Biochimica et Biophysica Acta 1813 (2011) 2133–2144

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Extreme calorie restriction and energy source starvation in *Saccharomyces cerevisiae* represent distinct physiological states

Léonie G.M. Boender [a,b], Marinka J.H. Almering [a,b], Madelon Dijk [a,b], Antonius J.A. van Maris [a,b], Johannes H. de Winde [a,b], Jack T. Pronk [a,b], Pascale Daran-Lapujade [a,b,*]

[a] *Kluyver Centre for Genomics of Industrial Fermentation, Julianalaan 67, 2628 BC Delft, The Netherlands*
[b] *Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands*

ARTICLE INFO

Article history:
Received 5 April 2011
Received in revised form 7 July 2011
Accepted 15 July 2011
Available online 22 July 2011

Keywords:
Retentostat
Saccharomyces cerevisiae
Calorie restriction
Carbon starvation
Reserve carbohydrates
Population heterogeneity

ABSTRACT

Cultivation methods used to investigate microbial calorie restriction often result in carbon and energy starvation. This study aims to dissect cellular responses to calorie restriction and starvation in *Saccharomyces cerevisiae* by using retentostat cultivation. In retentostats, cells are continuously supplied with a small, constant carbon and energy supply, sufficient for maintenance of cellular viability and integrity but insufficient for growth. When glucose-limited retentostats cultivated under extreme calorie restriction were subjected to glucose starvation, calorie-restricted and glucose-starved cells were found to share characteristics such as increased heat-shock tolerance and expression of quiescence-related genes. However, they also displayed strikingly different features. While calorie-restricted yeast cultures remained metabolically active and viable for prolonged periods of time, glucose starvation resulted in rapid consumption of reserve carbohydrates, population heterogeneity due to appearance of senescent cells and, ultimately, loss of viability. Moreover, during starvation, calculated rates of ATP synthesis from reserve carbohydrates were 2–3 orders of magnitude lower than steady-state ATP-turnover rates calculated under extreme calorie restriction in retentostats. Stringent reduction of ATP turnover during glucose starvation was accompanied by a strong down-regulation of genes involved in protein synthesis. These results demonstrate that extreme calorie restriction and carbon starvation represent different physiological states in *S. cerevisiae*.

1. Introduction

In the 1930s calorie restriction was first observed to significantly extend the lifespan of rats [30]. Calorie restriction (CR) is defined as a 25% to 60% reduction of calorie intake as compared to animals fed *ad libitum* that does not lead to malnutrition or starvation [52]. Effects of calorie restriction seem well conserved among eukaryotes and include extension of lifespan of organisms as distant as yeast [25], nematode [21] and rat [31].

In budding yeast, cellular aging is expressed as two distinct parameters. Replicative life span (RLS) is the number of budding events that a single mother cell can undergo before senescence, while chronological lifespan (CLS) indicates the time that a yeast cell can survive in a non-dividing stationary phase [9,17]. Replicative aging in yeast is considered a relevant model for actively dividing metazoan cells such as germ-line cells, while chronological aging constitutes a model for differentiated somatic cells [19].

The impact of calorie restriction on the CLS of yeast is typically investigated in aerobic glucose-grown shake-flask cultures. The CLS is then measured as the survival of yeast in stationary phase (SP). However, before reaching stationary phase, glucose-grown yeast cells go through three distinct growth phases: i) a lag-phase, where the necessary enzymes are synthesized, ii) an exponential growth phase during which glucose is dissimilated via a respiro-fermentative metabolism and ethanol and organic acids are formed, and iii) a slow-growth post-diauxic phase during which ethanol and organic acids (formed in the previous phase) are consumed. Between the exponential and the post-diauxic phases is a pause called the diauxic shift, which allows for the synthesis of enzymes needed for catabolism of ethanol and organic acids. When the medium can no longer sustain growth, usually because all carbon sources have been consumed, the SP starts, which is characterized by starvation and reduced metabolic activity. This cultivation method bears several drawbacks for studies on chronological aging and calorie restriction. First, the exponential respiro-fermentative growth phase on glucose cannot be easily uncoupled from the post-diauxic phase during which yeast catabolise ethanol respiratorily. It is therefore difficult to disentangle the effect of

Abbreviations: CR, calorie restriction; SP, stationary phase; ST, starvation; CLS, chronological lifespan; RLS, replicative lifespan

* Corresponding author at: Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands. Tel.: +31 15 2789965; fax: +31 15 278 23 55.
E-mail addresses: leonievandijk@gmail.com (L.G.M. Boender), m.j.h.almering@tudelft.nl (M.J.H. Almering), madelon_dijk@hotmail.com (M. Dijk), a.j.a.vanmaris@tudelft.nl (A.J.A. van Maris), j.h.dewinde@tudelft.nl (J.H. de Winde), j.t.pronk@tudelft.nl (J.T. Pronk), p.a.s.daran-lapujade@tudelft.nl (P. Daran-Lapujade).

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doi:10.1016/j.bbamcr.2011.07.008

carbon source from the effect of the type of metabolism (respiration or fermentation). Furthermore, during all these growth phases, concentrations of substrates and products, pH and oxygen availability change considerably and may cause secondary effects that cannot be dissociated from the primary effects of calorie restriction. In addition, due to the cultivation method, comparison of mutations leading to lower growth rates or impaired respiratory capacity lead to erroneous conclusions of the role of specific genes on CLS extension.

Retentostat cultures offer an alternative way to study calorie restriction. In retentostats, parameters such as agitation, aeration and pH can be tightly controlled and set at chosen values. Retentostats are a variation of the more popular chemostat cultivation [34]. In chemostats, the culture is continuously supplied with fresh medium at a fixed and steady flow rate, while the culture broth is continuously removed, keeping the culture volume constant. In contrast, in a retentostat, all cells are retained inside the fermentation vessel, *e.g.* by means of a filter placed in the effluent line [16,48]. As a consequence of cell retention the biomass concentration increases over time while the glucose supply remains constant, which results in a decreasing amount of glucose available for each individual cell [4]. Finally, a situation is attained where all the provided glucose is invested in the survival of the cells present in the vessel (*i.e.* maintenance processes) at the expense of cell division which eventually ceases [4]. This continuous minimum glucose supply closely resembles (extreme) calorie restriction as defined for metazoans, where the supply of energy substrate is sufficient to sustain the cells (maintenance of cellular homeostasis, turn-over of damaged protein and DNA, *etc.*) but does not lead to starvation. A recent study demonstrated that yeast cells cultivated under calorie restriction in retentostat display many hallmarks associated with yeast grown to stationary phase on YPD medium [5] such as the accumulation of storage compounds (glycogen and lipids) and the induction of genes involved in stress response and aging (*SIR2*, *SCH9*, *RIM15*, *MSN4*). Because commonly used cultivation procedures cannot dissect effects of calorie restriction from those of carbon starvation, it is unclear to what extent yeast responses to calorie restriction and calorie starvation differ or overlap.

The goal of the present study was to, for the first time, disentangle transcriptional and cellular responses to calorie restriction and starvation in *Saccharomyces cerevisiae*. To this end, the yeast was first grown for 14 days under extreme calorie restriction in anaerobic, glucose-limited retentostats. Subsequently, starvation was started by terminating the glucose feed. Yeast transcriptional reprogramming in response to calorie restriction and starvation was monitored by microarray analysis. Moreover, cellular physiology was explored in these two scenarios with an emphasis on quiescence hallmarks (population viability and metabolic activity, heat shock resistance, reserve carbohydrate and lipid contents accumulation) and cellular energetics.

2. Materials and methods

2.1. Yeast strain and media

The prototrophic laboratory strain *S. cerevisiae* CEN.PK113-7D (*MAT*a *MAL2-8*^{c}*SUC2*, obtained from Dr P. Kötter, Frankfurt, Germany) was used in the present study. The strain was grown overnight in YPD (yeast peptone dextrose) at pH 6 [41]. After addition of sterile glycerol (20% v.v^{-1}), 2 ml aliquots were stored in sterile vials at −80 °C. These frozen stock cultures were used to inoculate 500 ml shake flasks with 100 ml synthetic medium [51] at pH 6 with 2% glucose, which were used as pre-cultures for retentostat cultures. To keep medium composition constant during long-term cultivation, 40 l batches of medium were prepared, filter-sterilized and used for single retentostat experiments. Vitamins and the anaerobic growth factors ergosterol (final concentration, 10 mg.l^{-1}) and Tween-80 (final concentration, 420 mg.l^{-1}) were added to the medium reservoirs as described previously [50]. Furthermore, 1.5 ml of a 20% (wt.wt^{-1}) solution of the antifoaming agent Struktol J673 (Schill and Seilacher AG, Hamburg, Germany), sterilized separately at 120 °C, was added per liter of complete medium.

2.2. Retentostat cultivation and starvation

Anaerobic, glucose-limited retentostat cultivations were performed as described [4]. In short, glucose-limited anaerobic chemostat cultures were started at a dilution rate of 0.025 h^{-1}. When at least 5 volume changes had passed and dry weight, metabolite concentrations and carbon dioxide production varied by less than 2% over two consecutive volume changes, retentostat cultivation was started by redirecting the chemostat effluent through an AppliSense filter assembly (Applikon, Schiedam, the Netherlands) with a pore size of 0.22 μm. After 14 days of retentostat cultivation, when the estimated specific growth rate had decreased to 0.0016 h^{-1}, the supply of fresh medium was terminated. This caused immediate starvation since the residual glucose level in the glucose-limited retentostat was already extremely low [4]. All other parameters were kept the same; cultures were sparged with ultra-pure nitrogen at 0.7 l.min^{-1}, pH was kept at 5.0–5.1 by dual titration with 2 M KOH and 2 M H_2SO_4, and temperature was controlled at 30 °C. As a consequence of sampling, the culture volume decreased during the starvation phase (from 1.4 l to 0.75 l). Weight loss by evaporation (mostly ethanol), calculated by linear regression of the fermenter weight in between sampling points due to the continuous nitrogen flushing, was below 0.5 g.h^{-1}.

2.3. Analysis of metabolites, dry weight and cell concentration

Analysis of culture supernatant was performed by HPLC using a Bio-Rad Aminex HPX-87H column and detection by refractive index and wavelength absorbance detectors at 214 nm [4]. Dry weight was measured using pre-dried, weighed filters [38]. Cell concentration was determined with a Z2™ Coulter Counter (Beckman Coulter, Woerden, the Netherlands) using a 50 μm aperture. Particle volume was calibrated using 5 μm latex beads (Beckman Coulter) as recommended by the supplier. Appropriate dilutions were measured to have a coincidence between 5 and 10%. Glycogen was determined via alkali extraction by boiling in 0.25 M Na_2CO_3 and enzymatic conversion using amyloglucosidase preparation from *Aspergillus niger* (Sigma-Aldrich, Zwijndrecht, Netherlands)[35].

2.4. Calculation of metabolic fluxes

Biomass-specific metabolic fluxes in the retentostat cultures were calculated as described [4] with the following exceptions. Estimations of the specific growth rate were based on cell numbers instead of biomass concentration. Since only viable cells can grow, the increase of total cell numbers was divided by the number of viable cells estimated with CFDA/PI double staining (see below). The specific rate of ATP production by retentostat-grown cells was calculated from the specific rates of fermentation products (ethanol, glycerol, acetate, succinate, lactate and pyruvate) with yields of 1, −1, 1, 1, 1, 1 mole of ATP per mole of product, respectively assuming only viable biomass made products. Specific rates calculated during the retentostat phase are represented in Appendix A. During the starvation phase, the measurement of the (extremely low) production of metabolites was obscured by the inevitable ethanol and water evaporation from the culture vessel. Specific rates of product formation could therefore not be calculated. During the starvation phase, the specific rate of ATP turnover was therefore estimated from the decrease in glycogen content of the biomass. To this end, the glycogen content (g glycogen/g dry biomass) was fitted with an exponential decay function ($Y = (Y_0 - \text{Plateau})*e^{(-K*X)} + \text{Plateau}$, R^2 of 0.99). A yield of 3 moles of ATP per mole of glucose residues from glycogen was used, which represents phosphorolytic cleavage

of glycogen yielding glucose-1-P. This number is likely to result in an overestimation of the actual ATP generation rate as, depending on the degree of branching, the actual ATP yield per hexose unit may be closer to 2 [11,23].

2.5. Assessment of viability using flow cytometry

The viability of the culture was assayed using the *Fungal*ight CFDA, AM/propidium iodide yeast vitality kit (Invitrogen, Carlsbrad, CA) on a Cell Lab Quanta™ SC MPL flow cytometer (Beckman Coulter, Woerden, Nederland). Propidium iodide (PI) staining is an indicator for cell membrane integrity. When the membrane is compromised, the dye can diffuse into the cell and intercalate with DNA, which gives rise to red fluorescence. The acetoxymethyl ester of 5-carboxy-fluorescein diacetate (CFDA, AM in DMSO) can permeate through the membrane. In metabolically active cells, lipophilic diacetate groups are cleaved off by cytosolic non-specific esterases, yielding a charged green fluorescent product. Culture samples were diluted to give 1.0×10^7 cells.ml^{-1} in IsotonII® diluent (Beckman Coulter, Woerden, Netherlands). Single-stained, double-stained and unstained samples were prepared in IsotonII® diluent (Beckman Coulter, Woerden, Netherlands). After 15 min incubation at room temperature in the dark, the samples were diluted 10-fold using IsotonII diluent in Vicell sample cups (Beckman Coulter, Woerden, Netherlands) and measured in triplicate on a Quanta flow-cytometer (Beckman Coulter, Woerden, Netherlands). Samples were excited with a 488 nm laser and parameters measured were FL1 (bandpass filter at 525 nm for CFDA), FL3 (bandpass filter at 620 nm for PI), Coulter volume and side scatter. Cells were gated on the electronic volume and a total of 10,000 cells were counted for each sample. PMT settings were regularly checked by using flow-set fluorospheres (Beckman Coulter, Woerden, Netherlands) and proper alignment of the flow cell was checked upon start-up using flow-check fluorospheres (Beckman Coulter, Woerden, Netherlands). Viability (metabolic activity) was calculated as the number of CFDA+ and PI− cells (region A4, see Appendix B) divided by the total number of cells. Culture viability was also measured by traditional colony-forming unit counts (CFU); 10-fold dilutions were made, three dilutions were plated, each dilution in triplicate. At each timepoint the appropriate dilution (single colonies) was counted, which resulted in at least 200 colony counts per time point. This count was divided by the total cell count as obtained with the Z2 Coulter Counter (see Section 2.3.).

2.6. Thermotolerance assays

Thermotolerance of yeast cultures was measured by adding culture samples to pre-heated (53 °C) Isoton II diluent to obtain a cell concentration of 1.0×10^7 cells.ml^{-1} and incubating them in a 53 °C water bath. Samples were taken at t = 0, 5, 10, 15, 20, 30 min for analysis of thermotolerance after 0 and 4 days of retentostat cultivation. Two additional samples taken after 60 and 80 min of incubation at 53 °C were included in analyses of older retentostat cultures and samples taken during the subsequent starvation phase. Samples were immediately put on ice and subsequently analyzed with the CFDA/PI staining as described in Section 2.5., except that only unstained and double stained samples were prepared. Control experiments showed that the presence of ethanol (~20 mM) in the assay from the culture supernatants did not affect heat-shock tolerance of exponentially growing cells (data not shown). The percentage of metabolically active cells that are lost per minute of incubation was calculated by a linear fit of at least three time points on the killing curve ($R^2>0.9$), except for 21 days of starvation samples ($R^2=0.7$), where low initial viabilities of samples taken after prolonged starvation hampered quantitative analysis.

2.7. Fluorescence microscopy

Lipid staining was essentially performed as described by [13]: 1×10^8 cells.ml^{-1} were washed once and resuspended in PBS buffer (3.3 mM NaH_2PO_4, 6.7 mM Na_2HPO_4, 0.2 mM EDTA, 130 mM NaCl). Nile Red (N3013, Sigma-Aldrich, St. Louis, MO), prepared as stock solution of 1 mg.ml^{-1} in DMSO, was added to a final concentration of 0.1 ml.ml^{-1}. Stock solutions were briefly centrifuged before use to avoid addition of Nile Red crystals. Samples were incubated for 10 min in the dark at room temperature. Yellow lipid droplets and red membrane staining were visualized with a Imager-D1 fluorescence microscope (Carl-Zeiss, Oberkochen, Germany) equipped with Filter Set 09 (fluorescein isothiocyanate long-pass filter; excitation band-pass filter width from 450 to 490 nm; 510-nm beam splitter [dichroic mirror]; emission long-pass filter at 515 nm; Carl-Zeiss).

Staining with Syto9, a cell permeable nucleic acid stain, was performed essentially as recommended by the supplier (LIVE/DEAD *BacLight* kit, Invitrogen, Carlsbad, CA). Cells were centrifuged (6150 x *g*; 5 min at 4 °C). After discarding the supernatant, cells were resuspended in Isoton II diluent (Beckman Coulter). Subsequently, 1.5 μl of 3.34 mM Syto9 in DMSO was added to a final concentration of 5 μM and cell suspensions were incubated for 15 min at room temperature in the dark. Fluorescence was evaluated with the Imager-D1 fluorescence microscope with Filter Set 10 (fluorescein isothiocyanate band-pass filter; excitation band-pass filter width from 450 to 490 nm; 510-nm beam splitter [dichroic mirror]; emission band-pass filter 515–585 nm; Carl-Zeiss).

2.8. Transcriptome analysis

Two datasets were used in this study: one during the acquisition of calorie restriction in two independent duplicate retentostat cultures and the second during the starvation event. The first dataset is part of a large dataset described in [5] and accessible at Gene Expression Omnibus (GEO) with the series accession number GSE22574 (http://www.ncbi.nlm.nih.gov). The transcriptome data showed in this previous paper contain the retentostat samples taken at 0 days, 2 days, 9 days, 16 days and 22 days of retentostat cultivation, a total of 11 arrays (0 days in triplo, other time points in duplo). Procedures for the preparation of the Affymetrix S98 microarrays (Affymetrix, Santa Clara, CA, USA) can be found in [5].

The second dataset are culture samples taken for transcriptome analysis with microarrays from independent duplicate starvation experiments at t = 0 min (retentostat culture at 14 days just before switching off medium supply), 10 min, 30 min, 60 min and 120 min and instantly frozen in liquid nitrogen. Probe preparation and hybridization to 10 Affymetrix GeneChip S98 microarrays (Affymetrix, Santa Clara, CA, USA) was performed as described in [36] with the following modifications. The concentration of biomass for total RNA isolation, using hot-phenol chloroform extraction, was increased to 50 mg for the retentostat sample (t = 0) and for the starvation samples 100 mg was used. Total RNA was isolated and checked for quality (Total RNA chip, Agilent). Poly(A)$^+$ RNA was enriched from total RNA using an oligotex kit (Qiagen Benelux B.V, Venlo, Netherlands). Double-stranded cDNA synthesis was carried out using 15 μg poly(A)$^+$ RNA and the components of the One cycle cDNA Synthesis kit (Affymetrix). The double-stranded cDNA was purified (Genechip Sample cleanup Module, Qiagen) before *in vitro* transcription and labeling (GeneChip IVT Labeling Kit, Affymetrix). Labeled cRNA was purified (GeneChip Sample cleanup Module, Qiagen) followed by fragmentation and hybridization of 15 μg of biotinylated cRNA. Data acquisition, quantification of array images and data filtering were performed with Affymetrix GeneChip® Operating Software version 1.2. All arrays were globally scaled to 300 using the average signal from all transcripts. Scaling factors were between 1.04 and 1.94. Expression levels below 12 were considered

insignificant and were set to 12 as previously described [36]. From the 9335 probesets on the GeneChip, 6383 probesets corresponding to genes in *S. cerevisiae* were obtained. All microarray data used in this study are available via GEO series accession number GSE22602. The starvation dataset is also available in Appendix C. Genes with average expression levels below 20 over all 10 arrays were removed before statistical time course analysis with EDGE (University of Washington Seattle, USA), using the time points as covariate age, independent sampling and 2000 iterations [42]. Coefficients of variation (CV) of microarray analysis on independent replicate starvation experiments were between 15 and 19%, which is similar to the variation observed in chemostat-based microarray analysis [36]. Furthermore, transcript levels of widely used house-keeping genes (*ACT1* and *PDA1*) and other genes for which the expression has recently been shown to be steady throughout a variety of cultivation conditions (*ALG9*, *TAF10*, *TFC1* and *UBC6*, [45]) remained remarkably constant during the starvation runs (Coefficient of variation below 20%, Appendix D). These results indicate that 120 min starvation did not cause changes in the mRNA pools that precluded use of the standard normalization protocol. Conversely, mRNA and arrays prepared from samples taken after 26 h of starvation revealed a substantial mRNA degradation and perturbation in house-keeping gene levels, precluding the use of this time-point for transcriptome analysis (data not shown). A q-value cut-off of 0.02 was used after visual inspection of the q-p plot and π_0 (percentage of unchanged genes), resulting in differential expression of 549 genes. K-means clustering (positive correlation, sampling using bootstrap, 1000 iterations) was performed after item-wise half-normalization with Genedata Expressionist® v5.3 (Genedata AG, Basel, Switzerland). The resulting gene clusters were analyzed for enrichment in functional annotation (Munich Information Centre for Protein Sequences (MIPS)), GO categories (*Saccharomyces* Genome Database (SGD)) and transcription factor binding [14] employing a previously described hypergeometric distribution test [20].

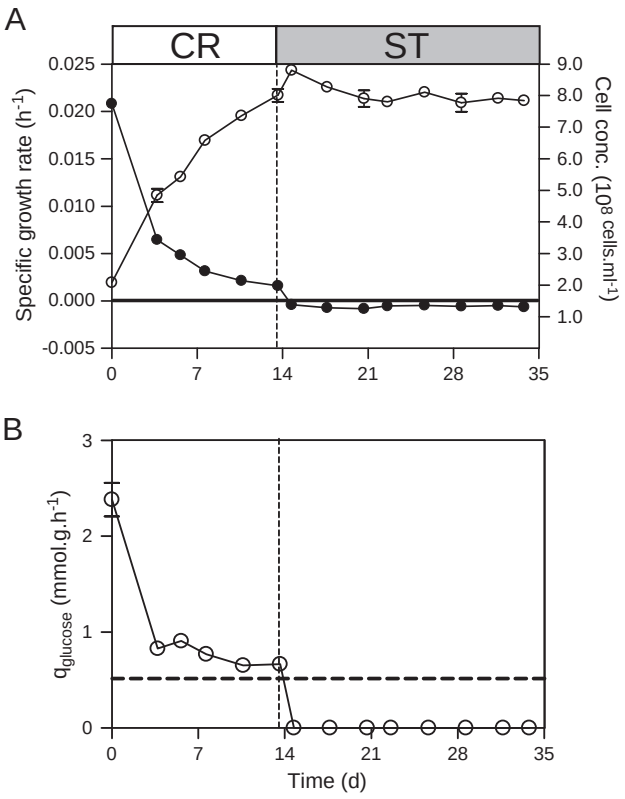


Fig. 1. Extreme calorie restriction (CR) in anaerobic *S. cerevisiae* retentostat cultures and subsequent starvation (ST). In panels A and B the dashed vertical line indicates the start of the starvation. (A) The average cell concentration (○) and the average specific growth rate (h^{-1}) (●) of two cultivations, error bars indicate the standard error of the mean. (B) The specific consumption rate of glucose in mmol.(g biomass)$^{-1}$.h^{-1} during CR and ST. The horizontal dashed line indicates the maintenance energy coefficient determined from exponentially growing yeast in anaerobic glucose-limited chemostat cultures [4].

3. Results

3.1. Achievement of calorie restriction and starvation

Calorie restriction was achieved using an anaerobic, glucose-limited retentostat cultivation set-up [4]. During retentostat cultivation, the supply of glucose to the fermenter stayed constant, while the cell concentration increased (Fig. 1A). This caused the specific glucose consumption rate to decrease close to the amount of glucose needed for maintenance of the cell population (Fig. 1B). Consequently, no glucose was available for production of new cells, as reflected in the extremely slow doubling time of 18 days (μ below 0.002 h^{-1}, Fig. 1A) after 14 days of retentostat cultivation. To compare the physiological and transcriptional response of cells to starvation, the supply of glucose was terminated after 14 days, resulting in immediate glucose starvation. The specific glucose consumption rate became zero and the specific growth rate determined from the cell concentration became slightly negative (Fig. 1, A and B).

3.2. Glucose-starved cells dramatically reduce ATP turnover rates

Glycogen accumulated to high levels during extreme caloric restriction in anaerobic retentostat cultures [4] and is known to be mobilized during glucose starvation [24,47]. Conversely trehalose remained below detection limit during both calorie restriction and starvation. Trehalose contents in *S. cerevisiae* are notoriously low under anaerobic conditions [15,47]. Lipids have been suggested to be a main source of energy in aerobic starvation experiments [53]. During calorie restriction in anaerobic retentostats, yeast cells accumulated lipids as intracellular lipid droplets (Fig. 2A). However, anaerobic conditions precluded their use as an energy reserve because β-oxidation and respiration both require oxygen. Therefore, quantitative analysis of glycogen contents during a transition from extreme caloric restriction to glucose starvation may provide insights into cellular energy metabolism under these two conditions.

Consistent with a previous publication [5] glycogen accumulated intracellularly to levels of up to 100 mg of glycogen per g dry weight during anaerobic retentostat cultivation. When the glucose supply to the retentostats was terminated, glycogen stores were slowly consumed and decreased to circa 20% of their original levels after one week of starvation (Fig. 2B). After three weeks of starvation, glycogen stores were entirely depleted (Fig. 2B).

In anaerobic retentostat cultures, rates of ATP turnover can be accurately estimated from the concentrations of fermentation products. Under the conditions of extreme calorie restriction that were reached during prolonged retentostat cultivation, the specific rate of ATP turnover equalled 1 mmol.(g biomass)$^{-1}$.h^{-1}. During starvation, ATP can only be generated from glycogen by substrate level phosphorylation. Upon starvation (t = 0 days) the estimated rate of ATP production from glycogen fermentation was as low as 0.013 mmol of ATP.(g biomass)$^{-1}$.h^{-1} and further decreased to 0.0002 mmol of ATP.(g biomass)$^{-1}$.h^{-1} after 21 days of starvation (Fig. 2C). While these estimated rates of ATP production are already 2–3 orders of magnitude lower than those in cells subjected to extreme caloric restriction, they are probably overestimations, since glycogen degradation via glucose rather than via glucose-1-phosphate only yields 2 moles of ATP per mole glucose.

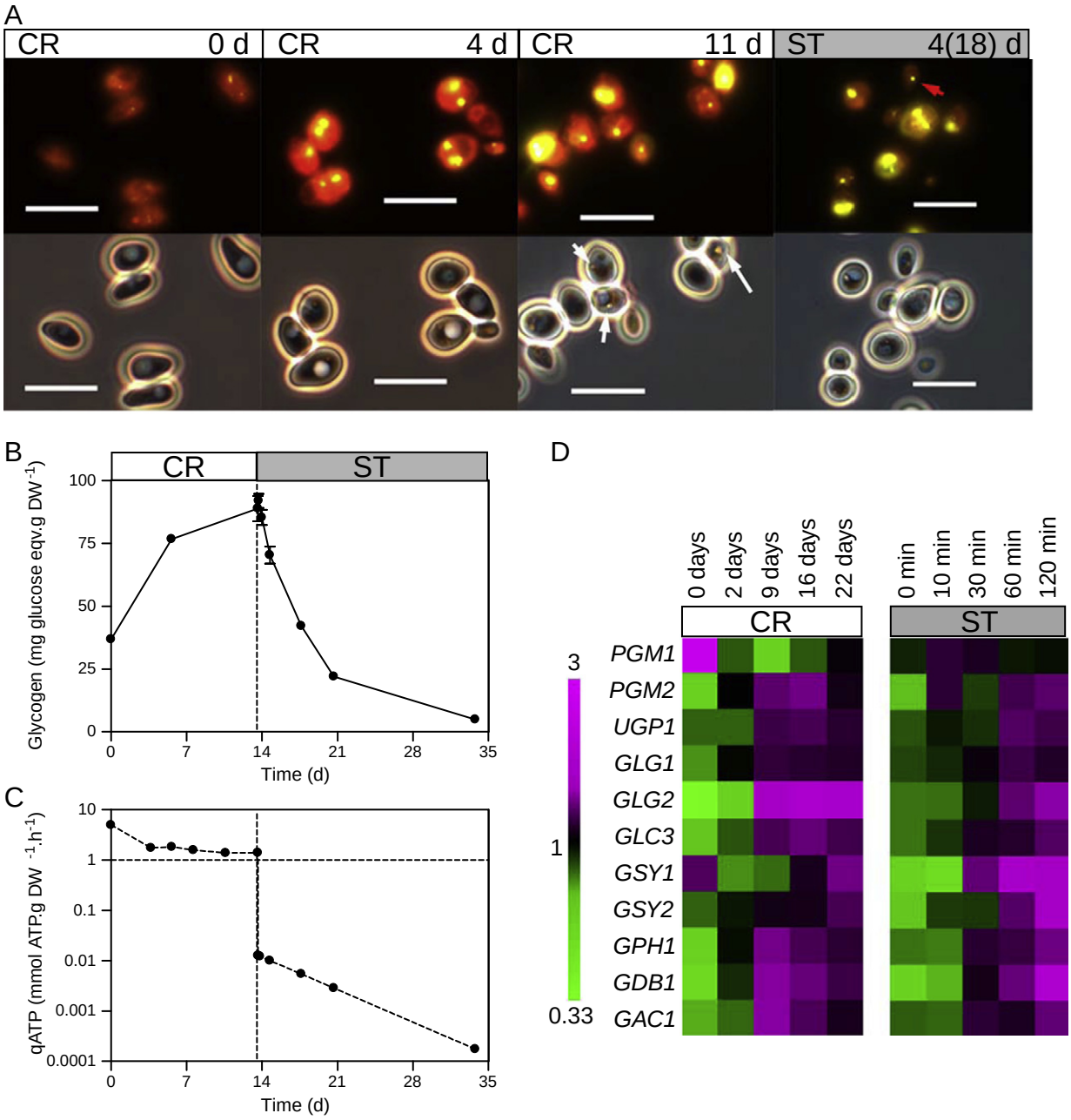


Fig. 2. Storage metabolism in *S. cerevisiae* during calorie restriction (CR) in retentostat cultures and subsequent starvation (ST). (A) Nile-red staining for intracellular lipid accumulation. The top images are fluorescent images, below the corresponding phase-contrast images. Marker bars indicate 10 μm. White arrows indicate lipid droplets on phase contrast and the red arrow indicates a lipid droplet in a phase-contrast dark (PCD) cell. (B) Glycogen content in mg glucose equivalents. (g biomass)$^{-1}$. (C) Estimated specific rates of ATP generation during CR and starvation (see Section 2.4. for calculation procedures). The horizontal dashed line indicates the maintenance energy coefficient calculated from anaerobic chemostat cultures [4]. In panels B and C the vertical dashed line indicates the time point at which glucose starvation was started, the data points represent the average of two independent cultivations and error bars indicate the average deviation of the mean. (D) Mean-normalized transcript levels of key genes involved in glycogen metabolism.

3.3. Transcriptional regulation of key genes in glycogen metabolism

During retentostat cultivation and the subsequent starvation phase, genes involved in glycogen synthesis and degradation showed increased transcript levels compared to those in exponentially growing chemostat cultures (D = 0.025 h^{-1}) (Fig. 2D). *GDB1*, which encodes the glycogen-debranching enzyme involved in glycogen breakdown, was induced under both conditions. Expression of genes involved in glycogen synthesis differed when glycogen was accumulated (calorie restriction) or degraded (starvation). When glycogen accumulated during retentostat cultivation, *GAC1*, which encodes a regulator of glycogen synthase 2 and *GLG2*, which encodes the self-glycosylating initiator of glycogen synthesis, were induced. *GSY2*, the target of Gac1, and *GSY1* remained at constant expression level. Conversely, during starvation, where glycogen was consumed, *GAC1* expression remained constant but its target *GSY2* was induced. This poor correlation between regulation of gene expression and regulation of glycogen metabolism is not surprising as the activity of the enzymes involved in glycogen accumulation and degradation are regulated by post-translational phosphorylation and dephosphorylation reactions [11,15].

3.4. Glucose starvation leads to a concerted transcriptional down-regulation of genes involved in protein synthesis

Protein synthesis is the most energy-expensive process in growing cells [43]. As a result of the extreme calorie restriction in the retentostat cultures, the yeast cells did not (or hardly) divide. Still, cells retained a high viability (CFU) and metabolic activity (above 70%), and no indications of major protein damage were observed [4]. This implies that protein synthesis still occurred to replace the damaged and degraded proteins (*i.e.* protein turn-over) and that a fraction of the ATP used for cellular maintenance was used for protein synthesis. In response to the decreased demand in *de novo* protein synthesis, expression of genes involved in protein synthesis strongly decreased in retentostat cultures as compared to exponentially growing cells [5].

Upon the switch from extreme calorie restriction to glucose starvation, a fast (within 30 min) and massive down-regulation of genes involved in protein synthesis was observed. As many as 109 out of the 409 genes that were down-regulated upon starvation encoded proteins involved in protein synthesis, especially ribosomal proteins (p-value 5E-25, Fig. 3A). Furthermore genes encoding proteins implicated in RNA binding (p-value 5E-8, Fig. 3B) and transcription, in particular rRNA processing (p-value 8E-20, Fig. 3C) were strongly overrepresented among the cluster of genes whose transcript levels decreased upon the onset of starvation.

Consistent with a concerted down-regulation of genes involved in protein synthesis, the cluster of genes whose transcript levels decreased during starvation showed a strong overrepresentation of promoter-region binding motifs for the transcription factors Sfp1 (p-value 5E-9), Fhl1 (p-value 2E-23) and Rap1 (p-value 4E-13) (see Section 2.8.). Both Sfp1 and Fhl1 transcription factors operate at the crossroads of TOR and PKA signaling to properly pass on information on nutrient availability to the repression of ribosomal proteins [7].

3.5. Cellular damage and extensive loss of viability occurs during glucose starvation but not during extreme calorie restriction

When *de novo* protein synthesis rates are reduced, damaged proteins may accumulate. Autofluorescence of cells at green wavelengths (525 nm) is an indicator for macromolecular damage [44] which has, for example, been observed in stationary-phase batch cultures of *S. cerevisiae* [3]. Green autofluorescence remained constant and very low during extreme calorie restriction in retentostat cultures, but increased substantially during the subsequent starvation phase (Fig. 3D).

During starvation the culture progressively lost viability (Fig. 4). After 7 days of starvation the viability (CFU counts) had decreased from 70% to 40% and after 21 days of starvation only 15% of the cell population was viable (Fig. 4). In comparison during long-term calorie restriction the viability (CFU) was still at 60% after 22 days of cultivation and metabolic activity was above 70% [4]. A similar chronological lifespan of 25 days was found in yeast incubated in water, grown to stationary phase in minimal synthetic medium [9]. While both CFU counts and viable stains revealed similar trends in viability during calorie restriction (slow loss of viability) and early starvation (rapid loss of viability during the first 7 days), they suggested an uncoupling between ability to divide (CFU count) and metabolic activity (viable stains) in the last 14 days of starvation. Indeed, while ca. 33% of the cells remained metabolically active after 20 days of starvation, only 15% of the population were still able to

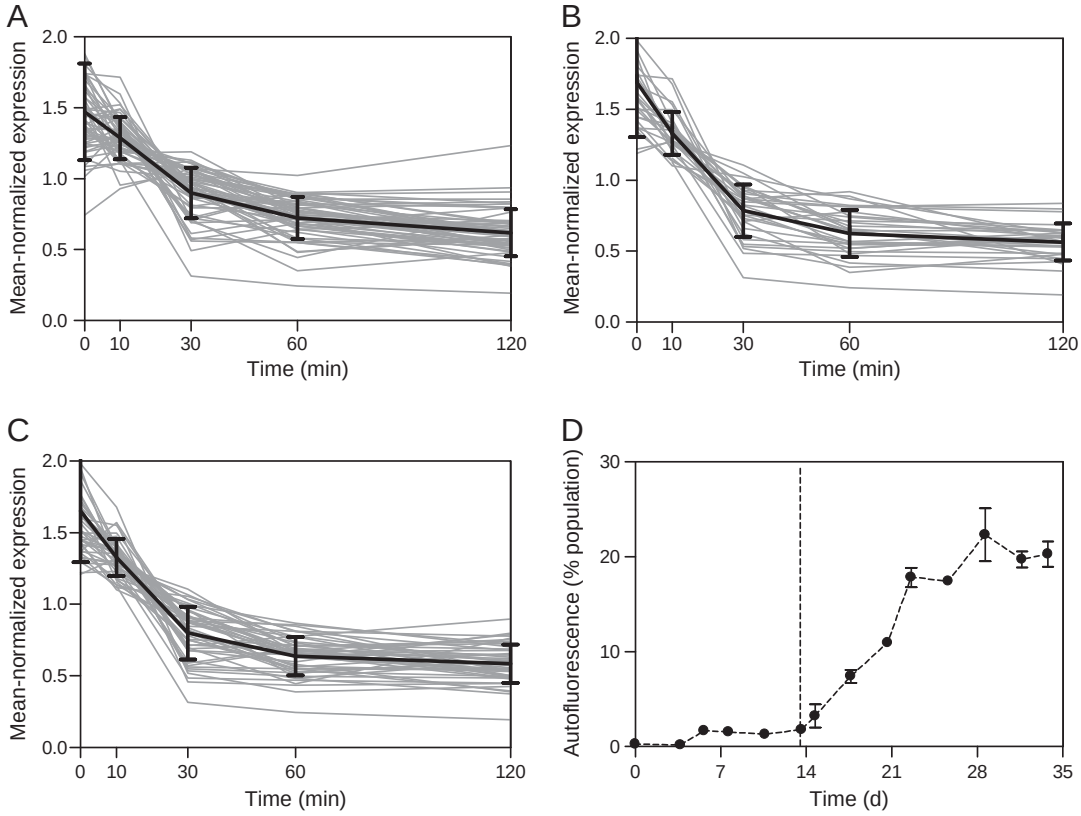


Fig. 3. Panels A to C represent the mean-normalized expression of differentially expressed genes during the first 120 min of the starvation phase. (A) MIPS category Ribosomal proteins (58 genes). (B) MIPS category RNA-binding proteins (30 genes). (C) MIPS category rRNA processing (47 genes). Solid lines indicate the average expression of all differentially expressed genes in that category and the standard deviation of the mean. In panel D is represented the increase in the percentage of unstained cells that emit green fluorescence as measured by flow cytometry during calorie restriction and starvation. Green autofluorescence is an indicator of macromolecular damage [44].

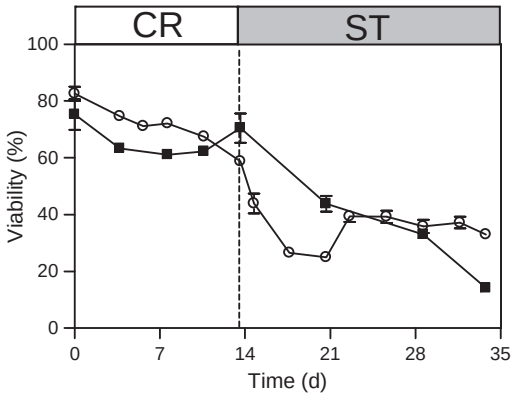


Fig. 4. The average viability of two cultures during calorie restriction (CR) and starvation (ST). Viability as measured with CFU/total count (■). Viability as measured with double-staining (CFDA–PI) by flow cytometry (○). Only the viable A4 population characterized by CFDA+ (metabolically active) and PI− (intact plasma membrane) staining is represented (see Appendix B). Error bars indicate the standard deviation (see Section 2.5.).

divide. The incapacity of cells to divide while remaining metabolically active may result from various factors, such as the irreversible degradation of macromolecules necessary for duplication, or the inability to pass the check-points necessary to exit from G_0 and start a new cycle.

3.6. Starvation, but not calorie restriction, leads to accumulation of phase-contrast dark cells

Propidium iodide (PI) and the acetoxymethyl ester of carboxy-fluorescein diacetate (CFDA) were used to assay membrane integrity and metabolic activity (active cytosolic esterases) cells respectively. In growing yeast cultures, these two dyes typically counterstain, most cells being either metabolic active (CFDA+) with intact membrane (PI−) or metabolically inactive (CFDA−) and with a damaged membrane (PI+). This population distribution was also observed during retentostat cultivation (Fig. 5A). However, immediately upon starvation, many cells stained with neither CFDA nor PI (CFDA−,PI−, Fig. 5B). Combining fluorescence and phase-contrast microscopy further showed that these non-staining cells were much darker in phase-contrast than dead cells (CFDA−,PI+) or metabolically active cells (CFDA+,PI−), and therefore were termed phase-contrast dark (PCD) cells (Fig. 5C, black arrow).

Several hypotheses might explain why PCD cells did not stain with either CFDA or PI: i) the metabolic activity is so low that CFDA is not converted, while the membrane is still intact, ii) both dyes could have reduced diffusion over the cell wall and membrane, iii) the cells are not-metabolically active and the amount of nucleic acids and especially DNA is reduced in the cell. To investigate these hypotheses another staining was performed with Syto9, a cell membrane permeable nucleic acid staining dye. Syto9 did permeate into the cells, but showed a strongly reduced staining of the PCD cells as compared to phase-contrast light (PCL) cells (Fig. 5C, Syto9), thereby supporting the first and last hypotheses. Furthermore, cells were treated by triton, a surfactant used to permeabilize the cell membrane (0.2% triton, 10 min incubation at room temperature). Permeabilization of PCD cells with triton did not lead to an increase in the staining of PCD cells with CFDA or PI (data not shown). Altogether these results indicate that PCD cells are dead and, at least partially, lysed cells. Other characteristics of PCD cells were the presence of a single small lipid droplet (Fig. 2A, red arrow) and a smaller average size and different shapes than PCL cells (data not shown). Flow-cytometric analysis showed that after 26 h of starvation, PCD cells already accounted for 43% of the population (Fig. 5D). This heterogeneity and separation between two distinct populations has been previously reported for yeast cultures in stationary phase [1,54].

3.7. Heat-shock tolerance is similar in calorie restricted- and starved cells

Heat-shock tolerance is a hallmark of quiescent yeast cells, and survival upon heat-shock has been shown to increase at low specific growth rates in chemostat cultures [27]. Furthermore, expression of heat-shock responsive genes in *S. cerevisiae* is negatively correlated with the specific growth rate in anaerobic chemostat and retentostat cultures [5,10]. Their transcripts stabilized at high levels during retentostat cultivation (Fig. 6A), suggesting that cells that experience extreme calorie restriction become hyper-resistant to heat shock [5,10]. Indeed, the rate at which these cells lost metabolic activity upon heat shock was directly correlated to the specific growth rate and the induction of heat-shock responsive genes (Fig. 6B). The heat shock tolerance reached its maximum level at a specific growth rates below 0.004 h^{-1}. With the exception of *SSE2*, the onset of starvation did not cause a further transcriptional up-regulation of heat-shock responsive genes (Fig. 6C), nor did it substantially increase heat-shock tolerance (Fig. 6B).

It is noteworthy that two heat shock proteins of the HSP70 family responded differently during retentostat culture. While most heat-shock responsive genes were up-regulated as growth rate decreased under calorie restriction, the expression of *SSA2* and *SSB2* was down-regulated (Fig. 6A). This negative correlation of expression with specific growth rate is in agreement with earlier transcriptome studies performed at various growth rates in aerobic and anaerobic chemostats [10,40]. A peculiar response of *SSA2* and *SSB2* was also previously reported in response to heat shock, *SSA2* expression being hardly affected and *SSB2* being down-regulated upon temperature elevation [6,26]. The expression of both genes was however not further changed upon starvation.

3.8. Transcriptional responses specific to starvation

K-means clustering of the 549 genes differentially expressed upon starvation (q-value above 0.02, see Section 2.8.) identified only two clusters, inclusion of more clusters did not lead to an improved gap-statistic. The first cluster contained 140 genes that were up-regulated during starvation as compared to calorie restriction. The second cluster contained 409 genes that were down-regulated (Fig. 7A). While the down-regulated genes showed a strong enrichment for genes encoding proteins involved at different levels in protein synthesis (109 genes, p-value 2E-32) and in the synthesis of amino acids (30 genes, 3E-4) as discussed in Section 3.4., the function of the up-regulated genes was not so obvious. A closer inspection of these up-regulated genes revealed an enrichment for genes encoding dehydrogenases (such as *MDH3*, *DLD1*, *BDH1*, *BDH2*, *SDH1*, *ALD4* and *CIT3*), which may indicate a need for cellular detoxification and redox balancing in starving cells. Among the genes induced upon starvation were also *SNZ1*, *YAK1*, *PDE1*, which are known to be important for entry and maintenance of stationary phase [53]. Of the nine SP genes (identified to be necessary for entrance and maintenance of SP, [29]) that are not required for growth on non-fermentable carbon-sources, four (*HBT1, FMP45, SPG4, SPG1)* were up-regulated upon starvation.

Several previous studies have shown that specific growth rate has a large impact on the transcriptome of *S. cerevisiae* [10,39]. To investigate which genes specifically responded to starvation and not to calorie restriction or growth rate, the current dataset was compared to the datasets of Boender *et al.* and Fazio *et al.* [5,10] (Fig. 7B). Of the 409 down-regulated transcripts the expression of 91 genes responded specifically to starvation. Among these an enrichment of previously identified MIPS categories of rRNA processing (p-value 8.3E-20) and proteins with RNA binding function (p-value 5.0E-8) was found. Furthermore, an enrichment (p-value 8.2E-3) for targets of transcription factor Abf1, a DNA binding protein with possible chromatin-

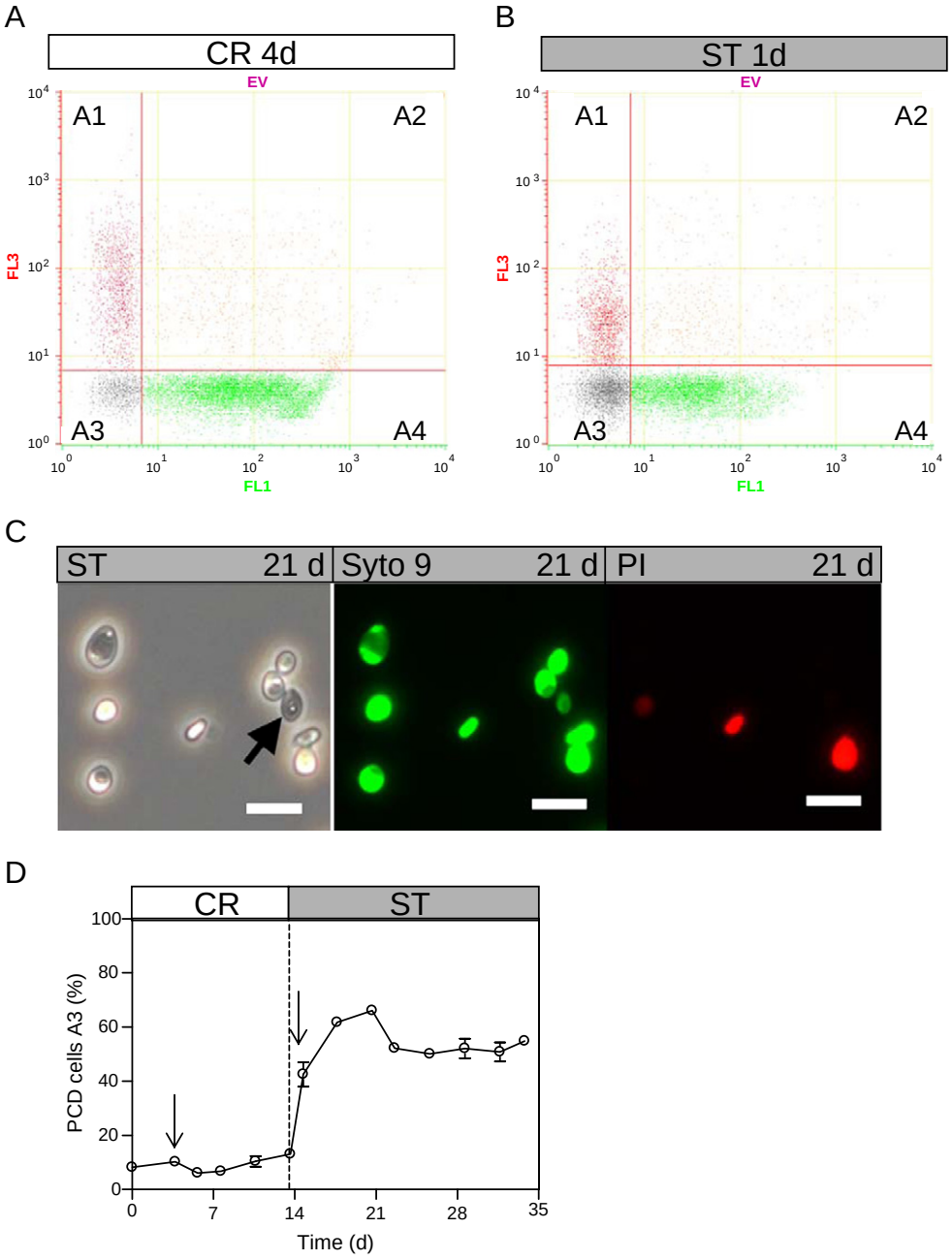


Fig. 5. Appearance of phase-contrast dark cells (PCD) upon starvation. (A) and (B) Typical flow-cytometric charts after 4 days of calorie restriction and 1 day of starvation respectively. 10,000 cells were analyzed for each time point. The A3 quadrant represents the PCD cells that are neither stained by PI nor by CFDA. (C) Fluorescent microscopy of cells double stained with propidium iodide and Syto9 at 21 days of starvation: (1) phase-contrast image with a black arrow pointing at a PCD cell, (2) corresponding green fluorescent image showing the Syto9 staining, (3) corresponding red fluorescent image showing the propidium iodide staining. White bar denotes 10 μm. (D) Percentage of PCD cells in the culture measured by flow-cytometric analysis (cells in A3 in A and B). The arrows indicate the sampling points corresponding to the scatter plots represented in panels A and B.

reorganizing activity involved in transcriptional activation, gene silencing, and DNA replication and repair, was observed [33]. Among the transcripts specifically down-regulated upon starvation was *AAH1*, encoding an adenine deaminase, which was shown to be tightly down-regulated upon SP entrance [8]. Aah1 is specifically targeted for proteasome-dependent degradation by Saf1. The *SAF1* gene was also induced during starvation even though it did not meet the stringency of our statistical test. Interestingly, the expression of 14 genes increased specifically upon starvation (Fig. 7B). These 14 transcripts included *OPI1* and *OPI3*, whose encoded proteins function in phospholipid biosynthesis. *OPI3* has been shown to be important for stationary phase since *opi3* mutants rapidly lose viability upon entry into stationary phase [32].

4. Discussion

The present study represents a first systematic dissection of the cellular responses of *S. cerevisiae* to calorie restriction and energy-source starvation. Anaerobic retentostat cultivation enabled a clear separation of severely calorie-restricted growth phase, during which virtually all glucose provided to the cultures was used for cellular maintenance and a starvation phase, during which no external energy substrate was available.

In comparison to exponentially growing cultures, several physiological and transcriptional responses were shared by calorie-restricted and starving cultures. While over 2000 genes were differentially

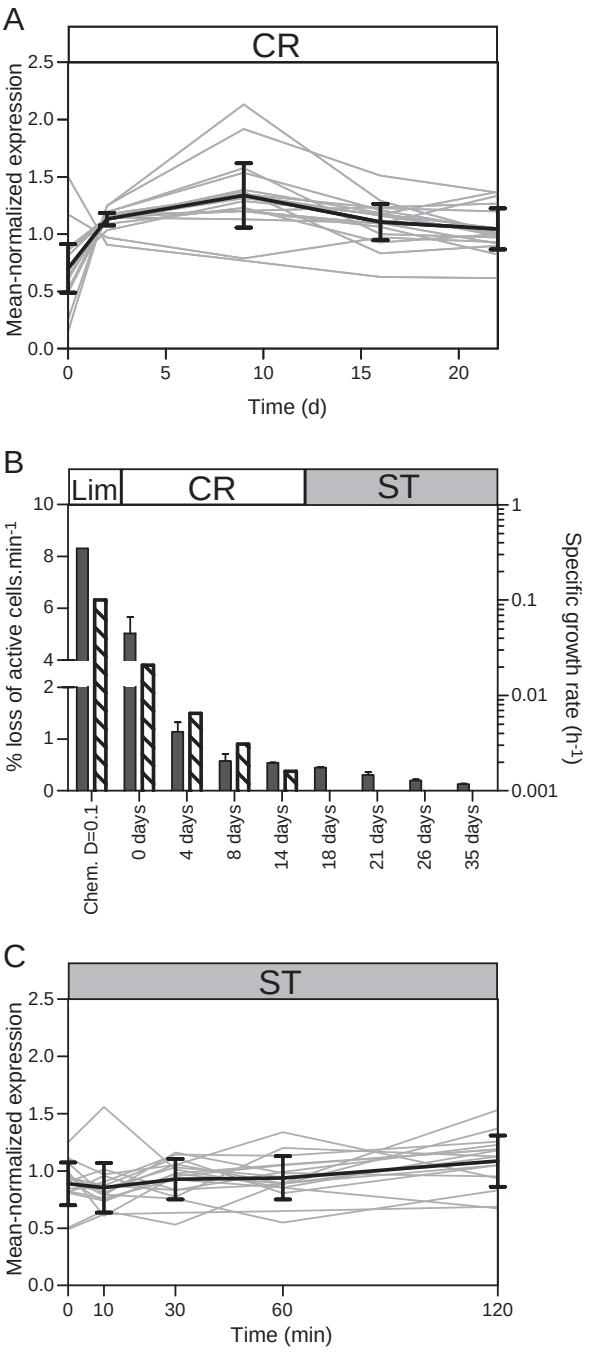


Fig. 6. Expression of heat-shock responsive genes and heat-shock tolerance during calorie restriction and starvation. (A) Mean-normalized expression of genes involved in heat-shock response (*HSP150*, *SSC1*, *HSP60*, *HSP82*, *SSA3*, *HSP26*, *HSP30*, *HSP42*, *ECM10*, *SSA4*, *HSP12*, *HSF1*, *SSE2*, *HSP10*, *SSA1*, *SSA2*, *SSB2*) during calorie restriction in the retentostat. (B) Tolerance of *Saccharomyces cerevisiae* to a heat-shock at 53 °C of two independent retentostat and starvation cultures. The percentage of metabolically active cells that are lost per minute of incubation (see Section 2.6.) is shown as black bars; error bars represent the standard deviation. At least three time points on the killing curve were used to calculate the rates based on linear regression (R^2>0.9, except for 21 days R^2 of 0.7). The specific growth rate is plotted as striped bars. As a reference for exponentially growing cells one carbon-limited anaerobic chemostats at higher specific growth rate of 0.1 h^{-1} is shown (Lim). (C) Mean-normalized expression of the same genes as in panel A during the starvation. Both CR and ST datasets were normalized separately.

expressed when comparing calorie-restricted and exponentially growing cells [5], a much smaller set of genes was expressed differentially during starvation and calorie restriction (549 genes). Common features included the up-regulation of genes involved in quiescence including many heat-shock proteins (*SNZ1*, *SSE2*, *RIM15*, *SIR2*, *HSP12*, *HSP26*, *SSA3*) and increased heat-shock tolerance, which is considered a hallmark of quiescent and slow-growing cells (Fig. 6, [27]). However, the comparatively small transcriptional response that was found after the shift to glucose starvation was accompanied by pronounced and specific cellular responses that were absent in calorie-restricted cultures.

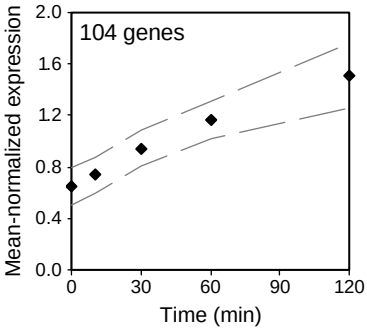
During prolonged cultivation in extremely calorie-restricted retentostat cultures, yeast cultures retained a high viability (above 60% viability after 22 days of calorie restriction (Fig. 4 and [4])). Conversely, starvation led to a progressive loss of viability (15% viability based on colony-forming units after 20 days of starvation, Fig. 4). In contrast to the calorie-restricted retentostat cultures, starving cultures exhibited a marked culture heterogeneity. In particular, the incidence of phase-contrast-dark cells increased to ca. 40% of the yeast population. In a previous study on yeast starvation [1], phase-contrast-dark cells were found to be apoptotic cells with increased metacaspase activity [54]. The absence of staining with propiodium iodide, Syto9 and CFDA, with and without permeabilization of the cellular membrane and an increased autofluorescence all support the notion that the phase-contrast-dark cells observed in the present study were indeed dead or dying and closely resembled the apoptotic cells previously observed under glucose starvation [1,2,12]. Although it is tempting to consider that the PCD cells observed in the current anaerobic starvation are physiologically similar to those previously described in the presence of oxygen, the current data are unfortunately not sufficient to conclude that the appearance of PCD cells results from programmed cell death. It is relevant to note that known pathways for inducing yeast apoptosis involve reactive oxygen species [28] and that apoptosis has hitherto not been demonstrated in anaerobic yeast cultures.

Cellular energetics during retentostat cultivation and the subsequent starvation phase were found to be strikingly different. During prolonged anaerobic, glucose-limited retentostat cultivation, the specific rate of alcoholic fermentation approached a constant, low value [4]. In a previous study, we showed that the corresponding rate of ATP turnover exactly matches the maintenance-energy requirement estimated from a series of anaerobic, glucose-limited cultures grown at different specific growth rates [4]. This correspondence suggested that the maintenance energy requirement of *S. cerevisiae* is growth-rate independent [4,37]. In contrast, the present study shows that, under glucose starvation, *S. cerevisiae* is able to dramatically decrease its rate of ATP turnover rate. During anaerobic starvation, where glycogen is the sole metabolically available energy storage material, estimated ATP turnover rates were two to three orders of magnitude lower than during the preceding phase of extreme calorie restriction (Fig. 2C).

At the transcriptional level, this response was accompanied by a strong downregulation of many genes involved in protein biosynthesis and gene expression. During calorie restriction, a large number of genes encoding proteins with growth-associated functions (translation and amino-acid, lipid and nucleotide synthesis) were already transcriptionally down-regulated relative to exponentially growing cells. Most of these genes showed a further decrease of their transcript levels during starvation. In addition, over 100 genes involved in rRNA processing, synthesis of ribosomal proteins and RNA binding were specifically down-regulated during starvation (Fig. 3). Since protein synthesis is the single most ATP-intensive process in living cells [43], down-regulation of these processes during starvation is likely to contribute to the observed reduction of ATP turnover. However, reduction of ATP turnover rate in starving cells may well involve other mechanisms. The experimental set-up described in this study enables further quantitative studies on ATP and protein turnover rates and on the regulation of these processes under anaerobic conditions.

The present study indicates that, during energy starvation, *S. cerevisiae* restricts its rate of glycogen metabolism to an extremely low value, which

A



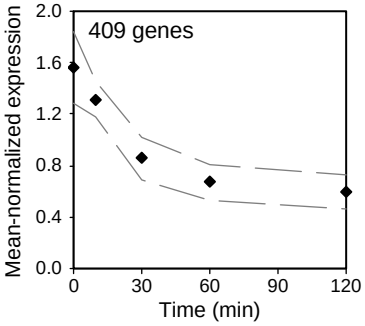


Figure 7. MIPS categories of genes differentially expressed during starvation (q<0.02)

MIPS category	Gene no.	p value
Cluster 1 UP during starvation 140 genes		
C-compound and carbohydrate metabolism	24	2.58^{-4}
sugar, glucoside, polyol and carboxylate metabolism	8	4.69^{-4}
sugar, glucoside, polyol and carboxylate catabolism	8	3.98^{-4}
carrier (electrochemical potential-driven transport)	4	4.23^{-4}
Cluster 2 DOWN during starvation 409 genes		
amino acid metabolism	30	3.56^{-4}
metabolism of the aspartate family	15	8.51^{-6}
metabolism of methionine	10	5.87^{-5}
biosynthesis of methionine	5	1.30^{-3}
TRANSCRIPTION	98	1.93^{-5}
rRNA synthesis	13	3.80^{-5}
RNA processing	58	8.47^{-10}
rRNA processing	49	8.30^{-20}
RNA modification	17	3.99^{-7}
rRNA modification	9	2.56^{-7}
PROTEIN SYNTHESIS	109	2.23^{-32}
ribosome biogenesis	90	8.61^{-34}
ribosomal proteins	70	4.90^{-25}
PROTEIN WITH BINDING FUNCTION	102	3.47^{-6}
nucleic acid binding	54	3.91^{-10}
DNA binding	21	1.15^{-3}
RNA binding	34	4.97^{-8}

B

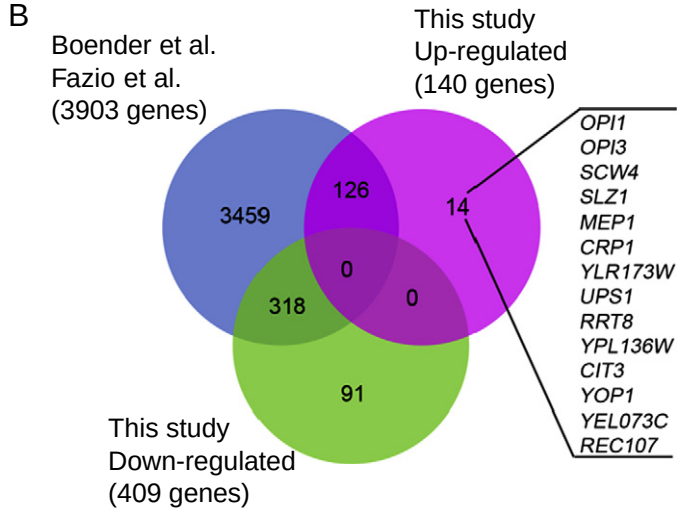


Fig. 7. (A) Transcriptional response of *Saccharomyces cerevisiae* to starvation in retentostat. Two profiles indicate the two clusters that were obtained with k-means clustering of the data, up- and down-regulated genes. For each cluster the enrichment for MIPS categories was calculated (see Section 2.8.) a p-value cut-off of 1.3E-3 was employed. (B) Dissecting the transcriptional response to starvation. The numbers show the amount of genes in common or specific for genes up-regulated and down-regulated during starvation in comparison with the anaerobic chemostat dataset at various growth rates, *i.e.* genes responding to a change in the specific growth rate.

is orders of magnitude lower than maintenance-energy requirements previously estimated from growing yeast cultures [4,50]. From an evolutionary perspective, the progressive loss of viability during long-term starvation may represent an acceptable price to maximize the chronological life span of the survivors. Future research should resolve whether the variable chronological life span during starvation is purely stochastic or whether it can be attributed to pre-existing heterogeneity in the calorie-restricted cultures, *e.g.* with respect to glycogen content or biosynthetic capacity.

Although, to our knowledge, previous studies have not quantitatively analyzed glycogen consumption and ATP turnover during starvation, studies on stationary phase in aerobic cultures [18,24] indicate even longer chronological life spans and slower glycogen consumption than found in this study. Presence of oxygen enables the use of lipids as an energy storage material and, moreover, substantially increases the yield of ATP on glycogen (6 to 8 fold higher than in anaerobic cultures, assuming an *in vivo* P/O ratio of 1 for *S. cerevisiae*; [49]). If glycogen turnover rates in aerobic starvation experiments are

indeed lower, this would indicate that an increased energy yield from storage materials compensate for increased damage by reactive oxygen species, which are considered to be a major factor in aging under aerobic conditions [22]. Furthermore, it would indicate that starving yeast cultures can tightly regulate glycogen mobilization to meet minimum demands for ATP and precursors. Since transcriptome analysis did not reveal pronounced changes in the transcript levels of genes involved in glycogen metabolism during the shift from calorie restriction to starvation, such regulation is likely to occur post-transcriptionally, *e.g.* via phosphorylation-dephosphorylation of glycogen synthase and glycogen phosphorylase [46]. Experiments with aerobic retentostats, followed by aerobic as well as anaerobic starvation phases, should enable these issues to be addressed experimentally.

In addition to identifying a stringent reduction of ATP-turnover and, in all likelihood, protein synthesis as specific responses of *S. cerevisiae* to energy starvation, this study demonstrates the usefulness of retentostat cultures as a tool for studies on calorie restriction, chronological aging and starvation. Although more technically complicated than conventional experimental cultivation systems, the tightly controlled cultivation conditions in retentostats enable a much more quantitative and specific analysis of cellular responses in this twilight zone between growth and cell death.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.07.008.

Acknowledgements

The PhD project of L.G.M.B. was sponsored by the Netherlands Genomics Initiative. We are grateful to Dr. Dick de Ridder for his help in the statistical treatment of the microarrays data. We thank our colleagues from the joint zero-growth project of the Kluyver Centre for Genomics of Industrial Fermentation for stimulating discussions.

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