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Novel function of transcription factor Uga3 as an activator of branched-chain amino acid permease *BAP2* gene expression

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Abstract

Gene regulation in yeast occurs at the transcription level, i.e. the basal level of expression is very low and increased transcription requires gene-specific transcription factors allowing the recruitment of basal transcriptional machinery. *Saccharomyces cerevisiae BAP2* gene encodes the permease responsible for most uptake of leucine, valine and isoleucine, amino acids that this yeast can use as nitrogen sources. Moreover, *BAP2* expression is known to be induced by the presence of amino acids such as leucine. In this context, the results presented in this paper show that *BAP2* is an inducible gene in the presence of nitrogen-non-preferred source proline but exhibits high constitutive non-inducible expression in nitrogen-preferred source ammonium. *BAP2* expression is regulated by the SPS sensor system and transcription factors Leu3, Gcn4 and Dal81. This can be achieved or not through a direct binding to the promoter depending on the quality of the nitrogen source. We further demonstrate here that an interaction occurs *in vivo* between Uga3 – the transcriptional activator responsible for γ-aminobutyric acid (GABA)-dependent induction of the GABA genes – and the regulatory region of the *BAP2* gene, which leads to an increase in *BAP2* transcription.

INTRODUCTION

Saccharomyces cerevisiae can use branched-chain amino acids as nitrogen sources whose transport from the extracellular medium is mainly mediated by permeases Gap1, Bap2, Bap3 and Agp1 [1]. The branched-chain amino acid permease Bap2 contains 12 transmembrane domains, has a molecular weight of 68 kDa and bears 68% homology to Gap1, a general amino acid permease [2]. Its expression depends on Ssy1, Ptr3 and Ssy5, the proteins which constitute the SPS amino acid sensor [3] and transcription factors Stp1 and Stp2, which act downstream in the SPS pathway [4, 5]. In response to amino acids, these factors are activated by endoproteolytic removal of their N-terminal domains [6]. It has been shown that Stp1 and Stp2 transcription factors can bind *in vivo* and *in vitro* between bases −590 and −526 with respect to the ATG start codon of *BAP2* and that this region contains a PuCGGC-N3-PuCGGC element similar to the UAS_{AA} element identified in *BAP3* (GCCGPy-N4-PuCGGC) [4, 7]. A binding consensus site for transcription factor Leu3, 5′-CGGAACCGG-3′, located between bases −385 and −377 with respect to the ATG start codon, has also been reported in the regulatory region of *BAP2* [1, 4], together with a key role of Leu3 in producing the highest levels of *BAP2* induction [4]. Putative binding consensus sites for transcription factor Gcn4 have also been found in the regulatory region of *BAP2*, although these sites do not seem to be functional according to several authors [1, 4]. The expression of *BAP2* also depends on the presence of pleiotropic transcription factor Dal81 [8, 9].

BAP2 regulation by nitrogen source quality has rendered controversial results and remains unclear. A significant induction of *BAP2* expression by 0.23 mM leucine was detected in cells grown on ammonium [1, 4], which may indicate that permease Bap2 is not subject to Nitrogen Catabolite Repression (NCR); Bap2 has been postulated as the permease responsible for the incorporation of branched-chain amino acids present in the extracellular medium when a preferred source of nitrogen is available [1, 4, 7, 10]. However, Forsberg *et al.* did not detect any *BAP2* mRNA in cells grown on ammonium as a nitrogen source either with or without 0.15 mM leucine [11]. On the other hand, when cells were grown on proline or urea, two non-preferred nitrogen sources, Bernard and Andre detected *BAP2* induction using 5 mM leucine [8], while Didion *et al.* failed to detect induction using 0.23 mM leucine [1].

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Abbreviations: GABA, gamma-aminobutyric acid; NCR, Nitrogen Catabolite Repression.

One supplementary figure is available with the online version of this article.

In this report, we analysed the transcriptional regulation of *BAP2* in response to extracellular leucine in the presence of both a preferred and a non-preferred nitrogen source. Our results show that *BAP2* expression was induced by leucine in the non-preferred nitrogen source proline, and that *BAP2* expression was constitutive in the preferred nitrogen source ammonium, with high values unaltered by the addition of leucine. We thus conclude that the transcription factors involved in *BAP2* regulation affect *BAP2* expression to different extents depending on the quality of the nitrogen source. We also demonstrate for the first time that Uga3, until now known as a transcriptional activator responsible for the induction of γ-aminobutyric acid (GABA) genes, is one of the main positive regulators of *BAP2* transcription.

METHODS

Strains and media

The *Saccharomyces cerevisiae* strains used in this study are isogenic to the wild-type Σ1278b and are listed in Table 1.

Cells were grown on minimal medium containing 0.17% Difco yeast nitrogen base (yeast nitrogen base without amino acids and ammonium sulfate) with 2% glucose as a carbon source and 10 mM proline or ammonium sulfate as a nitrogen source. The final concentration of leucine added to induce *BAP2* expression was 1.3 mM.

All yeast transformations were carried out using the lithium method [12].

Table 1. Strains used in this work

Strain	Genotype	Source or reference
Σ1278b	*Matα*	[38]
23344c	*Matα ura3*	[39]
KW018	*Matα ura3 stp1Δ*	[40]
KW021	*Matα ura3 stp2Δ*	[40]
KW022	*Matα ura3 stp1Δ stp2Δ*	[40]
30995b	*Matα ura3 ssy1Δ::kanMX2*	[41]
26790a	*Matα ura3 uga3Δ*	[37]
SBCY01	*Matα ura3 leu3Δ::kanMX4*	[23]
SBCY03	*Matα ura3 gcn4Δ::kanMX4*	[23]
SBCY17	*Matα ura3 dal81Δ::natMX4*	[23]
SBCY02	*Matα ura3 LEU3-3HA-kanMX6*	[23]
SBCY13	*Matα ura3 6HA-UGA3*	[23]
SBCY20	*Matα ura3 dal81Δ::natMX4 leu3Δ::kanMX4*	[23]
DEBY01	*Matα ura3 STP1-3HA-KanMX6*	[9]

Plasmids

All procedures for manipulating DNA were standard ones [13].

To construct the Yep357-*BAP2-lacZ* plasmid, the 5′-regulatory region and part of the coding regions of the *BAP2* gene (−800 to +21, with respect to the ATG initiation codon) were fused in-frame to the *lacZ* gene lacking its first seven codons, in the plasmid YEP357 [14]. DNA fragments were generated by PCR amplification using Σ1278b genomic DNA as template. Primers used in this construction were F-Eco-BAP2 (CGCGGAATTCAGCATTAATGCAAGTCGAGAA) and R-Bam-BAP2 (CGCGGATCCTCCAAAATCTTCTGA AGATAG). All fusion plasmids were verified by DNA sequence analysis. *Escherichia coli* DH5α was used to amplify and maintain the plasmids. pMB10 (*13xMYC-STP1-6xHA* in pRS316) expresses Stp1 flanked by 13 cMyc and 6 HA tags at the amino and carboxyl termini, respectively [15].

β-galactosidase activity assay

Cells grown on minimal medium up to an optical density at 600 nm of 0.5–0.9 were harvested and transferred to fresh medium with or without 1.3 mM leucine. After 1 h incubation, an aliquot (10 ml) of each culture was collected by centrifugation and resuspended in buffer Z [16]. *β*-galactosidase activity was expressed as Miller units [16]. Results are shown as the mean of triplicates within a representative assay. At least duplicate assays were performed for each of two independent transformants. The deviation of these values from the mean was less than 15%.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) experiments were performed according to Cardillo *et al.* [17]. Cells (a 100 ml culture) were grown to an optical density at 600 nm (OD_{600}) of 0.8 and, after different treatments, were fixed for 20 min at room temperature in the presence of 1% formaldehyde. Glycine was then added to give a final concentration of 125 mM and incubated for 5 min. Cells were harvested, washed with ice-cold 125 mM Tris-buffered saline (TBS)-glycine and ice-cold TBS and resuspended in 0.4 ml of FA lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and 2 mM phenylmethylsulfonyl fluoride). An equal volume of glass beads (0.5 mm in diameter; Sigma) was added, and the cells were disrupted by vortexing them for 40 min at 4 °C (4×10 min with intervals on ice). The lysates were separated from the glass beads, and the chromatin was then pelleted by centrifugation (17000 ***g*** for 30 min) and resuspended in 0.4 ml of fresh FA lysis buffer. Samples were sonicated to obtain DNA fragments with an average size of 500 bp (Branson Sonifier; 3×10 s at 15% amplitude) and clarified by centrifugation at 17000 ***g*** for 30 min. Protein content was measured using the Bradford assay, and 1 mg of protein was used for each immunoprecipitation. Samples were stored at 80 °C. Normal mouse IgG (Santa Cruz) or monoclonal anti-haemagglutinin (anti-HA) antibody (HA probe (F-7), Santa Cruz) was added to 25 µl of pre-blocked (1 mg ml^{-1} salmon sperm DNA and 1 mg ml^{-1} bovine serum albumin) magnetic

beads coupled to protein G (Dynal). After 5 h incubation, beads were added to each lysate and incubated overnight at 4 °C in a rotator. Immune complexes were sequentially washed five times with FA lysis buffer, four times with FA lysis buffer containing 500 mM NaCl, five times with wash buffer (10 mM Tris-Cl, pH 8.0, 0.25 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate) and twice with Tris-EDTA (TE) buffer. Bound proteins were eluted from the beads by adding 150 µl elution buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 1% SDS) and incubating for 15 min at 65 °C. Cross-linking was reversed by overnight incubation at 65 °C in the presence of proteinase K (0.25 mg ml^{-1}). DNA was purified using a QIAquick PCR purification kit (Qiagen). Real-time quantitative PCR was carried out in an Opticon Monitor 3 (BioRad) with primers amplifying the promoter regions of the *BAP2* gene (F-*BAP2*-qPCR AGGAGGCTACT-GACACTGC; R-*BAP2*-qPCR GCTGACATATTTACCG TTGAAGG). A pair of primers amplifying a region of the *TBP1* gene (F-*TBP1*-qPCR TATAACCCCAAGCGTTTTGC; R-*TBP1*-qPCR GCCAGCTTTGAGTCATCCTC) was used as an unbound control. ChIP DNA was normalized to input DNA and calculated as a signal-to-noise ratio over an IgG control ChIP. The ΔΔCt method was used to calculate fold changes in binding to the promoter of interest [18]. Results are expressed as the mean±SEM of three independent experiments.

Cell extracts and immunoblotting

Cells transformed with the pMB10 plasmid were grown on the indicated media and harvested by centrifugation. Protein extraction was immediately carried out as previously described [19]. Briefly, total proteins were prepared by lysing yeast cells in 1.85 N NaOH-7.5% β-mercaptoethanol on ice for 10 min, followed by precipitation with trichloroacetic acid (TCA) at a final concentration of 8%. The TCA pellets were neutralized with 1 M unbuffered Tris and resuspended in sodium dodecyl sulfate (SDS) loading buffer. Proteins were separated on a 7% SDS-PAGE, transferred to PVDF membranes and detected using the rat monoclonal anti-HA antibody (anti-HA high affinity 3E10, Roche) and the secondary goat anti-rat IgG antibody conjugated to horseradish peroxidase (Santa Cruz). Chemiluminescence immunodetection was performed on a FUJIFILM LAS-1000 reader and immunoreactive bands were analysed by digital imaging.

Statistical analyses

Statistical analyses were carried out with the statistical programming language R [20]. Specifically, ANOVA was performed with the required fixed factors for each case, including strain and leucine treatment (yes or no), and every interaction between them. When model assumptions of normality or homoscedasticity were not met, data were transformed by the natural logarithm or the square root. Tukey multiple comparisons tests were carried out with the emmeans package [21].

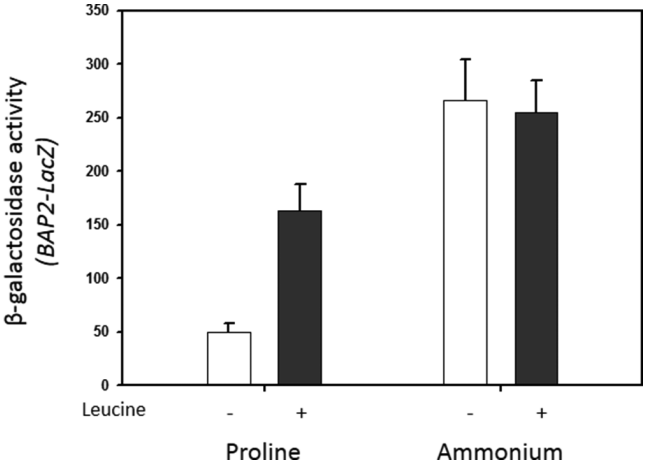

Fig. 1. Effect of the nitrogen source on *BAP2* expression. Wild-type (23344c) cells transformed with the Yep357-*BAP2*-lacZ plasmid were grown on 10 mM proline or ammonium sulfate and incubated (grey bars) or not (white bars) with 1.3 mM leucine. After 1 h, cells were harvested and β-galactosidase activity was measured. Results expressed as Miller units represent the mean±SD of triplicates within a representative assay.

RESULTS

In order to study the regulation of *BAP2* transcription, the expression of *LacZ* driven by the regulatory region of the *BAP2* gene was measured in cells grown on minimal media with ammonium or proline as the sole nitrogen source. Cells grown using the non-preferred nitrogen source proline showed low *BAP2* expression, which increased more than three times upon cell incubation with 1.3 mM leucine for 60 min (P<0.001) (Fig. 1). In turn, cells grown using the preferred nitrogen source ammonium showed a significantly higher *BAP2* expression (P<0.001) which was insensitive to extracellular leucine (Fig. 1). These results confirmed that *BAP2* is not subject to nitrogen repression, but also suggested a different regulatory mechanism depending on the quality of the nitrogen source, as the *BAP2* gene was inducible by leucine only in the presence of a non-preferred source.

To establish the regulatory mechanisms of *BAP2* under both nitrogen conditions, we analysed the participation of several regulatory proteins involved in the use of amino acids as nitrogen sources. Using ammonium cells, it has been demonstrated that *BAP2* induction depends on transcription factors Stp1 and, to a lesser extent, Stp2 [4], both of which are synthesized as latent cytoplasmic proteins with N-terminal regulatory domains. Upon induction by extracellular amino acids, the plasma membrane SPS sensor catalyses an endo-proteolytic processing event that cleaves away the regulatory N-terminal domains [22]. Before analysing the effect of the SPS pathway on *BAP2* expression in proline cells, we needed to confirm that the SPS system was functional in proline in the way described for cells grown in ammonium [22], which led us to analyse the cleavage of Stp1, assuming that Stp2 will be processed in the same way. To this end, wild-type and *ssy1*Δ

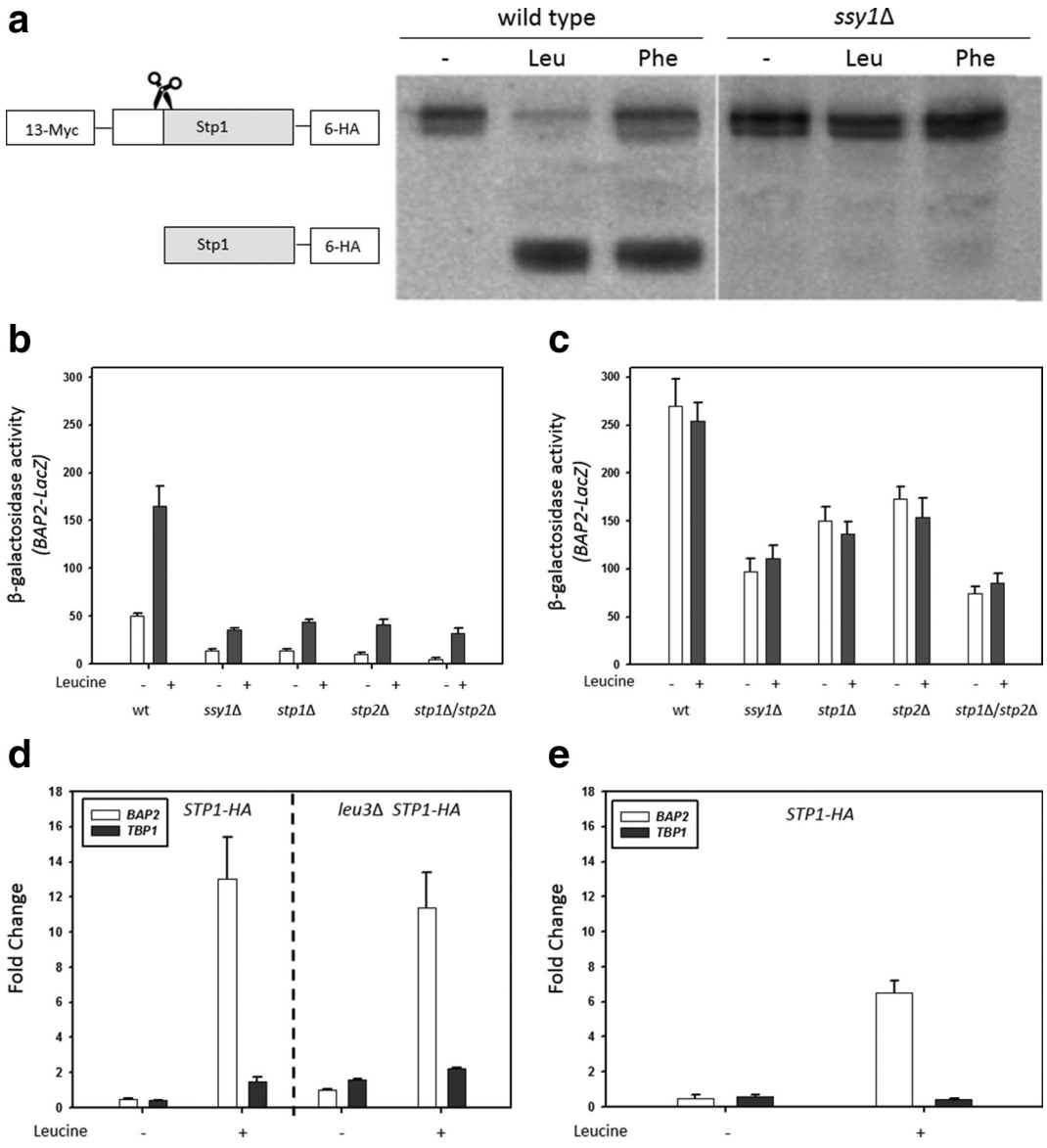

Fig. 2. SPS pathway and *BAP2* gene regulation. (a). Endoproteolytic processing of Stp1. Wild-type (23344c) and *ssy1*Δ (30995b) transformed with the plasmid pMB10 were grown on 10 mM proline and incubated or not with 1.3 mM leucine and phenylalanine for 5 min. Total cell extracts were analysed by immunoblotting with anti-HA antibodies. (b) and (c). Expression of *BAP2*. Wild-type (23344c), *ssy1*Δ (30995b), *stp1*Δ (KW018), *stp2*Δ (KW021) and *stp1*Δ *stp2*Δ (KW022) cells transformed with the plasmid Yep357-*BAP2*-lacZ were grown on 10 mM proline (b) or 10 mM ammonium sulfate (c) and incubated (grey bars) or not (white bars) with 1.3 mM leucine. After 1 h, cells were harvested and β-galactosidase activity was measured. Results expressed as Miller units represent the mean±SD of triplicates within a representative assay. (d) and (e). Interaction between Stp1-HA and the promoter of *BAP2*. Wild-type and *leu3*Δ cells expressing the Stp1-HA fusion protein were grown on 10 mM proline (d) or 10 mM ammonium sulfate (e) and incubated or not with 1.3 mM leucine for 1 h as indicated. ChIP assays were carried out using antibodies against the HA epitope. qPCR was performed with specific primers that amplify a region of *BAP2* promoter (white bars) and *TBP1* promoter used as a negative control (grey bars). Results are expressed as the fold change of binding to the *BAP2* promoter and are the mean±SEM of three independent experiments.

cells were incubated with leucine and phenylalanine for 5 min and then total protein extracts were prepared and analysed by immunoblotting. In proline cells, only after incubation with the amino acids tested, the cleaved form of Stp1 was detected in wild-type cells but not in the *ssy1*Δ mutant (Fig. 2a). This result is similar to that observed in ammonium cells (Fig. S1, available in the online version of this article), as previously demonstrated by Andreasson and Ljungdahl [22]. We then analysed *BAP2* expression in cells deficient in the SPS system. To such end, we measured the activity of β-galactosidase in *ssy1*Δ cells that failed to activate Stp1/Stp2 and in cells deficient in Stp1 and/or Stp2 lacking one or both of these transcription factors. In proline, the activity measured in cells before incubation with leucine in the four different mutants

was significantly lower than in the wild type, with $P<0.001$ in all cases (Fig. 2b). Incubation with leucine led to an increase in *BAP2* expression in these mutants ($P<0.001$), although the expression values reached upon induction were lower than those measured in the wild type (Fig. 2b). In ammonium, the activity of β-galactosidase was not altered by the presence of leucine in the mutant or wild type cells tested (Fig. 2c). On the other hand, the activity measured in the four SPS mutants was lower than in the wild type ($P<0.001$ in all cases) (Fig. 2c).

As described above, the expression of *BAP2* decreased in uninduced *ssy1*Δ and *stp1*Δ/*stp2*Δ cells (Fig. 2b, c). This result was unexpected, since Stp1 was detected bound to the *BAP2* promoter only after incubation with leucine under both nitrogen conditions (Fig. 2d, e). How *BAP2* expression is modified by the lack of Stp1 in conditions where Stp1 does not interact with the *BAP2* gene remains to be elucidated. It must be noted, however, that the binding of Stp1 to the promoter in ammonium cells (Fig. 2e) did not lead to *BAP2* induction (Fig. 2c).

Nielsen *et al.* proposed that Leu3 is involved in *BAP2* regulation [4]. We found that the absence of Leu3 drastically diminished *BAP2* expression under ammonium growth conditions (Fig. 3b) and in uninduced proline cells (Fig. 3a), ($P<0.001$). However, this factor produced no effect on *BAP2* induction in proline cells (Fig. 3a). On the other hand, we did detect Leu3 bound to the regulatory region of *BAP2* in proline-grown cells after incubation with leucine (Fig. 3c), but were unable to detect any interaction between Leu3 and *BAP2* in ammonium cells (data not shown). These results suggest an indirect effect of Leu3 on *BAP2* expression accomplished without a direct interaction between this transcription factor and the promoter. As synergy between Leu3 and Stp1 was

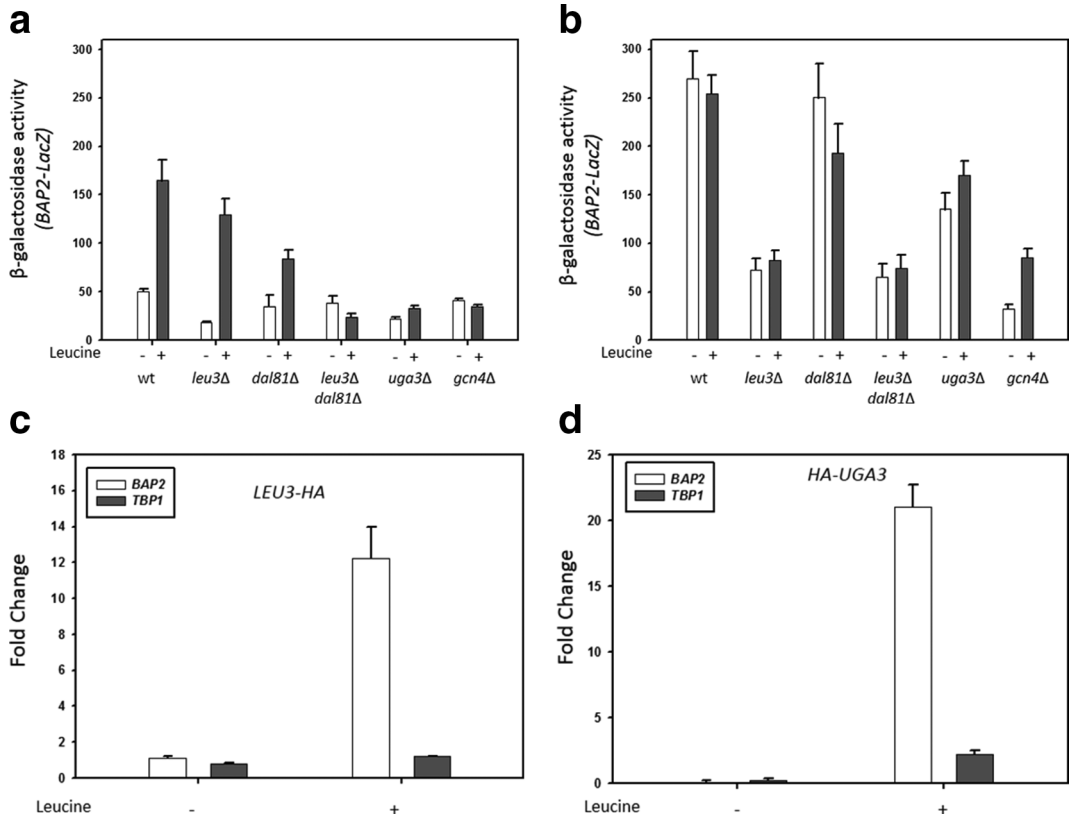

Fig. 3. *BAP2* gene regulation. (a) and (b). Expression of *BAP2*. Wild-type (23344c), *leu3*Δ (SBCY01), *dal81*Δ (SBCY17), *leu3*Δ *dal81*Δ (SBCY20), *uga3*Δ (26790) and *gcn4*Δ (SBCY03) cells transformed with the Yep-357-*BAP2*-lacZ plasmid were grown on 10 mM proline (a) or 10 mM ammonium sulfate (b) and incubated (grey bars) or not (white bars) with 1.3 mM leucine. After 1 h, cells were harvested and β-galactosidase activity was measured. Results expressed as Miller units represent the mean±SD of triplicates within a representative assay. (c). Interaction between Leu3-HA and the promoter of *BAP2*. Cells expressing the Leu3-HA (SBCY02) fusion protein were grown on 10 mM proline and incubated or not with 1.3 mM leucine for 1 h as indicated. ChIP assays were carried out using antibodies against the HA epitope. qPCR was performed with specific primers that amplify a region of *BAP2* promoter (white bars) and *TBP1* promoter used as a negative control (grey bars). Results are expressed as the fold change of binding to the *BAP2* promoter and are the mean±SEM of three independent experiments. (d). Interaction between HA-Uga3 and the promoter of *BAP2*. Cells expressing the HA-Uga3 (SBCY13) fusion protein were grown on 10 mM proline and incubated or not with 1.3 mM leucine for 1 h as indicated. ChIP assays were carried out using antibodies against the HA epitope. qPCR was performed with specific primers that amplify a region of *BAP2* promoter (white bars) and the *TBP1* promoter used as a negative control (grey bars). Results are expressed as the fold change of binding to the *BAP2* promoter and are the mean±SEM of three independent experiments.

previously proposed [4], and given that we detected Leu3 and Stp1 bound to the *BAP2* promoter under the same conditions (i.e. induced proline cells), we analysed whether their binding was dependent on each other. We found that the interaction of Stp1 with the *BAP2* promoter observed in wild-type cells after incubation with leucine occurred in cells lacking Leu3 (Fig. 2d), which shows that the interaction between Stp1 and *BAP2* was independent of the presence of Leu3.

It has been previously reported that Dal81 positively regulates the transcription of *BAP2* (8, 9). Accordingly, the *β*-galactosidase activity measured in leucine-induced proline cells was significantly lower in *dal81*Δ than in wild-type cells ($P<0.001$) (Fig. 3a). However, no effect of Dal81 was detected on *BAP2* in ammonium cells or in uninduced proline cells (Fig. 3a and b). The effect of Leu3 deficiency produced on *BAP2* expression in proline cells became more evident when Dal81 was also absent, indicating that the activity of Dal81 somehow masked the effect of Leu3 deficiency. Of note, no binding was detected between Dal81 and *BAP2* (data not shown).

With a view to understanding the indirect effect of Leu3 on *BAP2* regulation, we analysed *BAP2* expression in cells deficient in Uga3 and Gcn4, two transcription factors reportedly regulated by Leu3 [23–25]. Indeed, the expression of *UGA3*, the gene encoding a transcription factor described as specific to the *UGA* regulon, significantly diminished in the absence of Leu3 [23]. In our studies, the expression of *BAP2* in proline *uga3*Δ cells was significantly lower than in wild-type cells ($P<0.001$) (Fig. 3a), indicating that Uga3 may act as an activator of *BAP2*. Therefore, the decrease in *BAP2* expression in *leu3*Δ cells could have been produced by the decrease in Uga3 levels; however, as the expression values measured in ammonium were lower in *leu3*Δ cells than in *uga3*Δ cells, the effect of Leu3 might have been only partly mediated by Uga3.

Furthermore, we found that the addition of leucine produced no changes in the activity of *β*-galactosidase in proline *gcn4*Δ cells (Fig. 3a), which indicates that Gcn4 is involved in *BAP2* induction in proline cells. Moreover, the activity significantly decreased in the absence of Gcn4 in ammonium cells ($P<0.001$) (Fig. 3b). Taken together, these results confirm that Gcn4 is an activator of *BAP2* transcription [26]. While direct binding of Gcn4 to the *BAP2* promoter has been reported [27, 28], other authors have claimed that this effect is not direct [25]. Gcn4 and Leu3 have been also shown essential for full expression of *UGA3*, with expression levels being fourfold lower in cells lacking Gcn4 or Leu3 than in wild-type cells (unpublished results), as demonstrated for Gcn4 by others authors [29]. As a result, and taking into account the strongly positive effect observed for Uga3 on *BAP2* expression (Fig. 3a, b), an indirect effect of Gcn4 and/or Leu3 on *BAP2* could be explained by the decrease in *UGA3* expression.

As our results indicated that the indirect effect of Leu3 and Gcn4 could be mediated by Uga3, we analysed the interaction of Uga3 with the *BAP2* promoter. Although no interaction was detected between Uga3 and *BAP2* in ammonium cells (data not shown), Uga3 was found to interact with the *BAP2* promoter in proline cells in a leucine-dependent manner (Fig. 3d). Hence, we demonstrated that Uga3 is an activator of *BAP2* and that its activity is a consequence of a direct binding to the *BAP2* promoter.

DISCUSSION

In this report, we analysed the transcriptional regulation of *BAP2* in cells grown in the presence of two nitrogen sources. We found that *BAP2* is a leucine-inducible gene in cells grown in the poor nitrogen source proline, whereas a constitutive high expression insensitive to leucine was observed in the rich nitrogen source ammonium.

The effect of leucine on *BAP2* expression in proline cells had also been documented by Bernard and Andre [8]. In contrast, Didion *et al.* detected no *BAP2* expression regardless of the presence of leucine [1], although it should be pointed out that the leucine concentration used by these authors (0.23 mM) was lower than that used in the present work (1.3 mM) and in that by Bernard and Andre (5 mM).

Bernard and Andre [8] and Nielsen *et al.* [4] postulated that *BAP2* transcription is not subject to NCR based on the degree of induction of *BAP2* in ammonium, which finds support in our current results. However, no induction was detected in the present study, as high levels of *BAP2* expression were observed even before the addition of the inducer leucine. Nielsen *et al.* did not detect *BAP2* mRNA in uninduced ammonium cells but showed that it rapidly accumulates after leucine addition [4], in line with results obtained by Didion *et al.* using reporter gene assays [1]. On the other hand, Forsberg *et al.* found no *BAP2* transcripts in ammonium cells either before or after the addition of leucine [11]. Such variations in *BAP2* expression profiles lead us to postulate that the genetic background and/or growth media used have substantial impact on the regulatory mechanism of *BAP2*.

We also demonstrate here that an active SPS pathway is necessary for *BAP2* expression in both nitrogen conditions and in the presence or absence of the inducer leucine, which indicates that Stp1/2 is involved not only in *BAP2* induction but also in basal leucine-independent *BAP2* expression. We did not detect Stp1 bound to the *BAP2* promoter in the absence of leucine, which shows that the effects of Stp1/2 on *BAP2* regulation are mediated both by a direct binding of this factor to DNA and also by another means.

In addition, the mild decrease detected in *BAP2* expression in *leu3*Δ cells probably resulted from the positive activity of Dal81 and/or Gcn4, as the absence of Leu3 is known to mimic amino acid-starvation conditions inducing the translation of Gcn4 [25].

We demonstrated that Leu3 and Stp1 act on *BAP2* regulation independently of each other. This result is in agreement with the finding that Stp1 is capable of binding to the *BAP2* promoter regardless of the presence of a functional Leu3 binding site [4]. As a matter of fact, Kirkpatrick and Shimmel have proposed that Leu3 may be permanently bound to

its regulatory sites, poised to activate transcription upon receiving the appropriate signal [30]. However, our studies rendered leucine-dependent interaction between Leu3 and the *BAP2* promoter.

A certain degree of *BAP2* induction was still detected in the absence of Leu3 or when the SPS pathway was inactive. This remaining induction could respond to the activity of Leu3 in SPS-deficient cells, to the SPS pathway in *leu3*Δ cells and/or to other factors such as Dal81, Uga3 and Gcn4, since we also found that these transcription factors act as activators on *BAP2* transcription. Didion *et al.* showed that mutating the Gcn4 binding site does not affect *BAP2* expression [1], which means that this indirect effect, that is not dependent on the binding of Gcn4 to de DNA, could at least partly involve Uga3, whose expression diminishes in cells lacking Gcn4 (our unpublished results). Leu3 could also be responsible for the indirect effect of Gcn4 on *BAP2*, as Gcn4 has been reported to induce *LEU3* expression [25]. Although, to our knowledge, this regulation has not been directly demonstrated so far, transcriptional regulation is known to be dependent not only on DNA binding but also on interaction with other proteins or effector molecules [25].

Studying the three genes of the *UGA* regulon involved in the transport and catabolism of GABA, we have previously demonstrated that tight gene regulation is the result of an interplay among transcription factors Leu3, Dal81 and Uga3 [23, 31–34]. In the present work, we further show that transcription factor Uga3, until now known as a transcriptional activator responsible for GABA-dependent induction of GABA genes *UGA1, UGA2* and *UGA4* [35–37] is one of the main regulators of *BAP2* transcription. Uga3 is involved in both uninduced and leucine-induced expression of *BAP2*, although it seems to interact with the promoter only in induction conditions. *BAP2*, a gene expressed under environmental conditions quite different from those of *UGA* expression, is also regulated by these three factors together with Gcn4 and Stp1/2.

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Author contributions
M.B-.M. and S.C-.G. designed the research; S.A.M., J.F.G. and J.V-.G. performed the experiments and figures; M.B-.M. and S.C-.G. analyzed the data, conceived and coordinated the study, and wrote the article.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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