Opposite Effects of Tor1 and Tor2 on Nitrogen Starvation Responses in Fission Yeast

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ABSTRACT

The TOR protein kinases exhibit a conserved role in regulating cellular growth and proliferation. In the fission yeast two TOR homologs are present. $tor1^+$ is required for starvation and stress responses, while $tor2^+$ is essential. We report here that Tor2 depleted cells show a phenotype very similar to that of wild-type cells starved for nitrogen, including arrest at the G_1 phase of the cell cycle, induction of nitrogen-starvation-specific genes, and entrance into the sexual development pathway. The phenotype of $tor2$ mutants is in a striking contrast to the failure of $tor1$ mutants to initiate sexual development or arrest in G_1 under nitrogen starvation conditions. Tsc1 and Tsc2, the genes mutated in the human tuberous sclerosis complex syndrome, negatively regulate the mammalian TOR via inactivation of the GTPase Rheb. We analyzed the genetic relationship between the two TOR genes and the *Schizosaccharomyces pombe* orthologs of TSC1, TSC2, and Rheb. Our data suggest that like in higher eukaryotes, the Tsc1–2 complex negatively regulates Tor2. In contrast, the Tsc1–2 complex and Tor1 appear to work in parallel, both positively regulating amino acid uptake through the control of expression of amino acid permeases. Additionally, either Tsc1/2 or Tor1 are required for growth on a poor nitrogen source such as proline. Mutants lacking Tsc1 or Tsc2 are highly sensitive to rapamycin under poor nitrogen conditions, suggesting that the function of Tor1 under such conditions is sensitive to rapamycin. We discuss the complex genetic interactions between $tor1^+$, $tor2^+$, and $tsc1/2^+$ and the implications for rapamycin sensitivity in $tsc1$ or $tsc2$ mutants.

T OR proteins, the central protein kinases that control cell growth and proliferation, were first identified in the yeast *Saccharomyces cerevisiae* as the targets for the immunosuppressive and potential anticancerous drug rapamycin (WULLSCHLEGER *et al.* 2006). Rapamycin binds the small FKBP12 protein and the resulting protein–drug complex binds and inhibits TOR-dependent activities. Two TOR homologs are present in *S. cerevisiae*, *TOR1* and *TOR2*. These are found at the core of two different evolutionary conserved complexes (LOEWITH *et al.* 2002). Although a single TOR gene is found in higher eukaryotes, two distinct physical and functional TOR complexes are also present in mammalian and Drosophila cells (KIM *et al.* 2003; JACINTO *et al.* 2004; SARBASSOV *et al.* 2004). The mammalian mTORC1 complex regulates cell growth, in part, via phosphorylation of the two translation initiation factors, p70 S6 kinase and 4E-BP1 proteins (HAY and SONENBERG 2004; TEE and BLENIS 2005). mTORC2 regulates the actin cytoskeleton (JACINTO *et al.* 2004) and the cell growth and survival regulator Akt/PKB (SARBASSOV *et al.* 2005).

In accord with the suggestion that TOR regulates cell growth in response to nutrient availability, *S. cerevisiae* cells lacking both *TOR1* and *TOR2* exhibit many features characteristics of starved cells (BARBET *et al.* 1996; BECK and HALL 1999; DI COMO and ARNDT 1996; NODA and OHSUMI 1998; ZARAGOZA *et al.* 1998). Similarly, loss of function of the TOR proteins in worms, flies, and mice results in phenotypes like those of cells suffering from nutrient deprivation (OLDHAM *et al.* 2000; ZHANG *et al.* 2000; LONG *et al.* 2002; GANGLOFF *et al.* 2004; MURAKAMI *et al.* 2004).

In higher eukaryotes, TOR proteins are negatively regulated by the TSC1–TSC2 heterodimer. Mutations in either TSC1 or TSC2 cause a human syndrome, known as tuberous sclerosis complex (TSC), which is characterized by benign tumors and severe neurological defects. TSC2 encodes a GTPase-activating protein (GAP) and together with TSC1 converts the small GTPase Rheb into its GDP-bound inactive form. Rheb binds mTOR and positively regulates its activity (MANNING and CANTLEY 2003; LI *et al.* 2004). More recent results demonstrated that TSC1/2 also has positive effects on TOR signaling, suggesting the presence of a negative feedback loop (HARRINGTON *et al.* 2004; SHAH *et al.* 2004; UM *et al.* 2004).

The fission yeast, but not the budding yeast, contains homologs for TSC1/2, known as $tsc1^+$ and $tsc2^+$.

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TABLE 1

S. pombe strains used in this study

Strain	Genotype	Source
TA001	972 h^-	Lab stock
TA007	*leu1-32/ leu1-32 ura4-D18/ ura4-D18 ade6-M210/ade6-M216 h^- /h^+*	Lab stock
TA016	*ade6-M216 leu1-32 ura4-D18 h^{90}*	Lab stock
TA157	*tor1::ura4$^+$ ade6-M216 leu1-32 ura4-D18 h^{90}*	WEISMAN and CHODER (2001)
TA313	*tor2::ura$^+$ ura4-D18 leu1-32 ade6 pREP81-tor2$^+$ h^-*	This study
TA390	*tor1::ura4$^+$*	WEISMAN and CHODER (2001)
TA417	*leu1-32 ura4-D18 ade6-216 his7-366 h^-*	M. van Slegtenhorst
TA418	*tsc2::kan$^+$ leu1-32 ura4-D18 ade6-216 his7-366 h^-*	M. van Slegtenhorst
TA419	*tsc1::kan$^+$ leu1-32 ura4-D18 ade6-210 his7-366 h^+*	M. van Slegtenhorst
TA426	*tor1::ura4$^+$ tor2::his1$^+$ his1-102 ura4-D18 leu1-32 ade6-M210 pREP81-tor2$^+$ h^-*	This study
TA430	*tor2::ura$^+$ ura4-D18 leu1-32 ade6 pREP81-tor2$^+$ h^{90}*	This study
TA434	*rhb1$^+$/rhb::ura4$^+$ ade6-M210/ade-M216 ura4-D18/ura4-D18 leu1-32/ leu1-32*	This study
TA443	*rhb1::ura4$^+$ ade6 ura4-D18 leu1-32 pREP81-rhb1$^+$*	This study
TA449	*tsc1::G418*	This study
TA450	*tsc2::G418*	This study
TA459	*tor1::ura4$^+$ tsc2::kan$^+$ ura4-D18*	This study
TA467	*tsc1::G418 fkh1::ura4$^+$*	This study
TA468	*tsc2::G418 fkh1::ura4$^+$*	This study
TA481	*tor1::ura4$^+$ tsc1::kan$^+$ ura4-D18*	This study
TA502	*tor2::his1$^+$ his1-102 ura4-D18 leu1-32 ade6-M210 pREP1-tor2$^+$ h^{90}*	This study
TA561	*tor1::ura4$^+$ ade6 leu1-32 ura4D-18 h$^-$ pREP81-tor2$^+$*	This study
TA568	*leu1-32 pREP1-tor2$^+$ h^-*	This study
TA569	*tor1::ura4$^+$ tor2::his1$^+$ his1-102 ura4-D18 leu1-32 ade6-M210 pREP1-tor2$^+$ h^{90}*	This study
TA597	*fkh1::ura4$^+$*	This study

Schizosaccharomyces pombe Tsc1 and Tsc2 proteins also work as a complex (MATSUMOTO *et al.* 2002) and missense mutations that cause TSC in humans abolish the activity of the *S. pombe* homologs (VAN SLEGTENHORST *et al.* 2004). Disruption of *tsc1$^+$* or *tsc2$^+$* leads to amino acid uptake defects, impairs sexual development, and affects gene induction upon nitrogen starvation (MATSUMOTO *et al.* 2002; VAN SLEGTENHORST *et al.* 2004; NAKASE *et al.* 2006). Several findings demonstrate that the Tsc1–2 complex negatively regulates Rhb1, the *S. pombe* Rheb homolog: *rhb1* mutants exhibit a phenotype opposite to that of *tsc1/2* (YANG *et al.* 2001; URANO *et al.* 2005) and mutations that impair Rhb1 activity can reverse the amino acid uptake defect in *tsc1/2* mutants (VAN SLEGTENHORST *et al.* 2004; NAKASE *et al.* 2006). The *rhb1$^+$* gene is essential and cells disrupted for *rhb1$^+$* arrest at the G_1 phase of the cell cycle with the morphology and physiology of nitrogen-starved cells (MACH *et al.* 2000; YANG *et al.* 2001). The nitrogen-starved phenotype of *rhb1* mutants suggests that *rhb1$^+$* participates in signaling nitrogen availability.

The *S. pombe tor1$^+$* gene is required under starvation and stress conditions (KAWAI *et al.* 2001; WEISMAN and CHODER 2001). Δ*tor1* cells die quickly once they exit the logarithmic phase and do not initiate sexual development in response to starvation conditions. Δ*tor1* cells are also highly sensitive to osmotic and oxidative stress. Given the conserved role of TOR proteins in regulating growth, the finding that Tor1 is required mainly under starvation, when little growth is required, is surprising. However, Tor1 exerts its functions in stress and starvation via phosphorylation of the *S. pombe* p70 S6 kinase homolog, *gad8$^+$* (MATSUO *et al.* 2003). Thus, the substrate and mode of action of Tor1 is highly conserved in evolution. Rapamycin does not inhibit growth, entrance into stationary phase, or stress responses in fission yeast (WEISMAN *et al.* 1997; WEISMAN 2004). The only rapamycin-sensitive TOR-dependent function reported to date in *S. pombe* is amino acid uptake, which is mediated via a Tor1-dependent function (WEISMAN *et al.* 2005).

In contrast to our detailed knowledge on *tor1$^+$* activity, no description of Tor2 loss of function has so far been reported. Here we show that disruption of *tor2$^+$* results in a phenotype that highly resembles that of nitrogen-starved cells and that the Tsc1/2–Rhb1 module has different effects on Tor1 and Tor2 functions. While loss of function of Tor2 highly resembles that of *rhb1* mutants, loss of function of Tsc1/2 shares similar defects with Tor1 disruption. We discuss the complex genetic interactions between *tor1$^+$*, *tor2$^+$*, and *tsc1/2$^+$*.

MATERIALS AND METHODS

Yeast strains, media, and general techniques: Yeast strains are described in Table 1. Growth media were prepared as in MORENO *et al.* (1991). YES is YE supplemented with 75 μg/ml adenine and uracil. EMM (Edinburgh minimal medium) is defined as medium in which the sole nitrogen source is

ammonium chloride (STETTLER *et al.* 1996). Minimal medium was supplemented as required. Leucine, histidine, adenine, and uracil were supplemented at 75 μg/ml, unless otherwise indicated. For proline (Pro) and glutamic acid (Glu) plates, ammonium chloride in the EMM medium was replaced with 10–30 mM proline and 20 mM glutamic acid, respectively (YOUNG and FANTES 1987). EMM − N contains no nitrogen. Rapamycin was used at a final concentration of 100 ng/ml as described previously (WEISMAN *et al.* 1997). Transformation of *S. pombe* cells was performed by electroporation (PRENTICE 1992). Assays for mating efficiency were carried out as described (WEISMAN *et al.* 1997).

tor2⁺ plasmid constructs: For expression of *tor2⁺* from its own promoter on a multicopy plasmid, a fragment of 7936 bp was amplified, using the Expand long template PCR system (Roche Applied Science). This fragment, containing the *tor2⁺* ORF flanked by 413 and 509 bp, was cloned into the *LEU2*-based plasmid pIRT2 (BOOHER and BEACH 1986). For expression under the regulation of the *nmt1* promoter, a fragment of 7523 bp containing the *tor2⁺* ORF and 509 bp downstream of it was amplified. This fragment was cloned into *LEU1*-based pREP1 or pREP81 plasmids (MAUNDRELL 1993).

Measurement of leucine uptake: Leucine uptake was performed as previously described in WEISMAN *et al.* (2005). Cells were grown to log phase in minimal medium. One-half milliliter of logarithmic cells was harvested and resuspended in 0.5 ml minimal medium containing 0.01 mM leucine together with ³H-labeled leucine (1–5 μCi of [L-4,5-3H(N)]-leucine, 50 Ci/mmol; New England Nuclear Life Science Products, Boston). Cells were incubated at 30° and samples were taken at 6 min and mixed with chilled minimal medium containing 10 mM leucine. Cells were then washed three times before being resuspended in water containing 0.5% SDS. Experiments were done in duplicates.

Northern blot analysis and semiquantitative RT–PCR: Yeast RNA was extracted from logarithmic growing cells and the *isp5⁺* and *adh1⁺* mRNA were detected as previously described in WEISMAN *et al.* (2005). For semiquantitative RT–PCR analysis 50 ng yeast RNA were treated with DNAse I (Promega, Madison, WI) and used as template in one-step RT–PCR (QIAGEN, Valencia, CA). Samples were taken following 20, 25, and 30 cycles of 45 sec at 94°, 45 sec at 53°, and 90 sec at 72°.

Fluorescence-activated cell sorter analysis: Cells were stained with propidium iodide and analyzed by a Becton Dickinson FACSort as described in SNAITH and FORSBURG (1999). Data were analyzed by Cell Quest software for Macintosh.

Disruption of *S. pombe rhb1⁺*: A fragment containing 771 bp of the *rhb1⁺* ORF and an additional 118 bp upstream of the stop codon was amplified by PCR from genomic DNA with primers P280 (GGAATTCCATATGGCTCCTATTAAATCTCG TAG) and P281 (TTTGTGTGCAAAGTATCACCTC) and subcloned into a pGEM-T vector (Promega) to give pGEMrhb1. A 350-bp *Cla*I–*Xho*I fragment of pGEMrhb1 was replaced with the *ura4⁺* gene, resulting in the plasmid pGEMrhb1::ura4+. Primers P280 and P281 were then used to amplify a 2.2-kb disruption fragment that was transformed into diploid TA007. Disruption was confirmed by PCR and Southern blot analysis.
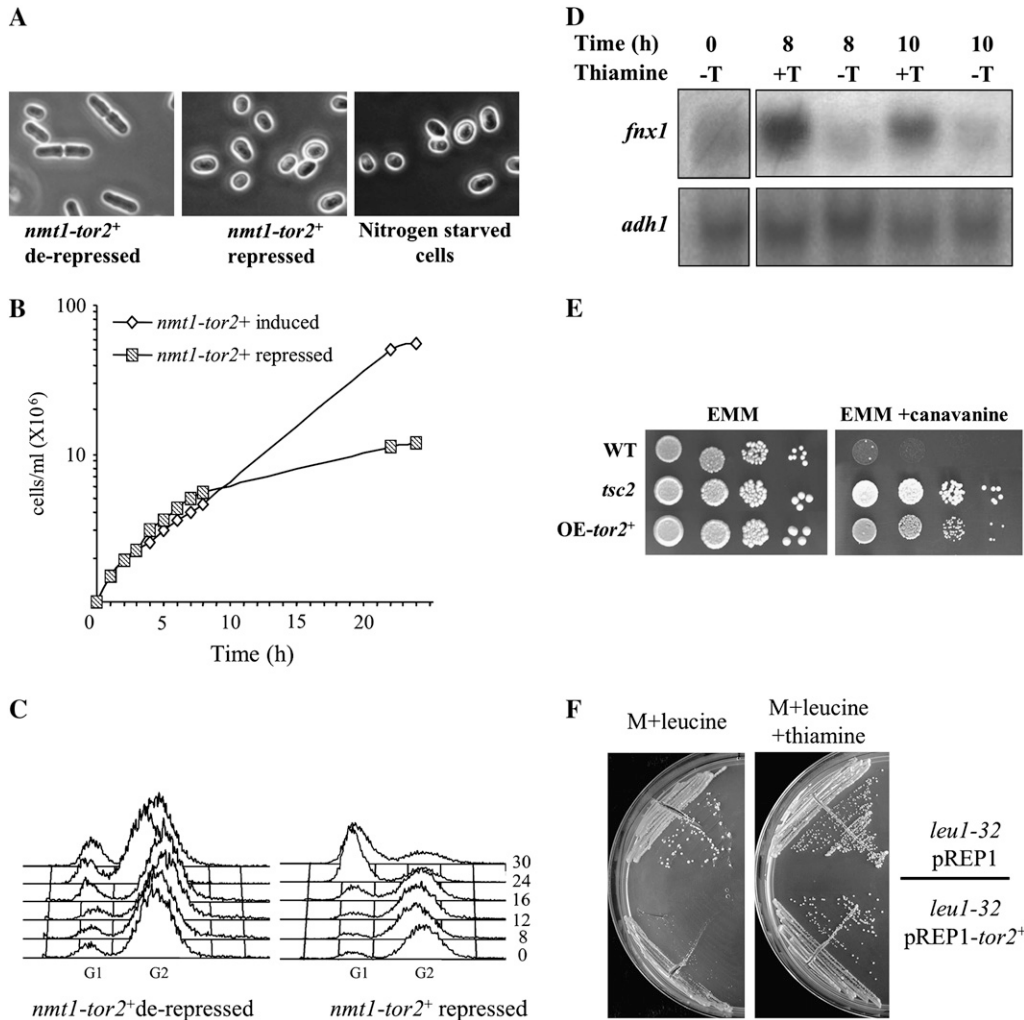
RESULTS

Cells depleted for Tor2 arrest with a phenotype that highly resembles nitrogen-starved cells: *tor2⁺* is an essential gene (WEISMAN and CHODER 2001). To further study the cellular role of *tor2⁺*, we cloned *tor2⁺* under the regulation of the *nmt1* promoter. This promoter is turned off in the presence of thiamine (MAUNDRELL

1993). Cells carrying disruption of *tor2⁺* at the chromosomal locus and expressing the *nmt1-tor2⁺* construct were grown to midlog phase and then shifted to repressing conditions and samples were taken for cell number counting and cell morphology examination. As seen in Figure 1, A and B, cells shifted to the repressed conditions arrested after three to four divisions as small, rounded cells, similar to wild-type cells starved for nitrogen. Under nitrogen starvation, cells undergo mitosis at a shorter cell length and thus give rise to small cells (FANTES and NURSE 1977; YOUNG and FANTES 1987). Nitrogen starvation of wild-type cells is also characterized by accumulation of cells in the G_1 phase of the cell cycle (FANTES and NURSE 1977; YOUNG and FANTES 1987). FACS analysis revealed that depletion of Tor2 leads to growth arrest with a G_1 DNA content (Figure 1C), very similar to the arrest seen in wild-type cells following nitrogen starvation (WEISMAN and CHODER 2001).

We also found that the nitrogen-starved-like phenotype of Tor2-depleted cells and the arrest in G_1 are very similar to the phenotype of cells depleted of Rhb1 (MACH *et al.* 2000). Cells depleted of Rhb1 are characterized by small cell size morphology, G_1 arrest, and induction of the nitrogen-starvation-specific gene, *fnx1⁺* (MACH *et al.* 2000). Northern blot analysis of total RNA from cells that were repressed for *tor2⁺* revealed that *fnx1⁺* mRNA was also increased when *tor2⁺* was repressed (Figure 1D). Peak levels were seen 8 hr after *tor2⁺* was repressed, at the time just before cell growth was dramatically reduced.

Another hallmark of the response to nitrogen starvation is initiation of sexual development, which is completely repressed in rich medium (WEISMAN 2004). Cells disrupted for *tor2⁺* were patched on rich YE medium, which contains sufficient thiamine to repress transcription from the *nmt1* promoter. Under these conditions no colonies are formed, but microscopic examination revealed that ∼30% of the cells underwent mating, meiosis, and sporulation. No meiosis was observed in isogenic wild-type cells, as expected. The ability of cells to enter sexual development on rich medium characterizes mutants of the cAMP pathway, such as cells lacking the adenylate cyclase, Cyr1/Git2, or the protein kinase A, Pka1 (STIEFEL *et al.* 2004; HOFFMAN 2005). While the cAMP-dependent pathway is thought to regulate growth and developmental decisions mainly in response to glucose sensing, the nitrogen-starved phenotype of Tor2 depletion suggests that *tor2⁺* regulates growth and development in response to nitrogen availability.

Overexpression of Tor2, like hyperactivation of Rhb1 or loss of function of Tsc1/2, results in resistance to canavanine: Loss of function of Rhb1 results in a phenotype that resembles nitrogen starvation, while hyperactive Rhb1 mutants confer resistance to the arginine toxic analog canavanine, apparently because of downregulated uptake (URANO *et al.* 2005). We tested whether increasing Tor2 activity may also result in canavanine

A

nmt1-tor2⁺
de-repressed

nmt1-tor2⁺
repressed

Nitrogen starved
cells

B

C

nmt1-tor2⁺de-repressed

nmt1-tor2⁺ repressed

D

Time (h)	0	8	8	10	10
Thiamine	-T	+T	-T	+T	-T

fnx1

adh1

E

	EMM	EMM +canavanine
WT		
tsc2		
OE-tor2⁺		

F

M+leucine

M+leucine
+thiamine

leu1-32
pREP1

leu1-32
pREP1-tor2⁺

FIGURE 1.—tor2 pheno-types. (A) Left and middle: *tor2⁻* cells expressing *nmt1-tor2⁺* (TA313) were grown to midlog phase and transferred to fresh minimal medium in the absence (derepressed) or presence (repressed) of thiamine. After 12 hr, cells were visualized by light micros-copy. Right: wild-type cells (TA001) were starved in minimal medium contain-ing no nitrogen for 21 hr. (B) Δ*tor2* mutant cells (TA313) were grown as de-scribed above. At time zero thiamine was added and cells were counted. (C) FACS analysis of the cells sampled in B. (D) Northern blot analysis. Total RNA was prepared from samples taken at the indicated time points (hours). Northern blots were probed with *fnx1⁺* and with *adh1⁺* as a loading control. (E) Wild-type (TA001), Δ*tsc2* (TA450), and Δ*tor2* carrying *nmt1-tor2⁺* (TA313) cells were grown in EMM to mid-log phase. Four different dilutions were spotted on EMM plates with and with-out 60 μg/ml canavanine. Plates were incubated at 30° for 3 days. (F) Cells auxotrophic for leucine were transformed with vector only or with vector containing the *nmt1-tor2⁺* construct. Slow-growth phenotype is observed only when the *nmt1* promoter is derepressed (no thiamine). Cells were streaked on minimal (M) plates supplemented with 75 μg/ml leucine and incubated at 30° for 4 days.

resistance. As shown in Figure 1E, overexpression of *tor2⁺* induces canavanine resistance in wild-type cells. Thus, while *rhb1* or *tor2* mutants exhibit a nitrogen-starvation phenotype, their overactivation leads to cana-vanine resistance, consistent with the hypothesis that their overexpression results in signaling of nutrient/ nitrogen sufficiency, leading to reduced uptake of nitro-genous compounds such as amino acids. Overexpres-sion of *tor2⁺* in cells that are auxotrophic for leucine leads to slow growth on minimal plates that are nor-mally supplemented for leucine (Figure 1F). This phenotype is similar to loss of function of *tsc1⁺* or *tsc2⁺* (MATSUMOTO *et al.* 2002). The similarity between *tor2* and *rhb1* mutants and the opposite effects of *tor2* and *tsc1/2* mutants on leucine auxotrophs suggest that they work on the same signaling pathway and that like in higher eukaryotes *rhb1⁺* positively regulate *tor2⁺*. This possibility is schematically presented in Figure 5C and is further discussed below.

tor1 and tor2 null mutants exhibit opposite pheno-types with respect to response to nitrogen availability: Under nitrogen starvation, *tor1* mutants show an abnor-mal phenotype as cells remain elongated and do not accelerate entrance into mitosis, do not arrest in G_1, and are completely sterile (WEISMAN and CHODER 2001). In contrast, cells depleted of Tor2 are small, arrested in G_1, and readily enter sexual development in the presence of rich medium. Thus, while Tor2-depleted cells appear as "always starved for nitrogen," cells disrupted for *tor1⁺* appear as "never starved for nitrogen" (Figure 2A). The opposite effects of two closely related homologs are clearly unexpected. An intriguing possibility is that the two homologs are connected to each other through a negative feedback loop. In such a scenario, absence of Tor1 may lead to overactivation of Tor2 and thus results in a phenotype of never starved for nitrogen. Yet, this hypothesis is highly speculative at present and awaits further experimental data.

To examine the genetic relationship between *tor1+* and *tor2+*, we constructed a double-mutant strain Δ*tor1* Δ*tor2* expressing the conditional *nmt1-tor2+* allele. When shifted to repressing conditions, Δ*tor1* Δ*tor2* cells divided for three to four divisions before growth arrest. The double-mutant cells did not reach the small cell size observed in repressed single *tor2* mutants, although some cell size reduction occurred (Figure 2B). FACS analysis revealed that *tor1 tor2* double mutants arrested at G_2 with only a small fraction of cells at G_1 (Figure 2C). Thus, the terminal phenotype of Δ*tor1* Δ*tor2* is intermediate compared with that of each of the single mutants. One possibility is that the *tor2* mutation is epistatic to *tor1*, but since the initial cell length of Δ*tor1* cells is slightly longer than wild type, the Δ*tor1* Δ*tor2* cells reach a longer terminal size. Alternatively, Tor1 and Tor2 may regulate cell size and cell cycle progression independently.

We also examined the ability of Δ*tor1* Δ*tor2* *nmt1-tor2+* cells to enter the sexual development pathway upon depletion of Tor2. Upon shutoff of *tor2+* expression, we found no mating activity either in rich or in minimal medium. Thus, Tor1 acts in regulating sexual development either downstream of Tor2 or independently.

Taken together, our analysis of the Δ*tor1* Δ*tor2* double mutant reveals complex interactions, which may only partially support the suggestion that *tor1+* loss of function leads to overactivation of Tor2. It suggests that Tor1 exerts at least some of its functions independently of Tor2.

Tor1 and the Tsc1–Tsc2 complex regulate leucine uptake and transcription of amino acid permeases by two separate mechanisms: It has previously been shown that Tsc1/2 and Tor1 are both required for efficient amino acid uptake and expression of three different amino acid permeases: *isp5+*, 7G5.06, and c869.10 (VAN SLEGTENHORST *et al.* 2004; WEISMAN *et al.* 2005). To explore the genetic relationships between *tor1+* and *tsc1/2+* we determined the expression of these permeases in the double mutants *tor1 tsc1* and *tor1 tsc2*. Since transcription of *isp5+* is almost undetectable by Northern blotting in the presence of ammonia, we compared *isp5+* transcription 15 min after a shift to proline-based medium. As expected, expression of *isp5+* is reduced in the single *tsc2* or *tor1* mutants. Further reduction is detected in the double mutant (Figure 3A), suggesting that Tor1 and Tsc2 regulate *isp5+* transcription by parallel mechanisms. Similar results were found for Δ*tsc1* and Δ*tor1* Δ*tsc1*, respectively (data not shown). Using semiquantitative RT–PCR analysis we also observed an additive decrease in the expression of the 7G5.06 permease in the double mutant *tor1 tsc2* compared to the single mutants (Figure 3A). Finally, we directly measured the uptake of radioactive leucine. Figure 3B shows that leucine uptake is reduced in the double mutants, compared to single mutants, in accord with the additive effects on transcription of amino acid permeases.
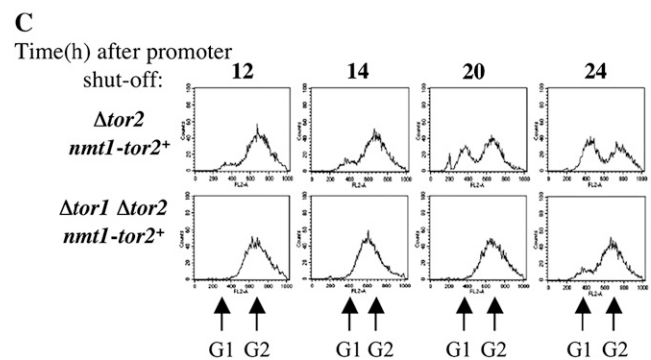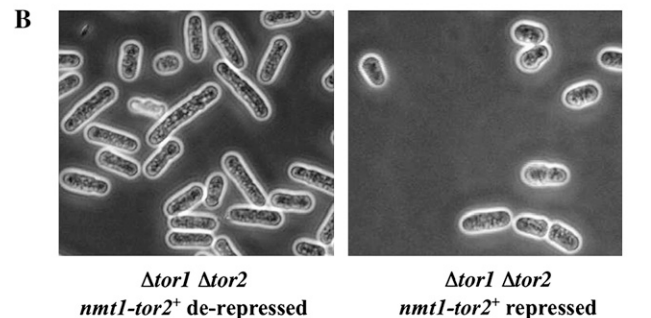
FIGURE 2.—Analysis of Δ*tor1* Δ*tor2* double mutants. (A) Schematic of the opposite phenotypes of Δ*tor1* and Δ*tor2* mutations. (B) Terminal phenotype of Δ*tor1* Δ*tor2* cells expressing the *nmt1-tor2+* construct (TA426) in the presence or absence of thiamine. (C) FACS analysis. Δ*tor2* or Δ*tor1* Δ*tor2* mutant cells expressing *nmt1-tor2+* (TA313 and TA426, respectively) were grown to midlog phase and thiamine was added.

Either Tor1 or Tsc1–Tsc2 is required for growth when the nitrogen source in the medium is proline: The defect in leucine uptake in Δ*tor1* cells or rapamycin-treated cells can be suppressed by replacing the rich nitrogen source in the medium (ammonia) by a poorer source, such as proline (WEISMAN *et al.* 2005). This suppression likely involves transcriptional induction of amino acid permeases in the presence of a poor nitrogen source such as proline (WEISMAN *et al.* 2005). Proline also suppresses the amino acid uptake defect of *tsc1* or *tsc2* mutants, enabling auxotrophic *tsc1 tsc2* mutants to grow on proline, but not on ammonia plates that are normally supplemented with the required amino acids (Figure 4A). However, unexpectedly, the growth of prototrophic *tsc1* or *tsc2* mutants was highly sensitive to rapamycin on proline plates, but not on ammonia plates (Figure 4B). Rapamycin inhibited the growth of
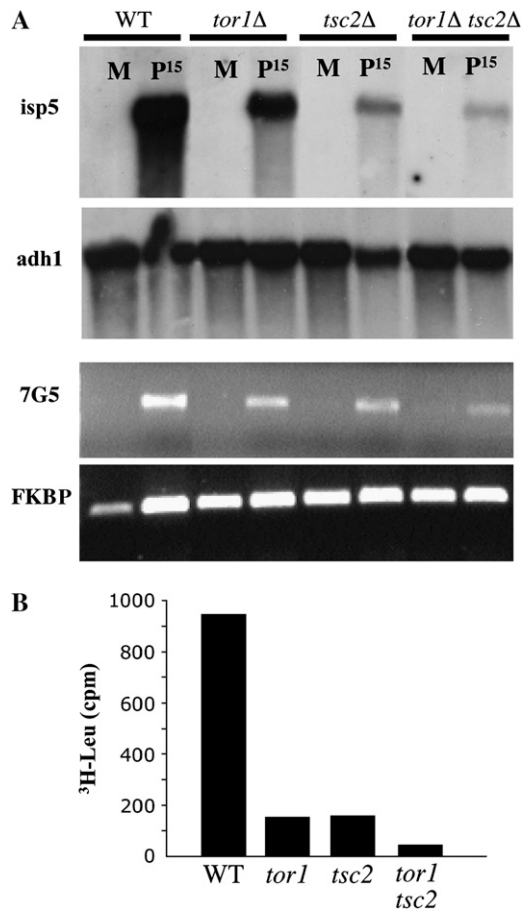
FIGURE 3.—Additive effects of Δ*tor1* and Δ*tsc2* mutations. (A) Wild-type (TA001), Δ*tor1* (TA390), Δ*tsc2* (TA450), and Δ*tor1* Δ*tsc2* (TA459) cells were grown to midlog phase in EMM (M) medium, washed, resuspended in proline (P) medium, and further incubated for 15 min at 30°. RNA was extracted and subjected to Northern blot (top two sections) or semiquantitative RT–PCR analyses (bottom two sections). Northern blots were probed with *isp5+* and *adh1+* (loading control). For RT–PCR analysis, primers were specific for the amino acid permease 7G5.06 or FKBP12 (control). (B) Δ*tor1* (TA390), Δ*tsc2* (TA450), and Δ*tor1* Δ*tsc2* (TA459) cells were grown to midlog phase in EMM medium. Incorporation of L-³H-leucine was measured after 6 min using the protocol detailed in MATERIALS AND METHODS.

tsc1/2 mutants on proline plates, but not when the nitrogen source in the medium was glutamic acid, a different amino acid that is more easily metaboilized by yeasts (Figure 4B). Thus, sensitivity of *tsc1/2* to rapamycin is specific to a poor nitrogen source and may represent inability to metabolize proline, rather then inability to uptake amino acids.

tsc2 mutants that also carried a mutation in the *S. pombe* FKBP12 gene, *fkh1+*, were completely resistant to growth inhibition by rapamycin on proline (Figure 4B). Similar results were seen for the *tsc1 fkh1* double mutant (data not shown). The FKBP12-dependent rapamycin sensitivity of *tsc1/2* suggests inhibition of a TOR-dependent function. We next examined the possibility that rapamycin may inhibit a Tor1-dependent function, by exam-

ining the ability of *tor1 tsc1* or *tor1 tsc2* mutants to grow on proline. As seen in Figure 4C the double mutants *tor1 tsc1* or *tor1 tsc2* failed to grow on proline plates, suggesting that Tsc1/2 and Tor1 act separately to regulate growth in the presence of a poor nitrogen source and that this function of Tor1 is sensitive to rapamycin.

The small cell size phenotype of *tsc1* or *tsc2* cells is reversed by *tor1* disruption: While both Tor1 and Tsc1/2 positively regulate amino acid uptake, cell size appears to be oppositely regulated by Tor1 and Tsc1/2. Δ*tor1* cells are elongated (WEISMAN and CHODER 2001; MATSUO *et al.* 2003); in contrast, we noted that single *tsc1* or *tsc2* mutants are slightly smaller than isogenic wild-type cells (Figure 4D). We examined cell length of the double mutants *tor1 tsc1* or *tor1 tsc2* and found that the *tor1* mutation reverses the short cell size phenotype of *tsc1/2* mutants (Figure 4D, data are shown for the *tsc2* mutation only). Thus, with respect to cell size, Tor1 may act downstream or independently of Tsc1/2.

When cells are shifted to less favorable growth conditions, such as poor nitrogen sources, mitosis is advanced and cells divide at a smaller size (FANTES and NURSE 1977; YOUNG and FANTES 1987). Shift of *tsc2* mutants from ammonia to proline medium (nutritional downshift) resulted in further cell size shortening and in the appearance of a small G_1 peak (Figure 4E). This G_1 peak likely reflects the small cell size of the *tsc1/2* mutants and thus the activation of a cell-size G_1/S checkpoint. Shift to proline in the presence of rapamycin resulted in further shortening of cell size and accumulation of even more cells at the G_1 phase (Figure 4E). Similar data were observed for *tsc1* mutants (data not shown). These findings indicate that *tsc1/2* mutants still respond to shift to proline by cell-size shortening and that loss of function of *tsc1/2* is additive to the effect of rapamycin. The target of rapamycin that causes this cell size shortening is yet unclear. Since loss of function of Tor2, but not of Tor1, leads to short cell size, it is possible that Tor2 inactivation leads to rapamycin-induced cell-size shortening. Growth arrest of *tsc2* mutants in proline medium occurs irrespective of their terminal cell size, since *tsc1/2* mutants die as small cells on proline in the presence of rapamycin, but as elongated cells when combined with the *tor1* mutation.

Rapamycin treatment, like loss of function of *tsc1* or *tsc2*, confers thialysine and canavanine resistance: One of the more detailed analyzed characteristics of *tsc* mutants is their inefficient amino acid uptake, which leads to canavanine resistance (VAN SLEGTENHORST *et al.* 2004; NAKASE *et al.* 2006). Since rapamycin treatment of wild-type cells leads to inefficient leucine uptake (WEISMAN *et al.* 2005), we examined their sensitivity in the presence of rapamycin to canavanine and thialysine, the later a toxic analog of lysine. We found that treatment with rapamycin results in resistance to these drugs, similar to the phenotype caused by deletion of *tsc1/2* (Figure 5A). The rapamycin-induced resistance to
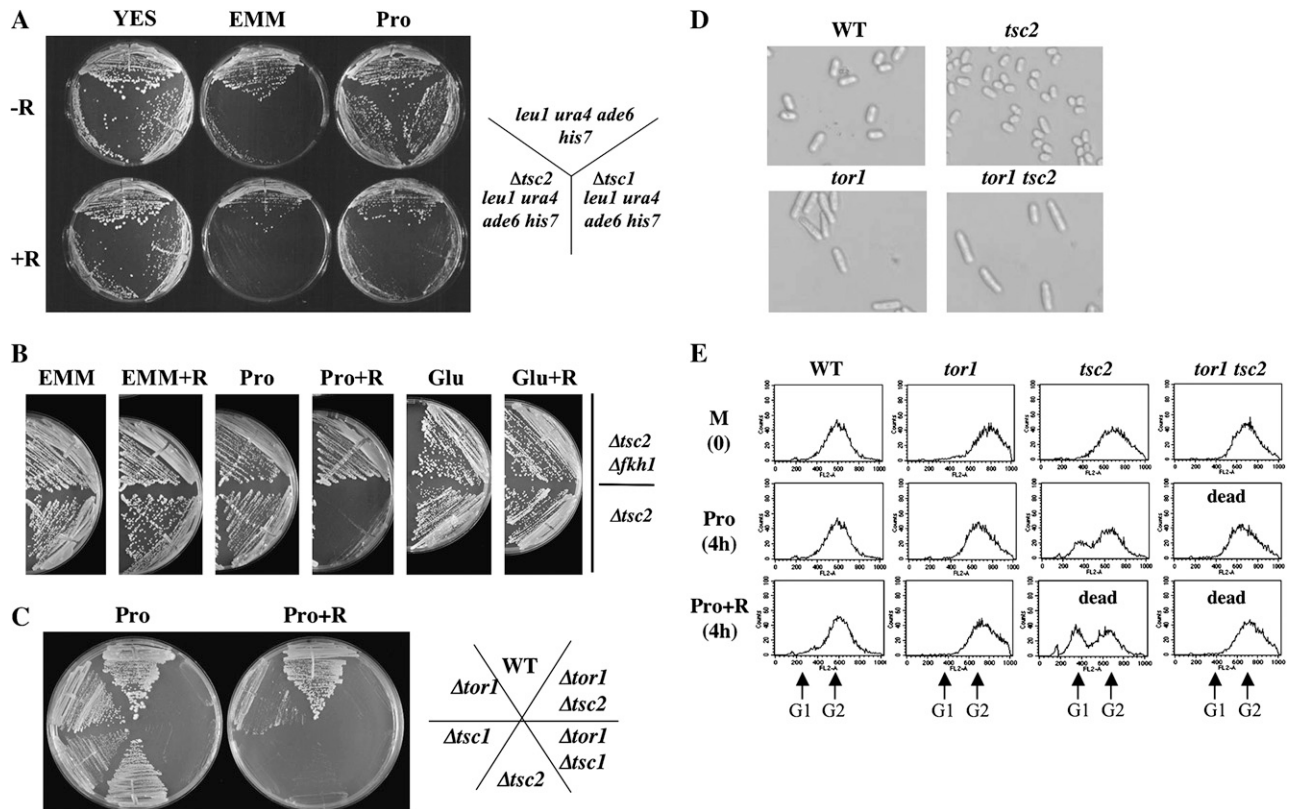
FIGURE 4.—Δ*tsc2* mutants are sensitive to rapamycin on medium containing a poor nitrogen source. (A) Δ*tsc1* or Δ*tsc2* mutants (TA419 and TA418, respectively) were streaked onto rich yeast extract (YE) or minimal plates containing ammonia (EMM) or proline (Pro) as the sole nitrogen source. Addition of rapamycin to the proline medium inhibited the growth on proline but not on rich medium. (B) Sensitivity to rapamycin on proline medium requires FKBP12. Prototrophic Δ*tsc2* mutants (TA450) or Δ*tsc2* Δ*fkh1* double mutants (TA468) were streaked on yeast extract (YE), proline (Pro), or glutamic acid (Glu) plates in the presence or the absence of 0.1 μg/ml rapamycin. Plates were incubated for 4 days at 30°. (C) Δ*tsc2* mutant cells were unable to grow on proline plates when combined with the Δ*tor1* mutation. The indicated strains, corresponding to TA001, TA390, TA449, TA481, TA450, and TA459, were streaked on proline plates in the presence or the absence of 0.1 μg/ml rapamycin. (D) Deletion of *tor1*⁺ reverses the small cell size and G₁ accumulation of Δ*tsc2* mutants. Wild-type (TA001), Δ*tor1* (TA390), Δ*tsc2* (TA450), and Δ*tor1* Δ*tsc2* (TA459) cells were grown to late log phase in minimal EMM media at 30° before being photographed. Deletion of *tor1*⁺ also reverses the small cell size and G₁ accumulation of Δ*tsc1*mutants (data not shown). (E) FACS analysis of the strains indicated in D. Cells were grown to midlog phase in EMM (M) media and then shifted to either proline (Pro) or proline with 0.1 mg/ml rapamycin (Pro + R).

canavanine or thialysine is abolished in cells carrying disruption of the FKBP12 gene (Figure 5A and data not shown), suggesting that rapamycin acts via inhibition of a TOR-dependent function. Either the FRB of Tor1 or that of Tor2 can bind the FKBP12-rapamycin complex, as we have shown by two-hybrid assays (WEISMAN 2004). However, as overexpression of Tor2 leads to canavanine resistance (Figure 1E), it is unlikely that the effect of rapamycin is due to direct inhibition of Tor2. A plausible mechanism to explain this effect of rapamycin would be the inhibition of a Tor1-dependent function. However, we found that *tor1* mutants do not show resistance to canavanine and are slightly more sensitive to thialysine compared to wild-type cells (Figure 5B). Using higher concentrations of canavanine or a different incubation time we also observed that *tor1* mutants are more sensitive to canavanine compared to wild-type cells (data not shown). This may be indicative of an increase in uptake

of the toxic analogs in *tor1* mutants that differs from the previously described uptake of leucine, histidine, or uracil. Since *tor1* mutants are highly sensitive to two different toxic amino acid analogs, we prefer an alternative explanation in which the sensitivity of *tor1* mutants to canavanine or thialysine reflects translational or protein folding defects.

At present the target of rapamycin in inducing canavanine or thialysine resistance is unclear. The possibility that rapamycin completely inhibits Tor1 activity is unlikely, since Δ*tor1* mutant do not show canavanine or thialysine resistance. Yet, it is possible that rapamycin inhibits certain Tor1-dependent activities, leading to reduced amino acid uptake. As noted above, Tor1 may negatively regulate Tor2, via a negative feedback loop, and thus its inhibition by rapamycin may remove the negative feedback loop, leading to overactivation of Tor2 and, thus, a decrease in amino acid uptake (Figure 5C).

DISCUSSION

Disruption of TOR activity in budding yeast, nematodes, Drosophila, mice, and human cells leads to features that are characteristic of starved cells. These starvation-like phenotypes have suggested that TOR plays a conserved role in regulating cell growth in response to nutrient availability. So far, studies of TOR signaling in *S. pombe* did not fit into such a model since the only characterized TOR homolog in fission yeast, Tor1, is

mainly required for starvation and stress responses and its absence results in inability to respond to starvation, rather then exhibiting a starvation-like phenotype. In contrast with Tor1 loss of function, we show here that disruption of *tor2*+ results in a phenotype that highly resembles nitrogen starvation. Cells depleted of Tor2 arrest their growth at the G_1 phase of the cell cycle as small and rounded cells, induce expression of nitrogen-starvation-specific genes, and are derepressed for sexual development. Most importantly, this phenotype highly resembles that of disruption of *rhb1*+, suggesting that the link between Rheb and TOR signaling is conserved in evolution from *S. pombe* to human. Previously, a physical interaction was described between Tor2 and hyperactivated Rhb1 in fission yeast (URANO *et al.* 2005). We suggest that Tor2 lies downstream of Rhb1. Yet, since overexpression of *tor2*+ did not suppress *rhb1*+ deletion (data not shown), final proof awaits isolation of hyperactivated Tor2. While this article was under review two articles (ALVAREZ and MORENO 2006; URITANI *et al.* 2006) were published that support our findings. Both demonstrate that Tor2 depletion leads to a phenotype of nitrogen-starved cells, including arrest as small rounded cells, induction of nitrogen-specific genes, such as *isp6*+, and entrance into sexual development under rich medium conditions.

The always starved for nitrogen phenotype of *tor2* mutants is in striking contrast to the never starved for nitrogen phenotype of *tor1* cells (Figure 2A). Since Tor1 and Tor2 appear to regulate similar processes in an opposite fashion, it is tempting to speculate that their functions are interconnected. One possibility is that Tor1 negatively inhibits Tor2; thus in the absence of Tor1, Tor2 may become hyperactivated, signaling availability of nitrogen even in the absence of a nitrogen source. Yet other inhibitory links between the two TOR-dependent pathways may explain these opposite effects. Tor1 is involved in additional, Tor2-independent processes. For example, stress response and acquisition of long-term viability in stationary phase are two functions regulated by Tor1, but not by Tor2 (Figure 5C).

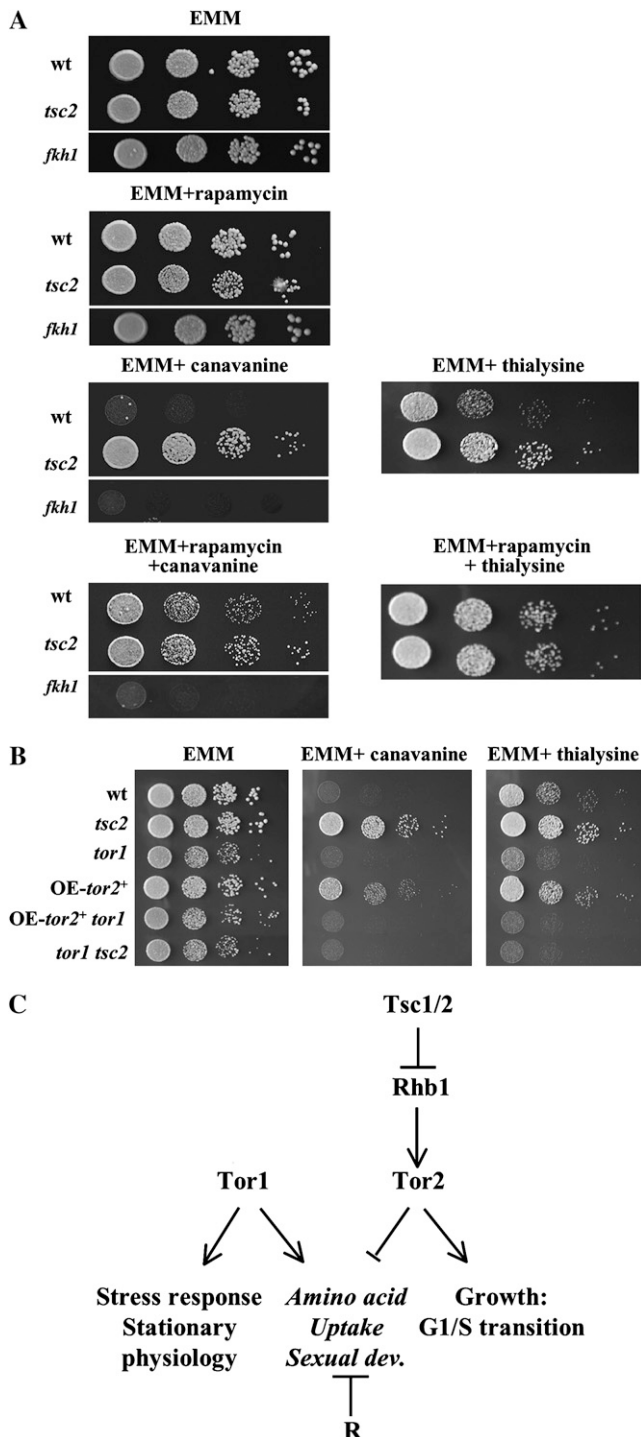While Tor2 is likely to be positively regulated by Rhb1, Tor1 appears to act in parallel with Tsc1/2 with respect

FIGURE 5.—Rapamycin confers canavanine and thialysine resistance, while deletion of *tor1*+ confers hypersensitivity to these toxic amino acid analogs. (A) Serial dilutions of exponentially growing wild-type (TA001), Δ*tsc2* (TA450), or Δ*fkh1* (TA597) cells were spotted on EMM medium containing canavanine or thialysine in the absence or presence of rapamycin. (B) Serial dilutions of TA001, TA450, TA390, TA568, TA561, and TA459 were spotted on EMM plates in the presence of either canavanine or thialysine. (C) Working model. Tor2 positively regulates growth, allowing the G_1/S transition under conditions of sufficient nutrients. Tor2 lies downstream of Rhb1 and is negatively regulated by Tsc1–2, while Tor1 acts in parallel with the Tsc1/2–Rhb1 module. The functions that are inhibited by rapamycin (R) are indicated in italics.

to amino acid uptake, regulation of amino acid permeases, and in regulating growth when proline is used as the nitrogen source. Most recently, YANG et al. (2006) found that the Drosophila Rheb and mammalian TSC1/2 have opposite effects on TORC1 and TORC2. These opposite effects are reminiscent of the opposite effects of TSC1/2–Rhb1 on Tor1 and Tor2. Our results show additive effects of the tor1 and tsc1/2 mutations: either tor1+ or tsc1/2+ is required for growth on poor nitrogen source and downregulation of amino acid permeases or leucine uptake is more pronounced in the double mutant tsc1 tor1 compared to the relevant single mutants. These findings argue against a direct effect of Tsc1/2–Rhb1 on Tor1. Rather, Tsc1/2–Rhb1 and Tor1 may act in two parallel pathways.

In many organisms, treatment with rapamycin results in TOR loss of function, often leading to a G_1 cell cycle arrest and to a phenotype of starved cells. In contrast, rapamycin does not affect wild-type S. pombe cells grown on rich medium. We have previously shown that auxotrophic S. pombe strains are sensitive to rapamycin due to inhibition of a Tor1-dependent function that positively regulates amino acid uptake. Here we identify tsc1/2 mutants as sensitive to rapamycin when grown on proline medium. This is clearly unexpected if Tsc1/2 in S. pombe were acting solely as negative regulators of TOR signaling. Accumulation of data now suggests that the phenotype of loss of function of tsc1/2+ is very similar to treatment with rapamycin. Both tsc1/2 mutants and rapamycin treatment result in partial sterility, decrease in leucine uptake, downregulation of permeases, resistance to canavanine, small cell size, and accumulation in G_1 (WEISMAN et al. 1997, 2005; MATSUMOTO et al. 2002; VAN SLEGTENHORST et al. 2004; this study). Tsc1/2 may act in parallel with a Tor1-dependent rapamycin-sensitive function. Yet, cells deleted for tor1+ are still sensitive to rapamycin in an FKBP12-dependent manner (WEISMAN 2004). Thus, whether certain Tor2 functions are also inhibited by rapamycin is as yet an open question. Our findings that rapamycin treatment can lead to cellular effects that mimic loss of function of Tsc1/2, rather than reversing the effects of mutations in Tsc1/2, draw attention to the complexity of the TOR signaling pathway and stress the need for more detailed information on the link between TSC and TOR.

LITERATURE CITED

ALVAREZ, B., and S. MORENO, 2006 Fission yeast Tor2 promotes cell growth and represses cell differentiation. J. Cell Sci. 119: 4475–4485.

BARBET, N. C., U. SCHNEIDER, S. B. HELLIWELL, I. STANSFIELD, M. F. TUITE et al., 1996 TOR controls translation initiation and early G1 progression in yeast. Mol. Biol. Cell 7: 25–42.

BECK, T., and M. N. HALL, 1999 The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402: 689–692.

BOOHER, R., and D. BEACH, 1986 Site-specific mutagenesis of cdc2+, a cell cycle control gene of the fission yeast Schizosaccharomyces pombe. Mol. Cell. Biol. 6: 3523–3530.

DI COMO, C. J., and K. T. ARNDT, 1996 Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. Genes Dev. 10: 1904–1916.

FANTES, P., and P. NURSE, 1977 Control of cell size at division in fission yeast by a growth-modulated size control over nuclear division. Exp. Cell Res. 107: 377–386.

GANGLOFF, Y. G., M. MUELLER, S. G. DANN, P. SVOBODA, M. STICKER et al., 2004 Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. Mol. Cell. Biol. 24: 9508–9516.

HARRINGTON, L. S., G. M. FINDLAY, A. GRAY, T. TOLKACHEVA, S. WIGFIELD et al., 2004 The TSC1–2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J. Cell Biol. 166: 213–223.

HAY, N., and N. SONENBERG, 2004 Upstream and downstream of mTOR. Genes Dev. 18: 1926–1945.

HOFFMAN, C. S., 2005 Glucose sensing via the protein kinase A pathway in Schizosaccharomyces pombe. Biochem. Soc. Trans. 33: 257–260.

JACINTO, E., R. LOEWITH, A. SCHMIDT, S. LIN, M. A. RUEGG et al., 2004 Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat. Cell Biol. 6: 1122–1128.

KAWAI, M., A. NAKASHIMA, M. UENO, T. USHIMARU, K. AIBA et al., 2001 Fission yeast tor1 functions in response to various stresses including nitrogen starvation, high osmolarity, and high temperature. Curr. Genet. 39: 166–174.

KIM, D. H., D. SARBASSOV DOS, S. M. ALI, R. R. LATEK, K. V. GUNTUR et al., 2003 GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol. Cell 11: 895–904.

LI, Y., M. N. CORRADETTI, K. INOKI and K. L. GUAN, 2004 TSC2: filling the GAP in the mTOR signaling pathway. Trends Biochem. Sci. 29: 32–38.

LOEWITH, R., E. JACINTO, S. WULLSCHLEGER, A. LORBERG, J. L. CRESPO et al., 2002 Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol. Cell 10: 457–468.

LONG, X., C. SPYCHER, Z. S. HAN, A. M. ROSE, F. MULLER et al., 2002 TOR deficiency in C. elegans causes developmental arrest and intestinal atrophy by inhibition of mRNA translation. Curr. Biol. 12: 1448–1461.

MACH, K. E., K. A. FURGE and C. F. ALBRIGHT, 2000 Loss of Rhb1, a Rheb-related GTPase in fission yeast, causes growth arrest with a terminal phenotype similar to that caused by nitrogen starvation. Genetics 155: 611–622.

MANNING, B. D., and L. C. CANTLEY, 2003 Rheb fills a GAP between TSC and TOR. Trends Biochem. Sci. 28: 573–576.

MATSUMOTO, S., A. BANDYOPADHYAY, D. J. KWIATKOWSKI, U. MAITRA and T. MATSUMOTO, 2002 Role of the Tsc1-Tsc2 complex in signaling and transport across the cell membrane in the fission yeast Schizosaccharomyces pombe. Genetics 161: 1053–1063.

MATSUO, T., Y. KUBO, Y. WATANABE and M. YAMAMOTO, 2003 Schizosaccharomyces pombe AGC family kinase Gad8p forms a conserved signaling module with TOR and PDK1-like kinases. EMBO J. 22: 3073–3083.

MAUNDRELL, K., 1993 Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene 123: 127–130.

MORENO, S., A. KLAR and P. NURSE, 1991 Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. 194: 795–823.

MURAKAMI, M., T. ICHISAKA, M. MAEDA, N. OSHIRO, K. HARA et al., 2004 mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. Mol. Cell. Biol. 24: 6710–6718.

NAKASE, Y., K. FUKUDA, Y. CHIKASHIGE, C. TSUTSUMI, D. MORITA et al., 2006 A defect in protein farnesylation suppresses a loss of

Schizosaccharomyces pombe tsc2⁺, a homolog of the human gene predisposing tuberous sclerosis complex. Genetics **173:** 569–578.

NODA, T., and Y. OHSUMI, 1998   Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J. Biol. Chem. **273:** 3963–3966.

OLDHAM, S., J. MONTAGNE, T. RADIMERSKI, G. THOMAS and E. HAFEN, 2000   Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. Genes Dev. **14:** 2689–2694.

PRENTICE, H. L., 1992   High efficiency transformation of Schizosaccharomyces pombe by electroporation. Nucleic Acids Res. **20:** 621.

SARBASSOV, D. D., S. M. ALI, D. H. KIM, D. A. GUERTIN, R. R. LATEK *et al.*, 2004   Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr. Biol. **14:** 1296–1302.

SARBASSOV, D. D., D. A. GUERTIN, S. M. ALI and D. M. SABATINI, 2005   Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science **307:** 1098–1101.

SHAH, O. J., Z. WANG and T. HUNTER, 2004   Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. Curr. Biol. **14:** 1650–1656.

SNAITH, H. A., and S. L. FORSBURG, 1999   Rereplication phenomenon in fission yeast requires MCM proteins and other S phase genes. Genetics **152:** 839–851.

STETTLER, S., E. WARBRICK, S. PROCHNIK, S. MACKIE and P. FANTES, 1996   The wis1 signal transduction pathway is required for expression of cAMP-repressed genes in fission yeast. J. Cell Sci. **109**(7): 1927–1935.

STIEFEL, J., L. WANG, D. A. KELLY, R. T. JANOO, J. SEITZ *et al.*, 2004   Suppressors of an adenylate cyclase deletion in the fission yeast Schizosaccharomyces pombe. Eukaryot. Cell **3:** 610–619.

TEE, A. R., and J. BLENIS, 2005   mTOR, translational control and human disease. Semin. Cell Dev. Biol. **16:** 29–37.

UM, S. H., F. FRIGERIO, M. WATANABE, F. PICARD, M. JOAQUIN *et al.*, 2004   Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature **431:** 200–205.

URANO, J., M. J. COMISO, L. GUO, P. J. ASPURIA, R. DENISKIN *et al.*, 2005   Identification of novel single amino acid changes that result in hyperactivation of the unique GTPase, Rheb, in fission yeast. Mol. Microbiol. **58:** 1074–1086.

URITANI, M., H. HIDAKA, Y. HOTTA, M. UENO, T. USHIMARU *et al.*, 2006   Fission yeast Tor2 links nitrogen signals to cell proliferation and acts downstream of the Rheb GTPase. Genes Cells **11:** 1367–1379.

VAN SLEGTENHORST, M., E. CARR, R. STOYANOVA, W. KRUGER and E. P. HENSKE, 2004   Tsc1+ and tsc2+ regulate arginine uptake and metabolism in Schizosaccharomyces pombe. J. Biol. Chem. **279:** 12706–12713.

WEISMAN, R., 2004   The fission yeast TOR proteins and the rapamycin response: an unexpected tale. Curr. Top. Microbiol. Immunol. **279:** 85–95.

WEISMAN, R., and M. CHODER, 2001   The fission yeast TOR homolog, tor1+, is required for the response to starvation and other stresses via a conserved serine. J. Biol. Chem. **276:** 7027–7032.

WEISMAN, R., M. CHODER and Y. KOLTIN, 1997   Rapamycin specifically interferes with the developmental response of fission yeast to starvation. J. Bacteriol. **179:** 6325–6334.

WEISMAN, R., I. ROITBURG, T. NAHARI and M. KUPIEC, 2005   Regulation of leucine uptake by *tor1*⁺ in *Schizosaccharomyces pombe* is sensitive to rapamycin. Genetics **169:** 539–550.

WULLSCHLEGER, S., R. LOEWITH and M. N. HALL, 2006   TOR signaling in growth and metabolism. Cell **124:** 471–484.

YANG, Q., K. INOKI, E. KIM and K. L. GUAN, 2006   TSC1/TSC2 and Rheb have different effects on TORC1 and TORC2 activity. Proc. Natl. Acad. Sci. USA **103:** 6811–6816.

YANG, W., A. P. TABANCAY, JR., J. URANO and F. TAMANOI, 2001   Failure to farnesylate Rheb protein contributes to the enrichment of G0/G1 phase cells in the Schizosaccharomyces pombe farnesyltransferase mutant. Mol. Microbiol. **41:** 1339–1347.

YOUNG, P. G., and P. A. FANTES, 1987   Schizosaccharomyces pombe mutants affected in their division response to starvation. J. Cell Sci. **88**(3): 295–304.

ZARAGOZA, D., A. GHAVIDEL, J. HEITMAN and M. C. SCHULTZ, 1998   Rapamycin induces the G0 program of transcriptional repression in yeast by interfering with the TOR signaling pathway. Mol. Cell. Biol. **18:** 4463–4470.

ZHANG, H., J. P. STALLOCK, J. C. NG, C. REINHARD and T. P. NEUFELD, 2000   Regulation of cellular growth by the Drosophila target of rapamycin dTOR. Genes Dev. **14:** 2712–2724.

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