Molecular Cell, Vol. 8, 1017–1026, November, 2001, Copyright ©2001 by Cell Press

# TIP41 Interacts with TAP42 and Negatively Regulates the TOR Signaling Pathway

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## Summary

**In *Saccharomyces cerevisiae*, the rapamycin-sensitive TOR kinases negatively regulate the type 2A-related phosphatase SIT4 by promoting the association of this phosphatase with the inhibitor TAP42. Here, we describe TIP41, a conserved TAP42-interacting protein involved in the regulation of SIT4. Deletion of the *TIP41* gene confers rapamycin resistance, suppresses a *tap42* mutation, and prevents dissociation of SIT4 from TAP42. Furthermore, a *TIP41* deletion prevents SIT4-dependent events such as dephosphorylation of the kinase NPR1 and nuclear translocation of the transcription factor GLN3. Thus, TIP41 negatively regulates the TOR pathway by binding and inhibiting TAP42. The binding of TIP41 to TAP42 is stimulated upon rapamycin treatment via SIT4-dependent dephosphorylation of TIP41, suggesting that TIP41 is part of a feedback loop that rapidly amplifies SIT4 phosphatase activity under TOR-inactivating conditions.**

## Introduction

The rapamycin-sensitive TOR kinases, TOR1 and TOR2, control cell growth in *Saccharomyces cerevisiae* in response to nutrients (Schmelzle and Hall, 2000; Gingras et al., 2001). Growth control by the TOR signaling pathway includes the TOR-effector TAP42 and the type 2A-related phosphatase SIT4. In the presence of a good nitrogen source such as ammonium or glutamine, TOR negatively regulates SIT4 by promoting the binding of the inhibitor protein TAP42 to SIT4. Under starvation conditions or rapamycin treatment, TOR is inactive, and TAP42 dissociates from SIT4 (Di Como and Arndt, 1996). Thus, SIT4 is active under conditions in which TOR is inactive (Beck and Hall, 1999). TAP42-unbound SIT4 associates with the high molecular weight SAP proteins SAP155, SAP185, SAP190, and possibly SAP4 (Di Como and Arndt, 1996; Luke et al., 1996; Sutton et al., 1991). Based on sequence similarities, the SAPs are categorized into two subgroups, SAP155-SAP4 and SAP185-SAP190. The SAPs associate with SIT4 individually and may thereby confer different target specificity on SIT4 (Luke et al., 1996). It is unclear how the SAPs may relate to SIT4 function in the TOR pathway. The mechanism by which TOR promotes formation of the TAP42-SIT4 complex is also unknown. TOR also promotes the association of TAP42 with the type 2A phosphatase (PP2A) catalytic subunits PPH21 and PPH22 (Di Como and Arndt, 1996).

TAP42 controls several downstream effectors of the TOR pathway. By binding and inhibiting SIT4, TAP42 maintains the GATA transcription factor GLN3 in a phosphorylated and inactive state (Beck and Hall, 1999; Cardenas et al., 1999; Hardwick et al., 1999). Phosphorylated GLN3 is inactive because it binds the cytoplasmic protein URE2 and is thereby prevented from translocating into the nucleus. Upon TOR inactivation, due to rapamycin treatment or nitrogen limitation, SIT4 is released from TAP42, leading to dephosphorylation and nuclear translocation of GLN3. TAP42 similarly controls the phosphorylation and activation of the Ser/Thr protein kinase NPR1 (Schmidt et al., 1998). Phosphorylated NPR1 is inactive, resulting in stabilization of specific amino acid permeases such as the tryptophan permease TAT2 and destabilization of the general amino acid permease GAP1 (Beck et al., 1999; Schmidt et al., 1998; Vandenbol et al., 1990). Upon rapamycin treatment or nitrogen limitation, NPR1 is rapidly dephosphorylated and activated. Active NPR1 mediates the destabilization of TAT2 and the stabilization of GAP1. The mechanism by which TAP42 controls the phosphorylation of NPR1 is not known but may also involve SIT4, as described above for GLN3. The activation of GLN3 and NPR1 upon nitrogen starvation leads to transcriptional and posttranscriptional induction of processes involved in scavenging secondary nitrogen sources.

To investigate the mechanism by which TAP42 is controlled, we screened by two-hybrid analysis for candidate TAP42 regulators. We identified an uncharacterized, conserved protein called TIP41 that binds to TAP42 and thereby negatively regulates TOR signaling.

## Results

### TIP41 Is a TAP42-Interacting Protein

To identify candidate regulators of TAP42, we employed two-hybrid analysis with TAP42 as bait. The bait hybrid contained the entire TAP42 protein fused to either the GAL4-DNA binding domain (GAL4-DBD) or lexA. A total of 8 million transformants were screened using both the GAL4-DBD-TAP42 and lexA-TAP42 as baits. The majority of interactors were identified as type 2A phosphatase catalytic subunits (data not shown). An uncharacterized protein encoded by open reading frame YPR040w containing 356 codons, which we named TIP41 (TAP42-interacting protein of 41 kDa), was also isolated (Figure 1A). TIP41 was obtained three times, once with the GAL4 activation domain (GAL4-AD) replacing the N-terminal 10 amino acids of TIP41 and twice with the activation domain replacing the N-terminal 30 amino acids. Expression of the β-galactosidase reporter gene upon coexpression of GAL4-DBD-TAP42 and

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A

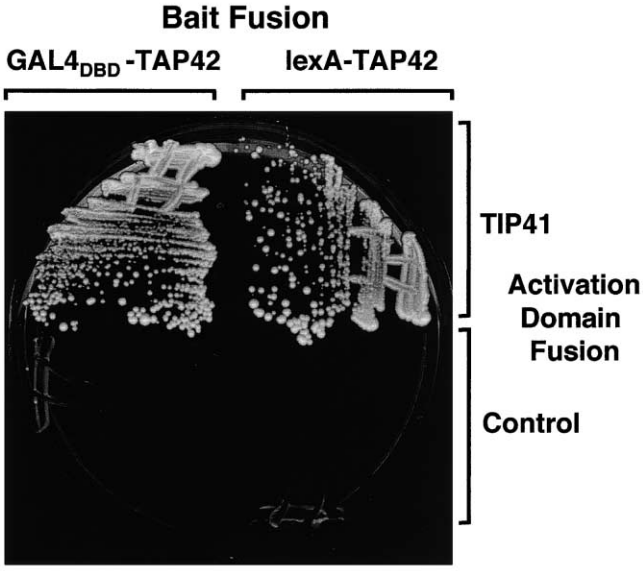



Figure 1. TIP41 Is a TAP42-Interacting Protein

(A) Strains containing *HIS3* coding sequences regulated either by GAL4 binding sites or by lexA binding sites and also containing the indicated bait fusion and activation domain fusion were streaked on synthetic complete medium lacking histidine and containing 30 mM 3-aminotriazole. Growth, indicating an interaction between the fusion proteins, is shown after 3 days at 30°C.

(B) β-galactosidase assays were performed on extracts prepared from strains containing the *lacZ* gene regulated by GAL4 binding sites and the indicated fusion proteins.

B

| $GAL4_{DBD}$ Fusion | Act. Dom. Fusion | β-gal units |
|---|---|---|
| TAP42 | TIP41, aa 31 | 480 |
| TAP42 | TIP41, aa 11 | 585 |
| TAP42 | no insert | 8 |
| no insert | TIP41, aa 11 | 5 |
| eIF-4E | TIP41, aa 11 | 6 |
| eIF-4E | eIF-4G | 850 |

GAL4-AD-TIP41 (Figure 1B) was dependent on both TAP42 and TIP41 fusion proteins. The translation initiation factors eIF-4E and eIF-4G fused to GAL4-DBD and GAL4-AD, respectively, were used as a positive control (Figure 1B). The interaction between TIP41 and TAP42 was confirmed biochemically using a GST pull-down assay, as described further below.

TIP41, like TAP42, is evolutionarily conserved (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/8/5/1017/DC1). TIP41 has 35% identity to a protein encoded by the human open reading frame HS.137576, 27% identity to the *D. melanogaster* CG9578, 31% identity to *S. pombe* SPCC4B3.16, and 34% identity to *C. elegans* C02C2.6. All these putative proteins are as yet uncharacterized.

### Overexpression of TIP41 Inhibits Growth, whereas Deletion of *TIP41* Suppresses a *tap42*$^{ts}$ Mutation and Confers Rapamycin Resistance

Deletion of *TIP41* did not confer a detectable growth defect on standard rich or minimal medium at various temperatures (data not shown), indicating that TIP41 is not essential for growth. To determine if overexpression of TIP41 confers a phenotype, we expressed *TIP41* under control of a galactose-inducible and glucose-repressible promoter. Induction of TIP41 by galactose caused a severe growth defect (Figure 2A).

The physical interaction between TIP41 and TAP42 and the growth inhibition caused by overexpression of TIP41 led us to examine a possible involvement of TIP41 in the TOR pathway. First, we investigated a genetic interaction between *TIP41* and *TAP42*. TAP42 is an essential protein, and a strain containing the temperature-sensitive *tap42-11* mutation grows very poorly at 30°C (Di Como and Arndt, 1996). A *TIP41* deletion was introduced into a strain containing the temperature-sensitive *tap42-11* allele. The *tap42-11 tip41* mutant, unlike the parental *tap42-11* strain, formed colonies at 30°C (Figure 2B). This suppression of the *tap42-11* growth defect by *tip41* suggests that TIP41 antagonizes TAP42 function in vivo. To investigate further a role for TIP41 in the TOR signaling pathway, we examined the rapamycin resistance of cells deleted for *TIP41*. Consistent with the above suggestion that TIP41 antagonizes TAP42, deletion of *TIP41* conferred partial resistance to rapamycin (Figure 2C). Thus, TIP41 appears to act as a negative regulator of the TOR pathway in vivo.

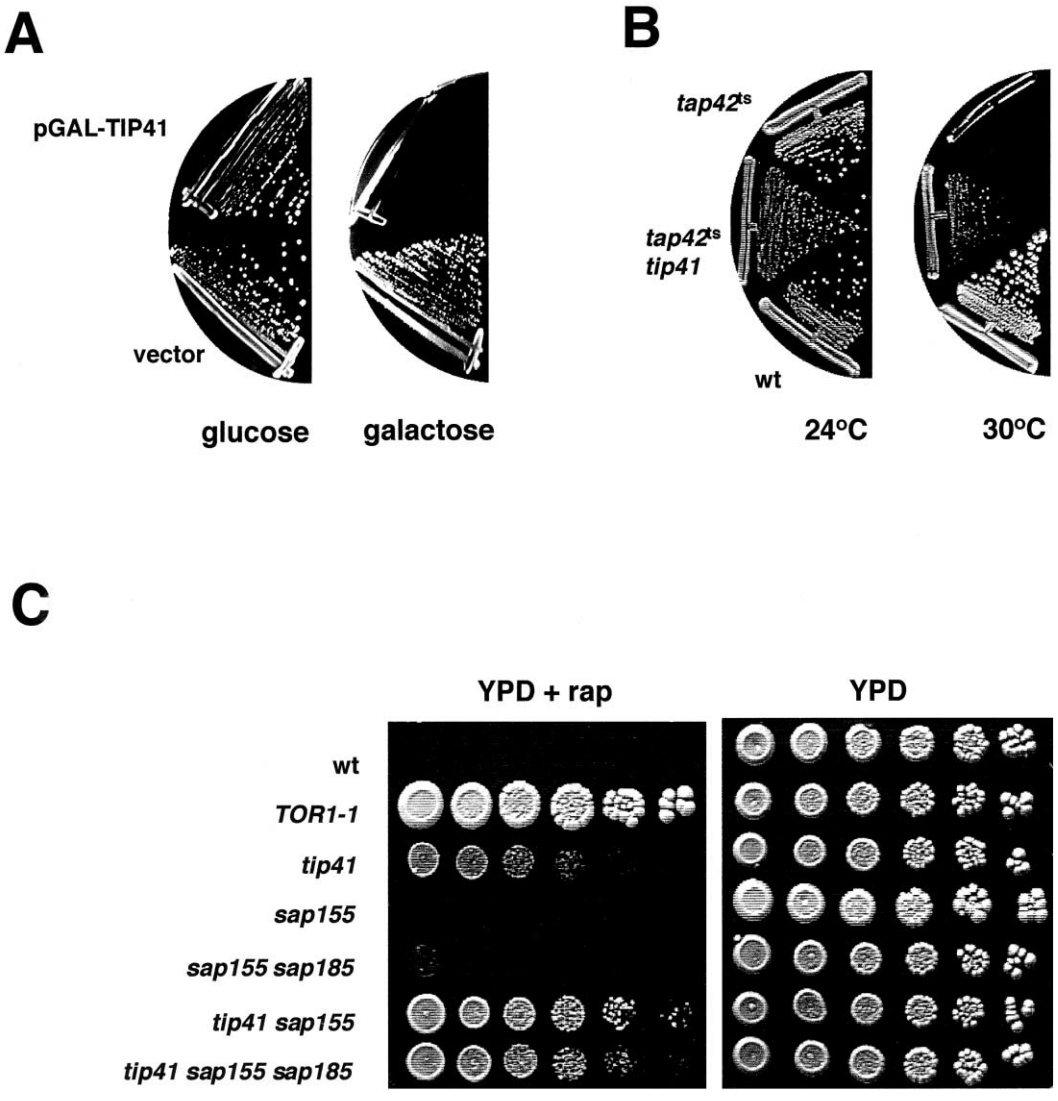



Figure 2. Overexpression of TIP41 Inhibits Growth, whereas Deletion of *TIP41* Suppresses a *tap42ts* Mutation and Confers Rapamycin Resistance

(A) Wild-type (JK9-3da) cells carrying a control vector (pWAB4) or pGAL-TIP41(pWAB3) were streaked on glucose (SD) and galactose (SGal) media. Plates were incubated at 30°C for 4 days.

(B) Wild-type (wt) (JK9-3da), *tap42ts* (TS54-5a), and *tap42ts tip41* (EJ152-4a) cells were streaked on YPD and incubated at 24°C or 30°C for 4 days.

(C) Wild-type (wt) (JK9-3da), *TOR1-1* (JH11-1c), *tip41* (EJ91-1d), *sap155* (EJ96-2d), *sap155 sap185* (EJ100-6d), *tip41 sap155* (EJ126-8a), and *tip41 sap155 sap185* (EJ124-3a) were serially diluted in YPD liquid medium and spotted on YPD or YPD containing 20 ng/ml rapamycin (rap). Cells were grown for 4 days at 30°C.

As the role of TAP42 is to inhibit SIT4, we next examined if combining the deletion of *TIP41* with a deletion of any of the *SAP* genes leads to increased rapamycin resistance. This analysis was performed with the *SAP* genes rather than with *SIT4* because, unlike deletion of *SIT4*, deletion of the *SAP* genes individually does not cause a growth defect (Luke et al., 1996). Strains deleted individually for *SAP155*, *SAP185*, *SAP190*, or *SAP4* did not display rapamycin resistance (Figure 2C and data not shown). Combined deletion of *SAP155* and *SAP185* conferred little to no rapamycin resistance. However, combined deletion of *TIP41* and *SAP155* conferred greater rapamycin resistance than deletion of *TIP41* alone (Figure 2C). This enhanced rapamycin resistance was specific to the *tip41 sap155* combination, as no increased rapamycin resistance was observed with any other *tip41 sap* pairwise combination. Furthermore, the additional deletion of *SAP185* or *SAP190* did not enhance the rapamycin resistance of the *tip41 sap155* mutant, nor did the *tip41 sap185 sap190* mutant display enhanced rapamycin resistance (Figure 2C and data not shown). The finding that deletion of *SAP155* enhances the rapamycin resistance of a *tip41* mutant suggests that TIP41 antagonizes TAP42-mediated inhibition of SIT4. The observation that deletion of only *SAP155* enhances the rapamycin resistance of a *tip41* mutant may indicate that SAP155-associated SIT4 is particularly important to the TOR pathway.

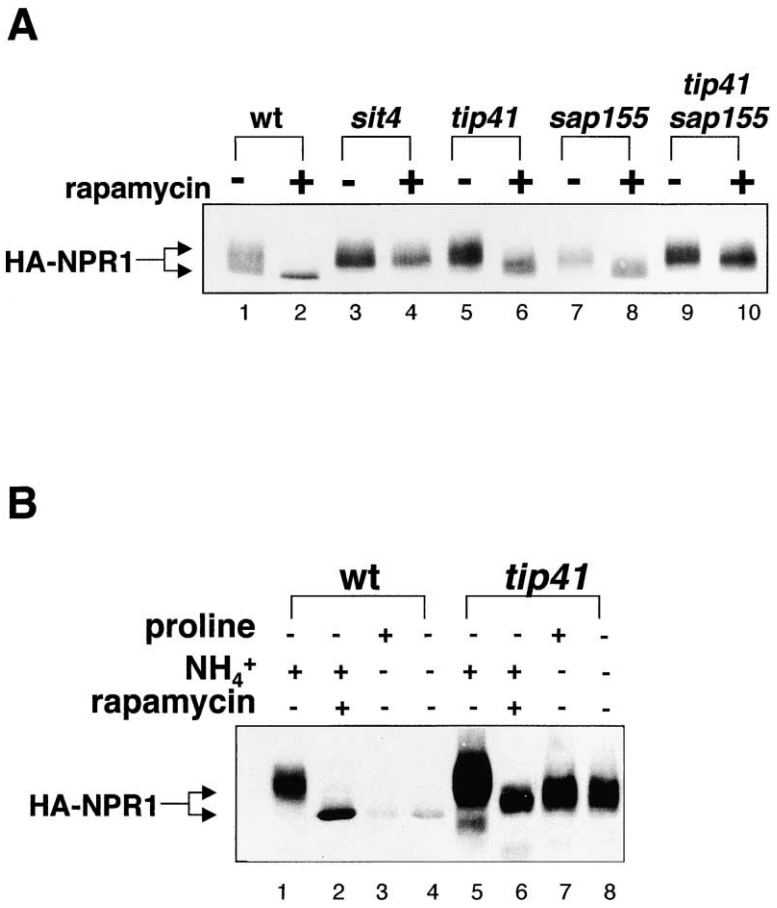



Figure 3. NPR1 Is Hyperphosphorylated and More Abundant in *tip41* Cells

(A) Wild-type (wt) (JK9-3da), *sit4* (TS64-1a), *tip41* (EJ91-1d), *sap155* (EJ96-2d), or *tip41 sap155* (EJ126-8a) cells carrying the plasmid pHA-NPR1 (pEJ23) were grown to log phase at 30°C and then treated for 30 min with either 100 ng/ml rapamycin or drug vehicle alone. Cells were harvested and lysed. Total cellular extracts were fractionated on 7.5% SDS-PAGE, transferred to Immobilon, and blotted using anti-HA antibody. Lanes 1–4 were each loaded with 50 μg total protein, lanes 5–10 with 10 μg total protein.

(B) Wild-type (wt) (TB50a) or *tip41* (EJ91-1d) cells carrying the plasmid pHA-NPR1 (pEJ23) were grown to log phase at 30°C and then either treated for 30 min with 100 ng/ml rapamycin or drug vehicle alone or nitrogen starved for 1 hr, as described in Experimental Procedures. Cells were then processed as described in (A). Each lane was loaded with 50 μg total protein.

### NPR1 Is Hyperphosphorylated and More Abundant in *tip41* Cells

The suggestion that TIP41 antagonizes TAP42-mediated inhibition of SIT4 led us to investigate SIT4-dependent events in a *tip41* mutant. If TIP41 indeed antagonizes TAP42-mediated inhibition of SIT4, SIT4-dependent events should not occur in a *tip41* mutant. We first examined NPR1 dephosphorylation. Upon nitrogen starvation or rapamycin treatment, NPR1 is dephosphorylated and activated (Schmidt et al., 1998). In a *sit4* mutant, NPR1 was hyperphosphorylated and was not dephosphorylated upon rapamycin treatment (Figure 3A, lanes 1–4), indicating that the dephosphorylation of NPR1 is SIT4 dependent. The dephosphorylation of NPR1 occurred normally in a type 2A phosphatase mutant (*pph3 pph21 pph22*$^{ts}$ mutant shifted to nonpermissive temperature), indicating that the dephosphorylation of NPR1 is SIT4 specific (data not shown). In *tip41* and *sap155* mutants, NPR1 was also hyperphosphorylated and only partly dephosphorylated in response to rapamycin (Figure 3A, compare lanes 1 and 2 to lanes 5–8). In a *tip41 sap155* double mutant, like in the *sit4* mutant, NPR1 was hyperphosphorylated (Figure 3A, lanes 1 and 9) and was not significantly dephosphorylated upon rapamycin treatment (Figure 3A, lanes 9 and 10). Thus, TIP41 is required for NPR1 dephosphorylation, providing further evidence that TIP41 is required for SIT4 activity.

Interestingly, the abundance, in addition to the phosphorylation, of NPR1 is affected by TIP41 and SAP155. The amount of NPR1 protein increased approximately 5-fold in *tip41*, *sap155*, and *tip41 sap155* mutants as compared to wild-type and a *sit4* mutant. Note that in lanes 5–10 of Figure 3A, 5-fold less protein was loaded compared with lanes 1–4. The reason for this increase in the amount of NPR1 is unclear but could be attributed to changes in the stability or expression of NPR1. NPR1 contains several PEST sequences that mediate protein degradation (Rechsteiner and Rogers, 1996; Vandenbol et al., 1990). However, it is not known if NPR1 levels are indeed controlled at the level of stability. It also remains to be determined why *tip41* and *sap155* mutants but not *sit4* mutants display increased NPR1 levels.

To determine if TIP41 is involved in the dephosphorylation of NPR1 that normally occurs in response to nitrogen limitation, we examined NPR1 dephosphorylation in cells grown in medium lacking a nitrogen source or containing only a poor nitrogen source (proline). When wild-type cells were shifted from a good nitrogen source to nitrogen-limiting conditions (minus $NH_4^+$ or only proline), NPR1 was dephosphorylated and markedly reduced in abundance (Figure 3B, compare lane 1 to lanes 3 and 4). This suggests that changes in NPR1 levels (as described above) may play a role in the regulation of NPR1. In a nitrogen-limited *tip41* mutant, compared to wild-type, NPR1 remained phosphorylated and abundant (Figure 3B, lanes 3 and 4 versus lanes 7 and 8). Thus, TIP41 appears to be involved in the regulation of NPR1 that occurs in response to the nitrogen source.

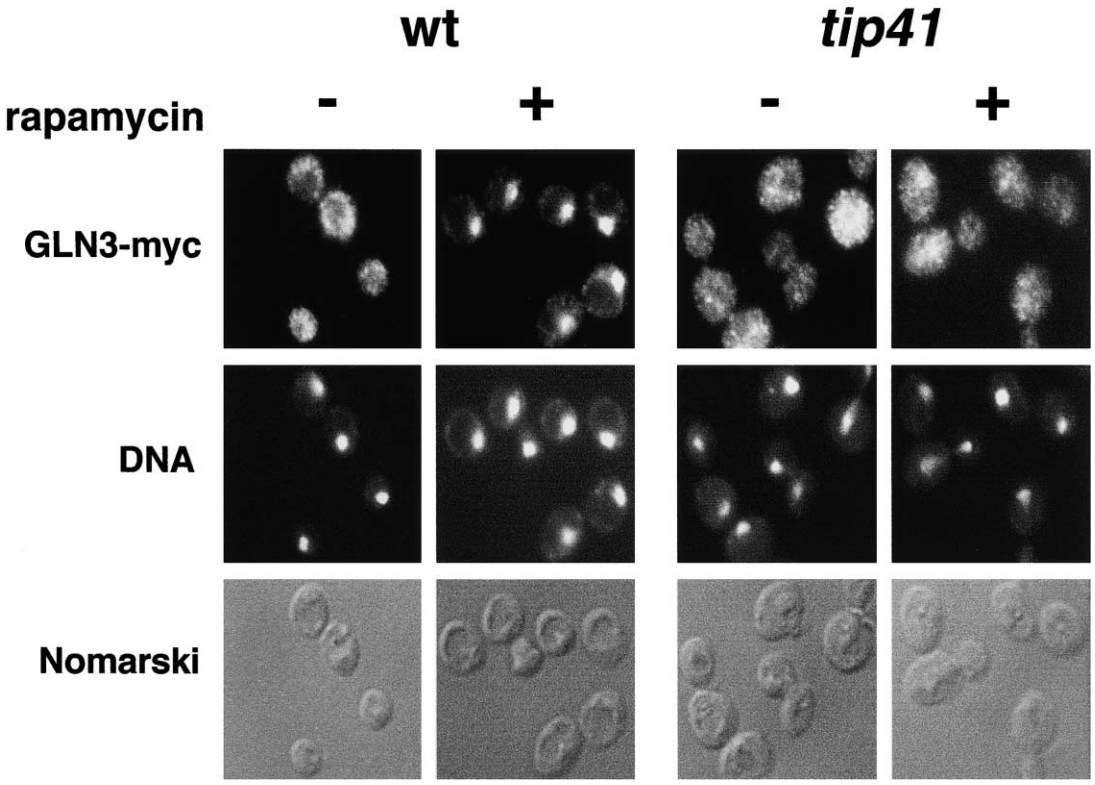



Figure 4. GLN3 Transcription Factor Remains in the Cytoplasm in *tip41* Cells

Wild-type (wt) (HM4-5d) and *tip41* (EJ104-3a) mutant cells, both expressing genomically myc-tagged GLN3, were grown to log phase at 30°C in YPD. Cultures were treated for 30 min with 100 ng/ml rapamycin or drug vehicle alone. Cells were then processed for indirect immunofluorescence. Localization of GLN3 was visualized using anti-myc antibody.

### GLN3 Transcription Factor Remains in the Cytoplasm in *tip41* Cells

To investigate a second SIT4-dependent event, we examined nuclear translocation of the GLN3 transcription factor in rapamycin-treated wild-type cells and *tip41* mutant cells. In rapamycin-treated wild-type cells, GLN3 rapidly translocates into the nucleus (Beck and Hall, 1999). In contrast, in rapamycin-treated *tip41* mutant cells, GLN3 remained in the cytoplasm (Figure 4). Thus, TIP41 is required for nuclear translocation of GLN3. The above results on NPR1 dephosphorylation and GLN3 nuclear translocation confirm that TIP41 is required for SIT4 activity, most likely by antagonizing TAP42.

### SIT4 and TAP42 Fail to Dissociate in *tip41* Cells

The above observations suggest, first, that TIP41 binds and inhibits TAP42 and, second, that TIP41 activates SIT4. Are these two activities of TIP41 related? To determine if the TIP41 requirement for SIT4 activity involves the action of TIP41 on TAP42, we examined the association of SIT4 and TAP42 in a *tip41* mutant. The presence of TAP42 protein was assayed in SIT4-HA immunoprecipitates from rapamycin-treated *tip41* mutant cells and wild-type cells. As reported previously (Di Como and Arndt, 1996), rapamycin treatment induced dissociation of the TAP42-SIT4 complex in wild-type cells. In contrast, no significant dissociation of the TAP42-SIT4 complex was detected in *tip41* cells (Figure 5), suggesting that TIP41 is required for the dissociation of TAP42 from SIT4. Thus, TIP41 appears to activate SIT4 by antagonizing the inhibitor TAP42.

### TOR-Inactivating Conditions Enhance the Interaction of TIP41 with TAP42

Is the binding of TIP41 to TAP42 regulated? To answer this question, we performed a GST pull-down assay with

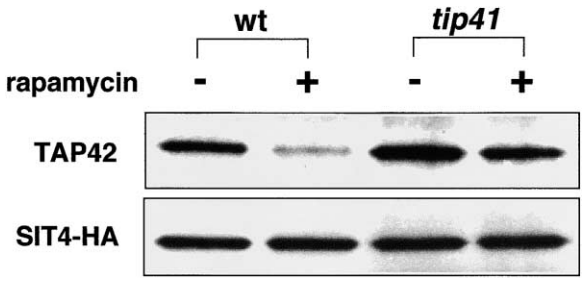



Figure 5. TIP41 Is Required for TAP42-SIT4 Dissociation

Wild-type (CY1258) or *tip41* (CY6323) cells were grown in YPD to log phase at 30°C and then treated with 100 ng/ml rapamycin or drug vehicle alone for 30 min. Cells were harvested, and protein extracts were prepared. SIT4-HA was immunoprecipitated from extracts, and the immunoprecipitates were analyzed by Western blot with anti-TAP42 and anti-HA antibodies. The same result was obtained with a 1 hr rapamycin treatment.

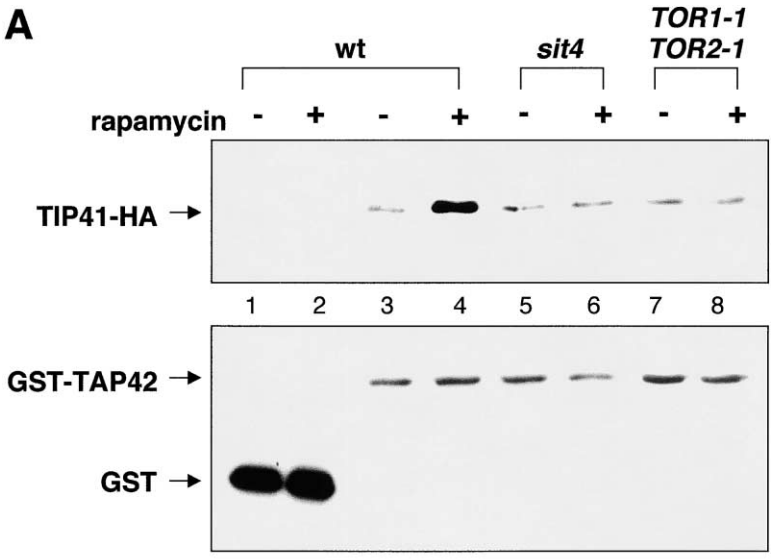
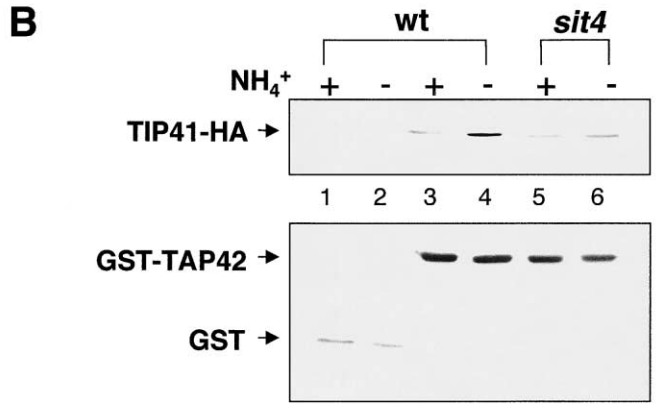



Figure 6. TOR-Inactivating Conditions Enhance the Interaction of TIP41 with TAP42

(A) Wild-type (wt) (JK9-3da), *sit4* (TS64-1a), or rapamycin-resistant *TOR1-1 TOR2-1* (JH18-3b) cells coexpressing HA-tagged TIP41 (pEJ120) and GST-TAP42 (pEJ27) or GAL-GST (pEG-KT) were grown to log phase at 30°C. Cultures were treated with 100 ng/ml rapamycin or drug vehicle alone for 30 min, harvested, and processed for extract preparation. A GST pull-down assay was performed, and bound TIP41-HA was detected by anti-HA immunoblotting (upper panel). Total amount of GST and GST-TAP42 was compared in all lanes by subsequent blotting using anti-GST antibody (lower panel). Equal amounts of TIP41-HA were detected in all extracts by a separate immunoblot (not shown). (B) Wild-type (wt) (TB50a) or *sit4* (TS64-1a) mutant cells coexpressing TIP41-HA and GST-TAP42 were starved for ammonium for 1 hr, as in Figure 3B. GST pull-down and anti-HA immunoblotting was performed as in (A).




extracts prepared from cells coexpressing GST-TAP42 and TIP41-HA. Extracts were prepared from rapamycin-treated and -untreated cells. Weak binding was detected in extracts from rapamycin-untreated cells (Figure 6A, lane 3). An average of 6-fold greater binding was observed with extracts from rapamycin-treated cells (Figure 6A, compare lanes 3 and 4). The enhanced binding observed upon rapamycin treatment was eliminated when the experiment was performed with a rapamycin-resistant *TOR1-1 TOR2-1* mutant in which TOR1 and TOR2 are insensitive to rapamycin (Heitman et al., 1991) (Figure 6A, lanes 7 and 8). The interaction of TIP41-HA with GST-TAP42 is specific, since TIP41-HA did not interact with GST alone (Figure 6, lanes 1 and 2). To further investigate the physiological relevance of the TIP41-TAP42 interaction, the pull-down experiment was performed with cells shifted to medium lacking a nitrogen source. Nitrogen deprivation, like rapamycin treatment, caused enhanced binding of TIP41 to TAP42 (Figure 6B, lanes 3 and 4). These findings indicate that the binding of TIP41 to TAP42 is regulated. More explicitly, the binding is enhanced upon inactivation of the TOR pathway. Thus, TIP41 is also controlled by the TOR pathway in addition to its role as a negative regulator of TOR signaling.

### Association of TIP41 and TAP42 Is SIT4 Dependent

The above findings suggest that TIP41 may control SIT4 via TAP42 as part of a feedback loop. To investigate this possibility, we examined the association of TIP41 and TAP42 in a *sit4* mutant. The GST pull-down assay, as described above, was performed with an extract prepared from a *sit4* mutant. The *sit4* mutation prevented the enhanced binding of TIP41 to TAP42 normally observed upon rapamycin treatment or nitrogen limitation (Figure 6, lanes 5 and 6). Thus, as SIT4 is required for enhanced binding of TIP41 to TAP42 and TIP41 positively controls SIT4, TIP41 appears to be part of a feedback loop that amplifies SIT4 activity when TOR is inactivated.

### Dephosphorylation of TIP41 Is SIT4 Dependent

The finding that the association of TIP41 with TAP42 is dependent on a phosphatase, SIT4, suggested that this association is controlled by phosphorylation. To investigate this possibility, we first examined if TIP41 is a phosphoprotein, using two-dimensional gel electrophoresis (Duncan and Song, 1999; Radimerski et al., 2000). TIP41-HA immunoprecipitated from wild-type cells migrated as five major distinct spots (Figure 7A, a–f) in an esti-

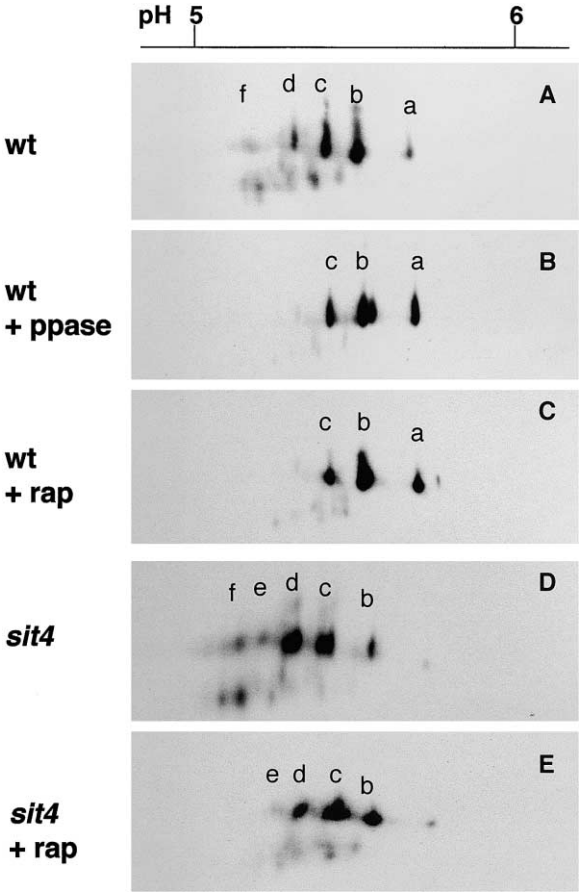



Figure 7. Dephosphorylation of TIP41 Is SIT4 Dependent

(A–E) Wild-type (wt) (TB50a) or *sit4* mutant (TS64-1a) cells were grown to log phase at 30°C and then treated for 30 min with 100 ng/ml rapamycin (rap) or drug vehicle alone. TIP41-HA immunoprecipitates were incubated either with or without alkaline phosphatase (ppase) at 37°C for 1 hr. First dimension analysis was performed by isoelectric focusing on a pH 3–10 NL gradient strip followed by a second dimension analysis on 10% SDS-PAGE. TIP41-HA was detected by immunoblotting using anti-HA antibody. Figure panels encompass pH 5–6 from left to right.

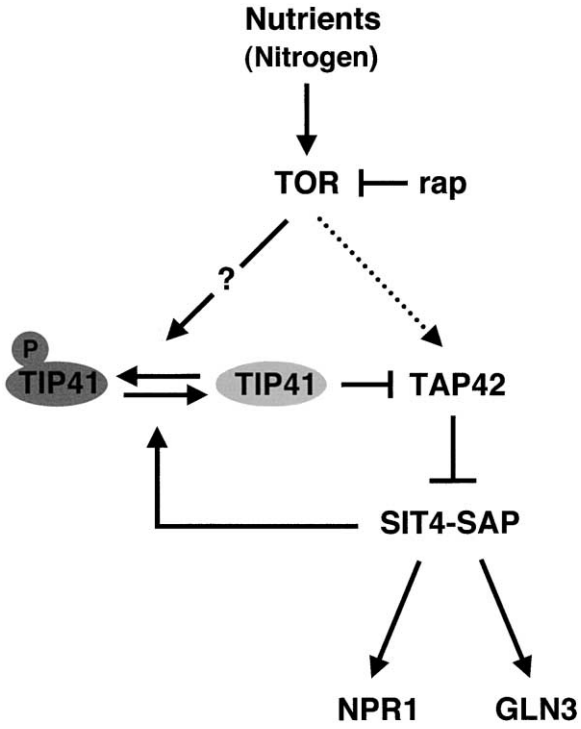



Figure 8. Model of TIP41 Action in the TOR Signaling Pathway

Arrows represent positive regulation; bars represent negative regulation. Rap, rapamycin. TOR refers to TOR1 or TOR2. The dotted arrow between TOR and TAP42 indicates that the phosphorylation of TAP42 by TOR (Jiang and Broach, 1999) plays a minor role in the regulation of SIT4. See Discussion for details.

mated pI range of 5.0–6.0, at the expected molecular weight of 41 kDa, suggesting that TIP41 is modified. To determine if TIP41 is modified by phosphorylation, immunoprecipitated TIP41-HA was treated with alkaline phosphatase. Phosphatase treatment caused two of the five spots to disappear (spots d and f) (Figure 7B), while spots a and b became more intense, indicating that TIP41 is indeed phosphorylated, most likely on multiple sites.

Next, we investigated if TIP41 is dephosphorylated in a SIT4-dependent manner. As SIT4 is activated upon rapamycin treatment, we examined if TIP41 is dephosphorylated upon rapamycin treatment. TIP41-HA immunoprecipitated from rapamycin-treated cells displayed altered mobility similar to that observed upon phosphatase treatment (Figures 7B and 7C), suggesting that SIT4 mediates the dephosphorylation of TIP41. To confirm that SIT4 is involved in TIP41 dephosphorylation, TIP41-HA was immunoprecipitated from a rapamycin-treated and -untreated *sit4* mutant. The more highly phosphorylated forms of TIP41 (lower pI forms, spots d–f) were more abundant in rapamycin-untreated *sit4* cells than in wild-type cells (Figures 7A and 7D). Furthermore, rapamycin treatment of the *sit4* mutant failed to cause the full dephosphorylation of TIP41 typically observed with rapamycin-treated wild-type cells (Figures 7C and 7E). Thus, SIT4 is required for rapamycin-induced dephosphorylation of TIP41 in addition to enhanced binding of TIP41 to TAP42, suggesting that the dephosphorylation of TIP41 controls the binding of TIP41 to TAP42.

## Discussion

Here, we present the identification of TIP41 and show that it is a component of the TOR signaling pathway. The rapamycin-sensitive TOR pathway controls cell growth in response to nutrients via different effector branches (Schmelzle and Hall, 2000). A particularly important TOR effector branch consists of TAP42-mediated inhibition of the type 2A-related phosphatase SIT4. In the presence of nutrients (or absence of rapamycin), TOR promotes the association of TAP42 with SIT4, which in turn results in SIT4 inhibition (Beck and Hall, 1999; Di Como and Arndt, 1996; Jiang and Broach, 1999). Upon nitrogen deprivation or rapamycin treatment, conditions that inactivate TOR, SIT4 is released from TAP42 and thereby activated. SIT4 subsequently dephosphorylates and activates a number of target proteins, such as the transcription factor GLN3 and the Ser/Thr kinase

NPR, involved in scavenging secondary nitrogen sources (Beck and Hall, 1999; Schmidt et al., 1998). The essential and conserved TAP42 protein also binds the PP2A catalytic subunits, encoded by *PPH* genes (Di Como and Arndt, 1996; Jiang and Broach, 1999), and may control several other downstream effectors (Shamji et al., 2000). TIP41 binds directly to and inhibits TAP42, thereby negatively regulating the TOR pathway. Interestingly, the binding of TIP41 to TAP42 is also regulated by the TOR pathway. Dephosphorylation of TIP41 by SIT4, direct or indirect, enhances association of TIP41 with TAP42. Thus, TIP41 appears to be part of a feedback loop in the TOR pathway, the role of which would be to rapidly amplify SIT4, and possibly PP2A, basal activity as TOR signaling diminishes. Such a feedback loop could account for the exceedingly rapid dephosphorylation of NPR1 that occurs in response to rapamycin treatment (Schmidt et al., 1998). A model summarizing our findings is illustrated in Figure 8.

TOR controls the association of TAP42 with the PP2A catalytic subunits and possibly with SIT4 by phosphorylating TAP42 directly (Jiang and Broach, 1999). However, it is unlikely that TAP42 phosphorylation/dephosphorylation plays a major role in SIT4 regulation, as TAP42 is dephosphorylated upon rapamycin treatment (Jiang and Broach, 1999) much more slowly than SIT4 appears to be activated (Schmidt et al., 1998; Beck and Hall, 1999) and also much more slowly than the SIT4-TAP42 complex dissociates (Di Como and Arndt, 1996). TOR may control the association of TAP42 and SIT4, and thus SIT4 activity, primarily via phosphorylation of TIP41 (Figure 8). TOR would thus negatively regulate SIT4 and possibly PP2A, both by phosphorylating TIP41 and by preventing dephosphorylation of TIP41. It remains to be determined if TOR indeed phosphorylates TIP41, directly or indirectly.

The association of TIP41 to TAP42 enhances SIT4 phosphatase activity possibly by preventing reassociation of TAP42 with SIT4, thus allowing free SIT4 subunits to associate with SAPs. Indeed, deletion of both *TIP41* and *SAP155* results in hyperphosphorylation of NPR1, similar to deletion of *sit4*. The lack of SIT4 phosphatase activity in the *tip41 sap155* mutant may also explain the enhanced rapamycin resistance of these cells.

The inhibition of phosphatase activity appears to be a major mechanism of TOR signaling. In yeast, TAP42, by inhibiting or altering the specificity of phosphatases, mediates TOR signaling to transcription, translation, and protein turnover (Beck and Hall, 1999; Di Como and Arndt, 1996; Powers and Walter, 1999; Schmidt et al., 1998; Jiang and Broach, 1999; Shamji et al., 2000). In mammalian cells, the TAP42 homolog $\alpha$4 may also mediate TOR signaling leading to translation initiation. $\alpha$4 associates with PP2A catalytic subunits (Chen et al., 1998; Nanahoshi et al., 1998). Although the rapamycin sensitivity of the $\alpha$4-PP2A complex is controversial (Chen et al., 1998; Inui et al., 1998; Murata et al., 1997), inactivation of mTOR (also known as FRAP or RAFT) causes rapid dephosphorylation of the ribosomal S6 kinase, $p70^{s6k}$, by PP2A (Dennis et al., 1999; Peterson et al., 1999). It will be of interest to determine if the mammalian homolog of TIP41 plays a role in mTOR signaling, particularly since human TIP41 coimmunoprecipitates with $\alpha$4 (our unpublished data). Should mammalian TIP41 inhibit mTOR signaling, like TIP41 in yeast, a small molecule that mimics TIP41 action could have rapamycin-like immunosuppressive or anti-cancer activity. Furthermore, the above findings suggest that mTIP41 may act as a tumor suppressor much like PTEN, which negatively regulates TOR-related signaling in mammalian cells (Gingras et al., 2001).

## Experimental Procedures

### Strains and Media

*Saccharomyces cerevisiae* strains used in these studies are listed online (see Supplemental Table S2 at http://www.molecule.org/cgi/content/full/8/5/1017/DC1). The composition of rich medium (YPD) and synthetic minimal media (SD and SGal) complemented with the appropriate amino acid mixture was as described (Beck et al., 1999). Media using ammonium or proline as the sole nitrogen source were prepared by using yeast nitrogen base without ammonium sulfate and without amino acids, supplemented with 10 mM ammonium sulfate or 1 mg/mL proline, together with 2% glucose, 40 mg/mL tryptophan, 20 mg/mL histidine, and 100 mg/mL leucine. A 1 mg/mL stock solution of rapamycin (gift from Abbott Laboratories) was dissolved in 90% ethanol and 10% Tween. Rapamycin treatments were for 30 min and at a final concentration of 100 ng/mL, unless otherwise indicated.

### Plasmids

pGAL-TIP41 (pWAB3) contains the TIP41 coding region (BamHI/Bsu36I fragment) inserted by blunt ligation into the BamHI site of the vector FB1533 (Mumberg et al., 1994). pWAB4 is as described for pWAB3, but containing the BamHI/Bsu36I fragment of TIP41 in the inverse orientation. pHA-NPR1 (pEJ23) is YEplac181 (2 $\mu$, *LEU2*) expressing functional HA-NPR1 under its own promoter (Schmidt et al., 1998). pGST-TAP42 (pEJ27) is YEplac195 expressing functional N-terminally GST-tagged TAP42 under its own promoter. A 0.2 kb PCR-generated fragment of theTAP42 promoter was first subcloned into the EcoRI-PstI sites of YEplac195. A 1.8 kb fragment of GST-TAP42 was then subcloned at the PstI site (GST-TAP42 was obtained by PCR from pEJ20; pEJ20 contains 1.2 kb PCR-generated fragment of the TAP42 coding region and terminator sequences, subcloned in-frame at the BamHI site of YCplac111-GAL-pro-GST). pTIP41-HA (pEJ120) is pHAC181 (2 $\mu$, *LEU2*) expressing C-terminally 3$\times$HA-tagged TIP41 under its own promoter. A 1.5 kb BamHI-SalI fragment containing the *TIP41* ORF and promoter region was generated by PCR using the primers 5′AATGGATCCAGCGTCGTTCTTCT CAGC and 3′ACACTTTATTAAGTCAAGCAGCTGGCC, and then was subcloned into pHAC181. pHAC181 is YEplac181 containing an insert encoding the 3$\times$HA tag and the CYC1 transcription terminator adjacent to the multiple cloning site. YCp50::*SIT4-HA* is as described (Sutton et al., 1991).

### Gene Disruption

*TIP41* was disrupted by replacing the entire open reading frame with PCR-generated *kan*MX4 cassettes, as described (Wach et al., 1994). *SAP155* (codons 10–945) and *SAP185* (codons 1–1045) were similarly disrupted using *HIS3*MX6 (Longtine et al., 1998) and *kan*MX4 cassettes, respectively. For strain CY6323, *TIP41* was disrupted by deletion of the coding sequence between AccI and PstI sites in *TIP41* and insertion of the *LEU2* gene by blunt ligation.

### Preparation of Cell Extracts and Immunoblotting

Yeast strains were typically grown to OD = 0.4 at 30°C. For nitrogen starvation experiments, cells were grown overnight in SD supplemented with 10 mM ammonium sulfate plus tryptophan, histidine, and leucine (see Strains and Media). Cells were then resuspended for 1 hr in fresh media in the presence or absence of a nitrogen source. Cells (40 OD total) were harvested by centrifugation (3500 rpm). Whole cell lysates for SDS-PAGE and Western analysis were prepared by resuspending cells in lysis buffer (PBS + 1% NP-40) with protease (2 $\mu$g/mL each of aprotinin, leupeptin, pepstatin A, and 1 mM PMSF) and phosphatase inhibitors (10 mM each of NaF, $NaN_3$, pNPP, NaPPi, and $\beta$-glycerophosphate). Cells were lysed by

mechanical disruption in a bead-beater (FastPrep, Savant) with 6 × 30 s pulses. Supernatants were recovered by centrifugation at 500 g for 10 min at 4°C. 50 μg of total protein was analyzed by SDS-PAGE and Western analysis using either anti-HA (1: 5000; BabCO), anti-GST (1:5000; Pharmacia), or anti-TAP42 (Di Como and Arndt, 1996) as a primary antibody, followed by the secondary HRP-conjugated anti-mouse or anti-rabbit IgG antibody (1:10000; Amersham Pharmacia Biotech). Proteins were detected using the ECL detection system (Amersham Pharmacia Biotech).

### Immunoprecipitation and GST Pull-Down Assay

Immunoprecipitations of epitope-tagged proteins were performed by incubating 5 mg of extract with 2 ml of anti-HA antibody at 4°C. After 1 hr, 15 μl of Protein G beads (Pharmacia) was added, and the samples were rocked at 4°C for another hour. For GST-pull down assay, 10 mg of extracts was incubated with 20 μl of glutathione Sepharose beads (Amersham Pharmacia Biotech) for 3 hr at 4°C. Beads were washed six times with lysis buffer (without protease and phosphatase inhibitors). Proteins were fractionated by SDS-PAGE, followed by Western transfer and immunoblotting using either anti-HA or anti-GST antibody.

### Indirect Immunofluorescence

Cells were fixed in the growth medium for 2 hr with formaldehyde (3.7%) and potassium phosphate buffer (100 mM [pH 6.5]). Cells were washed and resuspended in sorbitol buffer (SB) (1.2 M sorbitol and 100 mM potassium phosphate [pH 6.5]). Cell walls were digested for 45 min at 37°C using SB + 20 mM β-mercaptoethanol and Zymolyase 20T (0.1 mg/ml; Seigagaku Corp.). Fixed spheroplasts were attached to poly-L-lysine-coated glass slides and permeabilized with PBT (53 mM $Na_2HPO_4$, 13 mM $NaH_2PO_4$, 75 mM NaCl, and 0.1% Triton X-100). Cells were incubated with anti-myc antibody at a final dilution of 1:500 in PBT for 2 hr. After washing repeatedly with PBS, cells were incubated with Cy3-conjugated rabbit anti-mouse IgG (Molecular Probes, Inc.) at a final dilution of 1:500 in PBT for 1 hr. Washed cells were examined for immunofluorescence using a Zeiss Axiophot microscope (100× objective) and a video imaging system (MWG Biotech).

### Two-Dimensional Gel Analysis

Cells (total OD = 50) were lysed in 1 ml TNE lysis buffer (0.1 M Tris-Cl [pH 8], 1% NP-40, and 1 mM EDTA) (plus protease and phosphatase inhibitors, 0.1 μg/mL RNase A), then mechanically disrupted using a bead beater for 6 × 30 s. TIP41-HA was immunoprecipitated from 8 mg of total protein after incubation with 0.5 μl anti-HA and 40 μl Protein G Sepharose (Pharmacia) for 3 hr at 4°C. Immunoprecipitates were washed three times with 800 μl TSNE (TNE + 0.15M NaCl), then resuspended in 150 μl of CIP buffer (50 mM Tris-Cl [pH 8.5], 5 mM $MgCl_2$), with or without 100 units of alkaline phosphatase (New England Biolabs). The reaction was incubated at 37°C for 1 hr and then washed twice with 1 mL TNE. Samples were resuspended in 440 μl TNE, with the addition of 322 mg Urea, 117 mg Thiourea, and 6.6 μl β-mercaptoethanol, and then incubated at 37°C for 30 min. Samples were then centrifuged, and the supernatant was recovered. 242 μl of Buffer B (21.4% CHAPS, 107 mM DTT, and 21.4 μl IEF buffer NL 3-10) (IEF buffer NL 3-10 was from Pharmacia) and a few crystals of bromophenol blue were added to the supernatant. 300 μl of the sample was loaded into the IPG chamber, overlaid with an 18 cm, pH 3–10 NL IPG strip (Amersham Pharmacia Biotech), and covered with mineral oil. First dimension isoelectric focusing was performed on an IPGPhor apparatus (Pharmacia Biotech). Strips were rehydrated followed by isolectric focusing for 12 hr at 22°C, using the following protocol: 500 V 1 hr, 1000 V 1 hr, 8000 V 48 hr, and 500 V 99 hr, 50 mA per strip. Typically, a total of 185,000 Volthours was reached. Strips were equilibrated five times for 3 min in 5 ml SDS equilibration buffer (50 mM Tris-Cl [pH 6.8], 6 M Urea, 30% glycerol, 2% SDS, and few crystals of Coomassie blue), then electrophoresed in the second dimension on a 10% SDS-PAGE resolving gel, covered with liquefied 1% agarose in SDS running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS). Proteins were detected by Western blotting using anti-HA antibody.

### Acknowledgments

We thank Paul Jenö and Guy Werlen for comments on the manuscript, Debora Bonenfant and Florian Schaerer for help with 2-D gels, and Helena Morales and Dave Evans for providing strains. This work was supported by National Institutes of Health grant GM45179 (K.T.A.) and by grants from the Cancer Research Institute (E.J.), the Boehringer Ingelheim Foundation (T.S.), the Swiss National Science Foundation, and the Canton of Basel (M.N.H.).

Received February 20, 2001; revised June 22, 2001.

### References

Beck, T., and Hall, M.N. (1999). The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature *402*, 689–692.

Beck, T., Schmidt, A., and Hall, M.N. (1999). Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. J. Cell Biol. *146*, 1227–1238.

Cardenas, M.E., Cutler, N.S., Lorenz, M.C., Di Como, C.J., and Heitman, J. (1999). The TOR signaling cascade regulates gene expression in response to nutrients. Genes Dev. *13*, 3271–3279.

Chen, J., Peterson, R.T., and Schreiber, S.L. (1998). Alpha 4 associates with protein phosphatases 2A, 4, and 6. Biochem. Biophys. Res. Commun. *247*, 827–832.

Dennis, P.B., Fumagalli, S., and Thomas, G. (1999). Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. Curr. Opin. Genet. Dev. *9*, 49–54.

Di Como, C.J., and Arndt, K.T. (1996). Nutrients, via the TOR proteins, stimulate the association of TAP42 with type 2A phosphatases. Genes Dev. *10*, 1904–1916.

Duncan, R.F., and Song, H.J. (1999). Striking multiplicity of eIF4E–BP1 phosphorylated isoforms identified by 2D gel electrophoresis regulation by heat shock. Eur. J. Biochem. *265*, 728–743.

Gingras, A.C., Raught, B., and Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. Genes Dev. *15*, 807–826.

Hardwick, J.S., Kuruvilla, F.G., Tong, J.K., Shamji, A.F., and Schreiber, S.L. (1999). Rapamycin-modulated transcription defines the subset of nutrient- sensitive signaling pathways directly controlled by the TOR proteins. Proc. Natl. Acad. Sci. USA *96*, 14866–14870.

Heitman, J., Movva, N.R., and Hall, M.N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science *253*, 905–909.

Inui, S., Sanjo, H., Maeda, K., Yamamoto, H., Miyamoto, E., and Sakaguchi, N. (1998). Ig receptor binding protein 1 (α4) is associated with a rapamycin-sensitive signal transduction in lymphocytes through direct binding to the catalytic subunit of protein phosphatase 2A. Blood *92*, 539–546.

Jiang, Y., and Broach, J.R. (1999). TOR proteins and protein phosphatase 2A reciprocally regulate TAP42 in controlling cell growth in yeast. EMBO J. *18*, 2782–2792.

Longtine, M.S., McKenzie, A., III, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast *14*, 953–961.

Luke, M.M., Della Seta, F., Di Como, C.J., Sugimoto, H., Kobayashi, R., and Arndt, K.T. (1996). The SAPs, a new family of proteins, associate and function positively with the SIT4 phosphatase. Mol. Cell. Biol. *16*, 2744–2755.

Mumberg, D., Muller, R., and Funk, M. (1994). Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res. *22*, 5767–5768.

Murata, K., Wu, J., and Brautigan, D.L. (1997). B cell receptor-associated protein α4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein phosphatase 2A. Proc. Natl. Acad. Sci. USA *94*, 10624–10629.

Nanahoshi, M., Nishiuma, T., Tsujishita, Y., Hara, K., Inui, S., Saka-

guchi, N., and Yonezawa, K. (1998). Regulation of protein phosphatase 2A catalytic activity by alpha4 protein and its yeast homolog TAP42. Biochem. Biophys. Res. Commun. *251*, 520–526.

Peterson, R.T., Desai, B.N., Hardwick, J.S., and Schreiber, S.L. (1999). Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. Proc. Natl. Acad. Sci. USA *96*, 4438–4442.

Powers, T., and Walter, P. (1999). Regulation of ribosome biogenesis by the rapamycin-sensitive TOR- signaling pathway in *Saccharomyces cerevisiae*. Mol. Biol. Cell *10*, 987–1000.

Radimerski, T., Mini, T., Schneider, U., Wettenhall, R.E., Thomas, G., and Jeno, P. (2000). Identification of insulin-induced sites of ribosomal protein S6 phosphorylation in *Drosophila melanogaster*. Biochemistry *39*, 5766–5774.

Rechsteiner, M., and Rogers, S.W. (1996). PEST sequences and regulation by proteolysis. Trends Biochem. Sci. *21*, 267–271.

Schmelzle, T., and Hall, M.N. (2000). TOR, a central controller of cell growth. Cell *103*, 253–262.

Schmidt, A., Beck, T., Koller, A., Kunz, J., and Hall, M.N. (1998). The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease. EMBO J. *17*, 6924–6931.

Shamji, A.F., Kuruvilla, F.G., and Schreiber, S.L. (2000). Partitioning the transcriptional program induced by rapamycin among the effectors of the TOR proteins. Curr. Biol. *10*, 1574–1581.

Sutton, A., Immanuel, D., and Arndt, K.T. (1991). The SIT4 protein phosphatase functions in late G1 for progression into S phase. Mol. Cell. Biol. *11*, 2133–2148.

Vandenbol, M., Jauniaux, J.C., and Grenson, M. (1990). The *Saccharomyces cerevisiae* NPR1 gene required for the activity of ammonia-sensitive amino acid permeases encodes a protein kinase homologue. Mol. Gen. Genet. *222*, 393–399.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast *10*, 1793–1808.