Regulation of Cellular Senescence in Human Fibroblasts

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Abstract

Regulation of Cellular Senescence in Human Fibroblasts

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Normal mammalian cells only divide a limited number of times in culture before they undergo a permanent cell cycle arrest termed cellular senescence. In addition, expression of oncogenes and exposure to oxidative stress can elicit a similar response. The pathways that regulate senescence are disrupted in all cancer cells, suggesting that senescence evolved as a mechanism of tumor supression that protects cells against potential oncogenic changes. In human fibroblasts, the proliferative lifespan is limited primarily by the length of telomeres at the ends of chromosomes. Progressive telomere shortening eventually triggers a p53-mediated DNA damage response that leads to cell cycle arrest. Although the pathway from telomere shortening to cell cycle arrest has been studied in great detail, it has remained unclear how phenotypic changes that accompany senescence are regulated. In this work, I identified a novel transcription factor, APA-1, that regulates one aspect of the functional changes in senescent fibroblasts. Studies on the regulation of APA-1 have led to the discovery that senescence-associated changes such as matrix-remodeling are regulated, at least in part, through a telomere length-independent pathway. APA-1 was originally identified as a protein that interacts with the

tumor suppressor ARF, a gene involved in senescence in murine fibroblasts. I have examined the functional consequences of this interaction and found that in some settings, ARF antagonizes the transcriptional activation function of APA-1. Finally, the study of APA-1 regulation led to the discovery that freshly isolated human fibroblasts are resistant to senescence induced by oncogenic RAS. Moreover, I found that exposure of fibroblasts to extended passaging in culture senesitizes cells to RAS-induced senescence, through telomere-independent upregulation of the cyclin dependent kinase inhibitor p16Ink4a. These findings challenge the belief that all normal cells senesce in response to RAS expression, and suggest that oxidative stress is a necessary cofactor that sensitizes cells to oncogene-induced senescence.

Table of Contents

List of Figures

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Introduction

One of the primary barriers to the development of cancer is the limited replicative potential of normal human cells, which was first observed by Hayflick in the 1960s (Hanahan and Weinberg, 2000). In his landmark experiments, Hayflick showed that normal human fibroblasts only divide a finite number of times *in vitro* before undergoing a permanent arrest termed cellular senescence (Hayflick and Moorhead, 1961). In contrast, cancer cells were found to proliferate indefinitely, and were considered immortal (Hayflick, 1997). These findings led to the idea that senescence acts as a tumor suppression mechanism and that immortalization is an essential step in the transformation of normal cells (Campisi, 2001).

Senescence has been studied primarily in cultured cells however there is also evidence that it occurs *in vivo*. Cells expressing a marker of cellular senescence, senescence-associated β-galactosidase (SA-βgal), are seen more frequently in aged tissue than young tissue (Dimri et al., 1995), and studies in mice indicate that senescence can be initiated following some types of chemotherapeutic treatment (Schmitt et al., 2002). Moreover, senescent cells can be found at sites of liver regeneration in mice that have undergone partial hepatectomy, demonstrating a connection between senescence and extensive cellular proliferation *in vivo* (Satyanarayana et al., 2003).

Senescence is characterized by an irreversible arrest in the G1 phase of the cell cycle. Senescent cells remain alive and metabolically active but, in contrast to quiescent cells, they can not be stimulated to re-enter the cell cycle. The pathways that lead to

senescence differ between cell types, however the common end result is a lack of G1 cyclin-cdk kinase activity and hypophosphorylation of the tumor suppressor protein Rb. In its hypophosphorylated state, Rb binds to the E2F transcription factors and blocks E2F-mediated transcriptional activation of genes that are required for cells to enter S phase. Consistent with a permanent cell cycle arrest, several positive-acting cell cycle genes, including Cdc2, cyclin A, cyclin E and E2F1, are downregulated in senescent cells (Campisi et al., 1996), and cell cycle inhibitory factors such as p21 and p16 are increased (Alcorta et al., 1996; Hara et al., 1996; Stein et al., 1999). In addition to being arrested in the cell cycle, senescent cells undergo cell type-specific functional changes (Campisi et al., 1996) and acquire SA-βgal activity that is thought to represent an increase in lysosomal enzymes (Kurz et al., 2000).

Passage-induced senescence, often referred to as replicative senescence, is best understood in human fibroblasts. The primary factor limiting the lifespan of human fibroblasts is telomere length. Telomeres are nucleoprotein complexes, consisting of repetitive DNA sequences and associated proteins that cap the ends of linear chromosomes and distinguish them from double strand breaks, thus preventing degradation and end-to-end fusions (de Lange, 2002). With each round of DNA replication, 50-200 base pairs are lost from each telomere and eventually this shortening leads to a disruption of telomeric structure and cell cycle arrest (Mathon and Lloyd, 2001). Telomere-induced senescence is thought to be similar to a DNA damage response, since the tumor suppressor p53 is activated and cells lacking p53 activity continue to proliferate for several population doublings past the time when control cells

undergo senescence (Bond et al., 1994; Rogan et al., 1995; Bond et al., 1999). Activation of p53 results in induction of the cyclin-dependent kinase inhibitor p21, which inhibits cyclin-cdk activity and causes Rb-mediated cell cycle arrest as described above (Figure I.1) (Hengst and Reed, 1998). In support of telomeres being the critical factor leading to senescence in human fibroblasts, expression of the catalytic subunit of the enzyme telomerase, hTert, results in the lengthening of telomeres and the immortalization of fibroblasts (Bodnar et al., 1998; Counter et al., 1998; Vaziri and Benchimol, 1998).

The lifespan of human epithelial cells is also limited by telomere shortening, however epithelial cells have an additional barrier to overcome in order to be immortalized in culture. Human foreskin keratinocytes and mammary epithelial cells arrest after relatively few population doublings due to elevated levels of the cyclin dependent kinase inhibitor p16 (Kiyono et al., 1998; Foster et al., 1998; Brenner et al., 1998). p16 belongs to the Ink4 family of cyclin dependent kinase inhibitors, which bind directly to the G1 cyclin dependent kinases Cdk4 and Cdk6 and disrupt their association with D-type cyclins, thus preventing Rb phosphorylation and S phase entry (Figure I.2) (Ruas and Peters, 1998). Silencing of the p16 promoter by methylation, or expression of the HPV E7 oncoprotein, which disrupts the Rb pathway, allows epithelial cells to bypass p16-mediated growth arrest and continue proliferating until their telomeres reach a critcal length (Kiyono et al., 1998; Dickson et al., 2000). Because of this additional barrier, both disruption of the Rb pathway and expression of telomerase are required to immortalize human epithelial cells in culture.

Culturing keratinocytes on feeder layers or altering growth media can delay the accumulation of p16, suggesting that p16 is upregulated in response to culture-imposed stress (Ramirez et al., 2001; Benanti and Galloway, unpublished results). Moreover, p16 levels increase in human fibroblasts as they grow in culture, in a telomere-length independent manner (Kiyono et al., 1998; Benanti et al., 2002). In the case of most fibroblasts, p16 does not reach high enough levels to arrest cells, however the lifespans of WI-38 and IMR90 fibroblasts are limited by p16 and can be extended by reducing p16 levels (Itahana et al., 2003; Benanti and Galloway, unpublished results). These findings argue that in all human cells p16 levels increase with the passage in culture, yet p16 accumulation does not always limit lifespan.

Unlike human cells, rodent cells have extremely long telomeres and often express telomerase therefore they are not limited by telomere length. However, mouse embryo fibroblasts (MEFs) senesce after just a few population doublings in culture (Sherr and DePinho, 2000). In a process analogous to p16 upregulation in human cells, passaging of rodent cells in culture leads to the upregulation of a second cell cycle inhibitor encoded by the p16 locus, the ARF tumor suppressor protein (Zindy et al., 1998). ARF (p19ARF in mice, p14ARF in humans) shares two exons with p16, however it has a unique first exon and is translated in an alternative reading frame (Fig I.3) (Ruas and Peters, 1998). While p16 arrests cells by regulating Rb activity, high ARF levels induce p53-mediated growth arrest. ARF activates p53 indirectly, by binding to a negative regulator of p53, Mdm2 (Kamijo et al., 1998; Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998). In the absence of ARF, Mdm2 ubiqutinates p53 and targets it for proteasome-mediated

degradation. Following ARF expression, Mdm2 is inactivated and p53 levels rise. Although ARF has other cellular targets (Weber et al., 2000a; Eymin et al., 2001; Martelli et al., 2001; Rocha et al., 2003; Vivo et al., 2001), p53 upregulation appears to be the primary mechanism of passage-induced senescence in mouse cells, since MEFs established from either ARF or p53 null mice do not undergo senescence and are immortal in culture (Kamijo et al., 1997). Moreover, cells lacking Bmi-1, a negative regulator of ARF transcription, have elevated levels of ARF and senesce prematurely (Jacobs et al., 1999).

In addition to passage-induced senescence, other types of cellular stress, such as oncogenic signals and oxidative stress, can trigger a similar response. The best characterized example of oncogene-induced senescence is the response of normal cells to overexpression of an activated allele of H-*ras* (H-*ras*V12). Normal ras proteins are involved in transducing mitogenic signals in the cell and are mutated to constitutively activated forms in approximately 20% of human cancers (Bos, 1989). While H-*ras*V12 (RAS) can promote transformation in cells that have disrupted p53 and/or Rb pathways (Liu et al., 1994; Michalovitz et al., 1987; Phelps et al., 1988; Ruley, 1983), RAS expression in normal cells leads to a senescent cell cycle arrest (Serrano et al., 1997). RAS-induced senescence is believed to act through the downstream effectors Raf and Mek, as activated forms of these genes can also cause senescence (Lin et al., 1998; Zhu et al., 1998). In addition to RAS, overexpression of the transcription factor E2F1, which can inappropriately drive cells into S-phase, induces a senescent arrest (Dimri et al., 2000).

Exposure to oxidative stress can also induce a senescent arrest, often referred to as stress-induced premature senescence (SIPS) (Toussaint et al., 2000). SIPS is observed following either acute treatment, such as a brief exposure to H_2O_2, or chronic treatment, such as growth in hyperoxic conditions, and has many common features making it indistinguishable from replicative senescence. In addition, growth of human fibroblasts in low oxygen concentrations that are closer to the oxygen tensions cells are exposed to *in vivo*, lengthens *in vitro* lifespan (Saito et al., 1995). Recent work suggests that passage-induced senescence of MEFs also acts through oxidative stress. MEFs appear to be more sensitive to oxidative stress than human cells and growing MEFs in low oxygen conditions (3% instead of 20%) prevents senescence and allows immortalization (Parrinello et al., 2003). These observations may explain some of the known differences between senescence in human and mouse fibroblasts.

It is likely that all triggers of cellular senescence share some common signaling pathways. It was recently demonstrated that the stress-activated MAPK, p38, is a common downstream effector of replicative, RAS-induced, and oxidative stress-induced senescence (Iwasa et al., 2003). Constitutive activation of p38 itself, by expression of its upstream kinase MKK6, can also induce a senescent arrest (Wang et al., 2002; Haq et al., 2002). In addition, there may be other connections between senescence pathways. RAS has been shown to lead to an increase in oxidative stress (Lee et al., 1999), and oxidative stress has been shown to accelerate telomere shortening (von Zglinicki, 2002).

The fact that a wide variety of stimuli can induce cellular senescence suggests that senescence has evolved to protect cells against stress-induced damage and changes that

lead to tumorigenesis. Interestingly, a recent study suggested that although senescence may have tumor suppressive functions, it may also have a role in tumor promotion in older individuals. Senescent cells all undergo functional changes that are specific to the cell type of origin. These changes have been best described in human fibroblasts, which express an array of genes that are normally only expressed when a fibroblast becomes activated *in vivo*, in a wound-healing response (Campisi et al., 1996). These include secreted proteins that remodel the extracellular matrix and growth factors that can stimulate the proliferation of nearby epithelial cells. Consistent with this expression profile, when senescent fibroblasts are co-cultured with epithelial cells they in fact promote epithelial cell proliferation (Krtolica et al., 2001). This has led to the hypothesis that the accumulation of senescent cells in aged individuals may contribute to an increased incidence of cancer (Krtolica and Campisi, 2002). Although the functional changes in senescent fibroblasts have been well described, it remains unclear how these changes are regulated upon senescence.

The primary goal of this study was to understand how different aspects of cellular senescence are regulated in human fibroblasts. While a lot is known about how telomere shortening regulates the senescent cell cycle arrest, it has remained unclear if telomere shortening regulates all features of cellular senescence. In this work, the novel transcription factor APA-1 was identified as a regulator of gene expression changes in senescent human fibroblasts. Studies on APA-1 regulation have provided the first evidence that senescence-associated functional changes are regulated by a telomere length-independent mechanism. A potential role for the tumor suppressor ARF in

regulation of senescence-associated functional changes was also examined, as APA-1 was originally identified as an ARF-interacting protein. Finally, this study has led to the unexpected finding that passage-induced accumulation of the cyclin dependent kinase inhibitor p16 is required to sensitize cells to senescence induced by oncogenic RAS. Freshly established fibroblasts were found to be resistant to senescence induced by RAS, challenging a long held belief that all normal cells senesce in response to RAS, and suggesting that oxidative stress may be a necessary cofactor in oncogene-induced arrest. Taken together, this work has provided evidence that telomere length-independent factors, such as accumulation of APA-1 and p16 proteins, have important functional consequences during fibroblast aging *in vitro*.

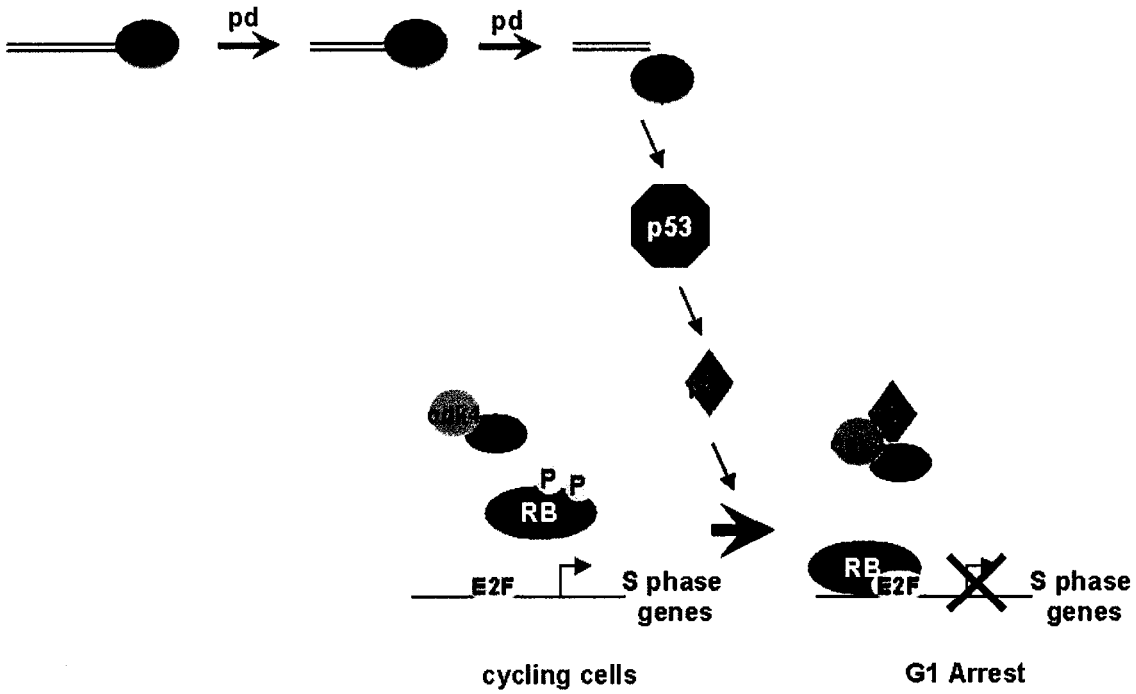


Figure I.1. Telomere shortening leads to p53-mediated arrest. As population doublings (pd) increase, telomeres shorten until they reach a critical length at which the telomeric structure is disrupted. This disruption of the telomere is sensed as a DNA-damage signal which leads to upregulation of p53 and its downstream target p21. p21 then binds to the cdk4/cyclinD (cycD) complex and inhibits its activity, thus preventing phosphorylation of Rb, E2F-mediated transcription, and entry into S phase.

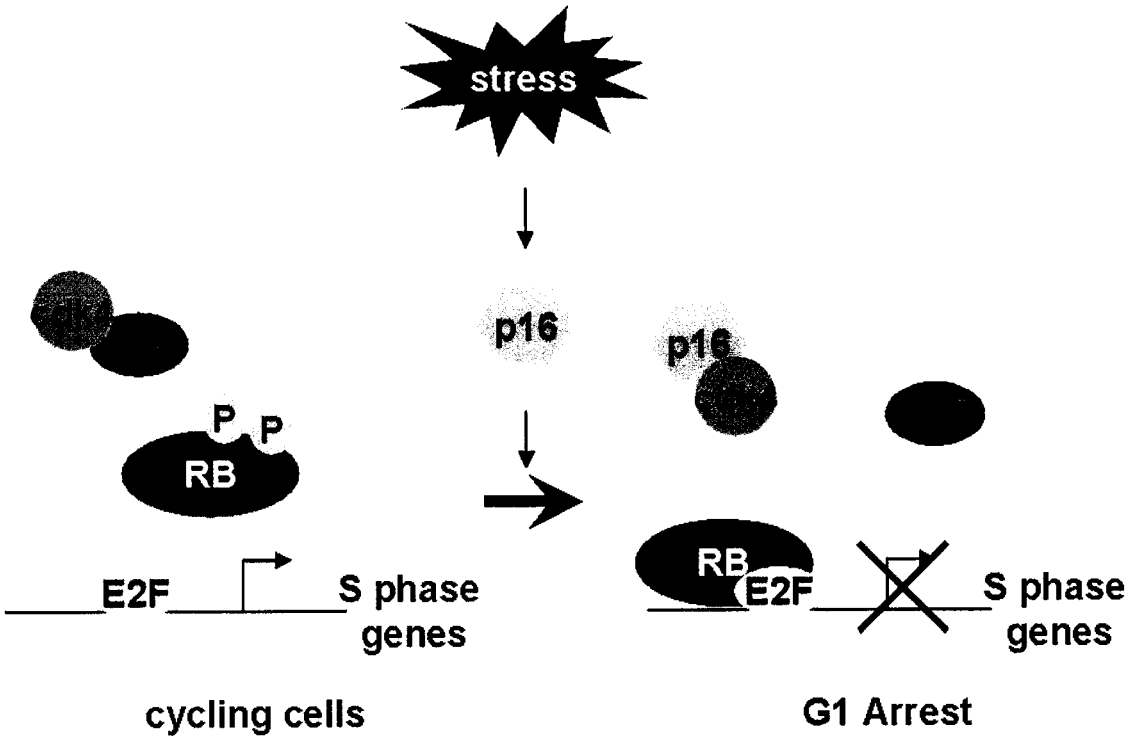


Figure I.2. Mechanism of p16-mediated arrest. In human cells, p16 is upregulated in response to culture-imposed stress. p16 can then bind to cdk4 and disrupt the cdk4/cycD complex, thereby inactivating the complex and causing a G1 cell cycle arrest.

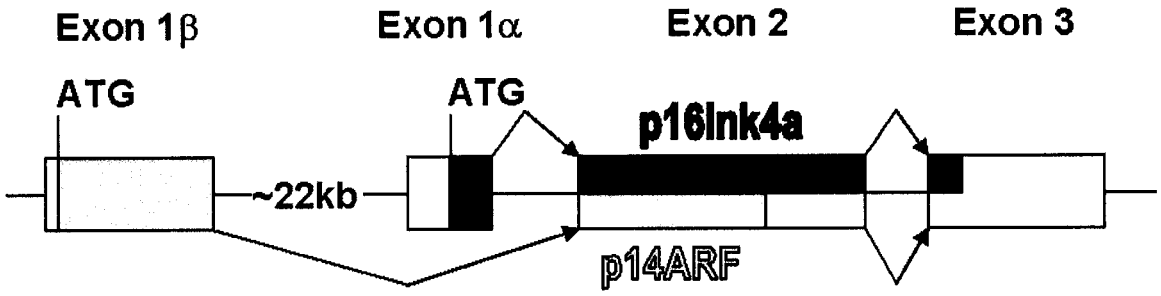


Figure I.3. Structure of the Ink4a/ARF locus. The ARF tumor suppressor protein is the product of an alternative transcript encoded by the p16Ink4a locus. While the p16 transcript include sequences from exon 1α, 2 and 3, the ARF mRNA has an alternate first exon (exon 1β) that is located approximately 22kb upstream from exon 1α.

Chapter 1: Induction of Extracellular Matrix-Remodeling Genes by the Senescence-Associated Protein APA-1

Summary

Human fibroblasts undergo cellular senescence after a finite number of divisions, in response to the erosion of telomeres. In addition to being terminally arrested in the cell cycle, senescent fibroblasts express genes that are normally induced upon wounding, including genes that remodel the extracellular matrix. I identified the novel zinc finger protein APA-1, whose expression increased in senescent human fibroblasts independent of telomere shortening. Extended passage, telomerase immortalized fibroblasts had increased levels of APA-1, as well as the cyclin dependent kinase inhibitor p16. In fibroblasts, APA-1 was modified by the ubiquitin-like protein SUMO-1, which increased APA-1 half-life, possibly by blocking ubiquitin mediated degradation. Overexpression of APA-1 did not cause cell cycle arrest; however it induced transcription of the extracellular matrix-remodeling genes *MMP1* and *PAI2* that are associated with fibroblast senescence. *MMP1* and *PAI2* transcript levels also increased in telomerase immortalized fibroblasts that had high levels of APA-1, demonstrating that the matrix-remodeling phenotype of senescent fibroblasts was not induced by telomere attrition alone. APA-1 was able to transactivate and bind to the *MMP1* promoter, suggesting that APA-1 is a transcription factor that regulates expression of matrix-remodeling genes during fibroblast senescence.

Introduction

One defining characteristic of tumor cells is that they proliferate indefinitely when grown in culture. In contrast, most normal mammalian cells have a limited lifespan and undergo cellular senescence, an irreversible cell cycle arrest, after a defined number of population doublings. This terminal arrest is one mechanism of tumor suppression that cells must overcome during the transformation process (Campisi, 2001).

Cellular senescence is initiated in different ways, depending on the cell type and growth conditions. In human cells, telomere length is a critical determinant of cellular lifespan (Serrano and Blasco, 2001). With each division, telomeres at the ends of chromosomes get incrementally shorter, eventually sending a DNA damage signal that initiates cell cycle arrest. Human fibroblasts will divide 70-90 times in culture until their telomeres reach a critically short length. Fibroblasts can be immortalized if telomeres are lengthened through expression of the enzyme telomerase (Bodnar et al., 1998).

Human epithelial cells reach an additional block to immortalization before telomeres become critically short. Both keratinocytes and mammary epithelial cells arrest after fewer than 30 population doublings due to elevated levels of the cyclin-dependent kinase inhibitor p16. If epithelial cells repress transcription of p16 through methylation of the p16 promoter, or express the human papillomavirus oncogene E7 that disrupts the Rb pathway, they can bypass this early arrest and continue dividing until their telomeres reach a critical length (Kiyono et al., 1998). Induction of p16 can also be delayed if epithelial cells are grown on feeder layers, leaving telomere length as the only barrier to immortalization (Ramirez et al., 2001).

Telomere length is not a factor in senescence of all cell types. Mouse embryo fibroblasts (MEF), which arrest after very few passages in culture, have extremely long telomeres that do not shorten significantly before cells reach senescence (Sherr and DePinho, 2000). Instead, mouse fibroblasts accumulate cell cycle inhibitors as they are passaged and arrest due to induction of the ARF-p53 pathway. Cells from *ARF*-/- or *p53*-/- mice, as well as cells that acquire mutations and lose function of either gene, can bypass senescence and divide continually (Kamijo et al., 1997). The signals that induce ARF and p53 in mouse fibroblasts, or p16 in human epithelial cells, are not known, but may result from the accumulated stress from growth in culture. Primary cells can also undergo senescence in response to activation of oncogenes, such as RAS (Serrano and Blasco, 2001). Broadly defined, cellular senescence can be triggered by both internal signals, such as telomere attrition and oncogne activation, or by external signals such as growth conditions. Senescence limits the number of divisions a cell can undergo and therefore acts as a block to transformation.

In addition to being arrested in the cell cycle, senescent cells show altered differentiation functions (Campisi, 1996). In the case of human fibroblasts, cells can remain metabolically active for extended periods of time, however they show an altered pattern of gene expression. Senescent fibroblasts express genes consistent with an activated, or wound-healing function; they express growth factors, cytokines and enzymes that remodel the extracellular matrix (Campisi et al., 1996). Experiments utilizing cDNA microarrays have confirmed this relationship between fibroblast senescence and wound-healing, as there is considerable overlap between transcriptional

profiles of senescent cells and cells stimulated with serum (Shelton et al., 1999; Iyer et al., 1999). The identities of the factors that induce transcription of wound-healing genes during senescence are not known, but senescent cells have an altered complement of transcription factors that may contribute to gene expression changes (Dimri and Campisi, 1994).

These phenotypic changes in senescent fibroblasts are an important component of cellular senescence, even though they have not been linked to telomere erosion or cell cycle arrest. Recent studies have demonstrated that senescent, but not pre-senescent, fibroblasts can stimulate the proliferation of nearby, initiated epithelial cells, perhaps through expression of secreted proteins (Krtolica et al., 2001). This secretory phenotype has also been described in fibroblasts isolated adjacent to tumors in vivo (Olumi et al., 1999), suggesting that senescent cells may stimulate tumorigenesis in vivo through the misexpression of wound-healing genes. Although this seems to contradict the model in which senescence acts as a tumor suppression mechanism, some evidence argues that limiting cellular lifespan may act to both prevent cancer formation early in the life of an organism, and to promote tumorigenesis later in life (Campisi, 2001).

A great deal is known about which genes change expression upon fibroblast senescence, however the regulatory molecules that translate the number of cell divisions into an altered phenotype remain to be discovered. It also remains to be determined if telomere attrition and cell cycle arrest are necessary for induction of wound-healing genes upon senescence. While searching for proteins that interact with the tumor suppressor p14ARF, I discovered an uncharacterized zinc finger protein whose

expression increased in senescent human fibroblasts. This zinc finger protein, called APA-1 (Another Partner for ARF), resembled a transcription factor but had no characterized homologs. APA-1 levels increased both in senescent fibroblasts and telomerase (hTert) immortalized fibroblasts, arguing that its upregulation was independent of telomere length. APA-1 was regulated post-transcriptionally in fibroblasts, through modification by the ubiquitin-like protein SUMO-1, which increased APA-1 half-life. Overexpression of APA-1 did not affect the cell cycle, however it did induce transcription of the matrix-remodeling genes *MMP1* and *PAI2* that are associated with fibroblast senescence. APA-1 both transactivated and bound to the promoter of *MMP1*, suggesting that it is a transcription factor that acts directly on these promoters. I also found that extended passage, hTert immortalized fibroblasts expressing high levels of APA-1 had elevated levels of MMP1 and PAI2, suggesting that APA-1 transcriptionally regulates a senescent phenotype in fibroblasts through a telomere-independent pathway.

Materials and Methods

Plasmids. The APA-1 cDNA clone (clone ID 23667 or Genbank Acc. No. U90919), originally described by Soares et al. (Soares et al., 1994), and obtained from Wei Yu (Baylor College of Medicine), was subcloned into pGEMT-Easy (Promega) for in vitro translation, pCDNA3 (with and without an HA tag) for transfection and LXSN for retroviral transduction. The mouse APA-1 cDNA (Genbank Acc. No. AF295806) was obtained from ATCC (IMAGE clone #1884982). Human p14ARF was cloned by RT-

PCR from human mammary epithelial cells and subcloned into LXSN for retroviral transduction. The p16 cDNA (originally from Yue Xiong, UNC) was also subcloned into LXSN. Plasmids for producing retroviruses (pJK3, pCMV/tat, pL-VSV-G) were provided by the laboratory of Michael Emerman (FHCRC) (Bartz and Vodicka, 1997). HA-tagged ubiquitin, pHA-ubi, was provided by James Roberts (FHCRC). MMP1 promoter fragments (Genbank Acc. No. AF023338), MMP1-1606 and MMP1-624, were cloned by PCR from human fibroblast genomic DNA and subcloned into pGL3-Basic (Promega) for luciferase assays. Quick change mutagenesis (Stratagene) was used to introduce mutations in the APA-1 binding site within pGL3-MMP1-624, generating pGL3-MMP1-624m.

Cell Culture. Human fibroblasts (HFF) and keratinocytes (HFK) were derived from neonatal foreskins. LXSN and LXSN/hTert expressing HFFs were described previously (Kiyono et al., 1998). SV40 transformed fibroblasts were provided by Harvey Ozer (UMDNJ) and MEFs were provided by Chris Kemp (FHCRC). All human fibroblasts and their SV40 derivates (Banga et al., 1997), ARF-/- MEF, 293T, and HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco-BRL) containing 10% fetal bovine serum and penicillin-streptomycin. HFKs were grown in Keratinocyte Serum Free Media with supplied supplements (Gibco-BRL). U2OS cells (ATCC) were grown in McCoy's 5a medium with penicillin-streptomycin and 10% fetal bovine serum.

Western Blotting. Lysates were prepared for western blotting by trypsinizing cells, washing with PBS, and resuspending in WE16th lysis buffer (50mM Tris-HCl pH7.5, 250mM NaCl, 5mM NaCl, 1% NP40, 0.1% SDS, 20% glycerol, complete protease

inhibitor tablet, Roche). Lysates were then sonicated and clarified by centrifugation. Protein concentrations were determined using the DC protein assay (Biorad). 40-80μg of protein lysates were electrophoresed on 8-15% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Western blots were performed with mouse anti-human p16 (Pharmingen), goat anti-actin (Santa Cruz Biotechnology, I-19), mouse anti-p21 (Oncogene Science, WAF1 Ab1), mouse anti-HA (BabCo, 16B12), mouse anti-GMP-1 (SUMO-1, Zymed), mouse anti-p53 (Oncogene Science, Ab6), and goat anti-p14ARF (Santa Cruz Biotechnology, C-18). APA-1 antiserum was generated in rabbits by injection of recombinant, his-tagged APA-1 protein.

Northern Blotting. Total cellular RNA was prepared using Qiagen's RNeasy kit. 20-60μg of total RNA was electrophoresed on 1% agarose-formaldehyde gels, transferred to Hybond-N membranes (Amersham) and hybridized to ^{32}P-labeled probes. Probes for APA-1 were generated by digesting and gel purifying the 5' half of the APA-1 cDNA and labeling by primer extension with a single antisense primer. All other probes were labeled by random hexamer priming of cDNAs (Roche). 36B4 loading control probe has been described before (Kiyono et al., 1998). IMAGE clones were obtained from ATCC for MMP1 (clone #589115), PAI2 (clone #70692), MMP12 (clone #196612), and MMP2 (clone #1474174) probes. Signals were quantified by phosphorimaging.

Immunoprecipitations. Denaturing immunoprecipitations were used to examine sumoylated APA-1 and HA-ubiquitin conjugated proteins. Cells were trypsinized, rinsed in PBS, resuspended in 2% SDS/TBS and boiled for 10 minutes. After boiling, lysates were cooled on ice and 8 volumes of TBS added. Lysates were then sonicated on ice,

and precleared by the addition of 50μl Protein A/G-Agarose (Roche) and rotating at 4C for 30 minutes. After clarifying by centrifugation, immunoprecipitations were done overnight at 4C, and then purified by adding Protein A/G-Agarose for 1 hour. Immunocomplexes were washed once with 0.5M LiCl/TBS and twice with TBS, then eluted into sample buffer. Elutions were electrophoresed on SDS-PAGE and western blotted as described above. In HA-ubiquitin immunoprecipitation experiments, U2OS cells were plated in 15cm plates and transfected with 10μg pCDNA/APA-1 and 10μg pHA-ubi (or empty vector control) using Fugene6 transfection reagent (Roche). After 20 hours, 25μM MG132 (Calbiochem) was added to cells. Lysates were collected 24 hours after transfection.

Cycloheximide and Proteasome Inhibitor Treatment. For half-life analysis, HFFs were treated with 25μM cycloheximide (Calbiochem) and harvested in WE16th lysis buffer at indicated time points. Protein levels were then examined by western blotting for APA-1 as described above. In proteasome inihibitor experiments, cells were treated with 25μM MG132 (Calbiochem), N-acetyl-leu-leu-norleu-al (ALLN, Sigma), N-acetyl-leu-leu-met-al (ALLM, Sigma) or an equal volume of DMSO (solvent control) for 4 hours.

Retroviral Infections. Retroviruses expressing APA-1, p16, p14ARF or LXSN were produced and concentrated as previously described (Bartz and Vodicka, 1997). Concentrated retroviruses were then used to infect HFFs. 24 hours after infection cells were expanded 1:2 into complete media containing 1mg/ml G418. 10-11 days after infection, when selection was complete, cells were harvested for analysis. For cell cycle analysis, cells were pulse labeled for 4 hours with 10μM BrdU, then trypsinized and

fixed in 70% ethanol. Nuclei were isolated from fixed cells, stained with FITC-conjugated anti-BrdU antibody (Becton Dickenson), and resuspended in 50μg/ml propidium iodide. Nuclei were then analyzed on a FACScan instrument (Becton Dickenson) and cell cycle fractions quantified using CellQuest software (Becton Dickenson). Senescence-associated β-galactosidase staining was carried out as previously described (Dimri et al., 1995).

Luciferase Assays. Early passage ARF-/- mouse embryo fibroblasts (MEF) were plated in 6 well dishes at approximately 50% confluence. The following day, cells were cotransfected with 0.1μg reporter construct (pGL3-MMP1-624, pGL3-MMP1-1606, pGL3-MMP1-624m) and 2μg empty vector (pCDNA3) or pCDNA3-APA1. Each condition was carried out in triplicate. Twenty four hours after transfection cells were lysed in Reporter Lysis Buffer (Promega), luciferase activity measured (Luciferase Assay Substrate, Promega), and protein levels quantitated (DC Protein Assay, Biorad).

Gel Shifts. Probes were generated by radiolabeling, annealing, and gel purifying complementary oligonucleotides containing sequences from the MMP1 promoter (Genbank Acc. No. AF02338). Sequences correspond to bases 4305-4349 for probe A, 4328-4372 for probe B, 4349-4394 for probe C, 4373-4418 for probe D and 4394-4439 for probe E. Four base pairs were changed in the sequence of probe C (TATTGGA to GATGAGC) to generate the APA-1 binding site mutation. Extracts were prepared from U2OS cells transiently transfected with pHA-APA-1 or empty vector (pCDNA3). Cells were washed with rinse buffer (40mM Tris-HCl pH 7.4, 1mM EDTA, 0.15M NaCl) then scraped into resuspension buffer (40mM HEPES-KOH pH 7.9, 0.4M KCl, 1mM DTT,

10% glycerol, 0.5mM sodium orthovanadate, 80mM β-glycerophosphate, 50mM sodium fluoride, complete protease inhibitors (Roche)). Lysates were freeze thawed three times, clarified by centrifugation and 2.5μl were added to 20μl binding reactions containing 25mM HEPES pH 7.6, 10% glycerol, 5μM zinc chloride, 5mM magnesium chloride, 50mM potassium chloride, 0.1mg/ml bovine serum albumin, 500ng poly(dI-dC)_poly(dI-dC) (Amersham Pharmacia Biotech) and 5000cpm radiolabeled probe. For antibody competition 1μl of IgG purified pre-immune, IgG purified anti-APA-1, or rat anti-HA (3F10, Roche) antibodies were added. Protein-DNA complexes were resolved on 5% polyacrylamide gels in 0.5X Tris-glycine buffer.

Results

Identification of APA-1. In order to search for ARF interacting proteins, a yeast two-hybrid screen was carried out with the human protein p14ARF. The complete ARF coding sequence was fused to the yeast Gal4 DNA binding domain and this construct was cotransformed, along with the Gal4 activation domain fused to a HeLa cell cDNA library, into a yeast reporter strain (Clonetech Matchmaker system). Several clones were obtained that grew on media without histidine and expressed β-galactosidase, indicating an interaction between the two fusion proteins. One clone encoded a novel C_2H_2-type zinc finger protein that I named Another Partner for ARF (APA-1). The ARF- APA-1 interaction was confirmed by in vitro binding experiments (presented in Chapter 2); however no apparent affect of APA-1 on ARF-mediated growth arrest could be ascertained (data not shown). For this reason, I decided to investigate the functions of

APA-1 independently of ARF, before continuing to pursue the consequences of the ARF-APA-1 interaction.

The APA-1 cDNA encoded a 478 amino acid protein predicted to contain 5 C_2H_2-type zinc fingers and a leucine zipper motif (Figure 1.1A). The zinc finger domain was highly similar to those found in many zinc finger transcription factors. No similarity was found between the N terminal half of APA-1 and other known proteins. However, a mouse EST with APA-1 sequence identity was obtained and sequenced. Mouse APA-1 had a predicted 94% amino acid identity to human APA-1 (Figure 1.1B). APA-1 mRNA appeared to be expressed ubiquitously, as it has been isolated from a large number of human tissues (NCBI-UniGene ID Hs.7137).

APA-1 expression correlates with senescence of human fibroblasts. In order to characterize APA-1 expression, polyclonal antiserum was raised against recombinant, his-tagged APA-1 protein and then used to investigate the expression of APA-1 in human cells. Human foreskin fibroblasts (HFF) were isolated from neonatal foreskins and passaged in culture until they underwent senescence at population doubling level (PDL) 76. HFFs expressed APA-1 protein of approximately 67kD in mass that increased substantially as cells approached senescence (Figure 1.2A). Northern blot analysis revealed a single band of approximately 2.2 kb corresponding to the APA-1 transcript (Figure 1.2B). APA-1 mRNA remained relatively constant at each PDL, demonstrating that increased protein levels in late passage cells were due to post-transcriptional regulation.

The expression pattern of APA-1 suggested that it may be involved in cellular senescence so APA-1 expression was analyzed in fibroblasts immortalized by expression of the catalytic subunit of telomerase, hTert. Fibroblasts immortalized by hTert expression bypass senescence but do not adopt a transformed phenotype (Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999). Telomerase expressing fibroblasts were established previously and shown to bypass senescence, as well as extend the length of telomeres past that of control cells (Kiyono et al., 1998). LXSN control cells reached senescence at PDL 44 after selection, while hTert expressing cells continued dividing past 109 population doublings. Surprisingly, APA-1 levels increased with population doubling level in hTert immortalized cells (Figure 1.2C). APA-1 levels were lower in hTert cells than LXSN cells at comparable passages, and levels in senescent LXSN cells (PDL 44) were equivalent to extended passage hTert cells (PDL 109). The cyclin dependent kinase inhibitor p16 was also found to increase in hTert immortalized fibroblasts (Figure 1.2C). In contrast to APA-1, p16 levels increased at similar rates in LXSN and hTert cells, with even higher levels of p16 in extended passage hTert cells (PDL 109) than senescent controls (LXSN PDL 44). The fact that APA-1 increased in both normal and hTert immortalized fibroblasts suggested that increased APA-1 protein levels were not due to progressive telomere shortening. Instead, a different mechanism is likely to regulate p16 and APA-1 levels in fibroblasts.

In order to clarify the relationship between APA-1 expression and senescence or immortality, I examined APA-1 levels in a variety of primary and transformed cell types. As shown in Figure 1.2D, APA-1 was expressed at high levels in several types of pre-

senescent human fibroblasts including IMR90 fetal lung fibroblasts, a second foreskin fibroblast line (HSF43) and normal bone marrow stromal fibroblasts (HS74). In contrast (Figure 1.2E, data not shown), APA-1 expression was reduced in primary human epithelial cells (HFK, human mammary epithelial cells). I also examined a previously described line of SV40 transformed primary fibroblasts and several other immortal cell lines. SV40 transformed fibroblasts were generated by expression of an origin-deficient mutant of SV40 in normal HS74 fibroblasts (Neufeld et al., 1987), resulting in cells with an extended lifespan (SV/HF-5). These cells subsequently underwent crisis and rare immortal clones were isolated. One clone, Cl39, divided slowly at early passages, but the doubling time decreased at later passages. At p30 subclones were isolated, one of which was designated Cl39 T. The transformants had numerous genetic alterations (Neufeld et al., 1987), a telomerase-independent mechanism of stabilizing their telomeres (Small et al., 1996), and had an altered morphology with some epithelial-like features. Reduced APA-1 levels were seen in SV40 transformants, with the largest decrease in the Cl39T subclone (Figure 1.2D). APA-1 levels were also significantly reduced in other transformed cell lines tested, including U2OS, HeLa, C33A, SiHa, MDA-MB-231, ZR75-1, HCT116, 293, and 293T (Figure 2E, data not shown). Taken together, these data suggest that APA-1 may be related to fibroblast-specific functions, and that expression increases with prolonged passage in culture.

APA-1 is modified by the ubiquitin-like protein SUMO-1. In addition to our observation that transformed cell lines and primary epithelial cells had reduced levels of the 67kD APA-1 protein, I noticed that some cell types (HFK, U2OS) expressed a

smaller form of the protein, of approximately 49kD. Also, a smaller form of APA-1 was expressed following transduction with an APA-1 expressing retrovirus (Figure 1.4A, 1.5B, 1.6A). This smaller form of APA-1 ran closer in molecular weight to the predicted molecular weight of 52kD, and in vitro translated APA-1, although an intermediate band was detected in the in vitro translation reaction (Figure 1.2E). This suggested that APA-1 was undergoing a large modification in cells, and a different, smaller modification in rabbit reticulocyte lysate.

To rule out the possibility that the large form of APA-1 detected in cells was a cross reacting protein and confirm that the 67kD band was in fact APA-1, a stable line of U2OS cells expressing HA-tagged APA-1 was generated. An APA-1 western blot revealed that the stable transfectant was in fact overexpressing both forms of APA-1 that had been detected in cells, although the molecular weights were slightly different due to the HA tag (Figure 1.3A). Occasionally, a band of greater than 67kD was dectected by the APA-1 antibody, however this band varied between experiments and the connection to APA-1 remains unknown. An anti-HA western blot confirmed that two overexpressed forms of APA-1 detected with APA-1 antibody resulted from the exogenous HA-APA-1 cDNA, and that APA-1 was undergoing a modification of approximately 18kD in cells.

Several types of protein modifications result in large molecular weight shifts of target proteins. One of these is modification by the ubiquitin-like protein SUMO-1. SUMO-1 is an 11kD protein, however when added to targets it can result in a shift of approximately 20kD in apparent molecular mass during electrophoresis (Melchior and Hengst, 2000). To test whether APA-1 was modified by SUMO-1 in HFFs, denaturing

immunoprecipitations were carried out with APA-1 antibody. APA-1 immunoprecipitates and pre-immune controls were then analyzed by western blotting with an antibody specific to SUMO-1. As shown in Figure 1.3B, the large form of APA-1 was recognized by a SUMO-1 antibody, demonstrating APA-1 is modified by SUMO-1 in vivo.

Sumoylation increases APA-1 half-life. SUMO-1 modification affects the function of target proteins in several ways, depending on the target. In the case of Mdm2 and NFκB, SUMO-1 conjugation blocks the addition of poly-ubiquitin chains and increases the half-life of the proteins (Buschmann et al., 2000; Desterro et al., 1998). To test whether sumoylation affects the stability of APA-1, the half-life of sumoylated APA-1 was compared to that of unmodified APA-1. HFFs expressed high levels of sumoylated APA-1 and almost undetectable levels of unmodified APA-1. However, introduction of the APA-1 cDNA into HFFs resulted in overexpression of the unmodified form (Figure 1.4A, 0 hrs). To determine relative half-life, time course experiments were carried out in cycloheximide using control (LXSN) and APA-1 overexpressing HFFs. As shown in figure 1.4A, sumoylated APA-1 (APA-1-S) remained stable and highly expressed in both cell populations throughout the course of the experiment. In contrast, unmodified APA-1 had a half-life of approximately 2 hours. The data suggest that sumoylation increases the half-life of APA-1.

One mechanism by which sumolyation could increase APA-1 half-life is that it may block ubiquitin-mediated degradation of APA-1. In order to determine if APA-1 is normally degraded by the ubiquitin-proteasome pathway, HFFs were treated with the

proteasome inhibitors MG132 and ALLN. In addition to acting on the proteasome, both of these inhibitors can also block the activity of cysteine proteases and calpains. For this reason the cysteine protease/calpain-specific inhibitor ALLM was also tested, in order to demonstrate proteasome-specific effects (Lee and Goldberg, 1998). As shown in figure 1.4B, when HFFs were treated with MG132 and ALLN there was an increase in the level of unmodified APA-1 protein compared to the solvent control. In addition, there was no effect upon treatment by ALLM, demonstrating that increased APA-1 stability resulted specifically from inhibition of the proteasome. As expected, levels of sumoylated APA-1 were not affected by proteasome inhibitor treatment. p53 is known to be degraded by the ubiquitin-proteasome pathway (Chowdary et al., 1994; Maki et al., 1996) and was also increased in MG132 and ALLN treated cells.

In most cases, proteins targeted for degradation in the proteasome are first tagged by the addition of poly-ubiquitin chains (Ciechanover, 1998). I tested whether APA-1 could be poly-ubiquitinated in vivo by transfecting cells with a plasmid encoding HA-tagged ubiquitin, followed by immunoprecipitation and western blotting (Treier et al., 1994). As shown in figure 1.4C, when HA immunoprecipitates were analyzed with APA-1 antibody, a ladder of bands larger than unmodified APA-1 was evident. This modification could also be demonstrated by immunoprecipitating APA-1 and western blotting for HA-ubiquitin. As previously reported for c-Jun and cyclin E proteins (Treier et al., 1994; Clurman et al., 1996), this assay results in different patterns of high molecular weight species, depending on the antibody used in the western blot. This is

presumed to occur because the anti-HA antibody will preferentially detect higher molecular weight forms that contain greater numbers of attached HA epitopes.

APA-1 overexpression does not induce premature senescence in fibroblasts.

Although the data suggested that increases in APA-1 were associated with increased passaging in culture and not specifically a senescent arrest, I still thought it possible that overexpression of APA-1 in fibroblasts might induce a senescence-like cell cycle arrest, similar to what is seen when p16 is expressed at supraphysiological levels (McConnell et al., 1998). In addition, I wanted to test whether induction of premature, telomere-independent senescence through overexpression of p16 or p14ARF could affect APA-1 levels. To address these questions APA-1, p16 and p14ARF expressing retroviruses, as well as empty vector (LXSN) control virus, were produced and used to infect HFFs. After selection, cells were harvested for western blots, labeled with BrdU and analyzed for cell cycle arrest, or stained for senescence-associated β-galactosidase expression, a marker of senescent cells (Dimri et al., 1995). Infections were repeated three times, and a representative experiment is shown in figure 1.5. Overexpression of all three proteins was seen in western blots (Figure 1.5A); as noted previously, APA-1 transduced cells overexpressed primarily unmodified protein. As expected, expression of p16 and p14ARF resulted in a senescence-like cell cycle arrest with increased p53 and p21 (Figure 1.5A-C). In both cases cells failed to incorporate BrdU into their DNA and stained positive for senescence-associated β-galactosidase activity. Induction of premature senescence by p16 and p14ARF led to a modest increase in APA-1 protein, however the increase in protein was not as great as in late passage HFFs. In contrast to

the p16 and p14ARF expressing cells, APA-1 overexpressing cells did not arrest in the cell cycle or express detectable β-galactosidase activity, despite slight increases in p53 and p21 proteins (Figure 1.5A-C). These data argue that APA-1 is not involved in the cell cycle arrest of senescent cells, but that it can be induced in response to induction of senescence in a telomere-independent manner.

APA-1 overexpression induces transcription of matrix-remodeling genes that are associated with fibroblast senescence. In addition to undergoing a cell cycle arrest, senescent fibroblasts are known to express genes in a pattern consistent with activation, or wound repair (Campisi et al., 1996). In order to determine if APA-1 overexpression altered the expression of any genes known to be associated with senescence, transcriptional profiles were analyzed using cDNA microarrays. RNA isolated from control (LXSN) and APA-1 overexpressing HFFs were labeled and co-hybridized to spotted cDNA microarrays that assay 18,000 human genes. Upon examination of genes whose expression changed more than two-fold in two experiments, I identified collagenase-1 (*MMP1*) and plasminogen activator inhibitor-2 (*PAI2*), which were induced upon APA-1 overexpression (data not shown) and have been reported to be upregulated in senescent human fibroblasts (West et al., 1989; Millis et al., 1992; West et al., 1996; Shelton et al., 1999). The matrix-remodeling gene metalloelastase (*MMP12*) was also induced, though it had not previously been associated with fibroblast senescence.

To confirm induction of these genes by APA-1, overexpression was repeated in a second line of HFFs and mRNA levels were analyzed by northern blotting. Levels of

gelatinase-A (*MMP2*), a matrix-remodeling gene not associated with fibroblast senescence, were examined for comparison. Early passage and senescent HFF samples were also included. First, APA-1 protein levels were examined by western blot (Figure 1.6A). In this experiment, both the sumoylated and unmodified forms of APA-1 were increased in APA-1 overexpressing cells. Northern blotting confirmed that *MMP1*, *PAI2* and *MMP12* transcription were all induced in cells overexpressing APA-1 (Figure 1.6B). Interestingly, increases in *MMP1* and *PAI2* mRNAs resulting from APA-1 overexpression were even greater than the induction in senescent HFFs, and although *MMP12* was induced by APA-1, no senescence associated increased in *MMP12* mRNA was seen. In contrast to the senescence-associated genes that were examined, transcription of *MMP2* was not significantly altered in APA-1 expressing cells or in senescent HFFs (Figure 1.6B). These data suggest that APA-1 may transcriptionally induce genes in the wound repair pathway that are associated with fibroblast senescence.

MMP1 and PAI2 increase in hTert immortalized fibroblasts. I would also expect that APA-1 would induce transcription of matrix-remodeling genes in extended passage, hTert immortalized fibroblasts that have high levels of APA-1 protein. To test this, *MMP1* and *PAI2* mRNA levels were examined in late passage hTert expressing HFFs, and compared to LXSN control cells as well as Cl39T transformed fibroblasts. hTert immortalized cells were analyzed at a passage when APA-1 levels were comparable to senescent LXSN cells (Figure 1.2D). For both genes, the increase in transcript level upon senescence in LXSN cells was similar to what was seen in previous experiments (Figure 1.6B-C). Late passage hTert (PDL 109) cells showed increased levels of both *MMP1* and

PAI2 mRNA by comparison to early passage hTert cells (PDL 11), however the increases were not as large as in senescent cells (Figure 1.6C). Compared to the corresponding early passage time points, *MMP1* increased 2.3 fold upon senescence of controls and just under 2 fold in late passage hTert cells. Similarly, *PAI2* increased 5 fold in senescent controls and 3 fold in late passage hTert cells. Cl39T cells, which have low levels of APA-1 (Figure 1.2C), had intermediate levels of *PAI2* and did not express any detectable *MMP1*.

APA-1 transactivates and binds to the MMP1 Promoter. In order to determine if APA-1 acts as a transcription factor and binds directly to promoters to activate transcription, I examined the effects of APA-1 overexpression on the *MMP1* promoter. First the ability of APA-1 to transactivate the *MMP1* promoter in reporter assays was examined. Luciferase reporter constructs containing 624 or 1606 base pairs upstream of the translation start site were created. *ARF*-/- mouse embryo fibroblasts were used to assay transactivation since these cells transfect easily, are immortal in culture, and are primary fibroblasts. Upon cotransfection of APA-1 and either reporter construct, luciferase expression increased 2.5-3 fold compared to vector controls (Figure 1.7A). This suggested that APA-1 was acting on the 624 base pair region of the *MMP1* promoter, just upstream of the translation start site.

Many C_2H_2 zinc finger containing proteins are sequence-specific DNA binding proteins (Wolfe et al., 2000), suggesting that APA-1 may bind directly to the promoter sequence of the *MMP1* gene. To test this the 624 base pair region of the *MMP1* promoter was divided into 5 fragments, from 137-196 base pairs in length, and these were used as

probes in gel shift assays with extracts from either control U2OS cells , expressing very low levels of endogenous APA-1, or U2OS cells transiently overexpressing HA-tagged APA-1. APA-1 could specifically shift a probe containing the 137 base pairs directly upstream of the translation start site (data not shown). The 137 base pair region was then narrowed down by analyzing five overlapping probes (Figure 1.7B). Each probe showed several shifts that were common between control (pCDNA3) and HA-APA-1 transfected cells, however probe C showed one band that was detected only upon addition of HA-APA-1 expressing extract (Figure 1.7C). This band was specific for APA-1, as it was competed by addition of either polyclonal anti-APA-1 antibody or an antibody that recognizes the HA tag. This was particularly interesting because probes B and D, which contain all of the sequence included in probe C, did not show similar shifts, suggesting that APA-1 bound to a region downstream of the PEA3 site and at the junction of the B and D fragments (Figure 1.7B).

Much work has been done to investigate sequence recognition by C_2H_2 zinc finger proteins. Structural studies have shown that each zinc finger adopts a conserved $\beta\beta\alpha$ fold that forms a finger-like structure, and that residues in the α-helix contact base pairs in the major groove of DNA (Pavletich and Pabo, 1991). Mutagenesis of residues within the α-helix, as well as studies of natural occuring zinc finger proteins have led to a suggested recognition code that predicts which base pairs a particular finger may recognize (Choo and Klug, 1997; Wolfe et al., 2000). I examined the sequence of the zinc fingers in APA-1 and attempted to predict a DNA sequence that it might bind to. Following the suggested recognition code, the sequence –X-(C/T)-X_2-(C/T)-G-X-A-X-

(where X is any amino acid) was predicted as a putative APA-1 binding site, assuming that three of the five zinc fingers bind to DNA in a canonical fashion.

Upon examination of gel shift probe C, I found a putative APA-1 binding site, overlapping the junction of probes B and D. To test if the predicted residues were important for APA-1 binding, I made base pair substitutions in probe C, changing the sequence ATATTGGAG to AgATgaGcG (lower case represents base pair substitutions) and tested the mutated sequence in a gel shift assay. As seen previously, extract from HA-APA-1 expressing cells, but not control extract, resulted in a specific shift of probe C that was competed by anti-APA-1 and anti-HA antibodies, but not a pre-immune antibody control (Figure 1.7D). After mutation of probe C, the APA-1 specific shift was no longer detectable upon addition of HA-APA-1 extract, indicating that the residues APA-1 was predicted to bind to were essential for the APA-1 specific shift, and further supporting the role of APA-1 as a sequence-specific DNA binding protein.

In order to determine if binding to the *MMP1* promoter is necessary for transactivation by APA-1, the four base pair changes that abolished APA-1 binding in the gel shift assay were introduced into the 624 base pair *MMP1* promoter- luciferase construct (MMP1-624m). These base pair substitutions diminished transactivation of the *MMP1* promoter by APA-1 (Figure 1.7E). This data indicates that APA-1 is a transcription factor that acts directly on the *MMP1* promoter to induce transcription.

Discussion

In this study, I have begun characterization of APA-1, a zinc finger protein found to be associated with fibroblast senescence. These experiments support several conclusions about the regulation and function of APA-1 during the replicative lifespan of human fibroblasts. First, I found that APA-1 protein increases with population doubling level in human fibroblasts, and that this upregulation is independent of telomere length. Since erosion of telomeres initiates senescence-associated cell cycle arrest in fibroblasts, I analyzed expression of APA-1 in cells immortalized through expression of hTert. APA-1 increased to comparable levels in both control and hTert immortalized cells, similar to the expression pattern of the cyclin dependent kinase inhibitor p16. These data argue that there is more than one signaling mechanism involved in the aging of human cells in culture. p16 levels are increased in senescent cells due to altered levels of its transcriptional regulators Ets1 and Id1 (Ohtani et al., 2001). Although the factors that alter Ets1/Id1 levels are not known, some studies suggest p16 increases when cells are exposed to a stress imposed by growth in cell culture. Fibroblasts can be induced to arrest after fewer divisions, with elevated p16 levels, if they are grown in chemically defined media with low serum (Ramirez et al., 2001). These findings argue that telomeres progressively shorten until they signal a cell cycle arrest and an additional signal, possibly an accumulation of stress, leads to increases in p16 and APA-1. The finding that APA-1 induces transcription of several matrix-remodeling genes suggested that some of the phenotypes associated with senescent fibroblasts may be the result of this second signaling mechanism and not due to telomere shortening (Figure 1.8B). In

fact, I found that expression of the matrix-remodeling genes *MMP1* and *PAI2* were increased in hTert immortalized cells that have high levels of APA-1. Increases in *MMP1* and *PAI2* were not as great in late passage hTert cells as in senescent controls therefore it is likely there are other factors involved in their transcriptional regulation as well. However, these data are consistent with a model in which APA-1 is one factor regulating transcription of these genes in late passage fibroblasts. In addition, these data demonstrate that telomerase immortalized cells may have important differences from their pre-senescent counterparts.

The connection between APA-1 and senescence is further strengthened by observations that APA-1 is induced in situations upon which fibroblasts undergo telomere-independent senescence. A senescence program can be induced in fibroblasts in response to overexpression of oncogenes and induction of the p16 and ARF tumor suppressor pathways (McConnell et al., 1998; Dimri et al., 2000; Wei et al., 2001). I found that induction of premature senescence due to the overexpression of p16 or ARF led to increased levels of APA-1 protein. In addition, the murine APA-1 protein increased upon senescence of mouse embryo fibroblasts (data not shown), a process known to be independent of telomere shortening. These data further support the model that telomere-independent factors lead to senescence-associated changes in the cell.

I have also shown that APA-1 levels are much lower in primary epithelial cells, transformed cell lines, and fibroblasts transformed by the DNA tumor virus SV40. These findings support the model that APA-1 is a transcription factor that regulates genes important for fibroblast-specific functions, such as extracellular-matrix remodeling.

Epithelial cells and transformed cell lines do not carry out these fibroblast-specific functions, and do not express high levels of APA-1.

Third, I have demonstrated that APA-1 is modified and regulated by the ubiquitin-like protein SUMO-1. Although two predominant forms of APA-1 protein were detected in cells, all fibroblasts examined expressed exclusively the larger form of the protein. This form was modified by SUMO-1 in vivo (Figure 1.4C) and recombinant APA-1 can be modified by SUMO-1 in vitro (data not shown). Addition of SUMO-1 to target proteins can have a range of effects including altering subcellular localization, enhancing transcriptional activation, and increasing protein half-life by blocking ubiquitin-mediated degradation (Muller et al., 2001). Both forms of APA-1 are found in the cytoplasm and the nucleus, suggesting that sumoylation does not alter APA-1 localization (data not shown). However, sumoylation of APA-1 increased the half-life of the protein substantially. In addition, unmodified APA-1 was degraded by the proteasome and was poly-ubiquitinated in vivo. These data argue sumoylation of APA-1 may act to block ubiqutination and increase protein stability, although alternate explanations cannot yet be eliminated.

One hurdle in studying sumoylated proteins has been that it is often difficult to overexpress sumoylated forms. It is possible that sumoylation may affect the transcriptional activity of APA-1, and that the unmodified form is more active than the sumoylated form. However, it is difficult to address this question without being able to overexpress sumoylated APA-1 in cells. Recently, several groups have discovered a number of SUMO conjugating enzymes, with different substrate specificities (Jackson,

2001; Kahyo et al., 2001; Sachdev et al., 2001). Although it is not yet clear how many proteins can act as SUMO ligases, it may be possible to find a SUMO ligase that targets APA-1, thus allowing us to examine the effects of sumoylation more directly.

Finally, I have shown that overexpression of APA-1 does not result in a senescence-like cell cycle arrest, but induces transcription of extracellular matrix-remodeling genes that are associated with fibroblast senescence. APA-1 was originally identified as an ARF binding protein, which suggested that its expression may affect the cell cycle. However, APA-1 overexpression did not have a discernable effect on the cell cycle, and did not appear to modulate any known ARF function. For these reasons, I began to examine the functions of APA-1 independent of ARF and discovered that it was capable of regulating the transcription of matrix-remodeling genes that are associated with senescence. It is important to note that induction of these genes by APA-1 was even greater than what was observed in senescent cells, and that in general, senescence-associated increases were not as large as have been reported by others (West et al., 1989; Millis et al., 1992; West et al., 1996; Shelton et al., 1999). It is likely that this is partly due to the experimental conditions. In studies where large differences have been seen, cells were grown in low serum media prior to comparison, while in this study cells were grown in 10% serum. Since transcription of wound-healing genes are stimulated by growth factors, it is possible that mRNA levels are artificially elevated in early passage cells grown in 10% serum. It also remains possible that the overexpressed, unmodified APA-1 is more transcriptionally active than the endogenous, sumoylated protein, and

therefore there is more APA-1 activity in cells overexpressing APA-1 than senescent HFFs.

The amino acid sequence of APA-1 suggested that the protein may bind to DNA and directly acitvate transcription of target genes. I analyzed the promoter of one APA-1 inducible gene, *MMP1*, and found that APA-1 was capable of both transactivating the promoter in reporter assays, and binding to the promoter in gel shift assays. Using gel shift analysis the APA-1 binding site was narrowed to a region of the promoter just downstream of the TATA box and a PEA3 site. Furthermore, mutation of residues necessary for APA-1 binding diminished its ability to transactivate a MMP1 reporter construct. These data demonstrate that APA-1 can act as a transcription factor and support a role for APA-1 regulation of *MMP1* during senescence.

Further mutagenesis of the *MMP1* promoter to determine the sequence elements required for APA-1 binding will allow screening of the promoters of other senescence-associated genes for potential APA-1 binding sites. Although the promoter of *MMP1* has been well characterized (Rutter et al., 1997), APA-1 does not appear to bind to any previously-defined sequence element. It is important to note that there are many sequence similarities in the promoters of matrix-remodeling genes that are known to be involved in coordinate transcriptional regulation in response to certain stimuli (Westermarck and Kahari, 1999). Most of the MMP gene promoters, as well as the *PAI2* promoter, contain several AP-1 sites that bind to the fos/jun family of transcription factors (Kruithof and Cousin, 1988). These sites are important for induction of the MMP genes during growth factor stimulation and wound healing. The promoters also contain

PEA3 elements that bind the Ets family of transcription factors. Ets factors can function as co-activators for the AP-1 transcription complexes in MMP promoters (Buttice et al., 1996). Expression levels of the Ets proteins Ets1, Ets2 and Id1 are also known to be altered upon senescence and to regulate p16 expression (Ohtani et al., 2001). In addition, the APA-1 binding site in the *MMP1* promoter is adjacent to one PEA3 site, raising the possibility that Ets factors cooperate with APA-1 to induce transcription of matrix-remodeling genes during senescence.

In conclusion, I have shown that the zinc finger protein APA-1 is upregulated in a telomere-independent manner in senescent human fibroblasts. Although overexpression of APA-1 does not induce a senescent-like cell cycle arrest, it induces transcription of matrix-remodeling genes that are associated with fibroblast senescence. These findings are the first to suggest that senescence-associated phenotypes, such as extracellular matrix-remodeling in human fibroblasts, are independent of telomere erosion in human cells.

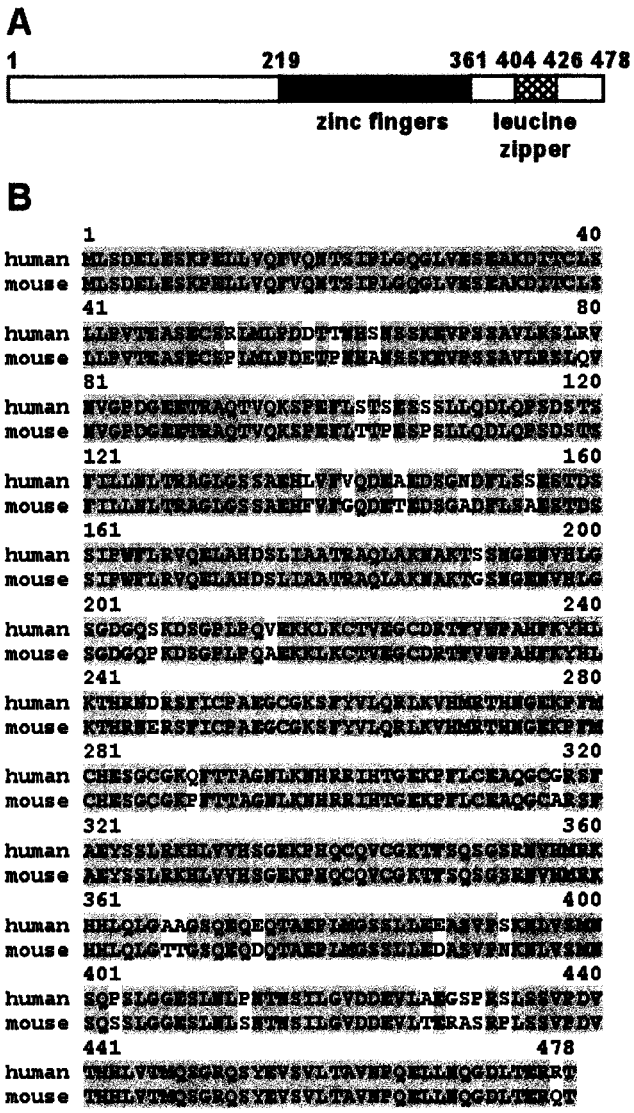


Figure 1.1. Sequence of APA-1. (A) Schematic diagram of APA-1. APA-1 contains 478 amino acids with 5 C_2H_2-type zinc fingers between amino acids 219 and 361 and a leucine zipper between amino acids 404 and 426. **(B)** Alignment of human and mouse APA-1 proteins. Identical residues are shaded grey. The two sequences are 94% identical.

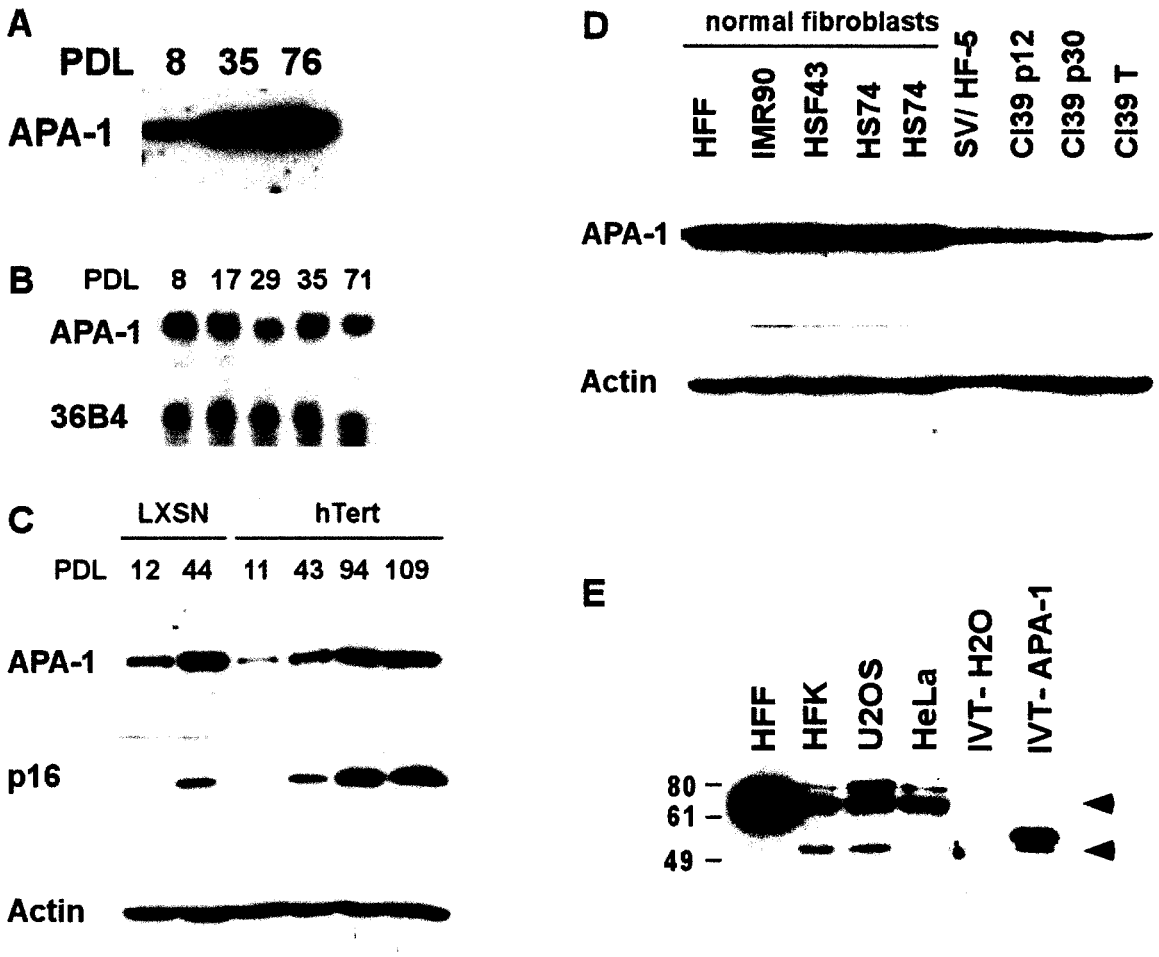


Figure 1.2. APA-1 expression in human fibroblasts. **(A)** Western blot of APA-1 protein in HFFs at increasing population doubling levels (PDL). Cells were senescent at PDL 76. **(B)** Constant APA-1 mRNA in HFFs throughout their lifespan. 20μg of total RNA from HFFs at indicated PDLs were analzyed by northern blot with probes to APA-1 and 36B4 (loading control). **(C)** Western blots of control (LXSN) and hTert transduced HFFs. Cells were transduced mid-lifespan, PDL represent population doublings after selection. LXSN cells reached senescence at PDL 44 and hTert cells continuted proliferating past 109 PDL. Lysates were examined for expression of APA-1, p16 and actin. **(D)** Lysates were collected from several normal human fibroblast types (HFF, IMR90, HSF43, HS74) as well as cells containing SV40 (SV/HF-5, Cl39 p12 and p30, Cl39T) and analyzed by western blot for APA-1 and actin. **(E)** Western blot of lysates from HFF, HFK, U2OS and HeLa cells, as well as in vitro translated APA-1 (IVT-APA-1) and a negative control (IVT- H2O, rabbit reticulocyte lysate alone). The two predominant forms of APA-1 protein run at approximately 67kD and 49kD and are indicated with arrowheads.

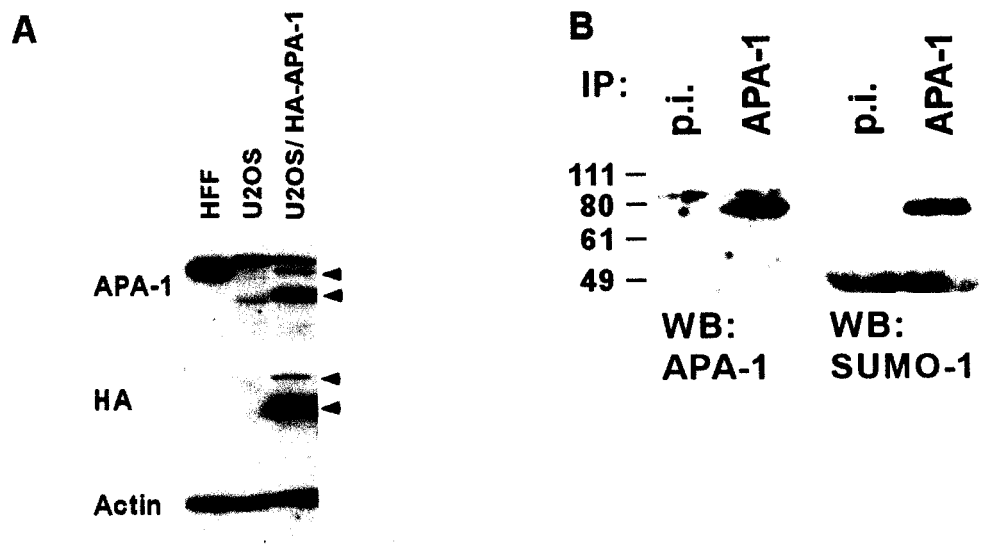


Figure 1.3. APA-1 is modified by SUMO-1 *in vivo*. **(A)** U2OS transfectants express two forms of APA-1. U2OS cells were transfected with HA-tagged APA-1. After selection the pool of cells was compared to untransfected U2OS cells and HFFs for expression of APA-1. Two forms of APA-1 are overexpressed in the HA-APA-1 cells (arrowheads), one of which runs close to the 67kD form seen in fibroblasts. These are observed by western blot with both anti-APA-1 and anti-HA antibodies. **(B)** APA-1 in HFFs is modified by SUMO-1. HFFs were lysed by denaturing lysis in SDS, diluted and immunoprecipitated with anti-APA-1 antiserum or pre-immune serum (p.i.). Immunoprecipitates were eluted in sample buffer, split in half and loaded onto two gels. These were western blotted for APA-1 or SUMO-1. The 67kD form of APA-1 was immunoprecipitated and was recognized by both antibodies.

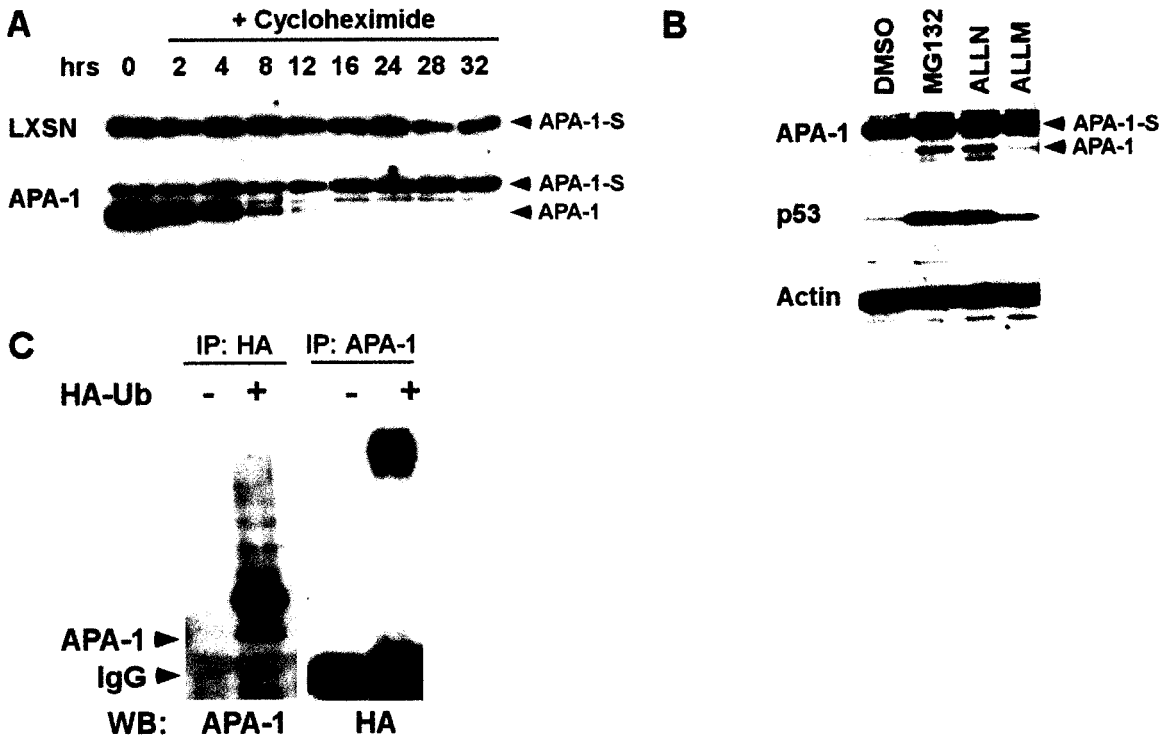


Figure 1.4. Sumoylation increases the half-life of APA-1. (A) Cycloheximide time course comparing the stability of unmodified and sumoylated APA-1. HFFs were transduced with either empty vector (LXSN) or APA-1. After selection, cells were plated in 10cm dishes and treated with 25μM cycloheximide to block protein synthesis. Lysates were collected at the indicated time points between 0 and 32 hours (hrs). Protein concentrations were determined and 40μg of each lysate was analyzed by western blotting for APA-1. Arrowheads represent sumoylated (APA-1-S) and unmodified (APA-1) APA-1 proteins. Approximate protein levels were quantitated using Fluor-S-Multiimager (Bio-Rad) and the half-life of unmodified APA-1 was determined to be approximately 2 hours. **(B)** Proteasome inhibitors increase levels of unmodified APA-1 in HFFs. HFFs were treated with 25μM of the proteasome inhibitors MG132, ALLN, the calpain inhibitor ALLM or an equivalent volume of DMSO for 4 hours and lysates were collected. 40μg total protein was analyzed for levels of APA-1, p53, and actin. **(C)** APA-1 is poly-ubiquitinated in vivo. U2OS cells were transfected with APA-1 and HA-tagged ubiqutin (HA-Ub) or an empty vector control. Denaturing lysates were made, diluted and immunoprecipitated with antibodies against HA or APA-1, as indicated. HA immunoprecipitates were analyzed by western blotting for APA-1 and APA-1 immunoprecipitates were analyzed by western blotting for HA. The positions of unmodified APA-1 and immunoglobulin heavy chaing (IgG) are indicated by arrowheads.

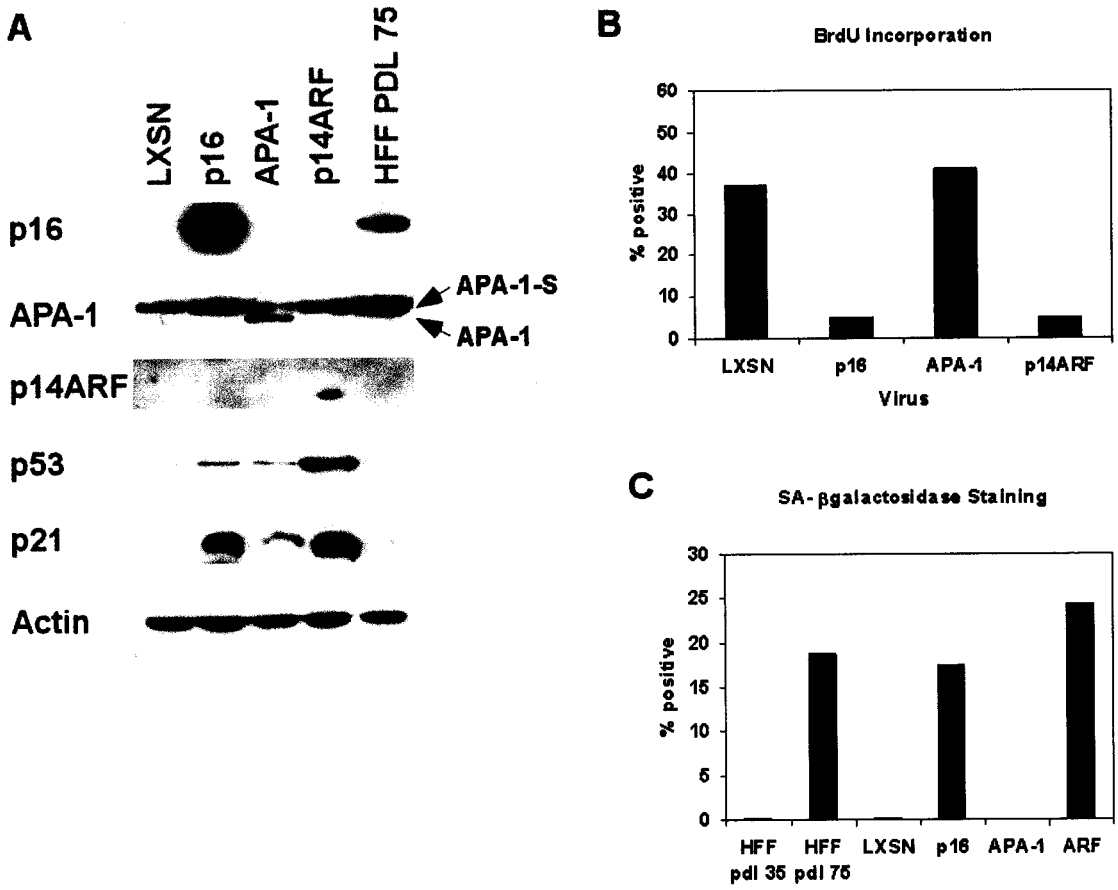


Figure 1.5. APA-1 overexpression does not induce senescence. (A) Western blots of transduced HFFs. Mid-life HFFs were infected with concentrated retroviruses expressing empty vector (LXSN), p16, APA-1, or p14ARF. After selection, lysates were collected and protein concentrations measured. 40μg total protein was analyzed by western blot for expression of p16, APA-1, p14ARF, p53, p21 and actin. Overexpression of p16, p14ARF and APA-1 was confirmed in the corresponding infections. **(B)** After selection, cells from (A) were labeled with 10μM BrdU for 4 hours and then fixed in 70% ethanol. Nuclei were labeled with anti-BrdU-FITC and propidium iodide, then analyzed by flow cytometry. Percent of BrdU positive cells are represented. **(C)** Cells from (A), along with early passage (HFF pdl 35) and senescent (HFF pdl 75) controls, were stained for senescence-associated β-galactosidase. Percent of positive cells are represented.

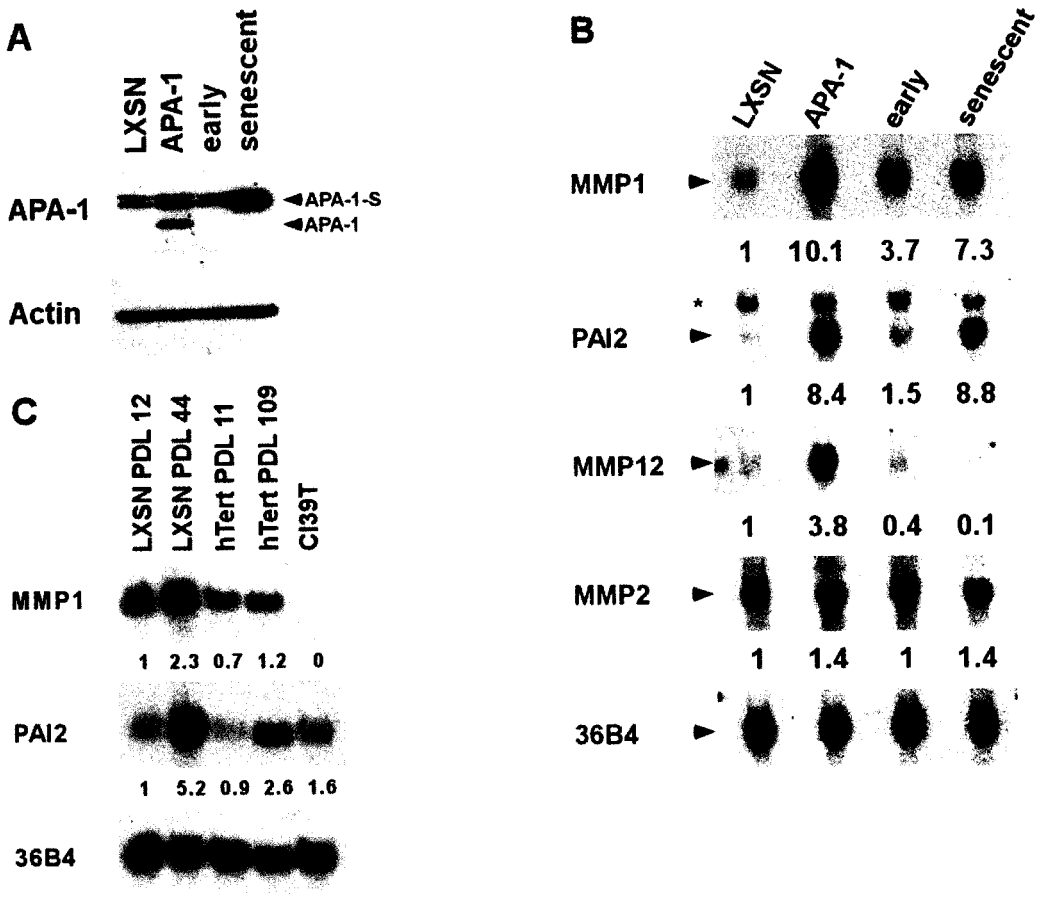
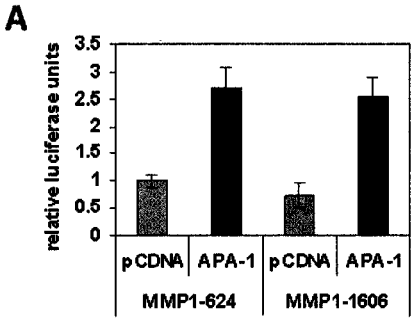


Figure 1.6. APA-1 overexpression induces transcription of extracellular matrix-remodeling genes. (A) Early passage HFFs were infected with concentrated retrovirus expressing empty vector (LXSN) or APA-1 and selected as before. APA-1 protein was analzyed by western blot and compared to uninfected early passage (early) and senescent HFFs. Arrowheads represent the two forms of APA1. **(B)** APA-1 induces transcription of senescence-associated matrix-remodelling genes. Total RNA was harvested from cells described in (A) and analyzed by northern blotting for MMP1, PAI2, MMP12, MMP2 and 36B4 (loading control). Specific band are represented by arrowheads, the asterick (*) represents residual signal on the PAI2 blot from a previous probe. Signals were quantified by phosphorimaging and relative signal (with respect to 36B4 loading control) is indicated by numbers beneath each blot. **(C)** hTert immortalized fibroblasts have increased levels of MMP1 and PAI2 mRNA, similar to senescent controls. RNA was harvested from cells described in Figure 2D as well as Cl39T cells. 30μg total RNA was then analyzed by northern blotting for MMP1, PAI2 and 36B4. Relative signals as determined by phophorimaging are indicated by numbers beneath each blot.

Figure 1.7. APA-1 transactivates and binds to the MMP1 promoter. (A) Early passage ARF-/- mouse embryo fibroblasts (MEF) were transfected with the indicated reporter plasmid (MMP1-624 or MMP1-1606) and empty vector (pCDNA) or APA-1. Luciferase values were normalized for protein concentration and graphed relative to pCDNA3 control for the MMP1-624 promoter fragment. Shown is an average of three experiments carried out in triplicate. **(B)** Schematic diagram the MMP1 promoter. Boxes indicate previously described TATA (grey) and PEA3 (black) elements, as well as the putative APA-1 binding site (hatched). Overlapping probes (A through E) used for gel shift analysis are indicated. **(C)** APA-1 binds to the MMP1 promoter. MMP1 gel shift probes B-D were used in binding reactions with control extracts (pCDNA) or extracts expressing HA-APA-1 as indicated. APA-1 antibody (A) or anti-HA antibody (H) only competed away binding of the APA-1 specific shift of probe C. **(D)** Four base pair substitutions disrupt APA-1 binding. Gel shift analysis of probe C with wild type sequence (ATATTGGAG) or with mutations in the putative APA-1 binding site (AGATGAGCG, with substitutions in outlined text) and control (C) or HA-APA-1 (A) expressing extracts as indicated. The APA-1 specific shift is not competed by addition of pre-immune antibody (P) but is competed by addition of APA-1 (A) or anti-HA (H) antibodies. **(E)** APA-1 binding is necessary for transactivation of the MMP1 promoter. ARF-/- MEF were transfected with MMP1-624 reporter plasmid or MMP1-624m reporter plasmid containing the same base pair changes as in (D) and vector control (pCDNA) or APA-1 expression plasmid. Luciferase values were normalized for protein concentration and graphed relative to pCDNA3 control for the MMP1-624 reporter. Shown is an average of three experiments carried out in triplicate.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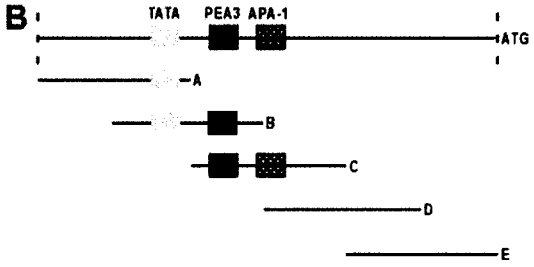
MMP1-1606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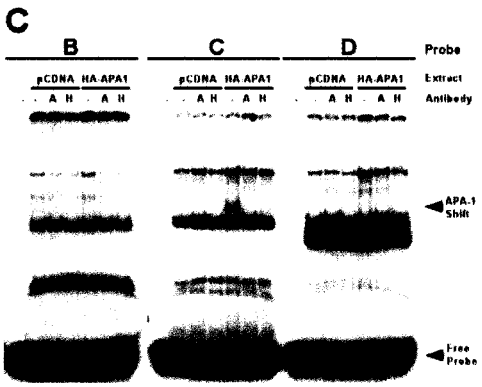
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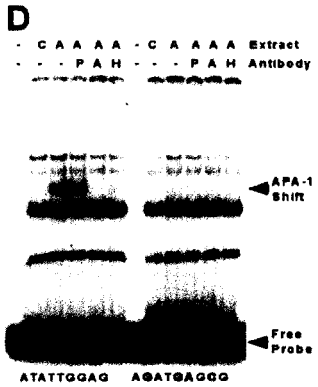
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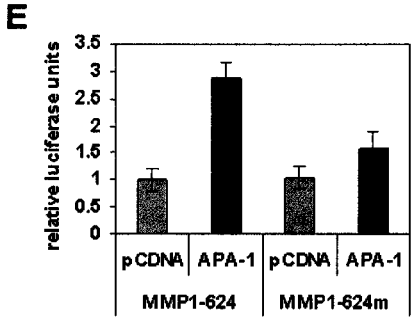
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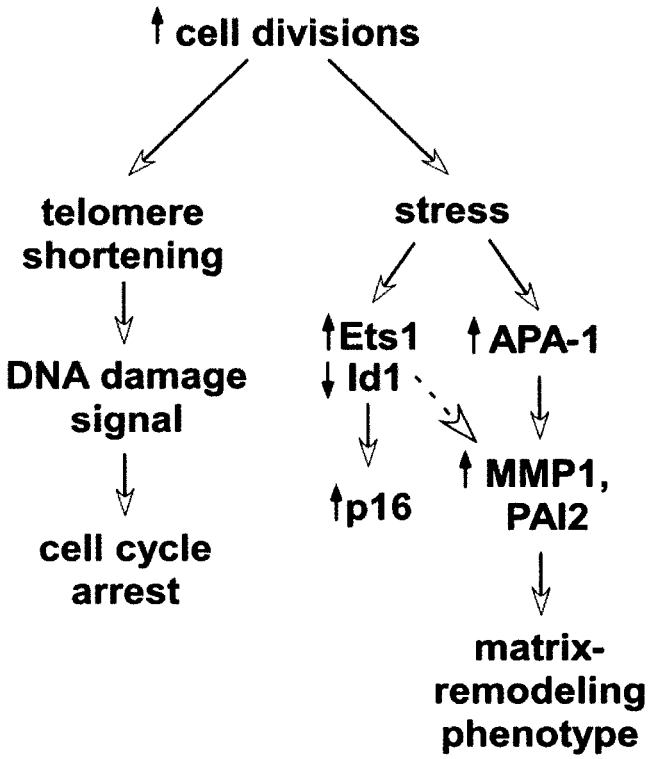

Figure 1.8. Model for induction of matrix-remodeling genes during fibroblast senescence. Although telomere shortening induces the senescent cell cycle arrest in fibroblasts, an additional signal, possible resulting an accumulation of stress, leads to upregulation of p16 and APA-1 proteins. If APA-1 is necessary for induction of matrix-remodeling genes in late passage cells, this suggests that the matrix-remodeling phenotype is separable from the telomere-induced cell cycle arrest during senescence.

Chapter 2: APA-1 Interacts with the Tumor Suppressor ARF

Summary

Senescence in murine fibroblasts is regulated by accumulation of the tumor suppressor protein ARF, which activates the p53 pathway and causes cell cycle arrest. The role of ARF in senescence of human fibroblasts has remained unclear, since telomere shortening is the primary regulator of senescence in human cells. In order to investigate a role for ARF in senescence of human cells, a screen was carried out to identify proteins that could interact with human ARF and the senescence-associated zinc finger protein APA-1 was isolated. In Chapter 1, I found that APA-1 increases in senescent human fibroblasts, in a telomere-length independent manner, and activates transcription of extracellular matrix-remodeling genes that are also increased in senescent cells. In the studies presented here in Chapter 2, I found that ARF interfered with binding of APA-1 to the *MMP1* promoter and blocked APA-1 mediated transactivation in reporter assays. These data suggested that ARF may be capable of blocking APA-1 mediated induction of extracellular matrix-remodeling genes. To test this, ARF was expressed in primary fibroblasts harboring disruptions in the p53 pathway, and APA-1 function was tested in ARF expressing cells. APA-1 induced expression of target genes equivalently in cells with and without ARF, suggesting that ARF may not interfere with APA-1 function *in vivo*.

Introduction

The tumor suppressor ARF was orginally identified as an alternative reading frame product of the *p16Ink4a* locus (Stone et al., 1995; Duro et al., 1995; Mao et al., 1995; Quelle et al., 1995). Like p16, ARF has growth suppressive properties and appears to act as a tumor suppressor gene (Quelle et al., 1995). While p16 arrests cells by acting on the Rb tumor suppressor gene pathway, ARF expression results in p53 induction through its interaction with the p53 negative regulatory protein Mdm2 (Zhang and Xiong, 2001).

In murine cells, ARF has shown to be essential for passage-induced senescence. ARF protein increases quickly as MEFs are passaged in culture and is required for the accumulation of p53 in senescent cells (Zindy et al., 1998). Cells lacking either *ARF* or *p53* do not undergo passage-induced senescence and are immortal in culture (Kamijo et al., 1997). In addition, cells lacking *Bmi1*, a negative regulator of ARF transcription, accumulate more ARF than control cells and undergo premature senescence. Overexpression of Bmi1 downregulates ARF and immortalizes MEFs in culture (Jacobs et al., 1999).

In other cell types, the role of ARF in senescence has remained unclear. ARF mRNA has been shown to increase in rat oligodendrocyte precursor cells (Tang et al., 2001), schwann cells, and fibroblasts (Mathon et al., 2001), however these cells are immortal in culture so ARF accumulation does not cause a senescent arrest. Moreover, conflicting findings have been reported in human fibroblasts. Unlike rodent cells, human fibroblasts are known to senesce in response to induction of the p53 pathway following

telomere shortening, however ARF disruption is not required for immortalization. Furthremore, *ARF* mRNA has been shown to increase in senescent WI-38 human fibroblasts (Dimri et al., 2000), but not in senescent LF1 human fibroblasts (Wei et al., 2001). Overexpression of ARF in human cells does lead to p53 activation and a senescent cell cycle arrest (Dimri et al., 2000; Wei et al., 2001).

Soon after the identification of ARF, it was found to activate the p53 pathway in response to oncogenic signals. In murine cells ARF is upregulated in response to RAS expression (Palmero et al., 1998) and in the absence of ARF cells are transformed by RAS (Kamijo et al., 1997). In addition, expression of either c-Myc or E1A leads to increased ARF, and ARF is required for apoptosis induced by these signals (Zindy et al., 1998; de Stanchina et al., 1998). Following these discoveries, ARF was shown to be regulated by the transcription factor E2F (Bates et al., 1998), which led to the model that ARF acts to link the Rb and p53 tumor suppressor gene pathways in cells. This model predicts that oncogenes such as RAS, c-Myc and E1A result in the phosphorylation and inhibition of the Rb protein, an increase in E2F-mediated transcription, and an increase in ARF. ARF may then activate the p53 pathway and serve as a link between aberrant E2F activation and p53-mediated growth arrest (Sherr and DePinho, 2000). However, this model may oversimplify the situation, as E2F can induce p53-mediated cell cycle arrest and apoptosis in cells lacking ARF (Palmero et al., 2002; Russell et al., 2002; Tolbert et al., 2002). Moreover, results from human cells differ from what has been seen in murine cells. There is evidence for E2F induction of ARF in human cells (Bates et al., 1998;

Dimri et al., 2000), but ARF is not induced following expression of RAS (Wei et al., 2001).

There is now a great deal of evidence that ARF has functions that are independent of p53. This was first suggested by data from mouse models. The ARF-Mdm2-p53 pathway is disrupted in pre-B and B-cell lymphomas that arise in mice expressing c-Myc from the immunoglobulin heavy chain enhancer (Eμ-myc mice). While most tumors contain an *ARF* deletion, *p53* mutation or *Mdm2* overexpression, some tumors lack ARF or p53 and overexpress Mdm2 (Eischen et al., 1999). In addition, tumor cells lacking ARF and p53 proliferate faster than cells lacking either protein alone, suggesting that they do not have completely overlapping functions. Comparison of the tumor spectra from *ARF* and *p53* knock out mice also points to p53-independent functions of ARF. Approximately 70% of *p53*-/- mice develop T-cell lymphomas and the remainder develop sarcomas (Jacks et al., 1994). In contrast, only 25% of *ARF*-/- mice develop T-cell lymphomas, 50% develop sarcomas, and the remainder develop other tumor types never seen in *p53*-/- mice (Kamijo et al., 1997; Kamijo et al., 1999). Furthermore, mice lacking both p53 and ARF develop a greater spectrum of tumors than either *p53*-/- or *ARF*-/- individually (Weber et al., 2000a).

Recently, several additional proteins have been shown to interact physically and functionally with ARF (Weber et al., 2000a; Eymin et al., 2001; Martelli et al., 2001; Vivo et al., 2001; Rocha et al., 2003). It is still not known whether these interactions contribute to tumor development *in vivo*. Our laboratory used a yeast two-hybrid screen to identify additional ARF interacting proteins. From this screen, the senescence-

associated transcription factor APA-1 was identified. APA-1 increases in senescent human fibroblasts and regulates transcription of extracellular matrix-remodeling genes that are associated with senescence, including *MMP1*, *PAI2* and *PAI1* (Benanti et al., 2002; Benanti and Galloway, unpublished results). Extracellular matrix-remodeling is also an important step in tumorigenesis, as expression of matrix-remodeling enzymes allows cells to remodel their surrounding environment and migrate to other tissues (Westermarck and Kahari, 1999). These findings led to the hypothesis that ARF may be able to act as a tumor suppressor by blocking APA-1 function, and inhibiting expression of extracellular matrix-remodeling genes. In this way, ARF could have a p53-independent effect on tumor progression. I tested this hypothesis and found that ARF could block APA-1 mediated transactivation of an *MMP1* reporter and could reduce the ability of APA-1 to bind to DNA. However, there was no evidence that ARF could conteract APA-1 function in cells, suggesting that ARF may not functionally interact with APA-1 *in vivo*.

Materials and Methods

Plasmids. pCDNA/APA-1, pCDNA/HA-APA-1, LXSN, LXSH, LXSN/APA-1, LXSN/p16, pGL3-MMP1-624, and pGL3-MMP1-1606 constructs have been described previously (Benanti et al., 2002). Truncations of APA-1 were generated by PCR and subcloned into pGEMT- Easy for in vitro translation. Human p14ARF (Benanti et al., 2002) was subcloned into pGEX2T (Pharmacia), pCMV with and without 3 N-terminal hemagglutinin (HA) tags (Kiyono et al., 1994), and pBABE-puro vectors. Dominant

negative p53 (p53DD) was provided in LXSN by Karl Munger (Harvard Medical School) and subcloned into LXSH.

Cell Culture. U2OS, 293T and HFF cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and penicillin-streptomycin. Retrovirus was produced and infections carried out as previously described (Bartz and Vodicka, 1997).

In vitro binding assays. GST and GST-ARF proteins were produced in E. Coli (BL21), purified by binding to Glutathione-Sepharose 4B (Amersham Pharmacia Biotech), and dialyzed against PBS containing 1% Nonidet P-40 (NP-40) and protease inhibitors (Complete, EDTA free protease inhibitor tablets, Roche). His-tagged APA-1 was purified similarly on Ni-NTA agarose (Qiagen). APA-1 binding was assayed by combining of equivalent amounts of APA-1 with GST and GST-p14ARF (concentrations were estimated by commassie gels) and incubating overnight in PBS + 1% NP40. Complexes were then purified on Glutathione-sepahrose 4B, eluted into sample buffer and run on SDS-PAGE. Gels were transferred and western blotted for APA-1. In vitro translations were carried out using Promega's Rabbit Reticulocyte Lysate coupled TNT kit and ^{35}S- cysteine. Mapping experiments were done by combining 10μl of in vitro translated protein with equivalent amounts of GST or GST-p14ARF in binding buffer (PBS + 1% NP-40). Reactions were incubated on ice for 30 minutes. Binding buffer was added to a final volume of 0.5ml and reactions were incubated while rotating at 4°C for 30 minutes. Complexes were then isolated by adding glutathione-sepharose 4B and rotating at 4°C for an additional 1 hour. Beads were washed 4 times in binding buffer

and proteins eluted by the addition of SDS sample buffer and boiling. Reactions were run, along with input controls, on SDS- polyacrylamide gels. Fluorography was carried out with 20% PPO in acetic acid to enhance the ^{35}S signal. Gels were then dried, and exposed to film (Biomax MR, Kodak).

Immunoprecipitations. U2OS cells were transfected with pCMV or pCMV/3HA-ARF cDNAs and after 24 hours lysates were preparaed by trypsinizing cells, washing in PBS, and resuspending in WE16th lysis buffer (50mM Tris-Cl pH 7.5, 250mM NaCl, 5mM NaCl, 1% NP-40, 20% glycerol, 1mM DTT, complete protease inhibitor tablet, Roche). Lysates were then sonicated on ice, clarified by centrifugation, precleared with Protein A-Agarose (Roche) and incubated with primary antibodies (rabbit-anti- APA-1 or corresponding preimmune serum) overnight. Complexes were purified on Protein A-Agarose, run on SDS-polyacrylamide gels and analyzed by western blotted for APA-1 and HA-ARF.

Western Blots. Western blots were performed as previously described (Benanti et al., 2002) with rabbit anti-APA-1, mouse anti-human p16 (Pharmingen), goat anti-actin (Santa Cruz Biotechnology, I-19), mouse anti-p21 (Oncogene Science, WAF1 Ab1), mouse anti-HA (BabCo, 16B12), mouse anti-p53 (Oncogene Science, Ab6), and goat anti-p14ARF (Santa Cruz Biotechnology, C-18).

Immunofluorescence. U2OS cells were plated on glass coverslips and transfected with pCMV/3HA-ARF. Cells were fixed with 2% paraformaldehyde, permeabilized with methanol:acetone, and blocked in 5% fetal bovine serum in PBS. Fixed cells were then

stained with mouse-anti-HA and rabbit-anti-APA-1 primary antibodies, followed by goat-anti-mouse-texas red and goat-anti-rabbit-FITC antibodies and DAPI to visualize nuclei.

APA-1 Functional Assays. Luciferase assays, gel shift assays, and northern blots were performed as previously described (Benanti et al., 2002). Gel shifts were carried out with extracts from U2OS cells transfected with pCDNA3, pCDNA/HA-APA-1, and pCMV/ARF as indicated. Northern blots were probed with radiolabeled cDNAs corresponding to *MMP1* (IMAGE clone 589115), *PAI2* (IMAGE clone 70692) and *PAI1* (provided by David Cobrinik, Columbia).

Results

APA-1 was originally identified in a yeast two-hybrid screen with the human p14ARF protein (Benanti et al., 2002). The interaction between ARF and APA-1 was confirmed in several ways. First, recombinant, his-tagged APA-1 was found to interact specifically with recombinant GST-p14ARF but not GST alone (Figure 2.1A). In addition, HA-tagged ARF coimmunoprecipitated with endogenous APA-1 in U2OS cells (Figure 2.1B). The localization of ARF and APA-1 was also examined. ARF has been reported to be located both in the nucleolus and throughout the nucleoplasm (Weber et al., 1999; Llanos et al., 2001). Following transfection into U2OS cells I found that ARF localized in three different patterns. In some cells ARF showed diffuse nucleoplasmic staining and appeared to be excluded from the nucleolus (Figure 2.1C, yellow circles), while in other cells ARF was located throughout the nucleus as well as the nucleolus (Figure 2.1C, green circles). ARF was also occasionally found in nuclear speckles that

did not coincide with the nucleolus (Figure 2.1C, red circles). In all cases, APA-1 exhibited a diffuse nuclear staining that overlapped, at least in part, with ARF localization (Figure 2.1C).

In order to determine which region of the APA-1 protein was interacting with ARF, several APA-1 deletion mutants were constructed. Truncations were made from both the N and C termini, in addition to an internal deletion of the five zinc fingers (Figure 2.2A). APA-1 mutants were then in vitro translated and tested for their ability to bind to GST and GST-p14ARF. All APA-1 proteins containing the zinc finger domain bound to ARF, while the N- terminal half alone (Δ219-478) and the zinc finger deletion (Δ219-368) did not. These data suggested that ARF bound to the zinc fingers of APA-1. Attempts were also made to localize the binding site of APA-1 on ARF. However, interactions were detected between APA-1 and both the N and C terminal halves of ARF, so the binding site could not be easily determined (data not shown). Mdm2 has also been shown to contact multiple sites on the ARF protein (Lohrum et al., 2000; Weber et al., 2000b; Clark et al., 2002) and it is possible that APA-1 interacts in a similar fashion.

Previous findings suggested that APA-1 activates transcription of extracellular matrix-remodeling genes through a direct interaction between the zinc finger domain of APA-1 and promoter DNA (Benanti et al., 2002; Benanti and Galloway, unpublished results). Since ARF was found to interact with the zinc fingers of APA-1, it seemed possible that ARF binding to APA-1 could interfere with DNA binding. Extracts were made from U2OS cells transfected with an empty vector, APA-1, ARF, or both APA-1 and ARF, and these were tested for APA-1 specific binding to the *MMP1* promoter by gel

shift analysis. As previously observed, extracts from cells expressing APA-1 caused a specific shift of the probe (Figure 2.3A). Interestingly, coexpression of ARF reduced the amount of APA-1 bound probe without affecting overall APA-1 protein levels (Figure 2.3). I also tested whether or not ARF could interfere with APA-1 mediated transactivation of an *MMP1* promoter-reporter. Two reporter constructs previously found to be transactivated by APA-1 were tested. In both cases, APA-1 expression resulted in an approximately 2.5 fold increase in luciferase expression compared to controls, and coexpression of ARF reversed this effect (Figure 2.4). Taken together, these findings suggest that ARF may inhibit APA-1 function.

In order to determine if ARF could affect the function of APA-1 in a more relevant cellular context, the effect of APA-1 on endogenous target genes was analyzed in primary fibroblasts expressing ARF. Since ARF overexpression induces cell cycle arrest in cells with an intact p53 pathway, p53 function was first disrupted by expression of either the HPV E6 oncoprotein or a dominant negative p53 (p53DD). E6 and p53DD cells were then infected with control (pB) or ARF expressing retroviruses. The resulting cell lines were then used to test whether APA-1 overexpression could induce transcription of the target genes *MMP1* and *PAI1*. In contrast to what was observed in gel shift analyses and reporter assays, APA-1 could induce target gene expression in cells expressing ARF to a similar extent as in control cells (Figure 2.5A).

There could be several reasons that might explain the discrepencies between different assays for APA-1 function. One possibility is that direct overexpression of unmodified APA-1 in fibroblasts results in a stronger activation signal when compared to

the accumulation of sumoylated APA-1 in senescent fibroblasts, and that the amount of ARF protein present is not sufficient to have an effect. Previous data suggested that direct APA-1 expression caused greater increases in *MMP1* expression than what is observed during normal replicative senescence (Benanti et al., 2002). For these reasons, I tested whether ARF could antagonize the upregulation of APA-1 target genes during senescence that is induced by expression of the cyclin dependent kinase inhibitor p16. Following p16 expression, p53DD and E6 expressing cells with and without ARF arrested in the cell cycle and adopted a senescent-like morphology (data not shown). APA-1 levels increased in all cells types following p16 expression, regardless of ARF status (Figure 2.5B). However, when senescence was induced by p16 expression there was no effect on *MMP1* mRNA levels (data not shown), and although *PAI1* levels did increase in p16 arrested cells, ARF expression had no effect. These data suggest that ARF does not have an effect on the expression of matrix-remodeling genes induced by either APA-1 overexpression or p16-induced senescence.

Discussion

APA-1 was originally identified by its ability to bind to the tumor suppressor ARF, and was subsequently shown to be a transcription factor that increases due to culture-imposed stress in human fibroblasts. Likewise, ARF is required for passage-induced senescence in murine fibroblasts, and also increases in response to culture-imposed stress. These observations suggest that there may be a functional interaction between the two proteins. ARF is known to have tumor suppressor functions

independent of its effect on p53 (Eischen et al., 1999; Weber et al., 2000a) and it is possible that ARF may block the transcriptional activation function of APA-1, interfering with expression of extracelluar matrix-remodeling genes that may have a role in tumor progression.

One problem with this model is that there is an inverse correlation between the expression of APA-1 and ARF in human cells. APA-1 is expressed at very high levels in primary human fibroblasts and is expressed at much lower levels in epithelial cells and transformed cell lines (Benanti et al., 2002). Meanwhile, ARF protein is not detectable in primary human fibroblasts (Dimri et al., 2000; Wei et al., 2001), but it is expressed at easily detectable levels in many transformed cell lines. Despite this, an interaction between exogenous ARF and endogenous APA-1 can be detected in transformed U2OS cells. This raises the possiblity that in cells lacking p53 and expressing high levels of ARF, ARF may antagonize APA-1 and inhibit transcription of APA-1 target genes that could be involved in transformation.

Evidence from reporter assays and gel shift analysis supports this model. Coexpression of ARF blocked transactivation by APA-1 in an *MMP1* reporter assay, and also diminished APA-1 binding to DNA. However, in primary fibroblasts engineered to overexpress ARF, there was no evidence that transcription of endogenous target genes were affected following overexpression of APA-1 or induction of senescence by p16. There are several potential problems with these experiments however. First, APA-1 is modified by the ubiquitin-like protein SUMO-1 in fibroblasts (Benanti et al., 2002), and it is unclear if the overexpressed, unmodified protein has the same activity as the

endogenous, sumoylated protein. I have not been able to test this directly because at this time the necessary cofactors for APA-1 sumoylation have not been identified, and it has not been possible to overexpress sumoylated APA-1 in cells. One possibility is that unmodified APA-1 has greater activity that sumoylated APA-1, and the levels of ARF expressed in fibroblasts are not sufficient to counteract APA-1 in this form. For this reason, I induced increased levels of sumoylated APA-1 protein by overexpression of p16, and tested whether ARF had an effect on transcription of APA-1 target genes. Unfortunately, there was no evidence for an ARF effect in this setting either. It remains possible that a functional interaction may exist, however it will be difficult to address this question until more is known about APA-1 function and regulation; more specifically, how APA-1 is sumoylated *in vivo* and what functions sumoylation has.

Another important question is understanding how ARF is expressed in human cells. Until this is better understood, it will be difficult to examine how ARF affects APA-1 in a cellular context. Although there is a great deal of convincing data showing that ARF is upregulated in primary murine cells in response to culture-imposed stress or expression of oncogenes (Palmero et al., 1998; Zindy et al., 1998; de Stanchina et al., 1998), the same signals do not seem to have the same effect on the human ARF protein (Wei et al., 2001). In cases when ARF mRNA is seen to increase in human cells, protein levels are still below detection and far lower that the level of ARF seen in transformed cells lines. This raises the question of what normal stimuli may induce ARF protein in human cells. If ARF protein is only expressed in cells after they have undergone the initiating events in tumorigenesis and have lost the p53 pathway, then the p53-

independent functions of ARF may play a greater part in its role as a tumor suppressor gene.

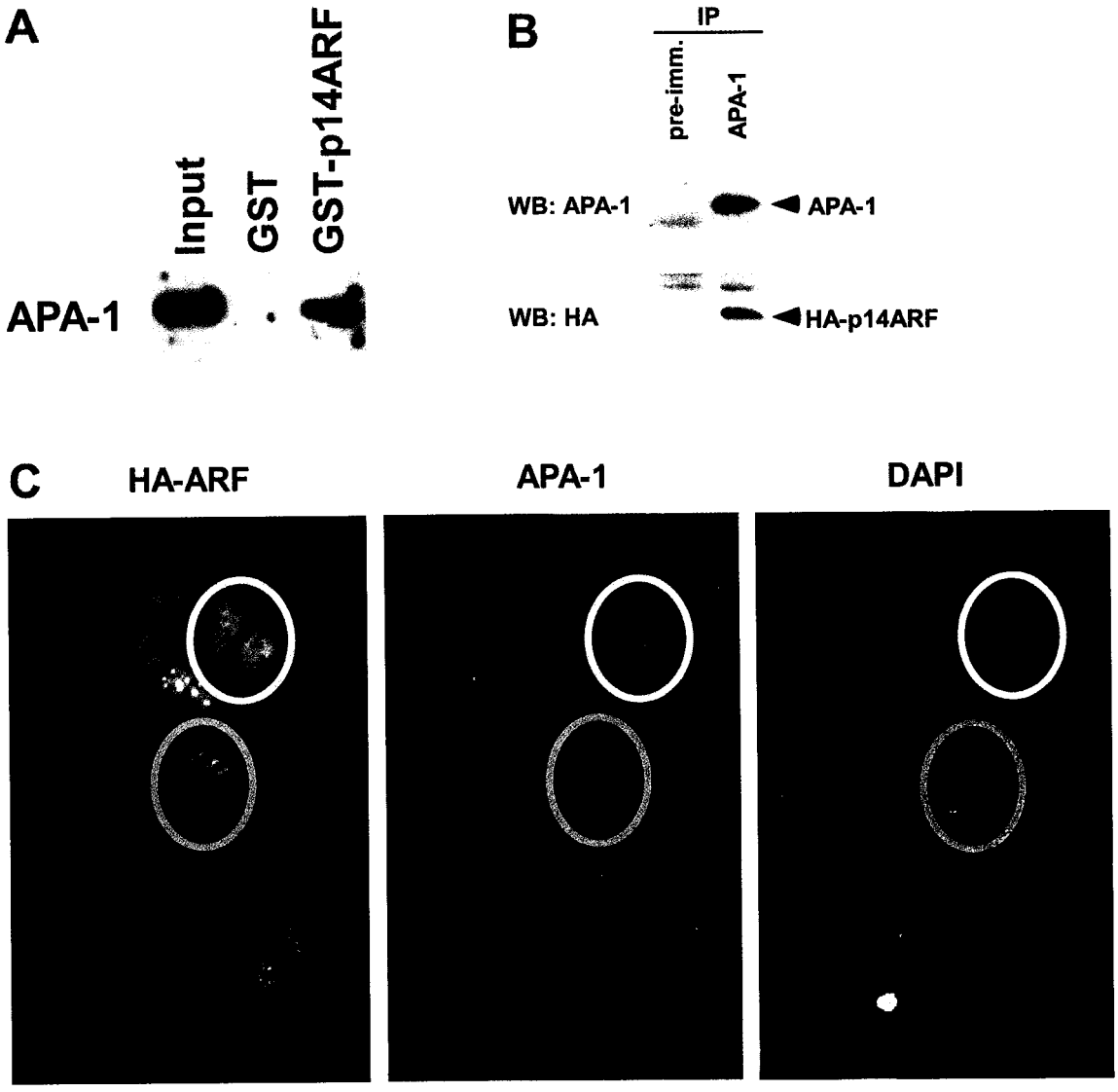

Figure 2.1. APA-1 interacts with ARF *in vitro* and *in vivo*. **(A)** Recombinant His-tagged APA-1 was incubated with GST or GST-p14ARF and purified on glutathione-sepharose. Western blot of APA-1 bound to p14ARF and 5% input is shown. **(B)** Coimmunoprecipitation of APA-1 and HA-tagged ARF. U2OS cells were transfected with HA-tagged ARF and immunoprecipitations carried out with anti-APA-1 antibody or preimmune serum control. Complexes were analyzed by western blot for APA-1 and HA. **(C)** U2OS cells from (B) were analyzed by immunofluorescence for HA-ARF and APA-1. Colored circles represent 3 different ARF localization patterns as described in the text. In all cases, ARF is found in the nucleoplasm, similar to APA-1.

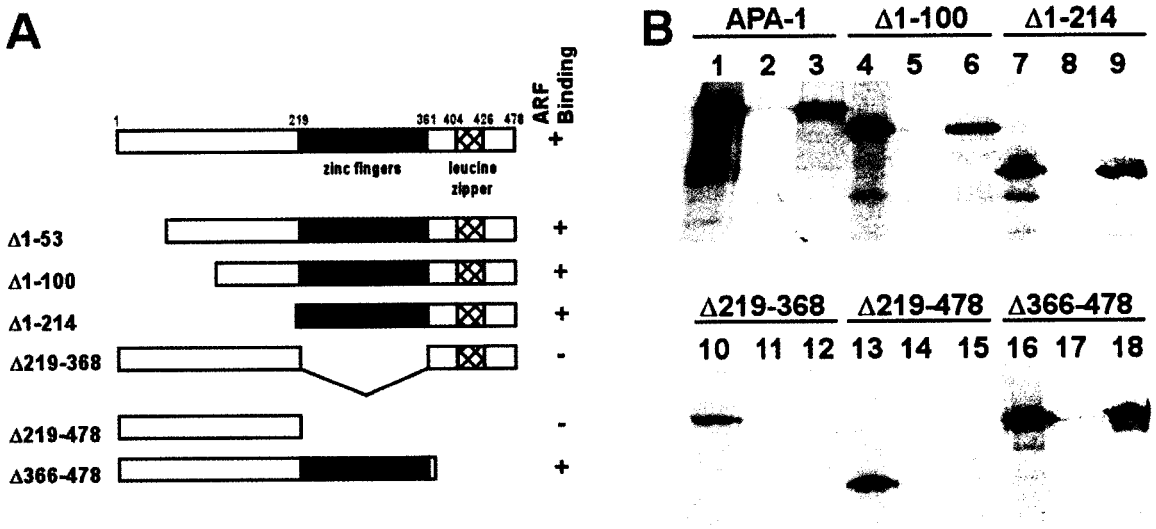

Figure 2.2. ARF binds to the zinc finger domain of APA-1. (A) Deletion mapping of the ARF interaction site on APA-1. Schematic diagram of mutants tested in GST-ARF pull down assays. Positive binding is indicated in the column on the right. **(B)** Binding of APA-1 deletions mutants to GST and GST-p14ARF. APA-1 deletion mutants were in vitro translated (lanes 1, 4, 7, 10, 13, 16) and tested for their binding to GST (lanes 2, 5, 8, 11, 14, 17) and GST-p14ARF (lanes 3, 6, 9, 12, 15, 18).

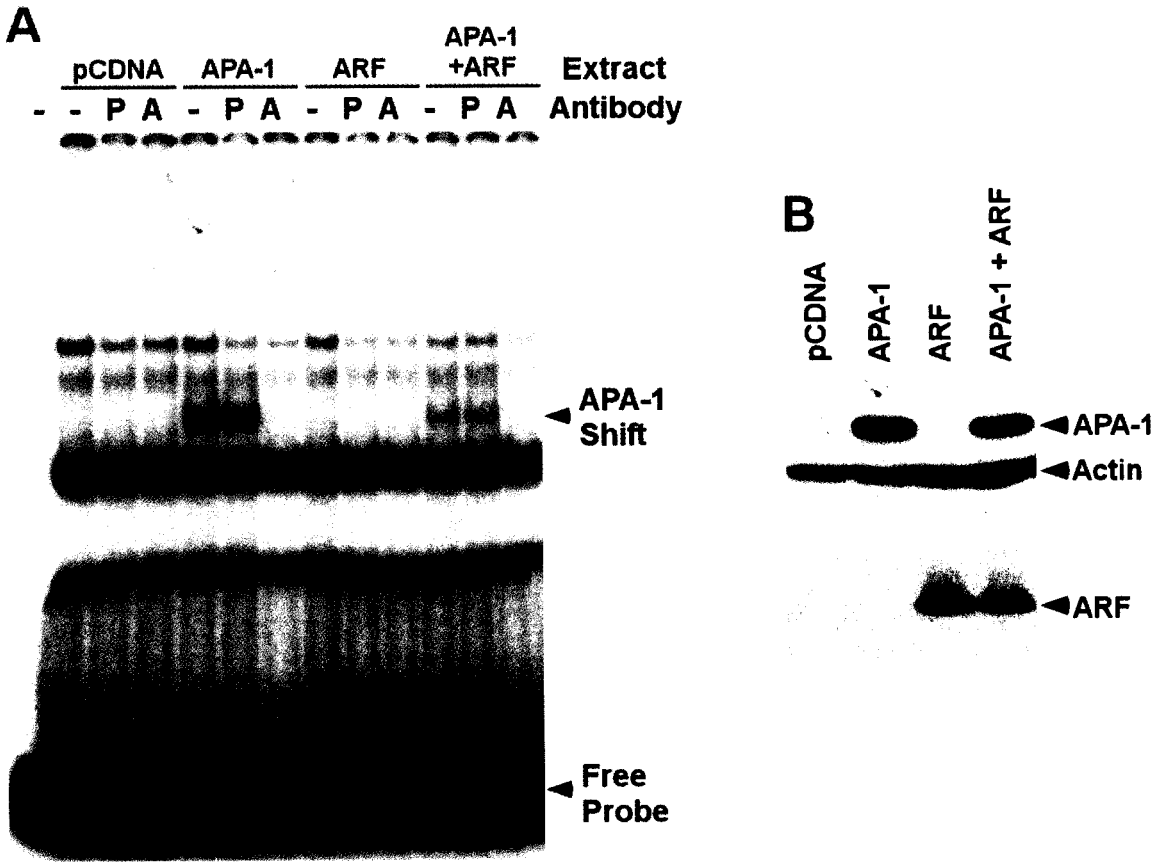

Figure 2.3. ARF inhibits APA-1 binding to the MMP1 promoter. (A) Gel shift of extracts from U2OS cells transfected with empty vector (pCDNA), APA-1, ARF or APA-1 and ARF. Extracts were incubated with radiolabeled probe and either preimmune (P) or anti-APA-1 (A) antibodies as indicated. APA-1 binding is reduced when ARF is coexpressed. **(B)** Western blot of APA-1, ARF, and actin in gel shift extracts.

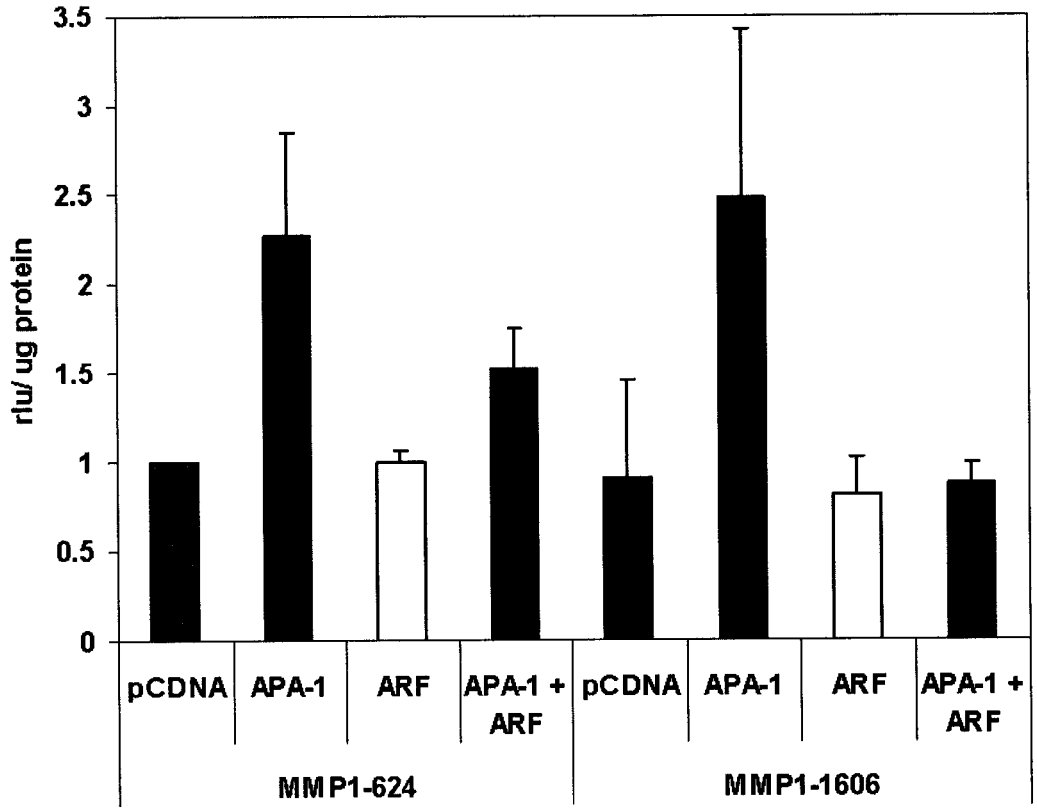

Figure 2.4. ARF blocks APA-1- mediated transactivation. Luciferase assay in ARF-/- MEFs transfected with pGL2-MMP1-624 or pGL3-MMP1-1606 reporter constructs along with empty vector (pCDNA), APA-1, ARF or APA-1 and ARF. Luciferase values were normalized to protein concentration and graphed relative to the pCDNA control for the MMP1-624 promoter fragment. Shown is the average of two experiments carried out in triplicate.

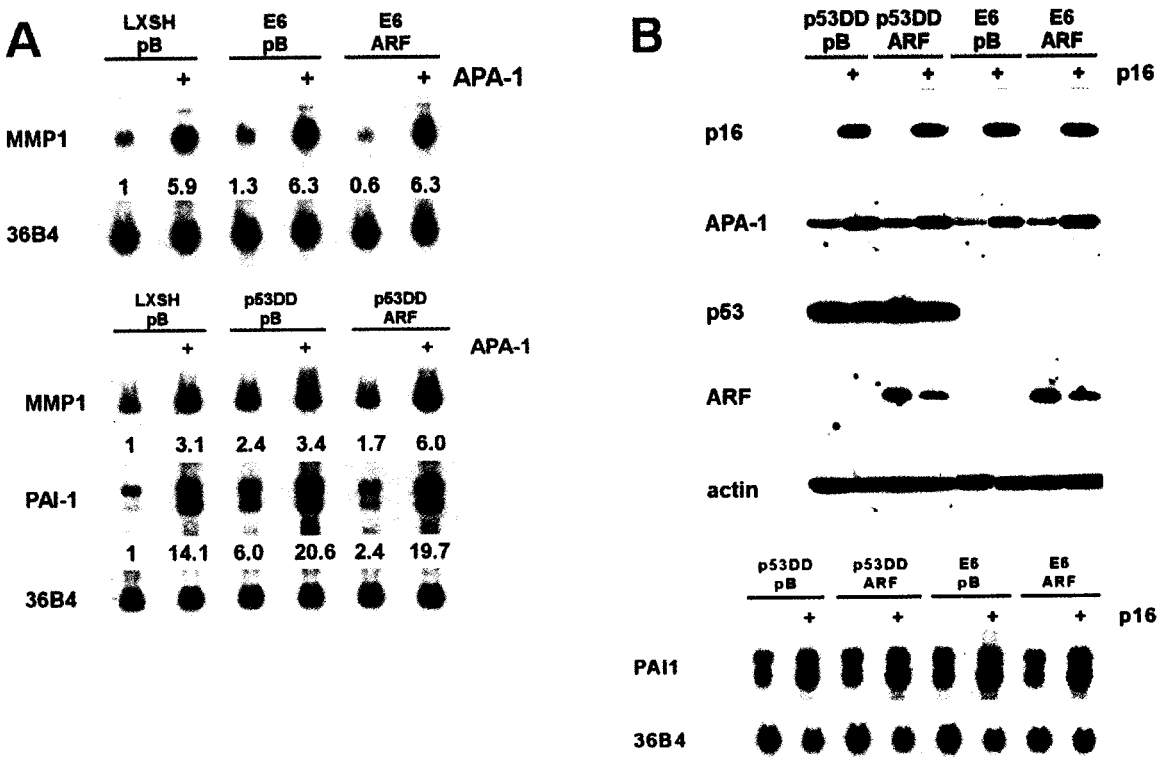

Figure 2.5. ARF can not inhibit induction of matrix-remodeling genes by APA-1. **(A)** HFFs expressing LXSH, E6, p53DD, pB and ARF as indicated were transduced with retrovirus expressing empty vector or APA-1. Following selection, cells were analyzed by northern blotting for the APA-1 target genes MMP1, PAI1 and 36B4 (loading control). Relative signal is shown beneath each lane. **(B)** HFFs expressing p53DD, E6, pB and ARF as indicated were transduced with retrovirus expressing empty vector or p16. Following selection lysates were analyzed by western blotting for p16, APA-1, p53, ARF and actin (upper panels) and PAI1 mRNA levels were analyzed by northern blotting (lower panels).

Chapter 3: Normal Human Fibroblasts are Resistant to RAS-induced Senescence

Summary

Oncogenic stimuli are thought to induce senescence in normal cells in order to protect against transformation, and to induce proliferation in cells with altered p53 and/or Rb pathways. In human fibroblasts RAS initiates senescence through upregulation of the cyclin dependent kinase inhibitor p16INK4A. I show here, that in contrast to cultured fibroblast strains, freshly isolated normal fibroblasts are resistant to RAS-induced senescence, and instead show some characteristics of transformation. RAS did not induce growth arrest or expression of senescence-associated β-galactosidase, and Rb remained hyperphosphorylated despite elevated levels of p16. Instead, RAS promoted anchorage-independent growth of normal fibroblasts, although expression of hTert with RAS increased colony formation and allowed normal fibroblasts to bypass contact inhibition. To test the hypothesis that p16 levels determine how cells respond to RAS, I expressed RAS in freshly isolated fibroblasts that expressed very low levels of p16, hTert-immortalized fibroblasts that had accumulated intermediate levels of p16, and IMR90 fibroblasts with high levels of p16. RAS induced growth arrest in cells with higher p16 levels and this effect was reversed by p16 knockdown in the hTert-immortalized fibroblasts. These findings indicate that culture-imposed stress sensitizes cells to RAS-induced arrest, whereas early passage cells do not arrest in response to RAS.

Introduction

Normal cells have several mechanisms in place to protect against uncontrolled proliferation and tumorigenesis. One line of defense is cellular senescence, a permanent growth arrest that occurs after extended periods of cell division, exposure to oxidative stress, or expression of activated oncogenes (Lundberg et al., 2000; Serrano and Blasco, 2001). Senescent cells remain alive and metabolically active, but are arrested in G1 of the cell cycle, are resistant to growth factor stimulation, and show common biochemical markers, such as expression of an acidic β-galactosidase activity (SA-βgal) (Campisi et al., 1996). In addition, senescent cells show altered differentiation functions that are cell type specific. For example, human fibroblasts secrete growth factors and extracellular matrix remodeling enzymes upon senescence (Campisi et al., 1996). While senescence has been characterized primarily in cultured cells, there is also evidence that it occurs *in vivo*. Cells expressing markers of senescence, such as SA-βgal, have been identified in normal tissues (Dimri et al., 1995), and senescence can be induced following some types of chemotherapeutic treatments (Schmitt et al., 2002).

The best characterized example of oncogene-induced senescence is the response of normal fibroblasts to expression of an activated allele of H-*ras* (H-*ras*V12). Normal ras proteins are important for transducing mitogenic signals in the cell and are mutated to constitutively active forms in approximately 20% of human cancers (Bos, 1989). These activated alleles contribute to transformation by increasing proliferation, angiogenesis, and invasion of tumors, as well as densensitizing cells to apoptosis (Downward, 2003).

All normal cells with intact p53 and Rb pathways are thought to senesce in response to RAS. Expression of viral oncoproteins that disrupt these pathways, such as SV40 T Ag (Michalovitz et al., 1987), HPV E6 or E7 (Liu et al., 1994; Phelps et al., 1988) or Adenovirus E1A (Ruley, 1983) block senescence and cooperate with RAS to transform cells. In murine cells, an intact p53/ARF pathway is required for both RAS- and passage-induced senescence (Kamijo et al., 1997; Serrano et al., 1997; Palmero et al., 1998). In human cells, the p16/Rb pathway plays a more significant role. Levels of p16 increase in response to either culture- (Ramirez et al., 2001; Benanti et al., 2002) or oncogene-induced (Serrano et al., 1997) stress, and high levels of p16 cause cell cycle arrest by inhibiting the G1 cyclin dependent kinases Cdk4 and Cdk6. Inhibition of these kinases by p16 prevents phosphorylation and inactivation of Rb, resulting in repression of E2F-dependent promoters and a G1 cell cycle arrest (Ruas and Peters, 1998). Recent studies have suggested that loss of p16 renders human fibroblasts resistant to RAS-induced senescence, since fibroblasts established from individuals carrying inactivating mutations in the *p16* gene do not arrest in response to RAS (Huot et al., 2002; Brookes et al., 2002). However, it remains controversial whether or not p16 loss is sufficient to prevent RAS-induced senescence. Data from other groups indicates that IMR90 (Serrano et al., 1997), BJ (Hahn et al., 2002), and LF1 (Wei et al., 2001; Wei et al., 2003) fibroblasts require disruption of both p16/Rb and p53/p21 pathways in order to prevent senescence induced by RAS.

It has been proposed that RAS-induced senescence in cultured cells models what happens when a normal cell acquires a RAS mutation *in vivo*, and that senescence

protects cells against RAS-mediated transformation. However, there is little evidence that expression of activated RAS *in vivo* leads to a senescence response. Several lines of transgenic mice expressing activated alleles of RAS have been generated and the tissues in these mice proliferate normally (Adams and Cory, 1991; Johnson et al., 2001; Guerra et al., 2003). This discrepency raises the question of whether cultured cells accurately represent cells *in vivo*, or if the process of establishment in culture somehow sensitizes cells to RAS-induced arrest.

In this study, I demonstrate that normal fibroblasts isolated directly from primary tissue are resistant to RAS-induced senescence because, unlike other cultured fibroblasts, they lack p16 expression at early passages. Furthermore, exposing normal fibroblasts to the stress of extended passaging in culture leads to an accumulation of p16, and increased p16 sensitizes cells to RAS-induced senescence. I also show that fibroblasts expressing RAS alone are capable of anchorage independent growth, and that co-expression of hTert increases the frequency of colony formation in soft agar and is required for cells to be able to bypass contact inhibition. These data suggest that expression of RAS in early passage normal fibroblasts is similar to what happens when a cell acquires an activating RAS mutation *in vivo*, and that RAS only causes senescence in cells that have been exposed to additional stress.

Materials and Methods

Cell Culture. Human foreskin fibroblasts (HFF1, HFF2) were isolated from neonatal human foreskins and infected with retroviruses within the first 20 population doublings.

IMR90 fibroblasts were obtained from ATCC and used within 10 population doublings. Extended passage HFFs expressing hTert have been described previously (Kiyono et al., 1998) and were infected with pBABE or 16-1 retroviruses at population doubling level 180 after selection. SV40 transformed Cl39T have also been described previously (Banga et al., 1997). All cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin.

Retroviral Infections. Retroviruses were produced and concentrated as previously described (Bartz and Vodicka, 1997). H-*ras*V12 was provided in pBABE-puro and pWZL-hygro retroviral vectors by Manuel Serrano (Spanish National Centre of Biotechnology) and subcloned into LXSN (LXSN/H-*ras*V12). pBABE-puro, LXSN, LXSH, and LXSN/p16 have been described elsewhere (Benanti et al., 2002). The hTert cDNA was cloned by PCR from a HeLa cDNA library (Clonetech) and was subsequently transferred into LXSN for retroviral expression. The p16 shRNA vector (16-1) was provided by Scott Lowe (Cold Spring Harbor Laboratory). 24 hours after infection cells were expanded 1:2 into complete media containing 1mg/ml G418, 1.5μg/ml puromycin, or 200μg/ml hygromycin, as appropriate. Selection in G418 or puromycin was usually complete within 3-5 days and selection in hygromycin was usually complete within 1 week. In all experiments, the day on which a parallel plate of uninfected target cells are completely killed in selective media is referred to as day 0. On day 0 cells were photographed, labeled with BrdU and fixed for immunofluorescence, and lysed for western blot analysis. In addition, on day 0 cells were split and replated for analysis at

later time points. In each case experiments were repeated multiple times and a representative experiment is shown.

BrdU staining. Cells were labeled with 40μM bromodeoxyuridine (BrdU) for 4 hours, fixed in 4% paraformaldehyde and permeablilized with methanol:acetone. Fixed cells were then analyzed by immunofluorescence using either mouse anti-BrdU antibodies provided with DNaseI from the manufacturer (Amersham-Pharmacia Biotech), or with mouse anti-BrdU (Sigma, BU-33) in blocking buffer (5% goat serum, 5% horse serum, 0.2% Tween-20 in PBS) and 20U/ml DNaseI (Sigma). Nuclei were also stained with DAPI (Sigma) in order to determine total number of cells in a given field. 100-500 nuclei per condition were photographed and the percentage of BrdU positive cells determined. Typically, 30-50% of nuclei in LXSN control populations were BrdU positive. For comparison, at each time point the percentage of BrdU positive cells in p16 and RAS expressing populations is shown relative to LXSN control cells.

Western Blotting. Preparation of whole cell lysates and western blotting were performed as previously described (Benanti et al., 2002). 40-80μg of protein were analyzed with antibodies to H-ras (Santa Cruz Biotechnology, F235), p16 (BD PharMingen), actin (Santa Cruz Biotechnology, I-19), APA-1 (Benanti et al., 2002), p53 (Oncogene Research Products, Ab6), p21 (Oncogene Research Products, Waf1 Ab1), Rb (BD PharMingen), p130 (Santa Cruz Biotechnology, C-20), and p27 (BD Transduction Laboratories, Clone 57).

Senescence-associated β-galactosidase staining. Acidic β-galactosidase staining was done as previously described (Dimri et al., 1995).

p16 and APA-1 immunofluorescence. Cells were grown on glass coverslips, fixed in 2% paraformaldehyde and premeablized with methanol:acetone. Fixed cells were then stained with mouse anti-p16 (E6H4, molecular tools in medicine) or rabbit anti-APA-1 (Benanti et al., 2002) and corresponding rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch). Cells were also stained with DAPI (Sigma) to visualize nuclei.

Telomerase activity. Telomerase activity was measured using the TRAPeze Telomerase Detection Kit (Serologicals Corporation).

Quiescence and cell cycle analysis. To arrest cells in G0, confluent cultures were incubated in complete growth media for 3 days, then refed with low serum media (DME containing 0.1% fetal bovine serum) and incubated for an additional 3 days. Cells were then replated at approximately 50% confluence in low serum media and incubated for an additional 2 days. At this stage (DS arrest) 3 plates of each cell type were labeled with 10μM BrdU for 4 hours, followed by fixation in 70% ethanol/PBS for flow cytometry. In addition, cells were harvested for western blot analysis as described above. Remaining plates were refed with complete media containing 10% serum and harvested in the same way after 24 hours. For cell cycle analysis, nuclei were isolated from fixed cells and stained with fluorescein isothiocyanate-conjugated anti-BrdU antibodies (Becton Dickinson) and propidium iodide as previously described (Benanti et al., 2002). Cell cycle fractions were quantitated with CellQuest software (Becton Dickinson).

In vitro transformation assays. To assay anchorage-independent growth, HFFs transduced with the indicated retroviruses were seeded into soft agar following

verification of RAS expression on day 11. 50,000 cells were resuspended in 0.3% noble agar/growth medium and plated, in triplicate, in 6-well dishes, on top of solidified 0.6% noble agar/growth medium. Fresh top agar was added every 4 days and cells were photographed and counted after 3 weeks. 3 fields of cells were counted in each of 3 wells per cell type. To assay contact inhibition, cells were plated in 6 well dishes at 100,000 cells/ well. Cells were trypsinized and counted in triplicate 4, 7 and 10 days following plating.

Results

Human foreskin fibroblasts do not senesce in response to RAS expression. While characterizing the response of freshly isolated, human foreskin fibroblasts (HFFs) to senescence induced by different signals, I found that, unlike other human fibroblasts, HFFs did not arrest following expression of RAS. These experiments were repeated in HFFs obtained from different donors with similar results (Figure 3.1, Figure 3.5, data not shown). I therefore decided to investigate the response of HFFs to RAS in greater detail.

Early passage HFFs were infected with retroviruses expressing empty vector (LXSN), p16 or RAS and cells were analyzed immediately following selection (day 0), as well as 5 and 13 days after selection. LXSN transduced cells resembled uninfected HFFs, and mitotic cells were evident in the population throughout the experiment (Figure 3.1A). In contrast, no mitotic cells could be detected in p16 infected cultures on day 0, and by day 5 most cells in the culture adopted an enlarged and flattened morphology, indicative of senescence. However, by day 13, the majority of p16-expressing cells appeared to have bypassed the senescent arrest and were indistinguishable from LXSN

controls. As reported by others, RAS-expressing cells appeared smaller and more refractile than controls immediately following selection, similar to cells transformed by RAS (Lin et al., 1998). However, unlike other fibroblasts that arrest approximately 5-6 days after selection (Lin et al., 1998), RAS-expressing HFFs did not appear senescent on day 5. A large number of small, dividing cells persisted in the culture throughout the experiment. Notably, the population was heterogeneous and large and flat cells were also present. This heterogeneity may be due in part to the heterogeneous nature of the population of fibroblasts that were established from the primary tissue.

I next labeled cells with BrdU and analyzed them by immunofluorescence. This type of analysis allowed me to determine if the flat, apparently senescent cells in the RAS expressing cultures were truly arrested, or if they were capable of entering S phase (BrdU+). On day 0 the majority of cells in the p16 infected culture were arrested, while RAS infected cells continued to proliferate (Figure 3.1B). As suggested by their appearance, on day 5 the p16 expressing cells remained arrested, while a large percentage of the RAS cells remained BrdU positive. BrdU incorporation in RAS cells persisted through day 13 and, consistent with their visual appearance, a large fraction of the p16 cells were also BrdU positive and appeared to have escaped cell cycle arrest on day 13. I also found that many of the senescent-looking, flat cells in the RAS infected population stained positive for BrdU (Figure 3.1C). Since senescence is defined by a G1 cell cycle arrest, I concluded that RAS-expressing HFFs were not senescent.

Western blotting confirmed that RAS protein levels were similar at each time point and showed the presence of p16 protein in RAS-expressing cells by days 5 and 13

(Figure 3.1D). Importantly, the amount of p16 expressed in RAS cells was significantly lower than the amount expressed directly from the p16 retrovirus, consistent with the fact that RAS expressing cells were not arrested in the cell cycle. In addition, on day 13 when the p16 cells appeared to resume cycling, the levels of p16 protein were decreased to levels similar to the amount of p16 in RAS cells. One possibility for the observed decrease in p16 levels on day 13 is that p16 expression levels may have been heterogeneous following retroviral infection, and cells expressing insufficient levels of p16 to cause cell cycle arrest had overtaken the culture. No similar decrease in RAS protein levels was observed in RAS-infected cultures at day 13, arguing that there was no selection against cells expressing high levels of RAS, and supporting the observation that RAS does not arrest HFFs. These data raised the possibility that RAS does not induce senescence in HFFs because p16 is not expressed at high enough levels to arrest the cell cycle.

The transcription factor APA-1 regulates transcription of extracellular matrix-remodeling genes during senescence and was examined as a marker of the functional changes that accompany senescence in human fibroblasts. As seen previously (Benanti et al., 2002), induction of premature senescence following p16 expression led to elevated APA-1 protein (Figure 3.1D). In contrast, RAS expression led to a reduction in APA-1, similar to what is observed in fibroblasts transformed by SV40 (Benanti et al., 2002). This pattern of APA-1 expression supported the model that RAS does not lead to senescence of HFFs, but instead causes changes indicative of transformation.

High levels of p16 in IMR90 cells correlate with sensitivity to RAS-induced senescence. In order to understand the reasons why HFFs were resistant to RAS-induced senescence, infection experiments were repeated in IMR90 fetal lung fibroblasts for comparison. Consistent with previous studies (Serrano et al., 1997; Lin et al., 1998), I found that IMR90 cells did undergo RAS-induced senescence (Figure 3.2). Similar to HFFs, RAS-expressing IMR90 cells were still cycling and appeared refractile immediately following selection (Fig 3.2A-B). However, on days 5 and 11, RAS cells appeared senescent and were arrested in the cell cycle, similar to the p16-expressing cells. IMR90 cells also differed from HFFs in their ability to bypass the growth arrest induced by p16 expression. On day 11 following selection, the majority of p16-expressing IMR90 cells still appeared senescent and were BrdU negative (Figure 3.2A-B), whereas in HFF populations, cells expressing lower levels of p16 grew out at later time points (Figure 3.1).

Expression of RAS and p16 in IMR90 cells was confirmed by western blotting (Figure 3.2C). Unlike HFFs that did not express any detectable p16 prior to infection, control IMR90 cells expressed p16 protein, which increased after infection with the p16 retrovirus. RAS expression also led to increased p16 levels on day 5 and day 11; however the increases were not as dramatic as in HFFs, probably due to their high level of p16 prior to RAS infection. As with HFFs, APA-1 was increased in IMR90 cells in response to p16 expression (Figure 3.2C). Surprisingly, APA-1 levels were again reduced following RAS expression in IMR90 cells, suggesting that downregulation of APA-1 by RAS overrides the upregulation of APA-1 in response to senescence.

Recent studies have shown that human fibroblasts with inactivating mutations in the p16 gene fail to undergo RAS-induced senescence (Huot et al., 2002; Brookes et al., 2002). This suggested that HFFs may have failed to arrest in response to RAS because p16 was not induced to sufficient levels. Direct comparison of HFF and IMR90 cells 5 days after selection supported this model. As shown in Figure 3.3A, a comparison of cells transduced with empty vector revealed that IMR90 control cells expressed p16 protein while none was detectable in HFFs. As noted above, p16 increased in both HFF and IMR90 cells following RAS expression. However, RAS-expressing HFFs had much lower levels of p16, even lower than control IMR90 cells. This data suggested that since IMR90 cells had high initial levels of p16, RAS expression increased p16 past a threshold that resulted in cell cycle arrest. The difference in p16 induction between cell types could not be explained by differences in RAS expression, as both expressed equivalent levels of RAS protein.

Rb expression was also compared in infected HFF and IMR90 cells 5 days after selection. During senescence induced by p16 or RAS, Cdk4/6 associated kinase activity should be inhibited and Rb in a hypophosphorylated, active form. Consistent with this model, Rb was exclusively hypophosphorylated in IMR90 cells expressing p16 or RAS (Figure 3.3A). Expression of p16 in HFFs also caused a shift in Rb phosphorylation to primarily the hypophosphorylated form. In contrast, the majority of Rb in RAS-expressing HFFs was hyperphosphorylated, similar to vector transduced controls, although overall levels of Rb were reduced.

The cyclin dependent kinase inhibitor p21 increases in response to RAS in human fibroblasts (Serrano et al., 1997; Wei et al., 2001). I examined p21 and p53 levels in HFF and IMR90 cells and found little difference between the cell types (Figure 3.3A). p21 increased slightly in both RAS-expressing HFFs and IMR90 cells, however HFFs were not arrested. This supported the model that p21 was not the critical factor leading to RAS-induced growth arrest.

Finally, I compared the expression of senescence-associated β-galactosidase (SA-βgal) expression in HFFs and IMR90 cells on day 5 following selection. IMR90 cells induced robust SA-βgal staining following expression of either p16 or RAS (Figure 3.3B). In HFFs, p16 expression also induced SA-βgal, although to a lesser extent than in IMR90 cells (19% compared to 38%, respectively). Consistent with other experiments, HFFs expressing RAS were primarily SA-βgal negative. Approximately 1% of HFFs with RAS stained positive, compared to 48% in IMR90 cells. These data supported the conclusion that RAS does not induce senescence in HFFs.

p16 levels sensitize HFFs to RAS-induced senescence. p16 levels increase when human fibroblasts are exposed to extended passaging in culture or to stress from sub-optimal growth conditions (Benanti et al., 2002; Ramirez et al., 2001). Since high p16 levels correlated with sensitivity to RAS-induced senescence, I hypothesized that *in vitro* aging may sensitize HFFs to RAS-induced senescence and provide a mechanistic explaination for the resistance seen in early passage cells. To address this possibility, I decided to examine the consequences of RAS expression in hTert-immortalized HFFs that were approximately 150 population doublings past the time when control HFFs

reached replicative senescence, and had elevated levels of p16 (Figure 3.4A). p16 was undetectable in early passage HFFs by immunofluorescence and appeared to be upregulated in almost all cells as they approached senescence, as well as in extended passage HFFs (Figure 3.4B). APA-1 levels also increased in all cells as they were passaged, indicating that culture-imposed changes were equivalent in all cells in the population.

The effects of RAS expression were next analyzed in extended passage HFFs. To confirm that p16 accumulation is the critical factor that sensitizes cells to RAS-mediated arrest, I also reduced p16 levels in extended passage HFFs by expression of a short hairpin RNA that specifically targets p16 (Narita et al., 2003). Control (pB) or p16 hairpin (16-1) expressing cells were transduced with retroviruses expressing a second empty vector (LXSH) or RAS. Cells were selected in hygromycin and analyzed immediately following selection (Day 0), 6 and 11 days later. As seen previously, all cells were cycling on day 0 (Figure 3.4C). However, on days 6 and 11 there were obvious differences between pB/RAS and 16-1/RAS cells. While small dividing cells were continually seen in the 16-1/RAS population, pB/RAS cells took on a senescent morphology and arrested in the cell cycle (data not shown, Figure 3.4C). Western blot analysis confirmed that pB control cells had increased levels of p16 protein on days 6 and 11 following RAS expression. In contrast, 16-1 cells had a reduced level of p16 protein at all time points, and it did not increase following expression of RAS (Figure 3.4D). These data demonstrated that p16 levels were the critical factor in determining whether or not HFFs will senesce in response to expression of RAS.

Growth properties of RAS-expressing HFFs. In most cell types, disruption of the p53 and Rb pathways allows cells to bypass RAS-induced senescence (Serrano et al., 1997). When RAS is expressed in these cell types, they exhibit properties indicative of transformed cells such as the ability to grow anchorage-independently and to bypass contact inhibition. In human cells, telomerase (hTert) is also often required for transformation, as the proliferation of many cell types is quickly limited by telomere length. Since early passage HFFs did not undergo senescence in response to RAS, I wished to test whether cells expressing RAS exhibited any properties of transformed cells. Although telomere length is not limiting in early passage HFFs, cells expressing hTert were also tested, in order to determine if telomerase could contribute to transformation by RAS.

Directly following isolation from the primary tissue, HFFs were infected with retroviruses expressing an empty vector (LXSN) or hTert. Following selection in G418, cells were infected with retroviruses expressing a second empty vector (pB) or RAS, selected in puromycin, and analyzed as described above. As seen before, small, dividing cells were observed immediately following selection (day 0), as well as on days 5 and 11 (Figure 3.5A). All cells incorporated BrdU into their DNA at similar levels (Figure 3.5B), and RAS expression led to increased p16 levels by day 5 following infection (Figure 3.5C). In addition, RAS expression had no effect on the amount of active telomerase in the cell (Figure 3.5D). All four cell types were continually passaged in culture until LXSN/pB and LXSN/RAS cells reached senescence after approximately 46 population doublings following selection. Therefore, RAS expression did not affect the

lifespan of HFFs. hTert/pB and hTert/RAS cells were passaged for approximately 20 additional population doublings and showed no signs of senescence when the experiment was ended.

I next determined whether RAS expression had an effect on the ability of cells to enter quiescence in response to contact inhibition and serum starvation. LXSN/pB, LXSN/RAS, hTert/pB and hTert/RAS fibroblasts were first held at confluence for 3 days in 10% serum, then refed with media containing 0.1% serum for 3 days, and finally replated at sub-confluent densities in 0.1% serum. Cells were harvested for western blotting and labeled with BrdU and fixed for cell cycle analysis. At the same time, a second set of cells were stimulated to reenter the cell cycle with 10% serum and analyzed 24 hours later. All four cell populations had approximately 2% of cells in S phase following this protocol, indicating that all cells could be arrested by a combination of density and serum starvation (Figure 3.6A). By comparison, by 24 hours following serum addition, approximately 20% of cells in all populations were in S phase.

Although RAS-expressing cells arrested following density and serum starvation, RAS altered the cell cycle profiles of the arrested cells. While 79% LXSN/pB control cells arrested with a 2N DNA content, consistent with a G0 arrest, only 56% of LXSN/RAS cells arrested in G0. Instead, 42% of LXSN/RAS cells arrested with a G2/M DNA content, compared to 19% in controls, suggesting that RAS expression may allow cells to bypass G0 arrest and arrest in G2/M. Surprisingly, it appeared that expression of hTert reversed the effects of RAS. hTert/RAS cells contained 84% of cells arrested in G0 and only 13% in G2/M, more similar to LXSN/pB and hTert/pB cells than cells

expressing RAS alone. An increased fraction of cells in G2/M was also observed in normally cycling LXSN/RAS cells that had reentered the cell cycle following serum addtion, however the differences were more apparent following DS arrest. RAS and p16 expression was similar in all cells, regardless of whether they were cycling or not (Figure 3.6B). In addition, all four cell types were found to have reduced Rb, increased p130 and increased p27 proteins follwing DS arrest. These changes are expected in quiescent cells (Dyson, 1998; Hengst and Reed, 1998). These results suggest that although RAS expression does not block quiescence, it alters normal cell cycle progression.

Early passage RAS-expressing cells were next seeded into soft agar and tested for their ability to grow in an anchorage independent manner. After 21 days in culture, both LXSN/RAS and hTert/RAS cells formed colonies of similar size (Figure 3.7A). However, hTert/RAS cells formed approximately twice as many colonies as LXSN/RAS cells (Figure 3.7B), suggesting that hTert could contribute to anchorage independent growth. Soft agar assays were initiated with cells at an early time point after selection, as well as cells that had been passaged for approximately 20 population doublings with simialr results.

RAS transformed cells can also bypass contact inhibition and form foci in culture dishes. To determine if RAS-expressing HFFs could bypass contact inhibition, cells were seeded into culture dishes and counted in triplicate on the indicated days. All cells appeared near confluent on day 4, although a few mitotic cells could be seen. HFFs expressing RAS alone were similar to vector alone controls (Figure 3.7C), demonstrating that expression of RAS alone did not allow cells ty bypass contact inhibition.

Surprisingly, at every time point hTert/RAS cells were considerably denser than cells expressing either hTert or RAS alone. Cells expressing hTert alone reached higher densities than controls at each time point, however they were always less dense than hTert/RAS cells, and were more similar in appearance to controls than to hTert/RAS cells (Figure 3.7D). By day 13 after plating, hTert/RAS cells did not form distinct foci but had grown in multiple layers on top of one another and came off of the plate in a sheet following trypsin treatment. It was not possible to achieve an accurate cell count at this time point, since hTert/RAS cells could not easily be dissociated from one another. This indicated that the hTert/RAS cells not only had bypassed contact inhibition, but were also capable of growing in the absence of adherence to a substratum. These growth characteristics were a striking contrast to control cells and cells expressing either RAS or hTert alone, which remained in monolayers. I observed that all HFF lines did not easily undergo contact inhibition, since even on day 13 mitotic cells were found in all cultures (Figure 3.7D). Experiments were repeated in cells at an early passage following selection as well as cells at approximately 24 population doublings after selection with similar results. These data demonstrated that although RAS-expressing HFFs have the ability to grow anchorage-independently, co-expression of hTert increases the frequency of colony formation in soft agar. In addition, expression of hTert was required for RAS-expressing HFFs to bypass contact inhibition.

Discussion

In this study I examined the response of freshly isolated human foreskin fibroblasts to expression of RAS and demonstrated that, in contrast to other isolates of normal cells, HFFs are resistant to RAS-induced senescence. While RAS arrests IMR90 fibroblasts by 5 days following selection, HFFs continue to proliferate. Consistent with this observation, RAS-expressing HFFs are capable of entering S phase, have hyperphosphorylated Rb, and are predominantly negative for senescence-associated β-galactosidase expression. In addition, these cells are capable of anchorage-independent growth, one hallmark of transformed cells.

Studies in other types of human fibroblasts have demonstrated that the cyclin dependent kinase inhibitor p16 is important for RAS-induced senescence. RAS expression is thought to activate the MAPK pathway, which leads to induction of the Ets1 and Ets2 transcription factors and activation of p16 transcription (Lin et al., 1998; Zhu et al., 1998; Ohtani et al., 2001). However, in fibroblasts that carry inactivating mutations in the p16 gene, RAS does not induce senescence and cells are able to grow in soft agar (Brookes et al., 2002; Huot et al., 2002). Our finding that HFFs are resistant to RAS-induced senescence is consistent with this model. Although p16 levels did increase in HFFs following RAS expression, our results suggest that the level of p16 was not sufficient to cause growth arrest. It is known that human fibroblasts vary in their basal level of p16 expression (Itahana et al., 2003), and therefore it is likely that fibroblasts will have variable responses following RAS expression. Similarly, the mechanism of passage-induced senescence in human fibroblasts varies depending on p16 levels. Some

strains of human fibroblasts, such as WI-38 and IMR90, express high levels of p16 and that p16 limits the lifespan of these cell types (Itahana et al., 2003; Benanti and Galloway, unpublished results). In contrast, strains such as BJ and HFFs express lower levels of p16 and their lifespans are limited primarily by telomere length (Itahana et al., 2003) .

One proposed explaination for these differences between fibroblast strains is that they are derived from different tissue types. Both IMR90 and WI-38 cells were derived from lung, whereas BJ cells, like HFFs, were derived from neonatal foreskin. However, tissue of origin can not explain the differences in response to RAS expression. BJ cells are known to senesce following RAS expression (Seger et al., 2002), yet HFFs do not, and they are derived from the same tissue type. A more likely explaination is that BJ cells, like WI-38 and IMR90, have been exposed to a greater amount of culture-induced stress when they were established. In support of this, early passage BJ cells have higher levels of p16 than HFFs (Figure 3.4A) and are sensitive to RAS-induced senescence. Freshly established HFFs have not been exposed to prolonged stress, do not express any detectable p16 and are therefore resistant to RAS-induced senescence. This idea is supported by our finding that extended passaging of HFFs leads to increased levels of p16 and sensitizes cells to RAS-mediated arrest. Some evidence suggests that this effect may be mediated by increased mitochondria and levels of oxidative stress during passage- and RAS-induced senescence (Lee et al., 1999; Xu and Finkel, 2002). It is possible that differences in mitochondrial function may explain the variable p16 levels in different fibroblast strains.

It is important to note that other investigators have found that both the p16/Rb and p53/p21 pathways need to be disrupted in order for human fibroblasts to be resistant to RAS-induced senescence. It has been reported that expression of RAS in IMR90 (Serrano et al., 1997) and LF1 (Wei et al., 2001; Wei et al., 2003) fibroblasts induces expression of p53 and p21, in addition to p16. However, I only observed a very weak induction of p53 and p21 in HFFs, and saw no increase in these proteins in IMR90 fibroblasts. The reason for this discrepency remains unclear, but it is likely that because the p53 pathway is not strongly activated following RAS expression in our system, disruption of this pathway is not required for cells to be resistant to RAS-induced senescence.

In other types of human fibroblasts, disruption of the p16/Rb pathway and expression of RAS are sufficient to induce the ability to grow in soft agar, however this is not sufficient to permit cells to form tumors in nude mice (Brookes et al., 2002; Seger et al., 2002). Consistent with these reports, early passage HFFs expressing RAS were capable of growth in soft agar. Surprisingly, I found that hTert expression increased the frequency of soft agar colony formation and appeared to be required for RAS to promote the bypass of contact inhibition. Although these cells show characteristics of transformation in culture, it is likely that they would not be tumorigenic in vivo. When compared to soft agar colonies formed by the transformed 293T cell lines (data not shown), RAS-expressing HFFs formed relatively small colonies. In addition, expression of RAS and hTert in Leiden fibroblasts lacking functional p16 protein allows growth in soft agar and the bypass of contact inhibition, however these are not sufficient for

tumorigenicity in vivo (Brookes et al., 2002). Moreover, LF1 and BJ fibroblasts expressing RAS along with disruptions in the p16 and p53 pathways are capable of producing small colonies in soft agar, but not tumors in mice (Seger et al., 2002; Wei et al., 2003). In BJ and LF1 cells, expression of another cooperating gene such as SV40 ST is required for cells to be able to form tumors in vivo (Hahn et al., 2002; Wei et al., 2003) and it is likely this would be the case in HFFs as well.

There is a growing body of evidence supporting a role for hTert in transformation, independent of its ability to lengthen telomeres and immortalize cells. Evidence from mice that overexpress mTert in skin or cardiac myocytes argues that forced telomerase expression can promote cell growth (Gonzalez-Suarez et al., 2001; Oh et al., 2001), and in human cells telomerase has been shown to affect transcription of growth promoting genes (Lindvall et al., 2003; Smith et al., 2003). In virus transformed human cells, telomerase has been shown to extend lifespan without net telomere lengthening (Zhu et al., 1999). In addition, human fibroblasts that are immortalized by a telomerase-independent mechanism are not transformed by RAS unless hTert is also expressed (Stewart et al., 2002). Early passage HFFs should provide a good system to study the contribution of hTert to transformation, since these cells proliferate for 70-90 population doublings in culture before telomere length becomes limiting. A second advantage to studying transformation in HFFs is that the p16/Rb pathway does not need to be inactivated to study the effects of RAS. I have found that RAS expression affects the cell cycle distribution of HFFs, but that it does not impair the ability of cells to enter quiescence. Moreover, coexpression of hTert modifies this cell cycle effect. It will be of

considerable interest to determine the mechanism of these cell cycle alterations and how hTert interacts with RAS in this setting.

In conclusion, I have shown that normal human fibroblasts do not undergo senescence in response to RAS expression. My findings are also consistent with what is known about RAS expression *in vivo*. Several cancer models have been developed by generating mice that express activated alleles of RAS. The tissues in these transgenic animals proliferate normally, suggesting that RAS may not in fact induce senescence *in vivo* (Adams and Cory, 1991; Johnson et al., 2001; Guerra et al., 2003). In addition, there is no evidence that RAS can cause senescence in human tissues. Activating mutations in RAS are believed to be early events during the development of human cancer (Bos, 1989). RAS mutations are thought to cause a burst of hyperproliferation, allowing for clonal expansion and an increased chance of acquiring additional mutations in tumor suppressor gene pathways. If a cell were to undergo senescence within a few population doublings following RAS activation, it would be improbable that additional growth-promoting mutations would arise and that RAS would have a tumor promoting effect (Jones et al., 2000). In this way, early passage human fibroblasts may be representative of cells *in vivo* and provide a good system to study the effects of RAS expression in human cells.

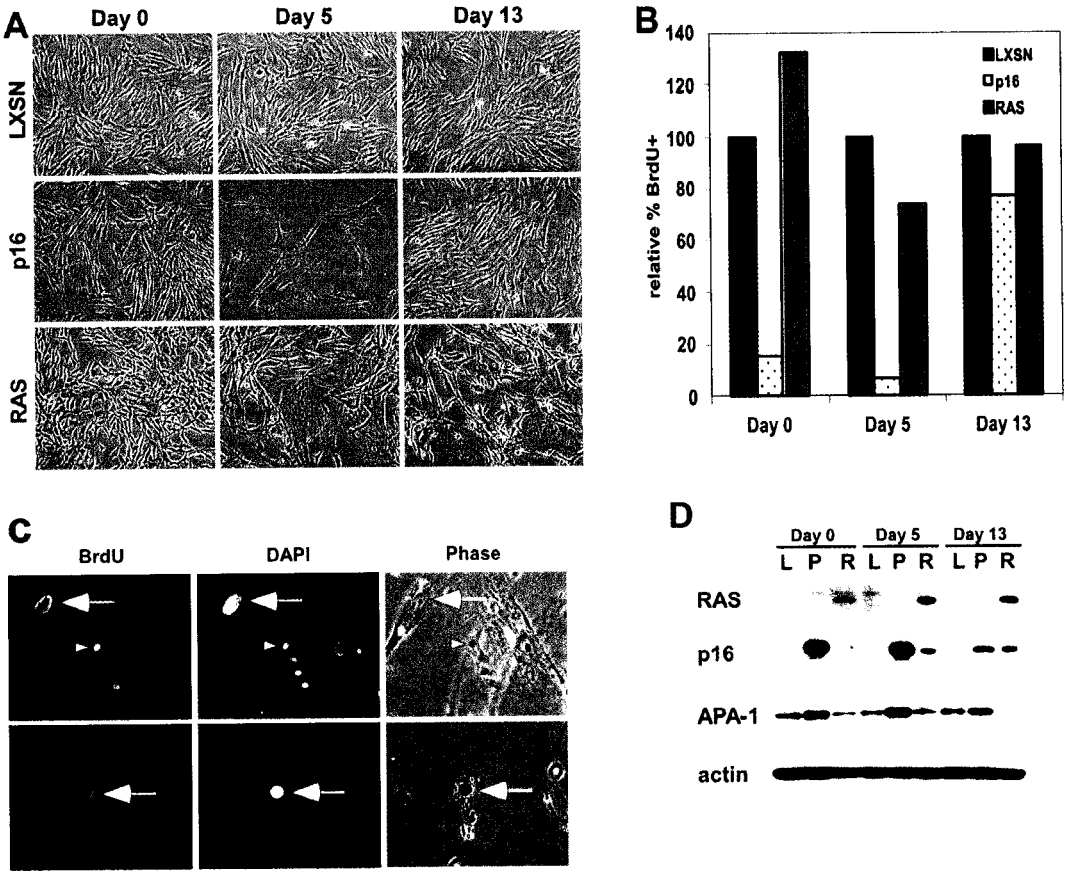

Figure 3.1. HFFs do not senesce in response to RAS expression. (A) Early passage HFF1 cells were infected with LXSN, p16 or RAS expressing retroviruses. Cells were photographed immediately following selection (Day 0), 5 and 13 days later. **(B)** LXSN cells (black bars), p16 cells (speckled bars) and RAS cells (gray bars) from (A) were labeled with BrdU, fixed, stained with anti-BrdU antibody, and counted. Percentage of BrdU positive cells, relative to LXSN controls, are shown. **(C)** Representative BrdU staining in RAS expressing HFFs on day 5. As seen in (A), RAS cultures were a heterogeneous population consisting of large, apparantly senescent nuclei (large arrows) and smaller nuclei (small arrowheads). A fraction of both large and small nuceli were BrdU positive. **(D)** LXSN (L), p16 (P), and RAS (R) expressing cells from (A) were harvested on days 0, 5 and 13. Levels of RAS, p16 and APA-1 proteins were examined by western blot. Actin is shown as a loading control.

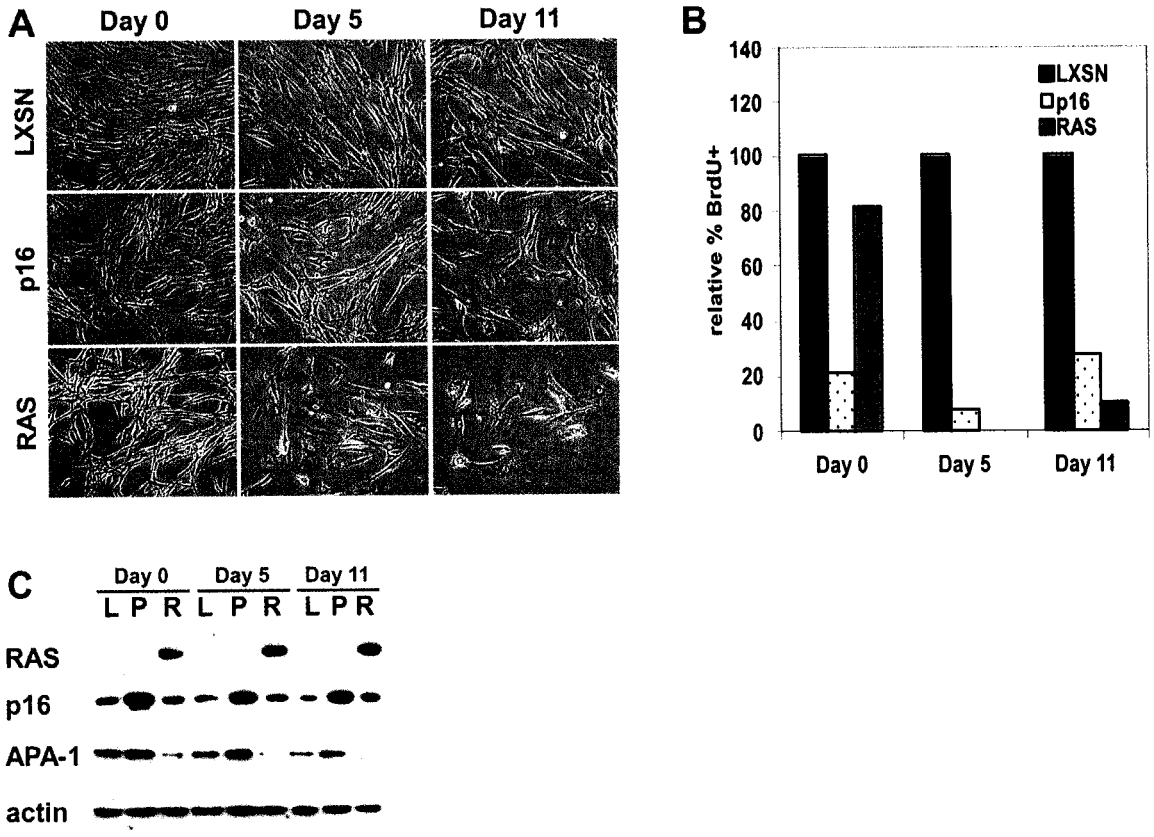

Figure 3.2. IMR90 cells arrest following RAS expression. (A) IMR90 cells were infected with LXSN, p16 or RAS expressing retroviruses. Cells were photographed immediately following selection (Day 0), 5 and 11 days later. **(B)** LXSN cells (black bars), p16 cells (speckled bars) and RAS cells (gray bars) from (A) were labeled with BrdU, fixed, stained with anti-BrdU antibody, and counted. Percentage of BrdU positive cells, relative to LXSN controls, are shown. **(C)** LXSN (L), p16 (P), and RAS (R) expressing cells from (A) were harvested on days 0, 5 and 13. Levels of RAS, p16 and APA-1 proteins were examined by western blot. Actin is shown as a loading control.

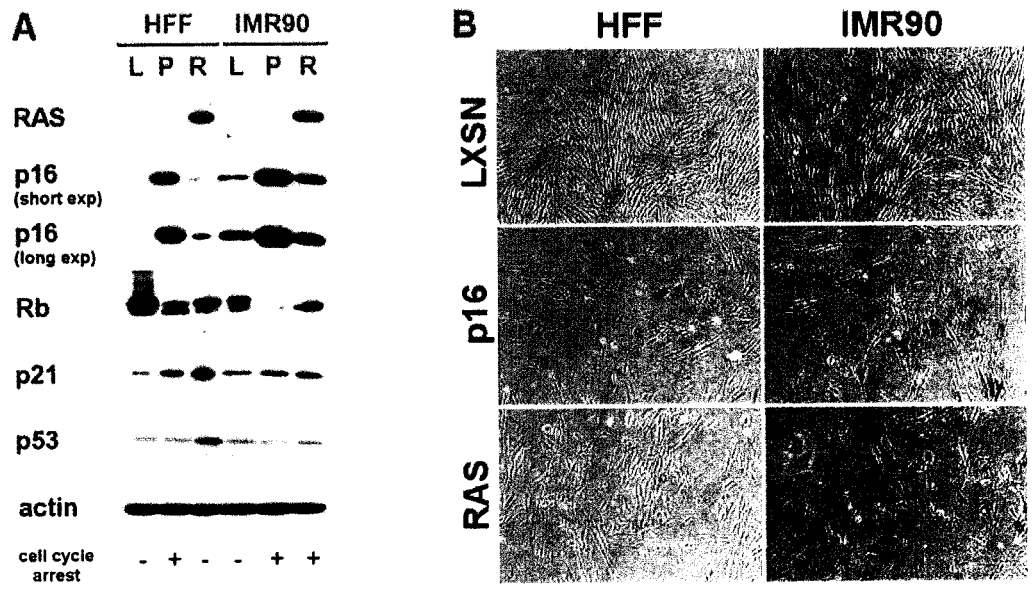

Figure 3.3. Comparison of HFF1 and IMR90 cells 5 days following selection. (A) Western blots of lysates from LXSN (L), p16 (P) and RAS (R) expressing HFF1 and IMR90 cells harvested 5 days following selection. Blots were probed with antibodies to RAS, p16 (short and long exposures are shown), Rb, p21, p53, and actin. Corresponding cell cycle arrest is indicated beneath the blots. **(B)** HFF1 and IMR90 fibroblasts infected with LXSN, p16 or RAS were fixed 5 days following selection and stained for senescence-associated β-galactosidase expression. Representative fields are shown.

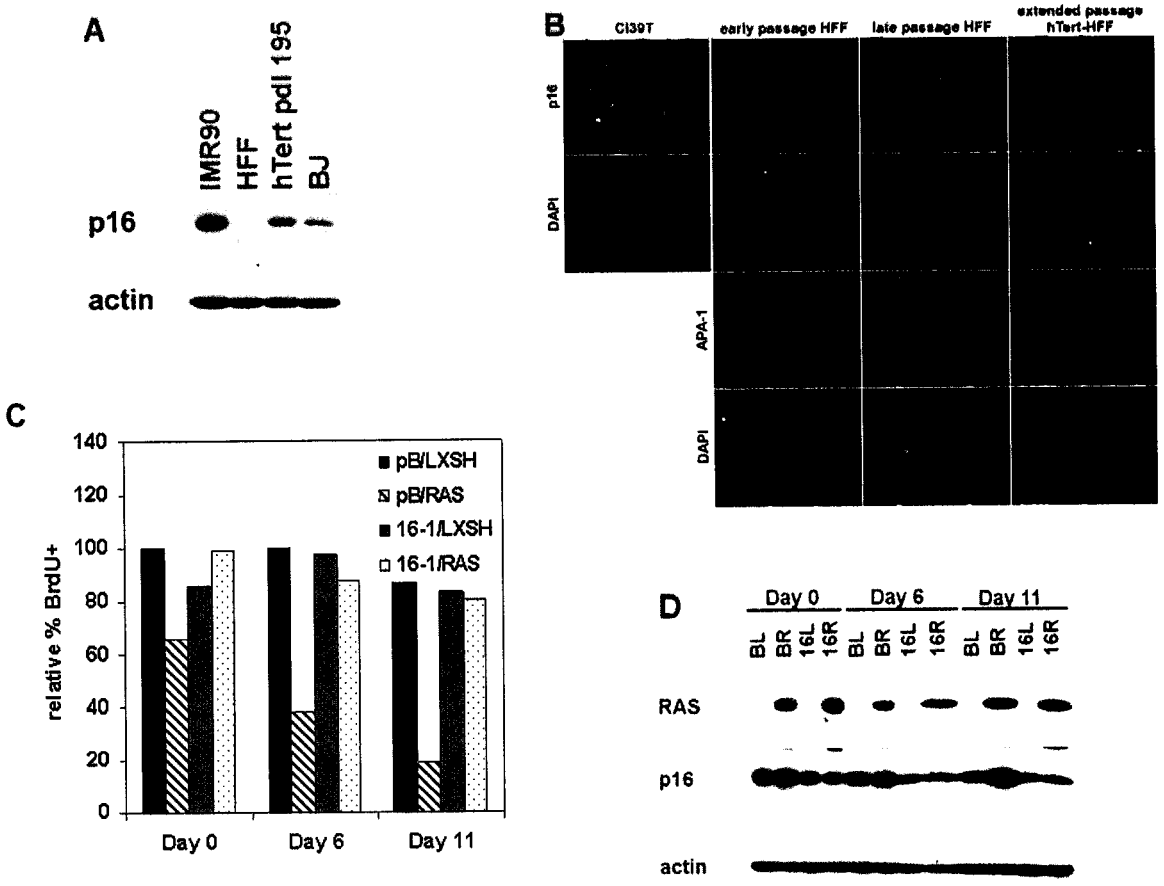

Figure 3.4. Expression of RAS in extended passage, hTert immortalized HFFs. (A) Western blot of p16 protein in IMR90 fibroblasts, early passage HFFs, hTert immortalized HFFs at 195 population doublings (pdl) after selection (corresponding control HFFs senesced at pdl 44), and BJ fibroblasts. Actin is shown as a loading control. **(B)** p16 and APA-1 immunofluorescence in C139T SV40 transformed fibroblasts, early passage HFFs, late passage HFFs (just before senescence), and hTert immortalized HFFs at 148 pdl after selection. Nuclei are visualized by corresponding DAPI staining. **(C)** pB/LXSH (black bars), pB/RAS (hatched bars), 16-1/LXSH (gray bars) and 16-1/RAS (speckled bars) cells from (A) were labeled with BrdU, fixed, stained with anti-BrdU antibody, and counted. Percentage of BrdU positive cells, relative to LXSN controls, are shown. **(D)** pB/LXSH (BL), pB/RAS (BR), 16-1/LXSH (16L), and 16-1/RAS (16R) cells from (A) were analyzed by western blotting on days 0, 6 and 11. Levels of RAS, p16 and actin are shown.

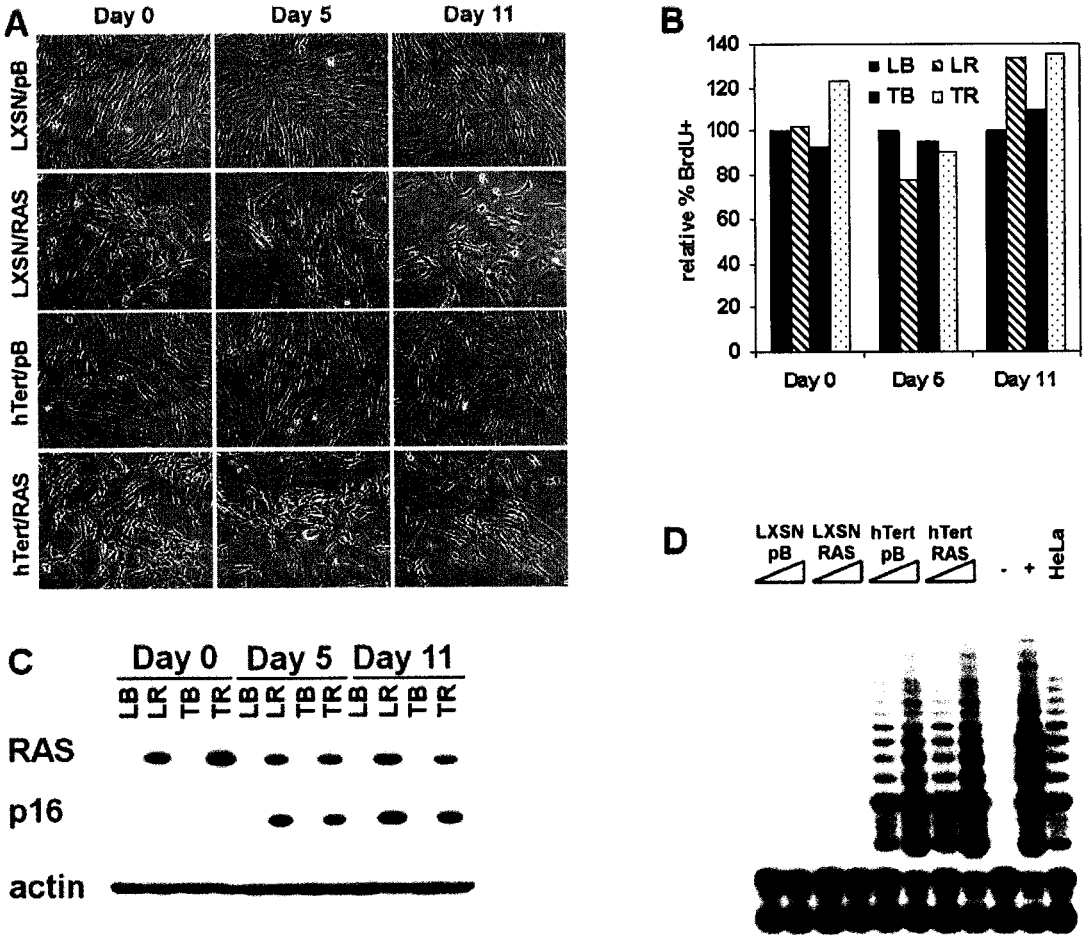

Figure 3.5. Expression of RAS in early passage HFF2 cells. (A) Early passage HFF2 fibroblasts expressing LXSN or hTert were infected with retroviruses expressing pB empty vector or pB/RAS. Cells were photographed immediately following selection (Day 0), 5 and 11 days later. **(B)** LXSN/pB (LB, black bars), LXSN/RAS (LR, hatched bars), hTert/pB (TB, gray bars) and hTert/RAS (TR, speckled bars) cells from (A) were labeled with BrdU, fixed, stained with anti-BrdU antibody, and counted. Percentage of BrdU positive cells, relative to LXSN controls, are shown. **(C)** RAS, p16 and actin protein levels were examined in cells described in (A) on day 0, 5 and 11, as indicated. **(D)** Telomerase activity in cells from (A) was analyzed 14 days following selection using the TRAPeze kit. 0.2 and 2μg of protein lysate was analyzed for each cell population and compared to a buffer alone control (-), the positive control provided with the kit (+), and 0.1μg of HeLa extract.

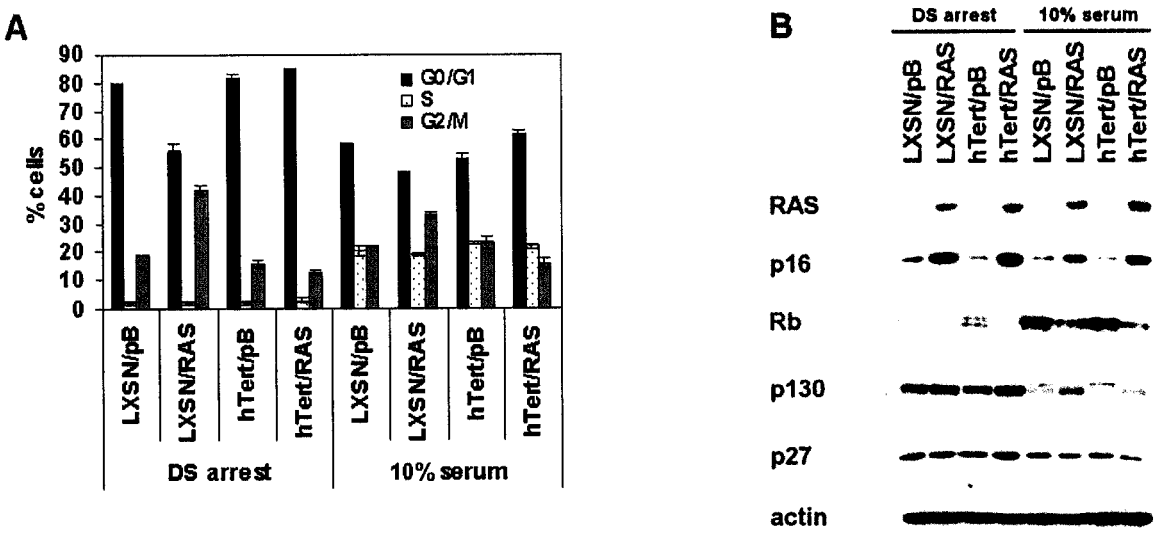

Figure 3.6. Quiescence in RAS-expressing HFFs. (A) Triplicate plates of cells described in Figure 5 were arrested by density and serum starvation as described in the text, labeled with BrdU, and fixed for cell cycle analysis (DS arrest). A second set of plates was stimulated to reenter the cell cycle by the addtion of 10% serum, and then harvested 24 hours later (10% serum). Percentage of cells in corresponding phases of the cell cycle are indicated; black bars represent G0/G1 cells with a 2N DNA content, speckled bars indicated BrdU positive cells that are in S phase, and grey bars indicate cells that are in G2/M and have a 4N DNA content. **(B)** An additional set of plates from (A) were harvested and analyzed by western blot analysis for RAS, p16, Rb, p130, p27 and actin.

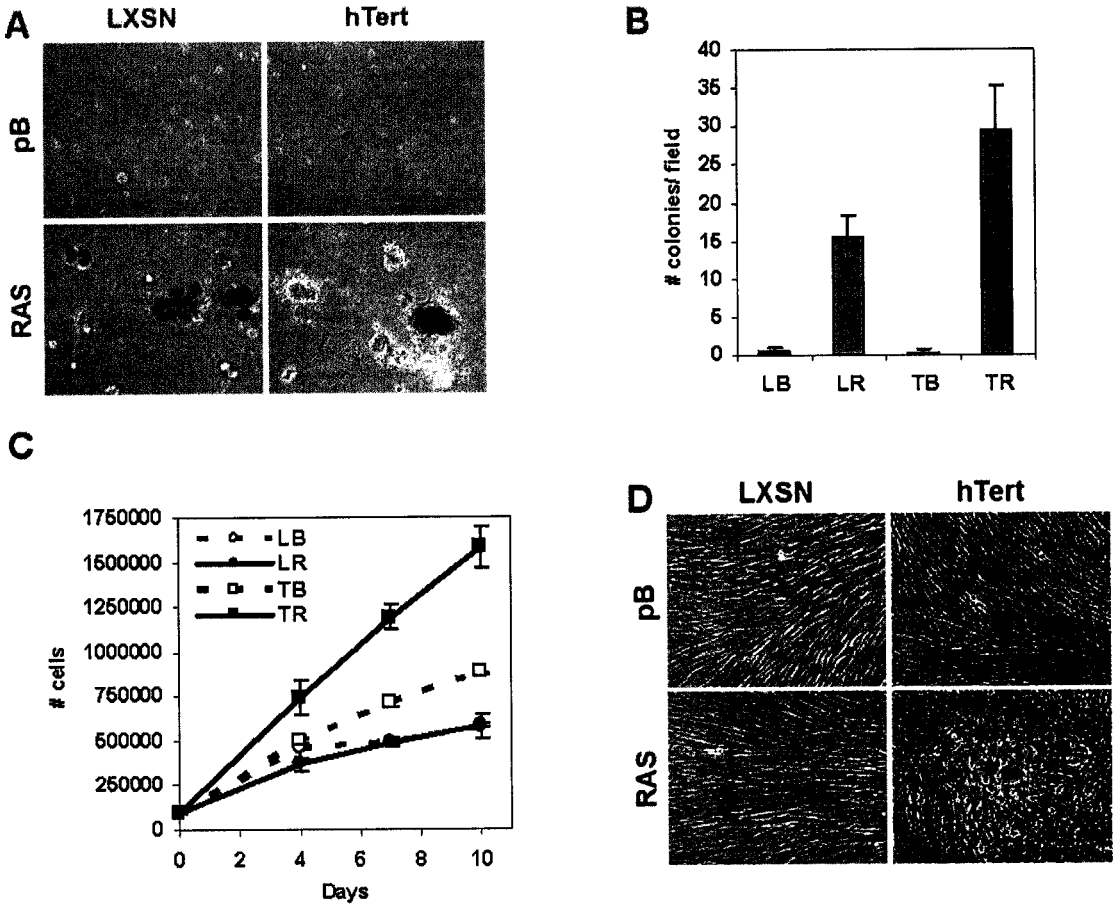

Figure 3.7. *In vitro* transformation of RAS-expressing HFFs. (A) Cells described in Figure 5 were seeded into soft agar at 50,000 cells/well and fed every 4 days. Cells were photographed after 21 days. **(B)** Quantitation of colonies from (A). Colonies in three representative fields per well were counted for LXSN/pB (LB), LXSN/RAS (LR), hTert/pB (TB), and hTert/RAS (TR) cells and averaged together. Average values from triplicate wells are represented in the graph. **(C)** Cells from Figure 5 were plated in 6 well dishes at 100,000 cells per well and fed every 3 days. Triplicate wells were counted on days 4, 7 and 10 after plating. Average cell numbers are indicated for LXSN/pB (LB; open circles, dashed line), LXSN/RAS (LR; filled circles, solid line), hTert/pB (TB; open squares, dashed line), and hTert/RAS (TR; filled squares, solid line) cells. **(D)** Photographs of cells from (C) on day 13 after plating. LXSN/pB, LXSN/RAS, and hTert/pB all appeared to be monolayers while hTert/RAS cells grew in multiple layers.

Conclusions and Future Directions

The primary goal of my research has been to understand how different aspects of cellular senescence are regulated in human fibroblasts. Since the limited proliferative capacity of normal cells acts as a barrier to cancer development, studying the pathways that regulate senescence should provide a better understanding of the changes that cells undergo as they become immortalized and are transformed. The role of telomere shortening in inducing the senescent cell cycle arrest in human fibroblasts has been studied in great detail (Mathon and Lloyd, 2001). My work suggests that there are also significant telomere-independent changes that occur during the aging of human fibroblasts. I identified a novel transcription factor, APA-1, that is involved in regulating gene expression changes during fibroblast senescence, and showed that APA-1 and its target genes are regulated in a telomere length-independent manner. APA-1 was originally identified by virtue of its interaction with the tumor suppressor ARF, a protein involved in senescence in murine fibroblasts. I have investigated the significance of this interaction and a potential role for ARF in regulation of APA-1 function. Finally, I discovered that the telomere-independent accumulation of p16 that occurs in aging human fibroblasts has important functional consequences, and is required to sensitize cells to senescence induced by oncogenic RAS. These findings suggest that both telomere-dependent and telomere-independent pathways control aspects of human fibroblast senescence, and that these pathways may act together to protect against transformation.

Regulation of senescence-associated gene expression changes by APA-1. Other groups have demonstrated that senescent fibroblasts have an altered profile of transcription factor-binding activities (Dimri and Campisi, 1994), and that the expression of transcription factors such as c-fos (Seshadri and Campisi, 1990), Id1, Id2 (Soares et al., 1994), and Ets1 (Ohtani et al., 2001) are altered in senescent cells. However, no factor has been identified that coordinately regulates senescence-associated genes during senescence. My results suggest that the transcrption factor APA-1 may act in this way. APA-1 regulates several genes involved in extracellular matrix-remodeling, a phenotype known to be associated with fibroblast senescence. I demonstrated that APA-1 interacts directly with the promoter of one gene, *MMP1*, at a sequence adjacent to the TATA box and just upstream of the translation initiation site. Although I have not analyzed the interaction between APA-1 and other promoters it is interesting to note that several other putative APA-1 target genes have large blocks of similar sequences in their promoters, including serum responsive elements (Westermarck and Kahari, 1999). In the future it will be important to carry out further mutagenesis of the *MMP1* promoter and identify a consensus APA-1 binding site. In addition, it will be useful to test for collaboration between APA-1 and other transcription factors with known binding sites in the promoters of APA-1 target genes.

The gene expression profile of senescent human fibroblasts resembles the profile of fibroblasts stimulated with serum (Shelton et al., 1999; Iyer et al., 1999) and suggests that fibroblasts become locked into an activated, or wound-healing response, when they undergo senescence. Since I found that APA-1 regulates transcription of several genes

involved in this process, it is also possible that APA-1 may have a role in transcriptional regulation during the wound healing process. I found that APA-1 is expressed at very high levels in several strains of primary human fibroblasts, and that is expressed at much lower levels in epithelial cells and transformed cell lines. These observations support the hypothesis that APA-1 may be important for a fibroblast-specific function *in vivo*, such as activating genes required for wound healing. It will be important to investigate this possibility further and determine if APA-1 is activated in response to serum stimulation or wounding.

Although senescence is primarily thought of as a tumor suppression mechanism, recent findings suggest that senescent fibroblasts promote proliferation and transformation of epithelial cells (Krtolica et al., 2001). This observation is consistent with the fact that senescent fibroblasts are similar to activated fibroblasts that secrete growth factors and extracellular matrix-remodeling enzymes, all of which can affect the tissue microenvironment as well as neighboring cells. Carcinoma-associated fibroblasts have been reported to exhibit a similar expression profile and also affect the tissue microenvironment. In fact, when cultured with initiated epithelial cells, carcinoma-associated fibroblasts stimulate epithelial tumorigenesis (Olumi et al., 1999). Since APA-1 appears to regulate at least a subset of these secreted proteins, it is possible that APA-1 could be activated in carcinoma-associated fibroblasts as well as senescent cells. It will be of considerable interest to determine if APA-1 is upregulated in carcinoma-associated fibroblasts, and if so, to determine if APA-1 is required for the observed stimulatory effect on epithelial cell proliferation.

Telomere-independent regulation of APA-1 and extracellular matrix-remodeling genes during senescence. Since senescence in fibroblasts is known to be regulated by telomere shortening, I examined APA-1 in telomerase-immortalized fibroblasts and found that APA-1 and its target genes are regulated, at least in part, by a telomere length-independent pathway. This is a significant finding as it provides the first evidence that senescence-associated gene expression changes and the senscent cell cycle arrest are regulated by separate pathways.

Some clues suggest that the telomere-independent pathway may involve an accumulation of oxidative stress. The tumor suppressor p16 is also upregulated in aging human fibroblasts, in a telomere-length independent manner, and altering growth media or oxygen levels alters the timing of p16 upregulation (Ramirez et al., 2001; Itahana et al., 2003). In addition, expression of the polycomb protein Bmi-1 (Itahana et al., 2003), Ets1, and Id1 transcription factors (Ohtani et al., 2001) are regulated in an age-dependent manner and activate p16 transcription during senescence. It remains to be determined if APA-1 accumulation is regulated by the same signals that regulate p16. Preliminary experiments suggest that Bmi-1 overexpression does not affect APA-1 (Benanti and Galloway, unpublished results), so although it remains possible that oxidative stress leads to APA-1 accumulation, it is likely that different factors are involved downstream of the oxidative stress signal. The role of oxidative stress in APA-1 regulation can be investigated by growing cells in low oxygen conditions or by exposing cells to an oxidative stress such as hydrogen peroxide, and determining if APA-1 levels are affected.

APA-1 does not appear to be regulated by age at a transcriptional level, since mRNA levels remain constant throughout the lifespan of fibroblasts. However, APA-1 protein increases substantially as cells are passaged. I found that the majority of APA-1 in fibroblasts is modified by the ubiquitin-like protein SUMO-1, and that sumoylation stabilizes APA-1 protein. A likely possibility is that the factors responsible for SUMO modification increase in fibroblasts as they age, and this results in APA-1 stabilization and accumulation. An important future question will be to determine what factors are required for APA-1 sumoylation. One way to identify such factors would be to purify tagged APA-1 and associated proteins from cells and then identify binding partners by mass spectrometry. This type of experiment may lead to the identification of factors that are known to be in the SUMO pathway and that can interact with APA-1. Candidate genes can then be tested to determine if they are involved in APA-1 sumoylation and if they are regulated in an age-dependent manner. It is also possible that there is a more general association between SUMO modification and senescence. Expression of PML has been shown to stabilize p53 and promote senescence in human fibroblasts (Bischof et al., 2002). Interestingly, both PML and p53 can be sumoylated (Sternsdorf et al., 1997; Kamitani et al., 1998; Muller et al., 1998; Gostissa et al., 1999; Rodriguez et al., 1999). An intriguing possibility is that there may be a general increase in SUMO modifications in older cells, and that increased sumoylation may lead to a general increase in protein stability.

Impact of ARF on transactivation by APA-1. At the time these studies began, the tumor suppressor ARF was known to be required for senescence in murine fibroblasts,

however its role in senescence of human fibroblasts was unknown. Murine fibroblasts senesce in response to a telomere-independent pathway and ARF is thought to be upregulated in response to culture-imposed stress (Sherr and DePinho, 2000). Since APA-1 was identified as an ARF interacting protein, and also appeared to be upregulated in senescent cells by a telomere-independent pathway, I hypothesized that there may be a functional interaction between ARF and APA-1.

Unfortunately, despite good evidence for a physical interaction between the two proteins, it remains unclear whether ARF can affect APA-1 function. Reporter assays and gel shift analyses suggested that ARF may inhibit APA-1-DNA binding and block APA-1 mediated transactivation. However, APA-1 was still capable of activating transcription of endogenous target genes in cells that were engineered to express ARF protein. It will be difficult to resolve these conflicting findings without a better understanding of the modification and regulation of APA-1. It is possible that ARF can affect one form of APA-1 and not the other; hopefully the experiments suggested above will help to clarify these issues. It also remains unclear how ARF is regulated in human cells. ARF is not normally expressed at detectable levels in fibroblasts, and although there is good evidence that oncogenes such as RAS can induce ARF in the mouse (Palmero et al., 1998), RAS has no effect on ARF expression in human cells (Wei et al., 2001). A better understanding of the signals that regulate ARF in human cells may clarify how it interacts with APA-1.

Functional consequences of p16 upregulation in aging fibroblasts. Previous work demonstrated that p16 levels increase in senescent human fibroblasts independent of

telomere shortening, however levels do not accumulate to an extent necessary to elicit cell cycle arrest (Kiyono et al., 1998). I found that passage-induced upregulation of p16 does have important functional consequences, and that it sensitizes fibroblasts to senescence induced by oncogenic RAS. When RAS was expressed in early passage foreskin fibroblasts, directly after isolation from the primary tissue, cells did not undergo senescence. However, after extended passaging in culutre, p16 levels increased and sensitized cells to RAS-induced arrest. Previously it was assumed that all normal cells with intact p53 and Rb pathways senesce following RAS expression (Serrano and Blasco, 2001). Based on this belief it has been proposed that many oncogenic signals can promote senescence, and that oncogene-induced senscence protects against transformation. My work suggests that oncogenes can induce senescence, but exposure to other types of stress may be a necessary cofactor for this arrest to occur. It will be important to determine if this observation can be extended to senescence induced by other oncogenes, such as E2F. It is possible that early passage HFFs may also be resistant to E2F-induced senescence and that other factors will be required for the senescence response.

hTert cooperates with RAS to transform human cells. Since RAS expression did not cause senescence in early passage HFFs, I tested whether RAS expression promoted transformation-associated properties. In addition, I tested whether coexpression of hTert could cooperate with RAS to transform cells, since many previous studies have examined RAS in cells immortalized by hTert. Interestingly, I found that RAS expression alone could promote anchorage-independent growth of HFFs, however other changes are

required for full transformation. Coexpression of hTert not only increased the frequency of colony formation, it was required to allow cells to bypass contact inhibition. There is precedent that hTert has telomere-length independent effects. Several groups have shown that hTert affects cell growth and expression of growth promoting genes (Gonzalez-Suarez et al., 2001; Oh et al., 2001; Lindvall et al., 2003; Smith et al., 2003). In addition, hTert expression is required for RAS-mediated transformation of fibroblasts that lengthen telomeres through a telomerase-independent mechanism (Stewart et al., 2002). My finding that this cooperative effect of RAS and hTert can be assayed easily in early passage HFFs will allow further investigation into the mechanism of this cooperation. One way to address this question would be to use microarray analysis to identify genes whose regulation is changed in cells expressing hTert and RAS, but not in cells expressing either gene alone. These genes, as well as downstream target genes of RAS and hTert can be tested to see if they are required for the collaborative effect. This could be accomplished in HFFs engineered to express siRNAs that knock-down expression of candidate genes.

The discovery that RAS can be expressed in normal cells with intact p53 and Rb pathways also allows a careful examination of the effects of RAS on normal cell cycle progression. Initial results suggest that cells expressing RAS can still be driven into quiescence, however the cell cycle profile of these cells is altered. A smaller percentage of RAS-expressing HFFs are in G1 of the cell cycle, with a corresponding increase in cells found in G2/M. It will be important to further characterize these cell cycle effects and determine how RAS acts to drive cells through G1.

In conclusion, my studies have elucidated factors that are involved in telomere length-independent regulation of senescence in human fibroblasts. These findings have implications for our understanding of senescence as a tumor suppression mechanism. My discovery that senescence-associated functional changes occur through a telomere-independent pathway suggests that these changes may be a more general consequence of aging, and also occur *in vivo* in cells that are immortalized by telomerase activation. These changes may have important implications for cancer development since fibroblasts that have this activated phenotype have been shown to promote transformation of epithelial cells. My work has also demonstrated that telomere-independent accumulation of p16 cooperates with expression of oncogenic RAS to induce senescence, and raises questions about whether expression of an oncogene is sufficient to induce senescence *in vivo*. Results of this study suggest that telomere-independent aging effects are likely to have an important role in the development of cancer.

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