REVIEW ARTICLE

Budding yeast as a model organism to study the effects of age

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Received 16 August 2013; revised 13 December 2013; accepted 6 January 2014. Final version published online 26 February 2014.

DOI: 10.1111/1574-6976.12060

Editor: Sophie Martin

Keywords
Saccharomyces cerevisiae; longevity; rejuvenation; aging factor; asymmetry; age metrics.

Abstract

Although a budding yeast culture can be propagated eternally, individual yeast cells age and eventually die. The detailed knowledge of this unicellular eukaryotic species as well as the powerful tools developed to study its physiology makes budding yeast an ideal model organism to study the mechanisms involved in aging. Considering both detrimental and positive aspects of age, we review changes occurring during aging both at the whole-cell level and at the intracellular level. The possible mechanisms allowing old cells to produce rejuvenated progeny are described in terms of accumulation and inheritance of aging factors. Based on the dynamic changes associated with age, we distinguish different stages of age: early age, during which changes do not impair cell growth; intermediate age, during which aging factors start to accumulate; and late age, which corresponds to the last divisions before death. For each aging factor, we examine its asymmetric segregation and whether it plays a causal role in aging. Using the example of caloric restriction, we describe how the aging process can be modulated at different levels and how changes in different organelles might interplay with each other. Finally, we discuss the beneficial aspects that might be associated with age.

Introduction

Every human being experiences the effects of getting older, a process called aging, and every gray hair, age spot or forgotten name serves as a reminder. But, although signs of age are evident in most multicellular organisms, unicellular species have long been mistaken as being immortal. For example, budding yeast can be propagated in culture indefinitely, similarly to mammalian immortalized cells. However, when A. Barton followed single 'mother' yeast cells, systematically removing every daughter cell produced, he discovered that single cells eventually die (Barton, 1950). The analysis of cell cohorts indicates that mortality increases during the life, a hallmark of aging (Fig. 1a; Mortimer & Johnston, 1959). Furthermore, cultures inoculated with the first or last daughters of the same mother cell are both able to grow (Barton, 1950). These early experiments demonstrated that, although yeast cells are mortal, their offspring do not inherit what kills their mother cells and are therefore born with a reset lifespan. This also speaks for the finite lifespan of yeast cells being the product of an aging process, rather than of some cellular disease. Because budding yeast has been characterized in great detail at the molecular level and powerful tools are readily available to alter and monitor cellular processes, this organism has emerged as an unexpected model species for studying aging.

In yeast, aging is studied using two main approaches. Replicative lifespan is defined as the number of buds produced before death. In practice, the replicative lifespan is measured by counting the number of divisions achieved by a cell whose buds are removed one by one by microdissection (Fig. 2a). Aging can be characterized based on the distribution of replicative lifespans. Alternatively, instead of focusing on divisions, the chronological lifespan is measured as the time a cell survives in a nondividing state, with survival being defined as cell wall integrity or as ability to form a colony. Aging is then characterized based on the distribution of chronological lifespans, obtained by measuring the decrease in survival with time in a stationary phase culture. Yeast replicative aging is thought to be comparable to aging phenomena observed in asymmetrically dividing cells of higher

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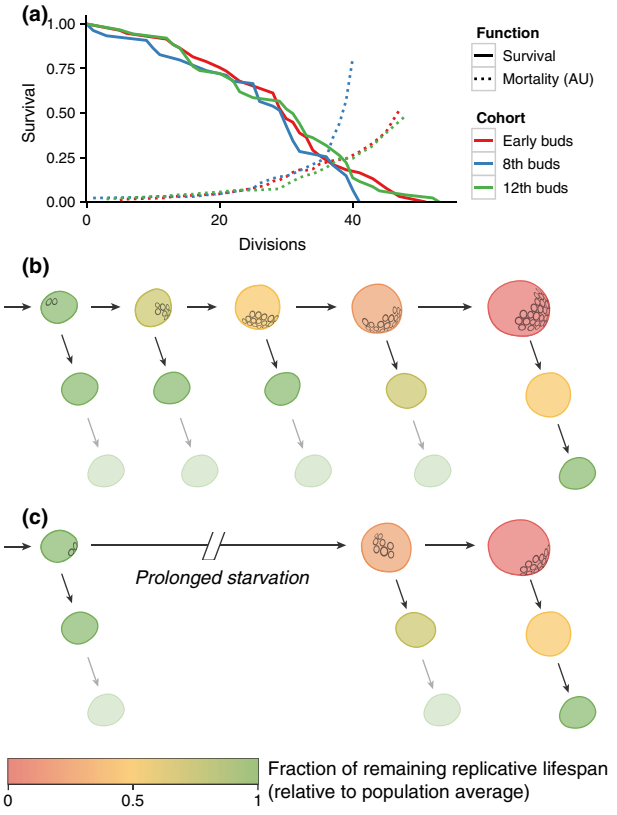

Fig. 1. Yeast cell age is asymmetrically segregated between mother and daughter cells. (a) The survival curve of a wild-type yeast population (solid red line) shows a sigmoidal shape indicating that the mortality increases with age. Based on this experimental survival curve (the probability $l(a)$ to survive up to age a; solid line), the mortality rate is obtained as $-\mathrm{d}\log l(a)/\mathrm{d}a$ (dashed line). Measuring the lifespan of cells produced by mother cells of different ages (in the first part of life) reveals that daughter cells have a full lifespan potential (blue and green line; replotted from Shcheprova *et al.*, 2008). (b) The rejuvenation process ensures a complete reset of the replicative lifespan potential during most of the lifespan. However, daughter cells of old mothers have a shorter lifespan. (c) When cells are maintained in starvation for a long period and hence accumulate chronological aging, their own replicative lifespan (as well the one of their buds) is decreased. In addition, the decrease in replicative lifespan of the buds produced either late in life or after prolonged starvation is not passed on to the next generations (b, c).

eukaryotes, such as stem cells. Yeast chronological aging is akin to the aging of nondividing cells such as neurons (Longo *et al.*, 2012). Finally, a third type of aging is observed in certain mutant strains that cannot be propagated eternally, a phenomenon called clonal senescence, which resembles the senescence process in telomerase deficient mammalian cells (Lundblad & Szostak, 1989; Singer & Gottschling, 1994). In this review, we focus on replicative aging in *Saccharomyces cerevisiae* as it has the longest history and has been most extensively characterized. Relevant observations associated with chronological aging are reported when available; we refer readers with specific interest in this process to dedicated reviews (Longo *et al.*, 2012; Piper, 2012).

Preparing large amount of cells that have undergone multiple divisions is intrinsically difficult, as old cells are diluted in their progeny during exponential growth; hence, replicative aging has been studied primarily by manual microdissection. However, this technique is both tedious and unsuitable for high-throughput studies. In the past years, new methods have been developed that overcome this limitation (Fig. 2). The first new technique is the Mother Enrichment Program (MEP), which uses a strain in which newly born daughters are prevented from dividing. This leads to a linear dilution (rather than exponential) of mothers in a population of arrested daughters and greatly facilitates the preparation of large populations of cells with a well-defined replicative age (Lindstrom & Gottschling, 2009). More recently, microfluidic devices have been used to follow the entire lifespans of yeast cells under a microscope (Lee *et al.*, 2012; Xie *et al.*, 2012). This allows the study of cellular events over the lifespan with unprecedented accuracy and can incorporate the use of fluorescent reporters. The different techniques used to study replicative aging are described in detail in the Appendix 1.

How do the aging phenomena observed in budding yeast relate to aging in other organisms? In general, aging is defined as 'any age-specific decline in variables associated with individual fitness, specifically mortality, reproduction and physiological performance' (Reznick *et al.*, 2004). These three components of aging are observed during replicative aging in budding yeast, as discussed below. Studies of biologic aging in different species share that aging is measured at the population level. This is not only a statistical requirement to reduce the measurement variability, but it comes more fundamentally from the fact that aging is a secondary trait which is measured as the age-specific change of primary traits. Two classes of hypotheses on the origin of aging are that it either follows a program selected by evolution and leading to cell death after a given time or that it results from the accumulation of damage during life. As any program-based explanation is unlikely to be general from an evolutionary point of view (Kirkwood & Melov, 2011), most attention has been given to damage accumulation. In particular, aging has been proposed early on to result from features selected for advantages they provide to the individual early in its life, but become deleterious later (Williams, 1957). Because of the unique insights we have into molecular mechanisms in yeast, this species may allow us to test how this hypothesis translates into molecular and cellular terms.

In this review, we describe the sequence of changes that occur in yeast cells as they age and discuss whether or not

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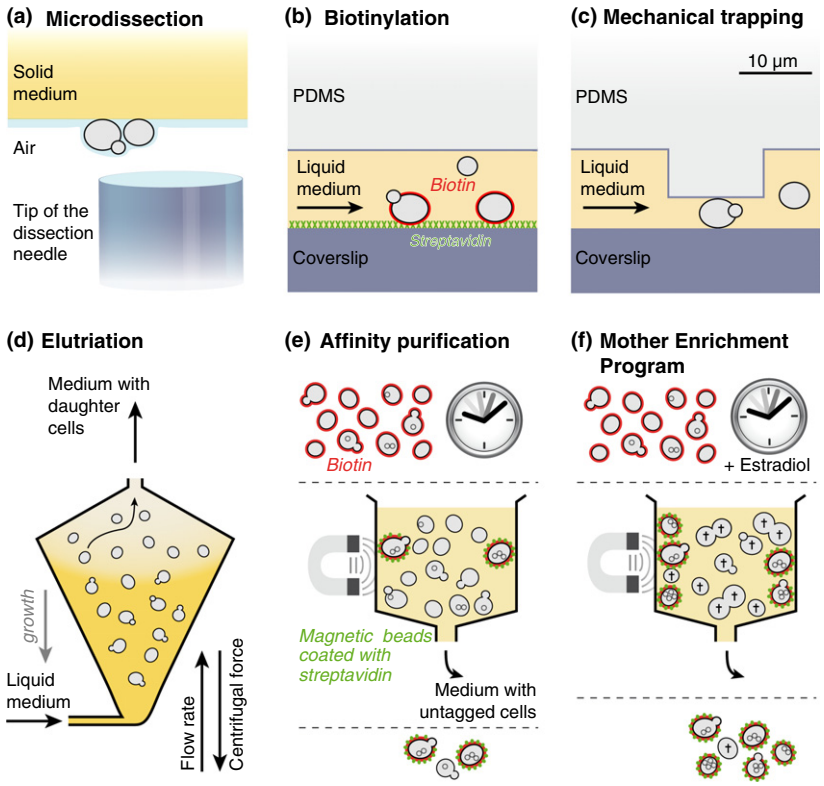

Fig. 2. Recent advances in techniques used to study age-associated changes. (a) At the single-cell level, the traditional life-long monitoring method, microdissection, consists in using a microscopic needle to remove all buds produced by a newborn cell. (b, c) Newly designed microfluidic devices allow to perform similar experiments and to quantify the intensity of fluorescent reporters during all the lifespan, based either on chemical trapping (b; adapted from Xie *et al.*, 2012) or on mechanical trapping (c; adapted from Lee *et al.*, 2012). (d) Elutriation sorts cells based on their size by removing the small buds, while mother cells are dividing in the centrifuge (adapted from Woldringh *et al.*, 1995). (e) Old cells of controlled age can be obtained by tagging mother cells with biotin, letting them divide and then sorting them on purification columns. (f) In the MEP, daughter cells cannot divide anymore upon estradiol addition, leading to linear rather than exponential growth of the population (Lindstrom & Gottschling, 2009). Old cells are further purified as in (e).

they are detrimental. We first report on the age-associated changes observed in traits affecting global properties of the cell, which is at the organism level. In a second part, we review the molecular changes observed within the different cellular compartments. The role of these changes in the process of aging itself is discussed in the context of damage accumulation. In a third section, we describe the known effects of caloric restriction to discuss how these changes integrate together. Finally, we discuss more general aspects of aging in the context of yeast, for example the link between aging and cellular asymmetry and whether positive effects could be associated with age.

Age-associated changes at the whole-cell level

Aging is defined as an age-specific increase in mortality, meaning that old cells are more likely to die than young cells. This property, when measured across a population, results in a sigmoidal survival curve (Fig. 1a) and has been reported in both haploid and diploid yeast strains from all genetic backgrounds studied so far, including natural isolates (Kaeberlein *et al.*, 2005b; Stumpferl *et al.*, 2012). Noticeably, homozygous diploid cells live longer than the corresponding haploids, although neither the causes nor the implications of this difference are understood.

Rejuvenation restores the bud lifespan potential

How do populations composed of aging individuals maintain viability over time? Under the simplistic assumptions that aging occurs and that the two individuals produced at mitosis are identical, one would expect that progeny have an increased age at birth, ultimately leading to population extinction. However, daughter cells are born with a full replicative lifespan potential, which is independent of the age of their mothers. Such a rejuvenation mechanism allows the maintenance of a lineage

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with full lifespan potential. This observation has been instrumental in characterizing the causes of aging in yeast: early studies showed that the last bud born to a specific mother is able to divide, which ruled out the possibility that genomic mutations play a causal role in aging (Johnston, 1966; Müller, 1971). Remarkably, rejuvenation becomes less effective as the mother ages: buds produced in the first third of the life of their mother live, on average, as long as their mothers. Buds born later display a progressively reduced lifespan (10–15% shorter for buds born at the middle of the life of their mother, *c.* 60% for the last daughters; Kennedy *et al.*, 1994). This indicates that rejuvenation occurs throughout the whole lifespan, although it is only partially complete later in life (Fig. 1b). Accordingly, the replicative lifespan distributions of buds born at the 8th and 12th divisions are identical to that of their mothers, although at the individual level, the bud lifespan is little influenced by that of its mother (Shcheprova *et al.*, 2008). Here as well, rejuvenation does not always fully reset age, and this effect is stochastic. This highlights that rejuvenation, like aging itself, is best described at the population level, but highly stochastic at the level of the individual cell.

Whole-cell phenotypes indicate the existence of aging factors

The impaired rejuvenation of late born buds suggests that old mothers start 'passing age' to their daughters, leading to the hypothesis that aging occurs through the progressive accumulation of aging factors. This view is supported by the observation that when cells of different ages are mated, the zygote's replicative lifespan is set by the age of the older haploid cell, indicating that age is a dominant phenotype (Müller, 1985). Under this hypothesis, rejuvenation corresponds to the retention of such aging factors in the mother at division, passively or actively. The decreased rejuvenation of daughters produced by very old mothers could reflect age-induced defects of the molecular machinery involved in retention or titration of the retention machinery by large amounts of the aging factors. Interestingly, while the replicative lifespan of the last bud is decreased, its first daughter and granddaughter show a gradual restoration of a normal lifespan (Kennedy *et al.*, 1994), suggesting that one or more factors must be diluted to achieve full rejuvenation. Together, these observations have led to the general paradigm that aging is caused by the accumulation of aging factors.

Aging factors must fulfill four basic requirements (Henderson & Gottschling, 2008). First, these factors must accumulate with age. Second, during mitosis, they must segregate asymmetrically to the older cell. These two-first requirements are shared between aging factors and any marker of aging that has no impact on the aging process. A third requirement, specific for *bona fide* aging factors, is that preventing or reducing the presence of an aging factor should lead to lifespan extension. Fourth, conditions that increase the levels of an aging factor should decrease lifespan. This definition places aging factors in the category of toxic damage that cells are unable to repair or eliminate. However, this definition does not explain why these factors are toxic, how they cause cell death, or whether their toxicity is dependent on environmental conditions.

One way to investigate the effects of aging factors on cellular physiology is to observe the effects of aging at the whole-cell level. For instance, cells maintained in stationary phase for long periods of time display a shorter replicative lifespan (Fig. 1c; Ashrafi *et al.*, 1999; Maskell *et al.*, 2003), indicating that aging factors accumulate during chronological aging and that these factors also affect the replicative lifespan. Hence, replicative and chronological aging may share common mechanisms and be partly coupled. Other examples of how aging factors affect cellular physiology at the organism level are given in the next sections.

Slower division time of old cells is not passed to their progeny

It was noticed early in microdissection studies that division time increases as the mother cell ages (Egilmez & Jazwinski, 1989). The division time also becomes more variable in old cells. Recently, life-long, continuous observation of yeast cells, using microfluidic devices coupled to microscopy, has enabled a more detailed description of growth and cell division (see Appendix 1). These studies demonstrate that division time increases primarily during the last five divisions before death, independent of the final age at death (Lee *et al.*, 2012; Xie *et al.*, 2012). From a demographic viewpoint, this increase in division time corresponds to an age-specific decline in reproduction, another facet of the definition of aging. Remarkably, the increase in division time observed in old mothers is only partially passed on to their daughters: the bud division time is not increased at the 18th division and increases by only 33% at the 26th division, when the mother division time is increased by 140%. Moreover, these slow-dividing daughters recover a normal division time within their first four budding cycles (Egilmez & Jazwinski, 1989). By analogy with lifespan rejuvenation, the restored division time of the buds is well explained by the retention of aging factors in the mother cells: this indicates that the accumulation of aging factors both shortens the lifespan and decreases growth. In addition, the progressive

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recovery of a normal division time by old cells' buds can be interpreted as the dilution of aging factors received from the old mother due to incomplete retention during prior divisions.

Rejuvenation occurs also during sporulation

Rejuvenation does not occur only during mitosis: when old diploid cells are sporulated, the spores produced by meiosis are also rejuvenated (Unal *et al.*, 2011). Noticeably, the four spores of a tetrad produced from an old diploid cell have similar lifespans. This is in sharp contrast to the mother and daughter cells produced by late mitotic divisions, in which age is asymmetrically segregated. In addition, the lifespans of spores are independent of the age of the sporulated cell. Intriguingly, Ndt80, a transcription factor that is both required for and specific to meiosis, can trigger the rejuvenation of old mother cells when artificially expressed in mitotic cells. These data suggest that Ndt80 controls a rejuvenation program in meiosis that actively clears damage. Nevertheless, the process of sporulation has asymmetrical aspects: during meiosis, important components of the mother nucleus are discarded (Fuchs & Loidl, 2004), and during packaging, only a small fraction of the mother cytoplasm is included into each spore (Neiman, 2005). Therefore, rejuvenation during sporulation could at least partly result from asymmetric segregation of damage to the excluded mother cytoplasm. Indeed, as it is ultimately discarded in the sporulation process, the cytoplasm is a good candidate compartment to retain aging factors. Therefore, understanding how this transcription factor triggers cell rejuvenation is an important step toward identifying aging factors and their mechanisms.

Cell morphology is changing with age

After each division, a chitin ring remains in the cell wall of the mother cell where the former bud neck was located. These scars are stable as the cell ages and are therefore useful to determine the replicative age of a given cell (Powell *et al.*, 2003) simply by staining and counting bud scars. Early studies led to the hypothesis that bud scars limit the surface available for the formation of new budding sites or for exchanges of solutes through the cell wall. This hypothesis was ruled out by the observation that artificially increasing the size of the cell, and hence the surface available, does not increase the lifespan.

Cell size increases during the entire life of the cell, and this increase is noticeably greater during the last few divisions. This has been reported in a number of different strains, using various methodologies: haploid cells purified by gradient centrifugation (Egilmez *et al.*, 1990), diploid cells followed by micromanipulation (Yang *et al.*, 2011), and haploid cells in microfluidics (Lee *et al.*, 2012). In this last study, the increase was quantified division after division and demonstrated to be moderate in the earlier part of the lifespan (+40% in 20 generations), but dramatic in the last two divisions (+80%). Could this massive change play a causal role in the aging of yeast cells? In other words, is size itself an aging factor? To test this hypothesis, the cell size has been manipulated in an age-independent manner by treating the cells with pheromone. Treatment did not significantly reduce the replicative lifespan (Kennedy *et al.*, 1994), indicating that cell size is not an aging factor. However, a recent set of experiments has revived this question by demonstrating that similar and longer pheromone treatments shorten the lifespan of the cells of a different long-lived haploid strain (Zadrag *et al.*, 2005). Furthermore, the replicative lifespan correlates with the size of the cell at birth; large buds have a shorter lifespan. Finally, in these studies, cell size at the last division showed little variation and was independent of birth size. These observations suggested that there is a size-threshold above which homeostasis cannot be maintained (Yang *et al.*, 2011; Ganley *et al.*, 2012). However, visualization of cells under microfluidics indicates that the size reached by the cells at the time they arrest dividing is largely variable (Lee *et al.*, 2012). Because of these conflicting results, whether and how cell size plays a causal role in aging remains an open question. In addition, the mechanism by which cell size increase would cause cell death remains unknown. A proposed mechanism should also reconcile the hypertrophy hypothesis with the observation that homozygous diploid cells have longer lifespans than haploid cells (Kaeberlein *et al.*, 2005b), even though diploid cells are substantially larger.

Responses to environmental changes are age-dependent

Other whole-cell effects that have been studied over the course of aging are physiological responses mounted when cells are exposed to diverse stimuli. The best-characterized case is the response to pheromone, which showed that yeast cells become sterile with age: after 20 divisions, the mating frequency drops to 5% (from 80% in young cells; Müller, 1985). In addition, old cells become insensitive to pheromone (only 35% respond to pheromone after 90% of their lifespan, while 80% do at the middle of their lifespan; Smeal *et al.*, 1996). Similarly, meiosis and subsequent spore formation, which are diploid-specific responses to nutrient deprivation, are also affected by age: cultures enriched in old cells (13.5

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divisions in average) display a very low sporulation efficiency (17% instead of 70% in young cells; Boselli *et al.*, 2009). Interestingly, these decreases in pheromone sensitivity and meiosis efficiency depend on the fraction of the replicative lifespan (i.e., the ratio of the number of divisions already accomplished to the total number of divisions to be completed by the cell), rather than on the replicative age (i.e., the absolute number of divisions already accomplished). Thus, aging may affect processes that modulate the ability of the cell to perceive and respond to its environment. This age-specific decline of physiological performance corresponds to another facet of the definition of aging given in the introduction; hence, all three facets (age-specific increase in mortality, decreases in reproduction and in physiological performance) are observed in budding yeast, which qualifies as a truly aging organism.

In contrast to pheromone response and sporulation ability, which both decrease with age, the survival to mild stresses do not always decrease with age. For instance, resistance to UV is optimal at intermediate ages (*c.* 8 divisions; Kale & Jazwinski, 1996). In contrast, resistance to a chemical mutagen such as ethyl methanesulfonate (EMS) decreases linearly with age. This difference may be due to the fact that UV, but not EMS, is encountered in the natural environment. Thus, stress response mechanisms are likely to have been selected to handle damage specifically caused by UV. A more radical idea is that old cells might handle stress more efficiently than young ones. Indeed, trehalose, a cytoplasmic compound that confers resistance to heat stress, accumulates in old cells (Levy *et al.*, 2012). However, this hypothesis has not been tested directly. Thus, aging might be accompanied by not only detrimental aspects, but also by positive changes that increase the ability of the cell to cope with its environment. Such beneficial acquired traits could have two different origins, which are not necessarily mutually exclusive. One possibility is that they may come from the activation of specific response pathways that the cell mobilizes as it adapts over time to its environment. Alternatively, beneficial acquired traits might be a secondary consequence of aging and reflect the response of the cell to internal stresses caused by age. These responses might in turn make the cell better able to cope with similar stresses of external origin. In any case, the idea that the cell matures as it ages is certainly worth more scrutiny, as well as the possibility that maturation and aging relate to each other.

Aging phenotypes vary between strains and between cells

How much is aging set by genes rather than by the history of the cell? The aging process and its variability are largely dependent on the genetic makeup of the strains, which itself might be adapted to a specific environment in the wild. When natural isolates, which are adapted to different conditions than laboratory strains, are crossed to a laboratory strain, the segregants display a large distribution of replicative lifespans (from −40% to +65% of the parents; Stumpferl *et al.*, 2012; Kwan *et al.*, 2013). Noticeably, the most important genetic factors identified explain only a small portion of this variability, highlighting the fact that longevity involves a number of cellular processes.

At the individual level, the aging and rejuvenation processes are highly variable: in fact, the variability of individual replicative lifespans is used to demonstrate that yeast cells do age. The variability in other age-associated phenotypes such as cell size and, to a lesser extent, division time has been described above. Variability is also observed late in the life of cells, as death can occur in at least two different ways, either as an unbudded cell or after failing to complete the last cytokinesis (Johnston, 1966; Lee *et al.*, 2012; Xie *et al.*, 2012). Remarkably, these two modes of death correlate with differences in lifespan, suggesting that they correspond to different modes of aging, which may in turn depend on the individual history of the cell.

Different stages of age in yeast

As discussed in the previous section, aging proceeds in highly variable ways, depending on a multitude of factors, such as the genetic background, the environment and the history of the cell. It is remarkable that the variability between individuals observed in age-associated phenotypes depends on the metrics used to measure individual age. One example of this is the division time increase observed in old cells, which increases primarily late in life when looking at individual data (Fig. 3a; data provided by S. S. Lee). However, when looking at the population average, if age is measured as the number of divisions since the cell was born ('replicative age'), the division time increases slowly after 10 divisions and more rapidly at the end of the lifespan (after 25 divisions). The variability between individuals is high throughout the lifespan. Alternatively, age can be defined as the number of divisions left before cell death; the division time is then constant up to 10 divisions before death, with a marked increase in the last five divisions (Fig. 3b). Noticeably, the variability is 2–3 times smaller using this second metrics than with the first one, which indicates that the mechanism governing division time increase depends more on the number of divisions before death than on the replicative age.

This difference in age-associated phenotypes depending on the metrics used to measure age suggests that we can

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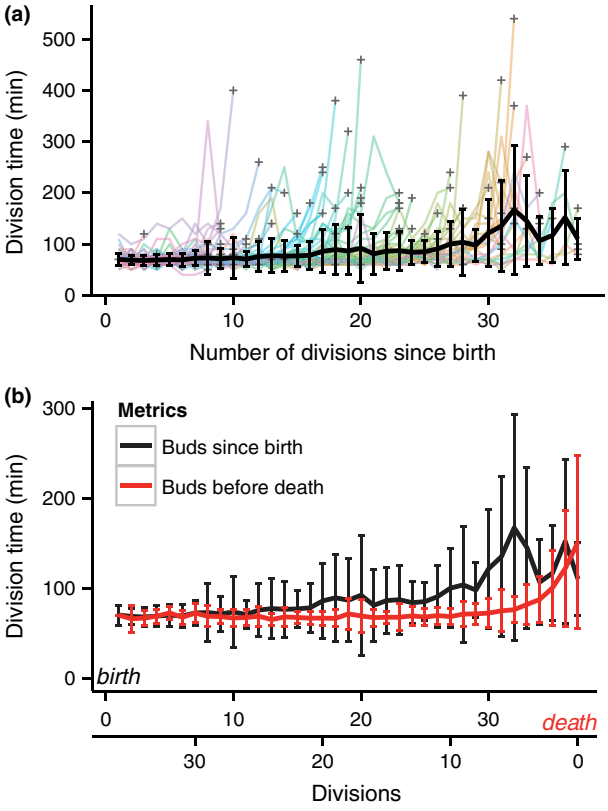

Fig. 3. Using two different age metrics allows to distinguish different stages of changes during aging (replotted from Lee *et al.*, 2012). (a) The division time of individual cells grown in a microfluidic device is shown in colors, with the average and standard deviation shown in black; crosses indicate death events. (b) Although in average, the division time increases with age counted as number of divisions since birth, individual traces reveal that the increase is more pronounced in the last divisions before death. As expected from this observation, plotting the same variable against age, now counted as the number of divisions remaining before cell death yields a sharper increase at the end and a much lower variability.

distinguish different stages in the lifespan of a cell (Fig. 5). The last stage is the easiest to describe, as late phenotypes of different cells tend to align well with each other when measured against the time before death. This observation suggests that each cell may take one of very few deterministic paths to its ultimate death. These paths are probably not reflective of the aging process itself, which occurs in a stochastic manner, but only its ultimate consequence. At the other end of the lifespan, we have to hypothesize early events that are not yet toxic and may even be beneficial to the cell (e.g., facilitating its adaptation to its environment). At intermediate age, it is the accumulation of these events that may inexorably trap the cell in one of the irreversible end-scenarios mentioned above.

Therefore, understanding aging is not only understanding from what and how the cell or organism dies, but which events contribute to death throughout the lifespan. Further complicating this analysis, some events that take place during the lifespan of yeast cells proceed in a more regular manner, such as the slow and progressive increase in cell size. Another example is the budding pattern of haploid cells, which gradually changes from axial to random as the cell ages (Jazwinski *et al.*, 1998). Are these events part of aging at all? Do they contribute to the loss of fitness or viability? Or are they age markers in its simplest sense: a manifestation that time passes and leaves its marks? Future studies of these processes will be required to provide a more mechanistic understanding of how each cell undergoes its own journey through a finite life.

Age-associated changes in the cell organelles

As described above, the physiology of the whole cell undergoes significant changes, while it ages. These observations lead to the questions of how physiology reflects changes at the intracellular level, and which putative aging factors contribute to these intracellular changes. We will address these questions perusing different organelles individually to describe the age-associated changes. Further, we will address which of these intracellular changes have a causal role in aging, ultimately leading to loss of viability, and which ones are simply aging markers without physiological consequences. To do this, we will review what is known about how aging affects the different compartments of the cell and which factors accumulate within the cell as it ages. Particularly, we will focus on how aging affects the nucleus (Fig. 4a), mitochondria (Fig. 4b), vacuole (Fig. 4c), endoplasmatic reticulum and cytoplasm (Fig. 4d).

Nucleus

Human diseases leading to premature aging phenotypes, including Hutchinson–Gilford progeria syndrome (HGPS) and Werner syndrome, provide important insights into the mechanisms of normal aging, as these diseases cause premature aging-associated phenotypes in several tissues (Martin & Oshima, 2000). HGPS is caused by a silent point mutation in the lamin A encoding gene, leading to an alternatively spliced variant of lamin A termed 'progerin'. Lamin A is a component of the nuclear lamina, and cells from HGPS patients show altered nuclear structure, thickening of the lamina and loss of peripheral heterochromatin (Dechat *et al.*, 2008). Werner syndrome results from a mutation in a helicase called WRN (Sgs1 in budding yeast), which is important for DNA integrity. Loss of function of WRN leads to defects in DNA double-strand break repair and increased aberrations at

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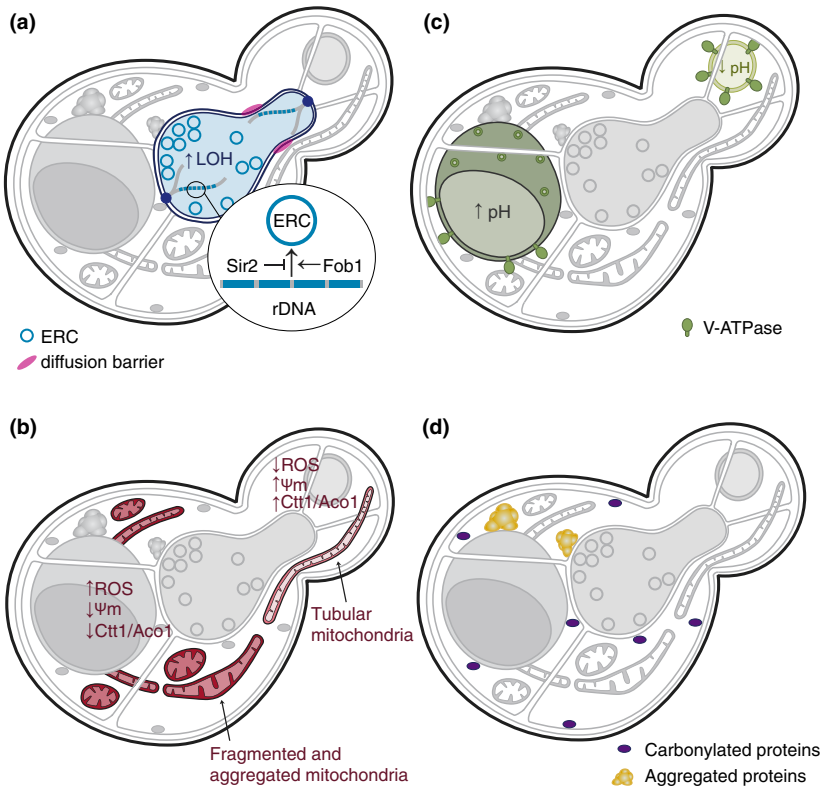

Fig. 4. Asymmetric distribution between mother and daughter cells of age-dependent changes in different organelles. (a) Nucleus: ERCs (blue circles) are formed by homologous recombination of rDNA repeats. The formation of ERCs is promoted by Fob1 and repressed by Sir2 (inset). ERCs are highly retained in the mother cell, and their retention depends on the geometry of the nucleus, speed of anaphase, and a diffusion barrier (pink) in the outer nuclear membrane at the bud neck. Additionally, loss of heterozygosity (LOH) increases with age. (b) Mitochondria: In old cells, mitochondria become fragmented and aggregated and their membrane potential (m) decreases (darker red). At the same time, the levels of ROS increase in old cells. The mitochondria in the bud contain low levels of ROS and show a high m (light red). This asymmetry is established by the retention of aggregated mitochondria in the mother cell and the increased activity of two detoxifying enzymes (Ctt1 and Aco1) in the daughter cell. (c) Vacuoles: As the cell ages, the size of the vacuole in the mother cell increases and acidity, which is established by the V-ATPase, drops (darker green). While acidity decreases rapidly with age, it is fully restored in daughter cells (light green). (d) Cytoplasm: Carbonylated proteins (violet) as well as aggregated proteins (yellow) are found in old cells and are segregated asymmetrically. Aggregated proteins are attachment to membranes (IPOD and JUNQ) and are diffusing rather slow (Hsp104-containing foci), which, together with the geometry of a dividing yeast cell, might explain their retention in the mother cell.

telomeres (Burtner & Kennedy, 2010). Together, these phenotypes suggest that loss of nuclear integrity leads to premature aging in humans, which might reflect a role of nuclear dysfunction in normal aging. Research in budding yeast shows that similar processes take place in this organism.

Chromosomal DNA

As described above, genomic DNA mutations cannot explain the aging process and the aging phenotypes, as daughter and granddaughter cells of old mother cells have a restored lifespan potential. However, epigenetic changes of the genomic DNA were observed. As yeast cells age, acetylation of histone H4 at lysine 16 (H4K16) increases. This increase has been observed mostly at subtelomeric regions and is accompanied by the loss of histones and decreased transcriptional silencing (Dang *et al.*, 2009). The authors simultaneously observed a decrease in protein levels of Sir2, the deacetylase of H4K16 (the role of Sir2 in aging is further discussed below). Together, this suggests that age-dependent decrease in Sir2 levels leads to increased histone acetylation and therefore to loss of histones at certain chromosomal loci, which might contribute to the aging process. Supporting this hypothesis, overexpression of histone H3 and H4 prolongs lifespan (Feser *et al.*, 2010). Therefore, the level of histones that can be incorporated into nucleosomes decreases with age and might reach a critical level in some old cells, impairing their viability.

Telomeres attracted the attention of aging researchers very early on: in mammalian cells that do not express

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telomerase, like fibroblasts, telomeres shorten with every cell cycle, leading to cellular senescence, whereas artificial expression of telomerase rescues their division potential (Bodnar *et al.*, 1998). These findings led to the hypothesis that the length of telomeres sets a clock for every cell. Although telomere length seems to be critical for a normal lifespan in mice, it is unclear whether telomere shortening is directly involved in the aging process (Rudolph *et al.*, 1999; Hornsby, 2007). In budding yeast, telomerase is constantly active, comparable to stem cells. Therefore, telomere length is unaffected during replicative and chronological aging, and telomere shortening cannot account for aging phenotypes in yeast (D'Mello & Jazwinski, 1991). Nevertheless, mutations in the telomerase encoding *EST* genes (ever shorter telomeres) or *TLC1*, the template RNA, lead to progressive telomere shortening and limit the propagation potential of the entire population over time. This process is distinct from cellular aging and is called clonal senescence (Lundblad & Szostak, 1989; Singer & Gottschling, 1994).

Extrachromosomal DNA circles

Sinclair *et al.* (1997) discovered that yeast cells lacking Sgs1 (WRN) show accelerated aging phenotypes, including shortened lifespan, increased cell size, earlier sterility, and an enlarged and fragmented nucleolus. The latter observation prompted them to examine the rDNA locus more carefully, which lead to the discovery that extrachromosomal rDNA circles (ERCs) play an important role in the aging process (Larionov *et al.*, 1980; Sinclair & Guarente, 1997).

ERCs fulfill all four requirements for an aging factor: they accumulate in old mother cells, they segregate highly asymmetrically toward the mother cell at mitosis, artificial introduction of ERCs into young cells shortens their replicative lifespan, and reducing their formation leads to lifespan extension (Sinclair & Guarente, 1997; Defossez *et al.*, 1999). Once an ERC is formed, it replicates once during S-phase due to the presence of an autonomous replication sequence. The replicated copies segregate asymmetrically, staying in the mother cell. As a consequence, ERCs accumulate exponentially in the mother cell over division cycles and thereby contribute to limiting the lifespan of the cell (Sinclair & Guarente, 1997).

ERCs are formed by homologous recombination in the rDNA array, which contains 80–150 tandem rDNA repeats. Due to the symmetry of the Holliday junction, the resolution of such a junction between two neighboring rDNA repeats leads to excision of one ERC 50% of the times. These recombination events are due to double-strand breaks. Chromosomal breakage in the rDNA is frequent during replication due to stalling of replication forks at the fork barriers that separate individual rDNA repeats (Takeuchi *et al.*, 2003). Consistently, removal of Fob1, a protein required to stall replication forks, dramatically reduces the frequency of double-strand breaks, decreases the rate of ERC formation, and extends lifespan (Defossez *et al.*, 1999). Although *fob1*Δ mutant cells are longer lived compared with wild-type cells, they still age, possibly due to ERC accumulation. Indeed, studies of cells harvested at different ages showed that *fob1*Δ cells produce ERCs, although later in their lifespan, and that ERCs accumulate exponentially in these cells (Lindstrom *et al.*, 2011).

The rate of homologous recombination within the rDNA locus is lower than expected based on recombination rates of two homologous sequences elsewhere in the genome. It was proposed that Sir2, a histone deacetylase, is required for this repression (Gottlieb & Esposito, 1989). Deletion of *SIR2* enhances intrachromosomal recombination within the rDNA and shortens lifespan, whereas moderate overexpression of Sir2 leads to lifespan extension (Gottlieb & Esposito, 1989; Kaeberlein *et al.*, 1999). As cells age, the levels of Sir2 decline and the rate of recombination increases (Dang *et al.*, 2009).This suggests that the rate of ERC formation increases as the cell ages. Indeed, mathematical modeling suggests that ERC formation increases quadratically with replicative age (Gillespie *et al.*, 2004). However, the age-dependent increase in recombination within the rDNA locus is not rescued by overexpression of Sir2, suggesting that rDNA recombination may be differently controlled in old and young cells (Lindstrom *et al.*, 2011). Nevertheless, deletion of *FOB1* rescues the short-lived phenotype of *sir2*Δ mutant cells back to wild-type levels. In these cells, the levels of ERCs are lower compared with wild-type, suggesting that, in cells containing fewer ERCs, deletion of *SIR2* shortens lifespan in an ERC-independent manner (Kaeberlein *et al.*, 1999).

Within the rDNA repeats, a bidirectional RNA polymerase II promoter was discovered, called E-pro, whose transcription is repressed by Sir2. In cells lacking Sir2, less cohesin is associated with rDNA and rDNA stability is decreased (Kobayashi *et al.*, 2004). These data led to the hypothesis that transcription of E-pro leads to increased rDNA instability. This model was tested by replacing E-pro with the *GAL1/10* promoter, which is repressed in glucose and activated in galactose-containing medium (Saka *et al.*, 2013). In support of the model, this strain shows increased rDNA stability, decreased ERC levels, and increased lifespan when grown in the presence of glucose. Conversely, growth on galactose increases rDNA instability, augments ERC accumulation, and shortens lifespan. Remarkably, rDNA instability and ERC formation are repressed in *sir2*Δ mutant cells when the promoter is arti-

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ficially turned off. Concurrently, lifespan is no longer shortened but is prolonged to the same extent as in *fob1*Δ cells (Saka *et al.*, 2013). This suggests that the short-lived phenotype of cells lacking Sir2 is mainly caused by rDNA instability and ERC formation.

Although the formation of ERCs is well characterized, the mechanism ensuring their retention is debated. Mathematical modeling predicted that a retention probability above 0.99 is required to simulate experimentally obtained aging curves (Gillespie *et al.*, 2004). Another modeling study revealed that the geometry of the nucleus and the speed of anaphase ensure a retention frequency of 0.75–0.90 (Gehlen *et al.*, 2011). Thus, mechanisms beyond geometry and speed of anaphase are likely to contribute to ERC retention. In particular, ERC retention was proposed to involve a diffusion barrier in the outer nuclear membrane (Shcheprova *et al.*, 2008). During early stages of nuclear division, the nucleoplasm is not compartmentalized, whereas diffusion between the mother and bud compartment of the nucleus is strongly impaired for proteins embedded in the outer nuclear membrane (Boettcher *et al.*, 2012). Interestingly, there is a very good correlation between the strength of this diffusion barrier and the retention of ERCs (Shcheprova *et al.*, 2008). Investigating ERC levels in diffusion barrier mutant cells at different ages revealed that ERCs are still formed but accumulate much more slowly (Lindstrom *et al.*, 2011). Accordingly, cells with a weak diffusion barrier are longer lived compared with wild-type cells (Shcheprova *et al.*, 2008). The prolonged lifespan is not extended by the deletion of *FOB1*, suggesting that ERC accumulation is no longer limiting lifespan. How the diffusion barrier in the outer nuclear membrane restricts the diffusion of ERCs in the nucleoplasm is currently debated. It was proposed that ERCs are attached to nuclear pores and the hypothesis arose that pre-existing nuclear pores are retained in the mother cell (Shcheprova *et al.*, 2008). However, newer reports show that the bulk of pre-existing pores is inefficiently retained in the mother cell and cannot alone account for ERC retention (Khmelinskii *et al.*, 2010). Therefore, the question remains how this diffusion barrier contributes to the retention of ERCs.

Why ERC accumulation becomes toxic and what causes old mother cells to die is unclear. It seems that the toxicity of ERCs is not caused by sequences specific of the rDNA, as every DNA circle studied so far that lacks a partitioning sequence (centromere or 2 μ plasmid) accumulates in mother cells and shortens their lifespan (Falcón & Aris, 2003). Therefore, titration of proteins that bind to any noncentromeric DNA circle, including ERCs, might explain the toxicity of these molecules. It was proposed that replication or transcription factors could be the titrated proteins (Sinclair & Guarente, 1997). Alternatively, if ERCs bind to a putative receptor in the nuclear envelope, ERCs might block this receptor. However, no experiments have addressed these hypotheses so far. Ganley *et al.* (2009) proposed that ERCs themselves are not deleterious, but rather that they have a negative effect on rDNA stability. Favoring this model, another study showed that increasing rDNA instability by deleting *HPR1*, a component of the RNA polymerase II complex, causes premature aging independent of ERC accumulation (Merker & Klein, 2002). However, both studies relied on mutations shortening the lifespan. Results from an ongoing project to determine the replicative lifespan of the entire deletion collection (where every nonessential ORF is deleted) indicate that 20% of all viable gene deletions shorten lifespan (Kaeberlein & Kennedy, 2005). The authors report that most of these strains show stochastic death events, proposing that these mutations cause stress, which indirectly shortens lifespan. Therefore, shortened lifespan may occur in an aging-independent context and should be interpreted with caution.

Taken together, these studies establish ERCs as a naturally occurring factor that is incidentally formed, and once formed, accumulates in the mother cell, and contributes to its aging. However, there are still many unresolved questions: Why are large amounts of ERCs toxic to the cells? Do ERCs induce rDNA instability or does increased rDNA instability induce ERC formation? Do ERCs titrate certain factors, and if so, which ones? Cells accumulating fewer ERCs (e.g., cells lacking Fob1 or cells deficient in the diffusion barrier) still age, raising the question of what other factors might contribute to the aging process.

Loss of heterozygosity

In diploid cells, the repair of double-strand breaks not only results in ERC formation but can also lead to loss of heterozygosity: the recombination of an initially heterozygous locus resulting in its homozygosity, which is a hallmark of mammalian cancer cells (Tuna *et al.*, 2009). Interestingly, in yeast, the frequency of loss of heterozygosity increases with age (McMurray & Gottschling, 2003; Carr & Gottschling, 2008; Lindstrom *et al.*, 2011). In most old cells, this age-dependent increase originates from loss of mitochondrial DNA (mtDNA; further discussed below). In respiration-competent cells, an age-dependent increases in loss of heterozygosity were observed at the rDNA locus on chromosome XII, but not on another locus on chromosome IV (Lindstrom *et al.*, 2011). These findings suggest that DNA stability is not globally affected but that the stability at the rDNA locus

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specifically decreases with age, possibly leading to the predicted increase in ERC formation with age. However, why rDNA locus stability is specifically affected during aging remains unclear.

Mitochondria

Mitochondrial integrity

A large number of studies have suggested that mitochondria also contribute to aging. Remarkably, a link between the nucleus and mitochondria was established when the increased rate of loss of heterozygosity in daughters of old mother cells was found to correlated with the formation of 'petite' daughter cells lacking mtDNA (also called ρ^0 cells; Veatch *et al.*, 2009). Even though mitochondrial function is essential for cell viability, respiration is not and yeast cells lacking mtDNA survive. These cells switch from respiration to fermentation, leading to a growth defect and their petite phenotype. Interestingly, the age-dependent formation of petite cells highly depends on the strain background: whereas 95% of mother cells from the originally used strain produced petite daughter cells when they became old, this was only the case for 35% of mother cells in another background (Lindstrom *et al.*, 2011). It was proposed that polymorphism in several genes leads to strain-dependent differences in the formation of petite cells (Dimitrov *et al.*, 2009).

The increased loss of heterozygosity in ρ^0 cells was proposed to be triggered by defective iron–sulfur (Fe-S) cluster biogenesis in cells lacking mtDNA (Veatch *et al.*, 2009). Fe-S clusters are synthesized in the mitochondria and act as cofactors for hundreds of proteins, many involved in DNA replication and repair (White & Dillingham, 2012). Additionally, the authors found that loss of mtDNA is accompanied by a cell-cycle arrest followed by spontaneous genetic changes leading to improved growth (Veatch *et al.*, 2009). This suggests that loss of mtDNA might be compensated by increased genetic rearrangements allowing for the survival and growth of ρ^0 cells. The observation that some old mother cells form ρ^0 daughter cells suggests that either old cells lose their mtDNA and therefore cannot pass mitochondria containing DNA to their daughters or that the DNA-containing mitochondria are retained in the old mother cell. However, little is known about the stability and partitioning of mtDNA in old yeast cells and why some strains are more defective in proper mtDNA segregation compared with others.

Upon damage, mitochondria are proposed to be segregated asymmetrically depending on their integrity: Lai *et al.* studied cells with defective mitochondria, using a temperature sensitive allele of *ATP2*, a subunit of the mitochondrial F1-ATP synthase. They found that at permissive temperature, mitochondrial potential (Ψm) decreases and mitochondrial morphology changes dramatically. This leads to accumulation of mitochondria in the mother cell and impaired segregation of active mitochondria to the daughter cell. Mother cells accumulating mitochondria fail to produce rejuvenated daughter cells (the replicative lifespan of the 7th daughter cell was seven generations shorter; Lai *et al.*, 2002; Jazwinski, 2004). Mitochondrial inheritance depends on Mmr1, a protein required for Myo2-dependent transport of mitochondria into the bud, and Phb1/Phb2, components of the prohibitin complex. Cells lacking Mmr1 or Phb1/Phb2 form mitochondria deficient buds, and these proteins were proposed to be involved in aging and rejuvenation (Piper *et al.*, 2002; McFaline-Figueroa *et al.*, 2011).

Studies using the MEP to investigate mitochondrial morphology at different time points throughout the yeast lifetime revealed that mitochondria, which are tubular in young cells, become fragmented early in the aging process (eight generation old cells) and form aggregates in older cells (17 generations), which persist for the rest of the lifespan (median of 25 generations). These mitochondria have a membrane potential that decreases with age (Hughes & Gottschling, 2012). In conclusion, mitochondria change dramatically through the lifetime of yeast cells. However, why this organelle is altered early in life and how these cells maintain viability despite these dramatic changes in mitochondrial morphology remain unclear. Additionally, damaged or nonfunctional mitochondria appear to be retained in the mother cell, possibly to ensure the generation of rejuvenated daughter cells containing only fully functional mitochondria. It will be interesting to investigate how damaged regions of mitochondria are specifically detected and retained in the mother cell.

Reactive oxygen species

Further studies of mitochondrial asymmetry revealed that mitochondria retained in the mother cell show a lower oxidizing redox potential and higher levels of reactive oxygen species (ROS) compared with the mitochondria inherited by the daughter cell (McFaline-Figueroa *et al.*, 2011). Additionally, ROS levels are elevated in old cells (Xie *et al.*, 2012). During the first five divisions, the mitochondrial redox potential declines in the mother cell and becomes more oxidizing, whereas the asymmetry between mother and bud is constant. Therefore, daughter cells produced from the 5th division inherit less-functional mitochondria (McFaline-Figueroa *et al.*, 2011). This is paradoxical, as these daughter cells are fully rejuvenated. One possible explanation is that the activity of a protein

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implicated in detoxification of mitochondrial ROS, the catalase Ctt1, is increased in daughter cells after cytokinesis (Erjavec & Nyström, 2007). Similarly, mitochondrial aconitase, Aco1, a protein containing an Fe-S cluster and involved in mtDNA maintenance, loses activity during normal replicative aging (Klinger *et al.*, 2010). Intriguingly, although the amount of Aco1 is split equally between old mother and daughter cells, the daughter cell primarily receives the active form of aconitase. Therefore, if daughter cells receive less-functional mitochondria, they might repair them more efficiently than old mother cells.

In 1956, Harman postulated the so-called Free Radical Theory of Aging, whereby increased metabolic rate leads to increased ROS formation, which would be harmful for the cells and cause aging. Although this theory gained popularity and is supported by experiments showing that ROS levels are increased in old mother cells (Laun *et al.*, 2001; Barros *et al.*, 2004), other reports showed that cells containing increased ROS levels show a prolonged lifespan under certain conditions (Sharma *et al.*, 2011). This latter finding led to the hypothesis that increased ROS levels can induce ROS defense and stress response mechanisms which prolong lifespan (Ristow & Zarse, 2010). This theory suggests that ROS act as a signal to activate the retrograde response, a response pathway that induces the transcription of stress response genes as a defense mechanism (see next paragraph). However, whether accumulation of ROS themselves shortens or prolongs lifespan is currently under debate (Kowaltowski *et al.*, 2009; Ristow & Schmeisser, 2011). Their effects could depend on their levels; mild ROS levels might activate stress response pathways which lead to prolonged lifespan, but higher ROS levels might be toxic and therefore shorten lifespan.

Retrograde response

The retrograde response is a pathway that signals from the mitochondria to the nucleus and is activated upon damage or loss of mtDNA. Activation of the retrograde response in ρ^0 cells leads to transcription of a specific set of genes encoding metabolic enzymes and stress response proteins in an Rtg2-dependent manner (Parikh *et al.*, 1987; Butow & Avadhani, 2004). The observation that the most long-lived cells in a population mainly consist of ρ^0 cells led to the hypothesis that ρ^0 cells show a prolonged lifespan. Indeed, in some strain background, loss of mtDNA leads to increased longevity. Further investigation revealed that the increased lifespan in ρ^0 cells depends on Rtg2, suggesting that activation of the retrograde response pathway leads to the lifespan extension observed in these cell (Kirchman *et al.*, 1999).

However, another aspect of the interplay between the nucleus and mitochondria is the fact that activation of the retrograde response also enhances ERC accumulation. At a first glance, it seems paradoxical that on one hand, this pathway activates longevity genes and on the other hand increases the accumulation of life-shortening ERCs (Borghouts *et al.*, 2004). The reason for this might be the dual role of Rtg2: it both is involved in transcriptional activation of retrograde-response-induced genes and suppresses ERC accumulation. It has been proposed that activation of the retrograde response by loss of mtDNA might require more Rtg2 for the transduction of the retrograde response signaling, leading to less repression of ERC formation (Jazwinski, 2005). In addition, it was proposed that ERCs signal back to the mitochondria via Tar1, a protein that is encoded on the antisense strand of the rDNA repeat and localizes to the mitochondria (Poole *et al.*, 2012). However, neither the function of Tar1 nor whether the amount of ERCs influences the levels of Tar1 is known.

Vacuole

Autophagy

Macroautophagy mediates the degradation and recycling of organelles through their engulfment into autophagosomes and targeting to the vacuole. The vacuole resembles the lysosome in metazoa and is required for both storage of ions and amino acids, and turnover of proteins and lipids (Armstrong, 2010). The turnover of macromolecules through autophagy is believed to be cytoprotective. Accordingly, survival of cells in several stress conditions depends on the autophagy machinery. In *Caenorhabditis elegans* and *Drosophila melanogaster*, several manipulations leading to prolonged lifespan enhanced autophagy, suggesting that autophagy has an anti-aging effect (Rubinsztein *et al.*, 2011). However, little is known about the role of autophagy during yeast aging. A genetic screen for shortened chronological lifespan revealed that many genes involved in autophagy are required for a normal lifespan (Fabrizio *et al.*, 2010; Matecic *et al.*, 2010). However, cells with defects in the autophagy pathways might suffer from stress that does not occur naturally during aging. Treating cells with spermidine, which activates autophagy, increases chronological lifespan. The effect of spermidine addition on replicative aging is less clear: treatment of young cells does not affect their lifespan but treating aged cells isolated by elutriation does (Eisenberg *et al.*, 2009). However, in this study, elutriation severely shortened lifespan even in the control cells, suggesting that spermidine might rather protect from the induced stress than prolonging the nor-

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mal replicative lifespan. Microfluidics could be used here as a tool to reinvestigate these questions.

Vacuoles and pH

As cells age, the vacuole grows drastically (Tang *et al.*, 2008; Lee *et al.*, 2012). Indeed, vacuolar morphology affects aging; cells defective in vacuolar fusion, *osh6*Δ and *erg6*Δ cells, exhibit shortened replicative lifespan, whereas overexpression of Osh6 prolongs lifespan (Tang *et al.*, 2008; Gebre *et al.*, 2012). Osh6 mediates vacuolar fusion by maintaining sterol levels in the vacuolar membrane, whereas overexpression of Osh6 depletes sterols from the plasma membrane. Similarly, Erg6 is directly involved in ergosterol biosynthesis. Therefore, these perturbations not only affect vacuolar fusion but also change sterol homeostasis, which might affect longevity through other pathways.

Insights into the age-associated changes occurring in the vacuole and their impact on mitochondrial physiology arise from work by Hughes and Gottschling. Acidity in the vacuole drops rapidly early in age (after four divisions) and is followed by changes in mitochondrial structure and membrane potential. Vacuolar acidity is established by the V-ATPase, and overexpression of Vma1, a subunit of the V-ATPase, delays the drop of acidity in the vacuole (Li & Kane, 2009; Hughes & Gottschling, 2012). Remarkably, the same perturbation also delays the dysfunction of mitochondria and extends lifespan. Furthermore, while acidity declines in mother cells as they age, the acidity in their daughter's vacuoles is reset. Together, these observations indicate that a very early change in the vacuolar pH affects mitochondrial function in the aging mother cell and contributes to the replicative aging process. However, why acidity drops so early in the yeast lifetime and how the effects on mitochondria lead to lifespan shortening remain unclear.

Cytoplasm

Carbonylated proteins

As previously discussed, ROS levels are elevated in old yeast mother cells (18 generations) compared with young cells (four generations; Laun *et al.*, 2001). This spurred the Nyström laboratory to investigate protein carbonylation, a form of irreversible oxidative damage to proteins (Stadtman, 2006). Carbonylated proteins segregate asymmetrically toward the mother cell and accumulate with replicative age. Interestingly, the asymmetry depends on Sir2 and the actin cytoskeleton (Aguilaniu *et al.*, 2003). Furthermore, age-induced carbonylated proteins interact with Hsp104, a chaperone involved in disassembly of protein aggregates, and the asymmetric distribution of carbonylated proteins depends on Hsp104 function. Cells lacking Sir2 exhibit increased carbonylation of different chaperones including Hsp104, which might impair their function and explain the increased symmetry of oxidized proteins. Accordingly, overexpression of Hsp104 rescues both the symmetric segregation of carbonylated proteins and the replicative lifespan of *sir2*Δ mutant cells (Erjavec *et al.*, 2007). How overexpression of Hsp104 rescues the lifespan of *sir2*Δ cells, which contain high ERC load, remains unclear. Furthermore, the retention of carbonylated proteins was proposed to be important for the rejuvenation of daughter cells; when aged mother cells were treated with latrunculin-A (Lat-A), a chemical compound that disrupts the actin cytoskeleton, more carbonylated proteins were passed to the daughter cell. The daughter cells that were produced during the Lat-A treatment displayed shortened lifespan, whereas the daughter cells born after removal of Lat-A were fully rejuvenated (Erjavec *et al.*, 2007). However, it is unclear whether the Lat-A-treated daughter cells suffer more from their higher load of carbonylated proteins or from the lack of actin-dependent transport during bud growth. Together, these experiments demonstrate that carbonylated proteins are retained in the mother cell, leading to their accumulation with age. However, they do not definitely clarify whether oxidative damage is indeed a lifespan determinant.

Protein aggregates

The Hsp104 chaperone facilitates the refolding of denatured and aggregated proteins (Parsell *et al.*, 1994). Unlike chaperones involved in the folding of newly synthesized proteins, Hsp104 is a disaggregase and interacts with aggregated proteins. Therefore, Hsp104 is frequently used as a marker of protein aggregation within the cell (Winkler *et al.*, 2012). To investigate how Hsp104 foci are segregated at mitosis, such foci have been induced through heat shock in young cells (42 °C, 30 min) and their behavior has been monitored by microscopy. Using this method, Zhou *et al.* describe their movement as rather slow and random, and mathematical modeling revealed that the geometry of dividing yeast cells might be sufficient to retain Hsp104 aggregates in the mother cell. The remarkably slow movement might suggest that these Hsp104 foci are not freely diffusing in the cytoplasm but are rather associated with an organelle. Using long-term microscopy, the dissolution of these heat induced aggregates was observed in both mother and bud (Zhou *et al.*, 2011). Using the same method, Liu *et al.* (2010) reported that, in *c.* 10% of the cells, Hsp104 foci

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moved in a seemingly directed manner from the bud to the mother cell. This retrograde transport depended on Sir2 and the polarisome. Hsp104 forms such foci even without heat stress as the cells age (Erjavec *et al.*, 2007; Liu *et al.*, 2010; Zhou *et al.*, 2011). Age-induced Hsp104 aggregates are not cleared by dissolution but are retained in the mother cell, and their diffusion is similar to that of heat induced ones (Liu *et al.*, 2010; Zhou *et al.*, 2011). However, little is known about age-induced Hsp104 aggregates, how they behave and to which extend they are comparable to the stress-induced aggregates. In addition, it is not known whether Hsp104 aggregates are toxic or have a protective function for the cell.

Analysis of protein aggregates in proteasome-deficient cells indicates that they are not all equivalent. Different reporter proteins chosen for their tendency to misfold show a two-step dynamics. They first aggregate into stress foci and then are sequestered into either one of two distinct compartments within the cell: soluble ubiquitinated proteins are targeted to the JUNQ for degradation by the proteasome and insoluble aggregates are deposited in a protective compartment called IPOD (Kaganovich *et al.*, 2008). Interestingly, the IPOD compartment is associated with the vacuole, and the JUNQ compartment localizes to the outer nuclear membrane where it is entrapped in ER membranes. Therefore, the movement of these deposits into the bud is constrained by their attachment to organelles. In cells lacking Hsp104, the stress foci are neither degraded nor deposited into IPOD or JUNQ and are no longer asymmetrically retained in the mother cell (Spokoini *et al.*, 2012). Accordingly, *hsp104*Δ mutant cells are short lived, indicating that Hsp104 function is important for normal lifespan (Erjavec *et al.*, 2007).

Together, studies on Hsp104-recruiting aggregates suggest that there are several parallel mechanisms ensuring their asymmetric segregation: (1) aggregates are efficiently retained in yeast mother cells through attachment to organelles, (2) once an aggregate is segregated into the bud, it can be either cleared by dissolution, or (3) possibly brought back into the mother cell by retrograde transport (Liu *et al.*, 2010; Zhou *et al.*, 2011; Spokoini *et al.*, 2012). It has to be noted that both heat shock treatment and blocking the proteasome machinery might not reflect the characteristics of aggregates arising throughout the aging process. Therefore, it will be interesting to study age-induced aggregates more carefully and ask which proteins are sequestered to these aggregates, when and how they are formed and whether different aggregates behave differently. Importantly, the question remains whether aggregates that appear with age act as aging factors or sequester aggregates from the cytoplasm, thereby ensuring a normal lifespan.

Modulation of the aging process by caloric restriction

Restriction in calorie intake is the most universal treatment known to prolong lifespan; originally found to extend lifespan of rodents (McCay *et al.*, 1935), caloric restriction was discovered to prolong both the replicative lifespan (Lin *et al.*, 2000) and the chronological lifespan (Smith *et al.*, 2007) of yeast cells, as well as the lifespan of many different other model organisms (Bishop & Guarente, 2007). Nevertheless, how this treatment extends lifespan is far from being understood, possibly because it affects different aspects of the aging process in parallel. As most of the previously described changes that occur in the cell during aging are affected by caloric restriction, we will discuss how the previously discussed changes are affected by high and low calorie diets.

In addition to caloric restriction, mutations mimicking low nutrient availability prolong the replicative lifespan, such as deletion of *HXK2*, a hexokinase catalyzing the entry of glucose into the glycolytic pathway (Lin *et al.*, 2000). Three nutrient-sensing kinases are affected by caloric restriction and regulate the response of the cell to nutrients availability: (1) Tor1, a subunit of the TORC1 complex, (2) Tpk1/2/3, the catalytic subunit of the cAMP-dependent protein kinase (PKA), and (3) Sch9, a functional ortholog of the human S6 kinase, which is involved in the insulin-like signaling. Deleting these kinases not only prolong lifespan similarly to caloric restriction, it also abolishes further lifespan extension by restricting caloric intake, strongly suggesting that caloric restriction acts through those kinases (Toda *et al.*, 1987; Lin *et al.*, 2000; Fabrizio *et al.*, 2001; Kaeberlein *et al.*, 2005c; Powers *et al.*, 2006). Inhibition of TOR enables nuclear localization of different transcription factors, including Gln3, Msn2/4, and Rtg1/3, and thereby modulates transcription of several hundred genes (Beck & Hall, 1999). Additionally, inactivation of TOR both downregulates ribosome biogenesis and inhibits translation initiation (Crespo & Hall, 2002). These comprehensive changes make the assignment of those that are relevant for lifespan extension extremely complex. Still many studies have tackled this challenge and asked whether putative aging factors are affected by caloric restriction.

Extrachromosomal rDNA circles

It is unclear whether caloric restriction prolongs aging by modulating ERC accumulation. The observation that recombination in the rDNA locus decreases in caloric restricted cells suggests that lifespan extension is a result of decreased ERC formation (Lamming *et al.*, 2005). Sup-

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porting this idea, inhibition of Tor1 increases association of Sir2 with the rDNA repeats, and caloric restriction does not prolong lifespan of cells lacking Sir2. Thus, caloric restriction may act through Sir2-dependent silencing at the rDNA locus (Lin *et al.*, 2000; Ha & Huh, 2011). This model has been questioned based on four arguments. (1) Caloric restriction still prolongs lifespan of *fob1*Δ mutant cells, which are long lived due to less ERC formation (Kaeberlein *et al.*, 2004). However, recent experiments show that *fob1*Δ mutant cells form ERCs later in their lifetime, but once formed, they still accumulate (Lindstrom *et al.*, 2011). Therefore, aging of *fob1*Δ mutant cells cannot be interpreted as completely ERC independent. (2) Cells lacking Sir2 show a high increase in ERC accumulation, and therefore, hyperaccumulation of ERCs might even conceal the beneficial effects of a low glucose diet (Kaeberlein & Powers, 2007). (3) Caloric diet prolongs lifespan in chronologically aged cells, which do not accumulate ERCs (Ashrafi *et al.*, 1999). However, in chronological aging, Sir2 might play a very different role than in replicative aging, as cells lacking Sir2 show a prolonged chronological lifespan, which is even further extended by caloric restriction (Fabrizio *et al.*, 2005). The difference between the role of Sir2 in chronological vs. replicative aging might result from the accumulation of acetic acid in the medium; acetic acid is produced by cells at the beginning of the chronological aging experiment and has been shown to be toxic. Acetic acid formation is clearly reduced in cells undergoing chronological aging under caloric restriction, which might explain the beneficial effect of this treatment under this aging regime (Burtner *et al.*, 2009). (4) Caloric restriction affects aging in higher eukaryotes as well and as the role of ERCs in the aging process has so far only been demonstrated in yeast, whether caloric restriction could modulate aging through ERC regulation in higher eukaryotes has been questioned. However, ERCs have been found to exist in many higher eukaryotes, including humans (Cohen *et al.*, 2010), and it would be interesting to know more about their effects in other model organisms. Taken together, it remains elusive whether caloric restriction prolongs lifespan through modulation of ERC levels. Therefore, the study of available nutrient amounts on ERC levels in old cells will be very insightful.

Reactive oxygen species

Similar to ERC formation, it is not clear whether the production of ROS is decreased or increased in cells grown under caloric restriction (Barros *et al.*, 2004; Sharma *et al.*, 2011). Cells grown in low glucose switch their carbon metabolism from fermentation to respiration, which favors ROS formation. Therefore, it has been suggested that moderate ROS levels caused by caloric restriction lead to increased stress response, which in turn prolongs lifespan (Pan, 2011; Sharma *et al.*, 2011). At the same time, increased respiration also augments the NAD^+/NADH ratio. This seems to be important for the lifespan extension under caloric restriction, as both disruption of the electron transport chain (*cyt1*Δ) and impaired NAD^+ synthesis (*npt1*Δ) prevent caloric restriction to extend longevity (Lin *et al.*, 2000). As the catalytic activity of Sir2 depends on NAD^+, increased NAD^+ levels during growth in low glucose may activate Sir2 and thereby promote longevity. However, the hypothesis that increased respiration and therefore enhanced Sir2 activity prolong lifespan under caloric restriction is debated, as discussed above, and has been challenged by the finding that caloric restriction prolongs lifespan in ρ^0 cells that is in the absence of respiration (Kaeberlein *et al.*, 2005a). To clarify whether changes in ROS contribute to the lifespan extension of caloric restricted cells, it will be important to investigate whether ROS levels are changed in old mother cells grown in media with different glucose concentrations.

Mitochondria and vacuole

As previously discussed, the acidity of the vacuole plays an important role in maintaining mitochondrial function and is required for a normal replicative lifespan. Interestingly, caloric restricted cells show an increase in vacuolar acidity and thereby largely delay age-dependent mitochondrial dysfunction (Hughes & Gottschling, 2012). Consistent with this, overexpression of the V-ATPase subunit Vma1, which leads to lifespan extension, does not further prolong lifespan under caloric restriction. This suggests that a low glucose diet extends replicative lifespan at least in part by preventing the early drop of acidity in the vacuole and the subsequent mitochondrial defects. The positive effects of caloric restriction on vacuolar acidity depend on the three described nutrient-sensing pathways (PKA, Sch9, and TOR). However, how these kinases promote vacuolar acidity is not known. Here, caloric restriction-dependent induction of autophagy might be involved (Noda & Ohsumi, 1998). Atg13, a regulatory component of the autophagy machinery, was recently shown to be a direct target of Tor1. It was proposed that inhibition of Tor1 by caloric restriction leads to dephosphorylation of Atg13, which induces autophagy (Kamada *et al.*, 2010). Supporting this, some autophagy genes were shown to be required for lifespan extension in low glucose (Tang *et al.*, 2008; Fabrizio *et al.*, 2010; Aris *et al.*, 2013). It will be interesting to uncover whether these observations are linked or whether low glucose intake acts on vacuolar acidity and the regulation of autophagy independently of each other.

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In general, most studies on the effects of caloric restriction have been performed on young cells, and the observations were then correlated with their effects on lifespan. Using new techniques will enables us to investigate the different changes occurring with age in caloric restricted cells. For example, the formation of protein aggregates during aging might be different in cells grown in high vs. low glucose. Additionally, many described changes might be linked to each other. We described how caloric restriction might activate autophagy pathways that could increase vacuolar acidity. Vacuolar function, in turn, has been shown to affect mitochondrial maintenance, and mitochondrial dysfunction leads to increased genomic instability and hence increased ERC accumulation. However, for most of these changes, we only have glimpses of how they develop and interplay with each other as the cells age.

Discussion

Following this overview of the changes that occur as budding yeast cells age, we would like to integrate the observations to understand how aging proceeds depending on individual histories, and how longevity can be shaped by evolution.

Revisiting the different stages of age in yeast

Is it possible to match the phenotypes observed at the whole-cell level with intracellular changes? Detrimental effects observed at the whole-cell level happen late in life, which could be interpreted to mean that these events are not related to aging. However, the replicative age of yeast cells is set from their first divisions (a population of cells that has undergone four divisions undergoes four divisions fewer on average than virgin daughters). Indeed, cellular activity is thought to produce damage continuously. Here, we re-examine the three stages in the life of a yeast cell that were defined previously, in an attempt to phrase them in molecular terms (Fig. 5). Early on, the cell is able to handle aging factors by clearing them and/or buffering their effects. Age is therefore set by the quantity of the remaining aging factors and/or of the byproducts of the repair activity. At intermediate ages, several types of aging factors increase simultaneously, with potentially synergistic effects (Kirkwood, 2005), and impair cellular activity. Also, loss of flexibility in regulatory networks might result in inappropriate responses to environmental changes, leading to the accumulation of new damage. Finally, after high levels of damage have been reached, this ultimately kills the cell, either as a direct effect or through induced secondary effects. This last stage correlates with a marked and continuous increase in general stress response transcription factors such as Msn2/4 (Xie *et al.*, 2012). Whether the transitions between these hypothetical stages are continuous or occur stepwise is unknown. As discussed previously, different metrics can be used to quantify age and each of them is better suited to describe the changes occurring at a given stage.

The observation that aging is variable even in well-controlled environments, such as microfluidic devices, suggests that stochastic intracellular events contribute to this process. Stochasticity accounts for the observation that progeny of a given (e.g., slow growing) cell rapidly re-establish a range of growth rates (Levy *et al.*, 2012). Stochasticity in the events underlying the three life stages of yeast could also explain the variability observed in individual lifespans, as well as to the different modes of death described previously. Although we do not understand how aging factor accumulation leads to cell death, different models can be proposed. First, factors required for cellular homeostasis could be titrated by abundant damage, such as replication factors could be titrated by ERCs, and chaperones by aggregated proteins. Second, damage accumulation could promote further damage. For example, aggregated proteins that titrate chaperones might lead to the accumulation of additional protein aggregates. In the case of ROS, futile damage control cycles could waste cellular resources. Finally, damage might produce irreversible changes in the aging cell that hinder its ability to handle new internal and external stimuli. The decreased UV resistance in old cells could be the result of such an irreversible loss in plasticity.

We anticipate that distinguishing between these different steps of aging will improve our understanding of aging mechanisms. However, the establishment and significance of these steps remain unclear. Aging is observed only in protected environments, in which the mortality is low enough for individuals to reach advanced age. The evolutionary theory of aging proposes that the fraction of resources dedicated to limiting damage is determined by the average lifespan that individuals achieve in the natural environment, where death is primarily due to extrinsic factors (Kirkwood, 2005). Past this age, selective pressures are proposed to decrease rapidly. When individuals are grown in protected environments with low extrinsic mortality (e.g., in the laboratory), they live longer and the ability to maintain and repair cellular structures decreases in old individuals, allowing the observation of aging. From this hypothesis, the early stage, which represents the majority of an exponentially growing population, corresponds to a period of selection for fast growth. Hence, aging factors are maintained at a level where they do not impact growth. At later ages, higher amounts of aging factors are harmful, as the repair machinery has been selected to process the lower levels of damage present in early life. In addition, the repair machinery might not be

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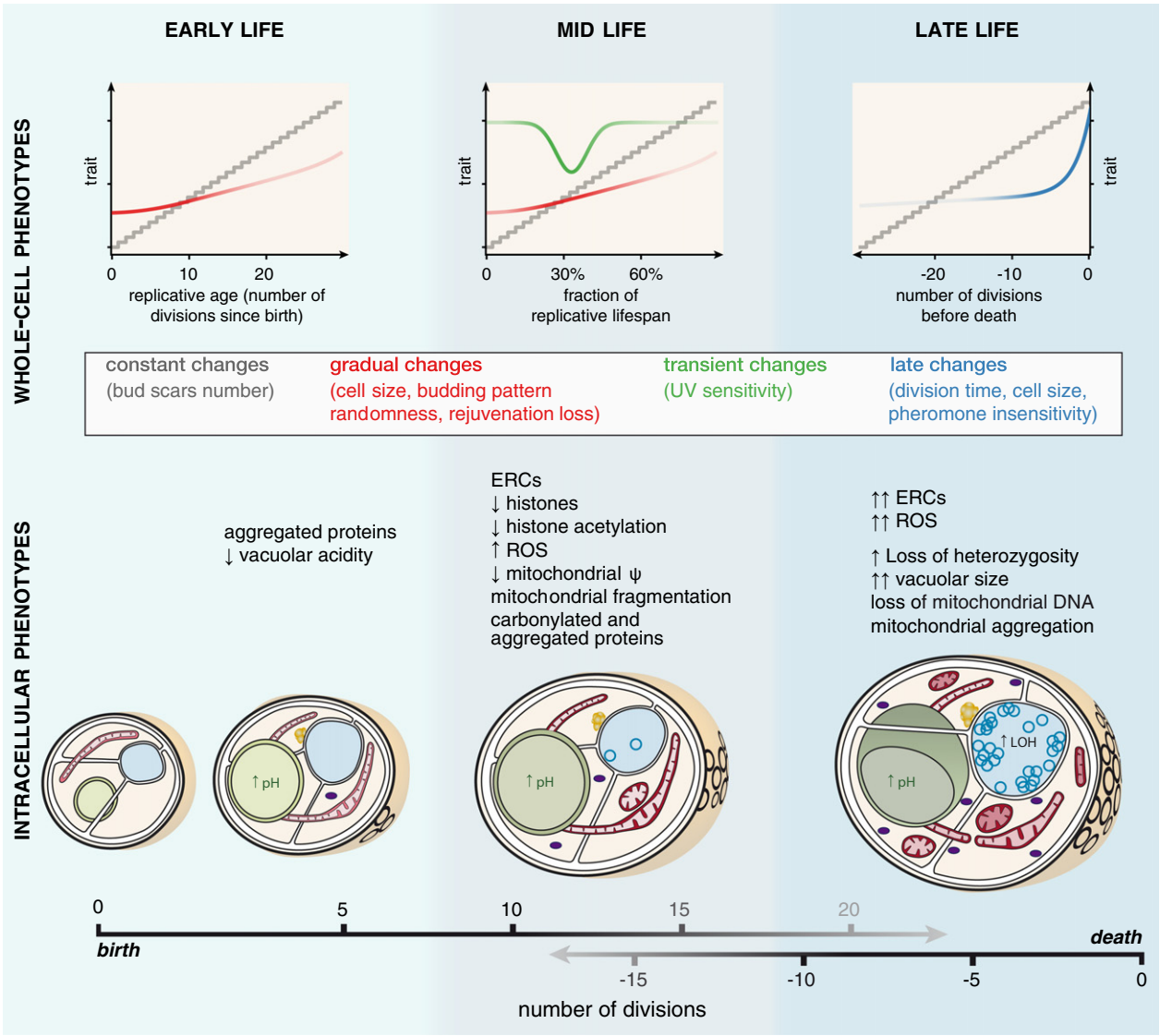

Fig. 5. Three stages in the life of budding yeast cells. The qualitative dynamics of age-associated phenotypes observed at the whole-cell level are depicted in the upper part. Noticeably a given phenotype can follow a combination of those dynamics, for instance size features both a slow gradual increase followed by a massive increase a few divisions before death. The drawings in the lower part illustrate the morphological and intracellular changes occurring with replicative age (symbols and colors as in Fig. 4). The different age metrics used in the upper graphs as well as the dual time axis in the drawings, counting divisions from birth or from death, illustrate how different age metrics can be used to describe different stages of age. Overall, this graphical abstract depicts three stages in the life of budding yeast cells: early age, during which changes do not impair cell growth; intermediate age, during which aging factors start to accumulate; and late age, which corresponds to the last divisions before death, during which damage accumulation ultimately leads to cell death. Importantly, the changes depicted here summarize the trends observed in average in a population of cells and are unlikely to happen in a stereotyped manner in all individual cells.

adapted to handle synergistic effects between different types of damage, as these effect occur too late to be seen by natural selection. Therefore, under the assumption that longevity is selected depending on extrinsic mortality, the transition from the early to intermediate stage is expected to happen at the age when selective pressures decrease – and when intrinsic mortality starts to increase. However, budding yeast has not yet been used as a model organism to challenge the predictions of the evolutionary theory of aging, which prevents us from understanding how the physiological changes observed as cells age relate to changes in selective pressures.

Future challenges include understanding how the longevity of budding yeast strains is shaped. For instance,

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strains evolved under controlled mortality regimes would allow researchers to correlate changes in extrinsic selection pressure with age-associated changes. Another approach is to characterize the determinants of longevity in different natural isolates from diverse environments, which presumably have different extrinsic pressures. Also, it will be instructive to study aging itself (primarily through age-specific changes in reproduction) in well-established longevity mutants. Finally, to understand how aging is shaped by different factors, future studies will also need to explore how these factors interact with each other and whether specific events are stereotyped or occur stochastically. The ability to isolate cells of specific age, coupled with life-long microscopy techniques, should enable these types of global studies.

How general is the aging process?

One remaining question is which aspects of replicative and chronological aging in yeast are shared. Clearly, one pathway that modulates both is the TOR nutrient response pathway. In contrast, some changes that are specific to replicative aging are linked to cell replication (ERC formation and accumulation) and can therefore be seen as byproducts of this activity. Chronological aging is primarily controlled by energy production (mitochondria) and cellular maintenance (autophagy). Although the chronological age has been shown to influence replicative age (Ashrafi *et al.*, 1999), it is not clear whether molecular changes observed during chronological aging play a role in replicative aging.

More generally, identifying aspects of aging that are shared between organisms is both an interesting evolutionary question and the first step toward applying the knowledge gained in yeast to the human aging. Despite the diversity present in species related to budding yeast regarding natural environments and division patterns, aging in these species has only been characterized superficially (carefully reviewed in Roux *et al.*, 2010). However, several multicellular organisms are well-established models to study aging. To apply our knowledge on yeast aging to these species, we must distinguish between the germline and the somatic cell lineages. Due to their replicative activity, dividing germline cells (and stem cells of the soma) are likely to accumulate specific aging factors, similar to those involved in yeast replicative aging. In particular, extrachromosomal circular DNA molecules resembling ERCs have been described in several multicellular organisms (Cohen, 2003; Cohen *et al.*, 2010). These circles are also composed of repeated sequences and are formed by homologous recombination. Although their role in aging has not been demonstrated, Werner syndrome proposes an intriguing link. Yeast cells lacking the WRN homolog, Sgs1, have shorter lifespans and accumulate ERCs. Although the precise link between ERCs and the reduced longevity of *sgs1*Δ mutant cells remains to be clarified, the homology with WRN suggests that extrachromosomal circular DNA may influence aging in multicellular organisms at both the cellular and systemic levels. A second conserved aspect of aging is the lifespan extension observed under caloric restriction. Notably, the genetic pathways that mediate this response, particularly the roles of PKA and TOR, are also conserved between yeasts and metazoans. These connections suggest that the aging process in yeast and multicellular organisms may share common mechanisms.

Linking aging to asymmetry

Rejuvenating one of the cells at the cost of the other is a simple way to perpetuate organisms that age. Although this asymmetry could occur due to sampling effects when small numbers of particles are involved, the accumulation of aging factors during life makes it likely that more refined partitioning mechanisms are involved in rejuvenation. Interestingly, these partitioning mechanisms may either have been selected directly for their role in rejuvenation, or they might retain aging factors as an indirect consequence of their primary activity. For instance, unicellular organisms are directly exposed to their environment, so that foreign DNA molecules can enter the cell. Although physical adaptations such as the cell wall offer some protection, asymmetric segregation of foreign DNA molecules provides an additional way to contain their spread. Hence, the mechanisms involved in the retention of ERCs in the mother cells might not be a dedicated one but rather correspond to cellular immunity against foreign noncentromeric DNA molecules.

A functional connection between asymmetry and aging has long been assumed. However, because of practical limitations, these two phenomena have not been studied simultaneously. Instead, factors that contribute to asymmetry were characterized in young cells, and their effects on replicative lifespan were measured separately. For instance, to understand how the retention of ERCs in mother cells plays a role in aging, measurement of asymmetric segregation of ERCs in young cells has been combined with measurements of replicative lifespan distributions in various strains and conditions (Sinclair & Guarente, 1997; Shcheprova *et al.*, 2008). Similarly, the effect on lifespan of mutations affecting mitochondrial segregation has been interpreted in the light of data characterizing this decrease in young cells (up to seven divisions; Piper *et al.*, 2002). However, the introduction of the MEP and microfluidics now offers the opportunity to test this assumption by directly studying asymmetry during the aging process.

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Aging and rejuvenation are thought to be caused by the accumulation and asymmetric segregation of aging factors. However, changes in longevity caused by changes in asymmetry are difficult to predict, even in the simplest case of a population at equilibrium in a given environment. For instance, increased longevity of mother cells can be interpreted in terms of higher damage transmission to daughter cells. However, higher transmission is also predicted to produce mother cells that have shortened lifespans, as these mothers themselves are born with more damage. Which of these two effects dominate the other can only be predicted in case where it is known whether the aging factor is diluted or accumulates over divisions. These dynamics are determined by the ratio of cell growth rate to aging factor accumulation rate (i.e., the net result of appearance and clearance processes), which are thus critical parameters to analyze the effects of asymmetry-related mutations on longevity. Furthermore, aging factors do not only affect longevity but also growth speed. Hence, more symmetric divisions would result in mother cells that live longer at the cost of their progeny growing slower from their first divisions (due to higher damage load at birth). Indeed, most mutations prolonging life span have been shown to have a negative effect on the strain fitness (Delaney *et al.*, 2011), which illustrates the trade-off between maintenance and growth. The interplay between damage accumulation, cell growth, and asymmetric division highlights the need for quantitative models of asymmetric damage segregation in the context of a population of dividing cells. Such models would allow the systematic study of different possible strategies to handle damage, as well as the relationship between longevity and fitness. Previous attempts to model aging in budding yeast have highlighted the lack of precise experimental estimates of critical parameters, such as the rates of appearance, amplification, and clearance of damage, and the asymmetry of damage inheritance (Gillespie *et al.*, 2004; Rashidi *et al.*, 2012). Notably, the obvious morphological asymmetry of budding yeast has led researchers to overlook these complex dynamics, with the assumption that asymmetry governed damage inheritance. This assumption did not exist in other, more symmetrically dividing cells, leading to more refined theoretical predictions of the effects of damage segregation on cell growth and aging (Chao, 2010). Finally, it is instructive to compare how damage is handled in the context of yeast replicative aging and in stem cells of multicellular organisms. Comparing several types of *Drosophila* stem cells revealed that damaged proteins are inherited by the cell with the shortest functional life span (Bufalino *et al.*, 2013). For instance, the germline stem cells retain damaged proteins and produce damage free cystoblasts, which have the potential to ultimately give rise to new organisms. In contrast, intestinal stem cells remain free of damaged proteins at the cost of their differentiating progeny, which are renewed every week. This study highlights that, although aging and rejuvenation occur through a limited number of mechanisms, they can be modulated to fit the needs of a specific cell type or strain.

One important counterpoint is that the asymmetric inheritance of cellular structures does not necessarily imply that individuals are themselves aging. For example, in *Schizosaccharomyces pombe*, many structures are asymmetrically segregated during mitosis, including the spindle pole body, cell poles, and protein aggregates. However, their inheritance does not increase the death probability of the recipient cell in the absence of environmental stress (Coelho *et al.*, 2013). Indeed, low damage levels in a population can be achieved either through asymmetric cell division or by clearing damage, as already discussed regarding the transcription factor Ndt80 and sporulation. In *S. pombe*, damage clearance might be efficient enough that no aging is observed as long as the stress response machinery is not challenged simultaneously by extrinsic stresses; instead, damage could be inherited randomly by one of the two progeny, where it would then either be cleared or trigger cell death in an age-independent manner. It is hoped that comparative aging studies between different microbial species under different environmental stress conditions will allow the identification of constraints that balance clearance vs. asymmetric segregation of cell damage.

The phenotypes of old individuals depend on their history

In this review, we have described the current knowledge of changes that affect yeast cells as they age. Most work to date has focused on how these changes impair physiological performance, but not all changes are necessarily detrimental. For example, the toxicity of a given aging factor may depend on the environment. Most hypotheses on the benefits of age-associated traits involve stress resistance mechanisms. For instance, if the concentration of protective compounds increases continuously over time, then old cells are expected to handle stress better; trehalose accumulation and its effect on heat resistance provide a good example of this mechanism, although it has not been demonstrated directly (Levy *et al.*, 2012). A second hypothesis is that a general increase in stress resistance may be induced by damage accumulation. At intermediate ages, the increased activation of stress responses would allow cells to both handle the low levels of age-associated damage present at that time, and to confer resistance to external stresses. This mechanism

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might explain the optimal resistance to UV at intermediate age (Kale & Jazwinski, 1996). A last hypothesis related to stress resistance holds in the case of fluctuating environments: after an initial challenge, cells would both respond to the perturbation and adapt to allow them to respond faster upon a second, similar challenge. If the adaptation persists over multiple division times and is asymmetrically inherited, old individuals would be predicted to respond more rapidly due to their history. This adaptation would have been beneficial during the evolution of yeast, as some environmental fluctuations, such as day/night temperature changes, are both repetitive and predictable.

If age-associated benefits are specific to old cells, factors that confer these benefits must be asymmetrically segregated. Although they have not been studied in old cells, conditions specifically promoting growth of mother cells are promising candidates to elucidate the identity of beneficial aging-related factors. One recently studied condition is growth in the presence of pheromone without mating partners, which results in mother-specific pheromone insensitivity and growth, mediated by the formation of Whi3 super-assemblies (Caudron & Barral, 2013). A second condition known to promote growth of older cells is medium-containing reduced metal concentration (Avraham *et al.*, 2013). In this case, the growth advantage arises from physiological constraints: when metal ions are scarce, the mother cell cannot share them with its daughter without compromising its own ability to divide. More generally, if old individuals are under selection when beneficial traits are expressed, these traits would counteract the effects of extrinsic mortality that sets the level of maintenance. Hence this selection would delay aging and promote longevity. Finally, it is tempting to hypothesize that mechanisms involved in rejuvenation are also used by cells to control the segregation of factors responsible for acquired traits. For instance, nuclear pores are involved in the asymmetric segregation of ERCs and play also a role in transcriptional memory, such as the expression of galactose catabolism or inositol synthesis genes (Brickner, 2009). Along similar lines, diffusion barriers involved in the retention of aggregated proteins (Caudron & Barral, 2009) could also retain specific aggregates required for the adaptation to previously encountered conditions.

Together, the different mechanisms envisioned here highlight the importance of individual history regarding both intracellular events (e.g., damage-induced stress resistance) and environmental conditions (e.g., memorization of pre-exposure). Indeed, the individual history can be seen as the integration of all stochastic events that occur during life, which ultimately determines both the phenotype of an individual and how it will age.

Acknowledgements

We thank Christine Weirich, Fabrice Caudron, Tom Kruitwagen, Andrzej Sliwa-Gonzalez and Gregory Paul for critical reading of the manuscript and stimulating discussions. We thank two anonymous reviewers for their comments on an earlier version of this manuscript. We are grateful to Sung Sik Lee for sharing raw data from previously published work. T.J. acknowledges a young researcher award from the Bettencourt Schueller Foundation.

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Appendix 1

Techniques to study age-dependent traits

The current understanding of aging in yeast relies heavily on the techniques available to characterize age-dependent traits. Here, we provide a brief overview of tools used to study replicative aging in *S. cerevisiae*. An overview of chronological aging protocols can be found in other reviews (Bitterman *et al.*, 2003; Longo *et al.*, 2012).

Microdissection

The first studies of senescence in yeast cells relied on microdissection (Schouten, 1935; Barton, 1950). This method was used to demonstrate that the death probability increases with the number of divisions (Mortimer & Johnston, 1959). It became the standard method to study yeast replicative aging and is still widely used (Sutphin *et al.*, 2012). This method consists in using a thin needle under a microscope (Fig. 2a) to sequentially remove all buds produced by a newborn cell (typically 10–40; Park *et al.*, 2002; Steffen *et al.*, 2009). Cells are grown on solid media and are most often kept at 4 °C overnight. Importantly, cooling (9 h day^{-1} at 10 °C) was shown not to affect replicative longevity (Müller *et al.*, 1980). Although this method allows the measurement of several age-dependent traits (growth rate, cell size, budding pattern, etc.), longevity has been the primary trait observed using this method. Studying multiple cells in parallel allows the determination of a distribution of longevities, whose median is commonly used as a proxy for the strain replicative lifespan. Unfortunately, the median lifespan of a strain is an oversimplified metric of aging and discards important information about damage accumulation. Finally, due to its labor intensiveness, microdissection has a limited throughput scale and cannot be used to purify large quantities of old cells.

Old cells purification

To overcome the limitations of microdissection, several methods have been devised to enrich old cells within a culture. Initially, based on the larger size of old cells, density gradients were used in combination with cell synchronization methods (Egilmez *et al.*, 1990). Although cells could be purified up to 20 divisions, the yield decreased sharply with cells older than 10 (typically 1–2% at 17 divisions, still yielding 10^8 cells in total) and the protocol involved multiple steps likely to perturb cell physiology and result in bacterial contamination.

This approach was replaced by the rise of counterflow centrifugation elutriation, a technique that separates cells by size by flowing them through a centrifugation chamber in a direction opposed to the centrifugal force (Fig. 2d; Diamond, 1991). Historically, the same technique had been used to synchronize cells (Gordon & Elliott, 1977). By growing cells in the device and thus continuously removing young daughters, Woldringh *et al.* (1995) demonstrated that old cells could be purified. Further protocol improvements allowed the isolation of old cells from exponentially growing cultures (Laun *et al.*, 2001). Later studies isolated large enough quantities of old cells to perform genome-wide gene expression studies (with median age close to 18 divisions; Lesur & Campbell, 2004).

In addition to these size-based methods, old cells can be enriched based on differential labeling and sorting, as the daughter cell wall is newly synthesized at each division and that the mother cell wall is stable over several divisions. In the standard enrichment protocol, young cells are biotinylated, grown to allow a number of divisions and further separated from their daughters using streptavidin affinity purification (Fig. 2e; Smeal *et al.*, 1996). This technique, allowing the purification of 10^8 cells per original cell after 20 divisions, was instrumental in demonstrating the existence of ERCs (Sinclair & Guarente, 1997). The proportion of old cells has been further improved using subsequent sorting steps such as fluorescence activated cell sorting, which relies on differential staining of cells labeled with streptavidin-PE (staining biotinylated cells only) or with WGA-FITC (which stains bud scars; Chen & Contreras, 2007). Unfortunately, these later purification steps are limited by the high variability of the WGA-FITC staining observed in cells of a given age.

These old cell purification methods face the same challenge: isolating with high accuracy rare old cells that have been diluted by exponential growth of their progeny. The expected proportion of cells of age n in an exponentially growing population is $1/2^n$. This translates to 1 : 1000 cells at age 10 and to 1 : 1 000 000 at age 20. To overcome the difficulty of isolating rare cells, genetic methods of preventing daughter cell division have been developed. In an early report, *CDC6*, which is

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essential for cell-cycle completion, was put under the control of a promoter expressed only in mother cells, the promoter of the HO endonuclease (Jarolim *et al.*, 2004). Although this effectively prevents the division of daughter cells, it unintentionally limits the median replicative lifespan of mother cells to four divisions. More recently, the 'Mother Enrichment Program' was reported: it is based on a strain in which the daughter cells division is inhibited by excising two essential genes, *CDC20* and *UBC9*, in a daughter-specific manner (Lindstrom & Gottschling, 2009). Excision is accomplished by the Cre recombinase, which is expressed only transiently in young buds as it is under the control of the daughter-specific *SCW11* promoter (Colman-Lerner *et al.*, 2001). Further, Cre is fused to an estradiol-binding domain (EDB), which allows its import into the nucleus only in the presence of estradiol and allows exponential growth in the absence of estradiol (Lindstrom & Gottschling, 2009). While this new method has already proven its strength, it must be noted that daughter cells are not killed by the Cre activity but remain as large, metabolically active, metaphase-arrested cells. Hence, rather than growing exponentially, the population of daughters grows linearly with time. It follows that the Cre activation by estradiol must be complemented by an affinity purification step (Fig. 2f). In addition, the number of cells that can be isolated at a given age is limited by mutations that allow the cells to escape the selection. These mutations either arise in the Cre-EDB gene itself, prevent localization of the EDB, or prevent the entry of estradiol in the cell. The rate of escape has been measured to be $\mu = 1.4 \times 10^{-6}$ per division in haploid cells and $\mu = 1.4 \times 10^{-8}$ per division in diploid cells (Lindstrom & Gottschling, 2009). This limits cell yield to roughly 1.7×10^{6} diploid cells that are 20 divisions old, or more generally $1/(2\ a\ \mu)$ cells of age a.

Life-long monitoring

The development of microfluidics has recently brought new tools relevant to the study of yeast aging. Last year, three methods for maintaining yeast cells during their entire lifespan under a microscope were reported. One is based on the binding of biotinylated young cells to a streptavidin-coated coverslip (Fig. 2b; Xie *et al.*, 2012). The other two are based on mechanical trapping of mothers under gel pads, with their buds being washed away by the channel flow (Fig. 2c; Lee *et al.*, 2012; Zhang *et al.*, 2012). These new methods not only allow longevity measurements (similar to microdissection) but also the characterization of age-dependent traits, in particular life-long observation of fluorescently tagged proteins. In practice, loss of cells during the experiments currently limits the throughput for longevity measurements, as only very few cells can be followed during their entire lifespan. In principle, this weakness could be overcome by survival analysis taking into account data corresponding to cells observed during a part of their lifespan only, instead of the classical comparison of median lifespan between strains and conditions.

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