SEACing the GAP that nEGOCiates TORC1 activation
Evolutionary conservation of Rag GTPase regulation

Nicolas Panchaud†, Marie-Pierre Péli-Gulli†, and Claudio De Virgilio*
Department of Biology; Division of Biochemistry; University of Fribourg; Fribourg, Switzerland
†These authors contributed equally to this work.

The target of rapamycin complex 1 (TORC1) regulates eukaryotic cell growth in response to a variety of input signals. In S. cerevisiae, amino acids activate TORC1 through the Rag guanosine triphosphatase (GTPase) heterodimer composed of Gtr1 and Gtr2 found together with Ego1 and Ego3 in the EGO complex (EGOC). The GTPase activity of Gtr1 is regulated by the SEA complex (SEAC). Specifically, SEACIT, a SEAC subcomplex containing Iml1, Npr2, and Npr3 functions as a GTPase activator (GAP) for Gtr1 to decrease the activity of TORC1 and, consequently, growth, after amino acid deprivation. Here, we present genetic epistasis data, which show that SEACAT, the other SEAC subcomplex, containing Seh1, Sea2–4, and Sec13, antagonizes the GAP function of SEACIT. Orthologs of EGOC (Ragulator), SEACIT (GATOR1), and SEACAT (GATOR2) are present in higher eukaryotes, highlighting the remarkable conservation, from yeast to man, of Rag GTPase and TORC1 regulation.

Introduction

The target of rapamycin complex 1 (TORC1) is a structurally and functionally conserved regulator of eukaryotic cell growth that adapts anabolic and catabolic processes in response to a variety of inputs, such as growth factors, cellular stress, energy, and nutrients. Amino acids, especially branched-chain amino acids like leucine, represent essential stimuli for TORC1 activation. Members of the conserved Rag family of guanosine triphosphatases (GTPases) mediate amino acid signaling to TORC1: in higher eukaryotes, RagA or RagB forms a heterodimer with RagC or RagD, whereas in S. cerevisiae, Gtr1 dimerizes with Gtr2. When RagA, RagB, or Gtr1 is bound to GTP, and RagC, RagD, or Gtr2 to GDP, the respective heterodimer is in its active, TORC1-stimulating conformation. In mammalian cells, Rag GTPases do not directly activate TORC1, but trigger TORC1 relocalization from the cytoplasm to the limiting membrane of the lysosome, where it can be activated by the GTPase Rheb. In S. cerevisiae, TORC1 remains associated with the limiting membrane of the vacuole (the yeast equivalent to the lysosome) irrespective of the presence or absence of leucine. Moreover, the yeast Rheb ortholog, Rhb1, is likely not required for the regulation of TORC1.

Thus, the mechanisms by which the Gtr1–Gtr2 heterodimer controls TORC1 function in S. cerevisiae remains mysterious. Gtr and Rag heterodimers are core switches that fulfill their function as part of larger protein complexes. In S. cerevisiae, Gtr1-Gtr2 associates with Ego1 and Ego3 to form the EGO complex (EGOC). Ego1 is N-terminally myristoylated and palmitoylated and thus tethers the EGOC to the vacuolar membrane. Ego3, the precise function of which remains unknown, forms homodimers that, like the C-terminal domains of Gtr1 and Gtr2, are structurally similar to members of the Roadblock/LC7 superfamily of proteins. In mammals, Rag GTPase heterodimers associate with the Ego1 equivalent p18 (LAMTOR1), the
Ego3-Ego3-related heterodimer p14-MP1 (LAMTOR2-LAMTOR3), C7orf59 (LAMTOR4), and HBXIP (LAMTOR5), which form the Ragulator complex. Like the EGOC, the Ragulator complex sits on the limiting membrane of the lysosome by virtue of lipodiation of p18, which is the only Rag–Ragulator subunit lacking structural resemblance with Roadblock domains (RDs). The entire pentameric Ragulator complex is proposed to act as the guanine nucleotide exchange factor (GEF) for RagA and RagB. Whether the EGOC possesses similar GEF activity remains questionable, because S. cerevisiae cells lack apparent orthologs of HBXIP and C7orf59, and guanine nucleotide exchange on Gtr1 is rather proposed to be stimulated by a Vam6-dependent mechanism.9 A GTPase-activating protein (GAP) that regulates Rag/Gtr proteins has, until recently, remained elusive.

Recently, subunits of the octameric vacuolar Seh1-associated complex (SEAC) were implicated in negative regulation of TORC1 in yeast. In an effort to clarify the relationship between SEAC and TORC1, we discovered in genetic epistasis analyses that the Iml1–Npr2–Npr3 SEAC subcomplex, which we now name SEACIT (for SEAC subcomplex Inhibiting TORC1 signaling), negatively regulates TORC1 through Gtr1 within the EGOC. Moreover, in line with our genetic data, we found that leucine deprivation triggered Iml1 to transiently interact with Gtr1 (in a Npr2- and Npr3-dependent manner) to stimulate its intrinsic GTPase activity. Of note, both Npr2 and Npr3 contain a N-terminal longin domain, the structure of which is closely related to RDs and may serve as platform for Rag GTPases. The GAP activity of SEACIT is conserved, as the orthologous complex in Drosophila and human cells (i.e., DEPDC5-Nprl2-Npr3), coined GATOR1, also acts as a GAP toward RagA and RagB. Intriguingly, various glioblastomas and ovarian cancers contain nonsense or frameshift mutations or truncating deletions in GATOR1-encoding genes, and a number of cancer cell lines with homozygous deletions in DEPDC5, NPRL2, or NPRL3 exhibit hyperactive mTORC1 that is insensitive to amino acid deprivation. Since these GATOR1-inactivating mutations also cause hypersensitivity to the TORC1 inhibitor rapamycin in mammalian cells, they may help to predict the therapeutic benefit of clinically approved TORC1 inhibitors in cancer treatments.

In addition to Iml1, Npr2, and Npr3 (SEACIT), the octameric SEAC also contains Sea2, Sea3, Sea4, Seh1, and Sec13, orthologs of the mammalian and Drosophila GATOR2 subcomplex proteins WDR24, WDR59, Mios, Seh1L, and Sec13, respectively. These proteins form the other SEAC-subcomplex, which we now name SEACAT (for SEAC subcomplex Activating TORC1 signaling). Except for Sec13, all of the GATOR2 components have been implicated in negative regulation of GATOR1 in higher eukaryotes. Similarly, yeast Sea2, Sea3, and Sea4 antagonize, although redundantly, the SEACIT-mediated TORC1 inhibition. However, roles for yeast Seh1, or either

**Figure 1.** Loss of Iml1 suppresses the TORC1 activation defect in sec13Δ (A) and seh1Δ (B) cells. Indicated (prototrophic) strains expressing a plasmid-based copy of Sch9HA5-HA, were grown exponentially at 25 °C (A) or 30 °C (B). Immunoblots detecting the level of phosphorylation within the C terminus of Sch9 were used to quantify in vivo TORC1 activity as previously described. Bar graphs refer to the mean ratio (± S.D.) of hyperphosphorylated/hypophosphorylated Sch9 from 3 independent experiments, normalized to the values for wild-type cells.

**Figure 2.** Conserved regulators of the Rag-family GTPases. The yeast SEAC is composed of 2 subcomplexes, SEACIT and SEACAT. SEACAT antagonizes the GAP-function of SEACIT. Vam6 is thought to be the GEF for Gtr1, which resides in the EGOC on the vacuolar membrane. Similarly, the mammalian (and Drosophila) GATOR complex is composed of the 2 subcomplexes GATOR1 and GATOR2. GATOR2 antagonizes the GAP-function of GATOR1. Whether or not mammalian Vam6 orthologs (i.e., the TGF-β receptor-associated protein 1 [TRAP1 or TGFBRAP1] and the TRAP1-like protein [TLP], aka hVPS39) act as a RagA/B GEF is unclear, rather the pentameric Ragulator complex, acting downstream of the vacuolar ATPase, is reported to serve this function. For details, please see text.
yeast or metazoan Sec13 upstream of the Rag GTPases are currently not reported.

Results and Discussion

To determine if Sec13, like other SEACAT components, controls TORC1 activity via SEACIT, we assayed TORC1 activity in a temperature-sensitive sec13 mutant. As is shown in Figure 1A, the sec13 mutant exhibited significantly reduced TORC1 activity when grown at the permissive temperature of 25 °C. This reduced TORC1 activity matches well with the observation that sec13-I is synthetic lethal when combined with a hypomorphic allele of LST8 (i.e., lst8-I for lethal with sec-thirteen), which encodes a stimulatory component in TOR-containing complexes. Importantly, loss of lml1 strongly activated TORC1 in both wild-type and sec13 mutant cells.

Similarly, we also observed that loss of seh1 resulted in a significant reduction of TORC1 activity, which was fully suppressed in the absence of lml1 (Fig. 1B). These genetic data therefore support a model in which Sec13 and Seh1, together with the other SEACAT components, promote TORC1 activity through inhibition of the GAP function of SEACIT. These results extend the remarkable evolutionary conservation of TORC1 regulation by Rag GTPases and delineate an inhibitory role for the pentameric SEACAT/GATOR2 subcomplex upstream of the SEACIT/GATOR1 subcomplex (Fig. 2).

Curiously, both Sec13 and Seh1 not only function within the SEACIT but also within the nuclear pore complex (NPC) as part of the conserved heptameric Nup84 subcomplex that is essential for the overall architecture of the NPC and consequently the transport of mRNAs and macromolecules (e.g., pre-ribosomes) across the nuclear membrane. Moreover, Sec13 also associates with Sec31 into a heterotetramer, which forms the outer shell of coatomer complex II (COPII) coated vesicles of the secretory pathway that bud off from the endoplasmic reticulum (ER). The occurrence of Sec13 and Seh1 in functionally different protein complexes suggests that their 3-dimensional structure, which is characterized, like those of all other SEACAT subunits, by the presence of WD-40 repeats that form β-propellers, renders them particularly well suited to serve as building and/or scaffolding blocks within larger protein complexes. Given these observations, it is tempting to speculate that Sec13/Seh1 serve to couple nuclear-to-cytoplasmic mRNA/protein transport or protein secretion to TORC1 control. For instance, compromised nuclear pore function or secretion may tie up or jam Seh1 and/or Sec13, thereby causing reduced SEAC assembly and, consequently, downregulation of TORC1. Interestingly, a genome-wide RNA interference screen by dsRNA reverse-transfection on living Drosophila cell microarrays identified nuclear pore components as TORC1 regulators. In a similar vein, alterations in the yeast secretory pathway have also been found to converge on TORC1 regulation. For instance, loss of the Golgi Ca2+/Mn2+ ATPase Pmr1 strongly increased the secretion of (heterologous) proteins that transit through the secretory pathway and, based on genetic experiments, also caused TORC1 activation (e.g., pmr1Δ suppressed the rapamycin-sensitive phenotype of the lst8-1 mutation). Conversely, addition of the secretory pathway inhibitor tunicamycin and inactivation of the Rab escort protein Mrp1 both strongly inhibited TORC1-dependent phosphorylation of Sch9. In sum, these observations lend support to a model in which both NPC function and secretory pathway flux are part of an increasing number of physiological cues (including v-ATPase activity, leucyl-tRNA synthetase function, glutaminolysis-driven production of α-ketoglutarate, glucose and amino acid levels, vesicle trafficking, or actin polarization), which may converge on Rag GTPase-mediated control of TORC1 (Fig. 3). Future studies should therefore aim at deciphering whether any of these cues may fine-tune TORC1 by regulating the GTP loading status of Rag GTPases through the SEACIT/GATOR1 and/or SEACIT/GATOR2 complexes.

Materials and Methods

Growth conditions, strains, and plasmids

Unless stated otherwise, prototrophic strains were pre-grown overnight in synthetic defined Dropout (SD; 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.2% [-adenine/-histidine/-leucine/-uracil/-tryptophan] dropout mix, and 2% glucose). For TORC1 activity assays, cells were diluted to an OD600 of 0.2 and further grown at 30 °C until they reached an OD600 of 0.8. The following isogenic S. cerevisiae strains (all wild-type for LYS2 and MET15 in the BY4741/2 background) were used in this study: MATα his3Δ1, leu2Δ0, ura3Δ0 (YL51; WT); MATα iml1Δ::kanMX, his3Δ1, leu2Δ0, ura3Δ0 (NP04-4C); MATα seh1Δ::kanMX, etc.
bi3Δ, leu2Δ, ura3Δ (MP308-7A); MATa seh1Δ::kanMX, iml1Δ::kanMX, his3Δ, leu2Δ, ura3Δ (MP308-8B); MATa sec13-Δ::kanMX, his3Δ, leu2Δ, ura3Δ (MP309-5D); and MATα sec13-Δ::kanMX, his3Δ, leu2Δ, ura3Δ (MP309-9A). The original sec13Δ (MATα sec13-1::kanMX, his3Δ, leu2Δ, ura3Δ, met15Δ)+6 and seh1Δ (MATα seh1Δ::kanMX, his3Δ, leu2Δ, ura3Δ, met15Δ)+10 mutants were rendered wild-type for MEF5 by backcrossing with YL515. Sequencing of the sec13Δ ORF revealed that this allele carries 2 mutations, which change Lys34 and Ser228 in Sec13 to Glu44 and Asp224, respectively. All strains carried the following plasmids: pRS413-Sch97570A-HA, pRS414.S1 and pRS416.S1.

TORC1 activity assays
TORC1 activity was determined by quantification of the phosphorylation of the C-terminal part of HA-tagged Sch97570A, which contains 5 TORC1 phosphorylation sites, as described previously. Briefly, following chemical cleavage with NTCl, extracts were separated by 7.5% SDS-PAGE, and membranes were probed with anti-HA antibodies (12CA5) and anti-mouse IgG antibodies coupled to HRP (Biorad).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Michael Costanzo for strains, Floriana Jaquier for technical assistance, and R Loewth for plasmids and critical and valuable comments on the manuscript. This research was supported by the Canton of Fribourg and the Swiss National Science Foundation (CDV).

References
1. Soulard A, Cohen A, Hall MN. TOR signaling in invertebrates. Curr Opin Cell Biol 2009; 21:825-36; PMID:19767189; http://dx.doi.org/10.1016/j.ceb.2009.08.007
2. Zoncu R, Efeyan A, Sabatini DM. mTOR: from lysosomes to disease. Trends Mol Med 2012; 18:524-33; PMID:22749019; http://dx.doi.org/10.1016/j.molmed.2012.05.007
3. Jessell TM, Russell RC, Guan KL. Amino acid signaling upstream of mTOR. Nat Rev Mol Cell Biol 2003; 14:133-9; PMID:12552354; http://dx.doi.org/10.1038/nrnm3522
4. Dodd KM, Tee AR. Leucine and mTORC1: a complex relationship. Am J Physiol Endocrinol Metab 2012; 302:E129-42; PMID:22354780; http://dx.doi.org/10.1152/ajpendo.00521.2011
5. Kim E, Gorokhova-Flicks P, Li L, Neufeld TP, Guan KL. Regulation of TORC1 by Rag GTPases in nutrient response. Nat Cell Biol 2008; 10:935-45; PMID:18604498; http://dx.doi.org/10.1038/ncb1753
6. Bindu M, Peli-Gulli MP, Bonfils G, Panchaud N, Urban J, Sturgell TW, Loewth R, De Virgilio C. The Van6 GEF controls TORC1 by activating the EGO complex. Mol Cell 2009; 35:563-73; PMID:19740853; http://dx.doi.org/10.1016/j.molcel.2009.07.009
7. Sanacik Y, Persetron TR, Shaal YD, Lindquist RA, Thoren CC, Bar-Peled L, Sabatini DM. The Rag GTPases bind raport and mediate amino acid signaling to mTORC1. Science 2008; 320:1496-501; PMID:18497260; http://dx.doi.org/10.1126/science.1157755
8. Kim E, Guan KL. Rag GTPases in nutrient-mediated TOR signaling pathway. Cell Cycle 2009; 8:1814-8; PMID:19270521; http://dx.doi.org/10.4161/cc.8.7.8124
9. Sanacik Y, Bar-Peled L, Zoncu R, Markward AL, Nada S, Sabatini DM. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 2010; 141:290-300; PMID:20381137; http://dx.doi.org/10.1016/j.cell.2010.02.024
10. Duhouo F, Deloche O, Wanke V, Cameroni E, De Virgilio C. The TOR and EGO protein complexes orchestrate microautophagy in yeast. Mol Cell 2005; 19:15-26; PMID:15989961; http://dx.doi.org/10.1016/j.molcel.2005.05.020
11. Bindu M, Bonfils G, Panchaud N, Peli-Gulli MP, De Virgilio C. An EGO-centric view of TORC1 signaling. Cell Cycle 2010; 9:221-2; PMID:20023374; http://dx.doi.org/10.4161/cc.9.2.10585
12. Kogan K, Spear ED, Kaiser CA, Fass D. Structural conservation of components in the amino acid sensing branch of the TOR pathway in yeast and mammals. J Mol Biol 2010; 402:388-98; PMID:20655927; http://dx.doi.org/10.1016/j.jmb.2010.07.034
13. De Virgilio C. The essence of yeast quiescence. FEMS Microbiol Rev 2012; 36:306-39; PMID:22168086; http://dx.doi.org/10.1111/j.1574-6976.2011.00287.x
14. Zhang T, Peli-Gulli MP, Yang H, De Virgilio C, Ding J. Ego3 functions as a homodimer to mediate the interaction between Gril2-Grl2 and Ego1 in the EGO complex to activate TORC1. Structure 2012; 20:2351-60; PMID:23213112; http://dx.doi.org/10.1016/j.str.2012.09.019
15. Bar-Peled L, Schweitzer LD, Zoncu R, Sabatini DM. Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. Cell 2012; 150:1196-208; PMID:22803980; http://dx.doi.org/10.1016/j.cell.2012.07.032
16. Zanetti G, Pahuja KB, Studer S, Shim S, Schekman R, COPII and the regulation of protein sorting in mammals. Nat Cell Biol 2011; 14:20-8; PMID:22193250; http://dx.doi.org/10.1038/ncb2390
17. Girkan C, Stagg SM, Laperonne P, Balch WE. The COPII cage: unifying principles of vesicle coat assembly. Nat Rev Mol Cell Biol 2006; 7:272-38; PMID:16990852; http://dx.doi.org/10.1038/nrm2025
32. Lindquist RA, Ortina KA, Wheeler DB, Hsu PP, Thoreen CC, Guertin DA, Ali SM, Sengupta S, Shaul YD, Lamprecht MR, et al. Genome-scale RNAi on living-cell microarrays identifies novel regulators of Drosophila melanogaster TORC1-S6K pathway signaling. Genome Res 2011; 21:433-46; PMID:21239877; http://dx.doi.org/10.1101/gr.114921.110

33. Devasahayam G, Rizr D, Hellwell SB, Burke DJ, Sturgill T W. Pmr1, a Golgi Ca2+/Mn2+-ATPase, is a regulator of the target of rapamycin (TOR) signaling pathway in yeast. Proc Natl Acad Sci U S A 2006; 103:17840-5; PMID:17995607; http://dx.doi.org/10.1073/pnas.0604303103

34. Rudolph HK, Antebi A, Fink GR, Buckley CM, Dorman TE, Levine J, Davudov LS, Mao JI, Moir JD, Schieffer KJ. The yeast secretory pathway is perturbed by DT. The yeast secretory pathway is perturbed by DT. The yeast secretory pathway is perturbed by DT, a member of a Ca2+ ATPase family. Cell 1989; 58:133-45; PMID:2526682; http://dx.doi.org/10.1016/0092-8674(89)90410-8

35. Lempainen H, Uotila A, Urban J, Dohnal I, Ammerer G, Loewith R, Shore D. Sfp1 interaction with TORC1 and Mrs6 reveals feedback regulation on TOR signaling. Mol Cell 2009; 33:704-16; PMID:19765946; http://dx.doi.org/10.1016/j.molcel.2009.01.034

36. Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H+-ATPase. Science 2011; 334:678-83; PMID:22153050; http://dx.doi.org/10.1126/science.1207956

37. Bonfils G, Jaenov M, Bontron S, Ostromicz C, Ungermann C, De Virgilio C, Leucyl-tRNA synthetase controls TORC1 via the EGO complex. Mol Cell 2011; 44:201-10; PMID:22045760; http://dx.doi.org/10.1016/j.molcel.2012.05.043

38. Flinn RJ, Backer JM. mTORC1 signals from late endosomes: taking a TOR of the endocytic system. Cell Cycle 2010; 9:1860-70; PMID:20436274; http://dx.doi.org/10.4161/cc.9.10.11679

39. Brachmann CB, Davies A, Cost GJ, Capuro E, Li J, Hieter P, Boeke JD. Deletion destruction of chromatin DNA in Saccharomyces cerevisiae. Genetics 2001; 154:109-22; PMID:11484994; http://dx.doi.org/10.1038/jcb.200102142

40. Goranov AI, Gulati A, Dephoure N, Takahara T, Maeda T, Gygi SP, Manalis S, Amon A. Changes in cell morphology are coordinated with cell growth through the TORC1 pathway. Curr Biol 2013; 23:1269-79; PMID:23810534; http://dx.doi.org/10.1016/j.cub.2013.05.035

41. Zuniga-Martinez SA, Puria R, Pan X, Boeke JD, Cardenas ME. Efficient TOR signaling requires a functional class C Vps protein complex in Saccharomyces cerevisiae. Genetics 2007; 176:2139-50; PMID:17565946; http://dx.doi.org/10.1053/genetics.107.072835

42. Efeyan A, Zoncu R, Chang S, Gumper I, Smirkin H, Wolfson RL, Kirak O, Sabatini DD, Sabatini DM. Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. Nature 2013; 493:679-83; PMID:23263183; http://dx.doi.org/10.1038/nature11745

43. Li L, Kim E, Yuan H, Inoki K, Goraksha-Hicks P, Schiefer RL, Neufeld TP, Guan KL. Regulation of mTORC1 by the Rab and Arl GTPases. J Biol Chem 2010; 285:19705-9; PMID:20457610; http://dx.doi.org/10.1074/jbc.C110.102483

44. Durán RV, Hall MN. Glutaminolysis feeds mTORC1. Cell Cycle 2012; 11:4107-8; PMID:23095634; http://dx.doi.org/10.4161/cc.22632

45. Woloson RL, Kirak O, Sabatini DD, Sabatini DM. The late endosome is essential for mTORC1 signaling. Cell 2013; 149:410-24; PMID:23810534; http://dx.doi.org/10.1016/j.cell.2012.02.044

46. Durán RV, Opplicher W, Robraillie AM, Heiserich L, Skendaj R, Gortlieb E, Hall MN. Glutaminolysis activates Rag-mTORC1 signaling. Mol Cell 2012; 47:349-58; PMID:22749228; http://dx.doi.org/10.1016/j.molcel.2012.05.043