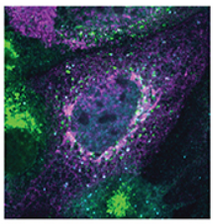
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Mitophagy as a guardian against cellular aging

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ABSTRACT

Mitophagy, the selective autophagic clearance of damaged mitochondria, is considered vital for maintaining mitochondrial quality and cellular homeostasis; however, its molecular mechanisms, particularly under basal conditions, and its role in cellular physiology remain poorly characterized. We recently demonstrated that basal mitophagy is a key feature of primary human cells and is downregulated by immortalization, suggesting its dependence on the primary cell state. Mechanistically, we demonstrated that the PINK1-PRKN-SQSTM1 pathway regulates basal mitophagy, with SQSTM1 sensing superoxide-enriched mitochondria through its redox-sensitive cysteine residues, which mediate SQSTM1 oligomerization and mitophagy activation. We developed STOCK1N–57534, a small molecule that targets and promotes this SQSTM1 activation mechanism. Treatment with STOCK1N–57534 reactivates mitophagy downregulated in senescent and naturally aged donor-derived primary cells, improving cellular senescence(–like) phenotypes. Our findings highlight that basal mitophagy is protective against cellular senescence and aging, positioning its pharmacological reactivation as a promising anti-aging strategy.

Abbreviation: IR: ionizing radiation; ROS: reactive oxygen species; SARs: selective autophagy receptors.

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Main

Macroautophagy/autophagy is a cellular catabolic process crucial for degrading surplus or damaged cellular components including dysfunctional mitochondria. Selective autophagy receptors (SARs) enable the selective degradation of targeted macromolecules by binding to ubiquitinated cargo and autophagy proteins. This facilitates the expansion and closure of phagophores to form autophagosomes, which subsequently fuse with lysosomes where their cargo is degraded. Mitophagy, the selective clearance of mitochondria by autophagy, is regarded as vital for mitochondrial quality control. In the PINK1-PRKN-mediated mitophagy pathway, PINK1 accumulates on damaged mitochondria and phosphorylates the E3 ubiquitin ligase PRKN, resulting in ubiquitination of mitochondrial surface proteins. Among SARs, CALCOCO2/NDP52, OPTN and, to a lesser extent, TAX1BP1, are recruited to ubiquitinated mitochondria and mediate damage-induced PINK1-PRKN mitophagy. Although mitophagy is implicated in both cellular homeostasis and age-related disease, its molecular mechanisms, particularly in the basal state, and its requirement for optimal cellular physiology are poorly defined. This gap may be partly due to low mitophagy activity observed in commonly used immortalized cell lines, requiring their exposure to excessive mitochondrial damage induced by mitochondrial toxins and uncouplers for mitophagy research. We determined that primary human cells, such as dermal fibroblasts, are characterized by highly active basal mitophagy compared to transformed cell lines such as mouse embryonic fibroblasts and the human cervical cancer cell line, HeLa. Additionally, immortalization of human fibroblasts by introducing human telomerase reverse transcriptase strongly suppresses mitophagy. These data indicate that reliance on basal mitophagy is a feature of primary cells.

We then tested whether the induction of senescence by ionizing radiation (IR) alters the mitophagy rate in primary human fibroblasts. We observed that basal mitophagy is strikingly suppressed as early as 2 h after IR and remains low over the course of 11 days, during which the cells display established hallmarks of senescence, including cell cycle arrest, increased expression of senescence-associated secretory phenotype/SASP markers and SA-GLB1/β-GAL staining, as well as mitochondrial alterations such as expansion of the mitochondrial network and inefficient respiration. Importantly, basal autophagy remains functional and even upregulated during senescence acquisition. Together, we conclude that the early mitophagy (but not bulk autophagy) downregulation occurs at the initial stage of the senescence program.

We next sought to identify the molecular mechanism underlying basal mitophagy. Mitophagy activation is typically associated with mitochondrial damage, such as mitochondrial depolarization and elevated mitochondrial reactive oxygen species (ROS). However, mitochondrial depolarization is detected following IR, indicating that suppressed mitophagy cannot be attributed to changes in mitochondrial membrane potential. Conversely, we observed that mitochondrial ROS levels correlate with basal mitophagy activity, as senescence

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induction reduces the number of superoxide-enriched mitochondria compared to proliferating cells. We hypothesized that increased mitochondrial network fusion, observed after IR, hinders the segregation of superoxide-rich mitochondria and mitophagy activation. Silencing the mitochondrial fusion regulator MFN2 results in the partial restoration of superoxide-rich mitochondria and mitophagy levels following IR. Additionally, paraquat, an inducer of mitochondrial superoxide production, increases mitophagy flux, whereas the mitochondrial superoxide scavenger MitoQ suppresses basal mitophagy and promotes cellular senescence with prolonged treatment. These findings suggest that mitochondrial ROS is crucial for triggering basal mitophagy and preventing senescence.

Further analyses revealed that basal mitophagy depends, at least in part, on the PINK1 and PRKN machinery. Interestingly, we found that SQSTM1/p62, but not CALCOCO2, OPTN, nor TAX1BP1 act as a SAR for PINK1-PRKN-mediated basal mitophagy. Accordingly, silencing PINK1, PRKN or SQSTM1 is sufficient to suppress basal mitophagy, and induce cellular senescence. We further demonstrated that superoxide-enriched mitochondria colocalize with PRKN and SQSTM1, suggesting that these mitochondria serve as initiation sites of PINK1-PRKN-SQSTM1-dependent basal mitophagy. To investigate this mechanism further, we employed redox-insensitive C105A C113A and oligomerization-deficient K7A D69A SQSTM1 mutants, because SQSTM1 is established to sense ROS and promote autophagy through its redox-sensitive cysteine residues and PB1-mediated oligomerization, respectively. Indeed, wild type, but not these mutants, can restore the suppression of basal mitophagy caused by SQSTM1 knockdown, signifying the importance of SQSTM1 oligomerization in basal mitophagy.

We therefore sought to target the SQSTM1 oligomerization process for mitophagy activation, and identified STOCK1N–57534, a small molecule that can stimulate SQSTM1-dependent mitophagy and rescue IR-induced cellular senescence. Mechanistically, STOCK1N–57534 binds to the ZZ domain of SQSTM1, facilitating the formation of SQSTM1-disulfide-linked conjugates/DLCs through its redox-sensitive C105 and C113 residues, which lead to SQSTM1 oligomerization and mitophagy activation. The small molecule is unable to rescue mitophagy in cells completely lacking, or re-expressing oxidation-insensitive SQSTM1 (C105A C113A). Interestingly, STOCK1N–57534 successfully rescues mitophagy impairments in cells with an oligomerization-deficient (K7A D69A) SQSTM1 mutant that retains ROS sensitivity. These results highlighted that the redox sensitivity of SQSTM1 is necessary and sufficient for basal mitophagy.

Finally, we analyzed fibroblasts obtained from young and older human donors to explore the association between basal mitophagy and aging. Although negative for SA-GLB1/β-GAL staining, aged fibroblasts display senescence-like phenotypes including cell cycle arrest, slower proliferation, impaired mitochondrial function and altered senescence-associated secretory phenotype profiles, indicating a potential pre-senescence state. Notably, mitophagy flux is significantly reduced in aged cells. A single treatment of STOCK1N–57534 is able to restore mitophagy levels to those detected in young cells, and improve cellular aging phenotypes. Our data suggest that suppression of mitophagy is a feature of cellular aging and can be targeted by pharmacological interventions.

In conclusion, these findings highlight that mitophagy is a safeguard against cellular senescence and aging (Figure 1) [1]. Mitophagy declines with age which is associated with the reduction of ROS signaling and redox regulation of SQSTM1. Targeting mitophagy by small molecule interventions, including our SQSTM1-targeting small molecule STOCK1N–57534, presents an approach for combating aging and, ultimately, extending lifespan. Given our identification of mechanisms specific to primary human cells, we propose that future studies using human-centric cell and tissue models will be essential to fully comprehend the biology underpinning human aging.

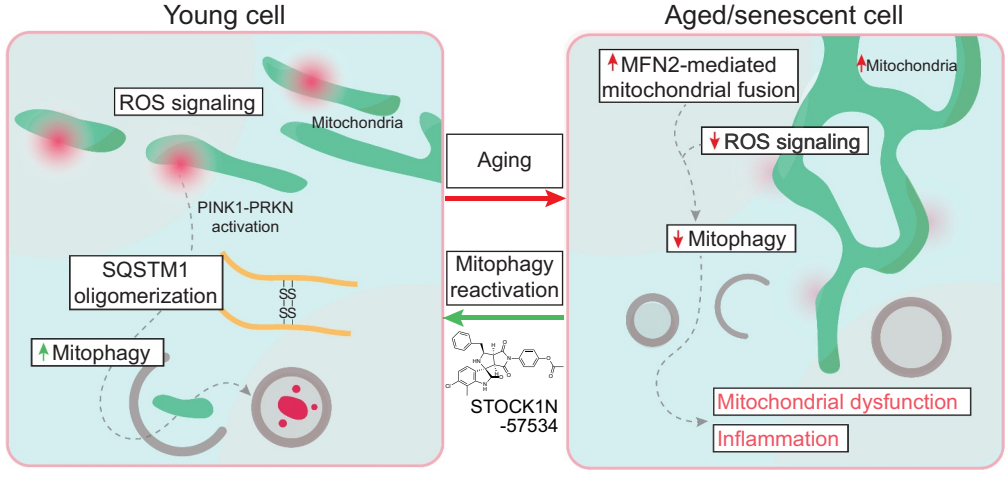


Figure 1. Basal mitophagy is a guardian against cellular senescence and aging. In young and proliferating human primary cells, basal mitophagy is highly active and acts as a safeguard against cellular senescence and aging phenotypes. The PINK1-PRKN-SQSTM1 pathway targets ROS-enriched mitochondria, via redox-sensitive cysteine residues of SQSTM1, leading to SQSTM1 oligomerization and subsequent mitophagy activation. In aged and senescent cells, alterations in the mitochondrial network, namely excessive mitochondrial fusion partially mediated by MFN2, hinders basal mitophagy, initiating cellular senescence programs. Small molecule interventions, such as STOCK1N-57534 that activates redox-regulated SQSTM1 oligomerization, can reactivate mitophagy in aged and senescent cells and improve cellular aging phenotypes.

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Disclosure statement

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