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Nitrogen Source Activates TOR (Target of Rapamycin) Complex 1 via Glutamine and Independently of Gtr/Rag Proteins*

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Background: Nutrients, in particular the nitrogen source, activate TORC1 signaling in yeast.
Results: The nitrogen source stimulates a rapid, transient activation of TORC1. Preferred nitrogen sources result in sustained TORC1 activity and growth via a mechanism dependent on glutamine accumulation and independent of the Gtr/Rag.
Conclusion: Nutrients activate yeast TORC1 via two distinct mechanisms.
Significance: Gtr/Rag is not the only mechanism to activate TORC1 in response to nutrients.

The evolutionary conserved TOR complex 1 (TORC1) activates cell growth in response to nutrients. In yeast, TORC1 responds to the nitrogen source via a poorly understood mechanism. Leucine, and perhaps other amino acids, activates TORC1 via the small GTPases Gtr1 and Gtr2, orthologs of the mammalian Rag GTPases. Here we investigate the activation of TORC1 by the nitrogen source and how this might be related to TORC1 activation by Gtr/Rag. The quality of the nitrogen source, as defined by its ability to promote growth and glutamine accumulation, directly correlates with its ability to activate TORC1 as measured by Sch9 phosphorylation. Preferred nitrogen sources stimulate rapid, sustained Sch9 phosphorylation and glutamine accumulation. Inhibition of glutamine synthesis reduces TORC1 activity and growth. Poor nitrogen sources stimulate rapid but transient Sch9 phosphorylation. A Gtr1 deficiency prevents the transient stimulation of TORC1 but does not affect the sustained TORC1 activity in response to good nitrogen sources. These findings suggest that the nitrogen source must be converted to glutamine, the preferred nitrogen source in yeast, to sustain TORC1 activity. Furthermore, sustained TORC1 activity is independent of Gtr/Rag. Thus, the nitrogen source and Gtr/Rag activate TORC1 via different mechanisms.

To avoid metabolic stress, unicellular organisms possess finely tuned regulatory systems to ensure that cell growth and replication are tightly coupled to nutrient availability (1). Nitrogen is an essential element required for synthesis of amino acids, nucleotides, and other cellular components. The budding yeast *Saccharomyces cerevisiae* can sense, take up, and assimilate several different nitrogen sources (N-sources) (2). Qualitatively better N-sources are assimilated before others. The quality of a N-source is generally defined by its ability (i) to sustain core nitrogen metabolism and thus growth and (ii) to activate or repress specific transcriptional programs that mediate selective nitrogen assimilation (3). To maintain growth, N-sources must sustain core nitrogen metabolism that involves four interconnected enzymatic reactions with α-ketoglutarate, glutamate, glutamine, and ammonia as key metabolites (4). Glutamine is considered the best N-source for yeast cells (4). In the presence of exogenous glutamine as the N-source, yeast cells grow and replicate at a high rate and maximally repress the metabolic pathways required for utilization of other N-sources (2, 5). Moreover, glutamine plays an essential role in anabolic metabolism, in particular in purine and pyrimidine synthesis, and in the biosynthesis of other amino acids after conversion to glutamate. Amino acids other than glutamate and glutamine can also serve as nitrogen sources but only by sustaining nitrogen core metabolism indirectly. These amino acids undergo deamination, liberating free ammonia that can then be used for glutamate or glutamine synthesis, or generate glutamate via transamination (6). Depending on the amino acid, the remaining carbon skeleton after deamination or transamination is either fed into carbon metabolism or converted through the Ehrlich pathway into fuse oils and secreted (7).

Metabolic adaptation to the nutritional environment is achieved via complex transcriptional, translational, and post-translational regulation, which relies on nutrient sensing and signal transduction (8, 9). The target of rapamycin (TOR)[2] pathway is a central regulator of cell growth in response to nutrients (10, 11). TOR is an evolutionary conserved protein kinase found in two highly conserved multiprotein complexes termed TOR complex 1 (TORC1) and TORC2. TORC1 is acutely sensitive to rapamycin and is activated by nutrients (12, 13). TORC1 couples nutrient sufficiency to growth by activating anabolic processes such as protein synthesis and ribosome biogenesis and repressing catabolic processes such as autophagy. Despite the many cellular functions in which TORC1 is involved, few direct TORC1 targets are known. The

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[2] The abbreviations used are: TOR, target of rapamycin; TORC1, TOR complex 1; EGOC, EGO complex; LeuRS, leucyl-tRNA synthetase; MSX, methionine sulfoximine; DHBB, 1,3-dihydro-1-hydroxy-2,1-benzoxaborole; YMM, yeast minimal medium; NTCB, 2-nitro-5-thiocyanatobenzoic acid.

best-characterized TORC1 direct target in yeast is Sch9, a kinase belonging to the AGC kinase family also containing PKA, PKG, and PKC. In the presence of nutrients, TORC1 phosphorylates at least six residues in the Sch9 C terminus and thereby activates Sch9 in a rapamycin-sensitive manner (14–16). Sch9 activates genes encoding ribosomal proteins and factors required for ribosome biogenesis (RiBi regulon) (17), ultimately controlling cell growth and longevity (18–20).

In yeast the so-called EGO complex (EGOC) mediates amino acid-dependent activation of TORC1 (21). EGOC consists of Gtr1, Gtr2, Ego1, and Ego3. The small GTP binding proteins Gtr1 and Gtr2 are orthologs of the mammalian RagA/B and RagC/D GTPases, respectively (22–24). Like their mammalian counterparts, Gtr1 and Gtr2 form a heterodimer that is active in the $Gtr1^{GTP}$-$Gtr2^{GDP}$ conformation. Ego1 and Ego3, the functional homologs of the mammalian Ragulator complex (25), are also required for activation of Gtr. The amino acid leucine stimulates $Gtr1^{GTP}$ interaction with Ego1, thereby activating EGOC and, in turn, TORC1 (22). Vam6 and the Npr2-Npr3-Iml1 complex were identified as the guanine nucleotide exchange factor and GTPase-activating protein, respectively, for Gtr1 (22, 26). The yeast leucyl-tRNA synthetase (LeuRS) was reported to positively influence the GTP loading of Gtr1 (27).

The rapamycin-induced response in yeast mimics the response to nitrogen starvation (28–30). This early observation led to the conclusion that the N-source activates TORC1. The mechanism by which the N-source, in the form of an amino acid or other nitrogenous compound, activates TORC1 is poorly understood. Early studies reported a prominent role for the intracellular level of glutamine in TORC1 activation, at least toward the transcription factors Gln3, Rtg1, and Rtg3 (31). Although increasing evidence suggests that intracellular leucine stimulates TORC1 via EGOC, it is not yet known if the N-source, including ammonium or other amino acids, activates TORC1 via EGOC. Cells expressing a constitutively active version of Gtr1 ($Gtr1^{Q65L}$) still down-regulate TORC1 activity in response to ammonium starvation (22), suggesting that the N-source signals to TORC1 independently of Gtr1.

Here we investigate the modulation of TORC1 activity by different N-sources *in vivo* using the phosphorylation state of Sch9 as readout. We show that preferred N-sources sustain TORC1 activity and growth rate by rapidly increasing glutamine synthesis. Moreover, we demonstrate that glutamine synthesis does not require Vam6 or Gtr1 for TORC1 activation. Thus, there are distinct molecular mechanisms by which nutrients activate TORC1.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmid, and Media—Yeast strains and plasmids used in this study are listed in Table 1 and 2. Yeast cultures were grown in flasks at 30 °C in liquid YMM containing only one source of nitrogen at a final concentration of 0.5 g/liter. Detailed composition of 1 liter YMM is as follows: 5 g of $(NH_4)_2SO_4$, 3 g of KH_2PO_4, 0.5 g of $MgSO_4{\cdot}7H_2O$, 1.5 mg of EDTA, 4.5 mg of $ZnSO_4{\cdot}7H_2O$, 0.3 mg of $CoCl_2{\cdot}6H_2O$, 1 mg of $MnCl_2{\cdot}4H_2O$, 0.3 mg of $CuSO_4{\cdot}5H_2O$, 4.5 mg of $CaCl_2{\cdot}2H_2O$, 3 mg of $FeSO_4{\cdot}7H_2O$, 0.4 mg of $NaMoO_4{\cdot}2H_2O$, 1 mg of H_3BO_3, 0.1 mg of KI, 5 μg of biotin, 100 μg of calcium pantothenate, 100 μg of nicotinic acid, 2.5 mg of inositol, 100 μg of pyridoxine; 20 μg of *p*-aminobenzoic acid, 100 μg of thiamine, 20 g/liter glucose, 0.5 g/liter N-source to 1 liter with 100 mM potassium hydrogen phthalate·H_2O, pH 5.

TABLE 1
Strains used in this study

Strain	Genotype	Source
YSBN9	MATα *FY3 ho::Ble ura3-52*	Canelas *et al.* (40)
YSBN9 *vam6*Δ	MATα *FY3 ho::Ble ura3-52* *VAM6::KanMX*	This study
YSBN9 *gtr1*Δ	MATα *FY3 ho::Ble ura3-52* *GTR1::KanMX*	This study
YSBN9 *gtr2*Δ	MATα *FY3 ho::Ble ura3-52* *GTR2::KanMX*	This study
YSBN9 *DOT6*-3HA	MATα *FY3 ho::Ble ura3–52* *DOT6*-3xHA::*KanMX*	This study

TABLE 2
Plasmids used in this study

Plasmid	Vector; insert	Source
pRS416	cen *URA3*	
pJU733	pRS416; cen *URA3* *SCH9*-3xHA	Urban *et al.* (16)
pMB1393	YCplac33; cen *URA3* Tet-on-*GTR1*	Binda *et al.* (22)
pMB1394	YCplac33; cen *URA3* Tet-on-*GTR1*-Q65L	Binda *et al.* (22)
pMB1395	YCplac33; cen *URA3* Tet-on-*GTR1*-S20L	Binda *et al.* (22)

Growth Conditions and Cell Extracts—Typically cells were grown in YMM supplemented with the first N-source at a concentration of 0.5 g/liter in shaking flasks at 30 °C until mid log phase ($A_{600} \approx 1.0$). In the case of nutrient upshift, the second N-source was added at time point 0 at the final concentration of 0.5 g/liter directly in the media. When performing nutrient downshifts, cells were gently pelleted by spinning for 2 min at 1000 × *g* and re-suspended at time point 0 in fresh YMM media containing L-glutamine at the final concentration of 0.5 g/liter. For each time point, 9 ml of culture were taken, quenched with cold trichloroacetic acid (10% final concentration), and incubated on ice for 5 min. Cells were then pelleted at 3500 × *g* for 5 min, washed twice with cold acetone, and dried in a SpeedVac. The cellular pellet was resuspended in 100 μl of urea buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 6 M urea, 1% SDS) and mixed with 200 μl of glass beads. Cell lysis was performed using a bead-beater (Biospec Products), beating 5 times for 45 s at maximum speed with 3 min intervals on ice. The extract was collected and heated to 65 °C for 10 min. Unbroken cells and debris were removed by centrifugation at 13,000 × *g* for 5 min. Protein concentration was determined using the Pierce BCA protein assay (Thermo Fisher Scientific, Rockford, IL).

Chemical Fragmentation and Phosphorylation Quantification of Sch9—Cell extracts were subjected to 2-nitro-5-thiocyanatobenzoic acid (NTCB) cleavage as described previously (16). Further analysis was done by SDS-PAGE and immunoblotting using anti-HA antibody (HA-tag (6E2) mouse mAb #2367, Cell Signaling Technology, Danvers, MA). To quantify Sch9 phosphorylation, we used the software ImageJ (32). We measured the integrated density of the uppermost band (phosphorylated protein) and divided it by the sum of the integrated density of

the uppermost plus the lowermost band (unphosphorylated protein).

Metabolite Extraction—All samples were supplemented with 50 μl of ^{13}C-labeled internal standard and extracted one time with 1 ml of 75%(v/v) ethanol buffered with 10 mM ammonium acetate, pH 7.5, at 78 °C for 3 min. After the extraction step biomass was separated by centrifugation for 3 min at 5000 rpm at −9 °C. The liquid extract of each sample was dried at 10^{-1} mbar to complete dryness in a RapidVac and then stored at −80 °C until resuspension.

Metabolite Mass Spectrometry—Liquid chromatography separation of compounds was achieved by an ion pairing-reverse phase method developed for ultra high performance liquid chromatography Q3 systems (33) based on previously published high pressure methods (34–36) and implemented on a Waters Acquity UPLC (Waters Corp., Milford, MA) using a Waters Acquity T3 end-capped reverse phase column with dimensions 150 × 2.1 mm × 1.8 μm (Waters Corp.). Selective and sensitive detection of compounds was achieved by coupling liquid chromatography to a Thermo TSQ Quantum Ultra QQQ mass spectrometer from Thermo Fisher Scientific (Waltham, MA) using a heated electrospray ionization source. The mass spectrometer was operated in negative mode with selected reaction monitoring. Fragmentation parameters were optimized individually for all compounds (33). Both acquisition and peak integration were performed with the Xcalibur software Version 2.07 SP1 (Thermo Fisher Scientific). Peak areas were normalized to fully ^{13}C-labeled internal standards (37) and the amount of biomass.

RESULTS

TORC1 Rapidly Phosphorylates Sch9 in Response to the Nitrogen Source—We initially sought to establish a system using the phosphorylation state of the Sch9 C terminus as a readout to monitor changes in TORC1 activity in response to nutrients *in vivo*. Moreover, we reasoned that signaling events associated with nutrient availability would be triggered very rapidly, as in the case of glucose-induced cAMP production in yeast cells (38, 39). To capture fast-acting signaling events, Sch9 phosphorylation was assayed as early as 20 s and up to 4 h after stimulation with nutrients. We used the strain YSBN9 (40), which we made prototrophic by transformation with the centromeric plasmid p*SCH9*-3HA containing the selectable marker *URA3* in addition to expressing C-terminally 3×HA-tagged Sch9 under the control of its own promoter. By working with a prototrophic strain we were able to grow cells in medium supplemented with a single nitrogen source, thereby avoiding the confounding effect of the simultaneous presence of different N-sources.

We first validated our system by pharmacologically inhibiting TORC1. Rapamycin treatment of yeast cells, grown in the presence of glutamine, resulted in an acute decrease in Sch9 C-terminal phosphorylation. Compared with untreated cells, Sch9 phosphorylation decreased to minimal levels within 5 min and remained low for the remainder of the experiment (Fig. 1, *A* and *B*). We next sought to determine the effect of changes in the nutritional environment on TORC1 activity by varying the quality of nutrients from good to poor (nutrient downshift) or from poor to good (nutrient upshift). When performing nutrient downshift experiments, cells were grown in the presence of the preferred N-source glutamine and switched to medium containing a less preferred N-source such as proline. As observed for rapamycin treatment, Sch9 phosphorylation decreased to minimal levels within minutes after nutrient downshift (Fig. 1, *C* and *D*). These data underscore the similarity between pharmacological and physiological inhibition of TORC1. In the case of nutrient upshift experiments, cells were grown in the presence of proline and stimulated with a good N-source. When using glutamine as the good N-source, Sch9 phosphorylation levels increased >2-fold already within 1 min after the addition of glutamine (Fig. 1, *E* and *F*). After this initial increase, Sch9 phosphorylation decreased to rise again at 15 min. Pretreating cells with rapamycin for 10 min was sufficient to block the increase in Sch9 phosphorylation induced by glutamine (Fig. 1*G*), demonstrating that Sch9 was phosphorylated by TORC1 during the nutrient upshift. To determine whether the increase in Sch9 phosphorylation was caused exclusively by the presence of the newly introduced N-source, we performed mock upshift experiments without increasing the nitrogen quality. As expected, no increase in Sch9 phosphorylation was observed when cells were treated with water or the same medium in which they were growing (Fig. 1, *H* and *I*). Overall, these findings indicate that our *in vivo* system is valid and that the N-source rapidly stimulates TORC1 activity.

The Quality of the Nitrogen Source Determines TORC1 Activity—To gain mechanistic insight into the nutrient-induced activation of TORC1, we quantified the extent of TORC1 activity over time in response to different N-sources. We determined the dynamics of Sch9 phosphorylation during nutrient upshift experiments in response to various nitrogen sources including growth-promoting amino acids and ammonium (Fig. 2). All N-sources tested induced a rapid increase in Sch9 phosphorylation within 20 s and peaking at 2–3 min after stimulation, although with different amplitudes. As observed above for glutamine, this rapid increase was transient as Sch9 phosphorylation then decreased, often to near basal levels. Upon comparing the change in Sch9 phosphorylation at 30 min and up to 4 h after shift, the N-sources clustered into two discrete groups. The first group contained the so-called high-end N-sources, able to re-stimulate and sustain high TORC1 activity ($p < 0.05$). The second group contained the low-end N-sources where Sch9 phosphorylation remained low (Fig. 2*A*). Arginine, asparagine, glutamine, and ammonium belonged to the high-end N-sources (Fig. 2*B*). Serine, threonine, the branched chain amino acids (isoleucine, leucine, and valine), the negatively charged amino acids (aspartate and glutamate), and the hydrophobic amino acids (alanine, methionine, phenylalanine, and tryptophan) fell in the low-end group (Fig. 2*C*). Tyrosine was not included in the study because of its low solubility in water. All the N-sources belonging to the high-end group were previously characterized as preferred N-sources (3). Similarly, the N-sources belonging to the low-end group were previously classified as less preferred, with the exception of alanine, serine, aspartate, and glutamate. We conclude that sustained TORC1 activity after nutrient upshift correlates with the quality of the N-source.

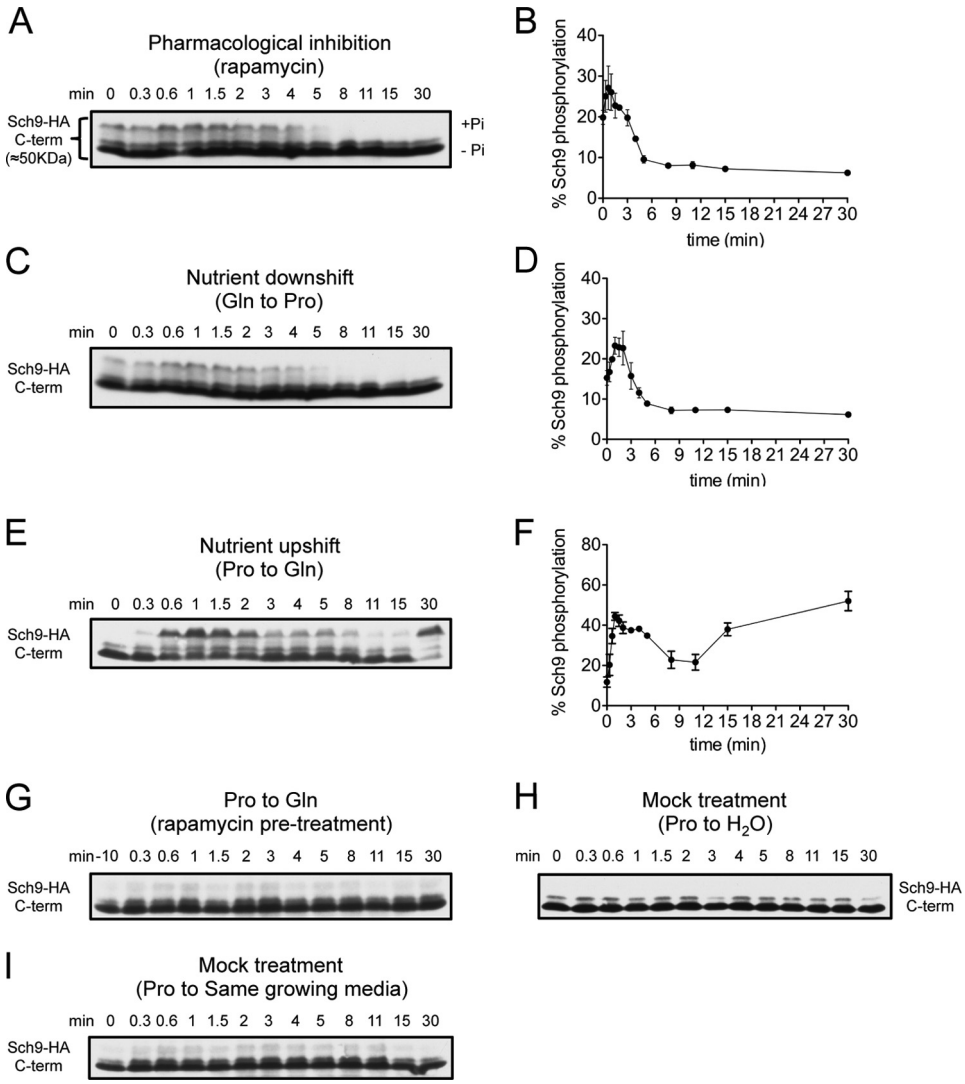


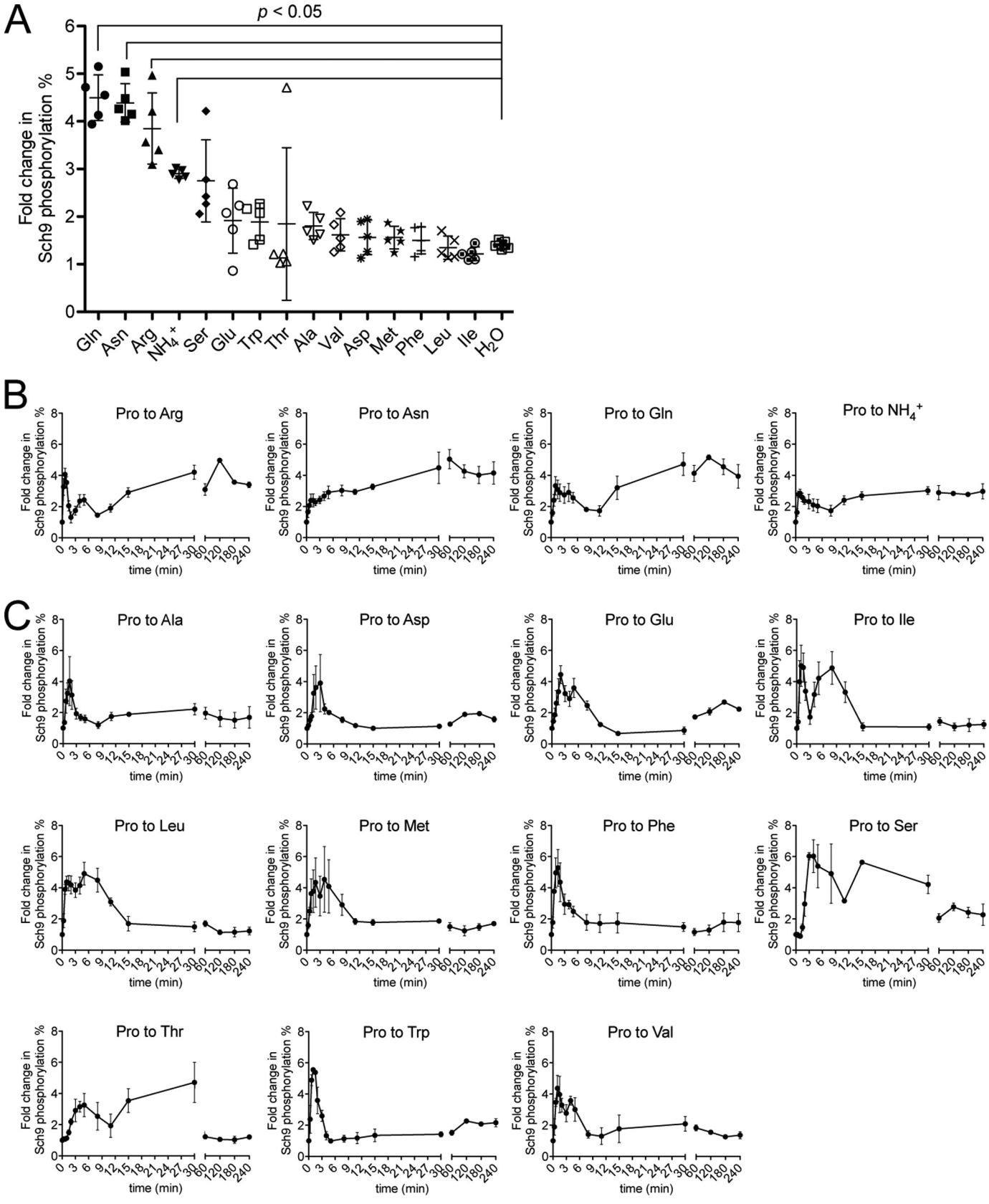
FIGURE 1. **TORC1 rapidly phosphorylates Sch9 in response to the nitrogen source.** Sch9 C-terminal phosphorylation decreased upon rapamycin treatment (200 nM) (*A* and *B*) and during nutrient downshift (glutamine to proline) (*C* and *D*). *D* and *E*, Sch9 C-terminal phosphorylation increased during nutrient upshift (proline to glutamine). Sch9 phosphorylation was unaffected when cells were subjected to either nutrient upshift (Pro to Gln) after rapamycin pretreatment (200 nM, 10 min; *G*) or mock-treated with water (*H*) or with the same medium in which they were growing (*I*). Protein samples were chemically cleaved with NTCB. Electrophoretic mobility shift was induced by the Sch9 C-terminal phosphorylation state, assessed by Western blot (*panel A*, *C*, *E*, *G*, *H*, and *I*). Relative quantification of Sch9 C-terminal phosphorylation (*panel B*, *D*, and *F*) is expressed as the mean ± S.E. of at least three independent biological experiments.

We next asked whether the quality of the initial N-source would affect the extent of TORC1 stimulation upon nutrient upshift. To this end we compared the ability of glutamine and ammonium, two preferred N-sources, to stimulate TORC1 activity in cells initially grown in the presence of proline or leucine, an intermediate and a non-preferred N-source, respectively. We observed that the glutamine- or ammonium-induced increase in Sch9 phosphorylation was equal in cells originally grown in the presence of proline or leucine as the only N-source (data not shown). Therefore, TORC1 activity is solely dependent on the quality of the N-source added during nutrient upshift. Collectively, we conclude that the extent of TORC1 stimulation *in vivo* parallels the quality of the N-source. In general, preferred N-sources stimulate and sustain TORC1 activity, whereas poor N-sources do not.

The Quality of the Nitrogen Source Couples TORC1 Activation to Cell Growth—Phosphorylation of Sch9 influences its kinase activity *in vitro* and *in vivo* (16, 17). We next asked whether the Sch9 phosphorylation increase observed during nutrient upshift would result in increased Sch9 kinase activity. To monitor Sch9 kinase activity *in vivo*, we measured the phosphorylation state of Dot6, a direct target of Sch9 involved in ribosome biogenesis (17). As assayed by immunoblotting, nutrient upshifts with glutamine, leucine, or ammonium resulted in the appearance of a slow-migrating form of C-terminally 3×HA-tagged Dot6 (Fig. 3*A*). As expected, no detectable change in Dot6 phosphorylation was observed when supplementing cells with water (Fig. 3*A*). In all three nutrient upshift experiments, Dot6 phosphorylation increased already at 2–3 min after stimulation, consistent with the rapid increase in Sch9 phosphorylation described above. Interestingly, Dot6 phosphorylation was sustained up to 30 min after stimulation with the preferred N-source glutamine or ammonium, whereas Dot6 phosphorylation decreased starting at 8 min after shifting to the less preferred N-source leucine. For unknown reasons, shift to leucine also caused a

decrease in Dot6 protein levels at 30 min after the shift. These data suggest that nutrient stimulation rapidly activates Sch9 downstream of TORC1.

Active Sch9 positively regulates ribosome biogenesis, thereby increasing cell growth potential (16, 41). We tested the physiological impact of TORC1 activation upon nutrient



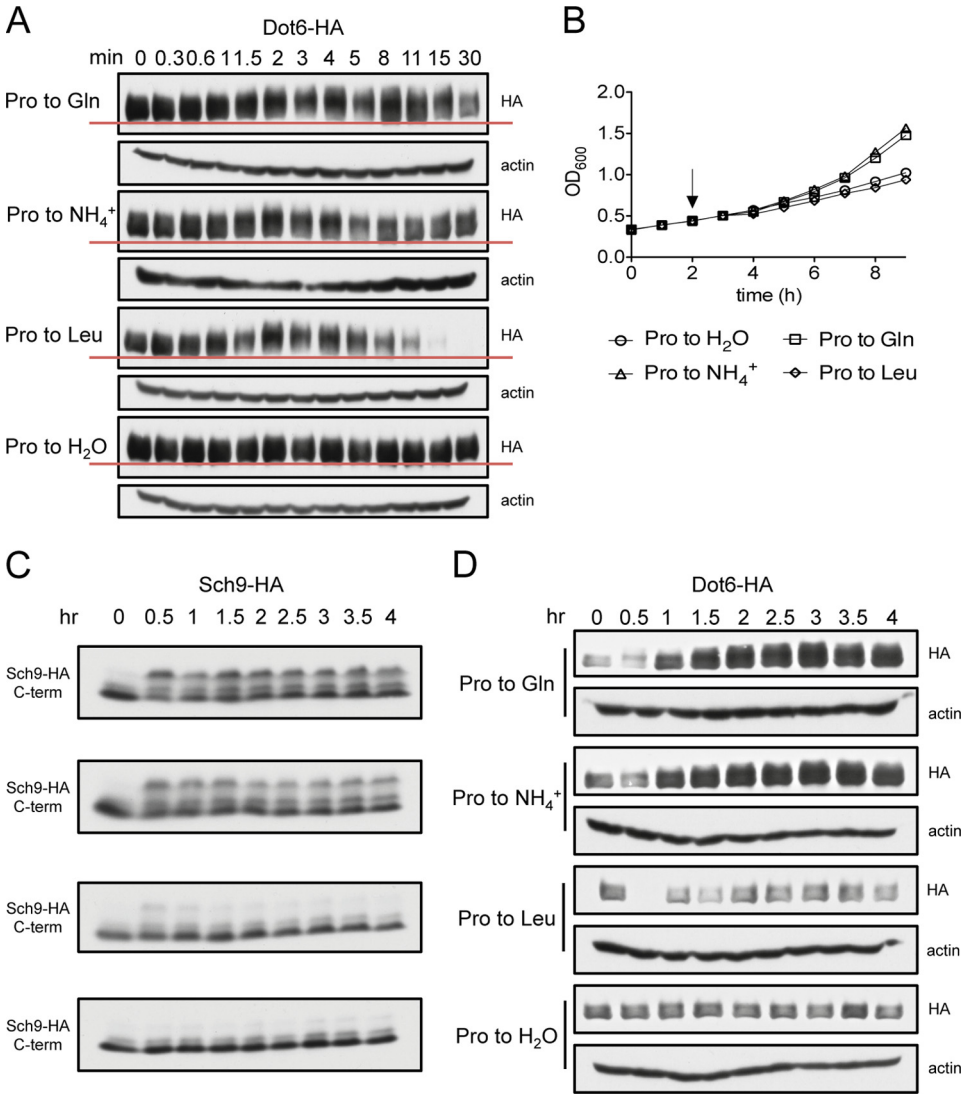


FIGURE 3. **The quality of the nitrogen source couples TORC1 activation to cell growth.** Sch9 is activated after nutrient upshift. *A*, the phosphorylation of the Sch9 direct target Dot6 increases during nutrient upshift from proline to glutamine, leucine, and ammonium but not when treating cells with water. A representative Western blot of protein extracts is shown. The *red line* indicates the fast migrating band of Dot6, which corresponds to the hypophosphorylated form. *B*, cell growth is increased after glutamine and ammonium upshift but not when using leucine or in cells treated with water. A representative growth curve of three independent replicates is shown. Increased phosphorylation of Sch9 C terminus (*C*) and Dot6 correlate (*D*) with the N-source induced increased in cell growth.

upshift on cell growth. Yeast cells grown in the presence of proline were supplemented with glutamine, ammonium, leucine, or water (Fig. 3*B*). After glutamine or ammonium stimulation, growth increased within 2–3 h after treatment compared with unstimulated cells. The observed increase in growth rates paralleled sustained Sch9 and Dot6 phosphorylation up to 4 h after treatment (Fig. 3, *C* and *D*). Conversely, growth rate was unaffected when cells were supplemented with leucine or water, and Sch9 and Dot6 phosphorylation remained low. Thus, stimulation with preferred N-sources confers an increase in TORC1 activity and a corresponding increase in proliferation.

Intracellular Glutamine Is a Metabolic Input to Activate TORC1—As described above, prolonged stimulation of TORC1 by preferred N-sources correlates with increased growth rate. These data suggest that the transient TORC1 activation elicited in the first minutes of nutrient upshift, common to all N-sources tested, is not sufficient to maintain growth. We

FIGURE 2. **The quality of the nitrogen source determines TORC1 activity.** N-sources can be grouped based on the increase in Sch9 C-terminal phosphorylation upon nutrient upshift. *A*, classification of the N-sources based on their ability to sustain TORC1 activity for up to 4 h after shift. The -fold change mean value ± S.D. of Sch9 phosphorylation at five different time points (30, 60, 120, 180, and 240 min after shift) for each N-source is shown in the graph. For statistical analysis we compared in a two-way analysis of variance analysis the values of these five time points for each N-source to the respective values obtained in mock shift experiments with water. In four cases (Arg, Asn, Gln, and NH_4^+) the difference was significant ($p < 0.05$). *B*, -fold change of Sch9 C-terminal relative phosphorylation after nutrient upshift from proline to individual high-end N-sources (arginine, asparagine, glutamine, and ammonium). Relative quantifications are expressed as the mean ± S.E. of at least three independent biological experiments. *C*, -fold change of Sch9 C-terminal relative phosphorylation after nutrient upshift from proline to individual low-end N-sources (alanine, aspartate, glutamate, isoleucine, leucine, methionine, phenylalanine, serine, threonine, tryptophan, and valine). Relative quantifications are expressed as the mean ± S.E. of at least three independent biological experiments.

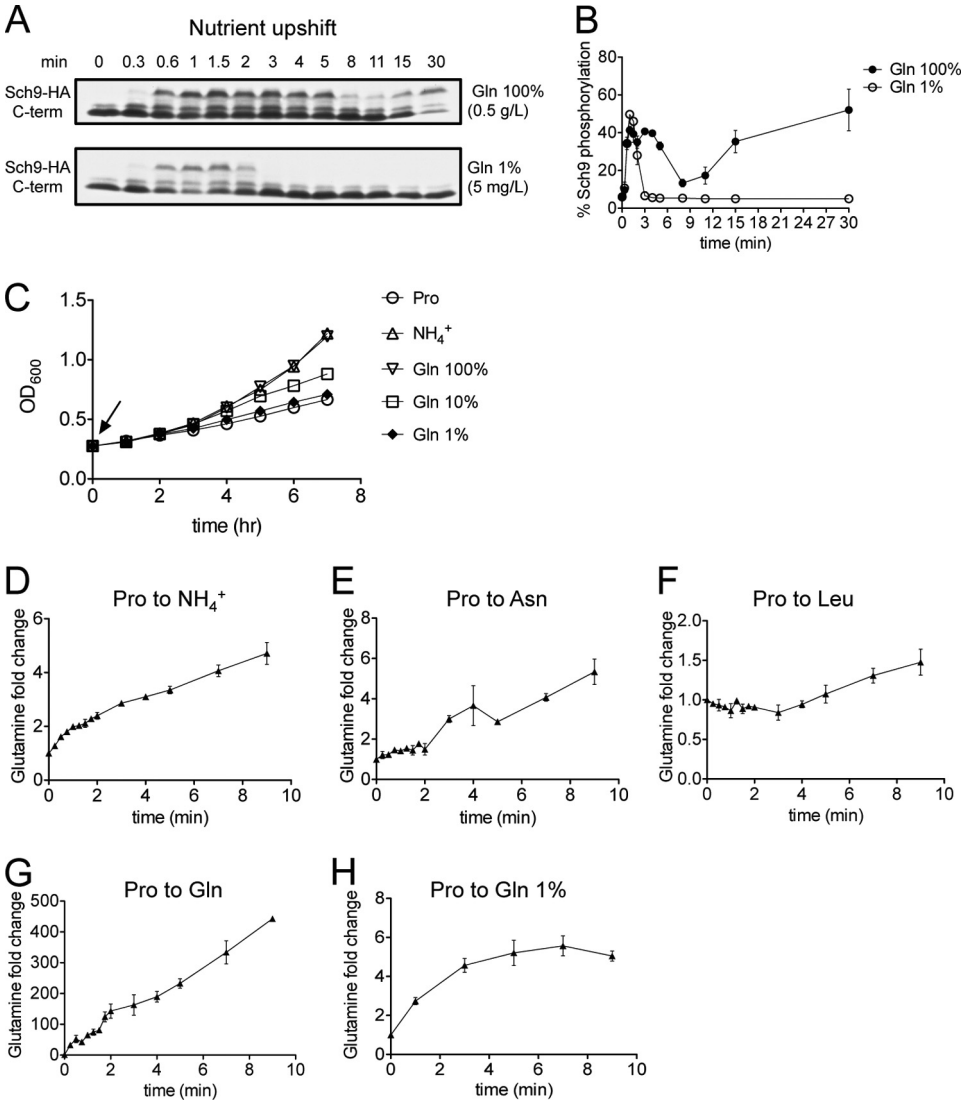


FIGURE 4. **Intracellular glutamine constitutes a metabolic input to activate TORC1.** The increase in cell growth rate after nutrient upshift depends on the quality and quantity of the given N-source. *A* and *B*, nutrient upshift with 1% glutamine (final concentration 5 mg/liter) results in low Sch9 C-terminal phosphorylation at 30 min after shift compared with nominal (100%) glutamine concentration (final concentration 0.5 g/liter). Relative Sch9 C-terminal phosphorylation quantification is expressed as the mean ± S.E. *C*, cell growth rate with different N-sources at different concentrations. Where not specified or when 100%, the final concentration of the N-source was 0.5 g/liter. The *black arrow* indicates the time of stimulation. A representative growth curve of three independent replicates is shown. Glutamine synthesis and accumulation within the first 10 min after nutrient upshift are key features of high-end, preferred N-sources. Shown are intracellular glutamine levels measured by LC-MS/MS during nutrient upshifts with ammonium (*D*), asparagine (*E*), leucine (*F*), and glutamine (*G*) at the final concentration of 0.5 g/liter (100%) and glutamine (*H*) at the final concentration of 5 mg/liter (glutamine 1%). -Fold changes of relative glutamine levels are expressed as the mean ± S.E.

tested this hypothesis by titrating the concentration of glutamine used for the nutrient upshift and monitoring the effects on Sch9 phosphorylation and growth rate. Indeed, a lower final concentration of glutamine (5 mg/liter, Gln 1%) was still able to rapidly induce TORC1 activity but failed to sustain Sch9 phosphorylation over time (Fig. 4, *A* and *B*). The low glutamine concentration also failed to enhance the growth rate of cells grown on proline, whereas an intermediate concentration of glutamine (50 mg/liter, Gln 10%) only partially increased growth (Fig. 4*C*). In conclusion, low concentrations of glutamine, unable to sustain TORC1 activity over time and consequently growth, still trigger an early activation wave of TORC1.

The above data suggest two distinct mechanisms for how N-sources stimulate TORC1. The first mechanism, responsible for the rapid, transient activation of TORC1, is triggered by all N-sources independent of quality and is unable to sustain growth. The second mechanism, in response to good N-sources, mediates sustained TORC1 activity and growth. The sustained stimulation of TORC1 could be dependent on a "metabolic input" that a poor N-source is not able to generate or maintain. To investigate the nature of a potential metabolic input, we measured the changes in relative levels of several metabolites by mass spectrometry, focusing on the first 10 min after nutrient upshift. We reasoned that if a common metabolic input exists, its relative level would increase/decrease within the first minutes after nutrient upshift and upon reaching a certain threshold would sustain TORC1 activity and growth. We consistently found that the intracellular level of glutamine increased in cells stimulated with a preferred N-source such as ammonium, asparagine, or glutamine itself

(Fig. 4*D*, *E*, *G*, and *H*). Interestingly, glutamine levels increased 2-fold already within 2 min after ammonium and asparagine addition, but remained below the 2-fold threshold for up to 9 min after the addition of leucine (Fig. 4*F*). When stimulating with a lower amount of glutamine (1%), we observed a corresponding smaller and transient increase of intracellular glutamine (Fig. 4*H*). These data suggest that intracellular glutamine may be the common metabolite required for TORC1 activation.

Next we investigated the role of glutamine synthesis in TORC1 activation during nutrient upshift. To this end we used the drug methionine sulfoximine (MSX), a specific inhibitor of glutamine synthetase. MSX treatment of cells growing in glutamine-free media leads to an arrest in cell growth due to decreased intracellular glutamine levels and decreased TORC1 signaling (31). As previously reported, MSX treatment compromised cell grown in the presence of ammonium or leucine but not in the presence of glutamine (data not shown). We tested the effect of blocking glutamine synthesis on activation of TORC1 by treating cells for 1 h with MSX before the addition of an N-source. Pretreatment with MSX prevented Sch9 phosphorylation when stimulating with leucine or ammonium (Fig. 5, *D* and *E*.). When stimulating with ammonium, Sch9 phosphorylation was significantly impaired in both the initial and delayed phases of TORC1 activation ($p < 0.01$) (Fig. 5*D*). MSX decreased the transient stimulation observed with leucine ($p < 0.05$) (Fig. 5*E*). However, no significant difference in the increase in Sch9 phosphorylation was observed in cells stimulated with glutamine (Fig. 5*F*). These data suggest that ammonium assimilation into glutamine is essential for ammonium to be sensed and translated into the metabolic input activating TORC1. We conclude that the intracellular level of glutamine is important for TORC1 activation by the N-source. Intracellular accumulation of glutamine constitutes a metabolic input for TORC1 activation in response to a good N-source.

The Metabolic Input Acts Independently of Gtr/Rag in TORC1 Activation—Next, we addressed the mechanism by which the N-source activates TORC1 and how this is related to amino acid-dependent activation of TORC1. Leucine and possibly other amino acids activate TORC1 via EGOC (see the Introduction), whereas the mechanism of TORC1 activation by N-sources such as ammonium and glutamine is unknown. To address the role of EGOC in TORC1 signaling during N-source upshift, we examined glutamine stimulation in strains lacking *GTR1* or *VAM6*. Glutamine failed to stimulate rapid Sch9 phosphorylation in *gtr1*Δ and *vam6*Δ strains but was still able to induce the delayed, sustained Sch9 phosphorylation (Fig. 6, *A* and *B*). The delayed Sch9 phosphorylation in the deletion strains was comparable with that observed in a wild-type strain (Fig. 6, *A* and *B*). We also tested the effect of inhibiting Gtr1 using 1,3-dihydro-1-hydroxy-2,1-benzoxaborole (DHBB). DHBB occupies the editing site in LeuRS, inhibiting the interaction of LeuRS with Gtr1 and favoring hydrolysis of Gtr1-bound GTP (27). DHBB pretreatment had no effect on Sch9 phosphorylation after glutamine stimulation but decreased TORC1 activation upon stimulation with leucine (Fig. 6, *C–F*). These data are consistent with a positive role for EGOC in the rapid, transient activation of TORC1 and at the same time suggest that glutamine accumulation is able to stimulate and sustain TORC1 activity even in the absence of a functional EGOC. Moreover, LeuRS appears to play a role in the specific activation of TORC1 by leucine and is dispensable for the N-source input.

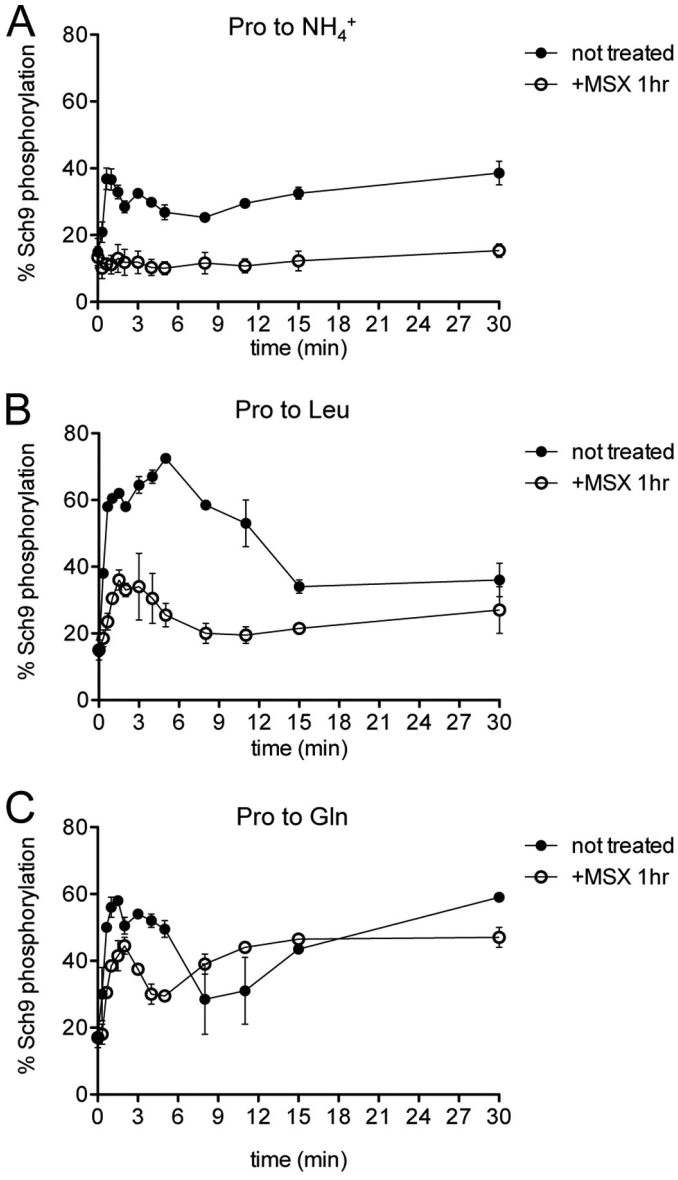


FIGURE 5. **MSX treatment blocks the metabolic input to TORC1.** Glutamine synthesis and accumulation constitute a metabolic input signal to TORC1. MSX pretreatment (2 mM, 1 h) reduced the Sch9 C-terminal phosphorylation increase during nutrient upshift with ammonium (*A*) and leucine (*B*) but not when using glutamine (*C*). Relative quantifications are expressed as the mean ± S.E. Statistical analysis was performed using two-way analysis of variance.

To further investigate the requirement of EGOC for growth, we compared growth of a wild-type and several EGOC mutant strains on solid YMM media supplemented with only one nitrogen source. We used *vam6*Δ, *gtr1*Δ, and *gtr2*Δ strains and strains in which we complemented *GTR1* loss by ectopically expressing wild-type *GTR1* or allelic versions of *GTR1* encoding inactive GDP-bound (*GTR1*^{S20L}) or active GTP-bound Gtr1 (*GTR1*^{Q65L}). Strains lacking *GTR2* grew worse than other EGOC-deficient strains (Fig. 6*G*), indicating a dominant role for *GTR2* over *GTR1* as previously reported (22). This may be

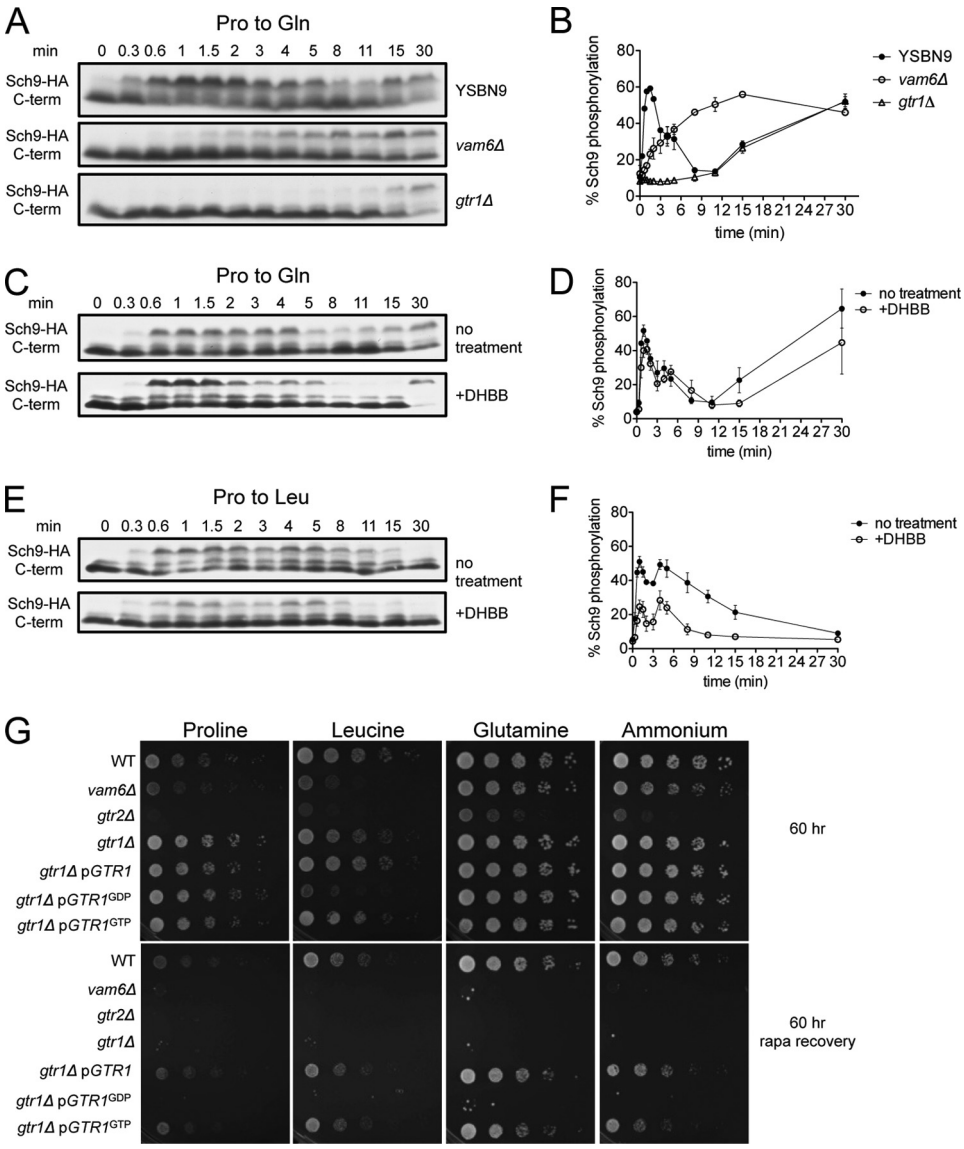


FIGURE 6. **The metabolic input acts independently of the EGO complex in TORC1 activation.** *A* and *B*, after glutamine upshift, only the rapid, transient increase in Sch9 C-terminal phosphorylation is impaired in the *vam6*Δ and *gtr1*Δ strains compared with wild type. DHBB pre treatment (10 μM, 30 min) has no effect on Sch9 phosphorylation after glutamine upshift (*C* and *D*), whereas it affects TORC1 activation in response to leucine stimulation (*E* and *F*). Western blot of NTCB-treated samples (*panel A*, *C*, and *E*) and relative quantifications are expressed as the mean ± S.E. (*panel B*, *D*, and *F*). *G*, growth assay on YMM solid media containing the indicated N-sources at the final concentration of 2.5 mg/ml. In the *lower panel*, cells were plated after rapamycin treatment (200 nM, 6 h). All the indicated strains were initially grown in YMM supplemented with ammonium at the final concentration of 0.5 g/liter and spotted on solid media after serial dilutions (1:5) starting at A_{600} of 0.2.

due to a more prominent role of Gtr2 in permease sorting, although both *GTR2* and *GTR1* were reported to be required for the plasma membrane sorting of Gap1 and Put4 (42). Consistent with this explanation, we found that the *gtr2*Δ strain failed to grow on proline as the sole N-source. Conversely, the *gtr1*Δ strain was viable on proline medium (Fig. 6*G*), indicating that at least in our background (S288c-derived prototroph) Gtr1 is not required for permease sorting to the plasma membrane. Deletion of *GTR1* negatively affected growth only when cells were forced to use leucine as the sole N-source. *GTR1* was dispensable for growth in the presence of the preferred N-source glutamine or ammonium (Fig. 6*G*). Expression of wild-type or constitutively active Gtr1 restored growth of *gtr1*Δ cells on leucine. Expression of inactive GDP-bound Gtr1 further impaired growth on leucine but had no remarkable effect when cells were grown in the presence of glutamine or ammonium (Fig. 6*G*). Also, growth of a *vam6*Δ strain was rescued in the presence of glutamine or ammonium. When grown on proline or leucine, a *vam6*Δ strain displayed a phenotype intermediate to that exhibited by *gtr1*Δ and *gtr2*Δ strains (Fig. 6*G*), suggesting a role for Vam6 in growth regulation in addition to its role as a guanine nucleotide exchange factor for Gtr1. We conclude that preferred N-sources, such as glutamine and ammonium, sustain growth independently of Gtr/Rag, whereas cells cultured in the presence of leucine as the only N-source require functional Gtr/Rag.

The EGOC components Gtr2, Ego1, and Ego3 were initially identified as necessary for restoration of growth after rapamycin treatment (43). Indeed, we found that *vam6*Δ, *gtr1*Δ, and *gtr2*Δ strains, independently of the N-source, were unable to

resume growth after rapamycin treatment (Fig. 6*G*, *lower panel*). In the *gtr1*Δ strain, growth was restored by expression of wild-type as well as constitutively active Gtr1. These data suggest that EGOC is essential for restoration of growth after rapamycin treatment independent of the nutritional environment. Overall, we show that glutamine activates TORC1 independently of Gtr/Rag.

DISCUSSION

Here we investigated *in vivo* activation of TORC1 by N-sources and whether the underlying molecular mechanism requires Gtr/Rag. Both high (glutamine or ammonium) and low quality (leucine) N-sources stimulated a rapid, transient activation of TORC1 in a Gtr/Rag-dependent manner. However, only high quality N-sources sustained TORC1 activity and growth. The preferred N-sources ultimately sustained TORC1 activity via glutamine synthesis and/or accumulation. Intracellular glutamine sustained TORC1 activity independently of Gtr/Rag. These findings suggest that nutrients activate TORC1 via different molecular mechanisms and explain why Gtr/Rag is not essential for TORC1 signaling or viability in the presence of preferred N-sources.

Several observations support our suggestion that Gtr/Rag is not the only mechanism of TORC1 activation by nutrients. First, constitutively active GTP-bound Gtr1 is unable to sustain TORC1 activity after ammonium starvation (22). Second, mutations that likely increase the intracellular levels of glutamine suppress the inability of EGOC mutants to resume growth after rapamycin treatment (43). Third, we show that glutamine but not leucine sustains growth of *vam6* and *gtr1* strains defective in EGOC function. Fourth, we also show that EGOC is required for an initial, transient stimulation of TORC1 in response to all examined nutrients but is not required for sustained TORC1 signaling in response to a good N-source. Thus, a good N-source such as glutamine activates TORC1 via a mechanism independent of EGOC. The decrease in Sch9 phosphorylation between the transient and sustained peaks of TORC1 activity might reflect the alternate onset of the EGO- and the glutamine-dependent mechanisms of TORC1 activation. The mechanism by which glutamine activates TORC1 in the absence of EGOC remains to be determined.

In mammalian cells two models have been proposed to explain how amino acids influence the GTP loading of Rags. In one model, amino acids stimulate RagA/B^{GTP} by a so-called lysosomal inside-out mechanism dependent on the vacuolar ATPase but independent of a proton gradient (44). In a second model glutamine and leucine stimulate glutaminolysis and thereby α-ketoglutarate production that enhances GTP loading of RagA/B, which ultimately activates mTORC1 (45, 46). Strikingly, in yeast, glutamine synthesis and accumulation, rather than glutaminolysis, positively regulate TORC1 activity. This may reflect the metabolic differences between mammalian cells and budding yeast. In proliferating mammalian cells, ATP is produced mainly via mitochondrial oxidative phosphorylation. In this case the flux through the TCA cycle is required to replenish the NADH pool to maintain respiration. Thus, in mammalian cells, the equilibrium α-ketoglutarate ↔ glutamate ↔ glutamine is pushed to the left, requiring α-ketoglutarate to sustain high flux through the TCA cycle (47). Conversely, exponentially growing *Saccharomyces cerevisiae* provided with a fermentable sugar favor glycolysis over respiration even in aerobic conditions and even though glycolysis is less efficient at producing ATP (48, 49). Glucose also affects the expression of genes involved in nitrogen metabolism, such as the NADP-dependent glutamate dehydrogenase encoding genes *GDH1* and *GDH3* (9, 50). In the presence of glucose, *GDH1* is highly expressed and accounts for the reductive amination of α-ketoglutarate to glutamate. Thus, glutamate levels are kept high in the presence of glucose, but ammonium is then required to convert glutamate to glutamine. In this situation, ammonium is limiting for growth (51). Preferred N-sources such as ammonium or glutamine, by favoring glutamine synthesis or accumulation, provide a richer nutritional environment leading to increased TORC1 signaling and faster growth. Non-preferred N-sources, unable to drive glutamine synthesis and accumulation, result in lower TORC1 activation and slower growth.

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Nitrogen Source Activates TOR (Target of Rapamycin) Complex 1 via Glutamine and Independently of Gtr/Rag Proteins

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