

REVIEW**The p16^{INK4a}-RB pathway : molecular link between cellular senescence and tumor suppression**

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Abstract : The p16^{INK4a} tumor suppressor protein functions as an inhibitor of CDK4 and CDK6, the D-type cyclin-dependent kinases that initiate the phosphorylation of the retinoblastoma tumor suppressor protein, RB. Thus, p16^{INK4a} has the capacity to arrest cells in the G1-phase of the cell cycle and its probable physiological role is in the implementation of irreversible growth arrest termed cellular senescence. Cellular senescence is a state of permanent growth arrest that can be induced by a variety of stresses such as DNA-damage and aberrant mitogenic signaling in human primary cells. In contrast to normal cells, the function of the p16^{INK4a} gene or its downstream mediators is frequently deregulated in many types of human cancers, illustrating the importance of cellular senescence in tumor suppression. Here we discuss the molecular mechanisms that direct cellular senescence and reveal its potential for tumor suppression. *J. Med. Invest.* 51 : 146-153, August, 2004

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INTRODUCTION

Cell division is a fundamental process whose error-free execution is essential for the continuity of all living organisms. In higher eukaryotes, there is an additional need for cell to stop dividing in order to differentiate and to maintain the correct balance of tissues and cell types (1). Aberrant cell growth therefore underlies many hypo- and hyper-proliferative disorders, including cancer, and a better understanding of the mechanisms involved could lead to new strategies for treatment and prevention of cancer (2).

In contrast to cancer cells, most human normal somatic cells permanently stop dividing after a finite number of cell divisions in culture and enter a state termed cellular or replicative senescence (3). These cells are irreversibly arrested in the G1 phase of the

cell cycle and no longer able to divide despite remaining viable and metabolically active for long periods of time (Figure 1). Most tumors contain cells that appear to have bypassed this limit and evaded replicative senescence. Immortality, or even an extended replicative lifespan, greatly increases susceptibility to malignant progression because it permits the extensive cell divisions needed to acquire successive mutations. Thus, cellular senescence may act as a barrier to cancer and play an important role in tumor suppression (4).

A number of hypotheses have been proposed to explain the mechanisms of cellular senescence, and they can be grouped into two broad categories (5). One set proposes that the loss of proliferative potential is due to random accumulation of damage or stress, while the other proposes that genetically programmed processes. Although numbers of reports suggest that that senescence in human cells is genetically controlled, at least, through a cell division counting mechanism (telomere-shortening) (6), accumulating evidences strongly suggest that it can also be induced by variety of extrinsic physiological stresses (7-9). Thus, the truth

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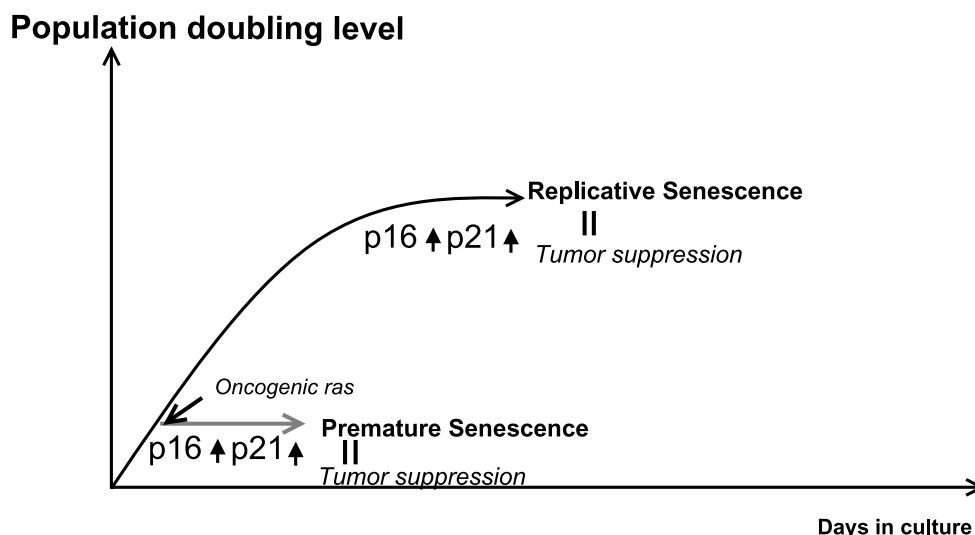


Figure 1. Tumor suppression by cellular senescence
Expression of CDK inhibitors, p16^{INK4a} and p21^{Cip1}, is induced in both replicative senescence and premature senescence.

probably lies somewhere in between. Indeed, it has become apparent that the p16^{INK4a}-RB tumor suppressor pathway is activated by a variety of distinct physiological stresses, and is playing a central role in induction and maintenance of cellular senescence.

In this review, we will discuss possible mechanisms that induce cellular senescence and elucidate how these mechanisms are perturbed in cancer cells. We also discuss the biological role of cellular senescence in living organism.

TELOMERE SHORTENING AND SENESCENCE

At the end of eukaryotic chromosomes specialized DNA structure called telomeres stabilize the chromosome ends and prevent chromosomal abnormalities (6). Telomeres are maintained by a specific enzyme called telomerase, which is not expressed in most normal human somatic cells (10). In these cells, telomere length is sequentially reduced at each cell division due to the end-replication problem. When the telomere reaches a critical length, it is thought to initiate DNA damage response signals and activate p53-dependent checkpoints that contribute to onset of cellular senescence (11). Telomere length therefore functions as a mitotic clock that counts the cell division number in human normal somatic cells. In accord with this model, ectopic expression of the catalytic subunit of telomerase enzyme, hTERT, in some human fibroblasts, halts the erosion of telomeres and prevents the onset of cellular senescence (12, 13). This is suggesting that telomere shortening is indeed a mechanism of onset of cellular

senescence. Recent studies have suggested, however, that the altered telomere state rather than loss of telomeric DNA induces cellular senescence (14). The structure of telomeres, the nucleoproteins complex that serves as a protective “cap” for the chromosome end, may undergo changes during cellular proliferation, possibly as a result of telomere-shortening. More recently, unexpected role of endogenous telomerase has been uncovered in normal human somatic cells (15). It has long been believed that normal human somatic cells do not express detectable level of hTERT gene and therefore do not express substantial level of telomerase activity. However, Matsutomi *et al* (2003) has shown that hTERT is actually expressed in normal human fibroblasts and plays important role in maintenance of proper structure of the chromosome end. Indeed, RNA interference (RNAi)-mediated inhibition of endogenous hTERT rapidly induced senescence-like cell cycle arrest in human primary fibroblasts (15). These evidences are also indicating that the “capping” state of telomeres, but not the overall length of telomeres, correlates with the induction of cellular senescence.

While telomere shortening occurs in normal human cells, telomere length is maintained either by overexpression of telomerase or by a mechanism known as alternative lengthening of telomeres (ALT) in most human cancer cells (16). Whatever mechanism, it is clear that telomere maintenance is essential for immortality and its control might be a possible therapeutic target in human cancer.

TELOMERE-INDEPENDENT MECHANISMS FOR SENESCENCE

In contrast to human cells, there is no strong evidence in rodent cells that cellular senescence is dependent on telomere erosion (8). Telomere in rodent cells are very long, and most rodent somatic cells have significant level of telomerase expression. Moreover, cellular senescence in rodent cells occurs without detectable telomere-shortening (8). How is cellular senescence induced without telomere shortening in rodent cells? This telomere-independent proliferation block may reflect a cell cycle checkpoint response to inappropriate cell culture condition rather than an intrinsic limitation imposed by a cell-division counting mechanism. It is likely therefore that there is a telomere-independent cellular senescence mechanism in higher eukaryotic cells. Indeed, accumulating evidences have demonstrated that various physiological stresses, such as oxidative stress and aberrant mitogenic stimulation, are involved in cellular senescence. For example, it was recently reported that when mouse embryonic fibroblasts (MEFs) are cultured in low (3%) oxygen, rather than normal (20%) oxygen condition, MEFs avoid cellular senescence and continue to proliferate, like a immortalized cells (17). This study also reported that the rapid accumulation of DNA damage was observed in MEFs, when grown in 20% oxygen, but not

in 3% oxygen condition. This is consistent with previous observation that human primary fibroblasts are able to undergo more cell divisions prior to cellular senescence when cells were cultured in low (1-3%) oxygen condition (18, 19, 29). These evidences, therefore, suggest that extrinsic physiological stress, such as oxidative stress, is a common factor which induces cellular senescence in both human and rodent cells. Taken together, it is clear that telomere shortening is not a sole mechanism that cause cellular senescence (21, 22).

p16^{INK4a}-RB PATHWAY IN HUMAN CANCER

In higher eukaryotes, the retinoblastoma (RB) and p53 tumors suppressor proteins are crucial gatekeeper of cellular senescence (23, 24). The activities of RB and p53 are tightly regulated by various post-translational modifications, such as phosphorylation, acetylation and ubiquitination (25, 26). RB is thought to impose a block on G1 progression that is alleviated by phosphorylation. In particular, the cyclin-dependent kinases, CDK 2, CDK 4 and CDK 6 play a critical role in phosphorylation of RB. When RB is phosphorylated by CDKs it loses its function and releases its target, the E2F family transcription factors (E2F1-3), resulting in the initiation of DNA replication (Figure 2) (27, 28). Deregulated activity of the D-type cyclin-dependent

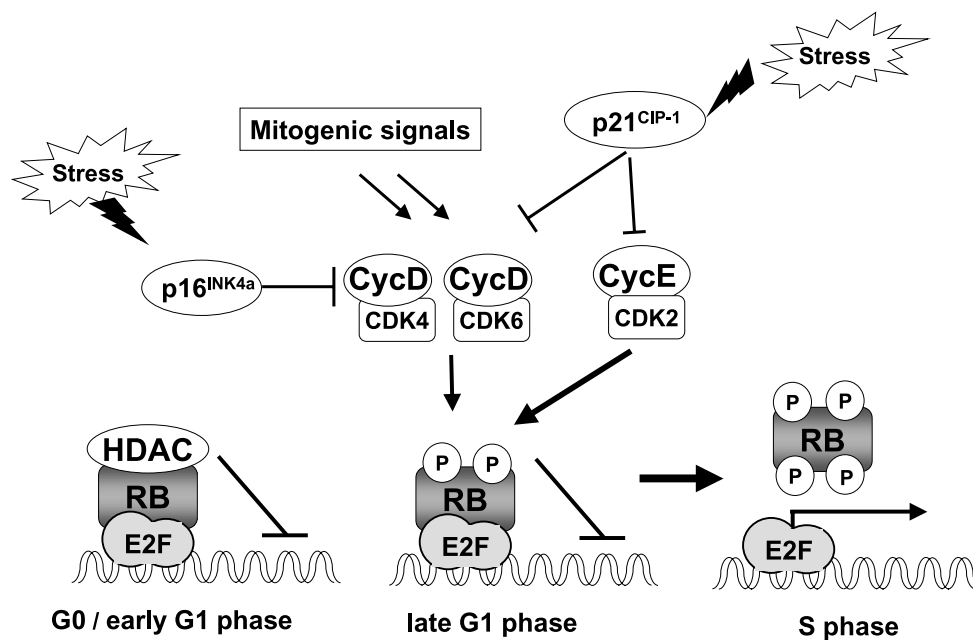


Figure 2. p16^{INK4a}/RB-pathway in cell cycle control
Following mitogenic stimulation, cyclinD and cyclinE in complex with CDK4/6 or CDK2 respectively, sequentially phosphorylate RB thus releasing it from its transcriptionally repressive complexes with E2Fs facilitating S phase progression. Expression of p16^{INK4a} and/or p21^{Cip1} is induced by distinct stresses, thereby inhibiting CDK activity.

kinases, CDK 4 and CDK 6, is widely observed in various tumor cells, illustrating their importance in controlling cell cycle (29). The cyclin D1 gene is amplified and over-expressed in a variety of human cancers, particularly breast cancer and squamous cell carcinomas, as well as being activated by chromosomal translocation in mantle cell lymphoma. Similarly, CDK4 is amplified in human sarcomas and more recently, a mutant (R24C) which does not bind to its negative regulators, the INK4 family proteins, is found in melanoma (29). More frequently, the p16^{INK4a} gene, a prototype of the INK 4 gene family, is inactivated by various mechanism in a wide range of human cancers (30). All these abnormalities cause deregulation of RB function. The p16^{INK4a}/CDK/RB pathway is therefore considered to be an attractive target for therapeutic intervention but more information is needed regarding the detailed mechanisms involved in order to identify the most appropriate strategies for tumor suppression.

We and others have shown that the p16^{INK4a} tumor suppressor gene plays a key role in cellular senescence in human cells. Although the level of p16^{INK4a} is extremely low in normal proliferating cells, the expression of p16^{INK4a} is significantly induced during cellular senescence (31, 32). Moreover, overexpression of p16^{INK4a} results in senescence-like growth arrest, identifying p16^{INK4a} as a strong candidate for mediating cellular senescence (33, 34) So, how is p16^{INK4a} expression induced in senescence? Although we cannot completely preclude the connection between p16^{INK4a} expression and telomere-shortening, its expression is more likely to be regulated by extrinsic stress signaling pathways. This is because accumulation of p16^{INK4a} is observed in both human and rodent senescent cells. Furthermore, Serrano *et al* (1997) have shown that aberrant growth signals provided by constitutive active form (oncogenic) Ras rapidly induce p16^{INK4a} expression without telomere shortening in both human and rodent cells (35)(Figure 1). In remarkable contrast to previous observations using established cell lines (36, 37), introduction of oncogenic Ras into normal primary cells results in the induction of various antiproliferative proteins, including the p16^{INK4a} and p53 tumor suppressors; the accompanying cell cycle arrest resembles cellular senescence and is termed "premature-senescence" (35)(Figure 1). Normal cells must therefore have a sensor that detects aberrant growth signals, such as too many cell divisions and/or oncogene activation, and that may induce p16^{INK4a} expression.

In rodent cells, targeted inactivation of RB or p53 gene overrides cellular senescence (38, 39). Moreover, triple knock-out MEFs lacking all Rb-family genes (Rb,

p107 and p130) do not senesce despite the presence of high levels of p53, suggesting that RB-family proteins play a role downstream of the p53 pathway in mouse cell senescence (40, 41). In human cells, however, inactivation of both RB and p53 is required to override cellular senescence in most cell types, suggesting that p53 and RB have overlapping, but distinct, roles in human cell senescence (23, 24).

TRANSCRIPTIONAL REGULATION OF p16^{INK4a} EXPRESSION

Previous experiments with Ras mutants, which are unable to activate one or another effector pathway of Ras signaling, have identified that the Ras/MEK signal transduction pathway is crucial for Ras-induced cellular senescence (42, 43). We have shown that p16^{INK4a} expression is controlled by the Ets 1 and Ets 2 (Ets1/2) transcription factor, which are down-stream mediators of the Ras/Raf/MEK-pathway (44). Normally, the Ets 1/2 transcription factor is phosphorylated by MAP kinase at certain points in the cell cycle. Id1, a transcriptional inhibitor, counter-balances this activity by binding to and inactivating Ets 1/2. In replicative senescence, which is provoked by cumulative cell divisions, expression levels of Ets 1 increase whilst Id 1 levels decline. Thus Ets1 likely to induce p16^{INK4a} expression aided by the concomitant down-regulation of Id1. In premature senescence, which is provoked by oncogenic Ras, Ets1/2 is constitutively activated by oncogenic Ras signals. This aberrant activation overrides the steady state and results in induction of p16^{INK4a} expression and premature senescence. This model is further supported by recent study using dermal fibroblasts, Q34 cells, from an individual carrying biallelic mutation in p16^{INK4a} gene (45). Both mutations alter the aminoacids sequence of p16^{INK4a} and functionally impaired the activity of p16^{INK4a} protein. In contrast, only one of the mutations affects the sequence of p14^{ARF} protein, causing an apparently innocuous changes near its carboxy terminus (Figure 3). Ectopic expression of oncogenic Ras or its downstream effectors Ets1 and Ets 2 failed to induce premature senescence in Q34 fibroblasts, although the similar levels of oncogenic ras or Est1/2s expression efficiently induced premature senescence in normal (wild type) human fibroblasts (45). This is suggesting that p16^{INK4a} is a critical downstream mediator for Ras/Ets-pathway in premature senescence. This data also imply that p 16^{INK4a}, but not p14^{ARF}, assumes the principal role in Ras-induced premature senescence in human fibroblasts. It is,

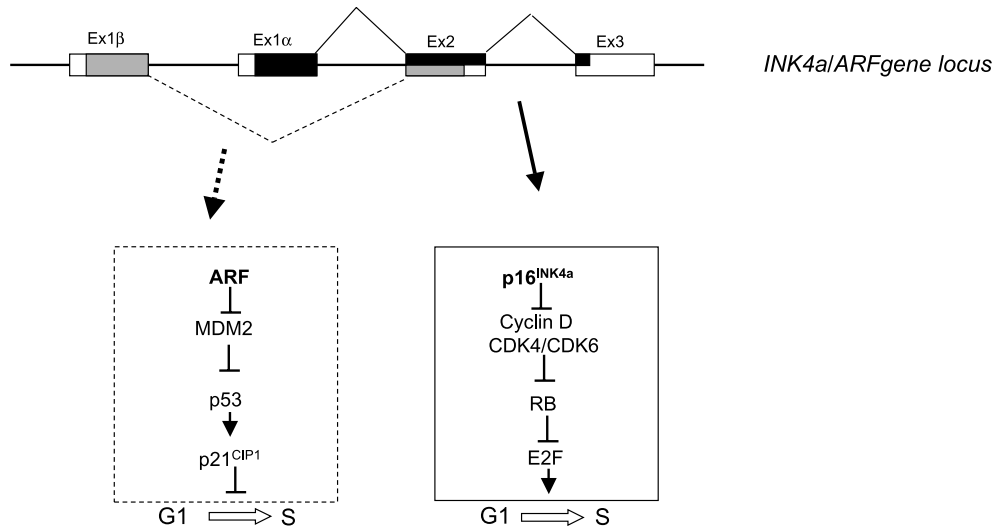


Figure 3. INK 4 a/ARF tumor suppressor gene locus regulates RB/p53 pathways
 INK4a/ARF locus encodes two proteins regulating RB or p53 tumor suppressor pathways. Although p14^{ARF} does not act as a CDK inhibitor, p14^{ARF} induces p21^{Cip1}, a CDK inhibitor, through activating p53-pathway.

however, important to note that other factors are involved in this process. In particular, chromatin remodeling factors such as SNF5 and BMI-1 are likely to be associated with Ras/Ets/p16^{INK4a}-pathway (46-49).

p16^{INK4a} VERSUS p14^{ARF} IN HUMAN CANCER

The INK4a/ARF gene locus has the unusual capacity to encode two structurally distinct proteins, p16^{INK4a} and p14^{ARF} (p19^{ARF} in mouse cells), by reading a shared second exon in different translational reading frames (Figure 3). Although p16^{INK4a} binds directly to and inhibits the activity of CDK4 and CDK6, p14^{ARF} binds directly to MDM2, but not CDKs, resulting in the stabilization and the activation of p53-target genes (30). In mouse model system, p19^{ARF} plays an important protective role from tumorigenicity whereas p16^{INK4a} loss causes only a limited induction of tumors (50). In MEFs, the levels of p19^{ARF} is significantly induced in both oncogenic Ras induced senescence and in replicative senescence (35). However, this is not the case in human primary fibroblasts, suggesting the differential importance of INK 4 a/ARF gene locus in human and mouse (51, 52). To directly assess the individual contribution of p16^{INK4a} and p14^{ARF} genes to tumor suppressor pathways in human, Voorthoeve and Agami (2003) have specifically blocked p16^{INK4a} and / or p14^{ARF} gene expression using RNAi mediated-gene silencing technology (53). Voorthoeve and Agami has shown that suppression of p16^{INK4a} expression, but not p14^{ARF} expression, synergize with p53 loss to accelerate growth

and causes transformation. These results are consistent with the notion that p16^{INK4a} is acting as a tumor suppressor gene in human cancer.

INACTIVATION OF p16^{INK4a}-RB PATHWAY IN HUMAN CANCER

It is evident that the expression of p16^{INK4a} gene is dramatically reduced in several human cancers due to the hyper-methylation of p16^{INK4a} gene promoter (54). Recent studies, however, indicated that a different mechanism blocking the p16^{INK4a} gene expression is existing in certain types of human cancers (55). The latent membrane protein 1 (LMP 1) oncoprotein of Epstein-Barr virus (EBV) is often present in EBV-associated proliferative diseases and is critical for the immortalizing and transforming activity of EBV. Unlike other DNA tumor virus oncoproteins which possess immortalizing activity, LMP1 does not bind to RB, but instead blocks the expression of the p16^{INK4a} gene (55). However, it has been unclear how LMP1 represses the p16^{INK4a} gene expression. We have now found that LMP 1 promotes the CRM1-dependent nuclear export of Ets2, thereby reducing the level of p16^{INK4a} gene expression (56). We further demonstrate that LMP1 also blocks the function of repressive E2Fs (E2F4 and E2F5), which primarily repress the E2F-target gene expression, through promoting their nuclear export in a CRM1-dependent manner (56). As E2F4 and E2F5 are essential downstream mediators for a p16^{INK4a}-induced cell cycle arrest (57), these results indicate that the action of LMP 1 on nuclear export

has two effects on the p16^{INK4a}-RB pathway : (i) repression of p16^{INK4a} expression and (ii) blocking the downstream mediator of the p16^{INK4a}-RB pathway. These results reveal a novel activity of LMP1 and increase an understanding of how viral oncoproteins perturb the p16^{INK4a}-RB pathway leading to cancer. Understanding the mechanisms of LMP1-induced nuclear export would help us to find a way of specific suppression of EBV associated cancers.

CONCLUDING REMARKS

It has recently become evident that cellular senescence, the final resting place of the cell, is a state induced by a variety of distinct physiological stresses including oncogene activation. These stresses activate cell cycle checkpoint response such as induction of p16^{INK4a} and cause permanent growth arrest. Although we still do not have definitive evidence that cellular senescence occurs *in vivo*, physiological stresses including oncogene activation, rather than cumulative cell divisions, could turn out to be a physiologically relevant senescence trigger. The identification of senescent cells, especially in the context of the human body, will provide valuable new insights into the development of cancer and open up new possibilities of its control.

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REFERENCES

1. Malumbres M, Barbacid M : To cycle or not to cycle : a critical decision in cancer. *Nat Rev Cancer* 1 : 222-231, 2001
2. Sherr CJ : Cancer cell cycle. *Science* 274 : 1672-1677, 1996
3. Hayflick L, Moorhead PS : The limited *in vitro* lifetime of human diploid cell strains. *Exp Cell Res* 25 : 585-621, 1961
4. Campisi J : Cellular senescence as a tumor-suppressor mechanism. *Trends Cell Biol* 11 : S27-S31, 2001
5. Campisi J, Dimri G, Hara E, : Control of replicative senescence. *Handbook of The Biology of Aging*, IV eds. Academic Press, New York, 1996, pp121-149
6. Wright WE, Shay JW : Telomere dynamics in cancer progression and prevention : fundamental differences in human and mouse telomere biology. *Nat Med* 6 : 849-851, 2000
7. Lundberg AS, Hahn WC, Gupta P, Weinberg RA : Genes involved in senescence and immortalization. *Curr Opin Cell Biol* 12 : 705-709, 2000
8. Serrano M, Blasco MA : Putting the stress on senescence. *Curr Opin Cell Biol* 13 : 748-753, 2001
9. Mathon NF, Lloyd AC : Cell senescence and cancer. *Nat Rev Cancer* 1 : 203-213, 2001
10. Nakayama J, Tahara H, Tahara E, Saito M, Ito K, Nakamura, H, Nakanishi T, Tahara E, Ide T, Ishikawa F : Telomerase activation by hTERT in human normal fibroblasts and hepatocellular carcinomas. *Nat Genet* 18 : 65-68, 1998
11. Smogorzewska A, de Lange T : Different telomere damage signaling pathways in human and mouse cells. *EMBO J* 21 : 4338-4348, 2002
12. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE : Extension of life-span by introduction of telomerase into normal human cells. *Science* 279 : 349-352, 1998
13. Vaziri H, Benchimol S : Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol* 8 : 279-282, 1998
14. Karlseder J, Smogorzewska A, de Lange T : Senescence induced by altered telomere state, not telomere loss. *Science* 295 : 2446-2449, 2002
15. Masutomi K, Yu EY, Khurts S, Ben-Porath I, Currier JL, Metz GB, Brooks MW, Kaneko S, Murakami S, DeCaprio JA, Weinberg RA, Stewart SA, Hahn WC. : Telomerase maintains telomere structure in normal human cells. *Cell* 114 : 241-253, 2003
16. Neumann AA, Reddel RR : Telomere maintenance and cancer-look, no telomerase. *Nat Review Cancer* 2 : 879-884, 2002
17. Parrinello S, samper E, Krtolica A, Goldstein J, Melov S, Campisi J : Oxygen sensitivity limits the replicative lifespan of murine fibroblasts. *Nat Cell Biol* 5 : 741-747, 2003
18. Packer L, Fuehr K : Low oxygen concentration extends the lifespan of cultured human diploid cells. *Nature* 267 : 423-425, 1977
19. Chen Q, Fischer A, Reagan JD, Yan LJ, Ames BN : Oxidative DNA damage and senescence of human diploid fibroblast cells. *Proc Natl Acad*

- Sci USA 92 : 4337-4341, 1995
20. von Zglinicki T, Saretzki G, Docke W, Lotze C : Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts : a model for senescence? *Exp Cell Res* 220 : 186-193, 1995
 21. Kiyono T., Foster S.A., Koop JI., McDougall JK, Galloway DA, and Klingelutz AD : Both Rb/p16^{INK4a} inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* 396 : 84-88, 1988
 22. Noble JR, Zhong Z-H, Neumann AA, Melki JR, Clark SJ, Reddel RR : Alterations in the p16^{INK4a} and p53 tumor suppressor genes of hTERT-immortalized human fibroblasts. *Oncogene* 23 : 3116-3121, 2004
 23. Hara E, Tsurui H, Shinozaki A, Oda K : Cooperative effect of antisense-Rb and antisense-p53 oligomers on the extension of lifespan in human diploid fibroblasts, TIG-1. *Biochem Biophys Res Commun* 179 : 528-534, 1991
 24. Shay JW, Pereira-Smith OM, Wright WE : A role for both Rb and p53 in the regulation of human cellular senescence. *Exp Cell Res* 196 : 33-39, 1991
 25. Classon M, Harlow E : The retinoblastoma tumour suppressor in development and cancer. *Nat Rev Cancer* 2 : 10-17, 2002
 26. Sharpless, NE, DePinho RA : p53 : good cop/bad cop. *Cell* 110 : 9-12, 2002
 27. Trimarchi JM, Lees JA : Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* 3 : 11-20, 2002
 28. Stevaux O, Dyson NJ : A revised picture of the E2F transcriptional network and RB function. *Curr Opin Cell Biol* 14 : 684-691, 2002
 29. Ortega S, Malumbres M, Barbacid M : Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim Biophys Acta* 1602 : 73-87, 2002
 30. Ruas M, Peters G : The p16^{INK4a}/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1378 : F115-F177, 1998
 31. Hara E, Smith R, Parry D, Tahara H, Stone S, Peters G : Regulation of p16^{CDKN2} expression and its implications for cell immortalization and senescence. *Mol Cell Biol* 16 : 859-867, 1996
 32. Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC : Involvement of the cyclin-dependent kinase inhibitor p 16(INK 4 a) in replicative senescence. *Proc Natl Acad Sci USA* 93 : 13742-13747, 1996
 33. McConnell BB, Starborg M, Brookes S, Peters G, Inhibition of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. *Curr Biol* 8 : 351-354, 1998
 34. Kato D, Miyazawa K, Ruas M, Starborg M, Wada I, Oka T, Sakai T, Peters G, Hara E : Features of replicative senescence induced by direct addition of antenapedia-p16 INK4A fusion protein to human diploid fibroblasts. *FEBS Lett* 427 : 203-208, 1998
 35. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW : Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and p16^{INK4a}. *Cell* 88 : 593-602, 1997
 36. Land H, Parada LF, Weinberg RA : Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* 304 : 596-602, 1983
 37. Ruley HE : Adenovirus early region1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature* 304 : 602-606, 1983
 38. Sage J, Miller AL, Perez-Mancera PA, Wysocki JM, Jacks T : Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature* 424 : 223-228, 2003
 39. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A : Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356 : 215-221, 1992
 40. Sage J, Mulligan GJ, Attardi LD, Miller A, Chen S, Williams B, Theodorou E, Jacks T : Targeted disruption of the three Rb-related genes leads to loss of G (1) control and immortalization. *Genes Dev* 14 : 3037-3050, 2000
 41. Dannenberg JH, van Rossum A, Schuijff L, te Riele H : Ablation of the retinoblastoma gene family deregulates G(1)control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev* 14 : 3051-3064, 2000
 42. Lin AW, Barradas M, Stone JC, van Aelst L, Serrano M, Lowe SW : Premature senescence involving p 53 and p16 is activated in response to constitutive MEK / MAPK mitogenic signaling. *Genes Dev* 12 : 3008-3019, 1998
 43. Zhu J, Woods D, McMahon M, Bishop JM : Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev* 12 : 2997-3007, 1998
 44. Ohtani N, Zebedee Z, Huot TJG, Stinson JA, Sugimoto M, Ohashi Y, Sharrocks AD, Peters G, Hara E : Opposing effects of Ets and Id proteins on p16^{INK4a} expression during cellular senescence. *Nature* 409 : 1067-1070, 2001
 45. Huot TJ, Rowe J, Harland H, Drayton S, Brookes S,

- Goptu C, Purkis P, Fried M, Bataille V, Hara E, Newton-Bishop J, Peters G : Biallelic mutations in p16^{INK4} confer resistance to Ras and Ets induced senescence in human diploid fibroblasts. *Mol Cell Biol* 22 : 8135-8143, 2002
46. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M : The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature* 397 : 164-168, 1999
47. Betz BL, Strobeck MW, Reisman DN, Knudsen ES, and Weissman BE : Re-expression of hSNF5/INI1/BAF47 in pediatric tumor cells leads to G1 arrest associated with induction of p16^{ink4a} and activation of RB. *Oncogene* 21 : 5193-5203, 2002
48. Versteeg I, Medjkane S, Rouillard D, Delattre O : A key role of the hSNF5/INI1 tumour suppressor in the control of the G1-S transition of the cell cycle. *Oncogene* 21 : 6403-6412, 2002
49. Oruetxebarria I, Venturini F, Kekarainen T, Houweling A, Zuijderduijn LM, Mohd-Sarip A, Vries RG, Hoeben RC, Verrijzer CP : P16^{INK4a} is required for hSNF5 chromatin remodeler-induced cellular senescence in malignant rhabdoid tumor cells. *J Biol Chem* 279 : 3807-3816, 2004
50. Drayton S, Peters G : Immortalisation and transformation revisited. *Curr Opin Genet Dev* 12 : 98-104, 2002
51. Wei W, Hemmer RM, Sedivy JM : Role of p14 (ARF) in replicative and induced senescence of human fibroblasts. *Mol Cell Biol* 21 : 6748-6757, 2001
52. Brooks 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. INK 4 a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. *EMBO J* 21 : 2936-2945, 2002
53. Voorhoeve PM, Agami R : The tumor-suppressive functions of the human INK4A locus. *Cancer Cell* 4 : 311-319, 2003
54. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, Sidransky D : 5'CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN 2/MTS 1 in human cancers. *Nat Med* 1 : 686-692, 1995
55. Yang X, He Z, Xin B, Cao L : LMP1 of Epstein-Barr virus suppresses cellular senescence associated with the inhibition of p16^{INK4a} expression. *Oncogene* 19 : 2002-2013, 2000
56. Ohtani N, Brennan P, Gaubatz S, Sanij E, Hertzong P, Wolvetang E, Ghysdael J, Rowe M, Hara E : Epstein-Barr virus LMP 1 blocks p 16^{INK4a} / RB-pathway through targeting E2F4/5 for nuclear export. *J Cell Biol* 162 : 173-183, 2003
57. Gaubatz S, Lindeman G J, Ishida S., Jakoi L, Nevins J R, Livingston DM, Rempel RE E2F4 and E2F5 play an essential role in pocket protein mediated G1 control. *Mol Cell* 6 : 729-735, 2000