Inappropriate translation inhibition and P-body formation cause cold-sensitivity in tryptophan- auxotroph yeast mutants

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1. Introduction

Adaptation to temperature downshifts is a critical event for the growth and survival of unicellular organisms. In the budding yeast Saccharomyces cerevisiae, cold influences, among others, the enzyme kinetics, increases the molecular order of membrane lipids, and stabilizes the secondary structures of RNAs [2]. This stabilization affects the transcriptional machinery, inhibits RNA degradation and reduces adversely the ribosome function, compromising the global translation [53]. Consistent with this, cold-shocked yeast cells temporally reduce protein synthesis [34], and increase the expression of transcription-related and ribosomal genes [59,61]. Nevertheless, the cold-instigated arrest of protein translation could also respond to additional mechanisms and have a protective role. Most types of stress reduce global translation whereby they prevent further protein damage, re-allocate their resources to repair processes and ensure cellular survival [34]. The physiological changes that cause the cold-mediated translational inhibition, the signaling pathways involved and its consequences in the ability of yeast cells to face with a downshift in temperature remain unclear.

In response to different adverse conditions, cells reduce protein translation through the phosphorylation of eIF2α (eukaryotic initiation factor 2α) by Gcn2, a highly conserved protein kinase. Gcn2 also controls the translation of Gcn4, a transcription factor involved in the induction of amino acid biosynthesis enzymes. Here, we studied the functional role of Gcn2 and Gcn2-regulating proteins, in controlling translation during temperature downshifts of TRP1 and trp1 yeast cells. Our results suggest that neither cold-instigated amino acid limitation nor Gcn2 are involved in the translation suppression at low temperature. However, loss of TRP1 causes increased eIF2α phosphorylation, Gcn2-dependent polysome disassembly and overactivity of Gcn4, which result in cold-sensitivity. Indeed, knock-out of GCN2 improves cold growth of trp1 cells. Likewise, mutation of several Gcn2-regulators and effectors results in cold-growth effects. Remarkably, we found that HOG1, the osmoreponsive MAPK, plays a role in the regulatory mechanism of Gcn2-eIF2α. Finally, we demonstrated that P-body formation responds to a downshift in temperature in a TRP1-dependent manner and is required for cold tolerance.

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2.2. Strains and plasmids

carried out at 15 or 12 °C for 8 days. Cold-growth experiments were conducted at 15 °C for the indicated times. The cells were spun at 3000 × g, washed with PBS and resuspended in 20 mM PBS, pH 7.4. Dcg2-GFP was observed under a confocal microscope (Zeiss). Image processing was done with Image J (http://rsb.info.nih.gov/ij/).

2.3. Preparation of protein extracts and Western blot analysis

Proteins were extracted, separated and analyzed by SDS-PAGE and Western blot as previously described [31]. The proteins tagged with 13Myc were visualized by using a mouse monoclonal antibody against human c-Myc (1:1000; cat#sc-40; Santa Cruz Biotechnology, Dallas, Texas). Anti-G6Pdh serum (1:3000; cat#8866; Cell Signaling, Danvers, MA) was used as a loading control. The phosphorylation of elf2α in response to either amino acid or glucose starvation [19] has been reported. Whether cold promotes the formation of P-bodies and whether this process is influenced by the limiting amino acid in auxotropic strains is a question that needs to be clarified.

We have investigated the functional role of the Gcn2-elf2α signaling and its effector kinases and phosphatases in the cold-induced translation regulation of TRP1 and trp1 yeast cells. Our data suggest that energy depletion is the triggering signal of the translational arrest in response to a downshift in temperature and that cold sensitivity in tryptophan biosynthesis mutant yeast cells is linked to overactivity of the Gcn2-Gcn4 regulatory module.

2. Materials and methods

2.1. Media, culture conditions, and stress sensitivity tests

Previously described standard methods were followed for media preparation [29]. Yeast cells were cultured at 30, 15 or 12 °C in YPD (1% yeast extract, 2% peptone and 2% glucose) or SCD (0.67% yeast nitrogen base without amino acids, DIFCO, plus 2% glucose) supplemented with the appropriate amino acid. Cells were transferred to the selected medium using a set of oligonucleotides (Table S2), designed to bind outside of the coding sequence. Correct gene disruption and tagging was done by diagnostic PCR [39], using a set of oligonucleotides (Table S2), designed to bind outside of the replaced gene sequence and within the marker module (data not shown).

2.2. Strains and plasmids

The S. cerevisiae strains, oligonucleotides and plasmids used in this study are listed in the supplementary material (Tables S1–S3). Tat1, Tat2 and Gap1 C-terminal tagging with 13-Myc epitope was carried out by PCR-based gene tagging using plasmid pFA6a-13Myc-His3MX6 (Table S3) as a template and appropriate target-gene specific plasmid pairs (Table S2). The TRP1 and HOG1 deletion strains were constructed by PCR-based gene replacement using the natMX4 cassette template (Table S3) and synthetic oligonucleotides (Table S2). Detection of the correct gene disruption and tagging was done by diagnostic PCR [39], using a set of oligonucleotides (Table S2), designed to bind outside of the replaced gene sequence and within the marker module (data not shown).

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Here, we have investigated the functional role of the Gcn2-elf2α signaling and its effector kinases and phosphatases in the cold-induced translation regulation of TRP1 and trp1 yeast cells. Our data suggest that energy depletion is the triggering signal of the translational arrest in response to a downshift in temperature and that cold sensitivity in tryptophan biosynthesis mutant yeast cells is linked to overactivity of the Gcn2-Gcn4 regulatory module.

2.4. ATP assay

SCD-grown overnight seed cultures of the BY4741 wild-type and trp1 mutant strain were refreshed at OD600 = 0.10 in the same medium and cultivated at 30 °C for 3 h. Aliquots were withdrawn for their immediate analysis (control), and cultures were split 1:2 and incubated at 30 or 12 °C until the OD600 reached values around 1.2. At different times during growth, 100 μl samples were analyzed for ATP levels using the CellTiter-Glo® Luminescent Assay following the manufacturer’s instructions (Promega). The ATP level in the cell suspensions was calculated after correcting for the reagent background using the signal produced by an ATP standard as reference. Values provided are expressed as nmol of ATP per OD600 and represent the mean (±SD) of triplicate assays. ATP kinetics for each strain was repeated at least twice times.

2.5. Polysomal analyses

For polysome profiling, 30 °C-SCD-grown cells (OD600 = 0.5) were incubated at 12 °C and at the indicated times, 80 ml samples were withdrawn, chilled for 5 min on ice in the presence of cycloheximide (0.1 mg/ml final concentration). Then, cell extracts were prepared, applied to 10–50% sucrose gradients, centrifuged and analyzed as described previously [24]. Each polysome gradient analysis was repeated at least twice times.

2.6. Microscopy

Cells were grown in SCD medium to mid-log phase and then shifted to 15 °C for the indicated times. The cells were spun at 3000 × g for 3 min and resuspended in 20 mM PBS, pH 7.4. Dcg2-GFP was observed under a Zeiss 510 Meta Confocal microscope with a 63 × Plan-Apochromat 1.4 NA Oil DIC objective lens (Zeiss). Image processing was done with Image J (http://rsb.info.nih.gov/ij/).
2.7. Statistical analysis

Sample averages were compared using a Student’s t-test. The samples denoted with * were significantly different with a p < 0.05.

3. Results and discussion

3.1. The tryptophan biosynthetic capacity influences polysome disassembly and eIF2α phosphorylation in cold-shocked yeast cells

Cold-shocked TRP1 yeast cells showed the typical features of translation initiation inhibition [15], including a decrease in the fraction of polysomes (Fig. 1A) and a rapid phosphorylation at Ser51 of eIF2α (Fig. 1B), the specific amino acid phosphorylated by the kinase Gcn2 [33]. We also observed that there was a relatively high level of remaining polysomes in cold-shocked cells (Fig. 1A; 2 h at 12 °C). In addition, the response was transient (Fig. 1A and B), suggesting that the global protein synthesis is not fully suppressed under the conditions tested. Indeed, previous reports have shown evidence of protein induction in cold-shocked cells of S. cerevisiae [8,41]. Compared with this, the repressive effect of a downshift in temperature on the bulk translation, as judged by polysome disassembly and eIF2α overphosphorylation, was more pronounced and persistent in the trp1 mutant strain (Fig. 1). The progressive polysome reassembly during cold-adaptation also took longer, and even after 24 h at 12 °C, the polysome profile was still altered in the tryptophan biosynthesis mutant (Fig. 1A).

3.2. Gcn2-dependent and -independent effects on polysome disassembly

The above results showed the activation of the Gcn2-eIF2α signaling pathway in response to cold, suggesting that amino acid starvation might be the primary signal to induce the translation inhibition under this condition. Amino acid uptake is strongly inhibited by cold-stress [71] and amino acid depletion upregulates Gcn2 function [33]. Thus, this regulatory mechanism might also explain the stronger effects on translational regulation caused by loss of TRP1. Tryptophan biosynthesis mutants depend exclusively on the external supply of tryptophan, and thus, cold effects on amino acid transport could be expected to reduce further amino acid intracellular levels. However, recent evidence in mammals, fission and budding yeast cells suggest that under a variety of stress conditions the initial translational inhibition is largely independent of GCN2 and eIF2α phosphorylation [34,42]. In agreement with this, we found that knock-out of GCN2 did not result in noticeable changes in the polysome profile of TRP1 yeast cells at either 30 or 12 °C (compare Figs. 1A and 2A). However, the loss of Gcn2 in the trp1 strain reduced the strong cold-instigated polysome disassembly caused by deletion of TRP1 (compare Figs. 1A and 2A; 2 h at 12 °C). Hence, our results suggest that yeast cells reduce protein synthesis in response to a downshift in temperature by pathways other than the cold-instigated Gcn2-eIF2αx, yet this mechanism plays an important role in the translational regulation of tryptophan biosynthesis mutant cells.

3.3. Cold triggers the inhibition of TORC1

Cold has been reported to cause energy depletion in mammalian cells, which results in the inhibition of mTOR activity [34]. mTOR like the yeast TORC1 (the Target of Rapamycin Complex 1), are sensitive to the energy status of the cell [32]. It has also been suggested that mTOR inhibition under stress conditions contribute to the fine-tuning of translation initiation by regulating the phosphorylating state of 43S preinitiation complex factors [37,56]. Thus, we first analyzed whether S. cerevisiae TORC1 is inhibited by cold. We followed the phosphorylation state in cold-shocked cells of the 40S ribosomal protein S6 (Rps6) at S232 and S233, a well established readout of TORC1-dependent signaling, via its direct targets Ypk1/Ypk3 [26,74]. As it is shown in Fig. 2B, the phospho-Rps6 signal began to decrease within 60 min after the transfer of yeast cells from 30 to 12 °C and almost disappeared at 120 min (Fig. 2B), suggesting that TORC1-Ypk1/Ypk3 signaling is

Fig. 1. Loss of TRP1 increases polysome disassembly and eIF2α phosphorylation after a downshift in temperature. A) Polysome profile of cold-shocked TRP1 and trp1 yeast cells of the BY4741 yeast background. SCD-cultures were incubated at 30 °C (OD600 ~0.5) and then transferred to 12 °C for the indicated times. Cell extracts were prepared and analyzed as described in Section 2. The positions in the gradient of polysomes and the ribosomal particle 80S (monosome) are indicated. The ratio of the area under the polysomal to 80S peaks is shown in brackets. B) Protein extracts from whole cells of the aforementioned strains were separated by SDS-PAGE and blots were probed with an antibody specific for phosphorylated (S51) eIF2α (P-eIF2α). The level of glucose 6-phosphate dehydrogenase (G6Pdh) was used as loading control. Spot intensities were quantified as earlier described [31]. The graph shows the relative values of P-eIF2α corrected with respect to that of G6Pdh. The highest relative signal for each strain, TRP1 and trp1, and sample analyzed was set at 100. A representative experiment out of the three is shown.
Cold triggers the inhibition of TORC1 and the Gcn2-independent polysome disassembly. A) Polysome disassembly was monitored in cold-shocked gcn2 mutant cells of the TRP1 and trp1 BY4741 yeast background. Cell extracts were prepared and analyzed as described in Fig. 1A. The positions in the gradient of polysomes and the ribosomal particle 80S are indicated. The ratio of the area under the polysomal to 80S sedimentation peaks is shown in brackets. B) Cell cultures of the indicated strains were subjected to cold shock at 12 °C for the indicated times, and total protein lysates were analyzed by Western blotting for phospho-Rps6 (P-Rps6) and total Rps6 as loading control. Relative signal levels (%) are shown. The highest relative signal for each strain was set at 100. A Western blotting for phospho-Rps6 (P-Rps6) and total Rps6 as loading control. Relative signal (%)

3.4. The turnover of tryptophan transporters is insensitive to low temperature

The finding that Gcn2 plays no major role as regulator of the translation initiation in wild-type cells during temperature downshifts, suggested that cold stress does not induces amino acid starvation. We sought to obtain further evidence of this by analyzing how low temperature could influence amino acid permeases abundance. In S. cerevisiae, the stability and sorting of the low and high affinity tryptophan permeases Tat1 and Tat2, is controlled by amino acid availability, and regulated inversely to that of the general amino acid permease Gap1 [9]. As shown in Fig. S1, myc-tagged Tat2 was clearly more abundant in 30 °C-grown trp1 mutant yeast cells than in TRP1 cells. On the contrary, the TRP1 prototroph strain displayed increased levels of Tat1 (Fig. S1). Thus, S. cerevisiae appears to regulate the abundance of low and high affinity transporters in response to its biosynthetic ability. On the other hand, cold exposure caused a downregulation of Tat2 in both TRP1 and trp1 strains that was evident 24 h after the shift of cells from 30 to 12 °C (Fig. S1). Comparing with this, a full degradation of Tat2 has been reported to occur in less than 60 min in rapamycin treated yeast cells [9]. Tat1 was also degraded at late time points, whereas Gap1 was induced in coincidence with the decrease in the level of tryptophan transporters (Fig. S1). Importantly, tryptophan auxotroph and prototroph strains showed again a similar kinetics of Tat1 and Gap1 regulation. Hence, a downshift in temperature does not seem to trigger a fast turnover of tryptophan transporters, even in the tryptophan biosynthesis mutant.

3.5. Amino acid uptake alone is not a limiting factor for growth at low temperature

We explored the effects of the overexpression of TAT2 on growth at low temperature. The study by Vicent et al. [71] demonstrated that a high-copy number expression of TAT2 is able to maintain tryptophan uptake at 10 °C to levels similar to the control strain at 28 °C. As shown in Fig. 3, excess Tat2 had no effect on the cold-growth of TRP1 yeast cells. Only in trp1 mutant cells, the overexpression of TAT2 stimulated the growth at 15 °C, yet the effect was limited and thus, growth differences between auxotrophic and prototrophic cells were still important (Fig. 3). Altogether, the results suggest that tryptophan uptake becomes a limiting factor for the cold growth of trp1 yeast cells, although this factor alone is not the main responsible for the extreme cold-sensitivity of this strain.

3.6. P-bodies formation responds to a downshift in temperature and is required for cold tolerance

The above results suggested that amino acid limitation is not the main reason why trp1 cold-stressed yeast cells show a strong growth defect. To further explore this idea, we analyzed the P-bodies formation after cold exposure. RNA processing bodies (P-bodies), which concentrate mRNA decay enzymes [5,12,36], are induced in response to certain stresses, including low glucose [58,67], but not by amino acid starvation.

![Fig. 2. Cold triggers the inhibition of TORC1 and the Gcn2-independent polysome disassembly.](image)

![Fig. 3. TAT2 overexpression effects on growth after a downshift in temperature.](image)
[37], and have been reported to play a role in cell survival and adaptation [6]. Accordingly, we examined the localization of P-bodies marker Dcp2 tagged with green fluorescent protein (GFP), in TRP1 and trp1 yeast cells exposed to low temperature (Fig. 4A). Dcp2 encodes a catalytic subunit of the Dcp1-Dcp2 mRNA decapping complex in yeast [23] and forms part of the decapping machinery that accumulates in P-bodies [13,66]. As it is shown in Fig. 4A, P-bodies increased in response to a downshift in temperature, suggesting again that amino acid starvation is not the primary signal mediating the cold-induced inhibition in translation initiation. We were unable to find significant differences in the number of P-bodies raised in cold-shocked TRP1 and trp1 yeast cells (data not shown). However, the Dcp2-GFP fluorescence pattern showed by wild-type and trp1 mutant strains differed. TRP1 cells formed large foci after 30 min at 15 °C (Fig. 4A). Instead, a more disperse cytoplasmic GFP pattern with smaller foci was observed in trp1 mutant cells (Fig. 4A). Moreover, a number of Dcp2-GFP foci were still evident during prolonged cold exposure of wild-type cells, whereas the reporter was hardly visible in the tryptophan biosynthesis mutant.

Finally, we analyzed whether impaired P-body formation might induce cold sensitivity. In E. coli, some of the main cold-shock proteins are RNA helicases and exoribonucleases that stimulate RNA degradation at low temperature through their RNA unwinding activity [53]. We examined the cold growth of TRP1 cells lacking Pat1, Ccr4 or Pop2. The protein Pat1 is a conserved core constituent of eukaryotic P-bodies that has been suggested to act as a scaffolding molecule during the assembly process [44,54]. Ccr4 and Pop2 form part of the major mRNA deadenylase complex in S. cerevisiae [69], and have been identified as enriched in yeast P-bodies [50]. In addition, ccr4 mutant cells have

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**Fig. 4. Inappropriate cold-instigated P-body formation and Gcn2 activity cause cold sensitivity.** A) P-bodies formation was analyzed by fluorescence microscopy of the marker protein Dcp2 tagged with green fluorescent protein (GFP), in TRP1 and trp1 yeast cells of the BY4741 wild-type (wt) strain exposed to low temperature. Cells were transformed with plasmid pRP1175 (Dcp2-GFP; [16]), cultured at 30 °C and then transferred to 15 °C for the indicated times. Aliquots of the cultures were withdrawn, and cells were visualized as described in Section 2. B) pat1, pop2 and ccr4 mutant cells of the wild-type (wt) BY4741 strain were pre-grown, spotted as mentioned in Fig. 3, and incubated at the indicated temperatures. C) TRP1 and trp1 derivatives of the BY4741 wild-type (wt) and gcn2 mutant strains were examined for growth in SCD at 30 and 15 °C, as mentioned in Fig. 3. In all cases, representative experiments are shown.
been reported as showing increased cold sensitivity in the D273-10B yeast background [10]. In agreement with this, BY4741 cells lacking Ccr4 displayed impaired growth at low temperature (Fig. 4B). Likewise, knock-out of POP2 and PAT1 caused strong cold sensitivity (Fig. 4B). Remarkably, growth of pat1 mutant cells was slowed down by cold-exposure at 15 °C (data not shown) and completely stopped at the temperature of 12 °C (Fig. 4B).

3.7. The activity of Gcn2 induces cold-sensitivity in trp1 yeast cells

Our study indicated that P-body assembly and disassembly is physiologically relevant for adaptation to low temperature in S. cerevisiae. We wonder whether the increased translation inhibition observed in cold-shocked trp1 yeast cells (Figs. 1A and 2A), could also contribute to their severe growth defect at low temperature. A previous study by Goossens et al. [27] had identified Gcn2 in a screening for negative factors in yeast salt stress tolerance. We found that lack of kinase activity provided by Gcn2 had no apparent effect on the cold growth of tryptophan prototroph wild-type cells (Fig. 4C). On the contrary, disruption of the Gcn2 gene stimulated the growth of the trp1 mutant at 15 °C, while no growth effects were observed at 30 °C by loss of Gcn2 (Fig. 4C). Likewise, mutation of other components of this regulatory circuit such as GCN1, GCN3 and GCN20 also resulted in improved cold growth (Supplementary material; Fig. S2). Only, the absence of Gcn4 caused a strong growth defect independently of the growth temperature tested (Fig. S2), a result also reported for salt exposed cells [27]. Hence, the tryptophan biosynthesis mutant trp1 shows Gcn2-dependent effects on translation regulation, and this effect correlates with cold sensitivity.

3.8. Overphosphorylation of eIF2α decreases the cold growth of yeast cells

We investigated whether mutations in regulators and effectors of the Gcn2-eIF2α signaling might alter the cold growth of wild-type cells. In S. cerevisiae, the GAAC pathway is regulated by different kinases (see a schematic representation in Fig. 5A), including TORC1, which inhibits Gcn2 activity in non-starved cells [19,21,22]. In addition, Snf1 acts as a negative regulator of two amino acid biosynthetic enzymes [33]. Elevated GCN1 expression, which in turn, is a negative regulator of the genes [68], which are important determinants of respiration rate, the role of the MAPK in cold tolerance might be linked to the cell’s energy status, which in turn, could affect the activity of TORC1 and Gcn2-eIF2α. More work is required to address this point and decipher the interesting relationship between Gcn2 and Hog1 in the stress response to low temperature.

3.9. Hog1 plays a role in the regulatory mechanism of Gcn2-eIF2α

Hog1, the MAPK of the High Osmolarity Glycerol (HOG) pathway [20,35,60], is required for yeast cells to adapt to low temperature [51]. On the other hand, Hog1 has been found to be physiologically relevant in modulating the translational response to NaCl in yeast cells [11]. Nevertheless, Hog1 does not appear to be involved in the initial inhibition of translation, but rather in reactivation of translation under stress [20,58,73]. Therefore, we were interested to investigate the importance of the MAPK in the translational regulation in response to a downshift in temperature. Loss of Hog1 increased the phosphorylation level of eIF2α in response to cold exposure (Fig. 6A), and impaired cold growth in either liquid (Fig. 6B) or solid (Supplementary material; Fig. S3) SCD medium. Thus, Hog1 appears to modulate directly or indirectly the phosphorylation state of eIF2α by increasing the activity of Gcn2 or downregulating eIF2α-targeting protein phosphatases. Indeed, previous work by Rodríguez-Hernández et al. [57], revealed the existence of a positive regulatory loop between Hog1 and Gcn2 protein kinases contributing to cell sensitivity to osmotic stress. However, the role of Hog1 in inhibiting eIF2α phosphorylation had no apparent effect on cold growth since the single hog1 and the double gcna2 hog1 mutant strains displayed a similar behavior at low temperature (Fig. 6B). Given that Hog1 appears to control the expression of mitochondrial pyruvate carrier genes [68], which are important determinants of respiration rate, the role of the MAPK in cold tolerance might be linked to the cell’s energy status, which in turn, could affect the activity of TORC1 and Gcn2-eIF2α. More work is required to address this point and decipher the interesting relationship between Gcn2 and Hog1 in the stress response to low temperature.

3.10. Cold sensitivity is linked to overactivity of Gcn4 and energy stress

The above results suggested a role of energy-sensitive pathways in the translational control during a cold shock. Amino acid biosynthesis mutant strains depend exclusively on energy-dependent transport, making them highly sensitive to energy stress. To analyze this possibility, we first measured ATP levels in TRP1 and trp1 yeast cultures incubated at low temperature. Aliquots of cells growing at 30 °C were transferred to 12 °C and the levels of ATP were followed at both temperatures until cultures reached an OD600 ~1.2. As can be seen in Fig. 7A, the ATP present in cells of the BY4741 TRP1 strain gradually increased as growth at 30 °C proceed, reaching a peak in coincidence with the mid-log-phase (OD600 ~0.7–0.8). The content of ATP in cells of the trp1 mutant showed a similar trend along the growth period analyzed, but values were always lower (Fig. 7A), suggesting that the tryptophan auxotrophy has an energetic cost for yeast cells. Comparing with this, cells exposed to low temperature showed a quite different profile of ATP (Fig. 7A). Except for a short period after the transfer to 12 °C, where ATP levels appeared to increase transiently, the ATP content in cold-shocked cells of the TRP1 strain were much lower than those found in control cells. For example, at OD600 ~1.0, ATP levels at 12 °C were <30% of those at 30 °C (Fig. 7A). Likewise, trp1 cells showed a continuous decrease in ATP content after their transfer to cold conditions. Furthermore, the ATP levels were again lower than those measured for the TRP1 counterpart at 12 °C (Fig. 7A).

We then examined whether increased energy wasting may explain the specific cold growth effects observed in trp1 cells. Indeed, trp1 yeast cells were more sensitive to the presence of metabolic inhibitors such as 2-DOG and sodium arsenate than the corresponding isogenic TRP1 strain (Fig. 7B). Given that the tryptophan biosynthesis mutant shows overphosphorylation of eIF2α (Fig. 1B), an energy consuming process could be the Gcn4-dependent expression of, among others, amino acid biosynthetic enzymes [33]. Elevated GCN4 expression in salt-exposed yeast cells has been suggested as the most likely reason explaining the salt-resistant phenotype of cells lacking Gcn2 [27]. Using a GCN4-lacZ reporter [47], we observed that cold exposure activated the Gcn2-dependent translational regulation of the GCN4 mRNA, and that the effect was larger (p < 0.05) in trp1 than in TRP1 yeast cells (Fig. 7C). Then, we analyzed whether the harmful effect of the loss of TRP1 on cold tolerance could be attributed to GCN4 overactivation. For this, we used two different genetic approaches. First, we investigated the cold phenotype of yeast cells lacking the eIF4E-associated protein Eap1 [18]. It has been proposed that upon TOR-inactivation, Eap1 attenuates the translation of GCN4 mRNA via a mechanism independent of eIF4E-binding [46]. Thus, deletion of EAPI
enhances GCN4 translation [46], and as it is shown in Fig. 7D, leads to cold sensitivity. In the second approach, wild-type yeast cells were transformed with plasmid p235, which contains a derepressed allele of GCN4 (uORF1-GCN4) that leads to increased transcription of Gcn4-regulated genes [28]. Transformants containing empty and wild-type Gcn4 expressing plasmids were used as control. As shown in Fig. 7E, overexpression of regulated wild-type GCN4 had no effect on the growth of the wild-type strain at either 30 or 15 °C. However, increased Gcn4 activity (Fig. 7E) caused cold sensitivity.

4. Concluding remarks

Phosphorylation of eIF2α by Gcn2 does not appear to cause effect on cold tolerance of tryptophan prototroph strains of S. cerevisiae. Neither...
the cold-instigated activation of Gcn2 explains the translation inhibition under these conditions. Instead, the cold-induced inhibition of TORC1 might account for the global downregulation of protein translation, although its role in this regulatory mechanism needs to be confirmed.

The idea that alterations in the cell’s energy status might be perceived as the primary signal downregulating translation initiation was not confirmed in our work. Indeed, we were unable to detect a sudden decrease of ATP levels in cold-shocked cells of either TRP1 or trp1 strain. Nevertheless, it is clear from our study that cold exposure causes ATP depletion and that this reduction in the cell’s energy reserves might influence also the activity of Gcn2 over the eIF2α translation factor. It is well known that protein translation is one of the most energy-demanding processes and that glucose depletion, the preferred energy-producing carbon source by yeast cells, causes a dramatic translation arrest. Our observation that Snf1/Glc7, central players in conveying energy- and nutrient-derived signals, inputs the translational machinery in cold-shocked cells indeed suggests an important role of the energetic metabolism in modulating the phosphorylation state of eIF2α and the level of translation initiation at low temperature.

Unlike prototroph strains, cells lacking TRP1 show Gcn2-dependent cold-sensitivity, inappropriate translation arrest and overactivity of the Gcn4 transcriptional factor. In addition, loss of Trp1 has an impact on the cold-induced formation of P-bodies. Both impaired P-bodies assembly/disassembly and energy-consuming Gcn4 activity appears to account for the extreme cold sensitivity phenotype of trp1 cells. Nonetheless, the mechanisms of translational regulation are very complex, and thus, additional factors could be involved. Our finding that Hog1 plays a role in the activity of the Gcn2-eIF2α, stresses this idea and adds new actors in the regulatory mechanisms of translation initiation.

**Conflict of interest**

The authors declare they have no conflicts of interest.

**Transparency document**

The Transparency document associated with this article can be found, in online version.

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Fig. 7. Cold sensitivity is linked to overactivity of Gcn4 and energy stress. A) Overnight SCD-grown cultures of TRP1 and trp1 derivatives of the wild-type (wt) BY4741 strain were refreshed in the same medium at 30°C and after 3 h, a portion of the culture was transferred to 12°C. ATP levels were measured at different times during growth using a recombinant firefly luciferase-based kit as described in Section 2. Values are expressed as nmol of ATP per unit of OD600, and represent the mean ± SD of triplicate assays. ATP kinetics for each strain was repeated at least two times. B) The same strains were examined for growth in SCD lacking or containing 2-deoxyglucose (2-DOG) or Na+-arsenate. Overnight SCD-grown cultures were adjusted and spotted as mentioned in Fig. 3. C) The mentioned strains were tested for the transcriptional activity of the GAAC pathway reporter GCN4:: lacZ. SCD-grown cells at 30°C were cold-shocked at 12°C for 3 h and processed for β-galactosidase activity. The values for the cold-shocked samples were divided by those of the 30°C-samples and represented as the relative fold change. The error bars represent the standard deviation of the mean values of three independent experiments. Statistical significance was determined by using the two-tailed Student's t-test. (*) were significantly different with a p < 0.05. D) Wild-type (wt) and lex1 mutant strains of the BY4741 yeast background were examined for growth at low temperature. E) BY4741 yeast cells harboring plasmids YCP50 (Control; URA3), p164 (GCN4) and p235 (uORF1-1-GCN4) were spotted on SCD-Ura plates and incubated at the indicated temperatures. Cells were pre-grown and spotted as mentioned in Fig. 3. A representative experiment is shown.

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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.2016.11.012.

References
