Loss of retinoblastoma but not p16 function allows bypass of replicative senescence in human fibroblasts

Wenyi Wei^{†*}, Utz Herbig^{*}, Shan Wei^{†*}, Annie Dutriaux[§] & John M. Sedivy^{†*}

Department of Molecular Biology, Cell Biology & Biochemistry, Brown University, Providence, Rhode Island, USA

Current models envision replicative senescence to be under dual control by the p53 and retinoblastoma (RB) tumour-suppressor pathways. The role of the p16^{INK4a}-RB pathway is controversial, and the function of RB in human cells has not been tested directly. We used targeted homologous recombination to knock out one copy of *RB* in presenescent human fibroblasts. During entry into senescence, *RB*^{+/-} cells underwent spontaneous loss of heterozygosity and the resultant *RB*^{-/-} clones bypassed senescence. The extended lifespan phase was eventually terminated by a crisis-like state. The same phenotype was documented for *p21*^{CIP1/WAF1} and *p53* heterozygous cells, indicating that loss of function of all three genes results in failure to establish senescence. By contrast, the abolition of p16 function by the expression of a p16-insensitive cyclin-dependent kinase 4 protein or siRNA-mediated knockdown provided only minimal lifespan extension that was terminated by senescence. We propose that p53, p21 and RB act in a linear genetic pathway to regulate cell entry into replicative senescence.

EMBO reports 4, 1061–1066 (2003)

doi:10.1038/sj.embor.7400001

INTRODUCTION

Telomere shortening is the molecular mechanism that counts elapsed divisions on an individual cell basis. In addition to replicative exhaustion, growth arrest states resembling senescence can be elicited by a variety of stresses and signalling imbalances (Drayton & Peters, 2002). With few exceptions (von Zglinicki, 2002), these premature senescence states are

independent of telomere attrition, and in some cases they might assume a predominant role (Sherr & DePinho, 2000). In culture conditions that minimize extrinsic stresses, telomere attrition appears to be the main driving force behind replicative senescence of human cells (Wright & Shay, 2002). Normal human diploid fibroblasts (HDFs) senesce predominantly in response to telomere attrition even under standard culture conditions (Bodnar *et al.*, 1998; Itahana *et al.*, 2003).

Cell entry into senescence is correlated with the upregulation of the cyclin-dependent kinase (CDK) inhibitors p21^{CIP1/WAF1} and p16^{INK4a} (hereafter p21 and p16, respectively). Early studies implicated RB, the retinoblastoma tumour-suppressor protein, and p53 in regulating this response (Shay *et al.*, 1991) by showing that expression of viral oncogenes (such as simian virus 40 large T antigen or human papilloma virus E6 and E7 proteins) resulted in an extended lifespan that was terminated by a period of active cell death designated as crisis. The interpretation of these studies is, however, uncertain because viral oncogenes might achieve only partial ablation and are also known to have multiple targets (Funk *et al.*, 1997). Gene targeting of *p21* resulted in escape from senescence (Brown *et al.*, 1997; Wei & Sedivy, 1999), whereas a bypass of p16 (by expression of a p16-insensitive CDK4) did not (Brookes *et al.*, 2002; Morris *et al.*, 2002). To analyse further the senescence regulatory circuit and to avoid the ambiguities associated with dominant-interfering proteins (viral or cellular), we chose to construct null alleles of *p53* and *RB* by gene targeting, and to downregulate *p16* by expressing short interfering RNA (siRNA).

RESULTS AND DISCUSSION

p21^{+/-} (heterozygous) cultures enter senescence normally, but on further incubation colonies of proliferating cells appear and grow out on top of the senescent cell monolayers (Brown *et al.*, 1997). Examination by Southern hybridization showed loss of heterozygosity (LOH) at the *p21* locus with preferential retention of the targeted (*p21* null) allele, and immunoblotting confirmed loss of p21 protein. On subculture the *p21*^{-/-} cells eventually underwent a proliferative decline with the characteristics of crisis, as evidenced by a high 5-bromodeoxyuridine (BrdU) labelling index (Wei & Sedivy, 1999). The *p21*^{-/-} cells retain a functional RB pathway (Wei *et al.*, 2001, 2003).

Department of Molecular Biology, Cell Biology & Biochemistry, Brown University, Providence, Rhode Island 02912, USA

[†]Present address: Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115, USA

[§]Present address: Siebel Systems, 2207 Bridgepointe Parkway, San Mateo, California 94404, USA

[§]Present address: Institut Jacques Monod, 2 Place Jussieu, 75251 Paris, France

*These authors contributed equally to this work

†Corresponding author. Tel: +1 401 863 7631; Fax: +1 401 863 9653;

E-mail: john_sedivy@brown.edu

Received 20 June 2003; revised 14 August 2003; accepted 8 September 2003

Published online 17 October 2003

To examine the generality of the LOH phenomenon we performed an analogous experiment with $p53^{+/-}$ HDFs that were also generated by gene targeting (Bunz *et al.*, 1998). A $p53^{+/-}$ culture was single-cell cloned after gene targeting, and individual colonies were expanded, verified for $p53$ status, and passaged until proliferation slowed down. In all cases proliferation resumed again, and examination revealed distinct colonies that were never observed in control dishes with $p53^{+/+}$ cells (Fig. 1A). Sampling at successive passages revealed a progressive loss of

the wild-type $p53$ allele (Fig. 1B), as well as $p53$ protein (Fig. 1C). Both RB and p16 proteins continued to be expressed normally (Fig. 1C), and the RB pathway appears to be functionally intact (Wei *et al.*, 2001, 2003). Cultures were propagated until no net increase in cell number occurred for a minimum of three weeks, and BrdU labelling was performed. The high labelling index (30–35%) indicated that the cultures were in a crisis-like state. HDFs from patients with Li–Fraumeni syndrome ($p53^{+/-}$) have also been documented to spontaneously give rise to clones endowed with extended lifespan (Rogan *et al.*, 1995). These results indicate that entry into an extended lifespan is under strong selection in senescent cultures, and that LOH occurs at a high enough frequency in HDFs to make such events readily observable during routine cell culture.

To address directly the role of RB in the regulation of senescence we knocked out one gene copy (Fig. 2A,B) in the embryo lung HDF strain LF1 that was used for the knockouts of $p21$ and $p53$. As an additional control we used an independently derived embryo skin HDF strain designated SF1. In both cases we obtained targeted clones with good efficiency and verified them using PCR and Southern hybridization (Fig. 2C). Cells heterozygous for all three genes ($p21$, $p53$ and RB) grow indistinguishably from parental cells of roughly comparable age. So, at least by this criterion, all three loss-of-function alleles appear to be fully recessive.

Both strains of $RB^{+/-}$ cells were passaged into senescence, as evidenced by a downturn in proliferation, positive staining for senescence-associated β -galactosidase (SA- β -gal; Fig. 3A) and a low BrdU labelling index (Fig. 4A). After continued incubation, however, colonies of apparently healthy and proliferating cells were observed, very similar to what was previously seen with $p53^{+/-}$ and $p21^{+/-}$ cultures (Fig. 3A). *In situ* immunocytochemical detection of RB protein was negative in several independent clones that displayed lifespan extension (Fig. 3B), and loss of RB expression was confirmed by immunoblotting (Fig. 3C). Notably, LOH at the RB locus is frequently observed *in vivo* during tumour development (Cairns *et al.*, 1991). The frequency of LOH, as estimated by the fraction of cultures that show LOH, and the frequency (number) of LOH colonies was in the order of $p53 > p21 > RB$ (Brown *et al.*, 1997; Bunz *et al.*, 1998). Two out of three RB cultures examined underwent LOH, with colonies in the range of 5–20 per 10^6 cells.

Clones that had entered into an extended lifespan phase eventually ceased proliferating. To address whether this downturn was caused by entry into senescence or into a crisis-like state we performed BrdU labelling. In all cases $RB^{+/-}$ clones displayed high BrdU labelling indexes (approximately 30%, Fig. 4A,B), which are in the same range as observed in analogous clones arising from $p21^{+/-}$ and $p53^{+/-}$ cultures. Terminally non-proliferative cultures contained cells with condensed and/or blebbing nuclei as evidenced by Hoechst dye staining, but we had insufficient material to perform more rigorous analyses of apoptosis. Cultures could not be kept for extended periods of time, and deteriorated slowly over a period of 4–6 weeks. Although these phenotypes are consistent with a crisis-like state, it is also possible that the cultures were arrested in one of the 'intermediate M' (M_{int}) states (Bond *et al.*, 1999; Morris *et al.*, 2002). The high BrdU labelling index is, however, clearly indicative of an escape from typical replicative senescence and entry into an extended lifespan phase.

Fig. 1 | Loss of heterozygosity in $p53^{+/-}$ human fibroblasts. (A) Stained 10-cm culture dishes. Cultures were propagated until cell numbers ceased to increase. At this time most of the cells had a flattened morphology and stained positive for senescence-associated β -galactosidase. Dishes were then further incubated with regular media changes for 3–5 weeks. (B) Southern hybridization analysis. Dishes with colonies (Ab) were trypsinized and subjected to a regular 1:4 passaging regimen (the first trypsinization was designated as passage 1). Cell-line designations and passage numbers are indicated above the lanes. Presenescent $p53^{+/+}$ and $p53^{+/-}$ cells were used as controls. Southern hybridization was performed with a unique chromosomal probe to the right (3') of the targeting vector (Fig. 2B). Untargeted wild-type (WT) and Neo vector-targeted (Neo) bands are indicated by arrows. (C) Immunoblot analysis. Left panel, extracts from cells treated as in (B) were harvested at successive passages and immunoblotted with an antibody to $p53$ protein. Right panel, expression of p16, p21, and RB before and after $p53$ loss of heterozygosity. Control, actin.

Fig. 2 | Gene targeting of the human retinoblastoma gene. **(A)** Overall experimental outline. Steps subsequent to gene targeting that resulted in the selection of clones that bypassed senescence are also indicated. **(B)** Design of targeting vectors. The genomic loci are shown underneath the vectors and are drawn approximately to scale; black boxes (numbered) represent exons. The grey bars underneath the genomic loci depict the location of Southern hybridization probes. **(C)** Knockout of one copy of the human *RB* gene. Southern hybridization was performed with a unique chromosomal probe to the left (5') of the targeting vector. Further verification of correct chromosomal configuration was performed by PCR of genomic DNA. The summary presents the number of colonies screened and the number of hits recovered. Note that two fibroblast cell strains (SF1 and LF1) were targeted in two independent experiments. SH18 is a derivative of SF1, and LH13 is a derivative of LF1. *RB*, retinoblastoma.

Fig. 3 | Loss of heterozygosity in *RB*^{+/-} human fibroblasts. **(A)** Photomicrographs taken at the time when colonies began to appear. **(a)** Senescent monolayer of *RB*^{+/-} cells; **(b)** a colony with proliferating cells. Both images were taken from the same plate that had been stained for senescence-associated β -galactosidase activity. **(B)** *In situ* immunohistochemical detection of *RB* protein. Colonies of proliferating cells were removed from the primary plates with cloning rings, placed in 24 microtitre wells and after several days of culture expanded into 6-cm dishes. Cells were processed for *in situ* immunodetection of *RB* protein after the cultures became established. **(a)** Presenescent, proliferating LH13 (*RB*^{+/-}) cells are shown as a positive control. **(b)** LH13-LOHc2 is a derivative of LH13, and **(c)** SH18-LOHc2 is a derivative of SH18. Arrowheads point to representative nuclei. **(C)** Immunoblot analysis. Upper panel, colonies of proliferative cells were picked and expanded as in **(B)** and immunoblotted with an antibody to *RB* protein. Cell-line designations are indicated above the lanes. LH13-LOHc1 is a derivative of LH13, and SH18-LOHc1 is a derivative of SH18. Lower panel, expression of p53, p107 and p16 before and after *RB* LOH. Control, actin; LOH, loss of heterozygosity; *RB*, retinoblastoma.

Fig. 4 | Effect of retinoblastoma loss of heterozygosity and p16 siRNA on establishment of senescence. **(A)** 5-Bromodeoxyuridine (BrdU) labelling of replicatively exhausted retinoblastoma loss of heterozygosity clones. Colonies were picked and expanded as indicated in Fig. 3. Cultures were incubated for 3–5 weeks after cell numbers ceased to increase before labelling. Labelling with BrdU was for 48 h. BrdU incorporation was detected immunohistochemically and scored by microscopic observation. Representative photomicrographs of stained plates are shown, with arrows indicating nuclei. **(B)** Quantification of BrdU incorporation presented in graphical format. **(C)** Quantification of p16 mRNA and protein in clones expressing p16 short-interfering (si)RNA. Bar graph, quantitative real-time reverse transcription–PCR (qPCR); lower panel, immunoblotting (IB). Clonal cell lines infected with target 2 (2-2, 2-5, 2-6) and target 4 (4-2, 4-6) siRNA are shown. The same cell lines are analysed in panels (C–E). Sen, noninfected senescent LF1 cells; EV-1, EV-5, cell lines infected with empty vector. **(D)** Lifespan extension of clones expressing p16 siRNA. Passage numbers were set to zero at the point when each clone was first expanded into a 10-cm dish. Each passage corresponds to approximately two population doublings. Four additional clones expressing empty vector senesced before the 10-cm dish stage and are not shown. **(E)** BrdU labelling of replicatively exhausted p16 siRNA-expressing clones. Experiment was performed as indicated in (A).

We followed numerous clones (five each from LH13 and SH18 cultures) until apparent growth arrest and determined the final number of cells. Assuming that each clone was founded by a single cell that converted to *RB*^{−/−} status, the population doublings (PD) required to reach the observed terminal cell number would be approximately 16 PD for eight clones, 19 PD for one clone and 23 PD for one clone. These values are minimum estimates, as it is unlikely that we recovered all the cells during ring cloning or that subsequent passaging was 100% efficient. The values are in the same range as the 20–30 PD lifespan extension previously documented for *p21*^{−/−} cells.

To investigate other factors that may have contributed to escape from replicative senescence, we determined the expression of p53, p21, p16, and the RB family members p107 and

p130 in the two clones (LH13-LOH-c1 and SH18-LOH-c2) that were previously analysed for RB expression. p53, p21 and p107 were expressed normally (Fig. 3C), and p130 expression was below the level of detection in all cells (including parental *RB*^{+/−} cells). As expected, p16 expression was increased in the absence of RB. Finally, we examined the expression of telomerase activity using the telomeric repeat amplification protocol (TRAP) and found no evidence for its upregulation following loss of RB expression.

We next investigated whether eliminating p16 function had the same effect as loss of RB. In spite of repeated attempts we failed to knock out *p16* by gene targeting. This is because the promoter-less method of gene targeting used in somatic cells requires that the drug-selectable marker (for example, Neo) in the vector be

expressed from the promoter of the chromosomal target locus (Sedivy & Dutriaux, 1999). In cases where the target gene is expressed at very low concentrations (as is *p16*) the homologously integrated vector does not express sufficient Neo protein. To phenocopy knockout of *p16* we first expressed a fusion protein between the *p16*-insensitive R24C mutant of CDK4 and cyclin D1 (DK; Wei *et al.*, 2003). Expression of DK in LF1 cells elicited a limited lifespan extension of 5–10 PD, at the end of which the cultures entered into a typical senescence-like state. LF1/DK cells were immune to the inhibitory effects of ectopic *p16* expression, indicating that the expressed DK protein had biological activity (Wei *et al.*, 2003), and the DK protein continued to be expressed in senescent cells. While this work was in progress, similar studies using R24C and wild-type CDK4, or naturally occurring point mutations in the *INK4a* gene, reached the same conclusions (Brookes *et al.*, 2002; Morris *et al.*, 2002).

Because the DK gain-of-function intervention could have unsuspected effects we sought to ablate *p16* mRNA expression using siRNA. To avoid affecting the expression of the *p14^{ARF}* protein also encoded by the *CDKN2A* locus, siRNA targets in the *p16^{INK4a}*-specific exon 1 α were chosen. The short hairpin RNA (shRNA)-encoding DNA templates were cloned in the retrovirus vector pRetroSuper (Brummelkamp *et al.*, 2002) and expressed in presenescent LF1 cells. All four siRNAs elicited *p16* mRNA knockdown in pools of drug-resistant cells, but to variable extents. To achieve higher and more consistent knockdown, the best two pools were single-cell cloned, and multiple clones of each were expanded into cell lines. Cells infected with empty vector and subjected to the same manipulations were used as controls. Expression of *p16* siRNA elicited >95% reduction of *p16* mRNA and protein (Fig. 4C) in individual clones. Reduction of *p16* expression was highly correlated with an extended lifespan (Fig. 4D). Lifespan extension was modest (5–10 PD) and was terminated by typical senescence, as evidenced by low BrdU incorporation (Fig. 4E). We therefore conclude that loss of *p16* expression is insufficient to allow escape from replicative senescence.

Importantly, different expression levels of *p21* and *p16* may elicit different cell-cycle responses, especially when combined with other proliferative or anti-proliferative signals. The ectopic expression of either *p16* or *p21* alone is sufficient, if the protein is present at high enough concentrations, to establish cell-cycle arrest (McConnell *et al.*, 1998). The concentrations of *p16* continued to increase in *p21^{-/-}* cells during the extended lifespan of the cells to levels beyond those seen in normal senescent cells, but the cultures continued to proliferate (albeit at reduced rates) (Brown *et al.*, 1997). However, a similar concentration of *p16* was strongly inhibitory in presenescent *p21^{+/-}* cells, as were further increases in *p16* concentration in *p21^{-/-}* cells (Wei *et al.*, 2001). Likewise, *p21* expression increased during the short lifespan extension of LF1/DK cells, and accumulated to higher levels than those seen in normal senescent cells. So, the failure of the loss of *p16* function to bypass senescence can be explained by the accumulation of *p21* to levels that are sufficiently high to inhibit all G1 CDKs, whereas the failure of endogenous *p16* to arrest *p21^{-/-}* cells can be attributed to insufficient accumulation in the window of opportunity preceding the onset of crisis. Because in both situations the outcome is dictated by the expression levels of *p16* or *p21*, it is easy to imagine that the situation could change in different cell types or under different culture

conditions. Nevertheless, in all situations the genetic hierarchy that best accommodates existing data is to place RB downstream of both *p16* and *p21*.

Replicative senescence, defined as the loss of proliferative capacity elicited by continuous subculture, is triggered largely by telomere shortening in HDFs, but by stress and/or signalling imbalances in mouse embryo fibroblasts (MEFs; Sherr & DePinho, 2000). In MEFs, the senescence response is regulated primarily by the Arf–p53 pathway, and neither Arf nor p53 knock-out MEFs undergo senescence. Although *p16* is induced in senescent MEFs, it apparently has a minor role, and *p16* knock-out MEFs do not immortalize with increased frequency (Sharpless *et al.*, 2001). Initial studies indicated that disruption of the *RB* gene did not appreciably affect the senescence response in MEFs, with only a concurrent inactivation of all three RB family members (*pRB*, *p107*, *p130*) eliciting spontaneous immortalization (Sage *et al.*, 2000). This requirement for concurrent activation is apparently caused by adaptation during embryonic development, given that an acute conditional deletion of *RB* in MEFs can overcome the senescence programme (Sage *et al.*, 2003). So, in the light of recent evidence, it appears that *p16* and *RB* influence the senescence of HDFs and MEFs in similar ways, in spite of the fact that the responses may be triggered by different stimuli.

In summary, replicative senescence has been typically described as being regulated by two pathways, the p53–p21 pathway and the *p16*–RB pathway, that act in concert to achieve growth arrest. On the basis of the genetic evidence presented in this communication we propose that the signalling cascade is better described by a linear relationship, with p53 at the top and RB at the bottom, and *p16* forming a branch that enters at the level of RB.

METHODS

LF1 and SF1 cells were cultured as previously described (Brown *et al.*, 1997). Gene targeting and retroviral transduction of human fibroblasts have been described in detail (Hemmer *et al.*, 2003; Wei *et al.*, 2003). The sources of antibodies were: anti-CDK4 (sc-260), anti-*p21* (sc-397), anti-p53 (sc-6243), anti-*p16* (sc-759), Santa Cruz; anti-RB (G3-245), PharMingen; anti-actin (N-350), Amersham; anti-BrdU (555 627), Becton Dickinson. Immunoblotting was performed as previously described (Wei *et al.*, 2001, 2003). Equivalent loading of lanes was determined by Coomassie or SYPRO staining of duplicate gels, and the efficiency of transfer was verified by direct staining of the membranes with Ponceau red. Where indicated, loading was further verified by immunoblotting for actin. BrdU labelling and SA- β -gal staining were performed as previously described (Wei & Sedivy, 1999). Quantitative real-time reverse transcription–PCR (qPCR) was performed using the SYBR green system in an Applied Biosystems 7700 Sequence Detector as previously described (O’Connell *et al.*, 2003). The *p16* target sequences were: target 1, CGCAC CGAATAGTTACGGT; target 2, TAGTTACGGTCGGAGGCCG; target 3, GGAGCAGCATGGAGCCTTC; target 4, GTGCTCGGAG TTAATAGCA.

ACKNOWLEDGEMENTS

This work was supported by grant AG16694 from the National Institutes of Health to J.M.S.

REFERENCES

- Bodnar, A.G. *et al.* (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science*, **279**, 349–352.
- Bond, J.A. *et al.* (1999) Control of replicative life span in human cells: barriers to clonal expansion intermediate between M1 senescence and M2 crisis. *Mol. Cell. Biol.*, **19**, 3103–3114.
- Brookes, S. *et al.* (2002) INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. *EMBO J.*, **21**, 2936–2945.
- Brown, J.P., Wei, W. & Sedivy, J.M. (1997) Bypass of senescence after disruption of p21^{CIP1/WAF1} gene in normal diploid human fibroblasts. *Science*, **277**, 831–834.
- Brummelkamp, T.R., Bernards, R. & Agami, R. (2002) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell*, **2**, 243–247.
- Bunz, F. *et al.* (1998) The induction of p21 by p53 is required for sustained G2 arrest following DNA damage. *Science*, **282**, 1497–1501.
- Cairns, P., Proctor, A.J. & Knowles, M.A. (1991) Loss of heterozygosity at the RB locus is frequent and correlates with muscle invasion in bladder carcinoma. *Oncogene*, **6**, 2305–2309.
- Drayton, S. & Peters, G. (2002) Immortalisation and transformation revisited. *Curr. Opin. Genet. Dev.*, **12**, 98–104.
- Funk, J.O. *et al.* (1997) Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. *Genes Dev.*, **11**, 2090–2100.
- Hemmer, R.M., Wei, W., Dutriaux, A. & Sedivy, J.M. (2003) in *Methods in Molecular Medicine*, Vol. 223 (ed. El-Deiry, W.S.) 187–206. Humana, Towata, New Jersey, USA.
- Itahana, K. *et al.* (2003) Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1. *Mol. Cell. Biol.*, **23**, 389–401.
- McConnell, B.B., Starborg, M., Brookes, S. & Peters, G. (1998) Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. *Curr. Biol.*, **8**, 351–356.
- Morris, M., Hepburn, P. & Wynford-Thomas, D. (2002) Sequential extension of proliferative lifespan in human fibroblasts induced by over-expression of CDK4 or 6 and loss of p53 function. *Oncogene*, **21**, 4277–4288.
- O'Connell, B.C. *et al.* (2003) A large scale genetic analysis of c-Myc-regulated gene expression patterns. *J. Biol. Chem.*, **278**, 12563–12573.
- Rogan, E.M. *et al.* (1995) Alterations in p53 and p16^{INK4} expression and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts. *Mol. Cell. Biol.*, **15**, 4745–4753.
- Sage, J. *et al.* (2000) Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev.*, **14**, 3037–3050.
- Sage, J., Miller, A.L., Perez-Macera, P.A., Wysocki, J.M. & Jacks, T. (2003) Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature*, **424**, 223–227.
- Sedivy, J.M. & Dutriaux, A. (1999) Gene targeting and somatic cell genetics: a rebirth or a coming of age? *Trends Genet.*, **14**, 88–90.
- Sharpless, N.E. *et al.* (2001) Loss of p16^{INK4a} with retention of p19^{ARF} predisposes mice to tumorigenesis. *Nature*, **413**, 86–91.
- Shay, J.W., Pereira-Smith, O.M. & Wright, W.E. (1991) A role for both RB and p53 in the regulation of human senescence. *Exp. Cell Res.*, **196**, 33–39.
- Sherr, C.J. & DePinho, R.A. (2000) Cellular senescence: mitotic clock or culture shock? *Cell*, **102**, 407–410.
- von Zglinicki, T. (2002) Oxidative stress shortens telomeres. *Trends Biochem. Sci.*, **27**, 339–344.
- Wei, W. & Sedivy, J.M. (1999) Differentiation between senescence (M1) and crisis (M2) in human fibroblast cultures. *Exp. Cell Res.*, **253**, 519–522.
- Wei, W., Hemmer, R.M. & Sedivy, J.M. (2001) Role of p14^{ARF} in replicative and induced senescence of human fibroblasts. *Mol. Cell. Biol.*, **21**, 6748–6757.
- Wei, W., Jobling, W.A., Chen, W., Hahn, W.C. & Sedivy, J.M. (2003) Abolition of cyclin-dependent kinase inhibitors p16^{INK4a} and p21^{CIP1/WAF1} functions permits Ras-induced anchorage-independent growth in telomerase-immortalized human fibroblasts. *Mol. Cell. Biol.*, **23**, 2859–2870.
- Wright, W.E. & Shay, J.W. (2002) Historical claims and current interpretations of replicative aging. *Nature Biotechnol.*, **20**, 682–688.