

The Vam6 GEF Controls TORC1 by Activating the EGO Complex

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SUMMARY

The target of rapamycin complex 1 (TORC1) is a central regulator of eukaryotic cell growth that is activated by a variety of hormones (e.g., insulin) and nutrients (e.g., amino acids) and is deregulated in various cancers. Here, we report that the yeast Rag GTPase homolog Gtr1, a component of the vacuolar-membrane-associated EGO complex (EGOC), interacts with and activates TORC1 in an amino-acid-sensitive manner. Expression of a constitutively active (GTP-bound) Gtr1^{GTP}, which interacted strongly with TORC1, rendered TORC1 partially resistant to leucine deprivation, whereas expression of a growth inhibitory, GDP-bound Gtr1^{GDP}, caused constitutively low TORC1 activity. We also show that the nucleotide-binding status of Gtr1 is regulated by the conserved guanine nucleotide exchange factor (GEF) Vam6. Thus, in addition to its regulatory role in homotypic vacuolar fusion and vacuole protein sorting within the HOPS complex, Vam6 also controls TORC1 function by activating the Gtr1 subunit of the EGO complex.

INTRODUCTION

The target of rapamycin complex 1 (TORC1) is a structurally and functionally conserved, essential multiprotein complex that positively regulates cell growth by promoting anabolic processes (e.g., protein synthesis) and inhibiting catabolic processes (e.g., autophagy) in response to diverse signals, including mitogenic growth factors, energy/ATP levels, and amino acids (De Virgilio and Loewith, 2006a; Wullschleger et al., 2006). The mechanisms that couple growth factors and energy levels to mammalian TORC1 (mTORC1) have been characterized in considerable detail and implicate the phosphatidylinositol 3-kinase (PI3K), Akt, TSC1/TSC2, and Rheb, a small guanosine triphosphate (GTP)-binding protein that directly binds to and stimulates mTORC1 (Sarbasov et al., 2005). The mechanisms through which amino acids signal to TORC1, in contrast, have largely remained elusive until very recently. Two complementary studies have reported that the conserved Rag GTPases act as upstream

regulators of TORC1 and play important roles in coupling amino-acid-derived signals to TORC1 in both *Drosophila* and mammalian cells (Kim et al., 2008; Sancak et al., 2008). RagA and RagB are very similar to each other and orthologous to yeast Gtr1, whereas RagC and RagD are similar and orthologous to yeast Gtr2 (Hirose et al., 1998; Schürmann et al., 1995; Sekiguchi et al., 2001). Rag and Gtr proteins function in heterodimeric complexes, which contain one Gtr1-like GTPase and one Gtr2-like GTPase (Nakashima et al., 1999; Sekiguchi et al., 2001). Importantly, amino acid signals are thought to impinge on GTP loading of RagA/B, but it is not known which factors regulate the GTP loading of these GTPases.

We previously reported that the EGO complex in yeast, consisting of Ego1/Meh1, Ego3/Slm4, Gtr2, and Gtr1, which was identified subsequently (Gao and Kaiser, 2006; Gao et al., 2005), may function upstream of TORC1 to mediate amino acid signaling (De Virgilio and Loewith, 2006a, 2006b; Dubouloz et al., 2005). In this study, we provide evidence that the EGO complex indeed functions directly upstream of TORC1. TORC1 activity is dictated by the nucleotide-bound state of Gtr1, and this is dependent on the presence of Tco89, a nonessential component of TORC1. Furthermore, we demonstrate that Vam6 colocalizes with the EGO complex/TORC1 at the limiting membrane of the vacuole and functions as a guanine nucleotide exchange factor (GEF) for Gtr1. Thus, in addition to its regulatory role in homotypic vacuolar fusion and vacuole protein sorting as part of the HOPS complex, Vam6 also controls the activity of TORC1 by activating the Gtr1 subunit of the EGO complex.

RESULTS AND DISCUSSION

EGOC Acts Upstream of TORC1

Loss of EGO complex (Ego1, Ego3, Gtr1, and Gtr2) or TORC1 (Tco89) subunits results in an inability to restart growth following exposure to rapamycin (Figure 1A; Dubouloz et al., 2005). Similar to wild-type cells treated with rapamycin or the more recently described TORC1 inhibitor caffeine (Kuranda et al., 2006; Reinke et al., 2006; Wanke et al., 2008), these mutants also possess decreased TORC1 activity as assessed by monitoring phosphorylation of the TORC1 substrate Sch9 (Urban et al., 2007) (Figure 1A). Moreover, the potent increase in TORC1 activity observed in wild-type cells treated with cycloheximide, a translation elongation inhibitor that may indirectly boost the levels of free intracellular amino acids (Beugnet et al., 2003; Urban

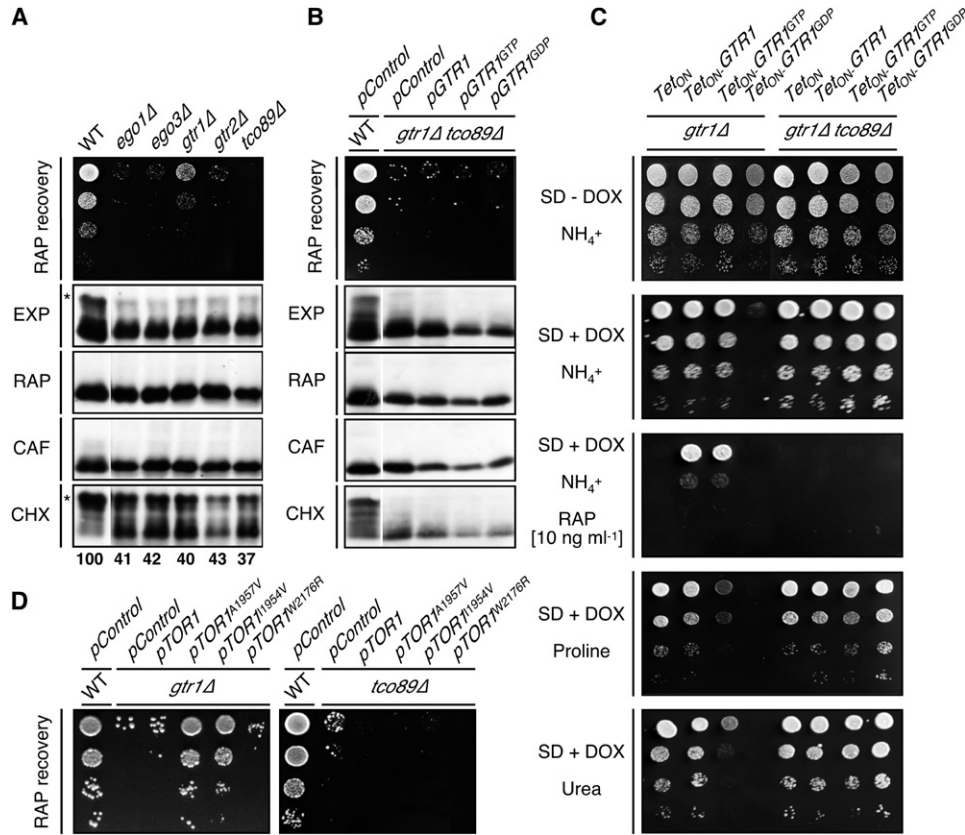


Figure 1. EGO Acts Upstream of TORC1

(A) Loss of TORC1 (Tco89) or EGO (Ego1, Ego3, Gtr1, and Gtr2) subunits causes a defect in recovery from rapamycin-induced growth arrest and decreases TORC1 activity. (Top) Wild-type and isogenic mutant cells were grown exponentially in YPD, treated for 6 hr with rapamycin (200 ng ml⁻¹), and then washed twice and spotted as 10-fold serial dilutions on YPD plates. (Bottom) Indicated strains expressing a plasmid-based copy of *SCH9*^{T570A}-HA₅ were grown exponentially (in YPD + 0.2% glutamine; EXP) and treated for 30 min with rapamycin (RAP; 200 ng ml⁻¹), caffeine (CAF; 20 mM), or cycloheximide (CHX; 25 μg ml⁻¹). Protein extracts were prepared and subsequently treated with NTCB to cleave polypeptide chains at cysteine residues (Urban et al., 2007). Aliquots were then analyzed by SDS-PAGE, and immunoblots were probed with anti-HA antibodies (only the migration pattern of the C terminus of Sch9 is shown). The extent of Sch9 phosphorylation (and, by proxy, TORC1 activity) was determined by quantifying the signal of the slowest migrating (most highly phosphorylated) species of Sch9 (*) and dividing this by the total signal observed for all species. For comparison, this ratio was set to 100% for wild-type cells and calculated correspondingly (in percent) for the indicated mutants (numbers below lowest chart; standard deviations were below 5% in each case).

(B) *Gtr1*^{GTP} suppresses neither the defects in recovery following rapamycin treatment nor the intrinsically low TORC1 activity in *tco89Δ* cells. Wild-type and double *gtr1Δ tco89Δ* mutant strains were transformed with an empty vector or vectors that express *GTR1*, *GTR1*^{GTP}, or *GTR1*^{GDP} from the endogenous promoter and were assayed as in (A).

(C) Growth inhibition following overproduction of *Gtr1*^{GDP} and growth inhibition on nitrogen poor (proline- and urea-containing) media following overproduction of *Gtr1*^{GTP} depend on the presence of Tco89. Single *gtr1Δ* and double *gtr1Δ tco89Δ* mutant strains were transformed with an empty vector or vectors that express *GTR1*, *GTR1*^{GTP}, or *GTR1*^{GDP} from the doxycycline-inducible *Tet*_{ON} promoter; were grown to exponential phase in doxycycline-containing media; and were spotted on plates containing, or not, doxycycline (DOX; 5 μg ml⁻¹) and rapamycin (RAP; 10 ng ml⁻¹) and either 75 mM NH₄⁺ (top three panels), 10 mM proline, or 10 mM urea as nitrogen source as indicated.

(D) Hyperactive *TOR1*^{A1957V} and *TOR1*^{I1954V} alleles, unlike wild-type or the hypoactive *TOR1*^{W2176R} allele, suppress the defect in recovery following rapamycin treatment in *gtr1Δ*, but not *tco89Δ* cells. Wild-type and single *gtr1Δ* and *tco89Δ* mutant strains were transformed with an empty vector or vectors that express *TOR1*, *TOR1*^{A1957V}, *TOR1*^{I1954V}, or *TOR1*^{W2176R} from the endogenous promoter and were assayed as in (A).

et al., 2007), was partially dependent on the presence of Ego1, Ego3, Gtr1, or Gtr2, further supporting the idea that the EGO complex may function upstream of TORC1 (Figure 1A). Observations from several genetic experiments are in line with this interpretation. First, expression of *Gtr1*^{GTP} or *Gtr1*^{GDP} alleles, which are predicted to be restricted to either a GTP- or GDP-bound conformation (Gao and Kaiser, 2006; Nakashima et al., 1999),² did not alter the intrinsically low TORC1 activity or the inability to recover following rapamycin treatment of *tco89Δ* cells

(Figure 1B). Second, overexpression of *Gtr1*^{GDP} reduced growth rate on rich media, whereas overexpression of *Gtr1*^{GTP} reduced growth rate on media containing poor nitrogen sources (proline and urea), and these phenotypes were suppressed in *tco89Δ* cells (Figure 1C). Third, hyperactive *TOR1*^{A1957V} and *TOR1*^{I1954V} alleles (Reinke et al., 2006), unlike wild-type *TOR1* or the hypoactive *TOR1*^{W2176R} allele, fully suppressed the defect in recovery following rapamycin treatment in *gtr1Δ* cells, but not in *tco89Δ* cells (Figure 1D). Lastly, TORC1 inhibition results in the activation

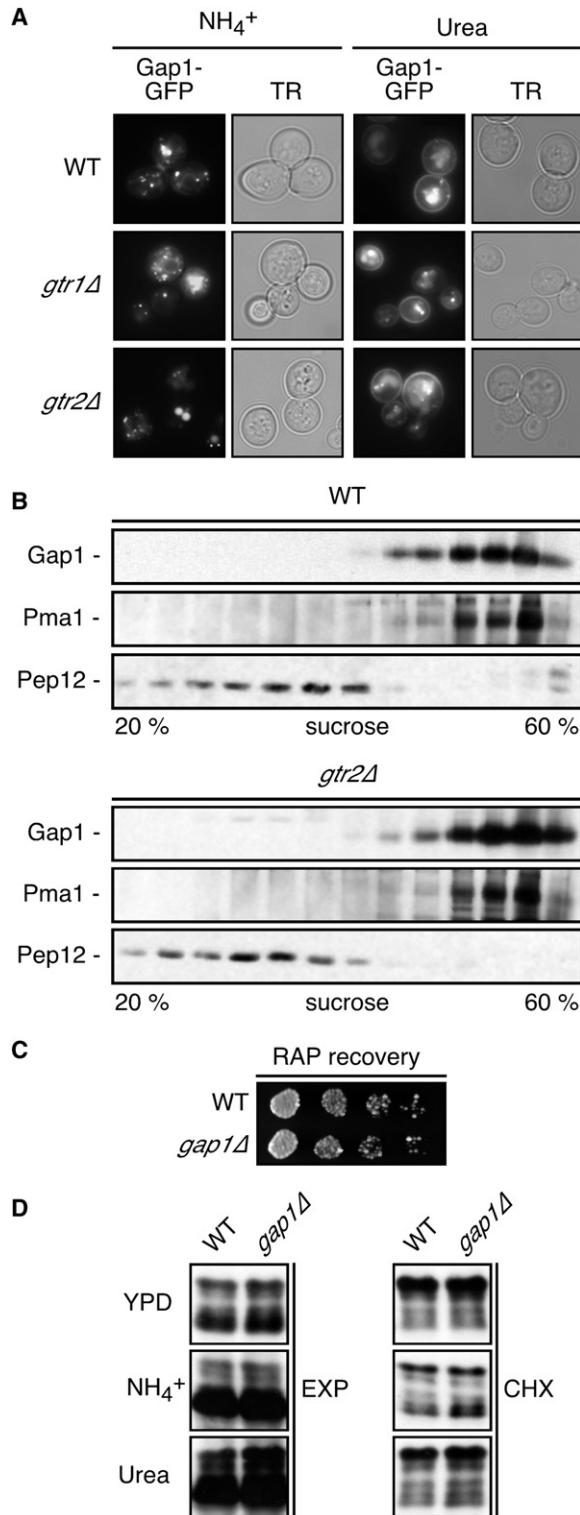


Figure 2. EGO Does Not Control TORC1 Indirectly via Gap1 Sorting

(A) EGO complex subunits (i.e., Gtr1 or Gtr2) are not required for sorting Gap1 to the plasma membrane. Prototrophic wild-type, *gtr1Δ*, and *gtr2Δ* strains expressing a plasmid-encoded Gap1-GFP under the control of the galactose-inducible *GAL1* promoter were pregrown on SD medium (containing 75 mM NH₄⁺ as nitrogen source and 2% raffinose/0.1% sucrose as carbon

and nuclear localization of the transcription factor Gln3 (Beck and Hall, 1999), an event that is growth inhibitory for cells. Deletion of *GLN3* suppressed the defect in recovery from rapamycin treatment, but not the TORC1 activity defect of EGO complex mutants (Figure S1 available online). Collectively, these observations support the idea that the EGO complex signals, both positively and negatively, to TORC1 and further suggest that this signal is mediated by the TORC1 component Tco89.

EGOC Does Not Control TORC1 Indirectly via Gap1 Sorting

In a previous study (Gao and Kaiser, 2006), all four subunits of the EGO complex were shown to be required for intracellular sorting of the general amino acid permease Gap1, a process that is controlled by the quality of the external nitrogen source. Accordingly, in wild-type cells grown on a relatively poor nitrogen source such as urea, Gap1 is sorted to the plasma membrane (where it is active for transport), whereas in cells grown on rich nitrogen sources such as glutamate/glutamine or ammonium (NH₄⁺), active Gap1 is internalized and directed to the vacuole for degradation (Springael and André, 1998), and newly synthesized Gap1 is directly sorted from the *trans*-Golgi to the vacuole (De Craene et al., 2001). Although we observed that loss of EGO affected TORC1 activity even when cells were grown on rich nitrogen sources, i.e., under conditions in which Gap1 is transcriptionally repressed and not sorted to the plasma membrane (De Craene et al., 2001; Jauniaux and Grenson, 1990), we decided to address the possibility that the EGO may indirectly affect TORC1 activity via a potential effect on Gap1 sorting. To our surprise, examination of the subcellular distribution of Gap1-GFP, which was transiently expressed from a galactose-inducible *GAL1* promoter (Nikko et al., 2003), revealed that sorting of Gap1 from exclusively intracellular compartments (in cells grown on NH₄⁺-containing medium) to the plasma membrane (in cells shifted to urea-containing medium) occurred normally in both wild-type and *egoc* mutant cells (Figure 2A). Similarly, when assayed by subcellular fractionation analysis, loss of Gtr2, which was suggested to be particularly important for Gap1 sorting (Gao and Kaiser, 2006), had no impact on the cells' ability to

source). Cells were then incubated for 2 hr in the presence of 2% galactose (to induce Gap1-GFP expression) and analyzed by fluorescence microscopy either directly (NH₄⁺) or following an additional 2 hr incubation on SD media containing 10 mM urea as nitrogen source (urea) and 2% glucose (to repress Gap1-GFP de novo synthesis). As reported previously, overproduction of Gap1-GFP from the *GAL1* promoter causes some accumulation of Gap1-GFP in the vacuole (De Craene et al., 2001). TR, transmission.

(B) Prototrophic wild-type and *gtr2Δ* cells growing on SD medium containing 10 mM urea as nitrogen source were lysed and fractionated by equilibrium density centrifugation on continuous 20%–60% sucrose gradients (Kaiser et al., 2002). The fractions were assayed for the presence of Gap1, the plasma membrane marker protein Pma1, or the endosomal marker protein Pep12 using specific antibodies that recognize the corresponding proteins.

(C) Gap1 is not required for recovery of cells following rapamycin treatment. Prototrophic wild-type and *gap1Δ* cells were treated as in Figure 1A.

(D) Gap1 is neither required for normal TORC1 activity in exponentially growing cells (EXP) nor for TORC1 activation by cycloheximide (CHX). Prototrophic wild-type and *gap1Δ* cells were grown on YPD (+ 0.2% glutamine), or on SD media containing 75 mM NH₄⁺ (NH₄⁺) or 10 mM urea (urea) as nitrogen source and were assayed for TORC1 activity as in Figure 1A.

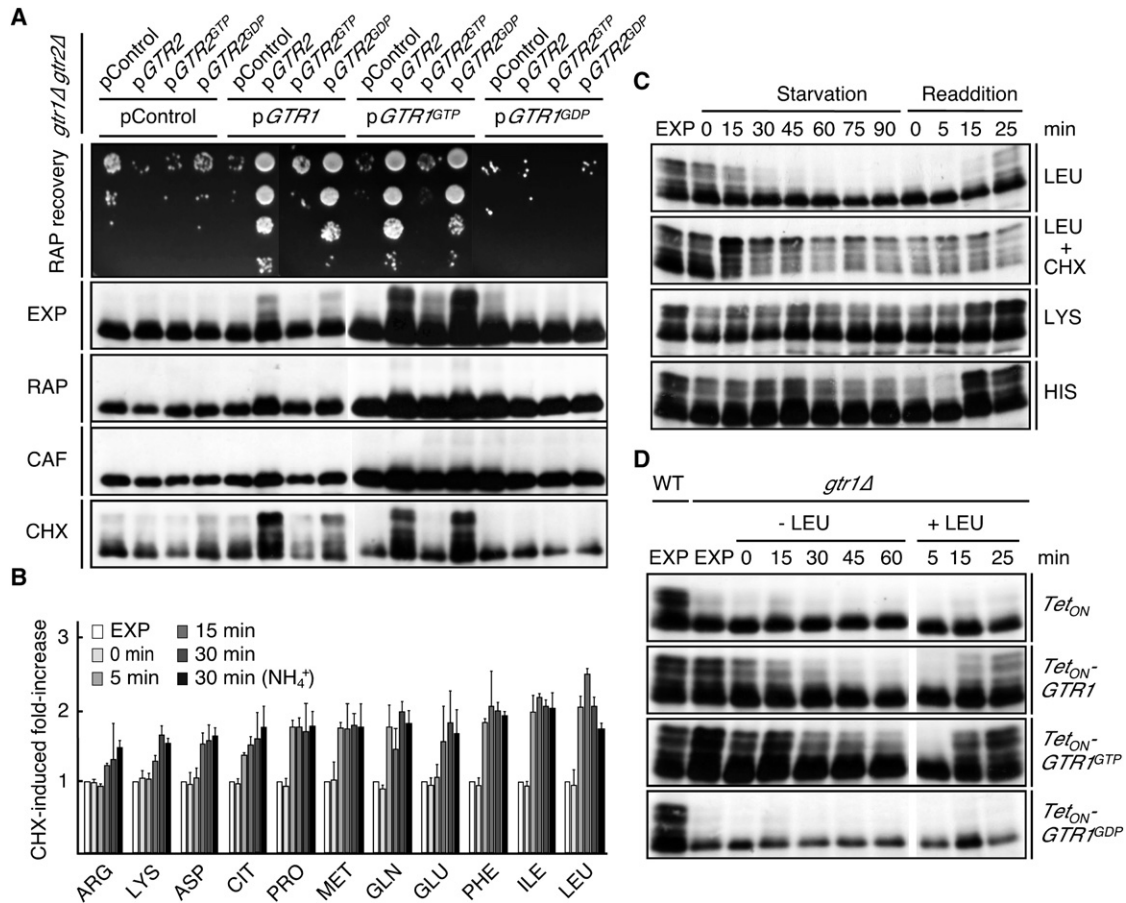


Figure 3. Alternative Nucleotide-Bound States of Gtr1 and Gtr2 Are Required for Amino-Acid-Dependent Control of TORC1

(A) The nucleotide-binding states of Gtr1 and Gtr2 control both the cell's ability to recover from rapamycin treatment and TORC1 activity. Rapamycin recovery and TORC1 activity assays (as in Figure 1A) were determined in a *gtr1Δ gtr2Δ* double-mutant strain carrying all combinations of plasmids between pGTR1, pGTR1^{GTP}, or pGTR1^{GDP} and pGTR2, pGTR2^{GTP}, or pGTR2^{GDP}.

(B) Intracellular pools of free amino acids increase rapidly following CHX treatment. Intracellular, free amino acids were determined in prototrophic wild-type cells subjected to CHX (25 μ g ml⁻¹) treatment for the times indicated and expressed as fold increase compared to the levels detected in exponentially growing (EXP) cells. Cells were grown on YPD (+ 0.2% glutamine) or (as indicated) on SD without amino acids (NH₄⁺). Experiments were done in triplicate and expressed as mean + SD. As assessed by one-way analysis of variance (ANOVA) followed by posttest analysis, the CHX-induced increases are statistically significant for all amino acids (except for arginine) with P values that are < 0.05 for glutamate and methionine and < 0.001 for all other amino acids.

(C) TORC1 is reversibly inactivated in response to amino acid starvation. Leucine (*leu2Δ*; top two panels), lysine (*lys2Δ*), and histidine (*his3Δ*) auxotrophic wild-type strains were grown to exponential phase (EXP) in medium containing leucine (LEU), lysine (LYS), or histidine (HIS), respectively, and were then transferred to a medium lacking the corresponding amino acid. Leucine (2.8 mM), lysine (0.4 mM), and histidine (0.5 mM) were readded after 90 min of starvation. Samples were taken at the times indicated following the medium changes and were assayed as in Figure 1A. The leucine starvation experiment was also carried out in the presence of cycloheximide (25 μ g ml⁻¹), which was added at time point 0 (before starvation; second panel from top).

(D) TORC1 is partially insensitive to leucine deprivation in cells expressing Gtr1^{GTP}. Leucine auxotrophic wild-type and *gtr1Δ* cells carrying the empty vector or vectors that express GTR1, GTR1^{GTP}, or GTR1^{GDP} from the doxycycline-inducible *Tet_{ON}* promoter were assayed for TORC1 activity (as in C).

sort Gap1 to the plasma membrane when grown on urea-containing medium (Figure 2B).

In trying to understand why our above results were so strikingly different from the previously reported observations, we noted that, in contrast to various wild-type strains, including Σ 1278b (Grenson, 1983), KT1960 (see below), and the S288C-derived BY4741/2 (Brachmann et al., 1998) used here, the wild-type strain used by the Kaiser group appears to be defective for NH₄⁺-inactivation of Gap1 (Gao and Kaiser, 2006). In this context, Roberg and colleagues have previously noted that this particular phenotype of their wild-type strain is due to a

loss-of-function allele at the *PER1* locus (Roberg et al., 1997), which apparently is closely linked to (and possibly within the same transcriptional unit as) the NADP⁺-dependent glutamate dehydrogenase-encoding *GDH1* gene (Courchesne and Magasanik, 1983). Remarkably, defects in *per1* are associated with a pleiotropic phenotype, including aberrant responses of various amino acid permeases (including Gap1) to environmental nitrogen signals (Courchesne and Magasanik, 1983). Although beyond the scope of this present study, it would be interesting to further characterize the potential synthetic interactions between EGOC/TORC1 and Per1/Gdh1 in future studies.

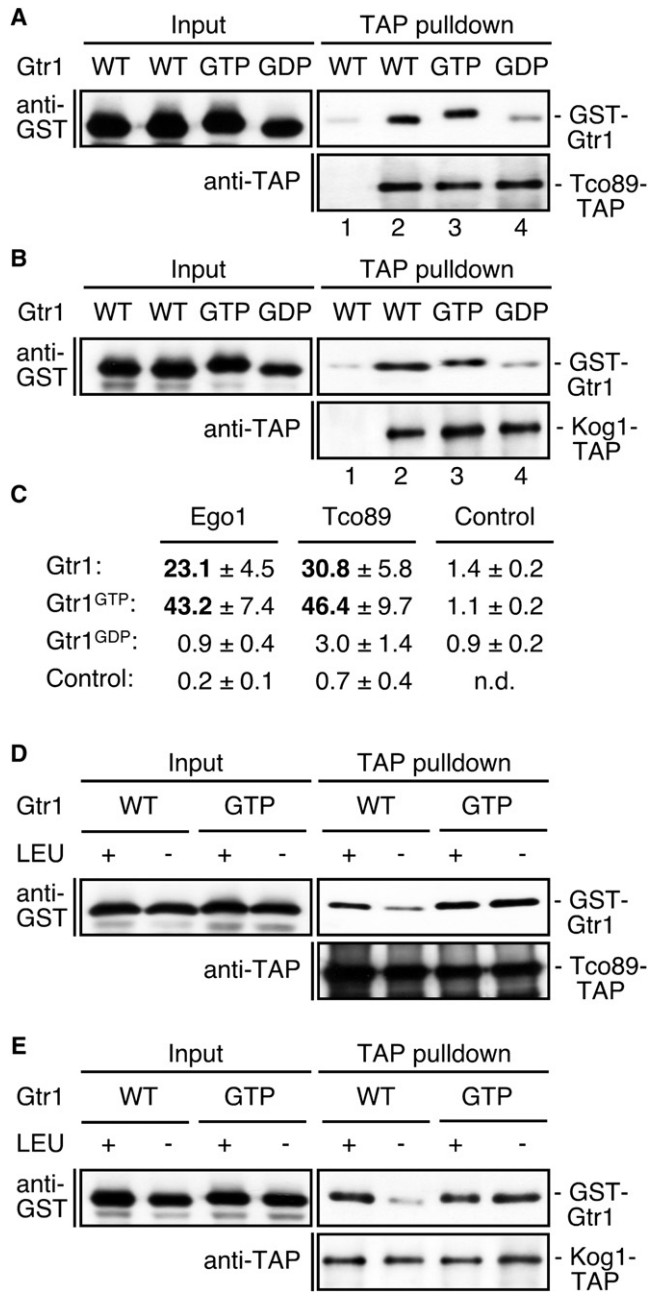


Figure 4. Gtr1, Preferentially in Its GTP-Bound Form, Physically Interacts with TORC1

(A and B) TORC1 subunits Tco89 and Kog1 preferentially interact with GTP-bound Gtr1^{GTP}. Wild-type control cells (A and B, lane 1) or wild-type cells expressing Tco89-TAP (A, lanes 2–4) or Kog1-TAP (B, lanes 2–4) were transformed with plasmids expressing GST-Gtr1 (A and B, lanes 1 and 2; WT), GST-Gtr1^{GTP} (A and B, lane 3; GTP), or GST-Gtr1^{GDP} (A and B, lane 4; GDP). Cell lysates (Input) and TAP pull-down fractions were subjected to SDS-PAGE, and immunoblots were probed with anti-GST or anti-protein A (anti-TAP) antibodies as indicated.

(C) Gtr1 and Gtr1^{GTP}, but not Gtr1^{GDP}, specifically interact with Ego1 and Tco89 in two-hybrid assays. Possible interactions between Gtr1 variants (expressed from the pPR3-N prey vector) and Ego1 or Tco89 (both expressed from the pCabWT bait vector) were evaluated using the membrane-based split

Regarding the present study, loss of Gap1 did not reduce the cells' ability to recover from a rapamycin treatment (Figure 2C) and had no impact on TORC1 activity in cells grown on rich (YPD + 0.2% glutamine or SD-NH₄⁺) or on poor (urea) nitrogen sources (Figure 2D). Taken together, these results, which were also reproducible in the completely unrelated KT1960 strain background (Pedruzzi et al., 2003; Stuart et al., 1994; data not shown), show that EGO does not affect TORC1 via Gap1 sorting.

Alternative Nucleotide-Bound States of Gtr1 and Gtr2 Are Required for Amino-Acid-Dependent Control of TORC1

To further examine the role of Gtr1 and Gtr2 in TORC1 regulation, we tested different combinations of nucleotide-restricted *GTR1* and *GTR2* alleles (Gao and Kaiser, 2006; Nakashima et al., 1999) for their effect on TORC1 activity and the cells' ability to recover from a rapamycin treatment. Expression of Gtr1^{GTP} combined with Gtr2 or Gtr2^{GDP} activated TORC1 (when compared to the corresponding heterodimers containing wild-type Gtr1) (Figure 3A). In contrast, expression of Gtr2^{GTP} combined with Gtr1 inhibited TORC1 (when compared to the wild-type heterodimer). Moreover, expression of Gtr1^{GDP}, irrespective of the nucleotide-binding status of Gtr2, was dominant-negative, as it abolished TORC1-controlled Sch9 phosphorylation under all conditions (including cycloheximide treatment) (Figure 3A). Consistent with previous studies (Kim et al., 2008; Sancak et al., 2008), these observations demonstrate that GTP-loaded Gtr1 and GDP-loaded Gtr2 stimulate TORC1, whereas GDP-loaded Gtr1 and GTP-loaded Gtr2 inhibit TORC1.

Activation of TORC1 by expression of Gtr1^{GTP} and by cycloheximide treatment was not additive, suggesting that amino-acid-dependent control of TORC1 may be controlled, at least in part, by the nucleotide-binding status of Gtr1 (Figure 3A). To explore this possibility further, we first sought to verify that cycloheximide treatment causes, as speculated, an increase in the pools of free intracellular amino acids. In prototrophic wild-type cells, we observed a significant accumulation of various amino acids within 5–15 min following cycloheximide treatment (Figure 3B). This effect (which was comparable in *gtr1*Δ cells; data not shown) and the corresponding activation of TORC1 were both observed to a similar extent in cells growing on YPD (+ 0.2% glutamine) and in cells growing on SD without amino acids (Figures 3B and S2), indicating that the accumulation of free amino acids following cycloheximide treatment does not depend on the uptake of extracellular amino acids. Interestingly, among the various amino acids, leucine appeared to accumulate most strongly and, hence, may play a particular role in TORC1

ubiquitin yeast two-hybrid system. Numbers represent mean β-galactosidase activities ± SD (in Miller units) from at least three different transformants for each pair of plasmids. Values that were at least 15-fold higher than the corresponding control (empty bait vector) are shown in bold.

(D and E) Gtr1-TORC1 interaction, but not Gtr1^{GTP}-TORC1 interaction, is sensitive to leucine starvation. Tco89-TAP (D) or Kog1-TAP (E) were precipitated from extracts prepared from wild-type cells coexpressing GST-Gtr1 or GST-Gtr1^{GTP}. Cells were either harvested in exponential growth phase prior to (+) or following a 60 min period of leucine starvation (–). Cell lysates (Input) and TAP pull-down fractions were subjected to SDS-PAGE, and immunoblots were probed with anti-GST or anti-protein A (anti-TAP) antibodies as indicated.

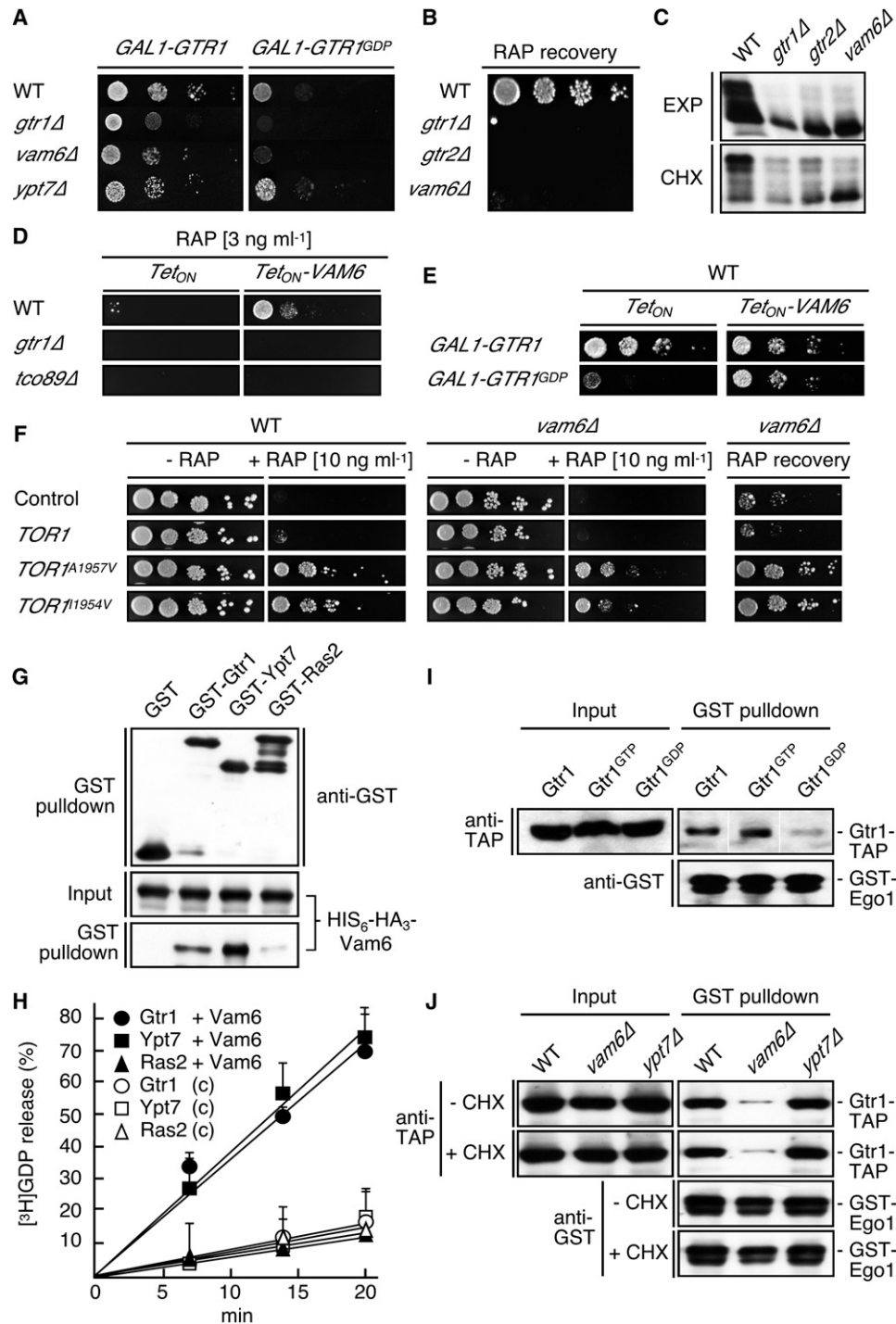


Figure 5. Vam6 Is a Gtr1 Nucleotide Exchange Factor

(A) Overproduction of Gtr1^{GDP} results in a synthetic growth defect when combined with *gtr1Δ* or *vam6Δ*, but not when combined with *ypt7Δ*. Indicated strains expressing *GTR1* or *GTR1^{GDP}* from the galactose-inducible *GAL1* promoter were grown overnight to exponential phase and spotted on galactose-containing plates.

(B and C) Loss of Vam6, like loss of the EGOC subunits Gtr1 and Gtr2, causes a defect in recovery from a rapamycin-induced growth arrest (B) and decreases TORC1 activity (C). For experimental details, see Figure 1A.

(D) Overproduction of Vam6 from a doxycycline-inducible *Tet_{ON}* promoter renders wild-type cells, but not *gtr1Δ* or *tco89Δ* strains, resistant to low rapamycin concentrations. Strains carrying the indicated plasmids were grown overnight to exponential phase and spotted (in serial 10-fold dilutions) on plates containing 5 μg ml⁻¹ doxycycline and 3 ng ml⁻¹ rapamycin.

activation in yeast (Figure 3B). In line with this interpretation, we found TORC1 to be reversibly inactivated in response to leucine (and less pronouncedly in response to lysine or histidine) deprivation (Figure 3C). Notably, inhibition of TORC1 activity following leucine starvation could be partially suppressed by either treatment of cells with cycloheximide or overproduction of Gtr1^{GTP} (Figures 3C and 3D); reactivation of TORC1 by readdition of leucine depended on Gtr1 but was abolished in the presence of Gtr1^{GDP} (Figure 3D). Because overproduction of Gtr1^{GTP} was unable to reverse TORC1 inactivation following NH₄⁺ starvation (Figure S3), we infer from this data set that Gtr1, within an intact EGO complex, likely mediates at least part of the amino acid signaling to TORC1.

Gtr1, Preferentially in Its GTP-Bound Form, Physically Interacts with TORC1

To examine whether Gtr1 may directly interact with TORC1, we coexpressed C terminally TAP-tagged versions of the TORC1 subunits Tco89 or Kog1 (the yeast homolog of mammalian raptor; Loewith et al., 2002) and different GST-tagged Gtr1 variants. Using coimmunoprecipitation assays, we were able to demonstrate that both TORC1 subunits specifically and preferentially interacted with GTP-bound Gtr1, whereas the corresponding interactions with Gtr1^{GDP} were close to background levels (Figures 4A and 4B). Employing a membrane-based two-hybrid system, we confirmed that Gtr1 and, more efficiently, Gtr1^{GTP}, but not Gtr1^{GDP}, specifically associated with Tco89 as well as with the EGO complex subunit Ego1 (Figure 4C). Given the role of Gtr1 in mediating an amino acid signal to TORC1, we also evaluated whether amino acids regulate the interaction between Gtr1 and TORC1. We found that the Gtr1-TORC1 interaction was sensitive to leucine starvation, whereas Gtr1^{GTP} remained associated with TORC1 under the same conditions (Figures 4D and 4E).

Vam6 Is a Gtr1 Nucleotide Exchange Factor

Our findings above indicate that Gtr1 controls TORC1 function and that amino acid signals may impinge on this process by

dictating the nucleotide-binding status of Gtr1. Accurate experimental determination of the Gtr1 nucleotide-binding status in vivo is not straightforward because Gtr1 and Gtr2 appear to be asymmetrically loaded with guanine nucleotides within heterodimeric complexes. To corroborate our model by alternative means, we therefore sought to isolate the Gtr1 GEF, which would allow us to more specifically modulate the nucleotide-binding status of Gtr1 within cells. Based on our finding that overproduction of Gtr1^{GDP} was semidominant with respect to growth inhibition, we reasoned that loss of the Gtr1 GEF, like loss of Gtr1 (Figure 5A), should yield a strong synthetic growth defect when combined with a construct that allows accumulation of Gtr1^{GDP}. Consequently, we carried out a systematic, genome-wide synthetic dosage lethal (SDL) screen (Measday et al., 2005) in which we overproduced Gtr1^{GDP} and tracked down corresponding synthetic growth defects. The two strongest positive hits in this screen included *gtr1Δ*—confirming the validity of our screening procedure—and *vam6Δ* (Figure 5A). The conserved Vam6 protein is a subunit of the homotypic fusion and vacuole protein sorting (HOPS/class C-Vps) complex in yeast (Caplan et al., 2001; Ostrowicz et al., 2008), which has recently also been suggested to control, via an unknown mechanism, TORC1 signaling in response to amino acids (Zurita-Martinez et al., 2007). Intriguingly, the HOPS complex is thought to facilitate the transition from tethering to *trans*-SNARE pairing during fusion at the vacuole, in part by Vam6-mediated nucleotide exchange on Ypt7, which is homologous to the mammalian Rab-7 GTPase (Wurmser et al., 2000). Combined with our data above (and the fact that Vam6, but not Ypt7, is required for cells to grow in the presence of elevated Gtr1^{GDP} levels; Figure 5A), this led us to speculate that Vam6 may have a dual role in activation of both Ypt7 and Gtr1.

In accordance with a model in which Vam6 activates Gtr1, we found that loss of Vam6, like loss of Gtr1 (or other EGOC subunits), resulted in a defect in recovery from rapamycin-induced growth arrest, constitutively reduced TORC1 activity, and reduced cycloheximide-induced TORC1 hyperactivation (Figures 5B and 5C). Moreover, overproduction of Vam6

(E) Overproduction of Vam6 suppresses the semidominant growth defect conferred by overproduction of Gtr1^{GDP}. Wild-type cells expressing *GTR1* or *GTR1^{GDP}* under the control of the galactose-inducible *GAL1* promoter were transformed with either an empty vector or a vector that expresses *VAM6* from the doxycycline-inducible *Tet_{ON}* promoter, grown overnight to exponential phase, and spotted (in serial 10-fold dilutions) on galactose- and doxycycline-containing plates.

(F) Expression of hyperactive *TOR1^{A1957V}* or *TOR1^{I1954V}* alleles renders wild-type and *vam6Δ* cells resistant to low rapamycin concentrations and enables *vam6Δ* cells to recover from a rapamycin-induced growth arrest. Wild-type and *vam6Δ* strains were transformed with an empty vector or vectors that express *TOR1*, *TOR1^{A1957V}*, or *TOR1^{I1954V}* from their endogenous promoter and were spotted on plates containing, or not, 10 ng ml⁻¹ rapamycin and/or assayed for their ability to recover from a rapamycin-induced growth arrest as in Figure 1A.

(G) Gtr1 interacts with Vam6. Equal amounts of recombinant GST-Gtr1, GST-Ypt7, GST-Ras2, and GST alone, preloaded on glutathione-sepharose 4B resin, were incubated with extracts prepared from wild-type cells expressing HIS₆-HA₃-Vam6. Cell lysate (Input) and GST pull-down fractions were then subjected to SDS-PAGE, and immunoblots were probed with anti-GST (top panel) or anti-HA (two lower panels) antibodies.

(H) Vam6 is a nucleotide exchange factor for Gtr1 and Ypt7, but not for Ras2. Recombinant GST-tagged GTP-binding proteins (i.e., 20 pMol of Gtr1 (●, ○), Ypt7 (■, □), or Ras2 (▲, △) were preloaded with [³H]GDP and incubated in the presence of HIS₆-HA₃-Vam6 (Vam6, filled symbols) or HIS₆-HA₃ (control [c], open symbols) that were purified from exponentially growing yeast. The time course of [³H]GDP release is shown (in percent). Prior heat inactivation of Vam6 (10 min at 95°) reduced its GDP release activity toward Gtr1 to background levels (data not shown). Experiments were done in triplicate and expressed as mean ± SD.

(I) Gtr1 and Gtr1^{GTP} interact strongly with Ego1. GST-Ego1 was precipitated from extracts prepared from wild-type cells coexpressing Gtr1-TAP, Gtr1^{GTP}-TAP, or Gtr1^{GDP}-TAP. Cell lysates (Input) and GST pull-down fractions were subjected to SDS-PAGE, and immunoblots were probed with anti-GST or anti-protein A (anti-TAP) antibodies as indicated.

(J) Loss of Vam6, but not loss of Ypt7, abolishes the interaction between Gtr1 and Ego1. GST-Ego1 was precipitated from extracts prepared from wild-type, *vam6Δ*, or *ypt7Δ* cells coexpressing Gtr1-TAP. Cells were harvested in exponential growth phase prior to (– CHX) or after a 30 min cycloheximide (25 μg ml⁻¹) treatment (+ CHX). For details, see Figures 4A and 4B.

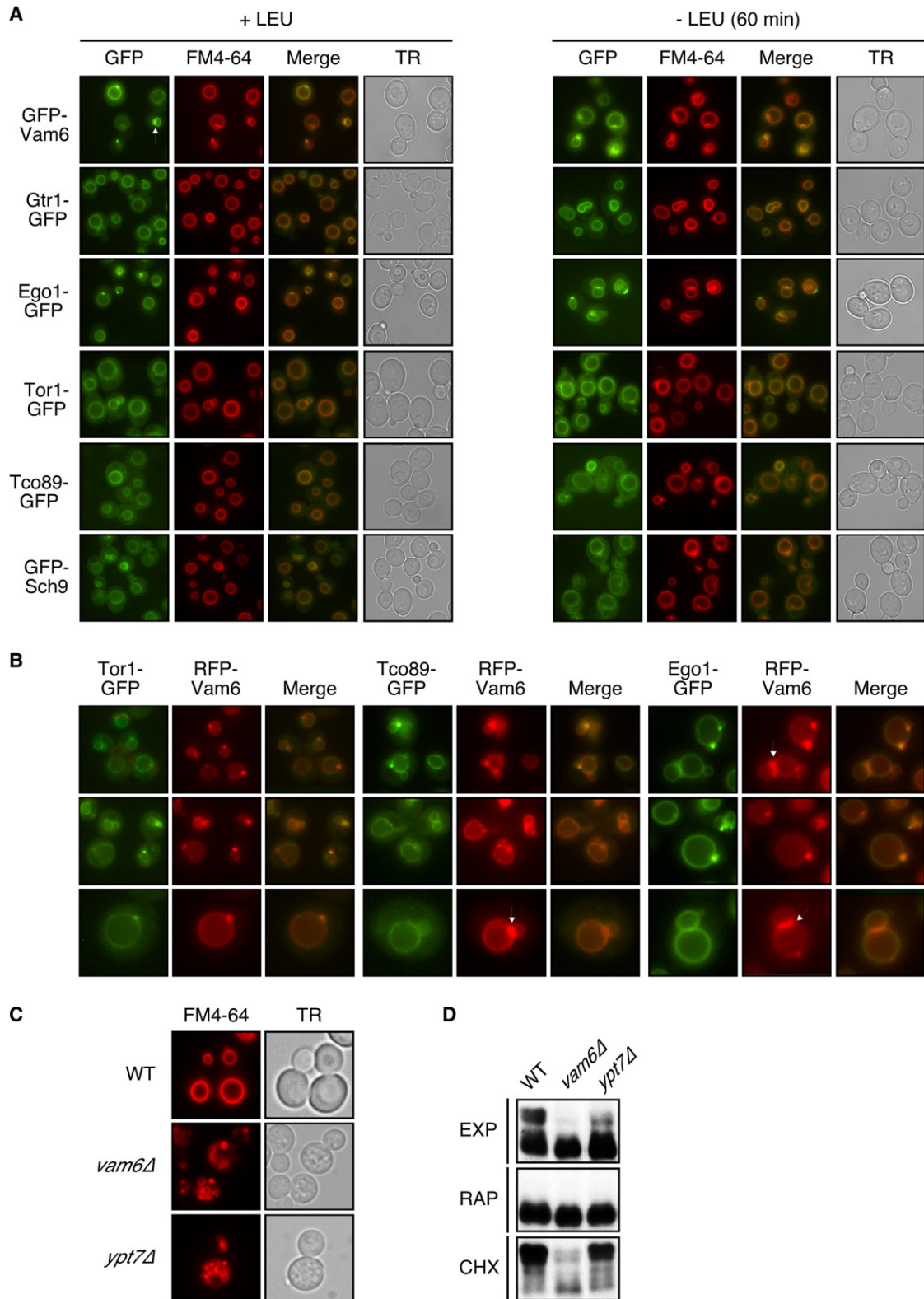


Figure 6. TORC1 and EGO Complex Subunits Colocalize with Vam6 at Vacuolar and Endosomal Membranes

(A) GFP-Vam6, Gtr1-GFP, Ego1-GFP, Tor1-GFP, Tco89-GFP, and GFP-Sch9 all localize to the limiting membrane of the vacuole. Leucine (*leu2Δ*) auxotrophic wild-type cells expressing functional versions of GFP-fusion proteins either from a plasmid (GFP-Sch9) or from the endogenous chromosomal locus (Gtr1-GFP,

rendered wild-type cells, but not *gtr1Δ* or *tco89Δ* cells, resistant to low rapamycin concentrations (Figure 5D) and suppressed the semidominant growth defect resulting from Gtr1^{GDP} overproduction (Figure 5E). Finally, expression of hyperactive TOR1^{A1957V} and TOR1^{I1954V} rendered rapamycin-sensitive *vam6Δ* cells as resistant to low rapamycin concentrations as wild-type cells and enabled *vam6Δ* cells to recover from a rapamycin-induced growth arrest (Figure 5F). Because these genetic experiments place Vam6 upstream of Gtr1, we determined whether Vam6 could interact with Gtr1. We found that Vam6 coprecipitated well with both GST-Gtr1 and the positive control GST-Ypt7; only very weakly with GST-Ras2, which is very closely related to Gtr1; and not at all with GST alone (Figure 5G). Further, Vam6 stimulated GDP release both from its known target Ypt7 and from Gtr1, but not from Ras2 (Figure 5H), indicating that Vam6 acts as a GEF for Gtr1 in vitro. Addition of nucleotide-free GST-Gtr2 did not significantly alter the Vam6-mediated GDP release on Gtr1 (data not shown). To explore whether Vam6 functions as a GEF for Gtr1 in vivo, we made use of the fact that Gtr1^{GTP}-TAP, but not Gtr1^{GDP}-TAP, specifically coprecipitates with GST-Ego1 (Figure 5I); hence, the level of Ego1-associated Gtr1 can be used to estimate the relative amount of Gtr1^{GTP} within cells. Using this assay, we noted that loss of Vam6, but not loss of Ypt7, severely reduced the interaction between Gtr1 and Ego1 (both prior to and following cycloheximide treatment), indicating that Vam6 also functions as a GEF for Gtr1 in vivo (Figure 5J). Taken together, our results show that Vam6 regulates the nucleotide-binding status of Gtr1 both in vitro and in vivo and suggest that Vam6 may integrate amino acid signals to coordinate the control of TORC1 activity and vacuolar fusion events.

TORC1 and EGO Complex Subunits Largely Colocalize with Vam6 at the Vacuolar Membrane

In line with previous reports (Araki et al., 2005; Gao and Kaiser, 2006; Jorgensen et al., 2004; Nakamura et al., 1997; Reinke et al., 2004; Sturgill et al., 2008; Urban et al., 2007), we found that GFP-Vam6, Gtr1-GFP, Ego1-GFP, Tor1-GFP, Tco89-GFP, and GFP-Sch9 all localized predominantly at the vacuolar membrane in both exponentially growing and leucine-starved cells (Figure 6A). In addition, all GFP fusion proteins (except for GFP-Sch9) occasionally localized to punctate structures adjacent to the vacuole, which in the case of Vam6, Gtr1, and Ego1 may correspond to late endosomes (Cabrera et al., 2009; Gao and Kaiser, 2006). Notably, RFP-Vam6 colocalized with TORC1 and EGO complex subunits (i.e., with Tor1-GFP, Tco89-GFP, and Ego1-GFP) at the vacuolar and the presumed endosomal membranes (Figure 6B). Unlike Tor1-GFP, Tco89-

GFP, or Ego1-GFP, however, RFP-Vam6 (and similarly GFP-Vam6) was found specifically enriched at the contact sites between two adjacent vacuoles (Figures 6A and 6B), which underscores the previously described role of Vam6 in vacuolar fusion. To exclude the possibility that the reduced TORC1 activity observed in *vam6Δ* cells is simply a result of their abnormal, fragmented vacuolar morphology (Figure 6C), we also measured TORC1 activity in *ypt7Δ* cells, which exhibit similarly fragmented vacuoles as *vam6Δ* cells (Figure 6C). The corresponding experiments clearly showed that only loss of Vam6, but not loss of Ypt7, seriously reduced TORC1 activity and prevented CHX-induced TORC1 activation, indicating that vacuolar fragmentation per se does not have an impact on TORC1 activity control (Figure 6D). Thus, together with the observation that the EGO complex is not required for vacuolar fusion (Dubouloz et al., 2005), it appears that Vam6 regulates two effector branches: one to control vacuolar fusion (via Ypt7) and one to control TORC1 (via the EGO complex subunit Gtr1).

Conclusions

In conclusion, we have demonstrated that Vam6 activates Gtr1 and that Gtr1 and, by extension, the EGO complex act upstream of TORC1 to regulate its activity. Thus, our present work extends and confirms the evolutionarily conserved importance of Rag family GTPases as upstream regulators of TORC1. However, we were surprised that, in contrast to the situation in mammalian cells in which GTP-locked RagB apparently completely uncouples mTORC1 from signals derived from leucine availability (Sancak et al., 2008), GTP-locked Gtr1 had only a partial effect. Perhaps in yeast, there are other signaling routes by which amino acid availability is communicated to TORC1. Alternatively, it is possible that GTP loading must be followed by hydrolysis for Gtr1 to fully activate TORC1 in yeast; this later reaction would not be possible with the GTP-locked Gtr1 protein. In this context, it is also worth noting that mammalian cells express, due to alternative mRNA splicing, two RagB isoforms (RagB/RagB^s and RagB^l), which are structurally significantly different from each other (Schürmann et al., 1995). It would, therefore, be of interest to also study the role of RagB^l in mTORC1 regulation.

Finally, our finding that leucine deprivation did not appreciably affect the localization of GFP-Vam6, Gtr1-GFP, Ego1-GFP, Tor1-GFP, Tco89-GFP, and GFP-Sch9 in yeast again contrasts with observations made in mammalian cells in which the Rag GTPases were proposed to mediate amino-acid-induced relocalization of mTOR within the endomembrane system to a compartment that contains the TORC1 activator Rheb (Sancak et al., 2008). Though this aspect of TORC1 regulation may have diverged during evolution—the budding yeast homolog of

Ego1-GFP, Tor1-GFP, and Tco89-GFP) or corresponding *leu2Δ vam6Δ* cells expressing functional GFP-Vam6 from a plasmid were labeled with the vacuolar membrane fluorescent dye FM4-64, and the localization of GFP fusion proteins was compared to FM4-64 staining by fluorescence microscopy. Cells were analyzed during exponential growth in leucine-containing SC medium (+ LEU) or following a 60 min leucine starvation period on SC medium lacking leucine (– LEU). TR, transmission.

(B) RFP-Vam6 colocalizes with Tor1-GFP, Tco89-GFP, and Ego1-GFP at vacuolar and presumed endosomal (green/red dots) membranes. In addition, RFP-Vam6 is specifically enriched at vacuolar fusion sites (arrows; see also GFP-Vam6 in A). Functional RFP-Vam6 was expressed from a plasmid. For details, see (A).

(C) Loss of Vam6 or Ypt7 results in fragmentation of vacuoles. Exponentially growing wild-type, *vam6Δ*, and *ypt7Δ* strains were stained with FM4-64 and analyzed as in (A). TR, transmission.

(D) Ypt7 is not required for TORC1 activation by cycloheximide (CHX). For experimental details, see Figure 1A.

Rheb is unlikely to perform a similar function in TORC1 activation (De Virgilio and Loewith, 2006a)—it will be most interesting to study whether Vam6 homologs are implicated in the control of TORC1 function by mediating nucleotide exchange on Rag GTPases in higher eukaryotes.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Plasmids

The *S. cerevisiae* strains used in this study are listed in Table S1. Unless stated otherwise, prototrophic strains were pregrown overnight in synthetic medium without amino acids (SD; 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% glucose). Before each experiment, cells were diluted to an OD₆₀₀ of 0.2 in YPD medium supplemented with 0.2% of glutamine and grown until they reached an OD₆₀₀ of 0.8. For amino acid deprivation experiments, strains that were specifically auxotrophic for one amino acid were grown to an OD₆₀₀ of 0.8 on complete synthetic medium (SC; i.e., SD plus all amino acids), washed twice, and resuspended in starvation medium (SC-Leu, SC-Lys, or SC-His lacking specifically one relevant amino acid). For nitrogen starvation, prototrophic cells were grown to an OD₆₀₀ of 1.0 on SD (without amino acids), washed twice, and resuspended in the same medium without ammonium sulfate. Low-quality nitrogen media were SD media, which contained 10 mM urea or proline instead of ammonium sulfate. The plasmids used in this study are listed in Table S2.

Sch9 Phosphorylation Analyses

To analyze Sch9^{T570A}-HA₅ C-terminal phosphorylation, we used the chemical fragmentation analysis as described previously (Urban et al., 2007; Wanke et al., 2008). For quantifications of Sch9 phosphorylation, NTCB-cleaved extracts were separated by 7.5% SDS-PAGE followed by immunoblotting with anti-HA antibody 12CA5. The anti-HA antibody was detected with far-red fluorescent Alexa Fluor 680 dye-labeled secondary anti-mouse antibody (Invitrogen, A21057), and fluorescence intensity was measured using the Odyssey Infrared Imaging System (LI-COR).

GDP Release Assay

To assay GDP release, 20 pmol of bacterially expressed GST-tagged G-proteins (i.e., GST-Gtr1, GST-Ypt7, and GST-Ras2) were preloaded by incubating with 40 pmol 5',8'-[³H]GDP (32.8 Ci mmol⁻¹; NEN) in preload buffer (20 mM HEPES [pH 7.2], 20 mM KOAc, 1 mM DTT, 5 mM EDTA, 1 μg μl⁻¹ BSA) for 15 min at 30°C as described (Jones et al., 2000). At the end of the incubation, samples were placed on ice, and MgCl₂ was added to 10 mM. Reactions were carried out in 50 μl containing 20 mM HEPES (pH 7.2), 5 mM Mg(OAc)₂, 0.5 mM GTP, 0.5 mM GDP, 1 mM DTT, 0.4 mg ml⁻¹ BSA, and HIS₆-HA₃-Vam6 or HIS₆-HA₃, both purified from exponentially growing yeast. Exchange reactions were initiated by the addition of 10 pmol of the preloaded G proteins. Incubations were carried out at 30°C for varying periods of time, as noted. At intervals, 5 μl samples were removed, added to 3 ml of ice-cold wash buffer (20 mM Tris-HCl [pH 7.5], 20 mM NaCl, 5 mM MgCl₂, 1 mM DTT), and filtered through nitrocellulose filters, which were then washed twice with 3 ml of ice cold wash buffer. Radioactivity bound to filters was quantified by liquid scintillation spectrometry using Filttron-X (National Diagnostics, LS-201) scintillation fluid. In all experiments, initial values were ~2–4 × 10³ dpm μl⁻¹.

Miscellaneous

Coimmunoprecipitation experiments were essentially done as described (Dubouloz et al., 2005; Loewith et al., 2002). Tco89-TAP and Kog1-TAP were purified from lysates with magnetic beads (Invitrogen) that were preloaded overnight in PBS with 1 mg ml⁻¹ IgG (Sigma) in the presence of 1 M (NH₄)₂SO₄ to facilitate the binding. For quantifications of free amino acids, cells (about 10 OD₆₀₀) were harvested by filtration, washed three times, and resuspended in distilled water. Following boiling (15 min) and centrifugation (10 min at 13,000 rpm), free amino acids were quantified in the supernatant by pulsed electrochemical detection after separation by anion exchange chromatography with an AAA-direct Dionex Amino Acid Analyzer, using a sodium acetate gradient to increase the ionic strength.

SUPPLEMENTAL DATA

Supplemental Data include two tables and three figures

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