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TOR Signaling in Budding Yeast

Yoshiharu Inoue and Wataru Nomura

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Abstract

TOR (*T*arget *o*f *R*apamycin) is a Ser/Thr kinase that was originally identified by genetic screening using the budding yeast *Saccharomyces cerevisiae.* The TOR protein forms two structurally and functionally distinct complexes (TOR complex 1, TORC1, and TOR complex 2, TORC2). TORC1 is involved in various cellular activities, such as cell growth, ribosome biogenesis, translation initiation, metabolism, stress response, aging, and autophagy. TORC2 is involved in actin organization, sphingolipid biogenesis, and endocytosis. TORC1 plays a central role in the signaling network in response to stimuli coupled to internal and external nutrient conditions, particularly an amino acid sufficiency. A dimeric complex of Rag GTPases, the activity of which is regulated by the guanine nucleotide-loading status, and some regulator proteins communicating with Rag GTPases are involved in the activation of TORC1 by amino acids. In TORC2 signaling, membrane stress appears to be a cue, in which some proteins associated with respective membrane compartments, such as eisosomes, play a role.

Keywords: TOR (*T*arget of *R*apamycin), small GTPase, signal transduction, protein kinase, *Saccharomyces cerevisiae*

1. Introduction

All heterotrophs must take organic compounds from outside of cells to gain energy for various biological activities. For example, since amino acids are components of proteins, an insufficiency in amino acids has serious effects on cellular functions. The bacterial feedback regulation of amino acid biosynthesis at the enzyme and gene expression levels is a well-known mechanism that controls intracellular amino acid levels. In this feedback-regulatory mechanism, an amino acid functions as a signaling molecule in the closed metabolic loop for the production of respective amino acids.

On the other hand, in higher Eukarya, an insufficiency in amino acids has been linked to various metabolic diseases; therefore, sensing amino acid amounts inside and outside of cells

through the transmission of signals needs to be strictly controlled. TOR (*T*arget *o*f *R*apamycin) is one of the nutritional signaling mechanisms that is evolutionarily conserved in eukaryotes from yeast to humans. TOR, a Ser/Thr kinase, is involved in two complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2), which are distinctly different structurally and functionally. TORC1 is in an active form when amino acids are abundant, namely conditions under which cellular activities that promote cell growth proceed dynamically. TORC1 signaling activates anabolic processes such as protein/lipid synthesis and ribosome biogenesis, which are linked to cell growth. TORC1 signaling also inhibits catabolic processes including autophagy, a bulk protein degradation system. Therefore, the breakdown of TORC1 signaling in humans has been linked to various diseases including cancers and metabolic disorders [1]. TORC2 is involved in the polarized organization of the actin cytoskeleton, endocytosis, and sphingolipid biosynthesis. Physiological cues to activate TORC2 signaling in mammalian cells are insulin and insulin-like growth factors; however, the mechanisms by which these hormones activate mammalian TORC2 have not yet been elucidated in detail. Furthermore, despite the evolutionary conservation of TORC2 in lower eukaryotes such as yeast, the growth factor-like hormonal-regulatory mechanism for the promotion of cell growth is not conserved in yeast. TORC1 signaling has been extensively examined using rapamycin, a potent inhibitor of TORC1 signaling, whereas TORC2 is insensitive to rapamycin. Therefore, although the mechanisms underlying the amino acid-induced activation of TORC1 signaling have been investigated in detail, limited information is currently available on the activation mechanisms of TORC2, which may be explained, at least in part, by the absence of TORC2-specific inhibitors, such as rapamycin for TORC1. However, many important insights into TORC2 signaling have been provided by the budding yeast *Saccharomyces cerevisiae*, which is an excellent model organism. This chapter overviews TOR signaling in the budding yeast, with a particular focus on the regulatory machinery and cues for the activation of TORC1 and TORC2, and compares it with that in mammalian cells. Since TOR is a master regulator of cell growth, the outputs of TOR signaling also cover a broad range of biological activities. More information on the downstream outputs of TOR signaling in yeast is available in recent reviews and references therein [2–4].

2. TOR: a master regulator for cell growth

2.1. Rapamycin and FKBP12

Rapamycin is a macrolide antifungal chemical that was identified from the bacterium *Streptomyces hygroscopicus*, which was isolated from a soil sample obtained on the Easter Islands, *Rapa Nui* in the local tongue, and, hence, it was named "rapamycin." Despite being an antifungal drug, rapamycin also exerted immunosuppressive effects; therefore, it was subsequently used as an immunosuppressant in organ transplantation [5]. Rapamycin and its derivatives also exerted antitumor effects, indicating their potential in the treatment of certain cancers [6].

The first approach to investigating the mode of action of rapamycin was biochemical. Since rapamycin was found to inhibit the mammalian immune system, molecule(s) with the ability to bind to rapamycin may be involved in the action of this drug as an immunosuppressant.

Rapamycin was shown to bind to a peptidyl-prolyl *cis-trans* isomerase, also known as FKBP12 (FK506-binding protein 12), which is one of the immunophilins [7]. Yeast has served as an excellent model organism of higher eukaryotes. Since the mechanisms underlying some biological events discovered in this unicellular microorganism to date are conserved among Eukarya, the mode of action of rapamycin in yeast is also expected to be evolutionarily conserved. Based on this concept, rapamycin-binding protein was purified from yeast cell extracts, and its amino acid sequence was partially elucidated. A reverse genetic approach was applied to clone the gene encoding the rapamycin-binding protein using a partial amino acid sequence, and the FKBP12 homologous gene *FPR1* (*F*KBP12 *p*roline *r*otamase) was obtained [8]. Fpr1 is a small protein that consists of only 114 amino acids (molecular weight, 12,157). Gene disruption experiments revealed that *FPR1* was dispensable for the growth of yeast cells [8–11]. However, since Fpr1 was a rapamycin-binding protein in yeast, the disruption of *FPR1* conferred resistance to rapamycin [8, 12]. These findings suggested that the formation of an Fpr1-rapamycin complex was involved in the mode of action of rapamycin in yeast, and this mode of action was observed in an immunophilin-immunosuppressant complex in mammalian cells.

2.2. Discovery of TOR

In order to identify the target of the Fpr1-rapamycin complex, genetic screening using *S. cerevisiae* with resistance to rapamycin was conducted, and consequently, three genes, that is, *TOR1*, *TOR2*, and *FPR1*, were identified [12]. As expected, most mutants (258 clones from 277 rapamycin-resistant mutants) contained recessive mutations in *FPR1* [8]. Similarly, deletion of *FPR1* conferred the recessive resistance to rapamycin, and expression of human FKBP12 restored sensitivity to rapamycin [13]. Two novel genes, *TOR1* and *TOR2* [8], which were also referred to as *DRR1* and *DRR2*, respectively, for dominant rapamycin resistance [13], were identified.

TOR1 and *TOR2* encode large-molecular-weight proteins (molecular weight, >280 kDa). The Tor1 (2470 amino acids) and Tor2 (2474 amino acids) proteins share 67% identity at the amino acid-sequence level and were initially considered to be lipid kinases (phosphatidylinositol kinases). However, neither proteins exhibited lipid kinase activity; they were later found to be phosphatidylinositol kinase-related kinases (PIKKs). Mutations occurring in *TOR1* (*TOR1-1*) and *TOR2* (*TOR2-1*) that conferred resistance to rapamycin were identified as a single amino acid substitution, that is, Ser1972Arg in Tor1 and Ser1975Ile in Tor2. In contrast to yeast, which possesses two *TOR* genes, mammalian cells have a single *TOR* (mTOR, mammalian TOR) gene. mTOR was initially designated as mammalian TOR, but has recently been referred to as mechanistic TOR, which includes not only mammalian TOR but also all other TORs, such as yeast Tor1 and Tor2.

Rapamycin itself does not directly bind to the TOR protein, whereas the Fpr1-rapamycin complex binds to the Tor1 or Tor2 protein, thereby inhibiting the protein kinase activity of TOR [14–17]. *TOR1-1* and *TOR2-1* produce Tor1 and Tor2 proteins, respectively, without affinity or with low affinity to the Fpr1-rapamycin complex; therefore, mutants with these alleles develop resistance to rapamycin. Similarly, the FKBP12-rapamycin complex binds to mTOR in order to inhibit its activity in mammalian cells [18].

2.3. Domain structure of TOR

The domain structures and amino acid sequences of all TOR proteins are evolutionarily conserved. Both Yeast Tor1 and Tor2 contain the following domains (in the direction from the N-terminus to the C-terminus): HEAT repeats, FAT, FRB, kinase, FIT, and FATC (**Figure 1**). These domains are also found in the mTOR protein in the same order. Each HEAT motif (originally identified in *H*untingtin, *e*longation factor 3, protein phosphatase 2*A* (PP2A), and *T*OR) consists of approximately 40 amino acid residues that form anti-parallel alpha-helices, and Tor1/Tor2 proteins contain ~20 tandemly repeated HEAT motifs between their N-terminal and central regions. Tor1 and Tor2 bind with their respective subunits that constitute distinct TOR complexes (see subsequent text) through the HEAT repeats. FAT, FRB, kinase, and FATC domains, which are located on the C-terminal to the HEAT repeats, are commonly found in PIKK family members [19–21]. The FAT domain, which was named to represent the main groups in PIKKs (*F*RAP, *A*TM, and *T*RRAP), consists of ~500 amino acid residues. The FRB (*F*KBP-*r*apamycin *b*inding) domain consists of ~100 amino acid residues, and the Fpr1-rapamycin complex binds to this region. The *TOR1-1* and *TOR2-1* mutations conferring resistance to rapamycin occur within the FRB domain, which demonstrates that the Fpr1-rapamycin complex is a true inhibitor of TOR kinase.

2.4. TOR complexes

Although the primary structures of Tor1 and Tor2 share strong similarities, their cellular functions are distinct [14, 22]. The *TOR1* null mutation is viable, whereas the *TOR2* null mutation is not. Previous studies reported that rapamycin treatments mimicked starvation, indicating that TOR is involved in cell growth control in response to nutrients [23, 24]. The findings of genetic analyses on *TOR1* and *TOR2* suggested that the roles of Tor1 and Tor2 are divided into two aspects, that is, some readouts in which TOR signaling is involved are redundantly regulated by Tor1 and Tor2, whereas some are specifically regulated by Tor2. Rapamycin was found to affect cellular events in which Tor1 and Tor2 functioned redundantly. For example, rapamycin inhibits protein synthesis and ribosome biogenesis, but induces autophagy, which occurs under nutrient-starved conditions, and both Tor1 and Tor2 are involved in these events [18]. Meanwhile, the regulation of actin organization, endocytosis, and sphingolipid biosynthesis is controlled by Tor2. The distinction between Tor1- and Tor2-related readouts is due to differences in the complexes in which Tor1 and Tor2 are involved.
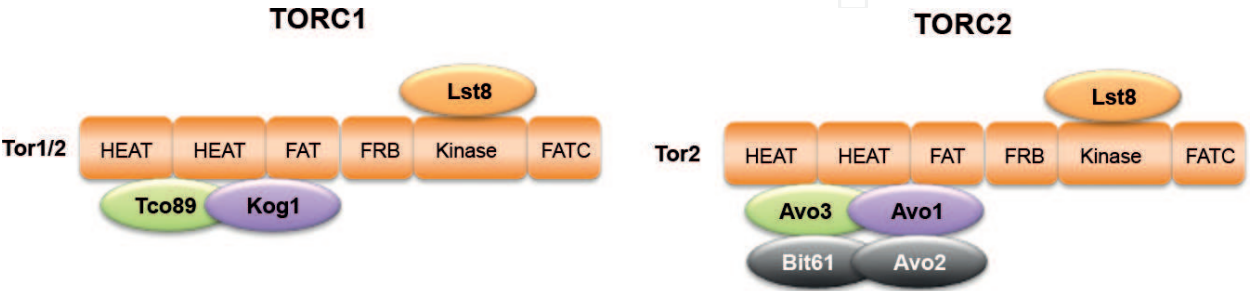

Figure 1. TOR complexes in *S. cerevisiae*.

TORC1 involves either Tor1 or Tor2 as the TOR protein, while Kog1, Tco89, and Lst8 are subunits. TORC2 involves Tor2 as the TOR protein with subunits of Avo1, Avo2, Avo3, Bit61, and Lst8. Readouts redundantly regulated by Tor1 and Tor2 are controlled by TORC1, which is sensitive to rapamycin, whereas the specific readouts of Tor2 are regulated by TORC2. TORC2 contains Tor2, in which the FRB domain exists; however, this TOR complex is not sensitive to rapamycin. This issue was resolved using crosslinking-mass spectrometric and electron microscopic analyses, that is, the C-terminal part of Avo3 was close to the FRB domain, which rendered the Fpr1-rapamycin complex incapable of accessing the FRB domain, resulting in TORC2 insensitivity to rapamycin [25].

3. TORC1

3.1. Subunit components

The following components constitute TORC1: Kog1, Tco89, Lst8, and either Tor1 or Tor2 [26–28] (**Figure 1**). Mammalian TORC1 (mTORC1) contains counterparts of each subunit of yeast TORC1, except for Tco89, instead mTORC1 contains PRAS40 (*p*roline-*r*ich *A*kt *s*ubstrate of *40* kDa) and DEPTOR (*D*isheveled, *E*gl-10, and *P*leckstrin domain-containing m*TOR*-interacting protein). mTORC1 forms a dimeric structure [29], and this also appears to be the case for yeast TORC1. The structural integrity of mTORC1 was disrupted by rapamycin [29], whereas all yeast TORC1 components were co-immunoprecipitated by FKBP12 [26], suggesting that rapamycin does not affect the structure of TORC1 in yeast.

Kog1 and mammalian ortholog Raptor (*r*egulatory-*a*ssociated *p*rotein of m*TOR*) contains the RNC (*R*aptor *N*-terminal *c*onserved) domain, through which Kog1/Raptor binds to the TOR protein and the substrates of TORC1. Kog1/Raptor contains three HEAT repeats in the proximity of the C-terminal of the RNC domain and also contains seven WD40 motifs in the C-terminus. Tco89 contains no obvious motifs. Lst8 (mLst8 in mTORC1) contains seven WD40 motifs.

3.2. Activation of TORC1 signaling

3.2.1. Rag GTPases (Gtr1 and Gtr2)

When cells are exposed to conditions that are unfavorable for growth, they cease division and remodel cellular metabolism and gene expression profiles to survive under these stressful conditions. The treatment of yeast cells with rapamycin causes multiple phenomena resembling those occurring in cells starved of nutrients, particularly amino acids. Therefore, one of the physiological cues for the activation of TORC1 signaling may be amino acid(s). Upstream module(s) that communicate with TORC1 were revealed by a genetic approach using *S. cerevisiae*. Mutants that are unable to recover cell growth when transferred from nutrient-depleted conditions to nutrient-rich conditions are expected to be defective in module(s) that communicate with TORC1. Since rapamycin mimics amino acid-starved conditions, mutants with the ability to recover from rapamycin-induced growth arrest were screened. *EGO* (*E*xit from rapamycin-induced *GrO*wth arrest) mutants were identified, in which the Ras-related GTPase (Rag) Gtr2 and the vacuolar membrane-associated proteins Ego1 and Ego3 were

included [30]. Another study revealed that modules involved in the trafficking of the general amino acid permease to the cytoplasmic membrane were Gtr1, Gtr2, Ego1, Ego3, and Ltv1 [31].

Gtr1 and Gtr2 belong to the Rag family. Orthologs of Gtr1 and Gtr2 in mammalian cells are RagA/RagB for Gtr1 and RagC/RagD for Gtr2. Amino acid-sequence similarities between RagA and RagB (90% identity) and between RagA/RagB and Gtr1 (48%) are high. This is also the case between RagC and RagD (81%) and between RagC/RagD and Gtr2 (46%). However, amino acid-sequence similarities between RagA/RagB and RagC/RagD and between Gtr1 and Gtr2 are low (approximately <25%) [32–34]. Rag GTPases function as heterodimers that are formed by a combination of one monomer of either RagA or RagB and one monomer of either RagC or RagD [33]. Similarly, Gtr1 and Gtr2 form a heterodimer [34]. Heterodimers with GTP-bound RagA/RagB and GDP-bound RagC/RagD exhibit full activity. This is also the case for *S. cerevisiae* Rag GTPase, that is, Gtr1^{GTP} and Gtr2^{GDP} are a dynamic combination that activate TORC1 in yeast (**Figure 2**).

3.2.2. EGO complex (ragulator)

Small GTPases are generally lipid-linked proteins, and lipid modifications enable these proteins to anchor to biological membranes. However, neither Gtr1 nor Gtr2 is modified by lipids.
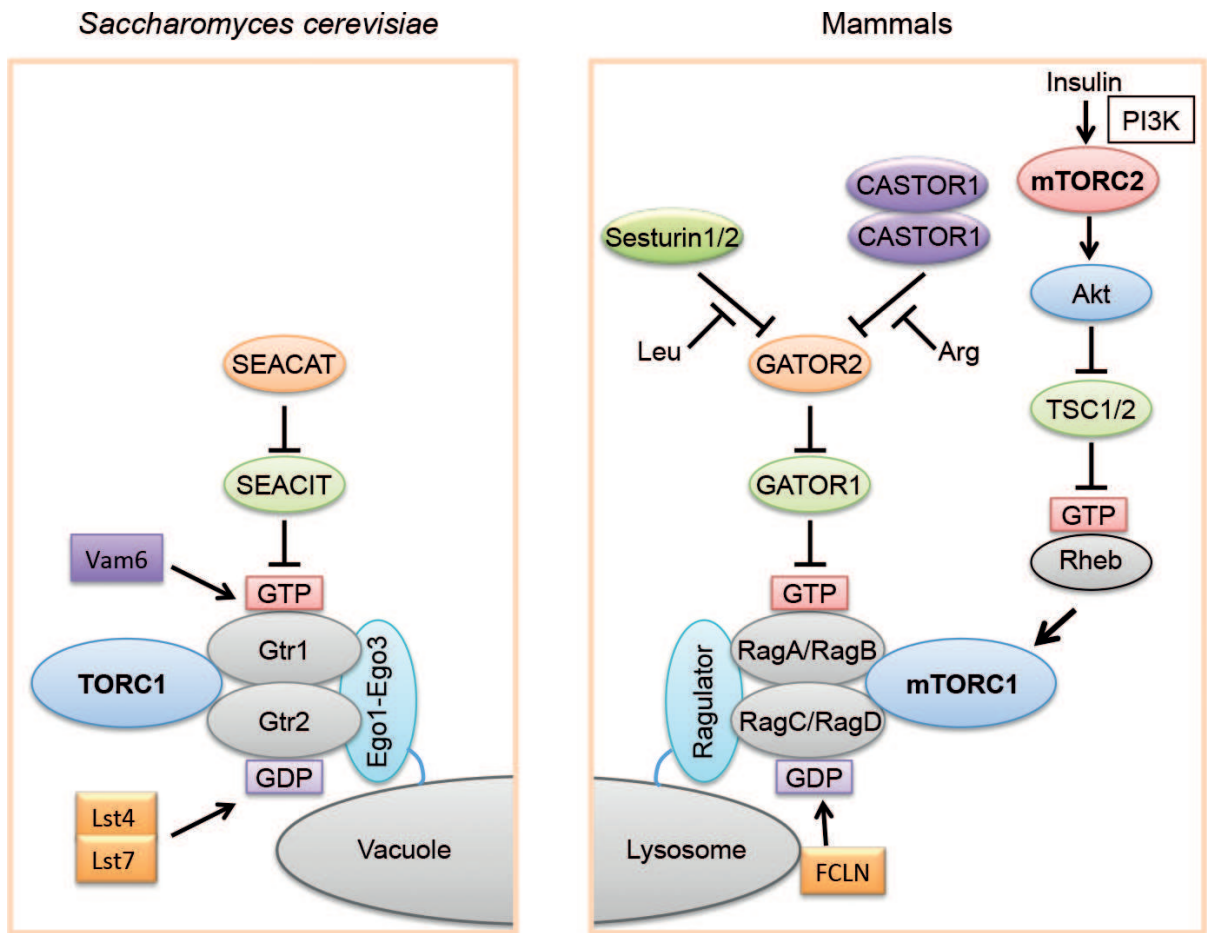

Figure 2. Activation of TORC1 signaling in yeast and mammals.

Ego1, Ego3, and Ego2 were recently found to form the EGO complex, which serves as a scaffold for the Gtr1-Gtr2 heterodimer to anchor to the vacuolar membrane in order to activate TORC1 in response to amino acids in *S. cerevisiae* [35]. Ego1 is a myristoylated and palmitoylated protein that is anchored to the vacuolar membrane through such lipids [36–39]. Ego2 and Ego3 bind to vacuolar membrane-anchored Ego1 [35].

Mammalian cells also contain a large protein complex that functions together with the heterodimeric Rag GTPase, designated "Ragulator" (Rag regulator). Ragulator consists of five subunits, that is, LAMTOR1-5 (LAMTOR, *L*ysosomal *A*daptor, and *M*itogen-activated protein kinase (MAPK), and m*TO*R). Ragulator is anchored to lysosomal membranes through lipid-modified LAMTOR1 [40]; therefore, LAMTOR1 may be a functional homolog of Ego1. LAMTOR 2 and LAMTOR3 form a heterodimer each with a monomer protein, which are structurally and functionally homologous to Ego3 [41]. LAMTOR4 and LAMTOR5 show high structural similarities with Ego2 and Ego4, a paralog of Ego2 [35, 42]. Ragulator and heterodimeric Rag GTPases, which consist of GTP-bound RagA/RgB and GDP-bound RagC/RagD, communicate the signal of an amino acid sufficiency to mTORC1 on lysosomal membranes in mammalian cells (**Figure 2**).

3.2.3. GEF and GAP for Rag GTPases

The activities of small GTPases are generally regulated by the status of the guanine nucleotide loaded, which is controlled by the guanine nucleotide exchange factor (GEF) and GTPase-activating protein (GAP). Gtr1-activating factors were screened using a genetic approach, and, as a result, Vam6 (also known as Vps39) was obtained [36]. Vam6/Vps39 exhibited Gtr1 GEF activity *in vitro* [36].

On the other hand, a breakthrough regarding the regulation of Gtr2 was achieved by the discovery that Folliculin (FLCN) tumor suppressor functioned as a positive regulator of RagC/RagD [43, 44]. FLCN forms a complex with either FNIP1 or FNIP2 and has the ability to recruit mTORC1 to lysosomal membranes in response to an amino acid stimulation, thereby activating mTORC1. FLCN-FNIP1/2 complexes are GAPs toward RagC/RagD. A similar mechanism was conserved in yeast, that is, Lst7 and Lst4 are counterparts of FLCN and FNIP1/2, respectively [45]. Lst4 and Lst7 form a stable complex, both of which are necessary for the activation of TORC1 in the presence of amino acids. The Lst4-Lst7 complex preferentially binds to $Gtr2^{GTP}$ in order to enhance the hydrolytic activity of its GTPase activity, thereby yielding $Gtr2^{GDP}$ to activate TORC1 in yeast upon an amino acid stimulation (**Figure 2**).

3.2.4. SEACIT and SEACAT (GATOR1 and GATOR2)

What is an upstream regulator(s) of Rag GTPases? In order to solve this question, genome-wide screening was conducted in yeast to discover negative effectors of TORC1 activity, and, as a result, Npr2 and Npr3 were identified [46]. Npr2 and Npr3 form a heterodimer [46]. The coatomer-related Seh1-associated complex (SEAC) that associates with vacuolar membranes was implicated in responses to nitrogen starvation [47–49]. Npr2 and Npr3 together with Iml1/Sea1 form a SEAC subcomplex, which negatively regulates Gtr1 within the EGO complex [50]. A biochemical analysis revealed that Iml1/Sea1 exhibited GAP activity toward Gtr1 *in vitro*.

Npr2 is phosphorylated by an unknown protein kinase and dephosphorylated by PP2A, the phosphorylation status of which correlates with the assembly of this SEAC subcomplex. The Iml1/Sea1-Npr2-Npr3 SAEC subcomplex, which was named SEACIT (for **SEAC** subcomplex-**I**nhibiting **T**ORC1 signaling), functions as GAP toward Gtr1, thereby inhibiting TORC1 signaling [50]. The GAP activity of SEACIT is conserved in higher eukaryotes, such as *Drosophila* and humans, that is, DEPDC5-NPRL2-NPRL3 corresponds to the yeast Iml1/Sea1-Npr2-Npr3, and DEPDC5 (ortholog of yeast Iml1/Sea1) directly binds to RagA and enhances the hydrolytic activity of GTP-bound RagA; therefore, the DEPDC5-NPRL2-NPRL3 complex was designated GATOR1 (for **G**AP **A**ctivity **TO**ward **R**ags **1**). GATOR1 inactivates mTORC1 in the absence of amino acids (**Figure 2**).

The SEAC of yeast is an octameric complex, that is, SEAC contains Sea2, Sea3, Sea4, Seh1, and Sec13 besides Iml1/Sea1-Npr2-Npr3, which constitutes SEACIT. These proteins constitute the other SEAC subcomplex, which binds to SEACIT in order to inhibit its Gtr1 GAP activity, and, thus, has been designated SEACAT (**SEAC** subcomplex-**A**ctivating **T**ORC1 signaling) [49]. Orthologs of components in SEACAT also exist in *Drosophila* and mammals, that is, WDR24, WDR59, Mios, Seh1L, and Sec13 in flies and humans, respectively, are Sea2, Sea3, Sea4, Seh1, and Sec13 in yeast, and this complex is referred to as GATOR2. All components in SEACAT and GATOR2 contain beta propeller-forming WD40 motifs, which are characteristic in membrane-coating proteins [51]. Sec13 is a component of COPII, which controls vesicle transport. In addition, Seh1/Seh1L and Sec13 are components of the nuclear pore complex [49].

3.2.5. Upstream modules of GATOR2 (Sestrins and CASTOR)

Amino acid sensors that function upstream of GATOR2 were identified in 2016, that is, Sestrin1/2 as a Leu sensor [52] and CASTOR as an Arg sensor [53]. Previous studies reported that Sestrins interacted with GATOR2 in order to inhibit mTORC1 signaling under amino acid-depleted conditions [54–56]. Wolfson et al. [52] demonstrated that Leu directly bound to Sestrin2 with a dissociation constant of 20 μM, and the binding of Leu to Sestrin2 disrupted the Sestrin2-GATOR2 interaction, thereby enabling GATOR2 to interact with GATOR1. The interaction between GATOR2 and GATOR1 inhibits the GAP activity of GATOR1 toward RagA/RagB, and, consequently, mTORC1 is activated.

The uncharacterized protein CASTOR1 binds to GATOR2, which inhibits GATOR2 binding to GATOR1. CASTOR1 forms a homodimer with CASTOR1 and a heterodimer with CASTOR2, a CASTOR1-related protein. Arginine specifically binds to CASTOR1 with a dissociation constant of ~30 μM, and the binding of Arg to CASTOR1 disrupts the CASTOR1-GATOR2 interaction, which turns CASTOR1 into a homodimer [53]. Liberated GATOR2 interacts with GATOR1 in order to inhibit its GAP activity toward RagA/RagB, which leads to the activation of mTORC1 (**Figure 2**).

Since no orthologs of Sestrins or CASTOR have been found in yeast, the mechanisms by which yeast senses intracellular amino acid availability currently remain unclear. A model in which tRNA functions as a negative regulator of TORC1 kinase activity in yeast was recently proposed [57]. Both amino acid-uncharged and amino acid-charged (aminoacylated) tRNAs inhibited TORC1 kinase activity in an *in vitro* kinase assay. Under nutrition-sufficient

conditions, aminoacylated tRNAs predominantly bind to ribosomes for protein synthesis; therefore, tRNAs have fewer opportunities to interact with TORC1 (i.e., TORC1 is active). Upon nutrition starvation, uncharged tRNAs are released from ribosomes and interact with TORC1 in order to inhibit its kinase activity.

Human and yeast cells depleted for Rag GTPase/Gtr remained the ability to respond to amino acid, particularly glutamine [58–60]. It was recently reported that phosphatidylinositol 3-kinase complex Vps34-Vps15, and a vacuolar membrane protein Pib2, which contains a phosphatidylinositol 3-phosphate-binding FYVE (Fab1, YOTB, Vac1, and EEA1) domain, played a role in sensing glutamine in the Gtr-independent activation of TORC1 in *S. cerevisiae* [61].

3.3. The TSC1/2-Rheb branch in the activation of mTORC1

In mammalian cells, mTORC1 is activated by another small GTPase Rheb (*R*as *h*omolog *e*nriched in *b*rain). Similar to other small GTPases, GTP-bound Rheb is a dynamic form in terms of the activation of mTORC1, and the guanine nucleotide status in Rheb is regulated by machinery downstream of growth factor signaling, such as the insulin-signaling pathway. Although the mechanisms by which Rheb^{GTP} stimulate mTORC1 have not yet been elucidated, mTORC1 activity is negatively regulated by the TSC complex, consisting of TSC1, TSC2, and TBC1D7, in which TSC2 functions as GAP toward Rheb^{GTP}. Upon a growth factor stimulation, Akt, a member of the AGC kinase family, is activated in a phosphatidylinositol 3-kinase-dependent manner, and activated Akt subsequently phosphorylates TSC2. The TSC complex is localized in the cytoplasm close to lysosomal membranes with which mTORC1 associates via Ragulator, and the phosphorylation of TSC2 alters the localization of the TSC complex away from the lysosome, thereby releasing Rheb from the inhibitory effects induced by the TSC complex [62]. On the other hand, a previous study reported that amino acid deprivation recruited the TSC complex to the lysosome [63], suggesting that the amino acid-dependent activation of mTORC1 is regulated by an interplay between the Rag GTPases-Ragulator branch and the TSC complex-Rheb branch. A recent study reported that Arg is required for the growth factor-dependent delocalization of the TSC complex from the lysosome, which leads to the activation of Rheb, and, thus, mTORC1 [64] (**Figure 2**).

S. cerevisiae does not contain the orthologs of TSC1/2, but has the Rheb homolog, Rhb1; however, there is currently no evidence to show that Rhb1 is a functional homolog of mammalian Rheb [36]. On the other hand, the fission yeast *Schizosaccharomyces pombe* was found to have homologs of TSC1/2 and Rheb [65]. Similar to the mammalian TSC complex, Tsc1 and Tsc2 in fission yeast form a complex, in which Tsc2 functions as a GAP toward Rhb1 GTPase [65–67]. Rhb1 physically interacts with Tor2 (Tor2 in fission yeast corresponds to Tor1 in budding yeast), thereby stimulating TORC1 (Tor2 is involved in TORC1 in fission yeast) activity [65, 68]. Therefore, an *rhb1* mutant showed some phenotypes that are displayed in cells starved of nitrogen [69, 70]. An epistatic analysis showed that Rhb1 functions upstream of Tor2, that is, the activated allele of *tor2*$^{+}$ suppressed the loss of function of *rhb1*$^{+}$ [71].

4. TORC2

4.1. Subunit components

The following components constitute the budding yeast TORC2: Tor2, Avo1, Avo2, Avo3, Bit61, and Lst8 (**Figure 1**). Avo1 has several conserved domains. Avo1 contains an RBD (a *R*as-*b*inding *d*omain) at the center of its molecule. At the C-terminal region of Avo1, an essential PH (*P*leckstrin *h*omology)-like domain exists, through which TORC2 may tether to the definite region of the plasma membrane called the MCT (*m*embrane *c*ompartment-containing *T*ORC2) [72]. The CRIM (*c*onserved *r*egion *i*n the *m*iddle) domain exists in proximity to the N-terminal side of RBD and has been implicated in binding to the substrates of TORC2 [73, 74]. Avo1 binds to the kinase domain of Tor2 via Lst8 [25].

Avo3 is the largest subunit of TORC2. It functions as a scaffold protein in order to maintain the integrity and function of TORC2 because the loss of Avo3 induced the disassembly of TORC2 [75]. Avo3 contains the ARM (*arm*adillo repeat)-like domain, which is a similar structure to the HEAT repeats, at the center of its molecule. Repeated ARM units fold together as a superhelical structure to provide a platform to interact with many proteins [76]. Avo3 also interacts with the FAT and kinase domains of Tor2 within TORC2. Avo3 has a RasGEFN domain, which is found in the N-terminal region of GEF proteins toward Ras-like GTPases; however, the function of this domain in Avo3 currently remains unknown. Since the FRB domain of Tor2 within TORC2 is masked by the C-terminal part of Avo3, the accessibility of the Fpr1 (FKBP12)-rapamycin complex to TORC2 is limited, which renders TORC2 insensitive to rapamycin.

Bit61 has a paralog Bit2. Although Bit61 binds to TORC2 through Avo1 and Avo3, it is not an essential subunit for the assembly of TORC2 [25, 75]. The specific functions of Bit61 have not yet been elucidated; however, mammalian orthologs of Bit61 and Bit2 exist (PRR5 also known as Protor-1, and PRR5L also known as Protor-2) and possess an HbrB domain that was found in a fungal *Aspergillus nidulans* protein required for filamentous growth [77].

Avo2 is a yeast TORC2-specific subunit, but is not essential. Avo2 contains ankyrin repeats. Avo2 and Bit61 have been reported to bind to Slm1 and Slm2 proteins, which are involved in the recruitment of Ypk1/Ypk2 to TORC2, thereby phosphorylating them [78].

The core subunits of mammalian TORC2 (mTORC2) include mTOR as the TOR protein, mSin1 (*s*tress-activated protein kinase-*in*teracting protein *1*) as the Avo1 ortholog, Rictor (*r*apamycin-*i*nsensitive *c*ompanion of m*TOR*) as the Avo3 ortholog, and mLst8 as the Lst8 ortholog. Analogous to the yeast counterpart, mSin1 contains RBD. mSin1 was originally cloned as a factor that interfered with *S. cerevisiae* Ras signaling [79]. mSin1 also contains the CRIM and PH domains, which function in the binding of substrates and tethering to the plasma membrane, respectively.

The ARM-like domain is conserved in Rictor and Avo3, while the RasGEFN domain is not conserved in Rictor. mTORC2 is also insensitive to an acute treatment with rapamycin, the mechanism of which is presumably the same as that elucidated in yeast TORC2. However, in some mammalian cell lines, a prolonged treatment with rapamycin was found to inhibit the interaction between newly synthesized mTOR and Rictor, and mTORC2-Akt signaling was subsequently reduced [80].

4.2. Activation of TORC2

4.2.1. Implication of GTPases

Small GTPase Rag complexes ($RagA^{GTP}/RagB^{GTP}$-$RagC^{GDP}/RagD^{GDP}$ in metazoans, and $Gtr1^{GTP}$-$Gtr2^{GDP}$ in yeast) play pivotal roles in the amino acid-induced activation of TORC1, as described in the previous sections. The other small GTPase Rheb is also involved in the growth factor-mediated activation of mTORC1. Do any small GTPases play roles in the activation of TORC2? In the fission yeast *S. pombe*, genetic screening revealed that the human Rab6 GTPase ortholog Ryh1 was involved in TORC2-Gad8 signaling [81]. *S. pombe* TORC2 phosphorylated Gad8, a member of the AGC kinase family, and a genetic mutation in *ryh1*+ markedly decreased the phosphorylation level of Gad8. *sat1*+ and *sat4*+ genes were predicted to code for GEFs toward Ryh1, and the mutational inactivation of these genes also induced a decrease in the phosphorylation level of Gad8, suggesting that $Rhy1^{GTP}$ is an active form in terms of the activation of TORC2-Gad8 signaling. GTP-locked Rhy1 facilitated the physical interaction between TORC2 and its substrate Gad8. Furthermore, the expression of human Rab6 functionally compensated for the loss of *ryh1*+ in *S. pombe* in terms of TORC2 signaling, which implied that Rab GTPase is involved in mTORC2-Akt signaling in mammals, similar to fission yeast. However, since *S. cerevisiae* does not possess the Rab6 ortholog, it currently remains unclear whether this regulatory system is generally conserved in eukaryotes. However, Avo1 and Avo3 contain the RBD and RasGEFN domains, respectively, both of which are related to Ras GTPase; therefore, some small GTPases may be involved in TORC2 signaling in *S. cerevisiae*. Previous studies demonstrated the participation of small GTPases in mTORC2 signaling. Rac1 GTPase was reported to bind directly to mTOR within mTORC1 and mTORC2, which led to the appropriate localization of these TOR complexes to the respective cellular membranes [82]. Rit, a Ras family GTPase, was shown to bind to mTORC2 and subsequently activate it in response to oxidative stress [83]. Since the oxidative stress-responsive activation of TORC2 was also observed in *S. cerevisiae* [84], a similar mechanism by which Ras family GTPase activates TORC2 may be conserved in budding yeast.

4.2.2. Posttranslational modifications in TORC2 components

mTOR is phosphorylated in the growth factor-mediated activation of mammalian TOR signaling. For example, Thr^{2173} in the kinase domain of the mTOR protein is phosphorylated by Akt, which appears to be the negative feedback regulation of mTORC2 signaling. This feedback regulation is also conserved in fission yeast TORC2-Gad8 signaling, that is, Gad8 phosphorylates Thr^{1972} in the ATP-binding domain to reduce Tor1 activity within TORC2 [85]. More than 20 potential phosphorylation sites have been assigned in Rictor [86]. Ser^{260} in the CRIM domain and Thr^{398} in the PH domain in mSin1 are also phosphorylated [87, 88]. A high-throughput phosphoproteomic analysis predicted numerous potential phosphorylation sites in Avo1-3 and Bit61 [89].

Besides phosphorylation, Rictor is known to be acetylated at Lys^{1116}, Lys^{1119}, and Lys^{1125} [90, 91], modifications to which may activate mTORC2 activity.

4.3. Activation of TORC2 signaling

4.3.1. Relationship between membrane tension and the activation of TORC2 signaling in yeast

When TORC2 was observed using GFP-tagged Avo1 or Avo3, its localization was visible as many dots just beneath the plasma membrane. The plasma membrane regions at which patchy TORC2 is located are referred to as the MCT [78]. Although Avo1 contains the PH domain, which has the potential to associate with membrane phospholipids, the underlying mechanisms by which TORC2 localizes to the plasma membrane remain unclear. Other regions on the yeast plasma membrane, referred to as eisosomes, are characterized by their distinctive shape, that is, they are furrows approximately 50-nm deep and 300-nm long on the surface of the plasma membrane [92]. The curvature of the membrane in eisosomes is formed by proteins possessing the BAR (*B*in/*a*mphiphysin/*R*vs) domain, that is, Pil1 and Lsp1. Eisosomes exist in close proximity to the MCT, but never overlap.

Slm1 and its paralog Slm2 are eisosome-residential proteins and are effectors as well as substrates of TORC2. Under normal turgor pressure conditions, Slm1 and Slm2 are predominantly localized in eisosomes; however, following an increase in membrane tension caused by, for example, hypotonic shock or some mechanical stress, Slm1 and Slm2 alter their localization from eisosomes to the MCT and then bind to TORC2 via its components Avo2 and Bit61. Slm1 and Slm2 may recruit Ypk1 to TORC2, and the interaction between TORC2 and its substrate Ypk1 promotes the phosphorylation of Ypk1 (**Figure 3**).

It has not yet been established whether there exist any natural conditions that change the tension of the plasma membrane in yeast. One of these conditions may induce a decrease in the levels of sphingolipids that constitute the yeast plasma membrane together with glycerophospholipids and ergosterols. The initial step in the biosynthetic pathway of sphingolipids is catalyzed by serine palmitoyltransferase. The activity of this enzyme is negatively regulated by Orm1 and its paralog Orm2, the functions of which are controlled through the phosphorylation by Ypk1, a TORC2 substrate, at Ser^{51}, Ser^{52}, and Ser^{53} in Orm1, and Ser^{46}, Ser^{47}, and Ser^{48} in Orm2 [93, 94]. Myriocin is a potent inhibitor of serine palmitoyltransferase; therefore, the treatment of yeast cells with this chemical reduces the production of sphingolipids, which causes feedback regulation to activate sphingolipid biosynthesis through
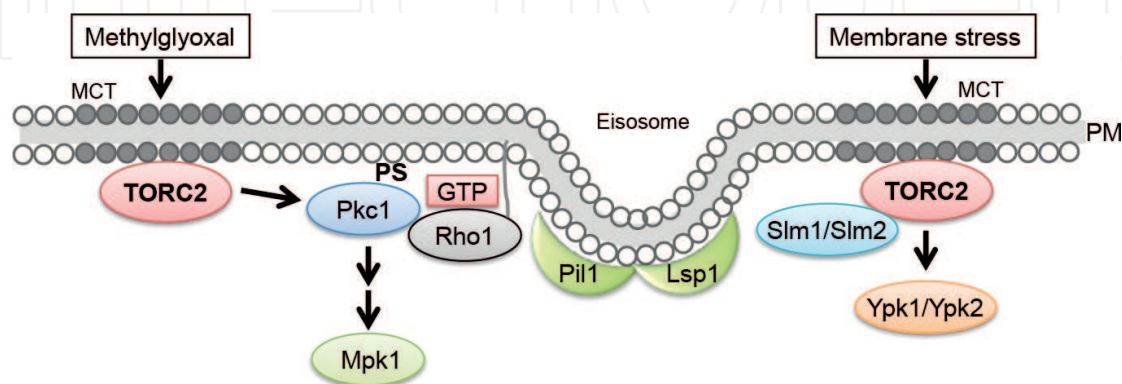

Figure 3. Activation of TORC2 signaling in *S. cerevisiae*.

the stimulation of TORC2-Ypk1 signaling. Orm1/2 is subsequently phosphorylated, and its inhibitory effects on serine palmitoyltransferase are then compromised. Aureobasidin A, a cyclic depsipeptide antibiotic drug, exerts similar effects on the yeast plasma membrane in terms of altering membrane tension because this chemical inhibits the synthesis of inositol-phosphoceramide, one of the sphingolipid species in yeast. Aureobasidin A and myriocin consistently induce the phosphorylation of Ypk1 at Thr^{662}, a target site of TORC2 [94].

4.3.2. Activation of TORC2 signaling by the metabolic cue methylglyoxal

In contrast to mammals, which possess many isozymes of protein kinase C and its related kinases, Pkc1 is the sole protein kinase C in budding yeast. Pkc1 is involved in numerous pivotal biological functions including the organization of the actin cytoskeleton and the maintenance of cell wall integrity (CWI). The Mpk1 MAPK cascade lies downstream of Pkc1, and the Pkc1-Mpk1 MAPK cascade constitutes the main stream of the CWI pathway [95]. Chemicals that provoke cell wall damage such as Congo red or heat-shock stress activate the CWI pathway. The small GTPase Rho1 plays a crucial role in the heat-shock stress-induced activation of the CWI pathway, that is, the transmembrane proteins Wsc1 and Mid2 on the plasma membrane sense heat shock and interact with Rom2, a GEF toward Rho1, to load GTP to Rho1. $Rho1^{GTP}$ physically interacts with Pkc1 to communicate the signal to the downstream Mpk1 MAPK cascade [96, 97]. A recent study reported that phosphatidylserine, one of the major glycerophospholipids prevailing in the plasma membrane, mediates the physical interaction between Pkc1 and $Rho1^{GTP}$ [98, 99]. On the other hand, methylglyoxal, a typical 2-oxoaldehyde derived from glycolysis [100], also activates the Pkc1-Mpk1 MAPK cascade; however, the methylglyoxal-induced activation of this pathway is not dependent on Wsc1/Mid2, whereas Rho1 is indispensable [101]. Besides Ypk1 and Ypk2, Pkc1 has also been identified as a direct substrate of TORC2 in *S. cerevisiae*, that is, Thr^{1125} within the turn motif and Ser^{1143} within the hydrophobic motif in Pkc1 are phosphorylated by TORC2 [101]. Methylglyoxal enhanced the phosphorylation levels of Pkc1 at Ser^{1143} in a TORC2-dependent manner [101] (**Figure 3**).

The methylglyoxal-induced activation of TORC2 is conserved in mammalian cells, that is, the phosphorylation levels of Ser^{473} within the hydrophobic motif in Akt, a substrate of mTORC2, were enhanced following the treatment of mouse 3 T3-L1 cells with methylglyoxal [101]. Collectively, these findings demonstrate that methylglyoxal activates (m)TORC2 signaling in yeast and mammalian cells; however, the underlying mechanisms have not yet been elucidated. Since methylglyoxal is a naturally occurring ubiquitous metabolite and is involved in type 2 diabetes and its complications [100], its involvement in the activation of (m)TORC2 signaling is of considerable interest in order to obtain insights into not only novel activation mechanisms of TORC2 but also the physiological significance of methylglyoxal.

4.3.3. Activation of mTORC2 signaling by growth factor

In mammalian cells, physiological cues for the activation of mTORC2 signaling are insulin and insulin-like growth factors [102]. Upon the capture of ligands by tyrosine kinase-type receptors, tyrosine-phosphorylated IRS (*i*nsulin *r*eceptor *s*ubstrate) undergoes the activation of phosphatidylinositol 3-kinase, which enhances the levels of phosphatidylinositol

(3,4,5)-trisphosphate (PtdIns(3,4,5)P_3). Two events are subsequently induced by this phosphoinositide near the plasma membrane: that is, the activation of PDK (*p*hosphoinositide-*d*ependent *k*inase) and the recruitment of Akt to the plasma membrane in which the PH domain of Akt binds to PtdIns(3,4,5)P_3. In turn, Akt at Thr^{308} within the activation loop and Ser^{473} within the hydrophobic motif are phosphorylated by PDK and mTORC2, respectively; however, the mechanisms by which insulin activates mTORC2 remain obscure.

5. Concluding remarks

Laboratory conditions for culturing yeast may be adequate for yeast cells to maintain cellular activities because ample amounts of glucose and amino acids are typically supplied in media. By contrast, nutritional conditions surrounding yeast cells that exist in the natural world are harsh and variable. Yeast cells have evolved mechanisms for sensing changes in nutritional conditions and transitioning the metabolic status and gene expression profile to adapt efficiently and survive inhospitable conditions. The TOR signaling system had been acquired as one of these signal network systems and has been evolutionarily conserved among eukaryotes. In higher eukaryotes, such as humans, dysfunctions in the TOR signaling network closely correlate with pathological conditions including diabetes, cancer, obesity, and neurodegeneration [1]; therefore, TOR is a target from a clinical point of view. Upstream and downstream processes of TORC1 signaling have been extensively investigated because rapamycin, a potent inhibitor for TORC1, was available. By contrast, studies on TORC2 signaling appear to be challenging because of the absence of TORC2-specific inhibitors. However, yeast was always a vanguard from the beginning of TOR studies (TOR was discovered by genetic screening using yeast) and will continue to be so in the future. Many issues remain to be solved in TOR signaling; however, since TOR is a central player in cell growth, studies on TOR will be nothing less than a study of the living system itself. Investigations on TOR will provide many insights for understanding "life."

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