2013

Defining the functions of a conserved hydrophobic domain in the ARF tumor suppressor

Aymen Hariz

Virginia Commonwealth University

DEFINING THE FUNCTIONS OF A CONSERVED HYDROPHOBIC DOMAIN IN THE ARF TUMOR SUPPRESSOR

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

Aymen Faris Hariz, B.Sc.

Lebanese University, 2004

Director: Steven R. Grossman, MD, PhD

Professor

Department of Hematology, Oncology and Palliative Care

Virginia Commonwealth University

Richmond, Virginia

April, 2013

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all the people who helped me with resources and guidance throughout this study.

I am grateful to my thesis advisor Dr. Steven Grossman for giving me this opportunity and supporting me in my quest to fulfill my research work under his supervision. I am indebted to Dr. Grossman for his time, extensive knowledge, patience and most of all for his care and advice. I am also thankful to the jury for their participation and invaluable suggestions.

I am especially appreciative to all the efforts and contributions that former and current members of the Grossman lab have provided me, in particular Dr. Priyadarshan Damle who was a second mentor to me during my research in the Grossman laboratory.

Above all, I am forever indebted to my wife and two daughters, for their understanding and support during the past two years and all the sacrifice they have made for my success.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α……. alpha

β…….beta

γ……..gamma

µg…… microgram

µl…….microliter

ml…… milliliter

V……. volt

rpm…… rotation per minute

rcf…….. relative centrifugal force

J……. joules

%..... Percent

-/-…….. Null

L…… leucine

D…… aspartic acid

S…… Serine

aa……. amino acid

CLL…… chronic lymphocytic leukemia

SLL…… small lymphocytic leukemia

ARF…… alternative reading frame

INK…….. Inhibitor of Kinase

ATM…… Ataxia Telangiectasia mutated

ATR…….Ataxia Telangiectasia and Rad3 related

hARF…… human alternative reading frame

mARF…… mouse alternative reading frame

PMEF…… Primary mouse embryonic fibroblast

H-Ras…… Harvey rat sarcoma viral oncogene homolog

smARF…….. small mitochondrial ARF

CHK1…… check point kinase

HDAC…….. Histone deacetylase

WRN…… Werner protein

Sumo…… small ubiquitin-like modifier

NADH……. Nicotine amide adenine dinucleotide hydride

HEK…… Human embryonic kidney

DMEM…… Dulbecco's modified eagle medium

FBS…….. Fetal bovine serum

EMT……. Epithelial to mesenchymal transition

GFP……... Green fluorescent protein

PcG ……... Polycomb protein complex

OIS…… Oncogene induced senescence

SAHF……. Senescence associated heterochromatin foci

γH2AX…… Phosphorylated variant of histone H2A

Ki67…… Cellular marker for proliferation

PAI-1……..Plasminogen activator inhibitor

P…….. Passage number

MAPK……. Mitogen activated protein kinase pathway

NSCLC…… Non small cell lung cancer

EDTA……. Ethylene-di-amine-tetra-acetic acid

Dox…… Doxorubicin

SA-β-gal…… Senescence associated beta galactosidase

EMT…….. Epithelial mesenchymal transition

CtBP…….. C-terminal binding protein

DMSO…… Dimethyl sulfoxide

ABSTRACT

DEFINING THE FUNCTIONS OF A CONSERVED HYDROPHOBIC DOMAIN IN THE ARF TUMOR SUPPRESSOR

By: AYMEN FARIS HARIZ

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2013

Director: Steven R. Grossman, MD, PhD
Professor
Department of Hematology, Oncology and Palliative Care

The INK4A-ARF locus encodes two tumor suppressors; p16 and $p19^{Arf}$, both of which restrain cell growth by regulating the functions of Retinoblastoma (Rb) and p53 respectively. Throughout development, $p19^{Arf}$ is kept at minimal levels, but under conditions of oncogenic stress, $p19^{Arf}$ expression is induced and its tumor suppressive activities are mediated through the stabilization of p53 or in a p53-independent manner. Introducing a point mutation (L46D) into the conserved hydrophobic domain (37-51) in $p19^{Arf}$ annulled ARF/CtBP2 interaction and mediated cell

survival by rendering cells irresponsive to apoptosis. In vivo analysis on the percentage of lymphoma free survivals in ARFL46D/L46D mice indicated that disrupting ARF/CtBP2 binding resulted in a tumor spectrum similar to that in ARF-null mice. In this study, we characterized the functions of the hydrophobic domain of ARF in MEF cells under genotoxic stress, ultra-violet irradiation and oncogene activation. We demonstrated that cells bearing the mutation showed decreased responsiveness to H-RasV12 induced senescence consistent with p21 deficiency. We speculate that the deficit in p21 expression is possibly caused by CtBP2 repressive and oncogene -like properties.

CHAPTER I

INTRODUCTION

I.1 Cancer: progression or suppression

Carcinogenesis is a process that develops through a series of insults at the genetic and epigenetic levels ultimately leading to the loss of homeostasis and control of cell division. Axiomatic requirements of cancer cells include: 1) capacity to proliferate irrespective of exogenous mitogens; refractoriness to growth inhibitory signals; resistance to apoptosis; unrestricted proliferation potential; capacity to recruit vasculature; ability to invade surrounding tissue and metastasize; reprogramming of energy metabolism and evading immune destruction (Hanahan & Weinberg, 2011). At the molecular level, interplay among tumor suppressors and proto-oncogenes regulates the progression of the cells throughout the cell cycle. In cases where tumor suppressor genes fail to function increase the risks of developing cancer. In this context, mutations in p53 and the INK4A/ARF locus are the two most common genetic lesions identified in human tumors (Ruas & Peters, 1998). A previous study on 37 patients with Adult T-cell leukemia (ATLL) identified inactivation deletion mutations in $p14^{ARF}$ associated with over 35% of the cases with poor prognosis and therefore reflecting the significance of the INK4A/ARF locus in tumor suppression and homeostasis.

Data from murine models with targeted deletions of $p16^{INK4a}$ and $p19^{Arf}$ suggests that mice with either deletion are prone to tumor formation and animals with both deletions are more

severely affected with histiocytic lymphoma similar to human Non-Hodgkin Lymphoma (NHL) comprising up to 90% of tumors (Sharpless, 2005). The tumor spectrum of ARF-null mice was similar to p53 knockout mice with the highest frequency of tumors being lymphocytic lymphoma of the thymus and/or lymph nodes (Table1). Although similar, p53 null mice developed T- cell lymphomas at a frequency of 70 % as opposed to small lymphocytic lymphoma arising in ARF-null mice at a lower frequency and longer mean latency of 38 weeks (Sharpless, 2005). Both tumor suppressor deletions also led to sarcoma formation as well (Ozenne, Eymin, Brambilla, & Gazzeri, 2010). Mitogen activated tumorigenesis revealed increased incidence in the frequency of all tumors in the various knockout mice models with additional tumor types (lung adenocarcinoma) now evident (Wang, Zhang, Kastens, Lubet, & You, 2003).

I.2 The INK4B - INK4A/ARF Locus

The INK4B-ARF-INK4A locus encodes two known tumor suppressors of the INK4 family proteins $p15^{INK4b}$ and $p16^{INK4a}$ respectively. The INK4A/ARF locus is unique in mammals in the sense that it encodes two structurally unrelated proteins CDKN2A ($p16^{INK4a}$) and $p19^{Arf}$ in mice / $p14^{ARF}$ in human, due to overlapping but frame shifted coding sequences (Gil & Peters, 2006). The locus is roughly 25Kb long, shorter than most mammalian loci, and consists of 4 exons (1α, 1 β, exon2 and exon3).

Transcription initiation proceeds at two distinct promoters upstream of exons 1 α and 1 β with 13Kb of intervening sequences separating the two exons in the mouse genome (Sherr, 1998). Both exons 1 α and 1 β share the same splice acceptor site with exon 2(Quelle, Zindy, Ashmun, & Sherr, 1995). Transcripts originating from exon 1 α lead to $p16^{INK4a}$ gene product whereas transcripts initiating from the promoter sequence upstream of exon 1β produces alternative reading frame transcripts ($p19^{Arf}$). Although exon 2 is fully transcribed in either gene product,

$p16^{INK4a}$ and $p19^{Arf}/p14^{ARF}$ share no homologous domains between their amino acid sequences (Figure 1). This variation is due to the initiation of transcription from overlapping reading frames and alternative splicing.

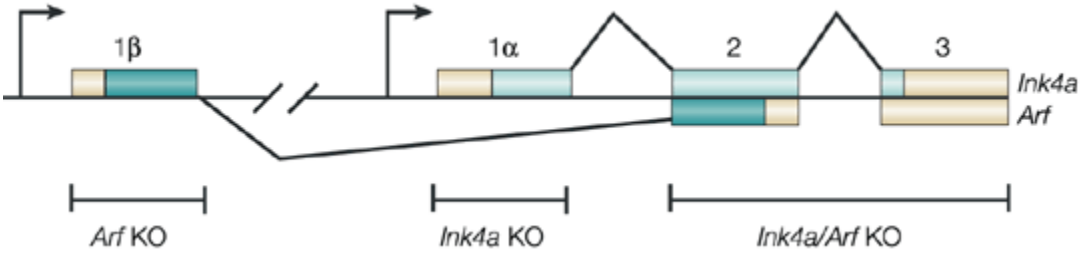


Figure 1. The INK4A-ARF locus. Arrows indicate transcription initiation sites. Segments indicate the genomic region(s) targeted to establish the required knock-outs. (Nature Reviews)

I.2.1 Evolutionary significance of INK4A/ARF locus

The INK4A/ARF is an unusual dual coding-region located on chromosome 9p21 in humans and on chromosome 4 in mice. From an evolutionary point of view, mammalian genomes are more complex than their marine ancestors. Genome analysis of Zebra fish and Fugu (Japanese puffer fish) showed that the INK4B-INK4A and INK4C-INK4D loci produce single p15/p16 and p18/p19 like member proteins. There is no evidence that ARF-like ortholog exists in the genome or the epigenome of Zebrafish and Fugu leading to the suggestion that ARF has been incorporated into the genome of vertebrates after a duplication event which ultimately led to splitting the INK4B-INK4A and INK4C-INK4D loci into 4 distinct coding regions (Sharpless, 2005). So why do vertebrates require an additional INK4 protein? Is there an evolutionary advantage in duplicating the INK4 loci and thereby introducing ARF into the genomic DNA? Is it economical for cells to initiate transcription from two separate promoters and therefore fuel more biological processes? Evidence from functional analysis in human and mice, document that the transition from water to land resulted in a shift in the potency of tumors, and thus provided more demand for tumor suppressors (Sharpless, 2005).

Table 1. Tumor profile in several INK4A/ARF knock-out mouse models

Genotype	Tumor spectrum and Penetrance		Spontaneous tumor mean latency in weeks	MEF properties
	Spontaneous	Mitogen Induced (DMBA)		
p53 -/-	Lymphoma 77%; Sarcoma23%	NA	20	Immortal
ARF -/-	Small Lymphocytic Lymphoma 37%; Sarcoma 33%	Small Lymph. Lymphoma 60% Lung carcinoma 40%	38	Immortal, Transformed by H-Ras V12
INK4A -/-	Sarcoma 52% Histiocytic Lymphoma 18%	Small Lymphocytic Lymphoma 39% Lung carcinoma 17%	68	Resemble Wild type; Undergo senescence
INK4A/ARF-/-	Histiocytic Lymphoma 90% Sarcoma 10%	NA	34	Immortal; Transformed by H-Ras V12

I.2.2 Transcriptional regulation of the INK4A/ARF locus

Regulation of transcription from the promoters of INK4A and ARF is kept under tight control in normal proliferating cells. In particular, early passage mouse and human embryonic cells show undetectable amounts of Arf mRNA and protein (Lowe & Sherr, 2003). Continuous passaging of mouse and human embryonic fibroblasts led to the buildup of both p16 and p19 with supporting evidence that the latter is more involved in protecting MEF cells from hyperproliferative/oncogenic signaling (Gil & Peters, 2006). As mentioned previously knockout mice for INK4A and ARF are more prone to tumor formation compared to INK4A or ARF knockout mice. The most accepted paradigm in characterizing the significance of each of the two tumor suppressors is that $p16^{INK4a}$ is more critical in inducing senescence and tumor suppression in human embryonic fibroblasts, whereas $p19^{Arf}$ is primarily the key player in these functions in mouse embryonic fibroblasts with compelling evidence from knock-out mouse models(Sharpless et al., 2001) . MEF cells from ARF-null mice bypassed spontaneous induced senescence in culture and rendered ARF-null cells immortal (Gil & Peters, 2006).

Several oncogenic activators have been identified in terms of their association with transcription initiation from the ARF promoter either directly or indirectly. For instance, mutant forms of Ras in MEF cells lead to ARF induced senescence by increasing the expression of ARF from its promoter indirectly through the activation of DMP1(cyclin D binding Myb-like protein) transcription factor that was found through chromatin immunoprecipitation studies bound to nonameric consensus sequences CCCG(G/T)ATG(T/C) in the ARF promoter dislodging repression caused by E2F-3b complex(Sreeramaneni, Chaudhry, McMahon, Sherr, & Inoue, 2005). Previous studies showed that the oncogenic signaling through H-Ras V12 activates DMP1

through the RAF-MEK-ERK pathway, which results in the activation of transcription factors that belong to the Fos and Jun family, and their subsequent binding to 5' leader sequence responsive elements in the mouse DMP1 promoter resulting in an increase in the DMP1 pool in the nucleus (Sreeramaneni et al., 2005).

Activation of transcription from the ARF promoter by DMP1 is a multistep process. The ARF promoter contains DMP1 and E2F binding sites. In normal unstressed cells, the E2F3b complex represses transcription of ARF (Asp, Acosta-Alvear, Tsikitis, van Oevelen, & Dynlacht, 2009) by binding polycomb protein Bmi-1 and thus establishing transcriptionally silent chromatin. In the presence of hyperproliferative/oncogenic stress caused by H-Ras V12, cyclin D is activated and assembles with CDK4, enabling the dissociation of E2F1,2 from Retinoblastoma (Rb) and thereby allowing DMP1 induced transcription from the ARF promoter (Sreeramaneni et al., 2005). In addition to the indirect transcriptional activation by H-Ras, transcription of ARF is mediated indirectly through other oncogenic proteins such as c-Myc, which in contrast to H-Ras, results in p53-independent apoptosis as opposed to premature senescence (Boone, Qi, Li, & Hann, 2011). Twist and Dermo two well-characterized transcriptional repressors of the ARF promoter, were identified in a screen for proteins that bypass Myc induced apoptosis (Table2). Moreover, INK4A/ARF-null mouse models compensated for the loss of Bmi-1 polycomb repressor protein, which is evidence that INK4A and ARF are downstream targets of Bmi-1 mediated transcriptional repression, and ARF is required to be expressed at least minimal levels for proper mouse development (Sharpless & DePinho, 1999).

Table 2. List of activators and repressors of the INK4A/ARF locus

Activator	Repressor
DMP1	pRb
E2F1,E2F2,E2F3	p53
Ras	Twist
Myc	TBX2,TBX3
c-Jun	ATM
ABL	E2F3b
B-catenin	JUND

I.2.3 The INK4A/ARF locus in tumor suppression and cell cycle regulation

Several lines of evidence suggest that the INK4A/ARF gene products $p16^{INK4a}$ and ARF (p19 in mouse, p14 in human) are potent tumor suppressors and involved in cell cycle regulation. Mutations targeting INK4A/ARF locus are common and are second in frequency to TP53 mutations in humans. Previous studies showed that homozygous and hemizygous deletions and epigenetic silencing of the $p14^{ARF}$ promoter were detected in 41% of individuals out of 100 surveyed patients diagnosed with primary breast carcinomas (Yi et al., 2004). Although p16 and p19 are encoded from the same locus, their expression is not the same in all tissues in human and mice. In a separate study, epigenetic silencing of $p14^{ARF}$ promoter was documented in human colorectal cancer cells (Esteller et al., 2001). Interestingly, in 14 out of 22 NSCLC with p16 mutations, p16 inactivation also resulted in $p14^{ARF}$ inactivation through homozygous deletions extending into the INK4A/ARF along with two point mutations in exon 2 (Sanchez-Cespedes et al., 1999). Recently, it has been shown that H3K27Me3, which is an indicator for transcriptional silencing was strongly associated with the ARF locus in T- ALL leukemia- initiating cells {{67 Volanakis,E.J. 2012}}. So far, no point mutations in exon 1 β have been reported. As mentioned previously, the INK4A locus encodes a CDK inhibitor known as $p16^{INK4a}$. The original paradigm

in defining the functional differences between $p19^{Arf}$ and $p16^{INK4a}$ was determined through studying changes in protein expression in human cells undergoing replicative or premature senescence and their association with cellular immortalization either due to cell culture shock or upon ectopic stress. These studies, led to the identification of retinoblastoma protein (Rb) and p53 as direct downstream targets of p16 and p19 respectively (Ruas & Peters, 1998). Based on that, two pathways have been identified with which signaling through either p16 or p19 takes place. Convincing evidence suggests that ARF is the primary tumor suppressor in mice encoded by the INK4A/ARF locus, since $p16^{INK4a}$-null MEFs are phenotypically undistinguishable from wild type primary mouse embryonic fibroblasts and are not sensitive to H-Ras V12 transformation (Ozenne et al., 2010). The $p16^{INK4a}$ - Rb pathway functions in tumor suppression by regulating cell proliferation through the repression of the genes which are responsible for the progression through the G1 phase of the cell cycle and entry into the S phase. Depending on the phosphorylation state of retinoblastoma, Rb binds E2F and inhibits the transcription of cell cycle proteins in its hypophosphorylated state by recruiting histone deacetylases thus making the promoters less accessible for transcription initiation. The phosphorylation status of Rb is regulated by cyclin dependent kinases 4 and 6 (CDK4,6), which themselves are activated in response to increasing levels of cyclin D1, and kept under tight regulation by the cdk inhibitory functions of p16INK4A (Sherr & McCormick, 2002). Mitogenic signaling through Ras/MAP kinase pathway, induces the transcription of D-type cyclins that form complexes with CDK4 and CDK6, thereby releasing E2F by phosphorylating Rb at Ser 795 and Ser807/811 phosphorylation sites, rendering it inactive and inducing the cells into entering the S-phase(Knudsen & Wang, 1996). Signaling through Ras-MAPK also results in inducing transcription of INK4A and ARF.

On the other hand, ARF functions in tumor suppression primarily through the p53 pathway. ARF is a nucleolar protein that binds nucleophosmin/B23 and localizes to the nucleolus to form a high molecular weight stable complex with a half-life time from 1 to 8 hours (Ozenne et al., 2010). It is a basic protein with more than 20% Arginine residues (pI >12) and possesses hydrophobic properties. $p14^{ARF}$ is 132aa long, whereas $p19^{Arf}$ is 169aa, with almost 50% sequence homology between the human and mouse orthologs. The first 62 amino acids in p19ARF are encoded by exon 1 β. Amino acids 26-37 encode a nucleolar localization sequence (NoLS) and cooperative MDM2 binding sites include amino acids 1-14 and 26-37 (Korgaonkar, Zhao, Modestou, & Quelle, 2002). $p19^{Arf}$ has only one lysine and there are none in $p14^{ARF}$ and no known secondary structures have been identified, unless associated with other molecules in complexes required for neutralizing its high basic properties (Chung, Wadhawan, Szklarczyk, Pond, & Nekrutenko, 2007) (Table 3).

Table 3. List of ARF-interacting proteins and their function(s)

Protein	Function
ARF-BP1	HECT- containing E3 ubiquitin ligase
E2F1	Transcription factor required for DNA synthesis
Myc	Transcription factor required for the transactivation and transrepression of several genes
CtBP1,CtBP2	Anti-apoptotic transcription co-repressors
PARF	Putative GTP-binding protein
Nucleophosmin B23	A nucleolar phosphoprotein required for rRNA synthesis
UBC9	An E2 conjugating enzyme required for sumoylation

Two internal methionine residues Met 45 and Met 48 have been identified in mouse and human ARF, respectively, and translation initiation from these two methionines results in shorter ARF molecules known as smARF that when overexpressed localize to the mitochondria (Balaburski, Hontz, & Murphy, 2010) since they lack nucleolar localizing sequences (NoLS) encoded by exon 1 β. This localization is a signal for autophagy, which is a mechanism with which cell die in response to low nutrients due to organelle autodigestion. However, the mechanism which smARF induces autophagy is still unclear.

ARF/p53 knockout mice have a greater risk of developing malignant tumors than an ARF or p53 single knockout. This suggests that ARF functions in tumor suppression through p53 dependent and independent mechanisms (Kawagishi et al., 2010). p53 dependent functions of ARF ultimately lead to the activation of p53 through the ARF-MDM2-p53 pathway resulting in cell cycle arrest, senescence or apoptosis. Overexpression of $p19^{Arf}$ ectopically, or upon hyperproliferative signals, results in cell cycle arrest either at G1 or G2 phases (Quelle et al., 1995). This primarily occurs due to the ability of full length ARF to neutralize the E3 ubiquitin ligase activity of the p53 antagonist murine double minute 2 (MDM2). After binding MDM2, ARF sequesters MDM2 into the nucleolus therefore inhibiting MDM2 mediated ubiquitination and proteasomal degradation of p53, thus rendering p53 more stable and active. p53 then induces cell cycle arrest by transcriptionally activating p53 downstream target genes with anti-proliferative functions such as p21CIP, which in turn inhibits the activity of CDK2 keeping retinoblastoma in a hypophosphorylated active state (Serrano, 2000).

I.2.4 p53- independent functions of ARF

As mentioned previously, ARF functions in tumor suppression in a p53 independent pathway, and debate on how ARF exerts its inhibitory control independently of p53 has not been fully resolved. Yeast two-hybrid system and immunoprecipitation studies showed that ARF directly binds a broad range of protein molecules involved in cell cycle progression (Figure2). In particular, ARF binds and represses the functions of Myc, E2F1, NF-κB, cyclin G1, WRN and B23 (Sherr et al., 2005) by sequestering them into the nucleolus and/or by disabling their binding to co-activator molecules. B23 is a key player in ribosomal biogenesis and shuttles between the nucleolus, nucleoplasm and the cytoplasm (Korgaonkar et al., 2005). Therefore, one way with which ARF functions as a tumor suppressor, is through impeding the translocation of B23 out of the nucleolus and hindering rRNA transcription and processing (Sherr, 2006). ARF has also been found to play an important role in sumoylation by associating with UBC9. This interaction is believed to enhance the transfer of sumo group to ARF binding proteins thereby triggering their proteasomal degradation (Sherr, 2006). Although ARF expression is not induced by DNA damaging agents, overexpression of $p14^{ARF}$ leads to activation of ATR/CHK1 dependent NF-kB phosphorylation which in turn leads to TNFα dependent apoptosis (Ozenne et al., 2010). Recently, the anti-apoptotic transcriptional co-repressor CtBP2 has been identified to bind the conserved hydrophobic domain of ARF at amino acid residues (46-51), and ARF targets this oncogenic co-repressor for proteasomal degradation in cells also exposed to stress such as UV (Paliwal et al., 2006) (Figure2). Moreover autophagy induced death upon smARF over-expression is considered a p53-independent function of ARF (Sherr, 2006).

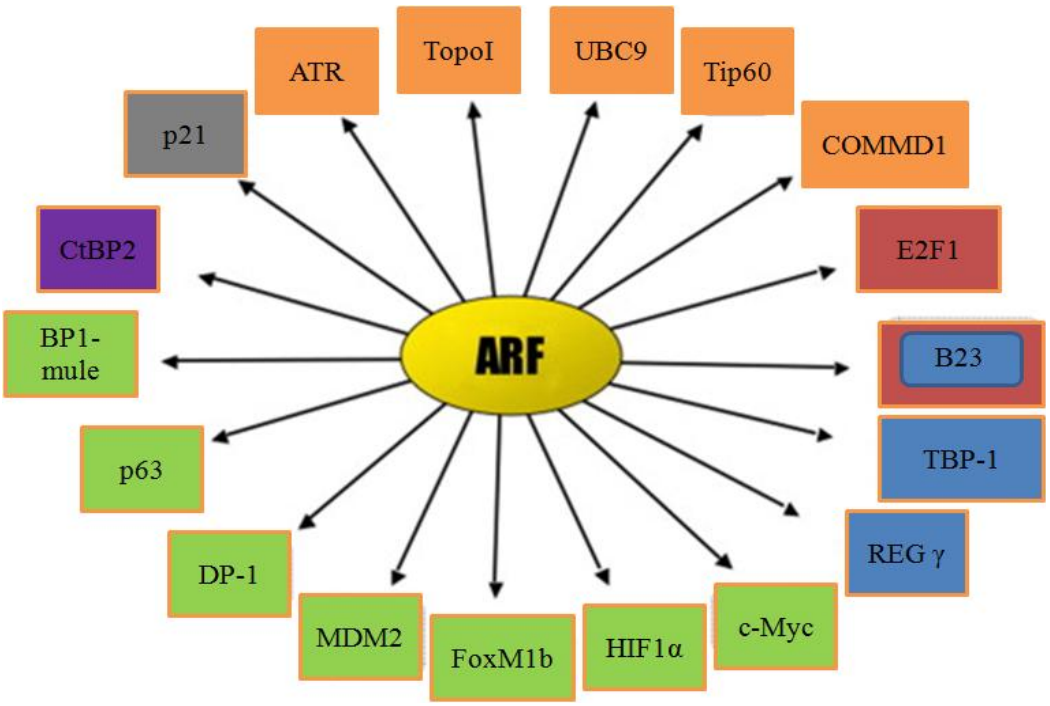


Figure 2. p53 dependent and independent functions of ARF. Grey is for proteins whose activity or stability is positively regulated by ARF through p53-dependent mechanisms. p53-independent activity of ARF: Purple is for binding partners induced to proteasome and ubiquitin- independent degradation by ARF. Green is for binding partners whose activity is blocked by ARF. Orange is for binding partners whose activity or stability is positively regulated by ARF. Red is for binding partners induced to proteasome and ubiquitin-dependent degradation by ARF. Blue is for binding partners which regulate ARF protein turnover. Adopted and modified from (91 Pollice,A. 2008).

I.3 C- terminal binding family proteins

C-terminal binding proteins are well-characterized transcriptional co-repressors known to play important roles in cell proliferation, differentiation and survival. In humans and mice C-terminal binding proteins consist primarily of two closely related proteins CtBP1 and CtBP2, which share 78% amino acid similarity (Bergman & Blaydes, 2006) . A third member of the family, CtBP3 has been identified as an N-terminal processed version of CtBP1, lacking the first 20 amino acids, and plays an important role in the fragmentation of the golgi membranes at the beginning of mitosis (Bonazzi et al., 2005) In humans, these proteins are encoded by two genes Ctbp1 and Ctbp2 located on chromosomal regions 4p16 and 10q26.13, respectively.

CtBP proteins were first identified as factors that bind the C-terminus of the adenoviral oncoprotein E1A at a consensus sequence PXDLS, and actively attenuate E1A's oncogenic properties. Subsequently, functional studies revealed that CtBP's function as transcriptional adaptors with both corepressor, and in some cases, coactivator functions (Bhambhani, Chang, Akey, & Cadigan, 2011).

Although CtBP1 and CtBP2 are highly similar in structure, function, and expression, several differences have been identified so far. In mice, CtBP1 is expressed in embryos and adults and can relocate between the nucleus and the cytoplasm, but CtBP2 is primarily expressed in embryos and exclusively locates to the nucleus (Barroilhet et al., 2012). Moreover, in developing embryos CtBP1 and CtBP2 are not expressed ubiquitously in all tissues (Furusawa, Moribe, Kondoh, & Higashi, 1999). Like INK4A/ARF locus, the Ctbp2 locus encodes another protein termed RIBEYE using an alternative promoter. RIBEYE is a synaptic ribbon protein involved in facilitating the release of neurotransmitters by the continuous docking of synaptic vesicles on presynaptic ribbons (Schmitz, Konigstorfer, & Sudhof, 2000). RIBEYE lacks the first 20 N-terminal amino acids of CtBP2, as a result of alternative splicing. Unlike the INK4A/ARF

locus, the expression of CtBP2/RIBEYE is in frame and tissue specific, occurring mainly in the retina (Chinnadurai, 2002).

I.3.1 CtBP structure and function

CtBP is a 48KDa phosphoprotein that has been evolutionary conserved among invertebrates and vertebrates whose activity has been shown to be regulated by stoichiometric variations in NADH/NAD ratio (Chen et al., 2009). As stated previously, CtBP protein possesses nuclear and cytoplasmic activities with the latter being more involved in maintaining vesicular membranes (Birts et al., 2010). Unlike ARF which possesses no known motifs, CtBP is a globular protein with peptide binding domain specific for PXDLS peptides (Nardini et al., 2006) responsible for its transcriptional activity (Figure 3), and a D2 hydroxy acid dehydrogenase domain responsible for NAD(H) binding and dimerization {{81 Nardini,M. 2006}}.

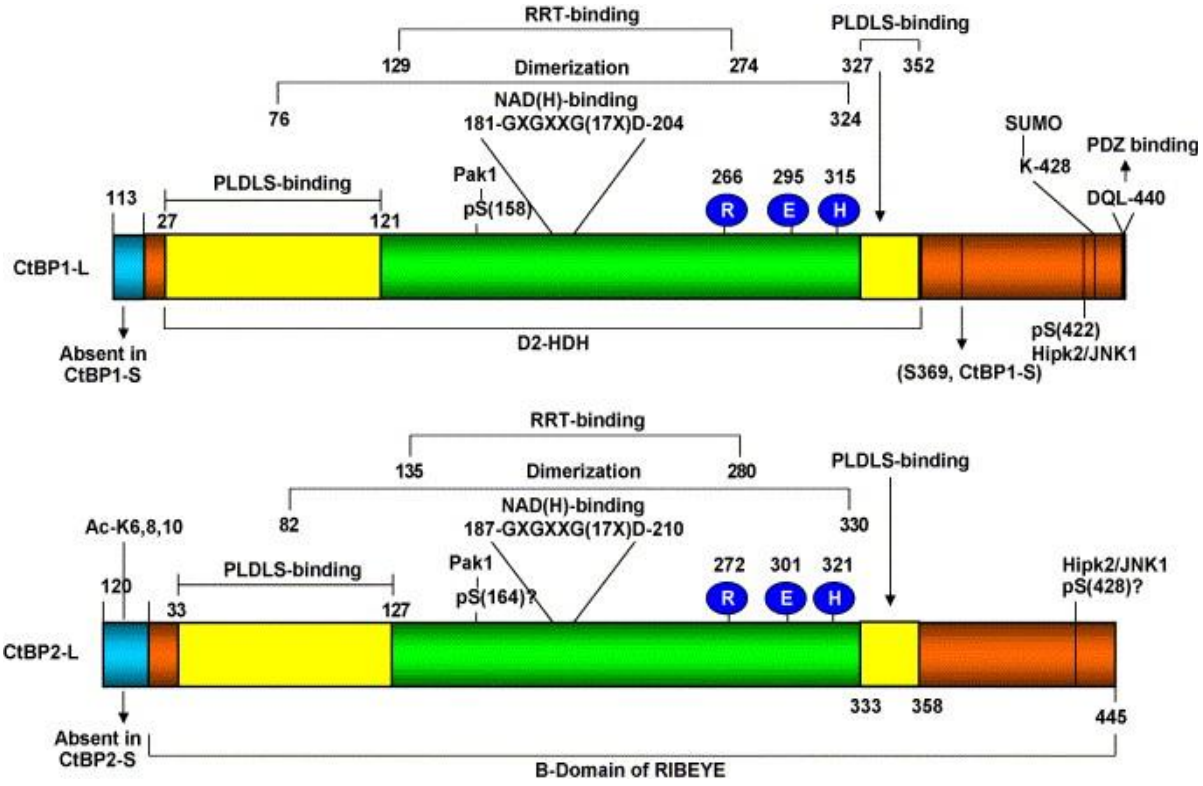


Figure 3. C- Terminal binding family protein functional domains (Chinnadurai, 2007)

The most accepted mechanism with which CtBP enforces repression is by recruiting DNA binding repressors through an HDAC dependent or independent manner. If HDACs are involved, the repression complex consists of either HDAC class I or class II and histone methyltransferases (HMTs) (Figure 4). In the absence of HDAC, CtBP recruits polycomb group complexes, or interacts with histone acetyltransferase co-activators (Bergman & Blaydes, 2006). In terms of developmental roles, ctbp1-null mice are runted but viable, whereas ctbp2 deficiency is embryonic lethal, with null embryos exhibiting multiple neurological and segmentation anomalies (Chinnadurai, 2003).

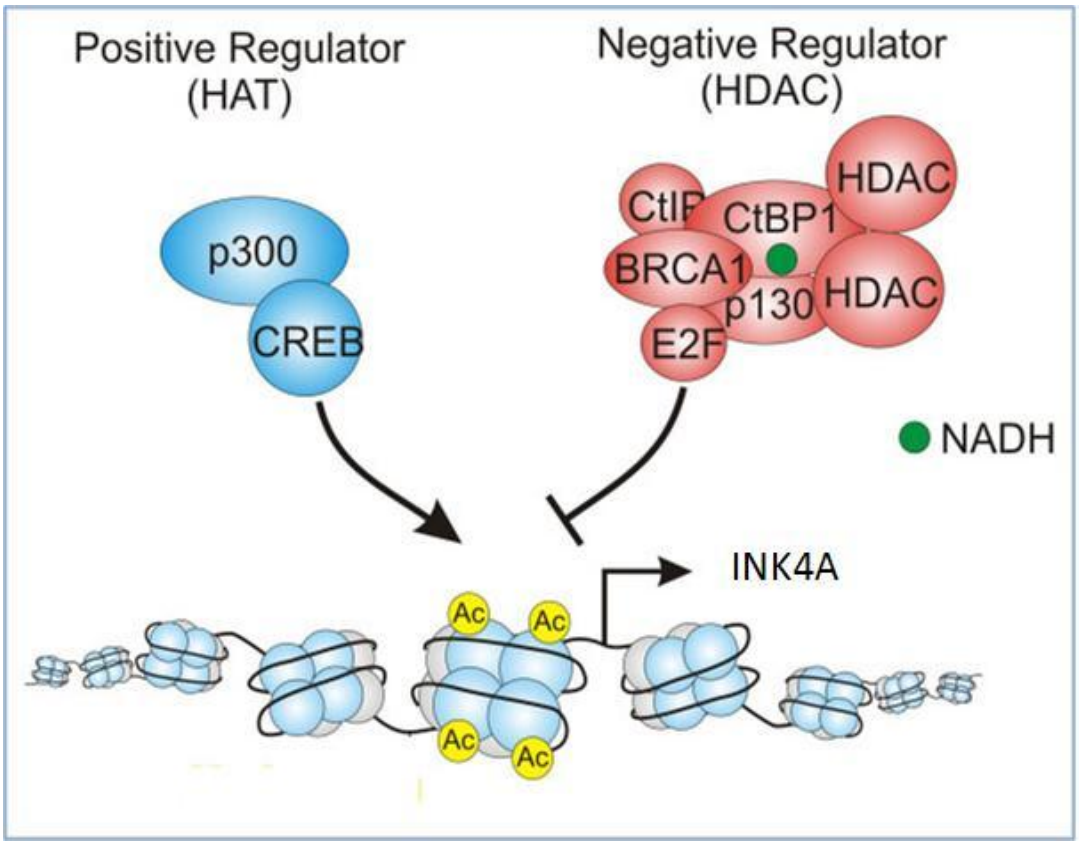


Figure4. Mechanism of CtBP1 transcriptional activities at the INK4A locus. Adopted and modified from (home.ccr.cancer.gov/inthejournals/gardner.asp).

I.3.2 CtBP possess oncogene like functions

Previous studies on CtBP double null mouse embryonic fibroblasts, revealed hypersensitivity to apoptosis in response to several ectopic stimuli such as genotoxic stress, loss of cell contact inhibition (anoikis) and FasI ligand and UV irradiation (Paliwal et al., 2006). Gene expression profiling on CtBP-null MEFs, indicated upregulation of two sets of cancer-related genes. The first set involved E-cadherin and cytokeratins, whereas the second included BH3-only genes (Noxa, Puma, Perp) (Bergman & Blaydes, 2006). These results indicate that CtBP is involved in cell survival and proliferation, and reintroduction of CtBP into CtBP1/2 double knockout MEFs, attenuated the activity of pro-apoptotic genes, tumor suppressor genes and induced cell survival. This suggests that in cancer, CtBP functions as apoptosis antagonist and also in repressing tumor suppressor genes, such as BH3 proteins. As mentioned previously, CtBP possesses a dimerization domain intrinsic to the dehydrogenase homology domain that allows homodimerization or heterodimerization of CtBP family proteins upon NADH binding. Therefore, CtBP family proteins function as a dimer, and adequate levels of NADH are important in eliciting the dimerization/activation of CtBP.

A major feature of solid tumors is poor vascularization due to rapid growth resulting in hypoxia. Hypoxia increases the levels of unbound NADH, which in turn activates the repressor activities of CtBP on the E-cadherin promoter allowing epithelial–mesenchymal transition, which is a normal process during embryogenesis, yet in cancer cells this is a step towards metastasis (Chinnadurai, 2009)(Q. Zhang et al., 2006). In a separate study, CtBP2 was shown to possess oncogene-like properties in ovarian cancer cells with high expression levels. siRNA mediated CtBP2 knockdown, resulted in a decrease in epithelial cancer cell proliferation, cell migration capacity and rendered ovarian cancer cells more responsive to HDAC inhibitors(Barroilhet et al., 2012).

I.3.3 Significance of ARF- CtBP interactions

Several studies showed that CtBP is upregulated in many human cancers including breast, ovarian and colon cancers (Barroilhet et al., 2012). Increased CtBP activity in response to metabolic changes in the tumor microenvironment is accompanied by transcriptional repression of tumor suppressor genes (E-cadherin, PTEN, etc.) (Chinnadurai, 2009) resulting in EMT, and the repression of INK4A leading to senescence in primary human fibroblasts (Mroz, Baird, Michaud, & Rocco, 2008). As an apoptosis antagonist, CtBP mediates the repression of BH3 Bik gene by recruiting KLF-8 to the Bik promoter and this repression is abrogated by ARF overexpression, indicating that the INK4A/ARF locus is both a target and a regulator of CtBP (Kovi, Paliwal, Pande, & Grossman, 2010).

Previous work in the Grossman laboratory, identified the carboxy-terminal domain of CtBP as a direct target of ARF and co-immunoprecipitation experiments identified the carboxyl terminal of CtBP2 as a platform for ARF binding, in particular amino acid residues 322-445, whereas ARF hydrophobic N-terminal amino acid residues 46-51 were necessary for that interaction. Introducing a point mutation in the conserved hydrophobic domain of ARF (L46D) abrogated the binding of ARF to CtBP without affecting ARF's nucleolar localization. As a tumor suppressor, hARF expression mediated proteosomal degradation of CtBP without involvement of ectopic stress, whereas mARF proteasome mediated degradation of CtBP was evident only after UV irradiation (Paliwal et al., 2006). This work highlighted one mechanism of p53- independent tumor suppression of ARF, via its antagonism of CtBP-driven cell survival and migratory/invasive activities at least in cell line models (Kovi et al., 2010).

I.4 Cellular senescence: An overview

Most human cells in vivo and in vitro possess an intrinsic property that limits their ability to divide indefinitely and lose homeostasis. This replication inhibitory phenomenon was described by Watson as the end replication problem since the replication machinery couldn't include the terminals of a linear DNA with every round of DNA synthesis. Therefore, telomere shortening is a normal process that occurs in human cells with every cell division and has been linked to senescence and aging. On the contrary, mouse primary cells, do not show telomere shortening due to the presence of very long telomeres compared to human telomeres, and the ability of DNA polymerases to maintain those terminal sequences. What MEFs and human fibroblasts share in common is the ability of cells to establish senescence in culture after several passages due to stress which is due in part to increasing levels of p19 and p16 (Espejel & Blasco, 2002). Loss of control over the cell cycle and continuous proliferation of human and mouse cells is a hallmark of cancer. Hence, senescence is a tumor suppressor mechanism that tends to maintain homeostasis by shifting cells towards a dormant state with metabolic activity.

Cellular senescence is an irreversible form of the cell cycle arrest or quiescence. Depending on the context, cellular senescence can be one of two forms, replicative or premature. Replicative cellular senescence is induced by telomere shortening, which results in a DNA damage response once the telomeric secondary structures are lost with shortening . Depending on the severity of the DNA damage, cells can shift from transient arrest to senescence or apoptosis. Replicative senescence is mediated by the activation of ATM/ATR leading to functional upregulation of p53 and CDC25 activity (Kuilman, Michaloglou, Mooi, & Peeper, 2010).On the other hand, cells entering premature senescence do not show attrition in their telomeres, yet they resemble replicative senescent cells in terms of high p16INK4A levels, large lysosomes and β-galactosidase expression, flat shape and large size, and senescence associated heterochromatin

foci (SAHF) which silence critical pro-proliferative genes(Rodier & Campisi, 2011). Premature senescence can be induced by stress due to shock in culture, oncogene expression, or due to the loss of tumor suppressor genes (Kuilman et al., 2010).

I.4.1 Oncogenic stress signals via H-rasV12 induces premature senescence in primary mouse embryonic fibroblasts

The Ras family proteins N-Ras, K-Ras and H-Ras are small GTP binding proteins with GTPase catalytic activity. Ras oscillates from an active form when bound to GTP to an inactive form when bound to GDP and plays a crucial role in mediating growth signals to transcription factors that regulate the expression of proteins involved in cell cycle and differentiation. The Ras family proteins are encoded by a 46 kbp proto-oncogene located on chromosome 12 in humans and chromosome 6 in mice and mutant forms are associated with more than 30 % of human cancers (McMahon & Woods, 2001). Ras family GTPases localize mainly to the cytoplasmic face of the phospholipid bilayer and are activated in response to signals that result in the phosphorylation of tyrosine kinases. Recent studies have shown that H-Ras can localize to the nucleus of non-transformed and primary mouse embryonic fibroblast, throughout the cell cycle (Contente, Yeh, & Friedman, 2011).

Activated Ras primarily signals through the MAPK pathway, which is a cascade of transducing kinases that result in the activation of cyclin dependent kinases, and hence the upregulation of E2F transcription factors leading to cell cycle progression. In cancer, control over the H-Ras activity is lost due to amplification of Ras oncogene or constitutive activation of Ras due to a G12V point mutation, which renders H-Ras incapable of hydrolyzing GTP into GDP hence maintaining an active state. This high activity of H-Ras V12 is enough to immortalize most mammalian cells, yet induces premature senescence in primary cells (Kuilman

et al., 2010). H-Ras V12 mediated premature senescence is tissue specific and pathways involved are not favored equally in humans and mice. In humans, the most accepted pathway involved in oncogene induced senescence (OIS) involves the expression of p16 and the hypophosphorylation of Rb. It is worth mentioning that freshly prepared human embryonic fibroblasts are resistant to H-Ras V12 induced senescence because of their low or insignificant levels of p16 (Schauble et al., 2012). On the other hand, $p19^{Arf}$ is the major sensor of OIS in MEF cells and primarily functions through the p53/p21 pathway, which is to date the most well characterized pathway in terms of inducing a permanent cell cycle arrest in PMEFs. This occurs through the p21-mediated inhibition through the inhibition of CDK2, maintaining Rb at a low phosphorylation state, and thus halting the cells at G1 phase. Although it is generally accepted that senescence is an irreversible form of cell dormancy, several studies showed that prematurely senescent cells were rescued upon the knockdown of tumor suppressor genes (Dirac & Bernards, 2003). For instance, human fibroblasts lacking p21 bypass H-RasV12 induced senescence and the disruption of either p16 or p53 was enough to evade the arrest in primary MEF cells (Serrano, Lin, McCurrach, Beach, & Lowe, 1997).

I.4.2 Tumor suppression: Interplay between senescence, cell cycle arrest and apoptosis

Senescence, cell cycle arrest, apoptosis and autophagy play a critical role in determining the fate of proliferating cells, and help maintain a state of equilibrium that once altered can lead to neoplasia. All of these mechanisms act as barriers to oncogenesis, and they are induced, in part, by DNA damaging agents such as UV irradiation, doxorubicin, and telomere shortening. At the molecular level, p53 appears to be involved in all of these mechanisms (Li et al., 2012), and what determines which mechanism stressed cells undergo vastly depends on the threshold of

DNA damage with cells exceeding a certain threshold of damage will be committed to either senescence, apoptosis or even autophagy(Kuilman et al., 2010).

I.5 Aims of this study

Mutations in proto-oncogenes, tumor suppressor genes and caretaker genes play an important role in carcinogenesis. Among the proteins that are encoded by proto-oncogenes is growth promoting signal proteins and their receptors, signal transduction proteins, transcription factors and anti-apoptotic proteins. In particular, mutant Ras protein (H-Ras V12) is an intracellular signal transduction protein that provides excessive growth promoting signals. Depending on the cell type, mutant H-Ras V12 activity has the tendency to induce cellular premature senescence, or immortalization/transformation. This solely depends on whether secondary mutations in tumor suppressor genes exist. The purpose of this thesis is to outline the functional significance of $p19^{Arf}$ as a barrier for lymphoma development upon oncogenic stress and to explore the significance of ARF/CtBP2 interaction in vitro by using primary MEF cells as a model that could explain at least in part the biological outcome at the molecular level when ARF/CtBP2 binding is disrupted through the point mutation L46D in ARF.

Chapter II

Materials and Methods

II.1 Preparation of MEF cells

Wild-type and ARF L46D/L46D mouse embryonic fibroblasts were explanted from E12.5-E13.5 embryos. Wild-type MEF cells were generated from embryos obtained from mating C57/BL6 wild-type mice. ARF L46D/L46D MEFs were established from embryos obtained from crossing C57/BL6 ARF+/ARF L46D heterozygote males and females. Pregnant females were sacrificed at day 12.5-13.5 following the standards set through IACUC. Uterine horns were rinsed in 70% alcohol then immersed in 1x PBS. Embryos were removed from the embryonic sac, placed in a sterile petri-dish and the head, red organs, tail and limbs were removed before mincing the trunk in 1 ml of 0.05% Trypsin–EDTA (Life technologies) using sterile surgical blade then pipetted into a 50ml conical tube and incubated at 37°C for 30 min and continuously pipetted in the hood every 5 min during the incubation period. Trypsin was inactivated by adding 1 volume of pre-warmed MEF medium. Genomic DNA was digested by adding 2µl of DNase I per 1 volume of Trypsin/Medium and the mix was incubated for additional 15-20 min at 37°C. MEF cells were collected by centrifugation at 1500rpm for 5min.Gelatin pre-coated 10cm culture plates for 2 hours, were used to seed the cells at passage 0(P0) and the medium was replaced the second day. Primary MEF cells were kept at 37°C under 5% CO_2 and passaged at a split ratio of 1:5 at 90% confluency. If not required immediately, MEF cells were frozen in 10%

DMSO in liquid nitrogen. MEF complete medium: DMEM (435ml), 10% FBS, PEN/Strep (5ml), L-Glutamine (5ml) and Non-essential amino acids (NEAA) (5ml). Embryos obtained upon crossing heterozygote males and females were genotyped by using a piece of the head for DNA extraction using QIAamp DNA mini Kit (Qiagen). Brain samples were incubated with lysis buffer (ALT) and proteinase-K at 55°C overnight. Genomic DNA was precipitated by ethanol on the spin column membrane and eluted by adding 200µl of Molecular Graded Water. A PCR reaction was performed using the following primers RL1, RL2 and RL3 (Table 4) followed by running the PCR products on 1.5% agarose gel for 30 min at 115V. Images were developed by exposing the gel to ultra violet light (UV) using alpha imager (Innotech). The expected product size is 250bp (Figure 5).

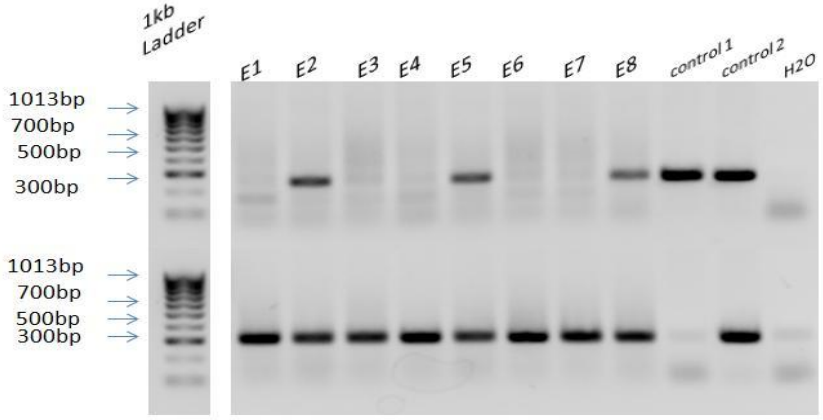


Figure 5. Representation of agarose gel electrophoresis for MEF genotyping. MEF cells were explanted from embryos upon mating C57/Bl6 ARF+/L46D female with C57/Bl6 ARF+/L46D male. Control 1 and control 2 determine whether the embryos are ARFL46D/L46D or ARF+/L46D mutants respectively.

Table 4. Primers used in genotyping primary MEF cells

Primer	Sequence	Properties
RL1	5'CTCTGGCTTTCGTGAACATGGAT3'	Amplifies ARF L46D allele
RL2	5'CGTGTGCAAAGTACTCCATCTCC3'	Reverse primer common to wild-type and ARF-L46D alleles
RL3	5'CTCTGGCTTTCGTGAACATGTTG3'	Amplifies Wild-type allele

II.2 Transient production of retroviral-based vectors for the transduction of MEF cells

Three different replication-defective retroviral expression vector stocks were prepared through transient Transfection of HEK293T packaging cell lines. The day before transfection, $5x10^5$ HEK293T cells (ATCC) were plated in 6cm culture plates in 5ml complete DMEM such that they reach 70% confluency on the day of transfection. Transfection of HEK293T cells was established through calcium-phosphate precipitation method. Prior to preparing the calcium phosphate precipitation mix, the medium was removed and replaced with 4.5ml incomplete medium, DMEM – P/S. Three transfer vectors were used separately, pBABE-puro, pBABE-HrasV12 and pBABE-GFP and simultaneously co-transfected with the packaging plasmid (pBABE- gag-pol) and the envelope plasmid (pMDG-VSV-g). A total of 10-15µg of DNA was added into a microcentrifuge tube, washed with water to a total volume of 225µl. The volume was adjusted to 250µl by adding 25µl of calcium chloride, and the mix was homogenized with finger tapping before adding it dropwise to 250µl of 2xHBS in a second microcentrifuge tube followed with vortexing at top speed for 3 seconds. The precipitation mix was incubated for 25 min at room temperature in the culture hood before adding it dropwise to the 293T cells. The medium was replaced 16-18hr after transfection with complete medium, and viral stocks were harvested 48-72hr post-transfection. The viral supernatant was supplemented with 10µl of 8mg/ml polybrene, and centrifuged at 1500rpm for 5 min in order to remove cellular debris. Excess viral stocks were frozen at -20°C.

II.3 Cell culture

Primary MEF cells including wild-type and ARF L46D/L46D MEFs were maintained in complete Dulbecco's modified eagle's medium containing 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine and 1% non essential amino acids. ARF-null MEF

cells, previously obtained from UMass were propagated in culture for over 20 passages. ARF-null, 3T3 MEFs and U2OS cell lines were maintained in complete DMEM medium. All cell lines were maintained in a humidified incubator under 5% CO_2 at 37°C.

II.4. DNA damage response

All MEF cells were treated with 1µM of Doxorubicin Hydrochloride stock 10mM (Sigma). $4x10^5$ passage 1 (P1) Wild-type MEF cells and $2.5x10^5$ passage 3 (P3) ARF L46D/L46D MEF cell were seeded in 6cm culture plates such that they reached 70-80% confluency the second day. The cells were treated for 2 hours, and then the medium was replaced again with complete DMEM. The cells were harvested at 1h, 2h, 4h, 8h and 24 hours post-pulse treatment for western blot analysis. Untreated cells (0h) were lysed for protein extraction during the 24h period.

II.5. Retroviral infections

Replication defective retroviral stocks stored at -20°C were thawed in a water bath a 37°C and 1ml or 2ml of viral supernatant were added to cells growing in 6cm or 10cm culture plates respectively and left under 5% CO_2 at 37°C. The viral cocktail was diluted 6-8 hours later with the appropriate volume of complete DMEM. On the second day, the cells were infected again for 6-8hours with the viral cocktail. For all cells lines, retroviral infections were done under extreme sanitizing conditions and once the viral cocktail was added, the plates were moved into a separate humidified incubator. For all assays, cells were infected for 48 hrs then selected with 2µg/ml of puromycin according to (Figure 6). NIH 3T3 cells were used as a control to determine the infection efficiency and were infected with retrovirus carrying green fluorescent protein transfer vector and selected with 2µg/ml of puromycin for 2 days and images were taken using AMG fluorescent microscope at a magnification of 40x.

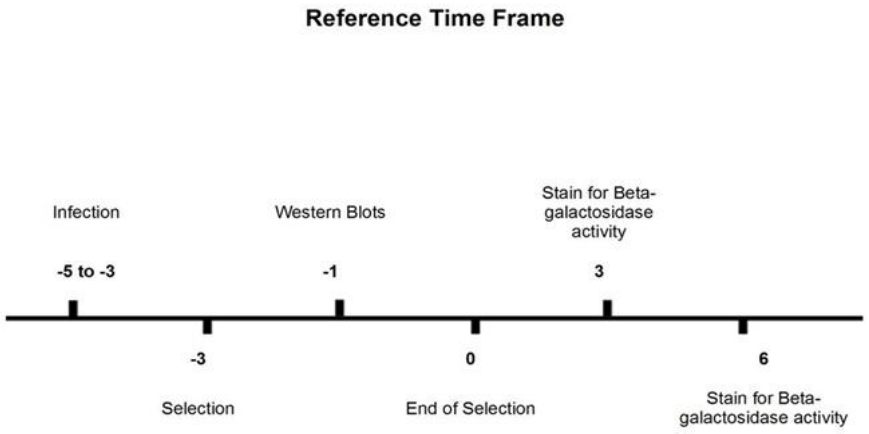


Figure 6. Experimental design and reference time-frame in days

II.6 Western blotting

P1 wild-type, P1 ARFL46D/L46D and ARF-null MEF cells transiently infected with retroviruses carrying an Empty vector or H-RasV12 were seeded at $8x10^5$, $5x10^5$ and $4x10^5$ in 10cm culture plates the day before transduction. Cells were infected for 2 days and selected for 2 days in 2µg/ml puromycin. Cells were washed twice in phosphate buffer saline (1x PBS), then lysed in RIPA lysis buffer (50mM Tris-HCL, 150mM NaCl, 1% NP-40, 0.5% sodium deoxychelate, 0.1% sodium dodecyl sulfate SDS) supplemented with Complete mini EDTA free protease inhibitor cocktail (Roche) on ice. Wild-type and ARF L46D/L46D MEFs were lysed in a total volume of 300µl whereas ARF-null MEFs were lysed in 500µl of the lysis buffer cocktail for a total of 10-15 min on ice. The plates were scraped with a plastic scraper and crude lysates were collected in a microcentrifuge tube and vortexed at low speed and left on ice for additional 5 minutes. The tubes were placed in a refrigerated centrifuge at 4°C and spun at a rotational

speed of 15000 rcf for 15 min. The supernatant was transferred into another microcentrifuge tubes and a bicinconinic acid (BCA) assay was performed in order to determine the lysate concentration. Into a 96well plate, 10 µl of each lysate was added in duplicates along with bovine serum albumin (BSA) standards of known concentrations, 0, 250, 500, 1000 and 2000 µg/ml. The BCA solution was prepared by mixing 50 parts of solution A and 1 part of solution B. A total of 200µl was added into each well, mixed by pipetting and incubated at 37°C for 30 min. The corresponding absorbances at 562nm were measured using Synergy H1 multi-mode microplate reader (Biotek). The concentrations of the lysates were calculated by plotting the concentrations of the BSA standards versus their measured absorbances. For cell cycle protein analysis of transduced cells, 20µg of total protein were loaded per well and proteins were resolved on a denaturing polyacrylamide gel NuPAGE (Bis-Tris 4-12%) for 35-50min at 200V in 1x NuPAGE-MES buffer. For DNA damage response and UV irradiation assays, 8-10µg of protein were loaded per well. The proteins were transferred to Immobilin polyvinylidine difluoride (PVDF) membranes 0.45µm (Millipore) which have been pre-activated in 100% methanol at 40V for 80min in 1X NuPAGE Transfer buffer (Life Technologies). The PVDF membranes were blocked in 0.2% blocking buffer (1volume of 1% casein, 4 volumes of 1x PBS), for 1hour and then incubated with primary antibodies at 4°C overnight while shaking. The membranes were washed with Tris-Buffered Saline 1x TBS-T (20% Tween-20) 5 times, 5 min each and incubated with the appropriate secondary antibody for 50 min at room temperature while shaking. A second wash with 1xTBS-T was performed 5x, 5 min each, and then the membranes were exposed to Odyssey Infra-red imager system (Licor). The following primary antibodies were used: Rabbit polyclonal anti-p53 antibody FL393 (sc6243) at 1:1000 dilution, rabbit polyclonal anti-p21 antibody M-19 (sc-471)at 1:1000, rabbit polyclonal anti-p16 antibody

M-156 (sc1207) at 1:1000 dilution, rabbit polyclonal anti- alpha actin antibody (A2066) Sigma Aldrich at 1:3000 dilution, rabbit polyclonal anti-H-Ras antibody C-20 (sc520) at 1:1000 dilution, mouse monoclonal anti-MDM2 antibody 2A10 (abcam) at 1:500 dilution, rabbit polyclonal anti-CDKN2A/p19ARF antibody ab80 (abcam) at 1:1000, rabbit polyclonal anti-pRb (ser795) antibody(sc21875-R) at 1:1000 dilution, mouse monoclonal anti-CtBP2 antibody 612044 (BD Transduction Laboratories) at 1:3000 dilution.

II.7 Localization assay

U2OS cells were seeded into BD Falcon four-well culture slides with polystyrene vessels at a density of $1x10^5$ cells in 1ml of complete medium per well and transfected using Lipofectamine 2000 transfection reagent with pcDNA3.1 plasmids encoding WT hARF, L50D hARF and pcMV-Bam-HDM2. Plasmid DNA (µg) to Lipofectamine 2000 transfection reagent (µL) ratio was 1:2 and a total of 0.8 µg of plasmid DNA was used per well. Plasmid DNA and Lipofectamine 2000 were diluted separately in 50µl of Opti-MEM- I reduced serum medium and incubated at room temperature for 5 min and then mixed to obtain a total volume of 100µl per transfection reaction. The combined volume was mixed gently and incubated for 20 min at room temperature. During the 20 min incubation period, the old growth medium was replaced with P/S free medium and then the cells were transfected by adding the 100µl complex dropwise to the culture vessels. Transfected U2OS cells were incubated under 5%CO_2 at 37°C for 24 hours and were not subjected to selection. U2OS cells were washed with 500µl, 1xPBS per well, then fixed with 4% paraformaldehyde (300µl/chamber) for 15min at room temperature. Chambers were washed again twice with 1xPBS before permeabilizing the membranes with 0.5% Ttiton X (300µl/well) for 20 min at room temperature. Another wash with 1xPBS was performed as described before, and then 300µl of 5% goat serum blocking buffer were added per

chamber for 1h at room temperature (RT). U2OS cells were stained overnight while shaking with the following primary antibodies: mouse monoclonal anti- p14 ARF antibody DCS241 (sc-53640), rabbit polyclonal anti-MDM2 antibody N-20 (sc813). Primary antibodies were diluted in (1% goat serum, 0.1% triton X) at a ratio of 1:100. The chambers were washed the second day and exposed for 50min without shaking and in the dark at RT to fluorescrein isothiocyanate (FITC) – conjugated goat anti-rabbit antibody (sc3839), and Texas red (TR)- conjugated goat anti-mouse antibody (sc362277) at 1:400 dilution. U2OS cells were washed again with 1xPBS and the chambers were physically detached from the glass slide before adding 10µl of the counter stain DAPI to the center of each well. The glass slides were covered with micro-coverslips (VWR INTERNATIONAL) and sealed with colorless nail polish. Images were taken using AMG fluorescent microscope at a magnification of 40x.

II.8 Senescence- associated β-Galactosidase assay

P1 Wild-type, P2 ARF L46D/L46D and ARF-null MEF cells were seeded into three separate 6-well plates in duplicates at the following cell densities: $5x10^4$, $7x10^4$ and $2x10^4$ cells/well respectively. In each 6-well plate, 2 wells were left untreated, whereas the remaining 4 wells were infected with retroviral cocktail containing either an Empty vector or H-Ras V12 and left for 6-8 hrs under 5% CO_2 at 37°C. Complete medium was added after the primary incubation with the viral supernatant (sup) and the infection process was repeated the second day. Infected cells were selected with 2µg/ml puromycin for 3 days and the selection medium was replaced by complete medium on the last day of selection referred to as day 0. Primary MEF cells were stained on day3 (P4) and day 6 (p5) post selection using 5-bromo-4-chloro-indolyl-Beta-D-galactopyranoside (X-gal) used as a substrate for β-galactosidase. MEF cells were washed twice in 1xPBS and fixed for 5 min in 2ml of fixing buffer (2% paraformaldehyde, 0.2%

glutaraldehyde in 1xPBS) per well and then washed again before adding 2ml of staining buffer. The staining buffer (20mg/ml X-gal in dimethyl formamide, 0.2M citric acid/ Na phosphate buffer pH6.0, 100mM potassium ferrocyanide, 100mM potassium ferricyanide, 5M sodium chloride, 1M Magnesium chloride and ddH2O) was prepared fresh prior to use. The cells were incubated at 37°C overnight and washed the second day with 1xPBS. Images were taken using AMG light microscope at a magnification of 4x.

II.9 UV irradiation

Wild-type MEF cells P1, ARF L46D/L46D MEF cells P2 and ARF-null MEF cells were seeded into 6cm culture plates as described before and incubated overnight under 5%CO_2 at 37°C until they reached 70-80% confluency. The medium was removed and the plates were washed in 1xPBS and exposed to 254nm UV light using the stratalinker (stratagene) at increasing amounts of energy: 0,5,10,20,30 J/m^2 . Complete medium was added immediately after irradiation and the plates were left in the incubator for 6 hours post-treatment. Cells were washed and lysed on ice as described before.

II.10 Statistical analysis

For western blots, densitometric analysis was performed using Image J software. Protein levels were quantitated and normalized against actin loading control. For senescence-associated β-galactosidase assay, 100-200 cells were scored in at least 2 fields, and cells were counted manually using Image J software. The percentages of β-galactosidase positive cells were obtained by dividing the number of blue-colored cells by the total number of scored cells. Comparison between two groups was done using a two-sided Student's t-test. P-value < 0.05 was considered statistically significant. All error bars represent the standard deviation of the mean from at least 2 independent biological replicates.

Chapter III

Results

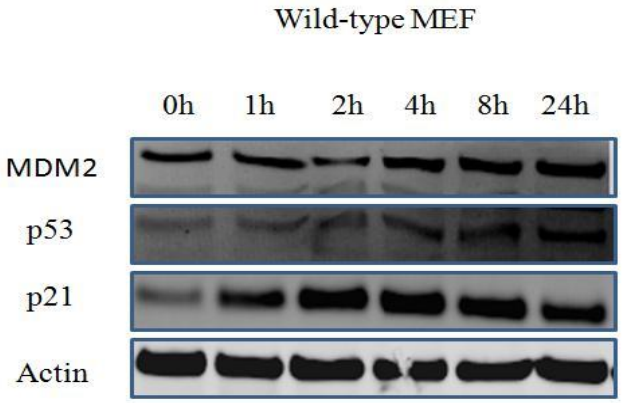
I endeavored to test whether cells with the L46D mutation in the ARF gene exhibited normal or abnormal properties relative to known ARF or p53 biologic responses. In order to accomplish that, I assessed the outcome of the mutation under DNA damage stress, ultraviolet irradiation and oncogene overexpression through studying changes in the expression of cell cycle proteins in canonical pathways under different stress conditions.

III.1 A conserved hydrophobic domain in p19Arf (37-51) is not required for the stabilization of p53 upon DNA damage stress.

The tumor suppressor p53 is a key transcription factor involved in the transcription of apoptotic as well as cell cycle arrest genes and is mutated in more than 50% of human tumors. As mentioned previously, mice lacking p53 are embryonically viable but develop T-cell and B-cell lymphomas as early as 20 weeks with 100% penetrance (Sharpless, 2005). Regardless of the molecular trigger, which can be oncogenic or DNA damage, p53 is stabilized indirectly by ARF or directly through phosphorylation events by ATM/ATR protein kinases (Kruse & Gu, 2009). Previous studies showed that $p14^{ARF}$ is neither upregulated nor required for p53 stabilization upon genotoxic stress caused by topoisomerase inhibitor doxorubicin (anthracyclins), but might be involved in enhancing DNA damage signaling in response to DNA damage caused by alkylating agents such as cyclophosphamide(Eymin et al., 2006). In order to confirm these

results in MEF cells and define whether the point mutation L46D in ARF would lead to novel outcome compared to the wild-type ARF, MEF cells were seeded in 6 cm plates at a confluency of 40-70% depending on the cell line, and treated with 1μM doxorubicin for 2 hours. The drug-containing medium was replaced after the 2 hour – treatment with complete medium, and the cells were lysed for protein extraction at different time points (See Materials and Methods). The choice of the proposed time points and drug concentration was solely based on previous studies which showed that higher concentrations of doxorubicin or treatments extending for prolonged periods of time would result in increased apoptosis and/or transcriptional repression of target genes involved in the DNA damage response pathway (Attardi et al., 2000). Western blot analysis clearly showed that the DNA damage response pathway was not influenced by the mutation in the conserved hydrophobic domain (L46D) of ARF, since p53 stabilization, p21 and MDM2 upregulation were equivalent in all cell lines (Figure 7).

(A)



(B)

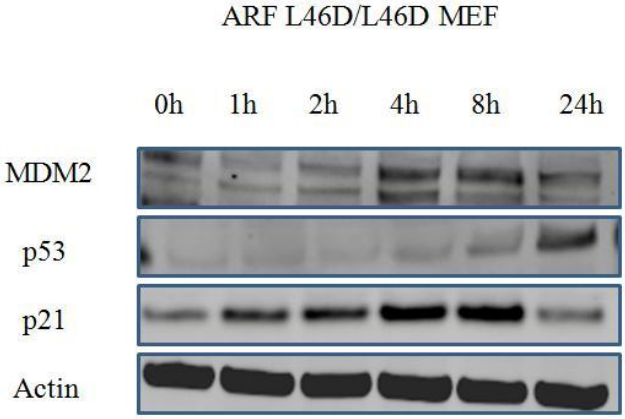


Figure 7. Time course analysis of MDM2, p53 and p21 levels in doxorubicin-treated MEF cells. (A) P1 wild-type and (B) P3 ARF L46D/L46D MEFs were treated with 1μM doxorubicin for 2hrs. Untreated cells (0h) were lysed for protein extraction during the 24h period. The blots were probed with actin as a loading control. Changes in MDM2, p53 and p21 expression levels were determined at the indicated time points by western blotting.

As expected these results confirmed that the role of ARF in DNA damage response is not essential, as abrogating CtBP2 binding to ARF through point mutation in the conserved hydrophobic domain did not lead to disruption of canonical DNA damage indicators, such as p53 stabilization or p21 upregulation.

III.2 Mutant p19ArfL46D maintains a nucleolar localization signal and fidelity to MDM2 sequestration

The most widely accepted dogma in defining the tumor suppressive functions of ARF is through its antagonistic activity against the murine double minute 2 E3 ligase (MDM2), whereby it exerts its effects in a p53-dependent pathway. Several studies have shown that endogenous MDM2 plays a role in ubiquitin mediated proteosomal degradation of p53 via its intrinsic E3 catalytic ligase activity serving to constrain p53 levels (Honda, Tanaka, & Yasuda, 1997). ARF is predominantly a nucleolar protein that is kept at basal levels upon binding to the nucleolar protein nucleophosmin-B23. Increasing nuclear levels of ARF results in MDM2 sequestration to the nucleolus, leading to p53 stabilization as described previously (Weber, Taylor, Roussel, Sherr, & Bar-Sagi, 1999). ARF residues 1-14 and 26-37 are required for MDM2 binding, but amino acids 26-37 also bind B23, suggesting that B23 can compete with MDM2 for ARF association therefore inhibiting its function (Korgaonkar et al., 2005)(Korgaonkar et al., 2002). Interestingly, in some situations, p14 ^{ARF} was found to exert its tumor suppressive functions by stabilizing p53 without relocating endogenous MDM2 from the nucleoplasm, suggesting that nucleolar homing of the ARF-MDM2 complex is not a necessity for the p53- dependent functions of ARF (Llanos, Clark, Rowe, & Peters, 2001). On the other hand, p53-independent

functions of ARF, which are still not fully characterized, were described previously where certain amino terminal deletion mutants in $p19^{Arf}$ sequestered MDM2 without stabilizing p53, and others didn't relocate MDM2 yet possessed growth inhibitory functions, suggesting that ARF exerts its effects in p53-dependent and independent pathways (Korgaonkar et al., 2002). To study the effects of the loss of ARF/CtBP2 interaction on the localization of mutant $p14^{ARFL50D}$ in humans (L46D in mice) and its ability to sequester MDM2 into the nucleolus, U2OS cells were transfected with pcDNA3.1-hARF, pcDNA3.1-L50D ARF, pcMV-Bam-HDM2 or co-transfected with plasmid DNA expressing the wild-type or mutant $p14^{ARFL50D}$ in combination with pcMV-Bam-HDM2. The subnucleolar localization of ARF and its mutant was ascertained through immunofluorescent staining (Materials and methods) using antibodies against $p14^{ARF}$, and HDM2. Wild-type ARF resided exclusively in the nucleolus and surprisingly did not co-localize with HDM2 with the latter being predominantly distributed in the nucleoplasm (Figure 8). On the other hand, mutant $p14^{ARFL50D}$ showed a broader distribution, which is the norm for wild-type ARF based on previous supporting information, and co-localized with HDM2 in doubly transfected U2OS cells (Figure 8). Co-localization of $p14^{ARFL50D}$ with HDM2 was seen both in the nucleoplasm and inside the nucleolus suggesting that abrogating CtBP2 binding to ARF has no influence on the nucleolar localization signal that governs the shuttling of ARF into the nucleolus, nor-on the domain that governs MDM2 binding. More importantly, both p14-ARF and L50D-ARF stabilized p53 as inferred from lanes 1 and 3, since U2OS cells transfected with plasmids expressing only wt-ARF or $p14^{ARFL50D}$ resulted in increased fluorescence activity upon staining for HDM2 when compared to lanes 7 and 8, which agrees with the negative feedback regulatory loop that defines the MDM2-p53 interaction (Moll & Petrenko, 2003)

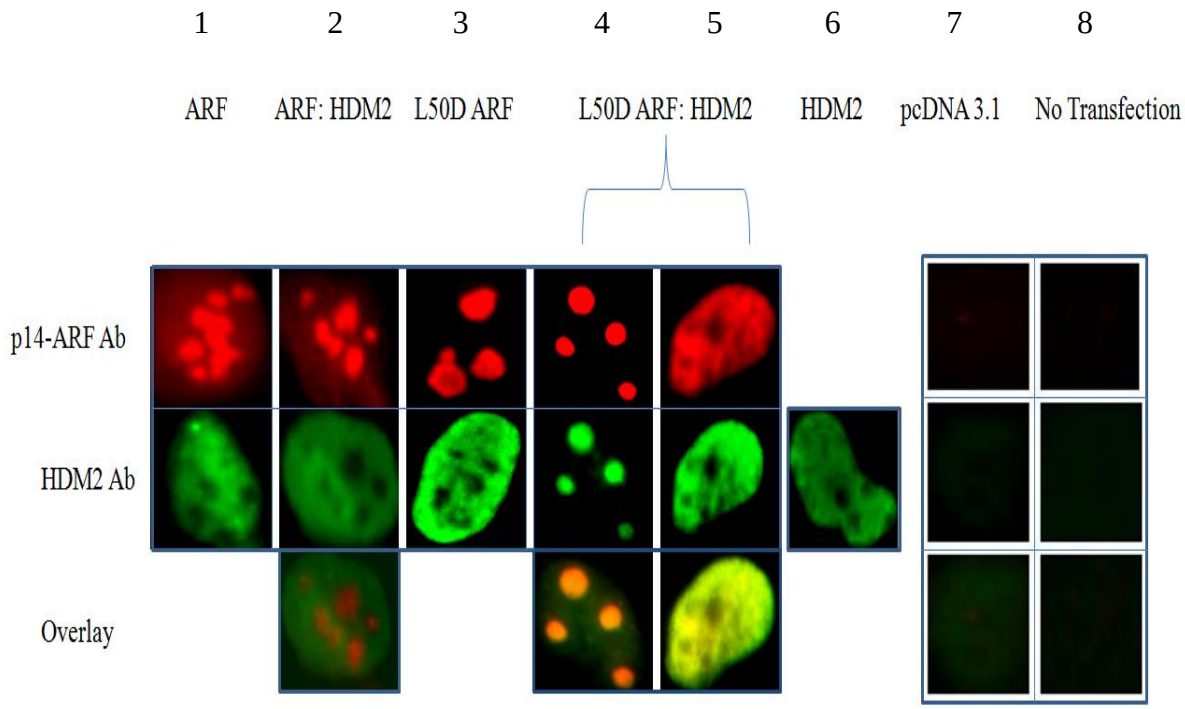


Figure 8. Localization of HDM2 after induction of L50D-ARF. U2OS cells were transfected with wild-type hARF, L50D-ARF and HDM2 expression plasmids (1,3,6) and co-transfected with hARF and HDM2 or L50D-ARF and HDM2 expression plasmids (2,4,5) respectively. Twenty-four hours after transfection, cells were immunostained with mouse monoclonal anti-$p14^{ARF}$ antibody and rabbit polyclonal anti-MDM2 antibody followed by detection with fluorescrein isothiocyanate (FITC) – conjugated goat anti-rabbit antibody, and Texas red (TR)-conjugated goat anti-mouse antibody. The merged image in (4) shows the co-localization of $p14^{ARFL50D}$ and HDM2 in the nucleolus.

III.3 Disruption of ARF/CtBP2 interaction through point mutation in the ARF conserved hydrophobic domain results in rescue from UV induced proteosomal degradation of CtBP2

It was reported in a previous study that the conserved hydrophobic domain (46-51) in $p19^{ARF}$ co-immunoprecipitate with CtBP2, and interestingly over-expression of ARF or the depletion of CtBP2 rescued the expression of Bik in HCT116 p53-/- cells leading to p53-independent apoptosis upon UV stress (Kovi et al., 2010), correlating with ARF mediated proteosomal degradation of CtBP2 (Paliwal et al., 2006) . As expected, $p19^{ArfL46D}$ over-expression didn't lead to Bik upregulation nor to apoptosis in HCT116 p53-/- cells (Kovi et al., 2010) thus supporting the belief that CtBP2 effects on cell survival are through its repression of pro-apoptotic Bcl-2 homology domain 3 (BH3)-only genes, and ARF/CtBP2 interaction is necessary for ARF tumor suppressor activity in the absence of p53. In order to determine whether the loss of ARF/CtBP2 interaction would disrupt proteosomal degradation of CtBP2 upon UV stress, which has been proposed to depend on ARF/CtBP2 interaction (Paliwal et al., 2006), MEF cells from 4 different cell lines (Wild-type, ARFL46D/L46D, ARF-null and p53-null) were seeded into 6 cm plates and irradiated with ultra-violet light and cells were lysed 6 hours later at a 70-80% confluency in order not to initiate contact inhibition signaling pathway that might lead to false positive result. The results shown in (Figure 9) confirm that the presence of full-length p19- ARF is required for CtBP2 destabilization after UV irradiation, since in ARF-null MEF cells CtBP2 levels increased with increasing energies. It can also be inferred from these results that ARF can target CtBP2 to degradation in the absence of p53 since CtBP2 decreased in p53-null MEFs with increasing energies similar to wild-type MEFs. As with ARF-null cells, the levels of CtBP2 in ARF L46D/L46D MEF lysates increased with increasing energies (Figure 9), thus supporting the concept that under UV stress CtBP2 levels decrease

when ARF is present, and abrogating ARF/CtBP2 with a point mutation in the conserved hydrophobic domain rescued CtBP2 from proteosomal degradation.

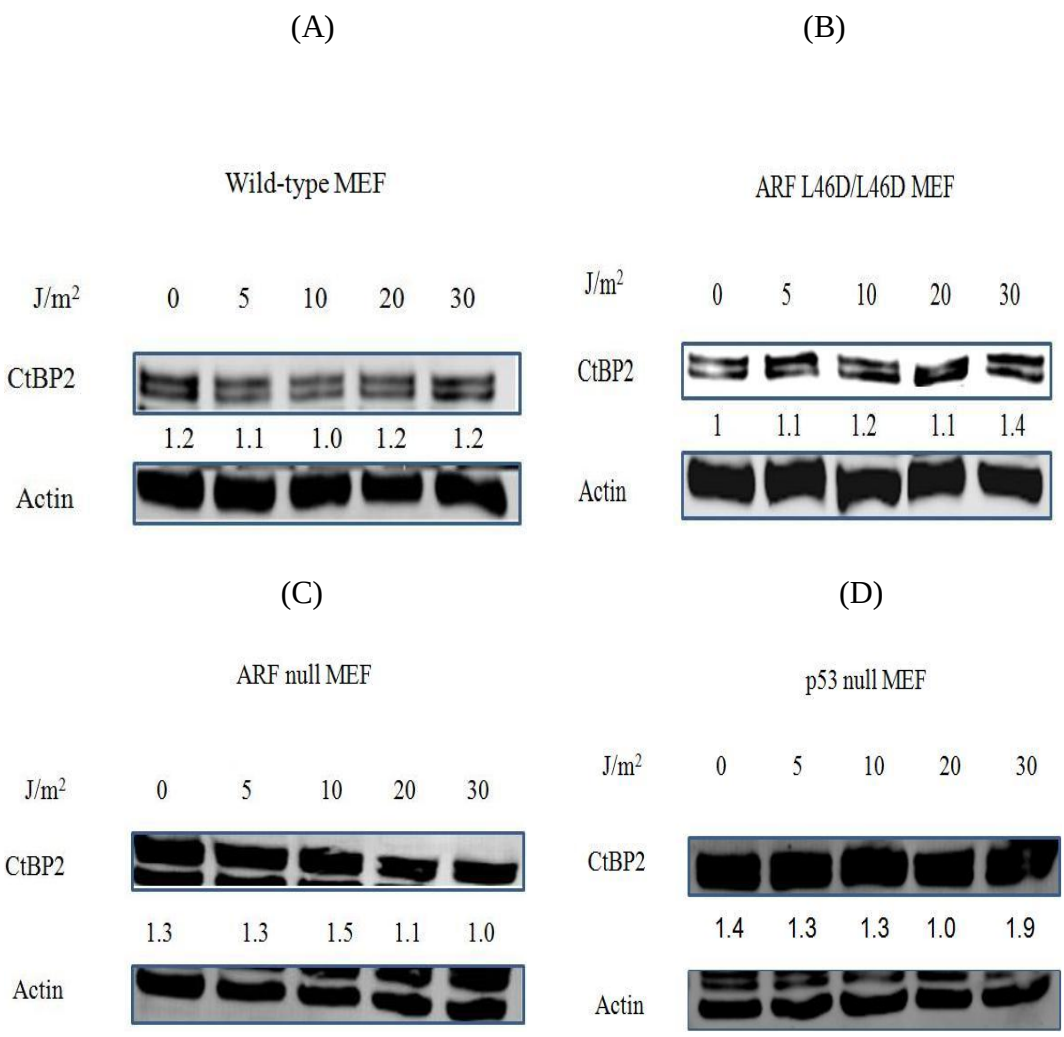


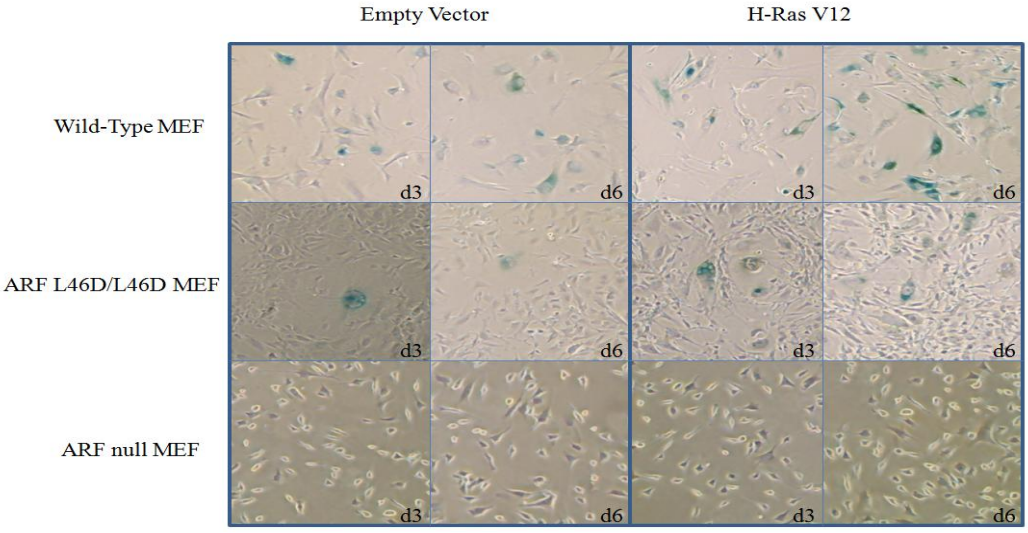
Figure 9. UV induced proteosomal degradation of CtBP2. P1Wild-type (A), P2 ARF L46D/L46D (B), ARF-null (C) and p53-null (D) MEF cells were irradiated with increasing doses (0-30J/m^2) of ultra-violet light (254nm). Changes in CtBP2 levels were determined by western blotting. CtBP2 levels were quantitated by densitometry and normalized to actin loading control.

III.4 The ARF conserved hydrophobic domain point mutation directs ARF functions in oncogene induced senescence

Premature-senescence is a signaling program that is activated independently of telomere shortening (Espejel & Blasco, 2002) and triggered through mechanisms involving DNA damage, oxidative stress, ionizing radiation and oncogene activation(Sherr & DePinho, 2000). Early studies on premature senescence induction through mitogenic signaling involving the MAPK pathway have identified p53 and p16 as key regulators of the senescence program (Serrano et al., 1997) (Lin et al., 1998). Unlike human fibroblasts, mouse embryonic fibroblasts favor the ARF-p53-p21 axis in tumor suppression upon oncogenic stress as opposed to the p16-Rb-E2F pathway (Sharpless, Ramsey, Balasubramanian, Castrillon, & DePinho, 2004). Synthesis of $p19^{Arf}$ is induced by overexpression of oncogenes such as c-myc, E1A, E2F1, and in particular, H-Ras V12 controls the expression of $p19^{Arf}$ and p16 from the INK4A/ARF locus (Palmero, Pantoja, & Serrano, 1998). Regardless of the molecular trigger, premature senescence resembles replicative senescence in terms of flat cellular morphology with larger size compared to the non-senescent counterpart cells and increasing SA-Beta gal activity due to the increase in the size of the lysosomes (Dimri et al., 1995). In order to define the functionality of the senescence program in ARF L46D/L46D MEF cells upon oncogenic stress in search for answers that might explain lymphoma development in wild-type, ARF L46D/L46D and ARF-null MEF cells were retrovirally infected with and empty vector control or HRas-V12 and selected in 2µg/ml puromycin medium. P1 Wild-type and P2 ARF L46D/L46D MEF cells were fixed and stained on day3 and day6 (Figure 6) at passage numbers (P3, P4) and (P4, P5) respectively. The percentage of wild-type MEF cells expressing β-galactosidase as indicated by the blue colored cells on day6 (Figure 10- (A)) was higher in H-RasV12 expressing population (53%) as opposed to empty vector infected cells (29%) (Figure 10-(B)). On the contrary, ARF-null MEF cells

showed no detectable expression of β-galactosidase either upon H-RasV12 over-expression, or when infected with an empty vector control. These results were expected since ARF-null MEFs were immortalized through a 3T9 cell immortalization assay (data not shown). Interestingly, the pool of ARF L46D/L46D MEF cells which expressed β-galactosidase was significantly lower than those in wild-type both on day 3 (7%) and day 6 (10%) (Figure 10-(B)), suggesting that disrupting the conserved hydrophobic domain responsible for ARF/CtBP2 interaction results in abrogation of the senescence program mediated by full-length ARF.

(A)



(B)

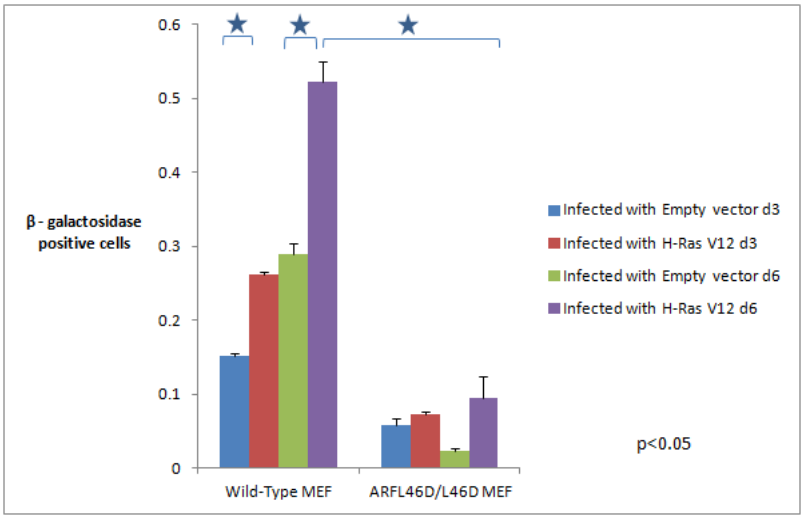


Figure 10. Oncogene induced senescence upon ectopic expression of H-RasV12. P1wild-type, P2 ARFL46D/L46D and ARF-null MEF cells were transduced with retroviruses expressing an empty vector or H-RasV12. (A) Acidic Beta-galactosidase staining performed on day 3 and day 6, (B) Bar diagram showing the percentage of β-galactosidase positive cells in wild-type and ARF L46D/L46D MEF cells. Error bars are SE.

III.5 H-RasV12 mediated induction of p21 is compromised in MEF cells harboring a point mutation in the conserved hydrophobic domain (37-51) in ARF.

In order to study the effects of disrupting ARF/CtBP2 binding on the expression of key senescence-associated cell cycle regulators, wild-type, and ARFL46D/L46D and ARF-null MEF cells were seeded in 10cm plates and were infected with retroviral cocktails expressing H-RasV12 or an empty vector control (Materials and Methods), and lysates were analyzed for p19-ARF, p53, p21, MDM2, p16, CtBP2 and phospho-Rb levels. Consistent with previous studies, H-RasV12 led to an increase in $p19^{Arf}$, MDM2, p53 and p21 in wild-type MEFs supporting the idea that in normal cells, stabilization of p53 leads to cell cycle arrest by inducing p21 expression (Figure 11). It was not unexpected to see CtBP2 levels not change in wild-type cells expressing H-RasV12, considering that though $p19^{Arf}$ was upregulated, there was no concomitant UV or other oxidative stress (Paliwal et al., 2006). ARF-null MEF expressing H-RasV12 surprisingly showed increasing levels of p53 and p21, although no $p19^{Arf}$ was detected. Increasing p53 stability could be partially explained by H-Ras V12 induced chromosomal instability leading to accumulation and phosphorylation of p53 (Woo & Poon, 2004). Failure to detect p16 expression in ARF-null MEFs expressing H-RasV12 or control empty vector was not expected (Figure11), since previous studies showed that p16 is expressed in ARF deficient cells (Kamijo et al., 1997). One explanation to the absence of p16 in ARF-null cells could be attributed to chromosomal instability due to continuous passaging in culture. Although no detectable levels of p16 were seen, using ser-795 phospho-specific antibody against Rb showed that hyperphosphorylated Rb was only and equally detected in ARF-null cells infected with an empty vector or H-RasV12, suggesting that in the absence of ARF and p16, CDK4 was therefore invariably functional regardless of H-RasV12 activity, correlating with high proliferation rate (data not shown).

Unlike ARF-null cells, ARF L46D/L46D MEF cells maintained a functional p16-Rb axis similar to wild-type MEFs, with upregulation of p16 and low phospho-Rb levels (Figure11). Paradoxically, H-RasV12 overexpression led to p53 stabilization in ARFL46D/L46D MEFs similarly to wild-type cells, significant p21 expression was not observed as compared to wild-type cells (Figure 11), suggesting that the ARF-p53 pathway is indeed defective in ARF L46D/L46D MEFs, and this correlates with a defect in oncogene induced senescence.

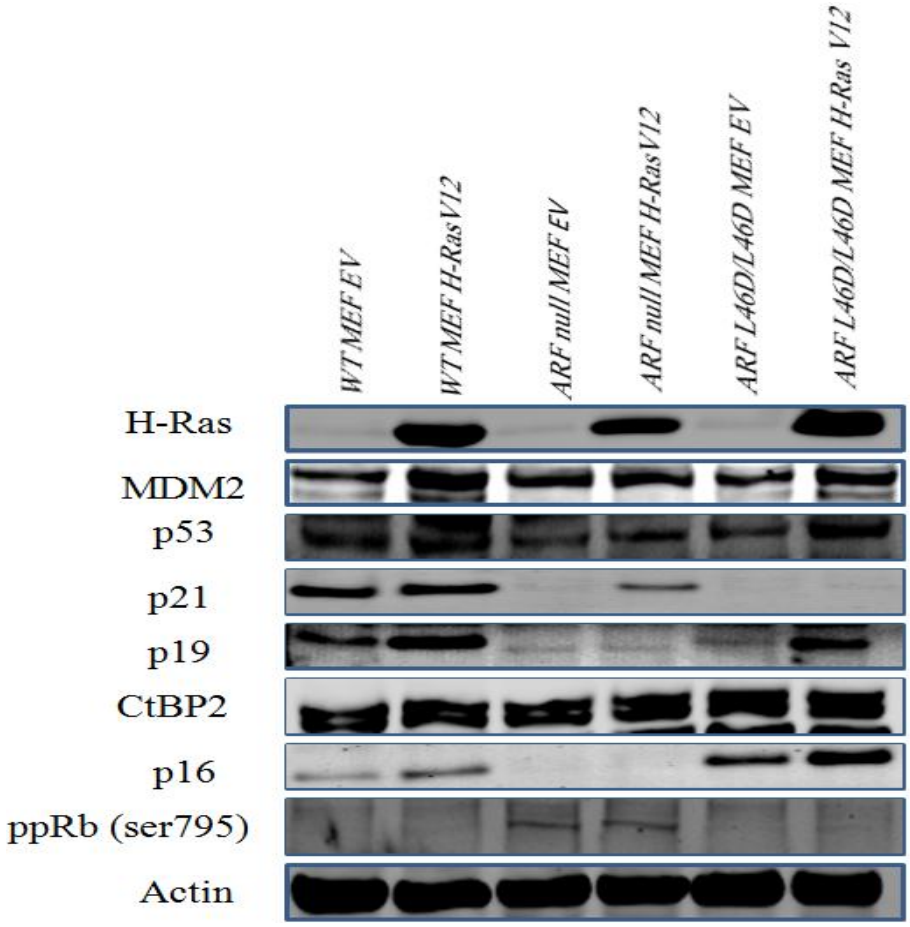


Figure 11. Ectopic expression of H-RasV12 does not induce p21 expression in ARFL46D/L46D MEF cells. P1wild-type, P1 ARFL46D/L46D and ARF-null MEFs were retrovirally transduced with an empty vector or H-RasV12 for 2 days and harvested on day 2 post-selection and lysates were analyzed by western blotting of the following proteins: MDM2, p53, p21, p19, p16, ppRb (ser795), and CtBP2.

Chapter IV

Discussion and Future Directions

Cellular aging (replicative senescence) and programmed cell death (apoptosis) are two normal biological processes that are intended to maintain homeostasis in mammals, and deregulation of either one may lead to several neurodegenerative disorders, diabetes and cancer. Hence, senescence and apoptosis act as safe-guard mechanisms, and failure to establish either process imposes increasing risks in cancer formation. Most anti-cancer therapeutics aim at triggering tumor cell death, but apoptosis-based therapeutic methods are limited by high toxicity and lack of selectivity between normal and tumor cells. In addition, many cancer cells are refractory to chemotherapeutic agents since available drugs primarily function by inducing cell death through apoptosis in fast dividing cells which can include pluripotent normal cells in the gastro-intestinal tract or bone marrow. Moreover, cancer stem-like cells have been found irresponsive to these chemical agents and radiotherapy, leading to disease progression despite therapeutic intervention (Morrison et al., 2011). Therefore, exploring senescence as an alternative method in treating cancer lead to new avenues of therapy to suppress chemo- and radio-resistant cancer stem-like cells that are believed to underlie therapy failures and relapse (Cahu, Bustany, & Sola, 2012).

The INK4A/ARF locus acts as a sensor of stress signals and expression of p16 and $p19^{Arf}$ in mice has been linked to tissue culture stress, oxidative stress and oncogene activation.

Supporting evidence suggests that in mouse embryonic fibroblasts, the loss of key downstream effector of $p19^{Arf}$, p53, results in cellular immortalization as opposed to the loss of pRb, the downstream target of p16 (Peeper, Dannenberg, Douma, te Riele, & Bernards, 2001). Moreover, the loss of all the Rb family proteins (pRb, p107, p130) would result in immortalization suggesting that the Rb family proteins act both upstream and downstream of the ARF-p53 axis(Sage et al., 2000). The extent to which the ARF-p53 pathway contributes to H-RasV12 induced premature senescence in MEF cells independent of p16 is relative, since CDK4-null MEFs were resistant to transformation in response to H-Ras V12 activation and p53 inhibition.(Zou et al., 2002). Interestingly, the same study showed that CDK4-null MEFs showed increasing levels of p21 upon H-RasV12 activation and p53 inhibition by dominant negative p53 (DNp53), and cells underwent senescence. This suggests that increasing H-RasV12 activity and depending on the cell context, would lead to premature senescence correlating with increase in the levels and stability of p21, even when the ARF-p53 axis is lost.

The major purpose of this study was to test the functions relevant to regulation of stress responses of the tumor suppressor ARF upon disrupting its binding to CtBP2 through a point mutation (L46D) in the conserved hydrophobic domain (37-51). We showed that $p19^{ArfL46D}$ did not disrupt p53 stabilization in MEF cells treated with the DNA damaging agent doxorubicin, nor did doxorubicin induce the transcriptional activation of ARF (data not shown). Using immunofluorescent techniques, we confirmed that the L46D mutation in the conserved hydrophobic domain did not interfere with the nucleolar localization sequence. Most importantly, ARF L46D/L46D maintained a functional MDM2 binding sequence as determined by the co-localization of mutant ARF and MDM2 in accordance with previous biochemical data (Paliwal et al., 2006). Previous work done in the Grossman Lab at UMass showed that CtBP2 is

targeted for proteosomal degradation upon UV irradiation in an ARF-dependent manner. Although it is not clear how $p19^{ARF}$ destabilizes CtBP2 in conditions of UV stress, ARF seems to mediate the delivery of ubiquitinated CtBP2 to the proteosome. Our results indicate that abrogating ARF/CtBP2 binding results in CtBP2 stabilization similar to what was shown previously in ARF null cells. Lastly, we assessed the behavior of MEF cells bearing the L46D mutation in response to oncogenic stress. Our data indicates that MEF cells with mutant ARF are not responsive to H-RasV12 induced senescence, and we have identified that p21 deficiency is most likely the underlying molecular defect which is promoting cell survival in ARFL46D/L46D MEF cells. Taken together, our results explain a possible molecular basis for lymphoma development in ARFL46D/L46D mice, as due to the loss of normal ARF-mediated oncogene induced senescence and p21 induction (Figure 11). Whether the defect impinges on a p53 dependent or independent pathway for ARF-induced senescence was not directly addressed in this study, though the involvement of p21 suggests a p53 dependent pathway. Indeed, a more in-depth testing on the underlying causes for p21 low expression levels is required, since these results were observed once from a single population of ARFL46D/L46D MEF cells and therefore maybe subject to variability (Appendix-I).

The ARFL46D/L46D knock-in mice were generated in order to characterize the physiologic significance of the interaction between ARF and CtBP2. Mice homozygous for the mutation in the conserved hydrophobic region of ARF showed a similar tumor profile to that seen in ARF-null mice, but with a longer mean tumor latency, suggesting that ARF L46D/L46D is contributing to tumor suppression possibly due to a combination of p53–dependent and independent mechanisms. In this study, we used mouse embryonic fibroblasts as a model to test for the outcomes of disrupting ARF/CtBP2 binding in cases of genotoxic and oncogenic stress.

Since all tumors that developed were lymphomas, then it is reasonable in the future, to study the effects of the L46D mutation in splenocytes, thymocytes or lymphoma cells under the same stress conditions used on MEF cells. Moreover, MEF cells don't share the same lineage with lymphocytes, which might account for changes in the identity of the mediators functioning in the senescence program. There is another advantage in shifting the study to lymphocytes. As discussed previously, no detectable changes in CtBP2 levels were seen in wild-type MEF cells expressing active H-Ras. Indeed, CtBP2 sequestration by ARF alone is not necessarily a signal for CtBP2 proteosomal degradation. However, since primary MEF cells are explanted from developing embryos, it is expected that CtBP2 levels are originally high, as CtBP2 has been associated with fine tuning the transcriptional response in development (Y. W. Zhang & Arnosti, 2011).

Gain of function mutations lead to abnormal functions and are dominant over the wild-type gene products. Based on that, it is critical to test whether the substitution of the hydrophobic Leucine with a hydrophilic aspartic acid in the L46D residue would result in a mutant $p19^{Arf}$ with novel properties. Accordingly, through immunoprecipitation and localization assays, we can further assess whether mutant ARF is sequestering p53 in conditions of oncogenic stress. Further testing on whether ARFL46D/L46D MEF cells behave similarly to ARF-null MEFs in regard to evasion of apoptosis upon UV irradiation would confirm our hypothesis and suggestion regarding CtBP2 stabilization when not sequestered by ARF.

Although SA-B-gal is one of the most widely used markers for senescence, there is no evidence that β-galactosidase itself is involved in modulating the senescence program. Further studies showed that it is not a legitimate but rather a surrogate marker for increased lysosomal activity and size, features common in aging cells (Lee et al., 2006). Based on this, using other

markers such as γH2AX, Ki67, PAI-1 in combination with X-gal in MEFs or lymphocytes would be a better approach for detecting senescence in response to oncogenic stress. Regardless of the drawback, our results indicate that a deterioration in the senescence program occurred in ARFL46D/L46D MEF cells. In order to further expand on the deficits in p21, chromatin immunoprecipitation (ChIP) study on the p21 promoter region and using antibodies specific for p53 (to ensure that there is no impact on p53 localization at the promoter) and CtBP2 (a known repressor of p21) (Madison & Lundblad, 2010) , would significantly discern key differences seen among wild-type and ARFL46D/L46D MEF cells. We hypothesize that CtBP2 is more abundantly occupying the promoter region of p21 and therefore antagonizing p53 mediated transcriptional initiation in cells where ARF cannot target CtBP for degradation. Real-time PCR preliminary results (data not shown) indicated low levels of p21 mRNA in ARFL46D/L46D MEF cells compared to the wild-type when infected with empty vector and H-RasV12, in accordance with the western blot results (Figure11). If CtBP2 was not found associating with p21 promoter, an alternative hypothesis is that oncogenic MDM2 is functioning as a negative regulator on p21 independent of p53. Again, ChIP of the p21 promoter region with MDM2 antibody followed by real-time PCR on the precipitated fragments from the different cell line is a good start point. Finally, since MTOB has proven efficiency against tumor cells, it is critical to test the drug in vivo once lymphoma is detected and at later stages in ARF-null and ARF L46D/L46D mice.

In conclusion, our data suggests that escape from senescence is a mechanism with which ARFL46D/L46D MEF cells are selected for survival and continuous proliferation, and a potential reason for lymphoma development in mice bearing the L46D mutation on both ARF alleles.

LIST OF REFERENCES

References

Asp, P., Acosta-Alvear, D., Tsikitis, M., van Oevelen, C., & Dynlacht, B. D. (2009). E2f3b plays an essential role in myogenic differentiation through isoform-specific gene regulation. *Genes & Development, 23*(1), 37-53.

Attardi, L. D., Reczek, E. E., Cosmas, C., Demicco, E. G., McCurrach, M. E., Lowe, S. W., et al. (2000). PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family. *Genes & Development, 14*(6), 704-718.

Balaburski, G. M., Hontz, R. D., & Murphy, M. E. (2010). p53 and ARF: Unexpected players in autophagy. *Trends in Cell Biology, 20*(6), 363-369.

Barroilhet, L., Yang, J., Hasselblatt, K., Paranal, R. M., Ng, S. K., Rauh-Hain, J. A., et al. (2012). C-terminal binding protein-2 regulates response of epithelial ovarian cancer cells to histone deacetylase inhibitors. *Oncogene,*

Bergman, L. M., & Blaydes, J. P. (2006). C-terminal binding proteins: Emerging roles in cell survival and tumorigenesis. *Apoptosis : An International Journal on Programmed Cell Death, 11*(6), 879-888.

Bhambhani, C., Chang, J. L., Akey, D. L., & Cadigan, K. M. (2011). The oligomeric state of CtBP determines its role as a transcriptional co-activator and co-repressor of wingless targets. *The EMBO Journal, 30*(10), 2031-2043.

Birts, C. N., Harding, R., Soosaipillai, G., Halder, T., Azim-Araghi, A., Darley, M., et al. (2010). Expression of CtBP family protein isoforms in breast cancer and their role in chemoresistance. *Biology of the Cell / Under the Auspices of the European Cell Biology Organization, 103*(1), 1-19.

Bonazzi, M., Spano, S., Turacchio, G., Cericola, C., Valente, C., Colanzi, A., et al. (2005). CtBP3/BARS drives membrane fission in dynamin-independent transport pathways. *Nature Cell Biology, 7*(6), 570-580.

Boone, D. N., Qi, Y., Li, Z., & Hann, S. R. (2011). Egr1 mediates p53-independent c-myc-induced apoptosis via a noncanonical ARF-dependent transcriptional mechanism. *Proceedings of the National Academy of Sciences of the United States of America, 108*(2), 632-637.

Cahu, J., Bustany, S., & Sola, B. (2012). Senescence-associated secretory phenotype favors the emergence of cancer stem-like cells. *Cell Death & Disease, 3,* e446.

Chen, S., Whetstine, J. R., Ghosh, S., Hanover, J. A., Gali, R. R., Grosu, P., et al. (2009). The conserved NAD(H)-dependent corepressor CTBP-1 regulates caenorhabditis elegans life span. *Proceedings of the National Academy of Sciences of the United States of America, 106*(5), 1496-1501.

Chinnadurai, G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Molecular Cell, 9*(2), 213-224.

Chinnadurai, G. (2003). CtBP family proteins: More than transcriptional corepressors. *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology, 25*(1), 9-12.

Chinnadurai, G. (2007). Transcriptional regulation by C-terminal binding proteins. *The International Journal of Biochemistry & Cell Biology, 39*(9), 1593-1607.

Chinnadurai, G. (2009). The transcriptional corepressor CtBP: A foe of multiple tumor suppressors. *Cancer Research, 69*(3), 731-734.

Chung, W. Y., Wadhawan, S., Szklarczyk, R., Pond, S. K., & Nekrutenko, A. (2007). A first look at ARFome: Dual-coding genes in mammalian genomes. *PLoS Computational Biology, 3*(5), e91.

Contente, S., Yeh, T. J., & Friedman, R. M. (2011). H-ras localizes to cell nuclei and varies with the cell cycle. *Genes & Cancer, 2*(2), 166-172.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proceedings of the National Academy of Sciences of the United States of America, 92*(20), 9363-9367.

Dirac, A. M., & Bernards, R. (2003). Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. *The Journal of Biological Chemistry, 278*(14), 11731-11734.

Espejel, S., & Blasco, M. A. (2002). Identification of telomere-dependent "senescence-like" arrest in mouse embryonic fibroblasts. *Experimental Cell Research, 276*(2), 242-248.

Esteller, M., Cordon-Cardo, C., Corn, P. G., Meltzer, S. J., Pohar, K. S., Watkins, D. N., et al. (2001). p14ARF silencing by promoter hypermethylation mediates abnormal intracellular localization of MDM2. *Cancer Research, 61*(7), 2816-2821.

Eymin, B., Claverie, P., Salon, C., Leduc, C., Col, E., Brambilla, E., et al. (2006). p14ARF activates a Tip60-dependent and p53-independent ATM/ATR/CHK pathway in response to genotoxic stress. *Molecular and Cellular Biology, 26*(11), 4339-4350.

Furusawa, T., Moribe, H., Kondoh, H., & Higashi, Y. (1999). Identification of CtBP1 and CtBP2 as corepressors of zinc finger-homeodomain factor deltaEF1. *Molecular and Cellular Biology, 19*(12), 8581-8590.

Gaidano, G., Foa, R., & Dalla-Favera, R. (2012). Molecular pathogenesis of chronic lymphocytic leukemia. *The Journal of Clinical Investigation, 122*(10), 3432-3438.

Gil, J., & Peters, G. (2006). Regulation of the INK4b-ARF-INK4a tumour suppressor locus: All for one or one for all. *Nature Reviews.Molecular Cell Biology, 7*(9), 667-677.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell, 144*(5), 646-674.

Honda, R., Tanaka, H., & Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Letters, 420*(1), 25-27.

Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., et al. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell, 91*(5), 649-659.

Kawagishi, H., Nakamura, H., Maruyama, M., Mizutani, S., Sugimoto, K., Takagi, M., et al. (2010). ARF suppresses tumor angiogenesis through translational control of VEGFA mRNA. *Cancer Research, 70*(11), 4749-4758.

Knudsen, E. S., & Wang, J. Y. (1996). Differential regulation of retinoblastoma protein function by specific cdk phosphorylation sites. *The Journal of Biological Chemistry, 271*(14), 8313-8320.

Korgaonkar, C., Hagen, J., Tompkins, V., Frazier, A. A., Allamargot, C., Quelle, F. W., et al. (2005). Nucleophosmin (B23) targets ARF to nucleoli and inhibits its function. *Molecular and Cellular Biology, 25*(4), 1258-1271.

Korgaonkar, C., Zhao, L., Modestou, M., & Quelle, D. E. (2002). ARF function does not require p53 stabilization or Mdm2 relocalization. *Molecular and Cellular Biology, 22*(1), 196-206.

Kovi, R. C., Paliwal, S., Pande, S., & Grossman, S. R. (2010). An ARF/CtBP2 complex regulates BH3-only gene expression and p53-independent apoptosis. *Cell Death and Differentiation, 17*(3), 513-521.

Kruse, J. P., & Gu, W. (2009). Modes of p53 regulation. *Cell, 137*(4), 609-622.

Kuilman, T., Michaloglou, C., Mooi, W. J., & Peeper, D. S. (2010). The essence of senescence. *Genes & Development, 24*(22), 2463-2479.

Lee, B. Y., Han, J. A., Im, J. S., Morrone, A., Johung, K., Goodwin, E. C., et al. (2006). Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell, 5*(2), 187-195.

Li, T., Kon, N., Jiang, L., Tan, M., Ludwig, T., Zhao, Y., et al. (2012). Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. *Cell, 149*(6), 1269-1283.

Lin, A. W., Barradas, M., Stone, J. C., van Aelst, L., Serrano, M., & Lowe, S. W. (1998). Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes & Development, 12*(19), 3008-3019.

Llanos, S., Clark, P. A., Rowe, J., & Peters, G. (2001). Stabilization of p53 by p14ARF without relocation of MDM2 to the nucleolus. *Nature Cell Biology, 3*(5), 445-452.

Lowe, S. W., & Sherr, C. J. (2003). Tumor suppression by Ink4a-arf: Progress and puzzles. *Current Opinion in Genetics & Development, 13*(1), 77-83.

Madison, D. L., & Lundblad, J. R. (2010). C-terminal binding protein and poly(ADP)ribose polymerase 1 contribute to repression of the p21(waf1/cip1) promoter. *Oncogene, 29*(45), 6027-6039.

McMahon, M., & Woods, D. (2001). Regulation of the p53 pathway by ras, the plot thickens. *Biochimica Et Biophysica Acta, 1471*(2), M63-71.

Moll, U. M., & Petrenko, O. (2003). The MDM2-p53 interaction. *Molecular Cancer Research : MCR, 1*(14), 1001-1008.

Morrison, R., Schleicher, S. M., Sun, Y., Niermann, K. J., Kim, S., Spratt, D. E., et al. (2011). Targeting the mechanisms of resistance to chemotherapy and radiotherapy with the cancer stem cell hypothesis. *Journal of Oncology, 2011*, 941876.

Mroz, E. A., Baird, A. H., Michaud, W. A., & Rocco, J. W. (2008). COOH-terminal binding protein regulates expression of the p16INK4A tumor suppressor and senescence in primary human cells. *Cancer Research, 68*(15), 6049-6053.

Nardini, M., Svergun, D., Konarev, P. V., Spano, S., Fasano, M., Bracco, C., et al. (2006). The C-terminal domain of the transcriptional corepressor CtBP is intrinsically unstructured. *Protein Science : A Publication of the Protein Society, 15*(5), 1042-1050.

Ozenne, P., Eymin, B., Brambilla, E., & Gazzeri, S. (2010). The ARF tumor suppressor: Structure, functions and status in cancer. *International Journal of Cancer.Journal International Du Cancer, 127*(10), 2239-2247.

Paliwal, S., Pande, S., Kovi, R. C., Sharpless, N. E., Bardeesy, N., & Grossman, S. R. (2006). Targeting of C-terminal binding protein (CtBP) by ARF results in p53-independent apoptosis. *Molecular and Cellular Biology, 26*(6), 2360-2372.

Palmero, I., Pantoja, C., & Serrano, M. (1998). p19ARF links the tumour suppressor p53 to ras. *Nature, 395*(6698), 125-126.

Peeper, D. S., Dannenberg, J. H., Douma, S., te Riele, H., & Bernards, R. (2001). Escape from premature senescence is not sufficient for oncogenic transformation by ras. *Nature Cell Biology, 3*(2), 198-203.

Quelle, D. E., Zindy, F., Ashmun, R. A., & Sherr, C. J. (1995). Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell, 83*(6), 993-1000.

Rodier, F., & Campisi, J. (2011). Four faces of cellular senescence. *The Journal of Cell Biology, 192*(4), 547-556.

Ruas, M., & Peters, G. (1998). The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochimica Et Biophysica Acta, 1378*(2), F115-77.

Sage, J., Mulligan, G. J., Attardi, L. D., Miller, A., Chen, S., Williams, B., et al. (2000). Targeted disruption of the three rb-related genes leads to loss of G(1) control and immortalization. *Genes & Development, 14*(23), 3037-3050.

Sanchez-Cespedes, M., Reed, A. L., Buta, M., Wu, L., Westra, W. H., Herman, J. G., et al. (1999). Inactivation of the INK4A/ARF locus frequently coexists with TP53 mutations in non-small cell lung cancer. *Oncogene, 18*(43), 5843-5849.

Schauble, S., Klement, K., Marthandan, S., Munch, S., Heiland, I., Schuster, S., et al. (2012). Quantitative model of cell cycle arrest and cellular senescence in primary human fibroblasts. *PloS One, 7*(8), e42150.

Schmitz, F., Konigstorfer, A., & Sudhof, T. C. (2000). RIBEYE, a component of synaptic ribbons: A protein's journey through evolution provides insight into synaptic ribbon function. *Neuron, 28*(3), 857-872.

Serrano, M. (2000). The INK4a/ARF locus in murine tumorigenesis. *Carcinogenesis, 21*(5), 865-869.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., & Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell, 88*(5), 593-602.

Sharpless, N. E. (2005). INK4a/ARF: A multifunctional tumor suppressor locus. *Mutation Research, 576*(1-2), 22-38.

Sharpless, N. E., Bardeesy, N., Lee, K. H., Carrasco, D., Castrillon, D. H., Aguirre, A. J., et al. (2001). Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature, 413*(6851), 86-91.

Sharpless, N. E., & DePinho, R. A. (1999). The INK4A/ARF locus and its two gene products. *Current Opinion in Genetics & Development, 9*(1), 22-30.

Sharpless, N. E., Ramsey, M. R., Balasubramanian, P., Castrillon, D. H., & DePinho, R. A. (2004). The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. *Oncogene, 23*(2), 379-385.

Sherr, C. J. (1998). Tumor surveillance via the ARF-p53 pathway. *Genes & Development, 12*(19), 2984-2991.

Sherr, C. J. (2006). Divorcing ARF and p53: An unsettled case. *Nature Reviews.Cancer, 6*(9), 663-673.

Sherr, C. J., Bertwistle, D., DEN Besten, W., Kuo, M. L., Sugimoto, M., Tago, K., et al. (2005). p53-dependent and -independent functions of the arf tumor suppressor. *Cold Spring Harbor Symposia on Quantitative Biology, 70*, 129-137.

Sherr, C. J., & DePinho, R. A. (2000). Cellular senescence: Mitotic clock or culture shock? *Cell, 102*(4), 407-410.

Sherr, C. J., & McCormick, F. (2002). The RB and p53 pathways in cancer. *Cancer Cell, 2*(2), 103-112.

Sreeramaneni, R., Chaudhry, A., McMahon, M., Sherr, C. J., & Inoue, K. (2005). Ras-raf-arf signaling critically depends on the Dmp1 transcription factor. *Molecular and Cellular Biology, 25*(1), 220-232.

Wang, Y., Zhang, Z., Kastens, E., Lubet, R. A., & You, M. (2003). Mice with alterations in both p53 and Ink4a/Arf display a striking increase in lung tumor multiplicity and progression: Differential chemopreventive effect of budesonide in wild-type and mutant A/J mice. *Cancer Research, 63*(15), 4389-4395.

Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J., & Bar-Sagi, D. (1999). Nucleolar arf sequesters Mdm2 and activates p53. *Nature Cell Biology, 1*(1), 20-26.

Woo, R. A., & Poon, R. Y. (2004). Activated oncogenes promote and cooperate with chromosomal instability for neoplastic transformation. *Genes & Development, 18*(11), 1317-1330.

Yi, Y., Shepard, A., Kittrell, F., Mulac-Jericevic, B., Medina, D., & Said, T. K. (2004). p19ARF determines the balance between normal cell proliferation rate and apoptosis during mammary gland development. *Molecular Biology of the Cell, 15*(5), 2302-2311.

Zhang, Q., Wang, S. Y., Nottke, A. C., Rocheleau, J. V., Piston, D. W., & Goodman, R. H. (2006). Redox sensor CtBP mediates hypoxia-induced tumor cell migration. *Proceedings of the National Academy of Sciences of the United States of America, 103*(24), 9029-9033.

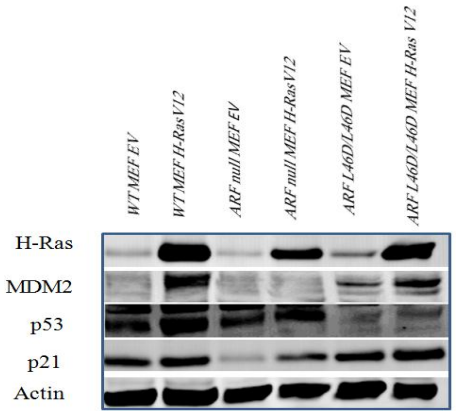
Zhang, Y. W., & Arnosti, D. N. (2011). Conserved catalytic and C-terminal regulatory domains of the C-terminal binding protein corepressor fine-tune the transcriptional response in development. *Molecular and Cellular Biology, 31*(2), 375-384.

Zou, X., Ray, D., Aziyu, A., Christov, K., Boiko, A. D., Gudkov, A. V., et al. (2002). Cdk4 disruption renders primary mouse cells resistant to oncogenic transformation, leading to Arf/p53-independent senescence. *Genes & Development, 16*(22), 2923-2934.

Appendix-I

p21 expression may be subject to variability among ARFL46D/L46D MEF cells

(A)



(B)

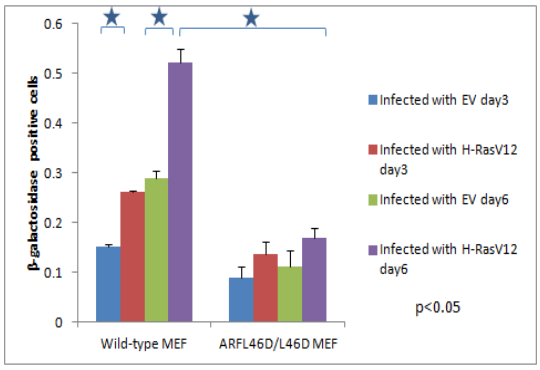


Figure A1. Ectopic expression of H-RasV12 induces p21 expression but not senescence in ARF L46D/L46D MEF cells of a particular lineage. P1 wild-type MEFs, P1 ARFL46D/L46D MEFs obtained from mating a homozygous male (ARFL46D/L46D) with a heterozygous female (ARF+/ARFL46D) and ARF null MEFs were retrovirally transduced with an empty vector or H-RasV12 as described before. (A) Western blot analysis on MDM2, p53 and p21. (B) Bar diagram showing the percentage of β-galactosidase positive cells in wild-type and ARFL46D/L46D MEF cells. Error bars are SE.

Vita

Aymen Faris Hariz was born on April 06, 1983, in Al-khobar, Saudi Arabia and is a Lebanese citizen. He graduated from Kernayel official high school, Mount Lebanon, Lebanon in 2000. He received his Bachelor of Science degree in Animal Biology from Lebanese University, Beirut, Lebanon in 2004 and subsequently taught in Lebanese public schools and then in the International School of Choueifat in Egypt for 6 years. He joined the Human Genetics department at Virginia Commonwealth University in 2011.