# A novel role for protein kinase Gcn2 in yeast tolerance to intracellular acid stress

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Intracellular pH conditions many cellular systems, but its mechanisms of regulation and perception are mostly unknown. We have identified two yeast genes important for tolerance to intracellular acidification caused by weak permeable acids. One corresponded to *LEU2* and functions by removing the dependency of the *leu2* mutant host strain on uptake of extracellular leucine. Leucine transport is inhibited by intracellular acidification, and either leucine oversupplementation or overexpression of the transporter gene *BAP2* improved acid growth. Another acid-tolerance gene is *GCN2*, encoding a protein kinase activated by uncharged tRNAs during amino acid starvation. Gcn2 phosphorylates eIF2$\alpha$ (eukaryotic initiation factor 2$\alpha$) (Sui2) at Ser[51] and this inhibits general translation, but activates that of Gcn4, a transcription factor for amino acid biosynthetic genes. Intracellular acidification activates Gcn2 probably by inhibition of aminoacyl-tRNA synthetases because we observed accumulation of uncharged tRNA$^{leu}$ without leucine depletion. Gcn2 is required for leucine transport and a *gcn2*-null mutant is sensitive to acid stress if auxotrophic for leucine. Gcn4 is required for neither leucine transport nor acid tolerance, but a S51A *sui2* mutant is acid-sensitive. This suggests that Gcn2, by phosphorylating eIF2$\alpha$, may activate translation of an unknown regulator of amino acid transporters different from Gcn4.

Key words: pH homoeostasis, signal transduction, Gcn2, *Saccharomyces cerevisiae*, amino acid transport.

## INTRODUCTION

The homoeostasis of intracellular pH is a fundamental activity of living cells [1,2] because this parameter affects most cellular functions, including growth [1,3,4] and death [5,6]. From an applied point of view, intracellular acidification is crucial for the action of weak acid food preservatives on spoilage micro-organisms [7,8]. Also, acid resistance of pathogenic bacteria is critical for survival in phagosomes and other acidic environments of animal cells [9].

Cellular responses to intracellular acidification may constitute ancestral signal transduction mechanisms and it has been proposed that acid stress generated by carboxylic acids during sugar fermentations determined the early evolution of proton pumps in primitive bacteria [10]. In yeast, the plasma membrane H$^+$-ATPase (Pma1) generates an electrochemical proton gradient that drives secondary active transport and regulates intracellular and extracellular pH [11]. This proton pump is activated by intracellular acidification [12] and its activity is crucial for tolerance to acid stress [7,8,13].

In addition to being a tightly regulated parameter with a permissive role for many cellular functions, intracellular pH may have a regulatory role as a second messenger of external stress conditions [4,14–16]. The concentration of protons in cells is in the range of those of calcium, a well-established second messenger. Protein domains specialized in calcium binding (EF hands and C2 motifs) act as calcium receptors, but protons bind to all proteins, whose histidine groups constitute a major cellular buffer [17]. Accordingly, very few proton receptors involved in cell regulation have been identified.

Yeast cells have two signalling pathways activated by intracellular acidification. Protein kinase A (Tpk1–Tpk3) is activated by cAMP produced by adenylate cyclase (Cyr1) in response to intracellular acidification via Ras1 [18]. The relevance of this pathway for tolerance to acid stress is not known. The Hog1 MAPK (mitogen-activated protein kinase) pathway is activated by several stresses (heat, osmotic and oxidative) and also by cytosolic acidification [19]. Most of the genes induced by intracellular acidification depend on the transcription factors Msn2 and Msn4, are regulated by the MAPK Hog1 and are part of the 'general stress response' [20–22]. Mutation of these genes (with the exception of *PDR12*) does not affect tolerance to the weak organic acids used to generate intracellular acidification [22]. Nevertheless, Hog1 is important because it phosphorylates and triggers endocytosis of Fps1, the porin used by acetic acid for entry into the cells [23].

As expected from the need for electrical balance during proton pumping, K$^+$ transport is also activated under conditions of intracellular acidification, and, in this case, the molecular mechanism is partially known. We have identified a pH-sensitive interaction between the protein phosphatase Ppz1 and its inhibitory subunit Hal3 [16]. At low intracellular pH, Hal3 binds and inhibits Ppz1, and this results in increased phosphorylation and activation of Trk1, a major high-affinity K$^+$ transporter inactivated by Ppz1 [24].

The screening of the yeast null-mutant collection [25,26] for sensitivity to intracellular acid stress generated by weak organic acids [22,27–29] has identified a group of yeast genes required for acid tolerance. The cellular functions represented in most studies are: tryptophan biosynthesis (*TRP1*, *TRP2*, *TRP5*), ergosterol biosynthesis (*ERG2*, *ERG3*, *ERG6*, *ERG28*), efflux of carboxylates (*PDR12*), potassium uptake (*TRK1*), leucine transport (*BAP2*), vacuolar proton pumping (subunits of vacuolar H$^+$-ATPase: *TFP1*, *VMA2*, *VMA22*), vesicle trafficking (*VPS16*, *VPS24*, *PEP5*), glycolysis (*PFK1*, *PFK2*, *TPD3*, *PDC1*) and transcription factors (*GAL11*, *WAR1*).

These results have confirmed some mechanisms of intracellular pH homoeostasis, such as the inhibition of tryptophan uptake

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by weak organic acids and the need for tryptophan biosynthesis under these conditions [30]. *PDR12* encodes an ABC (ATP-binding cassette) ATPase catalysing efflux of organic anions [31], and *WAR1* encodes a transcription factor specific for *PDR12* expression [22]. Ergosterol biosynthesis is required for plasma membrane localization of many transporters that could be important for acid tolerance [32] and vesicle trafficking is controlled by cellular pH [33]. Mutants in vacuolar $H^+$-ATPase do not grow in media with low pH values [11], and the important role of phosphofructokinase (*PFK1* and *PFK2*) has been anticipated by biochemical studies [7,34].

One problem with the knockout approach is that it cannot investigate the role of redundant or essential genes. For example, the essential plasma membrane $H^+$-ATPase (*PMA1*) is important for pH homoeostasis as demonstrated by partial loss of function [7,13,35] and the double mutant *hal4 hal5* is acid-sensitive, demonstrating the important role of the redundant protein kinases encoded by the *HAL4* and *HAL5* genes [36]. In addition, there are few coincidences between the results of different groups, and some genes important for tolerance to intracellular acid stress escaped the global screenings of the yeast null-mutant collection. These include *HAL3* (encoding an inhibitor of Ppz1 protein phosphatase [16]), *SPI1* (encoding a cell wall protein [37]), *BTN2* {encoding a v-SNARE (vacuolar soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) binding protein [38]} and *AQR1* (encoding a drug/$H^+$ antiporter [39]). Finally, in the same way that there is a 'general stress response' for the induction of a group of genes by different stresses [20–22], it is likely that mutation of many genes corresponding to basic cellular functions may result in non-specific sensitivity to many stresses (e.g. 'general stress sensitivity') without direct relevance to pH homoeostasis.

In order to identify novel regulatory components of intracellular pH homoeostasis, we have started a genetic analysis in the yeast model system by screening for genes that, upon overexpression from plasmids, increase tolerance to acid stress. This overexpression approach has the advantage of identifying rate-limiting steps in biological phenomena and it has been used successfully to dissect the mechanisms of salt tolerance [40]. Acid stress has two effects in yeast: mild acid stress (low concentrations of permeable weak organic acids), which transiently inhibits growth until cellular adaptation occurs [41], and strong acid stress (high concentrations of the acids), which induces programmed cell death [6] after release of mitochondrial cytochrome *c* to the cytosol and production of reactive oxygen species [42]. We have used acetic acid concentrations insufficient to induce significant cell death.

Our results indicate that the transport of leucine (and probably other amino acids and nutrients taken up by proton co-transport; see [30]) is an important toxicity target of intracellular acidification. The protein kinase Gcn2 has been shown previously to be activated by uncharged tRNAs and to phosphorylate eIF2$\alpha$ (eukaryotic initiation factor 2$\alpha$), promoting translation of the mRNA for the transcription factor Gcn4. We found that intracellular acidification activates Gcn2, probably by inhibition of aminoacyl-tRNA synthetases, and that it positively regulates amino acid transport by a novel mechanism independent of Gcn4, but requiring eIF2$\alpha$ phosphorylation.

## EXPERIMENTAL

### Yeast strains

Two strains of *Saccharomyces cerevisiae* were mostly used in the present work: BWG1-7A (*MAT**a** ade1-100 his4-*

*519 ura3-52 leu2-3,112*) [43] and BY4741 (*MAT**a** met15Δ0 his3Δ1 ura3Δ0 leu2Δ0*) [44]. The null mutants *gcn1*, *gcn2*, *gcn3*, *gcn4* and *gcn20* were derived from BY4741 by gene disruption with *kanMX4* [25,26]. The strain expressing from the chromosome locus a Bap2–GFP (green fluorescent protein) fusion derived from BY4741 by homologous recombination [45]. A disruption of the *GCN2* gene was made in these strains using the disruption cassette of the yeast deletant collection [25] .The diploid haploinsufficient strains deficient in different aminoacyl-tRNA synthetases were derived from BY4743 (*MAT**a**/α lys2Δ0/LYS met15Δ0/MET15 his3Δ1/his3Δ1 ura3Δ0/ura3Δ0 leu2Δ0/leu2Δ0*) by gene disruption of one of two copies of each gene with *kanMX4* [25] and they were obtained from EUROSCARF (European *Saccharomyces cerevisiae* Archive for Functional Analysis). The yeast diploid heterozygous *ade2* mutant was used as a wild-type control.

A strain expressing the S51A mutation of eIF2$\alpha$ (*SUI2* gene) was constructed as described in [46], with the following modifications. *SUI2* is an essential gene and therefore plasmid shuffling was required. The starting strain H1645 (*MAT**a** ura3-52 leu2-3,-112 trp1-Δ63 sui2Δ, p919[SUI2, URA3]*) has a null mutation of *SUI2* at the chromosome covered by a wild-type copy in a *URA3* plasmid (p919). This strain, however, is auxotrophic for tryptophan, and this may increase sensitivity to acid stress [30]. Also, the plasmids used for the shuffling (p1097 and p1098) had *LEU2* as a marker, also interfering with acid tolerance (see above). Therefore the BamHI inserts of 2.7 kb from plasmids p1097 and p1098 containing the wild-type *SUI2* gene and the *SUI2*-S51A mutant respectively were inserted at the unique BamHI site of the centromeric *TRP1* plasmid pRS414 (Stratagene), giving rise to plasmids pRS-65 (wild-type) and pRS-67 (mutant). Strain H1645 was transformed with these pRS414 plasmids containing *SUI2* or the *SUI2*-S51A mutation. The resulting transformants were plated on medium containing 0.2 % 5-FOA (5-fluoro-orotic acid) to evict the *URA3* plasmid containing *SUI2*.

### Media and assays for cell growth

The standard YPD [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose] and SD (synthetic dropout) media were used [47], buffered with 50 mM succinic acid taken to pH 4.0 with Tris base. SD medium was supplemented with the requirements of the strains. Cell growth was assayed in either liquid or solid medium. In the first case, cultures were grown overnight in YPD medium and then diluted to an attenuance at 600 nm ($D_{600}$) of 0.1 in fresh YPD medium with 0, 20, 40 and 60 mM acetic acid respectively. The acetic acid was buffered at pH 4.0 with Tris base. Growth was monitored in microtitre plates using the Bioscreen C microbiological workstation (Thermo Fisher Scientific). Half-maximal inhibitory concentrations ($IC_{50}$ values) were calculated using the SigmaPlot software ($P <$ 0.001). For assays in solid medium, overnight cultures were diluted 20–200 times and volumes of $\sim 3$ $\mu$l were dropped with a stainless steel replicator (Sigma) on plates containing 2 % Bacto-Agar (Difco). We have observed some variation in the inhibitory power of different stock solutions of acetic acid. Evaporation and some chemical degradation (mostly when pH was adjusted) may be part of the explanation.

### Screening of the overexpression library

Yeast cells (strain BWG1-7A) were transformed [48] with 50 $\mu$g of DNA from a genomic library in the multicopy plasmid YEp24 (2$\mu$ origin and *URA3* marker) [49]. Approximately 20 000

transformants were selected in 20 plates of SD medium without uracil. Transformed colonies were pooled and $\sim 10^6$ cells were distributed in ten plates with YPD medium supplemented with 30 mM acetic acid. The same amount of cells were plated on medium with 60 mM acetic acid. After 5 days, acid-resistant colonies were isolated. Plasmids were extracted and checked by re-transformation into strain BWG1-7A. Finally, one clone was isolated from the 30 mM acetic acid plates and three clones from the 60 mM plates. The first one contained a small insert of 1.9 kb (co-ordinates 431 670–433 568 of chromosome V) corresponding to a 3'-truncated version of the *GLC7* gene (*GLC7'*), very similar to the one isolated by Wek et al. [50]. The original clone was designated YEp-*GLC7'*. The other three clones contained overlapping inserts of 14.5, 15.7 and 15.8 kb with co-ordinates 81 156–95 673, 77 023–92 762 and 82 126–97 884 of chromosome III. The overlap region included the *LEU2* gene, and it was demonstrated that a multicopy plasmid with the *LEU2* gene (YEp351) [51] recapitulated the acid tolerance conferred by these three clones. This plasmid is referred to as YEp-*LEU2* hereafter.

### Plasmids

Plasmid pUN100 (centromeric, *LEU2*) [52] was used to complement the leucine auxotrophy of yeast strains. Plasmid YEp-*BAP2* was made starting from a clone of the genomic library in YEp24 [49] containing an 8.8 kb insert of chromosome II from co-ordinates 371 621 to 380 400. *BAP2* with its own promoter was amplified with primers upstream (5'-GATCAAG-ATCTCACAAAGCTTCCACCTTGCACC-3') and downstream (5'-GATCAAGATCTCGCTGGAAGGGATAGGCAAGAA-3'), digested with BglII and ligated to YEp24 digested with BamHI.

### Determination of intracellular pH

Strain BY4741 was transformed with plasmid pCB901YpHc [33] containing a pH-sensitive mutant of GFP called pHluorin. Cultures were grown on SD medium to mid-exponential phase ($D_{600}$ of 0.4–0.8), and acetic acid (40 mM, pH 4.0) or sorbic acid (0.4 mM) was added as indicated. Emission fluorescence intensity at 508 nm was recorded at excitation wavelengths of 405 and 485 nm with an LS 50B Luminescence Spectrometer (PerkinElmer). A calibration curve of the ratio of fluorescence intensity values (405 nm/485 nm) against pH was made as described in [33]. This calibration required a reduction of the concentration of the succinate buffer in the medium to 5 mM to facilitate changes of external pH.

### Determination of leucine and glutamate transport

Cultures of strain BY4741 and its mutant derivatives *gcn2*, *gcn4*, *gap1* and *bap2* were grown overnight in YPD medium to a $D_{600}$ of 4–5. Cells were harvested, washed with water and suspended at 20–25 mg of fresh weight/ml in a medium containing 2% glucose, 10 mM KCl and 50 mM succinic acid taken to pH 4.0 with Tris base. The final volume was 0.6 ml and, when indicated, acetic acid buffered at pH 4.0 was added to a final concentration of 56 mM. The cells were incubated for 6 min at 30°C before addition of L-[$\alpha$-$^{14}$C]leucine or L-[$\alpha$-$^{14}$C]glutamate (GE Healthcare) at 20 $\mu$M and 25 Ci/mol. Samples of 0.1 ml were taken at 1, 2, 3 and 4 min of incubation at 30°C, diluted with 10 ml of cold water, filtered on 2.5 cm glass fibre discs (Whatman GF/C) and washed on the filter with 10 ml of cold water. After drying, the radioactivity on the filters was determined with a liquid scintillator (Ready Safe, Beckman) and a scintillation counter (Beckman LS 9000) with efficiency greater than 90%. Controls for external non-washed

radioactivity were run without cells and amounted to less than 10% of transport values. Amino acid uptake was proportional to time in the range investigated.

### Immunoblot analysis

Strains were grown in liquid YPD medium to mid-exponential phase and, when indicated, treated with 60 mM acetic acid. For analysis of the Bap2–GFP fusion, cells were broken by vortex-mixing with glass beads in a medium containing 50 mM Tris/HCl (pH 7.6), 0.1 M KCl, 5 mM EDTA, 5 mM DTT (dithiothreitol), 20% glycerol and a cocktail of protease inhibitors (Roche). After centrifugation at 3000 *g* for 5 min at 4°C (Eppendorf 5415R), the supernatant was centrifuged further at 16 000 *g* for 30 min to obtain a membrane fraction that was suspended in Laemmli sample buffer. For analysis of eIF2$\alpha$ phosphorylation, cells were collected by centrifugation at 3000 *g* for 5 min, resuspended in 20% trichloroacetic acid and broken by vortex-mixing with glass beads. Insoluble protein extracts were pelleted by centrifugation at 3000 *g* for 5 min, washed with water and suspended in Laemmli sample buffer. In both cases, 20 $\mu$g of protein was subjected to SDS/PAGE and transferred on to nitrocellulose (Protran®, Schleicher & Schuell) filters. Uniform gel loading was confirmed by DB71 (Direct Blue 71) staining of membranes after transfer. GFP was detected using a mouse monoclonal antibody from Roche. Phosphorylated eIF2$\alpha$ was detected with an anti-phospho-eIF2$\alpha$ antibody (Ser[51]) from Cell Signaling Technology. Immunocomplexes were visualized by ECL (enhanced chemiluminescence) detection (GE Healthcare) using an HRP (horseradish peroxidase)-conjugated goat anti-(rabbit IgG) or anti-(mouse IgG) (Bio-Rad Laboratories). Representative experiments from at least three independent ones with essentially identical results are shown.

### Confocal fluorescence microscopy

The strains expressing a Bap2–GFP fusion (see above) were grown in YPD medium to mid-exponential phase ($D_{600}$ of 0.6–1.2) and samples of 2 $\mu$l were visualized with a TCS SL confocal microscope (Leica) with 40$\times$ objective, $\lambda_{ex} = 488$ nm and $\lambda_{em} = 500$–530 nm.

### RNA isolation and Northern blot analysis

Aminoacylated/non-aminoacylated tRNAs were prepared from exponential-phase yeast cells under acidic conditions (0.3 M sodium acetate, pH 4.5, and 10 mM EDTA) via glass bead lysis as described in [53]. RNAs were separated by electrophoresis on a 10% polyacrylamide (pH 4.5), 8 M urea gel. After transfer on to Hybond N membrane (GE Healthcare), RNAs were hybridized with an antisense oligonucleotide against tRNA$^{leu}$ (codon CCA; CTTGCATCTTACGATACCTGAGCTTG) terminally labelled with digoxigenin as described previously [54] and developed using a CSPD (chloro-5-substituted adamantyl-1,2-dioxetane phosphate) reagent kit (Roche).

### Determination of internal content of amino acids and of ATP

Cells were grown in YPD until reaching a $D_{600}$ of 0.4–0.8, harvested by centrifugation at 3000 *g* for 5 min and transferred into fresh YPD medium. The cells were then washed with ice-cold water and extracted by heating at 95°C for 12 min in 2% isocitrate buffer (adjusted to pH 2 with HCl). Then, 1:10 dilutions of these extractions were injected in a Biochrom 20 amino acid automatic analyser using a sodium citrate system and ninhydrin detection. This analysis was done in the Service of Protein Chemistry of the Centro de Investigaciones Biológicas, CSIC (Madrid).
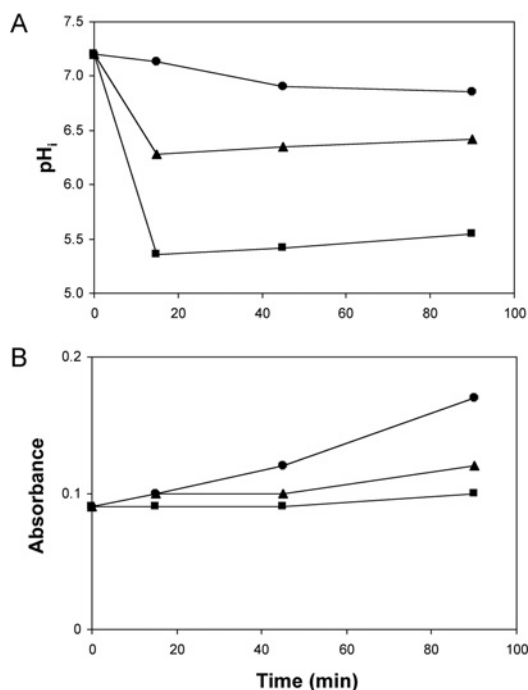
**Figure 1    Time course of intracellular pH (A) and cell growth (B) during acid stress caused by weak organic acids**

Yeast strain BY4741 transformed with plasmid pCB901YpHc containing pHluorin [33] was grown to exponential phase ($D_{600}$ of 0.08–0.09) in SD medium buffered at pH 4.0 (succinate/Tris buffer) and supplemented with leucine, methionine and histidine. At time zero either 0.4 mM sorbic acid (▲) or 40 mM acetic acid (pH 4.0 with Tris; ■) were added. A control without any addition is also shown (●). Intracellular pH was measured from the ratio of fluorescence intensities at 405 and 485 nm and a calibration curve made as described in [33].

For ATP determination, yeast cells were extracted with perchloric acid, neutralized with KOH/KHCO$_3$ and centrifuged at 3000 $g$ for 5 min, and ATP was determined in the supernatant as described in [55] using a Glomax 96 Microplate Luminometer (Promega).

## RESULTS

### Isolation of two genes important for tolerance to intracellular acid stress

pH homoeostasis, as with many biological phenomena, is better investigated under stress conditions. We have used intracellular acid stress imposed by weak permeable acids, such as acetic and sorbic acids used as food preservatives [7,8] to identify yeast genes important for pH homoeostasis. As indicated in Figure 1(A), these acids produce intracellular acidification, as measured with pHluorin, a pH-sensitive derivative of GFP [33], and delay cell growth (Figure 1B). Concentrations of acetic acid from 20 to 60 mM (buffered at pH 4.0) used in the present study inhibited the growth rate from 10 to 40% and extended the lag phase of the culture (approximately 2 h without acid stress) from 2- to 8-fold. Growth yield was also decreased by 10–40%. Cell death was less than 10% under these conditions, but becomes important at higher concentrations of the acids [6]. Measurements of ATP levels during acetic acid treatment indicate that, after 1 and 2 h of incubation in the presence of 50 mM acetic acid, growth was inhibited by 50%, but ATP levels in control and acid-treated cells were indistinguishable ($2.2 \pm 0.3$ nmol of ATP/mg of cells).

Therefore no energy stress occurred during this period of acid treatment.

We have identified two genes, *LEU2* and *GLC7′*, that, upon overexpression in a multicopy plasmid, increased growth in the presence of acetic acid (Figure 2). *LEU2* abolished the leucine requirement of the auxotrophic *leu2* yeast strain, and this suggested that uptake of leucine was inhibited by intracellular acid stress. *GLC7′* encodes a truncated protein phosphatase 1 that has a dominant-negative phenotype, reducing the activity of wild-type Glc7 and increasing the phosphorylation level of eIF2$\alpha$, the major substrate of Gcn2 [50]. This protein kinase may be involved in acid tolerance by some unknown mechanism. Both hypotheses were tested in the following experiments.

### Amino acid uptake is inhibited by intracellular acidification and is important for acid tolerance

Amino acid uptake in yeast occurs by an H$^+$-co-transport mechanism [11,56,57] and therefore intracellular acidification may result in product inhibition. Although this is clear from the thermodynamic point of view, the degree of inhibition of the initial rate may depend on the kinetic properties of H$^+$-co-transporters [58]. We have determined that, under our conditions (see the Experimental section), L-[$\alpha$-$^{14}$C]leucine uptake was inhibited by acetic acid to $14 \pm 2$% of control values. This inhibition is reversible because washing cells in fresh medium to remove the acetic acid after 3–8 h of incubation fully recovered transport rates (results not shown).

The relevance of this inhibition was demonstrated by the observation that overexpression of the gene for the major leucine transporter *BAP2* [59] (Figure 3A) and oversupplementation of growth medium with leucine improved growth in the presence of acetic acid (Figure 3B). We have observed that oversupplementation of medium with the other requirements of the strain (adenine, uracil and histidine) has no effect on acid tolerance. Therefore the improvement of acid tolerance caused by the *LEU2* gene can be explained by the suppression of the auxotrophy of the *leu2* strain because leucine transport becomes limiting for growth under intracellular acid stress. Accordingly, deletion of the *BAP2* gene results in sensitivity to sorbic [27] and acetic [28] acids. Similar results have been obtained with a tryptophan auxotrophic strain, where overexpression of the tryptophan permease gene *TAT2* improved growth under acid stress [30].

### Gcn2 is required for acid stress tolerance in leucine-auxotrophic strains and activates leucine transport

The acid tolerance conferred by *GLC7′* prompted an investigation of the role of Gcn2 in this phenotype because Glc7′ enhances the phosphorylation of eIF2$\alpha$, the substrate of this kinase [50]. Accordingly, a *gcn2*-null mutant is more sensitive to acetic acid than wild-type, and this phenotype is suppressed by transformation with the *LEU2* gene to correct for leucine auxotrophy (Figure 4A). This suggested that Gcn2 is probably required for leucine uptake. Interestingly, this novel function of Gcn2 is not mediated by its usual target Gcn4, which is dispensable for acid tolerance (Figure 4B). Gcn4 is a transcription factor whose translation is activated by Gcn2 during amino acid starvation and other stresses [60]. It induces the expression of genes related to amino acid, purine and vitamin biosynthesis, autophagy, peroxisomal and mitochondrial proteins, and amino acid transporters [61]. Clearly, the role of Gcn2 in acid tolerance does not follow this classical pathway. On the other hand, mutation
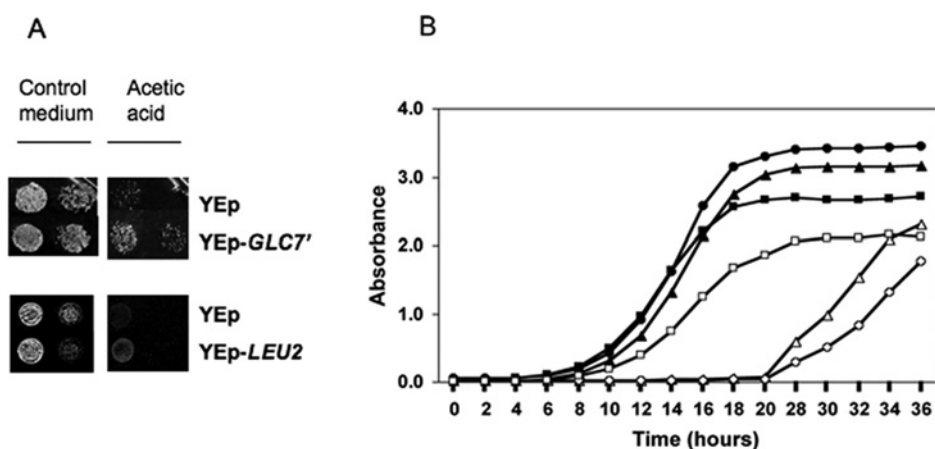
**Figure 2    Acid tolerance conferred by overexpression of *LEU2* and *GLC7′* in solid (A) and liquid (B) media**

YEp corresponds to yeast plasmid YEp24 and the yeast strain was BWG1-7A. (**A**) Overnight cultures in SD medium (supplemented with adenine, histidine and leucine) were diluted 1:20 and 1:100 and spotted on to control YPD plates and on to plates containing 40 mM acetic acid (pH 4.0). Growth was recorded after 2 days. (**B**) Experiment in liquid medium monitored with Bioscreen C. Closed symbols, control medium without acid; open symbols, medium with 40 mM acetic acid (pH 4.0); circles, cells transformed with YEp without insert; squares, cells with YEp-*LEU2*; triangles, cells with YEp-*GLC7′*.
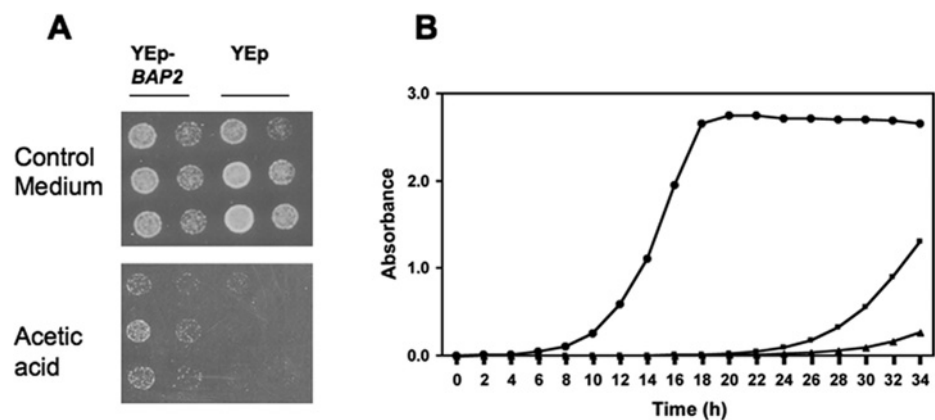


**Figure 3    Overexpression of *BAP2* (A) and oversupplementation of medium with leucine (B) improve yeast growth under intracellular acid stress**

The yeast strain was BWG1-7A. (**A**) Experiment in solid medium. Three overnight cultures in SD medium (supplemented with adenine, histidine and leucine) of cells transformed with empty plasmid YEp24 (YEp; control cells) and of cells with this plasmid containing the *BAP2* gene (YEp-*BAP2*) were diluted 1:20 and 1:100 and spotted on to control YPD plates and on to plates containing 40 mM acetic acid (pH 4.0). Growth was recorded after 2 days. (**B**) Experiment in liquid medium monitored with Bioscreen C. Overnight cultures as in (**A**) of control cells were diluted 1:200 in wells containing YPD medium (●), medium with 60 mM acetic acid (pH 4.0; ▲) and medium with the acid and 800 $\mu$g/ml leucine (■).

of *GCN1*, *GCN3* or, to a lesser extent, *GCN20*, known components required for Gcn2 activity [58], also result in acid sensitivity. In one experiment in liquid medium, growth for 40 h in the presence of 60 mM acetic acid was less than 5 % of wild-type in the case of the *gcn1* and *gcn2* mutants, 18 % of wild-type in the case of the *gcn3* mutant and 25 % of wild-type in the case of the *gcn20* mutant. In the absence of acetic acid, growth was similar to wild-type in all of the mutants.

The requirement of Gcn2 for amino acid transport was demonstrated by measuring the uptake of L-[$\alpha$-$^{14}$C]leucine and L-[$\alpha$-$^{14}$C]glutamate in different yeast strains. As indicated in Table 1, the uptake of leucine was inhibited more than 93 % by the *gcn2*-null mutation, and the uptake of glutamate was also inhibited more than 96 %. These two amino acids are transported by different systems: leucine by the hydrophobic amino acid permeases Bap2, Bap3, Agp1 and Gnp1, and glutamate by the dicarboxylic amino acid permease Dip5. In addition, both can be transported by the general amino acid permease Gap1 [59]. We have tested *bap2* and *gap1* mutants to ascertain the contribution

of these systems to leucine and glutamate transport under our experimental conditions. Lack of Gap1 had no effect on leucine uptake and, surprisingly, increased glutamate uptake. On the other hand, lack of Bap2 reduced leucine uptake to 50 % of control values and glutamate uptake to 60 % (Table 1). In any case, it seems that Gcn2 must regulate several amino acid permeases, including Bap2 and Dip5.

In the case of Bap2, we have investigated whether Gcn2 is required just for activity of the permease or for its expression at the protein level. By expressing in yeast a translational fusion of *BAP2* with the *GFP* gene (*BAP2–GFP*) and using an antibody against GFP, we have determined that the levels of fusion protein were similar in control and *gcn2* cells (Figure 5A). Also, it can be observed that acetic acid does not induce degradation of the Bap2–GFP fusion, in agreement with the reversible character of the inhibition of leucine transport by this acid (see above). Confocal fluorescence microscopy indicates that Bap2–GFP is located both in the plasma membrane and in the vacuole, without noticeable differences between control and *gcn2* cells (Figure 5B). Therefore

**Table 1  Rate of uptake of leucine and glutamate in mutants of the Gcn2 pathway and of amino acid permeases Bap2 and Gap1**

All of the strains were derived from BY4741 (wild-type). Overnight cultures were processed and the initial rate of uptake of L-[α-$^{14}$C]leucine and L-[α-$^{14}$C]glutamate measured as described in the Experimental section. Results are expressed as pmol/min per mg of cells (mean ± S.E.M. for three different experiments). n.m., not measured.

| Strain | Leucine uptake | Glutamate uptake |
|---|---|---|
| Wild-type | 53 ± 4 | 15 ± 2 |
| gcn2 | 4 ± 2 | 0.6 ± 0.2 |
| gcn4 | 54 ± 3 | n.m. |
| bap2 | 26 ± 4 | 9 ± 1 |
| gap1 | 55 ± 5 | 28 ± 5 |
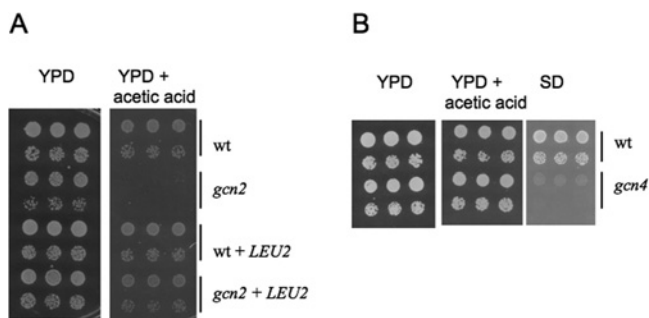


**Figure 4  Loss of function of GCN2 (A), but not of GCN4 (B), causes sensitivity to acid stress in a leu2-dependent manner**

Overnight cultures of strain BY4741 wild-type (wt), gcn2::kanMX mutant (gcn2), wild-type transformed with plasmid pUN100 containing the LEU2 gene (wt + LEU2), gcn2 transformed with plasmid pUN100 containing the LEU2 gene (gcn2 + LEU2) and gcn4::kanMX mutant (gcn4) were diluted 1:20 and 1:100 and spotted on to control YPD plates and on to plates containing 40 mM acetic acid (pH 4.0). Growth was recorded after 2 days. The far right-hand panel of (B) illustrates the poor growth of gcn4 mutant in minimal medium (SD) containing the required supplements (uracil, leucine, histidine and methionine), a typical phenotype of gcn4 mutants.

Gcn2 seems to regulate the activity of leucine transporter and not its amount or cellular location, although we cannot exclude the possibility that the Bap2–GFP fusion protein and the native Bap2 protein behave differently.

### Gcn2 is activated by intracellular acidification without depletion of intracellular amino acids

Given the facts that intracellular acidification inhibits amino acid uptake and that Gcn2 is important for tolerance to acid stress and for activation of several transporters, it was logical to test whether Gcn2 is activated by intracellular acidification. The activity of Gcn2 was followed by the phosphorylation state of its major substrate, eIF2α, analysed with antibodies specific for the phosphorylated form [62]. As indicated in Figure 6(A), the level of phosphorylation of eIF2α increases after 30–180 min of incubation with acetic acid, suggesting that Gcn2 has been activated by intracellular acidification. As indicated in Figure 6(B), this increase in eIF2α phosphorylation depends on Gcn2 and its accessory proteins Gcn1 and, to a lesser extent, Gcn20.

As Gcn2 responds to uncharged tRNAs generated by amino acid starvation [60], we have determined the intracellular pool of amino acids in control cells and in cells treated with acetic acid. The observed inhibition of leucine transport by acid stress suggested that this amino acid could be depleted under these
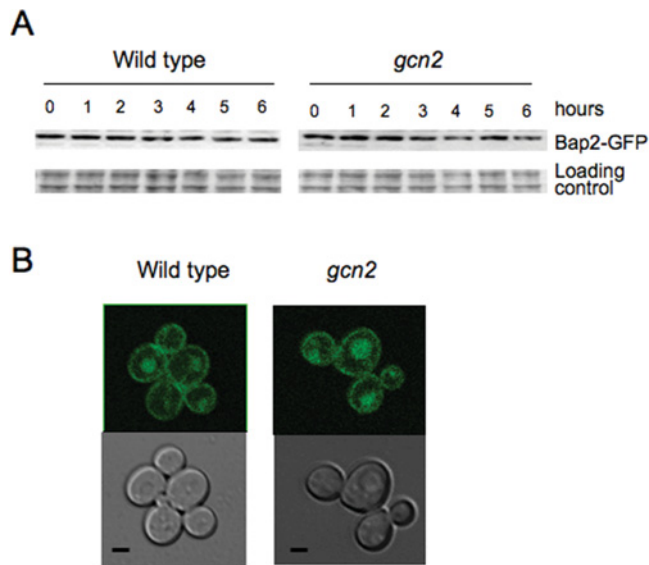


**Figure 5  The level (A) and localization (B) of a Bap2–GFP fusion is not affected by the gcn2 mutation**

The yeast strain was BY4741. (A) Membrane proteins from cells (wild-type and gcn2 mutant) expressing a Bap2–GFP fusion were analysed by Western blotting with antibodies against GFP. Times (in h) after addition of 60 mM acetic acid (pH 4.0) are shown in the upper panel and a loading control of two major proteins stained with DB71 is shown in the lower panel. (B) Cells (wild-type and gcn2 mutant) expressing a Bap2–GFP fusion were visualized by confocal fluorescence microscopy (upper panels), showing a dual location at both plasma membrane and vacuole in both strains. The lower panels show phase-contrast microscopy pictures. Scale bars, 3 μm.

circumstances. The inhibition of glutamate transport may be irrelevant because the yeast strain used is auxotrophic for leucine, but not for glutamate. It was a surprise, however, to observe that leucine levels were not decreased, but slightly increased, by acid stress (Table 2). Many other amino acids followed the same trend, suggesting that it is not related to the auxotrophy of the strain. A few amino acids (glutamic acid, aspartic acid, lysine and glycine) experienced a small decrease (10–30%) and only alanine was significantly reduced (by 60%). Therefore amino acid depletion may not be the cause of activation of Gcn2 during acid stress.

### Intracellular acidification induces the accumulation of uncharged tRNAs, and haploinsufficient mutants of some aminoacyl-tRNA synthetases are sensitive to acetic acid

The above results suggested that it was important to test whether during intracellular acidification there is accumulation of uncharged tRNAs. As a first approach, we specifically detected by hybridization the charged and uncharged forms of tRNA$^{leu}$ by Northern blot analysis. As indicated in Figure 6(C), acid stress triggers the uncharging of tRNA$^{leu}$. As leucine is not depleted (see above), this can be explained if aminoacyl-tRNA synthetases are inhibited not only by amino acid depletion [60], but also by intracellular acidification.

Although our results suggest that inhibition of amino acid transport may be a major cause of growth inhibition by acetic acid in auxotrophic strains, inhibition of aminoacyl-tRNA synthetases could also contribute to the effect of the acid. To test this possibility, we determined the half-maximal inhibitory concentration of acetic acid in haploinsufficient strains deficient in different aminoacyl-tRNA synthetases. Null mutations of yeast cytosolic synthetases are lethal, whereas null mutations
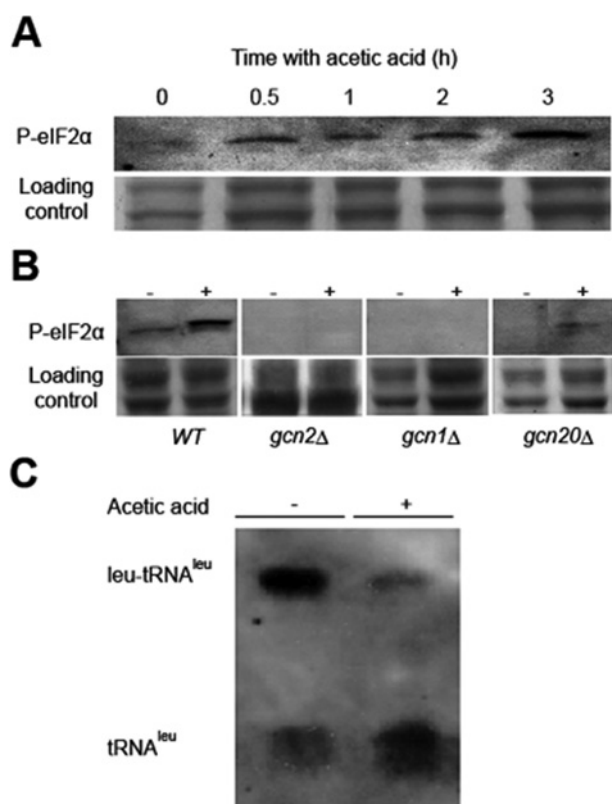
**Figure 6    Intracellular acid stress activates Gcn2 and induces accumulation of uncharged tRNA<sup>leu</sup>**

The yeast strain was BY4741. (**A**) Activation of Gcn2 by intracellular acidification was measured by phosphorylation of eIF2α at different times after addition of 60 mM acetic acid (pH 4.0) to growing cells. A loading control of two major proteins stained with DB71 is shown in the lower panel. (**B**) Acetic acid-induced phosphorylation of eIF2α depends on Gcn1, Gcn2 and Gcn20. The duration of incubation with 60 mM acetic acid was 3 h and a loading control was made as in (**A**). (**C**) Acetic acid induces the accumulation of uncharged tRNA<sup>leu</sup>. The duration of incubation with 60 mM acetic acid (pH 4.0) was 1 h and charged (leu-tRNA<sup>leu</sup>) and uncharged (tRNA<sup>leu</sup>) forms of the tRNA were detected by Northern blot analysis with specific oligonucleotide probes.

of yeast mitochondrial synthetases are viable. By using haploinsufficient diploid strains, we could compare all of the synthetase mutants with a reduction of 50 % of normal activity. The results shown in Table 3 indicate that approximately one-third of the aminoacyl-tRNA synthetase mutants are more sensitive to acetic acid than the wild-type, with reductions in the half-maximal inhibitory concentration of acetic acid from 5 to 20 %. The fact that reduction to 50 % of the activity of many aminoacyl-tRNA synthetases increases acid sensitivity supports the hypothesis that acid inhibition of these important enzymes contributes to inhibition of yeast growth.

## Phosphorylation of eIF2α at Ser⁵¹ is required for acid tolerance

Our results indicate that Gcn2, a protein kinase activated by uncharged tRNAs, is required for leucine transport and for acid tolerance of leucine-auxotrophic strains. The transcription factor Gcn4 is the classical downstream component of the Gcn2 pathway and it was therefore surprising that Gcn2, but not Gcn4, was required for acid tolerance. It was tempting to postulate that Gcn2 was acting on acid tolerance by a novel non-translational mechanism. However, the effect of mutations of aminoacyl-tRNA synthetases on acid tolerance (see above) suggested that translation could participate.

**Table 2    Effect of intracellular acidification on amino acid pools**

Results are expressed in nmol/mg of fresh weight cells (mean $\pm$ S.E.M. for three different experiments). Cultures of exponentially growing cells were divided into two portions and either supplemented with 60 mM acetic acid or not (control). After 45 min, the internal amino acids were extracted and quantified as described in the Experimental section. Tryptophan was not determined.

|  | Control | Acetic acid |
| --- | --- | --- |
| Amino acids decreased by acid stress |  |  |
| Alanine | 0.20 ± 0.01 | 0.082 ± 0.001 |
| Glycine | 0.26 ± 0.01 | 0.16 ± 0.01 |
| Glutamic acid, glutamine | 1.12 ± 0.12 | 0.76 ± 0.1 |
| Aspartic acid, asparagine | 0.23 ± 0.03 | 0.19 ± 0.017 |
| Lysine | 0.99 ± 0.15 | 0.88 ± 0.10 |
| Amino acids increased by acid stress |  |  |
| Threonine | 0.13 ± 0.04 | 0.16 ± 0.004 |
| Serine | 0.25 ± 0.05 | 0.41 ± 0.01 |
| Glutamic acid | 1.12 ± 0.12 | 0.76 ± 0.1 |
| Glycine | 0.26 ± 0.01 | 0.16 ± 0.01 |
| Cysteine | 0.015 ± 0.001 | 0.031 ± 0.006 |
| Valine | 0.046 ± 0.004 | 0.075 ± 0.006 |
| Methionine | 0.025 ± 0.002 | 0.067 ± 0.001 |
| Isoleucine | 0.056 ± 0.007 | 0.066 ± 0.0002 |
| Leucine | 0.07 ± 0.01 | 0.092 ± 0.005 |
| Tyrosine | 0.025 ± 0.004 | 0.059 ± 0.003 |
| Phenylalanine | 0.053 ± 0.007 | 0.062 ± 0.005 |
| Histidine | 0.095 ± 0.018 | 0.15 ± 0.001 |

Although Gcn4 is the only known protein whose translation is activated by Gcn2, the possibility exists that translation of other protein(s) required for amino acid transport and acid tolerance were activated by Gcn2. Many yeast genes have short uORFs (upstream open reading frames) within the 5′-untranslated region and, as in the case of *GCN4* mRNA, Gcn2 could phosphorylate eIF2α and promote bypassing of uORFs and re-initiation of the main open reading frame [60,63]. In order to test this possibility, we constructed a strain auxotrophic for leucine and with a mutation in the phosphorylation site of Sui2/eIF2α (S51A) [46]. The results of Figure 7 clearly indicate that this strain is very sensitive to both 3-aminotriazol (an inhibitor of histidine biosynthesis [46,60]) and acetic acid. Therefore the positive effect of Gcn2 on acid tolerance does not require Gcn4, but still operates at the translational level via phosphorylation of eIF2α. As expected, the sensitivity of the Sui2/eIF2α (S51A) mutant to acetic acid is alleviated by oversupplementation of medium with leucine (1 mg/ml final concentration; results not shown).

## DISCUSSION

Gcn2 is a protein kinase conserved in all eukaryotes that forms part of a stress-responsive pathway activated by amino acid starvation, purine starvation, glucose limitation, high salinity, DNA damage and, in the case of plants, wounding [64–66]. The mechanism of this pathway involves a unique translational control, where Gcn2 is activated by uncharged tRNAs and phosphorylates eIF2α. This causes some inhibition of general protein synthesis, but activation of translation of the bZIP (basic leucine zipper) transcription factors Gcn4 in yeast and ATF4 (activating transcription factor 4) in mammals. The mRNAs of these factors contain small open reading frames upstream of their coding regions and translate very inefficiently under normal conditions [60,65].

eIF2α and Gcn4 are the only known downstream components of Gcn2 signalling in yeast [60,61] and it has therefore been a surprise to find that Gcn2 is activated by intracellular acid stress and is required for tolerance to this stress independently of Gcn4

**Table 3   Half-maximal inhibitory concentrations (IC$_{50}$) of acetic acid in haploinsufficient strains deficient in different aminoacyl-tRNA synthetases**

Values are means ± S.E.M. (in mM acetic acid). Diploid strains are named from the gene which has one of the two copies deleted. Values marked with an asterisk (*) show a statistically significant ($P < 0.05$) reduction compared with the wild-type.

| Strain | IC$_{50}$ |
| --- | --- |
| Wild-type | 74.0 ± 0.1 |
| DPS1 | 77.9 ± 0.1 |
| CDC60 | 75.7 ± 0.1 |
| MSM1 | 75.3 ± 0.1 |
| MSD1 | 74.9 ± 0.1 |
| MSR1 | 74.7 ± 0.1 |
| WRS1 | 74.5 ± 0.1 |
| MSE1 | 74.4 ± 0.1 |
| MST1 | 74.4 ± 0.1 |
| NAM2 | 74.4 ± 0.1 |
| MSK1 | 74.3 ± 0.1 |
| THS1 | 74.3 ± 0.1 |
| ILS1 | 73.9 ± 0.1 |
| MSY1 | 73.9 ± 0.1 |
| YNL247W | 73.9 ± 0.1 |
| MES1 | 73.8 ± 0.1 |
| KRS1 | 73.8 ± 0.1 |
| MSW1 | 73.5 ± 0.1 |
| TYS1 | 73.5 ± 0.1 |
| YHR020W | 73.0 ± 5.1 |
| YER087W | 72.9 ± 0.1* |
| GRS1 | 72.7 ± 1.6* |
| SES1 | 71.8 ± 2.9 |
| ISM1 | 71.3 ± 5.2 |
| GLN4 | 70.8 ± 7.3 |
| GUS1 | 70.2 ± 2.3* |
| VAS1 | 69.8 ± 2.9* |
| HTS1 | 67.4 ± 0.3* |
| FRS2 | 66.9 ± 3.1* |
| ALA1 | 66.2 ± 0.2* |
| DED81 | 65.8 ± 2.1* |
| MSF1 | 64.3 ± 3.5* |
| DIA4 | 64.1 ± 0.1* |
| FRS1 | 62.8 ± 0.1* |
| YDR341C | 62.8 ± 1.5* |
| SLM5 | 58.0 ± 5.5* |
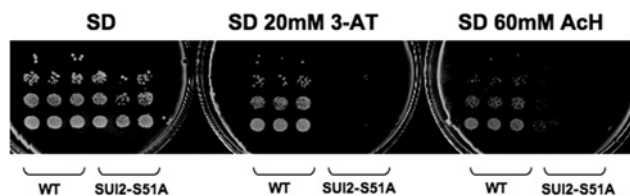


**Figure 7   The phosphorylated serine residue (Ser$^{51}$) of eIF2$\alpha$ is required for acid tolerance**

Derivatives of strain H1645 [46] expressing either wild-type *SUI2* (WT, the gene for eIF2$\alpha$ in yeast) or the S51A mutation (SUI2-S51A) were grown overnight in SD medium buffered at pH 4.0 and supplemented with uracil and leucine. After dilution, 1:10, 1:100, 1:1000 and 1:10 000, drops were spotted on to plates of the same medium and containing 20 mM 3-aminotriazol (3-AT) and 60 mM acetic acid (AcH) as indicated. Growth was recorded after 2 days.

(Figure 4). In addition, intracellular acid stress does not activate translation of Gcn4 as measured by the expression of a *GCN4-lacZ* fusion [60,67] (G. Hueso, J.R. Murguía and R. Serrano, unpublished work). Phosphorylation of eIF2$\alpha$ is required for acid tolerance (Figure 7) and therefore the role of the Gcn2/eIF2$\alpha$ pathway in acid stress may disclose a novel target of this important translational stress response.

A good candidate would be an activator system for leucine transport. The need for Gcn2 for tolerance to acid stress disappears when the yeast strain is transformed with the *LEU2* gene to restore leucine biosynthesis in the *leu2* mutant strain (Figure 4). This can be explained by the facts that leucine transport is strongly inhibited by intracellular acidification (to 14 % of non-stress values) and by the *gcn2* mutation (to 4 % of wild-type values). Combining acid stress and *gcn2* mutation, leucine transport is almost undetectable (0.4 % of non-stressed wild-type values). Overexpression of the leucine transporter Bap2 improves acid tolerance (Figure 3) and it is therefore plausible that Gcn2, through phosphorylation of eIF2$\alpha$, but without the need for Gcn4, activates transporters of leucine (Bap2, Bap3, Agp1 and Gnp1) and of other amino acids such as glutamate (Dip5) (Table 1). In the case of Bap2, the effect of Gcn2 and of acid stress is on the activity, not on the level of the transporter. In addition to the evidence obtained with a *BAP2–GFP* fusion (Figure 5), the expression of a *BAP2–lacZ* fusion is independent of both Gcn2 and acid stress (C. Montesinos and R. Serrano, unpublished work). Therefore a plausible mechanism is that the Gcn2/eIF2$\alpha$ pathway increases translation of a protein that activates several amino acid transporters.

Many yeast transporters are regulated by phosphorylation and subsequent ubiquitylation by the ubiquitin ligase Rsp5, followed by endocytosis and vacuolar targeting [68]. In the cases of the general amino acid permease Gap1 [69] and the leucine permease Bap2 [70], the protein kinase involved is Npr1. However, this mechanism modulates the level of transporters in the plasma membrane [68], whereas the regulation of leucine transport we have observed is at the activity level. Experiments are underway to investigate whether the activation of Bap2 by Gcn2 involves binding of the permease to some regulatory protein whose translation depends on the Gcn2/eIF2$\alpha$ pathway. Npr1 is still a candidate because it may phosphorylate and activate the transporters during acid stress, while endocytosis may be inhibited at low intracellular pH, stabilizing the transporters. Npr1 gives the name to a subfamily of yeast protein kinases [71] that seems to be dedicated to the regulation of plasma membrane transporters and that includes Ptk2 (regulator of plasma membrane H$^{+}$-ATPase [72]) and Hal4 and Hal5 (regulators of Trk1 K$^{+}$ transporter [37]). Within the small group of yeast genes containing putative regulatory short open reading frames in the mRNA 5′-untranslated region [63,73], none encodes amino acid permeases or putative regulators of them, such as Npr1. Some protein kinases putatively regulated by the Gcn2/eIF2$\alpha$ pathway are those encoded by the *BCK2*, *PKH2*, *CKA2* and *MKK1* genes and deserve further investigation.

The physiological significance of the activation of Gcn2 by intracellular acid stress can be explained by the inhibition of amino acid transport at low cellular pH and by the activation of amino acid transport by Gcn2. Amino acid transport in yeast operates by a H$^{+}$-symport mechanism [11] and intracellular acidification decreases the driving force of the electrochemical proton gradient and, as shown in the present study, inhibits amino acid uptake. The activation of Gcn2 would partially counteract this inhibition and in the case of *leu2* strains, it would improve leucine uptake and cell growth under acid stress.

The activation of Gcn2 by intracellular acid stress correlates with accumulation of uncharged tRNA$^{leu}$ (Figure 6). One obvious mechanism would start with inhibition of leucine uptake followed by cellular leucine starvation, inhibition of leucyl-tRNA synthetase and binding of uncharged tRNA$^{leu}$ to the HisRS-like regulatory domain of Gcn2 [60]. However, determination of the intracellular amino acid pools (Table 2) indicates that there is no leucine starvation and that the concentration of leucine, as that of most amino acids, increases upon acid stress. One possible

explanation is that uncharged tRNAs accumulate not because of amino acid starvation, but as a consequence of inhibition of aminoacyl-tRNA synthetases at low intracellular pH. The activity of these enzymes has a pH optimum at 7.5–8.0, with a dramatic fall at more acidic pH values. For example, at pH 6, the rate of reaction is only 8–9% of the optimum [74,75]. The importance of these essential enzymes as targets of intracellular acid stress is supported by our observation that reduction to 50% of the activity of many aminoacyl-tRNA synthetases increases acid sensitivity of the cells (Table 3).

The inhibition of aminoacyl-tRNA synthetases under stress conditions such as intracellular acidification may explain activation of Gcn2 by uncharged tRNAs without amino acid starvation. For example, in the case of NaCl stress [62,76], some aminoacyl-tRNA synthetases could be inhibited by high levels of intracellular $Na^+$, as observed for several intracellular enzymes [40]. Another possibility is that stress conditions cause intracellular acidification, as demonstrated in the case of heat shock [77], but not investigated during NaCl stress. The inhibition of aminoacyl-tRNA synthetases and activation of Gcn2 by intracellular acidification and other stresses may be of general relevance to plants and animals because the Gcn2/eIF2$\alpha$ pathway is conserved in eukaryotes [64–66].

In conclusion, we propose a model for pH homoeostasis in yeast where intracellular acid stress inhibits both amino acid transport and aminoacyl-tRNA synthetases. Uncharged tRNAs activate Gcn2, and eIF2$\alpha$ is phosphorylated and promotes translation of an unknown protein that activates amino acid transport.

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