CellPress
OPEN ACCESS

Cell
Leading Edge

Primer

Guidelines for minimal information on cellular senescence experimentation *in vivo*

Mikolaj Ogrodnik,[1,2,3,*] Juan Carlos Acosta,[4,5] Peter D. Adams,[6] Fabrizio d'Adda di Fagagna,[7,8] Darren J. Baker,[9] Cleo L. Bishop,[10] Tamir Chandra,[11] Manuel Collado,[12,13] Jesus Gil,[14,15] Vassilis Gorgoulis,[16,17,18,19,20] Florian Gruber,[21,22] Eiji Hara,[23,24] Pidder Jansen-Dürr,[25] Diana Jurk,[26] Sundeep Khosla,[27] James L. Kirkland,[28,29] Valery Krizhanovsky,[30]

(Author list continued on next page)

[1]Ludwig Boltzmann Research Group Senescence and Healing of Wounds, 1200 Vienna, Austria
[2]Ludwig Boltzmann Institute for Traumatology, The Research Centre in Cooperation with AUVA, 1200 Vienna, Austria
[3]Austrian Cluster for Tissue Regeneration, Vienna, Austria
[4]Cancer Research UK Scotland Centre, Institute of Genetics and Cancer, University of Edinburgh, Edinburgh EH4 2XR, UK
[5]Instituto de Biomedicina y Biotecnología de Cantabria, IBBTEC (CSIC, Universidad de Cantabria), C/ Albert Einstein 22, 39011 Santander, Spain
[6]Cancer Genome and Epigenetics Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA
[7]IFOM ETS – The AIRC Institute of Molecular Oncology, Milan, Italy
[8]Institute of Molecular Genetics IGM-CNR "Luigi Luca Cavalli-Sforza," Pavia, Italy
[9]Department of Biochemistry and Molecular Biology, Department of Pediatric and Adolescent Medicine, Robert and Arlene Kogod Center on Aging, Mayo Clinic, 200 First Steet SW, Rochester, MN 55905, USA
[10]Blizard Institute, Barts and The London Faculty of Medicine and Dentistry, Queen Mary University of London, 4 Newark Street, London E1 2AT, UK
[11]MRC Human Generics Unit, University of Edinburgh, Edinburgh, UK
[12]Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, Spain
[13]Department of Immunology and Oncology (DIO), Centro Nacional de Biotecnología (CNB-CSIC), Madrid, Spain
[14]MRC Laboratory of Medical Sciences (LMS), Du Cane Road, London W12 0NN, UK
[15]Institute of Clinical Sciences (ICS), Faculty of Medicine, Imperial College London, Du Cane Road, London W12 0NN, UK
[16]Molecular Carcinogenesis Group, Department of Histology and Embryology, Medical School, National and Kapodistrian University of Athens, 11527 Athens, Greece
[17]Biomedical Research Foundation, Academy of Athens, 11527 Athens, Greece
[18]Ninewells Hospital and Medical School, University of Dundee, Dundee DD19SY, UK
[19]Faculty Institute for Cancer Sciences, Manchester Academic Health Sciences Centre, University of Manchester, Manchester M20 4GJ, UK

(Affiliations continued on next page)

SUMMARY

Cellular senescence is a cell fate triggered in response to stress and is characterized by stable cell-cycle arrest and a hypersecretory state. It has diverse biological roles, ranging from tissue repair to chronic disease. The development of new tools to study senescence *in vivo* has paved the way for uncovering its physiological and pathological roles and testing senescent cells as a therapeutic target. However, the lack of specific and broadly applicable markers makes it difficult to identify and characterize senescent cells in tissues and living organisms. To address this, we provide practical guidelines called "minimum information for cellular senescence experimentation *in vivo*" (MICSE). It presents an overview of senescence markers in rodent tissues, transgenic models, non-mammalian systems, human tissues, and tumors and their use in the identification and specification of senescent cells. These guidelines provide a uniform, state-of-the-art, and accessible toolset to improve our understanding of cellular senescence *in vivo*.

INTRODUCTION

The first published description of cellular senescence by Hayflick and Moorhead[1] defined it as a limit to the proliferative capacity of human fibroblasts in cell culture. Since then, tremendous progress has been made in the discovery of inducers and the fundamental mechanistic properties of cellular senescence in cell culture systems. Further expansion of senescence characterization in animal models and human samples has enabled the discovery of the profound impact of cellular senescence on physiology and pathology.

CellPress
OPEN ACCESS

Tohru Minamino,[31,32] Laura J. Niedernhofer,[33] João F. Passos,[26] Nadja A.R. Ring,[1,2,3] Heinz Redl,[1,2,3] Paul D. Robbins,[33] Francis Rodier,[34,35,36] Karin Scharffetter-Kochanek,[37] John M. Sedivy,[38] Ewa Sikora,[39] Kenneth Witwer,[40,41] Thomas von Zglinicki,[42] Maximina H. Yun,[43,44,45] Johannes Grillari,[2,3,46,*] and Marco Demaria[47,*]
[20]Faculty of Health and Medical Sciences, University of Surrey, Surrey GU2 7YH, UK
[21]Department of Dermatology, Medical University of Vienna, Vienna, Austria
[22]Christian Doppler Laboratory for Skin Multimodal Imaging of Aging and Senescence – SKINMAGINE, Vienna, Austria
[23]Research Institute for Microbial Diseases (RIMD), Osaka University, Suita 565-0871, Japan
[24]Immunology Frontier Research Center (IFReC), Osaka University, Suita 565-0871, Japan
[25]Institute for Biomedical Aging Research, University of Innsbruck, and Center for Molecular Biosciences Innsbruck (CMBI), Innsbruck, Austria
[26]Mayo Clinic, Department of Physiology and Biomedical Engineering, Robert and Arlene Kogod Center on Aging, Rochester, MN, USA
[27]Kogod Center on Aging and Division of Endocrinology, Mayo Clinic, Rochester, MN, USA
[28]Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN, USA
[29]Division of General Internal Medicine, Department of Medicine, Mayo Clinic, Rochester, MN, USA
[30]Department of Molecular Cell Biology, The Weizmann Institute of Science, 7610001 Rehovot, Israel
[31]Department of Cardiovascular Biology and Medicine, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan
[32]Japan Agency for Medical Research and Development-Core Research for Evolutionary Medical Science and Technology (AMED-CREST), Japan Agency for Medical Research and Development, Tokyo 100-0004, Japan
[33]Institute on the Biology of Aging and Metabolism, Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 6-155 Jackson Hall, 321 Church Street, SE, Minneapolis, MN 55455, USA
[34]Centre de recherche du Centre hospitalier de l'Université de Montréal (CRCHUM), Montreal, QC, Canada
[35]Institut du cancer de Montréal, Montreal, QC, Canada
[36]Université de Montréal, Département de radiologie, radio-oncologie et médicine nucléaire, Montreal, QC, Canada
[37]Department f Dermatology and Allergic Diseases, Ulm University Hospital, Albert-Einstein-Allee 23, 89081 Ulm, Germany
[38]Department of Molecular, Cellular Biology and Biochemistry, Center on the Biology of Aging, Brown University, Providence, RI, USA
[39]Laboratory of Molecular Bases of Aging, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 02-093 Warsaw, Poland
[40]The Johns Hopkins University School of Medicine, Department of Molecular and Comparative Pathobiology, Baltimore, MD, USA
[41]The Johns Hopkins University School of Medicine, Department of Neurology, Baltimore, MD, USA
[42]Newcastle University Biosciences Institute, Ageing Biology Laboratories, Newcastle upon Tyne, UK
[43]Technische Universität Dresden, CRTD/Center for Regenerative Therapies Dresden, Dresden, Germany
[44]Max Planck Institute of Molecular Cellular Biology and Genetics, Dresden, Germany
[45]Physics of Life Excellence Cluster, Dresden, Germany
[46]Institute of Molecular Biotechnology, BOKU University, Vienna, Austria
[47]European Research Institute for the Biology of Ageing (ERIBA), University Medical Center Groningen (UMCG), University of Groningen (RUG), Groningen, the Netherlands
*Correspondence: mikolaj.ogrodnik@lbg.ac.at (M.O.), johannes.grillari@lbg.ac.at (J.G.), m.demaria@umcg.nl (M.D.)
https://doi.org/10.1016/j.cell.2024.05.059

However, the identification and characterization of senescent cells *in vivo* have several conceptual, methodological, and practical challenges. An important limitation is that classical markers of senescence, originally discovered and established using cell culture models, have limited utility for experimentation in the natural tissue microenvironment or "*in situ*." In addition, the lack of a specific biomarker for cellular senescence demands that multiple endpoints be measured, requiring multiplexing and measuring nucleic acids, proteins, and enzymatic activity in the same or adjacent tissue sections. Microarray and extracellular vesicle studies encounter similar challenges. The establishment of the so-called "minimum information for experimentation" (for example, "minimum information about a microarray experiment" [MIAME] or "minimal information for studies of extracellular vesicles" [MISEV]) led to an improvement in data reporting quality, harmonization, and reproducibility.[2,3]

Inspired by these guidelines and by the possibility to foster high quality and reproducibility in research on cellular senescence *in vivo*, we present the "minimum information for cellular senescence experimentation *in vivo*" (MICSE). These recommendations aim to:

- secure high-quality reporting and content;
- be accessible and easily understandable;
- be adaptable to progress and updates in the field; and
- be used for research but *not* for clinical trials.

The collection of practical guidelines and recommendations described here is primarily intended to facilitate the assessment of cellular senescence markers in living animals (*in vivo*) and *in situ*. Finally, this study focuses on cellular senescence research and does not aim to provide information on overcoming the general technological limitations of methodologies, such as autofluorescence for immunohistofluorescence (IHF) or mixed cell populations in tissue homogenates used for RT-qPCR or western blot (WB).

MICSE is a description of the *status quo* of the toolkit available for senescence detection and analysis and should be used as a guidebook for research on senescence *in vivo* and *in situ*.

FUNCTIONALITY, RELIABILITY, AND KNOW-HOW OF CELLULAR SENESCENCE MARKERS *IN SITU*

No single biomarker is sufficient to detect cellular senescence *in vivo*, and multiplexing remains a necessary requirement.

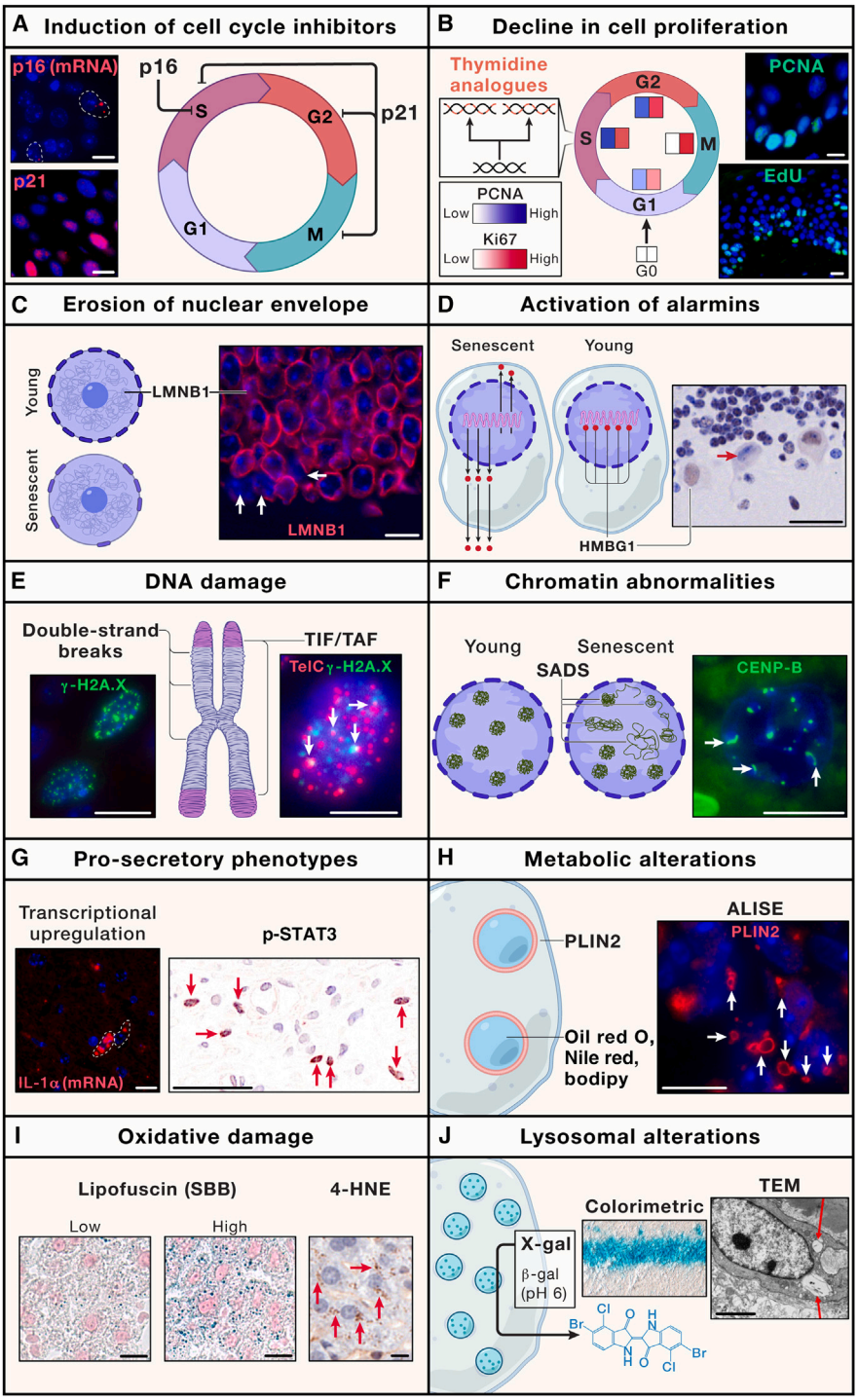

Figure 1. Markers of cellular senescence *in situ*

(A) Cell cycle inhibitors arrest cells in various phases: G1 → S (in the case of $p16^{Ink4a}$) and G1 → S, S → G2, and G2 → M (for $p21^{Cip1/Waf}$). Images show RNA *in situ* hybridization (RNA-ISH) for p16 of an aged brain and IHF staining against p21 in the wounded epidermis.

(B) Detection of cell proliferation can be performed using antibodies against Ki67 and PCNA or by visualizing the incorporation of thymidine analogs into DNA. The images show EdU incorporation over 6 h on the bottom right and PCNA staining on the top right, both wounded skin samples.

(C) Erosion of the nuclear envelope was visualized by detection of lamin B1 (LMNB1). The images show neurons in the dentate gyrus of an aged mouse. White arrows show cells negative for LMNB1.

(D) Senescent cells release Hmgb1 from chromatin, which translocates to the cytoplasm and outside the cell. The image shows the cerebellum of the aged mouse. The red arrow shows a Purkinje neuron negative for intranuclear HMGB1.

(legend continued on next page)

However, there is still uncertainty regarding which markers are suitable for *in situ/in vivo* experiments, how many markers are sufficient to define a cell as senescent, which markers should be prioritized to achieve the highest sensitivity and specificity, and what the most robust and reliable combinations of endpoints are to detect senescent cells across different tissues, disease conditions, and organismal ages. Here, we discuss the functional and practical aspects of senescence biomarkers *in situ* (Figure 1), focusing primarily on the markers for which there is the most evidence for their association with senescence in mouse tissues, as well as those that are most accessible to the global research community. Exemplary tissues/conditions that could be used as positive controls to prove the presence or absence of senescent cells and the recommended reagents are listed in Table 1.

Induction of cell cycle inhibitors

Background/function

Stable cell-cycle arrest has been one of the core features of cellular senescence,[23] and the expression of proteins involved in maintaining proliferative arrest can be used as the primary criterion when assessing senescence *in situ*. Although there are multiple cyclin-dependent kinase inhibitors (CDKIs), $p21^{Cip1/Waf}$ (*CDKN1A* locus; encoding $p21^{Cip1/Waf}$) and $p16^{Ink4a}$ (*CDKN2A* locus; encoding $p16^{Ink4a}$) are the inhibitors most commonly expressed by senescent cells *in vivo* (Figure 1A). Additional CDKIs, such as $p15^{Ink4b}$, $p19^{Ink4d}$, and $p27^{Kip1}$, could be potentially involved in the induction of cellular senescence,[24] but there is currently little evidence to support the use of their expression as markers of senescence *in situ* or *in vivo*.

Methodology and limitations

Measurements of *CDKN1A*/$p21^{Cip1/Waf}$ transcripts and proteins are well established, with several robust primers, probes, and antibodies available. By contrast, reliable detection of $p16^{Ink4a}$ poses challenges. One consideration for measuring $p16^{Ink4a}$ mRNA is that the *CDKN2A* locus encodes another mRNA coding for $p19^{Arf}$ (mouse)/$p14^{ARF}$ (human), which shares the second exon of *CDKN2A*, generating a region of sequence similarity between these two transcripts.[24] Thus, certain generic probes and primers designed to detect transcripts from the *CDKN2A* locus can simultaneously measure Arf and Ink4a mRNAs, making the validation of detection strategies essential. Although $p14/19^{Arf}$ and $p16^{Ink4a}$ have no overlapping amino acid sequences owing to the different reading frames of the transcripts, the detection of the $p16^{Ink4a}$ protein has proven difficult in mouse tissues. Nonetheless, several recent publications have successfully used anti-murine $p16^{Ink4a}$ antibodies in various applications.[25–27] Thus, while the community is working intensely to validate robust anti-mouse $p16^{Ink4a}$ antibodies, we highly recommend using a series of positive and negative controls, the latter ideally derived from p16-knockout (KO) mice. We also recommend the use of verified probes and primers for $p16^{Ink4a}$ transcripts. In addition, for both CDKIs, several transgenic reporter mouse models have been developed to aid senescence detection *in situ* and *in vivo*.

Finally, although the *stability* of cell-cycle arrest has been canonically associated with cultured senescent cells, this feature has not yet been verified as being associated with senescence *in situ* or *in vivo*. The available methodology for *in situ* and *in vivo* measurements offers only a snapshot of cell physiology and, apart from transgenic "tracker" animals, it is not yet possible to validate the persistence of $p16^{Ink4a}$ or $p21^{Cip1/Waf}$ expression *in situ* or *in vivo*. Therefore, while we recommend testing several time points to verify elevated levels of one or more cell cycle inhibitors, there are currently no easily accessible tools that allow researchers to provide evidence of persistent cell-cycle arrest. In summary, the cell cycle inhibitors $p21^{Cip1/Waf}$ and/or $p16^{Ink4a}$ are integral to senescence, both *in situ* and *in vivo*, and we recommend that every rodent study utilizes at least one of these.

Decline in cell proliferation

Background/function

Stable cell-cycle arrest is recognized as a core marker of cellular senescence. *In situ* cell proliferation can be approximated by measuring the levels of proteins involved in cell cycle progression, such as PCNA and KI67, as well as by measuring the incorporation of thymidine analogs into DNA (Figure 1B). Specifically, the levels of PCNA and KI67 provide an estimation of which cell cycle phase a cell is in, with PCNA being at the highest level in the S phase and KI67 in the M phase[28,29] (Figure 1B). Thymidine analogs, such as bromodeoxyuridine (BrdU), EdU, and IdU, are incorporated during DNA replication and can thus be used to label cells that have undergone DNA replication from the time the animal is exposed to the respective analog. Other methods to assess

(E) Double-strand breaks (DSBs) can occur at telomeres (TIF, telomere-induced foci; or TAF, telomere-associated foci) or anywhere else on the chromosomes. Images show IHF staining against γ-H2A.X and immunoFISH co-staining for γ-H2A.X and TelC in the wounded skin. White arrows show colocalization of γ-H2A.X and TelC signals, indicating TAF.
(F) Alteration of chromatin in senescent cells *in situ* can be visualized using FISH staining against sequences of peri-centromeric alpha satellites. The image shows the hepatocytes in an aged liver. White arrows show senescence-associated satellite decondensation of satellites (SADS).
(G) Senescence-associated secretory phenotype (SASP) can be visualized *in situ* by RNA-ISH (e.g., IL-1α in aged brain) or histologically by detecting the activation of pro-inflammatory pathways, for example, p-STAT3 (Y705) in wounded skin. White outlines show cells positive for IL-1α (left image) and red arrows show cells positive for p-STAT3 (right image).
(H) One facet of metabolic disruption in senescent cells involves an increased accumulation of lipid droplets (LDs) that can be visualized by staining against perilipin 2 (Plin2) or with dyes against neutral lipids such as Nile red, oil red O, or BODIPY. The image shows LDs in cells surrounding the lateral ventricle of an aged mouse brain. White arrows mark LDs.
(I) Increased production of reactive oxygen species (ROS) by senescent cells can be detected by visualizing oxidative damage, such as lipofuscin and 4-hydroxynonenal (4-HNE). Exemplary images show Sudan Black B staining in aged livers and IHC for 4-HNE in aged livers. Red arrows indicate clusters of 4-HNE-positive granules.
(J) Senescence-associated β-galactosidase (SA-β-gal) represents lysosomal activity and can be visualized in an enzymatic assay for β-galactosidase activity at acidic pH using colorimetric, fluorescent, or TEM-detectable reagents. Images show the hippocampal CA3 region from an aged brain and X-gal crystals visualized by TEM in a macrophage from an atherosclerotic plaque. Red arrows indicate the X-gal crystals.
Scale bars: 10 μm for (A)–(C), (F), (G, left), (H), and (I); 50 μm for (D) and (G, right); 5 μm for (E); 2 μm for (J, right).

Table 1. Positive control for *in situ* assessment of senescence in mouse tissues

Name/method	Tissue (cells)	Condition	Exemplary articles	Exemplary reagents
p16 (RT-qPCR, RNA-ISH)	abdominal fat, liver	aging, obesity	Baker et al.[4] and Ovadya et al.[5]	TaqMan probe: Thermo Fisher Mm.PT.58.42804808
p21 (IHC/IHF, RNA-ISH and WB)	skin (keratinocytes), liver (fibroblasts), lung (epithelial cells)	acute wounds, fibrosis, chronic inflammation	Ring et al.,[6] Sagiv et al.,[7] Levi et al.,[8] and Krizhanovsky et al.[9]	p21, Ab: RRID:AB_10891759; RRID:AB_2734729; RNA-ISH probe: Bio-Techne #408551
Ki67/PCNA (IHF/IHC), EdU (Click-it)	skin (epidermis), liver (hepatocytes)	acute wound, liver chemical and mechanical injury	Ring et al.,[6] Krizhanovsky et al.,[9] and Jurk et al.[10]	Ki67 Ab: RRID:AB_443209; PCNA Ab: RRID:AB_303394; EdU detection kit: EdU Click-488 Thermo Fisher C10337
LMNB1 reduction (IHF)	brain (neurons), skin (keratinocytes)	aging	Ogrodnik et al.[11] and Dreesen et al.[12]	LMNB1 Ab: RRID:AB_443298
γ-H2A.X/TAF (IHF)	liver (hepatocytes), lung (epithelial cells)	aging, obesity, chronic inflammation	Sagiv e al.,[7] Hewitt et al.,[13] and Ogrodnik et al.[14]	γ-H2A.X Ab: RRID:AB_2118009; TelC probe: PNA Bio (F1002)
SADS (FISH)	liver (hepatocytes), bone (osteoclasts)	aging	Ogrodnik et al.[14] and Farr et al.[15]	CENPB probe: PNA Bio (F3002)
ALISE (colorimetric/IHF)	brain (ependymal cells), liver (hepatocytes)	aging, obesity	Ogrodnik et al.[14,16]	Plin2 Ab: RRID:AB_2895086; BODIPY dyes (ThermoFisher)
Lipofuscin (fluorescence; colorimetric)	brain (neurons), heart (cardiomyocytes)	aging	Jurk et al.[17] and Li et al.[18]	fluorescent microscopy; Sudan Black B; SenTraGor
4-HNE (IHC/IHF)	brain (neurons), liver (hepatocytes)	aging	Jurk et al.[17] and Nelson et al.[19]	4-HNE Ab: RRID:AB_1106813
SA-β-gal (colorimetric, TEM)	abdominal fat, brain, heart (macrophages), lung (epithelial cells)	aging, obesity	Baker et al.[4] Sagiv et al.,[7] Levi et al.,[8] Ogrodnik et al.,[16] and Childs et al.[20]	individual reagents or a detection kit: Cell Signaling Staining Kit #9860
HMGB1 release	lung (epithelial cells), muscle	chronic inflammation, aging	Sagiv et al.[7] and Zhang et al.[21]	HMGB1 Ab: RRID:AB_444360
IL-1α (IHC)	liver	irradiation	Dou et al.[22]	IL-1α Ab: RRID:AB_354473

replication rates are available; however, to date, there has been limited characterization in the context of senescence *in vivo*.

Methodology and limitations

The level of cell proliferation is very different between cell culture and *in vivo* conditions. Senescence studies in culture are often based on fibroblasts or epithelial cells, both of which are characterized by active proliferation. In contrast, the majority of cells divide *in vivo* once every few weeks or months, and many cells are in a post-mitotic state. Thus, the lack of PCNA or KI67 staining or thymidine analog incorporation can be a strong indicator for senescence induction in most cell culture experiments, but the lack of proliferation *in vivo* is not sufficient to indicate stable cell-cycle arrest and cellular senescence. Therefore, we recommend using the reduction of cell proliferation markers as an auxiliary marker to be coupled to the expression level of cell cycle inhibitors.

Erosion of the nuclear envelope

Background/function

The nuclear envelope ensures structural integrity and chromatin stability. The induction of cellular senescence has been shown to cause a reduction in certain components of the nuclear envelope, especially the structural protein lamin B1[30] (LMNB1; Figure 1C). Although other lamins and components of the nuclear envelope could potentially be dysregulated during senescence in particular tissues and cell subpopulations, downregulation of LMNB1 expression is currently the most suitable nuclear envelope marker for *in situ* measurement of cellular senescence.

Methodology and limitations

A key advantage of measuring LMNB1 abundance *in situ* is the availability of highly specific antibodies. Moreover, predominant perinuclear localization facilitates the assessment of antibody specificity. A limitation for the use of LMNB1 as a marker of senescence is its variable baseline expression among different cell types, which makes the definition of "positive" and "negative" rather arbitrary. Thus, quantitative comparisons of LMNB1 in tissue homogenates must consider the potentially different proportions of cell types within a tissue. In addition, it is not clear how changes in the nuclear envelope develop *in situ* and whether LMNB1 expression decreases across the envelope or results in the appearance of "holes"—local absence of this protein.

Activation of alarmins

Background/function

Alarmins are endogenous proteins that are rapidly released from cells upon tissue stress or damage.[31] One of the most well-recognized alarmins, the high-mobility group box 1 (HMGB1) protein, acts as an architectural chromatin-binding factor that interacts with histones and transcription factors. Upon stress, HMGB1 is rapidly translocated to the cytoplasm and secreted into the extracellular space, where it has a paracrine effect on surrounding cells.[31] The release of HMGB1 from the nucleus is an early sign of senescence induction[32] and can be detected *in situ* (Figure 1D). The role of many additional proteins of the HMG superfamily is yet to be characterized in the context of cellular senescence *in vivo*.

Methodology and limitations

Akin to LMNB1, several highly specific antibodies against HMGB1 are commercially available. Reliable and unbiased quantification of this marker presents challenges similar to those of LMNB1, where the basal protein level differs between cell types and its nuclear localization in relation to senescence might be less pronounced. In addition, HMGB1 loss/release is triggered under various stress conditions, including necrosis and apoptosis,[6] making its use as a sole marker of senescence unfeasible. Overall, HMGB1 is a useful auxiliary marker of cellular senescence *in situ*, although the aforementioned limitations should be considered when drawing conclusions from the presence or absence of intranuclear HMGB1.

Damage to DNA

Background/function

Although senescent cells accumulate many forms of macromolecular damage,[33] a common and established marker of senescent cells, primarily in the context of pathology, is the presence of unresolved DNA damage, mainly in the form of double-strand breaks (DSBs). This leads to persistent activation of the DNA damage response (DDR), which contributes to stable cell-cycle arrest and transcriptional induction of pro-inflammatory senescence-associated secretory phenotype (SASP) factors.[34–36] DSBs can occur anywhere in chromosomes, but cellular senescence is associated with their preferential accumulation at telomeres because of the limited repair of telomeric DNA (Figure 1E). Telomeric DNA damage and attrition leads to DDR foci colocalizing with telomeric sequences known as telomere-associated foci (TAF) or telomere-induced foci (TIF).[13,37] A wide range of proteins are associated with DSBs and DDR, and for several components, highly specific tools are available for *in situ* experimentation. Currently, the most robust DDR markers employed in senescence studies are the phosphorylated forms of H2A histone family member X (γ-H2A.X) and p53-binding protein 1 (53BP1).

Methodology and limitations

The most apical components of the response to DSBs are preferentially localized to the site of the insult, and many DDR proteins appear as dots/foci upon immunodetection and high-magnification microscopy. γ-H2A.X is formed by post-translational modifications triggered by DSBs, while 53BP1 is recruited to DSBs in response to damage undergoing RNA-mediated condensation.[38] Thus, during senescence, γ-H2A.X accumulates, making it a suitable marker for immunohistochemistry (IHC) and IHF but also for methods relying on tissue homogenates, such as WB. However, DSBs are not specific to senescence, and it is currently unclear whether the number of DNA lesions can be used as a discriminant of senescence *in situ*. Telomeric DNA damage is more senescence specific and can be quantified by combining fluorescence *in situ* hybridization (FISH) for telomeric sequences and immunostaining (immuno-FISH) for DSB-associated proteins.[39] However, this remains a technically demanding procedure, and the measurement of telomeric DNA damage is time consuming. Altogether, we recommend using markers of DNA damage as auxiliary markers of senescence. It is crucial to carefully consider other potential inducers of DNA damage independent of senescence.

Decondensation of centromeric satellites

Background/function

Profound changes in chromatin structure and accessibility are associated with cellular senescence.[40] Although several methods are suitable for assessing epigenetic changes in senescence *in vitro*, most of these methods are not yet available for the assessment of cellular senescence *in situ*. One feature of senescence-associated changes in chromatin with an established methodology for *in situ* detection is senescence-associated decondensation of satellites[41] (SADS; Figure 1F). This phenotype measures changes occurring during senescence to constitutive heterochromatin, specifically within regions proximal to the centrosomes, the peri-centromeric satellites. This unfolding of chromatin is associated with changes in transcription and cell physiology. Other methods for epigenetic changes specific to cellular senescence *in situ* and *in vivo* are likely to be developed in the future.

Methodology and limitations

A unique feature of SADS detection is that it requires only a probe specific to the DNA sequence of peri-centromeric alpha satellites, which can be used for FISH assays with relatively low cost and high reliability. However, the quantification of SADS is rather sophisticated, as it requires the use of acquired three-dimensional (3D) projections and measurements of the frequency of decondensation (Figure 1F). Moreover, while decondensation occurs in essentially all centromeric satellites *in vitro*,[41] only a fraction of centromeric satellites undergo unwinding *in vivo*[14,15] (Figure 1F). Thus, the average frequency of SADS events per cell represents senescence more accurately than the number of positive cells does. Finally, although the causes and consequences of SADS *in vivo* have yet to be established, SADS has been shown to be a marker of senescent cells *in situ*. Therefore, we recommend it as a potential auxiliary marker for assessing senescence.

Secretory phenotypes

Background/function

Increased production and secretion of cytokines, growth factors, matrix-remodeling enzymes, lipids, extracellular vesicles, and miRNAs, generally defined as the SASP,[35,42] is a core feature of cellular senescence and a major mediator of the pathophysiological functions of senescent cells. SASP inducers are diverse; however, in most cases, they rely on the involvement of nuclear factor κB (NF-κB), CEBPβ, and/or STAT signaling.[43] Many SASP factors have been described in cultures of senescent cells, and it is currently being established which factors are senescence associated in different contexts and conditions *in vivo*.

Methodology and limitations

The standard approach to approximate SASP utilizes RT-qPCR to quantitate gene expression and enzyme-linked immunosorbent assay or WB to measure protein levels, either individually or in multiplex. However, these methods do not reveal the origin of the factors and whether they are secreted by senescent cells or by other cell types, including immune cells. The origin of SASP factors can be traced using histological methods; however, secreted factors are normally small proteins poorly retained within cells, which makes their detection using IHC/IHF highly challenging, although there has been some success, for example, with IHC against interleukin (IL)-1α[22] and IL-1β.[44] Another major challenge in establishing a single SASP factor as a reliable marker of senescence *in vivo* is that the secretory phenotype has been demonstrated to be highly variable, dynamic, and dependent on cell type, tissue, and environmental context. Detection of transcripts encoding SASP factors using methods such as RNA-ISH allows for the assessment of senescence in individual cells *in situ* (Figure 1G). However, as with all approaches of RNA measurements, the extent to which the transcript level reflects the protein level is not well established; thus, caution is advised when drawing conclusions from these quantitative analyses. Alternatively, antibodies can be used against post-translational modifications of SASP drivers, such as NF-κB (phospho-p65), p38 kinases, or STAT3, in assays such as IHC/IHF (Figure 1G). In-depth characterization of the SASP in individual subtypes of senescent cells *in situ* is ongoing. Although we recommend using hypersecretion as an auxiliary marker of senescence, individual SASP factors remain a valuable method for senescence specification.

Metabolic alterations

Background/function

Alteration of physiology in senescent cells leads to metabolic adjustments, which include an increase in the number and/or size of lipid droplets (LDs; Figure 1H), a phenotype termed accumulation of lipids in senescence (ALISE).[16] These cytoplasmic vesicles primarily contain neutral lipids surrounded by a single-layer lipid membrane enriched in proteins belonging to the family of perilipins (Plin). Accumulation of LDs is physiological for some cell types (such as adipocytes), but it is also exhibited by additional cell types when they enter a senescence state *in vivo*.

Methodology and limitations

Because LDs have a high content of neutral lipids, lipid-binding dyes, such as oil ed O, Nile red, and a series of BODIPY dyes, can be used to detect them in frozen tissue sections. In paraffin-based histology, processing of samples using alcohols removes most lipids, and the detection of LDs requires the measurement of proteins associated with the LD membrane, such as Plin2, using commercially available antibodies. Histological detection of LDs is straightforward, as they are sizable cellular organelles that are recognizable by their round shape and cytoplasmic location (Figure 1H); however, unbiased measurement methods are still being developed. Currently, senescence-associated LDs can be quantified by measuring the total number of positive signals (from dyes or an anti-Plin2 antibody) per cell or by assessing the frequency and size of the LDs. As mentioned previously, the presence of LDs is not specific to senescent cells. The use of LDs for senescence identification should be limited to tissues where LDs are most likely to be of ectopic origin and used only as an auxiliary marker for *in situ* assessments. Detection methods for other metabolic dysregulations observed in senescent cells in culture still lack reproducible, specific, and accessible *in situ* detection methods.

Oxidative damage

Background/function

Organelle dysregulation occurring in senescent cells, especially senescence-associated mitochondrial dysfunction (SAMD),[45] leads to increased generation of reactive oxygen species (ROS),

causing oxidative damage. Although many forms of oxidative damage result in relatively unstable and reactive intermediates, including lipid aldehydes like 4-hydroxynonenal (4-HNE), their reaction products are stable and readily detectable in tissues. Typically, lipid aldehydes react with proteins via Michael addition or Schiff base formation, causing the formation of long-lived lipid-protein aggregates. Lipofuscin, comprising heavily oxidized proteins/lipoproteins and metals, is resistant to hydrolysis by lysosomal enzymes and forms intra-lysosomal aggregates, eventually generating dysfunctional lysosomes and impaired autophagic flux.[46] As for other features associated with SAMD, such as a reduction in mitochondrial membrane potential, methods for *in vivo* senescence detection are still in development.

Methodology and limitations

IHC/IHF staining techniques for downstream products of lipid peroxidation reactions, including 4-HNE, enable robust semi-quantitative assessments of oxidative damage in tissues at cellular resolution (Figure 1I). However, as 4-HNE provides a relatively small epitope, the specificity of the antibody should be thoroughly established and verified using a series of suitable controls (for example, see Table 1). Mass spectroscopy-based imaging may provide a more quantitative approach for the spatial assessment of oxidized molecules (e.g., lysophosphatidylcholines)[47] without the need for antibody staining, although this method is still in development for senescence assessment. A variety of methods are applicable for the quantitative measurement of lipofuscin content, ranging from the detection of autofluorescent granules using light microscopy through lipophilic dyes such as Sudan Black B to commercially available reagents.[48,49] The main disadvantage of these oxidative damage markers is that they cannot provide information about the source of ROS. Moreover, it is possible that lipophilic dyes bind not only to lipofuscin granules but also to other structures of concentrated lipids, such as LDs, in tissue samples where lipids are preserved (e.g., frozen sections). Finally, similar to other forms of damage, oxidative damage is not specific to senescence and is therefore recommended only as an auxiliary marker.

Lysosomal alterations

Background/function

One of the most recognizable and common markers of cellular senescence is senescence-associated beta-galactosidase (SA-β-gal),[50] which reflects the activity of a lysosomal enzyme that cleaves terminal β-d-galactose residues from β-D-galactosides.[51] In senescent cells, the accumulation of this enzyme makes it possible to detect its activity at an otherwise suboptimal, less acidic pH by incubating samples with a substrate that is an analog of galactose, X-gal, resulting in its effective conversion into the deep blue 5,5′-dibromo-4,4′-dichloro-indigo-2 (Figure 1J). Although the causes and consequences of this phenotype *in vivo* are not entirely clear, SA-β-gal is commonly associated with alterations in autophagy flux and lysosomal biogenesis and function *in vitro*.[51,52] However, other assays that measure lysosomal alterations remain less reproducible and difficult to use *in situ*.

Methodology and limitations

SA-β-gal staining is feasible for any tissue material that preserves enzyme activity; however, fresh or frozen materials are arguably the most reliable. This staining is most commonly used in histology but has also been established for the detection of individual X-gal crystals using transmission electron microscopy (TEM),[4] nanoparticles,[53] and for fluorescence- or colorimetric-based detection in flow cytometry.[54] As for any enzymatic activity, the amount of generated product depends on the substrate provided, the temperature, and the duration of the reaction among various parameters, meaning that a well-standardized protocol including positive and negative controls needs to be implemented. As an alternative to measuring SA-β-gal using a colorimetric substrate, a fluorescent system to detect the lysosomal β-galactosidase enzyme (GLB1) has recently been developed.[55] Similar to other senescence markers, a major limitation of SA-β-gal is that it may reflect a wide range of metabolic adaptations of lysosomes—not only those occurring during senescence. Even in cell culture, SA-β-gal staining results in false-positive staining of starved or confluent cells, whereas for *in vivo* and *in situ* conditions, the range of specificity of SA-β-gal is unknown. In addition, *in vivo*, a number of cells, such as macrophages, might have an inherently high β-galactosidase activity due to the high mass and/or number of lysosomes.[56] Therefore, readouts for lysosomal alterations can function as useful auxiliaries but not as sole markers of senescence.

To summarize, a wide range of markers can be used to identify and characterize senescent cells *in situ*, each with its own advantages and limitations. Based on published evidence and agreement of the research community working on this article, we categorized these markers as primary and auxiliary. The first is the presence of p21 and/or $p16^{Ink4a}$, two markers that have guided research on senescence *in vivo* in the last two decades. Auxiliary markers pertain to the core features of senescence, including erosion of the nuclear envelope, a decline in proliferation markers, activation of alarmins, DNA damage, chromatin abnormalities, pro-secretory phenotypes, metabolic alterations, oxidative damage, and lysosomal alterations (Figure 1).

In addition, there are several other promising markers of cellular senescence, such as a wide range of epigenetic modifications, distortions in the shape and size of the cell soma and nucleus, mitochondrial dysfunction, presence of cytoplasmic chromatin fragments, reactivation of dormant endogenous retroviral sequences, and an anti-apoptotic phenotype. Methodologies for the reliable and globally accessible detection of these features *in situ* and/or *in vivo* are currently under development and, pending validation *in situ*, could eventually join auxiliary markers.

HETEROGENEITY OF CELLULAR SENESCENCE ACROSS ORGANS AND CELL POPULATIONS

An important layer of complexity in senescence assessment *in situ*/*in vivo* is the sheer heterogeneity of senescence phenotypes across different organs and cell types. Figure 2 provides a glimpse of the diversity of markers associated with senescence *in situ*. When assessing senescence markers in a given tissue/condition, the following conceptual guidelines should be considered.

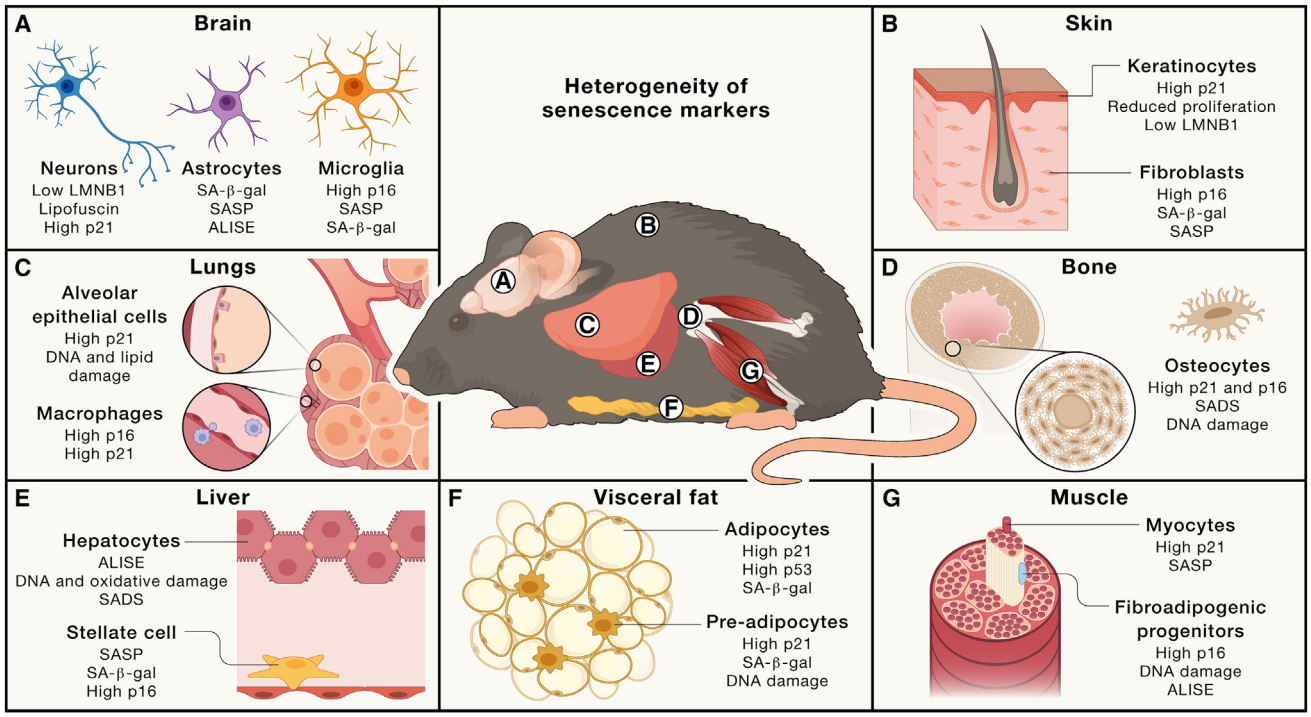

Figure 2. Heterogeneity of senescence markers in cell types and organs *in situ*

Senescent cells found in different organs show heterogeneous phenotypes, and many have been reported to express only some markers of cellular senescence that are attributed to senescent cells *in vitro*. Similarities and differences between phenotypes associated with cellular senescence can be observed across mouse tissues, including the (A) brain, (B) skin, (C) lungs, (D) bone, (E) liver, (F) adipose tissue, and (G) muscle.

- The senescent phenotype is heterogeneous, and no single marker is sufficient to confirm the state of senescence.
- Different cell types may be intrinsically resistant or susceptible to senescence (depending on the stimuli).
- Different cell types may exhibit different senescence phenotypes (expressing different senescence-associated markers).

Below, we provide examples of the heterogeneity and characteristics of cellular senescence in the selected murine tissues.

Brain

Both proliferation-competent glial cells (which constitute approximately 50% of brain cells) and post-mitotic neurons can be positive for markers of cellular senescence in aging, obesity, traumatic injury, and neurodegeneration,[11,16,17,57–59] among other conditions. An important point to consider when assessing senescence in the brain is that cells and the content of cell types differ, not only between their types (Figure 2A) but also between regions (e.g., neurons in the hippocampus, cortex, or cerebellum). However, it is not yet clear whether senescence markers reflect these differences. Moreover, while the presence of senescence markers has been proven even in post-mitotic cells, it is currently unclear how many of the properties of senescent cells known for fibroblasts *in vitro* can be extrapolated to these cells. Finally, certain senescence markers, such as SA-β-gal, despite being more intense in senescence-high conditions, such as aging, are also detectable in the neurons of young and healthy mice,[17,60] raising questions about the suitability of this marker for the assessment of senescence in the brain. However, under such conditions, it is also possible that these markers indicate that senescent cells are associated with murine growth and development.

Skin

The outer layer of the animal body is readily accessible for sample collection and is suitable as an index organ for aging and age-related pathologies of internal organs, such as the cardiovascular system, bones, and brain,[61–63] among others. Historically, being one of the first and most researched organs for its association with senescence in animal and human samples, assessment of senescence in skin remains challenging due to the highly histogenetically distinct populations of cell types. Numerous markers of senescence (including p21, $p16^{Ink4a}$, γ-H2A.X, and TAF/TIF) have been identified in skin cell types, such as keratinocytes and fibroblasts (Figure 2B), in conditions such as injury, aging, and accelerated aging.[6,64–67] Keratinocytes and fibroblasts can be further divided into different subtypes. Keratinocytes display different properties across different strata of the epidermis, and fibroblasts present not only papillary and reticular phenotypes but also heterogeneous populations if studied at the single-cell level.[68,69] Therefore, we expect differences in the signature and role of the senescent phenotype across different subpopulations of skin cells. In contrast to *in vitro* conditions, skin fibroblasts *in situ* are much smaller and only a fraction of their volume can be visualized by histology.

Moreover, the detection of transcripts and proteins *in situ* can sometimes be hindered by the presence of autofluorescent extracellular matrix components in the dermal compartment and chemically cross-linked macromolecules that form during the terminal differentiation of keratinocytes. In this context, it is worth mentioning that when detecting proteins with low expression in skin sections, autofluorescent signals should be reduced (e.g., Troy et al.[70] and Mansfield et al.[71]) or signals should be detected using far-red or near-infrared detection, as demonstrated in a number of studies (e.g., Ring et al.,[6] Keyes et al.,[72] and Ge et al.[73]). Finally, because keratinocytes and fibroblasts differ dramatically in replication dynamics, capacity, and lifespan *in vivo*, molecules that serve as valid markers of cellular senescence in the skin need to be clarified. Overall, there are a number of challenges and exciting perspectives on the horizon for cellular senescence in skin research.

Lungs

Like many other tissues, there are numerous distinct cell types within the lung required for creating functional substructures; thus, there is also heterogeneity of senescence phenotypes across lung cell types[7,8,74–78] (Figure 2C). The lung can be driven into pathological conditions by a variety of external stimuli, including smoke exposure[74] and viral infections.[75,79] In animal models of idiopathic pulmonary fibrosis (IPF), bleomycin-induced injury[76] and telomere deficiency[80] drive fibroblasts and epithelial cells toward increased levels of senescence, a feature also observed in the lung tissue of patients with IPF. In the context of KRAS-induced lung tumorigenesis, both resident[77] and monocyte-derived[78] macrophages exhibit senescence-related alterations. In bronchial epithelial cells exposed to chronic inflammation, senescence is detected by increased SA-β-gal activity, p21, and γ-H2A.X levels.[7,8] Therefore, identifying the cell types that become senescent with age is of great interest for future studies.

Bone

There is considerable evidence that senescent cells accumulate with aging and skeletal injury in the bone microenvironment.[15,81–84] With aging, there was a marked increase in *CDKN2A* mRNA levels in the osteocyte-enriched bone samples (Figure 2D), with a modest increase in *CDKN1A* mRNA levels.[81] In aged vs. young mouse bones, there is an increase in $p16^+$, $KI67^-$, $BCL2^+$, and $SASP^+$ cells (and an analogous increase in the $p21^+$ population) in late osteoblast/osteocytic and bone mesenchymal cells.[25] This heterogeneity in senescence markers might reflect how different subpopulations of senescent cells impact the physiology and pathology of bone. For example, following skeletal injury using radiation, clearance of $p21^+$ senescent cells, but not $p16^+$ senescent cells, prevents radiation-induced bone loss,[85] indicating that, in contrast to aging, $p21^+$ senescent cells may be more important following skeletal injury. Bone is an especially hard tissue that requires special processing and presents several methodological challenges for the detection of senescence markers, particularly those that rely on histology. In addition, bone has a very specific resorption-deposition cycle and extensive endocrine regulation. The impact of cellular senescence on these processes is currently being investigated.

Liver

Numerous liver cell types, including hepatocytes, endothelial cells, hepatic stellate cells, and cholangiocytes, display markers associated with senescence during aging, injury and regeneration, obesity, and age-related diseases, with different cell types displaying different senescence markers (Figure 2E). Age-related changes in murine liver cells include increased SA-β-gal activity,[86] DNA damage, TAFs,[13,37,86] karyomegaly, SADS,[14] ALISE,[14] LINE-1 elements,[87] and SASP factors,[4] some of them specified to occur in hepatocytes.[13,14] In various murine liver injury models, stellate cells can acquire senescence markers, including p21, p16, and SA-β-gal, and express certain SASP factors.[9,88–90] Given the diversity of cell types displaying different signatures of cellular senescence, it is unclear how distinct senescent cell types contribute to age-related changes in the liver or to the crosstalk between different liver cell types induced by senescence.

Adipose tissue

Mature adipose tissue is composed of >50%–90% terminally differentiated post-mitotic adipocytes, ~10% preadipocytes, and ~10% immune cells, including macrophages and lymphocytes. Although SA-β-gal staining is a common method for detecting senescent cells in adipose tissue, numerous other senescence markers, such as p21, p16, SASP factors, and DNA damage, are found across populations of adipose tissue cells[16,26,91–94] (Figure 2F). There is evidence that various pathologies may induce different subtypes of senescent cells in the fat. For example, $p16^+$ cells may predominate in aged adipose tissue, whereas p21 predominates in obesity.[91,92] Surprisingly, most $p21^+$ senescent cells are not $p16^+$ in either context.[26] Elimination of $p16^+$ cells in the adipose tissue of transgenic mice reduces overall tissue senescence,[93,94] making it necessary to clarify the distinct roles of $p16^+$ and $p21^+$ cells. Moreover, adipose tissue differs greatly in cellular composition based on anatomical location, sex, and degree of obesity, which undoubtedly contributes to the heterogeneity of cellular senescence. Therefore, comprehensive analysis of senescent cells using various markers is required.

Skeletal muscle

Muscle is a complex tissue composed of post-mitotic multinucleated cells, referred to as myofibers, and a heterogeneous population of mononuclear cells, encompassing satellite cells, fibroadipogenic progenitors (FAPs), endothelial cells, and macrophages (Figure 2G). A recent study investigated senescence-associated markers at the single-cell level in aged murine skeletal muscle using single-cell RNA sequencing (scRNA-seq) as well as spatially resolved methods such as RNAScope, Immuno-FISH, and IHF.[21] A distinct subset of FAPs is the predominant source of *CDKN2A* mRNA in aged muscle. However, FAPs also exhibit pro-inflammatory SASP, TAFs, and SADS. Interestingly, p21 increased with age in a subset of terminally differentiated myofibers, which also displayed other senescence-associated markers such as TAF, SADS, nuclear loss of LMNB1, HMGB1, and a unique SASP.[21,95] An independent study also showed increased senescence-associated markers in myofibers in aged mice.[96] Overall, the characteristics and role of cellular senescence in muscle cells are only beginning to be discovered, and exciting results are expected in the near future.

Immune system

With aging, the immune system loses its ability to mount an effective immune response against invading pathogens and cancer cells, a phenomenon termed immunosenescence.[97] These age-related changes are characterized by altered ratios of naive and memory T cells, lymphoid to myeloid cells, $CD4^+$ to $CD8^+$ cells, and thymic atrophy. In addition, many immune cell types and subtypes develop a senescence-like state, with increased expression of p16, p21, and SASP factors.[98,99] These changes limit the ability of the immune system to respond to pathogens or vaccinations.[100] However, given that the functions of certain aged immune cells can be stimulated under specific conditions, it is still unclear whether immune cell subsets are truly senescent or show signs of exhaustion, especially $CD8^+$ T cells. Interestingly, adaptive immune function can be improved by short-term treatment with rapamycin,[101] which reduces the markers of senescence and SASP in peripheral blood mononuclear cells (PBMCs).[99] To detect the senescence-like state in immune cell populations, an assay based on analysis of *CDKN2A* mRNA in PBMCs or selected subsets such as $CD3^+$ T cells was developed and shown to correlate with biological age and exposure to stress such as smoking or diet.[102] In addition, fluorescent SPiDER-ß-Gal has been used to identify senescent-like subsets of immune cells by flow cytometry with high sensitivity. It is important to note that activated macrophages, including tissue-infiltrating macrophages, express higher levels of p16 and stain positive for SA-β-gal, even though they may not be senescent.[56]

TRANSGENIC MOUSE MODELS FOR STUDYING CELLULAR SENESCENCE

Measurements of cellular senescence in animals and assessment of the impact of senescent cells on physiology and pathology can be aided by a wide range of transgenic mouse models. Here, although not exhaustive, we highlight transgenic models that drive or report senescent cells through the manipulation of genes involved in cell-cycle arrest, telomere biology, or DNA repair. It is also possible to drive senescence *in vivo* by using a variety of drugs that promote cellular, organelle, or macromolecular stress. However, we focus on former models that indicate when and where senescence is induced by aging and when protective mechanisms are genetically perturbed. Animal models that interrogate the (patho)physiological consequences of senescent cells by targeting specific SASP factors or regulators are of great interest. In this section, we review the applications, strengths, and limitations of available transgenic mouse models developed for senescence research. Despite these recent efforts, it is imperative to develop additional animal models to advance our understanding of senescent cell biology *in vivo*.

Models modulating crucial regulators of the senescence program

Background

Historically, some of the first transgenic animals used for senescence research were KO models of the major cell cycle inhibitors, p16-KO and p21-KO mice.[103,104] These models were primarily used to show how the absence of a single regulator of the senescence program affects physiology and pathology. Although the majority of currently available models are constitutive (active from conception), models of inducible KO or overexpression of senescence modulators are in development.

Limitations

Although working with these models, it is important to be aware that they affect only a single component of the senescence machinery and thus might not reflect the whole biology of cellular senescence. Moreover, p21, p16, TP53 (p53), and other senescence-associated proteins are involved in physiology that is distinct from cellular senescence. Therefore, at least some of the observed phenotypes of transgenic models with overexpression or KO of these factors might be unrelated to cellular senescence. Finally, in constitutive models, it is likely that the phenotype of adult mice is affected by alterations during embryogenesis and development (dependent or independent of cellular senescence). For this, we recommend, when available, the use of models where senescence regulators are affected in an inducible manner and ideally specific to one cell type by using specific Cre transgenes and a floxed allele of the senescence regulator.

Models of premature accumulation of cellular senescence

Background

In accordance with research on fibroblasts *in vitro*, senescence can be induced efficiently by accelerated telomere shortening, disruption of the cell division machinery, and increased DNA damage levels *in vivo*. Mouse models of rapid telomere shortening, dysfunctional mitosis, or impaired DNA repair (*Terc*, *BubR1*, and *Ercc1* mutant mice, respectively), display high levels of cellular senescence and accelerated aging.[93,105,106] These and other models, for which it has been shown that accelerated aging depends, at least partly, on cellular senescence, are valuable for studying the impact of senescence on health and lifespan or for testing approaches to eliminate senescent cells, as their accumulation occurs on average 5–6 times faster than in wild-type mice.[107] When crossed with models deprived of key senescence regulators, these mice can be used to verify the extent to which their age-related pathology is due to cellular senescence.

Limitations

Genes that induce accelerated senescence upon genetic depletion play pleiotropic roles in normal physiology. Thus, at least some of their accelerated aging pathologies can be ascribed to mechanisms distinct from senescence. For example, while the elimination of senescent cells alleviates age-related pathologies in *BubR1* and *Ercc1* mutant mice, it does not restore their lifespan to that of wild-type animals.[93,108] Moreover, because these mutations increase the burden of senescent cells *in vivo*, this does not mean that they are the physiological source of senescence under normal conditions. Hence, it is unclear whether subsets of senescent cells in these transgenic models are identical to those found in wild-type animals. Nonetheless, we recommend these models as reasonable first-approach models for linking cellular senescence to pathologies—senopathies[109]—and testing senotherapeutics.

Table 2. Exemplary transgenic mouse models to visualize senescent cells *in vivo* and/or *in situ*

Name	Reporter system	Promoter	Exemplary publications
p21-luc	firefly luciferase	2.5-kb segment of the human p21 promoter containing two p53 binding sites (transgenic)	Yoshimoto et al.[90] and Ohtani et al.[110]
p16-luc	firefly luciferase	195-kb segment of the human chromosome containing $p16^{Ink4a}$ gene locus (transgenic)	Kawamoto et al.[27] and Yamakoshi et al.[111]
p16-ATTAC	EGFP	2,617-bp segment of the murine $p16^{Ink4a}$ (transgenic)	Baker et al.[4,93]
p16-luciferase	luciferase	native murine $p16^{Ink4a}$ promoter (knockin)	Burd et al.[112]
p16-3MR	mRFP, renela luciferase	50-kb segment of the murine $p16^{Ink4a}$ (transgenic)	Demaria et al.[65]
p21-ATTAC	EGFP	3.2-kb segment of the mouse $p21^{Cip1/Waf}$ (transgenic)	Chandra et al.[85]
p16-tdTomato	tdTomato	native murine $p16^{Ink4a}$ (knockin)	Liu et al.[113]
INKBRITE	three copies of GFP	50-kb segment of the murine $p16^{Ink4a}$ (transgenic)	Reyes et al.[116]

Models for detection of senescent cells *in situ* and *in vivo*

Background

Several transgenic mouse models have been developed in which core modulators of senescence, such as p21 and p16, are tagged with a reporter, enabling the visualization and quantification of senescent cells *in situ* and *in vivo* (for example, see Table 2). Traditionally, these models contain luminescent or fluorescent reporter systems yielding increased signal, dependent upon the expression level of p21 or p16.[27,90,110–113] Reporters using EGFP or tdTomato fluorescence enable the detection of $p21^+$ or $p16^+$ cells *in situ*, whereas models with luciferase enable the approximation of senescent cells in live animals by measuring the amount of light emitted after the administration of an enzymatic substrate for the luciferase reporter. Models that enable the long-term tracking of senescent cell fate are also available.[114,115]

Limitations

Although these models are suitable for estimating the quantity of a particular type of senescent cell, the results must be validated with additional senescence markers because single molecular endpoints are not specific for senescence. By definition, the models underestimate the senescent cell burden *in vivo* because they only look at a single senescent cell type (e.g., p16 expressing) and many senescence markers exhibit low expression levels, leading to low signal-to-noise ratios. In such cases, the signal may be amplified by immunodetection of reporter proteins. Whether selection cassettes were deleted should be clearly reported and considered when comparing data from different reporter mouse models. Luminescence signals are strongly influenced by tissue density and light penetration, which may bias the measurement of the senescent cell burden.

In addition to the caveats of various reporter genes, reporter construct design also influences the reporter signal. For example, the inclusion of selection cassettes, necessary in the process of generating recombinant mouse embryonic stem cells, can potentially interfere with the expression of the reporter transgene of interest, as observed for p16,[115,117] possibly because of unintended promoter activity. Therefore, it is important to report whether the selection cassettes were deleted when comparing data from different reporter mouse models. Additionally, the location of the transgene should be described in as much detail as possible.

Furthermore, many models for senescence reporting utilize transgenes driven by different parts of the native promoter of the senescence regulatory gene (Table 2), making it challenging to compare the results between models.

CDKN2A mRNA levels are regulated by transcription and mRNA stability.[113,118] Therefore, the use of native promoters in reporter mice may not always accurately reflect endogenous *CDKN2A* mRNA levels, and combining reporter detection with other methods for detecting endogenous *CDKN2A* mRNA expression is highly recommended. In summary, senescence reporter mice are recommended for detecting senescent cells *in vivo* and for studying the dynamics of senescence over time or in disease models. However, reporter data should be complemented by direct biochemical and histological assessments of endogenous senescence biomarkers.

Models for pharmacogenetic elimination of senescent cells

Background

Over the past decade, several transgenic models have been developed that, unlike KO models, do not prevent senescence from occurring but rather allow the selective and timely removal of existing senescent cells. In these mice, an inactive form of an apoptosis-inducing gene is expressed from the promoter of a key senescence regulator, such as p21 or p16, and administration of a drug to the mice drives the elimination of $p21^+$ or $p16^+$ cells. These models are typically designed to not only enable drug-inducible elimination of senescent cells but also to report the level of cellular senescence *in situ* and/or *in vivo*. Some of the most commonly used models are INK-ATTAC, p16-3MR, and p21-ATTAC,[65,85,93] but others have been and are being generated (Table 2).

Limitations

When assessing the impact of elimination of senescent cells using these models, especially for a pathology specific to a given tissue, it should be acknowledged that the transgene is expressed throughout the body. Therefore, it is possible that the elimination of senescence in other tissues contributed to the observed phenotype. To address this limitation, transgenic

mice with cell-type-specific elimination of senescent cells were recently developed: p16-LOX-ATTAC mice.[83] Similar to other transgenic models used in senescence research, the activity of the transgene is dependent on a single gene, which is inadequate for fully defining the senescent cell burden *in vivo*. Moreover, as mentioned above, the promoter activity of p16 is generally weak and, if the neomycin cassette is not removed, its strong promoter may affect the p16 promoter, which could account for the differences observed between studies.[115,117] Finally, the threshold for the quantity of pro-apoptotic transgenic protein needed to drive the death of senescent cells is still unclear. In other words, it is unknown what happens if a cell expresses some quantity of the pro-apoptotic transgenic protein, but it is not enough to induce apoptosis. Despite their limitations, these models are currently state-of-the-art for the specific and selective elimination of subtypes of senescent cells, and we recommend their utilization.

In summary, several transgenic mouse models are available to induce, reduce, and visualize cellular senescence. Although this is a relatively large set of models that should be readily available to most researchers via collaborations and animal suppliers, the use of alternative models and other means to modulate cellular senescence *in vivo* includes, but is not limited to, pharmacological, dietary, wounding, transplantation, tissue explants, and psychosocial stress models.

Questions to be answered

The use of transgenic animals might provide the most mechanistic approach to investigate cellular senescence *in vivo* and generate preclinical data to support translation. Thus, the community is working extensively to improve and expand available models. One example is the generation of transgenic animals that enable the detection and/or manipulation of cells expressing more than one senescence marker, such as p21 and p16. Second, the community would benefit from models to study the temporal dynamics of senescent cells, for example, improved models of senescence lineage-tracing and isolation. Moreover, while transgenic mice with accelerated aging are suitable for studying some aspects of senescence, research could be improved if we had access to models for studying spontaneous senescence, which is likely to reflect the physiological process of aging. Finally, human physiology and many aspects of aging are better modeled in rats than in mice,[119] and with the recent availability of genome editing in rats,[120] transgenic reporter rats are being developed for senescence research.

STUDYING CELLULAR SENESCENCE IN NON-MAMMALIAN MODELS

Cellular senescence is not a mammalian-specific phenomenon but is widespread across the animal kingdom, including vertebrate and invertebrate organisms. Markers of senescence have been detected in birds (e.g., quail[121]), amphibians (e.g., axolotls,[122] xenopus,[123] and newts[124]), fish (e.g., zebrafish[125] and killifish[126]), and invertebrates (e.g., hydra[127] and *Drosophila*[128]). Senescence in non-mammals impacts on many aspects of organismal physiology, from development[123,129] to regeneration (e.g., salamander limbs,[124] zebrafish fins,[125] and whole hydra polyps[127]) and aging.[126,130,131] Understanding the full extent of senescent cell involvement in animal physiology is central to evolutionary theories of senescence. Knowledge of how senescent cells influence processes that are not conserved in humans, such as appendage or organ regeneration, is necessary to determine the features and functional scope of beneficial forms of cell senescence. However, our understanding of senescence in non-mammals is limited by the shortage of reagents for molecular analysis in many of these organisms.

Specifics of senescence research in non-mammalian animals

Although cells from non-mammals are capable of undergoing cell-cycle arrest that resembles cellular senescence, it might be driven by alternative mechanisms. For example, several vertebrate groups, including fish and amphibians, lack p16.[129,132] In contrast, p21 is elevated in salamander cells in regenerating limbs[133] and in the regenerating fins of zebrafish.[125] Other markers of cellular senescence, such as SA-β-gal, appear to function similarly across different animals, and their use has been instrumental in determining the presence of senescence *in vivo* across organisms. The availability of SA-β-gal as a senescence marker outside mammalian systems has also enabled the use of galacto-nanoparticles[134] to label, isolate, or eliminate SA-β-gal cells *in vivo* when delivered systemically.[133] Nevertheless, it is unknown how specific SA-β-gal is for cellular senescence as a whole; therefore, its staining should be supported by auxiliary markers. Some biochemical markers of senescence, including lack of incorporation of thymidine analogs as a reporter of reduced proliferation or increased ROS abundance and macromolecular oxidative damage, are established for several species.[126,127,131] Therefore, it is possible to use a combination of at least two markers to assess senescence more reliably. Another tool that can aid in the identification of senescent cells in non-mammalian species is senolytic treatment to eliminate senescent cells. This has been successfully applied in fish[125] and salamanders,[124,133] enabling both the functional assessment and validation of senescence through targeted reduction.

The community is currently testing antibodies and biochemical assays to further refine the detection of senescence in non-mammalian species. The development of new transgenic models will be particularly helpful, such as the recent generation of the p21-GFP transgenic reporter zebrafish.[135] Furthermore, at least for species whose genomes have already been sequenced, research will be accelerated by leveraging omics approaches, including at the single-cell level.[133]

The impact of senescent cells on the biology of non-mammalian species has been mainly investigated in the context of development, tissue remodeling, and regeneration, while conditions such as disease and aging still need to be further explored. To aid with these endeavors, the research community has promoted the development of tools and transgenic models for the detection, visualization, induction, and reduction of senescent cells. Finally, further progress is expected on how these models respond to senolytics and which markers most accurately report senolytic activity.

In summary, research on cellular senescence should be further expanded to animals other than mice, as each species

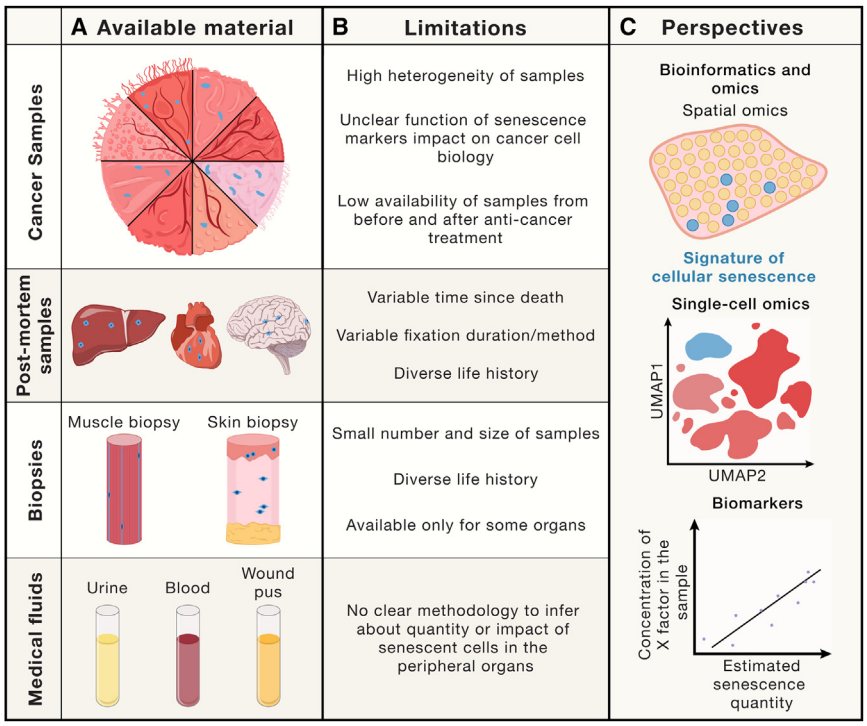

Figure 3. Approaches, limitations, and perspectives for research on cellular senescence in human samples

(A) Examples of human materials available for senescence research include cancer samples, post-mortem organ pieces, biopsies, and medical fluids.

(B) Limitations that challenge experimental approaches to work with human senescence. Senescent cells present a high level of heterogeneity across different types of cancer and across various tissue types. An additional layer of heterogeneity arises from the methodology used to obtain or process the samples prior to the detection of senescence markers. Finally, medical fluids can provide indirect information on senescence in organs; however, the methodology for making accurate predictions is still under development.

(C) Perspectives for exploration of the properties and roles of senescent cells in human health and disease. With the recent advancements in single-cell and spatial omics, it is anticipated that these methods will provide accurate data on the characterization and prevalence of senescent cells *in situ*. Information derived from samples collected in a non-invasive manner (e.g., medical fluids) can be used as a biomarker to indirectly measure the quantity of senescent cells in peripheral organs.

can teach us about the evolution and functional scope of cellular senescence. Research on senescence in non-mammalian animals is a highly promising emerging area. Therefore, additional proof of senescence markers, beyond classical SA-β-gal staining, is required. Due to species specificity, we do not provide any specific recommendations for research on non-mammalian animals at this point in time and strongly recommend the use of multiple senescence markers for the same biospecimen.

THE CURRENT STATE OF BIOINFORMATICS FOR ASSESSMENT OF CELLULAR SENESCENCE IN ANIMAL SAMPLES

The omics revolution enables in-depth characterization of the macromolecular composition of biological samples. Senescent fibroblasts produced *in vitro* have been thoroughly characterized using epigenomic, transcriptomic, proteomic, lipidomic, metabolomic, and phenomic approaches. Single-cell transcriptomics have revealed immense heterogeneity among senescent cells, even under relatively homogeneous conditions of two-dimensional (2D) cell culture.[136] Applying omics to assess the macromolecular composition of tissues, in principle, permits the unambiguous identification of senescent cells and their detailed characterization. Furthermore, spatial omics platforms allow for the correlation of an omics dataset (currently either whole genome transcriptomics or targeted transcriptomics or proteomics) with spatial location within a tissue and have the potential to revolutionize *in vivo* senescence research. Both bulk and single-cell omics have been applied to *in vivo* senescence research; however, the analysis and interpretation of results pose challenges.

Challenges and limitations

Based primarily on the frequency of $p16^{+}$ cells *in situ*, most conditions driving senescence, including age, yield a relatively small number of senescent cells, often estimated to be <10%.[137] This makes it practically difficult to reliably study senescence using standard, bulk omics methods because the detection of rare cells requires technically more challenging and expensive single-cell or spatial omics approaches. It is currently limited to unbiased transcriptomics and targeted proteomics on commercial platforms, although other omics modalities are in development (e.g., single-cell proteomics[138]). Spatial transcriptomics is less sensitive than scRNA-seq and can introduce bias based on methods of either detection of the 3′ end of poly(A) tail or nucleic acid ISH probes, which do not always distinguish between splice variants of CDKI genes such as *CDKN2A*. Single-cell transcriptomics requires a large number of intact cells. Furthermore, multiple sources of bias can be introduced through tissue dissociation (loss of fragile cells), microfluidics (loss of large cells), data analysis, and statistics. Therefore, a clear and complete reporting of these methodological steps is a prerequisite for reproducibility. Spatial omics has not yet been used extensively for senescence research but is in development.[139–143]

In the bioinformatics analysis of single-cell and spatial omics datasets, one goal is to define a "cellular senescence signature" (Figure 3). These signatures are often compared using gene set enrichment analysis (GSEA), although other statistical methods are available or are in development. Notably, senescent cell identification by scRNA-seq has been successful in multiple *in vivo* studies, providing valuable insights into proper study design.[78,136,144,145] A senescence transcriptional signature

could enable rapid detection of senescent cells, and multiple such gene sets have been described.[139–143,146] However, caution is warranted as these signatures are not applicable to all tissues, cell types, and inducers of senescence. Significant correlations may be detected for large gene lists based on the differential expression of only a small fraction of genes. Understanding the methods by which a signature is developed is also critical for identifying potential biases or limitations; for example, a SASP-heavy signature may not be accurate for some subsets of immune cells. Finally, there is no evidence that cellular senescence is regulated at a transcriptional level. Hence, although the transcriptional profile of cells or tissue regions of interest can be used to approximate the burden of senescence, the conclusions are not definitive in the absence of orthogonal validation, ideally measuring non-mRNA endpoints. Therefore, our current recommendations are to use omics as a supplemental tool (or discovery tool) to investigate senescent cell identity and phenotypes, and to always use *in situ* and/or *in vivo* validation to complement *in silico* findings.

Perspectives for senescence omics

Much research has been conducted on cellular senescence *in vivo* using omics and bioinformatics. First, the community is working to define cellular senescence signature(s) that most accurately represent senescent cells of various types *in vivo* (Figure 3). This will inevitably be an iterative process, as we do not yet have a state of the ground truth *in vivo*. Conceptual advancements are being made to support this aim by expanding the breadth of omics approaches, such as non-coding RNAs, epigenomics, proteomics, and lipidomics. Researchers are also working to refine bioinformatic approaches to integrate omics datasets (e.g., single-cell transcriptomics with proteomics data) with the aim of detecting complex cell phenotypes such as senescence.

Recent technological advances are expected to further support the aim of improving the detection and omics-based characterization of senescent cells. The development of reporter transgenic models has enabled researchers to use fluorescence-activated cell sorting (FACS) to enrich $p16^{high}$ and $p21^{high}$ cells and combine this with downstream omics analysis and single-cell omics transcriptomics (for example, Omori et al.[115]). A deeper understanding of the surface markers of senescent cells *in vivo* is being actively pursued. This knowledge will enable the more sophisticated isolation or enrichment of intact, viable senescent cells using flow cytometry. This approach will facilitate the assessment of senescent cell heterogeneity, either through the multiplexing of a panel of surface markers or through the isolation of subpopulations expressing different sets of "surfaceome markers." It will also enable the incorporation of cell-type-specific surface markers and have utility in non-transgenic models (e.g., human samples), further supporting insights from the full breadth of omics-based approaches, including epigenetics, scRNA-seq, and proteomics. In principle, this strategy could then be coupled with either bulk approaches (increasing throughput and reducing costs) or single-cell omics approaches.

Spatial omics provides exciting insights into senescent cell heterogeneity *in situ*. Certain platforms now enable bespoke design of isoform-specific probe sets. Emerging technologies also aim to enable the integration of biomics, such as transcriptomics and multiplex proteomics, within the same tissue section. These developments have the potential to address some of the limitations highlighted above.

Changes in nuclear and cellular morphology are largely conserved across senescent cells, both *in vitro* and *in vivo*. Advances in high-throughput imaging, computational tools, and machine learning have fostered the use of multiparameter morphological analysis as a robust, unbiased, and quantifiable marker of senescence. These new senescence classifiers can detect senescence *in vivo* in aging, fibrosis, preclinical models of cancer,[147,148] and a range of clinical samples.[148,149] As this is a microscopy-based approach, it could, in principle, be combined with other omics-based approaches such as spatial or multiplex microscopy for deeper insights. While still in its infancy, the use of phenomics to define senescence-imaging-based predictors offers a new direction for future research.

Finally, advanced analysis tools, such as high-content image analysis, approaches to integrate multi-omics datasets, statistical learning tools, and machine learning are being developed to facilitate the identification and quantification of senescent cells. These tools are being applied to tissues or 3D organoid samples, for example, from mice after senolytic interventions, to refine the senescence signature, but also to identify off-target effects of such interventions. The current expansion of the spectrum and depth of omics-based datasets, together with their integration, aims to illuminate senescent cell heterogeneity in an unbiased manner through careful cataloging of the breadth of senescent phenotypes in different cell types *in vivo*. This offers the potential to provide novel routes for senescent cell detection and, thus, assessment of senescent cell burden, potentially in a cell-type-specific manner, in a range of contexts and disease settings. Consequently, the functionality and potential applications of omics technologies are diverse. Given the relatively low abundance of senescent cells, single-cell approaches are likely required for senescent cell detection. For example, this could take the form of probe-based panels based on refined epigenetic clocks. Alternatively, a gene set derived through scRNA-seq approaches, ideally deployed as a validated multiplex protein readout, could be utilized.

Thus, although bioinformatic approaches are powerful tools to support research on cellular senescence *in vivo*, they have not yet been adequately developed to replace standard methods for detecting senescence *in situ* or *in vivo*. We consider omics analysis to be an indispensable component of current discovery research in cellular senescence, and we hope that, in the near future, omics will be advanced enough to become integral to future MICSE iterations. Accordingly, the current version of the MICSE guidelines does not provide any specific recommendations for the bioinformatic assessment of cellular senescence profiling or quantification. However, we encourage researchers to generate and share new omics datasets while also developing new analytical methodologies, reporting methodologies in exquisite detail, and collaborating with others to discover and overcome the difficulties in defining senescence signatures that work *in vivo*.

ASSESSMENT OF SENESCENCE IN CANCER SAMPLES

Senescence in cancer tissues

Cellular senescence is closely associated with the biology of cancer. Induction of senescence is a tumor suppressor mechanism[150–152] and a side effect of several chemotherapeutic drugs that induce a phenotype that might be considered an acquired premature progeroid syndrome in both normal and cancer cells.[153] More recently, it has become clear that targeting senescent cells may be a promising strategy to reduce the growth and metastatic potential of tumors by altering the senescent tumor microenvironment.[154] Although it remains unclear whether cancer cell senescence is irreversible, primary cancer therapeutics that trigger a senescence-like phenotype in cancer cells allow further targeting of these cells using a one-two-punch approach (senescence-senolysis) to eliminate surviving cancer cells.[155,156] Thus, in general, senescent cells in tumors present the same challenges as those in normal tissues: they need to be properly identified, and the impact of their modulation or elimination must be functionally evaluated in tumor tissues. In addition to cancer cells, tumors are composed of an array of non-malignant cells that support cancer tissue growth, and the presence of such senescent cells in tumors presents new research avenues. Unlike the cancer cells discussed below, senescence biomarkers in these non-malignant cells residing within tumors can be assessed using the same approach as for non-malignant tissues, including cell identity markers to localize them spatially.

Senescence markers in cancer cells

Samples from mouse models of oncogenic activation, as well as samples of benign tumors, such as prostate intraepithelial neoplasia or melanocytic nevi, are often enriched in non-proliferative senescent cells expressing all the expected senescent markers and SASP factors.[154] Senescent cancer cells express numerous markers of cellular senescence, including cell cycle inhibitors, SASP factors, and SA-β-gal.[154] However, the detection of these markers in human cancers can be challenging because of the universal limitations of human sample analysis. During the early phases of cancer progression, mutated cells display a genuine senescent phenotype, as a consequence of oncogene-induced senescence.[157] However, as cancer progresses, features of senescence are lost or mutated in cancer cells, including the expression levels of the tumor suppressor p53 and *CDKN2A/CDKN2B* loci gene products, $p16^{Ink4a}$, $p14/p19^{ARF}$, and $p15^{INK4b}$. Indeed, $p16^{Ink4a}$ is often used as a prognostic marker in some cancers, such as breast, colon, or head and neck cancer; however, in cancers, p16 rarely induces cell-cycle arrest.[158] For example, p16 overexpression serves as a marker for highly proliferative HPV-16-positive cervical cancer cells, driven by RB1 (pRB) inhibition of the viral protein E7.[158] Other senescence markers have been shown to be overexpressed or activated in cancer.[159] Thus, unlike primary cells, which *acquire* senescence markers as part of their senescence stress response program, cancer cells may *adopt* senescence markers in a manner unrelated to their function during senescence. However, these phenotypes are also associated with cancer survival. Except in some mouse models of senescence reactivation, such as through p53 restoration,[160] cells within a tumor are heterogeneous, with only a fraction becoming positive for cellular senescence markers. Although the impact of tumor cell senescence on tumor progression is currently not well defined, it is clearly context dependent. Although we currently do not have the tools to clearly define a cancer cell as senescent, we recommend using a combination of senescence markers for the detection and quantification of senescence in cancer. It is unknown whether targeting senescent tumor cells using interventions established for non-malignant pathologies will be beneficial.

Availability and diversity of samples

Although post-mortem tumor biospecimens are abundantly available, it would be ideal to have paired samples from one subject collected before and after cancer therapy to better define the role of senescent cell induction in cancer treatment responses. The sheer diversity of cancer types (Figure 3) and the lack of multiple established senescence markers related to cell cycle control to define cancer cell senescence in these diverse tumor types remain challenging. Features such as reduced proliferation, pro-inflammatory and angiogenic factors, and resistance to apoptosis provide promising markers of senescence in cancer, but will remain context dependent for each cancer, or even for each patient within a cohort, as very few cancers present with a homogeneous mutational background. Among potential biomarkers, the relevance of pro-survival proteins is that they enable cancer cells to avoid cell death upon acute stress (e.g., during chemotherapy or radiotherapy) and are targetable for cancer senolysis.[155,156] Methods for detecting anti-apoptotic proteins *in situ* or *in vivo* are not yet well established but would provide targetable senescence biomarkers in the context of cancer.

In summary, the next steps in research on senescence in cancer are to identify markers that specifically detect senescent cells in different types of cancer and to reconcile the impact of these senescence biomarkers on cancer cell function relative to non-malignant cells expressing the same biomarkers (Figure 3). Because of the very high heterogeneity between diseases of the same pathological subtype, we cannot offer universal recommendations for detecting senescent cancer cells, apart from using multiple biomarkers and referring to senescent cancer cells as having senescence-like phenotypes. This should include markers of cell-cycle arrest other than p21 and p16, unless it is validated that the cancer tissue under study possesses a functional version of these genes and their downstream molecular targets, which is rarely the case.

ASSESSMENT OF SENESCENCE IN HUMAN SAMPLES

Senescent cells are enriched in a wide range of human diseases and disease-associated conditions.[161,162] Human samples provide the best source of information about how cellular senescence contributes to human physiology and pathology, relative to inbred and specified pathogen-free mice. These include tissue biopsies, post-mortem tissue collection, and biological fluids. Each has its own set of limitations, affecting which markers and features of senescence can be investigated and quantified (Figure 3).

Senescence markers in human samples

In principle, well-preserved human samples can be analyzed using the same set of senescence markers developed for mice. Some markers of senescence, such as p16, are easier to detect in humans than in murine samples (because of the availability of a reliable antibody against human $p16^{Ink4a}$ developed as a clinical prognostic tool for cancer). Elevated expression of p16 in melanocytes[163] and epidermal and dermal cells[164,165] is associated with human aging. However, given the ethical concerns associated with human tissues and the fact that they are often not collected in the research laboratory, it is challenging to collect, properly preserve, and stabilize human tissues in a standardized manner, leading to increased variability in the post hoc analyses of macromolecules and metabolites. It is likely that certain senescence markers, such as those based on post-translational modifications, enzymatic activity, or protein translocations, are more sensitive to tissue collection times and fixation methods.[166,167] Similarly, metabolically active and lipid-rich tissues are more vulnerable to rapid *ex vivo* degradation. Therefore, when properly fixed tissue samples are not available, we recommend initiating the assessment of senescence in human samples by measuring the total amount of protein (e.g., $p16^{Ink4a}$, p21, LMNB1) or larger cytoplasmic inclusions (e.g., lipofuscin or LDs), which should be stable for a prolonged period of time. Finally, as clinical trials on the targeted elimination of senescent cells were initiated only relatively recently[162] and the molecular targets of many senolytics are either unknown or pleiotropic, it is still unclear which markers of senescence are most informative about the burden of senescence in humans. In the long term, the development of senescence biomarkers compatible with minimally invasive methodologies, such as blood or urine samples, would greatly enhance access to healthy human populations.

Variability of samples

Assessing and interpreting senescence in human samples is challenging for multiple reasons beyond biospecimen handling. Genetic heterogeneity between individuals in the human population will likely influence the distribution of senescence biomarkers from person to person. Aging also drives incredible heterogeneity among individuals, which has not been observed in young individuals. Furthermore, unlike laboratory models, there is little control over variables in humans that affect senescent cell burden, such as occult disease, body mass index, environmental factors, genetic factors impacting genome instability and telomere length, and levels of psychosocial stress. Often, human samples, especially those collected from live subjects, are small and low in number, and this, combined with the high interindividual variability, poses problems in reaching statistical significance. Although surrogate biomarkers of senescence burden from liquid biopsies, preferably from minimally invasive sampling such as blood, saliva, wound pus, urine, synovial, or cerebrospinal fluid, would provide a necessary and more accessible alternative, there is currently limited knowledge about SASP factors in these liquid biopsies. Thus, it is unclear whether and how single biomarkers or signatures of biomarkers in these fluids would correlate with senescent cell burden in organs and tissues. The community is working on establishing biomarkers of senescence, which are factors associated with the presence of senescent cells in readily accessible tissues that can be sampled easily and repeatedly and act as a surrogate for internal organ senescence, predict frailty, or disease susceptibility. Given that the genetics and environment of humans are more variable than those of mice, we recommend cautious power analyses and using a sufficiently large number of samples to draw conclusions about the presence and effect of senescent cells in human samples.

In summary, research on cellular senescence in human samples provides direct insight into the impact of senescent cells on human health and disease and is essential for establishing safe and efficacious therapeutic interventions. However, our knowledge of the functional impact of the burden of senescent cells in human samples is significantly less well established than that in mice. As of 2024, the scientific community does not have definitive recommendations for assessing cellular senescence in human tissues, except for the suggestion of using a large sample size and investigating several markers, with a preference for those that are highly stable.

MICSE

Over the last few decades, technological and methodological advancements have tremendously improved our ability to detect senescent cells in living organisms and our understanding of the biological functions of these cells in physiology and pathology. To continue this trend of excellent science and progress in senescence research *in vivo*, we believe that there is a need to standardize how to report and describe cellular senescence in animals, complex models, and human tissues. The aim of this effort is to enhance comparability and contextualization of results—a necessity when targeting senescent cells to improve health is being translated to humans. Therefore, we summarized the current recommendations for the minimum information for cellular senescence experimentation *in vivo*.

(1) Recommendations for senescence markers to support statements regarding the presence/absence of senescent cells:

Evidence of the expression of at least three senescence markers representing different properties of cellular senescence in a given tissue strongly supports the notion that the observed phenomena are indeed related to cellular senescence.

At least one of these markers needs to be evidence of stable cell cycle inhibition in the form of increased p21 or p16 expression. This does not apply to non-mammalian animals, where a reduction of proliferation with other markers should be shown, and to cancer cells, where p16 and p21 do not always correlate with proliferative arrest; however, a reduction in proliferation with other markers should also be shown.

There should be at least one experiment showing two markers co-detected in the same sample and at the same time, ideally colocalized in single cells.

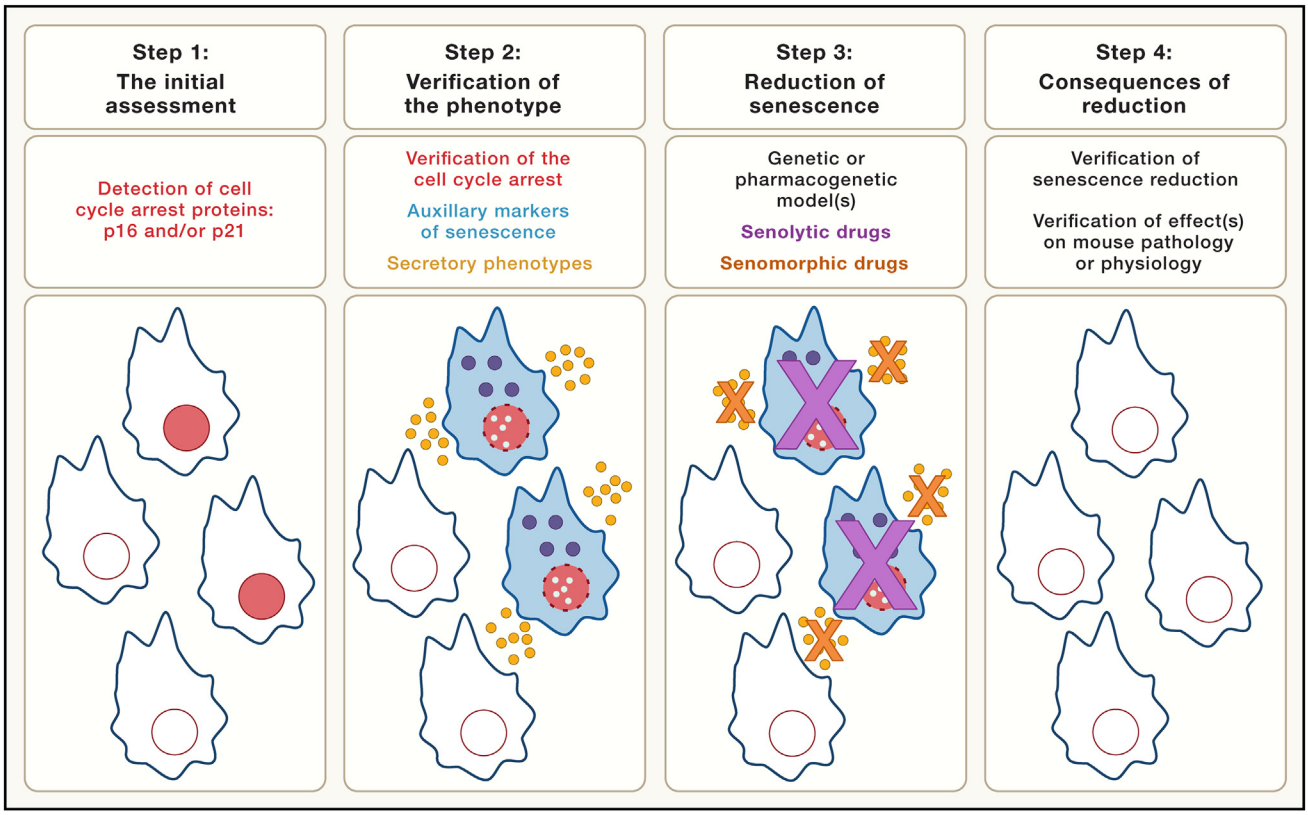

Figure 4. Sequential approach to study senescence *in vivo*

To accurately assess the relevance of cellular senescence to a specific phenotype, we suggest beginning with a general evaluation of the core markers for cellular senescence: p21 and/or p16 (step 1). Next, it is advisable to assess auxiliary markers of cellular senescence, including DNA damage, LMNB1, ALISE, oxidative damage, SASP, and SA-β-gal (step 2). The use of at least two auxiliary markers is recommended. Although these initial steps are adequate for determining the presence or absence of senescence in a particular tissue or condition, to ascertain whether senescent cells impact a physiological process or pathology, we recommend reducing the quantity and/or activity of senescent cells using available transgenic models and/or drugs (step 3). Finally, to provide conclusive evidence that senescent cells influence a phenotype, we suggest confirming that a chosen drug/model has decreased the frequency of targeted senescent cells and that this intervention has led to the expected alteration in the studied phenotype (step 4).

Assessment of senescence markers in a given tissue can be supplemented by co-identification of the cell-type identity and senescence markers that are more cell-type-specific (e.g., individual SASP factors).

(2) Providing information on the impact of senescence on a given condition:

Using means to reduce senescent cell abundance or their phenotype in tissues, ideally using two independent approaches (e.g., genetic and pharmacological).

Providing evidence that the intervention reduced senescent cell abundance or activity and coincided with a change in the (patho)physiological phenotype.

(3) Providing information to increase reproducibility and reliability of senescence research:

Reporting negative results and those that showed outcomes opposite to the hypothesis.

For results supporting a hypothesis, reporting on how many cohorts in which the results were observed and whether there were situations when the experiment did not work (and the possible reasons for this).

Reporting "failed" generation of transgenic animals, where outcomes were opposite/different from the expected.

THE SEQUENTIAL APPROACH TO DEMONSTRATE THAT SENESCENT CELLS CONTRIBUTE TO *IN VIVO* PHENOTYPES

Based on these MICSE recommendations, we provide an easy-to-follow set of guidelines for identifying senescent cells *in vivo* and demonstrate their roles in organismal phenotypes. In the current version, these are designed for investigations in mouse models, but the same recommendations are applicable to humans and other models. We propose a sequential approach for cellular senescence studies (Figure 4).

Step 1: Detection of the cell-cycle arrest proteins p21 and p16

Cell-cycle arrest machinery is widely considered necessary for the induction of senescence. Although it is possible that there

are some senescent cells expressing cell cycle inhibitors other than p21 and p16, or even no proteins from this family; there is currently no strong *in vivo* evidence to support this. With a wide range of methods available for the detection of p21 and p16 *in situ* (Figure 1), strong evidence of the presence and relevance of these proteins in murine tissues (Figure 2), and numerous transgenic mouse models developed to study these proteins and cells expressing them (Table 2), we recommend starting each senescence-research-dedicated mouse study for the detection and quantification of p21 and/or p16 at the mRNA or protein level.

Step 2: Verification of the presence of senescent cells

Although high levels of p21 and p16 provide the strongest evidence for the presence or absence of cellular senescence in mammals, these markers are not sufficiently specific. Therefore, for the second step, we recommend providing an in-depth characterization of at least two auxiliary markers of cellular senescence, LMNB1, HMGB1, SASP factors, SADS, ALISE, DNA damage, and SA-β-gal (see Figure 1), using histology, biochemical methods, FACS, or other methods. We also suggest that the quantities of senescent cells measured by different markers be compared with one another to make the most accurate predictions of the relative abundance of senescent cells. Finally, as senescence markers differ between cell types, quantification of the number of senescent cells is most reliable if performed on a single cell type obtained either by sorting or co-staining a senescence marker and a cell lineage/identity marker.

Step 3: Targeted senescence reduction

The first two steps provide strong evidence that senescent cells are present in a tissue/condition, but to claim causality of cellular senescence for a given pathological or physiological process, it is important to perform experiments on the targeted reduction of senescence. The phenotypes of existing senescent cells can be attenuated by using senomorphic or senostatic compounds. Senescent cells can be eliminated genetically (transgenic/KO mouse models) or pharmacogenetically using senolytic drugs. One noteworthy point is that different approaches to clear senescent cells yield different outcomes for numerous reasons, such as transgenic construct and drug pharmacokinetic properties. Finally, each of these approaches to reducing senescent cells has both known and unknown side effects or consequences, which may be unrelated to senescent cell ablation. Therefore, we recommend using at least two independent approaches to reduce the activity or levels of senescent cells.

Step 4: Verification of reduction in senescence and the consequent effect on phenotype

The final step for demonstrating the impact of cellular senescence on a given biological phenotype is to establish the relationship between the reduction in quantity/activity of senescent cells and an effect on the phenotype. To verify that an intervention aimed at reducing senescence *in vivo* actually did so, we recommend at least partially repeating the experiments from steps 1 and 2 post-intervention, including reporting more than one senescence endpoint. Ideally, the severity of the phenotype should be correlated with the number of senescent cells per animal. Establishing that an intervention reduces cellular senescence and modulates a given pathological feature provides reliable evidence supporting the causal contribution of cellular senescence to that pathology.

CONCLUDING REMARKS

The number of publications on cellular senescence *in vivo* has grown exponentially over the last two decades, illustrating the important role of senescent cells in numerous aspects of physiology, pathology, and aging. However, the science of cellular senescence is complex and challenging to approach experimentally. Thus, underneath this rapid progress lies the danger of publishing incomplete, overemphasized, or irreproducible results. Using MICSE, we aim to improve the quality of reporting ground-breaking findings regarding the features and functions of cellular senescence *in vivo*. Here, we assembled a brief description of the state-of-the-art for senescence research, accompanied by descriptions of the methods, sample types, and models. The essence of these is summarized in the MICSE list and accompanied by step-by-step guidelines to research senescent cells in living organisms. Importantly, the majority of our specific recommendations are applicable to mouse models, as studies in human samples, non-mammalian animals, and tumors require further research before we feel confident in providing clear recommendations without being too restrictive for research progress. Finally, the present version of MICSE is based on current knowledge and will need to be updated as our understanding of senescence *in vivo* increases. We aim to revise the MICSE guidelines every 5 years (starting 2029), incorporating the contributions from an expanded number of experts on cellular senescence *in vivo*, and peer review and publish the updated version of the MICSE.

ACKNOWLEDGMENTS

We thank Eirini Klinaki, who developed original illustrations for Figures 2 and 3.

We thank the Society of the Advancement of Research in Shock/Sepsis and Tissue Regeneration and Trauma Care Consult for supporting the "Wiggers-Bernard Conference: Senescence *in vivo*" and the MICSE workshop in Vienna.

The Research Group Senescence and Healing of Wounds (H.R., M.O., and N.A.R.R.) is a collaboration between the Ludwig Boltzmann Gesellschaft and the Austrian Workers' Compensation Board, with support from the Austrian Nationalstiftung.

M.O. is supported by Austrian Science Fund (Der Österreichischer Wissenschaftsfonds; FWF) grant DOIs https://doi.org/10.55776/P35382, https://doi.org/10.55776/P36483, and https://doi.org/10.55776/P37321 and a Federation of European Biochemical Societies Excellence (FEBS) Award.

N.A.R.R. is supported by Austrian Science Fund (Der Österreichischer Wissenschaftsfonds; FWF) grant DOI: https://doi.org/10.55776/P36483 and a Federation of European Biochemical Societies Excellence (FEBS) Award.

P.A. is supported by NIH research grants R01 AG071861, U54 AG079758, and P01 AG031862.

Core support from MRC (MC_U120085810) and a grant from CRUK (C15075/A28647) funded research in J. Gil's laboratory.

The research of K.S.-K. is funded by the German Research Foundation within the Collaborative Research Center CRC1506 (project ID 450627322) "Aging at Interfaces" and the Graduate Training Group GRK 1789 "Cellular and Molecular Mechanisms in Ageing (CEMMA)."

Work in the laboratory of M.C. is funded by grant PID2021-125479OB-I00 from MCINN/AEI/FEDER, UE.

J. Grillari kindly acknowledges support by the Austrian Science Fund (Der Österreichischer Wissenschaftsfonds; FWF) and 'Herzfelder'sche Familienstiftung project P35268-B.

F.d.d.F's laboratory is supported by ERC advanced grant (TELORN AGING—835103); AIRC-IG (21762); Telethon (GGP17111); AIRC 5X1000 (21091); Progetti di Ricerca di Interesse Nazionale (PRIN) 2015 "ATR and ATM-mediated control of chromosome integrity and cell plasticity"; Progetti di Ricerca di Interesse Nazionale (PRIN) 2017 "RNA and genome Instability"; Progetto AriSLA 2021 "DDR & ALS"; POR FESR 2014-2020 Regione Lombardia (InterSLA project); FRRB—Fondazione Regionale per la Ricerca Biomedica—under the frame of EJP RD, the European Joint Programme on Rare Diseases with funding from the European Union's Horizon 2020 research and innovation program under the EJP RD COFUND-EJP NO 825575; co-funding European Union – Next Generation EU, in the context of The National Recovery and Resilience Plan, Investment Partenariato Esteso PE8 "Conseguenze e sfide dell'invecchiamento," Project Age-It (Ageing Well in an Ageing Society), and National Center for Gene Therapy and Drugs based on RNA Technology, CN00000041.

C.L.B. is funded by the Biotechnology and Biological Sciences Research Council (BBSRC, grant BB/X006972/1)

L.J.N. and P.D.R. are supported by P01 AG043376, U19 AG056278, RO1 AG063543, P01 AG062413, U54 AG079754, and U54 AG076041 from the National Institutes of Health and an Aligning Science Across Parkinson's grant ASAP-000592 from the Michael J. Fox Foundation.

F.R is supported by grants from the Canadian Institute for Health Research (PJT173341), the Natural Sciences and Engineering Research Council (NSERC – RGPIN-2022-04385) by a FRQS junior I-II-senior career awards (22624, 33070, and 281706), and the Cancer Research Society (CRS) in partnership with Ovarian Cancer Canada (20087).

T.v.Z. is supported by BBSRC grant no. BB/S006710/1 and H2020 WIDESPREAD Project 857524.

D.J.B. is supported by grants from the National Institutes of Health (R01AG053229, R01AG068076, and U54AG079779) and the Glenn Foundation for Medical Research.

E.S. was supported by the National Science Center, grant UMO-2019/35/B/NZ4/01920.

F.G. gratefully acknowledge the financial support of the Federal Ministry for Labour and Economy of Austria and the National Foundation for Research, Technology, and Development of Austria to the Christian Doppler Laboratory for Skin Multimodal Imaging of Aging and Senescence.

Work in the Jansen-Dürr lab was supported by the Austrian Science Funds (FWF Project FG 2400 B, SENIOPROM) and the European Union (MSCA Project COFUND-ARDRE).

J.F.P. would like to acknowledge funding from the NIH grants R01AG068048, R01AG82708, UG3CA268103, and The Glenn Foundation For Medical Research.

D.J. would like to acknowledge funding from NIH grant R01 AG068182 and Hevolution/AFAR.

M.H.Y. is supported by DFG (22137416, 450807335, and 497658823) grants and TUD & CRTD funds.

J.L.K. is supported by NIH grants R37AG013925 and R33AG061456; The Connor Fund, Robert J. and Theresa W. Ryan, and the Noaber Foundation.

V.G. is supported by the National Public Investment Program of the Ministry of Development and Investment/General Secretariat for Research and Technology, in the framework of the Flagship Initiative to address SARS-CoV-2 (2020ΣΕ01300001); the European Regional Development Fund of the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH–CREATE–INNOVATE (project codes: T2EDK-02939 and T2EDK-03266); Sonia Kotopoulos donation; P. Galanis and Co. donation (lab supplies); and NKUA-SARG grant 70/3/8916.

V.K. is supported by European Research Council grant 856487, Israel Science Foundation grants 2633/17 and 1626/20, and DFG-CRC 1506 "Aging at Interfaces."

M.D. is funded by the Dutch Research Council (NWO) Talent Program (VIDI 09150172010029), Dutch Organization for Health Research and Development (ZonMw), Dutch Cancer Foundation (KWF), Health Holland, American Federation for Aging Research (AFAR), and Hevolution Foundation.

We are grateful to Barbara Bachmann, Teresa Brandtner, Helene Dworak, Ines Fischer, Eirini Klinaki, Razieh Khoshnevisan, Javier Orihuel, Tomaz Rozmaric, Cornelia Schneider, Paul Slezak, Tanja Szikora, Karla Valdivieso, and Susanne Windwarder for their help in organizing the "Wiggers-Bernard Conference: Senescence *in vivo*" and the MICSE workshop.

AUTHOR CONTRIBUTIONS

M.O. conceptualized and wrote the first draft of the manuscript. M.O., J. Grillari, and M.D. revised and finalized the manuscript; J.C.A., P.D.A., F.d.d.F., D.J.B., C.L.B., T.C., M.C., J. Gil, V.G., F.G., E.H., P.J.-D., D.J., S.K., J.L.K., V.K., T.M., J.F.P., N.A.R.R., F.R., H.R., K.S.-K., E.S., T.v.Z., L.J.N., K.W., P.D.R., and M.H.Y. contributed to writing of individual parts. M.O., D.J., T.v.Z., E.S., and D.J.B. contributed to the microscopy images shown in Figure 1. All the authors actively participated in discussions about MICSE at the "Wiggers-Bernard Conference: Senescence *in vivo*" and the MICSE workshop.

DECLARATION OF INTERESTS

D.J.B. has potential financial interests related to this study. He is a co-inventor of patents held by the Mayo Clinic, patent applications licensed to or filed by Unity Biotechnology, and a Unity Biotechnology shareholder. Research in the Baker laboratory has been reviewed by the Mayo Clinic Conflict of Interest Review Board and is being conducted in compliance with Mayo Clinic Conflict of Interest policies.

J. Gil has acted as a consultant for Unity Biotechnology, Geras Bio, Myricx Pharma Ltd., and Merck KGaA; owns equity in Geras Bio and share options in Myricx Pharma Ltd.; and is a named inventor in MRC and Imperial College patents related to senolytic therapies. J. Gil currently receives funding from Pfizer. Unity Biotechnology funded research on senolytics in J. Gil's laboratory in the past.

SenTraGor and GLF16 senescence detection compounds are under patent applications: EP3475287B1, and 20240100309 (Greek patent application) along with GB2406749.8 (UK patent application), respectively.

J.M.S. is a co-inventor on patents held by Brown University on methods to inhibit retrotransposon activation in age-related diseases. He is the scientific co-founder of Transposon Therapeutics, chair of their scientific advisory board, and a consultant and holds stock options. He is also a consultant and holds equity in Atropos Therapeutics. Research in the Sedivy laboratory has been reviewed by the Brown University Conflict of Interest Review Board and is being conducted in compliance with Brown University Conflict of Interest policies.

F.d.d.F. is an inventor on the patent applications PCT/EP2013/059753 and PCT/EP2016/068162.

M.D. is co-inventor on patents held by the Buck Institute for Research on Aging. He is the scientific co-founder of Cleara Biotech and consultant for Oisin Biotechnologies. M.D.'s laboratory currently receives research funding from Ono Pharmaceuticals.

J. Grillari is co-inventor on patents held by BOKU and is a co-founder and scientific advisor to TAmiRNA and Rockfish Bio.

REFERENCES

1. Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. Exp. Cell Res. *25*, 585–621. https://doi.org/10.1016/0014-4827(61)90192-6.

2. Théry, C., Witwer, K.W., Aikawa, E., Alcaraz, M.J., Anderson, J.D., Andriantsitohaina, R., Antoniou, A., Arab, T., Archer, F., Atkin-Smith, G.K., et al. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines.

J. Extracell. Vesicles *7*, 1535750. https://doi.org/10.1080/20013078.2018.1535750.

3. Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C., Aach, J., Ansorge, W., Ball, C.A., Causton, H.C., et al. (2001). Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat. Genet. *29*, 365–371. https://doi.org/10.1038/ng1201-365.
4. Baker, D.J., Childs, B.G., Durik, M., Wijers, M.E., Sieben, C.J., Zhong, J., Saltness, R.A., Jeganathan, K.B., Verzosa, G.C., Pezeshki, A., et al. (2016). Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. Nature *530*, 184–189. https://doi.org/10.1038/nature16932.
5. Ovadya, Y., Landsberger, T., Leins, H., Vadai, E., Gal, H., Biran, A., Yosef, R., Sagiv, A., Agrawal, A., Shapira, A., et al. (2018). Impaired immune surveillance accelerates accumulation of senescent cells and aging. Nat. Commun. *9*, 5435. https://doi.org/10.1038/s41467-018-07825-3.
6. Ring, N.A.R., Dworak, H., Bachmann, B., Schädl, B., Valdivieso, K., Rozmaric, T., Heimel, P., Fischer, I., Klinaki, E., Gutasi, A., et al. (2023). The p-rpS6-zone delineates wounding responses and the healing process. Dev. Cell *58*, 981–992.e6. https://doi.org/10.1016/j.devcel.2023.04.001.
7. Sagiv, A., Bar-Shai, A., Levi, N., Hatzav, M., Zada, L., Ovadya, Y., Roitman, L., Manella, G., Regev, O., Majewska, J., et al. (2018). p53 in Bronchial Club Cells Facilitates Chronic Lung Inflammation by Promoting Senescence. Cell Rep. *22*, 3468–3479. https://doi.org/10.1016/j.celrep.2018.03.009.
8. Levi, N., Papismadov, N., Majewska, J., Roitman, L., Wigoda, N., Eilam, R., Tsoory, M., Rotkopf, R., Ovadya, Y., Akiva, H., et al. (2023). p21 facilitates chronic lung inflammation via epithelial and endothelial cells. Aging (Albany, NY) *15*, 2395–2417. https://doi.org/10.18632/aging.204622.
9. Krizhanovsky, V., Yon, M., Dickins, R.A., Hearn, S., Simon, J., Miething, C., Yee, H., Zender, L., and Lowe, S.W. (2008). Senescence of activated stellate cells limits liver fibrosis. Cell *134*, 657–667. https://doi.org/10.1016/j.cell.2008.06.049.
10. Jurk, D., Wilson, C., Passos, J.F., Oakley, F., Correia-Melo, C., Greaves, L., Saretzki, G., Fox, C., Lawless, C., Anderson, R., et al. (2014). Chronic inflammation induces telomere dysfunction and accelerates ageing in mice. Nat. Commun. *2*, 4172. https://doi.org/10.1038/ncomms5172.
11. Ogrodnik, M., Evans, S.A., Fielder, E., Victorelli, S., Kruger, P., Salmonowicz, H., Weigand, B.M., Patel, A.D., Pirtskhalava, T., Inman, C.L., et al. (2021). Whole-body senescent cell clearance alleviates age-related brain inflammation and cognitive impairment in mice. Aging Cell *20*, e13296. https://doi.org/10.1111/acel.13296.
12. Dreesen, O., Chojnowski, A., Ong, P.F., Zhao, T.Y., Common, J.E., Lunny, D., Lane, E.B., Lee, S.J., Vardy, L.A., Stewart, C.L., and Colman, A. (2013). Lamin B1 fluctuations have differential effects on cellular proliferation and senescence. J. Cell Biol. *200*, 605–617. https://doi.org/10.1083/jcb.201206121.
13. Hewitt, G., Jurk, D., Marques, F.D.M., Correia-Melo, C., Hardy, T., Gackowska, A., Anderson, R., Taschuk, M., Mann, J., and Passos, J.F. (2012). Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. Nat. Commun. *3*, 708. https://doi.org/10.1038/ncomms1708.
14. Ogrodnik, M., Miwa, S., Tchkonia, T., Tiniakos, D., Wilson, C.L., Lahat, A., Day, C.P., Burt, A., Palmer, A., Anstee, Q.M., et al. (2017). Cellular senescence drives age-dependent hepatic steatosis. Nat. Commun. *8*, 15691. https://doi.org/10.1038/ncomms15691.
15. Farr, J.N., Xu, M., Weivoda, M.M., Monroe, D.G., Fraser, D.G., Onken, J.L., Negley, B.A., Sfeir, J.G., Ogrodnik, M.B., Hachfeld, C.M., et al. (2017). Targeting cellular senescence prevents age-related bone loss in mice. Nat. Med. *23*, 1072–1079. https://doi.org/10.1038/nm.4385.
16. Ogrodnik, M., Zhu, Y., Langhi, L.G.P., Tchkonia, T., Krüger, P., Fielder, E., Victorelli, S., Ruswhandi, R.A., Giorgadze, N., Pirtskhalava, T., et al. (2019). Obesity-Induced Cellular Senescence Drives Anxiety and Impairs Neurogenesis. Cell Metab. *29*, 1061–1077.e8. https://doi.org/10.1016/j.cmet.2018.12.008.
17. Jurk, D., Wang, C., Miwa, S., Maddick, M., Korolchuk, V., Tsolou, A., Gonos, E.S., Thrasivoulou, C., Saffrey, M.J., Cameron, K., and von Zglinicki, T. (2012). Postmitotic neurons develop a p21-dependent senescence-like phenotype driven by a DNA damage response. Aging Cell *11*, 996–1004. https://doi.org/10.1111/j.1474-9726.2012.00870.x.
18. Li, W.W., Wang, H.J., Tan, Y.Z., Wang, Y.L., Yu, S.N., and Li, Z.H. (2021). Reducing lipofuscin accumulation and cardiomyocytic senescence of aging heart by enhancing autophagy. Exp. Cell Res. *403*, 112585. https://doi.org/10.1016/j.yexcr.2021.112585.
19. Nelson, G., Wordsworth, J., Wang, C., Jurk, D., Lawless, C., Martin-Ruiz, C., and von Zglinicki, T. (2012). A senescent cell bystander effect: senescence-induced senescence. Aging Cell *11*, 345–349. https://doi.org/10.1111/j.1474-9726.2012.00795.x.
20. Childs, B.G., Baker, D.J., Wijshake, T., Conover, C.A., Campisi, J., and van Deursen, J.M. (2016). Senescent intimal foam cells are deleterious at all stages of atherosclerosis. Science *354*, 472–477. https://doi.org/10.1126/science.aaf6659.
21. Zhang, X., Habiballa, L., Aversa, Z., Ng, Y.E., Sakamoto, A.E., Englund, D.A., Pearsall, V.M., White, T.A., Robinson, M.M., Rivas, D.A., et al. (2022). Characterization of cellular senescence in aging skeletal muscle. Nat Aging *2*, 601–615. https://doi.org/10.1038/s43587-022-00250-8.
22. Dou, Z., Ghosh, K., Vizioli, M.G., Zhu, J., Sen, P., Wangensteen, K.J., Simithy, J., Lan, Y., Lin, Y., Zhou, Z., et al. (2017). Cytoplasmic chromatin triggers inflammation in senescence and cancer. Nature *550*, 402–406. https://doi.org/10.1038/nature24050.
23. Bringold, F., and Serrano, M. (2000). Tumor suppressors and oncogenes in cellular senescence. Exp. Gerontol. *35*, 317–329. https://doi.org/10.1016/s0531-5565(00)00083-8.
24. Kim, W.Y., and Sharpless, N.E. (2006). The regulation of INK4/ARF in cancer and aging. Cell *127*, 265–275. https://doi.org/10.1016/j.cell.2006.10.003.
25. Doolittle, M.L., Saul, D., Kaur, J., Rowsey, J.L., Vos, S.J., Pavelko, K.D., Farr, J.N., Monroe, D.G., and Khosla, S. (2023). Multiparametric senescent cell phenotyping reveals targets of senolytic therapy in the aged murine skeleton. Nat. Commun. *14*, 4587. https://doi.org/10.1038/s41467-023-40393-9.
26. Wang, L., Wang, B., Gasek, N.S., Zhou, Y., Cohn, R.L., Martin, D.E., Zuo, W., Flynn, W.F., Guo, C., Jellison, E.R., et al. (2022). Targeting $p21^{Cip1}$ highly expressing cells in adipose tissue alleviates insulin resistance in obesity. Cell Metab. *34*, 75–89.e8. https://doi.org/10.1016/j.cmet.2021.11.002.
27. Kawamoto, S., Uemura, K., Hori, N., Takayasu, L., Konishi, Y., Katoh, K., Matsumoto, T., Suzuki, M., Sakai, Y., Matsudaira, T., et al. (2023). Bacterial induction of B cell senescence promotes age-related changes in the gut microbiota. Nat. Cell Biol. *25*, 865–876. https://doi.org/10.1038/s41556-023-01145-5.
28. Sun, X., and Kaufman, P.D. (2018). Ki-67: more than a proliferation marker. Chromosoma *127*, 175–186. https://doi.org/10.1007/s00412-018-0659-8.
29. Mansilla, S.F., de la Vega, M.B., Calzetta, N.L., Siri, S.O., and Gottifredi, V. (2020). CDK-Independent and PCNA-Dependent Functions of p21 in DNA Replication. Genes (Basel) *11*. https://doi.org/10.3390/genes11060593.
30. Freund, A., Laberge, R.M., Demaria, M., and Campisi, J. (2012). Lamin B1 loss is a senescence-associated biomarker. Mol. Biol. Cell *23*, 2066–2075. https://doi.org/10.1091/mbc.E11-10-0884.
31. Andersson, U., Yang, H., and Harris, H. (2018). High-mobility group box 1 protein (HMGB1) operates as an alarmin outside as well as inside cells. Semin. Immunol. *38*, 40–48. https://doi.org/10.1016/j.smim.2018.02.011.
32. Davalos, A.R., Kawahara, M., Malhotra, G.K., Schaum, N., Huang, J., Ved, U., Beausejour, C.M., Coppe, J.P., Rodier, F., and Campisi, J. (2013). p53-dependent release of Alarmin HMGB1 is a central mediator

of senescent phenotypes. J. Cell Biol. *201*, 613–629. https://doi.org/10.1083/jcb.201206006.

33. Ogrodnik, M., Salmonowicz, H., and Gladyshev, V.N. (2019). Integrating cellular senescence with the concept of damage accumulation in aging: Relevance for clearance of senescent cells. Aging Cell *18*, e12841. https://doi.org/10.1111/acel.12841.
34. d'Adda di Fagagna, F. (2008). Living on a break: cellular senescence as a DNA-damage response. Nat. Rev. Cancer *8*, 512–522. https://doi.org/10.1038/nrc2440.
35. Coppé, J.P., Patil, C.K., Rodier, F., Sun, Y., Muñoz, D.P., Goldstein, J., Nelson, P.S., Desprez, P.Y., and Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol. *6*, 2853–2868. https://doi.org/10.1371/journal.pbio.0060301.
36. Rodier, F., Coppé, J.P., Patil, C.K., Hoeijmakers, W.A.M., Muñoz, D.P., Raza, S.R., Freund, A., Campeau, E., Davalos, A.R., and Campisi, J. (2009). Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. Nat. Cell Biol. *11*, 973–979. https://doi.org/10.1038/ncb1909.
37. Fumagalli, M., Rossiello, F., Clerici, M., Barozzi, S., Cittaro, D., Kaplunov, J.M., Bucci, G., Dobreva, M., Matti, V., Beausejour, C.M., et al. (2012). Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. Nat. Cell Biol. *14*, 355–365. https://doi.org/10.1038/ncb2466.
38. Pessina, F., Giavazzi, F., Yin, Y., Gioia, U., Vitelli, V., Galbiati, A., Barozzi, S., Garre, M., Oldani, A., Flaus, A., et al. (2019). Functional transcription promoters at DNA double-strand breaks mediate RNA-driven phase separation of damage-response factors. Nat. Cell Biol. *21*, 1286–1299. https://doi.org/10.1038/s41556-019-0392-4.
39. Rossiello, F., Jurk, D., Passos, J.F., and d'Adda di Fagagna, F. (2022). Telomere dysfunction in ageing and age-related diseases. Nat. Cell Biol. *24*, 135–147. https://doi.org/10.1038/s41556-022-00842-x.
40. Sen, P., Shah, P.P., Nativio, R., and Berger, S.L. (2016). Epigenetic Mechanisms of Longevity and Aging. Cell *166*, 822–839. https://doi.org/10.1016/j.cell.2016.07.050.
41. Swanson, E.C., Manning, B., Zhang, H., and Lawrence, J.B. (2013). Higher-order unfolding of satellite heterochromatin is a consistent and early event in cell senescence. J. Cell Biol. *203*, 929–942. https://doi.org/10.1083/jcb.201306073.
42. Terlecki-Zaniewicz, L., Lämmermann, I., Latreille, J., Bobbili, M.R., Pils, V., Schosserer, M., Weinmüllner, R., Dellago, H., Skalicky, S., Pum, D., et al. (2018). Small extracellular vesicles and their miRNA cargo are anti-apoptotic members of the senescence-associated secretory phenotype. Aging (Albany, NY) *10*, 1103–1132. https://doi.org/10.18632/aging.101452.
43. Birch, J., and Gil, J. (2020). Senescence and the SASP: many therapeutic avenues. Genes Dev. *34*, 1565–1576. https://doi.org/10.1101/gad.343129.120.
44. Hari, P., Millar, F.R., Tarrats, N., Birch, J., Quintanilla, A., Rink, C.J., Fernández-Duran, I., Muir, M., Finch, A.J., Brunton, V.G., et al. (2019). The innate immune sensor Toll-like receptor 2 controls the senescence-associated secretory phenotype. Sci. Adv. *5*, eaaw0254. https://doi.org/10.1126/sciadv.aaw0254.
45. Miwa, S., Kashyap, S., Chini, E., and von Zglinicki, T. (2022). Mitochondrial dysfunction in cell senescence and aging. J. Clin. Invest. *132*, e158447. https://doi.org/10.1172/JCI158447.
46. Gorgoulis, V., Adams, P.D., Alimonti, A., Bennett, D.C., Bischof, O., Bishop, C., Campisi, J., Collado, M., Evangelou, K., Ferbeyre, G., et al. (2019). Cellular Senescence: Defining a Path Forward. Cell *179*, 813–827. https://doi.org/10.1016/j.cell.2019.10.005.
47. Narzt, M.S., Pils, V., Kremslehner, C., Nagelreiter, I.M., Schosserer, M., Bessonova, E., Bayer, A., Reifschneider, R., Terlecki-Zaniewicz, L., Waidhofer-Söllner, P., et al. (2021). Epilipidomics of Senescent Dermal Fibroblasts Identify Lysophosphatidylcholines as Pleiotropic Senescence-Associated Secretory Phenotype (SASP) Factors. J. Invest. Dermatol. *141*, 993–1006.e15. https://doi.org/10.1016/j.jid.2020.11.020.
48. Magkouta, S., Veroutis, D., Pousias, A., Papaspyropoulos, A., Pippa, N., Lougiakis, N., Kambas, K., Lagopati, N., Polyzou, A., Georgiou, M., et al. (2023). A fluorophore-conjugated reagent enabling rapid detection, isolation and live tracking of senescent cells. Mol. Cell *83*, 3558–3573.e7. https://doi.org/10.1016/j.molcel.2023.09.006.
49. Evangelou, K., and Gorgoulis, V.G. (2017). Sudan Black B, The Specific Histochemical Stain for Lipofuscin: A Novel Method to Detect Senescent Cells. Methods Mol. Biol. *1534*, 111–119. https://doi.org/10.1007/978-1-4939-6670-7_10.
50. Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., and Pereira-Smith, O. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA *92*, 9363–9367. https://doi.org/10.1073/pnas.92.20.9363.
51. Lee, B.Y., Han, J.A., Im, J.S., Morrone, A., Johung, K., Goodwin, E.C., Kleijer, W.J., DiMaio, D., and Hwang, E.S. (2006). Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. Aging Cell *5*, 187–195. https://doi.org/10.1111/j.1474-9726.2006.00199.x.
52. Kurz, D.J., Decary, S., Hong, Y., and Erusalimsky, J.D. (2000). Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. J. Cell Sci. *113*, 3613–3622. https://doi.org/10.1242/jcs.113.20.3613.
53. González-Gualda, E., Pàez-Ribes, M., Lozano-Torres, B., Macias, D., Wilson, J.R., 3rd, González-López, C., Ou, H.L., Mirón-Barroso, S., Zhang, Z., Lérida-Viso, A., et al. (2020). Galacto-conjugation of Navitoclax as an efficient strategy to increase senolytic specificity and reduce platelet toxicity. Aging Cell *19*, e13142. https://doi.org/10.1111/acel.13142.
54. Biran, A., Zada, L., Abou Karam, P., Vadai, E., Roitman, L., Ovadya, Y., Porat, Z., and Krizhanovsky, V. (2017). Quantitative identification of senescent cells in aging and disease. Aging Cell *16*, 661–671. https://doi.org/10.1111/acel.12592.
55. Sun, J., Wang, M., Zhong, Y., Ma, X., Sun, S., Xu, C., Peng, L., Li, G., Zhang, L., Liu, Z., et al. (2022). A Glb1-2A-mCherry reporter monitors systemic aging and predicts lifespan in middle-aged mice. Nat. Commun. *13*, 7028. https://doi.org/10.1038/s41467-022-34801-9.
56. Behmoaras, J., and Gil, J. (2021). Similarities and interplay between senescent cells and macrophages. J. Cell Biol. *220*. https://doi.org/10.1083/jcb.202010162.
57. Tominaga, T., Shimada, R., Okada, Y., Kawamata, T., and Kibayashi, K. (2019). Senescence-associated-beta-galactosidase staining following traumatic brain injury in the mouse cerebrum. PLoS One *14*, e0213673. https://doi.org/10.1371/journal.pone.0213673.
58. Krzystyniak, A., Wesierska, M., Petrazzo, G., Gadecka, A., Dudkowska, M., Bielak-Zmijewska, A., Mosieniak, G., Figiel, I., Wlodarczyk, J., and Sikora, E. (2022). Combination of dasatinib and quercetin improves cognitive abilities in aged male Wistar rats, alleviates inflammation and changes hippocampal synaptic plasticity and histone H3 methylation profile. Aging (Albany, NY) *14*, 572–595. https://doi.org/10.18632/aging.203835.
59. Bussian, T.J., Aziz, A., Meyer, C.F., Swenson, B.L., van Deursen, J.M., and Baker, D.J. (2018). Clearance of senescent glial cells prevents tau-dependent pathology and cognitive decline. Nature *562*, 578–582. https://doi.org/10.1038/s41586-018-0543-y.
60. Piechota, M., Sunderland, P., Wysocka, A., Nalberczak, M., Sliwinska, M.A., Radwanska, K., and Sikora, E. (2016). Is senescence-associated beta-galactosidase a marker of neuronal senescence? Oncotarget *7*, 81099–81109. https://doi.org/10.18632/oncotarget.12752.
61. Gunn, D.A., de Craen, A.J.M., Dick, J.L., Tomlin, C.C., van Heemst, D., Catt, S.D., Griffiths, T., Ogden, S., Maier, A.B., Murray, P.G., et al.

(2013). Facial appearance reflects human familial longevity and cardiovascular disease risk in healthy individuals. J. Gerontol. A Biol. Sci. Med. Sci. *68*, 145–152. https://doi.org/10.1093/gerona/gls154.

62. Makrantonaki, E., Schönknecht, P., Hossini, A.M., Kaiser, E., Katsouli, M.M., Adjaye, J., Schröder, J., and Zouboulis, C.C. (2010). Skin and brain age together: The role of hormones in the ageing process. Exp. Gerontol. *45*, 801–813. https://doi.org/10.1016/j.exger.2010.08.005.

63. Castelo-Branco, C., Pons, F., Gratacós, E., Fortuny, A., Vanrell, J.A., and González-Merlo, J. (1994). Relationship between skin collagen and bone changes during aging. Maturitas *18*, 199–206. https://doi.org/10.1016/0378-5122(94)90126-0.

64. Gruber, F., Kremslehner, C., Eckhart, L., and Tschachler, E. (2020). Cell aging and cellular senescence in skin aging - Recent advances in fibroblast and keratinocyte biology. Exp. Gerontol. *130*, 110780. https://doi.org/10.1016/j.exger.2019.110780.

65. Demaria, M., Ohtani, N., Youssef, S.A., Rodier, F., Toussaint, W., Mitchell, J.R., Laberge, R.M., Vijg, J., Van Steeg, H., Dollé, M.E.T., et al. (2014). An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. Dev. Cell *31*, 722–733. https://doi.org/10.1016/j.devcel.2014.11.012.

66. Treiber, N., Maity, P., Singh, K., Kohn, M., Keist, A.F., Ferchiu, F., Sante, L., Frese, S., Bloch, W., Kreppel, F., et al. (2011). Accelerated aging phenotype in mice with conditional deficiency for mitochondrial superoxide dismutase in the connective tissue. Aging Cell *10*, 239–254. https://doi.org/10.1111/j.1474-9726.2010.00658.x.

67. Maity, P., Singh, K., Krug, L., Koroma, A., Hainzl, A., Bloch, W., Kochanek, S., Wlaschek, M., Schorpp-Kistner, M., Angel, P., et al. (2021). Persistent JunB activation in fibroblasts disrupts stem cell niche interactions enforcing skin aging. Cell Rep. *36*, 109634. https://doi.org/10.1016/j.celrep.2021.109634.

68. Solé-Boldo, L., Raddatz, G., Schütz, S., Mallm, J.P., Rippe, K., Lonsdorf, A.S., Rodríguez-Paredes, M., and Lyko, F. (2020). Single-cell transcriptomes of the human skin reveal age-related loss of fibroblast priming. Commun. Biol. *3*, 188. https://doi.org/10.1038/s42003-020-0922-4.

69. Zou, Z., Long, X., Zhao, Q., Zheng, Y., Song, M., Ma, S., Jing, Y., Wang, S., He, Y., Esteban, C.R., et al. (2021). A Single-Cell Transcriptomic Atlas of Human Skin Aging. Dev. Cell *56*, 383–397.e8. https://doi.org/10.1016/j.devcel.2020.11.002.

70. Troy, T., Jekic-McMullen, D., Sambucetti, L., and Rice, B. (2004). Quantitative comparison of the sensitivity of detection of fluorescent and bioluminescent reporters in animal models. Mol. Imaging *3*, 9–23. https://doi.org/10.1162/15353500200403196.

71. Mansfield, J.R., Gossage, K.W., Hoyt, C.C., and Levenson, R.M. (2005). Autofluorescence removal, multiplexing, and automated analysis methods for in-vivo fluorescence imaging. J. Biomed. Opt. *10*, 41207. https://doi.org/10.1117/1.2032458.

72. Keyes, B.E., Liu, S., Asare, A., Naik, S., Levorse, J., Polak, L., Lu, C.P., Nikolova, M., Pasolli, H.A., and Fuchs, E. (2016). Impaired Epidermal to Dendritic T Cell Signaling Slows Wound Repair in Aged Skin. Cell *167*, 1323–1338.e14. https://doi.org/10.1016/j.cell.2016.10.052.

73. Ge, Y., Miao, Y., Gur-Cohen, S., Gomez, N., Yang, H., Nikolova, M., Polak, L., Hu, Y., Verma, A., Elemento, O., et al. (2020). The aging skin microenvironment dictates stem cell behavior. Proc. Natl. Acad. Sci. USA *117*, 5339–5350. https://doi.org/10.1073/pnas.1901720117.

74. Birch, J., Anderson, R.K., Correia-Melo, C., Jurk, D., Hewitt, G., Marques, F.M., Green, N.J., Moisey, E., Birrell, M.A., Belvisi, M.G., et al. (2015). DNA damage response at telomeres contributes to lung aging and chronic obstructive pulmonary disease. Am. J. Physiol. Lung Cell. Mol. Physiol. *309*, L1124–L1137. https://doi.org/10.1152/ajplung.00293.2015.

75. Lee, S., Yu, Y., Trimpert, J., Benthani, F., Mairhofer, M., Richter-Pechanska, P., Wyler, E., Belenki, D., Kaltenbrunner, S., Pammer, M., et al. (2021). Virus-induced senescence is a driver and therapeutic target in COVID-19. Nature *599*, 283–289. https://doi.org/10.1038/s41586-021-03995-1.

76. Schafer, M.J., White, T.A., Iijima, K., Haak, A.J., Ligresti, G., Atkinson, E.J., Oberg, A.L., Birch, J., Salmonowicz, H., Zhu, Y., et al. (2017). Cellular senescence mediates fibrotic pulmonary disease. Nat. Commun. *8*, 14532. https://doi.org/10.1038/ncomms14532.

77. Prieto, L.I., Sturmlechner, I., Graves, S.I., Zhang, C., Goplen, N.P., Yi, E.S., Sun, J., Li, H., and Baker, D.J. (2023). Senescent alveolar macrophages promote early-stage lung tumorigenesis. Cancer Cell *41*, 1261–1275.e6. https://doi.org/10.1016/j.ccell.2023.05.006.

78. Haston, S., Gonzalez-Gualda, E., Morsli, S., Ge, J., Reen, V., Calderwood, A., Moutsopoulos, I., Panousopoulos, L., Deletic, P., Carreno, G., et al. (2023). Clearance of senescent macrophages ameliorates tumorigenesis in KRAS-driven lung cancer. Cancer Cell *41*, 1242–1260.e6. https://doi.org/10.1016/j.ccell.2023.05.004.

79. Gioia, U., Tavella, S., Martínez-Orellana, P., Cicio, G., Colliva, A., Ceccon, M., Cabrini, M., Henriques, A.C., Fumagalli, V., Paldino, A., et al. (2023). SARS-CoV-2 infection induces DNA damage, through CHK1 degradation and impaired 53BP1 recruitment, and cellular senescence. Nat. Cell Biol. *25*, 550–564. https://doi.org/10.1038/s41556-023-01096-x.

80. Piñeiro-Hermida, S., Autilio, C., Martínez, P., Bosch, F., Pérez-Gil, J., and Blasco, M.A. (2020). Telomerase treatment prevents lung profibrotic pathologies associated with physiological aging. J. Cell Biol. *219*, e202002120. https://doi.org/10.1083/jcb.202002120.

81. Farr, J.N., Fraser, D.G., Wang, H., Jaehn, K., Ogrodnik, M.B., Weivoda, M.M., Drake, M.T., Tchkonia, T., LeBrasseur, N.K., Kirkland, J.L., et al. (2016). Identification of Senescent Cells in the Bone Microenvironment. J. Bone Miner. Res. *31*, 1920–1929. https://doi.org/10.1002/jbmr.2892.

82. Kim, H.N., Xiong, J., MacLeod, R.S., Iyer, S., Fujiwara, Y., Cawley, K.M., Han, L., He, Y., Thostenson, J.D., Ferreira, E., et al. (2020). Osteocyte RANKL is required for cortical bone loss with age and is induced by senescence. JCI Insight *5*, e138815. https://doi.org/10.1172/jci.insight.138815.

83. Farr, J.N., Saul, D., Doolittle, M.L., Kaur, J., Rowsey, J.L., Vos, S.J., Froemming, M.N., Lagnado, A.B., Zhu, Y., Weivoda, M., et al. (2023). Local senolysis in aged mice only partially replicates the benefits of systemic senolysis. J. Clin. Invest. *133*, e162519. https://doi.org/10.1172/JCI162519.

84. Saul, D., Monroe, D.G., Rowsey, J.L., Kosinsky, R.L., Vos, S.J., Doolittle, M.L., Farr, J.N., and Khosla, S. (2021). Modulation of fracture healing by the transient accumulation of senescent cells. eLife *10*. https://doi.org/10.7554/eLife.69958.

85. Chandra, A., Lagnado, A.B., Farr, J.N., Doolittle, M., Tchkonia, T., Kirkland, J.L., LeBrasseur, N.K., Robbins, P.D., Niedernhofer, L.J., Ikeno, Y., et al. (2022). Targeted clearance of p21- but not p16-positive senescent cells prevents radiation-induced osteoporosis and increased marrow adiposity. Aging Cell *21*, e13602. https://doi.org/10.1111/acel.13602.

86. Wang, C., Jurk, D., Maddick, M., Nelson, G., Martin-Ruiz, C., and von Zglinicki, T. (2009). DNA damage response and cellular senescence in tissues of aging mice. Aging Cell *8*, 311–323. https://doi.org/10.1111/j.1474-9726.2009.00481.x.

87. De Cecco, M., Ito, T., Petrashen, A.P., Elias, A.E., Skvir, N.J., Criscione, S.W., Caligiana, A., Brocculi, G., Adney, E.M., Boeke, J.D., et al. (2019). L1 drives IFN in senescent cells and promotes age-associated inflammation. Nature *566*, 73–78. https://doi.org/10.1038/s41586-018-0784-9.

88. Cheng, N., Kim, K.H., and Lau, L.F. (2022). Senescent hepatic stellate cells promote liver regeneration through IL-6 and ligands of CXCR2. JCI Insight *7*, e158207. https://doi.org/10.1172/jci.insight.158207.

89. Yamagishi, R., Kamachi, F., Nakamura, M., Yamazaki, S., Kamiya, T., Takasugi, M., Cheng, Y., Nonaka, Y., Yukawa-Muto, Y., Thuy, L.T.T., et al. (2022). Gasdermin D-mediated release of IL-33 from senescent hepatic stellate cells promotes obesity-associated hepatocellular carcinoma. Sci. Immunol. *7*, eabl7209. https://doi.org/10.1126/sciimmunol.abl7209.

90. Yoshimoto, S., Loo, T.M., Atarashi, K., Kanda, H., Sato, S., Oyadomari, S., Iwakura, Y., Oshima, K., Morita, H., Hattori, M., et al. (2013). Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. Nature *499*, 97–101. https://doi.org/10.1038/nature12347.

91. Wang, B., Wang, L., Gasek, N.S., Zhou, Y., Kim, T., Guo, C., Jellison, E.R., Haynes, L., Yadav, S., Tchkonia, T., et al. (2021). An inducible p21-Cre mouse model to monitor and manipulate p21-highly-expressing senescent cells in vivo. Nat. Aging *1*, 962–973. https://doi.org/10.1038/s43587-021-00107-6.

92. Minamino, T., Orimo, M., Shimizu, I., Kunieda, T., Yokoyama, M., Ito, T., Nojima, A., Nabetani, A., Oike, Y., Matsubara, H., et al. (2009). A crucial role for adipose tissue p53 in the regulation of insulin resistance. Nat. Med. *15*, 1082–1087. https://doi.org/10.1038/nm.2014.

93. Baker, D.J., Wijshake, T., Tchkonia, T., LeBrasseur, N.K., Childs, B.G., van de Sluis, B., Kirkland, J.L., and van Deursen, J.M. (2011). Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature *479*, 232–236. https://doi.org/10.1038/nature10600.

94. Palmer, A.K., Xu, M., Zhu, Y., Pirtskhalava, T., Weivoda, M.M., Hachfeld, C.M., Prata, L.G., van Dijk, T.H., Verkade, E., Casaclang-Verzosa, G., et al. (2019). Targeting senescent cells alleviates obesity-induced metabolic dysfunction. Aging Cell *18*, e12950. https://doi.org/10.1111/acel.12950.

95. Englund, D.A., Jolliffe, A., Aversa, Z., Zhang, X., Sturmlechner, I., Sakamoto, A.E., Zeidler, J.D., Warner, G.M., McNinch, C., White, T.A., et al. (2023). p21 induces a senescence program and skeletal muscle dysfunction. Mol. Metab. *67*, 101652. https://doi.org/10.1016/j.molmet.2022.101652.

96. da Silva, P.F.L., Ogrodnik, M., Kucheryavenko, O., Glibert, J., Miwa, S., Cameron, K., Ishaq, A., Saretzki, G., Nagaraja-Grellscheid, S., Nelson, G., and von Zglinicki, T. (2019). The bystander effect contributes to the accumulation of senescent cells in vivo. Aging Cell *18*, e12848. https://doi.org/10.1111/acel.12848.

97. Lee, K.A., Flores, R.R., Jang, I.H., Saathoff, A., and Robbins, P.D. (2022). Immune Senescence, Immunosenescence and Aging. Front. Aging *3*, 900028. https://doi.org/10.3389/fragi.2022.900028.

98. Vicente, R., Mausset-Bonnefont, A.L., Jorgensen, C., Louis-Plence, P., and Brondello, J.M. (2016). Cellular senescence impact on immune cell fate and function. Aging Cell *15*, 400–406. https://doi.org/10.1111/acel.12455.

99. Yousefzadeh, M.J., Flores, R.R., Zhu, Y., Schmiechen, Z.C., Brooks, R.W., Trussoni, C.E., Cui, Y., Angelini, L., Lee, K.A., McGowan, S.J., et al. (2021). An aged immune system drives senescence and ageing of solid organs. Nature *594*, 100–105. https://doi.org/10.1038/s41586-021-03547-7.

100. Goronzy, J.J., and Weyand, C.M. (2013). Understanding immunosenescence to improve responses to vaccines. Nat. Immunol. *14*, 428–436. https://doi.org/10.1038/ni.2588.

101. Tanabe, M., Sekiguchi, T., Kaneko, N., Kobayashi, S., Takeuchi, T., Coutinho, A., Tateno, S., Maruyama, K., and Okazaki, I. (1989). Elevation of laminin and beta-subunit of prolyl 4-hydroxylase in the sera of human subjects with Schistosomiasis mansoni. Jpn. J. Exp. Med. *59*, 109–119.

102. Liu, Y., Sanoff, H.K., Cho, H., Burd, C.E., Torrice, C., Ibrahim, J.G., Thomas, N.E., and Sharpless, N.E. (2009). Expression of p16(INK4a) in peripheral blood T-cells is a biomarker of human aging. Aging Cell *8*, 439–448. https://doi.org/10.1111/j.1474-9726.2009.00489.x.

103. Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell *82*, 675–684. https://doi.org/10.1016/0092-8674(95)90039-x.

104. Sharpless, N.E., Ramsey, M.R., Balasubramanian, P., Castrillon, D.H., and DePinho, R.A. (2004). The differential impact of p16(INK4a) or p19(ARF) deficiency on cell growth and tumorigenesis. Oncogene *23*, 379–385. https://doi.org/10.1038/sj.onc.1207074.

105. Blasco, M.A., Lee, H.W., Hande, M.P., Samper, E., Lansdorp, P.M., DePinho, R.A., and Greider, C.W. (1997). Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell *91*, 25–34. https://doi.org/10.1016/s0092-8674(01)80006-4.

106. Wong, A., Kieu, T., and Robbins, P.D. (2020). The Ercc1$^{-/\Delta}$ mouse model of accelerated senescence and aging for identification and testing of novel senotherapeutic interventions. Aging (Albany, NY) *12*, 24481–24483. https://doi.org/10.18632/aging.202321.

107. Yousefzadeh, M.J., Zhao, J., Bukata, C., Wade, E.A., McGowan, S.J., Angelini, L.A., Bank, M.P., Gurkar, A.U., McGuckian, C.A., Calubag, M.F., et al. (2020). Tissue specificity of senescent cell accumulation during physiologic and accelerated aging of mice. Aging Cell *19*, e13094. https://doi.org/10.1111/acel.13094.

108. Yousefzadeh, M.J., Zhu, Y., McGowan, S.J., Angelini, L., Fuhrmann-Stroissnigg, H., Xu, M., Ling, Y.Y., Melos, K.I., Pirtskhalava, T., Inman, C.L., et al. (2018). Fisetin is a senotherapeutic that extends health and lifespan. EBioMedicine *36*, 18–28. https://doi.org/10.1016/j.ebiom.2018.09.015.

109. Lushchak, O., Schosserer, M., and Grillari, J. (2023). Senopathies-Diseases Associated with Cellular Senescence. Biomolecules *13*, 966. https://doi.org/10.3390/biom13060966.

110. Ohtani, N., Imamura, Y., Yamakoshi, K., Hirota, F., Nakayama, R., Kubo, Y., Ishimaru, N., Takahashi, A., Hirao, A., Shimizu, T., et al. (2007). Visualizing the dynamics of p21(Waf1/Cip1) cyclin-dependent kinase inhibitor expression in living animals. Proc. Natl. Acad. Sci. USA *104*, 15034–15039. https://doi.org/10.1073/pnas.0706949104.

111. Yamakoshi, K., Takahashi, A., Hirota, F., Nakayama, R., Ishimaru, N., Kubo, Y., Mann, D.J., Ohmura, M., Hirao, A., Saya, H., et al. (2009). Real-time in vivo imaging of p16Ink4a reveals cross talk with p53. J. Cell Biol. *186*, 393–407. https://doi.org/10.1083/jcb.200904105.

112. Burd, C.E., Sorrentino, J.A., Clark, K.S., Darr, D.B., Krishnamurthy, J., Deal, A.M., Bardeesy, N., Castrillon, D.H., Beach, D.H., and Sharpless, N.E. (2013). Monitoring tumorigenesis and senescence in vivo with a p16(INK4a)-luciferase model. Cell *152*, 340–351. https://doi.org/10.1016/j.cell.2012.12.010.

113. Liu, J.Y., Souroullas, G.P., Diekman, B.O., Krishnamurthy, J., Hall, B.M., Sorrentino, J.A., Parker, J.S., Sessions, G.A., Gudkov, A.V., and Sharpless, N.E. (2019). Cells exhibiting strong p16^{INK4a} promoter activation in vivo display features of senescence. Proc. Natl. Acad. Sci. USA *116*, 2603–2611. https://doi.org/10.1073/pnas.1818313116.

114. Li, Y., Zhao, H., Huang, X., Tang, J., Zhang, S., Li, Y., Liu, X., He, L., Ju, Z., Lui, K.O., and Zhou, B. (2018). Embryonic senescent cells re-enter cell cycle and contribute to tissues after birth. Cell Res. *28*, 775–778. https://doi.org/10.1038/s41422-018-0050-6.

115. Omori, S., Wang, T.W., Johmura, Y., Kanai, T., Nakano, Y., Kido, T., Susaki, E.A., Nakajima, T., Shichino, S., Ueha, S., et al. (2020). Generation of a p16 Reporter Mouse and Its Use to Characterize and Target p16^{high} Cells In Vivo. Cell Metab. *32*, 814–828.e6. https://doi.org/10.1016/j.cmet.2020.09.006.

116. Reyes, N.S., Krasilnikov, M., Allen, N.C., Lee, J.Y., Hyams, B., Zhou, M., Ravishankar, S., Cassandras, M., Wang, C., Khan, I., et al. (2022). p16^{INK4a+} cells in the basement membrane form a reparative niche in the lung. Science *378*, 192–201. https://doi.org/10.1126/science.abf3326.

117. Grosse, L., Wagner, N., Emelyanov, A., Molina, C., Lacas-Gervais, S., Wagner, K.D., and Bulavin, D.V. (2020). Defined p16^{High} Senescent Cell Types Are Indispensable for Mouse Healthspan. Cell Metab. *32*, 87–99.e6. https://doi.org/10.1016/j.cmet.2020.05.002.

118. Wang, W., Martindale, J.L., Yang, X., Chrest, F.J., and Gorospe, M. (2005). Increased stability of the p16 mRNA with replicative senescence. EMBO Rep. *6*, 158–164. https://doi.org/10.1038/sj.embor.7400346.

119. Mitchell, S.J., Scheibye-Knudsen, M., Longo, D.L., and de Cabo, R. (2015). Animal models of aging research: implications for human aging and age-related diseases. Annu. Rev. Anim. Biosci. *3*, 283–303. https://doi.org/10.1146/annurev-animal-022114-110829.

120. Chenouard, V., Remy, S., Tesson, L., Ménoret, S., Ouisse, L.H., Cherifi, Y., and Anegon, I. (2021). Advances in Genome Editing and Application to the Generation of Genetically Modified Rat Models. Front. Genet. *12*, 615491. https://doi.org/10.3389/fgene.2021.615491.

121. Nacher, V., Carretero, A., Navarro, M., Armengol, C., Llombart, C., Rodríguez, A., Herrero-Fresneda, I., Ayuso, E., and Ruberte, J. (2006). The quail mesonephros: a new model for renal senescence? J. Vasc. Res. *43*, 581–586. https://doi.org/10.1159/000096076.

122. Yun, M.H., Davaapil, H., and Brockes, J.P. (2015). Recurrent turnover of senescent cells during regeneration of a complex structure. eLife *4*, e05505. https://doi.org/10.7554/eLife.05505.

123. Davaapil, H., Brockes, J.P., and Yun, M.H. (2017). Conserved and novel functions of programmed cellular senescence during vertebrate development. Development *144*, 106–114. https://doi.org/10.1242/dev.138222.

124. Walters, H.E., Troyanovskiy, K.E., Graf, A.M., and Yun, M.H. (2023). Senescent cells enhance newt limb regeneration by promoting muscle dedifferentiation. Aging Cell *22*, e13826. https://doi.org/10.1111/acel.13826.

125. Da Silva-Álvarez, S., Guerra-Varela, J., Sobrido-Cameán, D., Quelle, A., Barreiro-Iglesias, A., Sánchez, L., and Collado, M. (2020). Cell senescence contributes to tissue regeneration in zebrafish. Aging Cell *19*, e13052. https://doi.org/10.1111/acel.13052.

126. Van Houcke, J., Mariën, V., Zandecki, C., Ayana, R., Pepermans, E., Boonen, K., Seuntjens, E., Baggerman, G., and Arckens, L. (2023). A short dasatinib and quercetin treatment is sufficient to reinstate potent adult neuroregenesis in the aged killifish. NPJ Regen. Med. *8*, 31. https://doi.org/10.1038/s41536-023-00304-4.

127. Salinas-Saavedra, M., Febrimarsa, Krasovec, G., Horkan, H.R., Baxevanis, A.D., and Frank, U. (2023). Senescence-induced cellular reprogramming drives cnidarian whole-body regeneration. Cell Rep. *42*, 112687. https://doi.org/10.1016/j.celrep.2023.112687.

128. Nakamura, M., Ohsawa, S., and Igaki, T. (2014). Mitochondrial defects trigger proliferation of neighbouring cells via a senescence-associated secretory phenotype in Drosophila. Nat. Commun. *5*, 5264. https://doi.org/10.1038/ncomms6264.

129. Czarkwiani, A., and Yun, M.H. (2018). Out with the old, in with the new: senescence in development. Curr. Opin. Cell Biol. *55*, 74–80. https://doi.org/10.1016/j.ceb.2018.05.014.

130. Novoa, B., Pereiro, P., López-Muñoz, A., Varela, M., Forn-Cuní, G., Anchelin, M., Dios, S., Romero, A., Martinez-López, A., Medina-Gali, R.M., et al. (2019). Rag1 immunodeficiency-induced early aging and senescence in zebrafish are dependent on chronic inflammation and oxidative stress. Aging Cell *18*, e13020. https://doi.org/10.1111/acel.13020.

131. Vanhunsel, S., Bergmans, S., Beckers, A., Etienne, I., Van Houcke, J., Seuntjens, E., Arckens, L., De Groef, L., and Moons, L. (2021). The killifish visual system as an in vivo model to study brain aging and rejuvenation. NPJ Aging Mech. Dis. *7*, 22. https://doi.org/10.1038/s41514-021-00077-4.

132. Kim, S.H., Mitchell, M., Fujii, H., Llanos, S., and Peters, G. (2003). Absence of p16INK4a and truncation of ARF tumor suppressors in chickens. Proc. Natl. Acad. Sci. USA *100*, 211–216. https://doi.org/10.1073/pnas.0135557100.

133. Yu, Q., Walters, H.E., Pasquini, G., Pal Singh, S., Lachnit, M., Oliveira, C.R., León-Periñán, D., Petzold, A., Kesavan, P., Subiran Adrados, C., et al. (2023). Cellular senescence promotes progenitor cell expansion during axolotl limb regeneration. Dev. Cell *58*, 2416–2427.e7. https://doi.org/10.1016/j.devcel.2023.09.009.

134. Muñoz-Espín, D., Rovira, M., Galiana, I., Giménez, C., Lozano-Torres, B., Paez-Ribes, M., Llanos, S., Chaib, S., Muñoz-Martín, M., Ucero, A.C., et al. (2018). A versatile drug delivery system targeting senescent cells. EMBO Mol. Med. *10*, e9355. https://doi.org/10.15252/emmm.201809355.

135. Morsli, S., Henriques, C.M., Ellis, P.S., Mortiboys, H., Baxendale, S., Loynes, C.A., Renshaw, S.A., and Bellantuono, I. (2023). A p21-GFP zebrafish model of senescence for rapid testing of senolytics in vivo. Aging Cell *22*, e13835. https://doi.org/10.1111/acel.13835.

136. Teo, Y.V., Rattanavirotkul, N., Olova, N., Salzano, A., Quintanilla, A., Tarrats, N., Kiourtis, C., Müller, M., Green, A.R., Adams, P.D., et al. (2019). Notch Signaling Mediates Secondary Senescence. Cell Rep. *27*, 997–1007.e5. https://doi.org/10.1016/j.celrep.2019.03.104.

137. Ogrodnik, M. (2021). Cellular aging beyond cellular senescence: markers of senescence prior to cell cycle arrest in vitro and in vivo. Aging Cell *20*, e13338. https://doi.org/10.1111/acel.13338.

138. Mund, A., Coscia, F., Kriston, A., Hollandi, R., Kovács, F., Brunner, A.D., Migh, E., Schweizer, L., Santos, A., Bzorek, M., et al. (2022). Deep Visual Proteomics defines single-cell identity and heterogeneity. Nat. Biotechnol. *40*, 1231–1240. https://doi.org/10.1038/s41587-022-01302-5.

139. SenNet Consortium (2022). NIH SenNet Consortium to map senescent cells throughout the human lifespan to understand physiological health. Nat. Aging *2*, 1090–1100. https://doi.org/10.1038/s43587-022-00326-5.

140. Kiss, T., Nyúl-Tóth, Á., DelFavero, J., Balasubramanian, P., Tarantini, S., Faakye, J., Gulej, R., Ahire, C., Ungvari, A., Yabluchanskiy, A., et al. (2022). Spatial transcriptomic analysis reveals inflammatory foci defined by senescent cells in the white matter, hippocampi and cortical grey matter in the aged mouse brain. GeroScience *44*, 661–681. https://doi.org/10.1007/s11357-022-00521-7.

141. Vandereyken, K., Sifrim, A., Thienpont, B., and Voet, T. (2023). Methods and applications for single-cell and spatial multi-omics. Nat. Rev. Genet. *24*, 494–515. https://doi.org/10.1038/s41576-023-00580-2.

142. Garbarino, O., Lambroia, L., Basso, G., Marrella, V., Franceschini, B., Soldani, C., Pasqualini, F., Giuliano, D., Costa, G., Peano, C., et al. (2023). Spatial resolution of cellular senescence dynamics in human colorectal liver metastasis. Aging Cell *22*, e13853. https://doi.org/10.1111/acel.13853.

143. Gurkar, A.U., Gerencser, A.A., Mora, A.L., Nelson, A.C., Zhang, A.R., Lagnado, A.B., Enninful, A., Benz, C., Furman, D., Beaulieu, D., et al. (2023). Spatial mapping of cellular senescence: emerging challenges and opportunities. Nat. Aging *3*, 776–790. https://doi.org/10.1038/s43587-023-00446-6.

144. Saul, D., Kosinsky, R.L., Atkinson, E.J., Doolittle, M.L., Zhang, X., LeBrasseur, N.K., Pignolo, R.J., Robbins, P.D., Niedernhofer, L.J., Ikeno, Y., et al. (2022). A new gene set identifies senescent cells and predicts senescence-associated pathways across tissues. Nat. Commun. *13*, 4827. https://doi.org/10.1038/s41467-022-32552-1.

145. O'Sullivan, E.D., Mylonas, K.J., Bell, R., Carvalho, C., Baird, D.P., Cairns, C., Gallagher, K.M., Campbell, R., Docherty, M., Laird, A., et al. (2022). Single-cell analysis of senescent epithelia reveals targetable mechanisms promoting fibrosis. JCI Insight *7*, e154124. https://doi.org/10.1172/jci.insight.154124.

146. Hernandez-Segura, A., de Jong, T.V., Melov, S., Guryev, V., Campisi, J., and Demaria, M. (2017). Unmasking transcriptional heterogeneity in senescent cells. Curr. Biol. *27*, 2652–2660.e4. https://doi.org/10.1016/j.cub.2017.07.033.

147. Wallis, R., Milligan, D., Hughes, B., Mizen, H., López-Domínguez, J.A., Eduputa, U., Tyler, E.J., Serrano, M., and Bishop, C.L. (2022). Senescence-associated morphological profiles (SAMPs): an image-based phenotypic profiling method for evaluating the inter and intra model heterogeneity of senescence. Aging (Albany, NY) *14*, 4220–4246. https://doi.org/10.18632/aging.204072.

148. Duran, I., Pombo, J., Sun, B., Gallage, S., Kudo, H., McHugh, D., Bousset, L., Barragan Avila, J.E., Forlano, R., Manousou, P., et al. (2024). Detection of senescence using machine learning algorithms based on nuclear features. Nat. Commun. *15*, 1041. https://doi.org/10.1038/s41467-024-45421-w.

149. Heckenbach, I., Mkrtchyan, G.V., Ezra, M.B., Bakula, D., Madsen, J.S., Nielsen, M.H., Oró, D., Osborne, B., Covarrubias, A.J., Idda, M.L., et al. (2022). Nuclear morphology is a deep learning biomarker of cellular

senescence. Nat. Aging *2*, 742–755. https://doi.org/10.1038/s43587-022-00263-3.

150. Campisi, J. (2013). Aging, cellular senescence, and cancer. Annu. Rev. Physiol. *75*, 685–705. https://doi.org/10.1146/annurev-physiol-030212-183653.
151. Schmitt, C.A., Fridman, J.S., Yang, M., Lee, S., Baranov, E., Hoffman, R.M., and Lowe, S.W. (2002). A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. Cell *109*, 335–346. https://doi.org/10.1016/s0092-8674(02)00734-1.
152. Collado, M., and Serrano, M. (2010). Senescence in tumours: evidence from mice and humans. Nat. Rev. Cancer *10*, 51–57. https://doi.org/10.1038/nrc2772.
153. Grillari, J., Katinger, H., and Voglauer, R. (2007). Contributions of DNA interstrand cross-links to aging of cells and organisms. Nucleic Acids Res. *35*, 7566–7576. https://doi.org/10.1093/nar/gkm1065.
154. Lee, S., and Schmitt, C.A. (2019). The dynamic nature of senescence in cancer. Nat. Cell Biol. *21*, 94–101. https://doi.org/10.1038/s41556-018-0249-2.
155. Fleury, H., Malaquin, N., Tu, V., Gilbert, S., Martinez, A., Olivier, M.A., Sauriol, S.A., Communal, L., Leclerc-Desaulniers, K., Carmona, E., et al. (2019). Exploiting interconnected synthetic lethal interactions between PARP inhibition and cancer cell reversible senescence. Nat. Commun. *10*, 2556. https://doi.org/10.1038/s41467-019-10460-1.
156. Wang, C., Vegna, S., Jin, H., Benedict, B., Lieftink, C., Ramirez, C., de Oliveira, R.L., Morris, B., Gadiot, J., Wang, W., et al. (2019). Inducing and exploiting vulnerabilities for the treatment of liver cancer. Nature *574*, 268–272. https://doi.org/10.1038/s41586-019-1607-3.
157. Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593–602. https://doi.org/10.1016/s0092-8674(00)81902-9.
158. Romagosa, C., Simonetti, S., López-Vicente, L., Mazo, A., Lleonart, M.E., Castellvi, J., and Ramon y Cajal, S. (2011). p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. Oncogene *30*, 2087–2097. https://doi.org/10.1038/onc.2010.614.
159. Valieva, Y., Ivanova, E., Fayzullin, A., Kurkov, A., and Igrunkova, A. (2022). Senescence-Associated beta-Galactosidase Detection in Pathology. Diagnostics (Basel) *12*, 2309. https://doi.org/10.3390/diagnostics12102309.
160. Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C., and Lowe, S.W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature *445*, 656–660. https://doi.org/10.1038/nature05529.
161. Evangelou, K., Belogiannis, K., Papaspyropoulos, A., Petty, R., and Gorgoulis, V.G. (2023). Escape from senescence: molecular basis and therapeutic ramifications. J. Pathol. *260*, 649–665. https://doi.org/10.1002/path.6164.
162. Chaib, S., Tchkonia, T., and Kirkland, J.L. (2022). Cellular senescence and senolytics: the path to the clinic. Nat. Med. *28*, 1556–1568. https://doi.org/10.1038/s41591-022-01923-y.
163. Victorelli, S., Lagnado, A., Halim, J., Moore, W., Talbot, D., Barrett, K., Chapman, J., Birch, J., Ogrodnik, M., Meves, A., et al. (2019). Senescent human melanocytes drive skin ageing via paracrine telomere dysfunction. EMBO J. *38*, e101982. https://doi.org/10.15252/embj.2019101982.
164. Ressler, S., Bartkova, J., Niederegger, H., Bartek, J., Scharffetter-Kochanek, K., Jansen-Dürr, P., and Wlaschek, M. (2006). p16INK4A is a robust in vivo biomarker of cellular aging in human skin. Aging Cell *5*, 379–389. https://doi.org/10.1111/j.1474-9726.2006.00231.x.
165. Sindrilaru, A., Peters, T., Wieschalka, S., Baican, C., Baican, A., Peter, H., Hainzl, A., Schatz, S., Qi, Y., Schlecht, A., et al. (2011). An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. J. Clin. Invest. *121*, 985–997. https://doi.org/10.1172/JCI44490.
166. Kultima, K., Sköld, K., and Borén, M. (2011). Biomarkers of disease and post-mortem changes - Heat stabilization, a necessary tool for measurement of protein regulation. J. Proteomics *75*, 145–159. https://doi.org/10.1016/j.jprot.2011.06.009.
167. Wang, Y., Zhang, Y., Hu, W., Xie, S., Gong, C.X., Iqbal, K., and Liu, F. (2015). Rapid alteration of protein phosphorylation during postmortem: implication in the study of protein phosphorylation. Sci. Rep. *5*, 15709. https://doi.org/10.1038/srep15709.