Cellular senescence: defining a path forward

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ABBREVIATIONS

DDR: DNA damage response, CDKs: cyclin-dependent kinases, ROS: Reactive oxygen species, MMPs: Matrix metalloproteinases, TGFβ; Transforming growth factor-β, SASP: Senescent associated secretory phenotype, SMS; Senescence messaging secretome, mTOR: Mammalian target of rapamycin, CCF: Cytoplasmic chromatic fragments, cGAS–STING: cyclic GMP–AMP synthase linked to stimulator of interferon genes, DAMPs: Damage-associated molecular patterns, scRNA-Seq: Single cell RNA-Sequencing, DSB: Double-strand break, TIFs: Telomere dysfunction-induced foci, TAFs: Telomere-associated foci, OIS: Oncogene-induced senescence, DNA-SCARS: DNA segments with chromatin alterations reinforcing senescence, PTP: Protein tyrosine phosphatases, DUSP: Dual specificity phosphatases, ERK: Extracellular signal regulated kinases, BPH: Benign prostatic hyperplasia, UPS: Ubiquitin proteasome system, PML: Promyelocytic leukemia protein, 4-HNE: 4-hydroxy-2-nonenal, EPA: eicosapentaenoate, 7-HOCA: 7-alpha-hydroxy-3-oxo-4-cholestenoate, TCA: tricarboxylic acid, ETC: Electron TH chain, AMPK: AMP-activated protein kinase, TASCC: TOR-autophagy spatial-coupling-compartment, SA-β-gal: senescence-associated β-galactosidase, CDK: cyclin-dependent kinase 4 and 6, HUCA: Mammalian histone chaperone complex composed of HIRA: Histone cell cycle regulation defective homolog A protein/UBN-1: Ubinuclein-1/CABIN11: Calcineurin-binding protein cabin1/ASF1a: Anti-silencing function protein 1, SAHF: Senescence-associated heterochromatin foci, SADS: senescence-associated distension of satellites, Hi-C: genome-wide mapping of chromatin contacts, miRNAs: microRNAs, PcG: Polycomb group, AGO2: Argonaute 2 (also known as eukaryotic translation initiation factor 2C, let-7f: member of the let-7 miRNA family, lncRNAs: Long-non coding RNAs, HGPS: Hutchinson-Gilford progeria syndrome, TTD: trichothiodystrophy, DRI: D-retro inverso, CYTOF: Cytometry by Time-Of-Flight, SBB: Sudan Black B, HRS cells: Hodgkin and Reed-Sternberg cells, cHL: classical Hodgkin Lymphoma, ssDNA: single stranded DNA.

ABSTRACT

Cellular senescence is a cell state implicated in various physiological processes and a wide spectrum of age-related diseases. Thus, accurate detection of senescent cells, especially *in vivo*, is essential especially since the field of senotherapeutics is growing rapidly. Here, we present a consensus from the International Cell Senescence Association (ICSA), defining and discussing key cellular and molecular features of senescence and offering recommendation on how to use them as biomarkers. We also present a resource tool to facilitate the identification of genes linked with senescence (SeneQuest, available at http://Senequest.net). Lastly, we propose an algorithm to accurately assess and quantify senescence, both in cultured cells and *in vivo*.

MAIN TEXT

1. Cellular senescence: walking a line between life and death

Cell states link both physiological and stress signals to tissue homeostasis and organismal health. In both cases, the outcomes vary and are determined by the signal characteristics (*type*, *magnitude* and *duration*), spatiotemporal parameters (*where* and *when*) and cellular capacity to respond (Gorgoulis et al., 2018). In the case of potentially damaging stress, damage can be reversed and cells restored structural and functional integrity. Alternatively, damage can be irreversible and cells activate death mechanisms mainly to restrict the impact on tissue degeneration. Between these extremes, cells can acquire other states, often associated with survival, but also with permanent structural and functional changes. An example is the non-proliferative but viable state, distinct from G0 quiescence and terminal differentiation, termed cellular senescence (Rodier and Campisi, 2011). Formally described in 1961 by Hayflick and colleagues, cellular *senescenc*e derived from the latin word *"senex"* meaning "old" (Hayflick and Moorhead, 1961)**,** was originally observed in normal diploid cells that ceased to proliferate after a finite number of divisions (*Hayflick limit*), later attributed to telomere shortening **(**see section "*Cell cycle withdrawal"*).

Cellular senescence has since been identified as a response to numerous stressors, including exposure to genotoxic agents, nutrient deprivation, hypoxia, mitochondrial dysfunction and oncogene activation **(Table 1: Senescence inducers)**. Over the last decade, improved experimental tools and the development of reporter/ablation mouse models have significantly advanced our knowledge about causes and phenotypic consequences of senescent cells. However, the lack of specific markers and absence of a consensus definition senescent cells are lacking. Further, although a link to organismal aging is clear, aging and senescence are not

synonymous (Rodier and Campisi, 2011)**.** Indeed, cells can undergo senescence, regardless of organismal age, due to myriad signals, including those independent of telomere shortening. Consequently, senescent cells are detected at any life stage, from embryogenesis, where they contribute to tissue development, to adulthood, where they prevent the propagation of damaged cells and contribute to tissue repair and tumor suppression. Thus, cellular senescence might be an example of evolutionary antagonistic pleiotropy or an abortive cellular program with detrimental effects. Here, we clarify the nature of cellular senescence by: **i)** presenting key features of senescent cells; **ii)** providing a comprehensive definition of senescence, **iii)** including means to identify senescent cells; **iv)** delineating their role in physiological and pathological processes, and **v**) paving the way for new therapeutic strategies.

2. Definition and characteristics of cellular senescence

Cellular senescence is a cell state triggered by stressful insults and certain physiological processes, characterized by a prolonged –and generally irreversible- cell-cycle arrest with secretory features, macromolecular damage and altered metabolism (**Figures 1-2**). These features can be inter-dependent but for clarity are described here separately.

- *Cell cycle arrest* (**Figures 1 and 2**)

One common feature of senescent cells is an essentially irreversible cell cycle arrest which can be an alarm-response instigated by deleterious stimuli or aberrant proliferation. This cell cycle withdrawal differs from quiescence and terminal differentiation (He and Sharpless, 2017). Quiescence is a temporary arrest state, with proliferation re-instated by appropriate stimuli; terminal differentiation is the acquisition of specific cellular functions, accompanied by a durable cell cycle arrest mediated by pathways distinct from those of cellular senescence

(**Figure 1**). In turn, senescent cells acquire a new phenotype, which can lead to an abortive differentiation program. Although the senescence cell cycle arrest is generally irreversible, cell cycle re-entry can occur under certain circumstances, particularly in tumor cells (Galanos et al., 2016; Milanovic et al., 2018; Patel et al., 2016; Saleh et al., 2019) (**Figure 1**).

In mammalian cells, the retinoblastoma (RB) family and p53 protein are important for establishing the senescence arrest (Rodier and Campisi, 2011). RB1 and its family members p107 (RBL1) and p130 (RBL2) are phosphorylated by specific CDKs (CDK4, CDK6, CDK2). This phosphorylation reduces the ability of RB family members to repress E2F-family transcription factor activity, which is required for cell cycle progression (Sharpless and Sherr, 2015). In senescent cells, however, the CDK2 inhibitor $p21^{WAF1/Cip1}$ (CDKN1A) and CDK4/6 inhibitor $p16^{INK4A}$ (CDKN2A) accumulate. This accumulation results in persistent activation of RB-family proteins, inhibition of E2F transactivation and consequent cell cycle arrest, which, in time, cannot be reversed by subsequent inactivation of RB-family proteins or p53 (Beausejour et al., 2003). This persistence is enforced by heterochromatinization of E2F target genes (Salama et al., 2014), the effects of cytokines secreted by senescent cells (Rodier and Campisi, 2011), and/or enduring ROS production (Takahashi et al., 2006). Notably, in senescent murine cells, ARF, an alternate reading frame protein of the $p16^{INK4a}$ gene locus that activates p53, also has an important role in regulating cell cycle arrest (Sharpless and Sherr, 2015).

Additional features of the senescent cell-cycle arrest include ribosome biogenesis defects and derepression of retrotransposons (De Cecco et al., 2019; Lessard et al., 2018). However, currently no specific marker of the senescent cell-cycle arrest has been identified (Hernandez-Segura et al., 2017). For example, RB and p53 activation also occurs in other forms of cell-cycle arrest (Rodier and Campisi, 2011). Even $p16^{INK4A}$, which is considered more specific to

senescence, is expressed in certain non-senescent cells (Sharpless and Sherr, 2015), and is not expressed by all senescent cells (Hernandez-Segura et al., 2017). Thus, detecting a senescence-associated cell cycle arrest requires quantification of multiple factors/features.

- *Secretion* (**Figure 2**)

Senescent cells secrete a plethora of factors, including pro-inflammatory cytokines and chemokines, growth modulators, angiogenic factors and matrix metalloproteinases (MMPs), collectively termed the Senescent Associated Secretory Phenotype (SASP or Senescence Messaging Secretome (SMS) (**Table 2**) (Coppe et al., 2010; Kuilman and Peeper, 2009). The SASP constitutes a hallmark of senescent cells and mediates many of their patho-physiological effects. For example, the SASP reinforces and spreads senescence in autocrine and paracrine fashions (Acosta et al., 2013; Coppe et al., 2010; Kuilman and Peeper, 2009), and activates immune responses that eliminate senescent cells (Krizhanovsky et al., 2008a; Munoz-Espin and Serrano, 2014). SASP factors mediate developmental senescence (Munoz-Espin et al., 2013; Storer et al., 2013), wound healing (Demaria et al., 2014) and tissue plasticity (Mosteiro et al., 2016), and contribute to persistent chronic inflammation (known as inflammaging) (Franceschi and Campisi, 2014). Thus, the SASP can explain some of the deleterious, pro-aging effects of senescent cells. Further, the SASP can recruit immature immune-suppressive myeloid cells to prostate and liver tumors (Di Mitri et al., 2014; Eggert et al., 2016) and stimulate tumorigenesis by driving angiogenesis and metastasis (Coppe et al., 2010).

While the senescent cell cycle arrest is regulated by the p53 and $p16^{INK4A}$/Rb tumor suppressor pathways, the SASP is controlled by enhancer remodeling and activation of transcription factors such as NF-κB, C/EBPβ and GATA4 (Ito et al., 2017; Kang et al., 2015; Kuilman and Peeper, 2009; Salama et al., 2014), and the mTOR (mammalian target of

rapamycin) and p38MAPK signaling pathways (Freund et al., 2011; Ito et al., 2017; Kuilman and Peeper, 2009). Upstream signals triggering SASP activation are multiple, and differ depending on the senescence inducer, but include DNA damage, cytoplasmic chromatin fragments (CCFs) that trigger a type1 interferon response, and damage-associated molecular patterns (DAMPs) that activate the inflammasome (Acosta et al., 2013; Davalos et al., 2013; Li and Chen, 2018).

The SASP composition and strength varies substantially, depending on the duration of senescence, origin of the pro-senescence stimulus and cell type (Childs et al., 2015). Further, single cell RNA-Seq reveals considerable cell-to-cell variability of SASP expression (Wiley et al., 2017b). For example, transition from an early TGF-β-dependent to a pro-inflammatory secretome is governed by fluctuation of Notch1 activity (Ito et al., 2017). Moreover, an interferon type I response occurs as a later event, and is driven in part by derepression of LINE-1 retrotransposable elements (De Cecco et al., 2019). Senescent cells also communicate with their microenvironment through juxtacrine NOTCH/JAG1 signalling (Ito et al., 2017), release of ROS (Kuilman et al., 2010), cytoplasmic bridges (**Suppl. Video 1**) (Biran et al., 2015) and extracellular vesicles, such as exosomes (Takasugi et al., 2017). Overall, defining the senescent secretome in each biological context will help identify senescence-based molecular signatures.

- *Macromolecular damage* (**Figure 2**)

DNA damage

The first molecular feature associated with senescence was telomere shortening, a result of the *DNA end-replication problem*, during serial passages (Shay and Wright, 2019). Telomeres are repetitive DNA structures, found in terminal loops at chromosomal ends, and stabilized by the Shelterin protein complex. This organization renders telomeres unrecognizable by the DDR and DSB repair pathways (de Lange, 2018; Shay and Wright, 2019). Telomerase, the enzyme

that maintains telomere length, is not expressed by most normal somatic (non-stem) cells, but is expressed by most cancer cells that have overcome senescence. Moreover, telomerase activity reconstitution in normal cells leads to telomere elongation, extending their replicative life-span in culture (Bodnar et al., 1998; Shay and Wright, 2019).

Telomere shortening during proliferation culminates in telomeric DNA loop destabilization and telomere uncapping, generating Telomere dysfunction-Induced Foci (TIFs) that activate the DDR, eventually causing cell-cycle arrest. This response can also be elicited by inhibiting or altering genes involved in telomere maintenance (d'Adda di Fagagna, 2008). Another form of DNA damage, termed Telomere-Associated Foci (TAFs), can exist at telomeres due to oxidative DNA damage at telomeric G-reach repeats, irrespective of telomere length or Shelterin loss (de Lange, 2018; Shay and Wright, 2019).

Although half the persistent DNA damage foci in senescent cells localize to telomeres, other stressful subcytotoxic insults can trigger senescence by inducing irreparable DNA damage. Numerous genotoxic agents, including radiation (ionizing and UV), pharmacological agents (e.g., certain chemotherapeutics), oxidative stress and others trigger senescence by causing DNA damage. Moreover, activated oncogenes can induce senescence (known as OIS) as a tumor suppressive response, restricting the uncontrolled proliferation of potentially oncogenic cells. OIS is often mediated by the tumor suppressors $p16^{INK4A}$ and ARF, both encoded by the C*DKN2A* locus, imposing a cell-cycle arrest (Kuilman et al., 2010; Serrano et al., 1997). But the DDR also plays a major role in triggering OIS (Gorgoulis and Halazonetis, 2010; Gorgoulis et al., 2018; Halazonetis et al., 2008). In this case, the damage signal originates at collapsed replication forks as a result of oncogene-driven hyperproliferation. Recently, it was shown that

249 the DDR and ARF pathways can act in concert during OIS with the former requiring a lower
250 oncogenic load than the latter (Gorgoulis et al., 2018).

251 Senescent cells harbor persistent nuclear DNA damage foci termed DNA-SCARS. DNA-
252 SCARS are distinct from transient damage foci; unlike transient foci, they specifically associate
253 with promyelocytic leukemian (PML) nuclear bodies, lack the DNA repair proteins RPA and
254 RAD51 and ssDNA and contain activated forms of the DDR mediators CHK2 and p53 (Rodier
255 et al., 2011). DNA-SCARS are dynamic structures, with the potential to regulate multiple
256 aspects of the senescent cells, including the growth arrest and SASP (Rodier et al., 2011).
257 However, as not all senescence-inducing stimuli generate a persistent DNA damage response,
258 DNA-SCARS are not a global feature of the senescent cells. CCF are another type of DNA
259 damage in senescent cells (Ivanov et al., 2013). These cytoplasmic chromatin fragments activate
260 a proinflammatory response, mediated by the cGAS–cGAMP–STING pathway (Ivanov et al.,
261 2013; Li and Chen, 2018), that can serve as another non-inclusive senescence-associated marker.

262 *Protein damage*

263 Proteotoxicity is a hallmark of aging and cellular senescence (Kaushik and Cuervo,
264 2015). Hence, damaged proteins help identify senescent cells. A prominent source of protein
265 damage is ROS, which oxidize both methionine and cysteine residues and alter protein folding
266 and function (Hohn et al., 2017). Many protein tyrosine phosphatases (PTPs) contain cysteine
267 residues in their active sites that can be inactivated by oxidation. This inactivation can trigger
268 senescence by hyperactivating ERK signaling, similar to the effect of activated oncogenes
269 (Deschenes-Simard et al., 2013). High phospho-ERK levels were detected in pre-neoplastic
270 lesions, rich in senescent cells, such as melanocytic nevi and benign prostatic hyperplasia (BPH)
271 (Deschenes-Simard et al., 2013) and are a characteristic of therapy-induced senescence

(Haugstetter et al., 2010). The PTP oxidation pattern (the oxPTPome) can be revealed by a monoclonal antibody that recognizes oxidized cysteine (Karisch et al., 2011).

ROS, in the presence of metals, can carbonylate proline, threonine, lysine and arginine residues. Protein carbonylation exposes hydrophobic surfaces, leading to unfolding and aggregation, and protein carbonyl residues can be specifically detected using antibodies (Nystrom, 2005). Moreover, carbonyl residues can react with amino groups to form Schiff-bases, contributing to protein aggregation. Subsequent cross-linking with sugars and lipids forms insoluble aggregates, termed lipofuscin from the Greek "lipo" meaning fat and "fuscus" meaning dark. Lipofuscin can be visualized in lysosomes by light microscopy or a histochemical method using a biotinylated Sudan Black-B analogue (GL13) (Evangelou et al., 2017). The latter is emerging as a another indicator of senescent cells in culture and *in vivo* (Evangelou et al., 2017; Gorgoulis et al., 2018; Myrianthopoulos et al., 2019*)*. It should be noted that damage accumulation continues, even when cell division ceases, and can continue for months or even years.

Most protein oxidative damage is not reversible, and degradation by the ubiquitin proteasome system (UPS) or autophagy often eliminates these proteins. As UPS (Deschenes-Simard et al., 2013) and autophagy are active in senescent cells, they could prove to be useful in chacterizing the senescent state (Ogrodnik et al., 2019a). Similarly, PML bodies act as sensors of reactive oxygen species and oxidative damage (Niwa-Kawakita et al., 2017) and can also be non-exclusive biomarkers of cellular senescence (Vernier et al., 2011).

Lipid damage

Lipids are essential for cell membrane integrity, energy production and signal transduction. Some age-related diseases are characterized by altered lipid metabolism, resulting

295 in lipid profile changes (Ademowo et al., 2017). Although, senescent cells are marked by
296 changes in lipid metabolism, it is unclear how this contributes to the senescent phenotype.

297 Mitochondrial dysfunction during senescence can result in ROS-driven lipid damage,
298 lipid deposits (Correia-Melo et al., 2016; Ogrodnik et al., 2017) and lipofucin accumulation
299 (Gorgoulis et al., 2018). Apart from oxidation, modifications, such as lipid-derived aldehydes
300 [e.g., 4-hydroxy-2-nonenal (4-HNE)] have been reported in senescent cells (Ademowo et al.,
301 2017; Jurk et al., 2012).

302 Lipid accumulation in senescent cells can be visualized using various commercial dyes
303 and assays (Ogrodnik et al., 2017) or immunostaining for lipid associated proteins such as
304 Perilipin 2 (Ogrodnik et al., 2017). Importantly, genetic or pharmacological clearance of
305 senescent cells in obese and aging mice reduced lipid deposits in liver (Ogrodnik et al., 2017)
306 and brain (Ogrodnik et al., 2019b).

307 Despite the association with lipid accumulation, our knowledge about specific lipid
308 metabolite composition in senescent cells is sparse. Fatty acids, their precursors and
309 phospholipid catabolites, such as eicosapentaenoate (EPA), malonate, 7-alpha-hydroxy-3-oxo-4-
310 cholestenoate (7-HOCA) and 1-stearoylglycerophosphoinositol increase in senescent fibroblasts,
311 whereas linoleate, dihomo-linoleate and 10-heptadecenoate decline (James et al., 2015).
312 Moreover, free cholesterol rises, accompanied by reduced phospholipids and cholesteryl esters
313 derived from acetate, while fatty acid synthase and stearoyl-CoA desaturase-1 declines (Maeda
314 et al., 2009). Several methods are available to detect lipid changes in tissues and cells, but their
315 use as senescence biomarker remains limited due to high variability of the senescence-associated
316 lipid profile. For example, lipid metabolites vary significantly between oncogene-induced
317 senescence and replicative senescence (Quijano et al., 2012).

- *Deregulated metabolic profile*

Mitochondria

Senescent cells exhibit several changes in mitochondrial function, dynamics and morphology. Mitochondria in senescent cells are less functional, showing decreased membrane potential, increased proton leak, reduced fusion and fission rates, increased mass and abundance of tricarboxylic acid (TCA) cycle metabolites (Kaplon et al., 2013; Passos et al., 2010). While mitochondrial are more abundant, it appears their ability to produce ATP is compromised (Birch and Passos, 2017; Korolchuk et al., 2017). In contrast, senescent cells often produce more ROS, which can cause protein and lipid damage, as discussed in previous sections (see '*protein damage*' and '*lipid damage*'), but also telomere shortening and DDR activation (Passos et al., 2007). Targeting aspects of mitochondrial biology, such as the electron transport chain (ETC), complex I assembly, mitochondrial fission rates and biogenesis, mitochondrial sirtuins and/or disruption of the TCA cycle can trigger senescence (Correia- Melo et al., 2016; Jiang et al., 2013; Kaplon et al., 2013; Miwa et al., 2014; Moiseeva et al., 2009; Park et al., 2010; Wiley et al., 2016). Altered AMP:ATP and ADP:ATP ratios during senescence contribute to cell-cycle withdrawal by activating AMPK, a main sensor of energy deprivation (Birch and Passos, 2017).

Mitochondrial dysfunction during senescence is also implicated in SASP regulation. Mitophagy (mitochondrial clearance) in senescent cells appears to suppress the SASP (Correia- Melo et al., 2016). Genetic or pharmacological inhibition of the ETC can induce senescence, with cells lacking expression of key pro-inflammatory SASP factors, such as IL-6 and IL-8 (Wiley et al., 2016). NAD^+/NADH ratios are reduced in senescent cells (Wiley et al., 2016), which could alter the activity of poly-ADP ribose polymerase (PARP) and sirtuins, both involved in activation of the SASP-regulator NF-kB (Birch and Passos, 2017).

While substantial data support a role for mitochondria in senescence in culture, less is known *in vivo*. Mouse models of mitochondrial dysfunction and enhanced oxidative stress show increased senescence (Wiley et al., 2016), but a detailed characterization of mitochondrial function in senescent cells *in vivo* is lacking. Because mitochondrial dysfunction characterizes other cellular processes (Eisner et al., 2018), like others, it is not a consistent biomarker of senescence. Finally, it is not clear whether senescent cells contribute to declined mitochondrial function observed during aging and age-related diseases (Srivastava, 2017).

Lysosomes

Secretion requires simultaneous activation of anabolic and catabolic processes (see "Secretion") (Salama et al., 2014). Increased catabolism provides energy and raw materials, and is favored by the lysosome, the end-degradation compartment of phagocytosis, endocytosis and autophagy (Settembre and Ballabio, 2014)**.** Lysosome biogenesis is transcriptionally-driven, and depends on the cellular energetic or degradative needs (Settembre and Ballabio, 2014). Intriguingly, when amino acid levels in the lysosomal lumen are high, mTOR1 is recruited and activated and *vice versa* (Settembre and Ballabio, 2014)**.** Additionally, lysosomes interact with mitochondria to preserve mitochondrial homeostasis (see *"Mitochondria"*) (Park et al., 2018).

Lysosomes in senescent cells increase in number and size, evident by the cytoplasmic granularity seen microscopically (Robbins et al., 1970); **Suppl Video 1,** for non-senescent cells see **Suppl Video 2**). The increased lysosomal number might reflect an attempt to balance the gradual accumulation of dysfunctional lysosomes by producing more new lysosomes. Thus, the balance between anabolism and catabolism, vital for secretion, is extended. This balance is maintained during OIS through TOR-autophagy spatial-coupling-compartment (TASCC), which coordinates the production of SASP factors (Salama et al., 2014).

364 The elevated lysosomal content does not necessarily reflect increased activity, as the
365 degradation stage of autophagy also declines (Park et al., 2018). Thus, the lysosome-
366 mitochondrial axis degrades, leading to pathological mitochondrial turnover that increases ROS
367 production. Subsequently, ROS targets cellular structures, including lysosomes, forming a
368 vicious feedback loop that induces more damage (Park et al., 2018). The increased lysosomal
369 mass has been linked to SA-β-gal activity (Hernandez-Segura et al., 2018), a senescence
370 biomarker. However, although the SA-β-gal is prominent in senescent cells (Dimri et al., 1995;
371 Hernandez-Segura et al., 2018), it is neither required nor a determinant of the senescent
372 phenotype (Hernandez-Segura et al., 2018). From a therapeutic viewpoint, the enlarged
373 lysosomal compartment offers an increased capacity to trap drugs that can be protonated, such as
374 the selective CDK4/6 inhibitors palbociclib, ribociclib and abemaciclib. This capacity reduces
375 their effective concentration in the cytosol and nucleus, but counteracted by the slow release of
376 the drugs from the lysosomes, thereby increasing drug exposure time (Llanos et al., 2019).
377 Another senescence trait, related to lysosomal malfunction, is the intra-lysosomal accumulation
378 of lipofuscin aggresomes (see "*Protein damage*" and "*Lipid damage*", reviewed in (Gorgoulis et
379 al., 2018). Interestingly, lipofuscin was reported to stimulate expression of the anti-apoptotic
380 factor Bcl-2, conferring resistance to apoptosis, another characteristic of senescent cells
381 (McHugh and Gil, 2018). Lysosomes in senescent cells also participate in chromatin processing
382 (CCFs) (see *"DNA damage"* and "*Secretion"*) (Ivanov et al., 2013).
383
384 **3.Senescence-associated (epi)-genetic and gene expression changes (Figure 2)**
385 The features listed above are associated with changes in gene expression, determined by
386 transcriptional regulation of coding and non-coding RNAs, which can be exploited for

senescence detection. Here, we discuss such major alterations, and describe a novel database that can aid the identification of genes associated with senescence, termed SeneQuest (http://Senequest.net) [see **Supplementary Information and Suppl. Table 1**].

- *Chromatin landscape*

Epigenetic modifications occur during senescence, but are mostly context-dependent (Cheng et al., 2017). For example, replicative senescence has been correlated with global loss of DNA methylation at CpG sites (Cheng et al., 2017). In addition to the global loss of DNA methylation, cellular senescence is entails focal increases in DNA methylation at certain CpG islands (Cruickshanks et al., 2013). Interestingly, this DNA methylation profile somewhat resembles the cancer- and aging-associated methylome patterns (Cruickshanks et al., 2013; Xie et al., 2018). Cells undergoing OIS fail to show such alterations in DNA methylation (Xie et al., 2018), reinforcing the diverse nature of epigenetic alterations during senescence.

Senescent cells also exhibit a global increase in chromatin accessibility, but the genome-wide profile varies depending on the stimulus (De Cecco et al., 2013). Individual histone modifications and variants (Cheng et al., 2017; Hernandez-Segura et al., 2018; Rai et al., 2014) demonstrate alterations during senescence. For instance, H4K16ac is often enriched at active promoters in senescent, but not proliferating, cells (Rai et al., 2014). Its accumulation correlates closely with histone variant H3.3, which is deposited into chromatin in a DNA replication-independent manner by the HIRA/UBN1/CABIN1 and ASF1a chaperones (Rai et al., 2014). Notably, N-terminus proteolytic cleavage of H3.3 correlates with gene repression in a different subset of genes during senescence (Ivanov et al., 2013). Global loss of linker histone H1 is another senescence feature (Funayama et al., 2006). Certain histone modifications are vital, such as elevated H4K20me3 and H3K9me3, which contribute to the proliferation arrest (Cheng et al.,

2017; Di Micco et al., 2011; Salama et al., 2014), whereas elevated H3K27ac at gene enhancers promotes a SASP (Hernandez-Segura et al., 2018).

Senescence is also associated with chromatin morphological changes. Senescence-associated heterochromatin foci (SAHF), visualized as DAPI-dense foci, are enriched in Heterochromatin Protein (HP) 1. SAHFs derive from chromatin factors, including RB, histone variant macroH2A, high mobility group A proteins, the HIRA/UBN1/CABIN1 and ASF1a chaperones, and increased nuclear pore density (Boumendil et al., 2019; Salama et al., 2014). SAHFs were initially hypothesized to contribute to gene regulation (Salama et al., 2014). However, SAHFs were since shown to comprise largely late-replicating gene poor heterochromatic regions, even in proliferating cells, suggesting a small role in senescence-associated gene expression (Salama et al., 2014). Senescence is also correlated with global loss of linker histone H1 (Funayama et al., 2006). Notably, SAHFs seem to be cell type- and stimulus-dependent, as they are not seen in all senescent cells (Di Micco et al., 2011; Kennedy et al., 2010; Sharpless and Sherr, 2015), rendering them useful for senescence identification, while the functional significance remains to be elucidated

Another chromatin feature termed, senescence-associated distension of satellites (SADS), corresponds to de-compaction of (peri-)centric constitutive heterochromatin (Cruickshanks et al., 2013; De Cecco et al., 2013; Swanson et al., 2013). SADS precede SAHF formation and might be widely linked to senescence (Swanson et al., 2013). Retrotransposable elements are another type of constitutive heterochromatin related to senescence. The normally-repressed retrotransposon Line 1 (L1) are activated, stimulating the cGAS-STING pathway that elicits a type I interferon response (see ”*Secretion*”) (De Cecco et al., 2013). Hence, in addition to triggering genomic instability, these elements fuel the SASP (Criscione et al., 2016).

Downregulation of lamin B1, a major component of the nuclear lamina, is another key feature of senescence (Dou et al., 2015; Freund et al., 2012; Shah et al., 2013; Shimi et al., 2011). Lamin B1 loss correlates with epigenetic profiles (Salama et al., 2014), as well as senescence-associated chromatin structures (SAHF and SADS) (Salama et al., 2014; Swanson et al., 2013). Its reduction occurs predominantly at H3K9me3-rich regions, a process that appears to liberate H3K9me3 from the nuclear lamina promoting spatial rearrangement of H3K9me3-heterochromatin to form SAHF (Salama et al., 2014). Hi-C analysis (genome-wide mapping of chromatin contacts) in OIS revealed a reduction in local connectivity at regions enriched for H3K9me3 and lamin B1, perturbing these long-range interactions (Chandra et al., 2015). Replicative senescence, on the other hand, showed loss of long-range and gain of short-range interactions within chromosomes (Criscione et al., 2016), implying that the nature of senescence-associated high-order chromatin interactions is stimulus and context-dependent (Zirkel et al., 2018). Furthermore, lamin B1 loss and reduced nuclear integrity is suggested to fuel the SASP by contributing to CCF formation (Dou et al., 2015; Ivanov et al., 2013), thereby stimulating the cGAS-STING pathway and interferon response (see "*Secretion*") ") (Li and Chen, 2018). Autophagy-mediated CCF formation (Dou et al., 2015) together with reduced histone synthesis (O'Sullivan et al., 2010) might also lead to a global loss of core histones during senescence, affecting the chromatin landscape (Chan and Narita, 2019; Ivanov et al., 2013).

- *Transcriptional signatures*

Several genes linked to the cell cycle arrest and SASP are frequently interrogated in combination with other biomarkers to validate the senescence phenotype or type of senescence. For example, increased expression of the cyclin-dependent kinase inhibitors CDKN1A ($p21^{WAF1/Cip1}$), CDKN2A ($p16^{INK4A}$) and CDK2B ($p15^{INK4B}$) and a subset of SASP genes, along

with decreased expression of cyclins CCNA2 and CCNE2 and LMNB1 should be determined. In addition, the transcriptome of putative senescent cells should be established, which can then be compared with the increasing number of existing senescence transcriptomes (Hernandez-Segura et al., 2018).

Whole-transcriptome studies have been instrumental in defining major signaling pathways involved in establishing senescence phenotypes, and in some cases predicting drug targets (Zhu et al., 2015). A set of 13 genes was differentially regulated in several cell types undergoing distinct forms of senescence, including oncogene-, replicative- and DNA damage-induced senescence (Hernandez-Segura et al., 2017). More recently, a similar study, which considered only fibroblasts and endothelial cells, also attempted at defining senescence-associated transcriptome signatures (Casella et al., 2019). Due to the current paucity of transcriptome data sets, and the availability of more single-cell studies that allow evaluation of intra-population variability (Wiley et al., 2017a; Zirkel et al., 2018), these gene signatures will likely change in coming years. But ultimately a senescence gene expression signature will prove valuable for identifying senescence under many conditions in culture and *in vivo*.

- *miRNAs and non-coding RNAs*

Non-coding RNAs, particularly microRNAs (miRNAs), can influence the senescence program, alone or in concert. Functional studies revealed several miRNAs that directly or indirectly modulate the abundance of key senescence effectors, including p53, $p21^{WAF1/Cip1}$ and SIRT1 (Suh, 2018). miR-504 targets the p53 3'UTR, reducing p53 abundance and activity (Hu et al., 2010). Also, Gld2-mediated stabilization of miR-122 enables its binding to the CBEP 3'UTR, resulting in decreased p53 mRNA polyadenylation and translation (Burns et al., 2011). Conversely, miR-605 targets MDM2, triggering p53-mediated senescence (Xiao et al., 2011),

and multiple miRNAs downregulate $p21^{WAF1/Cip1}$, including 28 miRNAs that block OIS (Borgdorff et al., 2010). Likewise, miR-24 suppresses $p16^{INK4a}$ in cells (Lal et al., 2008) and disease models, including osteoarthritis (Philipot et al., 2014). Intricate miRNA feedback loops can modulate senescence programs. For example, a p53/miRNA/CCNA2 pathway drives senescence independently of the p53/$p21^{WAF1/Cip1}$ axis (Xu et al., 2019). Similarly, p53-dependent upregulation of miR-34a/b/c downregulates cell proliferation and survival factors (Hermeking, 2010). Non-coding RNAs also regulate the SASP (Panda et al., 2017). MiR-146a/b, for example, increases weeks after senescence induction and dampens a proinflammatory arm of the SASP (Bhaumik et al., 2009). miRNAs also downregulate repressors of senescence, including Polycomb Group (PcG) members CBX7, EED, EZH2 and SUZ12 (miR-26b, 181a, 210 and 424), leading to $p16^{INK4a}$ derepression and senescence initiation (Overhoff et al., 2014). Finally, the role of miRNAs in senescence extends beyond their classical functions. For example, Argonaute 2 (AGO2) binds let-7f in the nucleus, forming a complex with RB1 (pRB), resulting in repressive chromatin at CDC2 and CDCA8 promoters (Benhamed et al., 2012). Silencing these E2F target genes is required for senescence initiation.

Long non-coding RNAs (lncRNAs) (> 200 nt) can bind RNA, DNA or proteins to regulate senescence. For example, ANRIL, a 30-40kb antisense transcript encoded by theCDKN2A locus, binds CBX7 to repress INK4b/ARF/INK4a expression (Kim et al., 2017). Likewise, the lncRNA PANDA recruits PcG complexes, suppressing senescence-promoting genes (Kim et al., 2017), whereas silencing of GUARDIN, a p53-responsive lncRNA, causes senescence or apoptosis (Hu et al., 2018). By contrast, following OIS induced by RAF, the lncRNA VAD preserves senescence by decreasing repressive H2A.Z deposition at INK promoters (Kim et al., 2017). Also, lncRNA UCA1 disrupts association of the RNA binding protein hnRNP A1 with

502 $p16^{INK4A}$, but not $p14^{ARF}$, transcripts (Kim et al., 2017). In addition, non-coding RNA profiling,
503 with a focus on miRNAs, provides a senescence signature (Suh, 2018). Intriguingly, the miRNA
504 content of small extracellular vesicles released by senescent cells varies, evolving over time
505 (Terlecki-Zaniewicz et al., 2018).

506 • *Immune-regulation and anti-apoptotic proteins*

507 The search for senescent protein markers started in OIS. In addition to identifying known
508 cell cycle regulators, these studies identified DCR2 as a common marker of senescence (Collado
509 et al., 2005), later shown to characterize other types of senescence. DCR2 is a decoy death
510 receptor that protects senescent cells from immunity-mediated apoptosis, thus blocking immune
511 surveillance of senescent cells (Sagiv et al., 2013). Similarly, the natural killer (NK) cell
512 activating receptor (NKG2D) ligands MICA and ULBP2 increase upon replicative, OIS and
513 DNA damage-induced senescence (Krizhanovsky et al., 2008b; Sagiv et al., 2016). Cell surface
514 markers are of special interest because they should allow quantification, isolation and single cell
515 transcriptional analysis of senescent cells extracted from tissues. However, DCR2 and NKG2D
516 ligands are not conserved among species, making mouse/human comparisons not possible.
517 Recently, two additional upregulated cell surface markers, Notch1 in OIS and DPP4 in
518 replicative and OIS, were identified (Hoare et al., 2016). Both proteins have roles in regulating
519 the SASP. Furthermore, an oxidized form of membrane-bound vimentin was identified as a
520 senescence marker, which could be used to target these cells by the adaptive immune system
521 (Frescas et al., 2017). Finally, senescent cells are resistant to apoptosis, which can be mediated
522 by increased expression of anti-apoptotic BCL-2 family members (Yosef et al., 2016).

523

524 **4. *In vivo* models to study cellular senescence**

- *Senescence reporter mice*

Several transgenic mice were developed to estimate *p16*^{Ink4a} expression *in vivo* or *ex vivo* using luciferase or fluorescent protein reporters. Measuring luciferase activity longitudinally revealed an increase in *p16*^{INK4A} expression as mice age, as well as an age-dependent increase in inter-animal variability, whereas isolation of fluorescent p16+ cells allowed phenotyping (Liu et al., 2019; Ohtani et al., 2010). This approach allows the endogenous *p16*^{INK4A} promoter to drive signals, but causes p16 hemizygosity. Another mouse (p16-3MR) used a luciferase (rLUC), monomeric Red Fluorescent Protein (mRFP) and Herpes simplex Virus-Thymidine Kinase (HSV-TK) fusion protein driven by the *p16*^{INK4A} promoter present on a bacterial artificial chromosome, integrated into the mouse genome (Demaria et al., 2014). This approach allows detection and killing of senescent cells, and does not perturb the endogenous CDKN2A locus. Finally, INK-ATTAC mice express a FKBP-Caspase 8 fusion-protein and eGFP reporter to kill and detect p16^{+} cells, driven from a 1.6 kB fragment of the *p16*^{INK4A} promoter (Baker et al., 2011; Folgueras et al., 2018). Despite differences between these mice, they have been valuable in showing that senescent cells contribute to a wide range of age-related pathologies (Calcinotto et al., 2019). Mice expressing luciferase and eGFP from *p21*$^{WAF1/Cip1}$ promoter are also available (Ohtani et al., 2007).

- *Murine models of accelerated senescence and aging*

Several progeric mouse models have been developed to mimic human progeric syndromes, including DNA repair and genome integrity deficiencies (Folgueras et al., 2018). Progeroid mice with accelerated senescence and shortened lifespans are also useful for assessing the role of cellular senescence in aging and testing senotherapeutics. For example, the demonstration that ablation of *p16*^{INK4A} expressing cells slowed age-related declines in progeroid

548 *BubR1*$^{H/H}$ mice provided the first evidence that senescent cells are causal for certain aging
549 phenotypes (Baker et al., 2011; Folgueras et al., 2018). BUBR1 is important for the mitotic
550 spindle assembly checkpoint (Guo et al., 2012). *BubR1*$^{H/H}$ mice, which express 10% of the
551 normal level of BUBR1, have increased aneuploidy, several progeroid features and increased
552 expression of senescence markers in several organs (Folgueras AR et al., 2018). Selective
553 removal of *p16*$^{INK4A+}$ cells from *BubR1*$^{H/H}$-INK-ATTAC mice delays kyphosis, cataracts and
554 muscle atrophy, but not cardiac arrhythmias and arterial wall stiffening, nor does it extend
555 lifespan (Baker et al., 2011; Folgueras et al., 2018).

556 Similarly, *Ercc1*$^{-/\Delta}$ progeroid mice, harboring a DNA repair defect, prematurely develop
557 multiple morbidities associated with age, driven in part by accelerated accumulation of senescent
558 cells in numerous tissues (Folgueras AR et al., 2018). *Ercc1*$^{-/\Delta}$ mice (Folgueras AR et al., 2018)
559 express 5% of the normal level of the endonuclease ERCC1-XPF, important for nucleotide
560 excision, interstrand crosslink and double-strand break repair. These mice develop numerous
561 age-related histopathologic lesions in virtually every tissue (Folgueras AR et al., 2018), and
562 accumulate oxidative DNA damage faster than wild-type mice (Wang et al., 2012). Treatment of
563 *Ercc1*$^{-/\Delta}$ mice with senolytic drugs reduces senescence markers and extends health span
564 (Fuhrmann-Stroissnigg et al., 2017; Yousefzadeh et al., 2018; Zhu et al., 2015). Cross-breeding
565 of these models with the *p16*^{INK4A} reporter transgenes permits monitoring senescent cell burden
566 longitudinally in live animals (Robinson et al., 2018; Yousefzadeh et al., 2018).

567 Hutchinson-Gilford Progeria Syndrome (HGPS) is a segmental or tissue-specific
568 progeria, caused by mutations that compromise lamin A processing (Cau et al., 2014). Mice
569 with altered or deleted LMNA develop HGPS-like phenotypes. They also accumulate senescent
570 cells, as determined by SA-ß-gal staining and mRNA levels of senescence markers, in skeletal

muscle and heart, consistent with sites of age-related pathology and disease (Folgueras AR et al., 2018). Similarly, in a mouse model of HGPS that recapitulates the pathogenic LMN splicing mutation, *Lmna*$^{G609G/G609G}$ mice, senescence in the liver and kidney was observed (Osorio et al., 2011). However, senescent cells have not yet been shown to be causative for HGPS pathology.

A mouse model of trichothiodystrophy (TTD) (Andressoo et al., 2006), caused by a specific mutation in the *Xpd* gene, also indicated a role for senescent cells in premature aging. Here the role of senescence in driving aging in the *Xpd*$^{TTD/TTD}$ was clearly documented by the fact that treatment with a D-retro inverso (DRI)-isoform peptide of FOXO4 able to disrupt FOXO4 interaction with p53. Treatment with the FOXO4-DRI peptide reduced lethargy in *Xpd*$^{TTD/TTD}$ mice and improved fur density, running wheel activity, and physical responses to stimuli (Baar et al., 2017).

Loss of Cu/Zn-superoxide dismutase (*Sod1*) in mice accelerates aging (Zhang et al., 2017). *Sod1*$^{-/-}$ mice show increased oxidative DNA damage, senescence (*p16*^{INK4A}, *p21*$^{WAF1/Cip1}$), SASP factors (*Il1β*, *Il6*), SA-βgal^{+} cells and age-associated pathology in kidneys (Zhang et al., 2017). To date, senescence has not been demonstrated to drive pathology in *Sod1*$^{-/-}$ mice.

Deletion of the nfkb1 subunit of the transcription factor NF-κB induces premature ageing in mice. These mice have been shown to experience chronic, progressive low-grade inflammation which contributes to a wide spectrum of ageing phenotypes and early mortality (however, in contrast to some of the widely used progeria mouse models these mice have a maximum lifespan of approximately 20 months). Furthermore, these mice show increased incidence of senescent cells in multiple tissues (Jurk et al., 2014).

Finally, the selective inbreeding of AKR/J mice resulted in numerous senescence-accelerated mouse (SAMP) strains including SAMP1-3 and SAMP6-11 (Takeda et al., 1997). Although

these mice have increased senescence and thus can be used for testing senotherapeutics, it remains unclear which mutant genes drive senescence in these strains.

5. Identification of cellular senescence *in vivo*

- *A simplified algorithm for detecting senescent cells in situ*

In vivo, senescent cells reside in complex tissues. Their impact on tissue function can be local or global due to the SASP (Xu et al., 2018). To understand how senescence affects tissue function, tissue remodeling and aging, we need tools to identify senescent cells in tissues.

Single cell analyses can be performed on most tissues. Common techniques include immunostaining, in-situ hybridization and multicolour (imaging) flow cytometry. Even higher numbers of markers can be assessed by mass cytometry (Cytometry by Time-Of-Flight, CYTOF) (Abdelaal et al., 2019). Although promising, limitations include loss of information about spatial associations and variable efficiency of isolation of different cell types, including senescent vs non-senescent cells. Therefore, microscopic imaging remains a preferred method for *in situ* senescence detection.

As mentioned, there is currently no single marker with absolute specificity for senescent cells. Marker specificity varies, depending on cell type, tissue, organismal developmental stage, species and other factors. However, some markers have more global/universal value/validity while others are related to specific senescence types. Therefore, we advise a multi-marker approach, encompassing/combining broader and more specific markers for more robust detection of senescent cells *in situ* (**Figure 3**).

- *Challenges to detect senescent cells in humans*

The role of senescence in human disease is clear from cellular studies, while *in vivo* evidence is only now catching up (Childs et al., 2015; He and Sharpless, 2017; Munoz-Espin and Serrano, 2014). OIS, initially described in culture, was the first type of senescence validated in humans (Serrano et al., 1997). OIS or senescence induced by loss of a tumor suppressor was verified *in vivo* in human preneoplastic lesions (Collado et al., 2005; Gorgoulis and Halazonetis, 2010; Kuilman and Peeper, 2009) and primary or treated neoplasias (Haugstetter et al., 2010). Later reports on the diverse activities of the senescence secretome (see "Secretion") led to the recognition of its pro-tumorigenic properties, establishing what is now accepted as the dual role of senescence in carcinogenesis (Lee and Schmitt, 2019). Evidence linking senescence to other common age-associated human diseases has recently emerged. These diseases include neurodegenerative disorders, glaucoma, cataract, atherosclerosis/cardiovascular disease, diabetes, osteoarthritis, pulmonary, and renal and liver fibrosis (Childs et al., 2015; He and Sharpless, 2017; Munoz-Espin and Serrano, 2014) (**Suppl Table 2**).

In most studies, senescence is assessed in *ex vivo* cultures or fresh samples by SA-β-gal staining or indirect markers in formalin-fixed tissues (Haugstetter et al., 2010; He and Sharpless, 2017; Kuilman and Peeper, 2009; Munoz-Espin and Serrano, 2014; Serrano et al., 1997). Since SA-β-gal is not suitable for fixed tissues, analyzing senescence in human samples is challenging. The recently developed assay and reagent Sudan Black-B (SBB) interacts with lipofuscin, another hallmark of senescent cells (Georgakopoulou et al., 2013). Lipofuscin is preserved in fixed material (Georgakopoulou et al., 2013) and resilient, as it was isolated from a 210,000 year old human fossil (Harvati et al., 2019; Myrianthopoulos et al., 2019). The test reagent is amenable to immunohistochemistry (Evangelou et al., 2017), and identified senescent Hodgkin and Reed-Sternberg (HRS) cells in Hodgkin lymphomas (cHL), where they predicted poor

639 prognosis (Myrianthopoulos et al., 2019). These cells are giant in size, with a large occasionally
640 multilobular nucleus - indication of an abortive cell cycle -, increased secretory activities,
641 embedded within an inflammatory milieu, a histological pattern strongly reflecting features of
642 the senescence phenotype (Kuppers et al., 2012) (**Figure 2)**. Another method for identifying
643 and quantifying senescent cells *in vivo* is SA-β-gal staining combined with ImageStream X
644 analysis (Biran et al., 2017).

645 Despite promising results that each marker provides, no marker is completely
646 senescence-specific (Sharpless and Sherr, 2015) (Sharpless and Sherr, 2015). We recommend
647 combining cytoplasmic (e.g., SA-β-gal, lipofuscin), nuclear (e.g., $p16^{INK4A}$, $p21^{WAF1/Cip1}$, Ki67)
648 and context/cell type-specific markers (Childs et al., 2015) (**Figure 3**).
649

650 **6. Conclusions, open questions and perspectives**

651 From the first description of cellular senescence by Hayflick and colleagues almost 60
652 years ago, significant progress has been made in understanding the characteristics and functions
653 of senescent cells. A limitation, particularly for studying biospecimens, remains the absence of
654 specific markers. To overcome this obstacle, we propose a multi-marker approach **(Figure 3).**
655 This strategy could also be used to evaluate the efficacy of senolysis, an emerging therapeutic
656 approach recently entered clinical trials for treatment of various age-related pathologies
657 (Myrianthopoulos et al., 2019).

658 Conceptually, senescence can be considered a non-linear, multivariable [F(x,y)=z]
659 function where the dependent variable (outcome) z depends on the independent variables x
660 (stimulus) and y (environment). The non-linear processing is dictated by dynamic genetic and
661 epigenetic processes that can lead to reprogramming cycles until a steady-state is achieved. At

first glance, the outcomes appear to be cell cycle withdrawal and secretion of bioactive molecules. However, recent evidence suggest that the cell cycle arrest is not always a necessary outcome, as post-mitotic cells, already unable to proliferate, can assume senescence-like features, and under certain conditions senescent cells can re-enter the cell cycle. The SASP appears a common senescence-associated feature, but it is highly heterogeneous. Thus, to understand the pleiotropic phenotypes of senescent cells, a shift from traditional reductionism to more systematic, multi-parametric approaches is needed. The development of sophisticated high throughput methods and machine learning tools that can handle multi–omics data will help achieve this goal (Vougas et al., 2019). Although "old and new" have pros and cons, we can combine the best to achieve a "de profundis" analysis of senescent phenotypes. This approach will likely unveil more specific senescence-associated signatures to address important unanswered questions: What causes and regulates the SASP? How do genetic and epigenetic determinants interact with triggering stimuli and cellular microenvironments? Which genomic repair systems act in different senescence scenarios? What causes cells to evade the growth arrest, and what phenotypes do 'escaped' senescent cells acquire? Answers to these and other questions will help develop specific panels of markers for each senescence subtype (step 3 in the workflow) and guide the evolving field of senotherapy (van Deursen, 2019), achieving the best outcome within the spirit of precision medicine.

CONFLICT OF INTEREST

The authors declare conflicts of interest related to this work.

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FIGURE LEGENDS

Figure 1. Cell cycle withdrawal in senescent, quiescent and terminally differentiated cells. Depicted are differences in cell cycle arrest reversibility, activated signals **(see text),** secretory functions and macromolecular damage that allow discrimination between these cellular states. Macromolecular damage is a common feature of senescence. Secretion is another common feature of senescence and is context-dependent on differentiation state. Cell cycle arrest is generally considered irreversible during senescence and terminal differentiation, although cell cycle re-entry can occur under certain conditions. Green color: active/present, red color: inactive/absent.

Figure 2. The hallmarks of the senescence phenotype. Senescent cells exhibit four inter-dependent (shown by the dashed thin outer cycle and bidirectional arrows) hallmarks: 1) cell cycle withdrawal, 2) macromolecular damage, 3) Secretory Phenotype (SASP) and 4) deregulated metabolism, as depicted in the outer circle (see text). The inner cycle includes distinct morphological and functional features that reflect the proposed hallmarks. Several of these traits are strongly evident in the malignant entity, the classical Hodgkin Lymphoma (see section 5). Multilobular nuclei commonly present in (senescent) HRS cells, as a result of S/M

phase dissociation, are linked to cell cycle withdrawal ($p21^{WAF1/Cip1}$ immunopositivity-left image) while the inflammatory milieu is associated with SASP. Lipofouscin accumulation assessed with GL13 staining (brown cytoplasmic staining-right image) reflects macromolecular damage leading to increased granularity (left centered image). Altered/increased gene expression (right centered image) that is also accompanied by increased transcriptional "noise" also confers to macromolecular damage (Schmoller and Skotheim, 2015; Ogrodnik et al., 2019).

Figure 3. A multi-marker, three-step workflow for detecting senescent cells. The first step of the proposed workflow includes assessing senescence-associated beta-galactosidasde (SA-β-gal) activity and/or lipofuscin accumulation (SBB or GL13 staining). Secondly, co-staining with other markers frequently observed in ($p16^{INK4A}$, $p21^{WAF1/Cip1}$) or absent from (proliferation markers, Lamin B1) senescent cells. In the third step, identification of factors anticipated to be altered in specific senescence contexts should be identified. This multi-marker workflow can lead to the recognition of senescent cells with the highest accuracy.

REFERENCES

Abdelaal, T., Hollt, T., van Unen, V., Lelieveldt, B.P.F., Koning, F., Reinders, M.J.T., and Mahfouz, A. (2019). CyTOFmerge: Integrating mass cytometry data across multiple panels. Bioinformatics.

Acosta, J.C., Banito, A., Wuestefeld, T., Georgilis, A., Janich, P., Morton, J.P., Athineos, D., Kang, T.W., Lasitschka, F., Andrulis, M., *et al*. (2013). A complex secretory program orchestrated by the inflammasome controls paracrine senescence. Nat Cell Biol *15*, 978-990.

Ademowo, O.S., Dias, H.K.I., Burton, D.G.A., and Griffiths, H.R. (2017). Lipid (per) oxidation in mitochondria: an emerging target in the ageing process? Biogerontology *18*, 859-879.

Andressoo, J.O., Mitchell, J.R., de Wit, J., Hoogstraten, D., Volker, M., Toussaint, W., Speksnijder, E., Beems, R.B., van Steeg, H., Jans, J., *et al*. (2006). An Xpd mouse model for the combined xeroderma pigmentosum/Cockayne syndrome exhibiting both cancer predisposition and segmental progeria. Cancer Cell *10*, 121-132.

Baar, M.P., Brandt, R.M.C., Putavet, D.A., Klein, J.D.D., Derks, K.W.J., Bourgeois, B.R.M., Stryeck, S., Rijksen, Y., van Willigenburg, H., Feijtel, D.A., *et al*. (2017). Targeted Apoptosis of Senescent Cells Restores Tissue Homeostasis in Response to Chemotoxicity and Aging. Cell *169*, 132-147 e116.

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.

Beausejour, C.M., Krtolica, A., Galimi, F., Narita, M., Lowe, S.W., Yaswen, P., and Campisi, J. (2003). Reversal of human cellular senescence: roles of the p53 and p16 pathways. Embo j *22*, 4212-4222.

Benhamed, M., Herbig, U., Ye, T., Dejean, A., and Bischof, O. (2012). Senescence is an endogenous trigger for microRNA-directed transcriptional gene silencing in human cells. Nat Cell Biol *14*, 266-275.

Bhaumik, D., Scott, G.K., Schokrpur, S., Patil, C.K., Orjalo, A.V., Rodier, F., Lithgow, G.J., and Campisi, J. (2009). MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8. Aging (Albany NY) *1*, 402-411.

Biran, A., Perelmutter, M., Gal, H., Burton, D.G., Ovadya, Y., Vadai, E., Geiger, T., and Krizhanovsky, V. (2015). Senescent cells communicate via intercellular protein transfer. Genes Dev *29*, 791-802.

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.

Birch, J., and Passos, J.F. (2017). Targeting the SASP to combat ageing: Mitochondria as possible intracellular allies? BioEssays *39*.

Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., and Wright, W.E. (1998). Extension of life-span by introduction of telomerase into normal human cells. Science *279*, 349-352.

Borgdorff, V., Lleonart, M.E., Bishop, C.L., Fessart, D., Bergin, A.H., Overhoff, M.G., and Beach, D.H. (2010). Multiple microRNAs rescue from Ras-induced senescence by inhibiting p21(Waf1/Cip1). Oncogene *29*, 2262-2271.

Boumendil, C., Hari, P., Olsen, K.C.F., Acosta, J.C., and Bickmore, W.A. (2019). Nuclear pore density controls heterochromatin reorganization during senescence. Genes Dev *33*, 144-149.

Burns, D.M., D'Ambrogio, A., Nottrott, S., and Richter, J.D. (2011). CPEB and two poly(A) polymerases control miR-122 stability and p53 mRNA translation. Nature *473*, 105-108.
Calcinotto, A., Kohli, J., Zagato, E., Pellegrini, L., Demaria, M., and Alimonti, A. (2019). Cellular Senescence: Aging, Cancer, and Injury. Physiol Rev *99*, 1047-1078.
Casella, G., Munk, R., Kim, K.M., Piao, Y., De, S., Abdelmohsen, K., and Gorospe, M. (2019). Transcriptome signature of cellular senescence. Nucleic Acids Res *47*, 7294-7305.
Chan, A.S.L., and Narita, M. (2019). Short-term gain, long-term pain: the senescence life cycle and cancer. Genes Dev *33*, 127-143.
Chandra, T., Ewels, P.A., Schoenfelder, S., Furlan-Magaril, M., Wingett, S.W., Kirschner, K., Thuret, J.Y., Andrews, S., Fraser, P., and Reik, W. (2015). Global reorganization of the nuclear landscape in senescent cells. Cell Rep *10*, 471-483.
Cheng, L.Q., Zhang, Z.Q., Chen, H.Z., and Liu, D.P. (2017). Epigenetic regulation in cell senescence. J Mol Med (Berl) *95*, 1257-1268.
Childs, B.G., Durik, M., Baker, D.J., and van Deursen, J.M. (2015). Cellular senescence in aging and age-related disease: from mechanisms to therapy. Nat Med *21*, 1424-1435.
Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A.J., Barradas, M., Benguria, A., Zaballos, A., Flores, J.M., Barbacid, M., *et al*. (2005). Tumour biology: senescence in premalignant tumours. Nature *436*, 642.
Coppe, J.P., Desprez, P.Y., Krtolica, A., and Campisi, J. (2010). The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol *5*, 99-118.
Correia-Melo, C., Marques, F.D.M., Anderson, R., Hewitt, G., Hewitt, R., Cole, J., Carroll, B.M., Miwa, S., Birch, J., Merz, A., *et al*. (2016). Mitochondria are required for pro-ageing features of the senescent phenotype. The EMBO journal *35*, 724-742.
Correia- Melo, C., Marques, F.D.M., Anderson, R., Hewitt, G., Hewitt, R., Cole, J., Carroll, B.M., Miwa, S., Birch, J., Merz, A., *et al*. (2016). Mitochondria are required for pro- ageing features of the senescent phenotype. The EMBO Journal *35*, 724.
Criscione, S.W., Teo, Y.V., and Neretti, N. (2016). The Chromatin Landscape of Cellular Senescence. Trends Genet *32*, 751-761.
Cruickshanks, H.A., McBryan, T., Nelson, D.M., Vanderkraats, N.D., Shah, P.P., van Tuyn, J., Singh Rai, T., Brock, C., Donahue, G., Dunican, D.S., *et al*. (2013). Senescent cells harbour features of the cancer epigenome. Nat Cell Biol *15*, 1495-1506.
d'Adda di Fagagna, F. (2008). Living on a break: cellular senescence as a DNA-damage response. Nat Rev Cancer *8*, 512-522.
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.
De Cecco, M., Criscione, S.W., Peckham, E.J., Hillenmeyer, S., Hamm, E.A., Manivannan, J., Peterson, A.L., Kreiling, J.A., Neretti, N., and Sedivy, J.M. (2013). Genomes of replicatively senescent cells undergo global epigenetic changes leading to gene silencing and activation of transposable elements. Aging Cell *12*, 247-256.
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.
de Lange, T. (2018). Shelterin-Mediated Telomere Protection. Annu Rev Genet *52*, 223-247.

Demaria, M., Ohtani, N., Youssef, S.A., Rodier, F., Toussaint, W., Mitchell, J.R., Laberge, R.M., Vijg, J., Van Steeg, H., Dolle, M.E., *et al*. (2014). An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. Dev Cell *31*, 722-733.

Deschenes-Simard, X., Gaumont-Leclerc, M.F., Bourdeau, V., Lessard, F., Moiseeva, O., Forest, V., Igelmann, S., Mallette, F.A., Saba-El-Leil, M.K., Meloche, S., *et al*. (2013). Tumor suppressor activity of the ERK/MAPK pathway by promoting selective protein degradation. Genes Dev *27*, 900-915.

Di Micco, R., Sulli, G., Dobreva, M., Liontos, M., Botrugno, O.A., Gargiulo, G., dal Zuffo, R., Matti, V., d'Ario, G., Montani, E., *et al*. (2011). Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. Nat Cell Biol *13*, 292-302.

Di Mitri, D., Toso, A., Chen, J.J., Sarti, M., Pinton, S., Jost, T.R., D'Antuono, R., Montani, E., Garcia-Escudero, R., Guccini, I., *et al*. (2014). Tumour-infiltrating Gr-1+ myeloid cells antagonize senescence in cancer. Nature *515*, 134-137.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., *et al*. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A *92*, 9363-9367.

Dou, Z., Xu, C., Donahue, G., Shimi, T., Pan, J.A., Zhu, J., Ivanov, A., Capell, B.C., Drake, A.M., Shah, P.P., *et al*. (2015). Autophagy mediates degradation of nuclear lamina. Nature *527*, 105-109.

Eggert, T., Wolter, K., Ji, J., Ma, C., Yevsa, T., Klotz, S., Medina-Echeverz, J., Longerich, T., Forgues, M., Reisinger, F., *et al*. (2016). Distinct Functions of Senescence-Associated Immune Responses in Liver Tumor Surveillance and Tumor Progression. Cancer Cell *30*, 533-547.

Eisner, V., Picard, M., and Hajnoczky, G. (2018). Mitochondrial dynamics in adaptive and maladaptive cellular stress responses. Nat Cell Biol *20*, 755-765.

Evangelou, K., Lougiakis, N., Rizou, S.V., Kotsinas, A., Kletsas, D., Munoz-Espin, D., Kastrinakis, N.G., Pouli, N., Marakos, P., Townsend, P., *et al*. (2017). Robust, universal biomarker assay to detect senescent cells in biological specimens. Aging Cell *16*, 192-197.

Folgueras, A.R., Freitas-Rodriguez, S., Velasco, G., and Lopez-Otin, C. (2018). Mouse Models to Disentangle the Hallmarks of Human Aging. Circ Res *123*, 905-924.

Franceschi, C., and Campisi, J. (2014). Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. J Gerontol A Biol Sci Med Sci *69 Suppl 1*, S4-9.

Frescas, D., Roux, C.M., Aygun-Sunar, S., Gleiberman, A.S., Krasnov, P., Kurnasov, O.V., Strom, E., Virtuoso, L.P., Wrobel, M., Osterman, A.L., *et al*. (2017). Senescent cells expose and secrete an oxidized form of membrane-bound vimentin as revealed by a natural polyreactive antibody. Proc Natl Acad Sci U S A *114*, E1668-E1677.

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.

Freund, A., Patil, C.K., and Campisi, J. (2011). p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. EMBO J *30*, 1536-1548.

Fuhrmann-Stroissnigg, H., Ling, Y.Y., Zhao, J., McGowan, S.J., Zhu, Y., Brooks, R.W., Grassi, D., Gregg, S.Q., Stripay, J.L., Dorronsoro, A., *et al*. (2017). Identification of HSP90 inhibitors as a novel class of senolytics. Nat Commun *8*, 422.

Funayama, R., Saito, M., Tanobe, H., and Ishikawa, F. (2006). Loss of linker histone H1 in cellular senescence. J Cell Biol *175*, 869-880.

Galanos, P., Vougas, K., Walter, D., Polyzos, A., Maya-Mendoza, A., Haagensen, E.J., Kokkalis, A., Roumelioti, F.M., Gagos, S., Tzetis, M., *et al*. (2016). Chronic p53-independent p21 expression causes genomic instability by deregulating replication licensing. Nat Cell Biol *18*, 777-789.

Georgakopoulou, E.A., Tsimaratou, K., Evangelou, K., Fernandez Marcos, P.J., Zoumpourlis, V., Trougakos, I.P., Kletsas, D., Bartek, J., Serrano, M., and Gorgoulis, V.G. (2013). Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence. A method applicable in cryo-preserved and archival tissues. Aging *5*, 37-50.

Gorgoulis, V.G., and Halazonetis, T.D. (2010). Oncogene-induced senescence: the bright and dark side of the response. Curr Opin Cell Biol *22*, 816-827.

Gorgoulis, V.G., Pefani, D.E., Pateras, I.S., and Trougakos, I.P. (2018). Integrating the DNA damage and protein stress responses during cancer development and treatment. J Pathol *246*, 12-40.

Halazonetis, T.D., Gorgoulis, V.G., and Bartek, J. (2008). An oncogene-induced DNA damage model for cancer development. Science *319*, 1352-1355.

Harvati, K., Roding, C., Bosman, A.M., Karakostis, F.A., Grun, R., Stringer, C., Karkanas, P., Thompson, N.C., Koutoulidis, V., Moulopoulos, L.A., *et al*. (2019). Apidima Cave fossils provide earliest evidence of Homo sapiens in Eurasia. Nature *571*, 500-504.

Haugstetter, A.M., Loddenkemper, C., Lenze, D., Grone, J., Standfuss, C., Petersen, I., Dorken, B., and Schmitt, C.A. (2010). Cellular senescence predicts treatment outcome in metastasised colorectal cancer. Br J Cancer *103*, 505-509.

Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. Exp Cell Res *25*, 585-621.

He, S., and Sharpless, N.E. (2017). Senescence in Health and Disease. Cell *169*, 1000-1011.

Hermeking, H. (2010). The miR-34 family in cancer and apoptosis. Cell Death Differ *17*, 193-199.

Hernandez-Segura, A., de Jong, T.V., Melov, S., Guryev, V., Campisi, J., and Demaria, M. (2017). Unmasking Transcriptional Heterogeneity in Senescent Cells. Current biology : CB *27*, 2652-2660.e2654.

Hernandez-Segura, A., Nehme, J., and Demaria, M. (2018). Hallmarks of Cellular Senescence. Trends Cell Biol.

Hoare, M., Ito, Y., Kang, T.W., Weekes, M.P., Matheson, N.J., Patten, D.A., Shetty, S., Parry, A.J., Menon, S., Salama, R., *et al*. (2016). NOTCH1 mediates a switch between two distinct secretomes during senescence. Nat Cell Biol *18*, 979-992.

Hohn, A., Weber, D., Jung, T., Ott, C., Hugo, M., Kochlik, B., Kehm, R., Konig, J., Grune, T., and Castro, J.P. (2017). Happily (n)ever after: Aging in the context of oxidative stress, proteostasis loss and cellular senescence. Redox Biol *11*, 482-501.

Hu, W., Chan, C.S., Wu, R., Zhang, C., Sun, Y., Song, J.S., Tang, L.H., Levine, A.J., and Feng, Z. (2010). Negative regulation of tumor suppressor p53 by microRNA miR-504. Mol Cell *38*, 689-699.

Hu, W.L., Jin, L., Xu, A., Wang, Y.F., Thorne, R.F., Zhang, X.D., and Wu, M. (2018). GUARDIN is a p53-responsive long non-coding RNA that is essential for genomic stability. Nat Cell Biol *20*, 492-502.

Ito, Y., Hoare, M., and Narita, M. (2017). Spatial and Temporal Control of Senescence. Trends Cell Biol *27*, 820-832.

Ivanov, A., Pawlikowski, J., Manoharan, I., van Tuyn, J., Nelson, D.M., Rai, T.S., Shah, P.P., Hewitt, G., Korolchuk, V.I., Passos, J.F., *et al*. (2013). Lysosome-mediated processing of chromatin in senescence. J Cell Biol *202*, 129-143.
James, E.L., Michalek, R.D., Pitiyage, G.N., de Castro, A.M., Vignola, K.S., Jones, J., Mohney, R.P., Karoly, E.D., Prime, S.S., and Parkinson, E.K. (2015). Senescent Human Fibroblasts Show Increased Glycolysis and Redox Homeostasis with Extracellular Metabolomes That Overlap with Those of Irreparable DNA Damage, Aging, and Disease. Journal of Proteome Research *14*, 1854-1871.
Jiang, P., Du, W., Mancuso, A., Wellen, K.E., and Yang, X. (2013). Reciprocal regulation of p53 and malic enzymes modulates metabolism and senescence. Nature *493*, 689-693.
Jurk, D., Wang, C., Miwa, S., Maddick, M., Korolchuk, V., Tsolou, A., Gonos, E.S., Thrasivoulou, C., Jill Saffrey, M., Cameron, K., *et al*. (2012). Postmitotic neurons develop a p21-dependent senescence-like phenotype driven by a DNA damage response. Aging Cell *11*, 996-1004.
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.
Kang, C., Xu, Q., Martin, T.D., Li, M.Z., Demaria, M., Aron, L., Lu, T., Yankner, B.A., Campisi, J., and Elledge, S.J. (2015). The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4. Science *349*, aaa5612.
Kaplon, J., Zheng, L., Meissl, K., Chaneton, B., Selivanov, V.A., Mackay, G., van der Burg, S.H., Verdegaal, E.M.E., Cascante, M., Shlomi, T., *et al*. (2013). A key role for mitochondrial gatekeeper pyruvate dehydrogenase in oncogene-induced senescence. Nature *498*, 109-112.
Karisch, R., Fernandez, M., Taylor, P., Virtanen, C., St-Germain, J.R., Jin, L.L., Harris, I.S., Mori, J., Mak, T.W., Senis, Y.A., *et al*. (2011). Global proteomic assessment of the classical protein-tyrosine phosphatome and "Redoxome". Cell *146*, 826-840.
Kaushik, S., and Cuervo, A.M. (2015). Proteostasis and aging. Nat Med *21*, 1406-1415.
Kennedy, A.L., McBryan, T., Enders, G.H., Johnson, F.B., Zhang, R., and Adams, P.D. (2010). Senescent mouse cells fail to overtly regulate the HIRA histone chaperone and do not form robust Senescence Associated Heterochromatin Foci. Cell Div *5*, 16.
Kim, K.M., Noh, J.H., Bodogai, M., Martindale, J.L., Yang, X., Indig, F.E., Basu, S.K., Ohnuma, K., Morimoto, C., Johnson, P.F., *et al*. (2017). Identification of senescent cell surface targetable protein DPP4. Genes Dev *31*, 1529-1534.
Korolchuk, V.I., Miwa, S., Carroll, B., and von Zglinicki, T. (2017). Mitochondria in Cell Senescence: Is Mitophagy the Weakest Link? EBioMedicine *21*, 7-13.
Krizhanovsky, V., Xue, W., Zender, L., Yon, M., Hernando, E., and Lowe, S.W. (2008a). Implications of cellular senescence in tissue damage response, tumor suppression, and stem cell biology. Cold Spring Harb Symp Quant Biol *73*, 513-522.
Krizhanovsky, V., Yon, M., Dickins, R.A., Hearn, S., Simon, J., Miething, C., Yee, H., Zender, L., and Lowe, S.W. (2008b). Senescence of activated stellate cells limits liver fibrosis. Cell *134*, 657-667.
Kuilman, T., Michaloglou, C., Mooi, W.J., and Peeper, D.S. (2010). The essence of senescence. Genes Dev *24*, 2463-2479.
Kuilman, T., and Peeper, D.S. (2009). Senescence-messaging secretome: SMS-ing cellular stress. Nat Rev Cancer *9*, 81-94.

Kuppers, R., Engert, A., and Hansmann, M.L. (2012). Hodgkin lymphoma. J Clin Invest *122*, 3439-3447.
Lal, A., Kim, H.H., Abdelmohsen, K., Kuwano, Y., Pullmann, R., Jr., Srikantan, S., Subrahmanyam, R., Martindale, J.L., Yang, X., Ahmed, F., *et al*. (2008). p16(INK4a) translation suppressed by miR-24. PLoS One *3*, e1864.
Lee, S., and Schmitt, C.A. (2019). The dynamic nature of senescence in cancer. Nat Cell Biol *21*, 94-101.
Lessard, F., Igelmann, S., Trahan, C., Huot, G., Saint-Germain, E., Mignacca, L., Del Toro, N., Lopes-Paciencia, S., Le Calve, B., Montero, M., *et al*. (2018). Senescence-associated ribosome biogenesis defects contributes to cell cycle arrest through the Rb pathway. Nat Cell Biol *20*, 789-799.
Li, T., and Chen, Z.J. (2018). The cGAS-cGAMP-STING pathway connects DNA damage to inflammation, senescence, and cancer. J Exp Med *215*, 1287-1299.
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 U S A.
Llanos, S., Megias, D., Blanco-Aparicio, C., Hernandez-Encinas, E., Rovira, M., Pietrocola, F., and Serrano, M. (2019). Lysosomal trapping of palbociclib and its functional implications. Oncogene.
Maeda, M., Scaglia, N., and Igal, R.A. (2009). Regulation of fatty acid synthesis and Δ9-desaturation in senescence of human fibroblasts. Life Sciences *84*, 119-124.
McHugh, D., and Gil, J. (2018). Senescence and aging: Causes, consequences, and therapeutic avenues. J Cell Biol *217*, 65-77.
Milanovic, M., Fan, D.N.Y., Belenki, D., Dabritz, J.H.M., Zhao, Z., Yu, Y., Dorr, J.R., Dimitrova, L., Lenze, D., Monteiro Barbosa, I.A., *et al*. (2018). Senescence-associated reprogramming promotes cancer stemness. Nature *553*, 96-100.
Miwa, S., Jow, H., Baty, K., Johnson, A., Czapiewski, R., Saretzki, G., Treumann, A., and von Zglinicki, T. (2014). Low abundance of the matrix arm of complex I in mitochondria predicts longevity in mice. Nat Commun *5*, 3837.
Moiseeva, O., Bourdeau, V., Roux, A., Deschenes-Simard, X., and Ferbeyre, G. (2009). Mitochondrial Dysfunction Contributes to Oncogene-Induced Senescence. Mol Cell Biol *29*, 4495-4507.
Mosteiro, L., Pantoja, C., Alcazar, N., Marion, R.M., Chondronasiou, D., Rovira, M., Fernandez-Marcos, P.J., Munoz-Martin, M., Blanco-Aparicio, C., Pastor, J., *et al*. (2016). Tissue damage and senescence provide critical signals for cellular reprogramming in vivo. Science *354*.
Munoz-Espin, D., Canamero, M., Maraver, A., Gomez-Lopez, G., Contreras, J., Murillo-Cuesta, S., Rodriguez-Baeza, A., Varela-Nieto, I., Ruberte, J., Collado, M., *et al*. (2013). Programmed cell senescence during mammalian embryonic development. Cell *155*, 1104-1118.
Munoz-Espin, D., and Serrano, M. (2014). Cellular senescence: from physiology to pathology. Nat Rev Mol Cell Biol *15*, 482-496.
Myrianthopoulos, V., Evangelou, K., Vasileiou, P.V.S., Cooks, T., Vassilakopoulos, T.P., Pangalis, G.A., Kouloukoussa, M., Kittas, C., Georgakilas, A.G., and Gorgoulis, V.G. (2019). Senescence and senotherapeutics: a new field in cancer therapy. Pharmacol Ther *193*, 31-49.

Niwa-Kawakita, M., Ferhi, O., Soilihi, H., Le Bras, M., Lallemand-Breitenbach, V., and de The, H. (2017). PML is a ROS sensor activating p53 upon oxidative stress. J Exp Med *214*, 3197-3206.
Nystrom, T. (2005). Role of oxidative carbonylation in protein quality control and senescence. EMBO J *24*, 1311-1317.
O'Sullivan, R.J., Kubicek, S., Schreiber, S.L., and Karlseder, J. (2010). Reduced histone biosynthesis and chromatin changes arising from a damage signal at telomeres. Nat Struct Mol Biol *17*, 1218-1225.
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. Nature Communications *8*, 15691.
Ogrodnik, M., Salmonowicz, H., and Gladyshev, V.N. (2019a). Integrating cellular senescence with the concept of damage accumulation in aging: Relevance for clearance of senescent cells. Aging Cell *18*, e12841.
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*. (2019b). Obesity-Induced Cellular Senescence Drives Anxiety and Impairs Neurogenesis. Cell Metabolism.
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 U S A *104*, 15034-15039.
Ohtani, N., Yamakoshi, K., Takahashi, A., and Hara, E. (2010). Real-time in vivo imaging of p16gene expression: a new approach to study senescence stress signaling in living animals. Cell Div *5*, 1.
Osorio, F.G., Navarro, C.L., Cadinanos, J., Lopez-Mejia, I.C., Quiros, P.M., Bartoli, C., Rivera, J., Tazi, J., Guzman, G., Varela, I., *et al*. (2011). Splicing-directed therapy in a new mouse model of human accelerated aging. Sci Transl Med *3*, 106ra107.
Overhoff, M.G., Garbe, J.C., Koh, J., Stampfer, M.R., Beach, D.H., and Bishop, C.L. (2014). Cellular senescence mediated by p16INK4A-coupled miRNA pathways. Nucleic Acids Res *42*, 1606-1618.
Panda, A.C., Abdelmohsen, K., and Gorospe, M. (2017). SASP regulation by noncoding RNA. Mech Ageing Dev *168*, 37-43.
Park, J.T., Lee, Y.S., Cho, K.A., and Park, S.C. (2018). Adjustment of the lysosomal-mitochondrial axis for control of cellular senescence. Ageing Res Rev *47*, 176-182.
Park, Y.-Y., Lee, S., Karbowski, M., Neutzner, A., Youle, R.J., and Cho, H. (2010). Loss of MARCH5 mitochondrial E3 ubiquitin ligase induces cellular senescence through dynamin-related protein 1 and mitofusin 1. Journal of Cell Science *123*, 619-626.
Passos, J.F., Nelson, G., Wang, C., Richter, T., Simillion, C., Proctor, C.J., Miwa, S., Olijslagers, S., Hallinan, J., Wipat, A., *et al*. (2010). Feedback between p21 and reactive oxygen production is necessary for cell senescence. Mol Syst Biol *6*, 347.
Passos, J.F., Saretzki, G., Ahmed, S., Nelson, G., Richter, T., Peters, H., Wappler, I., Birkett, M., Harold, G., Schaeuble, K., *et al*. (2007). Mitochondrial Dysfunction Accounts for the Stochastic Heterogeneity In Telomere-Dependent Senescence. PLoS Biology *5*, e110.
Patel, P.L., Suram, A., Mirani, N., Bischof, O., and Herbig, U. (2016). Derepression of hTERT gene expression promotes escape from oncogene-induced cellular senescence. Proc Natl Acad Sci U S A *113*, E5024-5033.

Philipot, D., Guerit, D., Platano, D., Chuchana, P., Olivotto, E., Espinoza, F., Dorandeu, A., Pers, Y.M., Piette, J., Borzi, R.M., *et al*. (2014). p16INK4a and its regulator miR-24 link senescence and chondrocyte terminal differentiation-associated matrix remodeling in osteoarthritis. Arthritis Res Ther *16*, R58.
Quijano, C., Cao, L., Fergusson, M.M., Romero, H., Liu, J., Gutkind, S., Rovira, I.I., Mohney, R.P., Karoly, E.D., and Finkel, T. (2012). Oncogene-induced senescence results in marked metabolic and bioenergetic alterations. Cell Cycle *11*, 1383-1392.
Rai, T.S., Cole, J.J., Nelson, D.M., Dikovskaya, D., Faller, W.J., Vizioli, M.G., Hewitt, R.N., Anannya, O., McBryan, T., Manoharan, I., *et al*. (2014). HIRA orchestrates a dynamic chromatin landscape in senescence and is required for suppression of neoplasia. Genes Dev *28*, 2712-2725.
Robbins, E., Levine, E.M., and Eagle, H. (1970). Morphologic changes accompanying senescence of cultured human diploid cells. J Exp Med *131*, 1211-1222.
Robinson, A.R., Yousefzadeh, M.J., Rozgaja, T.A., Wang, J., Li, X., Tilstra, J.S., Feldman, C.H., Gregg, S.Q., Johnson, C.H., Skoda, E.M., *et al*. (2018). Spontaneous DNA damage to the nuclear genome promotes senescence, redox imbalance and aging. Redox Biol *17*, 259-273.
Rodier, F., and Campisi, J. (2011). Four faces of cellular senescence. J Cell Biol *192*, 547-556.
Rodier, F., Munoz, D.P., Teachenor, R., Chu, V., Le, O., Bhaumik, D., Coppe, J.P., Campeau, E., Beausejour, C.M., Kim, S.H., *et al*. (2011). DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. J Cell Sci *124*, 68-81.
Sagiv, A., Biran, A., Yon, M., Simon, J., Lowe, S.W., and Krizhanovsky, V. (2013). Granule exocytosis mediates immune surveillance of senescent cells. Oncogene *32*, 1971-1977.
Sagiv, A., Burton, D.G., Moshayev, Z., Vadai, E., Wensveen, F., Ben-Dor, S., Golani, O., Polic, B., and Krizhanovsky, V. (2016). NKG2D ligands mediate immunosurveillance of senescent cells. Aging (Albany NY) *8*, 328-344.
Salama, R., Sadaie, M., Hoare, M., and Narita, M. (2014). Cellular senescence and its effector programs. Genes Dev *28*, 99-114.
Saleh, T., Tyutyunyk-Massey, L., and Gewirtz, D.A. (2019). Tumor Cell Escape from Therapy-Induced Senescence as a Model of Disease Recurrence after Dormancy. Cancer Res *79*, 1044-1046.
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.
Settembre, C., and Ballabio, A. (2014). Lysosomal adaptation: how the lysosome responds to external cues. Cold Spring Harb Perspect Biol *6*.
Shah, P.P., Donahue, G., Otte, G.L., Capell, B.C., Nelson, D.M., Cao, K., Aggarwala, V., Cruickshanks, H.A., Rai, T.S., McBryan, T., *et al*. (2013). Lamin B1 depletion in senescent cells triggers large-scale changes in gene expression and the chromatin landscape. Genes Dev *27*, 1787-1799.
Sharpless, N.E., and Sherr, C.J. (2015). Forging a signature of in vivo senescence. Nat Rev Cancer *15*, 397-408.
Shay, J.W., and Wright, W.E. (2019). Telomeres and telomerase: three decades of progress. Nat Rev Genet *20*, 299-309.
Shimi, T., Butin-Israeli, V., Adam, S.A., Hamanaka, R.B., Goldman, A.E., Lucas, C.A., Shumaker, D.K., Kosak, S.T., Chandel, N.S., and Goldman, R.D. (2011). The role of nuclear lamin B1 in cell proliferation and senescence. Genes Dev *25*, 2579-2593.

Srivastava, S. (2017). The Mitochondrial Basis of Aging and Age-Related Disorders. Genes (Basel) *8*.
Storer, M., Mas, A., Robert-Moreno, A., Pecoraro, M., Ortells, M.C., Di Giacomo, V., Yosef, R., Pilpel, N., Krizhanovsky, V., Sharpe, J., *et al*. (2013). Senescence is a developmental mechanism that contributes to embryonic growth and patterning. Cell *155*, 1119-1130.
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.
Takahashi, A., Ohtani, N., Yamakoshi, K., Iida, S., Tahara, H., Nakayama, K., Nakayama, K.I., Ide, T., Saya, H., and Hara, E. (2006). Mitogenic signalling and the p16INK4a-Rb pathway cooperate to enforce irreversible cellular senescence. Nat Cell Biol *8*, 1291-1297.
Takasugi, M., Okada, R., Takahashi, A., Virya Chen, D., Watanabe, S., and Hara, E. (2017). Small extracellular vesicles secreted from senescent cells promote cancer cell proliferation through EphA2. Nat Commun *8*, 15729.
Takeda, T., Hosokawa, M., and Higuchi, K. (1997). Senescence-accelerated mouse (SAM): a novel murine model of senescence. Exp Gerontol *32*, 105-109.
Terlecki-Zaniewicz, L., Lammermann, I., Latreille, J., Bobbili, M.R., Pils, V., Schosserer, M., Weinmullner, 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.
van Deursen, J.M. (2019). Senolytic therapies for healthy longevity. Science *364*, 636-637.
Vernier, M., Bourdeau, V., Gaumont-Leclerc, M.F., Moiseeva, O., Begin, V., Saad, F., Mes-Masson, A.M., and Ferbeyre, G. (2011). Regulation of E2Fs and senescence by PML nuclear bodies. Genes Dev *25*, 41-50.
Vougas, K., Sakellaropoulos, T., Kotsinas, A., Foukas, G.P., Ntargaras, A., Koinis, F., Polyzos, A., Myrianthopoulos, V., Zhou, H., Narang, S., *et al*. (2019). Machine learning and data mining frameworks for predicting drug response in cancer: An overview and a novel in silico screening process based on association rule mining. Pharmacol Ther, 107395.
Wang, J., Clauson, C.L., Robbins, P.D., Niedernhofer, L.J., and Wang, Y. (2012). The oxidative DNA lesions 8,5'-cyclopurines accumulate with aging in a tissue-specific manner. Aging Cell *11*, 714-716.
Wiley, C.D., Flynn, J.M., Morrissey, C., Lebofsky, R., Shuga, J., Dong, X., Unger, M.A., Vijg, J., Melov, S., and Campisi, J. (2017a). Analysis of individual cells identifies cell-to-cell variability following induction of cellular senescence. Aging Cell.
Wiley, C.D., Flynn, J.M., Morrissey, C., Lebofsky, R., Shuga, J., Dong, X., Unger, M.A., Vijg, J., Melov, S., and Campisi, J. (2017b). Analysis of individual cells identifies cell-to-cell variability following induction of cellular senescence. Aging Cell *16*, 1043-1050.
Wiley, C.D., Velarde, M.C., Lecot, P., Liu, S., Sarnoski, E.A., Freund, A., Shirakawa, K., Lim, H.W., Davis, S.S., Ramanathan, A., *et al*. (2016). Mitochondrial Dysfunction Induces Senescence with a Distinct Secretory Phenotype. Cell Metab *23*, 303-314.
Xiao, J., Lin, H., Luo, X., Luo, X., and Wang, Z. (2011). miR-605 joins p53 network to form a p53:miR-605:Mdm2 positive feedback loop in response to stress. EMBO J *30*, 524-532.
Xie, W., Kagiampakis, I., Pan, L., Zhang, Y.W., Murphy, L., Tao, Y., Kong, X., Kang, B., Xia, L., Carvalho, F.L.F., *et al*. (2018). DNA Methylation Patterns Separate Senescence from Transformation Potential and Indicate Cancer Risk. Cancer Cell *33*, 309-321 e305.

Xu, M., Pirtskhalava, T., Farr, J.N., Weigand, B.M., Palmer, A.K., Weivoda, M.M., Inman, C.L., Ogrodnik, M.B., Hachfeld, C.M., Fraser, D.G., *et al*. (2018). Senolytics improve physical function and increase lifespan in old age. Nat Med *24*, 1246-1256.

Xu, S., Wu, W., Huang, H., Huang, R., Xie, L., Su, A., Liu, S., Zheng, R., Yuan, Y., Zheng, H.L., *et al*. (2019). The p53/miRNAs/Ccna2 pathway serves as a novel regulator of cellular senescence: Complement of the canonical p53/p21 pathway. Aging Cell *18*, e12918.

Yosef, R., Pilpel, N., Tokarsky-Amiel, R., Biran, A., Ovadya, Y., Cohen, S., Vadai, E., Dassa, L., Shahar, E., Condiotti, R., *et al*. (2016). Directed elimination of senescent cells by inhibition of BCL-W and BCL-XL. Nature communications *7*, 11190.

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.

Zhang, Y., Unnikrishnan, A., Deepa, S.S., Liu, Y., Li, Y., Ikeno, Y., Sosnowska, D., Van Remmen, H., and Richardson, A. (2017). A new role for oxidative stress in aging: The accelerated aging phenotype in Sod1(-/)(-) mice is correlated to increased cellular senescence. Redox Biol *11*, 30-37.

Zhu, Y., Tchkonia, T., Pirtskhalava, T., Gower, A.C., Ding, H., Giorgadze, N., Palmer, A.K., Ikeno, Y., Hubbard, G.B., Lenburg, M., *et al*. (2015). The Achilles' heel of senescent cells: from transcriptome to senolytic drugs. Aging Cell *14*, 644-658.

Zirkel, A., Nikolic, M., Sofiadis, K., Mallm, J.P., Brackley, C.A., Gothe, H., Drechsel, O., Becker, C., Altmuller, J., Josipovic, N., *et al*. (2018). HMGB2 Loss upon Senescence Entry Disrupts Genomic Organization and Induces CTCF Clustering across Cell Types. Mol Cell *70*, 730-744 e736.

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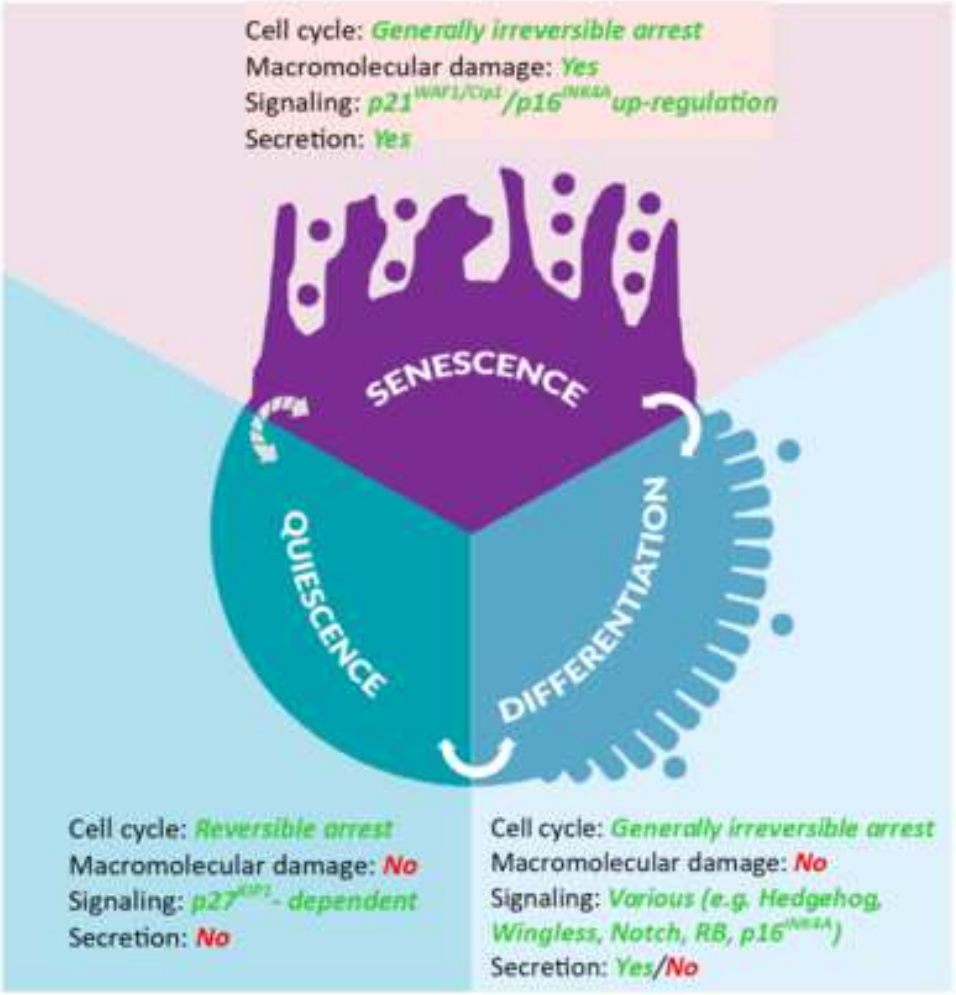

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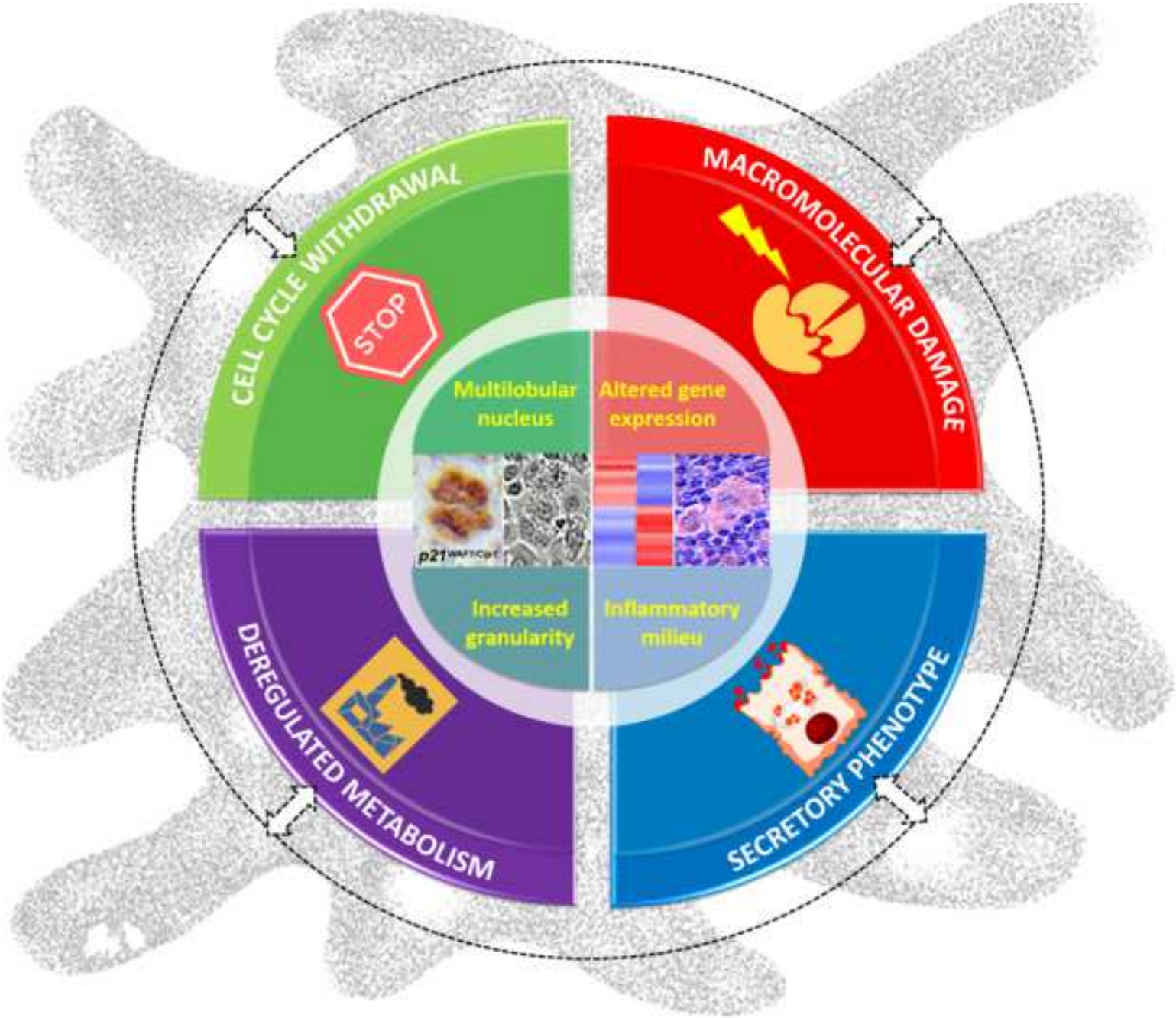

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