# Molecular aspects of cellular senescence in human diploid fibroblasts

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**A growing body of knowledge supports the idea that the replicative lifespan of individual cells is closely related to the *in vivo* lifespan of humans. Cultured human diploid fibroblasts normally undergo a limited number of population doublings before they activate an intrinsic growth-limiting program culminating in cellular senescence. Molecular studies of this aging program have begun to reveal some details of the underlying mechanisms and have suggested how their abrogation contributes to cellular immortalization and tumorigenesis. This review examines the role of some positive and negative growth-regulatory processes in the human diploid fibroblast model of cellular senescence that are being examined in our group. Evidence that hyperphosphorylation of serum response factor underlies the senescence-associated loss of Fos transcription factor activity is discussed. The role of key regulators of cell–cycle progression in senescence is reviewed, including the elevated expression of D-type cyclins and the augmented expression and/or activity of 'tumor suppressor' genes whose functions are lost in tumorigenesis. The role and regulation of attrition of telomeres in the initiation of the senescent state is also discussed.**

HUMAN diploid fibroblasts are mortal cells that normally undergo a limited number of mean population doublings (MPDs) *in vitro* before proliferation progressively slows and ceases[1]. This state of cellular (or replicative) senescence appears to reflect the activation of an intrinsic, growth-limiting program by a mechanism that counts the number of cell divisions experienced by the cells. Cellular senescence manifests itself as a dominant phenotype, as fusion with senescent cells or microinjection with their mRNA inhibits replication in cells otherwise competent for replication[2]. The relevance of this phenomenon to the aging of organisms is supported by observations such as the inverse correlation seen between the average number of MPDs experienced by cells before senescence and the age of the donor from which they were derived[3,4]. Moreover, the maximum number of MPDs that cells of various species can attain before becoming senescent is directly related to the average lifespan of that species[5]. In addition, certain senescence-associated gene products are expressed in cells from individuals of advanced age[6]. Cells from individuals with premature aging syndromes such as progeria and Werner's syndrome[7], or with deficiencies in certain DNA repair functions[8], also display premature senescence in culture. Both aging and premature aging syndromes also predispose to various tumors, presumably reflecting the escape of tumor cells from growth inhibitory influences that include the activation of a cellular senescence 'program'.

Cellular senescence superficially resembles quiescence induced by either serum starvation or contact inhibition in that cells become arrested at a similar phase of the cell cycle[9,10]. The cell cycle (Figure 1) includes a gap phase ($G_1$), during which the commitment to cell cycle progression and preparation for DNA synthesis occurs; S phase, during which DNA synthesis occurs; a second gap phase ($G_2$) wherein preparation for cell division occurs; and mitosis (M), in which cell division is completed. Under mitogenic conditions, daughter cells then progress once more through $G_1$ phase, whereas in quiescence or senescence, the cells become locked in $G_1$-like states referred to as $G_0$ and $G_S$, respectively. $G_S$ is fundamentally different from $G_0$ in that senescent cells lose the ability to initiate cell cycle entry under otherwise mitogenic conditions. It is not completely clear how cells become arrested in $G_S$, but the process appears to involve loss of growth-promoting mitogenic signalling, activation of growth inhibitors such as tumor suppressor genes and aberrant expression of some cell cycle regulators.

An emerging model of cellular senescence that is increasingly supported by experimental observations suggests that growing human fibroblasts are normally destined to experience a loss of growth potential and enter a senescent state via a 'mortality' (M1) pathway (Figure 2). Fibroblasts can be induced to bypass this M1 pathway, either by directly disrupting tumor suppressor genes or by introducing viral oncoproteins, but the extended proliferative lifespan experienced by those cells is also limited. Cells in this alternative 'M2' pathway soon enter a state termed crisis that somewhat resembles apoptosis, the programmed cell death pathway that

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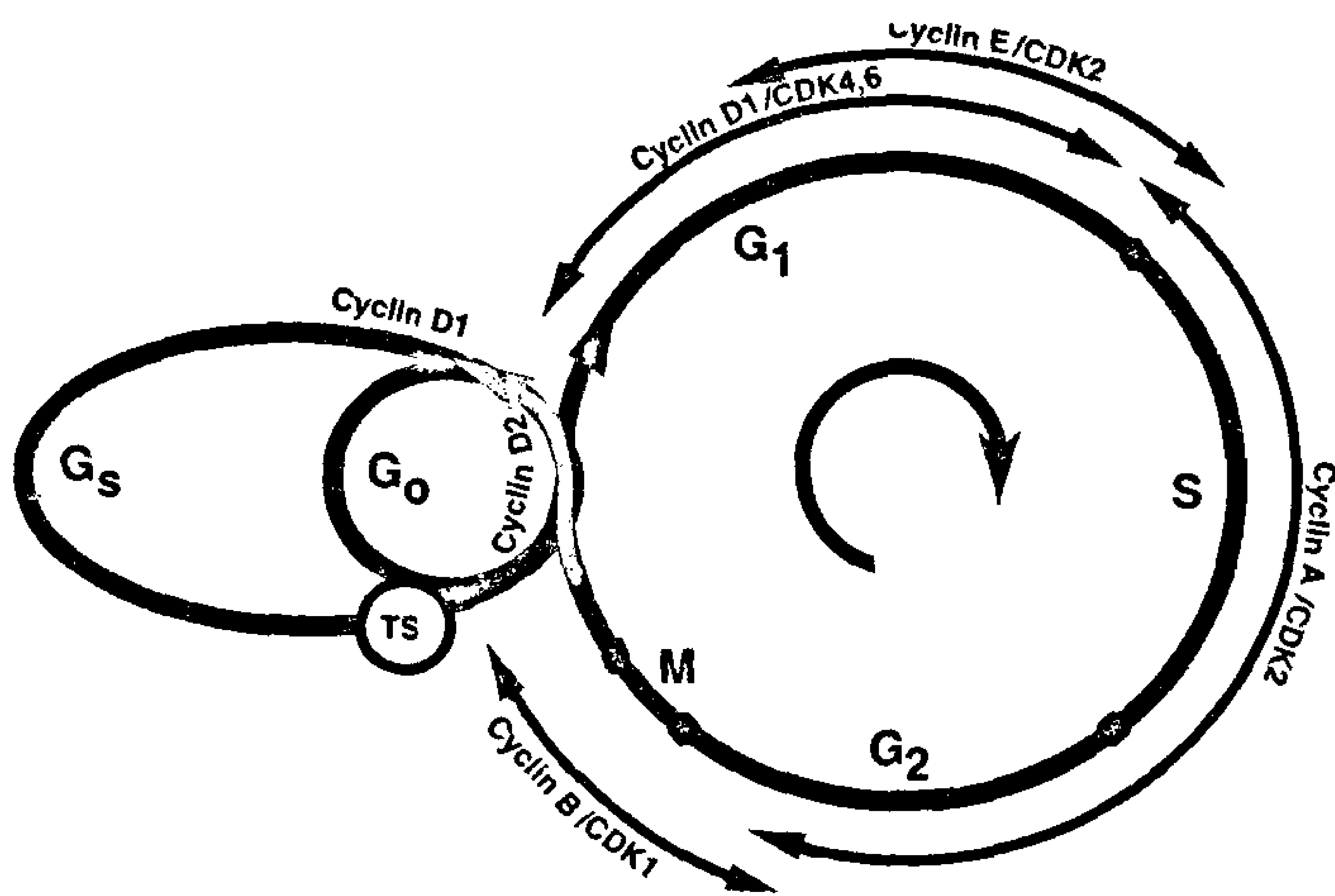


**Figure 1.** Cell-cycle associations of cyclin/CDK complexes. Cyclin D1 is expressed in $G_1$ phase and forms kinase complexes with CDK4 and 6. Cyclin E is expressed in mid $G_1$/S phases and forms active kinase complexes with CDK2. Cyclin A/CDK2 complexes are active in late $G_1$/S phases, and cyclin B/CDK1 complexes are active in late $G_2$/M phases. Cyclin D2 expression is maximal in $G_0$ and $G_S$, while cyclin in D1 expression is elevated in $G_S$ but not $G_0$. However, both are associated with inactive CDK2 in $G_S$. The constitutive activation of tumor suppressors (TS) such as Rb and p53 act to prevent exit from $G_S$.

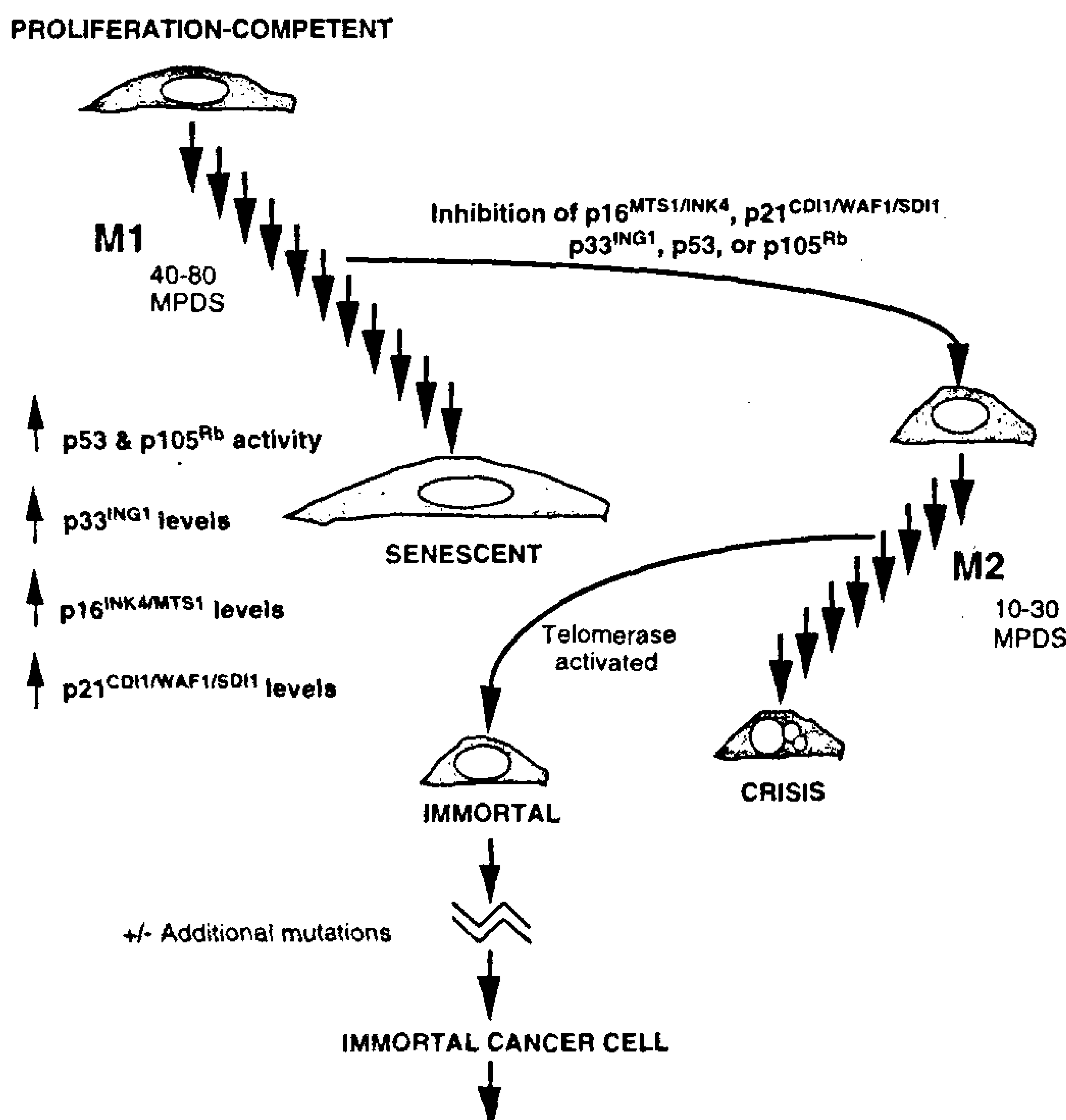


**Figure 2.** Senescence and its abrogation in human diploid fibroblasts. Fibroblasts normally undergo 40–80 mean population doublings before activation of a senescence program in a 'Mortality 1' (M1) pathway. The growth arrest characteristic of senescence is accompanied by changes in gene expression and activity indicated in the figure. Cells can escape from this M1 fate by blocking certain growth inhibitors, either directly or by addition of transforming oncogenes, allowing an additional 10–30 MPDs before cells enter 'crisis' and die. Cells can also infrequently escape this 'M2 pathway', acquire telomerase activity and become immortal during progression to malignant cancer.

normally acts to remove cells during embryonic development and in various proliferative tissues of mature organisms, which can be activated in cells by DNA damage[11]. Additional perturbations are believed to be required for the acquisition of immortality that is characteristic of most cancers *in vivo* and of all established cell lines. At least one of these additional events might be the activation of telomerase, an enzyme required to synthesize repetitive DNA elements at the ends of chromosomes where DNA polymerase is unable to function[12,13]. In fact, the lack of telomerase activity in normal human fibroblasts may also play an important role as part of the molecular clock that activates senescence, as chromosomal shortening through successive rounds of replication might signal the onset of senescence[14].

The remainder of this review covers some molecular aspects of replicative senescence that are currently being examined in our laboratory.

## Transcription factors in cellular aging: loss of positive growth regulators

The rapid induction of cellular 'immediate early' gene transcription mediated by modification of pre-existing transcription factors in mitogen-stimulated cells suggests a role for these genes as a molecular switch that activates subsequent cell cycle events in quiescent cells. For example, addition of mitogens to serum-starved, growth-competent cells induces rapid transcriptional activation of the c-*fos* immediate early gene which itself encodes a transcriptional activator with growth-promoting properties[15]. The Fos protein forms a part of the Activating Protein 1 (AP1) complex that binds to the AP1 concensus sequence in promoters of various downstream effectors and regulates their expression. Fos activity has been shown in several independent studies to be required for cell growth[16–20], but this activity is lost in senescent fibroblasts as they become refractory to mitogenic stimulation[21–27], an effect that appears to be due, in part, to modification of transcription factors that regulate the promoter of the c-*fos* gene[27].

Transcriptional regulation of c-*fos* occurs through several elements present in the promoter of this gene, but the element that appears most affected by cellular aging is the serum response element (SRE), a 20-bp sequence that binds to a variety of proteins and which is both necessary and sufficient for response to mitogens[28,29]. Perhaps the best-characterized protein that regulates SRE activity is the serum response factor (SRF), a 67 kDa protein that specifically binds to the SRE as a homodimer and itself is regulated by its association with a variety of other nuclear proteins[30,31]. The reduced levels of c-*fos* transcription in senescent cells is associated with a dramatic reduction in the binding of

SRF to SRE[27]. While SRF protein level is maintained during senescence, its ability to bind SRE elements is lost, most likely due to hyperphosphorylation of SRF. Since the SRF protein contains an unusually large number of phosphorylatable residues[30,31], it may be the target of multiple kinases and phosphatases that are affected by cellular aging. Current studies are directed towards distinguishing the residues of SRF that serve as substrates for senescence-associated hyperphosphorylation and identifying the senescence-associated kinases or kinase regulators that direct hyperphosphorylation and inactivation of SRF.

## Senescence and the cell cycle machinery

The cell cycle is regulated at various phases by the activity of the cyclin gene family, whose functions are conserved in organisms as broad ranging as yeasts and humans[32]. Many of the cyclin genes are expressed and act within a narrow window at characteristic phases of the cell cycle (Figure 1). The encoded proteins are thought to drive the cell cycle by binding and activating specific cyclin dependent protein kinases (CDKs), which then phosphorylate specific targets at specific phases of the cycle. For example, mammalian D-type cyclins (cyclins D1, D2, D3) share some homology with yeast $G_1$ cyclins and associate with CDK2, 4 and 6 (ref. 33). Expression of cyclin D1 is mitogen responsive[34–36] and microinjection of cyclin D1 antibodies has been reported to inhibit progression through $G_1$ (ref. 37–39), suggesting that this cyclin acts as a positive regulator early in the cell cycle. Cyclins E (mid G1/S phases), A (late G1/S phase), and B (M) associate with mostly distinct sets of CDKs (CDK2, CDK2 and CDK1, respectively; see Figure 1).

A variety of other growth-related proteins act by directly modifying the activity of cyclins/CDKs. For example, the related p21(SDI/CIP/WAF), p27(KIP) and p57(KIP2) proteins contain domains that can bind and inhibit cyclin/CDK complexes, thereby inhibiting cell-cycle progression[40,41]. Transcription of the *p21* gene is also induced by the activity of the p53 tumor suppressor protein[42], suggesting that it is an effector of p53-mediated cell cycle arrest (see below). Indeed, ectopic overexpression of p21 or p27 can also inhibit cell cycle progression. However, more recent evidence suggests that the effects of p21 and related proteins on the activity of cyclins/CDKs are dependent on stoichiometry, with low concentrations of these proteins actually activating cyclin/CDK complexes[43]. A second family of cyclin inhibitors are exemplified by the p16 (INK4/MTS1) protein which binds to CDK4 and CDK6, preventing association with, and activation by, D-type cyclins[41,44]. The growth-inhibitory role of p16 may be less ambiguous than that of p21, as the *p16* gene is also considered a strong candidate tumor suppressor gene[45–48].

The D-type cyclins have traditionally been viewed as growth stimulatory genes since cyclin D/CDK complexes are thought to contribute to the hyperphosphorylation of the retinoblastoma protein (Rb) that occurs progressively throughout late $G_1$ and S phases (see below). Consistent with this idea, both cell cycle progression and Rb hyperphosphorylation fail to occur in senescent cells[26,49,50]. However, the expression of D-type cyclins is actually increased in senescence[51–54] and transient ectopic overexpression of cyclin D1 can also inhibit proliferation in otherwise competent cells[53,55]. Our data so far indicate that senescence-associated upregulation of cyclin D1 occurs via a transcriptional mechanism, and studies to identify the relevant promoter elements are currently underway. We also find that cyclin D2 is upregulated in senescence and is growth inhibitory when ectopically overexpressed[56]. Unlike other D-type cyclins, however, cyclin D2 is also upregulated during quiescence induced by serum starvation or by contact inhibition, and so it appears to play a growth-inhibitory role in both $G_0$ and $G_S$ (Figure 1). It remains to be determined whether this effect of D-type cyclins represents a stoichiometry-dependent effect, similar to that suggested for p21 (ref. 43), which might suggest that the degradation of $G_1$ cyclins in cyclin/CDK complexes, like those of $G_2$ cyclins in M-phase complexes[57], is required for progression of the cell cycle. On the other hand, some functions of D-type cyclins may not directly involve CDKs. For example, inhibition of the major catalytic partner of cyclin D1, CDK4, with a dominant negative mutant has been reported to have little effect on cell-cycle distribution[58]. In fact, much of the cyclin D1 and D2 in senescent cells actually appears to be associated with inactive CDK2 (ref. 51, 56).

The increased expression of cyclin D1 and the decreased expression of mitotic cyclins[53,54,56] during cellular aging support an arrest in a $G_1$-like state. Nevertheless, cyclin D2 levels more closely resemble those seen in $G_0$ (ref. 56), and some S-phase gene expression has also been reported in senescent cells[59]. Hence, $G_S$ shares some characteristics of $G_0$, $G_1$ and S phases despite the loss of proliferative potential in senescent cells.

Other factors that are likely to contribute to growth arrest in senescent cells include elevated expression of both the p21 and p16 CDK inhibitors[53,54,60,61]. The overexpression of p21 in senescent cells could conceivably surpass the stoichiometry required for its synergistic effects on cyclin D/CDK activity, whereas titration of CDKs by p16 might also make D-type cyclins available for association with atypical CDK partners. Interestingly, homozygous deletion of the p21 gene in human fibroblasts has also recently been reported to allow cells to bypass the M1 pathway[62], thus further implicating its elevated expression as an active component of the senescence phenotype. However, given the positive regu-

latory role of low p21 levels[43], the subtlety of effects observed in mice lacking a functional *p21* gene[63], and the paucity of data indicating its loss in human tumors, it seems unlikely that *p21* functions as a tumor suppressor.

## A role for the Rb and p53 tumor suppressor genes in senescence

The study of tumor suppressor genes originated with a mathematical model that described a protective role for a gene lost during the progression of the hereditary eye cancer, retinoblastoma[64]. The isolation of the *Rb* gene and manipulation of this gene in the mouse has confirmed that loss or mutation of the *Rb* gene also predisposes mice to some additional forms of cancer[65]. During cell-cycle entry of quiescent cells, the Rb nuclear protein becomes progressively phosphorylated beginning in $G_1$ and reaching a maximum in late $G_1$ or early S phase, an event that is likely due, in part, to the activation of various cyclin/CDK complexes acting at these phases of the cell cycle[66]. This is believed to dissociate Rb-containing protein complexes and allow further cell-cycle progression. Indeed, the mechanisms used by some viral oncoproteins to promote cell immortalization include binding and inactivating tumor suppressor proteins such as Rb and p53 (Figure 2). Hence, p53 and Rb play important growth limiting roles that must be overcome during progression to cellular immortality.

The *p53* gene is perhaps the quintessential tumor suppressor gene as its somatic mutations cause as many as 50–60% of spontaneous human cancers, and germ-line mutations of *p53* are responsible for Li-Fraumeni syndrome, an inherited predisposition to various cancers[67]. The negative growth-regulatory role of the p53 nuclear phosphoprotein is thought to be an important factor in various 'checkpoints' throughout the cell cycle where progression can be paused to effect repairs[68]. The mechanisms employed by p53 appear to be numerous and include a general inhibition of transcription as well as site-specific activation of certain target genes.

Consistent with a role for Rb and p53 in cellular aging, both proteins are constitutively active in senescent cells[8,26,49,50,69], and selective inhibition of Rb and/or p53 function can extend the proliferative lifespan of cultured human diploid fibroblasts[70–74]. One plausible mechanism by which p53 may contribute to senescence-associated growth arrest is by upregulating the expression of certain target genes, including those encoding insulin-like growth factor-binding protein 3 (ref. 75), cyclin D1 (ref. 76) and p21 (ref. 42).

## The *ING1* candidate tumor suppressor gene in senescence and tumorigenesis

We recently cloned a novel growth inhibitor and candidate tumor suppressor gene, called *ING1* (for *IN*hibitor of *G*rowth) using a method that combined subtractive hybridization of cDNAs from normal and cancerous cells with an *in vivo* selection assay[77]. The encoded 33 kDa nuclear protein bears little homology to other known proteins, but like other growth suppressive genes, its ectopic overexpression inhibits cell cycle progression. *ING1* is located on chromosome 13q33–34 (ref. 78), a site that has also previously been implicated in the progression of various tumors[79–82]. It also appears to have a role in cell death as its expression confers sensitivity to apoptosis mediated by expression of c-*myc* in P19 teratocarcinoma cells whereas antisense expression protects them from c-*myc*-induced apoptosis[83]. Expression of *ING1* is regulated through the cell cycle, decreasing as cells exit $G_0$ and increasing again during late $G_1$ to reach a plateau in S phase[84].

A role for *ING1* in cell aging was first suggested by the dramatic accumulation of transcripts and protein as human fibroblasts approached senescence[84]. Antisense inhibition of *ING1* expression was subsequently found to extend the proliferative lifespan of these cells to an extent similar to that observed when either p53, Rb, p21 or p16 functions are inhibited. Hence, functional loss of *ING1* might contribute to tumorigenesis either by decreasing the capacity of the cells to undergo apoptosis and/or by allowing cells to escape the M1 pathway. Consistent with this idea, we have found that although mutation of *ING1* may be rare, the levels of *ING1* expression are dramatically down-regulated in different cancers, including brain tumors wherein at least 50% of primary tumors show complete loss of *ING1* expression[85,86]. Assessment of the possible role of *ING1* in tumorigenesis should also be assisted by studies to further clarify the activities and interactions of this unique gene.

## Telomeres and telomerase in cell aging and immortality

Maintenance of chromosomal termini through successive rounds of cell division requires the activity of telomerase, an unusual protein- and RNA-containing reverse transcriptase which synthesizes telomeric repeats (TTAGGG) *de novo* onto chromosomal termini[12]. While telomerase is expressed in most immortal cells, developmental repression of telomerase activity results in division-dependent attrition of telomeric DNA in human somatic cells[13]. Thus, progressive telomere shortening in these cells might somehow serve to couple the activation of a cellular aging program to the cells' proliferative history. Moreover, activation of telomerase may be a key step in the acquisition of unlimited proliferative ability by preventing progressive chromosomal degradation as cells divide[14].

We have focused on the identification of regulatory pathways controlling telomerase activity during differ-

entiation and growth arrest of immortal cells. Many immortal cell lines which retain the ability to differentiate also repress telomerase activity in response to differentiating agents[87,88], and hence they may represent useful model systems to study the regulation of telomerase activity. Our results to date indicate that repression of telomerase activity during differentiation is a complex process involving both cell cycle-correlated and cell cycle-independent mechanisms. In fact, telomerase activity is also down-regulated in some tumor-cell types that continue to proliferate and remain immortal in a state of induced differentiation, while regulation of telomerase activity in other cell lines more closely parallels that of the cell cycle. Presumably, these conflicting mechanisms might reflect cell type-specific differences in the regulation of telomerase activity. This also suggests the possibility that cell type-specific telomerase inhibitors could be used to selectively inactivate telomerase in tumor cells while sparing self-renewing, immortal stem cells that also rely on telomerase.

Further support for cell type-specific telomerase regulation has come from a more detailed study of the multipotential HL-60 promyelocytic leukemia cell line in which the regulation of telomerase activity has been followed during differentiation along various hematopoietic lineages. In these cells, repression of telomerase activity can be blocked with a potent serine protease inhibitor during monocytic, but not granulocytic, differentiation. Kinetic studies with the protein synthesis inhibitor, cycloheximide, further support a role for proteolysis in the repression of telomerase activity during monocytic differentiation.

In order to facilitate more detailed studies of the mechanisms underlying regulation of telomerase activity, we are using a yeast 3-hybrid screen[89], which identifies RNA-binding proteins (similar to the yeast two-hybrid system for identifying interacting proteins), to identify possible negative regulators of telomerase which bind specifically to its RNA component. Five strong candidates for human telomerase RNA-binding proteins have so far been isolated and are presently being analysed for expression patterns in mortal and immortal cells.

## Summary

Molecular analyses of cellular senescence in human diploid fibroblasts are beginning to reveal details of the growth-inhibitory mechanisms that underlie this phenomenon. These include several interrelated processes such as: 1) reduced expression of some growth-promoting genes, exemplified by the Fos transcription factor; 2) aberrant regulation of many cell cycle regulators, such as cyclins, CDKs and CDK inhibitors,whose relative levels appear to be important during senescence; 3) the enhanced expression or activity of growth inhibitors\tumor suppressors such as p53, Rb, ING1, p21 and p16, and 4) down-regulation of telomerase activity leading to cell division-dependent attrition of telomeres that may act to initiate senescence. Many other genes whose growth-regulatory roles are less readily distinguished are also regulated differentially in senescence. Therefore, further studies of their expression in senescence should also reveal as yet undiscovered mechanisms contributing to the growth arrest characteristic of senescence. It is becoming clear that many of the gene products contributing to cellular senescence are also those whose altered functions contribute to progression of malignancy. Hence, the growing model of their functions and interactions should also prove useful in the future to design rational therapies for human cancers.


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