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BRIEF REPORT

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A New Crosslinking Assay to Study Guanine Nucleotide Binding in the Gtr Heterodimer of *S. cerevisiae*

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ABSTRACT

The mechanistic target of rapamycin (mTOR) complex is responsible for coordinating nutrient availability with eukaryotic cell growth. Amino acid signals are transmitted towards mTOR via the Rag/Gtr heterodimers. Due to the obligatory heterodimeric architecture of the Rag/Gtr GTPases, investigating their biochemical properties has been challenging. Here, we describe an updated assay that allows us to probe the guanine nucleotide-binding affinity and kinetics to the Gtr heterodimers in *Saccharomyces cerevisiae*. We first identified the structural element that Gtr2p lacks to enable crosslinking. By using a sequence conservation-based mutation, we restored the crosslinking between Gtr2p and the bound nucleotides. Using this construct, we determined the nucleotide-binding affinities of the Gtr heterodimer, and found that it operates under a different form of intersubunit communication than human Rag GTPases. Our study defines the evolutionary divergence of the Gtr/Rag-mTOR axis of nutrient sensing.

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Introduction

The mechanistic Target of Rapamycin Complex 1 (mTORC1) is a serine/threonine kinase complex central to regulating cellular growth and metabolic pathways [1–4]. It senses growth factors [5], glucose [6,7], and amino acid availability [4,8–15], and coordinates these signals with downstream effectors. Under nutrient-poor conditions, mTORC1 activates catabolic pathways such as autophagy [16], while under nutrient-rich conditions, mTORC1 stimulates anabolic reactions such as protein synthesis [17], lipid synthesis [18,19], and cell proliferation [20–23].

In human cells, the mTORC1 signalling pathway is activated when both amino acids and growth factors are present. Amino acids activate the Rag GTPase heterodimer, which recruits the mTORC1 complex onto the lysosome [24,25]. At the same time, if growth factor signals are presented via the tuberous sclerosis proteins (TSC) branch, the kinase activity of mTORC1 will be turned on by the Rheb GTPase on the lysosomal surface [26–28]. The Rag GTPases employ a unique architecture compared with other signalling GTPases. They are obligate heterodimers formed between a RagA/B subunit and a RagC/D subunit. Each subunit within the heterodimer is capable of binding and hydrolysing GTP [29,30]. In the active state, RagA/B binds GTP and RagC/D binds GDP, while in the inactivated state, RagA/B binds GDP and RagC/D binds GTP [31,32]. Intrinsically, the Rag GTPase heterodimer operates with extensive intersubunit communication: When one subunit binds GTP, it becomes the dominant subunit by preventing the other from binding GTP, and in case of accidental binding that leads to a dual GTP-loaded state, stimulating the hydrolysis of the later bound GTP [32]. These mechanisms (termed 'locking') ensure that only one subunit is occupied by GTP, so that only defined signals are transmitted towards the downstream effector, mTORC1.

The nucleotide loading state of the Rag GTPase heterodimer is strictly regulated by its upstream regulators, such as GATOR1 and FLCN-FNIP2, which are two GTPase activating proteins (GAPs) [14,33–35], and SLC38A9 and the Ragulator complex, which are two guanine nucleotide exchange factors (GEFs) [9–11,36]. These GAPs and GEFs receive and process the amino acid signals from their upstream regulators, such as GATOR2 and KICSTOR, to ensure that the Rag GTPases adopt the correct nucleotide loading state and conformation based on the availability of amino acids.

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The Rag GTPases are obligatory heterodimers, so we need an assay that can monitor nucleotide binding to individual subunits. We have previously developed a crosslinking assay to measure the nucleotide binding to human Rag GTPases [32,37]. We first incubate the Rag GTPases with radioactively labelled GTP or GDP, and use ultraviolet (UV) light to induce crosslinking between the Rag subunit and the bound nucleotide. As RagA and RagC have different molecular weights, they can be separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the bound nucleotide can be quantified by their radioactivity. This assay is used to determine the binding kinetics of human Rag heterodimers and can be used to understand the regulation of the mTORC1 pathway in greater detail [32]. However, whether this assay can apply to Rag homologs in other species is unknown.

Despite the high sequence homology to the RagA-RagC heterodimer, the *Saccharomyces cerevisiae* homologs of the Rag GTPases have been underexplored. The Gtr heterodimer is most similar to the RagA-RagC heterodimer in the primary sequence [32,38], but it is unknown if the Gtr heterodimer functions via the same mechanism of intersubunit communication as human Rag GTPases, or if they even have similar biochemical properties to the Rag GTPases. Therefore, we set out to answer these questions in the following study.

Materials and Methods

Purification of the Gtr heterodimers

The Gtr heterodimers were purified based on a previously described method [37]. In short, a pETDuet vector containing C-terminally His-tagged Gtr1p-Gtr2p heterodimer was transformed into *E. coli*, and plated on an LB agar plate containing 100 μg/mL ampicillin. A single colony was inoculated in 100 mL of LB with 100 μg/mL of ampicillin and incubated in a shaker overnight. The culture was diluted 1:100 into flasks containing 2 L of LB with 100 μg/mL ampicillin. When the culture reached an OD_{600} of 0.4–0.5, the temperature was reduced to 18°C. Protein expression was induced with 0.5 mM IPTG overnight.

Cells were centrifuged at 5,000 × *g* for 15 minutes, and the cell pellets were resuspended in a buffer containing 50 mM NaHEPES (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 0.5 mM TCEP, 0.5 mM PMSF, 0.05% Triton, 0.1 mM ATP, 0.1 mM GDP, and 1:100 Protease Inhibitor Cocktail. They were then incubated with DNase and lysozyme for 30 minutes before passing through a microfluidizer at 18,000psi three times. The lysate was cleared by spinning at 40,000 × *g* for 40 minutes, and the cleared lysate was incubated with Ni-NTA resin for an hour. The resin was packed and washed before the protein was eluted off of the beads.

The protein was concentrated down and ultracentrifuged at 100,000 × *g* for 30 minutes. The protein was then loaded on an FPLC and purified using a monoQ column (Cytiva). The eluate from the monoQ was mixed with EDTA to a final concentration of 20 mM to strip nucleotides off of the Gtr heterodimer, and the mixture was incubated at room temperature for 1 hour. The proteins were then concentrated and loaded on a Superose 6 column (Cytiva). The purified proteins were then concentrated, mixed with glycerol to ~10%, and flash frozen in liquid nitrogen.

Nucleotide Binding Assay

An aliquot of Gtr heterodimer was thawed on ice and then ultracentrifuged at 200,000 × *g* for 30 minutes to remove aggregated protein. The concentration was taken using Bradford assay (Bio-Rad). Reactions were carried out in Assay Buffer (50 mM NaHEPES, pH 7.4; 100 mM KOAc; 2 mM $MgCl_2$; 2 mM DTT; 0.1% CHAPS). A total of 5 nM α-^{32}P-GTP (Perkin Elmer) and 5 nM–5 μM Gtr heterodimer were incubated for 4 hours on ice. The mixture was transferred to a chilled metal block covered in parafilm. The reaction was exposed to 0.3 Joule of 254 nm wavelength UV light to induce crosslinking (Spectronics). The reaction was then mixed with SDS loading dye, boiled, and run on a 12% Tris-glycine gel. The gel was fixed for 15 minutes and dried for 1 hour in a gel dryer (Bio-Rad). The dried gel was exposed to a phosphorimaging screen, which was then imaged and the band intensities were quantified.

Nucleotide Loading Assay

Wild-type Gtr1p(S20N)-Gtr2p and Gtr1p(S20N)-Gtr2p (F167W) were thawed on ice and ultracentrifuged at 200,000 × *g* for 30 minutes prior to use. Reactions were carried out in Assay Buffer, where 300 nM Gtr heterodimer was incubated with 5 nM α-^{32}P-GTP in a final volume of 200 μl at 4°C for an hour. A total of 30 μl of Ni-NTA beads were then added to the mixture and incubated at 4°C for an hour. The beads were washed three times with Assay Buffer supplemented with 300 mM NaCl and then resuspended in Ultra Gold scintillation counting solution (Perkin Elmer). The bound α-^{32}P-GTP was quantified using an LS6500 scintillation counter (Beckman Coulter).

Results

Wild-type Gtr2p fails to crosslink with nucleotides

We attempted to determine the nucleotide-binding affinity of Gtr1p-Gtr2p using a previously established method for human Rag GTPases. First, we utilized an *E. coli*-based expression system to generate the Gtr heterodimers from *S. cerevisiae* and *S. pombe* in high purity for assays (Figure 1a, see Material and Methods for details). These heterodimers were incubated with a trace amount of radioactively-labelled nucleotides, and after 4 hours they were crosslinked using 254 nm UV light. The crosslinked products were then resolved by SDS-PAGE and visualized using autoradiography to quantify the amount of nucleotide bound to each subunit (Figure 1b).

When we carried out this experiment with wild-type *S. cerevisiae* (*Sc*) and *S. pombe* (*Sp*) Gtr heterodimers, we observed the crosslinked product corresponding to Gtr1p bound to GTP (Figure 1b, left panels) and GDP (Figure 1b, right panels), but we were unable to observe that of Gtr2p, even at a very high protein concentration (2 μM). By quantifying the band intensities for Gtr1p bound to GTP or GDP at different concentrations, we were able to fit the data to a single-site binding equation (Figure 1 c and d) and determine the dissociation constants (K_d; summarized in Table 1). We found that the K_d for GTP was ~14 times tighter than that for GDP in *Sc*Gtr heterodimers and ~15 times in *Sp*Gtr heterodimers. This contrasts with human Rag GTPases, which have comparable binding affinities for GDP and GTP (Table 1).

To reduce the background and further corroborate this result, we harnessed a point mutation that specifically abrogates GTP-binding capacity in P-loop GTPases. This mutation retains GDP-binding capacity and has been widely used in the case of Ras GTPase (S17N) [39] and human Rag GTPases [RagA(T21N)

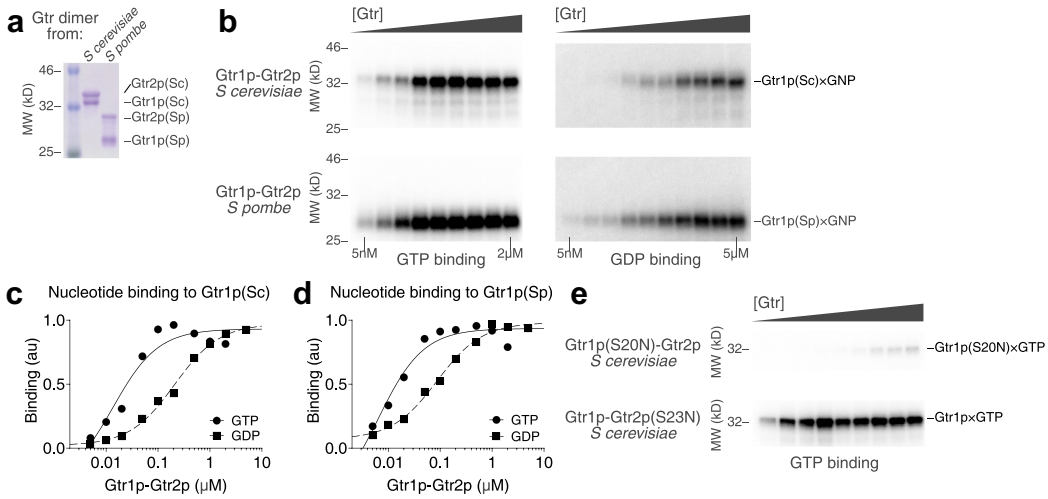

Figure 1. Wild-type Gtr2p is incapable of crosslinking to nucleotides. (a) A Coomassie stained gel showing the purified Gtr heterodimers from *S. cerevisiae* and *S. pombe*. (b) Crosslinking assays probing the binding of GTP (left panels) or GDP (right panels) to wild-type Gtr heterodimers from *S. cerevisiae* and *S. pombe*. Gtr1p is capable of being resolved in all cases, but not Gtr2p. (c) Quantification of GTP (circle) and GDP (square) binding to *S. cerevisiae* Gtr1p, fit to a single-site binding equation. (d) Quantification of GTP (circle) and GDP (square) binding to *S. pombe* Gtr1p, fit to a single-site binding equation. (e) Crosslinking assays probing the binding of GTP to GTP-binding deficient Gtr mutants, Gtr1p(S20N)-Gtr2p and Gtr1p-Gtr2p(S23N). When Gtr1p is the only subunit capable of binding GTP, we can resolve the crosslinked bands. When Gtr2p is the only subunit capable of binding GTP, no obvious crosslinked bands are observed.

Table 1. Summary of nucleotide-binding constants (K_d) at 4°C.

	Gtr1p/RagA		Gtr2p/RagC	
K_d (nM)	GTP	GDP	GTP	GDP
*Sc*Gtr1p-Gtr2p	14 ± 4	210 ± 38	-	-
*Sp*Gtr1p-Gtr2p	6.4 ± 1	87 ± 9	-	-
*Sc*Gtr1p-Gtr2p$_{R}$	26 ± 8	212 ± 22	8 ± 1	105 ± 15
*Sc*Gtr1p-Gtr2p$_{R}$(S23N)	33 ± 15	155 ± 18	-	115 ± 16
*Sc*Gtr1p(S20N)-Gtr2p$_{R}$	-	215 ± 20	181 ± 10	205 ± 29
*Hs*RagA-RagC[†]	47	76	11	41
*Hs*RagA-RagC(S75N)[†]	44	34	-	48
*Hs*RagA(T21N)-RagC[†]	-	84	17	39

[†]The data for human RagA-RagC were taken from Shen et al., Mol Cell, 2017.

and RagC(S75N)] [31]. When we introduced a similar mutation in the *Sc*Gtr heterodimers, S20N on *Sc*Gtr1p or S23N on *Sc*Gtr2p, we expected to specifically disrupt GTP binding to the corresponding subunit so that we can unequivocally distinguish nucleotide binding to the other subunit. Indeed, disrupting the binding between Gtr1p and GTP left us with an almost blank gel (Figure 1e, top panel), suggesting Gtr2p fails to crosslink with nucleotides. As a control, disrupting Gtr2p does not affect the crosslinking of nucleotides with Gtr1p (Figure 1e, bottom panel). These results further suggest that the current crosslinking assay is unable to detect GTP bound to wild-type Gtr2p.

Establishing a Gtr2p mutant capable of nucleotide crosslinking

Two possibilities could explain the failure of Gtr2p to crosslink with nucleotides. First, Gtr2p could lack nucleotide-binding capacity. Second, Gtr2p can bind to nucleotides, but lacks the structural element that confers crosslinking. As previous crystal structures of Gtr heterodimer have clearly captured the bound nucleotide [40–42], the second possibility is more likely. To probe the structural element that confers crosslinking, we examined a high-resolution crystal structure of human RagC bound to GppNHp, a non-hydrolysable GTP analogue (PDB: 3LLU). We observed a tyrosine residue localizing near the nucleotide-binding pocket of RagC, right next to the guanine moiety of the bound GppNHp (Figure 2a). This tyrosine residue corresponds to a conserved tryptophan residue in Gtr1p and in human RagA/B (Figure 2b, top). However, in Gtr2p, a phenylalanine residue occupies this position (Figure 2b, bottom). As both tryptophan and tyrosine have strong UV absorptions, but phenylalanine has weaker absorption, this difference in amino acid identity could be responsible for the loss of crosslinking between Gtr2p and the bound nucleotide.

To test if the phenylalanine residue is the missing factor to crosslinking with the nucleotides, we changed it to either a tyrosine or tryptophan residue. Here, when we disrupted GTP binding to *Sc*Gtr1p using the S20N mutation (Figure 2c, bottom panels), we clearly resolved a new crosslinking product at a higher molecular weight, which sharply contrasts the wild-type case scenario (cf. Figure 1e). The band intensity is dependent on the concentration of *Sc*Gtr protein. Accordingly, we assigned this band to the crosslinked product between Gtr2p and the bound nucleotide. As both mutant proteins were capable of crosslinking with guanine nucleotides, we chose the F167W mutant in the assays below (denoted as $Gtr_{\mathbf{R}}$, R: restoration of crosslinking).

To test the functional equivalence between wild-type Gtr2p and Gtr2p(F167W), we designed a nucleotide loading assay (Figure 2d), in which we first incubated equal amounts of Gtr1p(S20N)-Gtr2p or Gtr1p(S20N)-Gtr2p(F167W) with radiolabeled GTP. As Gtr1p contains the S20N mutation, GTP would not bind to it, so the only subunit capable of binding GTP is Gtr2p. After the nucleotide was loaded, we pulled down the protein using Ni-NTA beads, and washed the beads extensively with buffer. The amount of radioactively labelled GTP that remained bound to the GTPase was then measured using a scintillation counter. We found that Gtr1p(S20N)-Gtr2p and Gtr1p(S20N)-Gtr2p(F167W) bind to similar amounts of GTP, suggesting the F167W mutation does not impair the nucleotide-binding capacity of Gtr2p (Figure 2e). Importantly, this binding can be readily chased away by unlabelled GTP, suggesting the specificity of GTP binding. These data validated that the Gtr2p(F167W) mutant is able to bind and release GTP to the same extent as the wild-type Gtr2p.

With this newly designed construct in hand, we were able to measure nucleotide binding to the two subunits within the *Sc*Gtr heterodimer simultaneously (Figure 2f). We clearly visualized the two bands that correspond to Gtr1p and Gtr2p crosslinked with GTP or GDP (Figure 2f, top row), and we were able to fit the band intensity to a binding equation to extract the K_d values (Figure 2 g and j, summarized in Table 1). The measured K_d for Gtr1p is similar to that of wild-type Gtr1p-Gtr2p, and we found that Gtr2p behaves similarly to Gtr1p, with a strong preference towards GTP over GDP (Table 1). As a side note, we noticed that the band intensity for Gtr1p crosslinked with GTP is much higher than that for Gtr2p crosslinked with GTP (Figure 2f, top left panel). This is likely due to the local conformation near the nucleotide-binding pocket of Gtr1p that better posits the nucleotide so that the crosslinking efficiency is higher. However, as we mostly rely on the relative intensity across different protein concentrations, the measured binding affinity is independent of the absolute band intensity [35,37].

We then proceeded to measure the nucleotide-binding affinity of the GTP-binding defective mutants, namely *Sc*Gtr1p(S20N)-Gtr2p and *Sc*Gtr1p-Gtr2p(S23N) (Figure 2f, bottom two rows). In human Rag GTPases, we have previously shown that mutation on one subunit does not change the GTP-binding affinity of the other (cf. Table 1, *Hs*RagA-RagC) [35]. Consistent with this notion, when we tested the Gtr1p-Gtr2p(S23N) mutant, we observed that the S23N mutation on Gtr2p does not alter the nucleotide-binding

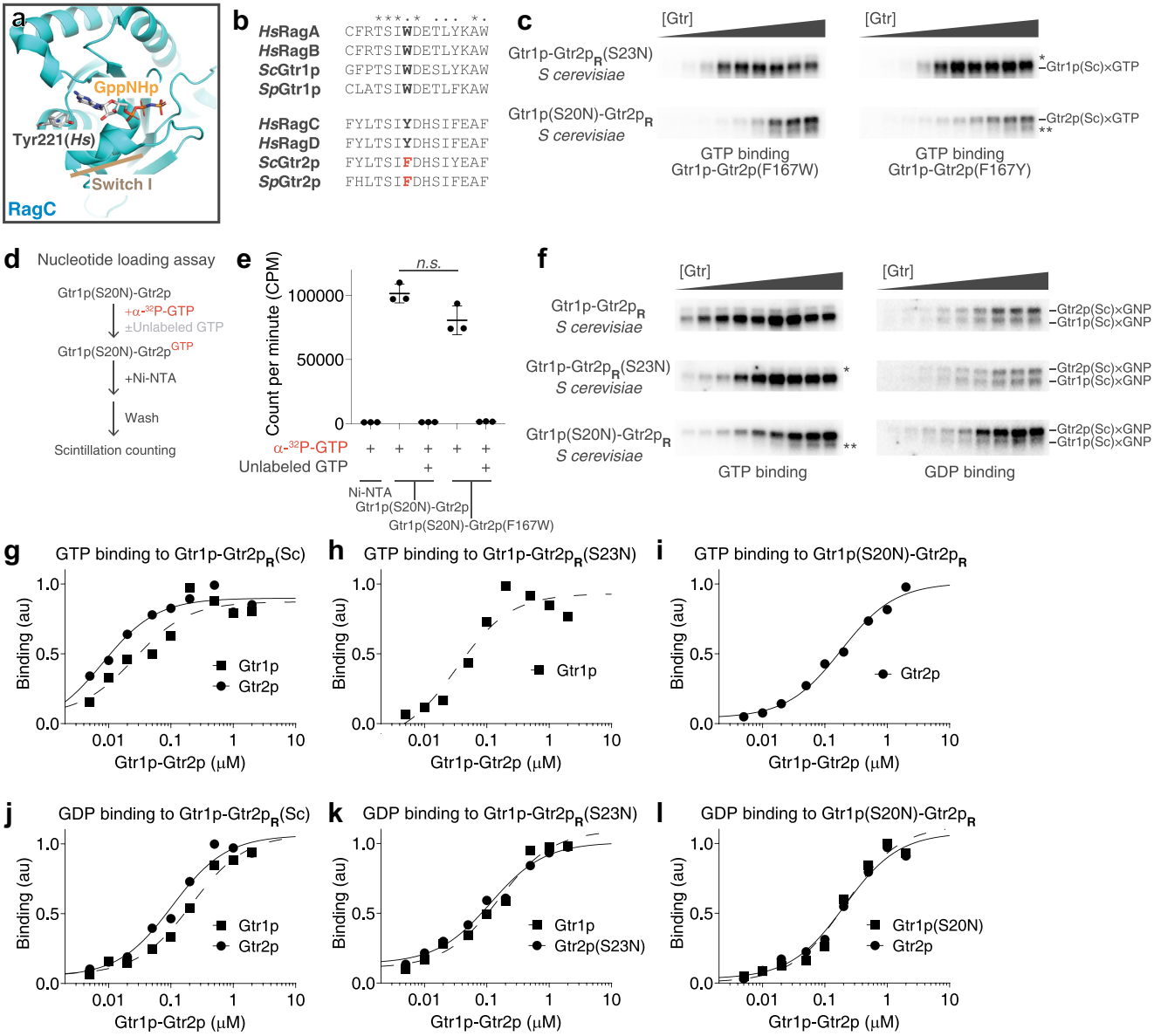

Figure 2. A mutation on Gtr2p restores the nucleotide crosslinking capabilities. (a) Crystal structure of the nucleotide-binding domain of human RagC (3LLU) reveals a tyrosine residue (Tyr221) in close proximity to the bound nucleotides. (b) Sequence conservation analysis of the Tyr221 residue on human RagC. A conserved aromatic residue occupies this position in Rag/Gtr GTPases. (c) GTP-binding assays using Gtr heterodimers carrying F167W (left panels) or F167Y (right panels) mutation on Gtr2p. The crosslinked bands corresponding to Gtr2p bound to GTP can be clearly resolved here. (d) Reaction scheme for the GTP loading experiment. (e) Quantification of the amounts of radioactively labelled GTP that remain bound to the GTPase by scintillation counting. Ni-NTA column is the blank control without any Gtr1p-Gtr2p. Columns 2 and 4: radioactively labelled GTP loading onto Gtr1p(S20N)-Gtr2p and Gtr1p(S20N)-Gtr2p(F167W) heterodimer, respectively. No significant change was observed. Columns 3 and 5: radioactively labelled GTP loading onto Gtr1p(S20N)-Gtr2p and Gtr1p(S20N)-Gtr2p(F167W) heterodimer in the presence of unlabelled GTP as a competitor. No radioactively labelled GTP was detected, suggesting the specificity of the binding event. (f) Nucleotide binding to Gtr1p-Gtr2p$_R$. *S. cerevisiae* wild-type, Gtr1p(S20N)-Gtr2p, and Gtr1p-Gtr2p(S23N) heterodimers carrying F167W mutation were used. (g-l) Quantifications of the nucleotide-binding assays shown in panel D. The data were fit to a single-site binding equation.

affinities on Gtr1p (Figure 2h, k, and Table 1). However, for Gtr1p(S20N)-Gtr2p, we observed that the binding affinity for GTP to Gtr2p is weakened by ~20-fold, and the binding affinity for GDP is weakened by ~2-fold (Figure 2i, l, and Table 1). This suggests that the Gtr heterodimer may operate under a different mechanism of intersubunit communication from the Rag GTPases, as the symmetry between the two

subunits is broken. Rather, strong directionality from Gtr1p to Gtr2p may dominate the behaviour of the Gtr heterodimer.

Discussion

As a critical mediator for the amino acid signals, the Rag/Gtr GTPase heterodimer occupies a central position in the mTOR pathway. Here, we present an updated approach to studying the biochemical properties of the Gtr heterodimer. We identified the structural element lacking in wild-type Gtr2p that confers cross-linking to the bound guanine nucleotide and restored it by sequence homology-based mutation. We are then able to explore the biochemical underpinnings of the metabolic network in yeast.

The Rag/Gtr GTPase heterodimer is a conserved GTPase unit that interacts and co-evolves with mTOR. In human cells, upstream regulators and amino acid sensors manipulate the nucleotide loading state of the Rag GTPases based on the amino acid concentration. The Rag GTPases then determine the subcellular localization of mTORC1 and thus control its activity. In *S. cerevisiae*, however, such a flow of information is not conserved, as TORC1 localization does not depend on the amino acid or nitrogen concentration [43], suggesting a distinctive molecular mechanism. Here, using the newly designed *Sc*Gtr construct, we found that, in a similar manner to how the human Rag GTPases operate, the *Sc*Gtr heterodimer utilizes extensive intersubunit communication, as a mutation in one subunit affects the biochemical properties of the other. However, this communication seems directional, from Gtr1p to Gtr2p, which sharply contrasts human Rag GTPases, as whichever Rag subunit bound to GTP is the dominant subunit and dictates the behaviour of the entire heterodimer.

What causes the difference between yeast Gtr heterodimer and human Rag GTPases? We proposed the following hypotheses. First, based on the primary sequence, the Switch I region of Gtr1p/RagA is highly conserved evolutionarily, while that of Gtr2p/RagC is less so [29,30]. This structural difference may alter the information communicated in between the two subunits. Second, as different species face various nutrient resources, their regulators and amino acid sensors upstream of the Rag/Gtr GTPases are different. For example, human GATOR1 and GATOR2 are separate complexes, while in yeast, they fuse into the SEACAT-SEACIT complex [44]. This may require the Rag/Gtr heterodimers to process signals of different origins, and therefore they may operate under a distinct molecular mechanism. Third, the activation process of TORC1 in yeast is different from that in human, as the loss of human Rheb homolog, Rhb1, has no effect on TORC1 activity in yeast [45,46]. Therefore, the Gtr heterodimer may have additional functional roles in regulating TORC1 activity, which may require a directional flow of information from Gtr1p to Gtr2p. Our new approach and results suggest the possibility and necessity to further explore the biochemical properties of the Gtr heterodimer in order to uncover its unique regulatory role in TORC1 signalling in yeast.

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Author contributions

D.D.D. and K.S. conceptualized the project. D.D.D. and K. V. performed the experiments and analysed the data. D.D. D. and K.S. wrote and edited the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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