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ATM-dependent telomere loss in aging human diploid fibroblasts and DNA damage lead to the post-translational activation of p53 protein involving poly(ADP-ribose) polymerase

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Telomere loss has been proposed as a mechanism for counting cell divisions during aging in normal somatic cells. How such a mitotic clock initiates the intracellular signalling events that culminate in G_1 cell cycle arrest and senescence to restrict the lifespan of normal human cells is not known. We investigated the possibility that critically short telomere length activates a DNA damage response pathway involving p53 and $p21^{WAF1}$ in aging cells. We show that the DNA binding and transcriptional activity of p53 protein increases with cell age in the absence of any marked increase in the level of p53 protein, and that $p21^{WAF1}$ promoter activity in senescent cells is dependent on both p53 and the transcriptional co-activator p300. Moreover, we detected increased specific activity of p53 protein in AT fibroblasts, which exhibit accelerated telomere loss and undergo premature senescence, compared with normal fibroblasts. We investigated the possibility that poly(ADP-ribose) polymerase is involved in the post-translational activation of p53 protein in aging cells. We show that p53 protein can associate with PARP and inhibition of PARP activity leads to abrogation of p21 and mdm2 expression in response to DNA damage. Moreover, inhibition of PARP activity leads to extension of cellular lifespan. In contrast, hyperoxia, an activator of PARP, is associated with accelerated telomere loss, activation of p53 and premature senescence. We propose that p53 is post-translationally activated not only in response to DNA damage but also in response to the critical shortening of telomeres that occurs during cellular aging.

Keywords: ATM/p53/$p21^{WAF1}$/PARP/telomeres

Introduction

Normal human diploid fibroblasts (HDFs) are mortal and undergo a limited number of population doublings in culture (Hayflick and Moorhead, 1961), after which they enter a viable but non-proliferative phase known as senescence. Senescent cells arrest primarily in the G_1 phase of the cell cycle (Sherwood *et al.*, 1988). This proliferative block can be overcome upon expression of the SV40 large T antigen or the HPV-16 E6/E7 proteins, resulting in an extension of cellular lifespan (Shay *et al.*, 1993). SV40 large T antigen is known to bind the p53 tumor suppressor protein and to interfere with its DNA binding and transcriptional activation function. HPV-16 E6 protein also binds p53 protein and promotes its degradation by the ubiquitin-dependent proteolytic pathway. Hence, both proteins disrupt the function of p53, suggesting that p53 protein likely serves as a key regulator of cellular senescence. Consistent with this idea are the findings that spontaneous loss of *p53* alleles in HDFs is associated with extension of cellular lifespan (Rogan *et al.*, 1995) and that expression of certain dominant negative *p53* alleles can extend the cellular lifespan of human (Bond *et al.*, 1994; Gollahon and Shay, 1996) and rat fibroblasts (Rovinski and Benchimol, 1988).

Aging in HDFs (Harley *et al.*, 1990; Lindsey *et al.*, 1991; Allsopp *et al.*, 1992) and in human hematopoietic cells (Hastie *et al.*, 1990; Vaziri *et al.*, 1993, 1994; Metcalfe *et al.*, 1996) is accompanied by the progressive loss of telomeric DNA. Expression of the enzyme telomerase in immortal cells provides one mechanism for the prevention of telomere shortening (Morin, 1989; Counter *et al.*, 1992; Kim *et al.*, 1994). Persistent loss of telomeres in the absence of telomerase could lead to a critically short telomere length, cell cycle arrest and senescence (Allsopp and Harley, 1995). However, it is unclear how shortened telomeres give rise to the anti-proliferative signals that result in senescence. One clue was provided by Benn (1976) and Sherwood *et al.* (1988), who reported an increase in the incidence of dicentric chromosomes in aging cell populations. Dicentric chromosomes are likely the result of recombination occurring at the shortened and exposed telomeric ends of chromosomes. We suggested previously that dicentric chromosomes, upon breakage at mitosis, might activate a p53-dependent DNA damage pathway leading to cell cycle arrest (Vaziri and Benchimol, 1996). Several lines of evidence are consistent with this model. First, the $p21^{WAF1}$ gene, which encodes an inhibitor of cyclin-dependent kinases (CDK) (Harper *et al.*, 1993; Dulic *et al.*, 1994), is transcriptionally regulated by p53 (El-Deiry *et al.*, 1993) and is over-expressed in senescent cells (Noda *et al.*, 1994). Overexpression of a transfected $p21^{WAF1}$ gene in recipient cells results in G_1 cell cycle arrest (Yang *et al.*, 1995). Second, exposure of HDFs to γ-irradiation leads to a p53-dependent, prolonged G_1 arrest and induction of $p21^{WAF1}$ expression that is reminiscent of senescence (Di Leonardo *et al.*, 1994). Third, DNA binding experiments indicate that binding of p53 protein to the p53-responsive element present on the ribosomal gene cluster (RGC) DNA fragment increases in senescent cells (Atadja *et al.*, 1995). Together, these results suggest that p53 protein may be activated during cell aging

to promote transcription of the *p21*^{WAF1} gene. *p21*^{WAF1} expression, however, is known to be regulated through p53-dependent and p53-independent pathways (Michieli *et al.*, 1994; Datto *et al.*, 1995). In this study we have investigated further the role of p53 and *p21*^{WAF1} in cell aging and demonstrate that the DNA binding activity of p53 protein on the p53-responsive element present in the human *p21*^{WAF1} promoter increases during cell aging. In addition, we show that *p21*^{WAF1} expression at senescence is dependent on p53 as well as on the transcriptional co-activator p300.

A number of genes encode proteins that may be capable of sensing DNA damage. ATM and poly(ADP-ribose) polymerase (PARP) represent two such proteins and both have been implicated in the p53-dependent DNA damage response pathway (Kastan *et al.*, 1992; Lu and Lane, 1993; Wesierska *et al.*, 1996). HDFs from individuals with ataxia telangiectasia (AT), an autosomal recessive disease characterized by sensitivity to ionizing radiation, sterility, immune dysfunction, telangiectasia and premature aging, undergo premature senescence in culture (Shiloh *et al.*, 1982). The gene that is defective in AT patients, called *ATM*, is related to the *TEL1* gene of *Saccharomyces cerevisiae* and encodes a protein with similarity to the phosphatidylinositol 3-kinase family of proteins. Inactivation of *TEL1* in *S.cerevisiae* leads to rapid telomere loss (Greenwell *et al.*, 1995). Furthermore, fibroblasts from mice lacking ATM undergo premature senescence in culture (Xu and Baltimore, 1996). We demonstrate that telomeric DNA is rapidly lost in human AT fibroblasts and that this telomere shortening is associated with premature senescence and increased DNA binding activity of p53 protein. PARP is strongly activated by binding to DNA strand breaks produced by various DNA damaging agents, including hydrogen peroxide, and undergoes rapid automodification by synthesizing long branches of poly(ADP-ribose) (Lindahl *et al.*, 1995). We report that PARP and p53 protein associate *in vitro* and *in vivo*. Hyperoxia, which is known to activate PARP (Zhang *et al.*, 1994), leads to accelerated loss of telomeres, activation of p53 protein and premature senescence. Furthermore, inactivation of either p53 or PARP in HDFs leads to extension of cellular lifespan. Together, these data implicate ATM, PARP, p53 and *p21*^{WAF1} as key components in a telomere loss/DNA damage signaling pathway that is activated in human cells during cell aging.

Results

DNA binding activity of p53 protein is altered during cellular aging

Nuclear injection of linearized plasmid DNA or circular DNA with a large gap is sufficient to induce p53-mediated growth arrest, a process that is known to depend on the DNA binding activity of p53 (Huang *et al.*, 1996). Moreover, the generation of double-stranded DNA breaks in response to ionizing radiation has been shown to increase the DNA binding activity of p53 (Reed *et al.*, 1995). If double-stranded breaks or large gaps in chromosomal DNA occur during the process of cellular aging in culture, one might expect to see increased DNA binding activity of p53 as a consequence of such DNA damage. Therefore, we examined the DNA binding activity of p53 protein using an electrophoretic mobility shift assay (EMSA) with a ^{32}P-labeled double-stranded oligonucleotide containing the p53 consensus binding sequence (p53CON) (Funk *et al.*, 1992) and nuclear extracts prepared from the human fibroblast strain MRC-5 at different population doublings (PDs). Inclusion of the p53-specific monoclonal antibody PAb421 in the binding reaction facilitated visualization of the p53–DNA complex. An increase in DNA binding activity of p53 protein was observed as MRC-5 cells approached senescence (Figure 1A and B). This observation was confirmed in two other human fibroblast strains, WI-38 and BJ (Figure 1C). Competition with an excess of unlabled p53CON oligonucleotide but not with an equivalent amount of unrelated oligonucleotide with a similar nucleotide composition to p53CON confirmed that binding was specific (Figure 1A). As a further control for specificity, binding to p53 was observed when an extract from the p53-overexpressing cell line SF1 was used (Figure 1A) but not when an extract prepared from p53-null cells was used (data not shown).

When the DNA binding activity was normalized to the amount of p53 protein present in extracts from young and old cells by Western blotting (Figure 1D), we found a 1.9-fold increase in the DNA binding activity of p53 in old MRC-5 cells ($n = 2$), a 5.5-fold increase in old WI-38 and a 1.5-fold increase in old BJ cells relative to their young counterparts (Figure 1E). Hence, the increase in DNA binding cannot be ascribed to elevated levels of p53 protein and could reflect an age-related post-translational change in p53 protein.

The frequency of dicentric chromosomes was shown previously to increase in aging MRC-5 cultures (Benn, 1976), possibly as the result of telomere shortening (Harley, 1991). In Figure 1F we superimpose our data showing age-related changes in the DNA binding activity of p53 protein with the frequency of dicentric chromosomes in aging MRC-5 cells. The DNA binding data was obtained from five independent experiments. The activity peaked in near senescent cultures as cellular proliferation declined and then decreased in cells that had reached senescence. The incidence of dicentric chromosomes near senescence peaks at the same time as the DNA binding activity of p53 (Figure 1F). It is of interest to note that extracts prepared from aging WI-38 cells, which have a higher incidence of dicentric chromosomes than MRC-5 cells (Benn, 1976), also exhibit a higher level of DNA binding by p53 after normalization for p53 protein content (Figure 1E). Since dicentric chromosomes undergo breakage and fusion cycles during mitosis, it is possible that the resulting DNA strand breaks activate the latent DNA binding activity of p53 protein. We suggest that senescence-associated DNA damage (Figure 1F), acting through p53, could initiate the events that lead to cellular senescence.

Since the increased DNA binding activity of p53 was measured in the presence of PAb421 antibodies, we considered the possibility that the DNA binding assay reflected greater accessibility of p53 protein to PAb421 antibodies, perhaps due to a conformational change and/or post-translation modification of p53 in old cells. While

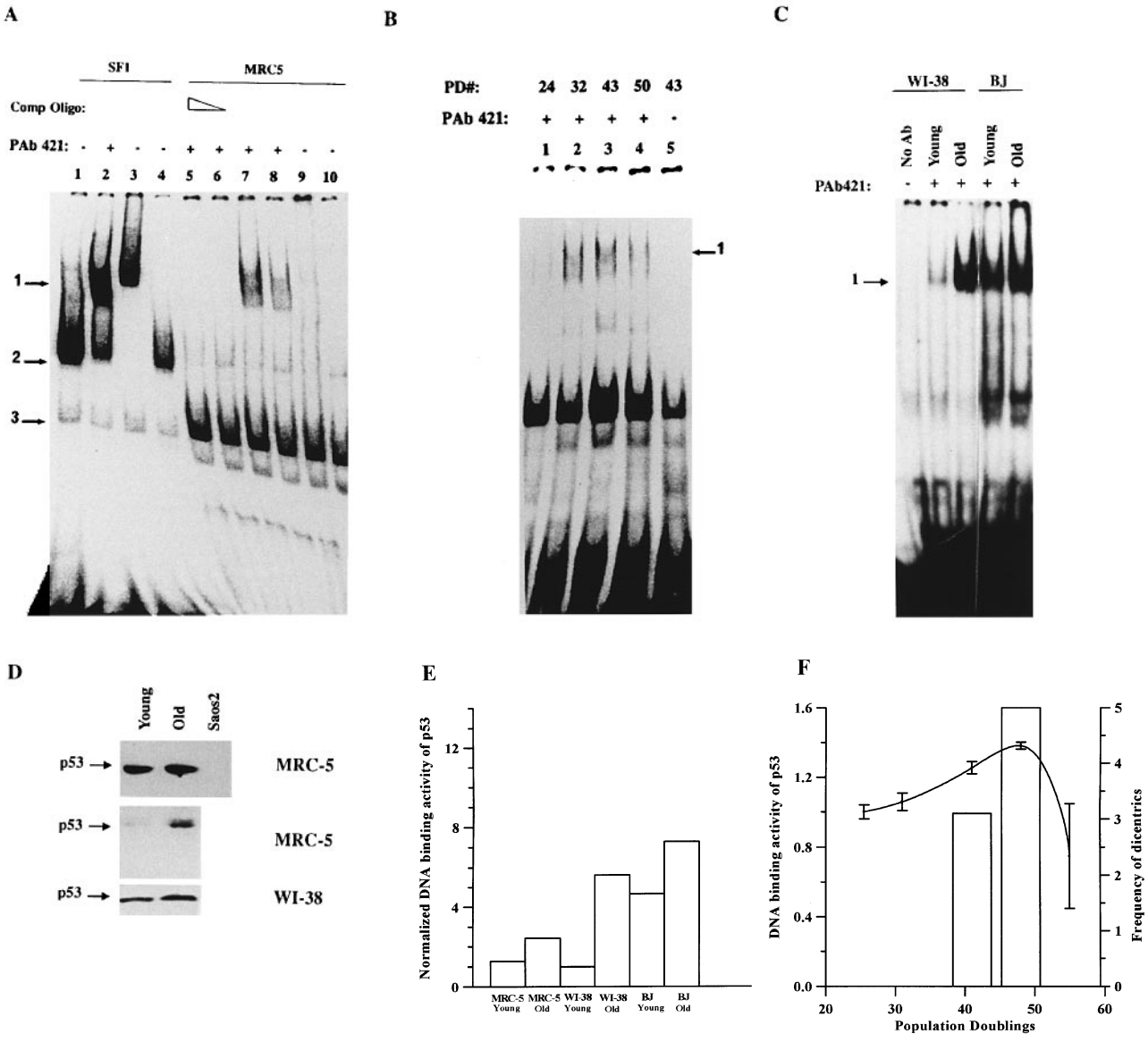


Fig. 1. DNA binding activity of p53 protein increases with cell age. (**A**) Nuclear extracts (5 μg) prepared from SF1 or MRC-5 cells were incubated with a ^{32}P-labeled double-stranded oligonucleotide containing the p53 consensus binding sequence (p53CON) with (+) or without (–) the p53-specific monoclonal antibody PAb421 and analyzed by EMSA. SF1 cells which overexpress p53 protein were used as controls in lanes 1–4. MRC-5 cells at PD32 were used in lanes 8 and 10 and at PD47 in lanes 5–7 and 9. The binding reactions were supplemented with the human p53-specific monoclonal antibody PAb1801 (lane 3) or control IgG antibodies (lane 4). Lanes 5 and 6 contained cold competitor p53CON at 50-fold and 10-fold molar excess over the labeled p53CON substrate; lanes 7 and 8 contained a 50-fold excess of an unrelated, cold double-stranded mutated oligonucleotide with the same nucleotide composition as p53CON. Arrow 1 on the left indicates the position of the supershifted PAb421–p53 protein–DNA complex, arrow 2 is the p53 protein–DNA complex and arrow 3 is a non-specific band. (**B**) Nuclear extracts prepared from MRC-5 cells at different PDs were tested for p53 DNA binding activity; arrow 1 indicates the position of the PAb421–p53 protein–DNA complex. (**C**) DNA binding activity of p53 in nuclear extracts prepared from young and old WI-38 and BJ cells; arrow 1 indicates the position of the PAb421–p53 protein–DNA complex. (**D**) Western immunoblot analysis of p53 protein in young and old MRC-5 cells (top) and in young and old WI-38 cells (bottom). The p53-negative cell line Saos2 was included as a control. Total cell lysates (300 μg protein) were resolved by SDS–PAGE; p53 protein was detected by immunoblotting with PAb1801 and visualized by enhanced chemiluminescence. (Middle) p53 was immunoprecipitated with PAb421 from young and old MRC-5 cells prior to Western blotting. (**E**) The p53 DNA binding activity in young and old cells was determined by EMSA and normalized to the amount of p53 protein present in the nuclear extracts by Western blotting. (**F**) Nuclear extracts were prepared from MRC-5 cells at different population doublings on five separate occasions. Each mean value shown on the curve is based on five independent samples (5 μg protein) each measured for DNA binding activity at least once within the linear range of the DNA binding assay. Binding activity was determined by measuring the amount of radioactivity present in the PAb421 supershifted complex using a Molecular Dynamics PhosphorImager and ImageQuant software. DNA binding activity is expressed relative to that seen at the earliest time point (PD24–27). Error bars represent the SEM. Also shown is the frequency of dicentric chromosomes (bars), which increase during cell aging in culture. The data for the frequency of dicentric chromosomes in aging MRC-5 cells was obtained from Benn (1976). At the latest time point (PD50–60) no dicentric chromosome data were available.

Western blotting revealed similar levels of p53 protein in young and old cells, immunoprecipitation analysis revealed increased levels of PAb421-reactive p53 protein in old cells (Figure 1D, middle). We turned, therefore, to studies of *p21*^{WAF1} expression and transcriptional reporter assays in which RNA expression, while dependent on the DNA binding activity of p53 *in vivo*, occurs independently of added PAb421 antibodies.

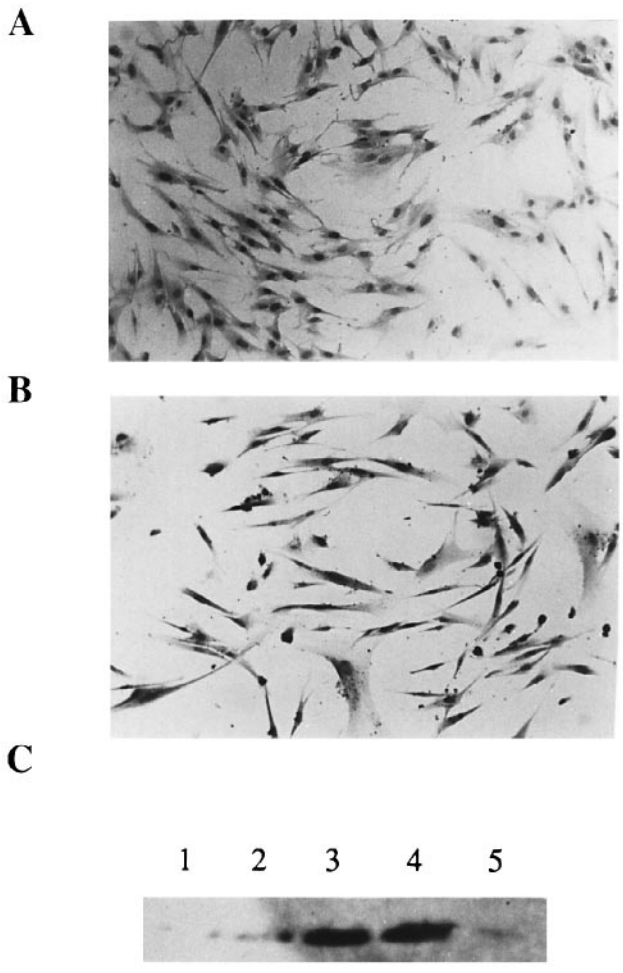


Fig. 2. p21 protein expression during cell aging. (**A**) Young and (**B**) near senescent MRC-5 cells were fixed and p21 protein was detected immunohistochemically using SC-187 monoclonal antibody and biotinylated HRP-conjugated secondary antibody. (**C**) p21 protein from S1C cell extracts (1 mg protein) was immunoprecipitated with SC-187 antibody and detected by immunoblotting and ECL using a rabbit polyclonal antibody against p21 (Pharmingen) and HRP-conjugated anti-rabbit antibodies. Lanes 1 and 2, near senescent (PD58) cultures of two clones transfected with HPV-16 E6; lanes 3 and 4, two near senescent cultures of S1C cells; lane 5, young S1C cells (PD25).

Involvement of p53 and p300 in the regulation of p21^{WAF1} promoter activity in aging human fibroblast strains

p21^{WAF1} mRNA levels were shown previously to increase as HDFs approached senescence (Noda *et al.*, 1994). Analysis of p21 protein levels in MRC-5 cells by immunohistochemistry (Figure 2A and B) and in S1C cells by Western immunoblotting (Figure 2C) revealed an accumulation of p21 protein in near senescent cells. While p21 protein was localized primarily in the nucleus of young cells, both nuclear and cytoplasmic staining were seen in the older cells.

To examine the involvement of p53 in regulating p21 levels in aging fibroblasts, clones of the human fibroblast strain S1C expressing HPV-16 E6 were generated and p21 protein levels were measured. S1C/E6 clones produced less p21 protein compared with non-transfected S1C cells at the same population doubling (Figure 2C). p21 protein expression, however, was not completely abolished in these cells, possibly due to residual p53 activity or the existence of a p53-independent pathway for *p21*^{WAF1} induction. It should be noted that S1C/E6 cells underwent senescence and did not exhibit a significant extension of their lifespan.

In order to examine the mechanism underlying transcriptional activation of the *p21*^{WAF1} gene in aging cells, transient expression studies were performed using a vector (p21P-luc) in which the human *p21*^{WAF1} promoter (2.4 kb fragment) containing a resident p53-responsive element was linked upstream of a luciferase reporter gene. A related reporter plasmid in which a 72 bp region encompassing the p53 consensus sequence was deleted from the *p21*^{WAF1} promoter (p21ΔP-luc) was used in parallel. All measurements of luciferase activity were normalized with respect to plasmid copy number in the transfected cells, using a modified Hirt assay, to control for variations in transfection efficiency. Measurement of luciferase activity 72 h after transfection in young and old cells revealed ~3-fold higher levels of luciferase in cells transfected with the full-length promoter compared with cells transfected with the deleted promoter (Figure 3A). Hence, the 72 bp fragment of the *p21*^{WAF1} promoter that contains a p53-responsive element is required for maximal luciferase activity in both young and old cells. Luciferase activity was ~2-fold higher in old cells compared with young cells (Figure 3A and D). Elevated expression of luciferase in old cells was observed with both the full-length and truncated promoters, suggesting that induction of luciferase activity in old cells might involve sequences that lie outside the 72 bp fragment of the luciferase promoter. El-Deiry *et al.* (1993) identified a second p53 recognition element in the human *p21*^{WAF1} promoter that may be involved in this age-related induction. However, it remains possible that the age-related increase in *p21* promoter activity involves a p53-independent pathway. To address this further, we examined the Box A promoter of the *IGF-BP3* gene, which is reported to be a target for p53 (Buckbinder *et al.*, 1995) and to be over-expressed in senescent HDFs (Goldstein *et al.*, 1991). Luciferase expression directed by the *IGF-BP3* promoter was 10 times higher in old MRC-5 cells compared with younger cells (Figure 3A). The SV40 promoter, which does not contain a p53 consensus binding site, provided a control for specificity in this series of transfection experiments and showed no age-related activation.

The ability of the 72 bp fragment containing the p53 consensus binding site to confer maximal activity on the *p21*^{WAF1} promoter was confirmed through the use of MRC-5 clones stably transfected with pSV2neo and either p21P-luc or p21ΔP-luc. Luciferase activity in pooled, stable, G418^{R} clones was 9-fold higher in cells that contained an intact *p21*^{WAF1} promoter compared with cells that contained the deleted promoter (Figure 3B).

To test directly the involvement of p53 protein in regulating the activity of the *p21*^{WAF1} promoter present in p21P-luc, we chose to disrupt endogenous p53 function in near senescent MRC-5 cells through transient overexpression of various dominant negative mutant p53 cDNAs. *p21*^{WAF1} promoter activity was significantly repressed upon expression of p53Ala143, p53His175 and p53Tyr275 polypeptides but not by wild-type p53 expression (Figure 3C).

An alternative means of disrupting endogenous p53

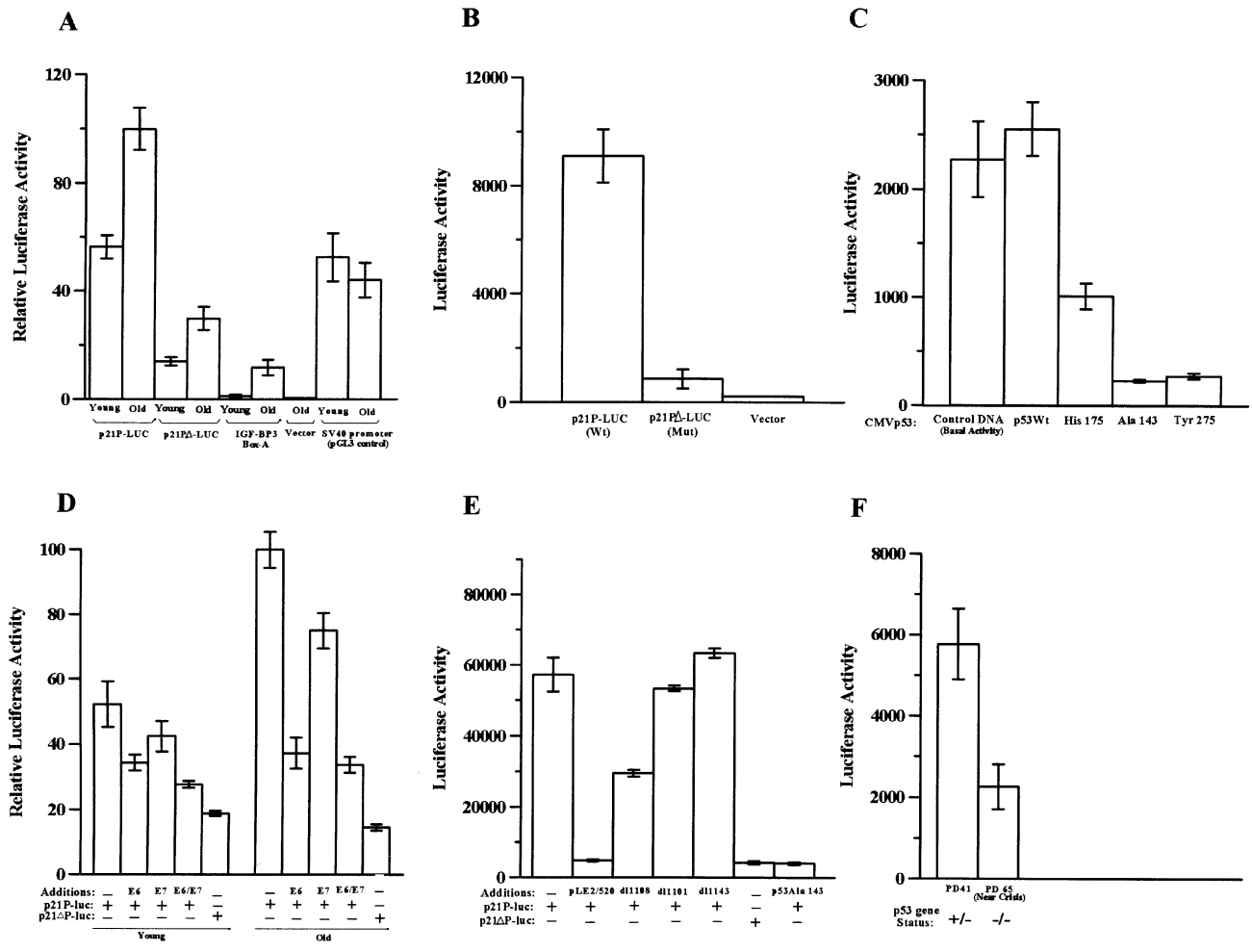


Fig. 3. Activation of the p21 promoter requires both p53 and p300. (**A**) Young and old MRC-5 cells were transfected with 5 μg reporter plasmid in which the luciferase coding sequence was placed downstream of the wild-type *p21* promoter (p21P-luc), a mutant *p21* promoter (p21ΔP-luc), the *IGF-BP3* promoter element bearing Box A, no promoter (vector) or the SV40/pGL3 control promoter. Luciferase activity was measured in triplicate, 72 h after transfection, using a luminometer and normalized for differences in plasmid copy number determined using a modified Hirt procedure as described in Materials and methods. The highest level of normalized luciferase activity was seen in old cells. This value was set at 100% and all other values are expressed proportionately. Error bars represent the SEM. (**B**) Young MRC-5 cells were stably transfected with wild-type or mutant *p21* promoter–luciferase plasmids. After selection in G418 the resulting 25 mutant promoter clones and 20 wild-type promoter clones were pooled and luciferase activity was measured. (**C**) MRC-5 cells nearing senescence were co-transfected with the *p21* promoter–luciferase reporter plasmid (p21P-luc) and one of various human *p53* cDNA plasmids (2 μg) in which expression was controlled by the CMV promoter. Normalized values of luciferase activity were determined as described in (A) and are expressed in relative light units (RLU). (**D**) Young and old cells were co-transfected with 5 μg p21P-luc plasmid together with 10 μg of one of the following expression plasmids: pSV2-E6, pSV2-E7, pSV2-E6/E7 or control vector plasmid. Seventy-two hours after transfection, luciferase activity was measured as described in (A). (**E**) Cells nearing senescence were transfected with the p21P-luc plasmid together with plasmids expressing wild-type or mutant Ad E1A protein. dl1108 can bind p300 but fails to bind pRB or p107; dl1101 and dl1143 both fail to bind to p300 but do bind to pRB and p107. pLE2/520 expresses wild-type E1A protein. (**F**) The cell strain 2675T, derived from a Li–Fraumeni patient, bears a heterozygous *p53* gene mutation. Upon prolonged passage in culture, these cells lose the wild-type *p53* allele. The *p53* gene status of these cells at PD41 and at PD65 was examined and found to be heterozygous (+/–) at the earlier passage with loss of heterozygosity (–/–) occurring at the later passage. Cells at PD41 and at PD65 were transfected with p21P-luc and luciferase activity was measured as described in Materials and methods.

function involves expression of HPV-16 E6 protein, which is known to promote ubiquitin-dependent degradation of p53 protein. Accordingly, young and old cells were co-transfected with p21P-luc and one of either E6, E7 or a combination of E6 and E7. E6 reduced $p21^{WAF1}$ promoter activity more profoundly in old cells than in young cells (Figure 3D). E7 had no significant effect on $p21^{WAF1}$ promoter activity in young cells but a reproducible repression of $p21^{WAF1}$ promoter activity was observed in old cells. The combination of E6 and E7 in old cells was no better than E6 alone in repressing luciferase expression. E7 protein is known to bind to a number of cellular proteins, including pRB, pRB-related proteins and the transcriptional adapter protein p300. To determine if any of these proteins is involved in regulating $p21^{WAF1}$ promoter activity, we made use of several adenovirus E1A protein variants that have either lost the ability to bind pRB and pRB-related proteins yet retain the ability to bind p300 (dl1108) or lost the ability to bind p300 while retaining the ability to bind pRB and pRB-related proteins (dl1101 and dl1143) (Howe *et al.*, 1990). Of the three E1A variants tested, only that encoded by dl1108 repressed the $p21^{WAF1}$ promoter in old cells (Figure 3E). Wild-type E1A (pLE2/520) repressed the activity of the $p21^{WAF1}$ promoter as effectively as p53Ala143. Wild-type E1A and the dl1108 variant also share the ability to induce DNA

synthesis in quiescent fibroblasts (Howe *et al.*, 1990). Together these data implicate both p300 (or other p300-related molecules) and p53 proteins in regulation of the $p21^{WAF1}$ promoter in human fibroblasts.

Loss of a wild-type p53 allele leads to reduced $p21^{WAF1}$ promoter activity and extension of cellular lifespan

Expression of SV40 large T antigen or HPV-16 E6/E7 proteins in human fibroblasts leads to an extension of cellular lifespan. These cells bypass the senescence checkpoint but eventually enter a second phase of arrest termed crisis that, unlike senescence, is associated with cell death. An elevation of $p21^{WAF1}$ RNA levels has been observed in cells entering crisis, as well as in cells entering senescence (Rubelj and Pereira-Smith, 1994). Having shown that p53 protein is involved in the regulation of $p21^{WAF1}$ gene transcription in aging fibroblasts, we next wished to determine if p53 played any role in induction of $p21^{WAF1}$ mRNA at crisis. We made use of fibroblasts obtained from an individual with Li–Fraumeni syndrome. Fibroblasts from Li–Fraumeni patients commonly contain one wild-type p53 allele and one mutant, non-functional p53 allele. When placed in culture, these cells have been shown to bypass senescence (Bischoff *et al.*, 1990) upon loss of the remaining wild-type p53 allele (Rogan *et al.*, 1995). The 2675T strain carries a heterozygous mutation at codon 245 that converts glycine to aspartic acid (Srivastava *et al.*, 1990; Mirzayans *et al.*, 1995). The p53Asp245 protein is stable, displays an altered conformation and fails to bind DNA (Friend, 1994). The 2800T strain was obtained from a normal individual, however, it carries a heterozygous p53 mutation at codon 234 that replaces tyrosine with cysteine (Mirzayans *et al.*, 1995). The p53Cys234 protein appears to have retained a normal conformation (Friend, 1994). We cultured the 2800T and 2675T strains and noticed that 2800T entered senescence after PD56, while 2675T continued to divide for a longer period of time prior to entering crisis after PD68. Analysis of the *p53* gene revealed that the senescent 2800T cells remained heterozygous at the *p53* locus, while 2675T lost the normal *p53* allele at some point prior to entering crisis between PD41 and PD65. The 2675T cells remained in crisis for 230 days and no immortal (i.e. post-crisis) clones arose from this strain. 2675T cells at PD41 and PD65 were transfected with p21P-luc and luciferase was measured 72 h later. Luciferase activity was lower at PD65 than at PD41 (Figure 3F). These data indicate that complete loss of wild-type *p53* gene expression in human fibroblasts is associated with a reduction in $p21^{WAF1}$ promoter activity and with extension of cellular lifespan.

Short telomeres, premature senescence and increased DNA binding activity of p53 protein in AT strains

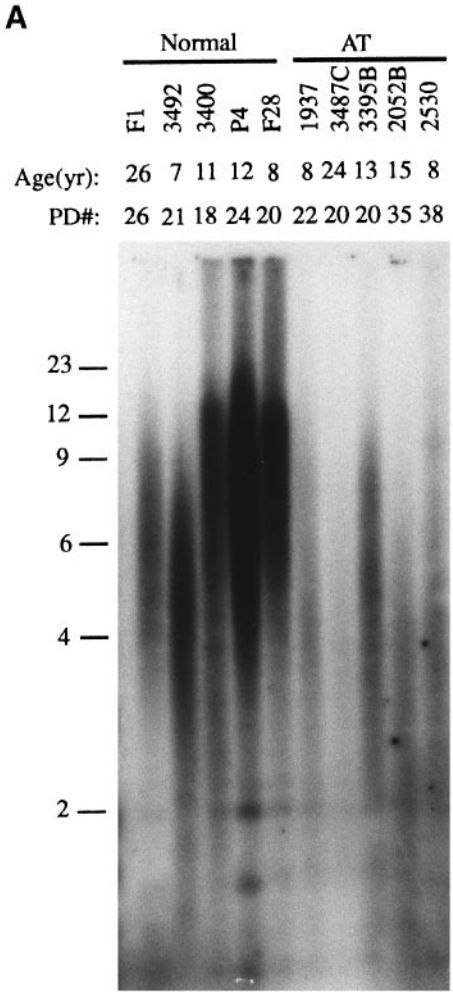
The *ATM* gene product has been proposed to lie upstream of p53 protein in the DNA damage response pathway that leads to cell cycle arrest at the G_1/S boundary (Kastan *et al.*, 1991; Lu and Lane, 1993). HDFs from individuals with ataxia telangiectasia display increased sensitivity to γ-irradiation and certain strains undergo accelerated senescence in culture (Shiloh *et al.*, 1982). In addition, disruption of the yeast *TEL1* gene, which shows homology with the human *ATM* gene, results in accelerated loss of telomeric DNA (Greenwell *et al.*, 1995). As a result of these findings, we wished to investigate further the connection between p53 function, aging and telomere loss in AT cells.

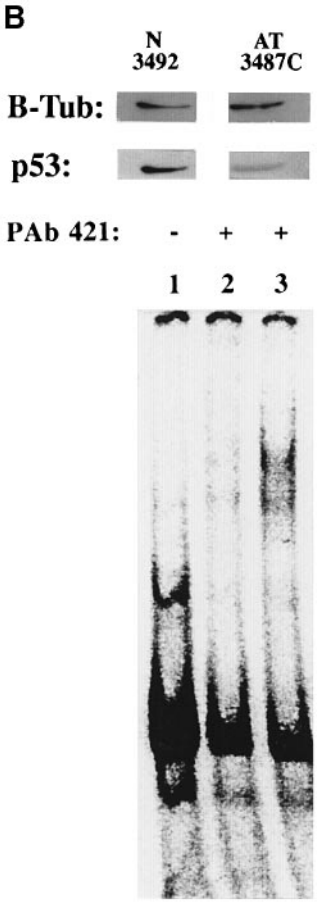
Fibroblasts were obtained from five AT patients and compared with fibroblasts from either their normal siblings or unrelated age-matched controls. For each cell strain we determined the number of population doublings that preceded senescence, mean terminal restriction fragment (TRF) length, the amount of p53 protein and the DNA binding activity of p53 protein. The results are presented in Figure 4. As expected, all the AT fibroblast strains reached senescence earlier than the normal strains. The mean TRF length, which reflects the length of telomeres, was shorter at the same PD in the AT strains 3487C and 1937B than in their sibling control 3492 and 3400 cells (Figure 4A and D). Of the remaining three AT strains, 3395B had a short TRF length and displayed premature senescence while 2052B and 2530 had longer telomeres (longer than controls) at early passage and continued to divide until they reached PD35 and PD38 respectively, at which time shortened telomeres were present (Figure 4D). This finding prompted us to compare the rate of telomere loss in AT strain 2530 with its age-matched normal control 3400. Accelerated telomere loss was observed in 2530 (267 bp/PD) compared with 3400 (60 bp/PD) cells. While these data strengthen the association between AT and the senescent phenotype, they indicate that involvement of the *ATM* gene in regulating telomere length and replicative senescence is likely to be complex.

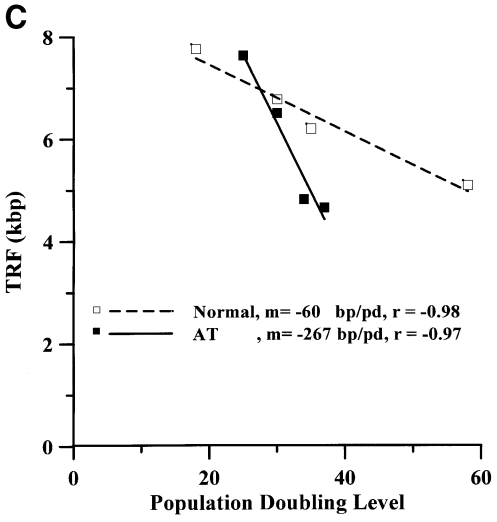
In Figure 1 we showed that the DNA binding activity of p53 protein increased during cell aging in the absence of a marked increase in the steady-state level of p53 protein. Since certain AT strains have short telomeres and display accelerated aging in culture, we compared the DNA binding activity and steady-state level of p53 protein in the 3487C AT strain with its age-matched, normal sibling strain 3492 at the same population doubling. Enhanced DNA binding activity was evident in 3487C even though these AT cells contain less p53 protein than the normal sibling control 3492 (Figure 4B). These results indicate that the specific DNA binding activity of p53 protein is higher in the 3487C AT strain than in the normal sibling 3492 strain. Increased DNA binding activity was similarly observed in 1937B AT cells compared with the age-matched sibling control 3400 cells (data not shown). These data support the view that ATM is involved in the regulation of telomere length and that the DNA binding activity of p53 protein is post-translationally activated in aging cells.

Hyperoxia leads to accelerated telomere loss, functional activation of p53 protein and premature senescence

The cellular lifespan of human diploid fibroblasts can be affected by the oxygen concentration at which the cells are cultured. Cells have an extended lifespan under low oxygen conditions and a shortened lifespan under high oxygen (Chen *et al.*, 1995; Saito *et al.*, 1995). In addition, telomeres of HDFs grown under hyperoxia have been shown to undergo an accelerated rate of shortening (von Zglinicki *et al.*, 1995). We wished to determine if the







D

Cell Strain	Lane#	Age (yrs)	Status	PD matched TRF (kbp)	PD matched SD (n=2)	PD matched PD#	Senescence TRF (kbp)	Senescence PD Max
F1	1	26	N	7.0	0.1	26	–	>52
3492	2	7	N	5.6	0.1	21	–	>36
3400	3	11	N	7.4	0.3	18	–	>40
P4	4	12	N	8.5	0.8	24	–	>62
F28	5	8	N	8.9	1.4	20	–	>56
3487C	6	8	AT	5.1	0.1	22	–	23
1937B	7	24	AT	5.0	0.4	20	–	22
3395	8	13	AT	5.5	0.2	20	–	29
2052B	9	15	AT	6.7	1.3	23	4.5	35
2530	10	8	AT	9.0	1.3	25	4.9	38

Fig. 4. Analysis of replicative lifespan, telomeric DNA length and DNA binding activity of p53 protein in AT HDFs. (**A**) Genomic DNA from five normal (lanes 1–5) and five AT strains (lanes 6–10) were digested with *Hin*fI/*Rsa*I and telomeric DNA was detected using a γ-^{32}P-labeled $(C_3TA_2)_3$ probe. The mean TRF length was determined from these data as described in Materials and methods and are indicated in (D). (**B**) (Bottom) EMSAs were performed with 5 μg nuclear extract from an AT strain (3487C) and its normal age-matched sibling (3492). Nuclear extracts were prepared at PD23 for both cell strains and were added to reactions containing end-labeled p53CON oligonucleotide and the p53-specific antibody PAb421. Lane 1, 3487C cell extract without antibody; lane 2, 3492 cell extract; lane 3, 3487C cell extract. (Top) A Western blot in which cell lysates (300 μg protein) were subjected to electrophoresis on a SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and subsequently blotted with DO-1 antibody to detect p53 and a β-tubulin control antibody. (**C**) Mean TRF length of AT strain 2530 was measured throughout its lifespan and compared with an age-matched normal control strain 3400. (**D**) Summary of data collected on normal and AT strains. Three AT strains had shorter telomeres and were entering senescence, while two AT strains with longer telomeres continued to proliferate further before entering premature senescence (indicated by PD max).

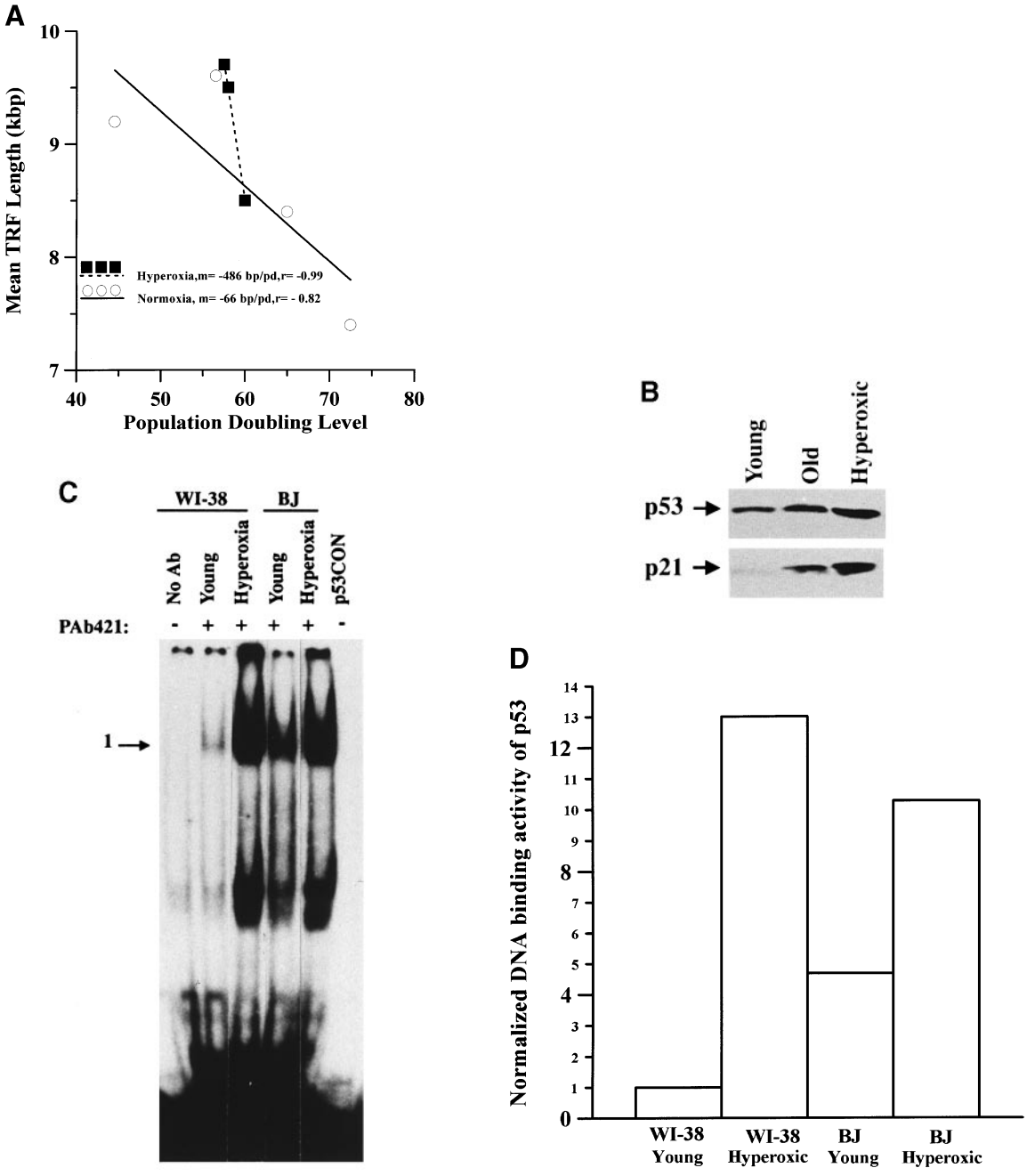


Fig. 5. Hyperoxia leads to accelerated telomere loss, activation of p53 protein and premature senescence. (**A**) *Hin*fI/*Rsa*I-digested genomic DNA (1 μg) from WI-38 and BJ cells under normoxia and hyperoxia was resolved on a 0.5% agarose gel and subsequently probed with a γ-^{32}P-labeled $(C_3TA_2)_3$ probe. Quantification of mean TRF length over the indicated PD range is shown for BJ cells under hyperoxia (dotted line and solid squares) and under normal oxygen (solid line and open circles). The rate of TRF loss observed under normal oxygen was 66 bp/PD (r = –0.82) and under hyperoxia, 486 bp/PD (r = –0.99). (**B**) Immunoblot analysis of p53 (DO-1) and p21 (SC-187) protein in young WI-38 cells under normal conditions and hyperoxic conditions and in old cells under normal conditions. (**C**) EMSA showing the increase in DNA binding activity of p53 in young and hyperoxic cells as described in Figure 1. Arrow 1 indicates the position of the supershifted band. (**D**) Quantification of DNA binding activity of p53 under normal and hyperoxic conditions. Values obtained by integration of the supershifted band (band 1) in Figure 5C and normalized to the values obtained from quantification of p53 protein in Figure 5B.

association between shortened telomeres, p53 activation and senescence could also be demonstrated in cells grown under hyperoxic conditions. WI-38 and BJ cells were cultured under normal oxygen (20%) or exposed to hyperoxia (40% oxygen). WI-38 and BJ cells were exposed to hyperoxia at PD27 and PD57 respectively and cultured under hyperoxic conditions for the duration of their lifespan. Both WI-38 and BJ cells lost telomeric DNA at an accelerated rate under hyperoxia and entered senescence prematurely (Figure 5A). BJ cells lost telomeric DNA at a rate of 66 bp/PD when grown at 20% O_2 or at a rate of 486 bp/PD in hyperoxia (Figure 5A); WI-38 cells lost telomeric DNA at a rate of 70 bp/PD under normal oxygen or at a rate of 240 bp/PD in hyperoxia (data not shown). We then measured the relative level of p53 protein by Western blot analysis and the DNA binding activity of p53 by EMSA in cells grown under normal or hyperoxic conditions (Figure 5B and C). Normalization of the DNA

binding values to the level of p53 protein revealed a 13- and a 2-fold increase in the specific DNA binding activity of p53 protein in WI-38 cells and BJ cells respectively upon exposure to hyperoxia. Consistent with this finding, the amount of p21 protein increased in hyperoxic cells to a level even higher than that seen in old cells (Figure 5B). Together these results indicate that hyperoxia leads to an accelerated loss of telomeric DNA beyond the level which can be explained by the end replication problem. The loss of telomeric DNA is associated with an increase in the specific activity of p53 protein, elevation of p21 protein expression and premature senescence.

Poly(ADP-ribose) polymerase and p53 protein interact in vitro and in vivo

The senescence-associated activation of p53 protein function seen in aging fibroblasts, AT fibroblasts and hyperoxic fibroblasts may be analogous to the post-translational activation of p53 protein that is observed in cells exposed to DNA damaging agents. Modifying enzymes that are activated in response to DNA damage, such as DNA-PK, SAPK and PARP, have been shown to modify p53 protein *in vitro* (Anderson, 1993; Wesierska *et al.*, 1996) and, hence, represent potential physiological activators of p53 protein during aging or after DNA damage.

To investigate the interaction of p53 and PARP, purified full-length human p53 was mixed with purified full-length PARP (Huletsky *et al.*, 1989). The mixture was subjected to immunoprecipitation with CM1 polyclonal antibody against p53, resolved by PAGE and processed for Western blotting to detect p53 (with a mixture of PAb7 and PAb240 antibodies) and bound PARP (C2-10 antibody). The immunoblot presented in Figure 6A shows that PARP co-immunoprecipitated with p53. In order to map the region of p53 that interacts with PARP, several truncated forms of p53 protein were prepared and mixed with full-length PARP for immunoprecipitation/Western blot analysis. As shown in Figure 6B, both an N-terminal p53 fragment (amino acids 1–72) and a C-terminal p53 fragment (311–393), but not the DNA binding domain of p53 (82–292), were able to interact with PARP. The binding with PARP was much stronger with the N-terminal fragment of p53.

To investigate this interaction *in vivo*, cell extracts were prepared from the wild-type p53-expressing human cell line OCI/AML-3 (Fu *et al.*, 1996) and subjected to immunoprecipitation with antibodies against p53 (PAb1801, PAb240 or PAb421) or PARP (318). The immunoprecipitates were resolved by PAGE, proteins were transferred to nitrocellulose membranes and probed with a mixture of antibodies against PARP (C2-10 and 318) or p53 (PAb7). The results of this co-immunoprecipitation/Western blot analysis indicate that p53 protein and PARP form a complex *in vivo*. Three antibodies directed against different epitopes of p53 co-immunoprecipitated bound PARP (Figure 6C, top) in addition to p53 protein (Figure 6C, bottom). Moreover, in the reciprocal experiment antibodies against PARP co-immunoprecipitated bound p53. An extract prepared from the *p53*-null cell line SKOV3 served as a control. Although certain extracts used for this experiment were prepared from γ-irradiated cells (2 or 6 Gy) in order to increase the amount of p53 protein and facilitate detection of complexes with PARP, the interaction between p53 and PARP was also seen in non-irradiated cells. Of the three p53 antibodies used, PAb1801 was the least effective in co-immunoprecipitating the bound PARP. One explanation for this observation is afforded by the peptide mapping experiments, which revealed that PARP bound tightly to the N-terminal fragment of p53. PAb1801 also binds to the N-terminus of p53 and could disrupt the interaction between PARP and p53. Alternatively the interaction between p53 and PARP may prevent access of PAb1801 to its binding site. Two other antibodies that recognize epitopes at the N-terminus of p53, DO-1 and DO-7, failed to co-immunoprecipitate PARP (data not shown).

The PARP–p53 interaction was investigated further using gel mobility shift experiments to assay for p53–DNA binding activity to the previously described p53 binding sequence p53CON. Nuclear extracts from young and old HDFs were incubated with p53CON. The binding reactions were supplemented with PAb421 or a mixture of PAb421 and 318 antibodies. The results presented in Figure 6D show that p53–DNA complexes can be recognized and supershifted with antibodies to p53 (band B) and that these supershifted complexes can be further shifted upon incubation with antibodies to PARP (band A). Inclusion of the PARP antibody alone in the binding reaction resulted in a supershifted band with a mobility very similar to that seen with the PAb421 antibody (data not shown). These results confirm the existence *in vivo* of a p53–PARP complex with DNA binding activity.

Inactivation of PARP leads to extinction of p21 and mdm2 expression in response to DNA damage

If the interaction with PARP regulates the activity of p53 protein in response to DNA damage, then it is possible that inhibition of PARP leads to abrogation of p53–DNA binding activity and of the expression of downstream targets of p53. Cells irradiated with 2 Gy showed an increase in the amount of p53 protein and an elevation in the level of p21 and mdm2 proteins (Figure 6E). However, prior treatment of the cells with the specific PARP inhibitor 1,5-dihydroxyisoquinoline (IQ), prevented the increase in expression of p21 and mdm2 after irradiation without affecting accumulation of p53 protein. Treatment with IQ also diminished and delayed the increased DNA binding activity that is normally seen after irradiation (data not shown). This indicates that mechanisms involved in accumulation of p53 protein in response to DNA damage are uncoupled from those affecting its activation.

Inactivation of PARP leads to an extension of lifespan in HDFs

The demonstration that p53 is involved in cellular senescence coupled with the observation that PARP, an enzyme which is strongly activated by binding to DNA single- or double-strand breaks, can associate with p53 protein *in vivo* raises the question of PARP involvement in modulating cellular lifespan. PARP may sense DNA damage directly and relay the signal to p53 protein, resulting in functional activation of p53. One prediction of this model is that in the presence of a PARP inhibitor p53 may not become activated and cells would have an extended lifespan. To test this model, HDFs were treated with inhibitors of PARP, namely 3-aminobenzamide (3AB), having an IC_{50} of 23 μM, and the more specific inhibitor IQ, having an

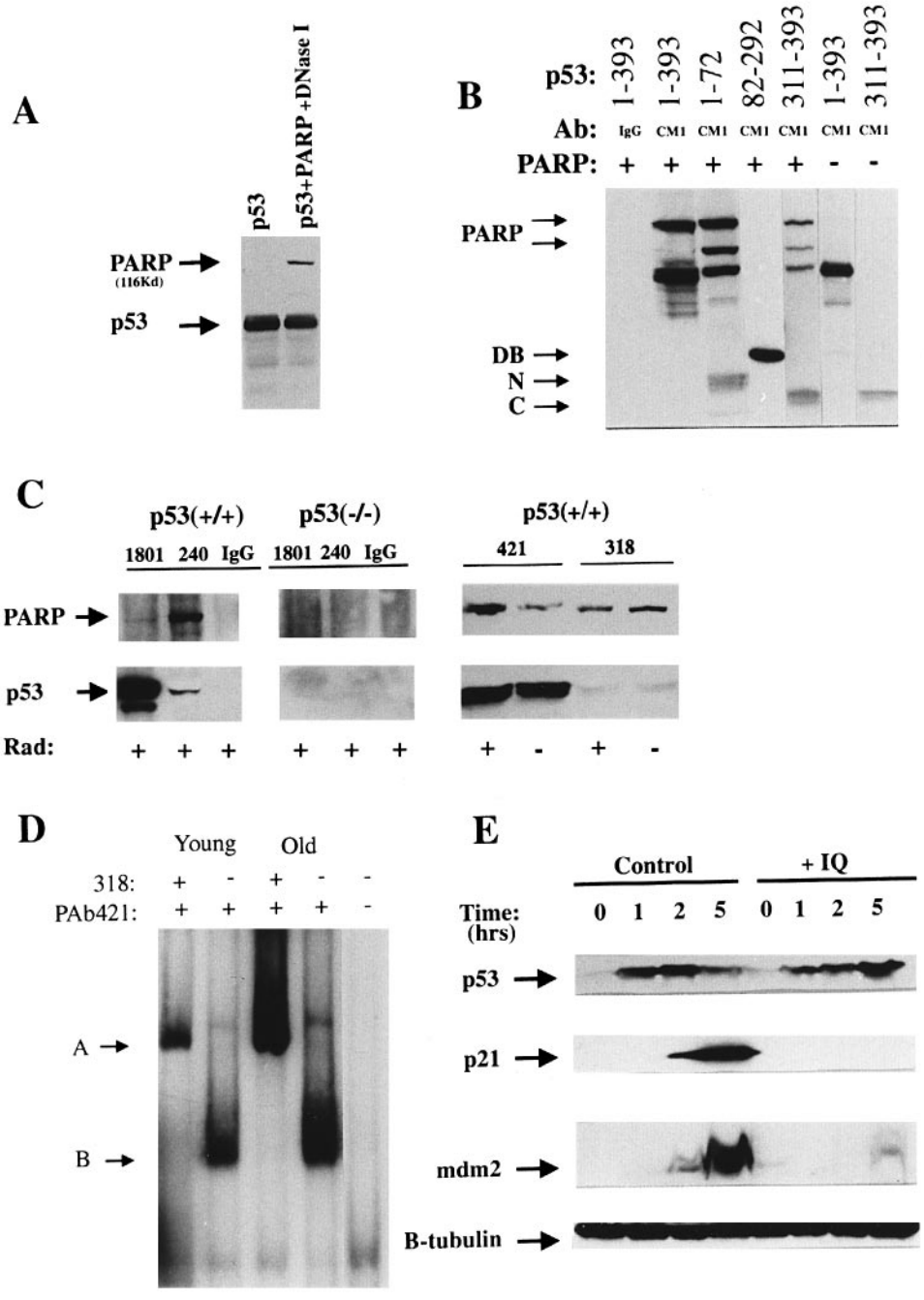


Fig. 6. (**A**) Interaction of full-length human p53 with PARP *in vitro*. p53 (0.5 μg) and PARP were mixed, incubated and subsequently immunoprecipitated with CM1 antibody against p53 as described in Materials and methods. Immune complexes were subjected to immunoblotting with PAb7 and C2-10 to detect p53 and PARP respectively. (**B**) Full-length purified PARP was mixed with different purified fragments of p53 protein as indicated and subjected to immunoprecipitation and immunoblotting with PAb7, PAb240 and C2-10. DB, DNA binding domain, residues 82–292; N, residues 1–72; C, residues 311–393. (**C**) Interaction between PARP and p53 protein *in vivo*. Cell extracts were prepared from untreated or γ-irradiated wild-type p53-expressing cells (OCI/AML-3) or p53-null cells (SKOV3), 1–2.5 h after treatment with a dose of 2 or 6 Gy. Proteins were immunoprecipitated with antibodies against p53 (PAb1801, PAb240 and PAb421), antibodies against PARP (318) or control IgG antibodies. Immunoprecipitated proteins were resolved by SDS–PAGE, transferred to nitrocellulose membranes and blotted with a mix of C2-10 and 318 antibodies specific for PARP (top) or with PAb7 antibodies directed against p53 (bottom). (**D**) Nuclear extracts were prepared in low pH buffer from young and old cells and mixed with ^{32}P-labeled p53CON. The DNA binding reactions were supplemented with PAb421 antibodies or a mixture of PAb421 and 318 antibodies and analyzed on a native polyacrylamide gel (pH 6.8). (**E**) OCI/AML-3 cells were incubated in the presence of 200 μM IQ or in DMSO (control) for 1 h prior to γ-irradiation (2 Gy). Twenty minutes after irradiation, a second equivalent dose of IQ was added to the cells that had received IQ earlier. At different times after irradiation cell extracts were prepared and samples containing 400 μg protein were subjected to sequential immunoblotting with the following antibodies: PAb1801 (p53), SC-187 (p21), 2A10 (mdm2) and anti-β tubulin.

IC_{50} of 0.39 μM (Banasik *et al.*, 1992). We recognize the possibility that these two inhibitors may target NAD-dependent enzymes other than PARP. However, it has been shown previously that IQ has specificity for PARP *in vivo* and that it does not significantly change the NAD^+ pool *in vivo* (Shah *et al.*, 1996). 3AB-treated cells showed

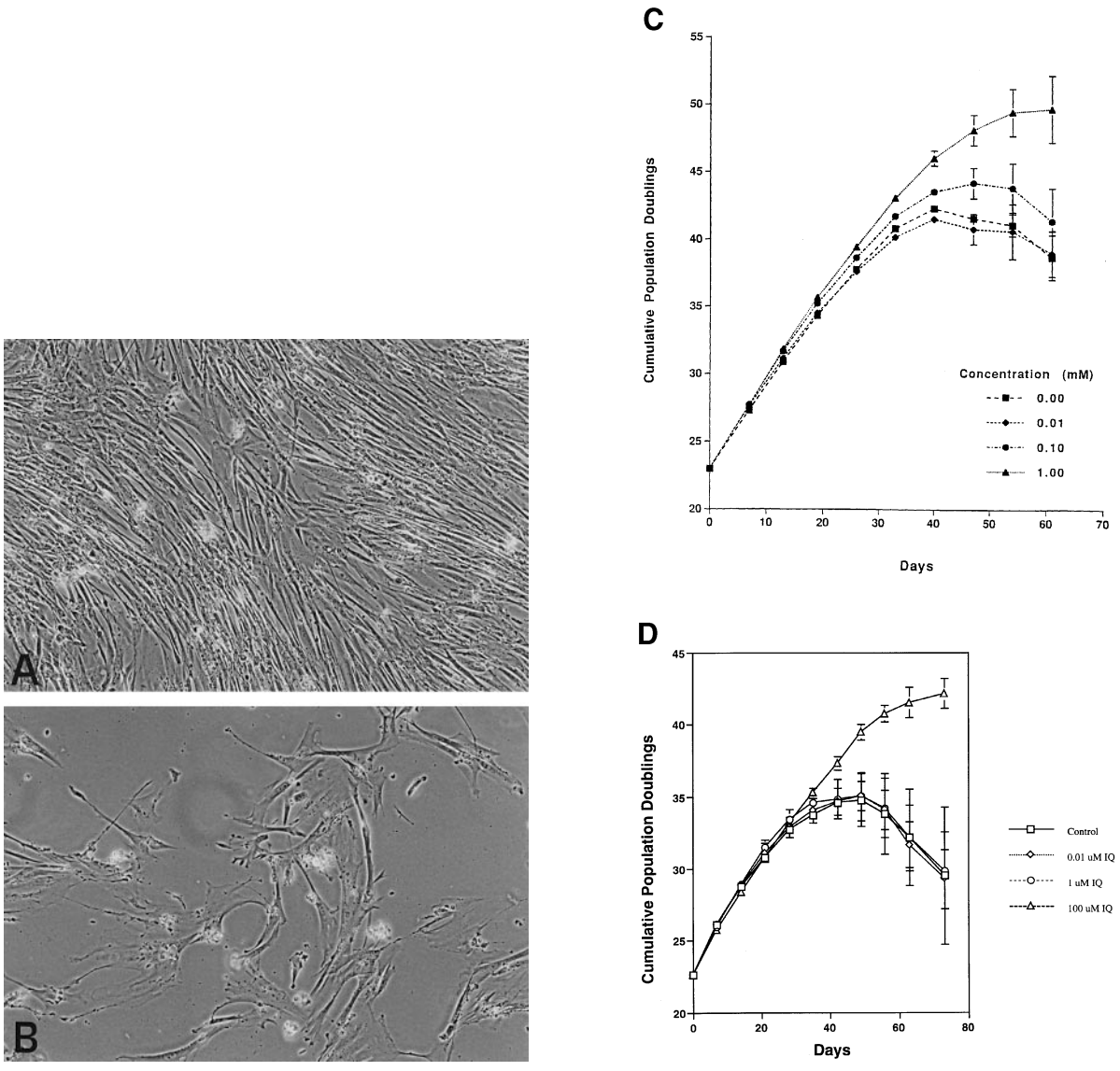


Fig. 7. Extension of cellular lifespan by PARP inhibitors. WI-38 cells were grown in the presence of 100 μm IQ (**A**) or in the absence of IQ (**B**). Growth curves of WI-38 cells incubated in the presence of different concentrations of 3AB (**C**) or IQ (**D**).

a significant extension of cellular lifespan, as did the IQ-treated cells (Figure 7). Removal of the PARP inhibitors led to restoration of normal lifespan and subsequent senescence (data not shown). Hence, functional inactivation of PARP or functional inactivation of p53 leads to a common phenotype, namely extension of cellular lifespan. This supports the idea that PARP and p53 are both components of a senescence-determining pathway.

Discussion

There is substantial evidence that cellular senescence is associated with elevated expression of the *p21*^{WAF1} gene and with a decrease in the size of telomeres. The p53 nuclear phosphoprotein has also been implicated in senescence, since it can activate expression of the *p21*^{WAF1} gene by binding to the p53-responsive element within the *p21*^{WAF1} promoter. Moreover, the DNA binding activity of p53 has been shown to increase in aging fibroblasts and disruption of p53 protein function has been reported to extend the proliferative lifespan of human fibroblasts. The data we present here extend these observations and demonstrate that binding of p53 to a physiologically relevant binding site in the *p21*^{WAF1} promoter increases in old cells. We show that expression of the *p21*^{WAF1} gene in aging fibroblasts is regulated by p53 and by the transcriptional co-activator p300, which was previously shown to regulate the *p21*^{WAF1} promoter independently of p53 (Missero *et al.*, 1995). The increased p53-dependent transcriptional activity of the *IGF-BP3* promoter in old cells (Figure 3A) also provides an explanation for the up-regulation of *IGF-BP3* with cell age (Goldstein *et al.*, 1991). In agreement with previous studies we find that the steady-state level of p53 protein does not change markedly as cells age in culture. These observations suggest that p53 protein is post-translationally activated in aging cells and that one of its functions is to control expression of *p21*^{WAF1}.

We and others have detected higher levels of p53 protein in old cells compared with young cells when extracts were immunoprecipitated with PAb1801 or PAb421 antibodies prior to Western blotting (Kulju and Lehman, 1995; Vaziri

and Benchimol, 1996). Immunoblotting without prior immunoprecipitation revealed similar levels of p53 protein in young and old cells. These observations suggest that p53 protein undergoes a change in conformation during cellular aging that exposes epitopes at the termini of the molecule. The altered conformation may have relevance with respect to the increased DNA binding (Figure 1) and transcriptional activity of p53 (Figure 3).

In AT cells that have short telomeres and consequently reach senescence after fewer population doublings we found that p53 protein had increased DNA binding activity compared with age-matched normal controls at the same population doubling. These data are consistent with the results obtained with aging normal fibroblasts and demonstrate an association between the DNA binding activity of p53 and cellular lifespan that is independent of p53 protein level.

Amongst the five AT fibroblast strains examined, we found heterogeneity with respect to telomere length. Three strains had short telomeres and senesced prematurely, while two strains with longer telomeres at the time of analysis displayed a higher proliferative capacity in culture. Nevertheless, these strains had an accelerated rate of telomere shortening and underwent premature senescence. These data are consistent with the idea that the *ATM* gene plays a role in determining cellular lifespan. However, the involvement of *ATM* in regulating telomere length is likely to be complex.

A number of studies have concluded that the p53-dependent DNA damage response is defective or attenuated in γ-irradiated AT cells (Kastan *et al.*, 1991; Lu and Lane, 1993). It may not be appropriate, however, to compare the activity of p53 protein in response to γ-irradiation in AT and normal cells or even the radiosensitivity of AT cells with normal cells. AT cells have a shorter lifespan than normal cells and, hence, at the time of analysis these cells will have completed a far greater proportion of their total lifespan than normal cells. AT cells may be approaching the end of their proliferative lifespan, while normal cells at the same population doubling will still retain considerable proliferative potential. It has been shown, for example, that the radiation sensitivity of normal fibroblasts could change with age in culture (Holliday, 1991).

Hyperoxia results in premature senescence and an accelerated rate of telomere shortening that cannot be accounted for by the end replication problem associated with the inability to fully replicate DNA at the ends of chromosomes during each round of DNA replication. Premature senescence resulting from hyperoxia, like the premature senescence seen in AT fibroblasts, is associated with shortened telomeres and with the post-translational activation of p53 protein.

We provide several lines of evidence to show that p53 and PARP interact: (i) binding occurs *in vitro* using purified components; (ii) binding *in vivo* can be demonstrated by co-immunoprecipitation of PARP using three antibodies against distinct epitopes of p53 and by co-immunoprecipitation of p53 with an antibody against PARP; (iii) PARP can be detected on p53–DNA complexes by antibody supershift experiments. An association between p53 and PARP *in vitro* was recently reported (Wesierska *et al.*, 1996). Chemical inhibition of PARP activity with IQ

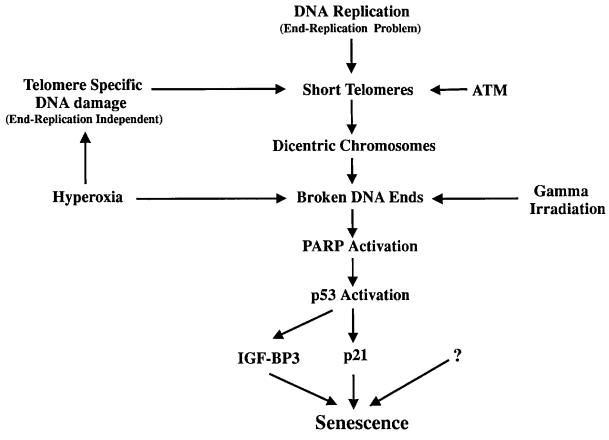


Fig. 8. Model connecting telomere loss signal and senescence-associated growth arrest. Telomere loss as a consequence of an end replication problem or direct damage to telomeres by free oxygen radicals may initiate a series of events leading to formation of dicentric chromosomes, which upon subsequent breakage activate PARP and p53 protein. p53-dependent/independent transcriptional activation of genes such as *p21* and *IGF-BP3* leads to cessation of proliferation and the cell cycle arrest associated with senescence.

resulted in abrogation of the p53-mediated induction of p21 and mdm2 expression normally seen in γ-irradiated cells. Our findings are consistent with the observation that the p53 response to DNA damage is defective in cells with PARP deficiency (Whitacre *et al.*, 1995). These data indicate that the interaction between PARP and p53 is critical for p53 function in response to DNA damage signals. While p53 activity was disrupted by the inhibition of PARP activity, accumulation of p53 protein in response to irradiation was not greatly affected. This provides compelling evidence that accumulation and activation of p53 protein in response to irradiation represent separate events that can be completely uncoupled. PARP activity is required for p53 protein activation but is dispensable for p53 protein accumulation.

Since PARP is known to become activated in response to DNA damage, it is possible that PARP acts as a DNA damage sensor that relays the telomere loss signal to p53. To investigate the presumed involvement of PARP in senescence, we rendered cells deficient in PARP activity through the use of two chemical inhibitors, 3-AB and IQ. Both inhibitors were effective in extending cellular lifespan. We conclude that PARP is involved in cellular aging. Hence, inactivation of at least two proteins, p53 and PARP, leads to a similar phenotype, namely extension of cellular lifespan. These findings suggest a model in which PARP, in response to the DNA ends which accumulate in aging cells, possibly as a consequence of dicentric chromosome breakage, activates p53 protein (Figure 8).

The p53–PARP interaction may affect p53 protein function in at least two ways. First, it is possible that p53 is ADP-ribosylated by PARP in response to DNA damage or cellular aging. ADP-ribosylation may be the mechanism through which p53 protein is post-translationally activated in aging cells or in cells that have acquired DNA damage. Inhibition of poly(ADP-ribose) synthesis would prevent the activation of p53 and, hence, no mdm2 or p21 expression would be triggered. In a second, alternative model, PARP may regulate p53 function in the absence of enzymatic modification. PARP is known to bind tightly

to DNA ends and strand breaks and requires automodification (i.e. ADP-ribosylation) for release from DNA. Binding of p53 to PARP at such sites will similarly sequester p53 and prevent it from acting as a transcriptional factor. The addition of an inhibitor of poly(ADP-ribose) synthesis will prevent the release of PARP and associated proteins such as p53 from the DNA ends. Under these conditions p53 would be incapable of promoting transcription. Further experiments are needed to determine which of these two models is correct. It will be important, for example, to determine if p53 protein is ADP-ribosylated *in vivo*.

Mice deficient in PARP activity have been generated (Wang *et al.*, 1995). These mice are born healthy and fertile. However, proliferation of primary embryonic fibroblasts in culture was impaired and proliferation of thymocytes *in vivo* following γ-irradiation was delayed. While the normal lifespan of these mice may seem to be at odds with the model presented in Figure 8, we believe that it is inappropriate to compare the lifespan of inbred mice having long telomeres in excess of 100 kb with that of human cells which have much shorter telomeres. It should be noted in this context that PARP activity is positively correlated with the lifespan of various species (Grube and Burkle, 1992). PARP activity may be dispensable in animals with a short lifespan.

In summary, our results provide a model for events which lead to activation of the genetic program of cellular aging and identify several key molecules involved in the p53 pathway for growth arrest. Knowledge of the molecular mechanisms involved in cell aging will be important for future drug design to extend the lifespan of normal cells and for the therapeutic intervention in and treatment of aging-associated diseases. Furthermore, these studies have relevance for the therapeutic eradication of immortal tumor cells through re-initiation of the senescence pathway.

Materials and methods

Cell culture and transfection

Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air. All cells were cultured in α-minimal essential medium supplemented with 10% fetal bovine serum (FBS), except for AT strains, which were grown in 20% FBS. Subconfluent cultures were split 1:8 in early passage and 1:4 or 1:2 in mid to late passage, using 0.25% trypsin/EDTA. Phosphate buffered saline (PBS) contained no calcium or magnesium. The normal HDFs used in this study included MRC-5 (ATCC), WI-38 (ATCC), S1C (age 45 years), F1 (age 26 years), P4 (age 12 years), F28 (age 8 years) and BJ (fetal foreskin). The 3400 (age 11 years) and 3492 (age 7 years) cells were obtained from the Coriell Institute. The following AT strains were obtained from the Coriell Institute: 1937B (age 24 years), 3395B (age 13 years), 3487C (age 8 years), 2530 (age 8 years) and 2052B (age 15 years). The 2675T and 2800T fibroblasts strains were derived from members of a Li–Fraumeni syndrome family and were kindly provided by Dr M.C.Paterson (University of Alberta). OCI/AML-3 is a wild-type p53-expressing cell line established from the primary blasts of a patient with acute myelogenous leukemia (Fu *et al.*, 1996). SF1 is an SV40-immortalized HDF cell line.

Cells were defined as being young if they had completed <30–40% of their lifespan and as being old if they had completed >85–90% of their lifespan. Senescence was defined as the inability to divide over a 3 week period. FACS analysis by propidium iodide staining and BrdU pulse labeling was used to confirm that old cells were arrested at the G_1/S and G_2/M boundaries as described previously (Sherwood *et al.*, 1988).

Transfection experiments were carried out by electroporation (to generate stable clones) or by the DEAE–dextran method (Kriegler, 1991) using triplicate dishes per plasmid DNA for transient expression studies. For electroporation HDFs, when 70% confluent, were trypsinized, washed in PBS, collected and mixed with the appropriate plasmids, p21P-luc, p21ΔP-luc or pSV2-E6 (20 μg), in combination with pSV_2neo (2 μg). Cells were electroporated using a Bio-Rad gene pulser at 150–300 V, 960 μF. Cells were selected in medium containing 400 μg/ml G418 and clones were isolated using cloning rings.

For inhibition of PARP varying amounts of 3-AB (Sigma) (0, 10 or 100 μM or 1 mM) or IQ (Aldrich) (0, 0.01, 1 or 100 μM) were added to the cells, which were plated at a density of 10^5 cells/cm^2 in 6-well plates.

Hyperoxic conditions

A tri-gas water-jacketed incubator from Forma Scientific (Model 3327) was used to culture cells under hyperoxia. BJ and WI-38 cells were exposed to hyperoxia (40% oxygen) at 50% confluency at PD57 and PD27 respectively. After several weeks in culture the BJ and WI-38 cells stopped dividing at PD60 and PD32 respectively. Control cells were maintained under normal oxygen conditions. Cells were grown in DMEM-M199 medium supplemented with 10% FBS and gentamicin (50 mg/l). Cells were re-fed weekly and split 1:2 upon achieving confluency.

Plasmid constructs

All plasmids were purified using Qiagen columns and quantified with a fluorometer (Turner Model 450) used within the linear range. A 2.4 kb DNA fragment derived from the endogenous human *p21*^{WAF1} promoter present in the plasmid wwp-luc (El-Deiry *et al.*, 1993) was digested with *Hin*dIII and subcloned in the *Hin*dIII site of the pGL3-Basic vector (Promega) in the correct orientation, to generate p21P-luc. p21ΔP-luc was constructed by digestion of p21P-luc with *Sac*I, which cuts once within the *p21*^{WAF1} promoter at the extreme 5′-end and once within the polylinker of the vector, followed by re-ligation of the plasmid. The plasmids were sequenced and deletion of a 72 bp fragment in p21ΔP-luc containing the p53 consensus binding site was confirmed. The pGL3-control vector expresses luciferase under control of the SV40 promoter. CMV-wtp53, p53Ala143 and p53Tyr275 are plasmids containing full-length p53 cDNA under control of the CMV promoter. pSV2-E6, pSV2-E7 and pSV2-E6/E7 contain genes derived from HPV-16 under control of the SV40 promoter (Watanabe *et al.*, 1989). pLE2/520 expresses wild-type E1A; dl1101 and dl1143 express E1A deletion mutants competent for binding to pRB but unable to bind p300; dl1108 expresses a mutant E1A protein which binds to p300 but is unable to bind pRB (Barbeau *et al.*, 1994).

Plasmid construction and purification of human p53 proteins

The nucleotide sequences encoding residues 1–72, 82–292 and 311–393 of wild-type human p53 were subcloned into the pET19b vector using standard techniques and their correct incorporation confirmed by sequencing. The resulting plasmids express 10 histidine residues followed by a linker containing an enterokinase cleavage site and a His-Met dipeptide immediately N-terminal to the p53 sequence. *Escherichia coli* BL21(DE3)-(pLys-S) harboring the desired plasmid were grown in 2 l batches of Luria broth at 37°C (p53:1–72 and p53:311–393) or 25°C (p53:82–292) and protein production was induced with 1 mM isopropyl-thiogalactose at an optical density (600 nm) of 0.6–0.7. Three hours post-induction cells were harvested by centrifugation at 6000 *g* for 30 min. His-tagged proteins were purified from cell extracts by Nickel affinity chromatography and dialyzed for 24 h at 4°C against 4 l buffer (25 mM sodium phosphate, 100 mM NaCl, pH 7.0 or 7.4) using Spectra/por 3000 molecular weight cut-off dialysis membrane. Protein concentrations were determined spectrophotometrically using the appropriate theoretical extinction coefficient.

Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared from cells washed with PBS and immediately lysed on ice in buffer A [20 mM Tris, pH 7.4, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 0.1% NP-40, 2 mM Pefabloc (Boehringer Mannheim), 70 μg/ml aprotinin, 50 μg/ml leupeptin]. The nuclear pellet was resuspended in buffer B (as buffer A except for 100 mM NaCl, 1% NP-40). The mixture was kept on ice for 15 min, spun and the supernatant used immediately. The binding reaction contained 0.1 μg PAb421, 1 μg poly(dI-dC), 5 μl nuclear extract (4 μg protein), ^{32}P-labeled double-stranded p53CON oligonucleotide (10^5 c.p.m.) containing the p53 consensus binding site (Funk *et al.*, 1992), shown underlined (GGATCCAAGCTTGGACATGCCCGGGCATGTCCCTCGAGGGATCC), in a final concentration of 100 mM NaCl, 5 mM EDTA, 20 mM Tris, pH 7.4. A fill-in reaction was used to

label the oligonucleotide using Klenow DNA polymerase, [α-^{32}P]dCTP and the antisense oligonucleotide (GGATCCCTCGAG). In some experiments a competitor oligonucleotide of the same base composition and length as p53CON was used. The reactions were incubated at room temperature for 20 min and samples were analyzed on a 4% non-denaturing polyacrylamide gel run at 200 V for 3 h. Gels were dried and exposed to a PhosphorImager screen for 3–5 days. Supershifted bands generated within the linear range of the DNA binding assay were quantified on a PhosphorImager (Molecular Dynamics) using ImageQuant software.

Immunoprecipitation, Western blot analysis and immunohistochemistry

Cells washed on ice with PBS were lysed either in lysis buffer C [1% NP-40, 150 mM NaCl, 20 mM Tris, pH 8.0, 2 mM Pefabloc (Boehringer Mannheim), 70 μg/ml aprotinin, 50 μg/ml leupeptin, 20 μg/ml pepstatin A, 500 mM EDTA] or SUG buffer (3% SDS, 125 mM Tris, pH 6.8, 6% urea, 10% glycerol and all of the above protease inhibitors). For immunoprecipitations lysates (1 mg) prepared using lysis buffer C were mixed with one of the following antibodies: PAb1801 (human p53-specific); PAb421 (panspecific p53); SC-187 (p21-specific); 318 (PARP-specific polyclonal). The immune complexes were collected with 100 μl protein G–Sepharose beads (Pharmacia). For analysis of PARP the samples were subjected to sonication for 20 s prior to immunoprecipitation. Protein quantification was performed using a modified Lowry assay (Sigma). An equal volume of 2× protein sample buffer was added to cell extracts adjusted to contain equivalent amounts of protein, boiled and loaded on 10% polyacrylamide gels containing SDS. Proteins were transferred to nitrocellulose membranes. The p53 protein was detected using DO-1, PAb1801 or PAb7 antibodies (Oncogene Science). CM1 is a polyclonal rabbit antibody raised against human p53 (Dimension Labs). PARP was detected using C2-10 monoclonal antibody. The protein–antibody complexes were detected using an HRP-conjugated secondary antibody using the super-signal enhanced chemiluminescence system (Pierce).

p21 protein was detected by immunohistochemistry using the Vectastain ABC kit (Dimension Labs) as suggested by the manufacturer. In brief, cells were fixed with 70% ethanol, blocked with 5% horse serum and washed with PBS containing 0.05% Tween-20. After incubation in a 1:200 dilution of SC-187 primary antibody (Santa Cruz) for 30 min, cells were incubated with a biotinylated secondary antibody for 10 min and treated with 3% H_2O_2 for 1 min.

Luciferase assay

Cells were lysed 72 h after transfection with p21P-luc or p21ΔP-luc and the protein content of each extract was determined. Luciferase activity was measured using the luciferase assay reagent (Promega). Light emission over a 30 s interval was measured in a Berthold LB 9507 luminometer. A modified Hirt procedure (Matsuoka *et al.*, 1990) was used to extract plasmid DNA from cells. Linearized plasmid DNA was run on a 0.8% agarose gel; the gel was dried and subsequently probed with a γ-^{32}P-end-labeled oligonucleotide (5′ATTACCAGGGATTTCAGTCG) specific for the luciferase coding sequence. Band intensities were quantified on a PhosphorImager (Molecular Dynamics) to provide an estimate of relative plasmid copy number in the transfected cells and, hence, a measure of transfection efficiency. This is preferred over co-transfection with a β-galactosidase vector, for example, for several reasons: endogenous β-galactosidase activity in HDFs increases with age (Dimri *et al.*, 1995); measurement of β-galactosidase activity provides only an indirect measure of transfection efficiency; p53 is known to modulate the activity of a large number of different promoters in co-transfection experiments. The RLUs (relative light units) obtained from the luciferase assay were normalized for differences in transfection efficiency in this way.

Isolation of DNA, SSCP analysis and measurement of telomeric DNA length

Cells were washed three times in PBS. Cell pellets was resuspended in proteinase K digestion buffer (100 mM NaCl, 10 mM Tris, pH 8, 5 mM EDTA, 0.5% SDS) containing proteinase K at a final concentration of 0.5 mg/ml. After incubation at 48°C overnight, the DNA was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform. DNA was precipitated with 95% ethanol and dissolved in TE (10 mM Tris, pH 8, 1 mM EDTA, pH 8).

DNA (10 μg) was digested with *Hin*fI and *Rsa*I (20 U each; BRL), extracted as described above, precipitated with 95% ethanol, washed in 70% ethanol, resuspended in TE and quantified by fluorometry using a Turner model 450 fluorometer. One microgram of digested DNA was resolved by electrophoresis on a 0.5% agarose gel poured on a Gel bound membrane (FMC Bioproducts) and run for 700 V h. Gels were dried, denatured, neutralized and probed with a γ-^{32}P-5′-end-labeled $(CCCTAA)_3$ oligonucleotide as described (Vaziri *et al.*, 1993). Gels were exposed to a PhosphorImager screen and the hybridization signals were digitized and subdivided into 1 kbp to 21 kbp for calculation of the mean TRF length (L) using the formula $L = \Sigma(OD_i{*}L_i)/\Sigma OD_i$, where OD_i is the integrated photon signal in interval i and L_i is the TRF length at the midpoint of interval i.

SSCP analysis of p53 genomic DNA from Li–Fraumeni cells was performed as previously described (Mitsudomi *et al.*, 1992) in young cells and during senescence and crisis.

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