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

Catherine Tesnière*, Martine Pradal, Chloé Bessière, Isabelle Sanchez, Bruno Blondin, and Frédéric Bigey
SPO, Université Montpellier, INRA, Montpellier SupAgro, 34060 Montpellier, France

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.

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 et al., 2008). 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 conservation 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...
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 replenishment (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 reported to 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 replenition, 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 strong 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 replenishment. Metabolic labeling of mRNA with [3H]uracil 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 prompt 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 sensor (Van Nuland et al., 2006). Since ammonium triggered some destabilization of PGK1 mRNAs, we examined whether ammonium sensing through Mep2 and its metabolism 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 metabolism, we used in combination a mutant and
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 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.

| Gene          | log(FC)\(^a\) | Adj. \(p\) value\(^b\) | log(FC)\(^a\) | Adj. \(p\) value\(^b\) | Enzyme name                      |
|---------------|----------------|--------------------------|----------------|--------------------------|----------------------------------|
| 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          |
| PG1           | −1.03          | 0.0007                   | −0.66          | 0.0021                   | Phosphoglucomutase               |
| 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\(_2\)O) 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.
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

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 gdh1Δ mutant, (D) water on wt yeast grown on ethanol, (E) 4 mM glutamine on wt yeast grown on ethanol, (F) 20 mM ammonium on rgt2Δ mutant, (G) 20 mM ammonium on snf3Δ mutant, and (H) 20 mM ammonium on gpr1Δ 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.
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 (Chijwiwa 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 HSPl26 (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 HSPl26 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 RM1, 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).

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 (xrn1Δ, Figure 4C). A similar result was observed for deactivating the deadenylylase complex using a ccr4 mutant (ccr4Δ; Figure 4D). Every time that the genetic background was different (BY4741 was used for xrn1Δ and ccr4Δ), 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
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 degradation 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

**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 xrn1Δ mutant (BY4741 background; inset: transcript level after addition of ammonium on BY4741 yeast), (D) 20 mM ammonium on ccr4Δ mutant (BY4741 background; inset: transcript level after addition of ammonium on BY4741 yeast), (E) water on igo1Δ mutant, (F) 20 mM ammonium on igo1Δ mutant, (G) water on igo2Δ mutant, and (H) 20 mM ammonium on igo2Δ 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.
of mRNAs in a situation of nutrient relief has been reported by Airoltdt 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; Baumgarten et al., 2011). We were able to show that PGK1 mRNA 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 3′-5′ 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). Igol1/2, 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 transcript 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 (GHD1 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 3 h. A constant cell population (7 × 10⁶ 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. Amounts (3 µg) of total RNA were separated by electrophoresis on 1.5% agarose, 2% formaldehyde denaturing gels in 1X MOPS buffer and transferred by capillarity to nylon membranes (Hybond). RNA extraction and Northern blot hybridization and quantification were done to 32P-labeled probes. PCR DNA fragments of PGK1, SCRT1, or RPL11B genes were labeled using the Megaprime DNA labeling system (Amersham). Hybridization was performed at 65°C using 2.5 × 10⁷ 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 PhosphorImager (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-ΔΔCt method (Livak and Schmittgen, 2001). We used SCR7 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 × 10⁶ 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 NH₄ 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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