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Review

Hallmarks of Cellular Senescence

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Cellular senescence is a permanent state of cell cycle arrest that promotes tissue remodeling during development and after injury, but can also contribute to the decline of the regenerative potential and function of tissues, to inflammation, and to tumorigenesis in aged organisms. Therefore, the identification, characterization, and pharmacological elimination of senescent cells have gained attention in the field of aging research. However, the nonspecificity of current senescence markers and the existence of different senescence programs strongly limit these tasks. Here, we describe the molecular regulators of senescence phenotypes and how they are used for identifying senescent cells *in vitro* and *in vivo*. We also highlight the importance that these levels of regulations have in the development of therapeutic targets.

Highlights

The phenotype associated with cellular senescence is highly variable and heterogeneous.

Senescent cells show common marks, but mechanisms behind these marks are not widely conserved among all the senescence programs.

Lack of universal or program-specific markers is a major limitation for the identification and the targeting of senescent cells *in vitro* and *in vivo*.

Technological advancements or more systematic approaches need to address difficulties associated with the study of cellular senescence.

The Complexity of the Senescence Phenotype

The functional decline of an organism throughout its life affects multiple organs and is accompanied by the appearance of several diseases. This general decline of functional capabilities is known as **aging** (see Glossary) and is fairly conserved among species [1].

A main feature of aged organisms is the accumulation of cellular senescence [1], a state of permanent cell cycle arrest in response to different damaging stimuli [2] (Box 1). Excessive and aberrant accumulation of senescent cells in tissues can negatively affect regenerative capacities and create a proinflammatory milieu favorable for the onset and progression of various age-related diseases, including cancer [3,4]. However, senescent cells have several beneficial functions for the organism. Due to the activation of an irreversible proliferation arrest, cellular senescence is seen as a strong safeguard against tumorigenesis [3]. Moreover, senescent cells can act via both cell and non-cell autonomous mechanisms as positive regulators of tissue remodeling and repair during development and adulthood [5,6]. Deleterious functions of senescent cells are potentially powerful targets for antiaging approaches [7], but the existence of beneficial senescence programs complicates the development of interventions without incurring toxicities.

The senescence phenotype is often characterized by the activation of a chronic **DNA damage response** (DDR), the engagement of various cyclin-dependent kinase inhibitors (CDKi), enhanced secretion of proinflammatory and tissue-remodeling factors, induction of antiapoptotic genes, altered metabolic rates, and endoplasmic reticulum (ER) stress (Figure 1, Key Figure). As a consequence of these signaling pathways, senescent cells show structural aberrations, from enlarged and more flattened morphology, altered composition of the plasma membrane (PM), accumulation of lysosomes and mitochondria, and nuclear changes (Figure 2).

The understanding of how these different hallmarks are regulated and how they are present in non-senescence states is essential to choosing the right methods to measure them. However, there are two important problems for the identification, isolation, and characterization of senescent cells. First, many of the senescence-associated molecular and morphological

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Box 1. Types of Senescence

In vitro senescence can be induced by different stimuli [2]. Whether all these 'types of senescence' actually occur in *in vivo* is not yet known. Here, we describe the main models of senescence used in research. At least two other biological events, namely wound healing and development, are known to rely on senescence [3]. However, these types have been less described and, thus, are beyond the scope of this review. **Replicative senescence:** this refers to the decrease in proliferation potential observed after multiple cell divisions that ultimately leads to total arrest [160]. The shortening of telomeres as a consequence of multiple cell divisions in nontransformed cells has been blamed for this type of senescence [3].

DNA damage-induced senescence: irreparable DNA damage can induce either senescence or apoptosis, depending on the magnitude of the damage [2]. *In vitro*, multiple DNA-damaging agents are used to induce this type of senescence, including radiation (ionizing and UV) or multiple drugs (see 'chemotherapy-induced senescence') [2].

Oncogene-induced senescence (OIS): the activation of oncogenes, such as Ras or BRAF, or the inactivation of tumor suppressors, such as PTEN, can lead to OIS [2,3].

Oxidative stress-induced senescence: either oxidizing products of the cell metabolism or known oxidative agents (e.g., H2O2) can cause senescence [49]. Although oxidizing agents exert their effect partly through DNA damage, other cellular components and processes are also affected.

Chemotherapy-induced senescence: multiple anticancer drugs are able to induce senescence. Some (such as bleomycin or doxorubicin) induce DNA damage, while others can act through different mechanisms, such as inhibition of CDKs (e.g., abemaciclib and palbociclib) [163].

Mitochondrial dysfunction-associated senescence (MiDAS): it was recently reported that induction of mitochondrial dysfunction also leads to senescence [56]. The phenotype, particularly the SASP, appears to be characteristic of this type of senescence [56].

Epigenetically induced senescence: inhibitors of DNA methylases (e.g., 5-aza-2′-deoxycytidine) or histone deacetylases (e.g., suberoylanilide hydroxamic acid and sodium butyrate) are also known to cause senescence [163].

Paracrine senescence: senescence induced by the SASP produced by a primary senescent cell [70].

features are present in other cellular states and conditions. Second, the phenotype of senescent cells is highly heterogeneous and dynamic, possibly a consequence of various distinct senescence programs. These limitations have to be taken into account carefully for the generation of therapies targeting senescent cells.

Here, we describe the main hallmarks of senescent cells, the methods used to measure them, and the limitations for their use as markers. Finally, we discuss how these senescence-associated hallmarks are currently being exploited for antisenescence interventions.

Signaling pathways as Hallmarks of Senescence

DNA Damage Response

In the presence of DNA damage, cells activate a robust response, the DDR. Double-strand DNA breaks (DSBs) are powerful activators of DDR, and can lead to cellular senescence when unresolved. DSBs promote the recruitment and binding of ATM kinase to the DNA damage site [8,9]. This recruitment drives phosphorylation of the histone H2AX, which facilitates the assembly of specific DNA repair complexes (Figure 1) [10]. Histone methylation can also contribute to the assembly of damage response components; a complex of kap-1, HP1, and the H3K9 methyltransferase suv39h1 is loaded directly onto the chromatin at DSBs, leading to the methylation of H3K9. H3K9me3 functions as a binding site and activates the acetyltransferase Tip60, which subsequently acetylates and activates ATM [11]. Therefore, H3K9 methylation is required for ATM-mediated DNA damage signaling during early stages of

Glossary

Aging: functional decline or an organism throughout life [1].
Alternative splicing: process that allows a gene to encode different mRNA products by differentially using exons and excluding introns in a primary transcript to give rise to different processed mRNAs.
Apoptosis: normal physiological form of cell death [82].
Autophagy: intracellular degradation system. It can degrade nonspecific (general autophagy) or specific (selective autophagy) targets [149].
Caveolae: cholesterol-enriched microdomains of the plasma membrane [119].
Cellular senescence: state of permanent cell cycle arrest in response to different damaging stimuli [2].
DNA damage response (DDR): robust response of the cells to the presence of DNA damage.
Epigenetic: regulatory mechanism of genes that affects their transcription. It includes methylation of DNA, post-translational modification of histones, and other chromatin-remodeling events [1].
Heterochromatin: chromatin enriched in repressive marks.
Inflammasome: protein complex formed by caspase-1 and adaptor proteins.
Metabolism: set of chemical reactions that occurs in an organism to obtain energy and building materials or to use them to build different and more complex structures.
Mitochondrial dysfunction: condition in which the regulation of mitochondrial homeostasis, production of mitochondrial metabolites, mitochondrial membrane potential, and ROS generation are altered [95,143].
Mitophagy: selective autophagy of mitochondria [146].
Nuclear lamina: protein structure surrounding the interior of the nuclear membrane that supports the structural integrity and shape of the nucleus [154].
'Omics techniques: high-throughput techniques including genomics, proteomics, transcriptomics, metabolomics, and so on.

the DDR, but H3K9 methylation has to be later reversed to promote the repair process. DDR provokes the degradation of G9a/GLP methyltransferase, which causes a global reduction in H3K9 dimethylation, including that of IL-6 and IL-8 promoters, two components of the senescence-associated **secretory phenotype** (SASP; discussed below) [12]. Many substrates are phosphorylated by ATM, including the two essential kinases CHK1 and CHK2, which propagate the signal by further phosphorylating their substrates [13,14]. The persistence of DDR induces the phosphorylation of p53 at multiple serine residues, which enhances the ability of p53 to induce the transcription of many genes [15].

Inductions of γ-H2AX nuclear foci or phosphorylated p53 are commonly used as markers of senescence. However, the DDR is activated by a variety of DNA-damaging stimuli that do not lead cells into a senescent state. Moreover, not all senescence programs are a consequence of DDRs.

Cyclin-Dependent Kinase Inhibitors and Cell Cycle Arrest

CDKs phosphorylate and regulate multiple proteins involved in cell cycle progression (Figure 1). Main drivers of the cell cycle arrest in senescence are the CDKis encoded in the CDKN2A ($p16^{INK4a}$, hereafter p16), CDKN2B ($p15^{INK4b}$, hereafter p15)m and CDKN1A ($p21^{CIP}$, hereafter p21) loci.

P16 comprises a 136-kb protein that directly interacts and inhibits CDK4/6. P16 is often used as a unique and specific marker for senescence (Box 2), and its transcriptional activation has been used extensively to report the presence of senescent cells *in vivo* [6,16,17]. Experimental evidence suggests that the main inducers of P16 levels are epigenetic changes, but other regulators, from promoter accessibility to protein stability, have been described.

The methyl-transferase DNMT3b is responsible for the *de novo* methylation of the p16 promoter [18], while DNMT1 maintains existing methylation. Inhibitors of DNMT1 cause demethylation of the p16 promoter and a senescence-like phenotype [19–21]. However, methylation levels do not always correlate with *p16* gene expression [22]. The Polycomb group repressive complexes 1 and 2 (PRC1 and PRC2) are also responsible for the deposition of repressive histone modifications at the CDKN2A locus [23], and can be recruited to the p16 promoter by the antisense long noncoding RNA for p16, ANRIL [24]. Other epigenetic marks, such as the repressive histone variant macroH2A1, are enriched in the inactive, but depleted in the active, p16 locus [25].

Transcription factors, such as Sp1, Ets, AP1 (particularly JunB subunit), and PPARγ [26–29], bind to the p16 promoter and trigger its transcription, while repressor mechanisms, such as the INK4A transcription silence element (ITSE), YB1, ID1, and AP-1 (c-Jun subunit), balance the activation of p16 [23,27,30,31].

The RNA-binding proteins hnRNP A1 and A2 promote the stability of p16 transcripts [32], while the ribonuclear protein AUF1 binds p16 mRNA and promotes its degradation [33]. Interestingly, there are also hints that p16 can suppress the expression of AUF1 [34]. Translation of the p16 mRNA can be modulated through a region on its 5′ untranslated region (UTR) end, which contains an internal ribosome entry site (IRES) [35], and the affinity of p16 for CDK4 can be modulated by Ser140 phosphorylation and Arg138 methylation [36]. Finally, p16 protein is degraded by N terminus polyubiquitinylation and, ultimately, the proteasome and this elimination is favored upon conditions of subconfluence [37,38].

Post-translational regulation: regulatory mechanism in which a protein suffers modifications (such as methylations, phosphorylations, acetylations, shedding, etc.) that affect its function.
qPCR: molecular biology technique used to amplify and detect small amounts of mRNA from a particular protein and to quantify them [161].
Reactive oxygen species (ROS): by-products of mitochondrial respiration [143].
Senescence-Associated-β galactosidase (SA-βGal): activity of the β-galactosidase enzyme detectable at pH 6.0
Senescence-associated heterochromatic foci (SAHFs): 4′,6-diamidino-2-phenylindole (DAPI) intense nuclear foci enriched in repressive epigenetic marks [153].
Secretory phenotype: set of molecules secreted by senescent cells. It includes extracellular matrix-remodeling enzymes and inflammatory molecules [3].
Shedding: proteolytic release of the extracellular domain of transmembrane proteins [75].
Transactivation: increased expression of a gene requiring the presence of another protein called a 'transactivator'.
Transcription: regulatory mechanism in which the transcription of the gene is either activated or repressed, for instance by transcription factors or repressors.
Translational regulation: regulatory mechanism in which the translation of an mRNA into protein is either enhanced or reduced.
Unfolded protein response (UPR): response of the ER against accumulation and aggregation of proteins that occurs as a consequence of multiple factors, such as oxidative stress, mutation, infection, and lack of chaperons [105].

Key Figure

Hallmarks of Senescence Regulation of Signaling Pathways

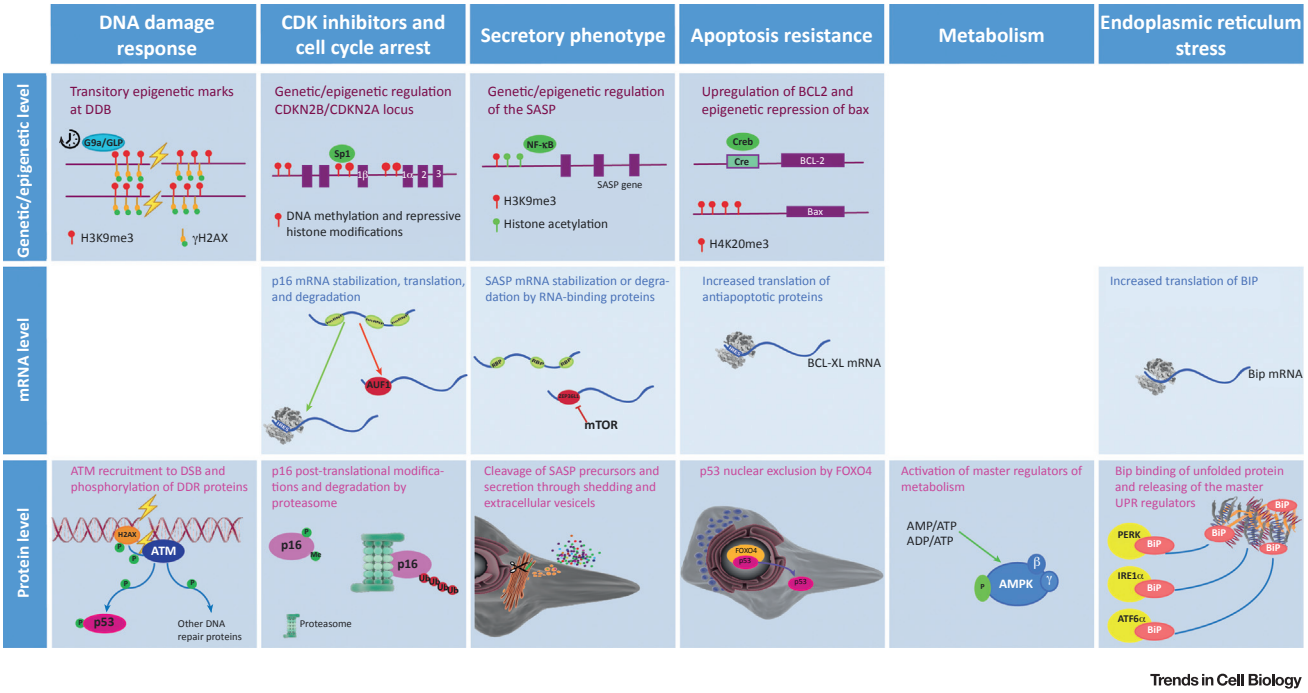

Figure 1. The main regulation steps for key molecular players and signaling pathways of senescence. The regulation is described at three different levels (i) genetic and/or epigenetic level, which includes mechanisms that modify transcription (for instance, histone modifications, DNA methylation, and recruitment of key transcription factors); (ii) mRNA level, which includes mechanisms such as mRNA stabilization or degradation and even recruitment of ribosomes, all of which affect translation; and (iii) protein level, which includes regulatory mechanisms that occur after translation (for instance, post-translational modifications, protein degradation, and protein transport). Abbreviations: CDK, cyclin-dependent kinase; DDR, DNA damage response; DSB, double-strand breaks; SASP, senescence-associated secretory phenotype; UPR, unfolded protein response.

P16 induction is often measured by mRNA levels. However, the complex nature of the CDKN2A locus hinders the design of specific primers. CDKN2A encodes not only p16, but also p14, p12, and p16-γ, all of which have a similar nucleotide and/or protein sequence [23]. Co-amplification of p16-γ or p12 is less problematic because p16-γ is speculated to have the same CDKI function as p16, and p12, despite acting as a transcription factor instead of a CDKI, is only expressed in pancreas [23]. However, p14 is ubiquitously expressed and has a completely different function, promoting p53 activation [39]. Thus, studies that base their conclusions on the expression of the whole locus or on assays co-amplifying p16 and p14 should be carefully interpreted. Even when the appropriate assay is used, changes in mRNA levels do not always reflect protein levels [40]. Moreover, measuring p16 protein levels can be challenging, particularly in mouse, where the lack of antibodies is a major complication.

p15 function and protein structure are similar to those of p16, but less attention has been paid to p15 in the context of senescence [23]. p15 is downstream of the Raf-Mek-Erk and the PI3K/AKT/FOXO3 pathways [41,42], mainly regulated epigenetically by PRC complexes and histone

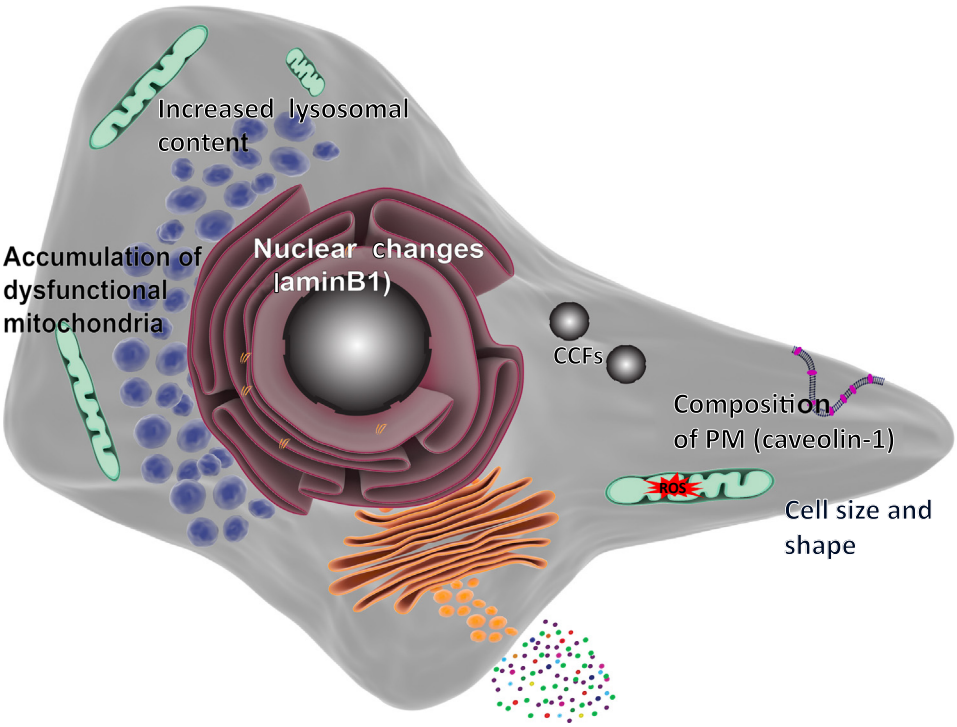

Figure 2. Hallmarks of Senescence Morphological Alterations. The molecular pathways of senescence result in morphological alterations. Senescent cells are enlarged and have an irregular shape; their nuclear integrity is compromised due to the loss of laminB1, which also leads to the appearance of cytoplasmic chromatin fragments (CCFs); they have an increased lysosomal content, which is often detected as high β-galactosidase activity; they have large but dysfunctional mitochondria that produce high levels of reactive oxygen species (ROS); and their plasma membrane (PM) changes its composition (for instance, upregulating caveolin-1).

modifications and transcriptionally by Sp1 and Miz-1 [43–45]. The latter is in turn negatively controlled by Myc and positively regulated by Arnt, a key effector in hypoxia-induced signaling. Regulation of p15 at the post-transcriptional level is less studied. However, p15 is at least partially regulated at the translational level by stabilization of its mRNA [46]. Moreover, p15 protein can be stabilized by TGF-β [47].

p21 is an 18-kDa protein that is capable of inhibiting a range of CDKs but, paradoxically, is also necessary for cell cycle progression [48]. Although p21 is consistently upregulated in response to different senescence-inducing stimuli [49], its expression is part of a more generic DDR and mainly regulated by direct **transactivation** via p53, which makes it difficult to use it as a unique senescence marker. P21 can also be activated in a p53-independent manner, guided by pathways such as TNF-β and by using Sp1 as a main transcription factor [43,48,50]. Other mechanisms of p21 regulation include: transcriptional repression via c-Myc, ID1, CTIP-2, CUT, and retinoid X receptor; inhibition of transcriptional elongation by disassembling elongation factors via Chk1; control of mRNA stability through binding of miRNAs (miR-17-92, miR-106a-363, and miR-106b-25) or RNA-binding proteins (HuD, HuR, RBM28, Msi-1, PCBP1/CP1/hnRNP E1, and TAX), or through phosphorylation (via Akt1/PKB, PKA, PKC, PIM-1, and GSK-β); and proteasomal degradation (through ubiquitination by the E3 ubiquitin ligases) [48]. AUF1 also targets p21 mRNA directly or indirectly by promoting degradation of some of its

Box 2. Current Methods to Detect Markers of Senescence

Multiple techniques have been used to identify senescent cells. Given the heterogeneity of senescent cells and the lack of specificity of the markers, a combination of different techniques is often used and encouraged for the detection of senescent cells.

DDR: the presence of γ-H2AX foci measured by immunostaining demonstrates continuous and unrepaired DNA damage [3]. Measurement of the level of phosphorylated p53, a key signaling player for the DDR, can be used [2].

Cell cycle arrest: two types of assay are normally used to show that cells have exited the cell cycle. The first includes direct measure of the proliferation potential of the cells via the measurement of their colony-formation potential or of the DNA synthesis rate by BrdU/EdU-incorporation assays. The second includes measuring the expression level of the CDKIs p16 and p21 [3,162].

Secretory phenotype: cytokines (e.g., IL-1a, IL-6,and IL-8), chemokines (e.g., CCL2) and metalloproteinases (e.g., MMP-1 and MMP-3) are used as markers [3,51]. It is also common practice to use either immunostaining or ELISA to measure the protein expression and secretion of some of these factors, particularly IL-6 [84].

Apoptosis resistance: the upregulation of the BCL-proteins Bcl-2, Bcl-w, or Bcl-xL has been used as a marker of senescence [81,85]. However, it is not yet regularly used.

Metabolism: due to the lack of information and consensus about the metabolic changes that cells undergo upon senescence induction, this feature is not often used as a marker of senescence.

ER stress: perhaps due to inconsistency in the particular branch of the UPR that is activated in different senescence-inducing stimuli, this feature is not often used as a marker of senescence [106–109].

Cell size: *in vitro*, the enlarged cell body and irregular shape of senescent cells is easily evaluated by regular bright-field microscopy [3]. Immunofluorescence targeting vimentin, actin, or other cytoplasmic proteins can be used to measure changes in cell shape [107].

Composition of PM: rarely measured in senescent cells and not used as a regular marker of senescence. Recently, an oxidized form of vimentin present at the PM and other membrane proteins, such as DEP1 and DPP4, have been proposed as markers [127–129].

Increased lysosomal content: SA-βgal is the most common marker of lysosomal activity and one of the first tests used to assess senescence [131]. Alternatively, SBB or its biotin-labeled analog (GL13) can be used to detect lipofuscins from old lysosomes, and LysoTrackers or orange acridine can reveal high lysosomal contents [137,144].

Accumulation of mitochondria: mitotrackers to measure the membrane potential of mitochondria and electronic microscopy to evaluate their cell shape (fusion/fission) have been used in some studies [143].

Nuclear changes: SAHF are observed as darker regions within the nucleus of senescent cells after DAPI staining and are enriched in markers of heterochromatin, such as H3K9me3 and HP1γ. However, this feature is not shared by all types of senescence and is not apparent in mouse cells [3]. Alternatively, downregulated levels of LaminB1 have become a common marker [157].

modulators (e.g., p53 and c-Myc). Therefore, p16 might indirectly regulate p21 expression via downregulation of AUF1 [34].

A general block of proliferation could be measured via EdU-incorporation or colony-formation assays. However, lack of proliferation is not a specific mark of senescent cells and is impractical for measuring senescence in postmitotic cells and inadequate for *in vivo* experiments.

Secretory Phenotype

Senescent cells secrete cytokines, chemokines, and proteinases, which are positively or negatively implicated in several biological processes, such as wound healing and cancer

progression [6,51,52]. The SASP is highly heterogeneous [49] and regulated at many levels, making the identification of more general regulatory mechanisms a challenge (Figure 1).

The SASP is, to a large extent, a transcriptional program mediated by the proinflammatory transcription factor NF-κB [53]. The major trigger for NF-κB activation is the DDR, but more recently the cGAS/STING pathway (see the section 'Nuclear Changes') has also been implicated [54,55]. In accordance with the hypothesis that DNA damage is an essential driver of the SASP, **mitochondrial dysfunction**-associated senescence (MiDAS) is associated with mild or no transcriptional induction of several SASP factors [56]. Additional transcription factors involved in the regulation of SASP genes are GATA4 and C/EBPβ [57,58]. Interestingly, NOTCH signaling is dynamically expressed upon senescence [59], which may explain the dynamic composition of the SASP [49], and counteracts C/EBPβ induction [59]. Notably, although NF-κB mainly induces the expression of inflammatory cytokines, other pathways, such as the Jak2/Stat3 pathway, upregulate a different set of cytokines, mainly immunosuppressive ones, such as CXCL1/CXCL2, GM-CSF, M-CSF, IL-10, and IL-13 [61]. In Pten-null prostate tumors, the Jak/Stat3 pathway is active and, therefore, it is hypothesized that inhibition of Jak/Stat3 would help tumor clearance [60].

Transcription of SASP genes can also be dependent on epigenetic changes. Repressive marks, such as H3K9me2, are reduced at the promoters of IL-6 and IL-8, two major SASP components, probably due to the degradation of the methyltransferase G9a via proteasomal degradation downstream of an ATM-dependent DDR signal [12]. By contrast, sirtuin 1 (SIRT1), a histone deacetylase, is downregulated during senescence and leads to increased expression of IL-6 and IL-8 via histone acetylation of the promoter regions [61]. The histone variant macroH2A1 has an interesting role in SASP expression. Although macroH2A1 is depleted from SASP-containing loci during senescence, it is required for the expression of several SASP factors, including IL-1A, IL-1B, IL-6, IL-8, and MMP-1, among others [62]. This apparent contradiction is explained by a feedback loop in which SASP expression causes ER stress, which in turn activates **reactive oxygen species** (ROS) production and DDR. The DDR (through the activity of ATM) promotes macro-H2A1 mobilization in an attempt to reduce SASP expression and, therefore, ER stress [62]. As mentioned above, the DDR (and particularly ATM) are known activators of NF-κB, which still poses a contradictory function for ATM and the DDR. Another histone variant, H2A.J, also positively regulates the transcription of SASP factors [63]. Finally, the 3D arrangement of the chromatin also influences the transcription of SASP genes [64], while chromatin-bound HMGB2 fine tunes SASP expression by avoiding **heterochromatin** spreading [65].

At the post-transcriptional level, mTOR regulates the SASP via two mechanisms: (i) promoting the translation of IL-1A, in turn, activating NF-κB and C/EBPβ [57,66]; and (ii) indirectly inhibiting the RNA-binding protein ZFP36L1, preventing it from degrading mRNA-encoding SASP factors [67]. In accordance, senescent endothelial cells that do not upregulate the mTOR pathway do not activate a SASP [68]. Moreover, the production of ROS can trigger the p38/MAPK pathway, which in turn phosphorylates and activates other RNA-binding proteins responsible for the stabilization of SASP-dependent mRNA [69].

Once synthesized, some SASP factors undergo post-translational modifications. For instance, the **inflammasome** (a protein complex formed by caspase 1 and accessory proteins) has an important role in the activation of the IL-1-signaling pathway, by cleaving and activating IL-1β [70]. Moreover, inhibition of either caspase-1 or the IL-1 receptor reduced the expression of SASP and partially prevented oncogene-Induced senescence (OIS) through inhibition of

paracrine senescence [70]. SASP factors, such as IL-1 and IL-6, can play cell autonomous functions and reinforce the senescence state [71]. However, many SASP factors exert a non-cell autonomous function and can alter the behavior of neighboring cells [72]. SASP factors have been shown to promote the reorganization of embryonic structures, participate in tissue remodeling and repair, and enhance immunosurveillance [5,6]. By contrast, the chronic presence of some proinflammatory and tissue-remodeling factors, such as interleukins and MMPs, has been associated with disease states and aging phenotypes [52]. Many SASP members are produced as soluble proteins that can be directly transported to the extracellular environment, but some SASP factors are initially expressed as transmembrane proteins and need to be released into the extracellular space by ectodomain **shedding** [73]. ADAM17, a sheddase, is upregulated in OIS and cancer and regulates the ectodomain shedding of several SASP factors from the cell membrane [74,75]. Furthermore, some SASP members are secreted via small extracellular (exosome-like) vesicles that, once released by senescent cells, can exert a more distal function, for instance enhancing the proliferation of cancer cells [76]. Notably, the biogenesis of these vesicles is p53 dependent [77].

It is clear that the SASP has an important role in the pathophysiological activity of senescent cells, but it is too unspecific and heterogeneous to be used as an unequivocal marker for senescence [49,52]. However, quantification of SASP composition could be used for the definition of different senescence programs. For example, senescent cells associated with tissue repair express several MMPs and growth factors, such as PDGF-A and VEGF [6,78], while age-associated or therapy-induced senescent cells are mainly associated with inflammatory factors [79,80].

Apoptosis Resistance

Senescent cells activate several prosurvival factors and become resistant to **apoptosis** [81]. Upon treatment with apoptosis inducers, senescent cells are unable to downregulate the antiapoptotic protein BCL-2 due to chronic activation of the transcription factor cAMP response element-binding protein (CREB), which prevents BCL-2 inhibition [82]. In addition, the proapoptotic gene *Bax* was shown to be enriched with the repressive histone mark H4K20me3 [83] (Figure 1).

More recently, additional prosurvival networks have been associated with senescence [84]. Key nodes of these networks include ephrins, PI3 K, p21, BCL-XL, and plasminogen activated inhibitor-2 [84]. Members of the BCL-2 family, specifically antiapoptotic BCL-XL and BCL-W, are essential for the survival of senescent cells [85]. BCL-W is transcriptionally upregulated during senescence, whereas BCL-XL shows a higher rate of translation mediated by an IRES motif [49,85]. FOXO4 is overexpressed at the mRNA and protein levels in senescent cells, and prevents cell death by sequestering p53 in the nucleus [86]. p21 protects senescent cells from death by restricting JNK and caspase signaling under persistent DNA damage [87]. Finally, HSP90 was shown to be a key protein for the survival of senescent cells via stabilization of P-AKT [88].

Induction of the expression of various BCL2 family members is a promising method to identify senescent cells. However, certain nonsenescent cells types, particularly blood cells, also show upregulation of these antiapoptotic regulators [89].

Metabolism

Senescent cells are metabolically active, and increases in the AMP:ATP and ADP:ATP ratios have been reported during senescence (Figure 1) [90]. AMP protects AMP-activated protein kinase (AMPK) from dephosphorylation and causes its allosteric activation [91]. Thus, AMPK

acts as a sensor of the reduced energetic state, further activating catabolic pathways while inhibiting biosynthetic ones, and regulating p53 and other targets [91].

p53 can further regulate cellular **metabolism** by leading to inhibition of glucose uptake and glycolysis, and promoting the tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and fatty-acid oxidation [56,92]. Indeed, high TCA cycle activity is essential for the tumor-suppressing action of senescent cells [93]. Many other proteins besides AMPK directly or indirectly regulate p53, for instance via post-translational modifications or direct interactions, including MDM2 and loss of PTEN [94]. Mitochondrial dysfunction and mTOR activation also generate metabolic changes. MiDAS activates AMPK and causes a reduction in the TCA cycle and NAD + [95] (a key cofactor for many other proteins [96]), which are both important players during senescence. Meanwhile, mTOR decreases **autophagy** and, therefore, influences protein homeostasis [97]. However, despite much evidence for the upregulation of mTOR during senescence [66,67,97], the autophagy status of senescent cells is less understood [98].

Finally, Rb, an essential player in cell cycle arrest during senescence, can also induce metabolic changes. In particular, it can increase oxidative phosphorylation by promoting the conversion of pyruvate into acetyl-coA via PDP2-mediated activation of the pyruvate dehydrogenase (PDH) [99]. Rb also inhibits the generation of deoxyribonucleotides through the regulation of key enzymes via the transcription factor E2F1 [100].

It is currently difficult to use metabolic changes as markers for senescence. First, these changes [101,102] can be either a cause or a consequence of several other hallmarks of senescent cells. Second, only a few studies have focused on the whole metabolome of senescent cells [103,104]. Additional characterizations could help to identify specific metabolites for use as senescence markers.

Endoplasmic Reticulum Stress

Multiple factors, such as oxidative stress, mutations, infections, and lack of chaperones, can cause ER stress, leading to the accumulation and aggregation of proteins. To cope with the stress, the ER initiates the **unfolded protein response** (UPR) that leads to a reduction in protein synthesis, enlargement of the ER, and export of misfolded proteins [105]. Indeed, senescent cells have an increased UPR, possibly in response to the increased protein synthesis demanded by the SASP [106,107].

Notably, the UPR appears to influence many other hallmarks of senescence, although not always using the same effectors (Figure 1) [106–109]. Indeed, the UPR comprises three pathways regulated by PERK, IRE1α and ATF6α, respectively [105]. BiP, an ER protein, is known to bind these three master regulators and inhibit their functions [110]. Upon ER stress, BiP binds to the misfolded and/or unfolded proteins instead, releasing its former partners and allowing UPR activation. Thus, BiP has a central role in the UPR and possibly in senescence. Unsurprisingly, BiP is tightly regulated, mainly at the translational level: it was demonstrated that the translational efficiency of BiP is largely increased upon UPR activation [111].

Remarkably, PERK and IRE1α are both transmembrane proteins with kinase activity that are also stabilized by heat-shock proteins (mainly HSP90 and HSP72) and whose activation is also influenced by the fluidity of the membrane [110]. ATF6α is a cAMP-dependent transcription factor that, when inactive, localizes to the ER as a transmembrane protein. Upon activation, ATF6α traffics to the nucleus, where it is cleaved to generate an active transcription factor. Therefore, it is also regulated by its cleaving enzymes S1P and S2P [110]. Although some

studies have monitored ER stress in senescence by **qPCR** of different downstream genes (*ATF4*, *GRP78*, *GADD153*, or spliced *XBP1*) [62], this is not a common practice in the senescence field and, therefore, there are no consensual markers.

Morphological Alterations as Hallmarks of Senescence

Cell Size and Shape

A key feature of *in vitro* senescence is the enlarged and irregularly shaped cell body. Activation of the mTOR pathway is necessary for the enlargement of the cell body of senescent endothelial cells [68]. mTORC1 is known to integrate various stress signals and to modulate cell growth accordingly [112], and mTORC1 activation occurs in response to senescence-inducing stimuli [113]. In normal aging, the decline in growth factors, such as GDF11, might also contribute to the activation of mTORC1 and the hypertrophy observed, particularly in cardiac cells [114]. mTORC1 is mainly modulated at the post-translational level by activation of its catalytic site by the GTP-ase Rheb and by a reconfiguration of the whole mTORC1 complex, which favors interaction with downstream effectors [115].

A contributor to the senescence-associated altered cell shape is rearrangement of the cytoskeleton, mainly of vimentin filaments [106,107,116]. When the changes in size and shape in senescent cells were decoupled, it was discovered that the ATF6α signaling pathway, one of the three branches of the UPR (see above), can control the size of the ER and the changes in cell shape during senescence [106,107]. Although the cyclooxygenase-2/prostaglandin-E2 pathway is a downstream effector of ATF6α that influences cell size [107], specific targets of ATF6α responsible for modifying cell shape are not fully known. ATF6α, as well as the other UPR branches, is able to activate NF-κB [110,117,118] and can directly bind to the vimentin promoter (Figure 2) [112].

Changes in size and morphology are easily measured with either normal or fluorescent microscopy, but difficult to detect and quantify *in vivo* or *in situ*.

Composition of the Plasma Membrane

The PM has a central role in communication with neighboring cells and the extracellular space. The most consistent change in the composition of the PM in senescent cells is the upregulation of caveolin-1 (Figure 2), an important component of cholesterol-enriched microdomains called **caveolae** [119]. A possible explanation for the senescence-promoting effects of caveolin-1 is its functional cooperation with the MAP kinase signaling pathway, a downstream effector of multiple senescence-inducing stimuli [120]. As a positive feedback loop, the p38 MAPK pathway appears to upregulate caveolin-1 both at the transcriptional level, by inducing Sp1 [121], and at the post-translational level, by direct phosphorylation [122].

In addition, caveolin-1 influences the morphology and the adherence of senescent cells [123,124]. It also contributes to an increase in p53 activity via the downregulation of SIRT1, activation of ATM, and inhibition of MDM2 [125]. The function of caveolin-1 during senescence is likely dependent on its localization within caveolae instead of within other cellular compartments, because inhibition of nuclear caveolin-1 failed to inhibit senescence in IMR90 fibroblasts [126].

Other PM proteins have also been reported to change their expression in senescence. Althubiti *et al.* [127] performed a proteomic screening of PM-associated proteins, and identified 107 of them as being specific for senescence; the authors developed a staining protocol using two of these proteins (DEP1 and B2MG) to detect senescent cells. Further optimization and mechanistic studies exploring the functional role of these proteins in senescence is still needed. Also, an oxidized form of vimentin is specifically expressed at the PM of senescent fibroblasts [128].

Remarkably, the surface protein DPP4 was demonstrated to sensitize senescent cells to elimination by natural killer cells [129].

Further validation of PM proteins is a promising strategy for the identification of novel markers with the unique advantage of allowing the sorting of senescent cells.

Increased Lysosomal Content

The senescence state is characterized by the upregulation of many lysosomal proteins and increased lysosomal content (Figure 2) [130]. The activity of the lysosomal enzyme **senescence-associated beta-galactosidase** (SA-βgal) activity is used as a surrogate marker for the enhanced lysosomal content of senescent cells. Since SA-βgal is upregulated during senescence, its residual activity can be measured at a suboptimal pH of 6.0 [131,132]. SA-βgal is one of multiple transcripts encoded by the *GLB1* gene [131]. Transcriptional and **post-translational regulation** of this enzyme include the presence of Sp1-binding sites on its promoter, the regulation of **alternative splicing**, and the cleavage of the translated peptide, resulting in two smaller peptides that associate with each other and conform the active enzyme [133–136]. However, the mechanisms governing its overexpression in senescence are surprisingly unexplored. Only recently was it shown that GLB1 is negatively regulated by the NOTCH1-pathway at the transcriptional level [59]. SA-βgal staining is arguably the most common marker of senescence. However, it cannot be used for paraffin-embedded tissue sections and live cells, which strongly limits its application.

Enhanced lysosomal content during senescence could be the result of the accumulation of old lysosomes or of enhanced lysosomal biogenesis. Residual bodies, namely lipofuscins, support the lack of lysosomal removal [137]. Lysosomal biogenesis is largely controlled by transcription factor EB (TFEB), known for coordinately regulating multiple lysosomal proteins and being a downstream effector of the mTOR signaling pathway. It has been shown that TFEB can bind the promoter of β-hexosaminidase, another lysosomal enzyme, in response to OIS [138]. However, claims that TFEB is either up- or downregulated in senescence or aging have also been reported, which make its use as senescence difficult [130,139]. An alternative marker for the detection of accumulating lysosomes is Sudan Black B (SBB). SBB selectively binds lipofuscins and labels reduced lysosomal degradation. Importantly, it can be used in paraffin-embedded tissue sections [137]; recently, a biotin-labeled analog (GL13) was synthesized that could be used to enhance detection using SBB [140].

Nevertheless, high lysosomal activity is not a specific senescence marker, and the constitutive expression of SA-βgal has been identified in nonsenescent cells [141].

Accumulation of Mitochondria

Senescent cells show an increased number of mitochondria (Figure 2) [142]. However, the membrane potential of these mitochondria is decreased, leading to the release of mitochondrial enzymes, such as endonuclease G (EndoG), and intensified ROS production [143,144].

The main source of the extra mitochondrial content is the accumulation of old and dysfunctional mitochondria due to reduced **mitophagy** [145]. This is, at least partly, a consequence of reduced mitochondrial fission and increased fusion [146], possibly as a mechanism to protect mitochondria from mitophagy and senescent cells from apoptosis [95].

A key step for triggering mitochondrial fission is the recruitment and translocation of Parkin to damaged mitochondria. PINK1 and Mfn2 recruit Parkin to the mitochondrial membrane, and a

deficiency in Mfn2 causes mitochondrial dysfunction in mouse embryonic fibroblasts and cardiomyocytes [147]. By contrast, cytoplasmic p53 interacts with Parkin, inhibiting its translocation to damaged mitochondria in doxorubicin-induced senescence in cardiomyocytes [148]. **Translational regulation** of Parkin cannot be excluded, since its expression is upregulated at the protein level [149].

It is unlikely that mitochondrial biogenesis has a significant role in the increase in mitochondrial content during senescence, because senescent cells show only a transient upregulation of PGC-1α and PGC-1β, two important regulators of mitochondrial biogenesis [150], followed by downregulation [146].

Nuclear Changes

A common mark of senescent cells is the loss of LaminB1, a structural protein of the **nuclear lamina** (Figure 2) [49,151]. The destabilization of the nuclear integrity caused by reduced LaminB1 results in other nuclear changes, such as the loss of condensation of constitutive heterochromatin and the appearance of cytoplasmic chromatin fragments (CCFs) enriched in epigenetic marks associated with DNA damage [152]. These CCFs can also be secreted to the extracellular environment via exosomes and activate DDR in other cells [12]. Moreover, senescence-associated heterochromatin foci [SAHFs; 4′,6-diamidino-2-phenylindole (DAPI) intense nuclear foci enriched in repressive epigenetic marks [153]] have been proposed as a compensatory mechanism to keep constitutive heterochromatin repressed [154]. However, SAHFs are not universal markers and are mainly observed in OIS [3].

LaminB1 is downregulated at different levels. Transcriptomics studies demonstrate that downregulation of LaminB1 mRNA is a widespread marker of senescence [49]. It was also reported that miRNA-23a, which is upregulated in senescent cells, can target LaminB1 mRNA, reducing its translation [155]. Interestingly, LaminB1 is exported to the nucleus together with the CCFs and aided by the autophagy protein LC3. Once in the nucleus, it is submitted for degradation by the lysosomes [156]. LaminB1 downregulation depends on p53 and p16, but is independent from other senescence-associated pathways, such as p38-MAPK, NF-kB, DDR, and ROS [157].

During irradiation-induced senescence, a sub-G1 population of cells with lower DNA content progressively appears [144]. This reduction in DNA content could be due not only to CCFs, but also to the release of mitochondrial EndoG and its translocation to the nucleus as a response to the loss of mitochondrial potential [144].

Finally, it was recently reported that senescent cells express many markers of ‘stemness’ [158]. For instance, the gene signature of Bcl2 lymphomas submitted to therapy-induced senescence resembles that of adult tissue stem cells. Moreover, senescent lymphoma cells were positive for other markers, such as Sca1 and H3K9me3, among others. Notably, if these senescent lymphoma cells manage to escape senescence by decreasing the expression of key senescence genes, their growth ability becomes larger than that of their nonsenescent counterparts. However, this is a recent finding that needs further confirmation in more cell types before it can be considered a senescence hallmark.

Implications for Senescence Interventions

Among the various biological functions in which **cellular senescence** is involved, its role in diseases such as cancer and aging has made it an attractive therapeutic target. Strategies to interfere with senescent cells are mostly based on the markers listed above (Figure 3). Two main

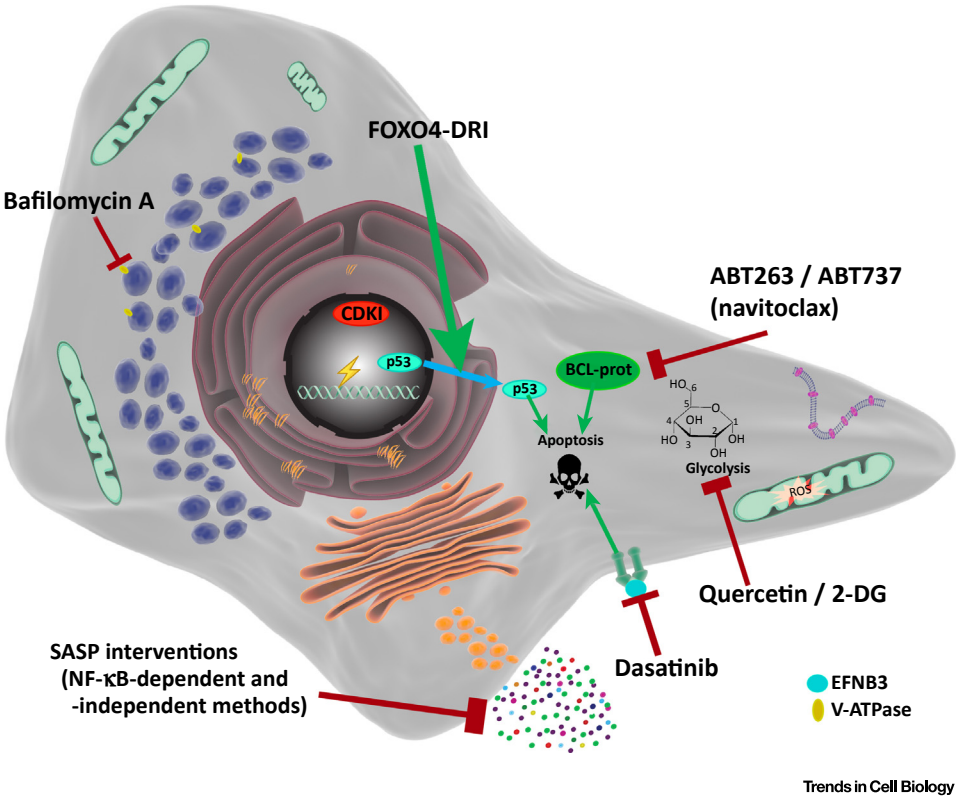

Figure 3. Senescence Interventions. The different therapies that target senescent cells are depicted, showing their main molecular targets. So-called 'senolytics' induce cell death specifically by targeting the Bcl-2 family of antiapoptotic proteins (e.g., ABT263 and ABT737), by promoting nuclear exclusion of p53 (FOXO4-DRI), by targeting glycolysis (quercetin, 2-DG) or other pathways that lead to apoptosis (dasatinib). Many other therapies target either one or multiple members of the senescence-associated secretory phenotype (SASP) by targeting NF-κB or other pathways controlling the secretory phenotype.

approaches are currently under development: (i) specific elimination of senescent cells; and (ii) inhibition of the SASP.

The first approach focuses on identifying compounds that can specifically induce senescent cells to die (also defined 'senolytics'). Given that apoptosis-resistance is a main feature of senescent cells, mechanisms involved in conferring this resistance are a preferential target of senolytics. Indeed, compounds discovered so far include ABT263 and ABT737, which are inhibitors of different members of the Bcl-2 family of antiapoptotic proteins; FOXO4-DRI, a peptide that forces the nuclear exclusion of p53; quercetin, a polyphenol with various functions, including inhibition of Pi3K; dasatinib, an anticancer drug with various functions, including inhibition of EFNB1 and B3; and 2-deoxyglucose (2-DG), a false substrate for glucose exokinase, which saturates the glycolytic flux [7]. Many of these drugs have intrinsic toxicities, which could limit their use for human purposes. Moreover, they target only subsets of senescent cells without discriminating among beneficial and deleterious senescence programs.

The second approach aims at reducing the negative effects of the SASP. Given that NF-κB is a major driver of the SASP, inhibiting its function or neutralizing some of its members is an effective strategy to reduce the expression of several proinflammatory factors in senescent cells

[7]. Interestingly, major life-extending compounds, such as rapamycin, resveratrol, and metformin, have been shown with variable degree of confidence to reduce the SASP [97,159]. A major limitation of SASP inhibitors is the nonspecificity for senescent cells, because most of these compounds are known anti-inflammatory drugs with severe toxicities when used long-term. Moreover, the SASP can have beneficial functions, and current SASP modulators are unable to spare these positive programs from inhibition.

Concluding Remarks

Since the discovery of senescent cells by Hayflick and Moorhead in 1961 [160], the scientific community has struggled to identify universal and unequivocal markers characterizing the senescence state. The difficulty in identifying such markers reflects the complexity of the senescence phenotype and the existence of highly heterogeneous senescence programs (see Outstanding Questions). Currently, the only possibility resides in combining the measurement of multiple hallmarks in the same sample [3]. For example, qPCR is a method of preference to measure many markers simultaneously at a relatively low cost. However, three problems arise with this choice. First, standard qPCR experiments use relative quantification methods relying on reference or housekeeping genes, such as cytoskeleton proteins (e.g., actin or tubulin) or metabolic enzymes (e.g., GAPDH), whose expression might vary in senescence [161,162]. Second, some hallmarks of senescence are regulated at the translational or post-translational level, making qPCR an inadequate measurement method. Third, qPCR is currently performed on whole populations, while the engagement of single cell techniques aimed at analyzing any cell in a certain tissue or biopsy would be preferable.

The use of **'omics techniques** to quantify various macromolecules, possibly at the single cell level to include intrapopulation variability, would be a preferred avenue for the discovery of novel markers [162]. Currently, the low sensitivity and high cost of such techniques are not suitable for the study of senescence, particularly for the characterization of senescent cells in tissues.

An alternative strategy is to systematically define the different senescence programs by identifying program-specific traits. This approach would not only offer a fresh and more complex understanding of the heterogeneity of cellular senescence, but also provide better hits for the design of therapeutic approaches aimed at interfering with detrimental senescence features while maintaining the beneficial components.

Outstanding Questions

Do universal mechanisms associated with cellular senescence exist?

Is the lack of universal markers a technical limitation that could be overcome by collecting more accurate data on the 'omics of senescent cells?

Are the various senescence programs independent and, if so, can we identify program-specific markers?

Could the discovery of program-specific markers help the development of less toxic and more potent antisenescence interventions?

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