

Sch9 Is a Major Target of TORC1 in *Saccharomyces cerevisiae*

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SUMMARY

The Target of Rapamycin (TOR) protein is a Ser/Thr kinase that functions in two distinct multiprotein complexes: TORC1 and TORC2. These conserved complexes regulate many different aspects of cell growth in response to intracellular and extracellular cues. Here we report that the AGC kinase Sch9 is a substrate of yeast TORC1. Six amino acids in the C terminus of Sch9 are directly phosphorylated by TORC1. Phosphorylation of these residues is lost upon rapamycin treatment as well as carbon or nitrogen starvation and transiently reduced following application of osmotic, oxidative, or thermal stress. TORC1-dependent phosphorylation is required for Sch9 activity, and replacement of residues phosphorylated by TORC1 with Asp/Glu renders Sch9 activity TORC1 independent. Sch9 is required for TORC1 to properly regulate ribosome biogenesis, translation initiation, and entry into G₀ phase, but not expression of Gln3-dependent genes. Our results suggest that Sch9 functions analogously to the mammalian TORC1 substrate S6K1 rather than the mTORC2 substrate PKB/Akt.

INTRODUCTION

In eukaryotes two distinct, conserved, multiprotein complexes known as TOR complex 1 (TORC1) and TORC2 function as major regulators of cell growth (Wullschleger et al., 2006). In both complexes, Target of Rapamycin (TOR), a large Ser/Thr protein kinase belonging to the family of phosphatidylinositol kinase-related kinases (Keith and Schreiber, 1995), functions as the catalytic subunit.

TORC1, but not TORC2, is directly inhibited by the macrocyclic lactone rapamycin (Jacinto et al., 2004; Loewith et al., 2002; Sarbassov et al., 2004).

S. cerevisiae TORC1 contains Lst8, Kog1, Tco89, and either Tor1 or Tor2 (Loewith et al., 2002; Reinke et al., 2004; Wedaman et al., 2003). Based primarily on the observed phenotypic similarities between rapamycin-treated and nutrient-starved cells it is generally believed that TORC1 couples nutrient cues to the cell growth machinery (Rohde et al., 2001). TORC1 activity also appears to be influenced by a number of other noxious stresses and, in metazoans, by extracellular mitogens (Crespo et al., 2001; Kim et al., 2002; Sarbassov and Sabatini, 2005). Under favorable conditions, yeast TORC1 promotes growth by stimulating translation initiation and, via transcription factors such as Iff1, Crf1, and Sfp1, expression of genes required for synthesis and assembly of the translation machinery. In addition to promoting anabolic processes, TORC1 also antagonizes entry into G₀ phase, the induction of stress response programs, and catabolic processes including autophagy and expression of gene products required for the metabolism of nonpreferred nutrients. Again, TORC1 regulates many of these processes by influencing the localization/activity of transcription factors including Gln3 (nitrogen discrimination pathway), Rtg1/Rtg3 (retrograde signaling), and Msn2/Msn4 (stress response) (De Virgilio and Loewith, 2006).

How TORC1 activity is linked to its diverse downstream targets is not well understood. Several processes are thought to be regulated through modulation of protein phosphatase 2A activity; however, the direct link between TORC1 and the phosphatases has not been clearly defined (Duvel et al., 2003). The lack of well-characterized TORC1 substrates with defined phosphorylation sites has also hampered the identification of physiological stimuli and upstream regulatory components that control TORC1 activity.

Activity of many members of the AGC protein kinase family (homologous to protein kinases A, G, and C)

requires both phosphorylation of the kinase domain activation loop by PDK1 and binding of the conserved “hydrophobic motif” (HM: F-X-X-F/Y-S/T-F/Y) found C terminal of the catalytic domain to a pocket in the kinase domain. The latter often depends on HM phosphorylation (Gold et al., 2006).

Mounting evidence suggests that TOR complexes phosphorylate the HM of several AGC kinases. In vivo and in vitro data show that mammalian TORC1 (mTORC1) and mTORC2 phosphorylate the HM in S6K1 and PKB/Akt, respectively (Burnett et al., 1998; Isotani et al., 1999; Sarbassov et al., 2005). *S. cerevisiae* TORC2 and a *Schizosaccharomyces pombe* TOR complex have also been reported to phosphorylate the HM in the AGC kinases Ypk1/2 and Gad8, respectively (Kamada et al., 2005; Matsuo et al., 2003). Both mammalian and yeast TOR complexes phosphorylate additional residues adjacent to the HM, including a sequence termed the “turn motif,” which are often followed by a Pro (Kamada et al., 2005; Matsuo et al., 2003; Montagne and Thomas, 2004). Phosphorylation of the turn motif is thought to further stabilize the interaction between the HM and its binding pocket (Gold et al., 2006).

In this study we queried whether the yeast AGC kinase Sch9 could be a direct substrate for TORC1. Previous studies have revealed phenotypic similarities between *sch9* cells and rapamycin-treated cells including nuclear localization and activation of the Rim15 kinase and decreased expression of genes encoding proteins required for ribosome biogenesis (Jorgensen et al., 2004; Pedruzzi et al., 2003). Furthermore, *tor1* and *sch9* cells also share increased lifespan (Kaeberlein et al., 2005). Lastly, a recent report suggested that Sch9 becomes partially dephosphorylated upon rapamycin treatment (Jorgensen et al., 2004).

Here we show that Sch9 is directly phosphorylated by TORC1 at multiple C-terminal sites and by the yeast PDK1 orthologs in the activation loop. Both phosphorylation events are independently required for Sch9 activity. Phosphorylation of TORC1 sites is abolished under either nitrogen or carbon starvation and transiently reduced when cells are subjected to various stress conditions. These observations support the notion that TORC1 activity is regulated by nutrient abundance and inhibited by noxious stress. Using TORC1-independent versions of Sch9, we found that Sch9 is a major effector of TORC1 that appears to function similarly to the mTORC1 substrate S6K1.

RESULTS

Chemical Fragmentation Reveals Multiple Rapamycin-Sensitive Phosphorylation Sites in the Sch9 C Terminus

Analysis of Sch9 phosphorylation using SDS-PAGE migration shifts has been complicated by both the large size of Sch9 (~100 kDa) and by the presence of multiple phosphorylation sites. To circumvent these challenges

we tested various chemical reagents used for fragmentation of proteins (Burgess et al., 2000), of which NTCB (2-nitro-5-thiocyanatobenzoic acid) proved to be particularly useful. NTCB selectively cyanylates Cys residues, and under alkaline conditions this is followed by chain cleavage at the modified residues. This allowed us to analyze smaller fragments that contained fewer phosphorylated residues and were better resolved by immunoblotting. Treatment of C-terminally HA-tagged Sch9 in a crude yeast extract yielded a highly reproducible cleavage at some of the nine cysteines found in Sch9 (Figure 1A).

Phosphorylation of Sch9 was decreased in cells treated with rapamycin (Rap) or wortmannin (WM) but was increased in the presence of a sublethal dose of cycloheximide (CHX). Cleavage with NTCB revealed that this included a dephosphorylation or hyperphosphorylation of the Sch9 C terminus, respectively (Figure 1A). Treatment with λ phosphatase showed that the ladder of bands migrating around 50 kDa in SDS-PAGE represents multiple phosphoisoforms of the same fragmentation product (Figure 1B). This was subsequently found to include the activation loop phosphorylation site T570 (Liu et al., 2005) and thus probably encompasses amino acids 554–824 of Sch9 and the 5HA tag (see Figures 2A and 2D).

We next tested whether physiological conditions predicted to regulate TORC1 activity also affect the phosphorylation of the Sch9 C terminus. Shifting cells from a medium containing glucose and Gln to media lacking either a carbon or nitrogen source caused a rapid dephosphorylation that was quickly reversed upon readdition of the missing nutrient. The centrifugation step required for the medium change also led to a transient partial dephosphorylation of the Sch9 C terminus (Figure 1C). Changing the nitrogen source from NH_4^+ to urea resulted in a rapid dephosphorylation followed by a complete rephosphorylation in less than 2 hr (data not shown). Transferring cells grown in a low-phosphate medium into a phosphate-free medium also caused a dephosphorylation, although with much slower kinetics (Figure 1D). Again, this was quickly reversed upon phosphate readdition. Further analysis showed that the C terminus of Sch9 was transiently dephosphorylated when cells were subjected to various stress conditions, including high salt, redox stress, or a shift to a higher temperature (Figure 1E).

We next wished to determine whether the extent of Sch9 C-terminal phosphorylation correlated with nutrient quality (Figure 1F). Sch9 was found to be slightly less phosphorylated when cells were supplied with raffinose or ethanol plus glycerol compared to glucose or galactose as carbon sources. NH_4^+ - and Pro-based media also supported slightly less phosphorylation compared to Gln- or urea-based media. However, the extent of Sch9 phosphorylation did not always correlate with growth rate; cells grew faster in NH_4^+ - versus Pro-based medium, but Sch9 C-terminal phosphorylation was similar under both conditions.

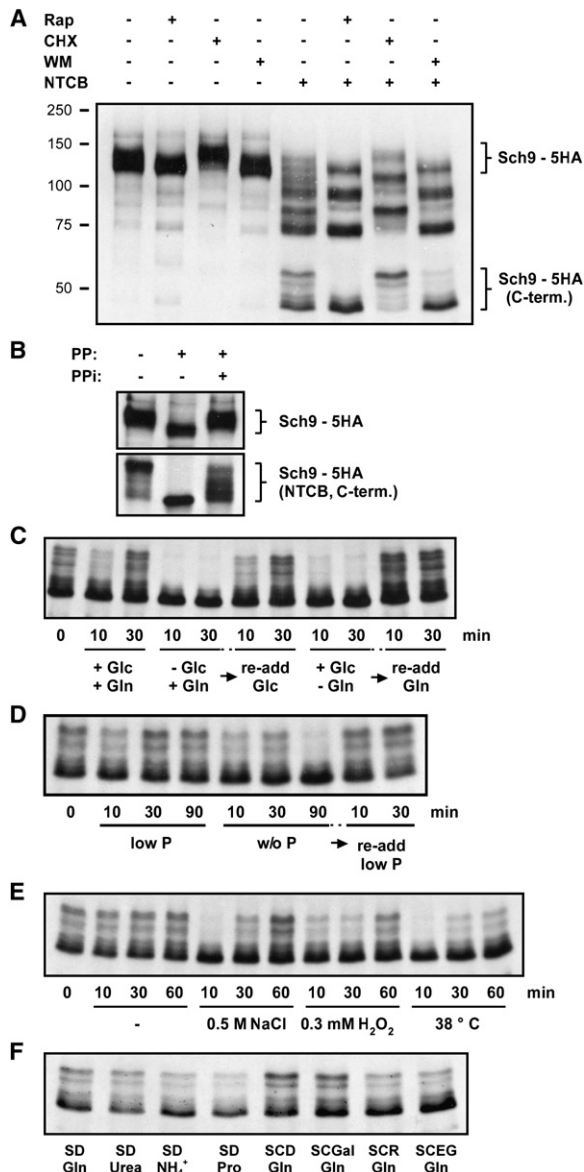


Figure 1. The Sch9 C Terminus Is Phosphorylated in a TORC1-, Nutrient-, and Stress-Dependent Manner

(A) Chemical fragmentation analysis using NTCB shows that the C terminus of Sch9 becomes dephosphorylated in response to rapamycin (Rap, 200 ng/ml) and wortmannin (WM, 5 μ M) and hyperphosphorylated in response to cycloheximide (CHX, 25 μ g/ml) treatment (all 30 min).

(B) Incubation with λ phosphatase (PP) in the absence or presence of phosphatase inhibitors (PPI) shows that the ladder of bands derived from the Sch9 C terminus represents differently phosphorylated isoforms of the same fragmentation product. Samples were prepared from CHX-treated cells.

(C) The Sch9 C terminus becomes reversibly dephosphorylated in response to carbon (–Glc) and nitrogen (–Gln) starvation. 2% Glc or 0.2% Gln was readded after 40 min of starvation. Aliquots were taken at the indicated times following medium change.

(D) Reversible dephosphorylation of Sch9 following transfer from low phosphate (0.37 mM) to phosphate-free medium. Phosphate (0.37 mM) was readded after 90 min of starvation.

TORC1 Phosphorylates Six Residues in the C Terminus of Sch9

To identify the sites phosphorylated in Sch9, we purified Sch9 from actively growing yeast and mapped potential phosphorylation sites by mass spectrometry (see Table S3 in the Supplemental Data available with this article online). Building on these results, we started an extensive mutational analysis that identified seven Ser/Thr residues in the C terminus that when changed to Ala caused obvious alterations in SDS-PAGE migration of fragmented Sch9 (Figure 2A). Cumulative substitution of these Ser/Thr residues to Ala led to a progressive loss of phosphorylated species, and a version of Sch9 lacking all seven sites yielded a C-terminal fragment upon NTCB cleavage that migrated as a single band (Figure 2B; uncropped image, Figure S1).

Experiments with a variety of constructs containing multiple Ser/Thr to Ala substitutions generally suggested that the various sites can be phosphorylated independently; only the phosphorylation of T723 seemed to depend to some extent on prior phosphorylation of S726 (data not shown). With the exception of T570 in the activation loop, phosphorylation of the remaining six C-terminal sites was sensitive to rapamycin treatment (Figure 2C, see also Figure 4A) demonstrating that it occurred in a TORC1-dependent manner.

Figure 2D shows the domain structure of Sch9 and the position of the phosphorylated residues in the C-terminal fragment. In addition to T570, the sites that were identified included T737 in the classical HM of AGC kinases and two Ser/Thr-Pro sites, T723 and S726. Two more sites were found in the C-terminal extension (CE) beyond the HM of Sch9 (S758 and S765). These show similarity to the HM, especially the presence of bulky hydrophobic residues at positions –4, +1, and +2. Finally, S711 was found to be phosphorylated as well. This residue is also followed by two hydrophobic amino acids but is not preceded by a hydrophobic residue at position –4.

To test whether Sch9 is a direct substrate for TORC1, we first queried whether TORC1 components can physically interact with Sch9. Although we were unable to coimmunoprecipitate TORC1 with Sch9, we detected a weak interaction between Tor1 and Sch9 using a two-hybrid approach (data not shown). Next we asked whether Sch9 is a substrate for TORC1 in vitro. Indeed, TORC1 purified from yeast phosphorylated recombinant Sch9 (Figure 2E). This phosphorylation was strongly diminished if TORC1 was obtained from cells treated with rapamycin or from cells expressing only a catalytically inactive version of Tor1 (Tor1^{D2275A}). Recombinant Sch9 lacking all six C-terminal sites (Sch9^{6A}) was much less phosphorylated compared

(E) The Sch9 C terminus becomes temporarily dephosphorylated in response to high salt, redox, and temperature stress.

(F) Phosphorylation of the Sch9 C terminus is reduced in cells growing on less preferred nitrogen or carbon sources. (A)–(F) show anti-HA immunoblots of untreated (A and B) and NTCB-treated (A–F) protein extracts obtained from WT cells containing a plasmid-based copy of SCH9-5HA. Only the C-terminal fragment is shown in (C)–(F).

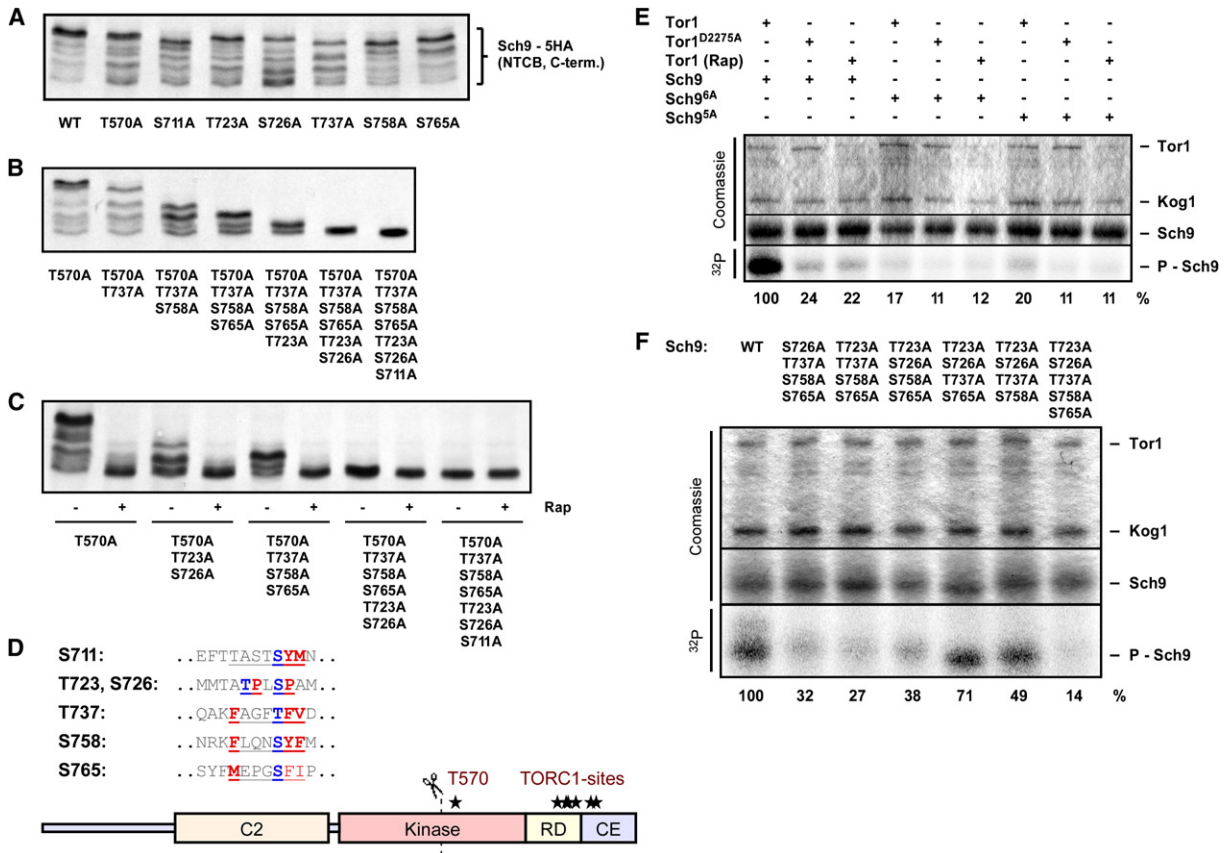


Figure 2. TORC1 Phosphorylates at Least Five C-Terminal Residues of Sch9

(A) Changing T570 or six other Ser/Thr residues to Ala alters the appearance of the C-terminal fragment.
 (B) Cumulative mutation of these sites reduces the number of phosphorylated species until the C-terminal fragment migrates as a single band. The uncropped picture is shown in Figure S1.
 (C) Phosphorylation of all six C-terminal sites is sensitive to rapamycin treatment. Samples for (A)–(C) were prepared from CHX-treated WT cells expressing Sch9-5HA. NTCB-treated protein extracts were analyzed by immunoblotting using anti-HA antibody. In (B) and (C), different amounts of protein were loaded per lane to get comparable signal intensity.
 (D) Domain structure of Sch9 and localization of the mapped phosphorylation sites and the putative NTCB cleavage site. A comparison of the surrounding amino acids reveals that the phosphorylated residues either lie in a sequence resembling the HM or are followed by a Pro. RD, regulatory domain; CE, C-terminal extension.
 (E) TORC1 phosphorylates bacterially expressed Sch9 in vitro, while TORC1 purified from cells expressing only inactive Tor1^{D2275A} or from rapamycin-treated cells has reduced activity toward Sch9. Phosphorylation is strongly diminished when the six C-terminal sites are replaced with Ala. Sch9^{5A} containing only S711 is not a good substrate for TORC1.
 (F) TORC1 phosphorylates recombinant Sch9 in vitro at T723, S726, T737, S758, and S765. Values in (E) and (F) show the relative amount of radioactivity incorporated into Sch9.

to Sch9^{WT}, suggesting that we have identified the majority of the residues in Sch9 that are modified by TORC1.

Phosphorylation of Sch9 was specific to TORC1: purified TORC2, although able to phosphorylate a physiological substrate, Ypk2 (Kamada et al., 2005), was unable to phosphorylate Sch9 in vitro (Figure S2).

To determine whether each of the six sites in the Sch9 C terminus could be phosphorylated by TORC1, we “added back” Ser/Thr residues to the Sch9^{5A} mutant and asked whether this improved their phosphorylation. A comparison between Sch9^{6A} and a version containing S711 (Sch9^{5A}) showed that S711 was poorly phosphorylated in the in vitro assay (Figure 2E). Sch9 versions containing

any of the other sites in addition to S711 were more strongly phosphorylated than Sch9^{5A}, indicating that at least five sites in the Sch9 C terminus can be directly phosphorylated by TORC1 (Figure 2F). Among these, the HM-like sites S758 and S765 in the CE appeared to be particularly good substrates in vitro while the Ser/Thr-Pro sites T723 and S726 were less used.

TORC1 Phosphorylation Sites Are Critical to Sch9 Function

To analyze the importance of TORC1-dependent phosphorylation of Sch9 in vivo, we took advantage of the observation that *sch9* cells grew slowly on YPD and not at all

on YPGal. Introduction of single-copy plasmids containing *SCH9* or the acidic residue-substituted alleles *SCH9^{3E}* (T737E, S758E, S765E) and *SCH9^{2D3E}* (T723D, S726D, T737E, S758E, S765E) into *sch9* cells restored normal growth on both YPD and YPGal, while the Ala-substituted allele *SCH9^{5A}* (T723A, S726A, T737A, S758A, S765A) failed to complement the growth defect. The *SCH9^{3E}* and *SCH9^{2D3E}* alleles also conferred a slight resistance to rapamycin (Figure 3A).

Because deletion of *GLN3* and *GAT1* renders cells resistant to low doses of rapamycin (Beck and Hall, 1999), we also performed our complementation studies in a (prototroph) *sch9 gat1 gln3* background. An added advantage of using this strain was that it appeared to be phenotypically more stable than *sch9* cells (data not shown). Compared to *sch9* cells, *sch9 gat1 gln3* cells grew markedly better on YPD and slowly on YPD + rapamycin but they still failed to grow on YPGal. Importantly, introduction of *SCH9^{3E}* and *SCH9^{2D3E}* alleles but not of *SCH9^{WT}* in this background allowed cells to grow on YPGal + rapamycin (Figure 3A). This shows that Sch9 function depends on TORC1-mediated phosphorylation of its C terminus and that substitution of the C-terminal TORC1 phosphorylation sites of Sch9 with acidic amino acids yields Sch9 proteins that appear to function independently of TORC1.

Further detailed analysis indicated that, with the possible exception of S711, all of the TORC1 phosphorylation sites in Sch9 play a positive role in Sch9 function with T737 in the HM being the most important site (Figure S3A). A version containing only a substitution of the HM site with glutamate (T737E) conferred rapamycin-resistant growth, but simultaneous replacement of several TORC1 sites with acidic residues (3E and 2D3E) resulted in a higher level of rapamycin resistance (Figure S3B).

In order to analyze how these mutations effect the Sch9 kinase activity, WT and mutated versions of Sch9-3HA were isolated from yeast cells treated with drug vehicle or rapamycin and tested for their ability to phosphorylate a peptide (GRPRTSSFAEG; Cross et al., 1995), which is known to be phosphorylated by various AGC kinases. *Sch9^{WT}* obtained from mock-treated cells was able to phosphorylate the peptide while no activity was measured when *Sch9^{WT}* was isolated from rapamycin-treated cells (Figure 3D). *Sch9^{k.d.}* (K441A; Morano and Thiele, 1999) and *Sch9^{5A}* showed no activity toward the substrate while *Sch9^{2D3E}* activity was, for unknown reasons, increased by prior rapamycin treatment. Together, these results demonstrate that phosphorylation by TORC1 is necessary for both Sch9 function in vivo and catalytic activity in vitro.

Pkh Kinases Phosphorylate T570 in the Activation Loop of Sch9

Activity of many AGC kinases requires phosphorylation of a Ser/Thr residue in the activation loop by PDK kinases (Mora et al., 2004). To analyze the phosphorylation in the Sch9 activation loop, phosphospecific antibodies against phospho-T570 were generated. Immunoblotting showed that these antibodies detected *Sch9^{WT}* expressed in

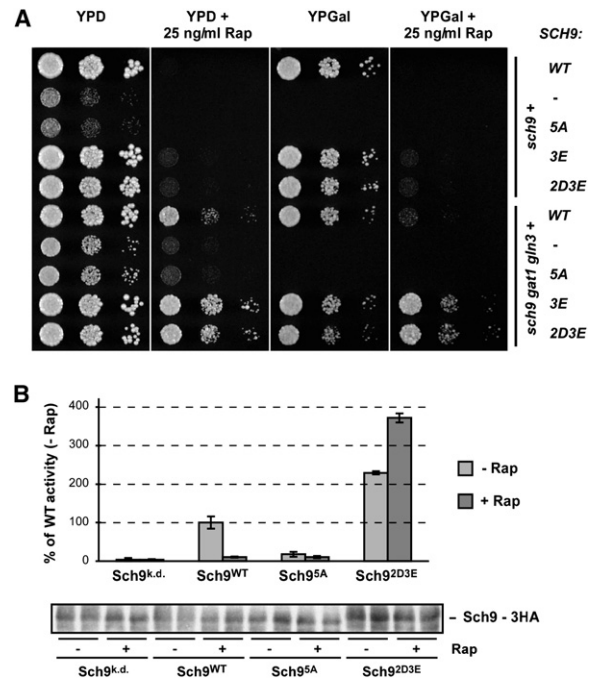


Figure 3. Mutation of Several TORC1 Phosphorylation Sites to Ala or Glu/Asp Renders Sch9 Inactive or Independent of TORC1

(A) *SCH9* alleles with multiple Ser/Thr to Ala mutations fail to suppress the growth defects of *sch9* and *sch9 gat1 gln3* cells on YPD and YPGal. Alleles with multiple Ser/Thr to Asp/Glu mutations yield rapamycin-resistant growth on YPGal in *sch9 gat1 gln3* cells. Serial 10-fold dilutions were spotted onto the indicated medium and incubated for 2 to 3 days at 30°C.

(B) *Sch9^{WT}* isolated from untreated, but not from rapamycin-treated, yeast shows activity toward the peptide GRPRTSSFAEG. *Sch9^{2D3E}* activity is rapamycin insensitive while *Sch9^{k.d.}* or *Sch9^{5A}* display little or no activity. Cultures were treated with CHX (10 min, 25 µg/ml) prior to harvest, and the kinase activity was determined in duplicate. Error bars represent the standard error.

sch9 gat1 gln3 cells, but not the Ala-substituted *Sch9^{T570A}* (Figure 4A). In vivo, T570 was phosphorylated similarly in *Sch9^{WT}*, the inactive *Sch9^{5A}*, or the TORC1-independent versions *Sch9^{3E}* and *Sch9^{2D3E}* as well as in *Sch9^{WT}* after rapamycin treatment (Figure 4A). This suggests that phosphorylation of the HM is not required to facilitate subsequent phosphorylation of the activation loop. The finding that both *Sch9^{T570A}* and *Sch9^{5A}* are inactive (see Figure 3B), although they still are phosphorylated by TORC1 and Pkh kinases, respectively (see Figures 2A and 4A), demonstrates that both activation loop phosphorylation and phosphorylation of the C terminus by TORC1 are independently required for Sch9 activity.

In yeast, PDKs are encoded by the *PKH1* and *PKH2* genes (Casamayor et al., 1999). To determine whether the activation loop in Sch9 is phosphorylated by Pkh kinases, we performed an in vitro kinase assay and found that recombinant GST-Sch9 was efficiently phosphorylated by GST-Pkh2 purified from yeast cytosol. A preparation

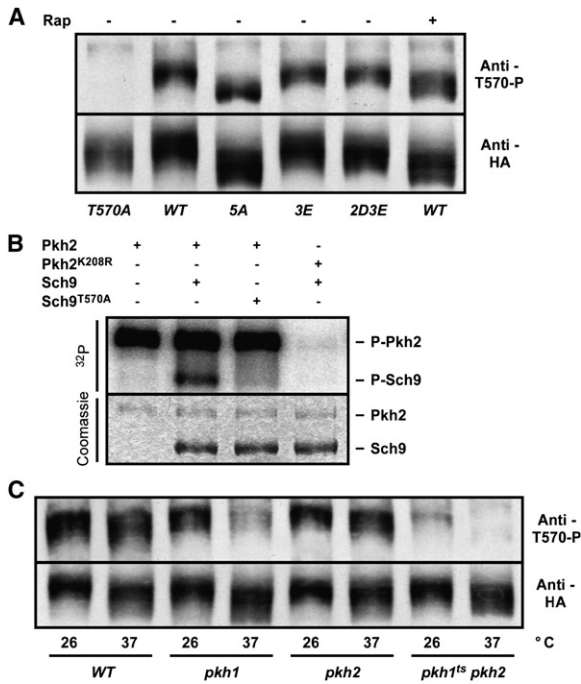


Figure 4. Pkh Kinases Phosphorylate the Activation Loop of Sch9

(A) Phosphospecific antibodies directed toward the activation loop specifically recognize Sch9 phosphorylated at T570. Mutating the C-terminal TORC1 sites to Ala or Asp/Glu or treating cells with rapamycin (30 min, 200 ng/ml) does not affect the phosphorylation of T570 in vivo. The indicated versions of plasmid-encoded 6HA-Sch9 were expressed in *sch9 gat1 gln3* cells, and protein extracts were analyzed by immunoblotting.

(B) Pkh2, but not inactive Pkh2^{K208R}, phosphorylates recombinant GST-Sch9 at T570 in vitro.

(C) Phosphorylation of T570 in 6HA-Sch9, expressed from the endogenous locus, is strongly reduced in *pkh1^{ts} pkh2* mutants at permissive temperature (26°C) and undetectable after a shift to nonpermissive temperature (20 min, 37°C).

of the catalytically inactive protein, Pkh2^{K208R} (Inagaki et al., 1999), did not show any activity toward Sch9, and the activation loop mutant Sch9^{T570A} was not a substrate for Pkh2, indicating that Pkh2 directly phosphorylates Sch9 at T570 (Figure 4B).

Immunoblot analysis showed that Pkh activity is also required for T570 phosphorylation in vivo. Relative to WT cells, at permissive temperature phosphorylation of T570 was reduced in *pkh1* cells and strongly reduced in *pkh1^{ts} pkh2* cells carrying the temperature-sensitive allele PKH1^{D398G} (Inagaki et al., 1999). After incubation at nonpermissive temperature, phosphorylation of the Sch9 activation loop was undetectable in *pkh1^{ts} pkh2* cells (Figure 4C). This shows that Sch9 is phosphorylated by both Pkh1 and Pkh2 in vivo.

TORC1 Is Active at the Vacuole

Sch9 was found previously to be concentrated at the vacuolar membrane, and this localization was shown to

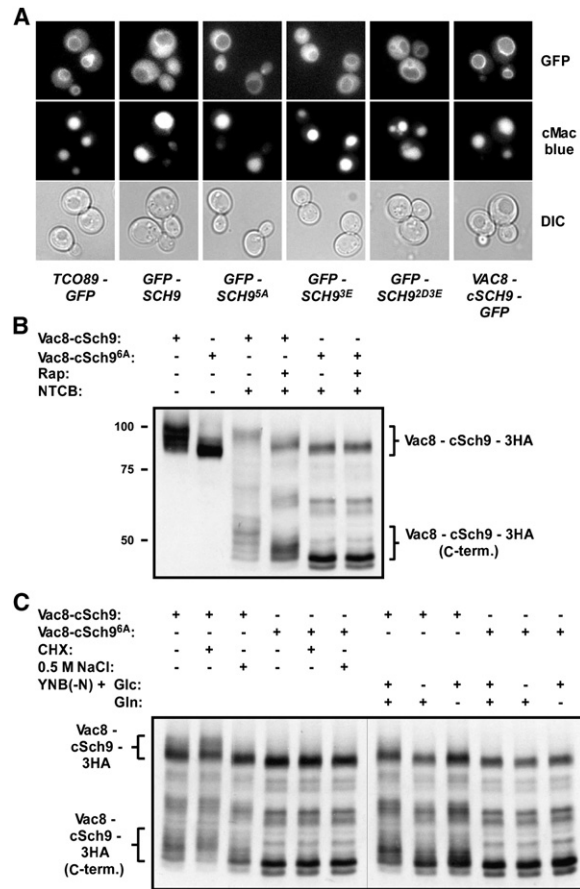


Figure 5. The C-Terminal Portion of Sch9 Artificially Tethered to the Surface of the Vacuole Is an Efficient Substrate for TORC1 Phosphorylation

(A) Tco89-GFP and versions of GFP-Sch9 are concentrated at the vacuolar surface. A fusion protein consisting of Vac8 and the Sch9 C-terminal 116 amino acids is found exclusively on the vacuole. The vacuolar lumen was stained with blue CMAC.

(B) Immunoblotting using anti-HA antibody of untreated and NTCB-treated protein extract shows that the Vac8-cSch9-3HA fusion protein is phosphorylated at its TORC1 sites in a rapamycin-sensitive manner. (C) Immunoblot of NTCB-treated protein extracts shows that phosphorylation of the TORC1 sites in Vac8-cSch9-3HA is stimulated by CHX (25 µg/ml, 10 min) and reduced in response to high salt treatment (0.5 M NaCl, 10 min). It is also reduced under carbon or nitrogen starvation (30 min).

be rapamycin insensitive (Jorgensen et al., 2004). Consistently, the localization of GFP-Sch9 was not significantly altered by the introduction of mutations at TORC1 phosphorylation sites (5A, 3E, and 2D3E) (Figure 5A). We found that functional, GFP-tagged TORC1 components Tco89 and Kog1 (Figure 5A and data not shown) also localized to the vacuolar membrane. Although the localization of TORC1 is currently debated, the existence of a pool of TORC1 at the vacuolar membrane is consistent with several reports (discussed in De Virgilio and Loewith, 2006). Thus, we wished to corroborate our localization data and determine whether TORC1 is active at the vacuole.

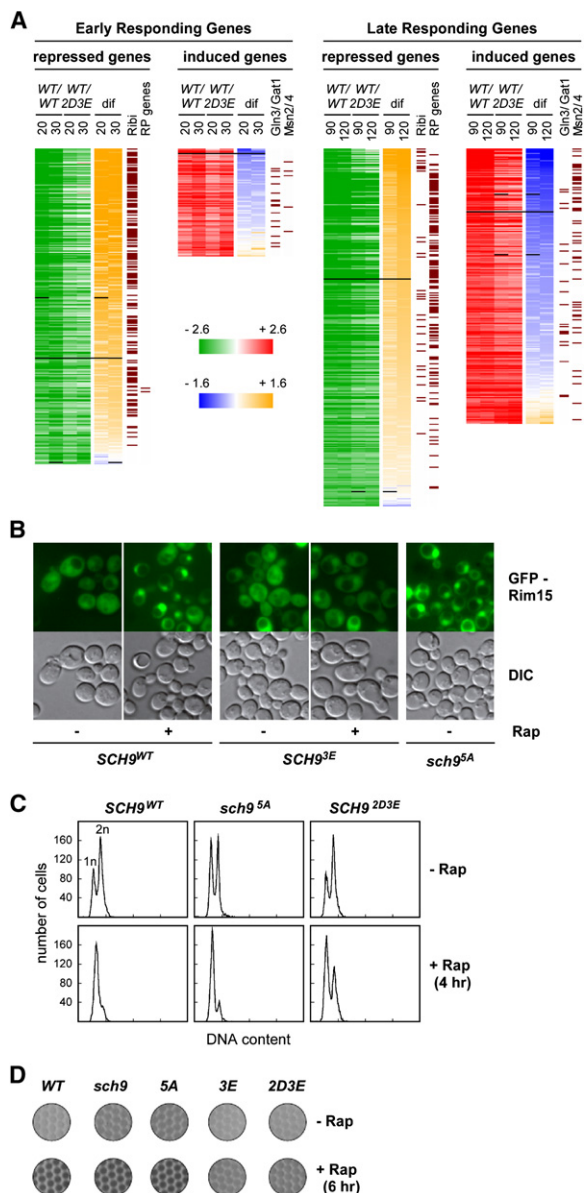


Figure 6. Sch9 Is Required for TORC1 to Properly Regulate Transcription and Entry into G₀ Phase

(A) Microarray analysis comparing WT cells (W303) containing a plasmid-based copy of *SCH9^{WT}* (WT/WT) or *SCH9^{2D3E}* (WT/2D3E) uncovers the Sch9 dependence of rapamycin-induced changes in transcription. Genes showing at least 3-fold change in expression were classified as either early- or late-responsive genes by comparing the rapamycin response in WT/WT cells at early (20/30 min) and late (90/120 min) time points. Genes were sorted according to the maximal Sch9 dependence of their response ($\text{dif} = \log_2 \text{WT}/2\text{D3E} - \log_2 \text{WT}/\text{WT}$). For genes above the black line the rapamycin-induced change in expression is diminished ≥ 2 -fold in WT/2D3E compared to WT/WT cells (i.e., $\text{dif} \geq 1$ or ≤ -1). Ribosome biosynthesis genes and genes encoding RPs as well as genes, whose expression is regulated by Gln3/Gat1 or Msn2/4, are indicated. Color codes show the log₂ of the expression change relative to untreated cells (green-red) and the corresponding dif value (blue-gold).

(B) The rapamycin-induced (200 ng/ml, 30 min) translocation of GFP-Rim15^{C1176Y} into the nucleus is blocked in *sch9 rim15* cells expressing

To test directly whether TORC1 can phosphorylate Sch9 at the vacuole, we fused the C-terminal portion of Sch9 (cSch9 = aa 709–824) and a tag onto the C terminus of Vac8, a palmitoylated protein that resides on the vacuolar membrane (Wang et al., 1998). Vac8-cSch9-GFP expressed in WT cells localized to the surface of the vacuole as expected (Figure 5A). Immunoblotting of untreated and NTCB-treated protein extracts showed that the C terminus of Vac8-cSch9-3HA became highly phosphorylated in a rapamycin-sensitive manner. No rapamycin-sensitive modification occurred in a construct containing alanines at all six TORC1 phosphorylation sites (Vac8-cSch9^{6A}-3HA; Figure 5B). Further analyses showed that the TORC1 sites in this construct were hyperphosphorylated upon treatment with CHX and dephosphorylated following high salt treatment as well as carbon or nitrogen starvation (Figure 5C). Following readdition of Gln to nitrogen-starved cells, Vac8-cSch9-3HA and Sch9-5HA were rephosphorylated with similar kinetics (data not shown). Similar results were obtained when the cSch9-GFP or cSch9-3HA sequences were fused to the first 134 amino acids of Sna4, a small proteolipid of the vacuolar membrane (data not shown), confirming that our findings are not specific for Vac8. These experiments indicate that the pool of TORC1 at the vacuolar surface is active. In the future, variants of these reporter constructs may be useful to probe for TORC1 activity at other loci.

TORC1 Regulates the Ribi and RP Regulons in Part via Sch9

To begin to investigate which of the many different TORC1 readouts are mediated by Sch9, we used global transcriptional analysis to compare the rapamycin response of WT cells (W303) expressing plasmid-encoded Sch9^{WT} (WT/WT) with those expressing plasmid-encoded Sch9^{2D3E} (WT/2D3E). We hypothesized that TORC1-independent Sch9^{2D3E} should act in a dominant manner to attenuate the response of genes that are regulated by TORC1 via Sch9. An analysis of all genes whose expression changed at least 3-fold after rapamycin addition revealed a biphasic response to rapamycin, with one set of genes responding maximally after 20 or 30 min and a second, essentially nonoverlapping set of genes responding maximally at later time points (Figure S4).

Thus we separately analyzed early- and late-responsive genes whose expression changed at least 3-fold in WT/WT cells (Figure 6A). At early time points following

plasmid-encoded Sch9^{2D3E}. In cells expressing Sch9^{5A}, Rim15 localizes constitutively to the nucleus.

(C) FACS analysis of *sch9* cells containing the indicated plasmid-based alleles of *SCH9* following treatment with rapamycin (4 hr, 200 ng/ml) or drug vehicle. Expression of Sch9^{2D3E} prevents the rapamycin-induced cell-cycle arrest, observed as accumulation of cells with a 1n DNA content. An enhanced portion of cells expressing Sch9^{5A} have a 1n DNA content even in the absence of rapamycin.

(D) *sch9 gat1 gln3* cells expressing Sch9^{3E} or Sch9^{2D3E} show a reduced glycogen accumulation upon rapamycin treatment (6 hr, 200 ng/ml) compared to cells expressing Sch9^{WT}.

rapamycin treatment the expression of 272 genes was reduced ≥ 3 -fold. Repression of a majority (181) of these genes was attenuated at least 2-fold in *WT/2D3E* cells compared to *WT/WT* cells, suggesting that TORC1-dependent phosphorylation of Sch9 contributes to the regulation of these genes. This group included predominantly genes that encode factors involved in the synthesis of ribosomes, tRNAs, and nucleotides (SGD), most of which have previously been assigned to the ribosome biogenesis (Ribi) regulon (Jorgensen et al., 2004). Quantitative PCR analyses confirmed that the effect of rapamycin on the expression of several Ribi genes (*PWP1*, *UTP13*, *DIP2*, and *CIC1*) was reduced in *WT/2D3E* compared to *WT/WT* cells (Figure S5A). Further experiments in the TB50 genetic background showed that the effect of rapamycin on the expression of these Ribi genes is reduced in *sch9 gat1 gln3* cells lacking Sch9 activity or expressing only TORC1-independent versions of Sch9 compared to cells expressing Sch9^{WT}. It is not known why rapamycin treatment caused a stronger reduction in Ribi gene expression in the W303 compared to the TB50 genetic background. The data for TB50 cells lacking Sch9 also revealed a strong Sch9-independent component in the regulation of Ribi gene expression upon rapamycin treatment (Figure S5B).

At later time points (≥ 90 min), the expression of 308 genes was downregulated ≥ 3 -fold in *WT/WT* cells. Repression of 113 of these genes was significantly (≥ 2 -fold) dependent on Sch9. Among these, genes encoding ribosomal proteins (RPs) figured prominently. These data are consistent with previous work (Jorgensen et al., 2004), which demonstrated that both TORC1 and Sch9 regulate the expression of Ribi and RP genes.

The expression of relatively few genes was increased more than 3-fold at early time points (93), and most of these appeared to be regulated independently of Sch9. The expression of 237 genes was increased at late time points. Among these, the upregulation of 55 genes was diminished more than 2-fold in *WT/2D3E* cells compared to *WT/WT* cells. Genes regulated by *Msn2/4* (<http://www.yeasttract.com>) were concentrated in this group, suggesting that TORC1 regulates *Msn2/4* activity (Beck and Hall, 1999) in part via Sch9 (Figure 6A).

Importantly, not all TORC1-regulated transcription programs appear to depend on Sch9. For example, most Gln3-regulated genes (Scherens et al., 2006), whose expression increased more than 3-fold following rapamycin treatment, did not show a significant dependence on Sch9 (Figure 6A). qPCR analyses confirmed that the rapamycin-induced expression of the Gln3/Gat1-regulated genes *GLN1* and *GAP1* was similar in *SCH9^{WT}*, *SCH9^{3E}*, and *SCH9^{2D3E}* cells (Figure S5C). The expression of the Rtg1/Rtg3-regulated gene *CIT2* was also similarly induced in *sch9 gat1 gln3* cells containing different alleles of *SCH9* (Figure S5D). Consistent with these results, both Gln3-13myc and Rtg1-GFP translocated normally into the nucleus after rapamycin treatment of *sch9* cells expressing Sch9^{3E} (data not shown). Thus Sch9 is not required for

TORC1 to negatively regulate the activity of Gln3/Gat1 (nitrogen discrimination pathway) or Rtg1/3 (retrograde signaling pathway).

TORC1 Inhibits G₀ Entry via Sch9

Both TORC1 and Sch9 prevent entry into G₀ by inhibiting nuclear translocation and activation of the Rim15 kinase (Pedruzzi et al., 2003). When treated with rapamycin, *SCH9^{WT}* cells contained nuclear Rim15 (Figure 6B), arrested with a 1n DNA content (Figure 6C), and exhibited G₀-specific phenotypes such as accumulation of the carbon reserve glycogen (Figure 6D). These readouts were partially blocked in *SCH9^{2D3E}* cells while cells lacking Sch9 or expressing Sch9^{5A} constitutively localized Rim15 to the nucleus and accumulated glycogen even in the absence of rapamycin. However, the expression of G₀-specific genes like *GRE1* following rapamycin treatment was only moderately reduced in cells expressing Sch9^{2D3E} in our microarray experiments. These results suggest that TORC1 inhibits G₀ entry in part, but not exclusively, via Sch9.

To analyze whether Sch9 phosphorylation by TORC1 is also required to complement the small cell phenotype of *sch9* cells (Jorgensen et al., 2004), we measured the peak volume of *sch9 gat1 gln3* cells expressing plasmid-encoded versions of Sch9. Cells expressing Sch9^{5A} were similar in size to cells containing a control plasmid (30.0 versus 31.0 μm^3) and significantly smaller than cells expressing Sch9^{WT} (43.1 μm^3). Cells expressing Sch9^{3E} and Sch9^{2D3E} yielded a peak volume of 40.2 and 36.6 μm^3 , respectively (data not shown).

TORC1 Regulates Translation Initiation in Part via Sch9

In yeast, rapamycin treatment leads to a rapid decrease of translation initiation (Barbet et al., 1996) and TORC1 has been proposed to regulate translation initiation via several potential targets including eIF2 α phosphorylation (Cherkasova and Hinnebusch, 2003) and eIF4G stability (Berset et al., 1998).

To investigate whether Sch9 is required for TORC1 to regulate translation initiation, we analyzed polysome profiles generated from mock- or rapamycin-treated *SCH9^{WT}*, *sch9*, *sch9^{5A}*, and *SCH9^{2D3E}* cells (Figure 7A). As expected, *SCH9^{WT}* cells showed a rapid arrest of translation initiation following rapamycin treatment as indicated by a 66% decrease in the polysome to 80S monosome (P/M) ratio compared to untreated cells. In cells expressing *SCH9^{2D3E}* only a slight reduction in the P/M ratio occurred upon rapamycin treatment (21% decrease). Protein synthesis appeared to be already compromised in untreated *sch9* and *sch9^{5A}* cells as judged by the reduced polysome content, and the P/M ratio of these cells was less reduced by rapamycin treatment (19% and 46% decrease, respectively) compared to *SCH9^{WT}* cells.

eIF2 α phosphorylation increased 4-fold in *sch9* cells expressing Sch9^{WT} upon treatment with rapamycin, but only 2.7-fold when rapamycin was added to cells expressing Sch9^{2D3E}. Furthermore, even in the absence of rapamycin,

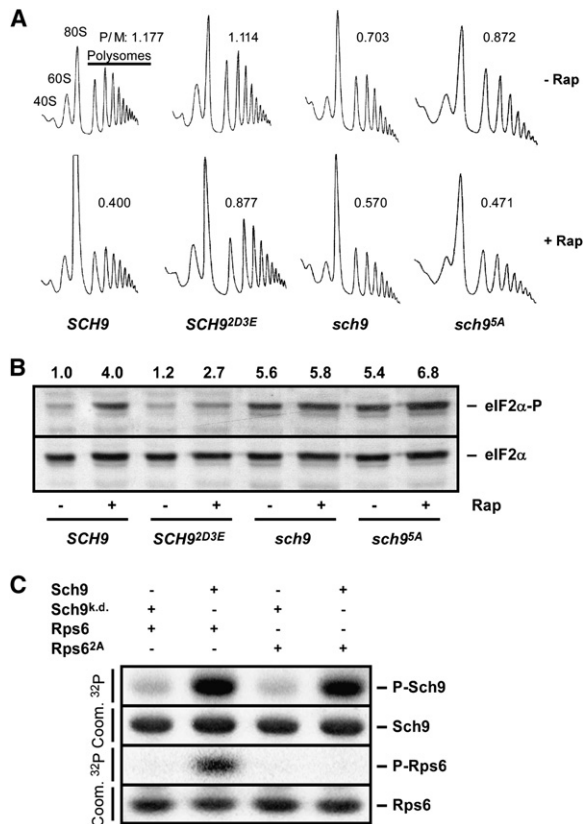


Figure 7. Sch9 Mediates Aspects of Translation Initiation Regulation by TORC1

(A) Changes in polysome profiles caused by rapamycin treatment (40 min, 200 ng/ml) are reduced in cells lacking Sch9 activity or expressing a TORC1-independent version of Sch9. The positions corresponding to the 40S and 60S subunits, the 80S monosomes, and polysomal ribosomes are indicated in the profile of untreated *SCH9^{WT}* cells. The ratio between polysome and 80S monosome peaks (P/M) is indicated. (B) Cells containing *SCH9^{2D3E}* show less rapamycin-induced (30 min, 200 ng/ml) phosphorylation of eIF2 α (Sui2) compared to *SCH9^{WT}* cells, while eIF2 α is constitutively phosphorylated in *sch9* and *sch9^{5A}* cells. Numbers indicate the increase of the eIF2 α -P/eIF2 α ratio relative to untreated WT cells. (C) Sch9 phosphorylates the yeast S6 ortholog Rps6, but not the Ala-substituted Rps6^{2A} in vitro.

phosphorylation of eIF2 α was strongly elevated in *sch9* and *sch9^{5A}* cells (5.6- and 5.4-fold, respectively, as compared to *SCH9^{WT}* cells) and this phosphorylation was only slightly further enhanced by rapamycin (Figure 7B).

In contrast, Sch9 does not seem to be involved in the rapamycin-induced turnover of the translation factor eIF4G, because eIF4G was equally abundant in *SCH9^{WT}*, *sch9*, *sch9^{5A}*, and *SCH9^{2D3E}* yeast and was degraded upon rapamycin treatment with similar kinetics in all of these cells (data not shown). Together, these results demonstrate that some but not all aspects of the regulation of translation initiation by TORC1 are mediated by Sch9.

Lastly, we wished to address whether Sch9 may function similarly to mammalian S6 kinase and phosphorylate

the yeast S6 ortholog (see Discussion). Sch9, but not catalytically inactive Sch9^{k.d.}, efficiently phosphorylated Rps6 in vitro (Figure 7C). Rps6^{2A}, which contains two amino acid substitutions (S232A and S233A) previously shown to abolish phosphorylation of Rps6 in vivo (Kruse et al., 1985), was not phosphorylated by Sch9. This suggests that Sch9 is indeed a genuine S6 kinase.

DISCUSSION

In this study, we have confirmed and extended previous work (Jorgensen et al., 2004) by showing that Sch9 is a direct substrate for TORC1 and a major component of the TORC1 signaling pathway in *S. cerevisiae*. TORC1 regulates ribosome biosynthesis and thus cell-size control in large part via Sch9. Sch9 is also required for TORC1 to properly regulate entry into stationary phase and translation initiation, while other processes like the expression of Gln3/Gat1 and Rtg1/3 target genes appear to be regulated by TORC1 independently of Sch9. This is consistent with previous studies that demonstrated that TORC1 uses distinct effector pathways to regulate the expression of Ribi/RP genes versus Gln3- and Rtg1/3- dependent genes (Duvel et al., 2003). In the future it will be very important to identify and characterize Sch9 substrates.

In addition to the seven sites described here, Sch9 is phosphorylated at many more residues in its N terminus, apparently due in part to autophosphorylation (J.U. and R.L., unpublished data). It is likely that inputs from other signaling pathways are integrated with those of TORC1 and Pkh kinases to regulate Sch9 activity. Indeed, crosstalk between TORC1 and other nutrient-responsive signaling pathways appears to be a recurring theme in cell growth control (Schneper et al., 2004). Last, but not least, localization and stability of Sch9 warrant further study, in particular the ligand-binding properties of its C2 domain.

How Is TORC1 Regulated?

Phosphorylation of the C terminus of Sch9 is sensitive to alterations in nutrient availability and application of various stresses. This probably reflects changes in TORC1 activity or localization, as similar results were observed with a construct that contains only the C-terminal 116 amino acids of Sch9 tethered to the vacuolar membrane. However, it is possible that changes in phosphatase activity contribute to the regulation of Sch9 phosphorylation as well. The next challenge will be to determine, at the molecular level, how growth cues regulate TORC1. An intriguing observation is the finding that at least a portion of TORC1 is active at the surface of the vacuole. The vacuole is a major nutrient reservoir in yeast, and therefore the vacuolar membrane would be an ideal location for a sensor of intracellular nutrients and for the compartmentalization of nutrient-responsive signaling pathways.

It is also noteworthy that Sch9 C-terminal phosphorylation does not always correlate with growth rate. Thus, although TORC1 activity is required for growth, factors in addition to TORC1 contribute to determine steady-state

growth rate. The reduction in TORC1 activity following nutrient starvation or the application of stress conditions elicits, in addition to a reduction in protein synthesis, a depression of genes encoding proteins required for the utilization of alternative nutrient sources and stress response factors. In this way TORC1 plays an important role in allowing cells to rapidly respond to changing growth conditions. Once cells have successfully adapted their metabolism to the availability of nutrients or acquired tolerance to environmental stress, TORC1 is reactivated and growth resumes. Developmental decisions such as entry into G₀ phase (Pedruzzi et al., 2003) or sporulation (Colomina et al., 2003) may require a prolonged inactivation of TORC1.

Is Sch9 an S6K1 Ortholog?

Many groups suggest that Sch9 is the yeast ortholog of mammalian PKB/Akt (reviewed in Sobko [2006]). However, for the following reasons we suggest that Sch9 function may be more closely related to that of mammalian S6K1.

(1) Like S6K1, Sch9 activity is regulated by TORC1. In contrast, Akt is regulated by mTORC2 (Sarbasov et al., 2005). S6K1 and Sch9 also share the unusual feature of having an extended sequence beyond their HMs. S6K1 mutants lacking this domain have been found to be inappropriately activated by mTORC2 (Ali and Sabatini, 2005).

(2) S6K1 and Sch9 apparently perform similar functions, most notably the regulation of translational initiation. Indeed, we have found that Sch9 is able to phosphorylate Rps6, the yeast ortholog of the mammalian RP S6, *in vitro*, and thus by definition is an S6 kinase.

We anticipate that further characterization of Sch9 function will not only enhance our understanding of TORC1 signaling in yeast but will also reveal additional functions of mammalian S6 kinase. For example, both TORC1 and Sch9 have been implicated in coupling nutrient excess to decreased lifespan (Fabrizio et al., 2005; Kaerberlein et al., 2005; Powers et al., 2006). This raises the interesting possibility that mTORC1 and S6K1 similarly influence longevity in mammals.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

Yeast strains and plasmids used in this study are listed in Tables S1 and S2. All strains except those used in Figures 4C, 6A, and 6B and Figure S4 were made prototroph for amino acids and nucleotides by introducing pJU 450 and a *URA3*-containing plasmid.

Western Blot and Chemical Fragmentation Analysis

PPI: 10 mM NaF, 10 mM NaN₃, 10 mM p-nitrophenylphosphate, 10 mM Na₂P₂O₄, and 10 mM β-glycerophosphate; PI: 1× Roche protease inhibitor cocktail and 1 mM PMSF.

Cultures were mixed with TCA (final concentration 6%) and put on ice for at least 5 min before cells were pelleted, washed twice with cold acetone, and dried in a speed-vac. Cell lysis was done in 100 μl of urea buffer (50 mM Tris [pH 7.5], 5 mM EDTA, 6 M urea, 1% SDS, 1 mM PMSF, and 0.5× PPI) with glass beads in a bead beater with subsequent heating for 10 min to 65°C. For NTCB cleavage 30 μl of 0.5 M CHES (pH 10.5) and 20 μl of NTCB (7.5 mM in H₂O) were added and

samples incubated over night at RT before 1 vol of 2× sample buffer (+20 mM TCEP and 0.5× PPI) was added. Further analysis was done by SDS-PAGE and immunoblotting using anti-HA antibody 12CA5 or anti-T570-P antiserum.

TORC1 Kinase Assay

Preparation of Recombinant Sch9

GST-Sch9 fusion proteins were expressed in *E. coli* from a pGEX-6P vector. After a 3 hr induction with 0.4 mM IPTG, a clarified bacterial lysate was prepared and the fusion protein was bound to glutathione Sepharose following standard procedures. Sch9 was cleaved from the GST moiety overnight at 4°C with 24 units PreScission protease (GE Healthcare) in 300 μl PreScission cleavage buffer containing 0.01% Tween 20 following manufacturer's instructions. The supernatant was dialyzed against (1× PBS, 20% glycerol, and 0.5% Tween 20), aliquotted, and frozen at -80°C.

Kinase Assay

TORC1 was purified from RL175-2d or RL176-1b cells treated with drug vehicle or 200 nM rapamycin for 30 min. Cells grown at 30°C in YPD (250 ml per assay point) to an OD₆₀₀ of ~1.0 were chilled on ice, collected by centrifugation, washed with H₂O, resuspended in lysis buffer (1× PBS, 10% [w/v] glycerol, 0.5% [v/v] Tween 20, PI, and PPI), transferred to 2 ml screw-cap tubes half-filled with glass beads (0.5 mm), and disrupted in a Fast Prep machine at 4°C (Bio101; 5× 30 s at max. speed). Crude lysates were cleared of debris with two 1000 × g spins and protein concentrations normalized as necessary. Extracts were precleared over CL-4B Sepharose before 7 μl of IgG Sepharose (GE Healthcare) per assay point was added and the mix rotated for 90 min at 4°C. Beads were collected in a column, washed with cold lysis buffer, and aliquotted to 1.5 ml tubes. Kinase reactions were performed in kinase buffer (1× PBS, 20% glycerol, 0.5% Tween 20, 4 mM MgCl₂, 10 mM DTT, 2 μg/ml heparin, and PI [-EDTA]) in a final volume of 30 μl containing ~350 ng of recombinant Sch9. Assays were started with the addition of 100 μM ATP and 50 μCi [γ-³²P]ATP, shaken for 20 min at 30°C, and terminated with the addition of 8 μl of 5× SDS-PAGE sample buffer. Samples were heated to 95°C for 5 min before being separated by SDS-PAGE, stained with Coomassie, and analyzed using a BioRad Molecular Imager.

Supplemental Data

Supplemental Data include five figures, three tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at <http://www.molecule.org/cgi/content/full/26/5/663/DC1/>.

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Author contributions: J.U., Figures 1, 2A–2D, 3A, 4A, 4C, and 5, and Figure S3; R.L., Figures 2E and 2F and Figure S2; A.H., Figures 3B and 7C; D.M., Figure 4B; S.L., Figure 6A and Figures S4 and S5A; A.S., Figures 6B and 6D and Figures S5B–S5D; V.W., Figure 6C; O.D., Figures 7A and 7B; D.A., Table S3; and manuscript preparation, J.U. and R.L.

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Accession Numbers

Microarray data have been deposited at <http://www.ncbi.nlm.nih.gov/geo/>. They are accessible with a series accession number of GSE7660. The complete data set is also available at http://puma.princeton.edu/cgi-bin/publication/viewPublication.pl?pub_no=509.