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Identification of the Lam-Gtr1-Gtr2 complex as a novel negative regulator of TOR complex 1 in fission yeast

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Graduate School of Biological Sciences　Doctoral Thesis Abstract

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Title	Identification of the Lam-Gtr1-Gtr2 complex as a novel negative regulator of TOR complex 1 in fission yeast		

The Target of Rapamycin (TOR) protein kinase forms an evolutionarily conserved high-molecular weight complex, TOR complex 1 (TORC1), which serves as a central hub to integrate nutrient signals to coordinate cell growth and metabolism. Several recent reports have proposed the molecular mechanism that mediates the mammalian TORC1 (mTORC1) activation in response to amino acid. In the proposed model, mTORC1 is recruited to lysosomal membranes upon amino acid stimuli, leading to its activation by Rheb, a key activator of mTORC1 that also resides to lysosomes. The translocation of mTORC1 to lysosomes is promoted by the heterodimeric Rag GTPases (RagA or RagB pairs with RagC or RagD), which are anchored to the lysosomal surface through a protein complex named Ragulator. On the other hand, depletion of amino acids causes mTORC1 to be released to the cytoplasm, leading to its inactivation. However, discrepant observations have also been reported, such as RagA/B-independent mTORC1 activation and amino-acid independence of guanine nucleotide loading to the Rag GTPases.

The TORC1 signaling pathway is well conserved in the fission yeast *Schizosaccharomyces pombe* and therefore, *S. pombe* is expected to serve as an excellent model system to study the regulatory mechanisms that control TORC1 signaling. Orthologs of the Rag GTPases have been found in fission yeast, called Gtr1 (a yeast homolog of RagA and RagB) and Gtr2 (a homolog of RagC and RagD). However, no apparent ortholog of the Ragulator components have been identified in fission yeast. In order to further elucidate the evolutionary conserved mechanism of TORC1 regulation in response to nutrient signals, I set out to dissect the Rag GTPases-TORC1 signaling by taking advantage of powerful genetic tools available in fission yeast.

Aiming to identify a Ragulator counterpart in fission yeast, the Gtr1-Gtr2 heterodimer was purified from *S. pombe* cell lysate using two successive immunoprecipitation procedures and the co-purified proteins were analyzed by mass spectrometry. This approach identified a protein complex composed of four proteins, which were named Lam1, Lam2, Lam3 and Lam4. Similar to Gtr1-Gtr2 heterodimer, the four Lam proteins were also found on vacuolar membranes. Importantly, disrupting any of the Lam genes caused mislocalization of Gtr1-Gtr2, suggesting that Gtr1-Gtr2 heterodimer is anchored to the vacuole surface through the Lam protein complex. These results

strongly suggest that the Lam protein complex is a fission yeast equivalent of the mammalian Ragulator complex.

It was noticed that the knockout mutants of the Lam proteins or Gtr1-Gtr2 exhibited severe growth defects on rich medium, indicating that Lam-Gtr1-Gtr2 machinery is required for *S. pombe* growth. In mammalian cells, the guanine nucleotide binding state of the Rag GTPases are important to control mTORC1 activation upon stimulation by amino acid; the GTP-bound form of RagA or RagB promotes the recruitment of mTORC1 to lyososomes. To test whether the nucleotide binding state of Gtr1 affects fission yeast growth, point mutations were introduced into the *gtr1*$^{+}$ gene to express GTP-locked and GDP-locked Gtr1. Surprisingly, the mutant expressing GTP-locked Gtr1 exhibited a growth defect similar to the defective phenotype of the Lam-Gtr1-Gtr2 null mutants, while the mutant expressing the GDP-locked Gtr1 grew normally, suggesting that the GDP-bound Gtr1 is required for growth. This result is somehow contradicting with the mammalian model, in which the GTP-bound RagA/B induces mTORC1 activation to promote cell growth. To examine whether TORC1 activity is regulated by the nucleotide binding state of Gtr1 in *S. pombe*, TORC1 signaling was accessed by the phosphorylation state of the TORC1 substrates, Psk1 and Sck1. The results clearly demonstrated that the mutant expressing GTP-locked Gtr1 and the Lam-Gtr1-Gtr2 null mutants exhibited hyperactivation of TORC1. Collectively, these results strongly suggest that Lam-Gtr1-Gtr2 are required to attenuate TORC1 signaling. Since TORC1 activation is known to promote cell growth, the growth defect phenotype induced by hyperactive TORC1 signaling is an unexpected observation. However, the Lam-Gtr1-Gtr2 null mutants were found to exhibit a defect in cellular uptake of amino acids, leading to the growth defect phenotype.

The Tsc-Rhb1 is a well-known signaling pathway upstream of TORC1. A protein complex consists of Tsc1 and Tsc2 has been reported as a negative regulator of TORC1 signaling by acting as GTPase-activating protein (GAP) for Rhb1, ortholog of the mammalian Rheb GTPase. To test the possibility that Lam-Gtr1-Gtr2 down-regulates TORC1 signaling through the Tsc-Rhb1 pathway, TORC1 activity of a mutant in which both *gtr1*$^{+}$ and *tsc2*$^{+}$ genes were deleted was evaluated. The double mutant exhibited an addictive effect in the TORC1 hyperactivation as compared to the individual single mutants, suggesting that the Lam-Gtr1-Gtr2 and Tsc-Rhb1 signaling pathways regulate TORC1 independently. Therefore, it appears that the Lam-Gtr1-Gtr2 mediated TORC1 attenuation utilizes a novel mechanism to suppress TORC1 signaling.

This study has identified a protein complex, the Lam complex, that serves as a fission yeast equivalent of the mammalian Ragulator complex. More importantly, this study also highlighted a novel negative regulation on TORC1 signaling by the Lam-Gtr1-Gtr2 machinery, which is important for optimal cellular response to nutrients. It is expected that further understanding of TORC1 signaling will contribute to the development of novel strategies to combat human diseases associated with deregulated TORC1 such as epilepsy and cancerous cell proliferation.

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1. Introduction

In multicellular organisms, individual cells sense the changes in their surrounding environment and respond to the changes appropriately for their growth and survival. Cells employ signaling pathways to sense and respond to diverse extracellular signals such as nutrients, stresses, growth factors and chemical signals emitted from other cells. Among such cellular signaling pathways, the TOR pathway has emerged as a pivotal signaling axis that attracts much attention lately.

1.1 The target of rapamycin (TOR)

The target of rapamycin (TOR) is a protein kinase initially discovered in the budding yeast *Saccharomyces cerevisiae* by Hall and his colleagues (Heitman et al., 1991). Mutations in *TOR1* and *TOR2* confer resistance to the toxicity of rapamycin, leading to the identification of the TOR proteins as cellular target of rapamycin (Heitman et al., 1991; Kunz et al., 1993; Cafferkey et al., 1993). Shortly thereafter, mammalian TOR (mTOR) was also identified through biochemical approaches (Brown et al., 1994; Chiu et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). TOR is a member of the phosphatidylinositol 3-kinase-related protein kinase (PIKK) family, and phosphorylates serine or threonine residues of its substrate proteins (reviewed in Loewith and Hall, 2011).

1.2 TOR complexes

In 2002, two distinct multiprotein complexes containing TOR were biochemically purified from budding yeast and named TOR complex 1 (TORC1) and TOR complex 2 (TORC2). TORC1 is sensitive to rapamycin, and it contains either Tor1 or Tor2 as its catalytic subunit. Rapamycin inhibits TORC1 activity by forming a complex with FKBP12 (FK506-binding protein, 12 kDa) that binds to the TOR kinase in TORC1. On the other hand, Tor2 is the core kinase of TORC2, and this complex is insensitive to rapamycin (Loewith et al., 2002). Shortly thereafter, TOR complexes were also identified in other eukaryotes, including worms and mammalian cells. Unlike budding yeast, mammalian cells only express a single mTOR kinase to serve as the catalytic subunit in mTORC1 and mTORC2 (Hara et al., 2002; Kim et al., 2002; Jacinto et al., 2004; Sarbassov et al., 2004).

1.2.1 TORC1

The budding yeast TORC1 is composed of the Kog1, Lst8 and Tco89 subunits that bind to either Tor1 or Tor2, while in mammalian cells, mTORC1 consists of Raptor, mLst8, PRAS40, DEPTOR, and mTOR (reviewed in Loewith and Hall, 2011). TORC1 responds to

growth factors, energy status, stresses, and nutrients such as amino acids. By phosphorylating its substrates, TORC1 regulates cellular metabolisms as well as transcription, translation, protein localization, ribosome and lysosome biogenesis, and autophagy (reviewed in Wullschleger et al., 2006; Loewith and Hall, 2011; Laplante and Sabatini, 2012). Recently, much attention has been paid to the factors involved in TORC1 signaling, because they are closely related to cancer, obesity, and aging. Indeed, rapamycin has been shown to be effective in the growth inhibition of several human cancers and also in the extension of murine lifespan (reviewed in Guertin and Sabatini, 2007; Laplante and Sabatini, 2012; Shimobayashi and Hall, 2016).

1.2.2 TORC2

Components of the budding yeast TORC2 includes Avo1, Avo2, Avo3, Bit61, Lst8 and Tor2. On the other hand, mTOR binds mSIN1, Rictor, mLST8, PRR5/Protor, and DEPTOR to form mTORC2 in mammalian cells (reviewed in Loewith and Hall, 2011). As mentioned above, TORC2 is insensitive to rapamycin, and a possible cause of rapamycin insensitivity in the budding yeast TORC2 has been proposed recently; the C-terminus of the Avo3 subunit sterically obstructs the binding of the rapamycin-FKBP complex to the FRB domain of TOR2 (Gaubitz et al., 2015). It remains to be determined if a similar mechanism is responsible for the rapamycin-insensitivity of TORC2 in other organisms. In mammalian cells, mTORC2 is known to respond to growth factors and hormones, such as insulin, through a mechanism that requires phosphatidylinositol 3-kinase (PI3K). Downstream effectors of mTORC2 includes SGK1 (serum- and glucocorticoid-induced protein kinase 1) and Akt, a well-known oncogene product (Sarbassov et al., 2005; Guertin et al., 2006; García-Martínez and Alessi, 2008). In budding yeast, TORC2 phosphorylates Ypk1 and Ypk2, an essential pair of homologous kinases that belong to the AGC kinase family (reviewed in Loewith and Hall, 2011; Laplante and Sabatini, 2012).

1.3 TORC1 as a master regulator in nutrient response

TORC1 appears to be a master regulator of the cellular response to nutrients, because TORC1 integrates nutrient signals and controls metabolism. The first indication that TORC1 links nutrient signals to cell growth was found in budding yeast. Because TORC1 controls both catabolic and anabolic processes, inactivation of TORC1 causes yeast cells to exhibit phenotypes similar to those under nutrient starvation, such as decreased protein synthesis, growth arrest and induction of autophagy (Barbet et al., 1996; reviewed in Loewith and Hall, 2011). Like its budding yeast counterpart, mTORC1 stimulates protein synthesis and suppresses autophagy to promote cell growth in response to nutrients. It phosphorylates S6

kinase 1 (S6K1) and translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) to promote protein synthesis (reviewed in Ma and Blenis, 2009). Active mTORC1 also suppresses autophagy by phosphorylating Atg13 and ULK1, components of the protein kinase complex required for initiation of autophagy (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009).

Following sections 1.3.1 and 1.3.2 describe the two major signaling axes upstream of TORC1.

1.3.1 The TSC-Rheb-mediated mTORC1 activation (Figure 1)

The Ras homolog enriched in brain (Rheb) small GTPase is an essential activator of mTORC1. Rheb localizes on lysosomal membranes and its GTP-bound form interacts with mTORC1 directly to stimulate the kinase activity of mTORC1 (Long et al., 2005; reviewed in Laplante and Sabatini, 2012). Another key upstream regulator is the TSC protein complex that is composed of tuberous sclerosis 1 (TSC1), TSC2, and TBC1D7. The TSC complex functions as a GTPase-activating protein (GAP) for Rheb, and the resulted GDP-bound form of Rheb cannot activate mTORC1 (Inoki et al., 2003; Tee et al., 2003). It is known that many of the upstream stimuli, such as growth factors and energy levels, impinge on the TSC-Rheb pathway to regulate mTORC1 activation. For instance, growth factor signals through the PI3K-PDK1-Akt pathway, and the activated Akt kinase then phosphorylates TSC2. Phosphorylation of TSC2 leads to dissociation of the TSC complex from the lysosome, thus resulting in mTORC1 activation (Menon et al., 2014; reviewed in Laplante and Sabatini, 2012; González and Hall, 2017). Because mTORC1 signaling controls cell growth, mutations in the genes that act upstream of mTORC1 signaling, such as *tsc1* and *tsc2*, are often observed in cancer cells (reviewed in Laplante and Sabatini, 2012). It should be noted that the budding yeast *S. cerevisiae* has no apparent TSC orthologs (reviewed in González and Hall, 2017).

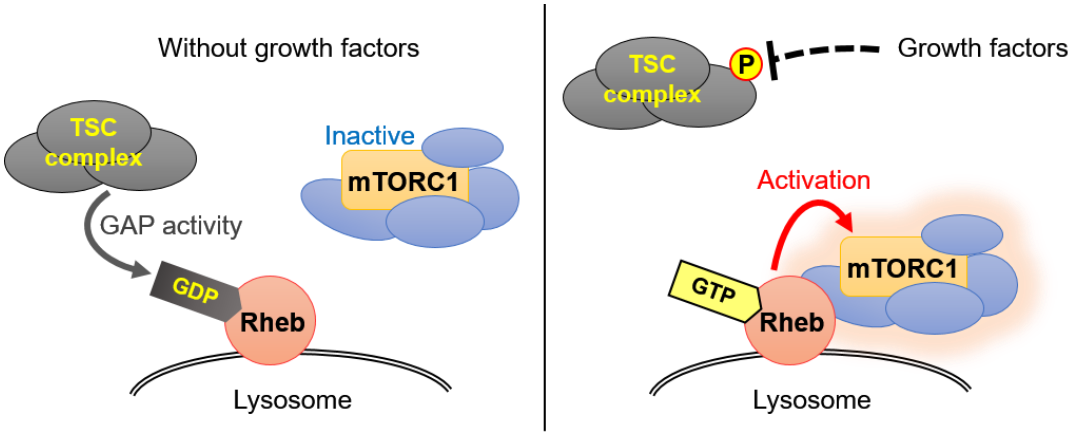

Figure 1. The TSC-Rheb-mediated mTORC1 activation.

1.3.2 The Rag GTPases-mediated TORC1 activation by amino acids

The molecular mechanism that mediates mTORC1 activation in response to amino acid stimuli has recently been a focus of very active research. In the currently prevailing model, a critical step in amino acid-induced activation of mTORC1 is its translocation to the surface of lysosomes, where the Rheb triggers mTORC1 activation (Figure 2). Recruitment of mTORC1 to lysosomes is mediated by the Rag GTPase heterodimer that is anchored to lysosomal membranes through a protein complex called Ragulator (Kim et al., 2008; Sancak et al., 2008; Sancak et al., 2010). The Rag heterodimer consists of RagA or RagB bound to either RagC or RagD, and it has been proposed that guanine nucleotide loading to RagA/B is regulated in response to amino acid signals; the Ragulator complex, which is composed of LAMTOR1/p18, LAMTOR2/p14, LAMTOR3/MP1, LAMTOR4/C7orf59 and LAMTOR5/HBXIP, functions as a guanine nucleotide exchange factor (GEF) for RagA/B (Bar-Peled et al., 2012). In the presence of amino acid stimuli, RagA/B are in the GTP-bound form and the Rag heterodimer can interact with the Raptor subunit of mTORC1 for lysosomal recruitment of mTORC1 (Sancak et al., 2008). On the other hand, the GATOR1 complex composed of DEPDC5, Nprl2, and Nprl3 has GAP activity toward RagA/B, whose GDP-bound form induces the release of mTORC1 from lysosomes in the absence of amino acids (Bar-Peled et al., 2013). Without functional GATOR1, mTORC1 signaling is resistant to amino acid starvation and, interestingly, inactivating mutations to the GATOR1 components have been found in human cancers. GATOR1 forms a larger GATOR complex together with GATOR2, which comprises WDR24, WDR59, MIOS, SEH1L and SEC13, and gene knockdown experiments suggest that the GATOR2 subcomplex negatively regulates the GAP activity of GATOR1 (Bar-Peled et al., 2013).

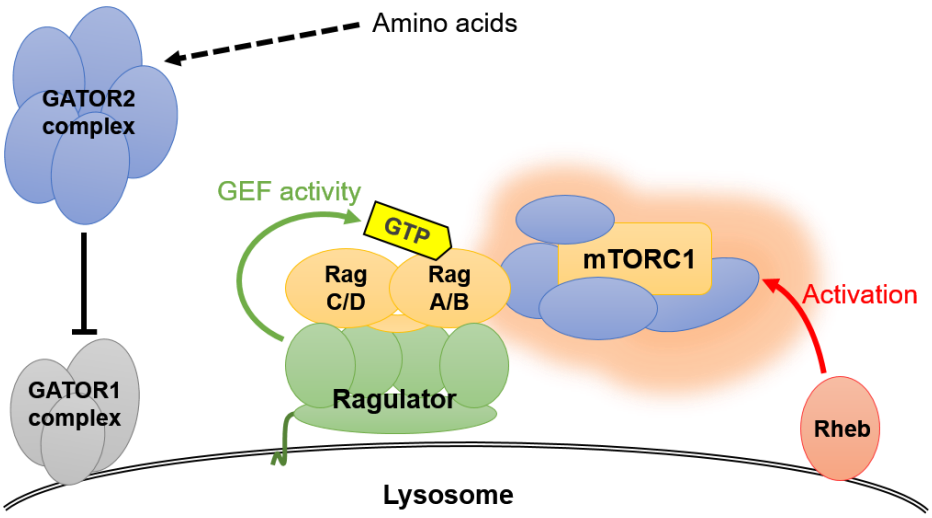

Figure 2. The Rag GTPases-mediated mTORC1 activation by amino acids.

The mTORC1 regulators enumerated above are conserved also in unicellular eukaryotes. The Gtr1-Gtr2 GTPase heterodimer is the budding yeast counterpart of RagA/B-RagC/D, interacting with the Ego1-Ego2-Ego3 complex that tethers Gtr1-Gtr2 to the membrane of the vacuole, a lysosome-like organelle (Nakashima et al., 1999; Dubouloz et al., 2005; Gao and Kaiser, 2006; Powis et al., 2015; Kira et al., 2016). The Ego ternary complex is likely to be the yeast equivalent of mammalian Ragulator, though their constituents share little sequence homology (Kogan et al., 2010). The yeast SEACIT and SEACAT complexes apparently correspond to mammalian GATOR1 and GATOR2, respectively (Neklesa and Davis, 2009; Dokudovskaya et al., 2011; Wu and Tu, 2011; Panchaud et al., 2013a; Panchaud et al., 2013b; Kira et al., 2014). However, these TORC1 regulators in budding yeast might function differently from those in mammals, because the Rheb GTPase, the primary mTORC1 activator, is not part of the TORC1 signaling pathway in budding yeast (Urano et al., 2000).

1.4 TORC1 signaling in the fission yeast *Schizosaccharomyces pombe*

The fission yeast *S. pombe* is a model eukaryote distantly related to budding yeast. As shown in Figure 3, the fission yeast TORC1 is composed of the Tor2 kinase associated with the regulatory subunits Mip1 and Wat1, which are orthologous to mammalian Raptor and mLST8, respectively (Álvarez and Moreno, 2006; Matsuo et al., 2007; Hayashi et al., 2007).

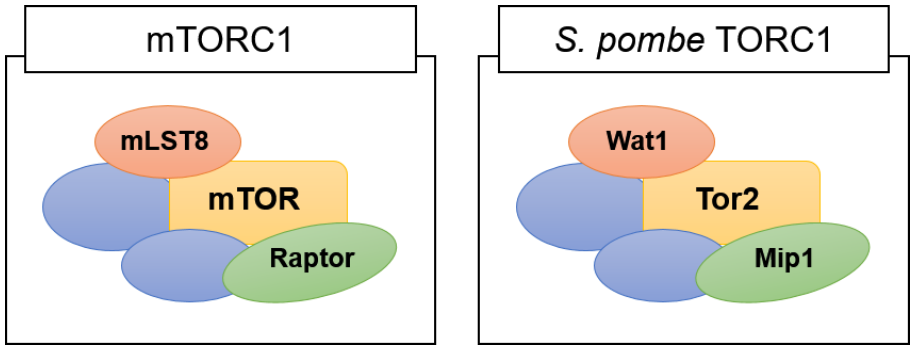

Figure 3. Comparison of the *S. pombe* TORC1 with the mTORC1.

Unlike budding yeast, the TSC-Rheb signaling is well-conserved in fission yeast, where the Rheb ortholog Rhb1 is an essential activator of TORC1. Moreover, Rhb1 is under the regulation of the Tsc1-Tsc2 complex that functions as GAP for Rhb1 (Mach et al., 2000; Urano et al., 2000; Urano et al., 2005; Matsumoto et al., 2002; van Slegtenhorst et al., 2004;

Uritani et al., 2006). Thus, *S. pombe* is expected to serve as an excellent experimental system to explore the TORC1 regulatory mechanisms that are conserved also in mammals. Interestingly, several recent studies have also reported that the heterodimeric Rag GTPases Gtr1-Gtr2 are also implicated in *S. pombe* TORC1 regulation (Valbuena et al., 2012; Ma et al., 2013; Laor et al., 2014; Ma et al., 2016). However, the exact role of Gtr1-Gtr2 is ambiguous, because the reported mutant phenotypes are not consistent among those studies.

1.5 Novel findings of this study

As mentioned above, the Rag-like heterodimeric Gtr1-Gtr2 GTPases are implicated in the regulation of *S. pombe* TORC1, but their exact role remains elusive. Therefore, our laboratory initiated studies to delve into the roles and relationships of the conserved regulatory factors in the control of TORC1 signaling. The objective of my study is to elucidate the exact role of the Gtr1-Gtr2 GTPases in TORC1 signaling of the fission yeast *S. pombe*.

In this study, I have identified a Ragulator-like complex in fission yeast, the Lam complex, which targets the Gtr1-Gtr2 heterodimer to vacuoles. Unexpectedly, however, this conserved regulatory machinery is required to attenuate TORC1 activity, and mutants lacking any of the components of the Lam complex show severe growth defects due to deregulated TORC1 activation. The data collected in this study suggest that TORC1 activation in *S. pombe* does not require the Rag-like GTPases and that they rather play an important role in moderating TORC1 activity on vacuolar membranes for optimal cellular response to nutrients.

2. Materials and methods

2.1 Reagents, yeast strains, and general techniques

Reagents used in this study were products of Nacalai Tesque, Japan or Wako Pure Chemical Industries, Japan unless specified otherwise. Enzymes used in this study were purchased from New England Biolabs (NEB), USA and Takara Bio, Japan. *S. pombe* strains used in this study are listed in Table 1. Antibiotics used in this study were 100 mg/L of Geneticin (G418) sulfate (Santa Cruz Biotechnology, USA), 100 ng/mL of rapamycin (LC Laboratories, USA), 100 mg/L of hygromycin B (Nacalai Tesque, Japan), and 100 mg/L of nourseothricin (Cosmo Bio, Japan).

2.2 Cultivation of yeast strains

Standard recipe for the yeast extract with supplements (YES) medium and the Edinburgh minimal medium (EMM) were used throughout the study, and cultivation of *S. pombe* cells were carried out at 30°C unless specified otherwise. Cryopreserved yeast cells were revived on YES agar medium for overnight, prior to inoculate into liquid medium. EMM agar and liquid was used to revive and cultivate all the mutant strains that exhibit growth defect on YES medium, respectively. A spectrophotometer (Shimadzu, Japan) was used to monitor the optical density of liquid cultures at 600 nm wavelength for growth determination.

2.3 Yeast genetic manipulations

Genetic manipulations for *S. pombe* have been described previously (Moreno et al., 1991; Shiozaki and Russell, 1997; Bähler et al., 1998), as briefly described in the following sections:

2.3.1 Genomic DNA extraction

Overnight cultured yeast cells (in 5 mL of YES or EMM liquid) were collected in a 1.5 mL tube by centrifugation at 900 × g for 5 min at room temperature. Cell pellets were suspended in 300 µL of lysis buffer [0.1 M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.001 M EDTA and 1% SDS] and 250 µL of phenol/chloroform solution, then vortexed vigorously with glass beads (Sigma Aldrich, USA) for 10 min. Approximately 250 µL of lysis buffer was added into the cell lysates, and the samples were centrifuged at 17700 × g for 10 min at room temperature. Aqueous phase of the solution was transferred to a new tube to perform phenol/chloroform extraction by mixing with 250 µL of phenol/chloroform solution, followed by centrifugation. The resulted aqueous phase of the solution was again transferred to a new

tube, and mixed gently with 30 µL of 3 M sodium acetate (pH 5.2) and 500 µL of ethanol. The mixture was incubated at room temperature for 5 min, and centrifuged at 17700 × *g* for 10 min. Precipitated DNA was rinse twice with 70% ethanol, dried up mildly and dissolve in TE buffer.

2.3.2 Transformation

S. pombe strain was cultured overnight in EMM liquid, and cell with a density equivalent to OD_{600} 1.0 was harvested by centrifugation and resuspended in 150 µL of 0.1 M lithium acetate (pH 4.9) for 1 hour at room temperature. DNA solution (less than 15 µL in total) was then added into the cell suspension and vortex vigorously for 10 seconds. Then, 350 µL of 50% PEG3350 was added and the mixture was vortexed vigorously for 30 seconds followed by 3 hours incubation at 30°C. After that, 1 mL of liquid YES was added into the mixture prior to subject to centrifugation at 900 × *g* for 5 min at room temperature. Cell pellet was then suspended in 100 µL of sterilized water and then spread on a YES agar plate. Cells were replicated on selection plates after 24 hours of incubation.

Because spontaneous suppressor mutations that mitigate the growth defects of the null mutants of the GATOR1 and Lam-Gtr1-Gtr2 complexes were detected with high frequency, rapamycin was added into all yeast extract with supplement (YES) medium agar for mutant selection procedures after transformation.

2.3.3 Gene disruption and gene tagging

The PCR-based gene targeting method that was established by Bähler and colleagues (1998) was used for gene tagging and gene disruption in this study. An example for gene tagging is described as the following:

Two DNA fragments of approximately 500 bp upstream and downstream of the 3' end of a target gene, respectively, were amplified using PrimeSTAR HS (Takara Bio, Japan) and *S. pombe* genomic DNA as template (Figure 4, Step 1). The reverse primer (D1) that was used to amplify DNA fragment ① was annealed with the upstream sequence of a pFA6a plasmid cassette (5'-TTAATTAACCCGGGGATCCG-3'), while the forward primer (U2) for DNA fragment ② was annealed with the downstream sequence of pFA6a plasmid cassette (5'-CAGATCCACTAGTGGCCTAT-3'). Next, PCR was performed using a pFA6a plasmid derivative with desired cassette as well as DNA fragment ① or ② as templates to amplify fragment ③ and ④, respectively (Figure 4, Step 2). The amplified DNA fragments ③ and ④ were then co-transformed into *S. pombe* for homologous recombination (Figure 4, Step 3). Lastly, the U0 and D0 primers were used in screening for successful mutants.

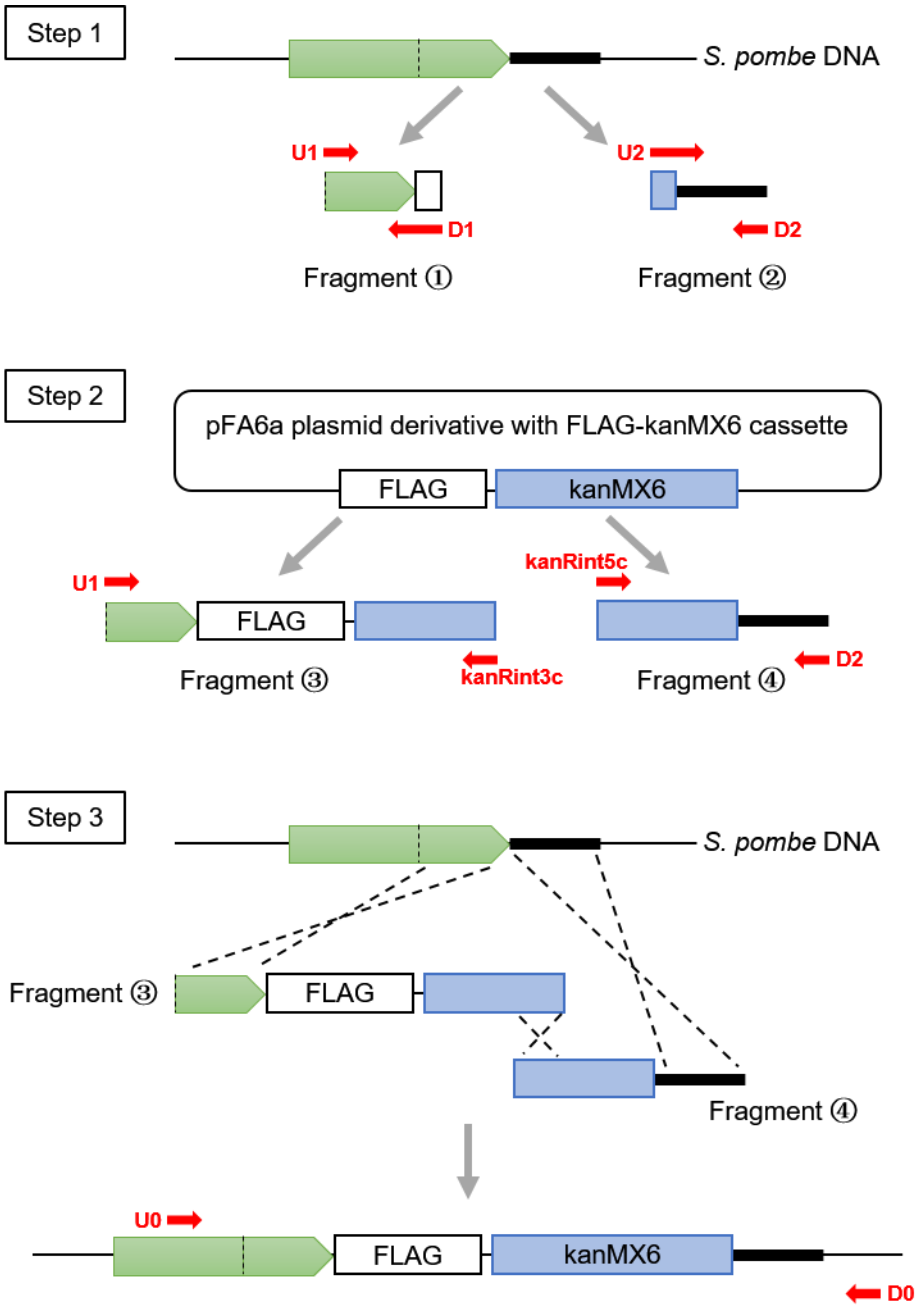

Figure 4. The PCR-based gene targeting method for gene tagging and gene disruption.

2.3.4 Site-directed mutagenesis

For site-directed mutagenesis, two primers that contain mutated or desired nucleotide sequence were used to amplify DNA fragment ① and ② using wild-type *S. pombe* genomic DNA as a template (Figure 5, Step 1). DNA fragment ① includes approximately 500 bp upstream sequence of the targeted gene, while DNA fragment ② covers 3' end of the targeted gene with the upstream sequence of a pFA6a plasmid cassette (5'-TTAATTAACCCGGGGATCCG-3'). Next, these two DNA fragments were used as templates to amplify the DNA fragment ③ (Figure 5, Step 2). On the other hand, DNA fragment ④ that contains the downstream sequence of a pFA6a plasmid cassette (5'-CAGATCCACTAGTGGCCTAT-3') was amplified using wild-type *S. pombe* genomic DNA as a template. The resulted DNA fragment ③ and ④ as well as a pFA6a plasmid derivative with desired cassette were then used as templates to amplify DNA fragments ⑤ and ⑥ (Figure 5, Step 3). The amplified DNA fragment ⑤ and ⑥ were then co-transformed into *S. pombe* for homologous recombination (Figure 5, Step 4). Lastly, the U0 and D0 primers were used in screening for successful mutants. Point mutation was also confirmed by DNA sequencing.

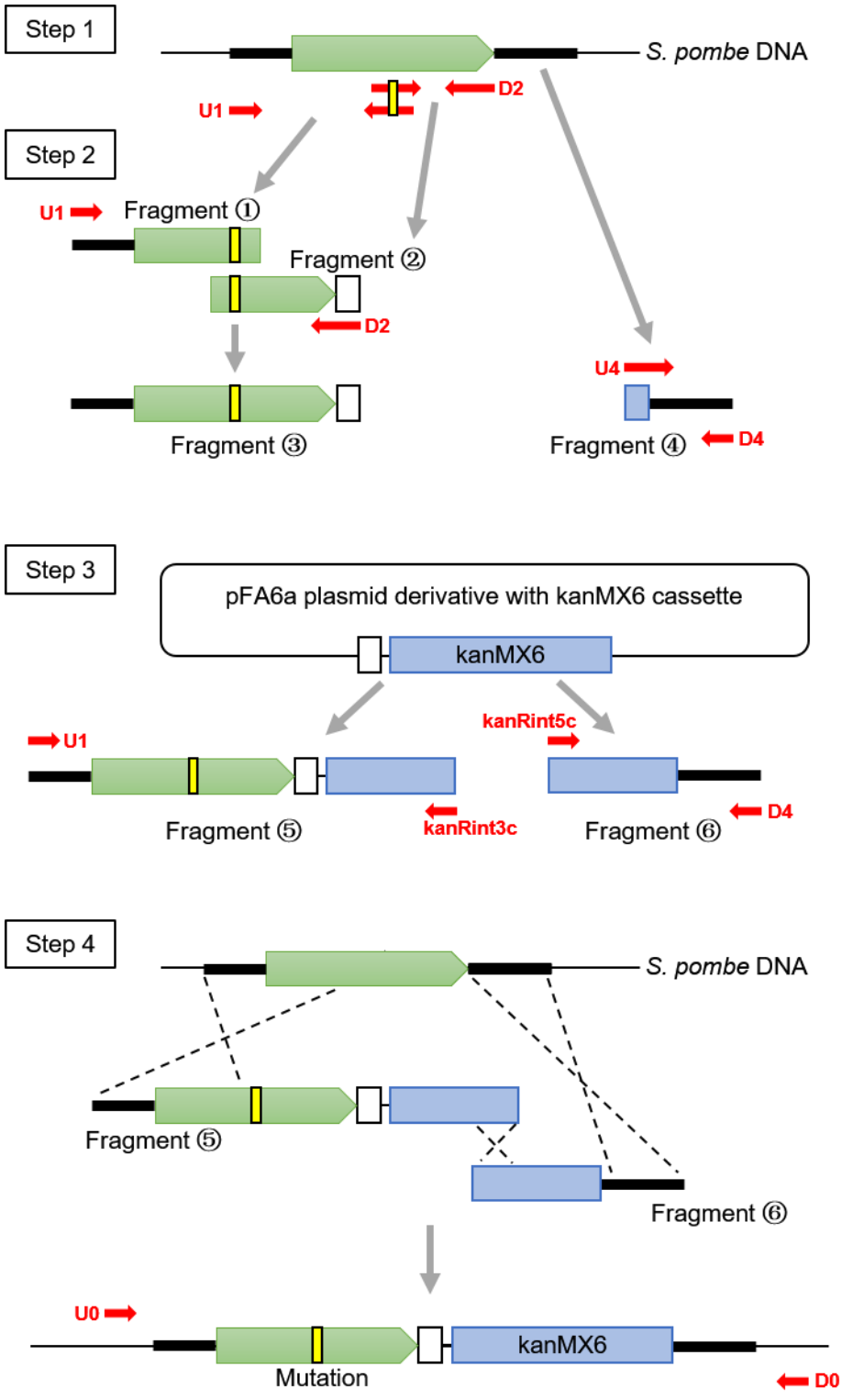

Figure 5. The PCR-based gene targeting method for site-directed mutagenesis.

2.4 Growth assay / spot test

EMM liquid cultures were adjusted to a cell density equivalent to OD_{600} 1.0. Serial dilutions of the cells were spotted onto solid media and incubated for 2~3 days. Images were captured by the LAS-4000 system (Fujifilm, Japan).

Solid media used in this study including YES, YES supplemented with 100 ng/mL rapamycin, YES supplemented with 5 mg/mL ammonium chloride, EMM supplemented with 20 mM arginine or proline, EMM without ammonium chloride (EMM–N) but supplemented with 20 mM arginine or proline, and EMM supplemented with 60 μg/ml canavanine.

2.5 Immunoprecipitation and mass spectrometry

Cells were grown to exponential phase in YES liquid (approximately OD_{600} of 0.4) and filtered onto 0.45 μm mixed cellulose ester membrane (Advantec, Japan). Subsequent steps were performed on ice. Filtered cells were disrupted in lysis buffer composed of 20 mM HEPES-KOH (pH 7.5), 150 mM potassium glutamate, 10% glycerol, 0.25% Tween-20, 10 mM sodium fluoride, 10 mM p-nitrophenylphosphate, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, and 0.1 mM sodium orthovanadate containing PMSF, leupeptin, and protease inhibitor cocktail (Sigma) with glass beads using a Multi-beads Shocker (Yasui Kikai, Japan).

For anti-FLAG immunoprecipitation, the recovered cell lysate was incubated with the Anti-Flag M2 magnetic beads (Sigma Aldrich, USA), which were prewashed with lysis buffer without protease inhibitor mix, and incubated with rotation for 2 hours at 4°C.

For anti-*myc* immunoprecipitation, the recovered cell lysate was incubated with the pre-washed EZview Red Anti-c-Myc affinity gels (Sigma Aldrich, USA) for 1 hour with rotation at 4°C.

The beads / gels were then washed three times using lysis buffer without protease inhibitor mix, and the immuno- or affinity-purified proteins were eluted by heating with the SDS-PAGE sample buffer for 10 min at 65°C. Samples were then analyzed by immunoblotting.

For mass spectrometric analysis, cell lysates were first subjected to anti-FLAG immunoprecipitation, eluted using 3X FLAG peptides (Sigma Aldrich, USA) and followed by anti-*myc* immunoprecipitation. Final eluted samples were then resolved in a 12% Mini-PROTEAN TGX precast gel (Bio-Rad, USA). Each lane was sliced into 4 pieces and digested with trypsin. Mass spectrometric analysis was performed using the LTQ-Orbitrap XL-HTC-PAL system. MS/MS spectra were analyzed by Mascot server (Matrix Science) and compared against NCBInr protein database (Taxonomy: *S. pombe*). For SDS-PAGE to be stained with

silver, samples were resolved in a 10% SDS-PAGE before staining with SilverQuest staining kit (Invitrogen, USA).

2.6 Nitrogen starvation

Yeast cells were grown to approximately OD_{600} of 0.2 in EMM liquid medium, then were filtered onto 0.45 µm mixed cellulose ester membrane (Advantec, USA). Subsequently, the filtered cells were rinsed with EMM–N, and resuspended in liquid EMM–N for indicated time course. Nitrogen starvation was considered began from the moment that cells were washed with EMM–N. Cells were harvested in 10% (v/v) trichloroacetic acid (TCA).

2.7 Preparation of crude lysate and immunoblotting

S. pombe cells were grown to approximately OD_{600} of 0.2 in EMM liquid medium and harvested in 10% (v/v) TCA by centrifugation. Cell pellets were then suspended in 10% (v/v) TCA and vortexed vigorously with glass beads (about 0.5 mm in diameter) for 10 min at 4°C. The cell suspensions were then centrifuged at 2400 × g for 5 min and the cell pellets were suspended in SDS-PAGE sample buffer prior boiling for 15 min at 65°C and centrifugation at 17700 × g for 15 min. The solubilized proteins in the supernatant were collected and the concentrations were normalized using Bradford Protein Assay (Bio-Rad, USA) before analyzed by immunoblotting.

Solubilized proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies as follows: anti-phospho-p70 S6K (Cell Signaling Technology, USA), anti-Psk1 (custom antibody raised in rabbits against the peptide "SDDEIAEEGYDFEELEAS") (Sigma Aldrich, Japan), anti-Spc1 (Tatebe and Shiozaki, 2003), anti-FLAG (M2, Sigma Aldrich, USA), anti-myc (9E10, Covance, USA; A-14, Santa Cruz Biotechnology, USA), and anti-GFP (Roche, Switzerland).

2.8 Fluorescence microscopy

Fluorescence microscopic analysis was carried out using DeltaVision Elite Microscopy System (GE Healthcare, UK). Cells grown exponentially in EMM liquid to approximately OD_{600} of 0.2, followed by staining with SynaptoRed C2 (FM4-64) dye (Invitrogen, USA; Biotium, USA) for vacuole visualization or Hoechst 33342 dye (Sigma Aldrich, USA) for DNA/nucleus visualization, if necessary. Vacuole staining was performed by adding 0.5 µL of 16 mM SynaptoRed C2 dye into 1 mL of culture for 30 min incubation in the dark. The stained cells were then recovered and transferred into 1.5 mL of fresh EMM medium for 90 min in the dark. For DNA staining, 1 µL of 1 mg/mL Hoechst 33342 solution

was added into 1 mL of culture for 15 min incubation in the dark. Prior analyzing, cells were concentrated and mounted on a thin EMM medium agarose film on glass slide. Z-axial images were taken at 0.4 μm with a 100X objective lens. Deconvolution of images was performed using DeltaVision SoftWoRx software.

Table 1. List of *S. pombe* strains used in this study.

Strain ID	Genotype	Source or reference
972/CA1	*h⁻*	Laboratory stock
M5-2g3/CA8106	*h⁻ rhb1DA4*	Murai et al., 2009
CA8667	*h⁻ lam2:13Myc(hph)*	This study
CA8741	*h⁻ gtr1:5FLAG(kanMX6) gtr2:13Myc(hph)*	This study
CA8744	*h⁻ gtr1:13Myc(hph) gtr2:5FLAG(kanMX6)*	This study
CA8829	*h⁻ tor2E2221K(kanMX6)*	This study
CA8910	*h⁻ tor2E2221K(kanMX6) rhb1Δ::hph*	This study
CA8914	*h⁻ tsc2Δ::kanMX6*	This study
CA9078	*h⁻ ura4-D18 sck1:5FLAG(ura4⁺)*	This study
CA9219	*h⁻ lam3:13Myc(hph)*	This study
CA9228	*h⁺ lam3:mEGFP(kanMX6)*	This study
CA9245	*h⁻ lam1:13Myc(hph)*	This study
CA9254	*h⁺ lam1:mEGFP(kanMX6)*	This study
CA9388	*h⁻ gtr1Δ::kanMX6*	This study
CA9390	*h⁻ gtr2Δ::kanMX6*	This study
CA9392	*h⁻ lam2Δ::kanMX6*	This study
CA9394	*h⁻ lam1Δ::kanMX6*	This study
CA9396	*h⁻ lam3Δ::kanMX6*	This study
CA9398	*h⁻ lam4Δ::kanMX6*	This study
CA9400	*h⁻ iml1Δ::kanMX6*	This study
CA9417	*h⁻ lam1:13Myc(hph) lam2:5FLAG(kanMX6)*	This study
CA9420	*h⁻ lam1:13Myc(hph) lam3:5FLAG(kanMX6)*	This study
CA9423	*h⁻ lam2:13Myc(hph) lam1:5FLAG(kanMX6)*	This study
CA9426	*h⁻ lam2:13Myc(hph) lam3:5FLAG(kanMX6)*	This study
CA9429	*h⁻ lam3:13Myc(hph) lam2:5FLAG(kanMX6)*	This study
CA9432	*h⁻ lam3:13Myc(hph) lam1:5FLAG(kanMX6)*	This study
CA9470	*h⁻ gtr1Q61L(kanMX6)*	This study
CA9473	*h⁻ gtr1S16N(kanMX6)*	This study
CA9497	*h⁻ lam4:5FLAG(kanMX6)*	This study
CA9505	*h⁺ lam4:mEGFP(kanMX6)*	This study
CA9557	*h⁻ tsc1Δ::kanMX6*	This study
CA9582	*h⁻ gtr1Δ::kanMX6 gtr2Δ::hph*	This study

CA9671	*h⁻ lam1Δ::kanMX6 gtr1Δ::hph*	This study
CA9673	*h⁻ lam2Δ::kanMX6 gtr1Δ::hph*	This study
CA9675	*h⁻ lam3Δ::kanMX6 gtr1Δ::hph*	This study
CA9677	*h⁻ lam4Δ::kanMX6 gtr1Δ::hph*	This study
CA9679	*h⁻ lam1Δ::kanMX6 gtr2Δ::hph*	This study
CA9681	*h⁻ lam2Δ::kanMX6 gtr2Δ::hph*	This study
CA9683	*h⁻ lam3Δ::kanMX6 gtr2Δ::hph*	This study
CA9685	*h⁻ lam4Δ::kanMX6 gtr2Δ::hph*	This study
CA9702	*h⁻ tsc2Δ::hph gtr1Δ::kanMX6*	This study
CA9722	*h⁻ tsc1Δ::kanMX6 gtr1Δ::hph*	This study
CA9724	*h⁻ tsc1Δ::kanMX6 iml1Δ::hph*	This study
CA9887	*h⁻ gtr1:mEGFP(kanMX6)*	This study
CA9889	*h⁻ gtr2:mEGFP(kanMX6)*	This study
CA9891	*h⁻ lam2:mEGFP(kanMX6)*	This study
CA9907	*h⁻ tsc2Δ::hph iml1Δ::kanMX6*	This study
CA9911	*h⁻ lam1:13Myc(hph) lam4:5FLAG(kanMX6)*	This study
CA9915	*h⁻ lam2:13Myc(hph) lam4:5FLAG(kanMX6)*	This study
CA9919	*h⁻ lam3:13Myc(hph) lam4:5FLAG(kanMX6)*	This study
CA10166	*h⁻ gtr1:mEGFP(kanMX6) lam1Δ::hph*	This study
CA10168	*h⁻ gtr2:mEGFP(kanMX6) lam1Δ::hph*	This study
CA10170	*h⁻ lam2:mEGFP(kanMX6) lam1Δ::hph*	This study
CA10172	*h⁺ lam3:mEGFP(kanMX6) lam1Δ::hph*	This study
CA10174	*h⁺ lam4:mEGFP(kanMX6) lam1Δ::hph*	This study
CA10191	*h⁻ gtr2Q60L(kanMX6)*	This study
CA10195	*h⁻ gtr2S17N(kanMX6)*	This study
CA10214	*h⁻ ura4-D18 gtr1Δ:: ura4⁺ tor2E2221K(kanMX6)*	This study
CA10216	*h⁻ ura4-D18 iml1Δ:: ura4⁺ tor2E2221K(kanMX6)*	This study
CA10224	*h⁻ gtr1:mEGFP(kanMX6) gtr2Δ::hph*	This study
CA10226	*h⁻ gtr1:mEGFP(kanMX6) lam2Δ::hph*	This study
CA10228	*h⁻ gtr1:mEGFP(kanMX6) lam3Δ::hph*	This study
CA10230	*h⁻ gtr1:mEGFP(kanMX6) lam4Δ::hph*	This study
CA10232	*h⁻ gtr2:mEGFP(kanMX6) gtr1Δ::hph*	This study
CA10234	*h⁻ gtr2:mEGFP(kanMX6) lam2Δ::hph*	This study
CA10236	*h⁻ gtr2:mEGFP(kanMX6) lam3Δ::hph*	This study
CA10238	*h⁻ gtr2:mEGFP(kanMX6) lam4Δ::hph*	This study
CA10240	*h⁺ lam1:mEGFP(kanMX6) gtr1Δ::hph*	This study
CA10242	*h⁺ lam1:mEGFP(kanMX6) gtr2Δ::hph*	This study
CA10244	*h⁺ lam1:mEGFP(kanMX6) lam2Δ::hph*	This study
CA10246	*h⁺ lam1:mEGFP(kanMX6) lam3Δ::hph*	This study
CA10248	*h⁺ lam1:mEGFP(kanMX6) lam4Δ::hph*	This study
CA10254	*h⁻ lam2:mEGFP(kanMX6) gtr1Δ::hph*	This study
CA10256	*h⁻ lam2:mEGFP(kanMX6) gtr2Δ::hph*	This study
CA10258	*h⁺ lam3:mEGFP(kanMX6) gtr1Δ::hph*	This study

CA10260	*h⁺ lam3:mEGFP(kanMX6) gtr2Δ::hph*	This study
CA10262	*h⁺ lam4:mEGFP(kanMX6) gtr1Δ::hph*	This study
CA10264	*h⁺ lam4:mEGFP(kanMX6) gtr2Δ::hph*	This study
CA10266	*h⁺ lam3:mEGFP(kanMX6) lam2Δ::hph*	This study
CA10268	*h⁺ lam4:mEGFP(kanMX6) lam2Δ::hph*	This study
CA10270	*h⁻ lam2:mEGFP(kanMX6) lam3Δ::hph*	This study
CA10272	*h⁺ lam4:mEGFP(kanMX6) lam3Δ::hph*	This study
CA10274	*h⁻ lam2:mEGFP(kanMX6) lam4Δ::hph*	This study
CA10276	*h⁺ lam3:mEGFP(kanMX6) lam4Δ::hph*	This study
CA10333	*h⁻ lam1G-A:GFPS65T(hph)*	This study
CA10336	*h⁻ lam1CC-AA:GFPS65T(hph)*	This study
CA10339	*h⁻ lam1LL-AA:GFPS65T(hph)*	This study
CA10373	*h⁻ gtr1:13Myc(hph) gtr2:13Myc(nat)*	This study
CA10377	*h⁻ gtr1:13Myc(hph) gtr2:13Myc(nat) lam1:5FLAG(kanMX6)*	This study
CA10380	*h⁻ gtr1:13Myc(hph) gtr2:13Myc(nat) lam2:5FLAG(kanMX6)*	This study
CA10383	*h⁻ gtr1:13Myc(hph) gtr2:13Myc(nat) lam3:5FLAG(kanMX6)*	This study
CA10386	*h⁻ gtr1:13Myc(hph) gtr2:13Myc(nat) lam4:5FLAG(kanMX6)*	This study
CA10708	*h⁻ lam1GCC-AAA:GFPS65T(hph)*	This study
CA11415	*h⁻ ura4-D18 gtr1Δ:: ura4⁺ tor2E2221K(kanMX6) rhb1Δ::hph*	This study
CA11417	*h⁻ ura4-D18 iml1Δ:: ura4⁺ tor2E2221K(kanMX6) rhb1Δ::hph*	This study
CA11433	*h⁻ lam1:NLS:GCC-AAA:mCherry(kanMX6) gtr2:GFPS65T(hph)*	This study
CA11459	*h⁺ mip1:mEGFP:GFP(hph)*	This study
CA11491	*h⁻ any1Δ::hph*	This study
CA11536	*h⁻ lam1:NLS:GCC-AAA:mCherry(kanMX6) gtr1:GFP(hph)*	This study
CA11799	*h⁻ mip1: mEGFP:GFP (hph) gtr1Δ::kanMX6*	This study
CA11801	*h⁻ mip1: mEGFP:GFP (hph) gtr2Δ::kanMX6*	This study
CA11805	*h⁻ mip1: mEGFP:GFP (hph) lam1Δ::kanMX6*	This study
CA12166	*h⁻ any1Δ::hph lam1Δ::kanMX6*	This study
CA12168	*h⁻ any1Δ::hph lam2Δ::kanMX6*	This study
CA12170	*h⁻ any1Δ::hph lam3Δ::kanMX6*	This study
CA12172	*h⁻ any1Δ::hph lam4Δ::kanMX6*	This study
CA13833	*h⁻ ura4-D18 sck1:5FLAG(ura4⁺) gtr1Δ::hph*	This study
CA13835	*h⁻ ura4-D18 sck1:5FLAG(ura4⁺) gtr2Δ::hph*	This study
CA13837	*h⁻ ura4-D18 sck1:5FLAG(ura4⁺) iml1Δ::hph*	This study
CA13838	*h⁻ ura4-D18 sck1:5FLAG(ura4⁺) tsc2Δ::hph*	This study

3. Results

3.1 Identification of proteins interacting with Gtr1-Gtr2 GTPases

The heterodimeric Rag GTPases in mammals (RagA/B-RagC/D) and the budding yeast *S. cerevisiae* (Gtr1-Gtr2) physically associate with the Ragulator complex and the Ego ternary complex, respectively, for their localization to lysosomes in mammals and vacuoles in budding yeast (Sancak et al., 2010; Bar-Peled et al., 2012; Dubouloz et al., 2005). The heterodimeric Gtr1-Gtr2 Rag GTPases in the fission yeast *S. pombe* also localize to vacuoles (Valbuena et al., 2012), and a recent study has identified Lam2, a fission yeast ortholog of the mammalian Ragulator component LAMTOR2, as a Gtr1-interacting protein (Ma et al., 2016). These reports suggest that the Ragulator complex might also be conserved in fission yeast. However, no apparent orthologs of other components of the Ragulator or the Ego ternary complex have been identified in *S. pombe*. Aiming to identify proteins that physically interact with the Gtr1-Gtr2 GTPases, the Gtr1-Gtr2 heterodimer complex was purified from strains that express FLAG- or *myc*-epitope fused Gtr1 and Gtr2 from their chromosomal loci. By using two successive immunoprecipitation procedures against FLAG and *myc* tags, the Gtr1-Gtr2 heterodimer was successfully purified, as shown in Figure 6. The resulted immunoprecipitates were then analyzed by mass spectrometry, and four co-purified proteins were reproducibly identified in all samples. These proteins are encoded by open reading frames SPBC1778.05c, SPBC29A10.17, SPAC222.19, and SPAC23D3.16 in the *S. pombe* genome database (Table 2).

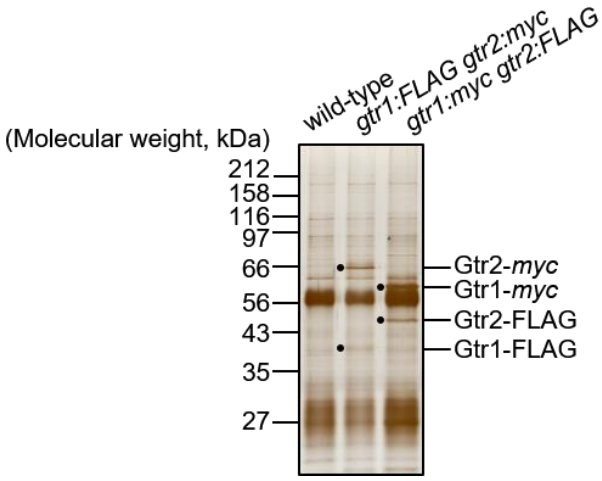

Figure 6. Affinity-purification of the Gtr1-Gtr2 GTPase heterodimer.

The heterodimer was purified from cell lysate of *gtr1:FLAG gtr2:myc* and *gtr1:myc gtr2:FLAG* strains by two successive immunoprecipitation procedures using anti-FLAG and anti-*myc* antibodies, and resolved on SDS-PAGE followed by silver staining. A wild-type strain was used as a negative control. The protein bands corresponding to the tagged Gtr1 and Gtr2 are indicated by black dots.

Table 2. Mass spectrometric analyses of proteins co-purified with the Gtr1-Gtr2 heterodimer.

Systematic ID	Protein	MW[1] (kDa)	Score[2]	Score[3]
SPBC337.13c	Gtr1	35.01	445	1346
SPCC777.05	Gtr2	35.59	407	513
SPBC1778.05c	Lam2	17.50	234	360
SPBC29A10.17	Unassigned (Lam1)	17.55	129	352
SPAC222.19	Unassigned (Lam3)	12.69	115	216
SPAC23D3.16	Unassigned (Lam4)	8.98	55	109

[1] Molecular weight

[2,3] The sum of peptide scores that exceed the 95% confidence level (P<0.05) for the proteins identified in immunoprecipitates obtained from cell lysates of *gtr1:FLAG gtr2:myc*[2] and *gtr1:myc gtr2:FLAG*[3] (refer to Figure 6). Two or more peptides were identified for each protein listed in the table.

Characterization of the proteins encoded by SPBC29A10.17, SPAC222.19, and SPAC23D3.16 has not been reported, and they show no apparent sequence homology to any known proteins. On the other hand, the protein product of SPBC1778.05c has been named Lam2, because of its sequence similarity to the mammalian Ragulator component LAMTOR2 (Ma et al., 2016). Interestingly, the predicted secondary structures of the proteins encoded by SPBC29A10.17, SPAC222.19, and SPAC23D3.16 exhibit some resemblance to those of the Ragulator and Ego components (Figure 7); therefore, I refer to those proteins as Lam1 (SPBC29A10.17), Lam3 (SPAC222.19), and Lam4 (SPAC23D3.16) hereafter.

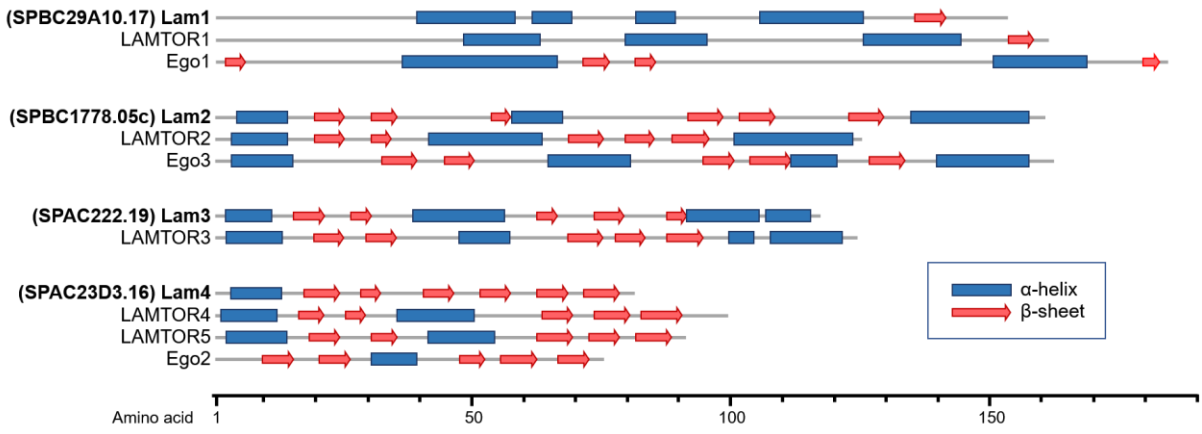

Figure 7. Comparison of the secondary structures of the Lam proteins and their putative counterparts in humans (LAMTOR1~5) and budding yeast (Ego1~3).

The secondary structures are predicted using the Jpred server (http://www.compbio.dundee.ac.uk/jpred/). Boxes and arrows indicate α-helix and β-sheet structures, respectively.

To verify the physical interaction between the Lam proteins with Gtr1-Gtr2, each of the Lam proteins with the FLAG tag was immunoprecipitated from the lysate of the strains expressing the *myc*-tagged Gtr1 and Gtr2. Both of the *myc*-tagged Gtr1 and Gtr2 were co-precipitated with any of the Lam proteins (Figure 8), confirmed that the Lam proteins physically interact with Gtr1-Gtr2 GTPases.

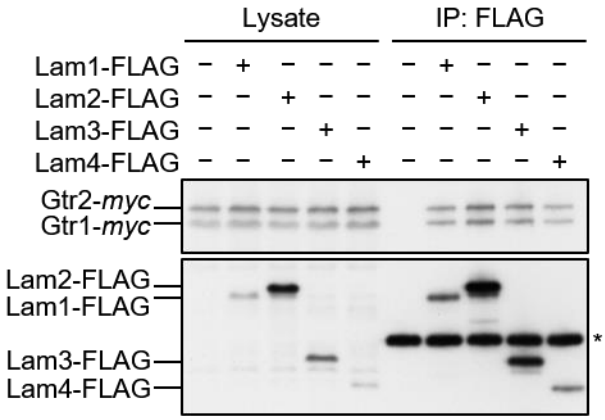

Figure 8. Confirmed physical interactions between the Lam proteins and Gtr1-Gtr2.

Crude lysate was prepared from *gtr1:myc gtr2:myc* strains expressing one of the FLAG-tagged Lam proteins from their chromosomal loci, and anti-FLAG immunoprecipitates ("IP:FLAG") were analyzed by immunoblotting. Immunoglobulin in the immunoprecipitates is asterisked.

3.2 The Lam proteins and the Gtr1-Gtr2 GTPases function in the same pathway

Because all the Lam proteins interact with the Gtr1-Gtr2 heterodimer, I further examined whether the Lam proteins interact with each other *in vivo*. Pairwise co-immunoprecipitation experiments were performed using strains expressing two of the Lam proteins with different epitope tags, FLAG and *myc*. As shown in Figure 9, co-precipitation was observed with all the pairs tested, suggesting that the Lam proteins physically interact with each other *in vivo*.

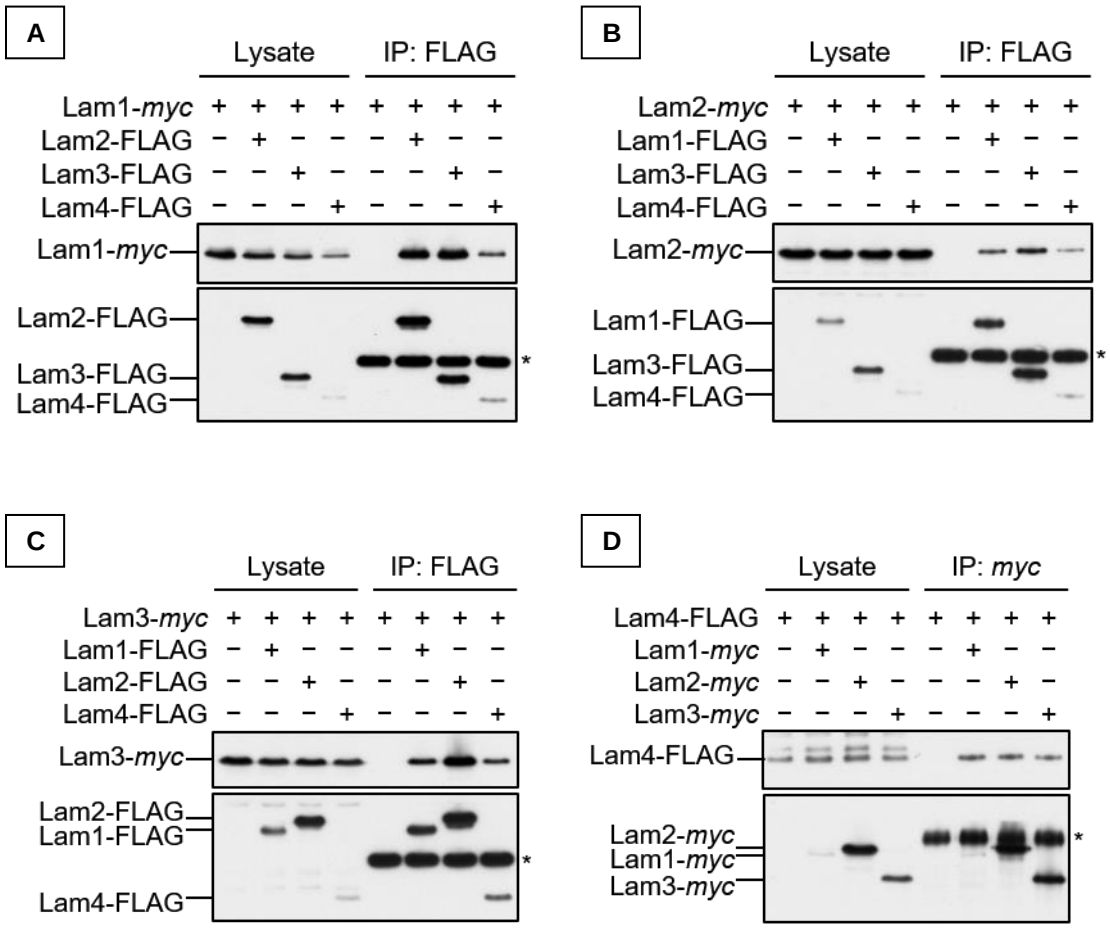

Figure 9. Lam proteins physically interact with each other *in vivo*.

lam1:myc (A), *lam2:myc* (B), *lam3:myc* (C) and *lam4:FLAG* (D) strains expressing one of the other Lam proteins with a different epitope tag were analyzed by anti-FLAG or anti-*myc* immunoprecipitation followed by immunoblotting. Immunoglobulin in the immunoprecipitates is asterisked.

Next, genetic interaction of the Lam proteins with Gtr1-Gtr2 GTPases was examined by comparing the growth of their single- and double-knockout mutants. In these experiments, cultured cells were serially diluted and spotted on Yeast Extract with Supplements (YES) and Edinburgh Minimal Medium (EMM) agar media (Figure 10). The cells lacking any of the *lam* genes exhibited a severe growth defect similar to that of the *gtr1* null (Δ) and *gtr2*Δ mutants on rich YES medium, while they could grow slowly on EMM medium. Importantly, any of the double mutants of *lam*Δ and *gtr*Δ exhibited no severer phenotype than the individual single mutants, indicating that the *lam*Δ and *gtr*Δ phenotypes are not additive. The results from both biochemical and genetic analyses suggest that the Gtr1-Gtr2 GTPases and the Lam1~4 proteins form a complex and function together to promote cell growth.

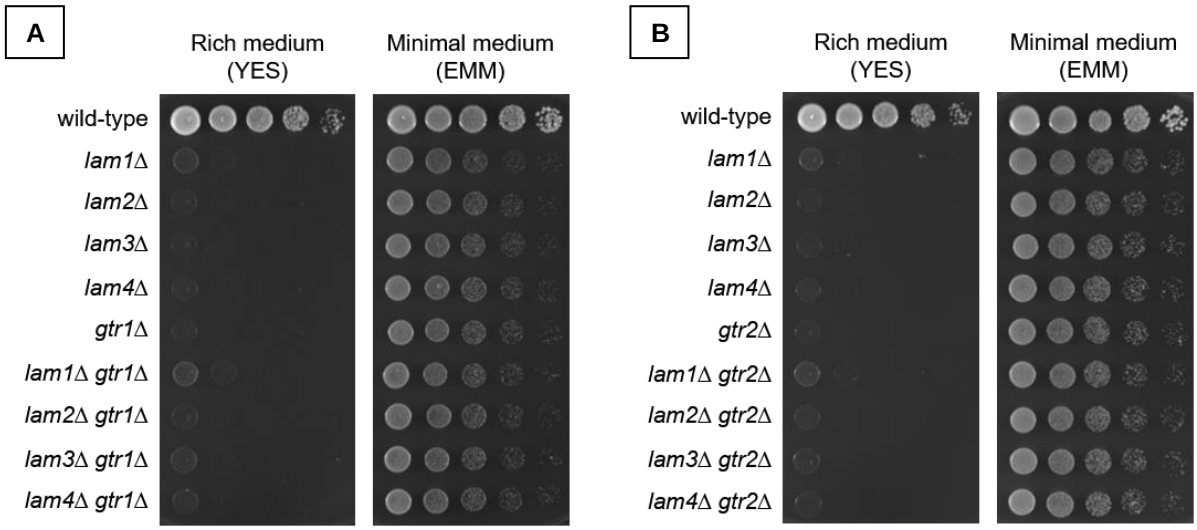

Figure 10. The *lam* and *gtr* genes function in the same pathway.

The indicated single and double mutants as well as a wild-type strain were grown in EMM liquid medium and their serial dilutions were spotted onto rich YES and EMM agar media for a growth assay at 30°C.

3.3 Lam proteins are essential for the vacuolar localization of Gtr1-Gtr2 GTPases

As mentioned above, the Gtr1-Gtr2 heterodimer localizes to the vacuolar membranes (Valbuena et al., 2012; Ma et al., 2016), and the Lam proteins physically interact with Gtr1-Gtr2. Therefore, it is likely that the Lam proteins are also localized to vacuoles. Fluorescence microscopy was employed to observe the cellular localization of the Lam proteins, using the strains in which their chromosomal *lam1*$^{+}$, *lam2*$^{+}$, *lam3*$^{+}$ and *lam4*$^{+}$ genes were tagged with the GFP-encoding sequence. As shown in Figure 11, the fluorescent signals of the Lam-GFP fusion proteins showed ring-shaped patterns similar to those of Gtr1-GFP, Gtr2-GFP, and the SynaptoRed C2 (FM4-64) fluorescent dye, which stains vacuolar membranes. Thus, it appears that the Lam proteins are localized to vacuolar membranes. A fraction of Lam2 was also detected in the cytoplasm, probably due to its higher protein abundance as compared to the other Lam proteins (Figure 8, cell lysates).

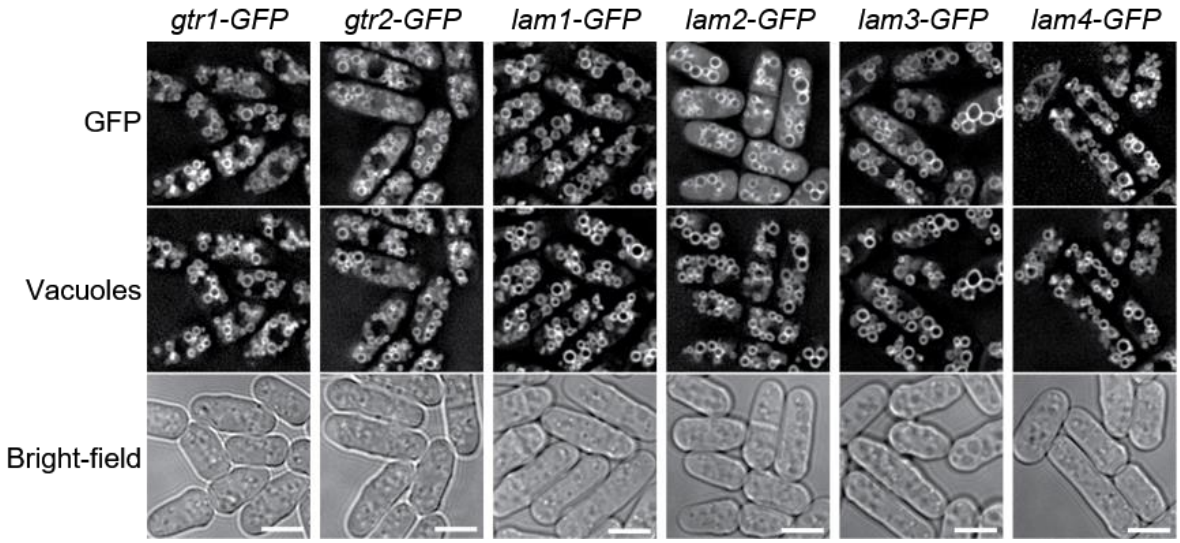

Figure 11. The Lam proteins and Gtr1-Gtr2 are localized to vacuolar membranes.

The chromosomal *gtr* and *lam* genes were tagged with the GFP sequence and the strains were grown in EMM at 30˚C, stained with SynaptoRed C2 fluorescent dye for vacuoles visualization and analyzed using fluorescence microscope. Z-axial images were collected and mid-section images after deconvolution are shown. Bars, 5 μm.

The results obtained so far strongly suggest that the Lam proteins form a complex with Gtr1-Gtr2, thus, raising the possibility that the Lam protein complex may serves as a fission yeast counterpart of the mammalian Ragulator and the budding yeast Ego ternary complex. I tested this possibility by analyzing the subcellular localization of GFP-tagged Gtr1 and Gtr2 in the absence of the Lam proteins. As expected, the vacuolar localization of the

Gtr1 (Figure 12A) and Gtr2 (Figure 12B) were disrupted in the *lam*Δ mutants, suggesting that Lam proteins are essential for the vacuolar localization of Gtr1-Gtr2. In addition, Gtr1 and Gtr2 were also mislocalized to the cytoplasm in the absence of the other, indicating that their heterodimer formation is important for the recruitment of Gtr1-Gtr2 to vacuoles.

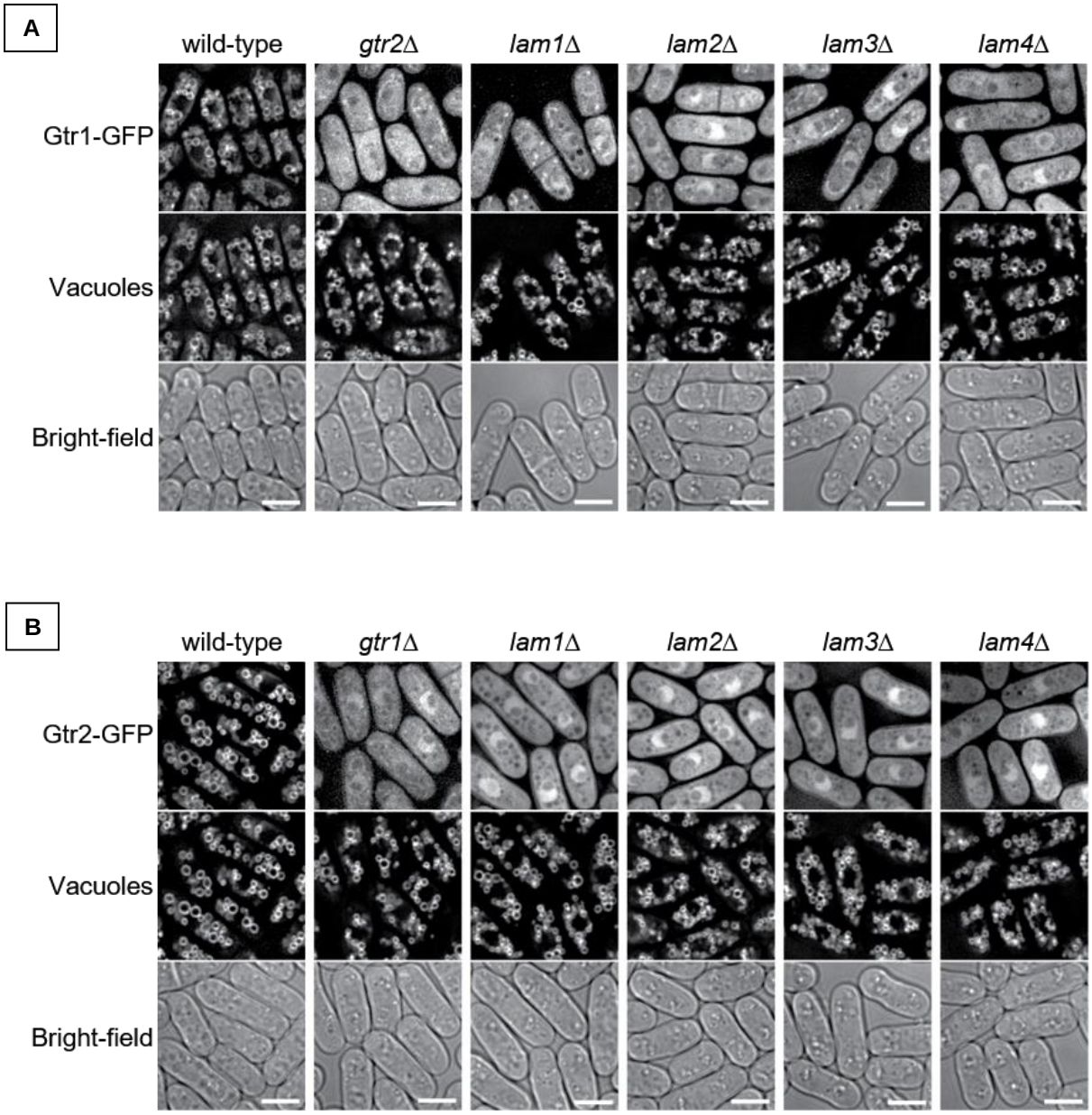

Figure 12. Heterodimer formation of the Gtr1-Gtr2 and Lam proteins are required for the vacuolar localization of Gtr1-Gtr2 GTPases.

Wild-type and the indicated mutant strains expressing GFP-tagged Gtr1 (A) or Gtr2 (B) from their chromosomal loci were analyzed by fluorescence microscopy as in Figure 11. Bars, 5 μm.

Next, I also examined whether the localization of Lam proteins require Gtr1-Gtr2 by following the localization of the Lam proteins in the absence of Gtr1-Gtr2. The vacuolar localization of Lam proteins remained unchanged in the *gtr* null mutants (Figure 13). Collectively, these observations strongly suggest that the Lam protein complex tethers Gtr1-Gtr2 GTPases to vacuoles, which serves as a fission yeast counterpart of the mammalian Ragulator complex and the budding yeast Ego ternary complex.

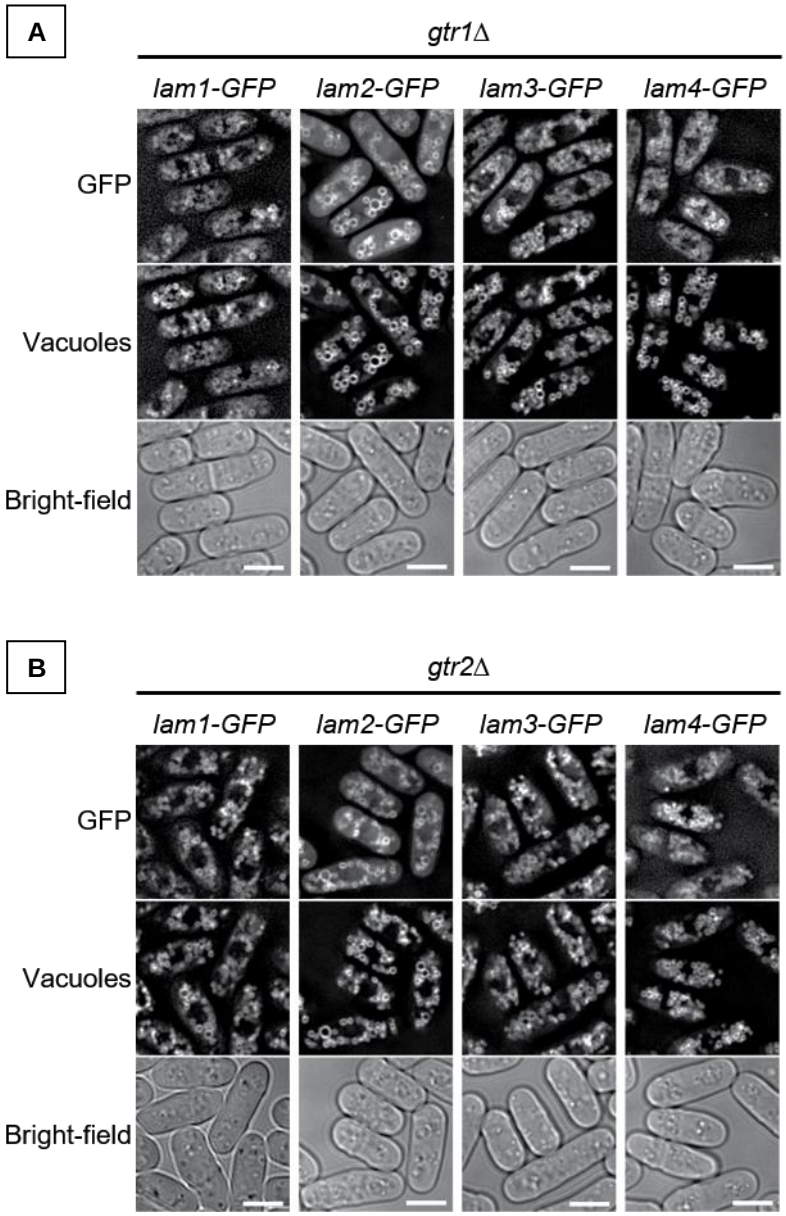

Figure 13. Vacuolar localization of the Lam proteins does not require Gtr1-Gtr2.

The *gtr1*Δ (A) and *gtr2*Δ (B) mutant strains expressing one of the GFP-tagged Lam proteins from their chromosomal loci were analyzed by fluorescence microscopy as in Figure 11. Bars, 5 μm.

3.4 Lam1 anchors the Lam complex to vacuolar membranes

To characterize the mechanism that determines the vacuolar localization of the Lam complex, localization of the GFP-tagged Lam proteins was analyzed in strains lacking one of the *lam* genes. Results showed that the vacuolar localization of Lam1 is not altered by the *lam2*Δ, *lam3*Δ, and *lam4*Δ mutations (Figure 14A), while Lam2, Lam3 and Lam4 were diffused throughout the cytoplasm in the absence of Lam1 ("*lam1*Δ" in Figures 14A and 14B), implying that Lam2~4 localize to vacuoles through Lam1. On the other hand, Lam4 might mediate association of Lam2 and Lam3 with Lam1, because Lam4 was observed on vacuoles even in the absence of Lam2 or Lam3 ("*lam4-GFP*" in Figure 14B) but not vice versa ("*lam2-GFP*" in Figures 14A and "*lam3-GFP*" in Figure 14B). Finally, Lam2 and Lam3 are mutually dependent for their vacuolar localization. A schematic diagram shown in Figure 15 represents a predicted architecture of the Lam-Gtr1-Gtr2 complex on the vacuolar surface.

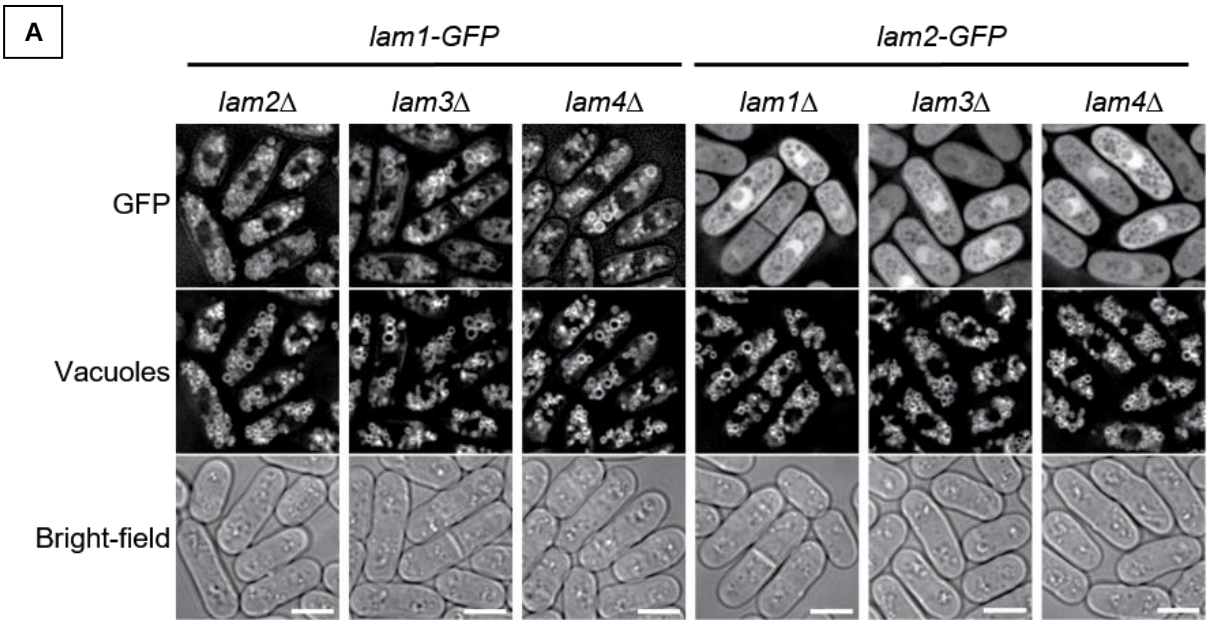

Figure 14A. Interdependent vacuolar localization of the Lam proteins. Vacuolar localization of Lam1 does not require other Lam proteins, but Lam2 requires Lam1~3 for its vacuolar localization.

The indicated mutant strains expressing Lam1-GFP or Lam2-GFP from their chromosomal loci were grown in EMM at 30˚C, stained with SynaptoRed C2 fluorescent dye for vacuole visualization, and analyzed using fluorescence microscope. Z-axial images were collected and mid-section images after deconvolution are shown. Bars, 5 μm.

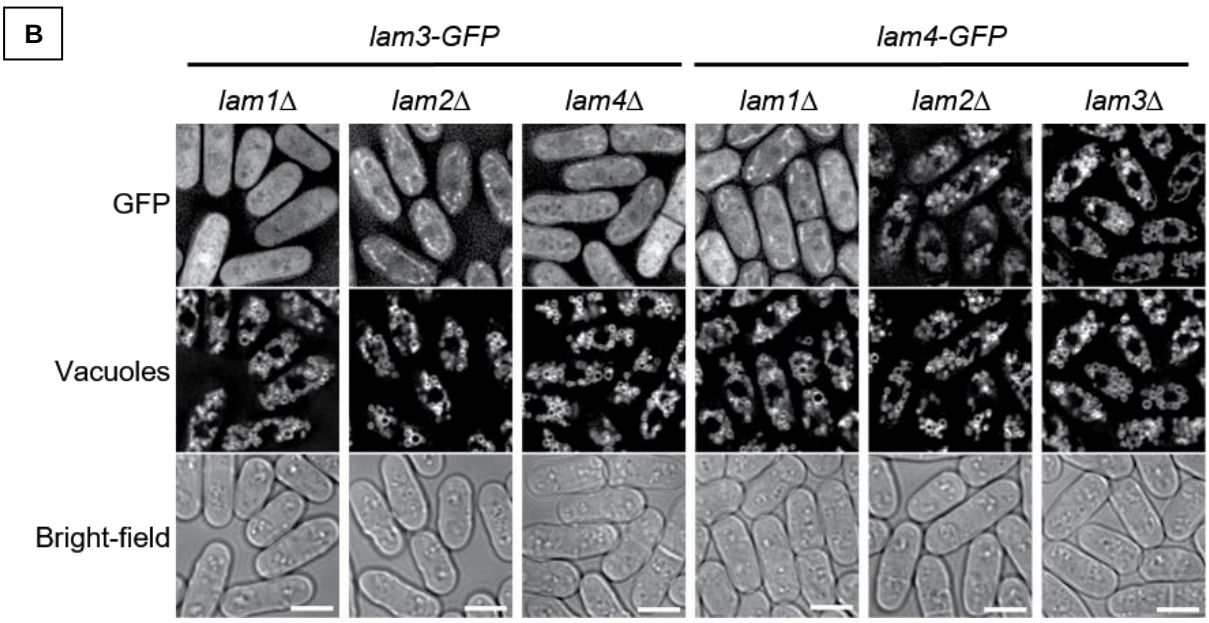

Figure 14B. Interdependent vacuolar localization of the Lam proteins. Vacuolar localization of Lam3 requires Lam1, Lam2 and Lam4, while the localization of Lam4 only depends on Lam1.

The indicated mutant strains expressing Lam3-GFP or Lam4-GFP from their chromosomal loci were analyzed by fluorescence microscopy as in Figure 14A. Bars, 5 μm.

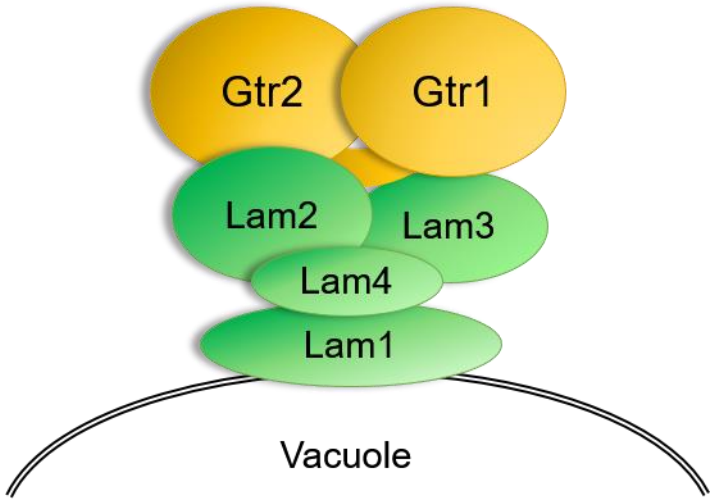

Figure 15. Schematic diagram of the predicted architecture of the Lam-Gtr1-Gtr2 complex on the vacuolar surface based on the interdependency among Lam and Gtr proteins for their vacuolar localization.

3.5 Lam1 has N-terminal lipid modification sites essential for its vacuolar localization

The mammalian Ragulator component LAMTOR1 and the budding yeast Ego1 possess potential lipid modification sites in their N-terminal region, the $MGX_{0-4}CX_{0-2}C$ motif with potential myristoylation (glycine) and palmitoylation (cysteine) sites (Kogan et al., 2010; Figure 16, highlighted in grey). Those potential modification sites are conserved in the N-terminal region of Lam1. Moreover, the putative vacuole/lysosome localization signal sequence, which is composed of acidic residues followed by di-leucine (Darsow et al., 1998), is also conserved in Lam1, LAMTOR1, and Ego1 (Figure 16, underlines), consistent with the inferred role of Lam1 as a vacuolar membrane anchor of the Lam-Gtr1-Gtr2 complex.

Figure 16. N-terminal sequences of the *S. pombe* Lam1 (UniProtKB ID: C6Y4C6), human LAMTOR1 (Q6IAA8) and *S. cerevisiae* Ego1 (Q02205).

The conserved Gly (black arrowhead) and Cys (red arrowheads) residues within the $MGX_{0-4}CX_{0-2}C$ motif (highlighted in grey) are potential sites for myristoylation and palmitoylation, respectively. Putative vacuole/lysosome localization signal sequences composed of acidic residues followed by a di-leucine motif (blue arrowheads) are underlined.

To test if those N-terminal sequence motifs are required for Lam1 targeting to vacuole membranes, the glycine residue, cysteine residues, and the di-leucine residues shown in Figure 16 were substituted with alanine, and localization of the mutated Lam1 proteins were analyzed by fluorescence microscopy. Simultaneous alanine substitutions of both myristorylation and palmitoylation sites in Lam1 resulted in its dispersal throughout the cytoplasm (Figure 17, *lam1GCCAAA*). Interestingly, Lam1 was distributed to both vacuolar and plasma membranes when the leucine residues of conserved acidic di-leucine motif were replaced by alanine residues (*lam1LLAA*). These observations suggest that specific targeting of Lam1 to vacuolar membranes requires its N-terminal lipid modifications as well as the di-leucine motif.

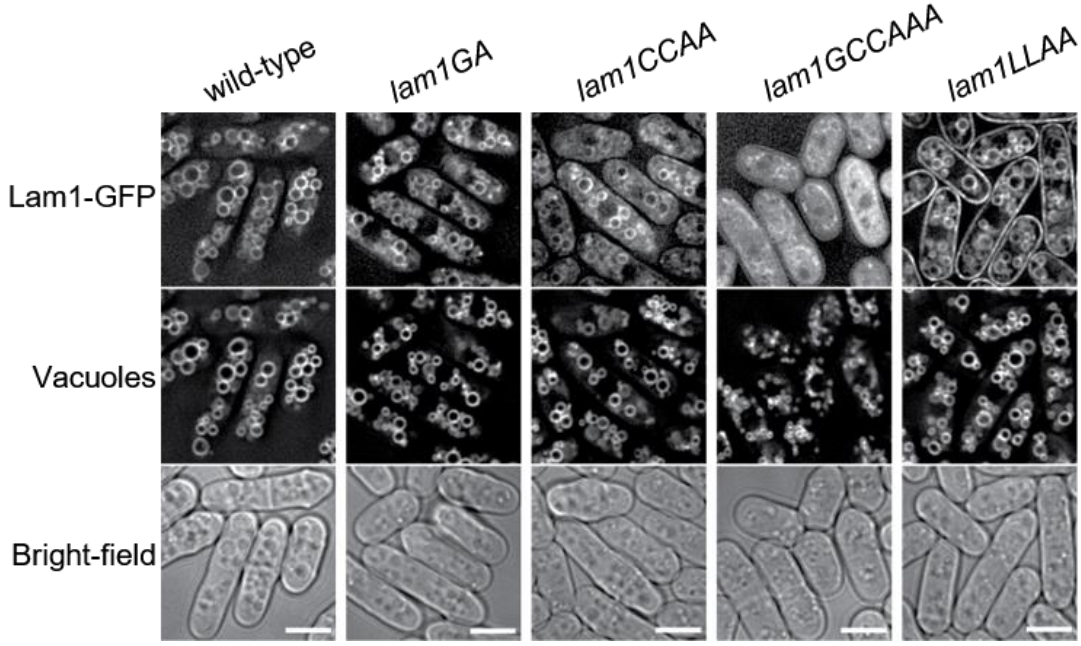

Figure 17. The conserved sequence motifs shown in Figure 14 are important for the vacuolar localization of Lam1.

The *lam1:GFP* strains with alanine-substitutions at the potential myristoylation site (GA), palmitoylation sites (CCAA), both of them (GCCAAA) or the di-leucine (LLAA) motif were grown in EMM at 30˚C, stained with SynaptoRed C2 fluorescent dye and analyzed using fluorescence microscope. Z-axial images were collected and mid-section images after deconvolution are shown. Bars, 5 µm.

3.6 Vacuolar localization of the Lam-Gtr1-Gtr2 complex is important for cell growth

The *lam* and *gtr* null mutants exhibit a severe growth defect on rich medium (Figure 10). In addition, loss of any of the Lam proteins causes the Gtr1-Gtr2 heterodimer to delocalize from the vacuolar surface to the cytoplasm (Figure 12), suggesting that vacuolar localization of Gtr1-Gtr2 is important for fission yeast growth. To further corroborate that vacuolar localization of the Lam-Gtr1-Gtr2 complex is important for cell growth under nutrient-rich conditions, a strain was constructed in which the Lam-Gtr1-Gtr2 complex was artificially localized to the nucleus.

To target the Lam-Gtr1-Gtr2 complex into the nucleus, the nuclear localization signal (NLS) sequence of the SV40 large T-antigen was fused to a mCherry-tagged version of the Lam1GCCAAA mutant protein that cannot localize to vacuoles (Figure 17). As shown in Figure 18A, Lam1 was successfully localized into the nucleus as its fluorescent signals showed similar patterns to that of DNA stained by the Hoechst dye. Consistent with the notion that Gtr1-Gtr2 forms a complex with the Lam proteins, the Gtr1-Gtr2 heterodimer was also detected in the nucleus of this strain (Figure 18B).

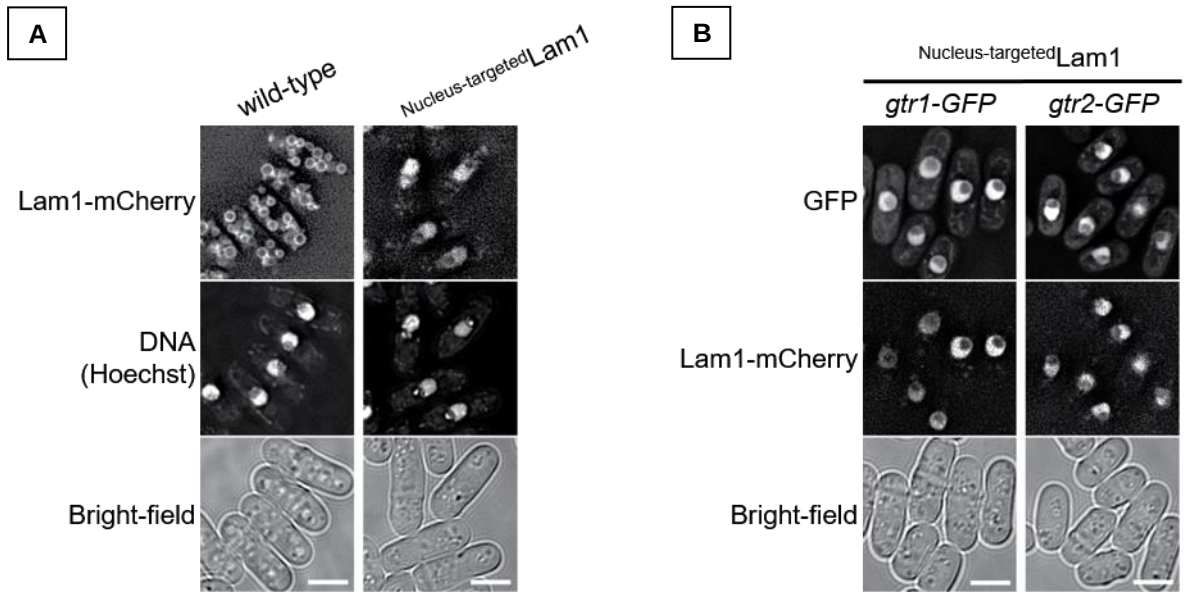

Figure 18. Artificial nuclear targeting of Lam1 translocates Gtr1-Gtr2 to the nucleus.

Lam1GCCAAA that cannot localize to vacuoles (Figure 15) was fused to the SV40 nuclear localization signal (NLS) and mCherry sequences (A), and expressed in the *gtr1:GFP* and *gtr2:GFP* (B) strains for fluorescence microscopy. DNA was stained by Hoechst dye for nucleus visualization. Bars, 5 μm.

Subsequently, growth of this mutant strain was assayed by spotting serially diluted culture on a rich YES and EMM agar media (Figure 19). The cells expressing the nucleus-targeted Lam1 showed little growth on YES, demonstrating that delocalization of Lam-Gtr1-Gtr2 complex from vacuolar membranes impair cell growth under this condition. On the other hand, the *lam1LLAA* mutant strain, in which a fraction of Lam1 still resides on vacuoles (Figure 17), exhibited normal growth comparable to that of the wild type. Collectively, these results confirmed that the vacuolar localization of the Lam-Gtr1-Gtr2 complex is important to promote cell growth.

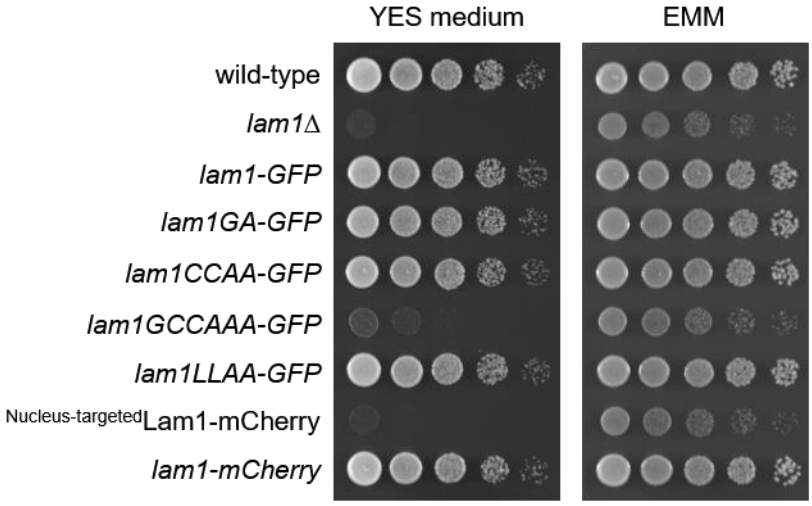

Figure 19. Vacuolar localization of the Lam-Gtr1-Gtr2 complex is important for yeast growth.

The indicated single and double mutants as well as a wild-type strain were grown in EMM liquid medium and their serial dilutions were spotted onto rich YES and EMM agar media for a growth assay at 30°C.

3.7 The GDP-bound form of Gtr1 is required for cell growth on rich medium

It has been proposed that guanine nucleotide loading to the Rag GTPases in higher eukaryotes is regulated in response to amino acids (Sancak et al., 2008). The GTP-bound RagA/B can interact with the *raptor* subunit of mTORC1 to recruit mTORC1 to the lysosomal surface for its activation by the Rheb GTPase. Consistent with this model, expression of the GTP-locked dRagA in *D. melanogaster* increased the cell size, while the GDP-locked dRagA decreased the cell size, suggesting that the guanine nucleotide-binding status of the Rag GTPases regulate cell growth (Kim et al., 2008). In order to test whether the nucleotide-loading status of Gtr1 and Gtr2 also regulate fission yeast growth, strains that express constitutively GTP- or GDP-bound Gtr1 were constructed by replacing the *gtr1*$^{+}$ locus with *gtr1Q61L* and *gtr1S16N*, respectively. Similar point mutations, *gtr2Q60L* and *gtr2S17N*, were employed to express GTP- and GDP-locked Gtr2, respectively (Nakashima et al., 1999; Valbuena et al., 2012).

Unexpectedly, the strain expressing Gtr1Q61L (Gtr1^{GTP}) exhibited a severe growth defect similar to that of the *gtr1*Δ mutant on YES medium. In contrast, the growth of the strain expressing Gtr1S16N (Gtr1^{GDP}) was comparable to that of wild-type cells (Figure 20), suggesting that the GDP-bound form of Gtr1 is important for cell growth. On the other hand, the nucleotide-loading status of Gtr2 did not significantly affect the cell growth. However, Gtr2 appears to play an important role for the Gtr1 function, because absence of Gtr2 impaired growth (Figures 10 and 20) as well as the vacuolar localization of Gtr1 (Figure 12A).

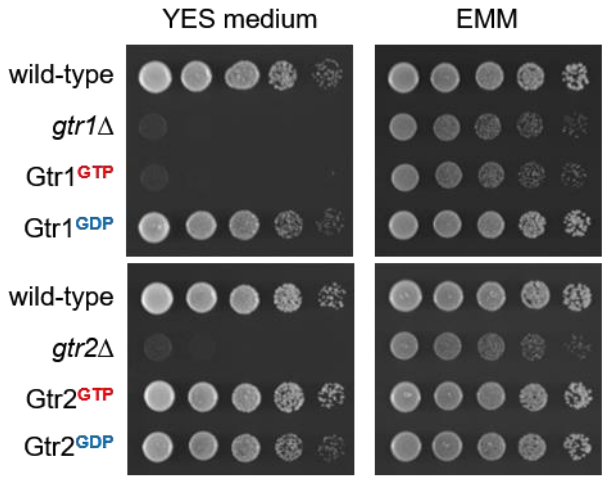

Figure 20. Growth phenotypes of the strains expressing the GTP-locked ("Gtr1^{GTP}" and "Gtr2^{GTP}") and GDP-locked ("Gtr1^{GDP}" and "Gtr2^{GDP}") mutants of the Gtr GTPases.

The indicated wild-type and mutant strains were grown in EMM liquid medium and their serial dilutions were spotted onto rich YES and EMM agar media for a growth assay at 30°C.

To confirm the defect phenotype of the strain expressing Gtr1^{GTP} is not due to disruption of the Lam-Gtr1-Gtr2 complex, the cellular localization of the GTP- and GDP-locked Gtr1 proteins was observed using their GFP fusions. The vacuolar localization of Gtr1 was not affected by the *gtr1Q61L* and *gtr1S16N* mutations (Figure 21), implying that Gtr1^{GTP} and Gtr1^{GDP} can form a complex with Gtr2 and the Lam proteins on the vacuolar surface.

Together these results strongly suggest an important function of the GDP-bound Gtr1 (Gtr1^{GDP}) in the Lam-Gtr1-Gtr2 complex to positively regulate fission yeast growth.

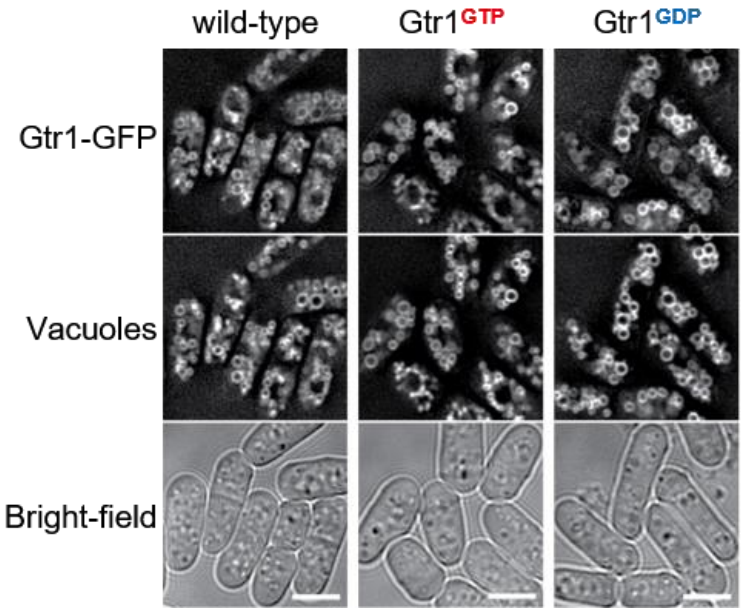

Figure 21. Vacuolar localization of Gtr1 is not affected by the *Q61L* (Gtr1^{GTP}) and *S16N* (Gtr1^{GDP}) mutations in *gtr1*$^{+}$.

The indicated wild-type and mutant strains expressing Gtr1^{GTP}-GFP or Gtr1^{GDP}-GFP from their chromosomal loci were grown in EMM at 30˚C, stained with SynaptoRed C2 fluorescent dye for vacuole visualization, and analyzed using fluorescence microscope. Z-axial images were collected and mid-section images after deconvolution are shown. Bars, 5 μm.

3.8 Vacuolar localization of TORC1 is independent of the Lam-Gtr1-Gtr2 complex

As described in the Introduction, the lysosomal recruitment of mTORC1 is mediated by the GTP-bound RagA/B in mammalian cells (Sancak et al., 2008). Because fission yeast TORC1 is localized on the vacuolar surface (Valbuena et al., 2012), I set out to determine whether the vacuolar localization of TORC1 is also mediated by the RagA/B ortholog Gtr1. The GFP sequence was inserted to the 3' end of the *mip1*$^{+}$ open reading frame in wild-type as well as *gtr1Q61L* and *gtr1S16N* strains in order to express GFP-fused Mip1, a *raptor* ortholog in fission yeast (Figure 3 in introduction). Mip1-GFP was mostly observed on vacuolar membranes in wild-type cells and, interestingly, also in the mutants expressing Gtr1^{GTP} and Gtr1^{GDP} (Figure 22A). Furthermore, Mip1-GFP was localized to vacuolar membranes in *lam*Δ and *gtr*Δ mutants (Figure 22B and 22C), as well as under the nitrogen starvation condition (Figure 23), where TORC1 is known to be inactivated (Nakashima et al., 2010; Nakashima et al., 2012; Hatano et al., 2015). Thus, fission yeast TORC1 localizes to vacuolar membranes in a manner independent of the Lam-Gtr1-Gtr2 complex and of the nutritional conditions.

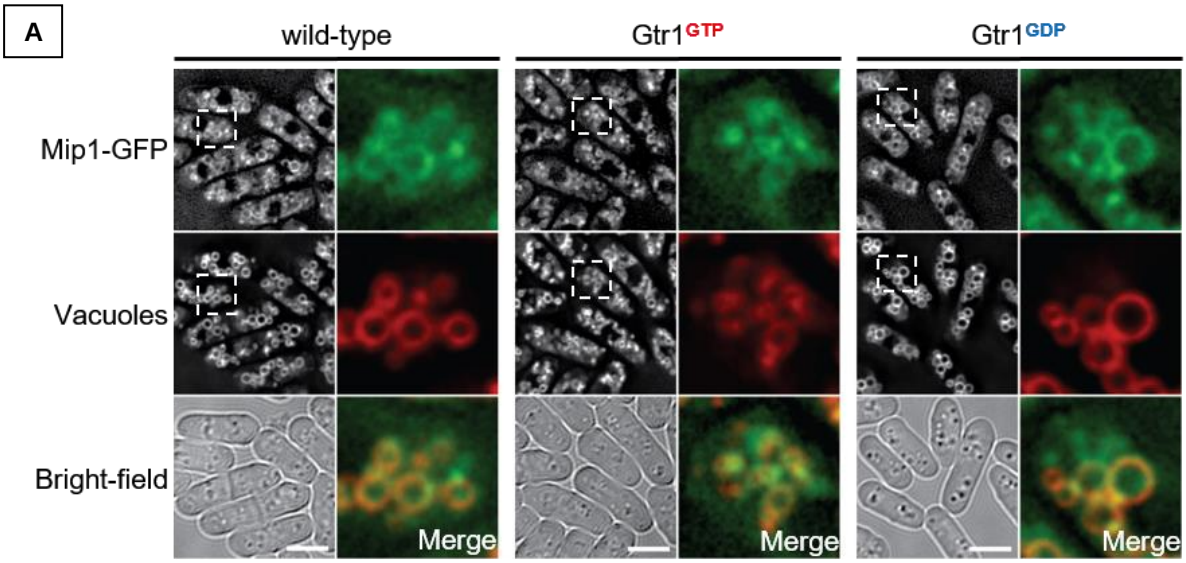

Figure 22A. Vacuolar localization of TORC1 is not affected by guanine nucleotide states of Gtr1.

The indicated wild-type and mutant strains expressing Mip1-GFP (green) from their chromosomal loci were grown in EMM at 30˚C, stained with SynaptoRed C2 fluorescent dye for vacuole visualization (red), and analyzed using fluorescence microscope. Z-axial images were collected and mid-section images after deconvolution are shown. Inset, section of micrographs that being enlarged four times and showed in colour. Merge, merged image of the enlarged sections. Bars, 5 μm, only applicable to all greyscale images.

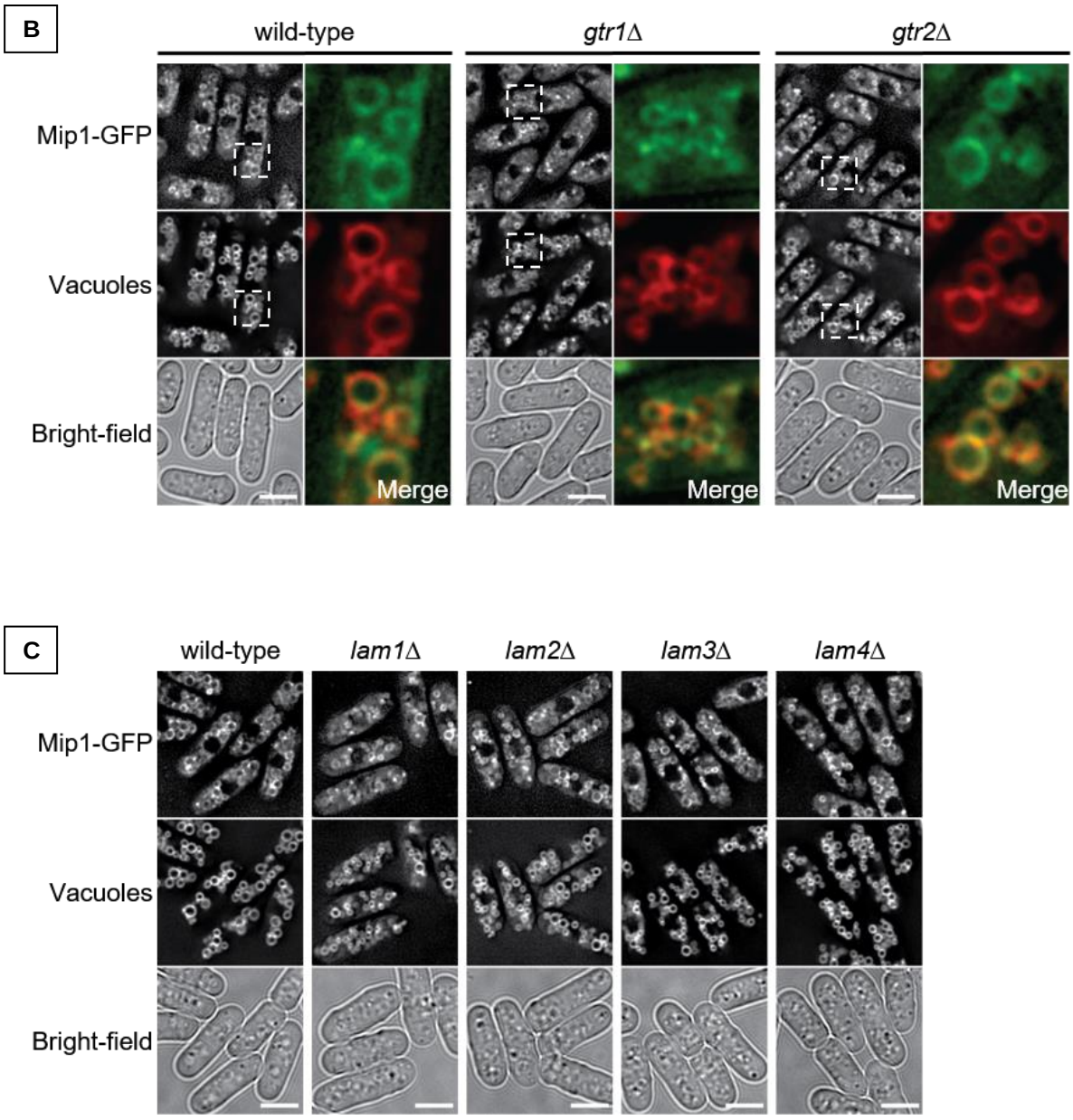

Figure 22B and C. The Lam-Gtr1-Gtr2 complex is not required for vacuolar localization of TORC1.

Wild-type as well as *gtr*Δ (B) and *lam*Δ (C) mutant strains expressing Mip1-GFP (green) from their chromosomal loci were stained with SynaptoRed C2 fluorescent dye for vacuole visualization (red), and analyzed by fluorescence microscopy as in Figure 22A. Inset, section of micrographs that being enlarged four times and showed in colour. Merge, merged image of the enlarged sections. Bars, 5 μm, only applicable to all greyscale images.

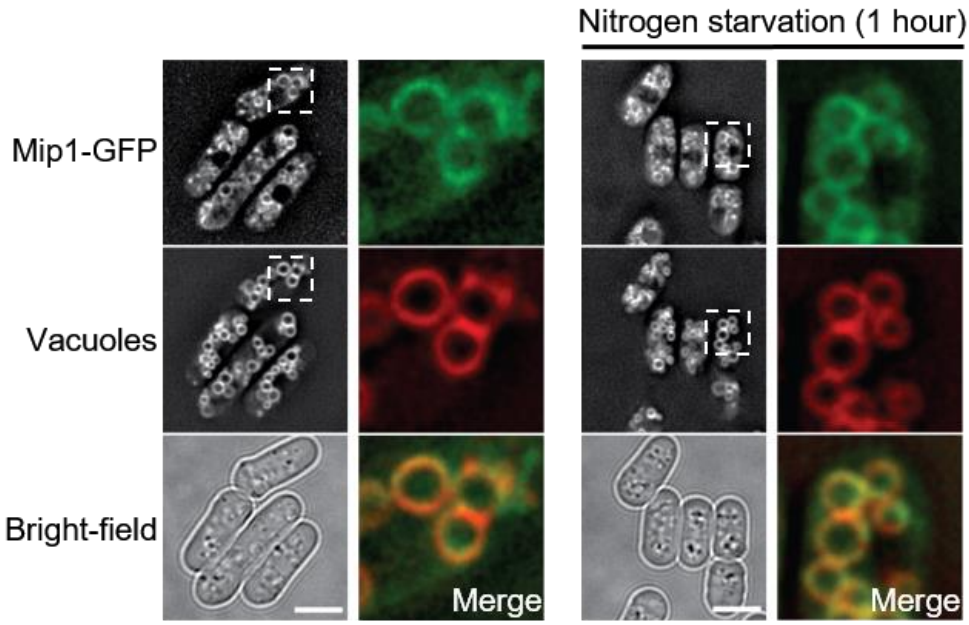

Figure 23. Vacuolar localization of TORC1 remains unchanged in nitrogen starvation.

Strain expressing Mip1-GFP from its chromosomal locus was grown in EMM at 30˚C, stained with SynaptoRed C2 fluorescent dye for vacuole visualization (red), and analyzed by fluorescence microscopy as in Figure 22A. For nitrogen starvation, vacuole-stained cells were filtered after 30 minutes of destaining, shifted into EMM without nitrogen source (EMM-N) for 1 hour, and analyzed by fluorescence microscopy. Inset, section of micrographs that being enlarged four times and showed in colour. Merge, merged image of the enlarged sections. Bars, 5 μm, only applicable to all greyscale images.

3.9 Swift inactivation of TORC1 upon nitrogen starvation requires GDP-bound Gtr1

Although the Lam-Gtr1-Gtr2 complex is dispensable for the vacuolar localization of TORC1, the genetic analyses described above strongly suggest that the Lam-Gtr1-Gtr2 complex is required for normal growth. To investigate how the Lam-Gtr1-Gtr2 complex regulates TORC1 signaling in fission yeast, TORC1 signaling was assessed by monitoring phosphorylation of Psk1, a *S. pombe* ortholog of human S6K1 (Figure 24A). The TORC1-dependent phosphorylation of Psk1 in its hydrophobic motif results in its reduced electrophoretic mobility and is also detectable using antibodies against phosphorylated S6K1 (Nakashima et al., 2012). Most of Psk1 was detected in its slow-migrating form in the presence of nutrients (Figure 24B, "Psk1" in "wild-type" at time 0), indicating that the majority of cellular Psk1 was phosphorylated under this condition. In response to nitrogen starvation, phosphorylated Psk1 ("pPsk1") became undetectable within 30 minutes, as reported previously (Hatano et al., 2015). In contrast, the phosphorylated form of Psk1 remained detectable in *gtr1*Δ, *gtr2*Δ and all of the *lam*Δ mutants until later time points (Figure 24B-D), indicating a significant delay in TORC1 inactivation in these strains. Consistent with the notion that the Lam proteins and the Gtr GTPases function together as a complex, the delay in TORC1 inactivation upon starvation in the *gtr1*Δ *gtr2*Δ and *gtr1*Δ *lam1*Δ double mutants was indistinguishable from that in the *gtr1*Δ single mutant (Figure 24E). In the absence of Gtr1-Gtr2, phosphorylated Psk1 was detectable even after 2 hours of nitrogen starvation (Figure 24F), suggesting that loss of the Lam-Gtr1-Gtr2 complex causes TORC1 to be partially insensitive to nitrogen starvation.

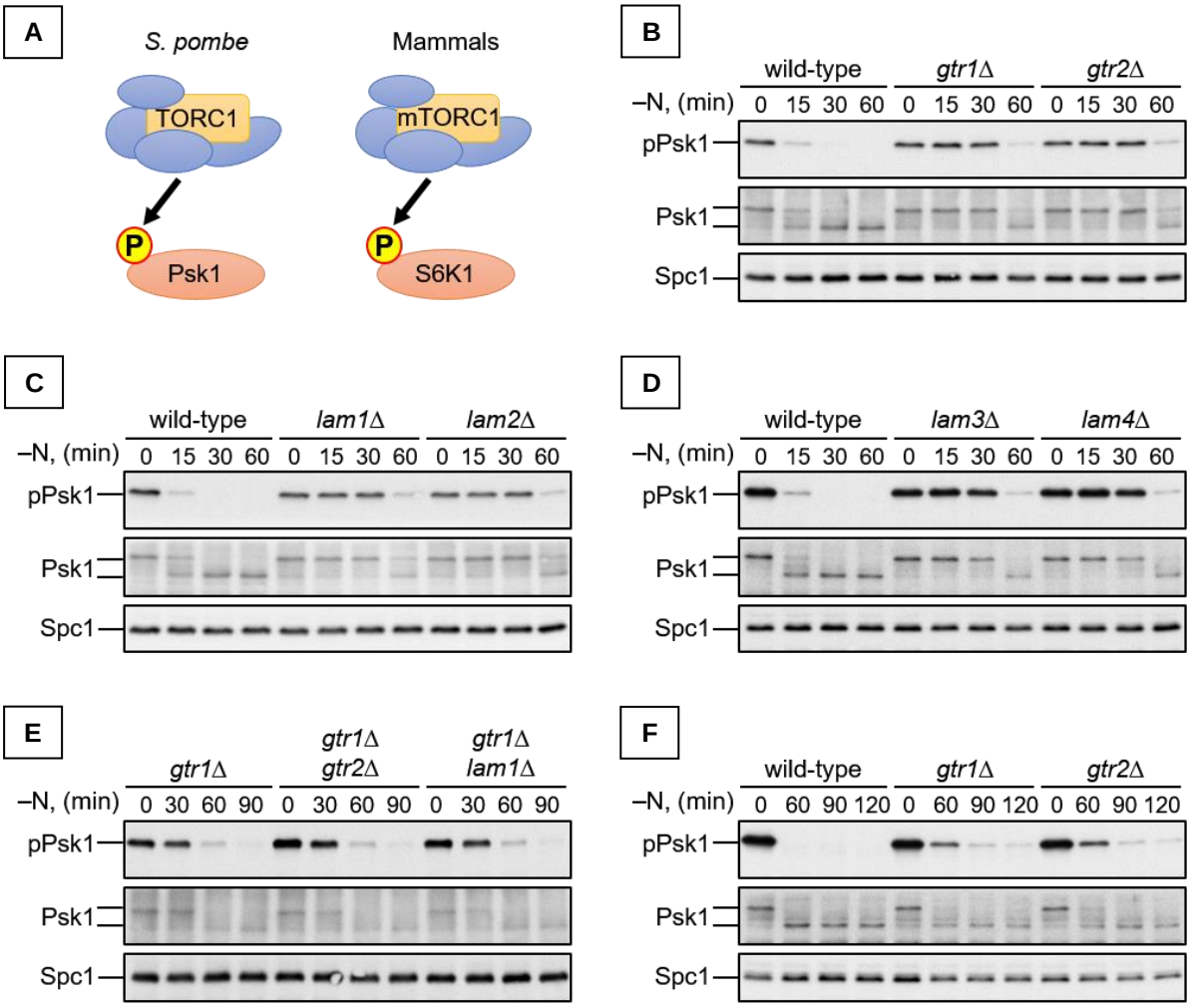

Figure 24. Cells lacking Lam-Gtr1-Gtr2 complex exhibit a delay in TORC1 inactivation during nitrogen starvation.

(A) Schematic diagram of TORC1 signaling in *S. pombe* and human, in which Psk1, an ortholog of the S6K1, is phosphorylated by fission yeast TORC1, while human mTORC1 phosphorylates S6K1. (B-F) TORC1 activity was monitored by immunoblotting to detect the TORC1-dependent phosphorylation of Psk1 ("pPsk1"), along the indicated time course after the indicated strains exponentially growing in EMM at 30˚C were shifted to the same medium without nitrogen source for starvation (–N). The samples were also probed with anti-Psk1 antibodies ("Psk1") as well as anti-Spc1 MAPK antibodies as loading controls. Inactivation of TORC1 after nitrogen starvation was delayed in the strains lacking Gtr1-Gtr2 (B), Lam1~4 (C and D), and both of Gtr1 and Lam1 (E). A longer time course of nitrogen starvation was performed using strains lacking the Gtr1-Gtr2 (F).

In order to determine whether the nucleotide-binding state of Gtr1 modulates TORC1 signaling, the Psk1 phosphorylation in the strains expressing GTP-locked Gtr1 ($Gtr1^{GTP}$) or GDP-locked Gtr1 ($Gtr1^{GDP}$) was also examined during nitrogen starvation. Intriguingly, the kinetics of TORC1 inactivation in the strain expressing $Gtr1^{GDP}$ was similar to that in the wild-type strain (Figure 25A), while the strain expressing $Gtr1^{GTP}$ exhibited an apparent delay comparable to that in the *gtr1*Δ mutant (Figure 25B). However, GDP-bound Gtr1 did not seem to be functional in the absence of the Lam complex, because the *gtr1S16N lam1*Δ double mutant showed a delay similar to the *lam1*Δ single mutant (Figure 25C), supporting the idea that the intact Lam-Gtr1-Gtr2 complex is required for the Gtr1 function. Collectively, these results suggest that the GDP-bound Gtr1 is required for the swift TORC1 inactivation during nitrogen starvation. Therefore, I hypothesized that the Lam-$Gtr1^{GDP}$-Gtr2 complex might function to suppress TORC1 signaling for optimal growth of fission yeast (Figure 25D).

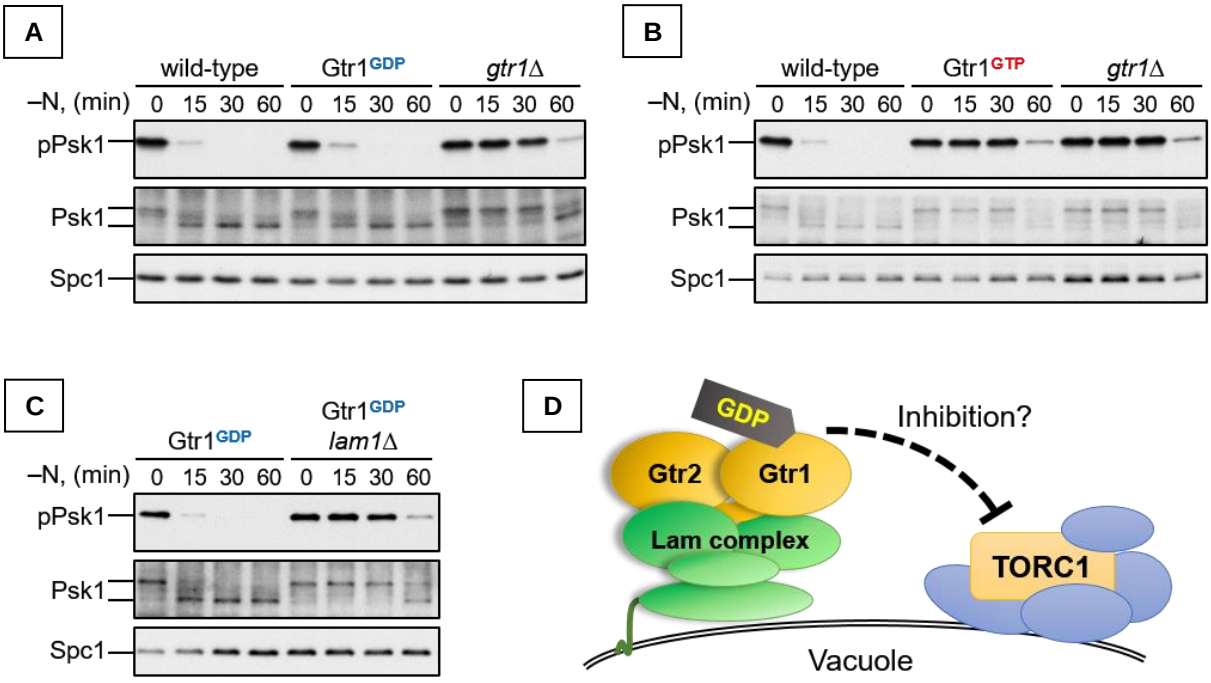

Figure 25. Loss of the $Gtr1^{GDP}$ causes a delay in TORC1 inactivation during nitrogen starvation.

(A-C) The indicated strains that growing exponentially in EMM at 30˚C were shifted to the same medium without nitrogen source for starvation (–N). TORC1 activity along the indicated time course was monitored by immunoblotting as in Figure 24. (D) Schematic diagram of a hypothesis in which $Gtr1^{GDP}$ functions to inhibit TORC1 in *S. pombe*.

3.10 TORC1 activity is elevated in the absence of GDP-bound Gtr1

Rapamycin inhibits TORC1 signaling without significantly affecting the growth of *S. pombe* (Takahara and Maeda, 2012). To test if the TORC1 signaling is hyperactive in strains lacking the Lam-Gtr1-Gtr2 complex, rapamycin was added to YES medium in the spot growth assay. As shown in Figure 26, the defective growth phenotype of the *gtr*Δ and *lam*Δ mutants was suppressed by rapamycin, implying that suppression of TORC1 activity in these mutants allows their growth on YES medium.

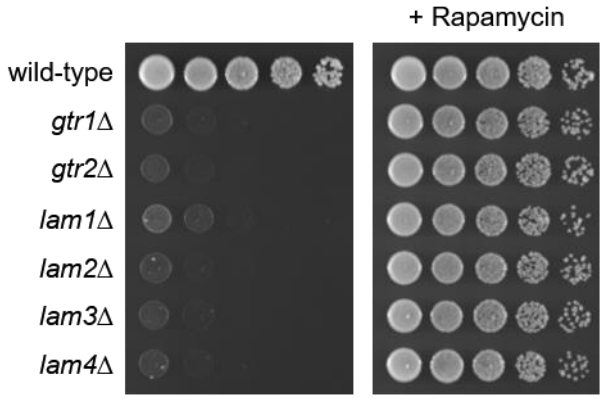

Figure 26. Rapamycin suppress the growth defects of the mutants lacking Lam-Gtr1-Gtr2.
The indicated wild-type and mutant strains were grown in EMM liquid medium and their growth were assayed at 30°C on rich YES medium agar with and without addition of 100 ng/mL rapamycin.

If the Lam-Gtr1-Gtr2 complex functions to suppress TORC1 signaling, hyperactivation of TORC1 in the absence of the complex would be consistent with the observed delay in the inactivation of TORC1 in the *lam*Δ and *gtr*Δ mutants after nitrogen starvation (Figures 24 and 25). Indeed, a similar delay in the starvation-induced inactivation of TORC1 was observed in strains lacking the Tsc1-Tsc2 complex (Figure 27A), a known negative regulator of TORC1 signaling (Figure 1 in introduction). A comparable delay was also apparent in strains carrying the activating mutations of Tor2 and Rhb1, *tor2E2221K* (Urano et al., 2007) and *rhb1-DA4* (Murai et al., 2009), respectively (Figure 27B). These results support the notion that the delayed TORC1 inactivation in nitrogen-starved cells lacking the Lam-Gtr1-Gtr2 complex is due to the hyperactivation of TORC1 signaling.

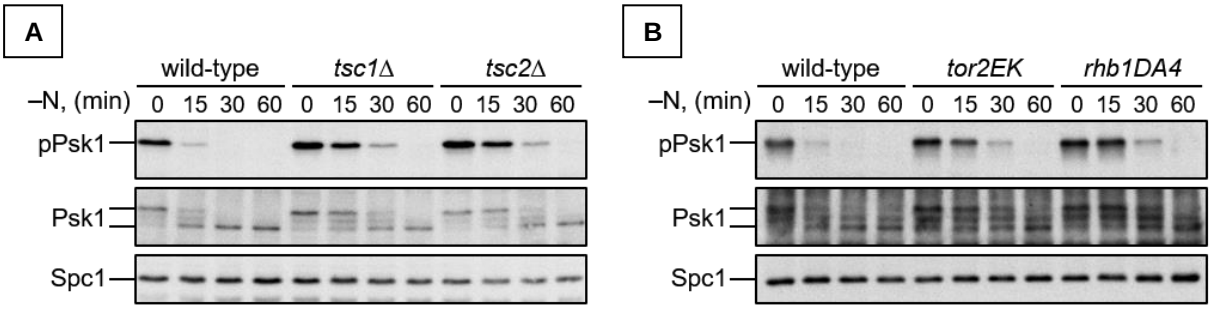

Figure 27. Inactivation of TORC1 during nitrogen starvation is also delayed in mutants carrying activated Tor2 and Rhb1, as well as in the strains lacking Tsc1-Tsc2 complex.

(A and B) TORC1 activity was monitored by immunoblotting to detect the TORC1-dependent phosphorylation of Psk1 ("pPsk1"), along the indicated time course after the indicated strains exponentially growing in EMM at 30˚C were shifted to the same medium without nitrogen source for starvation (–N). The samples were also probed with anti-Psk1 antibodies ("Psk1") as well as anti-Spc1 MAPK antibodies as loading controls.

In parallel with this study, Matsuda (2017) has identified GATOR1 in fission yeast, an evolutionarily conserved protein complex that functions as a GTPase-activating protein (GAP) for Gtr1 (Matsuda, 2017). He has shown that the GATOR1 null mutants also exhibit a growth defect on YES medium and that the phenotype is suppressed by rapamycin. Moreover, the GATOR1 null mutants exhibit a delay in the nitrogen-starvation response, consistent with the idea that TORC1 signaling is hyperactive in the absence of $Gtr1^{GDP}$. Therefore, I looked into the phosphorylation state of another known TORC1 substrate, Sck1, in strains lacking Gtr1-Gtr2 or GATOR1; the TORC1-dependent phosphorylation of Sck1 can be detected as reduced electrophoretic mobility (Nakashima et al., 2012). Interestingly, the slow-migrating form of FLAG-tagged Sck1 increased significantly in the *gtr*Δ mutants as well as in the *iml1*Δ mutant, which lacks the Iml1 subunit of GATOR1, when compared to that in wild-type cells (Figure 28A). This observation confirms that absence of $Gtr1^{GDP}$-Gtr2 results in elevated TORC1 activity even under nitrogen-replete conditions, and further supports my hypothesis that the Lam-Gtr1-Gtr2 complex is required for attenuation of TORC1 activity in fission yeast to promote cell growth (Figure 28B). On the other hand, loss of the Tsc2 seems to have little impact on Sck1 phosphorylation under the tested condition.

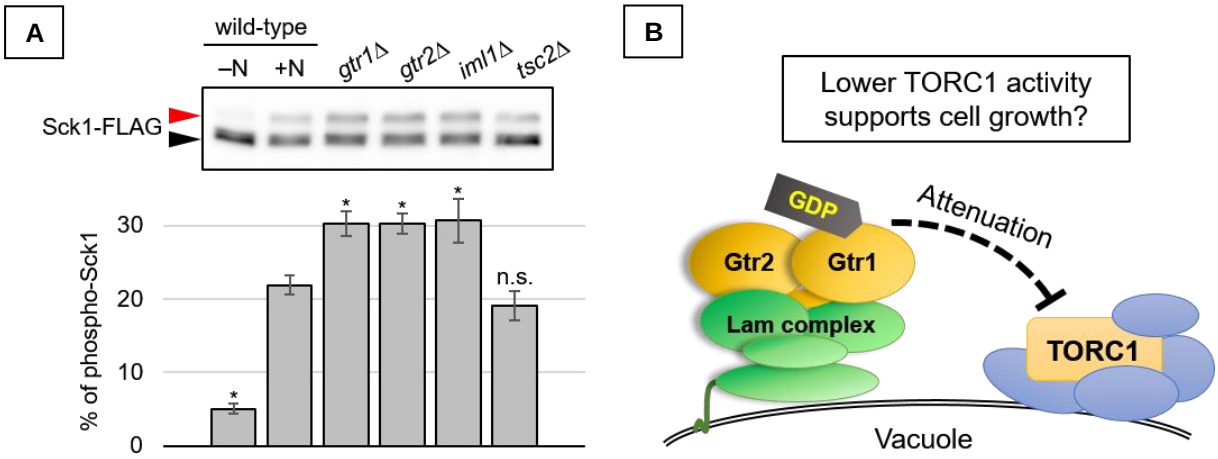

Figure 28. TORC1-dependent phosphorylation of Sck1 is augmented in the absence of $Gtr1^{GDP}$-Gtr2.

(A) The *sck1:FLAG* strains carrying the indicated mutations are grown in EMM at 30˚C and their lysate was analyzed by immunoblotting. As a negative control with inactive TORC1, a wild-type strain expressing Sck1-FLAG was starved for nitrogen for 1 hour ("–N"). The band intensity of the phosphorylated (red arrowhead) and unphosphorylated (black arrowhead) forms of Sck1-FLAG was quantified, and the percentage of the phosphorylated form to the total Sck1-FLAG level in each sample are presented in the bar graph as means ± SD (n = 4 independent experiments). *P<0.01; n.s., not significant, compared to the wild-type control using Student's t-test. (B) Schematic diagram of a hypothesis in which the Lam-$Gtr1^{GDP}$-Gtr2 functions to attenuate TORC1 to a level that supports *S. pombe* growth.

3.11 The growth defect in the absence of GDP-bound Gtr1 is partly attributed to impaired amino-acid uptake

Activated TORC1 signaling is known to promote cell growth and suppresses autophagy (reviewed in Wullschleger et al., 2006). However, despite their augmented TORC1 signaling, cells lacking the Lam-Gtr1-Gtr2 complex exhibit a severe growth defect. Genetic analysis by Fajar (2016) suggested that cellular uptake of amino acid seems to be impaired in the *gtr*Δ mutants. As was found with the *gtr*Δ mutants, I observed improved growth of the *lam*Δ mutants when YES medium was supplemented by ammonium (Figure 29). Therefore, cells lacking the intact Lam-Gtr1-Gtr2 complex may be starved of nitrogen on YES medium, probably because they cannot utilize amino acids in this medium; their growth defect can be ameliorated by adding ammonium as an alternative nitrogen source.

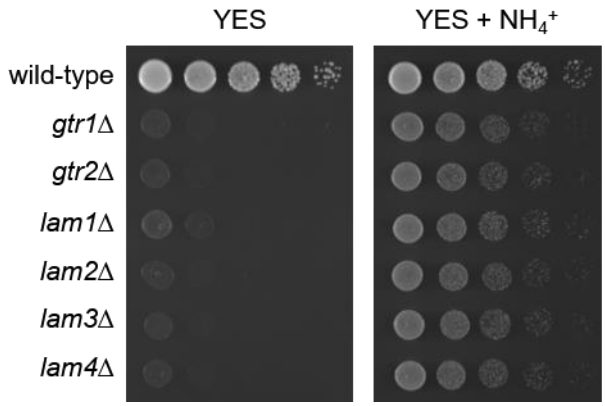

Figure 29. Ammonium alleviates the growth defects of the mutants lacking Lam-Gtr1-Gtr2 on rich YES medium.

The indicated wild-type and mutant strains were grown in EMM liquid medium and their growth were assayed at 30°C on rich YES medium agar with and without addition of 5 mg/mL ammonium chloride.

To test if cells lacking the intact Lam-Gtr1-Gtr2 complex are indeed starved of nitrogen in YES medium, I monitored the TORC1-dependent phosphorylation of Psk1 after wild-type and *gtr1*Δ cells were shifted from EMM medium, which contains ammonium as nitrogen source, to YES medium. Because TORC1 is responsive to nitrogen, dephosphorylation of Psk1 should be observed if cells are starved of nitrogen (see Figure 24). As expected, phosphorylated Psk1 was greatly reduced at 60 minutes after the shift from

EMM to YES medium in the *gtr1*Δ mutant, but not in the wild type (Figure 30). The *iml1*Δ mutant that lacks the GATOR1 complex also showed TORC1 inactivation after the shift to YES, suggesting that GDP-bound Gtr1 is required to prevent nitrogen starvation in YES medium.

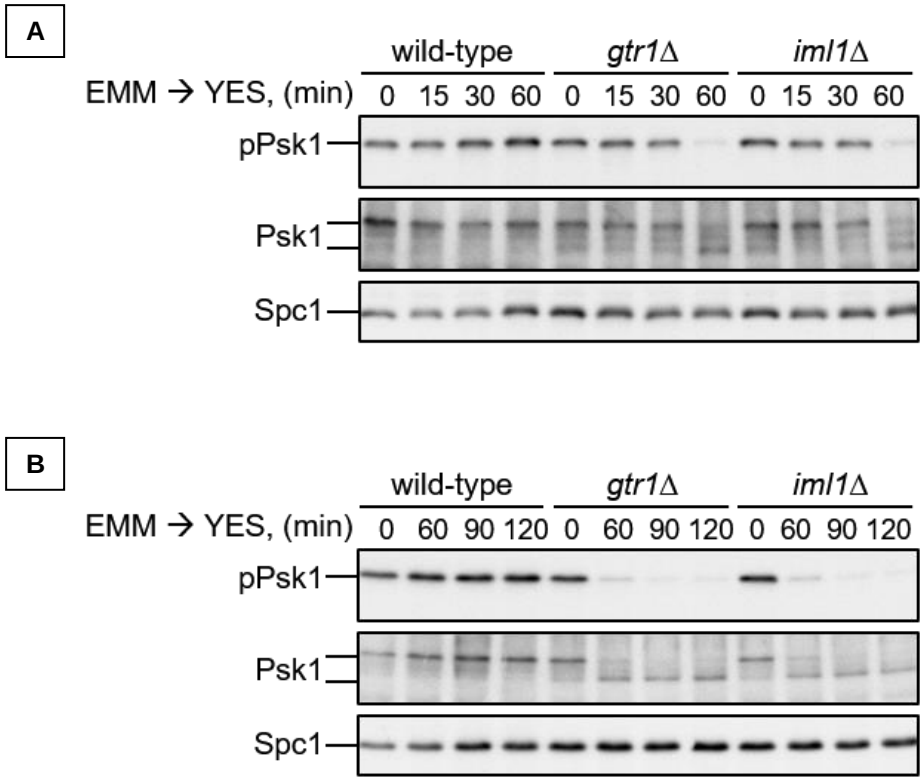

Figure 30. TORC1 signaling in the Gtr and GATOR mutants is inactivated in YES medium.
TORC1 activity was monitored by immunoblotting to detect the pPsk1, along the indicated time course after the indicated strains exponentially growing in EMM at 30˚C were shifted to YES liquid medium. The samples were also probed with anti-Psk1 antibodies ("Psk1") as well as anti-Spc1 MAPK antibodies as loading controls.

To further confirm defective amino-acid uptake in cells lacking the Lam-Gtr1-Gtr2 complex, their growth was tested on modified EMM medium, whose nitrogen source (ammonium) was replaced by amino acids, such as arginine or proline (Figure 31A). Both *gtr*Δ and *lam*Δ mutants failed to grow on such amino acid media, but their growth defect was partially suppressed by added ammonium. Moreover, the *gtr*Δ and *lam*Δ mutants were found

to be resistant to canavanine, a toxic analog of arginine (Figure 31B), suggesting the reduced ability of those mutants to uptake arginine.

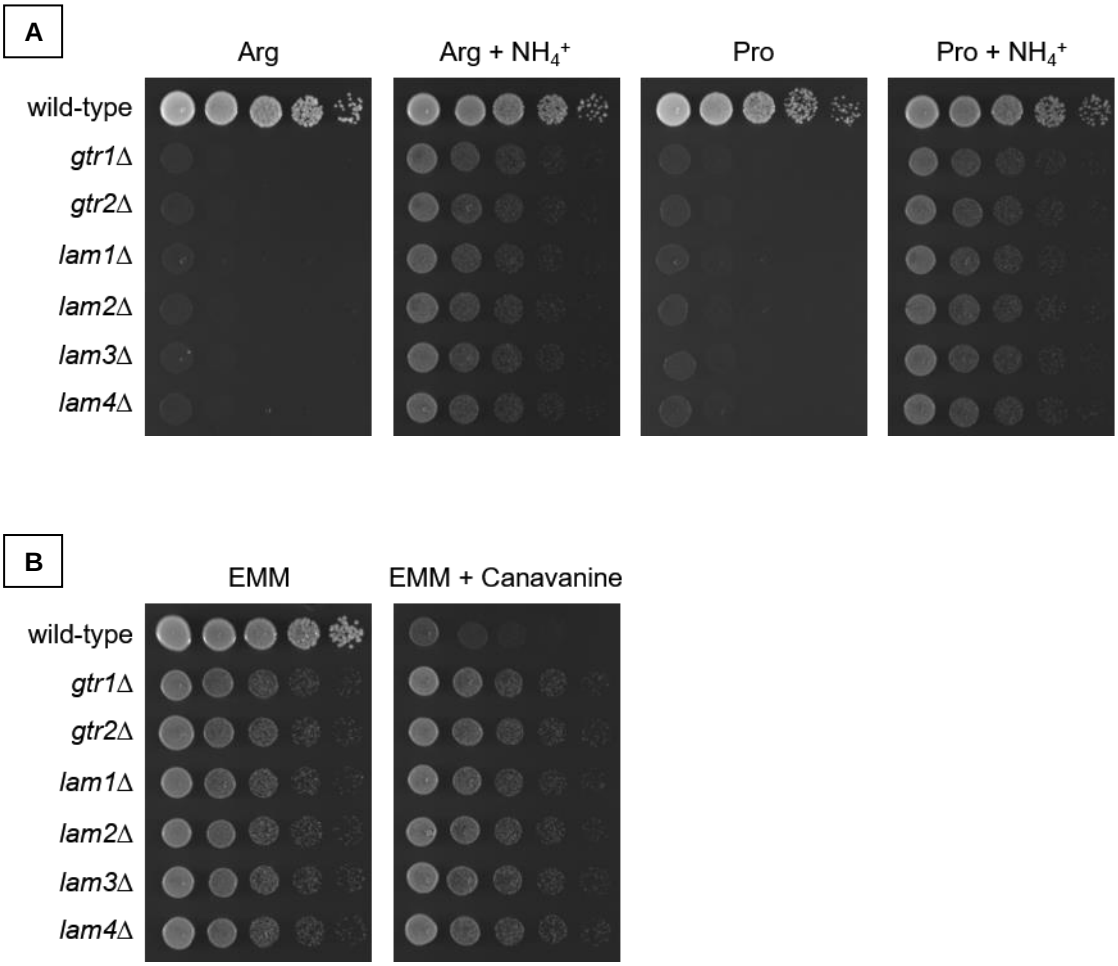

Figure 31. Loss of Lam-Gtr1-Gtr2 results in a defect in amino acid uptake.

The indicated strains were grown in EMM and their serial dilutions were spotted onto different agar media at 30˚C. (A) The Lam-Gtr1-Gtr2 mutants showed severe growth defects on EMM containing amino acid (20 mM Pro or Arg) as sole nitrogen source, while the phenotype was ameliorated by adding 5 mg/ml ammonium chloride. (B) In comparison to wild-type cells, those mutants were more resistant to canavanine ("Can", 60 μg/ml), a toxic analogs of arginine.

The Pub1 ubiquitin ligase and its arrestin-related adaptor, Any1, are reported to negatively regulate the plasma-membrane localization of amino acid transporters through endocytosis and their retention to Golgi/endosomes (Nakase et al., 2013; Nakashima et al.,

2014). Matsuda (2017) observed that the *pub1*Δ and *any1*Δ mutation complement the growth defect of the GATOR1 mutants on YES medium. As shown in Figure 32, the *any1*Δ mutation also suppressed the *lam*Δ phenotype on YES medium. In addition, the *any1*Δ mutation also abrogated the canavanine resistance phenotype of the *lam*Δ mutants. Thus, it appears that the growth defect of the Lam-Gtr1-Gtr2 mutants on YES medium is largely dependent on the inhibition of amino-acid uptake by Pub1-Any1.

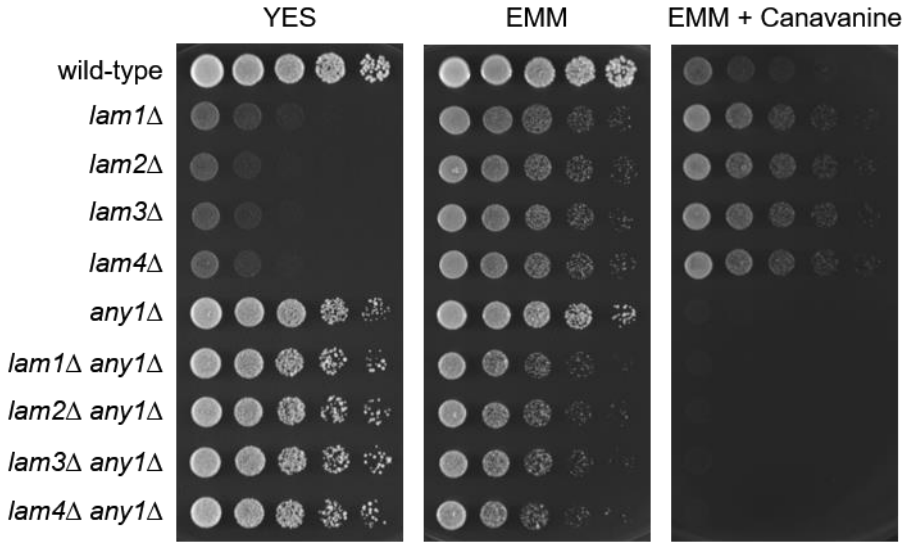

Figure 32. The *any1*Δ mutation restores the ability of amino acid uptake in mutants lacking Lam-Gtr1-Gtr2.

The indicated wild-type and mutant strains were grown in EMM liquid medium and their growth were assayed at 30°C on the indicated medium agar. Canavanine, "Can", was added into EMM medium at a concentration of 60 μg/ml.

3.12 The Lam-Gtr1-Gtr2 complex attenuates TORC1 activity independently of the Tsc-Rhb1 pathway

It has been established that TORC1 is negatively regulated by the Tsc1-Tsc2 complex that functions as GAP for Rhb1 (see Introduction; Matsumoto et al., 2002; van Slegtenhorst et al., 2004; Murai et al., 2009). To test the possibility that the Lam-Gtr1-Gtr2 complex down-regulates TORC1 signaling through the Tsc-Rhb1 pathway, the *tsc2*Δ mutation was introduced into the *gtr1*Δ and *iml1*Δ mutants, and their TORC1 response to nitrogen starvation was examined by monitoring the TORC1-dependent phosphorylation of Psk1. The *gtr1*Δ *tsc2*Δ and *iml1*Δ *tsc2*Δ double mutants exhibited a longer delay in TORC1 inactivation, as compared to their respective single mutants (Figure 33), suggesting that the Lam-Gtr1-Gtr2 and GATOR1 complexes negatively regulate TORC1 in parallel with the Tsc1-Tsc2 complex.

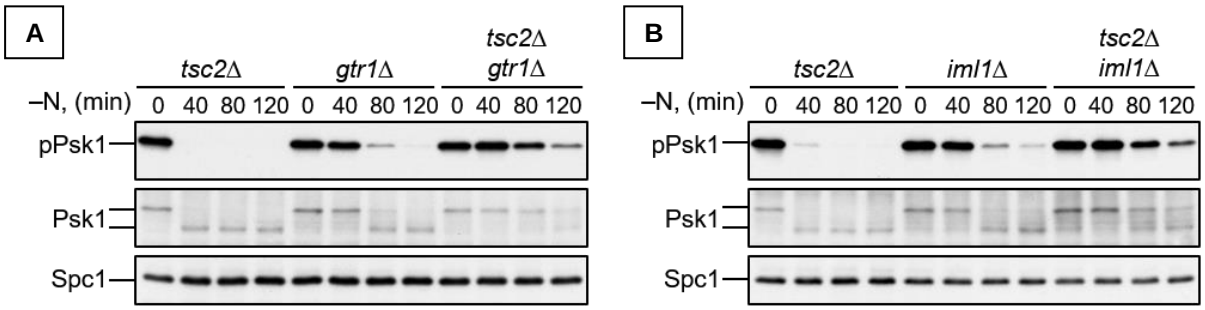

Figure 33. Introduction of the *tsc2*Δ mutation further delays the nitrogen starvation response of TORC1 in mutants lacking GATOR-Gtr machinery.

(A and B) TORC1 activity was monitored by immunoblotting to detect the TORC1-dependent phosphorylation of Psk1 ("pPsk1"), along the indicated time course after the indicated strains exponentially growing in EMM at 30˚C were shifted to the same medium without nitrogen source for starvation (–N). The samples were also probed with anti-Psk1 antibodies ("Psk1") as well as anti-Spc1 MAPK antibodies as loading controls.

Because the Tsc1-Tsc2 complex regulates TORC1 through the Rhb1 GTPase, an essential activator of TORC1, I further examined if the GATOR-Gtr pathway can regulate TORC1 independently of the Rhb1. It has been reported that the *rhb1*Δ mutant is viable only in the presence of an activated Tor2 allele, such as *tor2E2221K* (Mach et al., 2000; Urano et al., 2007). Therefore, *gtr1*Δ or *iml1*Δ mutant strains that carry the *tor2E2221K* (*tor2EK*) mutation, or the *tor2EK rhb1*Δ double mutations were constructed. As shown in Figure 34

(A), the *gtr1*Δ and *iml1*Δ mutants showed significant delays in TORC1 inactivation during nitrogen starvation even in the *tor2EK* background, indicating that TORC1 carrying the *tor2EK* mutation is still subject to the regulation by the GATOR1-Gtr pathway. Interestingly, introduction of the *gtr1*Δ and *iml1*Δ mutations into the *rhb1*Δ mutant also caused apparent delays in TORC1 inactivation during nitrogen starvation, as compared to the *rhb1*Δ mutant (Figure 34B). These results indicate that the GATOR-Gtr machinery can regulate TORC1 activity in a manner independent of Rhb1, at least in the *tor2E2221K* background. Therefore, it appears that the TORC1 attenuation by GATOR1 and Lam-Gtr1-Gtr2 utilizes a novel mechanism to suppress TORC1 signaling.

Taken together, the GATOR1-(Lam-Gtr1-Gtr2) pathway attenuates TORC1 in *S. pombe* for optimal cellular response to nutrients, and this regulation is independent of the Tsc-Rhb1 pathway.

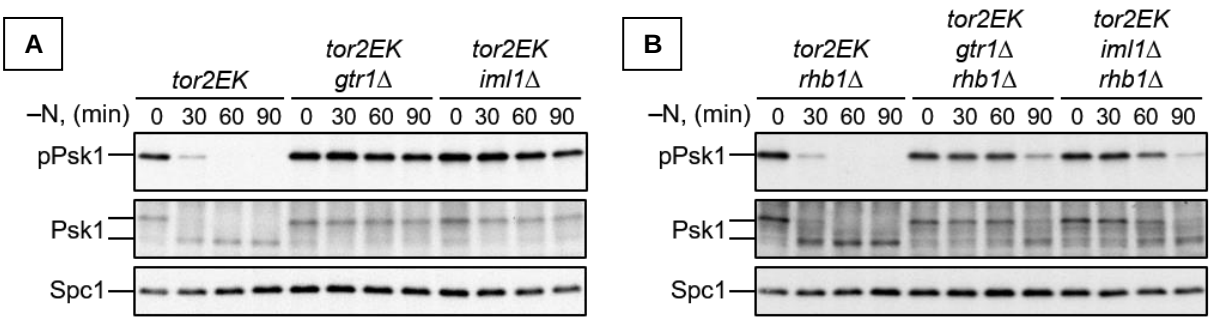

Figure 34. The GATOR-Gtr pathway can regulate TORC1 activity in the absence of Rhb1.

(A and B) TORC1 activity was monitored by immunoblotting to detect the TORC1-dependent phosphorylation of Psk1 ("pPsk1"), along the indicated time course after the indicated mutant strains exponentially growing in EMM at 30˚C were shifted to the same medium without nitrogen source for starvation (–N). The samples were also probed with anti-Psk1 antibodies ("Psk1") as well as anti-Spc1 MAPK antibodies as loading controls

4. Discussion

Growth factors and energy levels regulate the mammalian TORC1 (mTORC1) through the TSC-Rheb pathway (Garami et al., 2003; Inoki et al., 2003). The TSC-Rheb pathway is well-conserved in the fission yeast *S. pombe*. Like in mammalian cells, the Rheb GTPase Rhb1 is an essential activator of TORC1 in fission yeast (Mach et al., 2000; Urano et al., 2005; Matsumoto et al., 2002; van Slegtenhorst et al., 2004; Uritani et al., 2006). A more recent discovery of the Rag GTPases-dependent mechanism for mTORC1 activation in response to amino acids has also been attracting much attention (Kim et al., 2008; Sancak et al., 2008). Because the Rag-like heterodimeric Gtr1-Gtr2 GTPases are also implicated in the regulation of *S. pombe* TORC1 (Valbuena et al., 2012; Ma et al., 2013; Laor et al., 2014; Ma et al., 2016), our laboratory initiated studies to delve into the roles and relationships of these conserved regulatory factors in the control of TORC1 signaling. Fajar (2016) and Matsuda (2017) have identified the evolutionary conserved protein complex GATOR1 in *S. pombe*; GATOR1 is composed of the Iml1, Npr2 and Npr3 subunits and, like its mammalian counterpart, has GTPase-activating protein (GAP) activity for the RagA/B GTPase Gtr1.

In this study, I have identified and characterized the fission yeast counterpart of the mammalian Ragulator complex and the budding yeast Ego ternary complex, another key factor in the TORC1 regulation (Sancak et al., 2010; Bar-Peled et al., 2012; Dubouloz et al., 2005; Gao and Kaiser, 2006; Binda et al., 2009; Powis et al., 2015; Kira et al., 2016). Affinity-purification of the Gtr1-Gtr2 heterodimer followed by mass-spectrometry led to identification of four proteins, Lam1~4 (Figure 6). Lam1, Lam3 and Lam4 have never been characterized before with no apparent sequence homology to any known proteins. The observations in this study strongly suggest that the four Lam proteins form a complex and execute a function similar to that of the mammalian Ragulator complex, which tethers the RagA/B-RagC/D heterodimer to lysosomal membranes. First, among the components of the Lam protein complex, Lam2 shows detectable sequence similarity to the LAMTOR2, a component of mammalian Ragulator (Ma et al., 2016), while the predicted secondary structures of Lam1, Lam3 and Lam4 exhibit some resemblances to those of the LAMTOR1, LAMTOR3 and LAMTOR4 subunits of Ragulator (Figure 7). Second, the N-terminus of Lam1 contains the conserved motif with lipid modification sites as well as the lysosome/vacuole-targeting di-leucine motif, and these motifs are also found in the N-termini of mammalian LAMTOR1 and budding yeast Ego1 (Figure16). Third, the Lam proteins form a complex that localizes to the membranes of vacuoles, lysosome-like organelles in yeast, and

the Lam complex is essential to tether the Rag GTPases onto the vacuolar surface (Figures 11 and 12).

The fission yeast Lam protein complex is indispensable for vacuolar localization of the Gtr1-Gtr2 heterodimer. Lack of any of the Lam proteins causes Gtr1 and Gtr2 to delocalize from vacuolar membranes to the cytoplasm (Figure 12). Similarly, the budding yeast Ego ternary complex and the mammalian Ragulator are essential to tether the Rag GTPases onto vacuolar/lysosomal membranes (Powis et al., 2015; Sancak et al., 2010; Bar-Peled et al., 2012). Interestingly, the mammalian Ragulator complex functions not only as lysosomal anchor for the Rag GTPases, but also as guanine nucleotide exchange factor (GEF) for RagA/B to induce mTORC1 activation in response to amino acids (Bar-Peled et al., 2012). It remains unknown if the fission yeast Lam protein complex also functions as GEF for the Gtr1 GTPase. In this organism, TORC1 is active even in the absence of GTP-bound Gtr1 and therefore, the function of a Gtr1 GEF, if any, is cryptic under any of the culture conditions tested.

The most significant observation in this study is that the fission yeast TORC1 activity is elevated in the absence of the Lam-Gtr1-Gtr2 complex, suggesting that the complex represses TORC1 activity. Hyperactivation of TORC1 signaling in cells lacking the intact Lam-Gtr1-Gtr2 complex is suggested by the following observations:

(i) Inactivation of TORC1 in response to nitrogen starvation is delayed in the mutants lacking the Lam-Gtr1-Gtr2 complex (Figure 24). Similar delays in TORC1 inactivation are also known for strains with activating mutations of Tor2 or Rhb1, as well as for the mutants lacking the Tsc1-Tsc2 complex, a well-established negative regulator of TORC1 (Figure 27).

(ii) A significant increase in the level of phosphorylated Sck1, a substrate of TORC1, in the *gtr1*Δ and *gtr2*Δ mutants (Figure 28).

(iii) The growth defect phenotype of the mutants lacking Lam-Gtr1-Gtr2 is suppressed by the TORC1 inhibitor rapamycin (Figure 26).

In fission yeast, TORC1 localization to vacuolar membranes is not dependent on the Lam-Gtr1-Gtr2 complex (Figure 22). It is likely that the Gtr-independent targeting of TORC1 to the vacuolar surface renders TORC1 to activation by the Rhb1 GTPase, even in the absence of Lam-Gtr1-Gtr2. Rag-independent recruitment of mTORC1 to lysosomal membranes has also been reported (Jewell et al., 2015), and there might be a conserved mechanism for such TORC1 localization.

In the gene deletion experiments of this study, the null mutants of the Gtr1-Gtr2 GTPases and the Lam complex (Lam1, Lam2, Lam3 and Lam4) exhibit very similar growth defects, which are rescued by the TORC1 inhibitor rapamycin (Figure 26). Interestingly, Matsuda (2017) also observed a similar growth defect phenotype in the GATOR1 null mutants. He showed that, in the *gtr1*Δ and *iml1*Δ mutants, the plasma membrane amino acid transporter Cat1 is sequestered in the cytoplasm, and that the observed translocation of Cat1 is dependent on the Pub1 ubiquitin ligase and its adaptor, Any1 (Nakase et al., 2013; Nakashima et al., 2014). The phenotypes of the mutants lacking the Lam-Gtr1-Gtr2 complex also appear to be consistent with the idea that their ability to uptake amino acids is compromised (Figure 29-31). In addition, loss of Any1 restores the capability of the *lam*Δ mutants to uptake amino acids (Figure 32). Thus, the growth defect of the Lam-Gtr1-Gtr2 mutants on rich medium is dependent on Pub1 and Any1, which negatively regulate amino acid uptake by sequestering amino acid transporters from the plasma membrane.

The currently prevailing model based on studies in mammalian cells and budding yeast proposed that GTP-bound form of RagA/B (Gtr1 in yeast) plays an important role in TORC1 activation upon amino acid stimuli. In contrast, the GDP-bound form of Gtr1 ($Gtr1^{GDP}$) appears to be crucial in regulating fission yeast TORC1 signaling under nutrients replete conditions (Figure 20 and 25; Matsuda, 2017). It is currently unknown how $Gtr1^{GDP}$-Gtr2 suppresses TORC1, which is activated by the Rhb1 GTPase. In amino acid-starved mammalian cells, Tsc2 is recruited to lysosomal membranes by the Rag GTPases and inhibits Rheb, resulting in inactivation of mTORC1 (Demetriades et al., 2014). However, such a Tsc2-dependent mechanism cannot explain the observations that the effects of the *gtr1*Δ and *tsc2*Δ mutations are additive (Figure 33) and that the Gtr1-dependent attenuation of TORC1 appears to be operational even in the absence of Rhb1 (Figure 34).

It remains possible that GTP-bound Gtr1 ($Gtr1^{GTP}$) is capable of promoting TORC1 activation, as has been found for GTP-bound RagA/B in higher eukaryotes (Kim et al., 2008; Sancak et al., 2008; Sancak et al., 2010). However, such functionality of $Gtr1^{GTP}$ would be cryptic in *S. pombe*, because TORC1 can be strongly activated even in the *gtr1* null mutant (Figure 20).

4.1 Conclusion

In this study, a novel protein complex, the Lam complex, has been identified in fission yeast. This protein complex is composed of four proteins encoded by open reading frames SPBC1778.05c (Lam2), SPBC29A10.17 (Lam1), SPAC222.19 (Lam3), and SPAC23D3.16 (Lam4) in the *S. pombe* genome database. The Lam complex functions to recruit the heterodimeric Gtr1-Gtr2 GTPases to vacuolar membranes where TORC1 is localized. Notably, the GDP-bound form of Gtr1 is important to attenuate TORC1 signaling. Therefore, together with other data presented in this thesis, I propose that the $Gtr1^{GDP}$-Gtr2 heterodimer tethered onto the vacuolar surface by the Lam complex plays a role in moderating TORC1 activation induced by the Rhb1 GTPase. Although TORC1 activity is essential for cell viability in fission yeast (Álvarez and Moreno, 2006; Matsuo et al., 2007; Weisman and Choder, 2001), its attenuation by $Gtr1^{GDP}$-Gtr2 enables appropriate response to limitation in nitrogen source, allowing localization of the amino acid transporters to the plasma membrane. A model summarizes the findings in this study and those by Matsuda (2017) is shown in Figure 35.

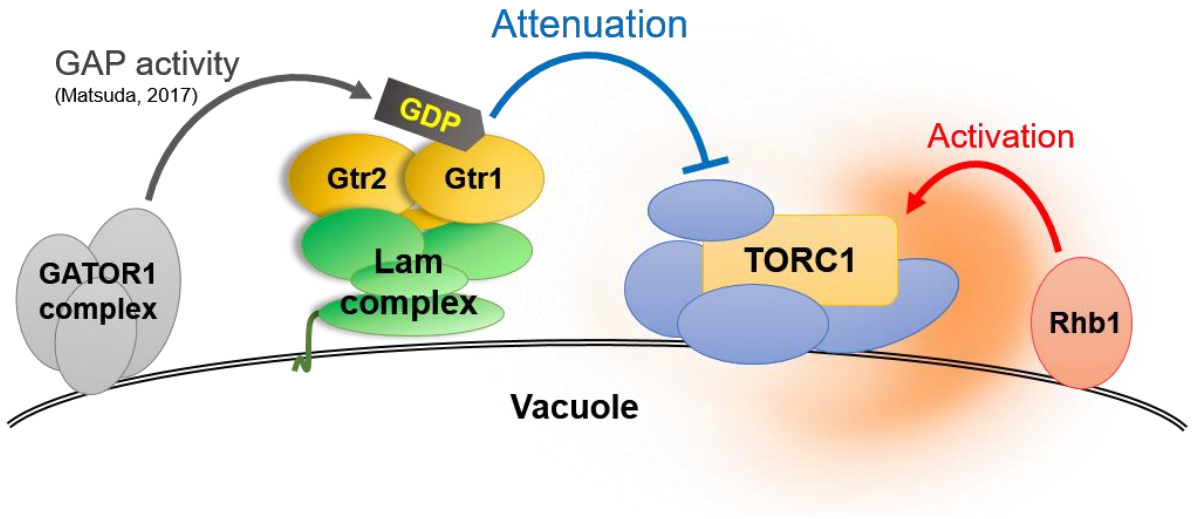

Figure 35. Schematic diagram depicting a model proposed in this study and those by Matsuda (2017). In fission yeast, the Lam complex tethers the heterodimeric Gtr1-Gtr2 GTPases onto vacuolar surface to form the Lam-Gtr1-Gtr2 complex. Another protein complex, the GATOR1, activates the intrinsic GTPase function of the Gtr1 to hydrolyze GTP to GDP. The resulted $Gtr1^{GDP}$ counteracts the Rhb1-dependent activation of TORC1 to ensure optimal cellular response to nutrients.

4.2 Future perspectives / suggestions

Hyperactivation of TORC1 signaling is implicated in cancerous cell proliferation and neurological disorders such as epilepsy and autism (Huang and Manning, 2008; Baldassari et al., 2016). The findings in this study is expected to provide a new insight into the molecular mechanisms that negatively regulate mTORC1. However, how $Gtr1^{GDP}$-Gtr2 down-regulate TORC1 activity in *S. pombe* remains unknown, and further studies to examine the different possibilities described below are necessary:

(i) $Gtr1^{GDP}$ inhibits TORC1 through direct physical interaction with TORC1?

Valbuena and colleagues (2012) reported the physical interaction between Gtr1 and Mip1, a *S. pombe* ortholog of *raptor*, in the presence of amino acids. Although the reported interaction between Gtr1 and Mip1 needs to be confirmed, it is likely that the $Gtr1^{GDP}$ is able to interact with TORC1. It is also interesting to know if the kinase activity of TORC1 is affected by Gtr1. To test this possibility, an *in vitro* kinase assay of *S. pombe* TORC1 should be established.

(ii) $Gtr1^{GDP}$ indirectly inhibits TORC1 via unknown protein(s) or other signaling pathways?

There might be unknown factor(s) that modulate TORC1 activity in a manner dependent on $Gtr1^{GDP}$. In order to identify the factors that mediate the Gtr1-dependent regulation of TORC1, a genetic screen can be employed to isolate mutants that show a growth defect similar to that of the GATOR1-Gtr1-Gtr2-Lam mutants on YES medium. By using a *gtr1S16N* strain expressing $Gtr1^{GDP}$ in this screen, re-isolation of GATOR1 mutants can be avoided.

A recent report suggests that a protein named Pib2 in budding yeast stimulates TORC1 activity (Kim and Cunningham, 2015). Interestingly, synthetic lethality is observed in the budding yeast strain carrying both *gtr1*Δ and *pib2*Δ mutations, implying that Pib2 is required to activate TORC1 in parallel with Gtr1. It would be of interest to examine if a Pib2 ortholog is also involved in TORC1 signaling in fission yeast.

(iii) The Lam proteins inhibit TORC1 in the presence of $Gtr1^{GDP}$?

It appears mysterious that the vacuolar localization of Gtr1-Gtr2 requires a four-protein complex such as the Lam complex; this complex might have an additional function other than tethering Gtr1-Gtr2 to the vacuolar surface. As pointed out above, $Gtr1^{GDP}$ might not directly inhibit TORC1; the Lam complex might be responsible for direct inhibition of TORC1 in the presence of $Gtr1^{GDP}$. By constructing *lam*Δ strains in which Gtr1-Gtr2 is

artificially targeted to vacuoles independently of the Lam complex, any unknown functions of the Lam proteins can be explored.

(iv) Ubiquitination of Gtr1 inhibits TORC1 activity?

Two independent studies have demonstrated that RagA is poly-ubiquitinated by two different E3 ligases, the RNF152 and the Skp2; poly-ubiquitination of RagA enhances its interaction with the GATOR1 complex, resulting mTORC1 inactivation (Deng et al., 2015; Jin et al., 2015). Indeed, knockout of the E3 ligases results in hyperactivation of mTORC1. It remains unknown if the *S. pombe* Gtr1 is also poly-ubiquitinated, although two of the four RNF152-targeted lysine residues in RagA are conserved in *S. pombe* Gtr1. Therefore, it would be interesting to examine if Gtr1 is also poly-ubiquitinated.

Remarks:

During this study, spontaneous suppressor mutations that mitigate the growth defects of the null mutants of the GATOR1 and Lam-Gtr1-Gtr2 complexes were detected with high frequency. This phenomenon may explain the inconsistent phenotypes described in the following studies:

(i) In the study reported by Valbuena et al. (2012), the *gtr1*Δ and *gtr2*Δ mutants showed different growth rates, which is in conflict with the idea that Gtr1 and Gtr2 function by forming a heterodimer. In contrast, this study has found that those two null mutants show indistinguishable phenotypes.

(ii) The *gtr1*Δ and *gtr2*Δ mutants used in Laor et al. (2014) exhibited no apparent growth defect on rich yeast extract medium, in contrast to the observations in my study (e.g., Figure 10). In addition, they reported that, like wild-type cells, those mutants are sensitive to canavanine, another observation implying suppressor mutations in their strains (see Figure 31B).

(iii) Ma et al. (2013) also reported the canavanine-sensitive phenotype of the *gtr1*Δ and *gtr2*Δ mutants.

(iv) However, the most recent study by the same group (Ma et al., 2016) withdrew their earlier conclusions, reporting the *gtr1*Δ and *gtr2*Δ phenotypes more consistent with what I found in this study.

To minimize selection pressure for such suppressor mutations, those null mutant strains should be grown in EMM, ammonium supplemented YES, or in the presence of rapamycin.

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