 minutes, followed by two washes in PBS. Staining was performed at 37°C for 12 hours in PBS (pH 5.5) with 5mM each of potassium ferrocyanide and potassium ferricyanide along with 1 mM $MgCl_2$ and 1 mg/ml X-gal. Sections were counterstained in nuclear fast red.

Cell lines and *in vitro* experiments. Cell lines were generated from sarcomas by mincing freshly extracted tissue with a razor blade followed by digesting with trypsin for 15 minutes at 37° C. Dissociated tissue was resuspended in DMEM (DME, 10% FBS, 2 mM glutamine) and resulting cell lines were subsequently passaged in this media. For lentivirus experiments target cells were selected in 5 ug/mL of puromyocin for 3 days following supernatant transfer. 2-4 days later cells were collected for analysis. 293T cells for virus production were grown in DMEM.

Lentiviral vectors and shRNA cloning. *In vitro* knockdown studies utilized a modified version of pSICO-Puro (Ventura et al. 2004), Puro- sh2.0, in which both 5' and 3' tata-lox sites were removed from pSICO-Puro, replacing the 5' site with a U6-shRNA cassette. For sarcoma induction in $K\text{-}ras^{LSL\text{-}G12D}$ mice a lentivirus containing pgkCre and a U6-shRNA cassette was used (a gift from M. Kumar and K. Lane, Massachusetts Institute of Technology KI, Cambridge, MA). Lung tumor formation in $K\text{-}ras^{FSF\text{-}G12D}$ relied on pSICO-Flpo, which was generated by amplifying pgkFlpo from pgkFlpobpA (Addgene) and cloning it into pSICO-Puro that had been digested previously to remove pgkPuro. Cloning details are available upon request. Target

sequences for shRNA knockdown were identified using pSICO Oligomaker V 1.5 (A. Ventura, Memorial Sloan Kettering Cancer Center, New York, NY). Cloning of DNA oligos into the U6-shRNA cassette in the above vectors was done as described previously (Ventura et al. 2004).

shRNA sequences:

Gene:	Target sequence:
Luciferase	GAGCTGTTTCTGAGGAGCC
$p19^{Arf}/p16^{Ink4a}$	GCTGGGTGGTCTTTGTGTA
Bmi-1	GTGATGACCTGGATTTGAA
Snf5	GGAAGAGGTGAATGATAAA

Lentivirus production and knockdown studies. For *in vitro* experiments 8.5 x 10^5 293T cells were plated onto 6 cm plates and the viral vector, HIV-1 packaging vector Δ8.2, and VSV-G encoding vector were co-transfected using Mirus transfection reagent (Mirus Bio LLC). 48 hours later the supernatant was filtered, supplemented with polybrene (to 10 μg/ml), and placed directly onto the target cells. To prepare virus for *in vivo* infections, 7.5 x 10^6 293T cells were seeded onto 15 cm plates, and the amounts of transfection reagent and plasmids were scaled up accordingly. Viral supernatants were collected and filtered at 48 and 72 hours post-transfection, and pooled collections were spun at 25,000 rpm for 1.5 hr at 4°C in an ultracentrifuge. Viral pellets were resuspended in 1X HBSS pH 7.4. 50-100 μl was administered either intratracheally or intramuscularly.

Protein extraction and immunoblots. Cell lines were lysed in RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Tx-100, .1% SDS, .5% sodium deoxycholate, 1 mM DTT) plus mini complete protease inhibitors (Roche) and phosphatase inhibitors (cocktails 1 and 2) (Sigma) for 10 minutes on ice. Snap-frozen tissue was finely minced with a razor blade on ice in TNE buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, supplemented with 1% Tx-100,

.1%SDS, 1mM DTT, and the same inhibitors mentioned above) and then rotated for 15 minutes at 4°C. Both *in vitro* and *in vivo* samples were centrifuged to remove insolubles and quantitated using a Bradford Assay (Bio-rad). Samples were then diluted in loading buffer and separated on 10-15% SDS-PAGE gels. Following transfer to PVDF membranes, the following antibodies were used: β-tubulin (#2146), Erk 1/2 (#9102), phospho-Erk 1/2 (#9101), Akt (#9272), phospho-Akt473 (#9271), cJun (#9162), phospho-cJun (#9261), phospho-p38 (#9211), p38 (#9212) (all from Cell Signaling Technology); actin (sc-1616), $p16^{Ink4a}$ (sc-1207), $p19^{Arf}$ (sc-32748), $p27^{Kip}$ (sc-528), Runx2 (sc-10758), K-ras (sc-30) (all from Santa Cruz Biotechnology); Snf5 (ab12167-Abcam); Hsp90 (610418-BD Biosciences); GAPDH (MAB374-Chemicon International); and GFP (NB 600-303-Novus Biologicals). HRP-conjugated secondary antibodies were used in conjunction with ECL+ detection systems (Amersham). Levels of Ras-GTP were determined with the Ras activation kit (Millipore).

mRNA isolation and qRT-PCR analysis. For some experiments, RNA was extracted using RNeasy kits (Qiagen). Briefly, tumor tissue was minced with razor blades and further homogenized using Qiashredder columns (Qiagen) before continuing with the manufacturer's instructions. In other experiments tumor tissue was homogenized with a dounce homogenizer in Trizol (Gibco), followed by RNA extraction according to the manufacturer's instructions. Once RNA was isolated, cDNA synthesis was performed on 1 μg of RNA using oligo dT primers and Superscript III (Invitrogen). cDNAs were analyzed by qPCR using either SYBR Green or Taqman detection systems in an ABI PRISM 7000 Sequence Detection System Thermo Cycler (Applied Biosystems). Relative mRNA levels were calculated using cycle threshold difference (ΔC_T) to control mRNAs (β-actin for SYBR Green and TBP for Taqman). In some instances normal lung tissue served as an additional baseline ($\Delta\Delta C_T$ method).

Primers for SYBR Green:

Locus:	Forward primer:	Reverse primer:
$p19^{Arf}$	GCCGCACCGGAATCCT	TTGAGCAGAAGAGCTGCTACGT
$p16^{Ink4a}$	AACTCTTTCGGTCGTACCCC	GCGTGCTTGAGCTGAAGCTA
Dec1	GGCGGGGAATAAAACGGAGCGA	CCTCACGGGCACAAGTCTGGAA
DcR2	AGCTAACCCAGCCCATAATCGTC	AGTTCCCTTCTGACAGGTACTGGC
β-actin	GGCACCACACCTTCTACAATG	GTGGTGGTGAAGCTGTAGCC

Gene:	Taqman probe:
E2F1	Mm00432936_m1
E2F3	Mm01138833_m1
Bmi-1	Mm00776122_gH
Ezh2	Mm00468449_m1
DMP1	Mm00516203_g1
TBP	Mm00446973_m1
Snf5	Mm00448776_m1
Jmjd3	Mm01332680_m1

PCR analysis on tumor DNA: Lung tumor DNA was prepared by Laser Capture Microscopy (LCM) as described previously (Gidekel Friedlander et al. 2009), while sarcoma DNA was prepared according to standard procedures. Both samples were then subjected to standard PCR analysis. PCR primers were as follows: Arf-1: AGTACAGCAGCGGGAGCATGG; Arf-2: TTGAGGAGGACCGTGAAGCCG; Neo-2: ACCACACTGCTCGACATTGGG.

Chromatin Immunoprecipiation (ChIP). For the *in vivo* ChIP, tumor tissue was cut up with razor blades in PBS, formaldehyde was added to 1%, and samples were incubated for 15 minutes at RT. Cross-linking was stopped by incubating with .125M glycine for 5 minutes, samples were washed once with cold PBS, pellets were resuspended in cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCL, .5% Igepal), homogenized in a dounce homogenizer, and incubated at 4°C for 10 minutes. After centrifugation pellets were resuspended in nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS) for 15 minutes on ice. Sonication was then done in a Branson 250

Sonifier to the appropriate shear length, debris was removed by centrifugation, and sheared chromatin was diluted 6X in Dilution Buffer (16.7 Tris, pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 1.1% Tx-100, .01% SDS) before incubating with Protein A beads (Sigma) to pre-clear. Samples were evenly split for overnight immunoprecipitations with 1-3 μg of the appropriate antibodies: Histone H3 tri-methyl K27 (ab6002-Abcam), Histone H3 tri-methyl K4 (04-745-Millipore), acetylated histone H3, (06-599-Millipore), Bmi-1 (supernatant provided by J. Lees, MIT KI, Cambridge, MA), and control IgGs (Santa Cruz). Inputs were taken from IgG samples before addition of Protein A beads the following day. Following incubation with beads for 1 hr at 4°C, beads were washed twice with Low Salt Immune Complex Wash Buffer (20 mM Tris, pH 8.1, 150 mM NaCl, 2mM EDTA, 1% Tx-100, .1% SDS), twice with LiCl Immune Complex Wash Buffer (10 mM Tris, pH 8.1, 1mM EDTA, 1% NP-40, 1% Na deoxycholate, .25 M LiCl), and twice with 1X TE, pH 8. DNA was eluted in 1X TE, 1% SDS, 150 mM NaCl, 5 mM DTT at 65°C, and cross-links were reversed overnight at 65°C. Proteinase K was added and samples were incubated at 55°C for 2hrs before purifying DNA with Qiagen PCR purification columns. For *in vitro* ChIP analyses, cells in 15 cm plates were fixed in 1% formaldehyde for 10 min at RT, followed by quenching in .125M glycine. Samples were washed twice in ice-cold PBS and lysed directly in nuclear lysis buffer before continuing with the protocol above. Snf5 (A301-087A-1-Bethyl Laboratories) was used for the indicated IPs.

Primers used for ChIP analysis:

Locus:	Forward primer:	Reverse primer:
$p19^{Arf}$	AAAGGGCGCAGCTACTGCTA	TCTTTGCTCCACGCCCATCT
$p16^{Ink4a}$	TTAGCGCTGTTTCAACGCCC	GCCACACTCTGCTCCTGACCT
p107	TTAGAGTCCGAGGTCCATCTTCT	GGGCTCGTCCTCGAACATATCC
cdc2	ACAGAGCTCAAGAGTCAGTTGGC	CGCCAATCCGATTGCACGTAGA

ACKNOWLEDGEMENTS

We are grateful to C. Sherr and F. Zindy for the generous gift of the Arf^{GFP} mice. We would also like to thank D. Crowley for histological preparations, R. Bronson and C. Fletcher for histopathological analyses, P. Iaquinta, B. Berstein, M. Ku, and T. Thruong for technical assistance, and D. Feldser and A. Cheung for critical reading of the manuscript. This work was supported in part y grant **5-U01-CA84306** from the National Institutes of Health, and partially by Cancer Center Support (core) grant P30-CA14051 from the National Cancer Institute. T.J. is a Howard Hughes Medical Institute Investigator and a Daniel K. Ludwig Scholar.

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CHAPTER 3

A new system for sequential mutagenesis reveals the importance of early p53 mutation in K-ras^{G12D}-driven sarcomagenesis

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Nathan Young performed all experiments in the laboratory of Tyler Jacks.

ABSTRACT

The development of cancer is a multi-step process largely driven by the accumulation of mutations in both oncogenes and tumor suppressors. While these alterations are thought to occur in a sequential manner, whether the specific order of events is important for tumor initiation and/or progression remains unknown. Here, we describe a mouse model for sequential mutagenesis that utilizes both Flp-Frt and Cre-LoxP recombination systems. By integrating a novel Flp-inducible allele of oncogenic K-ras with established methods for Cre-mediated p53 deletion, we are able to separately regulate these two commonly associated cancer genes *in vitro* and *in vivo*. Using this approach, we show that efficient tumor formation in a mouse model of soft-tissue sarcoma driven by K-ras^{G12D} activation and p53 loss is highly dependent on the order of these events. Interestingly, delaying p53 deletion relative to oncogenic K-ras induction reduces tumor burden, suggesting that p53 strongly inhibits very early steps in K-ras-dependent transformation in the muscle. Furthermore, using *in vivo* RNAi we implicate the p53 target gene p21 as a critical mediator in this process, highlighting cell-cycle arrest as an extremely potent tumor suppressor mechanism in muscle cells.

INTRODUCTION

Tumorigenesis is a multi-step process driven by the accumulation of both genetic and epigenetic alterations in oncogenes and tumor suppressor genes (Hanahan and Weinberg 2000; Vogelstein and Kinzler 2004). These individual changes occur in a sequential manner and are thought to drive distinct steps in the progression of normal cells to full malignancy (Kinzler and Vogelstein 1996). Although it is generally believed that the actions of these mutations combine to effect full transformation, it remains unclear if the order of events is important in this process. In addition, how specific oncogenic events dictate the transition from early to intermediate to late-stage disease states is largely unknown.

While mutations in the tumor suppressor gene *p53* comprise one of the most common occurrences in human cancer, the relative timing of these alterations seems to vary across tumor types (Baker et al. 1990; Blondal and Benchimol 1994; Greenblatt et al. 1994). In many cancers, such as those of the lung, colon, and pancreas, *p53* alterations have been documented in more advanced stages of tumor development, suggesting that p53 constrains progression of established tumors (Baker et al. 1990; Kohno et al. 1999; Yamasaki et al. 2000; Hezel et al. 2006). In contrast, the early onset of several cancer types in Li-Fraumeni patients, who inherit a germ-line mutation in *p53*, argues for a potential role of this tumor suppressor in inhibiting the first steps of transformation in some cell types (Kleihues et al. 1997). While these studies clearly illustrate that p53 is mutated at particular stages of tumor development, whether these differences underlie specific temporal requirements for p53 loss in distinct tissues remains to be determined.

p53's central role in tumor suppression derives from its ability to respond to oncogenic stress by inducing cell cycle arrest or apoptosis (Lowe et al. 2004; Zilfou and Lowe 2009). For

example, heterologous expression of oncogenic Ras leads to p53 stabilization and the induction of a permanent cell cycle arrest termed senescence (Serrano et al. 1997; Collado et al. 2005). Presumably, such a strong p53 response to oncogene Ras expression would block any incipient tumor cell growth and thus require a pre-exisitng mutation in p53 for transformation. Indeed, using a Cre-inducible allele of oncogenic K-ras ($K\text{-}ras^{LSL\text{-}G12D}$) in a mouse model of soft-tissue sarcoma, we demonstrated that tumor formation occurred only if accompanied by the simultaneous loss of p53 or its upstream activator $p19^{Arf}$ (Kirsch et al. 2007). However, many other cell types and tissues that express oncogenic K-ras at physiological levels are able to hyper-proliferate without p53 pathway abrogation, progressing to early or intermediate stages of tumor development (Tuveson et al. 2004; Collado et al. 2005). Therefore, both in humans and mice, p53 appears to play tissue-specific tumor suppressive roles. In some settings it inhibits the earliest stages of tumor initiation, whereas in other cases it slows the progression of established lesions. It is possible that depending on it's stage-specific function, the order and/or timing of *p53* mutations relative to other oncogenic events, such as $K\text{-}ras^{G12D}$ activation, might be an important determinant of tumor development in distinct cellular contexts.

Despite many advances in genetically engineered mouse models of cancer, most current models that involve multiple cancer-associated mutations are not designed to perform sequential mutagenesis and thus cannot directly test the importance of mutation timing or order. For example, the use of the conditional Cre-LoxP site-specific recombinase (SSR) system allows precise control of multiple gene activation or inactivation events, but these alterations occur simultaneously at the time of tumor initiation (Frese and Tuveson 2007). Additional SSR modalities, such as *S. cerivisae*-derived Flp-Frt, exist but have been used in a more limited fashion in mouse models (Vooijs et al. 1998; Awatramani et al. 2001). However, recent

improvements have increased its recombination efficiency in mammalian systems (Raymond and Soriano 2007). By combining different SSR systems within the same model one could achieve spatiotemporal control of distinct genetic events provided the recombinases are independently regulated (Branda and Dymecki 2004).

To establish a system for sequentially mutating the commonly-associated cancer genes *K-ras* and *p53*, we have developed a Flp-inducible allele of oncogenic K-ras expressed from its endogenous promoter. By combining this allele with an already well-established Cre-regulated p53 deletion allele (Jonkers et al. 2001), we could separately regulate these mutations, both in cells and in mice. Using this dual SSR strategy, we uncover new insights into the nature of p53-mediated tumor suppression by determining the temporal requirements of *K-ras* and *p53* mutation necessary for the development of soft-tissue sarcoma. We provide evidence that oncogenic K-ras rapidly induces robust p53 activity that limits the capacity of mutated cells to respond to subsequent p53 loss. Furthermore, using RNAi we show that the p53 target gene p21 is important for this response, implicating p53's canonical role in mediating cell cycle as a potent tumor suppression mechanism *in vivo*. Our data suggest that the particular order of two commonly-associated mutations can affect their ability to promote tumorigenesis.

RESULTS

Generation and Initial Characterization of K-ras$^{FSF-G12D}$ allele

In order to create an *in vivo* sequential mutagenesis model, we set out to combine the Flp-frt mediated mutagenesis system with already available tools from Cre-loxP systems. To this end, we first generated a Flp-recombinase inducible allele of oncogenic K-ras (*K-ras$^{FSF-G12D}$*), following a very similar strategy used to construct the well-studied Cre-inducible *K-ras$^{LSL-G12D}$* allele (Tuveson et al. 2004). Specifically, we targeted the endogenous locus of *Kras2* in ES cells

with a construct containing a transcriptional stop cassette flanked by Frt sites in intron 1 and an oncogenic point mutation in codon 12, such that expression of Flp would remove the stop cassette and permit expression of oncogenic K-ras at endogenous levels (Fig 1A). Southern blot analysis of ES cells identified correctly targeted clones (Fig 1B), one of which was used for chimera generation and subsequent germline transmission. To characterize the activity of this allele, we first generated mouse embryonic fibroblasts (MEFs) from $K\text{-}ras^{FSF\text{-}G12D/+}$ embryos. Introduction of an engineered thermostable version of Flp (Flpe) (Buchholz et al. 1998) but not Cre led to removal of the stop cassette, as demonstrated by PCR analysis (Fig 1C). In addition, Flpe expression in $K\text{-}ras^{FSF\text{-}G12D}$ MEFs induced high levels of active Ras-GTP, along with appropriate downstream signaling events, such as up-regulation of cyclin D1 (Fig 1D). Various functional attributes of MEFs expressing endogenous levels of K-ras^{G12D} have been reported from work with the similar Cre-inducible $K\text{-}ras^{LSL\text{-}G12D}$ allele. These include a spindle-like morphology and the ability to form colonies in soft agar when combined with *p53* deficiency (Tuveson et al. 2004). We were able to reproduce these phenotypes with the $K\text{-}ras^{FSF\text{-}G12D/+}$ MEFs in a Flpe-dependent manner (data not shown).

We next tested the activity of the $K\text{-}ras^{FSF\text{-}G12D}$ allele *in vivo*, which was of particular importance given the evidence that Flpe is much less efficient than Cre in mammalian systems (Raymond and Soriano 2007). While introduction of adenoviruses or lentiviruses expressing Cre (Ad-Cre or LV-Cre) into the lungs of $K\text{-}ras^{LSL\text{-}G12D}$ mice results in significant lung tumor formation (Jackson et al. 2001; DuPage et al. 2009), infection with Ade-Flpe and LV-Flpe failed to generate lung tumors in $K\text{-}ras^{FSF\text{-}G12D}$ animals (data not shown). However, using a mammalian codon-optimized version of Flp, termed Flpo (Raymond and Soriano 2007), we were able to initiate numerous lung tumors in $K\text{-}ras^{FSF\text{-}G12D}$ mice with intratracheal instillation of Ad-Flpo

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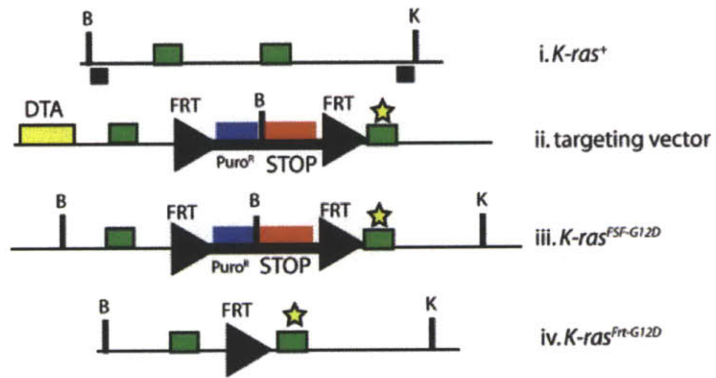

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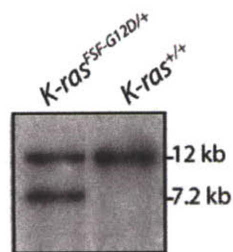

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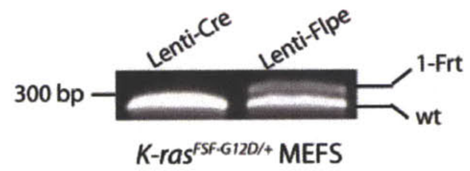

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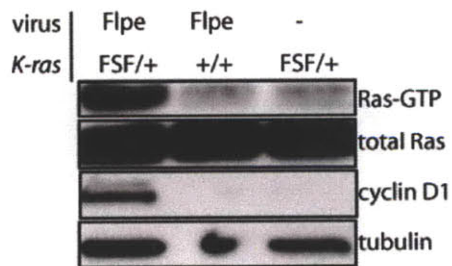

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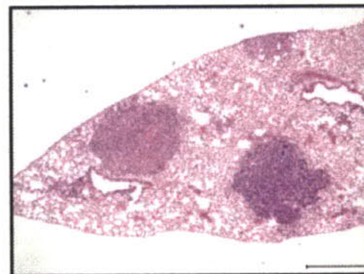


Figure 1: Construction and initial characterization of *K-ras*$^{FSF\text{-}G12D}$

A. Schematic of the targeting strategy for *K-ras*$^{FSF\text{-}G12D}$, including the **(i.)** endogenous *K-ras* locus, **(ii.)** targeting vector, **(iii.)** correctly targeted *K-ras*$^{FSF\text{-}G12D}$ locus, and **(iv.)** recombined *K-ras*$^{Frt\text{-}G12D}$ following Flp expression. Green boxes = exons; thick black bars = probes for Southern blot; star = oncogenic point mutation G12D; B = BamHI; K = KpnI. **B.** Southern blot of non-targeted (*K-ras*$^{+/+}$) and correctly targeted (*K-ras*$^{FSF\text{-}G12D/+}$) ES cell clones following BamHI/KpnI double digestion and hybridization with an external 5' probe. **C.** PCR analysis of genomic DNA isolated from *K-ras*$^{FSF\text{-}G12D/+}$ MEFs infected with lentiviruses expressing Cre (Lenti-Cre) or Flpe (Lenti-Flpe). **D.** Western blot of *K-ras*$^{+/+}$ and *K-ras*$^{FSF\text{-}G12D/+}$ MEFs either uninfected or infected with Lenti-Flpe showing up-regulation of Ras-GTP and cyclin D1, two hallmarks of Ras activation, in *K-ras*$^{FSF\text{-}G12D/+}$ MEFs in a Flpe-dependent manner. **E.** Representative hematoxylin and eosin (H&E)-stained section of a lung lobe from a *K-ras*$^{FSF\text{-}G12D/+}$ mouse 16 weeks post Ad-Flpo infection. Multiple lung tumors are evident. 4X magnification.

and LV-Flpo (Fig 1E and data not shown). Together, these data indicate that *K-ras*$^{FSF\text{-}G12D}$ is the functional equivalent of the *K-ras*$^{LSL\text{-}G12D}$ allele.

Sequential mutagenesis of K-ras and p53 in MEFs in vitro and in vivo

Given the well-known genetic interaction between K-ras and p53 in cellular transformation, we chose to combine mutations in these two genes for initial sequential mutagenesis experiments in MEFs. Previous work has demonstrated that while primary MEFs expressing endogenous K-ras^{G12D} have some characteristics of transformation, they are not fully transformed and require *p53* deletion to form tumors in immunocompromised mice (Tuveson et al. 2004). With the ability to separate *K-ras* and *p53* mutations in time, we set out to examine the consequences of delayed *p53* mutation in K-ras^{G12D}-expressing cells following transplantation into immunocompromised mice.

We constructed MEFs containing *K-ras*$^{FSF\text{-}G12D}$ and *Trp53*^{flox} alleles, as well as the *R26*$^{CreER\text{-}T2}$ allele, a tamoxifen-regulated version of Cre knocked into the ubiquitously expressed *Rosa26* locus (Ventura et al. 2007). Compound mutant MEFs of the following genotypes were used in these experiments: *K-ras*$^{FSF\text{-}G12D}$*; R26*$^{CreER\text{-}T2/+}$*; Trp53*$^{flox/flox}$ (FKCP) and *K-ras*$^{FSF\text{-}G12D}$*; R26*$^{CreER\text{-}T2/+}$*; Trp53*$^{flox/+}$ (FKC). Infection of these cells with Ad-Flpo led to activation of the *K-ras*$^{FSF\text{-}G12D}$ allele *in vitro* (designated FK*). FK*CP cells were injected subcutaneously into nu/nu mice and *p53* deletion was induced by i.p. tamoxifen injection at different time points thereafter. As a positive control, we included cells that had been treated with 4-hydroxytamoxifen (4-OHT) to inactivate p53 one week prior to injection.

As shown in Figure 2A, when mice injected with FK*CP cells started receiving tamoxifen on the same day of injection, efficient tumor formation was observed, similar to the positive control group. As expected, the resulting tumors showed complete recombination of

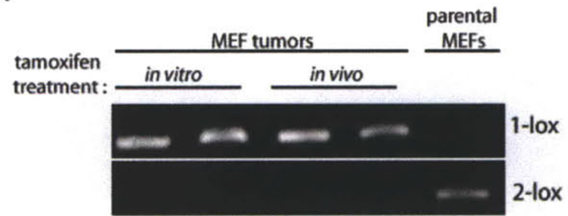
both *Trp53^flox^* alleles (Fig 2B). Importantly, this system showed no leakiness, as mice injected with FK*CP cells and treated with vehicle (corn-oil) failed to form tumors (Fig 2A). Additionally, the ability of tamoxifen treatment to promote tumor development depended on complete loss of p53, as shown by the lack of tumorigenecity of FK*C cells following tamoxifen administration (Fig 2A).

We next set out to investigate the fate of partially transformed FK*CP cells *in vivo* by addressing how long after the injection of cells could tamoxifen treatment still result in tumor formation. FK*CP cells were introduced into different groups of mice that varied in their tamoxifen treatment schedule: beginning 1 day (Group A), 1 week (Group B), or 3 weeks (Group C) following injection of the cells (Fig 2C). Strikingly, while tumor development was efficient in Groups A and B, almost no tumors formed in Group C (Fig 2D). These results suggest that injected FK*CP cells were either removed in a p53-dependent manner or became resistant to p53 loss sometime between one and three weeks following their introduction into the animal. Given the recent evidence that innate immune cells can clear other cells undergoing p53-dependent cell cycle arrest/senescence (Xue et al. 2007; Krizhanovsky et al. 2008), we wondered if FK*CP cells failed to form tumors in Group C because they were removed in such a manner. Therefore, we repeated these experiments in NOD/SCID mice, which are more immunocomprised than the nu/nu strain, lacking the cell populations implicated in senescent-cell clearance. The results of the time-course experiment in NOD/SCID were very similar to those in nu/nu mice (Fig 2E). This suggests that innate immune cell-mediated clearance of senescing cells does not explain the failure of the partially transformed FK*CP cells to remain responsive to *p53* deletion and form tumors following prolonged times *in vivo*. In summary, although primary MEFs expressing

A.

FK*CP			FK*C
corn-oil	tamoxifen		tamoxifen
	in vitro	*in vivo*	
0/6	4/4	6/6	0/6

B.



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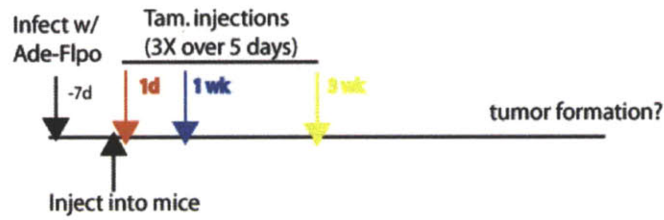

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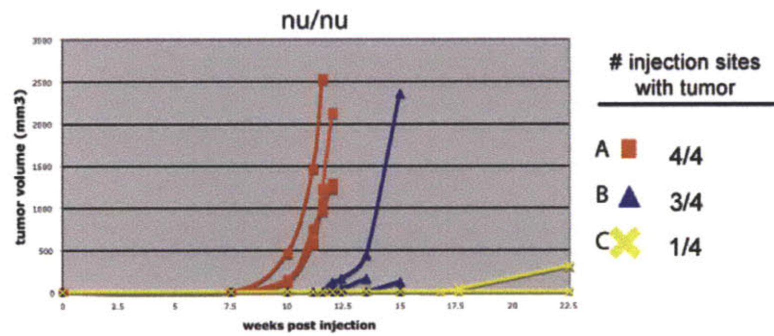

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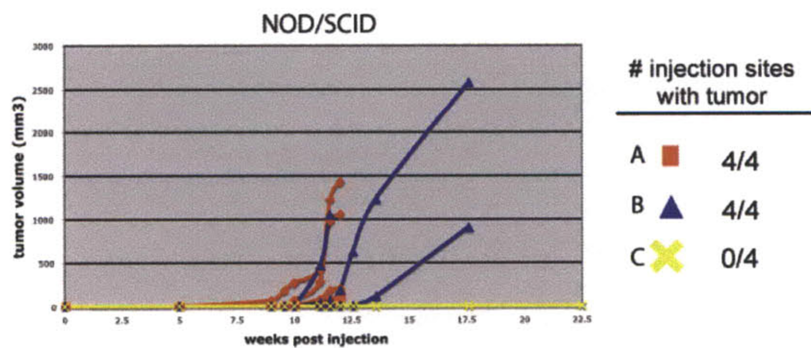


Figure 2: Sequential mutagenesis of K-ras and p53 in MEFs
A. Results of initial transplant experiments, represented as the number of injection sites presenting with tumors following introduction of different MEF lines and various treatment conditions (corn-oil or tamoxifen *in vivo*; 4-OHT *in vitro*). See text for experimental details. The system was sufficiently inducible and not leaky, as tumors formed only when tamoxifen was administered in the presence of two copies of $Trp53^{flox}$. FK*CP = $K\text{-}ras^{FSF\text{-}G12D/+}$; $R26^{CreER\text{-}T2/+}$; $Trp53^{flox/flox}$; FK*C = $K\text{-}ras^{FSF\text{-}G12D/+}$; $R26^{CreER\text{-}T2/+}$; $Trp53^{flox/+}$, * denotes activated K-ras^{G12D} **B.** Recombination analysis of $Trp53^{flox}$ alleles in resultant FKCP MEF tumors or the parental MEFs before injection into the animals. **C.** Schematic of sequential mutagenesis of K-ras and p53. See text for details. **D.** and **E.** Growth kinetics and overall incidence of tumors in which p53 was deleted 0 weeks (Group A-red square), 1 week (Group B-blue triangle), or 3 weeks (Group C-yellow X) post subQ injection of the cells into nu/nu (**D.**) or NOD/SCID (**E.**) mice. Delaying p53 loss until three weeks after injection severely inhibited the ability of partially transformed K-ras^{G12D}-expressing MEFs to subsequently form tumors.

endogenous levels of oncogenic K-ras proliferate continuously in cell culture conditions, transfer into an *in vivo* environment leads rapidly to engagement of the p53 pathway and irreversible tumor suppression.

Sequential mutagenesis of K-ras and p53 in a model of soft-tissue sarcoma

Having validated the dual SSR technology as means to perform sequential mutagenesis, we next applied the system to an endogenous tumor model. Previous work had shown that intramuscular infection of Ade-Cre into the limbs of *K-ras*$^{LSL-G12D}$; *Trp53*$^{flox/flox}$ animal resulted in efficient sarcomagenesis (Kirsch et al. 2007). However, the presence of just one wild-type allele of *p53* completely inhibited tumor formation. Furthermore, careful histological analysis of non-tumor bearing muscles of *K-ras*$^{LSL-G12D}$; *Trp53*$^{flox/+}$ animals several months after Ad-Cre infection failed to identify any microscopic lesions (data not shown). These results suggest that activation of endogenous K-ras^{G12D} in the muscle leads to rapid p53-dependent tumor suppression.

Despite these initial observations, the exact nature of the tumor suppression, and thus the fate of K-ras^{G12D}-expressing cells in the *Trp53*$^{flox/+}$background, remained unknown. One possibility was that oncogenic K-ras-positive cells persisted in the muscle, but were limited in their proliferative capacity due to p53-dependent growth arrest. In this case a secondary mutation in the p53 pathway could potentially unleash the oncogenicity of these latent cells. Another possible scenario was that K-ras^{G12D} activation resulted in a p53-dependent removal or irreversible alteration of the cells, such that subsequent p53 mutations would be ineffective in promoting full transformation of the initially targeted cells.

To distinguish between these two possibilities, we used a sequential mutagenesis strategy. Compound mutant mice of the genotype *K-ras*$^{FSF-G12D}$; *Trp53*$^{flox/flox}$; *R26*$^{CreER-T2/CreER-T2}$

were generated and separated into three groups. All three groups received simultaneous intramuscular Ad-Flpo infections to activate oncogenic K-ras, and then were separated based on their tamoxifen treatment schedule, and thus, time to *p53* deletion. Tamoxifen was administered on the same day of Ad-Flpo infection for Group A, on day 10 post infection (Group B), or day 21 post infection (Group C) (Fig 3A). If oncogenic K-ras expressing cells persisted and remained capable to respond to p53 loss, then all three groups would be expected to efficiently form sarcomas. In contrast, if mutant K-ras^{G12D} positive cells were somehow rendered refractory to delayed *p53* deletion, sarcomagenesis would be inhibited in Groups B and C. Remarkably, sarcoma formation varied greatly between three groups. While all mice in Group A presented with sarcomas, the percentage of effected mice was significantly lower in Group B (56%) and Group C (40%) (Fig 3B). Additionally, in separate experiments in which p53 loss was delayed until 5 weeks after K-ras activation, even fewer mice developed sarcomas (data not shown). Importantly, tumors from all groups displayed the expected recombination patterns at both *K-ras*$^{FSF\text{-}G12D}$ and *Trp53*^{flox} loci (Fig 3E). Of the sarcomas that did form in Group C, some appeared with delayed kinetics (time to tumor appearance after p53 loss) compared to Group A (Fig 3D). Taken together, these results suggest that the available pool of K-ras^{G12D} expressing cells in the muscle is significantly diminished over time, either in number or function, by a strong p53 response. Therefore, a mutation in *K-ras* must be soon followed by disengagement of the p53 pathway for efficient tumorigenesis to occur in the muscle. Alternatively, the inhibition of the p53 pathway would have to occur first in this cell type in order to render the cells sensitive to subsequent oncogene activation.

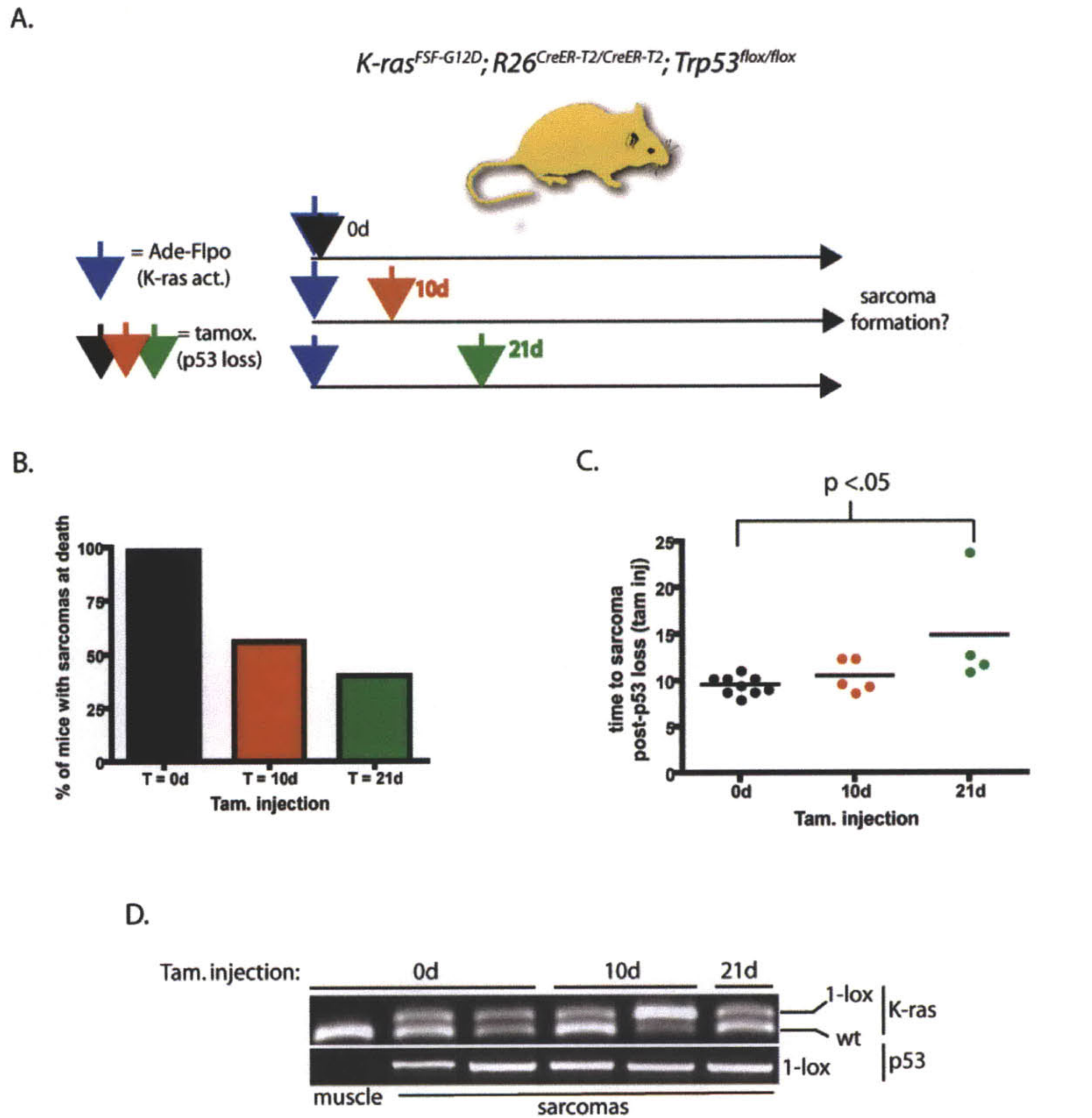


Figure 3: Delaying p53 loss relative to K-ras^{G12D} activation reduces tumor formation in a mouse model of soft-tissue sarcoma

A. Experimental outline of sequential mutagenesis strategy, showing the genotype of the compound mutant mice and the treatment regimen. See text for details. **B.** Bar graph illustrating tumor incidence in mice in which tamoxifen treatment began on day 0 (black bar), day 10 (red bar), or day 21 (green bar) following Ad-Flpo infection. **C.** Dot plot indicating the kinetics of sarcomagenesis as measured by the time to tumor formation post-p53 loss (tamoxifen treatment). Color scheme is same as in **(B)** **D.** PCR analysis of *K-ras* and *Trp53* loci. Tumors showed recombination at both loci while non-tumor bearing muscle from the same animals lack detectable recombination.

Genetic analysis of p53 response in K-ras^{G12D}-expressing cells in the muscle

We next sought to understand the mechanism of p53-mediated tumor suppression in the muscle. Through its ability to transcriptionally up-regulate a number of different genes under various stress conditions, p53 can induce distinct cell fates, such as apoptosis, cell cycle arrest, or senescence (Vousden and Lu 2002). Given that the sequential mutagenesis experiments did not distinguish between the clearance or retention of K-ras^{G12D}-positive cells, we considered all of these possibilities. In previous attempts to determine the mechanism of p53 action in the muscle, we studied mice of the genotype *K-ras*$^{LSL\text{-}G12D}$*; Bak1*$^{-/-}$*; Bax*$^{flox/-}$, and showed that deletion of the intrinsic pathway of apoptosis could not substitute for *p53* deletion in sarcoma development (Kirsch et al. 2007). This suggested that p53 is not functioning through the induction of apoptosis in this setting. Of note, deletion of *Arf*, an upstream regulator of p53, was as efficient as p53 loss in cooperation with oncogenic K-ras.

To address if p53 was acting through cell cycle arrest and/or senescence, we used a RNAi-mediated gene silencing method involving a lentiviral-based system for *in vivo* expression of Cre and a shRNA. In these experiments, it was necessary to use immunocompromised (*Rag2*$^{-/-}$) mice to avoid immune responses to lentiviral infection. Also, we employed mice heterozygous for a mutant *Arf* allele (*Arf*^{GFP}- see Chapter 2) to sensitize them to shRNA mediated p19^{Arf} inhibition. Accordingly, intramuscular lentiviral infection of *K-ras*$^{LSL\text{-}G12D}$*; Arf*$^{GFP/+}$*; Rag2*$^{-/-}$ animals did not result in tumors unless a shRNA to p19^{Arf} was included (Chapter 2). With this system, we could determine whether knockdown of core components of the senescence and/or cell cycle arrest machinery downstream of p53 was sufficient for sarcoma formation. To this end, we tested hairpins directed against the histone methyltransferase suv39h1. Despite not being a known p53 target gene, suv39h1 is one of the only factors known to play a direct role in

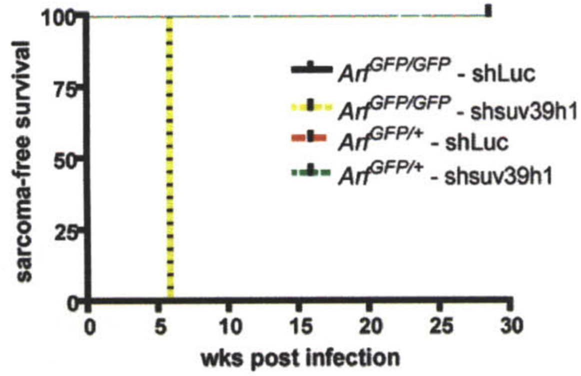
senescence in *in vivo* mouse models (Braig et al. 2005; Shamma et al. 2009). Infection of *K-ras$^{LSL-G12D}$; Arf$^{GFP/+}$; Rag2$^{-/-}$* animals with multiple shRNAs to suv39h1 failed to generate sarcomas, despite significant knockdown as assessed in control *K-ras$^{LSL-G12D}$; Arf$^{GFP/GFP}$; Rag2$^{-/-}$* sarcomas produced with the same vectors (Fig 4A and B). Therefore, if p53 is inducing senescence in this context, it is most likely suv39h1-independent.

One of the most well established p53 target genes is the cyclin dependent kinase inhibitor p21, which has been shown to be critical for the G1 cell cycle arrest elicited by p53 in a number of settings (Brugarolas et al. 1995; Deng et al. 1995; Efeyan et al. 2007). However, other studies using *p21$^{-/-}$* cells have called into question the importance of p21 in p53-mediated arrest downstream of oncogenic stress (Pantoja and Serrano 1999). To determine if p21 is important for p53-dependent tumor suppression in the muscle, we introduced a shRNA targeting p21 into the bifunctional lentiviral vector and infected *K-ras$^{LSL-G12D}$; Arf$^{GFP/+}$; Rag2$^{-/-}$* mice. Interestingly, knockdown of p21 was able to promote sarcoma formation (Fig 4C). Molecular analysis confirmed significant knockdown of p21 in the resulting tumors (Fig 4D). In addition, PCR analysis of tumor DNA indicated retention of the wild-type allele of *Arf*, suggesting no additional selective pressure for p53 pathway inactivation during tumor formation (data not shown). Together, these data indicate that p21 is required for the strong tumor suppressor activity of p53 in the muscle following expression of oncogenic K-ras. This suggests that the primary function of p53 in this context is the induction cell cycle arrest, and possibly, senescence.

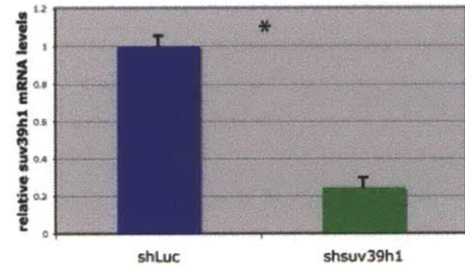
Further characterization of p53-and p21-dependent tumor suppression in the muscle

As described above, delaying p53 loss relative to K-ras^{G12D} activation hinders sarcoma formation, which implies that cells initially expressing oncogenic K-ras are either removed or

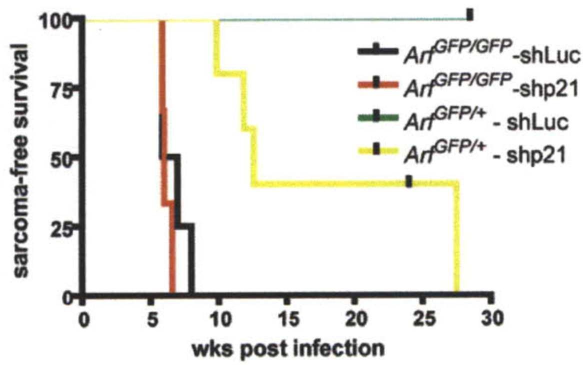
A.



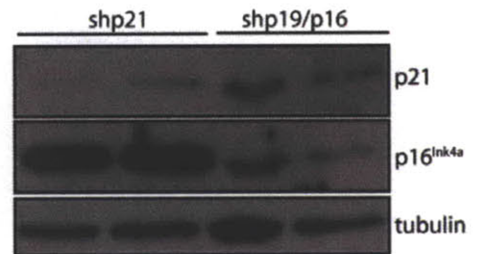
B.



C.



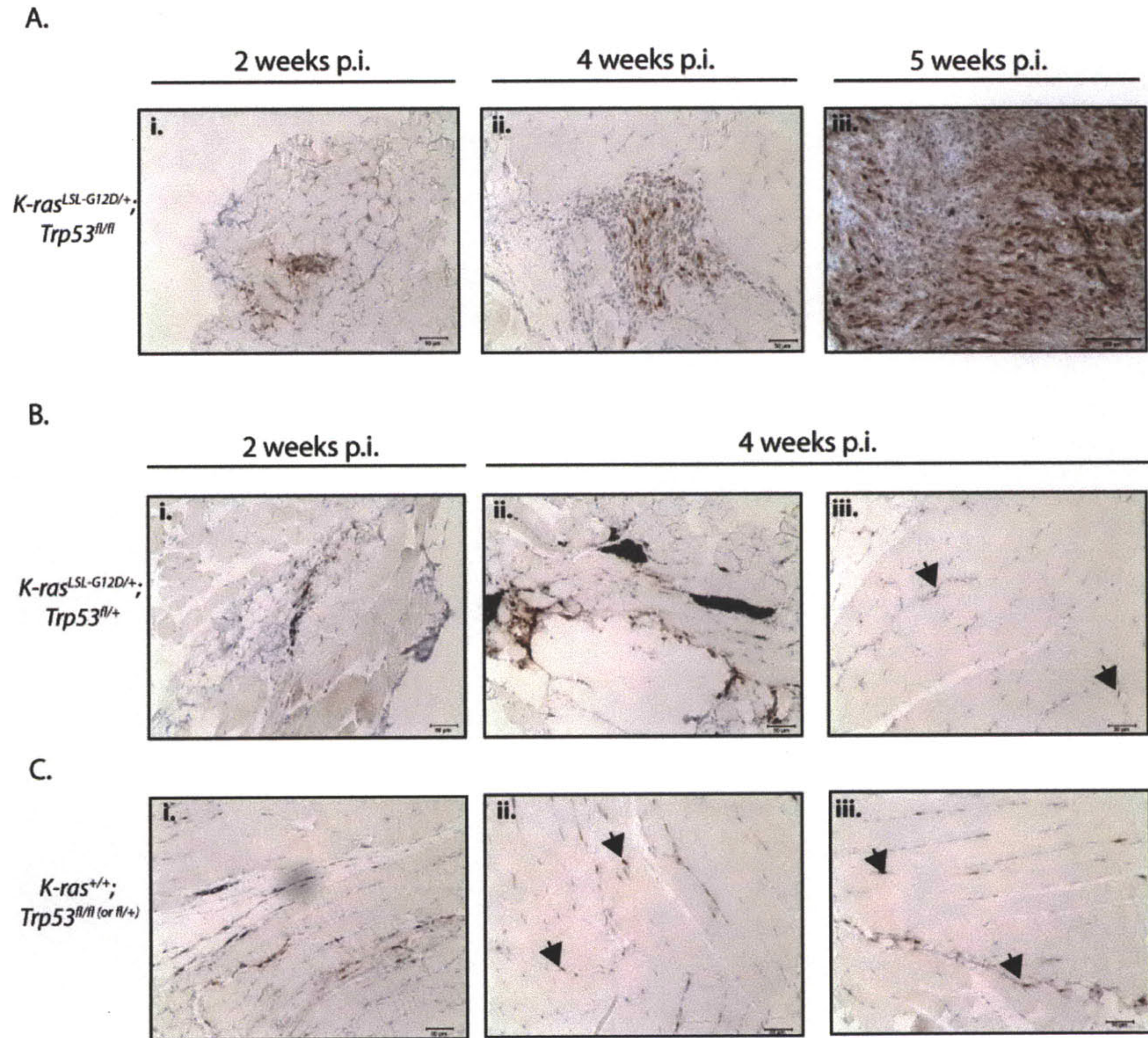
D.



<u>Figure 4:</u> Genetic analysis implicates p21 as a critical component of p53-dependent tumor suppression in the muscle.

A. Kaplan-Meier graph showing sarcoma-free survival in a cohort of $K\text{-}ras^{LSL\text{-}G12D}$; $Arf^{GFP/GFP}$; $Rag2^{-/-}$ (black and yellow lines) and $K\text{-}ras^{LSL\text{-}G12D}$; $Arf^{GFP/+}$; $Rag2^{-/-}$ (solid red and dotted green lines) mice infected with lentiviruses expressing Cre and a shRNA targeting either luciferase (solid black and red lines) or one of two shRNAs to suv39h1 (dotted yellow and green lines). For black bar, N = 2, yellow bar N = 4, red bar N = 4, green bar N = 8. **B.** qRT-PCR of suv39h1 mRNA levels in sarcomas from $K\text{-}ras^{LSL\text{-}G12D}$; $Arf^{GFP/GFP}$; $Rag2^{-/-}$ animals infected with lentiviruses expressing Cre and a shRNA to luciferase (blue bar) or one of two shRNAs to suv39h1 (green bar). N = 2 tumors for each group. *, P < .005. Error bars denote standard deviation. **C.** Kaplan-Meier graph showing sarcoma-free survival in a cohort of $K\text{-}ras^{LSL\text{-}G12D}$; $Arf^{GFP/GFP}$; $Rag2^{-/-}$ (red and black lines) and $K\text{-}ras^{LSL\text{-}G12D}$; $Arf^{GFP/+}$; $Rag2^{-/-}$ (yellow and green lines) mice infected with lentiviruses expressing Cre and a shRNA targeting either luciferase (black and green lines) or p21 (red and yellow lines). For black line, N = 4, red line N = 3, green line N = 4, and yellow line N = 5. **B.** Western blot analysis of $p16^{Ink4a}$ and p21 on a panel of sarcomas arising from infection of $K\text{-}ras^{LSL\text{-}G12D}$; $Arf^{GFP/+}$; $Rag2^{-/-}$ animals with either Cre-shp21 (shp21) or Cre-shp19/p16 (shp19/p16) lentiviruses.

become refractory to subsequent *p53* deletion. While previous genetic data indicated that apoptosis is not likely to be the mechanism of tumor suppression, affected cells could still be cleared from the body, perhaps through innate immune cell scavenging as has been shown for senescent cells (Xue et al. 2007). Alternatively, the cells could persist in a state of irreversible growth inhibition. Whether p53 can direct this type of permanent arrest *in vivo*, and if the continued presence of p53 is required, is unknown. To gain insight into the fate of K-ras^{G12D}-expressing cells, we pursued a cell marking strategy. Administering lentiviruses encoding Cre and GFP allowed us to mark infected cells using immunohistochemistry (IHC) for GFP. As a control, lentiviral infection of *K-ras*$^{LSL\text{-}G12D}$*; Trp53*$^{flox/flox}$*; Rag2*$^{-/-}$ animals efficiently generated sarcomas that stained for GFP (Fig 5A iii). Interestingly, an examination of infected muscles before macroscopic tumor formation revealed regions of GFP+ cells that increased in size over time, most likely corresponding to incipient tumor lesions (Fig 5A). However, while we could readily detect infected cells in *K-ras*$^{LSL\text{-}G12D}$*; Trp53*$^{flox/+}$*; Rag2*$^{-/-}$ mice, they never appeared to expand and frequently were seen scattered throughout the muscle (Fig 5B). This pattern was very similar to what was observed in mice with wild-type *K-ras* (Fig 5C). These data provide further evidence that p53 suppresses the very earliest events of K-ras^{G12D}-mediated transformation in muscle cells. So far, we have been unable to detect markers of senescence in any infected cells in *K-ras*$^{LSL\text{-}G12D}$*; Trp53*$^{flox/+}$*; Rag2*$^{-/-}$ animals (data not shown). Although we cannot rule out the possibility that some cells senesce and are cleared, the continued presence of GFP+ cells in *K-ras*$^{LSL\text{-}G12D}$*; Trp53*$^{flox/+}$*; Rag2*$^{-/-}$ animals indicates that some K-ras^{G12D}-positive cells remain in the muscle at time points at which *p53* deletion is unable to efficiently promote sarcomagenesis. This might suggest that p53 engages an irreversible cell cycle arrest in those cells.



<u>Figure 5:</u> Identification of GFP labeled cells in both tumor bearing and non-tumor bearing muscles at multiple time points after infection.

GFP IHC on formalin-fixed muscle tissue from *K-ras*$^{LSL\text{-}G12D}$; *Trp53*$^{flox/flox}$; *Rag2*$^{-/-}$ (**A**), *K-ras*$^{LSL\text{-}G12D}$; *Trp53*$^{flox/+}$; *Rag2*$^{-/-}$ (**B**), and *K-ras*$^{+/+}$; *Trp53*$^{flox/flox\ (or\ flox/+)}$; *Rag2*$^{-/-}$ animals at the indicated times post infection (p.i.). GFP staining is brown, and India Ink, which was used to mark the injection site, stains as black. While tufts of cells appear to grow into incipient tumors in *K-ras*$^{LSL\text{-}G12D}$; *Trp53*$^{flox/flox}$; *Rag2*$^{-/-}$ animals (**A**), non-tumor bearing animals contain dispersed cells either near the injection site (**B i** and **ii**, **C i**) or in isolated parts of the muscle (**B iii**, **C ii** and **iii**). Arrows indicate single GFP+ cells.

DISCUSSION

In the progression from normalcy to full malignancy, cells must overcome a number of restraints on growth and survival. It is generally accepted that the sequential accumulation of multiple mutations drives different parts of this process. While the cooperative nature of sets of mutations has been validated in a variety of experimental systems, the importance of their temporal relationship to one another has been largely unexplored, especially *in vivo*.

In the present study, we combined Flp-Frt and Cre-LoxP technologies in order to control different genetic events in a temporal fashion. Through the development of a Flp-inducible allele of endogenous oncogenic K-ras, we could then take advantage of already available tools for Cre-controlled p53 manipulation. This dual system allowed for the temporal separation of K-ras activation and p53 mutation. Using this strategy we gained new insights into the relationship between these commonly co-mutated genes. First, while escaping p53-mediated growth arrest during passage in cell culture, transfer into an *in vivo* environment rendered partially transformed K-ras^{G12D} –expressing MEFs susceptible to irreversible p53 tumor suppression. The signals converging on p53 specifically after *in vivo* transfer are unknown but this MEF system could be a useful model for understanding how p53 inhibits later stages of tumor progression, such as metastasis.

We next used sequential mutagenesis of *K-ras* and *p53* to understand the absolute requirement for both mutations in a mouse model of soft-tissue sarcoma. As shown previously, simultaneous mutation of both genes resulted in robust sarcomagenesis. Intriguingly, by delaying *p53* deletion relative to K-ras activation, we observed a decrease in the efficiency of sarcoma formation. This suggests that oncogenic K-ras induces a strong p53 response that compromises the tumorigenic potential of initially targeted cells, even when p53 is subsequently inactivated.

This immediate and severe p53-dependent response of muscle cells harboring an oncogenic Ras mutation might at least partially explain the low frequency of soft-tissue sarcomas compared to many other malignancies. Indeed, the incidence of soft-tissue sarcomas is dramatically elevated in Li-Fraumani patients inheriting a germ-line mutation of *p53* (Kleihues et al. 1997), indicating that pre-existing deficiencies in p53 function allow for muscle cell transformation, which is otherwise a rare occurrence. Moreover, somatic *p53* mutations have been proposed to be relatively early events in other forms of sarcomas (Taubert et al. 1998), further suggesting that muscle cells must overcome an early p53-dependent blockade during tumor initiation. As a result of this potent p53-dependent tumor suppression, spontaneous sarcomagenesis most likely selects for a particular sequence of events in which mutation of *p53* occurs very early. Because p53 alterations are rarely thought to be an initiating event (Hinkal et al. 2009), sarcoma development is limited.

Genetic dissection of pathways downstream of p53 implicated p21 in mediating this strong response, arguing that cell cycle arrest rather than apoptosis is the predominant route of tumor suppression in this context. Given that eventual p53 loss did not fully rescue the ability of K-ras mutant cells to form tumors, this arrest must be irreversible and/or lead to the clearance of affected cells. Currently, we cannot rule out either of these mechanisms. At later time points after K-ras^{G12D} activation in p53-proficient animals, we were still able to locate scattered infected cells, suggesting that at least some cells persist but cannot efficiently go on to form tumors. While we have not been unable to identify senescence markers, a further investigation of these cells is warranted.

Sequential mutagenesis of K-ras^{G12D} and p53 provided evidence that acute activity of p53 at tumor initiation was sufficient for significant tumor suppression. Similarly, Evans and

colleagues have demonstrated robust tumor suppression after a small window of p53 activity occurring shortly after irradiation (IR)-induced tumor initiation (Christophorou et al. 2006). Conversely, in a separate study, deleting p53 at different time points after IR revealed that the continued presence of p53 was required to prevent IR-induced tumorigenesis (Hinkal et al. 2009). In fact, once p53 was removed, tumor formation occurred with the same kinetics as seen in IR-treated *Trp53*$^{-/-}$ animals. Apparently, in this context p53 maintained pre-neoplastic cells in a reversible cell cycle arrest that was completely abrogated once p53 was deleted. This is in stark contrast to what we observed in K-ras^{G12D}-expressing muscle cells, where subsequent *p53* deletion failed to fully recover the tumorigenic capacity of mutated cells.

p53 mutation has been shown to exacerbate tumor phenotypes in a number of mouse models, validating its role as a critical tumor suppressor (Donehower and Lozano 2009). The precise stages of cancer development at which p53 function is most important are less clear. Some studies have suggested that p53 constrains the progression of already present lesions to more advanced states (Chen et al. 2005; Jackson et al. 2005; Hinkal et al. 2009). Consistent with a role in established tumors, restoration of p53 function has been shown to lead to robust tumor regression (Martins et al. 2006; Ventura et al. 2007; Xue et al. 2007). It is possible that in some settings sufficient p53 pathway activation requires additional stimuli apart from the initiating oncogenic lesion, resulting in delayed p53 function. In contrast, we found that even a relatively short time window of p53 activity soon after oncogenic K-ras expression in the muscle significantly reduced tumor burden, implying suppression of tumor initiation. This suggests that endogenous levels of K-ras^{G12D} are sufficient for functionally relevant p53 induction in this tissue. Interestingly, the same allele of *K-ras* fails to elicit significant p53 activity in the lung epithelium until much later during tumorigenesis. Accordingly, delaying *p53* mutation relative to

K-ras activation during lung tumor initiation generates tumors largely resembling those seen with a simultaneous mutagenesis strategy (data not shown). One explanation for this tissue-specific response could be differential regulation of p19^{Arf}, a major inducer of p53, in lung and muscle cells (Chapter 2). A further understanding of the factors governing p53's potent tumor suppression in the muscle might guide therapeutic strategies aimed at generating such a response in more resistant tissues.

Future studies using the sequential mutagenesis technology presented here will be able to address fundamental questions in tumor biology that have been difficult or impossible to accomplish using other systems. The ability to accurately model stepwise mutations inherent in tumorigenesis will allow one to test the functional relevance of particular orders of commonly co-mutated genes in a number of different tumor types. Additionally, the genetic dissection of pathways at different stages of tumor progression will yield insights that will be useful for tailoring therapeutic regimens to distinct stages of disease.

MATERIALS AND METHODS

Construction of *K-ras*$^{FSF\text{-}G12D}$ targeting vector

To construct the Frt-STOP-Frt (FSF) element, two separate Frt sites were individually inserted into pBluescript (pBS, Stratagene) by ligating annealed oligos encoding Frt sequences into digested pBS. Specifically, the 1st Frt site was placed into XhoI-KpnI digested pBS. 1Frt-pBS was then digested with XhoI and NotI and the 2nd Frt was inserted. A linker sequence containing unique PstI and HpaI sites was then placed in between the two Frt sites after digestion of 2Frt-pBS with XhoI. The resulting Frt-linker-Frt-pBS vector was cut with PstI and HpaI sites and ligated with the STOP element from LoxP-STOP-loxP (LSL-Tuv ref) that had been digested with Pst and ScaI, generating FSF-pBS. The FSF cassette was then subcloned into a TOPO vector (Invitrogen) creating FSF-TOPO. Finally, the FSF was removed from FSF-TOPO with a SalI digest and inserted into the previously generated, SalI-digested, pBS-DTA-K-ras^{G12D}, which contained the G12D point mutation and homolgy arms for targeting the *K-ras2* locus (Tuv ref). Correct orientation was checked by PCR. Additional details are available upon request.

Generation of *K-ras*$^{FSF\text{-}G12D}$ mice

The targeting vector was linearized by SfiI digest and electroporated into V26.2 C57BL/6 ES cells and puromycin-resistant clones were selected. Homologous recombination was assayed by Southern blot analysis on BamHI/KpnI doubly digested ES cell DNA, using both 5' and 3' probes lying outside of the targeting arms. A correctly targeted clone was injected into BALB/c derived blastocysts, which were then transplanted into a pseudopregnant female, and resulting black/white chimeras were crossed to C57BL/6 mice to monitor for germline transmission.

Genomic PCR was performed on black pups to ensure inheritance of the targeted allele. Genotyping details are available by request.

Mouse studies. *Trp53*^{flox} mice were provided by A. Berns (The Netherlands Cancer Institute, Amsterdam, the Netherlands), *Arf*^{GFP} mice were provided by C. Sherr (St. Jude Children's Hospital, Memphis, TN), and *Rag2*$^{-/-}$, nu/nu, and NOD/SCID mice were purchased from The Jackson Laboratory. *K-ras*$^{LSL\text{-}G12D}$ and *R26*^{CreER} were previously generated in our laboratory (refs). All animals (except for nu/nu and NOD/SCID) were maintained on a mixed background comprising 129S4/SvJae and C57BL/6 strains. Lung tumors were generated and processed as previously described (ref), substituting Ad-Flpo (University of Iowa, Gene Transfer Vector Core) for Ade-Cre. Intramuscular viral infections were done as previously shown (ref). For GFP marking experiments, needles were dipped in India Ink before injection in order to mark the needle track. Tamoxifen (Sigma) was dissolved in corn-oil at 10 mg/ml and injected intraperitoneally every other day for 5 days where applicable. Once masses were visible on the legs, tumors were processed for histology and molecular analysis as described below. For MEF tumor experiments, 1.5 x 10^6 cells were resuspended in 200 ul of PBS and injected subcutaneously. Mice were monitored every few days for tumor formation. Animal studies were approved by Massachusetts Institute of Technology's (MIT) Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals, and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory Animals (institutional animal welfare assurance number, A-3125-01).

Cell Culture. Primary MEFs of the indicated genotypes were isolated from E13.5 embryos and propagated in DMEM supplemented with 10% IFS, 5mM glutamine and penicillin/streptomycin. Where applicable 4-hydroxytamoxifen (Sigma) was added to the media at 100nM. Lentiviral infections were performed by directly transferring viral supernatant. For adenovirus infection *in vitro* Ad-Flpo was added to the media at an MOI of 10.

Genomic PCR analysis: DNA was prepared from MEFs or tumors and subjected to standard PCR analysis. PCR primers were as follows:

PCR name	forward primer	reverse primer
p53 2-lox	CACAAAAACAGGTTAAACCCA	GAAGACAGAAAAGGGGAGGG
p53 1-lox	AAGGGGTATGAGGGACAAGG	Same as above
Multiplex K-ras	GGGTAGGTGTTGGGATAGCTG	TCCGAATTCAGTGACTACAGATGTAC

Protein extraction and immunoblots. MEFs were lysed in RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Tx-100, .1% SDS, .5% sodium deoxycholate, 1 mM DTT) plus mini complete protease inhibitors (Roche) and phosphatase inhibitors (cocktails 1 and 2) (Sigma) for 10 minutes on ice. Snap-frozen tissue was finely minced with a razor blade on ice in TNE buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, supplemented with 1% Tx-100, .1%SDS, 1mM DTT, and the same inhibitors mentioned above) and then rotated for 15 minutes at 4°C. Both *in vitro* and *in vivo* samples were centrifuged to remove insolubles and quantitated using a Bradford Assay (Bio-rad). Samples were then diluted in loading buffer and separated on 10-15% SDS-PAGE gels. Following transfer to PVDF membranes, the following antibodies were used: anti-Pan Ras (#05-516), anti-cyclin D1 (#05-815-Millipore); anti-β-tubulin (#2146 – Cell Signaling Technology (CST)); anti-p21 (sc-6246), anti-p16^{Ink4a} (sc-1207- SantaCruz

Biotechnology). HRP-conjugated secondary antibodies were used in conjunction with ECL+ detection systems (Amersham). Levels of Ras-GTP were determined with the Ras activation kit (Millipore).

mRNA analysis mRNA isolation and qRT-PCR analysis. RNA was extracted using RNeasy kits (Qiagen). Briefly, tumor tissue was minced with razor blades and further homogenized using Qiashredder columns (Qiagen) before continuing with the manufacturer's instruction. cDNA synthesis was performed on 1 μg of RNA using oligo dT primers and Superscript III (Invitrogen). cDNAs were analyzed by qPCR using Taqman detection systems in an ABI PRISM 7000 Sequence Detection System Thermo Cycler (Applied Biosystems). Relative mRNA levels were calculated using cycle threshold difference (ΔC_T) comparing to TBP. The following Taqman probes were used: suv39h1 - Mm01347696_g1; TBP - Mm00446973_m1.

Lentiviral vectors and shRNA cloning. The lentivirus containing pgkCre and a U6-shRNA cassette was provided by from M. Kumar and K. Lane (Massachusetts Institute of Technology KI, Cambridge, MA), and the GFP-Cre lentivirus (Ubc-GFP; pgk-Cre) was provided by M. DuPage (Massachusetts Institute of Technology KI, Cambridge, MA). Cloning details are available on request. Target sequences for shRNA knockdown were identified using pSICO Oligomaker V 1.5 (A. Ventura, Memorial Sloan Kettering Cancer Center, New York, NY). Cloning of DNA oligos into the U6-shRNA cassette was done as described previously (ref). shRNA sequences were as follows: luciferase: 5'-GAGCTGTTTCTGAGGAGCC-3'; p21: 5'-GGAAGGGAATGTATATGCA-3'; suv39h1-1: 5'-GGATCACCGTGGAGAATGA-3'; suv39h1-2: 5'-GAACCAAGATCTAGGTATT-3'.

Lentiviral production. Lentivirus was produced as described previously (Rubinson et al 2003).

Histology and Immunohistochemistry. Tissues were fixed in 10% formalin for 6-8 hrs and further processed for histology as previously described (Johnson et al 1997). For immunohistochemistry, parraffin-embedded sections were dewaxed, followed by antigen retrieval in 10 mM citrate buffer (pH 6.0) in a pressure cooker. Slides were quenched in 3% hydrogen peroxide and washed in TBST. After blocking in TBST/5% goat serum for 1 hr, the primary antibody (rabbit mAb anti-GFP, #2956- CST) was diluted 1:100 in SignalStain Antibody diluent (#8112, CST) and incubated on slides overnight at 4°C. Detection was performed using a biotinylated goat anti-rabbit secondary antibody followed by the Vectastain ABC kit with diaminobenzadine (DAB) (Vector Labs). Slides were counterstained with haematoxylin before coverslipping.

ACKNOWLEDGEMENTS

We thank many members of the Jacks lab for helpful advice throughout the course of this project. We are grateful to D. Crowley for histology assistance and R. Bronson for histopathaological advice. This work was supported in part y grant **5-U01-CA84306** from the National Institutes of Health, and partially by Cancer Center Support (core) grant P30-CA14051 from the National Cancer Institute. T.J. is a Howard Hughes Medical Institute Investigator and a Daniel K. Ludwig Scholar.

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CHAPTER 4:

Discussion and Future Directions

Overview

The elaborate connectivity between pathways that promote cell growth and transformation with those that actively constrain tumorigenesis comprises a critical checkpoint system to protect against cancer. This oncogene-induced tumor suppression is typified by the activation of the potent $p19^{Arf}$-p53 tumor suppressor pathway by oncogenic Ras. The work presented in this thesis reveals novel insights into the relationship between these two highly relevant cancer pathways. I describe a tumor-type specific expression pattern of $p19^{Arf}$ in endogenous $K\text{-}ras^{G12D}$-driven lung tumors and sarcomas that is determined by inherent differences in chromatin regulation of the *Ink4/Arf* locus. Furthermore, the differential $p19^{Arf}$ expression correlates with the strength of tumor suppression enforced by the p53 pathway in lung and muscle cells. To illustrate this, I construct a new mouse model allowing for sequential mutagenesis and show that p53 robustly inhibits tumor initiation following oncogenic K-ras expression in the muscle, in contrast to its role in impeding later stages of lung tumor progression. These results demonstrate that distinct cell types vary in their engagement of tumor suppressors downstream of oncogenic insults. This has a profound influence on the susceptibility of different tissues to transformation by particular mutations, and also provides an example of how specific sequences of genomic alterations can dictate the tumor phenotype.

Tissue-specific $p19^{Arf}$ regulation

The concept of oncogene-induced tumor suppression originated from studies in which various oncogenes were retrovirally over-expressed in tissue culture (Serrano et al. 1997). As tumor suppressor induction was thought to be linked to hyperactivity of mitogenic signaling networks, it was possible that this phenomenon was the result of oncogene overexpression. Construction of endogenous alleles of oncogenes, most notably $K\text{-}ras^{G12D}$, permitted a direct comparison of physiological versus supraphysiological oncogenic signaling. Interestingly, the

extent of p19^{Arf}-p53 activation directly correlated with levels of oncogenic Ras (Tuveson et al. 2004). Additional studies manipulating both H-ras^{G21V} and c-myc *in vivo* came to the same conclusion that oncogene levels determine tumor suppressor pathway (specifically *Ink4a/Arf*) engagement (Sarkisian et al. 2007; Murphy et al. 2008). Accordingly, these data led some to argue that endogenous K-ras^{G12D} failed to effectively induce the p19^{Arf}-p53 pathway, allowing it to readily transform a variety of tissues (Tuveson et al. 2004).

Considering the highly specific tumor spectrum of *K-ras^{LA2}* animals (Johnson et al. 2001), I speculated that endogenous oncogenic K-ras could in fact engage p19^{Arf}-p53, albeit in a cell-type dependent manner. While the frequent lung tumors in *K-ras^{LA2}* animals suggested a lack of tumor suppressor up-regulation in this tissue, perhaps many more cell types undergo recombination and express endogenous K-ras^{G12D}, but preferentially activate p19^{Arf}-p53 and thus do not form tumors. To address this I crossed a p19^{Arf} reporter, *Arf^{GFP}* with the *K-ras^{LA2}* model, reasoning that removing p19^{Arf} function would allow any strongly suppressed cells to develop into tumors in which I could then compare p19^{Arf} activation to that seen in the lung tumors. In Chapter 2, I describe such an expansion of the tumor spectrum in *K-ras^{LA2}; Arf$^{GFP/GFP}$* mice and report a striking difference in p19^{Arf} levels between lung tumors and sarcomas, with sarcomas inducing p19^{Arf} to a much greater extent than lung tumors.

Oncogenic signaling levels

Given the previous data regarding oncogene levels and p19^{Arf} activation, I assessed both oncogenic K-ras levels as well as the associated signaling pathways linking K-ras to the *Ink4a/Arf* locus (Chapter 2). Despite the drastic difference in p19^{Arf} activation, these two tumor types expressed similar levels of K-ras. Signaling pathway analysis indicated similar levels of MAPK and PI3K activity as well. Interestingly, although signaling appeared equivalent, direct

activators of p19^{Arf}, such as E2F1/3 and cJun, were up-regulated in sarcomas. However, I was unable to functionally validate their role in p19^{Arf} induction, although redundancy and/or the importance of these genes in cellular proliferation could confound functional studies. Despite these uncertainties, the tumor-type specific p19^{Arf} induction in lung tumors and sarcomas cannot currently be explained by existing models implicating oncogene levels in the control of this locus.

Chromatin-modifying complexes

Chromatin-modifying complexes have recently been shown to play a critical role in *Ink4a/Arf* regulation. Most notable among these are members of PcG, which have been implicated in maintaining repression of this locus in a number of different cell types (Gil and Peters 2006). Considering the relatively low levels of p19^{Arf} and p16^{Ink4a} in lung tumors, I hypothesized that PcG was inhibiting expression from this locus. Indeed, ChIP analysis showed the PcG histone mark as well as Bmi-1 binding throughout *Ink4/Arf* in established lung tumors, and shRNA-mediated knockdown of Bmi-1 partially activated both p19^{Arf} and p16^{Ink4a} expression (Chapter 2). Intriguingly, sarcomas displayed much less PcG at *Ink4a/Arf*, providing a potential explanation for the differential expression of p19^{Arf} and p16^{Ink4a} between the two tumor types.

The importance of PcG in mediating *Ink4a/Arf* repression in lung tumors led me to search for additional chromatin modifiers that might mediate the activation of the locus in sarcomas. I speculated that this activation might rely on an antagonism of PcG, as the low level of PcG observed at *Ink4a/Arf* in sarcomas could be a remnant of the original regulation of this locus in the cell-origin of sarcomas, most of which is removed upon K-ras activation. Snf5, a member of the SWI/SNF nucleosomal remodeling complex, was recently shown to induce Ink4a/Arf by

evicting PcG (Kia et al. 2008). Interestingly, sarcoma cell lines displayed direct binding of Snf5 at *Ink4a/Arf* as well as reduced Arf-GFP levels after Snf5 knockdown. Most importantly, depletion of Snf5 *in vivo* caused tumor formation in a genetic background otherwise completely resistant to tumorigenesis owing to robust $p19^{Arf}$-p53 activation (Chapter 2). These results strongly implicated Snf5 as a critical factor linking oncogenic K-ras to $p19^{Arf}$ activation and further highlighted the importance of chromatin modifying factors in the regulation of this locus.

Differential activation thresholds

The observation that sarcomas and lung tumors exhibit distinct $p19^{Arf}$ induction despite similar oncogenic signaling patterns suggests that these two tissues have inherent differences in their activation threshold for *Ink4a/Arf*. The ability of comparable upstream signals to mediate such different gene expression programs could be due to unique chromatin configurations of this locus in lung and muscle cells both before and after oncogenic K-ras expression. Below I discuss two potential models regarding tissue-specific epigenetic regulation of *Ink4a/Arf* (Fig 4.1).

In Model 1, the respective cells-of-origin (COs) of lung tumors and sarcomas have a substantially different chromatin state under normal conditions. In this scenario, the different levels of PcG occupancy observed between lung tumors and sarcomas closely mirror the relative binding pattern present in a normal lung or muscle cell. Having less of a repressive state in a normal muscle cell might reduce the requirements for gene induction once the proper activation

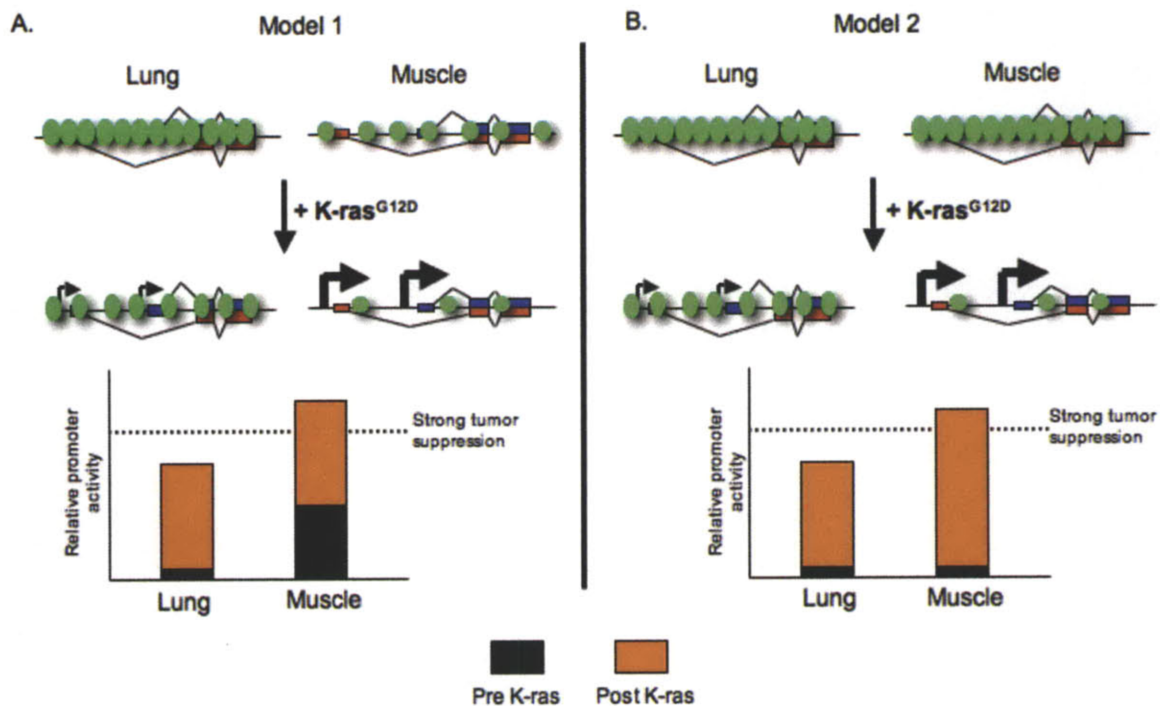


Figure 4.1: Two models to explain differential *Ink4a/Arf* activation in response to K-ras^{G12D}
A. Model 1, in which the cells-of-origin (CO) for lung tumors and sarcomas have drastically different levels of PcG occupancy before oncogenic K-ras expression. Both cell types have equivalent responses to oncogenic stress, which is consistent with Ras effector pathway analysis, but since the muscle cell started out with less repression, it activates Ink4a/Arf to a greater extent. **B.** Model 2 posits that both COs have similar levels of PcG repression pre-K-ras^{G12D}, but in response to the oncogenic insult muscle cells displace a large proportion of PcG, leading to robust activation of Ink4a/Arf. Since proximal signaling levels of canonical pathways downstream of oncogenic K-ras are equivalent in lung tumors and sarcomas, any differential response post-K-ras^{G12D} must be due to cell-type specificity in how oncogenic signals are interpreted, which could be dependent on the presence and/or activity of additional factors, such as SWI/SNF. The increased levels of E2Fs, c-Jun, and DMP-1 in sarcomas despite similar oncogenic pathway levels suggests that there can be such a disconnect between proximal and distal pathway components. Green circles = PcG. The genomic structure is the *Ink4a/Arf* locus. Black and orange bars represent the relative promoter activity at p19^{Arf} pre and post-K-ras, respectively.

signals are received. Conversely, more tightly packaged, PcG-dense chromatin in lung cells might be at least partially resistant to comparable signals converging on the locus following K-ras^{G12D} activation.

Such a model has been proposed to explain the different transforming capabilities of hematopoietic stem cells and/or progenitors compared to their more differentiated progeny (Williams and Sherr 2008). For example, introduction of the Notch-IC oncogene into bone marrow progenitors results in efficient transformation, while more committed T-cells progenitors require the additional deletion of *Arf* for leukemia development (Volanakis et al. 2009). As these cell types differ in the extent of PcG-mediated *Ink4a/Arf* repression (Valk-Lingbeek et al. 2004), a differential ability to activate p19^{Arf} following oncogene expression has been proposed as a mechanism, although this has not been tested.

Similarly, the differentiation status of lung tumor and sarcoma COs could explain a difference in basal levels of *Ink4/Arf*-localized PcG and hence ease of transformation by oncogenic K-ras. Bronchioalveolar stem cells (BASCs), a candidate CO for lung tumors, have been shown to depend on Bmi-1-mediated *Ink4a/Arf* repression for self-renewal (Dovey et al. 2008). While the identity of the cells that give rise to the sarcomas is completely unknown, they might be a more differentiated cell that lacks sufficient PcG-mediated repression of the locus to maintain low expression during oncogenic stress. In contrast to this differentiation status model, it could be that in general, PcG plays a more important role in *Ink4a/Arf* regulation in lung cells compared to muscle cells, which is supported by my observation of *Ink4a/Arf*-associated PcG marks in whole lung samples (Chapter 2). Moreover, *Bmi-1*$^{-/-}$ mice display p19^{Arf} up-regulation throughout the entire lung, suggesting that a large majority of lung cells rely on PcG for proper p19^{Arf} regulation (Dovey et al. 2008; Sacco et al. 2008). Similar experiments in the muscle have

yet to be performed due to technical limitations but most likely would be hard to interpret in the context of sarcoma COs as these cells are probably exceedingly rare in the whole muscle.

In an effort to make a meaningful comparison of lung cells versus muscles cells, one could use the newly discovered muscle stem cells (MSCs) as potential sarcoma COs (Sacco et al. 2008). To test their tumorigenic potential, one could isolate these cells from *K-ras*$^{LSL\text{-}G12D}$; *Trp53*$^{flox/flox}$ animals, infect them with Ad-Cre, and transplant them back into recipients to see if they form tumors. If these initial experiments were promising, one could perform various PcG-associated ChIPs on early passage BASCs and MSCs to compare relative PcG occupancy to see if BASCs have enhanced PcG-mediated repression of *Ink4a/Arf*. This system would also allow for extensive ChIP analysis of a variety of other chromatin regulators both pre-and post-K-ras^{G12D} activation, which would provide further insight into the mechanism of differential K-ras^{G12D}-induced p19^{Arf} activation in these two tissues.

An alternative model to explain distinct p19^{Arf} activation in the context of similar oncogenic signaling levels is that both lung tumor and sarcoma COs have very similar chromatin structures within *Ink4a/Arf* prior to oncongene activation but once K-ras^{G12D} is expressed, the cell types undergo distinct chromatin remodeling, which results in different p19^{Arf} expression (Fig 4.1). In other words, Model 2 postulates that both normal lung and muscle cells repress *Ink4a/Arf* via PcG, but following an oncogenic insult PcG is removed specifically from the muscle cells. Since proximal oncogenic signaling is similar, this tissue-specific response must derive from how the absolute signaling levels are interpreted and/or the presence of tissue-specific factors that cooperate with those signals to mediate PcG eviction in one cell type and not the other.

During normal development as well as under stress conditions, PcG is removed from genomic loci by several mechanisms (Schuettengruber et al. 2007). Identifying differences among lung tumors and sarcomas in any one of the factors contributing to PcG displacement would provide support for Model 2 as a mechanism for tissue-specific $p19^{Arf}$ expression. The simplest explanation for tumor-type specific PcG occupancy is differential expression of PcG components, but this was not observed (Chapter 2). Therefore, other mechanisms of PcG removal were investigated. The p38MAPK pathway, through the ability of its downstream kinase MAPKAP3 to directly phosphorylate Bmi-1, has been implicated in mediating chromatin dissociation of PcG (Voncken et al. 2005), most notably in a recent study showing that inhibition of p38MAPK activity partially blocked the age-associated decline in Bmi-1 occupancy at *Ink4a/Arf in vivo* (Wong et al. 2009). As mentioned previously, I have been unable to detect differences in oncogenic signaling pathways, including p38MAPK, in lung tumors versus sarcomas, arguing against this mechanism. Nonetheless, it still might be informative to immunoprecipitate (IP) Bmi-1 from lung tumors and sarcomas to see if there is a difference in its phosphorylation status. In addition, IP'ing PcG would allow for a comparison of subunit composition across tumor types, as inclusion or exclusion of different factors might affect overall complex function. Another critical process during PcG eviction is the removal of the H3K27me3 mark, mediated by the recently discovered histone demethylases, Jmjd3 and Utx, with Jmjd3 playing a key role in many settings of Ras-induced Ink4/Arf activation (Agger et al. 2009; Barradas et al. 2009). To explore this mechanism I assessed Jmjd3 levels in sarcomas and lung tumors, expecting to see higher levels in sarcomas. However, this expression pattern was not observed. Interestingly, Jmjd3 levels were equivalent in normal lung and lung tumors, providing

a possible explanation as to why lung tumors retain extensive PcG regulation of *Ink4a/Arf* in the context of oncogenic Ras.

A final class of potential PcG-displacing factors is the SWI/SNF family of chromatin remodelers, known to be important in the antagonism of PcG-mediated gene repression in fruit fly development (Tamkun et al. 1992; Gebuhr et al. 2000). In Chapter 2, I directly implicate Snf5 in p19^{Arf} activation in sarcomas. As Snf5 has been shown to evict PcG from *Ink4a/Arf* in malignant rhabdoid tumors (Kia et al. 2008), it is conceivable that a similar mechanism occurs in sarcomas, with sarcoma COs initially repressing p19^{Arf} but then activating Snf5, removing PcG, and inducing the locus. An experiment to test this model would be to compare PcG binding at *Ink4a/Arf* in Snf5 knockdown and control tumors. Enhanced PcG occupancy at *Ink4a/Arf* in knockdown tumors would be expected of there is Snf5-dependent PcG removal. Additional work should also clarify the relationship between oncogenic K-ras signaling and Snf5 recruitment to *Ink4a/Arf*, as very little is known about how SWI/SNF components localize to their targets. To determine if K-ras^{G12D} directly affects Snf5 localization, initial studies could examine whether acute oncogenic signaling pathway perturbations (using chemical inhibitors or shRNA-mediated knockdown) are sufficient to remove Snf5 from *Ink4a/Arf* in sarcoma cell lines.

If Snf5 is a critical factor mediating p19^{Arf} induction in sarcomas, could it explain the tumor-type specific p19^{Arf} expression pattern? While Snf5 expression is identical in the two tumor types, it is possible that expression differences in another critical SWI/SNF component or overall SWI/SNF activity/chromatin localization could account for sarcoma-specific function. Technical difficulties with *in vivo* ChIP of Snf5 have precluded the ability to obtain definitive results with respect to its *Ink4a/Arf* localization in lung tumors versus sarcomas. To investigate any potential tissue-specific SWI/SNF components and/or function, one could IP the SWI/SNF

complex from both tumor types to identify differences in subunit composition and post-translational modifications, as multiple subunits are regulated by phosphorylation (Sif et al. 1998; Simone et al. 2004). Moreover, the activity of lung tumor and sarcoma-derived SWI/SNF complexes could be compared using *in vitro* nucleosome remodeling assays.

Regardless of any tissue-specific differences seen with these biochemical approaches, the function of Snf5 in lung tumorigenesis should be assessed. To this end, $K\text{-}ras^{LSL\text{-}G12D}$ animals could be infected with the Cre-shSnf5 lentiviral vector used for sarcoma formation. If Snf5 plays a role in the modest $p19^{Arf}$ induction and tumor suppression observed during lung tumor progression, then Snf5 knockdown would be expected to lead to larger, more advanced tumors compared to control shRNA infections. Parallel infections of $K\text{-}ras^{LSL\text{-}G12D}$; $Trp53^{flox/flox}$ animals could be used as additional controls to check if any observed differences between Snf5 and control shRNAs were dependent on the p53 pathway.

Aside from SWI/SNF and PcG, it is possible that the differential $p19^{Arf}$ expression described here is mediated by unidentified factors. An unbiased approach to search for such regulators would be to use a technique called promoter trapping (Jiang et al. 2006). This would involve incubating nuclear extracts from lung tumors and sarcomas with a synthesized fragment of the $p19^{Arf}$ promoter and then purifying protein-DNA complexes on columns that specifically bind the introduced DNA fragment. Using mass spectrometry to compare promoter-bound proteins from the two extracts might identify tissue-specific factors that could then be functionally tested for roles in $p19^{Arf}$ regulation.

Models 1 and 2 need not be mutually exclusive. In fact, given the drastic differences between lung and muscle cells (and most likely, COs for lung tumors and sarcomas), it is highly

probable that a combination of both models underlies the distinct expression pattern of p19^{Arf} downstream of oncogenic K-ras in these two tumor types.

p19^{Arf} activation in the lung

Genetic analysis of *Arf* deficient lung tumors in Chapter 2 indicated that the p19^{Arf}-p53 pathway inhibits progression of established tumors, suggesting that these tumors eventually do up-regulate this pathway to some extent. In support of this notion, a subset of lung lesions display elevated levels of GFP expression, and a significant proportion of tumors in aged *K-ras^{LA2}; Arf$^{GFP/+}$* animals show LOH of the wild-type allele of *Arf*. Defining the secondary events that ultimately lead to lung tumor induction of p19^{Arf} and comparing it to the factors imparting high expression in sarcomas is an important future direction. In addition, determining the stage of disease progression in which this happens will further our understanding of lung tumor development. While a cursory examination of signaling pathways between GFP^{hi} and GFP^{low} failed to reveal any correlations, this should be done on a larger panel of tumors of different histological grades using immunohistochemistry (IHC), in which signaling pathways, direct transcriptional regulators, and p19^{Arf} expression changes could be readily associated with particular stages of lung tumorigenesis. Given the presumed importance of chromatin regulation in p19^{Arf} expression, it would be ideal to assess PcG and SWI/SNF in GFP^{hi} versus GFP^{low} lung tumors as well, but sorting out the GFP status prior to ChIP would be technically challenging.

Tissue-specific Ink4a/Arf regulation and tissue homeostasis

The inability of lung cells to fully engage the *Ink4a/Arf* locus during oncogenic stress might explain the relative sensitivity of this tissue to K-ras^{G12D}-driven tumorigenesis. Conversely, the robust up-regulation of p19^{Arf} in muscle cells following oncogenic K-ras

expression affords significant tumor suppression and might underscore the rarity of soft-tissue sarcomas. As *Ink4a/Arf* regulation appears to be a major determinant of tumor susceptibility, a critical question is why different cell types would vary in their inherent ability to induce this locus. I propose that differential requirements for the maintenance of proper tissue homeostasis critically influence the activation threshold of *Ink4a/Arf* in different cell types. Lung cells are constantly exposed to harsh environmental stimuli due to their role in respiration and therefore have the potential to undergo extensive damage and possible cell death. Accordingly, cells in the lung must be able to readily proliferate in order to counteract this damage, which is ensured by strong negative regulation of the growth inhibitory *Ink4a/Arf* locus. Indeed, such a homeostatic role for PcG-mediated repression of *Ink4a/Arf* has been documented in both BASCs of the lung as well as islet cells of the pancreas (Dovey et al. 2008; Chen et al. 2009; Dhawan et al. 2009). In both cases, Bmi-1-dependent silencing of $p19^{Arf}$ and $p16^{Ink4a}$ was shown to be important for cellular regeneration following injury. In contrast to these epithelial tissues, muscles normally undergo much less regeneration and therefore do not require a high proliferative potential. Consequently, *Ink4a/Arf* is not subject to the same constraints and can be more readily activated under stress conditions. This model suggests that increased proliferative potential might come at the cost of enhanced susceptibility to oncogenic insults.

Stage-specific p53-dependent tumor suppression occurs in different tissues

Although mutations in the p53 pathway are thought to occur in most, if not all, human tumors, the precise role of this tumor suppressor axis in different cancer types is relatively unknown. Mutational data from human tumors indicates that *p53* alterations can occur in both early and more advanced stages of tumor progression depending on the tumor type (Greenblatt et al. 1994), although the functional significance of this variability is unknown. In numerous

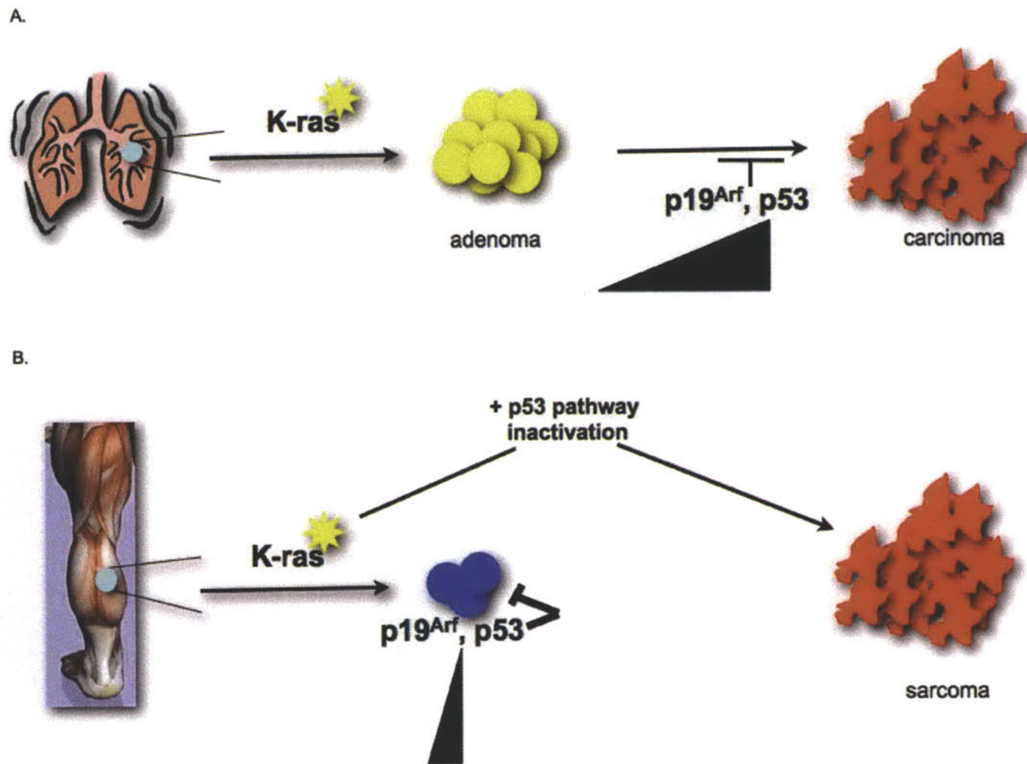
contexts, these mutations represent a relatively late event in the multi-step process to full malignancy. For example, in colon cancer, p53 disruptions have been linked to the transition from adenoma to carcinoma (Baker et al. 1990). Moreover, many mouse models of cancer incorporating *p53* mutations have documented equivalent tumor initiation but enhanced progression of lesions, further supporting a role for p53 in constraining later phases of tumorigenesis. These observations have been reported in tumor models of the lung, pancreas, and skin, among many others (Kemp et al. 1993; Hingorani et al. 2005; Jackson et al. 2005).

In contrast to these studies, in a mouse model of soft-tissue sarcoma driven by endogenous K-ras^{G12D}, p53 can have a strong suppressive role very early in tumor evolution. Initial generation of this model indicated that biallelic deletion of *Trp53* was required for oncogenic K-ras-driven tumor formation (Kirsch et al. 2007). Furthermore, as described in Chapters 2 and 3 of this thesis, careful analysis of animals in which was K-ras^{G12D} was induced in a p53-profient background suggested that p53 inhibits of the earliest steps of sarcomagenesis. This is most convincingly demonstrated in Chapter 3, when I delayeded p53 loss relative to oncogenic K-ras induction. Intriguingly, a short window of p53 activity severely limited the tumorigenicity of initially mutated cells, such that even after subsequent p53 deletion tumor burden was significantly reduced. These data indicate that p53 function is rapidly induced following an oncogenic insult in the muscle, which results in strong suppression of tumor initiation. Conversely, previous work on lung tumors initiated with the same oncogene (Jackson et al. 2005), as well as data presented in Chapter 2, suggest that the p53 pathway inhibits lung tumor progression. Thus, it appears that the timing and nature of p53-dependent tumor suppression varies across tumor types (Figure 4.2).

Strength of p53 function correlates with level of pathway activation

The simplest mechanistic explanation for the robust function of the p53 pathway in the muscle is that it is rapidly activated to a higher degree compared to those settings in which it has a weaker suppressive role (ie-the lung). Enhanced functional outputs of the p53 pathway have been linked to increased p53 levels previously. For example, Serrano and colleagues generated "super-p53" mice containing one extra copy of the p53 locus (Garcia-Cao et al. 2002). Following IR, thymocytes with three copies of p53 underwent more apoptosis than cells with two copies, indicating that higher levels of p53 allowed more cells to cross the threshold for induction of cell death. Importantly, super-p53 mice were also more resistant to two types of carcinogen-induced tumors, showing that cells with increased p53 can more effectively inhibit tumorigenesis *in vivo*. The sensitivity of output function to p53 levels was further highlighted by studies of endogenous versus overexpressed oncogenic Ras in MEFs. Despite the drastic phenotypic difference with respect to their growth properties, levels of p53 were only slightly altered between cells with different levels of Ras (Tuveson et al. 2004), implying that only small changes in pathway activation underlie the decision to senesce or proliferate.

The differential induction of $p19^{Arf}$ described in Chapter 2 and discussed in the previous section conveniently aligns with the hypothesis that sarcomas engage the p53 pathway to a



<u>Figure 4.2:</u> The p53 pathway suppresses different stages of lung tumor and sarcoma development.

A. p53 suppresses lung tumor progression. Following oncogenic K-ras expression, many hyperplasias and small adenomas form in the lung, and these initial steps towards malignancy are not appreciably altered in the context of p53 pathway mutations. However, more advanced stages of the disease, such as carcinoma development, rely on the loss of this pathway. This suggests that $p19^{Arf}$ and p53 block the progression of adenomas. This most likely results from their increased expression during this stage (indicated by the gradient - black triangle), although this has yet to be proven. **B.** p53 suppresses the initiation of sarcomagenesis. $K\text{-}ras^{G12D}$ activation must be soon accompanied by p53 loss in order for efficient sarcoma formation to take place, indicating that rapid and robust p53 pathway activation occurs in muscle cells following an oncogenic insult. As no lesions of any size can be found without the combined mutation of K-ras and p53, it appears that the earliest steps of transformation are inhibited by the p53 pathway.

greater extent than lung tumors. As p19^{Arf} is a critical inducer of p53, it follows that cells with more p19^{Arf} should have higher p53 levels as well. In support of this, reactivation of p53 in established lung tumors and sarcomas from *K-ras*^{LA2}*; Trp53*$^{LSL/LSL}$*; R26*$^{CreER/CreER}$ animals, which contain a LSL cassette in p53 that creates a null allele but allows for endogenous p53 expression following tamoxifen injection, led to much higher levels of p53 protein stabilization in sarcomas compared to lung tumors (D. Feldser, personal communication). Furthermore, reactivation of p53 in lung tumor cell lines, which contain much higher levels of p19^{Arf} than *in vivo* lung tumors, resulted in a robust p53-dependent arrest, suggesting that increasing p19^{Arf} can drive an enhanced p53 response. Because the p53 pathway strongly inhibits the earliest events of sarcomagenesis *in vivo*, high levels of p19^{Arf} should be present in muscle cells soon after oncogenic K-ras induction. While this hypothesis remains untested, it could be examined by performing p19^{Arf} (or GFP if using *Arf*^{GFP}) IHC on *K-ras*$^{LSL\text{-}G12D}$ muscles shortly after Cre infection. Conversely, as p53 appears to only effectively inhibit the later stages of lung tumorigenesis, only advanced lesions should be marked by increased p19^{Arf}-p53 levels. As explained in the previous section on p19^{Arf}, performing IHC on lung tumors of various grades would address this question.

How increased levels of p53 affect its growth suppressive abilities is unknown. It could simply be that more p53 results in greater induction of the same target genes, and their increased abundance more effectively mediates arrest or death. Alternatively, high p53 levels could engage entirely new genes that promote a more robust functional output. To address this, one would need a system allowing for careful adjustment of p53 levels that correlate with distinct functional outcomes. One possibility is the MEF system expressing different levels of oncogenic Ras as described above. Performing microarrays on cells expressing endogenous versus overexpressed

Ras (+/- p53 to identify p53-dependent changes) would generate a list of genes associated with high and low levels of p53 activity. Comparing gene sets and well as fold induction of particular transcripts between the groups would reveal whether levels and/or targets change most significantly. Regardless of the precise mechanism, it appears that the strength and nature of p53-dependent tumor suppression directly correlates with the intensity of pathway activation.

Other factors governing tissue-specific p53 functions

Aside from $p19^{Arf}$ driving different levels of activated p53, additional cell-autonomous and non-cell autonomous differences in p53 function could affect the nature and strength of tumor suppression. Although I have been unable to observe markers of senescence in the muscle, this program might still be engaged but below current levels of detection. Recent studies have shown that during senescence, p53 induces a unique gene expression program consisting of cytokines and ECM proteins that serve to activate the innate immune system to clear affected cells (Xue et al. 2007; Krizhanovsky et al. 2008). It is conceivable that muscle cells specifically induce these programs (cell autonomous), or that the microenvironment in the muscle is more conducive to immune cell interaction (non-cell autonomous). Microarray studies of $K\text{-}ras^{LA2}$; $Trp53^{LSL/LSL}$; $R26^{CreER/CreER}$ lung tumors and sarcomas post-tamoxifen would highlight any gene expression differences in regards to senescence programs. Apart from different levels of p53 driving distinct gene expression in lung and muscle cells (last section), cell-type specific post-translational modifications of p53 could also be responsible. This could be studied by applying mass spectrometry to IP'd p53 in the two cell types. Finally, to address potential non-cell autonomous factors mediating tissue-specific responses to p53, one could attempt to transplant $K\text{-}ras^{LA2}$; $Trp53^{LSL/LSL}$; $R26^{CreER/CreER}$ lung tumor and sarcoma cell lines into the lung and/or

muscle, activate p53, and assess the degree of immune cell interaction and/or cell clearance over time in the two different locations.

Transient p53 activity can provide significant protection through cell cycle arrest

Whether transient p53 activity affords significant tumor suppression, and if such a response is dependent on the stage of tumorigenesis, is largely unknown. Several recent studies reactivated p53 in established tumors and witnessed dramatic acute responses, and in some cases, long lasting tumor suppression through both cell cycle arrest and apoptotic mechanisms (Martins et al. 2006; Ventura et al. 2007; Xue et al. 2007). While the initiating oncogene was known for some of these tumors, it is unclear whether oncogene overexpression and/or additional stresses that accumulated during tumor progression played a role in engaging the robust p53 response. The sequential mutagenesis experiments in the muscle showed that maintaining p53 activity for only a week or two following oncogenic K-ras expression dramatically reduced the tumorigenecity of the initially targeted cells, even if p53 was subsequently removed. This suggests that in some settings, oncogenic signals downstream of endogenous K-ras^{G12D} are sufficient to fully induce strong p53 responses. In contrast, work from an IR-induced lymphomagenesis model has shown that the complete protection from tumorigenesis afforded by p53 in this context was entirely abolished if this tumor suppressor was deleted at any time after IR, indicating that targeted cells retained their tumorigenic potential and persisted within the animal, perhaps because they lacked the appropriate stimulus for significant p53 activation (Hinkal et al. 2009).

The ability of transient p53 activity to provide long-lasting protection in the muscle might relate to its mode of tumor suppression. While clearance of cells through apoptosis is an obvious route to irreversible tumor suppression, genetic analysis indicated that this is unlikely to be the

mechanism of p53-dependent protection in the muscle. Instead, p21, a mediator of cell cycle arrest/senescence, was implicated downstream of p53, suggesting that cell cycle arrest can have long-term tumor suppressive roles. Senescent cells have recently been shown to be cleared by the innate immune system in some contexts (Zilfou and Lowe 2009), and such a response could result in the loss of tumorigenic potential over time, as just a short pulse of p53 activity was shown to completely eradicate liver tumors through such a mechanism (Xue et al. 2007). Interestingly, *in vivo* restoration of p53 in $Trp53^{LSL/LSL}$; $R26^{CreER/CreER}$ sarcomas led to cell cycle arrest and subsequent tumor regression (Ventura et al. 2007), indicating that clearance of arrested cells might occur in this tissue as well. More detailed studies following the fate of targeted muscle cells need to be undertaken to determine if K-ras^{G12D}+ p53+ cells are progressively lost or maintained. For future experiments performed during tumor initiation, it will be helpful to separate viral infection from K-ras^{G12D} activation, as the innate immune system's response to the infection might make it difficult to interpret immune infiltrates and cell clearance. To this end, one could use a bifunctional lentivirus encoding GFP and CreER, such that oncogenic K-ras induction would be controlled by tamoxifen injection, which could take place weeks after the initial infection.

Although senescent-cell clearance may be important, initial cell fate experiments have identified infected cells in $K\text{-}ras^{LSL\text{-}G12D}$; $Trp53^{fl/+}$ animals at time points in which p53 deletion failed to promote sarcomagenesis in sequential mutagenesis experiments. This suggests that some cells persist but are unable to respond to p53 loss. As these cells might represent cells in an irreversible state of arrest, their further characterization, especially in regards to senescence markers, is warranted.

Temporal requirements for p53 pathway mutations might affect cancer prevalence

Simultaneous mutation of *K-ras* and *p53* efficiently promotes sarcomagenesis (Kirsch et al. 2007). Additionally, K-ras ^{G12D} can readily induce sarcomas in a *p53*-deficient background. In contrast, delaying p53 loss relative to K-ras^{G12D} activation limits tumorigenesis in the muscle (Chapter 3). Together, these results suggest that the timing of *p53* mutation influences sarcoma development, specifically implying that *p53* alterations need to occur early during the multi-step transformation of muscle cells. Does the human data support this claim? While p53 is frequently mutated in many types of sarcomas, such stage-specific information is lacking (Taubert et al. 1998). Typically, such studies are done by performing mutational or expression analyses in early versus more advanced tumor samples. Because early sarcoma lesions are rarely found, it is difficult to carry out these comparisons. However, the relatively high rate of sarcomas observed in Li-Fraumeni patients, who inherit a germline mutation in *p53*, does provide evidence that in the context of a pre-existing *p53* alteration, sarcomagenesis is much more prevalent (Kleihues et al. 1997). This might suggest that *p53* mutations are initiating events in sarcomas.

Requiring p53 as an initiating, or at least a very early mutation, might help to explain the rarity of sarcomas. Owing to its low expression under normal conditions, solely mutating *p53* does not provide a growth advantage, so this event is not selected via clonal expansion of cells *in vivo* (Kinzler and Vogelstein 1996). In contrast, other mutations that confer immediate hyperproliferation, like K-ras^{G12D}, often promote initial cellular expansion, thus creating a larger pool of cells as potential targets for additional hits and increasing the likelihood of overt transformation. In the muscle, such a pro-proliferative mutation might have to occur in a cell already mutant for *p53*, as targeting a *K-ras* mutation to a wild-type cell would result in robust tumor suppression and lack of hyperproliferation, or at least a very slow expansion that would

limit the possibility of secondary mutations. However, because initiating *p53* mutations fail to cause clonal selection, such a cell represents a very low frequency target for subsequent oncogene activation. Consequently, the chances of such an event occurring in this particular cell would be low, and therefore sarcoma development is rare.

This is in contrast to a number of other tissue types in which p53 pathway alterations comprise later events that control more advanced stages of disease progression, such as in the lung (Kohno et al. 1999; Yamasaki et al. 2000; Jackson et al. 2005). In these settings, cells can be targeted with an initiating mutation that provide an immediate growth advantage, such as K-ras^{G12D}, even in the presence of p53, presumably because this tumor suppressor is not significantly induced. This generates increased numbers of partially transformed cells, some of which might be responsive to secondary mutations and further progression towards malignancy.

In conclusion, the stage at which tumors require *p53* mutation relates to how strongly different cell types engage its associated tumor suppressor pathways at distinct stages during tumor evolution. Furthermore, specific temporal requirements for p53 pathway alterations might be one factor affecting the frequency of different cancers.

Next steps with *in vivo* shRNA technology

The ability to manipulate gene expression with shRNA technology has provided an efficient way to conduct loss-of-function studies. Additionally, the potential of scaling-up this approach has afforded the opportunity to carry out large-scale screens, which have been successfully performed *in vitro* and *in vivo*, although *in vivo* screens have relied on transplantations thus far (Zender et al. 2008; Bric et al. 2009; Meacham et al. 2009). In Chapters 2 and 3 I demonstrate that a lentiviral-based shRNA approach can promote tumor formation in

an autochthonous sarcoma model. This system could be more broadly used as a screening platform for tumor suppressor genes inhibiting K-ras^{G12D}-driven sarcoma development. Given the lack of any background with regards to tumor formation, this would be a highly sensitive, easily assayed screen. Using *K-ras$^{LSL-G12D}$; Rag2$^{-/-}$* animals, one could infect muscles with a pool of Cre-shRNA lentiviruses and monitor for macroscopic masses. The extent to which one could pool different viruses could be assessed by diluting a positive control virus (Cre-shp19^{Arf}/p16^{Ink4a}) and determining how dilute one could go while still achieving a reasonable time to tumor formation. While any number of genes and pathways might be implicated in such a screen, one could use a sensitized background (*K-ras$^{LSL-G12D}$; Arf$^{GFP/+}$; Rag2$^{-/-}$*) to bias the hits towards those affecting the p19^{Arf}-Mdm2-p53 pathway. Additionally, even more targeted screens could be performed, for example by focusing solely on putative transcriptional targets of p53 identified in gene expression studies from *K-ras^{LA2}; Trp53$^{LSL/LSL}$; R26$^{CreER/CreER}$* sarcomas in which p53 has been reactivated. Performing these types of screens could potentially identify novel genes in the p53 pathway that are specifically important in an *in vivo* setting.

Future applications of dual SSR technology

The dual SSR strategy employed in Chapter 3 provided new insights into the interaction between oncogenic K-ras and the p53 pathway. Combining *K-ras$^{FSF-G12D}$* with additional Cre-loxP regulated genes will allow many more genetic interactions to be investigated in a temporal fashion. However, due to technical problems regarding the ability to target both Flpo and Cre activity specifically to the same cells, the list of potential genes to include in such studies is severely limited. At present, only *R26$^{CreER-T2}$* has been used in conjunction with viral-Flpo, meaning that the Cre controlled gene is recombined throughout the organism. As a result, floxed

alleles of genes that are essential in particular adult tissues cannot be used. To circumvent this problem, one could generate a Flpo-inducible allele of CreER (FSF-CreER) targeted to a ubiquitously expressed locus, such as *R26*. In this way, CreER would only be expressed in cells that had already been exposed to Flpo, so systemic tamoxifen would recombine floxed alleles only in those cells that express oncogenic K-ras. Such a system for tumor-specific second hits would open up the possibility of efficiently deleting various K-ras effectors in established tumors, which is currently only feasible with partial shRNA-mediated knockdown (Appendix A). Being able to assess acute versus long-term effects of pathway inhibition will provide important mechanistic insights into Ras signaling in tumor maintenance. Additionally, one could combine this system with small molecule inhibitors targeting distinct signaling modalities in order to identify potential interactions among different pathways that could be harnessed therapeutically. To generate more advanced stages of lung tumorigenesis for these studies, a Frted allele of p53 ($Trp53^{Frt}$) could be incorporated with $K\text{-}ras^{FSF\text{-}G12D}$.

Some cancer-associated pathways are thought to play distinct roles in tumorigenesis at different stages of the disease. For example, alterations in the TGFβ pathway has been implicated in both tumor suppressive and oncogenic roles depending on whether they are an early or late event in tumor progression (Bierie and Moses 2006). Presently, such effects are difficult to model and must be inferred by assessing different time-points after a gene has been manipulated at tumor initiation. Now such stage-specific effects can be systematically studied in a variety of K-ras driven tumor models using $K\text{-}ras^{FSF\text{-}G12D}$ and already available Cre-LoxP strains, by initiating tumors and then subsequently deleting the second gene at different times during tumor development.

A very important future application of this technology will be to further test the functional significance of particular sequences of mutations on cancer initiation and progression. The multi-step nature of human tumorigenesis has been most carefully studied with regards to mutation of *APC* and *K-ras* in colon cancer, and Vogelstein and colleagues have proposed specific hypotheses relating to the importance of deleting *APC* prior to oncogenic K-ras activation (Kinzler and Vogelstein 1996). Combining $K\text{-}ras^{FSF\text{-}G12D}$ with a Cre-regulated allele of *APC* (APC^{2lox14}) (Colnot et al. 2004) would provide the opportunity to directly test their predictions. To ensure correct timing of the two mutations in the proper cells, additional mouse strains would have to be generated. Figure 4.3 contains an experimental outline.

While all of the potential uses of dual SSRs detailed thus far target two genes in the same cells, one could also spatially separate two events in completely different cell types. As there is accumulating evidence that mutations occurring in distinct cell types can drive tumor progression, such an approach will be extremely useful. For example, it has been suggested that p53 can have tumor suppressive functions outside of the cancer cells themselves (Kurose et al. 2002; Matsumoto et al. 2003). In one study involving a prostate cancer mouse model, a massive stromal cell response was noted during tumor progression, which was dependent on p53 mutation in the invading cells (Hill et al. 2005). While they performed functional analyses using germline p53 mutations, a less confounding approach would have been to investigate the specific contribution of p53 in particular cell types using specific Cre lines in combination with $Trp53^{flox}$. At present, such a strategy is feasible only with tumor models not reliant on Cre-LoxP for tumor initiation, limiting the number of models one could use. With $K\text{-}ras^{FSF\text{-}G12D}$ one could generate a variety of tumor types while maintaining the ability to use Cre-LoxP to target separate genes in non-tumor cells. This system could be very useful in studying tumor cell-immune cell

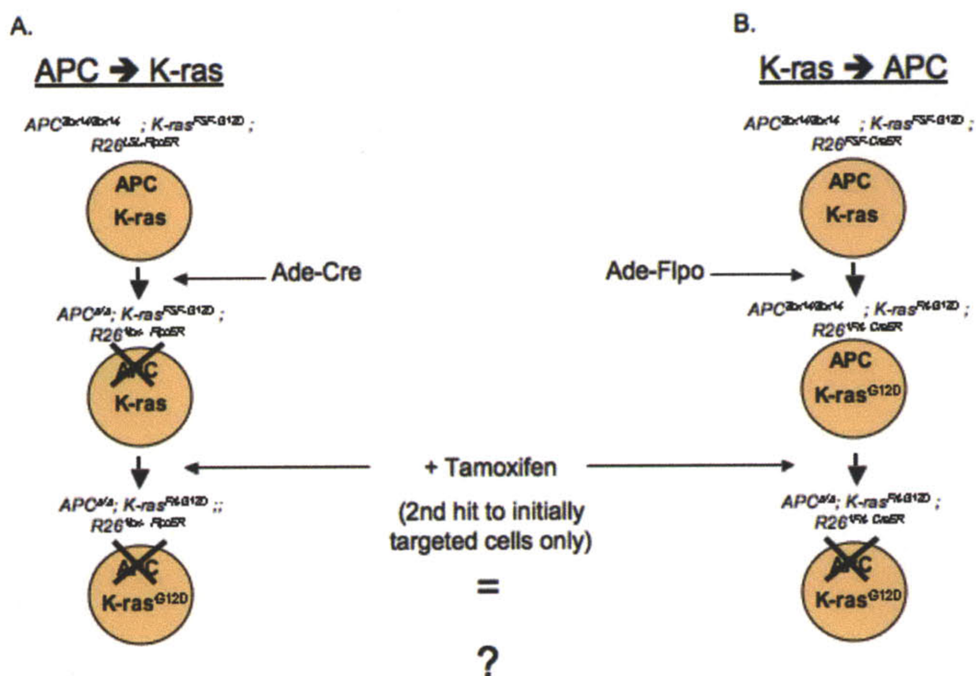


Figure 4.3: Experimental outline to test the importance of the order of *APC* and *K-ras* mutations during colon cancer initiation and progression.

A. Strategy for APC loss followed by K-ras^{G12D} activation. To initiate tumors, intracolonic Ad-Cre could be given to delete the floxed alleles of *APC* (*APC$^{2lox-14}$*) (Hung et al. 2010). *R26$^{LSL-FlpoER}$*, which would need to be generated, contains a tamoxifen regulated version of Flpo in the *R26* locus, and would initiate expression of Flpo in the same cells following Cre-mediated removal of the STOP cassette. Subsequent administration of tamoxifen would activate FlpoER in cells deficient in APC, thus turning on expression of oncogenic K-ras in these cells only. **B.** Strategy for K-ras^{G12D} activation followed by APC deletion. The same alleles of *APC* and *K-ras* would be used. For this sequence of events, Ad-Flpo infection would activate K-ras^{G12D} as well as induce transcription of *R26$^{FSF-CreER}$* by removal of the STOP cassette. Tamoxifen injection would promote Cre activity in oncogenic K-ras+ cells, leading to the deletion of floxed APC.

interactions, a subject of growing interest in the context of autochthonous tumor models. One could envision using *K-ras*$^{FSF\text{-}G12D}$ in conjunction with immune cell-type specific Cre lines to delete genes implicated in the dynamic interaction between tumor and immune cells. While this type of study could be done presently by transplanting cells from genetically modified mice, use of an endogenous immune system would offer important advantages.

Clearly, the ability to spatiotemporally control distinct genetic events creates the possibility for many applications. Hopefully, this potential will be realized, and these systems will provide important insights into tumor biology.

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APPENDIX A

Using conditional RNAi *in vivo* to interrogate Ras effectors in lung tumors maintenance

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Nathan Young performed all experiments except for intratracheal infections, which were done by Michel DuPage. All experiments were done in the laboratory of Tyler Jacks.

INTRODUCTION

Lung cancer, causing more than 1 million deaths annually, represents the leading cause of cancer mortality worldwide (Parkin et al. 2005). Among the various subtypes of the disease, one of the most common is adenocarcinoma, within the broader classification of non-small cell lung cancer NSCLC (Parkin et al. 2005). Activating mutations in the *Kras* proto-oncogene occur in approximately 30% of adenocarcinomas (Rodenhuis et al. 1988). In an attempt to model the disease in mice, our lab has engineered an inducible allele of the oncogenic K-ras mutation that when activated in the lung epithelium generates a tumor phenotype closely recapitulating many aspects of the human disease, including gene expression signature and histopathologic progression (Jackson et al. 2001; Sweet-Cordero et al. 2005). Introduction of a loss-of-function allele of the *Trp53* gene, another locus frequently mutated in NSCLC, drives tumor progression to more advanced stages such as metastasis, further validating the mouse model (Jackson et al. 2005).

Most conventional therapeutic strategies for NSCLC, such as platinum-based chemotherapeutics, have met with only limited success (Provencio et al. 2009). An emerging paradigm in cancer treatment is the use of targeted therapies, in which information regarding the underlying molecular genetic defects of the cancer cells informs specific intervention strategies that show improved efficacy and reduced general toxicity (Murdoch and Sager 2008). In the case of NSCLC, the high frequency of K-ras mutations makes this particular gene and the pathways it controls potential targets for new and improved treatments. Although numerous studies, both in human cells and in the mouse, have shown the therapeutic utility of disabling the driving oncogenic mutation (Chin et al. 1999; Fisher et al. 2001; Singh et al. 2009), the K-ras protein has

proven very difficult to target with small molecule compounds. K-ras sits near the top of a very complex array of overlapping signaling pathways such as MAPK, PI3K, and Ral, and it is conceivable that any number of these might be critically important for the ability of this oncogene to initiate and promote tumorigenesis (Downward 2003; Repasky et al. 2004; Karnoub and Weinberg 2008). Therefore, much of the therapeutic focus has shifted to these major effector pathways downstream of K-ras.

To date, most studies regarding the role of effector pathways in Ras-driven tumorigenesis have been impaired due to experimental limitations. Generally, investigators have used poorly controlled chemically-induced tumor models with germline effector knockouts or combined Cre-inducible tumor models with floxed alleles of genes of interest (Malliri et al. 2002; Gonzalez-Garcia et al. 2005; Kissil et al. 2007). While these studies have reported dramatic phenotypic consequences in the context of specific pathway inhibitions, the results have been restricted to tumor initiation due to experimental set-up. In contrast, by administering targeted therapies to established Ras-dependent malignancies, several groups have recently highlighted the benefits of pathway abrogation in developed tumors (Engelman et al. 2008; Johannessen et al. 2008). First, these studies have demonstrated a requirement for certain pathways in tumor maintenance, which is much more clinically relevant than effects on tumor initiation. In addition, they enabled detailed analyses of the mechanism of tumor inhibition, which are often difficult to perform if tumorigenesis is completely blocked at the earliest stages.

The sequential mutagenesis technology introduced in Chapters 2 and 3 permits secondary genetics events to be separated from oncogenic K-ras activation. In this Appendix this system is applied to the study of Ras effectors in lung tumor maintenance *in vivo*. Specifically, by combining the Flp-inducible allele of K-ras^{G12D} (*K-ras*$^{FSF-G12D}$) reported in Chapter 3 with a

lentiviral-based modality for Cre-regulated RNAi (introduced briefly in Chapter 2), gene knockdown can be induced after tumors have been established. This new technology has the potential to be used for the systematic genetic analysis of a large number of putative therapeutic targets.

RESULTS and DISCUSSION

A novel system to separate K-ras^{G12D} activation from Cre-inducible RNAi

The integration of established Cre-LoxP reagents with *K-ras$^{FSF\text{-}G12D}$* allows for unique spatiotemporal control of two distinct genomic alterations. While Chapter 3 highlighted the utility of this system when using germline floxed alleles as secondary events, this approach is hindered by the availability of such targeted alleles, as well as by the inability to sequester Cre activity specifically to tumor cells. We reasoned that substituting a Cre-controlled RNAi platform for floxed alleles would enable us to overcome these limitations. To this end, we began by introducing Flpo recombinase into a previously constructed lentiviral system for Cre-regulated shRNA-mediated knockdown (pSICO), creating pSICO-Flpo. This vector contains a LSL-shRNA element that prevents shRNA induction until Cre is expressed (Ventura et al. 2004). In addition, we generated compound mutant mice containing *K-ras$^{FSF\text{-}G12D}$* and *R26$^{CreER\text{-}T2}$* (Ventura et al. 2007), an allele which provides inducible Cre activity *in vitro* and *in vivo* (see Chapter 3). As shown in Figure 1A, infection of *K-ras$^{FSF\text{-}G12D}$; R26$^{CreER\text{-}T2}$* cells and/or animals with pSICO-Flpo will activate oncogenic K-ras, and the integrated lentivirus would subsequently express a shRNA following tamoxifen administration, but only in cells originally exposed to Flpo. In summary, this approach permits the separation of K-ras^{G12D}-dependent tumor initiation from tumor cell-specific gene knockdown.

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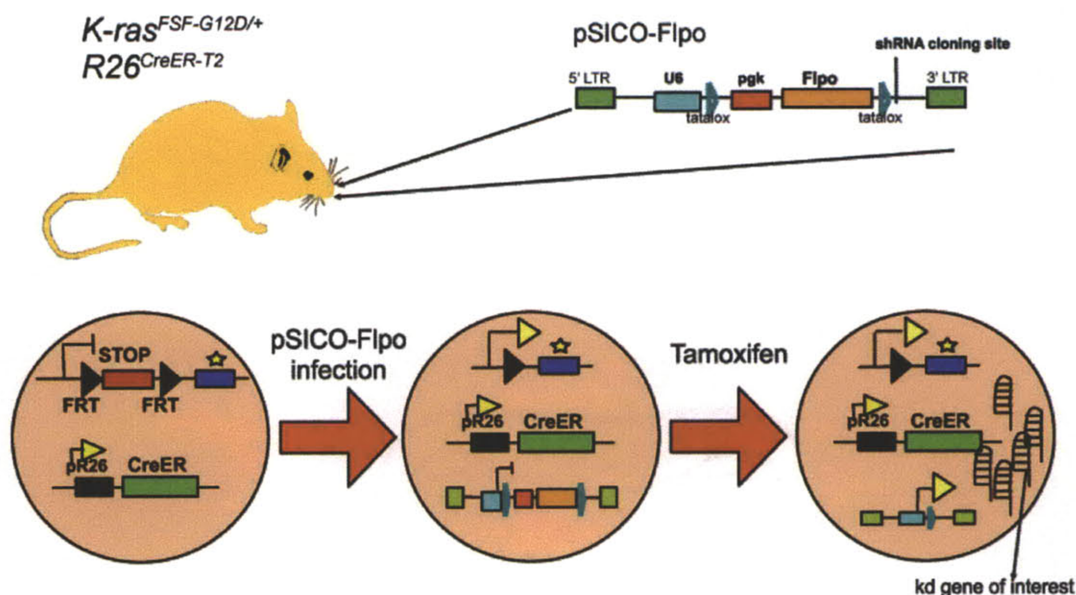

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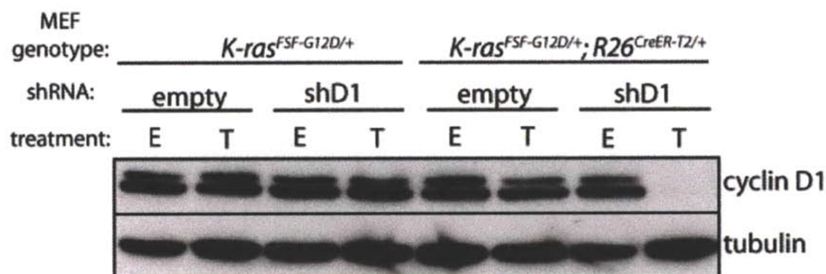

Figure 1: A new system for Cre-inducible RNAi following Flpo-mediated activation of K-ras^{G12D}.

(**A.**) Overview of the system, showing genotype of mice, the configuration of pSICO-Flpo, and a schematic of the ordered events in infected cells. See text for details. (**B.**) Western blot analysis of cyclin D1 levels in *K-ras$^{FSF\text{-}G12D/+}$* and *K-ras$^{FSF\text{-}G12D/+}$; R26$^{CreER\text{-}T2/+}$* MEFs infected with pSICO-Flpo without hairpin (empty) or pSICO with a hairpin to cyclin D1 (shD1). After infection cells were split and either treated with 4-OHT (T) or ethanol (E). Knockdown occurs with cyclin D1 shRNA in a CreER and 4-OHT-dependent manner.

To initially test this system, we performed sequential K-ras^{G12D} activation and gene knockdown experiments in MEFs. We infected *K-ras*$^{FSF\text{-}G12D/+}$ (FK) and *K-ras*$^{FSF\text{-}G12D/+}$*; R26*$^{CreER\text{-}T2/+}$ (FKC) MEFs with either empty pSICO-Flpo or one containing a shRNA to cyclin D1. Following infection, each was split into two groups that received either 4-hydroxytamoxifen (4-OHT) or vehicle control (ethanol). All cells up-regulated cyclin D1, indicating that Flpo activated K-ras^{G12D} (data not shown). Furthermore, as shown in Figure 1B, MEFs infected with the cyclin D1 hairpin achieved target knockdown in a CreER and 4-OHT-dependent manner. Together, these results demonstrate that this dual system for oncogenic K-ras activation and conditional RNAi system works efficiently *in vitro*.

Inducible knockdown of cyclin D1 in established lung tumors

We next tested this scheme in the context of lung tumor development *in vivo*. First, we ensured that in this setting the system would be highly inducible yet not leaky. As Cre-mediated induction of shRNA expression relies on recombination of the integrated lentivirus, we used multiplex PCR to monitor for this irreversible genomic rearrangement under different conditions. Accordingly, a group of *K-ras*$^{FSF\text{-}G12D/+}$*; R26*$^{CreER\text{-}T2/Cre\text{-}T2}$ mice with pSICO-Flpo-induced lung tumors were injected intraperitoneally (i.p.) with tamoxifen or vehicle control (corn-oil) for several days, and one week later tumors were harvested for genomic PCR analysis. While tumors from tamoxifen-treated mice displayed significant recombination, those in the corn-oil treated group showed no evidence of this genomic event, indicating that this system afforded inducibility without noticeable leakiness (Fig 2A).

When considering targets for initial knockdown studies *in vivo*, we noted that cyclin D1 was consistently induced in K-ras^{G12D}-driven lung tumors (Fig 2B). Given our ability to achieve robust depletion of cyclin D1 (Fig 1B), we decided to use this gene in proof-of-principle

experiments. We infected a cohort of *K-ras*$^{FSF\text{-}G12D/+}$; *R26*$^{CreER\text{-}T2/Cre\text{-}T2}$ mice with pSICO-Flpo containing no hairpin, a control hairpin to luciferase, or one targeting cyclin D1. After 20-24 weeks of tumor development, we administered several doses of tamoxifen to induce shRNA expression. One week later, one group of animals was sacrificed and tumors were harvested to assay for acute knockdown. Within this group, many tumors containing the cyclin D1 shRNA displayed decreased levels of the protein, although some variability was observed (Fig 2C). A separate group of mice was aged an additional 6 weeks after tamoxifen treatment to assess the stability of the knockdown. Importantly, these tumors also showed reduced cyclin D1, indicating that long-term knockdown was possible with this system (Fig 2D).

As cyclin D1 up-regulation is known to be important for the G1/S transition during cell cycle progression, we tested if the level of depletion we observed was sufficient to inhibit S-phase entry. To this end, mice were pulsed with BrdU to monitor for cells entering S-phase. Immunohistochemical analysis of BrdU+ cells revealed a statistically significant decrease in cells incorporating BrdU in tumors that had induced the cyclin D1 shRNA (Fig 2E-only short-term knockdown mice were analyzed). This suggests that the extent of knockdown provided by pSICO-Flpo can have functional effects in established tumors *in vivo*.

Targeting K-ras effectors

In light of these results showing that pSICO-Flpo provided inducible knockdown in established K-ras^{G12D}-driven lung tumors, we applied this technology to the study of Ras effectors in lung tumor maintenance. To begin, we analyzed the activation status of a variety of effector pathways in K-ras^{G12D} lung tumor models. Interestingly, both the PI3K and Ral

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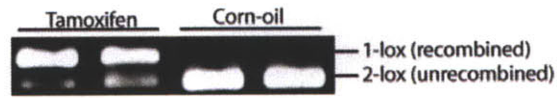

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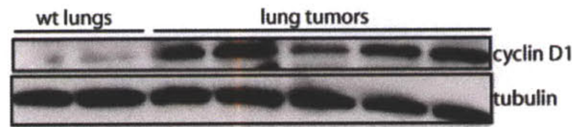

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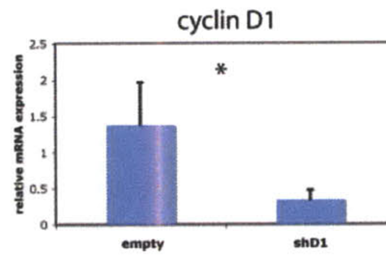

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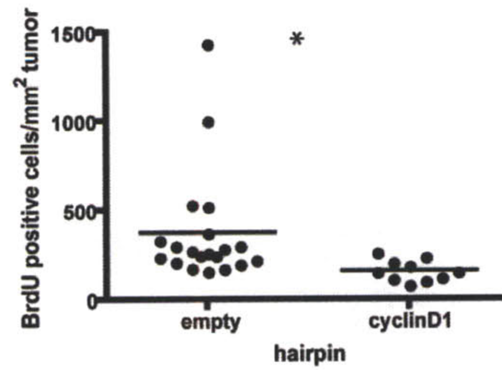


Figure 2: Inducible knockdown of cyclin D1 in established lung tumors.
(A.) PCR analysis of lentiviral recombination in lung tumors. *K-ras*$^{FSF\text{-}G12D/+}$; *R26*$^{CreER\text{-}T2/CreER\text{-}T2}$ (FKC) mice were infected with pSICO-Flpo to induce lung tumors and 20 weeks later were treated with tamoxifen or corn-oil. One week later tumors genomic DNA was harvested **(B.)** cyclin D1 immunoblot in lungs and oncogenic K-ras-driven lung tumors. **(C.)** Western blot analysis showing acute knockdown of cyclin D1 in established lung tumors. FKC mice were infected with pSICO-Flpo containing shLuc or sh cyclin D1 (shD1) and treated with tamoxifen 20 or 24 wks post infection. One week after the last treatment, lung tumors were harvested. The left panel shows two different areas (separated by the vertical line) of the same exposure of the same blot and is an example of consistent knockdown. The right panel shows the variability that sometimes occurred, with one tumor displaying substantial knockdown and another tumor from the same mouse showing none. **(D.)** qRT-PCR analysis of cyclin D1 mRNA levels in lung tumors with long-term shRNA expression. These experiments were performed with a separate cohort of mice from **C.**, which were aged 6 weeks after the final tamoxifen treatment. N = 2 (shLuc), N = 4 (shD1). *, $P < .05$. Error bars = standard deviation. **(E.)** Quantitation of BrdU+ cells in lung tumors from mice treated as in **C.** *, $P < .05$.

pathways were down-regulated in lung tumors when compared to whole lung lysates, while MAPK showed more variability (Fig 3). The reasons for this signaling attenuation are unknown but could be due to the induction of feedback inhibitors such as Sprouty2 or Mkp3, both of which have been shown to negatively regulate endogenous K-ras^{G12D}-associated signaling in other settings (Shaw et al. 2007; Haigis et al. 2008).

Despite our inability to detect consistent increases in signaling outputs among any of the pathways analyzed, we designed hairpins to a number of key components in these networks, reasoning that the absence of hyperactivation did not preclude the possibility that pathway inhibition would affect tumor cells. During initial tests, the two most efficient shRNAs targeted c-Raf and p110α, reducing their mRNA levels by more than 75% (Fig 4A and B). To gain insight into potential phenotypes to assay *in vivo*, we studied the effects of c-Raf and p110α knockdown on lung tumor cell lines. Biochemical analyses indicated that p110α depletion significantly reduced phospho-Akt levels as well as mTOR signaling, as shown by a strong reduction in phospho-S6 (Fig 4C). These results suggested that despite expressing both p110α and p110β (data not shown), inhibition of just one p110 family member disrupted PI3K signaling in K-ras^{G12D}-driven lung tumors. p110α knockdown also inhibited MAPK signaling, highlighting the extensive cross-talk known to occur among these pathways. Interestingly, although phospho-MEK decreased, its downstream target Erk remain phosphorylated (Fig 4C). Similar results were seen with c-Raf knockdown, illustrating that the lung tumor cell line used in these studies signals through a non-canonical MAPK pathway, which might also occur *in vivo*. Importantly, both shRNAs caused cellular phenotypes, including a robust cell cycle arrest (data not shown) as well as striking changes in cell morphology (Fig 4D). Together, these data indicated that both c-Raf and p110α were good candidates to test *in vivo*.

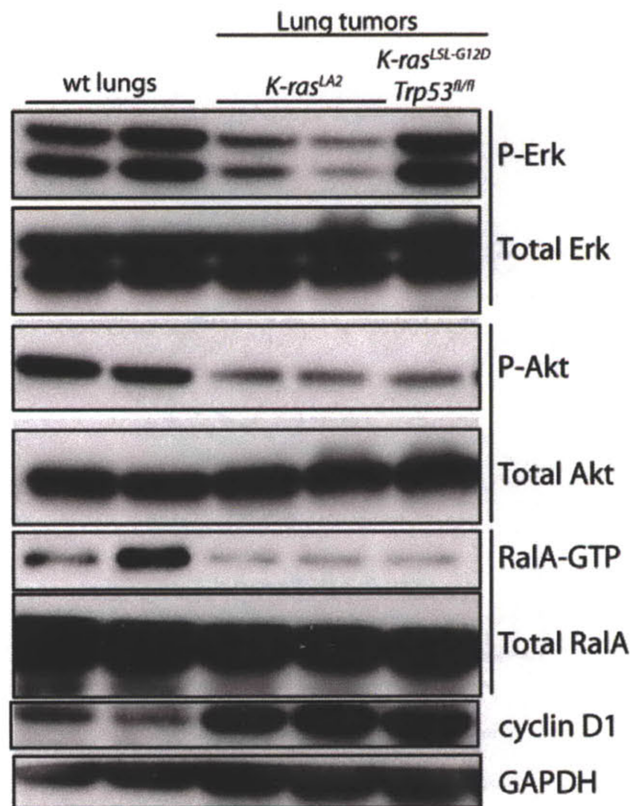
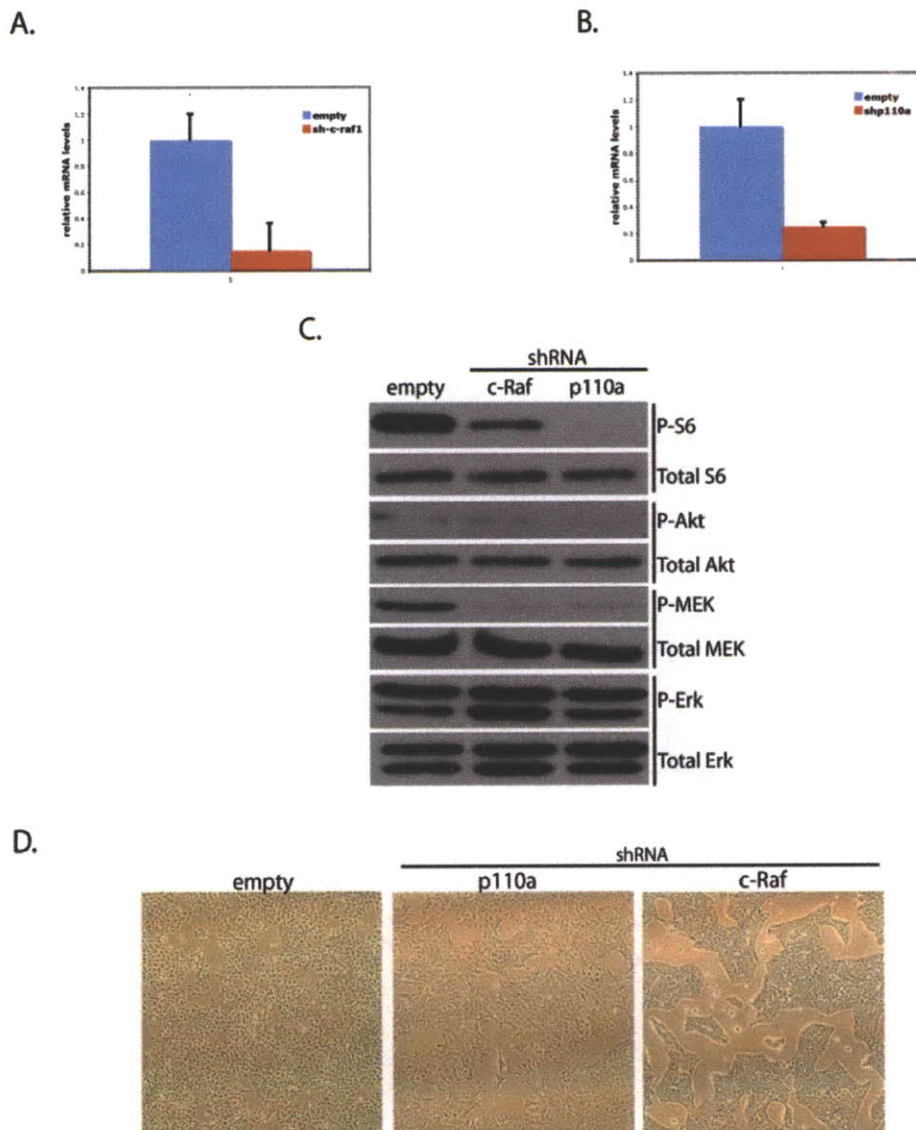


Figure 3: Ras effector pathway analysis in lung tumors
Representative western blot analysis of a MAPK (Erk), PI3K (Akt), and Ral (RalA) pathways in different types of K-ras^{G12D}-induced lung tumors. Wild-type lungs were used as a negative control. Despite the decreased signaling flux through these pathways, cyclin D1 is up-regulated in the lung tumors.



<u>Figure 4</u>: Characterization of c-Raf and p110α knockdown in a lung tumor cell line.
(**A.** and **B.**) qRT-PCR analysis of c-Raf (**A**) or p110α (**B**) in LKR13 cells infected with pSICO-PuroR either without a hairpin (blue bars) or with one containing a shRNA to c-Raf (**A**) or p110α (**B**) (red bars). Error bars = standard deviation. (**C.**) Western blots of various signaling pathways in LKR13 cells infected with control pSICO-PuroR (empty) or one containing a hairpin to c-Raf or p110α. (**D.**) Bright field images of cells used for the analysis in **C**. Cells in the "empty" group were split 1:5 before this picture was taken, while "p110α" and c-Raf" cells remained on their original plate, indicating that a substantial growth arrest had taken place after knockdown of p110α and c-Raf. Additionally, sh-p110α cells appear to be less refractile, while sh-c-Raf cells have undergone much more severe morphological changes and seem to have lost distinct cell boundaries with their neighbors.

Targeting c-Raf and p110α in established lung tumors

To assess the roles of c-Raf and p110α in K-ras^{G12D}-driven lung tumor maintenance, we intratracheally infected *K-ras*$^{FSF\text{-}G12D/+}$; *R26*$^{CreER\text{-}T2/Cre\text{-}T2}$ mice with pSICO-Flpo containing the relevant shRNAs (sh-c-Raf; sh p110α, and a control luciferase shRNA). At 20 weeks post-infection, tamoxifen was administered to induce shRNA expression, and mice were then aged an additional one or four weeks. Because most of the tumors were too small for mRNA or western blot analysis, we could not determine target knockdown in this study. However, immunohistochemistry (IHC) was performed to assess pathway perturbations. This analysis revealed that a number of tumors with the c-Raf shRNA had reduced staining for phospho-MEK, suggesting significant knockdown was occurring in at least some of the tumors (Fig 5A). Interestingly, this was not seen in sh-p110α tumors, highlighting a potential difference between *in vitro* and *in vivo* signaling mechanisms (Fig 4B and data not shown). Phospho-Akt was too low to detect in any of the tumors, precluding an analysis of PI3K signaling (data not shown).

To determine if c-Raf or p110α shRNA expression had functional effects on established tumors, we compared the number of hyperplasias and tumors at one week and four weeks post shRNA induction. While mice with control or c-Raf shRNAs had a relatively similar number of lesions following prolonged hairpin expression, those with sh-p110α dramatically reduced their tumor burden over the course of four weeks of knockdown (Fig 5B). Additionally, although the lesion number was similar in the sh-c-Raf group over the time course, the frequency of hyperplasias compared to more advanced tumors increased substantially, suggesting that c-Raf knockdown might block tumor progression (Fig 5B). Of the tumors that remained after four weeks of c-Raf or p110α shRNA expression, the largest of these had failed to recombine pSICO-Flpo (Fig 5C). This was in contrast to the significant recombination observed in tumors after one

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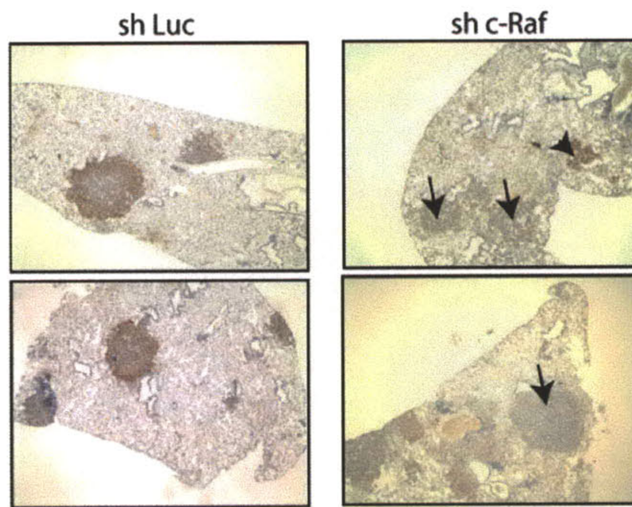

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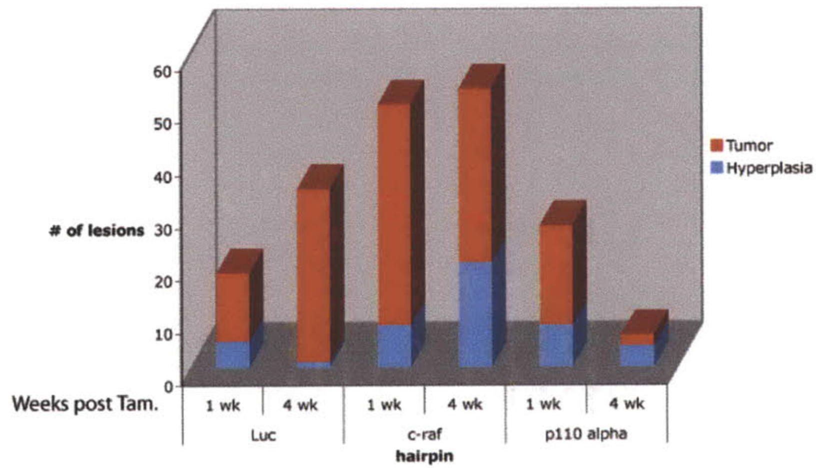

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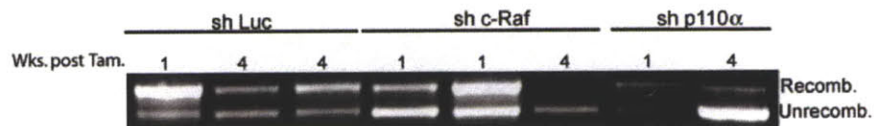

Figure 5: Effects of c-Raf and p110α knockdown *in vivo*
(**A.**) Phospho-MEK (P-MEK) IHC on lung sections from tumor-bearing FKC mice that were sacrificed one week after the last tamoxifen treatment. While the tumors with the control Luc shRNA stain for P-MEK, multiple lesions within the sh-c-Raf group display decreased staining (arrows). However, some tumors in sh-c-Raf animals maintained P-MEK (arrowhead), suggesting that either different tumors signaling through different Raf family members, or that not all tumors efficiently knocked down c-Raf. (**B.**) Quantitation of the number of hyperplasias and lung tumors in FKC mice infected with sh Luc, sh c-Raf, or sh p110α containing pSICO-Flpo viruses. 20 weeks post infection, shRNAs were induced with tamoxifen and animals were aged an additional one or four weeks. The number of lesions was determined by counting one representative section of the lungs from each mouse. N = 1 for each shRNA at each timepoint. (**C.**) PCR analysis of lentiviral recombination in representative tumors from **B.**

week of induction, indicating that perhaps over time there was selection for cells that did not induce hairpin expression. Importantly, this selection was not seen in tumors with the control hairpin (Fig 5C). Together, these data suggest that knockdown of p110α and c-Raf has deleterious effects on established lung tumors. A more detailed analysis of cell cycle progression and/or cell death at different time points following shRNA expression is warranted to gain further mechanistic insight into these effects.

Inefficient knockdown of a variety of K-ras effectors

While these initial experiments were ongoing, we set out to perform a comprehensive analysis of oncogenic K-ras^{G12D} effectors in lung tumor maintenance. To this end, we targeted a variety of components in numerous signaling pathways downstream of K-ras^{G12D}. In addition to studying other members of MAPK (b-Raf), and PI3K (p110β), we also targeted the Ral (RalA, RalB), mTOR (raptor, rictor), and Rho pathways (Rac1), as well as K-ras itself. While many hairpins displayed robust knockdown and produced *in vitro* effects in tumor cell lines (data not shown), very few worked efficiently *in vivo* in terms of knockdown or phenotypic consequences (Fig 6 and data not shown). Recombination analyses have shown extensive Cre-mediated removal of the STOP cassette within the tumors, indicating that they should be expressing the shRNAs (data not shown). However, we have yet to measure shRNA abundance in the tumors. Aside from technical problems such as inefficient expression or suboptimal design of our shRNAs, it is possible that many of the genes being targeted are absolutely essential for tumor cell viability, causing immediate selection against shRNA expression.

Nonetheless, we have achieved acute knockdown *in vivo* with cyclin D1 (Fig 2), CDK2 (data not shown), and Bmi-1 (Chapter 2). Our ability to target Bmi-1, which is thought to be

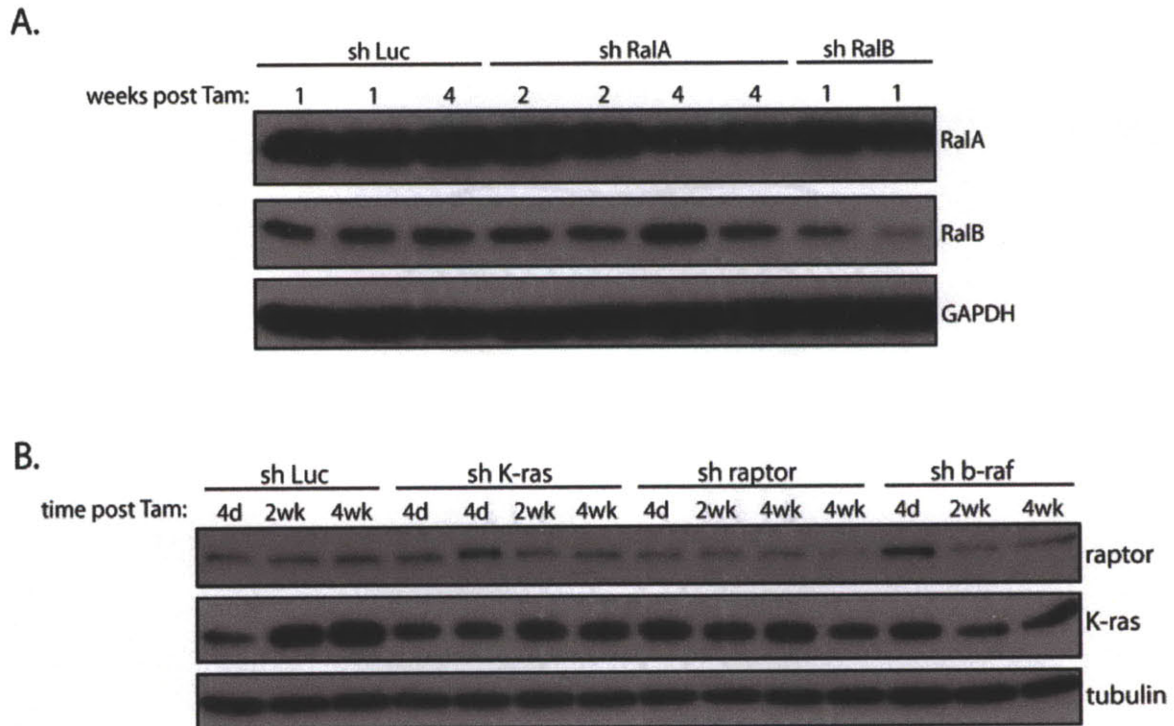

Figure 6: Examples of inefficient knockdown of a variety of K-ras effectors *in vivo*.
(**A.** and **B.**) Western blot analysis of numerous potential targets for shRNA-mediated knockdown in established tumors. **A** and **B** represent two separate cohorts of mice aged 20-24 weeks before tamoxifen administration. Only RalB showed any consistent decrease in levels, but no biological effects were observed.

important for lung tumorigenesis (Dovey et al. 2008), demonstrates that critical genes can be targeted in established tumors using pSICO-Flpo in combination with *K-ras*$^{FSF\text{-}G12D}$; *R26*$^{CreER\text{-}T2}$ mice.

With further optimization, this system might allow for efficient knockdown of Ras effectors, which would open up many interesting experimental possibilities. By varying the time at which shRNA expression is induced post-tumor initiation, one could discover stage-specific functions of pathways, which would further our understanding of lung tumor progression. In addition to studying canonical Ras effectors, an important future direction will be to test genes recently implicated as synthetic lethal interactors with mutant K-ras driven tumors (Barbie et al. 2009; Luo et al. 2009; Scholl et al. 2009). Finally, infection of other tissues will provide the opportunity to conduct these types of studies in other Ras-dependent malignancies. Comparing the relative functions of particular pathways in different Ras-driven tumor types will give important insights into Ras tumor biology as well as inform therapeutic strategies .

MATERIALS AND METHODS

Mouse studies. *R26*^{CreER} and *K-ras*$^{FSF\text{-}G12D}$ mice were generated in our laboratory. All animals were maintained on a mixed background comprising 129S4/SvJae and C57BL/6 strains. Lung tumors were induced by intratracheal instillation of pSICO-Flpo as previously described for Ade-Cre (DuPage et al. 2009). Tamoxifen (Sigma) was dissolved in corn-oil at 15 mg/ml and injected intraperitoneally every other day for 5 days. BrdU (Sigma) was dissolved in PBS at a concentration of 3 mg/ml and administered to mice 24 hours before sacrifice (10 μl/gram body weight). Animal studies were approved by Massachusetts Institute of Technology's (MIT) Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals, and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory Animals (institutional animal welfare assurance number, A-3125-01).

mRNA isolation and qRT-PCR analysis. RNA was extracted using Trizol (Gibco) according to the manufacturer's instructions. Once RNA was isolated, cDNA synthesis was performed on 1 μg of RNA using oligo dT primers and Superscript III (Invitrogen). cDNAs were analyzed by qPCR using Taqman detection systems in an ABI PRISM 7000 Sequence Detection System Thermo Cycler (Applied Biosystems). Relative mRNA levels were calculated using cycle threshold difference (ΔC_T) with TBP as an internal control.

Taqman probes:

cyclin D1	Mm00432360_m1
p110α	Mm00435673_m1
c-Raf	Mm00466513_m1
TBP	Mm00446973_m1

Protein extraction and immunoblots. Cell lines were lysed in RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Tx-100, .1% SDS, .5% sodium deoxycholate, 1 mM DTT) plus mini complete protease inhibitors (Roche) and phosphatase inhibitors (cocktails 1 and 2) (Sigma) for 10 minutes on ice. Snap-frozen tissue was finely minced with a razor blade on ice in TNE buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, supplemented with 1% Tx-100, .1%SDS, 1mM DTT, and the same inhibitors mentioned above) and then rotated for 15 minutes at 4°C. Both *in vitro* and *in vivo* samples were centrifuged to remove insolubles and quantitated using a Bradford Assay (Bio-rad). Samples were then diluted in loading buffer and separated on 10-15% SDS-PAGE gels. Following transfer to PVDF membranes, we probed with antibodies to β-tubulin, Erk 1/2, phospho-Erk 1/2, Akt, phospho-Akt473, phospho-S6, S6, raptor (from Cell Signaling Technology); actin, K-ras (from Santa Cruz Biotechnology); GAPDH (Chemicon International); RalA, RalB, and cyclin D1 (Millipore). HRP-conjugated secondary antibodies were used in conjunction with ECL+ detection systems (Amersham). Ral-GTP levels were assessed using the Ral activation kit according to the manufacturers instructions (Millipore).

Histology and Immunohistochemistry. Tissues were fixed in 10% formalin overnight and further processed for histology as previously described (Johnson et al. 1997). For immunohistochemistry, parraffin-embedded sections were dewaxed, followed by antigen retrieval in 10 mM citrate buffer (pH 6.0) in a pressure cooker. Slides were quenched in 3% hydrogen peroxide and washed in TBST. After blocking in TBST/5% serum for 1 hr, the primary antibodies (phospho-MEK, Cell Signaling Technology; BrdU-BD Biosciences) were incubated on slides overnight at 4°C. Detection was performed using a biotinylated secondary antibodies followed by the Vectastain ABC kit with diaminobenzadine (DAB) (Vector Labs). BrdU quantitation was done using Bioquant Image Analysis Software.

***In vitro* experiments.** MEFs were generated from E13.5 embryos using standard procedures. Cell lines (LKR13) and MEFs were grown in DMEM (DME, 10% FBS, 2 mM glutamine, pen/strep). 4-OHT (Sigma) was dissolved in ethanol and added to cells at 100 nM. For lentivirus experiments, target cells were selected in 5 ug/mL of puromyocin for 3 days following supernatant transfer. 2-4 days later cells were collected for analysis. 293T cells for virus production were grown in DMEM.

Lentivirus production and infections. Lentivirus was produced as described previously (Rubinson et al. 2003). For *in vivo* infections, viral pellets were resuspended in 1X HBSS pH 7.4, and 50-100 μl was administered intratracheally.

Lentiviral vectors and shRNA cloning. pSICO-PuroR was used for most *in vitro* knockdown experiments (Ventura et al. 2004). MEF infections and *in vivo* lung tumor formation in *K-ras*$^{FSF-G12D}$ animals relied on pSICO-Flpo, which was generated by amplifying pgkFlpo from pgkFlpobpA (Addgene) and cloning it into pSICO-Puro that had been digested previously to remove pgkPuro. Cloning details are available upon request. Target sequences for shRNA knockdown were identified using pSICO Oligomaker V 1.5 (A. Ventura, Memorial Sloan Kettering Cancer Center, New York, NY). Cloning of DNA oligos into the U6-shRNA cassette in the above vectors was done as described previously (Ventura et al. 2004).

shRNA sequences:

Gene:	Target sequence:
luciferase	GAGCTGTTTCTGAGGAGCC
cyclin D1	GAGCTGTTTCTGAGGAGCC
p110α	GGAATGAATGGCTGAATTA
c-Raf	GCAGCAGTCTCTACAAACA
b-Raf	GAAGAGGTATGGAATATCA
RalA	GCCAACGTTGACAAGGTA
RalB	GCTGTTCTCTTCATATTTA

K-ras	GAACAGTAGACACGAAACA
raptor	GAATCATGAGGTCATATAA

Lentiviral recombination analysis. Tumor DNA was extracted from lung tumors and subjected to multiplex PCR using the following primers:

pSICO-Flpo F	ATCGAGGAGTGGCAGCACATC
Loopout F	CTCGCACAGACTTGTGGGAG
Loopout R	GCAGCGTATCCACATAGCGTA

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