Molecular responses of *Saccharomyces cerevisiae* to near-zero growth rates

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Veur mien mam

Contents

1. General introduction

Saccharomyces cerevisiae*: from industrial workhorse…*

The application of yeast by humankind dates back millennia, as illustrated by ancient Egyptian drawings of leavening of bread and beer production (Fig. 1.1A), and references in the Bible to the use of yeast (Exodus XII:34;39). *Saccharomyces cerevisiae* is the most used yeast in baking, hence its common name baker's yeast. Leavening of dough is not the only application of yeast that dates back to ancient times, as indicated by its binomial name and other common name, brewer's yeast. The excellent fermentative capacities of *S. cerevisiae* and other, closely related *Saccharomyces* species are also used for production of alcoholic beverages such as wine and beer. This longstanding relation between humans and yeast (Fig. 1.1) has, in recent years, expanded beyond food biotechnology. Nowadays, *S. cerevisiae* is extensively used in 'industrial biotechnology': the industrial production of a wide variety of chemicals from renewable feedstock, with the aid of microbes and enzymes. Ethanol, a natural product of *S. cerevisiae* and an alternative transport fuel, is currently the single largest product in industrial biotechnology (85 billion liters in 2011,(Caspeta *et al.*, 2013)). However, ethanol is only one example of a large-scale product of industrial yeast biotechnology.

Through metabolic engineering, *S. cerevisiae* strains have been constructed that can produce bulk-chemicals such as organic acids, glycerol and the hydrocarbon farnesene (Asadollahi *et al.*, 2010; Cordier *et al.*, 2007; Curran *et al.*, 2013; Kirby and Keasling, 2008; Otero *et al.*, 2013; Zelle *et al.*, 2008), fine chemicals such as a key precursor for the anti-malaria drug artimisinin (Paddon *et al.*, 2013) and medicinally or industrially relevant peptides and proteins, such as insulin and amylase (Liu *et al.*, 2014; Nielsen, 2013; Thim *et al.*, 1987).

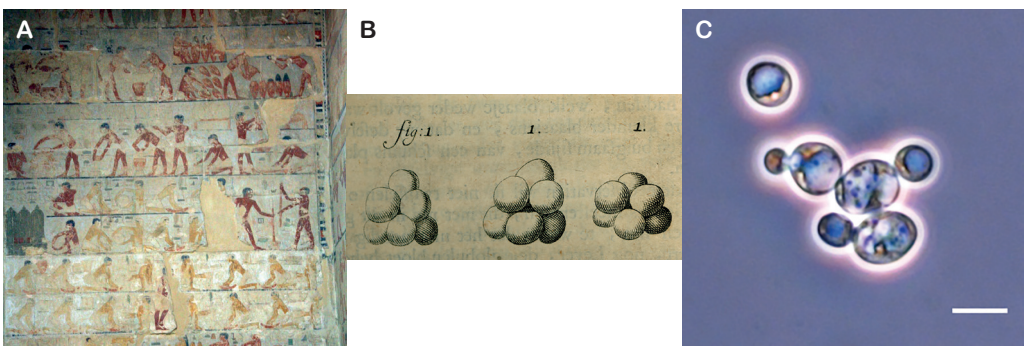


Figure 1.1 The long-standing relationship between yeast and man
(**A**) Yeast was already employed in ancient times as demonstrated by drawings on the Mastaba Tomb of Ty depicting the production of bread and beer, dated ca. 2500 BC (Image courtesy of Dr. Benderitter, copyright by www.osirisnet.net). (**B**) Millennia later, in 1680, Anthonie van Leeuwenhoek was the first to observe yeast cells, although he was not aware of their true nature. A drawing from his observations in a letter to the Royal Society of London (van Leeuwenhoek, 1684). (**C**) Phase contrast-micrograph of budding yeast *Saccharomyces cerevisiae*. Scale bar represents 5 µm.

Due to the long use of *S. cerevisiae* in several biotechnological processes and the ease of its cultivation, this unicellular fungus is now one of the most and best-studied eukaryotic organisms. Although its physiology under various conditions, including process and laboratory conditions, such as different temperatures and levels of nutrients, has been subject of study already for decades (Eaton and Klein, 1954; McManus, 1954; Stier, 1933), a turning point in yeast research took place in 1996. In that year, the full genome sequence of the laboratory strain S288C, the first full eukaryotic genome sequence, was published (Goffeau *et al.*, 1996). This milestone initiated a new era of systematic annotation of gene functions, mostly by deletion and overexpression studies. Progress in this area required and stimulated the development of new tools for analyzing and 'editing' the yeast genome. Today, the already rich tool-set for genetic modification of *S. cerevisiae* continues to expand at an enormous rate. Examples include the use of new, recyclable markers for gene deletion (Solis-Escalante *et al.*, 2013) and cloning of large genetic constructs using a procedure called Gibson Assembly (Gibson, 2012) or improvements thereof (Kuijpers *et al.*, 2013). While these techniques are being implemented, new and spectacular options, such as the CRISPR-CAS system, make an entry into yeast molecular biotechnology (DiCarlo *et al.*, 2013). These developments have led some authors to propose that *S. cerevisiae* may soon take over the role of the bacterium *Escherichia coli* as the universal laboratory work horse in molecular genetics research (Curtis *et al.*, 2013). In addition to this strong development of what nowadays is denoted as synthetic biology, i.e. the design or re-design of biological parts for useful purposes, the availability of the genome sequence also initiated the 'omics' era. A vast set of technologies has been developed to generate information on different levels of yeast biology, examples are genomics, transcriptomics, proteomics and metabolomics data (Kandpal *et al.*, 2009). Integration of the resulting, sometimes large datasets, allows the dissection of the different regulatory levels and more thorough understanding of the entire system. This integral, model-based approach to biology has been defined as systems biology (Castrillo and Oliver, 2011; Kowald and Wierling, 2011; Snyder and Gallagher, 2009).

Integration of synthetic and system biology approaches holds great potential for the optimization of existing industrial applications of *S. cerevisiae*. The aforementioned production of fuel ethanol forms a perfect example of this, as metabolic engineering has led to reduction of by-product formation (Guadalupe-Medina *et al.*, 2014), improvement of product yield (Basso *et al.*, 2011b) and expansion of the substrate range of *S. cerevisiae* to include C5-sugars (Farwick *et al.*, 2014; Kuyper *et al.*, 2005; Wisselink *et al.*, 2007). The latter development was an important step in enabling the use of non-food agricultural residues as feedstock for bioethanol production, a process that is currently being implemented at full industrial scale (POET-DSM, 2014). In addition to improving its current industrial applications, the increasing genetic accessibility of *S. cerevisiae* has played a major role in expanding its product portfolio.

… to eukaryotic model organism

The same characteristics - ease of cultivation and genetic manipulation - that contributed to its strong reputation as an industrial workhorse, have also firmly established *S. cerevisiae* as a model eukaryote for fundamental research. Many cellular processes are strongly conserved among eukaryotes, enabling the use of yeast as a model organism to study such processes. Strong functional homologies between *S. cerevisiae* and metazoans have even led to situation in which the discovery of fundamental biological mechanisms in yeast was followed by their detection in higher eukaryotes. A clear example is provided by the experiments with *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* that led to elucidation of key regulators in the eukaryotic cell cycle (Fig. 1.2), the cascade of events that cells undergo prior to and during cell division (Nurse *et al.*, 1998). In 2001, the importance of these discoveries was underlined with the Nobel Prize in Physiology or Medicine 2001 (Pulverer, 2001).

In addition to cell cycle control, large parts of other signaling cascades are also conserved between yeast and mammals (Smets *et al.*, 2010). Examples include the Target Of Rapamycin (TOR) signaling pathway (De Virgilio and Loewith, 2006), AMP-activated/Snf1 protein kinases (Hardie 2007) and cAMP-activated protein kinase A (PKA), and Sch9/PKB (Geyskens et al 2001). Although the actual inducing signals may differ between organisms (De Virgilio and Loewith, 2006), yeast has played and continues to play a central role in studies on eukaryotic signal transduction pathways (Smets *et al.*, 2010). Not only the signaling pathways, but also many of the processes that they control are conserved. An example is autophagy, the process that turns over organelles and proteins. This strongly conserved pathway is controlled by TOR and PKA and plays an important role in tumor suppression and protein aggregate clearance in humans (Stephan *et al.*, 2010).

While several cellular processes in eukaryotes were first discovered and studied in yeast, programmed cell death or apoptosis forms a notable exception. Apoptosis was long considered a purely metazoan mechanism until, in 1997, a mutant yeast strain was described to exhibit several hallmarks of apoptosis during cell death (Madeo *et al.*, 1997; Madeo *et al.*, 2002). Since then, yeast orthologs of key proteins involved in metazoan apoptosis have been identified. Currently, several research groups use *S. cerevisiae* to study apoptotic processes and inducers (Côrte-Real and Madeo, 2013; Madeo *et al.*, 2004).

Not only programmed cell death, but also cell division control and DNA repair are crucial processes for maintenance of healthy human tissues and alterations. Alterations in these processes can lead to transformation of normal cells into malignant tumor cells, i.e. carcinogenesis (Hanahan and Weinberg, 2000). Due to the homology of these processes between metazoans and *S. cerevisiae*, the latter can be used as an inexpensive, easy-to-cultivate model organism for studying specific hallmarks of carcinogenesis. The ease of cultivation also allows for fast screening and studying of responses, i.e. sensitivity or resistance of specific mutants to anti-cancer treatments and novel compounds (Matuo *et al.*,

2012). The excellent genetic accessibility of yeast can not only be employed to investigate the normal functioning and regulation of conserved processes, but also to 'humanize' parts of its catalytic and regulatory networks to generate models for specific human diseases. The expression of proteins such as alpha-synuclein and beta-amyloids, for example, resulted in humanized yeast models for Parkinson's and Alzheimer's disease, respectively (Braun *et al.*, 2010; Winderickx *et al.*, 2008). These models can not only be used to increase fundamental knowledge on the corresponding pathologies, but also for screening of compound libraries to generate 'leads' for possible therapeutics, as has been successfully done for compounds that reduce alpha-synuclein fibrilization toxicity (Griffioen *et al.*, 2006).

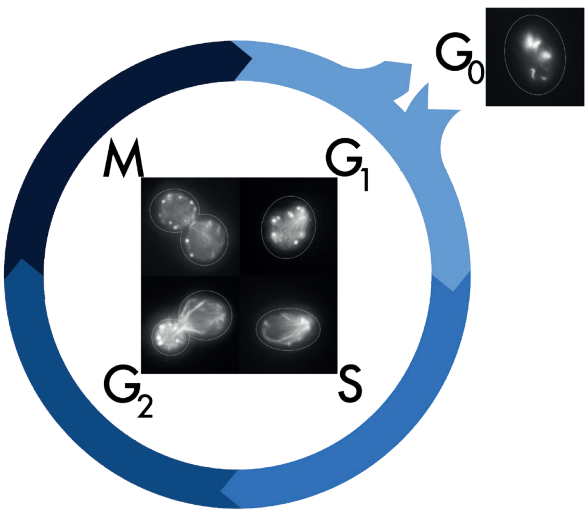


Figure 1.2 The yeast cell cycle
The active cell cycle consists of 4 phases, the gap1 (**G1**) phase during which growth and glycogen accumulation take place to prepare for the irreversible passage through Start. During the synthesis (**S**) phase, replication of the cellular DNA takes place. This phase is followed by a second gap (**G2**) during which cells prepare for the actual division, Mitosis (**M**). Additionally to this active cell division process, cells may exit the cell cycle to a gap 0 (**G0**) phase (see Box 1). Inserted micrographs show actin structures in *S. cerevisiae* (stained with Alexa-488-phalloidin conjugate) at different stages of the cell cycle.

Cancer and neurodegenerative diseases are especially prevalent in developed countries and age forms a major risk factor in these populations (Niccoli and Partridge, 2012). Although their average lifespan differs by several orders of magnitude, many aging-related processes are conserved between metazoan and yeast cells. Aging research performed in yeast has already uncovered the largest number of genes involved in this process in any organism, including man (Longo *et al.*, 2012). In addition to the abovementioned advantages of yeast in experimental design, the short lifespan of yeast as compared to laboratory animals such as mice provides an additional advantage in aging research. Furthermore, the asymmetric division of *S. cerevisiae* by budding allows dissection of replicative aging and chronological aging, i.e. of mother-cell specific aging and aging in non-dividing yeast cells respectively (Breitenbach *et al.*, 2012c).

Overall we can conclude that the long-term relationship between man and yeast has evolved from a rather prosaic exploitation of the latter, without any appreciation for its complexity, to a realization that we, as humans, share much more with this humble microbe than we would have considered possible a mere half century ago.

Relevance of zero growth studies

In (Western) society, growth is often considered as something positive, and even as an objective in its own right. Examples vary from personal growth or development to popular views on economic growth. However, growth may also have negative consequences. In different systems and contexts, drastic countermeasures have been implemented to curb uncontrolled growth. Examples vary from attempts to control growth of the Chinese population size by its government's one-child-policy to halting the growth of malignant tumors by a variety of medical interventions.

Although, at first glance, there seems to be a general paradigm that dictates all cells to grow and multiply, a state of no growth, sometimes referred to as quiescence, is at least as common in living cells as growth. For example, at any moment in time, ca. 60% of the microbial biomass on earth is estimated to be in a non-dividing state (Gray *et al.*, 2004). The absence of active growth is most often caused by external circumstances, i.e. physical factors or substrate availability. Also in the human body, a large fraction of the cells are in a viable, active, but non-dividing state. In some cells, this non-dividing state is irreversible, as in post-mitotic differentiated cells such as neurons, skeletal muscle cells and senescent cells. In others, it is reversible, as in mitotic cells such as fibroblasts and endothelial cells (Campisi and d'Adda di Fagagna, 2007).

… for industry…

In the field of industrial microbiology and biotechnology, active microbial growth has received much attention. Fast growth of biomass and/or increased yields of biomass on the industrial substrate are important performance indicators in various biomass production processes, such as for example the production of baker's yeast and yeast extracts (De Winde, 2006). Also in strain engineering approaches, growth is often a key performance indicator. For example, in laboratory evolution experiments, improved growth is generally the key selectable trait and mutants are selected that grow faster under specific conditions in comparison to the parental strain (Sauer, 2001; Vanee *et al.*, 2012). Using such strategies, yeast strains have been selected that consume pentose sugars faster, or co-consume these with glucose (Garcia Sanchez *et al.*, 2010; Wisselink *et al.*, 2009), and that are less inhibited by toxic compounds present in industrial fermentation media (Demeke *et al.*, 2013).

When evolutionary engineering, the application of laboratory evolution to select for industrially relevant phenotypes, is aimed at increased environmental robustness, evolution experiments can be designed to select for survival of non-growing cells under industrially stress conditions, such as freeze-thawing cycles (Çakar *et al.*, 2005). Microbial strains with a

strongly increased environmental robustness, i.e. resistance against a variety of stresses, are highly desirable, as adverse conditions are not uncommon in industrial processes. Moreover, in their industrial application, microbial cell factories, including yeast cells, often face periods of non-growth. In large-scale processes such as beer fermentation and bioethanol production, yeast biomass is often harvested and stored between fermentation runs, in some cases combined with treatments to kill or reduce contaminants (Fig. 1.3) (Basso *et al.*, 2011a). These cycles often involve periods during which yeast cells are stored under non-growing conditions until the next fermentation is initiated (Verbelen *et al.*, 2009). Growth arrest in such industrial cultures is usually caused by extremely low nutrient levels, usually the carbon- and energy source, resulting in starved or almost starved cultures. Besides a virtual absence of one or more essential nutrients, cells might also face additional stresses caused by for example low temperatures, i.e. cold storage or freezing (Hazelwood, 2009), low pH due to acid-wash cycles to combat contaminating bacteria (Della-Bianca and Gombert, 2013) or hyper osmotic stress during drying (Attfield, 1997).

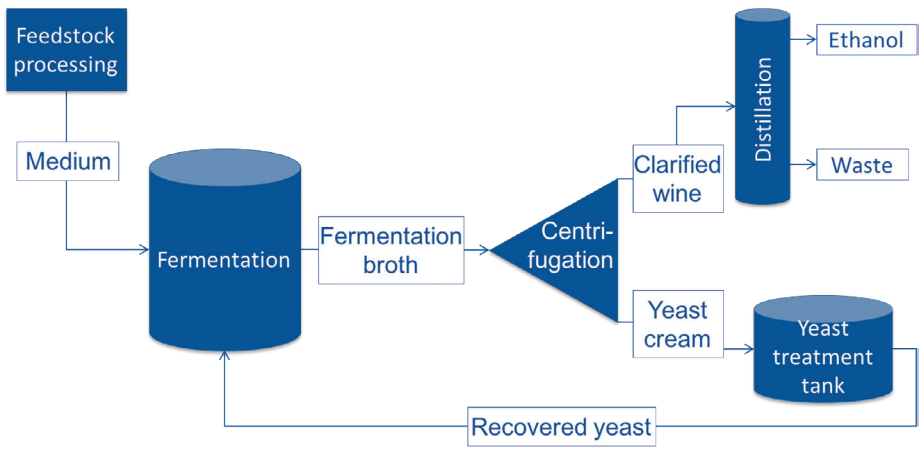


Figure 1.3 Schematic overview of the ethanol-production process
The large-scale production of ethanol from agricultural feedstock such as sugar-cane molasses and corn-starch consists of different steps. After pre-treatment of the agricultural feedstock to liberate fermentable carbon-sources, fermentation to ethanol takes place. The yeast is subsequently separated from the 'wine' or fermentation broth. From the culture liquid the biofuel ethanol is recovered. Sulphuric acid is added to the yeast cream in the yeast treatment tank to reduce the risk of bacterial contamination (Adapted from Basso *et al.*, 2011a).

Zero-growth is not only observed in these intermittent phases between culture cycles, but can also be an intrinsic part of the production process itself. This type of 'zero growth' is frequently encountered in food applications of microbes. Yeast-related examples include wine and beer production. After a relatively short initial phase of fast growth in must or wort, growth ceases as essential nutrients become limited. Yeast cells still strongly contribute to the development of flavor composition of the final products wine and beer under these near-zero growth conditions and during maturation (Boulton and Box, 2003; Swiegers and Pretorius, 2005).

Periodic phases in which growth is minimal or absent in industrial processes, i.e. storage of cells or severely nutrient-limited cultures in food-production, clearly indicate a strong relevance for zero-growth physiology in an industrial context. Still, growth has received major attention in scientific studies of industrial relevance, even while biomass is often not the product of interest. In the production of bulk chemicals, such as ethanol and dicarboxylic acids, and higher-value components, like medicinally relevant peptides and isoprenoids, biomass can be considered a byproduct. Increased levels of biomass formation, i.e. growth, result in reduced product yields on the carbohydrate feedstock, part of which is consumed for growth. Growth also causes higher down-stream processing costs, as the formed biomass needs to be removed and disposed. Especially in the case of genetically modified yeast, this biomass-waste stream may strongly influence the economic feasibility (Boender, 2011). Reducing or even completely abolishing growth in such industrial processes can lead to near-theoretical yields. However, a strong prerequisite for such an approach to be successful is that the metabolic activity, i.e. the productivity, of the cell factories is maintained in the absence of growth. In short, product formation and growth need to be uncoupled.

… and beyond

As outlined above, industrial biotechnology would strongly benefit from increased knowledge on non-growing yeast cultures. In view of the complexity of living cells, information on zero-growth biology should involve different levels of cellular information. Only by following a systems-biology approach including physiology, transcriptome and other 'omics', it may be possible to rationally improve process and storage conditions and, especially, to obtain robust, non-growing, yet metabolically active cultures to improve existing and newly-developed processes strongly with regards to product yields and cost-effectiveness. Industrial biotechnology is, however, not the only field of science that will profit from a much deeper understanding of the physiology of virtually non-growing cells. In any culture in which cell-division no longer occurs, replicative aging will come to a standstill, while individual cells continue to age chronologically even in the absence of growth. Studying reference and mutant strains of the eukaryotic model organism *S. cerevisiae* under non-growing, yet metabolically active conditions, may therefore further strengthen its role in chronological aging research.

At present, a number of cultivation systems are employed and being developed to study yeast, as well as other microorganisms, under non-growing conditions. The most widely employed and best-studied systems are stationary phase cultures. Stationary phase represents the final stage of batch cultures, during which biomass concentrations no longer increase as a consequence of either nutrient depletion or toxic effects of product accumulation (Herman, 2002; Werner-Washburne *et al.*, 1993).

Continuous cultivation systems, which generally allow a better control of process parameters such as nutrient levels and culture pH, are less straightforward to implement and consequently less employed. At present, two continuous systems are described in

literature that result in virtually non-growing yeast cultures. Both these systems are based on cell retention. In retentostats, planktonic microbial cells are retained in a continuously fed bioreactor by using an internal or external filter. In a recent study, an alternative continuous cultivation set-up was discussed. Cultivation of *S. cerevisiae* in alginate beads resulted in growth-arrest under carbon-excess conditions (Nagarajan *et al.*, 2014). In the following paragraphs the most extensively applied systems for studying near-zero growth in yeast, stationary phase batch cultures and retentostat cultures, are discussed.

Stationary phase cultures, starved but not dead (yet)

Stationary phase (SP) batch cultures of *S. cerevisiae* have been intensively investigated for several decades now (Lillie and Pringle, 1980) and are undoubtedly the most-studied non-growing yeast cultures. The apparent ease with which SP cultures can be obtained is probably the main reason for their prominent role in yeast research on zero-growth and chronological aging. Irrespective of whether cells are grown in 'simple' shake flasks/tubes or, alternatively, in bioreactors that allow a better control and monitoring of process conditions, SP represents the final phase of any batch culture (Fig. 1.4), during which the increase in biomass has come to a standstill.

Depending on the medium composition and yeast strain used, the cause of the growth arrest in batch cultures can vary from depletion of different nutrients, including auxotrophic markers, to acidification or accumulation of organic products. Of these factors, depletion of the carbon and energy source is most common. Cultures depleted for different essential nutrients share some characteristics, but cells in these SP cultures also display important differences, especially with regard to maintenance of viability and their ability to re-initiate growth (Werner-Washburne *et al.*, 1996). Currently available information indicates that carbon-source-depleted SP cultures exhibit the longest survival time. Some researchers have suggested that only carbon starvation represents a 'true' SP (Herman, 2002; Werner-Washburne *et al.*, 1996). Statements such as these reflect a more general ambiguity of the terminology used by researchers in the field to describe SP and the physiological state of yeast cells in SP cultures (see Box 1). Rather than just referring to cultures as SP, it is essential to exactly define the actual cause for growth arrest, as this strongly influences the outcomes and interpretation of experiments (Burtner *et al.*, 2009; Burtner *et al.*, 2011).

An important problem in the interpretation of SP cultures is specifically linked to aerobic, glucose-grown batch cultures of *S. cerevisiae*. In such cultures, glucose metabolism is predominantly fermentative. After an adaptation phase (the diauxic shift), ethanol and acetate are consumed during a second, respiratory growth phase (Fig. 1.4). Respiratory growth during the post-diauxic shift (PDS) phase can be extremely slow, especially when complex media such as yeast extract-peptone-dextrose (YPD) are used and when, moreover, the limited oxygen-transfer capacity of shake-flask cultures restricts growth. As a consequence, several studies are mistakenly performed with PDS cultures that have not yet reached SP, but are still slowly growing on ethanol (Herman, 2002). Unless stated otherwise, this paragraph

on SP cultures will only refer to cultures in which complete depletion of carbon sources has been experimentally verified.

Several characteristics distinguish cells in SP cultures from (fast) growing cells. These include increased cell wall thickness, increased resistance to a variety of stresses, including heat-shock and oxidative stress, increased levels of glycogen and trehalose, and condensed chromosomes. Furthermore, the majority of the cells are arrested in a non-budding phase of the cell cycle and their overall transcription and translation rates are reduced. An even stronger, and perhaps unique, identifier of cells in SP cultures is their ability to survive for long periods in the absence of an extracellular energy source (Gray *et al.*, 2004; Werner-Washburne *et al.*, 1993).

Besides these physiological differences between growing and SP cultures, differences at the transcriptome and proteome level have been described. A specific set of genes is transcriptionally up-regulated in SP cultures. In most cases this overexpression also results in increased protein levels. Examples of genes that belong to this set are *SNZ1, UBI4*, *HSP26*, *HSP104*, *SSA3* and *HSP12* (Braun *et al.*, 1996; Davidson *et al.*, 2011; Fuge *et al.*, 1994;

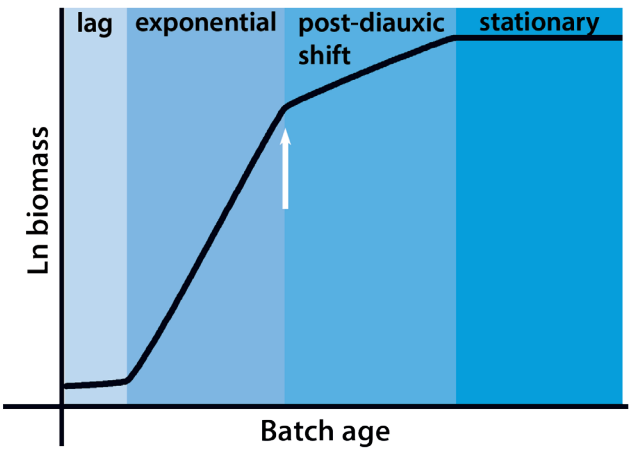


Figure 1.4 Growth phases during aerobic batch cultivation of* S. cerevisiae *on glucose
S. cerevisiae is a 'Crabtree'-positive yeast that, even in the presence of oxygen, ferments the majority of glucose under glucose-rich conditions (van Dijken *et al.*, 1993). This metabolic behavior results in a profile of 5 different phases of growth in aerobic, glucose-grown batch cultures. During the first phase, the so-called lag phase, cells adjust to the new environment and hardly any increase in total biomass is observed. The length of this phase strongly depends on the conditions under which the inoculum of the culture was prepared. Once adjusted, cells grow and divide at maximal rate during a subsequent exponential or logarithmic (log) phase. During this phase the fastest increase in biomass is observed and catabolism is predominantly fermentative. Besides biomass, mainly ethanol and CO_2 are produced. After glucose is depleted, a shift in metabolism occurs: the diauxic shift (white arrow). After this diauxic shift, a second growth phase takes place with a slower pace. During this post-diauxic shift (PDS) phase the previously produced non-fermentable carbon- and energy sources, i.e. ethanol, glycerol and organic acids, are consumed. As soon as one essential nutrient is depleted, often the carbon- and energy-source, growth stops and the culture enters stationary phase. The schematic growth profile shown here represents an 'ideal' batch culture, with all nutrients in excess compared to the carbon source. In practice the different phases may be harder to distinguish as growth and metabolism can be affected by changes in pH or factors other than carbon-source availability.

Martinez *et al.*, 2004; Webb *et al.*, 2013). The levels of the transcripts and proteins encoded by these genes generally already increase during the PDS phase and reach their maximum levels at the onset or during SP.

As entry into SP represents a drastic change in nutrient availability, it is not surprising that nutrient signaling pathways are essential in the transcriptional reprogramming that occurs upon entry of SP (Fig. 1.5). The PKA, TOR and Snf1 pathways are the key players in this respect (Galdieri *et al.*, 2010). The presence of nutrients, and especially glucose, activates the TOR and PKA-pathways, which negatively regulate entry into stationary phase (Galdieri *et al.*, 2010; Smets *et al.*, 2010). Snf1, on the other hand, is only active in the absence of glucose and activates the transcription of genes that are essential for growth on non-fermentable carbon-sources (Galdieri *et al.*, 2010). Down-stream of these signaling pathways, Sch9 and Rim15 are key players for entry into stationary phase. Sch9, under the control of the TOR pathway (Smets *et al.*, 2010) inhibits Rim15 function. Rim15 is a PAS-kinase that is activated upon nutrient depletion through the inactivation of the TOR and/or PKA signaling (Fig. 1.5). In wild-type yeast cells, the cell cycle is arrested at the G1 phase in response to nutrient depletion. When *RIM15* is deleted, cells are no longer able to properly arrest in the G1 phase of the cell cycle (Fig. 1.2), neither under nutrient depletion conditions nor in the presence of rapamycin (an inhibitor of the TOR pathway). Consequently, *rim15* mutants are unable to properly enter SP (Cameroni *et al.*, 2004; Pedruzzi *et al.*, 2003; Reinders *et al.*, 1998). When activated, Rim15 induces the expression of several stress-response related genes through the activation of transcription factors Msn2/Msn4, Gis1 and Hsf1 (Fig. 1.5) (Lee *et al.*, 2013; Zhang *et al.*, 2009). Msn2/4 regulates the expression of a large set of genes that carry the stress response element (STRE) in their promoter region. The recognition site for Gis1 is denoted as the post-diauxic shift (PDS) element. The sets of genes under control of these transcription factors show some overlap and many are involved in the observed increased stress resistance of SP cultures (Martínez-Pastor *et al.*, 1996).

SP cultures have been extensively used in fundamental research on chronological aging. In many of these studies, the focus was on identifying factors, such as genes or chemical components, that affect survival of cells during SP. In such experiments, the so-called chronological lifespan (CLS) is determined by measuring the time interval over which the fraction of the population that is able to reproduce (i.e. the number of 'colony forming units' divided by the total number of cells plated), drops below a certain threshold. Using this approach, several CLS enhancers and inhibitors have been identified. Limited glucose availability, often referred to as calorie restriction (see Box 1), is the most notable CLS enhancer, as it does not only increase the life-span of unicellular yeast, but also of higher eukaryotes. Calorie restriction is currently the only known non-genetic, non-pharmaceutical intervention that increases life-span in a wide range of organisms (Bishop and Guarente, 2007). Genetic and drug-based studies have furthermore shown that the life-span extension by calorie restriction is largely dependent on the same nutrient signaling cascades that ensure

proper entry into SP, TOR and downstream effectors Sch9 and Rim15 (Swinnen *et al.*, 2014; Wei *et al.*, 2008).

Research on SP yeast cultures has made important contributions to aging research (Longo *et al.*, 2012; Werner-Washburne *et al.*, 2012). The resulting understanding of the biology of non-dividing cells and, in particular, on the increased robustness of SP-cultures may also be highly relevant for the many industrial applications of *S. cerevisiae*. However, SP cultures suffer a number of inherent drawbacks that limit their usefulness in fundamental as well as in applied research. A first important drawback that has often been overlooked in early research is the heterogeneity of SP cultures. SP cultures consist of quiescent and non-quiescent cells (see Box 1 for explanation of terms). This heterogeneity, which has only been fully recognized in the past decade, has major consequences for the interpretation of whole-culture data derived from SP cultures. These consequences are now under intensive investigation (Allen *et al.*, 2006; Aragon *et al.*, 2008; Davidson *et al.*, 2011; Werner-Washburne *et al.*, 2012). The quiescent fraction, consisting mostly of virgin daughter cells, displays the most pronounced SP phenotype: quiescent cells are more robust, longer lived and more homogeneous than

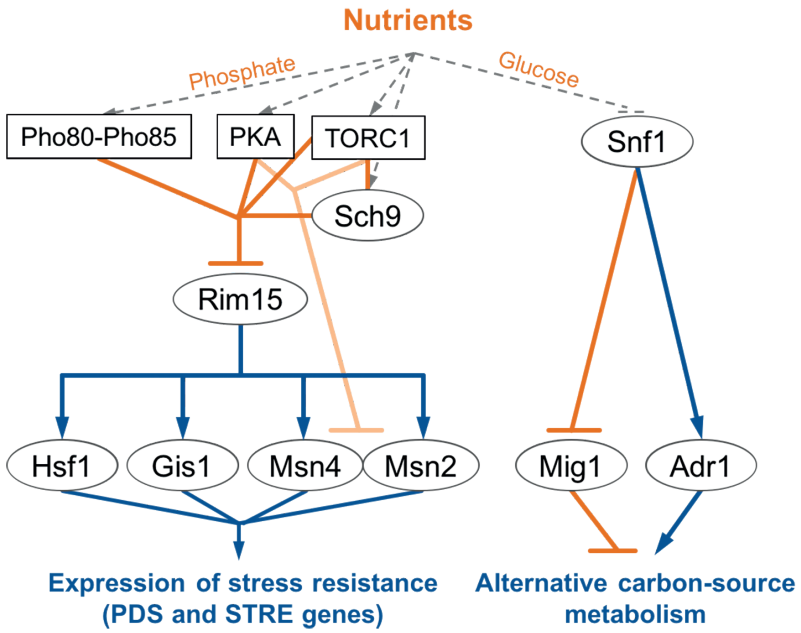


Figure 1.5 Nutrient signaling cascades in* S. cerevisiae *and their down-stream effectors
Nutrient signaling cascades play an essential role in growth arrest upon nutrient limitation or depletion. In the presence of nutrients, TOR, PKA and Pho80-Pho85 cascades are active and, partly via Sch9 inhibit the PAS-kinase Rim15. In the absence of one or more nutrients, Rim15 is activated. Rim15 subsequently activates transcription factors Msn2/4, Gis1, Hsf1 and putative other targets. Snf1 is an important player in glucose repression. Upon glucose depletion it is activated and via repression of Mig1 and activation of Adr1 induces expression of genes involved in alternative carbon-source utilization such as catabolism of other sugars, e.g. galactose, and non-fermentable carbon-sources, e.g. ethanol, and gluconeogenesis. (Adapted from Bisschops *et al.*, 2014; Galdieri *et al.*, 2010; Smets *et al.*, 2010.)

Box 1 Terminology in non-growing (yeast) cultures

The terminology used in research on non-growing yeast cultures, mostly stationary phase cultures, is often ambiguous. Here I present a number of important terms from the zero-growth field, each with a suggested definition. In doing so, I mostly followed the definitions as proposed by Werner-Washburne and co-workers in a series of reviews on the topic, giving priority to the most recent ones (Gray *et al.*, 2004;Werner-Washburne *et al.*, 1993;Werner-Washburne *et al.*, 2012;Werner-Washburne *et al.*, 1996)

Stationary phase (SP) – final stage of a batch culture in which no increase (or decrease) of biomass concentration, either measured as optical density (OD), mass per volume (g/L) or number of cells per volume (cells/mL) occurs. SP refers to a phase in the growth progression of a culture, not to the physiological state of the cells in that culture.

Quiescence – A cellular state that cells may enter following growth-arrest. Quiescent cells are unbudded and display a number of characteristics. Usually only a fraction of the cells in SP cultures (often about half of the population) becomes quiescent. Quiescence (and entry into G_0, see below) is generally assumed to occur only under carbon-limitation/starvation.

G_0 – a phase outside the active cell cycle (which consists of a G1, S, G2 and M-phase (Fig. 1.2)). Although G_0 has been extensively described for post-mitotic cells in higher eukaryotes, its existence in yeast is under debate, but quiescent yeast cells may have entered G_0.

Non-quiescent cells – SP cultures consist of two populations that can be separated based on their physiology, for example density and replicative age, and/or specific transcript and protein levels, for example *HSP12* and *HSP26*. The long-lived, stress-resistant, virgin daughter cells form the quiescent fraction, while non-quiescent cells are generally shorter-lived (old) mother cells.

Chronological life span (CLS) – the maximum chronological age cells reach in a culture. In practice this is most often the time cells survive during SP, usually in the absence of an extracellular energy-source. CLS is commonly measured as the decay of culture viability (measured by plating or fluorescent staining) in time. The conditions under which cells are incubated vary strongly among different studies. Common approaches include incubation in the spent medium or transfer of the cells to water.

Calorie restriction – an energy-source uptake rate that is not *ad libitum*, but close to minimal requirements for survival. Many authors consider a reduced initial glucose concentration, for example 0.5% w/v, compared to standard concentrations, i.e. 2% w/v, calorie restriction. However, in higher eukaryotic model organisms, calorie restriction reflects a reduced glucose uptake rate, which is not equal to a reduced initial glucose concentration. I therefore propose to use this term for glucose consumption rates that approximate the maintenance requirement.

Starvation – condition under which an essential nutrient is depleted. Strictly speaking this includes extracellular, such as glucose and ethanol, and intracellular resources, for example glycogen and trehalose, however the latter are usually not taken into account.

Maintenance energy – the metabolic energy (e.g., proton motive force, ATP) required and used by cells to maintain cellular integrity. Maintenance energy is used to fuel processes such as ion homeostasis, repair and turnover of macro-molecules like DNA and proteins, that do not result in a net increase of biomass.

their non-quiescent counterparts (Allen *et al.*, 2006). These phenotypic differences are also clearly reflected in the transcriptome and proteome of the two sub-populations (Aragon *et al.*, 2008; Davidson *et al.*, 2011; Webb *et al.*, 2013).

Another important drawback of SP cultures is a direct consequence of the conditions that lead to SP. Depleted from any extracellular energy-source, SP cultures display minimal metabolic activity, while slowly consuming their intracellular reserves (storage compounds such as glycogen, trehalose and, under aerobic condition, intracellular lipids). As soon as these are depleted, and cells are truly starved (Li *et al.*, 2013), cells lack energetic resources to maintain essential processes and deteriorate until viability is lost. This is a point where SP yeast cultures clearly differ from non-growing cells and tissues in higher eukaryotes (Breitenbach *et al.*, 2012c). SP cultures are, by definition, progressing towards energy depletion and, ultimately, to cell death due to depletion of endogenous reserves. In contrast, non-dividing cells in higher eukaryotes (metazoa) often display a high metabolic activity and are continuously supplied with nutrients. This not only limits the applicability of SP yeast cultures as a model for non-growing human cells. The low, sometimes virtually absent, metabolic activity of SP yeast cultures also implies a low productivity of any industrially relevant compound. With the notable exception of studies on shelf life of bakers' yeast and other starter cultures, this limits the relevance of SP cultures for industrial processes. For application in the yeast-based industrial production of chemical compounds, the ideal non-growing scenario would be one in which the absence of growth is coupled with a high productivity. This implies that, for industrially relevant studies on non-growing yeast cultures, experimental systems other than SP cultures need to be explored.

The retentostat: keeping non-growing cells alive and active

Non-growing yeast cultures that maintain high viability and activity over prolonged periods require a constant supply of an energy-source, i.e. glucose or an alternative carbon-source. This is by definition not possible in a 'closed' batch-cultivation system. Fed-batch cultures, to which an energy substrate is continuously added, could be considered as an alternative. However, such a continuous addition will eventually result in, at least, two problems. The continuous addition of energy substrate will result in an increasing culture volume that is constrained by the physical limits of the set-up, i.e. the bioreactor volume. Secondly, conversion of the substrate may result in accumulation of metabolites that become toxic at higher concentrations, such as for example ethanol and acetate. These two hurdles can be overcome by using a continuous cultivation set-up in which continuous addition of fresh medium is combined with the continuous removal of spent broth. The chemostat is the most widely applied mode of continuous cultivation and allows growth of micro-organisms under strictly controlled conditions (Novick and Szilard, 1950).

When the flow rate of the supply of fresh nutrient-limited medium and of the removal of culture liquid are identical and evaporation is negligible, the culture volume remains constant. In chemostat cultures, the dilution rate, which is defined as the outgoing flow

rate (liter per hour) divided by the culture volume (liter), dictates the specific growth rate of the microorganisms in the chemostat. The strict control of cultivation parameters makes chemostat cultivations highly reproducible and a powerful tool to investigate the effect of single variations in these parameters. Examples are studies on the impact of specific growth rate, under the same nutrient limitation or vice versa different nutrient limitations, for example carbon-, nitrogen- and phosphorus-limitation at a specific constant growth rate on physiology or gene expression levels (Daran-Lapujade *et al.*, 2008). In chemostats, due to the continuous removal of culture broth, including biomass, growth is required to maintain a constant biomass concentration. In practice, chemostat cultures can be used to study specific growth-rates down to circa 0.01 h^{-1}. At even lower specific growth rates, technical limitations prevent accurate measurements. Therefore, quantitative studies on (near) zero growth of microorganisms require an alternative cultivation technique.

Based on chemostat studies, a model for the energy distribution in growing cultures has been set-up in the early 1960s. This model, as shown in equation 1.1, predicts a linear correlation between specific growth rate and the biomass-specific energy-substrate consumption rate (under energy-substrate-limited conditions). Inspired by previous work by Monod and Duclaux, this model was postulated by Herbert and Pirt and is hence known as the Herbert-Pirt relation (Herbert, 1961; Pirt, 1965). The equation describes the relation between biomass-specific energy-substrate uptake rate (q_s), specific growth-rate (μ), maximum growth yield corrected for maintenance (Y_{sx}^{max}) and a growth-rate independent, biomass-specific rate of energy-substrate consumption for maintenance (m_s). This theorem predicts that when, under energy-substrate limited conditions, the specific energy-substrate uptake rate equals the maintenance requirement, growth is absent.

$$q_s = \frac{1}{Y_{sx}^{max}}\mu + m_s \qquad \textit{(Equation 1.1)}$$

To validate the growth-rate independency of the maintenance requirement, microbial cells need to be cultivated at (near) zero growth rate under energy-substrate-limited conditions. As discussed above, such a situation is very difficult to achieve in chemostats due to technical limitations. A different set-up for continuous cultivation, especially developed for studies at extremely low growth rates, was introduced in 1979. The recycling fermenter (Chesbro *et al.*, 1979) can be viewed as a modification of the chemostat. Instead of whole culture broth, including biomass, only the spent medium is withdrawn from the reactor and 100% of the biomass is retained in the reactor (Fig. 1.6A). This biomass retention is why, subsequently, this cultivation system is mostly referred to as 'retentostat'. Due to the complete biomass retention and continuous, constant energy-substrate feed, the biomass concentration in a retentostat initially increases. As a consequence, the biomass-specific rate of energy-substrate consumption will decrease over time (Fig. 1.6). According to the Herbert-Pirt relation, this should then finally result in a situation in which all energy substrate fed to a retentostat is used for maintenance ($q_s = m_s$) and growth ceases ($\mu = 0$) (Fig. 1.6B).

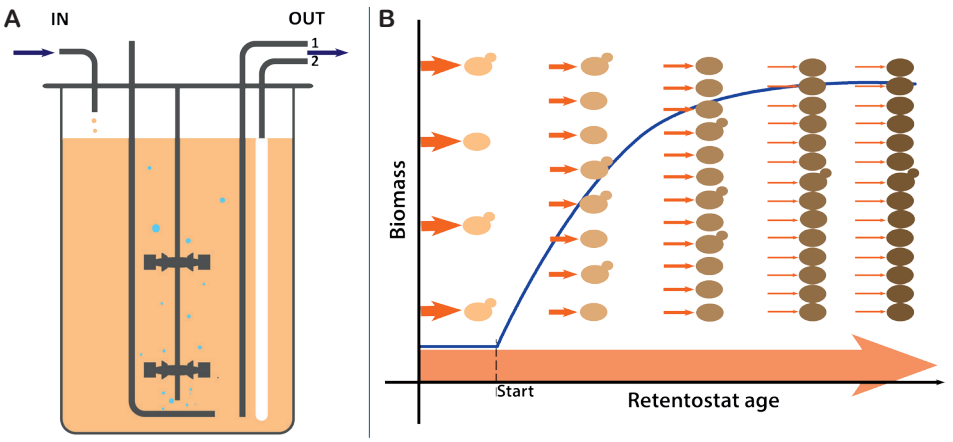


Figure 1.6 The retentostat: system and principle

(**A**) Schematic representation of the retentostat cultivation set-up. Carbon-limited medium is continuously supplied at a fixed flow rate (**IN**) and effluent is continuously removed (**OUT**) through a standard port (**1**) during the chemostat phase or a port equipped with a filter (**2**) during retentostat cultivation, such that biomass accumulates inside the reactor. (**B**) The principle of the retentostat, a continuous cultivation system with full biomass retention. While the medium flow (large arrow) remains constant, biomass (shown by the blue line and cells) increases in time. Subsequently the amount of substrate per cell (indicated by the thickness of the short arrows) decreases, in the case of energy-limited retentostats this results in severe calorie restriction and virtual absence of growth.

Extremely low growth rates have been achieved in retentostats. Surprisingly, the phylogenetically diverse bacteria grown in this system revealed a rather particular correlation between growth and energy distribution (Arbige and Chesbro, 1982; Bulthuis *et al.*, 1989; Chesbro *et al.*, 1979; Tappe *et al.*, 1999; Van Verseveld *et al.*, 1984). At moderately low to high specific growth rates, maintenance requirements were indeed found to be growth-rate independent. However, at extremely low growth rates, a decrease of the maintenance requirement was observed. These bacteria, including *Escherichia coli* responded to the extreme energy-limitations, i.e. calorie restriction, by a rearrangement of their metabolism and decreased their maintenance requirement, the so-called stringent response. Due to this stringent response, true zero-growth was not achieved in these retentostat cultures for several prokaryotic species examined (Arbige and Chesbro, 1982; Bulthuis *et al.*, 1989; Chesbro *et al.*, 1979; Van Verseveld *et al.*, 1984; Van Verseveld *et al.*, 1986).

The unique feature of the retentostat system, i.e. the ability to combine strictly controlled cultivation conditions with extremely low growth rates, under energy-limited conditions make it an extremely attractive tool to study yeast physiology at near-zero growth rates. Since, in yeasts and fungi, no stringent response has been documented, this opens up the possibility to approach zero growth in retentostat cultures of *S. cerevisiae*.

In a previous project at the Delft University of Technology, Boender and coworkers explored the use of glucose-limited retentostat cultures to study the physiology and transcriptional responses of the haploid *S. cerevisiae* reference strain CEN.PK113-7D

at near-zero growth (Boender *et al.*, 2009; Boender *et al.*, 2011b). Retentostat cultures were started from slow-growing anaerobic, glucose-limited chemostat cultures, grown at a dilution rate of 0.025 h^{-1}. Retentostat cultivation was then initiated by re-directing the effluent through an internal filter device (Fig. 1.6A). During the first nine days of retentostat cultivation, biomass retention resulted in a strong increase of the biomass concentration in the reactor and a concomitant decrease of the biomass-specific glucose-uptake rate and specific growth rate (Fig. 1.7A,B). After 21 days, specific growth rates close to zero (below 0.001 h^{-1}) were reproducibly achieved, with biomass doubling times corresponding to approximately one month (Boender *et al.*, 2009). Under these conditions of severe calorie restriction and near-zero growth, the specific glucose uptake rates closely corresponded to the maintenance requirements estimated from chemostat cultures grown at higher specific growth rates (Fig. 1.7B). This observation is consistent with the absence of a stringent response in anaerobic glucose-limited retentostat cultures of *S. cerevisiae*. Importantly, the viability and fraction of metabolically active cells remained high throughout the course of the retentostat (above 75%) and phase-dark cells, believed to be apoptotic cells, were virtually absent (Boender *et al.*, 2009; Boender *et al.*, 2011b)(Fig. 1.7C).

The work of Boender and coworkers indicated that yeast retentostat cultures might provide a means to overcome several major drawbacks of SP cultures, as viability and metabolic activity remained high. SP cultures display an increased resistance against a number of stresses, which is interesting for industrial applications. Cellular robustness was also assayed in retentostat cultures. Biomass samples taken at different time points during retentostat runs, i.e. at different specific growth rates, were subjected to heat stress and survival was monitored by flow cytometry. These experiments showed that, with decreasing growth-rate, the heat-shock resistance spectacularly increased (Boender *et al.*, 2011a). Analysis of genome-wide expression levels over a wider range of specific growth rates,

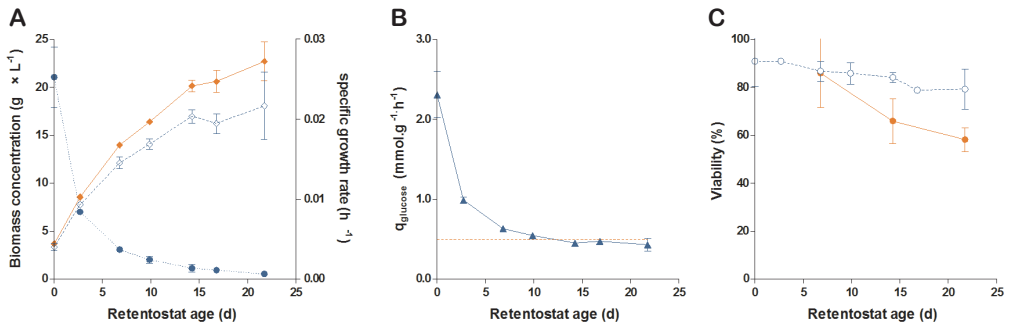


Figure 1.7 Some characteristics of anaerobic carbon-limited* S. cerevisiae *retentostat cultures
Data from anaerobic carbon-limited retentostat cultures of *S. cerevisiae* as published in (Boender *et al.*, 2009). (**A**) The increase in total (closed diamonds, ◆) and viable biomass (open diamonds, ◇) during the course of retentostat cultivation. With increasing biomass the growth rate (closed circles, ●) decreases. (**B**) The specific glucose uptake rate ($q_{glucose}$, ▲) decreases to the level of the maintenance requirement extrapolated from chemostat experiments (dashed line). (**C**) Viability, measured by fluorescent staining (open circles, ○) and by CFU (closed circles, ●), remained high throughout the course of the retentostat.

which involved the integration of data from chemostat and retentostat experiments, revealed a strong inverse correlation between specific growth rate and expression levels of genes involved in stress resistance (Boender *et al.*, 2011b). Similar trends were observed in other laboratories using transcriptome data from chemostat experiments only (Brauer *et al.*, 2008; Castrillo *et al.*, 2007; Regenberg *et al.*, 2006). Increased stress resistance was not the only characteristic of SP cultures that was observed to correlate with growth rate in continuous cultivation (retentostat and/or chemostat) experiments. Also the accumulation of glycogen and lipids that occurs in SP cultures was shown to exhibit an inverse correlation with growth rate (Boender *et al.*, 2009; Boender *et al.*, 2011b). Surprisingly, the expression of several genes induced in quiescent cells, such as the *SNO* and *SNZ* genes, but also *HSP12*, *HSP26* and *SSA3*, was also found to be negatively correlated with growth rate over a wide range of growth-rates and to peak at near-zero growth (Boender *et al.*, 2011b). Apparently, transition of cultures from growth to quiescence may not represent an 'on/off' switch, but rather a more gradual transition. This raises the tantalizing question whether extremely slow growing cultures may exhibit heterogeneity, with some of the cells already switched to quiescence while others are still actively growing.

Retentostat cultures resemble stationary phase cultures with respect to several cellular characteristics, including low (< 20%) budding indices (Boender *et al.*, 2011b). However, the continuous supply of substrate in retentostat cultures also results in key differences from SP cultures. Perhaps the most important of these is the high viability and metabolic activity in virtually non-growing retentostats. A direct comparison of retentostat cultures with starving cultures revealed additional differences. Despite their severely calorie restricted state, yeast cells in retentostat cultures continue to accumulate glycogen. When the glucose supply is stopped, these reserves are slowly consumed. Cessation of the substrate supply also caused a rise in the fraction of phase contrast dark cells, which are considered dying cells (Allen *et al.*, 2006; Boender *et al.*, 2011a). A very surprising result concerned the rate at which intracellular reserves, i.e. glycogen, were catabolized in starving cultures. Quantitative analysis indicated that, in anaerobic, energy-starved cultures, the rate of ATP turnover was at least 100-fold lower than in virtually non-growing retentostat cultures. This result indicated that *S. cerevisiae* could respond to energy starvation by a strong reduction in energy consumption. This response, which might be an eukaryotic counterpart of the stringent response, was mirrored at the transcriptome level by a further decrease in expression of genes involved in protein synthesis (Boender *et al.*, 2011a), one of the most energy expensive cellular processes (Stouthamer, 1973).

The increased robustness, but also the increased expression of genes previously shown to be involved in chronological aging and CLS extension, i.e. key-players under calorie restriction such as Rim15 and targets, combined with the maintained viability and higher metabolic activity make the virtually non-dividing retentostat cultures a promising alternative for the dying SP cultures. Not only in fundamental research on chronological aging, but also to explore strategies for zero-growth product formation in yeast-based industrial biotechnology.

Scope of this thesis

Boender and co-workers performed a first, thorough characterization of the physiological and transcriptional responses of *S. cerevisiae* retentostat cultures at near-zero growth rate (Boender *et al.*, 2009; Boender *et al.*, 2011a; Boender *et al.*, 2011b; Boender, 2011). These analyses implied that, under the severely calorie restricted conditions in anaerobic retentostats, nutrient signaling cascades play a crucial role. The overlap of the transcriptional responses in anaerobic retentostat cultures and aerobic SP cultures included many targets of the PAS-kinase Rim15. This observation suggests that Rim15, an integrator of several signaling cascades (Fig. 1.5) strongly influences the transcriptional and physiological adaptation of *S. cerevisiae* to near-zero growth rates. Rim15 has been shown to be crucial for a proper transition into stationary phase and, most probably, for transition of cells into quiescence (Box 1) in aerobic batch cultures (Cameroni *et al.*, 2004; Pedruzzi *et al.*, 2003; Reinders *et al.*, 1998). However, its role under severely calorie-restricted conditions has not been investigated. In **Chapter 2**, the physiological and genome-wide expression level of the reference strain *S. cerevisiae* CEN.PK113-7D and a congenic *rim15* knock-out strain are compared in anaerobic, glucose-limited chemostat and in retentostat cultures. The goal of this study was to investigate the role of Rim15 in transcriptional responses to near-zero growth and, in particular, its effect on heat-shock resistance, glycogen accumulation, cell cycle arrest and metabolic activity, four crucial parameters for industrial and fundamental applications of non-growing *S. cerevisiae* cultures.

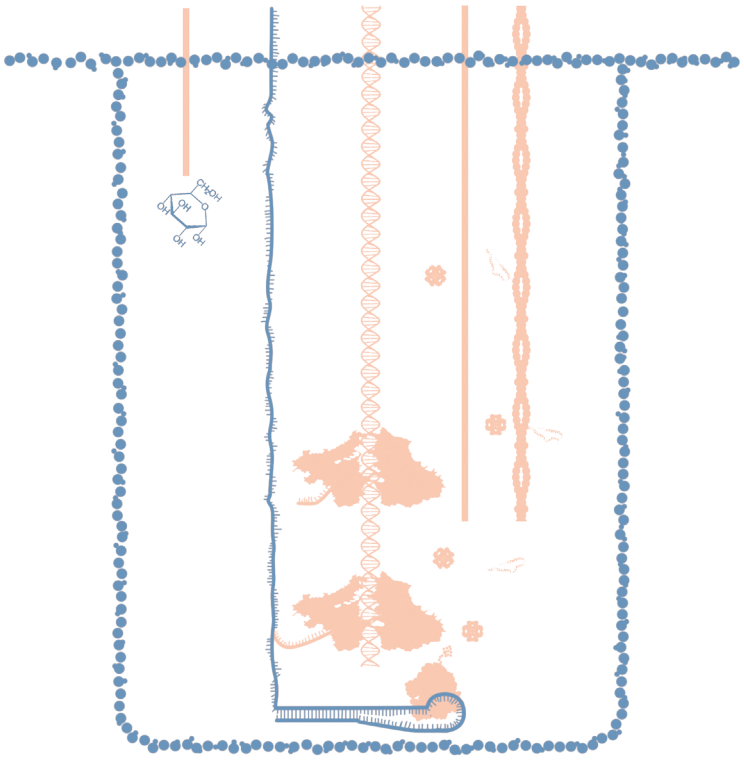
Transcriptome analysis of the reference strain *S. cerevisiae* CEN.PK113-7D showed a large set of genes responding to the decreasing glucose availability and the concomitantly dwindling growth rate (Boender *et al.*, 2011b). Transcription of genes is, however, an order of magnitude less energy-expensive than protein synthesis (Verduyn *et al.*, 1990a). It was therefore unclear to what extent the large transcriptional responses are reflected at the protein level under severe calorie restriction. In **Chapter 3** this question is addressed. In collaboration with the Heck Lab for Biomolecular Mass Spectometry and Proteomics at Utrecht University, a quantitative analysis was performed to identify changes in the proteome of *S. cerevisiae* under severe calorie restriction. Protein levels were followed over time in anaerobic retentostat cultures and compared with the transcriptional responses identified in a previous study (Boender *et al.*, 2011b). Based on these analyses, one specific protein, *Hsp30* was selected for further analysis. Both the transcript and protein levels encoded by *HSP30* increased strongly with decreasing growth rate. Hsp30 is a negative regulator of the plasma membrane ATPase 1 (Pma1) and may therefore, under energy-limited conditions, avoid unwanted expenditure of energy in the form of ATP. To test how Hsp30 influences the energetics of retentostat cultures, a congenic *hsp30* strain was compared with its parental strain in chemostat and retentostat cultures.

Retentostat cultures overcome one major drawback of SP cultures: their lack of metabolic activity and loss of viability due to the absence of extracellular energy substrate. Another drawback of SP cultures is their high degree of heterogeneity, which may strongly influence the interpretation of whole culture analyses (Werner-Washburne *et al.*, 2012). So far, retentostat cultures have only been analyzed at the whole culture level (Boender *et al.*, 2009; Boender *et al.*, 2011a; Boender *et al.*, 2011b). To investigate whether the observed responses may in fact

represent the average of very different responses in individual cells, it is essential to obtain information on responses a the single-cell level in the retentostat cultures. The anaerobic and severely calorie-restricted conditions in the retentostats represent an experimental challenge for such single-cell analysis. The fluorescent proteins most commonly used for such studies, i.e. green fluorescent protein and its derivatives, need molecular oxygen for proper chromophore formation (Remington, 2006) and are therefore not applicable under anaerobic conditions. Furthermore, effects of introducing a heterologous fluorescent protein on yeast physiology under severe calorie restriction are unknown. In order to address the question of population heterogeneity in anaerobic retentostat cultures, two different approaches based on fluorescence microscopy were followed (**Chapter 4**). Firstly, based on the observation of a quiescence-specific actin structure in yeast (Sagot *et al.*, 2006), actin structures in cells from retentostat cultures at different age were visualized to identify if yeast cells enter quiescence under severe calorie restriction and, if so, whether this quiescence entry is heterogeneous. A second, new method to analyze heterogeneity in anaerobic yeast retentostat cultures is fluorescent *in situ* hybridization (FISH) on mRNA (Itzkovitz and van Oudenaarden, 2011; Rahman and Zenklusen, 2013; Tan, 2010; Trcek *et al.*, 2012; Zenklusen and Singer, 2010). Using this technology, copy numbers of specific mRNA molecules can be measured in individual cells. In Chapter 4, experiments are described in which FISH was applied to monitor the expression of two quiescence-specific genes at the single cell level in anaerobic retentostat cultures.

Glucose-limited retentostat cultures of *S. cerevisiae* have so far been performed under anaerobic conditions, including the starvation experiments following retentostat cultivation (Boender *et al.*, 2011a). Aging studies in literature are, however, exclusively done under aerobic conditions. The presence of oxygen may strongly influence stationary phase characteristics, for example via differences in glucose metabolism and the ability to consume ethanol formed during the first phase of glucose growth. The role of oxygen in aging has mainly been addressed using mutants deficient in respiration, including mutants that lack mitochondrial DNA. **Chapter 5** describes experiments aimed at investigating the impact of aeration status on the survival of SP cultures. To this end, robustness of aerobic and anaerobic SP-cultures was compared at the physiological and transcriptome level. This study sought to not only identify the impact of oxygen on chronological aging, but also to obtain more insight into the anaerobic stationary phase in relation to anaerobic industrial applications of *S. cerevisiae*, such as the production of ethanol, beer and wine fermentation.

The work presented in this thesis was performed as part of the Zero Growth research programme of the Kluyver Centre for Industrial Genomics, a Netherlands-based research consortium involving several academic groups, linked to an international industrial platform. In this programme, not only *S. cerevisiae* but also several other microbial 'work horses' of modern industrial biotechnology were investigated under near-zero growth conditions using retentostats. In **Chapter 6**, transcriptional and physiological responses to near-zero growth are reviewed and compared for these organisms: the bacteria *Lactococcus lactis*, *Lactobacillus plantarum*, *Bacillus subtilis,* the yeast *S. cerevisiae* and the filamentous fungus *Aspergillus niger*.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2. To divide or not to divide: A key role of Rim15 in calorie-restricted yeast cultures

Markus M.M. Bisschops, Priscilla Zwartjens, Sebastiaan G.F. Keuter, Jack T. Pronk, Pascale Daran-Lapujade

Keywords: *Saccharomyces cerevisiae*, Rim15, cell cycle, calorie restriction, retentostat, robustness.

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The PAS kinase Rim15 is proposed to integrate signals from different nutrient-sensing pathways and to control transcriptional reprogramming of *Saccharomyces cerevisiae* upon nutrient depletion. Despite this proposed role, previous transcriptome analyses of *rim15* mutants solely focused on growing cultures. In the present work, retentostat cultivation enabled analysis of the role of Rim15 under severely calorie-restricted, virtually non-growing conditions. Under these conditions, deletion of *RIM15* affected transcription of over 10-fold more genes than in growing cultures. Transcriptional responses, metabolic rates and cellular morphology indicated a key role of Rim15 in controlled cell-cycle arrest upon nutrient depletion. Moreover, deletion of *rim15* reduced heat-shock tolerance in non-growing, but not in growing cultures. The failure of *rim15* cells to adapt to calorie restriction by entering a robust post-mitotic state resembles cancer cell physiology and shows that retentostat cultivation of yeast strains can provide relevant models for healthy post-mitotic and transformed human cells.

Introduction

Cellular health and fitness require tight regulation of the cell cycle in response to environmental conditions. Variations in nutrient supply are frequently encountered in nature and limited availability of one or more essential nutrients is a common environmental trigger for a programmed exit of the replicative cell cycle. In eukaryotes, exit from the mitotic cell cycle can lead to different viable, non-dividing physiological states, ranging from metabolically active post-mitotic mammalian cells to the virtually inactive dormant spores of fungi (Dahlberg and Etten, 1982; Valcourt *et al.*, 2012). Defects in regulatory mechanisms that control an exit from the replicative cell cycle result in either cellular transformation (in multicellular organisms), or dramatically reduced life span (particularly of unicellular organisms) (Gray *et al.*, 2004).

In eukaryotes, several signal-transduction and regulatory pathways are involved in coordinating cell cycle entry and arrest in response to nutritional status. Pathways such as those of the *t*arget *o*f *r*apamycin (TOR), which responds to nitrogen and carbon availability, and the cAMP-dependent *p*rotein *k*inase (PKA), which senses glucose availability, are highly conserved from fungi to mammals (De Virgilio and Loewith, 2006). TOR and PKA are also key nutrient signaling cascades in the model eukaryote *Saccharomyces cerevisiae* (Livas *et al.*, 2011; Rolland *et al.*, 2002). A growing body of evidence indicates that, in *S. cerevisiae*, various signaling cascades that sense nutritional status converge to a few key proteins that coordinate general responses such as cellular proliferation and stress resistance (De Virgilio, 2012).

Rim15, a PAS family protein kinase, has been proposed to integrate signals from various nutrient signaling networks (Cameroni *et al.*, 2004; Galdieri *et al.*, 2010) (Fig. 2.1). The regulatory activity and subcellular localization of Rim15 depend on nutrient sensing via TOR, PKA and the Pho80-Pho85 kinase (PHO) pathways, which sense nitrogen, sugar and phosphorus status, respectively, as well as on various environmental stresses (Swinnen *et al.*, 2006). A current working model proposes that Rim15 coordinates growth and, in particular, exit from the cell cycle into quiescence in response to signals from various sensing pathways (Smets *et al.*, 2010). Hitherto, the role of Rim15 has predominantly been investigated in glucose-grown shake-flask cultures. In such cultures, yeast cells undergo four distinct phases. After adaptation to the culture conditions in the lag phase, a fast exponential growth phase on glucose is followed by a slower diauxic growth phase on the fermentation products released in the first phase (mostly ethanol and organic acids) and by a final phase in which all carbon sources have been exhausted and the cells enter stationary phase. During stationary phase, cells enter a resting state that is commonly referred to as quiescence (Werner-Washburne *et al.*, 1993). While already expressed and activated during the diauxic phase, Rim15 appears to be especially important for survival and robustness during the subsequent stationary phase, in which cells are subjected to prolonged glucose starvation (Cameroni *et al.*, 2004; Wei *et al.*, 2008). Indeed, in stationary-phase cultures, deletion of *RIM15* causes a strongly

decreased accumulation of storage carbohydrates, reduced thermotolerance and reduced longevity, as well as an increase of the budding index (Reinders *et al.*, 1998; Talarek *et al.*, 2010; Watanabe *et al.*, 2012).

While the role of Rim15 has predominantly been associated with stationary phase, its impact on transcriptional regulation has not been investigated in non-growing cultures. This omission can probably be attributed to technical issues arising from the rapid degradation of mRNA in response to nutrient starvation (van de Peppel *et al.*, 2003). A transcriptome analysis in which the exponential growth phase on glucose of a *rim15* mutant and its parental strain were compared to the ethanol consumption phase of the two strains, identified a set of 152 Rim15-responsive transcripts. This dataset represents the largest reported response to the deletion of *RIM15*. This set showed an overrepresentation of genes involved in stress resistance (essentially heat shock and oxidative stress resistance), carbohydrate metabolism and respiration (Cameroni *et al.*, 2004). Most of these genes are under control of the transcription factors, Gis1, binding to the post-diauxic shift (PDS) element, and Msn2 and Msn4, two partially redundant transcriptional activators recognizing the stress response element (STRE) (Cameroni *et al.*, 2004; Zhang *et al.*, 2009) (Fig. 2.1). Other studies also reported changes in transcript levels in response to *RIM15* deletion, ranging from 11 genes

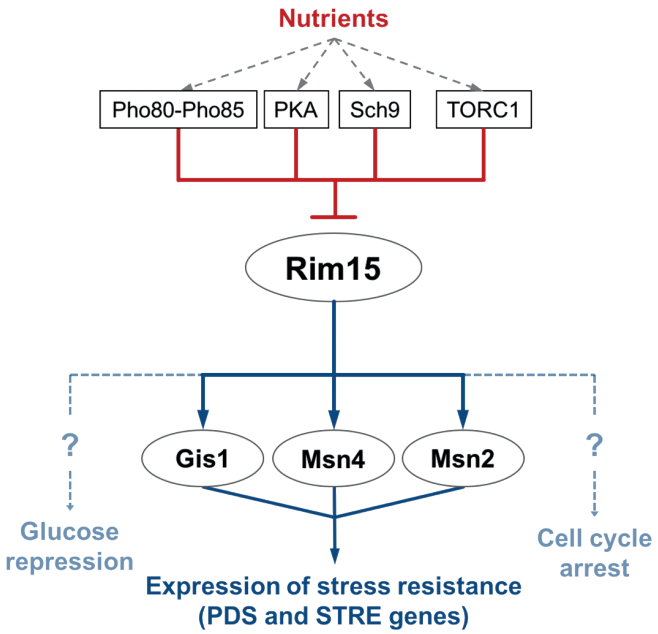


Figure 2.1 Regulatory cascades around the Rim15 PAS-kinase
The PAS-kinase Rim15 integrates signals from several nutrient sensing pathways, Pho80-Pho85, PKA, Sch9, and TORC1, to different effectors, including the transcription factors Gis1, Msn2 and Msn4, but putatively also factors that control glucose repression of genes and cell cycle arrest. Modified from Galdieri *et al.*, 2010; Smets *et al.*, 2010.

in exponentially growing cultures (van Wageningen *et al.*, 2010) to 54 genes in rapamycin treated cells (Talarek *et al.*, 2010). In the latter an overrepresentation of genes involved in stress response, carbohydrate metabolism and respiration was also found. The pronounced and pleiotropic phenotype of *rim15* mutants, as well as the proposed key role of Rim15 in quiescent cells, appears to be in contrast with the relatively small impact of a *RIM15* deletion. More specifically, the substantially decreased longevity and increased budding index of a *rim15* mutant during stationary phase were not reflected by the set of Rim15-responsive transcripts identified so far. These results suggest that the choice of cultivation conditions has not hitherto allowed scientists to capture the full scope of Rim15 regulatory functions.

We have recently implemented retentostat cultivation, which enables prolonged and tightly controlled cultivation of microorganisms under severe calorie restriction, as a tool for genome-wide transcriptome analysis of *S. cerevisiae* at near-zero growth rates (Boender *et al.*, 2011b). Physiological and transcriptome analyses revealed that yeast cells grown in retentostats adopt a physiological state that strongly resembles the G_0 phase of post-mitotic metazoan cells where growth is virtually absent but cells remain metabolically active. The physiological state of yeast under severe calorie restriction was shown to share many features with quiescent cells that are known to be orchestrated by Rim15, such as the transcriptional induction of the Rim15 target genes *SSA3*, *HSP12* and *HSP26*, the increased accumulation of glycogen and an increased robustness (Boender *et al.*, 2011a; Reinders *et al.*, 1998). We therefore anticipated that retentostat cultivation is ideally suited to explore the full scope of Rim15 functions in response to nutrient supply.

The aim of the present study was to shed light on the pleiotropic role of the PAS kinase Rim15 in the model yeast *S. cerevisiae* in response to nutrient supply. To this end, a prototrophic *rim15* deletion mutant was constructed in the widely used CEN.PK strain background (Nijkamp *et al.*, 2012) and grown under severe calorie restriction in retentostat cultures. The response of the *rim15* strain to prolonged cultivation under calorie restriction was investigated by physiological and transcriptional analyses and compared to that of a congenic *RIM15* reference strain.

Results

RIM15 deletion strongly affects yeast physiology under calorie-restricted conditions

The retentostat is a continuous cultivation set-up with a controlled, growth-limiting supply of the energy substrate, in which cells are trapped by a biomass retention system. During prolonged retentostat cultivation, cells divide until the energy-substrate availability per cell becomes too low to support cell division (Boender *et al.*, 2009; Van Verseveld *et al.*, 1986). Continued cultivation then results in a virtual non-growing, severely calorie-restricted situation, in which energy substrate is solely used for cellular maintenance processes (homeostasis of membrane potential and ion gradients, turn-over of macromolecules, etc.) (Stephanopoulos *et al.*, 1998a).

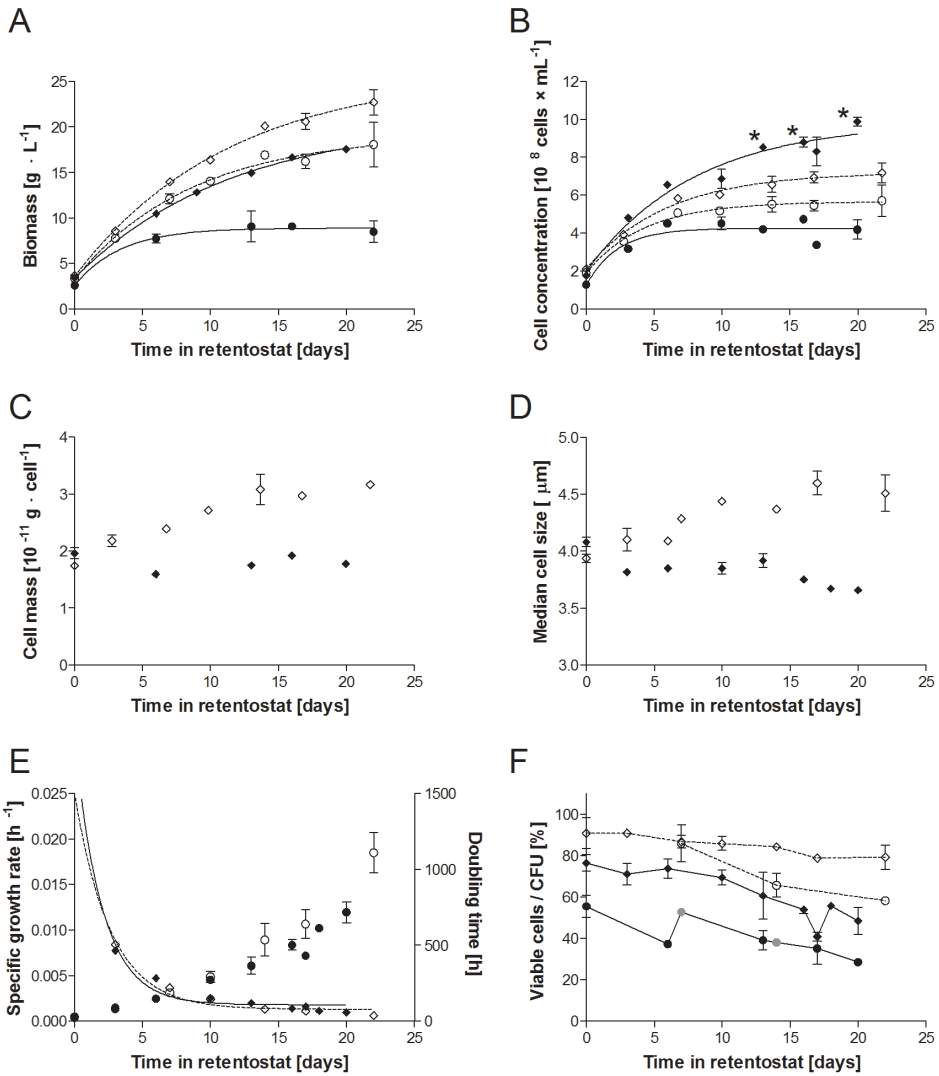


Figure 2.2 Biomass accumulation and viability of the* S. cerevisiae rim15 *mutant and the reference strain in retentostat cultures

Data for the *rim15* mutant IMK313 (closed symbols) and the reference strain CEN.PK113-7D (open symbols) are shown as mean values of at least duplicate cultures +/- SEM (Errors smaller than the symbol size are not visible). (**A**) Biomass measured as dry weight. (**B**) Biomass measured as cell concentration. (**A**) and (**B**), total biomass (diamonds) and biomass corrected for viability (circles). * indicates significantly different cell numbers (*p*-value below 0.05). (**C**) Average mass per cell. (**D**) Median cell size. (**E**) Specific growth rate (diamonds) and the corresponding doubling times (circles). The data shown are corrected for the viability. (**F**) Viability measured by flow cytometry (diamonds) and CFU (circles), shown as the number of viable cells. Data points shown in gray are obtained from a single culture.

As previously described, the initial biomass accumulation in anaerobic retentostat cultures of the *RIM15* reference strain *S. cerevisiae* CEN.PK113-7D (Fig. 2.2A) not only resulted from an increasing cell number (Fig. 2.2B) but also from increases in average cell mass and cell size (Fig. 2.2C,D) (Boender *et al.*, 2009). During prolonged retentostat cultivation, the specific growth rate decreased until, after 10 d (Fig. 2.2E), it had decreased to circa 0.002 h^{-1} (doubling time of circa 400 h). Under these severely calorie restricted conditions, cells retained metabolic activity and a high viability (above 79 % as measured by fluorescence staining, Fig. 2.2F) (Boender *et al.*, 2011a).

Retentostat cultivation of the *rim15* mutant IMK313 revealed striking differences with the reference strain. Firstly, the biomass dry weight remained lower than in the reference strain (22 % lower after 20 d, Fig. 2.2A). Conversely, cell numbers of IMK313 cultures were significantly higher than observed for the reference strain (up to 37 % higher after 20 d of retentostat cultivation; Fig. 2.2B). In contrast to the reference strain, the *rim15* strain did not show marked changes in cell size and cell mass during retentostat cultivation and displayed an abnormal morphology (Fig. 2.3). Moreover, the viability in retentostat cultures of strain IMK313 was significantly lower than that of the reference strain, both when measured as metabolic activity (fluorescence staining, Fig. 2.2F) and as ability to divide (CFU counts, Fig. 2.2F). During calorie restriction only 30 % of the *rim15* population retained the ability to divide, which is only half of the viability of the reference strain. Clearly, deletion of *RIM15*

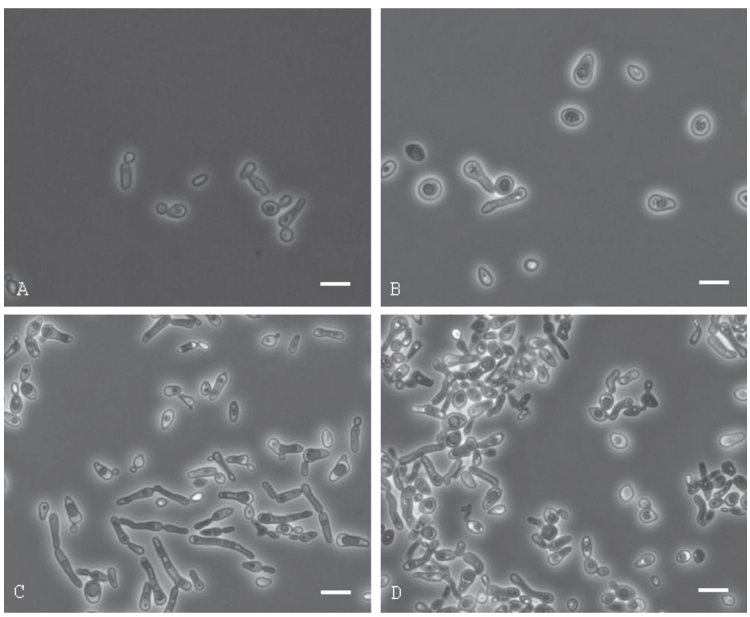


Figure 2.3 Phase contrast micrographs of the S. cerevisiae* rim15 *mutant and the reference strain under increasing calorie restriction
(**A**) and (**B**): Cells of 0 and 17 days old retentostat cultures of the reference strain, respectively. (**C**) and (**D**): Cells of 0 and 18 days old retentostat cultures of the *rim15* mutant IMK313. White marker bars represent 10 µm.

severely compromised the ability of yeast cells to maintain viability under calorie restricted conditions. Despite the differences in biomass concentration, cell numbers and viability, the profile by which the specific growth rate decreased in retentostat cultures was similar for the two strains (Fig. 2.2D).

Strong impact of *RIM15* deletion on transcriptome of calorie-restricted cultures

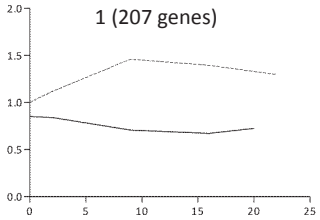
To investigate if the strong physiological impact of the *RIM15* deletion on calorie restricted cultures was reflected by changes in the transcriptome, genome-wide mRNA levels of the reference strain and the *rim15* mutant were measured and compared during anaerobic retentostat cultivation. Even though stringent statistical criteria were applied (*p*-value below 0.005), 1326 genes, corresponding to ca. 20 % of the yeast genome, were found to be differentially expressed between the two strains in calorie restricted retentostat cultures. This transcriptional response to *RIM15* deletion is considerably larger than previously reported. Transcriptome analysis of a *rim15* mutant and its parent during exponential growth on glucose and during diauxic growth on ethanol identified a set of only 152 differentially expressed genes (Cameroni *et al.*, 2004). An even smaller set of 49 genes was identified during glucose-limited chemostat cultivation (specific growth rate of 0.10 h^{-1}, aerobic) of a *rim15* mutant and its parental strain (Zhang *et al.*, 2009). Despite the differences in fraction of the genome that was affected by deletion of *RIM15*, genes involved in stress response, and more specifically oxidative stress response, were overrepresented in all three studies.

The present large calorie restriction dataset could be divided in a subset of 586 genes displaying a lower expression in the *rim15* mutant (Fig. 2.4, clusters 1, 2 and 3) and a set of 740 genes for which the expression was higher in the *rim15* mutant than in the reference strain (Fig. 2.4, clusters 4, 5 and 6). Genes involved in stress responses (*p*-value $2.4 \cdot 10^{-7}$, Fig. 2.4) were strongly overrepresented in the subset of genes whose transcript levels were lower in the *rim15* strain. Consistent with previous reports, this subset was enriched for targets of the STRE-binding proteins Msn2 and Msn4, two transcriptional activators acting downstream of Rim15 (Swinnen *et al.*, 2006). The subset of 740 genes that showed a higher

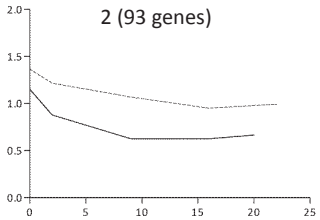
Figure 2.4 Clustering and enrichment analysis of differentially expressed genes in the S. cerevisiae* rim15 *as compared to the reference strain (>)

The 1326 genes differentially expressed between the *S. cerevisiae rim15* mutant IMK313 (solid line) and the reference strain CEN.PK113-7D (dashed line) were divided in 6 clusters (optimal number of clusters according to gap-statistics using K-means clustering). Graphs show the average mean-normalized expression of all genes in the cluster (*y*-axis) as function of time in retentostat (*x*-axis).The expression level of each gene was divided by the average expression of this gene across all 22 arrays in the retentostat dataset. The results of enrichment analysis of each cluster are shown below its respective clusters. The enrichment analysis at the bottom of the figure was performed using clusters grouped according to gene expression profiles: clusters 1, 2 and 3 that contain genes lower expressed in the *rim15* mutant and clusters 4, 5 and 6 that contain genes with higher expression in the *rim15* mutant. MIPS and Gene Ontology categories are shown in bold and italics respectively. Overrepresentation of binding motifs for specific transcription factors is indicated in standard font and for specific sets of genes described in literature in gray font; 1 (Badis *et al.*, 2008), 2 (Martínez-Pastor *et al.*, 1996), 3 (Bonner *et al.*, 1994), 4 (Boorstein *et al.*, 1990), 5 (Pedruzzi *et al.*, 2000). *p*-values are calculated according to Knijnenberg *et al.* and indicate the chance of random enrichment (Knijnenburg *et al.*, 2007).

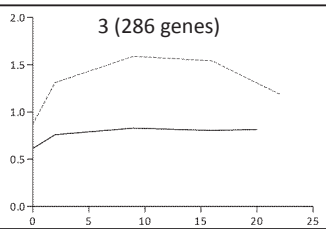
Lower



Description	# genes in cluster	Total # genes in category	p-value
STRE-element [1,2]	81	1786	2.77E-4
Oxidation Reduction	26	305	4.86E-6

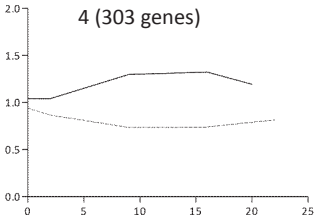


Description	# genes in cluster	Total # genes in category	p-value
Gcn4	12	182	1.11E-5
Ste12-Swi5	5	17	3.18E-6

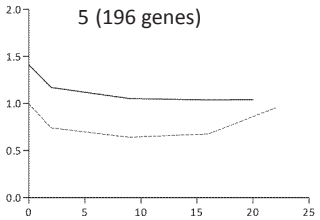


Description	# genes in cluster	Total # genes in category	p-value
Hsf1 [3]	59	435	2.85E-15
STRE-element [1,2]	133	1786	6.89E-12

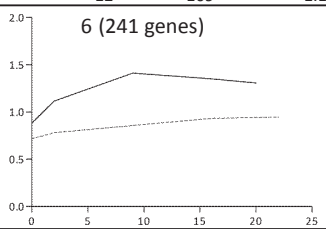
Higher



Description	# genes in cluster	Total # genes in category	p-value
Nucleotide binding	26	223	1.68E-5
Cell Cycle	75	1004	2.05E-5
rRNA processing	26	195	1.38E-6
Arg80	7	19	1.56E-5



Description	# genes in cluster	Total # genes in category	p-value
Swi4	21	144	1.93E-9
Mbp1	18	165	2.51E-6
Swi6	16	160	2.81E-5
Ndd1	12	109	1.15E-4



Description	# genes in cluster	Total # genes in category	p-value
Proteasome	8	37	5.49E-5
Electron transport chain	7	8	7.78E-10

Lower expression in *rim15* (cluster 1-3)

Description	# genes in cluster	Total # genes in category	p-value
Hsf1 [3]	94	435	2.51E-16
STRE-element [1,2]	251	1786	3.92E-16
PDS-element [4,5]	28	162	7.13E-4
Oxidative stress response	19	56	2.44E-7
Oxidation reduction	47	270	9.97E-6
Skn7	41	175	1.02E-8
Msn2	30	122	3.33E-7
Msn4	25	121	7.87E-5

Higher expression in *rim15* strain (cluster 4-6)

Description	# genes in cluster	Total # genes in category	p-value
Cell Cycle and DNA processing	179	1004	1.14E-10
Cell type differentiation	86	459	2.26E-6
Swi4	40	144	6.49E-8
Mbp1	43	165	1.55E-7
Mcm1	23	78	1.44E-5
Dig1	35	148	2.30E-5
Ndd1	28	109	3.09E-5
Arg80	9	19	1.13E-4

transcript level in the *rim15* strain showed a strong overrepresentation of genes involved in cell cycle, and more specifically of targets of the transcription factors Swi4, Swi6, Mbp1, Mcm1 and Ndd1 (Fig. 2.4).

Deletion of *RIM15* induces transcription of cell cycle-related genes

Among the 740 genes with increased transcript levels in the *rim15* strain, 179 (24 %) belonged to the MIPS functional category 'cell cycle and DNA processing' (*p*-value $1.1 \cdot 10^{-10}$, Fig. 2.4). Three separate gene clusters were defined based on the transcript profiles of the mutant and reference strain (Fig. 2.4). Transcript levels of the 303 genes in cluster 4 (Fig. 2.4) showed a positive correlation with specific growth rate in the reference strain, but a negative correlation with specific growth rate in the *rim15* strain. Of the three clusters with higher expression in IMK313, cluster 4 showed a strong overrepresentation of cell cycle-related functional categories (*p*-value $2.1 \cdot 10^{-5}$) and closer inspection showed overrepresentation of genes whose expression is cell cycle-dependent (*p*-value $1.3 \cdot 10^{-2}$) (Spellman *et al.*, 1998). A strong enrichment for genes involved in the G_1/S transition, including the cyclins Cln1 and Pcl1, was observed among the 740 genes with higher expression in IMK313 (*p*-value $1.4 \cdot 10^{-5}$) (Cho *et al.*, 1998). However, genes involved in other phases of the mitotic cell cycle, such as the cyclins, Clb1 and Clb2, were also found in this gene set. Increased expression, in the *rim15* mutant, of genes implicated in various stages of the cell cycle suggests that, in contrast to the reference strain, this mutant failed to efficiently curtail cell cycle activity under calorie restricted conditions.

Rim15 is essential for robustness, but not for glycogen accumulation under calorie restriction

As mentioned above, genes involved in stress resistance were overrepresented among the genes whose expression was reduced in calorie-restricted cultures of the *rim15* mutant (cluster 1 to 3, Fig. 2.4). Among these genes, an extremely strong overrepresentation was found for genes with a STRE binding motif in their promoter region (251 genes, *p*-value $2.5 \cdot 10^{-16}$), which is a target for the regulators Msn2 and Msn4. The PDS element (28 genes, *p*-value $7.1 \cdot 10^{-4}$), which is a target of Gis1, was similarly overrepresented in this gene set. This observation is consistent with the notion that Msn2, Msn4 and Gis1 act downstream of Rim15 in nutrient signalling cascades (Swinnen *et al.*, 2006). More specifically clusters 1 to 3, showed a strong overrepresentation of binding motifs of the Heat shock factor Hsf1. Although Hsf1 has not been demonstrated to be directly regulated by Rim15, this result shows that Rim15 is required for induction of many heat shock responsive genes in calorie restricted cultures. At the start of retentostat cultivation, where the specific growth rate was 0.025 h^{-1}, no difference in heat shock resistance between the two strains was observed (Fig. 2.5A). As specific growth rates approached zero during 10 days of retentostat cultivation, heat shock resistance increased by over 4 fold in the reference strain, in agreement with the transcript levels of Hsf1 targets (Fig. 2.5B). Conversely, over the same period, it hardly increased in the *rim15* mutant. Therefore, Rim15 is also essential for the induction of heat shock resistance

in calorie restricted *S. cerevisiae* cultures, as previously observed in nutrient-starved cells (Watanabe *et al.*, 2012; Wei *et al.*, 2008).

Reserve carbohydrates have been implicated in yeast robustness. Trehalose in particular is a stress protectant while trehalose and glycogen can act as energy storage compounds during starvation (François *et al.*, 2012). The reference strain strongly accumulated glycogen during calorie restriction in the retentostats (Fig. 2.5C). Under these conditions, intracellular trehalose remained below the detection limit for both strains. Under the same conditions the *rim15* mutant still accumulated glycogen, but glycogen levels were circa 50 % lower than in cultures of the reference strain. As previously observed with nutrient-starved yeast cells, Rim15 is a key factor for glycogen accumulation during calorie restriction of *S. cerevisiae* but is not essential (Pedruzzi *et al.*, 2003; Wanke *et al.*, 2005; Watanabe *et al.*, 2012).

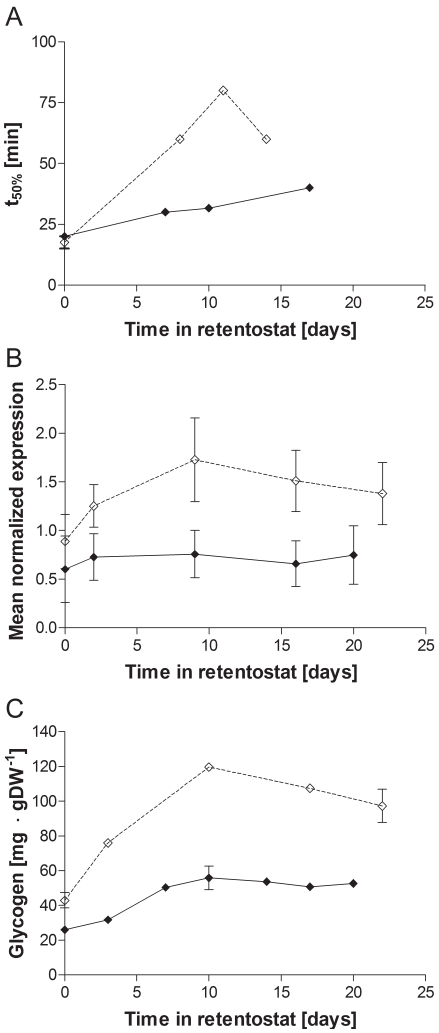


Figure 2.5 Effect of RIM15 deletion on stationary-phase features

(**A**) Heat shock resistance of the *S. cerevisiae rim15* strain IMK313 (closed symbols, ◆) and its parental strain CEN.PK113-7D (open symbols, ◇). $t_{50\%}$ is the time of incubation at 53°C at which the viability of the culture was reduced by 50% relative to that at the start of the experiment. Data are represented as mean values of duplicate retentostat cultures. (**B**) Averaged mean-normalized expression of genes involved in heat shock resistance during retentostat cultivation in the reference strain (open diamonds, ◇) and IMK313 (closed diamonds, ◆). Data are represented as the mean values +/- SD of the expression levels of *AUT7, CYC7, DDR48, ECI1, ECM4, GAD1, GDH3, GPH1, GTT1, HBT1, HSP12, MSC1, PCA1, PIR3, PNC1, PST2, RNR3, SDS24, SPI1, SSE2, STF2,TES1, TKL2, TPS1, TSA2, TSL1, YBL049W, YBR056W, YBR116C, YCL044C, YDR512C, YDR533C, YER079W, YGL047W, YGP1, YHL021C, YHR087W, YHR138C, YIR036C, YJL045W, YJR096W, YKL151C, YLR064W, YMR090W, YNL200C,YOR292C, YPL004C, YPL170W, YRO2, YSC84*, divided by the average expression of each gene across all 22 arrays in the retentostat dataset. (**C**) Cellular glycogen contents in the reference strain (open diamonds, ◇) and IMK313 (closed diamonds, ◆) during retentostat cultivation expressed as grams glucose equivalent per gram dry weight biomass. Data are represented as mean +/- SEM of duplicate retentostat cultures.

Rim15 is involved in carbon catabolite repression under severe calorie restriction

The growth-limiting glucose supply in retentostats leads to low and decreasing residual glucose concentrations. These, in turn, cause an alleviation of glucose repression and up-regulation of many glucose-sensitive genes in the reference strain (Boender *et al.*, 2011b). Although no significant differences in residual glucose levels were observed in retentostat cultures of the reference strain and the *rim15* mutant (Supplementary Fig. S2.2), the 1326 differentially expressed genes showed a strong overrepresentation of genes previously shown to be transcriptionally repressed by glucose (176 genes, *p*-value $7.6 \cdot 10^{-10}$) (Kresnowati *et al.*, 2006). Of these 176 genes, 125 were co-regulated and displayed a lower expression in the *rim15* strain (i.e. in clusters 1-3), indicating that, despite the similar residual glucose concentrations in cultures of the two strains, a stronger glucose repression occurred in the absence of Rim15. This subset of 125 genes showed a strong overrepresentation of Msn2/4 and Gis1 sequence motifs in their promoter regions (76 genes, *p*-value $1.1 \cdot 10^{-14}$, and 7 genes, *p*-value $3.9 \cdot 10^{-2}$, respectively). This regulation of glucose-responsive genes may therefore be mediated via Msn2/4 and Gis1; however 46 genes carried neither STRE nor PDS element.

Higher catabolic activity in calorie restricted cultures of a *rim15* mutant

In slow-growing, glucose-limited cultures, yeast cells have to divide the energy source over two major cellular activities, growth and cellular maintenance. In the retentostat cultures, the specific glucose uptake rate (q_s) decreased over time (Fig. 2.6), resulting in a progressively stronger calorie restriction. After 10 days, glucose was predominantly used for maintenance purposes and growth had virtually ceased. In the reference strain glucose consumption reproducibly stabilized at 0.5 mmol $\cdot$ $(g \cdot h)^{-1}$. Strikingly, despite the severe calorie restriction, the *rim15* mutant maintained a 40 % higher specific glucose uptake rate (0.72 mmol $\cdot$ $(g \cdot h)^{-1}$, Fig. 2.6) than the reference strain. In both strains, specific glucose uptake rates were based on viable cells. The results indicate that Rim15 plays a role in minimizing glucose uptake under conditions of extreme calorie restriction and that deletion of *RIM15* causes either increased maintenance requirements or excess glucose uptake.

Rim15 also plays a role in exponentially growing cells at slow growth rates

The proposed role of Rim15 has hitherto been associated with the transition from exponential growth to stationary phase. Zhang and coworkers identified a set of 49 genes differentially expressed in a *rim15* mutant during exponential growth in glucose-limited chemostat culture at a specific growth rate of 0.10 h^{-1} (Zhang *et al.*, 2009). Although, under these conditions, the *rim15* mutant did not show marked phenotypic differences from the reference strain, this transcriptional response to *RIM15* deletion suggested a role for Rim15 during exponential growth. Furthermore, earlier retentostat and chemostat experiments demonstrated that expression of *RIM15* and its targets (such as *SSA3* or *HSP12*) was negatively correlated with specific growth rate (Boender *et al.*, 2011b). To investigate whether Rim15 plays a role during exponential growth on glucose and whether this role is growth rate-dependent, the reference strain CEN.PK113-7D and the *rim15* strain IMK313

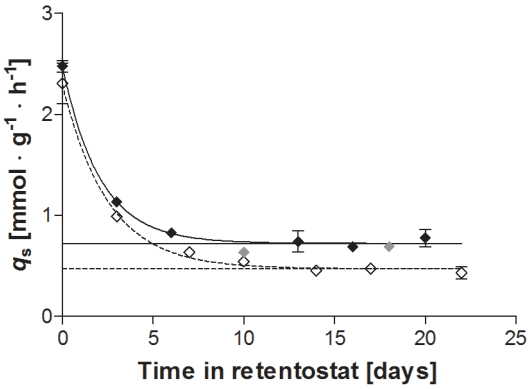


Figure 2.6 Specific glucose uptake rates of retentostat cultures
Specific glucose uptake rate (q_s) during retentostat cultivations of the *S. cerevisiae* reference strain (open diamonds, ◇) and the *rim15* mutant IMK313 (closed diamonds, ◆). Data were corrected for viability. The horizontal lines are the asymptotes corresponding to the specific glucose consumption rate in non-dividing, calorie-restricted cultures. These minimal specific glucose uptake rates, derived from fitting of the data using an exponential function (shown as the continuous line for the rim15 mutant and dashed line for the reference strain), are significantly different between the two strains (p-value below $1 \cdot 10^{-6}$). Data are represented as mean +/- SEM for at least 2 biological replicates, except for datapoints shown in gray that were obtained from a single culture. (Errors smaller than the symbol size are not visible.)

were grown in anaerobic, glucose-limited chemostats at specific growth rates ranging from 0.025 h^{-1} to 0.10 h^{-1}. In these chemostat cultures, viability, heat shock resistance, cell size and mass were not significantly different for the reference and mutant strain (Fig. 2.7A,B). However, the morphology of IMK313 was markedly elongated at all specific growth rates tested (Fig. 2.3C and data not shown). Furthermore, at the lowest specific growth rate tested (0.025 h^{-1}), the *rim15* mutant showed a 50 % lower glycogen content than the reference strain (Fig. 2.7C). These morphological and physiological differences between IMK313 and its parental strain demonstrated that Rim15 plays a role during exponential, glucose-limited growth. Microarray analysis of cultures of the *rim15* and reference strains grown exponentially at 0.025 h^{-1} also identified an impact at the transcriptional level. This response involved 10-fold fewer genes than observed in calorie-restricted cultures (120 differentially expressed genes as compared to 1326), but showed overrepresentation of the same functional categories (i.e., stress response, Msn2/Msn4 targets, etc., Supplementary Table S2.2). Although, at a specific growth rate of 0.025 h^{-1}, genes involved in heat shock resistance were expressed at a lower level in the *rim15* mutant, this transcriptional difference was not mirrored by a change in heat shock resistance (Fig. 2.7B). Although much narrower than that observed at near-zero growth rates in severely calorie-restricted cultures, the transcriptional response observed at a growth rate of 0.025 h^{-1} still involved more genes than previously observed at 0.10 h^{-1} (Zhang *et al.*, 2009). Together, these results show that the scope of the impact of Rim15 on transcriptional regulation is growth rate dependent.

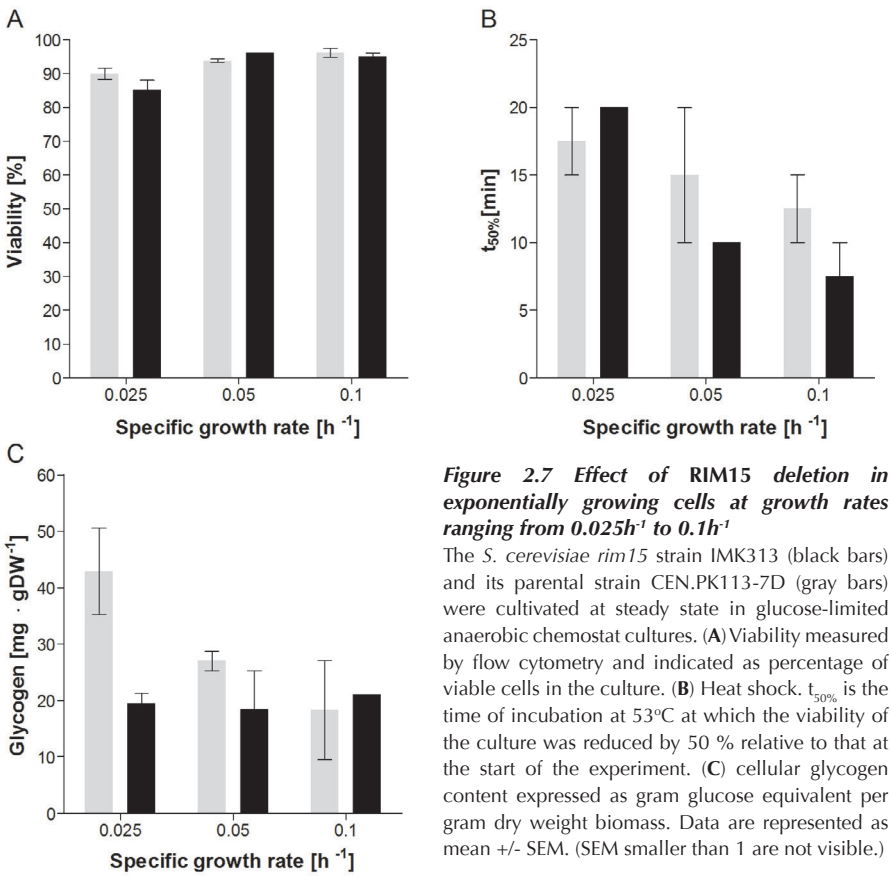


Figure 2.7 Effect of* RIM15 *deletion in exponentially growing cells at growth rates ranging from 0.025h^{-1} to 0.1h^{-1}

The *S. cerevisiae rim15* strain IMK313 (black bars) and its parental strain CEN.PK113-7D (gray bars) were cultivated at steady state in glucose-limited anaerobic chemostat cultures. (**A**) Viability measured by flow cytometry and indicated as percentage of viable cells in the culture. (**B**) Heat shock. $t_{50\%}$ is the time of incubation at 53°C at which the viability of the culture was reduced by 50 % relative to that at the start of the experiment. (**C**) cellular glycogen content expressed as gram glucose equivalent per gram dry weight biomass. Data are represented as mean +/- SEM. (SEM smaller than 1 are not visible.)

Discussion

Rim15 has a massive impact on transcriptional reprogramming for calorie-restricted, non-growing conditions

With one exception (Zhang *et al.*, 2009), previous investigations on the role Rim15 used shake flask cultures, either during diauxic growth on glucose or during the subsequent stationary phase. The strongest *rim15* phenotype was observed during stationary phase, where cells are effectively starved for carbon and energy (Smets *et al.*, 2010). However, mRNA degradation precludes accurate transcriptional analysis under those conditions (Boender *et al.*, 2011a; van de Peppel *et al.*, 2003). The transcriptional responses of a *rim15* mutant and a reference strain during exponential growth on glucose and the subsequent diauxic growth phase on ethanol have been compared previously (Cameroni *et al.*, 2004; Talarek *et al.*, 2010). Although growth of *S. cerevisiae* on ethanol is slower than on glucose (see Tahara *et al.*, 2013; Van Dijken *et al.*, 2000), both situations still represent active growth.

Use of retentostat cultures (Boender *et al.*, 2009; Van Verseveld *et al.*, 1986) enabled, for the first time, a comparison of transcriptional responses of a *rim15* strain and a congenic

reference strain of *S. cerevisiae* under controlled, severely calorie-restricted conditions. As specific growth rate in retentostat cultures decreased to virtually zero, previously reported responses associated with Rim15 intensified. These responses included glycogen accumulation, increased heat-shock resistance and increased expression of stationary-phase associated genes such as *SSA3*, *HSP12* and *HSP26* (Boender *et al.*, 2011a; Smets *et al.*, 2010). Deletion of *RIM15* led to disappearance or strong attenuation of these features.

Some transcriptional responses observed in retentostat cultures of the *rim15* strain were previously observed under different experimental conditions and in different strain backgrounds (Cameroni *et al.*, 2004; Reinders *et al.*, 1998; Talarek *et al.*, 2010; Watanabe *et al.*, 2012; Wei *et al.*, 2008). However, the calorie-restricted conditions used in this study revealed a spectacularly larger impact of Rim15 on the yeast transcriptome than previously reported. Even when applying stringent statistical criteria (*p*-value cut-off of 0.005), expression of one fifth of the yeast genome was affected by the deletion of *RIM15* under severe calorie restriction. This fraction is fivefold higher than that previously observed in post-diauxic-shift yeast cultures (Cameroni *et al.*, 2004). It can therefore be concluded that exponentially growing yeast cultures, even at sub-maximal growth rates such as the specific growth rate of 0.10 h^{-1} in a previous chemostat-based transcriptome analysis of a *rim15* mutant (Zhang *et al.*, 2009), only reveal a small fraction of the massive impact of Rim15 on the transcriptome of calorie-restricted, non-growing yeast cells.

While Rim15 has previously been associated with transcriptional activation, over half of the differentially expressed genes in retentostat cultures of the *rim15* mutant showed higher transcript levels than those in the control strain. This observation indicates that Rim15 is also, directly or indirectly, involved in transcriptional repression. Consistent with the reported mediation of Rim15 regulation by the transcription factors Msn2/Msn4 and Gis1 (Swinnen *et al.*, 2006), genes with STRE and/or PDS elements in their promoter regions were overrepresented among the gene sets that showed a lower transcript level in the *rim15* strain. Still, a substantial fraction (55 %) of this gene set did not harbor STRE or PDS binding sequences, nor did the majority (73 %) of genes displaying higher expression in *rim15* relative to the reference strain. While these differential expressions may result from secondary effects, the consistent co-regulation of genes belonging to specific functional categories, such as genes involved in cell cycle progression or responding to glucose catabolite repression, strongly suggest that as yet unidentified regulatory proteins function downstream of Rim15 to relay nutrient-sensing signals.

Other remarkable and new features of the *rim15* mutant revealed in the retentostat cultures were the absence of an increase of cell size and mass at low specific growth rates, an increased metabolic activity in non-growing cultures, a severe loss of viability at low specific growth rates and a peculiar morphology. Together with the transcriptional changes observed in a *rim15* strain, these phenotypes underline the vital role of the Rim15 kinase in reprogramming and preparing cells for cessation of growth due to calorie restriction.

Rim15 plays a key role in nutrient-status-mediated transition of the Start checkpoint

By integrating the results obtained in the present work, we can more precisely define the multifaceted role of Rim15. Earlier work led to the proposal that Rim15 is involved in the exit of yeast cells from the replicative cell cycle and entry into the so-called G_0 phase (Pedruzzi *et al.*, 2003; Reinders *et al.*, 1998; Watanabe *et al.*, 2012). Our results bring direct transcriptional and physiological evidence that Rim15 indeed affects cell cycle progression in yeast. Cell-cycle-related genes were strongly overrepresented among the genes that displayed a higher expression in calorie-restricted cultures of the *rim15* mutant (clusters 4-6, Fig. 2.4). The strongest overrepresentation *(p*-value from $2 \cdot 10^{-6}$ to $2 \cdot 10^{-9}$, Fig. 2.4) was observed for genes with promoter binding motifs for Swi4, Swi6, and Mbp1, which are subunits of the MBF and SBF complexes (Moll *et al.*, 1992). MBF and SBF transcriptionally activate many genes during the Start phase of the cell cycle, including the Cln1 and Cln2 cyclins that control the G_1/S transition (Koch *et al.*, 1993). The transcriptome data support the notion that, in the absence of Rim15, cells pass the Start checkpoint even when calorie restriction is so severe that it effectively precludes growth. Indeed, genes involved in different cycle phases showed elevated transcript levels in severely calorie restricted cultures of the *rim15* strain (Fig. 2.4). Because passing Start is irreversible, *rim15* cells are then compelled to proceed to complete their mitotic cycle (Charvin *et al.*, 2010). However, completing a mitotic cycle depends on metabolic energy and precursors, two requirements that cannot be fulfilled under severe calorie restriction. If severely calorie restricted cells nevertheless invest in formation of daughter cells, this inevitably compromises their ability to invest energy in maintaining cellular robustness and integrity. The increased rate of glucose metabolism in retentostat cultures of *rim15* strains, their reduced viability and robustness are fully consistent with a key role of Rim15 at the Start checkpoint. Furthermore, while the reference strain increases its cell size, mass, stress resistance and reserve carbohydrate content during calorie restriction, features characteristic of G_1 cells (Brauer *et al.*, 2008; Paalman *et al.*, 2003), these features remain strikingly stable in the *rim15* mutant. Also this observation is fully consistent with a model in which the reference strain remains in extended G_1 during severe calorie restriction, while *rim15* cells pass Start and proceed with cell division, thus spending less time in G_1. Further support for this model is provided by the observation that Rim15 is required for efficient G_1 arrest induced by the drug rapamycin or nutrient depletion (Pedruzzi *et al.*, 2003; Weinberger *et al.*, 2007). Unfortunately, the condition-dependent abnormal morphology of the *rim15* mutant observed in the present study (Fig. 2.3) precluded quantification of the budding index. While Rim15 clearly integrates nutritional status of yeast cells in the decision to proceed beyond Start, the mechanism by which this signal is transferred cannot be identified by transcriptional analysis. It will be of interest to assess whether Rim15, a PAS kinase, is able to phosphorylate key regulators active at the G_1/S interface. An alternative mode of action of Rim15 could be to primarily regulate the glucose uptake rate that, in turn, would control cell cycle progression. To test this scenario, the transcriptome of the *rim15*

mutant and the reference strain were compared using glucose uptake rate instead of growth rate as key identifier. This analysis also identified the strong up-regulation of genes involved in cell cycle progression and the down-regulation of glucose-repressed and stress-responsive genes in the *rim15* mutant (Supplementary Table S2.3) and supports the key role of Rim15 in cell cycle progression.

Role of Rim15 in exponentially growing cells

Transcriptome analyses on glucose-limited chemostat and retentostat cultures revealed an inverse correlation of *RIM15* expression with specific growth rate (Boender *et al.*, 2011b). The present study shows that the impact of *RIM15* deletion on transcriptome and physiology is also strongly growth-rate dependent. Indeed, the phenotype of the *rim15* strain IMK313 intensified at specific growth rates below 0.025 h^{-1}. At this growth rate, the phenotype of the *rim15* strain was restricted to a relatively narrow transcriptional difference with the reference strain, involving fewer than one tenth of the responsive genes found under severely calorie-restricted, non-growing conditions, and a reduced glycogen content.

Cell cycle-related genes were not overrepresented among the *rim15*-responsive genes in exponentially growing cultures. This is not surprising as, even at a low specific growth rate of 0.025 h^{-1}, cells do divide (ca. 1 generation in 28 h). Consequently, they pass Start and continuous glucose supply allows them to complete the replicative cell cycle, consistent with the high viability of *rim15* yeast cells under these conditions. Since, during retentostat cultivation, the glucose supply per cell and the specific growth rate decrease, the impact of the deletion of *RIM15* intensifies and peaks when severely calorie-restricted, virtually non-growing conditions are reached.

The peculiar morphology of the *rim15* mutant in chemostat cultures at growth rates ranging from 0.025 h^{-1} to 0.10 h^{-1} demonstrates that Rim15 does play a biologically relevant role in exponentially growing cells. Since this morphology was not observed in shake-flask cultures grown on excess glucose or ethanol (data not shown), it is probably related to nutrient-limited cultivation conditions.

Since growth rate in glucose-limited cultures is determined by the residual glucose concentration, intra- or extracellular glucose concentrations in such cultures set the degree of calorie sufficiency or restriction (Pirt, 1975). Such a direct link with nutrient concentration is consistent with the role of Rim15 in the interaction of signals from different nutrient sensing cascades. The apparent glucose repression response in severely glucose-limited cultures of the *rim15* strain IMK313 confirms that the 'overestimation' of glucose availability that occurs in the absence of Rim15 extends beyond cell-cycle related processes. Although no other nutrients were tested beyond glucose, the location of Rim15 downstream of various nutrient-signalling cascades (TOR, PKA and PHO), suggest that Rim15 could have a comparable tuneable activity in response to other nutrients (Swinnen *et al.*, 2006).

The gradual increase of the impact of Rim15 with decreasing specific growth rate indicates that this key regulator does not function as a rigid on-off switch between the mitotic

cell cycle and a separate G_0 phase. Rather, Rim15 appears to act as a cellular 'dimmer' that enables a gradual adaptation of the cell cycle and physiological make-up when yeast cells are exposed to increasingly stringent nutrient limitation. As long as requirements for maintenance of cellular integrity and viability are met, thereby preventing acute nutrient starvation, non-growing, metabolically active yeast cells appear to represent the end of the continuum rather than a distinct 'quiescent state'.

Retentostat cultures of yeasts: a model for post-mitotic mammalian cells

The present study on the role of Rim15 in *S. cerevisiae* illustrates how controlled cultivation in retentostat allows researchers to access a domain of yeast biology that cannot be accessed in conventional cultivation systems. Cultivation of *S. cerevisiae* in retentostat leads to a physiological status characterized by cell cycle arrest, maintenance of metabolic activity and robustness, features that are reminiscent of post-mitotic mammalian cells. Although yeast cells are already intensively used as models to study cellular aging, chronological aging is typically investigated in yeast cultures starved for carbon, in which cells are deteriorating and slowly dying (Breitenbach *et al.*, 2012a). The option to keep yeast cells alive and metabolically active in a non-dividing state for prolonged periods of time should make retentostat cultivation a valuable tool to investigate chronological aging.

The phenotype of *rim15* mutant includes imperfect control of cell cycle progression under calorie-restricted conditions. This lack of proper response to severely growth-limiting conditions resembles a major characteristic of cancer cells that are self-sufficient in growth signals and lack sensitivity to anti-growth signals (Hanahan and Weinberg, 2000). Yeast is not an uncommon model in cancer and anti-cancer treatment research (de Clare and Oliver, 2013; Kurtz *et al.*, 2004; Matuo *et al.*, 2012). In addition to uncontrolled cell cycle progression, the *rim15* mutant displays under severe calorie restriction substantially decreased robustness, a feature also shared with cancer cells. Retentostat cultures of the reference and *rim15* strain might therefore provide a valuable model to study the differential stress response (DSR) of healthy and malign mammalian cells under calorie restriction and its implications in cancer treatment (Lee and Longo, 2011; Raffaghello *et al.*, 2010).

Materials and Methods

Strains

The prototrophic *S. cerevisiae* strain CEN.PK113-7D (*MAT*a *MAL2*-8c *SUC2 RIM15*, obtained from Dr P. Kötter, Frankfurt, Germany) (Entian and Kötter, 2007; Nijkamp *et al.*, 2012) was used as a reference. Yeast transformations were performed using the Lithium-Acetate method described by Gietz and Schiestl (Gietz and Schiestl, 2007). For deletion of *RIM15* gene and marker removal the loxP-marker-loxP/Cre recombinase system was used. The knockout cassette was constructed based on plasmid pUG6 using primers RIM15-KO_FW and RIM15-KO_RV (for sequences see Supplementary Table S2.1) according to Güldener *et al.* (Güldener *et al.*, 1996). Correct integration in the genome of prototrophic *S. cerevisiae*

strain CEN.PK113-7D and removal of the cassette were confirmed by PCR using primers RIM15-KO_Ctrl1, RIM15-KO_Ctrl2 and RIM15-KO_Ctrl3 (for sequences see Supplementary Table S2.1). Primers were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). The resulting strain is the prototrophic, marker-free *rim15* strain IMK313. Strains were grown in YPD until late exponential phase and stored as glycerol stocks (20 % v/v) at -80°C.

Media and cultivation methods

Chemostat and retentostat cultures were grown on synthetic medium (Verduyn *et al.*, 1992) supplemented with the anaerobic growth factors ergosterol (10 mg · L^{-1}) and Tween 80 (420 mg · L^{-1}) according to Verduyn *et al.* (Verduyn *et al.*, 1990b) and the antifoam Struktol J673 (0.3 mg · L^{-1}). Glucose, the sole carbon and energy source, was the limiting nutrient for chemostat and retentostat cultures. All other nutrients, including the anaerobic growth factors Tween 80 and ergosterol, were supplied in excess. The glucose concentration in the feed was 50 g · L^{-1} for chemostats at growth rates of 0.025 h^{-1} and retentostat cultivations and 25 g · L^{-1} for chemostat cultures performed at dilution rates above 0.025 h^{-1}.

Anaerobic chemostat and retentostat cultivations were performed as described by Boender *et al.* (Boender *et al.*, 2009). Chemostats at the specific growth rate of 0.025 h^{-1} were performed in quintuplicate, and at the specific growth rates of 0.1 and 0.05 h^{-1} in duplicate, while retentostat cultures were run in triplicate. Cultures were kept anaerobic by sparging bioreactors (2 L with a 1.4 L liquid working volume) and medium reservoirs with ultrapure N_2 (5.0; Linde Gas Benelux, The Netherlands) (0.7 L · min^{-1}). Norprene tubing was used to prevent oxygen diffusion. Temperature was controlled at 30°C and pH at 5 by automated addition of 2 M KOH. Chemostat cultures were maintained until steady state criteria (Boender *et al.*, 2009) were met. Retentostat cultures were started from steady state chemostat cultivations with specific growth rate 0.025 h^{-1} by redirecting the effluent through a port equipped with an autoclavable AppliSense sample filter (0.2 µm pore diameter). The impact of sampling on calculated growth rates was kept below 2 %.

Determination of substrate, metabolite and biomass concentrations

Supernatants of samples were analysed using HPLC (Waters, Milford, MA) to determine concentrations of ethanol, glycerol, acetate, lactate, pyruvate and succinate, as described by Boender *et al.* (Boender *et al.*, 2009). After rapid quenching with cold steel beads, supernatant was analysed enzymatically to assay the residual glucose concentration (Roche kit no. 0716251) (Mashego *et al.*, 2003). Reserve carbohydrates were assayed as described by Boender *et al.* (Boender *et al.*, 2011a). Biomass concentrations were determined as culture dry weights according to the method of Postma *et al.* (Postma *et al.*, 1989) and by cell concentration measured with a Z2 Coulter counter (50 µm aperture, Beckman, Fullerton, CA). Mean cell sizes in cultures were based on the electronic volume measured by a Z2 Coulter counter. Exhaust gas from retentostat and chemostat cultivations was cooled (2°C) and dried (Perma Pure Dryer) and analysed online for carbon dioxide levels.

Viability and thermotolerance measurements

Viability of the cultures was determined according to Boender *et al.* (Boender *et al.*, 2011a). For flow cytometry-based assays the Fungalight CFDA, AM/propidium iodide yeast vitality kit (Invitrogen) was used. Cells stained green due to esterase activity on CFDA, AM were considered metabolic active and alive, whereas cells stained red with propidium iodide only or that did not stain at all were considered metabolically inactive and dead. Viability measured as ability to divide was determined using colony forming unit (CFU) assays.

Thermotolerance assays were performed as previously described (Boender *et al.*, 2011a) by monitoring viability of yeast cells incubated at 53°C at 5 minute intervals. Heat shock resistance is represented as the incubation time at which viability reaches 50 % or lower of the viability at the start of the assay.

Calculation of metabolic fluxes

Specific growth rates, consumption and production rates (q_i in mmol · (g · h)$^{-1}$) were calculated based on the methods of Boender *et al.* (Boender *et al.*, 2009). In short, total production and consumption rates (r_i in mmol · h^{-1}) were divided by the fraction of viable biomass ($C_{x,viable}$ in g) as only viable biomass contributes to these rates (Equation 2.1). Growth rates were calculated based on dry weight biomass measurements, unless otherwise stated. Viable biomass was calculated by multiplying the total measured biomass with the viability as determined by flow cytometry.

$$q_i = \frac{{}^{dC_i}/_{dt} - D(C_{i,in} - C_i)}{C_{x,viable}} = \frac{r_i}{C_{x,viable}} \qquad \textit{(Equation 2.1)}$$

Microscopy

Yeast cells in chemostat and retentostat culture samples were visualized by phase contrast microscopy with a Imager-D1 microscope equipped with an AxioCam MR camera (Carl-Zeiss, Oberkochen, Germany) using an EC Plan-Neofluar 40 x/0.75 Ph 2 M27 objective (Carl-Zeiss, Oberkochen, Germany).

Transcriptome analysis

Samples for microarray analysis were taken at 2, 9, 16 and 20 days of duplicate retentostat cultivations and from 3 steady state chemostat cultures at dilution rate 0.025 h^{-1} for the IMK313 strain. These array data can be retrieved from the Genome Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) with series number GSE46853. Data for the reference strain are part of a previously described dataset (Boender *et al.*, 2011b) with GEO series number GSE22574. Sampling from cultivations, preparation and hybridization of probes to Affymetrix S98 microarrays was performed following the method of de Nicola *et al.* (De Nicola *et al.*, 2007). Affymetrix GeneChip Operating Software (v1.2) was used for data acquisition, quantification of array images and data filtering. The via Genechip Operating Software generated .CEL files for all microarrays involved, for both reference strain and IMK313 were then used for robust multichip average (RMA) normalization (Irizarry *et al.*,

2003). 6383 open reading frames for yeast were extracted from the total transcript features on the arrays (Boer *et al.*, 2003).

Robust multichip averaged (RMA) normalized data of triplicate anaerobic chemostat cultivations at dilution rate 0.025 h^{-1} of the two strains IMK313 and CEN.PK113-7D (corresponding to the starting point of retentostat cultivations), were compared using significance analysis of microarrays (SAM version 4.0) add-in to Microsoft Excel (Tusher *et al.*, 2001). Fold-change threshold was set to 2 and the expected false discovery rate was 0.45 %. The entire retentostat datasets were analyzed using empirical analysis of digital gene expression data in R (EDGE, v 1.1.291). In EDGE a time-course differential expression analysis was performed to identify genes that show a different expression in time between the two strains. Baseline levels were included. Specific growth rate affects the expression of many genes (Boender *et al.*, 2011b; Castrillo *et al.*, 2007; Regenberg *et al.*, 2006), to avoid an artificial growth rate effect, average growth rates of each strain were used as time identifier. A *p*-value threshold of 0.005, (*q*-value below 0.009) was used to discriminate genes significantly changed according to EDGE. K-means clustering in Genedata Expressionist Pro (v3.1) of the significantly changed genes was performed as described by Boender *et al.* (Boender *et al.*, 2011b).

The resulting clusters were searched for enrichments in specific annotated functional categories or transcription factor (TF) binding based on the hypergeometric distribution analysis tool described by Knijnenburg *et al.* (Knijnenburg *et al.*, 2007). In addition to these previously described functional categories based on the Munich Information Centre for Protein Sequences (MIPS) database (http://mips.gsf.de/genre/proj/yeast), KEGG pathways (http://www.genome.ad.jp/kegg/pathway.html) and Gene Ontology (GO) (http://www.geneontology.org/) and transcription factor binding genes based on Harbison *et al.* (Harbison *et al.*, 2004), a number of additional categories were searched for enrichments. These consist of a set of genes down-regulated in response to a glucose pulse (Kresnowati *et al.*, 2006), sets of genes whose expression is cell cycle phase dependent (Cho *et al.*, 1998; Spellman *et al.*, 1998; Zhao *et al.*, 2009) and genes containing binding sites of transcription factor Gis1 (the post-diauxic shift element TWAGGGAT (Boorstein and Craig, 1990; Pedruzzi *et al.*, 2000)), of transcription factors Msn2 and Msn4 (the stress responsive element AGGGG (Badis *et al.*, 2008; Martínez-Pastor *et al.*, 1996)), or transcription factor Hsf1 (Bonner *et al.*, 1994) that were selected based on web-based Regulatory Sequence Analysis Tools (http://rsat.ulb.ac.be) (van Helden *et al.*, 2000). The resulting *p*-values indicate the chance of finding the same enrichment in a random set of n genes and are calculted according to (Knijnenburg *et al.*, 2007). To validate the microarray-based transcript analysis, RT-qPCR analysis was performed on six genes that showed different transcript levels in the IMK313 strain and the reference strain in the microarray experiments. *ACT1* was also included in this analysis. Although experimental variation was higher in the qPCR analyses, relative transcript levels of the seven transcripts in the two strains were consistent for the two analytical methods (Supplementary Fig. S2.1).

Acknowledgements

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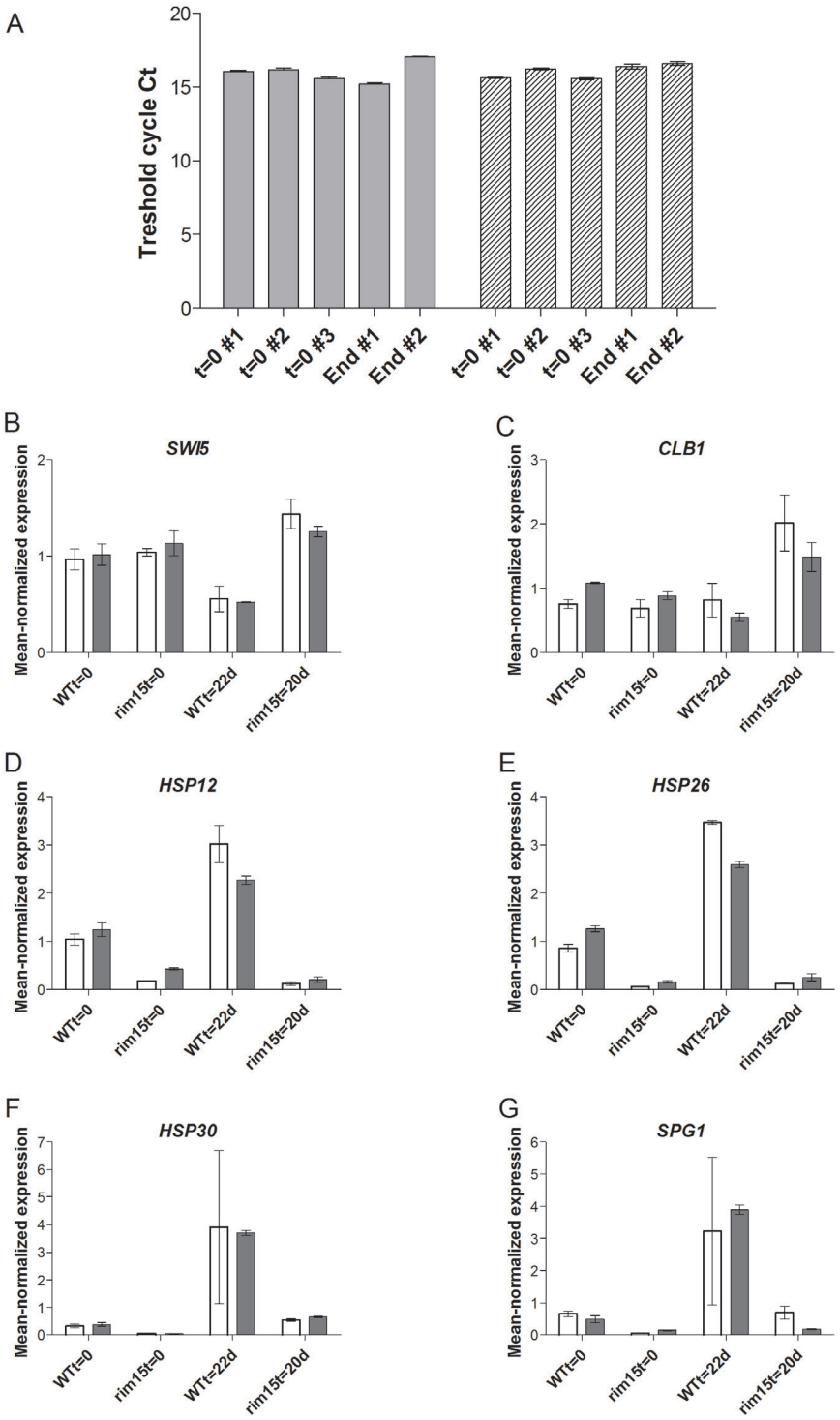
Supplementary materials Chapter 2

Supplementary materials are freely available online at the web-site of Biochimica et Biophysica Acta (BBA) - Molecular Cell Research in which the original publication appeared in Volume 1843, Issue 5, May 2014, Pages 1020–1030 (doi:10.1016/j.bbamcr.2014.01.026).

Supplementary Figure S2.1 Validation of micro-array results by RT-qPCR for 6 genes

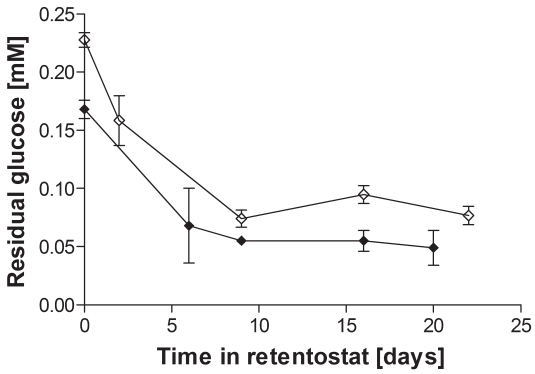
To validate the microarray-based transcript analysis, RT-qPCR analysis was performed on six genes that showed different transcript levels in the IMK313 strain and the reference strain in the microarray experiments. Genes were selected based on their expression profile and functional category. *CLB1* and *SWI5*, involved in cell cycle progression, showed a higher expression level in the *rim15* mutant while *HSP12*, *HSP26* and *HSP30*,involved in stress resistance, and the glucose repressed *SPG1*, displayed a lower expression in the mutant as compared to the parental strain.

The housekeeping gene *ACT1* was used for normalization and as control. cDNA was synthesised using 2 ug of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands) following the suppliers protocol. RT-qPCR on cDNA was performed as described previously (Beekwilder *et al.*, 2013), primers used, purchased from Sigma Aldrich (Zwijndrecht, The Netherlands), are *ACT1-FW* (TGTTACTCACGTCGTTCCAA), *ACT1-RV* (TTTCAGCAGTGGTGGAGAAA), *SWI5-FW* (AGATCGGCCATATTCCTGTG), *SWI5-RV* (CACAACCAAAGCGTCTTCTC), *CLB1-FW* (GAAACGTCCCAAGGACCATT), *CLB1-RV* (CATCGGCTCTCGAAACATCA), *HSP12-FW* (GACAAGGTCGCTGGTAAGGT), *HSP12-RV* (GCGGCTCCCATGTAATCTCT), *HSP26-FW* (CACACCCGCAAAGGATTCTA), *HSP26-RV* (AGGGAAACCGAAACCAGATG), *HSP30-FW* (ACAGGTGAAGTTCCCGGTAT), *HSP30-RV* (TATCCAAGGCGGAAATGTCG), *SPG1-FW* (AGAGGCACAAAGAGTTGTGA) and *SPG1-RV* (TACGCCGTTGATGTTGTCTA).


A
Treshold cycle Ct
20
15
10
5
0
t=0 #1
t=0 #2
t=0 #3
End #1
End #2
t=0 #1
t=0 #2
t=0 #3
End #1
End #2
B
SWI5
C
CLB1
D
HSP12
E
HSP26
F
HSP30
G
SPG1
Mean-normalized expression
WTt=0
rim15t=0
WTt=22d
rim15t=20d

(**A**): Threshold cycle numbers (+/- SEM) for house-keeping gene *ACT1* in all analysed samples of three chemostat cultures (corresponding to t=0) and final samples of duplicate retentostat cultures for the reference strain (filled bars) and the *rim15* mutant strain (dashed bars). Samples were analysed at least in duplicate.

(**B**) to (**G**): mean-normalized relative expression levels (+/- SEM) analysed by RT-qPCR (white bars, relative to *ACT1* expression) and mean-normalized expression levels analysed by Affymetrix GeneChips® (grey bars) at the start (t=0) and end (t=22d or t=20d) of reference strain (WT) and *rim15* mutant (rim15) retentostat cultures for the genes.



Supplementary Figure S2.2: Residual glucose levels of retentostat cultures

Residual glucose levels of retentostat cultures of the rim15 mutant IMK313 (closed diamonds, ◆) and the reference strain CEN.PK113-7D (open diamonds, ◇). Data are represented as the mean +/-SEM.

Supplementary Table S2.1 Primers used in construction of IMK313

Primer Name	Primer sequence
RIM15-KO_FW	*CTTGCCTCATTTGATAGAATAGATAAGCCCAGTAGAG GAAGACAGCAGCTGAAGCTTCGTACGC*
RIM15-KO_RV	*CAGTGCGTTTCATCAGAATCGCTCAATATAGTATGCT CTTCATCTGCATAGGCCACTAGTGGATCTGT*
RIM15-KO_Ctrl1	*GCTGAGCCACTTTGCCTTAC*
RIM15-KO_Ctrl2	*ACAAACCACCGTCAACAC*
RIM15-KO_Ctrl3	*TGAACGGGGGAAAATCCATG*

Supplementary Table S2.2

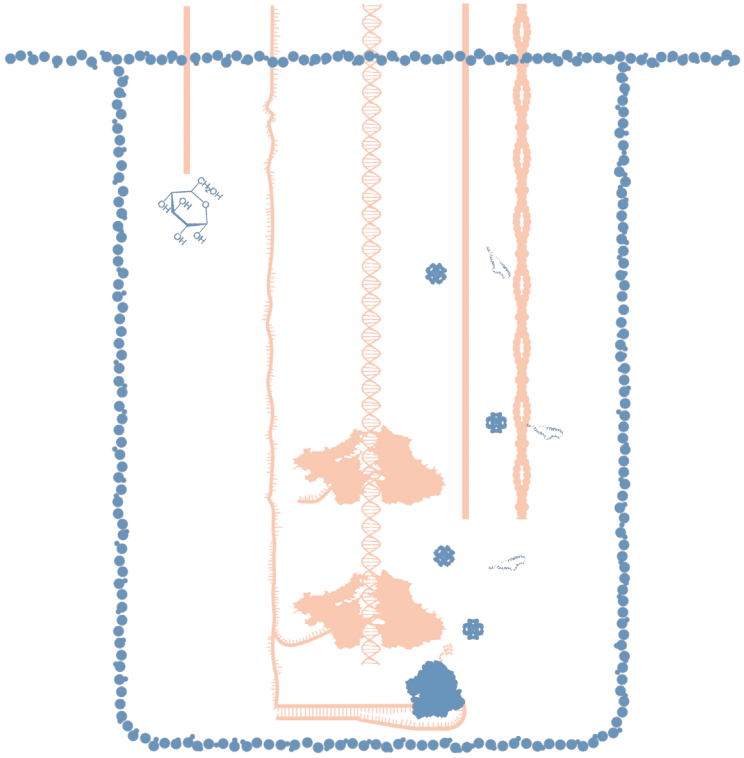
Functional enrichments found in the sets of genes whose expression is different between IMK313 and CEN.PK113-7D in anaerobic glucose limited chemostat cultivations at D = 0.025 h^{-1}. Of the in total 120 differently expressed genes (fold change threshold 2 and false discovery rate 0.5%), the expression of 91 genes was lower in IMK313, 29 genes showed higher expression. The categories shown are found in the MIPS (in bold) and Gene Ontology (in italics) databases, or show significant transcription factor binding (in standard font) or personal categories based on literature sets (in gray, see Materials and Methods).

Description	# genes in cluster	Total # genes in category	*p*-value
Enrichments among genes with lower expression in IMK313 (91 genes)			
STRE-element (Badis *et al.*, 2008; Martínez-Pastor *et al.*, 1996)	58	1786	$9.1 \cdot 10^{-13}$
PDS-element (Boorstein and Craig, 1990; Pedruzzi *et al.*, 2000)	8	162	$2.1 \cdot 10^{-3}$
Cell Rescue, Defense and Virulence	**26**	**558**	$\mathbf{3.0 \cdot 10^{-8}}$
Response to stress	*20*	*161*	$4.7 \cdot 10^{-14}$
Pentose-Phosphate shunt	*4*	*15*	$4.7 \cdot 10^{-5}$
MSN4	9	121	$5.0 \cdot 10^{-5}$
MSN2	8	122	$3.2 \cdot 10^{-4}$
Enrichments among genes with higher expression in IMK313 (29 genes)			
Respiratory electron transport chain	*6*	*8*	$1.4 \cdot 10^{-13}$
Fatty acid metabolic process	*5*	*53*	$7.6 \cdot 10^{-6}$
SPT2	3	33	$4.2 \cdot 10^{-4}$

Supplementary Table S2.3

Functional enrichments found in the sets of genes whose expression is different between IMK313 and CEN.PK113-7D in anaerobic glucose limited retentostat cultivations using specific glucose uptake rates as key identifiers. Of the in total 435 differently expressed genes (*p*-value below 0.005), the expression of 202 genes was lower in IMK313, 233 genes showed higher expression (based on K-means clustering into 2 clusters). The categories shown are found in the MIPS (in bold) and Gene Ontology (in italics) databases, or show significant transcription factor binding (in standard font) or personal categories based on literature sets (in gray, see Materials and Methods).

Description	# genes in cluster	Total # genes in category	*p*-value
Enrichments among genes with **lower** expression in IMK313 (202 genes)			
Hsf1 (Bonner *et al.*, 1994)	47	435	$2.2 \cdot 10^{-14}$
Glucose repressed genes (Kresnowati *et al.*, 2006)	66	565	$2.0 \cdot 10^{-22}$
STRE-element (Badis *et al.*, 2008; Martínez-Pastor *et al.*, 1996)	106	1786	$7.7 \cdot 10^{-14}$
PDS-element (Boorstein and Craig, 1990; Pedruzzi *et al.*, 2000)	14	162	$5.7 \cdot 10^{-4}$
Stress response	**28**	**453**	$\mathbf{4.3 \cdot 10^{-4}}$
Response to stress	*16*	*161*	$4.4 \cdot 10^{-5}$
Pentose-Phosphate shunt	*5*	*15*	$7.0 \cdot 10^{-5}$
MSN2	15	122	$5.9 \cdot 10^{-6}$
MSN4	13	121	$1.1 \cdot 10^{-5}$
Enrichments among genes with **higher** expression in IMK313 (233 genes)			
G2/M Boundary (Spellman *et al.*, 1998)	17	189	$5.0 \cdot 10^{-4}$
Late G1 phase (Cho *et al.*, 1998)	12	132	$3.0 \cdot 10^{-3}$
G1 phase (Spellman *et al.*, 1998)	19	285	$7.8 \cdot 10^{-3}$
G2 Phase (Cho *et al.*, 1998)	6	51	$1.0 \cdot 10^{-2}$
Fatty acid beta-oxidation	*4*	*9*	$1.9 \cdot 10^{-4}$
SWI4	19	144	$9.7 \cdot 10^{-7}$
NDD1	14	109	$3.7 \cdot 10^{-5}$
MBP1	17	165	$9.7 \cdot 10^{-5}$
MCM1	11	78	$1.1 \cdot 10^{-4}$



3. Proteome adaptation of *Saccharomyces cerevisiae* to severe calorie restriction in retentostat cultures

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Stationary-phase, carbon-starved shake-flask cultures of *Saccharomyces cerevisiae* are popular models for studying eukaryotic chronological aging. However, their nutrient-starved physiological status differs substantially from that of post-mitotic metazoan cells. Retentostat cultures offer an attractive alternative model system in which yeast cells, maintained under continuous calorie restriction, hardly divide but retain high metabolic activity and viability for prolonged periods of time. Using TMT labeling and UHPLC-MS/MS, the present study explores the proteome of yeast cultures during transition from exponential growth to near-zero growth in severely calorie-restricted retentostats. This transition elicited protein level changes in 20% of the yeast proteome. Increased abundance of heat-shock-related proteins correlated with increased transcript levels of the corresponding genes and was consistent with a strongly increased heat-shock tolerance of retentostat-grown cells. A sizeable fraction (43%) of the proteins with increased abundance under calorie restriction was involved in oxidative phosphorylation and in various mitochondrial functions that, under the anaerobic, non-growing conditions used, have a very limited role. Although it may seem surprising that yeast cells confronted with severe calorie restriction invest in the synthesis of proteins that, under those conditions, do not contribute to fitness, these responses may confer metabolic flexibility and thereby a selective advantage in fluctuating natural habitats.

Introduction

Over the past decade, the budding yeast *Saccharomyces cerevisiae* has emerged as an attractive model system to study lifespan and the mechanisms of aging (Mirisola *et al.*, 2014). This popularity can be explained by the similarity of its cell biology to that of higher eukaryotes, its tremendous genetic accessibility, and its simple laboratory cultivation techniques. This model function has been consolidated by the observation that calorie restriction and inactivation of Sch9 and Tor1, known to extend lifespan in higher eukaryotes, also increase yeast chronological life span (Fontana *et al.*, 2010). Yeast chronological life span (CLS), defined as the length of time that a non-dividing cell survives, is typically measured in stationary phase (SP) shake-flask cultures in which cells, depleted of carbon and energy source (typically glucose) after a period of fast growth, have ceased to divide (Hu *et al.*, 2013).

Although the similarity of cellular processes related to aging and longevity in yeast and higher eukaryotes support the potential of *S. cerevisiae* as model system (Longo *et al.*, 2012; Mirisola *et al.*, 2014), the use of SP cultures as models for longevity studies is debatable. Indeed, the physiology of SP cultures differs from aging metazoan cells in several important aspects. The first obvious difference is that the absence of carbon and energy sources in SP cultures inevitably causes loss of metabolic activity, cellular deterioration, and, ultimately, cell death. In SP yeast cultures, these processes occur over a period of days to weeks (Allen *et al.*, 2006). In contrast, post-mitotic metazoan cells are continuously supplied with nutrients. Even in a quiescent, non-dividing state, this nutrient availability enables them to retain metabolic activity and viability for prolonged periods of time, often even for decades. Second, before entering SP, *S. cerevisiae* undergoes major metabolic changes during respiro-fermentative and post-diauxic respiratory growth phases, which are typically not experienced by terminally differentiated metazoan cells.

Although widely used for yeast aging studies, growth and starvation in shake-flasks lead to strong variations in environmental factors such as culture pH, sugar concentration, oxygen availability and product toxicity, which combine to affect yeast life span and obscure data interpretation (Burtner *et al.*, 2011). While an increasing number of reports acknowledge the limitations of starving cultures as models for post-mitotic metazoan cells (Breitenbach *et al.*, 2012c; Mirisola *et al.*, 2014), few attempts have been made to explore alternative experimental approaches. This is not surprising, as it represents a considerable technical challenge to grow yeasts that do not, or hardly, divide but retain metabolic activity. To address this challenge, we have recently implemented retentostat cultivation in our research on the physiology of *S. cerevisiae* at near-zero growth rates (Boender *et al.*, 2009).

A retentostat is a continuous cultivation setup in which cells are retained in a bioreactor by a filter device in the effluent line. When a retentostat is fed with a constant flow of fresh medium that contains a growth-limiting amount of the energy substrate, cells will divide and accumulate in the culture vessel until the glucose supply per cell becomes too low to sustain

additional cell divisions (Boender *et al.*, 2009; Ercan *et al.*, 2013; Van Verseveld *et al.*, 1986). Upon prolonged retentostat cultivation, cells will therefore experience extreme calorie restriction and, eventually, stop growing and invest the limited glucose supply exclusively in maintenance processes required for survival (Stephanopoulos *et al.*, 1998b).

The essentially non-growing, metabolically active physiological state of yeast cells in retentostat cultures appears to be a much closer approximation of the physiological state of mitotically arrested long-lived metazoan cells, such as neurons and cardiac myocytes, than carbon-source starvation of yeast cells in SP shake-flask cultures (Terman *et al.*, 2009). While retentostat cultures of *S. cerevisiae* exhibited features reminiscent of SP cultures, such as enhanced resistance to heat stress and accumulation of carbon and energy reserves (lipids and carbohydrates), they also revealed marked differences with starved cultures (Boender *et al.*, 2011a; Boender *et al.*, 2011b; Gray *et al.*, 2004; Werner-Washburne *et al.*, 1993). When compared with actively growing yeast cultures (generation time of 40 h in anaerobic carbon-limited chemostats), the transcriptome of yeast cultivated in retentostat with generation times of ca. 48 days exhibited a pronounced transcriptional reprogramming, including a down-regulation of most growth-related processes (anabolic pathways, protein synthesis), and up-regulation of many genes involved in stress resistance (Boender *et al.*, 2011b). Although some transcriptional changes were visibly reflected in cell physiology (e.g., increased heat shock resistance), it is unclear to what extent this large transcriptional response observed during the transition from a dividing to a non-dividing state is mirrored at the proteome level.

Hitherto, four studies report the dynamics of protein levels during SP in *S. cerevisiae* (Davidson *et al.*, 2011; Fuge *et al.*, 1994; Webb *et al.*, 2013; Zakrajšek *et al.*, 2011). The most comprehensive yeast proteome study identified changes in levels of 842 out of 2663 quantified proteins (i.e., 31 %) when comparing quiescent and proliferating cells (Webb *et al.*, 2013). A flow-cytometry based approach using a yeast GFP-fusion library revealed a considerably smaller response, with the level of only 5 % of the 4159 identified proteins changed between exponential growth and stationary phase (Davidson *et al.*, 2011). Other previous proteomics studies on SP in *S. cerevisiae* were based on 2D gels, analyzing a restricted number of proteins (Fuge *et al.*, 1994; Zakrajšek *et al.*, 2011). A single study has addressed rates of protein synthesis and experimentally demonstrated that the overall rate of protein synthesis declined strongly upon entry into stationary phase in *S. cerevisiae* (Fuge *et al.*, 1994).

Up-regulation of hundreds of genes suggests that, despite severe calorie restriction, yeast cells grown in retentostats may invest many of their scarce resources in the synthesis of proteins, a substantial number of them arguably dispensable for survival (Boender *et al.*, 2011b). Such an apparent waste of resources may be prevented through down-regulation of protein synthesis, as suggested by proteome studies during SP. The present work, for the first time, explores the proteome of *S. cerevisiae* grown under severe calorie restriction and

investigates whether the large, previously described transcriptional reprogramming triggered by exposure to calorie restriction is accompanied by similar changes at the proteome level. Additionally, as retentostat cultures share some similar transcriptional and physiological responses with SP cultures, the present study investigates whether these resemblances extend to the proteome level.

Results

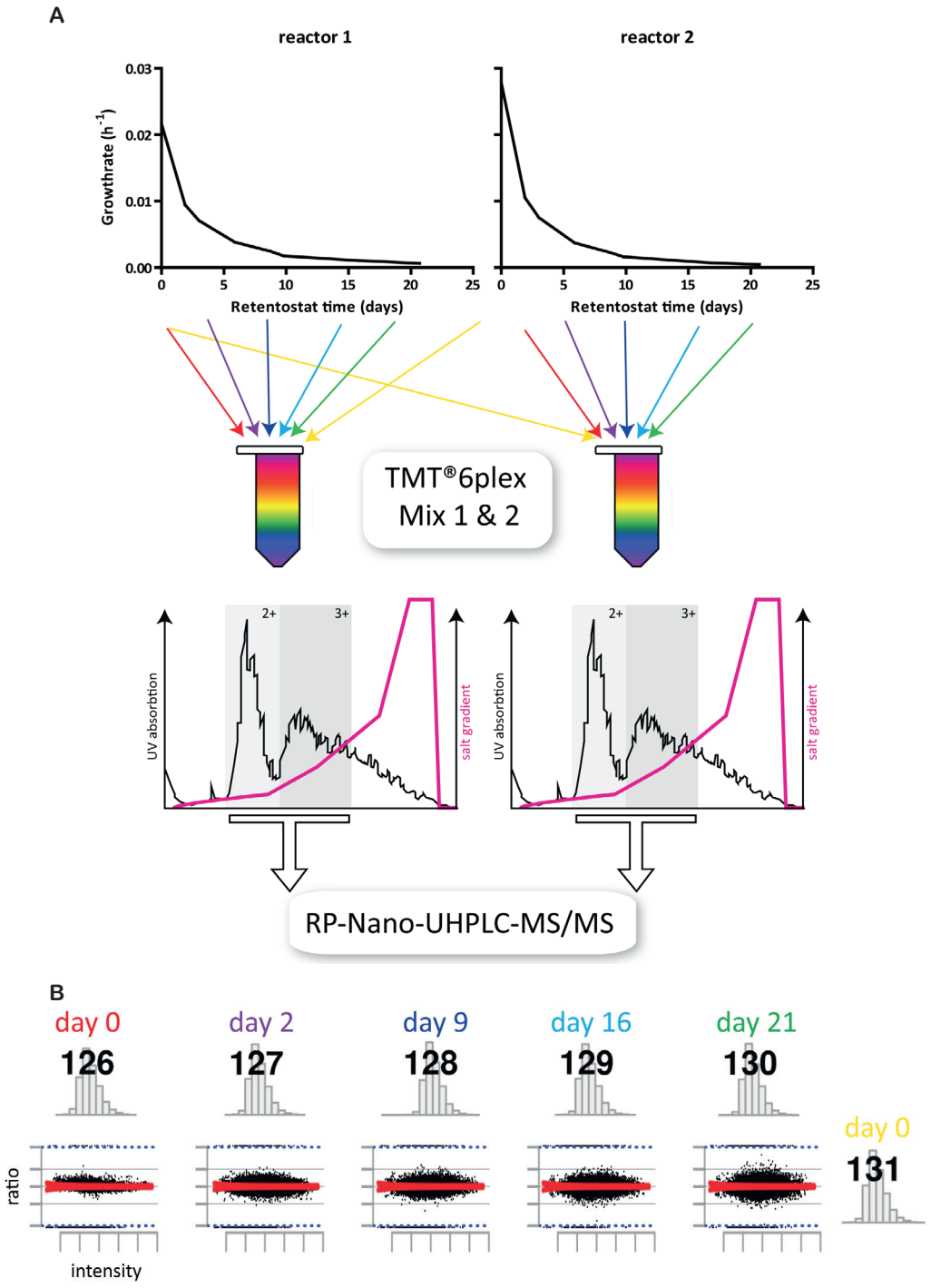
Levels of a quarter of the quantified proteins are changed as cells enter a non-dividing, calorie-restricted state

Retentostat cultures were started from slowly, but exponentially, growing cultures (the specific growth rate (μ) at t_0 was 0.025 h^{-1}). Biomass retention coupled to constant supply of a medium with glucose as the growth-limiting nutrient led to a rapid decrease of the specific growth rate during the first 9 days of cultivation, followed by stabilization of the specific growth rate at 0.0006 h^{-1} (Fig. 3.1A). At this stage, the average doubling time of the yeast cells in the retentostats was ca. 48 days. Although cells hardly divided, cultures retained a high viability and remained metabolically active due to the continuous, albeit severely limited, glucose supply (Boender *et al.*, 2009).

To investigate how the yeast proteome changed under increasing calorie restriction, relative protein levels were measured during the course of duplicate anaerobic retentostat cultures. Five samples from each retentostat culture, taken at the start of cell retention (t_0) and after 2, 9, 16, and 21 days, combined with the t_0 sample from the duplicate culture were, after digestion, labeled with the TMT 6-plex kit. These two mixes were then fractionated by SCX and analyzed using RPLC-MS/MS.

Analysis of the two mixes from duplicate retentostat cultures resulted in a total of 3813 detected protein groups, which corresponded to approximately 70 % of the *S. cerevisiae* proteome (according to MIPS, http://mips.helmholtz-muenchen.de/genre/proj/yeast) (Supplementary Tables S3.1 & S3.2). Compression of the calculated ratios from the reporter ion intensities due to co-isolation of precursor ions has been an issue in isobaric labeling experiments (Ting *et al.*, 2011). For our dataset, average PIF-scores of around 0.8 for each of the biological replicates demonstrated that co-isolation was within an acceptable range (Michalski *et al.*, 2011b) (Supplementary Table S3.3).

Integration of the data of the two culture replicates resulted in a robust set of 2435 protein groups, encompassing all cellular compartments, including membranes and nucleus, that were quantified at all five time points in both biological replicates (Supplementary Fig. S3.1A,B). To identify the proteins with altered relative levels during calorie restriction, ratios of the different time points to t_0 were calculated, and the data set was then submitted to statistical analysis. The global protein levels were increasingly diverging as severe calorie restriction was attained, with a maximum spread after 21 days (Fig. 3.1B). Comparison of replicate experiments at t_0 revealed high biological and analytic reproducibility. This


A
reactor 1
reactor 2
Growthrate (h-1)
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RP-Nano-UHPLC-MS/MS
B
day 0
126
day 2
127
day 9
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day 0
131
ratio
intensity

Figure 3.1 Experimental setup and quality of the data. (<)
(**A**) Schematic overview of the experimental setup, showing the time points of 0, 2, 9, 16, and 21 days of retentostat culture chosen for sampling cells for proteome analysis under severe calorie restriction. The growth curves of both biological replicates display the calculated specific growth rate at the sampling time points, indicating near-zero growth at days 16 and 21 of the retentostat cultures. Samples from each reactor were labeled with six different TMT labels and then combined to create mixes 1 and 2. Each peptide mix was fractionated with SCX and the fractions were analyzed with LC-MS/MS. (**B**) The signal intensity versus protein ratio plots from the isobar software output show the deviation of protein expression level of each sample compared with levels at day 0.

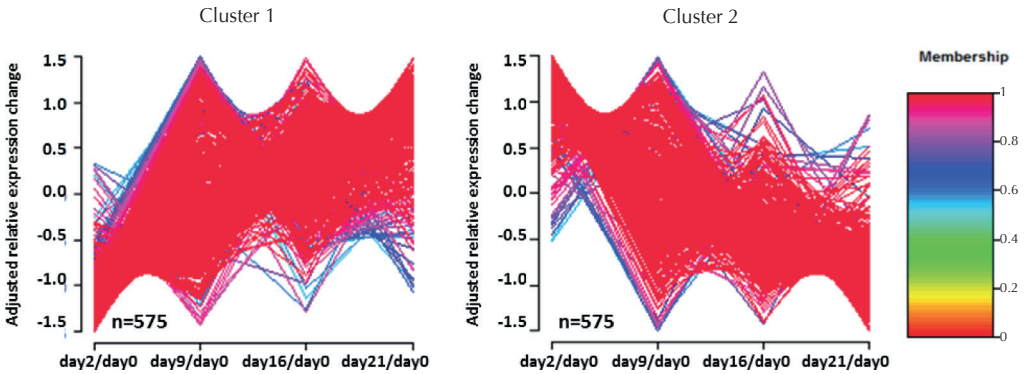

Figure 3.2 Clustering of the 575 changed proteins.
The 575 proteins changed in each of the replicate experiments (input = 1150 profiles) were clustered and 504 proteins showed similar trends in both experiments. These 504 proteins could be divided into a robust and consistent set of 252 proteins up-regulated in time in both experiments (**cluster 1**) and 252 proteins down-regulated in time (**cluster 2**).

Table 3.1 Functional enrichment analysis of proteins with increased abundance under severe calorie-restriction.*

Category	Number of proteins	% of total proteins in category	Benjamini-Hochberg corrected *p*-value
Mitochondrion (GO:0005739)	110	17	$1.6 \cdot 10^{-9}$
Oxidation reduction (GO:0055114)	53	21	$1.1 \cdot 10^{-4}$
Response to temperature stimulus (GO:0009266)	26	21	$4.1 \cdot 10^{-2}$

*Enrichment analysis of cluster 1, Fig. 3.2 was performed using DAVID software. Shown are the largest specific functional GO categories found enriched; additional and sub-categories can be found in supplementary table S3.4. For each enriched category the number of proteins belonging to that category is indicated as well as the fraction that these proteins represent compared to the total number of proteins in the respective category. The Benjamini-Hochberg corrected *p*-value is used as a measure of the statistical significance of the enrichment; a *p*-value of 0.05 was used as the threshold.

deviation between t_0 samples was used to calculate the background and significant changes in protein levels. Statistical analysis identified 575 proteins for which the abundance changed significantly in both replicates at one or more time points (*p*-value below 0.05), which corresponds to 24 % of the proteins quantified in both replicate cultures. Clustering of these 575 proteins in two broad groups according to their profiles, i.e., increased or decreased abundance in time, resulted in a set of 504 proteins that showed consistently increased or decreased levels in both replicate experiments. Remarkably, the level of half of these proteins (252 proteins) increased under severe calorie restriction (Fig. 3.2).

Increased abundance of proteins involved in mitochondrial processes under anaerobic calorie restriction

Functional enrichment analysis of the 252 proteins that show increased abundance in retentostats compared to their level in the slow growing chemostats revealed the over-representation of several functional categories (Table 3.1 and Supplementary Table S3.4). The most strongly over-represented were mitochondrial proteins and proteins involved in mitochondrial processes (*p*-value $1.6 \cdot 10^{-9}$), including mitochondrial ribosomal proteins (*p*-value $7.7 \cdot 10^{-4}$), and in respiration (*p*-value $1.1 \cdot 10^{-4}$).

These increased levels of proteins involved in respiratory catabolism were remarkable, as retentostats were operated under strictly anaerobic conditions. Of the 39 enzymes that are part of the TCA cycle and its carbon-supplementing reactions, 37 were quantified in at least one of the duplicate experiments, and 12 (31 %) were found up-regulated in both experiments (Fig. 3.3). The up-regulated proteins belong to the oxidative branch of the TCA-cycle, the glyoxylate cycle, and anaplerotic reactions (Fig. 3.3). Remarkably, five out of the six proteins involved in the succinate dehydrogenase (SDH) complex (Sdh1, Sdh2, Sdh3 and their paralogs Sdh1b, alias YJL045W (Colby *et al.*, 1998), and Shh3 (Szeto *et al.*, 2012)) were consistently up-regulated in response to calorie restriction, whereas the SDH complex is supposed to be inactive under anaerobic conditions (Camarasa *et al.*, 2003). Furthermore, at least two proteins of each multi-protein complex in the mitochondrial respiratory chain were consistently up-regulated under anaerobic severe calorie restriction (Fig. 3.3). Proteins of other mitochondrial complexes showed similarly increased levels under calorie restriction, including mitochondrial ribosomes (19 proteins, Supplementary Table S3.4). Out of the 18 proteins encoded by the mitochondrial genome, three were identified, and a single one (Cox1) was consistently up-regulated in both culture replicates. The vast majority of mitochondrial proteins are encoded by nuclear DNA and require import into the mitochondria from the cytosol. Although 33 proteins involved in mitochondrial protein trafficking (Gabriel and Pfanner, 2007) were quantified, no consistent co-regulation of these proteins was found under severe calorie restriction (three proteins showed increased levels, and four proteins showed decreased levels).

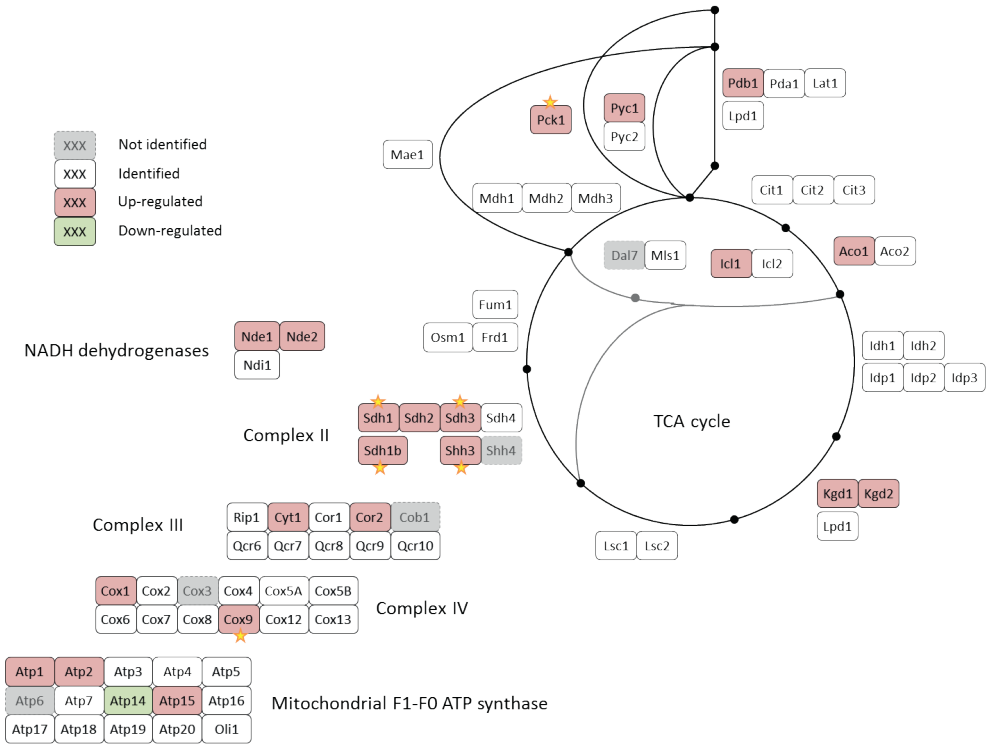

Figure 3.3 Several proteins in the respiratory metabolism are up-regulated under calorie restriction. A schematic overview shows the proteins involved in the TCA cycle, including anaplerotic routes and the complexes of the oxidative phosphorylation. Non-detected proteins are shown in gray; all other proteins were detected in at least one duplicate experiment. Red boxes indicate up-regulated proteins and green (Atp14), down-regulated. Proteins, whose corresponding transcript levels also changed significantly under calorie restriction are indicated with a star.

Strong up-regulation of Hsp30 is not essential for cell fitness and survival of calorie-restricted retentostat cultures

Proteins involved in response to temperature stimulus were enriched among the set of proteins that showed increased levels during retentostat cultivation (Table 3.1). The set of proteins with increased abundance in the severely calorie-restricted cultures encompassed nine proteins involved in heat shock responses and resistance (Fig. 3.4 and Supplementary Table S3.5). This increased abundance is consistent with the increased robustness of retentostat cultures challenged by heat shock (Boender *et al.*, 2011a).

Although most heat shock proteins showed a moderate, progressive increase during retentostat cultivation, levels of Hsp30 increased dramatically when extreme calorie restriction set in after 9 days of retentostat cultivation (Fig. 3.4). After 21 days of retentostat cultivation, Hsp30 levels had increased by 5-fold. Under the same conditions, *HSP30* transcript levels increased by ca. 10-fold (Boender *et al.*, 2011b). Expression of *HSP30* is induced in response to various stress conditions, including glucose exhaustion and Hsp30

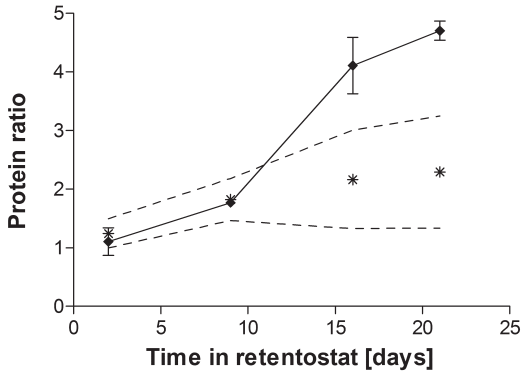

Figure 3.4 Up-regulation of heat shock proteins (HSP) in retentostat cultures
Asterisks (∗) indicate the average increase (dashed lines show standard deviation) in protein ratio of up-regulated HSP proteins: Hsp12, Hsp26, Hsp30, Hsp78, Ssa3, Ssc1, Sis1, and Ssq1. Diamonds (◆) show the strong increase in ratio of Hsp30 levels (averages of duplicate experiments +/- SEM).

has been shown to down-regulate activity of the plasma membrane ATPase Pma1 (Piper *et al.*, 1997). As such, hsp30 is assumed to be involved in energy conservation under prolonged stress or glucose shortage by limiting excessive ATP consumption by Pma1, which is responsible for substantial fraction of the ATP consumption for ion homeostasis (Piper *et al.*, 1997). To test whether this proposed role of Hsp30 had a substantial impact during extreme calorie restriction, the phenotype of the congenic *hsp30* deletion strain *S. cerevisiae* IMK381 was investigated in retentostat cultures. If, under these conditions, Hsp30 would prevent ATP "spillage" by the plasma membrane ATPase, its absence was anticipated to lead to an increase in the biomass-specific glucose uptake rate and/or to an increased loss of viability. However, after 21 days of retentostat cultivation, neither specific glucose uptake nor viability differed from those of the reference strain (Supplementary Fig. S3.2).

Decreased abundance of Like-Sm proteins, involved in mRNA processing, in retentostat cultures

Functional enrichment analysis did not reveal any enriched categories among the 252 proteins that showed reduced abundance in the retentostat cultures. However, close scrutiny of this subset revealed several interesting proteins. For instance, four out of 17 quantified proteins involved in ergosterol biosynthesis were among this set, whereas another four proteins involved in this process, although they did not pass the stringent statistical criteria, also displayed a slight but consistent decrease in abundance in the two experimental replicates (Supplementary Table S3.6).

Of the 14 quantified proteins that form the Like-Sm ribonucleoprotein core, 8 showed a decreased abundance in the retentostats, including the Like-Sm proteins Lsm1, Lsm2, Lsm3, Lsm4, Lsm5, and Lsm7. The Like-Sm proteins are involved in activating mRNA decapping (He and Parker, 2000). Interestingly, abundance of the mRNA decapping enzyme Dcp1, known to interact with the Like-Sm proteins (Parker and Sheth, 2007), was also decreased under severe calorie restriction.

Changes in proteome are only partially reflected at the transcript level

A previous genome-wide gene expression study on anaerobic retentostat cultures of the same yeast strain, grown under identical conditions, revealed a strong impact of severe calorie restriction on the transcriptome of *S. cerevisiae*. About 1400 genes were differentially expressed in retentostat cultures (*p*-value below 0.005) of which 629 genes showed down-regulation and 757 genes up-regulation relative to exponentially (slow) growing cells (Boender *et al.*, 2011b).

Among the genes that showed increased transcript levels in retentostat cultures, genes involved in respiration, mitochondrial translation and organization were overrepresented, as well as targets of the transcription factor Hsf1 involved in heat shock response. These categories were also overrepresented among the proteins with increased abundance during severe calorie restriction, showing that some cellular functions were regulated similarly at the transcript and protein level. Transcription of Hsf1 targets and many genes involved in stress response is known to be growth-rate dependent (Boender *et al.*, 2011b; Brauer *et al.*, 2008; Regenberg *et al.*, 2006) and most probably responded to the decreasing growth rate in retentostat. Conversely, transcript levels of genes involved in respiration and other

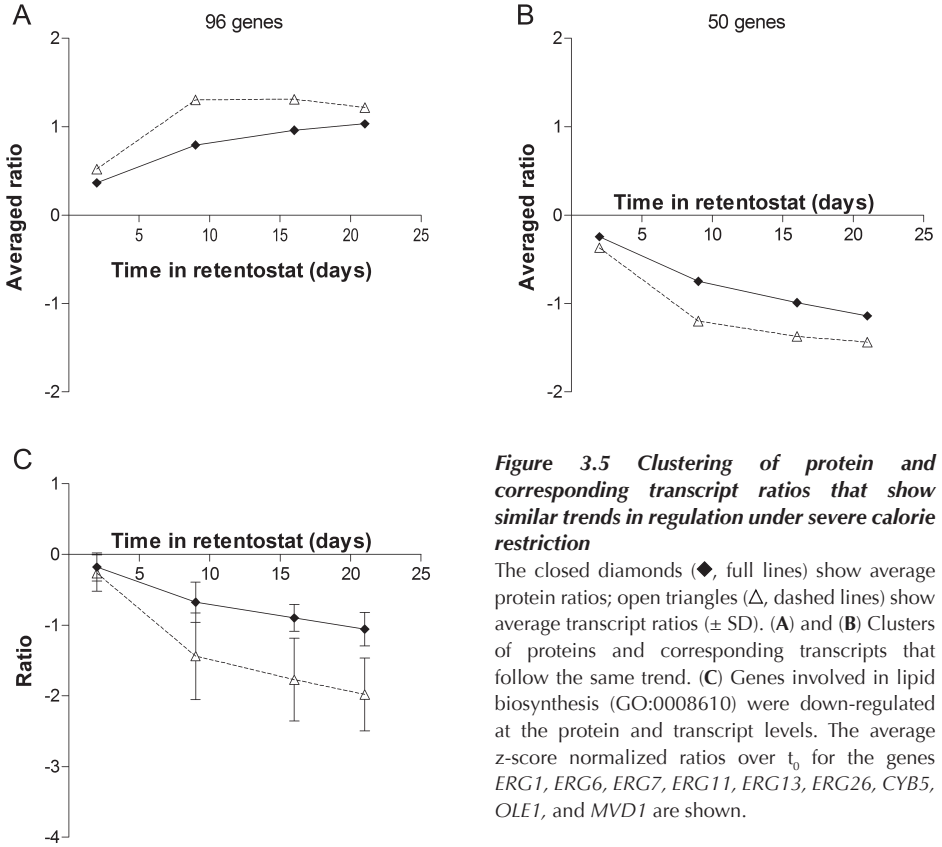

Figure 3.5 Clustering of protein and corresponding transcript ratios that show similar trends in regulation under severe calorie restriction

The closed diamonds (◆, full lines) show average protein ratios; open triangles (△, dashed lines) show average transcript ratios (± SD). (**A**) and (**B**) Clusters of proteins and corresponding transcripts that follow the same trend. (**C**) Genes involved in lipid biosynthesis (GO:0008610) were down-regulated at the protein and transcript levels. The average z-score normalized ratios over t_0 for the genes *ERG1, ERG6, ERG7, ERG11, ERG13, ERG26, CYB5, OLE1,* and *MVD1* are shown.

mitochondria-related functions were not growth-rate dependent for growth rates above 0.025 h^{-1} (the initial growth rate in the retentostats) and only occurred during retentostat cultivation (Boender *et al.*, 2011b). Severe calorie restriction led to a strong transcriptional down-regulation of hundreds of genes involved in growth-related processes, such as the biosynthesis of building blocks and translation. Although this transcriptional response appeared to be in good agreement with the decreased demand for biosynthetic processes in non-growing cells, this response was only partially observed at the protein level, indicating an uncoupling of transcript and protein regulation.

A statistical comparison of the entire transcriptome and proteome datasets revealed that correlations between changes of protein levels and of the corresponding transcript levels, for each analyzed time point, were low (Pearson correlations ranging from 0.2 - 0.3; Supplementary Fig. S3.3). Clustering the 575 proteins that showed a different abundance in exponentially growing and calorie restricted cultures and their corresponding transcripts based on expression profiles identified a set of 146 proteins whose abundances correlated well with their transcript levels during severe calorie restriction (Fig. 3.5A,B and Supplementary Fig. S3.4A). Among these, 96 genes consistently displayed higher transcript and protein levels during retentostat cultivation (Fig. 3.5A), corresponding to 38 % of the proteins that were present at increased levels in the retentostats, while 50 genes were expressed at a reduced level in the retentostats, corresponding to 20 % of the 252 proteins with decreased abundance (Fig. 3.5B).

Enrichment analysis showed that approximately one-fourth of the mitochondrial proteins (28 out of 110) and proteins involved in oxidative phosphorylation (11 out of 53) were regulated similarly at transcript and protein level. Proteins involved in heat-shock resistance suggested a stronger regulation at the transcriptional level, with 12 out of 26 proteins (46 %) showing a good correlation between changes in transcript and protein levels. Among the 50 genes with consistently decreased transcript and protein levels, loci involved in sterol and lipid synthesis were overrepresented, suggesting that this response occurs predominantly at the transcript level, as the transcript levels of the 4 genes with decreased protein levels also decreased (Fig. 3.5C). This finding is in good agreement with the expected down regulation of anabolic pathways upon severe calorie restriction.

Discussion

Proteome analysis indicates an unexpected use of resources in *S. cerevisiae* subjected to severe anaerobic calorie restriction

The present study represents the first proteome analysis of *S. cerevisiae* cultures grown under defined, severely calorie-restricted and essentially non-growing conditions. A representative sampling of the *S. cerevisiae* proteome revealed differential abundance of a sizable fraction of the yeast proteome (575 proteins, 24 % of the consistently identified proteins) in response to severe calorie restriction. Changes in the yeast proteome under these conditions are not surprising, as the retentostat cultivation protocol involved a 40-fold

reduction of the specific growth rate relative to the exponentially growing reference cultures. However, the observation that half of these proteins were present at increased levels during retentostat cultivation was unexpected. Under the severely calorie-restricted conditions in the retentostats, cell division comes to a virtual standstill, and the translation machinery as well as many biosynthetic processes operate far below maximum capacity. Moreover, protein synthesis is the single most ATP-intensive cellular process (19.6 mmol ATP · g yeast biomass^{-1} (Verduyn *et al.*, 1990a)), and abundances of many proteins involved in biosynthetic processes show a positive correlation with specific growth rate (Castrillo *et al.*, 2007), presumably to prevent allocation of resources to synthesis of proteins that do not contribute to fitness. Still in the present comparison with exponentially growing (μ = 0.025 h^{-1}) cultures, only 12 % of the analyzed proteins showed a reduced level in the virtually non-growing retentostat cultures.

Glycolytic proteins can account for up to 20 % of the soluble yeast proteome (Daran-Lapujade *et al.*, 2007). Even at moderate specific growth rates, this high-level expression of glycolytic proteins represents a significant energetic burden, as demonstrated by the observation that glycolytic overcapacity is rapidly lost due to mutation and selection upon prolonged glucose-limited cultivation in chemostats (Jansen *et al.*, 2005). However, glycolytic and other growth-related proteins were among the 76 % of the identified proteins whose levels remained stable under calorie restriction.

Of the 252 proteins that showed higher levels under calorie restriction, few are, according to current knowledge, essential for survival. Indeed, 110 of these proteins were mitochondrial, whereas, under the anaerobic, non-growing conditions used in the retentostats, respiration does not occur and mitochondria only have a limited role in biosynthesis. Proteins involved in heat-shock responses were clearly over-represented among the proteins that showed an increased level under severe calorie restriction. Among this functionally diverse group, Hsp30 was previously proposed to play a key role in energy conservation by preventing ATP-dissipation via the plasma membrane ATPase (Piper *et al.*, 1997). However, the lack of impact on fitness and survival of a *hsp30* mutant in retentostat cultures indicated that this role of Hsp30 was not essential for survival under calorie-restricted conditions.

To limit energy expenditure during extreme calorie restriction, cells might preferentially synthesize "cheaper" proteins of short lengths (for instance, Hsp26 and Hsp12, induced under carbon starvation, are short proteins of 26 and 12 KDa, respectively) or proteins with slow turnover. However, short proteins or proteins with a long turnover time (Helbig *et al.*, 2011) were not over-represented among the proteins that were present at elevated levels in retentostat cultures.

From an anthropomorphic viewpoint, it may seem surprising that yeast cells confronted with severe calorie restriction invest ATP and precursors in the synthesis of proteins that, under those conditions, do not contribute to fitness and may even represent an energetic burden. However, from an evolutionary perspective, some of these responses may confer a selective advantage by enabling fast adaptation and accelerating of growth when the nutrient limitation is relieved. Such an environmental 'preparedness' has been well described in

bacteria. In oligotrophic environments, *Escherichia coli* and other heterotrophic bacteria express a wide diversity of transporters and catabolic enzymes that do not contribute to fitness at the moment of induction but that increase metabolic flexibility (Egli, 2010; Ihssen and Egli, 2005). Bacteria and yeast can also display Pavlovian conditioning. For instance, in *E. coli*, transcriptional responses to temperature and oxygen perturbations are coupled, thereby mimicking the covariation in temperature and oxygen availability upon entry into the mammalian digestive tract (Mitchell *et al.*, 2009; Tagkopoulos *et al.*, 2008). These examples demonstrate that the production of proteins seemingly useless under well-defined and simplified laboratory conditions may confer an evolutionary advantage in fluctuating natural habitats.

Increased mitochondrial proteins under anaerobic conditions, not merely a response to reduced glucose availability?

The increased abundance of over a hundred mitochondrial proteins involved in a wide variety of mitochondrial processes, encompassing ribosome biogenesis, protein synthesis and modification, iron-sulfur cluster assembly and oxidative phosphorylation, represented a puzzling observation. It is generally accepted that the role of mitochondria in the absence of oxygen is restricted to a few anabolic reactions (e.g., amino acid synthesis and sterol uptake (Reiner *et al.*, 2006; Schatz, 1995). The very low demand for biosynthetic products in non-dividing cells further reduces the role of mitochondria under anaerobic calorie restriction. Following the line of reasoning in the previous paragraph, investing scarce resources in the mitochondrial respiration machinery, despite the absence of oxygen, might enable yeast cells to immediately start respiration when oxygen becomes available. In this respect it should be noted that, even at the relatively low efficiency of oxidative phosphorylation in *S. cerevisiae*, its ATP yield from respiratory sugar dissimilation is at least 8-fold higher than that from alcoholic fermentation.

Regulation of the synthesis of many mitochondrial proteins in *S. cerevisiae*, and especially proteins involved in oxidative phosphorylation, responds to two major environmental cues: oxygen and glucose (Lascaris *et al.*, 2002). In the absence of oxygen, remodeling of the mitochondrial proteome may therefore result from variations in the glucose concentration. Indeed, the residual glucose concentration slightly decreased in the first 9 days of retentostat cultivation and became constant during the final 12 days of the experiments (Boender *et al.*, 2011b). Of the 110 mitochondrial proteins whose expression increased during retentostat cultivation, 33 displayed an abundance profile that followed the residual glucose concentration. However, 77 others showed a continuous increase in abundance during the whole retentostat experiment. Furthermore, comparison with sets of proteins whose abundance is known to be regulated by the concentration of glucose (Giardina *et al.*, 2012; Kolkman *et al.*, 2006) revealed an overlap of only a few proteins. These findings indicate that the regulation of mitochondrial biogenesis in anaerobic, calorie-restricted cultures and its possible contribution to fitness in the absence of oxygen requires further study. Such further

work may reveal whether the key role of mitochondria in chronological aging under aerobic conditions, which has been mainly attributed to the formation of reactive oxygen species (Breitenbach *et al.*, 2012b; Pan, 2011), involves other functions of these organelles that do not depend on respiratory activity.

Limited transcriptional regulation of protein levels under calorie restriction

The limited overlap between the sets of genes changed at protein or transcript level (27 %) indicated that regulation at the transcript and protein level are largely uncoupled under severe calorie restriction. While a similar poor correlation between levels has been previously reported during stationary phase in *S. cerevisiae* and *Schizosaccharomyces pombe* (Fuge *et al.*, 1994; Marguerat *et al.*, 2012), it was particularly marked during severe calorie restriction and can be explained by different factors. One likely explanation lies in the different time constants for transcript and protein levels. In general protein half-lives are between 0.5 and over 20 h (Helbig *et al.*, 2011) and are significantly longer than mRNA half-lives that typically range between 3 min to 8 h (Wang *et al.*, 2002). This difference may be exacerbated in cells with limited resources already in growing cells considering the large difference in energy requirements of mRNA and protein synthesis (201 and 1957 mmol ATP · 100 g formed biomass^{-1} respectively) (Verduyn *et al.*, 1990a). Another factor may be the sequestration of mRNA into so called P-bodies, as observed in quiescent yeast cells. Such structures affect the accessibility of mRNAs for the translational machinery, which may affect the correlation between whole-cell transcript levels and protein levels (Aragon *et al.*, 2008; Li *et al.*, 2013). However the levels of Dcp1 and Lsm1-7, proteins commonly found in P-bodies were decreased under calorie restriction and of the 21 other proteins commonly found in these protein-mRNA aggregates, 16 were quantified but were not significantly changed (Supplementary Table S6) (Parker and Sheth, 2007), suggesting that P-bodies were not increased in number under calorie restriction and may therefore not contribute to the discrepancies between transcript and protein levels. In mammalian cells, metabolic gene products, transcripts and proteins, were found to be very stable (Schwanhäusser *et al.*, 2011). This could explain our observation of the down-regulation of anabolic pathways on transcript level but not yet on the protein level.

A better correlation between changes in mRNA and protein levels was found for the up-regulated rather than down-regulated loci. Although elevated transcript levels led to some extent to increased translation of several proteins, the vast majority of the transcripts with decreased abundance were not accompanied by a similar decrease in protein level. These observations underline the need for dissecting the impact of calorie restriction on protein synthesis and protein degradation. Quantitative analysis of proteome-wide turnover rates in calorie-restricted cultures represents a formidable challenge. Still, such experiments are highly relevant for addressing the question of whether and how the balance between synthesis and degradation of individual proteins is a function of carbon and energy source availability, as observed in SP cultures (Fuge *et al.*, 1994; Marguerat *et al.*, 2012).

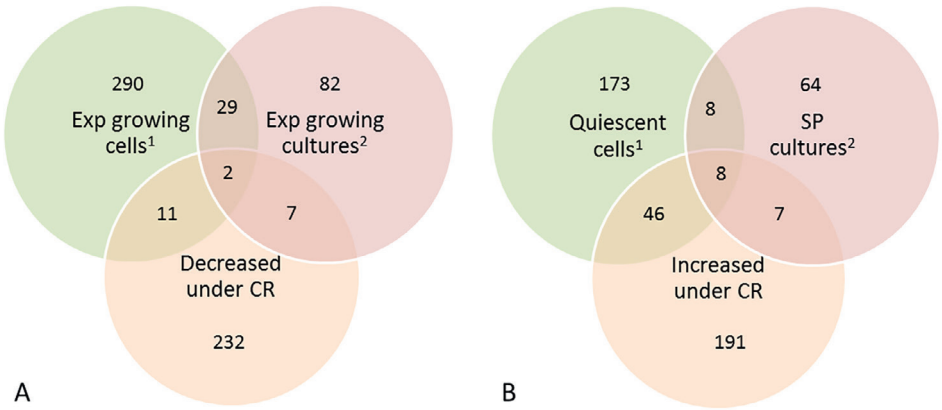

Figure 3.6 Overlap in proteins regulated upon calorie restriction and literature datasets of stationary phase (SP)-cultures.
(**A**) Overlap between proteins with decreased (cluster 1) levels under calorie restriction (CR) and proteins with increased levels in exponentially (exp) growing cultures and exp growing cells. (**B**) Overlap between proteins with increased levels under CR (cluster 2) and proteins whose abundance is specifically strongly increased in quiescent cells and SP cultures. Data for exponentially growing and quiescent cells[1] were derived from (Webb *et al.*, 2013), data for exp growing and SP cultures[2] from (Davidson *et al.*, 2011).

Comparison with other "aging" yeast cultures

Although this is the first proteomic study under severe calorie restriction, other authors have used different model systems of yeast in the context of aging. Cellular processes affected at the proteome level during starvation in SP in *S. cerevisiae* show a clear overlap with those identified in the present studied on severely calorie-restricted retentostat cultures, as evident from increased abundance of proteins involved in stress resistance and, more particularly, heat shock as well as respiratory metabolism in both systems (Davidson *et al.*, 2011; Fuge *et al.*, 1994; Webb *et al.*, 2013). However, closer inspection of the proteome data sets obtained under the two conditions presents a drastically different picture (Fig. 3.6). From the two most comprehensive proteome studies on SP, Davidson *et al.* reported the proteome of the whole SP population and sub-populations (Davidson *et al.*, 2011), while Webb *et al.* isolated quiescent long-lived cells from senescent cells by sucrose-gradient centrifugation before analysing their proteome (Webb *et al.*, 2013). Comparing the present retentostat proteome data set with these two SP data sets revealed only a very small overlap between the proteome responses to calorie restriction and starvation. Interestingly, the proteome of the calorie-restricted retentostat cultures more strongly resembled "healthy" quiescent cells than that of "average" SP cultures (Fig 3.6B). This is consistent with the notion that retentostat cultivation leads to a calorie-restricted, robust, and long-lived physiological state that is markedly different from starvation. Proteins involved in stress resistance (Hsp12, Hsp26, Ssa3, and Sod2) and respiration (Kgd1, Kgd2, Icl1, Shh3, Sdh1b, and Atp15) were increased in abundance both in quiescent and calorie-restricted cells (Webb *et al.*, 2013).

Remarkably, a number of proteins that were previously labeled as "markers" of stationary phase did not show a similar response during retentostat cultivation. For instance, the level of Snz1, involved in vitamin B6 synthesis, and its co-regulated and functionally related homologue Sno1, which show a clearly increased abundance under nutrient starvation (Braun *et al.*, 1996), displayed strongly reduced levels in the calorie-restricted retentostats. Abundance of Ssa2 remained constant throughout SP, in contrast with the decrease in its encoding transcript, whereas under calorie restriction, protein and transcript levels were both significantly decreased. The limited proteomic resemblance between stationary phase and retentostat cultures supports the previously proposed concept that calorie restriction and carbon starvation represent clearly distinct physiological states in *S. cerevisiae* (Boender *et al.*, 2011a).

Conclusion

Here, we took a step forward in identifying mechanisms that take place at the level of the *S. cerevisiae* proteome under calorie restriction and at near-zero-growth, as a model system of aging in eukaryotic cells. Quantitative profiling of the proteome over five time points revealed differential expression of about a quarter of the almost 2500 proteins monitored. Surprisingly, we observed that, under anaerobic conditions, a large number of mitochondrial proteins became more abundant. This may mean that mitochondria, which have been in implicated in aging under aerobic conditions, may also respond under anaerobic conditions. Moreover, several proteins known to be repressed by glucose, were observed to become more abundant in adaptation of chronologically long-lived cells to calorie restriction even when extracellular glucose levels steadied.

These findings reveal some of the unexpected mechanisms at the proteome level that are involved in the adaptation of *S. cerevisiae* towards calorie restriction and aging.

Materials and Methods

Strain, media, and cultivation

The haploid laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D (*MAT*a, *MAL*2-8^c, *SUC*2) (Entian and Kötter, 2007; Nijkamp *et al.*, 2012) and the prototrophic, marker-free *hsp30* deletion strain IMK381 (*MAT*a, *MAL2*-8^c *SUC*2 *hsp30*:: *loxP*) were used. Yeast transformations to construct the IMK381 strain were performed using the lithium acetate method described by Gietz and Schiestl (Gietz and Schiestl, 2007). The *loxP*-marker-*loxP*/Cre recombinase system was used for the deletion of the *HSP30* gene and subsequent marker removal. The knockout cassette was constructed on the basis of the plasmid pUG6 using primers with the sequences ACTTTAATATCTTTTGATTACTAAAAACAACAAATTTCAACCAGCTGAAGCTTCGTACGC and TGTGTTAAGCAAAGAATGATTAAGACAATCTCAAGCTGCTATAGGCCACTAGTGGATCTGA according to Güldener *et al*. (Güldener *et al.*, 1996). The correct integration of the knock-out cassette in the genome of *S. cerevisiae* strain CEN.PK113-7D and its subsequent removal were confirmed by PCR using primers CCTGAAAGGCAAAGGATGAG and

TCCCGATCCCGACTCTTATTCC. All primers were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Media and culture conditions were as described previously (Boender *et al.*, 2011b). Briefly, for anaerobic chemostat and retentostat cultivation, synthetic medium (Verduyn *et al.*, 1992), with *50 g · L^{-1}* glucose as limiting nutrient at pH 5, supplemented with anaerobic growth factors Tween 80 (240 mg · L^{-1}) and ergosterol (10 mg · L^{-1}) and antifoam agent Struktol J673 (0.3 g · L^{-1}) was used. For retentostat cultivation, chemostat cultures at a dilution rate of 0.025 h^{-1} were run until steady state was achieved according to the criteria described previously (Boender *et al.*, 2009); then the effluent was withdrawn through a 0.22 µm pore size filter to achieve full cell retention. Retentostats were followed over 3 weeks. Samples for proteome analysis were taken on days 2, 9, 16 and 21 of retentostat cultivation. Duplicate chemostat cultivations at a dilution rate of 0.025 h^{-1} were sampled at steady state, corresponding to day 0 of retentostat cultivations. The specific growth rate was calculated as described previously (Boender *et al.*, 2009).

Sample preparation

Cells were harvested from the duplicate bioreactors and immediately washed with ice-cold water, pelleted, and stored at -80°C, as described previously (Helbig *et al.*, 2011). Lyophilized cells were lysed in lysis buffer (6 M urea, 2 M thiourea, 200 mM DTT, 2× protease inhibitor tablets (Roche, Basel, Switzerland) in 20 mM Tris, pH 8). Cells were homogenized by beating them with 0.5 mm glass beads for 5 × 1 min in the bead beater dismembrator S (B.Braun, Melsungen, Germany). Lysates were cleared by centrifugation for 10 min at 20 000 *g* and 4°C. Supernatant was transferred into a new tube, and the protein amount was determined with the 2D Quant Kit (GE Healthcare, Zeist, The Netherlands). Next, proteins were digested according to a published method (Di Palma *et al.*, 2012). Briefly, 100 µg of lysed proteins were reduced and alkylated with DTT and iodoacetamide, respectively, and digested with trypsin (Promega, Madison, USA) at an enzyme/substrate ratio of 1/50. Resulting peptide mixtures were desalted using Seppak 1 cc columns (Waters Corporation, Milford, USA). The purified samples were chemically labeled with the TMT 6plex isobaric label kit according to manufacturer's instructions (Pierce, Rockford, IL, USA) and combined in a 1:1:1:1:1:1 ratio based on the average precursor ion area of each sample creating mix 1 and 2. Mix 1 consisted of equal protein quantities of protein lysates from bioreactor 1, sampling times from 0 to 21 days, containing the following TMT® 6plex labels: day 0 sample label 126, day 2 sample label 127, day 9 sample label 128, day 16 sample label 129, day 21 sample label 130. Label 131 was used for the day 0 sample from bioreactor 2. Likewise, mix 2 consisted of equal protein quantities of biological replicate 2 protein lysates (labels 126 to 130 used for day 0 to day 21 of the second retentostat) and label 131 for the day 0 sample of the biological replicate 1. Hence, the day 0 samples of both replicate retentostat cultures were directly compared within one MS-experiment and in duplicate.

Strong cation-exchange chromatography (SCX) and liquid chromatography (LC)-mass spectrometry (MS)

Strong cation-exchange chromatography was used as described earlier (Pinkse *et al.*, 2008). We applied an improved SCX system to fractionate the 6-plex TMT labeled sample. Briefly, an Agilent 1100 HPLC (Agilent, Santa Clara, CA, USA) system was equipped with a Opti-Lynx (Optimized Technologies, Portland, OR, USA) trapping cartridge and a Zorbax Bio-SCX II column (0.8 mm i.d. × 50 mm length, 3.5 µm, Agilent). Solvent A consisted of 0.05 % formic acid in 20 % acetonitrile, whereas solvent B contained 0.05 % formic acid and 0.5 M NaCl in 20 % acetonitrile. 600 µg of labeled peptide mixture was loaded, and a total of 50 fractions (1 min each, 50 µL elution volume) were collected and dried down in a vacuum concentrator. The salt gradient used for elution of the peptides was as follows: 0-0.01 min (0-2 % B); 0.01-8.01 min (2-3 % B); 8.01-14.01 min (3-8 % B); 14.01-28 min (8-20 % B); 28-38 min (20-40 % B); 38-48 min (40-90 % B); 48-54 min (90 % B); 54-60 min (0 % B). 2+ and 3+ fractions were reconstituted in 10 % formic acid and analyzed on a Q-Exactive mass spectrometer connected to an Easy UHPLC system (both Thermo Fisher Scientific, Bremen, Germany). The peptides were eluted from the reverse-phase column during a 3 h gradient and directly sprayed into the mass spectrometer with in-house-made gold-coated silica emitters. The columns were made in-house from either Aqua C18 (5 µm, Phenomenex, Torrance, CA, USA; 20 mm × 100 µm i.d.) for the trap column or Zorbax C18 (1.8 µm, Agilent; 38 cm x 50 µm i.d.) for the analytical column (Cristobal *et al.*, 2012). The mass spectrometer was operated in data-dependent acquisition mode using the following settings: ESI voltage, 1.7 kV; inlet capillary temperature, 275°C; full scan automatic gain control (AGC) target, 3E6 ions at 35 000 resolution; scan range, 350-1500 *m/z*; Orbitrap full-scan maximum injection time, 250 ms; MS2 scan AGC target, 5E4 ions at 17 500 resolution; maximum injection, 120 ms; normalized collision energy, 33; dynamic exclusion time, 30; isolation window 1.5 *m/z*; 10 MS2 scans per full scan.

Data analysis

The raw data obtained, were initially processed with Proteome Discoverer 1.3 (Thermo Fisher). The created peak lists were searched with Mascot (Matrix Science, Version 2.3) using the SGD database (containing 5779 entries) and the following parameters: 50 ppm precursor mass tolerance and 0.05 Da fragment ion tolerance. Up to two missed cleavages were accepted, oxidation of methionine was set as variable modification, and cysteine carbamidomethylation and the TMT label on lysines and the N-terminus were set as fixed modifications. The resulting .dat files were exported and filtered for < 1 % false discovery rate at the peptide level using the in-house developed software Rockerbox (version 2.0.1) utilizing the percolator algorithm (van den Toorn *et al.*, 2011). The filtering for significant changing proteins was done with the isobar software (v1.6.2) in R (v3.0.0, (R Core Team, 2013)) and a significance threshold of p-value ≤ 0.05. Isobar employs robust statistics that captures spectra and sample variability into a single statistical framework, which is

described in (Breitwieser *et al.*, 2011). Significantly changing proteins from both bioreplicate experiments were then clustered using GproX, a software that uses unsupervised clustering, based on the fuzzy c-means algorithm (Rigbolt *et al.*, 2011). Settings for the clustering were 1.25 for fuzzification, 100 iterations and minimum membership value of 0.1. Each of the data sets, from mix 1 and from mix 2 was loaded with the individual ratios into the GProX software tool and then analyzed together in one analysis. KEGG pathway and Gene Ontology functional categories enrichment analysis of the resulting clustered proteins was performed by using the online software tool DAVID (Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov)(Huang *et al.*, 2008), with all proteins identified in both experiments from this MS analysis as background. A Benjamini-Hochberg corrected *p*-value of 0.05 was used as a threshold to denote the significance of enrichment. To address the previously described issue of ratio compression within isobaric labeled datasets, we calculated the average precursor ion fraction (PIF) for both mixes using the isolation interference for each peptide (Michalski *et al.*, 2011a).

Integration of transcriptome and proteome

Transcriptome data from triplicate chemostat cultures at dilution rate 0.025 h^{-1} and retentostat cultures from a previous study were used and were obtained from the Gene Expression Omnibus under number GSE22574 (Boender *et al.*, 2011b). Using Empirical analysis of Digital Gene Expression data in R (EDGE, v 1.1.291), 823 genes were identified as differentially expressed in time (p-value cut-off of 0.0028).

The whole gene and protein profiles were both loaded into [R] and each dataset separately was filtered on interquartile range (IQR) > 0.5, to remove profiles that have no or only a little change in abundances in their profile. Subsequently, all profiles were scaled using the *z*-score transformation to account for differences in dynamic ranges for genomics and proteomics experiments. Both data sets were exported into tab-delimited files and merged into a single file keeping unique ID's for the genomics and proteomics profiles. The merged data file was subsequently imported into MeV (v4.8.2) (Saeed *et al.*, 2003). In MeV, the figure of merit was determined in order to get an estimate of the expected number of clusters for k-means clustering. Subsequently, k-means clustering was performed. For GO enrichment analysis, the two up- and down-regulated protein/gene clusters were combined.

Raw data access

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno *et al.*, 2013) with the dataset identifier PXD000161 and DOI 10.6019/PXD000161.

Acknowledgements

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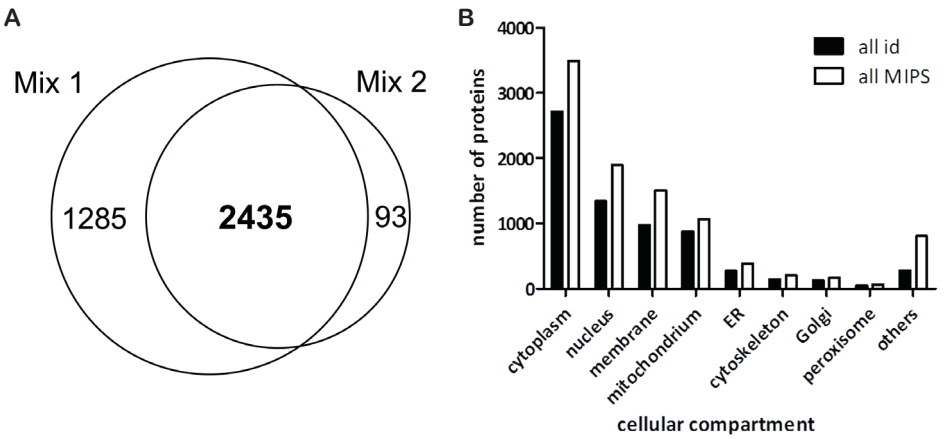
Supplementary materials Chapter 3

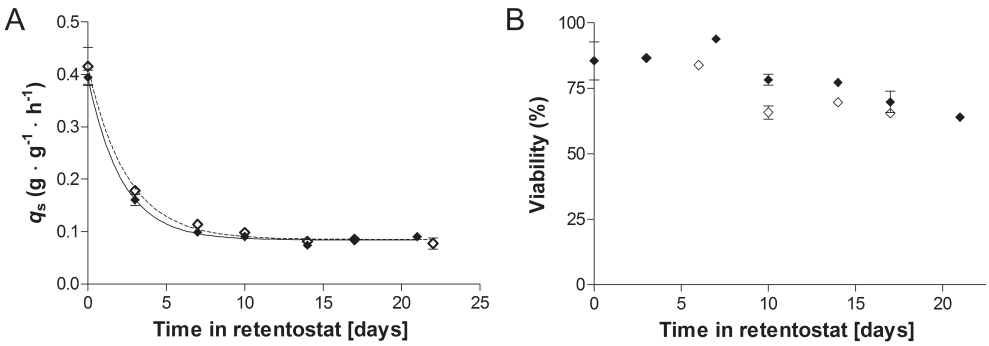
Supplementary materials are freely available online at the web-site of Journal of Proteome Research in which the original publication appeared in Volume 13, Issue 8, August 2014, Pages 3542-3553. (doi:10.1021/pr5003388).

Supplementary Figure S3.1



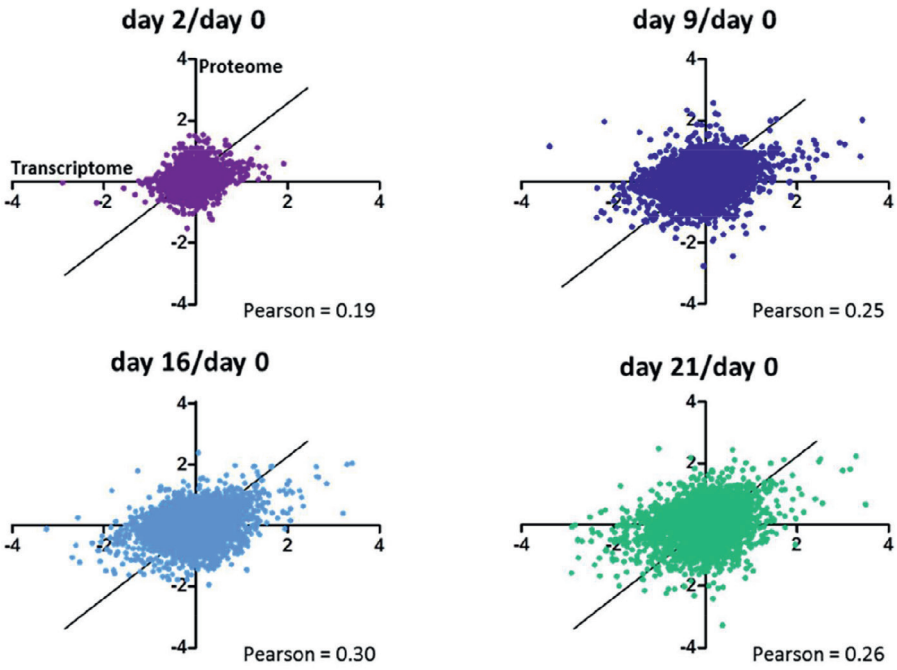
(**A**) The Venn diagram shows the overlap of 2435 proteins that were quantified in both biological replicates after LC-MS/MS analysis. Mix 1 shows 1285 and mix 2 shows 93 uniquely quantified proteins. (**B**) The distribution of identified proteins over various cellular compartments is shown by assigning all identified proteins from our study (black bars) to their GO annotations. As reference all entries from the MIPS database (5781 entries) were used (open bars).

Supplementary Figure S3.2



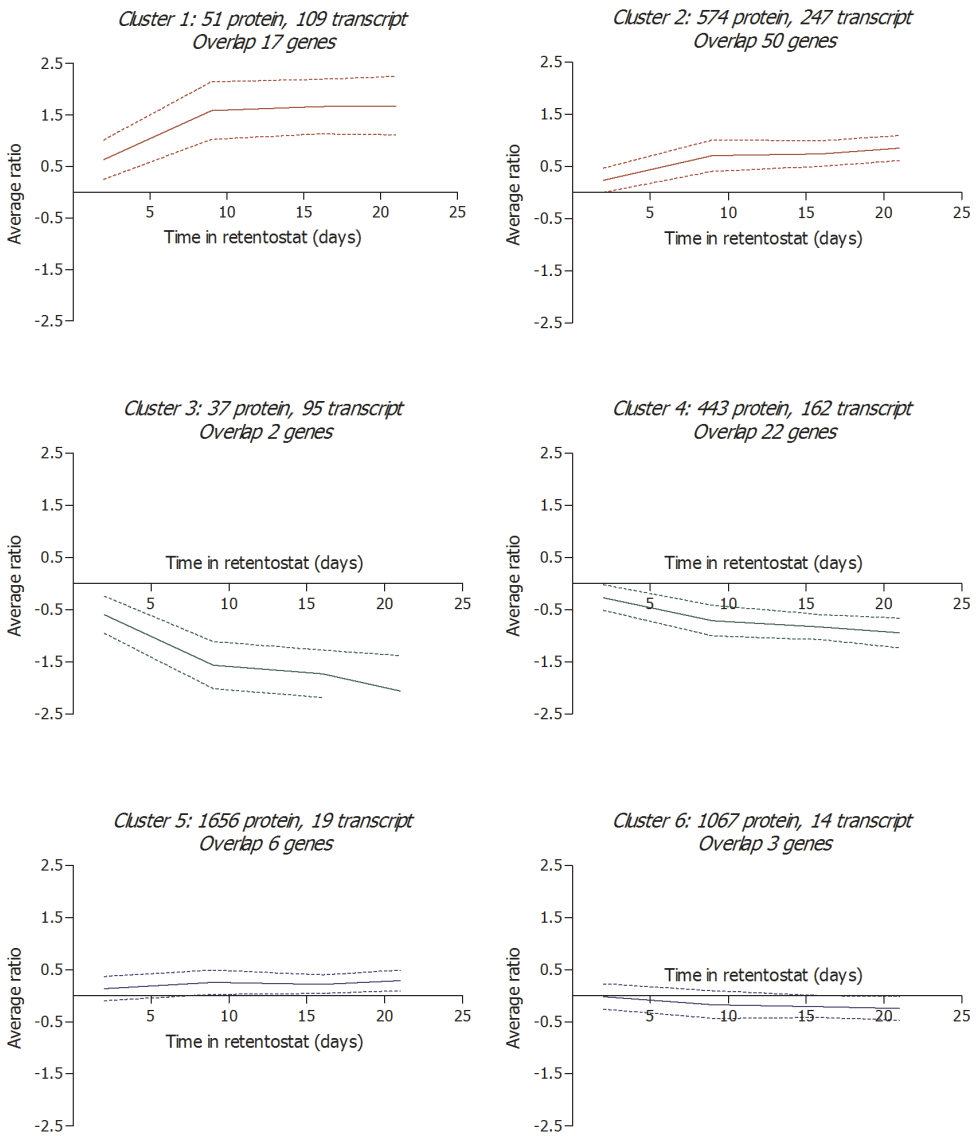
Characteristics of a *hsp30* deletion strain (closed diamonds, ◆) and the reference strain CEN.PK113-7D (open symbols, ◇) in anaerobic retentostat cultures. (**A**) Specific glucose uptake rates in g glucose per g biomass per hour. (**B**) Viability, measured as metabolically active cells, by flow cytometry expressed as percentage of the total number of cells measured. (Value are shown as mean +/- standard error of the mean, errors smaller than symbol size are not shown.)

Supplementary Figure S3.3



Transcript *versus* protein correlation plots for the individual time points reveal weak correlation. The log2 z-scores of transcripts and proteins were plotted. The diagonal line marks the perfect correlation. Pearson correlations were determined using GraphPad Prism software (v 4.03 GraphPad Software, La Jolla, CA).

Supplementary Figure S3.4



Clustering of protein and transcript ratios using k-means clustering. Ratios of all proteins quantified in at least one replicate culture and the ratios of corresponding and significantly changed transcripts were used as input. The figure of merit gave an optimal of six clusters. Shown are the averages (+/- st.dev. in dashed lines) for all ratios (i.e. protein and transcript) in the respective cluster. Above each plot the total numbers of proteins, transcripts and of genes for which transcript and protein levels were clustered together, present in the respective cluster are indicated. Cluster 1 and 2, and cluster 3 and 4 were combined to obtain the clusters shown in Figure 3.5A,B.

Supplementary Table S3.1

For the protein ratios of *S. cerevisiae* proteome analysis at near-zero growth rate the reader is referred to the Excel spread sheet that can be found online.

Supplementary Table S3.2

For the protein identifications of *S. cerevisiae* proteome analysis at near-zero growth rate the reader is referred to the Excel spread sheet that can be found online.

Supplementary Table S3.3

Peptide and protein identification data from both mixes.

Mix	Sample	Label	No. peptide groups	No. protein groups	Average PIF*
1	Reactor 1, t_0	126	27496	3721	0.84
	Reactor 1, t_2	127			
	Reactor 1, t_9	128			
	Reactor 1, t_{16}	129			
	Reactor 1, t_{21}	130			
	Reactor 2, t_0	131			
2	Reactor 2, t_0	126	12397	2528	0.80
	Reactor 2, t_2	127			
	Reactor 2, t_9	128			
	Reactor 2, t_{16}	129			
	Reactor 2, t_{21}	130			
	Reactor 1, t_0	131			

*PIF (precursor ion fraction)

Supplementary Table S3.4

Functional enrichments found among the 252 proteins with increased levels under calorie restriction.

Enrichment analysis was performed using DAVID and enrichments are called significant when the Benjamini-Hochberg corrected *p*-value was below 0.05. Most categories found enriched are related to mitochondria (highlighted in blue) and respiration and energy generation (highlighted in green). Categories were obtained from the Gene Ontology (GO) and KEGG databases.

Category	Number of proteins	% of total proteins in category	Benjamini-Hochberg corrected *p*-value
Mitochondrial part (GO:0044429)	79	23	$9.0 \cdot 10^{-12}$
Mitochondrion (GO:0005739)	110	17	$1.6 \cdot 10^{-9}$
Mitochondrial matrix (GO:0005759)	42	28	$4.1 \cdot 10^{-8}$
Mitochondrial inner membrane (GO:0005743)	37	29	$1.1 \cdot 10^{-7}$
Mitochondrial envelope (GO:0005740)	44	21	$1.1 \cdot 10^{-4}$
Oxidation reduction (GO:0055114)	53	21	$1.1 \cdot 10^{-4}$
Energy derivation by oxidation of organic compounds (GO:0015980)	31	27	$1.1 \cdot 10^{-4}$
Organelle envelope (GO:0031967)	51	18	$4.5 \cdot 10^{-4}$
Mitochondrial large ribosomal subunit (GO:0005762)	14	40	$4.9 \cdot 10^{-4}$
Mitochondrial ribosome (GO:0005761)	20	29	$7.7 \cdot 10^{-4}$
Citrate cycle (TCA cycle) (KEGG: sce00020)	11	39	$2.2 \cdot 10^{-3}$
Cellular respiration (GO:0045333)	22	29	$3.5 \cdot 10^{-3}$
Integral to membrane (GO:0016021)	53	17	$3.7 \cdot 10^{-3}$
Oxidative phosphorylation (KEGG:sce00190)	14	26	$6.4 \cdot 10^{-3}$
Aerobic respiration (GO:0009060)	19	28	$1.2 \cdot 10^{-2}$
Generation of precursor metabolites and energy (GO:0006091)	35	20	$1.2 \cdot 10^{-2}$
Co-factor metabolic process	31	21	$1.4 \cdot 10^{-2}$
Response to temperature stimulus (GO:0009266)	26	21	$4.1 \cdot 10^{-2}$
Co-enzyme metabolic process	24	22	$4.5 \cdot 10^{-2}$
Mitochondrial membrane part	19	22	$4.8 \cdot 10^{-2}$

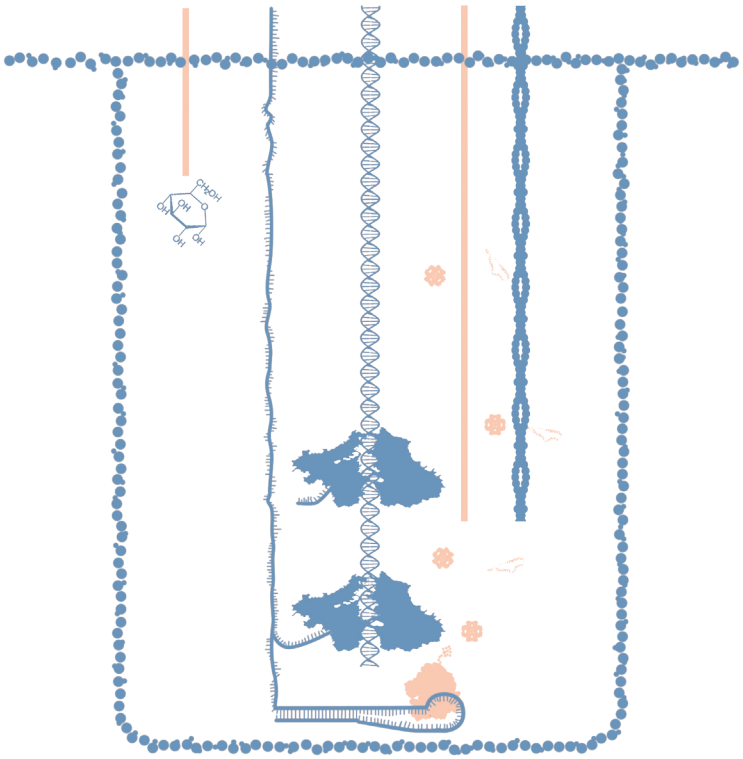
Supplementary Table S3.5

Heat shock proteins with various functions were up regulated during retentostat cultivation

Name	Function
Hsp12p	Involved in maintaining membrane organization in stress conditions, induced by entry into stationary phase, glucose depletion
Hsp26p	Small heat shock protein (sHSP) with chaperone activity involved in stress response
Hsp30p	Induced by entry into stationary phase
Hsp78p	Involved in folding of mitochondrial proteins
Ssa3p	ATPase involved in protein folding and the response to stress
Sis1p	Co-chaperone, protein abundance increases in response to DNA replication stress
Ssq1p	Mitochondrial Hsp70-type molecular chaperone, required for assembly of iron/sulfur clusters into proteins
Ssc1p	Mitochondrial Hsp70-related protein involved in protein translocation and folding
Mdj1p	Co-chaperone of Ssc1, protein folding/refolding in the mitochondrial matrix

Supplementary Table S3.6

For the two functional categories containing several proteins with lower expression under calorie restriction the reader is referred to the Excel spread sheet that can be found online.


CH2OH
OH
OH
OH
OH

4. Extreme calorie restriction in yeast retentostats induces uniform non-quiescent growth arrest

Markus M.M. Bisschops, Marijke A.H. Luttik, Anne Doerr,
Peter J.T. Verheijen, Jack T. Pronk, Frank J. Bruggeman, Pascale Daran-Lapujade

Keywords: Extreme calorie restriction, *Saccharomyces cerevisiae*, non-dividing, mRNA fluorescent *in situ* hybridization (FISH), F-actin, quiescence

Submitted for publication

Non-dividing *Saccharomyces cerevisiae* cultures are highly relevant for fundamental and applied studies. However, model systems in which non-dividing cells retain substantial metabolic activity are lacking. Unlike the current experimental paradigm for non-dividing yeast, stationary-phase (SP) batch cultures, cultivation under extreme calorie restriction (ECR) in retentostat enables non-dividing yeast cells to retain substantial metabolic activity and to prevent rapid cellular deterioration. Distribution of F-actin structures and single-cell levels of specific transcripts revealed that, contrary to SP cultures that differentiate into quiescent and non-quiescent subpopulations, cultivation under ECR yields highly homogeneous cultures. Combined with previous physiological studies, these results indicate that yeast cells subjected to ECR survive in an extended G_1 phase. Additionally, the present data suggest that *S. cerevisiae* adapts net transcription rates to natural variations in cell size. This study demonstrates that yeast cells exposed to ECR differ from carbon-starved cells and offer a promising experimental model for studying non-dividing, metabolically active, and robust eukaryotic cells.

Introduction

From both an applied and fundamental perspective, model systems in which cells do not divide and remain metabolically active are highly relevant. Since biomass is rarely the product of interest in microbial biotechnology, process conditions that force cells to invest resources in product formation, rather than in biomass formation, are economically appealing (Boender *et al.*, 2009). From an environmental point of view, the majority of microbial life on earth consists of slowly or non-dividing cells, mostly as a result of limited accessibility to nutrients (Gray *et al.*, 2004). The cellular and molecular mechanisms leading to the survival or senescence of cells in extremely nutrient-limited environments remain, however, poorly understood. This knowledge gap can readily be explained by the experimental challenges involved in maintaining microbial cultures in a physiological state in which cells are provided with sufficient nutrients to stay viable and active, but not enough to enable cell division. In the 1970's, the retentostat was designed as a bioreactor cultivation-system that enables the achievement of exactly this physiological state (Chesbro *et al.*, 1979). While technically more complicated than shake-flask cultivation, the retentostat is conceptually a simple system. A microbial culture in a bioreactor is continuously supplied, at a fixed rate, with a growth medium in which the energy substrate (typically glucose) is growth limiting. A filter-equipped effluent line enables the continuous withdrawal of spent medium, while retaining cells in the bioreactor. Trapped in the retentostat, biomass builds up until the culture reaches calorie restriction that is so extreme that all the energy substrate fed to the culture is used for maintaining cellular integrity and viability, and no energy source is available for further growth. This state of extreme calorie restriction (ECR) offers a unique opportunity to investigate the minimal energy requirement of cells and regulation of cellular function in non-growing cultures, but has so far been mostly used to study bacteria (Ercan *et al.*, 2015a). Beyond applied or ecological aspects, retentostat cultures are also extremely appealing for research on cellular aging, as cultures in which eukaryotic microbial cells do not divide but remain metabolically active may offer a good and easily accessible model system for non-dividing metazoan cells.

The budding yeast *Saccharomyces cerevisiae* is a well-established industrial workhorse and eukaryotic model organism. The most popular model system to study non-dividing yeast cells consists of stationary phase (SP) cultures, in which growth arrest is typically triggered by exhaustion of exogenous carbon and energy sources (Herman, 2002; Werner-Washburne *et al.*, 1993). This system presents several drawbacks, the most prominent of which is that, when cells are deprived of external energy and carbon source, they are hardly metabolically active, deteriorate and die (Breitenbach *et al.*, 2012c). Furthermore, entry of batch cultures into SP is a highly dynamic process and, in the Crabtree-positive yeast *S. cerevisiae*, encompasses several distinct phases. Aerobic batch cultivation of yeast cells on glucose starts with a glucose consumption phase during which fermentation products accumulate. This phase is followed by glucose depletion and a diauxic shift, after which fermentation products

(ethanol, acetate and glycerol) are consumed during a post-diauxic growth phase. Finally, when all utilizable carbon sources have been depleted, cultures enter into SP (Herman, 2002; Werner-Washburne *et al.*, 1993). The length of, and progression through, these phases is highly sensitive to culture conditions and strongly affects the subsequent survival of yeast cells during SP (Mirisola *et al.*, 2014). To address these complications, we have explored the potential of retentostat cultures to obtain and study non-growing but metabolically active yeast cultures. Previous studies with this system demonstrated that cells in retentostats can be reproducibly and stably cultivated under ECR for prolonged periods of time (Boender *et al.*, 2009). While ECR cultures share several features with carbon-starved SP cultures, such as high stress-tolerance, longevity under starvation conditions and the transcription and translation of SP-hallmark genes (including the heat shock proteins Hsp12 and Hsp26), they also have distinct characteristics (Binai *et al.*, 2014; Boender *et al.*, 2011a; Boender *et al.*, 2011b). Notably, ECR cells manifest substantially higher viability and metabolic activity than their SP counterparts, which can be explained by the low but continuous supply of exogenous energy source (Boender *et al.*, 2011a).

Several studies have reported that SP cultures are highly heterogeneous, consisting of subpopulations of quiescent and non-quiescent cells (Allen *et al.*, 2006; Aragon *et al.*, 2008; Li *et al.*, 2013). Quiescent cells have undergone a multitude of changes that were previously attributed to the whole SP population. These changes range from transcriptional reprogramming to sub-cellular reorganization, likely all contributing to an increased viability and stress-tolerance (i.e. robustness) of quiescent cells under nutrient-poor conditions (Allen *et al.*, 2006; Aragon *et al.*, 2008; Galdieri *et al.*, 2010; Gray *et al.*, 2004). Conversely, non-quiescent cells do not adapt adequately to the exogenous energy-source depletion, with senescence and death as ultimate consequences (Allen *et al.*, 2006).

Population heterogeneity has hitherto not been investigated in ECR cultures. It therefore remains unknown whether non-dividing, metabolically-active cells also differentiate into subpopulations of robust quiescent cells and senescent cells. The goal of the present work is to assess the heterogeneity under ECR in retentostat cultures to get insight in the mode of growth arrest and thereby, to evaluate the potential of yeast retentostat culture as model for non-dividing eukaryotic cells. To this end, we explored population heterogeneity in ECR cultures using two single-cell analytical approaches. F-actin staining enables visualization of the structural organization of actin in yeast cells and was used to monitor the progression of cells in or out of the active cell cycle, as shown for batch cultures (Sagot *et al.*, 2006). Absolute, single-cell transcript levels of *HSP12* and *HSP26*, two previously described hallmarks for quiescence in yeast (Aragon *et al.*, 2008; Davidson *et al.*, 2011), were monitored by single-molecule RNA fluorescent *in situ* hybridization (smRNA-FISH). Both techniques revealed a remarkable homogeneity in the transition from growth to cell cycle arrest triggered by calorie restriction, which is a striking difference between ECR and SP cultures.

Results

Extreme calorie restriction arrests cell division

Anaerobic retentostat cultures were started from glucose-limited chemostat cultures, i.e., continuously fed cultures from which both spent medium and cells are continuously withdrawn (Daran-Lapujade *et al.*, 2008; Ziv *et al.*, 2013). In the chemostat cultures, cells were steadily growing at a fixed specific growth rate of 0.025 h^{-1} (doubling time of 28 h). Switching these chemostat cultures to full cell retention enabled the smooth transition from slow but exponentially growing, glucose-limited cultures to non-growing and extremely calorie-restricted cultures. Over a period of 14 days cells accumulated, resulting in a progressive increase in calorie restriction and in a gradual decline of the specific growth rate to near-zero values ($\mu < 0.002$ h^{-1}; doubling time >500 h, Fig. 4.1A). In contrast to glucose-starved SP cultures (Bisschops *et al.*, 2015; Fabrizio *et al.*, 2001), culture viability remained high under ECR (above 65 % of viable cells, Fig 4.1B). This high viability is explained by the fact that calorie-restricted cells invest all available glucose in maintenance of cellular viability and integrity, at the expense of cell division.

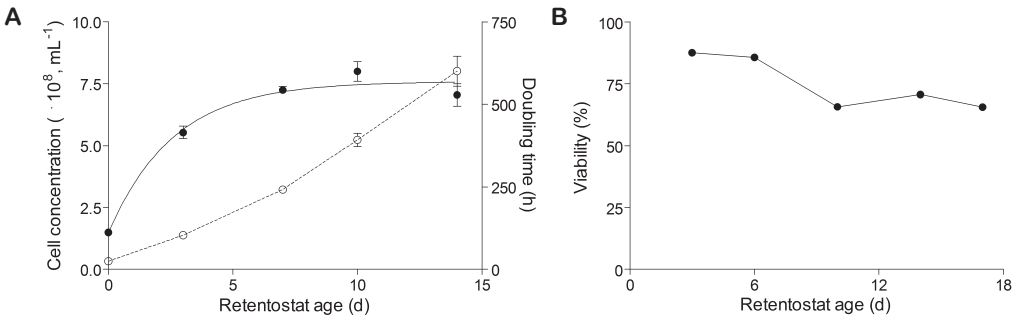


***Figure 4.1 Cell counts, doubling time and culture viability dynamics in retentostat cultures of* S. cerevisiae**

(**A**) Cell concentration (closed circles, ●) and doubling times (open circles, ○) in retentostat cultures as function of retentostat age. Doubling times corresponded to a decrease of the culture specific growth rate from 0.025 h^{-1} to below 0.002 h^{-1}. (**B**) Viability of retentostat cultures, measured by flow cytometry, during retentostat cultivation. Data are represented as the average and SEM of two independent culture replicates, SEM smaller than symbol size are not shown.

The fraction of cells containing replicated DNA (2n) decreased from 29 ± 6 % in growing cultures before switching to cell retention, to 12.2 ± 0.3 % after 14 days of retentostat cultivation (Fig 4.2). These numbers are in good agreement with the fraction of budded cells, which decreased from 35.0 ± 0.3 % to 18.2 ± 0.3 %, as previously reported for the same cultivation system (Boender *et al.*, 2011b). While this fraction of budded cells with replicated DNA may appear high for a non-growing population, it is consistent with previous reports for SP cultures (e.g. 10 ± 5 %, (Laporte *et al.*, 2011). These results indicate that growth arrest, upon a progressive increase of caloric restriction, predominantly occurs in a phase of the cell cycle that does not involve replicated DNA and suggest that cells from ECR and SP cultures arrest their cell cycle in a similar state.

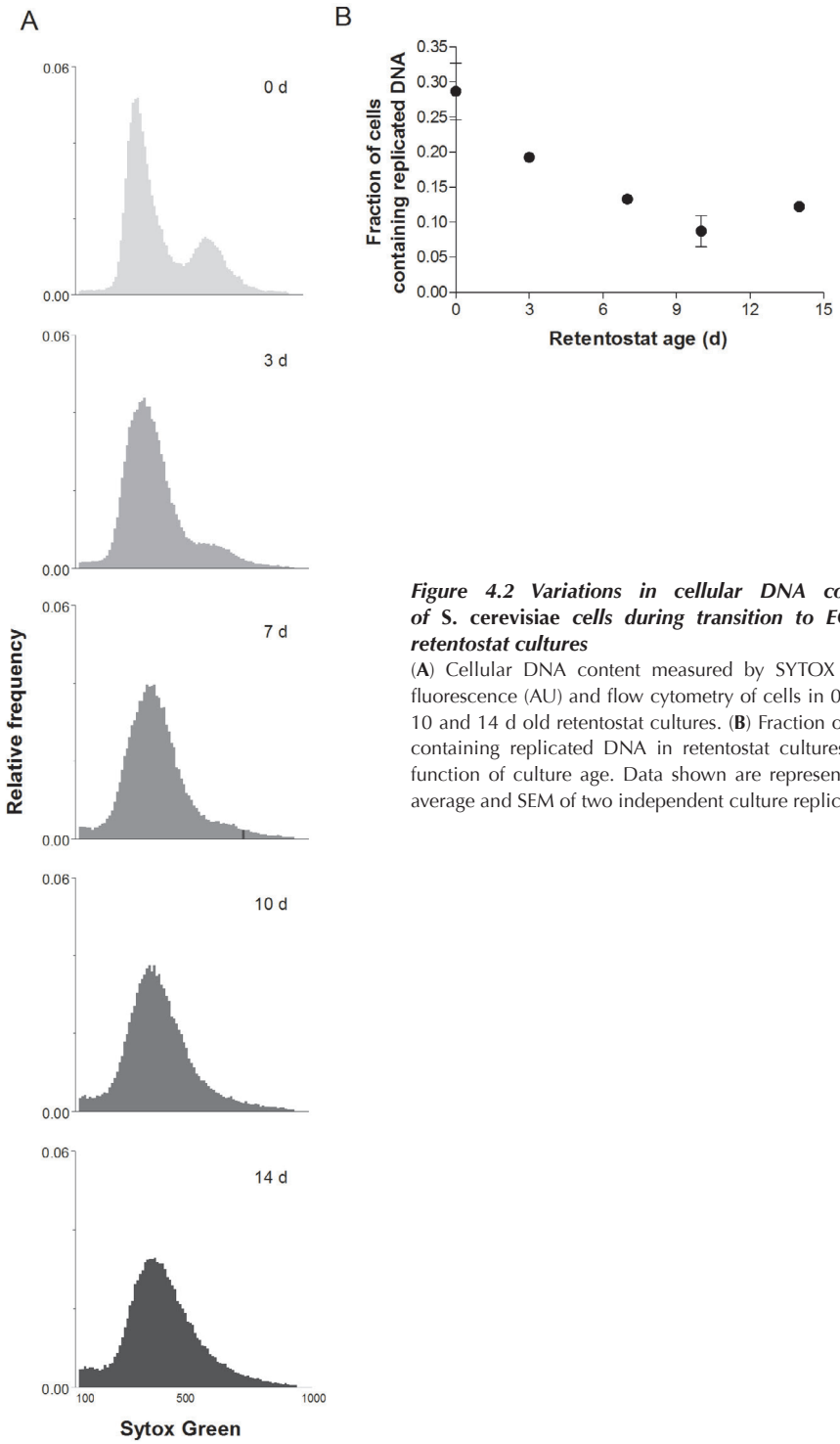


Figure 4.2 Variations in cellular DNA content of S. cerevisiae cells during transition to ECR in retentostat cultures

(**A**) Cellular DNA content measured by SYTOX green fluorescence (AU) and flow cytometry of cells in 0, 3, 7, 10 and 14 d old retentostat cultures. (**B**) Fraction of cells containing replicated DNA in retentostat cultures as a function of culture age. Data shown are represented as average and SEM of two independent culture replicates.

Growth arrest by extreme calorie restriction is characterized by uniform F-actin structures that differ from those observed in starved cells

Filamentous actin (F-actin) can adopt different structures and the transition between these structures is tightly orchestrated during the yeast cell cycle (Amberg, 1998). F-actin structure can therefore be used as an indicator of the cell cycle phase in which individual cells reside. F-actin patches, cables and rings, are three structures associated with active phases of the cell cycle (Amberg, 1998; Mishra *et al.*, 2014), while 'actin bodies', which are large dense amorphous structures, have been specifically observed in non-dividing yeast cells in SP batch cultures (Sagot *et al.*, 2006). Consistent with the results from the DNA-content analysis (Fig 4.2), the incidence of cells with clearly visible actin cables and polarized patches, which are associated with actively dividing cells (Mishra *et al.*, 2014; Sagot *et al.*, 2006), decreased from 27 % to below 2.0 % as growth in the retentostats gradually decelerated and stalled (Fig. 4.3). Simultaneously, the fraction of cells containing depolarized F-actin patches increased (Fig 4.3). During prolonged retentostat cultivation (21 d), the incidence of cells that did not show clear F-actin structures varies between 8 % to 18 %. Since actin is essential for cellular function (Mishra *et al.*, 2014) and these percentages fell within the fraction of cells that were estimated to have lost viability by fluorescent staining (Fig 4.1B), these cells were assumed to be non-viable.

The fraction of cells containing actin bodies, reminiscent of SP cultures, remained below 10 % and remarkably did not increase with increasing intensity of calorie restriction (Fig. 4.3). To verify whether our experimental conditions were misguidedly preventing the formation of actin bodies, we interrupted the glucose feed of our retentostat cultures, thereby triggering glucose starvation and culture entry into SP. Within 2 h after termination of the glucose feed, over 40 % of the cells in 21-day-old retentostat cultures contained clear actin-body structures (Fig. 4.3), starting from 7 %, thereby confirming that non-growing, extremely calorie-restricted retentostat cultures present a different physiological state from glucose-starved SP cultures (Boender *et al.*, 2011a; Laporte *et al.*, 2011; Sagot *et al.*, 2006). More specifically, the large majority of viable cells in the retentostat cultures gradually arrested cell division in a cell cycle stage characterized by depolarized actin patches.

Cellular *HSP12* and *HSP26* absolute transcript levels reveal increased population homogeneity during the transition to growth arrest.

Analyses of DNA content and actin structures suggested strong population homogeneity during the transition from growth to growth arrest under ECR. To more precisely identify if the population was differentiating into quiescent and non-quiescent cells as previously described for SP cultures (Allen *et al.*, 2006), we chose two quiescence-specific marker transcripts. Transition to SP triggers the expression of many genes, including genes involved in stress tolerance such as heat-shock proteins (Martinez *et al.*, 2004). The small heat-shock protein encoding genes *HSP12* and *HSP26* are strongly up-regulated in quiescent cells during SP (Aragon *et al.*, 2008). Hsp12 is a membrane-bound protein that is important for dietary-restriction-induced replicative lifespan extension and maintenance of membrane

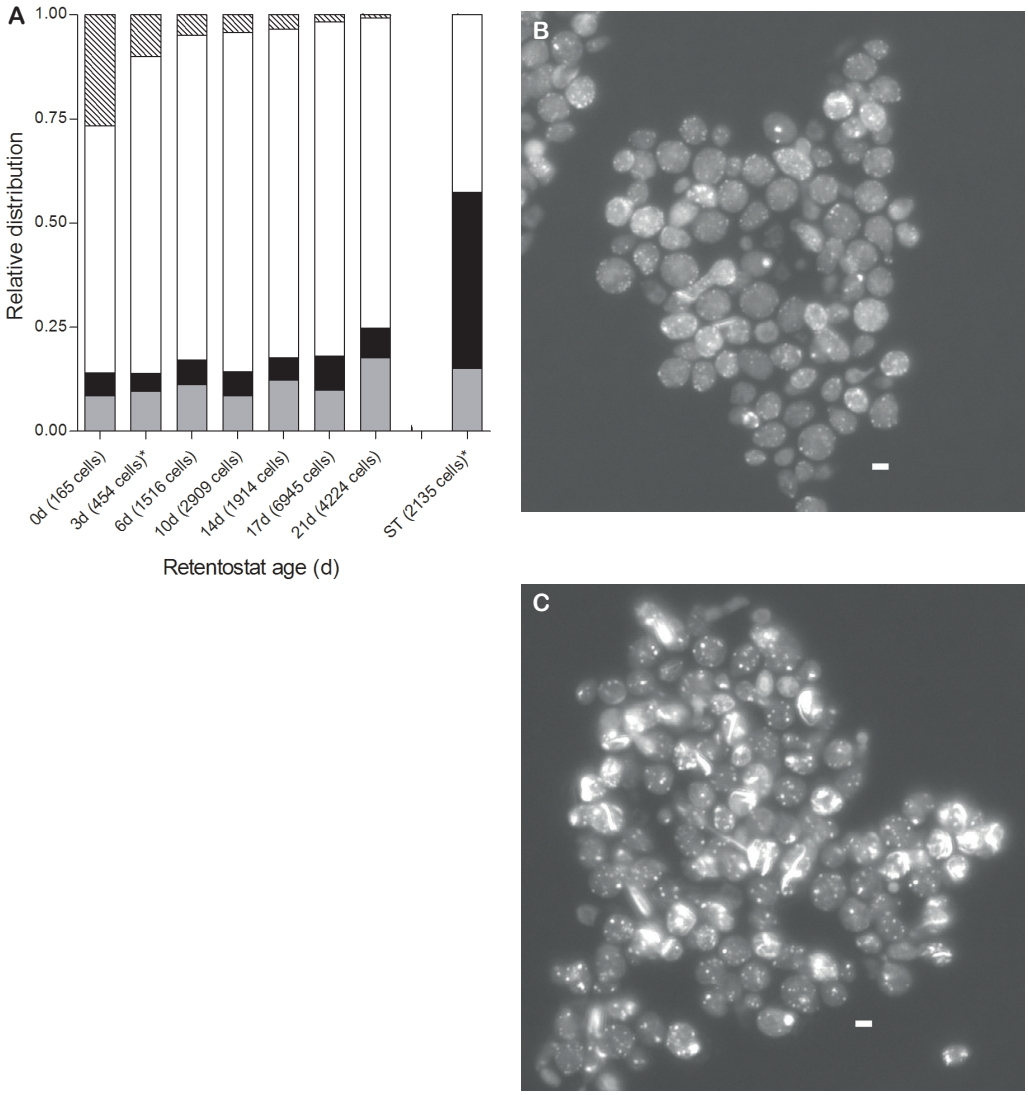


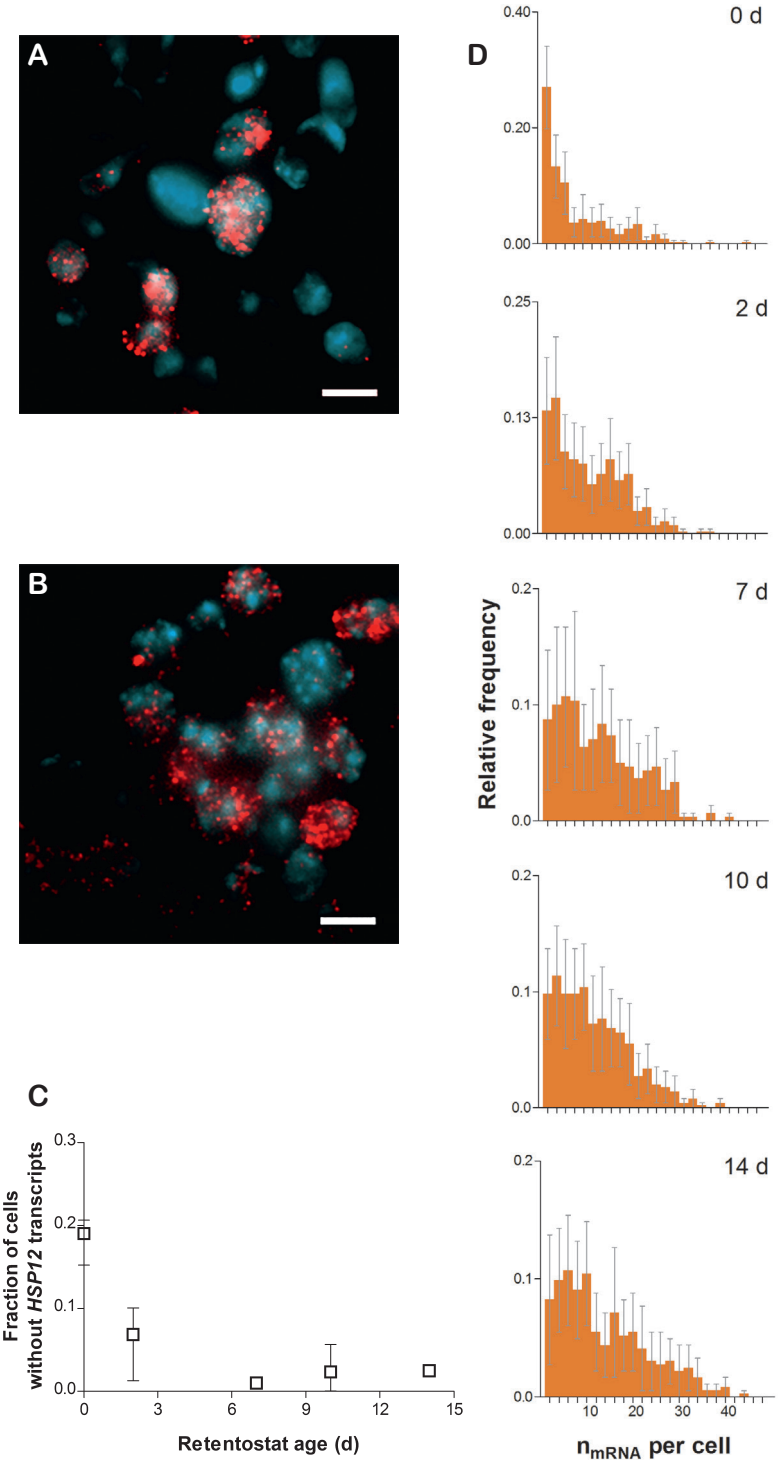
Figure 4.3 Actin structure dynamics during transition to ECR

(**A**) Distribution of cells containing either one of three observed F-actin structures; cables (hatched), depolarized patches (white) and bodies (black) or no apparent F-actin structure (grey) at 0, 3, 6, 10, 14, 21 d of retentostat cultivation and 2 h after feed termination (ST). Relative distributions were calculated from combined observations on two independent replicate cultures. Differences between individual cultures were below 4 %. Numbers in parenthesis indicate the numbers of cells analyzed for each time point. Time points indicated with * were analyzed in single cultures only. (**B**) Fluorescence micrograph of F-actin structures in yeast cells from 21 d retentostat cultures stained with Alexa488-Phalloidin conjugate. Scale bar represents 5 µm. (**C**) Fluorescence image of F-actin structures in yeast cells from 21 d retentostat cultures 2 h after feed was terminated, stained with Alexa488-phalloidin conjugate. Scale bar represents 5 µm.

function. Hsp12 protein levels are up to 37-fold higher in quiescent cells than in non-quiescent cells (Davidson *et al.*, 2011; Herbert *et al.*, 2012; Welker *et al.*, 2010). In addition to higher *HSP26* transcript levels in quiescent cells, also the protein levels of Hsp26, a chaperone that prevents protein aggregation, were significantly higher in quiescent cells than in non-quiescent cells (Burnie *et al.*, 2006; Haslbeck *et al.*, 1999; Webb *et al.*, 2013). Transcripts of *HSP12* and *HSP26* genes and the corresponding proteins were also found to be present at high levels in retentostat cultures at the whole population level (Binai *et al.*, 2014; Boender *et al.*, 2011b). *HSP12* and *HSP26* were therefore chosen as targets for mRNA FISH.

As also observed during SP, yeast cells subjected to ECR exhibit an increased cell wall thickness (Boender *et al.*, 2011b; Werner-Washburne *et al.*, 1993). Since the yeast cell wall is a permeability barrier for fluorescently labelled oligonucleotides (Scherrer *et al.*, 1974), cell wall degradation is a crucial step in yeast mRNA FISH protocols (Youk *et al.*, 2010; Zenklusen *et al.*, 2008). Special attention was therefore given to cell permeabilization. To degrade the cell wall of retentostat-grown yeast cells, lyticase treatments were extended to up to 135 min, while carefully monitoring spheroplast formation by phase-contrast microscopy (see Supplementary Fig. S4.1). Experiments yielded staining of above 90 % of the cells (Fig. 4.4), indicating successful cell processing.

At the onset of biomass retention, in slowly but exponentially growing cultures, single-cell mRNA copy-number distributions for *HSP12* and *HSP26* indicated that the majority of the cells have low transcript levels. At this stage, a substantial fraction of the cell population did not show any transcript (19 % and 27 %, respectively; Fig. 4.4C,G). As the growth rate in the retentostat cultures decreased, the percentage of cells containing *HSP12* mRNA molecules increased from 81 % to above 98 % (Fig. 4.4C). A similar trend was observed for *HSP26*, with the fraction of cells containing one or more copies of its transcript increasing from 73 % to 96 % (Fig. 4.4G). The trends in average expression of these two genes, as determined by mRNA FISH, were consistent with previous whole-culture micro-array-based transcriptome analysis (Supplementary Fig. S4.4). In addition to the increasing fraction of cells that contained transcripts of the respective genes, this increase in overall *HSP12* expression was caused by an increase of the average transcript number, from 7 to 13, in *HSP12*-expressing cells. Average *HSP26* transcript numbers in cells expressing *HSP26* showed a similar or even stronger increase, from 7 to 16 per cell. As the average transcript number per cell became higher, also the absolute variation in the single-cell transcript numbers increased (Fig. 4.4D,H). However, during the first days of retentostat cultivation, when the reduction in growth rate was most pronounced, coefficients of variation (CV) strongly decreased (Table 4.1), indicating that the relative population heterogeneity of transcript levels of these two stress-related genes decreased as the cultures proceeded towards growth arrest. The decrease in cells without *HSP12* or *HSP26* transcripts, as well as the absence of a clear bimodality in the relative frequency distributions of both transcripts (Fig. 4.4D,H) implied the absence of two clearly distinct populations with regard to *HSP12* and *HSP26* expression levels.


A
B
C
D
Fraction of cells without HSP12 transcripts
0.3
0.2
0.1
0.0
0
3
6
9
12
15
Retentostat age (d)
0 d
2 d
7 d
10 d
14 d
0.40
0.20
0.00
0.25
0.13
0.2
0.1
0.0
Relative frequency
10
20
30
40
nmRNA per cell

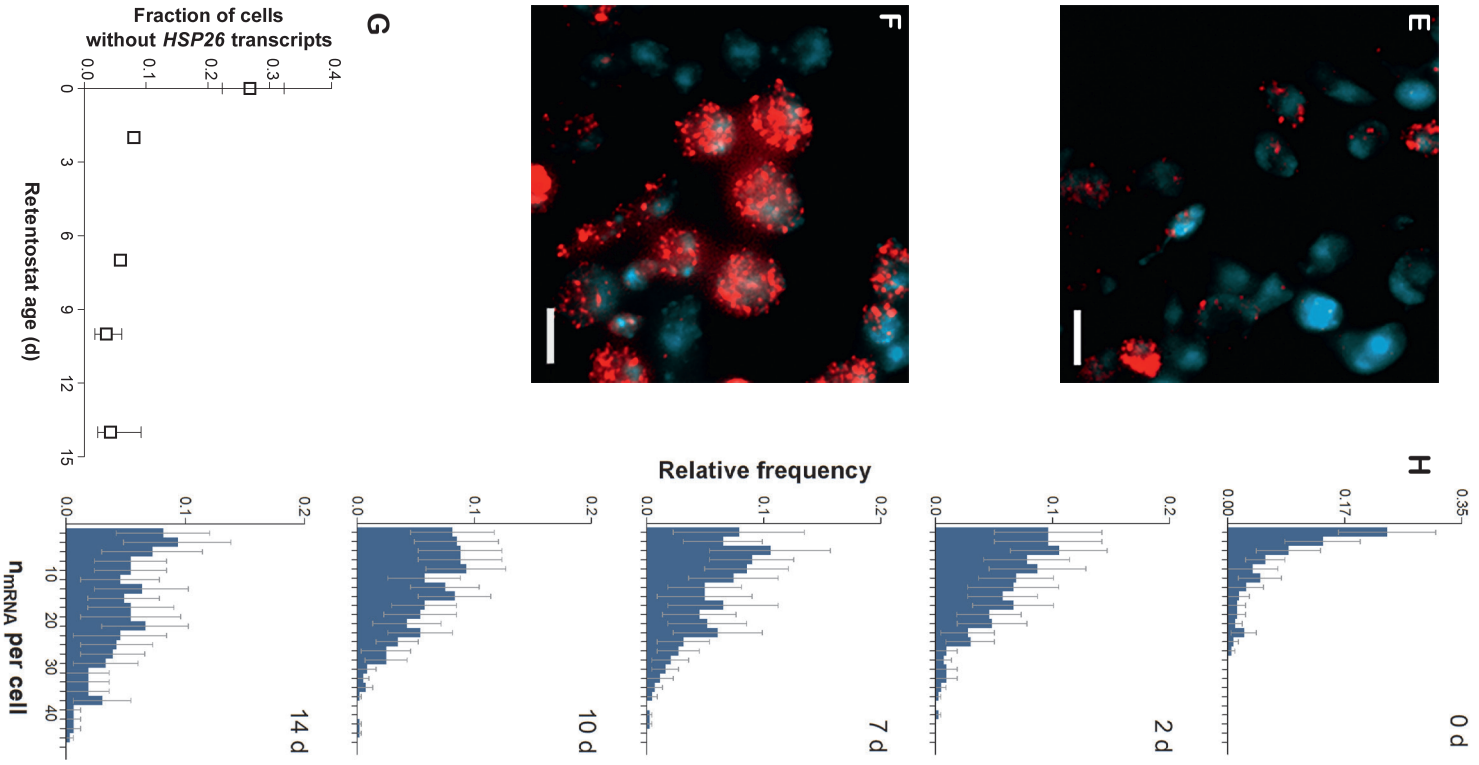


Figure 4.4 FISH of* HSP12 *and* HSP26 *transcripts during transition to ECR in retentostat cultures

Panels (**A**) to (**D**) represent the analysis of *HSP12* transcripts and (**E**) to (**H**) of *HSP26* transcripts. (**A**) and (**E**) show fluorescent imaging of cells from zero days old retentostat cultures stained by mRNA FISH. Transcripts are represented in red and DNA is visualized by DAPI staining in blue. The scale bar represents 5 µm. (**B**) and (**F**) show the same FISH analysis as (A) and (E) but for cells from 14 days old retentostat cultures. (**C**) and (**G**) show the fraction of cells devoid of transcripts in 0, 2, 7, 10 and 14 days old retentostat cultures. Data are shown as weighted average values of two independent culture replicates and error bars indicate the deviation from this value in the individual cultures. (**D**) and (**H**) report the frequency distributions of transcript number per cell of duplicate retentostat cultures of 0, 2, 7, 10 and 14 days old for cells containing at least one transcript. Data from two independent replicate cultures were combined. A bin width of two was used for the histograms. Error bars indicate the 95% confidence interval estimated by bootstrapping. Frequency distributions of the individual cultures can be found in Supplementary Fig. S4.2A for *HSP12* and in S4.3A for *HSP26*. The number of analyzed cells from 0, 2, 7, 10 and 14 days old retentostat cultures was 352, 452, 299, 511 and 364 respectively for *HSP12*, and 373, 436, 445, 614 and 332 respectively for *HSP26*.

Table 4.1 Single-cell transcript analysis of* HSP12 *and* HSP26 *in retentostat cultures: mRNA counts and concentrations.

	HSP12 transcripts					*HSP26* transcripts				
Culture age (d)	Number of cells analysed	mean number ($cell^{-1}$)[a]	CV[b]	mean concentration (μm^{-2})[c]	CV[d]	Number of cells analysed	mean number ($cell^{-1}$)[a]	CV[b]	mean concentration (μm^{-2})[c]	CV[d]
0	352	6.2	1.22	0.56 (± 0.03)	1.04**	373	4.9	1.27	0.49 (± 0.03)	1.14*
2	452	9.8	0.79	0.95 (± 0.03)	0.67**	436	11.0	0.78	0.93 (± 0.03)	0.63**
7	299	12.6	0.68	1.10 (± 0.03)	0.55**	445	12.9	0.72	1.01 (± 0.03)	0.59**
10	511	11.3	0.70	1.04 (± 0.03)	0.58**	614	12.7	0.68	1.08 (± 0.03)	0.58**
14	364	13.0	0.73	0.98 (± 0.03)	0.60**	332	15.6	0.73	1.09 (± 0.04)	0.60**

Cells from duplicate anaerobic retentostat cultures, sampled at different time points, were analyzed for *HSP12* or *HSP26* transcripts by mRNA-FISH. [a]For each transcript, the mean number of transcripts for all cells analyzed is shown, as well as the corresponding [b]coefficient of variation (CV). Transcript concentrations were calculated based on cross-sectional areas of cells and represented as [c]mean values and [d]CV. Asterisks indicate a significant reduction in CV of transcript concentrations compared to the CV of absolute transcript numbers per cell (*p-value below 0.03,**p-value below 0.005). Values in parentheses are the standard error of the mean values for the concentrations.

Absolute single-cell *HSP12* and *HSP26* mRNA numbers correlate with cell size

The cell size and, therefore, the cellular volume of *S. cerevisiae* is growth-rate dependent (Porro *et al.*, 2003; Silljé *et al.*, 1997). A tight link between gene expression and cell size has been observed using whole-population studies in several organisms (Marguerat and Bähler, 2012). During transition to zero growth in retentostat cultures, the specific cellular volume increases (Bisschops *et al.*, 2014), as also indicated by the cross-sectional areas of cells (Fig. 4.5). This approximately 1.5-fold increase in cell volume might contribute to the observed increase in the numbers of *HSP12* and *HSP26* transcripts in cells.

Although yeast cells are not perfectly spherical, the cross-sectional area gives an indication of actual cell size and volume and was used to test if transcript numbers correlated with cell size. Pearson's product-moment correlation coefficients (Pearson r coefficients) were calculated for transcript numbers and cross-sectional areas at different retentostat ages, resulting at all sampling points and for both genes in significant correlation coefficients ranging from 0.24 to 0.63 (p-value <0.0001) (Fig. 4.6A,B). Furthermore, for both genes and at all sampling points, normalization of the cellular transcript number for cross-sectional area, resulted in significantly lower CV values in transcript number distributions (Table 4.1). Changes during retentostat cultivation of the average transcript number per area were less pronounced than changes of the average absolute transcript numbers per cell (Fig. 4.6C,D). Average absolute transcript numbers per cell increased by 2.1 and 3.2 fold for *HSP12* and *HSP26* respectively, while the average transcript per area increased only by 1.6 and 2.0 fold, respectively. Transcript levels that are proportional to cellular volume are in line with data observed for human cells (Kempe *et al.*, 2015; Padovan-Merhar *et al.*, 2015). The frequency distributions of the area-normalized transcript numbers furthermore supported the conclusion that clearly distinct sub-populations within these ECR cultures were absent.

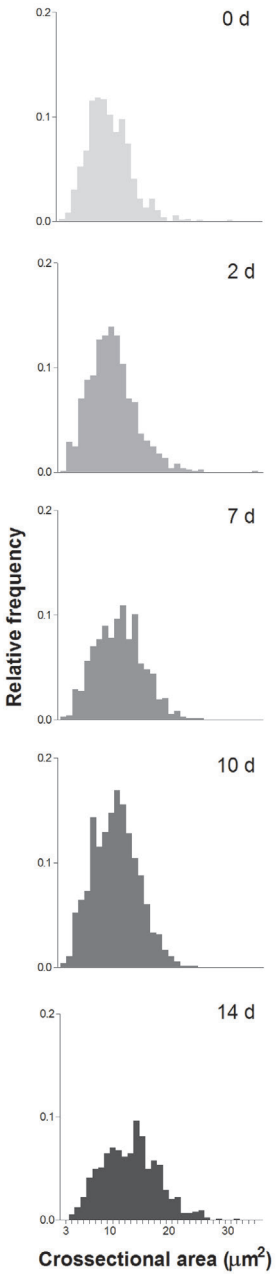


Figure 4.5 Cross-sectional areas of cells analyzed by mRNA FISH
Cross-sectional areas of yeast cells from duplicate retentostat cultures, sampled after 0, 2, 7, 10 and 14 d of retentostats cultivation, that were used for the mRNA FISH analysis presented in Fig. 4.4.

Discussion

Non-dividing but metabolically active cells remain in an extended G1 phase

Both F-actin and single-cell *HSP12* and *HSP26* transcript analyses in retentostat cultures revealed that, during the smooth transition from slow growth to virtual absence of cell division, the vast majority of cells arrested in a similar state. No evidence was obtained for the formation of clear subpopulations that has been reported for carbon-starved SP cultures (Allen *et al.*, 2006; Aragon *et al.*, 2008; Li *et al.*, 2013). Growth arrest during ECR in the form of mostly unbudded cells with non-replicated DNA suggested that cells resided in a relatively inactive phase of the cell cycle, most likely a specific stage in G_1, or became quiescent.

Another striking difference between retentostat and SP cultures was that only a small, stable fraction of fewer than 10 % of the cells contained actin bodies. The strong increase in the incidence of actin-body-containing cells that was observed when retentostat cultures were subjected to complete glucose starvation (Fig. 4.3C), showed that their absence in ECR cells was not due to the strain or anaerobic conditions used and strongly depended on the presence of extracellular energy and carbon source. The absence of actin bodies in over 90 % of the cells in ECR cultures therefore demonstrated that these cells neither experience complete carbon starvation nor enter quiescence. Depolarized patches of F-actin have previously been observed during transient growth arrest in non-starved *S. cerevisiae* cultures (Sagot *et al.*, 2006). Their presence in most cells under ECR is therefore in agreement with the survival of yeast cells in the form of non-dividing but non-quiescent cells. Together with previous studies (Amberg, 1998; Laporte *et al.*, 2011; Sagot *et al.*, 2006), the present work demonstrates that F-actin structures are useful, sensitive reporters of cell division in *S. cerevisiae*.

The conclusion, based on the absence of actin bodies and presence of actin patches of F-actin in the majority of cells in retentostat cultures, that ECR cells remain in an extended G_1-phase is in line with previous observations on glycogen accumulation, on cell-size increase and on the crucial role of the PAS-kinase Rim15 in ECR cultures (Bisschops *et al.*, 2014). Furthermore, it is consistent with the observation that cells under ECR do not completely shut down metabolic and cellular activity (Boender *et al.*, 2011a). The continuous supply of nutrients enabled cells to maintain a level of activity required to repair and turn-over macromolecules and organelles as well as to maintain cellular homeostasis and thereby prevent strong senescence and cell death.

Inferring *HSP12* and *HSP26* expression modes in growing and non-growing cells from transcript distributions

Gene expression is intrinsically stochastic due to the involvement of many events in, among others, transcription regulation, initiation and subsequent transport of mRNAs and translation initiation (Bajic and Poyatos, 2012; Kumar *et al.*, 2014; Murrugarra *et al.*, 2012; Raj and van Oudenaarden, 2008; Schwabe *et al.*, 2012). Gene expression can occur in transcription bursts, whereby the gene switches between an on- and off-state (Chubb *et al.*, 2006; Golding *et al.*, 2005; Kumar *et al.*, 2014; Raj *et al.*, 2006; Schwabe *et al.*, 2012; Suter *et al.*, 2011; Zenklusen *et al.*, 2008). Assuming that the yeast population in retentostat culture is isogenic and that all cells experience the same, albeit changing, environment, the frequency distributions of transcripts may provide information on the mode of transcriptional regulation of the corresponding genes. In the chemostat cultures, i.e. slow but steadily growing cultures, that were the starting point of retentostat cultures, a near-geometric distribution of *HSP12* and *HSP26* transcript numbers per cell was observed. Such a distribution suggested a stochastic expression, characterized by genes that, for most of the time, are in an off-state, which is incidentally interrupted by an on-state (Schwabe *et al.*, 2012; Suter *et al.*, 2011). The presence of *HSP12* and *HSP26*, considered as quiescence hallmarks in exponentially, albeit slowly, growing cells was surprising. The stochastic expression of these two genes encoding proteins involved in stress resistance may contribute to create a phenotypic heterogeneity that allows a part of the population to survive sudden environmental perturbations, such as heat-shock. This mode of expression of *HSP12* and *HSP26* might therefore be a conditional bet hedging strategy (de Jong *et al.*, 2011; Mineta *et al.*, 2015), providing a slowly growing population with a survival mechanism against sudden environmental cues without substantially affecting overall growth.

Transition to calorie restriction clearly modified the distribution of *HSP12* and *HSP26* transcripts among the cell population. As ECR set in, the distribution became broader (Fig. 4D,H), thus indicating that genes resided for longer periods of time and/or more often in an active state (Schwabe *et al.*, 2012). This increased transcriptional activity, and consequently increased transcript levels, in response to extreme calorie restriction is likely to reflect an enhanced activity of transcription factors affecting *HSP12* and *HSP26*. Both genes are under control of the transcription factors Msn2/Msn4 and Hsf1 (Karreman and Lindsey, 2005; Susek and Lindquist, 1990). These transcription factors are known to increase in number and activity upon nutrient limitation, amongst others through the Rim15 kinase (Smets *et al.*, 2010).

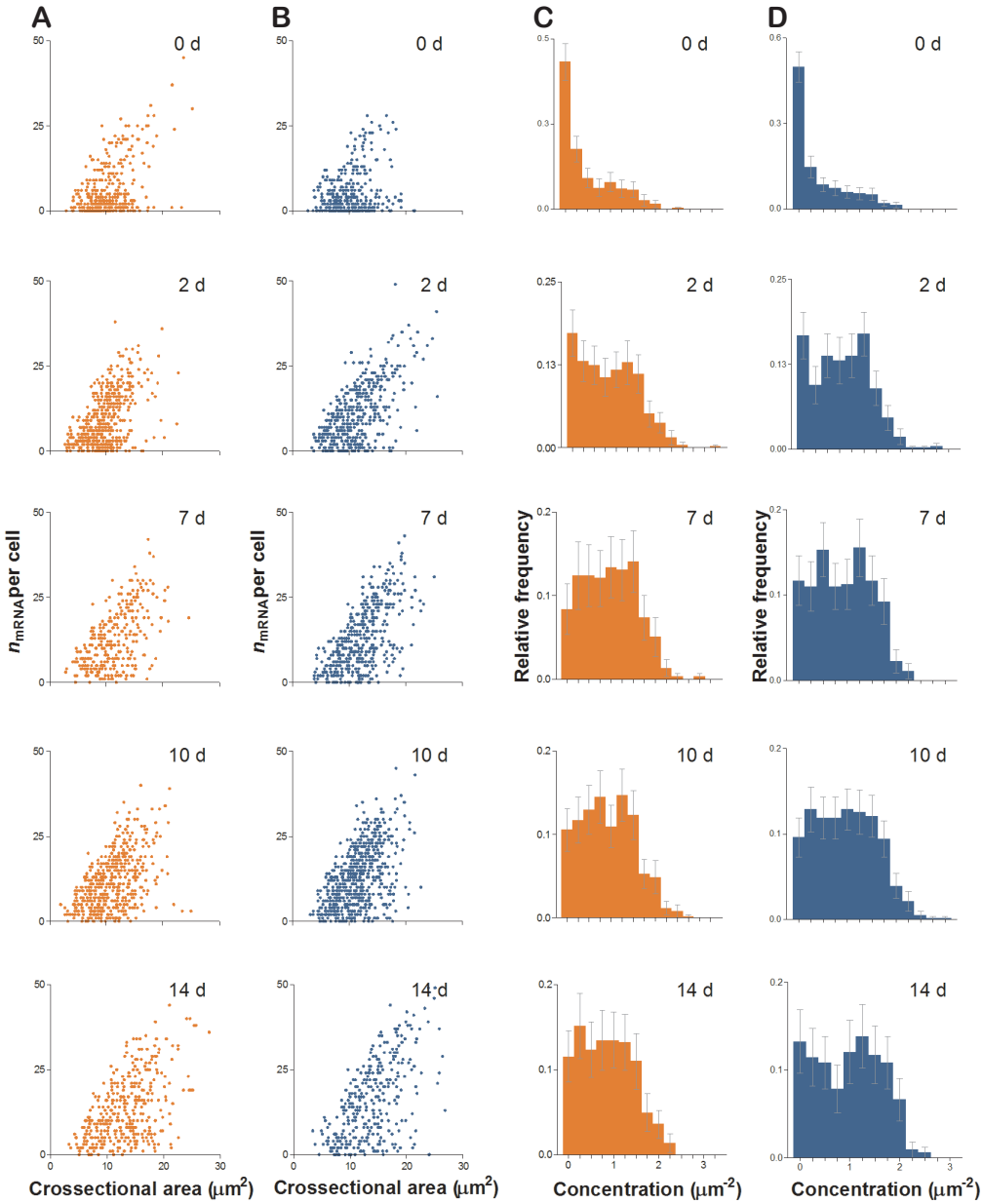


Figure 4.6 Correlation between mRNA copy numbers and cross-sectional area per cell in yeast retentostat cultures

(**A**) Correlation between the number of *HSP12* transcripts per cell and the cross-sectional area per cell (µm²) in 0, 2, 7, 10 and 14 d old retentostat cultures. *r* indicates significant Pearson r coefficient (*p*-value < 0.0001). (**B**) Same for the number of *HSP26* transcripts per cell. (**C**) Frequency distributions of *HSP12* transcript concentrations calculated based on cross-sectional areas. Distributions are shown for all cells analyzed from two independent replicate cultures, bin width is 0.25 µm^{-2}. Error bars indicate the 95% confidence interval, estimated by bootstrapping. (**B**) Idem for *HSP26* transcript concentrations. Distributions for the individual cultures can be found in Supplementary Fig. S4.2B and S4.3B.

Previous genome-wide expression analysis of retentostat cultures showed upregulation of Msn2/Msn4 and Hsf1 targets in response to ECR (Boender *et al.*, 2011b). *HSP12* and *HSP26* promoters harbor different numbers of binding sites for Msn2/Msn4 and Hsf1 (Karreman and Lindsey, 2005; Susek and Lindquist, 1990), which might explain differences, if any, in transcript frequency distributions observed for these two genes.

Transcript abundance is the net result of mRNA synthesis and decay rates. As demonstrated for cellular responses to nitrogen and glucose, cells can use several mechanism to optimize their response to nutritional changes by accelerating or decelerating the decay of specific sets of transcripts (Benard, 2004; Braun *et al.*, 2014; Braun and Young, 2014; Talarek *et al.*, 2010). While RNA synthesis is not as energetically expensive as protein synthesis, transcript synthesis requires cellular building blocks and energy (Verduyn *et al.*, 1990). Under ECR, to enable the expression of genes while resources are scarce, cells might tune down the decay rates of specific transcripts such as *HSP12* and *HSP26*, thereby increasing the cellular levels of these transcripts in a cost-efficient manner. In agreement with this hypothesis, ECR has been shown to trigger a decrease in abundance of several proteins involved in mRNA degradation, such as Lsm1-5 and Dcp1 (Binai *et al.*, 2014). These observations suggest the possibility that, in *S. cerevisiae*, a hitherto unidentified regulation mechanism down-regulates mRNA decay in response to calorie restriction.

Cell-size dependent gene expression in *S. cerevisiae*

The identification of significant, positive correlations between transcript numbers and cross-sectional areas suggested that the transcription of *HSP12* and *HSP26* is not solely responding to external cues, i.e. calorie restriction, but also to cell size (Fig. 4.6). Cell size is known to regulate transcription in a global manner in a wide variety of, if not all, organisms, including yeasts (Marguerat and Bähler, 2012). These correlations between cell size and gene expression in yeast species have been established using mutants with an altered cell size, such as cell-cycle mutants and polyploid strains (Wu *et al.*, 2010; Zhurinsky *et al.*, 2010). The correlation between cell size and transcription has consequently been strongly attributed to cellular growth, but also linked to cellular DNA content (Marguerat and Bähler, 2012; Shahrezaei and Marguerat, 2015). However, comparison of mutants or strains with various ploidies may bias the correlation between cell size and transcript concentration. Differences in transcript numbers and cell sizes between isogenic cells in the same environment have so far only been studied in cells of higher eukaryotes, i.e. of mammals or *Caenorhabditis elegans* (Kempe *et al.*, 2015; Padovan-Merhar *et al.*, 2015). These studies identified a strong correlation between transcript abundance and cell size and proposed putative mechanisms that allow cells to obtain similar concentrations in differently sized cells (Kempe *et al.*, 2015; Padovan-Merhar *et al.*, 2015). The absence of reports on similar observations in microbes might be partly explained by the still limited number of studies employing single-cell mRNA techniques in microorganisms, including few studies on yeast. Besides the work presented here and one previous study (van Werven *et al.*, 2012), all mRNA FISH analyses reported for yeast have been performed on growing cultures. Although growing cultures display a strong heterogeneity in cell size (Li *et al.*, 2013), this heterogeneity is mostly caused by the different cell cycle phases in which the cells reside (Jorgensen *et al.*, 2007). Transcriptional

and translational activities of genes vary between cell cycle phases and growth in both global and gene-specific modes (Schmoller *et al.*, 2015; Shahrezaei and Marguerat, 2015) and this influences the correlation between transcript numbers and cell size. In addition, the variation in size between cells that reside in the same cell cycle phase is usually limited and thereby reduces the detectability of a correlation between cell size and transcript numbers. Furthermore significant correlations between transcript numbers and cell size might be harder to detect for genes with low expression levels due to the relatively large molecular fluctuations. A large fraction of the yeast genes is actually expressed at low levels, roughly 40 % of protein-encoding genes are estimated to be expressed at an average of 2 or fewer mRNA copies per cell in *S. cerevisiae* and *Schizosaccharomyces pombe* (Marguerat *et al.*, 2012; Miura *et al.*, 2008). The ability to identify significant correlations between transcript levels and cell size might thus depend on the selected gene.

By eliminating cell cycle effects and offering a considerable variability in cell size, the use of non-dividing cultures, combined with an appropriate selection of target genes, may be highly useful for deciphering cellular mechanisms behind concentration homeostasis. Retentostat cultures fulfil these requirements (similar cell cycle phase shown in Fig. 4.2, variation in cell size shown in Fig. 4.5). The existence of such a correlation suggests that cells strive for optimal transcript concentrations rather than absolute numbers. The mechanism underlying cell-size dependent regulation of transcript levels are still not fully understood in higher eukaryotes and yet unexplored in yeast (Marguerat and Bähler, 2012; Padovan-Merhar *et al.*, 2015). Utilization of well-controlled cultivation systems such as retentostats may shed light on these mechanisms and identify whether in *S. cerevisiae* such size-dependent transcriptional regulation is gene-specific or is, as in higher eukaryotes (Padovan-Merhar *et al.*, 2015), a wide-spread mechanism. In the latter case this would have profound implications for the interpretation of transcriptome data as cell sizes would influence transcriptome sizes (Coate and Doyle, 2015).

Outlook

The present study unambiguously demonstrates that cells from non-growing *S. cerevisiae* cultures can very precisely tune their cellular and molecular responses to subtle variations in nutrient supply. Studying the transition in retentostat between exponential growth and non-dividing ECR and comparison with starved cultures should contribute to elucidate the mechanisms governing the critical cellular decisions to commit to a new cell cycle or to differentiate for survival in response to external stimuli. In addition, cultures stably maintained under ECR for prolonged periods of time offer a unique opportunity to identify and quantify the minimal, growth-independent cellular and molecular mechanisms required for cell survival such as organelles and macro-molecule turn-over. Explored in the easily accessible eukaryotic model *S. cerevisiae*, these poorly understood fundamental aspects should prove very useful for the understanding of non-dividing metazoan cells.

Materials and methods

Strains, media and cultivation methods

The prototrophic haploid laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D (*MAT*a *MAL2-8c SUC2* (Entian and Kötter, 2007; Nijkamp *et al.*, 2012)), obtained from Dr. P. Kötter (Frankfurt, Germany) was used for most experiments. For verification of DNA contents derived from cell-cycle distribution analyses (see below), the congenic prototrophic diploid laboratory strain CEN.PK122, obtained from the Euroscarf collection (Frankfurt, Germany), was used. Synthetic medium for glucose-limited anaerobic retentostat cultures, containing 50 $g \cdot L^{-1}$ glucose and the anaerobic growth factors ergosterol (10 $mg \cdot L^{-1}$) and Tween 80 (420 $mg \cdot L^{-1}$), was prepared according to Verduyn *et al*. (Verduyn *et al.*, 1992). Four anaerobic retentostat cultures, with controlled pH (5.0) and temperature (30 °C) and gassed with pure N_2 (Linde Gas Benelux The Netherlands), were performed as described previously (Boender *et al.*, 2009). In short, anaerobic chemostat cultures were run at a dilution rate of 0.025 h^{-1} until steady state was reached. Chemostat cultures were assumed to be in steady state when, after at least 5 volume changes, the culture dry weight and specific carbon-dioxide production rate changed by less than 3 % over 2 consecutive volume changes. When steady state was reached, the effluent was redirected through a 0.2 µm filter, resulting in full biomass retention in the bioreactor. Retentostat cultivation was then continued at a dilution rate of 0.025 h^{-1} for either 14 or 21 d. At the end of retentostat cultivation runs, cultures were switched from extreme calorie restriction to glucose starvation by terminating the medium feed.

Substrate, metabolite and biomass analyses

Culture samples for metabolite analysis were obtained by rapid quenching of the broth with cold steel beads (Mashego *et al.*, 2003). Concentrations of ethanol, glycerol, acetate, lactate, pyruvate and succinate in culture supernatants were determined by HPLC (Agilent, Santa Clara, CA) as described by Boender *et al*. (Boender *et al.*, 2009). Residual glucose concentrations were determined with a Bio-Rad Aminex HPX-87H column at 60°C with 5 mM sulfuric acid at an isocratic flow rate of 0.6 $mL \cdot min^{-1}$ using an Agilent HPLC coupled to a UV and RI detector. Exhaust gas from the cultures was cooled (2°C) to reduce evaporation and dried (Perma Pure Dryer) before carbon-dioxide levels were analyzed online as described previously (Boender *et al.*, 2009). Biomass concentrations were determined both as culture dry weight, according to the method of Postma *et al* (Postma *et al.*, 1989) and as cell counts, using a Z2 Coulter counter (Beckman Fullerton, CA).

Culture viability

Culture viability was measured as the fraction of cells positively stained by 5-CFDA,AM (Invitrogen, The Netherlands), as analyzed with a Quanta flow cytometer (Beckman, Fullerton, CA) following a previously described method (Boender *et al.*, 2011a).

Cell cycle distribution analysis

Cell cycle distribution of cells in retentostat cultures was determined based on the method described by Haase and Reed (Haase and Reed, 2001). Cells were sampled from 0, 3, 7, 10 and 14 d old retentostat cultures, washed with demineralized water, fixed in 70 % ethanol and stored at 4°C. To remove RNA, approximately $1 \cdot 10^7$ ethanol-fixed cells were washed using 50 mM Tris-HCl buffer (pH 7.5), suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mg · mL^{-1} RNase A and incubated at 37°C for 2 h. To remove proteins, trypsin (final concentration 3.3 mg · mL^{-1}) was added and cells were incubated for an additional 20 min at 37°C. Cells were subsequently washed, suspended in 50 mM Tris-HCl pH 7.5 with or without 1 µM Sytox Green and stored on ice. Prior to flow-cytometric analysis cells were sonicated at 6 µm peak-to-peak amplitude (MSE Soniprep 150, Fisher Scientific, Loughborough, UK) for 15 s and again stored on ice. Analysis was done on a BD-Accuri™ C6 flow cytometer equipped with a 488 nm excitation laser (Becton Dickinson, Franklin Lakes, NJ). After collection of a minimum of 30,000 events in fluorescence channel 1 (533/30 nm) with a threshold of 80,000 on forward scatter height (FSC-H) data were analyzed with the Cell Cycle platform in FlowJo v10.0.8 (FlowJo LLC, Ashland, OR) to determine fractions of cells with replicated DNA. Ranges for 1N and 2N peaks were constrained based on values of exponentially growing aerobic shake-flask cultures of the haploid *S. cerevisiae* strain CEN.PK113-7D and the diploid strain CEN.PK122.

Staining and imaging of actin structures

$1 \cdot 10^8$ cells were sampled directly from the bioreactor and fixed in 8 % formaldehyde in phosphate-buffered saline (PBS) buffer (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) solution pre-warmed to 30°C in a final 1:1 volume ratio after 0, 4, 8, 15 and 21 d of cultivation and after 2 h of subsequent starvation. Samples were incubated at least 10 min and no longer than 1 h at 30°C while shaking. Cells were then centrifuged (3824 · *g*) and resuspended in 1x PBS (pH 7.2) with sodium azide (0.3 mM) and stored at 4°C until staining. Staining of the actin cytoskeleton was done essentially as previously described (Amberg, 1998; Baggett *et al.*, 2001), with some minor modifications. $5 \cdot 10^7$ formaldehyde-fixed cells were resuspended in 1 mL of 0.2 % Triton X-100 and 4 % formaldehyde in PBS and incubated for 10 min. Cells were washed in PBS and suspended in 100 µL PBS. 10 µL of 200 U · mL^{-1} Alexa-488-phalloidin (A12379, Invitrogen, The Netherlands) was added and cells were stored in the dark for 1 h. After washing with 1x PBS buffer, cells were suspended in Gelvatol (prepared according to the protocol of the Center for Biologic Imaging, University of Pittsburgh, http://www.cbi.pitt.edu/protocols/gelvatol.htm) and stored overnight. Cells were imaged using a Zeiss D1 Imager using a 40X objective, HAL100 fluorescent lamp and Filterset 10 (Ex 450-490 nm/Em 515-565 nm) (Zeiss, Jena, Germany) equipped with an AxioCam HRm camera (Zeiss, Jena, Germany). Images were analyzed using ImageJ 1.46r (NIH, USA). Cells were scored based on F-actin structures: polarized patches and or filaments, depolarized patches, actin bodies or no clear actin structures. At least 400 cells per time-point and replicate were analyzed.

Fluorescent *in situ* hybridization of single mRNA molecules

Probe sets for mRNA fluorescent *in situ* hybridization (FISH) were designed using the Stellaris Probe Designer (Biosearch Technologies, Petaluma, CA) for the coding sequences of the *HSP12* and *HSP26* genes of the CEN.PK113-7D genome (Nijkamp *et al.*, 2012). Due to the short coding sequences, 327 nucleotides for *HSP12* and 642 nucleotides for *HSP26,* probe sets consisted of 16 and 28 19-mer probes labelled with the Quasar®-570 fluorophore by the supplier (BioSearch Technologies, Petaluma, CA) for *HSP12* and *HSP26*, respectively. Sequences of the probes are presented in Supplementary Table S4.1. Preparation of cells and hybridization was performed according to the method described by Schwabe *et al.* (Schwabe and Bruggeman, 2014), with minor modifications introduced to address differences in yeast strain and cultivation conditions. Approximately $4 \cdot 10^9$ cells were sampled directly in PBS + formaldehyde (4 % final concentration) from 0, 2, 7, 10 and 14 d old duplicate retentostat cultures. Cells in formaldehyde were incubated at 30°C for 20 min, under continuous shaking and subsequently stored overnight at 4°C. Cells were washed twice with spheroplasting buffer (1.2 M sorbitol, 0.1 M K_2HPO_4), suspended in 5.5 mL spheroplasting buffer containing 1 mg Lyticase (Sigma L5263) and incubated at 30°C with mild agitation. Progression of cell wall removal was monitored by phase-contrast microscopy. After approximately 50 % of the cells turned phase-contrast dark, i.e. after 105 to 135 min, cells were washed with ice-cold spheroplasting buffer and divided in 20 aliquots. After two additional washes with spheroplasting buffer, cells were suspended in 70 % ethanol and stored at 4°C. Cells were briefly rehydrated in washing buffer (10 % formamide in 1x SSC buffer) for 2 min at room temperature. After centrifugation ($380 \cdot g$, 5 min) hybridization buffer (washing buffer plus 1 mg $\cdot$ mL^{-1} *Escherichia coli* tRNA, 2 mM vanadyl ribonucleoside complex, 0.2 mg $\cdot$ mL^{-1} Bovine Serum Albumin and 0.1 g $\cdot$ mL^{-1} dextran sulfate (Sigma Aldrich, St. Louis, MO, USA)) containing the appropriate probe-set was added and hybridization took place overnight at 30°C. Cells were subsequently washed once using washing buffer ($380 \cdot g$, 5 min), suspended in the same buffer containing 5 ng $\cdot$ mL^{-1} DAPI and incubated for 30 min at 30°C under mild agitation. Subsequently cells were centrifuged, suspended in washing buffer and incubated for another 30 min at 30 °C under mild agitation. After centrifugation ($380 \cdot g$, 5 min) cells were suspended in gelvatol, mounted on glass slides and stored in the dark. Cells were imaged using a 150x 1.4 NA objective on an Olympus IX81 inverted stand Microscope (Olympus, Hamburg, Germany), equipped with an IXON3 897 EMCCD camera (Andor, Belfast, UK). Fluorescence of DAPI was detected using a 408 nm laser and Brightline filter set 11608B (Ex 380/396nm, Em 414/480nm) (Semrock, Rochester, NY), Quasar®-570 was imaged using a 561 nm Laser and Brightline filter set Cy3-4040C (Ex 508/554nm, Em 570/615nm) (Semrock, Rochester, NY). Cells were additionally imaged using differential interference contrast (DIC).

smRNA FISH image and data analysis

Image analysis was performed according to the method described by Raj and coworkers (Raj *et al.*, 2008), adjusted by Schwabe and coworkers (Schwabe and Bruggeman, 2014) with minor modifications. In short, DIC-images were used to manually create outlines of the cells. Only cells that showed a positive signal in the DAPI-channel were considered for analysis. Spot identification in Laplacian of Gaussian filtered images based on binarization was executed as described previously (Itzkovitz *et al.*, 2012). Thresholds showing the least variation were selected based on inverse coefficient of variation calculations over a window of 5 thresholds and corrected for small coefficients at variation (CV) at low thresholds by adding $\varepsilon = 2$ to the standard deviation. Settings, i.e. number of bins, minimal and maximal thresholds, were such that only few to zero spots were identified in cells that were processed without labelled probes as negative control in order to minimize false positives. As probes showed a loss in fluorescence intensity after freeze-thaw cycles, minimal intensity thresholds were lowered for analysis of images of the second replicate culture.

Bin sizes of the shown frequency distribution histograms were determined according to the Freedman-Diaconis rule (Freedman and Diaconis, 1981). Smallest bin sizes obtained per gene at all time points were used. Mean transcript numbers and the standard deviation were calculated per gene and per time point. Values for the mean and standard deviation were used to calculate CV. To obtain an estimate of the variance in the CV, a bootstrapping approach was followed. Bootstraps of the coefficients of variation were iterated 10,000 times using the standard bootstrap command in MatLab version R2015 (MathWorks, Natick, MA). For transcript numbers per cell divided by the cross-sectional area, i.e. the area within manually drawn outlines of cells, histogram bin size, mean and CV values were calculated similarly. Z-scores were calculated for the difference between CV of transcript numbers and CV of transcript concentrations based on variances obtained through bootstrapping. The probability of finding the observed difference in CV values while CV values are similar (p-values) were obtained assuming a normal distribution of CV. p-values below 0.05 were considered to indicate significant difference. Pearson correlation coefficients (Pearson, 1909) between cross-sectional areas and transcripts per cell were calculated using GraphPad Prism v 4.03 (GraphPad Software, La Jolla, CA) using a (two-tailed) p-value of 0.001 as threshold for significance.

Acknowledgements

We thank David Grunwald and Annette Ouwehand for advise in the experimental design of the mRNA FISH experiments, Joris Winderickx, Vanessa Franssens and Bernd Rieger for helpful discussions on F-actin structure imaging and analysis and Daniel Lam and the TUDelft Kavli Nanolab Imaging Center for help and access to their fluorescence microscopy facilities.

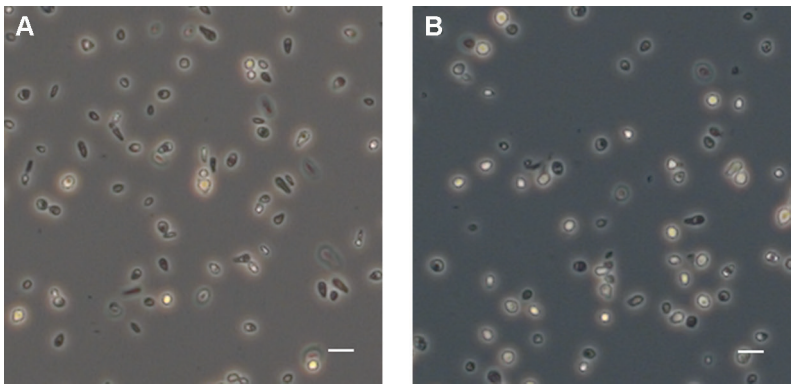
Supplementary materials Chapter 4

Supplementary Table S4.1 Sequences of probes used for FISH of *HSP12* and *HSP26* transcripts

Target	*HSP12*	*HSP26*
Sequences	ttctacctgcgtcagacat	ggcatcaacttcgttgttg
	agctttttcaccgaatcct	ttcacccagcaatctgtta
	gagtctggcttcaaagctt	tggtgcgtagcctcttaag
	accttgttcagcgtatgac	gtgtttgctaactgacgtc
	ttgtcagtgatgtattcct	cagtagaatcctttgcggg
	ttaccagcgaccttgtcgg	tggtctagcaacttccttg
	tgttgtcttctggttgaac	aaagagcgccagcatagtt
	gacaccttggaagacaccc	aggtttcatctctgggatc
	ccttttttcggcagagtcgt	attgtcgaaccaatcatct
	gaccttcagcgttatcctt	gatgggaacagggacaagt
	gtctgccaaagattcacct	ttctagggaaaccgaaacc
	cccatgtaatctctagctt	atcaactggaactgcgaca
	tcaacttggacttggcggc	tagttgttgtcatggtcca
	aacatattcgacggcatcg	accaggaaccacgactttc
	ttcaccgtggacacgaccg	atgtccttcttgcttttga
	tacttcttggttgggtctt	tggttcttgttttgatggt
		ggaatttcaccagaaacca
		actctcttcattcaaggta
		ttgaccttgaccttgtctt
		gaacttaccagagctgctc
		ggcaaagtgatgactctct
		catccacacctgggtagtc
		gtctgctttaatgttgtct
		gtcaaaacaccatttgcgt
		gcttcaattttggaactgt
		gttcttaccatccttctga
		acctcaatcttcttgacgt
		ccccacgattcttgagaag

Probes were labelled with Quasar®-570 Fluorophore and purchased from BioSearch Technologies, Novato, CA.

Supplementary Figure S4.1 Spheroplast formation of yeast cells from 0 and 14 day old retentostat cultures



Progression of cell wall removal was monitored by phase-contrast microscopy. After approximately 50% of cells became phase-dark, lyticase treatment was stopped. This was after 105 min for 0 d and 135 min for 14 d old retentostat cultures. Micrographs show the results of these treatments on 0 d (**A**) and 14 d (**B**) old cultures. Scale bar represents 5 µm.

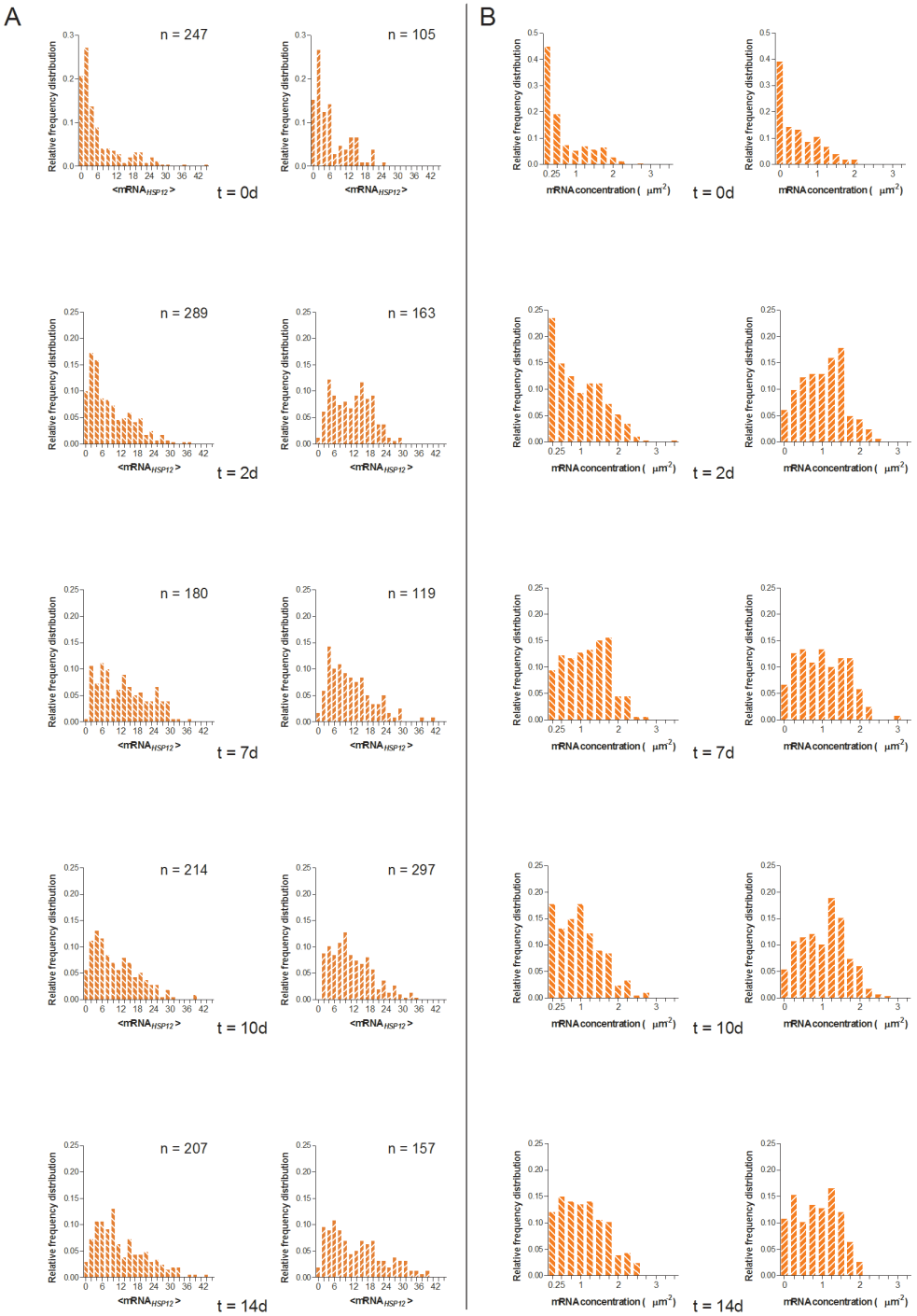
Supplementary Figure S4.2 *HSP12* transcript numbers and concentrations in individual replicate yeast retentostat cultures (p. 108)

Relative frequency distributions of the *HSP12* transcript numbers (**A**) and concentrations (**B**) measured in cells from 2 individual retentostat cultures. Bin widths are 2 (A) and 0.25 μm^{-2} (B) respectively. The combined data from these independent replicate cultures are shown in Fig. 4.4 and 4.6. Numbers indicate the numbers of cells analyzed per replicate.

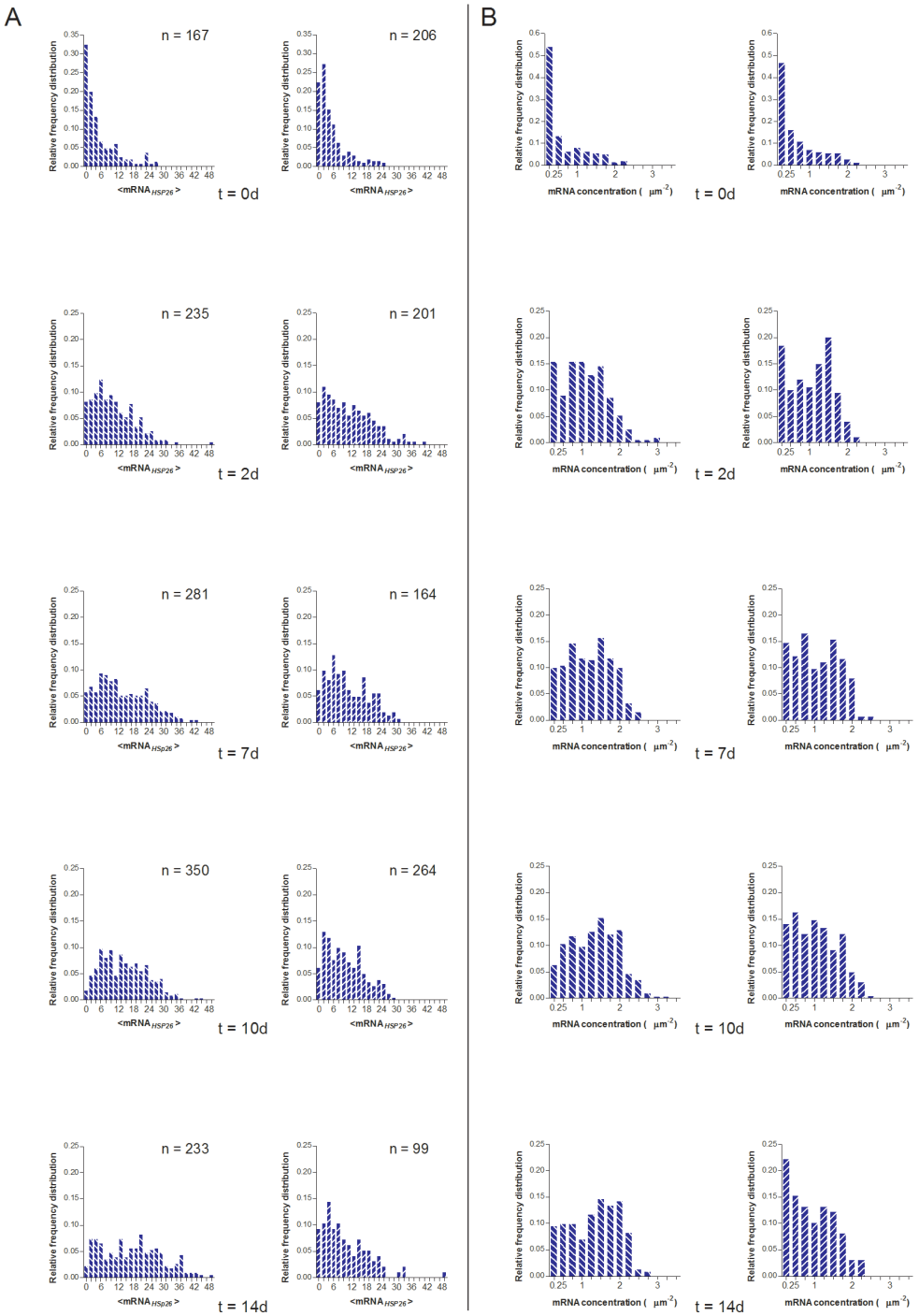
Supplementary Figure S4.3 *HSP26* transcript numbers and concentrations in individual replicate yeast retentostat cultures (p. 109)

Relative frequency distributions of the *HSP26* transcript numbers (**A**) and concentrations (**B**) measured in cells from 2 individual retentostat cultures. Bin widths are 2 (A) and 0.25 μm^{-2} (B) respectively. The combined data from these independent replicate cultures are shown in Fig. 4.4 and 4.6. Numbers indicate the numbers of cells analyzed per replicate.

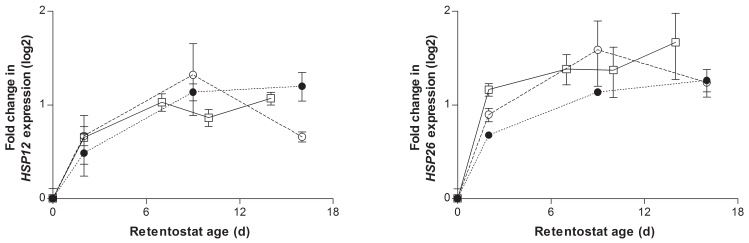
Supplementary Figure S4.2



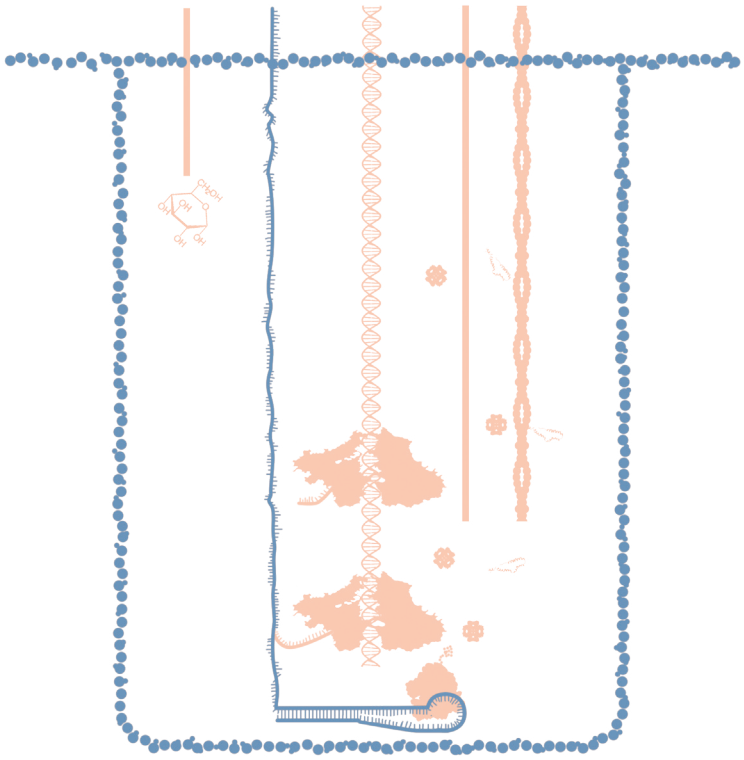
Supplementary Figure S4.3



Supplementary Figure S4.4 Trends in *HSP12* and *HSP26* expression in retentostat cultures as analyzed by 3 different approaches



The expression levels of *HSP12* (**A**) and *HSP26* (**B**) were analyzed previously at the whole-culture level at the transcript level (open circles, ○) (Boender *et al.,* 2011b) and protein level (closed circles, ●) (Binai *et al.,* 2014) and transcript levels in single cells (open squares, □) described in this study. Shown are average and SEM values for independent replicate retentostat cultures.



5. Oxygen availability strongly affects chronological lifespan and robustness in batch cultures of *Saccharomyces cerevisiae*

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Keywords: Chronological lifespan, thermotolerance, stationary phase, anaerobiosis, energetics, transcriptional response, conditioning

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Stationary-phase (SP) batch cultures of *Saccharomyces cerevisiae*, in which growth has been arrested by carbon-source depletion, are widely applied to study chronological lifespan, quiescence and SP-associated robustness. Based on this type of experiments, typically performed under aerobic conditions, several roles of oxygen in aging have been proposed. However, SP in anaerobic yeast cultures has not been investigated in detail. Here, we use the unique capability of *S. cerevisiae* to grow in the complete absence of oxygen to directly compare SP in aerobic and anaerobic bioreactor cultures. This comparison revealed strong positive effects of oxygen availability on adenylate energy charge, longevity and thermotolerance during SP. A low thermotolerance of anaerobic batch cultures was already evident during the exponential growth phase and, in contrast to the situation in aerobic cultures, was not substantially increased during transition into SP. A combination of physiological and transcriptome analysis showed that the slow post-diauxic growth phase on ethanol, which precedes SP in aerobic, but not in anaerobic cultures, endowed cells with the time and resources needed for inducing longevity and thermotolerance. When combined with literature data on acquisition of longevity and thermotolerance in retentostat cultures, the present study indicates that the fast transition from glucose excess to SP in anaerobic cultures precludes acquisition of longevity and thermotolerance. Moreover, this study demonstrates the importance of a preceding, calorie-restricted conditioning phase in the acquisition of longevity and stress tolerance in SP yeast cultures, irrespective of oxygen availability.

Introduction

Just like other living organisms, *Saccharomyces cerevisiae* cells age and have a finite chronological lifespan. The similarity of cellular processes in *S. cerevisiae* to those in higher eukaryotes and its accessibility to a wide range of experimental techniques have made this yeast a popular model for studying chronological aging of metazoan cells (Breitenbach *et al.*, 2012a; Denoth Lippuner *et al.*, 2014; Longo *et al.*, 2012; Mirisola *et al.*, 2014). Chronological aging of *S. cerevisiae* is typically studied in aerobic batch cultures, in which growth arrest and quiescence are triggered by exhaustion of the available carbon sources in the growth medium (Gray *et al.*, 2004; Longo and Fabrizio, 2012). Survival of individual yeast cells in such non-growing, stationary-phase (SP) cultures is then taken as a measure for their chronological lifespan (CLS). Over the past decade, studies on SP yeast cultures have contributed to our understanding of cellular mechanisms involved in aging, and several underlying cellular mechanisms were also found in higher eukaryotes (Fabrizio and Longo, 2003).

Calorie restriction has been shown to extend lifespan in organisms ranging from yeast to man, with studies on many organisms pointing at an important role of nutrient-signaling cascades (Sinclair, 2005). Turn-over of damaged macromolecules, and in particular proteins, has similarly been identified as a key process in aging in many organisms (Ryazanov and Nefsky, 2002). A third universal factor implicated in aging is respiration and, in particular, the associated formation of reactive oxygen species (ROS), which has been shown to enhance aging-related cellular deterioration in many organisms (Balaban *et al.*, 2005). However, ROS have also been implicated in beneficial effects. In particular, mild ROS stress has been proposed to contribute to CLS extension by inducing stress-resistance genes, a phenomenon known as hormesis (Ludovico and Burhans, 2014; Sharma *et al.*, 2011). Similarly, increased mitochondrial respiration and ROS production rates in calorie-restricted yeast cultures have been linked to CLS extension (Mesquita *et al.*, 2010; Ocampo *et al.*, 2012; Smith *et al.*, 2007).

ROS generation is not necessarily the only mechanism by which respiration and oxygen can affect CLS. In aerobic, glucose-grown batch cultures of *S. cerevisiae*, a fast and predominantly fermentative growth phase on glucose is followed by a second, respiratory growth phase in which the fermentation products ethanol and acetate are consumed (van Dijken *et al.*, 1993). This second growth phase, known as post-diauxic phase, is characterized by slow growth. During the post-diauxic phase, genes involved in SP are already expressed at an elevated level, as well as some features associated with SP cultures, such as increased stress resistance (Gray *et al.*, 2004). In anaerobic cultures of *S. cerevisiae*, the absence of oxygen prevents a respiratory post-diauxic growth phase. Instead, a phase of fast, fermentative exponential growth on glucose is immediately followed by SP, in which maintenance of viability and cellular integrity depends on metabolism of storage compounds. *S. cerevisiae* cells can contain two types of storage polymers: the storage carbohydrates trehalose and glycogen, and fatty acids, which are mostly stored in the form of di- and triacylglycerol esters

(François and Parrou, 2001; Kohei and Satoshi, 1984; Wilson and McLeod, 1976). In the absence of oxygen, yeast cells cannot catabolize fatty acids by β-oxidation and, moreover, conversion of storage carbohydrates via alcoholic fermentation yields 5-8 fold less ATP than their respiratory dissimilation (Boender *et al.*, 2011a).

Previous studies on the role of respiration in aging were predominantly based on the use of respiration-deficient *S. cerevisiae* mutants (e.g. ρ^0 strains and other mutants) (Aerts *et al.*, 2009; Barros *et al.*, 2010; Bonawitz *et al.*, 2007; Lin *et al.*, 2002; Tahara *et al.*, 2013) and respiratory inhibitors (Ocampo *et al.*, 2012). These approaches, however, have several drawbacks. Firstly, mitochondria are not only involved in respiration, but also in essential anabolic reactions (e.g., assembly of iron-sulfur complexes, amino acid biosynthesis and long-chain lipid biosynthesis (Breitenbach *et al.*, 2014)). Studies on petite or ρ^0 mutants may therefore cause unwanted 'side-effects' resulting from the absence or inefficiency of mitochondrial processes, rather than from direct effects of oxygen or respiration on aging. For example the absence of mitochondrial DNA influences crosstalk between these organelles and the nucleus (Woo and Poyton, 2009). Furthermore, inhibition of respiration may result in reduced ROS levels (Ocampo *et al.*, 2012), but can also result in ROS accumulation (Leadsham *et al.*, 2013), depending on the intervention chosen. In addition, ROS may still be produced by other oxygen-consuming processes in yeast, such as disulfide-bond formation during oxidative protein folding (Rosenfeld and Beauvoit, 2003).

Surprisingly, while *S. cerevisiae* is unique among yeasts and eukaryotes for its ability to grow fast under fully aerobic as well as strictly anaerobic conditions (Visser *et al.*, 1990), this ability has not been used to systematically investigate the impact of oxygen availability on entry into SP, on longevity and on robustness. The goal of the present study is therefore to investigate the impact of oxygen availability on yeast physiology in SP cultures. More specifically, we investigate whether the post-diauxic phase and respiratory mobilization of storage compounds in aerobic cultures affects CLS and thermotolerance during SP. To this end, aerobic and anaerobic bioreactor batch cultures of *S. cerevisiae* were grown into SP and subjected to detailed physiological and transcriptome analyses.

Results

Anaerobicity reduces chronological lifespan and stress resistance in stationary phase cultures

To investigate the impact of oxygen availability on chronological lifespan in SP cultures of *S. cerevisiae,* survival kinetics were analyzed during SP in aerobic and anaerobic, glucose-grown bioreactor cultures. In aerobic cultures, the percentage of cells capable of colony formation on complex-medium agar plates typically decreased to ca. 2 % in the 8 days following onset of SP, i.e. after exhaustion of all exogenous carbon sources including ethanol and organic acids (Fig. 5.1A). Viability of anaerobic cultures decreased much faster, to reach values below 1 % within 4.5 days after the onset of SP, that is after all exogenous glucose was consumed (Fig. 5.1A).

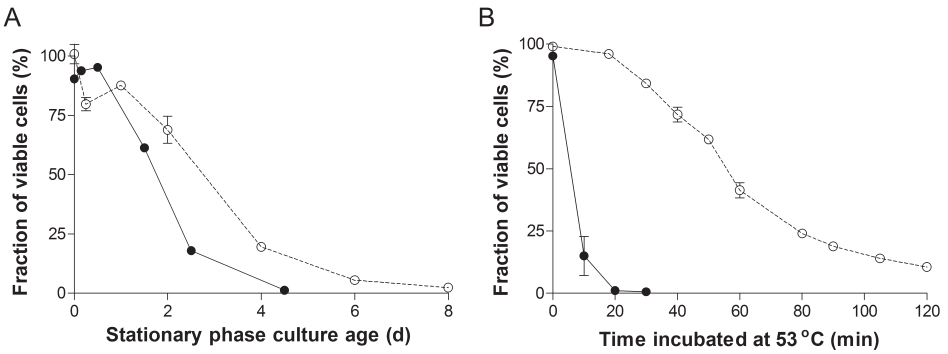


Figure 5.1 Chronological lifespan and thermotolerance of stationary-phase cultures is much lower under anaerobic than under aerobic conditions.
Chronological lifespan (**A**): survival expressed as ratio of colony forming units divided by the number of cells plated, during aerobic and anaerobic SP cultures. Time point zero indicates the onset of SP, which corresponds to exogenous glucose exhaustion in anaerobic cultures and exhaustion of all exogenous carbon sources including ethanol and organic acids in aerobic cultures. Thermotolerance (**B**): loss of viability after sudden exposure to 53°C of cells from aerobic and anaerobic cultures during early SP. Open symbols (○) represent aerobic cultures, closed symbols (●) anaerobic cultures. Data represent the average and SEM of measurements on independent duplicate cultures.

Increased thermotolerance is a well-documented characteristic of SP cultures of *S. cerevisiae* (Allen *et al.*, 2006; Werner-Washburne *et al.*, 1993). Indeed, half of the cells in samples from aerobic, early-SP cultures survived a 60-min exposure to 53°C. Notably, up to 20 minutes incubation at 53°C hardly affected viability, suggesting that cells were well capable of repairing heat-induced damage during this period. In contrast, fewer than 20 % of the cells from anaerobic early-SP cultures survived a 10-min incubation at this temperature (Fig. 5.1B). Implementation of anaerobic conditions during sampling and heat-shock assays did not significantly affect this difference, indicating that heat-induced loss of viability was not influenced by exposure of anaerobically grown cells to oxygen during the assays (data not shown). Furthermore, washing of cells prior to the heat-shock experiments did not influence heat-shock resistance, indicating that the presence of low (< 1 g · L^{-1}) ethanol concentrations in the assays did not cause the low thermotolerance of cells from anaerobic SP cultures.

Oxygen availability strongly affects the transcriptome of SP cultures

In aerobic yeast cultures, entry into SP is accompanied by a range of physiological changes that enhance survival in harsh, nutrient-poor environments (Werner-Washburne *et al.*, 1993). This adaptation coincides with a vast transcriptional reprogramming (Galdieri *et al.*, 2010; Martinez *et al.*, 2004; Wanichthanarak *et al.*, 2015) that includes up-regulation of genes involved in resistance mechanisms to a wide array of stresses. Currently, no transcriptome data are available in the literature on anaerobic SP cultures of *S. cerevisiae*.

A transcriptome analysis, performed on culture samples taken 4 h after the onset of SP, revealed that a quarter of the yeast genome (1452 genes, Supplementary Table S5.1) was differentially expressed (fold-change cut-off of 2.0 and adjusted *p*-value below 0.05)

in aerobic and anaerobic SP cultures. Among these genes were several genes known to be regulated by the heme and oxygen dependent transcription factors Hap1 and Rox1 (Harbison *et al.*, 2004; Keng, 1992; Ter Linde and Steensma, 2002). Approximately 40 % of the differentially expressed genes (574 genes, Supplementary Table S5.1) were transcribed at higher levels in the aerobic SP cultures. This gene set showed a strong overrepresentation of genes involved in fatty acid metabolism and, in particular, in β-oxidation (Table 5.1). This set of genes was also strongly enriched for genes that were up-regulated during SP in previous studies performed in shake flasks (Martinez *et al.*, 2004) (Table 5.1). Examples included the SP-genes *SPG1*, *SPG3*, *SPG4*, *SPG5*, and *SSA3*, which encodes a stress-induced ATPase. Furthermore, a significant number of genes (54) induced by the environmental stress response (Gasch *et al.*, 2000) was expressed at higher levels in aerobic SP cultures than in their anaerobic counterparts (Table 5.1).

Table 5.1 Functional categories overrepresented among genes with different expression levels in aerobic and anaerobic stationary-phase cultures.

Category description	*# of genes in dataset*	*# of genes in category*	*p-value*[a]
574 genes with higher expression in aerobic stationary phase cultures			
Genes induced in stationary phase (Martinez et al.*, 2004)*	53	122	$1.2 \cdot 10^{-22}$
Genes induced by environmental stress response (Gasch et al.*, 2000)*	54	281	$3.2 \cdot 10^{-6}$
Fatty acid metabolic process	13	29	$5.4 \cdot 10^{-4}$
Fatty acid beta-oxidation	7	9	$2.0 \cdot 10^{-3}$
Transmembrane transport	52	303	$3.8 \cdot 10^{-3}$
Glyoxylate cycle	6	8	$1.7 \cdot 10^{-2}$
878 genes with higher expression in anaerobic stationary phase cultures			
Translation	131	345	$4.1 \cdot 10^{-28}$
Genes induced by environmental stress response (Gasch et al.*, 2000)*	78	281	$1.7 \cdot 10^{-8}$
Mitochondrial translation	36	81	$1.9 \cdot 10^{-8}$
Oxidation reduction	74	270	$1.6 \cdot 10^{-6}$
Metabolic process	93	389	$2.6 \cdot 10^{-5}$
Response to stress	49	161	$3.2 \cdot 10^{-5}$
Heme biosynthetic process	10	12	$1.6 \cdot 10^{-4}$
Methionine metabolic process	11	15	$3.5 \cdot 10^{-4}$
Sulfate assimilation	9	11	$1.0 \cdot 10^{-3}$
Porphyrin biosynthetic process	8	9	$1.4 \cdot 10^{-3}$
Carbohydrate metabolic process	28	93	$4.2 \cdot 10^{-2}$
Glycolysis	14	32	$4.6 \cdot 10^{-2}$
Methionine biosynthetic process	14	32	$4.6 \cdot 10^{-2}$

[a]Bonferroni-corrected *p*-value cut-off of 0.05 was used and p-values indicate the probability of finding the same numbers of genes in a random set of genes. Functional categories are obtained from the Gene Ontology set or, in italic font, directly from literature references. Details can be found in Supplementary Table S5.2.

Most of the genes that were differentially expressed in aerobic and anaerobic SP cultures (60 %, 878 genes, Supplementary Table S5.1) showed a higher transcript level under anaerobic conditions. This gene set showed an overrepresentation of genes that were previously shown to be expressed at high levels in anaerobic yeast cultures and which were therefore not necessarily related to SP. This subgroup included genes involved in heme synthesis (Keng, 1992) and members of the multi-gene seripauperin family (Rachidi *et al.*, 2000) (Table 5.1 and Supplementary Table S5.2). Interestingly, a strong overrepresentation (131 out of 345 genes) was found for the GO category 'translation' (Table 5.1). This subset included many genes encoding cytosolic and mitochondrial ribosomal proteins (65 and 24 genes respectively). Furthermore, several genes involved in carbohydrate metabolism, including glycogen metabolism, were expressed at higher levels in anaerobic SP cultures (Table 5.1). Finally, the set of 878 genes with higher expression in anaerobic SP cultures showed a very strong overrepresentation of genes induced by the environmental stress response (Gasch *et al.*, 2000).

Anaerobicity negatively affects the energy status of SP cultures

Use of oxygen as an electron acceptor for respiration enables oxidative phosphorylation. As a consequence, ATP yields on glucose, glycogen and trehalose in respiratory cultures can be up to ca. 8-fold higher than in anaerobic, fermentative cultures (Boender *et al.*, 2011a; Verduyn *et al.*, 1990a). Furthermore, since anaerobic yeast cultures cannot catabolize fatty acids, their use as energy reserves is restricted to aerobic cultures. To investigate the impact of storage metabolism and energy status on the short CLS of anaerobic SP cultures, cellular contents of storage materials and adenylate energy charge (a measure for the energetic status of living cells (Atkinson, 1968)) were analysed in aerobic and anaerobic SP cultures.

In aerobic cultures, intracellular pools of trehalose and glycogen were depleted within 1 day after entry into SP (Fig. 5.2A,B). Cellular contents of the fatty acids palmitic and palmitoleic acid, also decreased during aerobic SP, but at a much slower rate than the storage carbohydrates and approached 6 % of the total dry biomass. This level is close to the membrane-associated fatty acid content previously reported in *S. cerevisiae* (Verduyn *et al.*, 1990b), indicating that yeast cells had exhausted most of their reserve lipids after 2 days in SP (Fig. 5.2C). Together with the increased expression of genes involved in β-oxidation in aerobic SP cultures (Table 5.1), this observation indicates that aerobic SP cells use part of their fatty acids as an endogenous carbon and energy source. In aerobic cultures, the adenylate energy charge was 0.70 (± 0.08) and the intracellular ATP concentration was 5.45 (± 0.76) mM at the onset of SP. These results are in good in agreement with published data (Ball and Atkinson, 1975; Choi and Lee, 2013). In the days after the onset of SP, both parameters gradually decreased. Two full days after the onset of SP, the adenylate energy charge was still above 0.25 (Fig. 5.3).

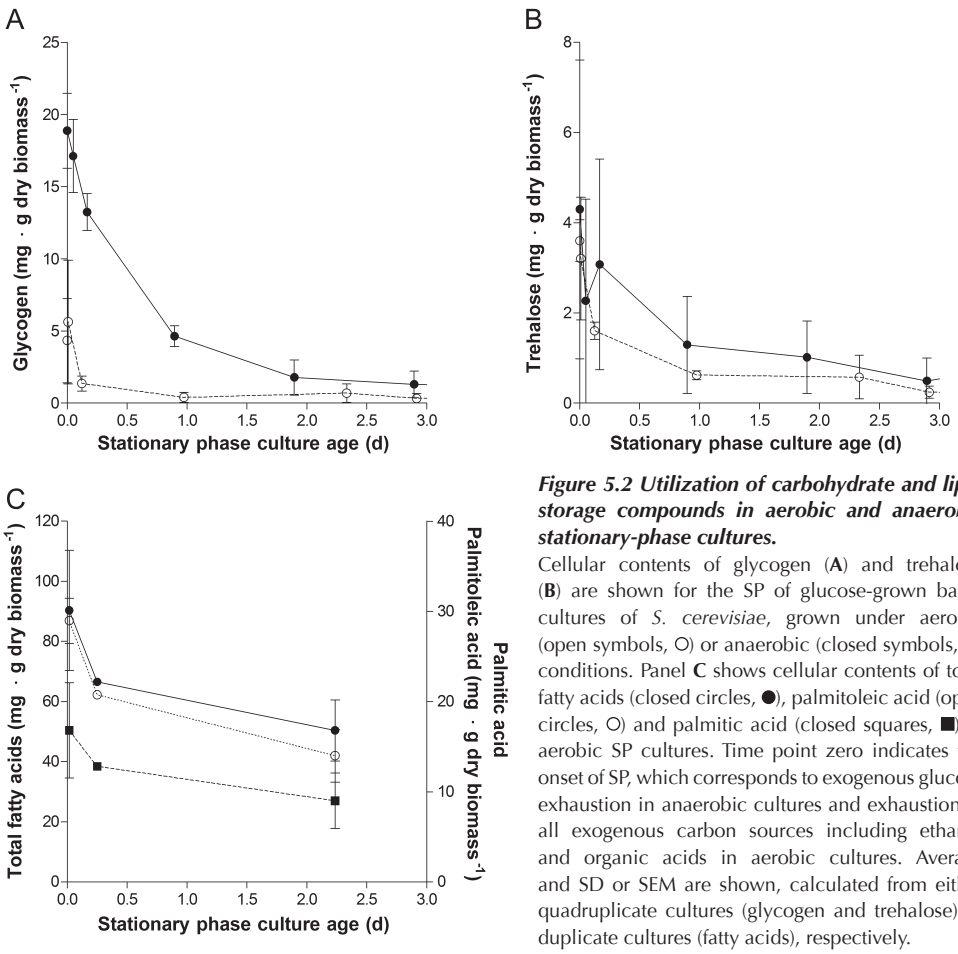


Figure 5.2 Utilization of carbohydrate and lipid storage compounds in aerobic and anaerobic stationary-phase cultures.

Cellular contents of glycogen (**A**) and trehalose (**B**) are shown for the SP of glucose-grown batch cultures of *S. cerevisiae*, grown under aerobic (open symbols, ○) or anaerobic (closed symbols, ●) conditions. Panel **C** shows cellular contents of total fatty acids (closed circles, ●), palmitoleic acid (open circles, ○) and palmitic acid (closed squares, ■) in aerobic SP cultures. Time point zero indicates the onset of SP, which corresponds to exogenous glucose exhaustion in anaerobic cultures and exhaustion of all exogenous carbon sources including ethanol and organic acids in aerobic cultures. Average and SD or SEM are shown, calculated from either quadruplicate cultures (glycogen and trehalose) or duplicate cultures (fatty acids), respectively.

Trehalose and glycogen are the only known carbon and energy reserves in anaerobic *S. cerevisiae* cultures. The initial trehalose content and utilization profile in anaerobic SP cultures strongly resembled those observed in aerobic cultures. The initial glycogen concentration in anaerobic SP cultures was ca. four-fold higher than in aerobic SP cultures. Nevertheless, intracellular glycogen was exhausted after 2 days in SP (Fig. 5.2A and 5.2B). At the onset of SP, intracellular ATP concentration and adenylate energy charge of anaerobic SP cultures were already lower than in aerobic cultures. Moreover, they decreased to very low levels within half a day after the onset of SP (Fig. 5.3).

Different entry trajectories into SP in aerobic and anaerobic cultures

The results described above reveal clear differences in transcriptome, energy status, thermotolerance and CLS of aerobic and anaerobic SP cultures. Some of these parameters already differed at the onset of SP, indicating the importance of different 'entry trajectories' of

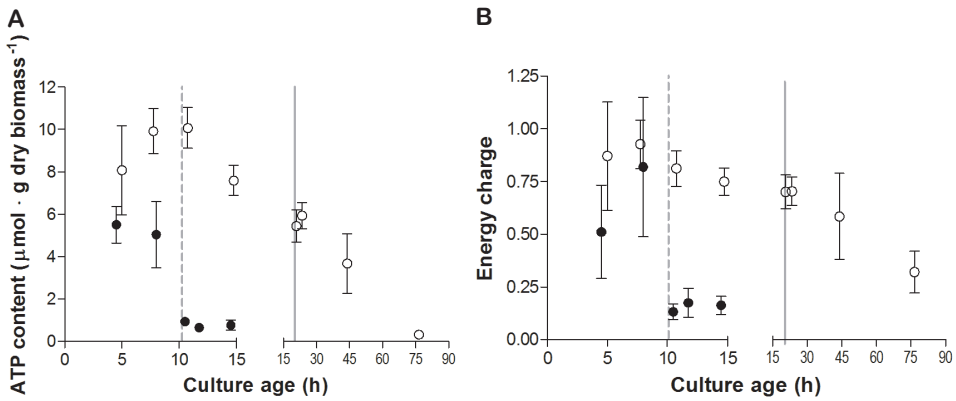


Figure 5.3 Intracellular ATP concentration and adenylate energy charge in aerobic and anaerobic stationary-phase cultures.
Cellular ATP content (**A**) and adenylate energy charge (**B**) in aerobic (open symbols, ○) and anaerobic (closed symbols, ●) cultures. The dashed vertical line represents glucose exhaustion and the onset of anaerobic SP, the solid vertical line represents ethanol exhaustion and the onset of aerobic SP. Values are shown as averages of duplicate cultures (+/- SEM).

aerobic and anaerobic cultures into SP. A major difference between aerobic and anaerobic batch cultures is the absence, in the latter, of a respiratory post-diauxic phase, in which ethanol and minor fermentation products acetate and glycerol are consumed. Growth in the post-diauxic phase, in which metabolism is completely respiratory, is slower than during the preceding glucose phase (Sonnleitner and Käppeli, 1986). In this study, the maximum specific growth rate of anaerobic cultures (0.31 ± 0.01 h^{-1}) during the glucose phase was lower than that of aerobic cultures (0.39 ± 0.02 h^{-1}, Fig. 5.4). In aerobic cultures, the specific growth rate during the post-diauxic phase (0.10 ± 0.01 h^{-1}) was ca. four-fold lower than during the fast growth phase on glucose. As a consequence, the specific growth rate in the hours preceding the onset of SP was ca. three-fold lower in aerobic cultures than in anaerobic cultures. To investigate whether the post-diauxic phase may have 'conditioned' aerobic cultures for entry into SP, analysis of aerobic and anaerobic batch cultures was extended to include the growth phases that precede SP.

A much higher thermotolerance in aerobic cultures was already evident in the exponential growth phase and further increased during the post-diauxic phase, to reach a maximum upon entry into SP (Fig. 5.5). Conversely, thermotolerance of anaerobic cultures did not increase during entry into SP and, consequently, remained much lower than that of aerobic cultures (Fig. 5.5). To further compare the different 'entry trajectories' into SP of aerobic and anaerobic batch cultures, transcriptome analyses were performed at different time points during exponential phase, post-diauxic phase (aerobic cultures only) and SP. Genes were grouped in 9 clusters, based on their time-dependent expression profiles in aerobic and anaerobic cultures (Fig. 5.6). A full dataset is available in supplementary material (Supplementary Tables S5.3 and S5.4).

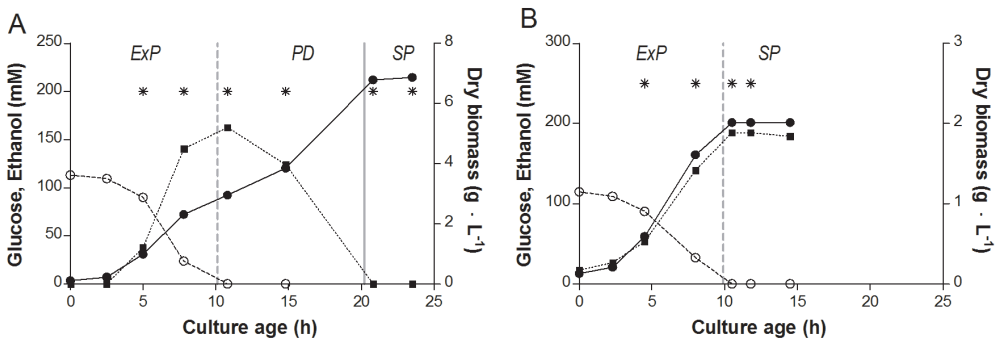


Figure 5.4 Growth phases in aerobic and anaerobic batch cultures.
Biomass (closed circles, ●), glucose (open circles, ○) and ethanol (closed squares, ■) concentration during the different growth phases of aerobic (**A**) and anaerobic (**B**) batch cultures of *S. cerevisiae*. The initial phase of exponential growth on glucose (**ExP**), the following post-diauxic phase of slower growth on non-fermentable carbon sources (**PD**) and final stationary phase (**SP**) are indicated. Values shown are from single representative batch cultures, independent replicate cultures yielded essentially the same results. Vertical lines indicate depletion of glucose (dashed line) and of fermentation products (solid line). Asterisks (*) indicate time points at which samples were taken for transcriptome analysis.

Less than one tenth (126 of 1452) of the genes that were differentially expressed in aerobic and anaerobic SP cultures (Table 5.1) already showed corresponding differences during the mid-exponential growth phase in aerobic and anaerobic cultures. For over half (834 of 1452) of the differentially expressed genes in SP cultures, the differences rose after glucose exhaustion, i.e. during the post-diauxic phase in aerobic cultures (Fig. 5.6A, clusters 1-3). Cluster 1 comprises genes whose expression increased during the aerobic and anaerobic exponential growth phases, with a further increase during the aerobic post-diauxic phase (Fig. 5.6A). Genes involved in fatty acid catabolism were overrepresented in this cluster, as well as genes that were previously shown to be induced in aerobic SP (including *SPG1*, *SPG3*, *SPG4*, *SPG5* and Hsp70-family-member *SSA3* (Martinez *et al.*, 2004)) (Fig. 5.6A and 5.7). Genes in cluster 2 showed similar transcript levels during the aerobic and anaerobic exponential growth phases on glucose. However, due to a pronounced decrease in expression during the post-diauxic phase in aerobic cultures, expression levels were higher in anaerobic SP cultures than in aerobic SP cultures. This cluster was markedly enriched for genes involved in amino acid synthesis and translation (Fig. 5.6A), suggesting that a down-regulation of protein synthesis occurred during the post-diauxic phase. Cluster 3 comprised of genes whose transcript levels increased during the exponential phase of both aerobic and anaerobic cultures but, subsequently, only decreased in aerobic SP cultures. Cluster 3 showed an overrepresentation of genes involved in mitochondrial translation and respiration (Fig. 5.6A).

For 595 of the 'oxygen-responsive in SP' genes listed in Table 5.1, differences in expression occurred already upon glucose exhaustion (Fig. 5.6B, cluster 4-8). Genes in cluster 5, characterized by a specific up-regulation upon entry into anaerobic SP, showed

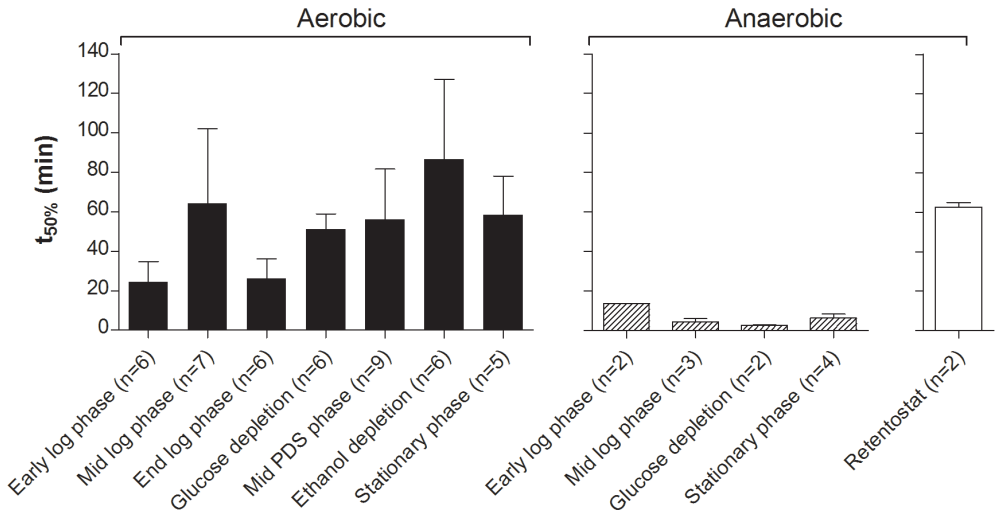
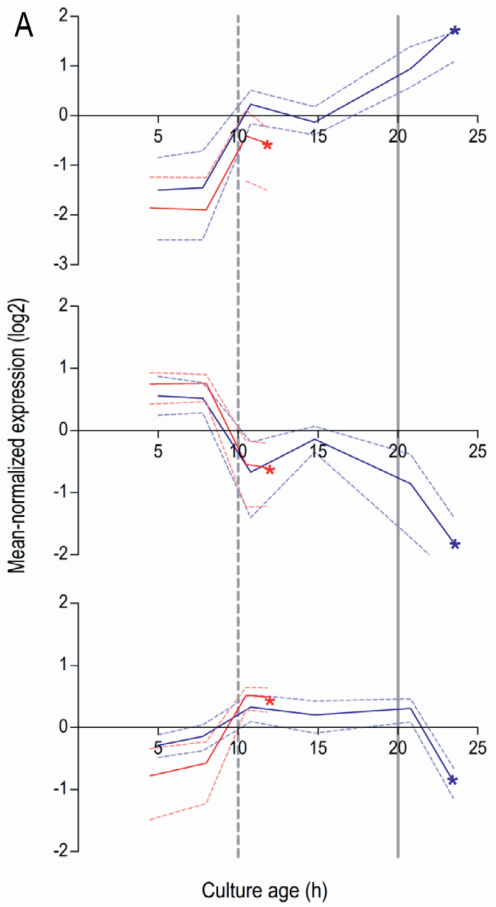


Figure 5.5 Thermotolerance of aerobic and anaerobic cultures during different growth phases. Thermotolerance of cells during different growth phases of aerobic (black bars) and anaerobic (hatched bars) batch cultures of *S. cerevisiae* (Fig. 5.4). The white bar depicts the thermotolerance of *S. cerevisiae* grown for 8 days in anaerobic retentostats (Boender *et al.*, 2011a). Thermotolerance was assayed by monitoring viability during incubation at 53°C and is shown as the incubation time resulting in a 50% decrease in viability ($t_{50\%}$) (see Materials and Methods for more details). The number of independent culture replicates for each of the growth phases are depicted on the *x*-axis labels.

an overrepresentation of stress-responsive genes (Fig. 5.6B, cluster 5). Several of these are known to be specifically expressed under anaerobic conditions (e.g., the cell-wall mannoprotein-encoding gene *DAN4* and members of the seripauperin family (Rachidi *et al.*, 2000)), but cluster 5 also included heat-shock genes whose expression is not specifically linked to anoxic conditions (e.g. *HSP30* and *SSA4*). Genes that showed a specific down-regulation during anaerobic SP, but a constant (Fig. 5.6B, cluster 6) or increased expression in aerobic SP (Fig. 5.6B, clusters 7 and 8) showed an overrepresentation of genes involved in transcription-related processes and carboxylic acid metabolism. The latter of which plays a role in the respiration of exogenous carboxylic acids during the post-diauxic phase (Fig. 5.6B, cluster 8). All 23 genes whose transcript levels were higher under anaerobic conditions, irrespective of the growth phase (Supplementary Table S5.4), were previously described to be up-regulated under anaerobic conditions. The majority (14) of these genes belonged to the seripauperin family (Rachidi *et al.*, 2000).

Two clusters (Fig. 5.6, cluster 1 and 5) comprised genes whose transcript levels increased in aerobic as well as in anaerobic batch cultivation, but to different final levels. These clusters were enriched for genes induced by the environmental stress response (Gasch *et al.*, 2000). The extreme differences in thermotolerance of aerobic and anaerobic SP cultures (Fig. 5.5) were therefore only partially mirrored at the transcript level, indicating that factors other than transcriptional reprogramming contribute to these differences.



Cluster 1 (316 genes)

Functional category	# in cluster	# in category	*p*-value[a]
Fatty acid metabolism	10	29	$1.8 \cdot 10^{-3}$
SP-genes [1]	46	122	$2.1 \cdot 10^{-27}$
ESR-induced genes [2]	51	281	$1.7 \cdot 10^{-14}$

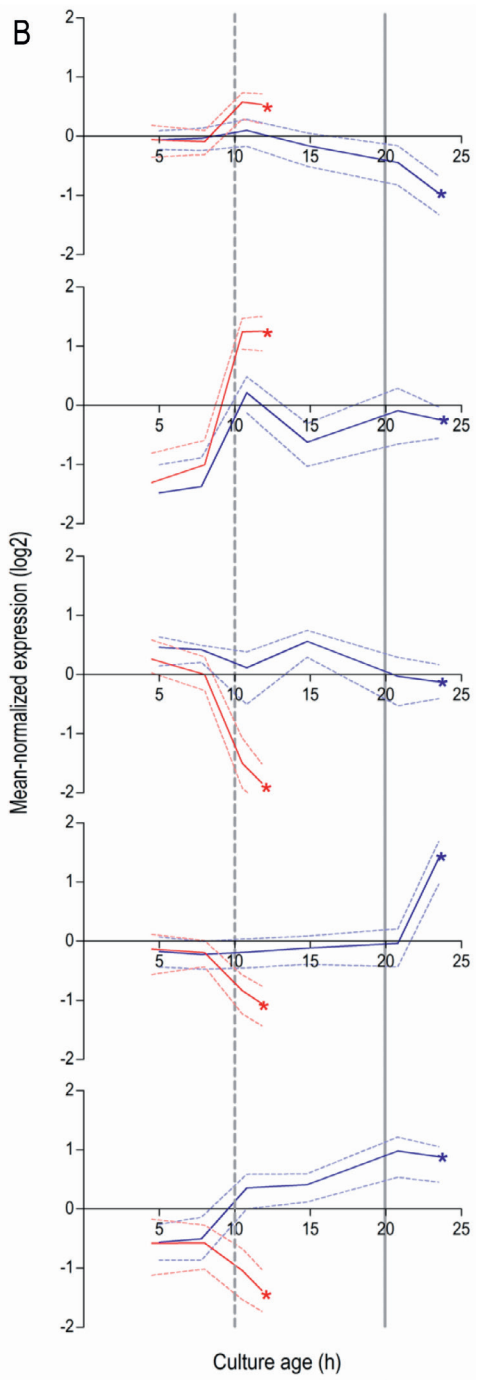
Cluster 2 (306 genes)

Functional category	# in cluster	# in category	*p*-value[a]
Translation	89	345	$3.2 \cdot 10^{-40}$
rRNA export from nucleus	17	48	$9.2 \cdot 10^{-8}$
Cellular amino acid biosynthetic process	22	101	$4.7 \cdot 10^{-6}$
ESR-repressed genes [2]	86	563	$9.6 \cdot 10^{-22}$

Cluster 3 (212 genes)

Functional category	# in cluster	# in category	*p*-value[a]
Mitochondrial translation	34	81	$2.5 \cdot 10^{-26}$
Translation	40	345	$4.9 \cdot 10^{-9}$
tRNA aminoacylation for protein translation	10	35	$4.1 \cdot 10^{-4}$
Aerobic respiration	12	71	$1.0 \cdot 10^{-2}$

Figure 5.6 Clustering of genes differentially expressed between aerobic and anaerobic SP cultures according to their expression profiles during the growth phases preceding SP.
Genes whose differential expression between aerobic and anaerobic SP cultures originated from changes after glucose depletion (**A** ∧). Clusters of genes whose differential expression between aerobic and anaerobic stationary phase cultures originated from changes upon glucose exhaustion (**B** >).
Each graph presents the expression profiles of genes from aerobic cultures (blue lines) and anaerobic cultures (red lines) in a particular gene cluster. The solid lines represent the average of the mean-normalized expression of all genes in the cluster. The dashed lines represent the first and third quartile of these mean-normalized expression values, giving information on the scatter in the expression of genes in the cluster. Asterisks (*) indicate the SP samples from anaerobic and aerobic batches. Vertical lines indicate glucose exhaustion (dashed line) and carbon exhaustion (solid line, for aerobic cultures only). For each cluster a table reports the overrepresentation of functional categories, including the number of genes in the cluster belonging to a specific functional category (# in cluster), the total number of genes in this functional category (# in category), and the [a]Bonferroni-corrected *p*-values that indicate the likelihood of obtaining such enrichment in a random set of genes. Only categories with a Bonferroni-corrected *p*-value below 0.05 were deemed significant and presented in the tables. Categories of SP-genes and environmental stress induced or repressed genes were obtained from 1: (Martinez *et al.*, 2004) and 2: (Gasch *et al.*, 2000) More details can be found in Supplementary Table S5.3.



Cluster 4 (83 genes)

Functional category	# in cluster	# in category	*p*-value[a]
No significant terms			

Cluster 5 (254 genes)

Functional category	# in cluster	# in category	*p*-value[a]
Response to stress	20	161	$1.7 \cdot 10^{-2}$
ESR-induced genes [2]	69	281	$2.9 \cdot 10^{-35}$

Cluster 6 (53 genes)

Functional category	# in cluster	# in category	*p*-value[a]
tRNA transcription from RNA polymerase III promoter	4	15	$1.5 \cdot 10^{-2}$

Cluster 7 (84 genes)

Functional category	# in cluster	# in category	p-value[a]
Regulation of transcription	20	505	$1.6 \cdot 10^{-2}$

Cluster 8 (121 genes)

Functional category	# in cluster	# in category	*p*-value[a]
Carboxylic acid metabolic process	4	9	$4.0 \cdot 10^{-2}$

Discussion

This study demonstrates a strong impact of oxygen availability on chronological lifespan and stress tolerance in SP batch cultures of *S. cerevisiae* and, thereby, confirms and extends earlier observations on its physiology in aerobic and anaerobic cultures (Mesquita *et al.*, 2010; Thomsson *et al.*, 2003; Wilson and McLeod, 1976). The CLS of anaerobically grown SP cultures was much shorter than that of their aerobic counterparts and an even more dramatic difference was observed for thermotolerance. As will be discussed below, these differences involved a different conditioning of aerobic and anaerobic cultures during the growth phases preceding SP, as well as energetic constraints imposed on yeast cells in anaerobic SP cultures.

The post-diauxic growth phase enables transcriptional conditioning of aerobic yeast cultures for stationary phase

Our transcriptome data revealed that 57 % of the transcriptional differences between aerobic and anaerobic SP cultures originated from transcriptional reprogramming during the aerobic post-diauxic growth phase. Although several well-known hallmark transcripts of SP cultures, previously identified in (semi-) aerobic shake-flask cultures, such as *SPG4*, *SPG5* and *SSA3* (Martinez *et al.*, 2004), showed increased levels in anaerobic SP cultures, their levels did not reach those observed in aerobic cultures (Fig. 5.7). Moreover, many genes involved in biosynthesis were strongly down-regulated during the aerobic post-diauxic phase and SP, but retained expression levels close to those in the exponential growth phase in anaerobic cultures (Fig. 5.6). These transcriptome data are consistent with the hypothesis that the post-diauxic phase in aerobic cultures conditions cells for entry into SP and that, conversely, absence of a post-diauxic phase prevents anaerobic batch cultures from adequately adapting to SP and starvation.

Hormesis could potentially explain the difference in robustness between aerobic and anaerobic cultures. Indeed respiration can generate low levels of ROS and thereby induce stress tolerance via increased expression of stress tolerance genes (Mesquita *et al.*, 2010; Tahara *et al.*, 2013). However, among a set of 22 genes encoding enzymes involved in ROS-protective mechanisms (Perrone *et al.*, 2008) including the superoxide dismutase genes *SOD1* and *SOD2*, whose expression is strongly upregulated during exposure to ROS (Gasch *et al.*, 2000; Mesquita *et al.*, 2010), only the peroxisomal catalase *CTA1* was higher expressed in aerobic SP cultures. These findings argue against a dominant role of ROS-based hormesis in the acquisition of increased robustness by aerobic SP cultures.

Caloric restriction: a key factor in conditioning yeast cells for stationary phase and starvation

Thermotolerance is negatively correlated with specific growth rate in *S. cerevisiae* as Lu *et al.* (2009) demonstrated in nutrient-limited chemostat cultures (Lu *et al.*, 2009). Although these authors did not evaluate the impact of specific growth rate on CLS nor investigate anaerobic growth, they showed that the negative correlation between thermotolerance and growth rate also held in a respiratory-deficient *S. cerevisiae* strain (Lu *et al.*, 2009). Our data are fully consistent with the hypothesis that the strong reduction of specific growth rate (from 0.39 to

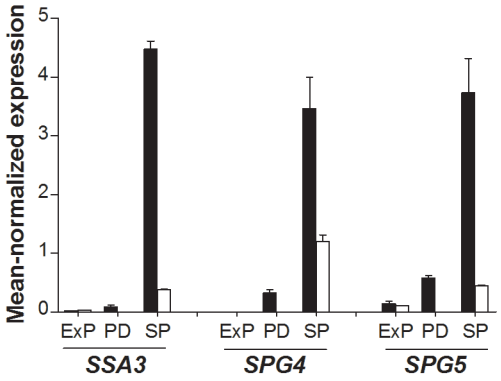


Figure 5.7. Expression levels of the SP-associated genes SSA3, SPG4 and SPG5 during different growth phases in aerobic and anaerobic batch cultures.
The mean-normalized expression values during exponential growth on glucose phase (ExP), post-diauxic growth phase (PD, only aerobic cultures) and stationary phase (SP, i.e., 4 hours after exhaustion of exogenous consumable carbon-sources) in aerobic (black bars) and anaerobic (white bars) cultures of *S. cerevisiae* of the genes *SSA3*, *SPG4* and *SPG5*. Average values of duplicate cultures are shown (± SEM).

0.10 h^{-1}) during transition from fast exponential growth on glucose to the post-diauxic phase in aerobic batch cultures could similarly trigger increased thermotolerance and extended CLS during the starvation phase. We have also recently shown that a gradual decrease of the specific growth rate to near-zero values in glucose-limited retentostats (Boender *et al.*, 2009) yielded yeast cells with a thermotolerance that is as high as that of aerobic SP cultures (Fig. 5.5), and with an even longer CLS during subsequent starvation (Boender *et al.*, 2011a). The transcriptional reprogramming observed in these anaerobic severely calorie-restricted cultures (Boender *et al.*, 2011b) strongly resembled the transcriptome changes observed in the present study for aerobic cultures entering SP and proteome analysis showed increased levels of proteins involved in stress resistance (Binai *et al.*, 2014). Deletion of Rim15, a kinase under control of several nutrient signaling pathways (Swinnen *et al.*, 2006), strongly reduced the acquisition of robustness in both anaerobic and aerobic calorie-restricted cultures (Bisschops *et al.*, 2014; Wei *et al.*, 2008), suggesting a strong role for nutrient signaling independent of oxygen availability.

The present study, combined with our previous retentostat studies, therefore clearly demonstrates that prior conditioning by a period of caloric restriction (e.g. by slow growth during the aerobic post-diauxic phase or in extremely glucose-limited cultures) is a prerequisite for acquisition of a prolonged CLS by non-growing, starving cultures of *S. cerevisiae*. This conclusion, which supports earlier proposals based on starvation experiments by Thomsson *et al*. (Thomsson *et al.*, 2003; Thomsson *et al.*, 2005), has important implications for the design and interpretation of yeast studies on chronological aging, for example when such studies involve mutants that are impeded in energy metabolism.

The low thermotolerance of exponentially growing anaerobic cultures does not correlate with expression of heat shock genes

Although both aerobic and anaerobic, non-growing yeast cultures can acquire a similar thermotolerance by an appropriate preceding conditioning phase, a drastic difference was observed in the thermotolerance of exponentially growing, aerobic and anaerobic cultures (Fig. 5.5). The similar adenylate energy charge and intracellular ATP concentrations of aerobic

and anaerobic cultures during exponential growth on glucose appear to rule out cellular energy status as a major cause of this difference. Since the high temperature (53°C) during the thermotolerance assays precludes *de novo* synthesis of mRNA or protein synthesis (Gibney *et al.*, 2013), this difference must already be expressed in the batch cultures themselves. Transcript levels of genes that were previously implicated in heat-shock resistance (including *HSP* genes (Westerheide and Morimoto, 2005) such as *HSP104* (Lindquist and Kim, 1996), *HSP26* (Haslbeck *et al.*, 1999), *HSP12* (Praekelt and Meacock, 1990), *SSA3* (Werner-Washburne *et al.*, 1987)) were similar during the exponential growth phase on glucose in aerobic and anaerobic cultures. Moreover, of 59 genes identified as essential for heat-shock survival by Gibney and coworkers (Gibney *et al.*, 2013), only one gene was differentially expressed, *LIA1*, and showed a higher transcript level in anaerobic cultures.

Oxygen availability strongly influences sterol and unsaturated fatty acid composition of yeast cells (Wilson and McLeod, 1976), especially because these compounds have to be added to growth media as anaerobic growth factors (Verduyn *et al.*, 1990b). These differences in membrane composition might partially explain the observed differences in thermotolerance between aerobically and anaerobically grown *S. cerevisiae* cells. The hypothesis that membrane composition is a key determinant in thermotolerance of *S. cerevisiae* (Dufourc, 2008; Guyot *et al.*, 2015) is consistent with a recent study, in which the acquisition of increased thermotolerance by laboratory-evolved strains was shown to be caused by changes in their sterol composition (Caspeta *et al.*, 2014).

A low energy status of anaerobic SP cultures limits metabolic flexibility

Consistent with earlier reports (Chester, 1963; Wilson and McLeod, 1976), anaerobic batch cultures *S. cerevisiae* displayed a substantially higher glycogen content than aerobic cultures. However, after the onset of SP, anaerobic cultures showed a much faster decrease of the adenylate energy charge. This difference can be attributed to several factors. Firstly, since *S. cerevisiae* cannot derive metabolic energy from lipids and amino acids in the absence of oxygen (van Roermund *et al.*, 2003), anaerobic cultures are entirely dependent on glycogen and trehalose as energy storage compounds and anaerobic catabolism of these storage carbohydrates yields less ATP than respiration. The estimated ATP synthesis rate from anaerobic glycogen dissimilation of ca. 5 µmol per g biomass dry weight per hour during the first day in SP (based on a maximum ATP yield of 3 ATP per glucose residue (Boender *et al.*, 2011a)), was two orders of magnitude lower than the cellular ATP demand for maintenance estimated from chemostat and retentostat cultures (m_{ATP} = 1 mmol ATP per g biomass dry weight per hour (Boender *et al.*, 2009)). A similar extreme reduction of ATP turnover rates was observed when anaerobic retentostat cultures were switched to carbon starvation (Boender *et al.*, 2011a). Together, these observations indicate that an extremely low ATP turnover is an intrinsic feature of anaerobic, starving yeast cultures. In addition to this extreme low ATP-turnover, it is even conceivable that the apparent inability of anaerobic batch cultures to efficiently down-regulate energy-consuming processes, including protein

synthesis, the single most expensive biosynthetic process in living cells (Stouthamer, 1973; Verduyn *et al.*, 1991), may have exacerbated the fast decline of their energy status after entry into SP (Fig. 5.3).

The low energy status of cells may at the same time have put strong constraints on these energy consuming processes. Proteome analyses should reveal whether the increased transcription of *HSP* genes implicated in thermotolerance, which took place late in the exponential growth phase (Fig. 5.6), was too late to enable synthesis of the corresponding proteins before the decline cellular energy status in anaerobic SP cultures. Such a scenario would explain the discrepancy between the oxygen-independent up-regulation of these genes (with notable exception of *SSA3*) and the absence of increased thermotolerance in anaerobic SP cultures.

Taken together, the results from the present study indicate that, in the short time lapse between the moment at which anaerobic cultures sense that glucose reaches critically low levels and the actual exhaustion of glucose, they lack the time and resources to perform the energy-intensive remodeling of their transcriptome and proteome required to robustly face starvation. Our data are therefore entirely consistent with the notion that the low CLS and thermotolerance of anaerobic SP cultures, in comparison with aerobic cultures, is due to the absence of a proper conditioning phase and a limited metabolic flexibility due to a lower cellular energy status.

Outlook

Many studies in which SP yeast cultures are used as a model system to investigate aspects of aging, still rely on shake-flask cultures. Due to their low and poorly controlled oxygen-transfer capacity, the aeration status of shake-flask cultures is generally unclear. The strong impact of oxygen availability on aging-related characteristics (Ocampo *et al.*, 2012) underlines the value of controlled cultivation techniques, e.g. in bioreactors including batch, chemostat and retentostat cultures (Boender *et al.*, 2009; Büchs, 2001; Nagarajan *et al.*, 2014; Ziv *et al.*, 2013) or flow-through cells (Lee *et al.*, 2012), in yeast-based aging studies. In particular, the use of anaerobic cultures as a model offers interesting possibilities to clarify the role of respiration and ROS in aging, apoptosis and longevity.

The short life span and low robustness of anaerobic SP cultures of *S. cerevisiae* is directly relevant for industrial applications. Robustness of SP cultures is especially important for processes in which biomass from anaerobic batch cultures is recycled, e.g. in industrial bioethanol production and beer brewing (Basso *et al.*, 2011a; Gibson *et al.*, 2007). Clearly, results from (semi-)aerobic shake-flask cultures cannot be used to predict the performance of such anaerobic processes and improvement of robustness in these industrial processes will have to be based on studies in anaerobic systems.

Saccharomyces yeasts have the capability, rare among eukaryotes, to grow fast in the complete absence of oxygen and it is often assumed that they are well adapted to anaerobic environments (Goddard and Greig, 2015; Hagman and Piškur, 2015; Visser *et al.*, 1990).

While the natural habitat of *S. cerevisiae* is still a matter of debate (Goddard and Greig, 2015), lower biomass concentrations frequently encountered in natural environments combined with the low affinity of yeast glucose transporters (Reifenberger *et al.*, 1997) may lead to a transition into SP that is sufficiently slow to enable acquisition of longevity and robustness under anaerobic conditions. Further research is therefore needed to investigate the ecological relevance of this laboratory study.

Materials and Methods

Strains and cultivation

The prototrophic *Saccharomyces cerevisiae* strain CEN.PK113-7D (*MAT*a *MAL*2-8c *SUC*2) used in this study is a congenic member of the CEN.PK family (Entian and Kötter, 2007; Nijkamp *et al.*, 2012). Stock cultures were grown at 30°C in shake flasks containing yeast extract (1 % w/v), peptone (2 % w/v) and dextrose (2 % w/v) (YPD) medium. Glycerol, final concentration 20 % (v/v), was added to overnight cultures and 1 mL aliquots were stored at -80°C.

Previously described synthetic medium (Verduyn *et al.*, 1992) was used with 20 g · L^{-1} glucose as sole carbon-source and 0.2 g · L^{-1} antifoam Emulsion C (Sigma, St. Louis, USA). In case of anaerobic cultivations, the medium was supplemented with anaerobic growth factors ergosterol (10 mg · L^{-1}) and Tween 80 (420 mg · L^{-1}) dissolved in ethanol. Inocula for batch fermentations consisted of 100 mL yeast culture grown overnight to an OD_{660} of 4 in synthetic medium with 20 g · L^{-1} glucose. Aerobic and anaerobic batch fermentations were carried out at 30°C in 2 L bioreactors (Applikon, Schiedam, The Netherlands), with a working volume of 1.4 L. Cultures were stirred at 800 rpm and sparged at a flow-rate of 700 mL · min^{-1} with either dried air or nitrogen gas (<10 ppm oxygen, Linde Gas Benelux, The Netherlands). The bioreactors were equipped with Norprene tubing (Saint-Gobain Performance Plastics, Courbevoie, France) and Viton O-rings (Eriks, Alkmaar, The Netherlands) to minimize diffusion of oxygen. During aerobic cultivations, dissolved oxygen levels remained above 40 % of the initial saturation level as measured by Clark electrodes (Mettler Toledo, Greifensee, Switzerland). The culture pH was maintained at 5.0 by automatic addition of 2 M KOH and 2 M H_2SO_4.

Analysis of biomass, metabolites, substrate and exhaust gas

Biomass concentration as culture dry weight was determined as described previously (Postma *et al.*, 1989).

For substrate and extracellular metabolite concentration determination, culture supernatants were obtained by centrifugation of culture samples (3 min at 20000 g) and analysed by high-performance liquid chromatograph (HPLC) analysis on a Waters Alliance 2690 HPLC (Waters, Milford, MA) equipped with a Bio-Rad HPX 87H ion exchange column (BioRad, Veenendaal, The Netherlands), operated at 60°C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL · min^{-1}. Detection was by means of a dual-wavelength

absorbance detector (Waters 2487) and a refractive index detector (Waters 2410).

The exhaust gas from batch cultures was cooled with a condenser (2°C) and dried with a PermaPure Dryer (model MD 110-8P-4; Inacom Instruments, Veenendaal, the Netherlands) prior to online analysis of carbon dioxide and oxygen with a Rosemount NGA 2000 Analyser (Baar, Switzerland).

Colony forming units

To determine culture viability, small aliquots of culture broth were taken from the reactor and cells were counted on a Z2 Coulter Counter (Beckman Coulter, Woerden, Netherlands) equipped with a 50 µm orifice (Multisizer II, Beckman Coulter, Woerden, Netherlands). Cells were diluted in 0.1 % peptone and 100 µL suspensions containing approximately 30, 300 and 3000 cells were plated on yeast extract (1 % w/v), peptone (2 % w/v) and dextrose (0.5 % w/v) (YPD) agar plates and incubated at 30°C for at least 3 days before counting the colonies. CFU was calculated as the number of colonies formed divided by the number of plated cells.

Thermotolerance assay

Cells from culture broth were counted with a Z2 Coulter Counter and diluted in pre-warmed (53°C) isotone diluent II (Beckman Coulter, Woerden, Netherlands) to yield 50 mL cell suspensions with a density of $1 \cdot 10^7$ cells/mL. Cell suspensions were incubated in a waterbath at 53°C and 4 mL aliquots were sampled in 10 min intervals. Samples were cooled on ice and assayed for viability using the *Funga*Light 5-CFDA, AM (acetoxymethyl ester 5-carboxyfluorescein diacetate)/propidum iodide (PI) yeast viability kit (Invitrogen, Carlsbrad, CA) by counting 10,000 cells on a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter, Woerden, Netherlands) as described previously (Boender *et al.*, 2011a). 5-CFDA, AM is a cell-permeant substrate for intracellular non-specific esterase activity. Hydrolytic cleavage of the lipophilic blocking acetoxymethyl and diacetate groups of 5-CFDA, AM results in a green fluorescent signal in metabolically active cells. Propidium iodide intercalates with DNA in cells with a compromised cell membrane, which results in red fluorescence. Cells stained with PI were considered dead cells. For each independent sample, the $t_{50\%}$ value (the time after which 50 % of the initial viable population was dead) was estimated by fitting the viability data with a sigmoidal dose-response curve in Graphpad Prism 4.03. Both measurements of viability, i.e. metabolic activity based on 5-CFDA, AM and membrane integrity based on PI gave similar $t_{50\%}$-values, therefore only results based on PI are shown.

Storage carbohydrate measurements

1.2 mL culture broth was quenched in 5 mL of cold methanol (-40°C) using a rapid sampling setup described previously (Canelas *et al.*, 2008), mixed and subsequently pelleted (4 400 · *g*, 5 min) at -19°C. Cells were washed with 5 mL of cold methanol (-40°C) and pellets stored at -80°C. Pellets were resuspended in Na_2CO_3 (0.25 M) and further processed according to a previously described procedure (Parrou and François, 1997).

Fatty acids measurements

Culture volumes corresponding to 50 mg biomass were sampled on ice, centrifuged (10 000 · *g*, 10 min at 4°C), washed twice, resuspended in 5 mL ice-cold water and stored at -20°C. Lipid extraction was performed as described previously (Johnson *et al.*, 2009). Aliquots of 0.15 mL were added to 15 mL tubes and 1.5 mL of a mixture of concentrated HCl and 1-propanol (1:4) and 1.5 mL of dichlorethane were added. 400 µg of myristic acid (a 15:0 fatty acid) was included as internal standard. Samples were incubated at 100°C for 2 h. Subsequently, 3 mL of water was added to cooled samples. 1 mL of the organic phase was filtered over water-free sodium sulfate into GC vials. The fatty acid propyl esters in the organic phase were analyzed by gas chromatography (model 6890N, Agilent, U.S.A.) using a DB-wax column (length, 30 m; inside diameter, 0.25 mm; film thickness, 0.25 µm; J&W Scientific, Folsom, CA) and helium as the carrier gas. The sample volume was 1 µL, and the split was set to 1:20. The injection temperature was 230°C, and the following temperature gradient was used: 120°C at the start, increasing at a rate of 10°C/min up to 240°C, and then 240°C for 8 min. The fatty acid propyl esters were detected using a flame ionization detector at 250°C.

Analysis of intracellular adenosine-phosphate concentrations

Samples for internal metabolite analysis were obtained by rapid sampling (Canelas *et al.*, 2008). 1.2 mL of culture broth was rapidly quenched into 5 mL of 100 % methanol, pre-cooled to -40°C. Samples were washed with cold methanol and extracted with boiling ethanol. Intracellular AMP and ADP were determined enzymatically, using a previously described assay based on myokinase, pyruvate kinase and lactate dehydrogenase reactions (Jaworek and Welsch, 1985). Assays were performed in white, flat bottom 96-well microtiter plates (Corning Inc., USA). NADH fluorescence was measured in a TECAN GENios Pro microtiterplate reader (Tecan, Männedorf, Switzerland) as previously described (Canelas *et al.*, 2008). Intracellular ATP was also assessed enzymatically. The assay contained 115 mM triethanolamine (pH 7.6), 11.5 mM $MgSO_4 \cdot 7H_2O$, 1.15 mM $NADP^+$ per well including sample extract, total volume was 205 µL per well. The reaction to measure ATP was initiated by adding 12 mM glucose and 30 U hexokinase (Sigma-Aldrich Chemie B.V, Zwijndrecht, The Netherlands). Assays were performed in black, flat bottom 96-well microtiter plates (Corning Inc., USA). NADPH fluorescence was measured in a TECAN GENios Pro microtiterplate reader. The adenylate energy charge was calculated according to the previously described (Atkinson, 1968) equation 5.1

$$EC = \frac{([\mathrm{ATP}] + 0.5[\mathrm{ADP}])}{([\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}])} \qquad \textit{(Equation 5.1)}$$

Transcriptome analysis

Independent duplicate aerobic and anaerobic batch cultures were sampled at six and four different time points respectively (see Fig. 5.4A,B) for microarray analysis, resulting in a total dataset of 20 microarrays. Sampling from batch cultures for transcriptome analysis was

performed using liquid nitrogen for rapid quenching of mRNA turnover (Piper *et al.*, 2002). Prior to RNA extraction, samples were stored in a mixture of phenol/chloroform and TEA buffer at -80°C. Total RNA extraction, isolation of mRNA, cDNA synthesis, cRNA synthesis, labelling and array hybridization was performed as previously described (Mendes *et al.*, 2013). The quality of total RNA, cDNA, aRNA and fragmented aRNA was checked using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Hybridization of labelled fragmented aRNA to the microarrays and staining, washing and scanning of the microarrays was performed according to Affymetrix instructions (EukGE_WS2v5).

The 6383 yeast open reading frames were extracted from the 9335 transcript features on the YG-S98 microarrays. To allow comparison, all expression data were normalized to a target value of 240 using the average signal from all gene features (Hebly *et al.*, 2014). The microarray data used in this study are available via GEO series accession number GSE69485. To eliminate variation in genes that are not expressed, genes with expression values below 12 were set to 12 and the gene features for which the maximum expression was below 20 for all 20 arrays were discarded (Boender *et al.*, 2011b). The average deviation of the mean transcript data of replicate batches was ca. 11 %, similar to the reproducibility usually observed in replicate steady state chemostat cultures (Daran-Lapujade *et al.*, 2004). The expression of housekeeping genes *ACT1, PDA1*, *TFC1, ALG9, TAF10* and *UBC6* (Teste *et al.*, 2009) remained stable for both conditions and all sample points (average coefficient of variation 17 % ± 5 %).

To identify genes that were differentially expressed between aerobic and anaerobic SP cultures, a pairwise comparison was performed between aerobic samples taken at time point 6 (Fig. 5.4A) and anaerobic samples taken at time point 4 (Fig 5.4B) as previously described (Hebly *et al.*, 2014). Similarly, genes differently expressed during growth on glucose under aerobic or anaerobic conditions were identified through a pairwise comparison of aerobic and anaerobic samples taken at time point 1 (Fig 5.4A,B). Differences with adjusted *p*-values lower than 0.05 and a fold difference of 2 or higher were considered statistically significant. Time-dependent expression profiles of selected genes were clustered according optimal k-means clustering using positive correlation as distance metric (Expressionist Pro version 3.1, Genedata, Basel, Switzerland) resulting in an optimal number of 9 clusters. For display of time-dependent transcript levels, expression values were normalized per gene by dividing single expression values by the average expression value of both conditions and all time points. Mean values of these average-normalized values for all genes in each cluster are shown, as well as the first and third quartile of average-normalized values.

Gene expression clusters were analysed for overrepresentation of functional annotation categories of the Gene Ontology (GO) database (http://www.geneontology.org/), based on a hypergeometric distribution analysis tool (Knijnenburg *et al.*, 2007). Additional categories describing genes expressed in SP cultures (Martinez *et al.*, 2004), genes commonly induced by several environmental stresses (Gasch *et al.*, 2000) or essential for heat-shock survival (Gibney *et al.*, 2013) were extracted from the respective references.

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Supplementary materials Chapter 5

Supplementary materials are freely available online at the web-site of Microbial Cell in which the original publication appeared in Volume 2, Number 11, October 2015, Pages 429–444. (doi: 10.15698/mic2015.11.238).

Supplementary Table S5.1

For the list of genes differentially expressed between aerobic and anaerobic stationary phase cultures the reader is referred to the Excel spread sheet that can be found online.

Supplementary Table S5.2

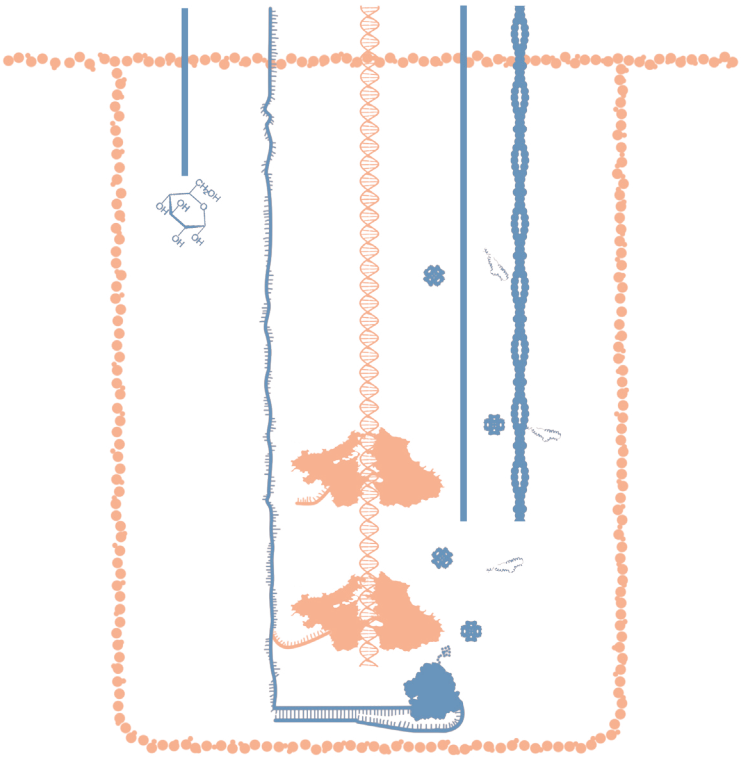
For the list of genes in functional categories overrepresented among genes with higher expression in (1) aerobic or (2) anaerobic stationary phase cultures the reader is referred to the Excel spread sheet that can be found online.

Supplementary Table S5.3

For the list of genes differentially expressed between aerobic and anaerobic SP cultures per cluster for clusters 1-8 the reader is referred to the Excel spread sheet that can be found online.

Supplementary Table S5.4

For the list of genes with constitutive higher expression in anaerobic batch cultures including stationary phase the reader is referred to the Excel spread sheet that can be found online.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6. Physiological and transcriptional responses of different industrial microbes at near-zero specific growth rates

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Keywords: Industrially relevant microbes, zero-growth, retentostat, quantitative physiology, transcriptome, calorie restriction

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Current knowledge of the physiology and gene expression of industrially relevant microorganisms is largely based on laboratory studies under conditions of rapid growth and high metabolic activity. However, in natural ecosystems and industrial processes, microbes frequently encounter severe calorie restriction. As a consequence, microbial growth rates in such settings can be extremely slow and even approach zero. Furthermore, uncoupling microbial growth from product formation, while cellular integrity and activity are maintained, offers economically highly interesting perspectives. Retentostat cultures have been employed to investigate microbial physiology at (near-)zero growth rates. This minireview compares information from recent physiological and gene-expression studies on retentostat cultures of the industrially relevant microorganisms *Lactobacillus plantarum*, *Lactococcus lactis*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Aspergillus niger*. Shared responses of these organisms to (near-)zero growth rates include increased stress tolerance and a down-regulation of genes involved in protein synthesis. Other adaptations, such as changes in morphology and (secondary) metabolite production, were species-specific. This comparison underlines the industrial and scientific significance of further research on microbial (near-)zero growth physiology.

Introduction

Most research in microbial physiology focuses on growing cells, although under natural and industrial conditions microbes frequently encounter a state in which neither growth nor deterioration of cells occurs. However, the experimental design to study microbes in this clearly relevant physiological state is far from trivial.

In this chapter, we define zero growth as a non-growing state in which viability and metabolic activity of a microbial culture are maintained over prolonged periods. As such, zero growth differs from starvation, which is coupled to cellular deterioration, loss of activity, and ultimately, cell death (Kunji *et al.*, 1993; Poolman *et al.*, 1987). Zero growth also differs from differentiated survival states, such as bacterial or fungal spores, in which metabolism comes to a standstill (Dahlberg and Etten, 1982). Conversely, under zero-growth conditions, microbes exclusively use available substrates for processes that contribute to maintenance of cellular integrity and homeostasis (Boender *et al.*, 2011b; Ercan *et al.*, 2013; Goffin *et al.*, 2010; Jørgensen *et al.*, 2010). Such processes include homeostasis of transmembrane gradients of protons and solutes, defense and repair systems, osmoregulation, and protein turn-over (Stephanopoulos *et al.*, 1998b; van Bodegom, 2007).

In classical food fermentation processes, (near-)zero growth occurs during prolonged periods of extremely restricted availability of energy substrates. Examples include cheese ripening by lactic acid bacteria (LAB) (Banks and Williams, 2004; Bergamini *et al.*, 2013; Smit *et al.*, 2005), wine fermentation by *Saccharomyces cerevisiae* (Mauricio *et al.*, 2001; Tudela *et al.*, 2012), and natto fermentation by *Bacillus subtilis* (Kiuchi and Watanabe, 2004). Despite the severely energy-limiting conditions, microbes manage to survive in these processes for many weeks, while continuing to produce aroma and flavor compounds in the product matrix (Banks and Williams, 2004; Erkus *et al.*, 2013; Kiuchi and Watanabe, 2004; Mauricio *et al.*, 2001). Another incentive for studying zero-growth physiology is related to application of microorganisms as cell factories for production of food ingredients, enzymes, chemicals, and biofuels. In such applications, biomass is essentially a undesirable by-product, especially when costs for inactivation of genetically modified biomass are incurred (Brandberg *et al.*, 2007; Hols *et al.*, 1999; Kuipers *et al.*, 1997; Papagianni, 2012). Ideally, product formation should be uncoupled from growth, using zero-growth cultures that retain high productivity and product yields for prolonged periods.

The physiology of non-growing microbes is mostly studied in batch cultures that proceed from exponential growth to stagnation of growth, stationary phase (Redon *et al.*, 2005; Werner-Washburne *et al.*, 1993). This fast transition in batch cultures does not allow researchers to capture the physiology of sustained zero growth. Slow growth of microbes under defined, constant conditions can be studied in chemostat cultures at low dilution rates (Finkel, 2006; Herbert, 1961; Lu *et al.*, 2009). However, chemostat cultures cannot be used to assess extremely low specific growth rates ($< 0.01\ h^{-1}$), due to technical limitations related to discontinuous substrate addition, the time required for reaching steady state, and the emergence and selection of adapted mutant variants (Bulthuis *et al.*, 1989; Daran-Lapujade *et al.*, 2008).

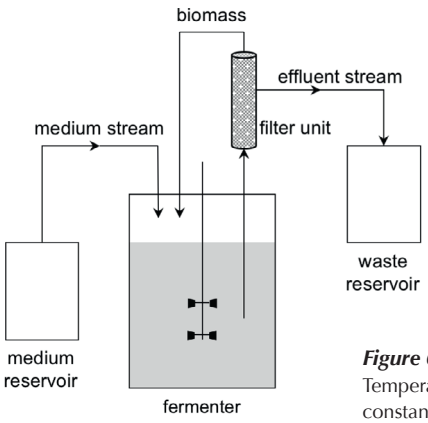

Figure 6.1 Basic scheme of retentostat cultivation set-up
Temperature, pH and working volume of cultures were kept constant during cultivations in all studies.

The retentostat, or recycling fermenter, is a continuous cultivation setup that was specifically developed to study microbial physiology at extremely low specific growth rates (Herbert, 1961; Van Verseveld *et al.*, 1986). In contrast to chemostats, biomass cannot leave a retentostat via the effluent line but, instead, is retained in the bioreactor by a membrane filter (Fig. 6.1). Prolonged energy source-limited retentostat cultivation at a constant dilution rate leads to a progressive buildup of biomass (Fig. 6.2) that coincides with a progressive lowering of the specific rate of substrate consumption. Eventually, this leads to stagnation of growth because all energy substrate provided to the culture is used to meet cellular maintenance requirements (Fig. 6.3) (Boender *et al.*, 2009; Ercan *et al.*, 2013; Goffin *et al.*, 2010; Van Verseveld *et al.*, 1986). Retentostat cultivation therefore allows a gradual transition from exponential growth to a (near-)zero growth state in which sufficient energy substrate is provided for maintaining cellular activity and integrity, thereby preventing cell death.

Before the advent of genomics techniques, retentostat cultivation was used to study microorganisms such as the bacteria *Escherichia coli* (Chesbro *et al.*, 1979), *Bacillus polymyxa* (Arbige and Chesbro, 1982), *Paracoccus denitrificans*, *Bacillus licheniformis* (Van Verseveld *et al.*, 1986), *Tetragenococcus halophila* (Röling and van Verseveld, 1997), *Nitrosomonas europaea*, and *Nitrobacter winogradskyi* (Tappe *et al.*, 1999) and the filamentous fungus *Aspergillus niger* (Schrickx *et al.*, 1995a; Schrickx *et al.*, 1995b; Schrickx *et al.*, 1991; van Verseveld *et al.*, 1991). More recently, the environmentally relevant bacteria *Desulfotomaculum putei* (Davidson *et al.*, 2009) and *Geobacter metallireducens* (Marozava *et al.*, 2014) have been characterized using retentostats. As a multi-laboratory collaboration, our groups have combined retentostat cultivation with quantitative physiological analysis and genome-wide expression studies to analyze zero-growth of the industrially relevant microbes *Lactococcus lactis* KF147, *Lactobacillus plantarum* WCFS1, *Bacillus subtilis* (*trpC2 sigF::spec*, *amyE::*P*rrnB-gfp+*, derivative of strain 168), *Saccharomyces cerevisiae* CEN. PK113-7D (*MAT*a *MAL* 2-8c *SUC2*), and *Aspergillus niger* N402 (*cspA*). The aim of this chapter is to compare physiological and gene expression responses of different industrially important microbes at (near-)zero growth rates in energy-limited-growth retentostat cultures.

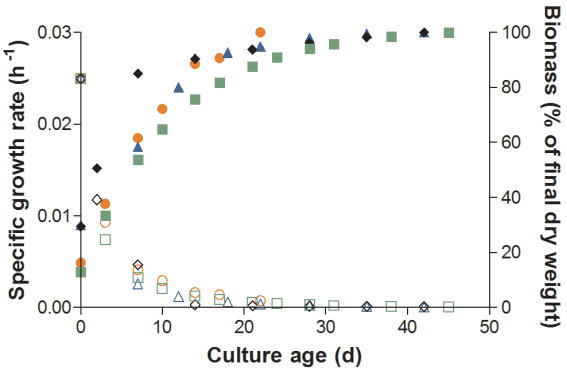

Figure 6.2 Specific growth rates and biomass accumulation during retentostat cultivation
Specific growth rates (open symbols) and levels of biomass accumulation (closed symbols) of *L. lactis* KF147 (diamonds, ◆ ◇), *L. plantarum* WCFS1 (squares, ■ □), *B. subtilis* 168 *sigF*::Sp^r *amyE*::P$_{rrnB}$-*gfp*$^+$ (triangles, ▲ △), and *S. cerevisiae* CEN.PK113-7D (circles, ● ○) during prolonged retentostat cultivation. Biomass concentrations at the end of retentostat cultivations are depicted as 100%. The *L. lactis* KF147, *L. plantarum* WCFS1, *B. subtilis* 168 168 *sigF*::Sp^r *amyE*::P$_{rrnB}$-*gfp*$^+$, and *S. cerevisiae* CEN.PK113-7D reached their maximal levels of 3.7, 6.6, 3.2, and 22.7 g · L^{-1} after 42, 45, 42, and 22 days of retentostat cultivation, respectively.

Physiology of microbes in retentostat cultures

Retentostat cultures are generally started as energy-limited chemostat cultures. After establishing steady-state growth, the effluent line is connected to a biomass retention device and the specific growth rate starts decreasing (Van Verseveld *et al.*, 1986). Depending on the dilution rate of the system and the physiological characteristics of the microbe, reaching extremely low specific growth rates can take several days. Growth rate profiles and quantitative data on specific growth rates for carbon-limited retentostats of different industrial microorganisms (Fig. 6.2, Table 6.1) show that growth rates below 0.001 h^{-1} (corresponding to doubling times of several hundred hours) can be reproducibly achieved in retentostats.

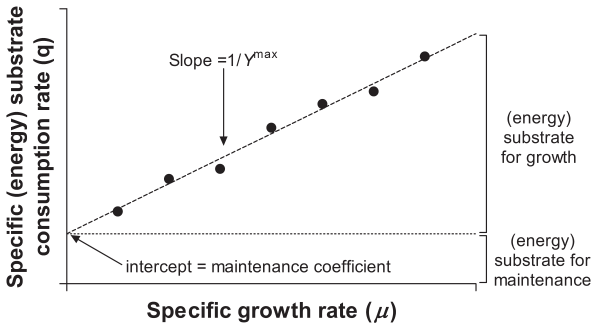

Figure 6.3 Distribution of energy and substrate for growth and maintenance
Determination of energy- and substrate-related coefficients with the plot of the specific substrate (energy) consumption rate (q) against growth rate in chemostat and/or retentostat cultivation. The intercept (extrapolation to a zero-growth condition) gives the maintenance coefficient (m), and the slope of the plot (dashed line) gives the inverse of the maximum growth yield (Y^{max}). Below the dotted line indicates metabolic energy or substrate for maintenance-related processes.

Table 6.1 Specific growth rate and doubling time of different microorganisms grown in energy-limited retentostat cultures.

Microorganism	Reference	Time in retentostat (days)	Specific growth rate (h^{-1})	Doubling time (days)
L. lactis KF147	(Ercan *et al.*, 2013)	42	0.00011	286
Lb. plantarum WCFS1	(Goffin *et al.*, 2010)	45	0.00006	472
B. subtilis 168 *sigF::*Sp^r *amyE::*P_{rrnB}*-gfp*$^+$	(Overkamp *et al.*, 2015)	42	0.00006	470
S. cerevisiae CEN.PK113-7D	(Boender *et al.*, 2009)	22	0.00063	46
A. niger N402	(Jørgensen *et al.*, 2010)	10	0.003	10

Due to the complete cell retention in retentostat cultures, even a low death rate is expected to lead to a significant accumulation of dead cells upon prolonged cultivation. Analysis of viability, for example by counting colony forming units (CFUs) and/or fluorescence-based live/dead staining (Boender *et al.*, 2009; Ercan *et al.*, 2013; Goffin *et al.*, 2010, Overkamp *et al.*, 2015) is therefore essential. After prolonged retentostat cultivation, the viability of both *L. lactis* and *L. plantarum* remained above 90 % (Ercan *et al.*, 2013; Goffin *et al.*, 2010). Remarkably, the viability of *B. subtilis* remained above 90 % as well, while the fraction of cultivable cells decreased down to 30 % (Overkamp *et al.*, 2015). This is most probably due to the observed cell-chain formation under these conditions, which strongly confounds accurate CFU enumeration, rather than to entry of these cells into a viable but non-culturable state (Oliver, 2005). Retentostat cultures of the yeast *S. cerevisiae* showed a slow accumulation of nonviable cells, leading to a viability of ca. 80 % after three weeks of retentostat cultivation (Boender *et al.*, 2009). Although cryptic growth cannot be fully excluded for all of the microorganisms, the cell debris that may be released from dead cells only provides no or minute amounts of consumable substrates that sustain growth. Cell turnover is therefore most probably minimal in these retentostat cultures. In many microorganisms, severe energy limitation triggers the onset of sporulation. Overkamp *et al.* (Overkamp *et al.*, 2015) and Boender *et al.* (Boender *et al.*, 2009) circumvented this problem by using a sporulation-negative strain of *B. subtilis* and a haploid strain of *S. cerevisiae*, respectively. However, in a study on maltose-limited retentostat cultivation of *A. niger*, extensive conidiation occurred already 4 days after the onset of biomass retention (Jørgensen *et al.*, 2010). Since conidiospores passed the biomass retention device, retentostat cultures of *A. niger* did not reach extremely low specific biomass production rates.

Microscopy of retentostat-grown cultures revealed significant morphological changes at (near-)zero specific growth rates, even in non-sporulating cultures (Fig. 6.4). The morphology of *L. lactis* changed from the typical coccoid form to a rod shape, which caused an approximate 2.4-fold increase of cell surface/volume ratio after 42 days of retentostat cultivation (estimated from flow-cytometry analysis), as the specific growth rate declined during retentostat cultivation (Ercan *et al.*, 2013) (Fig. 6.4A,B). Similarly, the morphology of

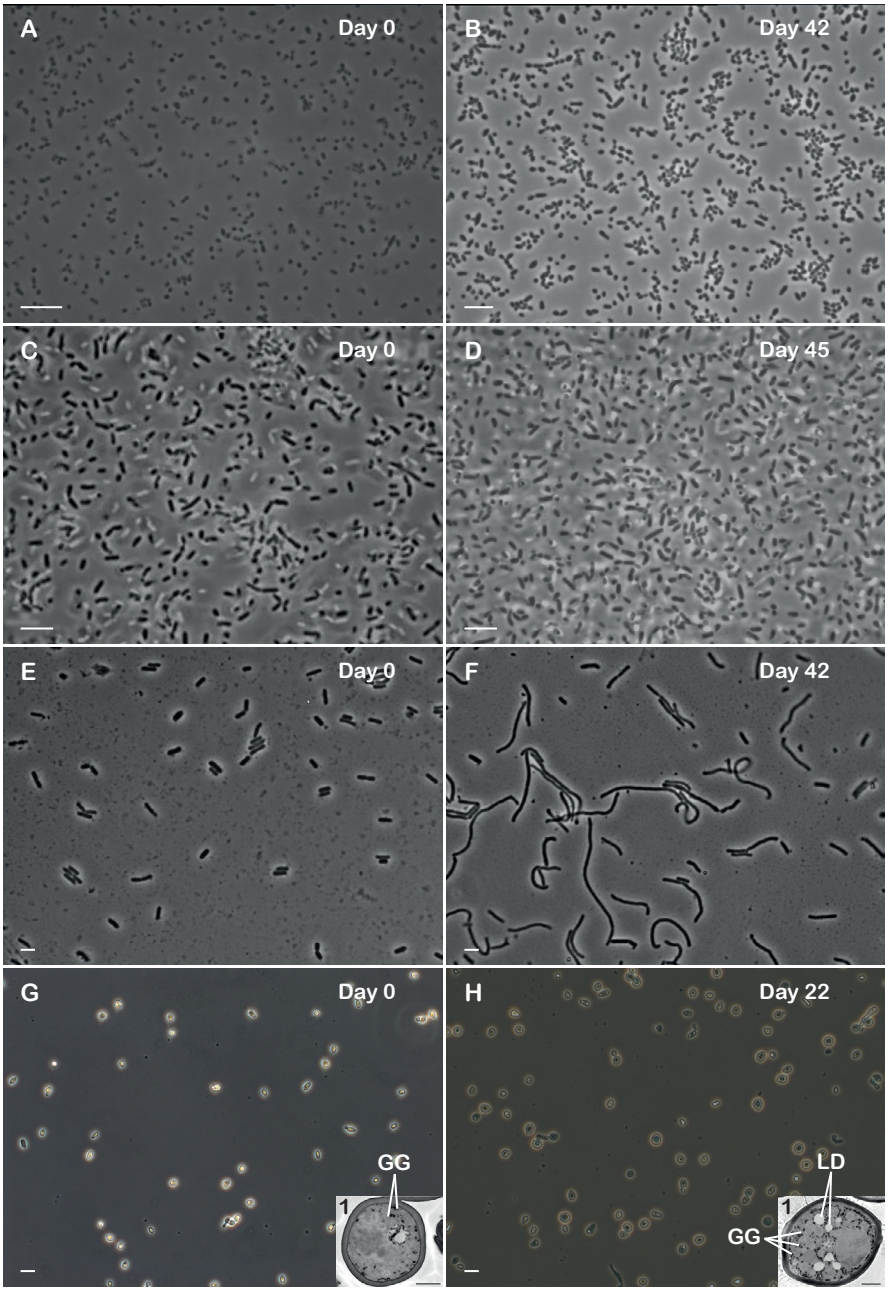

Figure 6.4 Morphology during retentostat cultivation
Phase-contrast microscopic images of chemostat cultures (left; day 0) and at the end of retentostat cultivations (right) of *L. lactis* KF147 (**A** and **B**), *L. plantarum* WCFS1 (**C** and **D**), *B. subtilis* 168 *sigF*::Sp^r *amyE*::P$_{rmB}$-*gfp*$^+$ (**E** and **F**), and *S. cerevisiae* CEN.PK113-7D (**G** and **H**). Scale bars indicate 10 µm. Electron micrographs of *S. cerevisiae* from chemostat (**G1**) and after 22 days in retentostat cultivation (**H1**) were previously published in (Boender *et al.*, 2011b). **GG**, glycogen granules; **LD**, lipid droplets. Scale bars in insets G1 and H1 represent 1 µm.

B. subtilis cells changed from the characteristic short rod shape to substantially elongated cells after 42 days of retentostat cultivation (Overkamp *et al.*, 2015)(Fig. 6.4E,F). Conversely, microscopic examination of chemostat- and retentostat-grown *Lb. plantarum* did not reveal significant changes in morphology (Goffin *et al.*, 2010) (Fig. 6.4C,D), showing that such a response is not a general phenomenon in firmicutes. Electron microscopy of retentostat-grown *S. cerevisiae* cells showed intracellular accumulation of glycogen and an increased content of lipid droplets in the cytosol (Boender *et al.*, 2011b) (Fig. 6.4H1), which likely contributed to a concomitant increase in cell size and mass. Notably, cell shape changes have been reported for several microbes when they enter the non-growing stationary phase of growth (Young, 2006). The changes reported under those stationary phase conditions, where cells become smaller and coccoid, are, however, opposite to the ones observed in retentostat cultures (Amy and Morita, 1983; Mason and Egli, 1993; Morita, 1993). This might reflect changes in the cell growth and cell division ratio. The low energy availability in retentostat cultures might inhibit the cell division machinery, leading to reduced septa formation and division frequencies. The continuous supply of carbon-source may, however, enable individual cells to increase their size and transport capacity by increasing the surface/volume ratio.

Energy metabolism at (near-)zero growth rates: impact of maintenance energy requirements

In heterotrophs, carbon and energy substrates are often used for both growth and maintenance processes. The distribution of substrate between these two processes can be derived from the relationship between the biomass-specific substrate consumption rate (q_s) and the specific growth rate (μ). When the q_s for maintenance processes (the so-called maintenance coefficient m_s) is growth-rate independent, this relation is linear and known as the Herbert-Pirt relation ($q_s = m_s + \mu/Y_{sx}^{max}$, where Y_{sx}^{max} is the maximum growth yield, the biomass yield on the growth-limiting nutrient at very high μ, where $m_s << q_s$) (Pirt, 1965; van Gulik *et al.*, 2001) (Fig. 6.3). Conventionally, m_s is predicted by extrapolation of measurements obtained from chemostat cultures grown at relatively high specific growth rates (e.g., $\mu \geq 0.05$ h^{-1}) and the slope of the linear relation represents $1/Y_{sx}^{max}$ (Boender *et al.*, 2009; Ercan *et al.*, 2013; Goffin *et al.*, 2010; Pirt, 1982).

While in microorganisms, growth rate-independent maintenance energy requirements adequately describe the stoichiometry of biomass and product formation at low to intermediate specific growth rates (Tijhuis *et al.*, 1993), it is unclear to what extent this concept is generally applicable at (near-)zero growth rates. Moreover, several prokaryotes activate a so-called stringent response at low specific growth rates. This alarmone-mediated response results in a down-regulation of energy-intensive cellular processes (e.g., protein turnover) at low specific growth rates, which reduces the ATP demand for maintenance processes (Chang *et al.*, 2002; Eymann *et al.*, 2002; Redon *et al.*, 2005). Retentostat cultivation enables a much closer approximation of a zero growth rate than chemostat cultivation and, thereby, a better assessment of the growth rate (in)dependence of m_s in different microorganisms.

In recent studies, m_s values were estimated from retentostat cultures of the industrial microorganisms *S. cerevisiae* (Boender *et al.*, 2009), *L. plantarum* (Goffin *et al.*, 2010), *L. lactis* (Ercan *et al.*, 2013), and *B. subtilis* (Overkamp *et al.*, 2015) (Table 6.2). Contrary to earlier retentostat studies employing other bacteria (Arbige and Chesbro, 1982; Chesbro *et al.*, 1979; Tappe *et al.*, 1999; Van Verseveld *et al.*, 1986), these experiments did not reveal a stringent response, i.e., m_s values calculated from (near-)zero growth retentostats were in good agreement with those estimated by extrapolation from chemostat cultivations at high dilution rates (Boender *et al.*, 2009; Ercan *et al.*, 2013; Goffin *et al.*, 2010). For all four microbes, the fraction of the carbon and energy source used for maintenance-associated processes increased from 13 to 31 % at the initial μ of 0.025 h^{-1} to above 98 % after prolonged retentostat cultivation (Table 6.2). This virtually exclusive allocation of energy to maintenance is consistent with the (near-)zero growth rates reached at the end of the retentostat experiments. Therefore, the results of the retentostat studies on these four microbes were entirely consistent with the concept of a growth rate-independent maintenance energy-requirement.

In *S. cerevisiae*, *B. subtilis*, and *L. plantarum* the relative concentrations of catabolic products remained essentially constant during the transition from chemostat to retentostat cultivation. Major catabolic products were lactate and low concentrations of acetate, formate, and ethanol for *L. plantarum* (Goffin *et al.*, 2010), CO_2 and H_2O for *B. subtilis* (Overkamp *et al.*, 2015), and ethanol and CO_2 for *S. cerevisiae* (Boender *et al.*, 2009). In contrast, substantial fluctuations in fermentation products were observed in *L. lactis* cultures that were also reflected by modulations in the expression of the corresponding genes. However, these metabolic changes were not predicted to influence the overall energy flux in these cells (Ercan *et al.*, 2013; Ercan *et al.*, 2015c).

Table 6.2: Substrate-related maintenance coefficient and maximum specific growth yield values and the relative distributions of energy and substrate costs of maintenance-associated processes during chemostat and extended retentostat cultivation[a].

Microorganism	Reference	Carbon source	m_s ($mmol_{carbon} \cdot g_{DW}^{-1} \cdot h^{-1}$)	Y_{sx}^{max} ($mg_{DW} \cdot mmol_{carbon}^{-1}$)	$Dist_{chemostat}$ (%)	$Dist_{retentostat}$ (%)
L. lactis KF147	(Ercan *et al.*, 2013)	Glucose and citrate	1.11	9.80	30.3	99.0
Lb. Plantarum WCFS1	(Goffin *et al.*, 2010)	Glucose and citrate	0.65	5.54	12.8	98.4
B. subtilis 168 *sigF*::Sp^r *amyE*::P_{rrnB}-*gfp*$^+$	(Overkamp *et al.*, 2015)	Glucose	1.43	7.92	31.4	98.2
S. cerevisiae CEN.PK113-7D	(Boender *et al.*, 2009)	Glucose	3.00	2.91	21.3	98.6

[a]Cultivations were performed under anaerobic conditions for all organisms except B. subtilis, for which aerobic conditions were used. m_s, maintenance coefficient; DW, dry weight; Y_{sx}^{max}, maximum specific growth yield; $Dist_{chemostat}$ and $Dist_{retentostat}$, relative distribution of energy and substrate costs to maintenance-associated processes during chemostat cultivation, with a dilution rate of 0.025 h^{-1}, and extended retentostat cultivation, respectively.

Although its growth was limited by carbohydrate substrate availability, *L. lactis* displayed major adaptations of its nitrogen metabolism during extended retentostat cultivation (Ercan *et al.*, 2015c). By integration of data of transcriptome analysis and metabolic modeling, *L. plantarum* was predicted to produce plant growth-stimulating metabolites, including indole compounds and phenylacetate, at (near-)zero growth rates (Goffin *et al.*, 2010). Short-term retentostat cultivation elicited significant metabolic changes in *A. niger*, including the production of the secondary metabolites flavasperone, aurasperone B, tensidol B, fumonisins B_2, B_4, and B_6 (Jørgensen *et al.*, 2011). These results show that in some, but not all (two of the five analyzed here), species (near-)zero growth elicits the production of secondary metabolites.

Similar transcriptional responses in pathways involved in nutrient sensing, anabolism and response to stress

Besides adaptations in metabolic fluxes, transcriptional adaptations were observed as a function of decreasing growth rate and nutrient availability (Fig. 6.5). Both in the eukaryotic and prokaryotic kingdoms, dedicated glucose sensing and signaling cascades (such as the protein kinase A (PKA) and TOR pathways in eukaryotes and catabolite control protein A (CcpA) in Gram-positive bacteria) result in transcriptional reprogramming, including the repression of genes involved in alternative carbon source utilization in glucose-rich environments (De Virgilio and Loewith, 2006; Kim *et al.*, 2005; Thevelein and de Winde, 1999; Zomer *et al.*, 2007). During retentostat cultivation, prolonged glucose limitation led to the progressive relief of repression of CcpA targets in prokaryotes and of glucose-repressible genes in *S. cerevisiae*, thereby demonstrating a gradual alleviation of carbon catabolite repression when approaching (near-)zero growth (Boender *et al.*, 2011b; Ercan *et al.*, 2015c; Goffin *et al.*, 2010; Overkamp *et al.*, 2015). In *L. lactis*, this carbon catabolite repression transcriptional relief was shown to enable retentostat-derived cultures to more rapidly ferment carbon sources other than glucose, illustrating the functional consequence of this transcriptional adaptation (Ercan *et al.*, 2015c). Notably, *A. niger* retentostat cultures displayed increased expression of genes involved in nutrient mobilization, including carbohydrate transporters (Jørgensen *et al.*, 2010). These evolutionarily conserved adaptive responses enable the microbes to rapidly scavenge alternative carbon and energy sources when they appear in their environment (Fig. 6.5).

When microbes approach (near-)zero growth rates, the cellular requirement for biosynthetic building blocks strongly decreases. This reduced anabolic demand is reflected in the transcriptome of most organisms by a decreased expression of genes involved in biosynthetic routes. Protein synthesis is the most energy-demanding biosynthetic process (Verduyn *et al.*, 1990a). Under the severe calorie restriction in retentostat cultures, *B. subtilis*, *S. cerevisiae*, and *A. niger* showed reduced expression of components of the translation machinery, including ribosomal proteins and amino acyl tRNA synthases (Boender *et al.*, 2011b; Jørgensen *et al.*, 2010; Overkamp *et al.*, 2015)(Fig. 6.5). Although chemostat

studies of *L. lactis* revealed the existence of a positive correlation between growth rate and expression of genes involved in translation at growth rates above 0.09 h^{-1} (Dressaire *et al.*, 2008), such a correlation was not observed in retentostat cultures of *L. lactis* or *L. plantarum* (Ercan *et al.*, 2015c; Goffin *et al.*, 2010). The absence of such a correlation could not be readily explained by differences in energy levels, as all unicellular organisms showed similar distributions of energy substrate to maintenance-associated processes (Table 6.2). This suggests an uncoupling between translation and growth rate at a growth rate between 0.025 and 0.09 h^{-1} in LAB. However, the use of different nutrient limitations (glucose and leucine in retentostats and chemostats, respectively) in the two *L. lactis* studies (Dressaire *et al.*, 2008; Ercan *et al.*, 2015c) may also have contributed to the observed differences.

The severe energy limitation in retentostats potentially is a stress factor for the culture. Indeed, a third conserved transcriptional response observed in the five microorganisms mentioned above encompassed an increased expression of genes involved in stress responses. Although observed during retentostat cultivation of all five microbes, responses were diverse. *L. lactis* and *S. cerevisiae* showed increased expression of genes involved in general stress resistance, including heat shock proteins (GroELS and DnaKJ protein complexes in *L. lactis* and Hsp12, Hsp26, Hsp30, Hsp78, and Ssa3 in *S. cerevisiae*). These responses were shown to confer increased tolerance of yeast to heat stress and of heat-, acid- and oxidative-stress conditions on *L. lactis* (Boender *et al.*, 2011a; Boender *et al.*, 2011b, O. Ercan, H.M.W. den Besten, E.J. Smid, and M. Kleerebezem, unpublished data). General stress response regulons were not significantly induced in retentostat cultures of *B. subtilis* and *L. plantarum* (Goffin *et*

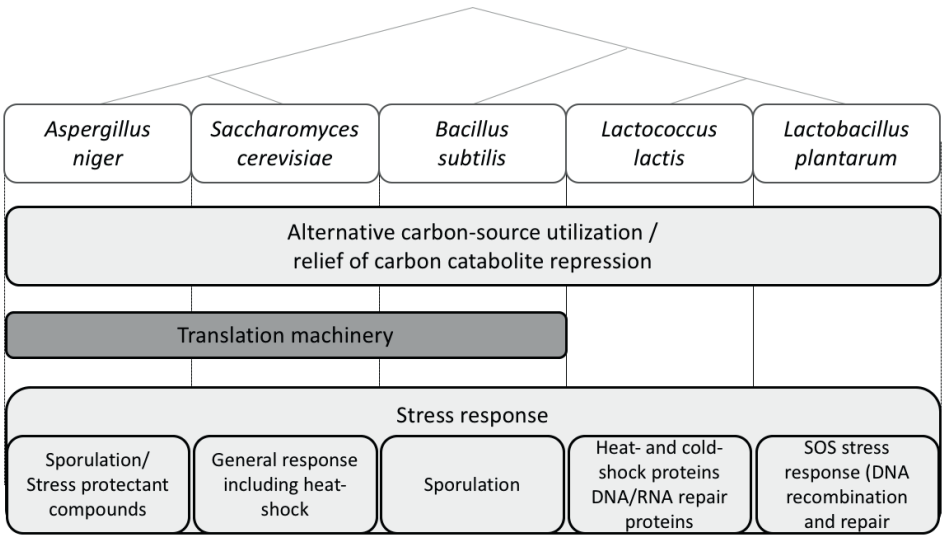

Fig. 6.5: Cross-kingdom-shared transcriptional adaptations to (near-)zero growth.
Increased and decreased expression levels of the functional categories during retentostat cultivation are indicated by light- and dark-gray boxes, respectively.

al., 2010; Overkamp *et al.*, 2015). The latter, however, did show induction of the expression of genes involved in the SOS response, including error-prone DNA-polymerases (Goffin *et al.*, 2010), which by increasing mutation rates might accelerate evolutionary adaptation and thereby increase chances of survival. However, the number of generations in the retentostat was too small to directly observe adaptive evolution or enrichment of fitness-improving mutations (Overkamp *et al.*, 2015). *A. niger* increased expression of genes involved in synthesis of stress-protectant compounds during retentostat cultures (Jørgensen *et al.*, 2010), while the induction of sporulation in this fungus can also be interpreted as an adaptation to the stress imposed by (near-)zero growth (Nicholson *et al.*, 2002; Wyatt *et al.*, 2013). True assessment of zero growth in *A. niger* may require the use of a nonsporulating mutant (Krijgsheld *et al*., 2013), analogous to what has been used for *B. subtilis*.

Microbe-specific transcriptional responses at (near-)zero growth rates

In addition to similar responses in two or more microorganisms, retentostat cultivation was also found to induce species-specific transcriptional responses. When exposed to nutrient limitation, *B. subtilis* cells are known to increase their expression of genes involved in motility and chemotaxis to enable migration to nutrient-rich environments (Rao *et al.*, 2008). However, *B. subtilis* retentostat cultures down-regulated expression of these genes, a response also observed in stationary-phase cultures (Blom *et al.*, 2011; Mirel *et al.*, 2000; Overkamp *et al.*, 2015). This could imply that the expression of these genes depends on a minimal carbon or energy source availability, which may not have been met in retentostat cultures. Reduced autolysin expression in growing cultures of *B. subtilis* was associated with cell chain formation and cell wall metabolism (Kearns and Losick, 2005; Overkamp *et al.*, 2015). Reduced expression of these functions during retentostat cultivation may explain the pronounced morphological changes in *B. subtilis*.

Exopolysaccharide production in LAB has been reported to be strongly growth related, which is in agreement with the repression of these genes during retentostat cultivation of *L. lactis*. Reduced exopolysaccharide expression at (near-)zero growth rates may increase mobility by reducing adhesion and/or biofilm formation and affect susceptibility to polysaccharide-recognizing bacteriophages (Laws *et al.*, 2001; Looijesteijn *et al.*, 2001). In addition to the aforementioned production of plant growth-stimulating compounds, the transcriptome of retentostat cultures of *L. plantarum* revealed further responses that could relate to plant environment-associated conditions, including the activation of genes that are shared with other plant-associated bacteria and putatively involved in the degradation of plant-derived material (Goffin *et al.*, 2010).

In anaerobic *S. cerevisiae* retentostat cultures, many genes involved in mitochondrial functions, including respiration, were up-regulated at both the transcript and protein level, despite the absence of oxygen (Binai *et al.*, 2014; Boender *et al.*, 2011b). This transcriptional reprogramming could not solely be attributed to the alleviation of glucose repression (Binai *et al.*, 2014; Boender *et al.*, 2011b) and therefore probably reflects a preparation for

environmental changes. In general, many yeast genes that were previously shown to be characteristic for quiescent cells (Werner-Washburne *et al.*, 1993), i.e., postmitotic cells, were gradually up-regulated during retentostat cultivation, suggesting a growth rate-dependent expression of these genes rather than an on-off switch of quiescence upon cessation of growth.

Consistent with the observed sporulation of *A. niger* retentostat cultures, genes involved in conidiation and related processes showed the strongest concerted up-regulation in retentostat cultures of this filamentous fungus. This response also included an increased expression of genes encoding small cysteine-rich proteins, such as hydrophobins, and of gene clusters involved in secondary metabolism. Conversely, expression of genes encoding secreted hydrolases was decreased (Jørgensen *et al.*, 2010), possibly to reduce the energy-intensive production of proteins that do not exclusively benefit the producing cells.

Conclusions and future perspectives

In this chapter, we compared physiological, metabolic, and genome-wide transcriptional adaptations of the industrially important microbes *L. lactis*, *L. plantarum*, *B. subtilis*, *S. cerevisiae,* and *A. niger* to (near-)zero growth rates imposed by energy-limited retentostat cultivation. Some cellular responses observed in retentostats, such as reduced expression of *B. subtilis* genes involved in motility and a strongly increased stress resistance of *S. cerevisiae* and *L. lactis*, resembled characteristics of stationary-phase, energy-starved cultures. However, many other responses seen in retentostat cultures differed strikingly from those observed in stationary-phase cultures. For example, the high and stable viability of bacterial and yeast retentostat cultures contrasted strongly with a reported loss of viability in stationary-phase, nutrient-starved cultures. Moreover, in *S. cerevisiae*, glycogen accumulated intracellularly during retentostat cultivation, while carbon and energy starvation leads to mobilization of this storage carbohydrate (Boender *et al.*, 2011a). In *B. subtilis* and *L. lactis* retentostat cultures, the cellular surface-to-volume ratio increased (Ercan *et al.*, 2013; Overkamp *et al.*, 2015), while this ratio decreases in starved bacterial cells (Amy and Morita, 1983; Mason and Egli, 1993; Morita, 1993; Young, 2006). Furthermore, transcriptional analyses showed an even stronger down-regulation of biosynthetic processes, such as translation, when *S. cerevisiae* and *L. lactis* retentostat cultures were completely deprived of extracellular energy sources (Boender *et al.*, 2011a; Ercan *et al.*, 2015b). These observations show that retentostat cultivation captures a physiological state that is distinct from starvation in stationary-phase cultures, which is still the most intensively used system for studying non-growing microorganisms.

A significant fraction of the huge amount of non-dividing microbial biomass on the planet (Gray *et al.*, 2004) resides in oligotrophic environments characterized by an extreme scarceness, but not a complete absence, of essential nutrients, including energy sources (Ferenci, 2001). Examples of such environments vary from nutrient-poor environments such as ocean water (Williams *et al.*, 2013) to natural biofilms, in which a high biomass density, low nutrient availability and diffusion limitation can together cause extremely low specific

growth rates (Evans and Holmes, 1987; Prosser *et al.*, 1987). We are convinced that the unique access that retentostat cultures provide to the largely uncharted but ecologically highly important "twilight zone" between growth and starvation amply rewards the efforts involved in setting up and running these rather labor-intensive cultivation systems. Additionally, planktonic growth of microbes in homogeneous retentostat cultures offers unique possibilities to further dissect effects of attached growth from those of the resulting (near)-zero growth rates in biofilms.

The uncoupling of microbial growth and product formation holds great potential for microbial biotechnology. This is exemplified by near-theoretical yields of the catabolic products ethanol and lactate in anaerobic retentostats of *S. cerevisiae* and LAB, respectively. For example, yields of yeast biomass and glycerol, which are the major by-products of bioethanol production by *S. cerevisiae* (Brandberg *et al.*, 2007), were negligible in retentostat cultures. An important challenge for future research is to combine these near-optimal product yields with a high biomass-specific fermentation rate, for example, by deliberate introduction of futile cycles that cause a net hydrolysis of ATP (Flores and Gancedo, 1997; Koebmann *et al.*, 2002a; Koebmann *et al.*, 2002b). Even greater challenges are associated with 'anabolic' products whose synthesis requires a net input of ATP, such as enzymes, proteins and peptides (Diers *et al.*, 1991; Papagianni, 2012; Van Verseveld *et al.*, 1986), as well as in some of the high-value flavor, texture, and health metabolite production processes that employ LAB and *S. cerevisiae* (Hols *et al.*, 1999; Kuipers *et al.*, 1997; Paddon *et al.*, 2013; Steidler *et al.*, 2000). Efficient production of anabolic products by non-growing cultures will need to overcome the observed down-regulation of the protein synthesis machinery during retentostat cultivation and the scarcity of ATP under calorie-restricted conditions. Strategies to genetically engineer strains for efficient conversion of carbon sources into the desirable end-product in a growth-uncoupled fashion have been demonstrated for *L. lactis* for the production of its endogenous metabolites (Bongers *et al.*, 2005; Gaspar *et al.*, 2004; Hugenholtz *et al.*, 2000; Pudlik and Lolkema, 2011), and the alternative metabolite L-alanine (Hols *et al.*, 1999). Moreover, retentostat cultures provide an excellent test bed for quantitative analysis of radical synthetic biology strategies aimed at uncoupling native and heterologous protein production by introduction of orthogonal translation systems (Hoesl and Budisa, 2012; Neumann, 2012). Also, retentostats are not the only means to uncouple growth and anabolic product formation. Growth limitation by a non-energy substrate can be explored, provided that overflow metabolism and reduced efficiency of energy source utilization under such energy excess conditions (Boer *et al.*, 2003; Schuurmans *et al.*, 2008) can be prevented. Moreover, cell division can be uncoupled from metabolic activity under glucose excess by continuous cultivation of *S. cerevisiae* in alginate beads (Nagarajan *et al.*, 2014).

In addition to the potential of retentostats to enable near-theoretical product yields, the strongly increased stress resistance of retentostat-grown *L. lactis* and *S. cerevisiae* provides leads for design of fermentation protocols that improve survival under subsequent industrial

processing and storage conditions. For example, eliciting an increased heat and/or oxidative stress tolerance by introducing a (near-)zero growth phase may be explored as a strategy to produce robust starter cultures.

Most of the observed responses under retentostat cultivation, e.g., stringent and SOS responses, etc., are specific to subsets of microbes. However, there are some conserved responses, such as activation of alternative carbon source utilization pathways and the generic chaperonin-type heat-shock proteins, and reorientation of energy metabolism. These common responses may be universally conserved in most microbes under (near-)zero growth conditions to strictly sustain maintenance-associated processes and not growth.

This study highlights the power of combining controlled cultivation in retentostats with genome-scale analytical techniques. The retentostat cultivation method yields quantitative information on an important domain of microbial physiology that is not experimentally accessible via other methods. We hope that our comparative study underlines both the versatility and additional value of this approach, not only for uncoupling growth and product formation in industrial microbes, but also for understanding microbial lifestyles and ecology in their natural habitats and, more tentatively, for the study of cellular aging (Bisschops *et al.*, 2014) of a wide variety of organisms.

Acknowledgements

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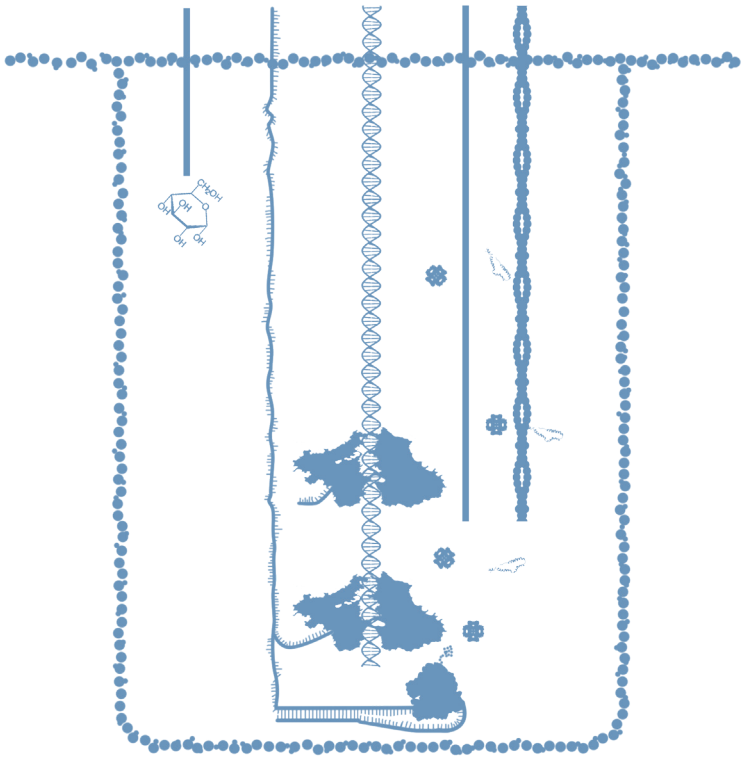

Summary / Samenvatting

Summary of the thesis:

"Molecular responses of Saccharomyces cerevisiae *to near-zero growth rates"*

Non-growing microbial cultures are at least as relevant as their far-better-studied growing counterparts. In industrial processes, the product of microbial growth, biomass, is often considered a byproduct that reduces yields and results in a, sometimes expensive, waste-stream. Uncoupling of growth and product formation would be of high interest for such processes. This, however, requires non-growing, yet highly metabolically active cultures. *Saccharomyces cerevisiae,* alias baker's yeast, is one of the most-used microorganisms in biotechnology to produce for example biofuels, chemicals and pharmaceutical proteins. In addition, *S. cerevisiae,* as unicellular eukaryote, has become an important model-organism to study cell biology and genetics of metazoans during the last decades. Amongst others, *S. cerevisiae* is used to assay the effect of specific diets and genes, of which often human homologs exist, on aging. An important aspect of aging in humans, chronological aging, is investigated using non-dividing, non-growing yeast cultures.

Currently, so-called stationary-phase (SP) cultures are the paradigm for non-growing yeast cultures. SP cultures are batch cultures in which growth is arrested due to depletion of one or more essential nutrients. Often the carbon and energy source is depleted, resulting in low metabolic activity and loss of viability over time within these yeast cultures. This state contrasts strongly with the physiology of human cells, for which they are used as a model. SP cultures are thus, despite the ease with which they can be obtained, not ideal to study chronological aging. Additionally, the virtual absence of metabolic activity and loss of viability also excludes their application in industrial production processes. An alternative cultivation method is therefore needed, in which cells are continuously supplied with an energy source, but not allowed to grow.

Retentostat cultures, a modification of chemostat cultures, a continuous microbial cultivation method, offers such an alternative. In both culture types fresh medium is continuously supplied, but in retentostats only the spent medium is removed and biomass is retained in the reactor by means of cell retention. Due to the accumulation of cells in a glucose-limited retentostat the amount of glucose available per cell dwindles to reach a level at which all available glucose is used for cellular maintenance, leaving no energy for growth. Previously, anaerobic retentostat cultures of *S. cerevisiae* have been studied in the Industrial Microbiology section of the TU Delft. In these retentostats indeed specific growth-rates close to zero (below 0.001 hour $^{-1}$) were obtained, while cells remained alive and metabolically active. Besides this important difference from aerobic SP cultures, also similarities were observed, such as a high thermotolerance and expression of genes previously associated with SP cultures. The truly different physiological state of cells in retentostat cultures was illustrated by glycogen metabolism. In retentostat cultures, yeast cells accumulated this reserve carbohydrate, whereas in SP consumption of glycogen was observed.

In this thesis the physiological state of cells under non-growing conditions, especially in retentostat cultures, will be further examined. The underlying goal of this research is to decipher the processes that are crucial to obtain robust, non-growing cultures of *S. cerevisiae*.

In retentostats, growth is limited due to the minimal availability of glucose, giving nutrient signaling cascades a crucial role in this gradual growth arrest. Several nutrient signaling cascades, such as the TOR and PKA pathways converge on Rim15, a PAS kinase, and modulate its activity. Rim15 in turn activates several transcription factors including the Msn2/Msn4 pair, Gis1 and Hsf1. The strong stress resistance, and high expression of genes under control of these transcription factors in retentostat cultures, suggest a key role for Rim15 in the attenuation of cell division in these cultures. To test this hypothesis, retentostat cultures of a *rim15* deletion mutant and a *RIM15* reference strain were compared at both the physiological and transcriptional level (**chapter 2)**. This study showed that Rim15 is indeed crucial for the increased stress resistance of *S. cerevisiae* at decreasing growth rate, as well as for accumulation of glycogen and the expression of many genes under control of Msn2/Msn4, Hsf1 and Gis1. More surprising results were also obtained. In *rim15*-mutant retentostat cultures viability decreased stronger compared to reference cultures. In addition, the mutant did not show the increase in cell size and mass observed for the reference strain at decreasing growth rate. Lastly, *rim15* cells showed an altered morphology. Together with the higher expression of genes involved in specific phases of the cell cycle, these results point towards a failure in the correct adjustment of cell division to glucose availability in *S. cerevisiae* cells lacking Rim15. Both this lack of cell division control and the increased stress sensitivity of *rim15* yeast cells under, normally, growth-restricting conditions, resembles characteristics of malignant human cells.

In glucose-limited retentostat cultures, yeast cells face extreme caloric restriction and one might expect yeast to reduce non-essential processes under such conditions. In apparent contradiction to this assumption, the transcript-levels of many genes involved in glucose respiration increased, although this strictly aerobic process could not occur in anaerobic retentostat cultures. However, transcription of genes requires relatively little energy compared to actual translation of mRNAs. To investigate whether, under extreme caloric restriction, mRNA levels correlated with the abundance of corresponding proteins, retentostat cultures were subjected to a proteome analysis (**chapter 3**). This proteome analysis showed that of the 2435 quantified proteins, 504 showed consistent changes in their levels during retentostat cultivation. The correlation between protein and transcript levels was however low. Only for 146 proteins a direct correlation with changes at the transcript levels was apparent. In full agreement with the observed increase in thermotolerance in retentostat cultures was the increased abundance of heat shock proteins (Hsp) and other proteins involved in thermotolerance. The levels of several enzymes that are part of the tri-carboxylic acid cycle and electron-transport chain, two processes partly or completely inactive under anaerobic conditions, increased. Yeast cells apparently invest, under extreme caloric restriction, scarce energy in, at first sight, non-relevant processes. From an evolutionary perspective, these

investments may however offer an advantage, as they could enable cells to rapidly respond to changes in the environment.

Transcriptome and proteome analyses of retentostat cultures showed several resemblances between these and SP cultures. SP cultures are known to be heterogeneous and consist of quiescent and non-quiescent cells. Quiescent cells are more robust; they survive longer in the absence of exogenous carbon and energy sources and are more stress resistant than non-quiescent cells. The degree of heterogeneity in retentostat cultures was unknown, but these cultures seemed to display characteristics of especially quiescent cells, such as robustness and expression of specific genes. Heterogeneity could have major implications for the interpretation of results from retentostat-based research, both for research of industrial fermentation processes as well as for aging research. In **chapter 4** we therefore set out to explore heterogeneity using flow-cytometric and fluorescence-microscopy based techniques. DNA content analysis showed that cells mostly arrest growth in a cell cycle phase with unreplicated DNA. Analysis of actin structures in cells also revealed a decrease in cells containing structures associated with active cell division. More surprisingly was the low and constant fraction of cells containing actin bodies, structures previously denoted specific for quiescent cells, when cells transit from low to zero growth rates. When cells were starved, these structures appeared rapidly, illustrating that their formation is merely a response to exogenous carbon or energy depletion and that cells in retentostats reside in a different physiological state. The single cell *HSP12* and *HSP26* transcript levels revealed that slow growing chemostat cultures were heterogeneous in *HSP12* and *HSP26* expression and that this heterogeneity reduced when cultures approached zero growth. The presence of *HSP12* and *HSP26* transcripts in a large fraction of the slow growing chemostat cultures suggested a conditional bet-hedging strategy. Transcript numbers per cell were furthermore found to correlate with cell sizes, suggesting that the transcription of these genes is not only condition but also size-dependent. *S. cerevisiae* may thus possess mechanisms to adjust transcript levels to variations in cell size, as has been described for mammalian cells. Overall the results in this chapter showed that retentostat cultures become highly homogenous and arrest growth in a non-quiescent phase.

The results in this thesis showed the existence of both similarities and crucial differences between retentostat and SP cultures. There is however an important difference in the experimental conditions used. All here and previously described retentostat cultures of *S. cerevisiae* have been performed under anaerobic conditions, while SP cultures have been mostly studied under aerobic conditions. This led to the question as to what extent aerobic and anaerobic SP cultures differ from each other and from anaerobic retentostat cultures. *Ergo*: how important is the availability of glucose and oxygen for the robustness of *S. cerevisiae*? To address this question, in **chapter 5** of this thesis, aerobic and anaerobic SP and the entrance therein were studied in detail using batch cultures. The focus was on robustness, i.e. stress resistance and viability, important characteristics of retentostat cultures. The absence of oxygen reduced the chronological lifespan of stationary phase cultures by

almost two-fold and had an even stronger effect on heat shock resistance. Surprisingly, genes involved in thermotolerance, such as *HSP* genes, were not overrepresented among genes with higher expression levels in aerobic SP cultures. Transcriptome analysis further showed that the known transcriptional reprogramming occurring in aerobic cultures entering SP, mostly took place during the respiratory, post-diauxic phase and only partially occurred in anaerobic cultures. Analysis of intracellular metabolites showed that upon glucose-depletion, cellular energy charge strongly decreased in anaerobic cultures, despite a higher glycogen content. Anaerobic cultures are apparently strongly limited in their energetic flexibility upon glucose-depletion. This energetic limitation may affect translation even stronger than transcription and contribute to the absence of thermotolerance and shorter chronological life span of cells in anaerobic SP cultures. In contrast to the abrupt transition into starvation of anaerobic SP cultures, the transient reduction in growth rate in anaerobic retentostat cultures did result in cells resistant against stress and starvation. Although other roles of oxygen cannot be excluded, these results illustrate that the energetic state of cells during the transition to growth arrest is an important factor regarding robustness of *S. cerevisiae* in the absence of exogenous energy sources.

Cells cultivated in retentostat cultures combine the positive aspects of SP cultures, such as the virtual absence of growth and high stress resistance, with high viability and metabolic activity. The adaptation to extreme caloric restriction, mediated through nutrient signaling cascades in *S. cerevisiae*, results in properties that are highly relevant for application in stressful industrial processes. *S. cerevisiae* is however not the only micro-organism widely used in biotechnology that has been studied in retentostat cultures. In recent years, research has been performed, within the Kluyver Centre for Genomics of Industrial Fermentation, at the physiological and molecular level of retentostat cultures of other industrially relevant micro-organisms, i.e. *Lactobacillus plantarum*, *Lactococcus lactis*, *Bacillus subtilis* and *Aspergillus niger*. In the last chapter of this thesis (**chapter 6**) an overview is presented of the adaptations of these different microorganisms to virtually non-growing conditions. This literature review showed among others that most of these microorganisms, including *S. cerevisiae*, induce one or more stress-responses transcriptionally when grown at extreme low growth rates. Furthermore pathways for alternative energy-substrates are induced in retentostat cultures. Besides shared responses the panel of studied microorganisms also displayed differences in their response to severe caloric restriction. For example, *A. niger* cultures sporulated, while in *B. subtilis* and *L. lactis* cultures pronounced changes in morphology and nitrogen metabolism were observed.

This thesis shows that retentostat cultures of *S. cerevisiae* as a model to study chronological aging are a promising alternative to the currently widely used SP cultures. In addition retentostat cultures provide a powerful tool for applied and fundamental microbiology to access a fascinating physiological state that is difficult to study in other ways: the twilight zone between growth and, starvation-induced, growth-arrest.

Samenvatting van het proefschrift:

"Molecular responses of Saccharomyces cerevisiae *to near-zero growth rates"*

Niet-groeiende cultures van micro-organismen zijn minstens zo relevant als hun veel beter bestudeerde, actief groeiende tegenhangers. In industriële processen is het product van microbiële groei, biomassa, vaak een bijproduct dat resulteert in lagere opbrengsten en een, soms dure, afvalstroom. Het ontkoppelen van groei en productvorming zou in zulke processen een zeer interessante optie zijn. Dit vereist echter niet-groeiende cultures, die wel een zeer actieve stofwisseling hebben. *Saccharomyces cerevisiae,* ofwel bakkersgist, is een van de meest gebruikte micro-organismen in de biotechnologie voor de productie van bijvoorbeeld biobrandstoffen, chemicaliën en farmaceutische eiwitten. Daarnaast is *S. cerevisiae*, als eencellige eukaryoot, in de afgelopen decennia ook een belangrijk model-organisme geworden voor de celbiologie en genetica van hogere organismen. Zo wordt *S. cerevisiae* onder andere gebruikt om de invloed van specifieke voedingsregimes en genen, waarvan vaak homologen aanwezig zijn in het menselijk genoom, op veroudering te bestuderen. Een belangrijk aspect van veroudering in de mens, chronologische veroudering, wordt onderzocht aan de hand van niet-delende, niet-groeiende cultures.

Tot nu toe waren deze niet-groeiende cultures voornamelijk stationaire-fase (SF) cultures. SF-cultures zijn batchcultures waarin groei gestopt is doordat een of meerdere essentiële nutriënten uitgeput zijn. Vaak betreft dit de koolstof- en energiebron, waardoor deze gistcultures nog maar weinig stofwisselingsactiviteit vertonen en afsterven. Deze toestand staat in scherp contrast met de fysiologie van de menselijke cellen waarvoor ze model staan. SF-cultures zijn dus, ondanks het feit dat ze technisch gezien makkelijk te verkrijgen zijn, niet de ideale gistcultures om veroudering te bestuderen. Daarnaast zijn ze door de praktische afwezigheid van stofwisselingsactiviteit en door afsterving ook niet inzetbaar in industriële productieprocessen. Een alternatief kweeksysteem, waarin cellen wel continu voorzien worden van een energiebron, maar niet de gelegenheid krijgen om te groeien, is daarom noodzakelijk.

Aanpassing van chemostaatcultures, waarin micro-organismen continu gekweekt kunnen worden, tot retentostaatcultures maakt dit mogelijk. In beide cultivatiesystemen is er een continue toevoer van vers medium, maar in de retentostaat wordt slechts het verbruikte medium verwijderd en blijft de biomassa in de cultuur. Door de ophoping van cellen in een glucose-gelimiteerde retentostaat neemt de hoeveelheid beschikbare glucose per cel sterk af, hetgeen uiteindelijk resulteert in de situatie waarin alle glucose gebruikt wordt voor onderhoud van de cel en er geen energie meer beschikbaar is voor groei. In de sectie Industriële Microbiologie van de TU Delft is eerder onderzoek verricht aan anaerobe retentostaatcultures van *S. cerevisiae*. In deze retentostaten werden groeisnelheden van vrijwel nul (lager dan 0.001 uur^{-1}) bereikt, terwijl de cellen levend en metabool actief blijven. Naast dit belangrijke

verschil met SF-cultures werden er ook overeenkomsten in de responses van gistcellen in beide kweeksystemen gevonden, zoals een hoge hittebestendigheid en de expressie van genen die eerder geassocieerd werden met SF-cultures. De wezenlijk andere fysiologische status van de cellen in retentostaatcultures werd geïllustreerd door de stofwisseling van glycogeen. In retentostaatcultures hoopten de gistcellen dit reservemateriaal op, terwijl in SF-cultures juist verbruik van glycogeen werd waargenomen.

In dit proefschrift wordt dieper ingegaan op fysiologische status van cellen onder niet-groeiende condities, en met name in retentostaatcultures. Het onderliggende doel van dit onderzoek is om inzicht te krijgen in processen die van cruciaal belang zijn om robuuste, niet-groeiende cultures van *S. cerevisiae* te verkrijgen.

In retentostaatcultures wordt groei beperkt door de minimale beschikbaarheid van glucose, waardoor zogenaamde nutriënt-signaleringsroutes een belangrijke rol spelen in de geleidelijk afname van groei. Verschillende nutriënt-signaleringsroutes, zoals de TOR- en PKA-routes, bepalen de activiteit van het PAS-kinase Rim15. Rim15 op haar beurt activeert bepaalde transcriptiefactoren zoals Msn2/Msn4, Gis1 en Hsf1. Afgaande op de stressbestendigheid van retentostaat-gekweekte cellen en de hoge expressie van genen die onder controle van deze transcriptiefactoren staan, leek het aannemelijk dat Rim15 een sleutelrol speelt in de afremming van celdeling in deze cultures. Om deze hypothese te testen, zijn retentostaatcultures van een *rim15*-deletiemutant en van een *RIM15*-referentie stam vergeleken op fysiologisch en transcriptioneel niveau (**hoofdstuk 2**). Dit onderzoek liet zien dat Rim15 inderdaad cruciaal is voor de toename in stressbestendigheid van *S. cerevisiae* die optreedt bij een dalende groeisnelheid, alsmede voor de ophoping van glycogeen en de toename in expressie van veel genen die onder controle staan van Msn2/Msn4, Hsf1 en Gis1. Hoofdstuk 2 leverde ook verrassende resultaten. Zo nam de fractie dode cellen in retentostaatcultures van de *rim15*-mutant sterker toe dan in de referentiestam. Verder vertoonde de mutant, in tegenstelling tot het wildtype, geen toename van celgrootte en -massa tijdens de daling van de groeisnelheid die typerend is voor retentostaatcultivatie. Bovendien hadden *rim15* cellen een andere morfologie. Samen met een hogere expressie van genen die betrokken zijn bij specifieke fases van de celcyclus, wijzen deze resultaten er op dat *S. cerevisiae* bij afwezigheid van Rim15, grote moeite heeft om de celdeling af te stemmen op de beschikbare hoeveelheid glucose. Het gebrek aan celdelingscontrole en de hogere stressgevoeligheid van de *rim15* gistcellen vertoont daarmee overeenkomsten met het gedrag van menselijke kankercellen.

In glucose-gelimiteerde retentostaatcultures worden gistcellen onderworpen aan extreme calorische beperking. Onder deze condities zou het een voordeel kunnen opleveren om niet-essentiële processen zo veel mogelijk te vermijden. Schijnbaar in tegenspraak met deze veronderstelling, namen de transcript-niveaus van veel genen betrokken bij de verademing van glucose, een strikt aëroob proces dat niet kan optreden in de anaërobe retentostaten, juist toe. Echter, transcriptie van genen vergt relatief weinig energie vergeleken

met de daadwerkelijke translatie van de gevormde boodschapper-RNA's. Om te onderzoeken of er, onder extreme calorische beperking, een correlatie was tussen de expressieniveaus van mRNA's en van de corresponderende eiwitten, werden retentostaatcultures op verschillende momenten onderworpen aan een proteoom-analyse (**hoofdstuk 3**). Deze proteoom-analyse toonde aan dat van de 2435 gemeten eiwitten, er 504 consistente veranderingen in hoeveelheid lieten zien tijdens retentostaatcultivatie. De correlatie tussen eiwit- en transcript-niveau was echter laag. Slechts voor 146 eiwitten was er sprake van een directe correlatie met de waargenomen veranderingen op transcriptieniveau. Geheel in overeenstemming met de toenemende stressbestendigheid tijdens retentostaatcultivatie, was de toename in hoeveelheid van eiwitten betrokken bij hittetolerantie, waaronder de heat shock proteins (Hsp's). De niveaus van verscheidene enzymen die onderdeel uitmaken van de citroenzuurcyclus en de elektron-transport-keten, twee processen die grotendeels of geheel inactief zijn onder anaerobe condities, namen ook toe. Gistcellen investeren dus kennelijk, tijdens groei in retentostaatcultures, schaarse energie in ogenschijnlijk niet relevante processen. Vanuit een evolutionair oogpunt zouden deze investeringen echter wel degelijk een voordeel kunnen bieden, omdat ze de cellen in staat kunnen stellen om sneller in te spelen op veranderingen in de omgeving.

Transcriptoom- en proteoom-analyses aan retentostaatcultures legden verschillende overeenkomsten met SF-cultures bloot. Van SF-cultures is bekend dat ze heterogeen zijn en bestaan uit cellen die wel of niet in ruststand zijn. De cellen in ruststand zijn robuuster; ze overleven langer in de afwezigheid van een extracellulaire koolstof- en energiebron en zijn stressbestendiger. Hoe heterogeen retentostaatcultures zijn was onbekend, maar ze lijken op transcript- en eiwit-niveau vooral overeenkomsten te vertonen met de cellen in rusttoestand. Eventuele heterogeniteit zou een grote invloed kunnen hebben op de interpretatie van resultaten uit retentostaatonderzoek, zowel voor onderzoek aan industriële fermentatieprocessen als voor verouderingsonderzoek. Met behulp van flowcytometrie en fluorescentiemicroscopie is daarom in **hoofdstuk 4** onderzocht hoe heterogeen retentostaatcultures zijn. Analyse van de cellulaire DNA-inhoud toonde aan dat cellen met name stoppen met delen in een fase van de celcyclus zonder gerepliceerd DNA. Analyse van de actine-structuren in cellen liet ook een afname in de fractie van cellen met celdeling-gerelateerde actine-structuren zien. Meer verrassend was de lage en constante fractie van cellen met 3 á 5 actinekernen, een structuur volgens de literatuur specifiek voor cellen in ruststand, tijdens de transitie van langzame groei naar nulgroei. Op het moment dat de cellen echter gehongerd werden vormden deze structuren zich snel. Daarmee lijkt de vorming van deze structuren voornamelijk een respons op de afwezigheid van extracellulaire koolstof- of energiebronnen. Dit toont daarnaast aan dat cellen in retentostaatcultures zich in een andere fysiologische toestand bevinden. De *HSP12* en *HSP26* transcript niveaus per cel lieten zien dat langzaam-groeiende chemostaatcultures heterogeen zijn wat betreft de expressie van deze twee genen, maar deze heterogeniteit nam af, naarmate de groeisnelheid afnam tot vrijwel nul. De aanwezigheid van *HSP12* en *HSP26*

transcripten in een groot deel van de langzaam-groeiende retetentostaatcultures suggereerde een conditionele bet-hedging stategie. Daarnaast bleken de aantallen transcripten per cel te correleren met het celvolume. Transcriptie van deze genen lijkt dus niet alleen af te hangen van omgevingsfactoren, maar ook van celgrootte. *S. cerevisiae* beschikt dus mogelijk over mechanismen om transcript niveaus aan te passen aan variaties in celgrootte, zoals eerder werd aangetoond voor menselijke cellen. Samengevat lieten de resultaten in dit hoofdstuk zien dat retentostaatcultures zeer homogeen worden en dat de meerderheid van de cellen zich niet in rusttoestand bevindt.

De resultaten in dit proefschrift laten zien dat er zowel overeenkomsten als cruciale verschillen zijn tussen retentostaat- en SF-cultures. Er is echter een belangrijk verschil in de gebruikte experimentele condities. Alle tot nu toe in de literatuur beschreven retentostaatcultures van *S. cerevisiae* zijn uitgevoerd onder anaerobe condities, terwijl SF-cultures voornamelijk onder aerobe condities zijn bestudeerd. De vraag die hieruit voort vloeit is in hoeverre aerobe en anaerobe SF-cultures met elkaar en met anaerobe retentostaatcultures vergelijkbaar zijn. Met andere woorden: hoe belangrijk is de beschikbaarheid van glucose en zuurstof voor de robuustheid van *S. cerevisiae*? Om deze vraag te beantwoorden zijn, in **hoofdstuk 5** van dit proefschrift, aerobe en anaerobe SF en de groei daaraan voorafgaand in detail bestudeerd. De aandacht was met name gericht op de positieve eigenschappen van retentostaatcultures: stressbestendigheid en hoge fracties levende cellen. De afwezigheid van zuurstof resulteerde in een halvering van de chronologische levensduur van SF-cultures en het effect van beluchting op hittetolerantie bleek zelfs nog groter. Verrassend genoeg waren genen betrokken bij hittetolerantie, zoals de *HSP* genen niet oververtegenwoordigd onder de genen met een hogere expressie in aerobe SF-cultures. De transcriptoom-analyse liet verder zien dat de bekende herprogrammering van het transcriptoom in aerobe cultures die SF naderen, voornamelijk plaats vond tijdens de post-diauxische, respiratoire groeifase en slechts gedeeltelijk gebeurde in anaerobe cultures. Analyse van intracellulaire metabolieten liet zien dat op het moment van glucosedepletie, de “energy charge” van de cellen sterk daalde in anaerobe cultures, ondanks hun hogere glycogeen-gehaltes. Anaerobe cultures zijn klaarblijkelijk op het moment van glucosedepletie sterk gelimiteerd in hun energetische mogelijkheden. Deze energetische limitatie heeft waarschijnlijk een nog sterker effect op translatie dan op transcriptie. Dit verschil zou kunnen bijdragen aan de afwezige stressbestendigheid en de kortere chronologische levensduur van anaerobe SF-cultures. In tegenstelling tot de abrupte overgang naar hongering die in anaerobe SF-cultures optrad, leidde de geleidelijke afname van de groeisnelheid in anaerobe retentostaatcultures wel tot stress- en hongerbestendige cellen. Hoewel een bijdrage van andere functies van zuurstof niet op voorhand kan worden uitgesloten, geven deze resultaten aan dat de energiestatus van cellen tijdens de transitie naar hongering, een belangrijke factor is voor de robuustheid van *S. cerevisiae* in afwezigheid van een extern energiesubstraat.

Cellen die gekweekt worden in retentostaten combineren de positieve eigenschappen met SF-cultures, zoals het vrijwel afwezig zijn van groei en een hoge stressbestendigheid met het behoud van metabole activiteit en voorkoming van afsterving. Deze aanpassing aan extreme calorische restrictie, die in *S. cerevisiae* wordt gemedieerd door nutriënt-signaleringsroutes, levert eigenschappen op die uitermate interessant zijn voor toepassing in stressvolle industriële processen. *S. cerevisiae* is echter niet het enige veelgebruikte micro-organisme in de biotechnologie dat bestudeerd is in retentostaatcultures. In de afgelopen jaren is er, binnen het Kluyver Centre for Genomics of Industrial Fermentation, op fysiologisch en moleculair niveau ook onderzoek gedaan aan retentostaatcultures van andere industrieel relevante micro-organismen, en met name aan *Lactobacillus plantarum*, *Lactococcus lactis*, *Bacillus subtilis* en *Aspergillus niger*. In het laatste hoofdstuk van dit proefschrift (**hoofdstuk 6**) wordt een overzicht gegeven van de aanpassingen van deze verschillende micro-organismen aan vrijwel niet-groeiende condities. Dit literatuuroverzicht liet onder andere zien dat de meeste van deze micro-organismen, waaronder ook *S. cerevisiae*, een of meer stress-responsen transcriptioneel induceren wanneer ze bij extreem lage groeisnelheden gekweekt worden. Bovendien worden in retentostaatcultures routes voor het gebruik van alternatieve energie-substraten geïnduceerd. Naast generieke responsen liet het onderzochte panel van micro-organismen ook verschillen zien in hun aanpassing aan extreme calorische restrictie. Zo lieten *Aspergillus niger* cultures bijvoorbeeld sporulatie zien, terwijl in cultures van *B. subtilis* en *L. lactis* uitgesproken wijzigingen in morfologie en in het stikstofmetabolisme voorkwamen.

Dit proefschrift toont aan dat retentostaatcultures van *S. cerevisiae*, als modelsysteem voor het bestuderen van chronologische veroudering, een veelbelovend alternatief vormen voor de nu alom gebruikte SF-cultures. Retentostaten vormen daarnaast een krachtig gereedschap voor de toegepaste en fundamentele microbiologie, dat toegang geeft tot een fascinerende fysiologische toestand die lastig op andere manieren te bestuderen is: de schemerzone tussen groei en, door hongering veroorzaakte, groeistop.

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Besides the recipe, tools and crust, the ingredients of the filling make a cake a success and fun. And it was definitely fun working with you IMB-members: **Alexey**, **Barbara** Kozak (for the threats ;)), **Bart** (voor de geweldige moppen), **Benjamin**, **Beth**, **Bianca** (por causar saudade do Brasil), **Dani** (for blocking the laminar flow), **Daniel**, **Dick** (zonder jou geen verantwoorde transcriptoom-analyse), **Eline** (voor de inspiratie en begeleiding), **Emilio**, **Filipa**,

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In addition to IMBers, also other people at the TU Delft spiced things up, to name a few: Thank you **Camilo**, **Camilo** & **Katelijne** (for keeping 1st years students in check) and **Peter** (voor dat significante verschil), **Jannie**, **Astrid** & **Apilena** (voor het wachten op de mediavaten), **Daniël** & **Tineke** (voor de hulp bij het vinden van de juiste focus), **Bernd** (voor de kijk in kwantitatieve microscopie), **David** & **Annette** (for introducing us into the world of FISH) and **Lesley** (for the fascinating stories about famous microbiologists). Daarnaast waren de 'kopjes' koffie (want dat hoort bij taart!) met **Tessa** van onschatbare waarde.

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Besides these "Delft" ingredients, this thesis also consists of and was made possible due to some more exotic ingredients. I would like to thank you for these fruitful collaborations from which I learned a lot in multiple perspectives: The Kluyver Centre Zero Growth Team: **Onur**, **Michiel**, **Wout**, **Oscar**, **Arthur** thank you very much for the invaluable discussions, glad to see our joint effort paid off in a nice review! **Joris** voor de goede discussies en het advies over onder andere Rim15. **Rubén** for your help in starving yeast, while discussing Spanish deli. **Nadine**, **Monique** and **Albert** for giving me a flavor of the wonderful world of proteomics. **Anne** & **Frank** for teaching me how to FISH and analyze the catch.

Daarnaast is er natuurlijk nog de slagroom naast de taart, die het leven zo aangenaam maakt. Vrienden en familie, hoewel niet (altijd) per se een inherent onderdeel van de taart, zonder jullie had ik het nooit volgehouden! Bedankt het voor het losrukken uit de micro-wereld en terugplaatsen in de macro-wereld, al dan niet met quasiwetenschappelijke discussies over de wetenschap.

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Zonder jouw steun en liefde was dit punt nooit bereikt, **Dennis**, mon cheri, jij bent waarlijk mijn kers op de taart!

Curriculum vitae

Curriculum vitae

Markus (Mark) Mattheus Maria Bisschops werd op 12 maart 1985 geboren te Heerlen en groeide op in Voerendaal. In 2003 behaalde hij zijn VWO diploma (*cum laude*) aan het Gymnasium van het Bernardinuscollege te Heerlen, om zich daarna toe te leggen op de studie Life Science Technology aan de Universiteit Leiden en Technische Universiteit (TU) Delft. De bachelor van deze opleiding werd (*cum laude*) afgesloten met een onderzoeksproject naar galacturonzuur en *Penicillium chrysogenum* binnen de Industriële Microbiologie (IMB) sectie van de TU Delft onder de begeleiding van Andreas Gombert, Jean-Marc Daran en Jack Pronk in 2007. Ook het afstudeeronderzoek van het daarop volgende masterprogramma "Cell Factory" werd binnen de IMB sectie uitgevoerd. Onder begeleiding van Eline Huisjes, Ton van Maris en Jack Pronk werd de uitdaging aangegaan om *Saccharomyces cerevisiae* galacturonzuur te laten consumeren. Voor het behalen van de ingenieurs- en Master of Science titel in 2010 werd nog een industriële stage vervuld binnen de Braziliaanse proeffabriek van het Amerikaanse biotechnologiebedrijf Amyris. Enthousiast geworden door het onderzoek binnen de IMB sectie werd eind 2009 vol overgave een promotieplaats onder begeleiding van Pascale Daran-Lapujade en Jack Pronk daarbinnen geaccepteerd. De resultaten van dit project waarin *S. cerevisiae* onder (vrijwel) niet-groeiende condities werd bestudeerd op verschillende cellulaire en moleculaire niveaus zijn beschreven in dit proefschrift. Na afronding van het praktisch werk aan dit project is Mark in 2014 gaan werken als onderzoeker binnen de Systems and Synthetic Biology division aan de technische universiteit Chalmers in Göteborg, Zweden onder begeleiding van Jens Nielsen.

Curriculum vitae

Markus (Mark) Mattheus Maria Bisschops was born in Heerlen, The Netherlands on March 12th 1985 and grew up in Voerendaal. In 2003 he finished his pre-university education (VWO) (with honours) at the Gymnasium of the Bernardinuscollege in Heerlen to start a study Life Science and Technology at Leiden University and Delft University of Technology. The Bachelor degree was obtained (with honours) in 2007 after a research project on *Penicillium chrysogenum* and galacturonic acid within the Industrial Microbiology (IMB) section of the Delft University of Technology under supervision of Andreas Gombert, Jean-Marc Daran and Jack Pronk. The Master end project of the subsequent Master Cell Factory was also performed within the IMB section. Under supervision of Eline Huisjes, Ton van Maris and Jack Pronk the challenge was accepted to make *Saccharomyces cerevisiae* consume galacturonic acid. Prior to obtaining the Master of Science degree in 2010, an industrial internship was completed in the Brazilian pilot plant of the American company Amyris. Inspired by the research in the IMB section a PhD-project under supervision of Pascale Daran-Lapujade and Jack Pronk in that same section was accepted enthusiastically. The results of this project in which *S. cerevisiae* has been studied under (virtually) non-growing conditions at the cellular and molecular level are described in this thesis. After completion of the experimental work of this PhD-project, Mark commenced working as a researcher within the Systems and Synthetic Biology division at Chalmers University of Technology in Gothenburg, Sweden under supervision of Jens Nielsen in 2014.

List of publications

List of publications

Bisschops MMM*, Vos T*, Martínez-Moreno R, de la Torre Cortes P, Pronk JT, Daran-Lapujade P. *Oxygen availability strongly affects chronological lifespan and thermotolerance in batch cultures of* Saccharomyces cerevisiae. Microbial Cell 2: 429-444

Ercan O*, **Bisschops MMM***, Overkamp W, Jørgensen TR, Ram AF, Smid EJ, Pronk JT, Kuipers OP, Daran-Lapujade P, Kleerebezem M. 2015. *Physiological and transcriptional responses of different industrial microbes at near-zero specific growth rates.* Applied and Environmental Microbiology. Applied and Environmental Microbiology 81:5662-5670

Binai NA, **Bisschops MMM**, van Breukelen B, Mohammed S, Loeff L, Pronk JT, Heck AJR, Daran-Lapujade P, Slijper M. 2014. *Proteome adaptation of* Saccharomyces cerevisiae *to severe calorie restriction in retentostat cultures*. Journal of Proteome Research 13: 3542-3553.

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*Authors contributed equally.

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Cover designed by Markus M. M. Bisschops; frontside shows actin structures in *S. cerevisiae* cells visualized by Alexa-488 phalloidin staining, on the back *HSP26* mRNA molecules are visualised by FISH (green) and DNA by DAPI (blue) staining in *S. cerevisiae* cells.

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