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# Expression of p16$^{INK4A}$ Variants in Senescent Human Fibroblasts Independent of Protein Phosphorylation

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**Abstract** Upregulation of the p16 tumor suppressor is a hallmark of senescence in human fibroblasts. In this study, we investigated potential protein modification of p16 in senescent human fibroblasts using 2D SDS–PAGE analysis. Three distinct p16 variants with isoelectric points of 5.2, 5.4, and 5.6, were consistently detected in normal human IMR90 fibroblasts that had undergone senescence due to forced expression of oncogenic H-ras or culture passage. Moreover, in contrast to short-term serum starvation, which induces quiescence, IMR90 fibroblasts cultured in low serum for a prolonged period exhibited senescent phenotypes and expression of the three p16 variants. All three p16 variants are unlikely phosphoproteins since they failed to react with antibodies against phospho-serine, and were resistant to the treatment with phosphatases. Functionally, co-immunoprecipitation assays using antibodies against cdk4 and/or cdk6 revealed that only the two most acidic p16 variants associated with cdk4/6. Moreover, senescence induced by the forced expression of p16 in early passage IMR90 fibroblasts or osteosarcoma U2OS cells was accompanied by expression of the two most acidic p16 variants, which also associated with cdk4/6. In summary, we report that prolonged serum starvation-induced senescence may provide an additional model for studying biochemical changes in senescence, including p16 regulation. Furthermore, induction of endogenous p16 in senescent human fibroblasts correlates with the expression of three distinct p16 variants independent of protein phosphorylation. Lastly, expression of the two cdk-bound variants is sufficient to induce senescence in human cells. J. Cell. Biochem. 94: 1135–1147, 2005. 

**Key words:** senescence; quiescence; oncogenic ras; human fibroblasts; p16; cdk; 2D SDS–PAGE; proteomics

## INTRODUCTION

The cyclin–cdk complexes play essential roles during cell cycle progression [Murray, 2004]. Members of the INK4 and KIP family proteins are inhibitors of cdks, thereby regulating cell cycle progression [Xiong et al., 1993; Polyak et al., 1994; Sherr and Roberts, 1995; Cunningham and Roussel, 2001]. The INK4 family includes p15$^{INK4B}$, p16$^{INK4A}$, p18$^{INK4C}$, and p19$^{INK4D}$, while the KIP family is composed of p21$^{CIP1}$, p27$^{KIP1}$, and p57$^{KIP2}$ [Cunningham and Roussel, 2001]. The INK4 family proteins are known to specifically bind to and inhibit cdk4 and cdk6, leading to the inactivation of cyclin D–cdk4/6 complexes, which are required for Rb phosphorylation during G1-S transition [Serrano et al., 1993; Medema et al., 1995; Lukas et al., 1995b; Ruas and Peters, 1998; Sherr, 2000]. Consequently, the Rb proteins remain underphosphorylated, leading to a G1 arrest. Among the cdk inhibitors, p16 is the only known bona fide tumor suppressor. Inactivation of p16 is a frequent event and is found in a wide range of human cancers [Okamoto et al., 1994; Aagaard et al., 1995; Rocco and Sidransky, 2001]. In contrast, induction of p16 in normal proliferating cells is a relatively rare event. Interestingly, elevated expression of p16 is one of the hallmarks of senescence in human cells, including fibroblasts, uroepithelial cells, and lymphocytes [Alcorta et al., 1996; Erickson et al., 1998; Puthenveettil et al., 1999].

Abbreviations used: cdk, cyclin dependent kinase; CHAPS, (3-[3-cholamidopropyl dimethylammonio]-1-propanesulfonte); 2D SDS–PAGE, two dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis; IPG, immobilized pH gradient; pI, isoelectric point(s); and SA-β-gal, senescent-associated β galactosidase.

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Numerous in vitro studies suggest that senescence acts to prevent cells from accumulating mutations that would otherwise lead to neoplastic transformation [Campisi, 2001; Mathon and Lloyd, 2001; Shay and Roninson, 2004]. In the case of replicative senescence, cells cease proliferation permanently after a defined number of population doublings, but remain metabolically active for a long period of time. Generally, senescent cells display characteristic changes in cell morphology and at the molecular level [Dimri and Campisi, 1994; Dimri et al., 1995; Campisi, 2003; Narita et al., 2003]. For instance, it has been shown that senescent cells exhibit elevated levels of the p53 and p16 tumor suppressors, and that inactivation of the p53 or p16/Rb tumor suppressor pathways can bypass senescence [Hara et al., 1991; Bond et al., 1995; Lin et al., 1998; Ferbeyre et al., 2002; Wei et al., 2003]. Consistent with these findings, p53, p16, or Rb are found to be frequently inactivated in a wide range of human cancers. Rising evidence demonstrates that senescence can be triggered by various stimuli, including telomere shortening, reactive oxygen species, DNA damage, and oncogenic stimuli [Chen et al., 1995; Serrano et al., 1997; Zhu et al., 1998; Macip et al., 2002; te Poele et al., 2002; Herbig et al., 2004]. Forced expression of oncogenic H-ras can induce senescence in normal cells, including normal human fibroblasts, primary mouse fibroblasts and skin keratinocytes [Serrano et al., 1997; Lin et al., 1998; Lin and Lowe, 2001]. The mechanisms by which oncogenic ras induces senescence depend on cellular context and signal intensity [Lin and Lowe, 2001; Sharpless et al., 2001; Deng et al., 2004]. For instance, the ARF/p53 pathway is essential for ras-induced senescence in mouse cells [Palmero et al., 1998; Lin and Lowe, 2001], while the p16/Rb pathway plays a critical role in ras-induced senescence in human fibroblasts [Beausejour et al., 2003; Drayton et al., 2003; Narita et al., 2003; Wei et al., 2003; Mallette et al., 2004]. Indeed, human fibroblasts that carry inactivating mutations in the *p16* gene are resistant to ras-induced senescence, suggesting that p16 plays a critical role in mediating senescence in human cells [Brookes et al., 2002; Huot et al., 2002; Narita et al., 2003].

The mechanisms by which p16 is inactivated in cancers is well studied. Point mutations, homozygous deletion, and DNA methylation of the p16 promoter have been shown to be highly associated with p16 inactivation [Ranade et al., 1995; Parry and Peters, 1996; Ruas et al., 1999; Florl et al., 2000]. In contrast, the activation mechanism for p16 is less characterized. Several studies have shown that p16 can be regulated transcriptionally by the balance between a positive factor, Id-1, and negative regulators Ets1/2 [Alani et al., 2001; Ohtani et al., 2001; Zebedee and Hara, 2001]. Additionally, Bmi-1, a member of the polycomb group gene product, has been shown to be a repressor of p16 expression [Jacobs et al., 1999; Itahana et al., 2003]. Post-translational regulation of p16 has only begun to be explored in recent years. A study using senescent prostatic epithelial cells detected phosphorylated p16 variants [Sandhu et al., 2000]. Additionally, proliferating normal human WI38 fibroblasts exhibit p16 protein phosphorylation [Gump et al., 2003]. Here, we investigate the expression pattern and potential modification of the p16 proteins in relation to its activity in senescent human cells induced by various triggers.

## MATERIALS AND METHODS

### Cell Culture

Early passage normal human diploid IMR90 fibroblasts, osteosarcoma U2OS cells, HeLa cells, and Pheonix retrovirus packaging cells were obtained from ATCC and maintained in DMEM (Mediatech, Herdon, VA) supplemented with 10% FBS (HyClone, Logan, UT), as described previously [Lin et al., 1998].

Replicative senescent cells were generated by culturing early passage normal human IMR90 fibroblasts in DMEM containing 10% FBS for approximately 25 passages (~60 population doublings). Senescence was determined based on the upregulation of Senescence associated-β-galactosidase (SA-β-gal) activity and p16 protein expression.

For serum starvation, early passage IMR90 fibroblasts were plated at low-density in DMEM with 10% FBS. After overnight incubation, cells were collected (designated as "Day 0") or washed with PBS three times and cultured in DMEM containing 0.1% FBS for the indicated days. Medium (containing 0.1% FBS) was replaced twice a week and cells were collected at the indicated days.

### Plasmids and Retroviral Gene Transfer

Retroviral constructs expressing H-RasV12 (H-RasV12-pBabe-puro or H-RasV12-pWzl-Hygro), human p16 (p16-pWzl-Hygro), or controls (pBabe-puro or pWzl-hygro) were used to transduce into cells via retroviral gene transfer [Serrano et al., 1997; Narita et al., 2003]. Briefly, helper virus-free retroviruses expressing H-RasV12 or p16 or empty vectors were produced by the Phoenix packaging cells and used to transfect early passage IMR90 fibroblasts or osteosarcoma U2OS cells, as described previously [Lin et al., 1998].

### SDS–PAGE and Western Blot Analysis

For one-dimensional (1D) SDS–PAGE analysis cells were lysed in Laemmli buffer [Laemmli, 1970] as described previously [Lin et al., 1998]. For two-dimensional (2D) SDS–PAGE analysis, cells were lysed in ice-cold lysis buffer (4% CHAPS, 2M thiourea, 7M urea, 40 mM Tris, 2 mM tributylphosphine, and 2% pharmalite) and centrifuged at 14,000*g* for 10 min at 4°C [Rabilloud, 2000]. Protein concentration of the cell extract was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). Typically, a total of 75–100 μg protein lysate was solubilized in the rehydration buffer (65 mM dithiothreitol, 8M urea, 2% CHAPS, 2% IPG buffer (pH 4–7), and 0.002% bromophenol blue) in a final volume of 125 μl. Isoelectric focusing was performed using 7 cm, 4–7 linear immobiline IPG strips on the IPGphore Isoelectric Focusing System following the manufacturer's instructions (Amersham, Piscataway, NJ). Briefly, immobiline strips were rehydrated with the solubilized sample in the strip holders for 16 h at 20°C. Subsequently, isoelectric focusing was carried out using a three-step protocol (500 V for 30 min, 1000 V for 30 min, and 5000 V for 2 h 40 min) to achieve a final of 8000 V hours. Following isoelectric focusing, IPG strips were first incubated in the equilibration buffer (50 mM Tris [pH 8.8], 6M urea, 30% glycerol, 2% SDS, 0.0002% bromophenol blue) with 65 mM dithiothreitol at room temperature for 30 min followed by incubating in the equilibration buffer with 135 mM iodoacetamide with agitation for an additional 30 min. After equilibration, IPG strips were mounted onto a 15% SDS–PAGE gel and electrophoresis was carried out at 20 mAmp/gel.

Western blot analysis was carried out as previously described [Serrano et al., 1997]. Briefly, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). Subsequently, blots and incubated with various primary antibodies (described below) followed by secondary antibody. Antibody binding was detected using ECL (Amersham) or Super Signal (Pierce, Rockford, IL).

### Antibodies

The following primary antibodies were used: p16 (C-20, Santa Cruz, Santa Cruz, CA), p16 (Delta BioLabs, Campbell, CA), p16 (Ab1, Oncogene, San Diego, CA), p16 (NCL-p16 mAb, Novocastra, Burlingame, CA), Rb (Pharmingen, Palo Alto, CA), p53 (CM1, Novocastra), Phospho-Ser$^{15}$ p53 (Cell Signaling, Beverly, CA), p53 (Ab-2, Oncogene), H-Ras (Oncogene), anti-α Tubulin (Sigma, St. Louis, MO), cdk4 (Upstate, Charlottsville, VA), cdk6 (Santa Cruz), and anti-p-Ser (Zymed, San Francisco, CA). HRP-conjugated anti-mouse or anti-rabbit antibodies (Amersham) were used as secondary antibody.

### Immunoprecipitation

Cells were collected, lysed in ice-cold immunoprecipitation buffer, sonicated on ice for three 10-s cycles (Sonic dismembrator-100, Fisher scientific, Pittsburg, PA) and centrifuged at 10,000*g* for 10 min at 4°C. NP-40 lysis buffer (0.1% NP-40, 150 mM NaCl, 50 mM Tris pH 8.0) was used as the immunoprecipitation buffer for immunoprecipitation of p16 (Delta BioLabs) or p53 (Ab-2, Oncogene). As a negative control, immunorprecipitation was also carried out using a normal rabbit serum (Biosource, Carmarillo, CA). For immunoprecipitation of cdk4 or cdk6, the immunoprecipitation buffer contained 50 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 0.1% Tween 20, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM $NaVO_4$, 1 mM PMSF, and 5 μg/ml leupeptin [Gump et al., 2003]. The supernatant was collected and protein concentration was measured as described above. In general, a total of 500–1000 μg of protein extract was subjected to immunoprecipitation with the indicated antibody. Antibody complexes were captured with protein G-Sepharose beads (Amersham) for 1 h at 4°C. The resulted immunocomplex was collected by centrifugation at 2,000*g* for 2 min at 4°C. The pellet was washed twice in ice-cold immunoprecipitation buffer followed by centrifugation. Subsequently, the pellet was resuspended in Laemmli buffer

and boiled for 4 min prior to 1D SDS–PAGE analysis. For 2D SDS–PAGE analysis, immunocomplex was eluted in rehydration buffer (2M thiourea, 8M urea, 2% CHAPS, 0.5% IPG buffer, 60 mM DTT, 0.002% bromophenol blue) and loaded onto 7-cm strip holders and rehydrated with pH 4–7 IPG strips (Amersham) overnight.

### Phosphatase Assay

Immunoprecipitation of p16, cdk4, or cdk6 was performed from 500 to 1000 μg of cellular extract as described above. For treatment using λ acid phosphatase (New England BioLabs), immunocomplexes were washed twice in lysis buffer and three times in λ acid phosphatase buffer (New England BioLabs). For treatment using potato acid phosphatase (Sigma), immunocomplexes were washed three times in washing buffer (50 mM PIPES, 1 mM DTT, pH 6) and subsequently, incubated with 100 U of λ acid phosphatase [Gump et al., 2003], or 12 U of potato acid phosphatase [Sandhu et al., 2000] for 3 h at 30°C. In parallel, immunocomplexes were incubated in the absence of the phosphatase as a negative control. α-casein and BSA were used as a positive and negative control, respectively.

### $^{32}$P Labeling of Cells

$^{32}$P labeling of oncogenic ras-induced senescent IMR90 fibroblasts (day 6 post selection) or HeLa cells was performed using $^{32}$P Orthophosphate (Amersham). Briefly, cells were washed in phosphate-free DMEM medium (GIBCO, Carlsbad, CA) three times and incubated in phosphate-free DMEM medium (GIBCO) containing 10% dialyzed FBS (GIBCO) for 30 min and the medium was replaced with medium containing 150 μCi/4 ml $^{32}$P Orthophosphate (Amersham) and incubated for 5 h at 37°C. Subsequently, cells were washed with ice-cold PBS and were lysed by incubating in ice-cold lysis buffer for 15 min on ice. Cell lysates were collected and centrifuged and the supernatant was subjected to immunoprecipitation with a p16 antibody (Delta BioLabs), a p53 antibody (Oncogene), or a normal rabbit serum (Biosource) as described above.

### SA-β-gal Activity Assay

SA-β-gal activity was analyzed as previously described [Serrano et al., 1997]. Briefly, cells were washed with PBS (pH 6.0) and fixed with 0.5% glutaraldehyde. After fixation, cells were washed with PBS (pH 6.0) supplemented with 1 mM $MgCl_2$ followed by incubation in X-Gal solution at 37°C. At least 200 cells were counted. Cell populations exhibiting more than 60% positive cells were considered senescent.

## RESULTS

### Oncogenic ras-Induced Senescent Human Fibroblasts and Replicative Senescent Fibroblasts Express Three Distinct p16 Variants

We investigated potential p16 protein modifications associated with senescence in human fibroblasts, including oncogenic H-ras-induced senescent cells and cells that had undergone replicative senescence. Early passage normal human IMR90 fibroblasts were transduced with oncogenic H-ras via retroviral gene transfer. By day 6 post-selection, cells that had been stably transduced with oncogenic H-ras exhibited flat and enlarged cell morphology and increased senescence-associated-β galactosidase (SA-β-gal) activity (Fig. 1A). Additionally, cells expressing oncogenic H-ras displayed elevated expression of p16 protein relative to the vector control (Fig. 1B). The activity of p16 is to block phosphorylation of the Rb proteins by binding to and inhibiting cdk4 and cdk6, leading to G1 arrest [Lukas et al., 1995a,b]. Consistent with this notion, oncogenic H-ras-induced senescent cells exhibited underphosphorylated and reduced Rb expression (Fig. 1B). Normal IMR90 fibroblasts that had been cultured for more than 25 passages also demonstrated flat and enlarged cell morphology, increased SA-β-gal activity and induction of p16 and were considered replicative senescent cells (R.S., Fig. 1A,B).

Western blot analysis of p16 following 1D SDS–PAGE consistently resolved only a single band that immunoreacted with the p16 antibody (Fig. 1B). However, it is known that a better resolution of protein variants can be achieved by 2D SDS–PAGE (2D SDS–PAGE). Hence, IMR90 cells expressing either oncogenic H-ras or vector control were subjected to 2D SDS–PAGE followed by Western blot analysis of p16. Strikingly, 2D SDS–PAGE analysis of oncogenic ras-induced senescent cells reproducibly resolved three distinct protein spots that were immunoreactive with a p16 polyclonal antibody, while no immunoreactive protein spots were detected in the lysates derived from cells expressing the empty vector (Fig. 1C). The

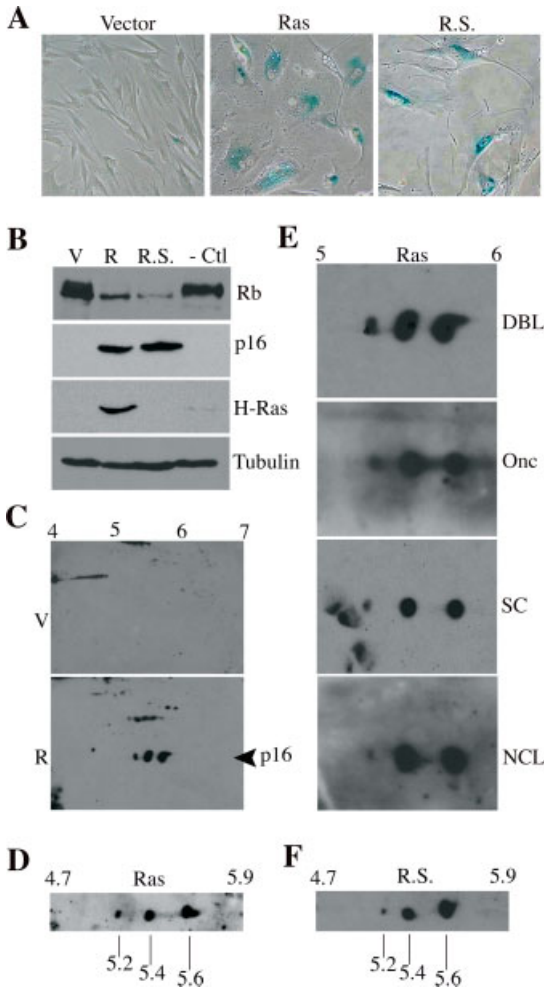


**Fig. 1.** Senescent human fibroblasts express three distinct p16 variants. **A**: Representative photomicrographs of the indicated cell populations stained for SA-β-gal activity. Vector: IMR90 cells expressing empty vector. Ras: IMR90 cells expressing oncogenic ras. R.S.: Replicative senescent IMR90 cells. **B**: Western blot analysis of the indicated proteins in IMR90 cells transduced with an empty vector (V), oncogenic ras (R), or replicative senescent IMR90 cells (R.S.). Osteosarcoma U2OS cells were used as a negative control (''− Ctl'') for p16 protein expression. **C**: 2D Western blot analysis of p16 in the indicated cell types. V: IMR90 cells transduced with an empty vector. R: IMR90 cells expressing oncogenic ras. IPG strips of pH 4–7 were used for isoelectric focusing. **D**: 2D Western blot analysis of p16 of IMR90 cells expressing oncogenic ras. A micro-range (pH 4.7–5.9) IPG strip was used for isolectric focusing. The three p16 variants exhibited pI of 5.2, 5.4, and 5.6, respectively. **E**: 2D Western blot analysis of IMR90 cells expressing oncogenic ras. Four antibodies specific for human p16 were used, including three rabbit polyclonal antibodies from different resources: Delta-Biolabs (DBL), Oncogene (Onc), Santa Cruz (SC), and one mouse monoclonal antibody from Novocastra (NCL). The regions between pH 5 and 6 were shown. **F**: Western blotting of p16 resolved by 2D SDS–PAGE analysis of replicative senescent IMR90 fibroblasts (R.S.). A micro-range (pH 4.7–5.9) IPG strip was used. The three p16 variants exhibited pI of 5.2, 5.4, and 5.6, respectively.

isoelectric points (pI) of these three protein spots span between pH 5 and 6, which coincides with the predicted pI of human p16. The pI of these three protein spots were more precisely determined as 5.2, 5.4, and 5.6, by using micro-range immobilized pH gradient strips (pH 4.7–5.9) (Fig. 1D). To further verify whether these three immunoreactive protein spots were indeed p16 species, three additional p16 antibodies were used for Western blot analysis following 2D electrophoresis. The results showed that the three protein spots reacted with all p16 antibodies tested, including three polyclonal antibodies and one monoclonal antibody (Fig. 1E), suggesting that these protein spots are likely bona fide p16 variants. Significantly, these three p16 variants were also reproducibly detected in IMR90 fibroblasts that had undergone replicative senescence (Fig. 1F). We have designated these three protein spots $p16^{(5.2)}$, $p16^{(5.4)}$, and $p16^{(5.6)}$. Of note, the occasionally observed vertically duplicated protein spots in this report were due to an artifact commonly seen in 2D SDS–PAGE analysis [Berkelman and Stestedt, 2004].

### Prolonged Serum Starvation Triggers an Irreversible Growth Arrest That is Associated With the Induction of Three p16 Variants

p16 upregulation in normal proliferating human cells is a rare event. However, it is highly specific to senescent cells, but not quiescent cells [Alcorta et al., 1996; McConnell et al., 1998]. In contrast to the irreversible nature of growth arrest in senescence, quiescent cells are able to re-enter the cell cycle once the trigger is removed. It is known that quiescence can be triggered by culturing cells in low serum condition [Burmer et al., 1984]. We next examined whether prolonged serum starvation can trigger an irreversible growth arrest or senescence. Early passage normal IMR90 fibroblasts were plated at low densities and cultured in DMEM containing 0.1% FBS for 14 or 21 days, with medium changed twice a week. Cells from various time points were analyzed for biochemical markers of senescence and for their ability to re-enter the cell cycle after being replenished with 10% serum. The results showed that serum starvation of IMR90 fibroblasts for 48 h was sufficient to trigger quiescence, as judged by G1 arrest and expression of under-phosphorylated Rb (data not shown and Fig. 2A). At this point, cells did not exhibit p16

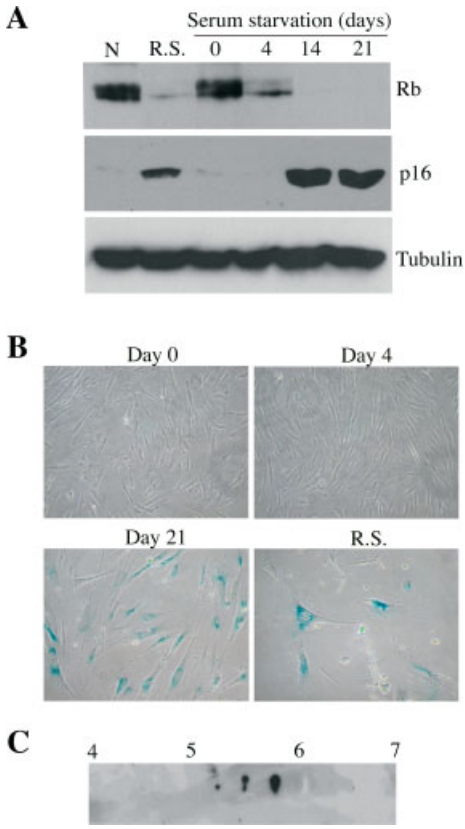


**Fig. 2.** Prolonged serum starvation-induced senescence is accompanied by the expression of three p16 variants in human IMR90 fibroblasts. **A**: Western blot analysis of the indicated proteins in early passage normal IMR90 cells (N), replicative senescent IMR90 cells (R.S.) or IMR90 cells cultured in low serum for the indicated days. **B**: Representative photomicrographs of cell populations stained for SA-β-gal activity. IMR90 cell populations were cultured in low serum for 4 days (Day 4) or 21 days (Day 21). IMR90 cells cultured in 10% FBS (Day 0) and replicative senescent IMR90 cells (R.S.) were used as a negative and positive control, respectively. **C**: 2D Western blot analysis of serum starvation-induced senescent IMR90 fibroblasts. A pH 4-7 IPG strip was used for isoelectric focusing.

induction and they maintained the ability to resume proliferation upon the addition of 10% serum (Fig. 2A and data not shown). In contrast, cells that were cultured in low serum for up to 14 or 21 days exhibited a significant induction of p16 (Fig. 2A). Moreover, these cells displayed elevated SA-β-gal activity and failed to re-enter the cell cycle even after the culture was replenished with 10% serum (Fig. 2B and data not shown). These results suggested that prolonged serum starvation could also induce a senescence phenotype in normal IMR90 fibroblasts. Significantly, the 2D SDS–PAGE analysis also resolved three p16 variants in serum starvation-induced senescent cells that reacted with the p16 antibody (Fig. 2C). Thus, prolonged serum starvation of normal IMR90 fibroblasts induced a senescence phenotype that was associated with the expression of three p16 variants.

### Forced Expression of p16 Induces Senescence in IMR90 Fibroblasts and Osteosarcoma U2OS Cells Associated With the Expression of Two p16 Variants

Upregulation of p16 is not merely a biomarker of senescence. Forced expression of p16 in early passage human fibroblasts can also lead to senescence [Medema et al., 1995; Lukas et al., 1995a; McConnell et al., 1998]. We next examined the expression pattern of p16 in senescent cells induced by forced expression of p16. Early passage IMR90 fibroblasts were transduced with p16 or an empty vector via retroviral gene transfer. Following selection, p16 expression pattern in these cells was assessed by 2D SDS–PAGE and Western blotting. Consistent with previous studies [Dimri and Campisi, 1994], IMR90 fibroblasts transduced with p16 exhibited biochemical and morphological changes that were characteristic of senescence (Fig. 3A). The 2D SDS–PAGE analysis of IMR90 cells transduced with p16 consistently resolved only two p16 variants, which aligned more closely with $p16^{(5.2)}$ and $p16^{(5.4)}$ (Fig. 3B). The least acidic variant of $p16^{(5.6)}$ that was highly expressed in oncogenic ras-induced senescent cells, replicative senescent cells and serum starvation-induced senescent cells was not detected in p16-induced senescent IMR90 cells. To further examine the expression pattern of p16 in a different cellular context, the p16-null osteosarcoma U2OS cells were transduced with p16 or and empty vector. As expected, U2OS cells transduced with p16, exhibited expression of underphosphorylated Rb (Fig. 3A). Moreover, forced expression of p16 in osteosarcoma U2OS cells also induced a senescence-like growth arrest, as evidenced by the flat and enlarged cell morphology and elevated SA-β-gal activity (Fig. 3A). Consistent with our finding in p16-induced senescent IMR90 cells, 2D SDS–PAGE analysis of U2OS cells expressing p16 also resolved only the two more acidic p16 variants, $p16^{(5.2)}$ and $p16^{(5.4)}$ (Fig. 3B).

The observation that overexpression of p16 resulted in only two p16 variants raised the question whether expression of the endogenous $p16^{(5.6)}$ was tissue-type specific. Therefore, we examined the expression pattern of p16 in a

different cell type that exhibits high levels of p16. Human cervical carcinoma HeLa cells are known to express high level of wild type functional p16, although Rb function in these cells is altered by the HPV E7 protein [Khleif et al., 1996]. 2D SDS–PAGE analysis of HeLa cells consistently resolved three p16 variants that exhibited pI indistinguishable from those detected in senescent IMR90 fibroblasts (Fig. 3C). These data suggest that expression of all three p16 variants may be common in cells that express high levels of endogenous p16 protein. Nevertheless, accumulation of the two more acidic p16 variants, $p16^{(5.2)}$ and $p16^{(5.4)}$, may be sufficient to mediate senescence.

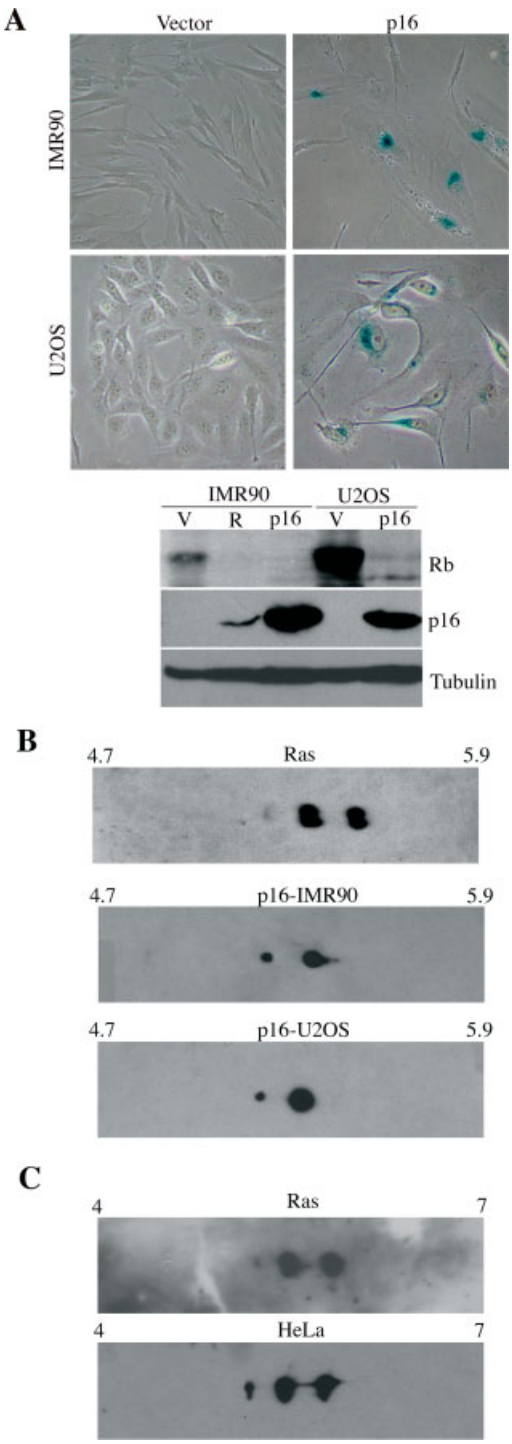


**Fig. 3.** Forced expression of p16 in IMR90 cells and osteosarcoma cells induces senescence accompanied by the expression of two p16 variants. **A** (**upper panel**): Representative photomicrographs of SA-β-gal activity analysis of IMR90 and U2OS cell populations transduced with an empty vector (Vector) or human p16 cDNA (p16). **Lower panel**: Western blot analysis of the indicated proteins in IMR90 cells or U2OS cells transduced with the indicated transgene. V: empty vector. R: oncogenic ras. p16: p16. **B**: 2D Western blot analysis of IMR90 cells transduced with oncogenic ras (Ras), p16 (p16-IMR90), or U2OS cells transduced with p16 (p16-U2OS). IPG strips of pH 4.7–5.9 were used. **C**: 2D Western blot analysis of p16 in HeLa cells (HeLa) as compared with that of IMR90 cells expressing oncogenic ras (Ras). IPG strips of pH 4–7 were used for isoelectric focusing.

### p16 Variants Arise via a Mechanism Independent of Protein Phosphorylation

Phosphorylation is one of the most common mechanisms for post-translational regulation of proteins. Based on evidence that p16 phosphorylation occurs in senescent prostatic epithelial cells and early passage WI 38 fibroblasts (51, [Gump et al., 2003], we examined the possibility that some of the p16 variants that were expressed in senescent IMR90 fibroblasts were modified through phosphorylation.

There are seven serine residues in human p16, of which serine 7, 8, and 43 are predicted to be phosphorylation residues (http://us.expasy.org/tools/pi_tool.htm). Hence, we first investigated the phosphorylation status of p16 in oncogenic ras-induced senescent cells using anti-phospho-Ser antibody. p16 proteins were immunoprecipitated from oncogenic H-ras-induced senescent cell lysates using p16 antibodies as previously described [Gump et al., 2003]. The immunocomplex was subjected to 2D SDS–PAGE analysis followed by Western blotting using antibodies against phospho-serine. Intriguingly, the antibody reacted with the positive control, α-casein, but failed to react with the immunoprecipitated p16 (Fig. 4A).

Although p16 is upregulated in senescent cells, the absolute amount of p16 protein present in these cells is low. To exclude the possibility that our inability to detect phosphorylation of p16 was due to low protein yield and/or insuf-

ficient sensitivity of the assays, we performed an additional more sensitive phosphatase assay. Immunoprecipitation of p16 from oncogenic H-ras-induced senescent fibroblasts was carried out as previously described [Sandhu et al., 2000]. The immunoprecipitated p16 was subjected to λ acid phosphatase or a control treatment, as described in "Materials and Methods" [Gump et al., 2003]. It is expected that phosphatase sensitive protein spots would exhibit changes in electrophoretic mobility (i.e., shift in molecular weight and/or pI) relative to the control. As a positive control, immunoprecipitation of p53 from HeLa cells was subjected to phosphatase treatment followed by Western blotting using antibodies against phospho-Ser$^{15}$ p53 and total p53, respectively. As expected, the phosphatase-treated p53 immunocomplex exhibited reduced immunoreactivity with the anti-phospho-Ser$^{15}$ antibody (Fig. 4B). Notably, total p53 protein levels remained unchanged in the presence of the phosphatase (Fig. 4B). In contrast, the p16 variants immunoprecipitated from oncogenic ras-induced senescent IMR90 fibroblasts did not show any appreciable change in their electrophoretic mobility following treatment with λ acid phosphatase (Fig. 4B) or potato acid phosphatase (data not shown).

We further investigated the potential phosphorylation of p16 by $^{32}$P orthophosphate labeling of Ras-induced senescent human fibroblasts. HeLa cells were also used since they exhibit high expression level of the three p16 variants that are expressed in senescent human

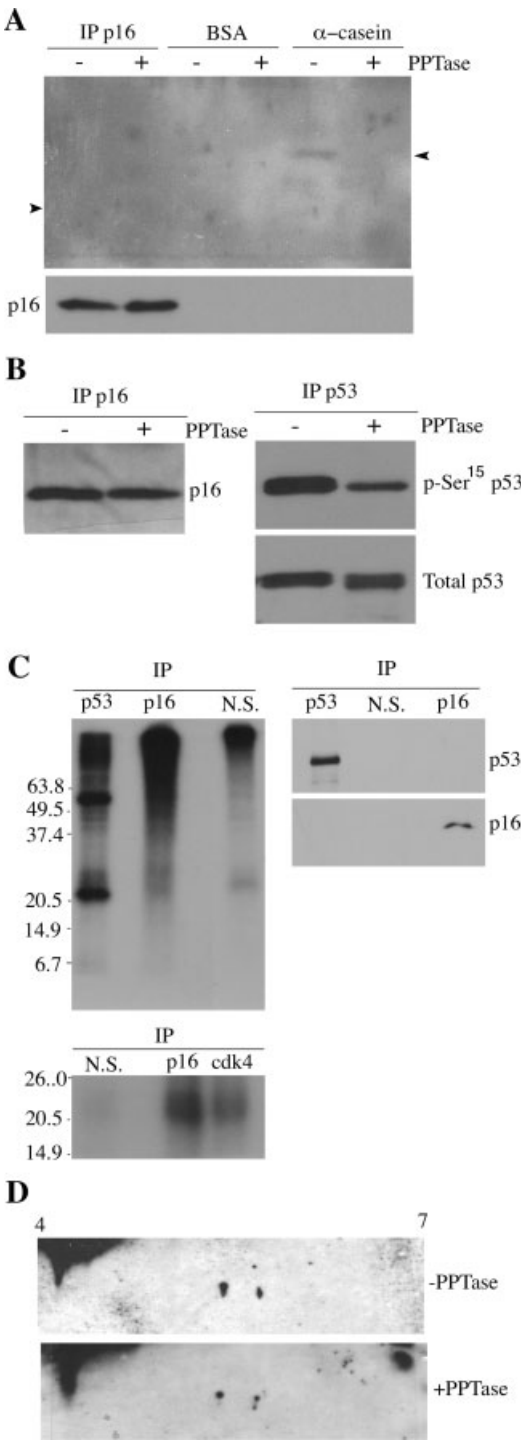


**Fig. 4.** Protein phosphorylation of p16 was not detected in IMR90 cells expressing oncogenic ras. **A**: Western blot analysis of p16 immunoprecipitated from IMR90 cells expressing oncogenic ras using an antibody specific for phospho-serine residues or p16. The immunocomplexes were treated with λ acid phosphatase ("+ PPTase") or a control ("− PPTase"). BSA and α-casein were used as a negative control and a positive control, respectively. **B**: Western blot analysis of p16 immunoprecipitated (IP 16) from IMR90 cells expressing oncogenic ras in the presence ("+ PPTase") or absence ("− PPTase") of phosphatase treatment. Immunoprecipitated p53 (IP p53) from HeLa cells was used as a control for phosphatase treatment. p53 protein levels and phosphorylation status were assessed using antibodies specific for p53 (Novocastra) or phospho-serine$^{15}$ p53 (Cell Signaling). **C**: SDS–PAGE analysis of immunoprecipitated p53 or p16 from $^{32}$P-labelled HeLa cells (**left top panel**) or Ras-induced senescent fibroblasts (**left bottom panel**) using antibodies against p53 (p53), p16 (p16), or cdk4 (cdk4). Gel was dried and exposed to the X-ray film for 50 h, and no radioactivity was detected in the immunoprecipitated p16 (left panel). As positive controls, the same samples were subjected to Western blot analysis for p16 and p53 using specific antibodies (**right panel**). Normal rabbit serum (N.S.) was used as a negative control for immunoprecipitation. Representative data from three independent experiments are shown. **D**: 2D Western blot analysis of immunoprecipitated p16. Immunoprecipitated p16 was prepared from IMR90 cells expressing oncogenic ras in the presence ("+ PPTase") or absence ("− PPTase") of phosphatase treatment. IPG strips of pH 4–7 were used for isoelectric focusing.

fibroblasts. Immunoprecipitated p53 from $^{32}$P-labelled HeLa cells demonstrated active incorporation of $^{32}$P to the p53 proteins (Fig. 4C, left panel). In contrast, immunoprecipitated p16 from $^{32}$P-radiolabelled HeLa cells (Fig. 4C, left top panel) or Ras-induced senescent IMR90 fibroblasts (Fig. 4C, left bottom panel) exhibited no detectable radioactivity. Taken together, these studies suggest that expression of the p16 variants in senescent fibroblasts does not involve protein phosphorylation.

### Selective Binding of p16 Variants to cdk4 and cdk6

p16 executes its growth inhibitory function by binding to and inhibiting the kinase activity of cdk4/6 [Serrano et al., 1993]. The function of p16 as a cdk inhibitor is critical in causing G1 arrest in senescent human cells [Medema et al., 1995]. Hence, we next examined the association of p16 variants with cdk4 and cdk6 in senescent cells by performing co-immunoprecipitation studies. p16, cdk4, or cdk6 protein was immunoprecipitated from total cell lysates of senescent cells, including oncogenic ras-induced and replicative senescent cells; normal rabbit serum was used as a control. The immunocomplexes were subjected to both 1D (Fig. 5A,B) and 2D SDS–PAGE (Fig. 5C,D) followed by Western blotting. Interestingly, while immunoprecipitation of p16 using a p16 polyclonal antibody consistently pulled down two major p16 variants, $p16^{(5.4)}$ and $p16^{(5.6)}$ (Fig. 5C,D), the cdk4 or cdk6 immunocomplex repeatedly pulled down $p16^{(5.4)}$ with a trace of $p16^{(5.2)}$ (Fig 5C,D). The low levels of $p16^{(5.2)}$ co-immunoprecipitated with cdk4 and cdk6 might be, in part, due to its low expression levels. The least acidic p16 variant, $p16^{(5.6)}$, which is expressed at high levels in senescent cells, consistently failed to co-immunoprecipitate with either cdk4 or cdk6 (Fig. 5C,D). To further confirm this observation, we examined whether the $p16^{(5.6)}$ variant remained in the cell extract following depletion of cdk4/6-bound p16. Cdk4- and cdk6-bound p16 was depleted by immunoprecipitation with a cocktail containing both cdk4 and cdk6 antibodies. The cdk4/6-depleted supernatant was then subjected to an additional p16 immunoprecipitation using a p16 polyclonal antibody. Consistent with data from cdk4/6 immunoprecipitation analysis (Fig. 5C,D), only one p16 variant remained unbound to cdk4 and cdk6 (Fig. 5E). The cdk-unbound p16 variant exhibited an approximate pI of 5.6, although a slight shift in pI was observed (Fig. 5E). Moreover, immunoprecipitation of cdk4/6 from U2OS cells or IMR90 cells transduced with p16 also pulled

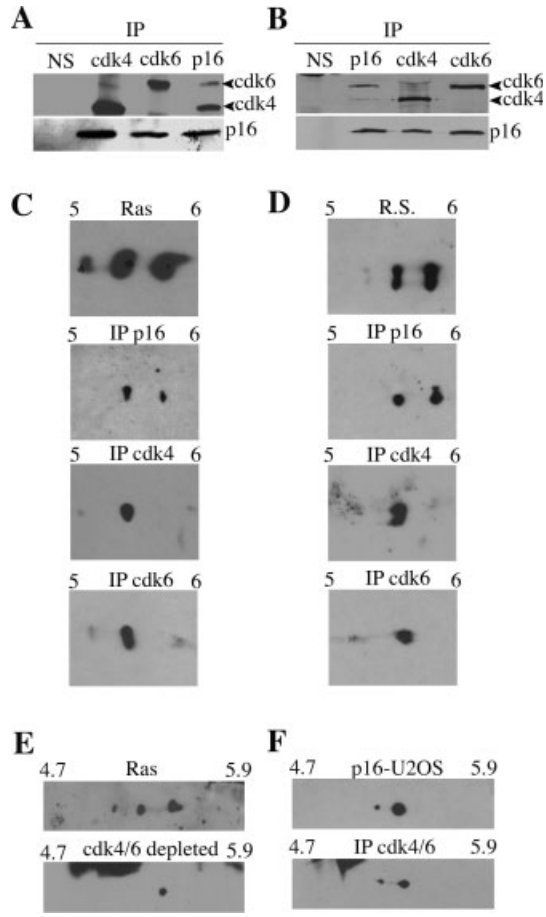


**Fig. 5.** Selective association of two specific p16 variants with cdk4 and cdk6 in senescent human fibroblasts. **A** and **B**: Western blot analysis of the indicated proteins immunoprecipitated (IP) from IMR90 cells expressing oncogenic ras (A) or replicative senescent cells (B). Immunocomplexes pulled down by antibodies specific for p16, cdk4, or cdk6, or normal serum (NS) were resolved by SDS–PAGE and blotted for cdk6, and cdk4, and p16. **C** and **D**: 2D Western blot analysis of total cell lysates or immunocomplexes prepared from IMR90 cells expressing oncogenic ras (C) or replicative senescent cells (D). Ras: IMR90 cells expressing oncogenic ras. R.S.: Replicative senescent cells. Immunocomplexes prepared from each cell type using antibodies specific for p16 (IP p16), cdk4 (IP cdk4), or cdk6 (IP cdk6) were resolved on 2D SDS–PAGE using pH 4–7 strips followed by Western blotting for p16. The regions between pH 5 and 6 were shown. **E**: 2D Western blot analysis of ras-expressing IMR90 cells (Ras) and cells depleted of cdk4/6 complexes (cdk4/6 depleted). IPG strips of pH 4.7–5.9 were used for isoelectric focusing. **F**: 2D Western blot analysis of p16 from total cell lysate (p16-U2OS) or immunocomplexes (IP cdk4/6) prepared from U2OS cells transduced with p16. IPG strips of pH 4.7–5.9 were used for isoelectric focusing. The resolved two p16 variants exhibited approximate pI of 5.2 and 5.4, respectively.

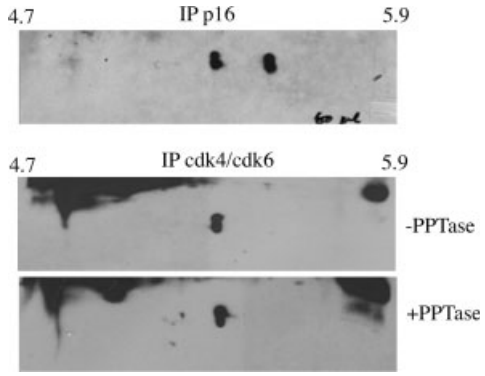


**Fig. 6.** Cdk4/6-bound p16 in senescent fibroblasts were resistant to phosphatase treatment. 2D Western blot analysis of immnocomplexes prepared from IMR90 cells expressing oncogenic ras. Immunocomplexes were prepared using an antibody specific for p16 (IP p16), or a cocktail containing antibodies specific for cdk4 or cdk6 (IP cdk4/6). Immnocomplex pulled down by cdk4/6 were treated with phosphatase (''+ PPTase'') or a control (''− PPTase'') prior to 2D SDS–PAGE analysis. IPG strips of pH 4.7–5.9 were used for isoelectric focusing.

down two p16 variants with pI of 5.2 and 5.4, respectively (Fig. 5F and data not shown). Lastly, cdk4/6-bound p16 from ras-induced senescent fibroblasts remained resistant to phosphatase treatment (Fig. 6), further supporting the notion that these p16 variants are not phosphorylated.

## DISCUSSION

Normal proliferating cells express p16 protein at extremely low or undetectable levels [Harland et al., 1997]. Unlike many other tumor suppressors, induction or activation of the p16 tumor suppressor is less characterized. This may be attributed, at least in part, to the fact that p16 induction is a relatively rare event, although it is highly specific in senescent cells [Alcorta et al., 1996; Hara et al., 1996; Serrano, 1997; Serrano et al., 1997]. In this study, we examined the expression pattern and potential protein modification of p16 in relation to its activity in senescent IMR90 fibroblasts induced to senesce by culture passage, expression of oncogenic ras, or prolonged serum starvation.

Expression of three distinct p16 variants was reproducibly detected in oncogenic ras-induced senescent fibroblasts and replicative senescent fibroblasts. Moreover, normal human fibroblasts cultured in low serum for an extended period of time also underwent senescence with associated expression of three p16 variants. This finding provides an additional example of stress-induced, and perhaps telomere-independent, senescence in normal human fibroblasts, and suggests that the observed p16 expression pattern was independent of the trigger of senescence. Additionally, human cervical carcinoma HeLa cells also expressed three p16 variants [Yeager et al., 1995; Khleif et al., 1996; Xiong et al., 1996; Jarrard et al., 1999] whose expression pattern was indistinguishable from that in senescent human fibroblasts, indicating that the expression of the three p16 variants was not limited to human fibroblasts.

Among the three p16 variants, the least acidic variant ($p16^{(5.6)}$) reproducibly appeared to be the most abundant form, while the most acidic variant ($p16^{(5.2)}$) is the least abundant form. Interestingly, the most abundant $p16^{(5.6)}$ variant showed no affinity for cdk4 and cdk6, and was not expressed in cells transduced with p16. One possibility is that expression of the $p16^{(5.6)}$ variant in senescent cells is regulated by an intrinsic post-transcriptional mechanism. This is unlikely since the reported p16 splicing variants are associated with melanoma and are shown to have arisen from germline splicing mutation [Loo et al., 2003]. At this stage, the basis on which the p16 cDNA construct only gave rise to two specific p16 variants remains unclear; however, our results suggest that expression of the two specific p16 variants is sufficient to induce a senescence phenotype.

p16 protein expressed in prostatic epithelial cells has been shown to be exclusively phosphorylated, with four phosphorylated p16 species identified [Sandhu et al., 2000]. Two of the identified phosphorylated p16 forms in the prostatic epithelial cells exhibit increased molecular weight and slightly alkaline pI relative to the native p16 protein. Additionally, phosphorylation of p16 at $Ser^{152}$ has been reported in proliferating WI38 fibroblasts, and the phosphorylated p16 detected in these cells exhibit more acidic pI compared to the unphosphorylated counterpart [Gump et al., 2003]. In contrast, we showed that senescent human fibroblasts and HeLa cells expressed three p16 variants and each exhibited a pI close to the predicted value of the p16 protein (i.e., 5.52) (http://us.expasy.org/tools/pi_tool.htm). However, no protein phosphorylation was detected in either the cdk4/6-bound or unbound p16 variants. Consistent with this result, phosphorylation of p16 detected in WI38 fibroblasts is not required for its binding activity to cdk4/6 [Gump et al., 2003]. Based on these observations, we

cannot rule out the possibility that differential protein modifications of p16 may exist depending on the cellular context. Nevertheless, protein phosphorylation does not appear to be essential for p16 to bind to cdk4/6. Indeed, p16 expressed in *E. coli* is able to interact effectively with cdk4 and cdk6 in in vitro binding assays [Parry and Peters, 1996; Ruas et al., 1999].

The expression pattern of p16 in senescent cells was closely associated with its activity. The two more acidic p16 variants, $p16^{(5.2)}$ and $p16^{(5.4)}$, exhibited binding activity to cdk4/6 and were consistently expressed in senescent cells generated by forced expression of p16. The function of the $p16^{(5.6)}$ variant is not yet understood, although it's likely that its activity is independent of cdk4/6 binding. A recent study suggests that p16 can also control the cell cycle progression through modulating the cdk-CTD kinase activity, which is crucial for transcription mediated by RNA polymerase II [Serizawa, 1998; Nishiwaki et al., 2000]. It remains to be examined which, if any, of these p16 variants play a role in suppressing transcription.

The biological mechanism by which these p16 variants were generated in senescent cells remains unclear. In addition to protein phosphorylation, protein modification such as acetylation, methylation or deamidation can potentially result in changes in pI [Bjellqvist et al., 1993, 1994]. However, the lack of lysine residues in human p16 rules out the possibility of protein acetylation. Deamidation of Asp or methylation of Arg in a protein can result in a reduction of pI [Aletta et al., 1998; Cimato et al., 2002]. However, deamidation is thought to be a modification mechanism favoring protein degradation, while the consequences of protein methylation are relatively unknown. Purification of these p16 variants from senescent cells would help to determine the mechanism(s) by which these variants are generated. However, while the p16 protein is upregulated in senescent cells, the expression level of p16 in these cells remains extremely low. The low expression level of p16, together with its low molecular weight, presents a practical barrier in further identifying these p16 variants. However, we anticipate this technical difficulty to be resolved in near future by using the more powerful and efficient Mass Spectrometry technique.

In conclusion, this study reports, for the first time, the identification of three p16 variants with distinct pI that were consistently associated with upregulation of endogenous p16 in human cells, including senescent human fibroblasts induced by various stimuli and tumor cells that exhibit high expression levels of p16. Additionally, we show that the expression of these p16 variants and their cdk binding activity is independent of protein phosphorylation. Lastly, expression of the two functional cdk4/6-binding variants of p16 correlates with the occurrence of the senescent phenotype in human cells triggered by various stimuli.

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## REFERENCES

Aagaard L, Lukas J, Bartkova J, Kjerulff AA, Strauss M, Bartek J. 1995. Aberrations of p16Ink4 and retinoblastoma tumour-suppressor genes occur in distinct sub-sets of human cancer cell lines. Int J Cancer 61:115–120.

Alani RM, Young AZ, Shifflett CB. 2001. Id1 regulation of cellular senescence through transcriptional repression of p16/Ink4a. Proc Natl Acad Sci USA 98:7812–7816.

Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC. 1996. Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proc Natl Acad Sci USA 93:13742–13747.

Aletta JM, Cimato TR, Ettinger MJ. 1998. Protein methylation: A signal event in post-translational modification. Trends Biochem Sci 23:89–91.

Beausejour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P, Campisi J. 2003. Reversal of human cellular senescence: Roles of the p53 and p16 pathways. Embo J 22:4212–4222.

Berkelman T, Stestedt T. 2004. 2-D electrophoresis principals and methods. Uppasala, Switzerland: Amersham Biosciences.

Bjellqvist B, Hughes GJ, Pasquali C, Paquet N, Ravier F, Sanchez JC, Frutiger S, Hochstrasser D. 1993. The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. Electrophoresis 14:1023–1031.

Bjellqvist B, Basse B, Olsen E, Celis JE. 1994. Reference points for comparisons of two-dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions. Electrophoresis 15:529–539.

Bond JA, Blaydes JP, Rowson J, Haughton MF, Smith JR, Wynford-Thomas D, Wyllie FS. 1995. Mutant p53 rescues human diploid cells from senescence without inhibiting the induction of SDI1/WAF1. Cancer Res 55:2404–2409.

Brookes S, Rowe J, Ruas M, Llanos S, Clark PA, Lomax M, James MC, Vatcheva R, Bates S, Vousden KH, Parry D, Gruis N, Smit N, Bergman W, Peters G. 2002. INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. Embo J 21:2936–2945.

Burmer GC, Rabinovitch PS, Norwood TH. 1984. Evidence for differences in the mechanism of cell cycle arrest between senescent and serum-deprived human fibroblasts: Heterokaryon and metabolic inhibitor studies. J Cell Physiol 118:97–103.

Campisi J. 2001. Cellular senescence as a tumor-suppressor mechanism. Trends Cell Biol 11:S27–S31.

Campisi J. 2003. Analysis of tumor suppressor gene-induced senescence. Methods Mol Biol 223:155–172.

Chen Q, Fischer A, Reagan JD, Yan LJ, Ames BN. 1995. Oxidative DNA damage and senescence of human diploid fibroblast cells. Proc Natl Acad Sci USA 92:4337–4341.

Cimato TR, Tang J, Xu Y, Guarnaccia C, Herschman HR, Pongor S, Aletta JM. 2002. Nerve growth factor-mediated increases in protein methylation occur predominantly at type I arginine methylation sites and involve protein arginine methyltransferase 1. J Neurosci Res 67:435–442.

Cunningham JJ, Roussel MF. 2001. Cyclin-dependent kinase inhibitors in the development of the central nervous system. Cell Growth Differ 12:387–396.

Deng Q, Liao R, Wu BL, Sun P. 2004. High intensity ras signaling induces premature senescence by activating p38 pathway in primary human fibroblasts. J Biol Chem 279:1050–1059.

Dimri GP, Campisi J. 1994. Molecular and cell biology of replicative senescence. Cold Spring Harb Symp Quant Biol 59:67–73.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, et al. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 92:9363–9367.

Drayton S, Rowe J, Jones R, Vatcheva R, Cuthbert-Heavens D, Marshall J, Fried M, Peters G. 2003. Tumor suppressor p16INK4a determines sensitivity of human cells to transformation by cooperating cellular oncogenes. Cancer Cell 4:301–310.

Erickson S, Sangfelt O, Heyman M, Castro J, Einhorn S, Grander D. 1998. Involvement of the Ink4 proteins p16 and p15 in T-lymphocyte senescence. Oncogene 17:595–602.

Ferbeyre G, de Stanchina E, Lin AW, Querido E, McCurrach ME, Hannon GJ, Lowe SW. 2002. Oncogenic ras and p53 cooperate to induce cellular senescence. Mol Cell Biol 22:3497–3508.

Florl AR, Franke KH, Niederacher D, Gerharz CD, Seifert HH, Schulz WA. 2000. DNA methylation and the mechanisms of CDKN2A inactivation in transitional cell carcinoma of the urinary bladder. Lab Invest 80: 1513–1522.

Gump J, Stokoe D, McCormick F. 2003. Phosphorylation of p16INK4A correlates with Cdk4 association. J Biol Chem 278:6619–6622.

Hara E, Tsurui H, Shinozaki A, Nakada S, Oda K. 1991. Cooperative effect of antisense-Rb and antisense-p53 oligomers on the extension of life span in human diploid fibroblasts, TIG-1. Biochem Biophys Res Commun 179: 528–534.

Hara E, Smith R, Parry D, Tahara H, Stone S, Peters G. 1996. Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. Mol Cell Biol 16:859–867.

Harland M, Meloni R, Gruis N, Pinney E, Brookes S, Spurr NK, Frischauf AM, Bataille V, Peters G, Cuzick J, Selby P, Bishop DT, Bishop JN. 1997. Germline mutations of the *CDKN2* gene in UK melanoma families. Hum Mol Genet 6:2061–2067.

Herbig U, Jobling WA, Chen BP, Chen DJ, Sedivy JM. 2004. Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). Mol Cell 14:501–513.

Huot TJ, Rowe J, Harland M, Drayton S, Brookes S, Gooptu C, Purkis P, Fried M, Bataille V, Hara E, Newton-Bishop J, Peters G. 2002. Biallelic mutations in p16(INK4a) confer resistance to Ras- and Ets-induced senescence in human diploid fibroblasts. Mol Cell Biol 22:8135–8143.

Itahana K, Zou Y, Itahana Y, Martinez JL, Beausejour C, Jacobs JJ, Van Lohuizen M, Band V, Campisi J, Dimri GP. 2003. Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1. Mol Cell Biol 23:389–401.

Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. 1999. The oncogene and Polycomb-group gene *bmi-1* regulates cell proliferation and senescence through the ink4a locus. Nature 397:164–168.

Jarrard DF, Sarkar S, Shi Y, Yeager TR, Magrane G, Kinoshita H, Nassif N, Meisner L, Newton MA, Waldman FM, Reznikoff CA. 1999. p16/pRb pathway alterations are required for bypassing senescence in human prostate epithelial cells. Cancer Res 59:2957–2964.

Khleif SN, DeGregori J, Yee CL, Otterson GA, Kaye FJ, Nevins JR, Howley PM. 1996. Inhibition of cyclin D-CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin kinase inhibitor activity. Proc Natl Acad Sci USA 93:4350–4354.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lin AW, Lowe SW. 2001. Oncogenic ras activates the ARF-p53 pathway to suppress epithelial cell transformation. Proc Natl Acad Sci USA 98:5025–5030.

Lin AW, Barradas M, Stone JC, van Aelst L, Serrano M, Lowe SW. 1998. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes Dev 12:3008–3019.

Loo JC, Liu L, Hao A, Gao L, Agatep R, Shennan M, Summers A, Goldstein AM, Tucker MA, Deters C, Fusaro R, Blazer K, Weitzel J, Lassam N, Lynch H, Hogg D. 2003. Germline splicing mutations of CDKN2A predispose to melanoma. Oncogene 22:6387–6394.

Lukas J, Aagaard L, Strauss M, Bartek J. 1995a. Oncogenic aberrations of p16INK4/CDKN2 and cyclin D1 cooperate to deregulate G1 control. Cancer Res 55:4818–4823.

Lukas J, Parry D, Aagaard L, Mann DJ, Bartkova J, Strauss M, Peters G, Bartek J. 1995b. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. Nature 375:503–506.

Macip S, Igarashi M, Fang L, Chen A, Pan ZQ, Lee SW, Aaronson SA. 2002. Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. Embo J 21:2180–2188.

Mallette FA, Goumard S, Gaumont-Leclerc MF, Moiseeva O, Ferbeyre G. 2004. Human fibroblasts require the Rb family of tumor suppressors, but not p53, for PML-induced senescence. Oncogene 23:91–99.

Mathon NF, Lloyd AC. 2001. Cell senescence and cancer. Nat Rev Cancer 1:203–213.

McConnell BB, Starborg M, Brookes S, Peters G. 1998. Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. Curr Biol 8:351–354.

Medema RH, Herrera RE, Lam F, Weinberg RA. 1995. Growth suppression by p16ink4 requires functional retinoblastoma protein. Proc Natl Acad Sci USA 92:6289–6293.

Murray AW. 2004. Recycling the cell cycle: Cyclins revisited. Cell 116:221–234.

Narita M, Nunez S, Heard E, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW. 2003. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. Cell 113:703–716.

Nishiwaki E, Turner SL, Harju S, Miyazaki S, Kashiwagi M, Koh J, Serizawa H. 2000. Regulation of CDK7-carboxyl-terminal domain kinase activity by the tumor suppressor p16(INK4A) contributes to cell cycle regulation. Mol Cell Biol 20:7726–7734.

Ohtani N, Zebedee Z, Huot TJ, Stinson JA, Sugimoto M, Ohashi Y, Sharrocks AD, Peters G, Hara E. 2001. Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. Nature 409:1067–1070.

Okamoto A, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP, Forrester K, Gerwin B, Serrano M, Beach DH, et al. 1994. Mutations and altered expression of p16INK4 in human cancer. Proc Natl Acad Sci USA 91:11045–11049.

Palmero I, Pantoja C, Serrano M. 1998. p19ARF links the tumour suppressor p53 to Ras. Nature 395:125–126.

Parry D, Peters G. 1996. Temperature-sensitive mutants of p16CDKN2 associated with familial melanoma. Mol Cell Biol 16:3844–3852.

Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P, Massague J. 1994. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 78:59–66.

Puthenveettil JA, Burger MS, Reznikoff CA. 1999. Replicative senescence in human uroepithelial cells. Adv Exp Med Biol 462:83–91.

Rabilloud T. 2000. Detecting proteins separated by 2-D gel electrophoresis. Anal Chem 72:48A–55A.

Ranade K, Hussussian CJ, Sikorski RS, Varmus HE, Goldstein AM, Tucker MA, Serrano M, Hannon GJ, Beach D, Dracopoli NC. 1995. Mutations associated with familial melanoma impair p16INK4 function. Nat Genet 10:114–116.

Rocco JW, Sidransky D. 2001. p16(MTS-1/CDKN2/INK4a) in cancer progression. Exp Cell Res 264:42–55.

Ruas M, Peters G. 1998. The p16INK4a/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta 1378: F115–F77.

Ruas M, Brookes S, McDonald NQ, Peters G. 1999. Functional evaluation of tumour-specific variants of p16INK4a/CDKN2A: Correlation with protein structure information. Oncogene 18:5423–5434.

Sandhu C, Peehl DM, Slingerland J. 2000. p16INK4A mediates cyclin dependent kinase 4 and 6 inhibition in senescent prostatic epithelial cells. Cancer Res 60:2616–2622.

Serizawa H. 1998. Cyclin-dependent kinase inhibitor p16INK4A inhibits phosphorylation of RNA polymerase II by general transcription factor TFIIH. J Biol Chem 273:5427–5430.

Serrano M. 1997. The tumor suppressor protein p16INK4a. Exp Cell Res 237:7–13.

Serrano M, Hannon GJ, Beach D. 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 366:704–707.

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88:593–602.

Sharpless NE, Bardeesy N, Lee KH, Carrasco D, Castrillon DH, Aguirre AJ, Wu EA, Horner JW, DePinho RA. 2001. Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. Nature 413: 86–91.

Shay JW, Roninson IB. 2004. Hallmarks of senescence in carcinogenesis and cancer therapy. Oncogene 23:2919–2933.

Sherr CJ. 2000. Cell cycle control and cancer. Harvey Lect 96:73–92.

Sherr CJ, Roberts JM. 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 9:1149–1163.

te Poele RH, Okorokov AL, Jardine L, Cummings J, Joel SP. 2002. DNA damage is able to induce senescence in tumor cells in vitro and in vivo. Cancer Res 62:1876–1883.

Wei W, Herbig U, Wei S, Dutriaux A, Sedivy JM. 2003. Loss of retinoblastoma but not p16 function allows bypass of replicative senescence in human fibroblasts. EMBO Rep 4:1061–1066.

Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. 1993. p21 is a universal inhibitor of cyclin kinases. Nature 366:701–704.

Xiong Y, Kuppuswamy D, Li Y, Livanos EM, Hixon M, White A, Beach D, Tlsty TD. 1996. Alteration of cell cycle kinase complexes in human papillomavirus E6- and E7-expressing fibroblasts precedes neoplastic transformation. J Virol 70:999–1008.

Yeager T, Stadler W, Belair C, Puthenveettil J, Olopade O, Reznikoff C. 1995. Increased p16 levels correlate with pRb alterations in human urothelial cells. Cancer Res 55:493–497.

Zebedee Z, Hara E. 2001. Id proteins in cell cycle control and cellular senescence. Oncogene 20:8317–8325.

Zhu J, Woods D, McMahon M, Bishop JM. 1998. Senescence of human fibroblasts induced by oncogenic Raf. Genes Dev 12:2997–3007.