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# Cooperative Tumor Suppression by ARF and p53

Jason Thomas Forys

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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Molecular Cell Biology

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Cooperative Tumor Suppression by ARF and p53

by

Jason Thomas Forys

A dissertation presented to the  
Graduate School of Arts and Sciences  
of Washington University in  
partial fulfillment of the  
requirements for the degree  
of Doctor of Philosophy

December 2013

St. Louis, Missouri

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## ABBREVIATIONS

<b>AgNOR</b>	<b>Argyrophillic nucleolar organizing region</b>
<b>AKT</b>	<b>v-Akt murine thymoma viral oncogene homolog 1</b>
<b>Anril</b>	<b>Antisense non-coding RNA in the INK4 locus</b>
<b>ARF</b>	<b>Alternative reading frame</b>
<b>ATF4</b>	<b>Activating transcription factor 4</b>
<b>ATM</b>	<b>Ataxia telangiectasia mutated 1</b>
<b>ATR</b>	<b>Ataxia telangiectasia and Rad3-related</b>
<b>BCR-Abl</b>	<b>Breakpoint cluster region-Abelson virus</b>
<b>BMI-1</b>	<b>B-lymphoma Mo-MLV insertion region</b>
<b>BrdU</b>	<b>Bromodeoxyuridine</b>
<b>BTG1</b>	<b>B-cell translocation gene 1</b>
<b>CAT</b>	<b>Chloroamphenicol acetyltransferase</b>
<b>CBX7</b>	<b>Chromobox protein homolog 7</b>
<b>CBX8</b>	<b>Chromobox protein homolog 8</b>
<b>CDK</b>	<b>Cyclin dependent kinase</b>
<b>CDKN2a</b>	<b>Cyclin dependent kinase inhibitor 2A</b>
<b>CML</b>	<b>Chronic myelogenous leukemia</b>
<b>Cre</b>	<b>Cre recombinase</b>
<b>CRISPR</b>	<b>Clustered regularly interspaced short palindromic repeats</b>
<b>DDX5</b>	<b>DEAD-box helicase 5</b>
<b>DHX33</b>	<b>DEAH box polypeptide 3</b>
<b>DKO</b>	<b>Double knockout</b>

<b>DMBA</b>	<b>9,10-dimethyl-1,2-benzanthracene</b>
<b>DMEM</b>	<b>Dulbecco's modified eagles medium</b>
<b>DMP1</b>	<b>Cyclin D-binding, Myb like protein 1</b>
<b>dp53</b>	<b>Deleted for p53</b>
<b>dp53R</b>	<b>Deleted for p53 and expressing RasV12</b>
<b>dsRNA</b>	<b>Double stranded RNA</b>
<b>E1A</b>	<b>Adenovirus early region 1A</b>
<b>ELISA</b>	<b>Enzyme-linked immunosorbent assay</b>
<b>ERK</b>	<b>Extracellular signal-regulated kinase</b>
<b>EZH2</b>	<b>Enhancer of zeste homolog 2</b>
<b>FLOX</b>	<b>Flanked by loxP sites</b>
<b>FOXO3A</b>	<b>Forkhead box protein O3A</b>
<b>GAS</b>	<b>Gamma-interferon activation sites</b>
<b>GFP</b>	<b>Green fluorescent protein</b>
<b>GTP</b>	<b>Guanosine triphosphate</b>
<b>H2A</b>	<b>Histone H2A</b>
<b>H3K27</b>	<b>Histone H3 Lysine 27</b>
<b>HDAC</b>	<b>Histone deacetylase</b>
<b>HER2</b>	<b>Human epidermal growth factor receptor 2</b>
<b>HERC</b>	<b>HECT and RLD domain containing E3 ubiquitin protein ligase</b>
<b>HIF-1<math>\alpha</math></b>	<b>Hypoxia-inducible factor 1<math>\alpha</math></b>
<b>HOTAIR</b>	<b>Hox antisense intergenic RNA</b>
<b>HPV</b>	<b>Human papilloma virus</b>

<b>hTERT</b>	<b>Human telomerase reverse transcriptase</b>
<b>HuR</b>	<b>Human antigen R</b>
<b>HVS</b>	<b>Hyaloid vascular system</b>
<b>IFIT3</b>	<b>Interferon-induced protein with tetratricopeptide repeats 3</b>
<b>IFN</b>	<b>Interferon</b>
<b>IFNAR</b>	<b>Interferon <math>\alpha/\beta</math> receptor</b>
<b>IHC</b>	<b>Immunohistochemistry</b>
<b>I<math>\kappa</math>B<math>\alpha</math></b>	<b>NF-kappa-B inhibitor alpha</b>
<b>IL-6</b>	<b>Interleukin-6</b>
<b>INK4</b>	<b>Inhibitor of cyclin dependent kinase 4</b>
<b>IRDS</b>	<b>Interferon-related DNA damage signature</b>
<b>IRF9</b>	<b>Interferon regulatory factor 9</b>
<b>ISG</b>	<b>Interferon sensitive genes</b>
<b>ISG15</b>	<b>Interferon sensitive gene 15</b>
<b>ISGF3</b>	<b>Interferon-stimulated gene factor 3</b>
<b>ISRE</b>	<b>Interferon sensitive response element</b>
<b>JAK</b>	<b>Janus kinase</b>
<b>JMJD3</b>	<b>Jumonji domain containing 3</b>
<b>Kb</b>	<b>Kilobase</b>
<b>lncRNA</b>	<b>Long non-coding RNA</b>
<b>LSL</b>	<b>LoxP-Stop-LoxP site</b>
<b>MAPK</b>	<b>Mitogen activated protein kinase</b>
<b>MAVS</b>	<b>Mitochondrial antiviral signaling protein</b>



<b>MDA5</b>	<b>Melanoma differentiation-associated protein 5</b>
<b>MDM2</b>	<b>Mouse double minute 2 homolog</b>
<b>MEF</b>	<b>Mouse embryonic fibroblast</b>
<b>miRNA</b>	<b>micro RNA</b>
<b>mRNA</b>	<b>Messenger ribonucleic acid</b>
<b>MRT</b>	<b>Malignant rhabdoid tumor</b>
<b>mTOR</b>	<b>mammalian target of rapamycin</b>
<b>NCL</b>	<b>Nucleolin</b>
<b>NEK-2</b>	<b>NIMA-related kinase 2</b>
<b>NF-kB</b>	<b>Nuclear factor kappa-light-chain-enhancer of activated B-cells</b>
<b>NPM</b>	<b>Nucleophosmin</b>
<b>NPMc</b>	<b>Nucleophosmin cytoplasmic mutant</b>
<b>NSAID</b>	<b>Non-steroidal anti-inflammatory drug</b>
<b>OAS</b>	<b>2'-5'-oligoadenylate synthetase 1</b>
<b>OASL</b>	<b>2'-5'-oligoadenylate synthetase-like 1</b>
<b>ODC</b>	<b>Ornithine decarboxylase</b>
<b>PcG</b>	<b>Polycomb group proteins</b>
<b>PDGFR</b>	<b>Platelet-derived growth factor receptor</b>
<b>PDX1</b>	<b>Pancreatic and duodenal homeobox 1</b>
<b>PI3K</b>	<b>Phosphatidylinositol-3-kinase</b>
<b>PIT-1</b>	<b>Pituitary-specific positive transcription factor 1</b>
<b>PP1</b>	<b>Protein Phosphatase 1</b>
<b>PP2A</b>	<b>Protein phosphatase 2A</b>

<b>PRC1</b>	<b>Polycomb repressive complex 1</b>
<b>PTEN</b>	<b>Phosphatase and tensin homolog</b>
<b>PUMA</b>	<b>p53 upregulated modulator of apoptosis</b>
<b>qRT-PCR</b>	<b>Quantitative real-time PCR</b>
<b>RAS</b>	<b>Rat sarcoma</b>
<b>Rb</b>	<b>Retinoblastoma</b>
<b>rDNA</b>	<b>Ribosomal deoxyribonucleic acid</b>
<b>RFP</b>	<b>Red fluorescent protein</b>
<b>RIG1</b>	<b>Retinoic acid-inducible gene 1</b>
<b>RING</b>	<b>Really interesting new gene</b>
<b>RNF2</b>	<b>Ring finger protein 2</b>
<b>SENP3</b>	<b>Sumo1/Sentrin/SMT3 specific peptidase 3</b>
<b>SMAD2</b>	<b>Sma and mad-related protein 2</b>
<b>smARF</b>	<b>Small ARF</b>
<b>STAT</b>	<b>Signal transducer and activator of transcription 1</b>
<b>SUMO</b>	<b>Small ubiquitin-like modifier</b>
<b>SUZ12</b>	<b>Suppressor of zeste 12</b>
<b>SV40</b>	<b>Simian virus 40</b>
<b>SWI/SNF</b>	<b>Switch/sucrose non-fermentable</b>
<b>TALEN</b>	<b>Transcription activator-like effector nucleases</b>
<b>TBK1</b>	<b>TANK-binding kinase 1</b>
<b>TBX2</b>	<b>T-box transcription factor 2</b>
<b>TCGA</b>	<b>The cancer genome atlas</b>

<b>TGFβ</b>	<b>Transforming growth factor beta</b>
<b>TIMP3</b>	<b>Tissue inhibitor of metalloproteinases-3</b>
<b>TKO</b>	<b>Triple knockout</b>
<b>TNBC</b>	<b>Triple-negative breast cancer</b>
<b>TSC2</b>	<b>Tuberous sclerosis 2</b>
<b>TTF-1</b>	<b>Transcription termination factor 1</b>
<b>TYK2</b>	<b>Tyrosine kinase 2</b>
<b>UBC8</b>	<b>Ubiquitin conjugating enzyme 8</b>
<b>UBE1L</b>	<b>Ubiquitin-like modifier activating enzyme 7</b>
<b>UBF</b>	<b>Upstream binding factor</b>
<b>ULF</b>	<b>Ubiquitin ligase for ARF</b>
<b>USP18</b>	<b>Ubiquitin-specific protease 18</b>
<b>UTR</b>	<b>Untranslated region</b>
<b>v-ABL</b>	<b>Abelson murine leukemia viral oncogene homolog 1</b>
<b>VEGF</b>	<b>Vascular endothelial growth factor</b>
<b>WRN</b>	<b>Werners helicase</b>
<b>Xist</b>	<b>X-inactive specific transcript</b>
<b>YFP</b>	<b>Yellow fluorescent protein</b>

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about science. Science is a momentous roller-coaster ride with numerous “steep” drops. Josh has served as a constant reminder that any finding (big or small) is new knowledge that no one else in the world has yet discovered.

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I dedicate this work to my family—all of you.

## ABSTRACT OF THE DISSERTATION

Cooperative Tumor Suppression by ARF and p53

by

Jason Thomas Forys

Doctor of Philosophy in Molecular Cell Biology

Washington University in St. Louis, 2013

Professor Jason Weber, Chairperson

Cancer is a complex genetic disease characterized by the inactivation of tumor suppressor genes and enhanced activity of oncogenes leading to deregulated cellular proliferation. Two tumor suppressor genes, p53 and Arf, play important roles in protecting cells against numerous biological stresses. In response to oncogenic signals, increased ARF expression leads to the activation of p53, which in turn leads to the cessation of cell division or induction of an apoptotic response. Interestingly, p53 coordinates repression of Arf transcription, setting up a negative feedback loop with currently unknown physiological significance. Cells that lack p53 express elevated levels of ARF, but it has been generally accepted that these levels serve no tumor suppressor function. This view has been challenged recently as numerous groups have demonstrated ARF can inhibit both cell growth and proliferation independently of p53. Additionally, co-inactivation of *p53* and *Arf* is frequently observed in human cancers, suggesting they do not function in a strictly linear genetic pathway.

The objective of my dissertation was to examine the biological functions of ARF in the absence of *p53*. I specifically wanted to understand why *p53*-deficient cells express elevated levels of ARF, and whether these increased levels are able to suppress tumorigenesis. By

addressing these questions, I hoped to provide a mechanistic explanation for the selective advantage cancer cells gain by inactivating both p53 and Arf, and ultimately uncover novel therapeutic approaches that could be used to treat patients whose tumors exhibit these specific genetic abnormalities.

My dissertation work utilized an *in vitro* system to study the role of ARF in cells lacking p53. I hypothesized that acute loss of p53 would lead to an upregulation of ARF which would exert a currently undefined tumor suppressor function. Indeed, I have demonstrated that loss of p53 leads to an induction of ARF, which is able to potently suppress tumorigenesis. Depletion of ARF in this genetic setting lead to the activation of a type I interferon response driven by interferon-beta and the STAT1 transcription factor. I further demonstrated that ARF and p53 cooperate to suppress the interferon response, and when both proteins are inactivated, interferon signaling can drive tumor cell proliferation. Additionally, I have shown that breast cancer cell lines lacking ARF and p53 are sensitive to STAT1 depletion, indicating targeted disruption of this signaling pathway can inhibit cancer cell growth. Finally, I identified a subtype of breast cancer, defined as ER-/PR-/HER2-, that exhibits activation of the interferon signaling pathway in the absence of p53 and ARF function.

This work has solidified ARF's role as a p53-independent tumor suppressor, and provides insight into the selective advantage cancer cells gain by co-inactivating these two tumor suppressor genes. As we enter an era of personalized cancer therapy, a detailed understanding of cancer cell vulnerabilities is imperative. The data presented in this dissertation has identified a subset of patients that would benefit from targeted inhibition of IFN- $\beta$  signaling. Equally as important, I have identified a novel oncogenic signaling pathway that could be promoting tumor growth in numerous other cancer types.

“For the scientist, at exactly the moment of discovery—that most unstable existential moment—the external world, nature itself, deeply confirms his innermost fantastic convictions. Anchored abruptly in the world, Leviathan gasping on his hook, he is saved from extreme mental disorder by the most profound affirmation of the real.”

~Richard Rhodes, *The Making of the Atomic Bomb*



## **Chapter 1:**

# **Introduction and Significance**

In 2013, the American Cancer Society estimates that 1.6 million new cases of cancer will be diagnosed in the United States and approximately 580,350 people will lose their lives battling this disease. While these numbers are certainly alarming, a more optimistic view indicates that there are over 13.7 million people alive today because of the efforts put forth by physicians and researchers over the past 100 years. Thus, we have come a long way since Ancient Greece when physicians such as Hippocrates believed cancer was caused by an excess of black bile (55). Today, we understand that cancer is a complex genetic disease caused by a multistep process involving specific alterations to cellular DNA. Changes to DNA that confer a proliferative advantage allow those cells to survive and divide in an uncontrolled manner, leading to tumor formation. Over time, the cells that sustain the most advantageous traits are able to endure the harsh intra-tumor environment and accumulate even more DNA alterations, eventually leading to their dissemination from the tumor to other sites in the human body. This process, called metastasis, is ultimately the point where patients lose the battle to cancer. Highlighting the complexity of cancer, there are over 200 distinct types which can be further sub-classified by molecular signatures, encompassing an immeasurable number of possibilities. The challenge for researchers today is to discover the most effective treatment options for all of these different types of disease. Unfortunately, there is not one simple remedy.

The ultimate goal of the research described in this dissertation was to enhance our knowledge of the molecular changes occurring within cancer cells, and to identify novel therapeutic targets to treat this devastating disease. Specifically, the work has significantly advanced our knowledge of the relationship between two of the most frequently mutated genes in human cancer—the Arf and p53 tumor suppressors. While originally thought to function in a linear genetic pathway, functional disruption of these two genes frequently co-exist in human

tumors, indicating our knowledge of their relationship is incomplete. This dissertation work has demonstrated that these two proteins cooperate to suppress a signaling pathway not normally considered to be oncogenic, but the experiments described here indicate otherwise. This novel finding will allow the opportunity for targeted therapeutic intervention in patients harboring defects in Arf and p53.

In this section I will not attempt to introduce all of the molecular intricacies of cancer cells. Rather, I will introduce tumor suppressors and oncogenes, briefly describe how normal cells become cancerous, and discuss where the field of cancer biology is headed in the 21<sup>st</sup> century. For an in depth review of the molecular characteristics that cancer cells possess, see the seminal work by Hanahan and Weinberg (109).

## **Oncogenes and Tumor Suppressors**

In 1914, Theodor Boveri, a German zoologist studying the effects of abnormal mitosis in sea urchins, hypothesized that there are both stimulatory and inhibitory chromosomes, essentially predicting the existence of what are now known as oncogenes and tumor suppressors (25). This was quite an astounding prediction considering that chromosomes (DNA) were not identified as the carrier of genetic information for 40 more years (114). The human genome encodes numerous “proto-oncogenes,” that play vital roles in promoting proliferation and cell growth both during development and throughout our lives. Without these genes, we would not develop properly and our bodies would not be capable of growing. Specific genetic changes like gain of function mutations, chromosomal amplifications, or translocation events can lead to inappropriate activity of proto-oncogenes, establishing them as *bona fide* oncogenes. Genetic

changes resulting in oncogenes are usually dominant in nature, meaning a change in just one allele is sufficient to produce the pro-tumor effect.

Evidence for the existence of oncogenes was first demonstrated in 1960 when Nowell and Hungerford observed a small chromosome that was present in cells from patients with chronic myelogenous leukemia (CML) but absent in normal cells (206). This piece of DNA, named the “Philadelphia Chromosome” after the city in which it was discovered, is the result of a translocation event between chromosomes 9 and 22. The chromosomal translocation event resulted in a fusion protein, joining the breakpoint cluster region (Bcr) to a receptor tyrosine kinase called Abl. As a result of the BCR-Abl fusion, proper regulation of Abl is lost, and constitutive downstream signaling occurs (146). The BCR-Abl oncogene was demonstrated to be sufficient to induce CML in mice, clearly indicating this abnormal protein is the driving force behind CML (50). Greater than 90% of CML patients exhibit this genetic abnormality, so a momentous effort was undertaken to generate BCR-Abl specific inhibitors. The drug that was eventually produced was called Gleevec, the first of its kind, and it increased the five year survival rate for patients with CML from 30% to 90% (75).

An equally important discovery made by Michael Bishop and Harold Varmus provided evidence for the existence of oncogenes. Bishop and Varmus were studying the Rous sarcoma virus which can induce tumors in chickens, and demonstrated that it was not the act of viral infection itself that caused tumor formation, but rather a specific piece of DNA within the viral genome (279). The piece of DNA, called *Src*, was actually a mammalian gene that the virus had incorporated into its own genome. A mutation in the *Src* sequence led to unregulated activation of the receptor tyrosine kinase that it encoded (210). Once expressed in the chicken cells, it initiated proliferative and survival-promoting pathways that led to tumor formation.

Perhaps the best example of an oncogene is RAS. It was originally identified by Harvey and Kirsten as a gene utilized by a murine retrovirus to induce sarcoma formation (RA Sarcoma virus). Similar to the *Src* proto-oncogene, *Ras* has mammalian homologs, including in humans, which suggests the virus hijacked the sequence from the mammalian genome and a mutation led to its ability to induce proliferation once expressed. This attribute is ideal for the virus, since it can infect rats and induce cells to divide, generating millions of new viral particles with each round of division (137). Today, it is known that mutations in the *Ras* proto-oncogene occur in a wide variety of tumors (251). As a small GTPase, RAS is active in the GTP-bound state, and activates downstream signaling pathways such as the mitogen-activated protein kinase pathway (MAPK), the RAL-guanine nucleotide dissociation stimulator pathway (RAL-GDS) and the phosphatidylinositol-3-kinase pathway (PI3K) (223). Normally, RAS transduces its proliferative signal as long as a stimulating growth factor is present. In the absence of growth factors, RAS hydrolyzes GTP to GDP, turning off the response. Cancer-associated mutations of the *Ras* gene result in RAS becoming “locked” in the GTP-bound state, leading to constitutive signaling through the above mentioned pathways (249). These pathways drive proliferation, growth, and survival, so mutant *Ras* is extremely beneficial to a cancer cell.

Boveri’s prediction of inhibitory chromosomes was validated in in the 1970’s by a physician named Alfred Knudson. While studying a heritable form of pediatric retinal cancer called Retinoblastoma, Knudson used an epidemiological approach to develop a hypothesis that the development of this cancer required inactivating mutations in two copies of a specific gene (145). This hypothesis, called the “two-hit model,” predicted that mutations of one allele of the gene (which was later cloned and coined *Rb* (159)), would be found in the germline of families with hereditary retinoblastoma. Likewise, tumors from retinoblastoma patients would exhibit

inactivation of the second allele. This was exactly what Knudson observed (17). Tumor suppressors such as Rb are responsible for monitoring and inhibiting the signaling pathways that oncogenes activate. A proper balance between the activities of these proteins ensures regulated proliferation in response to the appropriate cues. When this balance is lost, uncontrolled proliferation can occur.

An exception to the two-hit model of tumor suppression is found in one of the best characterized tumor suppressor proteins, p53. *TP53* (*Trp53* in the mouse) encodes a 53 kD protein that functions as a tetrameric transcription factor, and is the most frequently mutated gene in human cancer (162). Evidence characterizing p53 as a tumor suppressor was initially unclear. In fact, for several years researchers believed that p53 was an oncogene, due to its ability to interact with a viral oncoprotein (SV40 Large T-antigen) and the observation that many tumors had elevated levels of p53 (302). Later, it was demonstrated that mutations in p53 lead to increased stability, and can impart gain of function characteristics (29). Early experiments classifying p53 as an oncogene were actually using mutant forms of p53, which led to skewed interpretations. Additionally, we now know that the SV40 Large T-antigen expressed by tumor-causing polyomaviruses binds and inactivates p53, effectively ridding the cell of p53 function. Numerous other cancer-causing viruses inactivate p53, underscoring its important role in maintaining proper proliferation rates. HPV, the cause of cervical cancer, expresses an oncogene called E6 that binds and targets p53 for degradation (162). Like the tumor suppressor Rb, inactivating mutations in the *TP53* gene are associated with a hereditary cancer syndrome. Mutations in p53 are the causative defect in the hereditary cancer-predisposition syndrome, called Li-Fraumeni (182, 277). These patients develop multiple tumor types very early in life.

The p53 protein has been coined the “guardian of the genome” as it is responsible for maintaining genomic integrity (157). In this capacity, p53 is activated in response to DNA damage and can activate transcription of anti-proliferative genes such as p21 or pro-apoptotic genes like Puma and Noxa (83, 195, 203, 208). Thus, p53 is responsible for insuring proper genomic integrity before a cell is allowed to divide. If the damage is too severe, p53 activates pro-apoptotic programs to clear the cell from the organism. Other stresses such as hypoxia, reactive oxygen species, telomere shortening, or oncogenic stress, also lead to activation of p53, underscoring the immense selective pressure cancer cells face to mutate the *TP53* gene (162).

Mutations in p53 can lead to the formation of a dominant negative protein. Since p53 functions as a tetramer, having just one mutant molecule of p53 in the complex abrogates the wild-type transcriptional functions. In this regard, mutating one allele is sufficient to completely inactivate p53’s ability to respond to cellular stress (29). For this reason, p53 does not follow the classical two-hit hypothesis for tumor suppressor genes, but it is arguably our biggest asset in the defense against cancer. The discovery that cancer is a genetic disease, and an improper balance of oncogenic and tumor suppressive signaling is the driving force behind tumor formation has revolutionized the field of cancer biology. With this knowledge, researchers have been able to study the key requirements that make a normal cell cancerous.

### **From Normal to Abnormal—How do cells become cancerous?**

Human cells possess numerous defense mechanisms that protect against aberrant proliferation. Over the course of many years, cells accumulate mutations, and some of these stochastic events can affect a gene that is important in regulating cellular proliferation, growth, or survival. When this occurs, the cell gains an obvious advantage over its neighbor. In human

cells, one mutation is not sufficient to induce tumor formation. In fact, it is believed that at least 4-5 separate genetic events must occur to make a normal cell cancerous. This was demonstrated in a landmark publication by Hahn *et al.*, where the authors demonstrated a human cell could be transformed (a term used to describe a cell capable of forming a tumor) by the following genetic manipulations: 1) Inactivating the Rb and p53 tumor suppressor pathways 2) Inactivating the PP2A (protein phosphatase 2A) which negatively regulates several important proteins in proliferation such as c-MYC 3) Providing constitutive telomerase (hTERT) expression which ensures the ends of chromosomes do not shorten with each round of DNA replication and 4) Expressing a mutant form of the RAS proto-oncogene that imparts pro-proliferative and survival signals (107, 255). Obviously, these specific genetic perturbations are not the only route to cancer.

In reality, there are many ways of achieving the cancer end-point, but regardless of how cells accumulate the molecular changes required for tumorigenesis, cancer cells all possess the same general qualities. They must be capable of sustaining proliferative signaling, be able to divide indefinitely, evade growth suppressors, resist cell death, maintain an adequate blood supply, and invade surrounding tissues to metastasize. These “hallmarks,” as they have been coined by Hanahan and Weinberg, accurately depict the remarkable capabilities of a cancer cell (109). As research has continued to improve the mechanistic understanding of cancer, it is also now appreciated that the ability of cancer cells to avoid clearance by the immune system and to alter its cellular metabolism to keep up with proliferative demand are likely additional hallmarks. Additional tumor promoting mechanisms such as chronic inflammation, genome instability, and epigenetic modifications are also being actively researched (15, 104, 204).



If such a complicated series of genetic changes are required to induce transformation, then why do humans get cancer at all? While not an overly simple question to answer, it starts with the fact that around half of all cancers are preventable (48). Factors such as tobacco use, poor diet, obesity, and lack of exercise are all things that contribute to mutational rates and promote a physiological environment that are conducive to tumor growth. Most often, a lifetime of stochastic genetic changes are required to create the proper cellular environment for cancer formation, which is why an overwhelming amount of data indicates cancer incidence rapidly increases with age (57). The social and environmental factors listed above simply increase the chances that the harmful genetic changes will occur.

Our molecular understanding of cancer, elucidated over the past 50 years, is now allowing cancer researchers to make remarkable strides towards effectively treating the diverse spectrum of cancer sub-types. In recent years, an explosion in genome sequencing technology has allowed rapid and affordable analysis of human tumors. From these analyses, it is clear that most tumors possess targetable mutations (93). That is, they contain known “driver” oncogenes that drugs could target and inactivate. Therefore, the modern day goal for cancer therapeutics is personalized cancer therapy (52). With this approach, sequencing data from a patient’s tumor (compared to their normal tissue) would reveal a specific course of therapeutic action. This approach does not come without potential pitfalls. Tumor heterogeneity is a major obstacle to personalized therapy, as treatments need to clear all the unique clonal populations within a tumor, and sequencing methodologies must be able to accurately detect them (187). Additionally, there is still an extremely limited knowledge on the functional consequence of mutations in the noncoding genomic regions. While there is still much to be discovered, capable

scientists continue to uncover and explain the complexities of cancer, with the hope that one day effective treatments are available for all variations of this devastating disease.

## **THE ARF TUMOR SUPPRESSOR**

Most, perhaps all, cancer cells must navigate their way around two vital tumor suppressor pathways maintained by the Rb and p53 proteins. Without functional Rb and p53 pathways, cells are not capable of appropriately regulating their proliferation or responding to numerous physiological stresses, two hallmark characteristics of cancer cells. Interestingly, our genome contains a genetic locus known as *CDKN2a* that encodes two important regulators of the Rb and p53 pathways. In this section, I will introduce this unique genetic locus and discuss the two tumor suppressor genes encoded by it, p16 and ARF. The main focus of this section will be devoted to ARF, as my dissertation work has not concentrated on p16-mediated tumor suppression.

### **The CDKN2A Tumor Suppressor Locus**

Within a 35 kilobase (kb) span on chromosome *9p21*, the human genome encodes three tumor suppressor genes—*p15<sup>INK4b</sup>* (encoded by *CDKN2b*), *p16<sup>INK4a</sup>*, and *p14<sup>ARF</sup>* (collectively encoded by *CDKN2a*) (see Figure 1.1 for graphical depiction). Consequently, this stretch of DNA is one of the most frequently mutated, deleted, or epigenetically silenced genomic loci in human cancers (252). *p16<sup>INK4a</sup>* was the first gene in this region to be identified as a tumor suppressor. Serrano and colleagues identified p16 in a complex with the cyclin dependent kinase, CDK4, and demonstrated that p16 could inhibit the activity of this important cell cycle regulator (253). P16 is a member of the INK4 (Inhibitors of Cyclin Dependent Kinase 4) family of cell

cycle inhibitors. This family of proteins has three other members; p15<sup>INK4b</sup>, p18<sup>INK4c</sup>, and p19<sup>INK4d</sup> which are located on chromosome bands *9p21*, *1p32*, and *19p13* respectively (243).

The INK4 family of proteins participates in the regulation of cell cycle progression by binding and inhibiting the activities of cyclin D-dependent kinases, CDK4 and CDK6 (266). As cells progress through G1 phase of the cell cycle, cyclin D1 levels are transcriptionally induced and form complexes with CDK4 and CDK6 promoting their kinase activities (66). CDK4/6-cyclin-D1 complexes phosphorylate a tumor suppressor called the Retinoblastoma (Rb) protein. In resting cells, hypophosphorylated Rb normally binds and sequesters the E2F family of transcription factors which are important for enhancing expression of genes required for DNA synthesis (212). Therefore, Rb ensures cells do not proceed into S-phase of the cell cycle without the appropriate mitogenic signals. Phosphorylation of Rb by the CDK4/6-cyclin D1 complexes leads to the release of the E2F proteins and progression into S-phase. The INK4 proteins block the interaction between CDK4/6 and cyclin D1, effectively blocking entry into S-phase (265). Additionally, INK4-mediated disruption of the CDK4/6-cyclin D1 complexes leads to the release of two other CDK inhibitors, p21 and p27, which are required for proper cyclin D-CDK complex formation (45). Upon release, p21 and p27 inhibit the kinase activities of cyclin E-CDK2 and cyclin A-CDK2 complexes, thus providing complete inhibition of cell cycle progression (266). The G1 cell cycle arrest that subsequently occurs requires functional Rb, indicating the linear nature of this pathway (106, 172, 194). While all the members of the INK4 family are capable of inhibiting the activity of CDK's *in vitro* (265), p16 is by far the most commonly mutated family member, suggesting it is the most relevant tumor suppressor *in vivo* (245). Most, if not all, cancer cells must overcome the Rb-regulated cell cycle checkpoint in

order to maintain their enhanced proliferative capacity. This is most commonly achieved by deletion or mutation of p16 or Rb, or overexpression of the CDK's or Cyclin D1 (264).

The organization of the *CDKN2a* locus is distinctly unique. Shortly after the discovery of p16, it was noted that the *CDKN2a* locus actually encoded two transcripts (185, 280). This finding, along with a seminal paper published by Quelle and colleagues, demonstrated that the *CDKN2a* locus actually encoded two distinct proteins—p16 and ARF (228). p16 and ARF have distinct first exons, designated exon-1 $\alpha$  and exon-1 $\beta$  respectively, and are regulated by independent promoter sequences. Both exon-1 $\alpha$  and exon-1 $\beta$  splice into shared exons 2 and 3. However, due to the distinct ATG translation start site in exon-1 $\beta$  of the ARF transcript, exon 2 gets translated in an Alternative ReadinG Frame (Figure 1.1). As a result, ARF and p16 share absolutely no homology at the protein level. ARF, like p16, is able to induce cell cycle arrest, but does so primarily through a p53-dependent mechanism that will be discussed in a later section. To demonstrate the potency of this genetic locus, a mouse carrying an extra copy of the entire *Ink4a/Arf* locus was generated, and it displayed resistance to tumor formation (190).

The genomic organization of *CDKN2a* is conserved throughout mammals, but no examples of similarly constructed genomic loci have been found in humans. The only known mammalian exception is chickens. Chickens lack p16, and express a truncated form of ARF, representing only the first 60 amino acids of exon 1- $\beta$  (144). The significance of this will be discussed in a later section.

## Overview of the ARF protein

The exon-1 $\beta$  transcript encodes a 14 kD protein in humans (p14<sup>ARF</sup>) and a 19kD protein in mice (p19<sup>ARF</sup>). The mouse and human ARF proteins share 50% homology, and possess unique biochemical properties (228). ARF is a resident nucleolar protein and contains over 20% arginine residues. This feature of ARF makes it highly basic and results in an isoelectric point above 11 (170, 228). As a result, ARF is highly disordered at physiological pH, and is thought to require interaction with other proteins to neutralize its overall positive charge (261). Indeed, crystal structure evidence has indicated that the 37 amino acid N-terminal peptide of ARF is highly unstructured in aqueous solution but is capable of forming two alpha helical domains upon association with its binding partners (23, 68). As a result of ARF's unstructured and highly basic nature, it is capable of interacting with a growing list of proteins (Reviewed in (261)).

Another unusual feature of ARF is its apparent lack of lysine residues. Mouse ARF contains one lysine residue at position 26, and human ARF contains none. The half-life of ARF has been reported anywhere from 1-8 hours depending on the cell line studied, with ~6 hours being consistently found in mouse cells (43, 152, 221, 306). Since ARF is essentially a lysine-less protein, its turnover is regulated by a process called N-terminal polyubiquitination which is not dependent on lysine residues (152, 153). Rather, the free N-terminal amino group of ARF is used for conjugating ubiquitin. Yet another oddity of ARF can explain this phenomenon. Most eukaryotic proteins are acetylated at their N-termini, which would not permit conjugation of ubiquitin (220). ARF's N-terminal methionine is cleaved by a methionine aminopeptidase, leaving valine and glycine, respectively, as the N-terminal amino acids in human and mouse ARF. Valine and glycine are poor targets for acetylation. As a result, ARF's N-terminus is not acetylated and can therefore be targeted for degradation by the ubiquitin/proteasome system.

Until very recently, the search for an E3 ligase for ARF had been unsuccessful. Two proteins have been identified to be direct E3 ligases for ARF, named ULF (Ubiquitin Ligase for ARF) and SIVA1 (43, 306). Depletion of either ULF or SIVA1 in human cancer cell lines leads to an increase in ARF stability, and *in vitro* experiments indicate that ULF and SIVA1 can directly coordinate the transfer of ubiquitin to ARF (43, 306). ULF is overexpressed in several tumor types, indicating a possible mechanism for ARF inactivation in those cases (30, 89). No such evidence has yet been found for SIVA1. An unanswered question is whether ULF and SIVA1 are functionally redundant. Based on current research, this seems unlikely since depletion of either ULF or SIVA1 is sufficient to increase the half-life of ARF. The expression of ULF and SIVA1 may be tissue specific, or their effects could be additive. The consequence of depleting both these proteins would be useful in addressing these possibilities.

One final peculiarity of ARF structure is the presence of an internal methionine residue at amino acid number 48 in humans and 45 in mice. Reef and colleagues demonstrated that translation can be initiated from this methionine, resulting in a smaller version of ARF appropriately named smARF (236). Mouse ARF's nucleolar localization signal resides in amino acid residues 26-37 (309), so smARF does not localize to the nucleus. Rather, it localizes to mitochondria when overexpressed and can promote autophagy (235). The importance of this finding is a subject of debate since full length ARF is also capable of inducing autophagy from its resident nucleolar compartment (1, 31). In humans, two stretches of amino acid residues, 2-14 and 82-101 are required for proper nucleolar localization (170, 308, 328), so human smARF retains the N-terminal nucleolar signal. Regardless, overexpression of human smARF also localizes to the mitochondria (236). Unfortunately, most studies performed to date have required

gross overexpression of smARF to observe measurable phenotypes. Thus, the physiological role of smARF is still an area of active research.

The odd genetic and biochemical makeup of ARF make it challenging to manipulate experimentally. Great care must be taken in genetic experiments to ensure specific targeting of ARF or p16. Likewise, due to the basic nature of ARF protein, gross overexpression can often lead to non-physiological interactions. Regardless of these challenges, a tremendous amount of experimentation has established ARF's mechanistic role in tumor suppression—that is, the ability to sense oncogenic stress and activate p53-dependent cell senescence or apoptosis. I will first describe the experiments that led to the identification of ARF as a tumor suppressor and then introduce the p53-dependent functions of ARF.

### **ARF-mediated tumor suppression via p53**

Genetic experiments in mouse model systems have generated much of the data that classifies ARF as a *bona fide* tumor suppressor protein (252). Prior to the knowledge that ARF was encoded by *CDKN2a*, a knockout mouse was made that contained a deletion in exons 2 and 3. Without realizing it, Serrano and colleagues obliterated function of both ARF and p16 (254). The knockout mice were highly tumor prone and developed sarcomas or lymphomas by 9 months of age. The kinetics of tumor formation were significantly accelerated by treating the mice with two DNA damaging agents, UVB and 9,10-dimethyl-1,2-benzanthracene (DMBA) (56, 69). Even after these treatments, though, sarcomas and lymphomas dominated the observed tumor spectrum (254). Mouse embryonic fibroblasts (MEFs) harvested from these mice proliferated more rapidly than wild-type littermates and did not undergo culture-induced cellular senescence. Furthermore, the MEFs were capable of forming tumors in mice and forming

colonies in soft agar upon overexpression of mutant *Ras*<sup>V12</sup>, two defining characteristics of neoplastic transformation (122).

What is the mechanism of ARF-mediated tumor suppression? When Quelle et al. initially published their findings on ARF's existence, they also noted that ARF was capable of inducing cell cycle arrest at both the G1/S and G2/M checkpoints without the cooperation of p16 (228). They further demonstrated that ARF was not a CDK inhibitor, like the INK4 family of proteins. The *Arf*-knockout mouse cells were immortalized in cell culture even though they retained wild-type p53, a process that was previously considered to be dependent on p53 disruption (111, 136). This suggested that ARF and p53 might function in a linear genetic pathway. Indeed, elegant studies have subsequently demonstrated that ARF can activate the p53 tumor suppressor pathway (257) (Figure 1.2). A mouse model of B-cell lymphoma constitutively expressing oncogenic c-MYC was particularly useful in this discovery (2). In this model, clonal B-cell proliferation and expansion is initially offset by a high degree of p53-dependent apoptosis, before full-blown lymphoma develops (2, 250). Eischen and colleagues found that lymphomas arising in these mice frequently inactivate *p53* (28%) or *Arf* (24%) and others display overexpression of the E3 ubiquitin ligase for p53, MDM2 (82). When *Myc* transgenic mice were bred to contain only one functional *Arf* allele, the onset of lymphoma was greatly accelerated (mean survival of 11 weeks compared to 30 weeks in *Arf*<sup>+/+</sup> mice). Strikingly, over 80% of these tumors exhibited loss of the remaining *Arf* allele. Together, these data demonstrate that inactivating p53 or ARF is a pre-requisite requirement for *Myc*-induced lymphoma, and provide further evidence for ARF and p53 cooperation in tumor suppression.

The mouse model described above provides a nice transition into the mechanistic role of ARF as a tumor suppressor—sensing oncogenic stress. Numerous studies have indicated that



ARF lies upstream of p53, and promotes its stability by binding and sequestering the E3 ligase, MDM2 in the nucleolus (119, 308, 309, 328). The cellular levels of p53 are tightly regulated to insure inappropriate p53 signaling does not occur. As a result, the turnover of p53 protein is very rapid, with a half-life of about 20 minutes (178). ARF expression is increased in response to unperturbed oncogenic signaling, including RAS (165, 213) E1A (58), v-ABL (229), c-MYC (334), E2F1 (70, 332), NOTCH1 (299),  $\beta$ -CATENIN (51), MAPK (164), and mTOR (197). Once induced, ARF interacts with MDM2 via amino acids encoded by exon-1 $\beta$  (135), which uncovers a cryptic nucleolar localization domain in MDM2 (170, 308). Re-localization of MDM2 to the nucleolus, results in increased p53 protein stability leading to transcriptional activation of genes involved in cell cycle arrest or apoptosis. Remarkably, the first 14 amino acids of ARF are sufficient to bind MDM2 and induce p53-dependent cell cycle arrest (198). This highlights the importance of exon-1 $\beta$  and provides an explanation for the odd architecture of the *CDKN2a* locus in chickens where the only protein product expressed is the N-terminal portion of ARF (generating p7<sup>ARF</sup>) (144).

MDM2 is a proto-oncogene that is frequently amplified or overexpressed in human cancers (303). Besides possessing a RING-finger domain that allows it to ubiquitinate p53 (118), MDM2 can directly bind to p53's DNA-binding domain, inhibiting its ability to trans-activate target genes (199, 209). Additionally, MDM2 can shuttle p53 from the nucleus to the cytoplasm where it is degraded by the proteasome (287). Several groups have shown that ARF can activate p53-dependent cell cycle arrest without re-localizing MDM2 to the nucleolus (147, 168). In this model, ARF, a resident nucleolar protein, is capable of inhibiting MDM2's E3 ligase activity in the nucleus and also blocks MDM2's ability to shuttle to the cytoplasm (288). This alternative model was developed using data obtained from overexpressing ARF in human

and mouse cell lines (NIH3T3, HELA, and U2OS) that harbor tremendous genomic complexity due to p53 loss of function. Therefore, most researchers in the ARF field consider nucleolar sequestration of MDM2 to be the likely physiological mechanism by which ARF induces p53 activation.

While loss of p53 function is the most frequent event in human cancers, many have proposed that the selective pressure for this inactivation stems from p53's ability to respond to DNA damage. However, recent evidence indicates that oncogene sensing by the ARF-p53 pathway is actually the most relevant tumor suppressor function of p53 (80). First, Serrano's group utilized a previously characterized mouse where they engineered an additional copy of *p53*, along with its flanking genomic DNA sequence, into the mouse genome (called *p53<sup>super</sup>*) (92). These *p53<sup>super</sup>* mice had a lower incidence of tumor formation and exhibited an enhanced DNA damage response. To observe whether the DNA damage or oncogenic responses were more important for p53-mediated tumor suppression, they monitored tumor formation in *p53<sup>super</sup>;Arf-null* and *p53<sup>WT</sup>;Arf-null* mice. They hypothesized that the *p53<sup>super</sup>* mice that exhibit a more robust DNA damage response would be slightly less tumor prone. However, both sets of mice succumbed to the same spectrum of tumors under the same time frame (79). They also treated both sets of mice with a DNA damaging agent (3-methyl cholanthrene), and again observed no difference in tumor onset between cohorts. Importantly, the mice lacking *Arf* exhibited a normal DNA damage response, in support of previous reports (134, 282). Thus, even mice capable of enhanced p53 responses to DNA damage require ARF to actively suppress tumorigenesis, suggesting that ARF-mediated oncogenic sensing is the most relevant role of p53 in tumor suppression.

Another elegant study performed by Christophorou and colleagues utilized a tamoxifen-inducible mouse model of p53 restoration. Here, the mice are effectively *p53*-null in the absence of drug, but upon tamoxifen treatment, the p53 protein is capable of being regulated by normal endogenous signals. Six days prior to whole body  $\gamma$ -radiation treatment, mice were either treated with tamoxifen or left untreated (46). Normally, a wild-type p53 mouse will be protected from lymphoma development following irradiation, but a *p53*-null mouse will develop lymphoma by 24 weeks post treatment (140). The authors observed that restoring p53 function prior to irradiation did not have any tumor protective effect, even though a p53-dependent DNA damage response was evident following the genomic insult. Strikingly, restoring p53 function 8 days after whole body irradiation markedly enhanced tumor-free survival, and this effect was completely dependent on the presence of ARF. Taken together, the two studies described above indicate that ARF is absolutely required for p53-mediated tumor suppression. Since ARF does not typically respond to DNA damage, its ability to relay oncogenic signaling to p53 is likely playing a prominent role *in vivo*. These findings are in agreement with a recent study showing a p53 mouse model that is defective in its ability to respond to DNA damage is still resistant to spontaneous tumor formation (273). Certainly, the ability of p53 to respond to numerous stresses, including DNA damage, likely plays a role in suppressing tumorigenesis. I highlighted the two studies described above to simply make the point that the ability of ARF to sense hyper-proliferative and hyper-growth signals emanating from oncogenes is both an important and well-established pathway in human cancer prevention.

## **ARF and p16 in tumor suppression: which one is more important?**

When ARF was discovered, the obvious question of which gene (p16 or ARF) was the more pertinent tumor suppressor was posed. Kamijo and colleagues addressed this issue by creating a mouse harboring specific disruption of Arf's unique exon-1 $\beta$ , leaving p16 completely intact. Astonishingly, these *Arf*-specific knockout mice displayed all the tumorigenic qualities as the *Ink4a-Arf* knockout described in the previous section (136). Thus, at least in mice, ARF appears to be the primary tumor suppressor gene in the *CDKN2a* locus, but this conclusion is not so straightforward. Two independent groups created a *p16*-specific knockout (149, 258), and these mice did spontaneously develop tumors after a year of life, but at a much lower frequency than *Arf*-null mice. The *p16*-deficient mice were more susceptible to tumor formation caused by DMBA treatment compared to wild-type controls. Intriguingly, mutation of both *p16* alleles in combination with deletion of one copy of *Arf* led to the formation of metastatic melanoma (149). *CDKN2a* is frequently mutated in patients with melanoma (132, 245), so this finding suggested that ARF and p16 might cooperate in suppressing melanoma development.

While it is clear p16 can function as a tumor suppressor in the mouse, the two *p16*-knockout models indicated that p16 was not involved in replicative senescence, as *p16*-deficient MEFs underwent senescence after several passages (263). Furthermore, loss of *p16* is not capable of cooperating with *Ras*<sup>V12</sup> overexpression in oncogenic transformation assays. Therefore, in mice it appears that ARF is the more potent tumor suppressor and is directly involved in replicative senescence (259). Data from human cell lines, however, has added a layer of complexity to our understanding of tumor suppression by *CDKN2a*.

In human cells, it seems p16 is more responsive to oncogenic and stress signals than ARF. In response to RAS overexpression or passage in culture, a variety of human cell lines

upregulate p16 to induce senescence, and ARF levels remain low (28, 87, 310). Additionally, p16 inactivation (as opposed to *Arf*-inactivation in mice) is a cooperating genetic hit in oncogene-induced transformation of human cells (28, 74, 301). One caveat of these studies is that they all utilized human fibroblast cell lines in their experiments, so one could argue that human epithelial cells might behave differently. However, mammary epithelial cells display increased p16 expression with increasing population doublings, leading to a growth arrest. This arrest is overcome in clones that have epigenetically silenced the p16 promoter, leaving ARF intact (27). Interestingly, a combination of oncogenes such as B-catenin and Ras, or E2F1 and Ras are sufficient to induce ARF in human cells (18, 85). The findings described above suggest there are major differences between mouse and human cells in transcriptional regulation of *CDKN2a*, but this does not discredit ARF as being a potent suppressor of tumorigenesis in humans. Numerous studies have indicated ARF can inhibit the growth of human cancer cells in various ways (8, 86, 161, 163, 211, 325). Given that ARF activation leads to a p53-response, which usually leads to irreversible growth arrest or cell death, human cells might have adopted much tighter regulation of ARF, only allowing ARF to activate p53 in response to exceedingly high oncogenic signaling (i.e. the combination of oncogenic pathways mentioned above). This evolutionary trade-off might allow for larger and longer-lived organisms.

Analysis of patient tumor samples is arguably the best way to determine the relative importance of ARF and p16 in tumor suppression. Point mutations in p16 were initially identified in familial melanoma patients (117, 123, 132). The majority of these mutations occurred in exon 2 of *CDKN2a* which is shared between p16 and ARF. Functional analysis of these mutations in mice indicated these genetic changes do not affect the ability of ARF to induce cell cycle arrest (227). These studies were performed prior to the knowledge that human

ARF contains a second nucleolar localization domain in exon 2. It was later found that mutation in exon 2 of p16 can affect the localization and function of ARF (328). Furthermore, much of p16's coding sequence in exon 2 actually overlaps with the 3' UTR of ARF. Given the importance of 3'UTR-mediated regulation of gene expression, it is conceivable that any mutation in exon 2 of p16 could have dramatic effects on ARF expression (192, 314). Finally, there is some evidence for p14ARF-specific inactivating mutations or deletions in the germ-lines of familial melanoma patients (90, 232, 239).

In recent years, *The Cancer Genome Atlas* initiative has allowed large scale sequencing efforts to identify common genetic defects in numerous human cancers. These studies have found deletion of the entire *CDKN2a* locus in adenoid cystic carcinoma (115), bladder cancer (40), glioblastoma (34), head and neck squamous cell carcinoma (40), kidney renal papillary cell carcinoma (40), lung adenocarcinoma (71, 125), non-small cell lung carcinoma (99), sarcoma (14), melanoma (40), and stomach adenocarcinoma (40). These exhaustive analyses highlight the tendency of cancers to simply delete the entire *9p21* chromosomal arm that contains *CDKN2a*, and are in agreement with previous reports (extensively reviewed in (245, 248). While examples of specific inactivation of exon-1 $\beta$  or hypermethylation of the CpG island found in *Arf*'s promoter have been documented (72, 84, 158, 232, 248, 269, 298), there are also plenty of cases where p16 is seemingly the only gene altered (99, 256).

The *CDKN2a* locus is the second most frequently inactivated locus in human cancers, behind only p53 (245). This is not surprising given that inactivating this locus effectively knocks out the function of four tumor suppressor proteins: p16, ARF, p53, and Rb. Thus, answering the question of which tumor suppressor, p16 or ARF, is most important in human cancers seems futile. Rather, the frequent occurrence of *CDKN2a* deletion in virtually all types of human

cancers indicates a role for both these proteins in tumor suppression. Since my dissertation work has not provided any new insights into p16 biology, the remaining portion of this introduction will be dedicated to ARF.

### **The biological role of steady-state ARF levels**

Responding to oncogenic stress is undoubtedly ARF's most important function in tumor suppression. In this section, I will discuss the role of "basal" ARF levels in the cell. Work in this area of ARF biology has led to some surprising findings, and again, highlight the unique attributes of this protein.

Expression of ARF is normally kept very low in the cell. In fact, during development and even into adulthood, ARF levels are only detectable by sensitive methods such as quantitative real-time PCR in mouse tissue (335). Two exceptions to this rule, the eye and testes, will be discussed in more detail (105, 336). p16, on the other hand, is not detectably expressed during development and can only be found in tissues from older mice (150, 226, 335). If the cell is expending energy to make ARF protein then it likely serves some function. In the last 15 years, numerous functions of basal ARF have been discovered. The first of these is ARF's role in eye development. McKeller and colleagues found that ARF was required to promote the regression of the hyaloid vascular system (HVS) in the developing eye (193). In *Arf*-null mice, they observed an accumulation of endothelial and perivascular cells in the retrolental tissue. The phenotype was completely penetrant, and as a result of this abnormality *Arf*-null mice are blind (105, 193). Expression of ARF in the eye is enhanced beginning at postnatal day 1 (P1) in mouse development and remains until postnatal day 5 (P5). Since HVS regression occurs between P6 and P10 (128), and ARF expression precedes this, these data suggested that ARF might be

directly involved in promoting HVS regression. Interestingly, this role of ARF is completely p53-independent. Mice lacking *p53* do not have the HVS phenotype and are not blind (193).

In the years that followed McKeller's initial observation, the mechanism by which ARF regulates HVS regression was nicely demonstrated. During development, TGF $\beta$ 2 signaling induces ARF expression by promoting chromatin remodeling around the *Arf* promoter. This is dependent on the activities of p38MAPK and SMAD2/3 (91, 330, 331). The increased ARF levels inhibit PDGFR $\beta$  receptor tyrosine kinase expression, whose activity is required for pericyte accumulation (270, 313). Thus, in mice lacking *Arf*, PDGFR $\beta$  expression and activity is unrestricted leading to improper HVS regression.

As I alluded to, ARF also plays a role in the mouse testes. Gromley and colleagues generated an *Arf*-reporter mouse that expressed Cre-recombinase driven by the endogenous *Arf* promoter (105). It is important to note that this mouse was generated by knocking in Cre-recombinase to the endogenous *Arf* locus, so the result is an *Arf*-null mouse. They bred this mouse with mice containing a LSL-YFP (Lox-Stop-Lox-Yellow fluorescent protein) expression cassette knocked-in to the *Rosa-26* locus. If Cre-recombinase is present, the stop codon before YFP is excised and cells will subsequently express YFP. The authors were able to visualize YFP expression (a surrogate for ARF) in the testes, and noticed that the mice that were expressing YFP (and were therefore lacking ARF), exhibited testicular atrophy (105). As a result, sperm counts in the male mice were significantly lower than wild-type controls. Paradoxically, it was shown that ARF expression in the progenitor spermatogonia cells actually promoted the survival of the spermatocytes generated upon meiosis (47). ARF is normally thought to growth-inhibitory, so this finding was quite unexpected. At the very least, it explains why ARF



accumulates to such high levels in the testes, and certainly highlights the tissue-specific roles of ARF.

Another physiological role for ARF that was discovered by our lab is its ability to regulate protein synthesis (cellular growth), by monitoring nucleolar function. The nucleolus is a non-membrane bound organelle within the nucleus that forms around ribosomal DNA (rDNA) repeats. This structure is the center of ribosome biogenesis in the cell, and its function is directly tied to proliferative status (21). Cells that are dividing rapidly must increase rates of protein synthesis, which is dependent on ribosomal availability. For many years, pathologists have used a measure of nucleolar function known as AgNOR staining to assess the prognosis of certain cancer types (218). This staining method takes advantage of the fact that argyrophilic proteins (“silver-loving”) assemble around rDNA repeats, forming what are called nucleolar organizing regions (NORs) (176). Utilizing this AgNOR staining method, Apicelli *et al.* found that loss of *Arf*, both *in vitro* and *in vivo*, leads to increased numbers of nucleoli as well as abnormal morphology (7). Furthermore, cells from *Arf*-null mice exhibited increased rates of protein synthesis and accumulated more protein on a per cell basis. These gains in protein synthesis were due to enhanced rDNA transcription, rRNA processing, and rRNA export from the nucleus into the cytoplasm. ARF has previously been linked to all of these processes (248). ARF’s ability to regulate cell growth was entirely dependent on its ability to interact with a protein called Nucleophosmin (NPM) that plays a well characterized role in ribosome biogenesis (175). To further demonstrate the physiological role of ARF-regulated cell growth, the authors demonstrated osteoclasts from *Arf*-null mice were larger and exhibited enhanced bone-resorbing functions. As a result of dysfunctional osteoclasts, *Arf*-null mice do not properly regulate bone turnover ((233) and Crystal Winkeler unpublished observations). Taken together, the findings

presented above indicate that low levels of endogenous ARF are necessary to maintain proper nucleolar function.

The incidence of cancer increases in direct correlation with one's age (57). Thus, cancer is a disease that primarily affects an aging population who are in the decline of their reproductive potential. As a result, it is thought that tumor suppressor genes such as ARF, did not evolve to protect against cancer (262). Rather, they have adopted these secondary roles as a consequence of our long lifespans. The evolutionary conserved functions of ARF, therefore, are most likely to be the biological roles described in this section as well as others that have yet to be identified. As an added bonus, the Arf promoter is sensitive to oncogenic signals which allow our cells an opportunity to combat mutations or amplification of proto-oncogenes. Given these characteristics, the regulation of ARF expression must be complex, and I will discuss these intricacies in the following section.

### **Regulation of ARF expression: transcription, translation, and association**

I have mentioned that ARF levels normally kept low in cells to protect against aberrant p53 activation. How is this accomplished? Furthermore, how do cells relay oncogenic signaling to the Arf promoter leading to its induction? In this section I will provide answers to these questions. The regulation of ARF expression is complex, involving transcriptional and translational control, as well as stabilizing-interactions with binding partners. I will begin with a discussion of the factors involved in transcriptionally regulating the Arf promoter as well as those that aid in Arf mRNA translation.

### *Transcriptional and Translational regulation of Arf*

Some researchers have argued that the unique genomic organization of *CDKN2a* allows for coordinated control. By placing three tumor suppressor genes within a 35Kb stretch of DNA, this region of chromosome 9 can be blanketed with heterochromatin during development to insure cell cycle arrest is not induced (95). Indeed, transcriptional repression of *Arf* (as well as *Ink4a* and *Ink4b*) is primarily mediated by the polycomb group proteins (PcG). This group of histone-modifying proteins was initially characterized in *Drosophila* and consists of two distinct complexes called PRC1 and PRC2 (200). PRC2 catalyzes the tri-methylation of H3K27, which is dependent on the methylation activity of EZH2 (37, 201). Other components of PRC2 are SUZ12, which promotes EZH2 function (214), and HDAC1/2 which de-acetylate histones (200). The histone modification that PRC2 catalyzes serves as a signal for PRC1 binding. PRC1 consists of a catalytic subunit, RNF2 (also called RING1b), BMI-1, and several CBX proteins (271). RNF2 catalyzes the ubiquitination of histone H2A, and BMI-1 supports its E3-ligase function.

One interesting debate in the field of PcG proteins is how the PRC complexes get recruited to genomic loci. Recently, it was demonstrated that a long non-coding RNA (lncRNA) called *HOTAIR* could recruit PRC2 to complexes to the *HoxD* gene cluster (238). This is similar to the function of the lncRNA, *Xist*, which is involved in X-inactivation (329). Intriguingly, a lncRNA named *ANRIL* was identified that overlaps with *CDKN2a*, but is transcribed in the opposite direction (215). It was later demonstrated by Yap *et al.* that ANRIL can recruit PRC2 complexes to *CDKN2a* and silence the locus (4, 319).

The genetic evidence that implicates PCG proteins in the regulation of Arf expression is overwhelming. For example, the *Ring1b*-knockout mouse is embryonic lethal, but this

phenotype can be rescued by co-inactivation of *CDKN2a* (300). Likewise, cells lacking *Bmi-1* prematurely senesce in culture due to increased ARF and p16 expression (130). BMI-1 acts as an oncogene by suppressing ARF expression in the mouse. As a result, inactivation of *Bmi-1* can cooperate with RAS or MYC overexpression to enable tumor formation (54, 116, 131). Overexpression of BMI-1 is commonly found in human tumors since this effectively silences the *CDKN2a* (Reviewed in (36)). Other polycomb members including CBX7 (19), CBX8 (67), and EZH2 (44) both bind and repress the *Arf* locus. Cells lacking any of these proteins undergo premature senescence in culture, due to ARF de-repression.

Through the activities of the PcG proteins, access to the *Arf* promoter is greatly limited. An additional layer of inhibition is provided by the fact that the *Arf* promoter is a CpG island that can be hypermethylated (242). Moreover, several non-PcG proteins such as E2F3b (96), Pokemon (173), TBX2 (129), TBX3 (321), and ATF4 (120), and TWIST (174) can directly inhibit ARF expression. It is important to consider that not all of these factors are expressed in every given cell type at a given time. Differences in when and where these factors are expressed, as well as cellular environment, likely determine to what extent the *Arf* promoter is accessible. Clearly, human and mouse cells have evolved elaborate transcriptional networks to inhibit ARF expression. How, then, is ARF able to sense oncogenic signaling and become induced?

One way to counteract the effects of epigenetic silencing is to recruit ATP-dependent chromatin remodeling complexes such as SWI/SNF, and lysine demethylases to remove the inhibitory H3K27me3 chromatin marks. Indeed, both SWI/SNF and a demethylase called JMJD3 are important for Ras-mediated ARF induction (3, 13, 143). Using a mouse model expressing a mutant *K-ras*<sup>G12D</sup> allele as well as GFP knocked-in to exon-1β (336), Young *et al.* demonstrated that SNF5, a component of SWI/SNF was required for ARF upregulation in the

arising sarcomas (322). This suggests that in muscle tissue, ARF is activated by the chromatin remodeling activities of SNF5. SNF5, therefore, would be an ideal target for inactivation in cancer cells. Indeed, SNF5 inactivation is a frequent genetic occurrence in malignant rhabdoid tumors (241). Notably, GFP expression was not observed in the lung tumors that arose in *K-ras<sup>G12D</sup>;Arf<sup>GFP/GFP</sup>* mice, indicating that ARF was not induced in this tissue in response to RAS (322). These observations highlight the limited understanding that we have regarding Arf regulation *in vivo*. Why some tissues would respond to Ras by upregulating ARF and others would not remains unanswered. One possibility is that lung tissue is highly exposed to environmental stresses (i.e. smoke and other carcinogens), so it is possible that ARF expression in the lung is more tightly regulated than in muscle to prevent aberrant p53 activation. An additional mechanism for relieving PcG-mediated inhibition was recently shown by Liu *et al.* They showed that the PI3K-AKT pathway, specifically AKT1, was able to phosphorylate BMI-1 on Serine-316 which impaired its ability to associate with chromatin (167).

While it is necessary to evict PcG proteins from the *CDKN2a* locus to initiate Arf transcription, there must also be specific transcription factors that facilitate RNA polymerase II-mediated transcription. The best characterized transcription factor in this process is known as DMP1 (cyclin D-binding, Myb-like Protein 1). DMP1 was demonstrated to be a haplo-insufficient tumor suppressor, and *Dmp1*-null MEFs are immortalized in culture because they do not upregulate ARF (126, 127). The *Dmp1*-null mice were highly tumor prone to carcinogen-induced tumors and frequently retained Arf and p53, indicating loss of Dmp1 can phenocopy inactivation of the Arf/p53 pathway. Moreover, *Dmp1* deficiency cooperates with c-MYC overexpression to induce lymphoma formation in mice. Usually, c-MYC overexpression initiates the ARF/p53 pathway leading to apoptosis, and tumors can only arise when this

pathway has been disabled (82). In the c-MYC overexpressing *Dmp1*-null mice, loss of ARF or p53 was rarely seen indicating *Dmp1* loss can substitute for Arf/p53 inactivation (127). Finally, a mouse model of breast cancer expressing the HER2 oncogene displayed enhanced tumor formation when *Dmp1* was deleted, resulting from reduced ARF/p53 signaling (285). These data placed DMP1 upstream of the ARF/p53 pathway and led to the observation that DMP1 plays a critical role in relaying RAS-signaling to ARF (276).

In response to RAS overexpression, the *Dmp1* promoter is induced by activation of the mitogen-activated protein kinase cascade (MAPK). Specifically, activation of the RAF-MEK-ERK signaling cascade leads to activation of the c-Jun transcription factor which is capable of directly promoting *Dmp1* expression (276). DMP1 can then directly bind to the Arf promoter and induce its transcription. Interestingly, it was noticed that this linear pathway could not completely explain ARF induction because cells lacking *Dmp1* still exhibited some increases in ARF (276). Miceli and colleagues investigated this phenomenon and discovered that both transcriptional and translational upregulation is required for a complete ARF response. Arf mRNA translation was stimulated by activation of mTORC1 (197). A very recent publication has provided a further examination of this signaling pathway. Shin *et al.* demonstrated that it is ERK2 (not ERK1) downstream of MEK that is required for DMP1 upregulation. ERK2 is also responsible for phosphorylating the TSC2 complex (thereby inactivating it), which normally inhibits mTOR (267). Taken together, these data provide a linear model whereby RAS overexpression upregulates ARF by both transcriptional and translational means, leading to a p53 response that either halts proliferation or initiates cell death. The fact that ARF can be upregulated by mTOR suggests that ARF not only responds to hyper-proliferative signals induced by oncogenes, but also hyper-growth signals that mTOR coordinates.

Complicating this view, however, is the fact that expression of endogenous levels of mutant Ras does not engage the ARF/p53 pathway, most likely due to the lack of MAPK activation (41, 293). These cells proliferate more rapidly and exhibit morphological changes associated with transformation. Thus, it is not Ras-signaling *per se* that leads to ARF activation, but rather high RAS expression levels. A similar situation occurs with the c-MYC oncogene. Chen and colleagues showed that both low and high levels of c-MYC induce Arf mRNA expression, but only the high levels of c-MYC were capable of inducing ARF protein. They demonstrated that it was the ability of high c-MYC levels to inhibit one of ARF's E3 ligases, ULF, that led to the ARF induction and subsequent p53 response (42). The authors that demonstrated a lack of ARF induction in endogenously expressing mutant *Ras* cells did not measure mRNA levels, so it is certainly possible a similar situation exists. While it is clear there is much to discover in terms of understanding the physiological signals that mediate ARF upregulation, the frequency of *CDKN2a* in human cancers overwhelmingly indicates expression of genes at this locus are selected against during tumor progression.

Other transcription factors can directly activate the Arf promoter, including Pit-1 (65), Smad2 (91), FoxO3a (24), and E2F1 (70, 332). E2F1's ability to activate Arf transcription provides a direct link between the Rb and p53 pathways. Loss of Rb or overexpression of CDK4/6 would lead to increased E2F1 levels. Sustained activation of this pathway can therefore lead to ARF activation, yet another means by which ARF senses inappropriate proliferative signals. One final means of ARF regulation that I would like to mention was recently published by Kawagishi *et al.* These authors found that a protein called HuR (Human antigen R) was capable of binding to the 5' UTR of Arf mRNA and repressing its translation. This affect was specific to Arf, and not p16, since they do not share 5'UTR sequence (138). Very little is known

about regulation of Arf expression at its 3' UTR. Only one miRNA, miR-24, has been found to bind and repress translation of the Arf mRNA, but future studies will likely identify others (290).

#### *The ARF-NPM Stabilizing interaction*

Earlier in this introduction, I described the reasons for ARF's inherent disordered structure. As a result of its biochemical characteristics, ARF is thought to require interactions with other proteins for maintaining stability. One of ARF's best characterized interaction partners is the nucleo-cytoplasmic shuttling protein, Nucleophosmin (NPM) (22, 166). NPM is essential for normal cellular function, as knockout mice are embryonic lethal (102). It is a multifunctional protein, serving functions in ribosome biogenesis (175), centrosome duplication (305), and DNA damage (49). As I alluded to, the ARF-NPM interaction is required for ARF stability (49). ARF utilizes its N-terminal 14 amino acids to associate with NPM (20), the importance of which is underscored by the fact that *Arf* mutants lacking amino acids 1-14 exhibit rapid turnover (152). Mutations in NPM affecting the C-terminus (called NPMc) are commonly found in acute myeloid leukemia patients (39). These mutations result in an extra nuclear export signal, allowing NPM to rapidly shuttle between the nucleus and the cytosol. As a result of this "super-shuttling" capability, ARF becomes de-localized from the nucleolus allowing it to be targeted for degradation and inhibiting its ability to bind MDM2 to activate p53 (60).

Interestingly, it was also recently shown by Velemezi and colleagues that loss of ATM, a protein involved in propagating the DNA damage response pathway, leads to ARF upregulation. They demonstrated that ATM is able to phosphorylate protein phosphatase 1 (PP1), which results in inhibition of Nek-2-mediated phosphorylation of NPM. As a result, the ARF-NPM interaction is disrupted and ARF is able to be ubiquitinated by ULF. When ATM is inhibited, Nek-2



phosphorylates NPM and promotes the ARF-NPM interaction, offering ARF protection from ULF-mediated degradation. When *p53*-deficient cells were treated with an ATM inhibitor or an ATM-specific shRNA, the cells induced ARF and underwent a subsequent reduction in proliferation which was shown to be directly ARF-mediated. Furthermore, a panel of primary human lung tumors with *p53* mutation and low ATM expression, expressed high p14 ARF and exhibited lower proliferation (295). Taken together, the studies discussed in this section emphasize the importance of ARF's intracellular interactions, particularly with NPM.

#### *The p53 and ARF negative feedback loop*

A final intricacy of ARF regulation involves the *p53* protein. It has been observed since ARF's discovery that cells lacking *p53* contain elevated levels of ARF, suggesting a possible inhibitory feedback loop between *p53* and ARF (228, 281, 334). The feedback loop exists in both human and mouse cells (282), and has been demonstrated *in vivo* with mouse tumor models (12). For many years, only correlative data existed to link *p53* to the repression of *Arf* transcription. Re-introduction of *p53* cDNA into *p53*-null MEFs led to a reduction in ARF protein levels, and *p53* was able to inhibit expression of a chloramphenicol acetyltransferase (CAT) reporter construct regulated by *Arf* promoter sequence (135, 242). Mechanistically, it seems that *p53* is a direct transcriptional repressor of the *CDKN2a* promoter. Zeng *et al.* showed that *p53* could directly bind to a stretch of DNA upstream and downstream of exon-1 $\beta$  (324). No binding of *p53* was found in or around the p16 promoter. It was shown that histone deacetylase 1 (HDAC1) was recruited to the *Arf* locus by *p53* and its deacetylation of histones was required for *p53*-mediated repression. This finding was consistent with previous work that has found HDACs to be major regulators of *Arf* transcription (189, 321). Additionally, *p53* can recruit two

components of the PRC2 complex, EZH2 and SUZ12, leading to the inhibitory tri-methylation of H3K27.

The negative feedback loop between p53 and ARF likely exists to allow fine-tuning of the p53 response. Increased p53 stability as a result of DNA damage or oncogenic stress leads to an enhanced occupancy of p53 at the *Arf* promoter. This would repress *Arf* transcription and thus reduce ARF's inhibitory effects on MDM2. With a larger pool of available MDM2, the cell would be capable of turning off the p53 response, assuming the stress signal was no longer present. If the stress signal persists, p53 remains stabilized and can activate senescence or apoptosis.

A consequence of this negative feedback loop is that cells lacking proper p53 function (i.e. most human cancer cells), de-repress ARF resulting in its accumulation. Since a large portion of human cancers inactivate p53, the obvious question of whether ARF becomes induced to provide some protective affect can be posed. Does ARF possess p53-independent functions and can these functions suppress tumor growth in cancer cells that lack *p53* function? Given that *p53*-null mice succumb to tumor burden by 6 months of age, and that cells lacking *p53* proliferate rapidly, most people have assumed that ARF serves no tumor suppressor function in this context (73). However, mounting evidence indicates that this conclusion may not be entirely accurate.

### **ARF-mediated tumor suppression without p53?**

Experiments performed with mouse models provided the first evidence that ARF possesses p53-independent tumor suppressor functions. Eischen and colleagues compared the proliferation of bone marrow cells harvested from c-MYC overexpressing mice that harbored

deletions in *Arf*, *p53*, or *Arf/p53* double knockouts. Surprisingly, the cells lacking both *Arf* and *p53* had much higher proliferation rates than cells lacking either gene alone (82). *Arf*-null and *p53*-null mice both develop tumors, with sarcomas being most prevalent in *Arf*-null and T-cell lymphomas dominating in *p53*-null animals. *Arf*-null mice live slightly longer, with a mean latency of survival of 32 weeks compared to 19 weeks for *p53*-null animals (73, 133). Weber *et al.* generated *p53/Mdm2*-null (DKO), *Arf/p53/Mdm2*-null (TKO), and *Arf/p53*-null mice. All three strains developed tumors with a mean latency similar to that of single *p53*-null mice (307). Notably, 50% of the TKO mice and 30% of the *Arf/p53*-null mice developed tumors at multiple sites. Many of these tumors such as intestinal adenocarcinomas, renal cell carcinomas, and rhabdomyosarcomas have never been observed in *p53*-null mice. Therefore, loss of *Arf* in a *p53*-deficient mouse permits the formation of tumors that do not usually have time to surface in the lymphoma-prone *p53*-null mouse. Additionally, the authors demonstrate that overexpressing ARF in the MEFs harvested from the DKO and TKO mice leads to cell cycle arrest 72 hours post transduction (307). For reasons that are not yet clear, the *Arf/p53*-null mice were not as sensitive to ARF overexpression. It is possible that the presence of MDM2 might sequester the ability of ARF to interact with its p53-independent targets. This is unlikely since MDM2 expression is usually quite low in cells lacking *p53* due to p53's ability to positively regulate its transcription (184). Regardless of these uncertainties, the studies described here clearly demonstrate that ARF possesses p53-independent tumor suppressor functions.

Further work in mouse cell lines overexpressing ARF corroborated the findings by Weber *et al.* (38). These findings are not limited to mouse cells, as studies using human cell lines have also suggested ARF is capable of arresting cells that lack p53 function (76, 86, 163, 205, 320). From these experiments, it seems overexpression of ARF is able to arrest cells at both the G1/S

and G2/M cell cycle boundaries. Muniz and colleagues have recently shown that ARF overexpression can inhibit the growth and metastasis of a *p53*-mutant human pancreatic cell line in the mouse (202). Efforts to identify the important *p53*-independent targets of ARF have also been undertaken. Kuo *et al.* analyzed transcriptome changes in response to ARF overexpression. Many *p53*-dependent targets were found, but they also uncovered upregulated genes such as *Btg1* and *Btg2* that were capable of inhibiting the proliferation of *p53*-null MEFs (155). However, these have not been validated or explored further in recent years. A major caveat of all these studies is that they rely on gross overexpression of ARF in *p53*-deficient cells that already contain elevated ARF levels. By overloading the cells with a “sticky” protein such as ARF, there is a great potential for non-specific interactions that could result in cell-cycle arrest. As a result, direct evidence implicating ARF as a *p53*-independent tumor suppressor is currently lacking.

If *p53* can suppress tumorigenesis independently of *p53* in humans, then functional loss of ARF and *p53* should not be mutually exclusive. Rather, we should be able to find cases of human tumors where *p53* is mutated and *CDKN2a* has been deleted or epigenetically silenced. This would indicate those cancer cell clones selecting against a tumor suppressive function of ARF that is *p53*-independent. Indeed, cancers of the breast (177, 286), lung (35, 183), and pancreas (11, 244) as well as sarcomas (216) and glioblastomas (296) frequently harbor co-inactivating mutations or deletions of ARF and *p53*. Additionally, mouse tumor models suggest ARF and *p53* co-inactivation occurs. In a *Kras*<sup>LSL-G12D</sup>;*p53*<sup>Flox/Flox</sup> model of intrahepatic cholangiocarcinoma, Cre-recombinase expression specifically in the liver leads to Ras<sup>G12D</sup> expression, *p53* inactivation, and results in the formation of liver cancer (207). From the six tumors that were analyzed by western blot, 3 of them displayed no or low ARF expression. In

summary, mounting evidence indicates ARF is capable of suppressing the proliferation of incipient cancer cells independently of p53. Primary human tumor data also suggests that inactivation of ARF and p53 can occur within the same tumor cell.

### **The p53-independent functions of ARF**

A p53-independent function of ARF can be defined as any biological process in which ARF participates that does not require p53 activation. By this definition, I have already discussed three of these roles for ARF—its role in the developing eye, testes, and regulation of overall cellular growth. In this section, I will discuss other attributed p53-independent functions of ARF, and revisit cellular growth by offering mechanistic insights into its regulation. The functions that I will discuss are considered to be the most likely means by which ARF can suppress tumorigenesis in the absence of *p53*. For a graphical representation of the known p53-independent functions, see Figure 1.3.

#### *Cellular growth*

The ability of cells to generate new proteins directly influences its ability to traverse the cell cycle and divide. I have already discussed the growth-inhibitory role of steady-state ARF levels, but I have not provided any mechanistic explanations to validate this observation. This area is arguably ARF's best studied p53-independent function in the cell, and numerous groups have demonstrated a variety of steps in ribosome biogenesis that ARF regulates.

The ability of a cell to make ribosomes and transport them to the cytosol are rate limiting steps in the production of protein. Ribosome production begins in the nucleolus, a non-membrane bound organelle centered around ribosomal DNA repeats (112). The human genome

encodes approximately 400 of these rDNA repeats, organized in a head-to-tail fashion. Transcription of the 47S rDNA gene by RNA polymerase I generates the 47S rRNA precursor that is subsequently processed and chemically modified (2'O-methylation of the pentose sugar and pseudouridylation) to create the 18S, 5.8S, and 28S rRNA species. Along with the 5S rRNA which is transcribed independently by RNA Pol III, the processed rRNAs complex with numerous ribosomal proteins to form the 40S and 60S ribosomal subunits in the nucleus (156). Once assembled, the individual subunits can be exported to the cytoplasm to participate in protein synthesis. If the appropriate growth signals are present, the 40S and 60S subunits combine on a single mRNA to form an 80S unit that can then translate the message (169, 274). Multiple 80S complexes can be present on a single circularized mRNA which are then described as poly-ribosomes. This over-simplified view of an extremely complex process is essential for understanding where ARF imposes its regulatory functions.

As a resident nucleolar protein, ARF is ideally situated to regulate many of the steps of ribosome biogenesis, and therefore cell growth. I described in an earlier section how loss of ARF led to gains in rRNA transcription, rRNA processing, ribosomal export, and overall protein synthesis (7). The simplest possible explanation for this phenotype would be that ARF regulates the most upstream component in this pathway, rRNA transcription. Indeed, ARF can directly bind to rDNA promoters and inhibit the phosphorylation of the vital RNA Pol I transcription factor, upstream binding factor (UBF) (8, 9). Furthermore, Lessard and colleagues have demonstrated that ARF can inhibit the nucleolar localization and stability of another RNA Pol I transcription factor, TTF-1 (Transcription termination factor-1), resulting in reduced production of the 47S rRNA (160, 161). Finally, work from our lab by Saporita *et al.* showed that ARF can inhibit the nucleolar localization of an RNA helicase DDX5 which is important for rDNA

transcription. DDX5 was required for Ras-mediated transformation, validating it as a p53-independent tumor suppressor target of ARF (247).

In addition to controlling rDNA transcription, ARF is capable of inhibiting rRNA synthesis at the level of processing. This function is entirely independent of p53 and requires the first 14 amino acids of ARF (283). Also dependent on ARF's first 14 amino acids is its interaction with the ribosomal chaperone, NPM (20, 175). NPM is required for proper nuclear export of ribosomal subunits. Mice carrying hypomorphic *Npm* alleles are embryonic lethal, and display a striking phenotype where ribosomes are stuck at the nuclear membrane, unable to make it into the cytoplasm (175). ARF is able to bind NPM in the nucleolus and inhibit its ability to shuttle ribosomes from the nucleus to the cytoplasm. Interestingly, MDM2 overexpression can disrupt this interaction because ARF has a higher affinity for MDM2 (26). Thus, ARF is able to inhibit all the major steps of ribosome biogenesis. As a regulator of both cell growth and cell proliferation, ARF is positioned to coordinate these two processes. Cancer cells are unable to respond to anti-proliferative signals, and as a result must increase their synthesis of proteins to satisfy demand. Since ARF is a key downstream sensor of hyper-growth pathways such as mTOR and RAS, this provides a further selective pressure on cells to inactivate the *CDKN2a* locus.

### *Autophagy*

A link between ARF and autophagy was first suggested by Reef *et al.* when they demonstrated translation initiation from an internal methionine residue within Exon 2 of ARF results in a smaller version of the protein called smARF. They showed that smARF could localize to mitochondria, and when overexpressed, could induce autophagy (236, 260).

Autophagy is an evolutionarily conserved survival mechanism that cells utilize in poor nutrient environments. Cells undergoing autophagy digest cytoplasmic components to generate free amino acids, providing a temporary supply of nutrients that allows the cell to survive (151). It is now thought that both smARF and full length ARF are capable of inducing autophagy in a p53-independent manner (31). The involvement of this ARF function in tumor suppression is a subject of debate. Achieving the ARF-mediated autophagy response requires overexpressing ARF, a response that usually leads to cell cycle arrest or apoptosis. One group has shown that in cells lacking *p53* where ARF levels are increased, autophagy is induced. Depleting ARF expression in *p53*-null B-cell lymphoma cell lines led to a reduced ability of these cells to initiate tumor formation *in vivo* (10). These data suggest a tumor promoting role for ARF-mediated autophagy, but the physiological significance of this finding will require further validation.

### *Sumoylation*

ARF has been reported to interact with more than 30 different proteins (261). I have already discussed the importance of these interactions in maintaining ARF's stability, but the functional significance of these interactions is not as one-sided as it may seem. ARF can actually induce sumoylation of many proteins with which it interacts, including MDM2 (318), E2F1 (240), MIZ1 (113), HIF-1 $\alpha$  (240), WRN (316), and NPM (154). Sumoylation is a post-translational modification of proteins that conjugates the small-ubiquitin like modifier (SUMO) to lysine residues. There are 4 different members of the SUMO family, named SUMO1-4 (196). Conjugation of SUMO is performed by an activating enzyme (E1), a conjugating enzyme (E2), and an E3 ligase that provides target specificity (94). Sumoylation of proteins has pleiotropic effects, ranging from disrupting protein-protein interactions, promoting new interactions, to



changing sub-cellular localization (94). While many of the functional consequences associated with ARF-mediated sumoylation are not completely understood, progress has been made in mechanistically understanding how ARF promotes this modification. Kuo and colleagues have shown that ARF expression leads to the destabilization of the SUMO2,3-specific protease, SENP3. Similar to ARF, SENP3 protein stability depends on its ability to interact with NPM, so when ARF is overexpressed SENP3's ability to interact with NPM is reduced and it is rapidly degraded (154). Intriguingly, depletion of SENP3 in *p53/Mdm2/Arf*-null MEFs reduced proliferation in a manner similar to ARF overexpression. This suggests that ARF's ability to arrest p53-deficient cells might stem from its ability to inhibit expression of SENP3, leading to an increase in global sumoylation. An understanding of which newly sumoylated proteins lead to cell cycle arrest has not been elucidated, but NPM is a likely candidate given that sumoylation of NPM negatively affects its ability to promote rRNA processing (108).

#### *Transcriptional and Translational Regulation*

ARF is also capable of regulating the transcriptional activities of several proteins involved in promoting proliferation. Two independent groups demonstrated that ARF could inhibit the transcriptional activities of c-MYC (53, 225). The direct mechanism of this inhibition is not completely clear, as one group found ARF sequestered MYC in the nucleolus and the other demonstrated ARF directly inhibited MYC at target promoters by an inhibitory association. Regardless of the exact mechanism, inhibition of c-MYC is likely to affect ribosome biogenesis, as c-MYC can promote rRNA synthesis and overall protein synthesis, although this has not been experimentally tested (294). Likewise, Mason *et al.* demonstrated that ARF could directly bind to E2F1 and inhibit its transcriptional activity (188). Interestingly, this indicates that ARF is

both induced by E2F1 as I described earlier, and can directly inhibit its activities. Aberrant signaling by the CyclinD-CDK4/6 complexes is therefore carefully monitored by ARF. Finally, ARF can promote the transcription of TIMP3 (tissue inhibitor of metalloproteinase-3), a protein involved in inhibiting the migration of endothelial cells (224). Consequently, loss of ARF can lead to downregulation of TIMP3 and increased angiogenesis in tumors. This function of ARF was attributed to its ability to bind human MDM2 (HDM2), allowing SP1 to be released and activate TIMP3 transcription (325).

In addition to regulating gene expression, it has recently been shown that ARF can regulate the translation of specific mRNAs. Kawagashi and colleagues found that ARF could inhibit the association of VEGF mRNA with ribosomes, leading to decreased protein VEGF protein. VEGF plays an important role in stimulating angiogenesis, thereby insuring tumors have an adequate blood supply. Loss of ARF led to enhanced angiogenesis *in vivo*, and immunohistochemistry analysis of human colon carcinoma samples revealed an inverse correlation with blood vessel density and ARF expression (139). Our lab has demonstrated that ARF regulates the translation of the DROSHA and DHX33 mRNAs. DHX33 is a RNA helicase that promotes RNA polymerase I function, promoting the transcription of ribosomal RNAs (327). DROSHA is a multifunctional RNase III endonuclease that is best known for its involvement in micro-RNA (miRNA) biogenesis, but also contributes to rRNA processing (61, 317). Both DROSHA and DHX33 are required for RAS-mediated transformation of mouse cells, so in normal cells ARF would respond to RAS overexpression by reducing the expression of these two proteins. ARF is able to inhibit polyribosome formation on the DHX33 and DROSHA mRNAs, but the exact mechanism of this process has not been determined. Taken together, these studies implicate ARF in the translational regulation of select mRNAs. How

ARF accomplishes this is not known, but it is tempting to speculate that ARF's regulation of DROSHA might alter the cellular pool of miRNAs which would lead to changes in translation for many specific mRNAs.

### **The Type I Interferon Response in Cancer**

My dissertation work has steered me down an unexpected path involving several components of the type I interferon response pathway. As a result, I will introduce this signaling pathway and describe some of the members that specifically pertain to my work. I will detail the current understanding of the role these members play in cancer.

### **Canonical Interferon Signaling**

Interferon production is an innate immune response employed by a large variety of cell types to inhibit viral infection. The two most well characterized classes of interferon are type I (consisting of various IFN- $\alpha$  family members, IFN- $\beta$ , IFN- $\epsilon$ , IFN- $\kappa$ , and IFN- $\omega$ ) and type II (IFN- $\gamma$ ) (219). These cytokines activate signaling through the JAK/STAT pathway leading to the transcriptional upregulation of various interferon sensitive genes involved in the anti-viral response.

Type I interferons, in particular, bind to a heterodimeric membrane-bound receptor consisting of IFNAR1 and IFNAR2. Upon ligand binding, a conformational change allows autophosphorylation of receptor-bound JAK1 and TYK2. The activation of these kinases leads to phosphorylation of STAT1 and STAT2. Once phosphorylated, STAT1 and STAT2 form a heterodimer which further associates with IRF9 to form a complex known as IFN-stimulated

gene factor 3 (ISGF3). ISGF3 enters the nucleus and initiates transcription of genes containing IFN-stimulated response elements (ISREs) (278) (See Figure 1.4 for graphical depiction of this process).

It has been reported that more than 300 genes are stimulated by type I interferon (62). The function of many of these proteins is still unknown, but several well characterized proteins are absolutely required for an effective host-response. The family of OAS (2',5'-oligoadenylate synthetase) and OASL (OAS-Like) genes encode proteins that utilize ATP to catalyze the addition of adenosine to RNA molecules through a 2',5'-phosphodiester linkage (234). This specific modification is recognized by and activates RNaseL, which degrades single-stranded RNA molecules (246). Another interferon-sensitive gene family are the Mx-GTPases. The Mx proteins localize around the ER membrane and trap viral components as they bud off from this organelle (246). Numerous other important genes are induced following type I interferon, including ISG15, which I will discuss in more detail in a later section.

While activation of the STAT1/STAT2 heterodimer specifically activates ISREs, various other combinations of STAT proteins including STAT3 and STAT5 can promote the transcription of interferon response genes. However, these STAT complexes do not associate with IRF9 and are thus not able to bind to ISREs. Rather, they bind to specific promoter elements of interferon response genes containing an IFN- $\gamma$ -activated site (GAS). As the name implies, genes containing GAS elements are also the primary transcriptional target of activated STAT1 homodimers formed in response to type II interferon (219, 291). While much is known about the signal transduction pathway leading to activation of interferon response genes, the biological effect of many interferon sensitive genes remains poorly understood.

### *STAT1 in cancer*

In recent years, it has become evident that JAK/STAT signaling in response to interferon is much more than simply an anti-viral response. In fact, this signaling pathway plays a major role in the biology of various human diseases, including cancer (98). Inflammation, for example, is an emerging hallmark of cancer cells, and STAT3 activation is capable of inducing inflammation and promoting cell proliferation (110, 323). Numerous studies have implicated STAT3 as a driver of tumorigenesis, which is capable of responding to a variety of oncogenic stimuli, including the inflammatory promoting cytokine IL-6 (103, 323). In contrast, STAT1 is typically considered to have tumor suppressive functions and can evoke an immune response thought to inhibit tumor growth. More specifically, immune cells recruited to a developing tumor by cancer-specific antigens can secrete IFN- $\gamma$  as well as type I IFN to induce an anti-proliferative and pro-apoptotic response. Both of these responses are mediated through STAT1 (78). Paradoxically, a sustained immune response can lead to chronic inflammation which can promote tumorigenicity (104). Treatment of numerous types of cancers with type I interferon has been approved by the FDA, and many patients see a great survival benefit (78). Taken together, the data described above supports a multi-faceted and complex role for IFN in tumor development and progression. While IFN might initially be capable of eliminating incipient cancer cells, tumors that escape this “immune-surveillance” may benefit from an active immune response. This “dual” role of the immune system in cancer is similar to the roles being attributed to autophagy in cancer (191).

The response to type I interferon and STAT1 activation has been extensively characterized and is classically thought to induce a pro-apoptotic/anti-proliferative phenotype. This straightforward model has been challenged recently as mounting evidence suggests STAT1

activation might actually promote the progression of certain tumors (148). STAT1 and many of its transcriptional targets have been found to be overexpressed in breast, lung, leukemia, and cervical cancers (81, 100, 101, 121, 217, 230, 231, 275). While it could be argued that overexpression of STAT1 may be a “passenger” in human cancer, data from several human cancer studies indicate otherwise (142). An interferon related DNA damage signature (IRDS), which includes STAT1, can predict chemo and radiation therapy sensitivity in breast cancer patients (311). A similar signature is a predictive marker of poor survival outcome in the proneural subtype of glioblastoma multiforme (77). Overexpression of STAT1 also provides resistance to DNA damage-inducing drugs in a variety of cancer cell lines (141, 181). Furthermore, phosphorylation of Serine-727 on STAT1, which is a target of multiple kinases (including p38 MAPK) can promote the growth of specific tumor types by inhibiting NK-cell cytotoxicity (59, 97, 222, 289).

Finally, the unphosphorylated form of STAT1 was recently shown to negatively regulate Fas-mediated apoptosis and promote sarcoma development as a consequence (333). Thus, the biological functions of STAT1 signaling may be unique to cell types as well as specific genetic contexts. Understanding which of these contexts STAT1 acts in a tumor-promoting fashion would provide a very accessible therapeutic target in these patients, as there are already STAT1 specific drugs as well as cell-permeable peptide inhibitors under investigation (268, 304).

### *IFN- $\beta$ in cancer*

Interferon-Beta (IFN- $\beta$ ) is a member of the type I interferon family that is most well-known for its anti-viral effects. Recognition of viral infection by virtually any nucleated cell results in the secretion of IFN- $\beta$ . Subsequent activation of downstream Jak-Stat signaling results

in transcriptional activation of anti-viral genes which aid in preventing further infection (219). In the context of cancer biology, high dose IFN therapy treatment has been investigated as a potential therapeutic and has proven beneficial in certain hematological cancers. These clinical applications of IFN utilize a second member of the type I IFN family known as IFN- $\alpha$  (88). In solid tumors such as breast and ovarian cancer, attempts to utilize IFN- $\beta$  in this regard have not been successful (186, 237). Ultimately, little is known about the role IFN- $\beta$  might play in the tumor environment, and several reports actually indicate it might serve to promote survival of tumors. Tumor growth has previously been shown to be enhanced in response to low-levels of interferon, and a recent report suggests that autocrine IFN- $\beta$  signaling enhances the tumorigenicity of Ras-transformed cells (171, 292). Given that many tumors display activation of Jak-Stat signaling and overexpression of downstream target genes, further investigation of interferon signaling in the context of cancer biology is warranted.

### *ISG15 in cancer*

One well recognized gene activated downstream of IFN- $\beta$  signaling encodes the ubiquitin-like protein, ISG15. Similar to ubiquitin, ISG15 is conjugated to lysine residues in an enzymatic cascade involving an E1 activating enzyme (UBE1L), an E2 enzyme (UBC8), and finally an E3 ligase (HERC5 in humans and HERC6 in mouse) that facilitates targeting to specific proteins (33, 326). There are also de-ISGylating enzymes such as UBP43 (also called USP18), that can modulate levels of ISGylation (179). ISG15 has been well characterized in the context of innate immunity where it is conjugated to many proteins involved in the response to viral infection, and can promote or inhibit their function (272). One proposed mechanism of

enhancing protein activity is through blocking ubiquitin conjugation resulting in protein stability, but the true biological consequence of ISG15 conjugation is not well defined (180). Also, roles for free and secreted forms of ISG15 are now being appreciated. Werneke and colleagues demonstrated that *Ubell*-null mice, which are not capable of conjugating ISG15 to proteins, are equally sensitive to Chikungunya virus infection as wild-type mice (312). Further studies will be necessary to understand how un-conjugated ISG15 functions within the cell.

While ISG15 plays a key role in the antiviral response, many reports have begun to uncover a role for this protein in tumorigenesis (6). ISG15 has been found to be frequently overexpressed in pancreatic, bladder, breast, and oral cancers (5, 16, 124, 284, 297). A recent report describes a critical role for ISG15 conjugation in the tumorigenicity of mutant *Ras* containing breast cancer cells, suggesting inhibitors interfering with this process might be therapeutically beneficial (32). Indeed, Wood *et al* used mouse breast cancer models to demonstrate a vaccine against ISG15 could significantly reduce tumor burden and metastasis, underscoring the potential of targeted ISG15 therapy (315). Additionally, ISG15 overexpression in breast cancer cells can improve cell motility (64). How ISG15 would be able to promote tumor growth is unknown. However, one study identified ISG15 overexpression in several breast cancer cell lines and demonstrated that this resulted in defects in polyubiquitination and protein turnover. High ISG15 levels interfered with polyubiquitination and led to decreased protein turnover (63). While global defects in protein turnover are detrimental to the cell, it is possible that specific ISGylated proteins with increased half-lives could be serving tumor promoting functions. Together, these data indicate that ISG15 is tumor promoting in particular cancer types, but a mechanistic insight into this role is lacking.



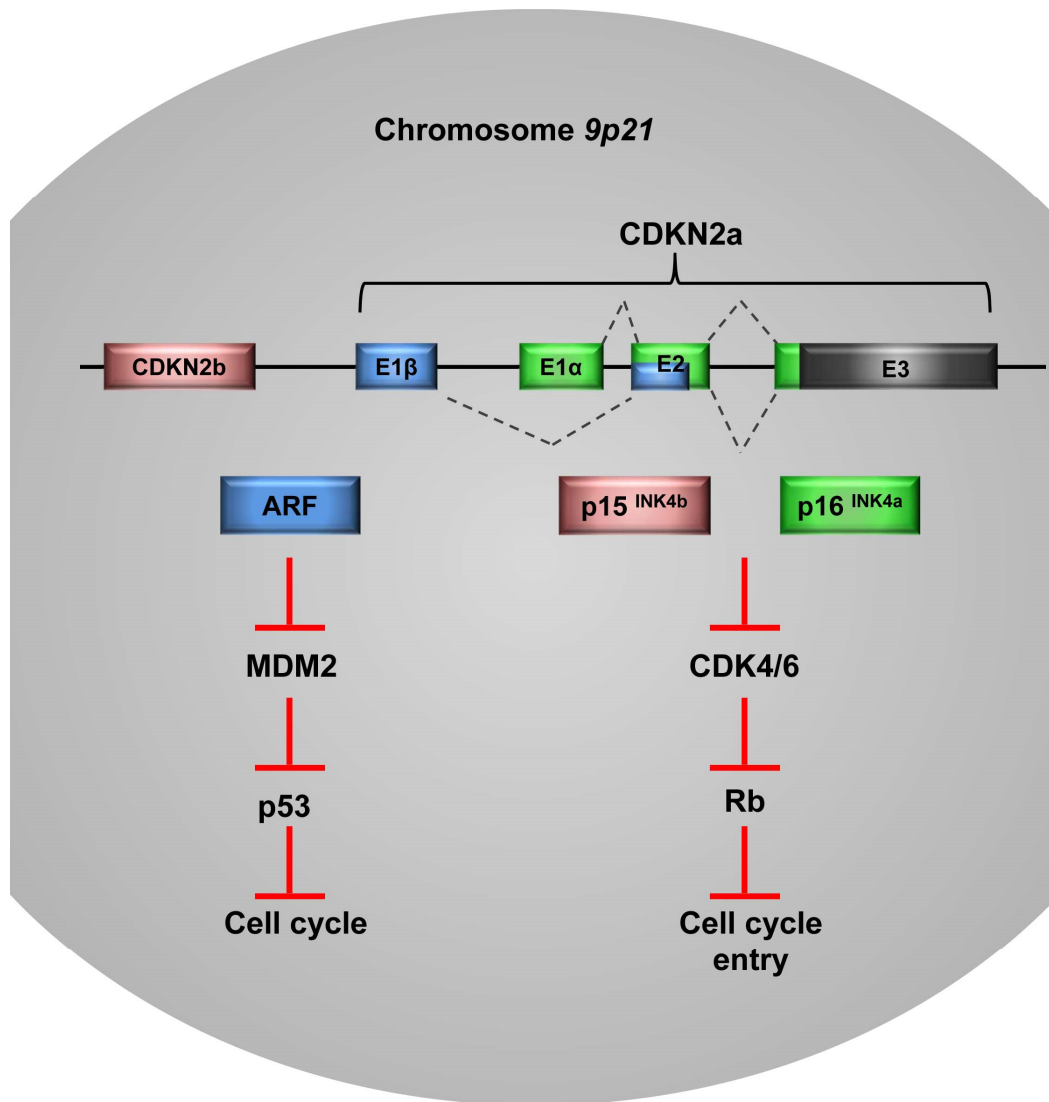
## Concluding thoughts and dissertation objectives

When my dissertation work began, we did not understand how ARF expression was regulated by p53. Additionally, a clear understanding of the role ARF plays in cells that lose p53 function was lacking. I began my dissertation work by asking two simple questions: 1) Does acute loss of p53 induce ARF, and 2) Do the induced ARF protein levels serve any tumor suppressive functions? Since numerous types of cancers exhibit co-inactivation of Arf and p53, these are important questions. Patients with tumors harboring these specific genetic defects would benefit from a detailed understanding of what signaling networks are dysfunctional. Likewise, by understanding the selective pressures that result in *CDKN2a* loss, we can learn a tremendous amount about the p53-independent functions of ARF.

I hypothesized that acute loss of p53 would result in an upregulation of ARF, and that these induced protein levels would be able to inhibit proliferation and tumorigenicity by ARF's well characterized role in regulating ribosome biogenesis. Experiments described in Chapter 2 support the hypothesis that loss of *p53* leads to robust increases in ARF expression. Moreover, these induced levels potently suppress the proliferation and tumorigenic potential of these cells. In Chapter 3, I outline experiments performed to test the hypothesis that regulation of cellular growth is the mechanism by which ARF suppresses the proliferation of p53-deficient cells. The results of these experiments were not supportive of the cell growth hypothesis, and other published p53-independent functions were experimentally tested and excluded. Chapter 4 discusses the unbiased experimental approach that I took to uncovering the novel p53-independent tumor suppressor function of ARF. The results of this approach led to the discovery of a novel pathway co-regulated by p53 and ARF. This pathway, the type I interferon response, is not widely appreciated as being pro-tumorigenic. The data presented in Chapter 4

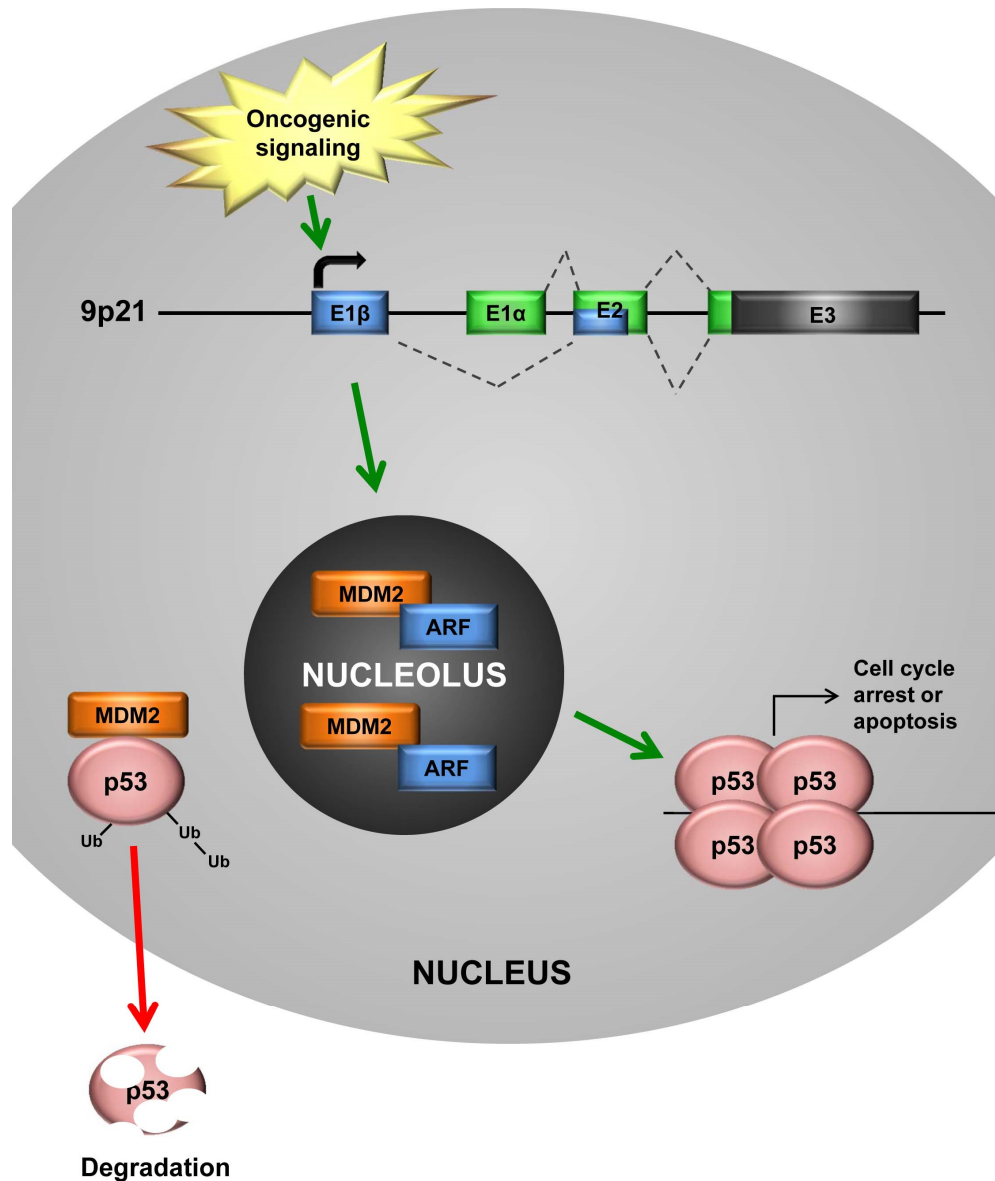
overwhelmingly supports the hypothesis that in a setting of Arf/p53-deficiency, components of the type I interferon response can actively promote proliferation and ultimately promote tumor progression. Finally, in Chapter 5 I discuss future directions that provide an opportunity to study the potential therapeutic applications of my findings.

## FIGURES



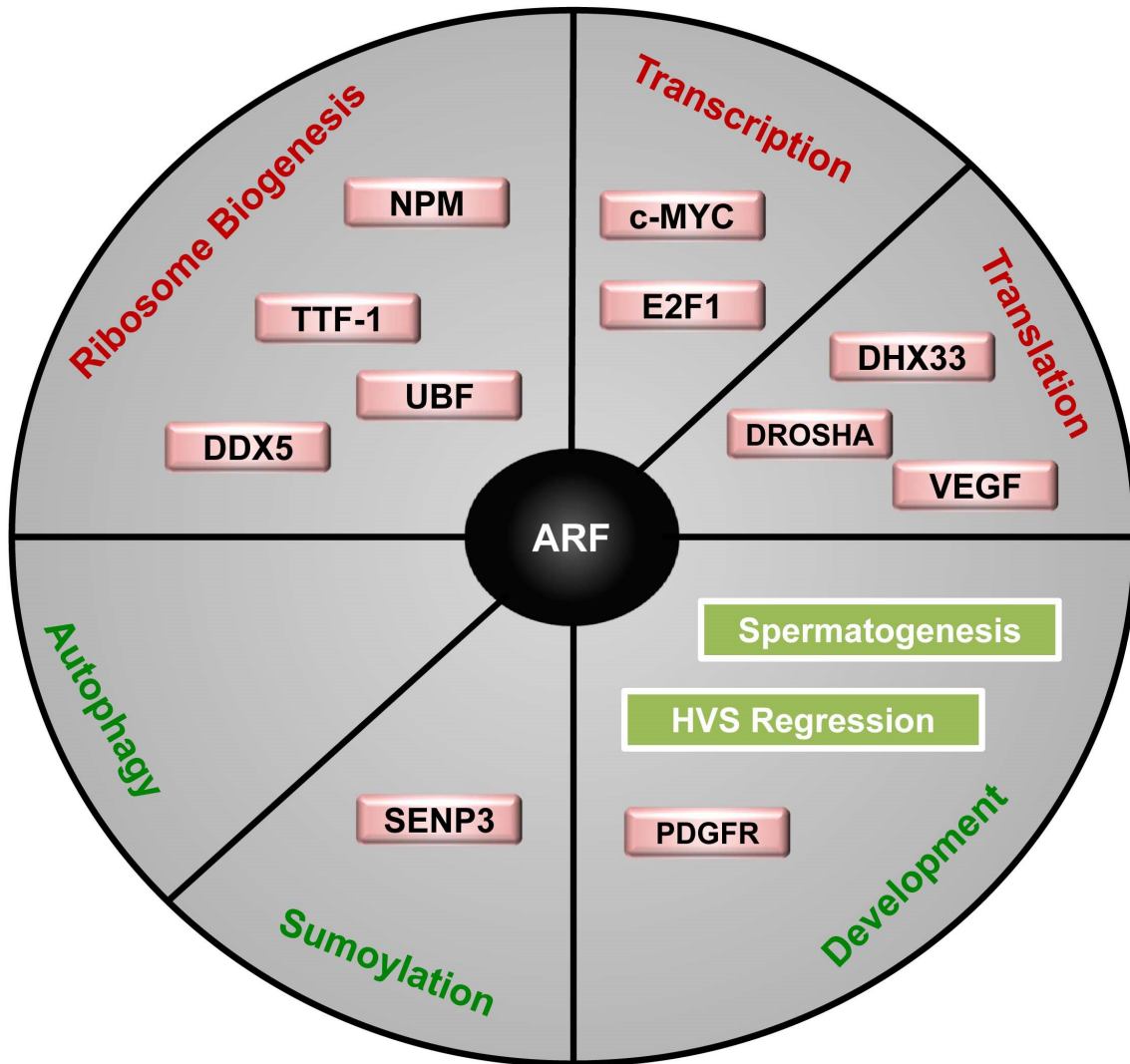
**Figure 1.1** Overview of the *CDKN2a* genomic locus.

*CDKN2a* encodes two tumor suppressor genes, ARF (blue) and p16 (green). The coding regions for each protein are indicated with the same colors. ARF participates in a p53-mediated checkpoint, and p16 can block cell cycle entry by inhibiting the activities of CDK4/6. A third tumor suppressor encoded by the *CDKN2b* gene, named p15 (pink), is located just upstream of ARF's distinct first exon.



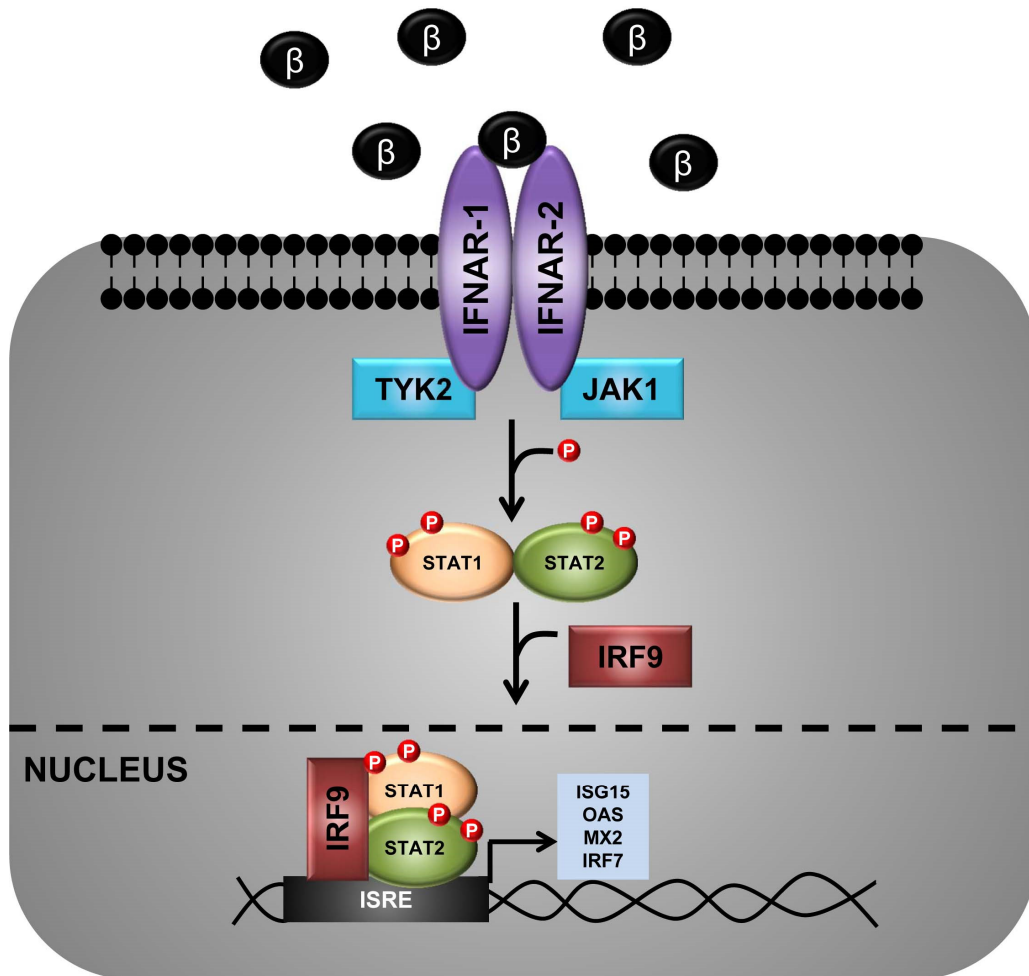
**Figure 1.2** Graphical depiction of p53-dependent tumor suppression by ARF.

In response to hyperproliferative signals emanating from unregulated oncogenic signaling, ARF transcription and translation (not shown) is induced. Increased ARF levels function to sequester MDM2 in the nucleolus, thereby relieving MDM2-mediated ubiquitination of p53. This leads to stabilization of p53 and the activation of p53-dependent transcriptional programs that initiate cell cycle arrest or apoptosis



**Figure 1.3** The p53-independent functions of ARF.

This figure highlights the p53-independent processes that ARF inhibits (Red) or promotes (Green). Pink boxes represent the specific proteins (or mRNAs in the case of translation) that ARF inhibits to elicit these processes.



**Figure 1.4** Overview of the Type I Interferon response

In response to a viral infection, cells are stimulated to secrete interferon- $\beta$  (black circles). IFN- $\beta$  binds to the heterodimer receptor composed of IFNAR-1 and IFNAR-2. Binding of IFN- $\beta$  leads to activation of the associated JAK kinase function, leading to STATs recruitment and their subsequent phosphorylation. Phosphorylation of STAT1 and STAT2 leads to heterodimerization and also recruits IRF9 to the complex. The STAT1/STAT2/IRF9 complex is known as ISGF3, and it translocates to the nucleus where it activates transcription of genes containing interferon sensitive response elements (ISREs).

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## **Chapter 2:**

# **ARF limits the proliferation and tumorigenicity of p53-deficient cells**

## ABSTRACT

The tumor suppressor, p19<sup>ARF</sup>, is classically considered to function in response to hyperproliferative signals by binding and sequestering MDM2 in the nucleolus, thereby activating p53. Steady-state levels of ARF are kept low to prevent activation of p53 and are thought to monitor nucleolar function. Interestingly, p53 itself inhibits the *Arf* locus by recruiting proteins involved in heterochromatin formation. Cell lines that lack functional *p53*, therefore, accumulate high amounts of ARF protein but proliferate rapidly due to the absence of proper cell cycle checkpoints. ARF and p53 are frequently co-inactivated in human cancers, suggesting this pathway is not strictly linear. Given that ARF has been reported to have p53-independent functions both *in vitro* and *in vivo*, we sought to investigate the function of ARF in the setting of acute *p53* loss. Consistent with previous findings, we observed that acute loss of *p53* leads to a transcriptional upregulation of ARF. We further demonstrate that while *p53*-deficient cells proliferate more rapidly than their wild-type counterparts, the induced levels of ARF are actually limiting maximal proliferation rates. By limiting the proliferation of *p53*-deficient cells, ARF is also able to suppress tumorigenicity when these cells are challenged with oncogenic *Ras*<sup>V12</sup>. These findings settle a long-standing debate on whether or not ARF functions as a tumor suppressor in a *p53*-null setting, and explain why certain types of cancers preferentially inactivate both ARF and p53. Upon *p53*-inactivation, ARF levels become induced to suppress tumorigenesis, which imposes a selective pressure for cancer cells to silence or delete the *Arf* locus.



## INTRODUCTION

The *Tp53* and *Cdkn2a* tumor suppressor genes are two of the most frequently inactivated genomic loci in human cancers (39). *Tp53* encodes a transcription factor that can promote or inhibit the expression of genes in response to various cellular cues (19). The p53 protein responds to numerous cellular stresses, including DNA damage, oncogenic activation, metabolism defects, hypoxia, oxidative stress, and telomere shortening (18). Depending on the level of damage, p53 can orchestrate cell-cycle arrest to give cells an opportunity to repair the defects, or can activate permanent programs like senescence or apoptosis (12). As a key sensor of stresses that cancer cells regularly face, it is not surprising that p53 is the most frequently mutated gene in human cancers (44).

*Cdkn2a* encodes two unrelated proteins p14<sup>ARF</sup> (p19<sup>ARF</sup> in mice) and p16<sup>INK4A</sup>, both of which function as tumor suppressors (28). There are two distinct first exons and promoters in *Cdkn2a*, called exon-1 $\beta$  and exon-1 $\alpha$ . Exon-1 $\alpha$  splices into exons 2 and 3 and once translated this mRNA encodes a 168 amino acid protein called p16. p16 is a well-characterized cyclin dependent kinase inhibitor, and functions to keep the Retinoblastoma protein (Rb) in a hypophosphorylated state—effectively blocking entry into S-phase of the cell cycle (32). Exon-1 $\beta$  splices into the identical splice acceptor sites in exons 2 and 3, but due to a frame shift in the first exon, this mRNA is translated in an Alternate ReadinG Frame, creating a 169 amino acid protein. Due to this unprecedented genomic organization in the mammalian genome, ARF and p16 share absolutely no sequence homology at the amino acid level, while sharing identical nucleotide sequences from exons 2 and 3 (28).

ARF is a nucleolar protein that is capable of sequestering the E3 ubiquitin ligase for p53, MDM2, in the nucleolus (42, 46). Under normal cellular conditions, ARF levels are kept low to prevent inappropriate activation of p53. However, in response to oncogenic stimuli such as

overexpression of c-MYC, RAS, or E1A, ARF expression is upregulated by both increased transcription and translation (6, 10, 23, 25, 48). Increased ARF levels promote the MDM2-ARF interaction, which leads to the stabilization p53 and subsequent activation of transcriptional programs leading to cell cycle arrest or apoptosis (29). Thus, ARF and p53 canonically function in a linear genetic pathway that functions to protect cells from inappropriate oncogenic signaling (38).

Since ARF's initial discovery, it has been observed that cells lacking p53 function contain elevated levels of ARF, suggesting a possible inhibitory feedback loop between p53 and ARF (28, 40, 48). For many years, only correlative data existed to link p53 to the repression of *Arf* transcription. Re-introduction of p53 cDNA into *p53*-null MEFs led to a reduction in ARF protein levels, and p53 was able to inhibit expression of a chloramphenicol acetyltransferase (CAT) reporter construct regulated by *Arf* promoter sequence (13, 31). A mechanistic explanation for this phenomenon surfaced when it was recently shown that p53 is a direct transcriptional repressor of the *Cdkn2a* promoter. Recruitment of histone deacetylases and polycomb group proteins by p53 renders the locus inaccessible to transcription factors (47). Thus, in the context of p53 loss of function, ARF transcription becomes de-repressed and protein levels become elevated. Given that *p53*-null mice succumb to tumor burden by 6 months of age, and that cells lacking p53 proliferate rapidly, it has been widely assumed that ARF serves no tumor suppressor function in this context (7). Restated, this line of thought concludes that the tumor suppressor functions of ARF are entirely dependent on signaling through p53.

The negative feedback loop between p53 and ARF likely exists to allow for fine-tuning of the p53 response. Increased p53 stability as a result of DNA damage or oncogenic stress leads would lead to an enhanced occupancy of p53 at the *Arf* promoter. This would repress *Arf*

transcription and thus reduce ARF's inhibitory effects on MDM2. With a larger pool of available MDM2, the cell would be capable of turning off the p53 response, assuming the stress signal was no longer occurring.

An alternative hypothesis is that p53 and ARF cooperate to suppress a pro-proliferative signal. Upon inactivation of p53, the cell is wired to turn on a "back-up." For this to be true, ARF must possess p53-independent tumor suppressor functions. Indeed, mounting evidence suggests ARF indeed possesses important p53-independent tumor suppressor functions. If ARF and p53 solely function in a linear genetic pathway, there would be no selective pressure for cancer cells to inactivate both of them. However, certain types of human cancers simultaneously harbor *p53* mutations and deletions in the *Arf* locus, including pancreatic, lung, sarcomas, breast, and glioblastomas (4, 22, 24, 26, 33, 34, 36, 37, 43). In agreement with these findings, mice lacking both *p53* and *Arf* are extremely susceptible to lymphoma formation by 6 months of age, but they will also frequently harbor multiple primary tumors including carcinomas that have never been observed in the *p53*-null mouse (45). Deleting *Arf* on a *p53*-null background, therefore, speeds up the formation of epithelial-cell tumors in mice that otherwise would succumb to lymphomas. Furthermore, several groups have shown that *p53*-null cells are sensitive to exogenous overexpression of ARF, demonstrating that ARF can function independently of p53 to inhibit proliferation (37, 39, 45). These studies, however, do not directly address the question of endogenous ARF function in the absence of *p53*.

We sought to address whether acute loss of p53 would lead to the upregulation of functional ARF. We show that acute p53 loss results in an induction of ARF protein expression and that this endogenous ARF accumulation functions to limit the proliferation and tumorigenicity of *p53*-deficient cells. Our data conclusively show that the negative feedback

loop between p53 and ARF has biological significance. The widely held assumption that the increased endogenous levels of ARF found in *p53*-deficient cells are non-functional is therefore, incorrect. This evidence also provides an explanation for why the inactivation of p53 and ARF are not mutually exclusive events in human cancers.

## MATERIALS AND METHODS

**Mice and cell culture.** *p53<sup>lox/lox</sup>* (FVB.129-*Trp53<sup>tm1Brn</sup>*) were obtained from the NCI Mouse Repository and have been previously described (11). Primary mouse embryonic fibroblasts were isolated as previously described (14). All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2mM glutamine, 0.1mM nonessential amino acids, 1mM sodium pyruvate, and 2ug/mL gentamicin. Unless otherwise indicated, cells were maintained in 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>. Rapamycin was purchased from LC Laboratories.

**Viral production and infections.** Adenoviruses expressing  $\beta$ -galactosidase (Ad-LacZ) or Cre recombinase (Ad-Cre) were purchased from the Gene Transfer Vector Core, University of Iowa. For Adenoviral infections,  $1 \times 10^6$  cells were plated in the presence of Ad-LacZ or Ad-Cre (MOI=50) and incubated for 8 hours. For mutant Ras<sup>V12</sup> overexpression, retrovirus was produced by transfecting 293T cells with either MSCV-HRAS<sup>V12</sup>-IRES-GFP plasmid or MSCV-IRES-GFP control, and the helper plasmid  $\psi$ -2. Virus-containing supernatants were harvested 48 hr post transfection. Collected retrovirus was used to infect  $1 \times 10^6$  MEFs in the presence of 10ug/mL polybrene. For the production of Lentiviral shRNAs, 293T cells were transfected using Lipofectamine 2000 (Invitrogen) with pCMV-VSV-G, pCMV- $\Delta$ R8.2, and pLKO.1-puro constructs. Viral supernatants were harvested 48hrs post transfection. Cells were infected with lentivirus for 8-12 hours in the presence of 10 ug/mL protamine sulfate. Puromycin was added to cell culture media at a concentration of 2 ug/mL for selection. The ARF (mouse specific) hairpin was described previously (1). The shARF-C7 hairpin sequence is 5'- GTC TTT GTG TAC CGC TGG GAA-3' and was obtained from the RNAi consortium library.

**Western Blotting.** Cell pellets were lysed and sonicated in EBC lysis buffer (50 mM Tris-Cl, pH7.4, 120mM NaCl, 0.5% NP-40, 1mM EDTA) containing HALT Protease and Phosphatase Inhibitor cocktail (Thermo Scientific) and 1mM phenylmethylsulfonyl fluoride (PMSF). Thirty  $\mu$ g of protein were separated on SDS-polyacrylamide gels. Proteins were transferred to PVDF (Millipore) and probed with antibodies. A comprehensive list of antibodies used in this study can be found in Table 2.1. Secondary horseradish peroxidase conjugated antibodies (Jackson Immunoresearch) were used and ECL plus was used to visualize the bands (GE Healthcare).

**Proliferation, BrdU and Foci Assays.** For proliferation assays,  $5-10 \times 10^4$  cells were plated in 6-well plates. Cells were lifted and counted using a hemocytometer at the indicated number of days post plating. For BrdU assays,  $1 \times 10^4$  cells were plated on glass coverslips and incubated overnight.  $10 \mu$ M BrdU-containing media was added to the cells for 4-6 hours. Cells were fixed with 10% formalin/methanol and BrdU staining was performed using an Anti-BrdU antibody (GE Healthcare) according to the manufacturer's instructions. For foci assays,  $3 \times 10^3$  cells were plated in 10cm dishes and cells were incubated for 10 days. Cells were fixed with 100% methanol and stained with Giemsa (Sigma Aldrich).

**Soft Agar Assay.** Cells were lifted and suspended in DMEM containing a final concentration of 0.4% Noble agar.  $1.5 \times 10^4$  cells were layered in triplicate onto 0.6% noble-agar/media bottom layer in 60mm plates. Plates were incubated for 20 days, feeding with media/0.4% agar mix every 6 days. Macroscopic colonies were visualized by staining with 0.005% Crystal Violet solution and colonies  $\geq 0.5$ mm were manually counted.

**Tumorigenesis Assay.**  $1.5 \times 10^6$  dp53R-shSCR or dp53R-shARF MEFs were suspended in PBS and injected into the flanks of female homozygous euthymic nude mice (*Foxn1<sup>nu</sup>/Foxn1<sup>nu</sup>*) obtained from Jackson Laboratories. Five mice per condition were used. Tumor size was monitored over the course of 20 days using calipers to measure in two dimensions. Tumor volume was calculated using the formula: Volume= [(height)<sup>2</sup> x length]/2 in which height equals the smaller of the two measurements.

**Quantitative Real-Time PCR.** qRT-PCR was performed as previously described (23). Fold change was measured using the  $\Delta\Delta C_T$  method (20). Primer sequences used for amplification can be found in Table 2.2.

**Immunofluorescence.** Sections were deparaffinized, rehydrated, washed in PBS, and blocked with serum-free Protein block (Dako) for 30 min at room temperature. Antigen retrieval which was performed in a food steamer using a 1x Reveal decloaker buffer (pH6.0) (Biocare Medical). Antibodies for the following markers were diluted in Antibody diluent (Dako) and applied overnight at 4°C: rat anti-p19ARF (1:400, Abcam), rabbit anti-E-cadherin (1:200, Cell Signaling Technology). A secondary antibody conjugated with Alexa Fluor 488 was placed on tissue sections for 1 hr at room temperature (1:300, Life Technologies). Nuclei were counterstained using Slow Fade Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies).

**Apoptosis analysis.** Equal numbers of cells were stained with FITC-Annexin V and propidium iodide using the Vybrant Apoptosis Assay kit according to the manufacturer's instructions (Life

Technologies). Stained cells were analyzed by flow cytometry using a FACSCalibur instrument, and data was analyzed using CELLQUEST analysis software (BD Biosciences). For a positive control, cells were treated with 50 $\mu$ M Etoposide (Sigma Aldrich) for 14 hours.

**Statistical Analysis.** Data are presented as means  $\pm$  s.d. Statistical differences between groups were determined with *P*-values obtained using two-sided, unpaired Student's *t*-test. All data points represent *n*=3. All images presented as “representative” were completed a minimum of three times.



## RESULTS

### Acute loss of p53 leads to transcriptional upregulation of ARF

It has long been assumed that the high levels of ARF found in p53-deficient cells are not tumor suppressive. To directly address this assumption, we utilized a previously characterized conditional mouse model of p53 inactivation where exons 2-10 are flanked by LoxP sites (11). Adenoviral (Ad) delivery of Cre-recombinase into  $p53^{lox/lox}$  mouse embryonic fibroblasts (MEFs) resulted in the efficient excision of p53 by 4 days post-infection as measured by Western Blot and quantitative real-time PCR (Figure 2.1 A and B). Importantly, an accumulation of ARF mRNA and protein by four days post-infection was observed, and these levels continued to rise over time and passage (Figure 2.1 A and B). These data are in agreement with previous findings that p53 directly binds to and is capable of repressing the ARF promoter by recruitment of Polycomb-group proteins and promoting heterochromatin formation (47). A transcriptional target of p53, MDM2, was reduced following excision of  $p53$  (Figure 2.1 A). Infection of wild-type MEFs with Ad-LacZ or Ad-Cre did not result in upregulation of either ARF or p53, indicating the observed ARF induction is specific to p53 loss (Figure 2.2). Furthermore, ARF induction following p53 loss is not dependent on tissue culture conditions, as performing the experiment in low serum or normoxic conditions had no effect on the level of induction (Figure 2.3 A and B).

The level of transcriptional upregulation of Arf mRNA ranged from 2-4 fold (Figure 2.1 B), while the fold change of protein expression we observed was frequently as high as 8-fold (Figure 2.1 A). These conflicting values led us to test whether there might be post-transcriptional or post-translational regulation of ARF following  $p53$  loss. We tested protein stability by treating Ad-LacZ or Ad-Cre infected  $p53^{lox/lox}$  MEFs with cycloheximide and measured the half-life of ARF protein. In agreement with previously reported findings, the half-

life of ARF in both Ad-LacZ and Ad-Cre conditions was 6 hours (Figure 2.4 A and B) (16). Thus, ARF protein stability is unchanged following p53 loss. It has also been reported that p53 can repress activation of the mTOR (mammalian Target Of Rapamycin) pathway by activating transcription of the tumor suppressors PTEN and TSC2 (9). Additionally, our lab has previously shown that activation of the mTOR pathway leads to an increase in translation of the Arf mRNA, so we hypothesized that *p53* loss would lead to increased signaling through mTOR and subsequent translational upregulation of ARF (23). As seen in Figure 2.5A, loss of *p53* led to activation of mTOR as measured by serine phosphorylation of p70 S6-kinase (a downstream target of mTOR) and its substrate, ribosomal protein S6. Levels of TSC2 were decreased, suggesting a mechanistic explanation for mTOR activation. Treatment of *p53*<sup>Δ/Δ</sup> MEFs with increasing concentrations of rapamycin, a pharmacological inhibitor of mTOR, did not reduce ARF protein expression (Figure 2.5 B). A selective increase in mRNA translation via mTOR, therefore, does not contribute to the elevated levels of ARF following *p53* loss. We chose to not pursue additional mechanisms of ARF induction, and instead focused our attention on the physiological function of ARF in the absence of *p53*.

We also wanted to determine whether induction of ARF in the absence of *p53* would occur *in vivo*. Under normal physiological conditions, ARF expression is maintained at very low levels and is difficult to detect by immunofluorescent (IF) staining. In mice, the only tissues expressing detectable ARF are the developing eye and testes (49, 50). We hypothesized that *p53*-knockout mice would have a “brake” relieved on the Arf promoter and would therefore express levels detectable by IF. We isolated mammary fat pads from three month old virgin wild-type, *Arf*-null, or *p53*-null mice. Immunofluorescent staining for ARF on formalin-fixed paraffin embedded sections showed evidence of punctate nucleolar staining only in the ductal

epithelial cells of *p53*-null mice (Figure 2.6). Interestingly, not every mammary gland stained positive for ARF, suggesting that *Arf* de-repression *in vivo* requires more than simply *p53* loss.

### **Adeno-Cre mediated excision of p53 leads to increases in cell proliferation**

It is widely appreciated that p53 is vital in maintaining proper cell cycle progression in response to a variety of cellular stresses (12). Activation of p53 can lead to checkpoints at both the G1/S and the G2/M transitions in the cell cycle, so its inactivation leads to unregulated progression through these boundaries (18). To validate our *in vitro* system of conditional p53 inactivation, we monitored the proliferation of Ad-LacZ or Ad-Cre infected *p53<sup>fllox/fllox</sup>* MEFs. As shown in Figure 2.7A, excision of p53 led to a marked proliferation advantage compared to the LacZ infected controls. Analysis of cell cycle progression by 5-bromodeoxyuridine (BrdU) incorporation showed a significantly increased population of Cre-treated cells had entered S-phase compared to controls (Figure 2.7 B). Additionally, cells lacking p53 were able to achieve much higher confluent densities and were capable of forming colonies when plated at low density (Figure 2.7 C and D). Together, these data validate our model of acute p53 loss, and support years of research that suggests p53-deficient cells proliferate rapidly even in the presence of elevated endogenous ARF.

### **Endogenous ARF limits the proliferation of p53-deficient cells**

As described above, loss of p53 leads to an increase in proliferation even though endogenous levels of another tumor suppressor, ARF, are robustly induced. This system of acute p53 loss allowed us to test the true p53-independent tumor suppressor functions of ARF without having the concern of confounding genomic changes that are often an issue in established cell

lines. We used an shRNA specifically targeting ARF to reduce ARF levels in these  $p53^{\Delta/\Delta}$  MEFs, hereafter referred to as dp53 MEFs (deleted for p53). As seen in Figure 2.8A, infection of dp53 MEFs with the ARF shRNA (shARF) leads to a reduction in ARF protein, but does not reduce p16 levels relative to the scrambled control (shSCR). Importantly, our ARF shRNA reduces ARF levels in dp53 MEFs back to wild-type levels observed in our Adeno-LacZ infected controls (Figure 2.8 A). This allowed us to specifically address the function of induced ARF levels in a p53-null setting. Infection of dp53 MEFs with shARF led to a modest, yet reproducible, increase in proliferation and long term colony formation (Figure 2.8 B-D). This data clearly indicates that endogenous ARF is able to limit the proliferation of cells faced with  $p53$  loss.

### **Endogenous ARF limits the tumorigenicity of $p53$ -deficient cells**

To test the tumor suppressive functions of ARF in the context of  $p53$  loss, we first transformed dp53 MEFs by overexpressing mutant H-Ras<sup>V12</sup>, and then depleted ARF (Figure 2.9 A). Ras<sup>V12</sup>-transformed dp53 MEFs (dp53R MEFs) were capable of forming colonies in soft agar (Figure 2.9 B, top left panel). However, depletion of ARF in the dp53R MEFs resulted in a tremendous increase in the size of soft agar colonies, indicating an increase in tumorigenic potential (Figures 2.9 B and C). The dp53R-shARF MEFs also exhibited higher proliferative rates, BrdU incorporation rates, and increased foci formation compared to dp53R-shSCR cells, supporting our observed tumorigenic phenotype (Figures 2.10 A-C). The observed increases in cell proliferation were not attributed to increased cell death in the shSCR cells or decreased cell death in the shARF cells, as measured by Annexin-V staining (Figure 2.10 D). We were unable to find a second shRNA that specifically targeted ARF without simultaneously depleting p16

levels. To show that p16 was not playing a role in inhibiting the tumorigenicity of dp53 MEFs, we infected these cells with a hairpin targeting ARF and p16, called shARF-C7. We hypothesized if p16 was also inhibitory, then depleting both ARF and p16 should enhance the transformation phenotype we observed. However, we did not observe an enhanced transformed phenotype suggesting p16 is not inhibiting the growth of p53-deficient cells (Figure 2.11).

To extend our findings *in vivo*, we injected the dp53R-shARF cells into the flanks of nude mice. We observed a striking enhancement in the growth kinetics of dp53R-shARF tumors relative to tumors formed with dp53R-shSCR cells (Figures 2.12 A and B). Taken together, these data demonstrate the endogenous ARF levels that accumulate following *p53* loss function to limit the tumorigenic potential of these cells.

## DISCUSSION

The ARF and p53 tumor suppressors are predominantly thought to function in a linear genetic pathway to protect cells from oncogenic stress. However, evidence from numerous cancer types, including pancreatic and lung cancers, indicates frequent co-inactivation of p53 and ARF suggesting the pathway may not be strictly linear (4, 33). It is also interesting that p53 is a known repressor of the Arf locus, and until now no one has understood the functional significance of this relationship. While numerous groups have attributed p53-independent tumor suppressive functions to ARF, most of these have been discovered by grossly overexpressing ARF in p53-deficient cells that already contain elevated levels of ARF (37). Therefore, there are currently two important unanswered questions in the field of ARF biology: 1) Does loss of p53 function lead to an increase in ARF levels capable of eliciting tumor suppressive functions, and 2) What are these physiologically relevant p53-independent functions of ARF? We believe these are important questions in the field of cancer biology as we enter an age of personalized cancer therapy (5). A genetic understanding of why there would be a selective pressure to inactivate both ARF and p53 in human tumors would provide therapeutic strategies currently unavailable to these patients.

We chose to address the first of the above-mentioned questions by using a murine tissue culture model of acute p53 loss. Loss of *p53* led to a robust induction of ARF. While our current data suggests this induction is mostly mediated at the level of transcription, we cannot conclusively rule out the involvement of post-transcriptional regulation. For instance, we were unable to establish a connection between the observed upregulation of the mTOR pathway initiated by p53 loss and increased ARF protein expression, but it is certainly possible that mTOR-independent increases in translation could be occurring. Numerous RNA binding proteins are involved in translational regulation, and one of these called HuR (Human antigen R)

was recently shown to repress the translation of the ARF mRNA (15). A scenario might exist where p53 promotes the transcription of HuR, and upon p53 loss HuR expression is reduced, leading to increased ARF translation.

Immunofluorescent staining of mouse mammary glands from wild-type, *Arf*-null, and *p53*-null mice supported our *in vitro* observations. The only glands where ARF was detectable were from *p53*-null mice. Interestingly, not all glands stained positive for ARF in a *p53*-null mouse, suggesting that *Arf* de-repression *in vivo* requires more than simply *p53* loss. We hypothesize that a threshold of oxidative or oncogenic stress must be achieved to fully re-organize the heterochromatin surrounding the *Arf* locus. From our cell culture model, it is clear that the stressful conditions used to maintain cells on plastic are sufficient to accomplish this re-organization in *p53*-null cells, since acute loss of *p53* leads to a rapid induction of ARF.

While acute p53 loss led to an induction of ARF, it also vastly enhanced the proliferation rate of the cells. At face value, this data would seem to indicate that ARF does not suppress the proliferation of these cells and, therefore, does not have p53-independent tumor suppressive functions. However, depletion of ARF in the dp53 MEFs using an ARF specific shRNA led to an even greater enhancement of both short and long-term proliferation. We further showed that dp53 MEFs transformed with mutant *Ras*<sup>V12</sup> are also being inhibited by the high levels of endogenous ARF. Lowering these high ARF levels with shRNAs resulted in dramatic increases both *in vitro* in soft-agar colony formation and *in vivo* in a mouse xenograft model. These effects were likely due to enhanced proliferation rates, as we were not able to detect any changes in cell death.

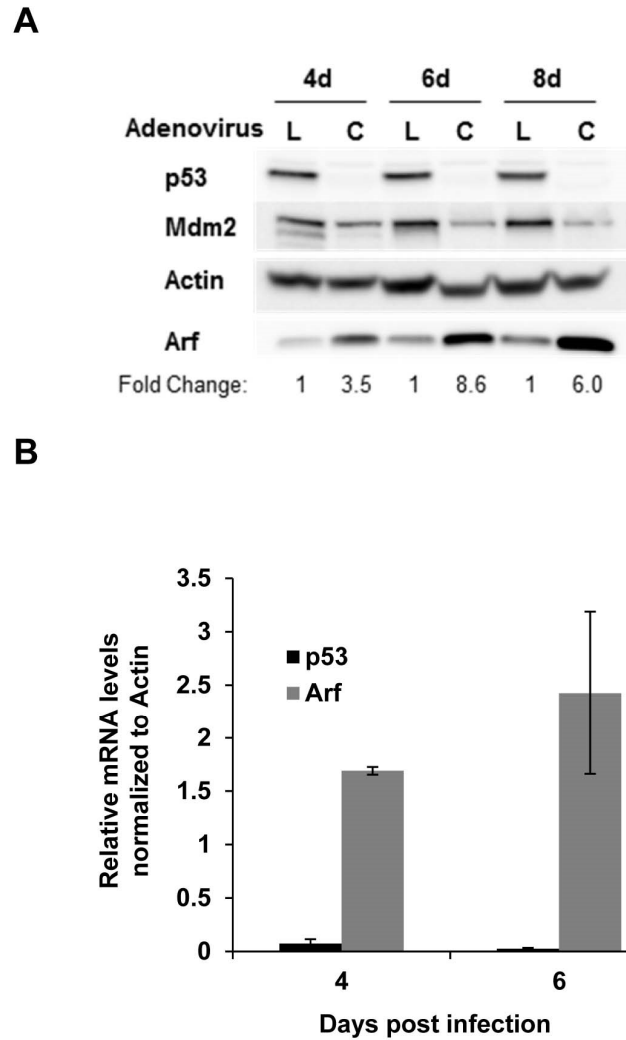
Mechanistically, ARF could be limiting the proliferation and tumorigenicity of p53-deficient cells in numerous ways. Our lab and others have shown that ARF is a key player in

ribosome biogenesis and mRNA translation (1-3, 17, 21, 30, 35, 41). As efficient protein synthesis is vital to maintain high proliferation rates, analysis of overall cellular growth in these cells is warranted. Alternatively, ARF has been shown to inhibit the activity of numerous cell cycle promoting transcription factors such as c-Myc and E2Fs (8, 27). Other previously reported p53-independent functions of ARF could be playing a role here as well, and these, along with the above-mentioned possibilities will be examined in Chapter 3.

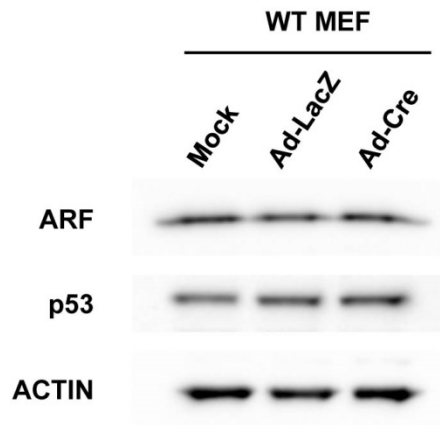
Our findings support a model whereby induction of ARF following p53 loss acts to prevent aberrant proliferation in the face of oncogenic stress. Thus, the functional links between p53 and ARF are far more important than anticipated. The p53-ARF network that we have identified provides tumor suppressive redundancy where none was thought to exist in cells. The question of why one tumor suppressor, p53, would inhibit the expression of another, ARF, has now been answered. As p53 loss of function is one of the most frequent events in human cancers, it would be advantageous for a cell to have an inhibitory signal propagated following this genetic event. Future work identifying ARF's relevant p53-independent targets using our cell culture system will provide potential therapeutic strategies for the aggressive tumors harboring p53 and ARF co-inactivation.



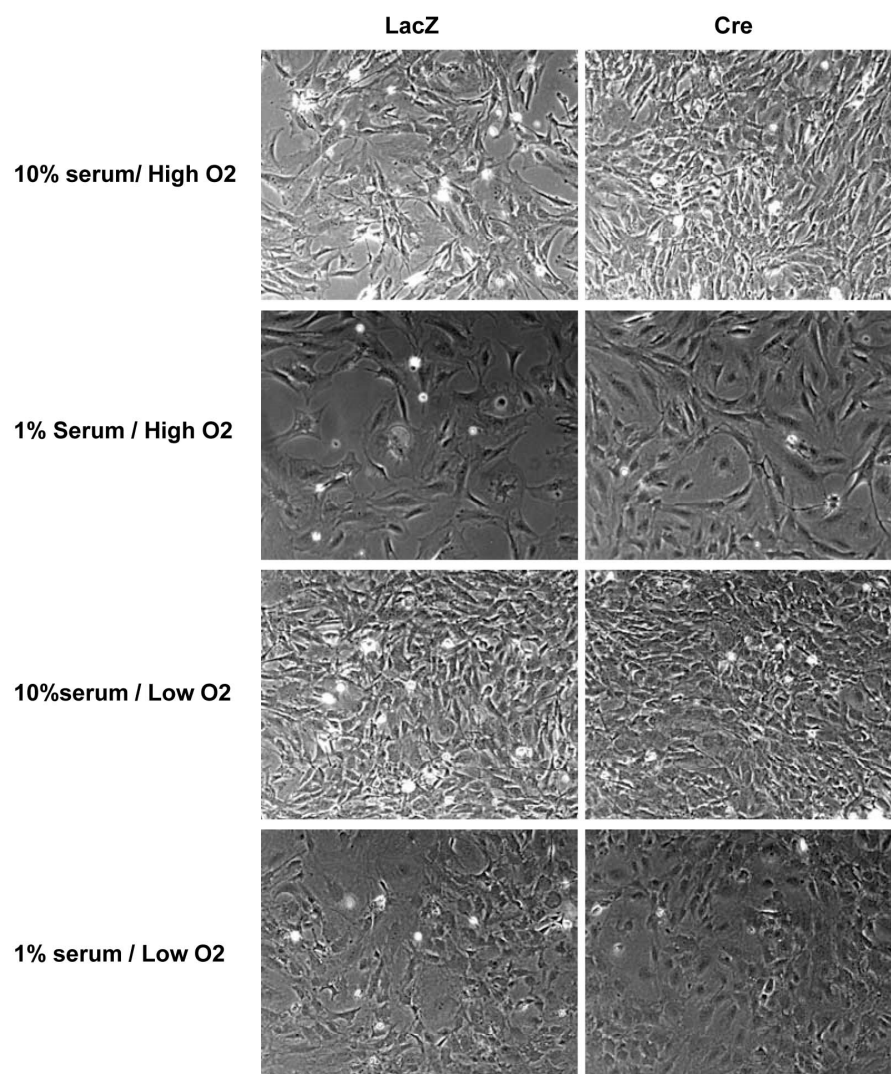
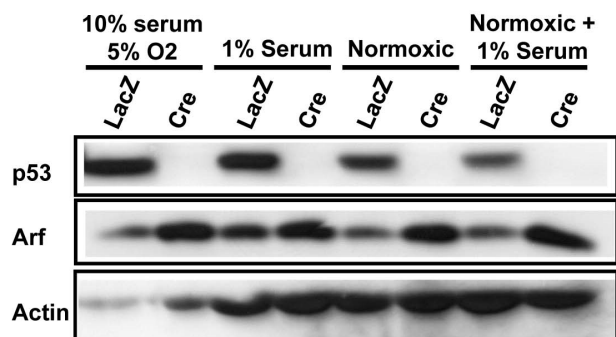
## FIGURES



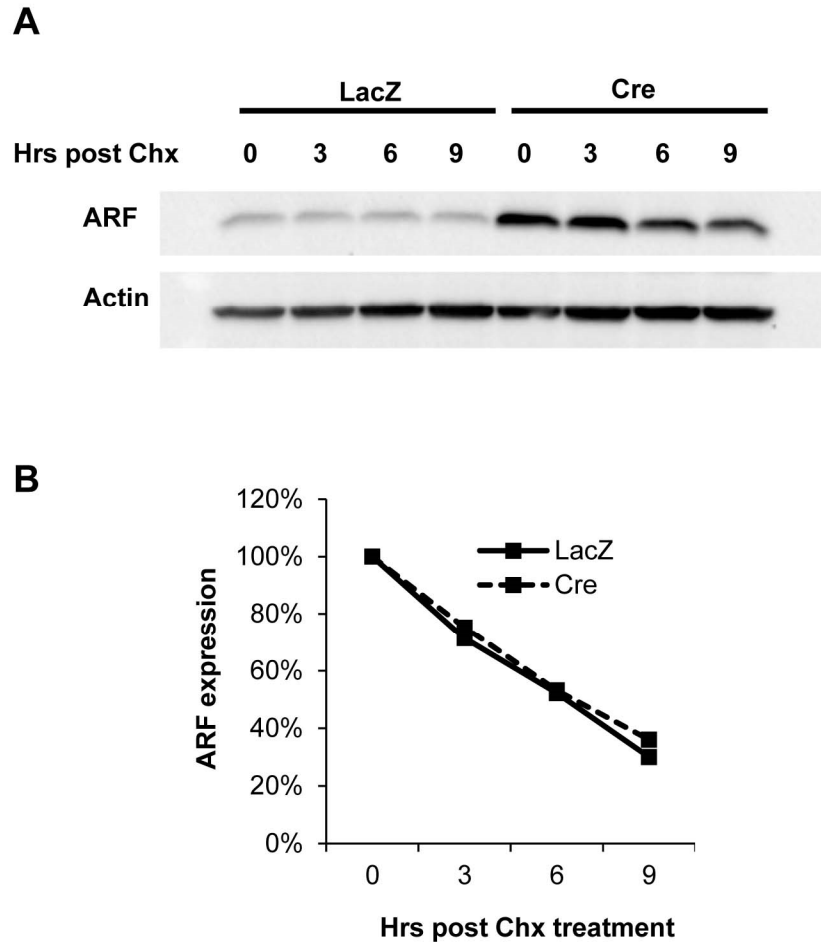
**Figure 2.1** Acute loss of p53 induces ARF. **(A)** Western blot analysis of cell lysates from  $p53^{flox/flox}$  MEFs infected with Ad-LacZ (L) or Ad-Cre (C) harvested at the indicated time points. Fold change of ARF levels are relative to Ad-LacZ control. **(B)** qRT-PCR analysis of p53 and ARF mRNA levels from  $p53^{flox/flox}$  MEFs infected with Ad-LacZ or Ad-Cre. mRNA levels were normalized to  $\beta$ -Actin and fold changes are relative to Ad-LacZ controls. Error bars represent s.d. for  $n=3$  from three independent experiments.



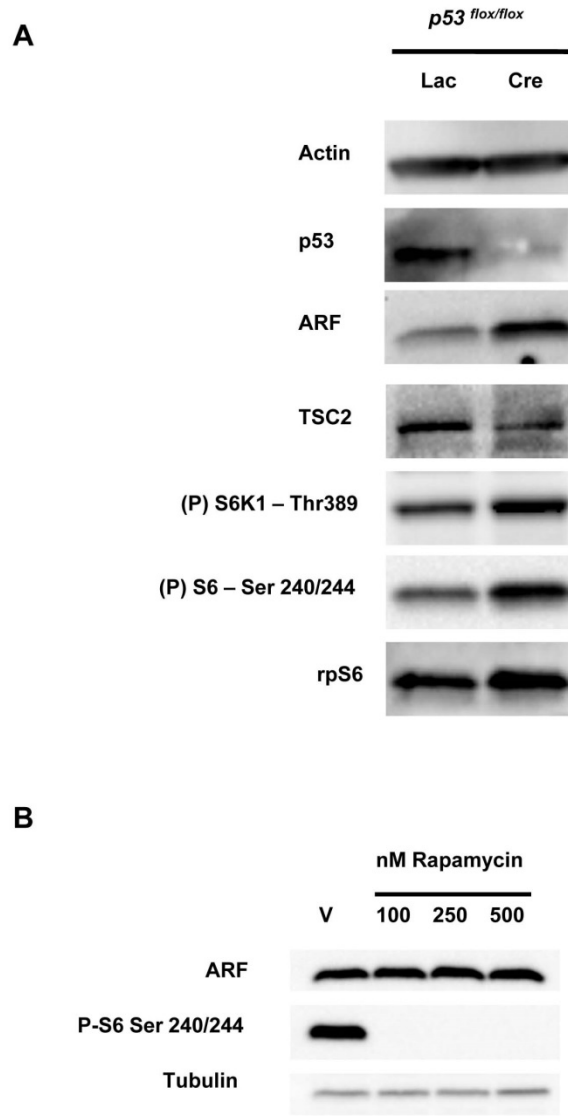
**Figure 2.2** Adenoviral infection does not induce ARF or p53 expression. Low passage Wild-type MEFs were either mock infected (no virus) or infected with Adeno-LacZ or Adeno-Cre (MOI=50). Four days post-infection, cells were harvested and Western blots were performed for the indicated antibodies.

**A****B**

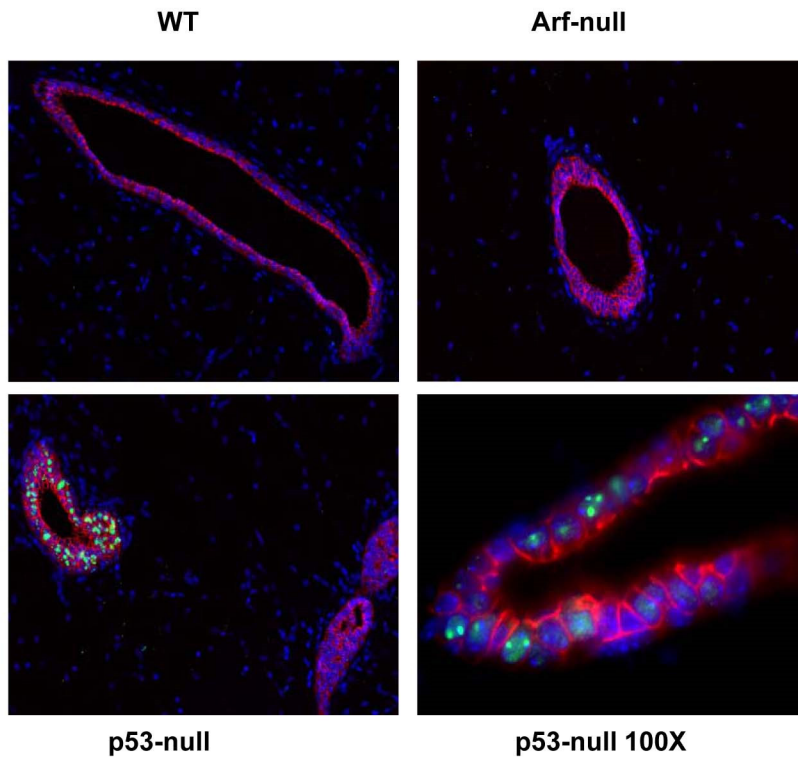
**Figure 2.3 ARF induction following p53 loss does not require high serum and high oxygen conditions.** (A) Light microscopy images of Ad-LacZ or Ad-Cre infected  $p53^{lox/lox}$  MEFs grown in the indicated tissue culture conditions. Images were taken six days post-infection. “High” O<sub>2</sub> indicates atmospheric oxygen concentrations (~21%) typically used for tissue culture and “Low” O<sub>2</sub> indicates a more physiologically relevant concentration of ~4%. (B) Western blot analysis of cells described in (A) at six days post-infection.



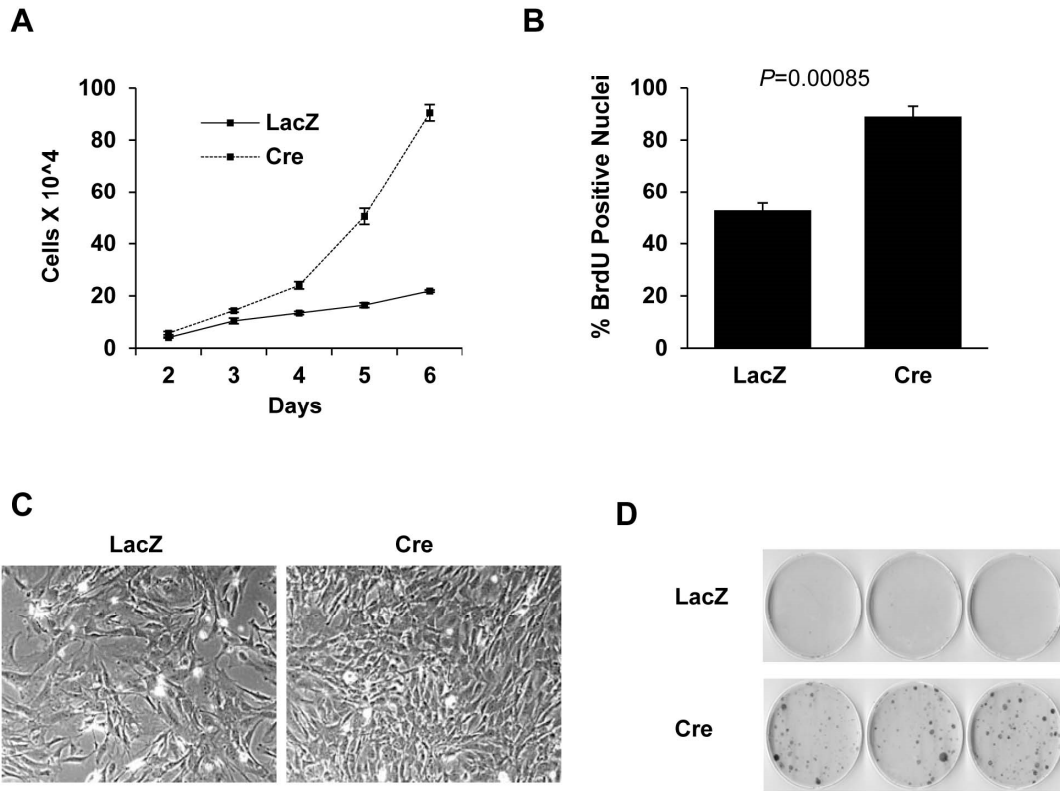
**Figure 2.4** ARF protein stability does not change following p53 loss. (A) Ad-LacZ or Ad-Cre infected  $p53^{fllox/fllox}$  MEFs were treated with 25  $\mu\text{g}/\text{mL}$  cycloheximide for the indicated amounts of time. Western blot analysis was performed for ARF and Actin was used as a loading control. Representative immunoblots are shown. (B) Determination of half-life was determined by using densitometry to quantify Actin-normalized ARF band intensities. Values are depicted as percent ARF remaining relative to the 0-hr time point. The graph is representative of three independent measurements.



**Figure 2.5** Loss of p53 leads to mTOR activation but mTOR is not required for the observed ARF induction. (A) Ad-LacZ or Ad-Cre infected *p53<sup>flx/flx</sup>* MEFs were analyzed by Western blot for the indicated antibodies. Representative immunoblots of three independent experiments are shown. (B) Ad-Cre infected *p53<sup>flx/flx</sup>* MEFs were treated with vehicle (V-Ethanol) or the indicated concentrations of Rapamycin for 14 hours. Western blot analysis was performed for ARF and phosphorylation of S6 was used to monitor the efficiency of Rapamycin treatment.



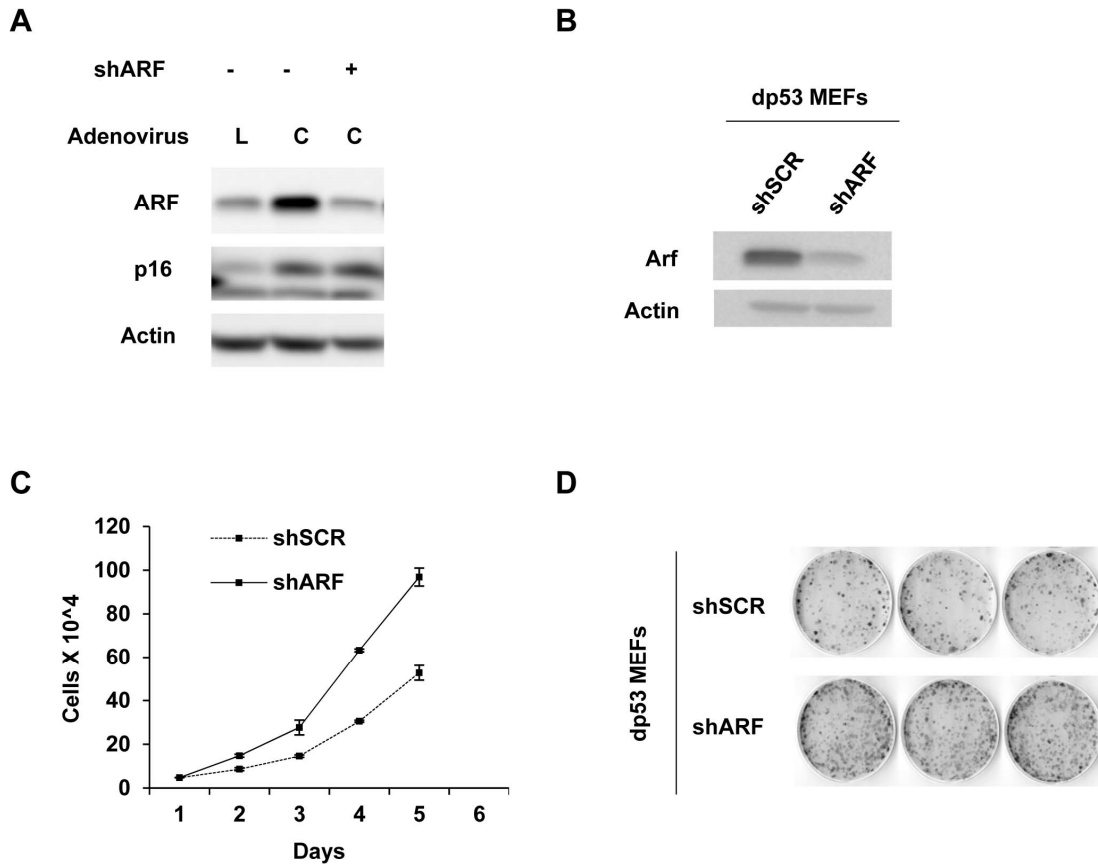
**Figure 2.6** Mammary glands from p53-deficient mice display increased ARF. Immunofluorescence was performed on age-matched, virgin murine mammary fat pads. ARF (green), E-Cadherin (Red), and Dapi (Blue). Representative mammary glands are shown.



**Figure 2.7 Loss of p53 leads to increased cell proliferation**

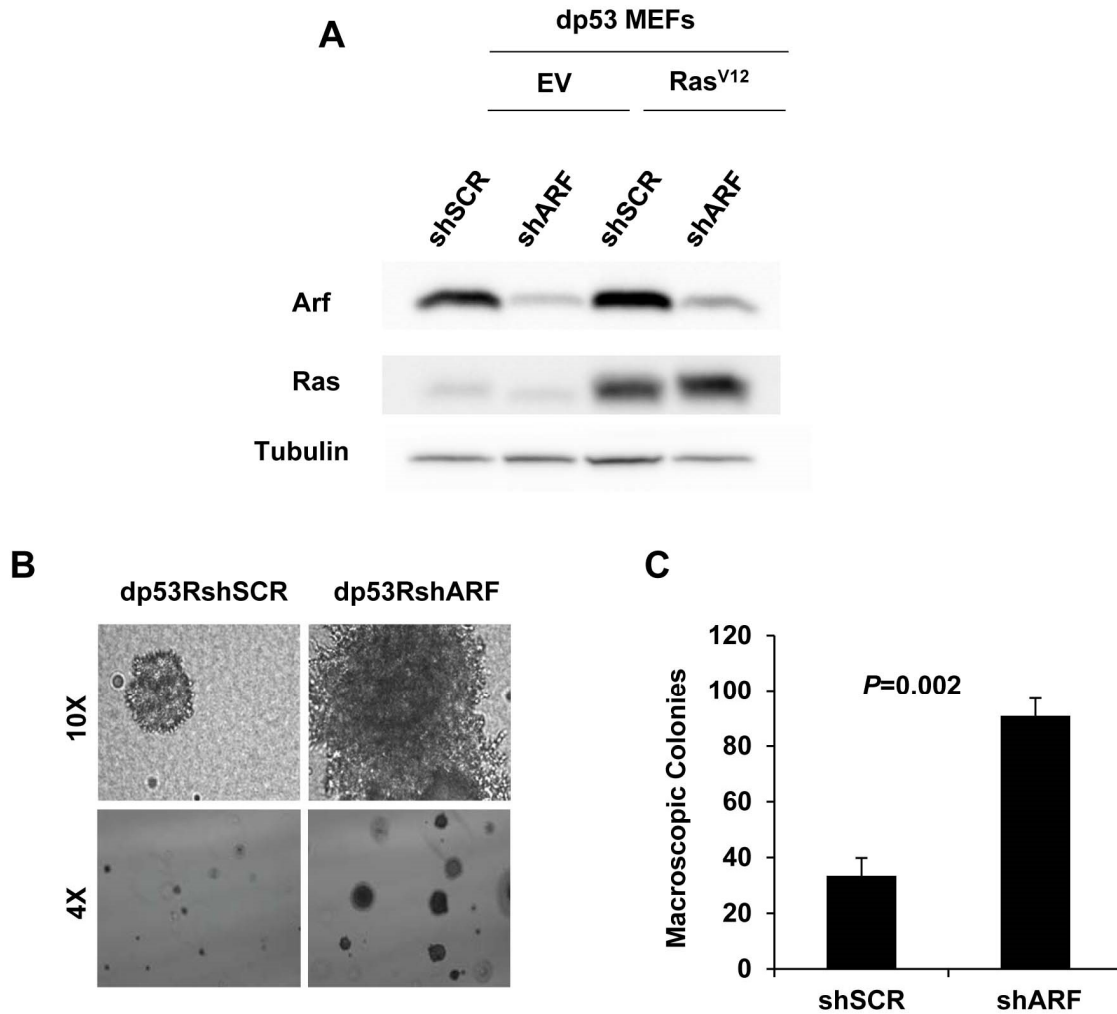
(A) Equal numbers of  $p53^{lox/lox}$  MEFs infected with Ad-LacZ or Ad-Cre were plated in six-well dishes and cell number was counted on the indicated number of days using a hemocytometer. Graph is representative of three independent experiments. (B) Ad-LacZ or Ad-Cre infected  $p53^{lox/lox}$  MEFs pulsed with BrdU for 4 hours. BrdU and DAPI positive nuclei were visualized using immunofluorescence, and data represents percent BrdU positive nuclei from three independent experiments. (C) Light microscopy image (4X objective) of confluent dishes containing cells described in (A) and (B). (D) Representative image of foci assay with Ad-LacZ or Ad-Cre infected  $p53^{lox/lox}$  MEFs.





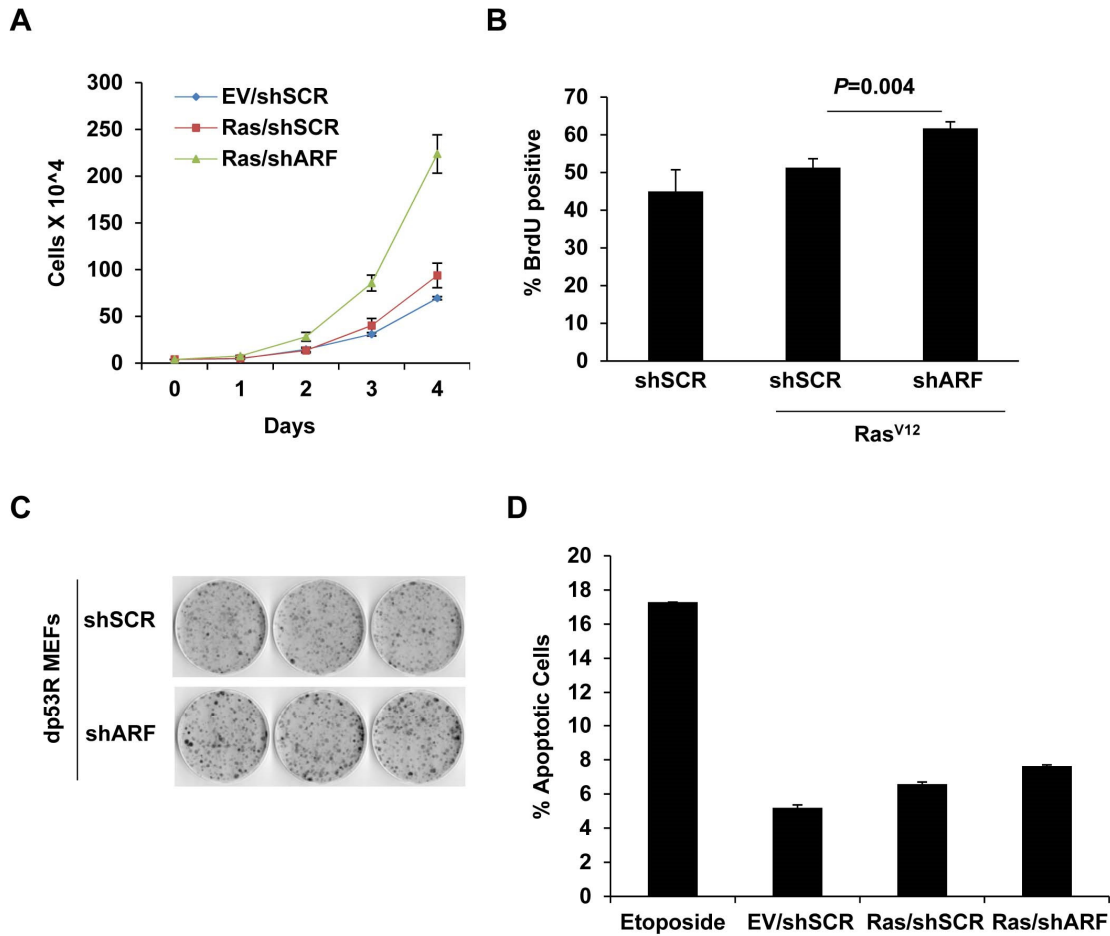
**Figure 2.8** Endogenous ARF limits the proliferation of p53-deficient cells.

(A) Western blot analysis of Ad-LacZ (L) or Ad-Cre (C) infected  $p53^{fllox/fllox}$  MEFs subsequently infected with an shRNA targeting ARF (+) or a scrambled control (-). (B) Western blot analysis of dp53 MEFs infected with shSCR or shARF. (C) Equal numbers of dp53 MEFs infected with shSCR or shARF were plated and manually counted on the indicated days. (D) Representative image of foci assay performed with dp53 MEFs expressing shSCR or shARF.



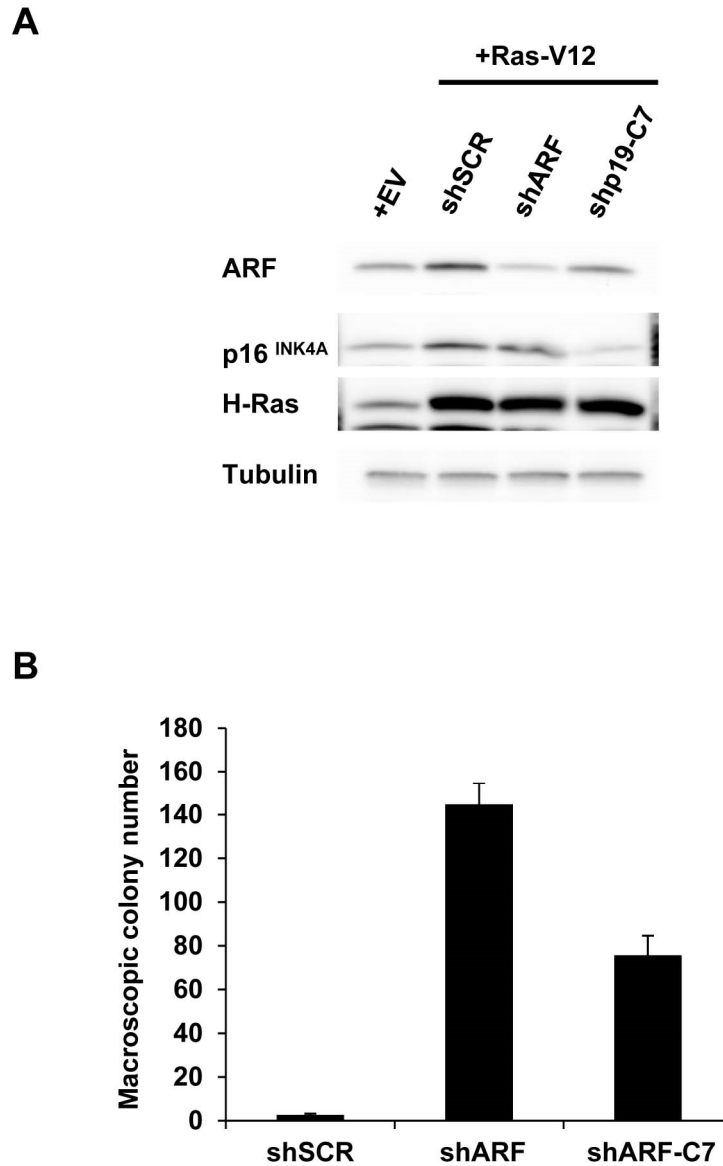
**Figure 2.9** Endogenous ARF limits the tumorigenicity of p53-deficient cells.

(A) Western blot analysis of dp53 MEFs expressing Ras<sup>V12</sup> (dp53R), and infected with shSCR or shARF. (B) Representative images of dp53R-shSCR or dp53R-shARF MEFs growing in soft agar. Macroscopic colonies were quantified in (C). Error bars represent s.d. of  $n=3$ .



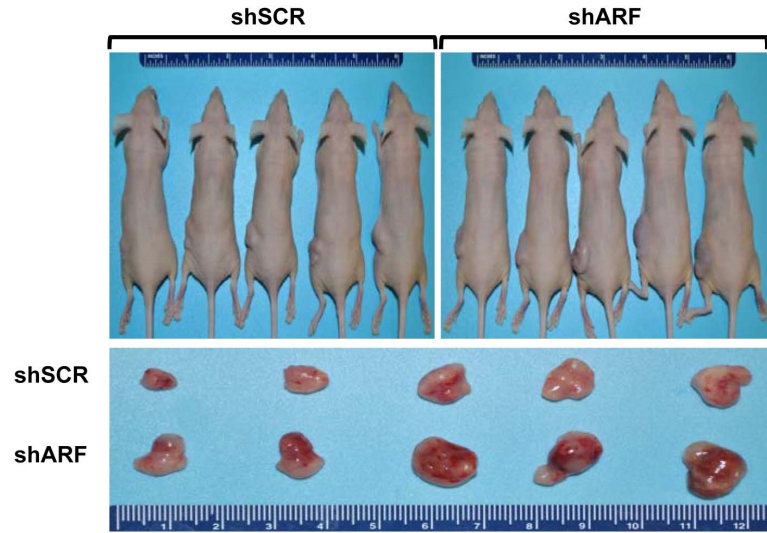
**Figure 2.10** ARF limits the proliferation of Ras-transformed p53-deficient cells.

(A) Proliferation assay of dp53 MEFs expressing empty vector or Ras<sup>V12</sup> and infected with shARF or shSCR control. Graph is representative of three independent experiments. (B) Percent BrdU positive nuclei of cells described in (A) following 4-hour pulse with BrdU. Error bars represent s.d. from three independent measurements of 100 nuclei. (C) Representative image of foci assay performed with dp53R MEFs expressing shSCR or shARF. (D) Annexin V staining was performed with the indicated genotypes using flow cytometry. Etoposide treated (50 $\mu$ M) dp53 MEFs were used as a positive control for apoptosis. Error bars represent the s.d. of three biological replicates.

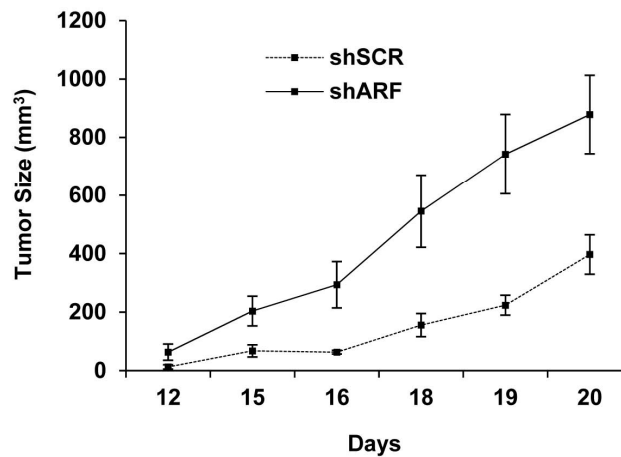


**Figure 2.11** p16 does not inhibit tumorigenicity of p53-deficient cells (A) dp53 MEFs overexpressing RasV12 were infected with shSCR, shARF or an shRNA targeting both p16 and ARF (C7). Western blot analysis was performed 96 hours post-infection to observe knockdown efficiency. (B) Cells described in (A) were plated in soft agar and incubated for 21 days. Macroscopic colony number was quantified. Error bars represent s.d. of three independent measurements.

**A**



**B**



**Figure 2.12** dp53R MEFs depleted of ARF form larger tumors in mice. (A) dp53R MEFs infected with shSCR or shARF were injected into the flanks of nude mice and incubated for 21 days. Images of tumor-bearing mice and excised tumors from allograft experiments using dp53R-shARF or shSCR MEFs are shown. (B) Tumor size was measured using calipers on the indicated days post-injection. Tumor size (volume) was calculated as described in the *Methods* section. Error bars represent s.d. of  $n=5$ .

<b>Gene</b>	<b>Company</b>	<b>Catalogue #</b>	<b>Dilution</b>
<b>p19ARF (mouse)</b>	<b>Santa Cruz</b>	<b>sc-32748</b>	<b>1:1000</b>
<b>p53 (mouse)</b>	<b>Cell Signaling</b>	<b>2524</b>	<b>1:1000</b>
<b>MDM2</b>	<b>Millipore</b>	<b>OP115</b>	<b>1:500</b>
<b>Actin</b>	<b>Santa Cruz</b>	<b>sc-8432</b>	<b>1:500</b>
<b>Gamma tubulin</b>	<b>Santa Cruz</b>	<b>sc-7396</b>	<b>1:1000</b>
<b>H-Ras</b>	<b>Santa Cruz</b>	<b>sc-520</b>	<b>1:2000</b>
<b>Gapdh</b>	<b>Bethyl</b>	<b>A300-641A</b>	<b>1:10000</b>
<b>Snf5</b>	<b>Bethyl</b>	<b>A301-087A-1</b>	<b>1:2000</b>
<b>p16Ink4a</b>	<b>Santa Cruz</b>	<b>SC-1207</b>	<b>1:1000</b>
<b>rpS6</b>	<b>Cell Signaling</b>	<b>2317S</b>	<b>1:1000</b>
<b>(P) S6 Ser240/244</b>	<b>Cell Signaling</b>	<b>2215S</b>	<b>1:5000</b>
<b>(P) S6K1 Thr 389</b>	<b>Cell Signaline</b>	<b>9205S</b>	<b>1:1000</b>

**Table 2.1. List of primary antibodies**

<b>ARF</b>	<b>FWD</b>	<b>5'-GAG TAC AGC AGC GGG AGC AT-3'</b>
	<b>REV</b>	<b>ATC ATC ATC ACC TGG TCC AGG ATT CC</b>
<b>p53</b>	<b>FWD</b>	<b>CAT CAC CTC ACT GCA TGG AC</b>
	<b>REV</b>	<b>AAA AGA TGA CAG GGG CCA TG</b>
<b>Actin</b>	<b>FWD</b>	<b>TCA CCC ACA CTG TGC CCA TCT A</b>
	<b>REV</b>	<b>TAC TCC TGC TTG CTG ATC CAC A</b>
<b>Histone 3.3</b>	<b>FWD</b>	<b>CGT GAA ATC AGA CGC TAG CAG AA</b>
	<b>REV</b>	<b>TCG CAC CAG ACG CTG AAA G</b>

**Table 2.2. List of qRT-PCR primers**

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## **Chapter 3:**

# **Investigating the function of endogenous ARF in the absence of p53**

## ABSTRACT

The ARF tumor suppressor functions to protect cells from hyper-proliferative and hyper-growth signals by activating p53-mediated cell cycle arrest or apoptosis. Genetic models in mice have indicated that ARF also possesses important p53-independent tumor suppressor functions, and these findings are supported by frequent co-inactivation of p53 and ARF in human tumors. While numerous p53-independent functions of ARF have been identified, the physiological relevance of these is not well understood. In Chapter 2 of this dissertation, we created and validated an *in vitro* system of acute p53 loss to monitor the functions of ARF. In this system, ARF became induced following p53 loss and potently inhibited tumorigenesis. In an effort to understand how ARF might be limiting tumorigenesis in the setting of *p53*-deficiency, we analyzed previously reported p53-independent function of ARF. We show that ARF does not affect rRNA transcription, protein synthesis, SENP3 stabilization, c-MYC driven transcription, or migration and invasion in cells faced with acute *p53* loss. Many of these p53-independent functions were discovered using ARF overexpression models, so our findings highlight the difficulties in interpreting these types of experimental systems. Our model uses acute loss of *p53* as the catalyst for ARF induction, allowing us to interrogate the importance of endogenous ARF in this genetic context. As such, we believe our system will allow us to confidently identify novel p53-independent targets of ARF that are required for tumor suppression in p53-deficient tumors.

## INTRODUCTION

The *CDKN2A* locus on human chromosome *9p21* encodes two tumor suppressor genes tasked with the job of maintaining appropriate rates of cellular proliferation (45). Deletion, mutation, or epigenetic silencing of *CDKN2A* is therefore a very common occurrence in human tumors (43). In fact, mutation of the well-known tumor suppressor, p53, is the only genetic abnormality that occurs more frequently (29). The two genes encoded by *CDKN2A* are p16<sup>Ink4a</sup> and p19<sup>ARF</sup>. Due to a unique genomic organization whereby p16<sup>Ink4a</sup> and p19<sup>ARF</sup> have distinct promoters and first exons (designated exon-1 $\alpha$  and exon 1- $\beta$  respectively), the encoded p16 and ARF proteins share no homology at the amino acid level. This is due to a frameshift in exon-1 $\beta$  of ARF that causes exon 2 to be translated in an alternate reading frame (40). As distinct proteins, ARF and p16 have very different tumor suppressor roles in the cell. p16, which is a member of the INK4 family of cell cycle inhibitors (p15INK4b, p18INK4c, p19INK4d are the other family members), is a cyclin-dependent kinase inhibitor that inhibits the association of CDK4/6 with Cyclin D (42). By inhibiting the formation of this complex, p16 is able to maintain the Retinoblastoma protein (Rb) in a hypo-phosphorylated state, thus keeping the cell cycle stuck in G1.

ARF is not a member of any known family of proteins, and its peculiarity at the genomic level is carried over to the amino acid level as well. The 169 amino acid protein contains 20% arginine residues, and as a result, has a pI around 12 (40). At physiological pH, therefore, ARF is highly disordered. However, upon association with binding partners, ARF can adopt alpha helical structures (13). Mouse ARF (p19) contains only one lysine residue, and human ARF (p14) contains none. As a result, the turnover of ARF protein ( $t_{1/2}$ = 6 hours) is maintained by a proteasome-targeting process that does not require lysine residues, called N-

terminal polyubiquitination (27). Finally, ARF is a resident of the nucleolus, a non-membrane bound organelle formed around actively transcribed ribosomal DNA (rDNA) loci (6, 46).

The canonical tumor suppressor function of ARF is the activation of p53-dependent cell cycle arrest in response to sustained oncogenic signaling. Increased levels or activity of oncogenes such as Ras, c-MYC, E1A, and E2F-1 lead to gains in both transcription and translation of Arf (4, 12, 34, 37, 65). Induced levels of ARF bind and sequester the E3 ubiquitin ligase for p53, MDM2, in the nucleolus (53, 58). p53 has a very short half-life (~20 minutes), so relief of ubiquitin-mediated degradation by the proteasome stabilizes p53 protein levels and allows it to participate in the transcriptional regulation of cell cycle inhibitory genes such as p21 (33). In this manner, ARF is able to protect cells from oncogenic transformation by activating p53-mediated arrest or apoptosis. In support of this model, *Arf*-deficient fibroblasts can be transformed by overexpression of RAS or c-MYC alone (30, 37, 65). Furthermore, in an *Eμ-Myc*-driven model of lymphoma, spontaneous inactivation of ARF or p53 occurred in 50% of the tumors and *Arf*<sup>+/-</sup>; *Eμ-Myc* exhibited increased rates of lymphoma development with the vast majority of these tumors inactivating the remaining *Arf* allele (14).

ARF also possesses p53-independent functions, but the physiological importance of these are not well understood (44). Evidence for the existence of p53-independent functions first surfaced when it was shown that enforced expression of ARF in p53-deficient cells can lead to cell cycle arrest, albeit with slower kinetics than wild-type cells (44, 57). Furthermore, mice lacking both *p53* and *Arf* develop multiple tumor types in addition to the typical lymphomas that arise as a result of *p53* loss alone (57). These findings have motivated researchers to find p53-independent targets of ARF, and in the last ten years, promising progress has been made. For instance, ARF can directly inhibit the transcriptional activities of c-MYC and E2F-1, limiting the

pro-proliferative gene expression programs that these factors promote (15, 39). ARF has also been shown to inhibit both the motility and invasiveness of cancer cells (9, 19, 35, 62). Finally, ARF can promote the conjugation of a ubiquitin-like moiety, SUMO, to many of its binding partners (52). Promotion of sumoylation is achieved by initiating the degradation of the SUMO-specific protease, SENP3 (28). The functional significance of these p53-independent functions (and others reviewed in (44)) in tumor suppression remain a subject of debate because the majority of these studies were performed by grossly overexpressing ARF, potentially leading to non-physiological interactions.

Arguably the most well-characterized p53-independent function of ARF is its ability to regulate ribosomal biogenesis and overall cellular growth (protein synthesis). This function was first reported by Sugimoto and colleagues, when they found ARF could inhibit the processing ribosomal RNA species in cells lacking functional p53 (49). Shortly thereafter, it was discovered that ARF could physically interact with the ribosomal chaperone, Nucleophosmin (NPM) (5, 32). Similar to its inhibition of MDM2, ARF sequesters NPM in the nucleolus, thus limiting NPM's ability to shuttle ribosomes to the cytosol (8). Our lab established that basal ARF levels are important in maintaining nucleolar integrity and function. Loss of ARF leads to gains in nucleolar size, rDNA transcription, rRNA processing, and ribosomal export. This led to the enhancement of overall protein synthesis and cell size ((2) and Crystal Winkeler unpublished data). Over the last 5 years, we and others have also shown ARF can regulate translation of specific mRNAs—namely Vegf-A, Pdgfr- $\beta$ , Drosha, and Dhx33 (24, 26, 59, 64). ARF, therefore, can affect multiple signaling pathways through general as well as selective translational control.



An interesting wrinkle in the ARF/p53 pathway is that a negative feedback loop exists between p53 and ARF, whereby p53 is capable of repressing transcription of *Arf* (61). Why would one tumor suppressor inhibit the expression of another? The most likely possibility is that this regulatory loop allows fine-tuning of the ARF-p53 response. By inducing expression of an inhibitor (MDM2), and suppressing expression of an activator (ARF), p53 can discriminate against normal physiological growth signals and oncogenic stress. If ARF gets induced in response to normal growth signals, a pulse in p53 activation would be allowed. However, the inducing signal would not be sustained long-term, so eventually ARF levels would become suppressed and MDM2 would be induced by p53 to return p53 back to steady-state levels. In response to sustained oncogenic activation, ARF would be constitutively transcribed and p53 would be outcompeted at the *Arf* promoter, leading to a durable p53 response. Of course, possessing this regulatory might also mean that upon p53 loss, ARF expression is unleashed and could function to limit oncogenic damage. In support of this possibility, we previously showed that acute loss of p53 led to a robust induction of ARF (Chapter 2). The increased ARF levels were capable of limiting both the proliferation and the tumorigenicity of these cells.

In this chapter, we tested whether the induced levels of ARF that occur following p53 loss are limiting proliferation and tumorigenicity through previously reported p53-independent tumor suppressive mechanisms. Endogenous ARF levels had no effect on rRNA transcription or overall protein synthesis in *p53*-null cells. Furthermore, ARF's involvement in sumoylation, invasion, and c-MYC-induced transcription did not appear to be important regulators of tumor suppression in the absence of p53. While we were unable to mechanistically explain ARF's ability to suppress tumorigenesis in cells facing acute *p53* loss of function, our data indicates that ARF is likely involved in novel tumor suppressor pathways that will necessitate further studies.

## **METHODS**

**Cytosolic Ribosome Fractionation.** Cells were treated with 10 µg/mL cycloheximide for 5 minutes before harvesting by trypsinization. Cell number was obtained using trypan-blue exclusion and a hemocytometer. For each condition,  $3 \times 10^6$  cells were pelleted and lysed. Cytosolic fractions were layered over 7%-47% sucrose gradients and ribosome fractionation and measurement was performed as previously described (36, 48)

**[<sup>35</sup>S]-Incorporation measurement of protein synthesis.** Cells ( $1 \times 10^5$ ) were plated in triplicate in 6-well dishes and starved of cysteine and methionine for 30 minutes. Cells were then pulsed with 150 µCi [<sup>35</sup>S]-protein labeling mix (Perkin Elmer) for various amounts of time. After pulsing, the cells were harvested by scraping in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid) containing 1X Halt Protease inhibitor cocktail (Thermo Scientific), and incubated on ice for 15 min. After sonication, supernatants were cleared by centrifugation at 14,000 x g for 5 minutes. Protein was precipitated with 10% trichloroacetic acid, and pellets were subjected to liquid scintillation counting to measure incorporated counts per minute (cpm).

**Immunofluorescence.** Cells ( $1 \times 10^5$ ) were seeded overnight on glass coverslips (Corning). Cells were fixed for 15 minutes at room temperature with 10% Methanol in Formalin. Membrane permeabilization was performed with 5 minute incubation in 1% NP-40. Cells were then blocked for 30 minutes with Dako serum-free protein block. Primary antibodies for ARF (1:400, Santa Cruz) were applied for 1 hour. A secondary antibody conjugated with Alexa Fluor 488 was placed on tissue sections for 1 hr at room temperature (1:300, Life Technologies).

Nuclei were counterstained using Slow Fade Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies).

**Immunoprecipitation and Western blotting.** Cell pellets were lysed in EBC lysis buffer (50 mM Tris-Cl, pH7.4, 120mM NaCl, 0.5% NP-40, 1mM EDTA) containing HALT Protease and Phosphatase Inhibitor cocktail (Thermo Scientific) and 1mM phenylmethylsulfonyl fluoride (PMSF). For immunoprecipitations, 400 µg of protein was incubated overnight with rabbit anti-p19ARF antibody in EBC. Immunocomplexes were precipitated with Protein-A Sepharose for 2 hours. Sepharose beads were washed three times with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid). Protein was eluted using 1X SDS loading buffer and resolved on SDS polyacrylamide gels. Following transfer to PVDF (Millipore), the primary antibodies listed in Table 3.1 were applied. Secondary horseradish peroxidase conjugated antibodies (Jackson Immunoresearch) were used and ECL plus was used to visualize the bands (GE Healthcare).

**Cell Culture and Reagents.** *p53<sup>flox/flox</sup>* (FVB.129-*Trp53<sup>tm1Brn</sup>*) were obtained from the NCI Mouse Repository and have been previously described (21). Primary mouse embryonic fibroblasts were isolated as previously described (23). All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2mM glutamine, 0.1mM nonessential amino acids, 1mM sodium pyruvate, and 2ug/mL gentamicin. Unless otherwise indicated, cells were maintained in 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>.

**AgNOR Staining.** AgNOR staining was performed exactly as described previously (2). Quantification of AgNOR/nucleus and %AgNOR Area/Nucleus was performed manually using Metamorph software (Molecular Devices). For each measurement, 100 nuclei were scored from three independent slides to obtain averages and standard deviations.

**47S rRNA transcription.** Analysis of 47S rRNA transcription was performed exactly as described previously (32). Briefly,  $1.5 \times 10^6$  cells were harvested and total RNA was isolated using a Nucleospin RNA II Kit (Machery-Nagel). Production of cDNA was performed using a Superscript III Kit (Invitrogen) and a mouse 47S specific primer. qRT-PCR was performed using 2X SYBR-Green mix (BioRad) and 47S rRNA specific primers. Amplification was performed on a BioRad CFX96 Real-Time System.

**Soft Agar.** Cells were lifted and suspended in DMEM containing a final concentration of 0.4% Noble agar.  $1.5 \times 10^4$  cells were layered in triplicate onto 0.6% noble-agar/media bottom layer in 60mm plates. Plates were incubated for 20 days, feeding with media/0.4% agar mix every 6 days. Macroscopic colonies were visualized by staining with 0.005% Crystal Violet solution and colonies  $\geq 0.5$ mm were manually counted.

**Scratch Assay.** Cells ( $8 \times 10^5$ ) were seeded overnight onto 60mm tissue culture dishes. A “scratch” was made down the center of the plate using a p200 pipette tip. Plates were then washed once with PBS to remove lifted cells and replenished with fresh media. Imaging was performed at  $t=0$  and  $t=8$  hrs post scratching, at three independent locations on the plate.

**Collagen Invasion Assay.** This assay was performed exactly as described previously (63). Briefly,  $1 \times 10^5$  cells were embedded in type I collagen (2 mg/mL) gel isolated from rat tail (BD Biosciences). Following incubation at 37°C to gel the collagen, the plug was suspended in 2 mg/mL collagen in a 24 well plate. Invasion into the collagen matrix was visualized 48 hours later with an inverted light microscope (4X Objective).

**Viral production and infections.** Adenoviruses expressing  $\beta$ -galactosidase (Ad-LacZ) or Cre recombinase (Ad-Cre) were purchased from the Gene Transfer Vector Core, University of Iowa. For Adenoviral infections,  $1 \times 10^6$  cells were plated in the presence of Ad-LacZ or Ad-Cre (MOI=50) and incubated for 8 hours. For mutant Ras<sup>V12</sup> overexpression, retrovirus was produced by transfecting 293T cells with either MSCV-HRAS<sup>V12</sup>-IRES-GFP plasmid or MSCV-IRES-GFP control, and the helper plasmid  $\psi$ -2. Virus-containing supernatants were harvested 48 hr post transfection. Collected retrovirus was used to infect  $1 \times 10^6$  MEFs in the presence of 10ug/mL polybrene. For the production of Lentiviral shRNAs, 293T cells were transfected using Lipofectamine 2000 (Invitrogen) with pCMV-VSV-G, pCMV- $\Delta$ R8.2, and pLKO.1-puro constructs. Viral supernatants were harvested 48hrs post transfection. Cells were infected with lentivirus for 8-12 hours in the presence of 10 ug/mL protamine sulfate. Puromycin was added to cell culture media at a concentration of 2 ug/mL for selection. The ARF (mouse specific) hairpin was described previously (2).

**qRT-PCR.** qRT-PCR was performed as previously described (34). Fold change was measured using the  $\Delta\Delta C_T$  method (31). Mouse-specific primer sequences used for amplification can be found in Table 3.2.

**Statistics.** Data are presented as means  $\pm$  s.d. Statistical differences between groups were determined with *P*-values obtained using two-sided, unpaired Student's *t*-test. All data points represent *n*=3. All images presented as “representative” were completed a minimum of three times.

## RESULTS

### ARF localizes to nucleoli and interacts with NPM in p53-deficient cells

Our results described in Chapter 2 of this dissertation demonstrated that endogenous ARF levels have a p53-independent tumor suppressor function. While numerous p53-independent functions have been attributed to ARF, only its regulation of cellular growth (i.e. macromolecular synthesis) has been demonstrated to be physiologically relevant. Thus, we hypothesized that p53 loss leads to an induction of ARF protein which functions to limit overall protein synthesis in the cell, limiting proliferative capacity.

To test this hypothesis, we first wanted to ensure ARF subcellular localization remained nucleolar in the absence of *p53*. Utilizing the mouse model of *p53*-inactivation we described in Chapter 2, we infected *p53<sup>lox/lox</sup>* MEFs with Adeno-LacZ or Adeno-Cre-recombinase. Six days post-infection, cells were stained for ARF. Both LacZ and Cre treated cells displayed punctate nuclear staining indicative of nucleolar structures, suggesting loss of *p53* does not affect the localization of ARF (Figure 3.1 A). Having demonstrated that ARF remains nucleolar in the absence of *p53*, we next wanted to test whether ARF was interacting with NPM in our system. As mentioned, ARF can sequester NPM in the nucleolus, leading to decreased ribosomal shuttling and cell growth (7, 32). We immunoprecipitated ARF complexes from day 6 Ad-LacZ or Ad-Cre infected *p53<sup>lox/lox</sup>* MEFs. Our inputs indicated that NPM expression does not change following p53 loss, but as expected, MDM2 levels are greatly diminished due to p53 being a positive transcriptional regulator of its expression (Figure 3.1 B) (3, 22). Markedly, in LacZ treated cells, ARF has a much greater affinity for associating with MDM2 than NPM, consistent with previous findings (7). In the Cre-treated MEFs, MDM2 levels are depleted and ARF appears to form more abundant complexes with NPM. Taken together, these data suggest ARF

can interact with NPM in the absence of *p53*, and are in support of the hypothesis that ARF might be inhibiting overall cellular growth to inhibit *p53*-null cell proliferation.

### **Dp53 MEFs display evidence of reduced overall cellular growth**

To test whether endogenous ARF was inhibiting cellular growth in *p53*-deficient cells, we first assessed the total amount of protein present in these cells. As shown in Figure 3.2A, Cre-treated *p53<sup>flox/flox</sup>* MEFs (hereafter referred to as dp53 MEFs—deleted for *p53*) had significantly lower amounts of total protein/cell both 4 days and 6 days post-infection. At these time points, ARF is robustly induced (Figure 3.2 E). Additionally, we assessed the level of active translation by monitoring cytosolic ribosome profiles in LacZ or Cre treated *p53<sup>flox/flox</sup>* MEFs. Cytosolic ribosome complexes were fractionated from sucrose gradients and rRNA was monitored using UV absorbance, allowing us to quantify the amount of 40S, 60S, 80S, and actively translating polyribosomes in the cells. In support of our protein/cell findings, we observed lower amounts of the 40S, 60S, 80S subunits, and actively translating polyribosomes in the dp53 cells (Figure 3.2 B). Lower amounts of 40S and 60S subunits could be due to a defect at any step in ribosome biogenesis: rRNA transcription, rRNA processing, assembly, or export of subunits into the cytosol. Having previously shown that ARF's basal role in the cell is maintaining proper rRNA synthesis and processing rates, we hypothesized that rRNA transcription was being affected by increased ARF levels (2). Using a quantitative real-time PCR method to detect the 47S precursor rRNA species, we found that dp53 cells with elevated ARF levels contained fewer copies of the 47S precursor rRNA, suggesting there are defects in rRNA transcription (Figure 3.2 C). Taken together, these data suggest that dp53 MEFs contain



less protein on a per cell basis and this could be due to a reduction in available ribosomal subunits as a result of decreased rRNA transcription.

Since rRNA transcription takes place in the nucleolus, we next wanted to assess the morphology of nucleoli since this can be a direct means of assessing nucleolar function. For many years, pathologists have analyzed the silver-staining nucleolar organizing regions (AgNORs) for readout of nucleolar function in human cancers. Higher AgNOR scores often correlate with increased aggressiveness of tumors (38). We performed AgNOR staining on LacZ or Cre treated *p53<sup>lox/lox</sup>* MEFs 6 days post-infection. We did not detect any changes in gross morphology of the NORs, but surprisingly, we did observe small but significant increases in the number of NORs/nucleus and the amount of nuclear area occupied by the NORs in dp53 MEFs (Figure 3.2 D). At face value, these findings are inconsistent with our previous cell growth measurements. However, the increase in AgNOR area/nucleus is likely explained by the fact that dp53 MEFs are smaller than their wild-type counterparts and, accordingly, their nuclei are smaller (Figure 3.2 D and data not shown). Since we did not normalize to nuclear size, the AgNOR area/nucleus is artificially increased due to smaller nuclei in dp53 MEFs. This does not, however, explain the increase in the number of AgNORs per nucleus, a finding that we will revisit in the discussion section.

### **Increased ARF levels in p53-deficient cells do not inhibit cellular growth**

Thus far, we have shown that p53-deficient cells display evidence of reduced protein synthesis as a result of rRNA transcription defects. In chapter 2, we showed that knocking down ARF in p53-deficient cells led to an increase in proliferation and an increase in tumorigenic

growth. Therefore, our hypothesis to explain this phenomenon is that loss of p53 leads to the induction of ARF, and the increased ARF levels are capable of inhibiting overall cellular growth, thus limiting proliferation and tumorigenicity. To directly test this hypothesis, we asked what effect depleting ARF from dp53 MEFs would have on cellular growth.

Knockdown of ARF in dp53 MEFs did not rescue the 47S rRNA levels or the reduced polyribosome formation (Figure 3.3 A-C). Likewise, in *Ras*<sup>V12</sup>-transformed dp53 MEFs (dp53R), ARF depletion had no effect on polyribosome formation or protein/cell (Figure 3.3 E and F). Additionally, we tested whether NPM function was specifically required for the increased tumorigenicity seen in *Ras*<sup>V12</sup>-transformed dp53 MEFs depleted for ARF (Chapter 2, Figure 2.9 B and C). As shown in Figure 3.4, these dp53R-shARF cells are equally sensitive to NPM depletion as the dp53R-shSCR controls (Figure 3.4 A-C). Together, these data indicate ARF does not limit the proliferation and tumorigenicity of p53-deficient cells through the regulation of overall cellular growth. In fact, when we performed a more quantitative measurement of active protein synthesis (<sup>35</sup>S-incorporation into newly made proteins), we observed significantly enhanced incorporation of <sup>35</sup>S in dp53 MEFs, regardless of high or low ARF expression (Figure 3.3 D).

To truly rule out ARF's regulation of cell growth in a p53-independent setting, we would need to utilize a post-mitotic cell system where cell-cycle differences would not affect our interpretation of experiments (e.g. *p53*-null osteoclasts) (54). We took an alternative approach by analyzing the effects of ARF knockdown prior to any increase in proliferation in dp53 MEFs. At 48 hours post shARF infection, we observed no increase in protein per cell or steady state levels of 47S rRNA even though ARF was significantly depleted (Figure 3.5 B-D). At this time

point, there are not yet proliferation differences between the shSCR and shARF cells (Figure 3.5 A, Day 2 time point).

### **Increased ARF levels do not dictate expression of the de-sumoylating enzyme SENP3 in dp53 MEFs.**

Having demonstrated that enhanced ARF levels were not regulating cellular growth in the absence of p53, we chose to examine other established p53-independent functions of ARF. Sumoylation is the process by which the small ubiquitin-related modifier (SUMO) is conjugated to lysine residues in target proteins (17). Sumoylation can affect cellular localization, enzymatic activity, or protein stability. Intriguingly, ARF can promote the sumoylation many proteins, including NPM, E2F-1, and HIF-1 $\alpha$  by inhibiting a de-sumoylating enzyme called SENP3 (28, 41, 51). Increased ARF expression leads to the decreased stability of the SENP3 protein (28). As sumoylation of NPM has been reported to inhibit its pro-proliferative functions, including rRNA processing, we hypothesized that increased ARF might be promoting an anti-proliferative effect in p53-deficient cells by promoting sumoylation as a result of SENP3 degradation (20). We monitored SENP3 expression levels during a time course of an Adeno-LacZ or Adeno-Cre infection of *p53<sup>fllox/fllox</sup>* MEFs. As seen in Figure 3.6A, loss of p53 and the subsequent ARF induction has no effect on SENP3 levels. Moreover, depleting ARF in *p53*-deficient cells (with or without *Ras<sup>V12</sup>*) did not significantly alter SENP3 expression (Figure 3.6 B). These results indicate that endogenous ARF in p53-deficient cells does not affect SENP3 protein stability, and is therefore, not likely to affect sumoylation.

### **ARF does not inhibit c-MYC transcriptional activity in dp53 MEFs**

As previously mentioned, ARF has been reported to interact with and inhibit the transcriptional activity of the proto-oncogene c-MYC (39). Amplification of the c-MYC gene is frequently found in human cancers, and overexpression of c-MYC in mice is sufficient to induce lymphoma formation (1, 11). Reportedly, ARF's inhibition of c-MYC does not require p53, so we asked whether the well-characterized c-MYC transcriptional targets ornithine decarboxylase (ODC) and nucleolin (NCL) are inhibited by endogenous ARF in dp53 MEFs (18, 56). Dp53 MEFs infected with an shRNA targeted against ARF did not display significant increases of ODC or NCL, while ARF mRNA levels were reduced by 40% (Figure 3.7). Thus, ARF does not inhibit c-MYC transcription in *p53*-deficient cells.

### **ARF does not inhibit the motility or invasiveness of p53-deficient cells**

Thus far, we have shown that ARF is not able to limit tumorigenicity of *p53*-deficient cells by inhibiting cellular growth, sumoylation, or c-MYC transcriptional inhibition. Lastly, we wanted to examine the role of ARF in motility and invasion, both of which have been described to be regulated by ARF in a *p53*-independent manner (9, 19, 35). We measured the motility of dp53R-shSCR (MEFs deleted for p53 expressing Ras<sup>V12</sup> and shSCR) or dp53R-shARF MEFS (MEFs deleted for p53 expressing Ras<sup>V12</sup> and shARF) with a classic “scratch assay,” and observed no change in the cells ability to migrate into the scratched area (Figure 3.8 A). To directly measure invasiveness, we used a collagen invasion assay that has been previously described (63). No differences were observed in the ability of dp53R-shSCR or dp53R-shARF cells to invade through the collagen matrix (Figure 3.8 B). Finally, it was recently shown that ARF could inhibit angiogenesis and tumor cell invasiveness by upregulating the mRNA

expression of the tissue inhibitor of metalloproteinase-3 (TIMP3) protein (62). When we depleted dp53R cells of ARF, we did not observe an appreciable decrease in TIMP3 expression, indicating it is unlikely ARF is functioning to promote TIMP3 mRNA expression in our system (Figure 3.8 C).

## DISCUSSION

In recent years, a much greater appreciation has been given to ARF's p53-independent functions in the cell. These are essentially defined as signaling networks ARF participates in that do not require p53. Extensive work has uncovered a role for ARF in regulating ribosome biogenesis, sumoylation, transcription, and even the metabolic stress response called autophagy (44). Many of these functions have been experimentally determined by overexpressing ARF in p53-deficient cells that already contain high ARF levels. ARF is fairly promiscuous with its interaction partners due to its high arginine content and a pI above 11 (44). Achieving non-physiological levels of ARF by exogenous overexpression may not be an accurate assessment of endogenous ARF functions. While much attention has been given to uncovering ARF-regulated processes that do not depend on p53, relatively little has been done to directly examine the tumor suppressor role (if any) in cells facing acute p53 loss. In this study, we wanted to uncover the p53-independent function that endogenous ARF was employing to limit the proliferation and tumorigenicity of cells encountering acute p53 loss, as described in Chapter 2. We were primarily focused on ARF's well characterized role in regulating ribosome biogenesis and mRNA translation (43). These functions have been demonstrated to be p53-independent processes that endogenous ARF regulates (2, 50).

We found that the induced levels of ARF following *p53* loss were appropriately localizing to nucleolar regions, and they were interacting with NPM. ARF's interaction with NPM has been reported to promote its stability, so it is tempting to speculate that this interaction might be contributing to the induced levels of ARF seen in *p53*-deficient cells (10). However, we observed no change in ARF's protein stability following *p53* loss in our system (Chapter 2, Figure 2.4). NPM is a ribosomal chaperone that actively shuttles between the nucleus and cytoplasm (32, 60). NPM, therefore, is required to ensure cytosolic ribosome levels remain sufficient to meet the translational demands of the cell. ARF can inhibit NPM function by sequestering it in the nucleolus, so observing a NPM-ARF interaction was in support of our hypothesis that ARF is limiting the tumorigenicity of *p53*-deficient cells by suppressing overall cellular growth.

Our initial cell growth measurements were also in support of this hypothesis. We observed a decrease in protein per cell, cytosolic ribosome content, and transcription of the 47S rRNA precursor following *p53* loss and subsequent ARF induction. These are in agreement with previous findings that identified ARF as being an important regulator of rRNA transcription, processing, and ribosome export (Reviewed in (43)). However, depletion of ARF in these *p53*-deficient cells did not rescue any of the above mentioned phenotypes, so the induced levels of ARF do not explain our observed reduction in cellular growth. In addition, NPM depletion significantly affected the proliferation and tumorigenicity of both shSCR and shARF expressing dp53 MEFs, suggesting enhanced NPM function was not specifically promoting the proliferation of cells depleted for ARF. Further experimentation actually revealed a flaw in our interpretation of the assays used to measure cell growth. Measurement of protein synthesis kinetics by pulsing cells with <sup>35</sup>S-methionine indicated cells that had lost *p53* were actually making protein at a

much faster rate than the wild-type controls. This finding was in agreement with our AgNOR staining that showed the dp53 cells had more AgNORs per nucleus.

How can cells that are making proteins at a faster rate have less protein per cell and fewer copies of the 47S rRNA precursor? We believe this phenomenon can be explained by proliferation differences between dp53 MEFs and wild-type controls. Under normal circumstances, cell proliferation (cell number) and cell growth (cell size) are tightly coupled such that cells appropriately accumulate enough protein to ensure daughter cells are roughly the same size (55). dp53 MEFs rapidly transit the cell cycle which means they spend shorter amounts of time in the growth phases (periods of macromolecular synthesis, G1 and G2) than their wild-type counterparts (see Chapter 2, Figure 2.7 A-C). Thus, when a “snap-shot” experiment is performed (i.e. protein/cell, 47S rRNA levels, ribosome profiling), we observe a decrease in these readouts. Like ARF, p53 can reportedly inhibit rRNA transcription. Furthermore, since *p53*-null so rapidly navigate the cell cycle, their nuclear envelope would be broken down much more frequently than wild-type cells. This would allow nuclear ribosomes to enter the cytoplasm without the chaperone function of NPM, and explains why the enhanced ARF-NPM interaction in this context does not seem to have a functional consequence. These assays are essentially “snap-shots” of an asynchronous population of cells. Cells that have lost important cell cycle checkpoints (e.g. p53) are able to navigate the cell cycle very rapidly compared to wild-type cells. Therefore, p53-deficient cells must ramp up protein synthesis to keep up with proliferative demands, but do not accumulate as much protein as wild-type cells. As a result, dp53 MEFs are smaller in size (Jason Forsys unpublished observation). In summary, while maintaining nucleolar function and protein synthesis are basal functions of ARF, the dramatic consequences of p53 loss

on cell cycle regulation appear to trump any anti-growth efforts stimulated by the induced ARF levels.

Having ruled out inhibition of cell growth as ARF's important p53-independent tumor suppressor function in our system, we tested other previously published roles of ARF. We showed that increased ARF levels as a result of p53 loss do not destabilize the de-sumoylating enzyme SENP3. It is believed that ARF and SENP3 compete for NPM binding, which promotes each of their stabilities. When ARF is overexpressed, it out-competes SENP3 for NPM binding, leading to the de-stabilization and subsequent reduction in SENP3 half-life (28). NPM is an extremely abundant protein, so even in p53-deficient cells that have increased endogenous ARF levels, the SENP3-NPM interaction must be sufficient to maintain steady-state levels of SENP3. We were also unable to validate the finding that ARF inhibits c-MYC transcriptional activation. We only analyzed two validated c-MYC target proteins, Nucleolin and Ornithine Decarboxylase, both of which are important for cellular growth and proliferation (16, 47). As c-MYC can reportedly trans-activate over 1000 genes, we cannot conclusively rule out an involvement of enhanced c-MYC function in the pro-tumorigenic phenotype of dp53 MEFs depleted for ARF (25). Finally, we observed no change in motility or invasiveness in the dp53R-shARF MEFs, indicating no role for these processes in the enhanced ability of these cells to grow in soft-agar and mice.

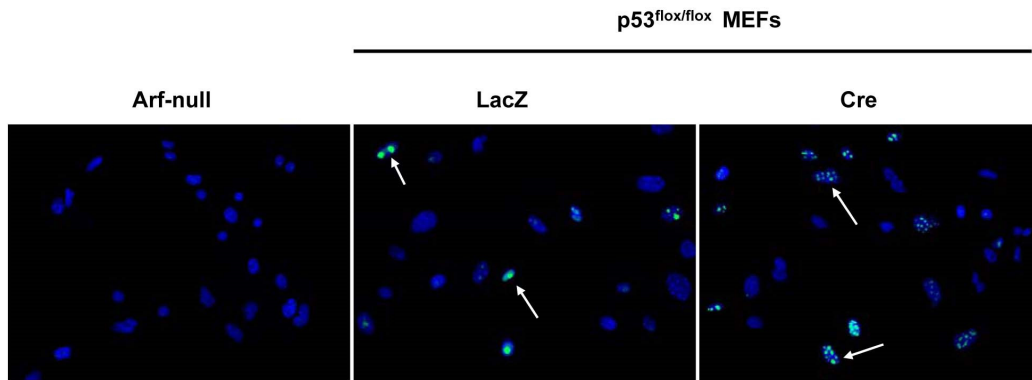
How is ARF able to inhibit proliferation/tumorigenicity of *p53*-deficient cells if not through the conventional p53-independent pathways? Our experimental efforts outlined in this chapter indicate that ARF is employing a novel p53-independent tumor suppressor function to limit the tumorigenicity of *p53*-deficient cells. We were able to show that many of the previously reported p53-independent functions of ARF are unlikely to suppress proliferation in a



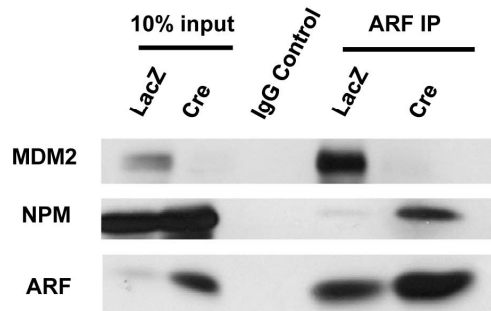
*p53*-null setting. Cautious interpretations should therefore be made in experiments utilizing gross overexpression of ARF. The model of acute *p53* loss that we have characterized will allow us to examine novel functions of ARF in a cell devoid of *p53*. As hypotheses based on previous findings have not proven fruitful, our future studies will utilize unbiased approaches to identify the role of ARF in the absence of *p53*. These analyses will ultimately provide insight into the selective pressures that *p53*-null cells face, and should help explain the perplexing observation of *p53* and ARF co-inactivation in human tumors.

## FIGURES

**A**



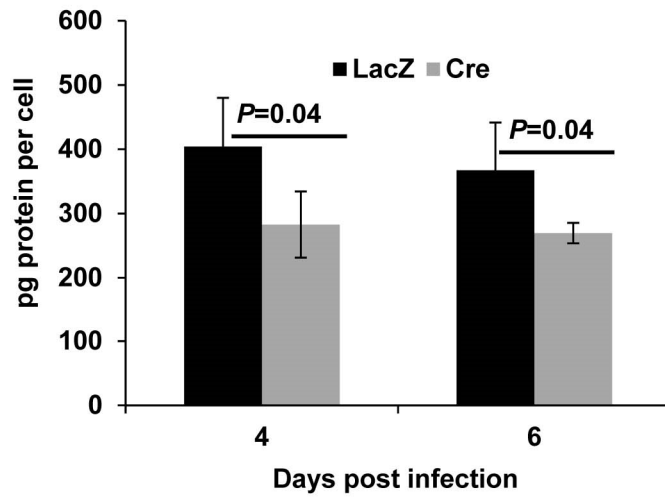
**B**



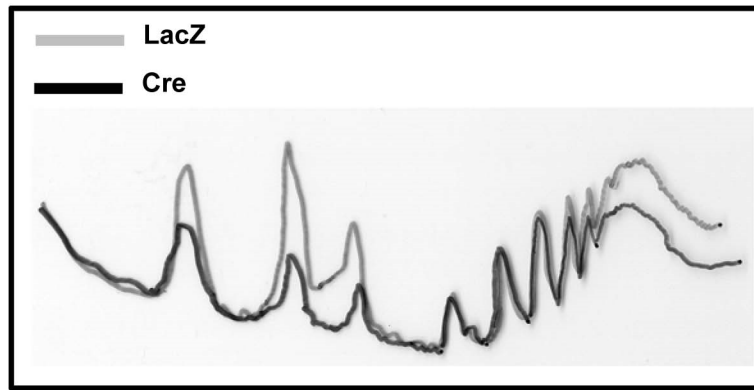
### **Figure 3.1** ARF localizes to nucleoli and interacts with NPM in *dp53* MEFs

(A) Equal numbers of *Arf*-null or *p53<sup>lox/lox</sup>* MEFs infected with Ad-LacZ or Ad-Cre were plated on coverslips and allowed to adhere overnight. Following MeOH/Formalin fixation, cells were stained for ARF (Green) or DAPI (Blue) to label nuclei. White arrows are indicating examples of cells exhibiting nucleolar localization of ARF. (B) Immunoprecipitation performed with Ad-LacZ or Ad-Cre infected *p53<sup>lox/lox</sup>* MEF whole cell lysate at t=6 days post infection. A rabbit polyclonal antibody against ARF was used for the IP, and normal Rabbit IgG was used as a control. 10% of the total protein used in the IP's was loaded as positive controls for Western blotting.

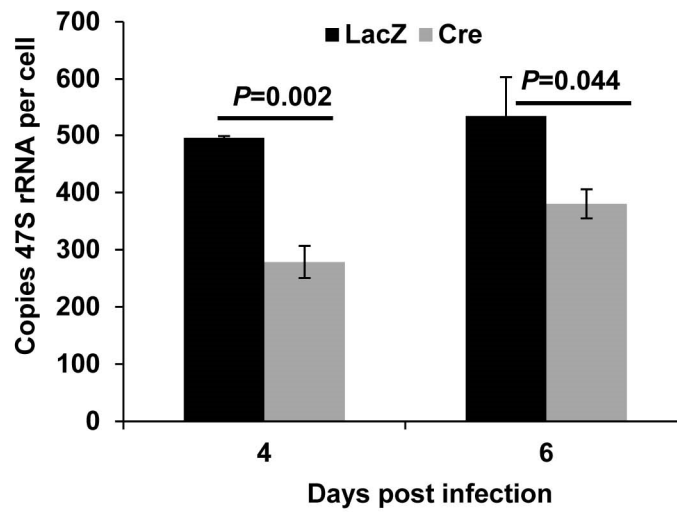
**A**

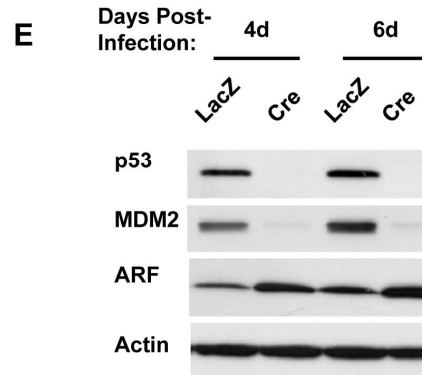
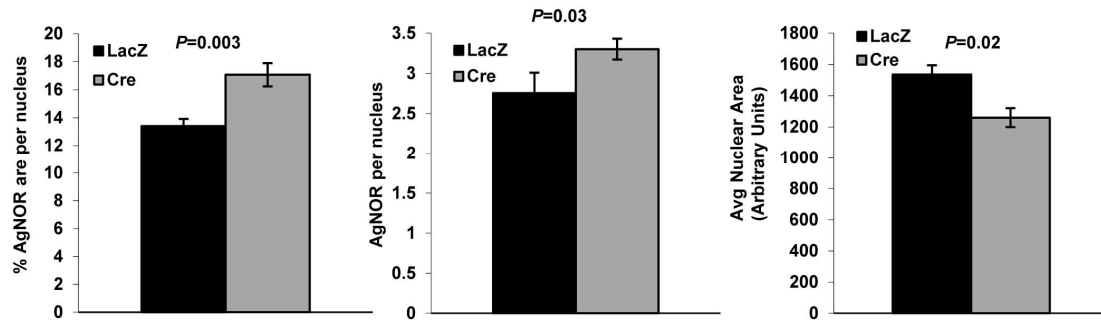


**B**



**C**

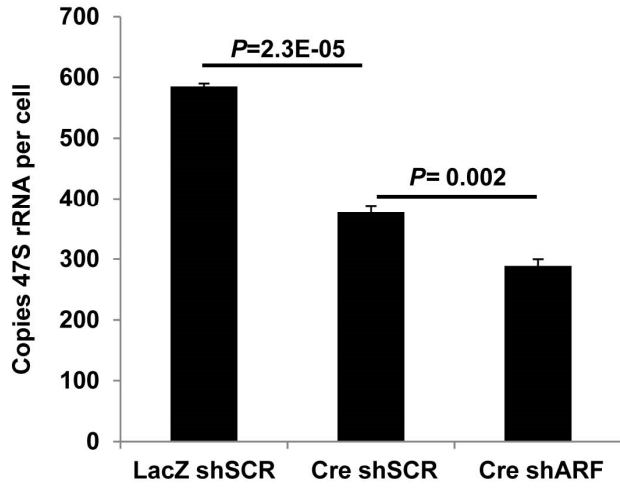
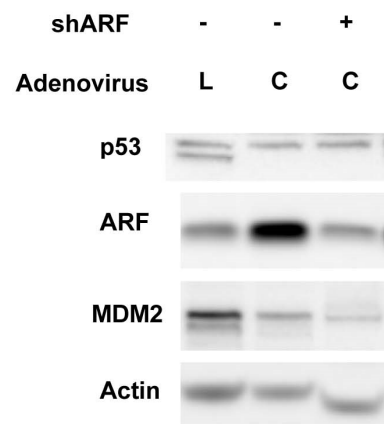
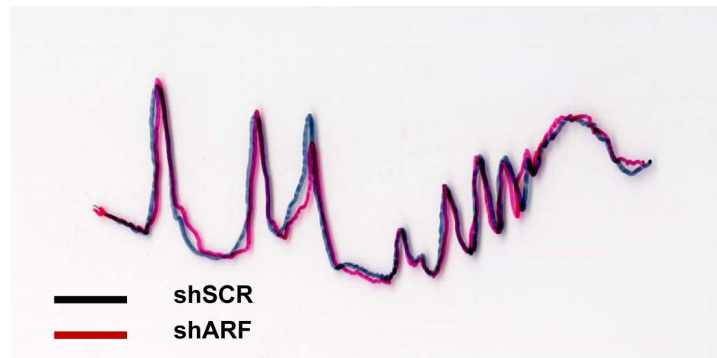
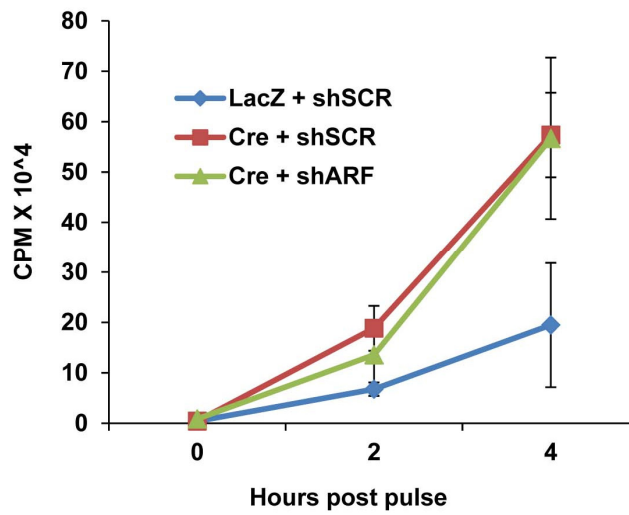


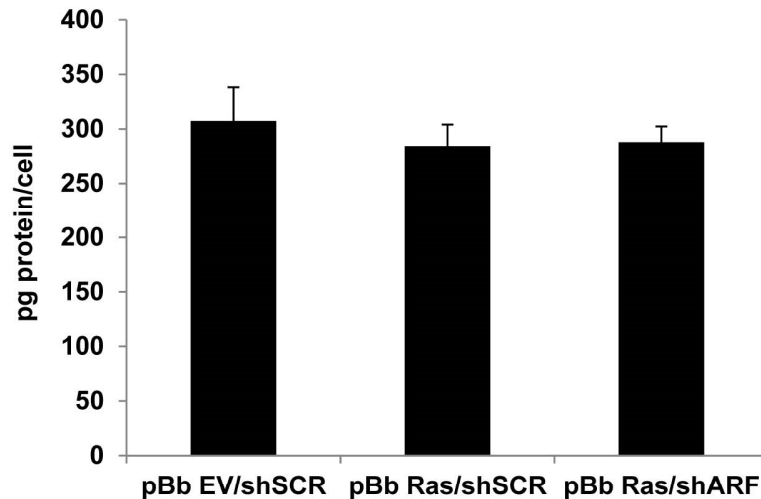
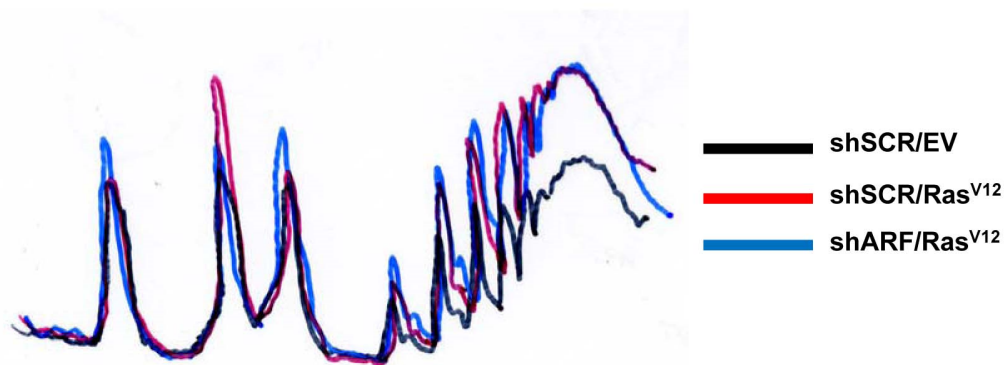
**D**

**Figure 3.2 dp53 MEFs display reduced readouts of overall cellular growth.**

(A) Equal numbers of  $p53^{lox/lox}$  MEFs infected with Ad-LacZ or Ad-Cre were lysed and total protein was quantified using a BioRad DC protein assay. Data was plotted as pg protein per cell and error bars represent s.d. of three independent measurements. (B) Ad-LacZ (Grey) or Ad-Cre (Black) infected  $p53^{lox/lox}$  MEF cytosolic ribosome content was analyzed by ultracentrifugation of cytoplasmic lysates in a sucrose gradient. Gradients were fractionated and RNA absorbance was measured at 254 nm. Plot is representative of three independent experiments. (C) Total RNA isolated from equal numbers of Ad-LacZ or Ad-Cre infected  $p53^{lox/lox}$  MEFs was analyzed by SYBR-green qRT-PCR for expression of the precursor rRNA 47S transcript. Results are plotted as the mean copy number of 47S per cell for  $n=3$ . (D)

Nucleolar morphology and number were measured by AgNOR staining. All values represent s.d. of means from three independent measurements of >100 nuclei. **(E)** Western blotting of LacZ or Cre infected *p53<sup>flox/flox</sup>* MEFs for the indicated proteins. Whole cell lysates were obtained 4 or 6 days post Adenoviral infection.

**A****B****C****D**

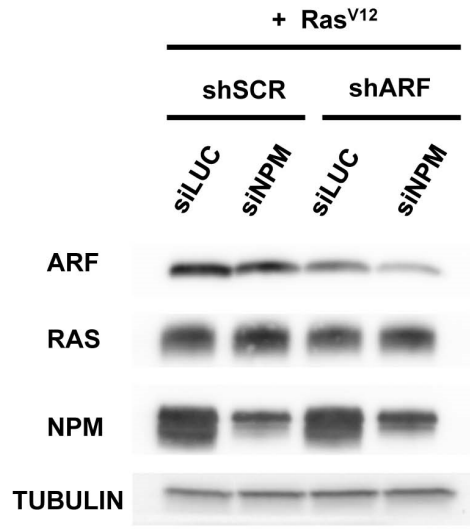
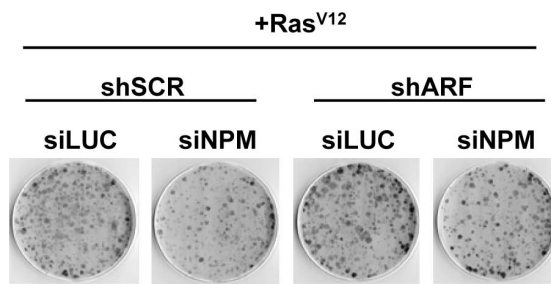
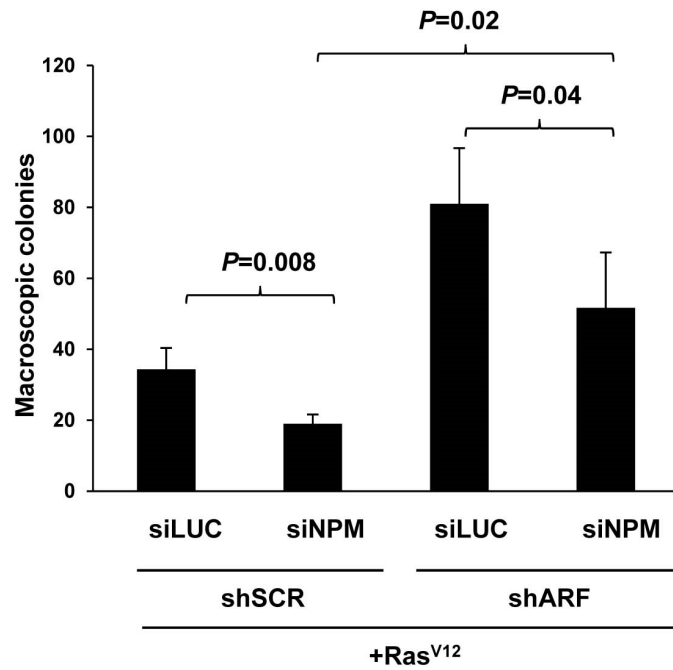
**E****F**

**Figure 3.3 Induced endogenous ARF levels in dp53 MEFs do not inhibit cellular growth.**

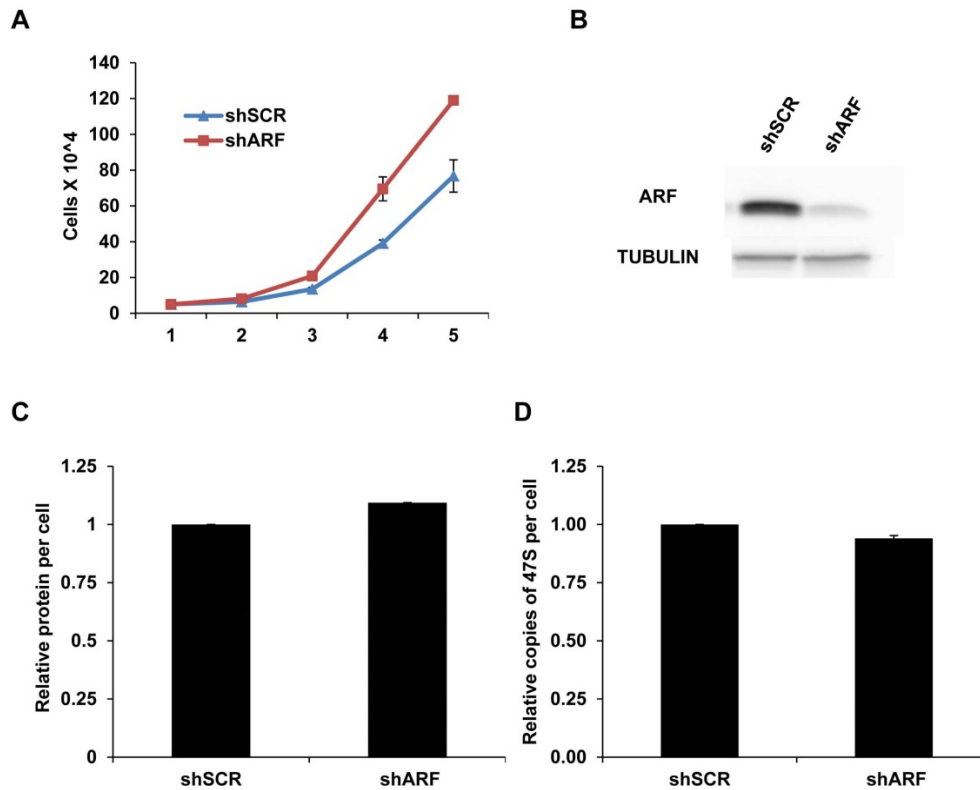
(A) Equal numbers ( $1.5 \times 10^6$ ) of LacZ or Cre treated  $p53^{lox/lox}$  MEFs infected with shRNAs targeting ARF (shARF) or a scrambled control (shSCR) were harvested and total RNA was extracted. Analysis of 47S precursor rRNA levels was performed using SYBR-green qRT-PCR. Results of copy number/cell are shown as means  $\pm$  s.d. for three independent measurements. (B) Western blot analysis of the cells described in (A) showing efficient excision of p53 and ARF knockdown. (C) Cytosolic ribosome fractionation was performed on dp53 MEFs expressing shSCR (Black) or shARF (Red) 4 days post infection. The trace is representative of three

independent experiments. **(D)** Cells with the indicated treatments were pulsed with  $^{35}\text{S}$ -Methionine for various amounts of time to monitor newly synthesized proteins. Equal numbers were lysed, protein was precipitated with Trichloroacetic acid, and counts per minute were obtained by liquid scintillation counting. Error bars represent s.d. of  $n=3$ . **(E)** dp53 MEFs expressing empty-vector or oncogenic *Ras*<sup>V12</sup> (dp53R) were infected with shSCR or shARF. Whole cell lysates from equal numbers of cells were obtained 4 days post-infection and total protein was analyzed using a BioRad DC Protein Assay. Results are displayed as pg/cell and error bars represent s.d. of  $n=3$ . **(F)** Cytosolic ribosome fractionation of the cells described in (E). The trace is representative of fractionations from three independent experiments.

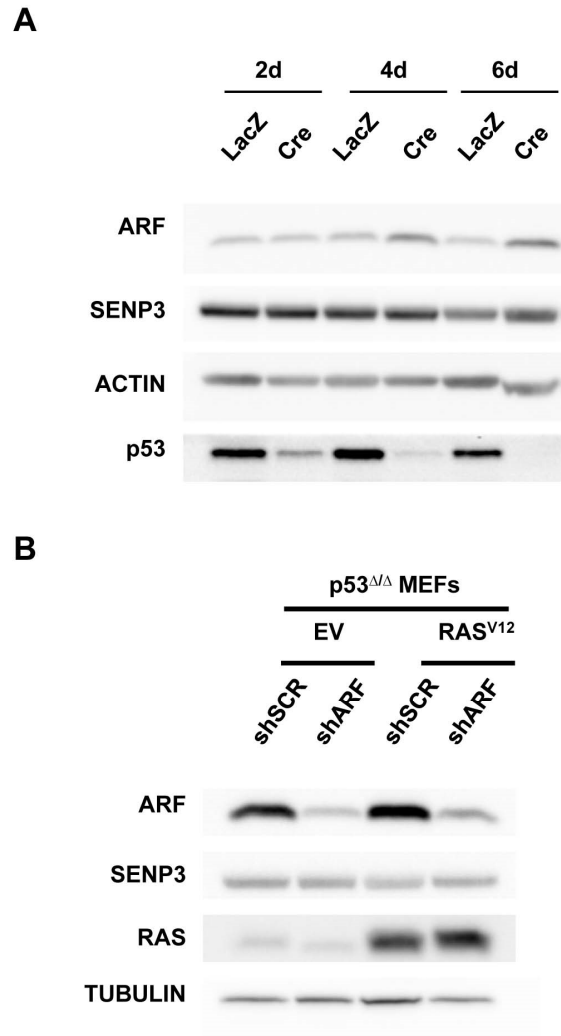


**A****B****C**

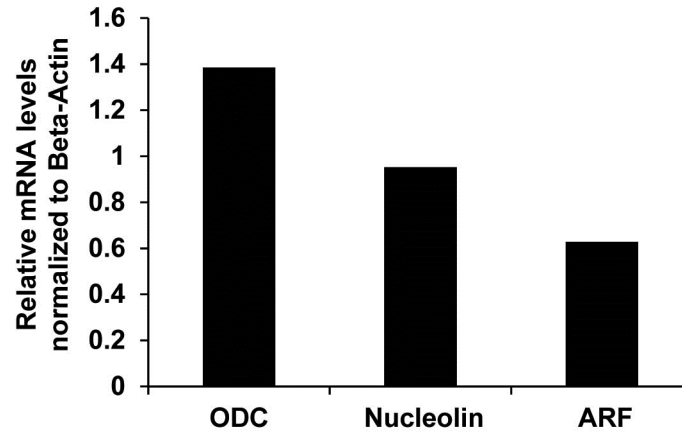
**Figure 3.4 dp53R-shARF MEFs are not uniquely dependent on NPM function for their enhanced tumorigenic phenotype.** (A) dp53R MEFs were first infected with shSCR or shARF. Following selection for the expression of these hairpins, the cells were infected with shLUC or shNPM. Western blot analysis from whole cell lysates was performed 4 days post-infection. (B) Long-term proliferation analysis was performed with cells described in (A).  $3 \times 10^3$  cells were plated in 100mm tissue culture dishes and grown for 10 days. Colonies were stained with Giemsa. Pictures are representative of three independent experiments. (C) Cells ( $1.5 \times 10^4$ ) described in (A) were plated in soft agar and incubated for three weeks. Colonies were stained with crystal violet and quantified manually. Data represents mean  $\pm$  s.d. of three independent experiments.



**Figure 3.5** ARF does not inhibit cellular growth of dp53 MEFs prior to their enhanced proliferation. **(A)** Equal numbers of dp53 MEFs expressing shSCR or shARF were plated on 6-well dishes in triplicate and counted on the indicated days post infection. Counts were performed using a hemocytometer and trypan blue exclusion. Error bars represent s.d. of  $n=3$ . **(B)** Western blot analysis of cells described in (A) at  $t=48$  hours (2 days) post-infection. **(C)** Equal numbers of dp53 MEFs expressing shSCR or shARF were harvested and lysed 2 days post-infection. At this time point, no increase in proliferation is evident. Protein in whole cell lysates was quantified using a DC protein assay. Data is plotted as relative protein per cell with the shSCR cells set to 1. Error bars represent s.d. of  $n=2$ . **(D)** Cells were treated and harvested exactly as described in (C). Total RNA was harvested from equal numbers of cells and 47S rRNA levels were quantified using SYBR-green qRT-PCR. Error bars represent s.d. of  $n=2$ .



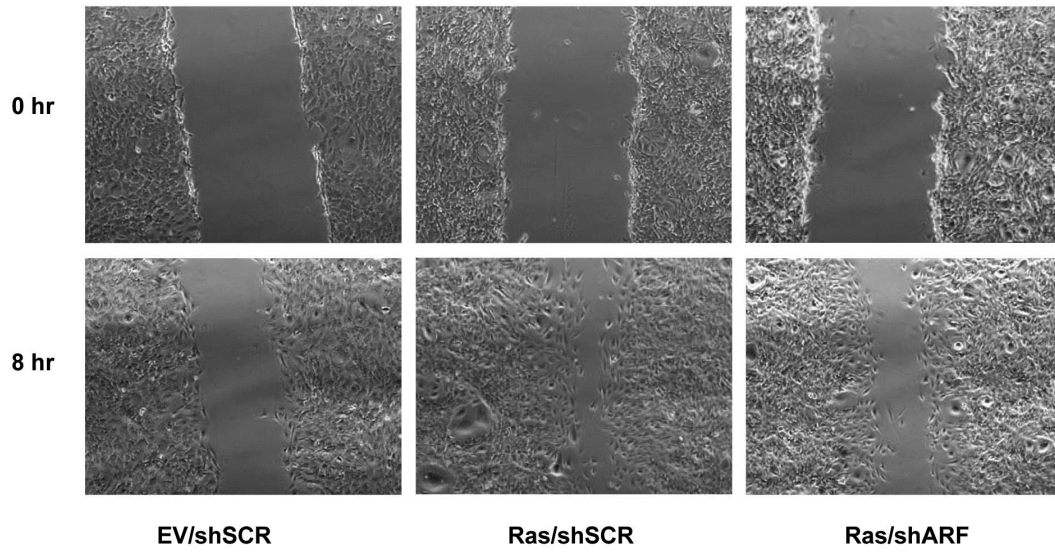
**Figure 3.6** Increased ARF levels do not dictate expression of the de-sumoylating enzyme SENP3 in dp53 MEFs. (A) Western blot analysis of  $p53^{lox/lox}$  MEFs infected with Ad-LacZ or Ad-Cre for the indicated number of days. (B) Western blot analysis of dp53 MEFs expressing empty-vector or  $Ras^{V12}$  infected with shSCR or shARF.



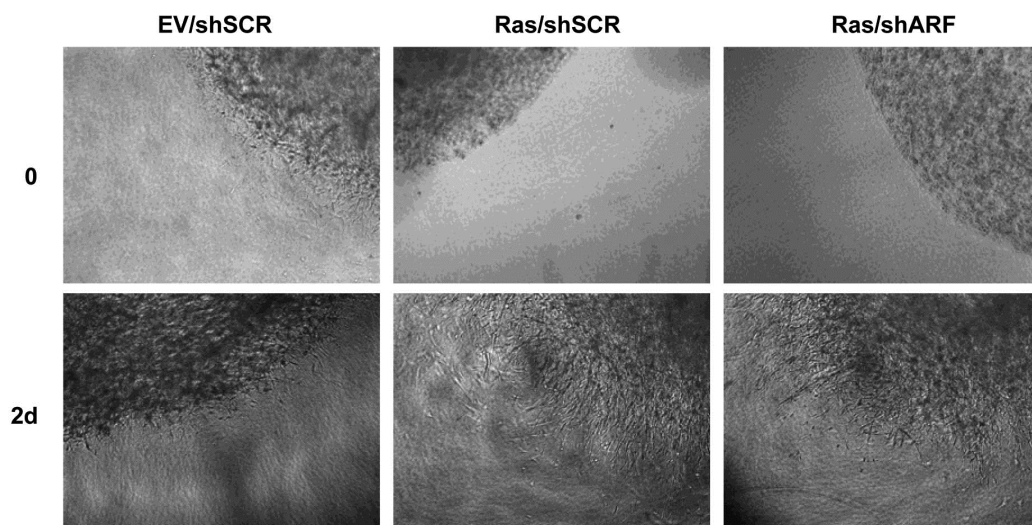
**Figure 3.7** ARF does not inhibit c-MYC transcriptional activity in dp53 MEFs.

Quantitative real-time PCR analysis of known c-MYC transcriptional targets was performed on total RNA extracted from dp53R-shSCR or dp53R-shARF MEFs 4 days after shRNA infection. Expression was normalized to  $\beta$ -Actin mRNA levels and is relative to shSCR expressing controls.

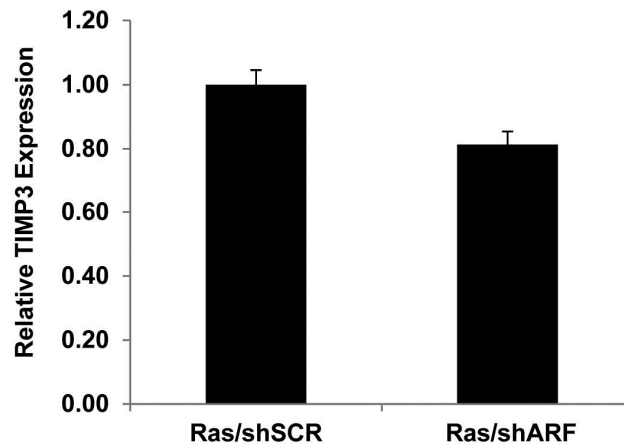
**A**



**B**



**C**



**Figure 3.8 ARF does not inhibit the motility or invasiveness of p53-deficient cells.**

(A) dp53 MEFs expressing empty-vector or *Ras*<sup>V12</sup> were infected with shRNAs targeting ARF or a scrambled control. Cells ( $8 \times 10^5$ ) were plated in 60mm dishes and allowed to adhere overnight. A scratch was made down the center of the dish with a p200 pipette tip and images were analyzed immediately following scratching or 8 hours later to observe cell motility. Light microscopy images are representative of three independent experiments. (B) Cells ( $1 \times 10^5$ ) described in (A) were embedded in type I collagen and invasion was monitored using light microscopy. An image was taken at  $t=0$ hr and 24 hr later to observe the movement of cells away from the cell “sphere.” (C) qRT-PCR analysis was performed to observe TIMP3 expression levels in dp53R-shSCR and dp53R-shARF MEFs. Expression was normalized to histone 3.3 mRNA and relative to shSCR controls.

<b>Gene</b>	<b>Company</b>	<b>Catalogue #</b>	<b>Dilution</b>
<b>p19ARF (mouse)</b>	<b>Santa Cruz</b>	<b>sc-32748</b>	<b>1:1000</b>
<b>p53 (mouse)</b>	<b>Cell Signaling</b>	<b>2524</b>	<b>1:1000</b>
<b>MDM2</b>	<b>Millipore</b>	<b>OP115</b>	<b>1:500</b>
<b>Actin</b>	<b>Santa Cruz</b>	<b>sc-8432</b>	<b>1:500</b>
<b>Gamma tubulin</b>	<b>Santa Cruz</b>	<b>sc-7396</b>	<b>1:1000</b>
<b>H-Ras</b>	<b>Santa Cruz</b>	<b>sc-520</b>	<b>1:2000</b>
<b>NPM</b>	<b>Invitrogen</b>	<b>325200</b>	<b>1:10000</b>
<b>SENP3</b>	<b>Cell Signaling</b>	<b>5591</b>	<b>1:1000</b>

**Table 3.1. List of primary antibodies used for Western blot**



<b>ARF</b>	<b>FWD</b>	<b>5'-GAG TAC AGC AGC GGG AGC AT-3'</b>
	<b>REV</b>	<b>ATC ATC ATC ACC TGG TCC AGG ATT CC</b>
<b>ODC</b>	<b>FWD</b>	<b>GGG TGA TTG GAT GCT GTT TG</b>
	<b>REV</b>	<b>TCT GGA TCT GCT TCA TGA GTT G</b>
<b>Nucleolin</b>	<b>FWD</b>	<b>GAG GTG GAA GAG GTG GAT TTG</b>
	<b>REV</b>	<b>AGG GAA AGA ATG GGA TGG AAG</b>
<b>TIMP3</b>	<b>FWD</b>	<b>TGA AGG CAA GAT GTA CAC AGG</b>
	<b>REV</b>	<b>GAG GTC ACA AAA CAA GGC AAG</b>

**Table 3.2. List of mouse specific primers used for qRT-PCR**

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## **Chapter 4:**

# **ARF and p53 coordinate tumor suppression of an oncogenic IFN- $\beta$ -STAT1-ISG15 signaling axis**

## ABSTRACT

The ARF and p53 tumor suppressors function in a linear genetic pathway to provide defense against oncogenic signaling. In Chapter 2 of this dissertation, we uncovered a novel role for the ARF tumor suppressor in limiting tumorigenic potential of cells that have lost p53-function. We proceeded to demonstrate, in Chapter 3, that the previously characterized p53-independent functions of ARF could not explain our observed increase in tumorigenicity in p53-deficient cells that were depleted of ARF. Here, we uncover mechanistic details on how ARF suppresses tumorigenesis in a completely p53-independent manner. As a result of *p53* loss, cells activate a type I interferon response pathway mediated by interferon-beta and the STAT1 transcription factor. ARF protein levels accumulate following *p53* loss and potently inhibit the interferon response. Depleting ARF leads to uncontrolled signaling through STAT1 which directly promotes an increase in proliferation. We further show that a downstream target of STAT1, the ubiquitin like protein ISG15, is absolutely required for these gains in proliferation. As proof of principle, human triple-negative breast cancer (TNBC) tumor samples with co-inactivation of p53 and ARF exhibit high expression of both STAT1 and ISG15, and TNBC cell lines are sensitive to STAT1 depletion. We propose that loss of p53 function and subsequent ARF induction creates a selective pressure to inactivate ARF, and propose that tumors harboring co-inactivation of ARF and p53 would benefit from therapies targeted against STAT1 and ISG15 activation.

## INTRODUCTION

A hallmark of cancer cell growth is their ability to traverse the cell cycle in an unregulated manner (23). To accomplish this feat, cancer cells must inactivate two tumor suppressor pathways mediated by p53 and the Retinoblastoma protein (Rb). The p53 tumor suppressor protein, coined as the “guardian of the genome” ten years ago, is responsible for maintaining genomic integrity (29). In this role, p53 responds to DNA damage that is sensed by upstream protein kinases named ataxia–telangiectasia mutated (ATM) and ataxia–telangiectasia and Rad3-related (ATR) (36). ATM/ATR dependent phosphorylation of the E3 ubiquitin ligase for p53, MDM2, leads to degradation of MDM2 and subsequent stabilization of p53 (58). Release of proteasome-mediated decay allows p53 to activate transcription of cell cycle inhibitors like p21 or pro-apoptotic proteins such as Puma and Noxa (17, 38, 41). Thus, p53 insures proper genomic integrity before allowing a cell to transit the cell cycle. If the damage is too severe, p53 activates pro-apoptotic programs to clear the cell from the organism. Other forms of cellular stress such as hypoxia, reactive oxygen species, telomere shortening, or oncogenic stress, also lead to activation of p53, underscoring the immense selective pressure cancer cells face to mutate the *TP53* gene (30).

Rb, on the other hand, directly influences the cells ability to enter the cell cycle by sequestering the E2F-DP transcription factor complex. The E2F-DP complex is required for activation of genes involved in DNA replication and cell cycle promotion, so by inhibiting this complex, Rb effectively decides when a cell can begin traversing the cell cycle (21). When appropriate growth factor signals are present, the CyclinD/CDK4/CDK6 complexes phosphorylate Rb leading to the release of E2F-DP (20).



Interestingly, two important regulators of p53 and Rb are encoded by a single genomic locus, *CDKN2A*. The *CDKN2A* locus, located on the short arm of chromosome 9 (*9p21*), was initially studied because it encodes a cyclin dependent kinase inhibitor, p16<sup>Ink4a</sup> (54). p16 can inhibit the interaction between CDK4/6 and Cyclin D, thus maintaining Rb in a hypophosphorylated state (50). A second protein product was discovered by Quelle et al, when they observed an alternate transcript with a distinct first exon and promoter, splicing into exon 2 of p16 (47). This transcript encodes a 169 amino acid nucleolar protein with absolutely no similarity to p16 because exon 2 is translated in an alternate reading frame. ARF, as it was aptly named, was also shown to possess potent cell cycle inhibitory functions, but these did not require Rb. Rather, ARF-mediated cell cycle arrest is dependent on the sequestration of MDM2 in the nucleolus, which stabilizes the short-lived p53 protein and its downstream transcriptional activity (61, 67).

ARF's canonical role as a tumor suppressor is to act as an intracellular sensor of oncogenic stress. Oncogenic stimuli such as RAS, c-MYC, v-ABL, or E1A overexpression leads to an induction of ARF, increased p53 stability, and subsequent activation of an irreversible exit from the cell cycle known as oncogene-induced senescence (8, 11, 12, 43, 71). This response requires ARF's ability to interact with MDM2. Increased activity of the E2F-DP complex can also induce ARF by binding to canonical E2F binding sites in the ARF promoter, thus providing a functional link between Rb and p53 (4). Therefore, ARF functions as a deterrent against hyperproliferative signals, and initiates permanent cell cycle arrest in cells harboring these abnormalities.

In the years that followed ARF's initial discovery, it became evident that the ARF-p53 pathway is more complex than described above. Cells lacking *p53* contain elevated levels of

ARF, and this is due to p53-mediated repression of the *Arf* locus (47, 59, 71). p53 first recruits the histone deacetylase, HDAC1, whose activity leads to the further recruitment of polycomb repressive complex 2 (PRC2). PRC2 members, SUZ12 and EZH2, promote the tri-methylation of H3K27, shutting down access of transcription factors to the region (69). Why would p53 inhibit expression of ARF? One explanation is that this feedback loop helps a cell turn off a p53 response if, for instance, a pulse in oncogenic activity has subsided. By lowering ARF levels, p53 would be able to free up its own negative regulator, MDM2, thus turning off the pathway. Alternatively, ARF and p53 might cooperate to promote some important tumor suppressor function, such that loss of *p53* would lead to an induction of the “backup,” ARF. For this hypothesis to be true, ARF would need to possess tumor suppressor functions independent of p53.

Indeed, mouse models have provided direct evidence that ARF possesses important p53-independent functions. Mice lacking *p53* develop primarily lymphomas by 6 months of age (13). Mice deficient for both *p53* and *Arf* are also susceptible to lymphoma development on the same time scale, but these mice frequently develop multiple primary tumors, including carcinomas that have never been observed in *p53*-null mice (66). This phenotype suggests loss of *Arf* in a *p53*-null background drives the formation of malignant tumors that normally do not have sufficient time to develop in a pure *p53*-null mouse.

Numerous groups have published p53-independent functions of ARF, primarily utilizing genetically unstable cell lines and enforced expression of ARF (55, 56). It is still unclear which reported functions of ARF are physiologically relevant. ARF is a “sticky” protein, containing 20% arginine residues and an isoelectric point above 12 (55). Gross overexpression often leads to misleading interactions that might not normally occur *in vivo*, making interpretation of these

studies difficult. Thus, there is an ongoing debate in the field of ARF biology over which, if any, reported p53-independent functions of ARF are relevant to human cancers. ARF and p53 co-inactivation in human tumors is frequently observed, which suggests a selective pressure to silence both ARF and p53 exists in cancer cells (3, 44, 53, 64). Therefore, a detailed understanding of this genetic abnormality would provide new therapeutic options to patients harboring these defects.

We previously showed that a cell culture model of acute *p53* loss led to a robust induction of ARF protein capable of limiting the proliferation and tumorigenicity of these cells (Chapter 2). While this data convincingly showed ARF possesses p53-independent tumor suppressive functions, we were unable to mechanistically explain this observation based on previous literature (Chapter 3). Here we show that a key p53-independent function of ARF is the inhibition of a pro-tumorigenic type I interferon response pathway. This pathway is induced by the secretion of interferon-beta and propagated by the STAT1 transcription factor. We extended these findings to human cancer samples, and show that triple-negative breast cancers are also under a selective pressure to inactivate both p53 and ARF, leading to an upregulation of the interferon response pathway. The identification of tumor promoting activities within this pathway provides a novel therapeutic avenue for treating patients harboring ARF and p53 genetic abnormalities.

## MATERIALS AND METHODS

**Mice and cell culture.** *p53<sup>lox/lox</sup>* (FVB.129-*Trp53<sup>tm1Brn</sup>*) were obtained from the NCI Mouse Repository and have been previously described (25). Primary mouse embryonic fibroblasts were isolated as previously described (26). All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2mM glutamine, 0.1mM nonessential amino acids, 1mM sodium pyruvate, and 2ug/mL gentamicin. Recombinant IFN- $\beta$  was obtained from PBL Interferon Source and used at the indicated concentrations.

**Viral production and infections.** Adenoviruses expressing  $\beta$ -galactosidase (Ad-LacZ) or Cre recombinase (Ad-Cre) were purchased from the Gene Transfer Vector Core, University of Iowa. For Adenoviral infections,  $1 \times 10^6$  cells were plated in the presence of Ad-LacZ or Ad-Cre (MOI=50) and incubated for 8 hours. For mutant Ras<sup>V12</sup> overexpression, retrovirus was produced by transfecting 293T cells with either MSCV-HRAS<sup>V12</sup>-IRES-GFP plasmid or MSCV-IRES-GFP control, and the helper plasmid  $\psi$ -2. Virus-containing supernatants were harvested 48 hr post transfection. Collected retrovirus was used to infect  $1 \times 10^6$  MEFs in the presence of 10ug/mL polybrene. The pBabe-Myr-Akt1 plasmid was obtained from Dr. Loren Michel at Washington University in St. Louis. For the production of Lentiviral shRNAs, 293T cells were transfected using Lipofectamine 2000 (Invitrogen) with pCMV-VSV-G, pCMV- $\Delta$ R8.2, and pLKO.1-puro constructs. Viral supernatants were harvested 48hrs post transfection. Cells were infected with lentivirus for 8-12 hours in the presence of 10 ug/mL protamine sulfate. Puromycin was added to cell culture media at a concentration of 2 ug/mL for selection. The

sequences of shRNAs are found in Table 4.1. The ARF (mouse specific) hairpin was described previously (2).

**Western Blotting.** Cell pellets were lysed and sonicated in EBC lysis buffer (50 mM Tris-Cl, pH7.4, 120mM NaCl, 0.5% NP-40, 1mM EDTA) containing HALT Protease and Phosphatase Inhibitor cocktail (Thermo Scientific) and 1mM phenylmethylsulfonyl fluoride (PMSF). Thirty  $\mu$ g of protein were separated on SDS-polyacrylamide gels. Proteins were transferred to PVDF (Millipore) and probed with antibodies. Primary antibodies used in this study can be found in Table 4.2. The mouse ISG15 antibody was a gift from Dr. Deborah Lenschow. Secondary horseradish peroxidase conjugated antibodies (Jackson Immunoresearch) were used and ECL plus was used to visualize the bands (GE Healthcare).

**Proliferation, BrdU and Foci Assays.** For proliferation assays,  $5-10 \times 10^4$  cells were plated in 6-well plates. Cells were lifted and counted using a hemocytometer at the indicated number of days post plating. For BrdU assays,  $1 \times 10^4$  cells were plated on glass coverslips and incubated overnight.  $10 \mu$ M BrdU-containing media was added to the cells for 4-6 hours. Cells were fixed with 10% formalin/methanol and BrdU staining was performed using an Anti-BrdU antibody (GE Healthcare) according to the manufacturer's instructions. For foci assays,  $3 \times 10^3$  cells were plated in 10cm dishes and cells were incubated for 10 days. Cells were fixed with 100% methanol and stained with Giemsa (Sigma Aldrich).

**Soft Agar Assay.** Cells were lifted and suspended in DMEM containing a final concentration of 0.4% Noble agar.  $1.5 \times 10^4$  cells were layered in triplicate onto 0.6% noble-agar/media bottom

layer in 60mm plates. Plates were incubated for 20 days, feeding with media/0.4% agar mix every 6 days. Macroscopic colonies were visualized by staining with 0.005% Crystal Violet solution and colonies  $\geq 0.5$ mm were manually counted.

**Microarray analysis.** RNA was isolated from dp53R-shSCR or dp53R-shARF MEFs using a Nucleospin RNA II Kit (Clontech). RNA samples from three independent experiments were submitted to the Genome Technology Access Center at Washington University School of Medicine for microarray analysis. Affymetrix Gene 1.0ST Arrays were used and data was processed in Affymetrix Expression Console (Affymetrix version) using RMA(Robust Multi-chip Average) algorithm. Differential expression analysis was performed using Significant Analysis of Microarrays (SAM) and a list of differentially expressed genes exhibiting fold changes greater than 2 was generated. Pathway analysis was performed using MetaCore software (Thomson Reuters). The NCBI Gene Expression Omnibus accession number for the microarray data reported in this chapter is GSE48315.

**Quantitative Real-Time PCR.** qRT-PCR was performed as previously described (37). Fold change was measured using the  $\Delta\Delta C_T$  method. Primer sequences used for amplification are listed in Table 4.3.

**IFN- $\beta$  ELISA.** Cell culture supernatants were concentrated using Vivaspin columns (GE Healthcare) according to manufacturer's instructions. Mouse IFN- $\beta$  levels were measured using the Verikine Mouse Interferon Beta ELISA Kit (PBL Interferon Source) according to the manufacturer's instructions.

**Immunohistochemistry.** Annotated breast cancer tissue arrays were obtained from US Biomax Inc (Cat#BR1503a). Staining was performed using the Dako EnVision+ System-HRP (DAB) according to the manufacturer's instructions. Rabbit anti-p14ARF (Bethyl) and Mouse anti-ISG15 (Santa Cruz) were used at a 1:200 dilution. Quantification was performed by two separate individuals by blindly scoring staining intensity on a 0-3 scale, with 0 being no staining and 3 being strong widespread staining. A score of 0-1 was considered "low/no" staining and a score of 2-3 was considered "high."

**Statistical Analysis.** Data are presented as means  $\pm$  s.d. Statistical differences between groups were determined with *P*-values obtained using two-sided, unpaired Student's *t*-test. All data points represent *n*=3. All images presented as "representative" were completed a minimum of three times.

## RESULTS

### ARF inhibits an interferon-sensitive gene signature induced upon *p53*-loss

Having demonstrated that the induced levels of ARF in *p53*-deficient cells serve a tumor suppressive function (Chapter 2), and that many of the previously described *p53*-independent functions of ARF cannot explain this observation (Chapter 3), we decided to take an unbiased approach to identify changes in global mRNA expression between dp53R-shSCR (MEFs deleted for p53 expressing Ras<sup>V12</sup> and shSCR) and dp53R-shARF (MEFs deleted for p53 expressing Ras<sup>V12</sup> and shARF) cells. Comparative microarray analysis yielded numerous upregulated immune response genes in the dp53R-shARF cells, including *Irf7*, *Oasl2*, *Ifit3*, *Usp18*, *Mx2*, and *Isg15* (Figure 4.1 A and B). Similar results were obtained when we overexpressed a constitutively active form of AKT1 in dp53 MEFs and depleted ARF (Figure 4.2 A, E-F). AKT overexpression was not sufficient to transform the dp53 MEFs, but similar to dp53R MEFs, depleting ARF significantly enhanced short and long-term proliferation (Figure 4.2 B-D). Pathway analysis indicated that the gene signature was most strongly associated with the innate immune response or type I interferon (IFN) response (Figure 4.1 C). The interferon sensitive gene (ISG) expression changes were independently validated by qRT-PCR (Figure 4.1 D). A second shRNA targeting ARF also resulted in upregulation of the ISGs (Figure 4.3 A and B).

As an important control, we analyzed ISG expression in our cell lines following infection with the various viral constructs used in our experiments and compared to mRNA levels in “mock” infected cells (no virus). Retroviral infection with empty vector or *Ras<sup>V12</sup>* did not induce ISG’s, and lentiviral infection of *Arf*-null MEFs with shSCR or shARF also had no effect on ISG mRNA levels, ruling out an off-target effect of the short hairpin (Figure 4.4 A and B). The only genetic setting where ARF depletion induced ISGs was in the context of *p53*-deficiency (Figure



4.4 C). Furthermore, a comparison of three different low passage (< passage 6) wild-type and *Arf*-null MEF lines showed no increase in ISG expression (Figure 4.4 D). Finally, we used a different control hairpin targeting red fluorescent protein (shRFP) to show that our scrambled control was appropriate for these experiments (Figure 4.4 E). Taken together, these data show the infections performed on our cells cannot explain the observed ISG response following ARF knockdown. Rather, ARF is specifically inhibiting ISG expression and this effect is entirely dependent on a *p53*-deficient genetic setting.

Given ARF's ability to inhibit ISG expression exclusively in the context of *p53*-deficiency, we hypothesized that loss of *p53* might be the driving force behind upregulation of the ISGs, and the induction of ARF would then serve as a biological "brake" to suppress the response. To test this hypothesis, we analyzed ISG mRNA expression following infection of *p53<sup>fllox/fllox</sup>* MEFs with Ad-Cre or -LacZ control. As shown in Figure 4.5A, expression of ISG15 and OASL2 are induced at 4 and 6 days post *p53*-loss. The ISG induction was specific to loss of *p53* and not due to Adeno-Cre expression (Figure 4.6). Consistent with our hypothesis, at 8 days post *p53*-loss when ARF protein levels are maximally induced, we no longer observed a significant induction of the ISGs (Figure 4.5 A and B). The suppression of ISG15 and OASL2 expression 8 days post *p53*-loss was completely relieved when ARF-specific shRNA was introduced. Therefore, the negative feedback *p53* imposes on ARF exists to allow ARF to respond to acute *p53* loss by inhibiting an induction of ISGs.

### **IFN- $\beta$ is necessary and sufficient for increased tumorigenicity in dp53R-shARF MEFs**

Our microarray data and pathway analysis indicated an activation of the type I interferon response, or more specifically, response to interferon-beta (IFN- $\beta$ ). Interferon-Beta (IFN- $\beta$ ) is a

member of the type I interferon family that is most well-known for its anti-viral effects. Recognition of viral infection by virtually any nucleated cell results in the secretion of IFN- $\beta$ . Subsequent activation of downstream Jak-Stat signaling results in transcriptional activation of anti-viral genes which aid in preventing further infection(46).

We analyzed IFN- $\beta$  mRNA expression using qRT-PCR in our dp53R-shARF MEFs and consistently observed a 2-3 fold induction (Figure 4.7 A). Additionally, this 3-fold induction of IFN- $\beta$  mRNA resulted in a nearly 11-fold increase in IFN- $\beta$  secretion in the media containing dp53R-shARF cells as measured by ELISA (Figure 4.7 B). Another member of the type I interferon family, IFN- $\alpha$  was not induced in our samples (Figure 4.7 C). To determine the requirement of secreted IFN- $\beta$  for cell proliferation, we knocked down IFN- $\beta$  in dp53R-shARF cells. This resulted in a significant decrease in IFN- $\beta$  expression, phosphorylated STAT1, and mRNA expression of an ISG called ISG15 (Figure 4.8 A and B). Long-term proliferation was significantly impaired in cells with reduced IFN- $\beta$  (Figure 4.8 C and D), indicating a requirement for IFN- $\beta$  production in dp53R-shARF cells.

Next, we sought to determine if enhanced production of IFN- $\beta$  was sufficient to promote the aberrant proliferation of dp53R cells in the presence of high ARF levels. Using concentrations of recombinant IFN- $\beta$  that matched the concentration range detected in the media of dp53R-shARF cells, we were able to stimulate ISG expression to the same level seen in dp53R-shARF cells (Figure 4.9 A). Markedly, we observed a significant increase in long-term proliferation of dp53R cells that was comparable to that seen in dp53R-shARF cells (Figure 4.9 B and C). An increase in short-term proliferation was also observed (Figure 4.9 D). Therefore, IFN- $\beta$  production alone is sufficient to phenocopy activation of this signaling pathway and the proliferative gains associated with ARF knockdown in dp53R cells.

### **ARF represses a pro-tumorigenic STAT1-ISG15 signaling cascade**

Canonical IFN- $\beta$  signaling occurs upon ligand binding to the membrane receptors IFNAR1/2. Upon ligand binding, a conformational change allows autophosphorylation of receptor-bound JAK1 and TYK2 (40). The activation of these kinases leads to phosphorylation of STAT1 and STAT2 proteins, which enables them to enter the nucleus. Once inside the nucleus, the STAT1/STAT2 heterodimer associates with IRF9 to form a complex known as IFN-stimulated gene factor 3 (ISGF3) which is fully capable of initiating transcription of genes containing IFN-stimulated response elements (ISREs) (46). Many of the genes in our ISG signature contain interferon response elements (ISRE) in their promoters (52), and it is well established that activation of the STAT1 transcription factor is required for upregulation of ISRE-containing genes (48). Therefore, we analyzed STAT1 status in dp53R-shARF cells and observed increases in the phosphorylation of both Tyrosine-701 and Serine-727 activation sites as well as an accumulation of total STAT1 levels (Figure 4.10 A). Neither STAT3 activation nor increased expression of its upstream cytokine, IL-6, were observed in the same genetic context (Figure 4.10 C and D). The increase in total STAT1 was due to an increase in mRNA levels, consistent with the observation that the STAT1 promoter contains an ISRE (Figure 4.10 B) (70).

To test whether STAT1-mediated signaling was required for the enhanced tumorigenicity of dp53R-shARF MEFs, we used shRNAs to deplete STAT1. Reducing total STAT1 protein levels led to a concomitant decrease in phosphorylation in dp53R-shARF MEFs (Figure 4.11 A). As shown in Figure 4.11B, mRNA expression of select ISGs was also reduced following STAT1 knockdown. Short and long-term proliferation of dp53R-shARF MEFs was inhibited and colony growth in soft agar was reduced (Figure 4.11 C-E). Taken together, these data indicate that ARF

protects p53-deficient cells from inappropriate STAT1 activation, and left unchecked, signaling through STAT1 can lead to increased tumorigenicity.

Interestingly, one of the IFN responsive genes upregulated following ARF depletion, *Isg15*, encodes a ubiquitin like protein that is conjugated to lysine residues and has recently been shown to be required for the tumorigenicity of select breast cancer cell lines (7). Increased ISG15 expression in dp53R-shARF MEFs is dependent upon STAT1 (Figure 4.11 B), so we hypothesized ISG15 might represent one of the pro-tumorigenic targets activated downstream of STAT1. Western blot analysis confirmed upregulation of both free and conjugated species of ISG15 in dp53R-shARF cells (Figure 4.12). Using an shRNA specific to ISG15, we observed a significant reduction in soft agar growth, foci formation, and proliferation in the dp53R-shARF MEFs upon ISG15 knockdown (Figure 4.13 A-E), indicating that elevated ISG15 is required for the tumorigenesis of dp53R-shARF cells. Depletion of ISG15 with the shRNA in non-transformed dp53 MEFs had no effect on proliferation, ruling out the possibility of off-target effects (Figure 4.14 A-C).

### **Analysis of TNBC patient samples and cell lines**

We have demonstrated that ARF protein induced by *p53* loss protects against the tumorigenic accumulation of an IFN-sensitive gene signature in a mouse model system. To investigate whether this pattern of regulation was conserved in human cells, we focused on triple negative breast cancer because over 80% of these patients harbor p53 mutations (18). We performed immunohistochemical analysis on an annotated breast cancer tissue array and scored the triple-negative cores. Whereas elevated expression of ARF would be expected in the presence of p53 mutation, we observed that eleven of the thirteen samples with p53 mutation

exhibited low or no ARF staining, suggesting co-inactivation of both ARF and p53. Further, six of eleven tissues with both ARF and p53 loss of function displayed intense staining of STAT1 and ISG15 (Figure 4.15 A and B).

Finally, we analyzed a panel of five triple-negative breast cancer cell lines. The HCC70 cell line, which displayed high ARF protein expression, was resistant to STAT1 depletion (Figure 4.16 A-C). The other four cell lines, which did not express ARF, were all extremely sensitive to STAT1 depletion, displaying signs of cytotoxicity (Figure 4.16 A-D). The short hairpins targeting STAT1 did not reduce STAT3, a known promoter of breast cancer tumorigenesis (35) (Figure 4.16 E), confirming the selective requirement of STAT1 activation in controlling the proliferation of these cells.

## DISCUSSION

Since its discovery nearly 20 years ago, numerous studies have reported p53-independent functions of the ARF tumor suppressor protein (55). These mechanistic insights have helped shape our current understanding of a common genetic occurrence in human cancers—co-inactivation of ARF and p53. Unfortunately, due to the heterogeneous nature of cultured cell lines, many of these findings have been difficult to reproduce, prompting the question: what are the true p53-independent functions of ARF? By using a murine model of acute *p53* inactivation, we sought to identify *bona fide* ARF functions that limit proliferation and tumorigenicity of *p53*-deficient cells.

Previous results discussed in Chapter 2 indicated that p53 loss leads to the upregulation of ARF, and this large pool of protein is able to limit Ras-mediated transformation. By depleting ARF with an shRNA, we hoped to gain insight into the mechanism of tumorigenic suppression. We performed gene expression analysis and to our surprise, depletion of ARF in *p53*-deficient cells led to an induction of interferon sensitive genes (ISGs). Our observed ISG induction was highly reproducible and was not a consequence of viral transduction. Further analysis revealed the ISG response was being activated by increased Interferon- $\beta$  expression and secretion.

Interferon signaling is predominantly considered to be tumor suppressive, so these results were initially puzzling. For instance, high dose IFN therapy treatment has been investigated as a potential therapeutic and has proven beneficial in certain hematological cancers. However, these clinical applications of IFN utilize a second member of the type I IFN family known as IFN- $\alpha$  (19). In solid tumors such as breast and ovarian cancer, attempts to utilize IFN- $\beta$  in this regard have not been successful (34, 49). Ultimately, little is known about the role IFN- $\beta$  might play in the tumor environment, and several reports actually indicate it might serve to

promote survival of tumors. Tumor growth has previously been shown to be enhanced in response to low-levels of interferon, and a recent report suggests that autocrine IFN- $\beta$  signaling enhances the tumorigenicity of Ras-transformed cells (31, 63). Given that many tumors display activation of Jak-Stat signaling and overexpression of downstream target genes, it is certainly possible that interferon signaling may play dual roles depending on genetic context (6, 14, 45, 70).

In agreement with this hypothesis, we were able to show that IFN- $\beta$  was necessary to maintain the tumorigenic phenotype of dp53R-shARF cells and sufficient to promote the proliferation of dp53R cells that contain high levels of ARF. Treatment of dp53 cells with exogenous IFN- $\beta$ , therefore, phenocopied depletion of ARF. Downstream of IFN- $\beta$ , the STAT1 transcription factor appeared to be absolutely required for both the enhanced transcription of ISGs and the tumorigenic phenotype of dp53R-shARF cells. Similar to interferons, STAT1 is considered to be tumor suppressive (10, 27). It is involved in a cancer immunosurveillance pathway that protects against neoplastic growth (15). However, these effects are mediated by a member of the type II interferon family of cytokines, interferon-gamma (IFN- $\gamma$ ). IFN- $\gamma$ -STAT1 signaling activates gene sets which contain GAS (IFN-gamma activation sites) elements in their promoters. These are distinct binding sites from the interferon sensitive response elements (ISREs) that type I interferon inducible genes harbor (46). Paradoxically, STAT1 is frequently found to be overexpressed in human cancers (6, 14, 45). In support of this observation, several groups have recently shown that STAT1 can be tumor promoting (28, 33, 62, 68, 70). These contradictory findings may suggest a dual role for STAT1 in cancer cells. Perhaps initial progress to a cancer phenotype is subdued by the IFN- $\gamma$ -STAT1 signaling axis, but prolonged

signaling stimulates inflammation, a process that is now widely accepted to be tumor promoting (22).

We were also able to identify an important STAT1 transcriptional target that is required for the increased tumorigenicity in dp53R-shARF cells—the ubiquitin like protein, ISG15. ISG15 has been well characterized in the context of innate immunity. It is conjugated to lysine residues in target proteins, many of which are involved in the type I Interferon response to viral infection and can promote or inhibit their function (57). One proposed mechanism of enhancing protein activity is through blocking ubiquitin conjugation resulting in protein stability, but the true biological consequence of ISG15 conjugation is not well defined (32). Interestingly, ISG15 has been found to be frequently overexpressed in pancreatic, bladder, breast, and oral cancers (1, 5, 24, 60, 65). A recent report defines a critical role for ISG15 conjugation in the tumorigenicity of mutant *Ras*-containing breast cancer cells, suggesting inhibitors interfering with this process might be therapeutically beneficial (7).

Our data further demonstrated a novel collaboration of p53 and ARF in suppressing STAT1 signaling activation and subsequent ISG transcriptional activation. Therefore, we propose that loss of p53 leads to two important events; induction of ISG's and the induction of ARF protein levels. Once ARF protein levels reach maximal expression, the transcription of ISG's is inhibited. In these cells, deletion or mutation of the *Arf* locus would predict an upregulation of the IFN gene signature and a subsequent growth advantage. Therefore, our results suggest a selective pressure does exist to co-inactivate both ARF and p53, a phenomenon that occurs in numerous cancer types (9, 39, 51, 53).

Finally, we identified a subset of triple negative breast cancer patients harboring co-inactivation of ARF and p53 alongside overexpression of STAT1 and ISG15. Additionally,

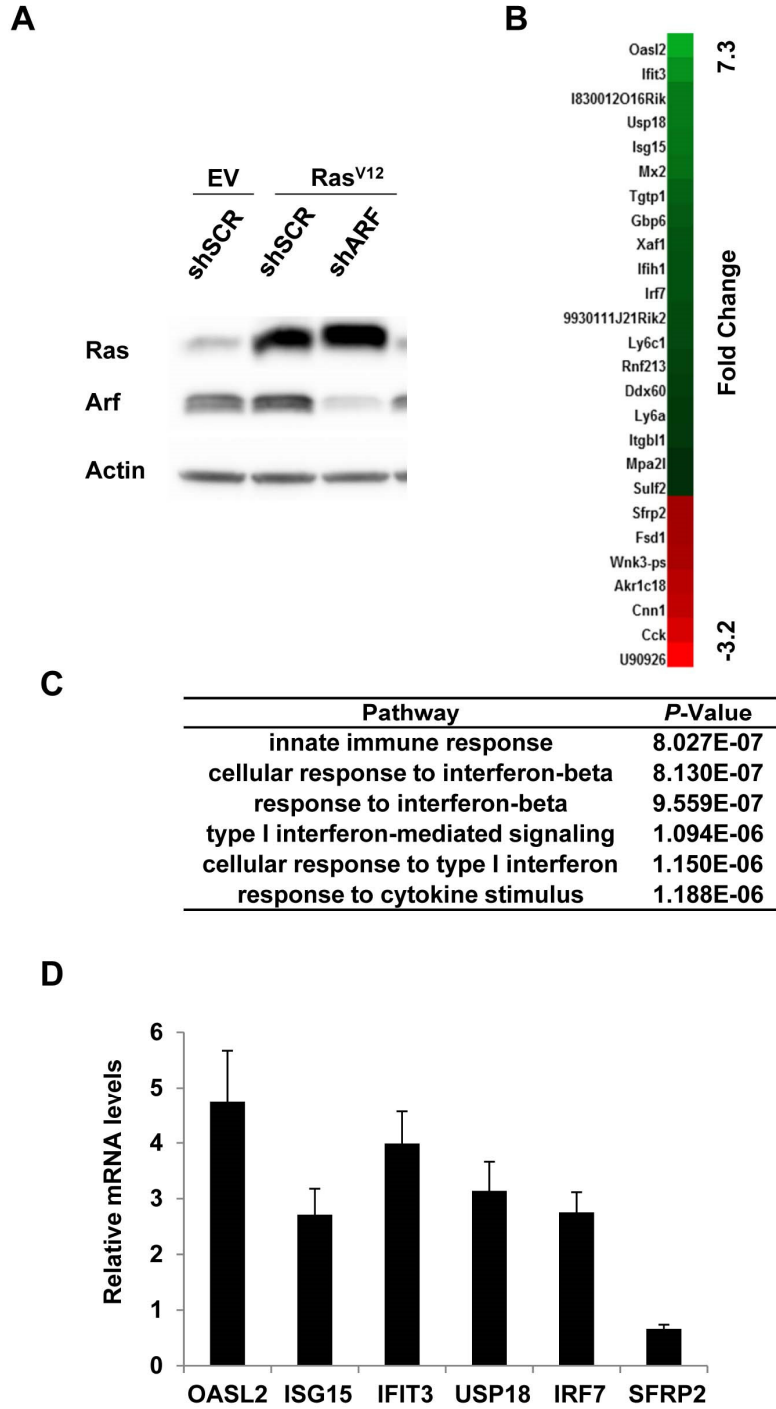


STAT1 depletion in a panel of p53-mutant TNBC cell lines showed that only cells lacking ARF expression were sensitive to the STAT1 shRNAs. Because existing mouse knockout models suggest that normal cells do not require the activity of STAT1 and ISG15 for viability (16, 42), targeted therapy of this pathway should be considered ideal for tumor reduction. Moreover, this IFN signaling axis need not be limited to triple negative breast cancer as numerous other cancers exhibit concomitant loss of function p53 and ARF.

The crosstalk between p53 and ARF has proven to be a multifaceted affair. ARF is induced in response to oncogenic signals to activate p53; ARF is also induced by loss of p53 to suppress STAT1 signaling. Our findings support a model whereby induction of ARF following p53 loss acts to prevent aberrant IFN- $\beta$  production and signaling to crucial downstream effectors. Thus, the functional links between p53 and ARF are far more imperative than anticipated. The complex p53-ARF network that we have identified provides tumor suppressive redundancy where none was thought to exist in cells.

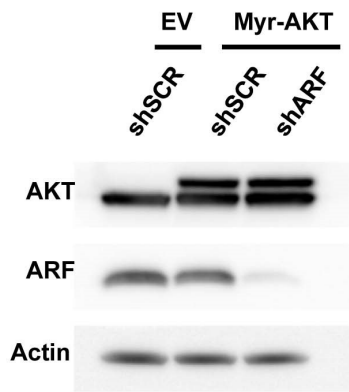
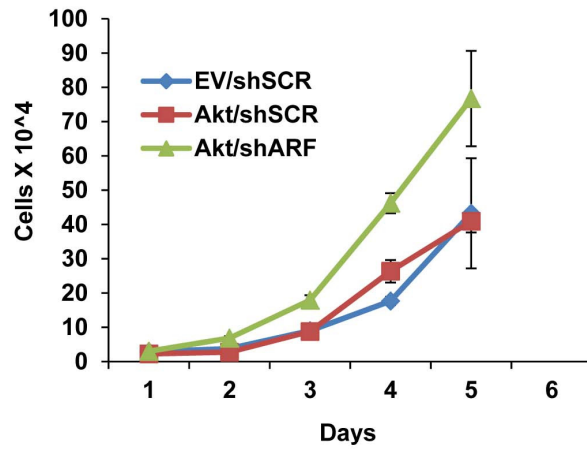
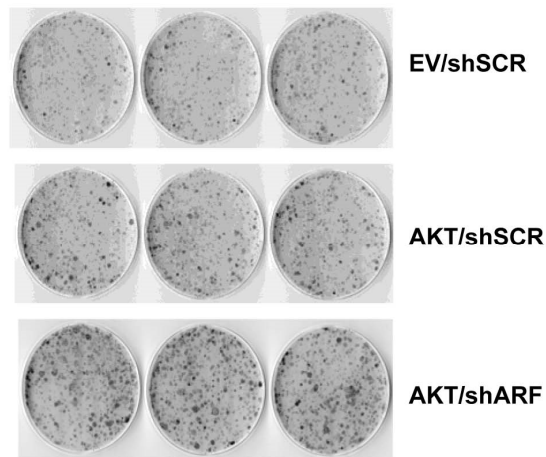
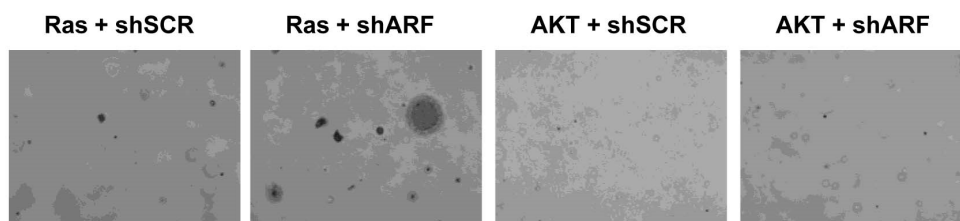
We believe our study, combined with several recent reports, indicate a need to more carefully examine the functional importance of interferon signaling in cancer cells to ensure the use of IFN as a treatment option does not produce an undesirable outcome (7, 63, 70). Moreover, our work suggests a subset of human cancer patients, those containing p53 and ARF mutations, might benefit from targeted inhibition of STAT1 or ISG15 activation.

# FIGURES

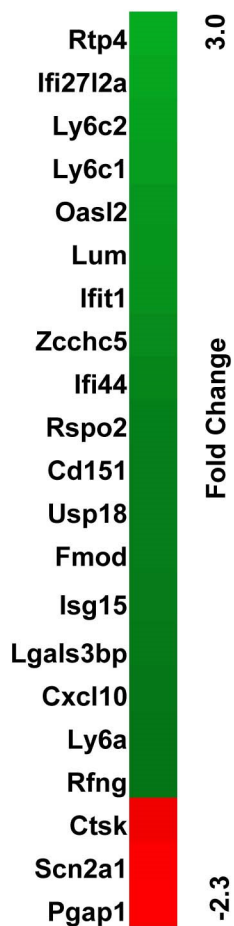


**Figure 4.1 Depletion of ARF in dp53R MEFs leads to increased ISG expression.**

(A) Western blot verifying overexpression of Ras<sup>V12</sup> and knockdown of ARF in dp53 MEFs. RNA from three independent experiments was submitted for microarray analysis. (B) Heat map showing significantly altered genes (>2-fold change). (C) Analysis showing significantly altered signaling pathways. (D) Validation of ISGs with qRT-PCR. Levels were normalized to Histone 3.3 mRNA and are relative to shSCR controls. Error bars represent s.d. from three independent experiments.

**A****B****C****D**

E

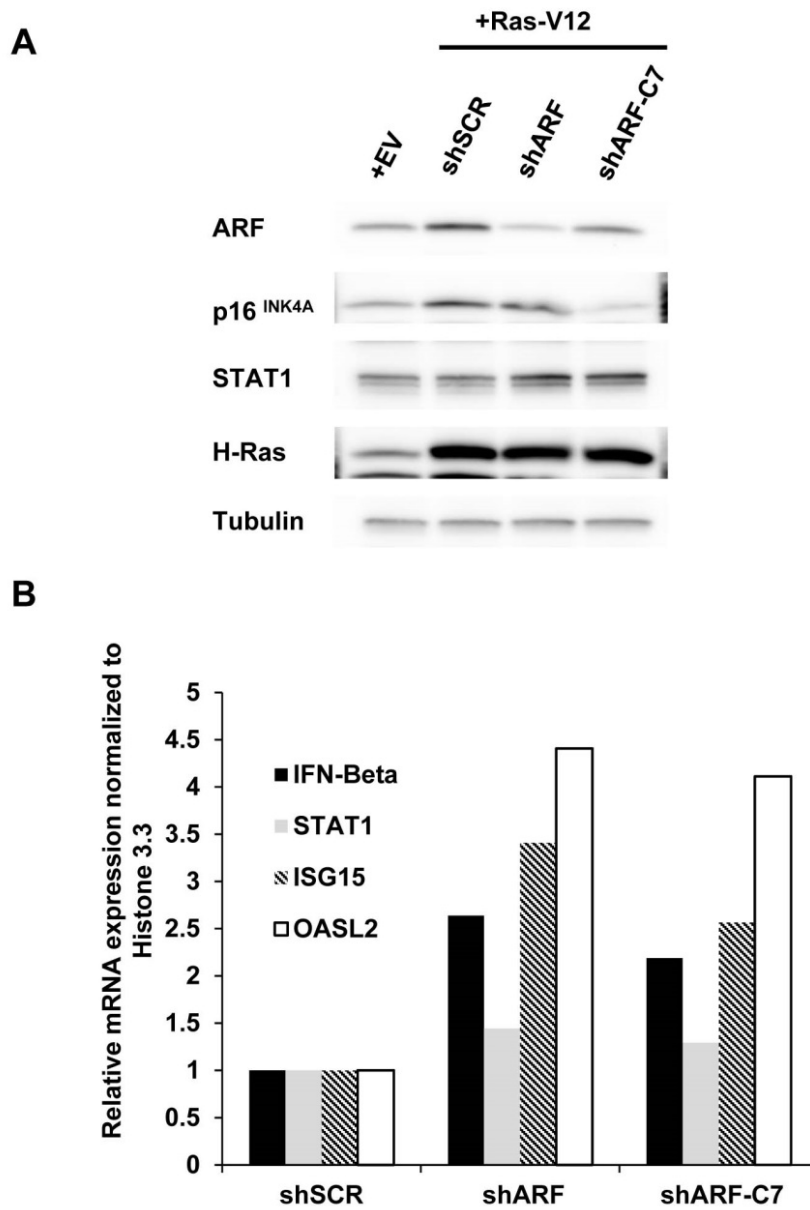


F

Pathway	P-Value
Immune response_IFN alpha/beta signaling pathway	7.888E-05
Cell adhesion_Role of tetraspanins in the integrin-mediated cell adhesion	2.198E-02
Development_Notch Signaling Pathway	2.551E-02
Immune response_TLR signaling pathways	3.312E-02
Chemotaxis_Leukocyte chemotaxis	4.415E-02
Immune response_IFN alpha/beta signaling pathway	7.888E-05

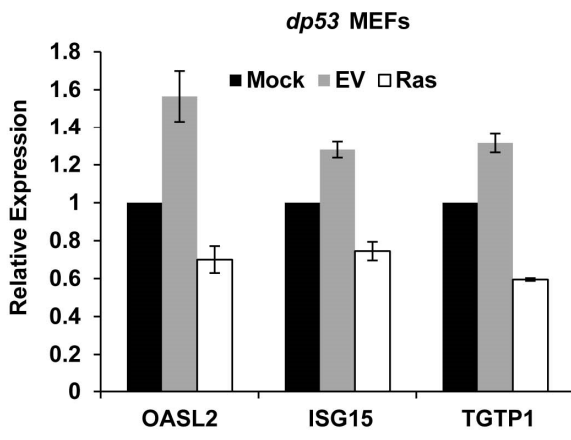
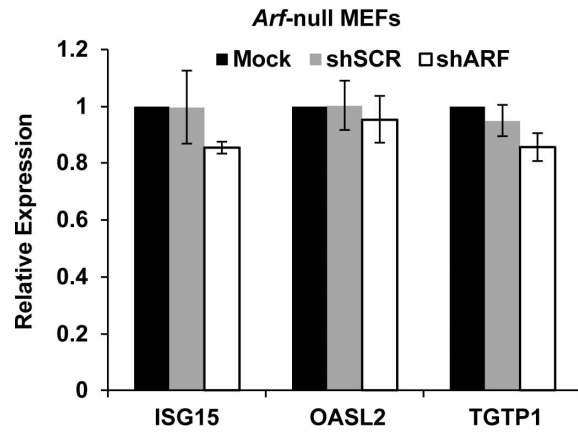
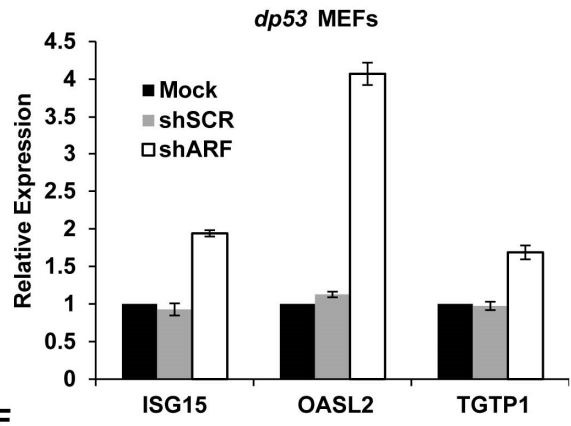
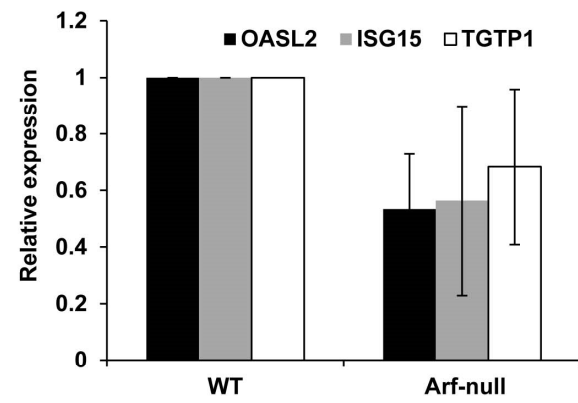
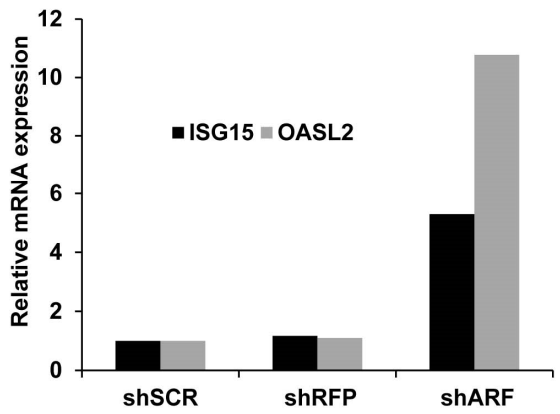
**Figure 4.2 dp53 MEFs overexpressing AKT1 and depleted for ARF exhibit a proliferation advantage and induce ISG expression.**

(A) Western blot analysis of dp53 MEFs expressing myristoylated-AKT1 and short hairpin RNAs targeting ARF or a scrambled control. (B) Proliferation assay from cells described in (A). This graph is representative of three independent experiments. (C) Representative image of foci assay performed with cells described in (A). The image is representative of three independent experiments (D) Light microscopy (4X objective) images of dp53 MEFs overexpressing the indicated constructs growing in soft agar. (E) Heat map showing significantly increased (green) or decreased (red) genes in dp53-AKT-shARF MEFs. Data is the average of three independent samples submitted for microarray analysis. (F) Pathway analysis was performed using the list of significantly altered genes.



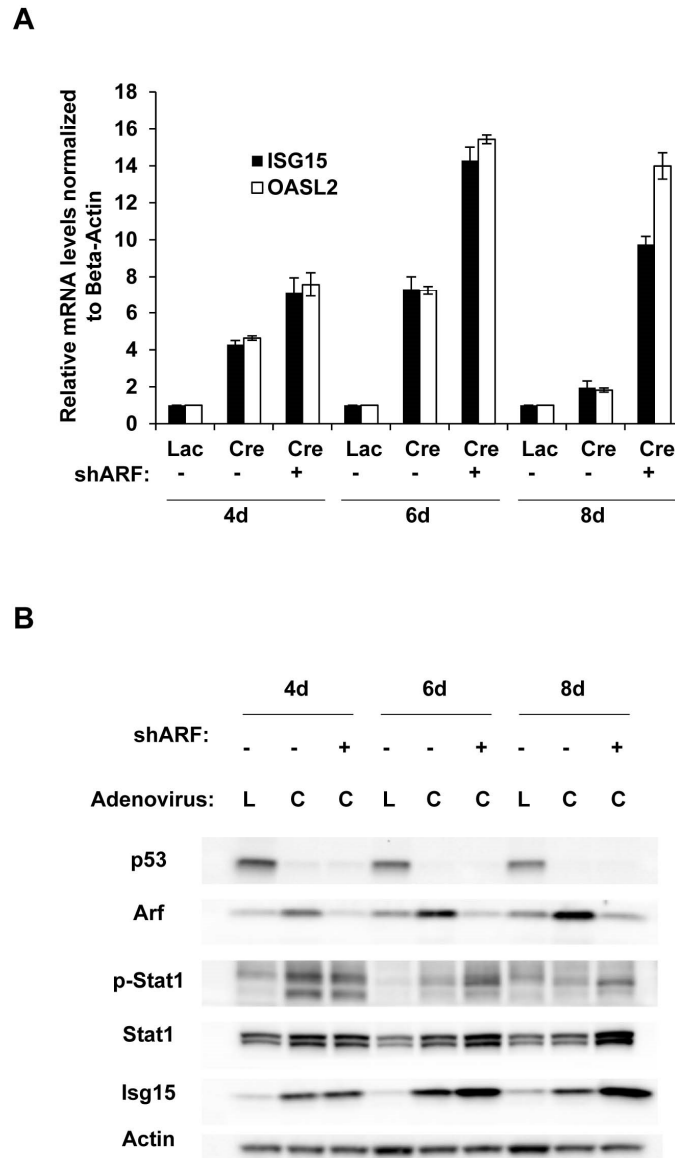
**Figure 4.3** dp53R MEFs expressing a second unique ARF shRNA induce ISGs.

(A) Western blot analysis of dp53 MEFs expressing *Ras*<sup>V12</sup> and two unique ARF shRNAs. (B) qRT-PCR analysis of cells described in (A) 5 days post-infection. mRNA levels were normalized to Histone 3.3 and relative to shSCR controls.

**A****B****C****D****E**

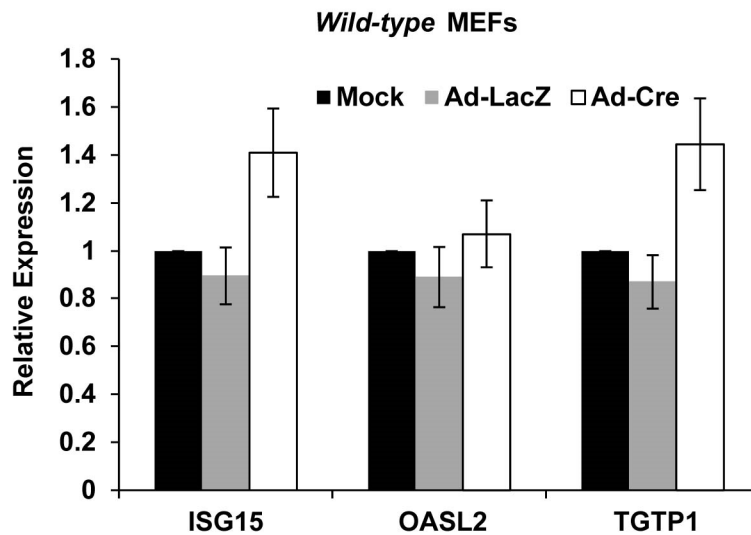


**Figure 4.4 ISG induction following ARF depletion is specific to *p53*-deficient setting.** (A) mRNA from dp53 MEFs infected with mock (no virus), empty vector, or Ras<sup>V12</sup> expressing retrovirus was analyzed by qRT-PCR. Relative mRNA expression levels were obtained by normalizing to Histone 3.3 mRNA. Fold changes are relative to mock-infected control. (B) mRNA from *Arf*-null MEFs infected with mock, shSCR, or shARF lentivirus was analyzed by qRT-PCR. Relative mRNA expression levels were obtained as described in (A). (C) mRNA from dp53 MEFs infected with mock (no virus), shSCR, or shARF lentivirus was analyzed by qRT-PCR. Relative mRNA expression levels were obtained as described in (A). (D) mRNA from three independent sets of low passage (<P6) Wild type or *Arf*-null MEFs was analyzed by qRT-PCR for the indicated genes. Relative mRNA expression was obtained as described in (A). (E) qRT-PCR analysis was performed on dp53R MEFs expressing shSCR, shRFP, or shARF. Cells were harvested 4 days post-infection and mRNA was normalized to Histone 3.3 and plotted relative to shSCR controls. All error bars represent s.d. of  $n=3$ .



**Figure 4.5** p53 and ARF cooperate to suppress ISG expression.

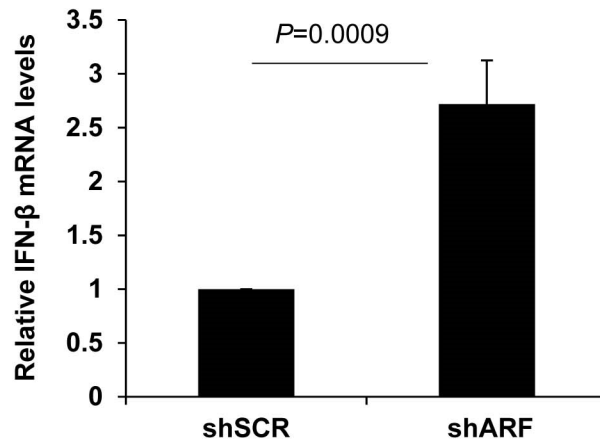
(A) qRT-PCR analysis of  $p53^{lox/lox}$  MEFs infected with Ad-LacZ or Ad-Cre from the indicated time points post-infection. Cells were all infected with shSCR(-) or shARF(+) 1 day post Cre-infection as indicated. mRNA levels are relative to Ad-LacZ-shSCR controls and error bars represent s.d. of three independent experiments. (B) Representative western blot analysis of cells described in (A).



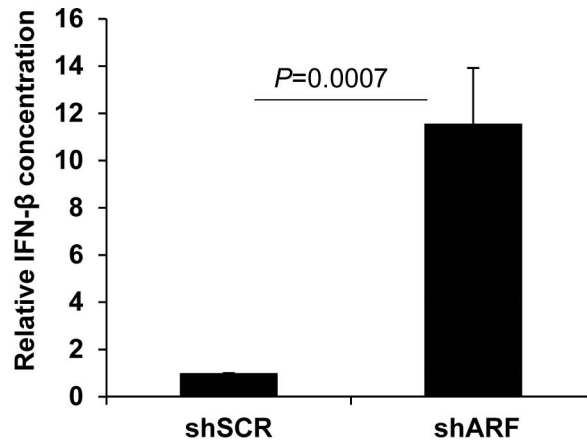
**Figure 4.6 Adenoviral infection does not induce ISGs.**

Low passage (<P4) wild-type MEFs were either mock (no virus) infected or infected with Adeno-LacZ or Adeno-Cre (MOI=50). Four days post-infection, cells were harvested and RNA was extracted. qRT-PCR analysis was performed and mRNA levels of the indicated genes were normalized to Histone 3.3 mRNA and are plotted relative to mock-infected controls.

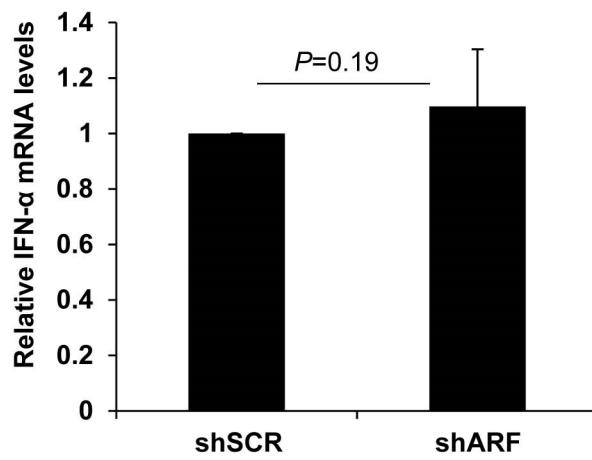
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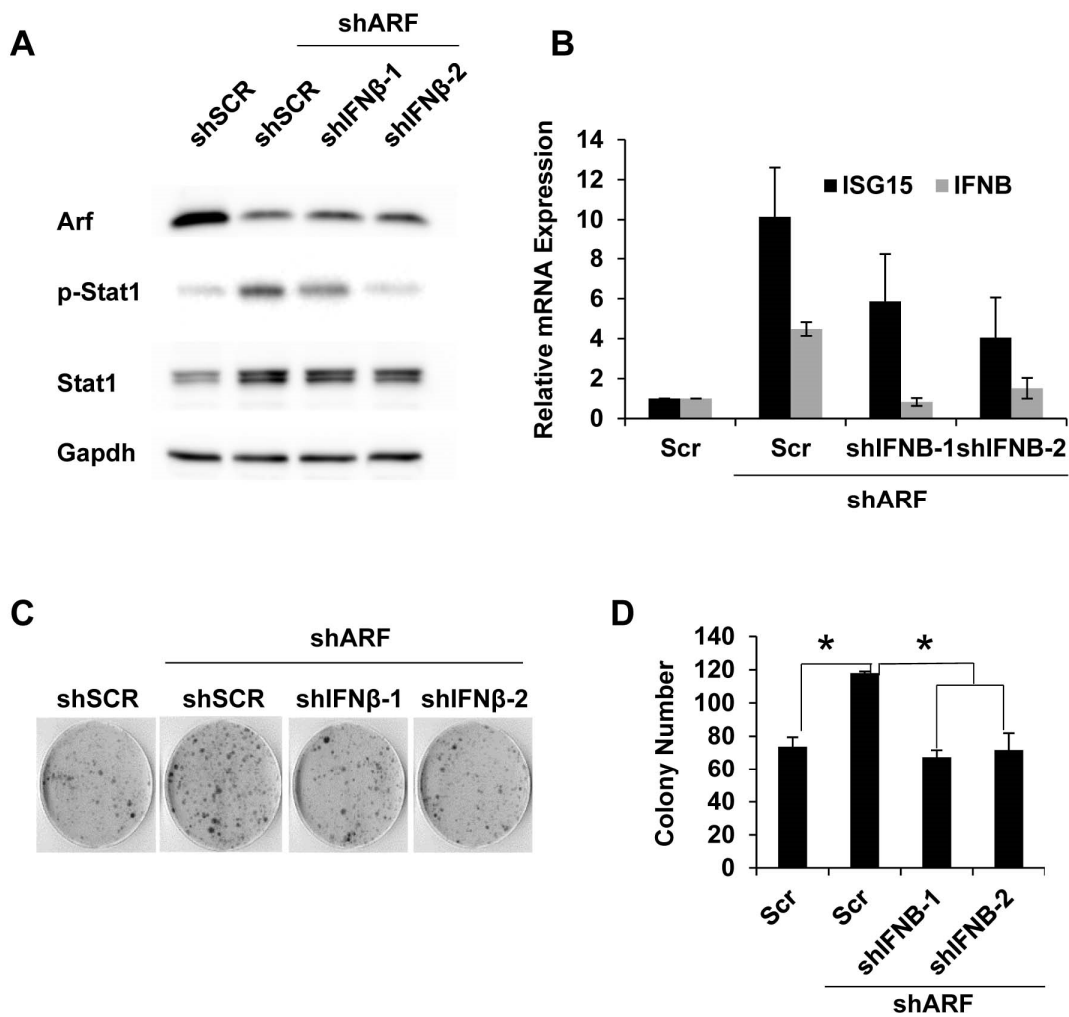
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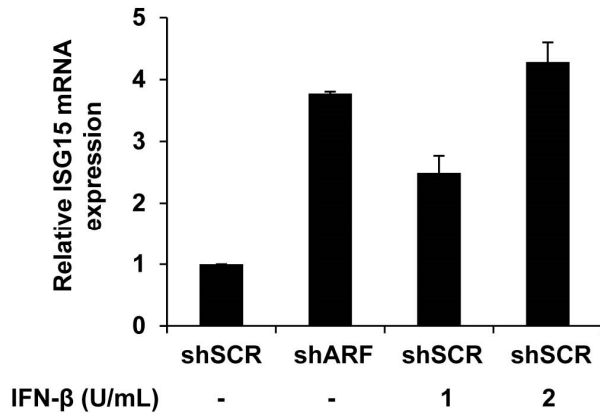


**Figure 4.7** Interferon-Beta expression and secretion is upregulated in dp53R-shARF MEFs. **(A)** qRT-PCR analysis of IFN- $\beta$  mRNA levels in dp53R-shARF MEFs. Levels are normalized to histone 3.3 mRNA and relative to shSCR controls. **(B)** Extracellular IFN- $\beta$  concentration measured by ELISA in dp53R-shARF MEF cell culture media. Values are fold changes relative to shSCR control. **(C)** qRT-PCR analysis performed with pan-IFN- $\alpha$  primers. All error bars represent s.d. of three independent experiments.

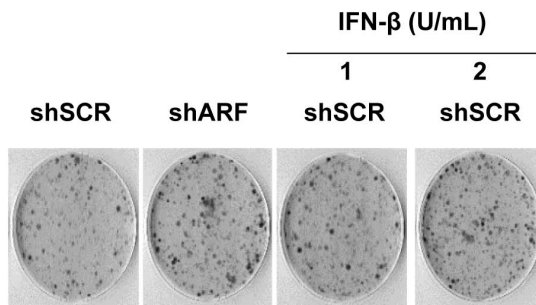


**Figure 4.8** IFN- $\beta$  signaling is necessary for the enhanced tumorigenicity of dp53R-shARF MEFs. **(A)** Western blot analysis of dp53R-shSCR or -shARF MEFs expressing two independent IFN- $\beta$  shRNAs. **(B)** qRT-PCR analysis of dp53R-shSCR or -shARF MEFs infected with two specific shRNAs targeting IFN- $\beta$ . Relative mRNA expression of ISG15 and IFN- $\beta$  was obtained by normalizing to Histone 3.3 mRNA. Error bars represent s.d. of three independent measurements. **(C)** Representative image of foci assay performed with dp53R-shARF or shSCR MEFs infected with two IFN- $\beta$ -specific shRNAs. Quantification of three independent measurements is shown in **(D)**.  $*=P<0.01$

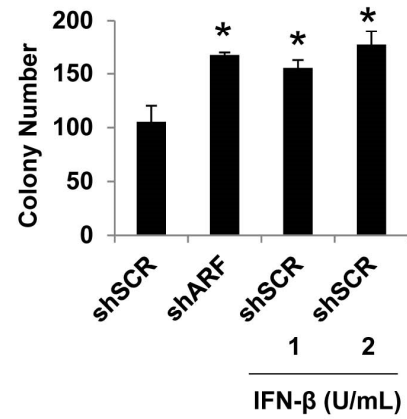
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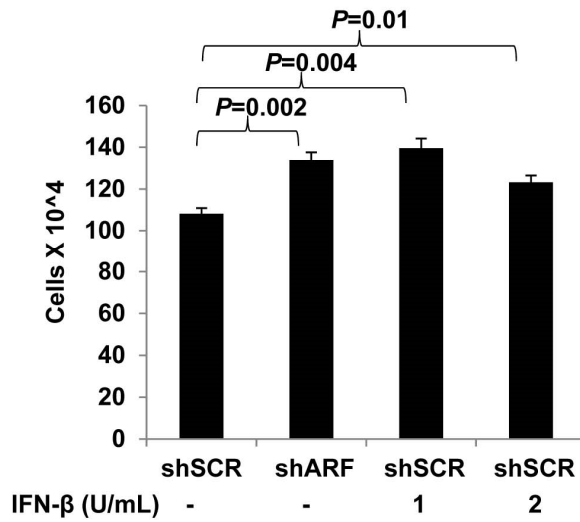
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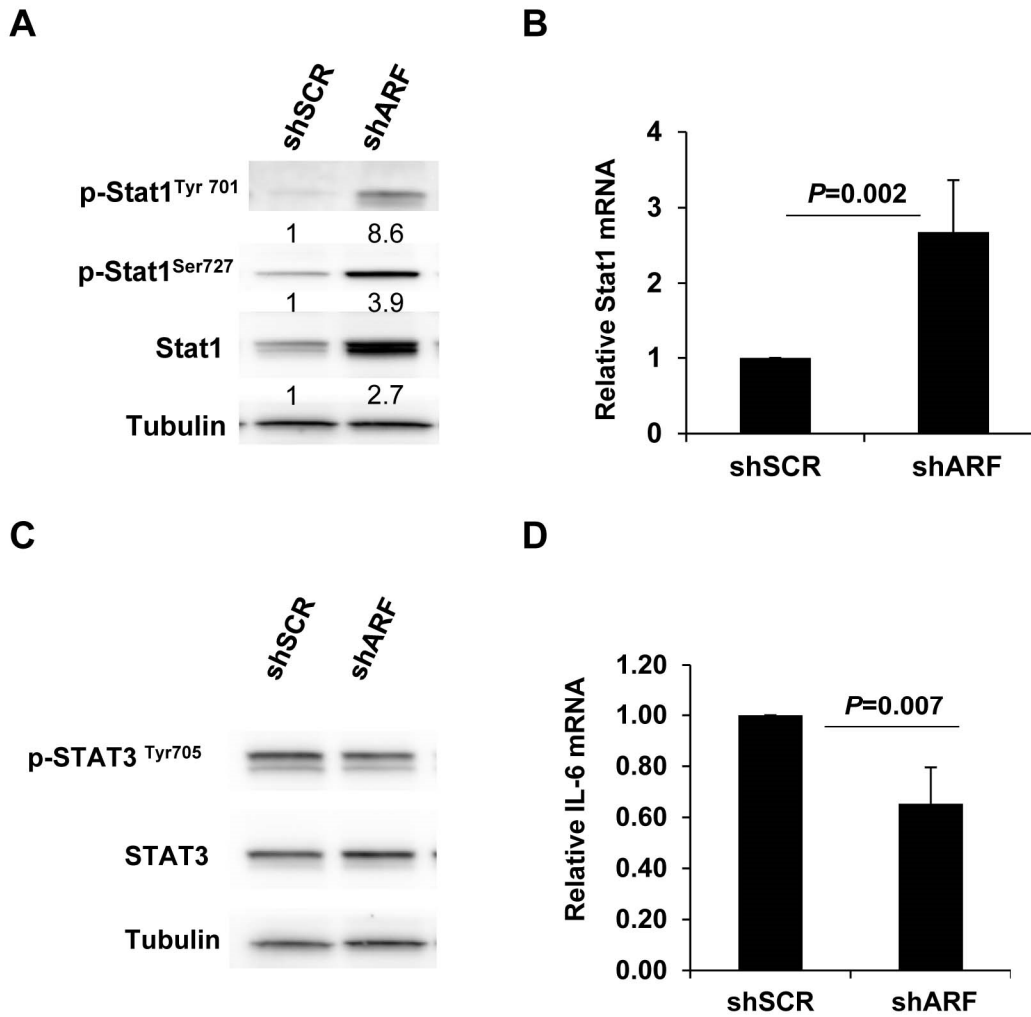
**D**



**Figure 4.9 Increased IFN- $\beta$  is sufficient to enhance proliferation in dp53R MEFs.**

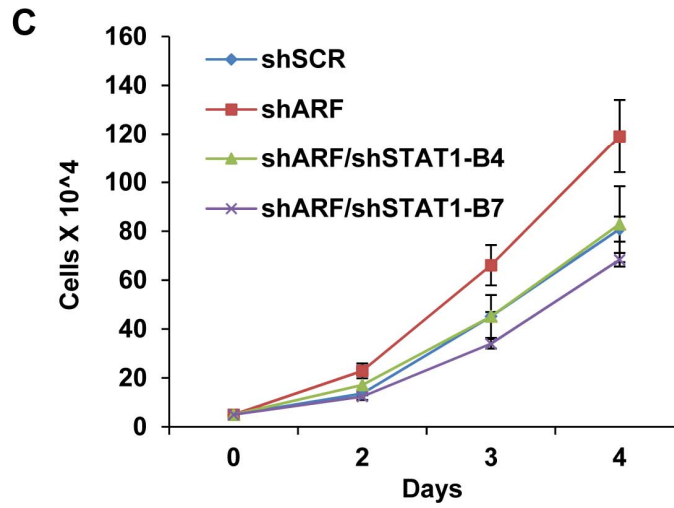
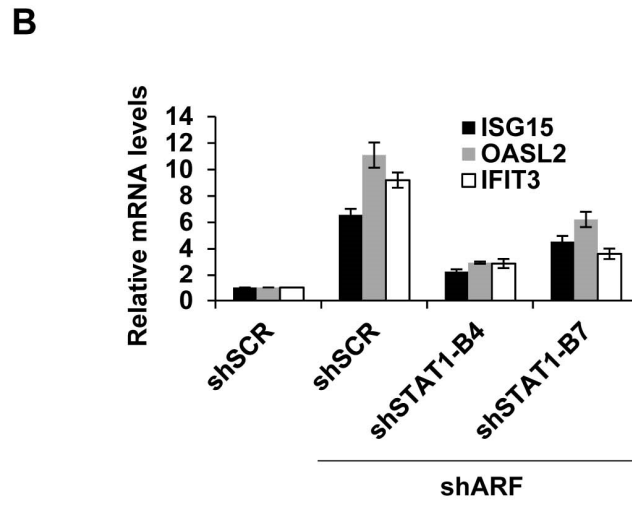
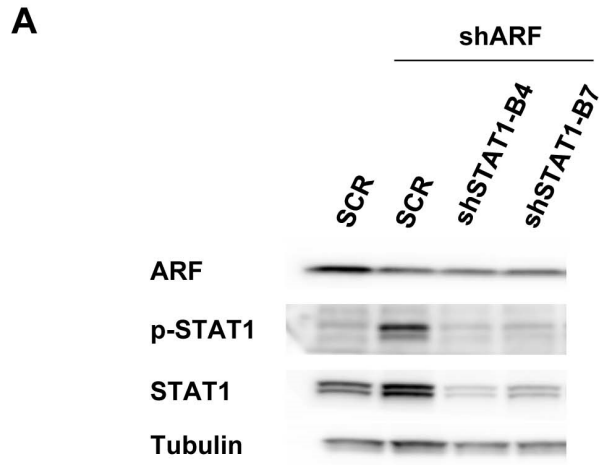
(A) qRT-PCR analysis of ISG15 mRNA in dp53R-shSCR or -shARF cells treated with the indicated concentration of IFN- $\beta$ . Error bars represent s.d. of values from three independent measurements. (B) Representative image of foci assay performed with dp53R-shARF or shSCR MEFs treated with the indicated concentration of recombinant IFN- $\beta$ . Quantification of colony number from three independent measurements is shown in (C)  $*=P<0.01$ . (D) Equal numbers of dp53R MEFs expressing shSCR or shARF were plated and treated with the indicated amounts of IFN- $\beta$ . Three days after treatment, the cells were trypsinized and cell number was obtained using trypan-blue exclusion. Error bars represent s.d. of three independent measurements.

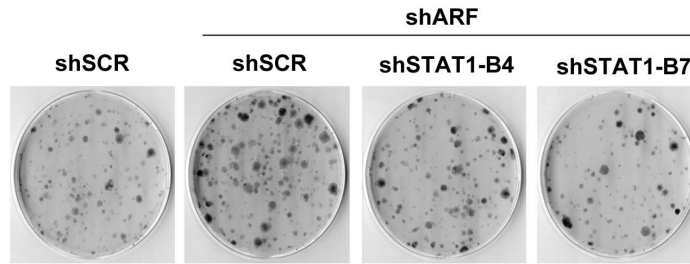
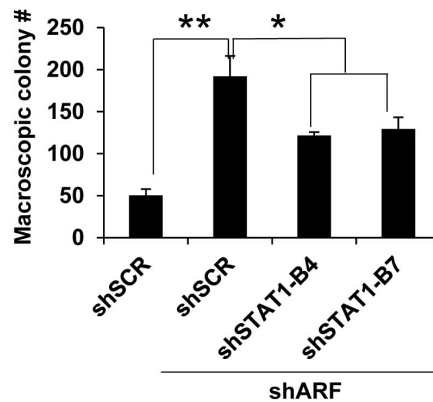




**Figure 4.10** Depletion of ARF in dp53 MEFs results in increased STAT1 phosphorylation.

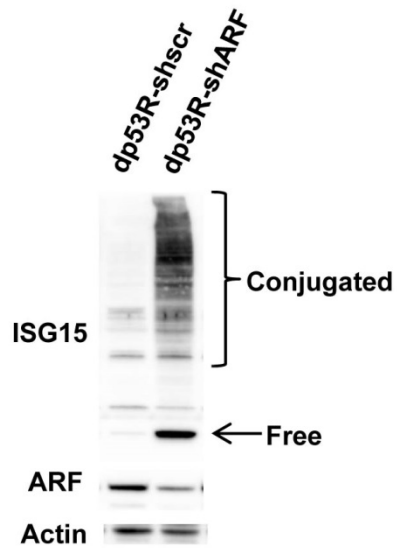
(A) Western blot analysis of dp53R-shARF or shSCR MEFs showing STAT1 activation. (B) qRT-PCR analysis of total STAT1 mRNA levels in dp53R-shARF MEFs. mRNA levels are relative to shSCR controls and normalized to Histone 3.3. (C) Western blot analysis of dp53R-shSCR or shARF MEFs for evidence of STAT3 activation. (D) qRT-PCR analysis of cells from (C) for expression of the cytokine, IL-6 normalized to Histone 3.3. Error bars represent s.d. of three independent measurements.



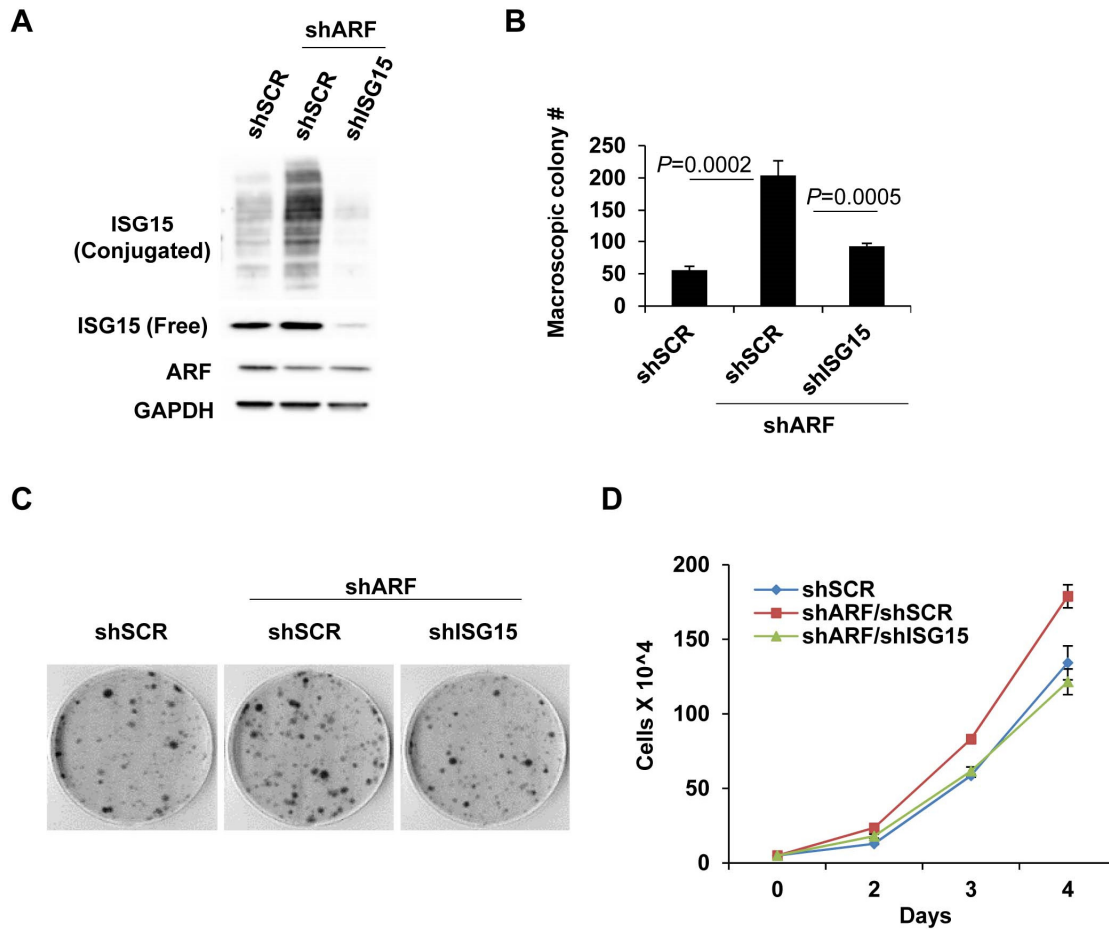
**D****E**

**Figure 4.11** STAT1 activation is required for increased tumorigenicity of dp53R-shARF

**MEFs.** (A) Western blot analysis of dp53R-shSCR or shARF MEFs infected with two different STAT1 shRNAs. (B) qRT-PCR analysis of various ISG mRNA levels in dp53R-shSCR or shARF MEFs infected with control or two different STAT1 shRNAs. (C) Proliferation assay of dp53R MEFs expressing the indicated shRNAs. Error bars represent s.d. of three independent measurements. (D) Representative images of foci assays with dp53R MEFs expressing indicated shRNAs. Images are representative of three independent experiments. (E) Soft agar quantification of STAT1 depleted dp53R-shARF MEFs. All error bars represent s.d. for  $n=3$ .  $*=P<0.0004$ ,  $**=P<0.009$ .



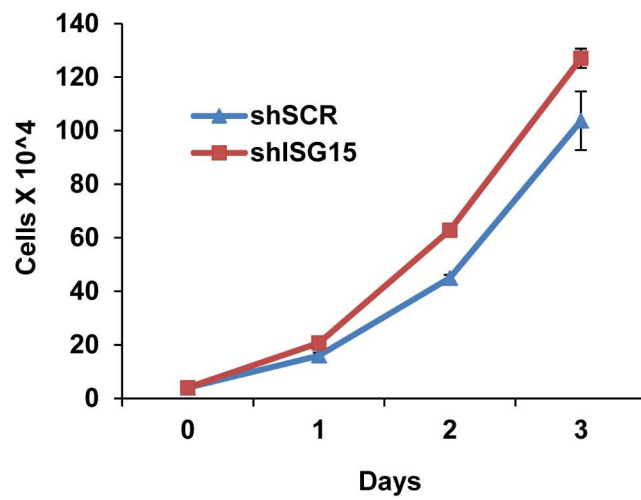
**Figure 4.12** Both free and conjugated forms of the ubiquitin-like protein, ISG15, are upregulated in dp53R-shARF cells. Western blot analysis of dp53R-shSCR or shARF MEFs performed with the indicated antibodies. Free and conjugated forms of ISG15 are indicated with an arrow and brackets, respectively.



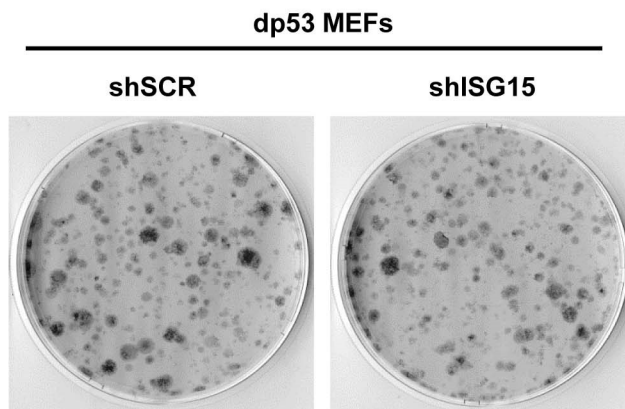
**Figure 4.13** ISG15 is required for increased tumorigenicity in dp53R-shARF MEFs.

(A) Western blot analysis of dp53R-shSCR or shARF MEFs expressing an shRNA specifically targeting ISG15. (B) Quantification of macroscopic soft agar colony number with cells described in (B). (C) Representative image of foci experiment from dp53R MEFs infected with the indicated shRNAs. (D) Proliferation assay for dp53R MEFs infected with the indicated shRNAs. All error bars represent s.d. of  $n=3$

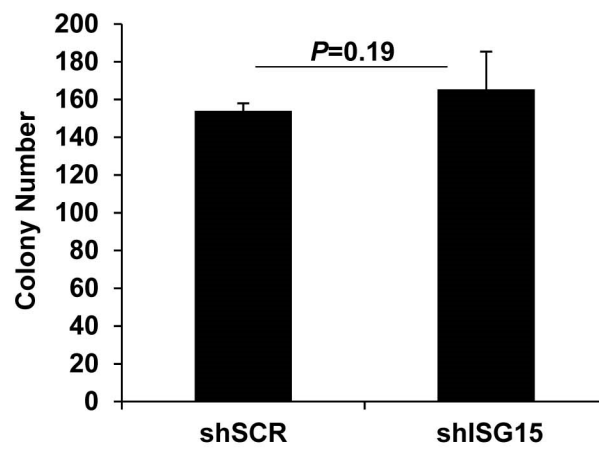
**A**



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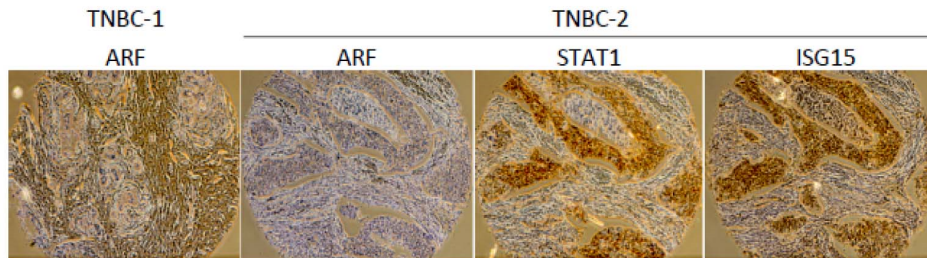


**Figure 4.14 Knockdown of ISG15 does not affect dp53 MEF proliferation.**

(A) dp53 MEFs were infected with shSCR or shISG15. Four days post infection, equal numbers of cells were plated and proliferation was measured on the days indicated by counting with a hemacytometer. (B) Representative image of foci assay performed with cells described in (A). (C) Quantification of foci assay. Error bars represent s.d. of  $n=3$ .

**A****TNBC IHC Statistics**

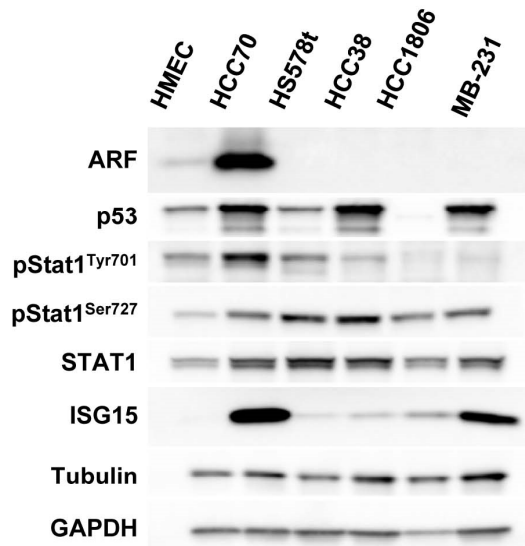
<b>p53<sup>mutant</sup> + High ARF</b>	<b>2/13 (15%)</b>
<b>p53<sup>mutant</sup> + Low/No ARF</b>	<b>11/13 (85%)</b>
<b>p53<sup>mutant</sup> + Low/No ARF + High STAT1/ISG15</b>	<b>6/11 (55%)</b>

**B****Figure 4.15 Immunohistochemistry analysis of TNBC samples**

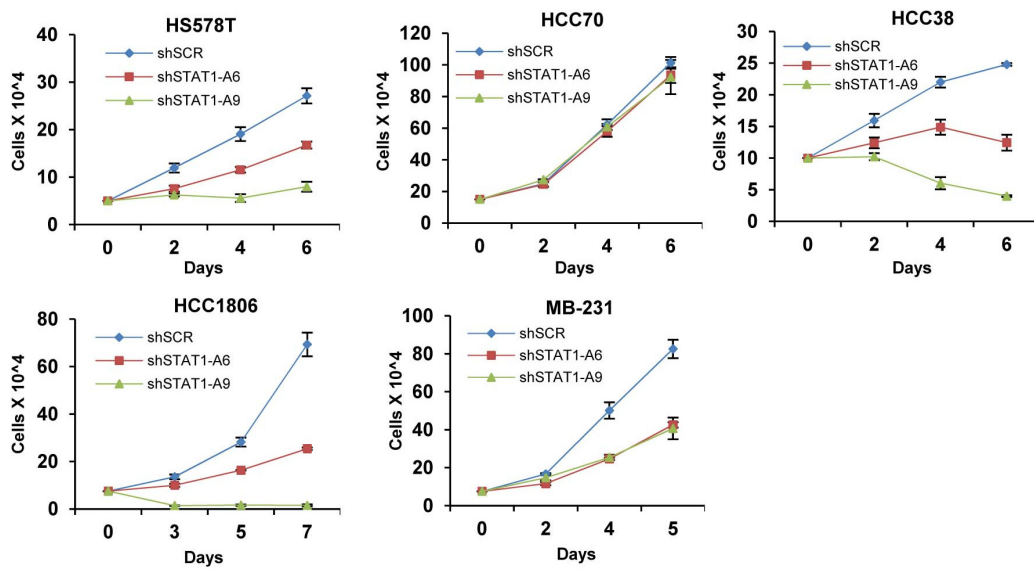
(A) Statistics from immunohistochemistry staining of human breast cancer tissue array. (B) Representative images from IHC displaying a section with high ARF staining (TNBC-1) and a section with low/no ARF and high ISG15/STAT1 (TNBC-2).



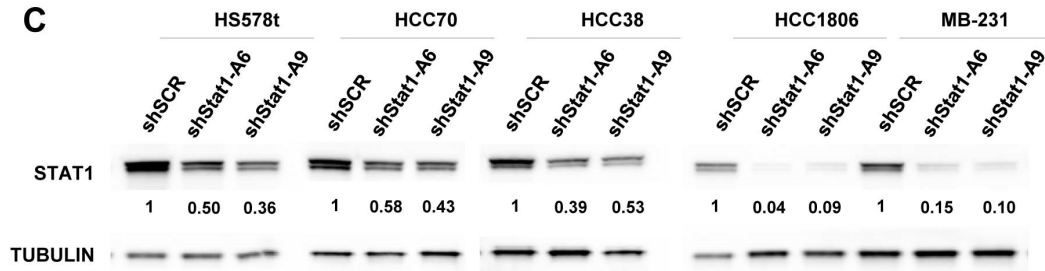
**A**

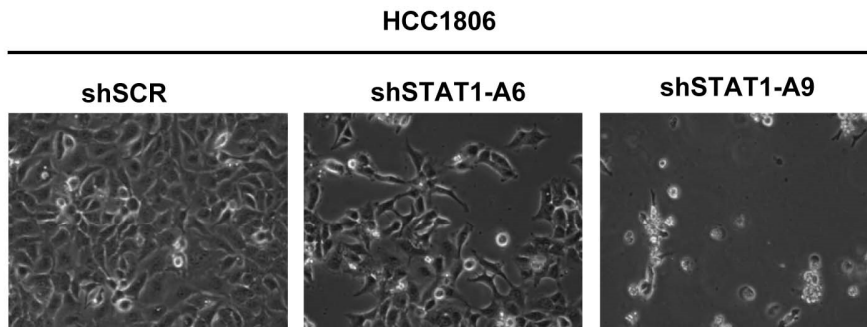
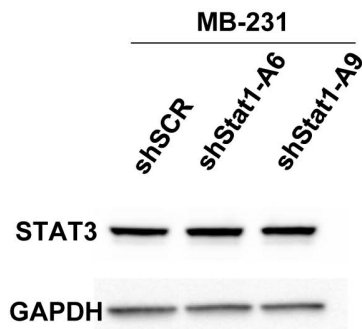


**B**

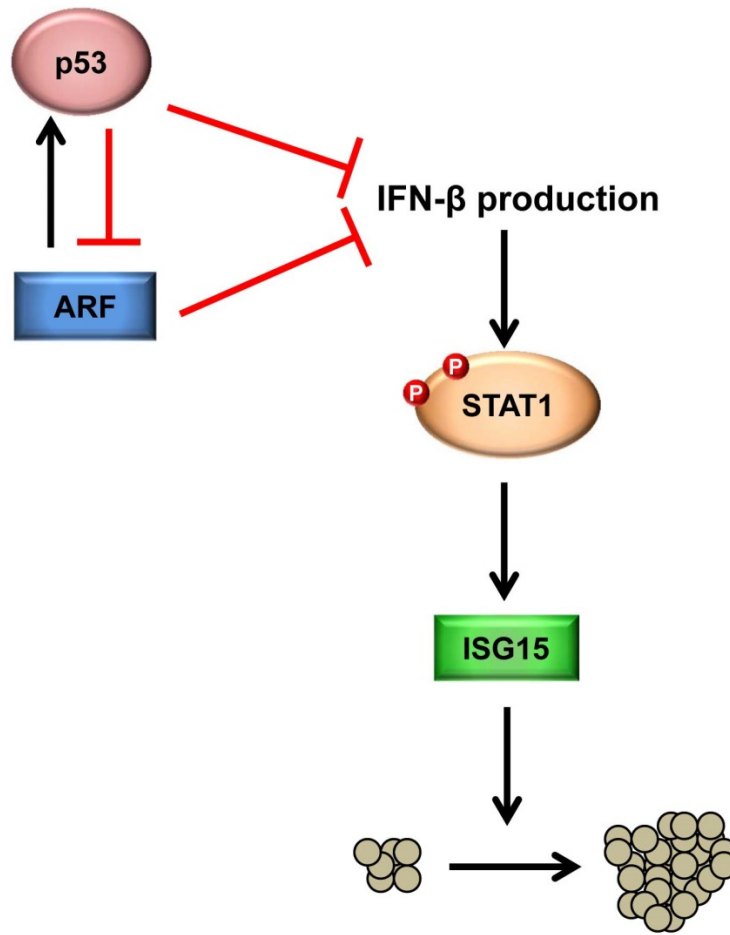


**C**



**D****E**

**Figure 4.16 Reducing STAT1 levels in ARF-deficient TNBC cell lines leads to proliferation defects.** (A) Western blot analysis of a panel of triple negative breast cancer cell lines blotted with indicated antibodies. Human mammary epithelial cells (HMECs) were used as a normal control. (B) Proliferation assays of the indicated triple negative breast cancer cell lines infected with two different STAT1 shRNAs. (C) Western blot analysis showing STAT1 depletion with shRNAs in various TNBC lines. Fold changes were calculated by normalizing to the tubulin loading control and are relative to shSCR controls. (D) Light microscopy images of HCC1806 cells infected with STAT1 hairpins displaying morphological evidence of apoptosis/necrosis. (E) Western blot in MB-231 cells depleted of STAT1 showing STAT3 levels are unaffected by the shRNAs.



**Figure 4.17 Working model**

ARF promotes p53 stability and p53 inhibits ARF mRNA expression. ARF and p53 cooperatively suppress IFN- $\beta$  production. Loss of *p53* initially leads to an upregulation of IFN signaling through STAT1, but the subsequent ARF induction functions to inhibit this response. Depleting ARF leads to unregulated IFN- $\beta$  production, STAT1 activation, and increased ISG15 expression. In the absence of *p53*, this signaling pathway can induce proliferation and promote tumorigenicity.

<b>Name</b>	<b>Species</b>	<b>Sequence</b>
<b>STAT1-B4</b>	<b>mouse</b>	<b>GCCGAGAACATACCAGAGAAT</b>
<b>STAT1-B7</b>	<b>mouse</b>	<b>GCTGTACTTTCCCAGATATT</b>
<b>STAT1-A6</b>	<b>human</b>	<b>GAACAGAAATACACCTACGAA</b>
<b>STAT1-A9</b>	<b>human</b>	<b>CTGGAAGATTTACAAGATGAA</b>
<b>ISG15</b>	<b>mouse</b>	<b>AGCACAGTGATGCTAGTGGTA</b>
<b>IFN<math>\beta</math>-1</b>	<b>mouse</b>	<b>GCAGAAGAGTTACACTGCCTT</b>
<b>IFN<math>\beta</math>-2</b>	<b>mouse</b>	<b>GCAGAGATCTTCAGGAACTTT</b>

**Table 4.1 shRNA sequences used in these studies.**

<b>Gene</b>	<b>Company</b>	<b>Catalogue #</b>	<b>Dilution</b>
<b>p19ARF (mouse)</b>	<b>Santa Cruz</b>	<b>sc-32748</b>	<b>1:1000</b>
<b>p14ARF (human)</b>	<b>Bethyl</b>	<b>A300-340A</b>	<b>1:500</b>
<b>p53 (mouse)</b>	<b>Cell Signaling</b>	<b>2524</b>	<b>1:1000</b>
<b>MDM2</b>	<b>Millipore</b>	<b>OP115</b>	<b>1:500</b>
<b>Actin</b>	<b>Santa Cruz</b>	<b>sc-8432</b>	<b>1:500</b>
<b>p53 (human)</b>	<b>Santa Cruz</b>	<b>sc-126</b>	<b>1:1000</b>
<b>Gamma tubulin</b>	<b>Santa Cruz</b>	<b>sc-7396</b>	<b>1:1000</b>
<b>H-Ras</b>	<b>Santa Cruz</b>	<b>sc-520</b>	<b>1:2000</b>
<b>Isg15 (human)</b>	<b>Santa Cruz</b>	<b>sc-166755</b>	<b>1:1000</b>
<b>Gapdh</b>	<b>Bethyl</b>	<b>A300-641A</b>	<b>1:10000</b>
<b>pStat1 Tyr 701</b>	<b>Cell Signaling</b>	<b>9167</b>	<b>1:500</b>
<b>pStat1 Ser727</b>	<b>Cell Signaling</b>	<b>8826</b>	<b>1:1000</b>
<b>Stat1</b>	<b>Santa Cruz</b>	<b>sc-346</b>	<b>1:1000</b>
<b>Stat3</b>	<b>Cell Signaling</b>	<b>9139</b>	<b>1:1000</b>

**Table 4.2 Primary antibodies used for Western blot.**

GENE NAME		SEQUENCE
ARF	FWD	5'-GAG TAC AGC AGC GGG AGC AT-3'
	REV	ATC ATC ATC ACC TGG TCC AGG ATT CC
p53	FWD	CAT CAC CTC ACT GCA TGG AC
	REV	AAA AGA TGA CAG GGG CCA TG
Actin	FWD	TCA CCC ACA CTG TGC CCA TCT A
	REV	TAC TCC TGC TTG CTG ATC CAC A
Histone 3.3	FWD	CGT GAA ATC AGA CGC TAG CAG AA
	REV	TCG CAC CAG ACG CTG AAA G
OASL2	FWD	ATC ATT GTC CTT ACC CAC AGA G
	REV	TGC TGG TTT TGA GTC TCT GG
ISG15	FWD	CTG ACT GTG AGA GCA AGC AGC
	REV	ACC AAT CTT CTG GGC AAT CTG
IFIT3	FWD	AGC ACA GAA ACA GAT CAC CAT
	REV	CAC CCT GTC TTC CAT ATG ACT G
USP18	FWD	TTC CCT CAG AGC TTG GAT TTC
	REV	CCG GAT GTA GGC ACA GTA ATG
IRF7	FWD	TTG ATC CGC ATA AGG TGT ACG
	REV	TTC CCT ATT TTC CGT GGC TG
SFRP2	FWD	GCC TGC AAA ACC AAG AAT GAG
	REV	GTC TTG CTC TTT GTC TCC AGG
STAT1	FWD	GCC GAG AAC ATA CCA GAG AAT C
	REV	GAT GTA TCC AGT TCG CTT AGG G
IFNB1	FWD	CCA CCA CAG CCC TCT CCA TCA ACT AT
	REV	CAA GTG GAG AGC AGT TGA GGA CAT C
IL-6	FWD	CAA AGC CAG AGT CCT TCA GAG
	REV	GTC CTT AGC CAC TCC TTC TG
TGTP1	FWD	CGA GTA CTG GGA AGC TTG AAA
	REV	ATC AGG AGA AGG GAA AGC ATG
IFN-alpha	FWD	CTT CCA CAG GAT CAC TGT GTA CCT
	REV	TTC TGC TCT GAC CAC CTC CC

**Figure 4.3** Primer sequences used for qRT-PCR.

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## **Chapter 5:**

### **Summary and Future Directions**

## SUMMARY

One of the challenges to personalized cancer treatment is knowledge of the molecular vulnerabilities based on the specific genetic alterations within a given tumor. With the explosion of efforts to generate genomic sequencing data for many human cancer types, cancer researchers now have the information at hand to directly address this challenge. My dissertation work has focused on understanding the selective advantage gained by cancer cells harboring co-inactivation of two tumor suppressor genes, ARF and p53. These two genes encode proteins that are canonically thought to function in a linear genetic pathway, where oncogenic signaling activates ARF which subsequently increases p53 stability by sequestering its E3-ligase, MDM2. The activity of this signaling pathway is absolutely vital in ensuring our cells do not inappropriately activate oncogenic signaling networks that could lead to aberrant proliferation. In recent years, it has become clear that ARF possesses p53-independent functions. Moreover, cells lacking p53 express high levels of ARF due to p53's ability to directly repress the Arf promoter. It is unclear why cells possess this negative feedback loop. Since many human cancers inactivate both p53 and ARF, I hypothesized that loss of *p53* leads to the induction of ARF which then elicits one of its p53-independent tumor suppressor functions. A selective pressure, therefore, exists to co-inactivate ARF. My work sought to address this hypothesis, and to identify the pertinent tumor suppressor role that ARF plays in *p53*-deficient cells.

The data presented in Chapter 2 of this dissertation provides clear evidence that acute loss of *p53* leads to a robust upregulation of ARF at both the mRNA and protein level. The cells lacking *p53* proliferated more rapidly than their wild-type counterparts, but shRNA-mediated depletion of ARF led to a further enhancement of proliferation. Furthermore, the tumorigenicity of *Ras*<sup>V12</sup>-transformed *p53*-null cells was greatly enhanced when ARF was knocked down. This observation was made in both soft agar assays as well as *in vivo* tumor allograft studies.

Mechanistically, increased proliferation, rather than lower rates of cell death, was likely the contributing factor in the enhanced tumorigenic potential of ARF-depleted cells. Taken together, the data presented in Chapter 2 support the hypothesis that loss of *p53* leads to increased ARF expression that is capable of suppressing tumorigenic potential.

In Chapter 3, I tested the hypothesis that ARF was utilizing its previously established role in regulating cell growth as the mechanism of tumor suppression in *p53*-null cells. In support of this hypothesis, *p53*-null cells displayed evidence of decreased cell growth. However, upon further interrogation, I was able to demonstrate that the observed defects were likely due to an abbreviated cell cycle in *p53*-null cells. The kinetics of overall protein synthesis was actually much faster in cells lacking *p53*, regardless of ARF status. Having ruled out enhanced cell growth as the mechanism for increased tumorigenicity, I examined other reported *p53*-independent functions of ARF such as sumoylation, inhibiting c-MYC transcription, and cell motility/invasion. I was unable to provide experimental evidence linking any of these functions to my observed phenotype, indicating ARF was likely performing a novel *p53*-independent tumor suppressor function.

Chapter 4 describes my effort to identify this novel *p53*-independent function of ARF, by performing an unbiased gene expression analysis of *p53*-null cells with or without ARF. To our surprise, the results of this experiment indicated ARF depleted cells were upregulating a type I interferon response pathway. I went on to show that ARF and *p53* cooperate to suppress the IFN response. Acute loss of *p53* induces the IFN signaling pathway, but the subsequent ARF induction acts to suppress it. Therefore, only upon inactivation of *p53* and ARF was the IFN response potently induced. My work further demonstrated that interferon-beta, STAT1, and the interferon-sensitive gene ISG15, are all required for the tumorigenic phenotype observed in *p53*-

deficient cells depleted of ARF. As direct evidence that interferon-beta signaling can promote cell proliferation, supplementing *p53*-deficient cell culture media with low levels of interferon-beta significantly induced proliferation even though they contained elevated ARF levels. I validated the physiological relevance of this signaling pathway by showing elevated expression of STAT1 and ISG15 in triple-negative breast cancer patients that harbored co-inactivation of *p53* and ARF. Furthermore, I showed that triple-negative breast cancer cell lines that lacked ARF and *p53* function were sensitive to STAT1 depletion. The data described in Chapter 4, therefore, identified the important *p53*-independent function of ARF that cancer cells overcome by deleting or silencing the *CDKN2a* locus.

The work described in this dissertation has uncovered a novel cooperation between ARF and *p53*. Cancer cells that inactivate both these genes gain a proliferative advantage due to an oncogenic IFN- $\beta$ -STAT1-ISG15 signaling axis. While I believe this finding defines targeted inhibition of this signaling pathway as a potential therapeutic option, future experiments are needed to address numerous questions that are raised by my findings. This chapter will address some of these questions, and explore future experimental directions that my work should catalyze.

## FUTURE DIRECTIONS

### Identify activators of Arf expression in response to *p53* loss.

I have demonstrated that acute loss of *p53* leads to an upregulation of ARF by 4 days post *p53* excision. Since we now know that *p53* represses Arf transcription by promoting heterochromatin formation (50), why is there a delayed Arf response to *p53* loss? Moreover, what are the transcription factors that promote Arf transcription when *p53* is lost? I initially hypothesized that chromatin remodeling complexes such as SWI/SNF would be required to open the Arf promoter up to transcription factors. An obvious candidate for this process is SNF5, which is a subunit of the ATP-dependent SWI/SNF complex that has been shown to be important for ARF induction in mouse muscle tissue as well as cultured human cells (20, 49). Additionally, loss of SNF5 function is a common event in malignant rhabdoid tumors (MRT), and this genetic event can cooperate with *p53* loss to promote MRT formation in the mouse (18). To test whether SNF5 activity was required for ARF induction in my system, I treated *p53*<sup>Flox/Flox</sup> MEFs with Adeno-Cre and then infected with shRNAs targeting SNF5. I analyzed ARF protein expression 6 days post Cre-infection. Depletion of SNF5 had no effect on ARF protein accumulation, suggesting chromatin remodeling may not be necessary in MEFs (Figure 5.1). One potential explanation for this result is that *p53* seems to be required to physically recruit the PcG proteins to the Arf promoter. Once *p53* is lost, these proteins would no longer be localized to this region. Since the repressive chromatin marks would not be maintained, several rounds of cell division would result in reduced heterochromatin formation.

Transcription of Arf mRNA is enhanced following *p53* loss, so what transcription factors mediate this response? Likely candidates would be known activators of Arf, such as DMP1, E2F1, SMAD2, and FOXO3a. Analysis of the requirement for each of these in Arf induction



following p53 loss could be tested with the shRNA approach I described above. If these experiments do not identify key regulators, then a large scale shRNA approach could be utilized. Our current *in vitro* system would not be amenable to such a screen since our cells do not carry a detectable marker for ARF expression. For this experiment,  $p53^{Flx/Flx}$  mice could be bred with the  $Arf^{GFP}$  mouse that contains a knocked-in *Gfp* allele to exon-1 $\beta$  of ARF (55). MEFs obtained from  $p53^{Flx/Flx}; Arf^{GFP/+}$  would be subjected to Cre-mediated excision of p53 followed by addition of a transcription factor shRNA library available from the RNAi Consortium. This experiment would allow GFP expression to be measured as readout for ARF transcription.

A more challenging question is how ARF is regulated *in vivo*. As I mentioned in Chapter 2, mouse mammary glands did not all stain positive for ARF in  $p53$ -null mice (Figure 2.6). Thus, inducing *Arf* *in vivo* requires more than simply  $p53$  loss. Cells grown on plastic are subjected to various stresses as a result of hyperoxic conditions and constant activation of growth factor pathways due to serum supplementation (21). As a result, ARF is induced after several passages and continues to rise until the cells undergo senescence. Being a key negative regulator of *Arf*,  $p53$  loss simply leads to a faster transcriptional induction. It is currently unknown which of the non-physiological conditions utilized in tissue culture directly induce ARF. Constitutive signaling through RAS as a result of high growth factor concentrations is certainly one possibility. Is oncogenic activation, such as *Ras* mutation, the inducing signal in ARF-positive mammary epithelial cells? This could be tested by co-staining the mammary glands with phospho-ERK or phospho-AKT antibodies. If activation of ERK or AKT is occurring in the same cells as those with induced ARF, then this suggests oncogenic signaling is initiating ARF transcription. To further validate this finding, a  $Blg-Cre;p53^{Flx/Flx}; LSL-Kras^{G12D}$  mouse could be generated to see whether, in the presence of activated RAS, all the mammary epithelial cells

would stain positively for ARF. If oncogenic signaling is not the physiological activator of ARF in epithelial cells, then ROS and DNA damage could also be measured using previously characterized staining techniques (29, 35).

While these experiments would certainly be informative for the regulation of mouse ARF, it would be beneficial to perform similar experiments in human cells. Very little is known about the physiological activators of Arf transcription in human cells and insight in this area would help explain the differential regulation of Arf in human and mouse cells. To uncover regulators of Arf transcription in human epithelial cells, I propose to use TL-HMECS, which are immortal human mammary epithelial cells expressing telomerase and Large T-antigen. These cells have been used extensively in large scale shRNA and cDNA overexpression screens (30, 42). With the help of new genome editing technologies, such as CRISPRs (7) or TALENS (4), a TL-HMEC line could be made where exon-1 $\beta$  is replaced by firefly luciferase. The TL-HMEC<sup>Arf-Luc</sup> cell line could be used for high throughput screens to identify activators or repressors of ARF transcription.

The experiments proposed in this section are intended to enhance our knowledge of Arf transcriptional regulation. A more detailed understanding of this process would help us explain the biological significance of the p53-Arf negative feedback loop. Furthermore, many p53 mutant tumors do not delete *CDKN2a* or exhibit overexpression of ARF. If we understood how Arf is induced in the absence of p53, perhaps we could identify a pharmacological approach to activating ARF and reducing tumor growth. Finally, analysis of Arf regulation in human cells would provide insight into the observation that p16 is the more responsive tumor suppressor at this genomic locus in humans. If ARF expression is kept under tight control in humans to allow

for increased growth, then finding ways to activate ARF might lead to potent inhibition of tumorigenesis.

**Determine whether overexpressing transcriptional inhibitors of Arf can phenocopy *CDKN2a* deletion.**

In human tumors, ARF and p53 co-inactivation is primarily thought to occur by mutating *p53* and deleting the entire *CDKN2a* locus. However, if de-repressed ARF levels serve p53-independent tumor suppressor functions, then *p53*-mutant tumors might utilize mechanisms besides *CDKN2a* deletion to evade those functions. For instance, overexpressing any of the PcG proteins, such as EZH2, BMI1, SUZ12, or RING1b might be able to re-silence the Arf promoter. This possibility is often overlooked since *CDKN2a* deletion is so common. However, in Figure 5.2 I have mined the TCGA database and found numerous cases in lung squamous cell carcinoma that overexpress these proteins without *CDKN2a* deletion. In fact, 20 additional *p53*-mutant tumors exhibit overexpression of one or more known Arf transcriptional inhibitors. I hypothesize that the combination of p53 mutation with either *CDKN2a* deletion or unchanged Arf mRNA levels, indicate negative selection against ARF.

To test this hypothesis *in vitro*, I would overexpress Arf's negative regulators immediately following p53-inactivation. I would predict that ARF levels would not become induced, and that if these cells were transformed by *Ras*<sup>V12</sup>, they would grow in soft agar similar to the dp53R-shARF MEFs. Of course, it is possible that p53 is absolutely required for recruiting these proteins to the *Arf* locus, and that their overexpression will not have an effect on ARF levels. If this is the case, I would overexpress more direct repressors of Arf transcription such as E2F3b (13) and ATF4 (17).

### **Determine how loss of p53 induces the IFN response.**

In Chapter 4, I demonstrated that loss of *p53* leads to an induction of the IFN response, followed by an ARF induction that suppresses IFN signaling (Figure 4.5). I have also shown that the IFN response is dependent upon complete loss of *p53* function, as *p53*-heterozygous MEFs (*p53*<sup>+/-</sup>) infected with shARF do not exhibit increased IFN signaling (Figure 5.3 A). I have developed three testable hypotheses to explain how *p53* loss can lead to an induction of the IFN response. The first of which has to do with the intriguing fact that the IFN- $\alpha$  and IFN- $\beta$  genes are located on chromosome 9p21, just 650 kilobases away from *CDKN2a* (37). Given that p53 represses Arf by promoting heterochromatin formation, it is possible that this heterochromatin spreads far enough to affect interferon transcription (45). To test this possibility, chromatin immunoprecipitation experiments could be performed in Adeno-LacZ or Adeno-Cre infected *p53*<sup>Flox/Flox</sup> MEFs using antibodies specific for the inhibitory H3K27me3 chromatin modification. RT-PCR analysis of several regions within the IFN gene cluster would identify whether the inhibitory mark is differentially expressed in Lac or Cre-treated cells. My hypothesis would be that Cre-treated cells would have fewer regions marked by the H3K27me3 modification. Alternatively, p53 could directly inhibit transcription of IFN $\beta$  or other genes involved in the IFN response. However, this possibility seems unlikely since restoring p53 function in a temperature sensitive *p53*-mutant cell line did not reduce expression of STAT1 or ISG15 in a 16 hour time period (Figure 5.3 B).

The second possibility is that defects in microRNA processing as a result of *p53* loss leads to activation of the dsRNA response, leading to IFN signaling. Previous work has identified numerous roles for p53 in miRNA biogenesis, at both the transcriptional level and

processing steps (43). If deregulated p53 function leads to inappropriate levels of pre-miRNAs in the cytoplasm, this could lead to the activation of a dsRNA response as demonstrated by Chiappinelli *et al.* (6). Cytosolic sensors such as RIG-1 and MDA-5 respond to dsRNA by signaling to downstream transcription factors that promote the expression of type I interferon (24). To test this hypothesis, miRNA expression arrays could be performed in Adeno-LacZ or – Cre treated  $p53^{Flx/Flx}$  MEFs to identify changes in levels of mature miRNAs. If a global or selective decrease in mature miRNAs is observed, then these miRNAs would have to be individually tested to see if the decreases are due to transcriptional or processing defects. Transcription would be tested by analyzing levels of the pri-miRNA. If levels of pri-miRNA are decreased in  $p53$ -deficient cells, then p53 is likely a transcriptional regulator. For miRNAs whose pri-miRNA levels either increase or do not change, pre-miRNA levels would be measured to assess if there are any processing defects. An increase of pre-miRNA levels in  $p53$ -deficient cells would suggest that a processing defect has occurred between the pre- and mature miRNA. These affects would presumably be rescued by Dicer1 overexpression, which is the key pre-miRNA processing enzyme (31). A decrease in pre-miRNA could also be due to a defect in export. However, a buildup of pre-miRNA in the nucleus would not be expected to initiate a dsRNA response.

More recently, it was shown by Schwitalla *et al.* that loss of p53 leads to NF- $\kappa$ B-mediated inflammation which promotes late-stage colorectal carcinogenesis (39). Several other studies have suggested a role for p53 in suppressing an inflammatory tumor microenvironment and inhibiting immune cell recruitment (15, 53). Additionally, the initial paper describing the creation of a  $p53$ -knockout mouse indicates that several mice lacking  $p53$  died from non-tumor associated causes. Upon examination, it was concluded that these mice died from unresolved

inflammatory reactions (10). Thus, a link between p53 and pro-tumorigenic inflammation is widely appreciated. One of the key mediators of inflammatory signaling is NF-kB (2, 19). Interestingly, p53 has been shown to inhibit NF-kB signaling (8, 40). Therefore, another hypothesis to explain IFN signaling in response to *p53* loss is the activation of NF-kB since it is a known activator of IFN- $\beta$  transcription (23). To examine this possibility, I would simply probe NF-kB activation in Cre-treated *p53<sup>Flox/Flox</sup>* MEFs by performing western blots for phosphorylation of p65 or I $\kappa$ B $\alpha$ . If evidence of NF-kB activation is observed, then shRNAs targeting p65 could be employed prior to Cre-mediated excision of p53. This would be predicted to completely protect p53-null cells from IFN-signaling. Pharmacological inhibition of NF-kB could also be employed.

#### **Determine the mechanism by which ARF inhibits the IFN response.**

My experimental data has indicated that ARF is able to suppress the IFN response induced by *p53*-loss. Since ARF is not an enzyme, the only known mechanism by which ARF regulates cellular targets is through physical association. Therefore, I hypothesized that ARF would interact with the STAT1 transcription factor to inhibit its ability to activate IFN-responsive genes. I performed immunoprecipitations with antibodies directed against ARF or STAT1 in dp53 MEFs 8 days post *p53*-excision. As shown in Figure 5.4A, ARF and STAT1 are capable of interacting in these cells. I also performed immunofluorescence staining of these cells to address the possibility that ARF might sequester STAT1 in the nucleolus, similar to its inhibition of NPM and MDM2. However, I was unable to detect co-localization of ARF and STAT1 (Figure 5.4 B). The observation that STAT1 and ARF can interact is certainly intriguing but further experiments are warranted. For example, I do not know if ARF interacts with un-

phosphorylated or phosphorylated STAT1. I hypothesize that if ARF is inhibiting STAT1 transcriptional function, then binding to phospho-STAT1 would be anticipated. However, there is evidence that un-phosphorylated STAT1 is also capable of entering the nucleus and activating transcription of immune response genes (5).

Additional experiments would be required to directly demonstrate that ARF inhibits STAT1 through physical binding. In particular, the amino acids that ARF utilizes to interact with STAT1 would be informative. ARF requires its N-terminal 14 amino acids for interaction with NPM and MDM2, so it is possible that ARF utilizes the same residues to interact with STAT1. I could therefore overexpress HA-tagged full length or  $\Delta$ 1-14 ARF in dp53 MEFs, perform IP's with an antibody against the HA-tag, and assess whether full length or  $\Delta$ 1-14 interacts with STAT1. I hypothesize that only full length ARF would be able to interact with STAT1. If  $\Delta$ 1-14 ARF is capable of interacting with STAT1, then a deletion panel of ARF would need to be generated to assess the required binding sites. If only full length ARF can interact with STAT1, then I would perform rescue experiments in dp53R-shARF cells using "wobble" mutants of ARF and  $\Delta$ 1-14 to demonstrate that only wild type ARF can suppress the IFN response. Additional studies such as STAT1 occupancy at IFN-responsive genes in the presence or absence of ARF would enhance my argument.

Of course, it is also possible that the ARF-STAT1 interaction is not biologically important. If this is the case, then other avenues of ARF-mediated IFN inhibition would need to be explored. I have already conducted an IP/Mass Spectrometry experiment in *p53*-null MEFs to discover novel ARF interacting proteins. As seen in Table 5.1, ARF interacts with a wide variety of proteins in *p53*-deficient cells. However, the experiment was clearly not exhaustive, as NPM and STAT1 are absent from the list of binding partners.

While none of the targets in Table 5.1 have established roles in the interferon response, the RNA helicases such as DDX50 and DHX30 are potentially interesting. RNA helicases play a major role in sensing viral infections and initiating the anti-viral response. Helicases such as RIG-1 and MDA-5 can bind to dsRNA, which allows them to interact with a mitochondrial associated protein called MAVS. This interaction leads to the activation of TBK1 and subsequent phosphorylation of the transcription factors IRF3, IRF7, and NF- $\kappa$ B (16). These transcription factors coordinate the upregulation of type I interferon, including IFN $\beta$ . ARF is known to interact with one RNA helicase, DDX5, and it also functions to inhibit mRNA translation of another, DHX33 (38, 54). DHX33 was recently shown to be involved in cytosolic viral RNA sensing, leading to the induction of NLRP3 inflammasome activation (27). Perhaps ARF regulates the expression, localization of function of RIG1 or MDA5. This hypothesis could easily be tested by immunoprecipitations and Western blots. The microarray data I generated by comparing dp53R-shSCR and dp53R-shARF MEFs shows a 2-fold induction of RIG1, but the standard deviation between my three experimental replicates was too high for it to be included in the final gene list. Thus, RIG1 mRNA induction could be validated by qRT-PCR, and western blots could be performed to assess RIG1 protein levels. If RIG1 mRNA and protein are both found to be elevated, then shRNAs targeting RIG1 could be used prior to ARF knockdown to test whether RIG1 induction is required for the IFN response.

### **Develop a mouse tumor model of ARF/p53 co-inactivation**

A mouse model of ARF/p53 co-inactivation would greatly accelerate our understanding of the pro-tumorigenic IFN signaling pathway that I have described in fibroblasts. Specifically, I propose to generate  $p53^{Flox/Flox}$  ;  $Arf^{Flox/Flox}$  ;  $Kras^{LSL-G12D}$  mice. These mice will be compared to



$p53^{+/+}$  ;  $Arf^{Flox/Flox}$  ;  $Kras^{LSL-G12D}$  ,  $p53^{Flox/Flox}$  ;  $Arf^{+/+}$  ;  $Kras^{LSL-G12D}$  , or  $p53^{+/+}$  ;  $Arf^{+/+}$  ;  $Kras^{LSL-G12D}$  controls. The easiest mouse tumor model to assess the consequences of loss of  $p53$ ,  $Arf$ , or both  $p53$  and  $Arf$  would be to utilize intra-nasal delivery of Adeno-Cre which would result in the inactivation (or activation) of these genes in the lung epithelium (11). This would mainly be due to the fact that we already have these animals at our facility. Mice would be monitored for tumor formation, and I would hypothesize that loss of both ARF and p53 would greatly accelerate lung tumorigenesis. Based on my *in vitro* data, I would predict that lung tumors that have lost both ARF and p53 would exhibit an upregulation of IFN signaling which could be measured by qRT-PCR and Western blot, as well as IHC.

Alternatively, a pancreatic cancer model could be developed by breeding the above mentioned mice to tamoxifen inducible  $Pdx1-Cre^{ER}$  mouse (52). While this would obviously be a more complicated breeding scheme, this model would be ideal to test novel therapeutics as this combination of genetic events occurs quite frequently in pancreatic tumors (36). Either of the above mentioned models would allow us to directly test whether loss of both p53 and ARF leads to enhanced tumorigenicity *in vivo*. While I am confident the mouse models would mirror my *in vitro* studies, it is certainly possible that equivalent tumor formation would be seen in mice having lost  $p53$  alone compared to  $p53/Arf$ . If this is the case, I could still look to see if activation of the IFN pathway is preferentially activated in the  $p53/Arf$  mice and use the models to try therapeutics that specifically target IFN $\beta$  signaling.

### **Determine how ISG15 promotes tumorigenesis in dp53R-shARF cells.**

In chapter 4, I demonstrated that ISG15 was required for the pro-tumorigenic phenotype in dp53R-shARF cells. However, I do not have a mechanistic understanding for how ISG15 can

promote this phenotype. Additionally, while most work has characterized the roles of conjugated forms of ISG15, increasing evidence suggests a key role for free ISG15 in cells (3). In my system, I have observed increases in both free and conjugated forms of ISG15 (Figure 4.12), so understanding which form of ISG15 is promoting proliferation is an important task. There are several ways in which this question could be addressed. First, UBE1L is the only known E1 enzyme for ISG15 conjugation, so depleting UBE1L from dp53R-shARF MEFs using shRNAs would directly test if conjugated ISG15 is required for the pro-tumorigenic phenotype (22). This approach could be challenging if the available shRNAs do not sufficiently reduce UBE1L levels. An alternative approach would be to perform rescue experiments of ISG15 depletion with ISG15 wild type or mutant cDNAs. Precursor ISG15 proteins are cleaved at their C-terminus revealing an LGLRGG motif that is required for conjugation to lysine residues (32). Rescue experiments in dp53R-shARF-shISG15 cells performed with wild type ISG15-LRLRGG, or a non-conjugatable mutant ISG15-LRLRAA, would specifically address whether free or conjugated ISG15. If the LRLRAA-ISG15 mutant is not capable of rescuing proliferation and growth in soft agar, then I would conclude the conjugated form of ISG15 is required.

ISG15 is also secreted from cells, although the physiological significance of this is not well understood (3). In my *in vitro* system, I do not know whether ISG15 is secreted from dp53R-shARF cells, so I would first need to measure ISG15 levels in cell culture supernatants using either ELISA or Western blot. If ISG15 is indeed secreted, then I would use neutralizing antibodies targeting ISG15 in dp53R-shARF cells to see if proliferation or soft agar growth was affected.

If the above mentioned experiments indicate that it is the conjugated form of ISG15 which is required for enhanced tumorigenicity of dp53R-shARF MEFs, then I would propose to

uncover the important ISGylated proteins. To accomplish this, I would perform ISG15 immunoprecipitations from dp53R-shSCR or dp53R-shARF MEFs to pull down ISG15 conjugated proteins. I would then submit these complexes for LC/MS analysis. Similar approaches have been used in the past to identify ISGylated proteins (12, 44). Identified proteins would be sorted based on their potential to affect tumorigenic properties. Since ISGylation can promote or inhibit protein function, both negative and positive regulators of tumorigenicity would be considered (51). I would knockdown the tumor promoting proteins with shRNAs, and attempt to rescue the tumorigenic phenotype with wild-type or mutants that are not able to be ISGylated. If ISGylation is inhibiting the function of a tumor suppressor protein, then overexpression of the protein should be able to reduce tumorigenicity. Knockdown-rescue experiments could also be employed with wild-type or mutant (unable to be ISGylated) proteins to test whether the non-ISGylated version is capable of reducing tumorigenicity.

**Explore other mechanisms to explain enhanced tumorigenicity mediated by IFN- $\beta$  signaling.**

As shown in Figure 4.13B, depletion of ISG15 in dp53R-shARF MEFs does not reduce macroscopic colony number to levels seen in cells expressing ARF. Thus, there are likely other contributing factors besides ISG15 to the enhanced tumorigenicity of dp53R-shARF MEFs. One observation that I consider worth exploring further is the phosphorylation of STAT1 on serine-727. Phosphorylation of this residue has been shown to be important in promoting Wilm's tumor growth (46), and has more recently been demonstrated to protect incipient cancer cells from NK-mediated cytotoxicity (34). Numerous kinases have been found to be capable of phosphorylating STAT1 on this site, including CDK8 (1), p38-MAPK (14), PI3K (28), and PKC- $\delta$  (48). I

propose to determine which of these kinases is responsible for serine-727 phosphorylation in dp53R-shARF cells by using both pharmacological and genetic approaches. I will first treat the dp53R-shARF MEFs with kinase inhibitors to see which of the above-mentioned pathways is responsible in my cell type. Once a kinase is identified, I will use shRNAs to validate the specific kinase is responsible for serine-727 phosphorylation.

My data suggests that serine-727 phosphorylation of STAT1 inversely correlates with ARF status in TNBC cell lines (Figure 5.5 A and B). I propose to analyze tumor microarray samples by IHC to determine whether this correlation exists in human tumor samples. Additionally, it would be interesting to see whether treatment of the TNBC cell lines with the kinase inhibitor discussed above would inhibit cell proliferation. These studies could uncover a potential therapeutic option for tumors lacking ARF and p53 function.

Another area that I would like to explore is the concept of inflammation. It is widely viewed that inflammation plays a vital role promoting tumorigenesis (9, 26). A recent study described how loss of *CKI $\alpha$*  in the mouse gut epithelium activates WNT-signaling and can induce a low-level inflammatory response that is associated with cellular senescence. The authors of this study went on to show that loss of *p53* in these cells allows for local invasion and carcinogenesis, which could be completely abrogated by NSAID (Non-steroidal anti-inflammatory drug) treatment (33). This study suggests that cells respond differently to inflammatory signals depending on p53 status. Perhaps dp53R-shARF cells are experiencing the effects of chronic inflammation and since they lack p53, the signaling is interpreted as pro-proliferative.

My microarray data obtained from dp53R-shARF MEFs did not contain any established pro-inflammatory cytokines. However, careful examination of the microarray data comparing

dp53R-shSCR to dp53R-shARF cells has revealed several interesting genes that were initially discarded due to stringent statistical approaches. Two of the genes in the heat-map in Chapter 4 (Figure 4.1), *Usp18* and *Irf7*, were not part of the initial upregulated gene list, but I was able to validate their increased expression by qRT-PCR from three independent experiments. Likewise, the pro-inflammatory chemokine, CXCL10, is 4-fold overexpressed in shARF cells, but was not included in the final list because the standard deviation value surpassed a stringent threshold. CXCL10 is a member of the CXC chemokine family, and binds to its receptor, CXCR3, to initiate a host of cellular responses. CXCL10 can activate signaling pathways involved in promoting cell proliferation such as MAPK and PI3K (25). Thus, I believe a more careful analysis of pro-inflammatory cytokines is warranted in dp53R-shARF MEFs. This could be performed using a qPCR array or an ELISA array from Qiagen.

**Determine whether inhibitors of the IFN- $\beta$ -STAT1-ISG15 signaling axis can inhibit proliferation of dp53R-shARF MEFs.**

I have demonstrated that the IFN- $\beta$ -STAT1-ISG15 signaling axis is oncogenic in the absence of both ARF and p53. Therefore, targeted inhibition of this pathway represents a potential therapeutic option for tumors harboring these specific genetic defects. I have obtained a selective JAK1/JAK2 inhibitor called Baricitinib that is in phase II clinical trials for the treatment of rheumatoid arthritis. Treatment of dp53R-shARF MEFs with increasing concentrations of Baricitinib reduces both phosphorylation of STAT1 and STAT3, validating its role as a JAK1/JAK2 inhibitor (Figure 5.7 B). I propose to test the effects of Baricitinib treatment on the proliferation and tumorigenicity of dp53R-shARF MEFs. I would perform these experiments *in vitro* and also utilize the mouse allograft model that I introduced in Chapter

2 (Figure 2.12). If treatment with Baricitinib alone does not produce a therapeutic effect, then combination with other drugs could be tested, such as those inhibiting PI3K or ERK. It would also be interesting to test the relative sensitivities of numerous cancer cell lines that exhibit activation of IFN signaling.

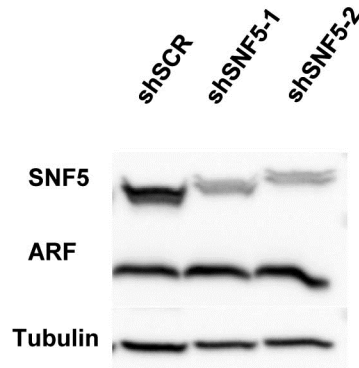
The fact that Baricitinib treatment reduces phosphorylation of STAT1 clearly indicates that either JAK1 or JAK2 is responsible for STAT1 phosphorylation. However, I have been unable to demonstrate increased activation of JAK1 or JAK2 in dp53R-shARF cells (Figure 5.6 A and Jason Forys unpublished observation). To directly test which JAK is required for the IFN response in dp53R-shARF MEFs, I would use specific shRNAs to knockdown JAK1, JAK2, JAK3, and TYK2.

Another approach to inhibiting interferon signaling is the use of neutralizing antibodies. I have performed experiments using interferon alpha/beta receptor-1 (IFNAR-1) neutralizing antibodies that were generated in Robert Schreiber's lab at Washington University (41). As seen in Figure 5.7 A-B, treatment of dp53R-shARF MEFs with anti-IFNAR1 antibodies (MARI-5A3) greatly reduces phosphorylation of STAT1 and expression of interferon-responsive genes. To our surprise, anti-IFNAR-1 treatment did not affect proliferation, growth in soft agar, or foci formation of the dp53R-shARF MEFs (Figure 5.7 C-E). Further studies will be required to explain this phenomenon, including the selective neutralization of IFN- $\alpha$  and IFN- $\beta$ . It is possible that targeting the IFN signaling pathway this far upstream will not be an effective therapeutic approach. Perhaps both pro- and anti-growth signals are occurring through the IFNAR receptor in these cells. As a result, when all the receptors are blocked there is no net change in proliferation. There is evidence suggesting interferon-alpha and -beta can activate distinct transcriptional programs even though they bind the same receptor (47). I have not been

able to detect IFN- $\alpha$  with a pan-qRT-PCR primer, but I have observed an upregulation of the IFN $\alpha$ 4 isoform in dp53R-shARF MEFs (unpublished observation).

Clearly, there is much work to be done before my work can be translated into a clinical setting. The experiments outlined in this section, as well as the other future directions that I have proposed, will provide insight into the selective advantage gained from IFN signaling. My expectation is that this signaling pathway can function as a driver of tumorigenesis in many tumor types, and likely does not strictly depend on ARF and p53 co-inactivation.

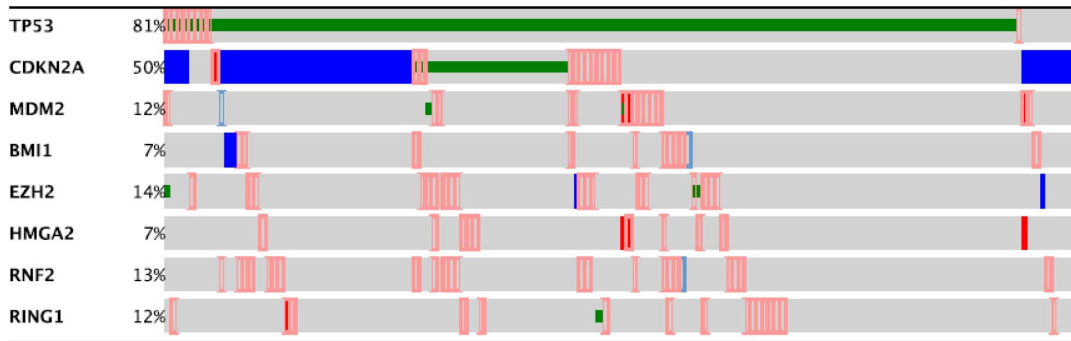
## FIGURES



**Figure 5.1** SNF5 is not required for ARF induction following p53 loss.

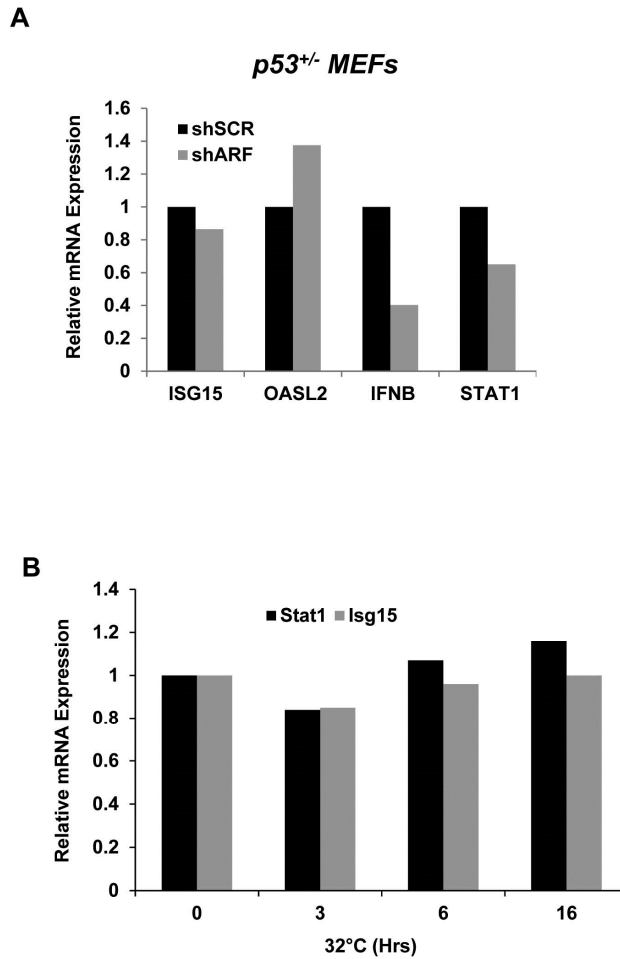
*p53<sup>Flox/Flox</sup>* MEFs were infected with Adeno-LacZ or Adeno-Cre. One day post Ad-Cre infection, cells were infected with the indicated shRNAs and selected with puromycin. Six days post Cre-infection, cells were harvested and analyzed by Western bot.





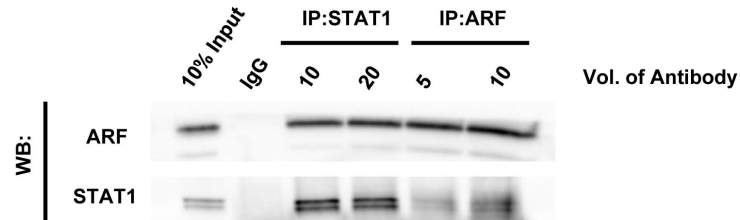
**Figure 5.2** Upregulation of Arf transcriptional repressors occurs in p53 mutant lung squamous cell carcinoma tumors.

The cBioPortal search tool was used to interrogate genetic status/expression levels of the indicated genes. Columns represent individual lung squamous cell carcinoma tumor samples. Green boxes indicate mutations, blue-filled boxes indicate homozygous deletions, red-filled boxes indicate amplification, and red outlined boxes indicate mRNA overexpression.

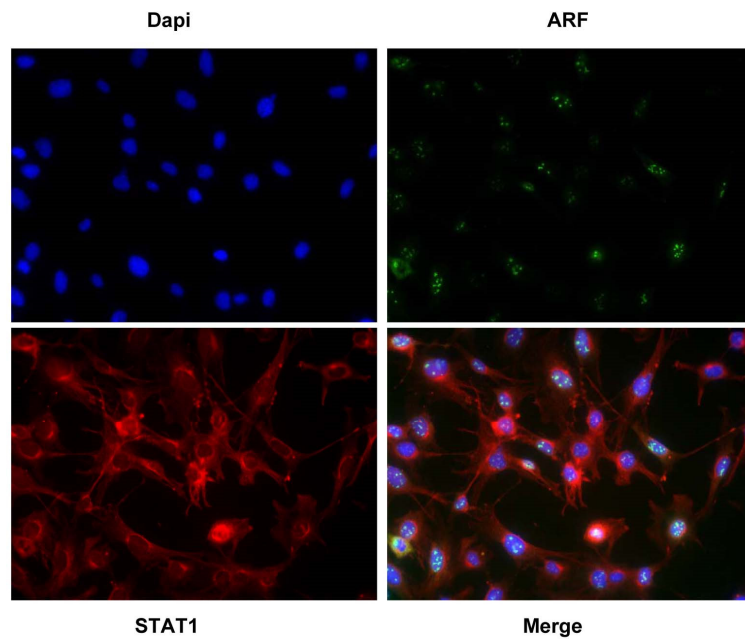


**Figure 5.3** Complete p53 loss is required to induce IFN signaling in Arf depleted cells, and p53 does not directly suppress IFN expression. (A) p53-heterozygous MEFs were infected with shSCR or shARF. Cells were harvested six days post-infection and RNA was analyzed by qRT-PCR for the indicated target genes. (B) A p53-null mouse leukemia cell line was transduced with empty-vector or a temperature sensitive p53-mutant. Both cell lines were shifted to the permissive temperature of 32°C for the indicated number of hours. RNA was extracted and qRT-PCR analysis for the indicated genes was performed. Data is presented relative to empty vector and normalized to histone 3.3 mRNA.

**A**



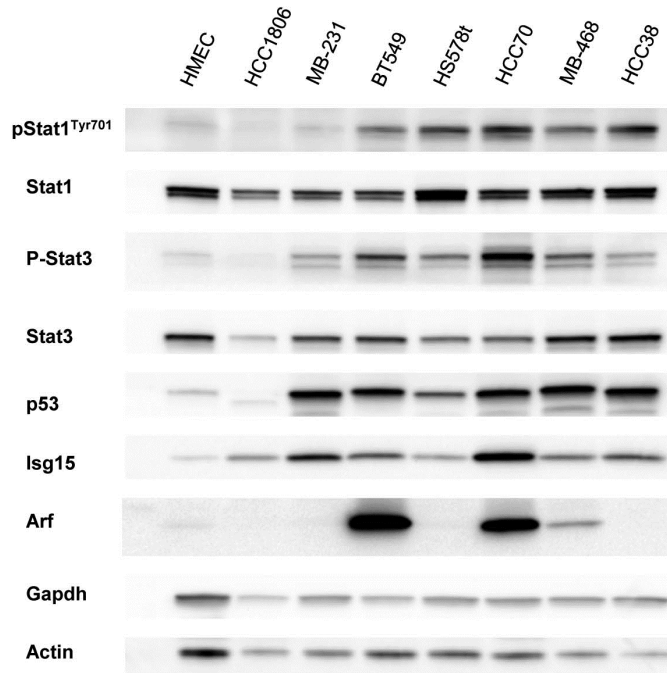
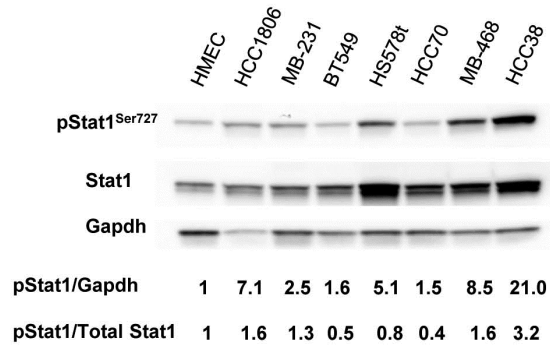
**B**



**Figure 5.4** ARF and STAT1 are capable of physically interacting, but ARF does not sequester STAT1 in the nucleolus (A) Immunoprecipitation of ARF or STAT1 was performed using lysate from dp53 MEFs, 8 days post Cre-mediated p53 excision. Immunoprecipitated complexes were analyzed by Western blot for the indicated proteins. (B) dp53 MEFs were fixed in methanol:acetone and stained using immunofluorescence. Blue=Dapi, Red=STAT1, Green=ARF.

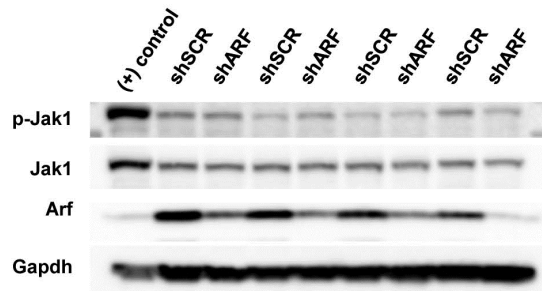
Gene	Description
Tubb2c	Tubulin-beta-2C chain
Rpl31	60S ribosomal protein L31
Rps7	40S ribosomal protein 7
Bclaf1	Bcl-2-associated transcription factor 1
Flnb	Filamin
Kif26b	Kinesin-like protein
Dhx30	Putative ATP-dependent RNA helicase 30
Acot9	Acyl-coenzyme A thioesterase 9
Igf2bp3	Insulin-like growth factor 2 mRNA binding protein 3
Rpl23a	60S ribosomal protein L23a
Ddx17	Probable ATP-dependent RNA helicase 17
Sfrs3	Splicing factor, arginine/serine rich 3
Ddx50	ATP-dependent RNA helicase 50
Rbm39	RNA-binding protein 39
Kiaa1370	Uncharacterized protein
Tuba3a	Tubulin alpha-3
Aldh18a1	Delta-1-pyrroline-5-carboxylate synthetase
Hnrnpul2	Heterogeneous nuclear ribonucleoprotein U-like protein
Eef1a1	Elongation factor 1-alpha 1
Myl6	Putative uncharacterized protein
Nop58	Nucleolar protein 58
Hnrnp1	Heterogeneous nuclear ribonucleoprotein H
Hspa9	Stress-70 protein, mitochondrial
Phb2	Prohibitin
Calm1	Calmodulin
Nxf1	Nuclear RNA export factor 1
Hadha	Trifunctional enzyme subunit alpha, mitochondrial
Fytd1	UAP56-interacting factor
Ywhaz	14-3-3 protein zeta/delta
Thrap3	Thyroid hormone receptor-associated protein3
Snrnp70	U1 small nuclear ribonucleoprotein 70
Sfrs1	Splicing factor, arginine/serine-rich 1
Flna	Filamin-A

**Table 5.1** List of ARF binding partners in dp53 MEFs

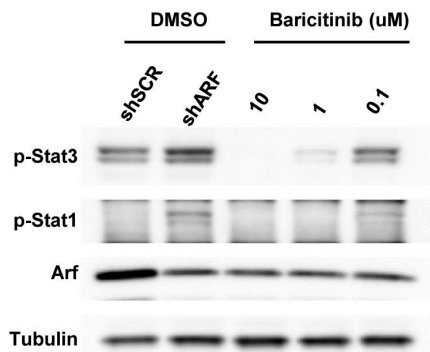
**A****B**

**Figure 5.5** STAT signaling in a panel of TNBC cell lines. **(A)** Western blot analysis of a panel of TNBC cell lines. Normal human mammary epithelial cells were used as a control. **(B)** Western blot analysis of same cell line panel described in (A). Phospho-STAT1 Ser-727 levels were quantified by first normalizing to GAPDH and then to total STAT1 levels.

**A**



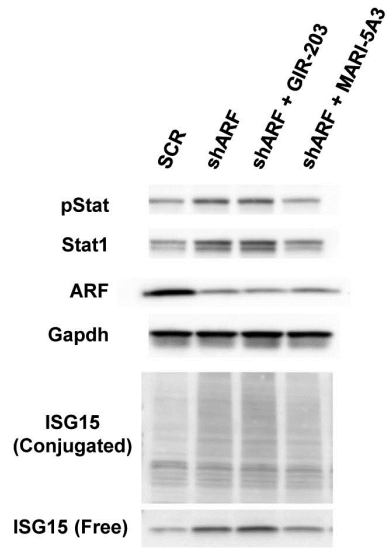
**B**



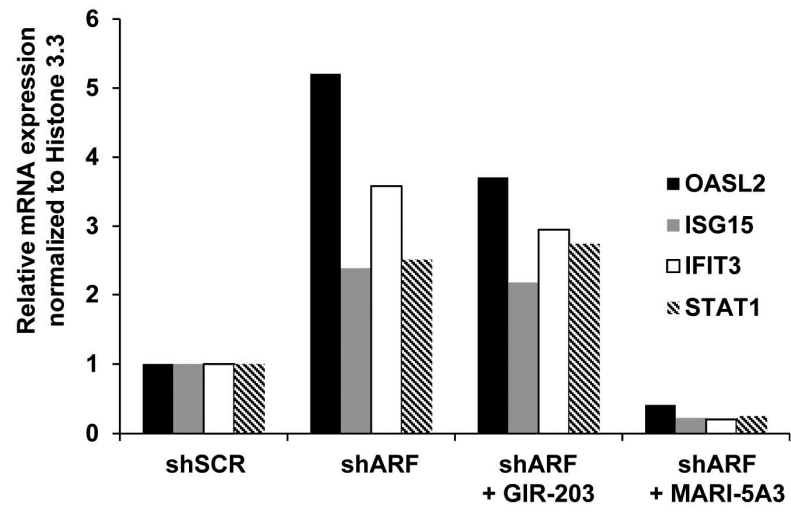
**Figure 5.6** Analysis of JAK1 activation and Baricitinib treatment in dp53R-shARF MEFs.

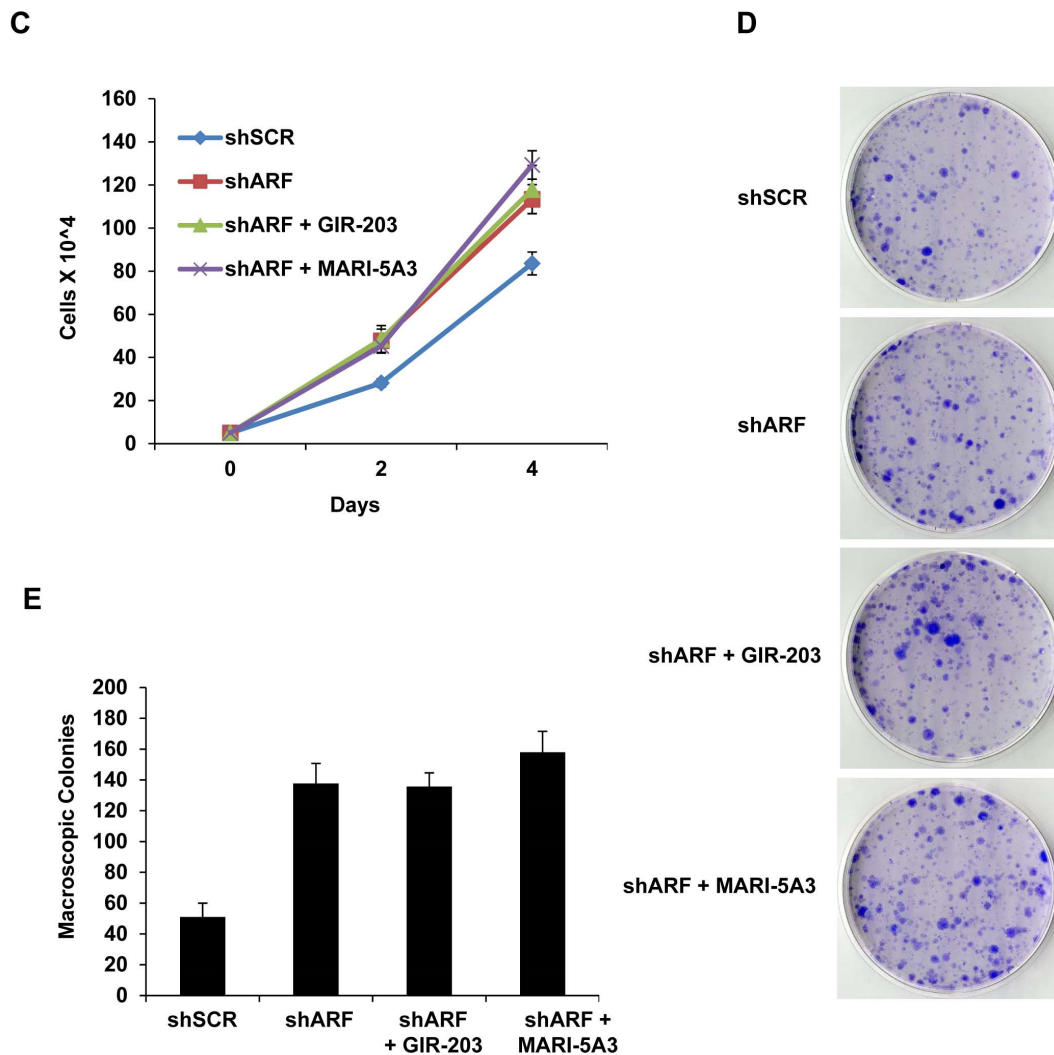
(A) Western blot analysis of JAK1 phosphorylation in four independent experiments comparing dp53R-shSCR and dp53R-shARF MEFs. As a positive control, dp53 MEFs were treated with 500U/mL recombinant IFN- $\beta$  for one hour. (B) dp53R-shARF MEFs were treated with the indicated amounts of Baricitinib overnight. Equivalent amounts of DMSO were added to cells as a control. Western blot analysis was performed to assess the phosphorylation status of STAT1 and STAT3.

**A**



**B**





**Figure 5.7** IFNAR-1 neutralizing antibodies do not affect the tumorigenesis of dp53R-shARF MEFs. (A) Western blot analysis of dp53R-shARF MEFs treated for 24 hours with 10 $\mu$ g/mL of a control IgG (GIR-203) or antibodies specific to IFNAR-1 (MARI-5A3). (B) Cells described in (A) were harvested and RNA was extracted. qRT-PCR analysis was performed to determine mRNA expression of the indicated genes. mRNA levels were normalized to histone 3.3 mRNA and fold-changes are relative to shSCR controls. (C) dp53R MEFs expressing shSCR or shARF were plated in 6-well dishes. Where indicated, cells were treated with 10 $\mu$ g/mL of GIR-203 or MARI-5A3. Antibodies were replenished every 2 days, and cell



number was measured by trypsinization and counting with a hemocytometer. **(D)** Cells (3000) were plated in 10 cm dishes and treated with antibodies as described in (C). 10 days post plating, colonies were fixed with methanol and stained with Giemsa. **(E)** Cells ( $1.5 \times 10^4$ ) were plated in soft agar that contained  $50 \mu\text{g}/\text{mL}$  of the indicated neutralizing antibodies. Fresh media containing  $10 \mu\text{g}/\text{mL}$  of antibody was replenished every 3 days. After incubating for 21 days, the soft agar plates were stained with crystal violet and macroscopic colonies were quantified. Error bars represent s.d. of  $n=3$ .

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