Endolysosomal Membrane Trafficking Complexes Drive Nutrient-Dependent TORC1 Signaling to Control Cell Growth in *Saccharomyces cerevisiae*

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ABSTRACT The rapamycin-sensitive and endomembrane-associated TORC1 pathway controls cell growth in response to nutrients in eukaryotes. Mutations in class C Vps (Vps-C) complexes are synthetically lethal with *tor1* mutations and confer rapamycin hypersensitivity in *Saccharomyces cerevisiae*, suggesting a role for these complexes in TORC1 signaling. Vps-C complexes are required for vesicular trafficking and fusion and comprise four distinct complexes: HOPS and CORVET and their minor intermediaries (*i*)-CORVET and *i*-HOPS. We show that at least one Vps-C complex is required to promote TORC1 activity, with the HOPS complex having the greatest input. The *vps-c* mutants fail to recover from rapamycin-induced growth arrest and show low levels of TORC1 activity. TORC1 promotes cell growth via Sch9, a $p70^{S6}$ kinase ortholog. Constitutively active *SCH9* or hyperactive *TOR1* alleles restored rapamycin recovery and TORC1 activity of *vps-c* mutants, supporting a role for the Vps-C complexes upstream of TORC1. The EGO GTPase complex **E**xit from $\mathbf{G_0}$ **C**omplex (EGOC) and its homologous Rag-GTPase complex convey amino acid signals to TORC1 in yeast and mammals, respectively. Expression of the activated EGOC GTPase subunits $Gtr1^{GTP}$ and $Gtr2^{GDP}$ partially suppressed *vps-c* mutant rapamycin recovery defects, and this suppression was enhanced by increased amino acid concentrations. Moreover, *vps-c* mutations disrupted EGOC–TORC1 interactions. TORC1 defects were more severe for *vps-c* mutants than those observed in EGOC mutants. Taken together, our results support a model in which distinct endolysosomal trafficking Vps-C complexes promote rapamycin-sensitive TORC1 activity via multiple inputs, one of which involves maintenance of amino acid homeostasis that is sensed and transmitted to TORC1 via interactions with EGOC.

THE **T**arget **o**f **r**apamycin (TOR) kinases are conserved across eukaryotes and orchestrate myriad cellular processes to control growth in response to nutrients and environmental signals. The Tor kinases form two evolutionarily conserved multi-protein complexes known as TORC1 (**Tor c**omplex) and TORC2. TORC1 is sensitive to the immunosuppressive and antiproliferative drug rapamycin, and in *Saccharomyces cerevisiae* is populated by Tor1 (or, to a lesser extent, Tor2), Kog1, Lst8, and Tco89. TORC1 controls cell growth when nutrients such as amino acids are abundant and serves to maintain robust nutrient transport, ribosome biogenesis, and protein synthesis and concomitantly inhibits autophagy (Heitman *et al.* 1991; Cardenas *et al.* 1999; Powers and Walter 1999; Loewith *et al.* 2002; Reinke *et al.* 2004; Wullschleger *et al.* 2006; Loewith and Hall 2011). TORC2 is rapamycin-insensitive and composed of Tor2, Lst8, Bit61/Bit2, Avo1, Avo2, and Avo3; TORC2 controls spatial growth via regulation of actin cytoskeleton polarization (Schmidt *et al.* 1996; Loewith *et al.* 2002). Amino acid levels are signaled to yeast TORC1, at least in part, via the leucyl-tRNA synthetase, which binds and influences the guanine nucleotide state of components of the Rag GTPase EGOC (**E**xit from $\mathbf{G_0}$ **C**omplex), formed by Ego1, Ego3, Gtr1, and Gtr2 (Bonfils *et al.* 2012). Specifically, the presence of amino acids promotes charging of Gtr1 and Gtr2 to their active GTP- and GDP-bound states, respectively, allowing binding and activation of TORC1 (Binda *et al.* 2009).

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Activated TORC1 in turn controls growth via phosphorylation of two main downstream effector branches: the Tap42 phosphatase complex and the protein kinase Sch9 (Di Como and Arndt 1996; Duvel *et al.* 2003; Urban *et al.* 2007).

To identify Tor1-specific roles in TORC1 activity, we screened for genes that, when mutated in combination with *tor1* mutation, result in a synthetically lethal or synthetic fitness defect (Zurita-Martinez *et al.* 2007). In addition to identification of EGOC components, the screen also identified genes involved in vacuolar protein sorting (Vps) and ribosomal and mitochondrial function. In particular, this screen identified all of the genes encoding components of the multi-subunit class C Vps (Vps-C) HOPS (**Ho**motypic vacuolar fusion and **p**rotein **s**orting) and CORVET (class **C** c**or**e **v**acuole-**e**ndosome **t**ransport) complexes. The HOPS complex acts as a tether to mediate late endosome-vacuole and vacuole-vacuole fusion via cooperation with the Ypt7 Rab GTPase, followed by a membrane fusion event mediated by SNARE proteins (Srivastava *et al.* 2000; Peterson and Emr 2001; Stroupe *et al.* 2006; Hickey *et al.* 2009; Nickerson *et al.* 2009; Ostrowicz *et al.* 2010; Epp *et al.* 2011). The CORVET complex interacts with the Rab GTPase Vps21 to mediate early-to-late endosomal trafficking and retrograde trafficking from the vacuole (Peplowska *et al.* 2007; Nickerson *et al.* 2009). Both the HOPS and the CORVET complexes are highly conserved from yeasts to metazoans (reviewed in Nickerson *et al.* 2009).

The *vps-c* mutants display highly fragmented vacuoles, which are the major amino acid storage reservoir in yeast, and show reduced levels of amino acids (Banta *et al.* 1988; Kitamoto *et al.* 1988b; Raymond *et al.* 1992). The *vps-c* mutants display severe rapamycin sensitivity and recovery defects and fail to recover from starvation-imposed arrest (Zurita-Martinez *et al.* 2007). Based on previous studies, TORC1 does not appear to execute a regulatory influence over the functions of the Vps-C complex (Zurita-Martinez *et al.* 2007).

EGOC, TORC1, and the downstream effector Sch9 are responsible for sensing and responding to amino acids, and all reside on the vacuolar membrane, congruent with the major role of the vacuole in amino acid storage (Cardenas and Heitman 1995; Huh *et al.* 2003; Wedaman *et al.* 2003; Jorgensen *et al.* 2004; Araki *et al.* 2005; Dubouloz *et al.* 2005; Gao and Kaiser 2006; Urban *et al.* 2007; Sturgill *et al.* 2008; Berchtold and Walther 2009; Binda *et al.* 2009). While mammalian (m)TORC1 is recruited to the lysosome (vacuole equivalent) and activated in response to amino acids (Sancak *et al.* 2008, 2010), yeast TORC1 is constitutively located on the vacuole (Binda *et al.* 2009) except under conditions of heat shock, whereupon TORC1 is sequestered into stress granules (Takahara and Maeda 2012). In mammals, the lysosomal membrane is also the location for sensing amino acids by a mechanism that involves conformational regulation of the vacuolar (v)-ATPase, transponded to mTORC1 via the ragulator complex (analogous to EGOC) (Zoncu *et al.* 2011). Thus, efficient function of TORC1, both in yeasts and in mammals, may also depend on the integrity of the endolysosomal membrane acting as a scaffold platform (reviewed in Rohde *et al.* 2008; Flinn and Backer 2010).

Here, we establish a role for the endomembrane vesicular trafficking system in the control of TORC1 activity. First, we demonstrate that the Vps-C HOPS, CORVET, and intermediary (*i*)-CORVET complexes all contribute to promote TORC1 activity. Epistasis analyses utilizing a hyperactive *TOR1* or *SCH9*, *GTR1*, and *GTR2* constitutively activated alleles show that the Vps-C complexes function to modulate TORC1 activity, in part, upstream of EGOC. Increasing amino acid concentrations partially suppressed the rapamycin sensitivity and recovery defects of *vps-c* mutants, as did conditions that increase cellular acidity that may affect amino acid homeostasis by modulating vacuolar amino acid transport (Russnak *et al.* 2001). Furthermore, *vps-c* mutation disrupted EGOC–TORC1 interactions that are important for amino acid-induced TORC1 activation. Our results demonstrate a role for distinct Vps-C complexes in maintaining amino acid homeostasis to promote TORC1 signaling and thereby to activate cell growth in a manner likely to be conserved in multicellular eukaryotes.

Materials and Methods

Strains, media, and growth conditions

Media consisted of yeast extract peptone dextrose (YPD), synthetic dextrose (SD), synthetic complete (SC), or variations of either synthetic medium with various amino acids and supplements omitted or added to complement auxotrophies or selected for plasmid maintenance (Sherman *et al.* 1974). When required, media were supplemented with rapamycin (stock was dissolved in 95% w/v ethanol/5% w/v tween 20; LC Laboratories), 100 μg/ml nourseothricin (ClonNAT, Werner BioAgents), 200 μg/ml G418 (AG Scientific), 200 μg/ml hygromycin (Calbiochem), or 1.5 μM natamycin (Sigma). Controlled media pH was buffered with 50 mM sodium phosphate solutions (NaH_2PO_4 and Na_2HPO_4). All cell cultures were incubated at 30°.

All strains used in this study were derived from BY4742 (Brachmann *et al.* 1998) and RL58-1a (Wullschleger *et al.* 2005) and are listed in Supporting Information, Table S1. Strains containing single-gene disruptions involving the kanMX4 cassette were typically obtained from the *Saccharomyces* Genome Deletion Project (distributed by Invitrogen, Carlsbad, CA) (Giaever *et al.* 2002). All other gene deletions were performed by the PCR-mediated overlap strategy using dominant drug markers and oligonucleotides listed in Table S3, as previously described (Wach *et al.* 1994; Goldstein and McCusker 1999). Strains expressing an empty vector or *VMA1* or *VPH2* from the *GPD1* promoter were constructed by integration into a neutral, intergenic region of chromosome 1 (199,456–199,457) of *Not*I-digested pAG306-GPD chromosome 1, pAG306-GPD-*VMA1* chromosome 1, or pAG306-GPD-*VPH2* chromosome 1, as described (Hughes and Gottschling 2012). Yeast transformations were performed by the lithium acetate transformation procedure (Gietz *et al.* 1995). All gene disruptions were confirmed by PCR.

Plasmid construction

All plasmids and their sources used in this study are listed in Table S2. Plasmid pJK28 was constructed as follows. Genomic *LEU2* plus 1 kb of 5′ upstream and 375 bp of 3′ downstream sequence were PCR-amplified (oligos JK172, JK173), cloned into *Sac*I/*Xma*I-digested pMKV002 by gap repair (Oldenburg *et al.* 1997). To construct pPC10, HA_3-*SCH9* was excised from pRS315-HA_3-*SCH9* following *Xba*I/*Sal*I digestion and ligated into *Xba*I/*Sal*I-digested pRS413. To construct pPC8a, a $GTR2^{GDP}$-containing *Pvu*II fragment was excised from pL263 (Wang *et al.* 2009) and ligated into pCR-Blunt II-TOPO (Invitrogen), from which the $GTR2^{GDP}$-containing *Eco*RI fragment was excised and ligated into *Eco*RI-digested pL112 (Nakashima *et al.* 1999). pPC9a was similarly constructed by ligating the *GTR2*-containing *Pvu*II fragment from pL140 (Wang *et al.* 2009) into pCR-Blunt II-TOPO, from which the *GTR2*-containing *Eco*RI fragment was isolated and ligated into the *Eco*RI site of pL63 (Nakashima *et al.* 1996). $GTR1^{GTP}$ and $GTR2^{GDP}$ and *GTR1* and *GTR2* are in the same orientation in pPC8a and pPC9a, respectively. All plasmids were confirmed by restriction enzyme and PCR analyses.

TORC1 kinase assays

For analysis of Sch9 phosphorylation, protein extracts were prepared as previously described (Urban *et al.* 2007) with minor modifications. Briefly, cells were typically grown to an optical density (OD_{600nm} wavelength) of 1 in 20 ml YPD or SC media + 2 mg/ml glutamine with pertinent amino acids omitted to ensure plasmid maintenance. Cultures were divided in half and treated without or with 200 nM rapamycin and incubated for a further 30 min. Incubation was terminated by the addition of 6% (v/v) trichloroacetic acid (TCA) to the culture. TCA cell pellets were harvested following incubation on ice for at least 30 min, and pellets were washed twice with acetone. Protein was extracted in 150 μl of urea buffer containing phosphatase inhibitors [50 mM Tris (pH 7.5), 5 mM EDTA, 6 M urea, 1% (w/v) SDS, 0.5 mM PMSF, 10 mM NaF, 10 mM NaN_3, 10 mM β-glycerophosphate, 1 mM Na pyrophosphate, 10 mM Na orthovanadate] using glass beads in a FastPrep machine (MP Biomedicals), followed by heating for 10 min at 65°. To monitor Sch9 phosphorylation using the anti-phospho-Thr737-Sch9 antibody, 50 μg of protein extract was separated via 7.5% SDS-PAGE (BioRad) and immunoblotted using standard procedures. Analysis of $Sch9^{T570A}$-HA_5 C-terminal phosphorylation by chemical fragmentation was performed as described previously (Urban *et al.* 2007). Signal was detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare). Quantification was performed with GeneSnap 7.12.06 imaging software of membranes and GeneTools 4.03 image analysis software (Syngene).

Protein extraction and immunoprecipitation

For protein immunoprecipitation, cell extracts were prepared by lysis with beads in lysis buffer [PBS (GIBCO) containing, 10% glycerol, 0.5% tween 20, 10 mM NaF, 10 mM NaN_3, 10 mM NaP_3O_7, 10 mM ρ-nitrophenylphosphate, 10 mM β-glycerophosphate, 1 mM PMSF, and protease inhibitor cocktail (Roche)]. Cell extracts were cleared by centrifugation at 14,000 × *g* for 10 min, protein concentration was determined by employing the BioRad protein quantification reagent, and 3–4 mg of protein was immunoprecipitated with a 50-μl 1:1 bead/volume suspension of immobilized FLAG antibody (Sigma). Immunoprecipitates were incubated for 1.5 hr with continued mixing at 4°, collected by brief centrifugation, and washed four times with lysis buffer. Immunoprecipitated proteins were eluted by incubating the washed beads in 60 μl of lysis buffer containing 200 μg/ml FLAG peptide (Sigma). Immunoprecipitated proteins were separated by SDS-PAGE and identified by Western blot analysis.

Antibodies employed in this study

The polyclonal anti-phospho-Thr737-Sch9 and anti-732-743-Sch9 antibodies were raised by injecting a rabbit with the peptide KFAGF(pT)FVDESAC (Sch9 sequence 732–743; New England Peptide). To separate the specific phospho-peptide antibody from the nonphosphorylated peptide antibodies, the antiserum was purified by several passes through an affinity column with immobilized nonphosphorylated peptide. The first eluate from the column contained a high titer of antibodies that efficiently reacted against the nonphosphorylated peptide while the phospho-peptide antibody was recovered from the last flow through of the column. The monoclonal anti-HA and anti-FLAG M2 antibodies were obtained from Babco and Sigma, respectively. The anti-Tor1 and FKBP12 antibodies were generated and characterized as described (Cardenas and Heitman 1995; Alarcon *et al.* 1996). ECL secondary mouse or rabbit antibodies conjugated with horseradish peroxidase were purchased from GE Healthcare.

Rapamycin Minimal Inhibitory Concentration (MIC) assays

Sensitivity of strains to rapamycin was determined in triplicate using MIC assays. Volumes of 20 μl from a 10× series of two-fold dilutions of rapamycin were aliquoted to 96-well microdilution plates. Working concentrations of rapamycin included 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0 nM. Strains were grown overnight in SC + 2 mg/ml glutamine. Cell density was determined using a hemocytometer, and cells were added to assay media at a density of ~1100 cfu/ml. A volume of 180 μl of the cell suspension was added to the rapamycin drug dilutions in wells (~200 cfu/well). Following 3 days of incubation at 30°, cells were resuspended in wells and OD_{600nM} readings were taken with a 96-well plate reader. MICs were defined as the concentration of rapamycin that inhibited strain growth ≥80% compared with the no-drug control.

Results

Distinct HOPS, CORVET, and intermediary i-CORVET complexes modulate TORC1 activity

Mutations of Vps-C HOPS complex components are synthetically lethal with *tor1* mutation and result in hypersensitivity

to rapamycin and a failure in growth recovery following rapamycin-induced cell cycle arrest (lanes 2–3 and 6–7 in Figure 1A) (Zurita-Martinez *et al.* 2007). These results are consistent with a role for the HOPS complex in TORC1 signaling. To determine if the HOPS complex regulates TORC1, we assessed the effect of HOPS complex disruption on TORC1 activity by monitoring the phosphorylation status of the major TORC1 substrate Sch9 with an antibody specific to a peptide encompassing phosphorylated Sch9-Thr737, a major residue targeted by the TORC1 kinase (Urban *et al.* 2007). A similar antibody was recently described (Takahara and Maeda 2012). Total Sch9 protein levels were monitored with an antibody specific to amino acids 732–743 of Sch9 (Figure S1). To ensure robust Sch9 signals for detection, we analyzed strains containing an HA_3-*SCH9* allele expressed from a centromeric plasmid.

All HOPS complex mutants showed a faster electrophoretic mobility of Sch9 and reduced levels (30–48%) of Sch9 phosphorylation compared with the wild type (WT) (lanes 1–3 and 6–7 in Figure 1B). TORC1 activity was also reduced in the EGOC *gtr1* mutant, which is known to compromise TORC1 activity (Binda *et al.* 2009). In validation of our phosphoantibody-based TORC1 kinase assay, similar results were obtained when Sch9 phosphorylation in the HOPS and *gtr1* mutants was monitored by an independent assay (Urban *et al.* 2007) (Figure S2). Moreover, phosphorylation of Sch9-Thr737 was completely abolished in all strains when exposed to rapamycin (Figure 1B). These results indicate that integrity of the HOPS complex is required for robust TORC1 kinase activity.

While Vps39 and Vps41 are specific subunits of the HOPS complex, the core Vps-C HOPS subunits (Pep3, Pep5, Vps16, and Vps33) also function as core components of the CORVET complex, together with the CORVET-specific components Vps3 and Vps8 and the Rab GTPase Vps21 (Peplowska *et al.* 2007; Ostrowicz *et al.* 2010) (Figure 1C). The HOPS complex functions in endosome-independent Golgi-to-vacuole, late-endosome-to-vacuole, autophagosome-to-vacuole, and vacuole-to-vacuole fusion events. The CORVET complex operates in fusing early endosomes and Golgi-derived vesicles to late endosomes and in retrograde vacuole-to-endosome traffic (reviewed in Nickerson *et al.* 2009) (Figure 6). Disruption of CORVET-specific subunits (Vps3, Vps8) also resulted in rapamycin sensitivity and TORC1 activity defects, although to a lesser degree than disruption of the analogous HOPS-specific subunits (Vps39, Vps41), suggesting a more important contribution of HOPS to TORC1 signaling (lanes 4–7 in Figure 1, A and B). In addition, disruption of the HOPS/CORVET shared core subunits Pep3 and Pep5 resulted in more severe defects in rapamycin sensitivity and recovery and somewhat reduced TORC1 activity compared with mutation of either CORVET- or HOPS-specific subunits (compare lanes 2–3 with 4–7 in Figure 1, A and B).

In addition to HOPS and CORVET, there is some evidence for minor occurrence of intermediary *i*-CORVET and *i*-HOPS complexes (Peplowska *et al.* 2007; Ostrowicz *et al.* 2010) (Figure 1C). However, the precise function of these complexes in endovesicular membrane trafficking remains to be determined. We considered two possible explanations as to why single *pep3* and *pep5* mutations confer greater defects on TORC1 activity and rapamycin sensitivity/recovery than disruption of HOPS-specific components. First, as shown above, the CORVET complex is also important for TORC1 activity. Second, Vps3 and Vps8 may partially compensate for Vsp39 and Vps41, respectively, as part of the hypothetical *i*-HOPS or the well-documented *i*-CORVET intermediary complexes (Figure 1C). To test these models, we compared the rapamycin sensitivity and recovery phenotypes and TORC1 activity of strains disrupted for all four possible HOPS and CORVET complexes (*vps3 vps39* and *vps8 vps41*) or containing only one intact complex: HOPS (*vps3 vps8*), CORVET (*vps39 vps41*), *i*-HOPS (*vps3 vps41*), or *i*-CORVET (*vps8 vps39*).

Remarkably, the *vps3 vps39* and *vps8 vps41* mutants disrupted for the four complexes and the *vps3 vps41* double mutant (only *i*-HOPS intact) showed as severe rapamycin sensitivity and recovery phenotypes as the core subunit *pep3* and *pep5* single mutants (compare lanes 8–9 and 11 with lanes 2–3 in Figure 1A). This result is in accord with limited evidence for the occurrence of the *i*-HOPS complex. The *vps8 vps39* double mutant containing an intact *i*-CORVET complex, which has been detected and suggested to modestly stimulate vacuole fusion (Peplowska *et al.* 2007; Ostrowicz *et al.* 2010), exhibits a degree of rapamycin recovery similar to the *vps39* single mutant (lanes 6 and 10 in Figure 1A). The *vps39 vps41* (intact CORVET) double mutant and *vps39* and *vps41* single mutants as well as the *vps3 vps8* (intact HOPS) double mutant and *vps3* single mutant had similar rapamycin recovery phenotypes, respectively, confirming that each pair of genes functions in a distinct complex (compare lanes 6–7 with lane 12 and lane 4 with lane 13 in Figure 1A). Single mutation of *vps3* and *vps8* resulted in slightly different rapamycin sensitivity phenotypes, suggesting that Vps8 and Vps3 could play additional CORVET-independent roles. Furthermore, the *vps3 vps8* double mutant with only an intact HOPS complex shows better rapamycin recovery compared to the *vps39 vps41* double mutant containing only an intact CORVET (lanes 12–13 in Figure 1A).

In general, all strains disrupted for Vps-C subunits showed reduced TORC1 activity compared with the WT strains. This effect was more marked in the *pep3* and *pep5* core subunit mutants and in the *vps3 vps39* and *vps8 vps41* double mutants disrupted for the four complexes (lanes 2–3, 8, and 11 in Figure 1B). In contrast, in the *vps3*, *vps8*, and *vps21* mutants affecting single complexes or in those retaining an intact CORVET (*vps39 vps41*) or HOPS (*vps3 vps8*) complex, the effects on TORC1 activity were less severe (lanes 4–5 and 12–14 in Figure 1B).

Mutation of the HOPS Rab GTPase *YPT7* resulted in increased rapamycin sensitivity and reduced TORC1 activity, whereas mutation of the CORVET Rab GTPase *VPS21* had a minimal effect on these TORC1-related phenotypes. However, the defects in these phenotypes and TORC1 activity of

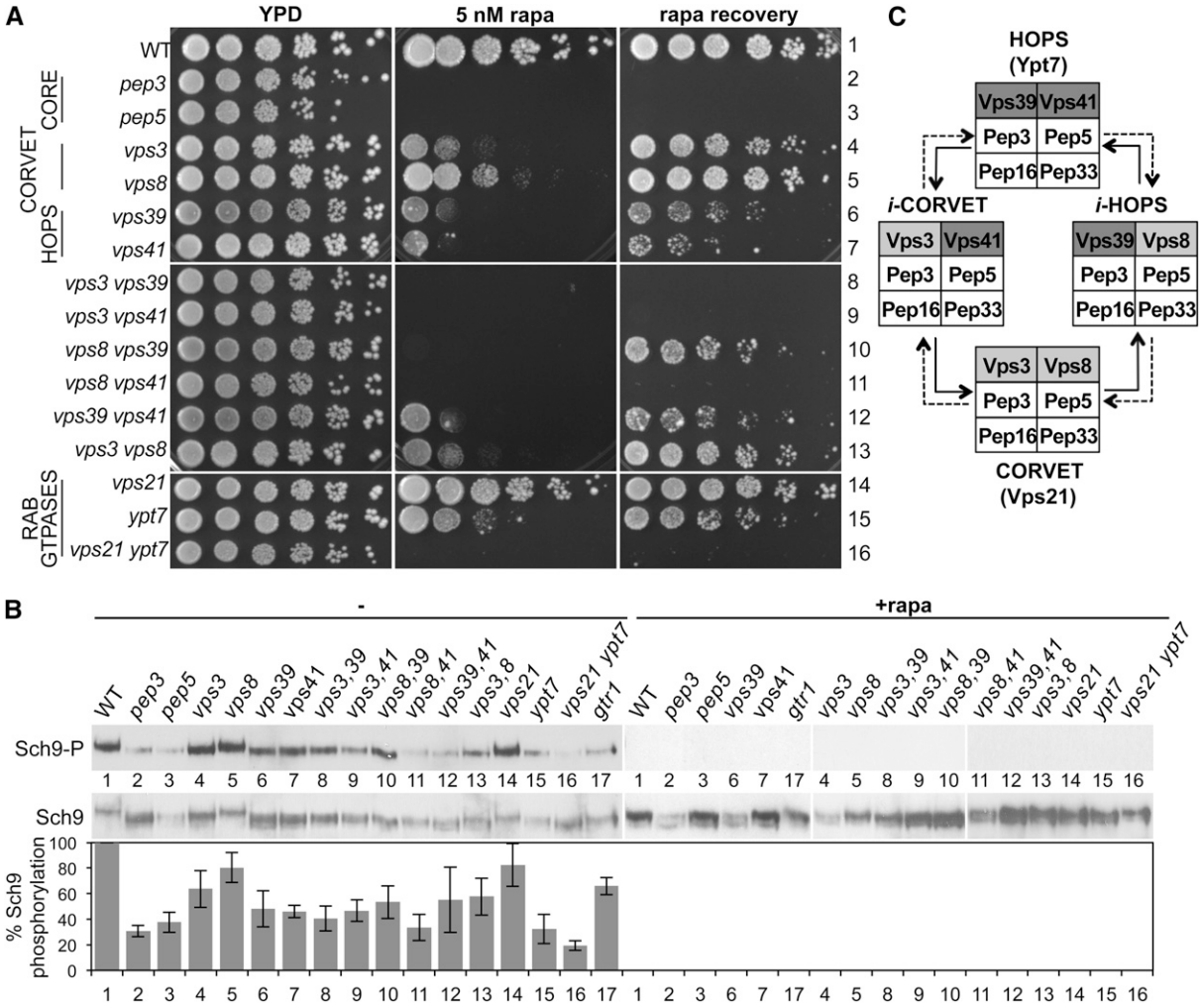

Figure 1 HOPS and CORVET complex mutations cause defects in TORC1 signaling. (A) Rapamycin sensitivity and recovery of *vps-c* complex mutants. The WT and strains mutated for components of the HOPS and CORVET complexes, which expressed plasmid-based HA_3-*SCH9* with all auxotrophic mutations complemented by pJK28, were grown to exponential phase in SC-ura, his, lys, leu + 2 mg/ml gln. Cultures were divided in half and treated with drug vehicle (control) or with 200 nM rapamycin for 6 hr at 30°. Rapamycin-treated cells were washed twice with water, and 5 μl of fivefold serial dilutions of cells were spotted onto YPD medium to test for rapamycin recovery. Drug vehicle control cultures were similarly diluted and plated on YPD without or with 5 nM rapamycin. Colonies were photographed following 3 days (YPD) or 4 days (5 nM rapamycin) incubation. Results are representative of at least three independent experiments. (B) TORC1 kinase activity of *vps-c* mutants. Strains described and grown as in A were treated for 30 min with (+rapa) or without (−rapa) 200 nM rapamycin. TORC1 activity was assessed by monitoring the phosphorylation status of Sch9 Thr737 and the overall protein levels of Sch9 by Western blot with the anti-phospho-Thr737-Sch9 (Sch9-P) and anti-732-743-Sch9 (Sch9) antibodies. Phosphorylation levels were normalized to Sch9 levels and expressed as an average percentage of WT Sch9 phosphorylation from three independent experiments, with error bars depicting the standard deviation. (C) Composition of HOPS, CORVET, and the hybrid complexes. The hybrid *i*-HOPS and *i*-CORVET complexes are hypothesized to be required for interconversion between the HOPS and CORVET complexes and, hence, antegrade and retrograde transport as depicted by the arrows (adapted from Nickerson *et al.* 2009).

the *ypt7 vps21* double mutant were as severe as those inflicted by mutation of *vps-c* core subunits (lanes 14–16 in Figure 1, A and B). Thus, composite loss of the HOPS, CORVET, and *i*-CORVET complexes is required to phenocopy the severity of TORC1-related defects imposed by mutation of the individual Vps-C core subunits Pep3 and Pep5. In addition, it has been proposed that the specific HOPS subunit Vps39 (also known as Vam6) functions as a GTP-exchange factor for the EGOC GTPase Gtr1 (Binda *et al.* 2009). However, the HOPS core component *pep3* and *pep5* mutations resulted in a more severe defect in TORC1 activity than mutation of *VPS39* (lanes 2–3 and 6 in Figure 1, A and B), suggesting that other Vps-C complex(es) in addition to HOPS and independently of Vps39 influence TORC1. Taken together, our results support a model in which the intact HOPS, CORVET, and, surprisingly, *i*-CORVET complexes are all capable of promoting TORC1 activity, although with different levels of efficiency, with HOPS being the most effective.

Vps-C complex functions upstream of TORC1

Epistasis analyses were conducted to establish the upstream position at which the Vps-C complex regulates TORC1

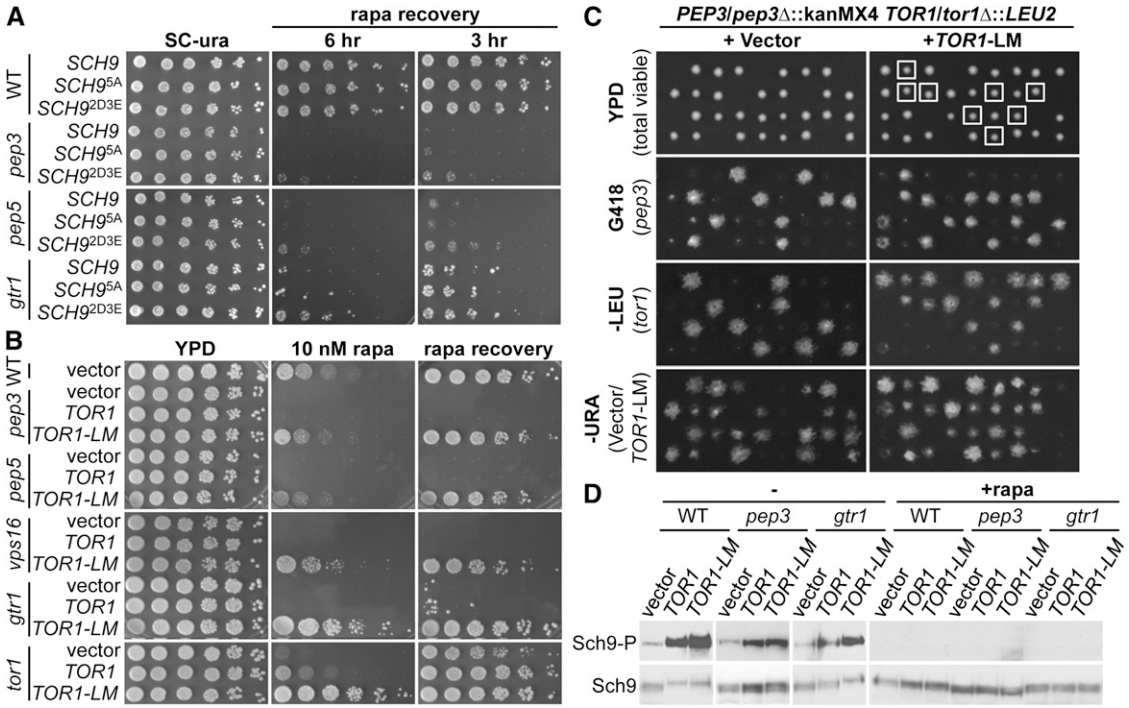

Figure 2 Expression of constitutively active *SCH9* and hyperactive *TOR1* alleles partially suppress TORC1 defects of *vps-c* mutants. (A) The constitutively active $SCH9^{2D3E}$ allele partially suppresses the rapamycin recovery defect of *vps-c* mutants. WT, *pep3*, *pep5*, and *gtr1* strains containing plasmids expressing *SCH9*, $SCH9^{5A}$, or $SCH9^{2D3E}$ were grown to exponential phase in SC-ura + 2 mg/ml gln and treated for 30 min with drug vehicle (left) or 200 nM rapamycin (center and right) for 6 or 3 hr. Culture aliquots were diluted, plated to SC-ura, and incubated, and colonies were photographed as indicated in Figure 1A. (B–D) The activated *TOR1-LM* allele suppresses (B) the rapamycin sensitivity and recovery defects, (C) synthetic lethality, and (D) TORC1 activity defects of *vps-c* mutants. (B) WT, *pep3*, *pep5*, *vps16*, *gtr1*, and *tor1* mutant strains containing a plasmid bearing $SCH9^{T570A}$-HA_5 and the empty vector, *TOR1*, or *TOR1-LM* were grown to exponential phase in SC-his-ura + 2 mg/ml gln and treated for 6 hr with drug vehicle ("YPD" and "10 nM rapa") or with 200 nM rapamycin ("rapa recovery"). Aliquots of serial dilutions of cultures were plated on YPD (left and right) and YPD + 10 nM rapamycin (center). Colonies were photographed after 3 days. (C) Heterozygous diploid *TOR1/tor1*Δ::*LEU2 PEP3/pep3*Δ::kanMX4 strains containing the empty vector or plasmid-based *TOR1-LM* were sporulated and dissected on YPD plates. Following 3 days of incubation, colonies were photographed and replica-plated to YPD+G418, SC-Leu, and SC-Ura media and incubated for 1 day to score genotypes. White boxes designate viable *tor1 pep3* (*TOR1-LM*) colonies. (D) WT, *pep3*, and *gtr1* strains containing vector, *TOR1*, or *TOR1-LM* plasmids as above, as well as pRS315-HA_3-*SCH9*, were grown to exponential phase in SC-his-ura + 2 mg/ml gln and treated for 30 min with 200 nM rapamycin or drug vehicle. TORC1 kinase activity was assayed as described in the legend to Figure 1B. All results are representative of at least three independent experiments.

signaling. First, we sought to further substantiate that the Vps-C complex acts upstream of Sch9. Sch9 is a major effector via which TORC1 activity controls cell growth (Urban *et al.* 2007). Substitution of all five major Sch9 phosphorylation sites targeted by the TORC1 kinase (T723, S726, T737, S758, and S765) with alanine ($SCH9^{5A}$) inactivates Sch9 (Urban *et al.* 2007). In contrast, the quintuple Sch9 phosphomimetic mutations [T723D, S726D, T737E, S758E, S765E ($SCH9^{2D3E}$)] render Sch9 activity TORC1-independent (Urban *et al.* 2007). Expression of the constitutively active $SCH9^{2D3E}$ allele, but not the WT *SCH9* or the hypoactive $SCH9^{5A}$ alleles, partially rescued the rapamycin recovery defects of *pep3*, *pep5*, and *gtr1* single-mutant strains (Figure 2A). These epistasis results support models in which the Vps-C complex acts via TORC1 and Sch9 to promote growth.

The *TOR1-LM* allele contains a mutation in the kinase domain ($Tor1^{L2134M}$) that renders TORC1 hyperactive and insensitive to upstream activation (Takahara and Maeda 2012). If the rapamycin sensitivity and recovery defects of *vps-c* mutants are due to reduced TORC1 activity, expression of the *TOR1-LM* allele in these mutants should suppress these defects. First, as a test of *TOR1-LM* function, introduction of this allele on a low-copy plasmid rendered a *tor1* mutant even more rapamycin-resistant than the WT, and the WT *TOR1* allele restored WT levels of rapamycin resistance as compared with the vector control (Figure 2B). In

Table 1 Minimum inhibitory concentrations of rapamycin

Strain	Genotype	Rapamycin MIC_{80} (nM)
BY4742	WT	10
Y4777	*pep3*	1.25
Y4778	*pep5*	1.25
Y4795	*gtr1*	5
Y4798	*gtr1 gtr2*	5
Y4796	*gtr2*	10
YJK3787	*pep3 gtr1 gtr2*	1.25
YJK3789	*pep5 gtr1 gtr2*	1.25

Medium for MICs comprised SC + 2 mg/ml glutamine, and results were recorded following 3 days incubation at 30°, as described in *Materials and Methods*.

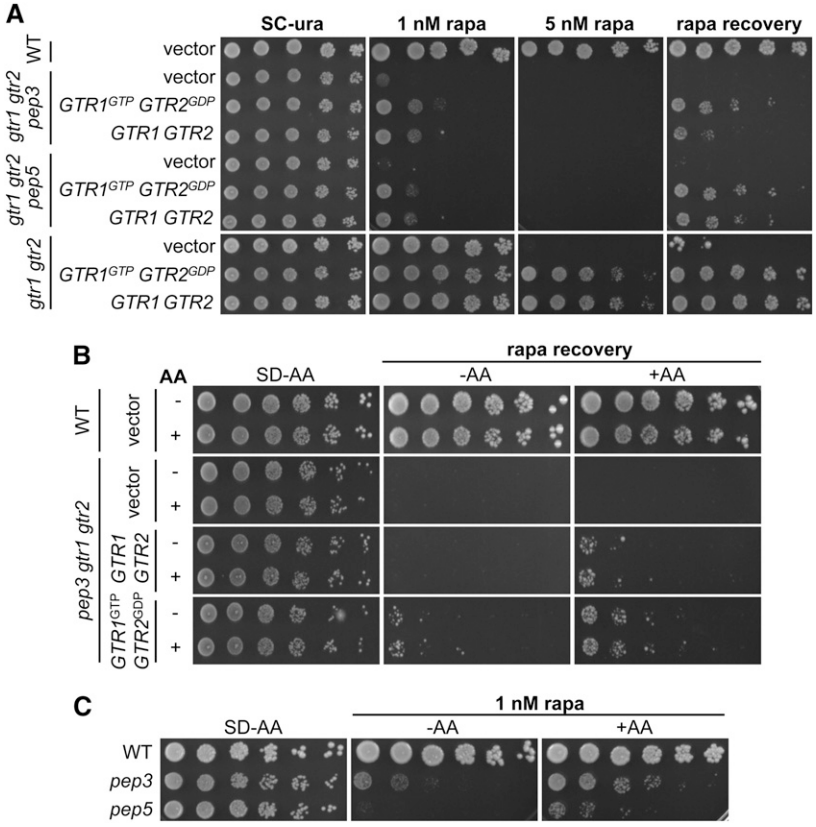

Figure 3 *GTR1*^{GTP} *GTR2*^{GDP}-locked activated allele expression partially suppresses *vps-c* mutant rapamycin sensitivity and TORC1 activity defects. (A) Rapamycin sensitivity (in 1 and 5 nM drug) and recovery (following treatment for 3 hr with 200 nM rapamycin) for WT, *gtr1 gtr2*, *gtr1 gtr2 pep3*, and *gtr1 gtr2 pep5* strains containing an empty vector or plasmids containing *GTR1* and *GTR2* or *GTR1*^{GTP} and *GTR2*^{GDP} expressed from their native promoters were assessed as indicated in Figure 2B. (B) Amino acids suppress the *vps-c* mutant rapamycin recovery defect. Cultures of WT and *gtr1 gtr2 pep3* strains containing pRS315-HA$_3$-Sch9 and the control vector or vectors expressing *GTR1* and *GTR2* or *GTR1*^{GTP} and *GTR2*^{GDP} were washed and resuspended in SD+his+lys supplemented without or with leu, gln, and arg (200, 200, and 40 mg/liter, respectively). Following 2 hr incubation, 200 nM rapamycin was added and incubation was continued. Prior to and 6 hr following rapamycin addition, aliquots were removed, washed, adjusted to OD_{600} of 0.5, and fivefold serial dilutions were plated on SD+his+lys media supplemented with no (−AA) or twofold leu, gln, and arg concentrations (+AA) as indicated above. Plates were photographed after 3 days ("SD-AA") or 7 days ("−AA" and "+AA"). (C) Amino acids suppress *vps-c* mutant rapamycin sensitivity. To test amino acid suppression of rapamycin sensitivity, WT, *pep3*, and *pep5* strains with all auxotrophies complemented by plasmids (pJK28, pPC10) were grown overnight in SC-ura-his-leu-lys + 2 mg/ml gln. Strains were adjusted to an OD_{600nm} of 0.5 and serially diluted fivefold, and 5-μl volumes of each dilution were plated to SD (−AA), SD + 1 nM rapamycin, supplemented without (−AA) or with (+AA) twofold leucine, glutamine, and arginine concentrations normally added to synthetic media (200, 200, and 40 mg/liter, respectively). Plates were photographed following 3 days incubation. Results are representative of three independent experiments.

addition, the *TOR1-LM* allele suppressed the rapamycin sensitivity and recovery defects of a *gtr1* mutant, as expected. Interestingly, both the rapamycin sensitivity and recovery defects of *pep3*, *pep5*, and *vps16* single mutants were also rescued by expression of the *TOR1-LM* allele, but not by expression of the WT *TOR1* allele or the vector control (Figure 2B). In addition, the *TOR1-LM* allele, but not the vector control, rescued the synthetic lethality of *pep3 tor1* segregants following dissection of a *PEP3/pep3 TOR1/tor1* diploid (Figure 2C). *TOR1-LM* expression also substantially elevated TORC1-dependent Sch9 phosphorylation in both *pep3* and *gtr1* mutants (Figure 2D). These findings further demonstrate a role for the Vps-C complex in activating events upstream of the TORC1 complex.

Vps-C complex controls TORC1 activity via EGOC by maintaining amino acid homeostasis

EGOC plays a major role in conveying amino acid signals to TORC1 (Binda *et al.* 2009). If the Vps-C complex functions in a linear pathway upstream of EGOC to activate TORC1, we predict that (1) EGOC and *vps-c* mutants will display similar rapamycin sensitivity and recovery defects and reduced levels of TORC1 activity; (2) combined disruption of EGOC and Vps-C complex components will result in the same rapamycin sensitivity and TORC1 activity phenotypes as those produced by disruption of either complex alone; and (3) concomitant overexpression of the *GTR1*^{GTP}- and *GTR2*^{GDP}-locked alleles, which constitutively activate TORC1 (regardless of amino acid levels) (Binda *et al.* 2009), should fully suppress *vps-c* mutant rapamycin sensitivity and TORC1 activity defects.

First, rather than showing the same rapamycin phenotypes, the *vps-c pep3* and *pep5* mutants were substantially more rapamycin-sensitive (fourfold lower MICs) (Table 1) and had lower levels of TORC1 kinase activity than the *gtr1* mutant (lanes 2–3 and 17 in Figure 1B). Second, disruption of *PEP3* or *PEP5* exacerbated the rapamycin sensitivity and recovery defects of the *gtr1 gtr2* double mutant (Figure 3A and Table 1). Third, although expression of the *GTR1*^{GTP}- and *GTR2*^{GDP}-locked alleles in *pep3 gtr1 gtr2* or *pep5 gtr1 gtr2* triple-mutant strains caused no increase in TORC1 activity compared with the vector control (Figure S3), it resulted in a partial, yet readily detectable, suppression of both rapamycin sensitivity and the rapamycin recovery defect (Figure 3A). Overexpression of the WT *GTR1 GTR2* alleles in the *pep3 gtr1 gtr2* and *pep5 gtr1 gtr2* triple-mutant strains also suppressed the rapamycin sensitivity and recovery defects compared with the vector control, although to a lesser degree than did the *GTR1*^{GTP}- and *GTR2*^{GDP}-locked alleles (Figure 3A). Taken together, these results support a model in which the Vps-C

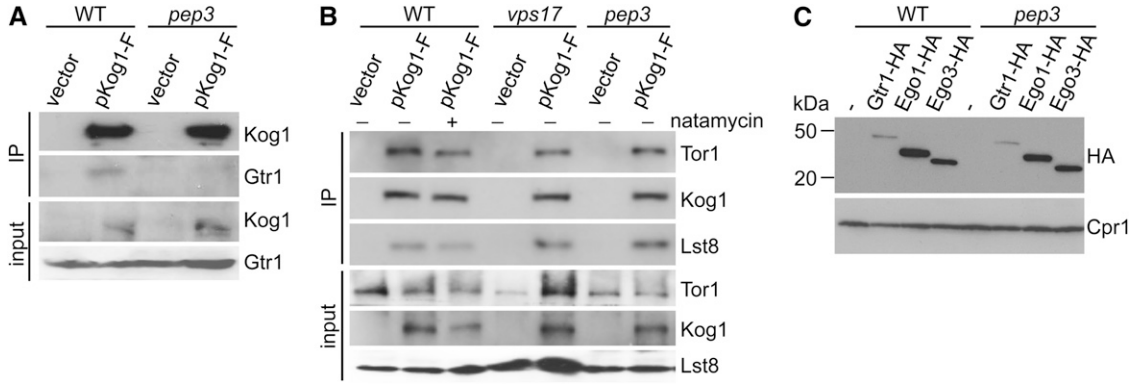

Figure 4 *vps-c* mutation perturbs EGOC–TORC1 interaction, but not TORC1 complex integrity or EGOC subunit stability. (A) Mutation of *PEP3* affects Kog1–Gtr1 interaction. WT and *pep3* mutant strains bearing a *GTR1-HA* allele and transformed with the vector or pRS314-*KOG1*-FLAG (pKog1-F) were grown to exponential phase. Protein cell extracts were prepared and immunoprecipitated with immobilized FLAG antibody. Bound proteins were eluted and analyzed by Western blot with anti-FLAG and anti-HA antibodies. (B) The *pep3* mutation or vacuolar fragmentation does not affect TORC1 stability. WT, *pep3*, and *vps17* strains containing an *LST8-HA* functional allele and transformed with the vector or pKog1-F plasmid were grown to exponential phase where indicated cells were treated with 1.5 μM natamycin for 5 hr prior to harvesting. Protein cell extracts were prepared and immunoprecipitated with immobilized FLAG antibody. Bound proteins were eluted and analyzed by Western blot with anti-Tor1, anti-FLAG, and anti-HA antibodies. (C) Mutation of *PEP3* has no effect on EGOC individual component protein levels. Protein extracts from WT and *pep3* strains expressing chromosomally HA_3-tagged *GTR1*, *EGO1*, *EGO3* or no tag (−) were analyzed by Western blot with anti-HA and anti-cyclophilin A (Cpr1) antibodies. All results presented in A–C are representative of two (A) and three (B-C) independent determinations.

complex signals in part via EGOC to TORC1. However, EGOC is not the sole conduit by which the Vps-C complex regulates TORC1, and there are additional inputs.

Previous studies, including our own, have shown that *vps-c* mutants have low amino acid concentrations (Kitamoto *et al.* 1988b; Zurita-Martinez *et al.* 2007). Because the Vps-C complex contributes to elicit TORC1 activity via the amino acid-responsive EGOC, this regulation may be effected through its role in maintaining amino acid homeostasis. To address this, we investigated whether elevated levels of amino acids could suppress the *vps-c* mutant TORC1-related defects. Supplementation of twofold the normal levels of leucine, glutamine, and arginine in the culture medium improved rapamycin recovery of the *pep3 gtr1 gtr2* mutant strain expressing either *GTR1 GTR2*- or $GTR1^{GTP}$ $GTR2^{GDP}$-locked alleles, but not the vector control (Figure 3B). Elevated levels of these amino acids also partially suppressed the rapamycin sensitivity of *pep3* and *pep5* mutants (Figure 3C). These results support that the Vps-C complex role in amino acid homeostasis is required to maintain full TORC1 activity via the EGOC.

Loss of function of the Vps-C complex impairs EGOC–TORC1 interaction

EGOC-dependent activation of TORC1 in response to amino acids is mediated via direct binding of EGOC with TORC1, which can be monitored by assaying Gtr1 interaction with the TORC1 subunits Kog1 and Tco89 (Binda *et al.* 2009). Thus, we examined whether the *vps-c* mutation affects Gtr1 interactions with TORC1. To this end, we expressed a functional, plasmid-borne *KOG1*-FLAG allele in the WT and *pep3* strains containing a chromosomal *GTR1-HA* allele and compared the levels of Kog1-Gtr1 that co-immunoprecipitated in FLAG affinity-captured samples. While Gtr1 was detected at similar levels in the total lysate from both strains and was detectable in the FLAG immunoprecipitates from WT cells, at best only traces of Gtr1 were detected in the FLAG immunoprecipitates from the *pep3* mutant (Figure 4A). This indicates that Gtr1-Kog1 binding is reduced in the *pep3* mutant background. Therefore, the *vps-c* mutation perturbs the EGOC–TORC1 interaction.

As shown above, the Vps-C complex has additional effects on TORC1 activity that are not mediated via EGOC. The HOPS complex controls membrane fusion with the vacuole, and thus mutation of HOPS components results in highly fragmented vacuoles (Banta *et al.* 1988; Kitamoto *et al.* 1988b; Raymond *et al.* 1992). Given that the vacuolar membrane serves as a platform for TORC1 activity, vacuolar fragmentation caused by *vps-c* mutations may destabilize TORC1 and thus affect EGOC–TORC1 interactions.

Next, we investigated whether TORC1 stability is compromised by *vps-c* mutation and thereby compromises EGOC–TORC1 interaction. Roughly equivalent proportions of the TORC1 components Tor1, Kog1, and Lst8 co-immunoprecipitated with Kog1-FLAG from WT and *pep3* mutants, indicating that the stability of TORC1 is comparable in the two strains (Figure 4B). Similarly, TORC1 was found to be equally stable when assayed in two other conditions that induce vacuolar fragmentation: *vps17* mutant cells (Rothman *et al.* 1989a) and WT cells treated with natamycin (inhibitor of vacuolar homotypic fusion via ergosterol binding) (te Welscher *et al.* 2010). We conclude that the *vps-c* mutation does not result in reduced TORC1 activity due to vacuolar fragmentation causing destabilization of the TORC1 complex and thereby affecting EGOC–TORC1 interaction. Thus, the EGOC-independent effects on TORC1 signaling remain to be determined and are under intensive study in our lab.

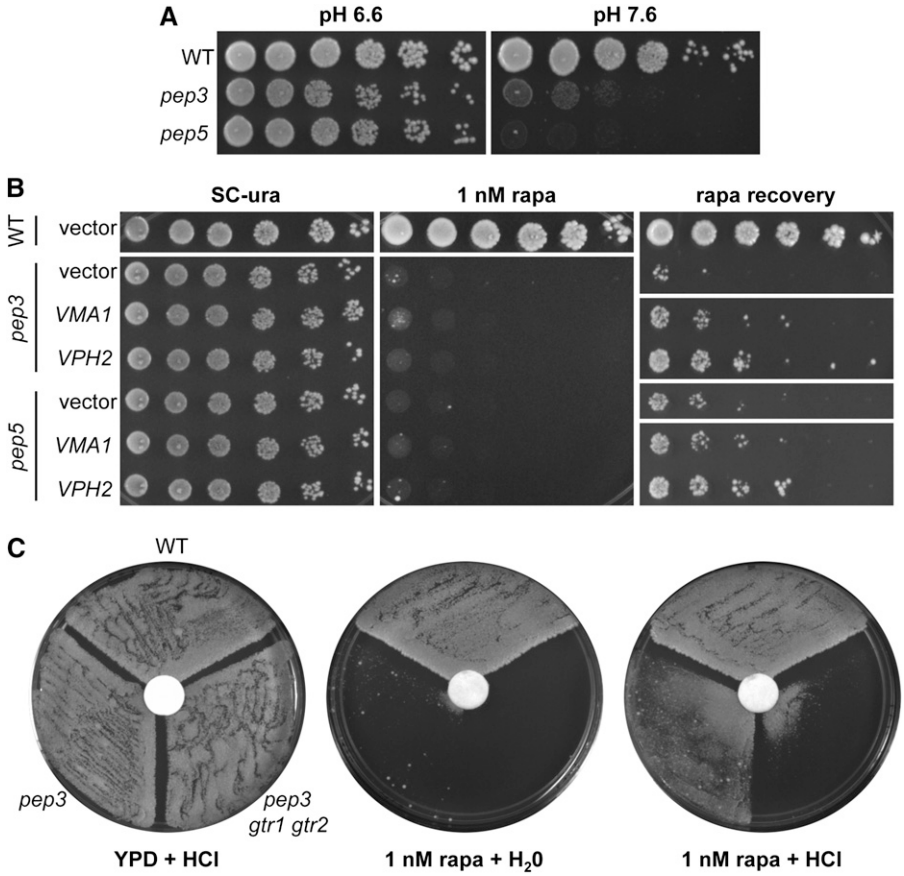

Figure 5 v-ATPase overexpression and acidic pH suppress *vps-c* mutant rapamycin phenotypes. (A) *vps-c* mutants are sensitive to alkaline pH. WT, *pep3*, and *pep5* strains were grown overnight in SC-ura-leu-lys-his + 2 mg/ml gln, resuspended to OD_{600nm} of 0.5, and serially diluted fivefold, and 5 μl of each dilution was plated to YPD buffered to pH 6.6 or 7.6. Plates were photographed after 2 days. (B) Rapamycin sensitivity (1 nM rapamycin) and recovery (following 3 hr rapamycin incubation) phenotypes were assessed for WT, *pep3*, and *pep5* strains carrying chromosomally integrated pAG306-GPD-empty (vector), pAG306-GPD-*VMA1*, or pAG306-GPD-*VPH2*. Plates were photographed after 5 days (SC-ura ± 1 nM rapamycin) or 6 days (SC-ura, rapamycin recovery). (C) Acidic pH ameliorates *pep3* rapamycin sensitivity. WT, *pep3*, or *pep3 gtr1 gtr2* strains were grown overnight in SC-ura-leu + 2 mg/ml gln. Culture aliquots were diluted to OD_{600nm} of 0.1 and evenly applied with a sterile cotton swab onto YPD plates with or without 1 nM rapamycin. Filter discs containing either 50 μl water or 1 M HCl were positioned at the plate centers, and plates were photographed after 3 days incubation. All the results presented are representative of three (A and C) and two (B) independent experiments.

Finally, it is known that individual disruption of EGOC components decreased levels of other complex members, thereby compromising EGOC stability (Gao and Kaiser 2006). Therefore, we compared individual protein levels of Gtr1, Ego1, and Ego3 in WT and *pep3* mutant backgrounds. Levels of each EGOC component were indistinguishable in the WT and mutant backgrounds (Figure 4C). This result demonstrates that the *vps-c* mutation does not grossly perturb the protein stability of individual EGOC components, and this is further corroborated by the observation that the extrachromosomally expressed EGOC subunits Gtr1/Gtr2 and $Gtr1^{GTP}$/$Gtr2^{GDP}$ are at least in part functional in *pep3* mutant strains (Figure 3, A and B).

Acidic pH or v-ATPase overexpression suppresses rapamycin sensitivity of vps-c mutants

The vacuolar H^+-ATPase (v-ATPase) is required to maintain a proton electrochemical gradient across the vacuolar membrane and in so doing plays a key role in regulating cellular pH (reviewed by Kane 2006). The *vps-c* mutants contain highly fragmented vacuoles and, similar to v-ATPase mutants, have defects in pH homeostasis, acidification of the vacuole, and assembly of the v-ATPase and display alkaline pH sensitivity (Rothman *et al.* 1989b; Sambade *et al.* 2005; Schauer *et al.* 2009; Garipler and Dunn 2013). We first verified that *pep3* and *pep5* mutants are sensitive to alkaline pH (pH 7.6) (Figure 5A), consistent with a role for the Vps-C complex in vacuolar acidification and pH homeostasis. Next, we investigated whether overexpression of genes important for maintaining vacuolar pH mitigates TORC1 signaling defects of *vps-c* mutants. *VMA1* encodes the v-ATPase catalytic subunit while *VPH2* regulates v-ATPase assembly (Hirata *et al.* 1990; Jackson and Stevens 1997) and their overexpression results in increased vacuolar acidity (Hughes and Gottschling 2012). Overexpression of either *VMA1* or *VPH2* partially suppressed *pep3* and *pep5* rapamycin recovery defects, with *VPH2* having the greater effect (Figure 5B). Furthermore, decreasing media pH was found to ameliorate the rapamycin hypersensitivity of the *pep3* mutant, and this effect was considerably reduced in the *pep3 gtr1 gtr2* triple mutant (Figure 5C). These results suggest a role for the Vps-C complex in controlling pH homeostasis, acting upstream of EGOC to promote TORC1 signaling.

Discussion

Our previous studies revealed that *vps-c* mutants are synthetically lethal with a *tor1* mutation and exquisitely rapamycin-sensitive and fail to recover from rapamycin-induced growth arrest, suggesting a function for the Vps-C complex in TORC1 regulation (Zurita-Martinez *et al.* 2007). Here, we characterized this role by demonstrating a requirement for distinct Vps-C complexes to promote TORC1 activity. We also show that Vps-C complex action is mediated in part

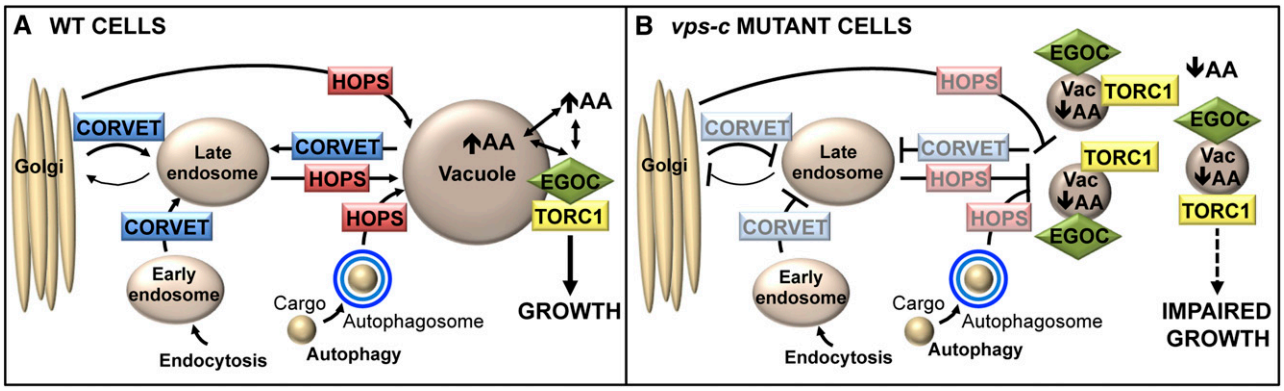

Figure 6 Proposed model for the role of Vps-C complex in promoting TORC1 signaling. (A) The Vps-C HOPS and CORVET complexes mediate multiple endomembrane fusion events, including endosomal-to-vacuole and vacuole-to-vacuole. The vacuole serves as the major site for amino acid storage in the cell, and TORC1 and EGOC reside on the vacuolar membrane. Distinct Vps-C complexes regulate TORC1 activity in part and indirectly by promoting amino acid homeostasis between the cytosol and the vacuole, which is sensed and transponded to TORC1 via interactions with EGOC to promote cell growth. (B) Disruption of Vps-C HOPS and CORVET complexes blocks endomembrane fusion events, resulting in fragmented vacuoles, and reduced amino acid levels (inside and outside the vacuole). In turn, diminished amino acid levels lead to reduced activation of EGOC and decreased EGOC–TORC1 interactions, resulting in impaired TORC1 activity and compromised cell growth.

via maintenance of amino acid homeostasis, transponded to TORC1 via interactions with the GTPase EGOC.

The Vps-C complexes include the well-characterized HOPS and CORVET complexes and two hypothesized complexes: *i*-HOPS and *i*-CORVET. Each of these endomembranous proteinaceous complexes contributes to different stages of vesicular trafficking (Figure 6) (Srivastava *et al.* 2000; Peterson and Emr 2001; Peplowska *et al.* 2007; Nickerson *et al.* 2009; Epp *et al.* 2011). We demonstrated that both HOPS and CORVET complexes individually promote TORC1 activity, with HOPS playing a greater role. Furthermore, composite loss of the HOPS, CORVET, and *i*-CORVET complexes was required to phenocopy the severity of TORC1-related defects imposed by mutation of the individual Vps-C core subunits Pep3 and Pep5. A similar effect was also observed with the concomitant disruption of the Rab GTPases Ypt7 and Vps21, which are required for membrane tethering by HOPS and CORVET, respectively, and presumably *i*-CORVET.

Surprisingly, our studies unveiled a role for *i*-CORVET in TORC1 signaling in the *vps8 vps39* double mutant, which is unable to form either a HOPS or CORVET complex. This suggests that *i*-CORVET may promote TORC1 signaling by (1) enabling retrograde trafficking from the vacuole or (2) in part compensating for other HOPS and/or CORVET-mediated trafficking functions. In contrast, we found no contributing role for the hypothetical *i*-HOPS complex in TORC1 activity. However, the precise roles for the *i*-HOPS and *i*-CORVET complexes in vesicular trafficking are presently unknown. That Vps39 interacts with Vps8, and Vps41 with Vps3, has previously supported models that hypothesize that both intermediary complexes may exist in the cell (Peplowska *et al.* 2007). The *i*-CORVET complex is detected in WT cells and is strongly enriched in *vps39* or *vps8* mutants or upon *VPS3* overexpression and able to bind activated Ypt7-GTP Rab GTPase to modestly stimulate vacuole fusion (Peplowska *et al.* 2007; Ostrowicz *et al.* 2010). This data are consistent with our findings of a role for the *i*-CORVET complex in providing HOPS/CORVET function. In contrast, there are no robust data supporting formation of an *i*-HOPS complex (Peplowska *et al.* 2007), suggesting that *i*-HOPS actually may not occur at levels that are biologically relevant. This result is in accord with our finding that the *vps3 vps41* double-mutant strain, which hypothetically can form only the *i*-HOPS complex, fails to recover from rapamycin arrest and shows low TORC1 activity. Taken together, our results support a model whereby distinct Vps-C complexes contribute multiple endolysosomal trafficking roles to provide robust TORC1 signaling (Figure 6).

Our epistasis analyses indicate that Vps-C complex modulation of TORC1 activity is elicited, at least in part, prior to the EGOC point of action in the pathway. First, expression of a constitutively active allele of the major TORC1 downstream effector Sch9, which uncouples Sch9 function from TORC1 activity, partially suppressed *vps-c* mutant rapamycin recovery. The inability of the constitutively active *SCH9* allele to fully support growth during rapamycin recovery is expected because Sch9 is not the only effector by which TORC1 controls cell growth. Second, expression of the hyperactive *TOR1-LM* allele fully suppressed *vps-c* mutant TORC1 activity defects. Finally, overexpression of the EGO GTPase *GTR1 GTR2*- and $GTR1^{GTP}$ $GTR2^{GDP}$-locked alleles partially suppressed *vps-c* mutant rapamycin sensitivity and recovery defects. In agreement with this result, Binda *et al.* (2009) noted that the $GTR1^{GTP}$-locked allele had only a partial effect in uncoupling TORC1 activity from leucine availability. This effect was attributed either to a requirement for subsequent GTP hydrolysis following $Gtr1^{GTP}$ loading for full TORC1 activation or to additional signaling routes by which amino acid availability is signaled to TORC1. The more severe rapamycin sensitivity and TORC1 activity defects exhibited by the *vps-c* mutants, compared with the EGOC mutants, evoke a model whereby the Vps-C complex control of TORC1 activity is only in part effected via EGOC and in which there are additional inputs.

Consistent with the Vps-C complex regulating TORC1 upstream of EGOC, the rapamycin hypersensitive and recovery phenotypes of *vps-c* mutants were partially suppressed by addition of exogenous amino acids. Disruption of the Vps-C complexes has previously been shown to reduce cellular levels of amino acids (Kitamoto *et al.* 1988b; Zurita-Martinez *et al.* 2007). The Vps-C complex is thought to promote amino acid homeostasis through several mechanisms. First, the Vps-C complex affects amino acid transporter expression and stability (Srivastava *et al.* 2000; Puria *et al.* 2008). Second, Vps-C complexes maintain vacuole integrity, and this organelle plays a key role in storage of amino acids that are acquired from the cytosol or produced directly at the vacuole by protein degradation during the normal recycling of proteins or autophagy (Wiemken and Durr 1974; Kitamoto *et al.* 1988a; Onodera and Ohsumi 2005). *VPS-C* disruption results in tiny and highly fragmented vacuoles, which are defective in proteolysis and autophagy, thereby significantly reducing both vacuolar amino acid levels and the capacity for vacuolar amino acid storage (Kitamoto *et al.* 1988b; Raymond *et al.* 1992; Klionsky 2005; Liang *et al.* 2008). Third, the acidic pH of the vacuole that is maintained by the v-ATPase is a prerequisite for vacuolar amino acid import and export (Russnak *et al.* 2001), and evidence suggests that *vps-c* mutants also have defects in vacuole acidification, assembly of the vacuolar v-ATPase, and pH homeostasis (Rothman *et al.* 1989b; Sambade *et al.* 2005; Schauer *et al.* 2009; Garipler and Dunn 2013). Therefore, the multiple mechanisms by which the Vps-C complex maintains cellular amino acid homeostasis likely contribute significantly to regulation of TORC1 signaling.

Interestingly, we demonstrated that both media acidification and overexpression of the v-ATPase assembly factor Vph2 partially rescue the *vps-c* mutant rapamycin sensitivity and/or recovery defects. Despite a reported role for the v-ATPase in mTORC1 regulation (Zoncu *et al.* 2011), we do not think that yeast *vps-c* mutant effects on TORC1 activity are mediated by fostering v-ATPase–TORC1 interactions because in *S. cerevisiae* v-ATPase mutants exhibit neither rapamycin hypersensitivity nor defects in rapamycin recovery (data not shown). Instead, we hypothesize that *vps-c* mutants have defects in v-ATPase assembly and that this can be alleviated by *VPH2* overexpression.

How else might the Vps-C complex regulate TORC1 activity? Sch9, TORC1, and EGOC all reside on the vacuolar membrane, which serves as a scaffolding platform for TORC1 activity (Cardenas and Heitman 1995; Huh *et al.* 2003; Wedaman *et al.* 2003; Jorgensen *et al.* 2004; Araki *et al.* 2005; Dubouloz *et al.* 2005; Gao and Kaiser 2006; Urban *et al.* 2007; Sturgill *et al.* 2008; Berchtold and Walther 2009; Binda *et al.* 2009). Vacuolar membrane fragmentation that results from Vps-C complex disruption may also contribute to the reduced EGOC–TORC1 complex interactions observed in the *pep3* mutant and also to other TORC1 interactions that foster its activity. Interestingly, a role for the endolysosomal membrane trafficking system has been suggested in mTORC1 signaling. Overexpression of the constitutively active Rab GTPase Rab5 (analogous to yeast Vps21) or small interfering RNA knockdown of hVps39 (mammalian HOPS complex component) perturbed early-to-late endosomal conversion leading to the accumulation of hybrid early/late endosomes (Flinn *et al.* 2010). This, in turn, blocked stimulation of mTORC1 signaling in response to insulin and amino acids, which was attributed to reduced interactions between mTORC1 and its GTPase activator Rheb (Flinn *et al.* 2010). Elucidating the roles for the yeast endolysosomal trafficking machinery in TORC1 signaling will provide insights to understand how this conserved and therapeutically relevant pathway is regulated in humans.

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GENETICS

Supporting Information
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Endolysosomal Membrane Trafficking Complexes Drive Nutrient-Dependent TORC1 Signaling to Control Cell Growth in *Saccharomyces cerevisiae*

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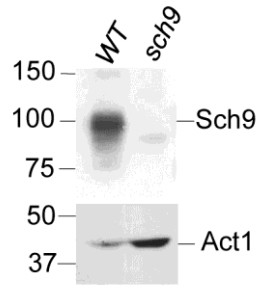

Figure S1 Specificity of the anti-732-743-Sch9 antibody. WT and *sch9* strains were grown to exponential phase in SC medium. Protein extracts were analyzed by SDS-PAGE, and immunoblots were probed with anti-732-743-Sch9 and anti-Act1 antibodies.

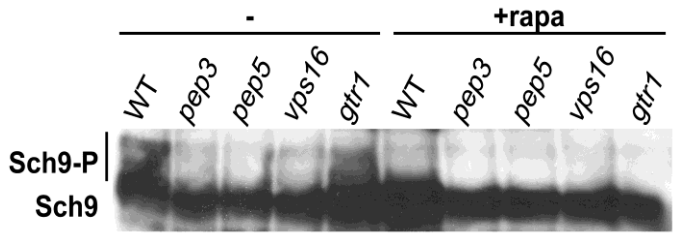

Figure S2 TORC1 activity is impaired in *vps-c* mutants. WT, *vps-c*, and *gtr1* mutants expressing a plasmid-based copy of *SCH9*^{T570A}-HA$_5$ (pJU1064) and pMKV002 were grown to exponential phase in YPD + 2 mg/ml gln and treated for 30 min with drug vehicle or with 200 nM rapamycin. Protein extracts were prepared and treated with NTCB to release the 50 kDa C-terminal phosphoacceptor domain of Sch9 (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 50 kDa C-terminal fragment of Sch9 is shown. The extent of Sch9 phosphorylation (and, by proxy, TORC1 activity) was indicated by the multiple phosphoisoforms migrating above 50 kDa.

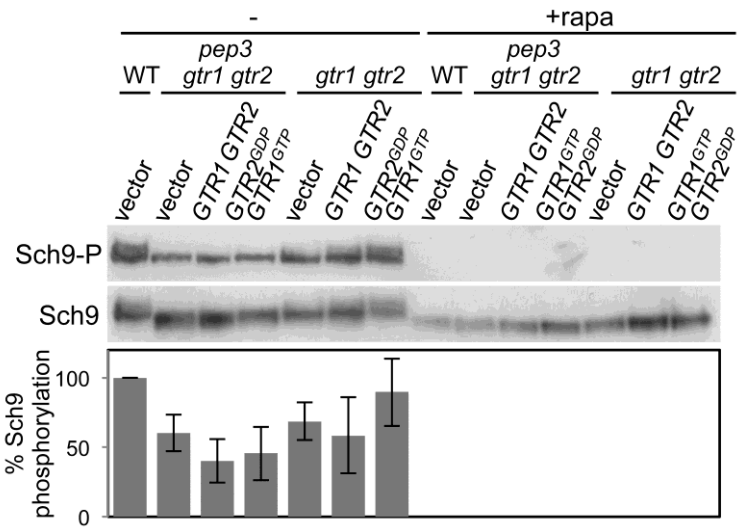

Figure S3 TORC1 kinase activity was determined as described in **Fig. 1B** legend for WT, *pep3 gtr1 gtr2*, and *gtr1 gtr2* strains containing control, *GTR1 GTR2*, or $GTR1^{GTP}$ $GTR2^{GDP}$ expressing plasmids and pRS315-HA_3-*SCH9* that had been grown to exponential phase in SC-his-ura + 2 mg/ml gln and treated without or with 200 nM rapamycin for 30 min. Results were the average of three independent experiments and error bars depict the standard deviation.

Table S1 Yeast strains used in this study. All strains listed are isogenic with BY4742 (BRACHMANN *et al.* 1998), or RL58-1a (WULLSCHLEGER *et al.* 2005) as indicated, and all except BY4742 and RL58-1a were constructed in this study.

Strain	Genotype	Figure
BY4742-derived strains		
BY4742	*MAT* α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Table 1, S1
YJK3814	pJK28 pPC10	1, 3C, 5A
YJK3815	*pep3*Δ::kanMX4 pJK28 pPC10	1, 3C, 5A
YJK3816	*pep5*Δ::kanMX4 pJK28 pPC10	1, 3C, 5A
YJK3817	*vps3*Δ::kanMX4 pJK28 pPC10	1
YJK3818	*vps8*Δ::natMX4 pJK28 pPC10	1
YJK3820	*vps21*Δ::kanMX4 pJK28 pPC10	1
YJK3822	*vps39*Δ::kanMX4 pJK28 pPC10	1
YJK3823	*vps41*Δ::kanMX4 pJK28 pPC10	1
YJK3824	*vps3*Δ::kanMX4 *vps39*Δ::natMX4 pJK28 pPC10	1
YJK3825	*vps3*Δ::kanMX4 *vps41*Δ::natMX4 pJK28 pPC10	1
YJK3826	*vps8*Δ::natMX4 *vps39*Δ::kanMX4 pJK28 pPC10	1
YJK3828	*ypt7*Δ::kanMX4 pJK28 pPC10	1
YJK3829	*gtr1*Δ::kanMX4 pJK28 pPC10	1
YJK4000	*ypt7*Δ::kanMX4 *vps21*Δ::natMX4 pJK28 pPC10	1
YJK4002	*vps39*Δ::kanMX4 *vps41*Δ::natMX4 pJK28 pPC10	1
YJK4006	*vps3*Δ::kanMX4 *vps8*Δ::natMX4 pJK28 pPC10	1
YJK4007	*vps8*Δ::natMX4 *vps41*Δ::kanMX4 pJK28 pPC10	1
YJK3504	pJU675	2A
YJK3506	pJU822	2A
YJK3508	pJU841	2A
YJK3510	*pep3*Δ::kanMX4 pJU675	2A
YJK3512	*pep3*Δ::kanMX4 pJU822	2A
YJK3514	*pep3*Δ::kanMX4 pJU841	2A
YJK3516	*pep5*Δ::kanMX4 pJU675	2A
YJK3518	*pep5*Δ::kanMX4 pJU822	2A
YJK3520	*pep5*Δ::kanMX4 pJU841	2A
YJK3586	*gtr1*Δ::kanMX4 pJU841	2A
YJK3587	*gtr1*Δ::kanMX4 pJU675	2A
YJK3588	*gtr1*Δ::kanMX4 pJU822	2A
YJK3243	*pep3*Δ::kanMX4 pJU1064 pRS416	2B
YJK3247	*pep3*Δ::kanMX4 pJU1064 pMY22	2B
YJK3249	*pep3*Δ::kanMX4 pJU1064 pMY28	2B
YJK3259	*pep5*Δ::kanMX4 pJU1064 pRS416	2B

YJK3263	*pep5*Δ::kanMX4 pJU1064 pMY22	2B
YJK3265	*pep5*Δ::kanMX4 pJU1064 pMY28	2B
YJK3267	*vps16*Δ::kanMX4 pJU1064 pRS416	2B
YJK3271	*vps16*Δ::kanMX4 pJU1064 pMY22	2B
YJK3273	*vps16*Δ::kanMX4 pJU1064 pMY28	2B
YJK3283	*tor1*Δ::kanMX4 pJU1064 pRS416	2B
YJK3287	*tor1*Δ::kanMX4 pJU1064 pMY22	2B
YJK3289	*tor1*Δ::kanMX4 pJU1064 pMY28	2B
YJK3291	pJU1064 pRS416	2B
YJK3299	*gtr1*Δ::kanMX4 pJU1064 pRS416	2B
YJK3303	*gtr1*Δ::kanMX4 pJU1064 pMY22	2B
YJK3305	*gtr1*Δ::kanMX4 pJU1064 pMY28	2B
YJK3898	pRS315-HA_3-*SCH9* pRS416	2D
YJK3899	*pep3*Δ::kanMX4 pRS315-HA_3-*SCH9* pRS416	2D
YJK3900	*pep3*Δ::kanMX4 pRS315-HA_3-*SCH9* pMY22	2D
YJK3901	*pep3*Δ::kanMX4 pRS315-HA_3-*SCH9* pMY28	2D
YJK3902	*gtr1*Δ::kanMX4 pRS315-HA_3-*SCH9* pRS416	2D
YJK3903	*gtr1*Δ::kanMX4 pRS315-HA_3-*SCH9* pMY22	2D
YJK3904	*gtr1*Δ::kanMX4 pRS315-HA_3-*SCH9* pMY28	2D
YJK4071	pRS315-HA_3-*SCH9* pMY22	2D
YJK4072	pRS315-HA_3-*SCH9* pMY28	2D
YJK4120	*MAT***a**/*MAT*α *his3*Δ*1*/*his3*Δ*1 leu2*Δ*0*/*leu2*Δ*0 met15*Δ*0*/*MET15 lys2*Δ*0*/*LYS2 ura3*Δ*0*/*ura3*Δ*0 PEP3*/*pep3*Δ::kanMX4 *TOR1*/*tor1*Δ::*LEU2* pRS416	2C
YJK4121	*MAT***a**/*MAT*α *his3*Δ*1*/*his3*Δ*1 leu2*Δ*0*/*leu2*Δ*0 met15*Δ*0*/*MET15 lys2*Δ*0*/*LYS2 ura3*Δ*0*/*ura3*Δ*0 PEP3*/*pep3*Δ::kanMX4 *TOR1*/*tor1*Δ::*LEU2* pMY28	2C
YJK3753	YEplac195	3A, 5C
YJK3797	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 *pep3*Δ::natMX4 YEplac195	3A, 5C
YJK3798	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 *pep3*Δ::natMX4 pPC8a	3A
YJK3800	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 *pep3*Δ::natMX4 pPC9a	3A
YJK3802	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 *pep5*Δ::natMX4 YEplac195	3A
YJK3803	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 *pep5*Δ::natMX4 pPC8a	3A
YJK3805	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 *pep5*Δ::natMX4 pPC9a	3A
YJK3807	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 YEplac195	3A
YJK3808	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 pPC8a	3A
YJK3810	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 pPC9a	3A
YJK3867	pRS315-HA_3-*SCH9* YEplac195	3B, S3
YJK3892	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 *pep3*Δ::natMX4 pRS315-HA_3-*SCH9* YEplac195	3B, S3
YJK3893	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 *pep3*Δ::natMX4 pRS315-HA_3-*SCH9* pPC9a	3B, S3
YJK3894	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 *pep3*Δ::natMX4 pRS315-HA_3-*SCH9* pPC8a	3B, S3

YJK3895	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 pRS315-HA_3-*SCH9* YEplac195	S3
YJK3896	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 pRS315-HA_3-*SCH9* pPC9a	S3
YJK3897	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 pRS315-HA_3-*SCH9* pPC8a	S3
YJK3428	*pep3*Δ::natMX4 *GTR1*-HA_3-kanMX6 *trp1*Δ::hphMX4 pRS314-*KOG1*-FLAG	4A
YJK3431	*GTR1*-HA_3-kanMX6 *trp1*Δ::hphMX4 pRS314-*KOG1*-FLAG	4A
YJK3432	*GTR1*-HA_3-kanMX6 *trp1*Δ::hphMX4 YCplac22	4A
YJK3435	*pep3*Δ::natMX4 *GTR1*-HA_3-kanMX6 *trp1*Δ::hphMX4 YCplac22	4A
NDY64	*pep3*Δ::natMX4	4C
NDY65	*pep3*Δ::natMX4 *GTR1*-HA_3-kanMX6	4C
NDY66	*pep3*Δ::natMX4 *EGO1*-HA_3-kanMX6	4C
NDY67	*pep3*Δ::natMX4 *EGO3*-HA_3-kanMX6	4C
NDY68	*GTR1*-HA_3-kanMX6	4C
NDY69	*EGO1*-HA_3-kanMX6	4C
NDY70	*EGO3*-HA_3-kanMX6	4C
YJK3948	chrI(199456-199457)::P_{GPD1}-$Term_{CYC1}$-*URA3* pRS315-HA_3-*SCH9*	5B
YJK3952	*pep3*Δ::kanMX4 chrI(199456-199457)::P_{GPD1}-$Term_{CYC1}$-*URA3* pRS315-HA_3-*SCH9*	5B
YJK3954	*pep3*Δ::kanMX4 chrI(199456-199457)::P_{GPD1}-*VMA1*-TermCYC1-*URA3* pRS315-HA_3-*SCH9*	5B
YJK3955	*pep3*Δ::kanMX4 chrI(199456-199457)::P_{GPD1}-*VPH2*-$Term_{CYC1}$-*URA3* pRS315-HA_3-*SCH9*	5B
YJK3956	*pep5*Δ::kanMX4 chrI(199456-199457)::P_{GPD1}-$Term_{CYC1}$-*URA3* pRS315-HA_3-*SCH9*	5B
YJK3958	*pep5*Δ::kanMX4 chrI(199456-199457)::P_{GPD1}-*VMA1*-$Term_{CYC1}$-*URA3* pRS315-HA_3-*SCH9*	5B
YJK3959	*pep5*Δ::kanMX4 chrI(199456-199457)::P_{GPD1}-*VPH2*-$Term_{CYC1}$-*URA3* pRS315-HA_3-*SCH9*	5B
YJK3856	*pep3*Δ::kanMX4 YEplac195	5C
Y5116	*sch9*Δ::kanMX4	S1
YJK3378	*pep3*Δ::kanMX4 pJU1064 pMKV002	S2
YJK3379	*pep5*Δ::kanMX4 pJU1064 pMKV002	S2
YJK3380	*vps16*Δ::kanMX4 pJU1064 pMKV002	S2
YJK3381	*gtr1*Δ::kanMX4 pJU1064 pMKV002	S2
YJK3382	pJU1064 pMKV002	S2
Y4777	*pep3*Δ::kanMX4	Table 1
Y4778	*pep5*Δ::kanMX4	Table 1
Y4795	*gtr1*Δ::kanMX4	Table 1
Y4796	*gtr2*Δ::kanMX4	Table 1
Y4798	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4	Table 1
YJK3787	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 *pep3*Δ::natMX4	Table 1
YJK3789	*gtr1*Δ::kanMX4 *gtr2*Δ::hphMX4 *pep5*Δ::natMX4	Table 1

RL58-1a derived strains

RL58-1a	*MAT***a** *leu2-3,112 ura3-52 trp1 HIS4 his3 rme1 HML***a** *LST8*-3HA-kanMX4	
YMC48	YCplac22	4B

YMC47	pRS314-*KOG1*-FLAG	4B
YJK3375	*vps17*Δ::natMX4 pRS314-*KOG1*-FLAG	4B
YJK3376	*vps17*Δ::natMX4 pRS314	4B
YMC50	*pep3*Δ::natMX4 pRS314	4B
YMC49	*pep3*Δ::natMX4 pRS314-*KOG1*-FLAG	4B

Table S2 Plasmids used in this study.

Plasmid	Description	(Reference) Source
pJK28	*CEN6 URA3 LYS2 LEU2*	This study
pJU675	*CEN6 URA3 SCH9*	(URBAN *et al.* 2007) R. Loewith
pJU822	*CEN6 URA3 SCH9* (*T723A, S726A, T737A, S758A, S765A*) (*SCH9^{5A}*)	(URBAN *et al.* 2007) R. Loewith
pJU841	*CEN6 URA3 SCH9* (*T723D, S726D, T737E, S758E, S765E*) (*SCH9^{2D3E}*)	(URBAN *et al.* 2007) R. Loewith
pJU1064	*CEN6 HIS3 SCH9*^{T570A}-HA_5	(BINDA *et al.* 2009) R. Loewith
pMKV002	*CEN6 URA3 LYS2*	J. McCusker
pMY22	*CEN6 URA3 TOR1*	T. Maeda
pMY28	*CEN6 URA3 TOR1-LM*	T. Maeda
pPC8a	*2μ URA3 gtr1-Q65L* (*GTR1*-GTP) *gtr2-S23N* (*GTR2*-GDP)	This study
pPC9a	*2μ URA3 GTR1 GTR2*	This study
pPC10	*CEN6 HIS3 HA_3-SCH9*	This study
pRS314	*CEN6 TRP1*	(SIKORSKI and HIETER 1989)
pRS314-*KOG1*-FLAG	*CEN6 TRP1 KOG1*-FLAG	(NAKASHIMA *et al.* 2008) Y. Kamada
pRS315-HA_3-*SCH9*	*CEN6 LEU2 HA_3-SCH9*	(MORANO and THIELE 1999) S. Zheng
pRS416	*CEN6 URA3*	(SIKORSKI and HIETER 1989)
YCplac22	*CEN4 TRP1*	(GIETZ and SUGINO 1988)
YEplac195	*2μ URA3*	(GIETZ and SUGINO 1988)
pAG306-GPD chr 1	P_{GPD1}-$Term_{CYC1}$ *URA3* chr1 (partial)	(HUGHES and GOTTSCHLING 2012) A. Hughes
pAG306-GPD-*VMA1* chr 1	P_{GPD1}-*VMA1*-$Term_{CYC1}$ *URA3* chr1 (partial)	(HUGHES and GOTTSCHLING 2012) A. Hughes
pAG306-GPD-*VPH2* chr 1	P_{GPD1}-*VPH2*-$Term_{CYC1}$ *URA3* chr1 (partial)	(HUGHES and GOTTSCHLING 2012) A. Hughes

Table S3 Oligonucleotides used in this study.

Name	Sequence (5'-3')[a]	Purpose
JK5	GTATTCTGGGCCTCCATGTC	Confirmation of gene disruption by MX4 cassette
JK6	GACATCATCTGCCCAGATGC	Confirmation of gene disruption by MX4 cassette
JK97	CCGTACTGTACCCTTAGTCAATCCATCTATCCTCTGAACACAGCTGAAGCTTCGTACGC	*VPS17* disruption with MX4 cassette
JK98	ACCTTGTTCAAAGGTATGAATTTTCTACTTTATATACGTAGCATAGGCCACTAGTGGATC TG	*VPS17* disruption with MX4 cassette
JK99	CTCATTATGTCAACCAGACC	*VPS17* disruption confirmation
JK100	GAGAAATAGCGGTAGACTTG	*VPS17* disruption confirmation
JK121	GCATTTTAACGAAGAGTATATACCTACTATTAGACATTAACAGCTGAAGCTTCGTACGC	*VPS41* disruption with MX4 cassette
JK122	AAGTGTACACTTGCCTTGTGTATTAAATGATGATTCGATAGCATAGGCCACTAGTGGATC TG	*VPS41* disruption with MX4 cassette
JK123	GATGTATACTTGCACCTGAC	*VPS41* disruption confirmation
JK124	CATCTTGAAGGTGCTGTTTC	*VPS41* disruption confirmation
JK125	GCAAAAACCCTTCAAAATATCAATTTATACCAAAAATTAACAGCTGAAGCTTCGTACGC	*VPS39* disruption with MX4 cassette
JK126	AAGAAATACTAACAACAATAACAGCAGCTGTTAAGGGATCGCATAGGCCACTAGTGGAT CTG	*VPS39* disruption with MX4 cassette
JK135	AAATATATATCTGCCGAGACCATTACTCATTACACCTAGACAGCTGAAGCTTCGTACGC	*VPS8* disruption with MX4 cassette
JK136	ACTTTTATGTAACCAAAGTTGTATTAAATATTTAGAAATGGCATAGGCCACTAGTGGATC TG	*VPS8* disruption with MX4 cassette
JK137	GGATATGGCTAAGGAAAAGC	*VPS8* disruption confirmation
JK164	TCCAAAAATCATAGCGTTTCATCTATAGGCACAGCAAATCCAGCTGAAGCTTCGTACGC	*PEP5* disruption with MX4 cassette
JK165	TTGTGACGCGGCGGCGACAATAGATCGTTATGATCCATCAGCATAGGCCACTAGTGGAT CTG	*PEP5* disruption with MX4 cassette
JK166	GAACTTGCCTGAACACATTG	*PEP5* disruption confirmation
JK167	CTTGCTGGTCGTTTATTCGT	*PEP5* disruption confirmation
JK172	GTGAATTGTAATACGACTCACTATAGGGCGAATTGGAGCTACATCGAGACCAAGAAGA AC	*LEU2* amplification for pJK28 construction
JK173	GACATGATCGGAACACCGTCTGGTAGAATTCCTGCAGCCCAATGGTCAGGTCATTGAGT G	*LEU2* amplification for pJK28 construction
JK236	TCTAAGCTATAAAAAAATATCCCTTTTATCACACAAAAAACAGCTGAAGCTTCGTACGC	*VPS21* disruption with MX4 cassette
JK237	TGCCCAGACTTTTTTTTTATATATATTTATTTTCCCCTCTGCATAGGCCACTAGTGGATCTG	*VPS21* disruption with MX4 cassette
JK238	GCTCACCATCAGGATCAAAC	*VPS21* disruption confirmation
JK239	GACACTGAAGATGATGCTGG	*VPS21* disruption confirmation
JK240	ACGATAGGGTATTGAACACC	*VPS39* disruption confirmation
JK241	ACGTCAAGGCCATCGAAATG	*VPS8* disruption confirmation

JK242	TCCTTCGACCACTAGAAATG	*VPS8* disruption confirmation
NDMC64	ATCTCACTGATTTCGATG	*GTR1* disruption confirmation
NDMC65	TCGCCATTGGTGACAATGC	*GTR1* disruption confirmation
NDMC71	AAGGCCATCAAAATCACGTTTATCAATCGACAATTTAGTACGGATCCCCGGGTTAATTAA	*GTR1* disruption with MX4 cassette
NDMC72	CAAACACTCAATTGCCGAATGTTTCGTCTACTCACCTCAGGAATTCGAGCTCGTTTAAAC	*GTR1* disruption with MX4 cassette
NDMC76	AATATGTGTAACTAAGCC	*VPS39* disruption confirmation
NDMC77	ACTGCGCTAATGGCCTAC	*VPS39* disruption confirmation
NDMC114	TTTACCACGATAAGTCGC	Amplification of *pep3*Δ::MX4 allele for disruption
NDMC115	TTATGTTGAACTTCAGCC	Amplification of *pep3*Δ::MX4 allele for disruption
NDMC116	AATAATAGAGGCACATCC	*PEP3* disruption confirmation

[a] Underlined sequence is homologous to vector sequence

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