The cytosolic form of aspartate aminotransferase is required for full activation of TOR complex 1 in fission yeast

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Activation of TORC1 by amino acids

Abstract
The evolutionarily conserved TOR complex 1 (TORC1) activates cell growth and proliferation in response to nutritional signals. In the fission yeast Schizosaccharomyces pombe, TORC1 is essential for vegetative growth, and its activity is regulated in response to nitrogen quantity and quality. Yet, how TORC1 senses nitrogen is poorly understood. Rapamycin, a specific TOR inhibitor, inhibits growth in S. pombe only under conditions in which the activity of TORC1 is compromised. In a genetic screen for rapamycin-sensitive mutations, we isolated caa1-1, a loss-of-function mutation of the cytosolic form of aspartate aminotransferase (Caa1). We demonstrate that loss of caa1+ partially mimics loss of TORC1 activity and that Caa1 is required for full TORC1 activity. Disruption of caa1+ resulted in aspartate auxotrophy, a finding that prompted us to assess the role of aspartate in TORC1 activation. We found that the amino acids glutamine, asparagine, arginine, aspartate, and serine activate TORC1 most efficiently following nitrogen starvation. The glutamine synthetase inhibitor L-methionine sulfoximine (MSX) abolished the ability of asparagine, arginine, aspartate, or serine, but not that of glutamine, to induce TORC1 activity, consistent with a central role for glutamine in activating TORC1.

TOR is an evolutionarily conserved serine/threonine protein kinase that coordinates cell growth with nutrient availability (1,2). TOR proteins were originally identified in the budding yeast Saccharomyces cerevisiae in a screen for mutations that conferred resistance to the growth inhibitory effect of rapamycin (3-5). Rapamycin is a macrolide compound that has immunosuppressive and anti-proliferative effects, which are beneficial in the treatment of transplant and cancer patients and possibly also in metabolic and neurological disorders (1,2,6). Rapamycin requires an intracellular co-factor for its toxicity, the peptidyl-prolyl cis/trans isomerase- FKBP12 (3). The FKBP12-rapamycin toxic complex binds the FRB (FKBP12-rapamycin binding) domain in TOR, thus inhibiting its activity ([5,7], reviewed in (8)).

TOR proteins are found in two distinct complexes, named TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (9). TORC1 is typically inhibited by rapamycin, while TORC2 is less sensitive to inhibition by the drug (9-11). In human cells, a single TOR gene exists, mTOR, which is found in association with the Raptor protein to form mTORC1, or with the Rictor and mSin1 proteins to form mTORC2 [reviewed in (2)]. Two TOR kinases are present in the fission yeast Schizosaccharomyces pombe, Tor1 and Tor2. These kinases act as the catalytic subunits of the two TOR complexes: S. pombe TORC1 contains mainly the Tor1 kinase, while S. pombe TORC2 contains mainly the Tor1 kinase (12-14). As in other eukaryotes, S. pombe TORC1 positively regulates many different growth-related processes, while inhibiting starvation responses (15).

TORC1 activity is sensitive to nutritional starvation. In particular, withdrawal of the glucose or nitrogen...
from the growth media leads to deactivation of TORC1 in fission yeast (16). The direct signal mechanisms for the activation of TORC1 are as yet unknown; however, a large body of evidence suggests that nitrogen source and amino acids (which may also serve as nitrogen source) play a key and conserved role in TORC1 activation (17,18). Nitrogen is an essential element required for synthesis of amino acids, nucleotides and other cellular components. Yeast cells can sense, take up and assimilate several sources of nitrogen. Studies in S. cerevisiae defined high quality nitrogen sources, such as glutamine, by their ability to promote rapid growth and suppress Nitrogen-Catabolite-Repression (NCR) genes (19). In fission yeast, withdrawal of the nitrogen source from the growth medium, or its replacement with a low quality source of nitrogen (for example, proline instead of ammonia), results in downregulation of TORC1 activity. This is best manifested by a dramatic reduction in the phosphorylation of Psk1, the kinase that lies directly downstream to S. pombe TORC1 (orthologue to human p70 S6 kinase, or in short p70S6K), or reduction in the phosphorylation of the substrate of Psk1, the ribosomal proteins Rps601 and Rps602 (S6 in human) (16,20). In return, S. pombe TORC1 regulates many nitrogen-starvation induced responses, including transcriptional programs (14), via the regulation of the transcription factors Gaf1 (21), Mei2 (22) or the SAGA (Spt-Ada-Gcn5 acetyltransferase) co-activator complex (23), as well as regulation of mitotic commitment and cell-size regulation (24,25). Nitrogen starvation is a strong cue for S. pombe cells to exit the cell cycle and enter the sexual development pathway. Consistent with the idea that nitrogen sufficiency is mediated via TORC1, cells mutated for TORC1 act as nitrogen-starved cells and enter the sexual development pathway under nutrient-rich conditions (12-14,23,26). S. pombe TORC1 is activated by two distinct, highly conserved guanosine triphosphate GTPases: Gtr (Rag in higher eukaryotes) and Rhb1 (Rheb in higher eukaryotes). Similar to their mammalian counterparts, these GTPases and their direct regulators are localized to the vacuolar membrane (the yeast equivalent to the lysosome), where they are found in association with TORC1 (27,28). Rhb1 is regulated by the Tsc1-Tsc2 complex that acts as a GTPase-activating protein (GAP) (29,30). Disruption of rhb1+ results in a nitrogen starvation-like phenotype, similar to disruption of TORC1, while disruption of tsc1+ or tsc2+ results in phenotypes associated with a nitrogen sufficiency signal (29).

In human cells, the TSC-Rheb module is a major hub for regulating TORC1 in response to cellular energy and growth factor signaling [reviewed in (18)]. The AMPK kinase that plays a central role in cellular energy homeostasis activates TSC1-TSC2 to inhibit mTORC1 via the control of Rheb (31). In fission yeast, the AMPK kinase, Ssp2, was shown to transmit a nitrogen stress signal (the shift from good to poor nitrogen source) to TORC1 via TSC-Rhb1, leading to regulation of mitotic commitment and cellular size (24). The Rag/Gtr proteins have been implicated in activation of TORC1 in response to specific amino acids by modulating their nucleotide binding status [reviewed in (17)]. The S. pombe Gtr1-Gtr2 complex appears to play both positive (27) and negative (28,32) roles in regulating TORC1 activity. However, Rag/Gtr1 proteins are not essential for regulating TORC1 in response to amino acids or nitrogen [(27) (33) (34), reviewed in (18)].
Interestingly, rapamycin does not inhibit growth of wild-type *S. pombe* cells, indicating that under normal growth conditions, the essential function of TORC1 is resistant to rapamycin (35). However, rapamycin inhibits growth and induces nitrogen starvation responses when the general activity of TORC1 is reduced. Thus, for example, the growth of *S. pombe* cells becomes sensitive to rapamycin when the activity of *tor2*+ is reduced (13) or in the presence of caffeine, which reduces the activity of TORC1 (36). Here we report the isolation of a loss of function mutation of *caa1*+, encoding the cytosolic isoform of aspartate aminotransferase, as a rapamycin sensitive mutation. We demonstrate that Caa1 is required for full activation of TORC1. We discuss our findings in view of the roles of specific amino acids in TORC1 activation following nitrogen starvation.

**Results**

**Isolation of *caa1-*1 as a rapamycin-sensitive mutation**

*S. pombe* TORC1 is essential for cell proliferation, yet rapamycin does not inhibit vegetative cell growth in this organism (35). Previous studies demonstrated that rapamycin can lead to growth arrest when TORC1 activity is compromised (20,36,37). To further explore TORC1 signaling and its response to rapamycin, we conducted a genetic screen to isolate mutant cells that are sensitive to rapamycin. Cells of the TA16 strain (*leu1-32 ura4-D18 ade6-M216 his*90) were mutagenized using UV irradiation. Following their growth on non-selective plates, cells were replica plated to 0.1 μg/ml rapamycin plates. A screen of 50,000 colonies led to the isolation of six rapamycin sensitive (RS) mutants. Rapamycin sensitivity was confirmed by re-streaking each candidate on nutrient-rich (YE) or minimal (EMM) medium in the presence or absence of rapamycin. Here we describe the characterization of one of these mutants, RS42.

As demonstrated in Fig. 1A, the RS42 mutant strain was sensitive to rapamycin on nutrient-rich plates (YE), compared with the parental strain used for mutagenesis, TA16. When examined on minimal plates (EMM, ammonia used as a nitrogen source), RS42 failed to grow either in the presence or absence of rapamycin. The parental strain shows rapamycin sensitivity on EMM plates due to leucine auxotrophy and the inhibitory effect of rapamycin on leucine uptake, as was previously shown (38).

The RS42 strain was crossed with the auxotrophic, but the otherwise wild-type strain TA2. Tetrad analysis of diploid cells resulting from the cross of TA2 and RS42 revealed a 2:2 segregation of small- versus normal-sized colonies (Fig. 1B). The small colonies were rapamycin-sensitive, while the large colonies were rapamycin-resistant (data not shown), indicating that the rapamycin-sensitive phenotype is dependent on a single mutation and co-segregates with the small-sized colony phenotype.

To identify the mutation that confers rapamycin sensitivity in RS42, RS42 cells were transformed with the *S. pombe* Norbury pREP3X-cDNA library and plated onto minimal medium supplemented with 50 ng/ml rapamycin. Following incubation of five days at 30°C, six out of 28,000 transformants formed colonies on the selective conditions. DNA sequencing of plasmids recovered from these cells showed that they all contained the SPAC10F6.13c gene, a predicted aspartate aminotransferase that shows high similarity with *AAT2*, the *S. cerevisiae* cytosolic aspartate aminotransferase gene (YLR027C).
We named the SPAC10F6.13c gene \( caa1^+ \), for cytosolic aspartate aminotransferase. The aspartate aminotransferase enzyme catalyzes the reversible transfer of the amino group from L-aspartate to 2-oxoglutarate (\( \alpha \)-ketoglutarate) to form oxaloacetate and L-glutamate (Fig. 1C). It plays a critical role in the metabolism of both carbon and nitrogen in all organisms (39,40). DNA sequencing of the \( caa1^+ \) gene in RS42 mutant revealed the presence of a mutation in nucleotide number 76 that results in a stop codon close to the N-terminal region of the gene; hereafter this mutation is referred to as \( caa1-1 \).

**Mutant \( caa1 \) cells display auxotrophy to aspartate**

*Saccharomyces cerevisiae* strains lacking AAT2 are viable in rich medium, but require aspartate in minimal medium (41). Therefore, we examined if the inability of the \( caa1-1 \) mutant cells to grow on minimal medium (Fig. 1A) is rescued by addition of aspartate. In order to avoid any possible complications in using auxotrophic mutant cells, we constructed a prototrophic \( caa1-1 \) mutant strain (TA637). We found that addition of aspartate, but not glutamate, rescued the inability of prototrophic \( caa1-1 \) mutant cells to grow on minimal plates (Fig. 1D). Deletion of the entire open reading frame of \( caa1^+ \) from the genome by replacing it with KanMX (\( Acaa1 \)) resulted in cells that showed the expected slow growth, aspartate auxotrophy and rapamycin sensitivity (Fig. 1E). Addition of aspartate alone (Fig. 1E), or aspartate and glutamate together (Fig. 1G), resulted in a similar phenotype and did not fully suppress the slow growth of \( caa1-1 \) or \( Acaa1 \)mutant cells. This may indicate that the \( caa1^+ \) gene contributes to cellular growth irrespective of its role in aspartate biosynthesis. In addition, supplementation of the medium with aspartate did not rescue the rapamycin-sensitive phenotype of cells lacking \( caa1^+ \) (Fig. 1F).

We next considered the possibility that rapamycin inhibited the uptake of aspartate, thus preventing the suppression of the rapamycin sensitivity by external aspartate. This option was particularly attractive since we previously showed that rapamycin inhibited leucine uptake (38). The sensitivity of leucine auxotrophs to rapamycin is suppressed by reducing the amount or quality of the nitrogen source, which leads to upregulation of general amino acid permeases and to an increase in amino acid uptake (38). Here, we found that addition of aspartate to a medium with lower concentration of ammonium chloride or to a medium in which ammonia was replaced with proline, a poor nitrogen source, did not rescue rapamycin sensitivity in prototrophic \( caa1-1 \) mutant cells (Fig. 1F). Thus, the inability of aspartate in the medium to suppress rapamycin sensitivity in \( caa1 \) mutant cells is unlikely to be due to a defect in aspartate uptake. Rather, loss of \( caa1 \) likely affects rapamycin sensitivity, at least partially, irrespective of aspartate biosynthesis.

**Deletion of the mitochondrial aspartate aminotransferase, \( maa1^+ \), does not result in rapamycin sensitivity**

In addition to the cytosolic form of the enzyme, mitochondrial isoenzymes exist both in *S. pombe* and *S. cerevisiae*. The *S. cerevisiae* mitochondrial isoenzyme is called AAT1 (YKL106W). We named the *S. pombe* homologue, SPBC725.01, \( maa1^+ \) for mitochondrial aspartate aminotransferase. In both yeasts the cytosolic and mitochondrial forms share about 48% identity (39,42). We examined whether \( maa1^+ \) is required
for rapamycin resistance by disrupting the entire open reading frame of the gene (\(\Delta maal\)). Cells deleted for maal\(^+\) were plated on nutrient-rich plates with or without rapamycin, together with wild-type and caa1-l cells as controls. In contrast to caa1-l, cells disrupted for maal\(^+\) are resistant to rapamycin (Fig. 2A), indicating that maal\(^+\) is not required for rapamycin resistance. \(\Delta maal\) cells were unable to grow on minimal medium (EMM), however, addition of aspartate or glutamate did not rescue this lethality (Fig. 2A). Thus, unlike caa1\(^+\), maal\(^+\) plays a different role in auxotrophy and rapamycin sensitivity.

The catalytic site of Caa1 is required for rapamycin resistance
Aspartate aminotransferases form a large protein subfamily with considerable diversity among its members. Yet, within the active catalytic site, 13 absolutely conserved residues have been identified (40,43). One of these residues is a conserved lysine that resides at the bottom of the active site that forms a Schiff base with the pyridoxal-phosphate (PLP) coenzyme. To test whether the catalytic activity of Caa1 is required for rapamycin resistance, we mutated the conserved lysine residue within the pyridoxal phosphate-binding site of Caa1, lysine 255, into arginine (Caa1\(^{K255R}\)). Cells carrying the caa1-\(K255R\) mutation exhibited a slow growth phenotype on nutrient-rich medium, failed to form isolated colonies on minimal medium and were sensitive to rapamycin (Fig. 2B). The protein level of Caa1\(^{K255R}\) is comparable to that of Caa1 (Fig. 2C). Thus, the enzymatic activity of Caa1 is required for rapid growth, aspartate biosynthesis and rapamycin resistance.

Rapamycin sensitivity in caa1 mutant cells is FKBP12-dependent
and results from inhibition of TORC1 and not TORC2
Since rapamycin inhibits TOR proteins only when it is found in a complex with the FKBP12 homolog (3), deletion of fkh1\(^+\), the S. pombe homolog of FKBP12, is expected to render cells resistant to rapamycin. Thus, for example, the rapamycin sensitivity of cells auxotrophic to leucine is suppressed by deletion of fkh1\(^+\) (38). In order to examine whether the rapamycin sensitivity of caa1-l mutant cells is dependent on the formation of the rapamycin-FKBP12 complex, we deleted the fkh1\(^+\) gene in caa1-l strain. Cells were plated on nutrient-rich medium with or without rapamycin, together with wild-type and caa1-l strains, as controls. Deletion of fkh1\(^+\) completely rescued the rapamycin sensitivity of caa1-l mutant cells (Fig. 3A), arguing that sensitivity to rapamycin in caa1 mutant cells is TOR-dependent and the result of inhibition of Tor1, Tor2 or both. A conserved serine residue within the FRB domain of either Tor1 or Tor2 is highly critical for the binding of the FKBP12-rapamycin complex (44). This serine is found at position 1834 or 1837 in Tor1 or Tor2, respectively. Mutations at the conserved serine residue confer rapamycin resistance by abolishing the binding of the FKBP12-rapamycin complex to the FRB domain (44,45). We used the rapamycin-binding defective alleles tor1S1834E (tor1SE) or tor2S1837E (tor2SE) to uncover the target of rapamycin in caa1 mutant cells. We constructed \(\Delta caa1\) tor1SE and \(\Delta caa1\) tor2SE double mutant strains and plated the cells on nutrient-rich medium with or without rapamycin. The mutation at the FRB domain in Tor2, but not the equivalent mutation in Tor1, rescued the inability of \(\Delta caa1\) mutant cells to grow in the presence of rapamycin...
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(Figure 3B). These findings demonstrate that the target for rapamycin inhibition in cells lacking the caa1+ gene is Tor2 (TORC1) and not Tor1 (TORC2).

Loss of function of Caa1 results in a hyper-mating phenotype

Microscopic examination of caa1-1 or Δcaa1 mutant cells revealed a hyper-mating phenotype, as cells underwent mating and sporulation in nutrient-rich medium, conditions that normally suppress sexual development. Thus, while wild-type cells of opposite mating types do not enter the sexual development pathway on nutrient-rich medium, Δcaa1 mutant cells exhibited about 6-7% mating efficiency under such conditions (see Fig. 3C). The activity of two central signaling pathways, protein kinase A (PKA) and TORC1, is known to suppress sexual development on rich medium. PKA is a signaling pathway that couples carbon availability to growth and proliferation and inhibits sexual differentiation (46). Accordingly, deletion of pka1+, the catalytic subunit of PKA, results in initiation of sexual development under nutrient-rich conditions (47,48). TORC1 couples nitrogen sufficiency to growth control and inactivation of TORC1 using conditional mutations of the catalytic subunit Tor2 or the auxiliary subunit Mip1 (Raptor in human cells) result in hyper-mating under rich medium conditions (12,14,26).

We used the hyper-mating defect in order to examine genetic interactions between Δcaa1 and mutations in the TORC1 or PKA pathways. We created the double mutant strain Δcaa1 Δpka1 and microscopically examined the mating efficiency under rich medium conditions. We observed that the double mutant Δcaa1 Δpka1 exhibited mating efficiency of about 30%, which is the sum of the mating efficiencies of each of the single mutations (~7% and 22%; Fig. 3C). This finding indicates an additive effect between Δcaa1 and Δpka1 and suggests that caa1+ and pka1+ act on two parallel signaling pathways that influence sexual development.

Next, we combined the Δcaa1 with the tor2 temperature-sensitive allele (tor2-51). The tor2-51 mutation results in a partial activity of tor2+ at the permissive temperature that leads to about 19% mating efficiency on rich medium (Fig. 3C). We observed that the mating efficiency of the double mutant cells tor2-51 Δcaa1 was similar to that of single Δcaa1 mutant cells (about 6%), indicating that the Δcaa1 mutation is epistatic to tor2-ts and suggesting that tor2+ and caa1+ act in the same signaling pathway.

Gtr1 and Gtr2, the S. pombe homologues of the mammalian Rag GTPases, form a heterodimer that functions upstream of TORC1 and mediates amino acid signaling. Cells disrupted for either gtr1+ or gtr2+ also show a hyper-mating phenotype on rich medium (27), presumably due to lower activity of Tor2 (TORC1). We detected about 3-4 % mating efficiency of Δgtr1 cells on nutrient-rich medium (Fig. 3C). The mating efficiencies of the double mutant cells Δcaa1 Δgtr1 were similar to the mating efficiency of single Δgtr1 mutant cells, supporting the possibility that Caa1 and TORC1 reside on that same signaling pathway. Taken together, our genetic interaction analyses support a model in which Caa1 acts in parallel with the PKA pathway, but resides on the same pathway as TORC1.

TORC1 activity is reduced in caa1 mutant cells

Psk1 is the fission yeast homolog of the human S6 kinase (p70S6K) and is phosphorylated directly by TORC1 (16). We used an antibody that...
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recognizes the mTORC1-phosphorylated form of p70S6K at Thr389, which cross-reacts with the Tor2-dependent phosphorylated form of Psk1 (15,28) (hereafter referred to as the anti-Psk1-P antibody). Prototrophic strains were used for all the experiments described below in order to avoid complications of having to add amino acids to supplement for auxotrophy. As expected, in wild-type cells, the anti-Psk1-P antibody detected a single band in rich, YE medium, which disappeared following a 60 minutes shift to EMM medium containing no-nitrogen source (Fig. 4A, WT). Adding aspartate to the no-nitrogen medium partially suppressed the loss of activity of TORC1 in wild-type cells (Fig. 4A, WT). The level of Psk1 phosphorylation in Δcaa1 mutant cells was dramatically reduced in rich medium (Fig. 4A, Δcaa1), indicating that Caa1 is required for full activation of TORC1. Addition of aspartate to no-nitrogen medium did not significantly improve TORC1 activity in Δcaa1 cells (Fig. 4A, Δcaa1). Thus, Caa1 is required for TORC1 activity at least partially irrespective of its role in aspartate biosynthesis, consistent with the inability of aspartate to suppress rapamycin sensitivity in Δaa1 mutant cells (Fig. 1E).

In parallel to using the anti-Psk1-P antibody, we also raised anti-Psk1 antibodies in order to detect the total level of Psk1 (see Experimental Procedures). The anti-Psk1 antibody detected two major bands, an upper band corresponding to the phosphorylated form of the protein, which was predominant in rich medium and a lower band, corresponding to the unphosphorylated form, which was predominant in no-nitrogen medium (see Psk1, Fig. 4A). In general, the results obtained using the anti-Psk1 antibody correlated well with the results obtained with the anti-Psk1-P antibody and supported a reduced level of TORC1 activity in caal mutant cells. However, repeated experiments showed the anti-Psk1-P antibody produced more reliable and consistent results for assessing the state of phosphorylation of Psk1. This is likely due to limitations of the band shift technique that may reflect multiple modifications. The anti-Psk1 blots were used to quantify the amount of phosphorylated Psk1 compared with total Psk1 (Fig. 4A).

Shifting cells from rich medium to media containing aspartate, glutamine, arginine or serine showed a moderate reduction in TORC1 activity in wild-type cells, as judged by the level of Psk1 (Fig. 4B). In contrast, Δcaa1 cells showed relatively low activity of TORC1 in rich medium, which remained low upon shift to media containing the above amino acids as nitrogen source (Fig. 4B). Similar results were obtained for Psk1-P in catalytic subunit mutant caal-K225 mutant cells (Fig. 4C). Taken together, our results demonstrate that without Caa1, or in catalytically compromised caal mutant cells, TORC1 activity is compromised.

Loss of Caa1 does not induce AMPK (Ssp2) activation

Branched-chain aminotransferases (BCATs) have recently been demonstrated to be required for full activation of TORC1 in S. cerevisiae (49). BCATs affect TORC1 activity by a mechanism involving the TCA cycle flux, glutamine levels and the AMPK kinase (49). The S. pombe AMPK homologue, Ssp2, has been shown to negatively regulate TORC1 in response to nitrogen stress (24). To determine whether loss of caal+ leads to activation of AMPK, we followed the level of phosphorylation of Ssp1 at Thr189, a well-known readout for activation of AMPK (24). However,
we did not observe activation of Ssp2 in \textit{caal} mutant cells (Fig. 4D). Thus, Caa1 does not appear to downregulate TORC1 via Ssp2.

A partial correlation between the ability of specific amino acids to re-activate TORC1 and their ability to support high growth rate

It was previously shown that glutamine was efficient in inducing re-phosphorylation of Psk1 following nitrogen starvation, while glutamate, proline or leucine failed to reactivate TORC1 (16). Since \textit{caal} mutant cells are rapamycin-sensitive, as well as auxotrophic to aspartate, we were interested in examining the role of aspartate, relative to other amino acids, in the activation of TORC1.

Wild-type cells were grown to mid-log phase before being subjected to nitrogen starvation for one hour, followed by addition of each amino acid at the final concentrations of either 1mM or 5mM for 20 minutes (Fig. 5A). We found that exposure of nitrogen-starved cultures to 5 mM of glutamine, asparagine, arginine, aspartate or serine induced re-phosphorylation of Psk1. No, or very minor activation of TORC1 was observed at the final concentration of 1 mM of any of the above amino acids tested, or when the nitrogen-starved cultures were exposed to glutamate, leucine, methionine, valine, isoleucine or threonine at 1 or 5 mM. Our findings showing a positive role for glutamine in the activation of TORC1, while no such effect is observed for glutamate or leucine, are consistent with previous reports (16).

Time-course experiments, which examined the phosphorylation of Psk1 following addition of amino acids for 5 or 20 minutes (at the concentration of 5 mM) demonstrated that glutamine, asparagine, arginine and aspartate strongly reactivated the phosphorylation of Psk1 as early as 5 minutes after exposure, while serine was able to re-activate TORC1 only following 20 minutes of exposure (Fig. 5B). Again, glutamate, methionine, leucine, valine, isoleucine or threonine did not support re-activation of TORC1 (Fig. 5B). Based on these findings, we divided the different amino acids into strong (glutamine, asparagine, arginine or aspartate), intermediate (serine) and poor (glutamate, methionine, leucine, valine, isoleucine and threonine) activators of TORC1 (Fig. 5C). Interestingly, in \textit{S. cerevisiae} glutamine, asparagine and arginine were also classified as the most efficient amino acids for stimulating rapid and sustained TORC1 activity (34). It was suggested that there is a correlation between the ability of these amino acids to act as preferable nitrogen sources and their ability to act as good activators of TORC1 (34). In \textit{S. pombe}, glutamic acid is often used as a proxy for a "good" nitrogen source, while proline is used as a "poor" nitrogen source (50). Yet a systematic examination of the different amino acids for their ability to support growth is lacking. We determined the growth rates of \textit{S. pombe} cells on media containing each of the above amino acids as a sole nitrogen source (Fig. 6A). We found that glutamine best supported \textit{S. pombe} growth, whereas ammonia, arginine, asparagine and aspartate closely followed. Glutamate supported an intermediate-to-fast growth rate, while the remaining amino acids could poorly support growth. Our data thus demonstrate a partial correlation between the quality of the nitrogen source and its ability to re-activate TORC1: glutamine asparagine, arginine and aspartate support fast growth and are good activators of TORC1; glutamate is a relatively good
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Nitrogen source, but a poor activator of TORC1; serine on the other hand, poorly supports growth, but is an intermediate activator of TORC1. Notably, aspartate is a good nitrogen source and a good activator of TORC1 in *S. pombe* (Fig. 6A), but not in *S. cerevisiae* (34). This may reflect metabolic differences between the two yeasts, which are yet to be determined.

**Re-activation of TORC1 by asparagine, arginine, aspartate or serine requires the activity of glutamine syntethase**

It was demonstrated in *S. cerevisiae* and later in *S. pombe* that methionine sulfoximine (MSX), a specific inhibitor of glutamine synthetase, diminishes TORC1 activity in ammonia-based medium (16,34,51). Inhibition of TORC1 activity by MSX was efficiently suppressed by addition of glutamine to the medium, leading to the suggestion that TORC1 activity in yeast responds to glutamine levels (16,34). As previously shown (16), MSX did not affect reactivation of TORC1 when glutamine was added to the medium (Fig. 6B). In contrast, MSX inhibited the reactivation of TORC1 by asparagine, arginine, aspartate or serine, suggesting that these amino acids must be converted to glutamine in order to induce TORC1 activity.

**Discussion**

The growth of *S. pombe* cells is resistant to inhibition by rapamycin, yet reduction of TORC1 signaling has been shown to lead to rapamycin sensitivity (13,36,52). Thus, genetic screens to identify rapamycin-sensitive mutant cells can help identify genes that are involved in TORC1 signaling. In this study, we describe the isolation and characterization of a rapamycin-sensitive mutant carrying a loss of function mutation in *caa1*+, the cytosolic isoform of the aspartate aminotransferase gene. Disruption of *caa1*+ leads to slow growth, hyper-mating and auxotrophy to aspartate. The rapamycin sensitivity and hyper-mating phenotypes observed in *caa1* mutant cells are reminiscent of the phenotype of loss of function of *S. pombe* TORC1 (12,13,26). Consistently, our results indicate that under rich nitrogen conditions, there is a decrease in TORC1 activity in *caa1* mutant cells compared to wild-type cells.

The mechanism by which aspartate aminotransferase regulates TORC1 in *S. pombe* cells is yet to be determined. However, cells carrying a catalytic site mutant, *caa1*-K255R, show reduced levels of TORC1 activity and are sensitive to rapamycin, similar to complete loss of *caa1*+. Thus, it appears that the loss of TORC1 activity depends on Caa1 catalytic activity.

Branched-chain aminotransferases (BCATs) are required for full activation of TORC1 in *S. cerevisiae*, via multiple modes of activation, including activation of the EGO-Gtr complex (equivalent to the mammalian Ragulator-RAG complex) (49). The equivalents in *S. pombe*, the Gtr1-Gtr2 GTPases, Ragulator and GATOR1-like complexes have been implicated in TORC1 regulation in response to amino acids (27,28,32,45,53). We have found epistatic relationship between Δgtr1 and Δcaa1 with respect to induction of sexual development under rich conditions (Fig. 3C), suggesting that Caa1 and Gtr1 may affect sexual development via the same mechanism. In *S. pombe*, the Ragulator-Gtr1-Gtr2 pathway has a dual role in regulating TORC1, as it positively regulates TORC1, but is also required to attenuate its activity (27,28,32,53). The relationship between Caa1 activity and the Gtr1-Gtr2 GTPases is thus
expected to be complex and is beyond the scope of this work. We classified eleven amino acids based on their ability to re-activate TORC1-dependent Psk1 phosphorylation. We identified glutamine, asparagine, arginine and aspartate as amino acids that strongly re-activate TORC1 following nitrogen starvation. Serine exhibited an intermediate capacity to re-activate TORC1, while glutamate, methionine, leucine, valine, isoleucine and threonine showed very poor or no activation of TORC1. Interestingly, glutamine, asparagine and arginine have also been identified as the amino acids that best supported induction of S. cerevisiae TORC1 activity (34). Stracka et al. suggested that the ability of an amino acid to act as a good activator of TORC1 correlated with its property as a preferable nitrogen source. The identification of glutamine, asparagine and arginine as good inducers of TORC1 and good nitrogen sources is in agreement with this suggestion. Also in agreement with studies in S. cerevisiae (34), we found that the inhibitor of glutamine synthetase (MSX) inhibits the ability of arginine, asparagine, aspartate or serine to induce TORC1 activity, suggesting that for ammonia to be sensed, it needs first to be assimilated into glutamine. Still, our findings indicate several differences in the two yeasts: glutamate is a relatively good nitrogen source (Fig. 6A), yet it does not induce TORC1 activity. On the other hand, serine is a good activator of S. pombe TORC1, but acts as a poor nitrogen source for growth. In addition, aspartate acts as a good activator of TORC1, but not in S. cerevisiae. To resolve the above inconsistencies and differences in the two yeasts, the mechanism by which glutamine induces TORC1 activity needs to be further explored. Importantly, addition of glutamine did not induce TORC1 activity in caa1 mutant cells. Therefore, for glutamine to be sensed by TORC1, cytosolic aspartate aminotransferase activity should be intact.

Experimental Procedures

Yeast strains and growth conditions. S. pombe strains are described in Table 1. All the media in this study are derived from the S. pombe media described in (50). Complex medium (YE) contains 3% glucose, 0.5% yeast extract, 150 mg/l adenine and 75 mg/l uracil. The minimal medium used (EMM, Edinburgh minimal medium) contains 2% glucose and 5 g/l NH4Cl; salts, minerals and vitamins as detailed in (54). Minimal media with either no nitrogen EMM-N (-N) or limited nitrogen source (lowEMM) are minimal medium as described above with either no ammonia added or 3 gr/L ammonia. For proline plates (Pro), ammonium chloride of the EMM medium was replaced with 10 mM proline. For the growth of auxotrophic strains, minimal media plates were supplemented to a final concentration of 75 mg/L histidine, 75 mg/L adenine, 80 mg/L uracil and 225 mg/L leucine. Aspartate or Glutamate was added at a final concentration of 100 mg/L. Rapamycin (Sigma) was used at a final concentration of 100 ng/ml.

Construction of gene disruptions and site-directed mutagenesis. Gene deletions were carried out by homologous recombination. PCR was performed using the pFA6a-kanMX6 plasmid as a template (55) with primers #1201 and #1202 for caa1 deletion, #1205 and #1206 for maa1 deletion. The PCR fragment was purified and transformed into wild-type strain. Correct integration at the indicated genes was validated by PCR. Site-directed mutagenesis of the conserved lysine residue in Caa1 into...
arginine (K255R) was carried out by PCR amplification with Phire DNA polymerase (Thermo Fisher Scientific), using the caa1-myc-KanMX cassette as template and complementary primers which contain the desired mutation. The caa1-K225R-myc-KanMX fragment was amplified and integrated into wild-type 972 h- strain and G418 resistant colonies were selected. The presence of the desired mutations was confirmed by DNA sequencing.

**Mating efficiency assays.** Mating efficiency was determined as follows: cells from a fresh patch of homothallic strains were grown to logarithmic phase to a density of approximately 5X10^6-1X10^7 cells/ml in rich medium. Then, the cultures were spotted on solid rich media at a density of 5 X 10^5 cells/ml and incubated at 30°C for three overnights. After the incubation, a toothpick was used to pick some of the cells from the center of each patch, and the cells were examined microscopically. The percentage of mating was calculated by dividing the number of zygotes, asci, and free spores by the number of total cells. One zygote or one ascus was counted as two cells, and one spore was counted as a half cell.

**Protein extraction.** 25ml of logarithmic growing cells were harvested and re-suspended in 200μl of 20% trichloroacetic acid (TCA). After addition of the same volume of glass beads, cells were broken by vortexing for 15' in a cold room (4°C). Glass beads were washed twice with 200μl of 5% TCA and the resulting extract was centrifuged for 10 minutes in 13,000 rpm at 4°C. The pellet was re-suspended in 200μl 2X Sample Buffer containing 4% Sodium-Dodecyl-Sulfate (SDS) 20% glycerol, 0.12% EDTA, 2.4% Tris-HCl pH 6.8, 34 mg/ml Dithiothreitol (DTT) and a small drop of Bromophenol blue. 100μl of 1M Tris base was added to each sample. The samples were boiled for 3' and centrifuged before loading.

**Western blotting.** Protein extracts were resolved on SDS-PAGE using 10-12% acrylamide gels. Gels were run using TG-SDS running buffer (Bio-lab) at 50 volts for 15 minutes and then at 150 volts for an additional hour. The proteins were transferred in TG buffer (Bio-lab) to nitrocellulose membranes (Amersham) using 400 mAmp for 90 minutes. Membranes were treated with blocking solution containing 5% milk in TBST buffer (Bio-lab) for 1 hour. Psk1 phosphorylation was detected using anti-phospho-p70S6 kinase Thr389 (Cell Signaling, catalog #9206), total Psk1 protein was detected by antibodies raised against the Psk1 phosphopeptide NCEFLSNNAVSNH (Bio Basic Canada Inc) and actin was detected using anti-actin antibody (MP-Biomedicals, catalog #691001). Phosphorylation of Ssp2 was detected using anti-phospho-AMPK-alpha-Thr172 (Cell Signaling, catalog #2535). Detection was carried out using the ECL SuperSignal detection system (Thermo Scientific).

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**Conflict of interest**
The authors declare that they have no conflicts of interest with the contents of this article.
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Table 1: *S. pombe* strains used in this study

| Strain  | Genotype                                   | Notes               |
|---------|--------------------------------------------|---------------------|
| TA0001  | 975 h+                                     | Lab stock           |
| TA0002  | *leu1-32 ura4-D18 ade6-M210 h*             | Lab stock           |
| TA0003  | *leu1-32 ura4-D18 ade6-M210 h*+            | Lab stock           |
| TA0016  | *leu1-32 ura4-D18 ade6-M216 h*             | Lab stock           |
| TA0267  | *caal-1 ura4-D18 leu1-32 ade6-M216 h*+     | This study          |
| TA0637  | *caal-1 h*                                 | This study          |
| TA0359  | *caal-1 fkh1::ura4 leu1-32 ura4-D18 ade6-M216 h*+ | This study          |
| TA0400  | *tsc2::G418 leu1-32 ura4-D18 ade6-210 his7-366 h* | Lab stock          |
| TA0505  | *pkal::ura4 leu1-32 ura4-D18 ade6-M216 h*+  | C. Hoffmann         |
| TA0657  | *tor2-51 ura4-D18 h*                       | S. Moreno (12)      |
| TA1397  | *tor2S1837E<kanMX6 h*                      | Lab stock (45)      |
| TA1463  | *tor1S1834E<kanMX6 h*                      | Lab stock (45)      |
| TA1547  | *gtr1::KanMX6 h*                           | S. Moreno (27)      |
| TA1719  | *tor2-ts6 leu1-32 ura4-D18 ade6-M216 h*+   | M. Yamamato (14)    |
| TA2867  | *caal::kanMX6 leu1-32 ura4-D18 ade6-M216 h* | This study         |
| TA2883  | *maa1::kanMX6 leu1-32 ura4-D18 ade6-M216 h*+ | This study         |
| TA2894  | *caal::kanMX6 leu1-32 ura4-D18 ade6-M216 h*+ | This study         |
| TA2908  | *caal::kanMX6 pkal::ura4 leu1-32 ura4-D18 ade6-M216 h*+ | This study |
| TA2909  | *caal::kanMX6 h*                           | This study          |
| TA2957  | *caal-13MYC<kanMX6 leu1-32 ura4-D18 ade6-M210 h* | This study |
| TA2968  | *tor2-51 ura4<caal::kanMX6 ura4-D18 leu1-32 ade6-M216 h*+ | This study |
| Strain   | Genotype                                | Study       |
|----------|-----------------------------------------|-------------|
| TA2971   | caa1::HYG leu1-32 ura4-D18 ade6-M216 h<sup>90</sup> | This study  |
| TA3020   | tsc2::G418 caa1::HYG leu1-32 ura4-D18 ade6-210 his7-366 h<sup>90</sup> | This study  |
| TA3054   | tsc2::G418 h<sup>90</sup>               | This study  |
| TA3055   | gtr1::G418 h<sup>90</sup>               | This study  |
| TA3093   | gtr1::KanMX6 leu1-32 ura4-D18 ade6-M210 h' | This study  |
| TA3215   | tor1S1834E<kanMX caa1::HYG leu1-32 ura4-D18 h' | This study  |
| TA3216   | tor2S1837E<kanMX6 caa1::HYG leu1-32 ura4-D18 h' | This study  |
| TA3218   | caa1::HYG gtr1::KanMX6 leu1-32 ura4-D18 ade6-M? h<sup>90</sup> | This study  |
| TA3278   | psk1-13MYC<HYG caa1::KanMX6 leu1-32 h' | This study  |
| TA3395   | caa1-Lys255Arg-13MYC<kanMX6 leu1-32 ura4-D18 ade6-M210 h' | This study  |
Figure legends

Figure 1. Isolation of caa1-1 as a rapamycin-sensitive mutation. (A) RS42 (TA267) and the otherwise isogenic wild-type strain (TA16) were plated on rich (YE) or minimal (EMM) media in the presence or absence of 0.1 μg/ml rapamycin. RS42 cells are rapamycin-sensitive on YE plates and do not form isolated colonies on minimal medium. (B) Tetrad analysis of a genetic cross between RS42 and wild type. RS42 carries a single mutation that leads to rapamycin sensitivity and small-sized colonies. (C) The enzymatic reaction catalyzed by amino acid aspartate. (D) The prototrophic caa1-1 (TA637) and a prototrophic wild type (TA1) strains were plated on YE, EMM and EMM supplemented with glutamate or aspartate. Only aspartate supports growth of caa1-1 on EMM (E) Addition of aspartate to minimal medium does not suppress the rapamycin sensitivity of caa1 mutant cells. Prototrophic caa1-1 (TA267), Δcaa1 (TA2867) and wild-type cells (TA2) were streaked on EMM or EMM containing aspartate with or without rapamycin. (F) Addition of aspartate to medium with lower concentration of ammonium chloride or proline as a sole nitrogen source does not rescue the rapamycin sensitivity of the caa1 mutant cells. caa1-1 (TA637) and wild-type cells (TA1) were plated on EMM plates containing aspartate with standard or low concentration of ammonium chloride (EMM 5gr/L and EMM 3gr/L ammonia, respectively) and EMM containing proline as the sole nitrogen source. (G) Addition of aspartate and glutamate results in a similar effect to addition of aspartate alone. Prototrophic caa1-1 (TA267), Δcaa1 (TA2867) and wild-type cells (TA2) were spotted onto the indicated plates following serial dilutions. All plates were incubated at 30°C for 4 days.

Figure 2. Δmaa1 is rapamycin resistant. (A) Wild type (TA16), Δmaa1 (TA2883), and caa1-1 (TA267) cells were streaked on YE in the presence or absence of rapamycin and on EMM plates with or without addition of aspartate or glutamate. The plates were incubated at 30°C for 4 days. (B) The catalytic site of Caa1 is required for rapamycin resistance. Wild type (TA2957), Δcaa1 (TA2867), Caa1-K255R (TA3395) cells were plated on YE plates with or without rapamycin and on EMM plates. The plates were incubated at 30°C for 4 days. (C) Western Blot analysis. Protein extracts of cells carrying Caa1-MYC or Caa1-K225R-MYC were analyzed with anti-MYC and anti-actin antibodies.

Figure 3. Caa1 is part of the TORC1 signaling pathway. (A) Deletion of fkh1+ rescued the rapamycin sensitivity of caa1-1 mutant cells. Wild-type (TA16), caa1-1 (TA267) and caa1-1 Δfkh1 (TA359) cells were plated on YE plates with or without rapamycin. The plates were incubated at 30°C for 4 days. (B) tor2SE (tor2ser1837glu), but not tor1SE (tor2ser1834glu) suppresses rapamycin sensitivity of caa1 mutant cells. Wild-type (TA16), Δcaa1 (TA2971), tor1SE (TA1463), tor2SE (TA1397), Δcaa1 tor1SE (TA3215) and Δcaa1 tor2SE (TA3216) cells were plated on YE plates with or without rapamycin. The plates were incubated at 30°C for 4 days. (C) Epistatic interactions between the Δcaa1 and tor2-ts mutations. Mating efficiencies in rich medium (YE) of Δcaa1 (TA2894), Δpka1 (TA0505), Δcaa1Δpka1 (TA2908), tor2-ts (TA1719), Δcaa1tor2-ts (TA2968), Δtsc2 (TA3054), Δcaa1Δtsc2 (TA3020), Δgtr1 (TA3055), and Δcaa1Δgtr1 (TA3218) cells.

Figure 4. TORC1 activity is reduced in caa1 mutant cells. (A) Prototrophic wild-type (TA0001) or Δcaa1 mutant cells (TA3965) were grown in YE to logarithmic
Activation of TORC1 by amino acids

...phase and then shifted into EMM-N (-N) with or without 5 mM aspartate (Asp). The extracted proteins were analyzed by immunoblotting with anti-phospho S6 kinase (Thr389) antibody (Psk1-P) or with anti-Psk1 antibody (Psk1). When anti-Psk1 antibodies were used, the upper band corresponds to the phosphorylated form of Psk1. Anti-actin antibodies were used for loading control. The intensities of the Psk1-P and Psk1 (phosphorylated and non-phosphorylated forms) bands were measured for three independent biological repeats. Only one representative blot is shown for each experiment. The numbers represent the ratio of Psk1-P to total Psk1, normalized to the same ratio in wild-type cells in rich (YE) medium, ± indicates standard deviations.

(B-C) Prototrophic Δcaa1 mutant cells (B) or caa1-K255R cells were grown in YE to logarithmic phase and then shifted for 60 minutes into EMM-N (-N) or EMM-N containing the indicated amino acids (aspartate, glutamine, asparagine, arginine or serine) at the final concentration of 5 mM. The ratio between Psk1-P and Psk1 was calculated as described above. (D) Western blot analysis of Ssp2-T189 phosphorylation in wild-type and Δcaa1 mutant cells. Cells were grown to mid-log phase and then shifted to minimal media containing glucose (2%) or with no glucose for one hour.

Figure 5. Amino acids stimulate TORC1 activity. TORC1 activity was assessed in wild-type cells using anti-Psk1-P and anti-Psk1 antibodies, as described in Fig. 4. (A) Reactivation of TORC1 in response to addition of low or high concentration of various amino acids. Cells were grown in minimal medium (EMM) to logarithmic phase, shifted to medium without nitrogen source (-N) for one hour, followed by addition of different amino acids at the indicated concentrations (1mM or 5mM for each amino acid) for 20 minutes. The intensities of the Psk1-P and Psk1 bands were measured. The numbers represent the ratio of Psk1-P to total Psk1, normalized to the same ratio in wild-type cells in EMM. (B) Reactivation of TORC1 in response to various amino acids as a function of time. Cells were grown in minimal medium (EMM) to logarithmic phase, shifted to medium without nitrogen source (-N) for one hour, followed by addition of different amino acids at 5 mM for 5 or 20 minutes. The ratio between Psk1-P and Psk1 was calculated as described above. (C) A table summarizing the efficiency by which single amino acid can re-activate TORC1.

Figure 6. Growth rates in cells growing with different amino acids as a nitrogen source. Prototrophic wild-type cells (TA0001) were grown to mid-log phase in EMM medium containing specific amino acids as nitrogen source. O.D measurements were taken every two hours. All amino acids were added to a final concentration of 20 mM, the standard concentration for EMM-Glu plates (50).

Figure 7. Reactivation of TORC1 requires increase in intracellular or extracellular glutamine levels. Prototrophic wild-type cells (TA0001) were grown in minimal medium (EMM) to logarithmic phase, shifted to medium without nitrogen source (-N) for one hour, in the presence or absence of the glutamine synthase inhibitor MSX, followed by addition of different amino acids (5mM) for 20 minutes. The numbers represent the ratio of Psk1-P to total Psk1, normalized to the same ratio in wild-type cells in EMM.
Activation of TORC1 by amino acids

Figure 1

A

EMM  YE

leu1 ura4
ade6 h90
RS42

-R

+R

leu1 ura4
ade6 h90
RS42

B

C

ASP + α-KG  Caal  GLU + OAA

D

EMM +Asp  EMM +Glu  EMM  YE

\[ \text{caal-1} \]

\[ \text{WT} \]

E

EMM  EMM +Asp  EMM +Asp +R

\[ \text{WT} \]

\[ \text{caal-1} \]

\[ \Delta \text{caal} \]

F

EMM +Asp  LowEMM +Asp +R  Pro +Asp +R

\[ \text{WT} \]

\[ \text{caal-1} \]

G

WT  EMM  EMM +Glu  EMM +Asp  EMM +Asp +Glu

\[ \Delta \text{caal} \]

\[ \text{caal-1} \]
Figure 2

A

YE  EMM  YE+R

EMM+Asp  EMM+Glu

caal-1  WT  Δmaa1

coa1-1  WT  Δmaa1

B

YE  YE+R  EMM

caal-K255R-myc
coa1-myc
Δcaal

C

No-tag  caal+  K255R

MYC

Act1

70 KDa
41 KDa

Activation of TORC1 by amino acids
Activation of TORC1 by amino acids

Figure 3

A

|          | YE     | YE+R   |
|----------|--------|--------|
| WT       | ![Image](image1.png) | ![Image](image2.png) |
| caa1-l   | ![Image](image3.png) | ![Image](image4.png) |
| caa1-l Δfkh1 | ![Image](image5.png) | ![Image](image6.png) |

B

|          | YE     | YE+R   |
|----------|--------|--------|
| WT       | ![Image](image7.png) | ![Image](image8.png) |
| tor1SE   | ![Image](image9.png) | ![Image](image10.png) |
| Δcaa1    | ![Image](image11.png) | ![Image](image12.png) |
| Δcaa1 tor2SE | ![Image](image13.png) | ![Image](image14.png) |

C

| Mating efficiency on YE (%) |          |
|-----------------------------|----------|
| WT                          | 0        |
| Δcaa1                       | 6.8±1.7  |
| Δpka1                       | 22±2.8   |
| Δcaa1Δpka1                  | 31.5±2.1 |
| tor2-ts                     | 18.7±1.2 |
| Δcaa1tor2-ts                | 6.3±1.5  |
| Δgtr1                       | 3.3±1.1  |
| Δcaa1Δgtr1                  | 3.2±1.3  |
Activation of TORC1 by amino acids

Figure 4

A

|        | WT   | Δcox1 |
|--------|------|-------|
| Asp    | -    | +     |
| Psck-P | -    | -     |
| Psck   | -    | +     |
| Act1   | 4.08 | 0.26  |
|       | 0.094| 0.028 |
|       | 0.086| 0.029 |
|       | 0.094| 0.079 |

B

|        | WT   | Δcox1 |
|--------|------|-------|
| Asp    | -    | -     |
| Psck-P | -    | -     |
| Psck   | -    | -     |
| Act1   | 5.91 | 0.26  |
|       | 0.094| 0.028 |
|       | 0.086| 0.029 |
|       | 0.094| 0.079 |

C

|        | WT   | Δcox1 |
|--------|------|-------|
| Asp    | -    | -     |
| Psck-P | -    | -     |
| Psck   | -    | -     |
| Act1   | 5.91 | 0.26  |
|       | 0.094| 0.028 |
|       | 0.086| 0.029 |
|       | 0.094| 0.079 |

D

|        | WT   | Δcox1 |
|--------|------|-------|
| Asp    | -    | -     |
| Psck-P | -    | -     |
| Psck   | -    | -     |
| Act1   | 5.91 | 0.26  |
|       | 0.094| 0.028 |
|       | 0.086| 0.029 |
|       | 0.094| 0.079 |

Glucose: + + -
Figure 5

A

|       | Glu | Arg | Asn | Met | Val | Ile |
|-------|-----|-----|-----|-----|-----|-----|
|       | 1mM | 5mM | 1mM | 5mM | 1mM | 5mM |
| EMM   | 1mM | 5mM | 1mM | 5mM | 1mM | 5mM |
| Psk1-P|     |     |     |     |     |     |
| Psk1  |     |     |     |     |     |     |
| Act1  |     |     |     |     |     |     |

B

|       | Glu | Arg | Asn | Met | Val | Ile |
|-------|-----|-----|-----|-----|-----|-----|
|       | 5   | 20  | 5   | 20  | 5   | 20  |
| Time [min]: EMM | -N |     |     |     |     |     |
| Psk1-P|     |     |     |     |     |     |
| Psk1  |     |     |     |     |     |     |
| Act1  |     |     |     |     |     |     |

C

| Strong  | Intermediate | Low or None |
|---------|--------------|-------------|
| Glutamine | Serine      | Glutamate   |
| Asparagine | Methionine  |             |
| Arginine  | Leucine      |             |
| Aspartate | Valine       |             |
|           | Isoleucine   |             |
|           | Threonine    |             |
|           | Proline (see ref. 16) |  |

Activation of TORC1 by amino acids
Activation of TORC1 by amino acids

Figure 6
Activation of TORC1 by amino acids

Figure 7

| EMM  | -N | Arg | Asn | Ser | Asp | Gln |
|------|----|-----|-----|-----|-----|-----|
| MSX: | -  | -   | +   | -   | +   | -   |
| Psk1-P | 53KD | 53KD | 53KD |
| Psk1 | 41KD | 41KD | 41KD |
| Act1 | 1   | 0.01| 0.06| 0.34| 0.09| 0.12| 0.166| 0.13| 0.12| 0.70| 0.13| 0.13| 0.70| 0.01| 0.72| 0.152| 0.152| 0.159|
The cytosolic form of aspartate aminotransferase is required for full activation of
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Sophie Reidman, Adiel Cohen, Martin Kupiec and Ronit Weisman

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