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Review

Aging and immortality in unicellular species

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ABSTRACT

It has been historically thought that in conditions that permit growth, most unicellular species do not to age. This was particularly thought to be the case for symmetrically dividing species, as such species lack a clear distinction between the soma and the germline. Despite this, studies of the symmetrically dividing species *Escherichia coli* and *Schizosaccharomyces pombe* have recently started to challenge this notion. They indicate that *E. coli* and *S. pombe* do age, but only when subjected to environmental stress. If true, this suggests that aging may be widespread among microbial species in general, and that studying aging in microbes may inform other long-standing questions in aging. This review examines the recent evidence for and against replicative aging in symmetrically dividing unicellular organisms, the mechanisms that underlie aging, why aging evolved in these species, and how microbial aging fits into the context of other questions in aging.

1. Introduction

In 1957, George Williams first proposed the theory of antagonistic pleiotropy of aging. This theory required a clear distinction between the soma and the germ line: "*The theory regards senescence as an evolved characteristic of the soma. We should find it where soma has evolved, but not elsewhere.*" (Williams, 1957). This was further expanded in the disposable soma theory (Kirkwood, 1977), which similarly requires a distinction between the expendable soma and the immortal germline. These theories form a cornerstone of the current thinking on evolution of aging (Gavrilov and Gavrilova, 2002; Ackermann et al., 2007a; Kirkwood, 2005) and predict that similarly to germ line cells, unicellular species such as *Escherichia coli* should not age (Williams, 1957). Despite these predictions, it has long been documented that some unicellular species do age, most notably *Saccharomyces cerevisiae* (Mortimer and Johnston, 1959) and *Paramecium tetraurelia* (Smith-Sonneborn, 1979; Aufderheide, 1987). Indeed, *S. cerevisiae* has been used as a model species for aging and has been extensively studied for decades (see Piper, 2006; Longo et al., 2012; Nyström and Liu, 2014b; Denoth Lippuner et al., 2014 for in-depth reviews). However, these species have attributes that make them arguably different from many other unicellular species. *P. tetraurelia*, unlike most other unicellular species, experiences clonal senescence when deprived of sexual reproduction (Aufderheide, 1987). *S. cerevisiae* replicates via asymmetric division – budding, where the aging mother cell is morphologically clearly distinct from the daughter cell (Mortimer and Johnston, 1959) and can be thought of as assuming the role of the soma. Importantly, for species that divide symmetrically, such distinction does not occur. *S. cerevisiae* and *P. tetraurelia* may therefore have been considered special cases, different from symmetrically dividing unicellular species and the germline of multicellular organisms. As a consequence of this view and the experimental difficulties of studying microbial aging, over the last 60 years, most unicellular species have generally been considered not to age (see Kirkwood and Austad, 2000; Nyström, 2003; Ackermann et al., 2007a). This review focuses on replicative aging in symmetrically dividing model species *E. coli* and *S. pombe*. Unlike *S. cerevisiae*, they have no clear morphological distinction between two daughters which would allow one daughter to assume the role of the soma – and thus, until recently, were predicted not to age. Importantly, throughout this review, "aging" refers to aging in environmental conditions that permit growth (replicative aging) which is conceptually distinct from senescence that occurs due to growth-limiting conditions (see below and Box 1. *Replicative aging vs conditional senescence*). For aging in the asymmetrically dividing *S. cerevisiae,* the reader is referred to one of the several excellent reviews (Steinkraus et al., 2008; Longo et al., 2012; Clay and Barral, 2013; Denoth Lippuner et al., 2014) and for senescence that occurs in growth-limiting conditions, the reader is referred to another set of great reviews (Longo et al., 2012; Gonidakis and Longo 2013; m 1999; m, 2002, 2003, 2007; m, 2002, 2003, 2007; m, 2002, 2003, 2007).

2. Do symmetrically dividing species age?

Senescence of both symmetrically and asymmetrically dividing microbes has been reported to occur in growth-limiting conditions since the 1960s (Burleigh and Dawes, 1967; also see Nyström, 2003;

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Box 1
Replicative aging vs conditional senescence.

Aging in microbial species has been studied in two main ways – in stationary phase, starvation, or other conditions that do not allow cells to divide (conditional senescence or chronological aging), and in conditions that permit active growth (replicative aging). In conditional senescence studies, the viability of the whole population is tracked, and longevity is measured using chronological time (chronological lifespan). In contrast, replicative aging studies mostly track cells on a single-cell level, and measure longevity as the number of divisions before death (replicative lifespan).

With the exception of S. cerevisiae, studies of conditional senescence predate studies of replicative aging, and mostly consist of studies where yeast or bacteria are growth-limited by growing them to stationary phase in complex medium, or in defined medium lacking key nutrients (Fredriksson and Nyström, 2006; Longo et al., 2012). In such conditions, the population-wide viability decreases with time, as seen by a decreasing number of colony forming units on agar after plating (although a number of other and more sophisticated methods have also been used to study this phenomenon) (Gonidakis and Longo, 2013; Longo et al., 2012). This process, termed chronological aging by yeast microbiologists and more carefully conditional senescence by bacterial microbiologist, is sometimes compared to aging of multicellular organisms (Fredriksson and Nyström, 2006; Gonidakis and Longo, 2013). While the definition of aging is not universally agreed upon (see Do symmetrically dividing species age?), this process is more similar to a stress response than to the process of mandatory aging seen in animals, as the growth arrest is caused by high cell density, starvation or toxic metabolites and results in upregulation of a number of stress-response genes (Nyström, 2003; Fredriksson and Nyström,2006). Indeed, it is not surprising that starvation or high stress would lead to a decrease in viability and ultimate death of any organism, irrespective of aging. Hence the term conditional senescence. Nevertheless, studies of conditional senescence should not be dismissed as uninformative to aging, nor viewed as strictly separate from replicative aging. A number of similarities exist between replicative aging and conditional senescence: in both, the affected cells show a time-dependent decrease in viability, accumulation of carbonylated proteins and reactive oxygen species (Gonidakis and Longo, 2013; Fredriksson and Nyström, 2006), and calorie restriction extends lifespan in both models (Fredriksson and Nyström, 2006; Longo et al., 2012). Furthermore, a number of genes have been identified through conditional senescence studies that have later been found to be important in aging across a number of species. These include the TOR/S6K pathway, Ras/adenylate cyclase pathway, PKA, SOD2, as well as a number of other downstream genes (Longo et al., 2012). Finally, as seen from this review, both are likely caused by environmental stress – although in the case of replicative senescence, the stress is not large enough to induce growth arrest.

However, important differences exist as well. It can be argued that replicative aging is a closer model to the constitutive aging seen in animals, as in this model the organisms are not affected by starvation or growth-inhibiting stress (Fredriksson and Nyström, 2006). On the other hand however, microbial populations are much more likely to experience growth-limiting conditions than a surplus of nutrients in nature, and it can be argued that studies of conditional senescence are employing a model more closer to the natural environments of microbes (Gonidakis and Longo, 2013). Therefore, the choice of the model depends on the specific question, the goal of the study, and the angle of view. Either way, considerable care should be taken before extrapolating results obtain from single-celled organisms to multicellular organisms.

Finally, an often overlooked aspect of replicative aging vs conditional senescence is that they describe two continuous, consecutive phases of the microbial life cycle. Replicative aging occurs during active growth in the logarithmic phase and transitions into conditional senescence during stationary phase, whereby the transition between the two phases is gradual. It is likely that replicative aging influences the cell fate later during conditional senescence. However it is unknown to what degree and in which manner, as at present, no studies have reported tracking cell aging continuously through both phases.

Fredriksson et al., 2006; Nyström, 2007; Ksiazek, 2010). In conditions where high cell density, starvation, presence of toxic metabolites or other factors keep cells from dividing, they undergo a time-dependent decrease in viability and eventually lose their capacity to proliferate – a process dubbed conditional senescence (Nyström, 1999; also known as chronological aging – see Box 1). Therefore, it is well established that under such conditions, unicellular species can senesce and die. However, whether this phenomenon should truly be considered aging has been debated (Nyström 2003; Fredriksson and Nyström,2006), as in this method, senescence is invariably induced by stressful extrinsic factors, while aging in animals and other organisms is caused by intrinsic changes that occur despite optimal environments. Until recently, it was thought that in conditions that support growth, most unicellular organisms do not age.

This was particularly true for symmetrically dividing species, which lack a morphological distinction between the mother and daughter cells. Indeed, the notion that symmetrically dividing species could age in general, even in optimal conditions, seems contradictory. If a cell divides into identical daughters, then if one daughter ages, the other must age as well – and if both daughters age similarly, it would ultimately lead to clonal aging and death of the whole population. Yet even in symmetrically dividing species, the two daughter cells are not completely identical. In *E. coli*, one cell pole is synthesized *de novo* during each division, and in the subsequent division, one daughter cell inherits the newly synthesized pole while the other inherits the old pole (Fig. 1). This creates an asymmetry between the two daughter cells, as their cell poles are of different ages. As a result, even though division is symmetrical and leads to apparently identical daughters, the daughters have different replicative ages due to the different ages of their cell poles (Fig. 1).

That aging may affect bacteria during active replication was first shown by the discovery of aging in *Caulobacter crescentus*, a bacterium in which a stalked mother cell attaches to the substrate and produces daughter cells via asymmetric division (see Figs. 2 and 3A) (Ackermann et al., 2003). In this species, stalked cells show a continuous decline in reproductive output and die on average after 200 hours (Ackermann et al., 2003). However, evidence that aging may also affect symmetrically dividing bacteria (and thus bacteria in general), came from subsequent studies of the symmetrically dividing *E. coli* (Stewart et al., 2005). In this study, fluorescence microscopy and automated tracking was used to track cells that received either the new or old poles in a colony growing on agarose (see Fig. 3A), which showed that cells that continuously received the old pole experience a small but significantly decreased division rate (Stewart et al., 2005). This was confirmed with similar observations a few years later (Lindner et al., 2008), providing support for aging in symmetrically dividing bacteria. However, as a key limitation, microscopy allowed the observation of only a limited number of divisions (approximately 10). When *E. coli* divisions were

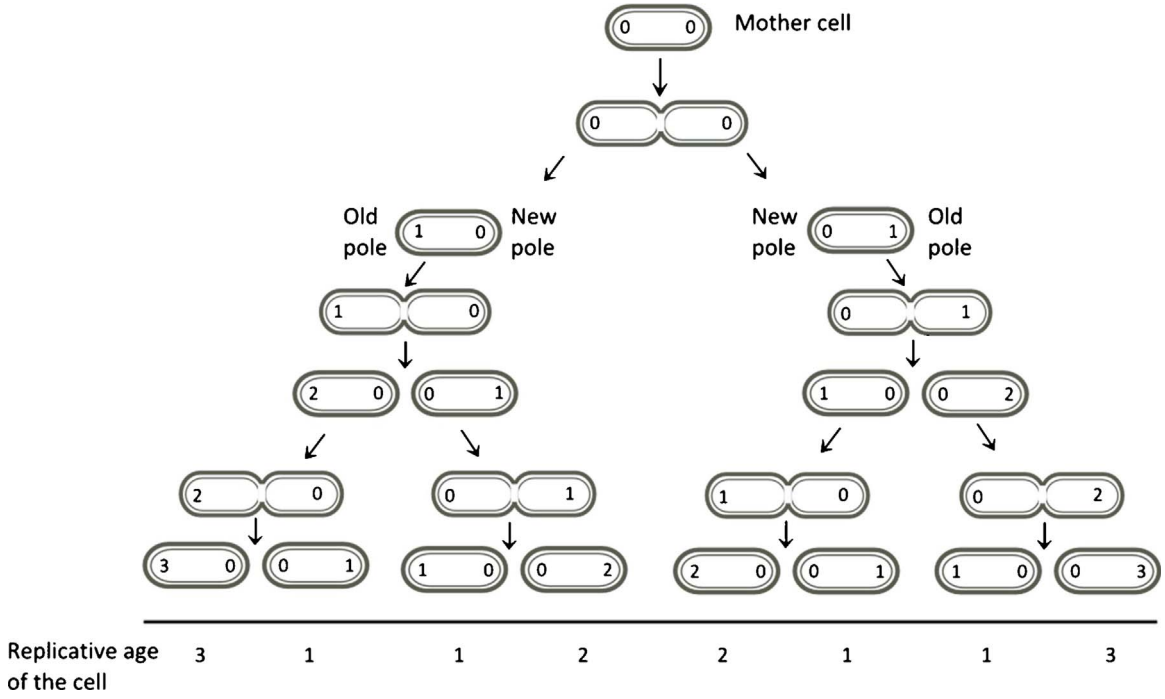

Fig. 1. Inheritance of cell poles in symmetrically dividing microbes. Both *E. coli* and *S. pombe* divide by symmetric fission, and are used as model organisms. Starting from a hypothetical completely rejuvenated cell in which all cell components are new (age of both poles is 0), during division one cell pole is synthesized anew whereas the other is inherited from the mother cell. The replicative age of the inherited pole increases with each division. After second division, one daughter inherits the new pole and the other the old pole, which creates a difference between the replicative ages of the daughters. The replicative age of a cell is equal to the replicative age of its oldest pole. Already after the first division, the two cell poles of the daughters are of different age − therefore asymmetry arises inevitably during cell division, and does not require a mother cell that is asymmetric. It is necessary to point out that for this reason, no cell in the population in which the age of both poles is 0 actually exist and is used here as an example only. Also, importantly, even old cells produce rejuvenated young daughter cells with replicative age of 1 at each division.

tracked long-term in a microfluidic device (see Fig. 3B), cells continuously receiving the old pole were shown to divide for over 200 generations with no decrease in division rate (Wang et al., 2010). This, on the opposite indicates that *E. coli* does not age. The exact reasons for this contradiction are not known. It is possible that the division rate of old pole cells decreases only initially, and these experiments observed different aspects of the same process, as fluorescence microscopy was used to track up to 10 divisions, (Stewart et al., 2005; Lindner et al., 2008), while microfluidics experiments excluded the first 10 divisions from the analysis but tracked subsequent divisions long-term (Wang

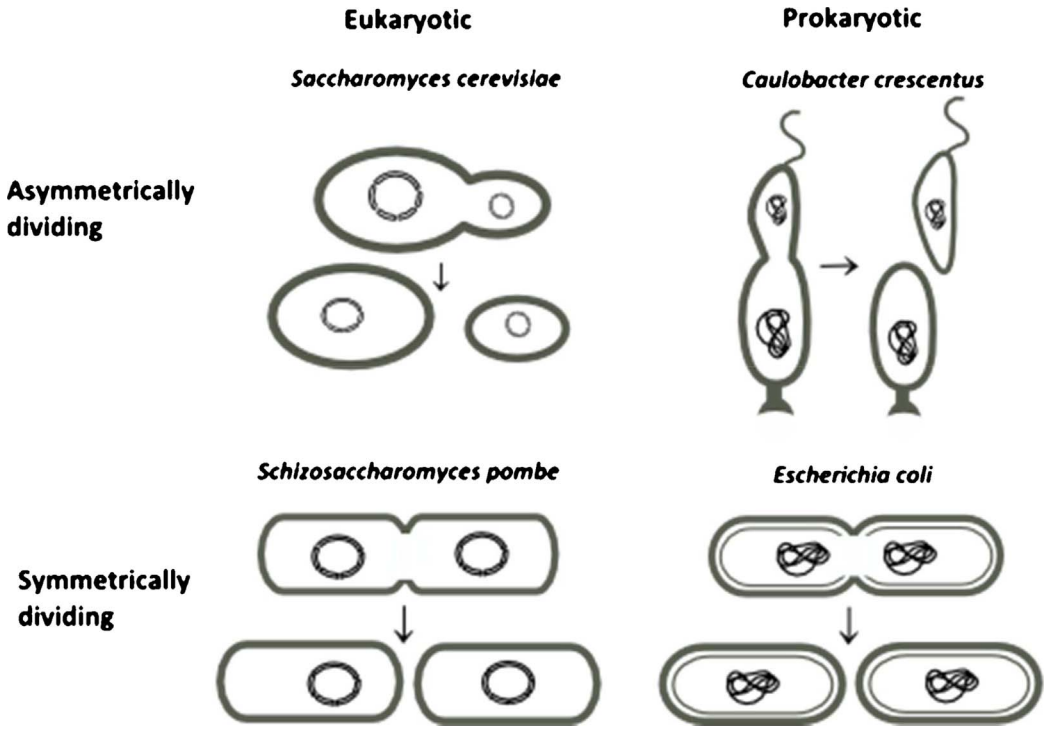

Fig. 2. The model unicellular species used for aging studies. Aging was first studied in the asymmetrically dividing yeast *Saccharomyces cerevisiae* (Mortimer and Johnston, 1959) and bacterium *Caulobacter crescentus* (Ackermann et al., 2003). *C. crescentus* reproduces by asymmetric fission of a stalked cell attached to a solid substrate, which produces a motile swarmer cell. Aging was then discovered in symmetrically reproducing species: the bacterium *Escherichia coli* (Stewart et al., 2005) and the fission yeast *Schizosaccharomyces pombe* (Coelho et al., 2013), both of which divide into morphologically similar daughters. Importantly, for symmetrically dividing species, no clear distinction between mother and daughter cell occurs, which was thought to preclude the possibility of aging.

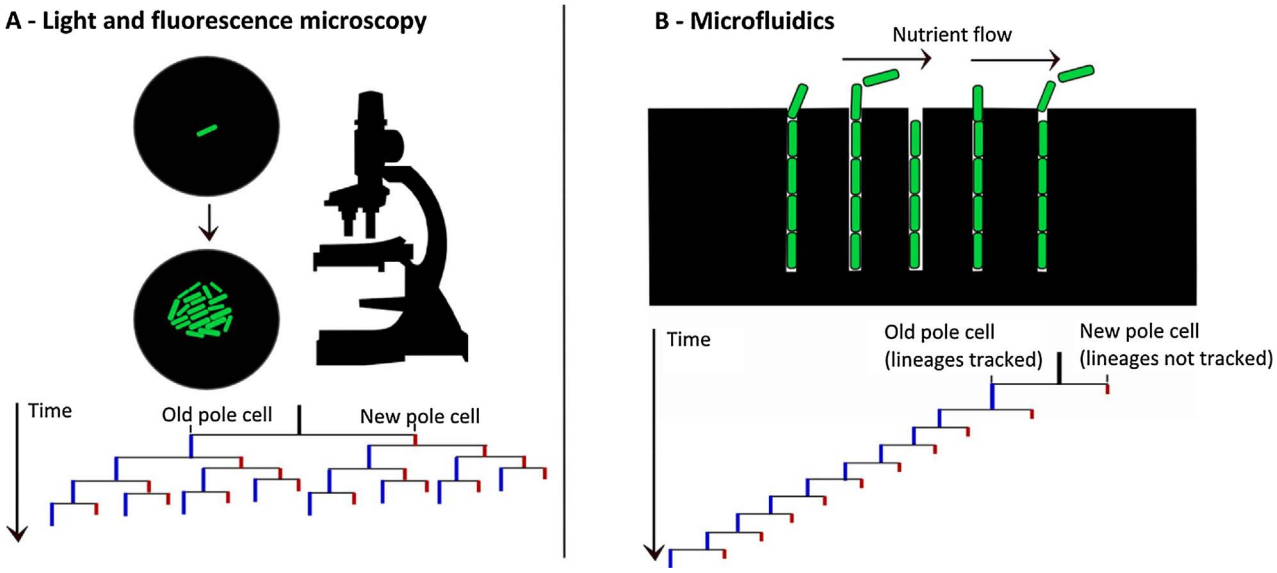

Fig. 3. Methods for studying aging in symmetrically dividing bacteria and yeast. (A) Light and fluorescence microscopy, used to study aging in *C. crescentus* (Ackermann et al., 2003), *S. pombe* (Barker and Walmsley, 1999; Coelho et al., 2013), *E. coli* (Stewart et al., 2005) and *S. cerevisiae* (Mortimer and Johnston, 1959). Using fluorescence microscopy and a strain expressing fluorescent proteins in conjunction with computational tracking, slight changes in cell division rates can be determined (Stewart et al., 2005). This allows comparisons of all cells, but is limited by the low number of divisions that can be observed (approximately 10). **(B)** Microfluidics with microscopy, used to study aging in *E. coli* and *S. pombe* long-term (Wang et al., 2010; Spivey et al., 2017). Nutrient is flown above the wells containing single cells, and growth is monitored via microscopy. While both new pole and old pole cells divide, old pole cells are retained at the bottom of the wells (bottom cell) and tracked for long periods of time while new pole cells (second to bottom and above) are extruded from the channel after a few divisions. This allows measurement of hundreds of consecutive divisions, but only allows tracking of old-pole lineages. Blue (left) and red (right) vertical bars indicate slower divisions of old pole cells and faster divisions of new-pole cells respectively.

et al., 2010). Alternatively, the differences could have been caused by differences in the experimental conditions, such as light intensity (which influences the generation of free radicals and ROS-damage; Greenbaum et al., 2000) or substrate (solid agarose vs liquid media).

In fact, there is evidence that both explanations may be correct. Studying aging in *E. coli*, Rang et al., 2011 developed a model to predict the behaviour of cells in a system where cells accumulate damage due to external stress and rejuvenate through continuous production of new cells. They see that in such system, cells that continuously receive the new pole (new pole lineage) converge to a state with a high division rate, while the cells that continuously receive the old pole (old pole lineage) fall to a state with a low division rate (see Fig. 4) (Rang et al., 2011). In these states, dubbed 'attractor' states, the division rates of both old pole and new pole lineages are stable, but the division rate of old pole lineage remains constantly lower than that of new pole lineage (Fig. 4). Importantly, the stable attractor states are predicted to be influenced by the level of extrinsic damage: the higher the extrinsic damage, the lower the division rate of old-pole cells is predicted to be (Rang et al., 2012). Were this model correct, it would explain the conflicting observations from fluorescence microscopy vs microfluidics experiments, as these two approaches used different media and photoactivation levels (which influences levels of extrinsic stress) and tracked cells over different timescales (Stewart et al., 2005; Wang et al., 2010).

Several lines of experiments indicate that this model may indeed be correct. Rang et al., 2012 show that there is a slight but significant difference between the division rates of old and new pole lineages in high antibiotic stress but not under low or no antibiotic stress. Further experiments show that under high levels of heat or antibiotic stress, old pole lineages show a high reduction in division rate compared with new pole lineages (Ni et al., 2012; Vedel et al., 2016). Combining experimental approaches with modelling, Vedel et al. further show that while some lineages converge to the old pole and new pole attractor states, these states represent the two extremes of division rates, with most cell having division rates between these two states (gray areas in Fig. 4) (Vedel et al., 2016). As a result, evidence indicates that in benign conditions, neither the new pole or old pole lineages of *E. coli* age. But when suffering from external damage, the old pole cell lineages of the population begin to suffer from a decreased division rate, proportionally to the level of damage (Fig. 4).

It therefore seems that aging in *E. coli* differs considerably from aging in most other species. In multicellular species, as well as unicellular species such as *S. cerevisiae* and *C. crescentus*, division rate decreases continuously after first reproduction until death (Mortimer and Johnston, 1959; Ackermann et al., 2003). This raises the question of whether the phenomenon seen in *E. coli* should be considered as aging at all. Importantly, this depends on one's definition of aging. Although the definition of aging is not universally agreed upon, in animal populations, aging is most commonly defined as a decreased fecundity and an increased probability of death with increasing age (Flatt, 2012). In conditional senescence studies, aging is commonly observed as decreased viability (as assayed by colony forming units on agar) of the bacterial cultures, and increased levels of stress and damage markers over time (Gonidakis and Longo, 2013). In the replicative aging studies of *E. coli* discussed above, most studies have used growth rate (i.e. fecundity) as their primary read-out for aging. While reduction in fecundity is often indicative of aging in other species, this is not always the case, and plateaued or even increasing fecundity is sometimes observed (Jones et al., 2014). This indicates that growth rate alone is not a sufficient proxy for aging, and should be accompanied by measurements of further parameters (probability of death, life span, viability and markers for damage, stress and senescence).

Nevertheless, while it is open to debate whether this phenomenon should be defined as aging, several observations argue that it indeed should. Firstly, in external stress, only the old pole lineages suffer from a decreased growth rate, while the new pole lineages replicate at a similar rate as cells without external stress (Rang et al., 2012). This is observed independently of the type of stress applied. Various sources of stress have been tested so far: streptomycin at different concentrations (Rang et al., 2012; Ni et al., 2012), kanamycin (Vedel et al., 2016), 42 °C heat shock (Ni et al., 2012; Vedel et al., 2016), hydrogen peroxide (in *S pombe*; Coelho et al., 2013) and potentially (inadvertently) also light-induced free radical damage as a result of fluorescence microscopy (Stewart et al., 2005). While there are some reports that different

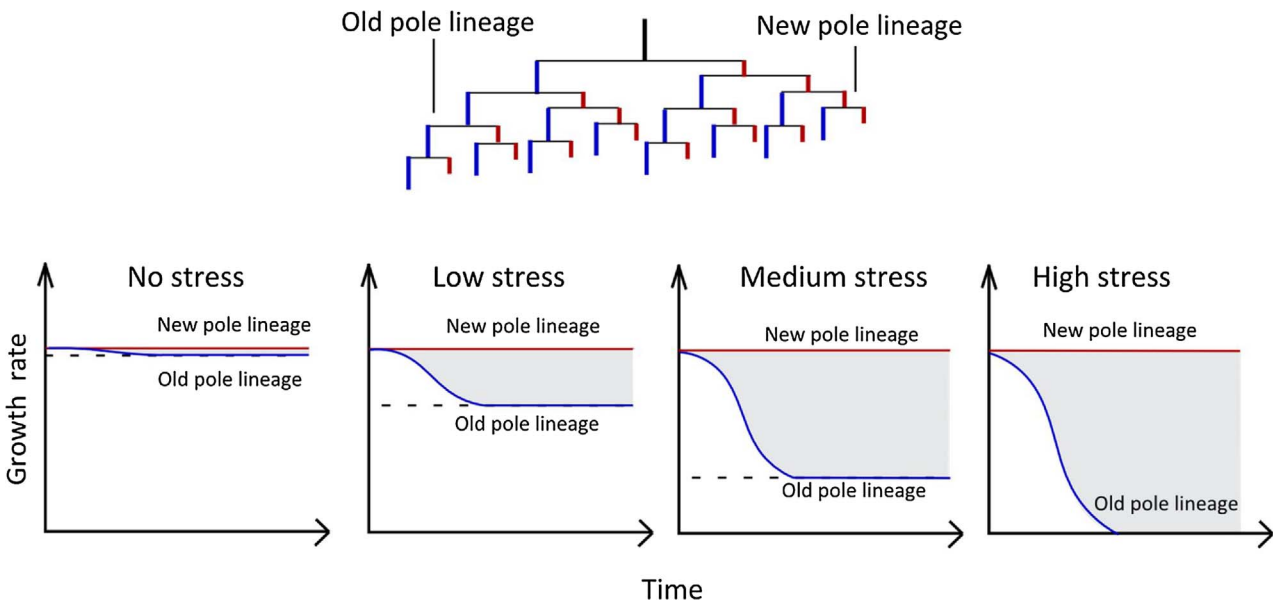

Fig. 4. Aging under different levels of stress. Growth rates of cell lineages receiving new pole vs old pole under no stress, low stress, medium stress and high stress. Growth starts from a rejuvenated cell. The rejuvenated new pole lineage retains a state with a high division rate, while the old pole lineage inherits more damage and suffers from decreased division rate. Both the new and old pole lineages reach states where division rate is stable, called attractor states. These states are dependent on the level of extrinsic stress − in low stress, the new pole and old pole lineages differ little, but at increased stress levels begin to differ considerably more. At high stress conditions, the old pole lineage division rate can decrease continuously until death. The two attractor states represent the extremes of high and low division rates, whereby most lineages occupy the space between them (gray area).

stressors may lead to somewhat different patterns of aging (see *Aging in S. pombe*), they all cause aging of only or mostly the old pole lineages. This asymmetry argues against simple stress-induced decline of division rate, which would be expected to affect all cell lineages equally. Secondly, although the division rate of *E. coli* did not decrease beyond a certain point in long-term experiments, an increase in probability of death with increasing age was nevertheless observed (Wang et al., 2010). This indicates that even when growth rate is stable, age-related damage still accumulates in the background, leading to eventual death of the cell. Finally, several studies have shown that markers indicative of damage and stress are present at higher levels in aged cells compared to rejuvenated daughters (see below in *Mechanistic causes of aging)*. Therefore, aging in *E. coli* seems to manifest itself as a decrease in division rate and an increased probability of death of old-pole lineages in response to stress.

3. Aging in *S. pombe*

Studies of aging in another symmetrically dividing species − the eukaryotic fission yeast *Schizosaccharomyces pombe* have yielded similar results. Reports of aging in *S. pombe* existed already before aging was discovered in *C. crescentus* (Barker and Walmsley, 1999), however whether *S. pombe* was truly aging was debated (see Minois et al., 2006). Since then however, considerable evidence has accumulated in support of aging in *S. pombe*. Aged cells show an increasing difference in morphology compared to new, rejuvenated daughters and die after a limited number of replications (Barker and Walmsley, 1999). Some cells in the population exhibit high levels of damaged proteins (Minois et al., 2006), with high damage levels being correlated with lower division rates (Erjavec et al., 2008). It was also shown that similarly to *E. coli, S. pombe* does not seem to age in favourable conditions, but does age under stress. In favourable conditions, cells showed no increase in mortality or decrease in division rate, but when treated with a heat shock or oxidative stress, showed a reduced division rate and an increased mortality in the aging cell lineage (Coelho et al., 2013). Interestingly, in *S. pombe*, aging was not correlated with old or new cell poles as was the case in *E. coli*, possibly because the new cell wall is synthesized from multiple areas along the whole cell wall in *S. pombe* (Coelho et al., 2013). Instead, aging was most correlated with inclusion bodies made of damaged proteins (see further below). The aging lineages in *S. pombe* therefore seem to consist of the daughter cells that continuously inherit protein or other macromolecular damage instead of a particular cell pole.

Finally, in an effort to track *S. pombe* divisions over long timespans, a recent study developed a microfluidics platform to continuously track *S. pombe* long-term (Spivey et al., 2017). The authors show that *S. pombe* can divide robustly for over 75 generations without changes in cell morphology, division rate or sibling health prior to sudden death (Spivey et al., 2017). Interestingly, they also observe that the replicative lifespan of *S. pombe* was increased by overexpression of sirtuins or treatment with rapamycin (Spivey et al., 2017). This indicates that similarly to *E. coli*, the division rates of *S. pombe* lineages remain stable in benign conditions, but that some damage is still accumulating in the background, and that this damage accumulation is reduced by these pro-longevity treatments. Considered together, these studies indicate that similarly to *E. coli, S. pombe* does not show conventional aging hallmarks in favourable conditions, but starts to age in response to external stress (Fig. 4).

4. Mechanistic causes of aging

The discovery of aging in these unicellular species raises the question of what ultimately causes aging on the cellular level. Damage to various cell components, including cell wall, proteins, organelles and DNA have been proposed. The majority of studies have evaluated protein damage (Lindner et al., 2008; Lindner and Demarez 2009; Villar-Pique et al., 2012; Coelho et al., 2013; Baig et al., 2014) and damage to the proteome certainly seems to play a role. In *S. pombe*, aging was best correlated with asymmetric segregation of large protein aggregates that formed as a result of external stress, rather than a cell pole (Coelho et al., 2013; Coelho et al., 2014). It is also notable that the pattern of protein aggregation and aging (decrease in division rate before death) differed between *S. pombe* cells treated with a heat shock or reactive oxygen species, indicating that different types of damage may cause aging in different ways (Coelho et al., 2013). Protein damage has also been implicated in the aging of *E. coli,* as old-pole cell lineages

have a higher probability of receiving protein aggregates, and aggregates alone (independent of old poles) explain approximately 30% of the decrease in division rate (Lindner et al., 2008; Winkler et al., 2010). How protein aggregates specifically bring about the reduction in fecundity and increased mortality is unknown, but it has been shown that aggregates can cause cellular toxicity through a variety of mechanisms, including increased production of reactive oxygen species, crowding of the cytoplasm, and an increasing recruitment of newly synthesized proteins to aggregates (see Bednarska et al., 2013 for a full review).

Despite the clear role of protein damage, aging in *E. coli* could not be explained by protein aggregates alone (Lindner et al., 2008), and it is likely that factors other than protein damage are involved. Although the old pole cell wall was the first cellular component to be tracked in *E. coli,* effects of damage to the cell wall seems not to have been studied extensively.

Another possibility is damage to DNA, which has been implicated to contribute to aging in yeast and multicellular species (Sinclair and Guarente, 1997; Garinis et al., 2008). If DNA damage were to also play a role in aging of *E. coli* and *S. pombe*, it would somehow have to be asymmetrically confined to one daughter cell. In *S. cerevisiae*, the old mother cell accumulates toxic rDNA circles, which are not passed on to the young daughter (Sinclair and Guarente, 1997), however rDNA circles have not been reported in *E. coli* or *S. pombe,* and whether and to what extent they are important in these species is unknown. In addition to rDNA circles, it has also been proposed that two DNA strands may be damaged differently, and that the damaged strand is selectively segregated during division to the aging cell (the immortal strand hypothesis − see Cairns, 1975). However, the existence of this is heavily debated even in mammalian stem cells in which it was first proposed (see Rando, 2007; Pine and Liu, 2014; Tomasetti and Bozic, 2015). Currently no empirical evidence exists to imply that this occurs in *E. coli*, *S. pombe* or any other unicellular organism.

Therefore, the majority of current evidence points towards damage to the proteome and subsequent asymmetric segregation of the damaged proteins as a major proximal cause of aging in *E. coli* and *S. pombe*. However, no mechanism that explains all of the observed aging effects have yet been reported, and it is likely that other factors are also important. In the well-studied *S. cerevisiae,* in addition to rDNA circles and damaged proteins (Zhou et al., 2014; Coelho and Tolić, 2015; Nyström and Liu, 2014a; Saarikangas and Barral, 2015; Saarikangas and Barral, 2016), the aging mother cells also show defects in vacuolar and cytoplasmic pH (Hughes and Gottschling, 2012; Henderson et al., 2014) and accumulates reactive oxygen species (Laun et al., 2004) and dysfunctional mitochondria (Scheckhuber et al., 2007; Winkler et al., 2010). These types of damage may also be important to symmetrically dividing unicellular species, however to what extent this is true is unknown. It is worthwhile to point out that in these studies, asymmetric segregation of damage did not lead to observable morphological differences between daughter cells. This indicates that asymmetric damage segregation does not necessarily lead to loss of symmetric divisions, defined as divisions that produce morphologically identical daughter cells as observed by microscopy. Therefore, symmetry of divisions can not be used as a proxy for damage segregation symmetry − whether damage is segregated asymmetrically or not must be determined with molecular tools specific to the type of damage in question.

5. Evolutionary benefits of asymmetric damage segregation

Observing asymmetric segregation of damage in the very distantly related *S. pombe* and *E. coli* suggests that it may have arisen due to some evolutionary advantage. Several models concerning various aspects of damage segregation have been created to estimate the evolutionary benefit of asymmetric damage segregation (Johnson and Mangel, 2006; Watve et al., 2006; Ackermann et al., 2007a; Erjavec et al., 2008; Chao 2010; Winkler et al., 2010; Rang et al., 2011; Rashidi et al., 2012; Clegg et al., 2014; Koleva and Hellweger, 2015; Lade et al., 2015; Vedel et al., 2016). These vary in their strategy, complexity and assumptions, however a common theme emerging from them is that under stressful conditions, populations in which cells segregate damage asymmetrically are predicted to tolerate higher levels of damage and outcompete the populations that do not. Some models even predict that asymmetric damage segregation may favour itself in a positive feedback loop (Vedel et al., 2016; Ni et al., 2012). As a result, multiple lines of evidence suggest that asymmetric damage segregation serves to limit macromolecular damage to only some cells of the population, and despite causing aging in these cells, leads to increased survival of the population as a whole. While population-level selection is unlikely to occur in animals, such selection can occur in microbes, as unlike in animals, the individual microbial cells are genetically almost identical. This conclusion is also reached when accounting for conditional senescence (see Gómez, 2010).

Of note, most models have not considered the role of active repair (degradation and re-synthesis of damaged proteins) in homeostasis, and contrary to most other models, a model proposed by Clegg et al. suggests that asymmetric segregation of damage is only advantageous when damage is abundant and toxic, and efficiency of repair is low or costly, while repair is the optimal strategy in other conditions (Clegg et al., 2014). Indeed, this is supported by the observation that in low levels of external damage, *E. coli* and *S. pombe* do not segregate damage unequally (Lindner et al., 2008; Erjavec et al., 2008; Vedel et al., 2016), indicating that damaged components are efficiently repaired or diluted during cell division. However, it must be noted that some types of protein damage (for example carbonylation) have been reported to be highly resistant to degradation in both species (Maisonneuve et al., 2008; Aguilaniu et al., 2003), and very long-lived protein junk has been found in *S. cerevisiae* (Thayer et al., 2014). Therefore while in most conditions repair may be the optimal strategy, the conditions in which asymmetric damage segregation has been observed to occur may indeed meet the requirements of abundant damage and low efficiency of repair as proposed by Clegg et al. (2014).

Given the likely evolutionary advantages of damage segregation, cells may have evolved mechanisms to actively control asymmetric segregation of damaged macromolecules. In *S. cerevisiae*, protein aggregates are tethered to mother cell organelles (Spokoini et al., 2012; Zhou et al., 2014) and have been shown to be actively transported via actin filaments by a machinery involving the polarisome (Fig. 5) (Liu et al., 2010; Liu et al., 2011; also see Coelho and Tolić, 2015). In *S. pombe* and *E. coli*, active transport of aggregates has not been reported. However, in *S. pombe*, it has been shown that large aggregates accumulate from smaller ones in a process requiring Hsp16 (Fig. 5) (Coelho et al., 2013, 2014), and that the formation of these aggregates leads to effective segregation of protein damage to only one daughter cell (Lade et al., 2015). In *E. coli*, formation of large aggregates similarly facilitates asymmetric damage segregation, as large aggregates are localized to one pole by diffusion and macromolecular crowding (Lindner et al., 2008; Coquel et al., 2013). Further studies indicate that in *E. coli*, asymmetric segregation of protein aggregates occurs as a result of the nucleoid region excluding the aggregates from the mid-cell region, which causes the aggregates to accumulate at the old cell pole (Gupta et al., 2014). This process occurs relatively robustly, although it can be affected by low temperatures (Gupta et al., 2014; Neeli-Venkata et al., 2016; Oliveira et al., 2016). Therefore, although active control of asymmetric damage segregation has not been reported for *E. coli* or *S. pombe*, it seems that mechanisms to ensure robust damage segregation have nevertheless evolved in these species as well.

The observation that *E. coli* and *S. pombe* only age under stress raises the question of why *C. crescentus* and *S. cerevisiae* age obligatively, even in benign environments. Unlike *E. coli* and *S. pombe*, *C. crescentus* and *S. cereivisiae* divide asymmetrically, which may offer a potential explanation. If asymmetric division somehow predisposes to constant segregation of damage into one cell, it is possible that obligative aging

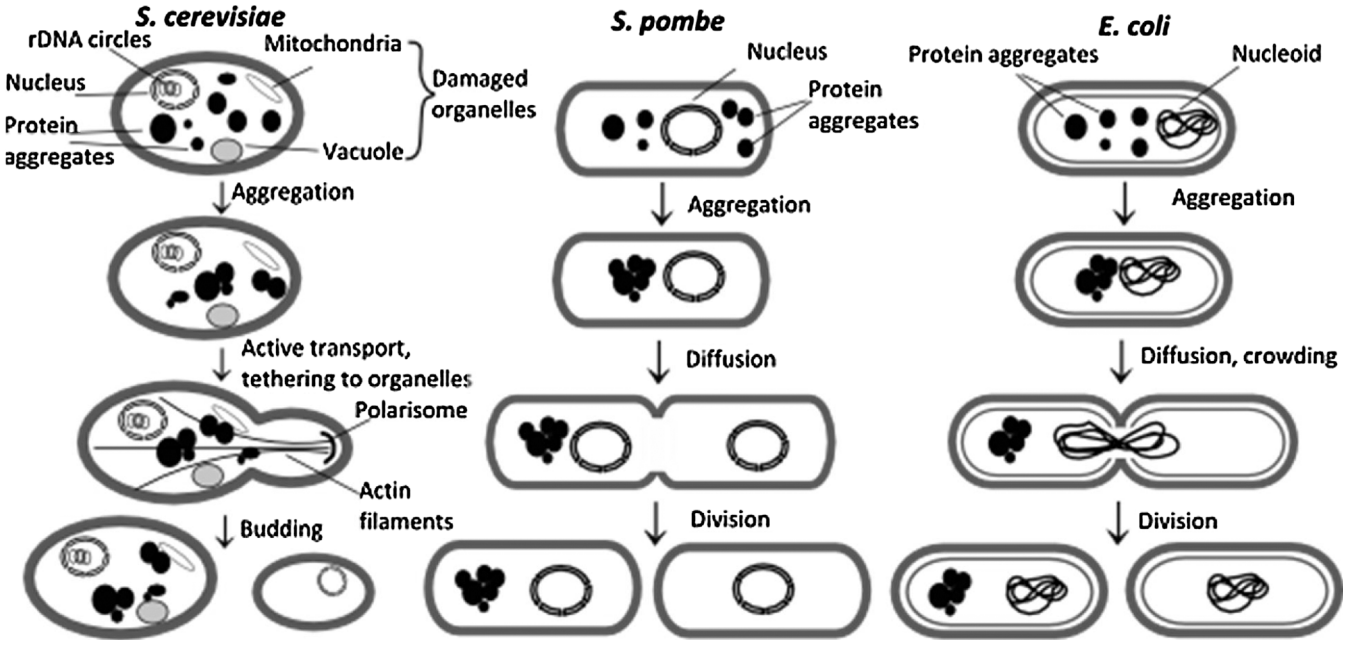

Fig. 5. Mechanisms of damage segregation in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Escherichia coli*. In *E. coli* and *S. pombe*, asymmetric segregation of protein aggregates is achieved by fusion of damaged proteins into large aggregates, which then segregate into one cell due to the effects of macromolecular crowding and diffusion (Lindner et al., 2008; Coquel et al., 2013; Coelho et al., 2014; Lade et al., 2015). In *S. pombe,* fusion of aggregates has also been shown to be Hsp16-dependent (Coelho et al., 2013; Coelho et al., 2014). In *S. cerevisiae*, protein aggregates have been shown to be tethered to mother cell organelles (nuclear membrane, mitochondria, vacuoles) (Spokoini et al., 2012; Zhou et al., 2014), and are actively transported along actin filaments to the mother cell in a process involving the polarisome (Liu et al., 2010; Liu et al., 2011; also see Coelho & Tolić 2015). Furthermore, damaged organelles and rDNA circles are also maintained in the mother cell through a variety of mechanisms (Denoth Lippuner et al., 2014). If and to what extent similar processes occur in *E. coli* or *S. pombe* is unknown. In all cases, damage segregation results in one damage-free new daughter cell and one damage-containing old daughter cell.

is simply the byproduct of morphologically asymmetric division. It is not known whether this is the case, however this could conceivably occur if the diffusion of damaged molecules into the daughter cell cytoplasm is restricted or if the daughter cell receives the majority of newly synthesized organelles or cell components. If so, obligative aging could be permitted to arise if the life span of cells in their natural environment is low and longevity is not selected for, or if the fitness cost of aging is low. Indeed, on average, yeast undergoes around 30 divisions and *C. crescentus* around 40 divisions before death (Mortimer and Johnston, 1959; Ackermann et al., 2003), meaning that in the absence of other causes of mortality, roughly only 1 in 2^{30} and 1 in 2^{40} cells in a population respectively die of aging (although this does not account for losses due to decreased fecundity). There is limited evidence that obligative aging may indeed be a byproduct. In *S. cerevisiae*, it was found that during division, a septin-dependent diffusion barrier forms in the nuclear envelope between the mother and daughter cells, which restricts the diffusion of rDNA circles into the daughter (Shcheprova et al., 2008). Furthermore, when populations of *C. crescentus* were subjected to strong early selection pressures (which was expected to lead to faster aging), the majority of populations surprisingly evolved lower rates of aging (Ackermann et al., 2007b). This indicates that increased longevity was a byproduct of some other mechanism (Ackermann et al., 2007b). However, very few studies have examined this question, and at present aging has been studied in too few unicellular species to determine whether asymmetric division is causatively linked to obligative aging.

6. Aging in other unicellular organisms

There are now well-studied examples of aging in the asymmetrically dividing *S. cerevisiae* and *C. crescentus* (Jazwinski 1993; Ackermann et al., 2003) as well as the symmetrically dividing *E. coli* and *S. pombe* (Stewart et al., 2005; Coelho et al., 2013). Discovery of aging in these species raises the question of how prevalent aging is among unicellular species in general. In addition to these four species, there are also limited reports of aging in *Paramecium tetraurelia* (Smith-Sonneborn, 1979; Aufderheide, 1987), *Mycobacterium* (Aldridge et al., 2012), *Bacillus subtilis* (Veening et al., 2008), *Methylobacterium extorquens* (Bergmiller and Ackermann 2011), diatom *Ditylum brightwellii* (Laney et al., 2012) and alga *Chlamydomonas reinhardtii* (Damodaran et al., 2015; Humby et al., 2013). These examples indicate that aging could be the rule, rather than exception in unicellular organisms. Furthermore, asymmetric division has been recorded in a large number of species (Kysela et al., 2013), and if asymmetric division is accompanied by obligative aging (as in *C. crescentus* and *S. cerevisiae*), then a large number of species would be expected to age. However, currently very little is known about the aging of the vast majority of unicellular species. Furthermore, aging has so far been tested only in simplified laboratory environments, due to the difficulties of detecting the subtle changes in phenotypes in more complex environments that would emulate natural conditions. This means that whether aging is important or occurs similarly in the context of varying environmental conditions, competition for food, predation, etc. is unknown. It is clear that large-scale studies are needed for a more comprehensive overview. Recently, a generalized data analysis method was proposed for the study of aging in unicellular organisms (Wu, 2013). This determines “old” and “young” cells probabilistically based on division rates and is not linked to cell poles, which may be useful to study aging in a larger variety of unicellular species (Wu, 2013).

7. Aging of the animal germ line

The discovery of aging and asymmetric damage segregation in unicellular species indicates that similar processes may occur in the germline of multicellular species. Similarly to unicellular species, the germline of multicellular species must remain functionally immortal. Indeed, germline stem cell (GSC) serial transplantations in mice have shown that male GSCs can sustain spermatogenesis longer than a single lifespan (Ryu et al., 2006). However, studies in humans, *Drosophila* and *C. elegans* demonstrate that the germline is nevertheless susceptible to

Box 2
Programmed vs non - programmed theories of aging.

In 1889, the prominent German biologist August Weismann described one of the first modern theories of aging. He viewed aging as an adaptive process, programmed into every animal, as '…the individual should be enabled to do its work towards the maintenance of species. This work is reproduction… As soon as the individual has performed its share ..., it ceases to be of any value to the species, and may die' (Weismann, 1889). Numerous theories of aging have since been proposed - Medvedev in 1990 could count over 300 (Medvedev 1990), however they can broadly be divided into non-programmed and programmed theories. Non-programmed theories state that aging is mainly caused by accumulation of damage through lifespan, the result of processes such as somatic mutations (Kennedy et al., 2012), reactive oxygen species (Hekimi et al., 2011), protein aggregation (Terman and Brunk, 2004), and others. Programmed theories of aging are broad, but generally state that aging arises due to genetic programs that control a sequence of events leading to aging. These events are not necessarily accompanied by damage accumulation, or cause damage accumulation over a short period of time.

Weismann's was the first of the 'adaptive' programmed theories of aging. However, since then, other 'mechanistic' programmed theories of aging have been proposed. It is important to draw a distinction between the two. 'Mechanistic' programmed theories state that aging proceeds by maladaptive or selectively neutral programs (for example, dysregulation or faults in normal cellular programs) and despite the similar terminology, are conceptually distinct from the 'adaptive' programmed theories, which propose that aging itself is adaptive and therefore controlled by a program. Although some proponents for 'adaptive' programmed theories exist (see Goldsmith, 2012), the current consensus is that with potential rare exceptions, aging itself is not adaptive (Kirkwood and Melov, 2011; López-Otín et al., 2013; Kenyon, 2010), as empirical evidence (Ricklefs, 2010) and convincing arguments (Kirkwood and Melov, 2011) provide a strong case against this idea. Namely, adaptive aging would require strong group selection in order to counter the individual fitness loss that accompanies aging, and this is rarely encountered (Kirkwood and Melov, 2011). However, although aging itself is not generally beneficial, programs that cause aging could nevertheless exist if they are pleiotropic and beneficial early in life (Williams, 1957) or when they exert their effects only late in life (Medawar 1952) when natural selection is inefficient at removing them. As the term 'programmed aging' is used loosely, different authors have understood it as different concepts, which has been the source of much confusion (Kirkwood and Melov, 2011, also see Austad 2004a; Bredesen 2004a; Austad 2004b; Bredesen 2004b). Because of this, it is important to specify that in the context of this work, programmed aging denotes 'mechanistic' programmed aging - the theoretical phenomenon where one or more genes lead to aging via maladaptive or dysregulated cellular programs, without the assumption that this is selectively advantageous.

There is evidence for both damage and 'mechanistic' programmed theories of aging. Damage is generally considered responsible for genome instability (Moskalev et al., 2013), mitochondrial dysfunction (Wallace, 2010), telomere loss (Blasco, 2007), loss of proteostasis (Powers et al., 2009), cell senescence, and other age-associated changes (López-Otín et al., 2013). Aspects of aging attributed mainly to programmed changes include NF-κB overexpression as a part of age-related inflammation (Salminen et al. 2012; Adler et al. 2007), maladaptive gene regulation by elt-3/elt-5/elt-6 GATA transcription circuit in *C. elegans* (Budovskaya et al., 2008) and menopause in human females (Shanley and Kirkwood, 2001) (for a full review, see López-Otín et al., 2013 and Kirkwood and Melov, 2011). On the organismal level, both types likely contribute to aging, and should be considered in a system-wide manner (Kirkwood, 2005). On the cellular level however, evidence from *E. coli* and *S. pombe* indicate that it is likely damage, not programs, that fundamentally causes aging (see Is aging caused by damage or programs?).

aging. Division rate, stem cell number and expression of self-renewal factors all decrease with age (Wallenfang et al., 2006; Boyle et al., 2007; also see Smelick and Ahmed, 2005; Fuller and Spradling, 2007). Furthermore, aging of the mammalian germ line is well documented as the female menopause in mice, rats, humans and other species (Tilly and Sinclair 2013). The mechanisms by which damage removal in GSCs is achieved are not known, but selection for healthy cells and increased cellular repair in stem cells or during differentiation have commonly been proposed (Hernebring, et al., 2006; Jones, 2007). However, results from *E. coli* and *S. pombe* imply that damage free progeny could alternatively be achieved through asymmetrical segregation of damage into some of the germ cells, which would maintain a pool of damage-free cells for the next generation.

Whether this indeed occurs seems not to have been systematically investigated. There is limited evidence suggesting that asymmetric damage segregation does occur in some types of stem cells. Asymmetric cell division has been reported in most stem cell types, and may concurrently favour asymmetric segregation of damage (Neumüller and Knoblich 2009). More specifically, asymmetric segregation of damaged proteins have been found in human and *Drosophila* intestinal stem cells, mouse and *Drosophila* neuronal stem cells and *Drosophila* female germline stem cells (Rujano et al., 2006; Bufalino et al., 2013; Moore et al., 2015). Notably, Fuentealba et al. (2008) found asymmetric segregation of damaged proteins in 80%-90% of human embryonic stem cell divisions (Fuentealba et al., 2008). Recently, it was found that inclusion bodies also segregate asymmetrically in several mammalian cell lines, and that this seems to be actively controlled by vimentin intermediary filaments (Ogrodnik et al., 2014). Standing on these results, a model for damage segregation in mammalian germ-line cells and tissues was developed, which suggests that asymmetrical damage segregation reduces overall damage loads and increases population longevity (Strandkvist et al., 2014). Taken together, these studies indicate that asymmetric damage segregation may play a role in germ line stem cells. However to which extent this is functionally important is unknown. If unequal damage segregation is indeed important, it is likely to function in conjunction with the other proposed mechanisms of germ line maintenance (see Jones, 2007).

8. Is aging caused by damage or programs?

The discovery of aging in symmetrically dividing microbes may have important consequences to other questions in aging. One of the longest debates in aging revolves around whether aging is caused by accumulation of damage or by genetic programs (Austad 2004a; Bredesen 2004a; Kirkwood and Melov 2011; Goldsmith 2012). Theories that support damage state that macromolecular or organellar damage, caused by accumulation of somatic mutations (Kennedy et al., 2012), reactive oxygen species (Hekimi et al., 2011), protein aggregates (Terman and Brunk, 2004), and other mechanisms is ultimately responsible for aging. Programmed theories of aging generally state that aging arises due to genetically controlled sequence of events, which are not necessarily accompanied by damage accumulation, or cause damage accumulation acutely over a short period of time (see Box 2. *Programmed vs non-programmed theories of aging*). With the exception of

S. cerevisiae, most studies focussing on the causes of aging have used multicellular animals (see Kenyon, 2010). Although they have located numerous genetic changes that considerably modulate aging, they have not been able to demonstrate what the fundamental causes of aging are, as no changes which would completely abolish aging have been found (Kirkwood and Melov 2011). However in the case of *E. coli* and *S. pombe*, we see two species that show no signs of aging in favourable conditions, yet age as a response to external stress (Stewart et al., 2005; Coelho et al., 2013). Aging in these species has been majorly attributed to accumulation of damage in the proteome or cell wall (Lindner et al., 2008; Erjavec et al., 2008; Coelho et al., 2013), and so far there is no evidence that a program is involved. Therefore, it seems that aging is the unfortunate side-product of damage accumulation that occurs after damage is selectively segregated into the aging old cell lineages. This can occur, since unlike individual animals in a population, individual cells in a microbial colony are genetically almost identical (the mutation rate in *E. coli* is approximately 10^{-3} and *S. cerevisiae* 10^{-1} per cell per generation; Lee et al., 2012; Zhu et al., 2014). *E. coli* and *S. pombe*, therefore provide perhaps the clearest evidence yet that different sources of damage, and not genetic programs, are the fundamental cause of aging at the cellular level.

9. Conclusion

E. coli and *S. pombe* embody the first known cases where otherwise non-aging species can be induced to age. Evidence suggests that they do not age in benign conditions, but start to age in response to external stress. In times of stress, damage is selectively segregated into a fraction of a population's cells, which sacrifices these cells to aging, but maintains the viability of the population as a whole.

These discoveries, in conjunction with the predicted fitness benefits of asymmetric damage segregation, suggest that aging may be ubiquitous among microorganisms in general. While aging has been confirmed in a handful of species (*E. coli*, *S. pombe, S. cerevisiae*, *C. crescentus*) and indicated in a few others, very little is known about aging in the vast majority of other unicellular species, and how aging influences their population dynamics in natural environments. In short, broad studies are needed to map the variation and mechanisms of aging in microbes. It is likely that such studies will unearth fundamentally new processes, and in the process, inform other questions in the field of aging as well.

Conflict of interest

The author declares no conflict of interests.

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