# Forging a signature of *in vivo* senescence

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Abstract | 'Cellular senescence', a term originally defining the characteristics of cultured cells that exceed their replicative limit, has been broadened to describe durable states of proliferative arrest induced by disparate stress factors. Proposed relationships between cellular senescence, tumour suppression, loss of tissue regenerative capacity and ageing suffer from lack of uniform definition and consistently applied criteria. Here, we highlight caveats in interpreting the importance of suboptimal senescence-associated biomarkers, expressed either alone or in combination. We advocate that more-specific descriptors be substituted for the now broadly applied umbrella term 'senescence' in defining the suite of diverse physiological responses to cellular stress.

'Cellular senescence' refers to the specific phenomenon wherein a proliferation-competent cell undergoes permanent growth arrest in response to various cellular stresses. The senescent state is accompanied by a failure to re-enter the cell division cycle in response to mitogenic stimulation and by an acquired resistance to oncogenic challenge. We believe that these properties — stress-induced irreversible proliferative arrest and resistance to both mitogenic and oncogenic stimuli — provide the best formal definition of the senescent state.

Notably, terminal differentiation of certain cell lineages may also lead to durable cell cycle arrest and a failure to respond to mitogenic or oncogenic stimuli. However, the terminally differentiated state represents a defined end-point of a developmental programme in which cells have become optimized to carry out particular functions. Although terminal differentiation is a programmed developmental response that is frequently accompanied by stable cell cycle exit, senescence is induced by stress signals that distort cellular homeostasis. The phenotypes of terminally differentiated cells in most tissues are readily apparent to histologists, whereas morphological features that describe the senescent state *in vivo* are few and poorly determined.

The concept of cellular senescence arose from early observations that human diploid cell strains have a finite replicative limit in culture, although they can remain viable and metabolically active after assuming a stable, non-dividing state[1,2]. With continued cell propagation, telomere attrition eventually leads to stable proliferative arrest[3,4]. Cultured primary embryo fibroblasts from laboratory mouse strains also exhibit a defined replicative capacity[5] and undergo senescence, although telomere attrition is not responsible[6]. Instead, chronic activation of particular tumour suppressors, such as the retinoblastoma protein (RB) and the transcription factor p53, can precipitate proliferative arrest in response to non-physiological conditions of tissue culture or oncogene challenge (herein referred to as oncogene-induced senescence (OIS))[7–11]. We now recognize that additional diverse senescence stimuli include reactive oxygen species (ROS), other DNA-damaging agents and the unfolded protein response[12–14] (FIG. 1).

Many stimuli that activate senescence are cancer-associated stresses, and the acquired resistance of senescent cells to oncogenic transformation supports a role for senescence in preventing tumour formation[12–16]. Inactivation of RB–p53 signalling in proliferating cells can bypass the onset of cellular senescence, whereas established senescent human cells in culture resist oncogenic insults that attempt to force cell cycle re-entry, such as the introduction of telomerase or the simian vacuolating virus 40 (SV40) large T antigen[17], which inactivates both the RB and the p53 pathways. By contrast, experimental co-inactivation of the G1 cyclin-dependent kinase (CDK) inhibitor $p16^{INK4A}$ and p53 in some senescent human cells[18] or conditional deletion of *Rb1* in mouse embryonic fibroblasts[19] have been reported to reverse senescence, permitting re-entry into the cell cycle. Whether the latter findings represent actual reversal of senescence or rather reflect the emergence of growth-arrested non-senescent subpopulations

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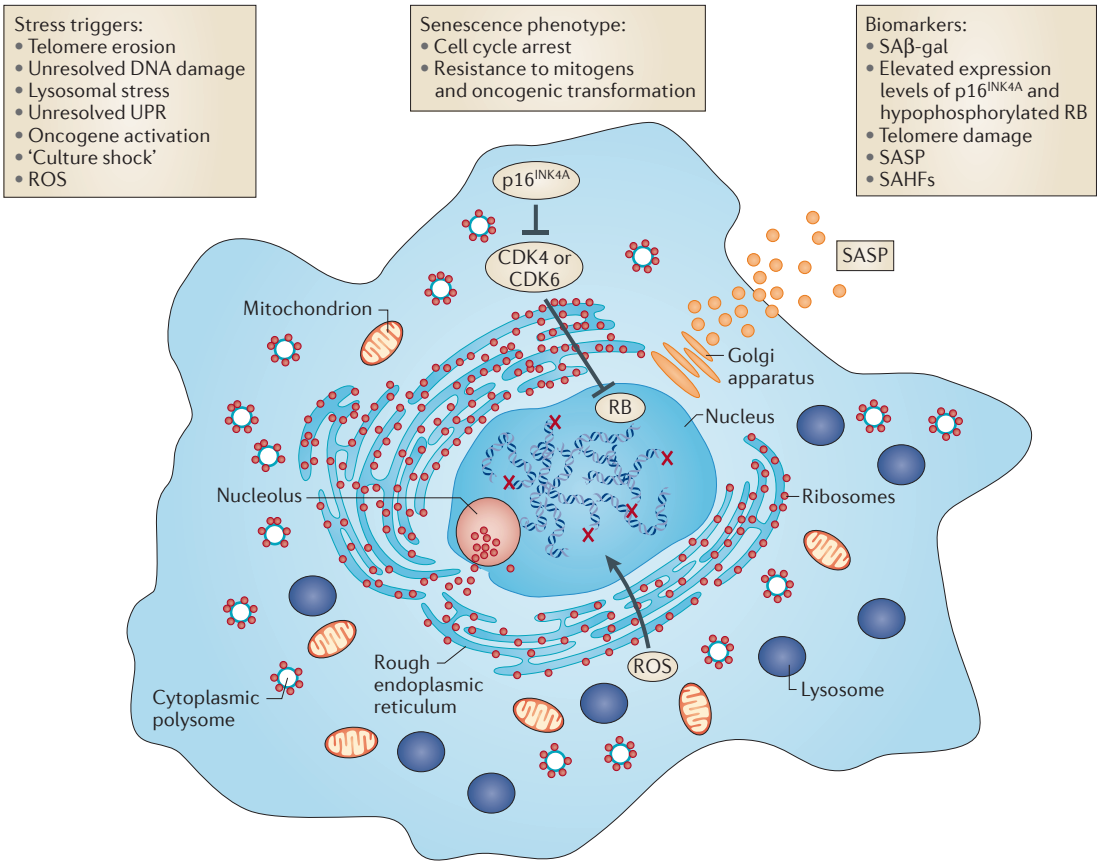


Figure 1 | **Stress triggers and biomarkers of senescence.** We define permanent growth arrest in response to various cellular stresses as the *sine qua non* of cellular senescence. The senescent state is accompanied by a failure to re-enter the cell division cycle in response to mitogens and by an acquired resistance to oncogenic challenge. Many forms of cellular stress can trigger cellular senescence and lead to the expression of the most commonly used biomarkers. The schematic depicts different organelles, including: the nucleus, containing damaged chromatin (X); the nucleolus; rough endoplasmic reticulum and 'free' cytoplasmic polysomes, each with ribosomes; mitochondria with internal cristae producing reactive oxygen species (ROS); and abundant lysosomes expressing β-galactosidase. Vesicles emanating from the Golgi apparatus contain secreted cytokines and chemokines that can impinge on surrounding cells (known as the senescence-associated secretory phenotype (SASP)), leading to paracrine signalling. The cyclin-dependent kinase (CDK) inhibitor $p16^{INK4A}$ in the cytoplasm prevents CDK4 and CDK6 from assembling into functional holoenzymes with their allosteric regulators, the D-type cyclins (not shown); $p16^{INK4A}$ binding to CDKs prevents the import of active kinases into the nucleus and inhibits the phosphorylation of nuclear retinoblastoma protein (RB). SAβ-gal, senescence-associated β-galactosidase; SAHFs, senescence-associated heterochromatic foci; UPR, unfolded protein response.

remains uncertain. Nonetheless, senescence is irreversible in the sense that known physiological stimuli cannot force senescent cells to re-enter the cell cycle[14].

Although senescence was first defined in cultured cells, additional lines of evidence in mice and humans have provocatively suggested a role for cellular senescence in mammalian ageing, wound healing, immunity and tissue remodelling, and even during mouse embryonic development. With senescence at the nexus of so many diverse research areas, and with a surfeit of high-profile papers focused on the subject, a troubling issue for the field has been obscured: namely, that *in vivo* markers to identify, quantify and characterize senescent cells in an intact organism are nonspecific and unreliable. This lack of a uniform definition of what constitutes *in vivo* senescence promotes confusion and controversy, and continues to raise numerous conceptual problems.

## Senescence versus quiescence

Senescence is thought to differ from less-durable forms of cell cycle arrest (for example, quiescence) in several ways (TABLE 1). When mitogen-dependent dividing cells are deprived of extracellular growth factors or exposed to anti-proliferative cytokines or contact inhibition, they exit the cell cycle into a non-proliferating quiescent state (G0). Although G0- and G1-phase cells cannot be distinguished by DNA content, quiescent cells comparatively produce reduced numbers of ribosomal RNA and proteins, have decreased metabolic activity, do not express G1 CDK activities, and tend to accumulate high steady-state levels of the CDK inhibitor $p27^{KIP1}$ (also known as CDKN1B)[20,21]. By contrast, adherent senescent cells emerging in culture in response to stress display an enlarged cell size and increased biomass, and they produce abundant stress granules[12–14]. Indeed, it has been

Table 1 | **Senescence versus quiescence**

| Feature | Senescence | Quiescence |
|---|---|---|
| Growth arrest | Permanent | Transient (mitogen responsive) |
| DNA content | 2N or 4N | 2N |
| Metabolism | High | Low (reduced ribosomal RNA and protein synthesis) |
| Molecular effectors | $p16^{INK4A}$, $p21^{CIP1}$, ARF, p53 and RB | $p21^{CIP1}$, $p27^{KIP1}$, p107, p130 and repressive E2Fs |
| Markers | • Short or dysfunctional telomeres<br>• SAβ-gal (lysosomal stress)<br>• $p16^{INK4A}$<br>• DNA-damage response<br>• SASP<br>• SAHFs | None |

2N, diploid; 4N, tetraploid; ARF, alternative reading frame protein; RB, retinoblastoma protein; SAβ-gal, senescence-associated β-galactosidase; SAHFs, senescence-associated heterochromatic foci; SASP, senescence-associated secretory phenotype.

suggested that high levels of nutrient-dependent mTOR and increased metabolic activity occurring in the face of stress-induced cell cycle arrest contribute to the conversion of non-proliferating cells to the senescent state[22]. Quiescent and senescent cells can be viably maintained in cell culture even after months of cell cycle arrest, but quiescent cells can re-enter the cell cycle in response to mitogenic and developmental cues, whereas senescent cells cannot. RB and $p16^{INK4A}$ are the cell cycle regulatory molecules most associated with maintaining irreversible cell cycle exit[8,18,23–26]. By contrast, regulators with similar biochemical activities more closely associated with quiescence (for example, the CDK inhibitor $p27^{KIP1}$ (REF. 27) and other RB family proteins, p130 (also known as RBL2) and p107 (also known as RBL1)[28]) have less clear roles in tumour suppression (TABLE 1).

Unlike the roles of RB, p53 and $p16^{INK4A}$, the role of the CDK inhibitor $p21^{CIP1}$ (also known as CDKN1A) in senescence and tumour suppression remains controversial. Stress-induced p53-dependent induction of $p21^{CIP1}$ inhibits interphase CDK activity to promote cell cycle withdrawal[29–31]. Expression of $p21^{CIP1}$ in response to transient DNA damage produces a reversible cell cycle pause that provides time for DNA repair and facilitates cell survival; correspondingly, tissues of $p21^{CIP1}$-deficient mice exhibit impressive sensitivity to apoptosis in response to DNA-damaging agents, such as ionizing radiation[32–34]. However, although mice lacking *Cdkn1a* are defective in G1 and G2 checkpoint control and develop more tumours than their wild-type counterparts late in life, they do not exhibit sensitivity to ionizing radiation-induced carcinogenesis[33,34]. Inactivation of $p21^{CIP1}$ does not abrogate senescence in commonly used *in vitro* model systems[35], cancer-prone human kindreds lacking $p21^{CIP1}$ have not been described, and somatic $p21^{CIP1}$ inactivation is rare in human malignancies. By contrast, consistent with observations that $p21^{CIP1}$ expression has been associated with prolonged proliferative arrest occurring in the setting of chronic DNA damage and p53 activation[36], more than 4 days of experimentally enforced $p21^{CIP1}$ expression has been reported to initiate senescence[37]. Although $p21^{CIP1}$ can trigger G1 arrest by inhibiting G1 CDK activity, the use of time-lapse microscopy and fluorescent sensors to monitor CDK2 activity in asynchronously dividing cells has recently suggested that levels of $p21^{CIP1}$ expressed during the G2 phase critically determine the length of the G1 interval in the subsequent cell division cycle[38]. Activation of high levels of $p21^{CIP1}$ by stress-induced p53 during the G2 phase also seems to facilitate senescence induction[38,39]. Even if $p21^{CIP1}$ is important for the initiation of senescence in some settings, its expression does not persist in senescent cells[40–42]. Therefore, $p21^{CIP1}$ cannot be used as a reliable marker of the senescence phenotype. By contrast, accumulation of high levels of $p16^{INK4A}$ prevents RB inactivation to maintain the senescent state[18,40,43], allowing the use of $p16^{INK4A}$ as a senescence marker.

Although it has been widely assumed that senescent cells exit the cell cycle in G0 or G1, this conclusion has been challenged by several lines of evidence[44]. Non-replicating mononuclear cells with tetraploid (4N) DNA content can arise during serial passage of cultured cells[45,46], and defects in cytokinesis and polyploidy have been noted in cells undergoing senescence[47,48]. Suggested mechanisms to explain these findings include ROS-induced defects in cytokinesis[48] and repression of the mitotic programme by non-canonical p53-induced E2F7 (REF. 49). Senescence-inducing stimuli, such as DNA damage, hydrogen peroxide and oncogenic RAS, can trigger p53–$p21^{CIP1}$ signalling in G2 to induce either G2 exit[44] or mitotic skipping[43], wherein mononuclear cells with 4N DNA content emerge and readapt to a G1-like state. This state is molecularly defined by activation of the E3 ubiquitin ligase APC/$C^{CDH1}$ (anaphase-promoting complex/cyclosome, in which CDC20-like protein 1 (CDH1; also known as FZR1) is the substrate adaptor co-activator protein) and the absence of cyclin A and cyclin B[39,43]. This sequence of events can mimic that seen in cells that skip mitosis in response to long-term exposure to microtubule inhibitors[50] or that have acute telomere deprotection and persistent DNA damage[51]. In short: $p21^{CIP1}$ can facilitate entry into senescence from either the G1 or the G2 phase of the cell cycle; senescent cells can exhibit diploid (2N) or 4N DNA content; regardless of their ploidy, senescent cells arrest in a G1 state; and the maintenance of senescence is not dependent on $p21^{CIP1}$ but relies heavily on persistent $p16^{INK4A}$ expression, CDK4 and CDK6 inactivation and active, hypophosphorylated RB.

## Markers of senescence

Adherent senescent cells attached to plastic culture dishes undergo morphological alterations, such as flattening, vacuolization and accumulation of stress granules[12–14]. As noted above, increases in cell size relative to proliferating cells in culture may reflect a continuation of anabolic processes, such as protein and membrane synthesis, in senescent cells that have exited the cell cycle. Senescent cells routinely express senescence-associated β-galactosidase (SAβ-gal) and $p16^{INK4A}$, and most secrete inflammatory cytokines and other signalling molecules — including interleukin-1 (IL-1), IL-6, IL-8, vascular endothelial growth factor A (VEGFA) and matrix metalloproteinases (MMPs) — as part of a senescence-associated secretory phenotype (SASP)[12–14,52,53]. Human cells undergoing OIS exhibit an unusual pattern of heterochromatin

mTOR
A serine/threonine kinase incorporated into mTOR complex 1 (mTORC1) and mTORC2, which act as nutrient sensors and regulators of translation.

APC/C
(Anaphase-promoting complex/cyclosome). A multi-subunit ubiquitin ligase complex that degrades cyclins A and B, depending on two alternative substrate selectivity factors, CDC20 and CDH1, that function during mitosis and G1 phase, respectively.

Foci
A nonspecific term frequently used to designate discrete, punctate topological sites (for example, of chromosomal DNA damage or heterochromatinization), which can also be described as speckles, detected by microscopy and usually with the aid of fluorescence-based antibodies.

*CDKN2A*
(Cyclin-dependent kinase inhibitor 2A; also known as the *INK4A–ARF* locus). Originally used to designate the gene encoding $p16^{INK4A}$, the locus is now recognized to encode a second, unrelated alternative reading frame (ARF) protein as well.

that is present in discrete nuclear subdomains, known as senescence-associated heterochromatic foci (SAHFs), which are associated with S-phase-promoting gene loci, such as E2F target genes[54]. Additionally, a prominent feature of many senescent cells is an activated and persistent DNA-damage response[55–58]. Although many of the features described above are widely but not uniformly observed in cultured senescent cells, numerous problems arise when trying to use them to describe senescence in living animals. This is because senescence *in vivo* yields more heterogeneous phenotypes, implying that multiple markers are required to define the senescent state with greater confidence. Below, we provide a brief description of the most commonly used markers of cellular senescence (FIG. 1), pointing to difficulties in detecting some of them and caveats in interpreting their expression *in vivo*.

***Acidic β-galactosidase.*** The most commonly used senescence marker, primarily because of its ease of detection in tissues, is SAβ-gal activity measured at pH 6.0 with the artificial substrate X-gal[59,60]. Endogenous β-galactosidase (encoded by the *GLB1* gene) in humans is a lysosomal enzyme optimally active at pH 4.0–4.5, so its detection at suboptimal pH 6.0 connotes its very high level of expression in senescent cells[61,62]. Substrates of β-galactosidase include gangliosides, keratin sulfate and various glycoproteins, and mutations in human *GLB1* cause GM1 gangliosidosis, a lysosome-storage disorder that predominantly affects nerve tissue. *Glb1*-knockout mouse strains are unavailable, probably because of embryonic lethality. In mice expressing transgenic p53–*lacZ*, endogenous β-galactosidase does not usually cause background detection problems because it exhibits greatly reduced activity under assay conditions at neutral pH[63]. Still, even under normal physiological circumstances, β-galactosidase activity is enriched in particular cell types, such as mature tissue macrophages and osteoclasts[64,65], and it is detected in cells undergoing increased lysosomal activity during autophagy[66–68]. Strong induction of endogenous β-galactosidase activity by ionizing radiation is seen in many tissues from different mouse strains, being detected within only 1 hour of exposure[63]. Even contact-inhibited quiescent cells maintained for prolonged periods in culture can upregulate β-galactosidase activity[60].

Such observations set clear limitations in interpreting the meaning of SAβ-gal expression detected at pH 6.0, which connotes exuberant lysosomal synthesis and activity observed in cells such as active phagocytes (including macrophages homing to sites of injury and inflammation and tumour-associated macrophages (TAMs)), as well as in resident Kupffer cells in the liver, microglia in the brain and cell types experiencing DNA damage. Despite routine reliance on this biomarker, it is dispensable for senescence, and its use as a senescence marker *in vivo* should be interpreted with caution, particularly in tumours or other inflammatory conditions, such as wound healing or after chemical insult, in which innate immune cells may predominate. Although investigators in the field acknowledge these caveats, most almost invariably use SAβ-gal activity to pinpoint senescent cells *in vivo*. A fair question is: can other potential markers (discussed below), either alone or in combination, definitively identify senescent cells without relying on SAβ-gal detection?

***p16$^{INK4A}$.*** The second most commonly used *in vivo* marker is the expression of $p16^{INK4A}$, a selective inhibitor of cyclin D-dependent CDK4 and CDK6 (REF. 69). The *CDKN2A* gene encoding $p16^{INK4A}$ is closely chromosomally linked to *CDKN2B*, which encodes a second INK4 family member, $p15^{INK4B}$ (REF. 70) (FIG. 2). Moreover, RNAs specified by exons 2 and 3 of the *CDKN2A* gene are co-opted into distinct transcripts originating from another upstream promoter and exon, where their coding sequences are translated in an alternative reading frame (ARF)[71]; the resulting protein, $p14^{ARF}$ in human and $p19^{ARF}$ in mouse, is also a potent tumour suppressor that activates p53 (REFS 9,72–74). Remarkably then, both RB and p53 are regulated by products of the *CDKN2A* locus: $p16^{INK4A}$ and ARF, respectively. The relative contributions of $p16^{INK4A}$ and ARF to senescence continue to be puzzling. For example, $p19^{ARF}$ expression is the more critical determinant of replicative senescence of cultured mouse embryonic fibroblasts[9], whereas $p16^{INK4A}$ is a key regulator of *in vitro* senescence in human cells[75]. Notably, deletion and silencing of the entire *CDKN2A–CDKN2B* locus and mutations inactivating $p16^{INK4A}$ are among the most frequent genetic events encountered in malignant human tumours[76,77], implying that, as with inactivation of *RB1* and *TP53*, loss of *CDKN2A–CDKN2B* enables cells to bypass tumour-suppressive restraints that are imposed by senescence.

The appeal of $p16^{INK4A}$ as a senescence marker is that its biochemical function in inducing cell cycle arrest is known and its expression is highly dynamic: it is generally absent in unstressed, healthy tissues in young animals but highly expressed in the setting of certain stresses and alterations of tissue architecture that occur with tumorigenesis, wounding and/or ageing. Despite these advantages, $p16^{INK4A}$ also has apparent limitations as an *in vivo* biomarker of senescence. First, there are forms of *in vitro* senescence that are not characterized by $p16^{INK4A}$ expression[18,78–80]. At least under some circumstances, the absence of $p16^{INK4A}$ expression might be compensated by upregulation of $p15^{INK4B}$ (REFS 81–83) or by the expression of *CDKN2C* (also known as *INK4C*)[84]. Perhaps of greater concern, there are situations *in vivo* during which $p16^{INK4A}$ is readily detected in non-senescent cells: for example, it is expressed at particularly high levels in cells with inactivated RB[24,69,85], including many cancer cells[85–88]. Hence, even if a senescence initiator is present and accompanied by $p16^{INK4A}$ expression, growth arrest can be blunted by mutations that inactivate downstream effectors of the senescence programme. Currently available antibodies are relatively poor at detecting $p16^{INK4A}$ by immunohistochemistry in mice, limiting the utility of this marker in experimental rodent studies *in vivo*. Bypassing this drawback, mice in which a cassette encoding a reporter gene (for example, luciferase (*Luc*)) under control of *Ink4a cis*-regulatory elements have been generated through both transgenic[80,89–91] and knock-in approaches[92]. These alleles not only confirm that *Ink4a*

promoter activity steadily increases as animals age[93,94] but also demonstrate its transient expression in settings such as wounding or clastogen exposure[90–92,95–98]. The meaning of p16$^{INK4A}$ expression in these transient settings followed by the disappearance of p16$^{INK4A}$-positive cells during healing and tissue repair raises the question of whether these cells were senescent and eliminated (for example, through clearance by the immune system) or whether non-senescent immune cells entering a wound are a source of p16$^{INK4A}$ expression, or both.

Using the LUC knock-in model (*p16$^{LUC}$* (REF. 92)), significant bioluminescence was detected within subcutaneous tumour masses that formed after LUC-negative tumorigenic cells were implanted into host *p16$^{LUC}$* mice, suggesting that *p16$^{INK4A}$* activation occurs in non-neoplastic cells within a nascent cancer. Transplantation of *p16$^{LUC}$* bone marrow cells into naive mice revealed that at least some of the LUC-positive cells populating an implanted subcutaneous tumour were derived from donor bone marrow and were, at least in part if not entirely, comprised of TAMs. It is unclear whether these tumour-associated haematopoietic elements are senescent or merely expressing p16$^{INK4A}$ as part of a stress response that is associated with inflammation and macrophage differentiation[83], analogous to p16$^{INK4A}$ activation with wounding[91,92,97–99]. These observations confirm that increases in p16$^{INK4A}$ expression occur in incipient tumours and with ageing, but they also underscore a problem with defining senescent cells on the basis of p16$^{INK4A}$ expression.

The expression of p16$^{INK4A}$ increases in lymphocytes as animals age. Abundant p16$^{INK4A}$ expression in peripheral blood T cells from aged humans and mice[100–102] is associated with a marked decline in their proliferative capacity, a defect that can, in part, be rescued through T cell-specific *p16* inactivation[102,103]. The expression of p16$^{INK4A}$ in lymphocytes can also be accelerated by serial culture[103,104], administration of cytotoxic chemotherapy[105,106] and chronic HIV infection[107]. Increases of p16$^{INK4A}$ with ageing in B cells and B cell progenitors are also well described, with a loss of regenerative capacity in these compartments being associated with a resistance to oncogene-induced transformation as long as the *Cdkn2a* locus remains functionally intact[108]. But, are p16$^{INK4A}$-expressing lymphocytes senescent? There is considerable scepticism in the immunology community regarding whether these hyporeplicative lymphocytes are senescent, exhausted or otherwise dysfunctional[100,103,109–113], with some investigators claiming that such cells are capable of re-entering the cell cycle when isolated and appropriately stimulated in culture[110,111]. Importantly, hyporeplicative T cells that accumulate with age do not robustly express other markers of senescence, such as SASP or SAHFs, and their numbers are difficult to quantify, extending the uncertainty of how they should be classified.

***Chromatin changes: DNA damage foci and SAHFs.*** DNA damage is intimately associated with most types of senescence *in vitro* and can be triggered *in vivo* by DNA replication errors and fork collapse during S phase, as well as by a plethora of genotoxic insults that are either intrinsic (for example, oxidative damage and telomere attrition) or environmental (for example, ultraviolet (UV) or ionizing radiation, chemotherapeutic drugs and many others). DNA-damage foci arise at dysfunctional telomeres that accompany replicative senescence[79,114,115]. Furthermore, many senescent cells express foci of DNA damage at non-telomeric sites, which similarly generate persistent ataxia telangiectasia mutated (ATM)–p53–p21$^{CIP1}$ signalling that is required for growth arrest[55–58,116]. In precancerous lesions, double-strand DNA breaks, possibly arising from unrepaired replication errors or oxidative DNA

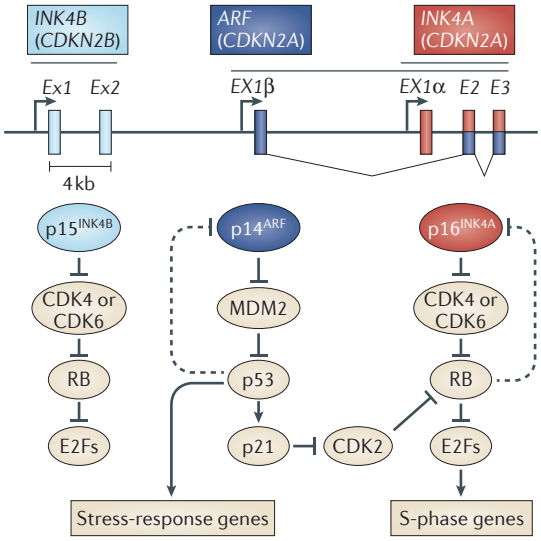


Figure 2 | **The *CDKN2A–CDKN2B* locus.** The cyclin-dependent kinase inhibitor 2A (*CDKN2A*)–*CDKN2B* locus, which is less than 50 kb in overall length, encodes three tumour-suppressor proteins. Exons within the locus are indicated by coloured vertical bars, and the three promoters are indicated by arrows. The *CDKN2B* gene (which encodes p15$^{INK4B}$) is specified by two exons (light blue). The *CDKN2A* gene encodes both p14$^{ARF}$ (p19$^{ARF}$ in mice; three dark blue exons) and p16$^{INK4A}$ (three red exons). RNAs transcribed from alternative first exons (designated Ex1β for *ARF* and Ex1α for *INK4A*) are spliced to mRNA sequences encoded by exons 2 and 3 of the *INK4A* gene, thereby generating two transcripts that are translated in alternative reading frames. The p16$^{INK4A}$ and p15$^{INK4B}$ proteins inhibit cyclin D-dependent CDK4 and CDK6 to prevent phosphorylation of the retinoblastoma protein (RB). The hypophosphorylated form of RB sequesters E2F transcription factors, preventing them from coordinately activating a suite of genes that are required for DNA replication (as shown for *INK4A* only). The ARF protein binds to the MDM2 E3 ubiquitin ligase to prevent p53 polyubiquitylation and to facilitate p53 activation. In turn, the p53 transcription factor regulates an extensive group of genes that are commonly induced by cellular stress. These include the CDK2 inhibitor p21$^{CIP1}$, which inhibits CDK2-mediated RB phosphorylation during progression through the G1 phase of the cell division cycle. Inactivation of p53 leads to *ARF* induction, whereas inactivation of RB induces *INK4A* expression. These negative feedback loops are depicted by dashed lines. Silencing of the *CDKN2A–CDKN2B* locus in stem cells[15,156,160,174], or its frequent deletion in cancer cells[75,76], abrogates the tumour-suppressive functions of RB and p53 to facilitate cellular self-renewal.

Ataxia telangiectasia mutated
(ATM). A serine/threonine kinase that acts as a sensor of DNA damage and that phosphorylates various substrates during the different phases of the cell cycle to activate checkpoint responses that arrest the growth of cells with DNA damage.

damage, activate p53 and p21$^{CIP1}$ to induce either apoptosis or senescence[117]. Although foci of DNA damage, detected through staining of γH2AX or p53-binding protein 1 (53BP1; also known as TP53BP1), are frequently visible in senescent cells, they are not invariant, as compounds such as chloroquine and histone deacetylase inhibitors that alter chromatin structure can also induce ATM–p53–p21$^{CIP1}$ signalling, p16$^{INK4A}$ and SAβ-gal without telomere dysfunction or overt DNA damage[118,119]. Likewise, most cells expressing markers of DNA damage are not senescent, so this marker has little specificity for the phenotype *in vivo*.

Human (but generally not mouse[120–122]) senescent cells in culture exhibit *in vitro* punctate 4′,6-diamidino-2-phenylindole (DAPI)-stained SAHFs associated with trimethylation of Lys9 on histone H3 (H3K9me3) and binding of heterochromatin protein 1 (HP1) to chromatin[54]. SAHFs per se may not serve as a senescence marker across species, but enrichment of H3K9me3 modifications and HP1 binding, particularly at E2F target genes, has been used biochemically to demonstrate enrichment of senescent cell populations in culture. Although p107 and p130 are the major RB family proteins bound to E2F-responsive promoters in quiescent and G1-phase cells[28,123], RB itself in association with HP1 and histone methyltransferases may be recruited to E2F promoters during senescence[23], contributing to the formation of SAHFs and having a key causal role in the establishment of senescence[54]. In contrast to the spreading of histone marks during cell differentiation, SAHFs contain static repressive marks and are presumed to be formed during spatial repositioning of pre-existing heterochromatin[124,125]. Enforced expression of p16$^{INK4A}$ *in vitro* can activate RB to induce SAHFs and downregulate E2F target genes, consistent with the idea that SAHFs are maintained in human senescent cells. However, these foci are not a common feature of many forms of senescence (that is, they are relatively specific to OIS), occur in a cell type-specific manner and are dispensable for cellular senescence[120,121]. Their detection *in vivo* relies primarily on histochemical staining for HP1 speckles, as opposed to on chromatin immunoprecipitation (ChIP) of selected HP1-modified genes. Therefore, SAHFs have limited utility in most settings as a biomarker of *in vivo* senescence.

***The SASP.*** One of the more intriguing phenotypes associated with senescent cells is the SASP. Senescent cells upregulate enzymes that degrade the extracellular matrix, and they secrete immune modulators and inflammatory cytokines, largely in response to nuclear factor-κB (NF-κB)-mediated signalling[126]. SASP factors are highly pleiotropic and induce a host of local activities, including reduced replicative capacity[127], recruitment of inflammatory cells, alterations of tissue composition and architecture[128–130], and promotion of wound healing[91,128–130] (FIG. 3). The effects of the SASP have been argued to be age-promoting and also to either facilitate[91,131] or inhibit[127,128,130,132] tumorigenesis. Cultured cells undergoing RAS-induced OIS secrete factors such as IL-1α, IL-6, IL-8 and transforming growth factor-β (TGFβ), at least some of which induce cell cycle arrest of neighbouring non-cancer cells. Surprisingly, co-culture with induced senescent cells leads naive cells to undergo oxidative cellular and DNA damage, as well as activate p16$^{INK4A}$, p21$^{CIP1}$, interleukins and chemokines, suggesting that senescence can spread in a paracrine manner[127]. Other examples of 'paracrine senescence' have been described in tumours and with ageing[133,134]. A recent review has compared SASP with ancient and evolutionarily conserved processes governing cellular competition and bidirectional paracrine signalling between weaker and fitter cells[135]. Under these circumstances, signalling between stressed, damaged cells and their neighbours might be expected to regulate tissue homeostasis. In several *in vivo* models of OIS, oncogene-expressing cells were surrounded by immune cells, many of which also expressed p16$^{INK4A}$ and p21$^{CIP1}$. Although of great potential interest, these bystander responses substantially complicate the interpretation of senescence-associated marker expression in tissues and raise further questions about how oncogene-stressed cells in animals influence the local stromal microenvironment and immune response. Moreover, expression of most, if not all, of the known components of the SASP is highly dynamic in settings independent of senescence, such as acute or chronic bacterial and viral infection, inflammation, wound healing and ongoing malignancies. For example, moderate senescence-associated increases (20- to 100-fold) in expression of IL-6, one of the best-described SASP components, are considerably less than the many-thousand-fold increases in serum IL-6 levels induced by some types of acute inflammation[136,137]. At present, no combination of SASP cytokines can reliably distinguish senescence from other forms of acute cellular stress characterized by NF-κB activation.

***Telomere shortening and dysfunction.*** Telomerase activity is not detected in human primary somatic cells, the inability of which to add telomeric repeats to chromosome ends eventually leads to telomere deprotection and a DNA-damage response that limits cellular proliferative lifespan[3,6,138–141]. In turn, enforced expression of telomerase can bypass replicative senescence and maintain chromosomal integrity[4]. Similarly, in inbred strains of laboratory mice engineered to undergo telomere attrition and exhibit premature ageing phenotypes, reactivation of telomerase can reverse degenerative traits[142]. In humans, telomere dysfunction associated with genetic disorders (for example, dyskeratosis congenita) is linked to early onset of some aspects of ageing, such as pulmonary fibrosis, bone marrow failure and cirrhosis[139,143], implying that assays of telomere attrition and integrity might be useful for measuring *in vivo* senescence in people. In practice, the most precise assays of telomere structure, such as flow-cytometric fluorescence *in situ* hybridization, require assessment of telomere integrity of each individual chromosome, and therefore are cumbersome and require substantial numbers of viable cells. Less-precise assays of telomere length, such as Southern blotting and PCR-based approaches, including sensitive single-telomere length analyses, are fraught by yet other technical issues that cloud their interpretation and utility[144]. As with the expression of p16$^{INK4A}$, telomere shortening and dysfunction can

γH2AX
A phosphorylated histone variant that decorates chromatin sites of DNA damage and is required for the assembly of repair proteins during the DNA-damage response.

Heterochromatin protein 1
(HP1). A family of proteins that bind to trimethylated Lys9 on histone H3, which is important in gene silencing.

Dyskeratosis congenita
A rare inherited disorder presenting with variable degenerative ageing phenotypes accompanied by reduced telomere maintenance and shortened lifespan; it is most commonly triggered by mutations affecting X-linked *DKC1*, which encodes the telomerase cofactor dyskerin.

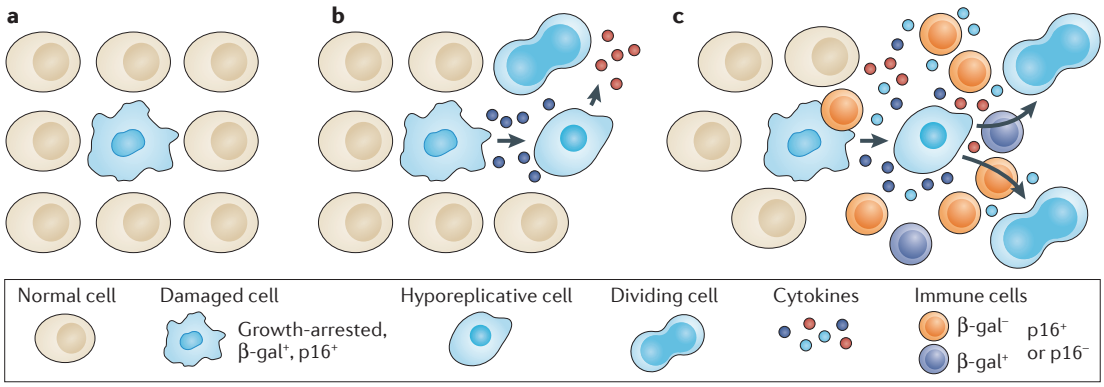


Figure 3 | **Senescence *in vivo*.** **a** | Stress-induced, damaged cells accumulate in a field of normal cells. Damaged cells undergo growth arrest and express $p16^{INK4A}$ and β-galactosidase (β-gal). **b** | Damaged and hyporeplicative cells secrete a complex suite of cytokines that produce multiple paracrine effects, potentially arresting some neighbouring cells but also stimulating others to divide. **c** | Damaged cells recruit functional immune cells, including macrophages and lymphocytes, some of which also express $p16^{INK4A}$ and β-gal. Immune cells also contribute to the extracellular secretory milieu. Many of these features are also observed in nascent tumours or within healing wounds. Note that damaged, growth-arrested cells exhibit both loss-of-function (proliferative arrest) and gain-of-function (robust paracrine secretion) activities. Chronic immune infiltration can exacerbate tissue damage, and demands for compensatory proliferation can accelerate the exhaustion of tissue-regenerative capacity associated with ageing or select for abnormal self-renewing cells that have mutations in tumour-suppressor genes associated with cancer.

occur in non-senescent cells, and senescence can be triggered by many stresses that are independent of telomere shortening in human cells *in vitro*. Telomeres seem to be favoured targets of random DNA damage, independent of telomerase activity, and age-dependent increases in the frequency of telomere-associated DNA-damage foci can occur in the gut and liver of mice regardless of telomere length[145]. However, when engineered to lack telomerase activity, laboratory-derived strains of mice that are endowed with long telomeres do not exhibit signs of ageing until they are intercrossed for several generations to allow telomere attrition[6,146]. It would seem that *in vivo* senescence ensuing in the most commonly used rodent models develops independently of telomere dysfunction. Therefore, although telomere deprotection clearly occurs *in vivo* and probably has a causative role in certain age-associated disease states, it is neither a sensitive nor a specific marker of senescence *in vivo*.

## Combining senescence markers

Given the shortcomings of isolated senescence biomarkers, as well as the fact that senescence can occur in response to diverse stimuli, act through different mechanisms and, seemingly, spread by intercellular signalling within stressed tissues, it becomes clear that no single marker will faithfully represent senescence *in vivo*. Hence, most investigators agree that expression of a combination of generally used senescence markers should be used to define senescent cells *in vivo*. Yet, there is no consensus on which, or how many, markers are required. One suggestion has been to affirm cell cycle arrest and then superimpose "at least two additional senescence markers, the choice of which may vary for different settings" (REF. 12). As arbitrary as this may seem, the most commonly used dyad is the expression of SAβ-gal and $p16^{INK4A}$; however, other combinations have been used, including the expression of $p16^{INK4A}$ and SASP markers, as well as the expression of multiple senescence-associated mRNA transcripts beyond *p16* (REF. 147). These difficulties in definition have been compounded by the use of additional but vague qualifying terms, such as 'deep', 'true', 'pre' and even 'post', to describe inconsistent phenotypic variations of senescence. In short, even the professionals actively playing on the senescence field have not yet agreed on the rules of the game.

It should be disconcerting that the most sensitive and oft-used marker of senescence (SAβ-gal) is an enzymatic activity found in many kinds of normal cells under physiological conditions, the activation of which is presumed to connote lysosomal stress. To see how expansive the topic of *in vivo* senescence will become when guided by SAβ-gal expression, consider the recently described entity of 'developmental senescence' (REFS 148,149). Here, the authors documented cell cycle arrest, SAβ-gal expression and diffuse heterochromatin changes in developing embryos. Specifically, SAβ-gal-positive cells were detected at defined organ sites, such as the regressing mesonephros, the inner ear, the neural roof plate, and the apical ectodermal ridge during limb formation. Inflammatory molecules that typically characterize SASP in adults were absent, whereas fibroblast growth factor 4 (FGF4), FGF8, TGFβ–SMAD and PI3K–forkhead box O (FOXO) signalling predominated in the embryo. Notably, developmental senescence was not associated with DNA damage, and it persisted in unaltered fashion in *Cdkn2a*-knockout and *Trp53*-knockout mice. The features of senescence in these embryos seem to rely on developmental cues that impinge on $p21^{CIP1}$, as loss of $p21^{CIP1}$ reduced the number of cells marked by SAβ-gal in the embryos. Although macrophages were reported to surround senescent cells at E13.5–E14.5, the mesonephros in *Cdkn1a*-null mice was free of macrophages that could potentially contribute to

SAβ-gal activity. Even in *Cdkn1a*-null mice, cells in both the apical ectodermal ridge and the mesonephros were eliminated by compensatory mechanisms, including macrophage infiltration and apoptosis[148,149]. Whether $p21^{CIP1}$ expression regulates a stable senescent state that cannot be detected in a setting of rapid cellular turnover or, alternatively, whether it triggers exit from the cell cycle in these tissues during their remodelling remains unresolved.

In contrasting studies, widespread and intense SAβ-gal staining in the early embryo was reported to also decorate the proliferating visceral endoderm of the post-implantation embryo at E5.5, eventually becoming restricted to extra-embryonic endoderm at E7.5 and to non-dividing cells in the derivative yolk sac at E9.5; at E5.5 and E7.5, no $p21^{CIP1}$ was detected, and highly SAβ-gal-positive cells were actively cycling[150]. Notably, the $p19^{ARF}$ tumour suppressor, but not $p16^{INK4A}$, is concordantly expressed in the proliferating embryonic visceral endoderm and extra-embryonic yolk sac[151]. Presumably, the early embryonic expression of these markers in these tissues connotes a senescence-independent process.

Together, these findings raise intriguing questions about the extent to which developmental senescence can be conceptually aligned with senescence in adult animals for which coordinated expression of multiple canonical biomarkers has been described. For example, in the case of OIS and in pre-malignant adenomas, oncogenic stress induces the expression of tumour suppressors ($p16^{INK4A}$ and p53), resulting in cell cycle arrest, the frequent appearance of DNA-damage foci and SAβ-gal expression[12,55,56,117,147,152,153]. By contrast, the developmental process seems to be very different, not featuring DNA damage, typical SASP components, telomere dysfunction or $p16^{INK4A}$ expression, and not being associated with preneoplastic conversion or stochastic cellular stresses. Do processes of tissue remodelling in the embryo mimic only selected features of senescent cells in adults? And, as some suggest, could developmental senescence reflect an evolutionary origin of the senescence programme? Or could these forms of senescence really reflect highly disparate forms of growth arrest during tissue remodelling, with little in common beyond the expression of SAβ-gal?

Taking things a step further, several studies of early neoplastic progression have seriously questioned the permanence of some of the best-characterized examples of *in vivo* senescence. Human and mouse melanocytes form growth-arrested naevi in response to oncogene activation (through BRAF-V600E), and these naevi are characterized by the expression of several markers of senescence, such as SAβ-gal and $p16^{INK4A}$ (REFS 152,153). However, in mice, these apparently senescent melanocytes can be readily coaxed back into the cell cycle[154,155]. An alternative interpretation is that $p16^{INK4A}$ is activated by BRAF-V600E to provide a barrier to malignant transformation, and that unrelenting oncogenic RAF signalling and the deranged tissue architecture present in a naevus in turn cause the production of senescence markers, including SAβ-gal and inflammatory cytokines, through activation of established pathways, such as those dependent on NF-κB. All such features could occur without some cells being permanently arrested, allowing a subpopulation that sustains inactivating lesions in tumour-suppressor genes to subsequently emerge as melanomas.

### *In vivo* senescence and ageing

Why do investigators in the field consider it important to measure senescent cells in an intact organism in the first place? The closest thing to a testable prediction that requires measurement of *in vivo* senescence is in the area of mammalian ageing research. Subscribers to the senescence theory of ageing contend that at least some features of organismal ageing may stem from a progressive accumulation of senescent cells in tissues over a lifetime[156–160], with the expectation that their enumeration *in vivo* might predict physiological age and longevity. Likewise, this model holds that 'senolytic' agents that are selectively toxic to senescent cells could trigger their clearance and reverse some aspects of ageing[161].

Many aspects of mammalian ageing reflect a functional decline in the ability to maintain tissue homeostasis and integrity, coupled with diminished responses to physiological demands under conditions of stress[156–160]. Age-associated declines of functional reserves include the loss of replicative capacity in cells with self-renewal potential (particularly stem and progenitor cells) as well as increased age-dependent secretion of pro-inflammatory cytokines that could exacerbate potentially deleterious inflammatory responses and contribute to tissue injury. The associated phenomena of interest are not in dispute: $p16^{INK4A}$, p53 and $p21^{CIP1}$ promote proliferative arrest; stressed and damaged cells can secrete pro-inflammatory cytokines and express robust β-galactosidase activity at pH 6.0; and chromatin alterations in histone and DNA methylation are widespread age-associated epigenetic processes. The expression of markers of telomere dysfunction, *p16* mRNA and cytokines such as IL-6 exponentially increase with ageing[93–95,101,102,162–164]. Moreover, the accumulation of $p16^{INK4A}$ has a causal role in some age-associated phenotypes of haematopoietic and neural stem cells, muscle satellite cells, pancreatic β-cells, and lymphoid cells[102,108,165–170], and measures to reduce the frequency of $p16^{INK4A}$-expressing cells in a range of tissues can rescue aspects of ageing[90,168,169,171,172]. Perhaps most remarkably, the experimental clearance of $p16^{INK4A}$-expressing cells in progeroid mice attenuated several age-associated phenotypes, such as sarcopenia and kyphosis[90]. Regulatory polymorphisms of telomerase reverse transcriptase (*TERT*), encoding the catalytic subunit of telomerase, and those found in close proximity to the *CDKN2A–CDKN2B* locus (encoding $p16^{INK4A}$, $p15^{INK4B}$ and ARF), have been linked to numerous age-associated diseases (such as myocardial infarction, aortic aneurysm, ischaemic stroke, type 2 diabetes, glaucoma, pulmonary fibrosis and several cancers) by large, unbiased, genome-wide association studies in humans (reviewed in REF. 173). Therefore, a wealth of data gained from humans and mice indicate that responses to cellular stress coupled with the expression of regulators of cell division, such as telomerase, p53 and $p16^{INK4A}$, are associated with phenotypic manifestations of mammalian ageing. In turn, cancer cells bypass these tumour-suppressive effects by acquiring genetic alterations that disrupt the

signalling of the RB–p53 network[12–19,174,175] (FIG. 2) and by circumventing chromosomal end attrition by either upregulating telomerase activity[140,141] or relying on alternative recombinational strategies to maintain telomere integrity[176–178].

The main issue is whether ageing stems from the cumulative unrepaired damage following a lifetime of exposure to various forms of cellular stress or from the accumulation of senescent cells per se. None of the aforementioned ageing phenomena strictly requires *in vivo* senescence, as rigorously defined by permanently growth-arrested cells *in situ*. Moreover, inflamed or stressed cells that express abundant β-galactosidase or SASP hormones *in vivo* may later re-enter the cell cycle, and cells expressing abundant $p16^{INK4A}$ may merely be hyporeplicative but not permanently arrested. In line with this view, several age-associated phenotypes caused by conditional $p16^{INK4A}$ overexpression readily revert when $p16^{INK4A}$ expression is extinguished *in vivo*[179]. This finding suggests that $p16^{INK4A}$ expression can promote some aspects of ageing without inducing durable growth arrest. Therefore, although the idea that senescence plays a part in ageing has some appeal, senescent cells cannot be unequivocally demonstrated to accumulate in ageing tissues, where their enumeration remains a formidable challenge and their causal role in the physiology of ageing remains unproven.

## Summary

There remains an urgent need to better define senescent cells based on consistent and rigorously applied criteria, in order to compare and contrast their roles in different settings and to functionally interrogate their contribution to disease and ageing. Perhaps emerging technologies for single-cell genome-wide expression and proteomic analysis will facilitate the discovery of more-precise markers of senescence (see, for example, REFS 147,180). However, rather than providing much-needed clarity, we suspect that such approaches will more likely reveal an even greater level of unexpected heterogeneity and phenotypic complexity among cells currently classified under the umbrella term senescence.

Given the many limitations with regard to senescence classification and detection *in vivo*, investigators should accept what are, in fact, widely acknowledged caveats in evaluating senescence biomarkers and accede to the idea that there are not, and may not be, 'magic' markers of senescence. We necessarily wonder, then, why many investigators qualify descriptions of seemingly diverse phenotypes by stating that 'there are many forms of senescence', or feel the need to ask, 'Is senescence one thing or many things?' Why yield to the proposition that senescent cells can be reversibly growth-arrested? Is there a quota of commonly used markers (and if so, how many?) that is required to define the senescent state? Why use terms like pre-senescence, acute or chronic senescence, deep senescence or post-senescence? This inexact nomenclature magnifies the problem.

What is the alternative? Perhaps the field should resist the convenience of what may be an inadequate catch-all definition and restrict the term senescence (as we have) to depicting states of irreversible stress-induced growth arrest. In turn, other empirical descriptions may be applied to better characterize the context-dependent nature of cells undergoing cell cycle exit *in vivo*. We suggest that, whenever possible, substitution of more accurate descriptors to define the phenotypes under study would prove far less confusing. Specific terms like cell cycle arrest, secretory activity, paracrine signalling, chromatin remodelling and others are more illuminating. As for associated molecular events, the detection of levels of $p16^{INK4A}$ and $p21^{CIP1}$, states of RB phosphorylation and telomere length, DNA-damage foci and secretion of specific cytokines are indisputable experimental findings that do not require extensive qualification. Rather than applying the term senescence to what are clearly complex and possibly distinct cellular states, we should aim for greater clarity by using judicious choices of more explicit terms. The senescence field would profit from greater precision, given the importance of, and widespread interest in, the many underlying biological phenomena and their implications for cancer, immunity and ageing research.


1. Hayflick, L. & Moorhead, P. S. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**, 585–621 (1961).
2. Hayflick, L. The limited *in vitro* lifetime of human diploid cell strains. *Exp. Cell Res.* **37**, 614–636 (1965).
3. Harley, C. B., Futcher, A. B. & Greider, C. W. Telomeres shorten during aging of human fibroblasts. *Nature* **345**, 458–460 (1990).
4. Bodnar, A. G. *et al.* Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349–352 (1998).
**This landmark paper provides direct evidence that experimental restoration of catalytically active telomerase in cultured human cells bypasses replicative senescence without causing oncogenic transformation.**
5. Todaro, G. J. & Green, H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* **17**, 299–313 (1963).
6. Blasco, M. A. *et al.* Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* **91**, 25–34 (1997).
**This paper demonstrates that, although laboratory strains of mice exhibit long telomeres, knocking out the RNA subunit of telomerase and interbreeding telomerase-deficient mice for sequential generations unmasks the potential effects of telomere attrition in this species.**
7. Harvey, D. M. & Levine, A. J. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. *Genes Dev.* **5**, 2375–2385 (1991).
8. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and $p16^{INK4a}$. *Cell* **88**, 593–602 (1997).
**The first demonstration of OIS associated with increased expression of $p16^{INK4A}$.**
9. Kamijo, T. *et al.* Tumor suppression at the mouse *INK4a* locus mediated by the alternative reading frame product $p19^{ARF}$. *Cell* **91**, 649–659 (1997).
**This paper was the first to describe epistasis between ARF and p53, and unveiled the role of ARF as a tumour suppressor.**
10. Wright, W. E. & Shay, J. W. Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology. *Nat. Med.* **6**, 849–851 (2000).
11. Sherr, C. J. & DePinho, R. A. Cellular senescence: mitotic clock or culture shock? *Cell* **102**, 407–410 (2000).
12. Kuilman, T., Michaloglou, C., Mooi, W. J. & Peeper, D. S. The essence of senescence. *Genes Dev.* **24**, 2463–2479 (2010).
13. Rodier, F. & Campisi, J. Four faces of cellular senescence. *J. Cell Biol.* **192**, 547–556 (2011).
14. Campisi, J. Aging, cellular senescence, and cancer. *Annu. Rev. Physiol.* **75**, 685–705 (2013).
15. Collado, M., Blasco, M. A. & Serrano, M. Cellular senescence in cancer and aging. *Cell* **130**, 223–233 (2007).
16. Kim, W. Y. & Sharpless, N. E. The regulation of *INK4/ARF* in cancer and aging. *Cell* **127**, 265–275 (2006).
17. Shay, J. W., Pereira-Smith, O. M. & Wright, W. E. A role for both RB and p53 in the regulation of human cellular senescence. *Exp. Cell Res.* **196**, 33–39 (1991).
18. Beauséjour, C. M. *et al.* Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J.* **22**, 4212–4222 (2003).
**This paper reveals that low threshold levels of $p16^{INK4A}$ expression in certain senescent human cell lines facilitate their re-entry into the cell division cycle.**
19. Sage, J., Miller, A. L., Pérez-Mancera, P. A., Wysocki, J. M. & Jacks, T. Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature* **424**, 223–228 (2003).

20. Pardee, A. B. G1 events and regulation of cell proliferation. *Science* **246**, 603–608 (1989).
21. Sherr, C. J. & Roberts, J. M. Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* **9**, 1149–1163 (1995).
22. Blagosklonny, M. V. Geroconversion: irreversible step to cellular senescence. *Cell Cycle* **13**, 3628–3635 (2014).
23. Blais, A., van Oevelen, C. J. C., Margueron, R., Acosta-Alvear, D. & Dynlacht, B. D. Retinoblastoma tumor suppressor protein-dependent methylation of histone H3 lysine 27 is associated with irreversible cell cycle exit. *J. Cell Biol.* **179**, 1399–1412 (2007).
24. Burkhart, D. L. & Sage, J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat. Rev. Cancer* **8**, 671–682 (2008).
25. Indovina, P., Marcelli, E., Casini, N., Rizzo, V. & Giordano, A. Emerging roles of RB family: new defense mechanisms against tumor progression. *J. Cell. Physiol.* **228**, 525–535 (2013).
26. Kareta, M. S. *et al.* Inhibition of pluripotency networks by the Rb tumor suppressor restricts reprogramming and tumorigenesis. *Cell Stem Cell* **16**, 39–50 (2015).
27. Polyak, K. *et al.* $P27^{Kip1}$, a cyclin–Cdk inhibitor, links transforming growth-factor-β and contact inhibition to cell-cycle arrest. *Genes Dev.* **8**, 9–22 (1994).
28. Balciunaite, E. *et al.* Pocket protein complexes are recruited to distinct targets in quiescent and proliferating cells. *Mol. Cell. Biol.* **25**, 8166–8178 (2005).
**This paper details elegant analyses using combined ChIP and DNA microarrays ('ChIP-on-ChIP') that define different mechanistic roles of RB and p130 in regulating E2F target genes in quiescent and proliferating cells.**
29. El-Deiry, W. S. *et al. WAF1*, a potential mediator of p53 tumor suppression. *Cell* **75**, 817–825 (1993).
30. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**, 805–816 (1993).
31. Xiong, Y. *et al.* p21 is a universal inhibitor of cyclin kinases. *Nature* **366**, 701–704 (1993).
32. Wang, Y. A., Elson, A. & Leder, P. Loss of p21 increases sensitivity to ionizing radiation and delays the onset of lymphoma in *atm*-deficient mice. *Proc. Natl Acad. Sci. USA* **94**, 14590–14595 (1997).
33. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J. & Leder, P. Mice lacking $p21^{CIP1/WAF1}$ undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**, 675–684 (1995).
34. Martín-Caballero, J., Flores, J. M., García-Palencia, P. & Serrano, M. Tumor susceptibility of *p21*$^{Waf1/Cip1}$-deficient mice. *Cancer Res.* **61**, 6234–6238 (2001).
35. Pantoja, C. & Serrano, M. Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic Ras. *Oncogene* **18**, 4974–4982 (1999).
36. Di Leonardo, A., Linke, S. P., Clarkin, K. & Wahl, G. M. DNA damage triggers a prolonged p53-dependent $G_1$ arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev.* **8**, 2540–2551 (1994).
37. Sang, L., Coller, H. A. & Roberts, J. M. Control of the reversibility of cellular quiescence by the transcriptional repressor HES1. *Science* **321**, 1095–1100 (2008).
38. Spencer, S. L. *et al.* The proliferation-quiescence decision is controlled by a bifurcation in CDK2 activity at mitotic exit. *Cell* **155**, 369–383 (2013).
39. Krenning, L., Feringa, F. M., Shaltiel, I. A., van den Berg, J. & Medema, R. H. Transient activation of p53 in G2 phase is sufficient to induce senescence. *Mol. Cell* **55**, 59–72 (2014).
40. Alcorta, D. A. *et al.* Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc. Natl Acad. Sci. USA* **93**, 13742–13747 (1996).
41. Robles, S. J. & Adami, G. R. Agents that cause DNA double strand breaks lead to $p16^{INK4a}$ enrichment and the premature senescence of normal fibrolasts. *Oncogene* **16**, 1113–1123 (1998).
42. Stein, G. H., Drullinger, L. F., Soulard, A. & Dulic, V. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol. Cell. Biol.* **19**, 2109–2117 (1999).
43. Johmura, Y. *et al.* Necessary and sufficient role for a mitosis skip in senescence induction. *Mol. Cell* **55**, 73–84 (2014).
44. Gire, V. & Dulic, V. Senescence from G2 arrest, revisited. *Cell Cycle* **14**, 297–304 (2015).
45. Sherwood, S. W., Rush, D., Ellsworth, J. L. & Schimke, R. T. Defining cellular senescence in IMR-90 cells — a flow cytometric analysis. *Proc. Natl Acad. Sci. USA* **85**, 9086–9090 (1988).
46. Mao, Z., Ke, Z., Gorbunova, V. & Seluanov, A. Replicatively senescent cells are arrested in G1 and G2 phases. *Aging* **4**, 431–435 (2012).
47. Wagner, M. *et al.* Replicative senescence of human endothelial cells *in vitro* involves G1 arrest, polyploidization and senescence-associated apoptosis. *Exp. Gerontol.* **36**, 1327–1347 (2001).
48. Takahashi, A. *et al.* Mitogenic signalling and the $p16^{INK4a}$–Rb pathway cooperate to enforce irreversible cellular senescence. *Nat. Cell Biol.* **8**, 1291–1297 (2006).
49. Aksoy, O. *et al.* The atypical E2F family member E2F7 couples the p53 and RB pathways during cellular senescence. *Genes Dev.* **26**, 1546–1557 (2012).
50. Lanni, J. S. & Jacks, T. Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. *Mol. Cell. Biol.* **18**, 1055–1064 (1998).
51. Hayashi, M. T. & Karlseder, J. DNA damage associated with mitosis and cytokinesis failure. *Oncogene* **32**, 4593–4601 (2013).
52. Coppe, J. P. *et al.* Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol.* **6**, 2853–2868 (2008).
53. Rodier, F. *et al.* Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat. Cell Biol.* **11**, 973–979 (2009).
54. Narita, M. *et al.* Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113**, 703–716 (2003).
**This paper provides the earliest description of RB-mediated SAHF formation and its effects in silencing E2F target genes.**
55. Bartkova, J. *et al.* Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* **444**, 633–637 (2006).
56. Di Micco, R. *et al.* Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* **444**, 638–642 (2006).
**References 55 and 56 illustrate the ability of oncogene-induced stress to activate DNA damage-dependent checkpoint responses that provide a barrier to tumour formation.**
57. Rodier, F. *et al.* DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *J. Cell Sci.* **124**, 68–81 (2011).
58. Fumagalli, M., Rossiello, F., Mondello, C. & di Fagagna, F. D. Stable cellular senescence is associated with persistent DDR activation. *PLoS ONE* **9**, e110969 (2014).
59. Dimri, G. P. *et al.* A biomarker that identifies senescent human-cells in culture and in aging skin *in vivo*. *Proc. Natl Acad. Sci. USA* **92**, 9363–9367 (1995).
**This paper gives the first description of an assay for SAβ-gal activity at pH 6.0, which is now widely used to denote senescent cells.**
60. Itahana, K., Campisi, J. & Dimri, G. P. Methods to detect biomarkers of cellular senescence: the senescence-associated β-galactosidase assay. *Methods Mol. Biol.* **371**, 21–31 (2007).
61. Kurz, D. J., Decary, S., Hong, Y. & Erusalimsky, J. D. Senescence-associated β-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J. Cell Sci.* **113**, 3613–3622 (2000).
62. Lee, B. Y. *et al.* Senescence-associated β-galactosidase is lysosomal β-galactosidase. *Aging Cell* **5**, 187–195 (2006).
63. Coates, P. J., Lorimore, S. A., Rigat, B. A., Lane, D. P. & Wright, E. G. Induction of endogenous β-galactosidase by ionizing radiation complicates the analysis of p53–*lacZ* transgenic mice. *Oncogene* **20**, 7096–7097 (2001).
64. Bursuker, I., Rhodes, J. M. & Goldman, R. β-galactosidase — an indicator of the maturational stage of mouse and human mononuclear phagocytes. *J. Cell. Physiol.* **112**, 385–390 (1982).
65. Kopp, H. G., Hooper, A. T., Shmelkov, S. V. & Rafii, S. β-galactosidase staining on bone marrow. The osteoclast pitfall. *Histol. Histopathol.* **22**, 971–976 (2007).
66. Young, A. R. J. & Narita, M. Connecting autophagy to senescence in pathophysiology. *Curr. Opin. Cell Biol.* **22**, 234–240 (2010).
67. Dorr, J. R. *et al.* Synthetic lethal metabolic targeting of cellular senescence in cancer therapy. *Nature* **501**, 421–425 (2013).
68. Ivanov, A. *et al.* Lysosome-mediated processing of chromatin in senescence. *J. Cell Biol.* **202**, 129–143 (2013).
69. Serrano, M., Hannon, G. J. & Beach, D. A new regulatory motif in cell-cycle control causing specific-inhibition of cyclin-D/CDK4. *Nature* **366**, 704–707 (1993).
**This paper describes the landmark discovery of $p16^{INK4A}$ and its role as a specific inhibitor of the cyclin D-dependent kinase CDK4.**
70. Hannon, G. J. & Beach, D. $p15^{INK4b}$ is a potential effector of TGF-β-induced cell-cycle arrest. *Nature* **371**, 257–261 (1994).
71. Quelle, D. E., Zindy, F., Ashmun, R. A. & Sherr, C. J. Alternative reading frames of the *INK4a* tumor-suppressor gene encode two unrelated proteins capable of inducing cell-cycle arrest. *Cell* **83**, 993–1000 (1995).
**This paper describes the surprising discovery of a second protein (ARF) that is encoded by the mouse *Cdkn2a* gene.**
72. Pomerantz, J. *et al.* The *Ink4a* tumor suppressor gene product, $19^{Arf}$, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* **92**, 713–723 (1998).
73. Zhang, Y., Xiong, Y. & Yarbrough, W. G. ARF promotes MDM2 degradation and stabilizes p53: *ARF–INK4a* locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* **92**, 725–734 (1998).
74. Kamijo, T. *et al.* Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. *Proc. Natl Acad. Sci. USA* **95**, 8292–8297 (1998).
75. Chandler, H. & Peters, G. Stressing the cell cycle in senescence and aging. *Curr. Opin. Cell Biol.* **25**, 765–771 (2013).
76. Bignell, G. R. *et al.* Signatures of mutation and selection in the cancer genome. *Nature* **463**, 893–898 (2010).
77. Beroukhim, R. *et al.* The landscape of somatic copy-number alteration across human cancers. *Nature* **463**, 899–905 (2010).
78. Rheinwald, J. G. *et al.* A two-stage, $p16^{INK4A}$- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. *Mol. Cell. Biol.* **22**, 6930–6930 (2002).
79. Herbig, U., Jobling, W. A., Chen, B. P. C., Chen, D. J. & Sedivy, J. M. Telomere shortening triggers senescence of human cells through a pathway involving ATM, 53, and $p21^{CIP1}$, but not $p16^{INK4A}$. *Mol. Cell* **14**, 501–513 (2004).
80. Yamakoshi, K. *et al.* Real-time *in vivo* imaging of $p16^{Ink4a}$ reveals cross talk with p53. *J. Cell Biol.* **186**, 393–407 (2009).
81. Krimpenfort, P. *et al.* $p15^{Ink4b}$ is a critical tumour suppressor in the absence of $p16^{Ink4a}$. *Nature* **448**, 943–946 (2007).
82. Kuilman, T. *et al.* Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* **133**, 1019–1031 (2008).
83. Cudejko, C. *et al.* $p16^{INK4a}$ deficiency promotes IL-4-induced polarization and inhibits proinflammatory signaling in macrophages. *Blood* **118**, 2556–2566 (2011).
84. Wiedemeyer, R. *et al.* Feedback circuit among INK4 tumor suppressors constrains human glioblastoma development. *Cancer Cell* **13**, 355–364 (2008).
85. Shapiro, G. I. *et al.* Reciprocal Rb inactivation and $p16^{INK4}$ expression in primary lung cancers and cell lines. *Cancer Res.* **55**, 505–509 (1995).
86. Khleif, S. N. *et al.* Inhibition of cyclin D–CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin kinase inhibitor activity. *Proc. Natl Acad. Sci. USA* **93**, 4350–4354 (1996).
87. Nakao, Y. *et al.* Induction of p16 during immortalization by HPV 16 and 18 and not during malignant transformation. *Br. J. Cancer* **75**, 1410–1416 (1997).
88. Witkiewicz, A. K., Knudsen, K. E., Dicker, A. P. & Knudsen, E. S. The meaning of $p16^{ink4a}$ expression in tumors: functional significance, clinical associations and future developments. *Cell Cycle* **10**, 2497–2503 (2011).

89. Ohtani, N., Yamakoshi, K., Takahashi, A. & Hara, E. Real-time *in vivo* imaging of *p16$^{Ink4a}$* gene expression: a new approach to study senescence stress signaling in living animals. *Cell Div.* **5**, 1 (2010).
90. Baker, D. J. *et al.* Clearance of p16$^{Ink4a}$-positive senescent cells delays ageing-associated disorders. *Nature* **479**, 232–236 (2011).
91. Demaria, M. *et al.* An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev. Cell* **31**, 722–733 (2014).
92. Burd, C. E. *et al.* Monitoring tumorigenesis and senescence *in vivo* with a p16$^{INK4a}$-luciferase model. *Cell* **152**, 340–351 (2013).
**References 80 and 89–92 describe the use of different p16 reporters in mice and demonstrate that increased expression of p16, together with other senescence markers such as SASP and SAβ-gal, marks the accumulation of various senescent cell types during inflammation, wound healing, ageing and tumorigenesis. Conditional removal of cells persistently expressing p16 can delay the onset of age-associated pathologies and attenuate already established age-associated disorders; by contrast, the transient appearance of p16- and SASP-positive cells can promote tissue repair.**
93. Zindy, F., Quelle, D. E., Roussel, M. F. & Sherr, C. J. Expression of the p16$^{INK4a}$ tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene* **15**, 203–211 (1997).
94. Krishnamurthy, J. *et al. Ink4a/Arf* expression is a biomarker of aging. *J. Clin. Invest.* **114**, 1299–1307 (2004).
95. Waaijer, M. E. *et al.* The number of p16INK4a positive cells in human skin reflects biological age. *Aging Cell* **11**, 722–725 (2012).
96. Sorrentino, J. A. *et al. p16$^{INK4a}$* reporter mice reveal age-promoting effects of environmental toxicants. *J. Clin. Invest.* **124**, 169–173 (2014).
97. Natarajan, E. *et al.* Co-expression of p16$^{INK4A}$ and laminin 5 γ2 by microinvasive and superficial squamous cell carcinomas *in vivo* and by migrating wound and senescent keratinocytes in culture. *Am. J. Pathol.* **163**, 477–491 (2003).
98. Jun, J. I. & Lau, L. F. Cellular senescence controls fibrosis in wound healing. *Aging* **2**, 627–631 (2010).
99. Jun, J. I. & Lau, L. F. The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. *Nat. Cell Biol.* **12**, 676–685 (2010).
100. Lemster, B. H. *et al.* Induction of CD56 and TCR-independent activation of T cells with aging. *J. Immunol.* **180**, 1979–1990 (2008).
101. Liu, Y. *et al.* Expression of *p16$^{INK4a}$* in peripheral blood T-cells is a biomarker of human aging. *Aging Cell* **8**, 439–448 (2009).
102. Liu, Y. *et al.* Expression of *p16$^{INK4a}$* prevents cancer and promotes aging in lymphocytes. *Blood* **117**, 3257–3267 (2011).
103. Migliaccio, M., Raj, K., Menzel, O. & Rufer, N. Mechanisms that limit the *in vitro* proliferative potential of human CD8$^+$ T lymphocytes. *J. Immunol.* **174**, 3335–3343 (2005).
104. Erickson, S. *et al.* Involvement of the Ink4 proteins p16 and p15 in T-lymphocyte senescence. *Oncogene* **17**, 595–602 (1998).
105. Schmitt, C. A. *et al.* A senescence program controlled by p53 and p16$^{INK4a}$ contributes to the outcome of cancer therapy. *Cell* **109**, 335–346 (2002).
106. Sanoff, H. K. *et al.* Effect of cytotoxic chemotherapy on markers of molecular age in patients with breast cancer. *J. Natl Cancer Inst.* **106**, dju057 (2014).
107. Nelson, J. A. E. *et al.* Expression of *p16$^{INK4a}$* as a biomarker of T-cell aging in HIV-infected patients prior to and during antiretroviral therapy. *Aging Cell* **11**, 916–918 (2012).
108. Signer, R. A. J., Montecino-Rodriguez, E., Witte, O. N. & Dorshkind, K. Aging and cancer resistance in lymphoid progenitors are linked processes conferred by p16$^{Ink4a}$ and Arf. *Genes Dev.* **22**, 3115–3120 (2008).
109. Jaruga, E., Skierski, J., Radziszewska, E. & Sikora, E. Proliferation and apoptosis of human T cells during replicative senescence — a critical approach. *Acta Biochim. Polon.* **47**, 293–300 (2000).
110. Shi, L. *et al.* KLRG1 impairs CD4$^+$ T cell responses via p16$^{ink4a}$ and p27$^{kip1}$ pathways: role in hepatitis B vaccine failure in individuals with hepatitis C virus infection. *J. Immunol.* **192**, 649–657 (2014).
111. Di Mitri, D. *et al.* Reversible senescence in human CD4$^+$CD45RA$^+$CD27$^-$ memory T cells. *J. Immunol.* **187**, 2093–2100 (2011).
112. Liu, Y. & Sharpless, N. E. Tumor suppressor mechanisms in immune aging. *Curr. Opin. Immunol.* **21**, 431–439 (2009).
113. Akbar, A. N. & Henson, S. M. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nat. Rev. Immunol.* **11**, 289–295 (2011).
114. d'Adda di Fagagna, F. *et al.* A DNA damage checkpoint response in telomere-initiated senescence. *Nature* **426**, 194–198 (2003).
115. Takai, H., Smogorzewska, A. & de Lange, T. DNA damage foci at dysfunctional telomeres. *Curr. Biol.* **13**, 1549–1556 (2003).
116. Nakamura, A. J. *et al.* Both telomeric and non-telomeric DNA damage are determinants of mammalian cellular senescence. *Epigenetics Chromatin* **1**, 6 (2008).
117. Halazonetis, T. D., Gorgoulis, V. G. & Bartek, J. An oncogene-induced DNA damage model for cancer development. *Science* **319**, 1352–1355 (2008).
118. Bakkenist, C. J. & Kastan, M. B. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* **421**, 499–506 (2003).
119. Munro, J., Barr, N. I., Ireland, H., Morrison, V. & Parkinson, E. K. Histone deacetylase inhibitors induce a senescence-like state in human cells by a p16-dependent mechanism that is independent of a mitotic clock. *Exp. Cell Res.* **295**, 525–538 (2004).
120. Kosar, M. *et al.* Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner, and follow expression of p16$^{ink4a}$. *Cell Cycle* **10**, 457–468 (2011).
121. Di Micco, R. *et al.* Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. *Nat. Cell Biol.* **13**, 292–302 (2011).
122. Kennedy, A. L. *et al.* Senescent mouse cells fail to overtly regulate the HIRA histone chaperone and do not form robust Senescence Associated Heterochromatin Foci. *Cell Div.* **5**, 16 (2010).
123. Trimarchi, J. M. & Lees, J. A. Sibling rivalry in the E2F family. *Nat. Rev. Mol. Cell Biol.* **3**, 11–20 (2002).
124. Chandra, T. *et al.* Independence of repressive histone marks and chromatin compaction during senescent heterochromatic layer formation. *Mol. Cell* **47**, 203–214 (2012).
125. Chandra, T. & Narita, M. High-order chromatin structure and the epigenome in SAHFs. *Nucleus* **4**, 23–28 (2013).
126. Chien, Y. C. *et al.* Control of the senescence-associated secretory phenotype by NF-κB promotes senescence and enhances chemosensitivity. *Genes Dev.* **25**, 2125–2136 (2011).
127. Acosta, J. C. *et al.* A complex secretory program orchestrated by the inflammasome controls paracrine senescence. *Nat. Cell Biol.* **15**, 978–990 (2013).
**This paper details an elegant study describing the paracrine spreading of SASP during OIS and control of SASP expression by inflammasome-mediated IL-1 expression.**
128. Xue, W. *et al.* Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* **445**, 656–660 (2007).
129. Krizhanovsky, V. *et al.* Senescence of activated stellate cells limits liver fibrosis. *Cell* **134**, 657–667 (2008).
130. Lujambio, A. *et al.* Non-cell-autonomous tumor suppression by p53. *Cell* **153**, 449–460 (2013).
**References 128–130 underscore how p53 activity in both tumour cells and non-cancer cells in the liver governs aspects of the innate immune response to regulate liver fibrosis and constrain tumorigenesis.**
131. Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y. & Campisi, J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc. Natl Acad. Sci. USA* **98**, 12072–12077 (2001).
132. Kang, T. W. *et al.* Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature* **479**, 547–551 (2011).
133. Ye, J. *et al.* Human regulatory T cells induce T-lymphocyte senescence. *Blood* **120**, 2021–2031 (2012).
134. Braumuller, H. *et al.* T-helper-1-cell cytokines drive cancer into senescence. *Nature* **494**, 361–365 (2013).
135. Neves, J., Demaria, M., Campisi, J. & Jasper, H. Of flies, mice, and men: evolutionarily conserved tissue damage responses and aging. *Dev. Cell* **32**, 9–18 (2015).
136. Wolf, J., Rose-John, S. & Garbers, C. Interleukin-6 and its receptors: a highly regulated and dynamic system. *Cytokine* **70**, 11–20 (2014).
137. Ataie-Kachoie, P., Pourgholami, M. H., Richardson, D. & Morris, D. L. Gene of the month: Interleukin 6 (IL-6). *J. Clin. Pathol.* **67**, 932–937 (2014).
138. Blackburn, E. H. Structure and function of telomeres. *Nature* **350**, 569–573 (1991).
139. Lansdorp, P. M. Telomeres and disease. *EMBO J.* **28**, 2532–2540 (2009).
140. Artandi, S. E. & DePinho, R. A. Telomeres and telomerase in cancer. *Carcinogenesis* **31**, 9–18 (2010).
141. Shay, J. W. & Wright, W. E. Role of telomeres and telomerase in cancer. *Semin. Cancer Biol.* **21**, 349–353 (2011).
142. Jaskelioff, M. *et al.* Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice. *Nature* **469**, 102–106 (2011).
**Following on earlier studies using cultured human cells (reference 4) and genetically engineered telomerase-deficient mice (reference 6), this study demonstrates that telomerase reactivation in adult mice reduces DNA damage and reverses degenerative phenotypes.**
143. Armanios, M. Telomeres and age-related disease: how telomere biology informs clinical paradigms. *J. Clin. Invest.* **123**, 996–1002 (2013).
144. Aubert, G., Hills, M. & Lansdorp, P. M. Telomere length measurement — caveats and a critical assessment of the available technologies and tools. *Mutat. Res.* **730**, 59–67 (2012).
145. Hewitt, G. *et al.* Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. *Nat. Commun.* **3**, 708 (2012).
146. Rudolph, K. L. *et al.* Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell* **96**, 701–712 (1999).
147. Collado, M. *et al.* Tumour biology — senescence in premalignant tumours. *Nature* **436**, 642–642 (2005).
148. Munoz-Espin, D. *et al.* Programmed cell senescence during mammalian embryonic development. *Cell* **155**, 1104–1118 (2013).
149. Storer, M. *et al.* Senescence is a developmental mechanism that contributes to embryonic growth and patterning. *Cell* **155**, 1119–1130 (2013).
**References 148 and 149 provocatively conclude that senescent cells transiently appear during various stages of mouse embryonic development, when they function to promote tissue remodelling. Is this the evolutionary origin of damage-induced senescence in adult animals?**
150. Huang, T. & Rivera-Pérez, J. A. Senescence-associated β-galactosidase activity marks the visceral endoderm of mouse embryos but is not indicative of senescence. *Genesis* **52**, 300–308 (2014).
151. Li, C., Finkelstein, D. & Sherr, C. J. *Arf* tumor suppressor and miR-205 regulate cell adhesion and formation of extraembryonic endoderm from pluripotent stem cells. *Proc. Natl Acad. Sci. USA* **110**, E1112–E1121 (2013).
152. Michaloglou, C. *et al.* BRAF$^{E600}$-associated senescence-like cell cycle arrest of human naevi. *Nature* **436**, 720–724 (2005).
153. Gray-Schopfer, V. C. *et al.* Cellular senescence in naevi and immortalisation in melanoma: a role for p16? *Br. J. Cancer* **95**, 496–505 (2006).
154. Vredeveld, L. C. *et al.* Abrogation of BRAF$^{V600E}$-induced senescence by PI3K pathway activation contributes to melanomagenesis. *Genes Dev.* **26**, 1055–1069 (2012).
155. Damsky, W. *et al.* mTORC1 activation blocks *Braf$^{V600E}$*-induced growth arrest but is insufficient for melanoma formation. *Cancer Cell* **27**, 41–56 (2015).
156. Sharpless, N. E. & Depinho, R. A. How stem cells age and why this makes us grow old. *Nat. Rev. Mol. Cell Biol.* **8**, 703–713 (2007).

157. Munoz-Espin, D. & Serrano, M. Cellular senescence: from physiology to pathology. *Nat. Rev. Mol. Cell Biol.* **15**, 482–496 (2014).
158. Martin, N., Beach, D. & Gill, J. Ageing as developmental decay: insights from p16$^{INK4a}$. *Trends Mol. Med.* **20**, 667–674 (2014).
159. van Deursen, J. M. The role of senescent cells in ageing. *Nature* **509**, 439–446 (2014).
160. Signer, R. A. J. & Morrison, S. J. Mechanisms that regulate stem cell aging and life span. *Cell Stem Cell* **12**, 152–165 (2013).
161. Zhu, Y. *et al.* The Achilles heel of senescent cells: from transcriptome to senolytic drugs. *Aging Cell* http://dx.doi.org/10.1111/acel.12344 (2015).
162. González-Navarro, H. *et al.* p19$^{ARF}$ deficiency reduces macrophage and vascular smooth muscle cell apoptosis and aggravates atherosclerosis. *J. Am. Coll. Cardiol.* **55**, 2258–2268 (2010).
163. Herbig, U., Ferreira, M., Condel, L., Carey, D. & Sedivy, J. M. Cellular senescence in aging primates. *Science* **311**, 1257 (2006).
164. Ershler, W. B. & Keller, E. T. Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. *Annu. Rev. Med.* **51**, 245–270 (2000).
165. Krishnamurthy, J. *et al.* p16$^{INK4a}$ induces an age-dependent decline in islet regenerative potential. *Nature* **443**, 453–457 (2006).
166. Janzen, V. *et al.* Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* **443**, 421–426 (2006).
167. Molofsky, A. V. *et al.* Increasing *p16$^{INK4a}$* expression decreases forebrain progenitors and neurogenesis during ageing. *Nature* **443**, 448–452 (2006).
168. Chen, H. *et al.* PDGF signalling controls age-dependent proliferation in pancreatic β-cells. *Nature* **478**, 349–355 (2011).
169. Cosgrove, B. D. *et al.* Rejuvenation of the muscle stem cell population restores strength to injured aged muscles. *Nat. Med.* **20**, 255–264 (2014).
170. Sousa-Victor, P. *et al.* Geriatric muscle stem cells switch reversible quiescence into senescence. *Nature* **506**, 316–321 (2014).
171. Berent-Maoz, B., Montecino-Rodriguez, E., Signer, R. A. J. & Dorshkind, K. Fibroblast growth factor-7 partially reverses murine thymocyte progenitor aging by repression of *Ink4a*. *Blood* **119**, 5715–5721 (2012).
172. Baker, D. J. *et al.* Opposing roles for p16$^{Ink4a}$ and p19$^{Arf}$ in senescence and ageing caused by BubR1 insufficiency. *Nat. Cell Biol.* **10**, 825–836 (2008).
173. Jeck, W. R., Siebold, A. P. & Sharpless, N. E. Review: a meta-analysis of GWAS and age-associated diseases. *Aging Cell* **11**, 727–731 (2012).
174. Sherr, C. J. *Ink4–Arf* locus in cancer and aging. *Wiley Interdiscip. Rev. Dev. Biol.* **1**, 731–741 (2012).
175. LaPak, K. M. & Burd, C. E. The molecular balancing act of p16$^{INK4a}$ in cancer and aging. *Mol. Cancer Res.* **12**, 167–183 (2014).
176. Henson, J. D., Neumann, A. A., Yeager, T. R. & Reddel, R. R. Alternative lengthening of telomeres in mammalian cells. *Oncogene* **21**, 598–610 (2002).
177. O'Sullivan, R. J. & Almouzni, G. Assembly of telomeric chromatin to create ALTernative endings. *Trends Cell Biol.* **24**, 675–685 (2014).
178. Cho, N. W., Dilley, R. L., Lampson, M. A. & Greenberg, R. A. Interchromosomal homology searches drive directional ALT telomere movement and synapsis. *Cell* **159**, 108–121 (2014).
179. Boquoi, A. *et al.* Reversible cell cycle inhibition and premature aging features imposed by conditional expression of p16$^{Ink4a}$. *Aging Cell* **14**, 139–147 (2015).
180. Althubiti, M. *et al.* Characterization of novel markers of senescence and their prognostic potential in cancer. *Cell Death Dis.* **5**, e1528 (2014).



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