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

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ABSTRACT

In response to different adverse conditions, most eukaryotic organisms, including *Saccharomyces cerevisiae*, downregulate protein synthesis 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 have 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 osmoresponsive 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.

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 the α subunit of the eukaryotic initiation factor-2 (eIF2α) by Gcn2. The protein kinase Gcn2 is virtually present in all eukaryotes and governs a regulatory module called the General Amino Acid Control (GAAC) pathway in *S. cerevisiae* [15,33,48]. When amino acid availability is low, uncharged tRNAs ($tRNA^{deacyl}$) accumulates in the cell leading to the stimulation of the protein kinase catalytic domain of Gcn2 and subsequent phosphorylation of eIF2α at Ser51 [33]. This modification blocks the translation initiation and simultaneously favors the preferential translation of specific mRNAs, such as that coding for Gcn4 [33], the transcriptional activator of a large number of genes involved in amino acid biosynthesis [49]. Increased levels of uncharged tRNAs caused by amino acid depletion are also proposed as the primary signal regulating Gcn2 in response to a variety of stress conditions such as high salinity [75], oxidizing conditions [45,63] and weak acids stress [38]. For example, it is well documented that both high Na^+ concentrations and downward shifts in temperature, cause a strong inhibition of amino acid uptake [1,52,71]. A link between amino acid limitation and translational inhibition might also be on the basis of the strong cold-sensitivity phenotype found in several amino acid transport and biosynthesis mutants [30], a phenotype especially severe in tryptophan auxotrophic strains of *S. cerevisiae* [62]. Recently, it has been reported that cold stimulates the Gcn2-mediated

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phosphorylation of eIF2α in mammals and *S. cerevisiae* cells, although the cold-induced translational depression was found to be largely independent of this event [34]. Thus, a role of Gcn2 in controlling translation during temperature downshift remains unclear. Whether the timing and duration of the cold-instigated translation arrest differs between prototrophic and auxotrophic yeast strains, and how this influences the growth of yeast cells at low temperature needs to be established.

The cold effects on protein translation may also be regulated by energy depletion. In mammal cells, downshifts in temperature reduce mitochondrial function leading to energy depletion and concomitant activation of AMP-activated protein kinase, AMPK [34]. It is also known that low temperature exposure induces an increase in the fermentative/oxidative ratio in *S. cerevisiae* [7,65] and that glucose starvation causes a rapid and robust inhibition of translation initiation [4], which is followed by mRNA P-bodies formation [43,67]. The P-bodies (processing bodies) consist of defined mRNA-containing granules that harbor much of the mRNA decay machinery [55,64]. It has been reported that Snf1, the yeast ortholog of AMPK, the central kinase of the catabolite repression pathway [17], regulates the phosphorylation state of eIF2α in response to either amino acid or glucose starvation [19]. However, the mechanism involved in each case differs. Snf1 promotes the formation of phospho-eIF2α by activating Gcn2 in histidine starved cells. Instead, Snf1 is required to inhibit the eIF2α-phosphatases Glc7 and Sit4, when cells are shifted from glucose to galactose [19]. Thus, Snf1 could play a role in regulating the translation arrest induced by low temperature exposure, although no evidence of this function has been reported. Whether cold promotes the formation of P-bodies and whether this process is influenced by the limiting amino acid in auxotrophic strains are questions that need to be clarified.

Here, we have investigated the functional role of the Gcn2-eIF2α 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 drop out (ForMedium, England). Yeast transformants carrying the geneticin (kanMX4) and nourseothricin (natMX4) resistant module were selected on YPD agar plates containing 200 mg/l of G-418 (Sigma) or 50 mg/l of nourseothricin (clonNAT, WERNER Bioagents, Germany), respectively [25,72]. *Escherichia coli* DH5α host strain was grown in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract and 0.5% NaCl) supplemented with ampicillin (50 mg/l). All amino acids, sugars and antibiotics were filter-sterilized and added to autoclaved medium. Solid media contained 2% agar. Yeast cells were transformed by the lithium acetate method [40].

For plate phenotype experiments, cultures were diluted to OD_{600} = 0.8 and 10-fold serial dilutions spotted (3 μl) onto SCD- or YPD-agar solid media. Unless otherwise indicated, colony growth was inspected after 2–4 days of incubation at 30 °C. Cold-growth experiments were carried out at 15 or 12 °C for 8–12 days.

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).

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 eIF2α was followed by using anti-phospho-S51 antibody (1:1000; cat#3597; Cell Signaling). Rabbit anti-phospho Rps6 (1:10,000; kindly provided by T. Moustafa) and rabbit polyclonal against Rps6 (1:1000; cat#ab40820; Abcam, Cambridge, UK), were used to check the activity of TORC1. Phospho-AMPKα (Thr172) rabbit monoclonal antibody (1:1000; cat#4188; Cell Signaling) was used to follow the phosphorylation state of Snf1. Total Snf1 was revealed by using a polyclonal rabbit antibody (1:1000; kindly provided by F. Estruch). The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit (1:2000; cat#7074; Cell Signaling) or rabbit anti-mouse (1:5000, cat#P0260; Dako, Carpinteria, CA). Blots were done and images were captured as described elsewhere [31].

2.4. ATP assay

SCD-grown overnight seed cultures of the BY4741 wild-type and *trp1* mutant strain were refreshed at OD_{600} = 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 OD_{600} 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 calculating 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 OD_{600} and represent the mean (±SD) of triplicate assays. ATP kinetics for each strain was repeated at least two times.

2.5. Polysomal analyses

For polysome profiling, 30 °C-SCD-grown cells (OD_{600} = 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 two 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-eIF2α, 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 mammal 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

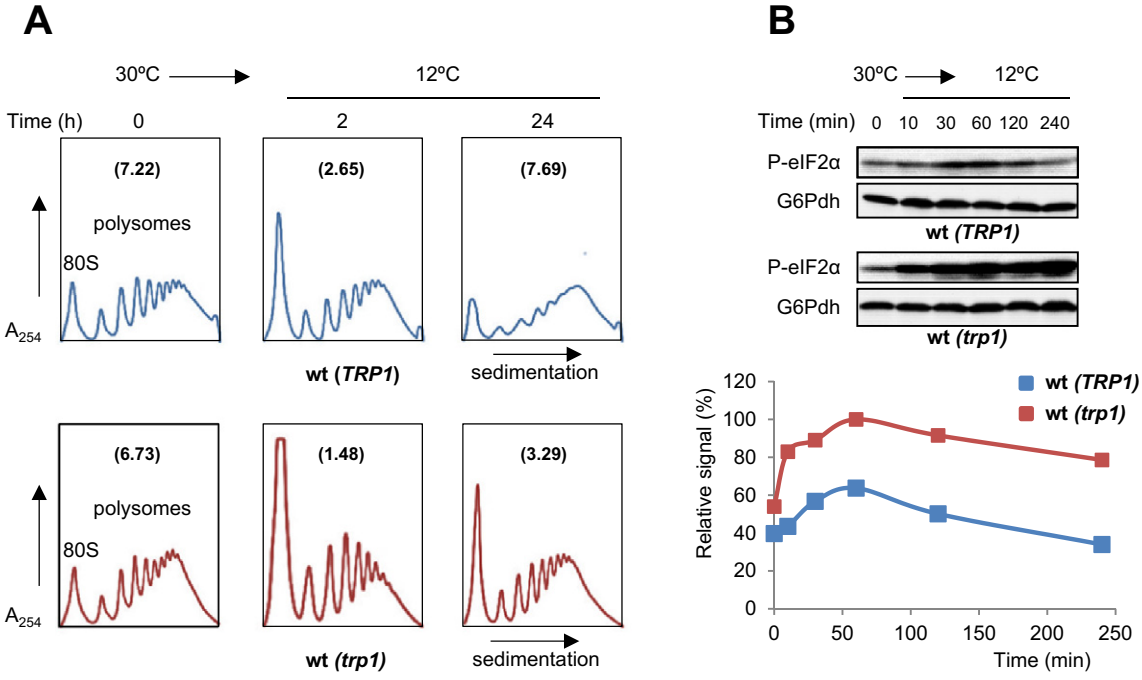

Fig. 1. Loss of *TRP1* increases polysome disassembly and eIF2α phosphorylation after a downshift in temperature. A) Polysome profile of cold-shocked *TRP1* and *trp1* yeast cells of the BY4741 yeast background. SCD-cultures were incubated at 30 °C (OD_{600} ~0.5) and then transferred to 12 °C for the 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.

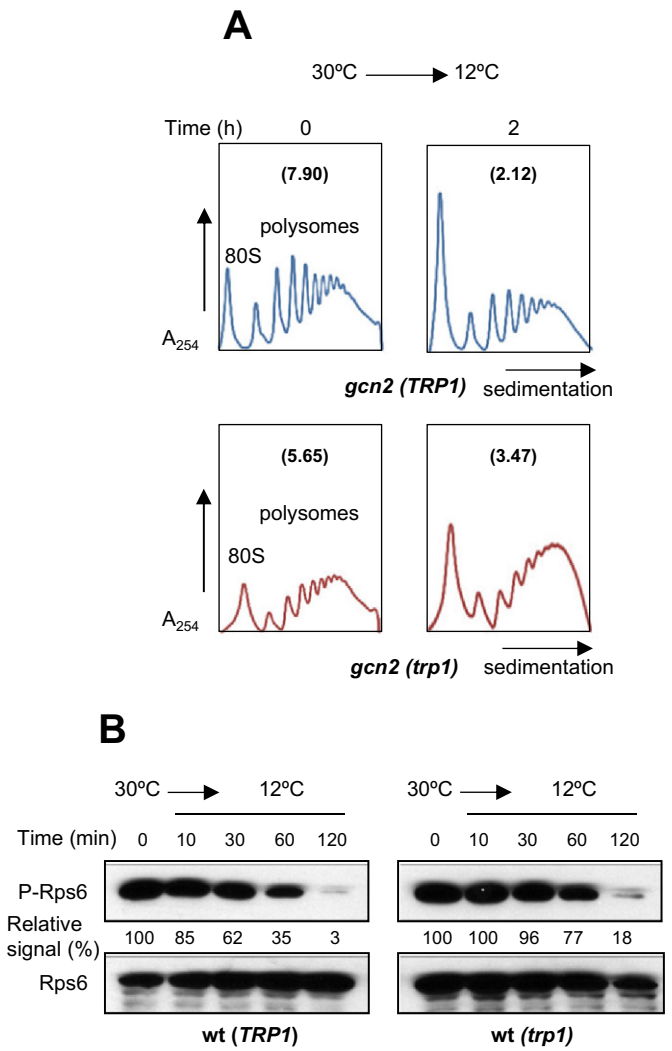

Fig. 2. Cold triggers the inhibition of TORC1 and the Gcn2-independent polysome disassembly. A) Polysome disassembly was monitored in cold-shocked *gcn2* mutant cells of the *TRP1* and *trp1* BY4741 yeast background. Cell extracts were prepared and analyzed as described in Fig. 1A. The positions in the gradient of polysomes and the ribosomal particle 80S are indicated. The ratio of the area under the polysomal to 80S 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 representative experiment out of the three is shown.

downregulated in response to low temperature. In addition, there were no major differences in the dephosphorylation kinetics of Rps6 in *TRP1* and *trp1* yeast cells (Fig. 2B). Thus, the results are consistent with the idea that TORC1 inhibition upon cold-shock may drive the Gcn2-independent translation downregulation observed in either *TRP1* or *trp1* yeast strains (Fig. 2A).

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

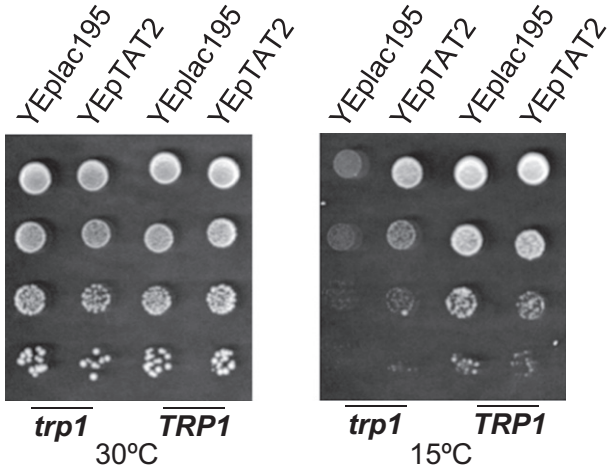

Fig. 3. *TAT2* overexpression effects on growth after a downshift in temperature. *TRP1* and *trp1* BY4741 yeast cells harboring empty (YEplac195; *URA3*) and *TAT2* (YEpTAT2) overexpressing plasmids were examined for growth at 30 and 15 °C. Exponentially growing cultures were adjusted to OD_{600} ~0.8, diluted ($1–10^{-3}$), spotted (3 μl) onto solid SCD-Ura medium, and incubated at the indicated temperature for 2 and 8 days, respectively. In all cases, representative experiments are shown.

[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

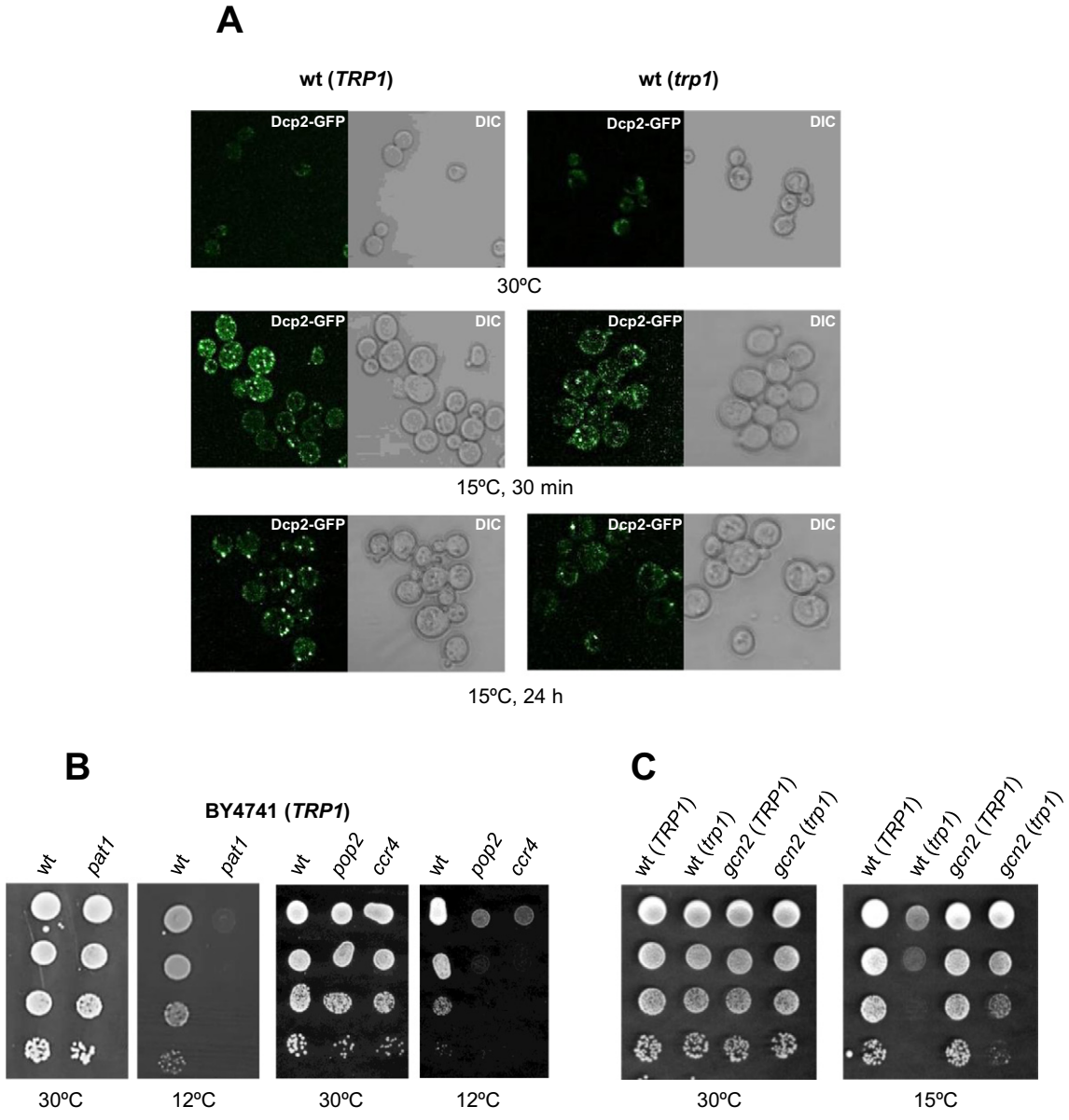

Fig. 4. Inappropriate cold-instigated P-body formation and Gcn2 activity cause cold sensitivity. A) P-bodies formation was analyzed by fluorescence microscopy of the marker protein Dcp2 tagged with green fluorescent protein (GFP), in *TRP1* and *trp1* yeast cells of the BY4741 wild-type (wt) strain exposed 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 [21,22], and the Snf1 protein kinase, a member of the AMP-activated protein kinase (AMPK) family [17]. Snf1 promotes the phosphorylation of eIF2α by stimulating the Gcn2 activity [19]. In addition, Snf1 acts as a negative regulator of two eIF2α phosphatases, Sit4, a PP2A-like enzyme [3], and Glc7, the protein phosphatase 1α, PP1α [14], which in turn, is a negative regulator of the Snf1 kinase activity [17]. As it is shown in Fig. 5B, loss of Snf1 reduced the growth of *TRP1* wild-type cells in SCD minimal medium at 30 °C. The Snf1 protein kinase is a key regulator of the transcriptional response to nutrient limitation [17]. However, a downshift in temperature resulted in a progressive recovery of *snf1* growth rate (Fig. 5B). Conversely, deletion of *SIT4* conferred cold-sensitivity (Fig. 5B). We also tested the phenotype of an allele of *GLC7* (*glc7-127*) which provides a phenotype of glucose repression insensitivity [70]. As expected, cells containing an integrated copy of this allele at the *GLC7* locus, showed increased abundance of phospho-Snf1 (Fig. 5C). In addition, *glc7-127* mutant cells displayed overphosphorylation of eIF2α (Fig. 5C), and reduced growth at low temperature in either minimal SCD or rich YPD medium (Fig. 5D). Again, our results support the idea that the aberrant activity of the Gcn2-eIF2α signaling module causes cold sensitivity.

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 Rodriguez-Hernandez 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 *gcn2 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 OD_{600} ~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 (OD_{600} ~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 OD_{600} ~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 *EAP1*

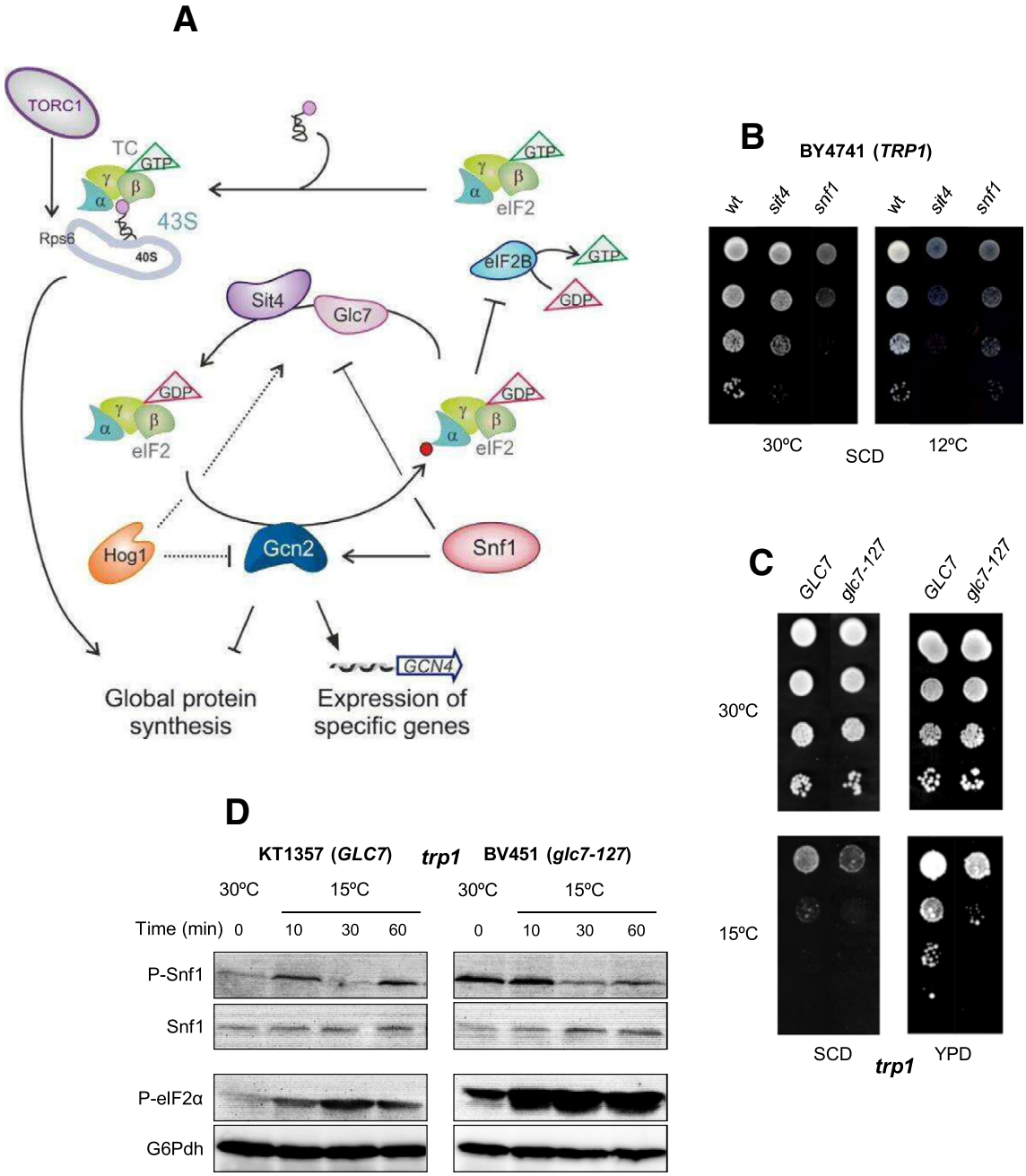

Fig. 5. The activity of different Gcn2 effectors influence cold-growth and eIF2α phosphorylation. A) Schematic representation and functional role of some regulators of the GAAC pathway in *S. cerevisiae*. When amino acid availability is low, the protein kinase Gcn2 phosphorylates the α subunit of the eukaryotic initiation factor-2 (eIF2α), a modification that blocks the translation initiation [33]. The protein kinase Snf1 regulates the phosphorylation state of eIF2α by stimulating the Gcn2 activity and inhibiting two eIF2α-phosphatases, Sit4 and Glc7 [19]. In addition the yeast TORC1 complex contributes to the fine-tuning of translation initiation by regulating the phosphorylating state of 43S preinitiation complex factors [37,56]. Available evidence from our study also indicates that the Hog1 MAPK participates in the regulation of Gcn2 activity and translation initiation. The red dot indicates a phosphate group. Positive effects are indicated by arrows, while inhibitory effects are denoted as T-bars. B) *sit4* and *snf1* mutant cells of the BY4741 strain were tested for growth at 30 and 12 °C. Overnight SCD-grown cultures were adjusted and spotted onto solid SCD medium as mentioned in Fig. 3. C) Cell cultures of the wild-type KT1357 strain (*trp1*, *GLC7*) and its corresponding mutant BV451, which contains an integrated copy of the mutant allele *glc7-127* at the *GLC7* locus, were subjected to cold shock at 15 °C for the indicated times, and total protein lysates were analyzed by Western blotting for phospho-Snf1 (P-Snf1) and total Snf1 as loading control. Blots were also probed with an antibody specific for phospho-eIF2α (P-eIF2α) and glucose 6-phosphate dehydrogenase (G6Pdh) as described in Fig. 1B. D) The same strains as in panel C were tested for growth at 30 and 15 °C. Cells were grown, diluted and spotted as described in Fig. 3. In all cases, representative experiments are shown.

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

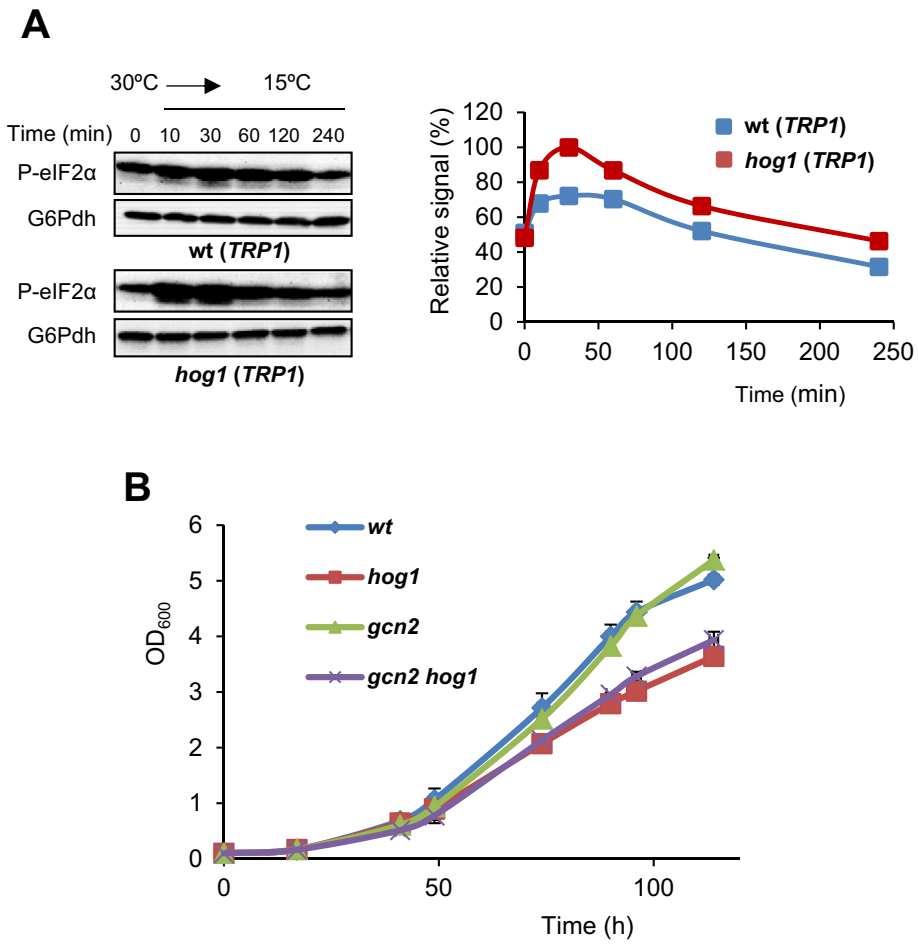

Fig. 6. Hog1 plays a functional role in the translation initial inhibition in response to a downshift in temperature. A) Protein extracts from whole cells of wild-type (wt; *TRP1*) and *hog1* mutant strains of the BY4741 yeast background were separated by SDS-PAGE and blots were probed with phospho-eIF2α (P-eIF2α) and glucose 6-phosphate dehydrogenase (G6Pdh) antibodies as described in Fig. 1B. The graph shows the relative values of P-eIF2α corrected with respect to that of G6Pdh, as described in Fig. 1B. B) Wild-type (wt), *hog1*, *gcn2* and *gcn2 hog1* mutant strains of the BY4741 yeast background were analyzed for growth in SCD liquid medium at 12 °C. The error bars represent the standard deviation of the mean values of three independent experiments.

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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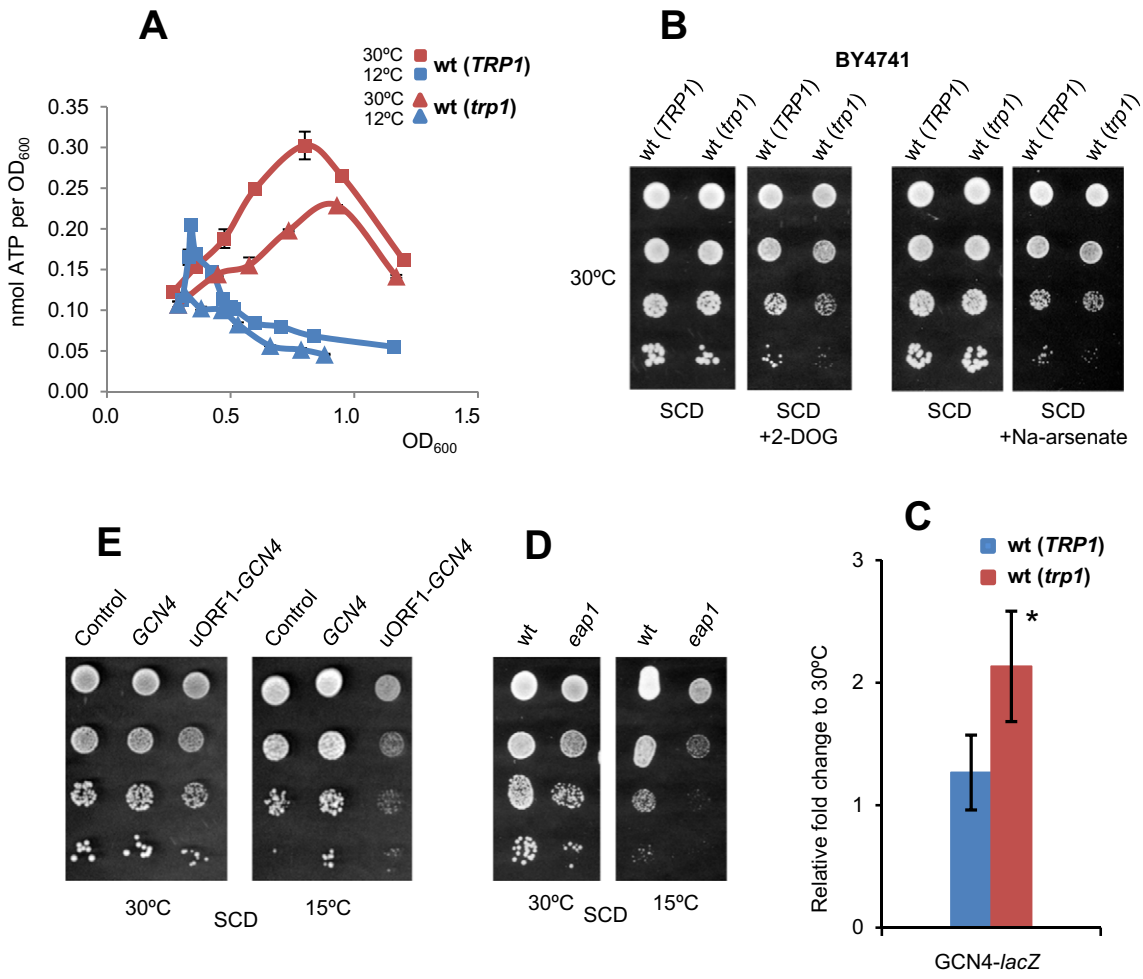

Fig. 7. Cold sensitivity is linked to overactivity of Gcn4 and energy stress. A) Overnight SCD-grown cultures of *TRP1* and *trp1* derivatives of the wild-type (wt) BY4741 strain were refreshed in the same medium at 30 °C and after 3 h, a portion of the culture was transferred to 12 °C. ATP levels were 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 OD_{600} 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 *eap1* 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-*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.

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