Nitrogen Regulates AMPK to Control TORC1 Signaling

Highlights
- Nitrogen stress actives AMPK and inhibits TORC1
- Nitrogen-stress-induced AMPK activation is independent of its β and γ subunits
- Hep3B cells activate mTORC1 in response to increasing ammonium levels
- Fission yeast is a model system to study conserved nutrient-sensing pathways

In Brief
Cells adjust their size to varying nutritional environments. Here, Davie et al. show that nitrogen stress activates AMPK to reduce target of rapamycin complex-1 (TORC1) activity. This AMPK activation does not require the β and γ regulatory subunits. Importantly, TORC1 regulation by the alternative nitrogen source ammonia is conserved in human cells.
Nitrogen Regulates AMPK to Control TORC1 Signaling

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Summary

Background: Cell growth and cell-cycle progression are tightly coordinated to enable cells to adjust their size (timing of division) to the demands of proliferation in varying nutritional environments. In fission yeast, nitrogen stress results in sustained proliferation at a reduced size.

Results: Here, we show that cells can sense nitrogen stress to reduce target of rapamycin complex-1 (TORC1) activity. Nitrogen-stress-induced TORC1 inhibition differs from amino-acid-dependent control of TORC1 and requires the Ssp2 (AMPK-α) kinase, the Tsc1/2 complex, and Rhb1 GTPase. Importantly, the β and γ regulatory subunits of AMPK are not required to control cell division in response to nitrogen stress, providing evidence for a nitrogen-sensing mechanism that is independent of changes in intracellular ATP/AMP levels. The CaMKK homolog Ssp1 is constitutively required for phosphorylation of the AMPKzSsp2 T loop. However, we find that a second homolog CaMKKzppk34 is specifically required to stimulate AMPKzSsp2 activation in response to nitrogen stress. Finally, ammonia also controls mTORC1 activity in human cells; mTORC1 is activated upon the addition of ammonium to glutamine-starved Hep3B cancer cells.

Conclusions: The alternative nitrogen source ammonia can simulate TORC1 activity to support growth and division under challenging nutrient settings, a situation often seen in cancer.

Introduction

Cells frequently need to coordinate their growth and division, and therefore size, with available resources in order to meet the demands of proliferation in a changing environment. In the presence of abundant nutrients, high levels of protein synthesis are maintained to increase biomass and promote division at an increased size. In eukaryotes, the target of rapamycin (TOR) network acts as a major nutrient sensor that couples cell growth and cell-cycle progression in response to environmental changes [1]. In all organisms, TOR comprises two structurally and functionally distinct multi-protein complexes, target of rapamycin complex-1 (TORC1) and TORC2. These complexes are defined by conserved, interacting components that are unique to each complex: raptor (Mip1 in S. pombe) defines TORC1, whereas rictor (Ste20 in S. pombe) defines TORC2 [2]. It is the rapamycin-sensitive TORC1 that acts as the major nutrient sensor to link environmental cues with cell growth. Amino acids play an essential role in activating TORC1 as cells respond to amino acid deprivation by inhibiting TORC1 to suppress cell growth [3]. Mammalian TORC1 (mTORC1) is activated by the small Rheb GTPase, which directly interacts with mTORC1 [4]. Rheb is thought to be essential for the regulation of mTORC1 activity in response to most environmental signals. However, AMP-activated protein kinase (AMPK) control of mTORC1 can also be regulated through direct phosphorylation of Raptor [5]. Many aspects of signaling upstream of mTORC1 are conserved in the fission yeast S. pombe. In contrast to S. cerevisiae, activation of TORC1 through Rheb is conserved in fission yeast, where the Rheb homolog Rhb1 interacts with Tor2 (TORC1) [6, 7], and rho1-defective mutants mimic tor2-defective cells [6, 8, 9]. In contrast to mammalian cells, fission yeast contains two TOR kinases: the nonessential Tor1 and the essential Tor2 [10]. TORC1 contains mainly Tor2, and TORC2 predominantly contains Tor1 [8, 11, 12]. Cells expressing hyperactive Tor2 can bypass the need for Rhb1 for growth [13]. In both mammals and fission yeast, a tumor suppressor complex consisting of tuberous sclerosis proteins Tsc1 and Tsc2 functions to negatively regulate Rheb activity [14–16]. Active Tsc1/2 is consequently a negative regulator of TORC1.

Despite extensive research, the molecular bases for the well-documented nutrient-sensing ability of TOR are not fully understood. In fission yeast, reduced carbon levels (that reduce cellular energy levels) and changes in amino acid concentrations are both actively sensed [17–19]. Fission yeast cells are also able to sense moderate reductions in nitrogen availability (nitrogen stress), which promote mitotic comment without cell-cycle arrest and therefore proliferate at a reduced cell size [20, 21]. In this case, negative regulation of TORC1 signaling boosts mitogen-activated protein kinase (MAPK) to promote mitotic commitment [21, 22]. This response contrasts with the response to complete removal of the nitrogen source (nitrogen starvation) in which cell division stops and cell-cycle progression arrests in G1. Following nutrient starvation, CaMKKzSsp1-dependent phosphorylation of AMPKzSsp2 promotes the translocation of the AMPK complex into the nucleus, and the ensuing accumulation of the Ste11 transcription factor promotes cell-cycle arrest and sexual differentiation [23].

AMPK is a well-characterized energy sensor that couples cellular responses to changes in environmental conditions. It is well established that activated AMPK inhibits TORC1 signaling following glucose and energy stress [24]. Activation of canonical AMPKs is dependent upon phosphorylation of the T-loop residue by upstream kinases. Mammalian AMPKz can be phosphorylated and activated by three kinases, LKB1, CaMKK, and TAK1 [24].

Here, we identify a role for S. pombe AMPKzSsp2 and the CaMKK homolog Ppk34 in regulating the response to nitrogen stress. We show that fission yeast cells can specifically sense nitrogen stress to activate AMPKzSsp2, reduce TORC1 activity, and promote cell division at a reduced cell size. We extend our observations to mammalian cells and show that changing the environmental nitrogen source in human hepatoma cell cultures to ammonia also regulates mTORC1.
Results

Nitrogen Stress Is Sensed to Control Mitotic Onset and Cell Size at Division

When the nitrogen source is changed from glutamate (good) to proline (poor), wild-type fission yeast cells adapt to advance mitosis and reduce their size at division by approximately 4 µm within 120 min of the change [20, 21] (Figure 1A). In contrast, cells maintain their size when transferred between media containing equal sources of nitrogen, i.e., from glutamate to glutamate (Figure 1A). In asynchronous populations, a reduction in the size threshold for division leads to a rapid increase in the proportion of dividing cells, as more cells in the population are now able to meet the newly defined size threshold. To explore whether advanced cell-cycle progression was limited to glutamate-to-proline downshifts, we transferred prototrophic wild-type cells from glutamate into another poor nitrogen source, uracil. This instigated a response that emulated that seen upon a glutamate-to-proline shift (Figure 1A). Alternatively, cells were grown with ammonium chloride as the sole nitrogen source and transferred into glutamate, proline, or uracil. These environmental changes also accelerated mitotic commitment to reduce cell size at division after 120 min (Figure 1B).

Changes in carbon availability can also be sensed to adjust cell size [18]. All of the minimal growth media used in this study contained identical glucose levels (2%) unless otherwise stated. Thus, the acceleration of mitosis observed should not arise from any alterations in carbon source. To verify this, we transferred cells from glutamate into a proline-based medium in which glucose levels were twice those of the initial medium (4%). Cells still advanced mitotic commitment and reduced their size at division under these conditions of abundant carbon, suggesting that the response to the change in medium was indeed nitrogen specific (Figure 1A).

Together, these data indicate that fission yeast cells can specifically sense the availability of nitrogen in their environment. Importantly, all these nitrogen-shifted cells are prototrophs, meaning that they can synthesize all of the amino acids they require. The glutamate or proline in our experimental media provides the sole external nitrogen source. These data show that nitrogen-regulated control of mitotic onset is not confined to shifts from glutamate to proline. However, since glutamate (C₅H₉NO₄) and proline (C₅H₉NO₂) supply an identical quantity of carbon atoms, all subsequent experiments utilize this downshift, which we shall refer to as nitrogen stress.

AMPKα<sup>Ssp2</sup> Is Required to Reduce Cell Size after Nitrogen Stress

Consistent with its status as a good nitrogen source for growth, glutamate is readily converted into ammonia and glutamine to provide the nitrogen required to carry out protein synthesis and other cellular processes. Glutamate also feeds into the energy-generating trichloroacetic acid (TCA) cycle. However, to utilize proline as a nitrogen source, cells must first convert it into glutamate and other useable metabolites [25]. In S. cerevisiae cultures, nitrogen stress or TORC1 inhibition induces bioenergy recovery mechanisms [26]. This suggests that the cellular energy status may be low following nitrogen stress. Given that TOR signaling is sensitive to alterations in energy status, we considered the possibility that nitrogen stress is initially sensed by the ensuing reduction in intracellular energy levels. Cellular ATP levels in wild-type nitrogen-stressed cultures were measured using a bioluminescence assay (Figure 1C). 5 min after nitrogen stress, ATP levels were reproducibly reduced (p < 0.01) compared with time point 0 min (Figure 1C). ATP levels had risen once more within 10 min of the initial stress, suggesting a rapid recovery, and therefore, a dynamic regulation of ATP homeostasis. These data support the hypothesis that a decline in environmental nitrogen availability invokes a transient drop in cellular energy levels.

We next explored the hypothesis that it is the reduction in cellular ATP levels that instigates the nitrogen-stress-dependent inhibition of TORC1 signaling. Energy stress reduces the ATP:AMP ratio to activate AMPK, a cellular energy sensor and inhibitor of TORC1 signaling [24]. To assess the involvement of AMPK in the nitrogen stress response, we shifted prototrophic ssp2Δ and ppsK9Δ (AMPKα homologs) cultures from glutamate to proline (Figure 2A). Cells deleted for AMPKα<sup>Δppsk9</sup> accelerated mitotic commitment in the same way as wild-type

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**Figure 1.** Nitrogen Stress Is Specifically Sensed to Control Mitotic Onset and Cell Size at Division

(A and B) Early exponential prototroph wild-type (wt) cells, grown at 28°C in glutamate or ammonium, were collected by filtration, washed, and re-suspended into glutamate, proline (Pro), uracil, or proline + 2x the initial glucose concentration. Samples were fixed in formaldehyde at the indicated time points. Division septa were stained with calcofluor white. Left: y axis shows average ±SEM, between repeat experiments) proportion of dividing cells (%). Right: y axis shows average ±SEM, between repeat experiments) change in cell length at division after 120 min in the indicated medium (µM).

(C) Cell pellets (3 × 10⁷ cells per pellet) were lysed, and the supernatants were analyzed for overall protein concentration, as well as overall ATP level. Asterisks represent statistical significance (p < 0.01) as determined by a Student’s t test.
cultures. However, cells lacking AMPKαssp2 were compromised in their ability to advance mitotic onset following nitrogen stress (Figure 2A). Similarly, AMPKαssp2Δ cells shifted from ammonium chloride to proline were less proficient than wild-type cultures at advancing the timing of mitotic onset and reducing cell size (Figure S1). Deletion of both AMPKαppk9 and AMPKαssp2 did not exhibit an additive phenotype, suggesting that the two *S. pombe* AMPKα subunits are functionally independent under these conditions and that only AMPKαssp2 is required for nitrogen-stress-induced control of cell size (Figure 2A).

**AMPKαssp2 Acts Upstream of TORC1**

Increased TOR signaling increases cell size at division [27]. Consistent with AMPK acting as an inhibitor of TORC1 [24], cells lacking AMPKαssp2 were 9% longer than wild-type cells (Figures 2B and 2C). Maf1, a repressor of RNA polymerase III, is a TORC1-specific substrate [28, 29]. Cells lacking AMPKαssp2 exhibited higher levels of Maf1.pk in its phosphorylated forms compared to wild-type cells (Figure 2D). This correlated with AMPKαssp2Δ mutant cells having lower basal levels of phosphorylated elf2α (low levels are indicative of increased TORC1 activity) (Figure 2D). Finally, the apparent increase in TORC1 signaling in AMPKαssp2Δ mutants conferred resistance to the TOR inhibitor Torin1 (Figure 2E) [30]. Hence, cells lacking AMPKαssp2 have increased TORC1 activity, establishing AMPKαssp2 as an upstream inhibitor of TORC1 signaling.

**AMPKαssp2 Is Not Required for the Amino-Acid-Dependent Control of TORC1**

As discussed above, because we used wild-type prototroph cells, glutamate and proline acted as sole external nitrogen sources in our nitrogen stress experiments. To further explore the role of AMPKαssp2 in environmental sensing, we exposed wild-type (*wt*) and AMPKαssp2Δ cells to external supplies of the amino acid (arginine) known to activate TOR signaling [3]. Following the addition this amino acid, cell length at division and Maf1 phosphorylation were increased in both *wt* and AMPKαssp2Δ cells (Figures 2C and 2F). This demonstrates that AMPKαssp2 is not required for amino-acid-dependent activation of TORC1 signaling. Therefore, it is likely that AMPKαssp2 is specifically involved in controlling TOR-regulated cell size in response to changes in nitrogen availability, but not in response to changes in amino acid levels.

**AMPKαssp2 Functions Upstream of TORC1 during Nitrogen Stress**

Following nitrogen stress, AMPKαssp2 might be responsible for the inhibition of TOR signaling that promotes mitotic entry to reduce cell size at division [21]. To probe whether
nitrogen-stress-induced TOR inhibition is a consequence of altered AMPKα<sup>ssp2</sup> regulation, we shifted AMPKα<sup>ssp2 Δ</sup> mutant cells from glutamate to proline and immediately treated them with either rapamycin or solvent control. Rapamycin treatment rescued the AMPKα<sup>ssp2 Δ</sup> mitotic commitment phenotype. Cells advanced into mitosis to the same degree as nitrogen-stressed wild-type cultures (Figure 3A), supporting the view that AMPKα<sup>ssp2</sup> acts upstream of TORC1.

AMPK has been shown to inhibit mTORC1 in part through promoting phosphorylation of TSC1/2 [31]. To further investigate whether AMPKα<sup>ssp2</sup> functions as part of a similar signaling module upstream of TORC1 following nitrogen stress, we subjected Tsc1/2 and Rhb1 mutants to nitrogen stress. The Rhb1 partial loss-of-function mutant rhb1.G63D-S165N [32] was severely compromised in its response to nitrogen stress (Figure 3B), suggesting that proper Rhb1 function is required for the regulation of TOR activity under these conditions. Similarly, cells lacking either Tsc1 or Tsc2 also failed to advance into mitosis and reduce their size at division following nitrogen stress (Figure 3C). Thus, AMPKα<sup>ssp2</sup>, Tsc1/2, and Rhb1 appear to function as a signaling module upstream of TORC1 under these conditions.

To determine whether it was only TORC1 activity and not TORC2 activity that was inhibited by nitrogen stress, we analyzed protein extracts from nitrogen-stressed cultures by immunoblotting for Maf1.pk. Maf1 phosphorylation was reduced after 30 min, as manifested by a collapse in the slower migrating phospho-forms of Maf1 (Figure 4A). In contrast, a control glutamate-to-glutamate shift showed no advancement into mitosis (Figure 1A) and no change in Maf1 phosphorylation status (Figure 4A). Similarly, when the non-responding tsc2Δ and rhb1.G63D-S165N mutants (Figures 3B and 3C) were exposed to nutrient stress, no collapse in the slower migrating phospho-forms of Maf1 was observed (Figure 4C). Conversely, phosphorylation on Gad8.S546 (AKT), a TORC2-specific site [33, 34], remained unchanged (Figure S2A). This latter observation is consistent with our previous finding that nutrient stress is regulated through the Tor1-containing TORC1-B

Figure 3. TORC1 Signaling Components, but Not the AMPK Regulatory Subunits, Function in the Response to Nitrogen Stress

(A–G) Early exponential cells from the indicated prototrophic strains, growing in medium containing glutamate, were shifted into medium containing proline to induce nitrogen stress. Percentage of dividing cells (left axis) and the average change in cell length at division (right axis) are shown.

(C) Leucine was added to prototroph tsc1Δ, tsc2Δ and the control wt culture to allow growth in EMMG. The TORC1 inhibitor Rapamycin was added at 300 ng/mL to nitrogen-stressed AMPKα<sup>ssp2 Δ</sup> cultures. Alternatively, solvent was added as a control.
complex [35]. These findings are reminiscent of the impact of rapamycin on wild-type steady-state cultures. Short-term rapamycin treatment reduced Maf1 phosphorylation to a similar extent as nitrogen stress and had no impact upon Gad8.S546 phosphorylation status [30]. As rapamycin is a specific inhibitor of TORC1 and does not impact upon TORC2 function [36–38], these results show that nitrogen stress specifically inhibits TORC1, but not TORC2, and that in fission yeast, AMPK \( \alpha \) signals to inhibit TORC1. The collapse in the slower migrating phospho-forms of Maf1 seen in wild-type cells was not observed in extracts from \( \text{AMPK}^{\alpha_{\text{Ssp2}}} \).\( \text{D} \) mutant cells following nitrogen stress (Figures 4A and 4B), indicating that TORC1 activity is not reduced to the same extent in the \( \text{AMPK}^{\alpha_{\text{Ssp2}}} \).\( \text{D} \) mutant. Together, our findings suggest that the ability of \( \text{AMPK}^{\alpha_{\text{Ssp2}}} \) to regulate cell size at division in response to changes in the nitrogen environment involves signaling to modulate TORC1 activity.

Nitrogen Stress Activates \( \text{AMPK}^{\alpha_{\text{Ssp2}}} \)

The AMPK catalytic \( \alpha \) subunit is activated through phosphorylation of the activating T-loop residue [39]. Nitrogen stress is therefore likely to promote \( \text{AMPK}^{\alpha_{\text{Ssp2}}} \) phosphorylation. Due to sequence conservation within the activation loops of \( \text{AMPK}_{z}^{\text{Ssp2}} \) and mammalian AMPK\( \alpha \), a commercial anti-phospho-AMPK\( \alpha \)Thr172 antibody was able to recognize phosphorylated \( \text{AMPK}^{\alpha_{\text{Ssp2}}} \). This commercial antibody was specific to \( \text{AMPK}^{\alpha_{\text{Ssp2}}} \) (Figure S2C) and did not also recognize

AMPK \( \beta \) and \( \gamma \) Are Not Required for the Response to Nitrogen Stress

We next explored the possibility that \( \text{AMPK}^{\alpha_{\text{Ssp2}}} \) was activated following nitrogen stress. Activation of mammalian AMPK can depend upon the binding of AMP/ADP to the AMPK regulatory \( \beta \) subunits to promote phosphorylation of Thr172 in the T loop, alongside a less potent allosteric activation of the phosphorylated kinase [24]. We therefore asked whether the regulatory \( \beta \) and \( \gamma \) subunits of \( S. \text{pombe} \) AMPK were involved in the nitrogen stress response. Surprisingly, cells lacking Amk2 (\( \beta \)) or Cbs2 (\( \gamma \)) did not alter the response to nitrogen stress in the same way as the \( \text{AMPK}^{\alpha_{\text{Ssp2}}} \).\( \text{D} \) mutant, as each mutant was able to advance into mitosis (Figure 3D). However, the response was slightly delayed compared with wild-type cells. A similar response was seen in the \( \text{AMPK}^{\alpha_{\text{Amk2}}}, \beta_{\text{Cbs2}} \) double mutant (Figure 3E), suggesting that \( \text{AMPK}^{\alpha_{\text{Amk2}}}, \beta_{\text{Cbs2}} \) act together within a single complex. These findings imply that the AMPK \( \beta \) and \( \gamma \) subunits in \( S. \text{pombe} \) are not essential for promoting mitotic entry during nitrogen stress. Importantly, \( \text{AMPK}^{\alpha_{\text{Ssp2}}} \) levels were reduced in the \( \text{AMPK}^{\alpha_{\text{Amk2}}}, \beta_{\text{Cbs2}} \).\( \text{D} \) double mutant (Figure S2B), which may account for the slight delay in the response of this mutant to nitrogen stress (Figure 3D). These data therefore raise the possibility of an ATP/AMP-independent regulation of the catalytic \( \alpha \) subunit following nutrient stress.

See supporting data in Figure S2.
AMPK<sub>a</sub> (Figure S2D). In addition, no signal was detected on a GST-AMPK<sub>a</sub>Spp2 fusion protein expressed in <i>E. coli</i>, suggesting that the antibody is phospho-specific (Figure S2E). Nitrogen stress led to an average 2.5-fold increase in AMPK<sub>a</sub>Spp2 Thr189 phosphorylation after 30 min (Figure 4D). An increase in AMPK<sub>a</sub>Spp2 Thr189 phosphorylation was also observed in the AMPK<sub>a</sub>camk2<sup>−/−</sup> AMPK<sub>a</sub>cbs2<sup>−/−</sup> double mutant (Figure S2F). An in vitro AMPK<sub>a</sub>Spp2 kinase assay using SAMS peptide as substrate confirmed AMPK<sub>a</sub>Spp2 activation following nutrient stress (Figure 4E).

AMPK<sub>a</sub>Spp2 has previously been implicated in the response to glucose starvation [19, 23]. Following glucose starvation, AMPK<sub>a</sub>Spp2 was hyper-phosphorylated. Importantly, phosphorylation at Thr189 was still increased when wild-type cells were shifted from glutamate into proline containing 4% (2 ×) glucose (Figure S2G), suggesting AMPK<sub>a</sub>Spp2 activation stems from nitrogen stress and is not an indirect consequence of a change in glucose levels.

The <i>cdc2.1w</i> mutant strain was used to address whether the increase in AMPK<sub>a</sub>Spp2 phosphorylation was a secondary consequence of accelerated mitotic commitment rather than the alteration in nitrogen supply. <i>cdc2.1w</i> cells are largely insensitive to Wee1 function and are unable to advance into mitosis in response to nitrogen stress [21]. Robust phosphorylation of AMPK<sub>a</sub>Spp2 on Thr189 followed nitrogen stress of <i>cdc2.1w</i> mutants (Figure S2H). Thus, AMPK<sub>a</sub>Spp2 regulation by the change in nitrogen environment is maintained, despite the inability of cells to initiate the downstream physiological advancement into mitosis.

The CaMKK Homologs Ssp1 and Ppk34 Are Both Required for Nitrogen-Stress-Induced AMPK<sub>a</sub>Spp2 Activation

Our data show that the β and γ subunits of AMPK are not required for nitrogen-stress-induced advancement into mitosis and the concomitant reduction in cell size at division. This suggests that the main nutrient-sensing mechanism following nitrogen stress may not be a change in ATP/AMP ratio (Figure 1C). Recently, the CaMKK homolog CaMKK<sub>sup1</sub> was found to be able to phosphorylate AMPK<sub>a</sub>Ssp2 [23]. Here, we found that cells lacking CaMKK<sub>sup1</sub> were unable to advance into mitosis following nitrogen stress (Figure 3F). However, despite this absence of a transient elevation in the frequency of dividing cells, CaMKK<sub>sup1</sub> mutant cells had reduced their length at division 2 hr after the stress. This suggests that, unlike any other mutants that we have studied so far, cell growth and cell division are uncoupled in CaMKK<sub>sup1</sub> mutants. In other words, following nitrogen stress, CaMKK<sub>sup1</sub> cells cease tip growth, which eventually leads to a reduction in their cell length at division over time. The previously reported role for CaMKK<sub>sup1</sub> in the control of the actin cytoskeleton may explain this additional observation [40]. Furthermore, the previously reported role for CaMKK<sub>sup1</sub> in control of mitotic entry through regulation of Cdr2/Wee1 is likely to explain the complete failure to advance mitosis following nutrient stress [41]. Consistent with the failure of CaMKK<sub>sup1</sub> cells to respond to nitrogen stress, AMPK<sub>a</sub>Spp2 Thr189 phosphorylation was completely absent from AMPK<sub>a</sub>Spp2<sup>camk2</sup><sup>−/−</sup> mutant cells, and no phosphorylation was seen following nitrogen stress (Figure 5A). To address whether AMPK<sub>a</sub>Spp2 Thr189 phosphorylation was also modulated by other CaMKK homologs, we tested the response of CaMKK<sub>papk34</sub> mutants. Under steady-state conditions, AMPK<sub>a</sub>Spp2 Thr189 phosphorylation was observed in both wt and CaMKK<sub>papk34</sub> mutants (Figure 5B). However, no increase in AMPK<sub>a</sub>Spp2 Thr189 phosphorylation was observed following nitrogen stress (Figure 5C). This observation predicts that, in a manner that is reminiscent of the AMPK<sub>a</sub>Spp2<sup>camk2</sup><sup>−/−</sup> mutant cells, CaMKK<sub>papk34</sub> cells would fail to advance into mitosis under these conditions. We exposed CaMKK<sub>papk34</sub> cultures to nitrogen stress and analyzed them for their ability to advance mitotic onset to test this prediction. Cells lacking CaMKK<sub>papk34</sub> failed to accelerate mitosis and reduce their cell size to the same degree as wild-type cultures (Figure 3G). We next examined the impact of rapamycin-induced TORC1 inhibition on the response of CaMKK<sub>papk34</sub> cells to nitrogen stress. Rapamycin treatment rescued the inability of CaMKK<sub>papk34</sub> cells to advance into mitosis at a reduced cell size (Figure S3A), supporting the notion that, in this context, CaMKK<sub>papk34</sub> functions upstream of TORC1.

Together, these observations suggest that CaMKK<sub>sup1</sub> is responsible for AMPK<sub>a</sub>Spp2 Thr189 phosphorylation but that CaMKK<sub>papk34</sub> specifically regulates the increase in AMPK<sub>a</sub>Spp2 Thr189 phosphorylation following nitrogen stress. Both AMPK<sub>a</sub>Spp2<sup>camk2</sup> and CaMKK<sub>sup1</sub> cells are resistant to Torin1-induced TOR inhibition. Thus, CaMKK<sub>sup1</sub>, but not CaMKK<sub>papk34</sub>, appears to play a direct role in AMPK<sub>a</sub>Spp2 Thr189 phosphorylation [23] and activation upstream of TORC1.

CaMKK<sub>papk34</sub> Regulates AMPK<sub>a</sub>Spp2 Phosphorylation Status

Because the steady-state level of AMPK<sub>a</sub>Spp2 Thr189 phosphorylation is unchanged in CaMKK<sub>papk34</sub> mutants (Figure 5B), it is likely that CaMKK<sub>papk34</sub> regulates AMPK<sub>a</sub>Spp2 Thr189 phosphorylation via an indirect mechanism. To assess CaMKK<sub>papk34</sub>-dependent regulation of AMPK<sub>a</sub>Spp2 phosphorylation more closely, we resolved protein extracts from nitrogen-stressed wild-type and CaMKK<sub>papk34</sub> cultures on Phos-tag gels that enhance the separation of differentially
phosphorylated forms. When the blot was probed with the anti-AMPKα2 antibody, a ladder of at least five bands was visible (Figure 5D). In wild-type glutamate-grown cells (time point 0 min), AMPKα2Sp2 was predominantly in the faster-migrating isoforms, with little protein in the slower-migrating bands. Importantly, 30 min after nitrogen stress, the band pattern shifted and AMPKα2Sp2 was now predominantly seen in the three slower-migrating bands (Figure 5D). This correlates with the increase in AMPKα2Sp2 T189 phosphorylation and activity after 30 min of nitrogen stress (Figures 4D and 4E). This shift in band mobility is in agreement with previous data, which showed an increase in the uppermost band following complete nitrogen starvation [23] (although only three forms of AMPKα2Sp2 were observed in this previous report). Interestingly, cells lacking CaMKKPK32 showed a different change in band pattern following nitrogen stress (Figure 5D), as the increase in the proportion of AMPKα2Sp2 in the slower-migrating forms that is seen in wild-type cells was absent in CaMKKPK32Δ mutants. Thus, nitrogen-stress-induced regulation of AMPKα2Sp2 phosphorylation is different in the absence of CaMKKPK32.

The PP2C Phosphatases Regulate Steady-State AMPKα2Sp2 Phosphorylation Status

We next asked whether CaMKKPK32Δ regulated AMPKα2Sp2 phosphorylation via an impact upon CaMKKSp1 function. However, no difference in CaMKKSp1 protein levels or phosphorylation status was observed in CaMKKPK32ΔΔ mutants compared with wild-type cells (Figures S4A and S4B). Furthermore, there was no additive impact of deleting CaMKKPK32ΔΔ in a CaMKKSp1 deletion background. Phosphorylation of AMPKα2Sp2 was identical in both the CaMKKSp1Δ and the CaMKKSp1Δ CaMKKPK32ΔΔ double mutants (Figures S4C and S4D). It is therefore unlikely that CaMKKPK32Δ directly regulates CaMKKSp1 or AMPKα2Sp2.

We next considered the possibility that CaMKKPK32Δ-dependent inhibition of an AMPK phosphatase could account for the increase in AMPKα2Sp2 activation following nitrogen stress. It has previously been shown that PP2C phosphatases regulate AMPK phosphorylation [42]. In fission yeast, four genes encode protein phosphatase 2C homologs. A strain deleted for ptc1–3 is sensitive to nitrogen stress due to lack of viability (Figure S4E) prompted us to assess the

Figure 6. The PP2C Phosphatases Regulate Steady-State AMPKα2Sp2 Phosphorylation Status

(A) Early exponential cells were analyzed for AMPKα2Sp2 T189 phosphorylation by western blotting, following both standard SDS-PAGE (top) and Phos-tag SDS-PAGE (bottom).

(B) Growth assay. Exponentially growing cells were spotted onto indicated medium.

(C and D) Samples from nitrogen-stressed ptc1,2,3Δ cells taken between 0 and 30 min (C) or 0 and 75 min (D) were used to perform western blot analysis to detect AMPKα2Sp2 T189 phosphorylation and total AMPKα2Sp2.

(E–G) wt and ptc1,2,3Δ cells were transformed with either Nourseothricin selectable empty pREP42 vector (+vector) or pREP42 expressing a copy of CaMKKPK32ΔΔ (CaMKKPK32ΔΔ). Transformed cells were grown with Nourseothricin selection to early exponential phase and then nitrogen stressed as previously described. Percentage of cell division and average cell lengths at division were both measured.

(F) Growth was assessed via 10-fold serial dilution and spotting onto solid medium containing 100 μg/mL Nourseothricin.

(G) Cell samples were taken for western blot analysis to detect AMPKα2Sp2 T189 phosphorylation and total AMPKα2Sp2 in wt (left) and ptc1,2,3Δ cells (right).

See supporting data in Figure S4.
and 120 min prior to harvesting cells.

**Figure 7. Ammonium Activates mTORC1 in HEP3B Heptoma Cancer Cells**

- **A** Human Hep3B cells were cultured in the presence (+) or in the absence (−) of leucine for 60 min or in the absence of leucine for 50 min prior to a 10-min leucine stimulation.
- **B** and **C** Western blot analyses were performed with anti-S6K (total) and anti-phospho-S6K (T389) (B). Human Hep3B cells were cultured in the presence (+) or in the absence (−) of glutamine for 180 min (C) or in the absence of glutamine (60-min starvation), followed by addition of NH₄Cl for 30, 60, and 120 min prior to harvesting cells.

consequences of overexpressing this phosphatase and to assess its impact on AMPKₐ²^Ssp2^Thr189 phosphorylation and the response to nutrient stress. No significant role for Ptc4 in control of Ssp2 Thr189 phosphorylation was observed (Figures S4F and S4G).

Together, these results suggest that although PP2C phosphatases appear to regulate steady-state levels of AMPK홈^Ssp2^Thr189 phosphorylation (Figure 6A), it is unlikely that these phosphatases control the nitrogen-induced activation of AMPKₐ²^Ssp2^.

**CaMKKᵖkp34 Overexpression Induces Growth Arrest**

To further explore the role of CaMKKᵖkp34 in nitrogen sensing and AMPKₐ²^Ssp2^ activation, we overexpressed CaMKKᵖkp34 in both wild-type and ptc1,2,3,Δ mutant cells. Increasing the levels of CaMKKᵖkp34 kinase in both strains reduced cell growth on the rich growth medium extract supplement (YES; a complex undefined media containing numerous nitrogen sources) (Figure 6F). Given that TOR inhibition can induce growth arrest, this CaMKKᵖkp34^overexpression reduces cell growth suggests that AMPKₐ²^Ssp2^ activity is elevated under these conditions. Indeed, an increase in AMPKₐ²^Ssp2^ Thr189 phosphorylation was observed in CaMKKᵖkp34^overexpressing^ wild-type cells grown in YES medium (Figure 6G). However, in the ptc1,2,3,Δ mutant cells, which already have elevated levels of AMPKₐ²^Ssp2^ Thr189 phosphorylation, these elevated levels did not increase further.

The growth inhibition following CaMKKᵖkp34 overexpression was dependent on the nutrient environment and was not evident when cells were plated on minimal medium (Edinburgh minimal media with glutamate [EMMG]) (Figure 6F). This suggests that the nutrient environment controls the expression or activity of key molecules that are regulated following CaMKKᵖkp34 overexpression.

Together, these results suggest that CaMKKᵖkp34^induced^ growth inhibition could in part stem from increased AMPKₐ²^Ssp2^ activity, which in turn would inhibit TORC1. However, AMPKₐ²^Ssp2^ activation is unlikely to be the only reason for the observed growth inhibition, as no increase in AMPKₐ²^Ssp2^ Thr189 phosphorylation was observed in the ptc1,2,3,Δ mutant, which already possessed elevated baseline levels of phosphorylation.

Overall, our data show that CaMKKᵖkp34 is required to induce AMPKₐ²^Ssp2^ activation following nitrogen stress (Figures 5C and 5D) and that overexpression of CaMKKᵖkp34 can promote AMPKₐ²^Ssp2^ activation through T189 phosphorylation.

**Ammonium Regulates mTORC1 in Hep3B Hepatomas**

It is well established that mammalian cells regulate mTORC1 in response to alterations in amino acid levels [3]. Here, we show that nitrogen stress can be sensed to regulate TORC1 signaling in fission yeast. It is unclear how fission cells sense nitrogen stress. Given that the generation of ammonia is a natural by-product in metabolism, we wondered whether internal levels of ammonia might be sensed. Such a mechanism would be consistent with EMM2 media (ammonia as nitrogen source) being a high-quality media and EMMP (proline as nitrogen source) being a low-quality media, as breakdown of proline to ammonia takes several enzymatic steps.

It is currently unclear whether mammalian cells share this ability to sense alterations in nitrogen supply (ammonia levels) to control mTORC1 signaling. To investigate this possibility, we exploited the finding that long-term survival and proliferation in Hep3B hepatoma cell lines are maintained when ammonia replaces glutamine in the media [44] and that a key role of liver cells is to maintain energy homeostasis [45]. Liver cells are therefore likely to be acutely sensitive to alterations in nutrient levels. Leucine starvation of Hep3B cells inhibited mTORC1 as observed in other cell lines. mTORC1 was activated following re-administration of leucine to the medium, as reflected in the changes in S6K phosphorylation, a well-established marker of mTORC1 activity [46] (Figure 7A). Interestingly, when Hep3B cells were starved of glutamine for 60 min and then supplemented with ammonia, we also observed mTORC1 inhibition followed by mTORC1 reactivation (Figures 7B and 7C). Therefore, the alternative nitrogen source ammonia in Hep3B cells regulates mTORC1 activity. This ammonia-induced regulation of mTORC1 in glutamine-starved cells (Figure 7C) is indicative of an internal sensor of ammonia levels in mammalian cells.

**Discussion**

It is well established that TORC1 signaling mediates the tight coordination of cell growth and division in response to changes in environmental conditions in a variety of organisms [27]. Exposure to an unfavorable nutrient environment leads to an inhibition of TORC1 to restrain anabolic processes and conserve nutrients [3]. Here, we report that cells sense nitrogen stress through a mechanism that is independent of the well-established carbon and amino-acid-sensing pathways. Exposure to nitrogen stress inhibited TORC1 activity and advanced mitotic commitment to promote division at a reduced cell size. This inhibition of TORC1 appears to stem from AMPK (Ssp2) activation. Extensive studies have linked AMPK and TORC1 activities [47, 48]. It is widely
recognized that mammalian AMPK can phosphorylate and activate Tsc1/2 to inhibit TORC1 activity via its control of the Rheb GTPase [31]. In agreement with this, we find that Tsc1/2 and Rhb1 are involved in the control of mitotic commitment following nitrogen stress in fission yeast, suggesting that an AMPK-Tsc-TORC1 signaling module exists in this model organism. Our data reveal that cells are able to inhibit TORC1 signaling in response to nitrogen stress even when glucose is in abundant supply (Figures 1A and S2G). We also find that removal of the AMPK hetero-trimer through the deletion of the regulatory subunits does not abolish the nitrogen stress response and reduction in cell size at division (Figure 3E). These results reveal a nitrogen stress response mechanism that is dependent on AMPK and yet likely to be independent of the mode by which this kinase senses changes in cellular ATP/AMP ratios. In stark contrast, the promotion of cell-cycle exit and cell differentiation in response to combined nitrogen and glucose starvation is reported to require all three subunits of the AMPK complex (Ssp2α, Amk2β, and Cbs2β), along with CaMKKβ and Ssp1 kinase. CaMKKβα is responsible for the activating phosphorylation of AMPKα on Thr180 (Figure 5A; [23]). Following nutrient starvation, CaMKKα-dependent phosphorylation of AMPKα has been reported to lead to the translocation of the AMPK complex into the nucleus and the accumulation of the Ste11 transcription factor [49]. We find no evidence of AMPKα/ß-GFP nuclear accumulation under the milder conditions of nitrogen stress we employ here (data not shown), supporting the idea that it is cytoplasmic AMPK activity that is increased by this milder form of stress. We also show that CaMKKαβ is required for nitrogen-stress-induced AMPKα activation. CaMKKαβ may regulate AMPKα/ß activity via inhibition of an AMPK phosphatase. However, this phosphatase is unlikely to be PP2C. Our preliminary data also exclude a role for the PP2A subunit Par1 (B56) (data not shown). Therefore, the identity of such a nitrogen-regulated AMPK phosphatase remains unclear at present and will form the focus of future studies. Importantly, the ability of cells to specifically sense and regulate TORC1 in response to nitrogen stress, together with the independent mode of TORC1 control that responds to alterations in amino acid levels, is likely to be conserved in mammals. Hep3B hepatoma cells are able to utilize ammonia as a nitrogen source for long-term survival and proliferation [44]. Furthermore, our results indicate that Hep3B cancer cells activate mTORC1 in response to increasing ammonium levels. Thus, an alternative nitrogen-sensing pathway that is akin to the pathway we have identified in fission yeast may exist in human cells. This response in mammalian cells could be particularly relevant for the survival of cancer cells. Because tumor cells regularly proliferate in environments with limited nutrient supply, they are generally nutritionally stressed and known to scavenge extracellular proteins in order to sustain growth through continual metabolic reprogramming [50]. It is therefore likely that the breakdown of extracellular protein increases ammonia levels in the cells, which can be exploited as an alternative nitrogen source. Interestingly, breast cancer cell lines that are insensitive to glutamine deprivation show increased expression of glutamine synthetase, an enzyme that utilizes both ammonia and glutamate to generate glutamine [51]. If true, such utilization of ammonia as a nitrogen source would considerably expand the survival mechanisms of cancer cells under nutrient stress.

Experimental Procedures

Strains used are listed in Table S1. Student’s t tests were used to calculate significant differences. SAMS peptide phosphorylation by AMPKαß was assessed with phospho-ACC antibodies on slot blots. For glutamine deple- tion/ammonium utilization of Hep3B cells, 0.8 mM NH4Cl was added to DMEM (10% dialyzed fetal bovine serum [FBS] without glutamine). Detailed experimental procedures can be found in the Supplemental Information.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.12.034.

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