# p53 Is Preferentially Recruited to the Promoters of Growth Arrest Genes *p21* and *GADD45* during Replicative Senescence of Normal Human Fibroblasts

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## Abstract

**Replicative senescence is the terminal growth arrest that most normal human cells enter into after a fixed number of divisions *in vitro*, limiting the proliferative potential of a cell and preventing genomic instability caused by critically short telomeres. Thus, senescence presents a tumor-suppressive mechanism and a barrier to tumor formation. However, senescent cells are inherently resistant to apoptosis and, as they accumulate in aging tissues, may contribute to organ dysfunction and promote tumor progression as part of the stromal environment. Replicative life span in normal human cells can be extended by inactivation of the tumor suppressor gene *p53* or its direct target, the cyclin-dependent kinase inhibitor *p21*, suggesting a direct role for this pathway in senescence. However, p53 recruitment to promoters of target genes during replicative senescence has not been shown in live cells. In this study, we used chromatin immunoprecipitation to determine that p53 preferentially occupied the promoters of growth arrest genes *p21* and *GADD45* in senescent normal human diploid fibroblasts but not the promoters of other target genes that recruited p53 following doxorubicin-induced DNA damage, such as apoptosis regulators *TNFRSF10b, TNFRSF6*, and *PUMA*. This differential recruitment of p53 in senescent versus doxorubicin-treated fibroblasts was accompanied by differences in post-translational modification of p53. These data provide mechanisms for both the growth arrest mediated by p53 and the resistant nature of senescent cells to apoptosis despite p53 activity.** (Cancer Res 2006; 66(17): 8356-60)

## Introduction

*p53* is the most commonly mutated gene in cancer, exerting its activity by binding DNA response elements and regulating transcription of specific genes after various stimuli, directing cells toward cell cycle arrest or apoptosis (1). After one such stimulus, genotoxic stress, p53 is phosphorylated and stabilized, resulting in accumulation of protein and formation of homotetramers that then activate specific gene promoters, including *p21* and *GADD45* for growth arrest and *PUMA, TNFRSF6/Fas-Apo*, and *TNFRSF10/Killer-DR5* for apoptosis.

Replicative senescence is thought to be a major barrier to tumor formation, as it limits the replicative potential of normal cells and preserves genomic integrity by preventing cells with short telomeres from entering the cell cycle (2). Experimental evidence suggests a role for p53 in cellular senescence (1). Inactivation of p53 using viral oncogenes (SV40 large T antigen or human papillomavirus E6 protein), gene targeting methods, or specific oligos allows a cell to bypass the initial arrest of replicative senescence, resulting in an extended replicative life span (reviewed in ref. 1). p53 also seems to be responsible for the maintenance of replicative senescence (3). Microinjection of anti-p53 antibodies into senescent cells (4) or introduction of large T antigen, mutant large T that binds only p53, or a dominant-negative acting p53 peptide each could induce senescent cells to enter S phase (3).

The cyclin-dependent kinase (cdk) inhibitor p21 is a major transcriptional target of p53. *p21* was originally identified as a gene from a cDNA library of senescent cells that inhibited DNA synthesis (5). Further study showed that p21 was up-regulated in numerous cell lines during senescence (6). p21 was also identified as an inhibitor of cyclin-cdk complexes, suggesting a mechanism of action for promoting cell cycle arrest (7). Additionally linking p21 to cellular senescence, adventitious expression of p21 was shown to induce a permanent growth arrest with characteristics of cellular senescence in several cancer cell types (8). Finally, normal human fibroblasts bypass replicative senescence when p21 has been deleted by gene targeting and eventually enter crisis (9).

p53 has been shown to bind the *p21* promoter *in vivo* following DNA damage in several cell lines, such as U2OS (10). However, despite the wealth of evidence showing *in vivo* p53 binding to the *p21* promoter after exogenous DNA damage, only a limited number of *in vitro* gel shift and reporter assay experiments have been done with senescent cells (11–13). Recent evidence suggests a disparity between these types of *in vitro* experiments relative to observations made *in vivo* that, as of yet, remains unresolved (14).

Previous studies have also suggested that increased p53 transcriptional activity in cells with DNA damage is due solely to increased protein levels (14). For replicative senescence, one study found increased p53 protein (15); however, numerous others have found no change (12, 13, 16, 17). The studies by Kaeser et al. (14) taken in context with those showing no change in p53 levels in replicative senescence would argue against a role for p53 transactivation of the *p21* gene in replicative senescence despite functional evidence to the contrary. Further, senescent cells are resistant to apoptosis despite the presumed activity of p53 in senescence and the known role of p53 in inducing apoptosis via gene regulation. Because of these unresolved questions, we examined p53 promoter dynamics in cells at replicative senescence compared with young cells at quiescence, in log growth, and treated with the DNA-damaging drug doxorubicin.

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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## Materials and Methods

**Antibodies and Western blotting.** p21 and p53 antibodies for Western blot were from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylated-specific p53 antibodies (Phospho-p53 Antibody Sampler kit) were from Cell Signaling (Danvers, MA). p53 acetylated K373/K382 was from Upstate Biotechnology (Charlottesville, VA). For Fig. 1, cells were harvested and immunoblotted exactly as described previously (18). In Fig. 3, nuclear lysates were prepared from cells [Nucleic Acids Res. 1991;19(9):2499] and 50 μg were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking in 5% milk (18), primary antibody [500:1, in TBS-Tween 20 solution (18)] was incubated overnight at 4°C followed by washing, secondary antibody incubation, and enhanced chemiluminescence (18).

**Cell culture.** Log-phase, quiescent, and senescent HCA2 (foreskin-derived fibroblast, also called MJ90) and IMR90 (lung-derived fibroblast) cells were cultured as described previously (19). Confluent cultures of young cells, population doubling (PD) 34 or lower, were made quiescent by incubation in 1% serum-containing medium for at least 7 days, conditions we have established previously and have confirmed typically result in <15% cells labeling after a 48-hour continuous bromodeoxyuridine exposure. HCA2 became senescent (failed to double after at least 2 weeks in culture, failed to proliferate after passage, and adopted a large, flattened morphology) at approximately PD 69. We confirmed that >90% of senescent cells were positive for senescence-associated β-galactosidase staining at pH 6, whereas <3% of young cells were positive. IMR90 cells became senescent at PD 51, and young cells used were at PD 35 or lower. Doxorubicin treatment was for 24 hours at 200 ng/mL (14).

**Chromatin immunoprecipitation.** Chromatin immunoprecipitations (ChIP) were done exactly as previously published (18) with p53 clone DO1 (Santa Cruz Biotechnology). Primer sequences used in real-time PCR and accession numbers of genes tested are published as Supplementary Data.

**Real-time reverse transcription-PCR.** Experiments were done exactly as previously published (18) using RPLP0 as the relative control; primer sequences are published as Supplementary Data.

## Results

**p53 levels do not change in senescent fibroblasts.** Western blot of lysates from HCA2 normal human diploid fibroblasts in quiescence (Q), growing asynchronously in log phase (L), treated with doxorubicin (T), and made senescent by extensive serial passage in culture (S) revealed that p53 protein was elevated following drug treatment but not in quiescent, log-phase, or senescent cells (Fig. 1, *top*). However, p21 levels were markedly increased in both young cells following doxorubicin treatment and in senescent cells (Fig. 1, *middle*), whereas actin levels were unchanged (Fig. 1, *bottom*). Similar results were obtained with IMR90 human fetal lung fibroblasts (data not shown).

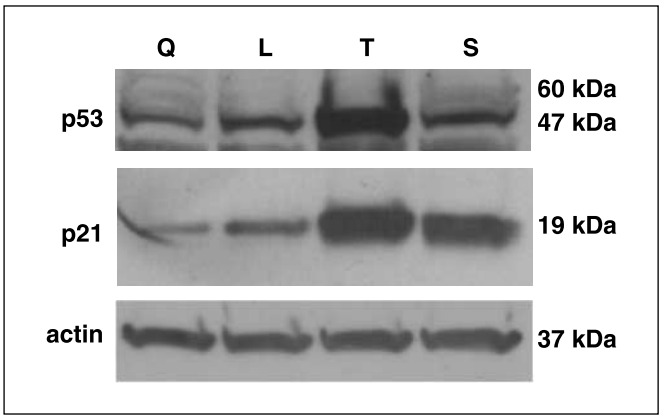


**Figure 1.** p53 and p21 expression in normal human fibroblasts. HCA2 cells at low passage number were incubated in 1% serum for 7 days to make them quiescent (*Q*), grown asynchronously in log phase to 70% confluence (*L*), and treated in log phase with 200 ng/mL doxorubicin for 24 hours (*T*), and cells made senescent by serial passage in culture (*S*) were harvested, protein extracted, and Western blotted for p53, p21, and actin control. Data are representative of at least two independent experiments.

**p53 binds growth arrest but not apoptotic gene promoters in senescent fibroblasts.** We next examined p53 occupancy of various target promoters by ChIP in HCA2 normal human diploid fibroblasts under the same conditions as in Fig. 1. p53 binding to specific promoters was detected at approximately four relative sensitivities compared with input levels. The highest relative amount of p53 was bound to the *p21* and *MDM2* promoters (Fig. 2*A*). There was increased p53 occupancy at both of these promoters following doxorubicin treatment, but p53 was enriched at only the *p21* promoter in senescent cells (Fig. 2*A*). As expected, p53 was not enriched at the acetylcholine receptor promoter (AchR), and control immunoprecipitations, done without primary antibody, did not precipitate *p21* or *MDM2* promoter DNA (Fig. 2*A, NoAb*).

Examining the second panel of p53 target promoters, we found that p53 bound the growth arrest gene *GADD45* both following drug treatment in young cells and in senescent cells. However, at other target genes, including those that regulate apoptosis, *TNFRSF10b* (*Killer-DR5*), *TNFRSF6* (*Fas-Apo*), and *PUMA* as well as *PLK3, MMP2*, and the uncharacterized gene *c12orf5*, p53 occupancy was increased only following drug treatment and not in senescent cells. At the third range of sensitivity, p53 bound each target promoter, *CCNG1, PRKAB, GPX, PHLDA3, 14-3-3, NOXA, Cardiotrophin* (*CAR*), *BTG2*, and *PIG3*, following drug treatment but not in senescence; p53 binding to *PERP* was unchanged (Fig. 2*A*). In a final panel of genes examined, each of which has also been previously reported to be p53 targets in various cell types or conditions (references included with Supplementary Data), we found a much smaller degree of enrichment of promoter DNA similar to background levels of enrichment in no-antibody controls and at the AchR. However, *BAX, AIP1*, and *CHEK1* seemed to have p53 present at their promoters at levels exceeding the controls but only following drug treatment. For the other genes examined, *BCL2, α1 acid glycoprotein* (*α1AGly*), *myoglobin* (*MYO*), *PTTG1a, PTTG1c, TGFα, IGFBP3, histidine decarboxylase* (*HisD*), the data were ambiguous or p53 clearly was not present at their promoters in any of the cell states. Similarly, in senescent IMR90 cells, p53 levels were elevated at the *p21* promoter, but p53 was not detected at promoters of apoptotic genes above the levels observed in young cells (data not shown). Thus, p53 bound only the promoters of *p21* and *GADD45* in senescent cells and not promoters for apoptotic genes or other growth arrest genes, such as *CCNG1* or *14-3-3*. These data are summarized in Table 1.

**Expression of p53 target genes.** We next examined mRNA levels of p53 targets. In senescent cells, we found that only p21 and GADD45 expression was increased (Fig. 2*B*) corresponding to increased p53 binding at those promoters (Fig. 2*A*). In doxorubicin-treated cells, we found that expression loosely correlated with p53 binding: genes that had increased p53 present at their promoters relative to untreated, *MDM2, TNFRSF10b, PRKAB1, PLK3, PIG3*, and *c12orf5* (Fig. 2*A*), had relatively higher expression levels of these genes after treatment (Fig. 2*B*). However, we observed some deviation from this trend as the PUMA promoter bound p53 following treatment but did not exhibit increased expression of mRNA (Fig. 2*B*). Further, PIG3 showed a marked increase in mRNA

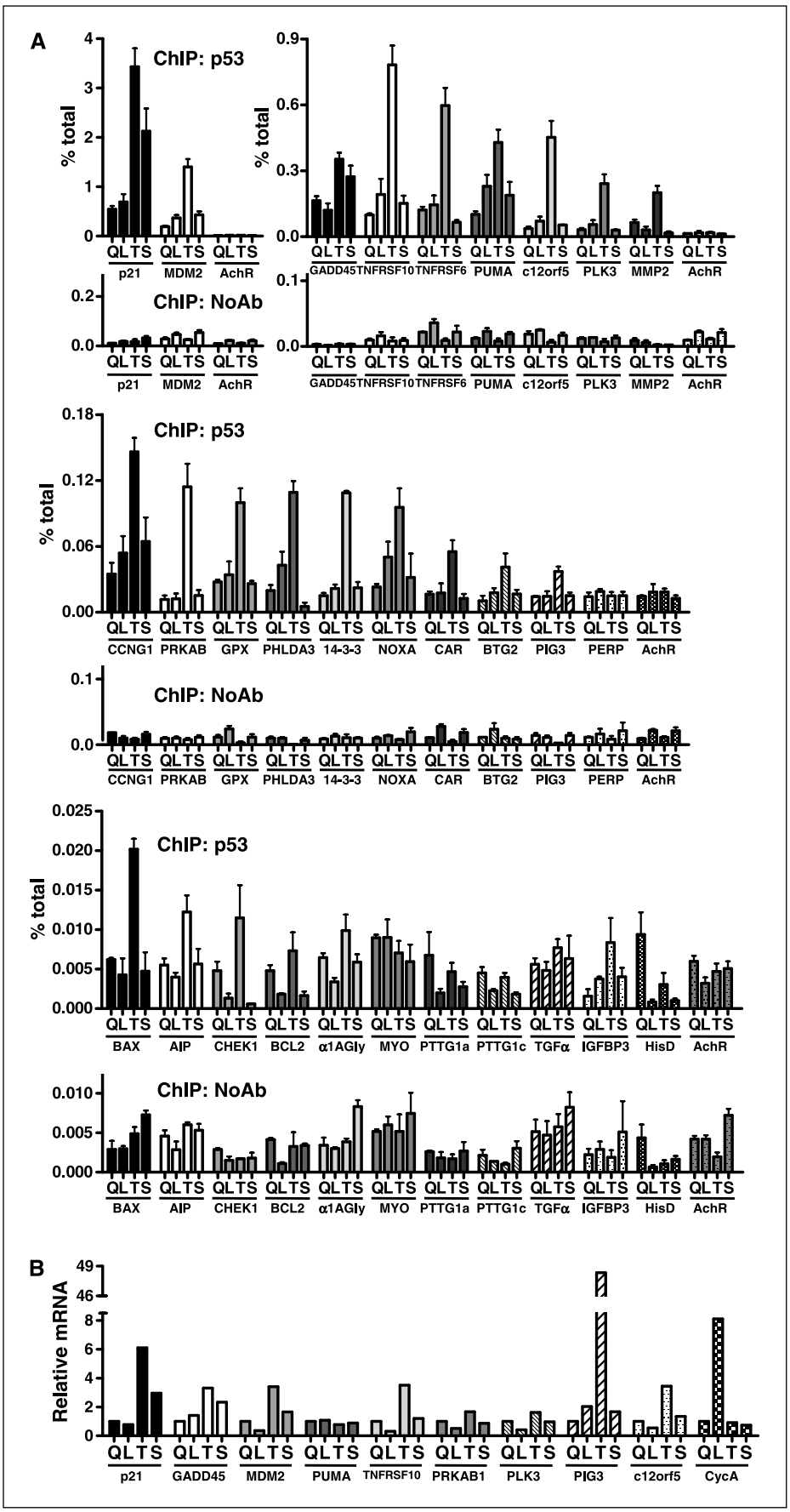


**Figure 2.** p53 recruitment to and expression of *p21* and *GADD45* growth arrest genes but not other target genes in senescent cells. *A,* HCA2 cells grown and treated as in Fig. 1 were harvested for ChIP, and real-time PCR was done with primers specific to promoter regions of genes indicated in the figure. Primer sequences and accession numbers of genes examined are provided in the Supplementary Data. Data are representative of two ChIP experiments. *B,* mRNA was extracted from HCA2 cells grown and treated as in Fig. 1, and reverse transcription was then done followed by PCR amplification in real time with primers as indicated in the figure (sequences are provided as Supplementary Data). mRNA levels were normalized to RPLP0, and values shown are relative to quiescent cells. Data are representative of at least two independent experiments.

**Table 1.** Data for ChIPs are presented as fold enrichment of p53 ChIPs over mean of no-antibody controls for each primer set

| | Gene promoter | Fold enrichment relative to mean no-antibody control ChIP | | | |
|---|---|---|---|---|---|
| | | Q | L | T | S |
| Growth arrest | *p21* | 27.92 | 35.47 | 175.55 | 108.81 |
| | *GADD45* | 57.82 | 42.64 | 124.79 | 96.68 |
| | *PLK3* | 2.80 | 4.86 | 21.26 | 2.60 |
| | *CCNG1* | 2.63 | 4.08 | 11.07 | 4.88 |
| | *14-3-3* | 1.42 | 2.04 | 10.22 | 2.10 |
| | *CHEK1* | 2.46 | 0.68 | 5.86 | 0.30 |
| | *BTG2* | 0.78 | 1.33 | 3.08 | 1.24 |
| Apoptosis | *TNFRSF10b* | 9.22 | 17.92 | 72.78 | 14.14 |
| | *PUMA* | 6.47 | 14.62 | 27.33 | 12.03 |
| | *TNFRSF6* | 5.52 | 6.58 | 27.02 | 2.96 |
| | *PRKAB1* | 1.21 | 1.27 | 11.90 | 1.59 |
| | *BAX* | 3.24 | 2.22 | 10.52 | 2.47 |
| | *NOXA* | 1.80 | 3.92 | 7.46 | 2.47 |
| | *PIG3* | 1.34 | 1.37 | 3.53 | 1.44 |
| | *IGFBP3* | 0.52 | 1.24 | 2.76 | 1.33 |
| | *P53AIP1* | 1.23 | 0.89 | 2.72 | 1.26 |
| | *BCL2* | 1.62 | 0.61 | 2.47 | 0.56 |
| | *PTTG1a* | 3.09 | 0.91 | 2.13 | 1.27 |
| | *PTTG1c* | 2.40 | 1.20 | 2.10 | 0.96 |
| | *PERP* | 0.99 | 1.32 | 1.04 | 1.05 |
| Other/ Unknown | *MMP2* | 12.95 | 6.12 | 39.81 | 3.51 |
| | *MDM2* | 4.96 | 9.58 | 36.40 | 11.18 |
| | *c12orf5* | 2.24 | 4.25 | 27.08 | 3.19 |
| | *PHLDA3* | 2.86 | 6.21 | 15.78 | 0.77 |
| | *GPX* | 2.20 | 2.71 | 7.95 | 2.07 |
| | *CAR* | 1.06 | 1.13 | 3.57 | 0.81 |
| | *α1AGly* | 1.39 | 0.73 | 2.13 | 1.27 |
| | *HisD* | 4.89 | 0.42 | 1.59 | 0.53 |
| | *TGFα* | 0.94 | 0.81 | 1.30 | 1.07 |
| | *MYO* | 1.51 | 1.51 | 1.19 | 1.00 |
| | *AchR* | 0.91 | 1.16 | 1.17 | 0.80 |

level but a moderate increase in an already low level of p53 binding (Fig. 2*A*). This occasional lack of correlation of p53 promoter binding and expression has been observed previously (14). mRNA levels were not systemically elevated in drug-treated cells, as cyclin A levels were depressed as expected in quiescent, senescent, and drug-treated cells and elevated in log-phase cells (Fig. 2*B*).

**p53 is differentially modified in senescent versus treated cells.** Previously, others have shown that, in senescent HCA2 fibroblasts, p53 has decreased phosphorylation of S392 and increased phosphorylation of S15 and S18 (17). Using modification-specific p53 antibodies, we confirmed the increase in S15 phosphorylation in senescent cells, whereas treated cells had increased phosphorylation or acetylation on each of the residues examined (Fig. 3). We were unable to detect any phosphorylation of S392 in untreated cells; thus, we cannot confirm a decrease in phosphorylation in senescent cells. We detected, however, decreased phosphorylation at residues S37 and S46 in senescent versus untreated cells. No phosphorylation of S6, S9, S20, and S392 or acetylation of K373/K382 was detected in senescent cells. Thus, taken with the Webley et al. study, these data suggest a possible combination of p53 modifications (increased S15 and S18 phosphorylation and decreased phosphorylation of S37, S46, and S392) that direct the preferential recruitment of p53 to only the *p21* and *GADD45* promoters in senescent cells without increasing protein levels.

In summary, we show that, in senescent fibroblasts, p53 was recruited exclusively to promoters of the growth arrest genes *p21* and *GADD45* but not to any of the other p53 target genes examined, including apoptotic genes. This difference in p53 stability and activity following doxorubicin-induced DNA damage and during replicative senescence might be explained by the difference in post-translational modification and lends mechanistic insight into the seemingly paradoxical resistance of senescent cells to apoptosis despite the known role of p53 in apoptosis.

## Discussion

p53 transactivation of growth arrest and apoptotic promoters after acute genotoxic stress has been well described, and abundant evidence exists indicating a role for both p53 and p21 in replicative senescence of normal human diploid fibroblasts (1). However, to our knowledge, p53 binding to the promoters of target genes *in vivo* during replicative senescence had not been shown previous to this study.

In agreement with others (12, 13, 16, 17), we found that levels of p53 protein were not increased in senescent cells but that p53 was converted from a latent, nonbinding form to an active form during senescence. This is in contrast to what was observed following

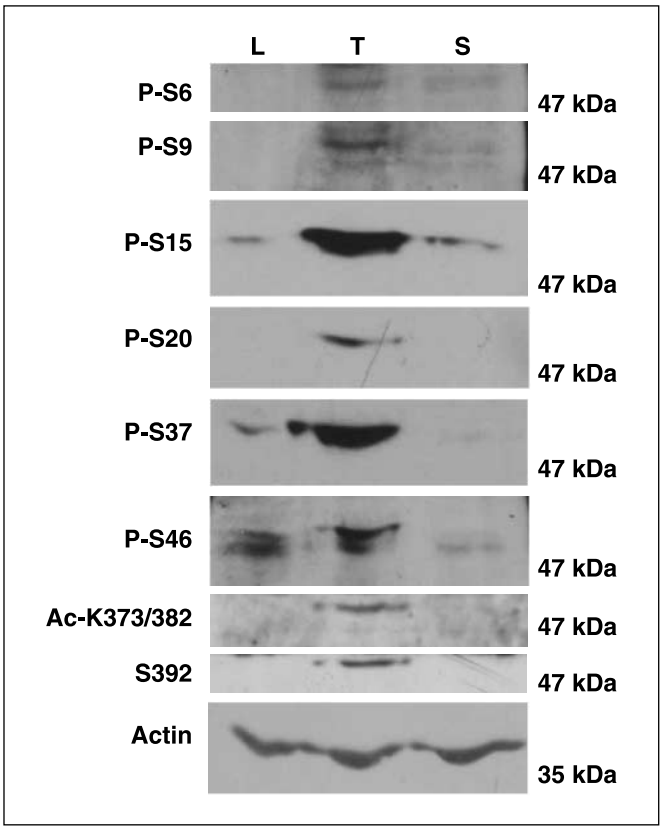


**Figure 3.** Differential post-translational modification of p53 in senescent versus treated cells. HCA2 cells were grown and treated as in Fig. 1, nuclear lysates were prepared, and parallel Western blots were done using the antibodies indicated in the figure. Data are representative of two independent experiments.

DNA damage by Kaeser et al. (14), where increased protein levels of p53 dictated increased binding to the p21 promoter. This difference could be explained by the distinct profiles of post-translational modifications observed in senescent versus drug-treated cells, which also could result in the association of p53 with different cofactors in senescent cells and the eventual recruitment to only the *p21* and *GADD45* promoters.

The increased binding activity of p53 at senescence is likely tumor suppressive, blocking the proliferation of cells with short telomeres. When cells bypass replicative senescence, telomeres continue to shorten with each division, eventually reaching a state where they form end to end fusions, creating dicentric chromosomes that lead to anaphase bridges and breakages, ultimately creating regions of amplification and deletion (20). The gross chromosomal abnormalities that ensue are similar to those observed in human epithelial cancers, leading to speculation that this breakage-fusion-bridge cycle occurs *in vivo* and contributes to malignant transformation (20). Thus, it is important that we understand the mechanisms of how cells are directed into the permanent arrest of replicative senescence and how this barrier might be bypassed in cancer formation. Accumulation of senescent cells that are inherently resistant to apoptosis, however, may also lead to organ dysfunction in the aged and, as part of the stromal environment, promote cancers (2). We provide data here elucidating how cells are directed to senescence by the tumor suppressor p53 and why these cells are resistant to apoptosis, allowing them to persist with deleterious effects, including aging phenotypes and tumor promotion in neighboring cells.

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