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SEACing the GAP that nEGOCiates TORC1 activation

Evolutionary conservation of Rag GTPase regulation

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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.

Keywords: Rag GTPases, TOR complex 1, EGO complex, SEA complex, amino acid signaling, Iml1-Npr2-Npr3 Rag GTPase GAP complex, GATOR1, GATOR2, Seh1, Sec13

Abbreviations: TOR Complex 1, (TORC1); EGO Complex, (EGOC); SEA Complex, (SEAC)

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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.[1-4] Amino acids, especially branched-chain amino acids like leucine, represent essential stimuli for TORC1 activation.[5-7] 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.[8-11] 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.[5,10,12] 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.[4,9] 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.[9,13-17] 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.[15,17] In mammals, Rag GTPase heterodimers associate with the Ego1 equivalent p18 (LAMTOR1), the

Ego3-Ego3-related heterodimer p14-MP1 (LAMTOR2-LAMTOR3),[17] C7orf59 (LAMTOR4), and HBXIP (LAMTOR5), which form the Ragulator complex.[12] Like the EGOC, the Ragulator complex sits on the limiting membrane of the lysosome by virtue of lipidation of p18, which is the only Rag–Ragulator subunit lacking structural resemblance with Roadblock domains (RDs).[5,6,12] The entire pentameric Ragulator complex is proposed to act as the guanine nucleotide exchange factor (GEF) for RagA and RagB.[18] 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.[19-22] 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.[23] 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.[24] The GAP activity of SEACIT is conserved, as the orthologous complex in *Drosophila* and human cells (i.e., DEPDC5-Nprl2-Nprl3), coined GATOR1, also acts as a GAP toward RagA and RagB.[25] 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.[25] 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.[25]

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.[25] Similarly, yeast Sea2, Sea3, and Sea4 antagonize, although redundantly, the SEACIT-mediated TORC1 inhibition.[23] However, roles for yeast Seh1, or either

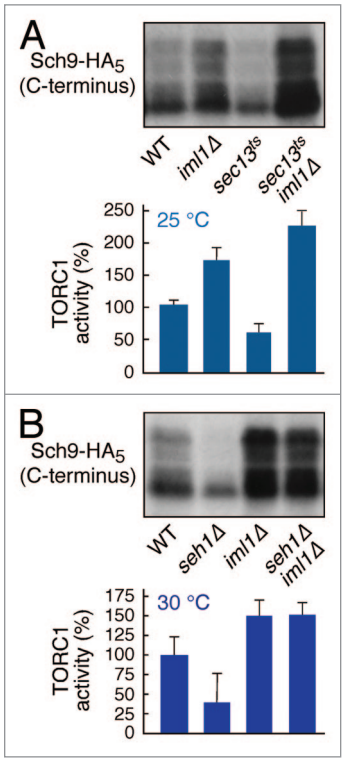

Figure 1. Loss of Iml1 suppresses the TORC1 activation defect in *sec13*^{ts} (**A**) and *seh1*Δ (**B**) cells. Indicated (prototrophic) strains expressing a plasmid-based copy of Sch9^{T570A}-HA$_5$ were grown exponentially at 25 °C (**A**) or 30 °C (**B**). Immunoblots detecting the level of phosphorylation within the C terminus of Sch9 were used to quantify in vivo TORC1 activity as previously described.[50] 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.

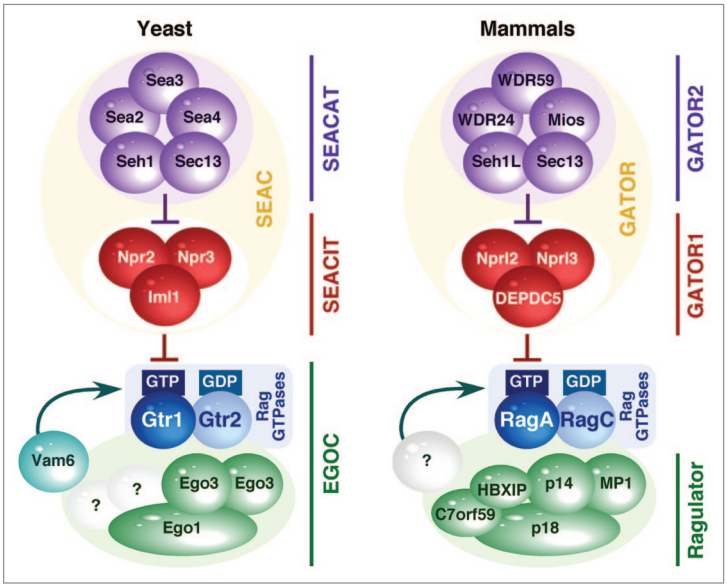

Figure 2. Conserved regulators of the Rag-family GTPases. The yeast SEAC is composed of 2 subcomplexes, SEACIT and SEACAT. SEACAT antagonizes the GAP-function of SEACIT. Vam6 is thought to be the GEF for Gtr1, which resides in the EGOC on the vacuolar membrane. Similarly, the mammalian (and *Drosophila*) 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)[45,46,52-54] 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*^{ts} (*sec13-1*)[26] mutant. As is shown in **Figure 1A**, the *sec13*^{ts} 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 *sec13-1* is synthetic lethal when combined with a hypomorphic allele of LST8 (i.e., *lst8-1* for lethal with sec-thirteen), which encodes a stimulatory component in TOR-containing complexes.[27,28] Importantly, loss of Iml1 strongly activated TORC1 in both wild-type and *sec13*^{ts} 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 Iml1 (**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 SEAC, 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.[29] Moreover, Sec13 also associates with Sec31 into a heterotetramer, which forms the outer shell of coatmer complex II (COPII) coated vesicles of the secretory pathway that bud off from the endoplasmic reticulum (ER).[30,31] 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,[19,21] 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-scale RNA interference screen by dsRNA reverse-transfection on living *Drosophila* cell microarrays identified nuclear pore components as TORC1 regulators.[32] 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 Ca^{2+}/Mn^{2+} 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., *pmr1*Δ suppressed the rapamycin-sensitive phenotype of the *lst8-1* mutation).[33,34] Conversely, addition of the secretory pathway inhibitor tunicamycin and inactivation of the Rab escort protein Mrs6 both strongly inhibited TORC1-dependent phosphorylation of Sch9.[28,35] 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),[9,36-46] 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 SEACAT/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 OD_{600} of 0.2 and further grown at 30 °C until they reached an OD_{600} of 0.8. The following isogenic *S. cerevisiae* strains (all wild-type for *LYS2* and *MET15* in the BY4741/2 background)[47] were used in this study: MATα *his3*Δ*1, leu2*Δ*0, ura3*Δ*0* (YL515; WT)[9]; MATα *iml1*Δ*::kanMX, his3*Δ*1, leu2*Δ*0, ura3*Δ*0* (NP04-4C)[23]; MATα *seh1*Δ*::kanMX,*

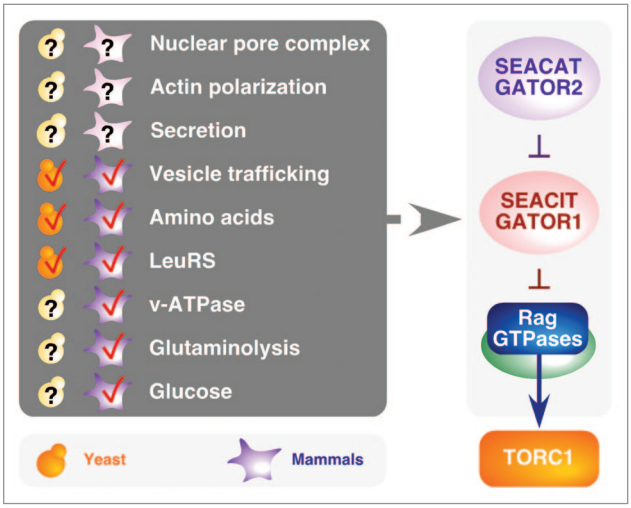

Figure 3. Physiological cues, which may regulate TORC1 through the Rag GTPase module. Red check marks indicate the existence of experimental data supporting (in yeast or mammalian cells) a model in which the respective cue impinges on Rag GTPase regulation (please see text for corresponding references). Currently speculative processes are denoted with a question mark.

his3Δ1, leu2Δ0, ura3Δ0 (MP308-7A); MATα *seh1Δ::kanMX, iml1Δ::kanMX, his3Δ1, leu2Δ0, ura3Δ0* (MP308-8B); MATα *sec13^{ts}-kanMX, his3Δ1, leu2Δ0, ura3Δ0* (MP309-5D); and MATα *sec13^{ts}-kanMX, iml1Δ::kanMX, his3Δ1, leu2Δ0, ura3Δ0* (MP309-9A). The original *sec13^{ts}* (MAT**a** *sec13-1-kanMX, his3Δ1, leu2Δ0, ura3Δ0, met15Δ0*)[48] and *seh1Δ* (MAT**a** *seh1Δ::kanMX, his3Δ1, leu2Δ0, ura3Δ0, met15Δ0*)[49] mutants were rendered wild-type for *MET15* by backcrossing with YL515. Sequencing of the *sec13^{ts}* ORF revealed that this allele carries 2 mutations, which change Lys[44] and Ser[224] in Sec13 to Glu[44] and Asp[224], respectively. All strains carried the following plasmids: pRS413-Sch9^{T570A}-HA$_5$,[50] pRS415,[51] and pRS416.[51]

TORC1 activity assays

TORC1 activity was determined by quantification of the phosphorylation of the C-terminal part of HA$_5$-tagged Sch9^{T570A}, which contains 5 TORC1 phosphorylation sites, as described previously.[9,50] Briefly, following chemical cleavage with NTCB, 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.

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