The impact of metabolism on aging and cell size in single yeast cells
Huberts, Daphne

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Chapter 1

Metabolism has a central role in yeast aging

Daphne H.E.W. Huberts
Introduction

Aging is the deterioration of an organism ultimately leading to death. Although the process is very common in living organisms, we still do not understand what drives the gradual decay of cellular functions in a cell. This lack of understanding is probably caused by the fact that aging is a very complex, multifaceted phenomenon that affects almost all aspects of a cell’s function. In humans, aging is an important risk factor for many diseases, such as cancer, type 2 diabetes and neurodegeneration (1). Understanding the mechanisms of aging and using this knowledge to slow down aging is therefore the prime target of aging research.

Yeast as a model in aging research

As humans are complex organisms with a long lifespan (2), aging research is mainly performed with model organisms (3). The simplest model organism in aging research is the yeast Saccharomyces cerevisiae, which is a unicellular eukaryote. S. cerevisiae replicates by asymmetric cell division with a mother cell producing a smaller daughter cell, which is also referred to as a bud. Although the mother cell ages, the daughter cells are generally born young and have a full life expectancy (4). Only in very old mother cells, age asymmetry is lost and daughter cells age prematurely.

Advantages of working with yeast compared to other aging models, such as the fruit fly Drosophila melanogaster or mice, are its short generation time, its ease of handling and that it can be kept under absolutely constant environmental conditions during its lifespan. In addition, a large fraction of its genes have been characterized and a large array of experimental tools exists to study this organism. For example, a gene deletion collection (5) and a strain collection with GFP tagged proteins are available (6). Finally, several proteins that modulate aging are conserved between yeast and humans. For example, Sgs1 and its human orthologue Wrn are both important in maintaining genome stability (7). Mutations in WRN lead to a hereditary premature aging disorder called Werner syndrome in humans (8), while deletion of sgs1 causes a shortened lifespan that is accompanied by a typical aging phenotype (increased cell size, increased division time and ERC accumulation) (9). Although the strength of S. cerevisiae as an aging model resides in its simplicity compared to multi-cellular aging models, this also provides disadvantages when it comes to applying any acquired knowledge to human aging. First, yeast aging omits the potential role of cell-to-cell communication and cell differentiation in aging inside a multi-cellular organism (10). Second, not all aging mechanisms identified in yeast may be relevant for aging in humans. For example, aging in yeast was shown to be associated with the accumulation of extrachromosomal ribosomal DNA circles (ERCs), which are formed when ribosomal DNA (rDNA) is excised from the genome (11). The age-associated accumulation of ERCs appears to be unique for yeast (12).
Different yeast aging models

In yeast, we generally distinguish between two forms of aging. These are called chronological aging and replicative aging: The chronological age is the time that a cell survives in the absence of nutrients (13, 14). The yeast chronological aging model is regarded as a model for non-dividing cells, e.g. terminally differentiated cells in a multi-cellular organism (15). To study chronological aging, yeast cells are typically grown to stationary phase and maintained in the spent medium, although several variations of this assay exist (16). Cell viability is then determined by re-introduction of nutrients and scoring the ability of a cell to re-enter cell cycle and divide. In contrast, replicative lifespan (RLS) is the number of daughter cells that a replicating cell produces before it dies. RLS is therefore different than the age measured in most other organisms, where age is expressed as survival time (17). The replicative aging model is considered a model for actively replicating cells in multicellular organisms, such as stem cells (14).

Although the chronological and replicative aging models both have their merits (15), we find the replicative model to be more suitable for our purposes. Chronologically aging yeast cells are nutrient-deprived, which causes them to stop dividing and to enter a non-dividing quiescence-like state during the assay (18, 19). Most post-mitotic cells in multicellular organisms are, however, not nutrient-deprived and reside in a high metabolically active state (14). This discrepancy may make the mechanisms involved in chronological aging in yeast less relevant for the aging of post-mitotic cells in humans.

Methods to study replicative aging in yeast

Studying replicative aging in yeast is difficult, because cell numbers increase exponentially during growth and mother cells are quickly overgrown by young daughter cells. For example, on glucose in an exponentially growing colony or culture, 54% of cells are daughter cells that have never budded before and around 27% have budded once (20). An average mother cell from a laboratory yeast strain produces 25 buds, which makes it impossible to directly study the phenotype of old yeast cells in a culture. Several methods were therefore developed to investigate replicative aging of yeast cells.

Population-based methods to study yeast aging

Aged mother cells can be isolated from a culture using magnetic cell sorting or centrifugal elutriation. Magnetic cell sorting requires the surface of the cells to be labeled with biotin at the start of the experiment. Since the cell membrane of daughter cells is synthesized de novo, biotin-labeling remains only on the mother cell’s membrane (21). Mother cells are separated from daughter cells using magnetic beads coated with streptavidin. Alternatively, aged mother cells can be isolated from a culture using centrifugal elutriation, which separates cells based on differences in cell size (22).
An advantage of centrifugal elutriation compared to magnetic cell sorting is that aged mother cells can be directly sorted from cultures without labeling, which could induce stress on the cells (23). In addition, there is a smaller amount of contamination from dead cells in elutriated samples compared to those obtained with magnetic sorting where dead cells are isolated together with aged cells due to the presence of the biotin labeling on the cell membrane (24). A disadvantage of both magnetic cell sorting as well as centrifugal elutriation is that it is difficult to obtain large amounts of old yeast cells, because these cells make up only a very small fraction of the cell population (20). Older mother cells can be obtained by combining cell sorting with the Mother Enrichment Program (MEP), which is an inducible genetic system that selectively eliminates the ability of daughter cells to replicate (25). With the MEP, cell numbers do not increase exponentially, but linearly, leading to increased fractions of aged cells in a culture and making it possible to isolate a larger number of aged cells. Nevertheless, it remains impossible to isolate large numbers of cells with an average replicative age of 25 or more buds (26).

**Classical microdissection technique**

Until recently, the microdissection technique was the only method available to measure the RLS of single yeast cells (27). In this method, cells are grown on agar plates and daughter cells are moved away from their mother cells every two hours using a needle. Each daughter cell removed from a mother cell is scored and when the mother cell dies, the total number of daughter cells produced is the RLS of this cell. Microdissection however suffers from several drawbacks. First, the method is extremely laborious, because each daughter cell needs to be removed manually. Second, due to the use of a thick opaque agar plate, the cells can only be poorly visualized.

**Microfluidic devices**

As an alternative to the microdissection technique, microfluidic devices were recently developed to study yeast replicative aging. These transparent devices are made from silicone, such as polydimethylsiloxane, and contain channels through which medium is pumped. Mother cells are trapped inside the channels in various manners, e.g. in cavities or underneath pads (28, 29). Daughter cells are typically removed automatically from their mothers by the flow of medium, which allows aging experiments to be performed under stable nutrient and temperature conditions. The automated removal of daughter cells makes RLS measurements less labor intensive and allows a greater number of cells to be studied per experiment.

Another important feature of microfluidic devices is the possibility to track individual cells in real time using high-resolution fluorescence imaging enabling the study of cell cycle control, organelle morphology and a wide variety of other molecular processes as a function of aging. These types of measurements are crucial to understand the interplay between various cellular processes and aging.
Several different versions of microfluidic devices were developed in the past few years, which differ mostly in the manner, in which mother cells are immobilized inside the device. Lee et al. 2012 (28) published the first microfluidic device, referred to as the microfluidic dissection platform that made it possible to track them during their entire RLS. Inside the microfluidic device, mother cells are held down between soft elastomer micropads and a thin cover glass, which allows high resolution fluorescent imaging of mother cells during their entire RLS, while daughter cells are washed away by a flow of medium (Table 1). Zhang et al. 2012 (30) quickly thereafter published a very similar microfluidic device using pensile columns instead of micropads. The design of this microfluidic device has a slight advantage over the design of Lee et al. 2012 (28), because it allows multiplexing of experiments into a single chip. The Charvin group developed a microfluidic chip called CLiC, in which cells are loaded into cavities alongside the main channel (29). An advantage of CLiC is that a very low number of mother cells are flushed away during the experiment compared to the microfluidic dissection platform (35% vs. 70% of loaded cells are flushed during an average experiment). A disadvantage of the method is that mother cells grow at the bottom of cavities, which are crowded with other cells. This may hamper the diffusion of medium to the mother cells. Crain et al. 2014 (31) developed the device ALCATRAS, which catches individual yeast cells using two pillars placed in a V-shape. The device can be used to track up to 1000 cells per experiment, but can only be run up to 67 hours after which the device clogs up with cells. Although 67 hours is sufficient to measure the average RLS of yeast cells (25 generations), it may not be sufficient to measure the RLS of longer lived cells. ALCATRAS seems therefore to be more suitable for measuring single cell events over shorter time periods in a great number of cells (31).

Table 1. Overview of characteristics of the different microfluidic devices published to measure RLS in *S. cerevisiae*.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Description</th>
<th>Experiments per chip</th>
<th>Cells per experiment</th>
<th>RLS WT (buds)</th>
<th>Additional comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lee et al. 2012 (28)</td>
<td>Microfluidic dissection platform</td>
<td>2</td>
<td>100</td>
<td>25.9</td>
<td></td>
</tr>
<tr>
<td>Zhang et al. 2012 (30)</td>
<td>Pensile columns</td>
<td>4</td>
<td>100</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Fehrmann et al. 2013 (29)</td>
<td>CLiC</td>
<td>1</td>
<td>25</td>
<td>25</td>
<td>Medium diffuses to cells in cavity</td>
</tr>
<tr>
<td>Crain et al. 2014 (31)</td>
<td>ALCATRAS</td>
<td>1</td>
<td>1000</td>
<td>24.7</td>
<td>Measurement max. 67h</td>
</tr>
</tbody>
</table>
Proposed theories of aging in yeast

The yeast aging field has uncovered many cellular processes and environmental factors that contribute to yeast aging. Below, we describe the most prominent theories for yeast aging.

Sir2 and the ERC theory of aging

The most accepted cause for aging in *Saccharomyces cerevisiae* is the accumulation of extrachromosomal rDNA circles (ERCs) (11). During aging, ERCs excise from the yeast rDNA locus, which consists of 100 to 200 tandem rDNA repeats. Each ERC contains an autonomously replicating sequence (ARS), which causes the ERCs to replicate with each cell division cycle. ERCs accumulate exponentially in mother cells due to their asymmetric segregation between mother and daughter cells (11). There are two main explanations for why ERCs segregate asymmetrically between mother and daughter cells. One model suggests that ERCs are anchored to nuclear pore complexes (32) and are actively retained in the mother cell due to the presence of a diffusion barrier. The other model suggests that ERCs are passively retained in the mother cell by geometric constraints of the bud neck and the short duration of mitosis (33).

A strong correlation between the accumulation of ERCs and RLS was reported. For example, artificial introduction of ERCs in young cells or the introduction of plasmids with an ARS was found to shorten RLS (11). However, it is still unknown why ERCs are lethal to the cell. One hypothesis states that ERCs accumulate to such high concentrations that they titrate away essential factors for DNA replication, such as transcription and replication factors (11, 34). Alternatively, it was proposed that the accumulation of ERCs promotes instability in the rDNA locus leading to cell death by a further unknown mechanism (35-37). ERCs may also bind to a putative receptor in the nuclear envelope blocking the function of this essential receptor and causing cell death (35, 38).

An important regulator of ERC accumulation is the NAD\(^+\)-dependent histone deacetylase Sir2 that silences chromatin at the rDNA locus (11) among other functions (39). Silencing of rDNA causes a more closed inaccessible chromatin structure and increases genome stability. In agreement with this notion, overexpression of Sir2 increases rDNA stability and increases RLS (40, 41), while deletion of sir2 causes an increased recombination frequency in the rDNA locus, accumulation of ERCs and a reduced RLS (40, 42). These findings make Sir2 a key regulator of yeast RLS in the ERC theory of aging.

Mitochondria and the free radical theory of aging

Aging in yeast is also associated with changes in mitochondrial morphology (43, 44), a loss of mitochondrial membrane potential (45), a loss of mitochondrial DNA (mtDNA) (46, 47) and an increase in ROS production (48). Thus, various studies suggest that mitochondria contribute to aging (39, 48-50). For example,
an increase in mitochondrial fusion delays the mitochondrial fragmentation that is observed during aging and subsequently increases RLS (44). Delaying mitochondrial dysfunction by suppressing the decline of vacuolar acidity also extends RLS (45). Furthermore, asymmetric segregation of mitochondria was found to be crucial for daughter cell rejuvenation. When the asymmetric inheritance of mitochondria is lost, daughter cells no longer inherit the fittest mitochondria and have a limited RLS (48). These observations suggest that mitochondria are important regulators of RLS.

Mitochondria were proposed to contribute to aging by different mechanisms. Mitochondria are a source of free reactive oxygen species (ROS) and mitochondria in older cells were shown to produce more ROS than those in younger cells (48, 51). ROS are highly reactive chemicals and inside the cell they can damage DNA, oxidize lipids and proteins. The accumulation of oxidative damage is considered to contribute to the aging process (52-54). In addition, ROS may increase genomic instability (45) and ERC accumulation (55). Damage or loss of mtDNA induces the retrograde response, which enables cells to compensate for the loss of respiration and encompasses the transcription of a broad range of nuclear genes and changes in metabolic, regulatory and stress-related pathways (56). The retrograde response was shown to increase RLS in some yeast strains (57, 58) and is therefore another mechanism by which mitochondrial function affects aging.

**Hypertrophy theory of aging**

As cells age, mother cells can easily be distinguished from their daughters (59), because their cell size increases steadily (28, 60). Consequently, it was suggested that cell size limits RLS (60-62). In fact, Yang et al. (60) suggested that cells die at a relatively constant size, meaning that cells cannot maintain themselves beyond a certain cell size. In support of this hypothesis, various mutations that decreased cell size extended RLS, while those that increased cell size shortened RLS, suggesting a correlation between cell size and RLS (60). Cell size was also shown to be dependent on nutritional conditions (63, 64). Cells growing rapidly on carbon sources, such as glucose and galactose, have a larger cell size compared to cells growing slowly on carbon sources, such as pyruvate and ethanol (65). Since cell size is thought to limit yeast RLS (60), altering cell size at the initial cell division should alter RLS. In agreement with this hypothesis, ethanol grown cells had a prolonged RLS compared to cells grown on glucose (17).

Cell size may limit RLS in various manners, such as dilution of essential factors such as DNA or cell cycle regulatory proteins that are expressed at low levels (66). Alternatively, the increase in cell surface may induce cell membrane stress (60) or the reduced surface-to-volume ratio may limit import of nutrients or export of waste products. Various metabolic changes have been observed in aging yeast cells, such as the age-dependent expression of hexose transporters and a metabolic shift from fermentation towards gluconeogenesis (67) despite cells being maintained in a constant environment. These metabolic changes suggest that the import of nutrients is reduced with replicative age implying that cell size may affect metabolism in aging cells. A further reduction in the amount of
nutrients that are imported may cause cell division to finally halt and lead to cell death.

Yet, there is also evidence that contradicts the hypothesis that cell size causes senescence. For example, deletion of fob1 increases RLS without altering cell size (68). Moreover, diploid yeast cells are larger than haploid yeast cells, but also have a longer RLS than their haploid counterparts (69) showing that an increased cell size does not necessarily correlate with an increased RLS. Consequently, it remains under debate whether cell size is a determinant of RLS.

Metabolism modulates replicative lifespan

Over 70 years ago, metabolism was shown to have a pivotal role in aging with the discovery that reducing the caloric intake of mice extended lifespan (70). A nutritious diet low in calories, commonly referred to as calorie restriction (CR), was subsequently shown to extend the lifespan of a broad range of organisms, such as yeast, worms, fruit flies, rats, and monkeys (71-74). As a result, a considerable amount of research has been performed to understand the mechanisms that regulate the lifespan extending effect of CR.

The role of NAD⁺ in CR-mediated lifespan extension

Initially, CR was thought to control aging by slowing down metabolism and thereby increasing the ratio between oxidized and reduced nicotinamide adenine dinucleotide (NAD⁺/NADH) (75) in the cell. Indeed, increasing NAD⁺ availability in the cell by deletion of NAD⁺ consuming enzymes was shown to extend RLS (76, 77). As the activity of Sir2 is dependent on levels of NAD⁺ (73), increasing NAD⁺ availability in the cell is thought to enhance Sir2 activity, which reduces genomic instability at the rDNA locus, reducing ERC accumulation and increasing RLS (78). However crucial aspects of this model of CR-mediated lifespan extension are currently disputed. For example, CR also extended the lifespan of cells that lack Sir2 (79, 80) and cells that are unable to respire (81). CR also did not increase intracellular NAD⁺ levels (82, 83).

Other studies suggest that the nicotinamidase Pnc1, which is part of the NAD⁺ salvage pathway, could mediate lifespan extension under CR conditions (82). For example, in a Δpnc1 strain, there is no lifespan extension under CR conditions showing that Pnc1 is essential for RLS extension under CR conditions. Moreover, overexpression of Pnc1 increases RLS and CR conditions were found to induce PNC1 expression (82). Pnc1 could exert its beneficial effect on RLS either by reducing the intracellular levels of nicotinamide, which inhibits RLS extension (Figure 1) (81) or by producing nicotinic acid, which is exported outside the cell in order to use it later in life (Figure 1) (84). However, it remains unclear how nicotinic acid or nicotinamide can modulate RLS.
The role of the Target of Rapamycin (TOR) pathway in CR-mediated lifespan extension

CR conditions may also elicit RLS extension via the target of rapamycin 1 (Tor1) kinase (Figure 1). Tor1 controls growth in response to nutrients by regulating translation, transcription, carbohydrate transport, ribosome biogenesis and autophagy via a complicated and extensive signaling network referred to as the TOR pathway (85-87). The TOR pathway is thought to function in CR-mediated lifespan extension, because deletion of tor1 in yeast results in lifespan extension and elicits a cellular response that is reminiscent of a cell experiencing nutrient deprivation, such as inhibition of protein synthesis (85, 88). In addition, calorie restriction does not extend lifespan in absence of tor1 suggesting that Tor1 mediates CR-mediated lifespan extension (89).

Tor1 is thought to increase RLS by suppressing ribosomal protein synthesis (85, 86) (Figure 1). Deletion of ribosomal proteins, such as rpl6b and rpl31a were shown to extend lifespan as well (89, 90) suggesting that ribosomal protein synthesis is an important regulator of lifespan (91). Reducing ribosomal protein synthesis may extend RLS by reducing replication initiation in the rDNA locus, which directly increases rDNA stability (34) and reduces ERC accumulation, or indirectly via an unknown pathway (90) (Figure 1).

Aim of the thesis

The aim of this thesis was to determine how metabolism affects yeast aging in single yeast cells using a novel microfluidic device. In particular, we investigated how changes in external nutrients affected yeast RLS and cell size.

Figure 1. Model of how CR conditions could induce lifespan extension. The grey dashed lines with a question mark indicate that the relevance of the link is in question among different research groups.
Outline of the thesis

In this thesis, we initially review how cells are able to sense nutrients in their environment (Chapter 2). We hypothesize that cells are able to regulate their metabolism by sensing their carbon uptake rate without discriminating between carbon sources. In Chapter 3, we describe the use of a newly developed microfluidic dissection platform that greatly improves our ability to study yeast aging. Using this microfluidic dissection platform, we studied the RLS of yeast cells growing under normal (2% glucose) and calorie restricted conditions (0.5% - 0.05% glucose). We did not detect any extension in RLS under CR conditions (Chapter 4). In contrast, CR conditions did extend RLS using the classical microdissection method suggesting that method-specific effects, instead of changes in carbon uptake rate, contribute to CR-mediated lifespan extension in yeast. In Chapter 5, we investigate how cell size increases with replicative age. We found that repeated rounds of protein overproduction after mitosis causes cell size to increase with replicative age. The rate of cell size increase with replicative age is dependent on the carbon source and can be explained by differences in protein synthesis rates between carbon sources causing more or less protein to be overproduced per cell division. In Chapter 6, we discuss our findings in respect to literature and our future expectations for yeast aging research.

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METABOLISM HAS A CENTRALE ROLE IN YEAST AGING


