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Review

Macroautophagy in quiescent and senescent cells: a pathway to longevity?

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Cellular quiescence – reversible exit from the cell cycle – is an important feature of many cell types important for organismal health. Aging and cellular dysfunction compromise the survival and reactivation of quiescent cells over time. Studies suggest that autophagic processes and lysosomal function are critical to maintaining the function of quiescent cells, especially adult stem cells, throughout life. Findings also point to both pro-senescence and anti-senescence functions for macroautophagy depending on context. In this review, we will discuss these findings, unanswered questions on the role of macroautophagy and lysosomal function in quiescent and senescent cells, and the possibility for interventions that stimulate macroautophagy and lysosomes to promote quiescent cell function and tissue regeneration.

Highlights

Cellular quiescence (reversible cell cycle arrest) and senescence (irreversible cell cycle arrest) are inter-related cell fates that govern many aspects of tissue homeostasis.

With age, quiescent cells lose reactivation efficiency, and senescent cells accumulate, possibly contributing to tissue dysfunction with age.

Lysosomes and macroautophagy are central to maintaining the function of quiescent cells, possibly by destroying damaged and toxic cellular components such as mitochondria.

'Basal' levels of macroautophagy mitigate quiescence-to-senescence transition, but high levels of macroautophagy may be detrimental. Therefore, autophagy-stimulating interventions should be evaluated for their effects on quiescent and senescent cells.

Quiescence and senescence: inter-related cell fates influencing health and longevity

Cellular quiescence and cellular senescence are, respectively, reversible and irreversible forms of cell cycle arrest that broadly influence tissue and organismal homeostasis throughout life. Conditions that are not suitable for growth induce cells to reversibly exit from the cell cycle, entering a G_0 state known as cellular quiescence. This is employed by adult stem cells such as neural stem cells (NSCs), hematopoietic stem cells (HSCs), and muscle stem cells (MuSCs), as well as lymphocytes, hepatocytes, and other cells [1]. Quiescent cells are not truly 'dormant' but employ active homeostatic mechanisms that influence their viability and re-entry into active proliferation [1–3]. Aging and disease can compromise these active homeostatic mechanisms, contributing to the reduced survival or reactivation of quiescent cells with age, potentially contributing to age-associated tissue dysfunction [4–12]. In contrast to reversibly arrested quiescent cells, senescent cells permanently exit the cell cycle and lose many of their cell-type-specific features and functions [13]. Cellular senescence is induced via a variety of cellular insults, including telomere shortening, DNA damage, and proteostasis dysfunction, and can serve as a barrier to oncogenesis by restricting the proliferation of damaged cells. Senescent cells accumulate with age and secrete a variety of factors, termed the senescence-associated secretory phenotype (SASP) that are hypothesized to contribute to inflammation and other pathologies associated with aging [13]. Hence, there is growing interest in the development of 'senolytics' that selectively kill senescent cells and may slow aging and disease. Quiescence and senescence may be inter-related cell states: over time, quiescent cell transcriptomes begin to resemble senescent cell transcriptomes, and active homeostatic mechanisms suppress this transition [14,15]. Thus, it is of increasing importance to understand the connections between these two inter-related cell fates that are crucial to human health and longevity.

Studies examining the survival and reactivation of quiescent cells in a variety of paradigms point to important roles for autophagy and lysosomes in quiescent cells [8,14–16]. Aging and disease compromise the functions of this system, possibly contributing to the age-associated decline in

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the reactivation of quiescent cells and subsequent tissue repair [17–20]. Macroautophagy and lysosomes have also been proposed to both contribute to and counteract senescence, depending on context [15,21,22]. In this review, we will evaluate the evidence supporting both positive and negative roles for macroautophagy and lysosomes in quiescent and senescent cells and whether increased macroautophagy is a pathway to longevity via modulation of quiescent and senescent cells.

Lysosomes and macroautophagy in quiescent cells

Lysosomes are acidic organelles where much of the superfluous or damaged components of cells are degraded via endocytosis, phagocytosis, and autophagy. A feature of quiescent cells is upregulation of lysosomal biogenesis and function and studies suggest that the function of their lysosomes is central to their regenerative potential [8,14,23,24]. Inhibition of the vacuolar-type ATPase (V-ATPase) of lysosomes, which causes lysosome de-acidification, thereby blocking autophagy and other lysosomal degradation processes, prevents the reactivation of quiescent NSCs and quiescent fibroblasts [8,14]. Conversely, stimulating lysosome biogenesis by overexpression of a constitutively active mutant form of the transcription factor EB (TFEB) or treating cells or mice with rapamycin, an inhibitor of mTORC1, leading to TFEB activation enhances the reactivation of aged quiescent NSCs [8]. Likewise, serum starvation of rat embryonic fibroblasts, which induces cellular quiescence, increases expression of lysosome-related genes over time, although hampered lysosomal function over time increases the quiescence depth of these cells and attenuates their reactivation [14]. In contrast to these findings are those concerning quiescent HSCs, where inhibiting protein degradation in lysosomes increases the potency with which they are reactivated in competitive transplantation experiments [24].

Macroautophagy is an ancient cellular process that engulfs large cellular cargos through the *de novo* formation of a double membrane structure termed an autophagosome and traffics them to lysosomes for destruction (Figure 1) [8,25]. Selective and nonselective forms of macroautophagy play fundamental roles in cellular and organismal functions, which include the removal of superfluous or damaged cellular components, remodeling cellular components during differentiation, and responding to challenges in metabolism or proteostasis. Given its fundamental role in cells, it is not surprising that null mutations in many of the genes encoding core macroautophagy machinery in *Caenorhabditis elegans* and mice are lethal or severely compromise development [26–34]. Macroautophagy is also thought to positively regulate lifespan [35]. With age, macroautophagy is compromised, and interventions that boost macroautophagy are correlated with a longer lifespan [36]. Furthermore, interventions that increase lifespan in *C. elegans* universally depend on macroautophagy [37,38]. Macroautophagy may contribute to lifespan by keeping cells tidy and free of things such as dysfunctional mitochondria or large protein aggregates that may contribute to cellular damage over time, eventually leading to compromised cell function later in life.

Genetic and pharmacological interventions that reduce macroautophagy compromise the survival, retention in a quiescent state, and/or reactivation of quiescent adult stem cells, whereas interventions that increase macroautophagic flux promote their survival, retention in a quiescent state, and subsequent re-entry into the cell cycle (Table 1) [8,15,25]. Inhibition of macroautophagy pathways causes depletion of HSC pools, likely both from increased cell death and HSCs inappropriately exiting quiescence [25]. Macroautophagy is also beneficial to the long-term maintenance of MuSCs by preventing an accumulation of dysfunctional mitochondria, which generate increased reactive oxygen species (ROS) [15]. In MuSCs, ROS can lead to premature senescence, and autophagy-deficient MuSCs are defective in regeneration of myofibrils, although this deficit could be explained in part by the important role of macroautophagy in the

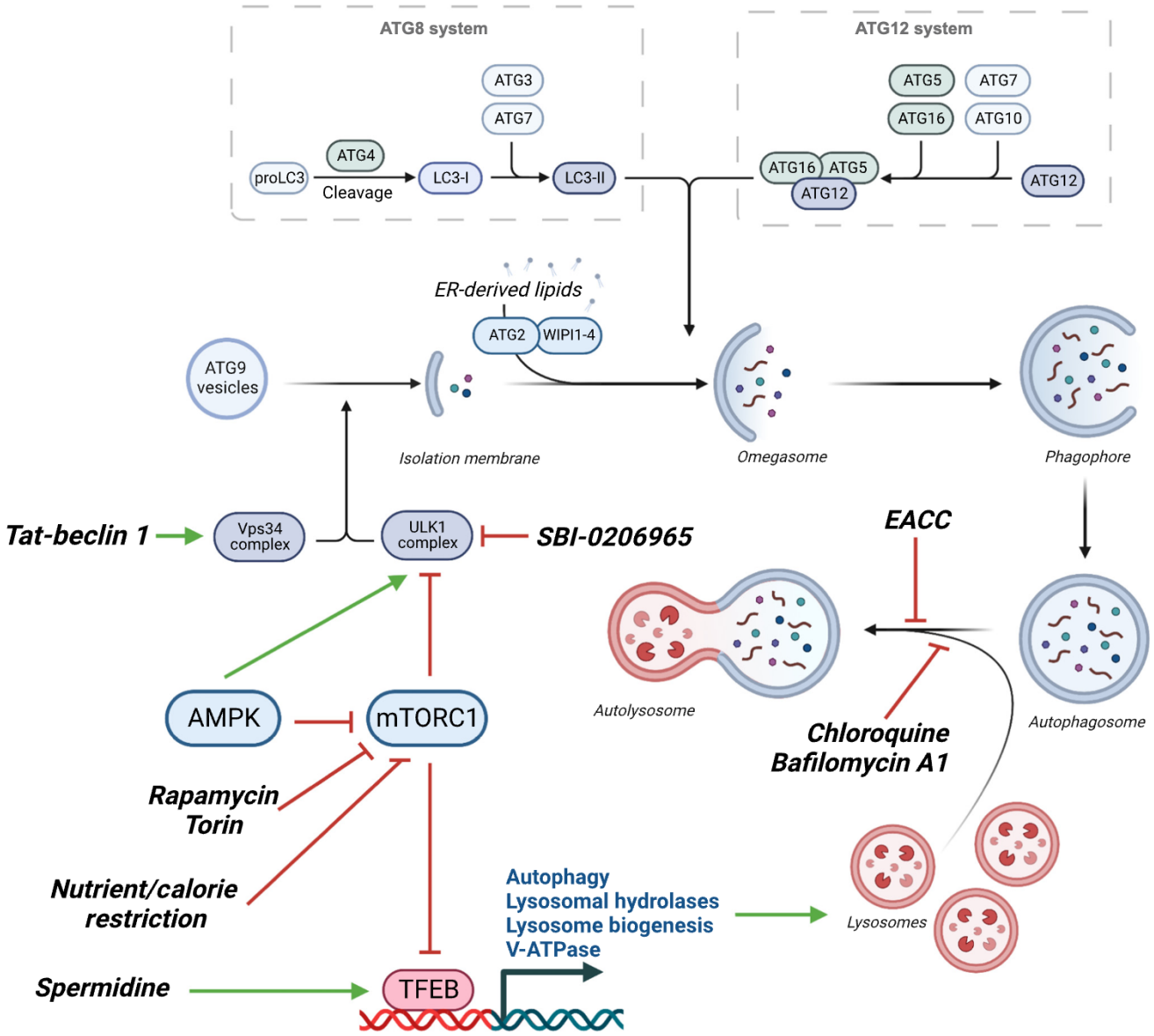

Figure 1. Mechanism and regulation of macroautophagy and lysosome biogenesis. Macroautophagy proceeds in a stepwise manner, starting with the recruitment of the ULK1 and phosphoinositide 3 phosphate kinase (PI3K) complexes to Atg9 vesicles. Activation of ULK1 is mediated by AMPK and inhibited by mTORC1. This step can be modulated by rapamycin and other mTORC1 inhibitors, nutrient or calorie deprivation, the ULK1 inhibitor SBI-0206965, and the Tat-beclin 1 cell-permeable peptide. Expansion of phagophore membranes requires phospholipid transport; the cleavage and lipidation of Atg8/LC3, leading to its association with phagophore membranes; and the recruitment of the Atg5-Atg12-Atg16 complex. Closure of the phagophore double membrane forms an autophagosome, which fuses with lysosomes/endolysosomes in a manner dependent on lysosome pH, which can be modulated with bafilomycin A1 (inhibits V-ATPase) and chloroquine/hydroxychloroquine (increases lysosome pH by accumulating in lysosomes as a deprotonated weak base) and Stx17-SNARE-mediated fusion (inhibited by EACC). mTORC1 also inhibits the transcription factor EB (TFEB), which increases the autophagic capacity of the cell by increasing expression of macroautophagy machinery and stimulating the biogenesis of increased numbers of functional lysosomes. Spermidine can stimulate autophagy via increasing translation of TFEB. Figure created using BioRender.com. Abbreviations: AMPK, 5′ AMP-activated protein kinase; EACC, ethyl [2-(5-nitrothiophene-2-carboxamido) thiophene-3-carbonyl] carbamate; ER, endoplasmic reticulum; V-ATPase, vacuolar-type ATPase.

reactivation of quiescent MuSCs [15,39]. While macroautophagy may help to eliminate dysfunctional mitochondria that generate increased ROS, it might, unabated, have broad-ranging effects on cellular metabolism that can affect reactivation of quiescent cells [24]. Spermidine, through

Table 1. The effect of macroautophagy-stimulating interventions on quiescent cells

Cell type	Intervention	Macroautophagy	Lysosome function	Macroautophagy dependent?	Result	Refs
NSC	Activated TFEB overexpression	Increased	Increased	Not determined	Increased reactivation *in vitro*	[8]
	Rapamycin	Increased	Increased	Not determined	Increased reactivation *in vivo*	[8]
	Nutrient deprivation	Increased	Increased	Not determined	Increased reactivation *in vitro*	[8]
	Whole-animal Beclin activation	Increased	Neutral	Not determined	Increased *ex vivo* expansion with age	[48]
MuSC	Rapamycin	Increased	Increased	Mostly	Reduced senescence, increased repopulation	[15]
	Spermidine	Increased	Increased	Mostly	Reduced senescence, increased repopulation	[15]
	Atg7 overexpression	Increased	Neutral	Not determined	Reduced senescence, increased repopulation	[15]
HSC	Spermidine	Increased	Increased	Not determined	No effect on HSCs or hematopoietic progeny	[40]
B cells	Spermidine	Increased	Increased	Yes	Restored macroautophagy and B cell responses with age	[40]
Fibroblasts	MiTF overexpression	Increased	Increased	Not determined	Shallower quiescence and enhanced reactivation	[14]

hypusination of TFEB, stimulates reactivation of memory B cells in a macroautophagy-dependent manner [40]. Most studies find that interventions stimulating macroautophagy are correlated with increased resilience and reactivation potential of quiescent cells, although the requirement of macroautophagy in these processes is not always defined (Table 1).

Lysosomes and macroautophagy in senescent cells

As a process that may contribute to cellular homeostasis by removing damaged or toxic cellular components, macroautophagy is generally thought to counteract the onset of senescence. Indeed, macroautophagy is downregulated and the burden of senescent cells increases with age. Supporting this theory, deletion of the core macroautophagy gene *Atg7* in MuSC of young mice leads to a premature increase in senescent MuSCs [15]. *In vitro*, knockdown of *Atg5* or *Atg7* in primary human fibroblasts leads to premature replicative senescence [22]. However, studies of oncogene-induced senescence (OIS) found that macroautophagy is induced by expression of oncogenes and macroautophagy genes play an important role in driving cells into senescence [21]. Macroautophagy is also upregulated in other models of senescence [41]. One explanation for these apparently contradictory findings is that some 'basal' amount of macroautophagy suppresses the onset of cellular senescence by, for example, removing dysfunctional, ROS-generating mitochondria, but that induced autophagy during oncogenesis exceeds this basal amount and through some means promotes and sustains cellular senescence.

Macroautophagy influences not only the onset of cellular senescence but influences the characteristics of senescent cells. Macroautophagy is upregulated in senescent cells arising from stress, replicative exhaustion, and OIS [41]. Paradoxically, mTORC1 activity is also upregulated in senescent cells in comparison to proliferating or quiescent cells and contributes to the generation of the SASP [41–43]. One means through which macroautophagy might promote senescence is by providing a key source of amino acids for sustained mTORC1 activation in senescent cells and may therefore contribute the persistence of senescent cells [41]. Thus, although macroautophagy may repress the development of senescence, it may also sustain it once it occurs. At the organismal level, inhibition of mTORC1 may increase lifespan by preventing the onset of senescence through increased quality control via stimulated macroautophagy and also by undermining the hyperactive mTORC1 signaling in senescent cells should these processes fail.

More recently, in studies using OIS to identify senolytic compounds, it was observed that senescent cells have lysosomes that harbor protein aggregates that damage their membranes [44]. Although not tested by the authors, one possible explanation for the senescence-inducing role of macroautophagy in OIS is that it delivers protein aggregates to lysosomes in quantities that are beyond their capacity to degrade, leading to lysosomal dysfunction that contributes to senescence. It may also be that cell cycle arrest may lead to the accumulation of aggregates in lysosomes in conditions beyond OIS: a study of quiescent NSCs found that their lysosomes also accumulate protein aggregates [8]. More protein aggregates accumulate in lysosomes of quiescent NSCs from old animals, which was correlated with a reduced capacity for reactivation and cell cycle re-entry. As is the case for OIS, it is not clear if the intralysosomal protein aggregates that accumulate in quiescent NSCs are delivered by macroautophagy, since genetic perturbations specifically targeting macroautophagy were not employed. A follow-up study using the same *in vitro* model for studying quiescent NSCs found that the reactivation of quiescent NSCs is fairly inefficient, with only a fraction returning to proliferation [45]. Since quiescent NSCs harbor intralysosomal protein aggregates like OIS cells, could some of them be on the path to senescence (Figure 2)? Furthermore, if the high levels of macroautophagy during OIS contribute to intralysosomal aggregates, will stimulating macroautophagy in quiescent cells prove advantageous or detrimental to their survival and future reactivation potential?

If some autophagy is good for quiescent cells, is more autophagy better?

In the aforementioned sections, we have mainly focused on the important role of basal macroautophagy in regulating the biology of quiescent and senescent cells. In the following sections, we will discuss studies that boost macroautophagy through a variety of means to improve aspects of quiescent cell function, mainly their ability to resist senescence and reactivate to repair damaged tissues. A central goal of our discussion is to critically examine whether increased flux through the macroautophagy pathway *per se* is beneficial to quiescent cells.

Several strategies have been employed to specifically increase macroautophagy in cells and animals (Table 1). Genetic means include overexpression of core macroautophagy gene *Atg5* and mutations in the autophagy initiator Beclin that prevent its inhibition and de-inhibit autophagosome formation [46,47]. In mice harboring these transgenes or mutations (expressed in all cells), the mice are leaner and live longer than control littermates. Related to quiescence, NSCs in the subventricular zone of mice with constitutively active Beclin 1 ($Becn1^{F121A/F121A}$) were protected with aging and were slightly more proliferative when grown as neurospheres *ex vivo* [48]. These results were correlative and did not examine whether increased autophagy was required for the phenotypes observed and a nonautophagy role for Beclin1 cannot be excluded. Overexpression of *Atg7* in MuSCs from geriatric mice improved their engraftment capacity and ability to generate muscle fibers when transplanted into damaged muscle [15]. Transient overexpression of constitutively active TFEB also boosts the reactivation of quiescent NSCs, possibly through macroautophagy and lysosome function [8].

Pharmacological and lifestyle interventions that increase autophagy have also been used to investigate whether increased autophagy protects quiescent cells (Table 1). mTORC1 plays a central role in regulating cellular autophagic capacity by inhibiting TFEB and Ulk1-dependent autophagosome formation. Inhibiting mTORC1 with small molecules such as rapamycin increases autophagy in cells. Rapamycin treatment has been found to increase the regenerative capacity of quiescent MuSCs and NSCs from aged animals [8,15]. In the case of NSCs, however, whether rapamycin exerted its effects through increased macroautophagy, and not through, for instance, reduced protein translation, is not clear. Transient or mild nutrient deprivation in quiescent cells, which stimulates macroautophagy and lysosome function, can also boost their subsequent

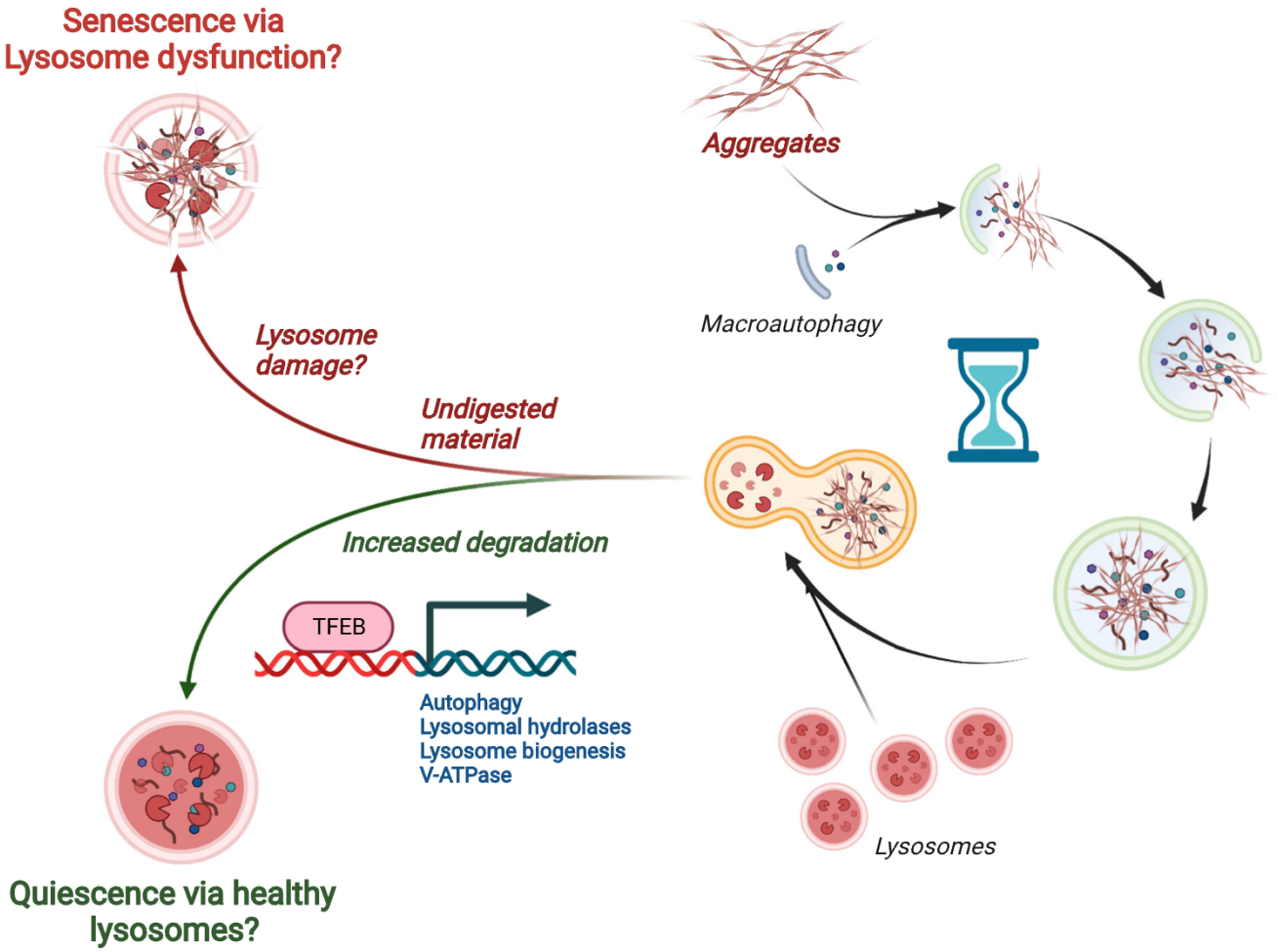

Figure 2. Hypothetical relationship between protein aggregates, macroautophagy, and lysosome function in quiescent and senescent cells. Protein aggregates accumulate in lysosomes during oncogene-induced senescence (OIS), leading to membrane damage. Macroautophagy is required for OIS, and thus may contribute to OIS by trafficking aggregates to lysosomes. Protein aggregates also accumulate in lysosomes of certain types of quiescent cells, which are negatively correlated with their ability to reactivate. Interventions that clear lysosomal protein aggregates also boost reactivation. Thus, the accumulation of protein aggregates in lysosomes may drive quiescent cells toward irreversible senescence. Further discussion of this hypothesis occurs in the main text and the 'Outstanding questions' section. Figure created using BioRender.com. Abbreviations: TFEB, transcription factor EB; V-ATPase, vacuolar-type ATPase.

reactivation [8]. This effect is dependent on functional lysosomes, but again, it is not clear whether it is through macroautophagy *per se*. More direct stimulation of macroautophagy can be achieved through cell-permeable peptides that compete with endogenous Beclin 1 for binding to GAPR-1, an autophagy inhibitor [49]. However, to our knowledge, the effect of these peptides on quiescent or senescent cells has not been examined.

It is important to note that macroautophagy is a fundamentally important process in animals from *C. elegans* to mammals. In *C. elegans*, *atg-7*, *atg-5*, and *atg-4.1*; *atg-4.2* (the two Atg4 homologs in *C. elegans*) null mutants are inviable [26,33,34]. Although some putative null mutations in core macroautophagy genes, such as *atg-2* and *atg-18*, are viable, they are, in our hands, very sickly and slow growing. Knockout mice targeting *Atg3*, *Atg5*, *Atg7*, *Atg12*, and *Atg16l1* die as neonates, and *Atg9a* knockout mice die as embryos [27–30,32,50]. Given macroautophagy's very

important or perhaps essential nature for the development of animals from *C. elegans* to mammals, the effects of completely abrogating it may assign too much importance to the role of increased macroautophagy in certain lifespan-extending conditions. For example, mTORC1 inhibition with rapamycin not only increases macroautophagy but also increases lysosome function via TFEB, reduces protein translation, and pre-stimulates subsequent mTORC1 reactivation via the TFEB-mediated transcriptional upregulation of *RagD* [58]. Conditional knockout of *Atg5* and *Atg7* is commonly used in quiescent stem cell populations *in vivo*, but if these cells are very dysfunctional it may be that no intervention, besides restoring some amount of macroautophagy through genetic complementation, can rescue them. It may, therefore, be the case that interventions such as rapamycin treatment might require macroautophagy to work but might exert their beneficial effects on quiescent cells through other processes. Knockout of macroautophagy genes also does not allow one to distinguish between the roles of macroautophagy in quiescent cells, reactivated cells, and subsequent progeny.

A more definitive demonstration of the effectiveness of increased macroautophagy in promoting the longevity and function of quiescent cells would be to reduce macroautophagy back to basal levels in interventions that, as one of their effects, increase macroautophagy levels. Commonly used inhibitors of autophagy are V-ATPase inhibitor bafilomycin A1 and ionophore chloroquine [51]. However, these compounds inhibit other aspects of lysosome function, and excluding these effects from interpretations is complicated. Other macroautophagy inhibitors such as the Ulk1 inhibitor SBI-0206965, which inhibits autophagosome formation, and ethyl [2-(5-nitrothiophene-2-carboxamido) thiophene-3-carbonyl] carbamate (EACC), which inhibits autophagosome fusion with lysosomes by inhibiting trafficking of the V-snare Stx17 to autophagosomes through a poorly understood mechanism, may be alternative means to acutely and reversibly inhibit macroautophagy [52,53]. If interventions that increase the longevity and reactivation potential of quiescent cells function primarily through increased macroautophagy, then reducing macroautophagy levels back to basal levels should eliminate the beneficial effects of those interventions. Conversely, reducing macroautophagy levels back to baseline might reveal that those interventions do not function through increased macroautophagy, and perhaps, might even do so despite it.

Pharmacological modulators of autophagy would affect many types of cells *in vivo*, complicating interpretation of their results. One alternative is a recently described mouse model where the core macroautophagy gene Atg5 is silenced by a doxycycline-inducible short hairpin RNA (shRNA) [54]. This system was capable of reversibly inhibiting macroautophagy and inhibiting macroautophagy dramatically reduced lifespan, with treated mice living only about one-third as long as control littermates. Although this system was employed throughout the body, it should be possible for the system to be in utilized in a tissue- or cell-type-specific manner. Similarly, inducible knockdown of *Atg7* in mice was recently described, which rapidly caused destruction of the pancreas, but other organs were not affected [31]. Another alternative is a recently generated mouse model using an inducible, dominant-negative variant of *Atg4B*, $Atg4B^{C74A}$, that enables temporal and cell-type-specific inhibition of macroautophagy [55]. It may, however, be difficult to titrate levels of autophagy inhibition in *Atg5* and *Atg7* shRNA and $Atg4B^{C74A}$ mouse models. Although it may be very difficult to titrate macroautophagy levels using these systems, one advantage of these genetic approaches over a cell-type-specific knockout is in their ability to temporally control macroautophagy inhibition *in vivo*, enabling one to restrict autophagy inhibition relative to perturbations and interventions and to also restrict macroautophagy inhibition to certain lineages. In the case of quiescent adult stem cells, knockout of macroautophagy genes using the Cre-Lox system in stem cells means that all their progeny are autophagy knockouts too: if macroautophagy is important to the function of their differentiated progeny, it may cause tissue dysfunction that

feeds back to regulate the behavior of stem cell populations, complicating interpretation of the effects of macroautophagy inhibition on these cells.

Concluding remarks

Macroautophagy, an ancient cellular mechanism to engulf and degrade cellular components, is a fundamental process inside eukaryotic cells. Together with proteasomes and other forms of autophagy, it serves as a major mechanism for the homeostatic turnover of cellular components and the remodeling of cellular function in response to challenges. With age, flux through the macroautophagy pathway is diminished, which has led to the hypothesis that restoration or boosting of macroautophagy will promote longevity. Several findings support this hypothesis: overexpression of rate-limiting components of macroautophagy leads to increased lifespan, as does rapamycin treatment and calorie restriction, both of which increase macroautophagy. However, there are several unanswered questions on the role of lysosomes and macroautophagy in quiescent and senescent cells (see Outstanding questions).

The function of adult stem cells, which often reside in a quiescent state, is diminished with age, concomitantly with a reduction in macroautophagy [5,7,8,10,15]. Consistently, knocking out autophagy genes can drive quiescent cells into premature senescence, and interventions that boost macroautophagy can restore the function of quiescent cells from aged animals (Table 1). However, the precise requirement for increased macroautophagy in these interventions should be more rigorously established. That is, when assessing the effects of autophagy-stimulating interventions on autophagy knockout cells, one cannot separate the role of increased macroautophagy in these interventions from the requirement for at least some amount of macroautophagy in these cells. What if these interventions increased the function and lifespan of quiescent cells not because of increased macroautophagy, but despite it? Furthermore, it should be considered whether ongoing macroautophagy in quiescent cells could be detrimental to their long-term function. Firstly, autophagic cell death has been reported to kill NSCs in response to certain stressors: could the autophagy-deficient NSCs in aged brains be the result of selection for cells that are resistant to autophagic cell death [56]? Secondly, circumstantial evidence points to a role for the accumulation of protein aggregates in lysosomes of senescent cells because of macroautophagy [21,44]. Protein aggregates also accumulate in the lysosomes of quiescent NSCs that reactivate inefficiently, suggesting that they might be on a pathway toward irreversible growth arrest [8,45]. Is the autophagic sequestration of protein aggregates beneficial to mitotic and terminally differentiated cells, but detrimental to quiescent cells? Conversely, mitophagy may suppress quiescence-to-senescence transition [15]. Might it be beneficial to stimulate autophagy of only certain kinds of cellular components in quiescent cells?

Quiescent cells, including adult stem cells, can serve as a reservoir of innate regenerative potential for aging individuals, but their function declines with age. Senescent cells accumulate with age and can lead to widespread tissue dysfunction through their inflammatory SASP, which has led to increased interest in senolytic medicines that kill them [13,57]. The retention or rejuvenation of functional quiescent cells, and the mitigation of senescent cells, then, may serve as useful and mutually supportive strategies for healthy aging and longevity. It is, therefore, critical to assess the pathways, including macroautophagy, that contribute to or counteract the generation and maintenance of these two inter-related cell fates.

Outstanding questions

What processes lead to the age-associated decline in lysosome and autophagy function in quiescent cells? Are these similar or distinct mechanisms when compared with other cells?

Is increased macroautophagy *per se* beneficial to the long-term maintenance and function of quiescent cells? Can it rejuvenate aged cells?

What are the cellular components (e.g., protein aggregates, mitochondria) that are targeted by macroautophagy that perturb or enhance quiescent cell functions and maintenance? Does targeted degradation of certain cellular components via macroautophagy have more beneficial effects than generally stimulated macroautophagy on quiescent cell function?

Is the intralysosomal accumulation of protein aggregates a process that contributes to senescence? What role does macroautophagy play in this process?

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Declaration of interests

The authors declare no competing interests.

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