

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 capacity⁵ and undergo senescence, although telomere attrition is not responsible⁶. 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 antigen¹⁷, 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 cells¹⁸ or conditional deletion of *Rb1* in mouse embryonic fibroblasts¹⁹ 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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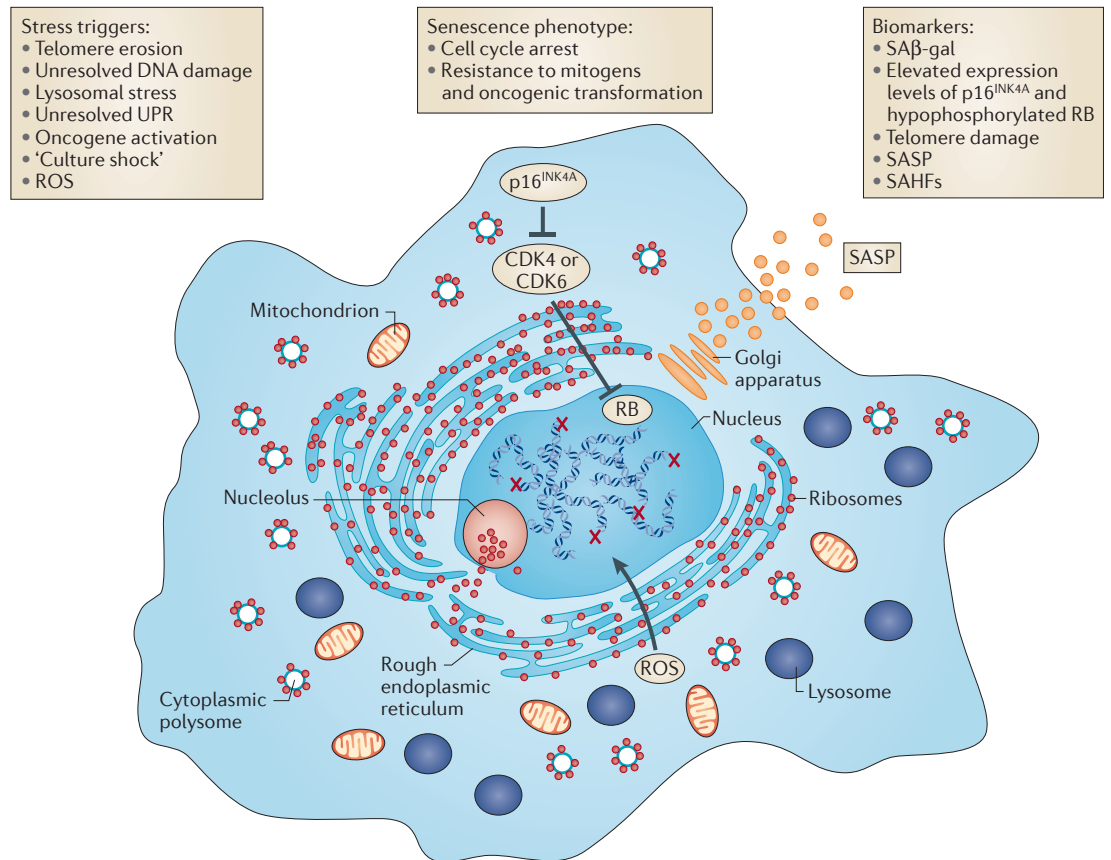


Figure 1 | Stress triggers and biomarkers of senescence. We define permanent growth arrest in response to various cellular stresses as the *sine qua non* of cellular senescence. The senescent state is accompanied by a failure to re-enter the cell division cycle in response to mitogens and by an acquired 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). SA β -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 cycle¹⁴.

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 • SAβ-gal (lysosomal stress) • p16^{INK4A} • DNA-damage response • SASP • 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 state²². 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 systems³⁵, 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 activation³⁶, more than 4 days of experimentally enforced p21^{CIP1} expression has been reported to initiate senescence³⁷. 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 cycle³⁸. 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 evidence⁴⁴. 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 cytokinesis⁴⁸ 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 exit⁴⁴ or mitotic skipping⁴³, 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 inhibitors⁵⁰ or that have acute telomere deprotection and persistent DNA damage⁵¹. 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 (SAHF), which are associated with S-phase-promoting gene loci, such as E2F target genes⁵⁴. 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 SA β -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 pH⁶³. 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 exposure⁶³. Even contact-inhibited quiescent cells maintained for prolonged periods in culture can upregulate β -galactosidase activity⁶⁰.

Such observations set clear limitations in interpreting the meaning of SA β -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 SA β -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 SA β -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 fibroblasts⁹, whereas p16^{INK4A} is a key regulator of *in vitro* senescence in human cells⁷⁵. 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 RB 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 approaches⁹². 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 differentiation⁸³, 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 infection¹⁰⁷. 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 intact¹⁰⁸. 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)

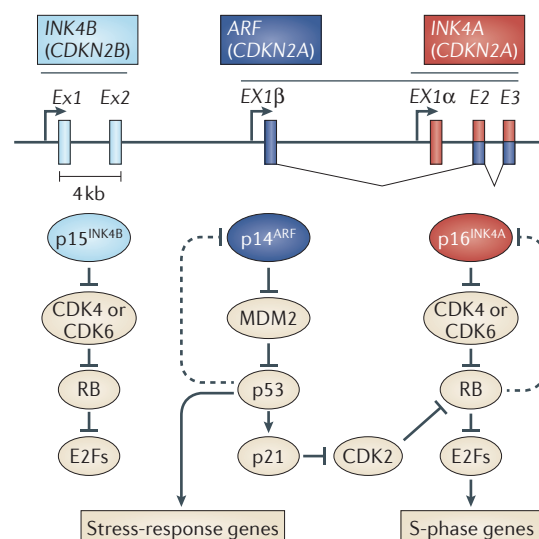


Figure 2 | The *CDKN2A*–*CDKN2B* locus. The cyclin-dependent kinase inhibitor 2A (*CDKN2A*)–*CDKN2B* locus, which is less than 50 kb in overall length, encodes three tumour-suppressor proteins. Exons within the locus are indicated by coloured vertical bars, and the three promoters are indicated by arrows. 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. Figure adapted with permission from REF. 71, Elsevier, and REF. 174, Wiley Periodicals.

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} signaling that is required for growth arrest^{55–58,116}. In precancerous lesions, double-strand DNA breaks, possibly arising

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.

from unrepaired replication errors or oxidative DNA damage, activate p53 and p21^{CIP1} to induce either apoptosis or senescence¹¹⁷. Although foci of DNA damage, detected through staining of γ H2AX 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 SA β -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 chromatin⁵⁴. 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 senescence²³, contributing to the formation of SAHFs and having a key causal role in the establishment of senescence⁵⁴. 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- κ B (NF- κ B)-mediated signalling¹²⁶. SASP factors are highly pleiotropic and induce a host of local activities, including reduced replicative capacity¹²⁷, 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-1 α , IL-6, IL-8 and transforming growth factor- β (TGF β), 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 manner¹²⁷. 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 cells¹³⁵. 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- κ B 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 integrity⁴. Similarly, in inbred strains of laboratory mice engineered to undergo telomere attrition and exhibit premature ageing phenotypes, reactivation of telomerase can reverse degenerative traits¹⁴². 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 utility¹⁴⁴. As with the

γ H2AX

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.

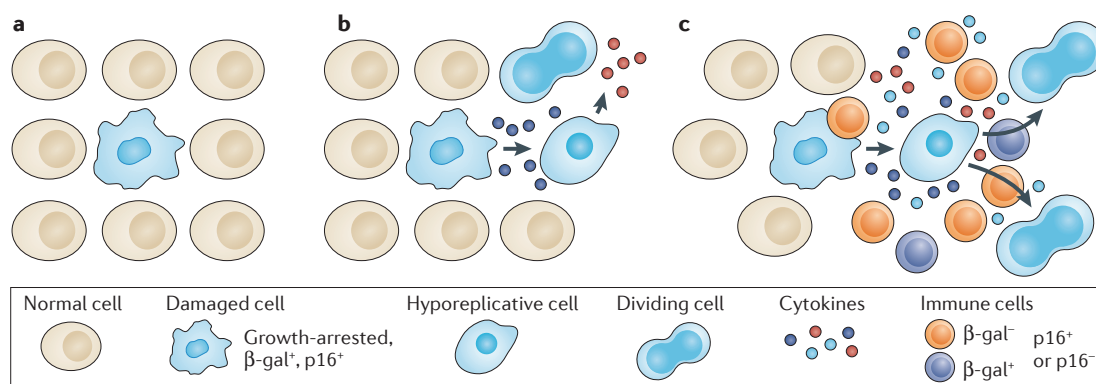


Figure 3 | Senescence in vivo. **a** | Stress-induced, damaged cells accumulate in a field of normal cells. Damaged cells undergo growth arrest and express p16^{INK4A} and β -galactosidase (β -gal). **b** | Damaged and hyporeplicative cells secrete a complex suite of cytokines that produce multiple paracrine 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.

expression of p16^{INK4A}, telomere shortening and dysfunction can 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 length¹⁴⁵. 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 SA β -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 (SA β -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 SA β -gal expression, consider the recently described entity of ‘developmental senescence’ (REFS 148,149). Here, the authors documented cell cycle arrest, SA β -gal expression and diffuse heterochromatin changes in developing embryos. Specifically, SA β -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, TGF β –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 SA β -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

SA β -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 SA β -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 SA β -gal-positive cells were actively cycling¹⁵⁰. Notably, the p19^{ARF} tumour suppressor, but not p16^{INK4A}, is concordantly expressed in the proliferating embryonic visceral endoderm and extra-embryonic yolk sac¹⁵¹. 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 SA β -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 pre-neoplastic 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 SA β -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 SA β -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 SA β -gal and inflammatory cytokines, through activation of established pathways, such as those dependent on NF- κ B. 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 ageing¹⁶¹.

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 kyphosis⁹⁰. 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.

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Competing interests statement

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ERRATUM

Forging a signature of *in vivo* senescence

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