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Defining the Role of CtBP2 in p53-Independent Tumor Suppressor Function of ARF: A Dissertation

Ramesh C. Kovi
University of Massachusetts Medical School

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Defining the Role of CtBP2 in p53-independent Tumor Suppressor Function of ARF

A Dissertation Presented

By

Ramesh C. Kovi

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

CANCER BIOLOGY

June 11, 2009

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The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the dissertation

Dr. Steven R. Grossman, M.D.Ph.D., Thesis Advisor

Dr. Stephen N. Jones, Ph.D., Member of Committee

Dr. Brian C. Lewis, Ph.D., Member of Committee

Dr. Sharon B. Cantor Ph.D., Member of Committee

Dr. Nabeel M. Bardeesy Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

Dr. Arthur M. Mercurio, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

Dr. Anthony Carruthers, Ph.D.,

Dean of the Graduate School of Biomedical Sciences

Cancer Biology

June 11, 2009

TO

THE CHERISHED MEMORY OF

MY MOTHER

Mrs. CHOWDAMMA

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Parts of this dissertation have appeared in separate publications:

Kovi RC, Paliwal S, Pande S and Grossman SR. 2009. An ARF/CtBP2 complex regulates BH3-only gene expression and p53-independent apoptosis. *Cell Death and Differentiation* (in press).

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Straza M, **Kovi RC**, Paliwal S, Messina M, Trench P and Grossman SR. 2009. C-terminal Binding Protein 2 is a cellular target of the cytotoxic compound 4-methylthio-2-oxobutyric acid (MTOB). *Cancer Research* (submitted).

Paliwal S, **Kovi RC**, Nath B, Chen YW, Lewis BC and Grossman SR. 2007. ARF antagonizes cancer cell migration via interaction with CtBP Corepressor. *Cancer Research*. 67 (19):9322-9.

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List of Abbreviations

APC	Adenomatosis polyposis coli
ARF	Alternate reading frame
BARS	brefeldin A-ADP ribosylated substrate
Bax	Bcl2-associated X protein
BH3	Bcl2 homology 3
BH3-only	Bcl2 homology only
Bik	Bcl2-interacting killer
BKLF	Basic Kruppel-like factor
CtBP	Carboxyl terminal binding protein
D2-HDH	D-isomer 2 hydroxy acid dehydrogenase
DMBA	7,12-Dimethyl benzanthracene
E1A	Early Region 1A
EMT	Epitheial-mesenchymal transition
Evi-1	Ecotropic viral integration site 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HIPK2	Homeodomain interacting protein kinase 2
MDM2	Murine double minute
MEFs	Mouse embryonic fibroblasts
NAD	Nicotinamide adenine dinucleotide
NPM	Nucleophosmin
PI3K	Phosphatidyl-inositol 3-kinase
PLDLS	Proline-leucine-aspartic acid-leucine-serine
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma
RT-PCR	Reverse transcriptase polymerase chain reaction
siRNA	Small interfering RNA
smArf	Short mitochondrial alternate reading frame
TGF- β	Transforming growth factor- β
UV	Ultra violet

Statement of Contribution

The research work presented in Chapters 2 and 3 were collaborations with Dr. Seema Paliwal, Instructor in Dr. Steven Grossman's Lab. Seema contributed to Figures 2.1A-B, 2.2A-B, 2.3 A-C, E, 2.5A and Figure 3.4A-C, F. Sandhya Pande performed the initial Apoptosis cDNA array for Chapter 3. I am responsible for all other experiments and findings discussed in Chapters 2, 3 and 4. We have acknowledged researchers who have contributed reagents and other support to each chapter at the end of each chapter.

Defining the Role of CtBP2 in p53-independent Tumor Suppressor Function of ARF

ABSTRACT

ARF, a potent tumor suppressor, positively regulates p53 by antagonizing MDM2, a negative regulator of p53, which in turn, results in either apoptosis or cell cycle arrest. ARF also suppresses the proliferation of cells lacking p53, and loss of ARF in p53-null mice, compared with ARF-null or p53-null mice, results in a broadened tumor spectrum and decreased tumor latency. This evidence suggests that ARF exerts both p53-dependent and p53-independent tumor suppressor activity. However, the molecular pathway and mechanism of ARF's p53-independent tumor suppressor activity is not understood.

The antiapoptotic, metabolically regulated, transcriptional corepressor C-terminal binding protein 2 (CtBP2) has been identified as a specific target of ARF's p53-independent tumor suppression. CtBPs are phosphoproteins with PLDLS-binding motif and NADH-binding central dehydrogenase domains. ARF interacts with CtBP1 and CtBP2 both *in vitro* and *in vivo*, and induces their proteasome-mediated degradation, resulting in p53-independent apoptosis in colon cancer cells. ARF's ability to target CtBP2 for degradation, and its induction of p53-independent apoptosis requires an intact interaction with CtBP2, and phosphorylation at S428 of CtBP2. As targets for inhibition by ARF, CtBPs are candidate oncogenes, and their expression is elevated in a majority of human colorectal adenocarcinomas specimens in comparison to normal adjacent tissue.

Relevant to its targeting by ARF, there is an inverse correlation between ARF and CtBP expression, and CtBP2 is completely absent in a subset of colorectal adenocarcinomas that retains high levels of ARF protein.

CtBPs are activated under conditions of metabolic stress, such as hypoxia, and they repress epithelial and proapoptotic genes. BH3-only genes such as Bik, Bim and Bmf have been identified as mediators of ARF-induced, CtBP2-mediated p53-indpendent apoptosis. CtBP2 repressed BH3-only genes in a tissue specific manner through BKLF (Basic kruppel like factor)-binding elements. ARF regulation of BH3-only genes also required intact interaction with CtBP2. ARF antagonism of CtBP repression of Bik and other BH3-only genes may play a critical role in ARF-induced p53-independent apoptosis, and in turn, tumor suppression.

To study the physiologic effect of ARF/CtBP2 interaction at the organismal level, the $p19^{ArfL46D}$ knock-in mice, in which the Arf/CtBP2 interaction was abrogated, was generated. Analysis of the primary cells derived from these mice, revealed that the Arf/CtBP2 interaction contributes to regulation of cell growth and cell migration. Overexpression of CtBP in human tumors, and ARF antagonism of CtBP repression of BH3-only gene expression and CtBP-mediated cell migration may therefore play a critical role in the p53-independent tumor suppressor function/s of ARF.

Chapter-I

Introduction

1.1 Tumor Suppression

Cancer is a genetic disease in which abnormal cells divide without control and are able to invade other tissues. Cancer arises through a multistep process whereby cancer cells accumulate several genetic and epigenetic changes that eventually lead to the acquisition of malignant characteristics. The properties of neoplastic cells that distinguish them from cognate normal cells of the same tissue have been categorized by Hanahan and Weinberg into six categories: 1) self sufficiency in growth signals, 2) insensitivity to growth-inhibitory signals, 3) evasion of apoptosis, 4) sustained angiogenesis, 5) invasion and metastasis and 6) capacity for sustained replicative potential [1]. In addition to these six hallmarks, there are other characteristics of tumor cells, which include evasion of immune surveillance, and stress phenotypes, such as DNA damage/replication stress, proteotoxic stress, mitotic stress, metabolic stress and oxidative stress [2, 3]. The mutations that may aid cancer cells in acquiring these capabilities occur in genes that encode a wide variety of proteins, often by modifying existing cellular programs normally used during development. The genes altered in cancer have been broadly categorized into two major classes: oncogenes and tumor suppressors.

It has been suggested that cancers arise as a result of accumulation of somatic mutations in cellular protooncogenes [4]. Deregulated (mutations in promoter, gene amplification, translocations or intragenic mutations) or abnormally overexpressed

protooncogenes govern several cellular processes such as cell proliferation, differentiation, and development. The mutations affecting these processes often constitutively deregulate specific signaling pathways. Studies involving somatic cell fusion and chromosome segregation have suggested that there are 'antioncogenes' which suppress tumorigenicity by opposing the effects of deregulated protooncogenes [5, 6].

There are three cardinal properties of 'classic' tumor suppressor genes; first, they are recessive, requiring inactivation of both the alleles to induce tumor formation [7]. Second, inheritance of a single mutant allele predisposes to tumor formation, as only one additional mutation is required for complete loss of function. Third, the same genes are frequently mutated in sporadic cancer [8]. Tumor suppressor genes regulate a wide range of normal cellular functions. Although their predominant function is not to protect against cancer, their involvement in cell cycle check point control, cell senescence, DNA damage response, hypoxia, apoptosis and other signaling pathways demonstrates the gamut of cellular processes that can be deregulated during tumorigenesis and tumor progression.

1.2 The *INK4a/ARF* locus

The *INK4a/ARF* locus on mouse chromosome 4 and the cognate locus *CDKN2A* on human chromosome band 9p21 both comprise four exons. The **a**lternate **r**eading **f**rame tumor suppressor protein (ARF, also known as $p14^{ARF}$ in human and $p19^{Arf}$ in mouse) was identified as an alternative transcript of the *INK4a* locus [9]. The structure of the *INK4a*/*ARF* locus is highly conserved among mammals [9, 10]. By virtue of unique first

exons, the $p16^{INK4a}$ and ARF transcripts are translated in alternative reading frames and encode proteins with no amino acid homology (Figure 1.1).

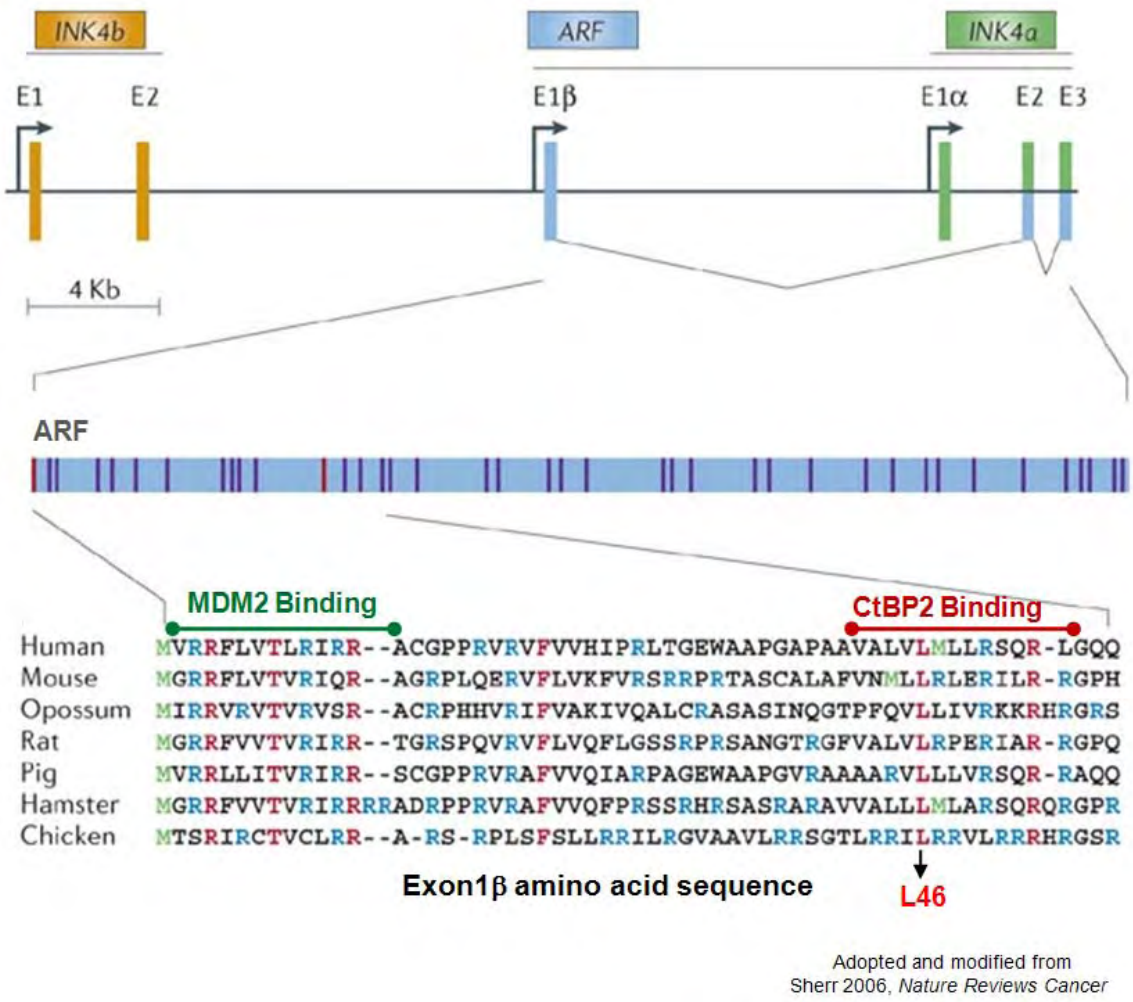
The *INK4a* gene encodes a polypeptide $p16^{INK4a}$ that inhibits cyclin D-dependent kinases CDK4 and CDK6 [11]. Further characterization of this locus revealed the presence of a similar *INK4a* gene, *INK4b*, which encodes a closely related kinase inhibitor $p15^{INK4b}$ (Figure 1.1). These kinase inhibitors exert their function by antagonizing the activities of G1 cyclin dependent kinases, which in turn prevents E2F-dependent transcription and cell proliferation [11]. The level of expression of $p16^{INK4a}$ and $p19^{Arf}$ is extremely low in most normal tissues. Therefore, these two proteins are not continuously restraining cell proliferation under physiological conditions, but rather, are activated in response to appropriate signals or additional stress. Oncogenes such as Ras, Myc, E1A and E2F1 differentially activate the expression of these proteins [12, 13, 14]. Transcriptional activation of the *INK4a/ARF* locus results in cell cycle arrest or apoptosis. The ARF tumor suppressor protein antagonizes the functions of MDM2, a negative regulator of p53 and stabilizes p53 which results in p53-dependent cell cycle arrest and apoptosis [15].

Not surprisingly, alterations in this locus affecting both $p16^{INK4a}$ and ARF are very common in human cancers [16]. Three common modes of inactivation of this locus have been reported: (i) Homozygous deletion occurs in about 14% of all human tumors and often, it also involves the *INK4b* gene [17]. (ii) Intragenic mutation accounts for 5% of tumors, especially point mutation in exon 2 that is shared by both genes. (iii) Promoter silencing by methylation, which results in complete inactivation of the two genes, has been found in about 19% of human tumors. Aberrant methylation of the *ARF* promoter

has also been found in human colorectal adenocarcinomas independently of *INK4a* promoter methylation [18]. Tumors that retain a functional *INK4a/ARF* locus exhibit dramatic upregulation of its expression due to continued presence of oncogenic stress and other alterations accumulated during tumorigenesis. It has been reported that $p16^{INK4a}$ is accumulated in skin, bladder and lung carcinomas [19].

The specific functions of *ARF* and *INK4a* in oncogenesis have been explored by targeted inactivation of the *INK4a/ARF* locus to generate whole animal and tissue-specific tumor models [20]. Mice deficient for both $p16^{INK4a}$ and $p19^{Arf}$ were generated by disruption of the shared exon 2 and 3 of the *INK4a* allele. Double-null mouse embryonic fibroblasts (MEFs) were able to overcome senescence at a faster rate compared to wild type MEFs and were highly susceptible to transformation with activated ras ($H\text{-}ras^{Val12}$) [21]. Consistent with the fact that the *INK4a/ARF* locus encodes two tumor suppressors, 69% of double-null mice developed spontaneous fibrosarcomas and lymphomas. Specific $p19^{Arf}$-/- mice were then created by targeting exon1β, and about 80% developed various malignant tumors, including sarcomas and lymphomas within the first year [22, 23] (Table 1.1). Additionally, $p19^{Arf}$-/- MEFs demonstrated increased proliferation and susceptibility to H-ras mediated transformation [22, 23]. In contrast MEFs derived from mice with exon 1α disruption, did not exhibit increased immortalization or susceptibility to H-ras mediated transformation as was observed in double-null or Arf-null MEFs [22, 24]. However, 25% of $p16^{Ink4a-/-}$ mice did develop tumors, including soft tissue sarcomas,

Figure 1.1 **The INK4b-ARF-INK4a locus**.
The locus consists of three tumor suppressor genes. Exons are indicated by letter E and colored rectangles and the promoters of the genes are designated by arrows. ARF is encoded by Exon1β, 2 and 3 with alternate reading frame in exon2. Both INK (INK4a and INK4b) genes encode inhibitors of cyclin D-dependent kinases CDK4 and CDK6 ($p16^{INK4a}$ and $p15^{INK4b}$ respectively). Lower panel is schematic of $p19^{Arf}$ protein and amino acid sequence of Exon 1β encoded region. MDM2 binding (2-14 residues) and CtBP2 binding (37-51) and all conserved residues including L46D are indicated.



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Sherr 2006, *Nature Reviews Cancer*

spleenic lymphomas and melanomas, though their incidence increases with mitogen treatment (Table 1.1) [24]. The majority of tumors in p53-null animals are lymphomas with a mean latency of 18-20 weeks, sarcomas being the second most common and carcinomas being rare [25, 26, 27]. Arf-null mice develop similar type of tumors but with mean latency of 38 weeks verses 20 weeks in p53-null. ARF functions upstream of p53, hence Arf-null mice phenocopy p53-null mice, and the extended latency in Arf-null is explained by the fact that p53 is induced by cell stress, including genotoxic stress and oncogene activation whereas Arf is induced by only oncogenic stress. More than 70% of the tumors arising in p53-null mice are T-cell lymphomas (Table 1.1), whereas Arf-nulls had lower incidence of lymphomas but more sarcomas and carcinomas. This suggests that the increased latency in the Arf-null animals may lead to a shift in the tumor spectrum to include a wide variety of tumors, which tend to develop in older mice. p53 and Mdm2 double-null mice developed tumors with the same latency and tumor spectrum as p53-null, suggesting that Mdm2 functions through p53 by antagonizing it [28, 29]. Interestingly, deletion of Arf in p53 and Mdm2 double-null mice results in a broader tumor spectrum with increased incidence of epithelial tumors, supporting the existence of an ARF tumor suppressor activity that functions independently of MDM2 and p53 [30, 31].

1.3 The ARF-MDM2-p53 pathway

The ARF tumor suppressor connects pathways regulated by canonical tumor suppressor proteins retinoblastoma (Rb) and p53. Inactivation of the Rb and p53 pathways is very common in human cancers [16]. Studies in murine and human cancers

suggested that these pathways are non-overlapping and their concomitant inactivation is cooperative during tumor progression. Upon sustained oncogenic stress such as overexpression of c-myc, v-abl, E2F or loss of Rb, ARF is transcriptionally induced [12, 13, 32, 33, 34]. Upon induction, ARF stabilizes p53 by antagonizing the E3 ligase activity of MDM2, sequestering MDM2 in the nucleolus, and by preventing MDM2-mediated nuclear export of p53 and subsequent degradation of p53 in the cytoplasm [35]. Stabilization of p53 leads to activation of p53 transcriptional target genes including MDM2, and results in either cell cycle arrest or apoptosis [36] (Figure 1.2). In the absence of p53, ARF levels are significantly elevated [10], however the reintroduction of wild type p53 into p53 null-cells can restore ARF levels to normal suggesting that there is a negative feedback regulation between ARF and p53 in vivo [37].

$p19^{Arf}$ expression in MEFs correlates with the onset of senescence and $p19^{Arf}$-null cells do not senesce in culture [23, 38]. However, $p14^{ARF}$ in normal human cells does not regulate replicative senescence [39, 40]. Despite this difference, $p14^{ARF}$ does function as a tumor suppressor in humans as it is targeted for inactivation either by chromosomal deletion or transcriptional silencing by promoter methylation [16]. ARF is frequently deleted in melanomas, biliary tumors, T- and B-cell ALLs, bladder, nasopharyngeal, and small cell lung carcinomas, and glioblastomas, strongly suggesting that ARF loss contributes significantly to human cancer [17, 41, 42]. Often, ARF is inactivated in p53 wildtype tumors as a putative alternative means of inactivating p53 function [41]. In addition to inactivating MDM2, ARF can also enhance p53 function by inhibiting E3 ligase activity of ARF-BP1/Mule, which directly interacts with and ubiquitinates p53 in

an MDM2-independent manner [43]. Further, ARF increases the effectiveness of the p53 pathway by promoting the phosphorylation and inhibition of transcriptional activity of the RelA subunit of NF-kB, which normally antagonizes p53 through induction of MDM2 and transcriptional repression of p53 [44].

ARF is a very unusual protein with more than 20% arginine residues and isoelectric point greater than 12. Mouse $p19^{Arf}$ contains one lysine (K26) whereas human $p14^{ARF}$ has none. There are no recognizable structural motifs in ARF, but $p14^{ARF}$ can form stable oligomers upon exposure to oxidizing agents [45]. Upon activation, ARF is localized to the nucleoli, an intranuclear organelle involved in ribosome biogenesis, and it forms high molecular weight complexes with nucleophosmin (NPM) [46, 47]. NPM inhibits ARF turnover by sequestering it in the nucleoli and it regulates both ribosome biogenesis and in turn, the growth phase of the cell cycle.

ARF stability and turnover are governed by N-terminal ubiquitination and proteasome-dependent degradation [48, 49]. ARF has been reported to interact with more than 30 proteins, some of which are implicated in p53-independent functions, however there is no evidence for ARF interaction with DNA or RNA directly [50]. Some of these ARF-interacting proteins are involved in ribosome biogenesis, transcriptional regulation, the DNA damage response, apoptosis and autophagy [50].

Figure 1.2 **The ARF-MDM2-p53 pathway.**
Mitogenic signal acting through Ras stimulate phosphorylation of Rb, in turn interrupts its interaction with E2Fs and promote S phase entry. Upon oncogenic stress, ARF gets activated and interferes with the activity of MDM2, resulting in p53 stabilization. Activated p53 turns on the p53-dependent transcriptional program by regulating several target genes to induce either cell cycle arrest or apoptosis in a context dependent manner. In addition to directly activating p53, oncogenic stress can also activate protective DNA damage response. Depending on the signal, DNA-damage responses activate the kinases ATM or ATR and these kinases phosphorylate p53 directly or through CHK kinases and these phosphorylations activate transcriptional activity of p53 [50, 51].

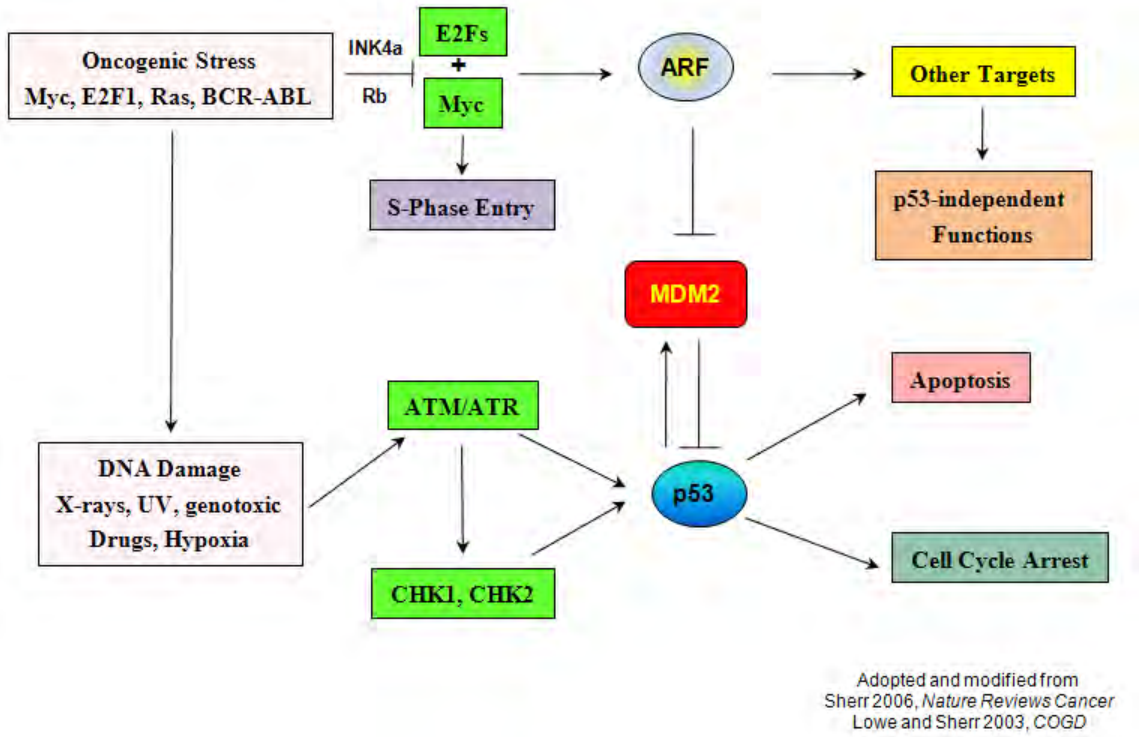


Table 1.1 **Spontaneous and DMBA-induced tumor spectra of various genotypes of genes in ARF-MDM2-p53 pathway**

Genotype	**Spontaneous Tumors**	**%**	**DMBA-induced**	**%**
Wildtype	Soft tissue Sarcomas	50	Small lymph. Lymphoma	69
	Lung Carcinoma	50	Soft tissue sarcoma	15
			Lung carcinoma	15
p16$^{INK4a-/-}$	Soft tissue sarcoma	52	Small lymph. Lymphoma	39
	Osteosarcoma	18	Soft tissue sarcoma	35
	Histiocytic lymphoma	18	Lung carcinoma	17
	Melanoma	12	Melanoma	9
p19$^{Arf-/-}$	Small lymph. Lymphoma	37	Small lymph. Lymphoma	60
	Soft tissue sarcoma	33	Lung Carcinomas	40
	Carcinomas	20	Soft tissue sarcoma	15
	Osteosarcoma	10		
INK4a/ARF-/-	Histiocytic lymphoma	90	Sarcomas	60
	Soft tissue sarcoma	9	Lymphoms	33
p53$^{-/-}$	Lymphomas	77		
	Sarcomas	23		

1.4 p53-independent functions of ARF

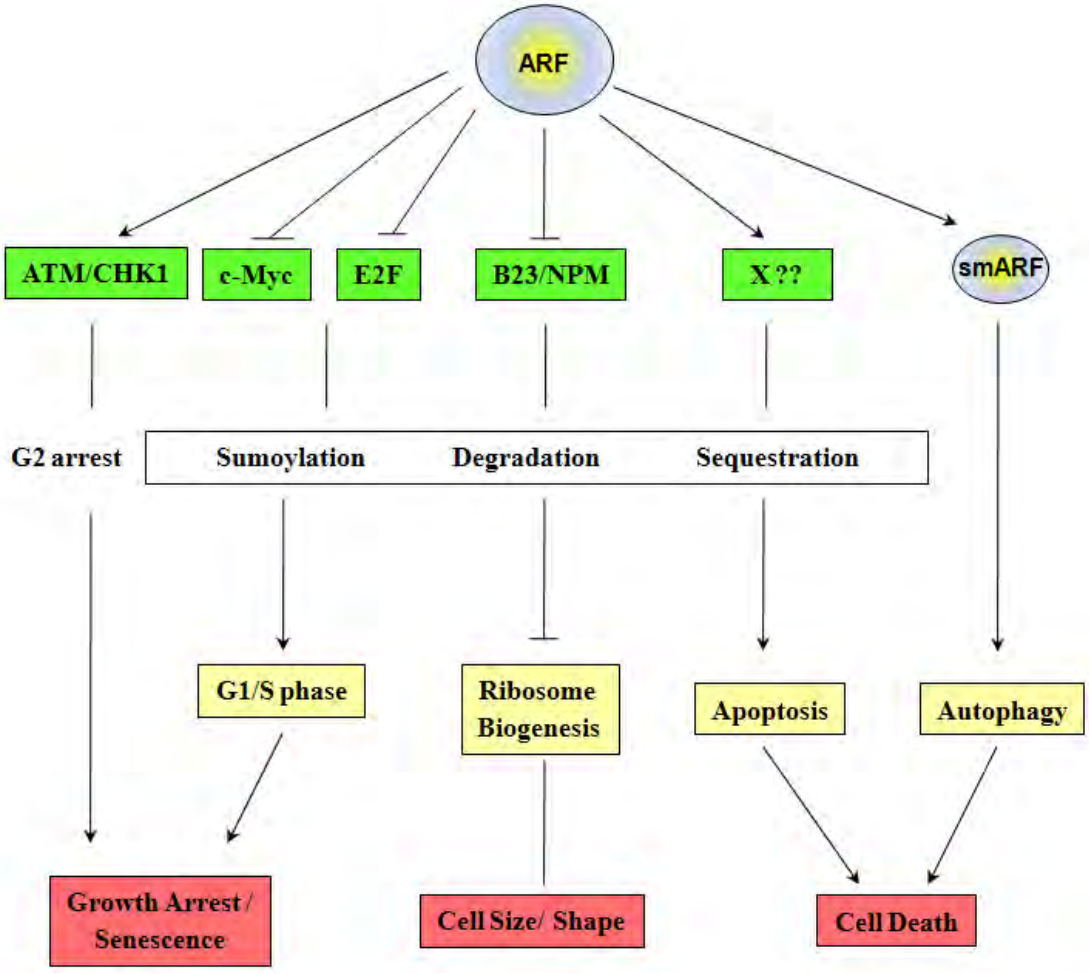
Besides its canonical role in the p53-dependent tumor suppressor pathway, there also appear to be p53-independent functions of ARF. Simultaneous inactivation of p53 and ARF in certain human tumors clearly suggests additional p53-indpendent tumor suppressor functions for ARF [52]. Given the difficulty in distinguishing the tumor suppressor contributions of the often concordantly disrupted $p16^{INK4a}$ and ARF genes in humans [16], analysis of *Arf* knockout mice has provided additional evidence for p53-independent functions of ARF. Epithelial tumors are rare in *p53* knockout mice, however 12%-28% *Arf* knockout mice developed epithelial tumors [23, 53]. Further, combined loss of *p53*, *Arf* and/or *Mdm2*, results in a substantially increased incidence of epithelial cancers, including those of the digestive tract [30, 31]. In fact, mice with loss of *p53* and *Mdm2* and retention of *Arf* display mainly mesenchymal tumors as in *p53* knockout mice [31, 54]. Additionally, in a transgenic K-ras skin cancer model, *Arf* loss accelerated tumor growth in a p53-independent manner [55].

At the cellular level, ARF can suppress the proliferation of p53-defective cells [31, 56]. There are two mechanisms by which ARF exerts this function. Firstly, ARF binds and inactivates both c-myc and E2F proteins (Figure 1.3), thus slowing the cell cycle progression through the G1/S transition, and in turn, suppressing proliferation [57, 58]. Secondly, ARF by localizing into nucleoli, forms a stoichiometric complex with nucleophosmin/B23, and disrupts ribosome biogenesis, and slows progression of cells through the growth phases of the cell cycle [47, 59, 60]. smArf (short mitochondrial ARF) is translated from internal translational initiation site at methionine 48 in human and 45 in

mouse. smARF has been reported to induce autophagy by altering the mitochondrial membrane potential in p53-independent manner [61].

ARF is not directly induced by DNA-damage signaling pathways, however ARF loss impairs the DNA-damage response indirectly, through its effects on Mdm2 and p53 [62]. ARF is reported to activate both ATM and ATR and concomitantly, CHK1 and CHK2 in cell lines lacking p53, in which the G1 checkpoint is defective, and upon $p14^{ARF}$ expression, cells arrested only in G2 phase [63]. ARF activates ATM/ATR by interacting with and stabilizing TIP60, which in turn acetylates ATM [64]. Upon DNA damage, the ARF/NPM complex gets disrupted and ARF is redistributed into the nucleoplasm and cytoplasm [65]. However, the exact role of ARF in the DNA-damage response is not clearly understood. Most recently, mutagenesis studies with $p14^{ARF}$ revealed that the Val^{24} residue is required for p53-independent growth suppression and multiple residues (Val^{24}, Thr^{31}, Ala^{41} and His^{60}) facilitated ARF's reversal of chromosomal instability in p53-null MEFs [66]. It is suggested that ARF can promote chromosomal stability independent of p53 through ATM which is a critical suppressor of chromosomal instability [67].

Figure 1.3 **p53-independent Functions of ARF.** ARF primarily functions as tumor suppressor by invariably inactivating its interacting proteins including MDM2 by sequestering them into nucleoli or altering their metabolic stability often inducing their degradation or by regulating their post-translational modifications such as sumoylation.



To investigate the molecular mediators and mechanisms of the p53-independent functions of ARF and relate this activity to its tumor suppression, we have attempted to identify additional cellular targets of ARF. By use of yeast two-hybrid screen, the C-terminal binding protein 2 (CtBP2) transcription regulator was identified as an ARF-interacting protein. ARF interaction with CtBP2 induced degradation of CtBP2 by the proteasome [68]. CtBP2 has been reported to function as antiapoptotic [69] and ARF-induces CtBP2 depletion resulted in apoptosis in a p53-independent manner suggested that CtBP2 may function as mediator of ARF-induced p53-independent apoptosis, and in turn, p53-independent tumor suppression by ARF. Though not yet proven, CtBP is likely to be linked to tumor progression, as it promotes both cell survival, cell migration and epithelial-mesenchymal transition by regulating proapoptotic and epithelial genes transcription [70].

1.5 C-terminal Binding Proteins (CtBPs)

The C-terminal binding protein (CtBP) family proteins are unique in invertebrates and in vertebrates. The invertebrates have a single CtBP gene while vertebrates have two genes, CtBP1 and CtBP2. In humans, these map to chromosome bands 4p16 and 10q26.13 respectively. CtPB1 and CtBP2 are expressed widely both during development and in adult tissue [71, 72]. The vertebrate CtBPs have highly conserved sequence and structural similarity with D-isomer specific 2-hydroxy acid dehydrogenases (D2-HDH). CtBP1 was identified in 1993 as a 48 kDa cellular phosphoprotein that bound to the C-terminal region of adenovirus E1A oncoprotein [73]. CtBPs were shown to bind to a five

amino acid motif PLDLS conserved at the C-terminus of E1A by mutational analysis [74]. CtBP2 protein was subsequently identified by EST sequence analysis and cloned by two hybrid screen against the transcription factor BKLF [75]. CtBP1 localizes to both the nucleus and the cytoplasm, whereas CtBP2 localizes predominantly to the nucleus. This differential cellular localization of CtBPs is attributed to the difference in their N-terminal 20 amino acids; CtBP2 has three N-terminal lysine residues which when acetylated by p300 confers nuclear localization on the protein [76].

1.6 Cellular Functions of CtBP

CtBP family members function in the nucleus as transcriptional corepressors by binding to a number of different DNA binding factors and modulating the expression of several genes that control development, oncogenesis, and apoptosis. In the cytosol, these proteins are involved in various functions associated with membrane trafficking, CNS synapse function and the regulation of microtubule cytoskeleton assembly [77, 78, 79].

A. Nuclear Function of CtBPs

i. Transcriptional Repression

CtBPs function predominantly as transcriptional corepressors in the nucleus in conjunction with a number of different DNA-binding repressors. Initial studies with dCtBP in *Drosophila* embryos provided strong evidence for its role in transcriptional repression [80]. Based on studies involving embryos deficient in maternal dCtBP and transgenic embryos containing repressors with CtBP-binding mutants, a number of

Drosophila repressors were identified which utilize dCtBP as a corepressor. The short range repressors such as Kruppel, Knirps, Snail, Giant and long range repressors such as Hairy and Groucho, require dCtBP for exerting their full repressor activity during embryonic development [80, 81, 82, 83].

CtBPs contribute to transcriptional repression through sequence specific DNA-binding core repressors, suggesting that CtBPs function as corepressors [84, 85]. So far, more than thirty different transcription factors have been suggested to exert their function through recruitment of CtBP [86]. In contrast, *C. elegans* homologue the ceCtBP can bind DNA directly through its N-terminal thanatos-associated protein (THAP) Zn^{2+} finger domain [87]. Vertebrate CtBP associates with type I histone deacetylases (HDAC) [88, 89], though short range repressors functions normally in HDAC mutant embryos in *Drosophila*. CtBPs are reported to play a direct role in repression mediated by polycomb group (PcG) proteins such Pleiohomeotic (PHO) in *Drosophila*, and YY1 and HPC2 in mammals [90, 91, 92].

Analysis of a tandem affinity tag purified (TAP) CtBP1 nuclear protein complex revealed that this complex is composed of a DNA-binding core repressor, CtBP and chromatin modifying factors. CtBP recruits most of these factors through its PLDLS-binding domain [93] (Figure 1.4). However, existence of such a high molecular mass complex suggests that there might be additional regulatory mechanisms and a very dynamic equilibrium in recognition of two PLDLS binding clefts of CtBP dimers (Figure 1.4B). In addition to the DNA-binding core repressors such as ZEB1/2, BKLF or Znf217, the CtBP complex contains the enzymes, which catalyze various modifications

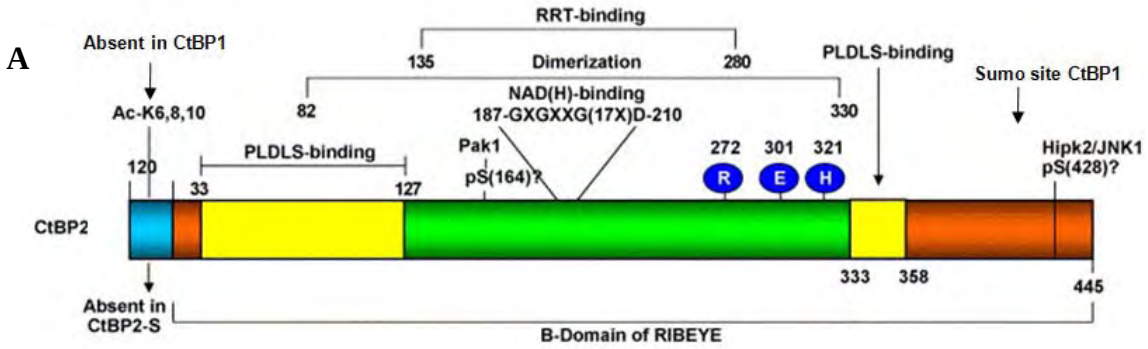
on histones. These include class I HDACs, histone methyl transferases (G9a and GLP), and lysine specific demethylases (LSD1). Components of the sumoylation enzyme machinery such as UBC9 (E2) and Hpc2 (sumo E3 ligase) are also part of the CtBP complex. Sumoylation of CtBP1 has been proposed to play a role in its nuclear retention. CtBP has been suggested to play a role in global repression by associating with bromodomain containing HAT coactivators (p300/CBP, GCN5) and basal transcription factors, including TAFIIF and TAFII-250 [94, 95].

ii. Transcriptional Activation

Although CtBP family members primarily function as transcriptional corepressors by association with DNA-binding repressors, they may function as transcriptional activators under certain conditions. dCtBP function as a transcriptional activator in a context dependent manner in different mammalian cells when dCtBP was tethered with Gal4 [96]. Further, studies with CtBP2-null mouse embryos revealed that one of the target genes of Wnt3A, *Brachyury* was transcriptionally repressed compared to wildtype embryos. In contrast to repression function, transcriptional activation seems to be an indirect effect. mTcf3 represses *Brachyury* through corepressor Gro, which contains two CtBP binding motifs. It is possible that CtBP manifests its coactivator function by interfering with the repressor function of Gro. However, CtBPs may function as transcriptional coactivators in a cell specific, spatial and temporal-dependent manner.

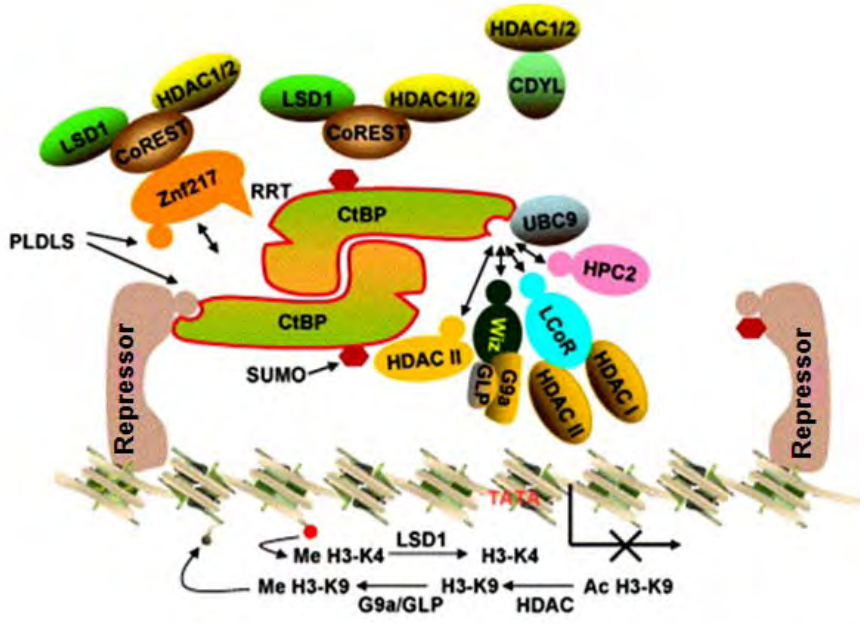
Figure 1.4 Schematic representations of CtBPs domain structure and Model for transcriptional repression by CtBP.

A. The physiologically relevant domains of CtBPs are PLDLS-binding motif, NADH binding dimerization domain which has the catalytic triad of D2-HDH activity. CtBPs nuclear localization is regulated by acetylation of K6, 8, 10 residues by p300. **B**. Model for gene specific transcriptional repressor function. CtBPs recruit core repressor and other factors associated with histone modification in to their PLDLS-binding clefts. Post-translational modification such as phosphorylation and sumoylation may regulate the stability of this complex. Histone modifying factors include HDACs and HMTases,



HATS and demethylases (LSD1).

B



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Chinnadurai 2007, *IJBCB*.

B. Cytosolic Functions of CtBP

Although CtBPs predominantly function as transcriptional corepressors, splice variants of the vertebrate CtBP1 and CtBP2 have been suggested to function in various biological processes in the cytoplasm. The CtBP1 splice variant, BARS-50 was identified as a target for ribosylation by the fungal toxin brefeldin A (BFA) in the Golgi apparatus [97]. Ribosylation of BARS-50 is correlated with disassembly of Golgi by BFA. Reconstitution studies with wildtype and dominant negative CtBP/BARS confirmed that CtBP is important for the mitotic fragmentation of the Golgi complex [98]. Further, CtBP1 was shown to be involved in membrane fission and transport in dynamin-independent endocytic and exocytic transport pathways [99]. However, there was no significant defects in Golgi apparatus partitioning in CtBP-null MEFs due to the possibility of compensatory mechanisms during embryonic development [72]. RIBEYE, a variant of CtBP2, is a major component of the ribbon synaptic complex in the central nervous system and it is highly conserved across the species [77]. CtBPs play both a structural role and a role in membrane turnover in the chemical synapse. Depletion of RIBEYE results in shorter synaptic ribbons. In addition to these cytosolic functions, the plant CtBP homolog AN also regulates microtubule cytoskeleton, and in turn, controls leaf shape by modulating the trichome (leaf hair) branching and polarized leaf cell expansion [78].

1.7 Role of CtBPs in Development

CtBPs play an important role during development both in invertebrates and vertebrates. Homozygous deletion or inactivation of the dCtBP gene is lethal in

Drosophila [81]. Progressive segmentation defects were observed with reduced levels of maternal dCtBP, which has been attributed to defects in the transcriptional repressor functions of short range repressors such as Hairy, Knirps, and Snail [81]. Further, there is a link between dCtBP and the Wg (mammalian Wnt) pathway during development [100]. Studies conducted in *Xenopus* embryos have revealed that CtBPs play a critical role in development by regulating the transcriptional activities of Tcf-3, FOG and ZEB-1/SIP1 [101, 102, 103]. A recent study of expression of CtBP1/2 in avian embryos suggests that these two genes may play functionally redundant roles in some tissues and unique roles in the development of others [104].

Genetic analysis of mice with mutations in the CtBP1 and CtBP2 genes have suggested that the two CtBP isoforms have unique, as well as redundant, functions during mouse development [72]. CtBP1-null mice are viable and fertile but are small and less robust, while homozygous inactivation of the CtBP2 locus results in embryonic lethality between E9 and E 10.5. The lethality in CtBP2-null mice was primarily due to vascular defects in the placenta, impairing the proper exchange of nutrients and waste. The phenotypic difference between $CtBP1^{-/-}$ and $CtBP2^{-/-}$ mice appears to be mainly due to differences in the tissue pattern of gene expression, as CtBP2 was the only CtBP expressed in placenta. Evidence for overlapping functions of CtBP1 and CtBP2 comes from genetic interaction studies between these genes by incorporating various combinations of CtBP1 and CtBP2 mutations. Embryos that are heterozygous for both genes are viable, however reducing the dosage of CtBP1 ($CtBP1^{-/-}$/ $CtBP2^{+/-}$) led to embryonic lethality with defects in vascular and skeletomuscular system development [72].

Some of the phenotypes associated with CtBP2 inactivation were attributed to a reduction in the expression of the T-box transcription factor *Brachyury*, which is a target of transcriptional coactivation by CtBPs.

E1A expression in several cancer cell lines reverses their oncogenic properties, due to activation of various epithelial genes, and CtBP was identified as an antagonist of the epithelial phenotype [105]. Indeed, a prominent phenotype associated with the deficiency of CtBP was the presence of extensive epithelial components in various tissues and organs. This is also consistent with the role of CtBP in repression of several genes important for conferring epithelial phenotype, such as E-cadherin [70]. The signaling pathways controlled by CtBPs during development remain to be elucidated. However, several groups suggested that signaling pathways such as Wnt and TGF-β/BMP might play critical roles in concert with CtBPs during development. Mouse models with functional knock-in mutants of CtBPs would greatly facilitate to understanding of roles of CtBPs in vertebrate development.

1.8 CtBPs in Oncogenesis and Apoptosis

The definitive role of CtBPs in oncogenesis is not clearly understood. Gene expression profiling of CtBP-null MEFs and CtBP rescued MEFs has also revealed that several epithelial (cytokeratins, tight junction components and lamins) and pro-apoptotic (such as Noxa, Bax and PERP) genes were activated in the absence of CtBPs [70]. CtBPs interact with the APC (adenomatous polyposis coli) tumor suppressor and regulate the expression of intestinal retinol dehydrogenases, and in turn, the differentiation of

intestinal cells. CtBP appears to inhibit the wnt signaling pathway though it paradoxically activated wnt [100, 106]. Adenomas from familial adenomatous polyposis patients showed high levels of CtBP protein in comparison to matching normal tissue. APC targets CtBP to proteasome-dependent degradation, however the mutated APC fails to do so, suggesting that CtBPs play an important role in colon adenoma formation.

A. CtBP in Epithelial-Mesenchymal Transition, Cell migration and invasion

During the progression of epithelial tumors, crosstalk between the often heterogeneous tumor cells, the tumor stroma, and extra cellular matrix is essential. The Epithelial-Mesenchymal Transition (EMT), where cells undergo a developmental switch from a polarized, epithelial phenotype to a highly motile mesenchymal phenotype, has emerged as a key step during embryonic development, cancer progression, metastasis and chronic inflammation [107]. Several signaling pathways including TGF-β, RTK/Ras, Wnt, Notch, Hedgehog and NF-kB are suggested to contribute to EMT. Phenotypic markers of EMT include an increased capacity for migration and invasion, as well as resistance to anoikis/apoptosis. Indeed, CtBPs contribute to all of these characteristics of EMT [105, 108, 109]. Repression of E-cadherin by transcriptional regulators such as Slug, Snail, Zeb or Twist in various cellular contexts, emerged as one of the critical steps driving EMT. CtBP exerts its transcriptional corepressor function through these core repressors, suggesting that CtBP is important in promoting EMT. Zeb 1 overexpression with low levels of E-cadherin has been reported in several human cancers. In human colorectal adenocarcinomas, high levels of Zeb1 and CtBP were highly correlated with low levels of

E-cadherin [110]. Studies from Dr. Grossman and Dr. Lewis lab have shown that CtBP2 promotes cancer cell migration under hypoxic conditions in cells defective for p53 and introduction of ARF inhibits CtBP2-mediated cell migration [108, 109]. Further, intact physical interaction of ARF/CtBP2 and NADH binding domain of CtBP2 were required for regulation of cancer cell migration [108, 109].

Increased cellular NADH levels, such as in the hypoxic environment seen in solid tumors has been shown to enhance the recruitment of CtBP to the E-cadherin promoter [111]. Depletion of CtBP leads to reduced cell migration and invasion suggesting that the effect was independent of HIF-1α or other E-cadherin repressors [108, 109].

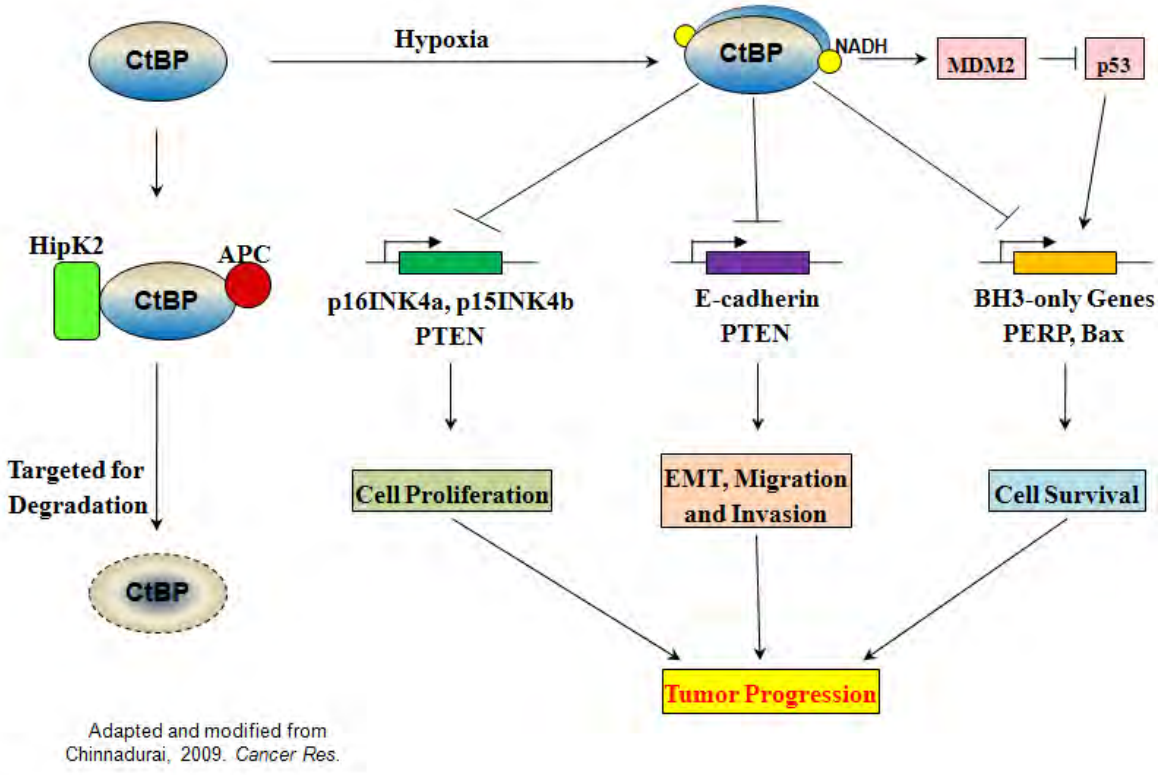
B. CtBP transcriptionally represses several tumor suppressors

CtBPs play a significant role in repression of E-cadherin, which is involved in EMT as discussed above. E-cadherin limits tumor progression by restricting cell migration and invasion, in turn preventing tumor cells gaining malignant properties. Studies with CtBP knockout MEFs suggested that the PTEN tumor suppressor gene is also one of the CtBP transcriptional targets [70]. The role of PTEN as a tumor suppressor has a direct link to its regulation of cell migration through the PI3K/Akt pathway. CtBP has also been suggested to play an important role in cell migration and invasion by targeting PTEN, and in turn, the PI3K signaling pathway [108, 109] (Figure 1.4). Furthermore, recent studies have shown that CtBP transcriptionally regulate the Ink4 family of tumor suppressors including $p16^{INK4a}$ and $p15^{INK4b}$ [112] (Figure 1.4).

C. CtBP as an Apoptosis Antagonist

The initial evidence for an anti-apoptotic function of CtBP comes from studies involving expression of E1A mutants (CtBP-binding defective) which induce apoptosis in mammalian cells [105].Mouse embryonic fibroblasts from CtBP-null embryos are hypersensitive to apoptosis in response to a wide variety of stress signals, including loss of cell-cell contact (anoikis), Fas ligand and genotoxic agents [70]. Gene expression profile analysis of CtBP-null MEFs revealed the repression of several proapoptotic genes including PERP (p53-effector related to pmp-22), PTEN, insulin-like growth factor binding proteins, Bax, Noxa and Id-1 [70]. Further, siRNA-mediated depletion of CtBP in human tumor cell lines was sufficient to induce apoptosis without any additional stress [113]. CtBP also directly suppresses p53 target gene transcription such as Bax, through its interaction with Mdm2 and can thus regulate p53-dependent apoptosis [114]. CtBPs are reported to associate with the Evi-1 repressor, which is a negative regulator of TGF-β signaling, and contributes to the progression of leukemias by inhibiting apoptosis [115]. In addition, CtBP also represses Id-1 expression by interacting with the inhibitory Smad, Smad6, which may contribute to an induction or suppression of apoptosis depending on the cellular context [103].

Figure 1.5 Role of CtBP in Oncogenesis and progression.
The hypoxic environment during tumor progression, results in reduced NAD+/NADH ratio inside the cell, which stimulates the activity of CtBPs by increasing dimerization and affinity of binding with DNA binding repressors. CTBP has been suggested to enhance cell proliferation by repressing the cell cycle inhibitors $p16^{INK4a}$ and $p15^{INK4b}$. Transcriptional repression of E-cadherin and PTEN results in promotion of Epithelial-Mesenchymal Transition (EMT), cell migration and invasion. By repressing proapoptotic genes such as PERP, Bax and Noxa, CtBP promotes cell survival. CtBP being a potential oncogene, is targeted for degradation by several tumor suppressor proteins such as HipK2, APC, all of which directly associate with CtBPs [116]. CtBPs also suppress transcription of p53 target genes through its interaction with Mdm2 [114].



Post-translational modifications, such as phosphorylation, of CtBPs play a critical role in the induction of apoptosis after UV-irradiation. High doses of UV activate homeodomain interacting protein kinase 2 (HIPK2), which phosphorylates CtBP1 at serine 422 (conserved in CtBP2 at S428) and targets CtBP for ubiquitin-dependent degradation by the proteasome [69, 113]. Other kinases may target CtBPs in cell-specific and context-dependent manner, and in turn, tighter regulation of CtBPs cellular functions.

MEFs explanted from CtBP-null embryos were hypersensitive to apoptosis and microarray analysis has shown up regulation of proapoptotic genes such as PERP, PTEN, Bax, BH3-only gene; Noxa and Id1 [70]. It has been also suggested that CtBP depletion by specific siRNA in human cancer cells was sufficient to induce apoptosis without any additional stress [68]. To identify the mediators of ARF-induced p53-independent apoptosis mediated through CtBP, a human apoptosis array was performed with CtBP2 depletion either by ARF overexpression or by CtBP2 siRNA. BH3-only genes were identified as critical mediators of ARF/CtBP2 mediated p53-independent apoptosis.

1.9 BH3-only proteins and Apoptosis

Programmed cell death or apoptosis is vital for the proper development and functioning of multicellular organisms. Tight regulation of apoptosis is essential for maintaining tissue homeostasis. The extent of tumor progression is determined not only by the rate of cell proliferation but also by the rate of cell death. Programmed cell death represents a major source of cell death as indicated from studies in mouse models,

cultured cells, and analysis of biopsied stages in human carcinogenesis. The acquired resistance towards apoptosis is a predominant trait of most, and perhaps all, types of cancers [1]. Many of the proapoptotic signals converge on the mitochondria, which respond by releasing cytochrome C, a potent catalyst of apoptosis [117]. Under several circumstances, a cell's fate to live or die is largely governed by the Bcl-2 family of interacting proteins [118, 119]. The Bcl-2 family is comprised of pro-survival members (Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1) and two groups of pro-apoptotic members; Bax group (Bax, Bak, and Bok) with three BH (Bcl-2 homology) domains, and the BH3-only proteins [118].

BH3-only proteins include Bim, Bid, Bad, Bik, Bmf, Puma, Hrk, and Noxa (Figure 1.5). Upon activation by cytotoxic signals, the BH3-only proteins interact with pro-survival members of the Bcl-2 family through their BH3 domain to prime apoptosis. In addition to BH3-only gene expression, subsequent activation of Bax or Bak is required for induction of apoptosis [120, 121]. Upon activation, BH3-only protein's activate Bax or Bak by dissociating them from anti-apoptotic bcl-2 family proteins, and allowing them to translocate to the outer mitochondrial membrane to form pores that allow the cytoplasmic release of cytochrome C [122, 123]. Amongst BH3-only genes, Bid is a critical mediator of apoptosis mediated by death receptor signaling [124], Bim is the determinant of paclitaxel (Taxol), ionomyin and cytokine deprivation responsiveness [125], Puma and Noxa are central mediators of p53-induced apoptosis [126] and Bad regulates apoptosis mediated by growth factor/cytokine signaling [127]. However, the cellular apoptotic stimuli that act

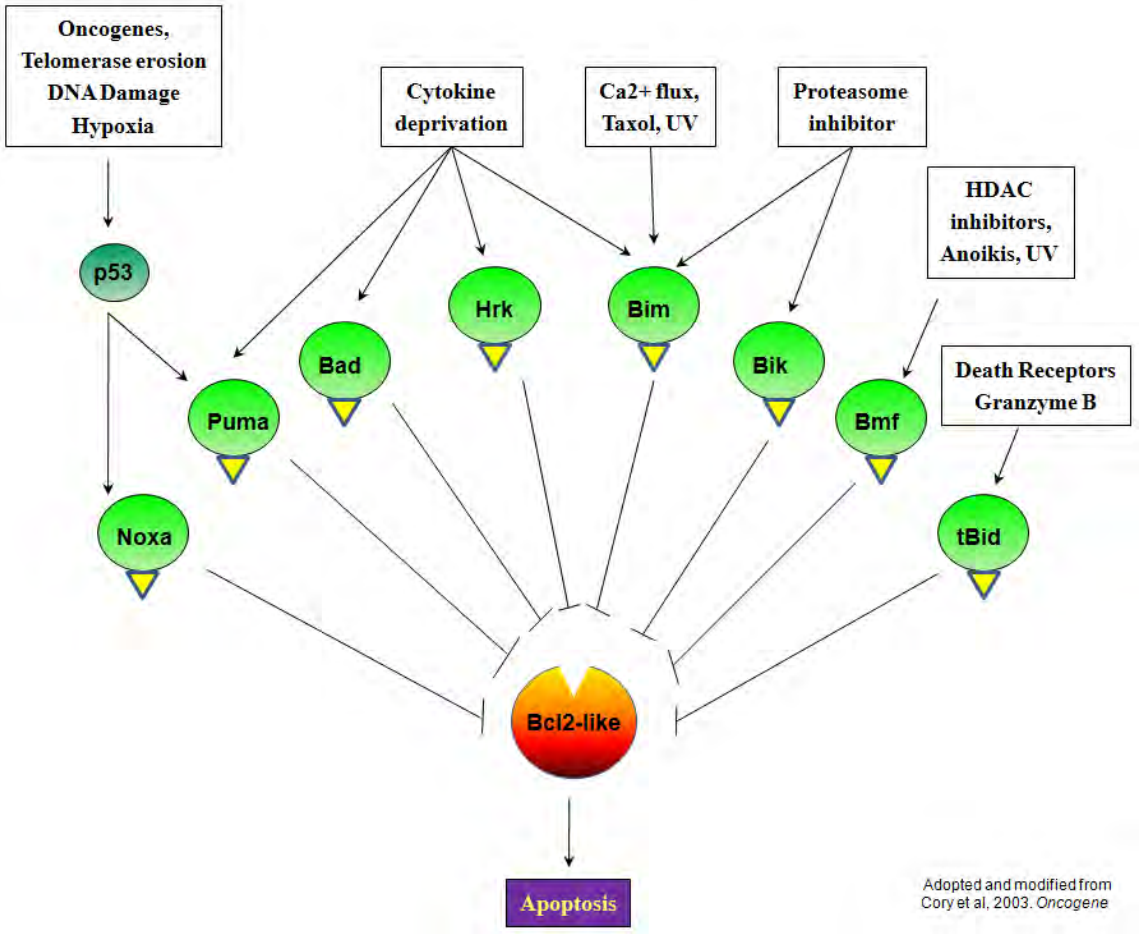
through Nbk/Bik, and the biologic functions of these genes in mammals, are not yet known, though they are induced upon proteasome inhibitor treatment of cells [128].

The presence of multiple BH3-only proteins and their complex regulation allows for tight control of apoptosis in mammalian cells. Studies using knockout mice indicated that one or several BH3-only proteins play a dominant role in the induction of apoptosis in response to diverse cytotoxic and oncogenic stress stimuli (Figure 1.5). To ensure proper tissue homeostasis by balancing cell death and cell proliferation, BH3-only proteins are restrained by multiple mechanisms [118, 119, 129]. Bim, Puma, Hrk and Noxa are regulated transcriptionally, whereas Bad, Bik, Bmf, and Bik, are regulated by phosphorylation [130]. BH3-only proteins exert their pro-apoptotic function by neutralizing pro-survival members of Bcl-2 family by associating with and sequestering them, or by directing them for proteasome-mediated degradation [131, 132].

The BH3-only proteins play a very important role during tumorigenesis. Bcl-2 and its homologues are very well recognized oncogenes [118]. In contrast, their BH3-only antagonists function as tumor suppressors. The apoptosis induced by DNA damage requires p53 and is critical for p53's tumor suppressor function [133]. Puma and Noxa are required for p53-dependent apoptosis [126]. BH3-only protein deficient mice developed various types of neoplastic conditions such as leukemias and lymphomas in bim-null, renal carcinomas in bik-null mice, diffuse large B cell lymphoma in bad-null mice and CML in bid-null mice [134, 135, 136, 137]. Other BH3-only proteins may likewise restrain oncogenesis in cell a specific manner. The development of peptide based or small molecule BH3 mimetics as novel targeted cancer therapeutics has been considered [118].

Bik is a BH3-only endoplasmic reticulum associated phosphoprotein, with its BH3 domain and phosphorylation is required for its apoptotic function [138]. Ectopic expression of Bik induces apoptosis in several mammalian cell lines and Bik interacts directly with Bcl-2 and Bcl-xL through its BH3 domain and inhibits their antiapoptotic functions resulting in the induction of apoptosis [139]. Bik is normally expressed in hematopoietic component and endothelial cells [140]. Bik is inducible by doxorubicin or γ-irradiation in a p53-dependent manner [141, 142]. Expression and activation upon certain apoptotic stimuli of Bik and Bim overlaps in the hematopoietic system, cardiomycocytes, and epithelial tissues of the kidney and mammary gland [140]. The bik and bim double-null mice studies indicated that both Bik and Bim share a critical role in spermatogenesis by regulating apoptosis, which is required for normal sperm development [143]. In contrast, bik-null mice did not protect hematopoietic cells *in vitro* from apoptosis induced by cytotoxic stimuli or cytokine withdrawal, suggesting that the function of Bik in programmed cell death may overlap with that of other BH3-only proteins [140].

Figure 1.6 **BH3-only proteins function in cellular homeostasis**.
Upon activation in response to a variety of cellular stresses, BH3-only proteins initiate apoptosis by interacting with and antagonizing Bcl-2 and Bcl-2 like pro-survival proteins via their BH3 domain (yellow triangle).



1.10 Aims of this Dissertation

ARF is silenced or deleted in a large number of human cancers. The role of ARF in tumor suppression has primarily been attributed to its role as a positive regulator of p53. However, multiple lines of evidence in mouse and human systems clearly suggest that ARF functions as a tumor suppressor apart from p53. The molecular pathways and mechanisms of this p53-independent tumor suppressor activity are not well understood. The transcriptional corepressor C-terminal Binding Protein (CtBP) has been identified as one of the interacting proteins involved in ARF-induced apoptosis in cells lacking p53. CtBPs are suggested to promote tumor progression by contributing to epithelial-mesenchymal transition (EMT) and also function as apoptosis antagonists. These findings have indicated that CtBPs are candidate oncogenes, and that they are targeted by tumor suppressors. Therefore, the main aims of this dissertation are to delineate the functional significance of the ARF/CtBP2 interaction, to determine the mechanism of ARF-induced p53-independent apoptosis, and to investigate the biological significance of the ARF/CtBP2 interaction *in vivo* by generating and characterizing a knock-in mouse containing a targeted mutation of the CtBP-interacting domain of *Arf*. A deeper understanding of the ARF/CtBP pathway in murine and human systems could allow development of targeted therapeutics that aim to restore the function of this pathway that might be lost in tumors that silence or delete ARF.

Preface

Part of this chapter has been published in or submitted to

Paliwal S, Pande S*, **Kovi RC***, Sharpless NE, Bardeesy N, and Grossman SR. 2006. Targeting of C-Terminal Binding Protein (CtBP) by ARF results in p53-Independent Apoptosis. *Molecular Cell Biology*. 26 (6); 2360-2372. *: Equal contribution.

Straza M, **Kovi RC**, Paliwal S, Messina M, Trench P and Grossman SR. 2009. C-terminal Binding Protein 2 is a cellular target of the cytotoxic compound 4-methylthio-2-oxobutyric acid (MTOB). *Cancer Research* (Submitted).

Chapter-II

ARF-induced CtBP2 degradation is part of a p53-independent tumor suppressor pathway targeted for inactivation in human cancer

Abstract

ARF, a potent tumor suppressor, stabilizes p53 by antagonizing its negative regulator MDM2. ARF also suppresses the proliferation of cells lacking p53, and loss of ARF in p53-null mice, compared with ARF-null or p53-null mice, results in a broadened tumor spectrum with decreased tumor latency. The transcriptional corepressor C-terminal binding protein 2 (CtBP2) has been identified as a putative target of p53-independent tumor suppression by ARF. ARF interacted with CtBP2 *in vitro* and *in vivo*. Interaction with ARF resulted in proteasome-dependent CtBP degradation, and required phosphorylation at serine 428. ARF-induced CtBP depletion led to p53-independent apoptosis in human colon cancer cells. Both ARF induced CtBP degradation and induction of apoptosis was dependent on its ability to interact with CtBP. As a target for inhibition by ARF, CtBP is a candidate oncogene, and its expression was deregulated in human colonic adenocarcinomas. CtBP expression level was elevated in more than 65% human colorectal adenocarcinomas tissue samples compared to their matching normal tissue. Moreover, CtBP expression was absent/decreased at the protein level in 25% of colorectal adenocarcinomas, and these tumors specifically expressed high levels of ARF. Overexpression of CtBP in human tumors may be selected for in oncogenesis to both overcome ARF-dependent degradation and inactivate other functions of ARF.

Introduction

ARF (mArf in mouse and hARF in human), a tumor suppressor product of the *INK4a/ARF* locus, functions by stabilizing p53 [36, 144, 145, 146, 147]. Given the fact that ARF stabilizes and activates p53 by antagonizing MDM2, inactivation of ARF has been attributed to inactivating the p53 pathway during tumorigenesis [23, 148]. The *INK4a/ARF* locus is frequently inactivated in human cancers by deletion or transcriptional silencing or mutation [16]. Arf knockout mice developed highly penetrant lymphomas or sarcomas, similar to the types of tumors observed in p53-deficient mice [23, 53]. The finding that simultaneous inactivation of p53 and *INK4a/ARF* occurs in certain human tumors, however, suggests that ARF may exert additional tumor suppressor function(s) independent of p53 [52].

The $p16^{INK4a}$ and ARF genes are often simultaneously deleted in humans, thus it is difficult to distinguish their contributions to tumor suppression [149, 150]. However, analysis of ARF-specific knockout mice has provided additional evidence for p53-independent functions of ARF. Epithelial tumors are rare in p53-null mice, but observed in about 20% of *Arf* knockout mice, and simultaneous targeting of both *Arf* and p53 results in an even higher frequency of epithelial tumors [53, 151, 152]. Further loss of MDM2, a negative regulator of p53, results in a substantially increased incidence of epithelial cancers, including those of the digestive tract [30, 31]. In contrast, mice with loss of p53 and MDM2, but retention of Arf, developed only mesenchymal tumors, as observed in p53 knockout mice [31]. This genetic evidence in mice also suggested that ARF exerts a tumor suppressor activity apart from stabilizing p53.

Studies in cell lines also revealed that ARF can suppress the proliferation of p53-defective cells [31, 147]**.** ARF executes this function by antagonizing c-myc and E2F (E2F1-3), accelerating their degradation via the proteasome, thus slowing progression of cells through the G1/S transition and decreasing proliferative rate [153, 154]. ARF can also disrupt ribosome biogenesis by interacting with nucleophosmin/B23 and/or by interfering with the export of ribosomal RNA [46, 47, 59, 60, 155]. This, in turn, would inhibit cell proliferation by retarding the progression of cells through the growth phases of the cell cycle.

ARF affects the function of its interacting proteins by sequestering them into nucleoli, targeting them for degradation, or affecting their state of post-translational modification [148]. E2Fs, c-*myc*, and MDM2 are sequestered into the nucleoli upon ARF expression [31, 57, 148, 156]. Other targets display changes in their post-translational modifications or localization: MDM2 and B23 become sumoylated [157, 158]. [109, 159]

In an attempt to understand the p53-independent functions of ARF and relate this function to tumor suppression, a two-hybrid screen of ARF interactors was performed, identifying *C-t*erminal *b*inding *p*rotein 2 (CtBP2) transcription regulator as an ARF-binding protein. ARF interaction caused proteasome-mediated CtBP degradation, dependent on CtBP phosphorylation. Depletion of CtBP either by ARF expression or by RNAi resulted in apoptosis in a p53-independent manner in human colon cancer cells that was dependent on ARF/CtBP interaction. Importantly, an inverse correlation between ARF and CtBP expression observed in human colorectal adenocarcinomas, validating results gained in biochemical and cell line studies.

Results

ARF and CtBP interact *in vitro and in vivo*

A yeast two-hybrid assay was employed in an attempt to identify the mediators of ARF's p53-independent functions. CtBP2 was thus identified as an ARF interacting protein. To characterize the putative ARF/CtBP interaction, purified GST or GST-mArf fusion proteins were incubated with U2OS cell lysates. GST-mArf, but not GST, was able to specifically recognize the endogenous 48 kDa CtBP2 protein from U2OS cell lysates, suggesting that these proteins can specifically interact *in vitro* (Figure 2.1A). To map the binding of mArf to CtBP2, V5-tagged N-terminus (1-321) and C-terminus (322-445) constructs of CtBP2 were transiently expressed in U2OS cells followed by analysis for binding of the V5-CtBP2 proteins to purified GST vs. GST-mArf. The C-terminal fragment (322-445aa), but not the N-terminal fragment of hCtBP2 (1-321aa), bound specifically to mArf (Figure 2.1A).

To determine the minimal region of mArf required for CtBP interaction, wild type mArf or deletion mutant mArf expression constructs were expressed along with CtBP2 in U2OS cells and immunoprecipitated with CtBP2 antibody. Wildtype mArf, and its mutants defective for Mdm2 binding (Δ8-32), or nucleolar localization (Δ26-37), all coimmunoprecipitated with CtBP2 (Figure 2.1B). However, the mArf mutants of a conserved but uncharacterized domain (L46D, Δ32-51, and Δ46-51) did not

coimmunoprecipitate with CtBP (Figure 2.1B). Thus, mArf residues between 37 and 51 were required for hCtBP2 interaction

To determine if ARF/CtBP interaction could be observed in human cells, U2OS cells were transfected with hCtBP2 and full-length hARF or $hARF^{L50D}$ (homologous to $mArf^{L46D}$) expression vectors, and the transfected cell lysates were immunoprecipitated with anti-CtBP2 or a control IgG antibodies, followed by immunoblotting with CtBP2 and ARF antibodies. ARF but not the $hARF^{L50D}$ mutant was evident in the anti-CtBP2 IP, which was consistent with mArf/CtBP interaction (Figure 2.1C).

$p19^{ArfL46D}$ and $p14^{ARFL50D}$ CtBP-binding defective mutants retain p53-dependent functions and nucleolar localization of ARF

By biochemical interaction assays, the minimal region of ARF required for CtBP2 interaction was localized within residues 37-51. This domain is highly conserved across the species (Figure 1.1) but uncharacterized for any functional significance. To determine whether this region contributes to p53-dependent functions, the CtBP interaction defective (L46D/L50D) ARF mutants and the MDM2-binding defective mutant constructs were transfected into U2OS cells along with p53-HA and MDM2 (Figure 2.1B). Loss of residues 46-53 or the L46D point had little or no effect on Arf-induced p53 stabilization in U2OS cells (Figure 2.2A) or Arf induced G1 cell cycle arrest (a p53-dependent function) in MEFs (Figure 2.2B). Consistent with previous reports, the MDM2 binding-defective Arf mutant Δ8-32 failed to stabilize p53 or induce cell cycle arrest (Figure 2.2A-B). Similarly, the $hARF^{L50D}$ mutant also retained its p53 stabilization

function when expressed in either U2OS or HCT116 cells (Figure 2.2D-E). Further, both the $mArf^{L46D}$ and $hARF^{L50D}$ mutants exhibited nucleolar localization similar to wildtype ARF (Figure 2.2C and F). These data suggest that disruption of the CtBP-binding domain of ARF does not disrupt its ability to interact with and antagonize MDM2, and in turn, stabilize and activate p53.

ARF induces CtBP degradation

ARF-interacting proteins can show major alterations in their metabolic stability. MDM2 is stabilized, whereas MDM-x, E2F1-3, and B23/nucleophosmin are destabilized when complexed with the ARF/MDM2 complex or ARF alone [127, 153, 160]. CtBP is degraded by the proteasome after UV exposure, resulting in induction of apoptosis [113]. Interestingly, the human cancer cell lines in which CtBP degradation was observed after UV, invariably expressed ARF [113], suggesting that a potential function of the ARF/CtBP interaction is to induce CtBP degradation.

To confirm the requirement of ARF for CtBP degradation, mArf wild-type (wt) or mutant alleles were introduced into ARF-negative U2OS cells (Figure 2.3A, E). Expression of Arf sensitized U2OS cells to UV-induced loss of hCtBP2 (Figure 2.3A-B). Consistent with a role of ARF interaction in directing CtBP degradation, expression of mArf mutants defective for hCtBP2 interaction (L46D, Δ46-51) did not induce hCtBP2 loss in UV-treated cells, whereas wt mArf or mArf(Δ8-32), which is defective for MDM2 interaction and nucleolar localization, were capable of targeting hCtBP2 for degradation after a UV dose of $10J/m^2$ (Figure 2.3A-B). Thus, the ability of ARF to form a

biochemical complex with CtBP2 correlated with its ability to direct CtBP2 degradation. ARF-induced CtBP2 degradation was blocked by a proteasome inhibitor (Figure 2.3E), which is consistent with a previous report that proteasome inhibition blocked UV-induced degradation of CtBP1 [69].

To confirm that human and mouse ARF have similar effects on CtBP metabolism, hARF was introduced into HCT116 colon cancer cells wt or null for p53 using a recombinant adenovirus [161, 162]. Surprisingly, hARF expression alone, without any additional stresses such as UV, resulted in a loss of hCtBP2 not seen in cells infected with a control virus, and irrespective of p53 status (Figure 2.3C). To rule out the possibility that ARF's effect on CtBP was transcriptional, a semi-quantitative RT-PCR was performed with CtBP2 primers on RNA isolated from control or ARF virus infected HCT116; p53-/- cells. Though there was a decrease in abundance of CtBP2 protein in ARF expressing cells, there was no significant change in CtBP mRNA levels, suggesting that the effect of ARF on CtBP2 protein level was posttranscriptional (Figure 2.3D).

To rule out the possibility of influence of viral infection of CtBP2 loss upon Ad-ARF transduction, hARF and L50D mutant expression constructs were transfected into HCT116; p53-/- cells. ARF was competent to cause CtBP depletion upon transfection, but the L50D mutant of hARF failed to do so, though it was expressed at similar levels. This clearly suggested that the intact interaction of ARF/CtBP2 is required for CtBP2 degradation (Figure 2.3F).

ARF induced CtBP2 degradation requires phosphorylation at serine 428.

UV-induced degradation of CtBP and ARF-induced degradation of CtBP presumably share a common mechanism based on the ARF requirement for UV-induced CtBP degradation [68]. HIPK2 or JNK1 phosphorylation of CtBP1 at S422 is required for UV-induced CtBP1 degradation [68], suggesting that CtBP phosphorylation is also required for non-stress ARF-induced degradation [68, 69, 163]. To answer this question, CtBP2 residue S428, paralogous to the S422 HIPK2 site in CtBP1, was mutated to alanine, which prevents phosphorylation when expressed *in vivo*. When ARF was cotransfected with CtBP2-V5 and CtBP2V5-S428A, there was a significant reduction in the levels of wt CtBP2-V5, whereas CtBP2-S428A was not affected by ARF coexpression (Figure 2.4A). This supports the requirement of S428 phosphorylation for ARF-induced degradation of CtBP2.

To rule out the possibility that ARF does not affect CtBP2-S428A due to a defect in ARF/CtBP2 interaction, these two proteins were assayed for binding in a coimmunoprecipitation assay. Lysates of U2OS cells transfected with ARF, CtBP2 or CtBP2-S428A constructs were used for immunoprecipitation using control IgG, anti-CtBP2 and anti-ARF antibodies. ARF showed similar avidities for both the wt CtBP2 and the CtBP2-S428A mutant (Figure 2.4B), suggesting that the resistance of CtBP2-S428A mutant to ARF-induced degradation was not due to loss of interaction between ARF and the mutant CtBP2.

CtBP depletion by ARF is sufficient to trigger p53-independent apoptosis

To determine whether ARF-induced CtBP depletion could be linked to its tumor suppression function, wild type or p53-null HCT116 cells were transduced with Ad-hARF or control Ad-lacZ virus and assayed for apoptosis. Both of the cell lines demonstrated a significant activation of the apoptotic marker caspase 3 (20-23 % of cells) after Ad-hARF infection regardless of p53 status (Figure 2.5A). To correlate ARF/CtBP interaction with ARF-induction of p53-indepdendent apoptosis, wt ARF and $hARF^{L50D}$ were compared for their ability to induce apoptosis. HCT116; p53-/- cells were infected with empty (Ev), ARF or L50D retroviruses, and analyzed for apoptosis induction by Annexin V-PE/7-AAD staining (Figure 2.5B). As expected, hARF expressing cells exhibited increased annexin V positivity (20%), whereas $hARF^{L50D}$ expressing cells exhibited annexin V positivity similar to that of empty vector control (11% versus 10%) (Figure 2.5B). Therefore, the ability of ARF to interact with and degrade CtBP correlates with its ability to induce p53-independent apoptosis.

CtBP and ARF levels vary coordinately in human colon tumors

ARF expression is lost by methylation in ~22-38% of colon cancers, suggesting that the remaining colon tumors express ARF to varying degrees, with the majority of ARF-expressing tumors likely to have disruption of p53 [164, 165]. One could imagine that high-level ARF expression, if detrimental to growth and progression of a cancer, even

after p53 loss, might be counteracted by other means than transcriptional silencing, such as overexpression or mutation of p53-independent targets like CtBP. Though by in vitro assay CtBP generally displays pro-oncogenic properties, it has never been proven to represent a cellular proto-oncogene. To test the idea that CtBP is targeted by ARF for inhibition during *in vivo* epithelial tumorigenesis, a series of 70 resected primary colon tumors with corresponding adjacent normal tissue were analyzed for CtBP1/2, hARF, and GAPDH protein levels by immunoblot (Figure 2.6A) and CtBP2 and GAPDH mRNA levels by RT-PCR (Figure 2.6B).

Three CtBP and ARF expression patterns were observed (Figure 2.6A; Class I-III; summarized in table). The majority of the tumors (65%) expressed substantially higher levels of CtBP1/2 than adjacent normal tissue, and ARF was undetectable in both normal and tumor tissue. However, 25 % of tumors demonstrated a striking absence of CtBP1/2 and specifically expressed high levels of ARF. Notably, the matched normal samples for Class II tumors invariably contained high levels of CtBP1/2 protein, as seen in matched normal samples from Class III tissues, but unlike the matched normal class I specimens. The inverse correlation between ARF and CtBP was significant with $p<0.00001$, when the Chi-square test was applied. RT-PCR analysis of these tissue samples for CtBP2 mRNA showed no significant difference between normal and tumor specimens where protein levels were disparate (Class I and Class II) (Figure 2.6B), suggesting that the loss of CtBP2 expression in tumors was post-transcriptional, possibly due to ARF-induced degradation. Thus, analysis of a series of colorectal cancer resection specimens suggested that majority of these tumors (Class I and II) demonstrate an inverse relationship between

ARF and CtBP (1 and 2) protein expression, which is consistent with findings from our cell line based studies.

Discussion

ARF functions as a tumor suppressor by p53-dependent and p53-independent mechanisms [10, 31, 148]. However, the molecular mechanism underlying the p53-independent tumor suppressor function/s of ARF remains unclear. Previous studies have shown that ARF can induce growth arrest or apoptosis in cells lacking p53 in a context dependent manner [31, 166]. In this study, we have identified CtBP2, a metabolically sensitive transcriptional corepressor, as a target for the p53-independent functions of ARF. ARF induced proteasome-mediated CtBP2 degradation, and in turn, efficient apoptosis in human colon cancer cells lacking p53. Both ARF-dependent CtBP2 degradation and the induction of apoptosis correlated with the ability of ARF to interact with CtBP. ARF-induced CtBP2 degradation also required the phosphorylation of CtBP2 at S428. Further, an inverse correlation between CtBPs and ARF protein expression levels was observed in human colorectal adenocarcinomas.

A conserved hydrophobic domain (37-51 residues) in exon 1β of ARF recognized the C-terminus of CtBP. The point mutation in this conserved hydrophobic region of ARF (L46D in mArf and L50D in hARF) abolished ARF/CtBP interaction, and in turn, ARF-induced p53-independent apoptosis. However, both mArfL46D and hARFL50D did not affect p53-dependent functions of Arf, suggesting that this domain is distinct from the

MDM2 binding region, and may not contribute to p53-dependent functions of ARF *in vivo*.

Upon UV irradiation, CtBP1 gets ubiquitinated and this is dependent on S422 phosphorylation (S428 in CtBP2) by HIPK2 kinase. Our data also suggest that hARF-induced CtBP degradation requires phosphorylation at S428 but this phosphorylation was not required for ARF/CtBP2 interaction, suggesting that S428 phosphorylation may regulate UV-induced or ARF-induced CtBP degradation at a step beyond the ability of CtBP and ARF to interact. That step could be a delivery of ubiquitinated substrates to the proteasome and CtBP2 phosphorylation at S428 may aid in this process. Further, our preliminary data suggests that ARF may function as a proteasome adaptor by physically associating with the S6a subunit of the 19s regulatory assembly of proteasome (Appendix I, Fig A1.1) [167] or may aid in recruiting other proteasome adaptors such as hPLIC and Rad23 proteins to ensure efficient degradation of ubiquitinated substrates [168]. hARF had a much more robust destabilizing effect on CtBP than mArf, which required additional UV stress to induce CtBP degradation. Though the CtBP binding region in ARF is well conserved across the species, the minor sequence differences between human and mouse ARF might contribute to their difference in causing CtBP degradation.

ARF expression in human colon cancer cells lacking p53 induced CtBP degradation, and in turn, efficient apoptosis. However, apoptosis has also been observed after CtBP depletion using specific siRNA alone [68]. This clearly suggests that ARF functions upstream of CtBP in this pathway. Repletion of CtBP in ARF expressing cells by exogenous expression has rescued cells from ARF-induced apoptosis, confirming a

direct role for CtBP in the apoptosis pathway activated by ARF in the absence of p53 [68]. Our results and previously reported work suggest that CtBP functions as antiapoptotic protein through repression of proapoptotic gene expression [113]. Targeting of CtBP by ARF may result in abrogation of this transcriptional repression, and in turn, induction of apoptosis. The proapoptotic genes regulated by CtBP2 may function as the mediators of ARF-induced p53-independent apoptosis.

ARF is a frequent target for silencing in a variety of human carcinomas including human colorectal adenocarcinomas [55, 164, 169, 170]. The tumor suppressor function of ARF has also been confirmed by Arf knockout mouse models [23, 152]. The molecular mechanisms by which ARF proteins suppress tumors remain unclear, and may depend on specific cellular and tissue contexts. Both hARF and mArf are associated with p53-dependent functions such as growth arrest, senescence and apoptosis, predominantly due to stabilization of p53 [10, 171]. These functions are critical for suppression of certain hematopoietic malignancies, such mouse Eμ-Myc transgene-driven B cell lymphomas, and likely human T-ALL [172, 173, 174, 175]. However, suppression of epithelial tumors in mice by ARF is at least partly p53-independent, and the underlying mechanism is unknown [55]. Our data from human colorectal adenocarcinomas showed an inverse correlation between ARF and CtBP expression levels. The majority of human colorectal adenocarcinomas displayed elevated levels of CtBPs with no detectable ARF, supporting the contention that CtBP may function as a cellular proto-oncogene. Interestingly, class II tumors displayed a striking absence of CtBP expression with retention of ARF expression. Thus, this group of tumors may harbor additional changes such as mutations

in PI3 kinase or upregulation of core repressors of CtBP or other changes, to enable these tumors to sustain the complete loss of CtBPs and continued presence of the ARF tumor suppressor. Normal specimens from class II and III invariably displayed high levels of CtBPs which may be due to the existence of precancerous but morphologically silent changes adjacent to tumors or specific differences in CtBP expression patterns in those groups of patients. Further study of truly 'normal' colon specimens could help resolve whether there is population based variation in normal CtBP expression pattern.

Loss of ARF has been linked to tumor invasiveness and metastasis in a mouse skin cancer model [55]. Evasion of apoptosis by tumor cells enables them to survive in an adverse microenvironment, and ultimately leads to invasion and metastasis [176, 177]. The ability of ARF to induce apoptosis by targeting CtBP may play a critical role in suppression of tumor invasion and metastasis, especially in tumors with p53 inactivation. Indeed, work from our lab has shown that CtBPs can promote the migration of cancer cells, and ARF can effectively inhibit CtBP2-mediated cell migration [109]. The mechanism for this regulation reflects the wide variety of genes regulated by CtBPs. In this case, ARF/CtBP2 regulates cancer cell migration by transcriptionally regulating PTEN phosphotase (Appendix II, Figure A2.1), and in turn, the modulating activity of the PI3 kinase pathway [109]. Selective pressure for ARF silencing in human epithelial cancers could thus derive, in part, from a p53-independent role in promoting tumor-cell apoptosis via CtBP degradation during the process of invasion and metastasis.

Figures

Figure 2.1 **ARF interacts with CtBP.**

A. GST or GST-mArf fusion proteins were conjugated to glutathione-agarose beads and incubated with U2OS cell lysates. Bound, endogenous, CtBP2 was assayed by western blot. Input lane shows 10% of the cell lysate. GST and GST-mArf migration positions in a GST immunoblot are indicated by arrows. **B.** Schematic representations of domains in mArf and mapping CtBP interaction domain of Arf. Lysates of U2OS cells transfected with indicated mArf constructs were immunoprecipitated with CtBP2 antibody, followed by immunoblotting with anti-ARF antibody. **C.** CtBP interacts with ARF in transfected cells. U2OS cells were transfected with V5 tagged hCtBP2 and hARF. Cell lysates were immunoprecipitated with control IgG, or anti-V5 antibodies, followed by Western blot analysis with anti-V5, anti-CtBP1 or anti-ARF antibody.

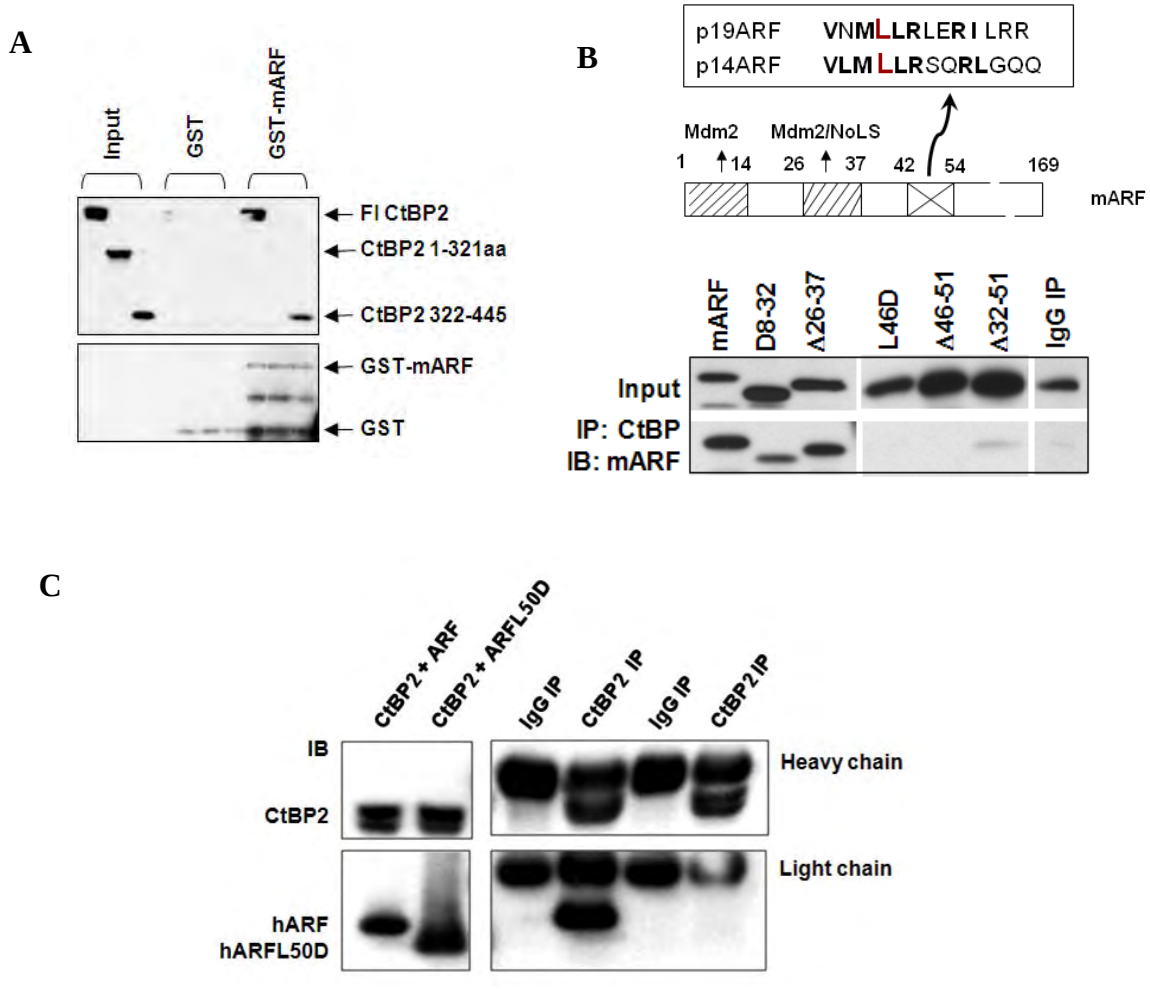
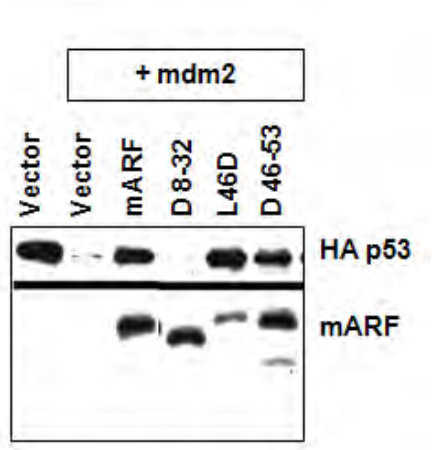


Figure 2.2 **p19^{ArfL46D} and p14^{ARFL50D} retains p53-dependent functions of ARF.** **A.** Mutations within the CtBP binding region of ARF do not disrupt p53 stabilization function. U2OS cells were transfected with HA-p53, MDM2 and the mArf plasmid constructs. Indicated proteins were detected by immunoblotting. **B.** Mutations within the CtBP-binding region of ARF do not grossly disrupt induction of G1 arrest. Mouse 3TC-D1 cells with wildtype p53, were transfected with the mArf constructs and sorted cells were analyzed for cell cycle profile by propidium iodide staining and FACS after 48 hrs of transfection. **C.** Mutations in the hydrophobic domain of ARF which interacts with CtBP do not disrupt nucleolar localization. U2OS cells were transfected with indicated mArf constructs and cells were immunostained with ARF antibody.

A



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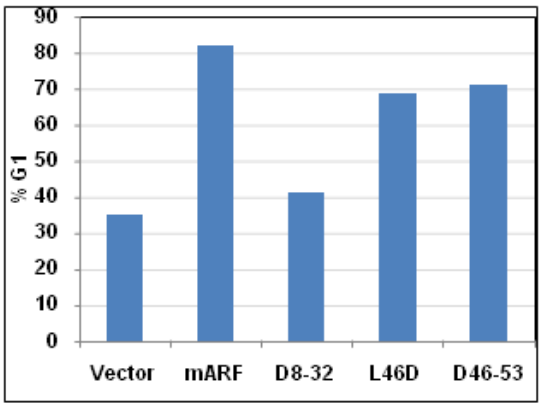
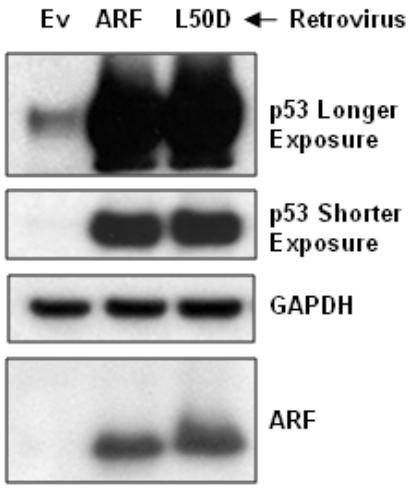


C

D-E. Mutation (L50D) within the hydrophobic region of $p14^{ARF}$ which interacts with CtBP2 does not disrupt p53 stabilization function. U2OS cells **(D)** were transduced with empty, ARF, and $p14^{ARFL50D}$ retroviruses and HCT 116 cells **(E)** were transiently transfected with the indicated expression constructs. The cell lysates were used for detection indicated proteins by immunoblotting after 24 hours of infection. **F.** L50D mutation in the CtBP-binding region of ARF does not disrupt nucleolar localization of ARF. U2OS cells were transfected with $p14^{ARF}$ and $p14^{ARFL50D}$ expression constructs and immunostained with ARF antibody after 24 hours of transfection.

D



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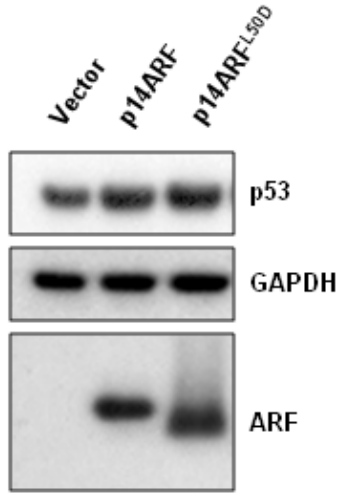

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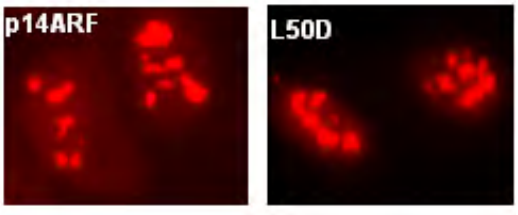
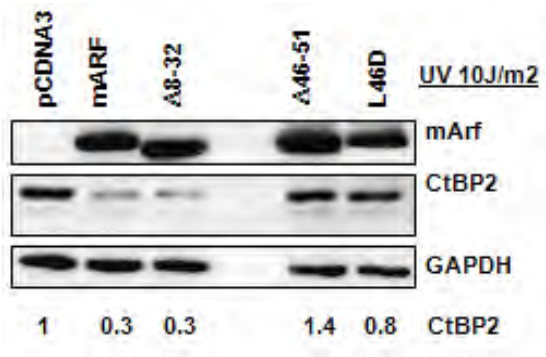


Figure 2.3 **ARF causes CtBP degradation.**
A. U2OS (ARF-silenced) cells were transfected with vector or mArf plasmids, followed by mock or UV treatment (UVC 10 J/m2) and CtBP2 levels were determined 6 hrs post UV treatment by immunoblot and **B.** relative CtBP2 protein level was quantified and normalization against GAPDH. **C.** hARF causes CtBP loss without additional stress. Lysates of HCT116 and HCT116;p53-/- cells obtained 24 hrs post-infection with Ad-lacZ or Ad-hARF were immunoblotted with ARF, CtBP2 or GAPDH specific antibodies. **D.** hARF does not affect hCtBP2 mRNA level. RT-PCR of RNA prepared from HCT116; p53 -/- cells infected with control or hARF retrovirus was carried out using CtBP2 and GAPDH specific primers. **E.** mCtBP2 is degraded by the proteasome in response to UV. MEFs (mArf $^{+}$ p53^{+}) were incubated with or without proteasome inhibitor (MG132) for 24 h after mock or UV (10 J/m^2) treatment. Cell lysates were analyzed for changes in CtBP2 level by Western blotting, followed by densitometry normalized to a GAPDH loading control. **F.** hARF^{L50D} does not degrade CtBP. HCT116 p53$^{-/-}$ cells were transfected with control vector, hARF, or L50D mutant expression plasmids, and 24 h after transfection, CtBP2, hARF, and GAPDH expression was detected by immunoblotting.

A



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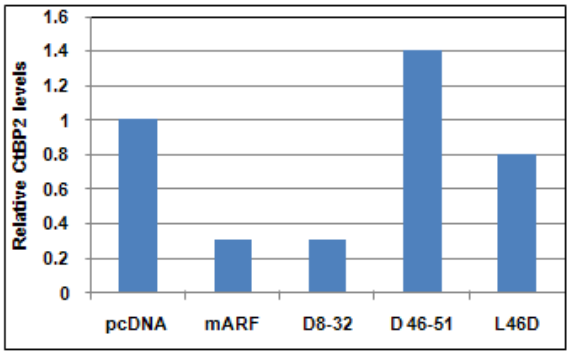

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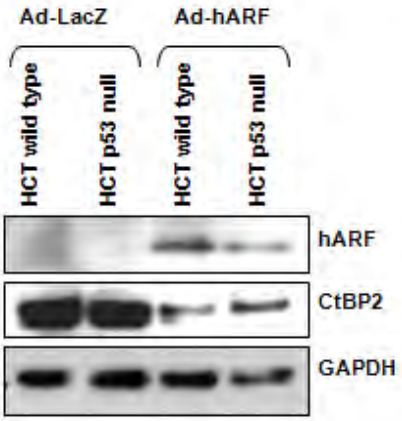
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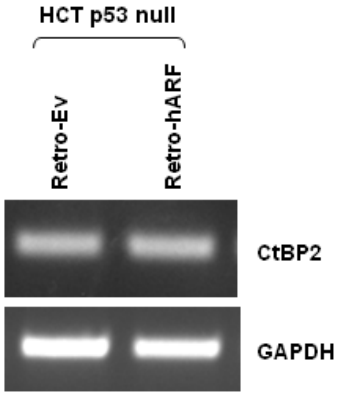
HCT p53 null
HCT wild type
HCT p53 null
hARF
CtBP2
GAPDH

D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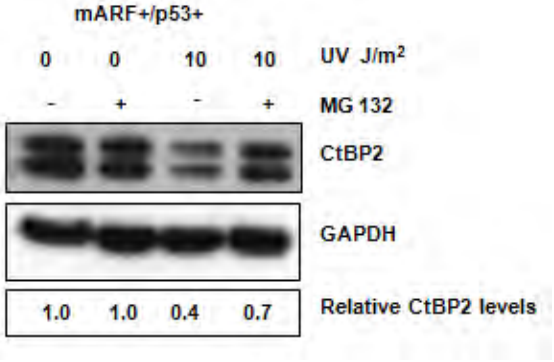
Retro-EV
Retro-hARF
CtBP2
GAPDH

E


mARF+/p53+
0 0 10 10 UV J/m2
- + - + MG 132
CtBP2
GAPDH
1.0 1.0 0.4 0.7 Relative CtBP2 levels

F

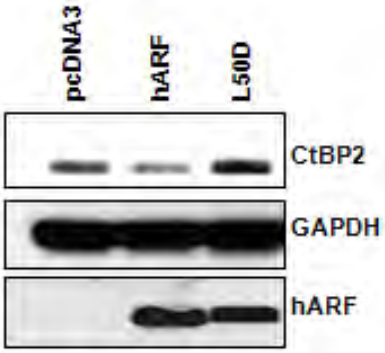
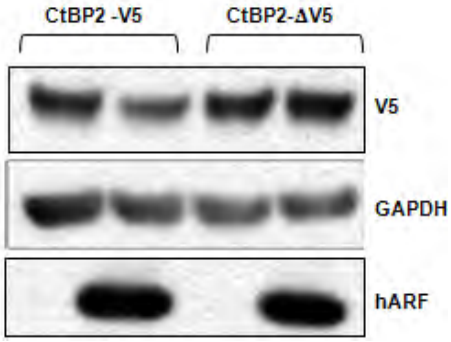

pcDNA3
hARF
L50D
CtBP2
GAPDH
hARF

Figure 2.4 **ARF-induced CtBP2 degradation requires a phosphorylation at S428** **A.** Effect of S428 mutation to alanine on ARF-induced depletion of exogenous CtBP2. U2OS cells were cotransfected with the full-length CBP2 (CtBP2-V5) or S428A mutant CtBP2 (CtBP2-ΔV5) expression plasmids and vector or ARF expression plasmid. Cell lysates were analyzed after 24 hrs of transfection by anti-V5, anti-ARF and anti-GAPDH immunoblot. **B.** S428A mutation does not affect ARF/CtBP2 interaction *in vivo*. U2OS cells were transfected with the indicated CtBP2 expression plasmids along with ARF expression plasmid. Lysates were immunoprecipitated with control (IgG) or anti-CtBP2 antibodies, and the IPs immunoblotted with anti-ARF and anti-CtBP2 antibodies.

A



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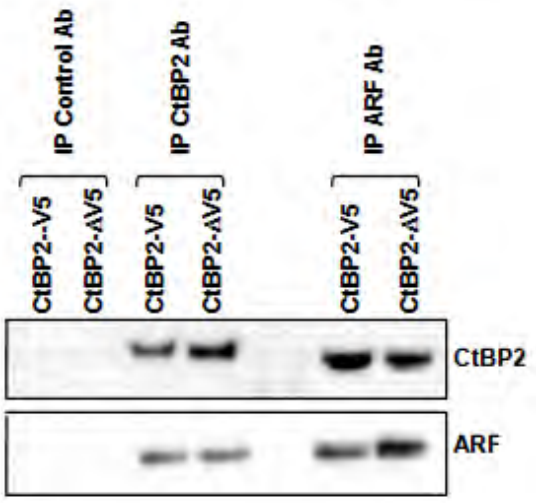
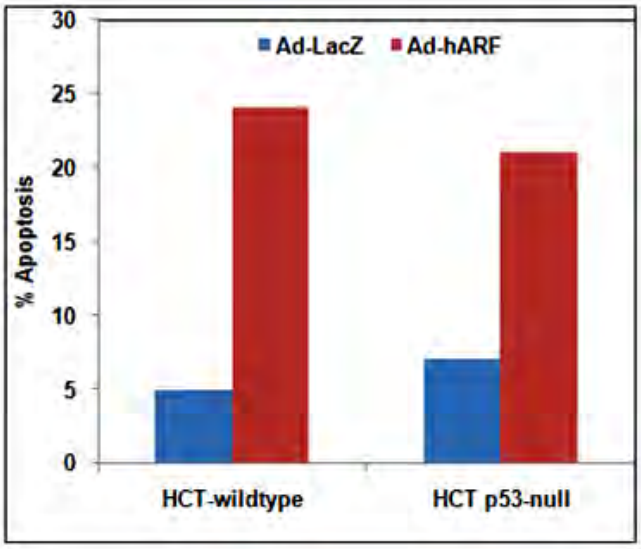


Figure 2.5 **ARF expression or CtBP depletion causes p53-independent apoptosis** **A.** ARF induces p53-independent apoptosis. 24 hrs after infection with Ad-lacZ or Ad-hARF, HCT116 wt or p53-null cells were labeled with FAM-DEVD-FMK and propidium iodide and assayed by FACS. The percentages of live cells in each sample with active caspase 3 were plotted. **B.** $p14^{ARFL50D}$ does not induce apoptosis. HCT116; p53-/- cells were infected with empty, ARF and L50D retrovirus were labeled for Annexin V-PE and 7-AAD and analyzed by FACS.

A



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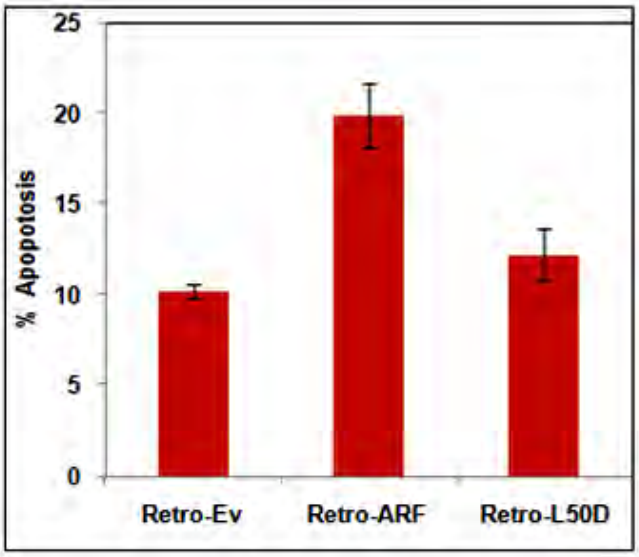
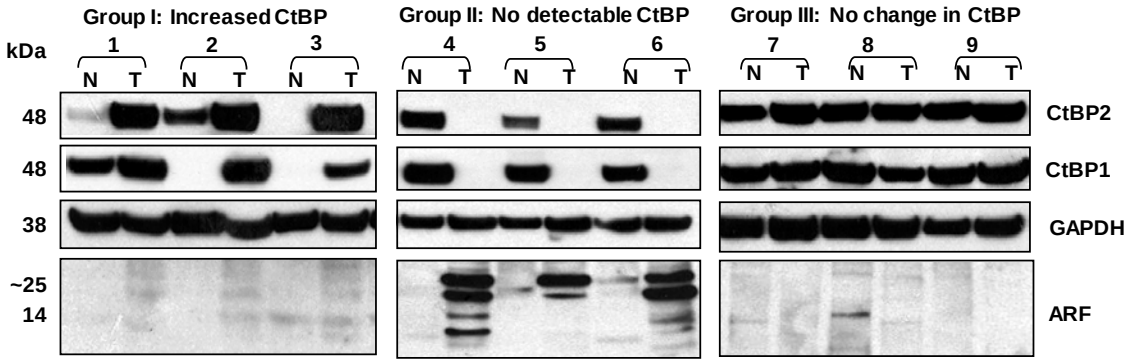


Figure 2.6 **CtBP and ARF regulated coordinately in human colon cancers.**
(A) Representative Immunoblot of protein Lysates from colonic adenocarcinomas resection specimens (T), including matching normal tissue (N) with CtBP1, CtBP2, ARF, and GAPDH specific antibodies. (B) RT-PCR with the Total RNA isolated from same set of tissue samples as in (A), RT-PCR with CtBP2 and GAPDH specific primers. (C) The percentage of each groups of all tumor samples screened compiled in the table format, the p-value was calculated by applying Chi square test, $p<0.00001$, when compared the correlation between ARF and CtBP expression levels.

A



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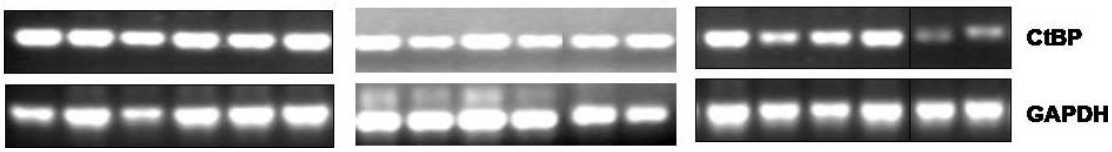

C

Tumor Groups	CtBP Levels # Samples / Total Samples (%)	ARF levels # of samples /samples in Group (%)
Group I: Increased CtBP, low ARF	45/70 (66.0)	6/45 (13)
Group II: No detectable CtBP, high ARF	17/70 (24.0)	17/17 (100)
Group III: No difference in CtBP, variable ARF	8/70 (10.0)	3/8 (37)

Materials and Methods

Cell Culture and Transfections

Human osteosarcoma cell line (U2OS) and human colon cancer cell line (HCT116: ARF silenced by promoter methylation) were grown in complete DMEM and McCoy's 5A medium respectively. Medium was supplemented with 10% fetal bovine serum and 100 units/ml penicillin and streptomycin, and incubated in humidified 5% CO2 at 37^{o}C. Expression plasmids were transfected using Fugene [111].

Plasmid Constructions

Full length CtBP2 was cloned from human lung carcinoma cDNA using pcDNA3.1 (Invitrogen), directional TOPO cloning, and specific sense (CACCATGGCCCTTGTGGATA) and antisense (TTGCTCGTTGGGGTGCTC) primers. CtBP deletion mutants were constructed in pcDNA 3.1 using specific PCR primers. pCD-mArf was generated by insertion of PCR amplified mArf coding sequence into pCDNA3. Missense and deletion mArf mutants were generated using PCR as per QuickChange protocol (Stratagene). A synthetic mArf gene with arginine codons recoded and optimized for bacterial usage was synthesized from overlapping oligonucleotides and PCR amplified prior to cloning into BamH1/EcoR1 sites of pGEX vector. The integrity of the plasmids and the coding sequences were confirmed by sequencing.

Antibodies, Western Blotting

Antibodies used were as follows: CtBP1 and 2 (BD Transduction Laboratories), $p19^{Arf}$ (ab80; AbCam), $p14^{ARF}$ (Novus Biologicals), GST (Z-5; Santa Cruz), V5 tag (Invitrogen), HA (12CA5, Roche), GAPDH (Advanced Immunochemical Inc.), PARP (BD Pharmingen) and cleaved caspase 3 (Cell Signaling). Anti-rabbit IgG-HRP or anti-mouse IgG-HRP conjugates (Amersham) were used with ECL detection (Amersham) for Western blots.

GST Pull-Down Assays

GST-mArf (codons optimized for *E. coli*) and GST were expressed in BL21 cells. Lysates from U2OS cells transiently transfected with CtBP full length and mutants were prepared as described [178] and incubated with GST or GST-conjugated mArf immobilized on glutathione-Sepharose beads. The beads were washed with wash buffer (10mM Tris pH8.0, 150 mM NaCl, 0.1% Triton –X-100, 2 mM MgCl2, 10µM ZnCl2, 10% glycerol) three times. Protein bound to beads was eluted with 20mM glutathione in elution buffer (10mM Tris pH8.0, 120 mM NaCl, 0.1% Triton X-100, 10% glycerol), separated by SDS PAGE and immunoblotted.

Coimmunoprecipitation

Cell lysates (100 µg of protein) from $1.5x10^7$ cells in lysis buffer (20mM Hepes, 10µM ZnCl2, 1mM MgCl2, 250 mM NaCl, 0.1% Triton X-100, 1mM DTT, 1mM PMSF, 10µg/ml Aprotinin and Leupeptin) were incubated at 4^oC for 1 hr with specific

antibody-conjugated Sepharose used for immunoprecipitation. Beads were washed 3 times in Wash Buffer (20mM Hepes, 10µM ZnCl2, 1mM MgCl2, 150 mM NaCl, 0.1% Triton X-100, 1mM PMSF, 10µg/ml Aprotinin and Leupeptin) followed by SDS-PAGE and Western blotting.

UV Irradiation

Cells were grown to 60% confluence in DMEM with supplements. The medium was removed and the dishes were exposed to UVC at a dose of 0-30 J/m^2 using a Stratalinker (Stratagene). The cells were collected 6 hrs post-treatment, washed with PBS and scraped in lysis buffer supplemented with protease inhibitors. The lysates were then analyzed by western blotting.

Adenoviral Infections

Ad-hARF [162] and Ad-lacZ high titer virus stocks were the generous gift of T. Kowalik. Cells (10^6) were plated in 6 well plates 24 hr before infection at confluency of 50%. Cells were washed with PBS once and infected with the Ad-lacZ or Ad-hARF virus in serum free medium at an MOI of 100 at 37^{o}C for 1 hr. Virus medium was removed and McCoy's medium was then added, and the cells were collected at 24 hrs post-infection and either lysed for western blotting or stained for caspase detection. Caspa Tag ™ Caspase-3/7 *In Situ* Assay kit (Chemicon International) was used for in situ detection of activated caspase 3 and 7 by FACS.

Immunofluorescence

U2OS cells grown on cover slips were transfected with the indicated ARF constructs, and 24 hrs after transfection cells were fixed and immunostained with anti-ARF antibody and anti-Rabbit IgG-rhodamine as described [153].

Tumor Sample Analysis

Whole tissue lysates were prepared using the PARIS® kit (Ambion Inc, TX). CtBP (both CtBP1 and CtBP2), ARF, GAPDH were immunoblotted using mentioned antibodies. The total RNA from each tumor and normal samples was prepared as per *PARIS® kit* (Ambion). RT-PCR was done using Stratascript ® RT kit. The primers used for RT-PCR were CTBP2- sense (132-155) 5'-CGAGACGAGAGTTTCATCAC CTTA-3' antisense (411-387)5'-GCGGATACCTTCACAAATTCTGTC-3' and GAPDH sense (219-240) 5'-ATCACCATCTTCCAGGAGCGA-3'antisense (690-670) 5'-GCCAG TGAG CTTCCCGTTCA-3'. The Institutional Review Board (IRB) at University of Massachusetts Medical School approved this tumor sample study.

Acknowledgements

I would like to thank Dr. Seema Paliwal; she has started this project and for her contributions to this chapter. I also want to thank Drs. Tim Kowalik for high-titer Ad-hARF and Ad-lacZ, Steve Lyle for help with tumor sample analysis, B. Vogelstein (Johns Hopkins, MD) and the Altieri lab for providing HCT116; p53-/- cells.

Preface

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Chapter- III

An ARF/CtBP2 complex regulates BH3-only gene expression and p53-independent apoptosis

Abstract

The ARF tumor suppressor exerts both p53-dependent and p53-independent functions. The corepressor *C-t*erminal *b*inding *p*rotein (CtBP) interacts with ARF, resulting in proteasome-mediated degradation of CtBP. ARF expression can induce p53-independent apoptosis in p53-null colon cancer cells, in a manner dependent on ARF interaction with CtBP. Bik was uniquely identified in an apoptotic gene array as coordinately upregulated in colon cancer cells after either CtBP2 knockdown or ARF overexpression. ARF expression led to robust induction of Bik mRNA and protein expression, and this activity required an intact CtBP binding domain. Analysis of the Bik promoter revealed binding sites for CtBP-interacting Basic Kruppel-like Factor (BKLF). A Bik promoter luciferase reporter was repressed by BKLF and CtBP2, and ARF reversed CtBP-associated repression. Chromatin immunoprecipitation analyses showed that CtBP is recruited to the Bik promoter largely by BKLF. Expression profiling of BH3-only gene expression in ARF-expressing or CtBP deficient cells revealed that Bik was uniquely regulated by ARF/CtBP in colon cancer cells, whereas additional BH3 proteins (Bim, Bmf) exhibited CtBP dependent repression in osteosarcoma cells. ARF antagonism of CtBP repression of Bik and other BH3-only gene expression may play a critical role in ARF-induced p53-independent apoptosis and tumor suppression.

Introduction

The tumor suppressor ARF is a product of the *INK4a/ARF* locus [10] that can act via p53-dependent or independent pathways [31, 148]. ARF is frequently inactivated in a wide spectrum of human cancer types, including colorectal, breast, and pancreatic adenocarcinomas, malignant glioma, melanoma, and non-Hodgkin's lymphoma [169, 179]. Germline homozygous knockout of *Arf* in mice results in the development of lymphomas and sarcomas similar to those observed in p53-deficient mice [31]. Simultaneous inactivation of *p53* and *Arf* results in a broader tumor spectrum and more aggressive tumors than are observed with either knockout alone, suggesting an additional mechanism for ARF tumor suppression apart from its canonical activation of p53 [31].

The transcription regulator CtBP has been identified as a specific target of the ARF tumor suppressor relevant to ARF's ability to induce apoptosis in cells lacking p53 [68]. ARF binds to CtBP, resulting in proteasome-mediated degradation and inactivation of CtBP [68]. CtBP proteins act as transcriptional repressors in conjunction with a wide range of DNA binding transcription factors, and are regulated and activated as repressors by NADH binding to their dehydrogenase domains [180].

CtBP1/2-null MEFs are hypersensitive to apoptosis in response to a wide variety of stimuli [70]. Microarray analysis has shown that epithelial specific and proapoptotic genes are upregulated in these MEFs [70], though the precise mechanism that links CtBP to the suppression of pro-apoptotic gene expression is not known. Separate evidence suggests that siRNA mediated CtBP knockdown in human tumor cell lines is sufficient to induce apoptosis in the absence of additional stress [68]. Though not yet proven, CtBP is

likely to be linked to tumor progression, as it promotes both cell survival and epithelial-mesenchymal transition by repressing the transcription of both proapoptotic and epithelial genes [70].

The effects of CtBP on cell survival have been linked specifically to its repression of pro-apoptotic BH3-only genes, of which Noxa and Puma were identified in a microarray comparison of wt and CtBP1/2 knockout MEFs [70]. Of note, the proapoptotic BH3-only proteins are critical mediators of death induced by cytokine deprivation, activated oncogenes, and various DNA damage stresses [118]. Their presumptive mechanism of action is to dissociate bax or bak from anti-apoptotic bcl-2 family proteins, allowing them to translocate to the outer mitochondrial membrane to form pores that allow the cytoplasmic release of cytochrome C [122, 123]. Of the 8 known BH3-only genes, Bid is a critical mediator of apoptosis mediated by death receptor signaling [124], Bim is the determinant of taxane responsiveness [125], Puma and Noxa are central mediators of p53-induced apoptosis [126], and Bad regulates apoptosis mediated by growth factor/cytokine signaling [127]. By contrast, the cellular apoptotic stimuli that act through Nbk/Bik, and the biologic functions of these genes in mammals, are not yet known [128].

In this study, the BH3-only protein Bik was identified as an ARF and BKLF/CtBP-regulated gene, and a critical mediator of ARF-induced, CtBP2-mediated p53-independent apoptosis in colon cancer cells. CtBP repression of Bik was directly antagonized by ARF, and CtBP was recruited to the Bik promoter via BKLF. Apoptosis induced by CtBP deficiency in the absence of p53 was substantially impaired when Bik expression was also reduced by RNA interference. Other BH3-only family members

besides Bik were co-regulated by CtBP in other cell types to suggest that p53-independent ARF tumor suppression may involve regulation of different sets of BH3-only proteins, dependent on tissue origin.

Results

Bik is upregulated after CtBP2 depletion or ARF overexpression

To identify the mediators of ARF/CtBP2-induced p53-independent apoptosis, a human cDNA apoptosis microarray was interrogated with mRNA obtained from HCT116;p53-/- cells infected with control or ARF adenovirus [68] or treated with control or CtBP2 siRNA. Genes with more than a two-fold change (compared to control) after either CtBP2 depletion or ARF overexpression were considered for further investigation. Though a number of TNF pathway genes were induced after both ARF expression and CtBP2 silencing—none were common between the two conditions (Figure 3.1A). The BH3-only gene Bik was the only common gene upregulated under both conditions (Figure 3.1A).

In order to more quantitatively assess the effects of CtBP2 depletion and ARF overexpression on Bik expression, Bik mRNA and protein levels were analyzed in HCT116; p53 -/- cells 24 hrs after either depletion of CtBP2 using siRNA or ARF overexpression by retroviral infection (Figure 3.1B). Real-time PCR analysis confirmed that Bik expression was increased upon either CtBP2 depletion (2.6-fold) or ARF overexpression (2.3 fold) in HCT116; p53-/- cells (Figure 3.1B). Similarly, the protein

levels of Bik were found to be upregulated in CtBP2 siRNA treated cells as compared to control siRNA treated cells (Figure 3.1C).

CtBP2 interaction is required for ARF regulation of Bik

Since the ability of ARF to interact with CtBP correlates with its ability to induce apoptosis in p53-null cells [68], Bik expression was analyzed in cells where ARF/CtBP interaction was either intact or abrogated. HCT116; p53 -/- cells infected with ARF, ARF^{L50D} (CtBP interaction defective, however it retains p53 stabilization function and nucleolar localization of $p14^{ARF}$, Figure 1.2D-F), or control retrovirus were analyzed for Bik protein levels by western blotting (Figure 1D). Both ARF and ARF^{L50D} were expressed at similar levels (Figure 3.1D). Bik was induced in ARF expressing cells, whereas ARF^{L50D} expressing cells showed no Bik induction, similar to that observed for cells infected with empty virus (Figure 3.1D). Thus, the ability of ARF to interact with CtBP was required for its induction of Bik expression.

Bik depletion rescues CtBP2-induced p53-independent apoptosis

To further investigate the hypothesis that Bik functions as an important mediator of ARF/CtBP induced apoptosis, Bik and CtBP2 were simultaneously knocked down in HCT116; p53-/- cells and induced to undergo apoptosis upon UV treatment. Despite comprehensive screening for effective Bik-specific si- and shRNA sequences, the best knockdown of Bik that could be achieved was only partial, though two independent shRNA sequences were obtained (Figure 3.2A). Annexin-V and trypan blue stains

documented apoptotic fraction (Figure 2A) and viability (Figure 2B), and cell lysates were also immunoblotted to monitor for PARP and caspase 3 cleavage (Figure 3.2B).

As expected, CtBP2 depletion led to increased Bik levels in cells with control shRNA, and even in Bik shRNA-expressing cells (Figure 3.2B). The basal apoptosis rate in low-dose (20 J/M2) UV treated cells with control si and shRNA's was 7%, and the overall non-viability rate was 12% (Figure 3.2C-D). siCtBP2 induced a more than doubling of apoptotic fraction to 16% (p= 0.04) and non-viability to 27% (p= 0.01). This effect was partially abrogated by shBik, with reduction of apoptotic fraction to 10% (7% basal level, p= 0.02) and non-viability to 17 % (12% basal level, p= 0.02; Figure 3.2C-D). A second Bik shRNA yielded essentially similar effects in all assays (Figure 3.2C-D). Results with annexin and trypan blue staining were mirrored in the abundance of PARP and caspase 3 cleavage products (Figure 3.2B). These data strongly support the hypothesis that Bik plays an important role in the induction of apoptosis after CtBP depletion in colon cancer cells. The partial rescue of apoptosis by Bik knockdown would be consistent with the partial knockdown of its expression by shRNA, suggesting that a more robust knockdown might have further suppressed apoptosis closer to baseline levels. However, the contribution of other proapoptotic proteins (BH3-only or other) to apoptosis in CtBP2 deficient cells cannot be completely ruled out.

ARF is generally not expressed at detectable levels in normal cells, and it is not induced by environmental stresses, except oncogenic signals. However, its ability to inhibit CtBP may potentiate the effects of stress such as UV or hypoxia. Additional stresses such as UV ($20J/m^2$) or hypoxia in human colon cancer induced more robust

apoptosis when they express ARF than control (15% vs. 26% with UV and 15% vs. 32% in hypoxia), whereas ARF^{L50D} expression in these cells did not enhance stress-induced apoptosis. These findings are consistent with the hypothesis that, ARF's interaction and inhibition of CtBP is required for it to both induce and potentiate apoptosis in cells lacking p53 (Figure 3.2E).

ARF/CtBP regulation of the Bik promoter through BKLF recognition elements

In silico analysis of the Bik promoter for recognition sites relevant to transcription factors that recruit CtBP as a corepressor revealed 4 sites with an exact match to BKLF (KLF8/ZNF741/BKLF3) recognition elements, including a tandem repeat [181] (Figure 3.3 A, B). Examination of the upstream (-1 to -2000) promoter regions of the other 7 known BH3-only genes revealed obvious BKLF sites upstream of the Noxa, Puma, Bmf and Bim genes (Figure 3.3B). To test the hypothesis that the recruitment of CtBP by BKLF represses Bik promoter activity, Bik promoter luciferase reporters containing either all wildtype BKLF binding sites, or with the two tandem sites mutated, were transfected into U2OS cells with BKLF, ARF, and CtBP2 expression vectors. Either BKLF or CtBP2, alone, repressed the wildtype promoter about 2.5-fold (p <0.01) (Figure 3.3C) while there was no effect on the mutant reporter (Figure 3.3D). CtBP2/BKLF coexpression further repressed Bik promoter activity another 2-fold (p <0.05) (Figure 3.3C). Overexpression of ARF had no effect on BKLF repression of Bik promoter activity (compare 1st and 3rd yellow bars; Figure 3.3C), but when ARF was cotransfected with CtBP2 and BKLF, ARF caused a near complete reversal of CtBP2-associated

repression (compare 2nd and 4th yellow bars; Figure 3.3C). Reversal of CtBP2-mediated repression by ARF is consistent with the finding that ARF degrades and/or sequesters CtBP in the nucleolus, abrogating its repressor activities [68]. Thus, BKLF elements are crucial for CtBP/BKLF-mediated repression of the Bik promoter. Consistent with ARF's known effects on CtBP, ARF reversed CtBP2/BKLF-mediated repression of the Bik promoter, but had no effect on BKLF-mediated repression in the absence of CtBP2.

CtBP2 is recruited to the Bik promoter

Previous studies have demonstrated that CtBP2 binds to BKLF and regulates expression of genes downstream of BKLF recognition elements [182]. To address whether CtBP is directly recruited to the Bik promoter, CtBP2 chromatin immunoprecipitation (ChIP) was performed using two sets of promoter specific primers including one set (PS1) that amplified a fragment which is near to the single BKLF binding site in the distal part of the Bik promoter or a PS2 set of primers amplified a fragment encompassing the 2 tandem BKLF binding sites in the Bik promoter (Figure 4A-B). Additionally, a negative control primer set (NS) that amplified a fragment 10 kb upstream of the promoter was also tested and E-cadherin promoter primers were utilized as a positive control [111]. CtBP2 was recruited to both the Bik (PS2) and E-cadherin promoters in chromatin obtained from H1299 human lung carcinoma cells, whereas no signal was seen in either of the CtBP2 ChIPs with control (NS) or PS1 primers that amplified fragments with no BKLF sites, nor was any signal detected in control or no antibody ChIPs with PS1 or PS2 primers (Figure 3.4B-C).

To test whether CtBP2 recruitment to the Bik promoter required BKLF, a CtBP2 ChIP assay was performed using chromatin from cells where BKLF was stably depleted using shRNA (knockdown ~50% by RT-PCR; Figure 3.4D) and primers specific for the Bik and E-cadherin promoters or control Bik intergenic region primers. When compared to control, BKLF shRNA expression led to increased Bik expression as determined by immunoblot, and as predicted by the reporter assays (Figure 3.4E, 3.3C). As expected, CtBP2 was present at the Bik and E-cadherin promoters in control shRNA expressing cells, but in the absence of BKLF, CtBP2 was no longer recruited to Bik promoter, despite remaining present at the E-cadherin promoter (Figure 3.4F). The specificity of the ChIP signals were bolstered by the lack of signal either in the control antibody ChIP or in the CtBP2 ChIP with control intergenic region Bik primers (Figure 3.4F). Thus, CtBP2 is recruited to the Bik promoter, largely via BKLF (Figure 3.4G).

Differential regulation of BH3-only pro-apoptotic genes

Though Bik plays an important role in ARF-induced p53-independent apoptosis in HCT116;p53-/- cells, several other BH3-only genes have been previously shown by genomic techniques (in MEFs) to be regulated by CtBP1 and 2 [70]. In order to study the potential regulation of the family of BH3-only genes by CtBP in cells of either epithelial or mesenchymal lineage, the mRNA and protein levels of Bik, Bim, Bmf, Noxa, and Puma were determined in HCT116;p53-/- colon carcinoma cells and U2OS osteosarcoma cells after treatment with control or CtBP2 siRNA. As predicted by the apoptosis gene array (Figure 3.1), Bik was the only BH3-only genes induced >2-fold at RNA level in

HCT116;p53-/- cells, while U2OS cells were more permissive for BH3-only gene expression with 2-2.5 fold induction of Bik, Bim, and Bmf, and 1.5-fold induction of Puma and Noxa after CtBP2 knockdown (Figure 3.5A, D). Where mRNA induction was >= 2-fold, protein expression was also increased as seen for Bik in HCT116;p53-/- cells, and Bik, Bim, and Bmf in U2OS cells.

ARF expression would be expected to phenocopy CtBP2 knockdown for regulation of BH3 gene expression, based on its antagonism of CtBP [68]. Indeed, ARF expression resulted in Bik induction as previously seen (Figure 1), but had little effect on protein levels of the other BH3-only proteins (Bim, Bmf, Puma and Noxa) in HCT116;p53-/- colon carcinoma cells (Figure 3.5C). ARF^{L50D} expression had little effect on the abundance of any BH3-only protein, including Bik, as already has been noted. Thus, ARF expression phenocopies the specific impact of CtBP2 depletion on BH3-only gene expression in colon cancer cells, supporting the hypothesis that ARF/CtBP complexes directly control BH3-only gene expression and thus, p53-independent apoptosis, in a cell-type specific manner.

Discussion

ARF overexpression, or depletion of CtBP2, induced mRNA and protein expression of the proapoptotic BH3-only gene Bik. Induction of Bik required ARF/CtBP interaction, and the induction of apoptosis by UV and CtBP2 depletion required physiologic levels of Bik. CtBP2 was recruited to the Bik promoter by the transcription factor BKLF, and ARF abrogated CtBP/BKLF repression of the Bik promoter.

Furthermore, the pattern of BH3-only gene regulation by CtBP appeared to depend on cell type context, suggesting that p53-independent tumor suppression by ARF may be more relevant to certain tumor types than others.

Bik is a proapoptotic protein of the 'BH3-only' family. Expression of Bik triggers apoptosis in breast, lung, prostate, and colon carcinoma, as well as glioma and melanoma-derived cell lines [123, 183, 184, 185, 186]. Consistent with a role for Bik in tumor suppression, 22p13.3, which contains Bik, is commonly deleted in human colorectal and breast cancers [187], and Bik mutations have been identified in renal cell carcinoma [188]. By contrast, in non-malignant cells, Bik functions may overlap with other BH3 proteins, as Bik is not essential for normal development [140]. In vitro, Bik knockout mouse T and B cells also did not exhibit an apoptotic defect, though epithelial cells were not examined in that study [140]. The absence of a mouse phenotype does not necessarily exclude a role in native tumor suppression, however, as the Bik knockout mice and cells were not exposed to an oncogenic stress to reveal a more subtle tumor or apoptotic phenotype [140].

Functionally, Bik is not a direct initiator of apoptosis, but acts upstream of the pro-survival Bcl-2- family members [189]. Recent studies also suggest that Bik plays a role in oxidative stress induced apoptosis [190]. Bik binds directly to BCL-2 or BCL-X_L via its BH3 domain, and inactivates their antiapoptotic functions. Therefore, an increase in Bik levels lowers the cellular apoptotic threshold by blocking the anti-apoptotic function of BCL-2-family proteins [191]. A competing hypothesis suggests that Bik might also activate the downstream effectors Bak or Bax directly to cause apoptosis [142]. Further study on the

apoptotic pathway downstream of Bik will be required to fully understand the role of Bik in CtBP2 induced p53-independent apoptosis.

CtBP has been described as a transcriptional regulator of apoptosis as it transcriptionally repress multiple proapoptotic genes, such as Noxa, Puma and PERP [70]. Many of these genes are also known transcriptional targets of p53. However, CtBP regulation of Bik, as shown in the current work, is p53-independent and likely acting through BKLF instead of p53. BKLF can recruit mCtBP2, via its PXDLS motif [192] to the β-globin promoter element, resulting in repression [75]. We have observed that CtBP2 is also recruited by BKLF to tandem CACCC elements in the Bik promoter, since knockdown of BKLF abrogated CtBP2 recruitment to the Bik promoter in colon cancer cells and mutation of the tandem repeat abrogated CtBP2 repression of a Bik reporter. The involvement of other related KLF transcription factors (KLF1/EKLF: erythroid Kruppel-like factor, KLF2/LKLF: lung Kruppel-like factor, KLF4/GKLF: gut-enriched, KLF5/IKLF: intestinal-enriched, KLF7/UKLF: ubiquitous KLF, among others) in CtBP2 recruitment cannot be ruled out in other cell contexts, as many KLF's function in a tissue dependent manner [193].

CtBP senses the metabolic state of the cell due to a requirement for NADH binding to its dehydrogenase domain to activate repressor function [180]. CtBP has been linked to the hypoxic activation of cell migration, and this effect may be due to its repression of other non-apoptosis pathway genes such as PTEN [70, 109]. Since hypoxia is fundamentally linked to tumor progression, CtBP may serve as a critical oncogenic link by which hypoxia leads to activation of key malignant characteristics such as enhanced

cell survival and increased motility and invasion [108, 109, 194]. Based on this hypothesis, the current data would support the idea that Bik might be especially important in tumor suppression in hypoxic cells, and moreover, ARF loss or mutation should specifically enhance cell survival in hypoxia by release of CtBP from any negative control. This, then, may also explain why ARF can so profoundly impact tumor progression in vivo with its loss promoting increased tumor aggressiveness [55, 108, 195].

This study demonstrates a role for the ARF/CtBP complex in the transcriptional regulation of Bik, and this pathway contributes to the apoptotic response in human colon cancer cells. The ARF/CtBP interaction may explain many of the p53-independent apoptotic effects of ARF. This work raises the possibility that tumorigenesis is enhanced in the absence of ARF due to an apoptotic defect in a parallel ARF-regulated tumor surveillance system that is completely independent of p53. With further understanding of the cellular consequences of ARF/CtBP interaction there is the distinct possibility of manipulating this pathway either through ARF-mimetics or CtBP inhibitors for therapeutic benefit in the substantial fraction of tumors that lack p53 and/or ARF function.

Figures

Figure 3.1 **Bik is upregulated upon ARF overexpression or CtBP depletion in p53-null human colon cancer cells.**

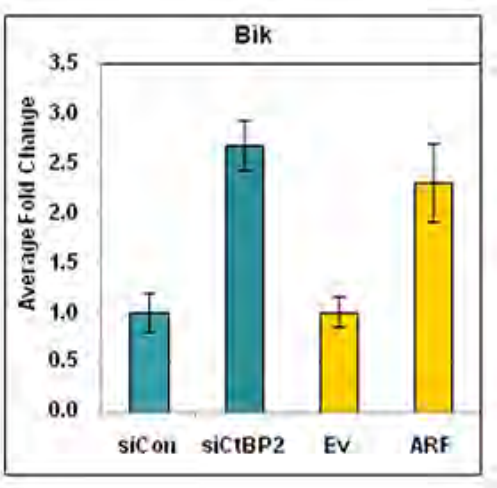
A. Total RNA isolated from HCT116; p53-/- cells following either ARF overexpression or CtBP knockdown was subjected to an apoptotic gene array (Superarray) analysis. The fold change in relative gene expression level was calculated by using GEArray Expression Analysis Suite (SA Biosciences). **B.** RNA prepared from HCT116; $p53^{-/-}$ cells with above treatment was to carry out quantitative real time PCR using Bik and GAPDH specific primers. Graph represents GAPDH-normalized average fold change of Bik in treated cells. Error bars represent +/- 1 standard deviation (S.D.) **C.** CtBP2 regulates Bik expression. HCT116; p53-/- cells were treated with control or CtBP2 siRNA duplexes, and CtBP2, Bik and GAPDH levels were determined by immunoblotting 24 hrs after transfection. **D.** CtBP2 interaction with ARF is required for regulation of Bik expression. HCT116; p53 -/- cells were infected with vector, hARF, or ARF (L50D) mutant lentivirus for 24 hrs. Cell lysates were analyzed for Bik, GAPDH, and ARF levels by immunoblotting.

A

CtBP2 knockdown	
Gene	**Fold Induction**
Bik	**2.0**
TNFSF12	1.5
TNF	1.6
ARF infection	
Bik	**6.0**
TRAIL	2.0
CD40L/TRAP	3.5

cell line: HCT116 $p53^{-/-}$
Apoptosis cDNA array (GE Array)

B



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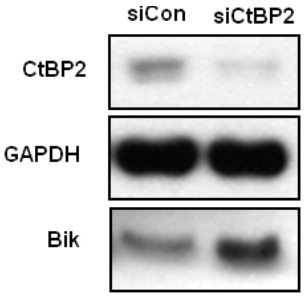

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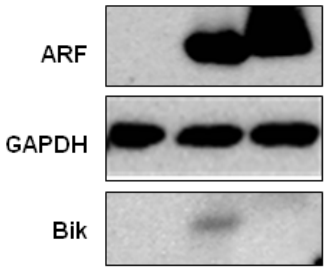
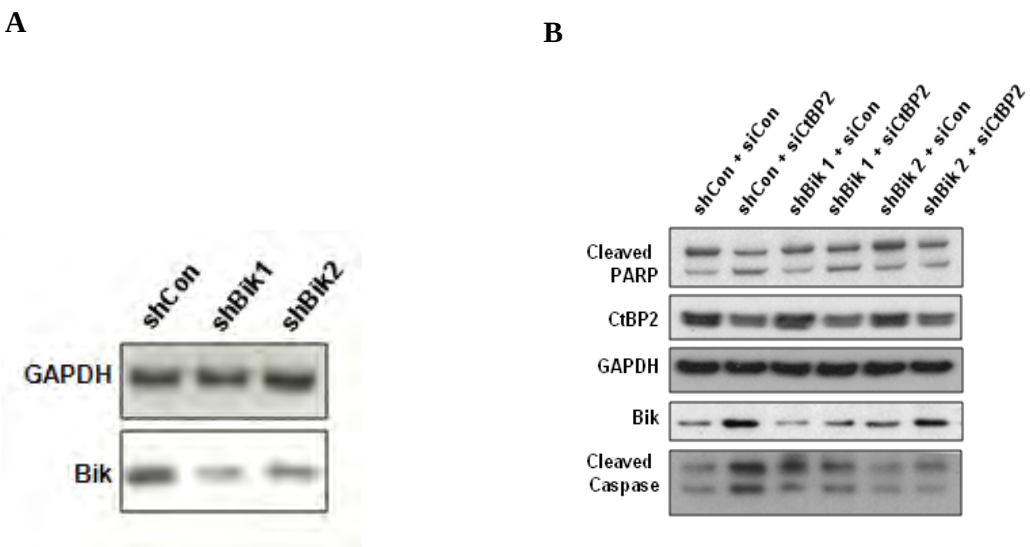


Figure 3.2 **Bik knockdown rescues ARF/CtBP2 induced p53-independent apoptosis.**

A. HCT116; p53-/- cells stably infected with Tet-inducible shBik1 and shBik2 lentiviruses and induced with 2 µg/ml doxycycline for 24 hours and immunoblotted for GAPDH and Bik protein levels. **B.** HCT116; p53-/- cells with stable Bik knockdown were transiently transfected with either control or CtBP2 siRNA, and then 20J/M2 UV-C treated. After 24 hours of transfection and UV treatment, apoptosis markers (cleaved caspase 3 and cleaved PARP), CtBP2, Bik and GAPDH levels in each of the treatment conditions were determined by immunoblotting. **C.** *Apoptosis Assay*: HCT 116: p53-/- cells were stably infected with shBik1 and shBik2 lentiviruses and transiently transfected with either control or CtBP2 siRNA. All cells were exposed to 20J/M^2 UV-C. After 24 hours of transfection and UV treatment, apoptosis was determined by Annexin V-PE/7-AAD staining. **D.** *Cell Viability Assay*: The percentage of viable cells was determined after treatment as in (C) by staining cells with 0.4 % Trypan Blue (Sigma). **E.** *ARF-induced apoptosis upon stress*. HCT116 p53-/- cells were infected with empty (Ev), ARF and L50D retrovirus. After 24 hours of infection, cells were exposed to either 20J/m2 of UV or hypoxia (0.5 % oxygen in hypoxia chamber) for 24 hours. Apoptosis was determined by Annexin V-PE and 7-AAD staining and FACS analysis. All experiments were performed in triplicate and the results expressed as Mean ± 1 S.D with the p-value <0.05.



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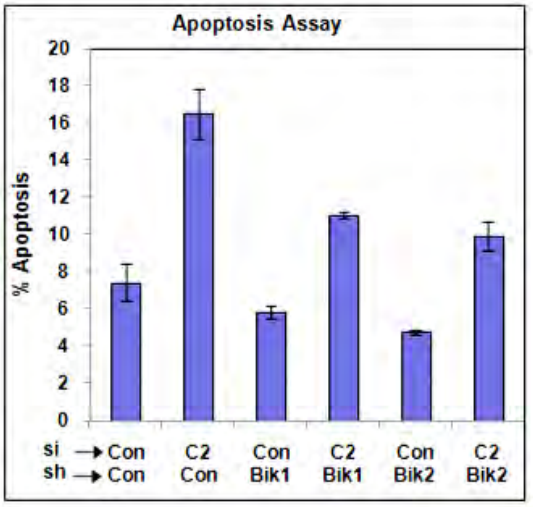
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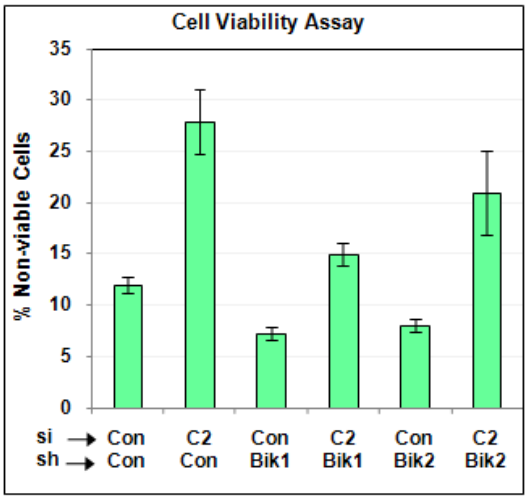
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C2 Bik2

E

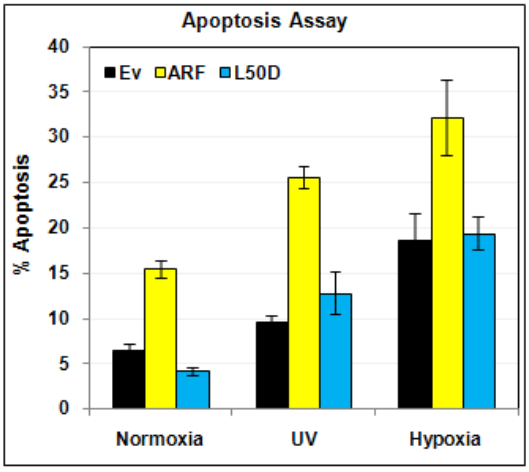
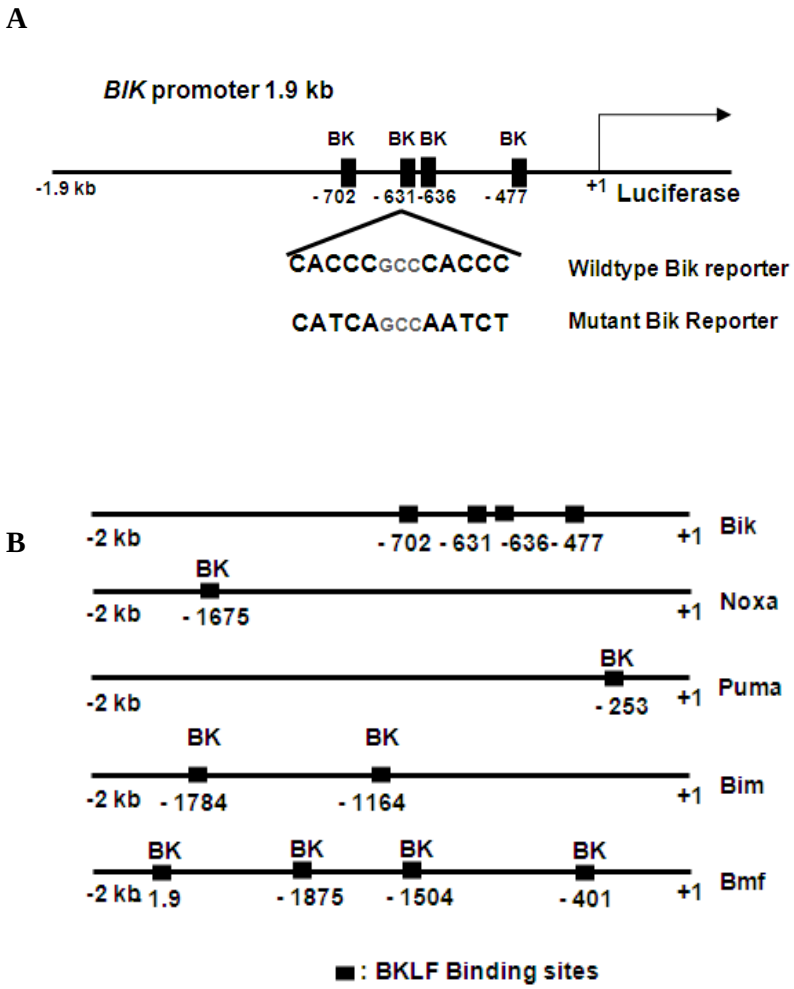
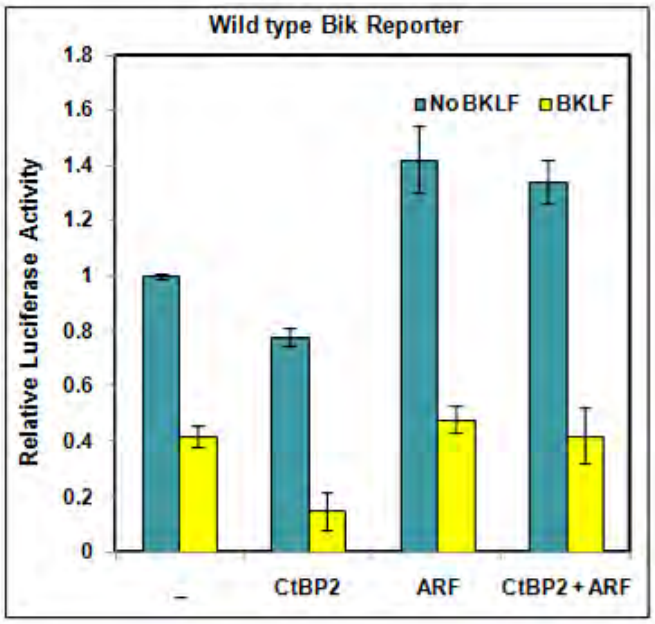

Apoptosis Assay
Ev
ARF
L50D
% Apoptosis
40
35
30
25
20
15
10
5
0
Normoxia
UV
Hypoxia

Figure 3.3 ARF/CtBP regulates the Bik promoter through BKLF recognition elements.

BH3-only genes contain BKLF recognition elements. **A.** Diagram of BKLF recognition elements in the Bik promoter. **B.** Alignment and BKLF element localization in the Noxa, Puma, Bim, Bmf and Bik promoters. **C.** ARF antagonizes CtBP/BKLF repression of the Bik promoter. Bik (pGL3-Bik-luc) was cotransfected with expression constructs for ARF, BKLF, or CtBP2 into U2OS cells along with a control reporter plasmid expressing *Renilla* luciferase (pRL-TK). **D.** A Bik-luciferase reporter with mutation of the tandem BKLF sites was cotransfected with expression constructs for ARF, BKLF, or CtBP2 into U2OS cells along with a control reporter plasmid expressing *Renilla* luciferase (pRL-TK). Normalized firefly luciferase activity from three independent experiments was averaged, and error bars indicate +/- 1 S.D and with the $p < 0.005$.



C


Wild type Bik Reporter
Relative Luciferase Activity
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
No BKLF
BKLF
–
CtBP2
ARF
CtBP2 + ARF

D

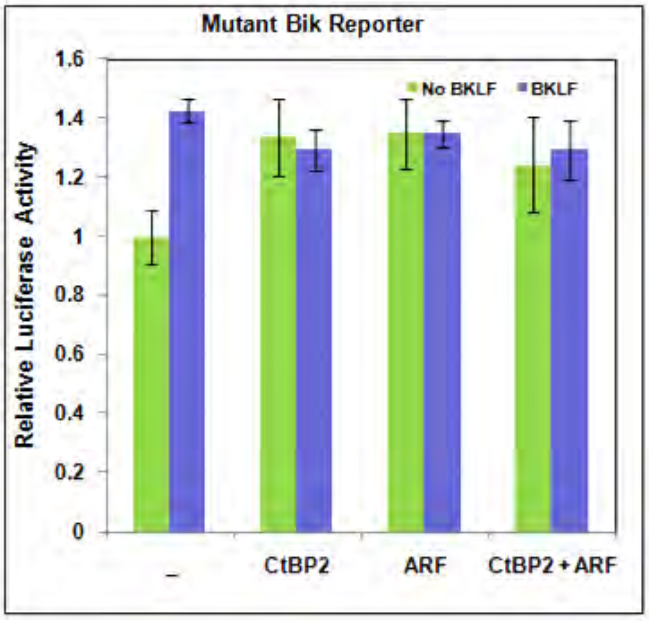
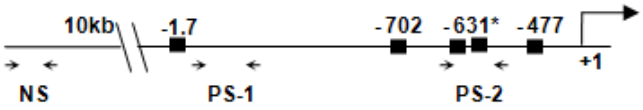
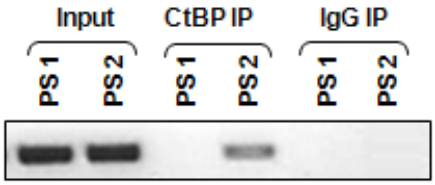

Mutant Bik Reporter
Relative Luciferase Activity
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
No BKLF
BKLF
–
CtBP2
ARF
CtBP2 + ARF

Figure 3.4 **BKLF mediated recruitment of CtBP to the Bik promoter.**
A. The location of the primers (arrows) relative to BKLF binding sites, primer set 1 (PS-1) is near to distal BKLF binding site, PS-2 is flanking the tandem BKLF binding site at -631, primer set NS is 10 kb downstream of start site of Bik promoter. **B and C.** *ChIP assay for CtBP*. H1299 chromatin was immunoprecipitated with control IgG or CtBP antibody (B). Immunoprecipitated and input DNAs were amplified by PCR using primers sets PS 1 and 2 (C and D) specific for the BKLF binding region of the Bik promoter (-693 to –551), non-specific (NS) primers that amplify a fragment ~10 kb upstream of the Bik transcription start site (-9500 to –9300; negative control), or E-cadherin promoter primers [111]. **D.** *Efficacy of BKLF shRNA*. shRNA targeting BKLF or GFP was stably expressed in H1299 cells. RT-PCR was performed to determine the knockdown of BKLF mRNA with GAPDH mRNA level as an internal control. RT-PCR products were electrophoresed in an agarose gel and stained with ethidium bromide. **E.** *BKLF regulation of Bik expression*. shBKLF or shGFP were stably expressed in HCT116: p53-/- cells, and protein levels of GAPDH and Bik determined by immunoblotting. **F.** CtBP recruitment to the Bik promoter requires BKLF. CtBP2 or control (No Ab) ChIP was performed with chromatin obtained from shGFP or shBKLF expressing cells. Immunoprecipitated DNA was analyzed by PCR using primers specific for Bik promoter, non-specific and E-Cadherin promoter primers as in (A). **G.** Model of BKLF/CtBP2 mediated transcription regulation of Bik expression

A



B



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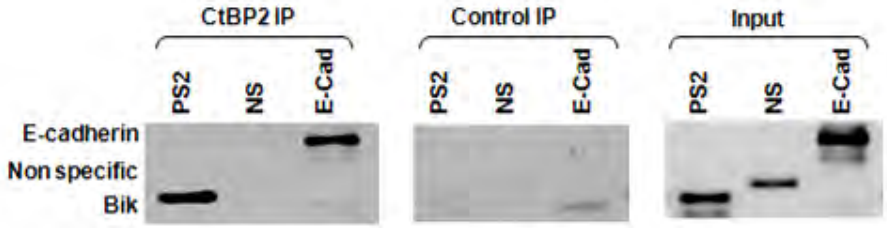
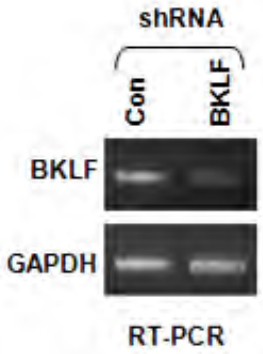
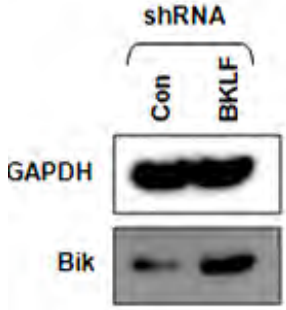

CtBP2 IP
Control IP
Input
PS2
NS
E-Cad
E-cadherin
Non specific
Bik

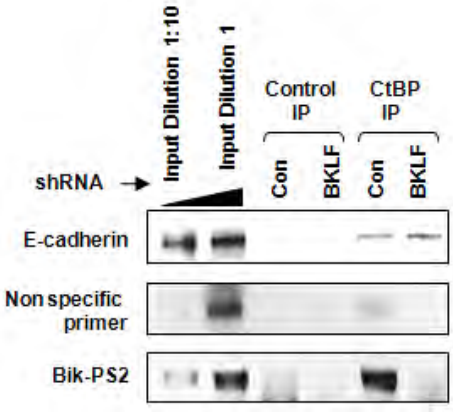
D


shRNA
Con
BKLF
BKLF
GAPDH
RT-PCR

E


shRNA
Con
BKLF
GAPDH
Bik

F


Input Dilution 1:10
Input Dilution 1
Control IP
CtBP IP
shRNA
Con
BKLF
Con
BKLF
E-cadherin
Non specific primer
Bik-PS2

G

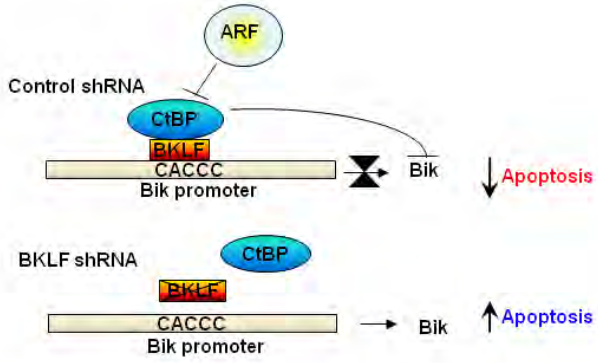
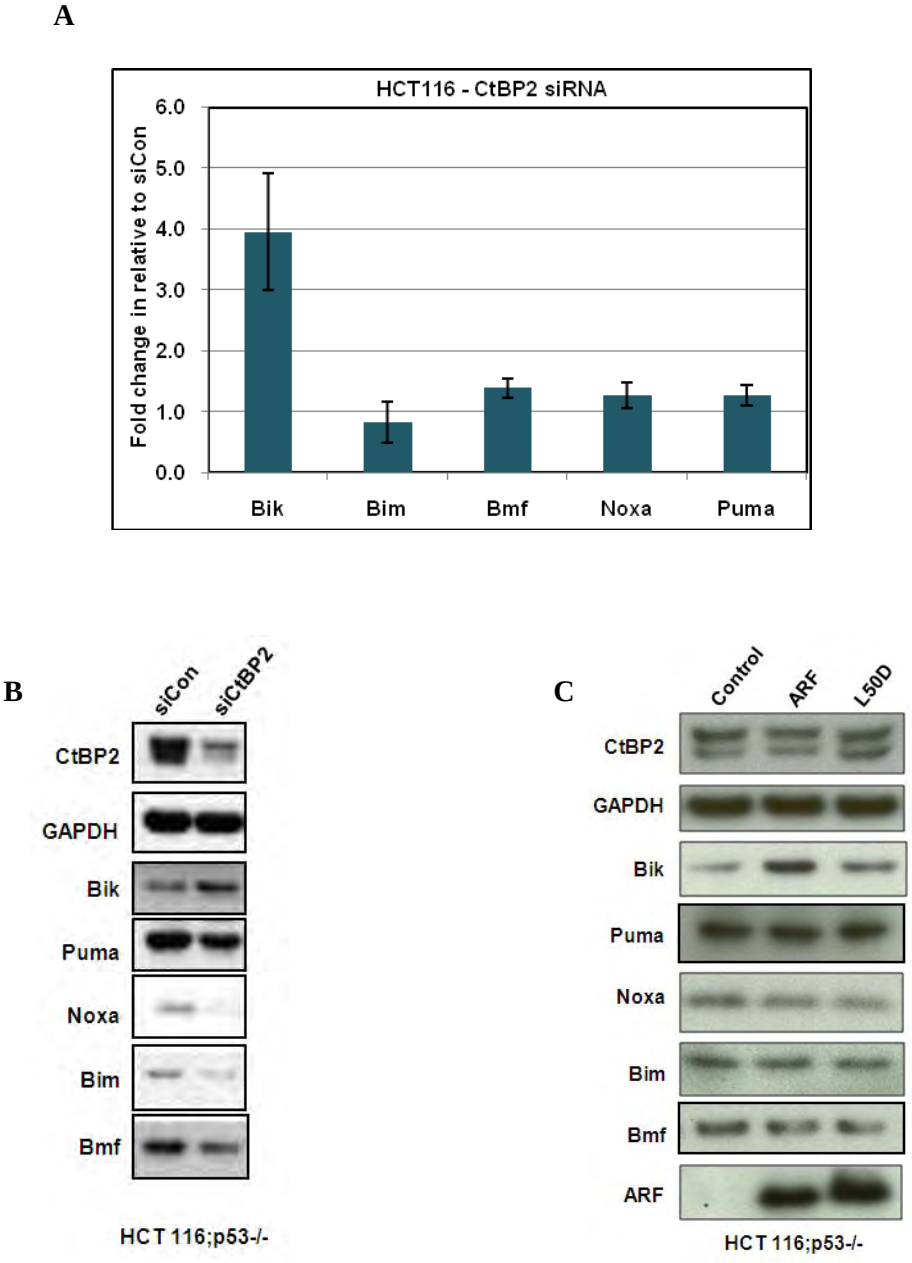
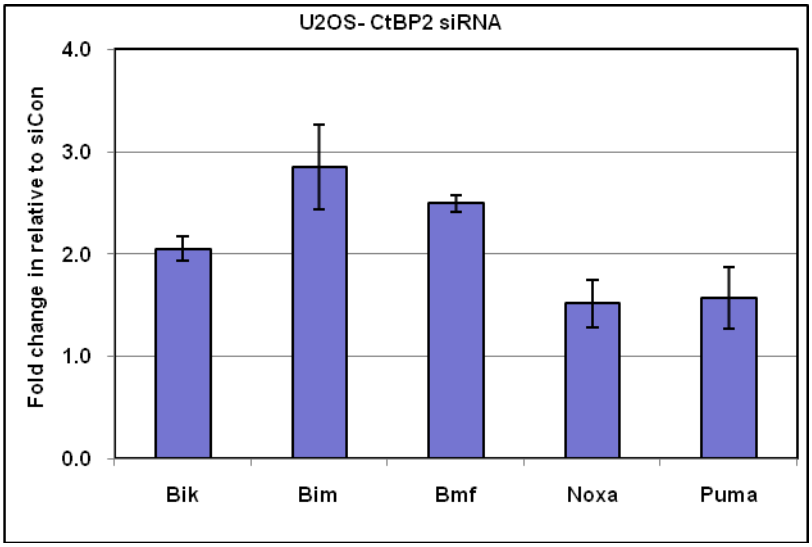

ARF
Control shRNA
CtBP
BKLF
CACCC
Bik promoter
Bik
Apoptosis
BKLF shRNA
CtBP
BKLF
CACCC
Bik promoter
Bik
Apoptosis

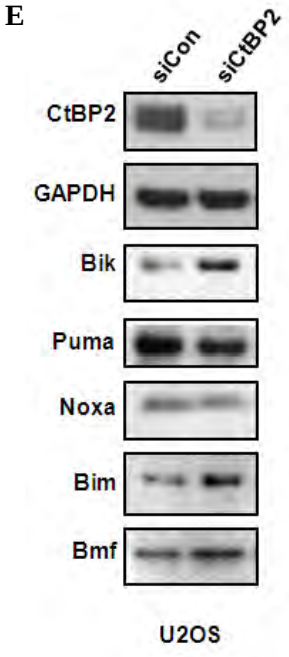
Figure 3.5 **Regulation of BH3-only genes by ARF and CtBP.**
RNA isolated after CtBP2 or control siRNA treatment of HCT116; p53-/- **(A)**, or U2OS **(D)** cells was subjected to RQ-PCR using GAPDH, β-Actin and Bik, Bim, Bmf, Puma and Noxa primers. Cell lysates from HCT116; p53-/- **(B)**, or U2OS **(E)** cells treated with control or CtBP2 siRNA and HCT116; p53-/- cells infected with either empty, ARF or ARF^{L50D} retroviruses **(C)** were analyzed for GAPDH, Bik, Bim, Bmf, Puma and Noxa protein levels by immunoblotting.



D


U2OS- CtBP2 siRNA
Fold change in relative to siCon
4.0
3.0
2.0
1.0
0.0
Bik
Bim
Bmf
Noxa
Puma

E


siCon
siCtBP2
CtBP2
GAPDH
Bik
Puma
Noxa
Bim
Bmf
U2OS

Materials and Methods

Cell culture and transfection

HCT116 human colon cancer cells (ARF silenced) [196] with targeted deletion of p53 were grown in McCoy's 5A medium. U2OS (human osteosarcoma) and H1299 (human lung cancer) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100µg of streptomycin and incubated in humidified 5% CO2 at 37°C. Mammalian expression plasmids were transfected using Fugene [111], and siRNA duplexes were transfected with Oligofectamine (Invitrogen), with a siRNA concentration of 40 nM. After retro/ lentiviral infections, cells were selected using puromycin at 2µg/ml.

Plasmids, siRNAs, shRNA and viral expression vectors

V5-tagged CtBP2 expression plasmid pcDNA-V5-CtBP2 has been described [68]. PcDNA-T7ARF was generated by insertion of a PCR amplified ARF coding sequence with a T7 tag sequence embedded in the 5' primer into pCDNA3. The expression plasmid containing the CtBP2-binding defective allele of (L50D) of ARF was generated from PcDNA-T7ARF using PCR as per the Quikchange protocol (Stratagene). pBabe-Puro ARF and pBabe-ARF^{L50D} were used to generate retroviruses. pLenti-ShGFP, pLenti-ShBik, pLenti-Puro-hARF and pLenti-Puro-hARF^{L50D} were generated using Gateway cloning System® (Invitrogen). Retro/lentiviruses were produced in HEK293T cells by transfecting ARF constructs along with packaging constructs (pol/gag and VSVG). siRNA sequence for human CtBP2 was AAGCGCCUUGGUCAGUAAUAG. shBKLF:

CTGGTCGATATGGATAAACTCA; shBik1: GGAGAAATGTCTGAAGTAA; shBik2: ACACTTAAGGAGAACATAA.

Immunoblotting

Cells were lysed in lysis buffer (20 mM HEPES pH 7.4, 0.5% Triton X-100, 2 mM $MgCl_2$, 10µM $ZnCl_2$, 2 mM NEM, 1 mM PMSF, 240mM NaCl) containing protease inhibitor tablets [111]. Antibodies used were as follows: CtBP2 (BD Biosciences), hARF (Novus), T7 tag (Novagen), GAPDH (Advanced Immuno), Noxa (Imgenex), Bik, Puma, Cleaved Caspase (Cell Signaling) PARP (Santa Cruz). Anti-rabbit IgG-horseradish peroxidase and anti-mouse IgG-horseradish peroxidase conjugates (Jackson Immunoresearch) were used with ECL detection (GE Healthcare) for immunoblotting.

cDNA array analysis of apoptosis-associated genes

Total RNA was extracted from HCT116; p53-/- cells after CtBP2 knockdown or ARF infection using RNeasy (Qiagen). Biotin labeled cDNA probes were generated with 5µg of total RNA using TrueLabeling-AMP™ 2.0 kit (SuperArray) according to manufacturer's instructions. cDNA probes were purified using an ArrayGrade cDNA cleanup kit (SuperArray). Biotinylated-cDNA probes were denatured, hybridized to GEArray® Human Apoptosis microarray (OHS-012, SuperArray) as per manufacturer's instructions. After overnight incubation at 60°C, the membranes were washed successively in 2x SSC-1% SDS and 0.1x SSC-0.5 % SDS for 15 min each. The arrays were developed using chemiluminescence detection kit (SuperArray) and the acquired

images were analyzed by using GE Array Expression Analysis Suite 1.1. The basic raw data was normalized for empty spot and housekeeping genes (GAPDH and β-actin).

Real-time quantitative PCR

mRNA transcripts for human Bik, Puma, Noxa and GAPDH were analyzed by real-time quantitative reverse transcription-PCR (RQ-PCR) using SYBR green (Applied Biosystems) and an ABI 7300 (Applied Biosystems). Relative amounts of the mRNA transcripts were calculated using the $\Delta\Delta C_T$ method with GAPDH and b-actin mRNA as internal references. The primer sets used were Bik (sense**:** TCCTATGGCTCTG CAATTGTCA, antisense: GGCAGGAGTGAATGGCTCTTC), Bim (sense: GCCCC TACCTCCCTACAGAC, antisense: ACTGTCGTATGGAAGCCATTG), Bmf (sense: CCACCAGCCAGGAAGACAAAG, antisense: TGCTCCCCAATGGGCAAGACT), Noxa (Sense: CTGCAGGACTGTTCGTGTTCA, antisense: GGAACCTCAGCCTCCA ACTG), Puma (sense: GGGCCCAGACTGTGAATCC, antisense: CGTCGCTCTCTCT AAACCTATGC), B-Actin (Sense: GCTCCTCCTGAGCGCAAGT, antisense: TCGTCA TACTCCTGCTTGCTGAT).RT-PCR for BKLF was done using the following BKLF primers: sense BKLF 5'AGGTGGCTCAATGCAGGTAT3'; antisense BKLF 5'CATGG GCAGAGACTGCACTA3'. GAPDH primers were sense 5'ATCACCATCTTCCAGGA GCGA-3'; antisense 5'-GCCAGTGAGCTTCCCGTTCA3'.

Apoptosis and cell viability Assays

For apoptosis analysis, cells were trypsinized after 48 hrs of transfection, washed with cold PBS, stained with Annexin V-PE and 7-AAD according to the manufacturer's instructions (BD Biosciences, USA), and subjected to FACS analysis. For viability analysis, cells were trypsinized, mixed with trypan blue solution and counted in the hemocytometer.

Bik promoter-luciferase reporter assay

A 1.9 kb region of human Bik promoter (−1710 to +203) was amplified by PCR from pBLCAT2 [181] and inserted into the firefly luciferase reporter pGL3 (Promega). U2OS cells were transfected with pGL3-Bik and a control plasmid expressing the *Renilla* luciferase (pRL-TK), CtBP2, ARF, and BKLF using Fugene [111]. The expression of reporter genes was determined by Dual Luciferase assay (Promega) after 36 hours of transfection.

Chromatin immunoprecipitation assay (ChIP)

Cells were plated for 24 hrs and approximately 10^8 cells were used for each ChIP assay. Cells were washed once in PBS and were treated with 1% formaldehyde in cold PBS for 10 min at 4°C with continuous shaking. Glycine (final concentration of 125mM) was added to quench the formaldehyde for 5 min at 4°C with continuous shaking. Cells were then harvested and washed twice with ice-cold PBS. Nuclei were isolated by incubating the cells in nucleus isolation buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0. 5%

NP40) for 20 to 30 min on ice. The nuclei were harvested at 4°C by centrifuging the cell suspension at 7,000g for 5 min and resuspended in 2 ml of RIPA buffer (150 mM NaCl, 1% v/v Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8. 0, 5 mM EDTA) containing protease inhibitors. Chromatin was fragmented to approximately 200 to 700 bp by sonication. Nuclear debris was removed by centrifuging the lysates at 4°C for 15 min at 14,000 rpm. The lysate was precleared by incubation with the protein G Sepharose beads for 30 min at 4°C. Immunoprecipitation was performed overnight at 4°C with the respective antibody. Protein G Sepharose beads were added and the immunocomplexes were allowed to bind to the beads for 2 h at 4°C. The beads were washed once each with RIPA buffer, RIPA buffer with 500 mM NaCl, immunoprecipitation wash buffer (10 mM Tris HCl pH 8.0, 250 mM LiCl, 0. 5% NP40, 0. 5% Sodium deoxycholate, 1 mM EDTA) and finally with Tris-EDTA. Beads were resuspended in 200 µl of elution buffer (50 mM Tris HCl, pH 8.0, 10 mM EDTA, 1% SDS) with Proteinase K and incubated overnight at 55°C. DNA was extracted by phenol-chloroform, precipitated in the presence of glycogen by ethanol, allowed to air dry, and dissolved in TE buffer pH 8.0. Immunoprecipitated DNA was diluted tenfold to keep the PCR in the linear range of amplification. The following set of primers was used to amplify different regions of the genes indicated: For the promoter region (-551bp to -693bp) of *Bik* where BKLF binding sites are present PS2; 5'TATACCAG GGCTGGAGTTAGGTCC3'and 5'-CTCACGTGCAGACCTGGTGAG A-3; primers set near distal BKLF binding site PS1 (-1504 to -1647); sense 5'-CTGCTAATGTTTACTG AACATCTC-3' and antisense 5'- AAATTGAGACAGGGTGGTAAAG-3' Non specific

primers (-9.5 kb to -9.3 kb) upstream of BKLF binding sites 5'CCTAAGAAGCTGGCC ACAGCTC3' and 5' CCATCATGTTGGCCAGAATGGTC TC3'; E-Cadherin primers 5'TAGCCTGGCGTGGTGGTGTGCACCTG3' and 5'GTGCGTGGCTGCAGCCAGG TGAGCC3' [111].

Acknowledgements

The authors wish to thank Dr. Seema Paliwal, who has contributed to ChIP assays, Sandhya Pande, who did the initial apoptosis array, Drs. T. Kowalik for high-titer Ad-hARF and Ad-lacZ, Eric Campeau for lentiviral shRNA vectors, and G. Chinnadurai for pBLCAT2 reporter construct. SRG was supported by an American Cancer Society Research Scholar Award and the Worcester Foundation Grant for Biomedical Research.

Chapter-IV

Functional analysis of $p19^{ArfL46D}$ knock-in mouse primary cells

Introduction

The $p16^{INK4a}$ and $p19^{Arf}$ tumor suppressor proteins encoded by the *INK4a/ARF* locus [10] exert their function in distinct anti-cancer pathways: $p16^{INK4a}$ regulates RB, and $p19^{Arf}$ regulates p53. The $p16^{INK4a}$ transcript is encoded from exons 1α, 2 and 3, whereas $p19^{Arf}$ is encoded by exons 1β, 2 and 3. Although both genes utilize common exons 2 and 3, they are controlled by distinct promoters, and exon 2 is transcribed in an alternate reading frame in $p19^{Arf}$. Therefore, $p16^{Ink4a}$ and $p19^{Arf}$ are completely divergent in protein sequence, and by inference, function. Ectopic expression of $p19^{Arf}$ in cells lacking $p16^{INK4a}$ induced a G_0/G_1 arrest, indicating $p19^{Arf}$ functions independently of $p16^{INK4a}$ [10]. Expression of $p19^{Arf}$ is upregulated upon hyperproliferative signals from Ras, Myc, E1A and E2F, and through the inhibition of Mdm2, results in either p53-dependent apoptosis or cell cycle arrest [12, 13, 14]. ARF can also suppress the proliferation of cells defective for p53 by antagonizing c-myc and E2F proteins [31, 56]. The increased incidence of epithelial tumors in Arf-null mice compared to p53-null mice, and the broader tumor spectrum observed in triple-null (p53/Arf/Mdm2-null) mice compared to that observed with loss of any one of these genes, has provided genetic evidence in the mouse to support the existence of an ARF tumor suppressor activity that is independent of p53.

ARF is generally not detectably expressed in normal cells or during mouse embryogenesis, and genetic targeting and disruption of the Arf gene has no effect on development [23, 38]. $p19^{Arf}$, $p16^{INK4a}$, p53, and p21 are induced with increasing passage in primary mouse embryonic fibroblasts (MEFs) in culture, and their accumulation inversely correlates with the cell proliferation rate [12]. Arf-null MEFs are immortal and can be transformed by oncogenic ras without a requirement for collaborating oncogenes such as adenovirus E1A or myc [23]. Arf-null MEFs display an increased rate of cell proliferation and exhibit reduced basal levels of $p21^{Cip1}$, a p53-regulated gene and a potent senescent cell-derived growth inhibitor [23, 197]. Paradoxically, they express higher levels of $p16^{INK4a}$ than their wildtype counterparts. Thus, ARF appears to exert its effects on growth control in fibroblasts mainly upstream of p53.

The idea that ARF might have tumor suppressor functions that are independent of p53 and Mdm2 has led to the identification of abundance of ARF interacting proteins [10, 50, 68]. ARF is a very unusual protein with more than 20% arginine residues, and likely unstructured when not complexed (Straza and Grossman unpublished), and it thus may interact promiscuously with several proteins. Thus, the challenge is how to validate *bona fide* ARF interacting proteins and distinguish them from non-specific candidates. ARF expression is induced by oncogenic stress, by ablation of p53, or by the non-physiological conditions of tissue culture in primary MEFS. Therefore, the molecular mechanism that governs ARF expression and its functional effects in cell culture may differ from its behavior *in vivo.* Thus, the development of mouse models is imperative to validate and understand the functional significance of ARF's interactions.

The transcriptional corepressor CtBP2 has now been identified and validated in cell based assays as a putative target of the p53-independent tumor suppression function of ARF. ARF interacted with and induced the proteasome-mediated degradation of CtBP, leading to the induction of p53-independent apoptosis [68]. A conserved hydrophobic region within Arf (residues 32-51 in mouse Arf) was identified as critical for CtBP interaction. A point mutation within this domain (L46D in mouse; L50D in human) abrogated CtBP interaction and the induction of apoptosis. Both ARF-induction of CtBP degradation and p53-independent apoptosis was dependent on its ability to interact with CtBP. Thus, CtBP proteins represent putative targets of ARF's p53-independent tumor suppressor activity.

The robust test for the function and physiologic relevance of the ARF/CtBP interaction is to develop a mouse model where the interaction is abrogated by mutation of either ARF or CtBP. Given that *Arf* is a single gene and the minimal region required for ARF/CtBP interaction has been mapped, while CtBP is a gene family with two highly conserved members, ARF would appear to be the logical gene to target in a mouse model designed to investigate function of the ARF/CtBP interaction. The $p19^{ArfL46D}$ mutation is defective in cell culture for CtBP-interaction and degradation, but retains properties of nucleolar localization, MDM2 binding, and p53 activation. Thus, the L46D mutation might be an ideal candidate mutation for generating a knock-in mouse. However, the possibility that other proteins may interact with this region of ARF and the functional consequences cannot be ruled out in this genetic model.

To investigate the physiologic function of ARF/CtBP interaction in tumor suppression *in vivo*, a $p19^{ArfL46D}$ knock-in mouse was generated by incorporating the point mutation into exon1β of *INK4a/ARF* locus. Analysis of $p19^{ArfL46D}$ (heterogzygote and homozygote) primary mouse embryonic fibroblasts (MEFs) revealed that the L46D point mutation in $p19^{Arf}$ increased the proliferation rate of MEFs, providing evidence in primary cells that the abrogation of p53-independent tumor suppressor activity of Arf leads to uncontrolled cell growth. However, L46D homozygous MEFs proliferate slower than Arf-null MEFs confirming the robustness of the residual p53-dependent effects of ARF. Although L46D (heterozygote/homozygote) MEFs grew with an increased proliferation rate, they failed to undergo spontaneous immortalization, suggesting that the p53-dependent functions of ARF remains intact in Arf L46D cells. Further, L46D homozygous and L46/- hemizygous MEFs demonstrated increased migration rates compared to their wildtype counter parts but, migrated slower than Arf-null MEFs consistent with contributions from both the CtBP and p53 pathways to this ARF function.

Results

Generation of $p19^{ArfL46D}$ knock-in mice

To investigate the specific contribution of the Arf/CtBP2 interaction to Arf tumor suppressor function *in vivo*, the L46D point mutation, which is defective for CtBP-interaction and degradation in cell culture, was introduced in to the mouse germline. To generate a $p19^{ArfL46D}$ targeting construct, a 1.0 kb genomic region containing exon1β was

amplified and the L46D point mutation with an *Mse* I unique restriction site was introduced into exon1β using PCR primers (Figure 4.1) and Quickchange protocol (Stratagene). 3.0 kb of 5' and 3' intronic genomic sequence flanking exon1β was PCR amplified and cloned 5' and 3' to the mutated exon1β. The targeting construct also contained a loxP flanked pGK-neo neomycin drug resistance marker to facilitate positive selection (Figure 4.1). The $p19^{ArfL46D}$ knock-in targeting construct was electroporated into AB2.2 embryonic stem (ES) cells, and underwent positive selection (G418). Four hundred ES cell clones were screened for homologous recombination of the target allele using Southern blot analysis. *EcoRV* digested genomic DNA, and a 3' flanking probe was used to identify the four clones that correctly displayed both the wildtype 30 kb and the mutant 17.3 kb bands (Figure 4.2). Further analysis with a 5' flanking probe demonstrated that those four ES cell clones correctly underwent homologous recombination by yielding 30 kb and 12.7 kb bands (Figure 4.2).

Three correctly targeted ES cell clones were injected into blastocysts, and subsequently transferred into foster mothers, resulting in four high-degree chimeras (2 each of 60% and 90%). Crossing the 90% chimeras to C57BL/6 females gave rise to *agouti* F1 progeny, and Southern blot (Figure 4.3A) and PCR/restriction enzyme *MseI* digestion (Figure 4.3B) analysis demonstrated germline transmission of the $p19^{ArfL46D\text{-}flox^{neo}}$ mutant allele.

$p19^{ArfL46D\text{-}flox\text{-}neo}$ heterozygous mice were viable, fertile, and appeared phenotypically normal. In order to excise the neo cassette from the heterozygous L46D allele, $p19^{ArfL46D\text{-}neo}$ mice were bred to protamine-Cre (PC3-Cre) transgenic mice [198].

$p19^{ArfL46D/+}$ heterozygous mice were generated from this cross, and heterozygous mice were intercrossed to obtain $p19^{ArfL46D/L46D}$ homozygous mice with the neo cassette excised. Excision of the neo cassette was confirmed by PCR using neo cassette flanking primers that yielded a larger fragment than wildtype in excised mice due to the residual loxP and polylinker sequence left behind. The wildtype allele yielded 107 bp fragment whereas, the homozygous L46D yielded a 210 bp fragment, and heterozygotes yielded both of these fragments (Figure 4.3C). The presence of the mutant allele was also confirmed by sequencing a PCR amplified fragment of exon1β. Genotyping of 50 pups from a heterozygous mating demonstrated that 14 were wild type, 24 were L46D/+, and 12 were L46D/L46D, consistent with the expected Mendelian ratios. L46D heterozygous and homozygous mice were indistinguishable from their littermates phenotypically. Western blot analysis of $p19^{Arf}$ protein harvested from mouse embryonic fibroblasts generated for each genotype revealed that the homozygous knock-in mutant retained the expression of $p19^{Arf}$ protein similar to that observed in wildtype littermate MEFs (Figure 4.4B).

Regulation of cell proliferation by the L46D point mutation

Mouse embryonic fibroblasts were explanted from wildtype, L46D heterozygous, L46D homozygous and Arf-null embryos to compare the proliferation rates of cells of different genotype. Two independent lines from each genotype were grown in 6 cm plates, and the cells counted every 24 hours for a 8 day period. Analysis of the growth rate of these MEFs revealed that the L46D homozygous MEFs proliferated significantly

faster than wildtype MEFs, but not as fast as Arf-null MEFs (Figure 4.4A). The L46D homozygote MEFs' proliferation rate was similar to that of $p19^{Arf}$+/- MEFs (data not shown) suggesting that the L46D mutation may not affect p53-dependent functions, which was consistent with the effect of L46D on p53-dependent ARF functions in cell based assays (Figure 2.2).

Arf-null MEFs grew with a higher proliferation rate with decreased $p21^{Cip1}$expression as reported previously [23] (Figure 4.4B). To determine the expression pattern of CtBP2, p16INK4a, $p19^{Arf}$, p21Cip1 and p53, the whole cell lysate harvested from first 4 passages was used from wildtype, Arf+/-, Arf-null, L46D/- hemizygous, L46D heterozygous, and L46D homozygous MEFs. There was no significant difference in CtBP2 protein levels in Arf-null and L46D MEFs, may be due to the requirement for an additional UV stress for efficient degradation of CtBP2 [68]. Further, there was no significant difference in the expression levels of p53, $p16^{INK4a}$, and $p21^{Cip1}$ in L46D homozygous, heterozygous and hemizygous MEFs, confirming that L46D does not affect p53-dependent functions (Figure 4.4B). These findings also suggest that p53-independent and CtBP2 dependent pathways might both regulate cell growth. However, to completely rule out the possibility of a contribution of p53 to the L46D phenotype, homozygote L46D/p53-null MEFs might be compared directly to L46D homozygote MEFs. This effect on cell growth may be completely due to cell proliferation, as MEFs does not easily undergo apoptosis under normal cell culture conditions. However, further analysis including BrdU incorporation and cell cycle analysis should be performed to completely rule out the contribution of apoptosis.

Immortalization of p19^{ArfL46D} MEFs

Spontaneous immortalization is an extremely rare event in human cells, however rodent cells spontaneously immortalize at much greater rates. Cellular immortalization of MEFs has been achieved by the inactivation of the p53 or Rb pathways [199, 200]. To determine the contribution of the ARF/CtBP interaction to cell immortalization, we carried out a 3T9 (modified 3T3) cell immortalization assay. MEFs harvested from wildtype, L46D/+, L46D homozygous, L46D/- hemizygous, Arf+/-, Arf-null embryos were cultured continuously as per the standard protocol [201]. As previously reported, Arf-null MEFs demonstrated a rapid rate of cell growth throughout the entire assay relative to wildtype MEFs [22] (Figure 4.4). L46D homozygous MEFs grew with a slower proliferation rate than Arf-null cells, but faster than wildtype MEFs, and they did finally enter senescence after the 15th passage. Thus, unlike Arf-null MEFs, which are phenotypically similar to p53-null MEFs in immortalization assays [202], Arf$^{L46D/L46D}$ MEFs showed high rate of proliferation initially but did not ultimately immortalize. Interestingly, L46D/- hemizygous cells continued to grow at much faster rate than L46D or wildtype MEFs and undergone spontaneous immortalization (Figure 4.4A), suggesting that there is crosstalk and cooperation between p53-dependent and p53-indpendent pathways controlled by ARF.

Loss of CtBP interaction with ARF results in increased cell migration in MEFs

To study the effect of ARF/CtBP interaction on cell migration in MEFs, a transwell cell migration assay was performed using MEFs harvested from wildtype, Arf-

null, L46D/- (hemizygous), L46D heterozygous, and homozygous embryos. It has been shown that CtBP2 promotes cell migration and invasion, and hARF effectively inhibits cancer cell migration by targeting CtBP2 for proteasome-mediated degradation [68, 109]. Consistent with the previous report [203], Arf-null MEFs migrated significantly faster than wildtype and other genotype MEFs used in this assay (Figure 4.6). The MEFs explanted from embryos carrying an L46D mutation demonstrated an increased migration rate compared to littermate wildtype MEFs, but slower than that of Arf-null MEFs (Figure 4.6). Interestingly, L46D/- hemizygous MEFs showed an increased migration rate compared to that of wildtype or L46D homozygote MEFs (Figure 4.6). These data suggest that the CtBP-binding domain of ARF contributes to ARF's ability to regulate cell migration.

Discussion

p53-null and Arf-null mice develop highly penetrant lymphomas and sarcomas with mean latencies for survival of 20 and 32 weeks respectively [25, 152]. In mice lacking p53, T-cell lymphomas are most common, with the remainder being sarcomas [27]. In contrast, Arf-null mice primarily develop sarcomas with a lower incidence of lymphomas, as well as a low incidence of carcinomas and gliomas [23, 152]. The increased tumor latency in Arf-null mice might allow the shift in tumor spectrum and emergence of tumor types such as carcinomas and gliomas, which were not normally seen in p53-null mice. Further, the incidence of carcinomas was increased in Arf/p53 double-null and Arf/p53/Mdm2 triple-null animals suggesting the existence of Mdm2- and p53-

independent tumor suppressor functions for ARF. However, the physiologic mechanisms of p53-independent tumor suppressor functions remain obscure. CtBP2 has been identified as a target for the p53-independent functions of ARF. ARF inhibits CtBP2 by inducing its degradation, sequestering it in the nucleolus, and CtBP's functional inhibition results in the induction of apoptosis and a reduction in cancer cell migration [68, 109].

In this study, we have generated the $p19^{ArfL46D}$ knock-in mice and primary mouse embryonic fibroblasts to study the role of ARF/CtBP interaction in cellular growth control to migration in a native physiologic setting. Primary MEFs were used to study the effects of L46D mutation on the regulation of cell survival and cell migration. MEFs explanted from L46D homozygote embryos showed an increased rate of cell proliferation. However, L46D MEFs failed to undergo spontaneous cell immortalization, though they continued to grow at a higher proliferation rate into late passage, than wildtype littermate MEFs, suggesting that L46D mutation may contribute to cell proliferation but not induce spontaneous immortalization. Further, L46D MEFs migrated faster than wildtype and more interestingly, L46D/- (hemizygous) MEFs migrated significantly faster than L46D homozygous MEFs, suggesting p53-independent functions of Arf also contribute to the regulation of cell migration.

Mouse embryonic fibroblasts from Arf-null embryos proliferated at significantly faster rates than their wildtype counterparts as previously reported [23]. L46D MEFs showed a higher proliferation rate than wildtype MEFs but slower than Arf-null MEFs. More interestingly, L46D/- hemizygous MEFs proliferated at rates similar to that of

Arf+/- MEFs and they continued to grow for more passages than L46D homozygous MEFs, suggesting that negative growth regulation by the p53-independent functions of ARF is not as robust as p53-dependent growth regulation. The increased proliferation rate of Arf-null MEFs was linked to a reduction in the expression of $p21^{Cip1}$, a p53 responsive gene, though the Arf-null MEFs displayed increased levels of $p16^{INK4a}$. In contrast, there was no significant difference in $p16^{INK4a}$ or $p21^{Cip1}$ expression levels in L46D MEFs, compared to their wildtype counterparts. However, the contribution of other pro-survival ARF/CtBP2 transcriptional targets such as $p15^{INK4a}$ or other uncharacterized targets to the growth effects of L46D mutation cannot be ruled out. Although L46D MEFs continued to grew with a higher proliferation rate than wildtype MEFs, they did not undergo spontaneous immortalization, which is a p53-dependent function, suggesting that the L46D mutation does not greatly affect the p53-dependent function of Arf in mouse primary cells as has been previously reported in cancer cell lines [68]. Interestingly, L46D/- hemizygous MEFS showed some inclination to undergo spontaneous immortalization compared to L46D/L46D MEFs, implying that both p53-dependent and p53-independent functions of Arf may exert additive effects on spontaneous immortalization. To rule out completely the possibility that L46D may contribute to the regulation of cell growth through p53, MEFs should generate from L46D/p53-null and compared to the single homozygotes background.

$p19^{Arf}$ has been shown to regulate cell migration via p53-dependent and p53-independent pathways [108, 109, 203]. Studies from our lab demonstrated that CtBP2 promotes cancer cell migration under hypoxic conditions and ARF inhibits CtBP2-mediated cancer

cell migration by targeting CtBP2 for degradation [68, 109]. Genetic disruption of Arf in MEFs enhances cell migration as shown previously [203]. Abrogation of Arf/CtBP2 interaction in MEFs (L46D) also increased cell migration significantly compared to wildtype MEFs but not as robustly as Arf deletion. L46D/- hemizygous MEFs demonstrated faster cell migration than L46D homozygous MEFs, confirming the contribution of both p53-dependent and p53-independent functions of Arf towards regulation of cell migration.

The data from our study demonstrated that p53-independent tumor suppressor function of ARF contribute to the inhibition of cell growth in primary mouse cells. Furthermore, abrogation of Arf/CtBP2 interaction correlated with increased cell migration. Further work will be needed to better understand and characterize the role of $p15^{INK4a}$ or PI3 kinase pathway components or uncharacterized transcriptional targets in ARF/CtBP regulation of cell proliferation, migration, apoptosis and tumorigenesis. Although analysis of primary mouse cells revealed the expected cellular phenotypes, the tumor spectrum, size, and latency of tumor formation and survival of L46D mice in comparison to Arf-null, p53-null, and wild type, would reveal the functional significance of ARF/CtBP interaction at an organismal level.

Figures

Figure 4.1 **Targeting strategy for the p19^{ArfL46D} knock-in mouse.**
Schematic of the *INK4b-ARF-INK4a* locus and the *p19$^{Arf\ L46D}$* knock-in construct. A 1.0 kb fragment containing exon 1β and cloned into pBluescript-II and L46D point mutation and Mse I restriction site was introduced as per mutagenesis protocol (Stratagene). Approximately 3.0 kb of 5' and 3' homologous sequence was amplified by PCR and cloned sequentially into pBluescript-ArfL46D construct. Finally, the neo cassette was inserted into the construct.

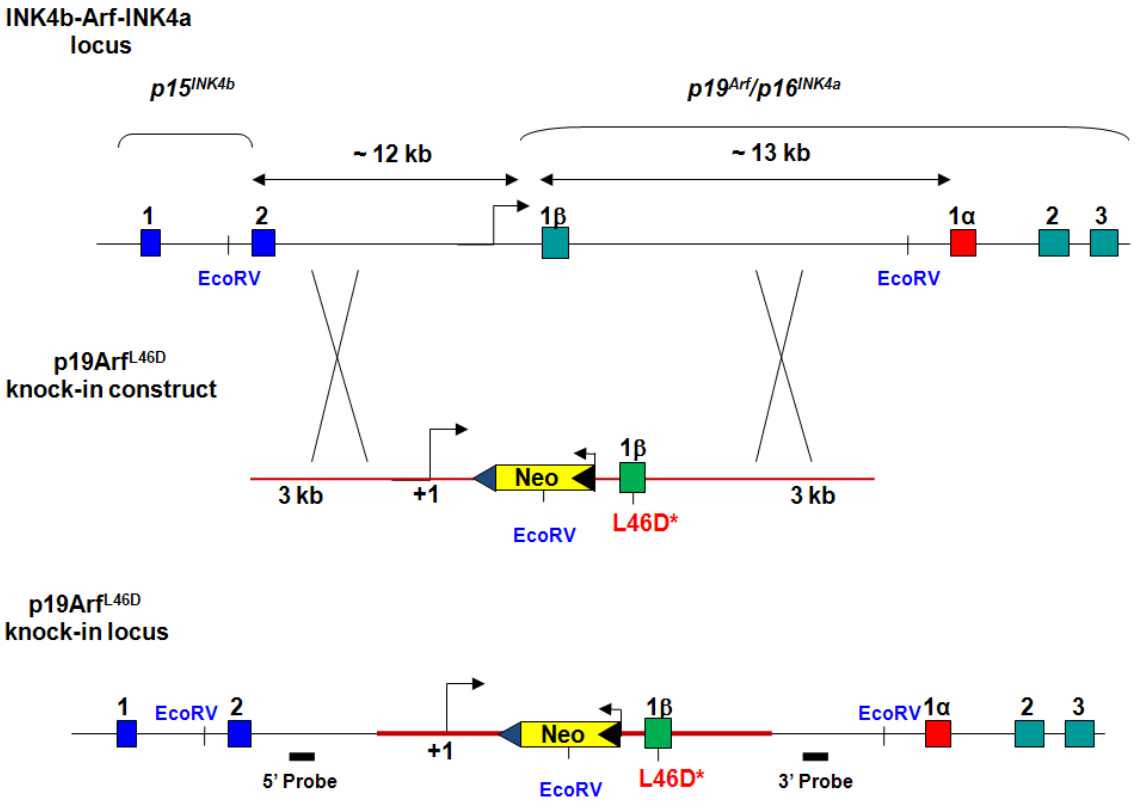


Figure 4.2 **Screening of targeted p19^{ArfL46D} ES cells.**
The DNA isolated from targeted ES cells was digested overnight with EcoRV and Southern blot analysis was performed. Probing with the 5' flanking probe yields a 30 kb band corresponding to the wildtype allele, and due to an additional EcoRV site in the neo cassette, the L46D mutant yield 17.3 kb band. Similarly, probing with a 3' flanking probe yields a 30 kb wildtype band and a 12.7 kb band for the mutant allele.

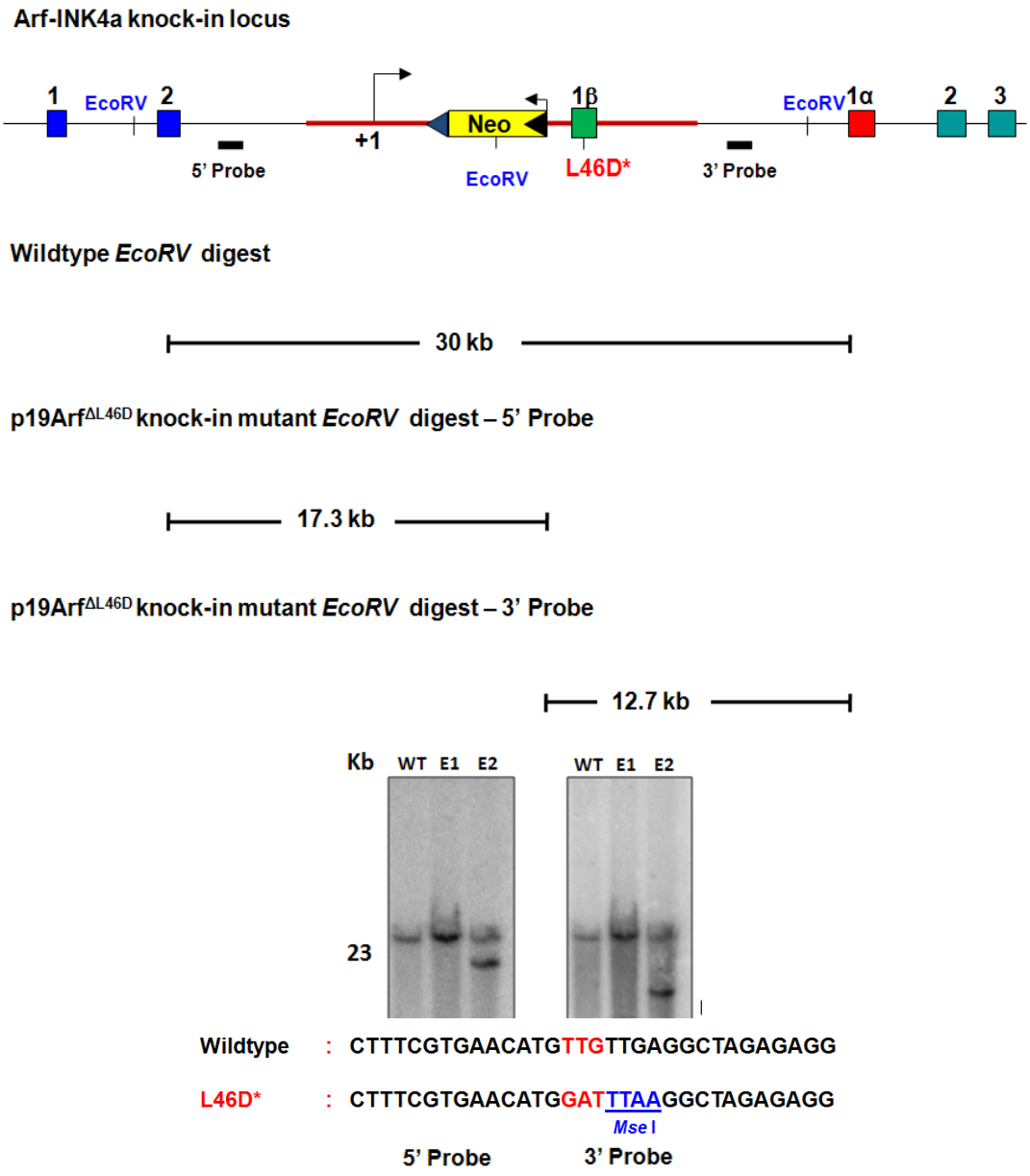
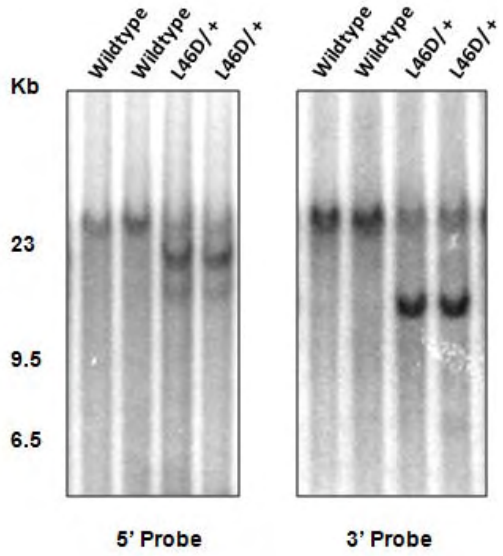
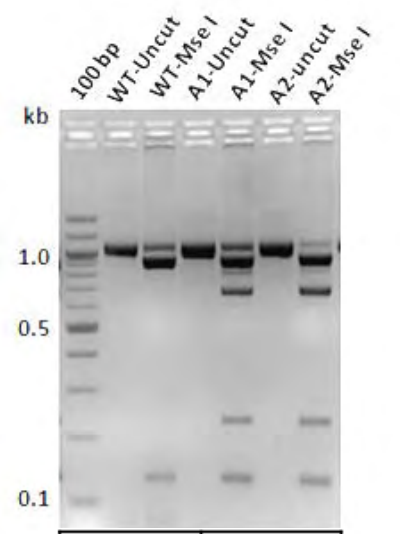


Figure 4.3 **Genotyping of the p19^{ArfL46D} knock-in mouse. A.** Tail DNA isolated from wildtype, L46D heterozygous and homozygous knock-in mice was digested with EcoRV and southern blot analysis was performed. Probing with the 5' flanking probe yields a 30 kb band corresponding to a wildtype allele, and due to an additional EcoRV site in the neo cassette, the mutant yields 17.3 kb band. Similarly, probing with a 3' flanking probe yielded a 30 kb band for the wildtype and a 12.7 kb band for the mutant allele. **B.** A 1.0 kb fragment containing Exon1β was PCR amplified and digested with Mse I restriction enzyme. Wildtype DNA yielded 910 bp and 120 bp bands whereas DNA from a heterozygous pup yielded 680 bp, 230 bp and 120 bp bands. A1 and A2 are L46D heterozygous mice. **C.** PCR to confirm neo cassette excision: DNA isolated from pups generated by crossing PC3-Cre and L46D-neo was used in PCR using neo cassette flanking primers. Wildtype yielded the expected 107 bp band; whereas L46D homozygous yielded a 120 bp band and heterozygotes yielded both the expected bands.





Wildtype	L46D/+
910 bp	
	680 bp
	230 bp
120 bp	120 bp

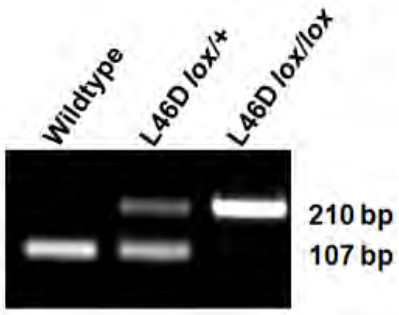
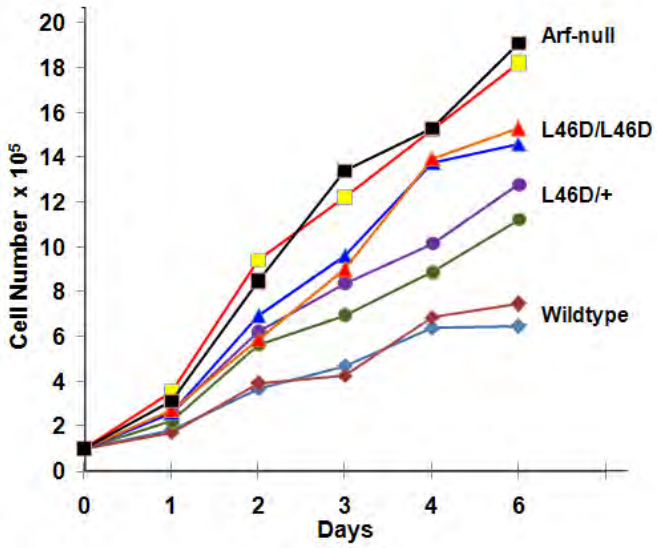


Figure 4.4 **Regulation of cell proliferative by p19^{ArfL46D}**

A. Two independent mouse embryonic fibroblasts (MEF) cell lines each of the Arf-null, L46D homozygous, L46D heterozygous, littermate wildtype of Arf-null (indicated by asterisk) and L46D littermate wildtype genotypes were plated in triplicate in 6 cm plates and the proliferation rate was analyzed by counting cells over 6 days. Each data point represents average of triplicate plates and three counts in each plate with p-value between Arf-null/WT p=0.001 and L46D/WT, p=0.001. **B.** MEFs isolated from the indicated genotypes were harvested up to four passages and immunoblotted with the indicated a bodies.

A



B

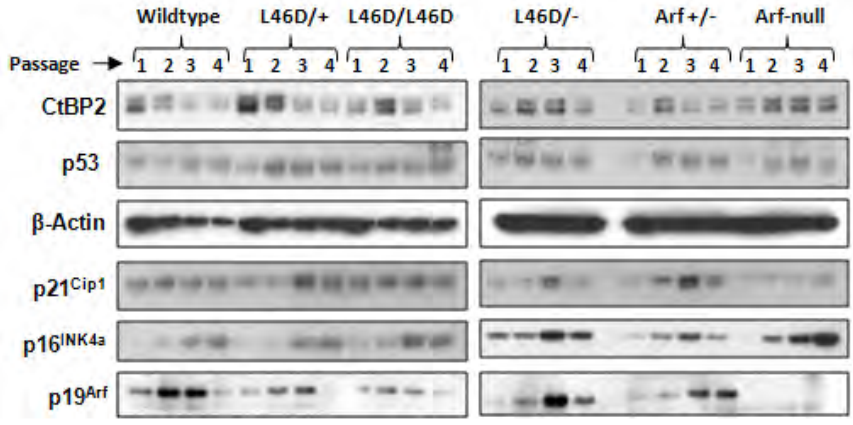


Figure 4.5 **Immortalization of p19^{ArfL46D} cells.**

Cell Immortalization (3T9) assay. At 3-day intervals, the total number of cells per two independent lines of MEFs from each genotype were determined prior to repassage of the cells at $3x10^6$ cells per 10cm plates. Each data point represents the average of number of cells from triplicates of two independent lines for each genotype. Wildtype littermate MEFs from the Arf-null and L46D backgrounds (two lines) were included in the assay as controls.

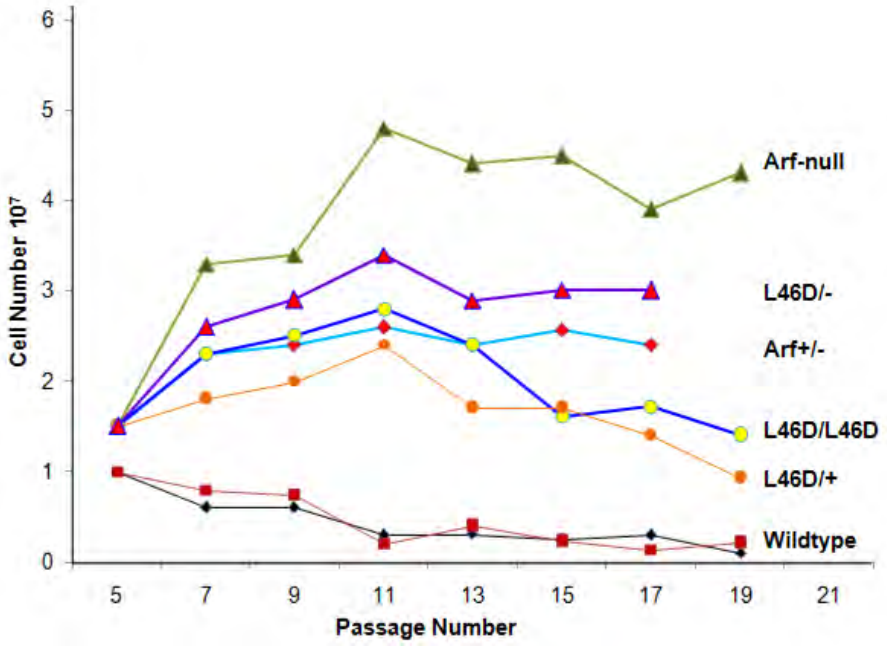
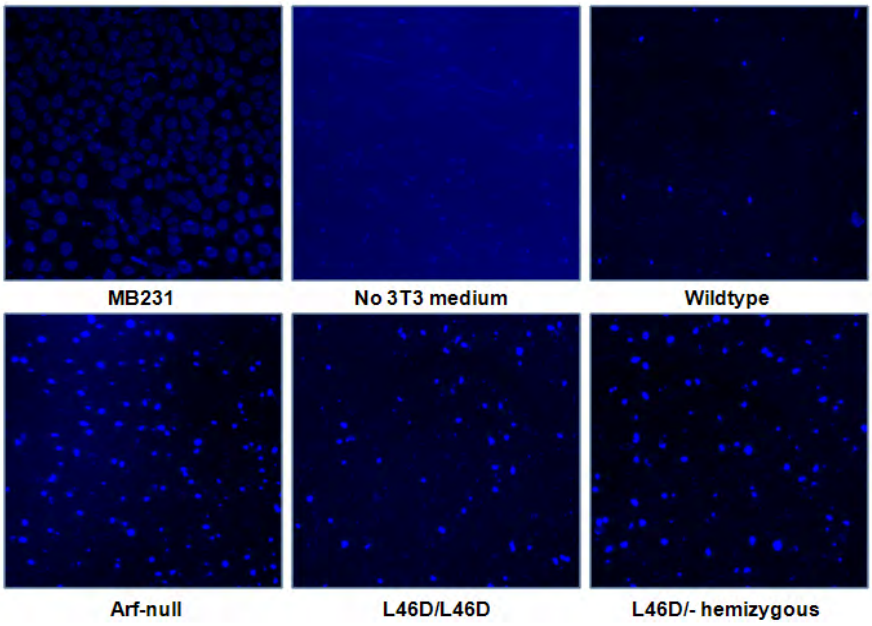


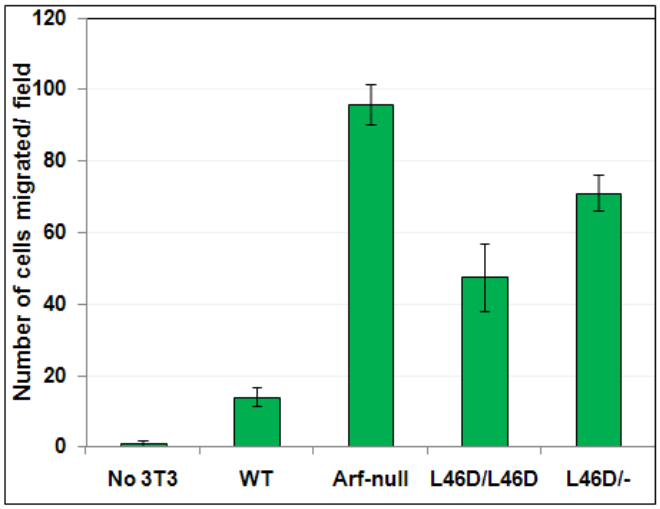
Figure 4.6 **Transwell cell migration assay**

MEFs explanted from embryos with wildtype, Arf-null, L46D homozygous and L46D/- hemizygous genotypes were used in a transwell cell migration assay. 3T3 conditioned medium was used as chemo attractant and the transwells were coated with collagen. A representative field for each genotype is shown **(A)**. The number of cells migrated/field was an average of the number of migrated cells counted from five microscopic fields after 1 hour of incubation at 37 ^{o}C from duplicate experiments and error bars indicate +/- 1 S.D **(B)**.

A



B



Materials and Methods

Generation of $p19^{ArfL46D}$ knock-in targeting construct

A 1.0 kb genomic fragment containing exon1β *Xho I-Sal I* was generated by PCR and cloned into pBluescript II KS (+) vector. The L46D point mutation and a restriction enzyme *Mse* I site was introduced using Quickchange protocol for mutagenesis (Stratagene). A 2.3 kb *Kpn I-Xho I* genomic fragment of 5' homology and a 2.4 kb *Sal I-Bgl II* genomic fragment 3' homology respectively were generated by PCR and then added into pBluescript-exon1β^{L46D}. To enable positive selection, PGK neomycin cassette was inserted upstream of exon 1β. The entire construct was sequenced using several primers to confirm the presence of L46D mutation and other region of genomic locus to enable homologous recombination.

Generation of $p19^{ArfL46D}$ knock-in ES cells and chimeras

The $p19^{ArfL46D}$ knock-in construct was linearized at a unique *Not* I site, gel purified and electroporated into AB2.3 embryonic stem (ES) cells. After positive selection with G418, clones were picked and screened by Southern blot analysis. ES cell DNA was isolated and digested with *EcoR*V overnight, electrophoresed on 1 % agarose gel, transferred on to Hybond® nitrocellulose membrane and probed with 5' flanking probe. Putative positive clones additionally screened with the 3' flanking probe. The L46D point mutation was confirmed by PCR and restriction digestion with *Mse I*. Correctly targeted ES cell clones were microinjected into C57BL/6 blastocysts and

implanted into pseudo pregnant foster mice using standard protocol. Chimeric offsprings are identified by presence of agouti coat color and the highest degree chimeras (> 70 %) were mated to C57BL/6 females to assay for germline transmission of the $p19^{ArfL46D}$ mutant allele.

Mice

In order to remove neomycin resistance cassette, the $p19^{ArfL46D}$ heterozygous female mice with neo cassette intact were crossed to PC3-Cre 129/C57 male mice. Excision of the neo cassette was confirmed using flanking PCR primers M1 (5'-AGATGGGCGTGGAGCAAAGATG-3') and M6 (5'-CTCACTGTGACAAGCGAGGT GAG). All mice were maintained and used, in accordance with federal guidelines and those established by Institution Animal Care and Use Committee (IACUC) at University of Massachusetts Medical School. Mice were monitored regularly for signs of any abnormalities or disease.

Cell Culture

Mouse embryonic fibroblasts (MEFs) were generated from D13.5-15 day embryos as described previously [204]. All studies were conducted using low passage MEFs (passage 2-4), maintained in a 5% CO2 incubator at 37 ^{0}C in a Dulbecco's modified Eagles medium (DMEM) supplemented with 10 % fetal bovine serum, penicillin and streptomycin.

Cell Proliferation Assay

In order to determine the rates of cell proliferation, two lines of wildtype, Arf-null, Arf-heterozygous, ArfL46D hemizygous, L46D heterozygous and L46D homozygous MEFs were seeded at $1x10^5$ cells per well in a 6well plate. Cells were harvested and counted every 24 hours using Z1 Coulter Particle Counter (Beckman Couture, FL). Cell proliferation assay was repeated twice in triplicates for each genotype and average number of cells was plotted in line diagram.

Immortalization Assay (3T9 Assay)

To determine the rate of spontaneous immortalization of MEFs, a 3T9 assay was carried out as described [201]. Briefly, $3x10^6$ cells from wildtype, L46D heterozygous and L46D homozygous MEFs were plated in complete DMEM on 10 cm plates every three days. Two separate lines of MEFs for each genotype were maintained in total of 3 plates with $3x10^6$ cells ($9x10^6$ cells). Cells were trypsinized prior to counting and plated at a density of $3x10^6$ cells per 10 cm plate every three days.

Cell Migration Assay

Cell migration assays were performed as previously described [205]. Briefly, 6.5 mm Transwell chambers (8µm pore size; Costar, Cambridge, MA) were coated on the underside with 25µg/ml type I collagen for 2hrs at 37^oC or overnight at 4^oC. Cells from

two lines for each genotypes were trypsinized, washed in serum-free medium and resuspended in DMEM medium with 0.25% heat inactivated BSA at a concentration of 10^6/ml. 10^5 cells were transferred to the top chamber of the transwell and 3T3 conditional medium was added to the lower chamber. Migration was allowed to proceed for 1 hour at 37 ^oC. Non-migrating cells were removed from the top surfaces of the Transwell membranes using cotton swabs. The membranes were then fixed in methanol and stained with DAPI. Average number of cells migrated was calculated by counting cells in five microscopic fields in triplicate Transwells for each lines.

Acknowledgements

We would like to thank Drs. Stephen Jones, Director of UMass transgenic core for all the help towards generation of $p19^{ArfL46D}$ knock-in mouse and generously providing PC3-Cre transgenic mice and p53-null mice, Michelle Kelliher and her lab members for letting me use radioactivity workstation and for other helpful discussions, Charles Sherr and NCI mouse repository for generously providing Arf-null mice.

Chapter-V

Final thoughts and Future Directions

Cancer cells acquire a common set of properties such as unlimited proliferation, resistance to apoptosis, and self-sufficiency, through a multistep, mutagenic process. Cancer cells gain these phenotypes by reactivating and modifying several cellular programs that are normally active during development. Cancer evolves through random mutations and epigenetic changes in oncogenes and tumor suppressors that alter cellular processes such as cell proliferation, migration, apoptosis, and differentiation, and selects those cells that can tolerate and survive under deleterious circumstances. In addition to frequent mutations in a number of oncogenes and tumor suppressors, there are large numbers of low frequency changes that contribute to tumorigenesis. Not surprisingly, individual mutations in as many as 20% of all kinases can play an important role during oncogenesis [206], although it remains to be elucidated, whether mutations in other gene classes will also enhance tumor formation as much as that of oncogenes or tumor suppressors. The CtBP family proteins are targets of tumor suppressor proteins for inhibition, and thus, may qualify as candidates for deregulation during tumorigenesis.

The ARF tumor suppressor gene is inactivated in a large number of human cancers. Several lines of evidence from murine and human systems suggest that ARF exerts tumor suppressor functions apart from p53. Most interesting and important is that

tumor progression is enhanced in the absence of ARF, due to an apoptotic defect in a tumor surveillance system that is completely independent of p53. Thus, I sought to identify the mediators of p53-independent functions of ARF. From my dissertation research, we have demonstrated that ARF/CtBP2 interaction explains many of the p53-independent effects of ARF. The ARF tumor suppressor protein appears to destabilize CtBP, allowing cancer cell to evade apoptosis. Indeed, CtBP levels are elevated in majority of human colorectal adenocarcinomas, and inversely correlated with ARF expression. Furthermore, we identified BH3-only genes such as Bik, Bim, and Bmf, as novel targets and the critical mediators of ARF-induced, CtBP2-mediated p53-independent apoptosis in colon cancer cells (Figure 5.1). Studies from our lab also revealed that CtBP2 promotes cancer cell migration by transcriptionally regulating PTEN phosphotase, and in turn, activating the PI3-kinase pathway. Introduction of ARF can therefore effectively inhibits CtBP2-mediated cell migration by abrogating CtBP2-mediated regulation of the PI3-kinase pathway [109]. Lastly, we have identified, and generated a knock-in mouse model with, a functional point mutation in $p19^{Arf}$ (L46D; Leucine to Aspartic acid) that is defective for CtBP2-binding, degradation, and induction of apoptosis. The cells harvested from knock-in mice revealed a cell growth and cell migration phenotype. Taken together, these findings suggest that CtBPs are novel downstream targets for p53-independent tumor suppression by ARF.

The molecular mechanism by which ARF affects the functions of its interacting proteins remains unclear, though the functional consequence is invariably inactivation

[148]. Indeed, ARF interacted with CtBP2 through a novel, conserved hydrophobic domain (37-51 residues in $p19^{Arf}$) in exon 1β and induced proteasome-mediated degradation of CtBP2, leading to apoptosis in cells lacking p53 [68]. No known functions of ARF have been assigned to this conserved hydrophobic region of ARF. The point mutation in this region of ARF (L46D in $p19^{Arf}$ and L50D in $p14^{ARF}$) both physically and functionally abrogated ARF/CtBP2 interaction. Though ARF inactivated CtBP2 functionally by altering its metabolic stability, the molecular mechanism by which ARF induced CtBP degradation remains unclear. Moreover, the point mutation in this conserved region of ARF did not affect p53-dependent functions of ARF suggesting that a novel function of ARF was inactivated by the L46D/L50D mutations.

CtBP family proteins are modulators of several essential cellular processes including cell proliferation, cell migration and apoptosis [116]. CtBP protein levels are tightly regulated by the ubiquitin-proteasome system. CtBP1 becomes ubiquitinated upon UV irradiation and this is dependent on phosphorylation of S422 by HipK2 kinase [69]. Studies from our lab showed that CtBP2 appeared to be constitutively ubiquitinated, and its ubiquitination status was unaffected by ARF expression [68]. Our preliminary studies on the mechanism of CtBP2 degradation demonstrated that phosphorylation at S428 (site for the stress sensitive kinase HipK2) was required for both UV-induced and ARF-induced degradation. The mutation that abolishes phosphorylation at this site rendered CtBP resistant to ARF-induced or UV-induced degradation. However, phosphorylation at serine 428 was not required for ARF and CtBP2 interaction, suggesting that

phosphorylation may play an important role in CtBP2 degradation in steps beyond ARF interaction. That step could be a delivery of ubiquitinated substrates to the proteasome and CtBP2 phosphorylation at S428 residue may aid in this process. The possibility that the S428 phosphorylation site may be targeted by other kinases that might regulate CtBP degradation under conditions other than UV-induced stress would be worth exploring. The effects of a phosphorylation mimic mutant of CtBP2 (S428D) on ARF-induced CtBP2 degradation would be worth considering for future studies. Further, the S428A mutant may serve as an ideal candidate for generation of knock-in mouse model with a CtBP2 mutation, which disrupts ARF/CtBP2 interaction at a functional level. Compared to p19Arf, p14ARF had a robust destabilizing effect on CtBP without any additional stress such as UV treatment. Thus, the minor amino acid sequence difference between human and mouse ARF might contribute to the difference in their ability to destabilize CtBP2. Further analysis using deletion mutants or chimeric ARF proteins would be required to address these functional differences between human and mouse ARF.

ARF-induced CtBP2 degradation depends on a functional 26S proteasome, but does not involve ARF-dependent modulation of CtBP ubiquitination [68]. CtBP1, but not CtBP2 is sumoylated, as CtBP2 lacks the SUMO conjugation consensus site, suggesting that SUMO modification is unlikely to play a role in ARF-induced CtBP2 degradation. Thus, the promotion of CtBP degradation in the absence of alteration in ubiquitination by ARF raises the important question of whether ARF plays the role of a ‘proteasome adaptor’. A previous report [167], along with our data, suggested that ARF interacts with the

S6a (TBP-1) ATPase subunit of the 19S proteasome regulatory particle (Appendix-I). Further, ARF and S6a coeluted with the 26S proteasome complex, but not with the 20S core particle of the proteasome assembly (Appendix-I). This finding implies that ARF, like other proteasome adaptor proteins such as hPLIC and Rad 23, may serve as a 'receptor' for the proteasome that recognizes ubiquitinated substrates that are ready for degradation [168]. Thus, it will be critical to determine whether CtBP2 and other ARF targets, such as E2F or NPM, interact with the proteasome in an ARF-dependent manner. Even though ARF interacts with a proteasome subunit, and coeluted with the proteasome regulatory assembly, it may still interact with the proteasome indirectly through a third protein, such as another proteasome adaptor or receptor. Our preliminary data with interaction studies using GST-S6a and p14ARF revealed that S6a and ARF interact directly. However, direct interaction of ARF with the proteasome should be determined using purified proteasomes and ARF. Ultimately, the biochemical link between ARF, CtBP, and the proteasome that results in CtBP degradation can be established by attempting *in vitro* proteasome degradation of CtBP2 with reconstitution of the reaction components. The potentially novel function of ARF as a 'proteasome adaptor' would explain its ability to inactivate its interacting proteins mainly by destabilizing them.

ARF expression in cells lacking p53 enhanced CtBP2 degradation resulting in induction of apoptosis. CtBP depletion using specific siRNA was alone sufficient to induce apoptosis, suggesting that ARF acts upstream of CtBP in this pathway. Moreover, repletion of CtBP in ARF expressing cells rescued cells from ARF-induced apoptosis,

confirming a direct role for CtBP in the apoptosis pathway activated by ARF in the absence of p53 [68]. CtBP2 functions as an antiapoptotic protein by transcriptionally repressing proapoptotic genes [113]. Inactivation of CtBP2 by ARF may result in abrogation of this transcriptional regulation, and in turn, induction of apoptosis. The CtBP2 target genes may therefore function as the mediators of ARF-induced p53-independent apoptosis.

CtBP-null mouse embryonic fibroblasts (MEFs) are hypersensitive to apoptosis, and microarray analysis of these cells revealed that there was significant upregulation of proapoptotic genes including the BH3-only gene Noxa [70]. We have identified BH3-only genes Bik in colon cancer cells and Bik, Bim, Bmf in osteosarcoma cells, as critical mediators of ARF-induced p53-independent apoptosis. Bik is a proapoptotic protein, which triggers apoptosis in breast, lung, prostate, colon adenocarcinoma, glioma, and melanoma derived cell lines. Consistent with its role in tumor suppression, the Bik locus (22p13.3) is commonly deleted in human colorectal and breast cancers [187]. Bik mutations have been identified in renal cell carcinoma [188]. By contrast, in non-malignant cells, Bik function may overlap with that of other BH3-only proteins, as Bik is not essential for normal development in mouse [143]. ARF overexpression, or depletion of CtBP2, induced mRNA and protein expression of BH3-only genes. The induction of BH3-only genes also required ARF/CtBP2 physical interaction, and the induction of efficient apoptosis by UV and CtBP2 depletion required physiologic levels of Bik. CtBP2 regulation BH3-only genes expression required the cooperation of the DNA-binding transcription factor Basic

Kruppel-like Factor (BKLF). However, simultaneous depletion of Bik and CtBP2 using RNAi, only partially rescued the apoptotic phenotype in colon cancer cells, suggesting that there might be more mediators of apoptosis after CtBP2 depletion, including other BH3-only genes that might be involved in this pathway in a cell-specific and context-dependent manner. Moreover, the involvement of other KLF transcription factors (EKLF: erythroid Kruppel-like factor, KLF2: Lung Kruppel-like factor, GKLF: gun-enriched Kruppel-like factor, UKLF: ubiquitous Kruppel-like factor) in CtBP2 recruitment to the target genes promoters cannot be ruled out. Many of the KLF factors are known to function as transcriptional repressors in a tissue-dependent and context-dependent manner [193].

ARF is transcriptionally silenced in a variety of human carcinomas including colorectal adenocarcinomas. ARF's target CtBP2 interacts with a wide range of transcription factors involved in developmental processes and tumorigenesis, such as AML1/Evi-1, MLL (mixed lineage leukemia) and AML1/FOG2 fusion proteins. CtBP, in turn, contributes to leukemiogenesis by cooperating with these oncogenic fusion proteins to promote abnormal hematopoietic growth and differentiation [95, 207, 208]. The data reported in this dissertation are relevant to understanding the development and progression of, at least, human colorectal cancer. There is an inverse correlation between ARF and CtBP2 expression levels in human primary colorectal adenocarcinomas, with the majority of tumors displaying elevated levels of CtBP2 with no detectable ARF. This supports the idea that CtBP may function as a cellular proto-oncogene. However, analysis

of a larger sample size, and other tumor types, including melanoma, breast cancer, and tumors of nervous system, would yield valuable information about the contribution of the ARF/CtBP2 interaction to tumorigenesis. Most of these tumors may select for ARF silencing, in part, to inactivate its ability to degrade and inactivate CtBP proteins.

CtBP proteins are uniquely sensitive to cellular metabolic conditions due to their intrinsic dehydrogenase activity and ability to bind NAD^+/NADH. Given that ARF is activated under conditions of oncogenic stress which frequently induce reactive oxygen species [209], the dehydrogenase domain may serve as a further sensor to determine whether the conditions are correct for ARF to induce CtBP degradation or not. Thus, a combination of both ARF expression and altered metabolic state might be required to induce CtBP degradation, apoptosis, and inhibition of migration/invasion (Figure 5.1). Indeed, studies from our lab suggest that the NADH-binding region of CtBP2 is required for the promotion of cancer cell migration under hypoxic conditions. CtBP has been linked to the hypoxic activation of cell migration, due to its transcriptional repression of other non-apoptotic pathway gene such as PTEN [70, 109]. Further, intact interaction of ARF/CtBP2 was essential for the inhibition of cell migration by ARF. Since hypoxia is fundamentally linked to malignant tumor progression, CtBP may serve as a critical oncogenic link by which hypoxia leads to activation of key malignant features such as enhanced cell survival, increased motility and invasion [108, 109, 194]. The promotion of hypoxic cell migration by ARF inactivation may also serve as a potent selective force for the epigenetic silencing of ARF expression early in tumorigenesis. Indeed, analysis of a

p53/Arf-double null mouse hepatocellular cancer model suggests that Arf can block both migration and invasion *in vitro* in a p53-indepenendent and CtBP2-dependent manner. ARF loss or mutation should specifically enhance cell survival in hypoxia by release of CtBP from any negative control and this then, may also explain why ARF can also so profoundly impact tumor progression *in vivo* with its loss promoting increased tumor aggressiveness [55, 108, 195].

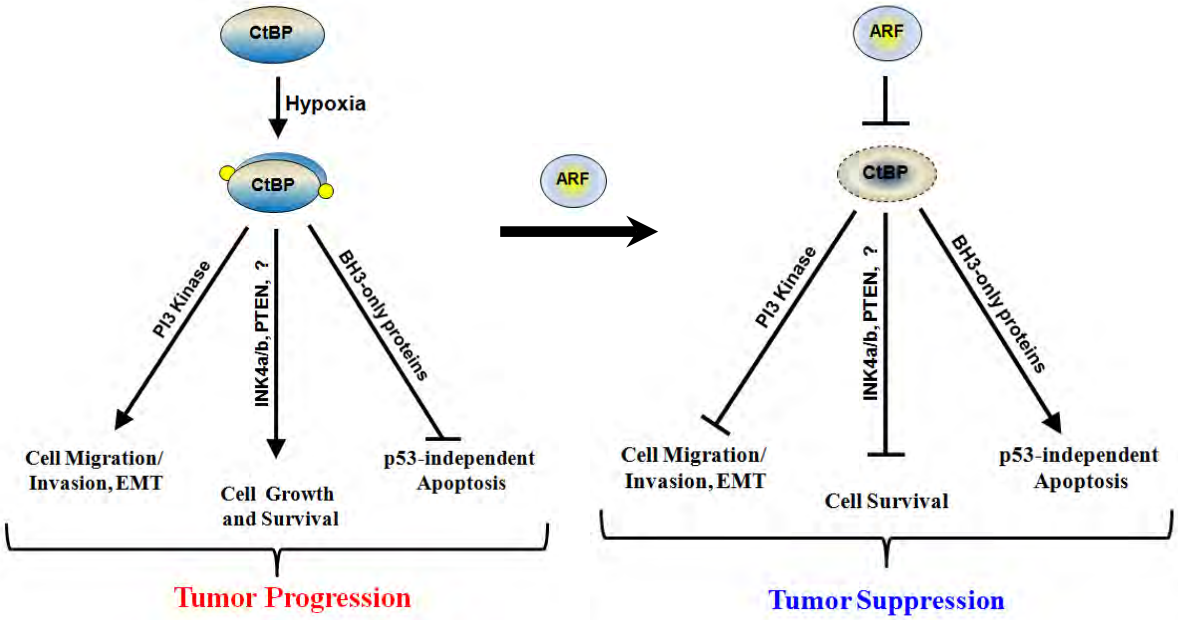
Evasion of apoptosis by tumor cells enables them survive in an adverse microenvironment, and may powerfully cooperate with invasion/migration mechanisms to promote metastasis by allowing survival through the various steps of metastatic process [210]. Loss of ARF has been linked to tumor invasiveness and metastasis in a mouse skin cancer model. Studies from our lab have shown that CtBP2 promotes the migration of cancer cells, and ARF can effectively inhibit CtBP2-mediated cancer cell migration [109]. The mechanism by which CtBP regulates this function reflects the wide variety of genes regulated by them. Transcriptional regulation of the phosphatase PTEN by CtBPs seems to play an important role by activating the PI3-Kinase pathway and in turn, inducing cancer cell migration [109]. Thus, selective pressure for ARF silencing or deletion in human epithelial cancers could due to p53-independent tumor cell apoptosis through CtBP degradation could also lead to the augmentation of the process of invasion and metastasis. Further understanding of role of CtBP in cell migration, invasion and EMT might also enable the development of targeted therapeutics.

p53-null and Arf-null mice develop highly penetrant lymphomas and sarcomas with mean latencies of 20 and 32 weeks respectively [25, 152]. Analysis of Arf/p53-double null and Arf/Mdm2/p53-triple null mice revealed that Arf functions as tumor suppressor independent of p53 and Mdm2, especially during epithelial tumorigenesis, as there was an increased incidence of carcinomas in these animals. In an attempt to understand the biological significance of ARF/CtBP2 interaction *in vivo,* we have generated p19Arf^{L46D} knock-in mice. Primary mouse embryonic fibroblasts (MEFs) were used to study the effects of abrogation of Arf/CtBP2 interaction on cell survival and migration. Homozygous L46D MEFs displayed an unexpected increased proliferation rate, proliferating at rates lower than Arf-null MEFs but higher than wildtype MEFs. The mechanism for this effect on proliferation rate is unclear as the increased proliferation rate of Arf-null MEFs is due to a reduction in the expression of p21^{Cip1}, a p53 target gene, while, there was no significant difference in p16^{INK4a} and p21^{Cip1} in L46D MEFs. However, the contribution of other pro-survival CtBP2 targets such as p15^{INK4b} or other uncharacterized targets to the growth advantage of L46D MEFs cannot be ruled out.

Although L46D MEFs continued grew at a higher proliferation rate than wildtype MEFs, they failed to undergo spontaneous immortalization, suggesting that the L46D mutation may not greatly affect the p53-dependent functions of ARF, such as spontaneous immortalization, in mouse primary embryonic fibroblasts. Interestingly, L46D/- hemizygous MEFs showed an increased tendency to undergo spontaneous immortalization compared to L46D/L46D homozygous MEFs. This clearly suggest that

Figure 5.1 **Model for ARF regulation of CtBP2 functions**

Hypoxic conditions in cells enhance CtBP dimerization, NADH binding and an efficient transcriptional repression of target genes such as PTEN, INK4a, BH3-only genes contributing to tumor progression. When cancer cells retain ARF expression, ARF induces CtBP degradation, and reverses the CtBP-mediated repression of target genes resulting in robust tumor suppression.



This clearly suggest that both p53-dependent and p53-independent functions of Arf may exert additive effects to cause spontaneous immortalization. To rule out completely the possibility that the L46D mutation in p19Arf may contribute to the regulation of cell growth through p53, MEFs should be generated from L46D/p53-null mice, and compared their cell proliferation rate to that of each single homozygote background. Further, the tumor spectrum in L46D/p53-null mice would be worth investigating to determine the possibility of cross talk between the p53-depenedent and p53-independent functions of ARF.

Unlike human ARF, $p19^{Arf}$-induced CtBP2 degradation required an additional UV stress in mouse cells. There was no significant difference in the CtBP2 protein levels in L46D homozygote cells or Arf-null MEFs compared to that of wildtype. It is quite possible that these cells will require additional UV stress to undergo ARF-induced CtBP degradation to appreciate any changes in CtBP2 protein levels. Since, no known functions of ARF have been assigned to the CtBP-binding hydrophobic region of ARF, the possibility of other proteins interacting with this domain and their contributions to the observed phenotype in L46D cells cannot be ruled out completely. To address this hypothesis, generation of knock-in mice with mutation in CtBPs that disrupt their interaction with ARF, should be considered for future studies. One such candidate mutation in CtBP2 is the HipK2 phosphorylation site mutation S428A, which functionally abrogates ARF/CtBP2 degradation.

Genetic disruption of Arf in MEFs enhances cell migration as shown previously [203]. MEFs isolated from $p19^{ArfL46D}$ embryos showed increased cell migration compared to wildtype MEFs but not as robust as Arf-null MEFs. More interestingly, loss of one complete allele of Arf and L46D mutation in another allele (L46D/- hemizygous mice) demonstrated an even more robust migratory phenotype than that of L46D MEFs, confirming the contribution of both p53-dependent and p53-independent functions of Arf towards regulation of cell migration [109].

Arf-null mice and $p19^{ArfL46D}$ knock-in mice are viable. Unlike Arf-null mice, L46D mice may not be susceptible to the typical and highly penetrant lymphomas and sarcomas as seen in Arf-null or Ink4a/Arf-null mice. These tumors arise due to a defect in p53-dependent apoptosis in tumor cells of these lineages [16]. Quite possibly, L46D homozygote mice will develop more carcinomas with much delayed latency than Arf-null mice, as the L46D allele retains p53-dependent functions. In Arf-null mice, early onset of sarcomas and lymphomas is believed to mask susceptibility to more latent carcinomas, that are more frequently seen in Arf/p53 double-null or Arf/p53/Mdm2 triple-null mice [31]. Thus, it will be critical to breed Arf^{L46D} homozygous mice to a p53-null background to see if the tumor spectrum seen in p53-null animals is widened to the same degree to include more epithelial tumors as seen in Arf/p53 double-null animals.

The tumor spectrum of L46D/- hemizygous mice and p53-null background would enable us to understand the contribution of the CtBP-binding hydrophobic region of

ARF, to p53-indpendent tumor suppressor function of Arf. If no additional tumors developed in the L46D and L46D/p53-null background, consideration should be given to induce epithelial tumors in specific tissues of these mice with mutagens such as DMBA for skin tumors, azoxymethane for intestinal tumors, or cross into tissue specific oncogene tumor models including melanoma, breast cancer or pancreatic cancer models [211]. However, the initial focus should be given to address the role of ARF/CtBP2 interaction in intestinal tumors, as this interaction was characterized in human colon cancer cells and human colorectal adenocarcinomas tissue samples [68, 109]. To study the impact of the abrogation of ARF/CtBP interaction on the development of intestinal tumors, L46D mice should be crossed with *min* intestinal adenoma-prone mice followed by the comparison of tumor latency, number, size of tumors and survival of mice between the wildtype and L46D background.

In conclusion, the data presented in this dissertation contributes to our understanding of a novel molecular mechanism governing the p53-independent tumor suppressor functions of ARF. In the past, p53-dependent pathways were exploited to develop effective therapeutics to treat cancer. However, p53 is mutated or inactivated in more than 50% of human cancers. Thus, it is important to study and understand p53-independent tumor suppressor pathways. The role of CtBP in modulating oncogenic outcomes through activation of migration/invasion/epithelial-mesenchymal transition and suppression of apoptosis raises the possibility that CtBPs may be useful anti-neoplastic drug targets. Further understanding of the cellular consequences of ARF/CtBP2

interaction will lead to increased ability in manipulating this pathway for therapeutic benefit in tumors that lack normal p53 function, which constitute the majority of human solid tumors.

Appendix

This appendix contains research work and publications to which I have contributed, but that are not included in the main part of my dissertation

Appendix-I

ARF may function as proteasome adapter via the S6a proteasome

UV treatment of mouse cells resulted in proteasome dependent degradation of CtBP2 only when ARF was present, suggesting that UV-mediated degradation of CtBP required an intact ARF/CtBP interaction. Interestingly, there was no significant change in levels of ubiquitination of CtBP2 upon ARF overexpression (Figure A1.1A). ARF also induces sumoylation of its interacting proteins [157], CtBP1 has been shown to get sumoylated at S428 [212]. However, CtBP2 lacks the CtBP1 sumoylation consensus sequence (Fig A1.1B), thus, excluding the possibility of sumoylation playing role in ARF-induced degradation of CtBP2. ARF has been described as an interaction partner of the S6a subunit of the 19s proteasome [167]. To determine whether ARF might act as an adaptor between ubiquitinated CtBP and the proteasome, the interaction of S6a and ARF was confirmed by CoIP [167]. Lysates of U2OS cells expressing T7-hARF and cDNA for either the V5-tagged S5a or S6a subunit of the 19s proteasome, were immunoprecipitated with normal IgG, V5 or T7 antibodies. As reported previously [167], robust interaction between ARF and S6a was noted, as both proteins were found in the V5 and T7 IPs, but neither was seen in the control IPs (Figure A1.1C). Interestingly, another subunit of the 19s regulatory particle, S5a, did not coIP with ARF when it was used as bait, although it was observed in the T7-ARF IP (Figure A1.1C) with lower stoichiometric relation than

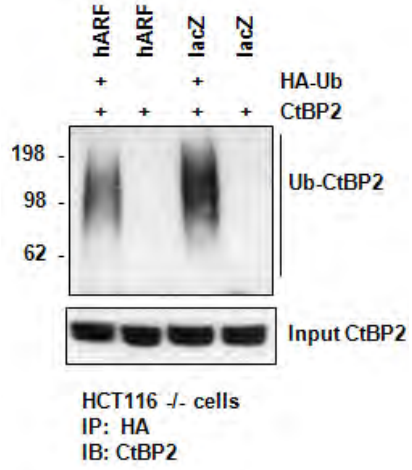
S6a, suggesting that the ARF-S5a interaction was weaker and indirect, possibly as part of the larger 19s proteasome assembly being in complex with ARF.

To pursue the idea that ARF may serve as a ‘receptor’ or proteasome adaptor for CtBP, its association with the proteasome complex has investigated. Lysates with intact proteasomes prepared from H1299 human lung cancer cells, which express endogenous ARF, were subjected to gel filtration chromatography. The fractions were analyzed for coelution of the S6a subunit, ARF and 20s proteasome subunits using anti-ARF, S6a and proteasome core subunit specific antibodies. Consistent with the *in vitro* binding assays (with the free forms S6a and ARF), both S6a and ARF coeluted with the 26s proteasome (Figure A1.1D). However, ARF did not coelute with the 20s proteasome suggesting that ARF interacts with only the 19s regulatory assembly that recruits it to the 26s complex. This preliminary evidence suggest that ARF may recruit ubiquitinated substrates such as CtBP, to the 26s proteasome for degradation through its interaction with S6a.

Figure A1.1 ARF may function as proteasome adapter via S6a subunit of proteasome.

A. *Effect of ARF on CtBP2 ubiquitination***.** Human colon cancer HCT116; p53-/- cells were transfected with CtBP2 and HA-ubiquitin, and after 16 hours of transfection cells were transduced with Ad-lacZ or Ad-ARF. Whole cell lysates were harvested and immunoprecipitated with anti-HA, followed by CtBP2 immunoblot. Ub-CtBP2 indicates the migration pattern of ubiquitinated CtBP2. **B.** CtBP1 has been reported to be sumoylated at S428; however CtBP2 lacks this sumoylation consensus sequence. **C.** *ARF interacts with the S6a subunit of the 19s proteasome regulatory assembly***.** Human osteosarcoma, U2OS cells were cotransfected with T7-ARF expression construct along with either S5a-V5 or S6a-V5 expression plasmids. Whole cell lysates harvested after 36 hours of transfection were immunoprecipitated with the control IgG, anti-V5 or anti-T7 antibodies and followed by immunoblotting with anti-V5 or –T7 antibodies. **D.** *ARF and the S6a subunit of the 19s proteasome coelute in gel filtration chromatography*. Whole cell lysates were harvested from H1299 human lung carcinoma cells using low salt lysis buffer and applied to a Superose 6 Column after pre-purification over Heparin Sepharose column.

A



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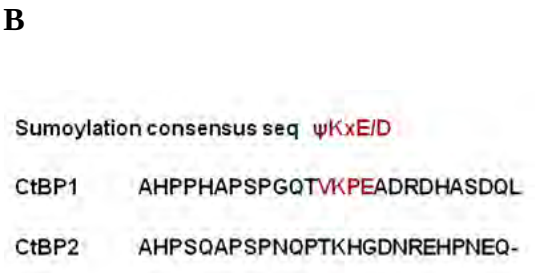

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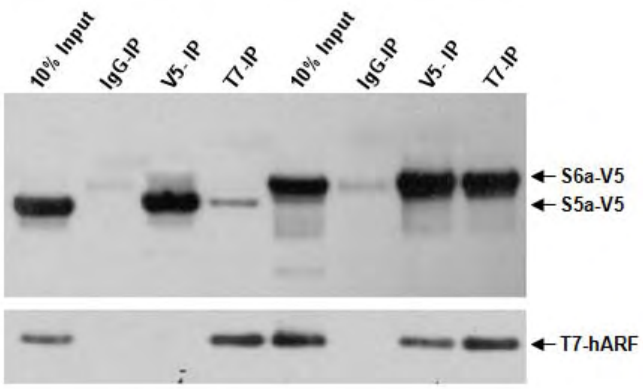
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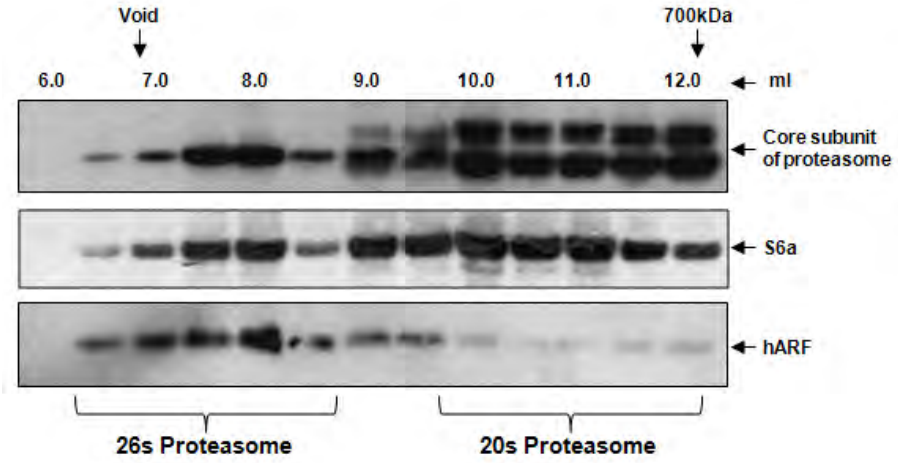
Void
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Core subunit of proteasome
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26s Proteasome
20s Proteasome

Appendix-II

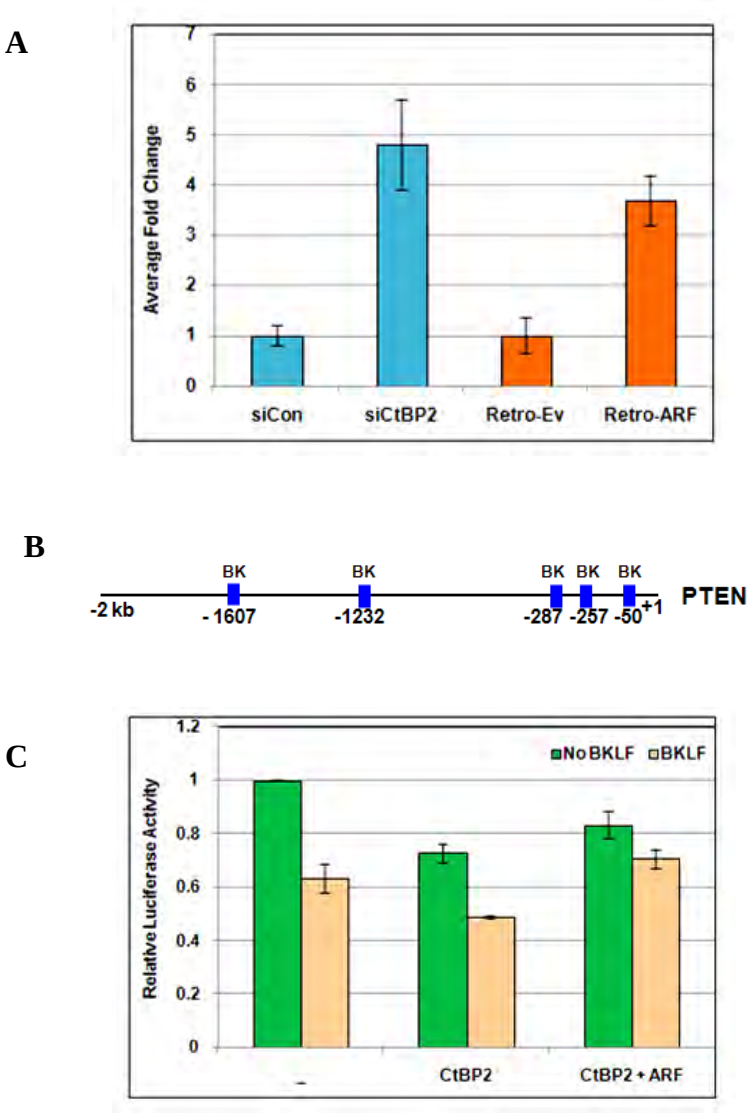
ARF/CtBP2 transcriptionally regulates PTEN tumor suppressor

CtBPs have been directly implicated in pro-oncogenic functions such as survival, hypoxia induced migration, and in turn, epithelial-mesenchymal transition [70, 105, 111]. Studies from our lab reported that CtBPs activated upon metabolic stress, such as, hypoxia, repress epithelial and proapoptotic genes and can promote hypoxia-induced migration of cancer cells. Further, introduction of ARF during hypoxia effectively inhibited hypoxia-induced cell migration [109]. Depletion of ARF or overexpression of CtBP2 in a p53-null human colon cancer cell line led to the activation of the PI3Kinase pathway and down regulation of PTEN expression [109]. PTEN was also found in a microarray analysis of CtBP repressed genes [72]. The PTEN promoter contains five consensus-binding sites for basic Kruppel-like factor (BKLF) (Figure A2.1A). BKLF as a core repressor that can recruit CtBPs and other factors, and mediate transcriptional repression. Thus, the transcriptional regulation of PTEN expression by ARF/CtBP2, was explored. To assess the effects of CtBP2 depletion and ARF overexpression on PTEN expression quantitatively, PTEN mRNA levels were analyzed in HCT116; p53 -/- cells 24 hrs after either depletion of CtBP2 using siRNA, or ARF overexpression by retroviral infection (Figure A2.1B). Real-time PCR analysis confirmed that PTEN expression was increased upon either CtBP2 depletion (4.8-fold) or ARF overexpression (3.7 fold) in HCT116; p53-/- cells (Figure A2.1B). Similarly, the protein levels of PTEN were found

to be upregulated in CtBP2 siRNA treated cells as compared to control siRNA treated cells [109].

To determine whether the recruitment of CtBP by BKLF at the PTEN promoter could repress PTEN promoter activity, a 2.0 kb PTEN promoter luciferase reporter construct was transfected into U2OS cells with a BKLF, ARF, and CtBP2 expression vectors, as indicated. Either BKLF or CtBP2, alone, reduced the reporter activity by 40% (Figure A2.1C 1st and 3rd bar), however CtBP2/BKLF coexpressed further reduced the PTEN reporter activity by 20% (Figure A2.1C 2nd and 4th bar). Overexpression of ARF caused a reversal of CtBP2-mediated repression, which is consistent with the finding that ARF degrades CtBP2, and inhibits its repressor activity.

Figure A2.1 ARF/CtBP2 transcriptionally regulates PTEN tumor suppressor.
A. RNA prepared from HCT116; $p53^{-/-}$ cells with either CtBP2 knockdown or ARF infection was used to carry out quantitative real time PCR using PTEN and GAPDH specific primers. Graph represents GAPDH-normalized average fold change of PTEN in treated cells. Error bars represent +/- 1 standard deviation (SD) **B.** Diagram of BKLF recognition elements in the PTEN promoter. **C.** ARF antagonizes CtBP/BKLF repression of the PTEN promoter. PTEN-luciferase reporter was cotransfected with expression constructs for ARF, BKLF, or CtBP2 into U2OS cells along with a control reporter plasmid expressing *Renilla* luciferase (pRL-TK). Normalized firefly luciferase activity from three independent experiments was averaged, and error bars indicate +/- 1 SD and with the $p < 0.05$.



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