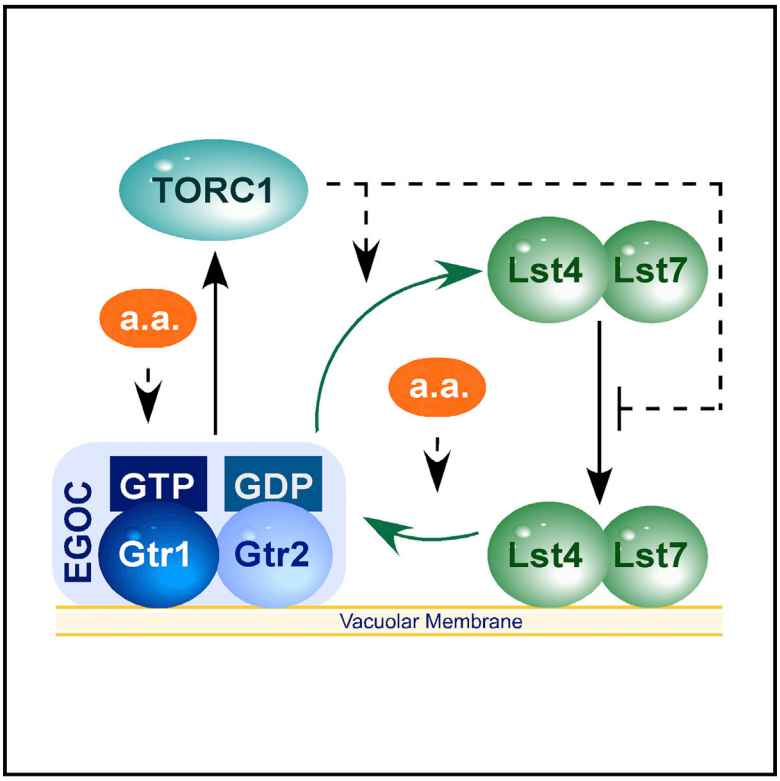
Cell Reports

Report

Amino Acids Stimulate TORC1 through Lst4-Lst7, a GTPase-Activating Protein Complex for the Rag Family GTPase Gtr2

Graphical Abstract



Authors

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In Brief

Amino acids represent primordial signals that modulate TORC1 and, consequently, eukaryotic cell growth through conserved Rag GTPases. Here, Péli-Gulli et al. show that the Lst4-Lst7 complex in yeast functions as a GAP for the Rag family GTPase Gtr2 to mediate amino-acid-dependent activation of TORC1.

Highlights

- The Lst4-Lst7 complex in yeast is necessary for TORC1 activation by amino acids
- Rag GTPases associate with Lst4-Lst7 in response to amino acid stimulation
- The Lst4-Lst7 complex functions as a GAP for the Rag family GTPase Gtr2
- A TORC1-dependent feedback mechanism attenuates Lst4-Lst7 function

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Amino Acids Stimulate TORC1 through Lst4-Lst7, a GTPase-Activating Protein Complex for the Rag Family GTPase Gtr2

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SUMMARY

Rag GTPases assemble into heterodimeric complexes consisting of RagA or RagB and RagC or RagD in higher eukaryotes, or Gtr1 and Gtr2 in yeast, to relay amino acid signals toward the growth-regulating target of rapamycin complex 1 (TORC1). The TORC1-stimulating state of Rag GTPase heterodimers, containing GTP- and GDP-loaded RagA/B/Gtr1 and RagC/D/Gtr2, respectively, is maintained in part by the FNIP-Folliculin RagC/D GAP complex in mammalian cells. Here, we report the existence of a similar Lst4-Lst7 complex in yeast that functions as a GAP for Gtr2 and that clusters at the vacuolar membrane in amino acid-starved cells. Refeeding of amino acids, such as glutamine, stimulated the Lst4-Lst7 complex to transiently bind and act on Gtr2, thereby entailing TORC1 activation and Lst4-Lst7 dispersal from the vacuolar membrane. Given the remarkable functional conservation of the RagC/D/Gtr2 GAP complexes, our findings could be relevant for understanding the glutamine addiction of mTORC1-dependent cancers.

INTRODUCTION

The target of rapamycin complex 1 (TORC1) plays a pivotal role in the control of eukaryotic cell growth by adjusting anabolic and catabolic processes to the nutritional status of organisms and of individual cells (Albert and Hall, 2015; Laplante and Sabatini, 2012). Amino acids represent primordial signals that modulate TORC1 activity through the conserved Rag family of GTPases (Jewell et al., 2013; Sancak and Sabatini, 2009), which assemble into heterodimeric complexes consisting of RagA or RagB and RagC or RagD in higher eukaryotes, or Gtr1 and Gtr2 in yeast (Binda et al., 2009; Kim et al., 2008; Sancak et al., 2008). The functionally active TORC1-stimulating state of these heterodimers contains guanosine 5′-triphosphate (GTP)-loaded RagA/B/Gtr1 and GDP-loaded RagC/D/Gtr2 and is maintained by an intricate interplay between distinct guanine nucleotide exchange factor (GEF) and GTPase-activating (GAP) protein complexes. In mammalian cells, these include (1) the pentameric Ragulator complex that tethers Rag heterodimers to the lysosomal membrane and acts as RagA/B GEF (Bar-Peled et al., 2012), (2) the heterotrimeric GATOR1 complex with RagA/B GAP activity (Bar-Peled et al., 2013), and (3) the heterodimeric FNIP-Folliculin complex that functions as RagC/D GAP (Petit et al., 2013; Tsun et al., 2013). The amino-acid-sensitive events upstream of these Rag GTPase regulators are currently poorly understood, but likely involve both lysosomal amino acid sensors, such as the v-ATPase and lysosomal amino acid transporter(s) (Rebsamen et al., 2015; Wang et al., 2015; Zoncu et al., 2011), and cytoplasmic amino acid sensors, such as the leucyl-tRNA synthetase (LeuRS) (Bonfils et al., 2012; Han et al., 2012).

Some of the regulatory mechanisms impinging on Rag GTPases have been remarkably conserved throughout evolution. Accordingly, yeast cells express a protein complex, coined the EGO complex (EGOC), that is structurally related to the Ragulator complex and that tethers Gtr1-Gtr2 to the vacuolar/lysosomal membrane, although it remains unknown whether it also exhibits Gtr1 GEF activity (Binda et al., 2009; Panchaud et al., 2013a, 2013b; Zhang et al., 2012b). In addition, the Gtr1 GAP complex termed SEACIT is functionally equivalent to GATOR1 and both GAP complexes are presumably inhibited in a similar manner by the yeast SEACAT and mammalian GATOR2 orthologous multi-subunit complexes (Bar-Peled et al., 2013; Panchaud et al., 2013a, 2013b). A Gtr2 GAP, however, has hitherto remained elusive.

Here, we report on our discovery that the heterodimeric Lst4-Lst7 complex in yeast functions as a GAP for Gtr2 to activate TORC1 following amino acid stimulation of cells. Like the functionally orthologous mammalian complex containing FNIP and Folliculin (Petit et al., 2013; Tsun et al., 2013), the Lst4-Lst7 complex is recruited to and released from the vacuolar surface upon amino acid starvation and refeeding, respectively. Our study suggests a model in which amino acids promote the Lst4-Lst7 complex to associate with and stimulate the GAP activity of the vacuolar membrane-resident fraction of Gtr2 in amino acid-starved cells, thereby triggering the activation of TORC1 and the release of Lst4-Lst7 from the vacuolar membrane.

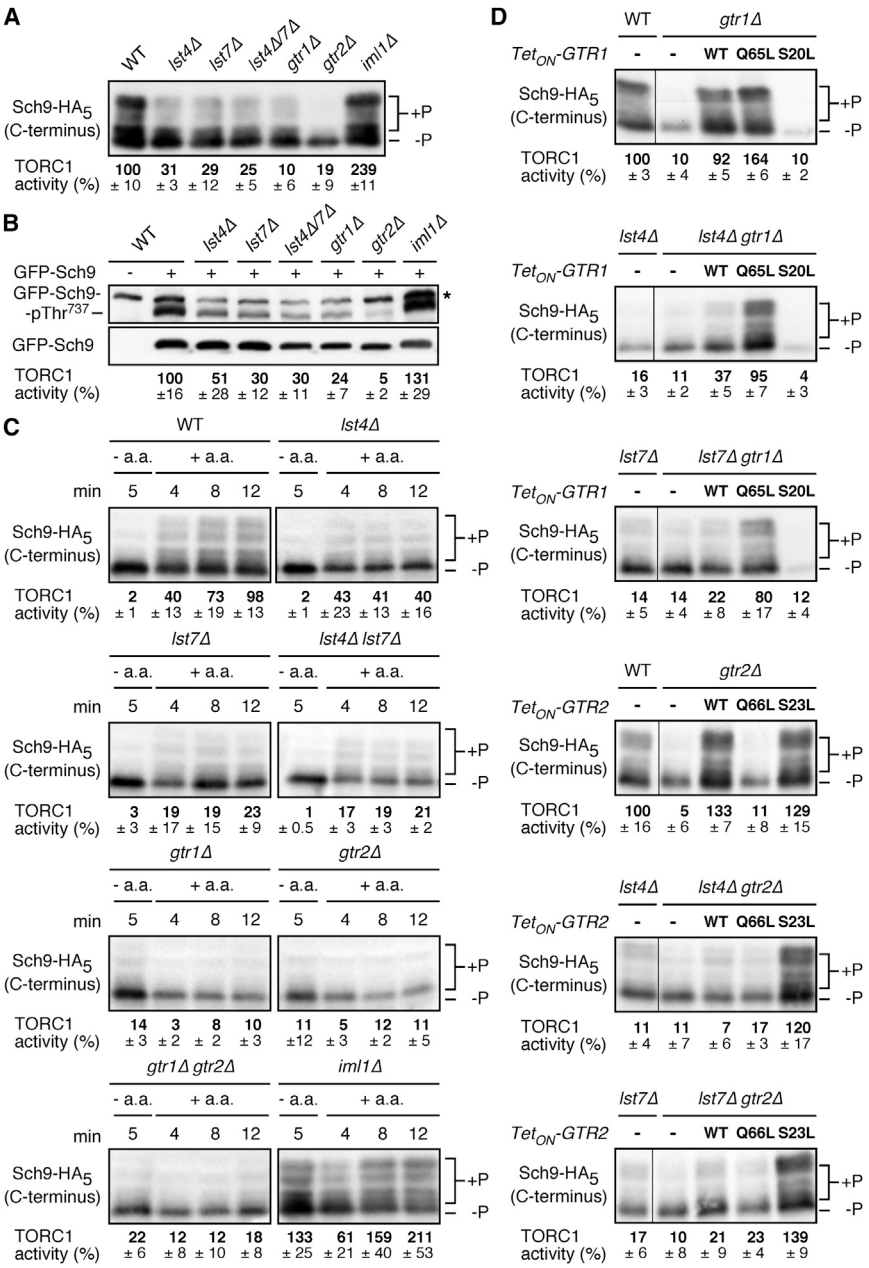

Figure 1. Lst4 and Lst7 Regulate Amino Acid Signaling to TORC1 through Gtr2

(A and B) Loss of Lst4 and/or Lst7 causes a decrease in TORC1 activity. In (A), indicated strains expressing Sch9^{T570A}-HA$_5$ were grown exponentially in synthetic dropout (SD) medium. A representative anti-HA immunoblot of NTCB-treated extracts is shown. The respective TORC1 activities were assessed as the ratio of hyper-phosphorylated (+P)/hypophosphorylated (−P) Sch9 C terminus and normalized to that of wild-type (WT) cells (set to 100%). In (B), similar results for TORC1 activities were obtained by using specific antibodies recognizing the phosphorylated Thr^{737} (pThr^{737}) of Sch9, a key target of TORC1 in yeast (Urban et al., 2007). Indicated strains expressing (+) or not (−) GFP-Sch9 were grown as in (A). Representative anti-pThr^{737} and anti-GFP immunoblots are shown together with respective TORC1 activities calculated as the ratio of pThr^{737} GFP-Sch9/total GFP-Sch9 and normalized to the TORC1 activity in exponentially growing WT cells (set to 100%). *, cross-reacting band. See also Figure S1 for further phenotypes associated with loss of Lst4 and/or Lst7.

(C) Loss of Lst4 and/or Lst7 renders TORC1 less responsive to amino acid stimulation. Strains (genotypes indicated) were grown to exponential phase in a synthetic complete medium devoid of ammonium sulfate, but containing a mixture of all amino acids as nitrogen source (SC w/o AS). TORC1 activities (normalized to the one in exponentially growing WT cells; set to 100%) were monitored as in (A) following starvation (− aa; 5 min) and readdition (+ aa; times indicated) of amino acids.

(D) Overproduction of signaling competent Gtr2^{S23L} fully rescues the TORC1 activity defects caused by loss of Lst4 or Lst7. Gtr1 or Gtr2 variants were overexpressed under the control of the *Tet$_{ON}$* promoter in the indicated mutants grown exponentially in SD medium. TORC1 activities were assayed as in (A) and normalized to the TORC1 activity in exponentially growing WT cells (set to 100%). Numbers in (A)–(D) are means ± SD from three independent experiments.

RESULTS AND DISCUSSION

Lst4 and Lst7 Are Necessary for TORC1 Activation via the Rag GTPase Gtr2

Recent studies employing highly sensitive methods for structural homology detection (e.g., HHpred) identified *Saccharomyces cerevisiae* Lst4 and Lst7 as potential orthologs of FNIP and Folliculin, respectively (Levine et al., 2013; Zhang et al., 2012a). Lst4 and Lst7 have been originally identified in a genetic screen designed to identify mutations, which exhibit synthetic lethality when combined with *sec13-1* (Roberg et al., 1997). Surprisingly, in addition to being part of both the nuclear pore complex and the outer shell of coatmer complex II coated vesicles (Hoelz et al., 2011), Sec13 is also a component of the SEACAT complex that is required for normal TORC1 activity (Panchaud et al., 2013a). Together with the observation that the presumed Lst7/Folliculin ortholog in *Schizosaccharomyces pombe* (i.e., BHD) reportedly stimulates TORC1 by unknown means (van Slegtenhorst et al., 2007), the current literature therefore suggests that Lst4 and Lst7 may, like FNIP and Folliculin, control TORC1 function via Rag GTPases. In support of this assumption, we found that loss of Lst4 or of Lst7, like loss of Gtr1 or of Gtr2, resulted in decreased TORC1 activity (Figures 1A and 1B). The

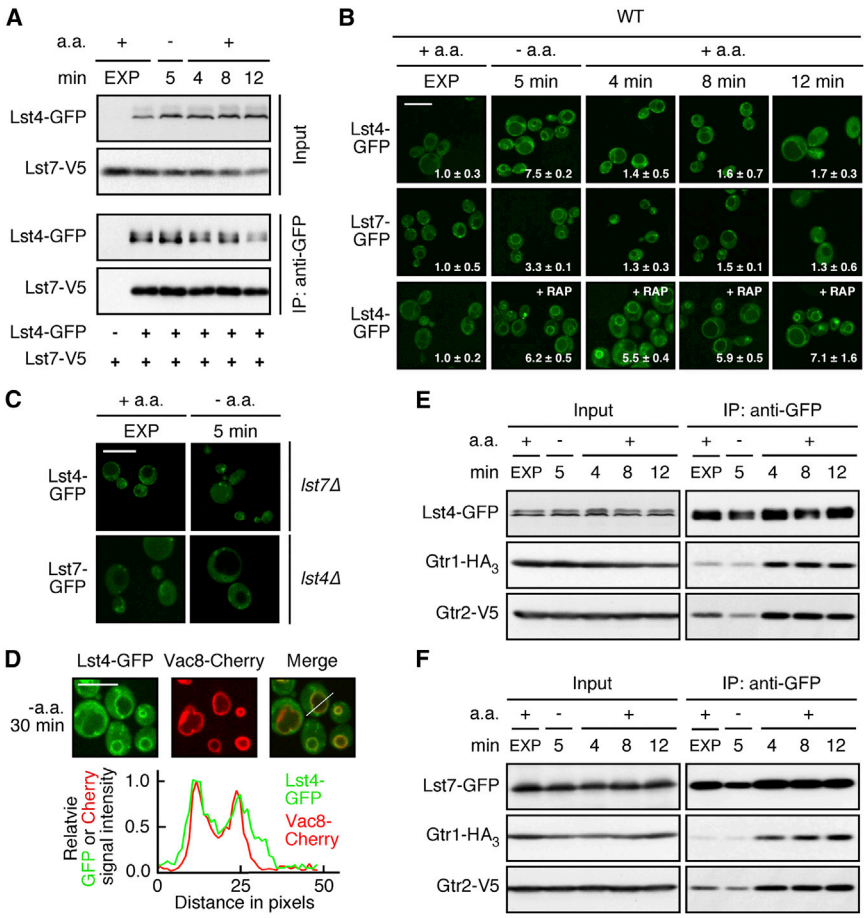

Figure 2. Lst4 and Lst7 Form a Complex that Assembles at the Vacuolar Membrane upon Amino Acid Starvation and Interacts with Rag GTPases in Response to Amino Acid Refeeding

(A) Lst4 stably binds Lst7 in the presence (aa: +) or absence (aa: −) of amino acids. Lst7-V5 cells co-expressing (+) or not (−) Lst4-GFP were grown as in Figure 1C. Lysates (input) and anti-GFP immunoprecipitates (IP: anti-GFP) were analyzed by immunoblotting with anti-GFP and anti-V5 antibodies.

(B) Amino acid starvation and readdition promote Lst4 and Lst7 recruitment to and redistribution from the vacuolar membrane, respectively. Representative pictures of GFP-tagged Lst4- or Lst7-expressing cells, cultured as in Figure 1C, are shown. Rapamycin (+ RAP) was also added to Lst4-GFP-expressing cells at the beginning of the amino acid starvation and maintained throughout the amino acid restimulation period. Numbers represent fold increases in the vacuolar membrane GFP signal intensity, normalized to respective signal in exponentially growing (EXP) cells (set to 1.0). Data are means ± SD from three independent experiments. Scale bar for all panels (white; top left), 5 μm.

(C) Lst4-GFP and Lst7-GFP depend on each other for their recruitment to the vacuolar membrane in both exponentially growing (+ aa; EXP) and amino-acid-starved (− aa; 5 min) cells. Scale bar for all panels (white; top left), 5 μm.

(D) Lst4-GFP colocalizes with the vacuolar membrane resident Vac8-Cherry. The lower graph shows the combined fluorescence intensity profiles of Lst4-GFP and Vac8-Cherry that were measured in an amino-acid-starved (30 min) cell along the defined line in the merged panel. Scale bar (white; first panel), 5 μm.

(E and F) Amino acids stimulate the interaction between Lst4-Lst7 and Gtr1-Gtr2 in amino-acid-starved cells. Lst4-GFP (E) or Lst7-GFP (F) were IPed in extracts from cells that co-expressed Gtr1-HA_3 and Gtr2-V5 and that were grown as in Figure 1C. Cell lysates (input) and anti-GFP immunoprecipitates (IP: anti-GFP) were analyzed by immunoblotting using anti-GFP, anti-HA, and anti-V5 antibodies.

See also Figure S2.

concomitant loss of Lst4 and Lst7 decreased TORC1 activity (and caused rapamycin sensitivity and a mild defect in recovery from rapamycin treatment) to a similar extent to the individual loss of Lst4 or Lst7 (Figures 1A, 1B, and S1), indicating that Lst4 and Lst7 may share a common biological function in TORC1 stimulation. Next, we studied the effect of amino acid readdition to wild-type, *lst4Δ*, *lst7Δ*, *lst4Δ lst7Δ*, *gtr1Δ*, *gtr2Δ*, *gtr1Δ gtr2Δ*, and *iml1Δ* cells that had been subjected to amino acid starvation (for 5 min), following which all strains exhibited very low TORC1 activity (except the *iml1Δ* control strain that is defective in SEACIT/Gtr1 GAP activity; Panchaud et al., 2013b). Readdition of amino acids strongly elicited TORC1 activity in wild-type cells within 4–12 min, while this effect was significantly reduced in the absence of Lst4 and/or Lst7 and virtually undetectable in the absence of Gtr1 and/or Gtr2 (Figure 1C). Thus, Lst4 and Lst7 are important for proper amino acid stimulation of TORC1, possibly through regulation of the Gtr1-Gtr2 heterodimer. Consistent with this idea, the reduced TORC1 activity in exponentially growing *lst4Δ* and *lst7Δ* cells could be suppressed by expression of either the GTP-locked $Gtr1^{Q65L}$ or the $Gtr2^{S23L}$ variant (which has low affinity for nucleotides; Figure 1D). Since TORC1 activity remained to some extent sensitive to the loss of Lst4 or Lst7 in the presence of the $Gtr1^{Q65L}$, but not in the presence of the $Gtr2^{S23L}$ form, our genetic data suggested that Lst4 and Lst7 specifically act upstream of Gtr2.

Lst4 and Lst7 Form a Complex that Interacts with Rag GTPases in Response to Amino Acid Stimulation of Cells

Our genetic epistasis analyses led us to examine next whether Lst4 interacted with Lst7 in cells using co-immunoprecipitation (coIP) assays. Lst4 specifically and stably bound Lst7 in exponentially growing cells, as well as in cells that were starved for and subsequently restimulated with amino acids (Figure 2A). Our analyses of functional GFP-fused Lst4 and Lst7 further indicated that both proteins were mainly present in the cytoplasm in exponentially growing cells with minor fractions being localized to the vacuolar membrane (Figure 2B), where EGOC and TORC1 primarily reside in yeast (Binda et al., 2009; Sturgill

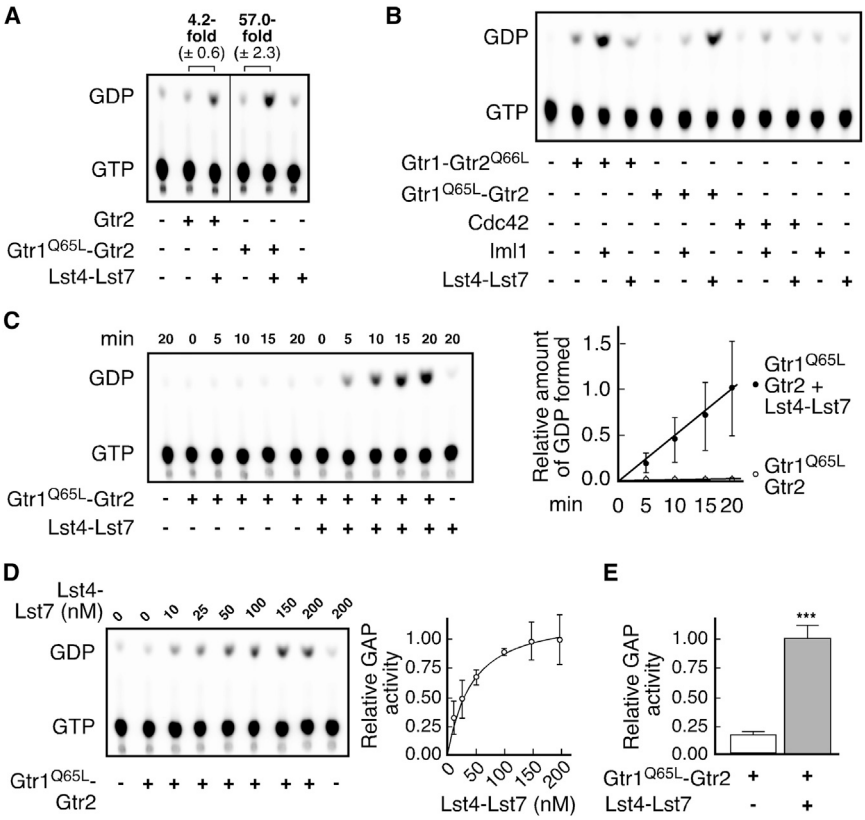

Figure 3. The Lst4-Lst7 Complex Functions as a GAP for Gtr2

(A) The Lst4-Lst7 complex stimulates the GTP hydrolysis activity by Gtr2 particularly within the Gtr1^{Q65L}-Gtr2 heterodimer. Purified Gtr2 and Gtr1^{Q65L}-Gtr2 were pre-loaded with [α-^{32}P] GTP. Following a 20-min incubation in the absence (–) or presence (+) of purified Lst4-Lst7, the extent of [α-^{32}P] GTP hydrolysis to [α-^{32}P] GDP was examined. One representative thin-layer chromatography (TLC) autoradiograph is shown. Numbers represent fold increases ± SD from three independent experiments. GTP was incubated either alone (first lane) or with the Lst4-Lst7 complex solely (last lane) in control experiments.

(B) The GAP activities of the Lst4-Lst7 complex and of Iml1 are specific for Gtr2 and Gtr1, respectively. GAP specificity was determined by comparing the GAP activities of the Lst4-Lst7 complex and of Iml1 toward Gtr1^{Q65L}-Gtr2 (with Gtr1 in its GTP-locked state), Gtr1-Gtr2^{Q66L} (with Gtr2 in its GTP-locked state), and the unrelated Rho GTPase Cdc42. Control experiments were as in (A).

(C and D) The Lst4-Lst7 complex stimulates GTP hydrolysis by Gtr1^{Q65L}-Gtr2 in a time- (C) and concentration- (D) dependent manner. GAP assays were performed as in (A) with indicated reaction times (C) or increasing concentrations of Lst4-Lst7 (D). Representative TLC autoradiographs and quantifications (± SD from three independent experiments) are shown.

(E) Single-turnover GAP assays on Gtr1^{Q65L}-Gtr2 in the absence (–) or presence (+) of Lst4-Lst7. Data are means ± SD from three independent experiments. ***$p < 0.003$, compared to the control assay without Lst4-Lst7 using an unpaired Student's t test.

et al., 2008). Provided that each of the two proteins was present, amino acid starvation rapidly provoked an enrichment of Lst4-GFP and Lst7-GFP at the vacuolar membrane, which was promptly reversed upon readdition of amino acids (Figures 2B–2D). Together with our genetic and biochemical data, these cell biological analyses suggested that Lst4 and Lst7 assemble into a complex that mediates amino acid regulation of TORC1 via Gtr2, within the Gtr1-Gtr2 module. In support of this notion, Gtr2 and Gtr1 coIPed with Lst4-GFP and Lst7-GFP in exponentially growing cells (Figures 2E and 2F). Moreover, amino acid starvation (for 5 min and up to 50 min) slightly weakened the interactions between Gtr1-Gtr2 and Lst4 or Lst7, while subsequent stimulation of starved cells with amino acids substantially strengthened the respective interactions (Figures 2E, 2F, and S2). Thus, amino acid starvation triggers the recruitment of the Lst4-Lst7 complex to the vacuolar membrane, where it is adjacent to, but not directly associated with Gtr1-Gtr2, while subsequent stimulation of cells with amino acids promotes the interaction between the Lst4-Lst7 complex and Gtr1-Gtr2, as well as the release of Lst4-Lst7 from the vacuolar membrane.

The Lst4-Lst7 Complex Is a GAP for Gtr2

Combined, our data suggested that the Lst4-Lst7 complex activates TORC1 following amino acid stimulation of cells indirectly, possibly by functioning as a GAP for Gtr2. To verify this assumption, we performed in vitro GAP assays with purified Lst4-Lst7 and Gtr2 alone or within a heterodimer containing GTP-locked Gtr1^{Q65L} (Binda et al., 2009). Lst4-Lst7 stimulated the rate of GTP hydrolysis by monomeric Gtr2 and heterodimeric Gtr1^{Q65L}-Gtr2 by factors of 4.2 and 57.0, respectively (Figure 3A). A control experiment with purified Lst4-Lst7 and free GTP showed minimal GTP hydrolysis, excluding the possibility that the observed GTP hydrolysis was due to contaminating phosphatases (Figure 3A). In addition, unlike the Gtr1 GAP Iml1 (Panchaud et al., 2013b), Lst4-Lst7 did not noticeably stimulate the rate of GTP hydrolysis by Gtr1 (within a Gtr1-Gtr2^{Q66L} complex containing the GTP-locked form of Gtr2) (Figure 3B). Conversely, Iml1, unlike Lst4-Lst7, only marginally affected the rate of GTP hydrolysis by Gtr2 (within the Gtr1^{Q65L}-Gtr2 complex), and both Iml1 and Lst4-Lst7 had little impact on the rate of GTP hydrolysis by the unrelated Rho GTPase Cdc42 (Figure 3B). Finally, in addition to stimulating the GTP hydrolysis by Gtr2 in a time- and dose-dependent manner in steady-state GAP assays (Figures 3C and 3D), Lst4-Lst7 also significantly accelerated the catalytic rate of Gtr2-mediated GTP hydrolysis in single-turnover GAP assays (Figure 3E). The Lst4-Lst7 complex therefore functions as a bona fide GAP for Gtr2, specifically within the context of the Gtr1-Gtr2 heterodimer.

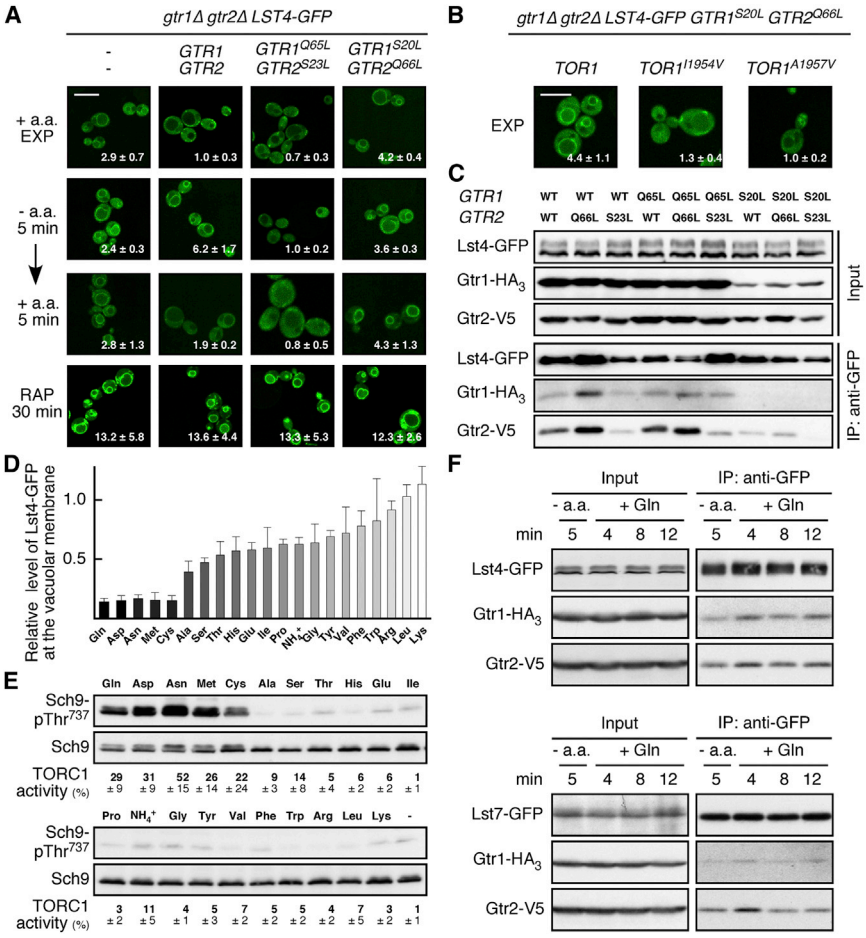

Figure 4. Amino Acids Act Upstream of the Lst4-Lst7 Complex

(A and B) Enrichment of Lst4-GFP at the vacuolar membrane does not depend on Rag GTPases and is antagonized by TORC1. The *gtr1*Δ *gtr2*Δ *LST4-GFP* strain carrying either empty plasmids (−/−) or the indicated combinations of plasmid-encoded alleles of *GTR1* and *GTR2* in the absence (A) or presence of a copy of the indicated *TOR1* alleles (B) were analyzed by fluorescence microscopy during exponential growth (+ aa; EXP), following amino acid starvation (− aa; 5 min) and subsequent amino acid replenishment (+ aa; 5 min) and following rapamycin treatment (+ RAP; 30 min). Numbers represent fold increases in the vacuolar membrane GFP-signal intensity, normalized to respective signal (set to 1.0) in control cells (i.e., exponentially growing *gtr1*Δ *gtr2*Δ *LST4-GFP* cells expressing WT *GTR1* and *GTR2* from plasmids). The scale bar (white; 5 μm) in the top left panel in (A) and in (B) applies to all panels in (A) and in (B), respectively.

(C) Lst4-GFP preferentially binds Gtr2 and $Gtr2^{Q66L}$. Lst4-GFP was IPed in extracts from exponentially growing cells that co-expressed the indicated variants of Gtr1-HA_3 and Gtr2-V5. Cell lysates (input) and anti-GFP immunoprecipitates (IP: anti-GFP) were analyzed by immunoblotting using anti-GFP, anti-HA, and anti-V5 antibodies.

(D and E) Glutamine (Gln), aspartate (Asp), asparagine (Asn), methionine (Met), and cysteine (Cys) are highly competent in displacing Lst4-GFP from the vacuolar membrane (D) and reactivating TORC1 (E) in amino-acid-starved cells. Lst4-GFP-expressing WT cells were starved for 5 min for all amino acids, restimulated for 12 min with individual amino acids (each at a final concentration of 3 mM) or NH_4^+ (37.7 mM), and then analyzed for Lst4-GFP localization and TORC1 activity. The Lst4-GFP signal at the vacuolar membrane was expressed relative to a control sample (set to 1.0) that received no amino acids and TORC1 activities (i.e., Sch9-$pThr^{737}$/total Sch9) were normalized to the ones of untreated exponentially growing cells (set to 100%). −aa, no amino acids added (E). All data are means ± SD from three independent experiments. See also Figure S3A.

(F) Glutamine transiently stimulates the interaction between Lst4 and Gtr2 in amino-acid-starved cells. Cells co-expressing Lst4-GFP, Gtr1-HA_3, and Gtr2-V5 were starved for amino acids (− aa; 5 min) and then restimulated with 3 mM glutamine (+ Gln) for the indicated times. Cell lysates (input) and anti-GFP immunoprecipitates (IP: anti-GFP) were analyzed by immunoblotting with anti-GFP, anti-HA, and anti-V5 antibodies.

See also Figures S3B and S3C.

TORC1 Antagonizes the Vacuolar Membrane Enrichment of the Lst4-Lst7 Complex

The mammalian FNIP-Folliculin complex preferentially associates with and docks to the lysosomal membrane via the inactive form of the Rag GTPase heterodimer that prevails under amino acid starvation conditions (Petit et al., 2013; Tsun et al., 2013). We were therefore surprised to find that the Lst4-Lst7 complex, despite its recruitment to the vacuolar membrane, appeared to be slightly compromised for Gtr1-Gtr2 binding in amino-acid-starved cells (Figures 2E and 2F). We therefore considered the possibility that the vacuolar membrane recruitment of the Lst4-Lst7 complex may not require the presence of Rag GTPases. This was indeed the case, as loss of Gtr1 and Gtr2 per se prompted the enrichment of Lst4-GFP at the vacuolar membrane in exponentially growing cells (Figure 4A). Conspicuously, expression of the active $Gtr1^{Q65L}$-$Gtr2^{S23L}$ heterodimer not only reduced the level of vacuolar membrane-resident Lst4-GFP in exponentially growing cells, but also prevented the accumulation of Lst4-GFP at the vacuolar membrane in amino-acid-starved cells (Figure 4A). Expression of the inactive $Gtr1^{S20L}$-$Gtr2^{Q66L}$ heterodimer, in contrast, resulted in constitutively high levels of Lst4-GFP recruitment to the vacuolar membrane, independently of whether the cells were starved for or fed with amino acids. An interpretation that fits well with all of these results is that the Rag GTPases in yeast regulate the vacuolar membrane localization of the Lst4-Lst7 complex indirectly via TORC1. Two sets of additional observations support this assumption. First, addition of rapamycin stimulated the accumulation of Lst4-GFP at the vacuolar membrane in exponentially growing cells (even in the presence of the active $Gtr1^{Q65L}$-$Gtr2^{S23L}$ heterodimer; Figure 4A) and precluded the redistribution of Lst4-GFP to the cytoplasm upon refeeding of

amino acids in starved cells (Figure 2B). Second, the expression of the hyperactive *TOR1*^{I1954V} and *TOR1*^{A1957V} alleles (Reinke et al., 2006), unlike wild-type *TOR1*, fully suppressed the constitutive vacuolar membrane-enrichment of Lst4-GFP in *gtr1Δ gtr2Δ* cells expressing *GTR1*^{S20L}/*GTR2*^{Q66L} (Figure 4B). Thus, the enrichment of Lst4-Lst7 at the vacuolar membrane does not require Rag GTPases and is antagonized by TORC1, which is apparently not the case for FNIP-Folliculin in mammalian cells (Petit et al., 2013; Tsun et al., 2013). Not surprisingly, therefore, the affinity of Lst4 to the different combinations of Rag GTPase alleles also did not recapitulate the reported preference of the FNIP-Folliculin module for the GDP-free RagA allele (Petit et al., 2013; Tsun et al., 2013). Accordingly, Lst4 only weakly bound Gtr1 or the rather unstable GDP-free Gtr1^{S20L} allele, but associated well with Gtr2, specifically in its GTP-locked state, which is a property that it shares with many other GAPs and their cognate GTPases (Figure 4C).

Our findings predicted that the Lst4-Lst7 complex, once tethered to the vacuolar membrane in amino-acid-starved cells, requires an amino-acid-dependent signal to activate TORC1 via Gtr2 and be released from the vacuolar membrane. To begin to study the respective mechanism(s), we asked whether the Lst4-Lst7 complex responds to certain amino acids more specifically. Interestingly, most amino acids (and NH_4^+) were, to some extent, able to displace Lst4-GFP from the vacuolar membrane (Figures 4D and S3A). However, glutamine (as well as aparagine and aspartate, which both can be specifically deaminated and readily be converted to glutamate/glutamine) and methionine (as well as cysteine, which can serve as precursor for methionine biosynthesis) (Ljungdahl and Daignan-Fornier, 2012) were exclusively potent in both displacing Lst4-GFP from the vacuolar membrane and reactivating TORC1 in amino-acid-starved cells (Figures 4D and 4E). Since glutamine plays an essential role in anabolic metabolism (e.g., in purine and pyrimidine synthesis) and is under homeostatic control by, and plays a pivotal role in, TORC1 regulation in yeast (Laxman et al., 2014; Ljungdahl and Daignan-Fornier, 2012), we specifically studied the effects of glutamine addition to amino-acid-starved cells. Accordingly, glutamine transiently reinforced the interaction between Lst4-Lst7 and Gtr2 and reactivated TORC1 in an Lst4-, Lst7-, and Gtr1-Gtr2-dependent manner when added to amino-acid-starved cells (Figures 4F, S3B, and S3C). All together, these data are best explained in a model in which glutamine (and presumably also other amino acids, such as methionine) stimulates the Lst4-Lst7 complex to act on the vacuolar membrane-resident fraction of Gtr2, thereby entailing its subsequent release into the cytoplasm. In this model, Rag GTPase-dependent activation of TORC1 is part of a feedback inhibitory loop that favors Lst4-Lst7 removal from or prevents Lst4-Lst7 docking to Gtr2-proximal sites at the vacuolar membrane. This could also elegantly explain the previously reported transient nature of the rapid Rag GTPase-dependent response of TORC1 to glutamine addition (Stracka et al., 2014).

Glutamine and glutamine-derived metabolite(s) activate TORC1 via Rag GTPase-dependent and/or Rag GTPase-independent ways (Durán et al., 2012; Jewell et al., 2015; Nicklin et al., 2009; Stracka et al., 2014), although the underlying mechanisms remain largely to be discovered. In this context, our present study pinpoints the Lst4-Lst7 complex as an important node that likely channels (among others) glutamine signals via the Rag GTPases to TORC1. Given the surprising functional conservation of the Lst4-Lst7 complex, it will therefore be interesting to determine whether the glutamine addiction of certain mTORC1-dependent cancers (Wise and Thompson, 2010) may in part be mediated by the FNIP-Folliculin complex.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Plasmids

Unless stated otherwise, prototrophic strains were pre-grown overnight in synthetic dropout (SD) medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% dropout mix [USBiological], and 2% glucose) to maintain plasmids. Before each experiment, cells were harvested by centrifugation and diluted to an OD_{600} (optical density at 600 nm) of 0.2 and further grown at 30°C in synthetic complete medium without ammonium sulfate (SC w/o AS; 0.17% yeast nitrogen base, 0.2% of the complete mix of all amino acids [i.e., dropout mix complete (USBiological)], and 2% glucose) until they reached an OD_{600} of 0.8. For amino acid deprivation experiments, cells were filtered and transferred to amino acid starvation medium (SM, which is SC w/o AS, but lacking all amino acids). For restimulation by all amino acids, cells in SM medium were filtered and transferred back to SC w/o AS. For restimulation with single compounds, cells in SM medium were supplemented with a final concentration of 37.7 mM ammonium sulfate or of 3 mM of the indicated amino acid. The *S. cerevisiae* strains and plasmids used in this study are listed in Tables S1 and S2, respectively.

TORC1 Activity Assays

TORC1 activity was quantified by assessing the phosphorylation of the C-terminal part of hemagglutinin (HA) -tagged Sch9^{T570A}, which contains five bona fide TORC1 phosphorylation sites and a mutation in the Pkh1/2-dependent activation loop residue Thr^{570}, as previously described (Urban et al., 2007). Alternatively, TORC1 activity was assessed as the ratio between the phosphorylation on Thr^{737} of full-length Sch9 (or GFP-Sch9) compared to the total abundance of Sch9 (or GFP-Sch9) using phosphospecific anti-pThr^{737}-Sch9 produced by GenScript and anti-Sch9 (or anti-GFP) antibodies, respectively.

GTP Hydrolysis Assays

GAP assays were performed as previously described (Panchaud et al., 2013b). Briefly, 100 nM of purified GTPase were incubated for 30 min at room temperature in loading buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA, and 1 mM DTT) in the presence of 40 nM [α-^{32}P]-GTP (Hartman Analytic; 3,000 Ci/mmol). Unless otherwise indicated, 200 nM of His_6-Lst4/His_6-Lst7 or Iml1-His_6 were then added to the mix, together with 10 mM $MgCl_2$ to initialize the reaction. Reactions were stopped after 20 min of incubation at room temperature by the addition of elution buffer (1% SDS, 25 mM EDTA, 5 mM GDP, and 5 mM GTP), and samples were then heat denatured for 2 min at 65°C. Single turnover GAP assays were performed as described above, except that 1.7 mM unlabeled GTP was added at the same time as $MgCl_2$. The concentration of His_6-Lst4/His_6-Lst7 was constant (200 nM), and samples were taken at times 0 and 30 min. [α-^{32}P]-GTP and [α-^{32}P]-GDP were separated by thin-layer chromatography (TLC) on PEI Cellulose F Plates (Merck) with buffer containing 1.0 M acetic acid and 0.8 M LiCl. Results were visualized using a phosphorimager and quantified with ImageQuant software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.059.

AUTHOR CONTRIBUTIONS

M.-P.P.-G., A.S., N.P., and C.D.V. are responsible for the conception and design of the study. M.-P.P.-G., A.S., N.P., and S.R. performed all the

experiments. C.D.V. directed the project, prepared all the figures, and wrote the manuscript. All authors discussed and interpreted the data together.

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