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Cellular senescence: all roads lead to mitochondria

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Abstract

Senescence is a multi-functional cell fate, characterized by an irreversible cell-cycle arrest and a pro-inflammatory phenotype, commonly known as the Senescence-Associated secretory Phenotype (SASP). Emerging evidence indicates that accumulation of senescent cells in multiple tissues, drives tissue dysfunction and several age-related conditions. This has spurred the academic community and industry to identify new therapeutic interventions targeting this process.

Mitochondrial dysfunction is an often-unappreciated hallmark of cellular senescence which plays important roles not only in the senescence growth arrest but also in the development of the SASP and resistance to cell-death. Here, we review the evidence that supports a role for mitochondria in the development of senescence and describe the underlying mechanisms. Finally, we propose that a detailed road map of mitochondrial biology in senescence will be crucial to guide the future development of senotherapies.

Graphical Abstract

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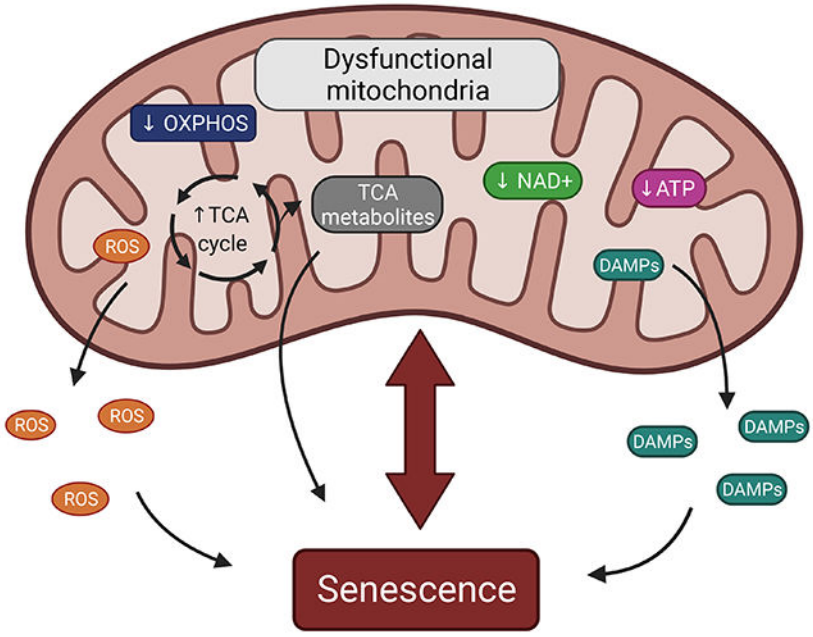

Mitochondria play a central role in the development of cellular senescence. Senescence is characterized by several mitochondrial functional changes such as a decrease in OXPHOS, reduced levels of NAD+ and ATP and accumulation of TCA cycle metabolites, DAMPs, and ROS. Here, we provide an overview of the recent findings demonstrating how these mitochondrial changes can contribute to the senescence-associated growth arrest and the SASP.

Keywords

Mitochondria; senescence; SASP; aging

1- Introduction

Cellular senescence is a process that imposes a permanent proliferative arrest on cells in response to various stressors. It is thought to have evolved as a tumor suppressive mechanism; however, recent data indicates that it may play other important physiological roles in embryonic development, wound healing, and tissue repair [1]. It is a complex phenotype, involving kinetic alterations in virtually all aspects of the cell's biology. These manifestations of the senescent phenotype are highly dependent on the cell-type, inducing stimuli and physiological context. This phenotypic and temporal heterogeneity makes it nearly impossible for the scientific community to find specific markers that are universal to all types of senescent cells [2].

While initially thought to be a process that exclusively occurs in mitotic cells, recent data indicates that many post-mitotic cells can activate a senescent program and exhibit many senescent markers common to proliferative cells [3].

Multiple studies have shown that senescent cells accumulate during aging in multiple tissues and in age-related diseases. To investigate whether senescence plays a causal role in aging, mouse models were developed which allow the specific elimination of senescent cells [4,5]. These models allowed the demonstration that clearance of senescent cells alleviates the onset of several pathologies in aging mice, supporting the hypothesis that accumulation of senescent cells is a driver of age-associated tissue dysfunction [6-9].

Senescent cells can impact on age-related tissue dysfunction in a cell-autonomous fashion by limiting the regenerative potential of tissues. However, senescent cells can secrete high levels of pro-inflammatory cytokines, chemokines, and extra-cellular matrix degrading proteins, which are collectively known as the senescence-associated secretory phenotype (SASP) [10]. The discovery of the SASP proposed a possible mechanism to explain why senescent cells, which are thought to accumulate in relatively low numbers in tissues during aging, have such large detrimental effects on tissue function and induce systemic effects. The SASP, when chronic, can transmit senescence to otherwise healthy cells [11,12], it can be pro-fibrotic [9,13], it can recruit and activate immune cells contributing to chronic inflammation [14,15] and can also impair the function of stem and progenitor cells [16]. Another important characteristic of senescence is their resistance to cell-death, thought to the result of up-regulation of several cell survival mechanisms, including anti-apoptotic pathways [7,17,18]. This combined with an age-dependent decline in the ability of the immune system to clear senescent cells [19], may contribute to their persistence in tissues during aging.

Senescence can be induced by several stimuli such as telomere dysfunction, DNA damaging agents, oncogene activation, mitochondrial dysfunction, chromatin modifications amongst others. Two main pathways are thought to be engaged in the senescence-associated growth arrest: the p53/p21 pathway, mostly triggered by a DNA damage response which results in the stabilization of p53 and upregulation of its transcriptional target p21 (a cyclin-dependent kinase inhibitor) and the $p16^{Ink4a}$ pathway which can act as a second barrier by preventing CDK4- and CDK6-mediated inactivation of RB (retinoblastoma protein) to block cell cycle progression [1].

Senescence is also characterized by dramatic changes in mitochondrial mass, dynamics, structure, and function. These changes are tightly interconnected to other features of cellular senescence and play an important role in both the cell-cycle arrest and the regulation of the SASP. Here we will review the literature indicating aspects of mitochondrial biology which are altered in senescent cells and how they impact on the senescence phenotype. Finally, we will discuss how targeting mitochondrial function may be a powerful strategy to counteract senescence during aging and age-related disease.

2- Mitochondrial phenotype in senescent cells

2.1 Mitochondrial dynamics, quality control and content in cellular senescence

The textbook representation of a mitochondrion is usually that of a static and isolated organelle. Advances in live-cell microscopy have revealed that mitochondria are in fact highly dynamic organelles, forming a densely interconnected network and frequently

undergoing fusion and fission events. These events are thought to be essential for the maintenance of mitochondrial function, quality control and mitochondrial inheritance [20].

Numerous studies indicate that cellular senescence is characterized by significant changes in mitochondrial dynamics and organization. It is generally believed that damaged mitochondria can fuse with healthy ones to dilute and rearrange the matrix or use fission to separate and subsequently degrade damaged mitochondria. Thus, to ensure healthy cellular function, the balance between fission and fusion must be tightly controlled [21]. Data indicate that senescence is associated with impaired mitochondrial dynamics. In different models of cellular senescence, mitochondria are found to be elongated, enlarged and hyperfused [22-26]. During senescence, an elongated and hyperfused mitochondrial network has been linked with reduced expression of the protein FIS1, a protein which participates in mitochondrial fission by recruiting DRP1 into mitochondria [27](Fig.1). Consistently, knock-down of FIS1 has been shown to lead to increased Reactive Oxygen Species (ROS) production and induce cellular senescence. In contrast, overexpression of FIS1 was shown to prevent mitochondrial elongation and reverse the senescent phenotype [27]. This work suggests that mitochondrial fission plays a protective role against cellular senescence. Consistent with this idea, Park and colleagues demonstrated that depletion of MARCH5, a mitochondrial E3 ubiquitin ligase that regulates mitofusin-1, leads mitochondrial elongation and cellular senescence *via* inhibition of the activity of the fission protein DRP1 [28].

The hyperfused mitochondrial network observed during senescence could be a protective mechanism contributing to apoptosis resistance [29]. In fact, studies demonstrate that senescent cells are protected from both intrinsic and extrinsic pro-apoptotic signals, likely due to the increased expression of anti-apoptotic proteins of the Bcl-2 family, which control the outer mitochondrial membrane permeability and the release of pro-apoptotic molecules such as cytochrome c by inhibiting BAX and BAK macropore formation [17,30]. Apoptosis resistance is recognized as one of the hallmarks of senescence and one of the targets for the development of interventions capable of specifically killing senescent cells, commonly named senolytic [19,31]. Apoptosis is closely linked with mitochondrial dynamics. During apoptosis, DRP1, a protein which regulates mitochondrial fission, has been shown to translocate from the cytosol to mitochondria, resulting in loss of mitochondrial membrane potential and cytochrome c release [32,33]. In contrast, OPA1, a profusion protein, has been shown to protect against apoptosis by preventing cytochrome c release [34]. Finally, decreasing fission by deletion of phosphoglycerate mutase 5 (PGAM5), a phosphatase which normally dephosphorylates Drp1 and induces fission, has been shown to induce cellular senescence *in vitro* and *in vivo*. This deletion leads to senescence by an increase of mTOR pathway and IRF/IFNβ signaling [35].

However, whether mitochondrial hyperfusion contributes to protection against apoptosis in senescent cells remains to be investigated.

Another mechanism that could explain mitochondrial phenotypes exhibited by senescent cells is impaired mitophagy (Fig.1). Mitophagy refers to the selective autophagic degradation of damaged or dysfunctional mitochondria and plays an important role in mitochondria quality control and homeostasis. Mitochondria are enclosed by

autophagosomes which fuse with lysosomes where they are eventually degraded [36]. Mitophagy is a complex process involving the activation of numerous pathways under different conditions. One of the most studied is the PINK1-Parkin pathway. When mitochondria are damaged or dysfunctional, the sentinel protein PTEN-induced kinase 1 (PINK1) accumulates in their outer membrane. This accumulation leads to the recruitment of the E3 ubiquitin ligase Parkin from the cytosol which then ubiquitinates numerous proteins localized on the outer mitochondrial membrane. This ubiquitination mediates the autophagosome formation and the lysosomal elimination of dysfunctional mitochondria [37,38]. Several studies indicate that senescent cells have impaired mitophagy and importantly that defects in mitophagy may play a role in the induction of cellular senescence [39-41]. For example, Ahmad and colleagues showed that exposure to cigarette smoke (CS), a stressor known to induce cellular senescence [42], leads to reduced Parkin translocation to damage mitochondria. This results in perinuclear accumulation of damaged mitochondria and is associated with the induction of senescence [43]. Using a similar model of CS-induced senescence, Araya and colleagues showed that while Parkin knock-down resulted in impaired mitophagy and induction of senescence, its overexpression was sufficient to induce mitophagy and attenuate cellular senescence [44]. Mechanistically, it has been suggested that during senescence, accumulation of the protein p53 in the cytosol leads to the sequestration of Parkin, inhibiting its translocation to the mitochondria. This process has been shown to maintain or reinforce the senescence phenotype [43,45,46].

Another important feature of cellular senescence is an increase in mitochondrial content (Fig.1). This has been shown to occur regardless of the inducing stimuli, whether senescence is induced by oncogenes [47], genotoxic stress [48], oxidative stress or extensive replication [24,25]. While the majority of these observations were performed in fibroblasts, increased mitochondrial content during senescence has also been shown in other cell-types such as adipose-derived mesenchymal stromal cells [49,50], cardiomyoblasts [46], hepatocytes [51], pancreatic β-cells [52] and others. This phenotype is however not universal to all types of cells. For instance, T CD8 cells display lower mitochondrial content compared to T CD4, which is associated with a faster acquisition of an immuno-senescent profile [53]. Cardiomyocytes isolated from aged mice show increased expression of senescence-associated markers, but decreased mRNA expression of mitochondrial genes [9]. Thus, similarly to other features of senescence, data supports that the senescence-associated mitochondrial phenotype is cell-type specific.

2.2. Mitochondrial metabolism and oxidative phosphorylation during cellular senescence

Mitochondria are the cellular main source of energy through the generation of ATP. To produce ATP, mitochondria use sugars, amino acids, and fatty acids as fuels. These nutrients are degraded and metabolized by the tricarboxylic acid (TCA) cycle, which leads to the production of 3 molecules of NADH and 1 molecule of FADH2 [54]. These byproducts are used secondarily by the electron transport chain (ETC) to create an electrochemical gradient characterized by an accumulation of protons (H^+) in the mitochondrial intermembrane space, which will ultimately result in the generation of ATP by the enzyme ATP synthase. The process of ATP production requires the presence of oxygen and is generally

called oxidative phosphorylation (OXPHOS). These two cell machineries, TCA cycle and OXPHOS are therefore tightly connected [55].

Senescent cells have been shown to be less efficient in producing ATP [56,57], leading to a bioenergetic imbalance with an increase in AMP/ATP ratio. This drop of ATP production could be explained, in senescent cells, by a decrease in the efficiency of OXPHOS (characterized by less H^+ in the intermembrane space) associated with a reduction of mitochondrial membrane potential (Fig.1). Consistently, a decrease in mitochondrial membrane potential has been described in the context of replicative senescence [25,58], stress-induced senescence [48] and oncogene-induced senescence (OIS) [47]. This decrease in mitochondrial membrane potential was associated with increased production of ROS, suggesting that mitochondria in senescent cells are dysfunctional [25,59]. Interestingly, mitochondrial uncoupling using carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) was show to induce premature senescence in human fibroblasts [60].

As previously mentioned, many types of senescent cells show increased mitochondrial mass and this is reflected by a significantly higher rate of oxygen consumption *per* cell. However, despite respiring more due to having more mitochondria, data indicates that senescent cells experience a shift towards glycolysis [39]. This phenomenon was already described several decades ago when Bittles and Harper reported that replicatively senescent cells exhibit a highly significant increase in glucose uptake and a corresponding increase in lactate production [61]. More recently, similar observations were made using more sophisticated analyses such as metabolomics or measures of mitochondrial oxygen flux and extracellular acidification rates in different senescent cell-types [39,62,63]. It should be noted however that this metabolic shift is not universal for all type of cells and senescent stimuli. For instance, in the context of oncogene induced senescence (OIS), no changes in glucose uptake were observed, but there was an increase in oxygen consumption rate and TCA activity, linked to an increase in pyruvate dehydrogenase activity [64]. Additionally, enhanced glycolysis via overexpression of glycolytic enzymes phosphoglycerate mutase or glucosephosphate isomerase was shown to bypass both replicative and oncogene-induced senescence [65]. Furthermore, replicative senescent human mammary epithelial cells did not show an overall glycolytic shift, with no changes in glucose consumption or lactate secretion [57]. This highlights the need to avoid overgeneralizations when it comes to metabolism during senescence, as it may be vary according to cell-type and physiological context.

Several studies have shown that senescent cells show an increase in TCA cycle metabolites [62,64,66,67] (Fig.1) and the TCA cycle and OXPHOS are tightly connected and in constant feedback with each other as reviewed here [55].

Moreover, different TCA metabolites have been shown to play numerous roles in diverse functions such as lymphangiogenesis, stem cell function, immune modulation, tumorigenesis, thermogenesis but also in chromatin modifications, DNA methylation and post-translational protein modifications [55]. Given that senescence is characterized by epigenetic and chromatin modifications, it is likely that modulation of these metabolites could have an impact on the senescence phenotype.

3- Mitochondria as inducers of senescence

As highlighted before, mitochondria are complex organelles where multiple biochemical reactions occur, and which play a role in various cellular functions. While previous data indicates that multiple aspects of mitochondrial biology are altered in cellular senescence, it is challenging to ascertain which factors, if any, contribute causally to the induction of the senescent phenotype.

To begin to address this question, our group first proposed a proof-of-principle experiment, in which we generated human fibroblasts which contained no mitochondria. This was based on prior observations that damaged mitochondria can be removed via the PINK-1/Parkin mitophagy pathway [36]. We found that ectopic expression of the ubiquitin E3 ligase Parkin combined with short-term treatment with mitochondrial uncoupler FCCP, leads to widespread mitophagy resulting in the generation of cells with virtually no mitochondria [68]. Using this tool, we found that removing mitochondria in different models of cellular senescence, attenuated their pro-inflammatory phenotype, however, cells did not resume the cell-cycle [51]. This work suggested that the SASP is dependent on mitochondria, however, the underlying mechanisms are not yet elucidated.

In this section of the review, we will summarize what is known about potential mechanisms by which mitochondria may contribute to the SASP during senescence. We will also review evidence that changes in mitochondria may contribute to the senescence-associated cell-cycle arrest.

3.1 Mitochondrial ROS as a driver of senescence

ROS is a general term used to describe several reactive molecules which are partially reduced metabolites of oxygen metabolism. These molecules can be generated by various enzymatic and metabolic reactions, including mitochondrial respiration [69]. Indeed, mitochondria have been found to be one of the major producers of ROS in the cell, notably during mitochondrial respiration where the leakage of electrons along the electron transport chain (ETC) produces the free radical superoxide anion (O2-•) [70,71]. Superoxide anions are converted in hydrogen peroxide (H_2O_2) by antioxidant enzyme superoxide dismutase (SOD) and finally transform in H_2O by catalase [72]. H_2O_2 can be then also be partially reduced and converted to another, more toxic free radical, the hydroxyl radical (•OH), by the Fenton reaction [70].

As mentioned above, senescent cells contain dysfunctional mitochondria, and several studies indicate that they produce excessive ROS [25,48]. ROS has been shown to contribute to damage to several macromolecules including proteins, lipids, and DNA (Fig.2). Importantly in the context of cellular senescence, mitochondrial ROS has been shown to induce DNA damage and accelerate telomere-induced senescence. Telomeres are genomic regions rich in guanine residues which are particularly susceptible to oxidative modifications and when exposed to mild oxidative stress have been shown to accumulate single-stranded breaks [73]. Upon cell division, these single-stranded breaks, result in accelerated telomere shortening and contribute to cellular senescence [74]. Consistent with a role for ROS in the process,

interventions known to reduce mitochondrial ROS, have been shown to decelerate telomere shortening and extend replicative lifespan [25,75].

Acute exposure to pro-oxidant agents also leads to premature senescence, however, this process was traditionally viewed as occurring independently of telomere shortening. More recent studies have indicated that acute exposure to pro-oxidant or DNA damaging agents can lead to DNA double stranded breaks (DSBs) at telomere regions, which occur independently of telomere length and cannot be rescued by telomerase [76,77]. In contrast to DSBs induced in non-telomeric regions, telomere damage elicits a persistent DDR (DNA Damage Response) which is thought to enforce checkpoints that maintain the senescence cell-cycle arrest. This occurs because telomere-binding proteins, such as TRF2, inhibit DNA repair [76-78] potentially to avoid chromosomal fusions and maintain the linear structure of chromosomes. Thus, as genomes are constantly exposed to endogenous or exogenous damage throughout the life of a cell, when damage occurs at telomeres, it will not be efficiently repaired, resulting in accumulation of telomere damage, and eventually leading to cellular senescence. Consistent with a role for ROS in mechanisms mediating telomere maintenance, recent studies have shown that oxidative modifications at telomeres can accelerate telomere dysfunction [79], impair telomerase activity [80] and disrupt the binding of telomere-associated proteins TRF1 and TRF2 which are important for telomere stability [81].

ROS produced by senescent cells can have both autocrine and paracrine effects (Fig.2). Mitochondrial ROS produced by senescent cells have been shown to contribute to the maintenance of cellular senescence, via a positive self-amplifying loop, which constantly generate DNA damage [48]. ROS produced by senescent cells have also been shown to induce a DNA damage response in neighboring cells via gap junctions [82]. Senescent melanocytes have been shown to induce telomere damage to surrounding keratinocytes via ROS signaling, potentially contributing to epidermal atrophy during aging [83].

Mitochondrial ROS in senescence does not affect solely DNA damage but can also contribute to oxidative modifications to other macromolecules such as proteins and lipids. Recently, Hamon and colleagues have used metabolomic and proteomic approaches to analyze oxidative modifications that occur during replicative senescence. They observed that numerous proteins are found carbonylated, 4-Hydroxynonenal (HNE)-modified (an aldehyde produced during lipid peroxidation) or advance glycation end product (AGE)-modified, with half of them localized to the mitochondria. Interestingly, these oxidized proteins were implicated in metabolic processes such as the tricarboxylic acid (TCA) cycle, oxidative phosphorylation and fatty acid β-oxidation [84]. Lipofuscin, a nondegradable aggregate of oxidized lipids, proteins, oligosaccharides and transition metals which accumulate in lysosomes, has been shown to accumulate in senescent cells [85] and has recently been proposed as a marker of senescent cells [86]. Interestingly, treatment with synthetic lipofuscin can by itself induce senescence [85], suggesting that lipofuscin accumulation can play a causal role in the process.

ROS in senescent cells do not necessarily originate from mitochondria. For instance, endothelial cell senescence was shown to be induced by endogenous NADPH oxidase

NOX4 [87], which in turn can contribute to mitochondrial dysfunction by inactivating complex I of the mitochondria [88]. NOX4 has also been shown to play a role in OIS [89], suggesting that ROS produced independently of mitochondria plays a role in senescence.

ROS has also been shown to regulate the SASP (Fig.2). Studies show that ROS can activate NF-κB (a major regulator of the SASP) [90] or lead to its activation indirectly *via* ROS-dependent induction of DNA damage and a DDR [48]. Inhibition of ATM (the main initiator of the DDR) has been shown to decrease the SASP [91]. Mechanistically, ATM has been shown to activate NEMO, IKK complex and nuclear translocation of NF-κB [92].

While considerable data support a role for mitochondrial ROS as a driver of senescence in culture, less is known *in vivo*, however, mouse models of mitochondrial dysfunction and enhanced oxidative stress show increased senescence in different tissues.

For instance, cardiomyocyte-specific overexpression of the mitochondrial enzyme monoamine oxydase A (MAO-A), which has been shown to increase during cardiac aging [46,93], leads to increased ROS and mitochondrial dysfunction [94]. Cardiomyocytes from TgMAO-A mice show increased telomere dysfunction and markers of cellular senescence, which can be rescued by treatment with antioxidant N-acetyl cysteine (NAC) [9]. More recently, it was shown that MAO-A driven cardiomyocyte senescence also induces paracrine senescence to cardiac stromal cells [95].

Additionally, deficiency of antioxidant enzyme mitochondrial superoxide dismutase 2 (SOD2), has been shown to induce cellular senescence in mouse skin [96] and result in an age-dependent accumulation of telomere associated foci (TAF) in cardiomyocytes [9]. Selective SOD2 deficiency in connective tissue was shown to induce accelerated aging in different tissues, such as muscle, bones and skin, associated with increased oxidative damage and induction of cellular senescence [97]. Moreover, deletion of the antioxidant enzyme cytosolic superoxide dismutase 1 (SOD1) was associated with increased oxidative stress and expression of senescence and SASP markers in kidney [98]. Finally, mice with reduced expression of DNA repair enzyme ERCC1-XPF endonuclease show increased levels of ROS and accumulate senescent cells in different tissues. Importantly, chronic treatment with a mitochondrial-targeted free radical scavenger was shown to attenuate oxidative damage and the accumulation of senescence cells, thus supporting a causal link between mitochondrial ROS and induction of cellular senescence *in vivo* [99]. Similarly, accelerated aging mouse model *nfkb1*$^{-/-}$ showed increased ROS and telomere dysfunction, which could be significantly reduced by treatment with antioxidant BHA [100].

All together, these studies support a central role for mitochondrial ROS in the induction and maintenance of senescence both *in vivo* and *in vitro*.

3.2 The role of mitochondrial metabolites in senescence

As previously discussed, senescent cells experience a bioenergetic imbalance characterized by an increase in AMP/ATP and ADP/ATP ratios [101]. Evidence suggests that these changes can contribute causally to cellular senescence. Increase of these ratios can lead to the activation of the AMP-activated protein kinase (AMPK), a central mediator of cellular

metabolism, which has been shown to play a role in senescence [56,102]. AMPK activation can lead to the direct phosphorylation and activation of p53 [103,104], down regulation of proliferative genes, such as cyclin A, or reduction of retinoblastoma protein (RB) phosphorylation, all of which have been shown to contribute to the senescence growth arrest [102]. AMPK activation can also increase the nuclear presence of RNA stabilizing factor human antigen R (HuR) protein, resulting in increased p16 expression but also inhibiting the stabilization of mRNAs encoding cyclins, leading to a senescent phenotype [101,105].

The nicotinamide adenine dinucleotide (NAD) has been shown to play important roles in metabolism and cellular senescence. This coenzyme can be found in 2 forms: the oxidized NAD^+ and the reduced NADH. Senescent cells are characterized by a decrease in the NAD^+/NADH ratio [106]. Nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme for NAD^+ salvage from nicotinamide, has been shown to decrease in senescent cells [107,108]. Importantly, decreased NAMPT activity induces senescence whereas its overexpression delays senescence induction [109]. Knock-down of the cytosolic malate dehydrogenase (MDH1), an enzyme that catalyzes the reversible reduction of oxaloacetate to malate, has been shown to a reduce NAD^+/NADH ratios, activate AMPK, and induce cellular senescence [110]. Low NAD^+/NADH levels have also been implicated in the regulation of the SASP. Wiley and colleagues described that mitochondrial dysfunction induced senescence resulted in an atypical SASP with no induction of commonly expressed SASP factors IL-1α, IL-1β, IL-6 and IL-8. This observation was confirmed *in vivo* in a progeroid mouse model (Polg mutator mouse) characterized by accumulation of mtDNA mutations and mitochondrial dysfunction in various tissues [111]. NAD seems to play different roles in the regulation of the SASP depending on the inducing stimuli. For instance, levels of NAD^+, NAD^+/NADH ratios and NAMPT expression differ significantly between replicative and oncogene-induced senescence (OIS) and this seems to impact on the extend of SASP expression [108]. In contrast to replicative senescence where NAMPT and NAD^+/NADH ratios decline, OIS is characterized by increased expression of NAMPT enzyme and higher NAD^+/NADH ratios. Nacarelli and colleagues propose that during OIS, NAMPT promotes the SASP through NAD^+-mediated suppression of AMPK kinase, which suppresses the p53-mediated inhibition of p38 MAPK to enhance NF-κB activity [108].

Recent evidence suggests other mechanistic links between NAD metabolism and the regulation of cellular senescence. It has been observed that NADase CD38 (cluster of differentiation 38) activity is increased during aging and it was suggested that this ecto-enzyme is responsible for the age-dependent decline in NAD^+ [112-114]. Recently, it was shown that senescent cells can stimulate the expression of CD38 in non-senescent cells, especially in immune cells, via the SASP [112,114,115]. This led to the hypothesis that accumulation of senescent cells and the SASP may contribute to the age-dependent decline in NAD^+ levels via up-regulation of CD38 in non-senescent cells. Furthermore, CD38 has also been shown, *in vivo*, to be an inducer of mitochondrial dysfunction, which could potentially contribute to further induction of senescence [115]. Interestingly, pharmacological inhibition of CD38 was shown not only to prevent the decline in NAD^+ levels during aging but also reduce telomere-associated DNA damage, a major driver of senescence [116].

As previously discussed, senescence is characterized by significant changes in the TCA cycle and several of its pathways and metabolites have been implicated in the regulation of senescence. Supraphysiological concentrations of pyruvate, which enters the mitochondrial matrix and is converted into Acetyl-CoA, have been shown to accelerate replicative senescence *in vitro* [117]. Enhanced usage of pyruvate via the enzyme pyruvate dehydrogenase (PDH), which links glycolysis to the TCA cycle, plays a key role in oncogene-induced senescence [64]. Acetyl-CoA production from acetate has been shown to accelerate chronological aging in yeast and induce senescence in human endothelial cells [118]. Acetyl-CoA can also be used in lipid synthesis and in fact, it has been shown that fatty acid synthase (FASN) which combines acetyl-CoA with malonyl-CoA to produce long-chain fatty acids plays a role in the initial stages of the senescence program [119].

Jiang and colleagues demonstrated a role for malic enzymes ME1 and ME2, two TCA cycle enzymes which recycle malate into pyruvate, in cellular senescence. Authors showed that p53 represses ME1 and ME2 expression, which reciprocally activate p53 through MDM2 and AMPK in a feed-forward manner [104].

Fumarate, a precursor to malate in the TCA cycle, has also been implicated in senescence. Accumulation of fumarate was shown to lead to the formation of succunicGSH, a covalent adduct between fumarate and glutathione, increasing oxidative stress and inducing senescence in epithelial cells and fibroblasts [120].

Fumarylacetoacetate hydrolase (FAH) domain containing protein 1 (FAHD1) is a mitochondrial metabolic enzyme which can hydrolyze acylpyruvates and decarboxylase oxaloacetate, a TCA cycle metabolite. Downregulation of this enzyme was shown to inhibit mitochondrial function and include premature senescence in human endothelial cells [121].

Glutamine metabolism and consumption has also been shown to be increase in senescence [122,123]. This pathway feeds the TCA cycle by increasing the levels of α-ketoglutarate by glutaminase and glutamate dehydrogenase activity [124]. Intriguingly, on one hand, pharmacological inhibition of glutaminolysis has been found to induce premature senescence in HUVECS [122] and, on the other hand, has been identified as a new senolytic treatment, improving health span, when administered to aged mice [123].

Interestingly, TCA cycle metabolites can also have effects that counteract senescence. For instance, α-ketoglutarate has been shown to act as a senomorphic by reducing the SASP in senescents fibroblasts and extend healthspan and lifespan in aged mice [125].

While the role of TCA cycle intermediates in non-metabolic signaling functions has been extensively studied in the context of cancer and inflammation [126], their role in senescence remains poorly understood. Given the interlink between senescence, cancer, and inflammation, understanding their role in senescence may lead to the identification of new therapeutic approaches to counteract aging and age-related diseases.

3.3 The role of mitochondrial DAMPs and mitochondrial derived peptides in senescence

As a response to stress, cells can release endogenous components to the extracellular space to trigger an immune response which are collectively called the damage-associated

molecular patterns (DAMPs). DAMPs are recognized by pattern-recognition receptors (PPR) often localized in immune cells, but also found in non-immunes cells, and promote inflammatory responses [127]. An emerging concept is that inflammation during aging and age-related diseases may arise because of the innate immune system identifying damaged components derived from mitochondria. Indeed, mitochondrial components such as cardiolipin, mitochondrial transcription factor A (TFAM), N-formyl peptide, ATP, succinate, cytochrome c and mitochondrial DNA (mtDNA) have been identified as a subclass of DAMPs, commonly referred to as mitochondrial DAMPs [128,129] (Fig.3). Whether these mitochondrial DAMPs play a role in senescence is still relatively understudied.

Mitochondrial dysfunction during senescence has been shown to contribute to the generation of cytosolic chromatin fragments (CCFs) which trigger the SASP though activation of cGAS-STING pathway (reviewed in [130]). It has been suggested that during senescence, mitochondrial ROS activate the stress-activated kinases JNK1/2, which interacts with the CCF suppressor 53BP1, leading to CCF formation [131]. CCF once in the cytosol are recognized by DNA sensor Cyclic GMP-AMP synthase (cGAS) which has been shown to be a major driver of the SASP [130]. Consistent with this idea, wide-spread clearance of mitochondria or suppression of mitochondrial ROS was shown to prevent formation of CCF and inhibit the SASP [51,131].

Mitochondrial DNA (mtDNA) is present at thousands of copies *per* cell and has been shown to be a potent inducer of inflammation once outside the mitochondrial matrix via different PPRs, including TLR9, cGAS-STING and the inflammasome [132]. Studies have shown that circulating cell-free mtDNA increases in the plasma during aging and in the context of several age-related diseases, leading to the hypothesis that it is a major contributor to inflammation [133]. A recent study has implicated senescent cells as major sources of cell-free mtDNA [134]. This study showed that elimination of senescent cells by senolytic drugs significantly reduced circulating cell-free mtDNA in aged mice. Additionally, senolytic treatment improved the survival of mice receiving transplants from aged mice [134].

The inflammasome has also been demonstrated to be activated in senescent cells *in vitro* and *in vivo* [11]. This pathway is mainly initiated by activation of the cytoplasmic NLR family pyrin domain containing 3 (NLRP3) by DAMPs, which by oligomerization forms the NLRP3 inflammasome and activates caspase-1. Once activated, caspase-1 matures pro-IL1-β and pro-IL18 into IL-1β and IL-18 respectively [135]. Interestingly, other mitochondrial components have been shown to activate the inflammasome such as mitochondrial ROS and cardiolipin [136,137]. Cardiolipin is a glycerophospholipid found exclusively in the inner mitochondrial membrane, but upon mitochondrial stress, can be exposed in the outer membrane where it can activate different pathways such as autophagy, apoptosis but also the inflammasome [137,138]. Importantly, it has been shown that senescent cells accumulate cardiolipin, and that cardiolipin can, in turn, also induce premature senescence in human fibroblast *in vitro* [139].

Mitochondrial derived peptides humanin and MOTS-c were also shown to be elevated in senescent cells. Interestingly, their administration to senescent cells resulted in higher mitochondrial respiration and a modest increase in selected components of the SASP [140].

The mitochondrial transcription factor A (TFAM), a member of HMGB family and essential for maintenance of mtDNA, has also be found to be immunostimulatory. For example, absence of TFAM can cause aberrant mtDNA packaging and mtDNA release into the cytosol where it activates the cGAS-STING pathway and inflammation [141]. Moreover, specific deletion of TFAM in T cells was shown to induce premature senescence in different tissues triggered by an increase of pro-inflammatory cytokines [142]. TFAM can also by itself or synergistically with other DAMPs, as N-formyl peptide, to induce an inflammatory response in immune cells [143,144].

4- Mitochondrial therapy- keeping senescence at bay

As we described in the previous sections, mitochondria are key regulators of the senescent phenotype by contributing to both the cell cycle arrest and the SASP. Another aspect we have not yet discussed is that mitochondria may hold the key to the survival of senescent cells, because they are major regulators of apoptosis. Mitochondrial outer membrane permeabilization (MOMP) is often essential for apoptosis and is regulated by interactions between pro- and anti-apoptotic members of the Bcl-2 protein family [145].

Currently, there is great enthusiasm regarding the development of pharmacological interventions targeting senescent cells, given their pivotal roles in aging and age-related diseases [146]. Two classes of senotherapies are currently being investigated: “senolytic” therapies that specifically induce cell-death of senescent cells and “senomorphic” therapies that suppress the SASP without affecting cell viability. We argue that obtaining a detailed road map to mitochondrial biology in senescence will be essential to guide the development of both classes of therapies for the following reasons:

First, many of the senolytic drugs being currently developed exert their effects by targeting anti-apoptotic proteins which are located at mitochondria and prevent mitochondrial-driven apoptosis. Senolytic drugs such as ABT-263 (navitoclax) and others in this family exert their senolytic effect by targeting the anti-apoptotic B-cell lymphoma 2 (BCL-2) family of proteins located in mitochondria [17,18]. More recently, mitochondria-targeted tamoxifen (MitoTam) was shown to induce apoptosis of senescent cells and reduce the expression of senescent markers p21 and p16 in kidney and lung of aged mice [147]. Furthermore, as we previously described mitochondrial apoptosis depends on processes such as mitochondrial fusion and fission and mitophagy both of which are altered in senescent cells.

Secondly, data supports that mitochondria play an essential role in the development of the SASP [51,131]. They are major sources of DAMPs and metabolites which have been shown to engage pro-inflammatory pathways. Senomorphic drugs such rapamycin and metformin which have been shown to suppress the SASP [148-150], have also been shown to improve mitochondrial function [51,151,152]. Therefore, uncovering the underlying mechanisms mediating mitochondrial dysfunction during senescence, will be important in the developing new therapeutic avenues targeting the SASP.

Given the wide-ranging alterations in mitochondria in senescent cells and their key role in regulating cell-death and inflammation, we hypothesize that screening for compounds

targeting different aspects of mitochondrial biology may lead to the identification new classes of senolytic and senomorphic drugs.

Conclusions and future perspectives

Evidence in the literature is clear in highlighting that senescent cells show significant alterations in mitochondrial function and metabolism. However, many of the reported observations are limited to a relatively small number of cell-types and may not be universal to all subtypes of senescent cells. In fact, as we discussed in this review, several mitochondrial and metabolic parameters differ depending on the senescent cell-type and stressor. Thus, a more in-depth characterization of mitochondrial function in different senescent cell-types is warranted.

Furthermore, while data indicates that mitochondria become dysfunctional in senescent cells cultured *in vitro*, very little is known about mitochondrial function, structure and dynamics in senescent cells present in tissues *in vivo*. To investigate this further, methods that allow the isolation of senescent live cells from different tissues need to be developed so that these cells can be further characterized *ex vivo*. While this may be currently feasible using mouse models expressing reporters of senescence-associated genes, isolation of senescent cells in human tissues will present further challenges.

Another important question in the field, is how much senescent cells contribute to the overall mitochondrial dysfunction observed in tissues during aging. Studies are consistent in showing that mitochondria isolated from aged tissues show decreased respiratory coupling and increased ROS production. The extent to which senescent cells contribute to these phenotypes is not yet known.

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Abbreviations

AMPK	5' AMP-activated protein kinase
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
BCL-2	B-cell lymphoma 2
CCF	cytoplasmic chromatin fragment
CD38	cluster of differentiation 38
cGAS	cyclic GMP-AMP synthase
DAMPs	damage-associated molecular patterns
DDR	DNA damage response

DSBs	DNA double stranded breaks
ETC	electron transport chain
H^+	protons
IL	interleukin
MAO-A	Monamine Oxidase A
MOMP	mitochondrial outer membrane permeabilisation
MitoTAM	mitochondrial targeted tamoxifen
MOTS-c	mitochondrial open reading frame of the 12S rRNA-c
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
NAD	nicotinamide adenine dinucleotide
NAMPT	nicotinamide phosphoribosyltransferase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OIS	oncogene-induced senescence
OXPHOS	oxidative phosphorylation
PINK1	PTEN induced kinase 1
RB	retinoblastoma protein
ROS	reactive oxygen species
SASP	senescence associated secretory phenotype
SOD	superoxide dismutase
STING	stimulator of interferon genes
TAF	telomere associated foci
TCA	tricarboxylic acid
TFAM	mitochondrial transcription factor A
TLR9	toll-like receptor-9

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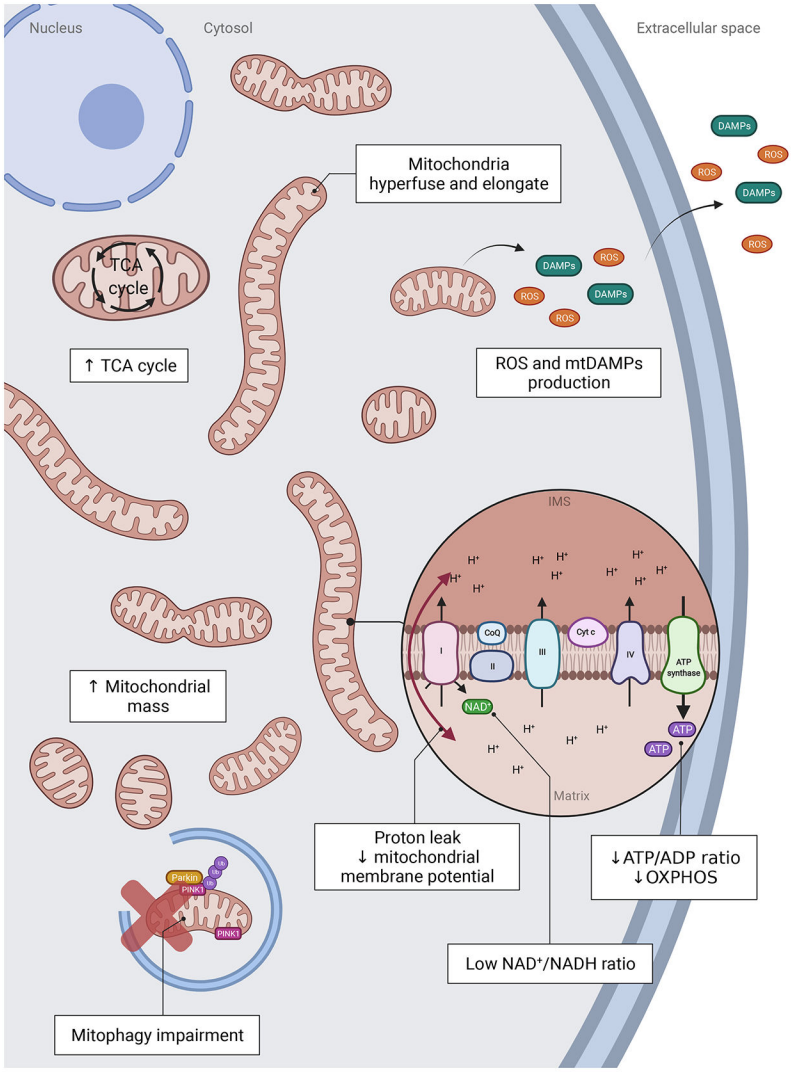

Figure 1: Mitochondria in senescent cells exhibit several changes in terms of function, structure, and dynamics.

Senescent cells show increased mitochondrial protein leak and decreased mitochondrial membrane potential as well as decreased ATP/ADP and NAD^+/NADH ratios. Mitochondrial mass is increased as well as the abundance of TCA cycle metabolites, and mitochondria become hyperfused and elongated. Mitophagy is decreased, potentially inhibiting the cell's ability to eliminate dysfunctional mitochondria. Mitochondria produce excessive amounts of ROS and DAMPs.

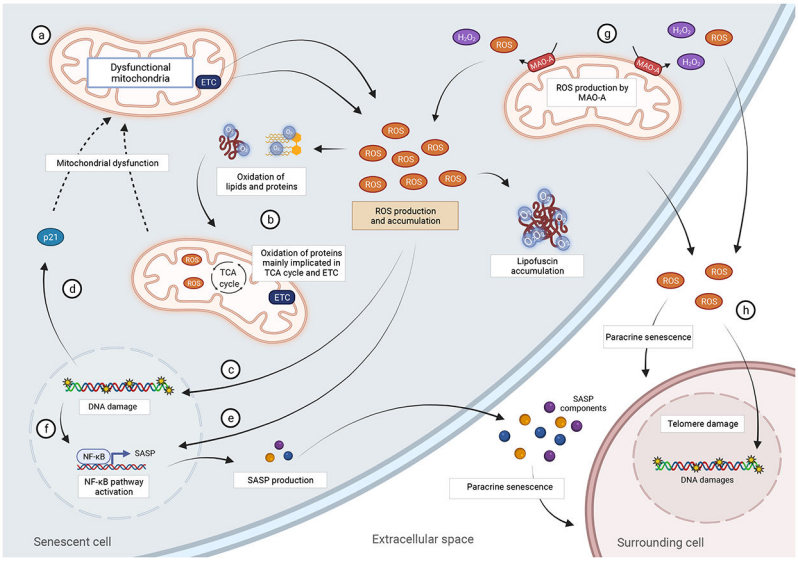

Figure 2: Mitochondrial ROS play important roles in senescence.

Dysfunctional mitochondria in senescent cells produce high ROS which can lead to the oxidation of proteins, lipids, and DNA. High ROS can induce both single- and double-stranded breaks in the genome, particularly at telomere regions, leading to senescence. High ROS production can activate NF-κB (a major regulator of the SASP) directly or via induction of a DNA damage Response. ROS can play important roles in spreading senescence to surrounding, otherwise healthy cells, via the induction of a DNA damage response.

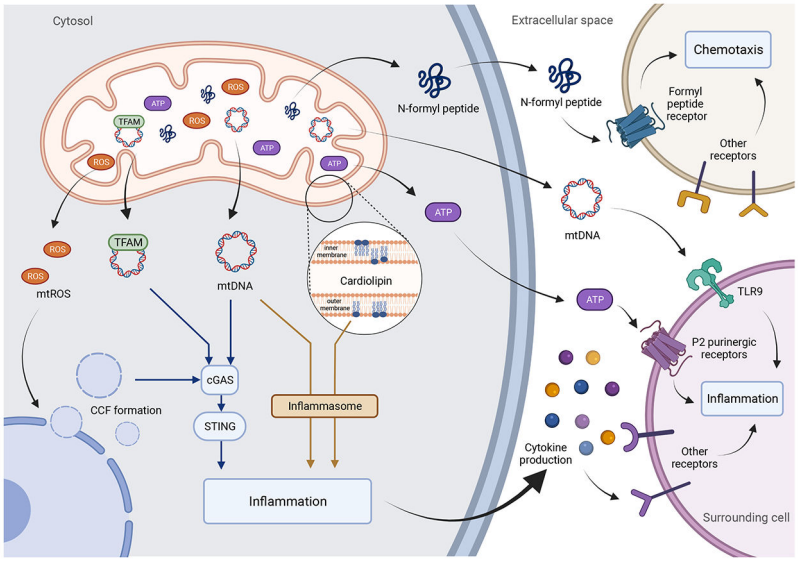

Figure 3: Mitochondria are a major source of DAMPs.

TFAM, mtDNA, cardiolipin, ATP and N-formyl peptides can be released from mitochondria into the cytosol or the extracellular space and engage pro-inflammatory pathways. Mitochondrial ROS has been shown to contribute to the nuclear release into the cytosol of CCF during senescence which can be recognized by DNA sensor cGAS and activate the STING.