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Short Article

Leucyl-tRNA Synthetase Controls TORC1 via the EGO Complex

Grégory Bonfils,[1] Malika Jaquenoud,[1] Séverine Bontron,[1] Clemens Ostrowicz,[2,3] Christian Ungermann,[2] and Claudio De Virgilio[1,*]
[1]Department of Biology, Division of Biochemistry, University of Fribourg, CH-1700 Fribourg, Switzerland
[2]Department of Biology and Chemistry, Biochemistry Section, University of Osnabrück, 49076 Osnabrück, Germany
[3]Present address: Helmholtz Centre for Infection Research, D-38124 Braunschweig, Germany
*Correspondence: claudio.devirgilio@unifr.ch
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SUMMARY

The target of rapamycin complex 1 (TORC1) is an essential regulator of eukaryotic cell growth that responds to growth factors, energy levels, and amino acids. The mechanisms through which the preeminent amino acid leucine signals to the TORC1-regulatory Rag GTPases, which activate TORC1 within the yeast EGO complex (EGOC) or the structurally related mammalian Rag-Ragulator complex, remain elusive. We find that the leucyl-tRNA synthetase (LeuRS) Cdc60 interacts with the Rag GTPase Gtr1 of the EGOC in a leucine-dependent manner. This interaction is necessary and sufficient to mediate leucine signaling to TORC1 and is disrupted by the engagement of Cdc60 in editing mischarged $tRNA^{Leu}$. Thus, the EGOC-TORC1 signaling module samples, via the LeuRS-intrinsic editing domain, the fidelity of $tRNA^{Leu}$ aminoacylation as a proxy for leucine availability.

INTRODUCTION

The structurally and functionally conserved target of rapamycin complex 1 (TORC1) plays a central role in the control of eukaryotic cell growth by promoting anabolic processes (e.g., protein synthesis) and inhibiting catabolic processes (e.g., autophagy) in response to a variety of signals, including hormones, growth factors, energy levels, and amino acids such as leucine (Avruch et al., 2009). The mechanism through which leucine signals to TORC1 relies on the highly conserved Gtr1/RagA/B and Gtr2/RagC/D Rag GTPases, which function in heterodimeric complexes containing one Gtr1-like and one Gtr2-like GTPase that are tethered, via the Ego1/3 subunits of the yeast EGO complex (EGOC) or the structurally related mammalian Ragulator complex, to the vacuolar or lysosomal membranes, respectively (Binda et al., 2009; Dubouloz et al., 2005; Kim et al., 2008; Kogan et al., 2010; Sancak et al., 2010; Sancak et al., 2008). Via unknown mechanisms, leucine promotes the GTP-bound state of Gtr1-like GTPases, which combine with GDP-bound Gtr2-like GTPases to interact with and activate TORC1 (Binda et al., 2009; Kim et al., 2008; Sancak et al., 2008). In mammalian cells, this involves Rag-Ragulator complex-dependent positioning of TORC1 in proximity to Rheb (Sancak et al., 2010; Sancak et al., 2008). How leucine signals to Gtr1/RagA/B to control TORC1, however, remains mysterious.

Here, we show that the leucyl-tRNA synthetase (LeuRS) Cdc60 interacts with the Rag GTPase Gtr1 of the EGOC in a leucine-dependent manner. This interaction, which is necessary and sufficient for TORC1 activation by leucine, is mediated by the nonessential amino acid-editing domain and does not require the functionally independent aminoacylation activity of Cdc60. We propose a simple model in which the conformational change adopted by the Cdc60 editing domain, which results from its engagement in editing mischarged $tRNA^{Leu}$, disrupts the Cdc60-Gtr1 interaction and consequently causes downregulation of TORC1.

RESULTS AND DISCUSSION

LeuRS Cdc60 Physically Interacts with the TORC1 Regulator Gtr1 in a Leucine-Dependent Manner

Based on both the observation that leucine is one of the most potent TORC1 activators (Avruch et al., 2009) and the assumption that proteins involved in signaling amino acid availability are likely to interact with Gtr1 in an amino acid-dependent manner, we purified Gtr1-TAP from yeast cells prior to and following leucine starvation and determined the coprecipitating proteins by mass spectrometry (MS). Remarkably, besides various proteins involved in fatty acid synthesis (e.g., Fas1, Faa4, and Acc1), we identified the LeuRS Cdc60 among the most prospective leucine-dependent, Gtr1-interacting candidate proteins (Table S1 available online). This finding, which we independently confirmed in coprecipitation assays using an endogenously tagged version of Cdc60 (Figures 1A, 1B, and S1), suggests that the LeuRS Cdc60 may play a role in signaling leucine availability to Gtr1-TORC1. Notably, eukaryotic LeuRSs exhibit two functionally separate activities, namely an essential $tRNA^{Leu}$ aminoacylation activity and an amino acid proofreading (editing) activity that involves recognition and hydrolysis of misacetylated $tRNA^{Leu}$ molecules (Ling et al., 2009). To study whether LeuRS-mediated aminoacylation impinges on TORC1, we first used a temperature-sensitive (ts) *cdc60*^{ts} strain (Figure 1C) that is defective in $tRNA^{Leu}$ aminoacylation and, therefore, accumulates uncharged $tRNA^{Leu}$ at the restrictive temperature (Hohmann and Thevelein, 1992). In control

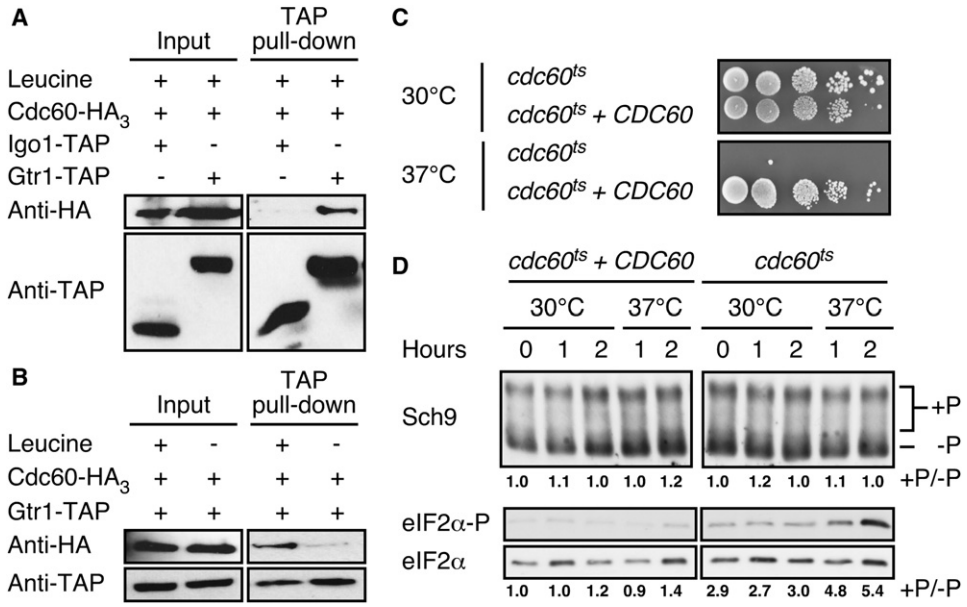

Figure 1. LeuRS Cdc60 Physically Interacts with the TORC1 Regulator Gtr1 in a Leucine-Dependent Manner, but Cdc60-Mediated tRNA^{Leu} Aminoacylation Is Not Required for Normal TORC1 Activity

(A and B) Gtr1-TAP or the control protein Igo1-TAP (A) was precipitated from extracts of Cdc60-HA$_3$-expressing cells. Cells were grown to exponential growth phase and harvested either prior to (+) or following a 60 min period of leucine starvation (−). Cell lysates (Input) and TAP pull-down fractions were subjected to SDS-PAGE, and immunoblots were probed with anti-HA or anti-protein A (anti-TAP) antibodies as indicated. See also Figure S1.

(C) Expression of *CDC60* rescues the temperature-sensitivity at 37°C of a *cdc60^{ts}* strain. Serial 10-fold dilutions of cells were spotted on YPD plates.

(D). Immunoblots detecting the extent of Sch9 phosphorylation were used to quantify in vivo TORC1 activity (Urban et al., 2007) in exponentially growing WT (*cdc60^{ts}* harboring a plasmid expressing *CDC60*) and *cdc60^{ts}* strains that were grown at the indicated temperatures (top); numbers below the blot refer to the mean ratio of hyperphosphorylated [+P]/hypophosphorylated [-P] Sch9 from three independent experiments, normalized to the values at time 0). Levels of eIF2α phosphorylation (on Ser^{51}) were used as a proxy for the accumulation of uncharged tRNA^{Leu} (Hinnebusch, 2005) (lower panels; numbers below the blot refer to the mean ratio of phosphorylated eIF2α-P/unphosphorylated eIF2α from three independent experiments).

experiments, phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α) at Ser^{51}—a sensitive indicator of the presence of uncharged tRNAs (tRNA^{Leu}) that stimulate the eIF2α-kinase Gcn2 (Hinnebusch, 2005)—strongly increased in *cdc60^{ts}* but not in wild-type (WT) cells when incubated for 1 or 2 hr at 37°C (Figure 1D). Under the same conditions, however, temperature-inactivation of Cdc60^{ts} had no significant impact on TORC1 activity, as assessed by monitoring the phosphorylation level of the TORC1 substrate Sch9 (Figure 1D) (Urban et al., 2007). These observations are consistent with similar experiments in Chinese hamster ovary cells (Wang et al., 2008), indicating that LeuRS-mediated aminoacylation, uncharged tRNAs, and Gcn2 kinase activation do not impinge on TORC1 regulation.

Trapping of tRNA^{Leu} within the LeuRS Editing Site Downregulates EGOC-TORC1 Signaling

To study whether the editing function of LeuRS may be implicated in TORC1 control, we used 1,3-dihydro-1-hydroxy-2,1-benzoxaborole (DHBB), an analog of the antifungal compound 5-fluoro-DHBB (aka AN2690), which inhibits cell growth by trapping uncharged tRNA^{Leu} in the editing active site within the connective peptide 1 (CP1) domain of LeuRS (Figure 2A) (Rock et al., 2007). Surprisingly, DHBB treatment, which did not noticeably alter Gtr1-GFP and Tor1-GFP localization (Figures S2A and S2B), resulted in significant downregulation of TORC1 activity in WT cells, but not in cells expressing the DHBB-resistant Cdc60^{D418R} variant (Rock et al., 2007; Yao et al., 2008) (Figures 2 and 2B). In addition, coexpression of the Gtr1^{GTP} and Gtr2^{GDP} alleles, which are predicted to be restricted to a GTP- and GDP-bound conformation (Binda et al., 2009; Gao and Kaiser, 2006), respectively, almost entirely suppressed the DHBB-mediated TORC1 inactivation without affecting the corresponding accumulation of uncharged tRNA^{Leu}, the activation of Gcn2, or inhibition of growth in DHBB-treated cells (Figures, 2B, and 2C). Moreover, DHBB disrupted, in a concentration-dependent manner, the Gtr1-Cdc60 (and Gtr1^{GTP}-Cdc60; Figure S2C), but not the Gtr1-Cdc60^{D418R} interaction (Figures 2D and 2E).

To explore whether DHBB treatment affects the GTP-loading status of Gtr1, we made use of the fact that Gtr1^{GTP}-TAP, but not Gtr1^{GDP}-TAP, specifically coprecipitates with the TORC1 subunit Kog1 (Binda et al., 2009); hence, the level of Kog1-associated Gtr1 can be used to estimate the relative amount of Gtr1^{GTP} within cells. Using this assay, we found that DHBB treatment, like leucine starvation, severely reduced the interaction between Gtr1 and Kog1 (Figure 2F). Together, these data evoke a simple model in which the conformational change adopted by the CP1 domain in Cdc60, which results from its engagement in editing mischarged tRNA^{Leu} (Tukalo et al., 2005) or from binding the DHBB-tRNA^{Leu} adduct

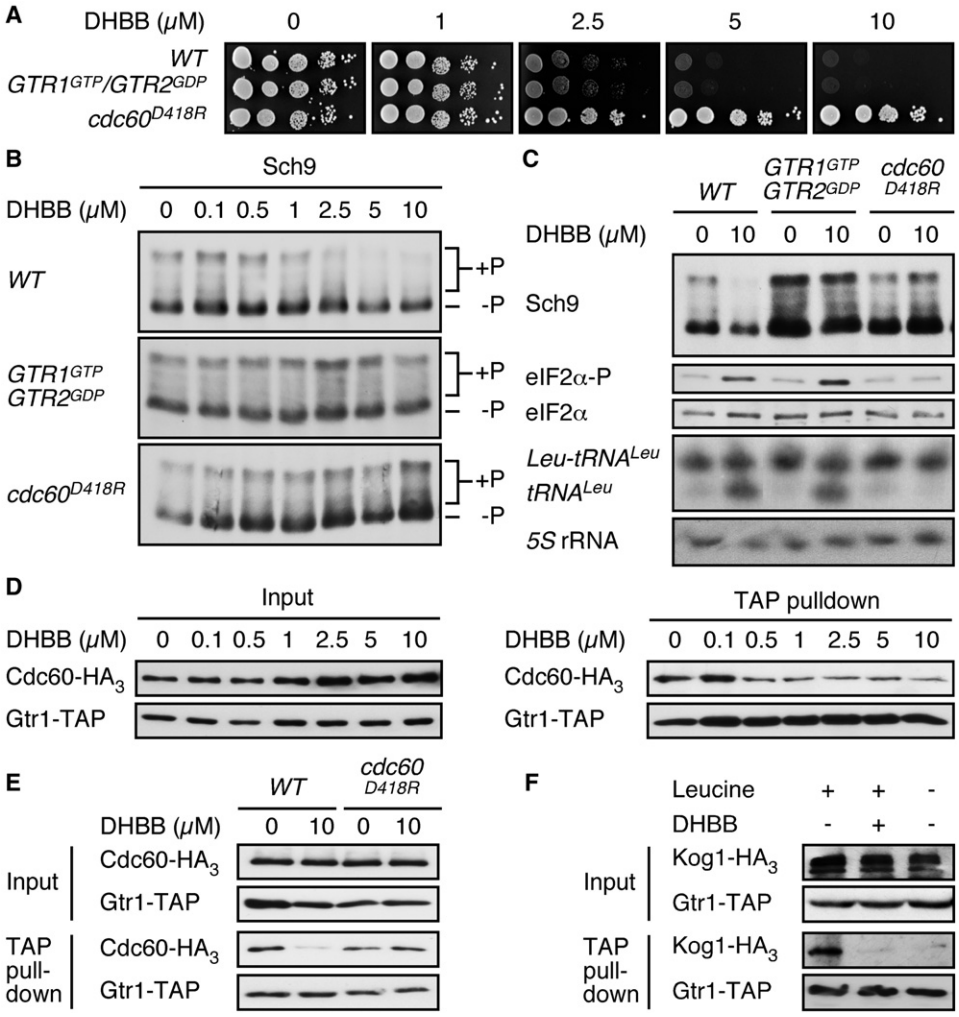

Figure 2. DHBB-Mediated Trapping of tRNA^{leu} within the LeuRS Editing Site Downregulates EGOC-TORC1 Signaling

(A) DHBB treatment inhibits growth of WT and Gtr1^{GTP}/Gtr2^{GDP}-expressing cells, but not of cells expressing the DHBB-resistant Cdc60^{D418R} variant. Serial 10-fold dilutions of cells were spotted on SD plates containing the indicated concentrations of DHBB.

(B and C) Expression of Gtr1^{GTP}/Gtr2^{GDP} prevents DHBB-induced inactivation of TORC1 (B), but not the accumulation of uncharged tRNA^{Leu} and consequent phosphorylation of eIF2α (C). Expression of Cdc60^{D418R} prevents DHBB-induced inactivation of TORC1 (B), as well as accumulation of uncharged tRNA^{Leu} and eIF2α phosphorylation (C). 5S rRNA served as loading control. DHHB treatments were done for 30 min in each case.

(D and E) DHBB treatment (30 min) disrupts the Cdc60-Gtr1 interaction (D), but not the Cdc60^{D418R}-Gtr1 interaction (E), in a concentration-dependent manner.

(F) Gtr1-Kog1 interaction is sensitive to both DHBB treatment and leucine starvation. Cells expressing Gtr1-TAP and Kog1-HA$_3$ were harvested either in exponential growth phase or following a 30 min period of DHBB treatment or leucine starvation. See also Figure S2.

(Rock et al., 2007), disrupts the Cdc60-Gtr1 interaction and consequently causes GTP hydrolysis within Gtr1 and downregulation of TORC1. Notably, a catalytically defective *cdc60^{D419A}* editing mutant responds normally to leucine starvation in terms of TORC1 inactivation (Yao et al., 2008; Figure S3A), indicating that the structural rearrangement of the CP1 domain, rather than the ensuing hydrolysis of mischarged tRNAs, primarily signals to EGOC-TORC1. Interestingly, the corresponding conformational change of the CP1 domain appears to depend on prior tRNA^{Leu} aminoacylation/misacetylation because temperature-inactivation of Cdc60^{ts} significantly protects TORC1 from leucine starvation-induced downregulation (Figure S3B).

The LeuRS Inhibitors Leucinol and Norvaline Oppositely Affect EGOC-TORC1 Signaling

To further substantiate our model, we made use of two leucine analogs, namely leucinol (LeuOH) and norvaline (Nva), which both competitively inhibit LeuRS (and therefore growth) in different ways. LeuOH cannot be charged onto tRNA^{Leu} (Rouget and Chapeville, 1968) and it blocks LeuRS-mediated aminoacylation (and growth; Figures 3A and 3B), thus impeding LeuRS from engaging in editing activities. In contrast, Nva is both charged and edited by LeuRS (Ataide and Ibba, 2006; Chen et al., 2011) and, as a result, sustains a futile cycle of charging and editing, limiting growth at higher Nva concentrations (Figure 3B). Consistent with our model and its mode of action toward

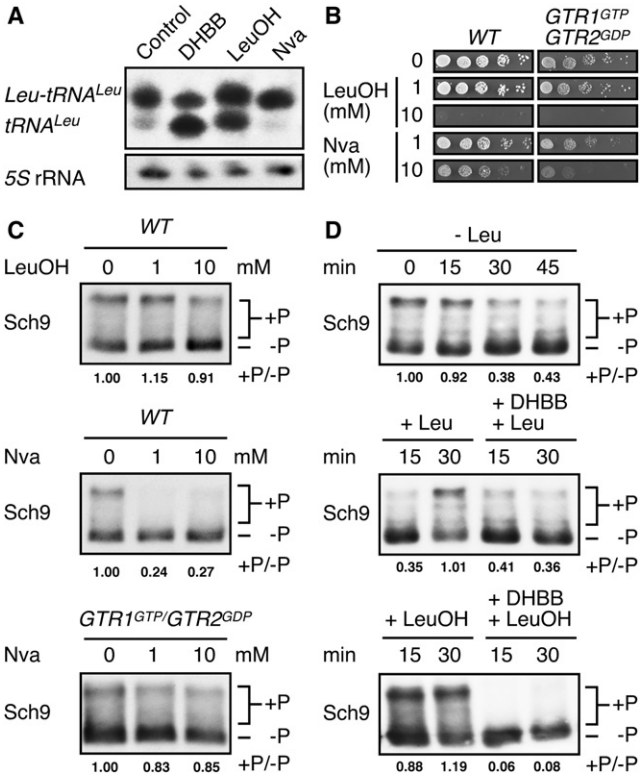

Figure 3. LeuRS Inhibitors Leucinol (LeuOH) and Norvaline (Nva) Dampen and Stimulate LeuRS Editing, Respectively, and Oppositely Affect EGOC-TORC1 Signaling

(A) The levels of charged and uncharged $tRNA^{Leu}$ were assayed in WT cells treated for 30 min with the indicated LeuRS inhibitors (DHBB [10 μM], LeuOH [10 mM], and Nva [10 mM]) or vehicle alone (control). 5S rRNA served as loading control.

(B) Both LeuOH and Nva inhibit growth of WT and $Gtr1^{GTP}/Gtr2^{GDP}$-expressing cells. Serial 10-fold dilutions of cells were spotted on SD plates containing the indicated concentrations of LeuRS inhibitors.

(C) Unlike LeuOH treatment (30 min), Nva treatment inactivates TORC1, which is significantly suppressed by $Gtr1^{GTP}/Gtr2^{GDP}$ expression.

(D) Leucine- (Leu) and LeuOH-mediated TORC1 activation in leucine-starved cells is abolished by prior (i.e., 30 min) addition of 10 μM DHBB. Leucine and LeuOH were added to final concentrations of 2.8 mM and 10 mM, respectively. For TORC1 quantifications (bold numbers below the blots in [C] and [D]), see legend of Figure 1D.

LeuRS, LeuOH did not cause TORC1 downregulation (Figure 3C). Instead, and in line with similar observations in *Xenopus laevis* oocytes (Christie et al., 2002), LeuOH was equally competent as leucine in activating TORC1 in leucine-starved WT, but not in *gtr1Δ* cells (Figure 3D; Binda et al., 2009 and data not shown). As expected, neither LeuOH nor leucine was able to restimulate TORC1 in the presence of DHBB (Figure 3D). The results with Nva were equally clear: Nva potently inhibited TORC1 in WT cells (without increasing the levels of uncharged $tRNA^{Leu}$; Figure 3A) even when applied in concentrations that are subinhibitory for growth (Figures 3B and 3C). Importantly, Nva-mediated downregulation of TORC1, but not the observed growth inhibition at higher Nva concentrations, was significantly suppressed by expression of the $Gtr1^{GTP}/Gtr2^{GDP}$-encoding alleles (Figures 3B and 3C). Together with the observations that 1) the addition of a disproportionate quantity of isoleucine causes transient TORC1 inactivation in WT cells (Figures S3C) and 2) LeuRS editing is specifically required for growth under leucine-limiting conditions (Figure S3D), these data corroborate a model in which $tRNA^{Leu}$ mischarging following leucine deprivation represents a key signal that impinges on EGOC-TORC1 signaling.

Mutation of Ser^{414} to Phe within the CP1 Domain of Cdc60 Disrupts Its Interaction with Gtr1

Our model predicts that mutations within Cdc60, which prevent it from binding Gtr1, may uncouple LeuRS-signaling from LeuRS-$tRNA^{Leu}$ charging. Conceivably, corresponding Cdc60 variants may grant a yet elusive GTPase activating protein access to Gtr1, thus provoking downregulation of TORC1. Based on this reasoning, we tried to identify *cdc60* alleles that confer rapamycin-sensitive growth by employing a classical plasmid-shuffling technique with a plasmid library of PCR-mutagenized *CDC60* genes (Forsburg, 2001). This approach allowed us to isolate the $cdc60^{S414F}$ allele that, similar to *gtr1Δ*, caused no obvious growth defect per se, but rendered cells defective for growth in the presence of low doses of rapamycin (Figure 4A). Because the rapamycin-sensitivity of $cdc60^{S414F}$ cells could be suppressed by expression of the $Gtr1^{GTP}/Gtr2^{GDP}$-encoding alleles (Figure 4A), we then used two-hybrid and coimmunoprecipitation (coIP) analyses to verify our assumption that the $Cdc60^{S414F}$ variant may be defective in binding Gtr1. These experiments not only revealed that the CP1 editing domain within Cdc60 ($CP1^{Cdc60}$) specifically interacted with Gtr1 (and not with Gtr2; Figure 4B), but also that the specific Ser^{414} to Phe mutation within this domain abolished the $CP1^{Cdc60}$-Gtr1 interaction (Figures 4B and 4C). Finally, in agreement with a model in which Cdc60 protects Gtr1 from a negative regulator, overproduction of $CP1^{Cdc60}$, but not $CP1^{Cdc60\text{-}S414F}$, significantly protected TORC1 from inactivation during leucine starvation (Figure 4D).

In conclusion, LeuRS binds the TORC1-regulator Gtr1 via its CP1 editing domain, which is necessary and sufficient to mediate leucine signaling to TORC1. Notably, comprehensive analyses of amino acid composition in eukaryotic genomes revealed that leucine represents the most frequently used amino acid (Echols et al., 2002), which, together with the fact that the LeuRS Cdc60 represents the most abundant aminoacyl-tRNA synthetase (Ghaemmaghami et al., 2003), also provides a rationale for the preeminent effect of leucine in TORC1 regulation. Because TORC1 is deregulated in common cancers (Guertin and Sabatini, 2007), it will be interesting to study whether the recently discovered contribution of human LeuRS (LARS1) to growth of human lung cancer cells (Shin et al., 2008) may also implicate Rag-Ragulator-complex-TORC1 signaling.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Plasmids

Unless stated otherwise, prototrophic strains were pre-grown overnight in synthetic medium without amino acids (SD; 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% glucose). Before each experiment, cells were diluted to an OD_{600} of 0.2 in SD until they reached an OD_{600} of 0.8. For leucine deprivation experiments, strains that were specifically auxotrophic

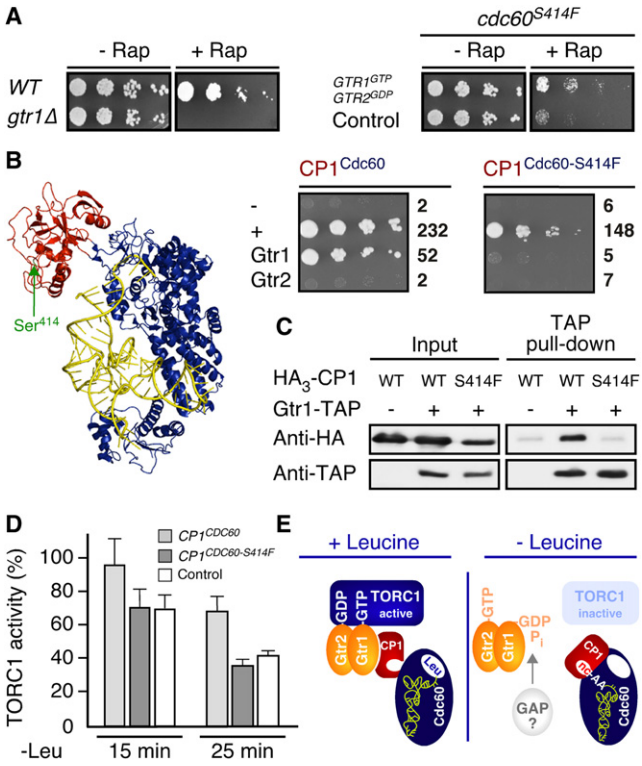

Figure 4. Mutation of Ser^{414} to Phe within the CP1 Domain of Cdc60 Disrupts Its Interaction with Gtr1

(A) Serial 10-fold dilutions of cells with the indicated genotypes were spotted and grown on either YDP control plates (-Rap) or plates containing low levels (5 ng ml^{-1}) of rapamycin (+Rap).

(B) CP1^{Cdc60}, but not CP1$^{Cdc60\text{-}S414F}$, specifically interacts with Gtr1 in a split-ubiquitin yeast two-hybrid assay. Interactions were tested by monitoring growth on plates lacking adenine or β-galactosidase activities (in Miller units; numbers on the right represent the mean of three independent experiments) of cells expressing Nub-Gtr1/2 and either CP1^{Cdc60}-Cub or CP1$^{Cdc60\text{-}S414F}$-Cub. pDL2-Alg5 and pAI-Alg5 vectors were used as negative (−) and positive (+) controls, respectively. Left, a ribbon model of the *P. horikoshii* LeuRS (dark blue; with the CP1 editing domain in red) in complex with tRNA^{Leu} (yellow) (Protein Data Bank, 1WZ2).

(C) HA$_3$-Cdc60^{CP1} (HA$_3$-CP1; WT) but not HA$_3$-Cdc60$^{CP1\text{-}S414F}$ (HA$_3$-CP1; S414F) coprecipitates with Gtr1-TAP. Cells were harvested in exponential growth phase, and pull-down experiments were carried out as in Figure 1B.

(D) Overexpression (from the *Tet$_{ON}$* promoter) of *CP1^{CDC60}*, but not *CP1$^{CDC60\text{-}S414F}$*, partially protects TORC1 (quantified as in Figure 1D) from inactivation during leucine starvation. Data are expressed as relative values with respect to the 0 time point and reported as averages (n = 3), with standard deviations indicated by the lines above each bar.

(E) Model for the role of LeuRS Cdc60-mediated editing in Gtr1-TORC1 signaling. Nc-AA, noncognate amino acid. For details see text. See also Figure S3.

for leucine were grown to an OD$_{600}$ of 0.8 on SD supplemented with leucine (0.37 mg ml^{-1}), washed twice, and resuspended in SD. The *S. cerevisiae* strains and plasmids used in this study are listed in Tables S2 and S3, respectively.

Extraction, Separation, and Analysis of Amino-Acylated tRNAs

Assessment of tRNA^{leu} charging was performed as described (Köhrer and Rajbhandary, 2008). Briefly, total RNA (from 10 OD$_{600}$ of cells) was extracted under acidic conditions (0.3 M NaOAc pH 4.5, 10 mM EDTA) in acetate-saturated phenol/chloroform. After quantification, 2 μg of total RNA were separated on a 6% denaturing acid/urea-acrylamide gel and, after transfer to a positively charged nylon membrane, were immobilized by UV-crosslinking. Hybridization was performed overnight at 42°C using P^{32}-labeled oligonucleotide probes that specifically bind tRNA^{Leu} (5′-GCATCTTACGATACCTG-3′) or 5S rRNA (5′-GGTCACCCACTACACTACTCGG-3′). The corresponding membranes were exposed at −80°C to X-ray films for autoradiography.

Tandem Affinity Purification (TAP) and IP Experiments

Using a standard TAP-tag purification protocol (Gelperin et al., 2005), Gtr1-TAP was purified from WT (YL515) cells harboring plasmid pMB1344-*GTR1-TAP*, which drives expression of Gtr1-TAP from its own promoter. Prior to protein extraction, cells were pre-grown on SD-URA and then washed and shifted for 30 min to SD-URA/-LEU. Gtr1-TAP preparations purified from cells prior and subsequent to leucine starvation were analyzed for coprecipitating partner proteins using MS/tandem MS analyses (Talarek et al., 2010). CoIP experiments were essentially done as described (Dubouloz et al., 2005; Loewith et al., 2002). Using IgG-coated sepharose beads, Gtr1-TAP and Igo1-TAP were purified from protein extracts that were prepared in lysis buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) containing protease inhibitor cocktail (Roche) and 0.5 mM PMSF. After cleavage by TEV protease, eluates were further purified on calmodulin beads in lysis buffer with 2 mM $CaCl_2$.

eIF2α and Sch9 Phosphorylation Analyses

For analysis of the phosphorylation status of eIF2α, cultures were mixed with TCA (final concentration 6%), put on ice for at least 5 min, pelleted, washed twice with cold acetone, and dried in a speed-vac. Cell lysis was done in 100 μl of urea buffer (50 mM Tris pH 7.5, 5 mM EDTA, 6 M urea, 1% SDS, 1 mM PMSF, and 0.5x PP$_i$) with glass beads in a bead beater, with subsequent heating for 10 min to 65°C. Equal amounts of total proteins from the different extracts were then resolved on 12% SDS-PAGE and subjected to immunoblotting using polyclonal antibodies specific for phosphorylated Ser^{51} in *S. cerevisiae* eIF2α (Invitrogen). The blots were then stripped and reprobed with polyclonal anti-eIF2α antibodies. To analyze Sch9^{T570A}-HA$_5$ C-terminal phosphorylation, we used the chemical fragmentation analysis as described previously (Urban et al., 2007; Wanke et al., 2008).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and three tables and can be found with this article online at doi:10.1016/j.molcel.2012.02.009.

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Supplemental Information

Leucyl-tRNA Synthetase Controls TORC1 via the EGO Complex

Grégory Bonfils, Malika Jaquenoud, Séverine Bontron, Clemens Ostrowicz, Christian Ungermann, and Claudio De Virgilio

Supplemental References

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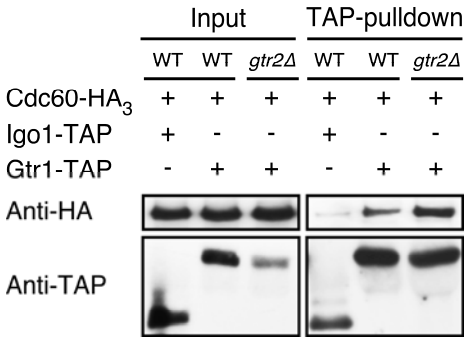

Figure S1. Cdc60-Gtr1 Interaction in the Absence of Gtr2, Related to Figure 1

Gtr1-TAP, or the control protein Igo1-TAP, was precipitated from extracts of Cdc60-HA_3-expressing wild-type (WT) or *gtr2*Δ cells that were grown to and harvested in exponential growth phase. Cell lysates (Input) and TAP pull-down fractions were subjected to SDS-PAGE and immunoblots were probed with anti-HA or anti-protein A (anti-TAP) antibodies as indicated.

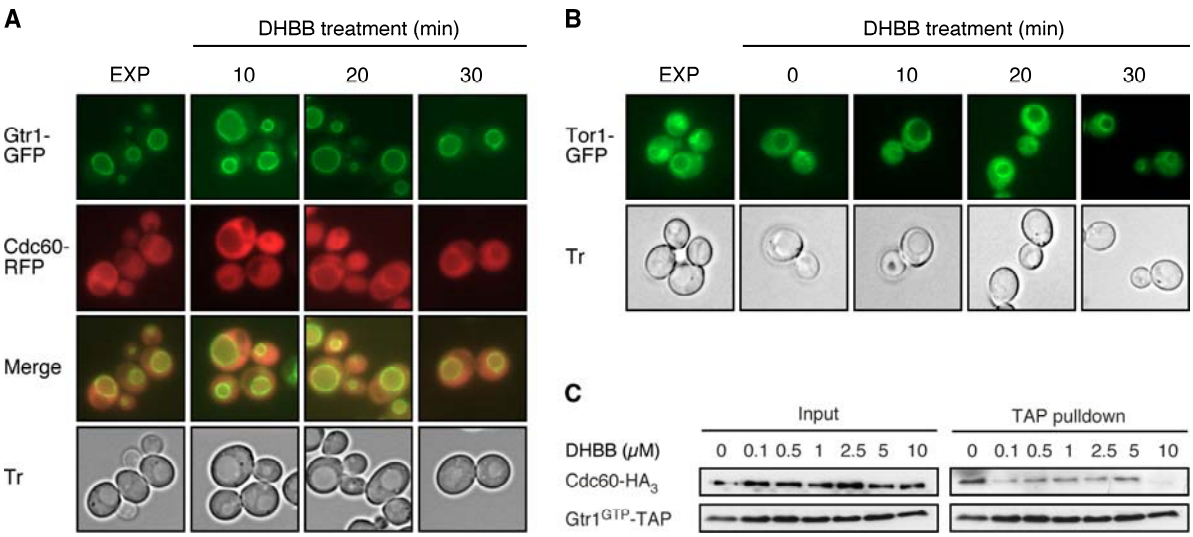

Figure S2. Additional Effects of DHBB Treatment, Related to Figure 2

(A and B) DHBB treatment does not affect the localization of Gtr1-GFP (A) or Tor1-GFP (B). Gtr1-GFP (A) and Tor1-GFP (B) mainly localize to the vacuolar membrane, while Cdc60-RFP (A) adjoins the limiting membrane of the vacuole, but mainly localizes to the cytoplasm in exponentially growing cells (EXP). DHBB (10 μM) treatment does not detectably alter the localization of Gtr1-GFP, Tor1-GFP, or Cdc60-RFP. Notably, given the high abundance of Cdc60-RFP within the cytoplasm, a potential DHBB-induced displacement of Cdc60-RFP from the vacuolar membrane may escape detection by standard fluorescence microscopic analyses. Tr, transmission.

(C) DHBB disrupts the Cdc60-Gtr1^{GTP} interaction in a concentration-dependent manner. Gtr1^{GTP}-TAP was precipitated from cells co-expressing Cdc60-HA$_3$. Cells were harvested in exponential growth phase either prior to (0) or following a 30-min period of treatment with the indicated DHBB concentrations. Cell lysates (Input) and TAP pull-down fractions were subjected to SDS-PAGE and immunoblots were probed with anti-HA or anti-protein A (anti-TAP) antibodies.

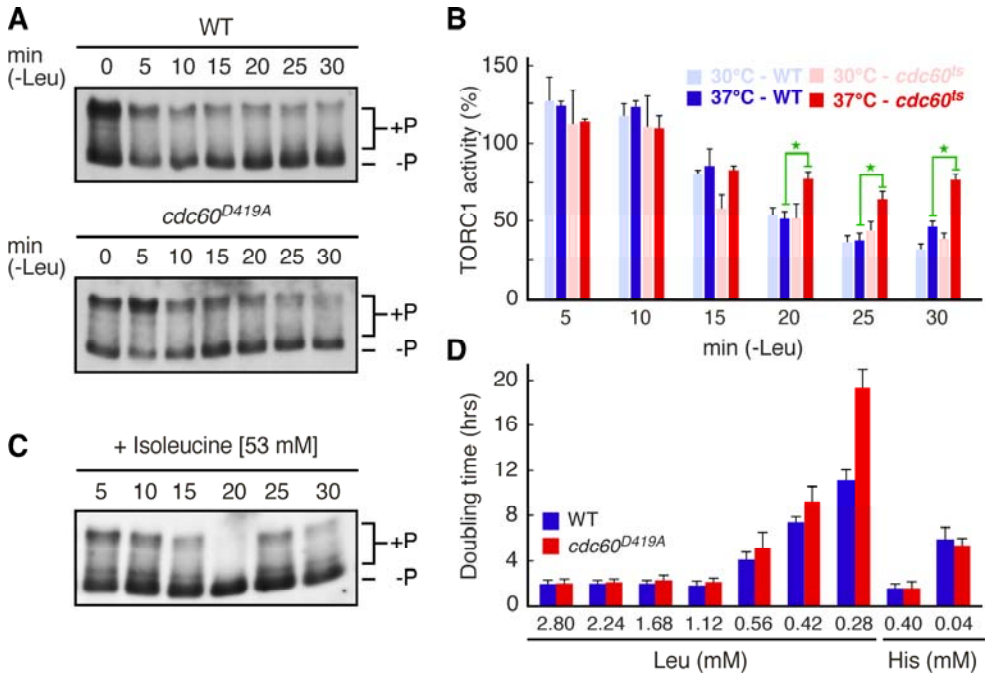

Figure S3. Physiological Relevance of LeuRS Editing, Related to Figure 4

(A) Leucine starvation causes TORC1 inactivation in both wild-type and *cdc60*^{D419A} mutant cells. Leucine (*leu2*Δ) auxotrophic wild-type (WT) and *cdc60*^{D419A} mutant cells were grown to exponential phase in medium containing leucine and then transferred to a medium lacking leucine (0 time point). Samples were taken at the times indicated following leucine starvation and TORC1 activity was assayed as in Figure 1D.

(B) Temperature-inactivation of Cdc60^{ts} protects TORC1 from leucine starvation-induced downregulation. Leucine (*leu2*Δ) auxotrophic wild-type and *cdc60*^{ts} mutant cells were grown to exponential phase in medium containing leucine, incubated for 1 hr at either 30°C or 37°C, and then transferred to a medium lacking leucine (0 time point). Samples were taken at the times indicated following leucine starvation (-Leu) and TORC1 activity was assayed as in Figure 1D. Data are expressed as relative values with respect to the 0 time point and reported as averages (n = 3), with standard deviations indicated by the lines above each bar. As assessed by two-way analysis of variance (ANOVA) followed by post-test analysis, the observed differences between wild-type and *cdc60*^{ts} cells at 37°C are statistically significant with p-values < 0.05 (indicated with one asterisk).

(C) Addition of isoleucine in disproportionate quantities causes transient TORC1 inactivation. Wild-type cells were grown (on SD medium) to exponential growth phase and treated with excessive amounts of isoleucine (*i.e.* final concentration of 53 mM). Samples were taken at the times indicated following isoleucine addition and TORC1 activity was assayed as in Figure 1D.

(D) LeuRS editing is specifically required for growth under leucine limiting conditions. Leucine (*leu2*Δ) and histidine (*his3*Δ) auxotrophic wild-type (blue bars) and LeuRS editing defective *cdc60*^{D419A} mutant (red bars) cells were grown in SD medium containing either 5 mM histidine and various levels of leucine (Leu [mM]), or 9 mM leucine and different levels of histidine (His [mM]) as indicated. Doubling times are reported as averages (n = 3), with standard deviations indicated by the lines above each bar.

Table S1. Proteins Identified in Gtr1-TAP Pull-Down Experiments, Related to Figure 1

Protein[1]	Function	No. of peptides (+ Leu)	No. of peptides (- Leu)
Rpl4A	Component of the large (60S) ribosomal subunit	9	0
Vas1	Mitochondrial and cytoplasmic valyl-tRNA synthetase	6	0
Ded1	DEAD-box helicase	6	0
Fas1	Fatty Acid synthetase	24	1
Cdc60	**Leucyl-tRNA synthetase**	11	1
Rpo21	DNA-directed RNA polymerase	9	1
Gnd1	6-Phosphogluconate dehydrogenase	8	1
Trr1	Thioredoxin reductase	6	1
Faa4	Fatty acyl-CoA synthetase	5	1
Acc1	Acetyl-CoA carboxylase	5	1
Pfk2	Phosphofructokinase	5	1
Rpn8	Regulatory subunit of the 26S proteasome	5	1
Fet5	Multicopper oxidase	5	1

[1]Proteins were identified by LC-MS-MS analysis of polypeptides in purified Gtr1-TAP preparations from exponentially growing (+Leu) or leucine-deprived (30 min; -Leu) cells. Only proteins for which at least one peptide was identified in the Gtr1-TAP preparations (confidence interval of 99.9%) and none in control preparations from non-tagged wild-type cells were retained for further analysis. Proteins for which at least 5 peptides were identified in the +Leu samples and none in the corresponding –Leu samples, or proteins for which the ratio of the number of peptides in the +Leu versus the -Leu samples was > than 5, were retained for this table.

Table S2. Strains Used in This Study

Strain	Genotype	Source	Figure
BY4741	*MAT***a**; *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	Euroscarf	S3B
BY4742	*MATα*; *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	Euroscarf	
Y08149	[BY4741] *MAT***a**; *cdc60^{ts}*	Li et al., 2011	S3B
YL515	[BY4741/2] *MATα*; *his3Δ1, leu2Δ0, ura3Δ0*	Binda et al., 2009	
YL516	[BY4741/2] *MAT***a**; *his3Δ1, leu2Δ0, ura3Δ0*	Binda et al., 2009	2A-C, 3A-D, 4C
GB2381	[YL516] *MAT***a**; *CDC60-HA$_3$::HIS3*	This study	1A/B, 2D/E, S1, S2C
GB2382	[YL516] *MAT***a**; *IGO1-TAP-kanMX4, CDC60-HA$_3$-HIS3*	This study	1A, S1
GB2549	[YL516] *MAT***a**; *CDC60-HA$_3$-HIS3, gtr2Δ::kanMX4*	This study	S1
GB2523	[YL516] *MAT***a**; *KOG1-HA::kanMX4*	This study	2F
MPG1630	[YL516] *MAT***a**; *gtr1Δ::kanMX4*, *gtr2Δ::kanMX4*	This study	2A-C, 3B/C
GB2378	[YL516] *MAT***a**; *cdc60^{D418R}*	This study	2A-C
GB2379	[YL516] *MAT***a**; *cdc60^{D418R}- HA$_3$::HIS3*	This study	2E
MB32	[YL516] *MAT***a**; *gtr1Δ::kanMX4*	Binda et al., 2009	4A
MJA2638	[YL515] *MATα; cdc60Δ::KanMX4* [YCplac111-*CDC60*]	This study	4A, S3A, S3D
MJA2786	[YL515] *MATα; cdc60Δ::KanMX4* [YCplac111-*CDC60^{D419A}*]	This study	S3A, S3D, S3C
MJA2784	[YL515] *MATα; cdc60Δ::KanMX4* [*CEN, HIS3, CDC60*]	This study	S3D
MJA2785	[YL515] *MATα; cdc60Δ::KanMX4* [*CEN, HIS3, CDC60^{D419A}*]	This study	S3D
MJA2604	[YL515] *MATα; cdc60Δ::KanMX4* [YCplac111-*cdc60^{S414F}*]	This study	4A
8003	*MATα*; *leu2Δ0, ura3, trp1, his3, ade8, cdc60^{ts}*	Hohmann and Thevelein, 1992	1C/D
MPG2389	*MAT***a**; *HIS3::GTR1-GFP, gtr1Δ::natMX4, ura3-52, leu2, trp1*	This study	S2A
MP52-2A	[YL516] *MAT***a**; *TOR1-D330-3xGFP*	Binda et al., 2009	S2B
NMY51	*MAT***a**; *his3Δ200, trp1-901, leu2-3,112, ade2, LYS::(lexAop)4-HIS3, ura3::(lexAop)8- lacZ, ade2::(lexAop)8-ADE2 GAL4*	Dualsystems	4B

Table S3. Plasmids Used in This Study

Plasmid	Description	Source	Figure
YCplac33	*CEN, URA3*	Gietz and Sugino, 1988	2A-C, 3A-D, 4A-D
YCplac111	*CEN, LEU2*	Gietz and Sugino, 1988	2A-C, 3A-C, 4A
pMB1344	YCplac33-*GTR1-TAP*	Binda et al., 2009	1A-B, 2D-F, 4C, S1
pCM264	*CEN, URA3,* Tet_{OFF}-HIS_6-HA_3	Arino and Herrero, 2003	1C/D, 4A
pGB1957	pCM264-Tet_{OFF}-HIS_6-*CDC60*	This study	1C/D
pJU1462	pRS413-$SCH9^{T570A}$-HA_5	Urban et al., 2007	1D, 2A-C, 3A-D, 4D, S3C
pMB1394	YCplac33-Tet_{ON}-$GTR1^{Q65L}$	Binda et al., 2009	2A-C, 3B/C, 4A
pPM1623	YCplac111-Tet_{ON}-$GTR2^{S23L}$	This study	2A-C, 3B/C, 4A
pMJ1974	YCplac111-*CDC60*	This study	4A, S3D
pMJ2113	YCplac111-$CDC60^{S414F}$	This study	4A
pDL2-Alg5	2µ, *ADH1-HA-NubG, TRP1*	Dualsystems	4B
pAI-Alg5	2µ, *ADH1-HA-NubI,TRP1*	Dualsystems	4B
pPR3-N	2µ, *CYC1-NubG-HA, TRP1*	Dualsystems	4B
pNP1689	pPR3-N-*CYC1-NubG-HA-GTR1*	Binda et al., 2009	4B
pNP1692	pPR3-N-*CYC1-NubG-HA-GTR2*	Binda et al., 2009	4B
pMJ1868[1]	pCabWT-$CYC1\text{-}Cub\text{-}LexA\text{-}Cdc60^{CP1}$	This study	4B
pMJ2115[1]	pCabWT-$CYC1\text{-}Cub\text{-}LexA\text{-}Cdc60^{CP1\text{-}S414F}$	This study	4B
pMPG1574	2µ, Tet_{ON}-HIS_6-HA_3, *URA3*	Binda et al., 2009	4C/D
pMJ2059[1]	pMPG1574-Tet_{ON}-HIS_6-HA_3-$CDC60^{CP1}$	This study	4C/D
pMJ2116[1]	pMPG1574-Tet_{ON}-HIS_6-HA_3-$CDC60^{CP1\text{-}S414F}$	This study	4C/D
pMB1372	*YCplac33-* $GTR1^{Q65L}$ *-TAP*	Binda et al., 2009	S2C
pMJA2192	pRS416-*CYC1-CDC60-RFP, URA3*	This study	S2A
pMJA2069	YCplac111-$CDC60^{D419A}$	This study	S3A, S3D
pJU1436	pRS416-$SCH9^{T570A}$-HA_5	Urban et al., 2007	S3C
pMJA2168	*CEN, HIS3, CDC60*	This study	S3C, S3D
pMJA2167	*CEN, HIS3,* $CDC60^{D419A}$	This study	S3D

[1]Plasmids pMJ1868/pMJ2115 and pMJ2059/pMJ2116 express the Cdc60 CP1 editing domain encompassing amino acids 263-548 and 263-530 of Cdc60, respectively.