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To divide or not to divide: A key role of Rim15 in calorie-restricted yeast cultures

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ABSTRACT

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.

1. 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 [14,55]. 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) [23].

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 target of rapamycin (TOR), which responds to nitrogen and carbon availability, and the cAMP-dependent protein kinase (PKA), which senses glucose availability, are highly conserved from fungi to mammals [18]. TOR and PKA are also key nutrient signaling cascades in the model eukaryote *Saccharomyces cerevisiae* [33,47]. 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 [17].

Rim15, a PAS family protein kinase has been proposed to integrate signals from various nutrient signaling networks [10,21] (Fig. 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 [51]. 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 [48]. 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 [67]. While already expressed and activated during the diauxic phase, Rim15 appears to be especially important

Abbreviations: CFU, colony forming units; EDGE, empirical analysis of digital gene expression data in R v 1.1.291; MIPS, Munich Information Centre for Protein Sequences; PDS, post-diauxic shift; PHO, Pho80–Pho85 kinase; PKA, cAMP-dependent protein kinase; q_s, specific glucose uptake rate; SAM, significance analysis of microarrays v 4.0; STRE, stress response element; TOR, target of rapamycin

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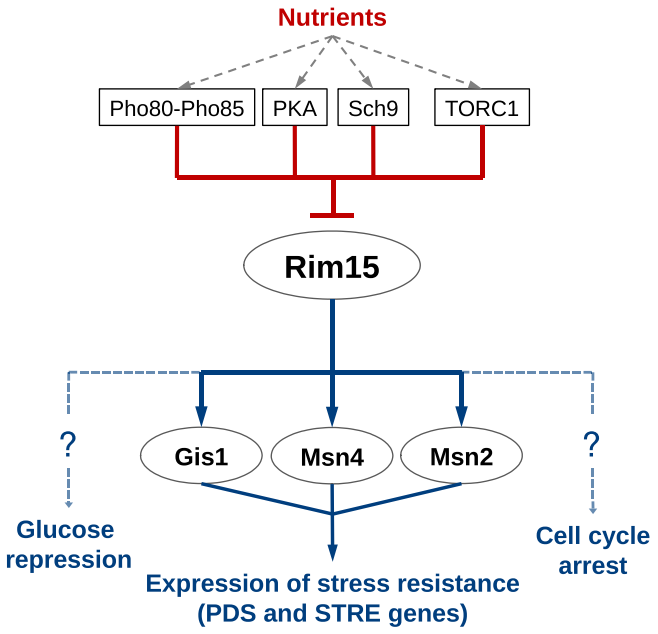


Fig. 1. Regulatory cascades around the Rim15 PAS-kinase. The PAS-kinase Rim15 integrates signals from several nutrient sensing pathways, Pho80-Pho85, PKA, Sch9 and TORC1, to different effectors, including the transcription factors Gis1, Msn2 and Msn4, but putatively also factors that control glucose repression of genes and cell cycle arrest. Modified from [21,48].

for survival and robustness during the subsequent stationary phase, in which cells are subjected to prolonged glucose starvation [10,65]. 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 [46,53,64].

While the role of Rim15 has been predominantly 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 [56]. 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 [10]. 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) [10,68] (Fig. 1). Other studies also reported changes in transcript levels in response to *RIM15* deletion, ranging from 11 genes in exponentially growing cultures [60] to 54 genes in rapamycin treated cells [53]. 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 [4]. 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 [2,46]. 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 [38] 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.

2. Materials and methods

2.1. Strains

The prototrophic *S. cerevisiae* strain CEN.PK113-7D (*MAT*a *MAL2*-8c *SUC2 RIM15*, obtained from Dr P. Kötter, Frankfurt, Germany) [19,38] was used as a reference. Yeast transformations were performed using the lithium-acetate method described by Gietz and Schiestl [22]. 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 Supplemental Table S2) according to Güldener et al. [24]. 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 Supplemental Table S1). 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.

2.2. Media and cultivation methods

Chemostat and retentostat cultures were grown on synthetic medium [62] supplemented with the anaerobic growth factors ergosterol (10 $mg \cdot L^{-1}$) and Tween 80 (420 $mg \cdot L^{-1}$) according to Verduyn et al. [61] and the antifoam Struktol J673 (0.3 $mg \cdot L^{-1}$). 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 $g \cdot L^{-1}$ for chemostats at growth rates of 0.025 h^{-1} and retentostat cultivations and 25 $g \cdot L^{-1}$ 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. [3]. 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 $L \cdot min^{-1}$). 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 [3] 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%.

2.3. Determination of substrate, metabolite and biomass concentrations

Supernatants of samples were analyzed using HPLC (Waters, Milford, MA) to determine concentrations of ethanol, glycerol, acetate, lactate, pyruvate and succinate, as described by Boender et al. [3]. After rapid quenching with cold steel beads supernatant was analyzed enzymatically to assay the residual glucose concentration (Roche kit no. 0716251) [35]. Reserve carbohydrates were assayed as described by Boender et al. [2]. Biomass concentrations were determined as culture dry weights according to the method of Postma et al. [43] 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 analyzed online for carbon dioxide levels.

2.4. Viability and thermotolerance measurements

Viability of the cultures was determined according to Boender et al. [2]. 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 metabolically 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 [2] 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.

2.5. Calculation of metabolic fluxes

Specific growth rates, consumption and production rates (q_i in mmol $(g \cdot h)^{-1}$) were calculated based on the methods of Boender et al. [3]. In short, total production and consumption rates (r_i in mmol $\cdot h^{-1}$) were divided by the fraction of viable biomass ($C_{x,viable}$ in g) as only viable biomass contributes to these rates (Eq. (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.

$$q_i = \frac{{}^{dC_i}/_{dt} - D\left(C_{i,in} - C_i\right)}{C_{x,viable}} = \frac{r_i}{C_{x,viable}} \quad (1)$$

2.6. 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×/0.75 Ph 2 M27 objective (Carl-Zeiss, Oberkochen, Germany).

2.7. Transcriptome analysis

Samples for microarray analysis were taken at 2, 9, 16 and 20 d 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 [4] 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. [16]. 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 [27]. 6383 open reading frames for yeast were extracted from the total transcript features on the arrays [5].

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 [54]. 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 [4,11,45], 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. [4].

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. [28]. 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. [26], a number of additional categories were searched for enrichments. These consist of a set of genes down–regulated in response to a glucose pulse [30], sets of genes whose expression is cell cycle phase dependent genes [13,49,69] and genes containing binding sites of transcription factor Gis1 (the post-diauxic shift element TWAGGGAT [7,40]), of transcription factors Msn2 and Msn4 (the stress responsive element AGGGG [1,34]), or transcription factor Hsf1 [6] that were selected based on web-based Regulatory Sequence Analysis Tools (http://rsat.ulb.ac.be) [58]. The resulting p-values indicate the chance of finding the same enrichment in a random set of n genes and are calculated according to [28]. 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 (Supplemental Fig. S1).

3. Results

3.1. 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 [3,59]. Continued cultivation then results in a virtually 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.) [50].

As previously described, the initial biomass accumulation in anaerobic retentostat cultures of the *RIM15* reference strain *S. cerevisiae* CEN.PK113-7D (Fig. 2A) not only resulted from an increasing cell number (Fig. 2B) but also from increases in average cell mass and cell size (Fig. 2C and D) [3]. During prolonged retentostat cultivation, the specific growth rate decreased until, after 10 d (Fig. 2E), it was reduced 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. 2F) [2].

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 aft 20 d, Fig. 2A). Conversely, cell numbers of IMK313 cultures were significantly higher than that observed for the reference strain (up to 37% higher after 20 d of retentostat cultivation; Fig. 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. 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. 2F) and as ability to divide (CFU counts, Fig. 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* 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. 2D).

3.2. 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

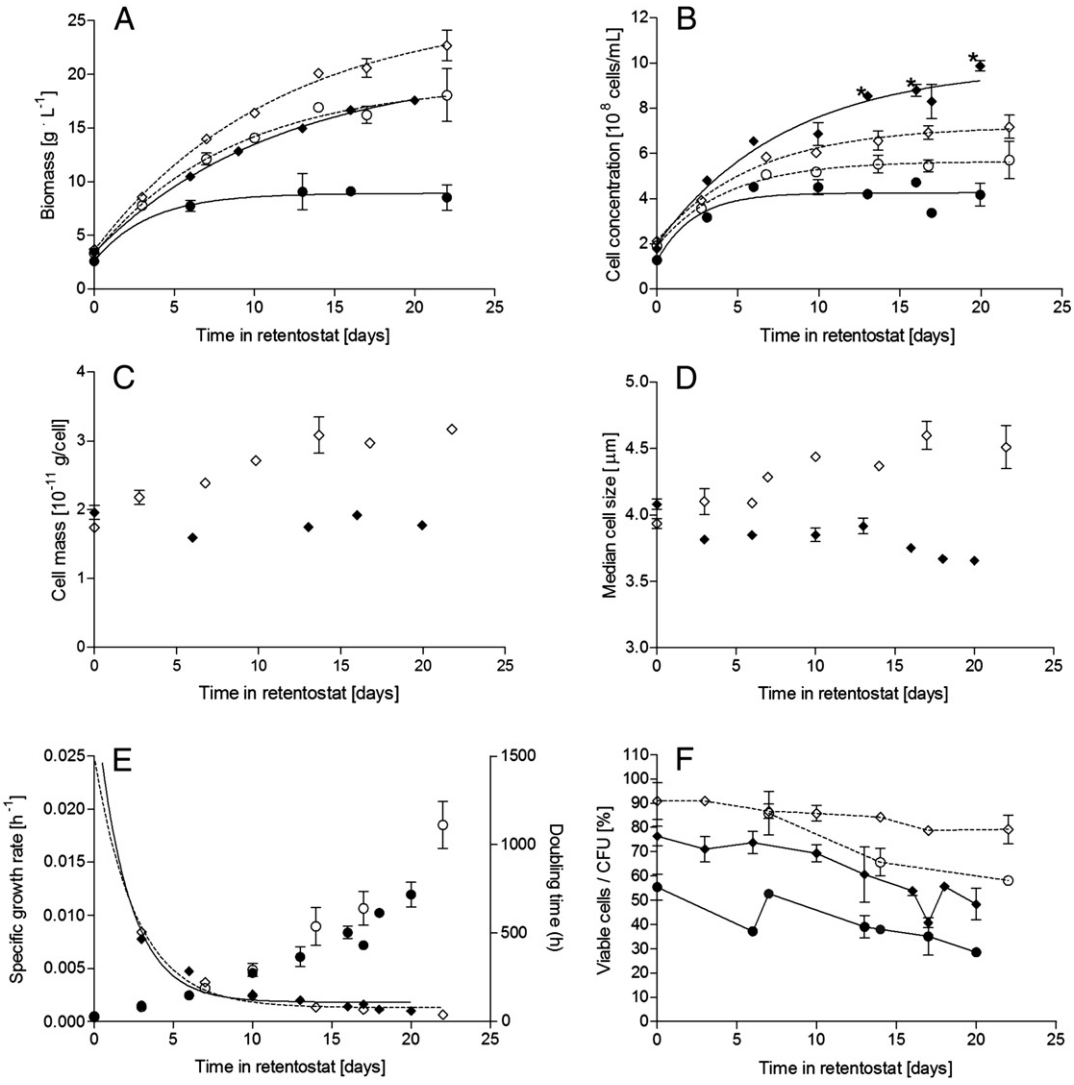


Fig. 2. Biomass accumulation and viability of the *S. cerevisiae rim15* mutant and the reference strain in retentostat cultures. Data for the *rim15* mutant IMK313 (closed symbols) and for the reference strain CEN.PK113-7D (open symbols) are shown as mean values of at least duplicate cultures +/− SEM (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.

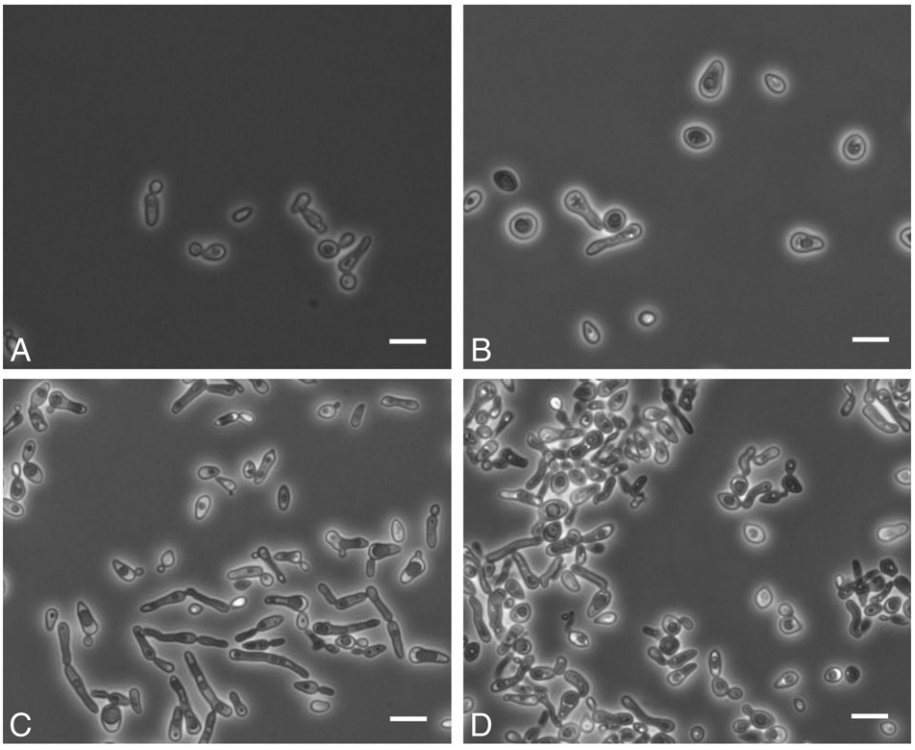


Fig. 3. Phase contrast micrographs of the *S. cerevisiae rim15* mutant and the reference strain under increasing calorie restriction. White marker bars represent 10 μm. A and B: Cells of 0 and 17 days old retentostat cultures of the reference strain, respectively. C and D: Cells of 0 and 18 days old retentostat cultures of the *rim15* mutant IMK313.

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 [10]. An even smaller set of 49 genes was identified during glucose-limited chemostat cultivation (specific growth rate of 0.1 h^{-1}, aerobic) of a *rim15* mutant and its parental strain [68]. 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. 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. 4, clusters 4, 5 and 6). Genes involved in stress responses (p-value 2.4E−7, Fig. 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 [51]. The subset of 740 genes that showed a higher 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. 4).

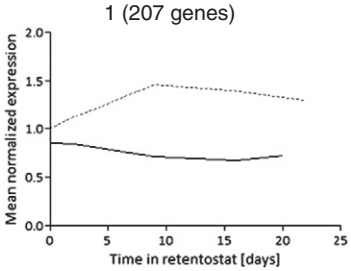
3.3. Deletion of RIM15 induces transcription of cell cycle-related genes

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 1.1E−10, Fig. 4). Three separate gene clusters were defined based on the transcript profiles of the mutant and reference strain (Fig. 4). Transcript levels of the 303 genes in cluster 4 (Fig. 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 of 2.1E−5) and closer inspection showed overrepresentation of genes whose expression is cell cycle-dependent (p-value of 1.3E−2) [49]. 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 1.4E−5) [13]. 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 suggest that, in contrast to the reference strain, this mutant failed to efficiently curtail cell cycle activity under calorie restricted conditions.

Fig. 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). 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. P-values are calculated according to Knijnenburg et al. and indicate the chance of random enrichment [28].

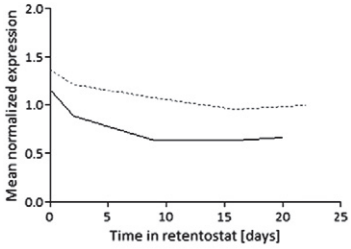
Lower

1 (207 genes)



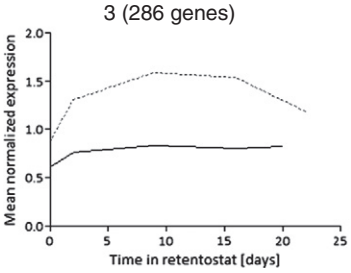
Description	# genes in cluster	Total # genes in category	p-value
STRE-element [1,34]	81	1786	2.77E-4
Oxidation Reduction	26	305	4.86E-6

2 (93 genes)



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

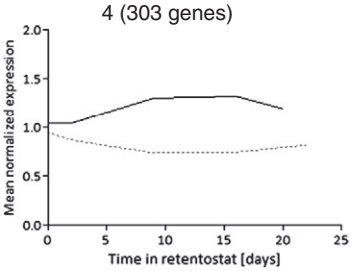
3 (286 genes)



Description	# genes in cluster	Total # genes in category	p-value
Hsf1 [6]	59	435	2.85E-15
STRE-element [1,34]	133	1786	6.89E-12

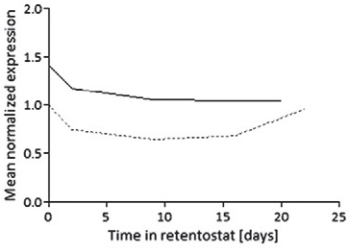
Higher

4 (303 genes)



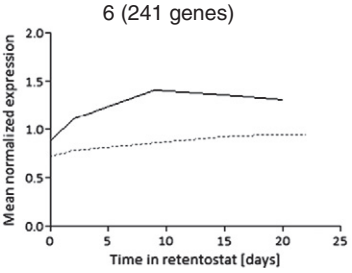
Description	# genes in cluster	Total # genes in category	p-value
Nucleotide binding	26	223	1.68E-5
Cell Cycle	75	1004	2.05E-5
rRNA processing	26	195	1.38E-6
Arg80	7	19	1.56E-5

5 (196 genes)



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

6 (241 genes)



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 [6]	94	435	2.51E-16
STRE-element [1,34]	251	1786	3.92E-16
PDS-element [7,40]	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

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

As mentioned in Section 3.2, 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. 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 2.5E−16), which is a target for the regulators Msn2 and Msn4. The PDS element (28 genes, p-value 7.1E−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 signaling cascades [51]. 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. 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. 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 [64,65].

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 [20]. The reference strain strongly accumulated glycogen during calorie restriction in the retentostats (Fig. 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 those 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 [41,63,64].

3.5. 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 [4]. Although no significant differences in residual glucose levels were observed in retentostat cultures of the reference strain and the *rim15* mutant (Supplemental Fig. S2), the 1326 differentially expressed genes showed a strong overrepresentation of genes previously shown to be transcriptionally repressed by glucose (176 genes, p-value = 7.6E−10) [30]. 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 1.1E−14, and 7 genes, p-value 3.9E−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.

3.6. 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. 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·g biomass^{-1}·h^{-1}. Strikingly, despite the severe calorie restriction, the *rim15* mutant maintained a 40% higher specific glucose uptake rate (0.72 mmol·g^{-1}·h^{-1}, Fig. 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.

3.7. 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 0.1 h^{-1} [68]. 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 [4]. 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 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. 7A and B). However, the morphology of IMK313 was markedly elongated at all specific growth rates tested (Fig. 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. 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., Supplemental Table S2). 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. 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 0.1 h^{-1} [68]. Together, these results show that the scope of the impact of Rim15 on transcriptional regulation is growth rate dependent.

4. Discussion

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

With one exception [68], 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

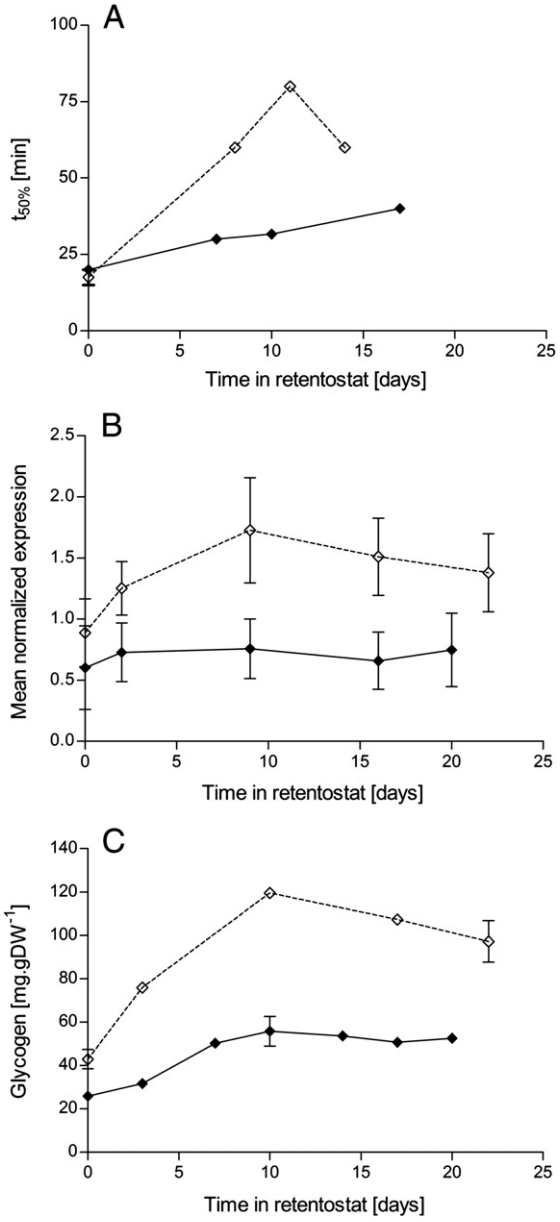


Fig. 5. Effect of *RIM15* deletion on stationary phase features. A: Heat shock resistance of the *S. cerevisiae rim15* strain IMK313 (closed symbols) and its parental strain CEN.PK113-7D (open symbols). $t_{50\%}$ is the time of incubation at 53 °C at which the viability of the culture was reduced by 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*, and *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.

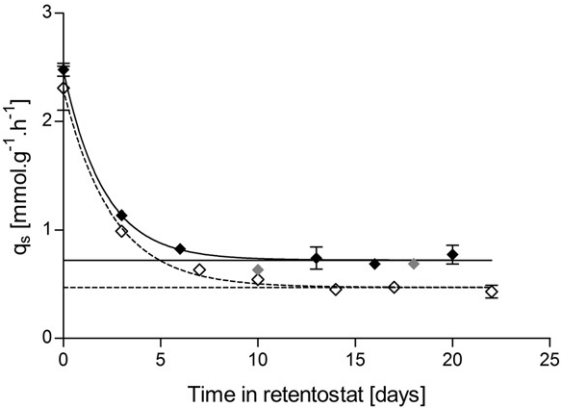


Fig. 6. Specific glucose uptake rates of retentostat cultures. Specific glucose uptake rate (q_s) during retentostat cultivations of the *S. cerevisiae* reference strain (open diamonds) and the *rim15* mutant IMK313 (closed symbols). Data were corrected for viability. The horizontal lines are the asymptotes 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 1E−6). Data are represented as mean +/− SEM for at least 2 biological replicates, except for data points shown in gray that were obtained from a single culture (errors smaller than the symbol size are not visible).

starved for carbon and energy [48]. However, mRNA degradation precludes accurate transcriptional analysis under those conditions [2,56]. 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 [10,53]. Although growth of *S. cerevisiae* on ethanol is slower than on glucose (see refs. [52,57]), both situations still represent active growth.

Use of retentostat cultures [3,59] 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* [2,48]. 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 [10,46,53,64,65]. 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 [10]. 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 [68], 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 [51], 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

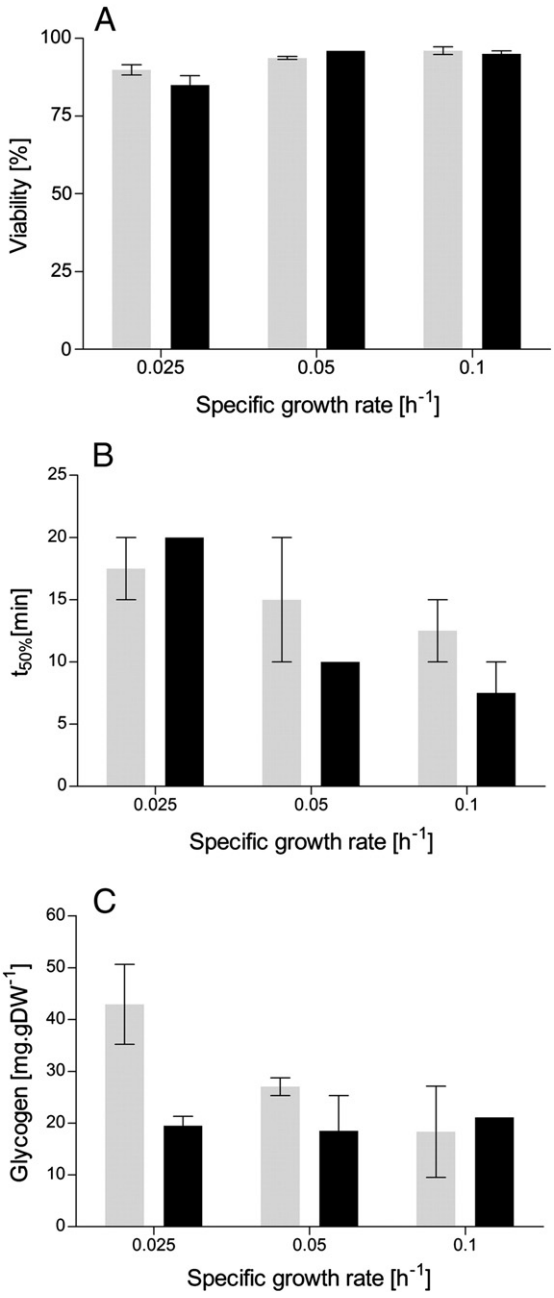


Fig. 7. Effect of *RIM15* deletion in exponentially growing cells at growth rates ranging from 0.025 h^{-1} to 0.1 h^{-1}. The *S. cerevisiae rim15* strain IMK313 (black bars) and its parental strain CEN.PK113-7D (gray bars) were cultivated at steady state in glucose-limited anaerobic chemostat cultures. 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 grams glucose equivalent per gram dry weight biomass. Data are represented as mean +/− SEM (SEM smaller than 1 is not visible).

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 results 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.

4.2. 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 [41,46,64]. 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. 4). The strongest overrepresentation (p-value from 2E − 06 to 2E − 09, Fig. 4) was observed for genes with promoter binding motifs for Swi4, Swi6, and Mbp1, which are subunits of the MBF and SBF complexes [37]. 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 [29]. 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. 4). Because passing Start is irreversible, *rim15* cells are then compelled to proceed to complete their mitotic cycle [12]. 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 [8,39], 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 [41,66]. Unfortunately, the condition-dependent abnormal morphoplogy of the *rim15* mutant observed in the present study (Fig. 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 genes and stress-responsive in the *rim15* mutant (Supplemental Table S3) and supports the key role of Rim15 in cell cycle progression.

4.3. 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 [4]. 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 [42]. 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-signaling cascades (TOR, PKA and PHO), suggest that Rim15 could have a comparable tunable activity in response to other nutrients [51].

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'.

4.4. 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 [9]. 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 [25]. Yeast is not an uncommon model in cancer and anti-cancer treatment research [15,31,36]. 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 [32,44].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.01.026.

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