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Review

The signals and pathways activating cellular senescence

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Abstract

Cellular senescence is a program activated by normal cells in response to various types of stress. These include telomere uncapping, DNA damage, oxidative stress, oncogene activity and others. Senescence can occur following a period of cellular proliferation or in a rapid manner in response to acute stress. Once cells have entered senescence, they cease to divide and undergo a series of dramatic morphologic and metabolic changes. Cellular senescence is thought to play an important role in tumor suppression and to contribute to organismal aging, but a detailed description of its physiologic occurrence in vivo is lacking. Recent studies have provided important insights regarding the manner by which different stresses and stimuli activate the signaling pathways leading to senescence. These studies reveal that a population of growing cells may suffer from a combination of different physiologic stresses acting simultaneously. The signaling pathways activated by these stresses are funneled to the p53 and Rb proteins, whose combined levels of activity determine whether cells enter senescence. Here we review recent advances in our understanding of the stimuli that trigger senescence, the molecular pathways activated by these stimuli, and the manner by which these signals determine the entry of a population of cells into senescence.
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Keywords: Senescence; p53; Rb; p16; ARF; Oxidative stress; Telomeres; DNA damage

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1. Senescence as a cellular stress response

The biologist attempting to experiment with normal mammalian cells will frequently encounter the phenomenon of cellular senescence. Be they of mouse or of human origin, of fibroblastic or of epithelial nature, most normal cells will ultimately undergo senescence. This may occur after an extended period of propagation in culture, or in response to inadequacy in growth conditions or physiologic stress. In fact, senescence is a major obstacle to the continued propagation of cells in culture, and a defining feature of almost all transformed cell lines is their inability to enter into this state.

It is difficult not to notice cellular senescence once it has occurred in the culture dish, as it involves dramatic changes in almost every aspect of cell function and morphology. Cells entering senescence cease to respond to mitogenic stimuli, undergo dramatic changes in chromatin structure and gene expression, and become enlarged and flattened (Fig. 1) (Shelton, Chang, Whittier, Choi, & Funk, 1999; Serrano & Blasco, 2001; Narita et al., 2003). Typically such cells acquire increased adhesion to the extracellular matrix while losing cell–cell contacts. Cells that have undergone senescence can remain in this viable, non-dividing state for months.

These observations suggest that cellular senescence is a fundamental cellular program that is activated in various situations of physiologic stress and acts to prevent further cell proliferation (Ben-Porath & Weinberg, 2004). This function places senescence as a program that parallels the well-studied phenomenon of programmed cell death—apoptosis. Studies performed in recent years, discussed below in detail, have shed much light on the mechanisms and molecular pathways responsible for the activation of the senescence program. However, the physiologic role of senescence remains poorly understood. We have yet to identify the situations in which it is activated in the living tissue, the molecular and cellular characteristics of senescence in vivo, and the molecular pathways executing the multitude of cellular changes associated with senescence.

Senescence has been viewed historically as a cell-intrinsic mechanism designed to restrict unlimited cell proliferation. This view was derived from the classical experiments performed by Leonard Hayflick in the 1960s, who found that normal human fibroblasts cease to divide following a period of proliferation in culture (Hayflick & Moorhead, 1961). Subsequently, it was found that the attrition of telomeres plays an important role in the senescence of certain cell types (Bodnar et al., 1998; Harley, Futcher, & Greider, 1990). Studies performed in recent years have revealed that normal cells undergo senescence in response to various types of stress, such as DNA damage, damage to chromatin structure and oxidative stress (Fig. 1) (Serrano & Blasco, 2001; Lloyd, 2002). In addition, cells undergo senescence in response to overexpression of activated oncoproteins, specifically Ras and its downstream effectors (Serrano, Lin, McCurrach, Beach, & Lowe, 1997). The activation of senescence in these situations

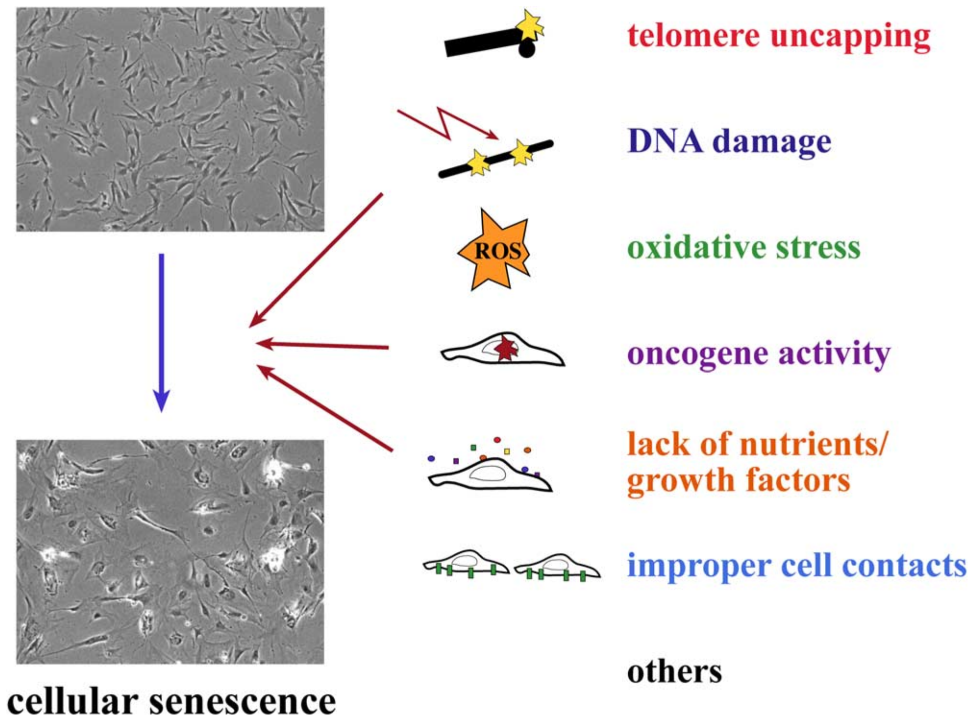

Fig. 1. The signals activating senescence. Multiple types of stress can induce cells to undergo senescence. The combined levels of stress determine how rapidly the entry into senescence will occur.

occurs relatively rapidly—in a manner of days. These findings have led to a distinction between "replicative senescence", a term referring to senescence that occurs following extended proliferation, presumably triggered by a cell-intrinsic mechanism, and "stress-induced premature senescence", a term referring to rapid senescence triggered by extrinsic stress.

As useful as this distinction may be, it probably does not refer to two independent cellular mechanisms, but, rather, to the fact that disparate signals can elicit a common cellular response. As discussed below, most studies indicate that the senescence program is activated once a cell has suffered a critical level of damage, whatever that damage may be. A population of propagated cells may simultaneously suffer from multiple stresses, which can exert a cumulative effect (Fig. 1). Moreover, extrinsic stresses, such as oxidative stress, may affect intrinsic factors, such as DNA damage accumulation and telomere shortening rates. Thus, a combination of various stressors may exert a complex cumulative effect on a heterogeneous population of cells, ultimately triggering the senescence of the population as a whole.

2. p53 and Rb as the central activating pathway of senescence

2.1. Parallel or linear senescence pathways?

The two paradigmatic tumor suppressor proteins, p53 and Rb, have been shown to play critical roles in the induction of senescence. Both p53 and Rb are activated upon the entry into senescence (Fig. 2). The p53 protein is stabilized and proceeds to activate its transcriptional targets, such as p21$^{CIP1/WAF1}$ (Kulju & Lehman, 1995). Rb is found at senescence in its active, hypophosphorylated form, in which it binds to the E2F protein family members to repress their transcriptional targets (Narita et al., 2003). These targets constitute the majority of effectors required for cell-cycle progression.

Numerous studies have exmined the relative roles of p53 and Rb in the induction of senescence by assessing the consequences of their inactivation. Various methods have been used to this end: overexpression of viral oncoproteins such as the SV40 large T-antigen and

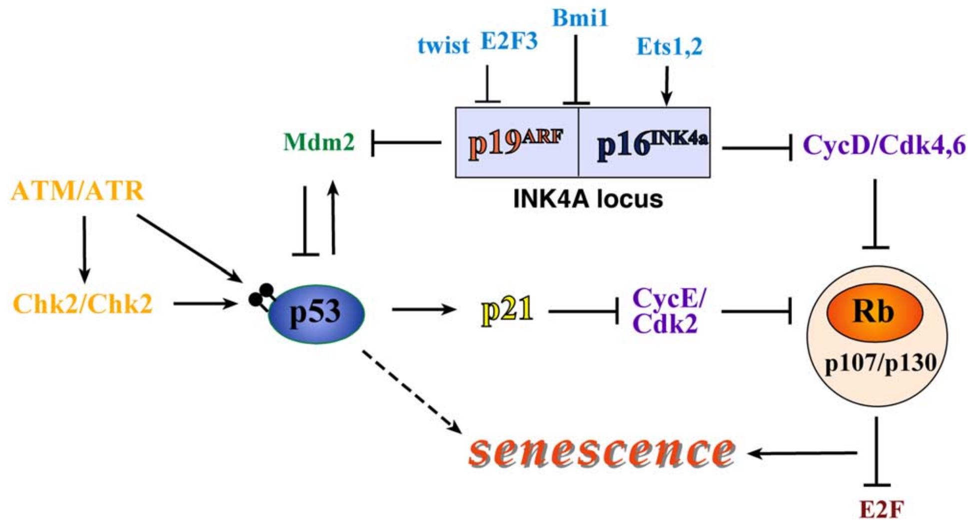

Fig. 2. The molecular circuitry of senescence. p53 and Rb are the main activators of senescence. p53 can activate senescence by activating Rb through p21 and other unknown proteins, and also, in human cells, can activate senescence independently of Rb. Rb activates senescence by shutting down the transcription of E2f target genes. Rb is activated either by p21, or by the $p16^{INK4a}$ product. p53 activation is achieved by phosphorylation, performed by the ATM/ATR and Chk1/Chk2 proteins, and by the $p19^{ARF}$ product of the INK4a locus, which sequesters Mdm2 in the nucleolus. The transcriptional control of the INK4a products is not fully elucidated, indicated are some of these regulators.

the human papillomavirus E6 and E7 proteins (Shay, Pereira-Smith, & Wright, 1991), germline homologous recombination in the mouse (Dannenberg, van Rossum, Schuijff, & te Riele, 2000; Sage et al., 2000) and somatic homologous recombination in human cells (Brown, Wei, & Sedivy, 1997; Wei, Herbig, Wei, Dutriaux, & Sedivy, 2003), nuclear injection of antibodies (Gire & Wynford-Thomas, 1998) dominant-negative forms of these proteins (Bond, Wyllie, & Wynford-Thomas, 1994; Beausejour et al., 2003), and, recently, RNAi expression knockdown methods (Voorhoeve & Agami, 2003; Wei et al., 2003).

A complex picture emerges from these studies. In mouse embryo fibroblasts (MEFs), inactivation of p53 is sufficient to prevent senescence, allowing these cells to divide indefinitely (Dirac & Bernards, 2003). Cells carrying a null mutation in the Rb gene senesce normally, yet inactivation of additional Rb family members – p107 and p130 – is sufficient to prevent senescence (Dannenberg et al., 2000; Sage et al., 2000). This indicates that both p53 and Rb are necessary for the initiation of senescence, but also that the other Rb family members are capable of providing redundancy for this function of Rb. Once MEFs have undergone senescence, the continued activity of both p53 and Rb is required to maintain this state—inactivation of either of these genes in senescent MEFs allows these cell population to resume a proliferating state (Dirac & Bernards, 2003; Sage, Miller, Perez-Mancera, Wysocki, & Jacks, 2003).

These findings suggest a model of a linear activation pathway, in which a stress signal activates p53, which in turn activates Rb (Fig. 2). The p21 protein, an inhibitor of cyclin E/Cdk2 complexes, is the natural candidate to mediate this linear activation of Rb by p53; however, surprisingly, MEFs carrying a null mutation in p21 undergo senescence normally (Pantoja & Serrano, 1999), indicating that p21 is not a crucial link in this linear chain, or that other proteins can compensate for its function. This model therefore carries a missing link between p53 and Rb, and the role of p21, whose levels are strongly induced during the senescence of these cells, is not fully clear.

Various experiments with human fibroblasts indicate that, unlike the behavior of mouse cells, the inactivation of both p53 and Rb is required to prevent the onset of senescence (Smogorzewska & de Lange, 2002). Accordingly, the inactivation of either protein alone generally only delays the onset of senescence. This suggests that in human cells, p53 and Rb are ac-

tivated in parallel (as opposed to the linear activation envisioned in mouse cells) and perform partially redundant roles (Fig. 2). Such a mechanism could provide an extra layer of protection against the bypass of senescence and thus against tumor development.

More recent experiments, however, provide examples of cases in which the inactivation of either p53 or Rb seems sufficient to prevent senescence or, at least, to significantly delay its onset. Most compellingly, inactivation of either p53 or Rb by somatic homologous recombination in human lung fibroblasts allows prevention of senescence, suggesting a linear activation pathway between these proteins, as in mouse (Wei et al., 2003). Moreover, in contrast to the mouse, inactivation of p21 by homologous recombination is sufficient to prevent senescence, supporting the proposed linear model linking p53 and Rb via p21 (Brown et al., 1997). In addition, it has been shown that inactivation of p53 alone in cells that have already entered replicative senescence, using a dominant-negative protein to do so, suffices to allow these cells to resume growth and division (Beausejour et al., 2003). This is true for some human fibroblast cell strains, but not for others, in which Rb growth-suppressive activity is apparently maintained independently of p53 (Beausejour et al., 2003).

2.2. The independent arm of p16^{INK4a}

Which is the correct model, then, the linear p53 → p21 → Rb model, or the parallel, p53/Rb activation model? It seems that both are correct. The key lies in the function of the p16^{INK4a} protein, which provides an additional activation input line feeding directly to Rb (Fig. 2). p16 is an inhibitor of cyclin D/Cdk4,6 complexes and is not normally expressed in adult tissues (Lowe & Sherr, 2003; Zindy, Quelle, Roussel, & Sherr, 1997). However, it is induced in various situations of stress and is highly expressed in senescent cells (Alcorta et al., 1996; Palmero et al., 1997). Rb can thus be activated by either the p53 → p21 linear arm, or by the p16 arm, or by both in parallel. As discussed below, the specific combination of stresses and their severity determine the levels of activation reached by p53 and Rb in different cell types. Thus, p53 and Rb function as a complex stress–signal integration and processing unit, whose input is the total level of stress and damage a cell is experiencing, and whose output is the activation of the appropriate cellular response.

Inactivation of p16 in human cells causes a delay in the onset of senescence, quite significantly in some cases (Brookes, Rowe, Gutierrez Del Arroyo, Bond, & Peters, 2004). The most dramatic effect of p16 can be observed in human epithelial cells, specifically mammary epithelial cells and keratinocytes. p16 is solely responsible for the induction of an early, stress-induced, senescence stage in these cell types, often termed M0 (Foster, Wong, Barrett, & Galloway, 1998; Kiyono et al., 1998; Rheinwald et al., 2002). However, cells can spontaneously emerge from M0 by silencing p16 expression through promoter methylation or deletion of the p16 locus, which permits them to continue dividing until they reach a subsequent, p53-dependent senescence stage termed M1. In these epithelial cells, the activation of senescence through p16 and p53 is thus separated in time, providing a clear demonstration of their independent modes of action.

The activation of Rb's growth-suppressive functions by p16 does indeed seem to represent an extra layer of safety that is significantly more active in human cells than in mouse cells. *p16* is one of the most frequently inactivated genes in human tumors (Rocco & Sidransky, 2001), to an extent matching only the frequency of *p53* mutations. In contrast, while inactivation of p53 in the mouse germline causes widespread tumor development, p16-null mice suffer from only a narrow set of tumor types (Krimpenfort, Quon, Mooi, Loonstra, & Berns, 2001; Sharpless et al., 2001). Expression of p16 is induced during the senescence of MEFs in culture, but its inactivation does not prevent or delay the senescence of these cells or of most other mouse cell types (Sharpless, Ramsey, Balasubramanian, Castrillon, & DePinho, 2004).

There are, however, examples demonstrating that the p16 signaling arm does function in the mouse in certain settings. Although p53 inactivation is sufficient for the immortalization of most mouse cell types, the additional inactivation of p16 is necessary for a subset of them, such as bone marrow-derived macrophages (Randle, Zindy, Sherr, & Roussel, 2001). Mouse lymphomas induced by Myc and Bcl-2 overexpression undergo senescence in vivo in response to chemotherapy, doing so in a p16-dependent manner (Schmitt et al., 2002). The wiring of the senescence circuitry seems therefore to be conserved between mouse and humans in its outline, but the importance of the p16 → Rb arm is clearly enhanced in humans.

To fully reconcile the linear and parallel models, however, one additional issue needs to be addressed. The finding that the senescence of human cells can, in some cases, be induced in the absence of the activity of Rb or its family members seems to indicate that p53, on its own, can induce senescence of human cells through a pathway independent of the Rb family (Smogorzewska & de Lange, 2002). What this pathway may be is unknown. However, it is possible that all three Rb family members were not fully inactivated in human cells.

2.3. The upstream controllers of p53 in senescence

In the context of senescence, p53 seems to be controlled by two major pathways (Wahl & Carr, 2001). One is the DNA damage-response pathway, mediated by the ATM/ATR and Chk1/Chk2 proteins, which cause the post-translational stabilization of p53 through its phosphorylation (Fig. 2). The other pathway acts through the $p19^{ARF}$ protein ($p14^{ARF}$ in human, ARF hereafter). ARF is encoded by an alternative transcript of the *INK4a* locus, which shares two exons with the $p16^{INK4a}$ transcript but has a separate promoter and first exon and is translated in a different reading frame (Lowe & Sherr, 2003). The ARF product activates p53 by sequestering Mdm2, an E3 ubiquitin ligase, in the nucleolus, thereby preventing the Mdm2-mediated targeting of p53 to proteolytic degradation (Fig. 2).

Like p16, ARF is induced in situations of stress and senescence. It is also activated by ectopic expression of oncoproteins, such as Myc, Ras, E2F1 and E1A (Lowe & Sherr, 2003). While the phosphorylation of p53 in response to DNA damage does not involve ARF, it has been shown to contribute to p53 stabilization in these situations, and its expression is induced in response to ionizing radiation (Khan, Moritsugu, & Wahl, 2000; Khan, Guevara, Fujii, & Parry, 2004). ARF thus seems to represent a stress-dependent pathway that activates p53. However, little is known about the physiologic contexts in which this pathway is activated.

In contrast to p16, ARF is a critical tumor suppressor in the mouse, and its inactivation in the mouse germline causes widespread tumor development, similar to that observed upon p53 inactivation (Kamijo et al., 1997). Its role as a tumor suppressor in humans is less well established, and it is not upregulated to the same extent at senescence in human cells as it is in mouse cells. Echoing the behavior of p53-null MEFs, MEFs deficient in p19ARF do not undergo senescence (Kamijo et al., 1997). It is commonly accepted that in the mouse, the ARF pathway of activation is more prominent than in the human, providing another example of human/mouse differences (Lowe & Sherr, 2003).

The INK4a locus and its two products are thus central mediators of senescence signals upstream to p53 and Rb. Surprisingly little is known about the manner in which the transcription of these genes is regulated. The Bmi1 protein, a member of the polycomb family, represses both genes, and is downregulated at senescence (Fig. 2) (Itahana et al., 2003). Its activity is thus a powerful means by which senescence may be bypassed. Members of the Ets transcription factor have been implicated as activators of p16, and the Twist and E2F3 proteins act as repressors of p19ARF transcription. Tbx2, Tbx3, JunB and other proteins have also been implicated in the regulation of the INK4a locus (Lowe & Sherr, 2003). However, these interactions remain poorly characterized. The transcriptional regulation of the INK4a locus and the molecular signaling pathways that control it remain an important question in awaiting further exploration.

3. The various triggers of senescence — Telomere uncapping

3.1. How do telomeres get uncapped?

Telomeres, the structures protecting chromosome ends, have received much attention as a trigger for replicative senescence. During the proliferation of human cells, a gradual shortening of the average length of telomeres (defined as the stretch containing tandem hexameric repeat units) is observed (Harley et al., 1990). The molecular causes of this shortening are thought to stem from the "end-replication problem"—the inability of the DNA replication machinery to complete DNA synthesis at the very beginning of the replicated lagging strand. However, the dynamics of telomere length regulation are quite complex, and other factors, not fully characterized, seem to contribute to this shortening process (Makarov, Hirose, & Langmore, 1997; von Zglinicki, Pilger, & Sitte, 2000; Huffman, Levene, Tesmer, Shay, &

Wright, 2000; Baird, Rowson, Wynford-Thomas, & Kipling, 2003).

There is great variability in telomere lengths within individual cells and in a population of dividing cells (Martens, Chavez, Poon, Schmoor, & Lansdorp, 2000; Baird et al., 2003; Martin-Ruiz et al., 2004). It has been suggested that the shortening of telomeres leads to eventual telomere "uncapping"—i.e. disruption of the proper structure of the protective cap at the end of the telomere, thus exposing its end (Blackburn, 2001). An uncapped telomere seems to be recognized as a double-stranded DNA break, which activates the DNA damage machinery. Evidence that such uncapping does occur at senescence has been provided by the observation that at senescence telomeres lose some of their single stranded portion—the telomeric overhang (Stewart et al., 2003). The overhang is an extension of several hundred nucleotides in length of the G-rich, 3′-ending, strand beyond the C-rich strand. This portion of the telomere is presumably crucial for the maintenance of the telomeric "T-loop", a DNA structure that may be essential for the formation of the protective telomeric cap (Stewart et al., 2003). Forced uncapping of telomeres through the inhibition of the TRF2 telomere-binding protein induces loss of the telomeric overhang and leads to the induction of senescence (van Steensel, Smogorzewska, & de Lange, 1998).

The direct causes of telomere uncapping at senescence, and the dependence of this event on telomere shortening remain unclear. It has recently been shown that normal human fibroblasts express low levels of activity of the telomerase enzyme, and that the inhibition of this activity leads to premature senescence (Masutomi et al., 2003). Strikingly, telomerase inhibition does not accelerate telomere shortening rates in these cells, but, rather, enhances the erosion of the telomeric overhang. This suggests that overhang erosion and telomere uncapping can occur independently of telomere shortening.

Similarly, it has been shown that overexpression of TRF2, which apparently plays an important role in telomere capping, accelerates telomere shortening without accelerating the onset of replicative senescence of fibroblasts (Karlseder, Smogorzewska, & de Lange, 2002). Overexpression of this protein appears to allow telomeres to shorten without undergoing uncapping. Telomerase activity and TRF2 expression thus seem to play a protective role against uncapping forces; the exact nature of these uncapping forces is unknown.

3.2. *Telomere uncapping causes a DNA damage response*

How is telomere uncapping detected once it has occurred, and what signaling pathways lead from this event to the activation of the senescence program? Several recent studies have revealed that DNA damage foci appear at the telomeres of senescent cells. These foci contain multiple DNA damage-response proteins, such as γ-H2AX, 53BP1, MDC1, NBS1, MRE11 and RAD17 (d'Adda di Fagagna et al., 2003; Herbig, Jobling, Chen, Chen, & Sedivy, 2004). Moreover, senescent cells express activated forms of the ATM and ATR protein and of their downstream targets, Chk1 and Chk2, which are the central activators of p53 in response to DNA damage (d'Adda di Fagagna et al., 2003; Herbig et al., 2004). These proteins also localize to the DNA damage foci at telomeres in senescence, and their inhibition allows the re-entry of cells into the cell cycle. These findings indicate that uncapped telomeres are indeed recognized as DNA damage and induce the activation of a cellular signaling response akin to that observed upon the exposure of cells to ionizing radiation.

The above results suggest that the molecular pathway that induces senescence upon telomere dysfunction acts through the DNA damage-response pathway – most prominently ATM and its targets Chk1 and Chk2 – to activate p53 (Fig. 3A). It may thus be expected that cells deficient in the ATM protein would be more resistant to senescence; however, this is not the case. In fact, ATM loss accelerates telomere shortening rate and telomeric damage (Tchirkov & Lansdorp, 2003; Wong et al., 2003). ATM-deficient human cells enter senescence like normal cells in response to telomere uncapping by TRF2 inhibition, although they display fewer DNA damage foci at telomeres (Takai, Smogorzewska, & de Lange, 2003). These findings suggest that the ATM protein is involved also in repair or protection of telomeres, and that in its absence, other signaling molecules, such as ATR or DNA-PKc, may take its place in the senescence-activating signaling cascade.

As discussed above, the ARF protein has been implicated in the activation of p53 at senescence.

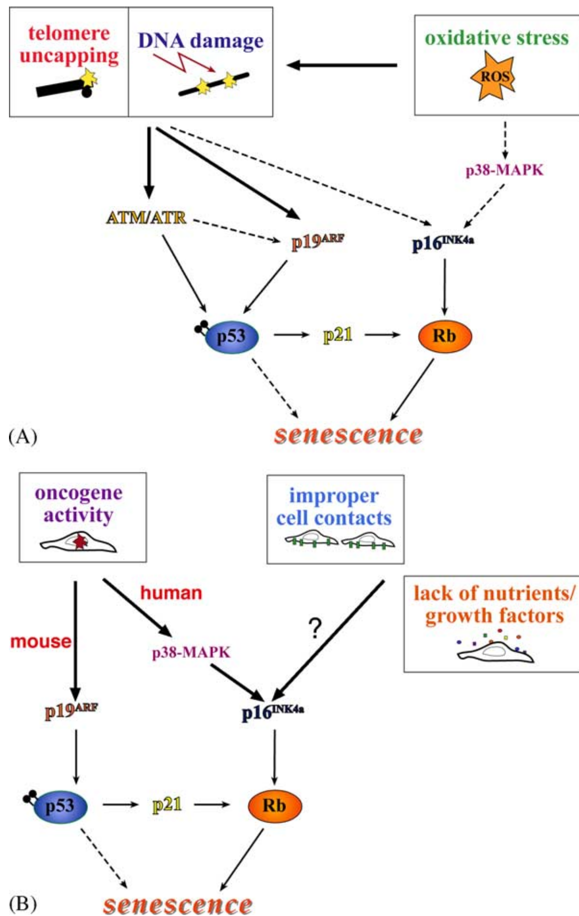

Fig. 3. Activation of the senescence program by different stimuli. (A) Telomere uncapping induces senescence mainly through the DNA damage pathway, activating the ATM/ATR pathway and Chk1/Chk2 to stabilize p53. In mouse this response is dependent on the activity of p19^{ARF}, while in humans the role of ARF in this response is not known. In human cells p16 is activated in certain settings in response to telomere uncapping, through unknown pathways. Direct DNA damage activates the senescence program mainly through p53, in essentially the sane manner as telomere uncapping. Oxidative stress induces DNA damage, and also accelerates telomere shortening, possibly leading to accelerated telomere uncapping. The downstream response is mediated through the DNA damage pathway and through p19^{ARF}. Activation of p16 by oxidative stress is seen in certain conditions mainly in human cells, and may be mediated through p38-MAPK. (B) *RAS* oncogene activation induces senescence through p19^{ARF} in the mouse, and mainly through p16^{INK4a} in human cells. p38-MAPK plays a role in mediating ras-induced senescence. p16^{INK4a} is activated by additional physiologic stresses to induce senescence, such as nutrient and growth factor deficiency, and improper cell–cell and cell–matrix contacts. The exact stimuli for this activation and the pathways mediating it are unknown.

Does ARF participate in the activation of p53 in response to telomere dysfunction? In mouse cells it clearly does, as ARF-deficient fibroblasts do not undergo senescence in response to forced telomere uncapping (Fig. 3A) (Smogorzewska & de Lange, 2002). Whether ARF plays a similar role in human cells is yet to be seen. The manner by which ARF is activated in response to telomere dysfunction is unknown.

3.3. Is the p16-Rb pathway activated in response to telomere uncapping?

As discussed above, the prevention of senescence of human cells often requires the inactivation of both the p53 and Rb pathways. If telomere dysfunction is the major cause for the replicative senescence of these cells, then it is expected that p16 and Rb would be activated by telomere uncapping, independently of p53. Indeed, forced telomere uncapping by TRF2 inhibition causes an induction of p16 and senescence, even in the absence of p53 (Smogorzewska & de Lange, 2002). This suggests the existence of a signaling pathway connecting p16 induction to the DNA damage response activated upon telomere dysfunction (Fig. 3A). This pathway does not seem to function in mouse cells (Smogorzewska & de Lange, 2002).

Recent studies suggest, however, that it is mainly, if not solely, the p53 pathway which triggers senescence in response to the telomere dysfunction that occurs during replicative senescence. It has been found that within populations of human fibroblasts there exists a subpopulation of cells which express p16, and that the number of such cells increases as the population approaches senescence (Itahana et al., 2003; Herbig et al., 2004). This subset of cells expressing p16 does not, however, overlap with the subset of cells displaying DNA damage foci at their telomeres, but the latter do express p21 (Herbig et al., 2004). This indicates that within a cell population there are some cells that suffer from telomere uncapping, and these appear to activate the linear p53 → p21 → Rb pathway, while other cells, suffering from other physiologic stresses, activate the parallel p16 → Rb pathway. As the population continues to be propagated in culture, more and more cells activate one or both of these pathways, and the population as a whole eventually undergoes senescence (Fig. 4) (Herbig et al., 2004). A population of human cells in culture is thus

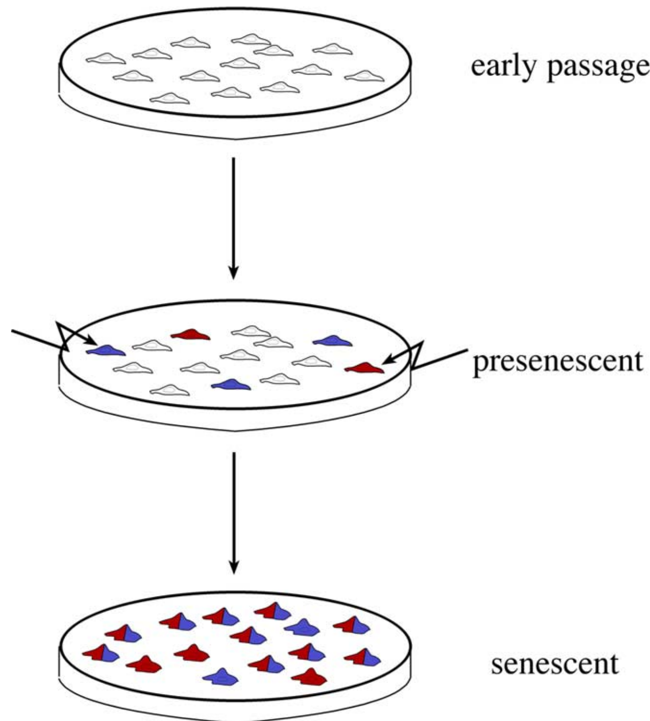

Fig. 4. Replicative senescence as a consequence of the cumulative effect of multiple stressors. As a population of cells is propagated in culture, cells are exposed to various extrinsic and intrinsic stresses. These lead to the activation of the p53 pathway in some cells (red) and of the p16 pathway in others (blue). As the population approaches senescence one of these pathways or both are activated in more and more cells, causing these cells to senescence, and the population as a whole to cease proliferating.

a mosaic of distinct subpopulations responding to different stresses.

Several findings provide further support to this model. First, inhibition of p16 by siRNA vectors or by overexpression of its repressor Bmi1 provides an extension of cellular lifespan, but not immortalization (Itahana et al., 2003; Wei et al., 2003). Furthermore, some normal human lines do not display significant levels of p16-expressing cells, and the senescence of these can be reversed by p53 inactivation solely (Beausejour et al., 2003). Ectopic expression of the telomerase catalytic subunit, hTERT, which prevents telomere uncapping, can immortalize those cell types that do not express high p16 levels, such as BJ cells, whereas fibroblast lines such as WI-38, IMR90 and MRC5, which do accumulate p16, eventually undergo senescence even in the presence of hTERT expression (Forsyth, Evans, Shay, & Wright, 2003). This indicates that the prevention of telomere uncapping does not prevent p16 induction by other stress signals in these cells.

4. Other triggers for senescence: oxidative stress, DNA damage and oncogene activity

4.1. Multiple stresses can have a cumulative effect

As mentioned above, the senescence of normal cells can be induced by various stresses other than telomere dysfunction (Fig. 1), and these may also have a cumulative gradual effect on triggering senescence or, in certain situations, a rapid acute effect. Three inducing stimuli of senescence have received the most attention: direct DNA damage, oxidative stress, and oncogene overexpression. These different triggers are not independent of one another or mutually exclusive. Oxidative stress can cause DNA damage and also accelerate telomere shortening rates (von Zglinicki, Saretzki, Docke, & Lotze, 1995; Forsyth et al., 2003; Parrinello et al., 2003). The induction of senescence by the *RAS* oncogene has been suggested to occur through the induction of reactive oxygen species (Lee et al.,

1999). These observations and others demonstrate the complexity of the cellular response to various stresses. It is of great interest to dissect the effects of these different stresses and to study the manner by which each activates the senescence program.

As discussed above, recent data suggests that the induction of p16 during the replicative senescence of fibroblasts is not a result of telomere dysfunction, but of other culture stresses. What specifically are the stresses that activate p16? Human mammary epithelial cells, when grown in a conventional growth medium, undergo a stage of senescence that is solely dependent on p16 (Foster et al., 1998). Growth in different media, or on a layer of feeder cells, has been reported to prevent or delay p16 induction and consequently prevent the onset of M0 (Herbert, Wright, & Shay, 2002). This points to factors such as nutrient composition, presence of growth factors and the nature of cell–cell and cell–matrix contacts as candidate stimuli for p16 induction in these cells (Fig. 3B). During tumor development, neoplastic mammary cells are likely to encounter some or all of these stresses, which may induce p16 expression.

4.2. Oxidative stress

Oxidative stress and the accumulation of intracellular reactive oxygen species (ROS) play an important role in the induction of senescence. Human fibroblasts undergo premature senescence when grown in high ambient oxygen conditions—40–50% oxygen. Conversely, the proliferative lifespan of these cells is extended when they are grown in low ambient oxygen, 2–3%, a condition more closely resembling physiologic oxygen levels (Chen, Fischer, Reagan, Yan, & Ames, 1995; Packer & Fuehr, 1977). Similarly, increase of intracellular ROS levels through hydrogen peroxide treatment or through the inhibition of ROS scavenging enzymes, such as superoxide dismutase Sod1, causes premature senescence (Blander, Machado De Oliveira, Conboy, Haigis, & Guarente, 2003). Internal ROS can damage cellular components through the oxidation of DNA, proteins and lipids (Chen et al., 1998; Sitte, Merker, Von Zglinicki, Grune, & Davies, 2000), and can also act directly as second messengers to regulate specific signaling pathways (Saitoh et al., 1998).

In fact, it has been shown that the reduction of ambient oxygen levels does not reduce the fraction of p16-expressing cells in a pre-senescent population of normal fibroblasts; rather, it is the proportion of p21-expresssing cells that is reduced (Itahana et al., 2003). This suggests that oxidative stress, like telomere dysfunction, acts through the p53 → p21 → Rb arm to induce senescence (Fig. 3A). Other studies do indicate, however, that p16 can be activated in response to oxidative stress, possibly through the action of the p38-MAPK protein, a member of the stress-activated protein kinase family (Fig. 3A) (Iwasa, Han, & Ishikawa, 2003).

The stress-activated protein kinase family (SAPK) is a group of kinases that respond to a variety of physiologic stresses, including oxidative stress, and induce apoptosis and senescence in response (Kyriakis & Avruch, 2001). This family includes JNK1/2/3 and p38-MAPKα/β/γ/δ, the latter of which have been mostly implicated in senescence. Members of this protein family and their upstream activators (MKKs) are excellent candidates for mediating the activation of p16 in response to a variety stress signals.

It has been shown that the rate of telomere shortening is accelerated in fibroblasts grown in high oxygen conditions (von Zglinicki et al., 1995; Forsyth et al., 2003). The mechanism by which this occurs is not known, although it has been suggested that oxidative stress induces single stranded breaks in telomeric DNA (von Zglinicki et al., 2000), which may lead to deletions of telomeric repeat stretches (Baird et al., 2003). This opens the possibility that ROS promote senescence through telomere dysfunction (Fig. 3A). However, oxidative stress does not induce loss of the telomeric overhang (Keys, Serra, Saretzki, & Von Zglinicki, 2004) and does not induce telomeric DNA foci (Sedelnikova et al., 2004). Another possibility is that it is the induction of damage to genomic DNA by ROS that accelerates the senescence of these cells, doing so through p53 activation. Indeed, DNA damage foci outside of telomeres do appear in response to hydrogen peroxide treatment (Sedelnikova et al., 2004).

The senescence of MEFs, which typically occurs after approximately 10 population doublings, has been recently shown to be a result of oxidative stress in culture (Parrinello et al., 2003). When these cells are grown in 3% oxygen instead of 20%, senescence is greatly delayed or avoided altogether. It has been shown

that these cells suffer from a high level of DNA damage in 20% oxygen conditions, suffering more DNA damage than human cells grown in the same conditions (Parrinello et al., 2003). This indicates that human cells are more capable of withstanding oxidative stress either through an increased ability to neutralize ROS or to monitor and repair DNA. The senescence response of MEFs to oxygen-induced DNA damage is mediated through p53, as p53-null MEFs do not senesce. The fact that p19^{ARF}-null MEFs are also resistant to senescence indicates that p19^{ARF} plays a central role in mediating this response. It is formally possible, however, that the activation of p19^{ARF} and senescence are not a result of the oxidative DNA damage experienced by these cells, but of another unknown consequence of oxidative stress.

4.3. DNA damage

The direct damaging of DNA, achieved either by irradiation of cells or by treatment with DNA-damaging agents, can induce cells to undergo senescence (Fig. 3A) (Wahl & Carr, 2001). Often though, the cellular response to such damage is cell death or reversible cell-cycle arrest, depending on the type of agent, the dosage administered, and the type of cell treated (Wahl & Carr, 2001). Inactivation of DNA repair genes such as Xrcc4, DNA ligase IV, Brca1 and others in the mouse germline typically induces the premature senescence of MEFs cultured from these mice, and of premature aging phenotypes in the mice themselves (Frank et al., 2000; Gao et al., 2000; Ongusaha et al., 2003). This type of premature senescence, both in the tissue culture and in the living mouse, can usually be prevented by p53 inactivation (Frank et al., 2000; Gao et al., 2000; Ongusaha et al., 2003).

Activation of p53 by DNA damage has been studied at length (Wahl & Carr, 2001). It is not clear how cells choose between senescence and apoptosis upon DNA damage-induced p53 activation, although it has been suggested that this is determined by differences in the post-translational modifications that p53 undergoes in response to different stimuli (Webley et al., 2000), by binding of different proteins to p53, and through the activation of different sets of transcriptional targets (Wahl & Carr, 2001). It is known that normal cells, in which all signaling pathways are intact, have a preference toward senescence when compared to transformed cells, and that senescence is usually induced by lower levels of damage than those leading to apoptosis. As discussed above, ARF has been shown to play an important role in the stabilization of p53 following DNA damage, and its expression is induced following ionizing radiation. It has been suggested that ARF plays a role in the long-term maintenance of p53 activity following the induction of damage (Khan et al., 2004). In some of the mice carrying mutations in DNA repair genes, such as Brca1 mutants, activation of p53 has been shown to depend on ARF activity (Somasundaram et al., 1999).

Interestingly, DNA damage can also activate Rb through the p16 pathway to induce senescence (Fig. 3A) (Robles & Adami, 1998; Shapiro, Edwards, Ewen, & Rollins, 1998). While the activities of p53 and p21 are induced to high levels immediately after damage, their levels decrease after several days. In contrast, p16 levels gradually increase during this period (Robles & Adami, 1998). This suggests that while p53 and p21 act to initiate the senescence response, p16 acts to maintain this state.

Perhaps the most interesting example of the role of p16 activation in response to DNA damage comes from a recent study in which mouse lymphomas were generated by overexpression of the Myc and Bcl-2 oncogenes (Schmitt et al., 2002). These tumors were treated in vivo with the alkylating agent cyclophosphamide, to which they responded by undergoing cellular senescence rather than apoptosis. Inactivation of either p53 or of both INK4a products – p19ARF and p16 – prevented this response. Interestingly, inactivation of ARF alone did not prevent senescence, indicating that it is p16 that plays an essential role in the senescence response to this type of DNA damage. The demonstration of a p16-dependent senescence response in an in vivo tumor model is especially compelling and underscores the role p16 plays as a mediator of senescence in tumor suppression. Interestingly, p16 does not seem to act in parallel to p53 in this model, but, rather, upstream to it.

4.4. Oncogene activation

Normal cells in culture undergo senescence in response to the overexpression of the *RAS* oncogene (Serrano et al., 1997) or of downstream effectors such as RAF, activated MAP kinase and the PML oncoprotein (Zhu, Woods, McMahon, & Bishop, 1998; Pearson et al., 2000). This response has been suggested to

represent a tumor-suppressive mechanism, by which cells prevent uncontrolled proliferation in response to the aberrant activation of proliferation-driving oncogenes. It seems, however, that extremely high levels of *RAS* expression and of its effectors are required for senescence to occur, indeed levels that may not be present in the majority of spontaneously arising human tumors, even those carrying *ras* mutations. In a recently described mouse tumor model carrying a single mutated copy of K-*Ras*, no signs of senescence were observed (Tuveson et al., 2004). However, there are examples of human tumors where senescence may occur in response to excessive *RAS* signaling. An interesting example is that of Spitz nevi, a benign melanocytic lesion that is considered a precursor for melanoma. Some of these nevi carry a mutated form of *HRAS* in multiple copies due to gene amplification (Maldonado, Timmerman, Fridlyand, & Bastian, 2004). Cells in these lesions express high levels of p16, and are growth arrested. Interestingly, Spitz nevi that carry a single copy of mutated *HRAS* do not express these high levels of p16 (Maldonado et al., 2004). These findings suggest that in melanocytic nevi p16 can be activated in response to high levels of *RAS* activity, inducing cell-cycle arrest and senescence. In fact, congenital melanocytic nevi express senescence-associated β-galactosidase activity (Dorothy Bennett, personal communication). Since most melanocytic nevi carry activating mutations of the *BRAF* or *NRAS* genes and express high levels of p16, this lesion may represent a general case of in vivo oncogene-induced senescence (Bennett, 2003; Pollock et al., 2003).

In MEFs, induction of senescence by *ras* occurs through an ARF → p53 → Rb pathway (Fig. 3B) (Serrano et al., 1997). This pathway seems to be distinct from the DNA damage response. In human cells, Ras causes parallel activation of p53 and p16 (Serrano et al., 1997). However, it is p16 that seems to play a more prominent role in this type of senescence (Fig. 3B). In some cell types its inactivation is sufficient to prevent this form of senescence (Brookes et al., 2002), and cells that express low initial levels of p16, such as freshly isolated primary human fibroblasts, do not respond to *RAS* overexpression by senescence at all, but instead undertake enhanced proliferation (Benanti & Galloway, 2004).

The p38-MAPK proteins have been shown to play a crucial role in mediating *ras*-induced senescence and in activating p16 and p53 (Wang et al., 2002; Iwasa et al., 2003). Interestingly, it has been suggested that *RAS* overexpression induces senescence through the generation of high levels of ROS (Lee et al., 1999), an effect that could lead to p38 activation. If this were the case, *RAS* would be expected to induce DNA damage, but this has not been documented. The role played by ROS in *RAS*-induced senescence awaits further exploration.

5. Conclusions

Cellular senescence is a program executed by cells in response to a variety of stresses. It seems to be a physiologic response of normal cells that must be overcome in order for tumor development in vivo or cell immortalization in culture to occur. Activation of this program can be triggered by multiple afferent signaling pathways, and its execution also involves multiple molecular pathways that have not been elucidated in detail. The p53 and Rb pathways function as a central integration point for these various signaling pathways.

p53 plays a prominent role in mediating the response to telomere dysfunction, DNA damage, and oxidative stress. This response is mediated through classic DNA damage pathways, and the ARF protein plays a central role in mediating these signals. ARF's function, however, seems more prominent in mouse cells than in human cells. p16 plays an important role in activating senescence as in a parallel manner to p53, and responds to all of the above stresses, yet to a lesser extent than p53. p16 also responds to additional physiologic stresses that have not been well characterized. The p16 response is more enhanced in human cells than in mouse cells, and provides an additional safety layer to prevent tumor development. All in all, relatively little is known about the factors directly responsible for inducing the expression of both *INK4a* products—p16 and ARF.

As a population of cells is propagated in culture it is exposed to a variety of stresses that lead to the progressive accumulation of p16 in some cells, to the activation p53 in others, and, ultimately, to the concomitant activation of both pathways in many cells (Fig. 4). Eventually, the cumulative effect of these stresses leads to the replicative senescence of the cell population as a whole. Different cell types differ in their sensitivity to different stressors, and therefore the relative contribu-

tion of the p53 and p16 pathways to the activation and maintenance of senescence differs among them.

Cellular senescence is strongly implicated as an important mechanism of tumor suppression—p53 and p16 are the most commonly mutated tumor suppressor genes, and their ability to mediate cell-division arrest is central to their activity in blocking tumor development (Rocco & Sidransky, 2001; Schmitt et al., 2002; Liu et al., 2004). Further work will be required to elucidate how cell senescence is manifested in vivo and how specific physiologic stressors activate this cellular response in living tissues.

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References

Alcorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., & Barrett, J. C. (1996). Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proceedings of the National Academic Science USA*, *93*, 13742–13747.

Baird, D. M., Rowson, J., Wynford-Thomas, D., & Kipling, D. (2003). Extensive allelic variation and ultrashort telomeres in senescent human cells. *Nature Genetics*, *33*, 203–207.

Beausejour, C. M., Krtolica, A., Galimi, F., Narita, M., Lowe, S. W., Yaswen, P., et al. (2003). Reversal of human cellular senescence: Roles of the p53 and p16 pathways. *EMBO Journal*, *22*, 4212–4222.

Benanti, J. A., & Galloway, D. A. (2004). Normal human fibroblasts are resistant to RAS-induced senescence. *Molecular and Cellular Biology*, *24*, 2842–2852.

Bennett, D. C. (2003). Human melanocyte senescence and melanoma susceptibility genes. *Oncogene*, *22*, 3063–3069.

Ben-Porath, I., & Weinberg, R. A. (2004). When cells get stressed: An integrative view of cellular senescence. *Journal of Clinical Investigation*, *113*, 8–13.

Blackburn, E. H. (2001). Switching and signaling at the telomere. *Cell*, *106*, 661–673.

Blander, G., Machado De Oliveira, R., Conboy, C. M., Haigis, M., & Guarente, L. (2003). SOD1 knock down induces senescence in human fibroblasts. *Journal of Biological Chemistry*, *18*, 18.

Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., et al. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science*, *279*, 349–352.

Bond, J. A., Wyllie, F. S., & Wynford-Thomas, D. (1994). Escape from senescence in human diploid fibroblasts induced directly by mutant p53. *Oncogene*, *9*, 1885–1889.

Brookes, S., Rowe, J., Gutierrez Del Arroyo, A., Bond, J., & Peters, G. (2004). Contribution of p16(INK4a) to replicative senescence of human fibroblasts. *Experimental Cell Research*, *298*, 549–559.

Brookes, S., Rowe, J., Ruas, M., Llanos, S., Clark, P. A., Lomax, M., et al. (2002). INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. *EMBO Journal*, *21*, 2936–2945.

Brown, J. P., Wei, W., & Sedivy, J. M. (1997). Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. *Science*, *277*, 831–834.

Campisi, J. (2000). Cancer, aging and cellular senescence. *In Vivo*, *14*, 183–188.

Chen, Q., Fischer, A., Reagan, J. D., Yan, L. J., & Ames, B. N. (1995). Oxidative DNA damage and senescence of human diploid fibroblast cells. *Proceedings of the National Academic Science USA*, *92*, 4337–4341.

Chen, Q. M., Bartholomew, J. C., Campisi, J., Acosta, M., Reagan, J. D., & Ames, B. N. (1998). Molecular analysis of H2O2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. *Biochemical Journal*, *332*, 43–50.

d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., et al. (2003). A DNA damage checkpoint response in telomere-initiated senescence. *Nature*, *426*, 194–198.

Dannenberg, J. H., van Rossum, A., Schuijff, L., & te Riele, H. (2000). Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes* & *Development*, *14*, 3051–3064.

Dirac, A. M., & Bernards, R. (2003). Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. *Journal of Biological Chemistry*, *278*, 11731–11734 (Epub 12003 Jan 11727).

Forsyth, N. R., Evans, A. P., Shay, J. W., & Wright, W. E. (2003). Developmental differences in the immortalization of lung fibroblasts by telomerase. *Aging Cell*, *2*, 235–243.

Foster, S. A., Wong, D. J., Barrett, M. T., & Galloway, D. A. (1998). Inactivation of p16 in human mammary epithelial cells by CpG island methylation. *Molecular and Cellular Biology*, *18*, 1793–1801.

Frank, K. M., Sharpless, N. E., Gao, Y., Sekiguchi, J. M., Ferguson, D. O., Zhu, C., et al. (2000). DNA ligase IV deficiency in mice leads to defective neurogenesis and embryonic lethality via the p53 pathway. *Molecular Cell*, *5*, 993–1002.

Gao, Y., Ferguson, D. O., Xie, W., Manis, J. P., Sekiguchi, J., Frank, K. M., et al. (2000). Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. *Nature*, *404*, 897–900.

Gire, V., & Wynford-Thomas, D. (1998). Reinitiation of DNA synthesis and cell division in senescent human fibroblasts by microinjection of anti-p53 antibodies. *Molecular and Cellular Biology*, *18*, 1611–1621.

Harley, C. B., Futcher, A. B., & Greider, C. W. (1990). Telomeres shorten during ageing of human fibroblasts. *Nature*, *345*, 458–460.

Harvey, M., Sands, A. T., Weiss, R. S., Hegi, M. E., Wiseman, R. W., Pantazis, P., et al. (1993). In vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene*, *8*, 2457–2467.

Hayflick, L., & Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. *Experimental Cell Research*, *25*, 585–621.

Herbert, B. S., Wright, W. E., & Shay, J. W. (2002). p16(INK4a) inactivation is not required to immortalize human mammary epithelial cells. *Oncogene*, *21*, 7897–7900.

Herbig, U., Jobling, W. A., Chen, B. P., Chen, D. J., & Sedivy, J. M. (2004). Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). *Molecular Cell*, *14*, 501–513.

Huffman, K. E., Levene, S. D., Tesmer, V. M., Shay, J. W., & Wright, W. E. (2000). Telomere shortening is proportional to the size of the G-rich telomeric 3′-overhang. *Journal of Biological Chemistry*, *275*, 19719–19722.

Itahana, K., Zou, Y., Itahana, Y., Martinez, J. L., Beausejour, C., Jacobs, J. J., et al. (2003). Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1. *Molecular and Cellular Biology*, *23*, 389–401.

Iwasa, H., Han, J., & Ishikawa, F. (2003). Mitogen-activated protein kinase p38 defines the common senescence-signalling pathway. *Genes Cells*, *8*, 131–144.

Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., et al. (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell*, *91*, 649–659.

Karlseder, J., Smogorzewska, A., & de Lange, T. (2002). Senescence induced by altered telomere state, not telomere loss. *Science*, *295*, 2446–2449.

Keys, B., Serra, V., Saretzki, G., & Von Zglinicki, T. (2004). Telomere shortening in human fibroblasts is not dependent on the size of the telomeric-3′-overhang. *Aging Cell*, *3*, 103–109.

Khan, S., Guevara, C., Fujii, G., & Parry, D. (2004). p14ARF is a component of the p53 response following ionizing irradiation of normal human fibroblasts. *Oncogene*, *14*, 14.

Khan, S. H., Moritsugu, J., & Wahl, G. M. (2000). Differential requirement for p19ARF in the p53-dependent arrest induced by DNA damage, microtubule disruption, and ribonucleotide depletion. *Proceedings of the National Academic Science USA*, *97*, 3266–3271.

Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J. K., Galloway, D. A., & Klingelhutz, A. J. (1998). Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature*, *396*, 84–88.

Krimpenfort, P., Quon, K. C., Mooi, W. J., Loonstra, A., & Berns, A. (2001). Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. *Nature*, *413*, 83–86.

Kulju, K. S., & Lehman, J. M. (1995). Increased p53 protein associated with aging in human diploid fibroblasts. *Experimental Cell Research*, *217*, 336–345.

Kyriakis, J. M., & Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiological Review*, *81*, 807–869.

Lee, A. C., Fenster, B. E., Ito, H., Takeda, K., Bae, N. S., Hirai, T., et al. (1999). Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. *Journal of Biological Chemistry*, *274*, 7936–7940.

Lin, A. W., Barradas, M., Stone, J. C., van Aelst, L., Serrano, M., & Lowe, S. W. (1998). Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes & Development*, *12*, 3008–3019.

Liu, G., Parant, J. M., Lang, G., Chau, P., Chavez-Reyes, A., El-Naggar, A. K., et al. (2004). Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. *Nature Genetics*, *36*, 63–68.

Lloyd, A. C. (2002). Limits to lifespan. *Nature Cell Biology*, *4*, E25–E27.

Lowe, S. W., & Sherr, C. J. (2003). Tumor suppression by Ink4a-Arf: Progress and puzzles. *Current Opinion in Genetics & Development*, *13*, 77–83.

Makarov, V. L., Hirose, Y., & Langmore, J. P. (1997). Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell*, *88*, 657–666.

Maldonado, J. L., Timmerman, L., Fridlyand, J., & Bastian, B. C. (2004). Mechanisms of cell-cycle arrest in Spitz nevi with constitutive activation of the MAP-kinase pathway. *American Journal of Pathology*, *164*, 1783–1787.

Martens, U. M., Chavez, E. A., Poon, S. S., Schmoor, C., & Lansdorp, P. M. (2000). Accumulation of short telomeres in human fibroblasts prior to replicative senescence. *Experimental Cell Research*, *256*, 291–299.

Martin-Ruiz, C., Saretzki, G., Petrie, J., Ladhoff, J., Jeyapalan, J., Wei, W., et al. (2004). Stochastic variation in telomere shortening rate causes heterogeneity of human fibroblast replicative life span. *Journal of Biological Chemistry*, *279*, 17826–17833 (Epub 12004 Feb 17812).

Masutomi, K., Yu, E. Y., Khurts, S., Ben-Porath, I., Currier, J. L., Metz, G. B., et al. (2003). Telomerase maintains telomere structure in normal human cells. *Cell*, *114*, 241–253.

Narita, M., Nunez, S., Heard, E., Lin, A. W., Hearn, S. A., Spector, D. L., et al. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell*, *113*, 703–716.

Ongusaha, P. P., Ouchi, T., Kim, K. T., Nytko, E., Kwak, J. C., Duda, R. B., et al. (2003). BRCA1 shifts p53-mediated cellular outcomes towards irreversible growth arrest. *Oncogene*, *22*, 3749–3758.

Packer, L., & Fuehr, K. (1977). Low oxygen concentration extends the lifespan of cultured human diploid cells. *Nature*, *267*, 423–425.

Palmero, I., McConnell, B., Parry, D., Brookes, S., Hara, E., Bates, S., et al. (1997). Accumulation of p16INK4a in mouse fibroblasts as a function of replicative senescence and not of retinoblastoma gene status. *Oncogene*, *15*, 495–503.

Pantoja, C., & Serrano, M. (1999). Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic Ras. *Oncogene*, *18*, 4974–4982.

Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S., & Campisi, J. (2003). Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nature Cell Biology*, *5*, 741–747.

Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., et al. (2000). PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. *Nature*, *406*, 207–210.

Pollock, P. M., Harper, U. L., Hansen, K. S., Yudt, L. M., Stark, M., Robbins, C. M., et al. (2003). High frequency of BRAF mutations in nevi. *Nature Genetics*, *33*, 19–20.

Randle, D. H., Zindy, F., Sherr, C. J., & Roussel, M. F. (2001). Differential effects of p19(Arf) and p16(Ink4a) loss on senescence of murine bone marrow-derived preB cells and macrophages. *Proceedings of the National Academic Science USA*, *98*, 9654–9659 (Epub 2001 Jul 9631).

Rheinwald, J. G., Hahn, W. C., Ramsey, M. R., Wu, J. Y., Guo, Z., Tsao, H., et al. (2002). A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. *Molecular and Cellular Biology*, *22*, 5157–5172.

Robles, S. J., & Adami, G. R. (1998). Agents that cause DNA double strand breaks lead to p16INK4a enrichment and the premature senescence of normal fibroblasts. *Oncogene*, *16*, 1113–1123.

Rocco, J. W., & Sidransky, D. (2001). p16(MTS-1/CDKN2/INK4a) in cancer progression. *Experimental Cell Research*, *264*, 42–55.

Sage, J., Miller, A. L., Perez-Mancera, P. A., Wysocki, J. M., & Jacks, T. (2003). Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature*, *424*, 223–228.

Sage, J., Mulligan, G. J., Attardi, L. D., Miller, A., Chen, S., Williams, B., et al. (2000). Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes & Development*, *14*, 3037–3050.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., et al. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO Journal*, *17*, 2596–2606.

Schmitt, C. A., Fridman, J. S., Yang, M., Lee, S., Baranov, E., Hoffman, R. M., et al. (2002). A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell*, *109*, 335–346.

Sedelnikova, O. A., Horikawa, I., Zimonjic, D. B., Popescu, N. C., Bonner, W. M., & Barrett, J. C. (2004). Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks. *Nature Cell Biology*, *6*, 168–170.

Serrano, M., & Blasco, M. A. (2001). Putting the stress on senescence. *Current Opinion in Cell Biology*, *13*, 748–753.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., & Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*, *88*, 593–602.

Shapiro, G. I., Edwards, C. D., Ewen, M. E., & Rollins, B. J. (1998). p16INK4A participates in a G1 arrest checkpoint in response to DNA damage. *Molecular and Cellular Biology*, *18*, 378–387.

Sharpless, N. E., Bardeesy, N., Lee, K. H., Carrasco, D., Castrillon, D. H., Aguirre, A. J., et al. (2001). Horner JW, and DePinho RA, Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature*, *413*, 86–91.

Sharpless, N. E., Ramsey, M. R., Balasubramanian, P., Castrillon, D. H., & DePinho, R. A. (2004). The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. *Oncogene*, *23*, 379–385.

Shay, J. W., Pereira-Smith, O. M., & Wright, W. E. (1991). A role for both RB and p53 in the regulation of human cellular senescence. *Experimental Cell Research*, *196*, 33–39.

Shelton, D. N., Chang, E., Whittier, P. S., Choi, D., & Funk, W. D. (1999). Microarray analysis of replicative senescence. *Current Biology*, *9*, 939–945.

Sitte, N., Merker, K., Von Zglinicki, T., Grune, T., & Davies, K. J. (2000). Protein oxidation and degradation during cellular senescence of human BJ fibroblasts. Part I. Effects of proliferative senescence. *FASEB Journal*, *14*, 2495–2502.

Smogorzewska, A., & de Lange, T. (2002). Different telomere damage signaling pathways in human and mouse cells. *EMBO Journal*, *21*, 4338–4348.

Somasundaram, K., MacLachlan, T. K., Burns, T. F., Sgagias, M., Cowan, K. H., Weber, B. L., et al. (1999). BRCA1 signals ARF-dependent stabilization and coactivation of p53. *Oncogene*, *18*, 6605–6614.

Stewart, S. A., Ben-Porath, I., Carey, V. J., O'Connor, B. F., Hahn, W. C., & Weinberg, R. A. (2003). Erosion of the telomeric single-strand overhang at replicative senescence. *Nature Genetics*, *33*, 492–496.

Takai, H., Smogorzewska, A., & de Lange, T. (2003). DNA damage foci at dysfunctional telomeres. *Current Biology*, *13*, 1549–1556.

Tchirkov, A., & Lansdorp, P. M. (2003). Role of oxidative stress in telomere shortening in cultured fibroblasts from normal individuals and patients with ataxia-telangiectasia. *Human Molecular Genetics*, *12*, 227–232.

Tuveson, D. A., Shaw, A. T., Willis, N. A., Silver, D. P., Jackson, E. L., Chang, S., et al. (2004). DePinho RA, and Jacks T. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell*, *5*, 375–387.

van Steensel, B., Smogorzewska, A., & de Lange, T. (1998). TRF2 protects human telomeres from end-to-end fusions. *Cell*, *92*, 401–413.

von Zglinicki, T., Pilger, R., & Sitte, N. (2000). Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. *Free Radical Biology and Medicine*, *28*, 64–74.

von Zglinicki, T., Saretzki, G., Docke, W., & Lotze, C. (1995). Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: A model for senescence? *Experimental Cell Research*, *220*, 186–193.

Voorhoeve, P. M., & Agami, R. (2003). The tumor-suppressive functions of the human INK4A locus. *Cancer Cell*, *4*, 311–319.

Wahl, G. M., & Carr, A. M. (2001). The evolution of diverse biological responses to DNA damage: Insights from yeast and p53. *Nature Cell Biology*, *3*, E277–E286.

Wang, W., Chen, J. X., Liao, R., Deng, Q., Zhou, J. J., Huang, S., et al. (2002). Sequential activation of the MEK-extracellular signal-regulated kinase and MKK3/6-p38 mitogen-activated protein kinase pathways mediates oncogenic ras-induced prema-

ture senescence. *Molecular and Cellular Biology*, *22*, 3389–3403.

Webley, K., Bond, J. A., Jones, C. J., Blaydes, J. P., Craig, A., Hupp, T., et al. (2000). Posttranslational modifications of p53 in replicative senescence overlapping but distinct from those induced by DNA damage. *Molecular and Cellular Biology*, *20*, 2803–2808.

Wei, W., Herbig, U., Wei, S., Dutriaux, A., & Sedivy, J. M. (2003). Loss of retinoblastoma but not p16 function allows bypass of replicative senescence in human fibroblasts. *EMBO Report*, *4*, 1061–1065.

Wong, K. K., Maser, R. S., Bachoo, R. M., Menon, J., Carrasco, D. R., Gu, Y., et al. (2003). Telomere dysfunction and ATM deficiency compromises organ homeostasis and accelerates ageing. *Nature*, *421*, 643–648.

Zhu, J., Woods, D., McMahon, M., & Bishop, J. M. (1998). Senescence of human fibroblasts induced by oncogenic Raf. *Genes & Development*, *12*, 2997–3007.

Zindy, F., Quelle, D. E., Roussel, M. F., & Sherr, C. J. (1997). Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene*, *15*, 203–211.