Relief from nitrogen starvation triggers transient destabilization of glycolytic mRNAs in *Saccharomyces cerevisiae* cells

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ABSTRACT **Nitrogen replenishment of nitrogen-starved yeast cells resulted in substantial transcriptome changes. There was an unexplained rapid, transient down-regulation of glycolytic genes. This unexpected result prompted us to search for the factors controlling these changes, among which is the possible involvement of different nutrient-sensing pathways such as the TORC1 and cAMP/PKA pathways. To that end, the effects of various gene deletions or chemical blocking agents were tested by investigating the expression of *PGK1*, one of the glycolytic genes most affected after nitrogen replenishment. We report here that several factors affected glycolytic mRNA stability, among which were glucose sensing, protein elongation, nitrogen metabolism, and TOR signaling. Ammonium sensing was not involved in the response, but ammonium metabolism was required. Thus, our results suggest that, in the presence of glucose, carbon/nitrogen cross-talk is likely involved in the response to nitrogen upshift. Our data suggest that posttranscriptional control of glycolytic gene expression may be an important response to nitrogen replenishment.**

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INTRODUCTION

Microorganisms' ability to adapt to changing nutritional conditions is important for their survival. Nitrogen is an important nutrient for yeast, and its availability has numerous consequences for gene expression (transcription level, mRNA decay/stability), signaling, and biosynthetic pathways or metabolism. For instance, whenever nitrogen is present in insufficient amounts, some degradation of intracellular components occurs to maintain essential cellular functions (Kraft *et al.*, 2008, 2010; Uttenweiler and Mayer, 2008). Upon nutrient replenishment, yeast cells immediately resume their growth and division cycle (Unger and Hartwell, 1976). In fact, the general response to nutrient repletion consists of a rapid induction of genes involved in mass accumulation and cell division along with a repression of genes involved in respiration, gluconeogenesis, and stress response (Slattery and Heideman, 2007). Nutrient refill could have an impact on cAMP-dependent protein kinase (PKA), SNF1/AMP-activated protein kinase (AMPK), and target of rapamycin (TOR) signaling pathways and lead to substantial transcriptome changes (Slattery *et al.*, 2008; Broach, 2012). While studying the effect of nitrogen replenishment on gene expression, we noticed a strong, transient down-regulation of some glycolytic mRNAs. This observation was surprising because genes encoding these mRNAs can be considered as housekeeping genes due to the high conservatism and evolutionary antiquity of the glycolysis process. Indeed, glycolytic mRNAs are generally stable and weakly regulated (Brown, 1989; Muhlrad *et al.*, 1995). This prompted us to examine in more detail the factors that led to the control of glycolytic mRNA down-regulation under conditions of nutrient relief after nitrogen starvation. Under nutrient modulation, PKA and TOR signaling pathways dictate the expression of ribosomal protein (RP) and ribosome biogenesis (RiBi) regulons (Jorgensen *et al.*, 2008). Our data suggested

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Abbreviations used: BSA, bovine serum albumin; cAMP, cyclic AMP; ESR, environmental stress response; MOPS, 3-(*N*-morpholino)propanesulfonic acid; qRT-PCR, quantitative real-time PCR; SSC, saline sodium citrate; TOR, target of rapamycin; YNB, yeast nitrogen base; YPD, yeast extract–peptone–dextrose.

that several factors were able to modify glycolytic mRNA stability and that, in the presence of glucose, carbon/nitrogen pathway cross-talk was likely involved.

RESULTS

Global transcriptome changes in conditions of nutrient relief after nitrogen starvation

We examined yeast cell response to nitrogen addition after 16 h of nitrogen starvation in glucose medium as described in *Materials and Methods*. Thirty minutes after ammonium sulfate replenishment of these nitrogen-starved cells, 1410 genes were significantly (p value < 0.05) up-regulated with a logarithm (base 2) of fold change ranging from 0.1 to 4.5 (Supplemental Figure S1). This gene set was enriched in genes related to rRNA processing, ribosome biogenesis, translation, and amino-acid biosynthesis (Supplemental Table S1). This up-regulation of the sets of genes involved in biosynthetic processes is consistent with previous observations on yeast response to nutrient replenishment (Conway *et al.*, 2012). Concurrently, 1564 genes were significantly down-regulated (logarithm of fold change ranging from –0.1 to –5.2; Supplemental Figure S1), including genes related to protein catabolic processes (ubiquitin-dependent and vacuolar protein degradation), stress response, and oxidation-reduction processes (Supplemental Table S1). The down-regulation of stress-responsive genes is in agreement with nutrient replenishment response and has a strong negative correlation with the expression of genes involved in ribosomal biogenesis (Levy *et al.*, 2007).

Surprisingly, a large subset of glycolytic genes (25 out of 34) displayed strong down-regulation 30 min after nitrogen repletion (Table 1 and Supplemental Figure S1). This down-regulation was unexpected because genes related to glycolysis are known to display very stable expression (Brown, 1989; Wang *et al.* 2002). In addition, the decoupling of the expression of glycolytic genes from that of RP and RiBi regulons and more generally genes related to protein synthesis seemed unique to the present experiment, as their expression is reportedly tightly correlated whenever nutrients are available (Deminoff and Santangelo, 2001; Levy *et al.*, 2007).

We more specifically assessed the response of *PGK1* (3-phosphoglycerate kinase) mRNAs through Northern blot hybridization and quantitative RT-PCR (Figure 1, A–C). These experiments confirmed the rapid down-regulation of *PGK1* mRNAs, with a decrease already observed within the first 15 min after repletion, and clear opposite behavior to that of the ribosomal gene *RPL11B* (Figure 1A). Monitoring of *PGK1* mRNAs over a longer duration highlighted the transient nature of the mRNA decrease, with a minimal level reached around 30 min after nitrogen replenishment. Thereafter we observed a straight increase of expression (threefold compared with $t = 0$), suggesting that *PGK1* mRNA level is reduced during nitrogen starvation. Examination of the response of four other glycolytic genes (*GPM1*, *TDH1*, *ENO2*, *TPI1*) by quantitative real-time (qRT)-PCR revealed similar transient down-regulation after ammonium addition, although the patterns displayed some variations (Supplemental Figure S2). The transient nature of the down-regulation of this set of glycolytic genes likely explains its previous overlooking; it is notable that it was no longer detectable 60 min after nitrogen addition.

To gain further insight into the mechanisms underlying the decrease in mRNA abundance, we measured the half-life of *PGK1* mRNA after nitrogen repletion. Metabolic labeling of mRNA with 4-thiouracil was used to measure *PGK1* mRNA half-life. A *PGK1* mRNA half-life of 6 min was calculated based on the kinetic data from the first 15 min after ammonium addition (Figure 1D). This is far below the published *PGK1* mRNA stability duration, which was estimated to be as high as 45 min (Muhlrad *et al.*, 1995; Wang *et al.*, 2002), suggesting that, in our case, *PGK1* transcripts produced during starvation are highly sensitive to destabilization.

Glycolytic mRNAs are not known to be regulated by nutrient signals; our data thus prompted us to search for factors controlling a specific transcription reduction in response to nitrogen relief and in particular to investigate the signaling pathways involved.

Signaling pathways and metabolism

To evaluate whether the destabilization of glycolytic genes was ammonium-specific or could be triggered by different nitrogen sources, we further tested cell response to a set of sources, using *PGK1* transcripts as a marker. A decrease in mRNA level was observed after replenishment with different concentrations of glutamine, with no dose-dependent effect (Supplemental Figure S3). A similar down-regulation of mRNA was observed with methionine, arginine, leucine, and urea (data not shown), indicating that *PGK1* mRNA destabilization did occur, whatever the nitrogen source.

To ascertain which pathways were contributing to the response to nutrient relief after nitrogen starvation, we blocked different pathways under nutrient-depleted conditions and then observed the changes in *PGK1* transcript levels after addition of ammonium or glutamine. We also measured the expression of the *RPL11B* gene, coding a ribosomal 60S subunit protein, and used as a marker of the expression of genes related to the translation machinery.

Both TOR signalization and nitrogen metabolism are involved. To evaluate whether the nitrogen-sensitive complex TORC1 was involved in the response to nitrogen replenishment, we investigated the effect of treatment with rapamycin (an inhibitor of the TORC1 complex) on global transcriptome changes (Supplemental Figure S1). We found that genes related to RP and RiBi regulons and more generally those related to protein synthesis were still up-regulated, whereas those related to stress response, proteolysis, and autophagy were down-regulated (Supplemental Table S1). Some glycolytic genes were down-regulated, but with smaller fold changes than in cells not treated with rapamycin (Table 1), suggesting involvement of the TOR pathway. Monitoring of *PGK1* mRNA by qRT-PCR confirmed the limitation of the transcript down-regulation by rapamycin, consistent with TORC1 inhibition (Figure 2, A and B). In addition, inactivation of the two downstream targets of TORC1, Ure2 and Gln3, did not impact *PGK1* transcript destabilization, ruling out their involvement in the process (unpublished data).

Ammonium sensing is not involved but metabolism of ammonium is required. Ammonium can be transported into *Saccharomyces cerevisiae* cells by a set of carriers, Mep1, Mep2, and Mep3, and sensed by Mep2, which has been described as an ammonium transceptor (Van Nuland *et al.*, 2006). Since ammonium triggered some destabilization of *PGK1* mRNAs, we examined whether ammonium sensing through Mep2 and its metabolization were involved in the process. Deletion of the *MEP2* gene reduced the decrease in *PGK1* mRNAs level, with altered kinetics from that in wild-type cells, pointing to a contribution of Mep2 (Supplemental Figure S4A). To discriminate between the sensing and transport functions of Mep2, we used methylamine, a nonmetabolizable ammonium analogue that nevertheless activates signaling through Mep2 sensing. As shown in Supplemental Figure S4B, no destabilization of *PGK1* mRNAs was observed after methylamine addition, indicating that ammonium sensing by Mep2 is not involved in signaling. To specify the role of ammonium metabolization, we used in combination a mutant and

Gene	– Rapamycin		+ Rapamycin		Enzyme name
	log(FC)[a]	Adj. *p* value[b]	log(FC)[a]	Adj. *p* value[b]	
PGK1	–5.24	0.0013	–2.52	0.0431	3-Phosphoglycerate kinase
GPM1	–3.10	0.0083			Glycerate phosphomutase
TDH2	–3.02	0.0063			Triose-phosphate dehydrogenase
TDH3	–2.47	0.0138			Triose-phosphate dehydrogenase
TDH1	–2.10	0.0014	–0.26	0.0048	Triose-phosphate dehydrogenase
ENO2	–2.06	0.0083	–0.32	0.0078	Enolase
PDC1	–1.97	0.0358			Pyruvate decarboxylase
TPI1	–1.91	0.0034			Triose-phosphate isomerase
ENO1	–1.63	0.0056			Enolase
PDC6	–1.54	0.0009	–0.57	0.0276	Pyruvate decarboxylase
MDH2	–1.36	0.0007	–0.23	0.0130	Malate dehydrogenase
PFK1	–1.17	0.0033	–0.51	0.0069	Phosphofructokinase
PCK1	–1.08	0.0007	–0.60	0.0066	Phosphoenolpyruvate carboxykinase
ALD2	–1.04	0.0055			Aldehyde dehydrogenase
PGI1	–1.03	0.0007	–0.66	0.0021	PhosphoglucoIsomerase
ALD4	–1.01	0.0012	–0.25	0.0164	Aldehyde dehydrogenase
GLK1	–0.93	0.0010	–0.53	0.0379	Glucokinase
CDC19	–0.87	0.0297			Cell division cycle
PFK2	–0.83	0.0239	–0.18	0.0409	Phosphofructokinase
ADH1	–0.47	0.0181			Alcohol dehydrogenase
HXK1	–0.46	0.0104	–0.22	0.0420	Hexokinase
PGM1	–0.38	0.0055	–0.15	0.0393	Phosphoglucomutase
PDA1	–0.29	0.0337			Pyruvate dehydrogenase alpha
FBP1	–0.29	0.0167	–0.32	0.0092	Fructose-1.6-bisphosphatase
ADH3	–0.26	0.0089	0.33	0.0401	Alcohol dehydrogenase
PYK2	–0.17	0.0242			Pyruvate kinase
ADH4	0.16	0.0284			Alcohol dehydrogenase
ADH5	0.56	0.0128			Alcohol dehydrogenase
PYC1	0.66	0.0016	1.29	0.0001	Pyruvate carboxylase
ALD5	0.81	0.0086	0.90	0.0021	Aldehyde dehydrogenase
ADH6	1.01	0.0023	0.84	0.0004	Alcohol dehydrogenase
ALD6	1.20	0.0073	0.75	0.0036	Aldehyde dehydrogenase
PYC2			0.36	0.0232	Pyruvate carboxylase

Total RNA was extracted from nitrogen-starved cells sampled 30 min after addition of either water (no relief) or 20 mM ammonium (nitrogen relief). To study the effect of rapamycin, cells were incubated with rapamycin for 60 min before the addition of water/nitrogen. Fold changes measure the shift of expression level between the condition of nitrogen relief (NH_4) and that of no relief (H_2O) after 30 min. Missing data correspond to genes not differentially expressed.
[a]Logarithm of fold change to base 2.
[b]Adjusted for multiple testing (Benjamini and Hochberg, 1995).

TABLE 1: Changes in glycolytic transcript levels as measured by microarray hybridization.

an inhibitor that completely abolish ammonium metabolism through the suppression of both glutamate dehydrogenase (Gdh1) and glutamine synthetase activities (Gln1). As *GLN1* deletion is lethal, we used a *gdh1* mutant together with a specific Gln1 inhibitor, that is, methionine sulfoximine (MSX). Under these conditions, no ammonium-induced decrease of *PGK1* transcripts amount was observed (Figure 2C), suggesting that ammonium metabolism is required for mRNA destabilization.

Carbon signaling is involved in transcript down-regulation. To assess the contribution of carbon metabolism to mRNA destabilization, different tests were performed. Growth and starvation on ethanol as carbon source only resulted in a weak impact on mRNA amount upon nitrogen repletion compared with that on glucose (Figure 2, D and E). Therefore, glucose was needed to trigger a full decrease in the *PGK1* transcripts. This prompted us to further investigate the role of the different glucose-sensing systems of

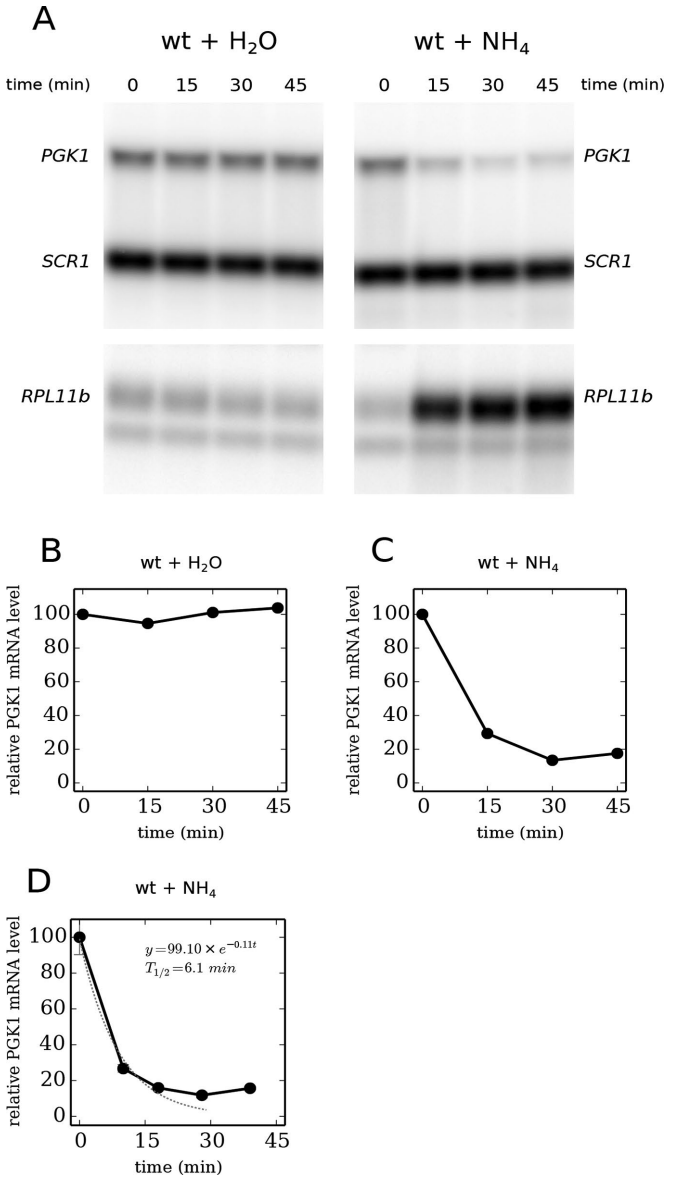

FIGURE 1: (A) Northern blot hybridization showing the evolution over time of *PGK1*, *SCR1*, and *RPL11B* transcript levels following addition of water (left panel) or 20 mM ammonium (right panel) on nitrogen-starved wild-type (wt) yeast cells. (B, C) *PGK1* mRNA levels were determined by quantification of the radioactive hybridization signals on blots (no replication). (D) Estimation of the decay rate of *PGK1* mRNA. Cells were labeled in the presence of 200 µM 4-thiouracil during nitrogen starvation (16 h) before addition of 20 mM ammonium and of 2 mM uracil at $t = 0$ min. Labeled *PGK1* mRNA level was determined by quantitative RT-PCR. Data represent mean and SD of three replicates. The curve (red) was fitted using an exponential decay model. Levels are expressed relative to those found before nitrogen addition ($t = 0$ min).

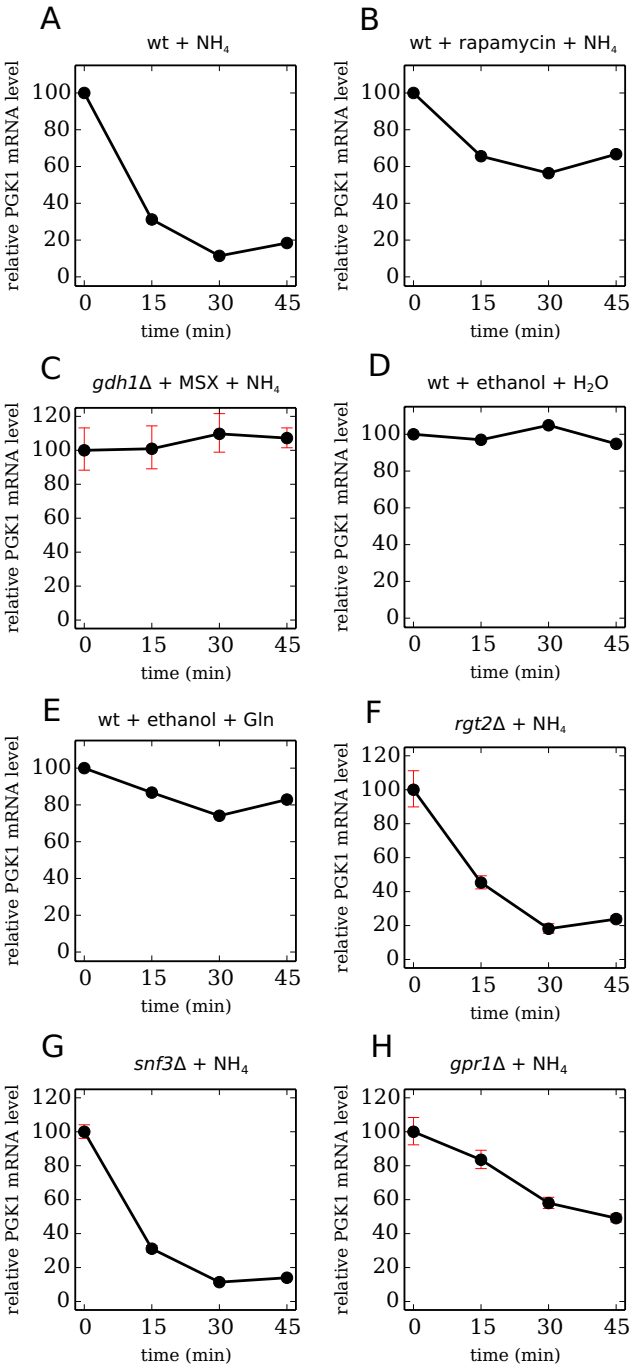

FIGURE 2: Change in *PGK1* transcript level in nitrogen-starved yeast cells after addition of (A) 20 mM ammonium on wt yeast, (B) 20 mM ammonium on rapamycin-treated wt yeast, (C) 20 mM ammonium on methionine sulfoximine (MSX)-treated *gdh1*Δ mutant, (D) water on wt yeast grown on ethanol, (E) 4 mM glutamine on wt yeast grown on ethanol, (F) 20 mM ammonium on *rtg2*Δ mutant, (G) 20 mM ammonium on *snf3*Δ mutant, and (H) 20 mM ammonium on *gpr1*Δ mutant. mRNA levels were determined by quantification of Northern blot hybridization radioactive signals (plots A–D, no replication) or by quantitative RT-PCR (plots E–H, triplicates). Levels are expressed relative to those found before nitrogen addition ($t = 0$ min). For qRT-PCR, data represent mean and SD of three replicates.

S. cerevisiae cells. We first examined the contribution of the plasma membrane glucose sensors Rgt2 and Snf3. We found that deletion of the genes *RGT2* and *SNF3* had no impact, suggesting that neither was involved in the destabilization of *PGK1* transcripts (Figure 2, F and G). The same conclusion was obtained for the AMP-activated S/T protein kinase Snf1, for which gene deletion had no effect (Supplemental Figure S4C). Deletion of *GPR1*, a part of the plasma membrane Gpr1/Gpa2 glucose sensing complex, impaired the down-regulation of *PGK1* mRNAs upon nitrogen replenishment (Figure 2H). However, *GPR1* deletion did not totally abolish *PGK1* mRNA decay, suggesting that another glucose-sensing system is

involved in the signaling. Since Gpr1/Gpa2 is known to signal through the cyclic AMP-dependent protein kinase (PKA) pathway, we assessed its involvement in the response.

We first inactivated *BCY1*, which encodes the negative regulator of the catalytic component of PKA (Tpk1-3). Correspondingly, *BCY1*-deleted strains have a high level of PKA activity (Toda *et al.*, 1987). Our results showed a gradual increase in *PGK1* transcripts following nitrogen repletion, indicating that high PKA activity prevented *PGK1* mRNA degradation (Figure 3, A and B). This suggests that PKA was not involved in the signaling, since greater destabilization would otherwise be expected. Treatment with the PKA inhibitor H89 (Chijiwa *et al.*, 1990) showed that *PGK1* mRNA reduction was still observed after nitrogen resupply (Supplemental Figure S4D), suggesting that reduced PKA activity did not impede destabilization. This is also consistent with the absence of PKA contribution to glycolytic mRNA destabilization. However, the effect of H89 inhibition on PKA is poorly understood, and it is generally advised that H89 should not be used as the single source of evidence for PKA involvement (Lochner and Moolman, 2006).

PKA activity in the different strains and conditions can be checked using genes whose expression is under its control. Under the high-glucose condition, PKA inhibits the nuclear localization of the stress-responsive transcriptional activator Msn2/4 (Lee *et al.*, 2013). We thus examined the effect of *BCY1* deletion on the expression of *HSP26* (coding a small heat shock protein with chaperone activity). As expected, expression level was down-regulated in wild-type cells upon nitrogen addition (Supplemental Figure S5, A and B), whereas it increased in the mutant slightly (Supplemental Figure S5, C and D). However, the *HSP26* transcript level was 6.3 times lower in the mutant than in the wild type (Supplemental Figure S6A), consistent with high PKA activity and Rim15 remaining in the cytoplasm impairing activation of Msn2/4. Similarly, we checked the effect of the *bcy1* mutation on the expression of *RPL11B*, whose protein is involved in translation. *RPL11B* transcription is known to be controlled by PKA and TORC1 (Slattery *et al.*, 2008). In contrast to controls (Supplemental Figure S7, A and B), no induction of *RPL11B* expression was observed in mutant *bcy1* (Supplemental Figure S7, C and D), but we observed that the *RPL11B* transcript level was already three times higher in the mutant than in the wild type before nitrogen addition (Supplemental Figure S6B). This increase in ribosomal protein mRNA levels after *BCY1* disruption was already observed by Klein and Struhl (1994) and is consistent with permanently high PKA activity.

We also addressed the impact of deleting *RIM1*, a gene coding for a protein kinase involved in cell proliferation in response to nutrients (carbon and nitrogen) and in the activation of the Msn2/4 transcription factor by direct phosphorylation (Lee *et al.*, 2013). Rim15 kinase is known to be negatively controlled by PKA and TOR. Addition of nitrogen resulted in a induction of *PGK1* transcripts in *rim15* mutant (Figure 3, C and D). This response is consistent with the involvement of Rim15 in the signaling of carbon or nitrogen upon repletion. We observed that *HSP26* expression was repressed in wild-type cells (Supplemental Figure S5, A and B), but was highly induced in the *rim15* mutant (Supplemental Figure S5, E and F). In fact, the *HSP26* transcript level was 14.7 times lower in mutant than in wild-type cells (Supplemental Figure S6A), consistent with a low stress response in mutant *rim15*. No *RPL11B* induction was observed in the mutant (Supplemental Figure S7, E and F) but the transcripts level was already 13 times higher than in the wild type before replenishment (Supplemental Figure S6B).

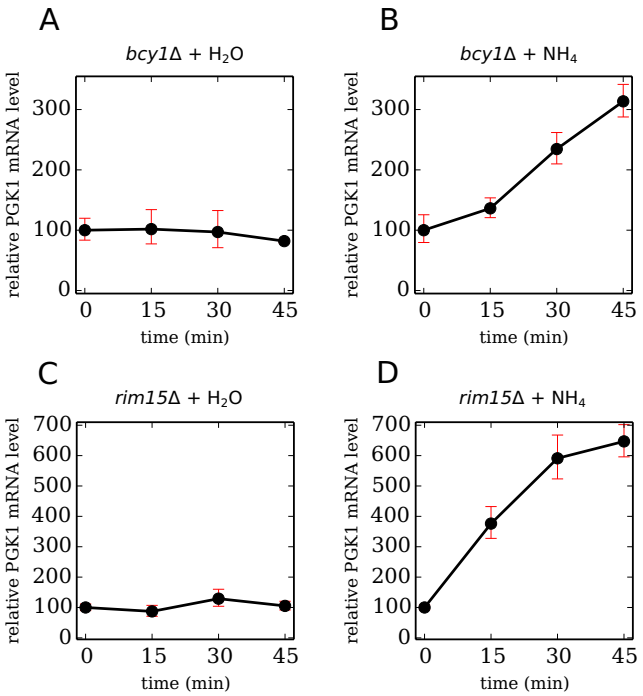

FIGURE 3: Change in *PGK1* transcript level on nitrogen-starved yeast cells after addition of (A) water on *bcy1*Δ mutant, (B) 20 mM ammonium on *bcy1*Δ mutant, (C) water on *rim15*Δ mutant, and (D) 20 mM ammonium on *rim15*Δ mutant. mRNA levels were quantified by quantitative RT-PCR. Levels are expressed relative to those found before nitrogen addition ($t = 0$ min). Data represent mean and SD of three replicates.

mRNA level reduction is achieved through the general mRNA decay pathway

We found that a cycloheximide treatment (CHX) impaired postrelief *PGK1* mRNA transcript reduction (Figure 4, A and B), although it did not affect the ribosomal protein *RPL11B* gene, whose transcription was induced as early as 15 min after nitrogen repletion (data not shown). Cycloheximide freezes ribosomes during elongation and is known to decrease decapping and mRNA decay (Beelman and Parker, 1994).

Suppressing the 5′-to-3′ degradation by the *Xrn1* exonuclease almost completely suppressed *PGK1* transcript reduction (*xrn1*Δ, Figure 4C). A similar result was observed for deactivating the deadenylase complex using a *ccr4* mutant (*ccr4*Δ; Figure 4D). Every time that the genetic background was different (BY4741 was used for *xrn1*Δ and *ccr4*Δ), we observed that *PGK1* mRNA level is higher in mutants (Figure S8), suggesting that the *PGK1* transcript is more stable when the mRNA decay pathway is impaired. These results suggest that the main mRNA decay pathway is involved in *PGK1* mRNA destabilization.

We further tested whether loss of either *Igo1* or *Igo2* had an effect on *PGK1* transcript reduction. These proteins prevent degradation of nutrient-regulated mRNAs by association with the decapping activator Dhh1 (Talarek *et al.*, 2010). In fact, their deletion had no major effect on *PGK1* mRNA stability (Figure 4, E and H). We also tested whether *PGK1* transcripts contained any 3′-end cis-elements that controlled transcript decay. We replaced the 3′-untranslated region of *PGK1* (3′-UTR) with that of *ERG1* (squalene epoxidase involved in the ergosterol biosynthesis pathway), whose transcript level was found to be stable in the above microarray study. Under

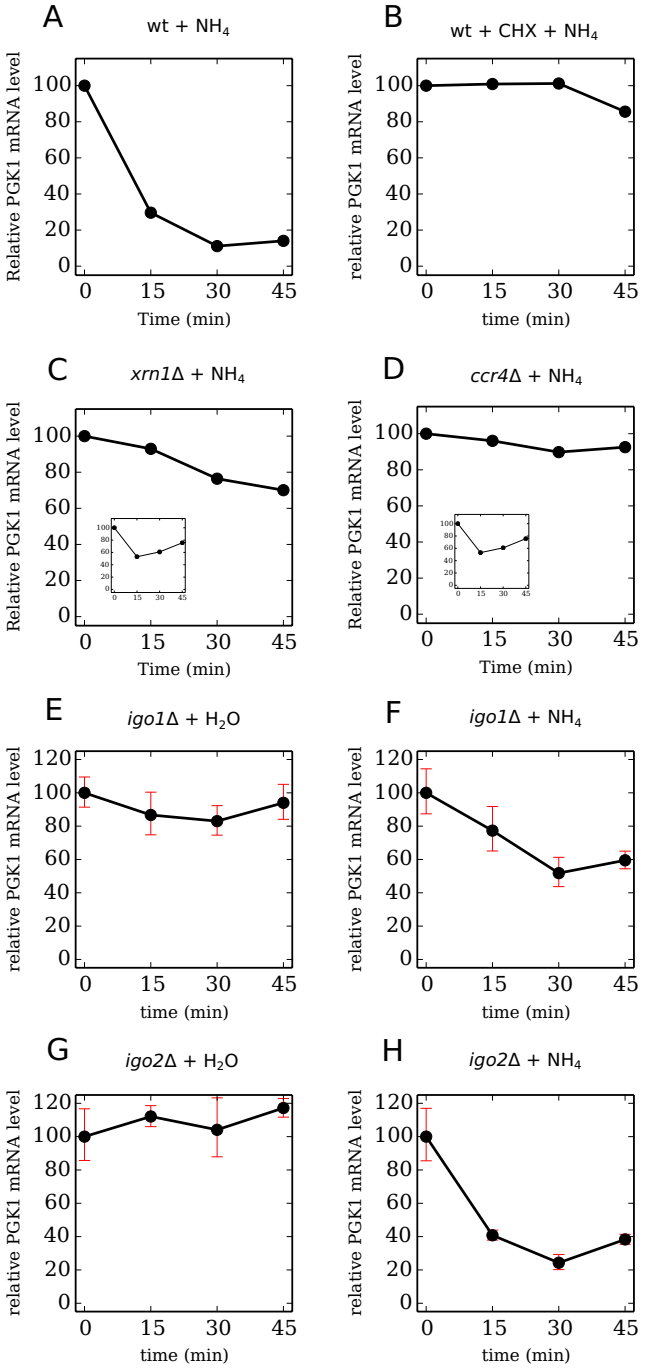

FIGURE 4: Change in *PGK1* transcript levels on nitrogen-starved yeast cells after addition of (A) 20 mM ammonium on wt yeast, (B) 20 mM ammonium on cycloheximide (CHX)-treated wt yeast, (C) 20 mM ammonium on *xrn1*Δ mutant (BY4741 background; inset: transcript level after addition of ammonium on BY4741 yeast), (D) 20 mM ammonium on *ccr4*Δ mutant (BY4741 background; inset: transcript level after addition of ammonium on BY4741 yeast), (E) water on *igo1*Δ mutant, (F) 20 mM ammonium on *igo1*Δ mutant, (G) water on *igo2*Δ mutant, and (H) 20 mM ammonium on *igo2*Δ mutant. The mRNA levels were determined by quantification of Northern blot hybridization radioactive signals (plots A–D, no replication) or by quantitative RT-PCR (plots E–H, triplicates). Levels are expressed relative to those found before nitrogen addition (t = 0 min). For qRT-PCR, data represent mean and SD of three replicates.

these conditions, *PGK1* transcript down-regulation was still observed (Supplemental Figure S4, E and F).

DISCUSSION

The transcriptional response of yeast to nutrient availability and the transition from a quiescent state to growth has been already extensively described (Martinez *et al.*, 2004; Levy *et al.*, 2007; Slattery *et al.*, 2008). The main features of the response include the induction of genes of protein synthesis, ribosome biogenesis, and cell division and the repression of genes necessary for resistance to stress and more broadly to environmental changes (ESR). We consistently observed these main aspects of the transcriptional responses in cells preparing for growth after a short-term response to nitrogen replenishment. In addition, we observed unexpected down-regulation of a set of genes encoding glycolytic enzymes. This change is rapid and transient (Supplemental Figure S2), which likely explains why it escaped previous genome-wide analysis. The decrease in mRNAs is rapid and significant for many genes; it does not correspond to a simple cessation of transcription but is consistent with destabilization of the transcript. The transient destabilization of glycolytic mRNAs observed just after nitrogen replenishment could be due in part to their low abundance, because they are poorly transcribed and/or they are highly unstable under nitrogen-depleted conditions. We could confirm that *PGK1* mRNAs, initially present before nitrogen addition, are highly unstable (6 min half-life; Figure 1). Given the similarity of behavior within the set of glycolytic genes, our data suggest that all the other glycolytic genes were also subject to degradation. This response is surprising, since these mRNAs are considered to be very stable (Wang *et al.*, 2002). Moreover, their destabilization in a situation where yeast is preparing to resume growth in a glucose medium, which will require an efficient glycolytic enzyme setup to metabolize sugar and produce energy, is counterintuitive. Indeed, in this situation, we observed an unusual uncoupling of the expression of glycolytic and ribosomal protein genes (strongly up-regulated). These two groups of genes are known to respond very similarly to the nutrient status in the environment. Clearly, within the time scale investigated, yeast cells give priority to the expression of genes involved in protein synthesis, while decreasing the potential for glycolytic protein synthesis.

We investigated several nutrient-sensing systems to test whether they are involved in the triggering of mRNA degradation following nitrogen replenishment (Supplemental Figure S9). Full destabilization of glycolytic mRNAs requires the activation of both nitrogen and carbon sensing systems. We observed that the TOR signaling pathway was involved in the response to a nitrogen upshift. Consistent with this role, the response was not specific to any nitrogen form, but could be observed with either ammonium or different amino acids. Glycolytic mRNA decay was not triggered by all carbon sources: glucose was an efficient trigger, but not ethanol (Figure 2, D and E). Our data suggest that the glucose signal was transduced through Gpr1, one of the two G-protein systems that control adenylate cyclase activity. However, we did not identify any contribution of the cAMP/PKA cascade to the destabilization of glycolytic mRNA. How glucose acts as a signal for mRNA destabilization is therefore still unclear. We showed that Rim15, which is known to integrate signals from at least three nutrient-sensory kinases, TOR, PKA, and Sch9, was involved in the signaling for mRNA degradation. Cross-talk between sugar and nitrogen metabolism or between different nutrient signals has already been described under conditions of nutrient replenishment (Wu *et al.*, 2004; Conway *et al.*, 2012). In addition, destabilization

of mRNAs in a situation of nutrient relief has been reported by Airoldi *et al*. (2016). They described a specific degradation of transcripts of nitrogen catabolite repression (NCR)–controlling genes and showed that it involved TOR pathway signaling. Our data show that TOR controls the stability of mRNAs of a large set of genes that are not usually considered as targets of its activity and thus expands the role of TOR.

There is increasing evidence that mRNA stability plays an important role in regulating gene expression programs (Puig *et al*., 2005; Bennett *et al*., 2008; Baumgartner *et al*., 2011). We were able to show that *PGK1* mRNAs destabilization takes place through the general decay pathway. In yeast, this pathway, common to stable and unstable transcripts, is a process in which deadenylation by the Ccr4/Pop2/Not complex leads to decapping by Dcp1/2. mRNAs are thereafter degraded by Xrn1, a 5′-to-3′ exonuclease (Sheth and Parker, 2003). Studies on the connections between the general pathway of mRNA decay and translation have usually assessed the effects of alterations in translation on mRNA stability (Huch and Nissan, 2014). Indeed, the inhibition of translation elongation with cycloheximide resulted in *PGK1* mRNA stabilization (Figure 4B). In fact, mRNA structures such as the 5′ cap and the 3′ poly(A) tail are potential factors that affect mRNA stability (Brown, 1989). Igo1/2, which prevents degradation of nutrient-regulated mRNAs (Luo *et al*., 2010; Talarek *et al*., 2010), is under control of Rim15. However, under our specific conditions, we found that their inactivation had a limited effect on *PGK1* mRNA stability (Figure 4, E–H).

Yeast cells remain metabolically active in the absence of nitrogen (Brice *et al*., 2014), although the cellular cycle is arrested in G1, corresponding to a stationary phase of growth, where transcription still occurs although at a reduced rate. Nitrogen addition to nondividing cells induced a massive transcription of genes related to the cellular translational machinery (RiBi and RP). These new transcripts are in competition with those already present, resulting in a sharp decrease in the translational rate of some of them. It has been reported that a decrease in the elongation rate is associated with mRNA destabilization (Huch and Nissan, 2014) and that changes in the ribosome transit rate represent a key event in the decapping and turnover of mRNA (Sweet *et al*., 2012). Thus, we can hypothesize that this degradation may allow a reallocation of ribosomes to transcripts required for growth and proliferation (Kief and Warner, 1981; Lee *et al*., 2011).

The rationale of a massive yeast glycolytic mRNA transient decay is likely linked to the amount of mRNA associated with this pathway. Indeed, glycolytic genes have a high expression level and their overall transcripts were estimated to constitute 5–13% (Fraenkel, 2003) of the total cell mRNA molecules. Moreover, proteins forming the glycolysis pathway represent more than 30% of soluble cell proteins (Moore *et al*., 1991), and it could be beneficial for the cell to preserve energy, limiting glycolysis-protein synthesis in favor of proteins required for the cellular translation machinery. A rapid decay of glycolytic mRNAs likely allows the yeast cell to orient the synthesis toward ribosomal components through a reallocation of ribosomes to transcripts required for growth.

The phenomenon observed here is likely to occur in many environments whenever yeast resumes growth. Although not described in detail, it is notable that strong down-regulation of glycolytic genes was reported after wine yeast *S. cerevisiae* inoculation in high-glucose medium (Rossignol *et al*., 2006); in germinating spores of *S. cerevisiae* (Brengues *et al*., 2002); and in *Schizosaccharomyces pombe* cells about to resume growth after nitrogen replenishment (Shimanuki *et al*., 2007). Degradation of glycolytic mRNA is therefore likely a common phenomenon in yeast when it prepares for growth.

MATERIALS AND METHODS

Yeast strains, growth conditions, and cell fixation

Several genes involved in nitrogen or carbon signaling pathways were deleted to study 1) glucose metabolism downstream on the transport pathway (*SNF3* deletion) or 2) glucose sensing (*GPR1*, *RGT2*, or *SNF3* deletions), 3) nitrogen signaling and metabolism (*GDH1* or *MEP2* deletions), and 4) the cAMP/PKA pathway (*BCY1* deletion) or the interconnection of TOR/PKA pathways (*RIM15* deletion). The characteristics of the strains used in this study are summarized in Supplemental Table S2.

After overnight culture in YPD (yeast extract–peptone–dextrose) medium, cells were transferred to YNB medium (yeast nitrogen base without amino acids 6.7 g/l, glucose 20 g/l) for 32 h. A constant cell population (7×10^9 cells) was then nitrogen-starved in 350 ml YNB medium (yeast nitrogen base without amino acids and ammonium, glucose 50 g/l) for 16 h.

To block the TOR signaling pathway, protein biosynthesis, or glutamine synthetase activity, the nitrogen-free medium was supplemented with rapamycin (0.2 µg/ml, 60 min before nitrogen addition), cycloheximide (100 µg/ml, 5 min before nitrogen addition), or methionine sulfoximine (MSX, 0.1 mM 15 min before nitrogen addition). Several nitrogen sources or water (control) were then added to the medium. Cells were sampled rapidly after 0, 15, 30, and 45 min as follows: cells were collected by filtration (0.45 µm membrane) and suspended in 1.5 ml DEPC-treated water. Water was removed by centrifugation and cells stored at -80°C before RNA extraction.

RNA extraction and Northern blot hybridization and quantification

RNA was extracted from cells as described previously (Chomczynski and Sacchi, 1987). RNA concentrations were determined by measuring absorbance at 260 nm with a Nanodrop apparatus. Equal amounts (3 µg) of total RNA were separated by electrophoresis on 1.5% agarose, 2% formaldehyde denaturing gels in 1X MOPS buffer and then transferred by capillarity to nylon membranes (Hybond; Amersham) using 20X SSC as transferring buffer. Membranes were dried and hybridized to ^{32}P-labeled probes. PCR DNA fragments of *PGK1*, *SCR1*, or *RPL11B* genes were labeled using the Megaprime DNA labeling system (Amersham). Hybridization was performed at 65°C using 2.5×10^7 cpm of labeled probe in 10 ml hybridization buffer (0.5 M sodium phosphate, pH 7, 1 mM EDTA, 7% SDS, bovine serum albumin [BSA] 10 mg/ml). Filters were washed twice (5 min each time) at room temperature in 2X SSC and three times at 65°C in 0.2X SSC (10 min each time). Membranes were dried and exposed on phosphor screens to record the hybridization signal. Screens were scanned using a Storm PhosphoImager (Amersham Biosciences), and the resulting images were analyzed with ImageQuant software. We used *SCR1* as a reference gene for normalization. Levels are expressed relative to those found before nitrogen addition.

Quantitative RT-PCR

Gene expression levels were analyzed using real-time, quantitative PCR. Reverse transcription was performed with SuperScript III reverse transcriptase (Invitrogen), using 0.8 µg of total RNA previously treated with DNase I (Invitrogen). The cDNA samples were then diluted 200-fold. The gene-specific primers designed using Primer Express software (Applied Biosystems) are shown in Supplemental

Table S3. PCRs (20 µl final volume) contained 5 µl of dilute cDNA, 250 nM of specific primers, and 10 µl of Power SYBR Green PCR Master Mix (Applied Biosystems). Real-time PCRs were carried out in a 7300 fast real-time PCR system (Applied Biosystems) as follows: an initial enzyme activation of 10 min at 95°C, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min. Primer specificity was confirmed by analyzing the dissociation curves of PCR amplification products. All cDNA samples to be compared for transcript levels were analyzed in triplicate in a single batch for each primer pair. The relative quantification of gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). We used *SCR1* as a reference gene for normalization. Levels were expressed relative to those found before nitrogen addition.

mRNA decay rate

This method relies on the uptake of 4-thiouracil (4-sU; Sigma) by yeast cells and its incorporation into newly synthesized mRNAs. For labeling, 7×10^9 yeast cells were nitrogen-starved for 16 h in 350 ml YNB medium devoid of nitrogen but containing 200 µM 4-sU. The day after, 2 mM uracil (to block labeling) and 20 mM NH_4 (for nitrogen repletion) were added. Cells were harvested after different times by filtration and total RNA was extracted as above. Isolation and purification of labeled mRNAs were performed as described in Zeiner *et al*. (2008). Briefly, labeled mRNAs were isolated by reversible conjugation to biotin and binding to streptavidin beads. After washing, the labeled RNA was recovered via application of the reducing agent, β-mercaptoethanol. The labeled mRNAs were then quantified by qRT-PCR as described above.

Labeled cDNA preparation, microarray hybridization, and data analysis

Retrotranscription and labeling were performed using a ChipShot direct labeling and clean-up system kit (Promega) according to the manufacturer's instructions. Microarray hybridizations were performed using a Pronto Universal Microarray kit (Corning) according to the manufacturer's instructions. Two biological replicates per condition were obtained, based on a dye swap design. Hybridization was performed on microarrays (UltraGap chips; Eurogentec) containing 5803 oligonucleotides (70-mer; Operon).

Microarrays were scanned using a GenePix pro 3 scanner (Axon Instruments). Data were processed using R (R Core Team, 2008) and the Limma package (Smyth and Speed, 2003; Smyth, 2005; Smyth *et al.*, 2005). Within- and between-array normalizations were performed respectively using the print-tip-loess and quantile methods. Differentially expressed genes were identified through a linear model approach and the Benjamini–Hochberg method was used to adjust *p* values (Benjamini and Hochberg, 1995). A gene was considered differentially expressed at a 5% level if its adjusted *p* value was lower than 0.05. The complete data set is available through the Gene Expression Omnibus under accession number GSE76778. Statistical analysis for overrepresentation of functional groups was performed using the Genecodis version 3 Web-based tool (Nogales-Cadenas *et al.*, 2009).

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REFERENCES

Airoldi EM, Miller D, Athanasiadou R, Brandt N, Abdul-Rahman F, Neymotin B, Hashimoto T, Bahmani T, Gresham D (2016). Steady-state and dynamic gene expression programs in *Saccharomyces cerevisiae* in response to variation in environmental nitrogen. Mol Biol Cell 27, 1383–1396.

Baumgartner BL, Bennett MR, Ferry M, Johnson TL, Tsimring LS, Hasty J (2011). Antagonistic gene transcripts regulate adaptation to new growth environments. Proc Natl Acad Sci USA 108, 21087–21092.

Beelman CA, Parker R (1994). Differential effects of translational inhibition in cis and in trans on the decay of the unstable yeast *MFA2* mRNA. J Biol Chem 269, 9687–9692.

Benjamini Y, Hochberg Y (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B Stat Methodol 57, 289–300.

Bennett MR, Pang WL, Ostroff NA, Baumgartner BL, Nayak S, Tsimring LS, Hasty J (2008). Metabolic gene regulation in a dynamically changing environment. Nature 454, 1119–1122.

Brengues M, Pintard L, Lapeyre B (2002). mRNA decay is rapidly induced after spore germination of *Saccharomyces cerevisiae*. J Biol Chem 277, 40505–40512.

Brice C, Sanchez I, Tesnière C, Blondin B (2014). Assessing the mechanisms responsible for differences between nitrogen requirements of *Saccharomyces cerevisiae* wine yeasts in alcoholic fermentation. Appl Environ Microbiol 80, 1330–1339.

Broach JR (2012). Nutritional control of growth and development in yeast. Genetics 192, 73–105.

Brown AJP (1989). Messenger RNA stability in yeast. Yeast 5, 239–257.

Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J Biol Chem 265, 5267–5272.

Chomczynski P, Sacchi N (1987). Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162, 156–159.

Conway MK, Grunwald D, Heideman W (2012). Glucose, nitrogen, and phosphate repletion in *Saccharomyces cerevisiae*: common transcriptional responses to different nutrient signals. G3 2, 1003–1017.

Deminoff SJ, Santangelo GM (2001). Rap1p requires Gcr1p and Gcr2p homodimers to activate ribosomal protein and glycolytic genes, respectively. Genetics 158, 133–143.

Fraenkel D (2003). The top genes: on the distance from transcript to function in yeast glycolysis. Curr Opin Microbiol 6, 198–201.

Huch S, Nissan T (2014). Interrelations between translation and general mRNA degradation in yeast. WIREs RNA 5, 747–763.

Jorgensen P, Rupes I, Sharom JR, Schneper L, Broach JR, Tyers M (2008). A dynamic transcriptional network communicates *growth potential* to *ribosome* synthesis and critical *cell size*. Genes Dev 18, 2491–2505.

Kief DR, Warner JR (1981). Coordinate control of syntheses of ribosomal ribonucleic acid and ribosomal proteins during nutritional shift-up in *Saccharomyces cerevisiae*. Mol Cell Biol 1, 1007–1015.

Klein C, Struhl K (1994). Protein kinase A mediates growth-regulated expression of yeast ribosomal protein genes by modulating RAP1 transcriptional activity. Mol Cell Biol 14, 1920–1928.

Kraft C, Deplazes A, Sohrmann M, Peter M (2008). Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3/Bre5p ubiquitin protease. Nat Cell Biol 10, 602–610.

Kraft C, Peter M, Hofmann K (2010). Selective autophagy: ubiquitin-mediated recognition and beyond. Nat Cell Biol 12, 836–841.

Lee MV, Topper SE, Hubler SL, Hose J, Wenger CD, Coon JJ, Gasch AP (2011). A dynamic model of proteome changes reveals new roles for transcript alteration in yeast. Mol Syst Biol 7, 514.

Lee P, Kim MS, Paik S-M, Choi S-H, Cho B-R, Hahn J-S (2013). Rim15-dependent activation of Hsf1 and Msn2/4 transcription factors by direct phosphorylation in *Saccharomyces cerevisiae*. FEBS Lett 587, 3648–3655.

Levy S, Ihmels J, Carmi M, Weinberger A, Friedlander G, Barkai N (2007). Strategy of transcription regulation in the budding yeast. PLoS One 2, e250.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25, 402–408.

Lochner A, Moolman JA (2006). The many faces of H89: a review. Cardiovasc Drug Rev 24, 261–274.
Luo X, Talarek N, de Virgilo C (2010). Initiation of the G0 program requires Igo1 and Igo2, which antagonize activation of decapping of specific nutrient-regulated mRNAs. RNA Biol 8, 14–17.
Martinez MJ, Roy S, Archuletta AB, Wentzell PD, Anna-Arriola SS, Rodriguez AL, Aragon AD, Quiñones GA, Allen C, Werner-Washburne M (2004). Genomic analysis of stationary-phase and exit in *Saccharomyces cerevisiae*: gene expression and identification of novel essential genes. Mol Biol Cell 15, 5295–5305.
Moore PA, Sagliocco FA, Wood RMC, Brown AJP (1991). Yeast glycolytic mRNAs are differentially regulated. Mol Cell Biol 11, 5330–5337.
Muhlrad D, Decker CJ, Parker R (1995). Turnover mechanisms of the stable yeast *PGK1* mRNA. Mol Cell Biol 15, 2145–2156.
Nogales-Cadenas R, Carmona-Saez P, Vazquez M, Vicente C, Yang X, Tirado F, Carazo JM, Pascual-Montano A (2009). GeneCodis: interpreting gene lists through enrichment analysis and integration of diverse biological information. Nucleic Acids Res 37, W317–W322.
Puig S, Askeland E, Thiele DJ (2005). Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation. Cell 120, 99–110.
R Core Team (2008). R: A Language and Environment for Statistical Computing, Vienna: R Foundation for Statistical Computing. URL: http://www.R-project.org (accessed 2017).
Rossignol T, Postaire O, Storaï J, Blondin B (2006). Analysis of the genomic response of a wine yeast to rehydration and inoculation. Appl Microbiol Biotechnol 71, 699–712.
Sheth U, Parker R (2003). Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science 300, 805–808.
Shimanuki M, Chung SY, Chikashige Y, Kawasaki Y, Uehara L, Tsutsumi C, Hatanaka M, Hiraoka Y, Nagao K, Yanagida M (2007). Two-step, extensive alterations in the transcriptome from G0 arrest to cell division in *Schizosaccharomyces pombe*. Genes Cells 12, 677–692.
Slattery MG, Heideman W (2007). Coordinated regulation of growth genes in *Saccharomyces cerevisiae*. Cell Cycle 6, 1210–1219.
Slattery MG, Liko D, Heideman W (2008). Protein kinase A, TOR, and glucose transport control the response to nutrient repletion in *Saccharomyces cerevisiae*. Eukaryot Cell 7, 358–367.
Smyth GK (2005). Limma: linear models for microarray data. In: Bioinformatics and Computational Biology Solutions Using R and Bioconductor, Statistics for Biology and Health, ed. R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, and W. Huber, New York: Springer, 397–420.
Smyth GK, Michaud J, Scott HS (2005). Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics 21, 2067–2075.
Smyth GK, Speed T (2003). Normalization of cDNA microarray data. Methods 31, 265–273.
Sweet T, Kovalak C, Coller J (2012). The DEAD-Box protein Dhh1 promotes decapping by slowing ribosome movement. PLoS Biol 10, e1001342.
Talarek N, Cameroni E, Jaquenoud M, Luo X, Bontron S, Lippman S, Devgan G, Snyder M, Broach JR, de Virgilo C (2010). Initiation of the TORC1-regulated G0 program requires Igo1/2, which license specific mRNAs to evade degradation via the 5′-3′ mRNA decay pathway. Mol Cell 38, 345–355.
Toda T, Cameron S, Sass P, Zoller M, Scott JD, McMullen B, Hurwitz M, Krebs EG, Wigler M (1987). Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae*. Mol Cell Biol 7, 1371–1377.
Unger MW, Hartwell LH (1976). Control of cell division in *Saccharomyces cerevisiae* by methionyl-tRNA. Proc Natl Acad Sci USA 73, 1664–1668.
Uttenweiler A, Mayer A (2008). Microautophagy in the yeast *Saccharomyces cerevisiae*. Methods Mol Biol 445, 245–259.
Van Nuland A, Vandormael P, Donaton M, Alenquer M, Lourenço A, Quintino E, Versele M, Thevelein JM (2006). Ammonium permease-based sensing mechanism for rapid ammonium activation of the protein kinase A pathway in yeast. Mol Microbiol 59, 1485–1505.
Wang Y, Liu CL, Storey JD, Tibshirani RJ, Herschlag D, Brown PO (2002). Precision and functional specificity in mRNA decay. Proc Natl Acad Sci USA 99, 5860–5865.
Wu J, Zhang N, Hayes A, Panoutsopoulou K, Oliver SG (2004). Global analysis of nutrient control of gene expression in *Saccharomyces cerevisiae* during growth and starvation. Proc Natl Acad Sci USA 101, 3148–3153.
Zeiner GM, Cleary MD, Fouts AE, Meiring CD, Mocarski ES, Boothroyd JC (2008). RNA analysis by biosynthetic tagging using 4-thiouracil and uracil phosphoribosyltransferase. Methods Mol Biol 419, 135–146.